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# Group II Intron-Mediated *Trans*-Splicing in the Gene-Rich Mitochondrial Genome of an Enigmatic Eukaryote, *Diphylleia rotans*

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## Abstract

Although mitochondria have evolved from a single endosymbiotic event, present day mitochondria of diverse eukaryotes display a great range of genome structures, content and features. Group I and group II introns are two features that are distributed broadly but patchily in mitochondrial genomes across branches of the tree of eukaryotes. While group I intron-mediated *trans*-splicing has been reported from some lineages distantly related to each other, findings of group II intron-mediated *trans*-splicing has been restricted to members of the Chloroplastida. In this study, we found the mitochondrial genome of the unicellular eukaryote *Diphylleia rotans* possesses currently the second largest gene repertoire. On the basis of a probable phylogenetic position of *Diphylleia*, which is located within Amorphea, current mosaic gene distribution in Amorphea must invoke parallel gene losses from mitochondrial genomes during evolution. Most notably, although the cytochrome c oxidase subunit (*cox*) 1 gene was split into four pieces which located at a distance to each other, we confirmed that a single mature mRNA that covered the entire coding region could be generated by group II intron-mediated *trans*-splicing. This is the first example of group II intron-mediated *trans*-splicing outside Chloroplastida. Similar *trans*-splicing mechanisms likely work for bipartitely split *cox2* and *nad3* genes to generate single mature mRNAs. We finally discuss origin and evolution of this type of *trans*-splicing in *D. rotans* as well as in eukaryotes.

**Key words:** Diphyllostia, introns, mitochondria, inverted repeats, *trans*-splicing.

## Main Text

Mitochondria in extant eukaryotic cells are direct descendants of endosymbiotic alpha-proteobacteria which were already integrated as organelles in the last common ancestor of extant eukaryotes (LECA) (Gray 2012). Mitochondrial genomes are streamlined in size and gene number when compared with current alpha-proteobacterial genomes (Gray et al.

2004). Nonessential genes in the ancestral endosymbiont genome were presumably lost prior to LECA, and, once a protein targeting system evolved, other genes were transferred to host nuclear genomes (Gray 1999; Adams and Palmer 2003). Comparative analysis of mitochondrial genomes have revealed that even closely related strains/species/lineages can have different gene repertoires, suggesting

that gene loss from mitochondrial genomes is still ongoing (e.g., Gray 1999; Adams and Palmer 2003; Hancock et al. 2010; Masuda et al. 2011; Kamikawa et al. 2014). The most gene-rich mitochondrial genomes are those of the excavate group Jakobida with 65–100 kb in length, which carry 61–66 protein genes and 30–34 RNA genes (Burger et al. 2013). The most gene-rich mitochondrial genome outside Jakobida so far was found in two heterolobosean *Naegleria* species of which 49 kb-long mitochondrial genomes carried 42 protein genes and 23 RNA genes (Herman et al. 2013). The other extreme, mitochondrial genomes of dinoflagellates, apicomplexan parasites, and their close relatives, that is, *Chromera velia* and *Vitrella brassicaformis*, retain only two or three protein genes and fragmented rRNA genes (Feagin 2000; Kamikawa et al. 2007; Kamikawa, Nishimura, et al. 2009; Flegontov et al. 2015). The mitochondrial genomes of apicomplexan parasites are also the smallest in size (6–7 kb; Feagin 2000).

In addition to gene repertoires and size, the diversity of mitochondrial genomes is also reflected in types of introns and splicing. Introns found in mitochondrial genomes are group I and group II introns with extensive RNA secondary structures. While all eukaryotes with few exceptions possess spliceosomal introns in their nuclear genomes, group I and group II introns in mitochondrial genomes are sparsely distributed in the tree of eukaryotes; many mitochondrial genomes completely lack them. It has been argued that this patchy distribution of group I and II introns in organellar genomes is the mainly the product of homing/retrohoming and transposition/retrotransposition mechanisms facilitated by endonucleases (e.g., Hardy and Clark-Walker 1991; Goddard and Burt 1999; Gogarten and Hilario 2006; Kamikawa, Masuda, et al. 2009; Nishimura et al. 2012, 2014; Zimmerly and Semper 2015). During splicing, introns are removed from a precursor RNA, and the concomitant ligation of exons results in the formation of a mature transcript. When this process of intramolecular ligation involves only a single RNA molecule it is called *cis*-splicing (Glanz and Kuck 2009). In cases that involve more than one primary transcript in an intermolecular ligation, the RNA is processed by *trans*-splicing (Glanz and Kuck 2009). This latter splicing reaction is a known variant mechanism of spliceosomal, group I and group II intron splicing (Dorn et al. 2001; Hastings 2005; Fischer et al. 2008; Glanz and Kuck 2009; Nilsen and Graveley 2010; Kamikawa et al. 2011). Group I intron *trans*-splicing was first discovered in mitochondria of placozoan animals (Burger et al. 2009) and a lycophyte plant (Grewe et al. 2009), but was later found to be also distributed in mitochondria of some green algae and fungi (Pombert and Keeling 2010; Nadimi et al. 2012; Pelin et al. 2012; Pombert et al. 2013). Group II intron *trans*-splicing was discovered more than 30 years ago from mitochondria of the liverwort *Marchantia polymorpha* (Fukuzawa et al. 1986) and plastids of the green alga *Chlamydomonas reinhardtii* (Kück et al. 1987). To date, group II intron-mediated *trans*-splicing

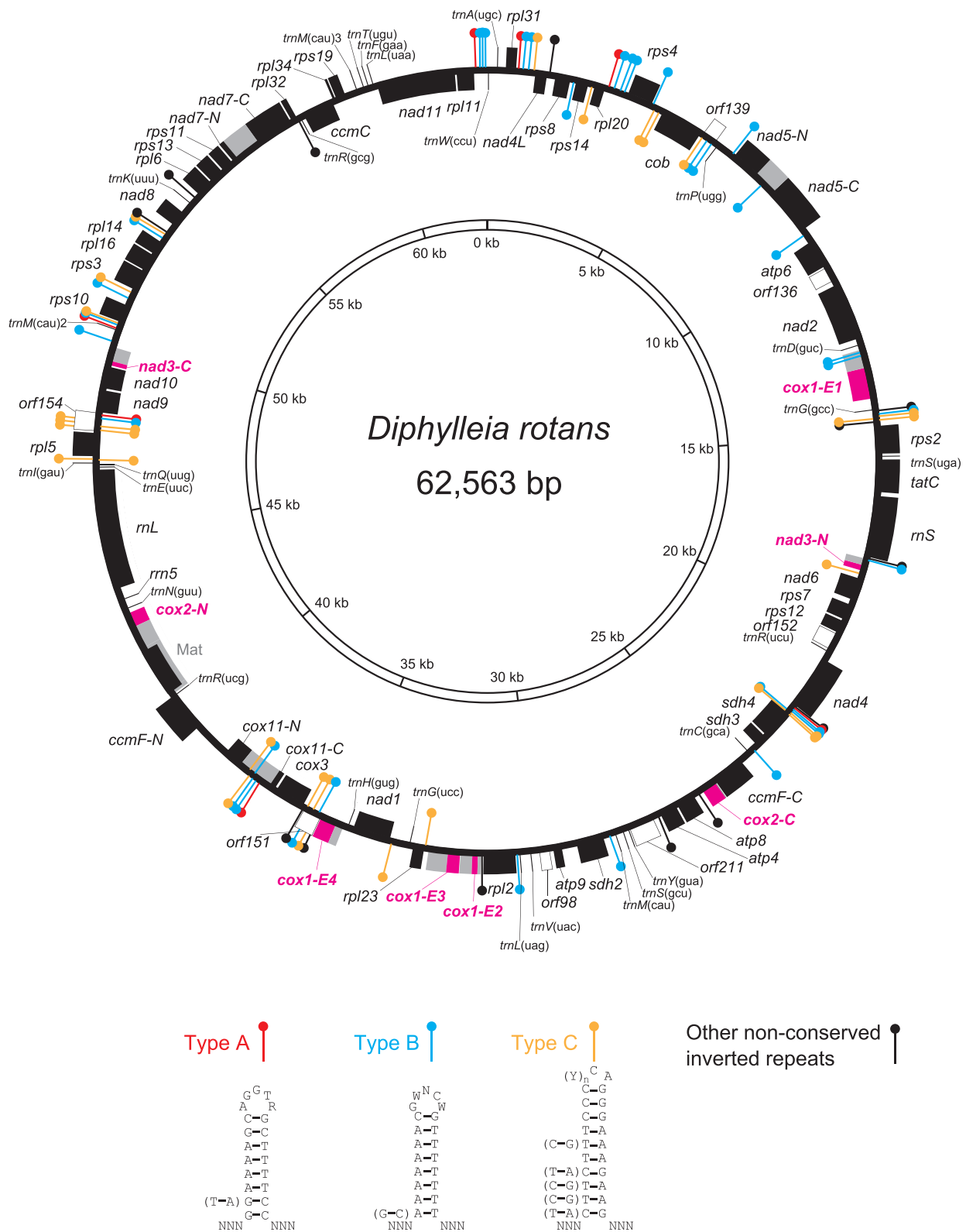
has only been found in the Chloroplastida assemblage composed of green algae and land plants (e.g., Bonen 2008; Glanz and Kuck 2009).

*Diphyllia rotans* is a bacterivorous, unicellular eukaryote with two flagella. Electron microscopic and molecular phylogenetic analyses suggested close relationship between *Diphyllia* and the early branching eukaryote *Colloidietyon* (Brugerolle et al. 2002; Zhao et al. 2012), resulting in a group that has been named Diphyllatia (Cavalier-Smith 2003). The precise phylogenetic position of Diphyllatia in the tree of eukaryotes remains unclear (Zhao et al. 2012). Recently, it was proposed that eukaryotes can be divided into three large clades called Amorphea, Excavata, and Diaphoretickes and the root of the eukaryotic tree of life lies between Amorphea on the one hand, and Excavata and Diaphoretickes on the other (Adl et al. 2012; Brown et al. 2013; Eme et al. 2014; Derelle et al. 2015). In this scheme, Diphyllatia is proposed to be more likely a member of Amorphea (Derelle et al. 2015).

We completely sequenced the mitochondrial genome sequence of the “deeply branching” protist *D. rotans* to gain insight into the early evolution of mitochondrial genomes in eukaryotes. We find that, consistent with an early-branching position of Diphyllatia, this organism has a relatively rich gene content, the second most “bacterial-like” mitochondrial gene content after the jakobids. Unexpectedly, we also found the first examples of group II intron-mediated *trans*-splicing from a eukaryote outside of the Chloroplastida.

## The Structure and Content of the Mitochondrial Genome of *D. rotans*

The complete sequence of the mitochondrial genome of *D. rotans* can be mapped as a circular molecule that is 62,563 bp in length (fig. 1). The overall A+T content is 65.6%. The coding region occupies 41,240 bp which is approximately 65.9% of the entire sequence. This genome encodes three rRNA genes and 27 tRNA genes, which is sufficient to translate codons corresponding to all the amino acids (supplementary table S1, Supplementary Material online). We identified 49 protein-coding genes by their sequence similarity to orthologues and 7 functionally uncharacterized open reading frames (orf98, orf136, orf139, orf151, orf152, orf154, and orf211). Two *cis*-spliced group I introns exist in *cox1* and *nad5*, and two *cis*-spliced group II introns occur in *cox11* and *nad7*. We confirmed these introns were spliced *in vivo* by RT-PCR and sequencing of the resulting amplicons (supplementary fig. S1, Supplementary Material online). In addition to the conventional introns, we also found three group II introns in multiple pieces in *cox1*, *cox2*, and *nad3* (discussed below in more detail). Almost all the *D. rotans* introns lack intronic open reading frames (ORFs). Of these, only *cox2* intron carries an intronic ORF that contains a maturase domain and lacks other domains typical of group II intron ORFs such as the reverse transcriptase domain, maturase domain, DNA-binding



**Fig. 1.**—The complete mitochondrial genome of *D. rotans*. Functionally identifiable protein-coding, SSU rRNA, and LSU rRNA genes are depicted as closed boxes whereas unidentified ORFs are as open boxes. 5S rRNA and tRNA genes are shown as lines. Intron sequences are colored in gray. Split genes are highlighted in magenta. Three types of conserved palindromic sequences and nonconserved palindromic sequences are indicated by different colors: type A in red, type B in blue, type C in yellow, and nonconserved type in black.

domain, and endonuclease domain (e.g., Zimmerly and Semper 2015). Unfortunately, the intron sequences in this genome are divergent, and therefore, their evolutionary origins are impossible to investigate using molecular phylogenetic analyses. Nevertheless, it is noteworthy that the two group I introns and one group II intron in *D. rotans* are located in homologous sites to those of other eukaryotes (supplementary table S2, Supplementary Material online).

## Abundant Palindromic Repeats in the *D. rotans* Mitochondrial Genome

Closer inspection of the *D. rotans* mitochondrial genome revealed a series of short palindromic repeats; the consensus sequences, complementary bases, and copy numbers of the palindromic elements are outlined in figure 1. The short palindromic repeats in the *D. rotans* mitochondrial genome are of 21–45 nt in length and restricted to intergenic and intronic regions, with the exception of the palindromic elements in unidentified orf139, orf151, and orf154. When contained within introns, the palindromic repeats are confined to the non-ORF portions of the group I and group II introns (fig. 1). In total, we detected 83 short palindromic repeats that could be divided into three general types (type A to C; fig. 1 and supplementary fig. S2, Supplementary Material online) except for 12 “nonconserved” elements. Within each repeat type, the nucleotide sequences are almost identical to each other (supplementary fig. S2, Supplementary Material online). Palindromic repeat elements in other mitochondrial genomes have been thought to be selfish, mobile DNA elements (Nakazono et al. 1994; Paquin et al. 2000). This hypothesis can simply explain our observation of many palindromes with similar nucleotide sequences over the mitochondrial genome. Currently, it is unclear what, if any, function the short palindromic sequences serve in the *D. rotans* mitochondrial genome. Some short palindromic repeats have been thought to contribute to regulation of transcription (Ohta et al. 1998) and they could be involved in similar functions in *D. rotans* mtDNA. In any case, a notable consequence of such repeats is that mediate genome rearrangements (Nedelcu and Lee 1998; Beaudet et al. 2013), as well as the fragmentation and scrambling of genes (Smith and Lee 2009); Hopefully, gathering more mitochondrial genome sequences from related diphyllatians will reveal whether they undergo rapid genome rearrangements mediated by the palindromes and whether such rearrangements resulted in generation of the split introns.

## A Large Set of Proteins Encoded on Mitochondrial DNA

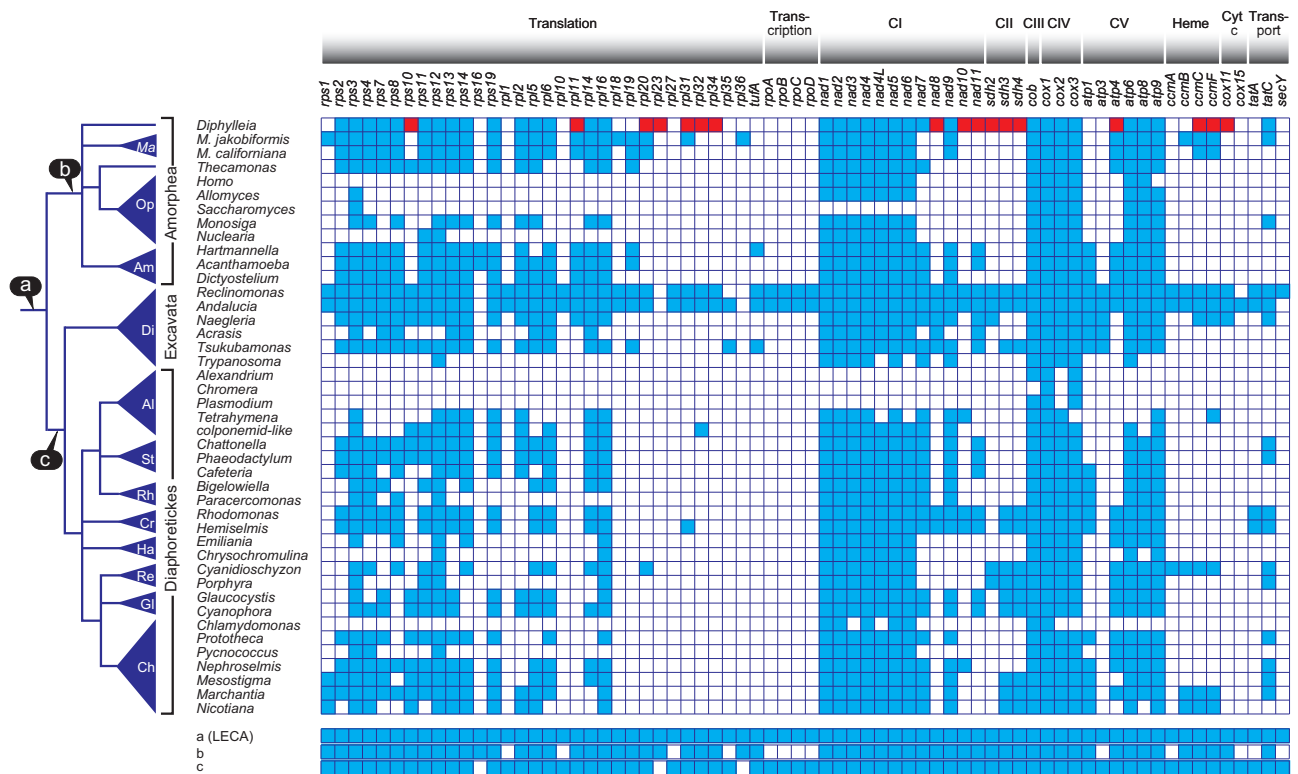
Although the mitochondrial gene repertoire of *D. rotans* is nearly a subset of that of the Jakobida species (fig. 2), it is conspicuously larger than that of *Naegleria* spp. (containing

42 protein genes; fig. 2). Thus, *D. rotans* has the most gene-rich mitochondrial genome outside Jakobida. In addition to the relatively common genes encoding subunits of complex I (*nad1-4,4L,5-7,9*), complex III (*cob*), complex IV (*cox1-3*), and complex V (*atp6,8,9*), *D. rotans* mitochondrial DNA encodes rarely found genes for complex I (*nad8,10,11*), complex II (*sdh2-4*), complex V (*atp4*), cytochrome *c* maturase (*ccmC* and *ccmF*), cytochrome *c* oxidase assembling protein (*cox11*), and ribosomal proteins (*rps10*, *rpl11,20,23,31,32,34*; fig. 2). Among the ribosomal protein genes, only *D. rotans* and Jakobida possess *rpl32*, and *rpl34* encoded on their mitochondrial genomes; *rpl23*, by contrast, is a gene which has been identified in no other mitochondrial genome to date (fig. 2).

To gain a better understanding of the mitochondrial genome content of the last eukaryotic common ancestor (LECA) and evolution of the gene repertoire in Diphyllatia and its close relatives, we mapped the mitochondrial genome content of diverse lineages across the tree of eukaryotes. Assuming only events of gene loss or gene transfer to the nucleus, the mitochondrial genome of LECA must have encoded at least 70 proteins (fig. 2; black balloon a). If the eukaryote root falls between Amorphea (A) on the one hand, and Excavata (E) and Diaphoretickes (D) on the other (Derelle et al. 2015), the common ancestor E + D likely had 67 genes after losing only three (balloon c in fig. 2). The lineage leading to the ancestor of Amorphea appears to have lost at least 13 genes resulting in 57 genes (balloon b in fig. 2). Curiously, the *D. rotans* mitochondrial genome possesses genes for *rpl23*, *rpl32*, *rpl34*, *nad8*, *nad10*, *sdh2-4*, and *cox11* whereas all other amorphean mitochondrial genomes lack them (fig. 2). There are other mitochondrial genomes within the Amorphea whose unique gene content implies massive parallel gene loss. For example, the mitochondrial genome of *Malawimonas jakobiformis* exclusively carries genes for *rpl1*, *rpl18*, *rpl36*, and *ccmB* (although *M. jakobiformis* branching within Amorphea remains controversial). Similarly, some, but not all, amoebozoan species possess *rps16*, *tufA*, and *atp1*. The requirement for massive parallel loss events in these cases does not significantly change if the root of eukaryotes is located in several alternative positions such as the branch leading to Jakobids (He et al. 2014) or that leading to Euglenozoa (Cavalier-Smith 2010). However, if the precise phylogenetic position of *D. rotans* is found to be outside Amorphea in future analyses, the gene loss events predicted here would require reevaluation.

## Group II Intron-Mediated *Trans*-Splicing

The *cox1* gene in the *D. rotans* mitochondrial genome is broken up into four fragments, that are scattered over the genome (fig. 1) that will henceforth be referred to as *cox1-E1* to *cox1-E4*. Two of the fragments, *cox1-E2* and *cox1-E3*,

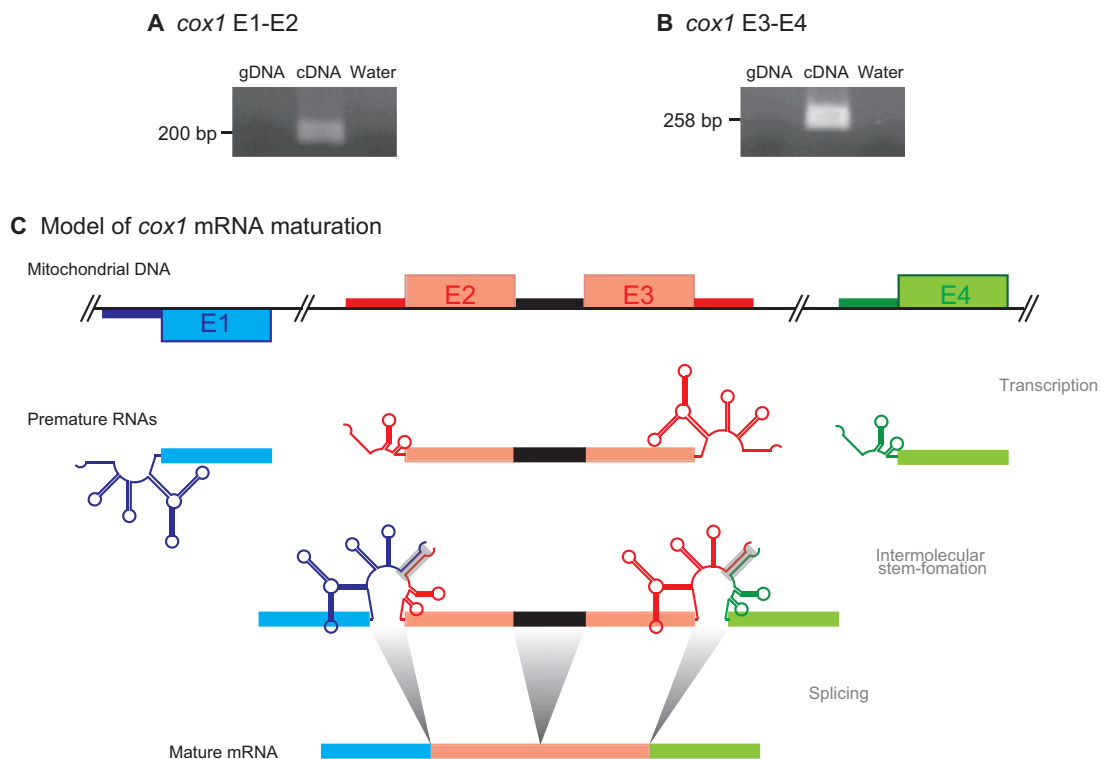


**Fig. 2.**—Distribution of protein coding genes in mitochondrial genomes. Presence and absence of corresponding genes in mitochondrial genomes of various eukaryotes is shown by closed and open boxes, respectively. The gene contents were determined from genome sequences retrieved from the GenBank. Rare genes found in the *D. rotans* mitochondrial genome are highlighted in red. Phylogenetic relationships of eukaryotes are based on Derelle et al. (2015), Brown et al. (2013), and Eme et al. (2014). The predicted protein gene contents of LECA (a), the last common ancestor of Amorphea (b), and that of Diaphoretickes and Excavata (c), are shown. Ma: *Malawimonas*; Op: Opisthokonta; Am: Amoebozoa; Di: Discoba; Al: Alveolata; St: Stramenopiles; Rh: Rhizaria; Cr: Cryptophyceae; Ha: Haptophyta; Re: Red algae (Rhodophyceae); Gl: Glaucophyta; Ch: Chloroplastida; CI–CV: electron transport chain complex I–V.

are interrupted by a *cis*-spliced group I intron as mentioned above and located between *rpl2* and *rpl23* (fig. 1, supplementary figs. S3 and S4, Supplementary Material online). *Cox1-E4* is located on the same strand as *cox1-E2/cox1-E3* but is separated by approximately 5 kb of DNA that encodes a total of four genes: *cox1-E4* is located downstream of *nad1* (fig. 1, supplementary figs. S3 and S4, Supplementary Material online). In contrast, *cox1-E1* is located upstream of *nad2* on the opposite strand from the other *cox1* fragments (fig. 1, supplementary figs. S3 and S4, Supplementary Material online). The curious fragmentation patterns and distribution over the genome are suggestive that a mature *cox1* transcript might be assembled via *trans*-splicing reactions. To provide evidence that the mitochondrial *cox1* pieces in *D. rotans* are processed into single mature mRNA *in vivo*, we conducted RT-PCR experiments, using primers located in the respective flanking exons (supplementary fig. S3, Supplementary Material online). Sequencing of the resulting PCR products confirmed that the exons of *cox1* were accurately ligated *in vivo* (fig. 3A and B), probably through *trans*-splicing.

Sequencing of the PCR products confirmed 100% sequence identity to the corresponding exons. No PCR product was obtained using total genomic DNA as the template (fig. 3A and B), ruling out the possibility that contiguous homologues exist in the nuclear genome.

Some *trans*-splicing phenomena in mitochondria are associated by split group I or group II introns (Glanz and Kuck 2009). In other cases, such as *trans*-splicing in *Diplonema* (Vlcek et al. 2011) and dinoflagellate mitochondrial mRNAs (Jackson and Waller 2013), the mechanisms are unknown. In both group I and group II intron-mediated *trans*-splicing, fragmented intron pieces flanking to a coding region of transcripts form a precise secondary structure, and are then excised to generate contiguous mRNA. *In silico* scanning of the *D. rotans* mitochondrial genome allowed us to identify partial group II intron fragments flanking the split *cox1* gene pieces; RNAweasel and Mfannot identified domain V, which is a highly conserved structural domain of group II introns (Zimmerly and Semper 2015), in the flanking regions of *cox1* fragments (data not shown). We found that 3'-flanking



**Fig. 3.**—*Trans*-splicing in *cox1* transcripts. (A) Reverse transcriptase PCR for transcripts of *cox1*-E1 and *cox1*-E2. Genomic DNA (left lane), cDNA (middle lane), and distilled water (right lane) were used as templates. (B) Reverse transcriptase PCR for transcripts of *cox1*-E3 and *cox1*-E4. Genomic DNA (left lane), cDNA (middle lane), and distilled water (right lane) were used as templates. (C) A model of *cox1* mRNA maturation. Mitochondrial DNA, *cox1* coding regions, and intron regions are depicted as thin lines, boxes, and thick lines, respectively. Independent gene fragments and their transcripts are distinguished by different colors, whereas the group I intron splitting *cox1*-E2 and *cox1*-E3 is colored in black. Four *cox1* gene fragments are located on the mitochondrial DNA separately. *Cox1*-E2 and *cox1*-E3 are separated by insertion of a *cis*-spliced group I intron. In this model, premature RNAs from separately distributed gene fragments for *cox1* are transcribed with flanking intron regions. Flanking intron regions form intermolecular stem structures that associated to form group II intron secondary structures.

region of *cox1*-E1 and 5'-flanking region of *cox1*-E2 had the potential to form an intermolecular stem structure (supplementary fig. S5A, Supplementary Material online). We also predicted a group II intron-like RNA secondary structure, that is, six stem-loop structures radiating from a central wheel (Zimmerly and Semper 2015; supplementary fig. S5A, Supplementary Material online), of the 3' flanking region of *cox1*-E1 and the 5'-flanking region of *cox1*-E2 as a whole. In this prediction, the intermolecular stem structure is tentatively identified as Domain IV. Similarly, we found that 3'-flanking region of *cox1*-E3 and 5'-flanking region of *cox1*-E4 also had the potential to form a group II intron RNA secondary structure with the intermolecular stem structure again within Domain IV (supplementary fig. S5B, Supplementary Material online). The RT-PCR results and predicted secondary structures of flanking regions strongly suggest that *cox1* of *D. rotans* uses group II intron-mediated *trans*-splicing for maturation. In what follows, we propose a model of the maturation process of the *cox1* transcripts (fig. 3C). First, each set of *cox1*-E1/flanking group II intron fragment and *cox1*-E4/flanking group

II intron fragment is transcribed independently whereas *cox1*-E2 and *cox1*-E3, separated by a *cis*-spliced group I intron, are transcribed as a single transcript with group II intron fragments at each end. The three transcripts then associate by forming intermolecular stem structures. Finally, each intron or intron fragment is spliced via either the group I (*cis*-spliced intron within the *cox1*-E2/*cox1*-E3 transcript) or group II intron splicing mechanisms, resulting in a single mature *cox1* mRNA. Again, to date, group II intron-mediated *trans*-splicing has been only found in organellar genomes of green algae and land plants (Bonen 2008) and thus this is the first report of group II intron-mediated *trans*-splicing in a species outside Chloroplastida.

The *cox2*, *nad3*, and *ccmF* genes of the *Diphyllieia* mitochondrial genome are also broken up into two fragments, and the fragments are scattered over the genome. Although the bipartite fragments of *ccmF* (*ccmF*-N and *ccmF*-C) and *nad3* (*nad3*-E1 and *nad3*-E2) are encoded on the same strand of the mitochondrial genome, they are located at a distance of more than 15 kb from each other. The bipartite fragments of *cox2*

are located on different strands from each other. We confirmed *cox2* and *nad3* were not pseudogenes but posttranscriptionally processed, without sequence modification, by sequencing of an RT-PCR product (supplementary figs. S5C–S5F, Supplementary Material online). We inferred a group II intron-like secondary structure with intermolecular stems between the 3′-flanking region of N-terminal coding region and 5′-flanking region of C-terminal coding region in *cox2* and *nad3* (supplementary figs. S5C–S5F, Supplementary Material online), strongly suggesting that the two genes are also spliced *in trans* by group II intron splicing mechanisms. However, the *cox2* and *nad3* introns are disrupted at sites within Domain V and Domain I, respectively, different from the Domain IV split sites that have been previously reported for *trans*-spliced introns (Bonen 2008) and found in the *cox1* split introns of *D. rotans* (supplementary fig. S5, Supplementary Material online). To verify our prediction that the *cox2* and *nad3* introns are truly split within Domain V and Domain VI, respectively, sequencing mitochondrial genomes of close relatives of *D. rotans* may reveal intact close homologs of these introns. Because the intermolecular stem structures we predict in these group II introns involve only a few dozen base-paired residues, we expect that *D. rotans* may possess proteins that stabilize these structures, as proposed for plant organellar *trans*-splicing (Bonen 2008).

In contrast, we find no evidence of mature mRNAs that cover both *ccmF-N* and *ccmF-C* in both PCR reactions with gDNA and cDNA (data not shown) and neither show intermolecular secondary structures flanking them, suggesting 1) the proteins may be truly fragmented into two functional peptides like the CcmF of the land plant *Arabidopsis thaliana* (Rayapuram et al. 2008) or 2) the *ccmF* pieces we detect are pseudogenes.

## Understanding the Evolutionary Origins of *Trans*-Splicing in Eukaryotes

In Chloroplastida, *trans*-spliced group II introns are thought to have been generated by physical splits of *cis*-spliced group II introns caused by genomic rearrangements in these mitochondrial genomes (Qiu and Palmer 2004). Similarly, it is possible that the palindromic repeats that are widely distributed over the *D. rotans* mitochondrial genome have mediated genomic rearrangements (e.g., Nedelcu and Lee 1998) in an ancestral diphylatian mitochondrial genome and this generated the gene fragments and *trans*-splicing introns we have observed in *D. rotans*. In support of this scenario, five of the split intron fragments and two *cis*-spliced introns are located in close proximity to and include short palindromic repeats, respectively (fig. 1). If the split introns in *D. rotans* recently evolved, the mitochondrial genomes of close relative of *D. rotans* could have contiguous introns that are homologous to the *D. rotans* split introns. Future determination of

complete mitochondrial genomes of other diphylatian may help clarify the origins of split introns in *D. rotans*.

*Diphylleia rotans* has the first mitochondrial genome outside the Chloroplastida where *trans*-splicing of introns is known to occur. We note that the apusozoan *Thecamonas trehans*, a sister lineage of Opisthokonta (Derelle et al. 2015), has a candidate split group II intron in its mitochondrial genome: its *nad3* is annotated as a bipartite split gene, the two fragments are not encoded next to each other, and one of the fragments is flanked by a region that has the potential to form a group II intron-like structure (NC\_026452; Valach et al. 2014). Clearly, group II intron-mediated *trans*-splicing is more widespread in the tree of eukaryotes than previously thought.

## Materials and Methods

### Culturing of *D. rotans*, DNA Extraction, and Mitochondrial DNA Sequencing

*Diphylleia rotans* NIES-3764 was cultivated with the cyanobacterial strain *Microcystis aeruginosa* NIES-298 as a food source, in C-Si medium (<http://mcc.nies.go.jp/02medium.html>; jsessionid=72A858BC9BF571F6DC50B3EC0896E57D#csi, last accessed February 9, 2016) at 20 °C under 10–50 micromole photons/m<sup>2</sup>/s with the 14 h light/10 h dark cycle. DNA was extracted from cells of *D. rotans* NIES-3764 together with the cyanobacterial cells using the Plant DNA extraction kit (Jena BioSciences) following the manufacturer's instructions. Total DNA was sent to Hokkaido System Science Co., Ltd (Japan) for 100-bp paired-end sequencing by the Illumina HiSeq2000 platform using the 350-bp library constructed with the Truseq Nano DNA LT Sample Prep Kit (illumina) following the manufacturer's instructions. The sequenced reads were filtered on the basis of fluorescence purity by Chastity [Chastity = Highest intensity/(Highest intensity + Next highest intensity)]: reads were passed if reads had no more than one cycle of a chastity below 0.6 within the first 25 cycles. Removal of adapter sequences, all the reads containing "N," and all the reads containing adapter sequences at the 3′-ends were performed using cutadapt ver. 1.1 (<https://cutadapt.readthedocs.org/en/stable/>, last accessed February 9, 2016), resulting in 21 million reads yielding 21 Gb of data where 90% of nucleotides had Q30 scores and of which the mean quality score was greater than 35. Subsequent assembly was performed with Velvet ver. 1.2.08 (Zerbino et al. 2009) with hash length 65. Seven contigs derived from the mitochondrial genome were detected by homology search using the protein and rRNA sequences from the jakobid *Andalucia godoyii* as queries. The mean coverage of these detected contigs was more than 60. We also performed an assembly with the same program with hash length 95 and there was no significant conflict detected between the contigs assembled with the different hash length parameters. After prediction of adjacent contigs



with paired-end information, gaps between the contigs generated with a hash length 65 were closed by PCR amplification (primers reported in [supplementary table S3, Supplementary Material](#) online) and subsequent Sanger sequencing of amplicons. Genes encoding proteins and rRNAs were identified by BLASTx and BLASTN searches against the nonredundant databases at the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, last accessed February 9, 2016; Altschul et al. 1990), which also confirmed the identified genes were not derived from misassemblies of cyanobacteria-derived reads. Transfer RNA-encoding genes were found by using tRNAscan-SE (Lowe et al. 1997). The mitochondrial genome sequence was deposited in the DNA Data Bank of Japan (GenBank/EMBL/DBJ; accession no. AP015014).

Annotation was also performed by MFANNOT (<http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl>, last accessed February 9, 2016) and RNAweasel (<http://megasun.bch.umontreal.ca/cgi-bin/RNAweasel/RNAweaselInterface.pl>, last accessed February 9, 2016) with the standard genetic code. Intramolecular interaction of split group II intron RNAs were predicted by Mfold (Zuker 2003) with the default settings, followed by manual modifications according to the model structure of these types of organellar introns. Palindromic sequence elements were detected by EMBOSS explorer (<http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome>, last accessed February 9, 2016) with the following parameters: Minimum length of palindromes was 8, maximum length of palindromes was 100, maximum gap between elements was 10, and no mismatch was allowed in palindrome. Some detected sequences were manually excluded if a loop region was longer than a stem region.

To confirm that *cox1*, *cox2*, *nad3*, and *ccmF* were split genes as assembled, we performed PCR assays for the regions between the split gene fragments and their adjacent protein-coding genes ([supplementary figs. S3 and S4, Supplementary Material](#) online). In order to confirm *in vivo* splicing reactions, we conducted reverse transcriptase PCR assays. RNA was extracted from cultures using Trizol (Invitrogen) following the manufacturer's instruction. cDNA was synthesized with random hexamers, total RNA, and 3'-rapid-amplification-of-cDNA-ends kit (Invitrogen). PCR was conducted with either gDNA, cDNA, or distilled water as the template and primers were designed for each exon ([supplementary fig. S3, Supplementary Material](#) online), and PCR products were sequenced by the Sanger method.

## Supplementary Material

Supplementary figures S1–S5 and tables S1–S3 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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