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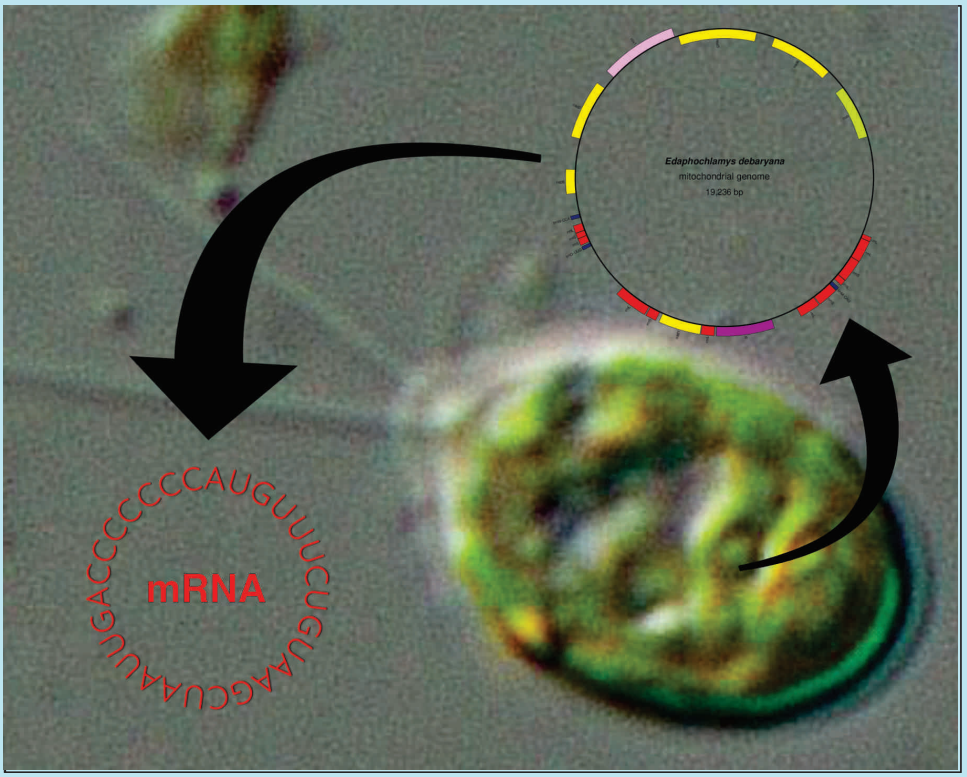


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**Cover Photograph**: The mitogenome of Edaphochlamys debaryana was sequenced and annotated. Analysis of its mitochondrial mRNAs revealed they are polycitydylated and circularized. Photograph © Robin Matthews.

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# The Mitochondrial Genome and mRNA Processing in an Appalachian Isolate of the Green Alga *Edaphochlamys debaryana* Prӧschold & Darienko (Goroschankin)

A. Bruce Cahoon[1]*, Ashar Khan[1], Robin Matthews[2]

**Abstract-** In this study we report the isolation and culture of the green microalga *Edaphochlamys debaryana* Prӧschold & Darienko (Goroschankin), from the mid-southern region of the Appalachian Mountains. Its organellar genomes were sequenced and archived, and aspects of mitochondrial mRNA processing were analyzed. Comparison of its mitogenome to all other Chlamydomonadales mitogenomes currently available demonstrated that its gene synteny is identical to *Chlamydomonas reinhardtii* P.A. Dangeard and its closest known relatives, *C. incerta* Pascher, and *C. schloesseri* Prӧschold & Darienko. Analyses of its mitochondrial mRNAs revealed that it cleaves them directly upstream of each start codon leaving no 5' UTR, has short yet variable 3' UTRs, polycitidylates some 3' termini, and circularizes mRNAs with full-length coding regions creating translatable circularized transcripts. These findings are consistent with observations from *C. reinhardtii* and *Pediastrum duplex* Meyen and provides another example of the conservation of these phenomena among Chlorophyta. *E. debaryana* has a worldwide distribution and it has been suggested that it could be a model for the study of micro-algal ecology making these molecular markers useful for future studies.

## Introduction

Mitochondria are semi-autonomous organelles that originated from the establishment of an endosymbiosis between a proteobacterium and a proto-eukaryotic cell (reviews in Gabaldón 2018, Roger et al. 2017). These organelles are considered semi-autonomous as they replicate by fission independently of the host cell cycle and have maintained a remnant of their original genome, called the mitogenome or chondriome. Mitogenomes are typically circular and carry genes coding protein subunits used by the mitochondrial electron transport chain as well as rRNAs and tRNAs (Johnston and Williams 2016). They have their own unique gene expression process including a T7 viral-like RNA polymerase, mRNA processing, and translation machineries distinct from the nucleo-cytoplasmic system (reviewed in D'Souza and Minczuk 2018).

Algal mitogenomes are much more diverse than those found in animals in terms of size, gene content, and gene synteny (Smith and Keeling 2015) and have their own variations of mitochondrial gene expression. The best understood of these algal mitogenomes is that of the green microalga *Chlamydomonas reinhardtii* P.A. Dangeard, which has been a genetic model system for decades (Harris 2009). Its mitochondrial genome is linear and compact with a size of ~15,750 bp carrying only 8 protein coding, 3 rRNA, and 3 tRNA genes (Gray and Boer 1988). This mitogenome is transcribed as two poly-cistronic primary transcripts, one for each strand, originating from a single control region. Each mRNA, tRNA, and rRNA is endonucleolytically cleaved from the primary transcript and the rRNAs must be trans-spliced to create working ribosomes. Each mRNA is removed from the primary transcript by endonucleolytic cleavage directly upstream of the AUG start codon resulting in no 5' un-

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translated regions (UTRs) (Duby et al. 2001, Tracy and Stern 1995). These mRNA do have 3' UTRs comprised of the nucleotides of the downstream intergenic region that remains when the downstream gene is removed. The termini of these 3' UTRs then may receive a polyC, polyU, or mixed polyC-U oligonucleotide additions (Cahoon and Qureshi 2018, Salinas-Giegé et al. 2017, Zimmer et al 2009). The final processing step is circularization of the mRNAs prior to translation (Cahoon and Qureshi 2018).

In this study we addressed the question: are the unusual aspects of mRNA processing in *C. reinhardtii* mitochondria, i.e. no 5' UTR and diverse 3' polynucleotide additions, an adaptation related to its unusually small and linear mitogenome? To investigate this topic, we sequenced the mitogenome and analyzed the mitochondrial mRNAs of a close relative, *Edaphochlamys debaryana* Pröschold & Darienko (Goroschankin), which has a larger circular mitogenome (~18,052 – 23,097). *E. debaryana* is an abundant green alga, previously known as *Chlamydomonas debaryana*, that was recently transferred to a new genus as *Chlamydomonas* is highly polyphyletic (Pröschold et al. 2001, 2018). The abundance and ubiquity of *E. debaryana*, at least across the Northern hemisphere, has led to the suggestion that it could serve as a model for the study of algal molecular ecology (Craig et al. 2021). Therefore, the production and analysis of molecular resources could be beneficial for a broad range of future studies.

## Materials and Methods

### Establishment of *Edaphochlamys debaryana* culture

A green alga was cultured from a small ephemeral pool on a preserve that is part of the UVa Wise campus (Wise, VA). This body of water and the cell isolation process was described in Hornberger et al. (2023). The isolate was found to thrive in/on both liquid and agarose versions of Bold's freshwater medium (Nichols and Bold 1965). It had a cellular morphology consistent with the Chlamydomonadales (Fig. 1), is maintained at UVa Wise, and is available upon request.

### Microscopy

Live, unstained cells were examined using wet mounts of cultured material placed on glass slides and compressed slightly using #1 coverslips. The cells were examined using a Nikon 80i microscope equipped with differential interference contrast objectives and polarizing filters. The digital images were captured as high resolution uncompressed tif files using a Nikon DS-Fi2 digital camera. Scale bars were stamped on the images using custom scripts created for the GNU Image Manipulation Program (GIMP 2.10-30). The lengths and widths of 20 mature cells were measured with GIMP using a pixel-to-micron conversion script and accuracy was checked using a stage micrometer.

### DNA and Organellar genome sequencing

Cells were grown in liquid Bold's medium, pelleted, and resuspended in the Genomic Lysis Buffer provided with ZymoResearch's (Irvine, CA) Quick-DNA Miniprep Kit. The cells were lysed using Zymo Research's 0.1 and 0.5 mm BashingBead Lysis Tubes and vortexed for at least 10 min. Cells were microscopically inspected for lysis and bead beating repeated until most cells were visibly disrupted. The cell lysate was removed from the bead beater tube and the kit instructions followed to complete the extraction. Whole genome sequencing was completed by Azenta (Plainfield, NJ) using their Illumina paired-end short-read non-human whole genome sequencing service. Raw sequences were de novo assembled using Geneious Prime (2021.2.1 BioMatters, Auckland, NZ). The mitogenome was identi-

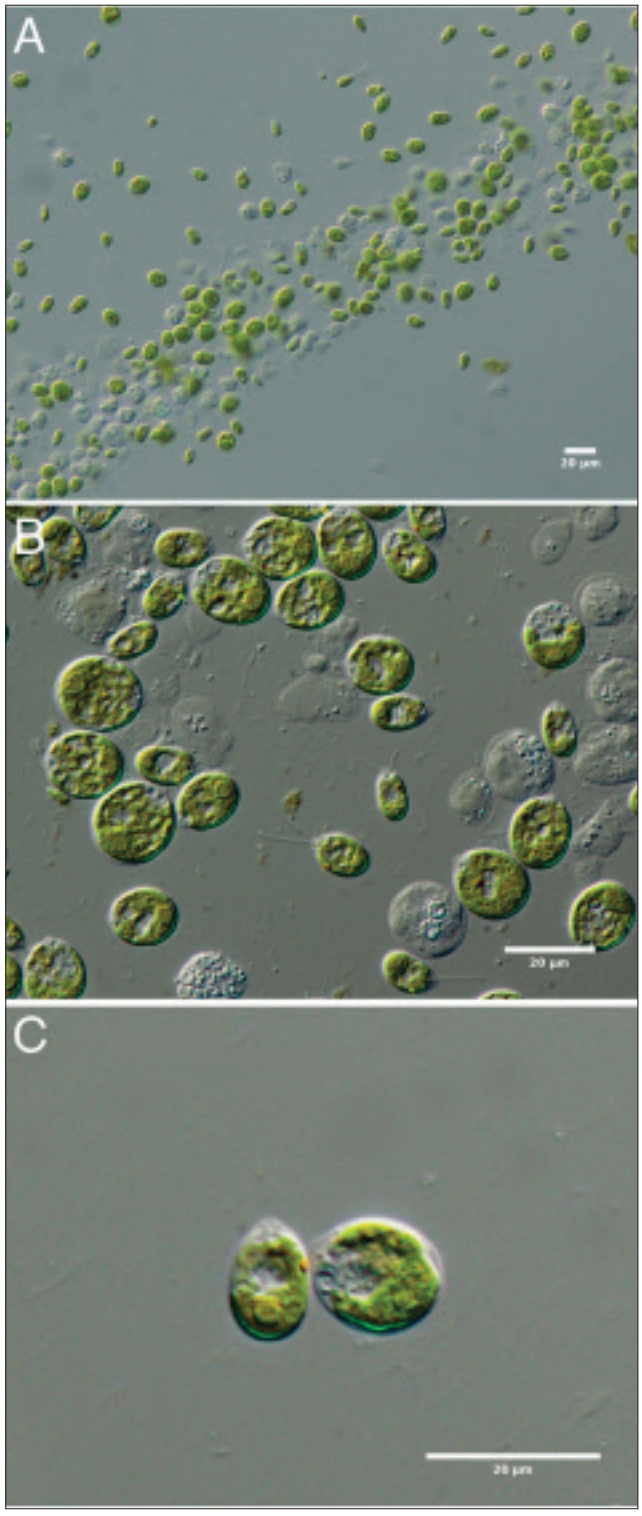


Figure 1. Light micrographs of the cultured *Edaphochlamys debaryana*.

fied from the de novo contig library through its homology to a *Chlamydomonas* sp. *coxI* gene. Once the isolate was identified as *E. debaryana* it was annotated by comparing open reading frames to other available *E. debaryana* mitogenomes. The mitogenome produced from this Wise, VA isolate is available in GenBank (OK514730). The chloroplast genome was also assembled and annotated and is available in Genbank (ON243965). Whole genome raw data are available through GenBank's Sequence Read Archive (PRJNA954744).

**Mitogenome Phylogenetics**

The protein coding genes common to all members of the Chlamydomonadales (*cob*, *cox1*, *nad1*, *nad2*, *nad4*, *nad5*, and *nad6*) with mitogenomes archived in GenBank were concatemerized using Geneious Prime (v. 2022.2, BioMatters, Ltd., Auckland, NZ), aligned using MUSCLE (v 5.1, Edgar 2004) with default settings, and the alignments were visually inspected for inconsistencies. Maximum Likelihood phylogenies were produced with IQTree, using the ModelFinder function, Ultrafast Bootstrapping with 1000 replicates, and FreeRate heterogeneity +R (Hoang et al. 2018, Kalyaanamoorthy et al. 2017, Soubrier et al. 2012, http://www.iqtree.org accessed January 2023). The model Finder function chose GTR+F+I+G4 as the best fit.

**circRT-PCR RACE and Amplicon sequencing**

RNA was extracted from *E. debaryana* using Qiagen's RNeasy Plant Mini kit (Valencia, CA). Cells were grown in liquid Bold's medium, pelleted by centrifugation and resuspended in the RLT buffer provided with the kit. Cells were lysed by bead beating as described above. The cell lysate was transferred to a Qiagen Qiashredder column and RNA extraction completed following Qiagen's protocol, including the optional DNase steps.

The 3' and 5' termini of each mRNA were determined using circRT-PCR to perform a Random Amplified cDNA Ends (RACE) assay using a protocol described in Cahoon and Qureshi (2018) and Mance et al. (2020). Briefly, total RNA was treated with T4 RNA ligase and cDNAs produced using gene specific primers (See Supplemental File 1, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-035-Cahoon-s1.pdf, primers labelled _R1) and MMLV reverse transcriptase (Promega, Madison, WI). These cDNAs were used as template for PCR using gene specific primers (See Supplemental File 1, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-035-Cahoon-s1.pdf, Primary PCR Pairs), Phusion DNA Polymerase (ThermoFisher, Waltham, MA), and a BioRad (Hercules, CA) C1000 thermal cycler, 95 °C 10 min (95 °C 30 secs, 55 °C 15 sec, 72 °C 60 sec) x 40 cycles, 72 °C 10 min, to individually amplify each cDNA fragment. Amplicon production was confirmed using gel electrophoresis and the products diluted 10x for use in a second round of PCR. Secondary PCR reactions used diluted primary reaction products as template and gene specific primers (See Supplemental File 1, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-035-Cahoon-s1.pdf). Amplicon production was confirmed using gel electrophoresis and products were prepared for sequencing using the GeneJET PCR purification kit (ThermoFisher). Amplicons produced from each mRNA were combined to form a single set of amplicons that were sequenced by Azenta (Plainfield, NJ) using their paired-end Illumina Amplicon EZ sequencing service. This process was completed twice for all genes beginning with two independently isolated total RNA samples. Naturally occurring circular mRNAs were also analyzed twice by leaving out the initial T4 RNA ligase step.

Sequence analysis was conducted using Geneious Prime software (v. 2022.2). Briefly, sequences were paired which removed bases < 95 % certainty. Sequences belonging to each

gene were sorted using the Map-to-Reference function. Like sequences from each gene were binned using the de novo assembly function and the contigs compared to the *E. debaryana* mitogenome sequence using BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to locate the 5' and 3' termini that had been joined by circularization.

**circRNA Quantification**

Circular mRNAs were quantified using the qRT-PCR assay described in Cahoon and Qureshi (2018) and Mance et al. (2020). Briefly, total RNA was treated with RNase-A, which indiscriminately degrades RNA, RNase-R, which degrades linear but not circular RNA, or left untreated. cDNAs were synthesized from these RNAs using random hexamers and MMLV (ThermoFisher). These cDNAs were used as templates for qPCR reactions using BioRad's cfx96 Real-Time PCR system, BioRad's SsoAdvanced Universal SYBR Green Supermix, and gene specific primers (See Supplemental File 1, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-035-Cahoon-s1.pdf). Assays were completed from three independent RNAs with three technical replicates.

## Results

**Morphological Features**

The cultured *E. debaryana* cells ranged from nearly spherical to elliptical, with an average cell length of 13.0 ± 2.16 μm (SD) and cell width of 8.3 ± 1.64 μm (SD). The cells had an apical papilla, cup-shaped chloroplast with a single, large, basal pyrenoid, and a central or apically located eyespot.

**Mitochondrial Genome**

The mitogenome of *E. debaryana* isolated in Wise, Virginia was 19,236 bp with 8 protein coding genes (PCGs), two rRNAs, and three tRNAs (Fig. 2A). The gene complement and synteny were identical to four other *E. debaryana* mitogenomes archived in Genbank. Phylogenetic analysis of seven concatemerized PCGs shared among all archived mitogenomes within the order Chlamydomonadales grouped the Wise, VA isolate within a clade of *E. debaryana* strains (Fig. 2B).

The fully sequenced mitogenomes of Chlamydomonadales reveal that they all have the same 7 PCGs (*cob*, *coxI*, *nad1*, *nad2*, *nad4*, *nad5*, and *nad6*), three tRNAs (*trnM*, *trnW*, and *trnQ*), and disconnected rRNAs that must be spliced to form functioning ribosomes. Gene synteny differs extensively within the Chlamydomonadales so it is significant that *E. debaryana* is identical to *C. reinhardtii*, *C. schloesseri*, and *C. incerta*. One PCG, rtl, is present in *E. debaryana* but missing from 15 of the 26 archived Chlamydomonadales mitogenomes. One other source of variation is the presence of introns in *cob*, *coxI* and *nad5*. Four of the five *E. debaryana* mitogenomes have intact versions of these genes with no introns. The fifth, strain NIES-2212, has introns in *nad5* but not *cob* or *coxI*. The presence/absence of these mitogenome differences generally follow evolutionary trends predicted by the phylogenetic analyses, and are noted in Figure 2B.

**mRNA Processing**

The 5' and 3' termini of mitochondrial mRNAs were determined using circRT-PCR. The 5' termini of all 8 PCGs occurred at the beginning of the AUG start codon, meaning they had no 5' untranslated regions (Fig. 3 and See Supplemental File 2A-E, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-035-Cahoon-s2.pdf). All of the PCGs had 3' untrans-

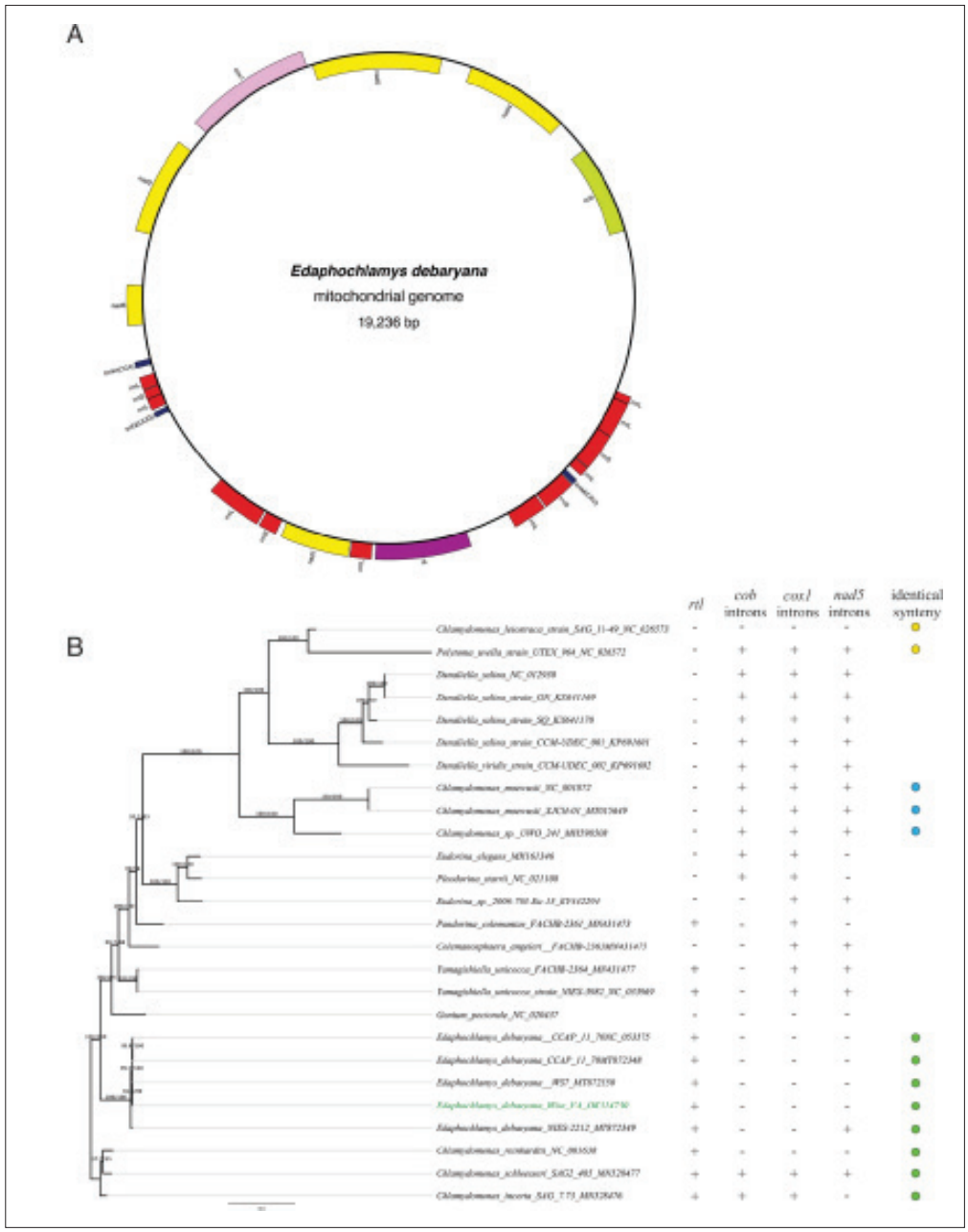


Figure 2. Mitogenome.

A. The mitogenome of an isolate of E. debaryana cultured from an ephemeral pool in Wise, VA. The map was produced using OGDraw (Lohse et al. 2013).

B. Maximum Likelihood tree produced using a concatemer of the protein coding genes shared among Chlamydomonadales with a full mitogenome archived in GenBank. Model Finder function chose GTR+F+I+G4 as the best fit. Branch support numbers are SH-aLRT %/ultrafast bootstrap with 1000 replicates. The scale represents number of substitutions. The table to the right of the phylogeny represents the presence/absence of rtl and introns in either cob, coxI, or nad5 which vary among Chlamydomonadales mitogenomes. Synteny refers to the gene order. Mitogenomes denoted with the same color circle share an identical synteny.

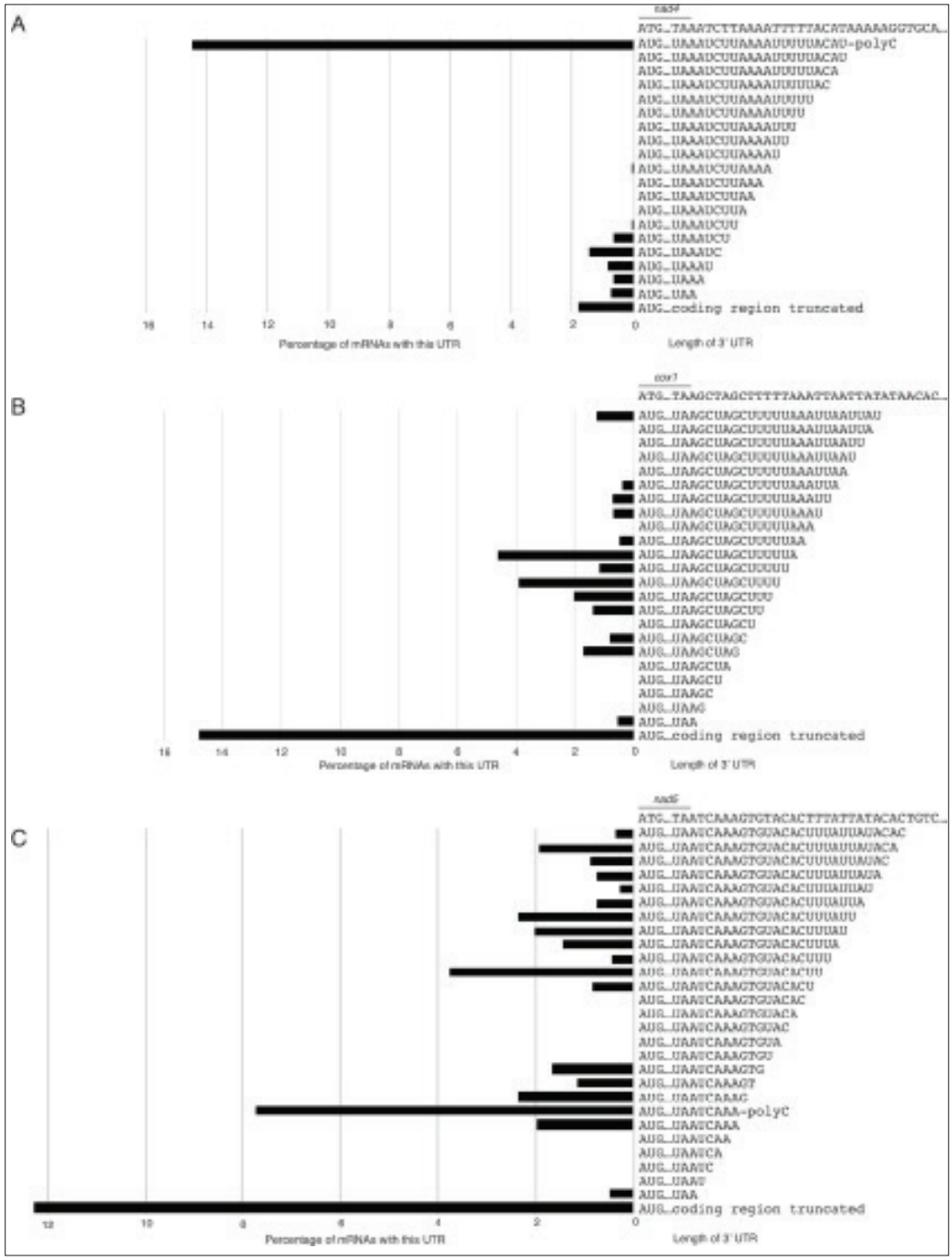


Figure 3. 5’ and 3’ termini of E. debaryana mitochondrial mRNAs. The mRNA termini of nad4 (A), cox1 (B) and nad5 (C). The sequence at the top of each represents the gene sequence while those below represent the mRNAs. Each gene’s start codon (AUG) occurs to the left of the ellipse and represents the 5’ termini as no 5’ UTRs were detected. The stop codon (UAA) is represented to the right of the ellipse and nucleotides following the stop codon represents the length of the 3’ UTR. The graph to the left of each set of mRNA sequences represents the percentage of that sequence detected among the sequenced circRT-PCR amplicons.

lated regions whose lengths were gene specific and varied from 0-25 nucleotides (Fig. 3 and See Supplemental File 2A-E, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-035-Cahoon-s2.pdf). Four of the PCGs were polycytidylated and the proportion of polyC mRNAs was gene specific – *cob* = 0.19 %, *nad1* = 6.22 %, *nad4* = 14.49 %, and *nad5* = 7.75 %. No 3' polynucleotide additions were detected on *cox1*, *nad2*, *nad6*, or *rtl*.

For six of the PCGs, the largest proportion of mRNAs had truncated 3' coding regions suggesting they were degradation products. The exceptions were *nad2* and *nad5* for which mRNAs with 5' UTRs comprised the majority of the products detected.

The circRT-PCR assays were completed on both ligated and non-ligated total RNA and similar results were collected for both suggesting all the mRNAs were naturally circular-

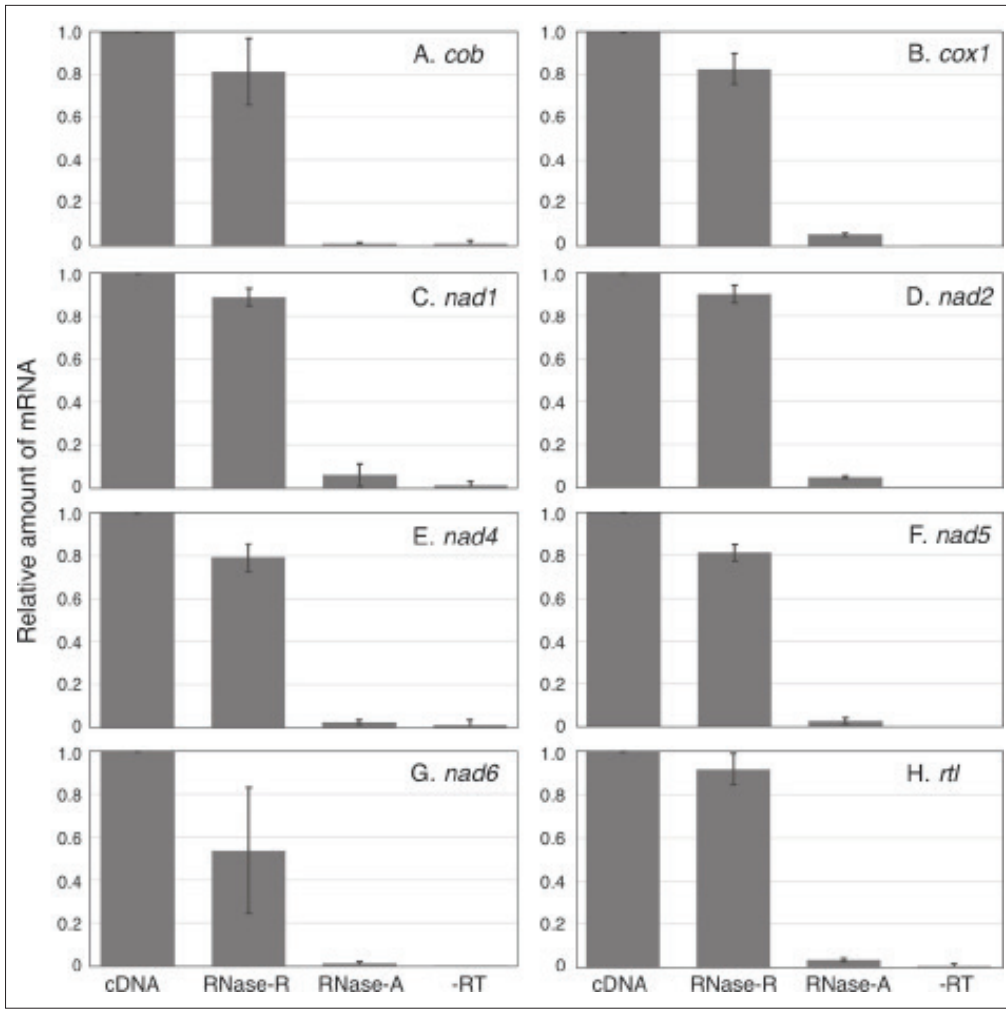


Figure 4. The proportion of circularized mRNAs detected for each mitochondrial gene using qRT-PCR. cDNA represents the amount of each mRNA detected from untreated total RNA. These amounts were scaled to represent a proportion of 1.0 and other assays compared to it. RNase-R degrades linear RNAs, leaving circularized versions. Those columns represent the proportion of each mRNA in the circular conformation. RNase-A degrades all RNA regardless of its conformation. -RT samples were not treated with Reverse Transcriptase prior to qPCR. Error bars represent the standard deviation from three biological replicates (n = 3) that were each amplified in triplicate (9 total reactions).

ized. Circular mRNAs were quantified using qRT-PCR and it was determined that for all mRNAs a majority of the total detectable number of cDNAs were circularized (Fig. 4).

## Discussion

### Mitogenome

In this study, we report the isolation and culture of *E. debaryana* from the upper-southern region of the Appalachian Mountains. The mitogenome was completed as part of the identification and characterization of the isolate and it was observed to have the same gene complement and synteny as *C. reinhardtii*, *C. incerta*, and *C. schloesseri*. Chlamydomonadales mitogenomes have the smallest number of genes among the Archaeplastida (Smith et al. 2013a, 2013b) with a conserved complement of the same 3 tRNAs, 7 or 8 protein coding genes, and 2 rRNAs. What does vary considerably within the order is gene synteny with very few examples of conserved gene order (Khani-Juyabad et al. 2019). This variation suggests that the observed conservation of synteny among these four species is noteworthy. The phylogenetic relationships among Chlamydomonadales species presented by Nakada et al. (2019) using a concatemer of nuclear 18S and plastid *atpB*, *psaA*, *psaB*, *psbC*, and *rbcL* and in Craig et al. (2021) who used 1,624 nuclear PCGs found that *E. debaryana* along with *C. reinhardtii*, *C. incerta*, and *C. schloesseri* formed a weakly supported group they called Metaclade-C. Our own phylogenetic analysis using all conserved mitochondrial PCGs among Chlamydomonadales also showed this clade. We believe that the addition of mitochondrial phylogenetic relationships and conserved gene synteny within this Metaclade-C provides more evidence that these four species are closely related.

### mRNA Processing

Our experiments demonstrated that the mitochondrial mRNAs of *E. debaryana* have no 5' UTRs, variable 3' UTRs, are polycitidylated, and circularized with full-length coding regions. This pattern of 5' and 3' termini was reported previously in the mitochondria of *C. reinhardtii*, and the more distantly related chlorophyte *Pediastrum duplex* (Cahoon and Qureshi 2018, Proulex et al. 2021). *C. reinhardtii* has an extremely compact mitogenome encoding 8 PCGs with small intergenic regions and it is possible that the 5' and 3' termini originate from a single endonucleolytic cleavage directly upstream of each mRNA, which leaves the remaining intergenic region attached to the upstream mRNA as the 3' UTR (Cahoon and Qureshi 2018). *P. duplex* has 12 PCGs but its mitogenome is more than 2.5 times larger than *C. reinhardtii* due to larger intergenic regions. Despite its larger size, *P. duplex* also produces mRNAs with no 5' upstream sequences and relatively small 3' UTRs suggesting these mRNAs are produced by two endonucleolytic cleavage events that coincide with the removal of tRNAs (Proulex et al. 2021). *E. debaryana* is more closely related to *C. reinhardtii* and has the exact same gene complement, but has a mitogenome about 4000 bases larger due to expanded intergenic regions. This similarity suggests that it also releases its mRNAs with two endonucleolytic cleavages, and presumably more than one endonuclease. Like *C. reinhardtii*, the release of mRNAs from the primary transcript does not coincide with the removal of tRNAs.

Four of the 8 mitochondrial PCGs were polycytidylated (*cob*, *nad1*, *nad4*, and *nad5*), a phenomenon that appears to be conserved among chlorophyte algal mitochondria (Cahoon and Qureshi 2018, Proulex et al. 2021, Salinas-Giegé 2017). The physiological roles of poly(C) in algal mitochondria are still unknown but their presence in other systems provides room for speculation. For instance, tracts of poly(C) can potentially fold into discrete secondary structures (reviewed in Zarudnaya et al. 2019). Also, some mamma-

lian viral mRNAs are polycitydylated and host cells have poly(C)-binding proteins that play roles in stabilization and translation of these mRNAs as well as replication of viral mRNAs (Carocci and Bakkali-Kassimi 2012, Lin et al. 2009, Makeyev and Liebhaber 2002, Serrano et al. 2006). Therefore, it is possible that green algae have mitochondria-localized poly(C) binding proteins that promote translation of mitochondrial mRNAs.

All eight *E. debaryana* mitochondrial mRNAs were observed to be circularized with full-length coding regions. Circular RNAs are very common in the nucleo-cytoplasmic gene expression system where they are typically non-coding fragments that play roles in numerous aspects of gene expression and cell physiology (reviewed in Liu and Chen 2022). However, this is not the case in the mitochondria of the green algae *C. reinhardtii* and *P. duplex* and the streptophytes *Chara vulgaris*, *Zea mays*, *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lcopersicum*, *Cucumis sativus*, and *Vitis vinifera* where mRNAs with full length coding regions are circularized (Cahoon and Qureshi 2018, Liao et al. 2022, Proulex et al. 2021). These circular mRNAs have been shown to be associated with ribosomes in both Chlorophyta and Streptophyta, demonstrating that they are translated. This process appears to be a conserved trait among the Archaeplastida (Cahoon and Qureshi 2018, Liao et al. 2022).

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