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Uncovering evolutionary changes in the mitochondrial genomes of lichenized fungi within the order Arthoniales

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Keywords: Lichen, Symbiosis, Mitochondria, Genomics

Abstract

Lichens are symbiotic organisms that are understudied despite their usefulness to a wide range of disciplines. Studying the genetics of lichen, will contribute to a better understanding of the genetic mechanisms behind symbiosis and fill in grey areas of taxonomic classification of lichens. The lichen order of Arthoniales is even less studied compared to its sister order Lecanorales. This study expands the genomic resources for the former order of lichen and reveals some novel genetic trends found among species within Arthoniales. Some genetic trends have yet to be identified in other lichen species such as the trans-splicing of nad4 among Arthonia species and lack of atp9 in select species in Opegrapha and Chrysothrix.

Trans-splicing of nad4 in Arthonia species is characterized by two exons of nad4 being split by thousands of base pairs. In some instances, the second is also on the opposite strand as the first exon. This was thought to be unique to the mitochondrial genomes of plants (Knoop, 1991) until trans-splicing was also identified in fungi in 2012 (Pelin, 2012). In plants, trans-splicing allows their extremely flexible genomes to rearrange without losing the function of essential genes.

The lack of atp9 in the mitochondrial mycobiont genome has proven to be a significant characteristic among certain species of lichen (Pogoda, 2018). In obligate symbiotic organisms, the exchange of genes between species allows core genomic processes to be streamlined. In the past study of genomic obligate symbiosis of lichen, the loss of atp9 occured in all species of a related genus. Our findings show the loss of atp9 among single species within a genus.

This research fills a gaping hole in the genetic research of lichenized fungi within the order Arthoniales and reveals unique genetic features yet to be seen among lichenized fungi.

Introduction

Lichens are obligate symbioses between a fungal species (mycobiont) and one or more photosynthetic organisms (photobiont), usually a green alga or cyanobacterium. Lichens are
ecologically important, successful in many different terrestrial habitats, inhabit many different niche environments and come in a variety of colors and growth forms (Fryday et al., 2007). The Arthoniales are the second largest order of lichenized fungi after Lecanorales. Despite the large number of extant species within Arthoniales, very little genomic information is available, especially whole organellar genomes. Of the 299 mitochondrial sequences associated with Arthoniales in NCBI’s GenBank, complete annotated mitochondrial resources are available only for Arthonia susa and Opegrapha vulgata. The ten mitochondrial mycobiont genomes we assembled and annotated during this study will comprise the majority of genomic resources available for species within this order. Using these annotations, we can determine variation between species’ genome length, conservation of gene order, and other unique features of these lichen species. With this information, we identify unusual trends and patterns among Arthoniales and their relevance to the orders evolutionary history.

Mitochondria in plant, animal, and fungal cells are the powerhouses that drive energy production needed for all biological processes. Containing protein-coding genes required for the electron transport chain as well as ATP synthase, mitochondria serve an essential role for complex eukaryotic life (Gray et al., 1999). The endosymbiotic theory states that mitochondria were once free-living alpha-proteobacteria, and their presence in eukaryotic cells today is due to an early symbioses event that caused a bacterium to be engulfed, but not digested by a eukaryotic cell (Zimorski, 2014). This view is supported by the similarity of the mitochondrial genome to that of Rickettsia prowazekii, an obligate intracellular parasite that causes typhus (Gray et al., 1999). By studying mitochondrial genomes, the evolutionary history of these species can be elucidated, including genetic changes that are defining characteristics for this order of lichenized fungi.

The mitochondrial genome of most lichenized fungi, contains 15, sometimes 14, protein-coding genes along with the small ribosomal subunit, large ribosomal subunit, and a number of tRNAs. One of the protein-coding genes, atp9, is absent in two of our ten Arthoniales mitochondrial genomes. The absence of atp9 has been seen in a previous paper with the absence being consistent with an entire genus (Pogoda, 2018). The present report may be the first to demonstrate atp9 missing among specific species within a genus. Prior studies show that the
absence of atp9 generally occurs within an entire genus. Of the ten species characterized here, *Opegrapha corticola* and *Chrysothrix susquehannensis* of the order Arthoniales are the only species missing atp9.

In addition, retrotransposons were identified in the genera Opegrapha and Chrysothrix but none in *Arthonia* species. Retrotransposable elements have been useful in distinguishing between large evolutionary lineages (Pogoda, 2019). They are parasitic homing endonucleases which find their way into the genomes introns and intein segments. Previous research hypothesized that lack of gene synteny could be linked to the presence of retrotransposons in the genome (Bennetzen, 2005). However, as mentioned before, only half of the ten Arthoniales species had retrotransposons present but all the species showed a lack of gene synteny.

Lastly, trans-splicing of nad4 in *Arthonia* was found with the exons for nad4 thousands of base pairs apart and occasionally on different strands depending on the species. Trans-splicing has been identified in metazoans, arbuscular mycorrhizal fungi, and a number of plant species but this is the first time trans-splicing has been identified in lichenized fungi. In plants, trans-splicing is thought to play a role in the mediating the impact of gene rearrangement on gene function (Knoop, 1991). Plant mitochondrial genomes are flexible and Arthoniales genomes also seem to be flexible based on gene synteny figures. Further research is needed to confidently say trans-splicing is a result of highly variable gene order.

**Methods**

**Sample Collection**

To study major lineage differences between Arthoniales and Lecanorales, ten species representing three genera were compared to ten previously annotated Lecanorales genomes. All the Arthoniales and Lecanorales species are native to the southern Appalachian Mountain biodiversity hotspot of eastern United States area and were collected in the wild during fieldwork between 2016 and 2018. Collected specimens were deposited in both the herbaria of the New York Botanical Garden (NY) and University of Colorado, Boulder (COLO). Tissue collection efforts were as follows: for macrolichens (lichens with larger structures/fruiting bodies), ca. 1 x 1
cm of tissue was removed, targeting the thallus margins and lobes. For microlichens (difficult to see structures without close examination), tissue was scraped from rock or tree substrates using a sterile razor blade. Tissue samples were then air dried in a laminar flow hood for 24 hours then kept frozen at -20°C until transport to the University of Colorado for subsequent DNA extraction and sequencing.

DNA Extraction and Sequencing

Genomic DNA was extracted from tissue samples of *A. rubella*, *A. ruana*, *A. quintaria*, *A. kermesina*, *A. cupressina*, *O. vulgata*, *O. moroziana*, *O. corticola*, *C. susquehannensis*, and *C. onokoensis* using a Qiagen DNeasy 96 plant kit (Qiagen, Germany) with a modified protocol by Cloe Pogoda and Kyle Keepers. This modified protocol included a 10 minute 65°C incubation step during the lysis phase. A 100% ethanol wash was included before finally drying the membrane before elution. Genomic libraries were prepared using Nextera® XT DNA library prep kits (Illumina®, California) and each sample was barcoded by the adapters Nextera® i5 and i7.

Samples that passed QC were processed for paired end 150 base pair reads on the Illumina NextSeq® sequencer at the University of Colorado’s BioFrontiers Institute Next-Generation Sequencing Facility in Boulder, Colorado.

Assembly and Annotation

The mitochondrial genomes for each species were assembled de novo using SPAdes v3.9 (Saint Petersburg State University, Russia). To identify the mitochondrial contigs, a BLAST search was conducted of the assemblies against known mycobiont mitochondrial protein coding genes. The contigs containing mitochondrial genes were then web BLASTed to identify if the contig belonged to the mycobiont or photobiont partner. Once enough contigs were identified to contain all the protein coding genes for a species, they were tiled together by referencing the reverse complement for each contig and the trimmed fastq files to find overlapping sequences and ultimately form a full circularized sequence. Once circularized, the mycobiont mitochondrial sequences were BLASTed against previously annotated sequences to determine the proper orientation and to standardize the sequences to start at *cox1*. Standardized sequences were error
corrected utilizing the SAMTOOLS suite to determine SNPs between the trimmed fastq files and the circularized mitochondrial sequence. Add in info about orientation, circularization and error correction (tview).

Identification of genomic content

Gene features of each assembly were annotated using DOGMA, NCBI BLAST (National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA), CHLOROBOX, and Sequin v15.1. DOGMA was used to approximately assign the gene order for each species. We modified the DOGMA parameters to mitochondrial genome type, gapped alignment, mold mitochondrial for genetic code for Blastx, and percent identity cutoff for both proteins and rRNA was set to 40. After using DOGMA to get a general idea of the gene order, the mitochondrial genomes were circularized and oriented to cytochrome c oxidase subunit I (cox1). By circularizing and orienting to cox1, all the species analyzed were standardized for further comparison.

For NCBI BLAST, either BLASTn or BLASTx were used depending on whether nucleotide FASTA sequences or the protein FASTA sequences were available. To locate a specific gene feature, the function “align two or more sequences” was used with the Genetic Code option “Mold Mitochondrial;...4”.

Parameters were modified for use of Chlorobox GeSeq. For our circular, mitochondrial genomes, BLAST searches were set to an identity of 40, and tRNAscan-SE v2.0 output was included in the analysis. Parameters were set to include organellar tRNAs, a genetic code for Mold/Protozoan/Coelenterate Mitochondrial, with a cut-off score of 40. Reference species used included Peltigera dolichorhiza, Peltigera malacea, Imshaugia aleurites, and Usnea ceratina from the order, Lecanorales.

Identification of “missing” atp9
For *Opegrapha corticola* and *Chrysothrix susquehannensis*, a command line tBLASTn was performed to locate matches of *atp9* within each species meta-assembly file. Contig matches were either associated with the mycobionts nuclear genome or a photobiont partner’s mitochondrion. Benchmarking Universal Single-Copy Orthologs (BUSCO) for fungi were utilized to determine which contigs were associated with the mycobionts nuclear genome. An online BLASTn confirmed each contigs similarity to algal species, allowing us to assume the contig that matched belonged to a photobiont partner.

**Homing Endonucleases**

Parasitic elements such as LAGLIDAGs and GIY-YIGs were identified using ORFfinder and NCBI SMART BLAST. After all of the features were annotated in Sequin, the mitogenome was submitted to NCBI GenBank and we were given temporary accession numbers which can be found near the conclusion of this paper.

**Phylogenetic analyses**

The phylogeny was created using ribosomal DNA sequences from our assemblies as well as large ribosomal subunit sequences from 17 other species within Arthoniales available on genbank (Ertz et al., 2009). Gene alignments created very discordant trees, while the highly conserved ribosomal sequences produced highly supported phylogenetic relationships. Sequences were aligned in MEGA7 utilizing the MUSCLE algorithm and curated in PhyDE (Kumar et al., 2015; Edgar, 2004). The tree was created utilizing the Maximum-likelihood method with 500 bootstraps and rooted with *Cladonia grayi* and *Cladonia uncialis*.

**Results**

All but two of the mitogenomes contained 15 protein-coding genes and genes for two subunit rRNAs: three cytochrome c oxidase subunits (*cox1-3*), seven subunits of NAD dehydrogenase (*nad1-6, nad4L*), three ATP synthases (*atp6,8,9*), one ribosomal protein (*rps3*), one cytochrome oxidase b (*cob*), and the large and small rRNA subunits (*LSU* and *SSU*). The
other two species, *Opegrapha corticola* and *Chrysothrix susquehannensis*, contained all of these features with the exception of ATP synthase subunit 9 (*atp9*). [See Figure 2]

The absence of *atp9* in the mitogenomes of *Opegrapha corticola* and *Chrysothrix susquehannensis* was associated with evidence of its presence elsewhere. A tBLASTn revealed matches for *atp9* in each of the mycobiont’s nuclear genome as well as within the mitogenome of their photobiont partners. For *O. corticola*, the contig associated with the mycobionts nuclear genome matched *atp9* with an E-value of 1e-08, and a contig with a similarity to algae matched with an E-value of 1e-04. Although the BLAST results only identified parts of *atp9*, the low E-value offers significant evidence that a copy of *atp9* may be present in either the mycobionts nuclear genome or the mitochondrial genome of its algal counterpart. For *C. susquehannensis*, the contig associated with the mycobionts nuclear genome matched *atp9* with an E-value of 2e-16, and a contig with a similarity to algae matched with an E-value of 3e-06.

Each annotated species within *Arthonia* was observed to contain a trans-spliced *nad4* gene. This gene was found in two exons, the location of which differed among species. In addition, there was no conserved pattern of which strand an exon was found on. Across the five *Arthonia* species, each exon was found on the plus and minus strands, and of the four combinations possible (plus/plus, minus/minus, plus/minus, minus/plus) all were observed except for both exons on the minus strand. The exons for *Arthonia* were separated by thousands of base pairs or found on different strands. This trans-splicing of *nad4* is present in all *Arthonia* species even through translocation. Trans-splicing had not been observed in any other lichenized fungi but trans-splicing has been observed in plant mitochondrial genomes, and associated with group II introns (Bonen, 2008).

A triple cotranscription was observed in *Arthonia kermesina* between *cob-cox1-cox2*, in that order (Table 1). Cotranscription is common within mitochondrial genomes, and has been frequently observed especially in plants (Gualberto et al., 1988; Hoffman et al., 1999; Itani et al., 1998). While cotranscription of *cob* and *cox2* is a common characterization of species within Lecornorales, this is the first evidence, to our knowledge, of a triple cotranscription event recorded for the mitochondrion of a lichenized fungus. Evolutionarily, cotranscription is
economically beneficial to an organism as it requires less energy to transcribe three protein-coding genes at once rather than having intergenic regions where errors could occur (Sneppen, 2010).

Arthonia is distinguished by a lack of introns and retrotransposable elements, although many are identified in Opegrapha and Chrysothrix. Of the species annotated within Arthonia, only *A. quintaria* contained a significant number of introns and retrotransposable elements compared to the other four which contained almost all of their protein coding genes as a single exon (with the exception of *nad4*). *A. quintaria* contained at least one intron in *cob, cox1, cox3, nad5,* and *rps3* while *A. kermesina, A. cupressina, A. rubella,* and *A. ruana* only contained an intron in *rps3.*

**Discussion**

Comparison of the genomes of the ten Arthoniales species to the genomes of previously annotated Lecanorales species demonstrated the order of their genes to be highly variable.

**Phylogenetic Analysis**

Previous studies produced similar trees to the one developed with the data obtained for this project. Opegrapha was shown to be paraphyletic and Arthonia monophyletic (Ertz et al., 2009; Nelsen et al., 2009). In addition, placement of *Arthonia cupressina* with *Chrysothrix xanthina* is supported by a high bootstrap value (81), as well as its close relationship to *Arthonia caesia* that was previously shown to group with *C. xanthina* (Nelsen et al., 2009). This finding presents evidence that *A. cupressina* may truly belong to the genus Chrysothrix, and further research may uncover other species of lichen that need to be reassigned. Development of phylogenetic trees based on the morphology of lichen using analysis of structural components, mode of reproduction, etc have limitations, but genomic resources are helpful in clarifying the grey areas of lichen phylogenetics (Nelsen et al., 2009).

**Evolutionary History in relation to gene synteny**
A number of hypotheses have been considered by lichenologists when trying to explain the observed difference between Lecanorales and Arthoniales. The most promising hypothesis considers the evolutionary history of the order, Arthoniales. Since within the Ascomycota tree, Arthoniales diverged much earlier than Lecanorales, it is inferred that there is a large number of extinct individuals in the Arthoniales evolutionary history (Grube, 1998; Ertz et al., 2009). As a number of species became extinct, gaps began to emerge in the phylogenetic tree for the Arthoniales. The extant Arthoniales species present today represent a very small percentage of the entire Arthoniales tree. Therefore, it is likely that the ten species we annotated are more divergent from one another than originally thought. This could explain why gene order is so variable from species to species among Arthonia, Chrysothrix, and Opegrapha.

**Gene synteny in relation to retrotransposable elements.**

Another hypothesis attempts to link inconsistent gene order to the presence of retrotransposable elements (Bennetzen et al., 2005; Beaudet et al., 2013). In fungi, high variability in the mitochondrial genome has been observed even within members of the same phylum (Aguileta et al., 2014). Changes within the mitochondrial genome have been associated with DNA polymerases and selfish retrotransposable elements such as homing endonucleases (Kanzi et al., 2016). These elements can cause shifts in gene order and changes in genome size through the movement of genetic elements (Beaudet et al., 2013; Bennetzen, 2005; Nadimi et al., 2015).

In the case of lichen species, a few common retrotransposable elements found are LAGLIDADG’s and GIY-YIG’s. Activation of these retrotransposons have been associated with environmental stressors and have been linked to variability in genome size (Grandbastien, 1998; Joardar et al., 2012). While this link may be true among Lecanorales, there are almost no retrotransposons in Arthonia species that exhibit clear gene disorder. However, these retrotransposons can be found in most Chrysothrix and Opegrapha species, which could potentially explain their gene disorder. The causes of gene reorganization within Arthonia are unclear. Introns and retrotransposable are widely discussed (Grandbastien, 1998; Joardar et al.,
2012; Aguileta et al., 2014; Bennetzen et al., 2005; Beaudet et al., 2013) as major contributors to rearrangement; a lack thereof may indicate another force driving divergence within *Arthonia*.

**Absence of atp9**

The absence of atp9 in the mycobiont mitochondrial genome is not a new finding and has been identified in a number of genera in the order Lecanorales (Pogoda et al., 2018). The latter authors explain how relocation of atp9 to the photobiont’s mitochondrial genome suggests that obligate symbiosis involves consolidating genome space among asexually reproducing lichens. Although energy efficiency and consolidation are possible factors, another important factor could be the symbiotic relationship itself. To better coordinate and regulate the symbiotic relationship, the partitioning of gene copies is a common attribute of symbiotic species (Khachane et al., 2007; Tsaousis et al., 2008; Corradi et al., 2010; Baumgarten et al., 2015). In lichens however, the partitioning of specific gene copies to the photobiont is completely dependent on the mode of sexual reproduction.

The life cycle of a sexually reproducing lichen exemplifies why the loss of atp9 is so unusual. Generally, a fungal spore is released from a mature lichen, but this spore does not include the photosynthetic symbiont. For the first stage of development, a sexually reproducing lichen thus develops as a fungus without it’s photosynthetic partner (Honegger, 1998). The young lichen requires ATP synthase in order to create energy but, without ATP synthase subunit 9 in the mycobiont mitochondrial genome, it is not clear how this species would develop independent from the photobiont partner.

Asexually reproducing lichen are more likely to partition specific genes from the mycobiont to the photobiont because they are never separated (Pogoda et al., 2018). Many asexually reproducing lichen multiply as propagules containing both the photosynthetic partner and the mycobiont. This allows the mycobiont to allocate a copy of atp9 to the photobiont because they are associated with the photobiont throughout their life history.

*Chrysothrix susquehannensis* and *Opegrapha corticola* are unique in having a portion of atp9 residing in the mycobiont nuclear genome and a copy present in the photobiont
mitochondrial genome. Further analysis is needed to confirm whether the “portion” of atp9 is a functional piece of the protein-coding gene or whether it is a remnant copy. The transfer of atp9 from the mitochondria to the nucleus has been revealed to impact the functionality of atp9 but serves a regulatory function rather than being lethal (Dequard-Chablat et al., 2011; Sellem et al., 2016; Bietenhader et al., 2012).

While the members from the genus *Usnea*, which belongs to the order Lecanorales, were missing *atp9*, only one species in the genera *Chrysothrix* and *Opegrapha* were missing *atp9* and each had a copy of *atp9* in its nuclear genome for both species. This finding suggests that a functional *atp9*-subunit may be produced within the nucleus and imported into the mitochondria (Bietenhader et al., 2012), which may reflect an evolutionary reduction within the mitochondrial genomes of these species of lichen through gene transfer to the nucleus. The majority of mitochondrial genes are encoded within the nuclear genomes, with the exceptions of cytochrome b, *cox1*, and most notably, *atp9* (Bietenhader et al., 2012). When *atp9* is transferred to the nucleus however, a heat shock response is activated (Bietenhader et al., 2012). Further research is necessary to examine whether this heat shock response might be beneficial to some lichens in specific regions of the world. Perhaps there may be an underlying ecological pressure for the transfer of *atp9* from the mitochondria to the nucleus in specific species.

This finding suggests that *C. susquehannensis* and *O. corticola* are evolving to further reduce their mitochondrial genomes through transfer of *atp9* into their nuclear genomes. Previous studies demonstrated loss of *atp9* was isolated to Lecanorales, Teloschistales and Ostropales (Pogoda 2018). The loss of *atp9* in *Chrysothrix susquehannensis* and *Opegrapha corticola* extends this feature to members of the order Arthoniales.

**Conclusions**

Lichens of the order Arthoniales are widespread and constitute a large portion of known lichenized fungi. The data presented provide genetic information on this order for which little such information had been available. Unique differences in the mitochondrial genomes of Arthoniales and Lecornorales raise new questions about the evolutionary history of these lichens. What drives the grand gene disorder in *Arthonia*? Does the transfer of *atp9* in *O. corticola* and *C.*
susquehannensis from the mycobiont mitochondrial genome to the mycobiont nucleus or photobiont mitochondria serve an ecological function? Arthoniales do appear to have a much different evolutionary history from previously studied orders of lichenized fungi. Future research should address additional methods of genome rearrangement, ecological impacts on lichen genomic trends, as well as processes acting towards obligate symbiosis.

Data Accessibility

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Chrysothrix and Opegrapha species assembled and annotated by Dustin Bailey. Arthonia species assembled and annotated by Arif Nadiadi

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Figures and Tables

Figure 1: Arthonia kermesina aka “Hot dots” (A), Arthonia cupressina (B), Arthonia quintaria (C), Arthonia ruana (D), Arthonia rubella (E), Chrysothrix onokoensis (F), Chrysothrix susquehannensis (G), Opegrapha corticola (H), Opegrapha vulgata (I). All pictures taken by Erin A. Tripp and James C. Lendemer.
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Table 1: Gene order of 15 protein-coding sequences oriented to cox1 across species annotated in this study as well as six species from the order Lecanorales. Within the order Arthoniales, high levels of genomic rearrangement is observed. However, certain gene orders were conserved such as nad2-nad3, nad5-nad4L, cox1-cox2, and cox2-cob. In contrast, the genera Cladonia and Usnea (belonging to the order Lecanorales) both show very conserved gene order across all species annotated (Pogoda et al., 2018). For trans-splicing in the case of Arthonia, only the first exon of nad4 is indicated. Created by Dustin Bailey and Arif Nadiadi.
Figure 3: This cladogram shows an estimation of the relationships between 33 species of lichen including the ten species annotated in this study. Utilizing rDNA from each species, this cladogram was created using the Maximum-likelihood method with 500 bootstraps and rooted with Cladonia grayi and Cladonia uncialis. Further
programs are being tested to produce a better phylogenetic representation of Arthoniales. Figure produced by Arif Nadiadi.

Figure 4: Mauve alignment of all ten species produced by Arif Nadiadi and Dustin Bailey