

Plastid Genome Degradation in the Endangered, Mycoheterotrophic, North American Orchid *Hexalectris warnockii*

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Abstract

Heterotrophic plants provide evolutionarily independent, natural experiments in the genomic consequences of radically altered nutritional regimes. Here, we have sequenced and annotated the plastid genome of the endangered mycoheterotrophic orchid *Hexalectris warnockii*. This orchid bears a plastid genome that is ~80% the total length of the leafy, photosynthetic *Phalaenopsis*, and contains just over half the number of putatively functional genes of the latter. The plastid genome of *H. warnockii* bears pseudogenes and has experienced losses of genes encoding proteins directly (e.g., *psa/psb*, *rbcl*) and indirectly involved in photosynthesis (*atp* genes), suggesting it has progressed beyond the initial stages of plastome degradation, based on previous models of plastid genome evolution. Several dispersed and tandem repeats were detected, that are potentially useful as conservation genetic markers. In addition, a 29-kb inversion and a significant contraction of the inverted repeat boundaries are observed in this plastome. The *Hexalectris warnockii* plastid genome adds to a growing body of data useful in refining evolutionary models in parasites, and provides a resource for conservation studies in these endangered orchids.

Key words: orchid, heterotroph, plastome, pseudogene, evolution, chloroplast.

Introduction

Plants that parasitize other plants or mycorrhizal fungi provide unique opportunities to study the genomic consequences of radically altered nutritional lifestyles and associated changes in selective regimes (Wolfe et al. 1992; Barrett et al. 2014; Wicke et al. 2016). In particular, plants that have become obligate parasites upon fungi for nutritional needs represent case studies of convergent evolution. Transitions to this lifestyle have occurred an estimated minimum of 30× in the orchid family alone, mostly due to their complete, parasitic dependence upon mycorrhizal fungi early in development, called “initial mycoheterotrophy” (Freudenstein and Barrett 2010; Merckx and Freudenstein 2010). Furthermore, many of these plants are rare or endangered (Freudenstein 1999; Merckx et al. 2013), and in many cases represent “ecological indicators” of undisturbed habitat, or may serve as “umbrella species” for conservation efforts (Taylor et al. 2013).

What happens to the genomes of organisms that have undergone such drastic changes in nutritional mode, from autotrophy to heterotrophy? Representative plastid genomes have been sequenced from plant lineages containing heterotrophs, allowing researchers to construct models of plastid genome degradation, including pseudogene formation (functional losses), physical gene losses, and increased substitution rates as a result of relaxed selective pressures on photosynthetic function (Wicke et al. 2011, 2016; Barrett and Davis 2012; Barrett et al. 2014; Graham et al. 2017). However, sampling gaps exist in these models, underscoring the need for more thorough representation of plant lineages containing nonphotosynthetic members, each representing an independent trajectory of plastome degradation.

One such lineage is the North American orchid genus *Hexalectris* Raf. Members of this genus are hypothesized to obtain most or all nutrients, including carbon, from their symbiotic mycorrhizal fungi (Taylor et al. 2003; Kennedy et al.

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2011), a situation called mycoheterotrophy. *Hexalectris* contains ten currently recognized species, many of which are rare and restricted to highly specific habitats (Catling and Engel 1993; Catling 2004; Kennedy and Watson 2010). *Hexalectris warnockii* Ames and Correll, or the Texas purple-spike, is an endangered member of the genus restricted to Texas, Arizona, and Mexico, where it grows in shaded oak-juniper-pinyon canyons near seasonally dry creek beds, or on calcareous soils under juniper scrub (IUCN Red List: Endangered D; Goedeke et al. 2015). It is known from ~24 sites in United States, including: Big Bend National Park, northeastern Texas (Dallas area), the Edwards Plateau, and Arizona; in Mexico it is found at a site in Coahuila and another at the southern tip of Baja California Sur (Catling 2004).

Here, we have sequenced, assembled, and annotated the plastid genome of *Hexalectris warnockii*. The goals of this study are: 1) to use genomic criteria—that is, extensive loss of photosynthesis-related genes—to determine if *H. warnockii* is nonphotosynthetic (fully mycoheterotrophic) or retains photosynthetic capability (partially mycoheterotrophic); 2) to compare the plastid genome of *H. warnockii* to those from members of other heterotrophic plant lineages; and 3) to provide a genomic resource for the development of plastid markers to facilitate studies of genetic diversity in populations of this endangered species.

Materials and Methods

Floral tissue of *H. warnockii* was collected from Brewster County, TX. A voucher specimen was deposited at The Miami University Willard Sherman Turrell Herbarium (Accession: Kennedy and Freeman #33). We extracted DNA using a CTAB protocol (Doyle and Doyle 1987), yielding 17.4 ng/ μ l based on a Qubit Fluorometer reading (ThermoFisher Scientific, Waltham, MA). Illumina libraries were prepared by shearing total genomic DNA to 350–400 bp fragments on a Covaris E220 ultrasonicator (Covaris, Woburn, MA), followed by the protocol of Glenn et al. (2016). Library concentrations and fragment sizes were calculated on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), pooled with 19 other libraries, and sequenced on two lanes of an Illumina HiSeq2000 for paired-end, 100-bp reads.

We carried out adapter removal and quality trimming with Trimmomatic v.0.36 (Bolger et al. 2014), using a 3-bp sliding window and a minimum PHRED score of 20 (1:100 error rate). The plastome was assembled from cleaned reads using NOVOPlasty v.2.6.3 (Dierckxsens et al. 2017), which uses a reference sequence as an initial seed (here, *rbcl* from the leafy, photosynthetic orchid *Phalaenopsis equestris*, GenBank# JF719062) and builds a circularized plastome. Reads were mapped with high stringency to the draft plastome produced by NOVOPlasty in Geneious v.8.1 to check for assembly errors (<http://www.geneious.com>, last accessed May 1, 2018; Kearse et al. 2012; 98% similarity, allowing

gaps up to 100 bp). The plastome was annotated initially in DOGMA (Wyman et al. 2004). Start/stop codons, exon/intron boundaries, inverted repeat (IR) boundaries, and putative loss-of-function pseudogenes were verified and adjusted by aligning the plastome to protein coding and RNA genes from *P. equestris* (GenBank accession JF719062), *Phoenix dactylifera* (Arecaceae, GU811709), and *Heliconia collinsiana* (Heliconiaceae, JX08866), as was done in Barrett et al. (2014).

The annotated *H. warnockii* plastome was aligned with that of *P. equestris* using the progressiveMAUVE (Darling et al. 2010) plugin for Geneious v. 8.1, which identifies syntenic regions between two or more genomes, thus allowing detection of genomic rearrangements. Putatively functional genes (with open reading frames or lacking drastic modifications in the case of RNA genes), pseudogenes (putative functional losses, i.e., those with interrupted reading frames or nontriplet insertions or deletions), and physical gene losses were recorded and compared with the plastome of the leafy, photosynthetic *P. equestris*. We also compared plastome size and functional gene content for a number of full mycoheterotrophs, partial mycoheterotrophs, holoparasites, hemiparasites, and other leafy, autotrophic species.

Genomic repeat type and abundance were calculated in REPuter (Kurtz et al. 2001), specifying a minimum length of 20 bp (for forward, reverse, palindromic, and reverse-complementary repeats), a Hamming distance of 3, and a maximum e-value of 1.0×10^{-3} . Tandem repeats were identified using the Phobos plugin for Geneious (Mayer 2010), specifying 2–50 bp motif length, a minimum total length of 10 bp, and allowing only perfect repeats. All results were plotted in R (R Core Development Team 2013) or PAST v.3.8 (Hammer et al. 2001). A linearized plastome map was created in OGDRAW (Lohse et al. 2013).

Results and Discussion

Illumina paired-end sequencing of *H. warnockii* yielded a total of 38,633,900 reads (after trimming), with an average insert size of 350 bp. Coverage depth of the finished plastome was 712.2 \times , representing 2.19% of the total read pool. The 119,057 bp plastome has a quadripartite structure as is typical for angiosperms (fig. 1), with a Large Single Copy region (LSC; 66,903 bp), Small Single Copy region (SSC; 17,490), and an Inverted Repeat (IR; 17,332) (table 1 and fig. 1). The *H. warnockii* plastome is thus 29,902 bp smaller than the leafy orchid *P. equestris*, or ~79.9% the total size of the latter (148,959 bp, representing a typical orchid plastome size). The largest physical reduction in the *H. warnockii* plastome was in the LSC region, which was 27.6% smaller than that of *P. equestris* due to several large deletions. There is a contraction of the inverted repeat (IR) in *H. warnockii*, representing a 33% difference in total IR length relative to *P. equestris*. This contraction resulted in the following genes, typically found in the IR, becoming part of the SSC: 16S rRNA, *trnI*^{GAU}, *trnA*^{UGC},

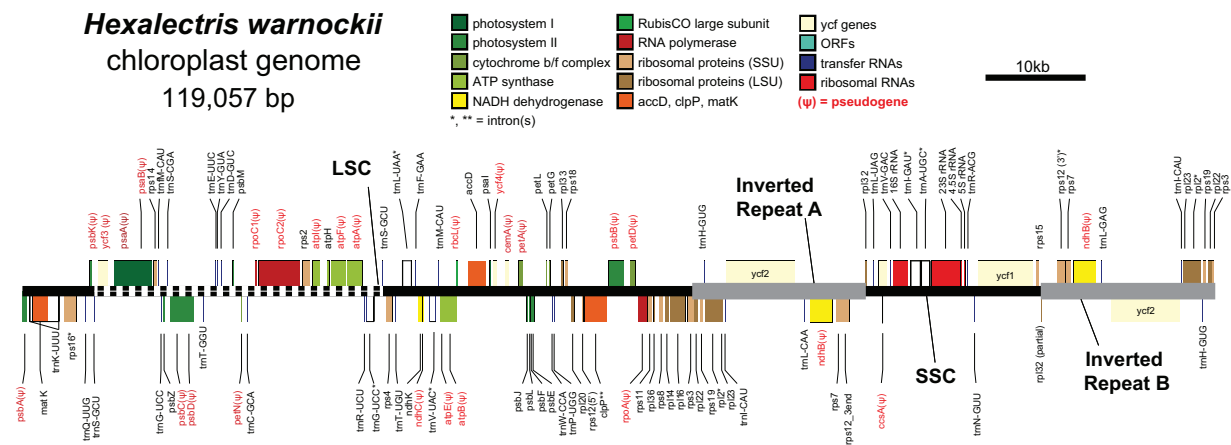


Fig. 1.—Map of the plastid genome of *Hexalectris warnockii*. LSC, large single copy region; SSC, small single copy region; gray, inverted repeat; dashed line in LSC = a 29-kb inversion. Red text indicates the presence of a pseudogene (ψ). “*” and “**” denote genes with one or two introns, respectively.

Table 1

Features of the Fully Mycoheterotrophic *Hexalectris warnockii* Plastid Genome Relative to That of the Leafy, Autotrophic *Phalaenopsis equestris* (GenBank accession JF719062)

	<i>Hexalectris warnockii</i>	<i>Phalaenopsis equestris</i>	% of <i>Phalaenopsis</i>
Total length (bp)	119,057	148,959	79.9
Large single copy (LSC)	66,903	85,967	77.8
Inverted repeat (IR)	17,332	25,846	67.1
Small single copy (SSC)	17,490	11,300	154.8
protein coding genes (CDS)	38	69	55.1
Pseudogenes (ψ)	25	3	833.3
Transfer RNA genes (tRNA)	30	30	93.3
Ribosomal RNA genes (rRNA)	4	4	100.0
Putatively functional	72	103	69.9
Total genes and pseudogenes	97	106	91.5

23S rRNA, 4.5S rRNA, 5S rRNA, *trnR*^{ACG}, *trnN*^{GUU}, and the 5' portion of *ycf1*. Total GC content is 36.9% after removing one copy of the IR, and similar to that of *Phalaenopsis* at 36.7%.

We identified 45 dispersed repeats across the genome passing our filters in REPuter: two were forward-compliment, 16 forward-forward, 22 palindromic, and five forward-reverse (table 2). We identified 419 tandem repeats with minimum motif lengths of 10 bp (table 2 and supplementary table S1, Supplementary Material online). The most abundant of these were hexanucleotide repeats (141) followed by pentanucleotide repeats (99). We identified three dinucleotide repeats, 17 trinucleotide repeats, and 50 tetranucleotide repeats. Thus, there are several options for the development of potentially variable satellite markers in *H. warnockii*, which will be useful in determining patterns of plastid genomic diversity across populations of this endangered orchid. Alignment with MAUVE detected a major genomic inversion of a ~29-kb region of the LSC relative to *P. equestris* with breakpoints spanning *trnS*^{GCU} and *trnS*^{GGA}; the entire collinear block detected by MAUVE contains 29 genes (fig. 1).

Table 2

Numbers of Dispersed and Tandem Repeats Detected with REPuter and Phobos, Respectively

Dispersed Repeats	
Repeat Type	Number
Forward-compliment	2
Forward-forward	16
Palindromic	22
Forward-reverse	5
Tandem repeats	
Motif Length (bp)	Number
2	3
3	17
4	50
5	99
6	141
7	50
8	19
9	15
>9	25

NOTE.—Tandem repeat sequences are listed in supplementary table S1, Supplementary Material online.

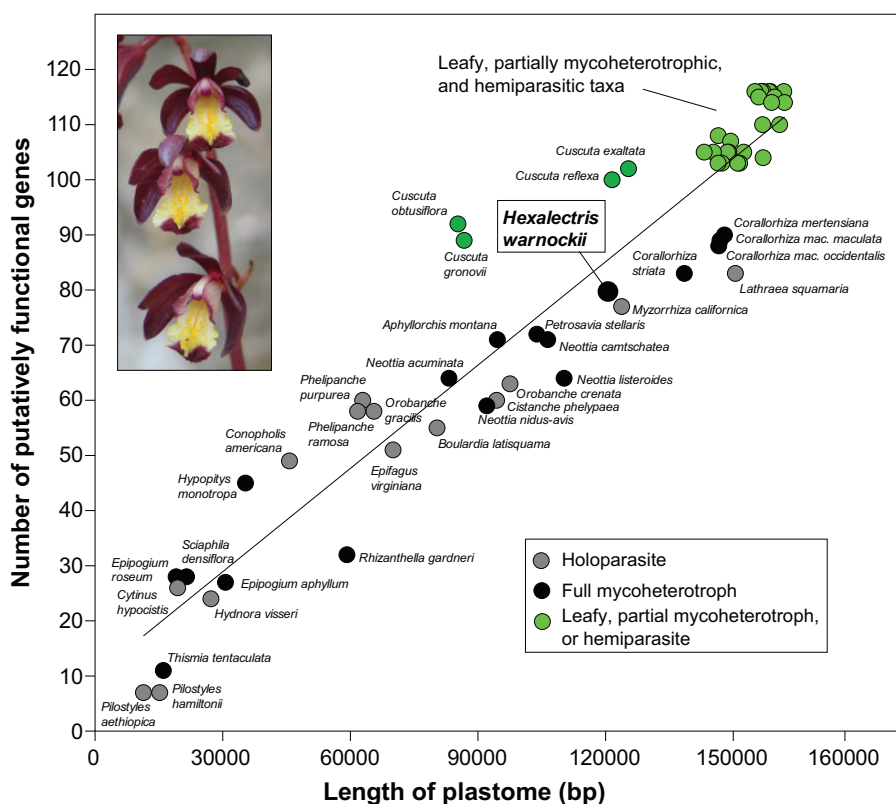


FIG. 2.—A comparison of plastid genome size and the number of putatively functional genes for sequenced holoparasites (gray), full mycoheterotrophs (black), and selected autotrophs (including partial mycoheterotrophs and hemiparasites; green), with trophic status based on whether or not photosynthetic genes are putatively functional in each. Inset: *Hexalectris warnockii* in flower (photo: A. Kennedy; Big Bend National Park, Brewster Co., TX).

The plastome of *H. warnockii* encodes 72 putatively functional genes (protein-coding, tRNA, and rRNA), compared with 103 in *P. equestris*, a 31.1% difference in functional gene content, composed of pseudogenes (i.e., functional losses, 25 in *H. warnockii* relative to *P. equestris*) and physical gene losses (11 in *H. warnockii* relative to *P. equestris*). The total plastome size reduction in *H. warnockii* is largely due to the deletion of regions containing photosynthesis-related genes (fig. 1), thus also reducing the gene count. Plastome size in *H. warnockii* is comparable to that of the mycoheterotrophic orchid *Corallorhiza striata* var. *vreelandii* at 137,505 bp (Barrett and Davis 2012), and to the holoparasite *Myzorhiza californica* at 120,840 bp (Wicke et al. 2013; see fig. 2). Overall there is a strong positive correlation between the number of putatively functional genes and plastome length among heterotrophic angiosperms (fig. 2; Pearson correlation $r = 0.953$, $P < 0.0001$); thus, physical gene loss is at least in part driving a reduction in plastome size.

Genes that are either functionally or physically lost conform to the models of Barrett and Davis (2012), Wicke et al. (2016) and Graham et al. (2017), and include: photosynthesis-related genes [Photosystem I and I subunits (*psa*, *psb*), Cytochrome subunits (*pet*), RuBisCO Large Subunit (*rbcl*), Photosystem Assembly Factors (*ycf3*, *ycf4*, also called *paf1* and *paf2*,

respectively; Wicke et al. 2011); subunits of the plastid-encoded RNA Polymerase (*rpo*); and subunits of the ATP synthase complex (*atp*)]. There are also substantial functional and physical losses among subunits of the NAD(P)H Dehydrogenase complex (*ndh*; all physically lost except *ndhK*, *ψndhB*, and *ψndhC*), but this is common in other orchids including *Phalaenopsis*, perhaps due to the tendency of orchids to occupy low-light environments (Lin et al. 2017).

Losses in subunits of these functional gene categories conform to “stage 4” of the model of plastome degradation by Barrett and Davis (2012), and are also in line with a recent mechanistic model of plastome evolution (Wicke et al. 2016). Functional loss of five out of six ATP Synthase subunit genes is significant, in that many parasitic lineages early in the process of plastome degradation tend to have preserved reading frames for *atp* genes despite having experienced major losses in photosynthesis-related and *rpo* genes (Barrett et al. 2014; Wicke et al. 2016; Braukmann et al. 2017; Graham et al. 2017). Thus, *H. warnockii* may have entered a new phase in plastome evolution following a period of evolutionary stasis, based on the “punctuated burst” model of plastome evolution put forth by Naumann et al. (2016).

The IR is hypothesized to function in plastid genome structural stability, but studies from highly rearranged genomes are

equivocal (Palmer 1985; Lam et al. 2015; Lim et al. 2016). Here, a 29-kb LSC inversion is found in conjunction with a drastic reduction of the IR (fig. 1). Repeats have been shown in parasitic Orobanchaceae to be associated with plastome structural rearrangements and shifts in IR boundaries (Wicke et al. 2013); thus additional sampling of *Hexalectris* spp. and related genera will allow for explicit tests among repeat content, structural rearrangements, and substitution rates.

The ancestor of *Hexalectris* may have been evolving under relaxed selective pressure for up to 32 Myr, based on a stem-node age estimate of *Hexalectris*, which also includes members of the closely related genera *Basiphyllaea* and *Bletia* (Sosa et al. 2016). *Hexalectris warnockii* is consistently placed as sister to the remaining members of genus *Hexalectris* in previous studies (Kennedy and Watson 2010; Sosa et al. 2016); thus it is unknown whether this species has undergone an independent transition to full mycoheterotrophy, or if this condition is shared by all species in the genus. Regardless, plastome degradation has been occurring in *H. warnockii* for an estimated 24 Myr, when the first divergence occurred within *Hexalectris* (Sosa et al. 2016). Sequencing of additional members of *Hexalectris*, and the closely related members of tribe Blettiinae (*Basiphyllaea*, *Bletia*) will allow fine-scale reconstruction of plastid genome degradation, and testing of the hypothesis of a single origin of full mycoheterotrophy/loss of photosynthesis in *Hexalectris*. Furthermore, sampling of multiple individuals per species may uncover substantial variation in plastomes across the geographic range of each species, as has been recently demonstrated in the fully mycoheterotrophic orchid *Corallorhiza striata* (Barrett et al. 2018).

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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