

The Plastomes of Two Species in the Endoparasite Genus *Pilostyles* (Apodanthaceae) Each Retain Just Five or Six Possibly Functional Genes

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Abstract

The 23 species of mycoheterotrophic or exoparasitic land plants (from 15 genera and 6 families) studied so far all retain a minimal set of 17 of the normally 116 plastome genes. Only *Rafflesia lagascae*, an endoparasite concealed in its host except when flowering, has been reported as perhaps lacking a plastome, although it still possesses plastid-like compartments. We analyzed two other endoparasites, the African Apodanthaceae *Pilostyles aethiopica* and the Australian *Pilostyles hamiltonii*, both living inside Fabaceae. Illumina and 454 data and Sanger resequencing yielded circularized plastomes of 11,348 and 15,167 bp length, with both species containing five possibly functional genes (*accD*, *rps3*, *rps4*, *rnr16*, *rnr23*) and two/three pseudogenes (*rpoC2* in *P. aethiopica* and *rpl2* and *rps12* in both species; *rps12* may be functional in *P. hamiltonii*). Previously known smallest land plant plastomes contain 27–29 genes, making these Apodanthaceae plastomes the most reduced in size and gene content. A similar extent of divergence might have caused the plastome of *Rafflesia* to escape detection. The higher plastome degeneration in both these families of endoparasites, Rafflesiaceae and Apodanthaceae, of similar high age, compared with exoparasites points to a difference of plastome function between those two modes of parasitic life.

Key words: endoparasite, chloroplast genome, photosynthesis, gene loss, minimal plastome.

INTRODUCTION

The loss of photosynthesis has occurred in parasitic lineages ranging from algae to angiosperms, but is not necessarily accompanied by a loss of plastid genomes (reviewed in Krause 2012). Which plastid genes may persist in nonphotosynthetic land plants has been studied in 24 parasitic or mycoheterotrophic *Cuscuta*, Orobanchaceae, Rafflesiaceae, Aneuraceae, Orchidaceae, Petrosaviaceae, and Triuridaceae (Wolfe et al. 1992; Funk et al. 2007; McNeal et al. 2007; Wickett et al. 2008; Delannoy et al. 2011; Logacheva et al. 2011; Barrett and Davis 2012; Wicke et al. 2013, Barrett et al. 2014; Logacheva et al. 2014; Molina et al. 2014; Lam et al. 2015; Schelkunov et al. 2015; table 1). Comparison of these 24 plastomes (table 1) shows that 23 of them still contain the same 17 genes (ten ribosomal proteins, four ribosomal RNAs, and three transfer RNAs), either because of global or lineage-dependant selective pressure or by inertia. The

exception is *R. lagascae* in which no putative plastid sequences have intact reading frames (Molina et al. 2014; and see Smith and Lee 2014 for a possible loss of the plastome in the algae *Polytomella*). This species is the only endoparasitic land plant investigated so far and seems to completely lack a functional or pseudogenized plastome (Molina et al. 2014). All the other parasitic land plants analyzed are exoparasites, meaning they connect to the host via haustoria from the outside or via fungal hyphae. Endoparasites, such as *Rafflesia*, instead live permanently inside the host as a network of parenchyma cells (Heide-Jorgensen 2008; Molina et al. 2014). Endoparasitism has evolved four times in land plants, namely in Rafflesiaceae (34 species in three genera), Cytinaceae (~10 species in two genera), Mitrastemonaceae (one or two species in one genus), and Apodanthaceae (10 species in two genera, *Apodanthes* and *Pilostyles*). The stem lineage of Rafflesiaceae is about 95 Myr old (Bendiksby et al. 2010), those of Cytinaceae 72 Myr,

Table 1

Plastome Content of Nonphotosynthetic Land Plants, and Age of Parasitism/Mycoheterotrophy

Name	Type of Parasitism	Number of Functional Genes/RNAs ^a	Source	Age of Parasitism or Mycoheterotrophy ^b (Myr)
<i>Pilostyles aethiopica</i> (Apodanthaceae)	Endo-holoparasite	5?	This study	< 81 (62-98), Bellot and Renner 2014b
<i>Pilostyles hamiltonii</i> (Apodanthaceae)	Endo-holoparasite	5?	This study	< 81 (62-98), Bellot and Renner 2014b
<i>Rafflesia lagascae</i> (Rafflesiaceae)	Endo-holoparasite	Plastome not found	Molina et al. 2014	< 95 (83-109), Bendiksby et al. 2010
<i>Aneura mirabilis</i> (Aneuraceae, Liverworts)	Exo-holomycotroph	92	Wickett et al. 2008	?
<i>Corallorhiza</i> var. <i>maculata</i> (Orchidaceae)	Exo-holomycotroph	89	Barrett et al. 2014	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Corallorhiza</i> var. <i>occidentalis</i> (Orchidaceae)	Exo-holomycotroph	88	Barrett et al. 2014	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Corallorhiza mertensiana</i> (Orchidaceae)	Exo-holomycotroph	90	Barrett et al. 2014	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Corallorhiza striata</i> (Orchidaceae)	Exo-holomycotroph	82	Barrett and Davis 2012	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Epipogium aphyllum</i> (Orchidaceae)	Exo-holomycotroph	38	Schelkunov et al. 2015	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Epipogium roseum</i> (Orchidaceae)	Exo-holomycotroph	30	Schelkunov et al. 2015	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Neottia nidus-avis</i> (Orchidaceae)	Exo-holomycotroph	59	Logacheva et al. 2011	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Petrosavia stellaris</i> (Petrosaviaceae)	Exo-holomycotroph	72	Logacheva et al. 2014	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Rhizantella gardneri</i> (Orchidaceae)	Exo-holomycotroph	32	Delannoy et al. 2011	≪ 49, Epidendroideae; Lovisa et al. 2010
<i>Sciaphila densiflora</i> (Triuridaceae)	Exo-holomycotroph	28	Lam et al. 2015	≪ 90-50 Triuridaceae; Mennes et al. 2013
<i>Boulardia latisquama</i> (Orobanchaceae)	Exo-holoparasite	54	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Cistanche deserticola</i> (Orobanchaceae)	Exo-holoparasite	62	Li et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Cistanche phelypaea</i> (Orobanchaceae)	Exo-holoparasite	60	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Conopholis americana</i> (Orobanchaceae)	Exo-holoparasite	49	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Cuscuta gronovii</i> (Convolvulaceae)	Exo-holoparasite	89	Funk et al. 2007	< 35 (13-57) Naumann et al. 2013
<i>Cuscuta obtusiflora</i> (Convolvulaceae)	Exo-holoparasite	92	McNeal et al. 2007	< 35 (13-57) Naumann et al. 2013
<i>Epifagus virginiana</i> (Orobanchaceae)	Exo-holoparasite	51	Wolfe et al. 1992	< 32 (13-52) Naumann et al. 2013
<i>Myzorrhiza californica</i> (Orobanchaceae)	Exo-holoparasite	78	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Orobanche crenata</i> (Orobanchaceae)	Exo-holoparasite	63	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Orobanche gracilis</i> (Orobanchaceae)	Exo-holoparasite	58	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Phelipanche purpurea</i> (Orobanchaceae)	Exo-holoparasite	60	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013
<i>Phelipanche ramosa</i> (Orobanchaceae)	Exo-holoparasite	57	Wicke et al. 2013	< 32 (13-52) Naumann et al. 2013

^aThe number of genes in a typical angiosperm is 116 (from Barrett et al. 2014), whereas for photosynthetic *Aneura* it is 121 (Wickett et al. 2008).

^bThe age of parasitism/mycoheterotrophy is younger than the stem age of the respective parasite/mycoheterotrophic clade.

Mitrastemonaceae 78 Myr, and Apodanthaceae about 100 Myr (Naumann et al. 2013).

Apodanthaceae occur in North and South America, Africa, Iran, and Australia (Bellot and Renner 2014b), and based on mitochondrial and nuclear sequences they belong in the Cucurbitales (Filipowicz and Renner 2010). Like other endoparasites, they lack leaves and stems, emerging only as small flowers that break through the host's bark once a year (fig. 1). No chloroplasts have ever been observed in their tissues (Rutherford 1970: *Pilostyles thurberi*; Dell et al. 1982; *P. hamiltonii*), although plastid-like compartments have been reported for *P. hamiltonii* (Dell et al. 1982). The plant's body consists of cell clusters inside their hosts, which are Fabaceae for *Pilostyles* in the Americas, Africa, Iran, and Australia, but Salicaceae for *Apodanthes* in South America.

In this study, we investigate the African *Pilostyles aethiopica* and the Australian *P. hamiltonii*, endoparasites that diverged from each other about 23–33 Myr ago and that live inside Fabaceae (Bellot and Renner 2014a), to test if they retain a



FIG. 1.—Flowers of *Pilostyles aethiopica* emerging from the host *Julbernardia globiflora* (Fabaceae) in Harare, Zimbabwe. Scale bar is 5 mm. Photo S. Bellot.

functional plastome. We used three kinds of evidence to infer the genomic location of any plastome-like DNA region: BLAST searches, flanking regions, and read depth differences. In normal photosynthetically active plants, copy number of plastome sequences is expected to be one to two orders of magnitude higher than that of mitochondrial sequences and two to four orders higher than that of nuclear sequences (Zoschke et al. 2007), although those proportions may change in nonphotosynthetic tissues.

MATERIALS AND METHODS

Taxon Sampling, DNA Sequencing, and Genome Size Measurements

Flower tissue from a female individual of *P. aethiopica* (voucher S. Bellot 28, deposited in the herbarium of Munich) was collected in the Mukuvisi woodlands of Harare, Zimbabwe on February 29, 2012 and kept frozen until DNA isolation with the kit DNeasy Plant Maxi Kit (Qiagen), following the manufacturer's protocol. One microliter of the DNA (17.2 ng/μl) was sent to Eurofins MWG Operon for precipitation and sequencing. One genomic shotgun library of insert sizes 160–310 bp was sequenced in one channel of Illumina HiSeq 2000, yielding 236,404,172 paired-end reads of 101 bp. The same DNA sample was also submitted by the same company to a run of pyrosequencing using a GS FLX+ sequencer (454 Life Science, Roche), yielding 864,474 reads of 640 bp in average, already trimmed based on quality.

DNA from silica-dried flowers of male and female *P. hamiltonii* collected near Perth in October 2010 (voucher K. Dixon 1039 in the herbarium PERTH) was isolated using the same approach, and the DNA was sent to the University of Vienna (C. Schlötterer's lab) for Illumina sequencing on a Genome Analyzer IIx platform. This yielded 80,223,076 paired-end reads of 101 bp.

The 1C values of *P. aethiopica* and *P. hamiltonii* were determined with flow cytometry, using the same batch of flowers as used for DNA sequencing. Flow cytometry relied on propidium iodide as the DNA stain and *Solanum pseudocapsicum* as the standard, following the protocol of Temsch et al. (2010). A CyFlow ML flow cytometer (Partec, Muenster, Germany) equipped with a green laser (100 mW, 532 nm, Cobolt Samba, Cobolt, Stockholm, Sweden) was used for the fluorescence measurements, with 5,000 particles measured per run and three runs performed per plant preparation. The C value was calculated according to the formula: $1C \text{ valueObject} = (\text{mean G1 nuclei fluorescence intensityObject} / \text{mean G1 nuclei fluorescence intensityStandard}) * 1C \text{ valueStandard}$.

Quality Control, Preprocessing, and Assembly of Reads

For *P. aethiopica*, following quality control of the Illumina reads with PRINSEQ (Schmieder and Edwards 2011) and

FASTQC (Andrews, <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>, last accessed December 27, 2015), adaptors were removed when necessary, using fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/, last accessed December 27, 2015), and sequences were trimmed at both ends using PRINSEQ to remove polyAT tails greater than 5 nt and all bases with a quality score less than 20, stopping at the first base with a quality greater than 20. The few sequences shorter than 60 bp were then removed as were sequences of a mean quality score less than 30, or greater than 1% of Ns, or an entropy less than 70. This left 180,443,106 reads. For *P. hamiltonii*, adaptors were removed from the reads using Cutadapt (Martin 2011), and the reads were then filtered and trimmed using PRINSEQ with similar stringency thresholds as for *P. aethiopica*. This left 69,000,257 reads.

De novo assemblies of the cleaned total reads of *P. aethiopica* and *P. hamiltonii* were performed on the CLC Genomics Workbench 7 (<http://www.clcbio.com>, last accessed December 27, 2015) using different word and bubble sizes, of which the automatic ones provided the best results, and improved with SSPACE (Boetzer et al. 2011), which remaps reads using the included Bowtie assembler and scaffolds the resulting contigs using the paired-end information. This produced 952,874 de novo contigs for *P. aethiopica* and 270,940 for *P. hamiltonii*, with N50 of 601 and 446 bp, and a maximum contig size of 61,798 and 33,472 bp. The 454 reads of *P. aethiopica* were also assembled with CLC, producing 54,793 contigs with a N50 of 558 bp and a maximum size of 37,200 bp.

Isolating and Assembling Reads with a Reference Plastid Genome

The Illumina reads of *P. aethiopica* and *P. hamiltonii* were mapped with low stringency against the plastome of *Cucumis sativus* (supplementary table S1, [Supplementary Material](#) online, lists all plastid genomes used in this study). For each gene, rRNA, or tRNA, the corresponding reads were extracted, as well as reads matching adjacent intergenic regions until the first gap in the mapping. Reads were then de novo assembled with Geneious R7 (Biomatters, <http://www.geneious.com/>, last accessed December 27, 2015), using the highest stringency to allow the separation of similar sequences mapping to the same region. Contigs were checked for ambiguities, and weakly covered consensus (with at least 3, 20, 40, or 80 reads, depending on the length of the region) were retained to avoid losing plastome-like nuclear regions or a plastid genome that might be present in a low copy number. In many cases, multiple contigs corresponded to a given gene. All retained contigs were aligned against the plastome of *C. sativus*, keeping only contigs with an e-value <0.00001 (BLASTn command of BLAST+ version

29; Camacho et al. 2008; <ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.2.29/>, last accessed December 27, 2015). This reduced the number of plastome-like contigs from 3,755 to 313 for *P. aethiopica* and from 873 to 215 for *P. hamiltonii*. Finally, we kept the de novo contigs (previous section) overlapping those reference-based contigs as possible candidates for forming a plastome.

Finding More Divergent Plastome-Like Sequences

Because the reference genome approach did not recover a *Pilostyles* plastid genome (Results), we used a second strategy, which consisted in blasting the de novo contigs of *Pilostyles* against the plastid genes of 701 organisms (including the nonphotosynthetic apicomplexan *Babesia microti*) for which plastomes were available on GenBank in January 2015, using low stringency (maximum e-value of 10, maximum number of target sequences equal to total number of genes, and “-task” option set to BLASTn with default word-size of 11, as was the case for the BLASTn analyses described below) and keeping all contigs to which at least one gene (or a tRNA or a rRNA) matched along $\geq 50\%$ of its length, sometimes in multiple high scoring pairs (gapped alignment). This allowed us to retrieve 60 more de novo contigs as plastome gene candidates in *P. aethiopica* and two more in *P. hamiltonii*, resulting in 176 and 121 candidates, respectively. In an even more conservative approach, the same analysis was conducted using tBLASTx and keeping all contigs matching with an e-value ≤ 0.01 , no matter the length of the match. The flanking regions of the plastome-like regions were retrieved using custom scripts, BEDtools (Quinlan and Hall 2010), and SAMtools (Li et al. 2009). Mean read depth was retrieved for every de novo contig after stringent (99% overlap, 99% identity) remapping of the reads using CLC and following removal of reads possibly resulting from polymerase chain reaction (PCR) duplicates (as inferred from their mapping coordinates) using SAMtools (command “rmdup” option “-S”). To confirm that borders between flanking and plastome-like regions were not unusually covered compared with the rest of the contig, we used the “Coverage analysis” option available in CLC, using a conservative minimum length (6 bp) and *P* value (0.05), which allowed identification of even small regions of low/high coverage (normal coverage being as the global coverage of the contig). From this analysis, we pooled the reads belonging to possibly misassembled contigs containing plastome-like regions with the reads from contigs selected as plastome candidates (Results), and reassembled them de novo using Geneious R7 at low, medium, and high stringency to check for alternative assemblies that would produce different plastome candidates. For *P. aethiopica*, the 454 reads were mapped to the borders when available, and they all matched perfectly whenever CLC inferred a normal coverage (data not shown).

Genomic and Phylogenetic Origin of Plastome-Like DNA Fragments

To infer the genomic placement of the candidate plastome contigs of both species of *Pilostyles*, each plastome-like region was aligned against the NCBI nucleotide database using BLASTn, with a maximum e-value of 10 and a maximum number of target sequences set to 5,000. Custom Python scripts were written to record hits bitscores and genomic compartments. Flanking regions of the plastome-like regions were submitted to the same analysis, and results were double-checked manually. The genomic location of plastome-like contigs was also inferred from their read depth in comparison to that of contigs known to belong to the mitochondrial and nuclear genomes. Mitochondrial contigs were identified by blasting all contigs against the mitochondrial genes of *Citrullus lanatus* (NC_014043), *Cucurbita pepo* (NC_014050; Alverson et al. 2010), and *C. sativus* (NC_016004, NC_016005, and NC_016006; Alverson et al. 2011), and keeping for each gene the contig matching with the highest bitscore, some of them carrying multiple mitochondrial genes. Nuclear contigs were identified the same way, using as a reference the nuclear protein-coding genes of *C. sativus* published by Li et al. (2011) and available in GenBank (BioProject number PRJNA80169).

The high divergence and short length of most sequences made their phylogenetic placement difficult, regardless whether using BLAST or alignments and trees searches. We used a script (<http://seqanswers.com/forums/showthread.php?t=40975>, last accessed December 27, 2015) to extract GenBank’s taxonomic classification information for each sequence and then recorded the bitscore of its first hit to Cucurbitales (the order of the parasite family Apodanthaceae), Fabales (the order of the host), Fagales or Rosales (the four orders form a monophylum), or to other orders. Contigs with parts most similar to Fabales were kept even when they were globally more similar to another order (including Cucurbitales) because they could represent cases of horizontal gene transfer from the hosts. Contigs that completely matched bacteria or Fabales (the order to which all African and Australian host species belong) were removed from further analyses after we had made sure that they could not represent parts of the *Pilostyles* plastome by looking at their gene content. We are confident that these removed contigs do not form part of any *Pilostyles* plastome because all their reads were included in the iterative remapping analyses performed to extend plastome candidates (next section); none of them allowed further extension. The genes found in the plastomes of *P. aethiopica* and *P. hamiltonii* (Results) were aligned, and we then performed maximum likelihood phylogenetic analyses to infer their relationships with other Viridiplantae, using 37–39 representative lineages of land plants and green algae as outgroups (supplementary table S1, [Supplementary Material](#) online). Alignments were generated with Geneious

R7 and MAFFT 7.017 (Katoh et al. 2002), and tree searches were conducted in RAxML 7.2.8 (Stamatakis 2006) with the GTR + G substitution model and 100 bootstrap replicates, all through Geneious R7.

Extension and Concatenation of the Plastome-Like Contigs

For each *Pilostyles* species, all reads were remapped iteratively at low stringency (requiring only 30% of length to match at 100%) onto the candidate plastome contigs (Results), using CLC. The ends of each contig were checked manually for possible further extension and/or alternative ends that would allow concatenation. After each extension, contigs were blasted against the pooled de novo contigs, to ensure that no plastid contig was missed, especially divergent noncoding regions.

To confirm the sequence of a few low-complexity repeated regions and the closure of the plastome of *P. aethiopica*, the genomic DNA used for the next-generation sequencing was used for PCRs using standard protocols (as in Bellot and Renner 2014a) and Sanger sequencing relying on the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3130-4 automated capillary sequencer. The same procedure was also applied to join the plastome contigs of *P. hamiltonii*, on a DNA sample (voucher: K. Thiele 4527, herbarium PERTH) collected at the same locality as the sample used for next-generation sequencing. We also designed a pair of primers to amplify the whole plastome of *P. aethiopica* by long-range PCR performed on the DNA used for Illumina sequencing, using the following conditions: 25 μ l of LongAmp Hot Start Taq 2X Master Mix (New England BioLabs), 2 μ l of each primer, 1 μ l (120 ng) of DNA, and 20 μ l of H₂O yielding an individual reaction volume of 50 μ l. Thermocycling conditions were 30s at 94 °C + 30 * (30 s at 94 °C + 60 s at 46 °C + 9 min at 65 °C) + 10 min at 65 °C. The primers were designed using Primer3Plus v. 2.3.6 (Untergasser et al. 2012) and are listed at the end of supplementary table S1, [Supplementary Material](#) online.

In Silico Assessment of Plastid Gene Functionality

Contigs bearing plastome-like regions were annotated using DOGMA (Wyman et al. 2004; <http://dogma.cccb.utexas.edu/html/cite.html>, last accessed December 27, 2015) at the lowest possible stringency (threshold of 25% identity, gapped and nongapped alignment), and amino acid or nucleotide sequences of all gene and rRNA fragments identified were aligned with the corresponding genes of *Cucumis*, manually compared for identity and searched for frameshifts and stop-codons (for protein-coding genes). For the plastomes, the results of DOGMA were refined using ORF Finder online (Tatusov and Tatusov; <http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi>, last accessed December 27, 2015) as well as manual BLASTn, tBLASTx, and BLASTp against Genbank. In

addition, the two plastomes were searched for tRNAs using tRNAscan-SE 1.21 (Lowe and Eddy 1997) at low stringency (Cove score 15), and secondary structure of the candidate tRNAs was investigated using the online program RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>, last accessed December 27, 2015).

RESULTS

Genome Size, Sequencing Coverage, and Genomic Location of Plastome-Like Regions

The 1C value of *P. aethiopica* is 1.5 pg, that of *P. hamiltonii* 5 pg, which corresponds to, respectively, 1.467 (1.50.978*10⁹) and 4.89 Gb. *Pilostyles aethiopica* has a chromosome number of $n = 29$ (Bellot 2015); the chromosome number of *P. hamiltonii* is unknown. The results from blasting the *de novo* contigs of *P. aethiopica* and *P. hamiltonii* against Genbank (Material and Methods) are presented in table 2 and tables S2 and S3, [Supplementary Material](#) online. To confirm or clarify the genomic locations inferred from blasting, we used coverage information (fig. 2). The length of the assembled *P. aethiopica* contigs is 498,766,256 bp, that of *P. hamiltonii* 117,704,350 bp, representing ca. 34% and 2.4% of their respective genomes; the expected read depth is thus 36 and 59 discounting copy number variation. Figure 2 shows the read depths of the mitochondrial and nuclear contigs as well as that of plastome-like contigs located in the mitochondrial genome as inferred from their flanking regions. For both species, the latter have a read-depth similar to that of contigs known to be mitochondrial. In a second step, the contigs of unclear location were assigned to a genomic compartment on the basis of expected differences in read depths and by performing further read mappings (supplementary tables S2 and S3, [Supplementary Material](#) online, provide details about the assignment of particular contigs). In *P. aethiopica*, 48 plastome-like regions are located in the chondriome, nine in the nucleus, and one contig may be part of the plastome. In *P. hamiltonii*, 55 plastome-like regions are located in the chondriome, seven in the nucleus, three may be in either the chondriome or the nuclear genome, and three may form a plastome (table 2).

Do the Plastome-Like Contigs in the Two Species of *Pilostyles* Form a Plastome?

The only plastid contig found in *P. aethiopica* (contig 177; arrow in fig. 2) had a mean read depth of 174, higher than any of the other *de novo* contigs. Contig 177 matches first to an *rrn16* region (supplementary table S2, [Supplementary Material](#) online) with high identity to those amplified by PCR from *P. hamiltonii* (Thiele et al. 2008) and *P. thurberi* (Nickrent et al. 1997). Read remapping as well as blasting of contig 177 at low stringency to all *de novo* contigs did not allow further extension, and showed homogeneous coverage except at the

Table 2

Contigs of *Pilostyles hamiltonii* and *Pilostyles aethiopica* Containing Plastome-Like Regions, with Their Genomic Location, Read Depth, and Gene Content

De Novo Contig	Genomic Location	Mean Coverage (in reads by bp)	Protein-Coding Genes	De Novo Contig	Genomic Location	Mean Coverage (in reads by bp)	Protein-Coding Genes
Pham_scaffold_3	MT	67.6		Paet_scaffold_1	MT	129.7	<i>orf42*</i>
Pham_scaffold_5	MT	68.9		Paet_scaffold_4	MT	131.6	<i>accD*, atpA*, psbT*</i>
Pham_scaffold_9	MT	87.1	<i>psbD*</i>	Paet_scaffold_5	MT	125.7	
Pham_scaffold_10	MT	87.1	<i>rpl23*</i>	Paet_scaffold_8	MT	127.3	
Pham_scaffold_16	MT	73.5		Paet_scaffold_10	MT	129.7	<i>psbE</i>
Pham_scaffold_17	MT	86.5		Paet_scaffold_13	MT	126.0	
Pham_scaffold_22	MT	79.4	<i>rpoC1*</i>	Paet_scaffold_17	MT	127.9	
Pham_scaffold_23	MT	81.8		Paet_scaffold_20	MT	128.7	
Pham_scaffold_25	MT	65.0		Paet_scaffold_24	MT	126.2	
Pham_scaffold_26	MT	84.5		Paet_scaffold_28	MT	128.7	
Pham_scaffold_29	MT	60.7		Paet_scaffold_29	MT	130.0	
Pham_scaffold_32	MT	79.5	<i>ndhC*, ndhK*</i>	Paet_scaffold_31	MT	128.6	
Pham_scaffold_34	MT	116.3		Paet_scaffold_37	MT	128.4	<i>rpl23*</i>
Pham_scaffold_37	MT	72.6	<i>infA*</i>	Paet_scaffold_42	MT	122.5	
Pham_scaffold_38	MT	84.5	<i>petB*, petD*, psbH, rpoA*, rps11</i>	Paet_scaffold_50	MT	133.8	<i>ccsA*, cemA*</i>
Pham_scaffold_40	MT	79.5		Paet_scaffold_51	MT	120.0	<i>atpB, psbC*, psbZ, rps7*</i>
Pham_scaffold_41	MT	95.6		Paet_scaffold_67	MT	131.5	<i>accD*, atpA*, atpF*, atpI*, atpB, atpE*</i>
Pham_scaffold_42	MT	77.1		Paet_scaffold_70	MT	127.8	
Pham_scaffold_44	MT	74.8		Paet_scaffold_72	MT	129.1	
Pham_scaffold_46	MT	79.5	<i>rbcl*</i>	Paet_scaffold_73	MT	117.3	
Pham_scaffold_50	MT	84.8	<i>rpoC1*</i>	Paet_scaffold_84	MT	126.5	
Pham_scaffold_58	MT	88.3		Paet_scaffold_85	MT	122.7	
Pham_scaffold_62	MT	67.3	<i>cemA*</i>	Paet_scaffold_93	MT	131.7	
Pham_scaffold_65	MT	74.9		Paet_scaffold_95	MT	132.2	<i>rps2*</i>
Pham_scaffold_66	MT	65.4		Paet_scaffold_106	MT	129.5	<i>accD*</i>
Pham_scaffold_69	MT	95.3	<i>psbE*, psbF*, psbJ, psbN*, rpl20*, ycf1*</i>	Paet_scaffold_107	MT	136.9	
Pham_scaffold_70	MT	76.5		Paet_scaffold_108	MT	134.6	
Pham_scaffold_72	MT	100.1		Paet_scaffold_122	MT	136.4	
Pham_scaffold_78	MT	67.1	<i>orf56</i>	Paet_scaffold_125	MT	138.8	<i>accD*, atpB, ndhJ*, ndhK*, rbcl*, rps4*</i>
Pham_scaffold_80	MT	79.5	<i>psaA*</i>	Paet_scaffold_126	MT	134.9	
Pham_scaffold_81	MT	87.5		Paet_scaffold_136	MT	133.7	<i>petA*</i>
Pham_scaffold_83	MT	73.8	<i>atpH*</i>	Paet_scaffold_157	MT	125.8	
Pham_scaffold_88	MT	60.1		Paet_scaffold_166	MT	123.3	<i>petG</i>
Pham_scaffold_113	MT	79.1		Paet_scaffold_177	CP?		<i>accD, rpl2*, rps3, rps4, rps12*, rrn16, rrn23</i>
Pham_scaffold_158	MT	76.7		Paet_scaffold_189	MT	126.1	
Pham_scaffold_160	MT	68.3	<i>psaC</i>	Paet_scaffold_213	MT	136.5	
Pham_scaffold_163	MT	59.4	<i>psaA*</i>	Paet_scaffold_226	MT	126.8	<i>rpoC1*</i>
Pham_scaffold_175	MT	69.3	<i>psbB*, psbT*</i>	Paet_scaffold_243	MT	124.6	
Pham_scaffold_180	MT	90.0	<i>rpoC2*</i>	Paet_scaffold_245	MT	113.8	
Pham_scaffold_198	MT	48.9		Paet_scaffold_267	MT	120.5	<i>psbA*</i>
Pham_scaffold_203	MT or NC	45.9	<i>ndhJ*</i>	Paet_scaffold_286	MT	117.2	
Pham_scaffold_205	MT	80.0		Paet_scaffold_299	MT	130.6	<i>psbN*</i>
Pham_scaffold_207	CP?	111.6	<i>rpl2*, rrn23</i>	Paet_scaffold_319	MT	123.4	

(continued)

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Table 2 Continued

De Novo Contig	Genomic Location	Mean Coverage (in reads by bp)	Protein-Coding Genes	De Novo Contig	Genomic Location	Mean Coverage (in reads by bp)	Protein-Coding Genes
Pham_scaffold_241	MT	61.4		Paet_scaffold_346	MT	137.6	
Pham_scaffold_244	MT or NC	52.7		Paet_scaffold_377	MT	129.9	<i>atpA*</i> , <i>ycf1*</i>
Pham_scaffold_251	MT or NC	59.8		Paet_scaffold_420	MT	133.1	
Pham_scaffold_261	CP?	89.3	<i>rrn16</i>	Paet_scaffold_512	MT	118.8	
Pham_scaffold_266	MT	71.2		Paet_scaffold_908	MT	118.6	<i>psbD*</i> , <i>rpoB*</i>
Pham_scaffold_270	MT	68.5	<i>clpP*</i>	Paet_scaffold_1807	NC	7.3	
Pham_scaffold_315	MT	67.8	<i>rps12*</i>	Paet_scaffold_2390	MT	128.9	
Pham_scaffold_462	MT	71.7		Paet_scaffold_19077	NC	5.4	
Pham_scaffold_491	MT	75.6		Paet_scaffold_53779	NC	7.2	<i>psaA*</i>
Pham_scaffold_503	MT	60.0		Paet_scaffold_77278	NC	7.6	
Pham_scaffold_506	MT	42.9		Paet_scaffold_113062	NC	5.3	<i>accD*</i>
Pham_scaffold_508	MT	48.7		Paet_scaffold_149204	NC	22.9	
Pham_scaffold_706	CP?	116.9	<i>accD</i>	Paet_scaffold_164148	NC	3.0	<i>atpH*</i>
Pham_scaffold_775	MT	92.5	<i>psaB*</i> , <i>rps14*</i>	Paet_scaffold_277960	NC	4.7	
Pham_scaffold_2613	NC	64.5		Paet_scaffold_605245	NC	4.4	
Pham_scaffold_14490	MT	63.6					
Pham_scaffold_22113	MT	134.5	<i>psbA*</i>				
Pham_scaffold_33324	NC	8.4					
Pham_scaffold_49221	NC	5.6					
Pham_scaffold_50886	NC	4.9	<i>ycf4*</i>				
Pham_scaffold_79191	NC	1.6					
Pham_scaffold_90224	NC	4.8					
Pham_scaffold_148584	MT	89.7	<i>rpl36</i>				
Pham_scaffold_220160	MT	78.2					
Pham_scaffold_223261	NC	4.6					

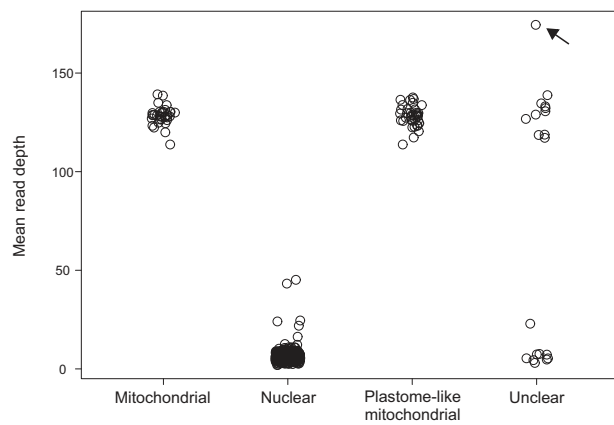
NOTE.—Genes marked with an asterisk are pseudogenes, whereas the others may be functional as inferred only from the DNA sequence (see [supplementary table S4, Supplementary Material](#) online, for details). Contigs without plastid genes show similarities with very small plastid gene fragments or with uncoding plastid regions. Genomic location of the plastid regions in contigs in bold was inferred from their flanking regions whereas for the others, it required coverage analysis and read remappings (see [supplementary tables S2 and S3, Supplementary Material](#) online, for details). MT, mitochondrial; NC, nuclear; CP?, possibly part of a chloroplast genome.

beginning and end of the contig and in two other small sections ([supplementary fig. S2A, Supplementary Material](#) online). Circularization of contig 177 of *P. aethiopica* is supported in silico by eight Illumina reads and 24 paired-end reads having one mate mapping to the start of the contig and one mate mapping to its end. Sanger sequencing using primers matching the ends of this contig was successful, confirming the existence of plastomes in which the ends are adjacent ([supplementary fig. S2B, Supplementary Material](#) online). Finally, long-range PCR using a primer pair designed on contig 177 ([fig. 2A](#)) yielded a product size of approximately 11 kb, corresponding to the size of contig 177 ([supplementary fig. S2B, Supplementary Material](#) online, inset). The contig with the highest read depth obtained from the assembly of the 454 data was identical to contig 177, with the same pattern of coverage distribution, albeit not opened (linearized) at the same place but instead in a well-covered repeated region. By Sanger resequencing we checked the assembly of contig 177 ([supplementary fig. S2C, Supplementary Material](#) online). These different types of evidence support that contig 177 forms the complete plastome of *P. aethiopica*, although it is probably not always circular.

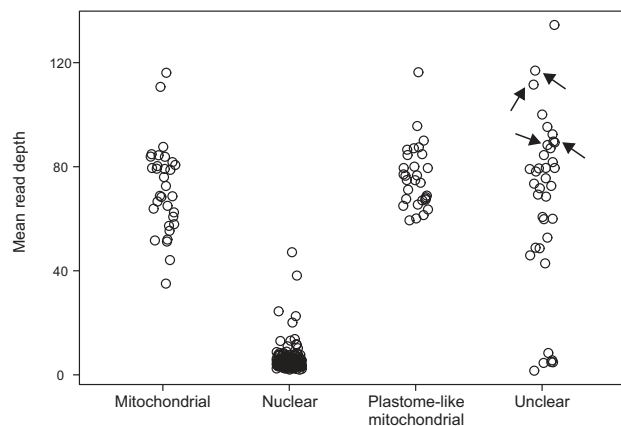
The plastid contigs found in *P. hamiltonii* (contigs 207, 261, and 706) align to different regions of the plastome of *P. aethiopica* and have read depths of, respectively, 112, 89, and 117, about the same as mitochondrial contigs ([table 2 and fig. 2](#)). Contigs 207 and 706 match to *Plasmodium* and green algae and then to angiosperm plastomes, and contig 261 matches the *rrn16* sequences of *P. hamiltonii* found in GenBank (EF446141 to EF446144 and EU512418 to EU512420; Thiele et al. 2008). Blasting of the de novo contigs of *P. hamiltonii* to the plastome of *P. aethiopica*, as well as iterative remappings of all the reads ([Material and Methods](#)), revealed six more contigs that overlapped contigs 207, 261, and 706 by at least a few reads. Those six contigs had mean read depths between 31 and 77, and two of them showed similarities with known plastid genes (below). PCR amplification and Sanger sequencing ([supplementary fig. S2D, Supplementary Material](#) online) suggest that all nine contigs form a circular plastome, collinear to that of *P. aethiopica*.

Possibly Functional Genes in the Plastomes of *Pilostyles*

The plastomes of *P. aethiopica* and *P. hamiltonii* are represented in [figure 3](#). Mapping of the reads to the plastomes



A *Pilostyles aethiopica*



B *Pilostyles hamiltonii*

FIG. 2.—Read depth of *Pilostyles* contigs with plastome-like regions. Unclear: Contigs with plastome-like regions whose flanking regions were too short to infer their genomic location. Arrows indicate contigs that are part of plastomes (Results). The means for *P. aethiopica* are based on 34 contigs located in the chondriome, 670 located in the nuclear genome, 38 inferred to be in the chondriome from their flanks, and 19 of unclear location. The means for *P. hamiltonii* are based on 32 contigs located in the chondriome, 369 located in the nuclear genome, 31 inferred to be in the chondriome from their flanks, and 37 of unclear location.

at various stringencies did not reveal the presence of the typical inverted repeat. In total, the plastome of *P. aethiopica* is 11,348 bp long, of which 52% (5,901 bp) are recognizable as functional or pseudogenized genes and rRNAs. The plastome of *P. hamiltonii* is 15,167 bp long, of which 41.6% (6,314 bp) are functional or pseudogenized genes; its larger size is thus mostly due to an increase of non-coding DNA. The mean GC content of the plastome of *P. aethiopica* is 24.2% and that of *P. hamiltonii* is 22.7% (fig. 3).

We found the genes *accD*, *rpl2*, *rps3*, *rps4*, *rps12*, *rrn16*, and *rrn23* in both species, and *rpoC2* only in *P. aethiopica*. At least *rpl2* and *rpoC2* are pseudogenes judging from their short size and lack of start codons, whereas *accD*, *rps3* and *rps4*

may be functional because they have long open reading frames (supplementary table S4, [Supplementary Material](#) online, provides details on the inference of gene function). The rRNAs are as long as in other plants, and BLAST searches of the *rrn23* and *rrn16* of *P. hamiltonii*, and *rrn23* of *P. aethiopica* against *P. thurberi* transcriptome data (Matasci et al. 2014; <https://www.bioinfodata.org/Blast4OneKP/>, last accessed December 27, 2015) recovered a hit for each of them. The *rrn16* of *P. aethiopica*, however, failed to match anything despite its similarity to *P. hamiltonii* (fig. 3). The *rps12* gene from both plastomes is as long as in photosynthetic plants, but consists of one piece instead of having three exons; it has a start codon in *P. hamiltonii* but apparently not in *P. aethiopica*.

The tRNA-like sequences found in the plastomes of *P. aethiopica* and *P. hamiltonii* by comparing them to *Cucumis melo* and the apicomplexan *B. microti* do not seem capable of forming a typical cloverleaf structure. When blasting (BLASTn, word size=7) the *trnE* genes of *Cucumis* and *Babesia* against the plastomes of *P. hamiltonii* and *P. aethiopica*, there was no match with a score greater than 25. The same searches against all de novo contigs hit a mitochondrial contig of *P. hamiltonii* (number 198; table 2) with a bitscore of 104, but again it does not seem capable of forming a cloverleaf secondary structure. No hit with a bitscore greater than 54 was found in *P. aethiopica*.

The Mitochondrial and Nuclear Genomes of *Pilostyles* Contain Plastid Pseudogenes

Supplementary table S4, [Supplementary Material](#) online, summarizes the presence/absence of plastid genes in the total de novo contig sets of *P. aethiopica* and *P. hamiltonii* compared with *C. sativus*. Of the 81 protein-coding genes present in *Cucumis*, 30 had detectable traces in *P. aethiopica* and 34 in *P. hamiltonii*, with 14 shared among them (not considering the genes located in the plastomes). In *P. aethiopica*, five mitochondrial contigs contained possibly functional plastid genes (three copies of *atpB*, and *petG*, *psbE*, and *psbZ*), as inferred from their length and absence of internal stop codons (supplementary table S4, [Supplementary Material](#) online). In *P. hamiltonii*, we also found five mitochondrial contigs containing possibly functional *orf56*, *psaC*, *psbH*, *psbJ*, *rpl36*, and *rps11* (table 2). However, none of these genes matched the transcriptome data from the American *P. thurberi* available online (Matasci et al. 2014; <https://www.bioinfodata.org/Blast4OneKP/>, last accessed December 27, 2015).

Many of the plastome-like sequences found in the nuclear and the mitochondrial genomes of *Pilostyles* have their first BLAST hit to Fabales (supplementary tables S2 and S3, [Supplementary Material](#) online), suggesting horizontal transfers from the host, but their shortness and high divergence prevented reliable assessment of their origin.

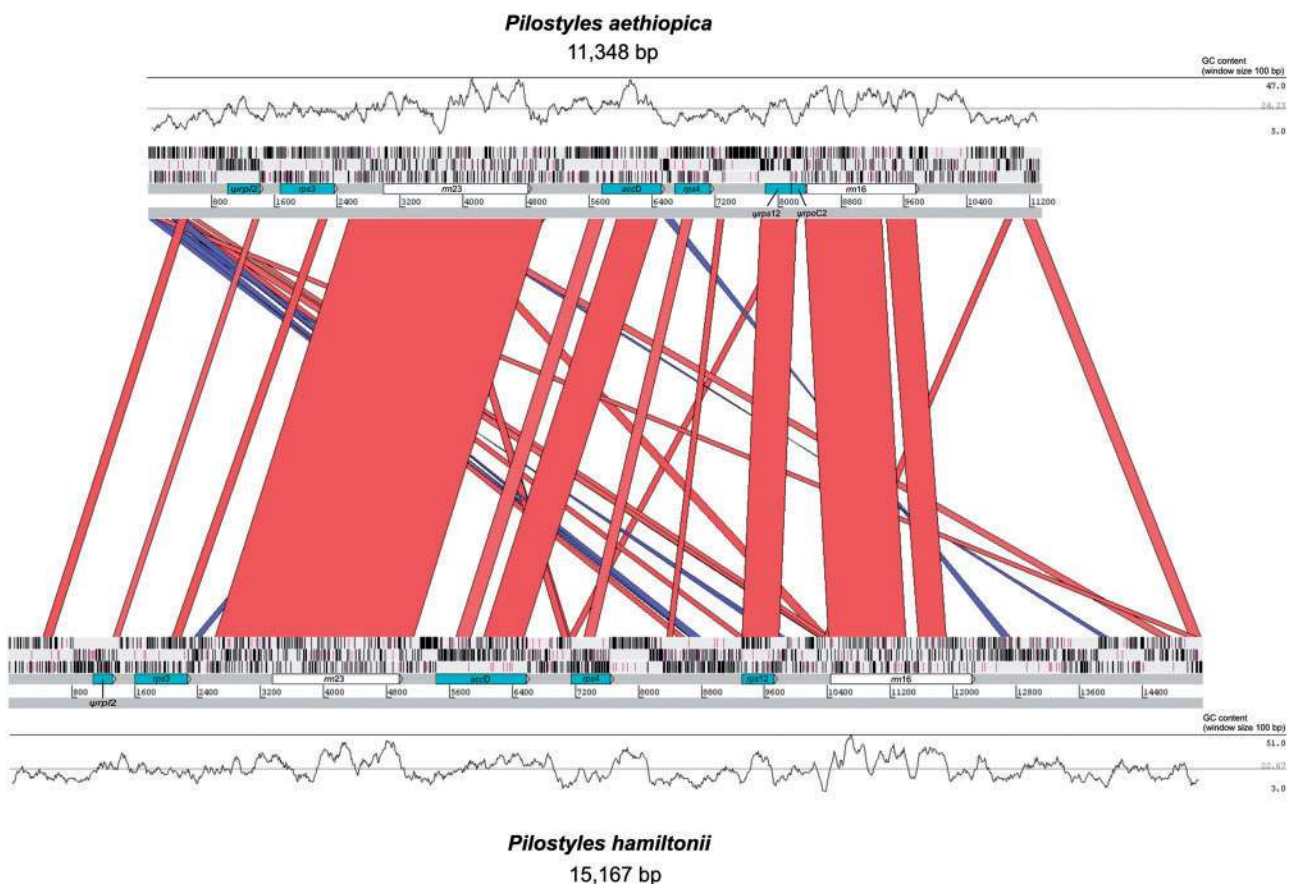


FIG. 3.—Map of the plastid genomes of *P. aethiopica* and *P. hamiltonii*. The skyline graphs represent the GC% with the minimum, mean, and maximum values indicated on the right. The blue and red bands indicate identity greater than 70% for bitscores greater than 100, red bands show a match in the same orientation whereas blue bands symbolize reversed-complement matches. The three bars above the gene labels refer to the reading frames; stop codons are represented by vertical black bars, and start codons (methionine) by purple vertical bars. Ψ means the gene is pseudogenized. Visualization obtained with the Artemis Comparison Tool (Carver et al. 2005).

No Detectable Horizontal and Internal Gene Transfers towards the *Pilostyles* Plastomes

The phylogenetic placement in Viridiplantae of each possibly functional plastome gene was assessed by maximum likelihood inference; results are shown in supplementary figure S1, [Supplementary Material](#) online. In all trees, the two *Pilostyles* species were sisters, with 100% or 90% (*rps4*) bootstrap support (BP), and the branches leading to *Pilostyles* are longer by at least one order of magnitude than other branches in the respective phylogenies. The genes *rps3*, *rps4*, or *rrm23* placed *Pilostyles* as sister to *Silene*, *Welwitschia*, or *Psilotum*, always with low support ($\leq 32\%$ BP). The tree obtained from the *rrm23* alignment does not fit currently accepted angiosperm relationships, while that from *rps12* shows *Pilostyles* as sister to land plants (with 76% BP) in a topology that otherwise more or less fits expected relationships. The *rrm16* gene places *Pilostyles* closer to *Cicer* (Fabales; with 62% BP), and together *Pilostyles* and *Cicer* are sister to the remaining

angiosperms (in which relationships are not well resolved). When we blasted the *rrm16* of *Pilostyles* against GenBank (BLASTn at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, last accessed April 2015), Fabales were not among the first hits, but instead other sequences of *Pilostyles*, then *Balanophora* (a holoparasite belonging to Santalales), and then *Plasmodium* (a nonphotosynthetic apicomplexan). The *accD* tree, finally, shows *Pilostyles* as sister to *Cucumis* (Cucurbitales; with 63% BP), and all three are located among other Rosales in a well-resolved angiosperm phylogeny. BLASTn of each gene against GenBank revealed the high divergence of the *Pilostyles* plastome genes, with every gene except *accD* having its first hit outside of angiosperms, always with a relatively low bitscore compared with the length of the gene (results available on request).

BLAST searches (BLASTn at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, last accessed April 2015) of the non-coding regions of the plastome of *P. aethiopica* revealed a 509 bp-long low-complexity region (12% GC) matching an

mitochondrial genomes of *Pilostyles* may thus reflect a Cucurbitales heritage rather than result from a parasitic way of life.

Structure and Function of the Plastome of the Two *Pilostyles* Species

Prior to this study, the most reduced plastomes known from nonphotosynthetic angiosperms were those of *Sciaphila densiflora* (Triuridaceae) and *Epipogium aphyllum* and *E. roseum* (Orchidaceae), with 27–29 functional genes and transfer RNAs (Lam et al. 2015; Schelkunov et al. 2015). The plastomes of *P. aethiopica* and *P. hamiltonii*, with five or six potentially functional genes (*accD*, *rps3*, *rps4*, *rrn16*, *rrn23* and *rps12* for *P. hamiltonii*) and two to three pseudogenes (*rpl2*, *rpoC2*, and *rps12* for *P. aethiopica*), retain only about one-fifth of the genes of these previous smallest plastomes. We were able to find these extremely reduced plastomes by combining contig blasting at low stringencies, read-depth analysis, analysis of flanking regions, and a range of assembly strategies. That Molina et al. (2014) failed to identify a plastid genome in *R. lagascae*, the only land plant so far reported as possibly having lost its plastome, could be due to a similarly divergent and small plastome that escaped detection; their inference was based on coverage information and blasting at relatively high stringencies. Successful PCR amplification of the *rrn16* gene from *Rafflesia* (Bendiksby et al. 2010) indeed points to the presence of a plastome. Under the microscope, cells of both Rafflesiaceae and Apodanthaceae have plastome-like compartments, suggesting a metabolic function (Dell et al. 1982; Molina et al. 2014), and in the American *Pilostyles thurberi*, the *rrn16* and the *rrn23* are expressed, despite their unusual secondary structure (Nickrent et al. 1997; Matasci et al. 2014).

Because the five or six potentially functional genes remaining in the *Pilostyles* plastome can all be transcribed by a nuclear-encoded polymerase (NEP), at least in tobacco (Liere et al. 2011), the loss of the *rpo* genes for the plastid-encoded polymerase in *Pilostyles* probably does not prevent their transcription. There is no consensus sequence for most types of NEP promoters so they are not easily identifiable from the DNA sequence only, and those of *Pilostyles* remain to be characterized. Because we found no trace of functional tRNAs in the *Pilostyles* plastomes, the tRNAs required for the expression of the five or six retained plastome genes must be imported from the cytosol, as seems to be the case for other parasites, such as *Epifagus virginiana* (Wolfe et al. 1992).

Do the Plastomes of Endo- and Exoparasites Differ in Their Function?

Plastids fulfill metabolic functions other than photosynthesis, and of the approximately 116 land plant plastome genes, only approximately 50 are involved in photosynthesis while most of

the remainder are involved in the modification of the RNAs or proteins encoded by the first 50 (Bock 2007; Wicke et al. 2011). Including the two Apodanthaceae species studied here, the plastid genomes of 26 nonphotosynthetic plants from seven families have now been investigated (table 1). Of these, 23 are exoparasites that still form vegetative shoots, whereas three are endoparasites without any shoots, namely the one *Rafflesia* (Molina et al. 2014) and the two *Pilostyles* species investigated here.

The plastomes of the exoparasites still contain 27 to 92 protein-coding genes and RNAs, depending on the lineage (fig. 4 adapted from Barrett et al. 2014), with 17 genes retained in all of them: three tRNAs (*trnE*-UUC, *trnM*-CAU, and *trnI*-CAU), ten ribosomal proteins, and four ribosomal RNAs. A plastid location of the *trnE* might be essential for biosynthesis of mitochondrial haem components (Barbrook et al. 2006), but why the other two tRNAs are retained is unclear. Other genes, namely *accD*, *clpP*, *ycf1*, and *ycf2* (Krause 2012), have also been considered the *raison d'être* of a plastome in nonphotosynthetic plants, even though they have been lost in some (Li et al. 2013; Wicke et al. 2013). Of these, *accD* functions in fatty-acids biosynthesis, *clpP* is likely a protease and also involved in the import of proteins into the plastid (Krause 2012), *ycf1* functions in “photosynthetic protein import, and [is] therefore essential for plant viability” (Kikuchi et al. 2013. p. 573), and *ycf2* has an unknown function.

The plastomes of the two endoparasites studied here contain a seemingly functional copy of the *accD* gene and lack a functional nuclear copy, supporting that *accD* may be essential to plastome maintenance; the gene appears to play a role in fatty-acid synthesis and leaf development (Kode et al. 2005). We could not detect a cloverleaf-forming *trnE* in the plastome of any *Pilostyles*, which leaves open the mechanism of haem synthesis in these endoparasites. No *trnE* has been detected in the only other endoparasite studied, *R. lagascae* (Molina et al. 2014).

Living completely embedded in a photosynthetic host might have reduced selection on plastome genes more than is the case in exoparasites. The four families of endoparasites, Apodanthaceae, Cytinaceae, Mitrastemonaceae, and Rafflesiaceae, are not closely related to each other or to any exoparasites, and there is so far no scenario for how endoparasitism evolved. Parasitism in the six families of exoparasites so far studied happens to be younger than it is in the two lineages of endoparasites (table 1), and so age per se might explain the less reduced plastomes in the former. Studies of the plastomes of ancient exo-holoparasites, such as Balanophoraceae with a stem age of 110 Myr, Cynomoriaceae with a stem age of 100 Myr, or Hydnoraceae with a stem age of 101 Myr (Naumann et al. 2013), are required to test if such old exoparasites have similarly reduced plastomes as Apodanthaceae.

Supplementary Material

Supplementary figures S1 and S2 and tables S1–S4 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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