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ORIGINAL ARTICLE

A comparative study across the parasitic plants of *Cuscuta* subgenus *Grammica* (Convolvulaceae) reveals a possible loss of the plastid genome in its section *Subulatae*

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Abstract

Main conclusion **Most species in *Cuscuta* subgenus *Grammica* retain many photosynthesis-related plastid genes, generally under purifying selection. A group of holoparasitic species in section *Subulatae* may have lost their plastid genomes entirely.**

Abstract The c. 153 species of plants belonging to *Cuscuta* subgenus *Grammica* are all obligate stem parasites. However, some have completely lost the ability to conduct photosynthesis while others retain photosynthetic machinery and genes. The plastid genome that primarily encodes key photosynthesis genes functions as a bellwether for how reliant plants are on primary production. This research assembles and analyses 17 plastomes across *Cuscuta* subgenus *Grammica* with the aim of characterizing the state of the plastome in each of its sections. By comparing the structure and content of plastid genomes across the subgenus, as well as by quantifying the selection acting upon each gene, we reconstructed the patterns of plastome change within the phylogenetic context for this group. We found that species in 13 of the 15 sections that comprise *Grammica* retain the bulk of plastid photosynthesis genes and are thus hemiparasitic. The complete loss of photosynthesis can be traced to two clades: the entire section *Subulatae* and a complex of three species within section *Ceratophorae*. We were unable to recover any significant plastome sequences from section *Subulatae*, suggesting that plastomes in these species are either drastically reduced or lost entirely.

Keywords *Cuscuta* · Dodder · *Grammica* · Heterotrophy · Parasite · Parasitic plants · Photosynthesis · Plastid · Plastome

Introduction

Parasitic plants forge direct vascular connections, known as haustoria, with their hosts through which they obtain water and nutrients (Kuijt 1969). In doing so, they reduce (or, in some cases, completely eliminate) their reliance on photosynthesis. Across angiosperms, 292 genera and c. 4750 species of haustorial parasites are currently recognized, and their origins have been traced to 12 independent transitions from autotrophy to heterotrophy (Nickrent 2020). These 12 lineages share a suite of morphological, ecological, and life-history changes. For example, leaves and roots are often reduced or absent, the ability to acquire water and nutrients from the soil is thus often limited or lost, chlorophyll accumulation may be diminished, etc. (Kuijt 1969; Heide-Jorgensen 2008). Parasitic plant lineages, therefore, show remarkable levels of convergent evolution in a phenomenon often referred to as the 'parasitic reduction syndrome' (Colwell 1994). This repeated shift away from photosynthesis resulting in similar phenotypic change is fertile ground for comparative genomics research to test hypotheses regarding shared evolutionary trajectories in parasitic angiosperm genomes.

Such research has mainly been focused on plastid genomes because of the importance of plastids as the sites of

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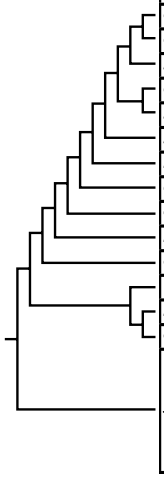
photosynthesis in the cell. Typical autotrophic plastomes are also highly conserved in terms of gene order and composition due to the high degree of purifying selection imposed by a dependence on photosynthesis. They are 135–165 kb long (e.g., *Nicotiana tabacum*: 155,939 bp, *Arabidopsis thaliana*: 154,478 bp) and have a four-part structure, with large and small single-copy regions separated by two inverted repeat regions (Shinozaki et al. 1986; Sato et al. 1999; Downie and Palmer 1992). They contain c. 79 protein-coding genes, the majority of which code for key portions of the photosynthetic apparatus, 30 tRNA genes, and four rRNA genes. However, in heterotrophic plants, the selection pressure due to photosynthesis is diminished and plastid genomes are able to evolve more freely. Generally, this has led to a reduction in plastome size and gene content (Wicke et al. 2011, 2013; Graham et al. 2017). The degree of this reduction tends to be correlated with the plant's position on the trophic continuum (Westwood et al. 2010). Published parasitic plastomes range from the intact molecules (171,851–177,797 bp) in the hemiparasitic genus *Krameria* (Banerjee et al. 2022) to the shortest assembled example (11,348 bp) in holoparasitic *Pilostyles aethiopica* (Apodanthaceae), containing only 5 genes, none of which have photosynthetic function (Bellot and Renner 2015). Plastid genomes are even thought to be lost entirely in two species in Rafflesiaceae (Molina et al. 2014; Cai et al. 2021). Coding sequence reduction and loss-of-function changes in plastid genomes are expected to be irreversible and, over time, as these plants adapt to their heterotrophic lifestyle, their plastomes are expected to slide down a 'slippery slope' and accumulate even further reduction (Stefanovic and Olmstead 2005).

Our knowledge of how plastomes change in parasitic plants has the benefit of breadth of data because at least one plastid genome from each parasitic angiosperm lineage has been sequenced (Funk et al. 2007; McNeal et al. 2007b; Wicke et al. 2013; Bellot and Renner 2015; Bellot et al. 2016; Naumann et al. 2016; Roquet et al. 2016; Wu et al. 2017; Schneider et al. 2018; Banerjee and Stefanović 2019; Gonçalves et al. 2019; Chen et al. 2020; Banerjee et al. 2022). However, the depth of our understanding, through fine-scale sampling within these different lineages, is still generally lacking. Among the 12 clades with independent origin of parasitism, each of which can be seen as a separate natural experiment regarding plastid evolution in light of reduced selective pressure, the genus *Cuscuta* (dodders, Convolvulaceae) represents a particularly tractable case system. *Cuscuta* is a group of c. 200 obligate stem parasites characterized by slender, pale, twining stems, scale-like leaves, and absent roots. Plants in this genus parasitize a wide range of woody and herbaceous plants, forging both xylem and phloem connections, and have a nearly cosmopolitan distribution with species found on every continent except Antarctica (Costea and Tardif 2006; Heide-Jorgensen 2008). The genus is of economic interest because several species have been identified as agricultural pests (Costea and Tardif 2006), but many dodders are also ecologically important and play keystone roles in plant communities (Press and Phoenix 2005; Kaiser et al. 2015; Li et al. 2020). Alongside the family Orobanchaceae and the order Santalales, *Cuscuta* is one of only three lineages of parasitic plants with both hemi- and holoparasitic members (Nickrent 2020). While no species are able to survive more than a few weeks without their hosts, some have been shown capable of limited and localized photosynthesis, especially in sepals and ovaries in fruiting flowers and the tips of unattached seedlings (Dawson et al. 1994; Hibberd et al. 1998; Choudhury and Sahu 1999; McNeal et al. 2007a). Other *Cuscuta* species, however, have been found to completely lack chlorophyll (van der Kooij et al. 2000) and many photosynthesis-related genes (Braukmann et al. 2013; Banerjee and Stefanović 2019), and likely retain no photosynthetic ability. This level of trophic diversity in a relatively young clade (stem age c. 35 My with a 95% highest posterior density interval of 13–57 My; Naumann et al. 2013) makes *Cuscuta* an excellent system for studying the transition away from photosynthesis.

Cuscuta also has a well-understood and supported phylogeny with well-resolved species relationships (Stefanovic et al. 2007; Garcia et al. 2014; Costea et al. 2015). The genus is circumscribed in four subgenera and 19 sections, although 15 of these sections fall within the largest subgenus *Grammica* (phylogeny in Fig. 1) which also contains roughly 75% (c. 153) of the species diversity (Costea et al. 2015). Despite the intensive scrutiny that *Cuscuta* biology has received over the last three decades, only four plastomes had been published: two from section *Cleistogrammica* in subgenus *Grammica* (82–85 kb long with 92 genes; Funk et al. 2007; McNeal et al. 2007b) and two from subgenus *Monogynella* (121–125 kb long with 103 genes; Funk et al. 2007; McNeal et al. 2007b). More recently, plastomes from the two remaining subgenera *Pachystigma* (105–114 kb with 91–96 genes) and *Cuscuta* (97–98 kpb with 95 genes) have been reported (Banerjee and Stefanović 2020), along with those from three additional *Grammica* sections—*C. californica* from section *Californicae* (81 kb; Lin et al. 2022b), *C. americana* from section *Obtusilobae* (78 kb; Lin et al. 2022b), and the comprehensive sampling of all eight confirmed species from section *Ceratophorae* (61–87 kb with 61–88 genes; Banerjee and Stefanović 2019). Altogether, plastid genomes are currently known for eight of the 19 sections that comprise the genus *Cuscuta*. The remaining 11 sections all belong to subgenus *Grammica* and are the subject of the research reported here.

Infrageneric classification in *Cuscuta* has gone through a few revisions (Choisy 1841; Engelmann 1859; Yuncker 1932) until the most recent research based on plastid and nuclear molecular markers circumscribed 153 recognized



Section	Species Sampled	Collection Number	Deposited To	GenBank Accession
Californicae	*C. pacifica*	*Stefanović SS-15-23*	TRTE	OP263625
Cleistogrammica	*C. obtusiflora**	GenBank	-	EU189133*
Racemosae	*C. micrantha*	*Muñoz 5131*	WLU	OP356701
Oxycarpae	*C. gronovii*	*Stefanović SS-02-03*	TRTE	OP448628
Denticulatae	*C. nevadensis*	*Lloyd 2639*	NY	OP390286
Partitae	*C. haughtii*	*Asplund 5618*	F	OP402843
Lobostigmae	*C. volcanica*	*Garcia Ruiz 5108*	IEB	OP402844
Obtusilobae	*C. macrocephala*	*Van Devender et al. 2001-758*	WLU	OP414597
Grammica	*C. chinensis*	*Carter 628*	CANB	OP414596
Prismaticae	*C. corymbosa stylosa*	*Van Devender et al. 2001-16*	WLU	OP414598
Ceratophorae	*C. chapalana**	GenBank	-	MK887214*
Umbellatae	*C. polyanthemos*	*Van Devender 2006-809*	WLU	OP441382
Indecorae	*C. indecora*	*UTM-1568*	TRTE	OP414599
Gracillimae	*C. vandervenderii*	*Van Devender et al. 98-1434*	WLU	OP414600
Subulatae	*C. argentinana*	*Olmstead et al. RGO-2007-15*	UWT	-
	C. microstyla	Muñoz 5165	WLU	-
	C. purpurata	*Muñoz 5144*	WLU	-
	C. foetida pycnantha	*Lira 13*	SGO	-
	C. kilimanjari	*Knox 5020*	IND	-

Fig. 1 A summary of sampling strategy for this project. Species selected from each of the 15 sections that comprise subgenus *Grammica* are listed, along with their collection numbers, deposition locations, and GenBank accessions. Plastomes for *Cuscuta obtusiflora* (sect. *Cleistogrammica*) and *C. chapalana* (sect. *Ceratophorae*) have been previously published and were taken from GenBank (*). All other plastomes with listed accession numbers were assembled in this research. Phylogenetic relationships between sections have been included on the left and are based on Fig. 3 in Garcia et al. (2014). Abbreviations of herbaria in which the vouchers are deposited follow Index Herbariorum

Grammica species into 15 well-supported sections (see Fig. 1 in Costea et al. 2015). From the standpoint of trophic diversity, subgenus *Grammica* epitomizes the complexity of the genus as a whole. Most species appear to retain the bulk of their photosynthetic genes (Braukmann et al. 2013) and have been theorized to be capable of localized photosynthesis despite their heterotrophic morphology at maturity (McNeal et al. 2007a). These "cryptically photosynthetic" (McNeal et al. 2007a) plants are analogous to hemiparasites on the trophic continuum. Other *Grammica* species, however, like three species from section *Ceratophorae*, contain plastids that lack many genes that are crucial parts of the photosynthetic apparatus (Braukmann et al. 2013; Banerjee and Stefanović 2019). It is hypothesized that photosynthesis is completely lost in these plants, and they are thus holoparasitic, dependent on their hosts for all of their nutrient intake.

In the present research, we sample and analyze plastid genomes from 17 species across 13 subgenus *Grammica* sections, including the 11 sections from which plastomes are currently unknown. In doing so, we complete the comprehensive investigation of plastomes in the genus by ensuring that at least one plastid genome from each section is assembled and examined. This will allow for fine-scale analyses of plastid evolution across *Cuscuta*, further establishing this lineage as a model system for the study of plastome evolution in heterotrophic plants.

Materials and methods

Taxon sampling, DNA extraction, and sequencing

Our *Grammica* taxon sampling was explicitly guided by the phylogeny of the genus (Costea et al. 2015) as well as Southern hybridization results from the broad plastid genome survey across *Cuscuta* (Braukmann et al. 2013). For 12 out of 15 *Grammica* sections, one species each was sampled to best capture the phylogenetic depth of this subgenus (Fig. 1). Section *Subulatae* is the largest in the genus (c. 30 species) and is positioned as the sister to the rest of *Grammica* (Garcia et al. 2014; Lin et al. 2022a). Members of this group have shown extensive losses of plastid genes from every category typically found in the plastome (Braukmann et al. 2013), and hence was represented by five species, chosen from across its phylogeny (Costea et al. 2021; Fig. 1). No additional sampling was needed for two sections, *Cleistogrammica* and *Ceratophorae*, for which multiple plastomes have been published previously (Funk et al. 2007; McNeal et al. 2007b; Banerjee and Stefanović 2019).

Total genomic DNA was isolated from fresh (for *Cuscuta indecora*) or silica-dried (for all other species) tissue using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987) and checked for quantity and quality using a Nano Drop 1000

Spectrophotometer (Thermo Fisher Scientific). Voucher information for each sample is listed in Fig. 1. These extractions were sequenced on an Illumina HiSeq 2000 platform (2 × 100 bp reads; McGill University and Genome Quebec, Montreal, Quebec) for *Cuscuta kilimanjari*, an Illumina MiSeq v2 platform (2 × 250 bp reads; The Centre for the Analysis of Genome Evolution and Function, University of Toronto, Toronto, Ontario) for *C. argentinana, C. purpurata*, *C. foetida pycnantha*, and *C. microstyla*, or an Illumina HiSeq 2500 platform (2 × 125 bp reads; The Centre for Applied Genomics, Sick Kids Hospital, Toronto, Ontario) for all other samples. Demultiplexing of raw reads and removal of indexing barcodes were performed at the sequencing facilities.

Plastome assembly, annotation, and computational methods

All reads were trimmed using Sickle v1.33 (Joshi and Fass 2011) with the threshold for quality set at a minimum PHRED score of 27. Trimmed reads were assembled de novo on both Geneious (vR9 or vR10; Biomatters, Auckland, New Zealand; 'produce scaffolds' and 'don't merge variants' boxes unchecked) and GetOrganelle v1.7.5 (Jin et al. 2020; -R set to 15, -k to 65, 115, and -w varied between 90 and 120) for each species. Initial annotation was conducted on Geneious R10 and then refined and confirmed manually using BLASTn (Altschul et al. 1990), BLASTx (Altschul et al. 1990) and tRNAscan-SE 2.0 (Lowe and Chan 2016) to confirm rRNA gene sequences, establish open reading frames, and to determine the boundaries of tRNA genes.

Additional approaches were attempted for assembling plastomes from *Cuscuta kilimanjari*, *C. argentinana*, *C. foetida pycnantha*, *C. microstyla*, and *C. purpurata*, all from section *Subulatae*. The coverage for putative plastid sequences in these species were found to range from very low to nonexistent. To enrich datasets for potential plastid reads over nuclear and mitochondrial reads, subsets of the raw initial read-set (between 2 and 10 million reads) were subjected to iterative BLASTn (Altschul et al. 1990) and HMMER (v3.3.1 and v3.3.2; www.hmmr.org) searches with plastome seed files from phylogenetically neighboring *C. vandervenderii* and *C. polyanthemos* used as query sequence libraries. Assemblies were then attempted using the refined read-set on Geneious R10 (both de novo and by reference mapping), GetOrganelle v1.7.5, and NOVOPlasty v4.3.1 (Dierckxsens et al. 2017).

The assembled and annotated plastomes newly obtained in this research were aligned using progressiveMauve (Darling et al. 2010) along with previously reported plastomes from sections *Cleistogrammica* (*Cuscuta obtusiflora*, GenBank accession EU189133; Fig. 1) and *Ceratophorae* (*C. chapalana*, MK887214), to identify any structural differences (McNeal et al. 2007b; Banerjee and Stefanović 2019). Selection analyses were conducted on all genes found as open reading frames or pseudogenes in the assembled plastomes. Gene sequences extracted from each species were aligned pairwise with the corresponding genes in *Ipomoea nil* (AP017304; Hoshino et al. 2016), a photosynthetic outgroup from Convolvulaceae, using Muscle (Madeira et al. 2019) on the Multiple Sequence Alignment (MSA) package v1.18 (Bodenhofer et al. 2015) on R v3.6.3 (R Core Team 2022). The ratio of substitution rates (dN/dS) for each gene was generated using the Analysis of Phylogenetic Evolution (APE) package v5.3 (Paradis et al. 2004; Popescu et al. 2012).

Results

Complete, circularly mapping plastid genomes were obtained for each species sampled from all sections in subgenus *Grammica* (Table 1) other than sect. *Subulatae* (see below). Assemblies from Geneious and GetOrganelle closely matched and corroborated one another for all species except for *Cuscuta indecora* whose plastome was only successfully assembled using GetOrganelle. The 2C genome size in *C. indecora* has been recorded to be 65.54 pg (McNeal et al. 2007a), which is an order of magnitude more than the average genome size in subg. *Grammica* and more than four times greater than the next largest species, *C. compacta* (McNeal et al. 2007a). As a result, relative coverage for plastid sequences was substantially lower in *C. indecora* and we hypothesize that this caused de novo assemblies on Geneious to fail to recover sizeable plastid contigs. Enrichment of reads by querying against seed files from other *Cuscuta* plastomes eventually yielded an adequate coverage on GetOrganelle and multiple successful assemblies were obtained.

No plastid sequences of any significant size were recovered from any of the five species sampled from section *Subulatae*. An extensive process of read-set enrichment using both BLASTn and HMMER with a variety of plastome seed files from *C. vandervenderii* and *C. polyanthemos* resulted in no plastid coverage at all during the vast majority of attempts, and a best-case coverage of 3.55 (*C. microstyla*), a value that is not sufficient for the software to go through to assembly. Assemblies run de novo on Geneious, GetOrganelle, and NOVOPlasty as well as those using reference mapping on Geneious failed to find plastid contigs.

The plastid genomes from the 14 remaining sections are between c. 78 and 87 kb long with 57–58 protein-coding genes, 23–24 tRNA genes, and the full complement of 4 rRNA genes (Table 1). They are mostly structurally identical, all maintaining a quadripartite architecture (IR-SSC-IR-LSC) and comprising inverted repeat regions typically

Table 1 Plastid genome size, structure, and content information for the 12 newly assembled and two previously published (*) species discussed in this research

Section	Species	Plastome size (bp)	Genes (protein/tRNA/rRNA)	GC$^{\Delta}$ (%)	IR$^{\pm}$ (bp)	IR$^{\pm}$ (bp %)
Californicae	*C. pacifica*	82,539	58/24/4	38.4	13,814	16.74
Cleistogrammica	*C. obtusiflora**	85,286	58/24/4	37.8	14,131	16.57
Racemosae	*C. micrantha*	85,736	58/24/4	37.3	14,445	16.85
Oxycarpae	*C. gronovii*	78,903	58/24/4	37.8	10,009	12.69
Denticulatae	*C. nevadensis*	83,906	58/24/4	37.1	14,557	17.35
Partitae	*C. haughtii*	82,941	58/24/4	36.8	13,858	16.71
Lobostigmae	*C. volcanica*	85,698	57/24/4	37.1	15,048	17.56
Obtusilobae	*C. macrocephala*	80,284	58/24/4	38.2	8,265	10.29
Grammica	*C. chinensis*	87,103	58/24/4	37.6	14,604	16.77
Prismaticae	*C. corymbosa stylosa*	79,684	58/24/4	38.7	8,685	10.90
Ceratophorae	*C. chapalana**	84,607	58/24/4	37.6	13,068	15.45
Umbellatae	*C. polyanthemos*	84,968	58/24/4	37.2	14,184	16.69
Indecorae	*C. indecora*	81,804	57/23/4	36.8	14,465	17.68
Gracillimae	*C. vandervenderii*	82,777	58/23/4	37.3	14,917	18.02

Plastomes were assembled using GetOrganelle v1.7.5 (Jin et al. 2020)

$^{\Delta}$GC% quantifies the frequency of guanine plus cytosine in the DNA sequence

$^{\pm}$IR stands for 'inverted repeat'

*Species with previously published plastomes

13–15 kb long which represent 15–18% of the total plastome size (Fig. 2, Table 1). However, in *Cuscuta gronovii*, *C. macrocephala*, and *C. corymbosa stylosa* (Fig. 2), a segment of the genome usually present in the inverted repeat region is instead present in the large single-copy region, thereby reducing the inverted repeat to 10.0, 8.3, and 8.7 kb, respectively. In *C. gronovii*, this segment is c. 3.6 kb long and includes the gene *trnI*-CAU and c. 65% of the *ycf2* gene. In *C. macrocephala* and *C. corymbosa stylosa*, this fragment is c. 6 kb long and includes *trnI*-CAU and all of the *ycf2* gene. Additionally, in *C. corymbosa stylosa*, the *trnL*-CAA and *rps7* genes are excluded from the inverted repeat region as well. *Cuscuta corymbosa stylosa* has also incurred the translocation of a c. 1.5 kb long segment of the large single-copy region, containing the genes *trnH*-GUG and *psbA*, into the inverted repeat region in between the *trnV*-GAC gene and the second exon of the *rps12* gene. Gene composition is consistent across subgenus *Grammica*, both for protein-coding genes (Fig. 3) and tRNA genes (Fig. 4), with few exceptions.

The results of the pairwise ratio of substitution rates (dN/dS or ω, calculated as the ratio of nonsynonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site for a given sequence) conducted for each gene present in the 12 plastomes newly assembled in this research are presented in Fig. 5. A table with ω values for each gene is included as Supplementary Table S1. The outgroup used for these pairwise analyses was *Ipomoea nil*, an autotrophic plant in the same family. On average, plastid gene families with bioenergetic function exhibit low ω values in all species, indicating that they remain under purifying selection. The 'housekeeping' ribosomal protein genes also generally appear to remain under purifying selection, although the genes *rps8*, *rps15*, and *rps18* appear to be under more relaxed selection than the others (average ω values of 0.59, 0.55, and 0.74, respectively). Genes with nonbioenergetic function appear to be evolving more neutrally. For example, integral membrane proteins *cemA*, *ycf1*, and *ycf2* show average ω values of 0.66, 1.02, and 1.23 in these species. The essential *accD* and *clpP* genes, known to be retained in even the most reduced plastomes because of their crucial roles in lipid biosynthesis and protein folding, respectively, exhibit ω values of 0.65 and 0.81 on average.

Discussion

Plastids in section Subulatae

Plastid genomes were successfully assembled for all species sampled in this project except for the five species from section *Subulatae*. For each of these five species, coverage of putative plastid sequences was too low to allow for assembly, even after enrichment of the sequenced reads against plastid references from *Cuscuta vandervenderii* and *C. polyanthemos*. The same read sets, however, without the need for any

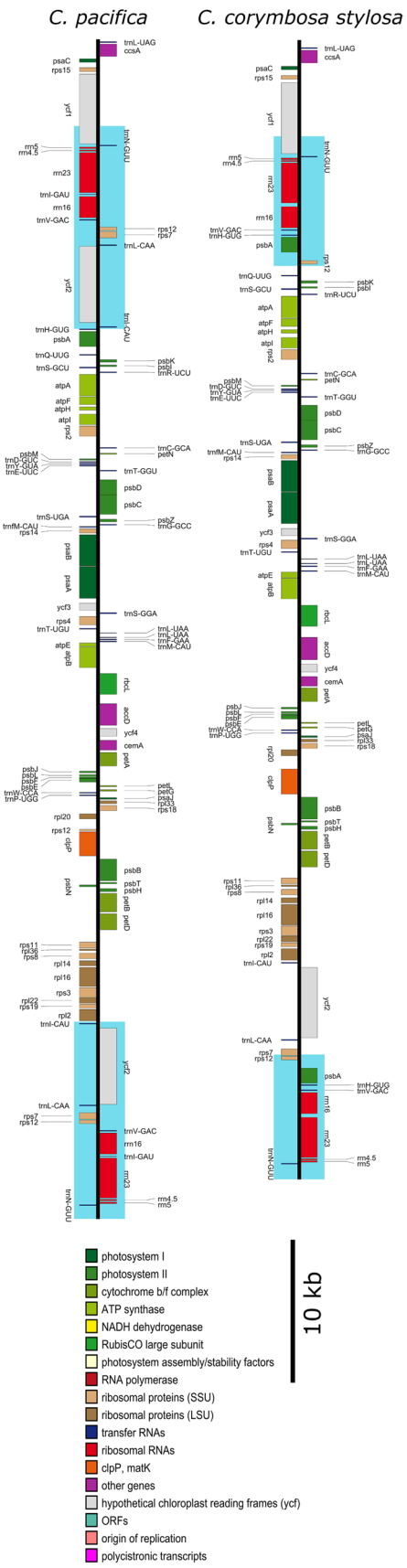


Fig. 2 Annotated plastid genomes for two species (*Cuscuta pacifica*, ▶ section *Californicae*, and *C. corymbosa stylosa*, sect. *Prismaticae*) as examples of the 12 plastomes newly assembled as part of this project. Blue boxes represent inverted repeat regions. This figure was created using OGDRAW (Greiner et al. 2019)

enrichment, were used successfully to assemble mitochondrial contigs (Lin et al. 2022a) as well as nuclear ribosomal arrays (unpublished) for all five species, indicating that the quality of the datasets is robust.

While our inability to find plastid genomes in section *Subulatae* is not proof in itself that they have been lost entirely in these species, it does indicate that if they remain, plastomes in this section may be heavily reduced, present in very low copy-number in the cell, or both. Our sampling of five species spanned the basal node of *Subulatae* (see Fig. 3 in Garcia et al. 2014) and thus covers the diversity of the clade. This is not the first indication of severe plastome sequence reduction in *Subulatae*: a slot-blot hybridization survey of 48 protein-coding genes conducted by Braukmann et al. (2013) across the genus failed to find strong positive signals for any plastid genes in 17 species they have sampled across this section. Given our inability to assemble any *Subulatae* plastid genomes, with either short-read (Illumina HiSeq; *C. kilimanjari*) or medium-read (Illumina MiSeq; *C. purpurata*, *C. microstyla*, *C. argentinana*, and *C. foetida pycnantha*) datasets, the next step in this research should be to perform long-read sequencing and to assemble total genomic scaffolds. This would allow us either to find plastomes in whatever state they are in these species, or to be closer to concluding that the plastid genome has been lost in this entire clade. While negative results are difficult to prove, this finding would be analogous to the conclusion drawn for Rafflesiaceae (Molina et al. 2014; Cai et al. 2021), and potentially only the second such case among all heterotrophic plants. As far as the research described here is concerned, it is also important to note that sect. *Subulatae* is sister to the rest of *Grammica*. In other words, all the other sections in *Grammica* form a clade and, therefore, analyses of plastome evolution as well as conclusions drawn about it in this subgenus are still robust and not confounded by the unavailability of *Subulatae* data.

Plastome evolution in the rest of subgenus Grammica

The structure and gene composition of plastid genomes from the rest of subgenus *Grammica* are summarized in Table 1 and Figs. 2, 3 and 4. In terms of structure, *Grammica* plastomes are similar to each other with the exceptions of translocation events in *Cuscuta gronovii*, *C. macrocephala*, and *C. corymbose stylosa* (e.g., Fig. 2) that have seen the movement of fragments from the inverted repeat region to the large

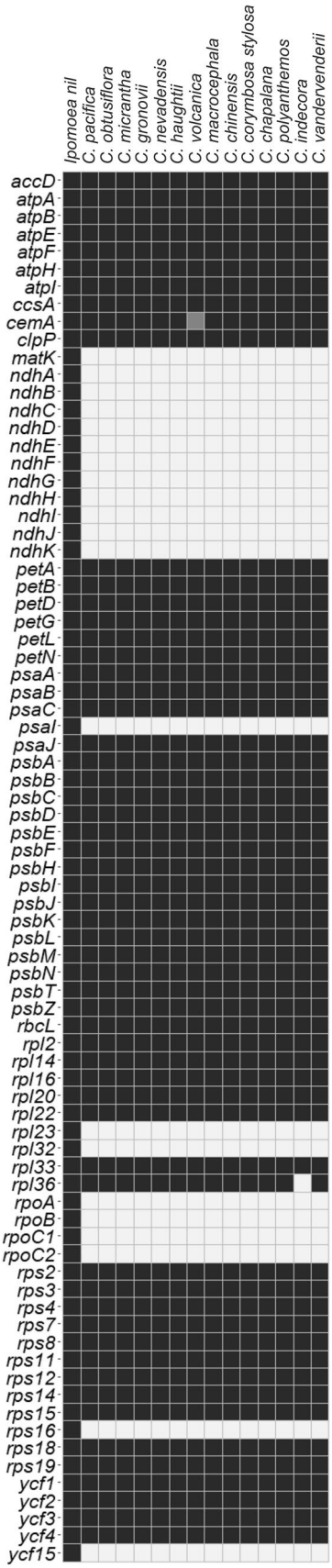


Fig. 3 A heatmap showing the presence and absence of protein-coding genes in the subgenus *Grammica* plastomes discussed in this research compared to the autotrophic outgroup *Ipomoea nil* (Convolvulaceae). Dark squares indicate that the genes are present as open reading frames and presumably functional, light squares indicate that the genes are absent, and gray squares indicate that the genes are present as pseudogenes

single-copy region. These changes have caused commensurate reductions in overall plastid size, and have presumably occurred in parallel given that these three species are not sister to one another (Fig. 1; Stefanovic et al. 2007; Garcia et al. 2014).

In terms of gene composition, protein-coding (Fig. 3), tRNA (Fig. 4), and rRNA gene content are consistent across all 14 sections, with three exceptions. First, the *cemA* gene appears to have been pseudogenized in *Cuscuta volcanica* through what appears to have been a frameshift resulting in multiple stop codons truncating the open reading frame to 171 bp for a gene that is typically 699 bp long. In a second exception, the *rpl36* gene is absent from the *C. indecora* plastome, and this absence is accompanied by a commensurate c. 150–200 bp reduction in the intergenic distance between the *rps11* and *rps8* genes relative to other section *Grammica* plastomes. Third, the *trnS*-GGA gene has been lost in both *C. indecora* and *C. vandervenderii* (Fig. 4), likely in a common ancestor of the two species given that they are representatives of sections sister to one another.

Otherwise, our results show that protein-coding sequence losses in *Grammica* are limited to those shared by the whole subgenus: the wholesale absence of the *ndh* and *rpo* gene families, the loss of the *matK* and *ycf15* genes, the loss of three ribosomal protein genes (*rpl23*, *rpl32*, and *rps16*), and the loss of the *psaI* gene. In fact, some of these losses (the *ndh* genes, *rpl23* and *rps16*) have been shown to be common to all *Cuscuta* species (McNeal et al. 2007a; Banerjee and Stefanović 2020). The *ndh* genes are primarily responsible for mitigating the effects of photo-oxidative stress through the regulation of electron flow (Peltier et al. 2016) but have been shown to be non-essential in normal, non-stress environments (Krause 2011) and are lost in most lineages of heterotrophic plants (Graham et al. 2017) and several lineages of autotrophs (Kim et al. 2015; Ruhlman et al. 2015; Sanderson et al. 2015; Silva et al. 2016; Sabater 2021). The *rpo* genes produce plastid-encoded polymerase and function in the expression of plastid genes, and are thus usually essential 'housekeeping' genes. However, Krause et al. (2003) have shown that the responsibility for the expression of subgenus *Grammica* plastid genes has been subsumed by nuclear-encoded polymerase in their absence. The gene *matK* is usually responsible for splicing group IIA introns, eight of which are typically present in the plastome. However, in *Grammica*, seven of these eight introns have been

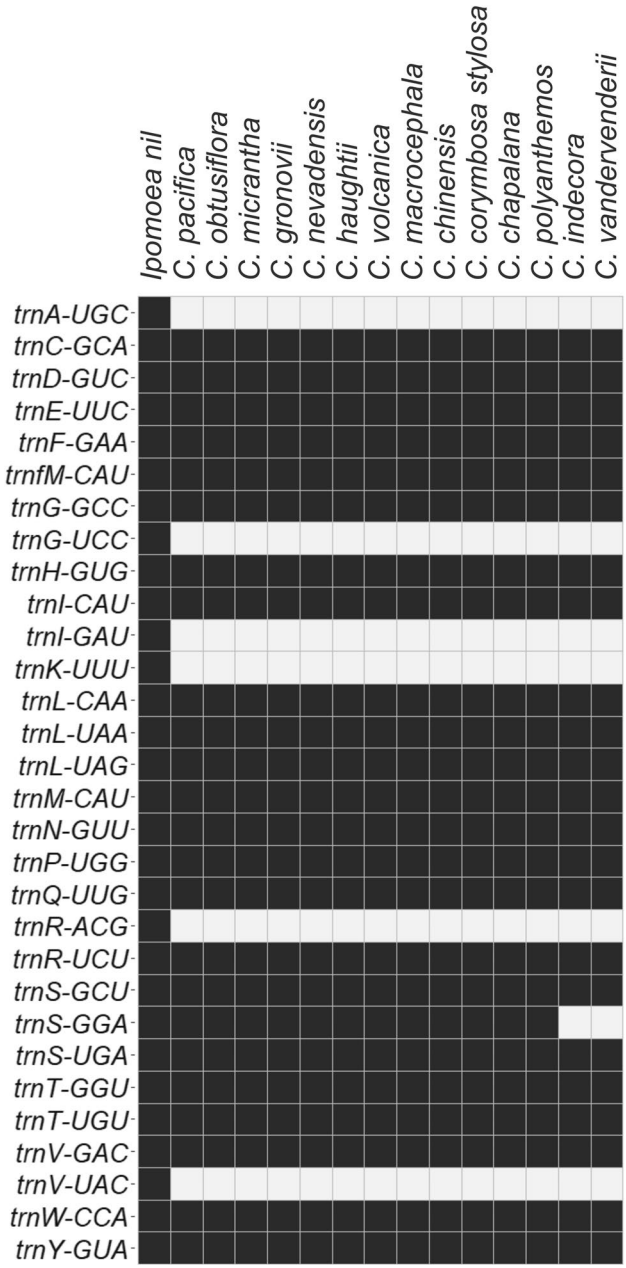


Fig. 4 A heatmap showing the presence and absence of tRNA genes in the subgenus *Grammica* plastomes discussed in this research compared to the autotrophic outgroup *Ipomoea nil* (Convolvulaceae). Dark squares indicate that the genes are present and presumably functional and light squares indicate that the genes are absent

lost (McNeal et al. 2009), leaving behind only the second intron in *clpP* which has been shown to be self-splicing (Zoschke et al. 2010), and thus, *matK*, having no role to fulfill, has followed suit (McNeal et al. 2009).

The only photosynthesis-related gene to have been lost across subgenus *Grammica* is *psaI*, a loss which has already been hypothesized based on the results of targeted amplification experiments (McNeal et al. 2007a) as well analyses of previously sequenced plastomes (Banerjee and Stefanović 2020). The *psaI* encodes one of the 14 subunits of photosystem I (Jensen et al. 2007). However, gene knockout experiments in *Nicotiana tabacum* have shown that the subunit encoded by this gene is non-essential for the energetic functions of photosystem I (Schöttler et al. 2017). Instead, it appears to play a role in stabilizing photosystem I during leaf senescence (Schöttler et al. 2017). Given that leaves in *Cuscuta* are reduced to vestigial scales as well as a heavily reduced reliance on photosynthesis in the plants of subgenus *Grammica*, the loss of such a gene would likely have no major phenotypic consequences, as appears to have been the case here.

Perhaps more striking than the genes that have been lost are those that remain. Across the subgenus, all other genes with photosynthetic function (i.e., *atp*, *pet*, *psa*, *psb*, *rbcL*, *ccsA*, *ycf3*, and *ycf4* genes) are retained. This comprehensive preservation of photosynthesis-related genes strongly suggests that *Grammica* species make use of the photosynthetic apparatus in at least some tissue and at some stage(s) in their life cycle, substantiating earlier conclusions that these plants are "cryptically photosynthetic" (McNeal et al. 2007a), and contradicting their apparent holoparasitic appearance. McNeal et al. (2007a) have suggested that photosynthetic gene products may be utilized in *Cuscuta* ovules for lipid synthesis and storage in seeds. It is also possible that limited photosynthesis may be conducted in *Cuscuta* seedlings before they attach to hosts, thus increasing their energy reserves and extending the time they have to establish haustorial connections. Whatever the reason, the continued presence of the bulk of photosynthesis genes in the subgenus *Grammica* plastomes assembled in this research means that three species in section *Ceratophorae* (*C. boldinghii*, *C. erosa*, and *C. strobilacea*; Banerjee and Stefanović 2019) along with all species in section *Subulatae* appear to be the only members of the genus *Cuscuta* to have entirely lost the ability to photosynthesize.

Selection analyses on plastome genes

The broad narrative illustrated by the dN/dS results depicted in Fig. 5 are consistent with trends observed in the other *Cuscuta* subgenera (Banerjee and Stefanović 2020), in other groups of hemiparasitic plants (Logacheva et al. 2016; Barrett et al. 2018; Banerjee et al. 2022), as well as in lineages of autotrophic plants (Guisinger et al. 2010; Wicke et al. 2011; Barnard-Kubow et al. 2014). However, there are a few noteworthy outliers. The gene *atpF* consistently exhibits higher ω values (with a range of 0.53 to 0.76) than the other *atp* genes in these species (Fig. 5). It is one of three genes involved in encoding the F_0 domain of the plastid ATP synthase complex (Wicke et al. 2011) and is thus usually considered an essential photosynthetic gene. However, *atpF*

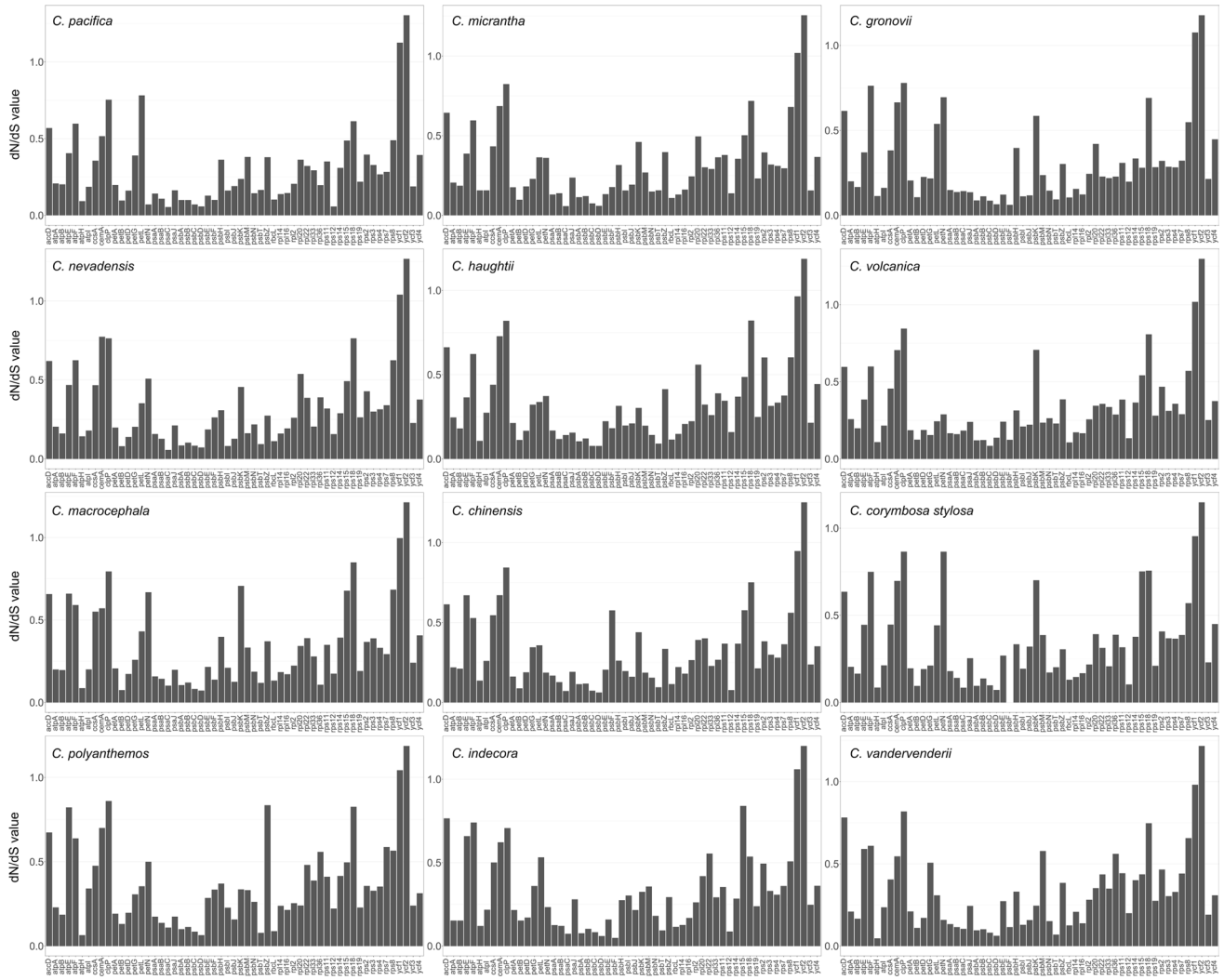


Fig. 5 Bar graphs showing the substitution ratio (dN/dS) values for all plastid genes present in the 12 newly assembled species from subgenus *Grammica* discussed in this research. The outgroup used for the pairwise analyses was the photosynthetic *Ipomoea nil* from the same family. Values below 1.0 indicate that the genes are under purifying selection, values greater than 1.0 indicate that the genes are under positive/diversifying selection, and values ≈ 1.0 indicate that selection is neutral

is the most commonly lost *atp* gene from the plastid genome (Mohanta et al. 2020), was found to have elevated ω levels relative to other *atp* genes in the genus *Krameria* (a lineage of obligately hemiparasitic plants; Banerjee et al. 2022), and was even found to be under positive selection in the autotrophic genus *Quercus* (Yin et al. 2018). Further study of selection on this gene in other groups of heterotrophic plants may be required to further establish if this elevated dN/dS ratio is a reliable trend.

In addition, four photosynthesis-related genes exhibit inconsistent ω values across *Grammica* species indicating variation in the strength of selection acting upon them in this subgenus. The *atpE* gene appears to be under relatively strong purifying selection in five species (ω values of 0.37–0.40) but a range of ascending ω values in the other species, peaking at 0.82 in *C. polyanthemos*, is indicative of more neutral selection. Similarly, *petL* and *petN*, genes encoding subunits of the cytochrome b6/f complex, feature ω values ranging from 0.24 to 0.78 and 0.07 to 0.87 respectively. The photosystem II gene *psbK* exhibits ω values ranging from 0.24 to 0.71. There appears to be no phylogenetic signal to this variation for any of these four genes, suggesting that this diversity is stochastic and idiosyncratic in nature. These results reveal that although the bulk of photosynthetic genes remain under purifying selection in *Grammica*, a small number of genes encoding parts of the photosynthetic apparatus are able to evolve more freely.

Conclusions

In summary, this research, added to previously published work (Funk et al. 2007; McNeal et al. 2007b; Banerjee and Stefanović 2019; Lin et al. 2022b), provides a near-complete picture of plastome evolution in subgenus *Grammica*. Plants in this subgenus (other than the holoparasitic species in sections *Ceratophorae* and *Subulatae*) share very similar plastid genomes with few structural and gene composition variations to differentiate them. They remain largely in stasis, retain the bulk of their photosynthetic genes, and have not progressed further down the 'slippery slope' of heterotrophic plastome evolution. The last piece of the puzzle that remains is the state of plastomes in sect. *Subulatae*, for which further research is still required to complete the examination of plastid evolution in the genus *Cuscuta*.

Author contribution statement AB and SS conceived the research conducted here. SS obtained the plant tissue and extracted DNA. AB prepared samples for sequencing and performed plastome assemblies and annotation. AB conducted the analyses and produced the first draft of the manuscript. Both authors have read and approved the final version of the manuscript.

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Data availability The plastid genomes assembled in this project were submitted to GenBank and can be accessed using the following NCBI accession numbers: *Cuscuta pacifica* OP263625, *C. micrantha* OP356701, *C. gronovii* OP448628, *C. nevadensis* OP390286, *C. haughtii* OP402843, *C. volcanica* OP402844, *C. macrocephala* OP414597, *C. chinensis* OP414596, *C. corymbosa stylosa* OP414598, *C. polyanthemos* OP441382, *C. indecora* OP414599, *C. vandervenderii* OP414600.

Declarations

Conflict of interest The authors declare no conflict of interest.

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