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INVITED REVIEW

Beyond parasitic convergence: unravelling the evolution of the organellar genomes in holoparasites

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- **Background** The molecular evolution of organellar genomes in angiosperms has been studied extensively, with some lineages, such as parasitic ones, displaying unique characteristics. Parasitism has emerged 12 times independently in angiosperm evolution. Holoparasitism is the most severe form of parasitism, and is found in ~10 % of parasitic angiosperms. Although a few holoparasitic species have been examined at the molecular level, most reports involve plastomes instead of mitogenomes. Parasitic plants establish vascular connections with their hosts through haustoria to obtain water and nutrients, which facilitates the exchange of genetic information, making them more susceptible to horizontal gene transfer (HGT). HGT is more prevalent in the mitochondria than in the chloroplast or nuclear compartments.
- **Scope** This review summarizes current knowledge on the plastid and mitochondrial genomes of holoparasitic angiosperms, compares the genomic features across the different lineages, and discusses their convergent evolutionary trajectories and distinctive features. We focused on Balanophoraceae (Santalales), which exhibits extraordinary traits in both their organelles.
- **Conclusions** Apart from morphological similarities, plastid genomes of holoparasitic plants also display other convergent features, such as rampant gene loss, biased nucleotide composition and accelerated evolutionary rates. In addition, the plastomes of Balanophoraceae have extremely low GC and gene content, and two unexpected changes in the genetic code. Limited data on the mitochondrial genomes of holoparasitic plants preclude thorough comparisons. Nonetheless, no obvious genomic features distinguish them from the mitochondria of free-living angiosperms, except for a higher incidence of HGT. HGT appears to be predominant in holoparasitic angiosperms with a long-lasting endophytic stage. Among the Balanophoraceae, mitochondrial genomes exhibit disparate evolutionary paths with notable levels of heteroplasmy in *Rhopalocnemis* and unprecedented levels of HGT in *Lophophytum*. Despite their differences, these Balanophoraceae share a multichromosomal mitogenome, a feature also found in a few free-living angiosperms.

Key words: Balanophoraceae, *Cuscuta*, horizontal gene transfer, *Lophophytum mirabile*, mitochondria, non-photosynthetic plastid, *Ombrophytum subterraneum*, Rafflesiaceae, *Rhopalocnemis phalloides*, Santalales.

INTRODUCTION

The genetic material within plant organelles contains genes necessary for photosynthesis and respiration, which are essential processes for plant survival. Examining the evolution of the plastid and mitochondrial genomes in parasitic plants helps to address key evolutionary questions, such as how parasitism developed independently many times in angiosperms and how holoparasitic plants have evolved to rely entirely on their host plants for survival. Furthermore, these studies improve our understanding of the cascading effects provoked by photosynthesis loss and the relaxation of plastid metabolism (Wicke and Naumann, 2018; Cai, 2023). Genomic and transcriptomic investigations provide information about the molecular and genomic changes that occurred during the shift from free-living to parasitic plants, such as those involving haustorium formation (Yoshida *et al.*, 2016; Teixeira-Costa, 2021), cytonuclear interactions (Ceriotti *et al.*, 2022), co-evolutionary dynamics between host and parasites, and the potential role of horizontal gene transfer (HGT) in driving molecular changes in both the nucleus and the organelles (Davis and Xi, 2015; Yang *et al.*, 2019; Wickell and Li, 2020).

EXPLORING THE DIVERSITY OF HETEROTROPHIC PLANTS: MYCOHETEROTROPHS AND PARASITES

Heterotrophy has originated multiple times throughout the evolution of the Archaeplastida, including green algae, red algae and land plants. In streptophytes, the greatest diversity of heterotrophy occurs among angiosperms (Hadariová *et al.*, 2018), where it exists in two different modes: mycoheterotrophy and parasitism. Mycoheterotrophs acquire nutrients directly from fungi or indirectly from another plant through a fungus (Graham *et al.*, 2017). Fully mycoheterotrophic plants lost their ability to photosynthesize and associate with mycorrhizal fungi, which act as a bridge between the mycoheterotroph and an autotrophic plant (Merckx, 2013). Mycoheterotrophic species evolved independently in ten angiosperm families, namely Burmanniaceae, Corsiaceae, Ericaceae, Gentianaceae, Iridaceae, Petrosaviaceae, Polygalaceae, Thismiaceae, Triuridaceae and Orchidaceae, which have undergone at least 30 independent transitions to mycoheterotrophy (Merckx and Freudenstein, 2010; Nickrent, 2020). Parasitic plants, on the other hand, obtain part or all of their nutrients from the host plant through a modified root known as haustorium, which connects the parasite with the host's vascular tissues (Kuijt, 1969; Nickrent, 2020). There are nearly 4750 species of parasitic angiosperms that feed on other plants, invading the roots or stems of their hosts (Heide-Jorgensen, 2008; Westwood *et al.*, 2010; Nickrent, 2020). Parasites are distributed across 12 angiosperm lineages, namely *Cassytha*, Cynomoriaceae, Cytinaceae, *Cuscuta*, Hydnoraceae, Krameriaceae, Lennoaceae, Mitrastemonaceae, Orobanchaceae, Santalales, Apodanthaceae and Rafflesiaceae (Fig. 1). While parasitic angiosperms can have a positive role in ecosystems, some of them, such as the Orobanchaceae *Striga* and *Orobanche* or the dodder *Cuscuta*, can cause crop damage and have been studied more intensively (Press and Phoenix, 2005; Nickrent, 2020; Zagorchev *et al.*, 2021; Albanova *et al.*, 2023).

The transition to a heterotrophic lifestyle is accompanied by numerous morphological and physiological changes that have occurred independently in distant angiosperm lineages, indicating the existence of highly convergent evolutionary trajectories. These changes include reductions in vegetative body, photosynthetic tissue and chlorophyll content. At the genetic level, convergent traits are also observed, particularly in the plastid genome. These convergent features are known as the parasitic reduction syndrome, and are shared by all heterotrophic plants, regardless of the feeding type (Wicke *et al.*, 2013; Graham *et al.*, 2017; Wicke and Naumann, 2018).

The phylogenetic affiliations of parasitic lineages have been a subject of conflict, but it is now widely accepted that the parasitic lifestyle originated 12 times independently in the evolution of angiosperms (Fig. 1). Parasitic lineages exhibit notable variability in terms of the number of species and range of nutritional modes, including hemiparasites (i.e. parasites able to photosynthesize) and holoparasites (i.e. completely non-photosynthetic parasites). For example, Orobanchaceae and Santalales have experienced remarkable expansion with more than 2000 described species, whereas others, such as Cynomoriaceae and Mitrastemonaceae, have only one or two described species (Nickrent, 2020). Among the 12 taxonomic lineages of parasitic plants, seven include exclusively holoparasites and two only contain hemiparasites (Krameriaceae and the genus *Cassytha*). The remaining three lineages (Santalales, Orobanchaceae and the genus *Cuscuta*) encompass both hemi- and holoparasites (Fig. 1).

The transition to a parasitic life form occurred at different times during the evolution of angiosperms, leading to the emergence of both older and more recent parasitic lineages. Santalales are the oldest (~109 Mya), along with Hydnoraceae (~101 Mya) and Cynomoriaceae (~100 Mya). These are followed by Rafflesiaceae (65–95 Mya), Cytinaceae (~72 Mya), Mitrastemonaceae (~78 Mya), Apodanthaceae (65-81 Mya), Krameriaceae (61.8 Mya), *Cassytha* (77 Mya) and Lennoaceae (40-67 Mya) (Naumann *et al.*, 2013; Xi *et al.*, 2013; Magallón *et al.*, 2015; Bellot and Renner, 2016; Rose *et al.*, 2018). The youngest parasitic lineages include *Cuscuta* and Orobanchaceae that originated independently 35–38 Mya (Naumann *et al.*, 2013; Magallón *et al.*, 2015; Xu *et al.*, 2022).

HOLOPARASITIC ANGIOSPERMS

Holoparasitism is the most extreme form of parasitism in plants, whereby parasitic plants lack photosynthetic activity and are completely reliant on their host for their nutritional needs. Even though ten of the 12 parasitic angiosperm lineages include holoparasitic taxa (Fig. 1), holoparasites represent ~10 % of the parasitic species, and invade either the stem or the roots of the host plants. Some holoparasites have an endophytic stage that dominates their life cycle, and four parasitic families are exclusively endoparasitic, i.e. they only grow within their host for the majority of their lifespan (Teixeira-Costa and Davis, 2021). Holoparasitism has arisen about 15 times during the evolution of angiosperms, once in Apodanthaceae, Cynomoriaceae, Cytinaceae, Hydnoraceae, Lennoaceae, Mitrastemonaceae and Rafflesiaceae, twice in *Cuscuta*, three to five times within the Orobanchaceae, and twice within the Santalales leading to Balanophoraceae *s.s.* and Mystropetalaceae (Banerjee and Stefanović, 2019; Nickrent, 2020; Xu *et al.*, 2022). Other Santalales approaching holoparasitism have also been described, such as the endoparasites *Tristerix aphyllus* (Loranthaceae) (Mauseth *et al.*, 1984), *Arceuthobium* spp. (Viscaceae) (Nickrent and García, 2009) and *Viscum minimum* (Viscaceae) (Mauseth and Rezaei, 2013). The genus *Cuscuta* encompasses ~200 species, a number of which are considered functionally holoparasitic (van der Kooij *et al.*, 2000; Banerjee and Stefanović, 2019).

Among the holoparasitic angiosperms, those in Balanophoraceae stand out for several reasons. First, it is one of the best studied holoparasitic lineages in terms of the number of characterized organellar genomes. Second, their plastomes exhibit unique features, even when considering all other living organisms: the highest AT content, the most biased codon usage and a novel genetic code change. Third, the mitochondrial genome of *Lophophytum mirabile* leads the ranking of functional horizontal transfers among those reported in the three domains of life.

The taxonomic complexity of the family Balanophoraceae: its position within the order Santalales and the phylogenetic relationships among its genera

The order Santalales is the largest lineage of parasitic plants comprising ~50 % of all parasitic angiosperms with 2428 species (Barkman *et al.*, 2007; Su *et al.*, 2015; Nickrent, 2020). It is the only order with more than one family of parasitic plants, consisting of ~15 families with worldwide distribution (Nickrent, 2020). The morphological losses and reductions that

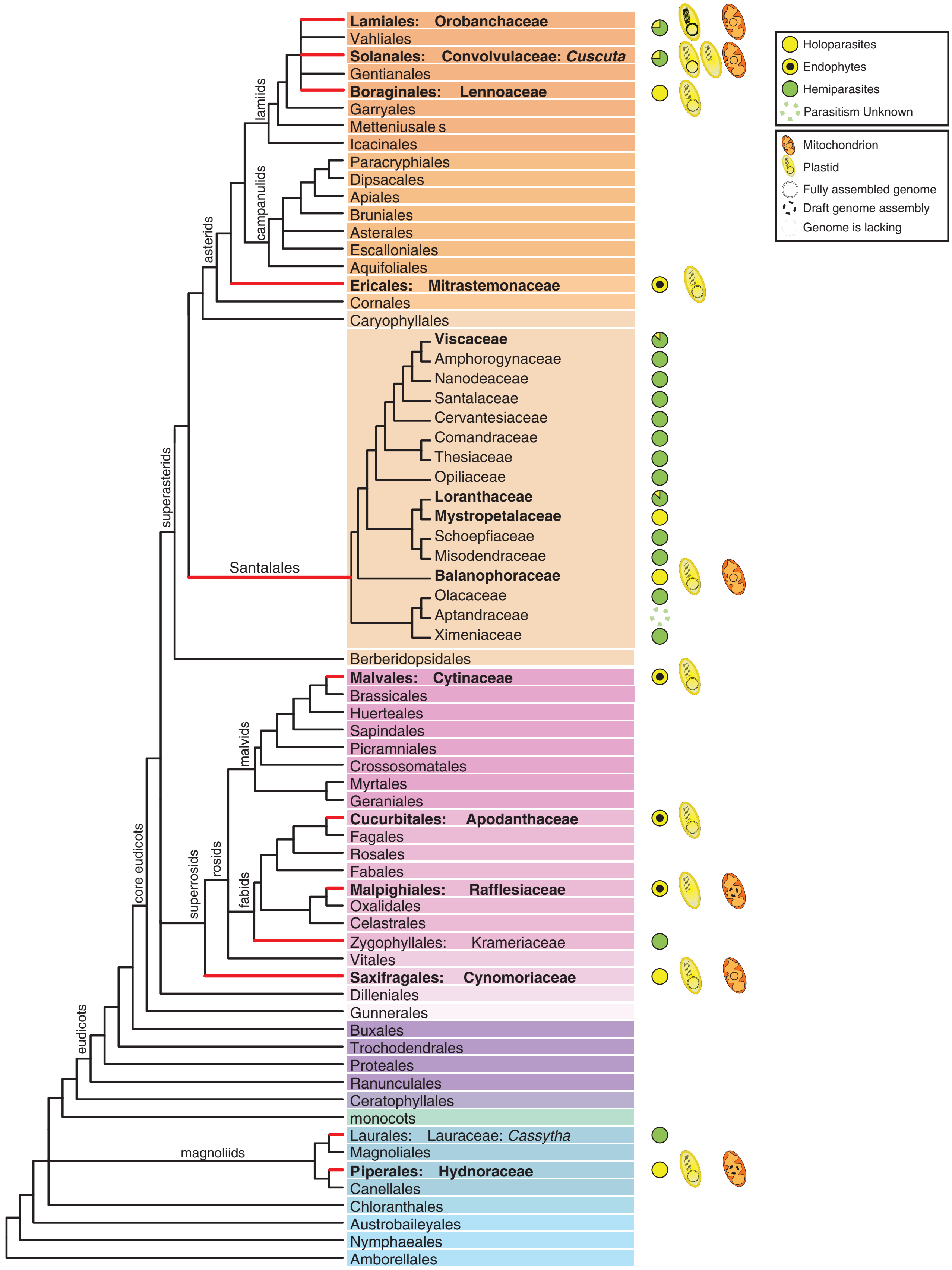

FIG. 1. Phylogeny of angiosperms depicting parasitic lineages. Red branches depict lineages including parasitic plants. Families that contain holoparasitic plants are in boldface. The availability of organellar genomic resources of holoparasites is indicated by a schematic plastid or mitochondrion. A pie chart next to the parasitic lineages shows the proportion of hemi- and holoparasitic taxa. The phylogeny follows Angiosperm Phylogeny Group IV (2016) classification and the phylogeny of Santalales is based on Nickrent (2020).

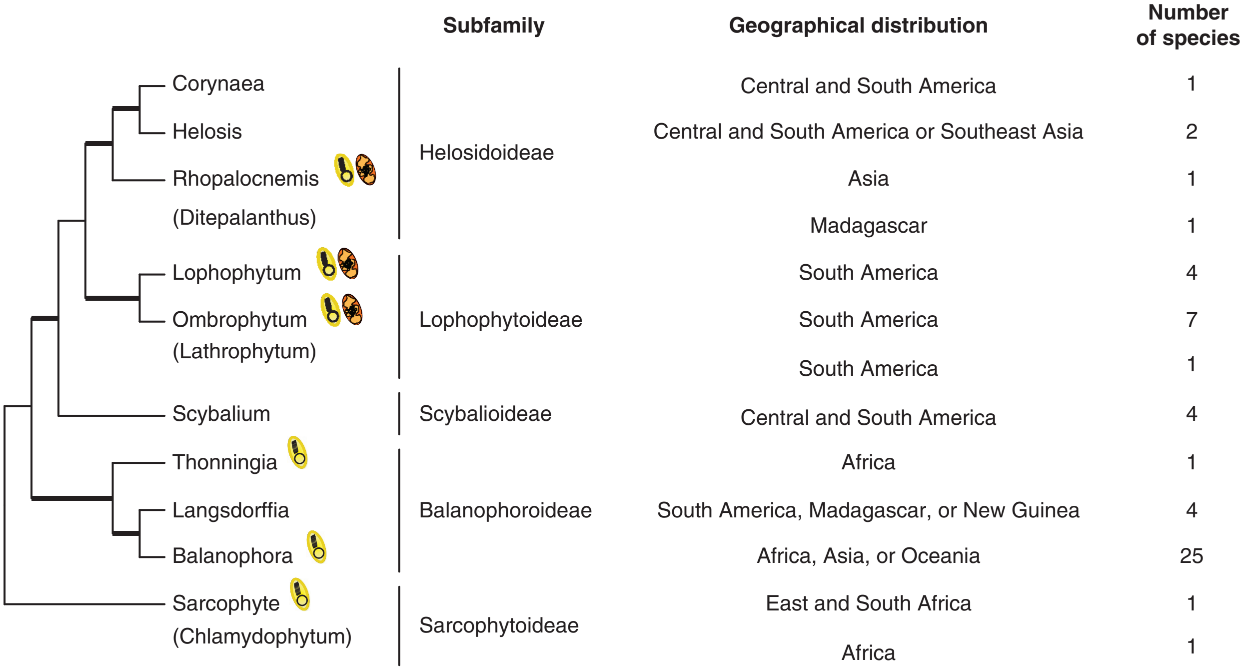

FIG. 2. Phylogeny and features of Balanophoraceae. Maximum-likelihood phylogenetic tree based on a concatenated alignment of nuclear (rDNA operon) and mitochondrial (*matR*) sequences. No molecular data are available for *Ditepalanthus*, *Lathrophytum* or *Chlamydophytum*. Thick and thin branches indicate strongly or weakly supported relationships with bootstrap support values above or below 90 %, respectively. Details of the phylogenetic analysis are shown in Supplementary Data Figure S1. The availability of organellar genomic resources of holoparasites is indicated by a schematic plastid (in yellow) or mitochondrion (in orange). The geographical distribution and number of species was taken from https://powo.science.kew.org/.

are characteristic of parasitic plants, especially in those that lost the ability to perform photosynthesis, led to remarkable convergent traits hindering an accurate phylogenetic classification. Therefore, despite strong support for the monophyly of the order (Soltis *et al.*, 1999, 2003), the placement of Santalales within the overall angiosperm phylogeny has been uncertain (Leebens-Mack *et al.*, 2019; Li *et al.*, 2019), and several reviews of their complex taxonomic history have been published (Kuijt, 1969; Nickrent *et al.*, 2010; Kuijt and Hansen, 2015; among others). The association of Balanophoraceae with Santalales dates back to the 19th century (Eichler, 1867; Van Tieghem, 1896), but it was not until the advent of molecular data that the relationships and position of Balanophoraceae began to be clarified (Nickrent and Duff, 1996; Su *et al.*, 2012, 2015; Ceriotti *et al.*, 2021). The analysis of several molecular markers from the nucleus, plastid and mitochondrial compartments supported the position of the family within Santalales, and the separation of Balanophoraceae *s.l.* into two clades, Balanophoraceae *s.s.* and Mystropetalaceae (Su *et al.*, 2015). The split of Balanophoraceae *s.l.* indicates that holoparasitism evolved independently in these two clades within Santalales, although their exact phylogenetic positions within the order remain to be elucidated.

The family Balanophoraceae *s.s.* consists entirely of root holoparasites with 13 genera (*Exorhopala* has been placed within *Helosis*; Eberwein and Weber, 2004) and 53 species distributed in tropical and subtropical regions. Unlike cormophytes, species of this family lack typical structures such as roots, stems and leaves. Instead, they develop an underground vegetative body called tuber (Gonzalez and Mauseth, 2010; Sato and Gonzalez, 2016). During reproduction, the endotrophic tissue develops a fleshy inflorescence, often cylindrical, elongated, with unisexual and tiny flowers, observed in both monoecious and dioecious species (Kuijt and Hansen, 2015; Gonzalez and Sato, 2016; Sato and Gonzalez, 2016). *Balanophora* is the most species-rich genus within the family, whereas most other genera are monotypic or encompass a low number of species (Fig. 2).

Relationships among the genera of Balanophoraceae based on molecular data (Su *et al.*, 2012, 2015; Ceriotti *et al.*, 2021; Kim *et al.*, 2023) are generally concordant with groups recognized in morphology-based classifications (Hansen, 1980; Takhtajan, 2009; Kuijt and Hansen, 2015), although a thorough analysis including all genera is still lacking. We performed a maximum-likelihood phylogenetic analysis (Fig. 2 and Supplementary Data Fig. S1) using a concatenated data set of nuclear (rDNA operon) and mitochondrial (*matR*) sequences, which represent all markers available in public databases encompassing most Balanophoraceae (Supplementary Data Table S1). The tree includes all genera except for *Chlamydophytum*, *Ditepalanthus* and *Lathrophytum*, for which no molecular data are available. The topology shown in Fig. 2 agrees with previous studies that included a smaller number of species or were based on smaller data sets (Su *et al.*, 2012, 2015; Schelkunov *et al.*, 2019; Ceriotti *et al.*, 2021; Kim *et al.*, 2023). The relationships among eight of the ten genera are well resolved, except for *Sarcophyte* and *Scybalium*, which show poorly supported affiliations. Overall, the tree shows that *Lophophytum* and *Ombrophytum* [and probably *Lathrophytum* based on morphological data (Hansen, 1980; Kuijt and Hansen, 2015)] represent a monophyletic group (named subfamily Lophophytoideae) sister to a clade formed by the subfamily Helosidoideae. On the other hand, *Balanophora*, *Langsdorffia* and *Thonningia* form a separate monophyletic group, recognized as the subfamily Balanophoroideae. *Balanophora* and *Langsdorffia* form a well-supported clade in the tree in contrast to previous classifications based on flower morphology that placed *Langsdorffia* as sister to *Thonningia* (Harms, 1935; Takhtajan, 2009). However, similarities in embryo sac development and the morphology of the pollen between *Balanophora* and *Langsdorffia* were recognized (Kuijt and Hansen, 2015) and agree with the molecular phylogeny.

Morphological analyses place *Sarcophyte* and *Chlamydophytum* as monophyletic due to their conspicuously branched inflorescences and sessile stigma (Hansen, 1980; Kuijt and Hansen, 2015). The phylogenetic tree shows the subfamily Sarcophytoideae as the earliest diverging branch, although with low bootstrap support. A recent phylogeny based on a large plastid gene data set also found *Sarcophyte* as sister to all other Balanophoraceae plastomes with strong support (Kim *et al.*, 2023). The affiliation of *Scybalium* as sister to the subfamilies Helosidoideae and Lophophytoideae recovered in the phylogenetic analysis with strong support agrees with the presence of shared morphological characters, such as the unbranched inflorescences and the presence of flowers with two stylodia and starch in tubers (Hansen, 1980; Kuijt and Hansen, 2015). The advent of DNA sequences, especially from plastids, from all the described genera in the family will help to fully resolve the speciation events in the family Balanophoraceae.

ORGANELLE GENOMES OF HOLOPARASITES

Over 50 holoparasitic genera have been identified (Nickrent, 2020), but only a few of them have been studied at the genomic level (Table 1 and Supplementary Data Note S1). Complete mitochondrial genomes (mtDNAs) have been sequenced from representatives of nine holoparasitic genera from four different lineages, whereas plastid genomes (cpDNAs) have been reported from representatives of 28 genera from nine lineages (Table 1).

Convergent traits in the plastomes of holoparasitic plants

The transition from an autotrophic to a heterotrophic lifestyle had a profound impact on plastid genomes, resulting in genomic features that differ significantly from those of free-living plants. This is particularly evident in holoparasitic plants, as the loss of photosynthetic capacity is associated with a relaxation of the evolutionary pressures on the plastome. These changes are essentially convergent and characterized by rampant gene loss, accelerations of molecular evolutionary rates and biased nucleotide compositions, in sharp contrast to the highly conserved plastid genomes of free-living plants (Wicke and Naumann, 2018). The reported plastomes from 28 holoparasitic genera share a reduction in gene content and genome size, primarily due to photosynthesis-related gene losses, and a decrease in overall GC content ranging between 11.56 % in Balanophoraceae and 38 % in Lennoaceae, *Cuscuta* and Orobanchaceae (Table 1). This contrasts with photosynthetic plants, which exhibit a genome size of 120–170 kb carrying 120–130 genes, and a GC content of 35–40 % (Ruhlman and Jansen, 2014). While the conserved large inverted repeats are believed to stabilize the plastome (Palmer and Thompson, 1982; Maréchal and Brisson, 2010), the typical quadripartite structure has been perturbed in species from seven holoparasitic lineages through the loss of the inverted repeats (Table 1). These changes often lead to genomic rearrangements (Wicke *et al.*, 2013; Bellot and Renner, 2016; Roquet *et al.*, 2016; Chen *et al.*, 2019; Schelkunov *et al.*, 2019; Su *et al.*, 2019; Ceriotti *et al.*, 2021; Jost *et al.*, 2022). In addition, the complete loss of the plastid genome has been described in the Rafflesiaceae (Molina *et al.*, 2014; Cai *et al.*, 2021) and recently suggested for species in section Subulatae within the genus *Cuscuta* subgenus *Grammica* (Banerjee and Stefanović, 2023).

Plastid evolution in Balanophoraceae: extreme nucleotide composition, strong gene content reduction, and genetic code changes. The complete loss of photosynthetic ability in Balanophoraceae led to the most extreme plastomes known to date. Initially, it was believed that Balanophoraceae had lost their plastids completely (Gedalovich-Shedletzky and Kuijt, 1990; Nickrent *et al.*, 1997). However, recent reports of the plastid genome in *Balanophora* and ultrastructural observations of these organelles (Chen *et al.*, 2019; Su *et al.*, 2019), followed by the sequencing of plastomes from *Rhopalocnemis* (Schelkunov *et al.*, 2019), *Lophophytum* and *Ombrophytum* (Ceriotti *et al.*, 2021), and *Sarcophyte* and *Thonningia* (Kim *et al.*, 2023), provide ample evidence to support the presence of reduced and aberrant plastids with even more odd plastomes (Fig. 3). Transmission electron micrographs of ovarian cells of *Lophophytum pyramidale* show the presence of non-photosynthetic plastids that accumulate starch (Fig. 3C), which are also observed with light microscopy of cells stained with iodine (Fig. 3D) (Sato and Gonzalez, 2016; Torres *et al.*, 2021). Starch-containing plastids in *Lophophytum* contrast with the description of *Balanophora* plastids that exhibit only oil droplets (Su *et al.*, 2019). Additionally, in the tissues of *Balanophora* and *Lophophytum*, large quantities of tannins were observed (Hsiao *et al.*, 1995; Torres *et al.*, 2021), which seem to have originated from plastids called tannosomes (Brillouet *et al.*, 2013).

To date, six genera of the Balanophoraceae have their plastomes sequenced (Table 2). These plastomes have undergone significant reductions in size and gene content, and their nucleotide compositions are highly biased (Chen *et al.*, 2019; Schelkunov *et al.*, 2019; Su *et al.*, 2019; Ceriotti *et al.*, 2021; Kim *et al.*, 2023). In fact, compared to hemiparasitic Santalales and free-living plants, more than 80 and 90 genes are missing, respectively. This severe gene loss probably took place in the common ancestor of Balanophoraceae, although additional gene losses occurred during species diversification (Ceriotti *et al.*, 2021; Kim *et al.*, 2023). It is worth noting that *Sarcophyte*, which diverged early in the evolution of the family (Kim *et al.*, 2023), presents the largest gene content among the examined Balanophoraceae plastomes (Table 2). Interestingly, *Balanophora* spp. achieved an exceptional level of genome compaction, resulting from shrunken intergenic regions, genes that overlap and the outright loss of *cis*-introns in the retained genes (Chen *et al.*, 2019; Su *et al.*, 2019).

A major feature shared by all plastomes of the family is the low GC content (Schelkunov *et al.*, 2019; Su *et al.*, 2019; Ceriotti *et al.*, 2021). The dramatic nucleotide bias probably

TABLE 1. *Organellar genomes in angiosperm holoparasitic lineages.*

Holoparasitic lineages and genera	Santalales, Balanophoraceae s.str.: 13 genera [c]	Santalales, Mystropetalaceae: ***Dactylanthus, Hachettea, Mystropetalon***	Piperales, Hydnoraceae: ***Hydnora, Prosopanche***	Saxifragales, Cynomoriaceae: ***Cynomorium***	Malpighiales, Rafflesiaceae s. str.: ***Rafflesia, Rhizanthes, Sapria***	Malvales, Cytinaceae: ***Cytinus, Bdallophytum*** (including ***Sanguisuga***)	Ericales, Mitrastemonaceae: ***Mitrastemon***	Cucurbitales, Apodanthaceae: ***Apodanthes, Pilostyles***	Boraginales, Lennoaceae: ***Lennoa, Pholisma***	Solanales, Convolvulaceae: ***Cuscuta*** (~200 spp) [d]	Lamiales, Orobanchaceae: 22 genera with holoparasitic species [e]
Type of parasitism	root parasite	root parasite	root parasite	root parasite	stem or root endophyte	root endophyte	root endophyte	stem endophyte	root parasite	stem parasite	root parasite
cpDNAs available in public databases?[a]	10 species in 6 genera	none	9 species in 2 genera	*Cynomorium coccineum*	Rafflesiaceae lacks a cpDNA	*Cytinus hypocistis*	*Mitrastemon kanehirai, M. yamamotoi*	*Pilostyles aethiopica, P. hamiltonii*	*Lennoa madreporoides*, *Pholisma arenarium*	34 species of *Cuscuta*	30 species in 13 genera
Main features of the cpDNAs	cpDNA size: 15,130 - 28,384 bp Gene content: 14 - 27 genes GC content: 11.56 - 21.2% Inverted repeats: absent *trnE:* present or absent Genetic code change: yes (TGA>Trp or TAG>Trp) or no	NA	cpDNA size: 24,479 - 28,658 bp Gene content: 25 - 27 genes GC content: 20.4 - 24.1% Inverted repeats: present or absent (*H. esculenta* and *Prosopanche spp.*) *trnE*: present Genetic code change: no	cpDNA size: 45,519 bp. Gene content: 27 genes GC content: 30% Inverted repeats: present *trnE*: present Genetic code change: no	NA	cpDNA size: 19,400 bp. Gene content: 23 genes GC content: 29.9% Inverted repeats: absent *trnE*: present Genetic code change: no	cpDNA size: 18,252 - 25,740 bp. Gene content: 19 - 24 genes GC content: 22.45 - 25.1% Inverted repeats: absent *trnE*: present Genetic code change: no	cpDNA size: 11,348 - 15,167 bp Gene content: 5 - 6 genes GC content: 22.7 - 24.2% Inverted repeats: absent *trnE:* absent Genetic code change: no	cpDNA size: 81,198 - 83,675 bp Gene content: 60 genes GC content: 37.1 - 38.1% Inverted repeats: present *trnE*: present Genetic code change: no	cpDNA size: 60,959 - 125,373 bp. Gene content: 84 -103 genes GC content: 35 - 38.3% Inverted repeats: present or absent (in *C. approximata* and *C. pedicellata*) *trnE*: present Genetic code change: no	cpDNA size: 45,673 - 150,504 bp Gene content: 21 - 48 genes GC content: 31.09 - 38.13% Inverted repeats: present or absent (in *Conopholis americana* and *Phelipanche ramosa*) *trnE*: present Genetic code change: No
Complete mtDNAs available in public databases?[a]	*Lophophytum mirabile*, *Ombrophytum subterraneum, Rhopalocnemis phalloides*	none	none	*Cynomorium coccineum*	none	none	none	none	none	*C. australis*, *C. campestris*, *C. epilinum*, *C. europea*, *C. japonica*	*Aeginetia indica*, *Boschniakia himalaica*, *B. rossica*, *Christisonia kwangtungensis*, *Cistanche spp.*

Table 1. *Continued*

Holoparasitic lineages and genera	Santalales, Balanophoraceae s.str.: 13 genera [c]	Santalales, Mystropetalaceae: ***Dactylanthus, Hachettea, Mystropetalon***	Piperales, Hydnoraceae: ***Hydnora, Prosopanche***	Saxifragales, Cynomoriaceae: ***Cynomorium***	Malpighiales, Rafflesiaceae s. str.: ***Rafflesia, Rhizanthes, Sapria***	Malvales, Cytinaceae: ***Cytinus, Bdallophytum*** (including ***Sanguisuga***)	Ericales, Mitrastemonaceae: ***Mitrastemon***	Cucurbitales, Apodanthaceae: ***Apodanthes, Pilostyles***	Boraginales, Lennoaceae: ***Lennoa, Pholisma***	Solanales, Convolvulaceae: ***Cuscuta*** (~200 spp) [d]	Lamiales, Orobanchaceae: 22 genera with holoparasitic species [e]
Main features of the mtDNAs[b]	mtDNA size: 170,713 - 821,919 bp Structure: multiple circular chromosomes GC content: 44.2 - 45% HGT: numerous genic and intergenic foreign regions from their hosts in *Lophophytum* spp. and *O. subterraneum*	NA	NA (HGT: 2 chimeric genes in draft mtDNAs)	mtDNA size: 1,106,389 bp Structure: multiple circular molecules GC content: 44.3% HGT: 5 foreign genes and intergenic DNA (MTPTs)	NA (HGT: several mitochondrial genes and intergenic regions, e.g. MTPTs in draft mtDNAs)	NA	NA	NA	NA	mtDNA size: 125,641 - 813,731 bp. Structure: circular GC content: 43.4 - 44.6% HGT: intergenic DNA	mtDNA size: 401,628 - 3,978,341 bp Structure: one or more circular molecules GC content: 43.5 - 47.5% HGT: intergenic DNA.

[a] Detailed information in Note S1.

[b] MTPTs refer to plastid derived DNA located within the mitochondrial genome.

[c] 13 genera: Balanophora, Chlamydophytum, Corynaea, Ditepalanthus, Helosis, Lathrophytum, Langsdorffia, Lophophytum, Ombrophytum, Rhopalocnemis, Sarcophyte, Scybalium, Thonningia.

[d] Some species of Cuscuta are considered functionally holoparasitic

[e] 22 genera: Aeginetia, Alectra (1sp; A. orobanchoides), Aphyllon, Boschniakia, Boulardia, Christisonia, Cistanche, Conopholis, Phelypaea, Epifagus, Eremitilla, Gleadovia, Harveya, Hyobanche, Kopsiopsis, Lathraea, Mannagettaea, Myzorrhiza, Orobanche, Phacellanthus, Phelipanche, Striga (some species).

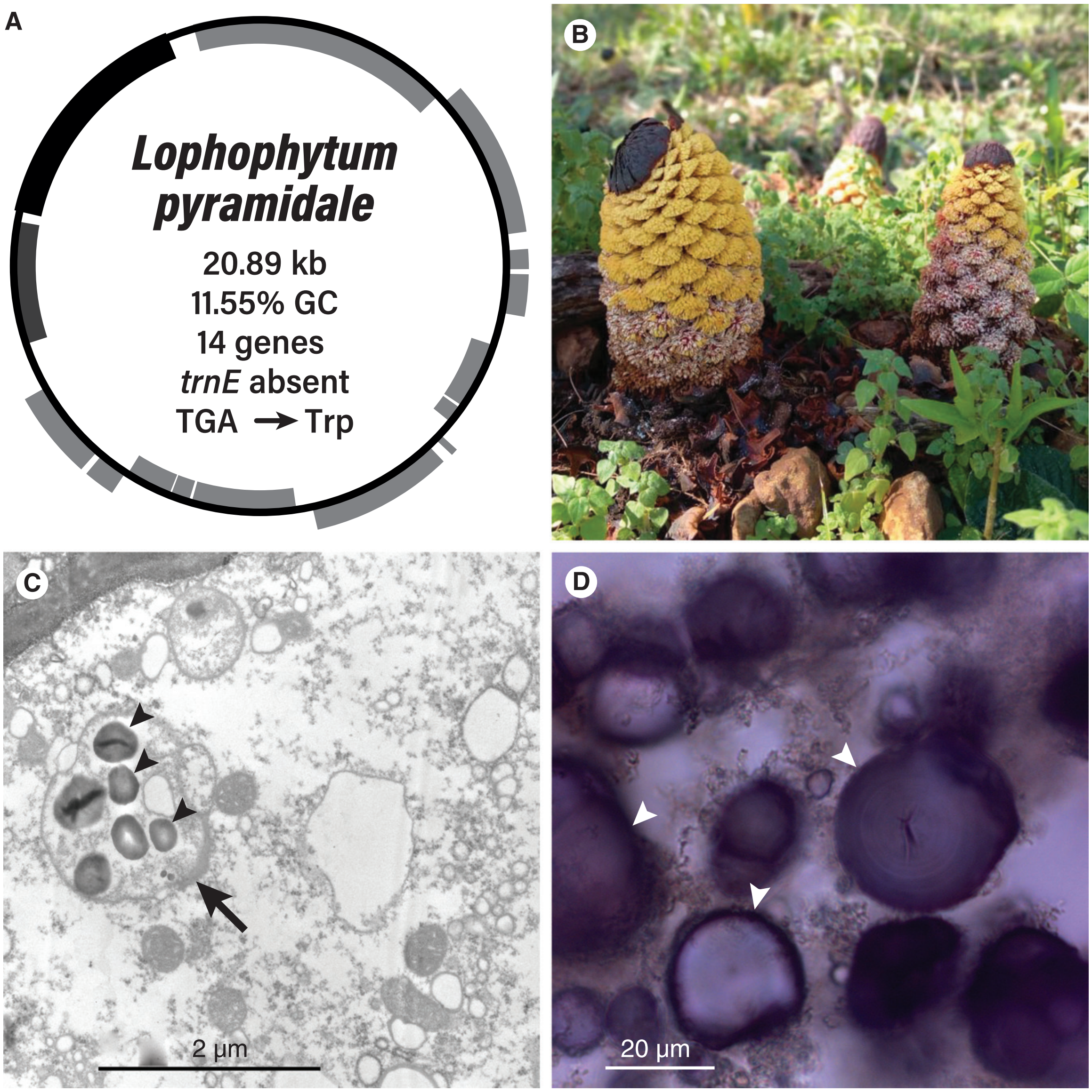

FIG. 3. *Lophophytum pyramidale*. (A) Map of the plastome with the main features shown in the centre. (B) Inflorescences of *L. pyramidale* growing in the province of Misiones (Argentina). Yellow and whitish flowers are male and female, respectively. (C) Transmission electron micrograph of an ovarian cell. The arrow points to the non-photosynthetic plastid. (D) Light micrograph of a cell from the female flower stained with iodine. Arrowheads depict starch granules.

occurred in the ancestor of the Balanophoraceae and became stronger after the divergence of *Sarcophyte*, as all other genera, except for *Thonningia*, exhibit GC contents < 14.05 % (Table 2) compared to the 36–38 % observed in other Santalales. This reduction in GC content has resulted in protein genes and proteins that are barely recognizable by comparative tools, and some Balanophoraceae exhibit the most biased codon usage and nucleotide compositions across the tree of life (Su *et al.*, 2019). Only the plastomes of Apicomplexa (apicoplasts) and some Proteobacteria show comparable levels of nucleotide bias (Wilson *et al.*, 1996; Su *et al.*, 2019). The bias in the Balanophoraceae plastomes is likely to be the result of mutational forces, probably due to a relaxation and subsequent erosion of the maintenance machinery (Schelkunov *et al.*, 2021; Ceriotti *et al.*, 2022) and to genetic drift (Su *et al.*, 2019; Ceriotti *et al.*, 2021).

Another outstanding finding is that the only two genetic code changes found in plants occur in Balanophoraceae. Specifically, TAG and TGA, which are typically stop codons, code for tryptophan in the plastomes of *Balanophora–Thonningia* and *Lophophytum–Ombrophytum*, respectively. The reassignment of the stop codon UGA to a tryptophan coding codon is the most common genetic code change in eukaryotes, and is mainly found in mitochondrial genomes (Yokobori *et al.*, 2010). In contrast, the *Balanophora–Thonningia* change has never been described before. The codon capture theory (Osawa and Jukes, 1989) has been favoured as the most plausible explanation for both genetic code changes (Su *et al.*, 2019; Ceriotti *et al.*, 2021). This hypothesis consists mainly of three steps: codon disappearance from the genome (probably due to gene loss and nucleotide bias), the stop codon is no longer recognized (which is supported by the loss of specific release factors in *Lophophytum* and *Balanophora*; Ceriotti, *et al.* 2021) and, finally, reassignment of the codons. For this last step, several unexplored complex scenarios involving tRNAs and related enzymes are possible, especially for *Balanophora* spp. and *Thonningia* (Su *et al.*, 2019; Ceriotti *et al.*, 2021). The family Balanophoraceae displays exceptional variation regarding plastid tRNA biology, which makes it a promising lineage to explore unknown aspects of tRNA metabolism in plastids and to capture the transitional steps that result in the functional replacement of plastid *trnE* and eventually lead to the loss of the entire cpDNA.

TABLE 2. *Plastid genomes of Balanophoraceae.*

Species	***Lophophytum pyramidale***	***Ombrophytum subterraneum***	***Rhopalocnemis phalloides***	***Balanophora spp.***[a]	***Thonningia sanguinea***[b]	***Sarcophyte sanguinea***[b]
cpDNA size	20,892 bp	17,313 bp	18,622 bp	15,130 - 16,056 bp	18,560 - 19,013 bp	28,372 - 28,384 bp
Gene content	14	15	15	18-20	22	27
GC (%)	11.56	14.05	13.21	11.61 - 12.95	20.4 - 21.2	19.1
Genetic code	UGA -> W	UGA -> W	*standard*	UAG -> W	UAG -> W	*standard*
Accession numbers	MT834848	MT834847	MK036331	MN414176, MN414177, KX784265, KX784266, MT901289	OQ810029-OQ810031	OQ810027- OQ810028
References	Ceriotti *et al.* 2021	Ceriotti *et al.* 2021	Schelkunov *et al.* 2019	Chen *et al.* 2020; Su *et al.* 2019	Kim *et al.* 2023	Kim *et al.* 2023

[a] Plastid genomes of five species of *Balanophora* are available: *B. fungosa, B. harlandii, B. laxiflora, B. reflexa, B. yakushimensis.*

[b] Three and two individuals of *Thonningia sanguinea* and *Sarcophyte sanguinea* are available, respectively.

The minimal plastome for survival. The characteristics of the minimum plastome needed for survival (if they exist) remain a matter of debate (Barbrook *et al.*, 2006; Molina *et al.*, 2014; Su *et al.*, 2019). To date, the complete loss of the cpDNA has been described in only a few non-photosynthetic eukaryotes, namely the Rafflesiaceae and *Cuscuta* subgenus Grammica section Subulatae among angiosperms (Molina *et al.*, 2014; Cai *et al.*, 2021; Banerjee and Stefanović, 2023), and four distantly related protists and algae: *Polytomella* (Chlorophyta; Smith and Lee, 2014), *Paraphysomonas* (Chrysophyta; Dorrell *et al.*, 2019), *Perkinsus* (Alveolata; Fernández Robledo *et al.*, 2011) and *Rhodelphis* (sister to Rhodophyta; Gawryluk *et al.*, 2019).

The plastid *trnE* gene is believed to be the *raison d'être* of maintaining a non-photosynthetic plastome due to its dual role in tetrapyrrole biosynthesis and translation (Barbrook *et al.*, 2006). However, *trnE* is missing from the plastomes of *Lophophytum*, *Ombrophytum* and *Rhopalocnemis* (Schelkunov *et al.*, 2019; Ceriotti *et al.*, 2021), marking the second independent loss of this gene in angiosperms along with the holoparasitic *Pilostyles* spp. (Bellot and Renner, 2016). A functional substitution of the plastid *trnE* by the nuclear homologue has been proposed for these Balanophoraceae (Ceriotti *et al.*, 2021), *Polytomella* (Smith and Lee, 2014) and *Paraphysomonas* (Dorrell *et al.*, 2019). In *Balanophora* spp., however, a divergent anticodonless *trnE* is present in all plastomes (Chen *et al.*, 2019; Su *et al.*, 2019), a feature only reported in the non-photosynthetic orchid *Pogoniopsis schenckii* (Klimpert *et al.*, 2022). It has been suggested that the plastid *trnE* gene in *Balanophora* and *Pogoniopsis* functions in tetrapyrrole biosynthesis but not in translation (Su *et al.*, 2019; Ceriotti *et al.*, 2021; Klimpert *et al.*, 2022), as evidenced by the loss of the anticodon and the conservation of most sequence determinants necessary for its recognition by tetrapyrrole synthesis enzymes (Ceriotti *et al.*, 2021).

Evolutionary trends in the mitochondrial genomes of holoparasitic plants

The mitochondrial genome of holoparasitic plants is expected to be impacted to a lesser extent than plastomes by the loss of photosynthesis and a parasitic lifestyle. Angiosperm mitochondrial genomes are widely conserved in terms of gene content (Richardson *et al.*, 2013), but their size and structure are dynamic, with genomes consisting of linear, circular or branched subgenomic molecules (Oldenburg and Bendich, 1996; Sloan, 2013; Kozik *et al.*, 2019) varying in size up to 11.3 Mb (Sloan *et al.*, 2012). Mitogenomes of parasitic plants have not been studied as extensively as plastomes. Complete mitogenomes are available from only nine genera that belong to four lineages of holoparasitic angiosperms, namely Cynomoriaceae, Balanophoraceae, Orobanchaceae and *Cuscuta* (Table 1). In addition, several partial mitochondrial assemblies have been reported (Table 1).

Comparisons of these mitogenomes show no evolutionary convergence in the structure, gene content, nucleotide composition, substitution rate or genome size as a result of their extreme lifestyle (Zervas *et al.*, 2019; Petersen *et al.*, 2020). Although initially, parasitic lineages were thought to exhibit accelerated substitution rates in all cell compartments (Bromham *et al.*, 2013), recent analyses revealed that most parasites have ordinary or only slightly elevated rates in their mitochondrial genes, with a few exceptions (Skippington *et al.*, 2015, 2017; Zervas *et al.*, 2019; Ceriotti *et al.*, 2022). Members of three holoparasitic lineages [Balanophoraceae, *Boschniakia himalaica* (Orobanchaceae) and *Cynomorium coccineum* (Cynomoriaceae)] exhibit a mitogenome assembled as multiple circular molecules (Bellot *et al.*, 2016; Sanchez-Puerta *et al.*, 2017; Roulet *et al.*, 2020; Yu *et al.*, 2022; Zhang *et al.*, 2023), which has also been observed in some free-living angiosperms (Alverson *et al.*, 2011; Sloan *et al.*, 2012; Rice *et al.*, 2013; Wu *et al.*, 2015).

One aspect shared by the mitochondria of holoparasites is the presence of foreign DNA (Barkman *et al.*, 2007; Xi *et al.*, 2013; Davis and Xi, 2015; Bellot *et al.*, 2016; Sanchez-Puerta *et al.*, 2017; Petersen *et al.*, 2020; Roulet *et al.*, 2020). While many free-living angiosperms have occasionally acquired mtDNA from other plants (Richardson and Palmer, 2007; Bock, 2010; Wickell and Li, 2020), the impact of HGT is higher in parasitic plant mitochondria (Petersen *et al.*, 2020). In particular, the Balanophoraceae *Lophophytum mirabile* takes foreign DNA content to new levels (see below).

Mitochondrial evolution in Balanophoraceae

Three mitogenomes of Balanophoraceae have been reported, revealing striking intrafamily differences (Table 3). In contrast to the radical features of their plastomes shared by all the Balanophoraceae analysed, the three mitogenomes investigated lack a nucleotide composition bias, have a similar gene content to that of free-living angiosperms and use the standard genetic code. Nevertheless, the mtDNAs exhibit other odd features that are either shared or variable within the family.

The three genera share a particular genomic structure, i.e. a multichromosomal arrangement of their mitochondrial genome (Sanchez-Puerta *et al.*, 2017; Roulet *et al.*, 2020; Yu *et al.*, 2022). The mitogenomes consist of 21–60 putatively autonomous circular chromosomes that range in size from 4.9 to 58 kb (Table 3). Similar to free-living species of *Silene* (Caryophyllaceae) (Wu *et al.*, 2015), the mitogenomes of Balanophoraceae present 2–36 chromosomes that lack known mitochondrial genes (Table 3). These chromosomes may carry unidentified functional elements that are relevant for mitochondrial functions (Rice *et al.*, 2013; Wu *et al.*, 2015; Sanchez-Puerta *et al.*, 2017). However, the variable presence of non-coding chromosomes across populations of *Silene noctiflora* (Caryophyllaceae) suggests that they are evolving by genetic drift (Wu *et al.*, 2015). Comparable studies are necessary to test these hypotheses in Balanophoraceae.

Mitochondrial chromosomes in *Rhopalocnemis* are fewer and smaller (<8 kb) than those in *Lophophytum* and *Ombrophytum* (Table 3), with a much smaller mitogenome (131 kb vs. 714–822 kb). The difference in genome sizes is easily explained by the remarkable horizontal acquisition of mtDNA in both ancestral and independent events in *Lophophytum* and *Ombrophytum* (Sanchez-Puerta *et al.*, 2017; Roulet *et al.*, 2020). In addition, chromosomes in *Rhopalocnemis* mitochondria display a conserved arrangement (Fig. 4). The 21 minicircular chromosomes share an identical non-coding region, which carries a sequence capable of forming a stem loop and is believed to be the replication origin for the rolling circle replication mechanism (Yu *et al.*, 2022). Minicircular organellar genomes with a similar structure, in which a replication origin is shared among chromosomes, were described in other eukaryotic lineages, such as the plastid of dinoflagellates (Zhang *et al.*, 1999) or the mitochondria of lice (Shao *et al.*, 2009) and nematodes (Gibson *et al.*, 2007), among others. Such a particular structure is the result of convergent evolution in widely disparate eukaryotic lineages. In contrast, the mitochondrial chromosomes in *Lophophytum* and *Ombrophytum* do not have conserved repeats and the replication mechanism remains unknown. Another outstanding feature exclusive to *Rhopalocnemis* is the extreme level of heteroplasmy, mainly due to short indels, which are observed across the mitogenome, including frameshift mutations in the coding regions. This heteroplasmy is registered in the majority of the DNA sequence reads and in the RNA transcripts, indicating that the functional protein coding transcripts are the minority (Yu *et al.*, 2022).

HGT IN HOLOPARASITIC PLANTS: A FREQUENT PROCESS IN MITOCHONDRIAL GENOMES

A hallmark of parasitic plants is the vascular connections established by haustoria, which allow for the transfer of water, nutrients and even nucleic acids, such as RNA and DNA, between parasitic plants and their hosts (Mower *et al.*, 2010; Xi *et al.*, 2013; Sanchez-Puerta, 2014; Davis and Xi, 2015; Góralski *et al.*, 2021). Holoparasites, in particular, typically exhibit both xylem and phloem connections with their hosts (Gonzalez and Sato, 2016; Těšitel, 2016), establishing an intimate contact that facilitates the exchange of genetic information making them more susceptible to HGT (Mower *et al.*, 2004, 2010; Davis *et al.*, 2005; Barkman *et al.*, 2007; Cusimano and Wicke, 2016; Yang *et al.*, 2016). In addition to rare parasite-to-host transfer events (Mower *et al.*, 2004, 2010; Davis *et al.*, 2005), several reports of host-to-parasite HGT have accumulated over the years (Mower *et al.*, 2004, 2010; Davis *et al.*, 2005; Barkman *et al.*, 2007; Xi *et al.*, 2013; Cusimano and Wicke, 2016; Yang *et al.*, 2016). The reasons for the larger impact of HGT on parasitic plants than on their hosts reside in the fact that parasites establish a connection with the host at an early stage of development and the acquired DNA may spread to germ cells and be transmitted to the mitochondria of offspring (Huang, 2012; Petersen *et al.*, 2020). Petersen *et al.* (2020) compiled a comprehensive list of HGT events involving parasitic angiosperms.

Among the three DNA-containing compartments in plant cells, plant-to-plant HGT is far more common in the

TABLE 3. *Mitochondrial genomes of Balanophoraceae.*

Species	***Lophophytum mirabile***	***Ombrophytum subterraneum***	***Rhopalocnemis phalloides***
mtDNA size	821,906 bp	713,777 bp	130,713 bp
Protein-coding genes (including duplicates)	35 (44)	36 (51)	36
Mitogenome structure	60 circular chromosomes	54 circular chromosomes	21 circular chromosomes
Non-coding chromosomes	36	20	2
GC (%)	44.5	44.22	45.0
Host-derived DNA (%)	from Fabaceae (59.1%), 29 foreign and 3 chimeric genes	from Fabaceae (15.4%); from Asteraceae (13.7%), 12 foreign genes	none reported
Heteroplasmy	no	no	yes
Accession numbers	KU992322-KU992380, KX792461	MT076267-MT076320	MZ269392-MZ269412
References	Sanchez Puerta *et al.* 2017; 2019; Roulet *et al.* 2020	Roulet *et al.* 2020	Yu *et al.* 2022

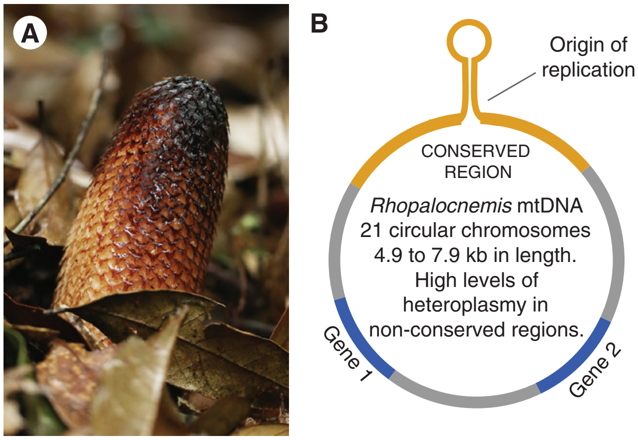

FIG. 4. *Rhopalocnemis phalloides*. (A) Photo of an inflorescence of *R. phalloides*, courtesy of Runxian Yu. (B) Map of a mitochondrial chromosome of *R. phalloides*, depicting the conserved region (in orange) shared across chromosomes, which includes a region that can fold into a stem loop and is considered the origin of replication. Genes are shown in blue.

mitochondrial than in the chloroplast or nuclear genomes (Xi *et al.*, 2013; Sanchez-Puerta, 2014; Davis and Xi, 2015). The lower frequency of HGT identified in the nuclear genome may be related to the limited availability of nuclear data. In fact, reports of HGT affecting nuclear genomes have increased in recent years (Dunning and Christin, 2020; Wickell and Li, 2020), while HGT in plastid genomes remains exceptional (Ma *et al.*, 2015; Gandini and Sanchez-Puerta, 2017; Burke *et al.*, 2018). Mitochondria from two free-living species, *Amborella trichopoda* (Rice *et al.*, 2013) and *Geranium brycei* (Park *et al.*, 2015), are particularly rich in HGT. *Amborella*, a flowering plant endemic to New Caledonia, stands out for having a 3900-kb mtDNA that carries foreign DNA equivalent to six mitochondrial genomes donated by green algae, mosses and other angiosperms, including parasitic Santalales (Rice *et al.*, 2013).

The mobile *cox1* intron

The most pervasive case of horizontal transfer among plant mitochondria involves an intron found in the mitochondrial *cox1* gene (cytochrome *c* oxidase subunit 1) of many diverse and disparately related angiosperms (Cho *et al.*, 1998). This group I intron was originally transferred to angiosperms from a fungal donor and subsequently spread among many diverse angiosperm lineages via hundreds (if not thousands) of plant-to-plant transfer events (Cho *et al.*, 1998; Sanchez-Puerta *et al.*, 2008, 2011). The *cox1* intron is commonly found in parasitic plants (Barkman *et al.*, 2007), including almost all holoparasites examined to date, with one exception in which an individual of *Cynomorium songaricum* has secondarily lost the intron (Sanchez-Puerta *et al.*, 2011). However, it is uncertain whether these plants acquired the intron horizontally from their hosts or inherited it vertically from free-living ancestors (Barkman *et al.*, 2007; Fan *et al.*, 2016). Studies of the Orobanchaceae have shown that the *cox1* intron was acquired by free-living ancestors (Fan *et al.*, 2016), while family-wide analyses of the Hydnoraceae revealed independent acquisitions in the two holoparasitic genera *Hydnora* and *Prosopanche*, but the donor lineages remain unknown (Yu *et al.*, 2023). Wider taxon sampling of free-living relatives is necessary to test these hypotheses across all plant parasitic lineages (Barkman *et al.*, 2007; Fan *et al.*, 2016).

Mitochondrial HGT in holoparasitic plants

Horizontal acquisition of mitochondrial genes from other plant mitochondria initially results in gene duplication in the recipient mtDNA. Foreign genes commonly coexist with native homologues leading to *duplicative HGT*, and the foreign copies generally become pseudogenes (Mower *et al.*, 2010; Rice *et al.*, 2013; Sanchez-Puerta, 2014). Occasionally, the foreign and native copies may undergo continuous or discontinuous gene conversion, generating chimeric gene copies, also known as *chimeric or partial replacement HGT*, which has been increasingly reported (Barkman *et al.*, 2007; Hao *et al.*, 2010; Mower *et al.*, 2010; Sanchez-Puerta *et al.*, 2019; Yu *et al.*, 2021, 2023; Darshetkar *et al.*, 2023). In rare cases, the native copy is lost and only the xenologue (foreign copy) remains, known as *full replacement HGT*. Despite the long list of horizontally acquired genes reported in angiosperm mitochondria, evidence of a functional role of these foreign genes is scarce (Bergthorsson *et al.*, 2003; Hao *et al.*, 2010; Garcia *et al.*, 2021). Again, *Lophophytum* stands out as the angiosperm with the highest number of functional foreign mitochondrial genes (see below).

The impact of HGT across complete or draft mitogenomes of holoparasites has mainly been assessed in coding regions or in plastid-derived sequences (MTPTs) or not evaluated at all (Table 1). These mitogenomes exhibit a wide range of foreign DNA content, including coding and non-coding DNA, although intergenic DNA has rarely been examined (Bellot *et al.*, 2016; Sanchez-Puerta *et al.*, 2019; Roulet *et al.*, 2020; Choi and Park, 2021; Lin *et al.*, 2022*a*, *b*; Zhong *et al.*, 2022). Among the holoparasites, the most striking examples are found within the Cynomoriaceae, the Rafflesiaceae and the Balanophoraceae exhibiting increasing levels of mitochondrial HGT. Foreign DNA might serve as a diagnostic of former host associations, as previously recognized in *Boschniakia* spp. (Zhang *et al.*, 2023), *Cynomorium coccineum* (Cusimano and Renner, 2019) and *Sapria himalayana* (Cai *et al.*, 2021).

Extent of mitochondrial HGT in holoparasitic lineages

The family Balanophoraceae exhibits divergent patterns of mitochondrial HGT, with no reported instances in *Rhopalocnemis*, and exceptional levels in the mitogenomes of *Lophophytum* and *Ombrophytum* (Sanchez-Puerta *et al.*, 2017; Roulet *et al.*, 2020). Extensive analyses of HGT across the entire mitogenomes of *Lophophytum* and *Ombrophytum* revealed that ~60 and 30 % of their mtDNA (including 29 and 12 foreign mitochondrial genes), respectively, was acquired from their hosts (Sanchez-Puerta *et al.*, 2019; Roulet *et al.*, 2020). However, these two species demonstrate disparate patterns in terms of the functional impact of HGT. While all foreign genes in *Ombrophytum* coexist with native homologues that presumably remain functional (Roulet *et al.*, 2020), *Lophophytum* lost 20 native genes, which were functionally replaced by foreign homologues (Roulet *et al.*, 2020; Garcia *et al.*, 2021). In addition, *Ombrophytum* exhibits foreign DNA from Fabaceae and Asteraceae donors, both of which are known hosts of this holoparasite. Legume-derived DNA tracts are fragmented across the genome and seem to result from older horizontal transfer events. In contrast, more recently acquired DNA from Asteraceae is concentrated in nine mitochondrial chromosomes (Roulet *et al.*, 2020).

Despite having only draft assemblies of their mitogenomes available, three species of Rafflesiaceae have been extensively studied. Foreign DNA in this lineage involves multiple mitochondrial genes, including five, five and 11 genes (28–41 %) acquired from their hosts by *Rafflesia cantleyi*, *R. tuan-mudae* and *Sapria himalayana*, respectively (Xi *et al.*, 2013). Many of these foreign genes are transcribed and appear to have replaced the native homologues, but further analyses are needed to evaluate their functionality in these parasites (Garcia *et al.*, 2021).

The mitogenome of the endophytic plant *Cynomorium coccineum* contains five foreign mitochondrial genes, as well as foreign plastid and nuclear-derived sequences obtained from two different plant host lineages (Bellot *et al.*, 2016). While three foreign genes coexist with native homologues, *atp1* and *atp8* may have replaced the native copies (Bellot *et al.*, 2016). However, the functionality of these genes was not analysed in depth. Evolutionary analyses of coding regions in the draft mitochondrial assemblies of the root holoparasites *Hydnora* and *Prosopanche* revealed a small incidence of HGT, with only short regions of the mitochondrial genes *cox1* and *atp8* replaced by foreign DNA (Yu *et al.*, 2023).

The impact of mitochondrial HGT on holoparasites of the family Orobanchaceae is very small and limited to non-coding regions with no foreign mitochondrial genes reported (Choi and Park, 2021; Zhang *et al.*, 2022, 2023; Zhong *et al.*, 2022). Finally, the complete mitogenomes of five species of *Cuscuta*, *C. australis*, *C. campestris* (Anderson *et al.*, 2021), *C. apilinum*, *C. europea* and *C. japonica* (Lin *et al.*, 2022*b*), and several draft mitochondrial assemblies (Lin *et al.*, 2022*b*) did not reveal any foreign genes. Nevertheless, mitochondrial intergenic regions of *C. japonica* were inferred as foreign based on similarity searches (Lin *et al.*, 2022*b*). In a wide gene survey of the genus *Cuscuta*, *atp1* was found to be chimeric in one of the 27 *Cuscuta* species examined (Lin *et al.*, 2022*a*).

All these findings provide further evidence of the potential functional impact of mitochondrial HGT on holoparasitic plants and highlight the importance of further research to better understand the mechanisms and implications of HGT in parasitic plant evolution. To date, the numerous cases of horizontally transferred intergenic or non-functional genes reported in holoparasitic mitochondria indicate that functional HGT is extremely rare. However, elevated rates of mitochondrial horizontal transfer may occasionally result in exceptional cases of functional foreign genes, as observed in *Lophophytum* (Garcia *et al.*, 2021).

Is mitochondrial HGT paralleled by nuclear HGT?

The large-scale horizontal transfer of mtDNA mainly from host plants to both *Lophophytum* and *Ombrophytum* (Sanchez-Puerta *et al.*, 2017; Roulet *et al.*, 2020) raises new questions that can be better addressed with the sequencing of nuclear genomes. Is the scale of nuclear HGT equivalent to that of mitochondrial HGT? Unfortunately, the nuclear genomes of species of Balanophoraceae have not been explored, and it does not seem to be an easy task since genome size estimations (e.g. 30 000 Mbp for *Rhopalocnemis phalloides*) indicate that their nuclear genomes are large (Schelkunov *et al.*, 2019). Alternatively, the impact of HGT in the nuclear compartment can be evaluated based on transcriptomic data. Next, the role, if any, of the foreign nuclear genes in the holoparasites could be determined. Given the extent of mitochondrial HGT, we expect large-scale nuclear HGT in *Lophophytum* or *Ombrophytum*, but this aspect has not yet been analysed in depth. Likewise, the impact of HGT in the nuclear genomes of Cynomoriaceae or Hydnoraceae remains unknown.

Evolutionary analyses of nuclear HGT in parasitic plants of the Orobanchaceae, Rafflesiaceae and *Cuscuta* uncovered the expression of dozens of horizontally transferred nuclear genes (Xi *et al.*, 2012; Yang *et al.*, 2016, 2019; Vogel *et al.*, 2018). Of those lineages, only Rafflesiaceae also exhibits massive mitochondrial HGT (Xi *et al.*, 2013). A transcriptomic assay of *Rafflesia cantleyi* revealed more than 40 foreign nuclear genes (Xi *et al.*, 2012), and ~1.2 % of the nuclear genome of

S. himalayana was acquired from its hosts (Cai *et al.*, 2021). Interestingly, while 84 nuclear genes were identified as foreign in the Orobanchaceae *Aegenetia indica* (Kado and Innan, 2018), its mitochondrial genome displays very limited evidence of host-to-parasite HGT and no mitochondrial genes are affected (Choi and Park, 2021; Zhong *et al.*, 2022). Similarly, the mitochondrial genomes of some *Cuscuta* species were expected to be highly impacted by HGT, based on the identification of dozens of foreign genes in the nuclear genome of *C. campestris*, which were probably acquired from different donors (Vogel *et al.*, 2018; Yang *et al.*, 2019). However, the reported impact of mitochondrial HGT in *Cuscuta* spp. is minimal, as described above. This unexpected disparity between mitochondrial and nuclear HGT levels within a single species or lineage is intriguing. Uncommon features of their mitochondrial genomes, such as a small genome size or reduced rates of DNA incorporation, may contribute to lower levels of mitochondrial HGT (Anderson *et al.*, 2021).

It has been proposed that many of the transferred genes in the nuclear genome may undergo neo-functionalization, such as roles related to invasive processes or to camouflage the invasion. Functional nuclear HGT events may represent an important evolutionary force, as they allow these organisms to expand their genetic tools and develop novel physiological capabilities (Yang *et al.*, 2019). In three members of the family Orobanchaceae, six horizontally transferred genes related to defence responses against infection or insect toxins were detected (Yang *et al.*, 2016). *Cuscuta* also has foreign genes that are related to enzymes that modify cell walls. These genes are expressed mainly in prehaustorial structures, suggesting a role in host invasion (Yang *et al.*, 2019). Comparisons between foreign nuclear genes in *Cuscuta* spp. and Orobanchaceae revealed functional and transcriptional convergence. Approximately 18 foreign nuclear genes were independently acquired from their hosts by these two parasitic lineages (Yang *et al.*, 2019). The extent of evolutionary convergence with other holoparasitic lineages remains to be elucidated.

THE CURIOUS CASE OF *LOPHOPHYTUM MIRABILE*

The mitogenome of *Lophophytum mirabile* (Balanophoraceae) stands out as being the first case of large-scale replacement HGT, in which two-thirds of the protein-coding genes have functionally supplanted the native homologues (Sanchez-Puerta *et al.*, 2017; Garcia *et al.*, 2021). Replacement HGT of the scale found in *Lophophytum* mtDNA is unprecedented for any genome or organism, including bacteria. A recipient cell faces potential barriers for the expression of foreign genes so that only rarely does a horizontally transferred gene become functional in a new genome. These barriers include recognizing a foreign promoter, accurately splicing foreign introns, and identifying and editing novel RNA editing sites. A total of 23 foreign protein-coding mitochondrial genes in *Lophophytum* have successfully overcome these challenges, leading to the functional replacement of native copies (Garcia *et al.*, 2021). The use of host-derived genes may have a positive effect on the host–parasite relationship, but could also be the result of non-adaptive forces that led to neutral replacement despite a minor reduction in fitness, or even be deleterious by generating cytonuclear incompatibilities (Garcia *et al.*, 2021; Gatica-Soria *et al.*, 2022). These replacements raise questions concerning the factors that might enable or promote such a remarkably high level of foreign gene takeover, despite the low likelihood of overcoming the aforementioned expression barriers and the expected reluctance due to the risk of disrupting coevolved cytonuclear interactions.

Doubly chimeric OXPHOS complexes in *Lophophytum*

Several of the functional foreign genes in *Lophophytum* encode subunits of mitochondrial complexes, which are also formed by other subunits encoded either in the mitochondrial or nuclear genomes. For example, the oxidative phosphorylation system (OXPHOS) of plants, responsible for cellular respiration, requires the interaction of up to 20 mitochondrial-encoded proteins and 71 nuclear-encoded proteins (Meyer *et al.*, 2019) generating multiple opportunities for mitonuclear coevolution. Thus, the replacement of coadapted subunits within OXPHOS is predicted to lead to cytonuclear incompatibilities. The complexity hypothesis establishes that genes that encode proteins that form part of multisubunit complexes are less likely to suffer functional replacement by HGT given the potential disruption of coevolved subunits (Jain *et al.*, 1999; Cohen *et al.*, 2011). In particular, proteins that have extensive molecular interactions are more recalcitrant to functional replacement. In contrast, other proteins present incredible tolerance to the functional replacement of native genes by foreign homologues, with no apparent negative effects (Woese *et al.*, 2000; Adams and Palmer, 2003; Rice and Palmer, 2006; Sloan *et al.*, 2022). That is, some systems are able to preserve the interchangeability of their components without generating incompatibilities (Sorek *et al.*, 2007; Sloan *et al.*, 2022). Four parameters that determine the incompatibility or interchangeability of systems have been proposed: the number of protein–protein interactions, the sensitivity to gene dosage, the evolutionary rate of the proteins and the relevance of the functional role (Sloan *et al.*, 2022). The multisubunit structure, the fundamental functional role of OXPHOS and the elevated expression levels contribute to the prediction that subunits of these complexes are less likely to be interchangeable and thus less likely to suffer functional replacement by foreign homologues (Park and Zhang, 2012; Sloan *et al.*, 2022). In fact, it has been shown that in hybridization events or in cytoplasmic hybrids (cybrids) produced in the laboratory, cytonuclear incompatibilities arise even between closely related species (Schmitz-Linneweber *et al.*, 2005; Meiklejohn *et al.*, 2013). A single nucleotide difference in mitochondrial genes could be responsible for strong phenotypic changes due to the disruption of OXPHOS complexes (Kim *et al.*, 2009; Meiklejohn *et al.*, 2013; Dahan *et al.*, 2014; Qi *et al.*, 2017).

Interestingly, the OXPHOS complexes are doubly chimeric in *Lophophytum* in terms of both the cell compartment in which the genes reside and the phylogenetic origin of the genes, as shown in Fig. 5B. Eleven OXPHOS subunits encoded in the mitochondrial genome of *Lophophytum* are partially or fully foreign, while eight mitochondrial-encoded subunits are native (Sanchez-Puerta *et al.*, 2017; Garcia *et al.*, 2021). Additionally, all but one (*sdh3*) of the nuclear-encoded subunits have a native origin (Gatica-Soria *et al.*, 2022). Despite the expected tight coordination between nuclear and mitochondrial genes, *Lophophytum* does not display signs of cytonuclear incompatibilities in the OXPHOS that may have arisen from the functional replacement of many mitochondrial subunits (Ceriotti *et al.*, 2022; Gatica-Soria *et al.*, 2022). Physiological studies have revealed that *Lophophytum* exhibits levels of canonical and alternative respiration comparable to those of free-living angiosperms (L. M. Gatica-Soria *et al.*, unpublished results), in contrast to hemiparasites of the genus *Viscum* (Santalales), which lack OXPHOS complex I and use alternative oxidases (Maclean *et al.*, 2018; Senkler *et al.*, 2018; Petersen *et al.*, 2022). This unexpected replacement of mitochondrial subunits without apparent disruptive consequences has been explained by the high similarity of the mitochondrial-encoded OXPHOS subunits donated by the host and those native to Balanophoraceae, which minimizes the incompatibilities in the assembly and functioning of these multiprotein complexes (Gatica-Soria *et al.*, 2022), as a low substitution rate has been proposed to mitigate the potential incompatibilities (Sloan *et al.*, 2022).

In contrast, nuclear-encoded subunits of the OXPHOS are less conserved and thus less likely to be replaced. Nonetheless,

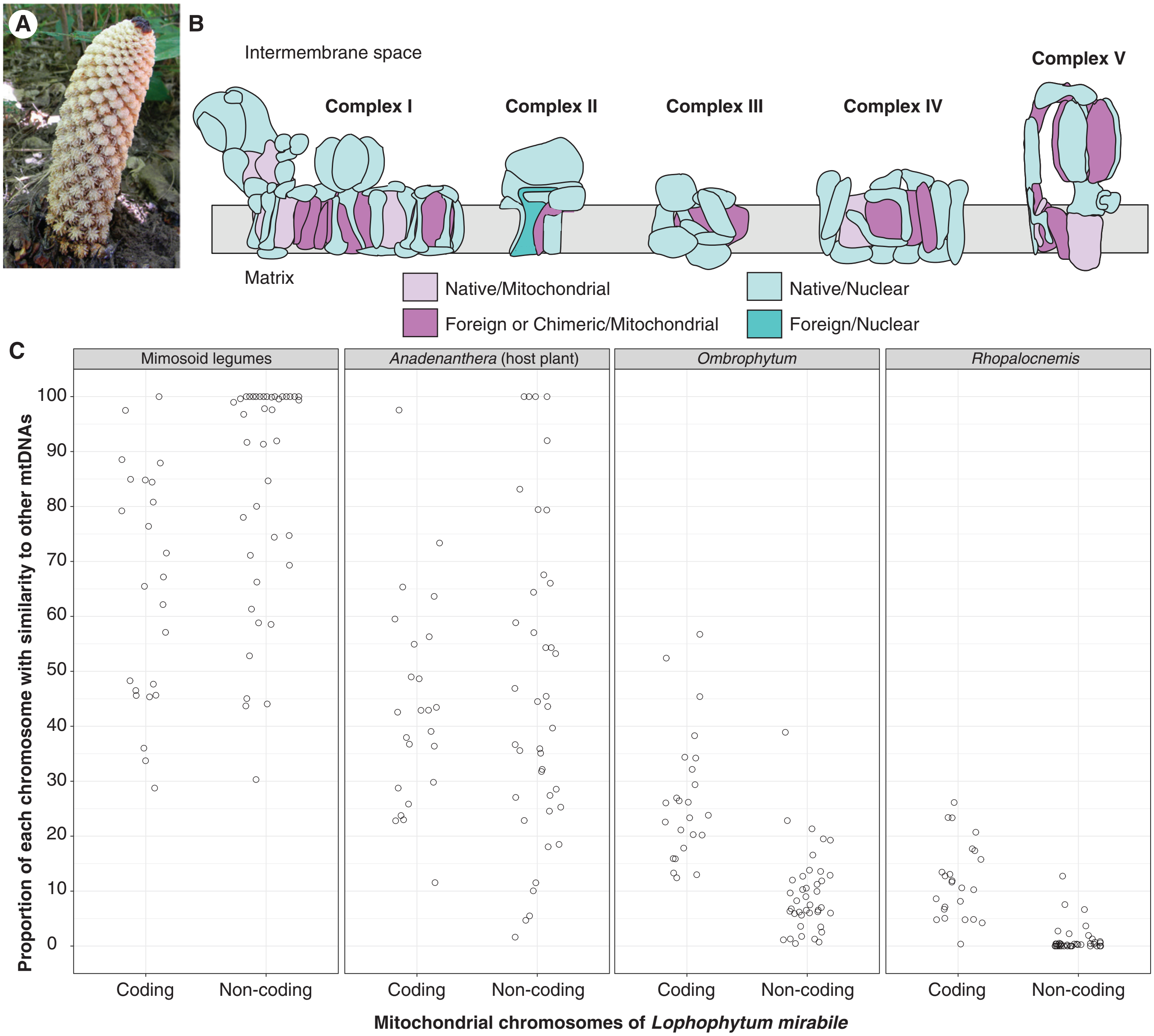

FIG. 5. *Lophophytum mirabile*. (A) Inflorescence of *L. mirabile* growing in Jujuy (Argentina). Only the staminate flowers emerged above the soil level; female flowers are located below them. (B) Diagram of the internal membrane of the mitochondria with the five OXPHOS complexes of *L. mirabile* depicting the subunits coloured according to their phylogenetic origin and the genome in which the genes are located. Figure modified from Gatica-Soria *et al.* (2022). (C) Proportion of each of the mitochondrial chromosomes of *L. mirabile* with similarity (>80 %) to the mitogenomes of mimosoids, the actual host plant *Anadenanthera colubrina*, or to the close relatives *Ombrophytum subterraneum* and *Rhopalocnemis phalloides*.

the foreign nuclear-encoded subunit (SDH3) has functionally replaced the native subunit in *Lophophytum* (Garcia *et al.*, 2021). Within OXPHOS complex II, SDH3 interacts extensively with SDH4, which is mitochondrial-encoded (SDH4) and foreign in *Lophophytum*. The cooccurrence of these two foreign genes acquired from mimosoid hosts could be the result of gene cooperation (Gatica-Soria *et al.*, 2022), as the replacement of coevolved subunits often requires the parallel replacement of other interacting subunits to preserve compatible interactions (Waller *et al.*, 2006; Monier *et al.*, 2009).

Foreign DNA in non-coding regions of the *Lophophytum* mitogenome

Deciphering the entire mitochondrial genome of *Lophophytum* also revealed that it acquired over 60 % of the non-coding sequences from mimosoid hosts, even with a single mimosoid mitogenome available for comparison (Choi *et al.*, 2019; Sanchez-Puerta *et al.*, 2019). Today, DNA from six mimosoid mitogenomes has been sequenced, including the actual host of *L. mirabile*, *Anadenanthera colubrina* (M.E. Roulet *et al.*, unpublished results). Similarity searches with each of the 60 mitochromosomes of *Lophophytum* against the mitogenomes of mimosoids, *Anadenanthera*, or the close relatives *Ombrophytum* and *Rhopalocnemis* uncovered a stunning picture (Fig. 5C). The proportion of mtDNA of *Lophophytum* with identity greater than 80 % when compared with their close relatives is strikingly low (4–10 % on average) in those chromosomes containing known genes and even lower in the non-coding chromosomes (0.7–6 % on average). These values are lower than expected for intrafamily comparisons of angiosperm mitochondrial genomes (Alverson *et al.*, 2010; Choi *et al.*, 2019; Gandini *et al.*, 2019). However, what is particularly shocking is the large number of chromosomes with most of their sequences shared with legume mitochondrial genomes. In fact, there are at least 15 mitochondrial chromosomes of *Lophophytum* composed fully of mimosoid-like mtDNA, which is especially observed among non-coding chromosomes. Although genus-wide and population-level studies are still missing in *Lophophytum*, horizontal events may have occurred not only recently, but also in the past from ancestral legume hosts. This is supported by the relatively low number of chromosomes shared with the actual host *Anadenanthera* in comparison to those shared with the six mimosoids combined (Fig. 5C).

A ‘mitochondrial fusion-compatibility’ model has been proposed to explain the horizontal transfer of DNA between plant mitochondria from unrelated land plants. This model suggests that complete mitochondria enter a foreign plant cell and that the mitogenomes recombine with the native ones (Rice *et al.*, 2013). In host–parasite relationships, entire organelles may move through the parasite haustoria, as observed in tissue grafts (Gurdon *et al.*, 2016; Hertle *et al.*, 2021). *Lophophytum* provides strong evidence for the fusion-compatibility model of mitochondrial HGT (Rice *et al.*, 2013). However, the foreign DNA in *Lophophytum* mitochondria may not always or immediately recombine with the resident genome and may be perpetuated as separate molecules (M. E. Roulet *et al.*, unpubl. data).

WHY IS THE EXTENT OF HGT DIFFERENT ACROSS HOLOPARASITES?

The intimate relationships of parasites and their hosts associated via vascular connections and the early establishment of this connection upon parasite germination provide ample opportunities for DNA transfer leading to expected high levels of HGT from host to parasite. Among parasitic plants, the degree of dependence on the host has been linked to a greater propensity for nuclear HGT (Yang *et al.*, 2016; Yoshida and Kee, 2021; Ashapkin *et al.*, 2023). Holoparasitic plants, which are in close contact with their host from an early stage of development and possess phloem connections, are more likely to experience HGT (Yang *et al.*, 2016). Indeed, the highest levels of nuclear HGT were reported for the holoparasite *Phelipanche* in comparison with other Orobanchaceae ranging from free-living to obligate parasites (Yang *et al.*, 2016; Kado and Innan, 2018; Yoshida and Kee, 2021). However, this correlation is not observed in the mitogenomes of the Orobanchaceae, which show very low levels of HGT, regardless of the degree of host dependency (Zhang *et al.*, 2022; Zhong *et al.*, 2022). A limited impact of HGT in the mitogenome has also been observed in other host–parasite relationships (Fan *et al.*, 2016; Skippington *et al.*, 2017; Zhang *et al.*, 2022; Yu *et al.*, 2023), while others have been strongly altered by HGT (Xi *et al.*, 2013; Bellot *et al.*, 2016; Sanchez-Puerta *et al.*, 2017; Roulet *et al.*, 2020).

Overall, the reported extent of mitochondrial HGT differs substantially across and within holoparasitic lineages. One possible explanation for this phenomenon is the type of parasitism, with endoparasites exhibiting higher levels of foreign DNA than exoparasites (Bellot *et al.*, 2016). This hypothesis is supported by the observation that endoparasites in the Rafflesiaceae and Cynomoriaceae carry numerous foreign mitochondrial genes. It is expected that high levels of HGT will be discovered in unexplored endoparasitic lineages, such as Apodanthaceae and Mitrastemonaceae.

Interestingly, *Lophophytum mirabile* and *Ombrophytum subterraneum* are not endoparasites but contain large amounts of mitochondrial foreign DNA (Sanchez-Puerta *et al.*, 2017; Roulet *et al.*, 2020). In fact, *Lophophytum* ranks highest for mitochondrial HGT (Sanchez-Puerta *et al.*, 2017, 2019). It is unclear why these taxa are particularly prone to HGT. Furthermore, the close relative *Rhopalocnemis* does not exhibit any evidence of foreign mtDNA (Yu *et al.*, 2022). The variability in the degree of HGT observed in the mitogenomes of root holoparasites within the family Balanophoraceae is intriguing.

The unprecedented levels of HGT observed in *Lophophytum* may be explained by its unique reproductive dynamics. Specifically, parenchymatous cells of *Lophophytum* form small groups within the cambium area of the host root. This nodule generates new tubers over time, representing a form of vegetative reproduction. For this, the initial nodule may grow through the host phloem and periderm, emerge from the root, and produce a tuber outside of the host that will eventually flower (Gonzalez and Sato, 2016). This nodule, considered the endophytic stage, acts as a reservoir of parasitic cells within the host root and exhibits intimate contact with direct exchange of substances across intercellular communications between parasite and host cells, facilitating the incorporation and inheritance of host DNA, in agreement with the weak-link model (Huang, 2012). A similar anatomical structure has been observed in *Ombrophytum* growing within the host root of Asteraceae (Mauseth *et al.*, 1992). However, the anatomy of *Rhopalocnemis* inside the host root remains unexplored to the best of our knowledge, preventing a further evaluation of the hypothesis for the differential mitochondrial HGT among Balanophoraceae.

We propose that parasitic plants with a stable endophytic phase, as described for *Lophophytum* and observed in those considered endophytes, may exhibit high levels of HGT, both nuclear and mitochondrial. Other factors that might contribute to increased levels of mitochondrial HGT include alteration of the DNA repair, replication and recombination processes, predisposition to undergo mitochondrial fusion with unrelated taxa, tolerance to fluctuations in mitochondrial genome size, phylogenetic distance between host and parasite, and occurrence of population bottlenecks. Within the mitogenome, foreign intergenic regions may be incorporated without negative consequences (except for the increase in genome size) and evolve neutrally under the whims of genetic drift. Thus, foreign non-coding regions are more likely to be found in parasitic plant mitochondria, making the examination of intergenic regions highly relevant to assess the degree of HGT.

CONCLUSIONS

In addition to their morphological similarities, plastid genomes of holoparasitic plants also display convergent features, such as biased nucleotide compositions, increased evolutionary rates and gene content reductions related to photosynthesis. In-depth comparisons among mitochondrial genomes of holoparasitic plants are limited by the amount of genomic data available. Based on the data at hand, there are no obvious genomic differences from mitogenomes of free-living angiosperms, except for a higher incidence of HGT, which is widely observed among holoparasites but is not universal.

The study of a greater diversity of parasitic lineages will increase our understanding of the evolutionary mechanisms and genomic changes experienced by parasites with variable degrees of dependence on the host. Furthermore, anatomical studies and detailed descriptions of the life cycles will be instrumental to understanding the evolutionary dynamics of HGT in holoparasitic species. Among the diversity of parasitic lineages, Balanophoraceae and endophytes, in particular, offer interesting evolutionary traits and intriguing features yet to be discovered.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following.

Figure S1. Maximum-likelihood phylogenetic analysis of Balanophoraceae based on a concatenated alignment of nuclear and mitochondrial sequences. **Table S1.** Accession numbers or references of the nuclear and mitochondrial sequences used for the phylogenetic analysis of Balanophoraceae. **Note S1.** List of species and accession numbers of taxa mentioned in Table 1.

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