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Functional resilience: An active oxidative phosphorylation system prevails amid foreign proteins in holoparasitic plants

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ABSTRACT

Mitonuclear incompatibility results from a breakdown of the coordinated function between co-evolved genes located in nuclear and mitochondrial compartments. Horizontal Gene Transfer (HGT), involving the acquisition of genes from unrelated species, can trigger mitonuclear incompatibilities when foreign gene products interact with native ones, particularly in multisubunit complexes. Recent findings highlighted rampant HGT in the mitochondrial genomes of holoparasitic plants of the genus *Lophophytum* (Balanophoraceae). In *Lophophytum*, some mitochondrial genes involved in the Oxidative Phosphorylation (OXPHOS) system were acquired from their legume hosts, unlike the nuclear-encoded OXPHOS subunits, which remain native. This unique configuration of a doubly chimeric OXPHOS, combining native nuclear-encoded subunits with both foreign and native mitochondrial-encoded subunits, raises questions regarding the potential effects of the interactions between native and foreign proteins on mitochondrial respiration activity in *Lophophytum*. We examined the mitochondrial ultrastructure, evaluated protein expression via Western blots, and analyzed cellular respiration through oxygen consumption rates and adenylate content in these holoparasitic plants. Surprisingly, our results revealed no disruption of the OXPHOS machinery or activity in *Lophophytum* despite the functional replacement of several native protein subunits by foreign homologs. Furthermore, there was no apparent impact on the OXPHOS system given their parasitic lifestyle and complete loss of photosynthesis.

1. Introduction

The mitochondria are essential organelles for eukaryotic life and play a critical role in providing the energy required to sustain a wide range of cellular processes. This energy is produced by the Oxidative Phosphorylation (OXPHOS) system, which involves a series of protein complexes located in the inner membrane of the mitochondria. Coordinated integration of dozens of proteins encoded by genes located in the mitochondrial (mtDNA) or nuclear (nucDNA) genomes is required to achieve a proper OXPHOS assembly and functionality [11,28]. Given its fundamental role in mitochondrial function, mitochondrial and nuclear (ie mitonuclear) coevolution of OXPHOS subunits represents a topic of profound interest in the field of cell and molecular biology [20].

Mitonuclear incompatibility refers to the disruption of the coordinated function between coevolved genes located in the two cellular compartments and can lead to adverse fitness effects [21,8]. This incompatibility has been documented across a range of eukaryotic organisms, from plants to animals [55], mainly affecting the functionality of OXPHOS and leading to modifications in ATP synthesis and in the production of reactive oxygen species [8].

Horizontal Gene Transfer (HGT), which involves the acquisition of genes from distantly related species, can trigger mitonuclear incompatibilities when the products of foreign genes interact with native ones to form multisubunit complexes [10,55]. Unlike hemiparasitic

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plants, holoparasites completely lack photosynthetic capacity, are completely dependent on their host plants, and are particularly prone to HGT [47]. Recently, rampant HGT was described in the mtDNA of holoparasitic plants of the family Balanophoraceae [46,48,49]. For example, the holoparasite *Lophophytum mirabile* presents a mtDNA composed of almost 75 % foreign DNA acquired from their legume hosts (Roulet et al., unpublished results). This includes fully or partially (ie chimeric) foreign mitochondrial genes encoding proteins involved in OXPHOS complexes that functionally replaced the native homologs [15]. In contrast, the numerous OXPHOS subunits encoded in the nucDNA of *L. mirabile* are native [16]. The presence of a doubly chimeric OXPHOS in *L. mirabile,* formed mainly by native nuclear-encoded subunits and foreign and native mitochondrial-encoded subunits, raises questions about the evolution and physiology of this parasitic plant. The interaction between native and foreign proteins may result in incompatibilities between mitochondrial subunits of different phylogenetic origin, as well as in mitonuclear interactions. These incompatibilities could, in turn, affect the functionality of OXPHOS and consequently lead to a reduction of *L. mirabile* cellular respiration.

In contrast, the closely related holoparasite, *Ombrophytum subterraneum*, carries all native functional mitochondrial genes despite large-scale HGT from their hosts [46]. The maintenance of co-evolved native subunits in both the mitochondria and nuclear genomes of *Ombrophytum* implies that the functionality of OXPHOS would not be affected by the mitochondrial HGT events. However, an intriguing consideration arises: the parasitic lifestyle or the loss of photosynthesis could affect or contribute to the relaxation of selection pressure on the OXPHOS system, resulting in an altered respiration activity in the holoparasitic Balanophoraceae. Some unicellular parasitic protists exhibit diminished respiratory capacities owing to the loss or drastic reduction of certain components of the OXPHOS [22,33,50]. Among parasitic angiosperms, studies on the OXPHOS system are rare, except for those on hemiparasitic plants of the genus *Viscum* (Santalales) that lost the genes encoding subunits of complex I [41,42,53]. Dysfunction of the OXPHOS in parasitic eukaryotes often leads to an increased dependence on alternative pathways in the electron transport chain [13,34,50]. On the other hand, the colorless chlorophyte *Polytomella* lost its alternative oxidase along with its photosynthetic activity and exhibits smaller respiratory complexes [27,45,63].

We aim to characterize the OXPHOS composition and activity in parasitic plants that completely lost photosynthesis and to evaluate the effect of HGT on the OXPHOS system in holoparasitic species with contrasting content of foreign functional genes. We included *Ombrophytum* with no functional foreign genes, and two species of *Lophophytum* that exhibit rampant replacement of native mitochondrial subunits by foreign homologs, which is expected to disrupt mitonuclear compatibility. These three holoparasites form a subterranean vegetative body that grows attached to the roots of their hosts and only the inflorescences emerge above the soil level [17]. We characterized the ultrastructure of the mitochondria, examined the expression of certain proteins that constitute the respiratory complexes by Western blot, evaluated cellular respiration through the analysis of oxygen consumption rate, and measured ATP production and total adenylate content. We also investigated the expression and phylogenetic origin of nuclear-encoded OXPHOS subunits and related genes (cytochrome c maturation -ccm- system and alternative respiratory pathways) in the holoparasites. Surprisingly, there is no evidence of a disruption in the OXPHOS structure or activity in these holoparasites. This indicates that neither the functional replacement of several OXPHOS native subunits by foreign ones in *Lophophytum* spp. nor their parasitic lifestyle or lack of photosynthesis have an adverse effect on mitonuclear compatibility and respiration activity.

2. Materials and methods

2.1. Parasitic plant sample collection

Individuals of *L. mirabile* were collected on December 29, 2021 in Calilegua, Jujuy, Argentina (23°56'26.5''S, 64°55'43.8''W), growing on roots of the mimosoid legume *Anadenanthera colubrina*. Individuals of *L. pyramidale* were collected on August 29, 2021 in San Ignacio, Misiones, Argentina (27°15'26.8''S, 55°32'43.9''W), parasitizing roots of the mimosoid legume *Parapiptadenia rigida*. Finally, individuals of *Ombrophytum subterraneum* were collected on April 19, 2022 in Rodeo, Jujuy, Argentina (22°15'57.39''S, 65°55'50.83''W), parasitizing roots of *Parastrephia* sp. (Asteraceae).

2.2. Transcriptomic data and BLAST searches

The RNA sequence data of *L. pyramidale* and *Ombrophytum* was taken from Roulet et al. (unpublished results) and Ceriotti et al. (unpublished results), respectively. Briefly, total RNA was extracted from male and female flowers of individuals of *L. pyramidale* and *Ombrophytum* collected in San Ignacio (Misiones, Argentina) and Rodeo (Jujuy, Argentina), respectively, using a CTAB protocol modified for highly viscous samples rich in polysaccharides [65] and purified using the RNAqueous kit (Invitrogen). Total RNA was depleted of rRNA using a custom kit at BGI. RNA sequencing was performed with DNBseq (100 bp PE reads, PCR-free library and 40 Gb clean data) technology at BGI (Hong Kong). The RNAseq data was curated using Trimmomatic [5], resulting in 240,855,027 and 240,268,982 reads from *L. pyramidale* and *Ombrophytum,* respectively. The RNA reads were assembled using Trinity v.2.8.4 [18].

We performed BLASTn searches using nuclear gene sequences from *L. mirabile* or Arabidopsis involved in OXPHOS complexes and genes related as bait to find homologous genes in the transcriptomes of *L. pyramidale* and *Ombrophytum*. We selected those transcripts whose hits had an identity greater than 60 % and a query coverage greater than 70 % to ensure we got all the homologs. We identified ORFs within the selected transcripts using the program GETORF (EMBOSS: GETORF). We also searched for the bridging domain present in complex I [27] in *L. mirabile* RNAseq data (Bioproject PRJNA601125), which was not included in a previous study [16].

2.3. Phylogenetic analyses

Multiple sequence alignments were prepared to infer the phylogenetic origin of nuclear genes of *Lophophytum* spp. and *Ombrophytum*. Nucleotide sequences from diverse angiosperms were obtained from GenBank databases by searching the subunits involved in OXPHOS and related genes listed by [36] and Klusch et al. [27] using the Arabidopsis gene identifier. Nucleotide sequences of genes were aligned using prank v.170427 (parameters: -codon -F) [32]. Misaligned regions were removed with BMGE v.1.12 (parameters: -t CODON -m BLOSUM) [12]. Maximum likelihood analyses were performed with RAxML v.8.2.11 [58] using the GTRGAMMA model and including 1000 rapid bootstrapping pseudoreplicates. Trees were visualized using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Transmission electron microscopy

The ultrastructure of the mitochondria in the holoparasite *L. pyramidale* was analyzed using transmission electron microscopy. Inflorescences of *L. pyramidale* were fixed in glutaraldehyde (2 %) - paraformaldehyde (2.5 %) in 0.1 M phosphate buffer, pH 7.2. The material was then placed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 2 h at 2 °C, followed by dehydration with alcohol solutions and inclusion in Spurr's resin. Ultrathin sections were contrasted with aqueous uranyl acetate and lead citrate [38] and observed with a Zeiss

EM 900 transmission electron microscope.

2.5. Western blot analysis

For Western blot analysis, 50 mg of inflorescences from parasitic plants and leaves from *Arabidopsis thaliana* (Col-0) were used to prepare total soluble protein extracts with three biological replicates, following [44]. Polyvinylidene difluoride (PVDF) membranes were hybridized with polyclonal rabbit antibodies against Cytochrome c (CYTc, AS08 343A, Agrisera; dilution 1:5000), mitochondrial Voltage-dependent anion-selective channel protein 1–5 (VDAC1-5, AS07 212, Agrisera; dilution 1:5000), Actin (AS 132640, Agrisera; dilution 1:10000), Cytochrome c oxidase subunit 1 (COXI, homemade, dilution 1:3000), Cytochrome c oxidase subunit 2 (COXII, homemade, dilution 1:3000), Beta subunit of ATP synthase (ATPB, AS 05 085-10, Agrisera; dilution 1:3000), and Alternative Oxidase proteins 1 and 2 (AOX1/2, AS 04054, Agrisera; dilution 1:3000), followed by detection with the HRP-conjugated secondary antibody anti-Rabbit (ThermoFisher Scientific A16110; dilution 1:50000) and SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA, USA). The incubation time for blockage and primary and secondary antibodies lasted 1 h. The antibodies and milk were diluted in 1X Tris Buffered Saline (TBS) containing 0.1 % Tween 20.

2.6. Respiration measurements

Inflorescences of parasitic plants (and leaves of *Arabidopsis thaliana* (Col-0) as a positive control) were used to evaluate oxygen consumption in a liquid-phase oxygraph system (Hansatech, Norfolk, UK), according to Racca et al. [44] with some modifications. Briefly, 20–60 mg of samples were kept in darkness for 30 min and transferred to the reaction vessel containing 2 mL of respiration buffer (10 mM potassium phosphate, pH 7.2) under agitation. Oxygen consumption was monitored at 25 °C using a Clark oxygen electrode (Hansatech, Norfolk, UK). The inhibitors, KCN (10 mM) or Salicylhydroxamic acid (SHAM) (10 mM), were directly added to the reaction vessel during the measurements. The capacity of the COX pathway was determined as the O_2 uptake sensitive to KCN in the presence of SHAM. The capacity of the alternative pathway was determined as the O_2 uptake sensitive to SHAM in the presence of KCN. Basal or residual respiration was measured in the presence of both inhibitors. Oxygen consumption was measured five times independently in inflorescences (with three biological replicates each) from the holoparasites *L. mirabile, L. pyramidale, Ombrophytum,* and in Arabidopsis leaves as control.

2.7. ATP and total adenylate measurements

Extraction and quantification of adenylates were done according to Racca et al. [44] with minor modifications. Briefly, 50 mg of flowers of the parasites were homogenized with 400 µl methanol:chloroform (1:1) and phase separation was induced by addition of 250 µl H_2O. The aqueous phase was used for adenylate quantification by High Performance Liquid Chromatography (HPLC) on a LC-2030 C Plus (Shimadzu, Japan), according to Menegollo et al. [35]. The samples (10 µl) were injected in a Luna Omega C18 column (150 mm × 4.6 mm×3 µm particle size; Phenomenex, USA) with a PS C18 SecurityGuard cartridge (Phenomenex). Separation was performed at 30 °C with a flow rate of 0.75 ML min^{-1} and a mobile phase consisting of 0.1 M $NH_4H_2PO_4$, pH 6.0 %, and 1 % (v/v) methanol. Peaks corresponding to ATP, ADP, and AMP were identified by adding standard compounds (Sigma-Aldrich). Quantification was performed with a standard curve of known concentrations and LabSolutions Lite software (Shimadzu) using absorbance at 254 nm. The adenylate energy charge is expressed as the proportion [(ATP + 0.5 ADP)]/ [(ATP + ADP + AMP)] and quantifies the amount of metabolic energy stored in the adenine nucleotide pool [60]. This value ranges from 0 (only AMP is in solution) to 1 (all AMP molecules have been converted to ATP).

2.8. Statistical analyses

Data were analyzed using a one-way ANOVA test (Tukey's or Sidak's post hoc test, $P < 0.05$). Comparisons between two samples were performed by Student's t-tests ($P < 0.05$).

3. Results

3.1. OXPHOS complexes in Lophophytum and Ombrophytum encompass all the standard subunits

A complete set of subunits conforming to the five classical OXPHOS complexes were identified in *Lophophytum* spp. and *Ombrophytum*, including those encoded in the nuclear or mitochondrial genomes (Fig. 1; Table S1). Also, the three holoparasites present homologs for most of the ccm system and the alternative respiratory electron transport pathways (alternative oxidases -AOX- and dehydrogenases -altND-) previously identified in Arabidopsis mitochondria (Fig. 1; Table S1). Three homologs for Arabidopsis AOX genes are present in *Lophophytum* spp. (two *aox1* and one *aox2*) and two in *Ombrophytum* (*aox1* and *aox2*). Also, three altND genes were detected in the three species (two *nda1/nda2* and one *ndb2/ndb3*). The ORF lengths within the transcripts of the holoparasites were comparable to those of Arabidopsis (Table S1). Thus, these plants present over 97 % of the subunits that make up the mitochondrial respiratory complexes in Arabidopsis, which include 71 nuclear-encoded subunits. The subunits missing in these transcriptomes are γ Carbonic anhydrase 3, SDH8, NDB1, NDB4, and NDC (Table S1). These missing subunits would not affect the functionality of OXPHOS because they are variably present across angiosperms [19,23] and are less expressed isoforms [26,61].

3.2. The nuclear-encoded genes of the OXPHOS system in the holoparasites are native

Phylogenetic analyses showed that all nuclear-encoded OXPHOS subunits and those proteins involved in alternative pathways from *Lophophytum* spp. and *Ombrophytum* are native, except for *sdh3* (Fig. 2; Fig. S1). The gene *sdh3* is foreign and nuclear-encoded in *Lophophytum* spp., while it is native and mitochondrial-encoded in *Ombrophytum* as reported by Roulet et al. [46]. The phylogenetic analysis showed that the *sdh3* gene of *Lophophytum* spp. exhibit high bootstrap support (90 %) with the nuclear-encoded *sdh3* from mimosoids, and are sister (BS 79 %) to the *sdh3* of *Anadenanthera colubrina* (Fig. 2). The sister relationship of *L. mirabile* and *L. pyramidale* in the *sdh3* phylogeny suggests that this gene was horizontally transferred from a mimosoid legume host to the ancestor of *Lophophytum* spp.

Native genes from the three holoparasites form strongly supported clades (generally with bootstrap support >90 %), in which the two species of *Lophophytum* are sister to *Ombrophytum* (Fig. S1). The native genes of the Balanophoraceae are expected to be sister to the asterid clade in phylogenetic terms [1,29] or to the rosids [39]. However, in most cases, the clade of Balanophoraceae does not affiliate with any other lineage with strong bootstrap support. A few duplicates of the OXPHOS genes were recognized in these holoparasites (Table S1), which result from recent or older gene duplication events. Duplicates of *cox6b* and *nda* in *L. pyramidale* and *Ombrophytum* are shared with *L. mirabile* [16] and other angiosperms (Fig. S1; Table S1). Recent duplicates of the genes *atp20, cytc,* and *aox1* in *Lophophytum* spp. are missing in *Ombrophytum* (Fig. S1; Table S1). *Ombrophytum* has additional copies of *sdh2* and *qcr6* compared to *Lophophytum* spp., which are shared with other angiosperms (Fig. S1; Table S1). The phylogenetic origin of the mitochondrial-encoded genes involved in OXPHOS (Fig. 1) has been previously inferred for the three holoparasites ([15,46], unpublished results).

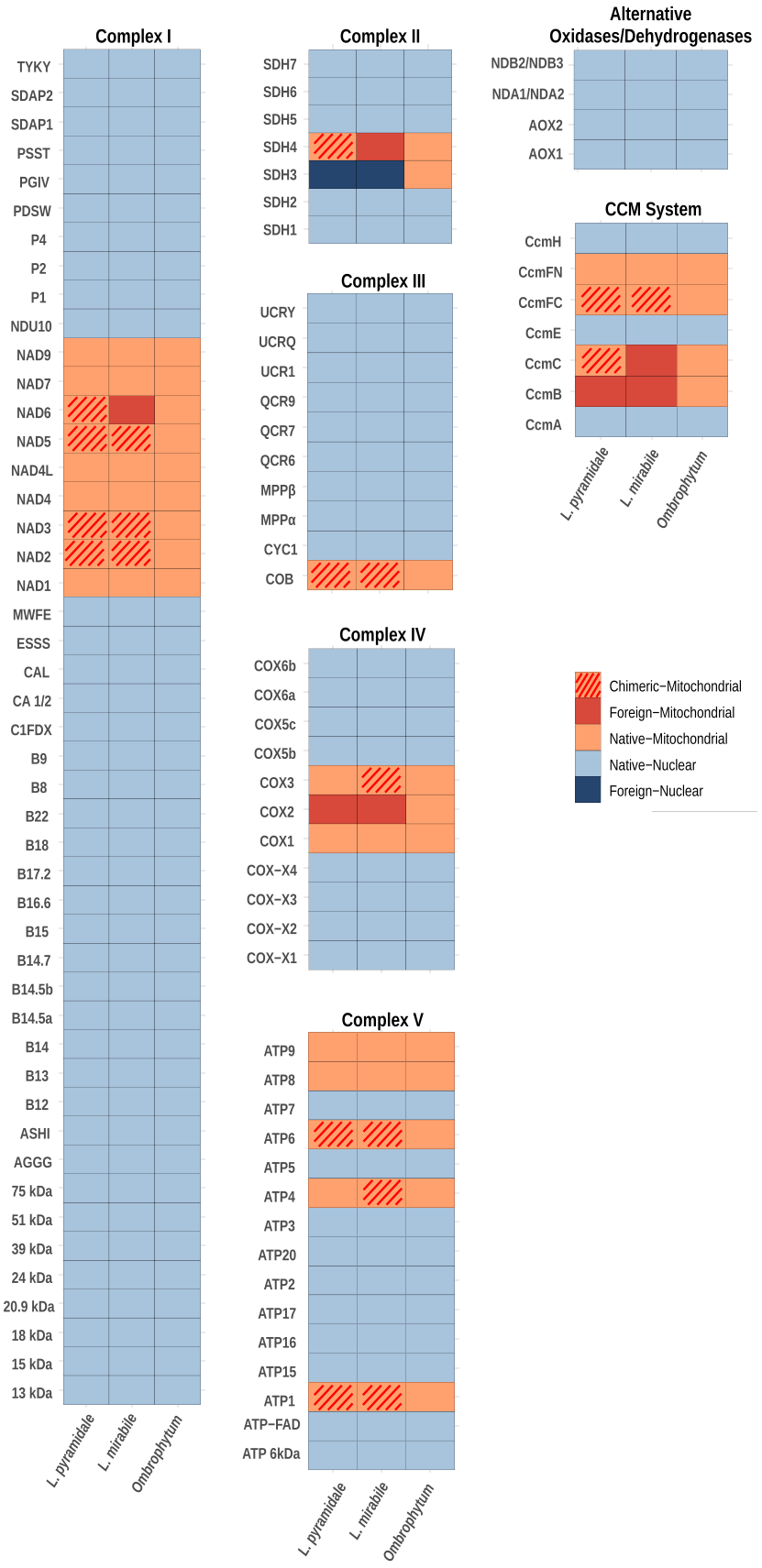

Fig. 1. Phylogenetic origin of genes related to OXPHOS in three holoparasitic species: *Lophophytum mirabile*, *Lophophytum pyramidale,* and *Ombrophytum subterraneum*. Foreign, chimeric, or native genes encoded in the mitochondrial genome are labeled as foreign-mitochondrial, chimeric-mitochondrial, and native-mitochondrial, respectively. Chimeric mitochondrial genes contain a mixture of native and foreign DNA. Foreign and native genes encoded in the nuclear genome are labeled as foreign-nuclear and native-nuclear, respectively.

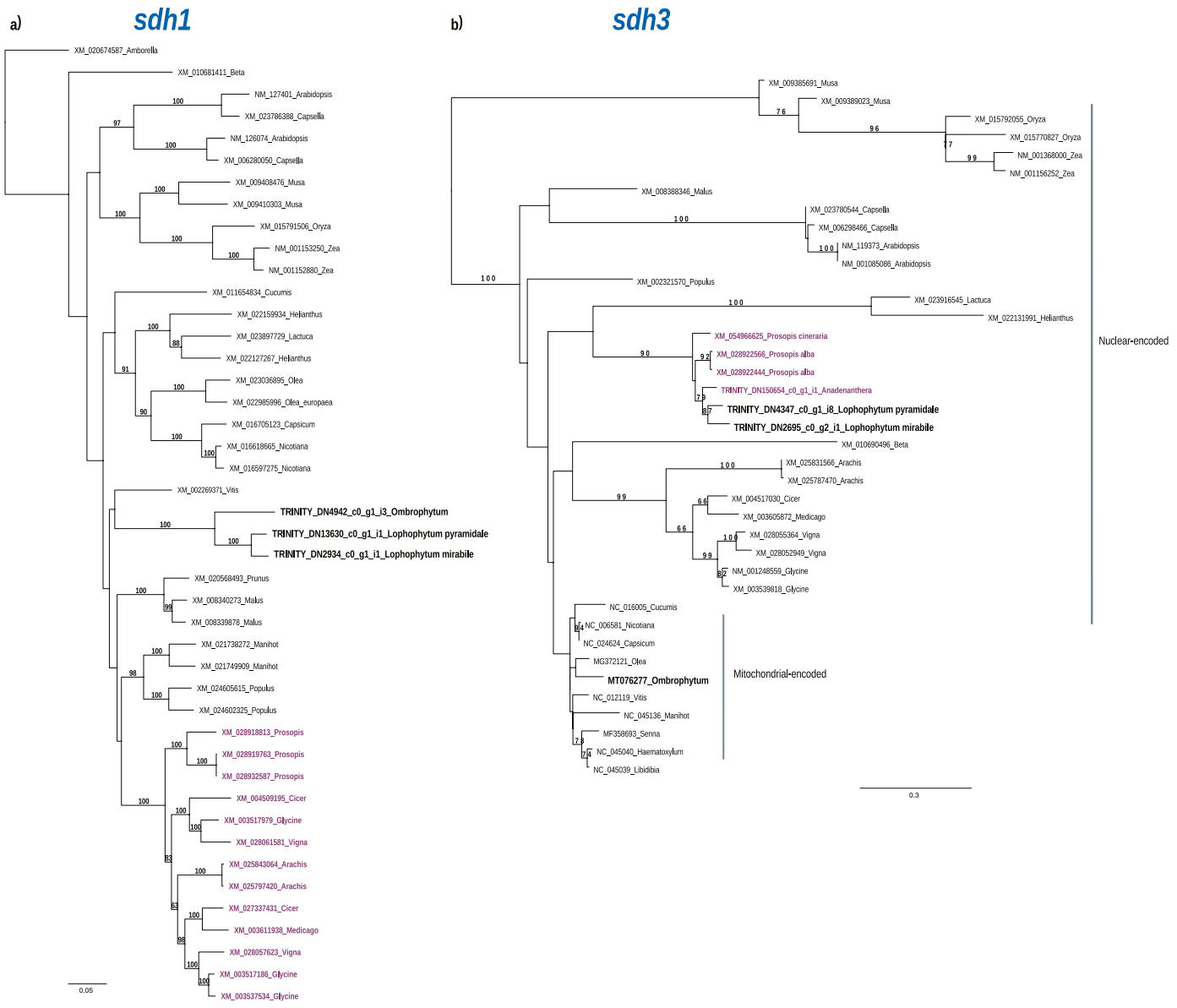

Fig. 2. Maximum Likelihood (ML) phylogenetic analyses of two nuclear-encoded genes in *Lophophytum mirabile* and *L. pyramidale*. **a)** Tree showing the native origin of *sdh1* in *Lophophytum* spp. **b)** Tree showing the foreign origin of *sdh3* in *Lophophytum* spp., as a result of a horizontal transfer from mimosoid hosts. Both genes are native in the holoparasite *Ombrophytum subterraneum.* ML bootstrap support values ≥50 % from 1000 bootstrap pseudoreplicates are shown above each branch. The scale bar corresponds to substitutions per site. Sequences from *L. mirabile, L. pyramidale,* and *Ombrophytum* (holoparasite plants, Balanophoraceae) are highlighted in light blue, while the sequences from mimosoids (hosts) are in purple. The gene *sdh3* is either nuclear or mitochondrial-encoded across angiosperms.

3.3. Abundant mitochondrial cristae were observed with the transmission electron microscope

Using transmission electron microscopy, we characterized the mitochondria and their corresponding mitochondrial cristae in *L. pyramidale*. Mitochondria present in ovary cells of *L. pyramidale* showed different shapes and sizes, ranging from 0.5 to 1 micrometer in length (Fig. 3). The staining of the mitochondrial matrix is prominent, indicating an active metabolism. In addition, abundant mitochondrial cristae are clearly observed, similar to those in free-living plants. These results contrast with those from the mitochondria of the parasitic plant *Viscum*, where a low number of mitochondrial cristae were recorded due to the absence of OXPHOS complex I, complex V dimers, and some supercomplexes, as reported by [52] and [34].

3.4. OXPHOS proteins were detected by Western blot

Western blot analyses were carried out to examine the expression of selected mitochondrial proteins, including components of complex IV (COX1 and COX2); a component of complex V (ATPβ), the hemoprotein CYTc, which acts as an electron carrier between complexes III and IV, and components of the alternative respiratory pathway (AOX1/2) (Fig. 4). Presence of the Voltage-dependent anion channel protein (VDAC1–5) located in the outer mitochondrial membrane could also be observed (Fig. 4), confirming the integrity of the mitochondrial membranes in these parasitic plants. Detection was made on PVDF membranes prepared with total protein extracts from inflorescences of the three holoparasitic plants by using antibodies directed against Arabidopsis homologs (Fig. 4). In this regard, extracts of total Arabidopsis proteins were used as a positive control for the identity of each of the proteins under analysis. The analysis was strictly qualitative and should not be used to compare expression levels between the analyzed species.

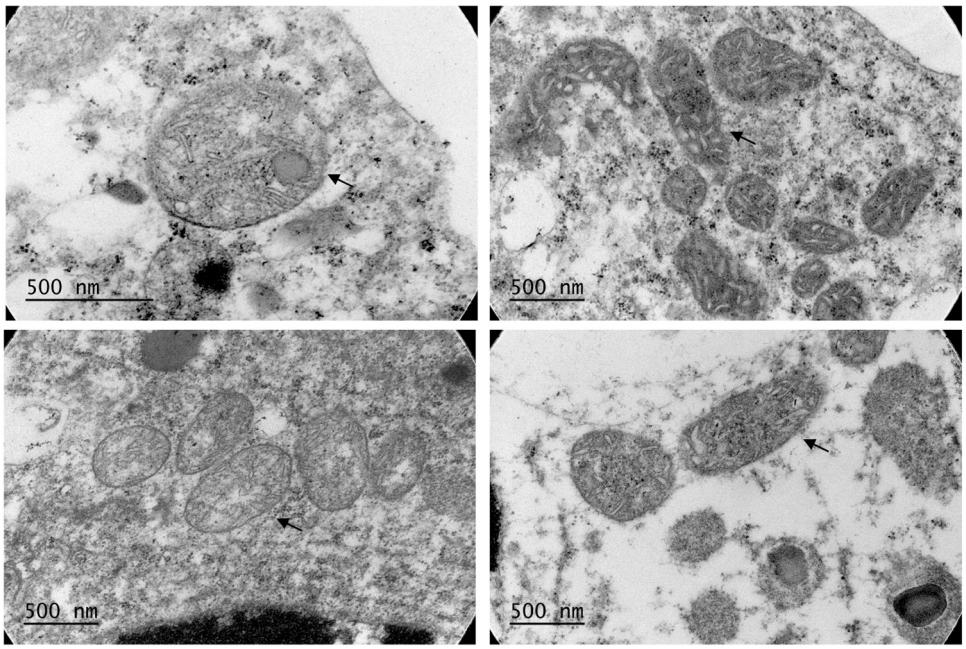

Fig. 3. Transmission electron micrographs of ovarian cells of *Lophophytum pyramidale*. Arrows point to the mitochondria. Scale bars are shown on the lower left of each photo.

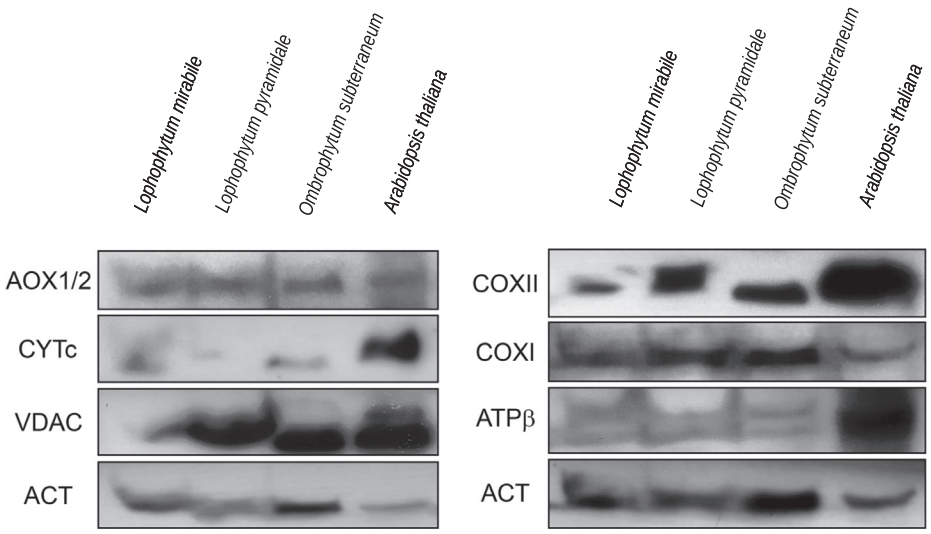

Fig. 4. Analysis of protein expression by Western blot in four different species. Components of complex IV: COXI, Cytochrome c oxidase subunit 1 (29.4 kDa) and COXII, Cytochrome c oxidase subunit 2 (30 kDa). Component of complex V: ATPβ, β-subunit of the ATP synthase (53 kDa). Electron-carrier: CYTc: cytochrome c (14 kDa). Alternative oxidases: AOX1/2, alternative oxidase proteins 1 and 2 (36–40 kDa). Mitochondrial protein from the outer mitochondrial membrane: VDAC1–5, Voltage-dependent anion-selective channel protein 1–5 (30 kDa). Loading control: ACT, Actin (41.6–45 kDa). Representative image of the three biological replicates. *Arabidopsis thaliana* was used as a control of antibody reactivity. The assay is for qualitative purposes and is not intended to compare expression levels between different species.

Thus, with different specificity degrees depending on the mitochondrial component and the analyzed parasitic species, our findings support the presence of proteins corresponding to the oxidative phosphorylation pathways in the mitochondria of these holoparasitic plants.

3.5. Oxygen consumption in the holoparasites is similar to that of free-living plants

The oxygen consumption of inflorescences from the three holoparasitic plants and leaves of Arabidopsis was experimentally evaluated with an oxygen meter in the presence or absence of the inhibitors of the CYTc-dependent respiratory pathway (KCN) and cyanide-resistant or alternative respiration pathway (SHAM). Total oxygen consumption rates in the holoparasites were comparable to that obtained for adult-leaves of Arabidopsis plants (Fig. 5a,b red columns).

Regarding the ATP-producing pathway, KCN inhibits respiration via COX, while the tissue may consume O_2 through the alternative-cyanide-resistant and residual pathways. SHAM inhibits respiration via the alternative pathway, and the tissue may respire via the COX pathway and residual respiration. The addition of KCN resulted in a significant decrease in oxygen consumption levels in all the species analyzed compared to total oxygen consumption rates, with *L. mirabile* exhibiting the greatest reduction (Fig. 5a). Conversely, the addition of SHAM had an opposite effect as *L. mirabile* showed the smallest decrease in oxygen

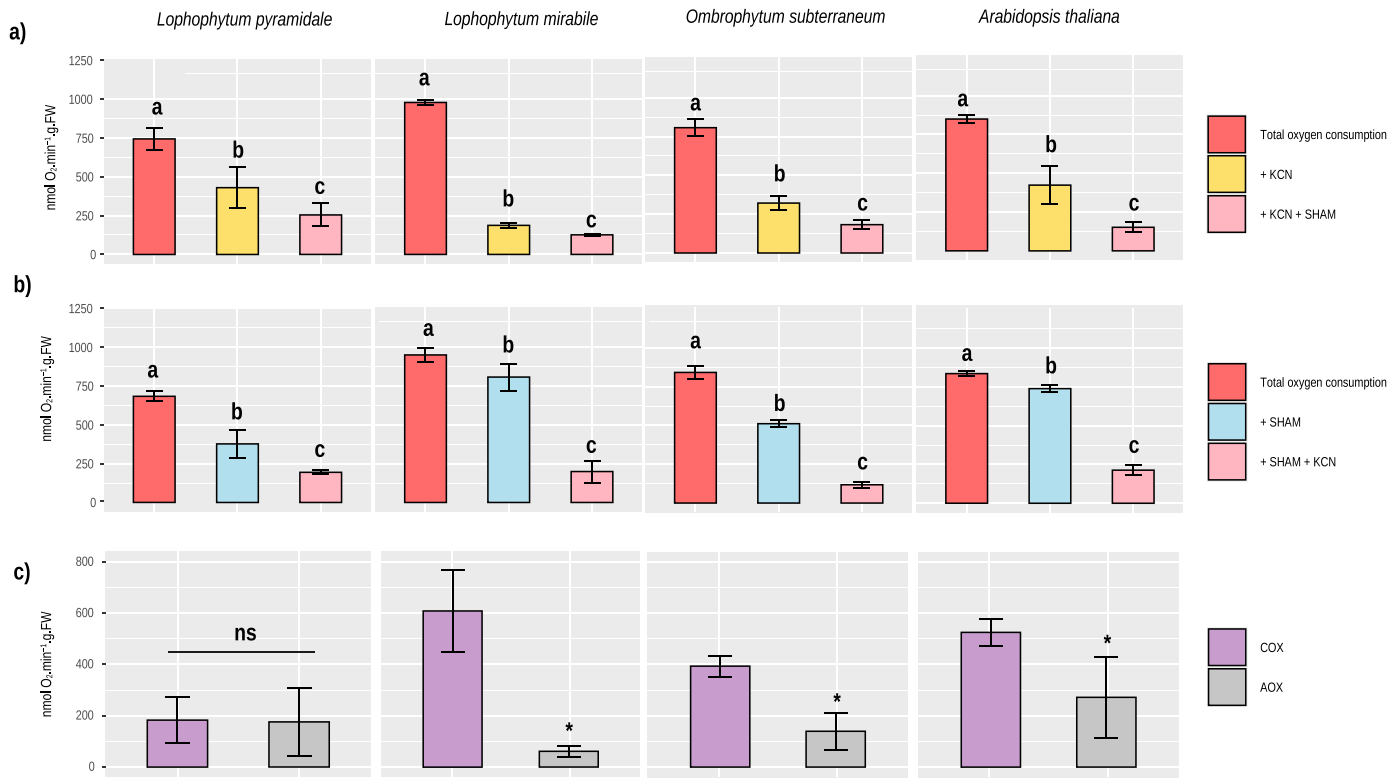

Fig. 5. Respiration measurements. Oxygen consumption in the presence and absence of the inhibitors KCN **(a)** or SHAM **(b),** followed by the addition of the second inhibitor. KCN and SHAM were added at 10 mM to the reaction vessel, when indicated. Values are referred to the fresh weight of the inflorescence included in each assay. All oxygen consumption measurements were performed at 25 °C. Different letters indicate significant differences based on Tukey Tests (ten biological replicates, $P < 0.05$). **c)** Respiration capacity corresponding to the COX or alternative pathway (AOX). The asterisk indicates significant differences between the two measures based on Student's t-tests ($P < 0.05$, $n = 10$). ns: not significant.

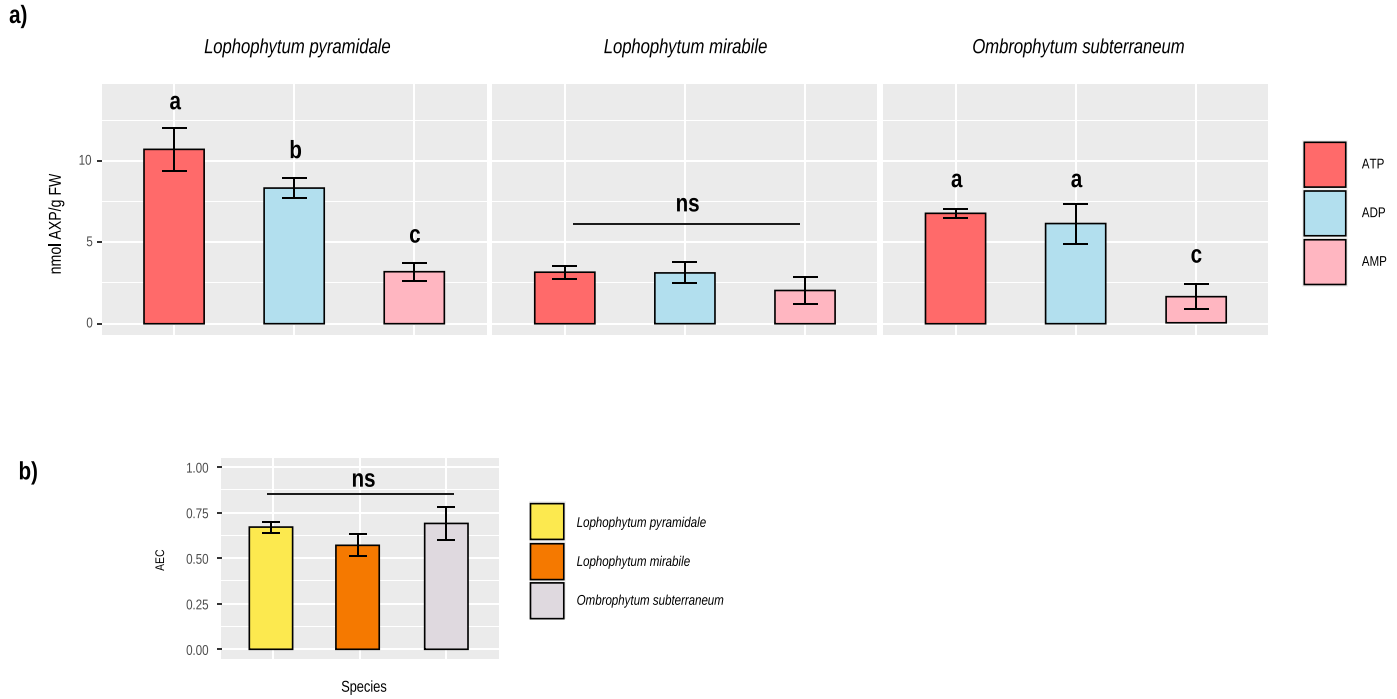

Fig. 6. Adenylate quantification in flowers of *Lophophytum* spp. and *Ombrophytum*. **a)** Quantification of individual adenylates (AXP: ATP, ADP or AMP). Different letters indicate significant differences based on Tukey Tests (four biological replicates, $P < 0.05$). ns: not significant. **b)** Changes in adenylate energy charge (AEC). AEC was calculated as (ATP + 0.5 ADP)/(ATP + ADP + AMP). ns: not significant.

consumption levels (Fig. 5b). On the other hand, our findings indicate that *L. pyramidale* possesses comparable levels of COX and AOX pathway respiratory capacity.

Finally, our results indicate that the COX pathway is prevalent in the holoparasites, as also observed in Arabidopsis (Fig. 5c). Inhibiting both KCN and SHAM results in low levels of residual respiration (Fig. 5a,b). Comparable values of total oxygen consumption rates (in the absence of inhibitors) and residual respiration were observed in the different holoparasites and *Arabidopsis thaliana* after adding the two inhibitors (KCN and SHAM).

3.6. Adenylate content in the holoparasitic plants is similar to that in Arabidopsis

Since we observe that the analyzed holoparasitic plants exhibited detectable levels of oxygen consumption rates, it was imperative to determine whether this consumption is associated with ATP production. Thus, ATP levels and total adenylate content were measured by HPLC. The inflorescences of the three holoparasites showed a total adenylate load of ATP, ADP, and AMP (Fig. 6a) comparable to previous quantifications in Arabidopsis [30,44]. In the three holoparasites analyzed, ATP and ADP levels are higher than AMP levels (Fig. 6a), suggesting a shortage of AMP in solution and a favorable energy state in the cells of these plants. Indeed, the adenylate energy charge (AEC), a parameter associated with cellular energy status, was greater than 0.5 (Fig. 6b), indicating that AMP molecules are being efficiently converted to ATP. This observation indicates an adequate ratio of ATP to ADP, reflecting good energy availability for cellular and metabolic functions.

4. Discussion

Although the protein complexes involved in OXPHOS have been extensively investigated in different species, the potential incompatibilities arising from interactions within these complexes have been limited [55,7]. In this context, studying holoparasitic plants of the genus *Lophophytum* (*L. mirabile* and *L. pyramidale*) provides a unique and fascinating perspective. Like most eukaryotes, the OXPHOS and ccm system of *Lophophytum* spp. is formed by subunits encoded by genes located in both the mtDNA and nucDNA. However, its uniqueness rests on the incorporation of 12–14 foreign mitochondrial-encoded genes and one foreign nuclear-encoded gene acquired via HGT from its mimosoid host, replacing, fully or partially, the native genes ([15,49]; Roulet et al. unpublished results). The introduction of foreign genes can negatively influence the fitness of an organism, leading to alterations in protein homeostasis, increased cytotoxicity, ineffective gene expression, and cytoplasmic incompatibility [2,3,40,4,59]. Therefore, the presence of subunits encoded by foreign or chimeric genes of the OXPHOS system in *Lophophytum* spp. raises questions regarding the functioning of OXPHOS in these holoparasitic plants. In contrast, no functional foreign mitochondrial genes have been identified in the close relative, the holoparasite *Ombrophytum subterraneum* ([46]). The contrasting pattern observed in these holoparasitic Balanophoraceae enables interesting comparisons to increase our understanding of the evolution and adaptation of OXPHOS in parasitic and non-photosynthetic eukaryotes.

Contrary to the expectations of disrupted respiration in *Lophophytum* spp., we describe a fully functional OXPHOS in the three holoparasites based on different lines of evidence. First, most genes (both mitochondrial and nuclear) encoding mitochondrial respiratory complex subunits were identified in the transcriptomes of the three species. Previous research shed light on the expression, editing, and splicing of the foreign mitochondrial genes, as well as their evolution under purifying selection, providing solid evidence for a functional role in the mitogenome of *Lophophytum* [15,16,49]. However, their actual translation into functional proteins had not been shown. Here, Western blot analysis revealed the presence of OXPHOS-related proteins (both mitochondrial or nuclear-encoded) (Fig. 4), confirming their correct translation in *Lophophytum* spp. and *Ombrophytum*. Furthermore, we provide experimental validation for the functioning of OXPHOS by measuring oxygen consumption (Fig. 5), ATP production, and total adenylate content (Fig. 6) in inflorescences of *Lophophytum* spp. and *Ombrophytum*. In addition, electron microscopy observations reveal that the ultrastructure of the mitochondria of *L. pyramidale* is similar to that of functional organelles in free-living plants (Fig. 3). All these complementary approaches have strengthened our understanding of the functionality of OXPHOS systems in these holoparasitic plants.

Our findings on the OXPHOS of the Balanophoraceae contrast with that of hemiparasitic plants of the genus *Viscum*, despite belonging to the same order Santalales and sharing a parasitic ancestor. *Viscum* lacks genes associated with complex I of the OXPHOS [41,42,53,54] and other subunits of complexes III and IV, which could be crucial for supercomplex formation and stability [42]. The altered or missing OXPHOS subunits are congruent with the observed reduction in the number of mitochondrial ridges in micrographs of *Viscum* cells [34,52]. This would limit the ability of *Viscum* mitochondria to generate ATP, which could be compensated by generating ATP through non-mitochondrial processes, such as glycolytic oxidation of imported host sugars [13,51]. In contrast, our findings corroborate that the three holoparasitic Balanophoraceae possess the ability to produce its own ATP via classical OXPHOS pathways despite the presence of mitochondrial proteins of foreign origin (in *Lophophytum* spp.) and their parasitic lifestyle. Furthermore, even though photosynthesis severely affects the OXPHOS system [6], the loss of photosynthesis in the holoparasites *Ombrophytum* and *Lophophytum* did not generate any obvious consequences considering the aspects analyzed in this study. Further exploration of the OXPHOS system in these holoparasites might reveal subtle divergences from free-living plants.

The incorporation of foreign subunits into the OXPHOS system of *Lophophytum* spp. poses an intriguing exception to what is known as the "complexity hypothesis". According to this hypothesis, first presented by [24], the probability of a gene being transferred is influenced by the number of protein-protein interactions that the gene product maintains. In simple terms, genes encoding proteins that are part of enzyme complexes would tend to show a lower propensity for a successful HGT event. This is because interactions between native and foreign proteins could generate incompatibilities that would decrease efficiency and even nullify the activity of the resulting multiprotein complex [10]. However, the situation of *Lophophytum* spp. challenges this hypothesis because, despite incorporating foreign proteins into its energetic machinery, OXPHOS remains functional. This unexpected evolutionary scenario may be explained by the low substitution rate of mitochondrial genes across angiosperms [14]. It has been proposed that genes involved in protein complexes but with low evolutionary rates may be susceptible to HGT and can functionally replace their homologs [25,31,37,55]. Likewise, genes transferred between closely related bacteria do not alter the function of protein complexes, as their similarity to native genes is remarkable [57,62]. Also, a negative correlation was identified between the number of HGT events and the evolutionary distance of donor and recipient species [43,56,64]. Even though *Lophophytum* spp. diverged from their mimosoid hosts about 125 mya [29], mitochondrial genes in both the holoparasites and the hosts exhibit a low substitution rate [14,9] and a high similarity between the native proteins in *Ombrophytum* and the fully or partially foreign proteins in *Lophophytum* spp. (Table 1). Therefore, a considerable similarity between native ancestral proteins and foreign proteins in *Lophophytum* spp. might have enabled appropriate protein–protein interactions, avoiding potential incompatibilities between foreign and native proteins. Finally, the co-acquisition of two foreign genes encoding subunits with extensive contact within complex II (*sdh3* and *shd4*) have likely cooperated to mitigate the disadvantages of breaking-up co-evolved subunits [16].

Table 1
Pairwise similarity (%) between protein sequences of mitochondrial-encoded genes in *Ombrophytum subterraneum* (native origin) and in *Lophophytum* spp. (fully or partially foreign).

Gene name	*Ombrophytum* vs *L. pyramidale*	*Ombrophytum* vs *L. mirabile*
ATP1	94.1	95.5
ATP4	84.2*	76.4
ATP6	93.4	91.7
CcmB	86.9	86.0
CcmC	92.2	93.8
CcmFC	80.0	78.1
COB	93.4	93.1
COX2	94.7	93.9
COX3	96.6*	97.0
NAD2	94.2	94.0
NAD3	92.4	92.4
NAD5	95.0	93.5
NAD6	88.7	85.3
SDH4	87.0	89.1

* These genes are native in both *Ombrophytum* and *L. pyramidale*.

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CRediT authorship contribution statement

Gatica-Soria LM: Investigation, Original draft preparation. **Canal MV:** Methodology, Formal analysis, Writing - Reviewing and Editing. **Roulet ME:** Methodology, Writing -Reviewing and Editing. **Sato H:** Methodology. **Gómez Villafañe V:** Methodology. **Welchen E:** Conceptualization, Resources, Writing - Reviewing and Editing, Funding acquisition. **Sanchez-Puerta MV:** Conceptualization, Resources, Writing - Reviewing and Editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2024.100322.

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