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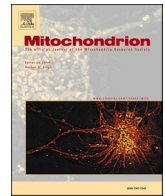
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Genes from oxidative phosphorylation complexes II-V and two dual-function subunits of complex I are transcribed in *Viscum album* despite absence of the entire mitochondrial holo-complex I

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ABSTRACT

Mistletoes (*Viscum*) and close relatives are unique among flowering plants in having a drastically altered electron transport chain. Lack of complex I genes has previously been reported for the mitochondrial genome, and here we report an almost complete absence of nuclear-encoded complex I genes in the transcriptome of *Viscum album*. Compared to *Arabidopsis* with approximately 40 nuclear complex I genes, we recover only transcripts of two dual-function genes: gamma carbonic anhydrase and L-galactono-1,4-lactone dehydrogenase. The complement of genes belonging to complexes II–V of the oxidative phosphorylation pathway appears to be in accordance with other vascular plants. Additionally, transcripts encoding alternative NAD(P)H dehydrogenases and alternative oxidase were found. Despite sequence divergence, structural modeling suggests that the encoded proteins are structurally conserved. Complex I loss is a special feature in *Viscum* species and relatives, as all other parasitic flowering plants investigated to date seem to have a complete OXPHOS system. Hence, *Viscum* offers a unique system for specifically investigating molecular consequences of complex I absence, such as the role of complex I subunits involved in secondary functions.

1. Introduction

Respiration is a fundamental cellular process that convert organic compounds into chemical energy in the form of ATP (Schertl and Braun, 2014). This process, which takes place in the mitochondria, is highly conserved among aerobic eukaryotes and involves five main protein complexes. Protein complexes I–IV constitute the electron transport chain (ETC) and include NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV). Complex I is the main entry point for electrons, that are transferred to molecular oxygen via the quinone pool (Meyer et al., 2019). The electron transport processes in complexes I, III and IV create an electrochemical proton gradient across the inner mitochondrial membrane, which allows for phosphorylation of ADP by

the ATP synthase complex (complex V). Together, the protein complexes constitute the core oxidative phosphorylation (OXPHOS) system that provide the cells with the majority of the ATP.

The OXPHOS system in plants appears to be more complex than in most other eukaryotes by comprising extra subunits in the protein complexes, totaling at least 100 proteins (Braun, 2020; Meyer et al., 2019). Furthermore, the OXPHOS system of photosynthesizing eukaryotes includes an especially large set of alternative oxidoreductases, including the alternative oxidase (AOX) and a number of alternative NAD(P)H dehydrogenases. These enzymes are absent in vertebrates and arthropods. The alternative NAD(P)H dehydrogenases serve as alternative entry points for electrons into the quinone pool and the AOX oxidizes the reduced form of quinone (quinol), thereby bypassing Complex III and IV. Electron transport by the alternative OXPHOS enzymes does

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not contribute to the formation of a proton gradient across the inner mitochondrial membrane (Del-Saz et al., 2018; Rasmussen et al., 2008).

The five protein complexes of the OXPHOS system are each composed of multiple subunits which are encoded by genes located in the mitochondrial as well as the nuclear genome (Braun, 2020; Braun et al., 2014, 1994; Heazlewood et al., 2003; Millar et al., 2004). In plants, the mitochondrial gene complement usually includes nine complex I genes (*nad*), two complex II genes (*sdh*), one complex III gene (*cob*), three complex IV genes (*cox*), and five complex V genes (*atp*) (Gualberto and Newton, 2017; Mower, 2020; Mower et al., 2012). One or both *sdh* genes may, however, be transferred to the nuclear genome and be absent from the mitochondrial genome (Adams et al., 2002; Mower et al., 2012). Additionally, functional transfer of *cox2* is known from some species in Fabaceae (Covello and Gray, 1992; Nugent and Palmer, 1991). The nuclear OXPHOS gene complement has been best characterized in the model plant *Arabidopsis thaliana* with >80 genes, approximately half of which code for subunits of complex I (see e.g. Braun, 2020; Meyer et al., 2019). But even in *Arabidopsis* the subunit composition of some complexes, complex IV in particular, is still debated (Mansilla et al., 2018; Meyer et al., 2019; Senkler et al., 2017). The recent presentation of cryoEM structures for complexes I, III and IV will make it easier to establish the complete set of subunits in these protein complexes (Klusch et al., 2021; Maldonado et al., 2021, 2020; Soufari et al., 2020). However, it remains to be determined how conserved the nuclear OXPHOS gene complement is across the plant kingdom.

In the hemiparasitic genus *Viscum*, the complete absence of all mitochondrial complex I genes was recently reported (Petersen et al., 2015a; Skippington et al., 2015) and most other mitochondrial OXPHOS genes are extremely divergent compared to those of other angiosperms (Petersen et al., 2015a; Skippington et al., 2015; 2017). Complex I gene loss coupled with extreme sequence divergence of other genes was also found in the related genus *Phoradendron* (Zervas et al., 2019) suggesting that the entire Viscaceae has a modified OXPHOS system.

Biochemical studies of the European mistletoe, *Viscum album*, have now confirmed the absence of a functional complex I and a reduced amount of other complexes compared to *Arabidopsis* (Maclean et al., 2018; Petersen et al., 2020; Senkler et al., 2018). At the same time the presence of alternative NAD(P)H dehydrogenases and an alternative oxidase was observed (Maclean et al., 2018; Senkler et al., 2018). In transcriptomes from *Viscum album* as well as other species of *Viscum* and *Phoradendron*, Zervas et al. (2021) report transcripts of the complex II–V OXPHOS genes previously recorded in the mitochondrial genomes, but no transcripts of mitochondrially encoded complex I genes are recovered. It cannot not be excluded that the mitochondrially encoded complex I genes have been transferred to the nucleus or that they are so highly divergent that they failed detection, but it is considered more probable that the mitochondrial complex I genes have been truly lost. The nuclear genomes of species of *Viscum* are among the largest of all plants, e.g. in *V. album* 2C = 205.8 pg (Zonneveld, 2010) corresponding to some 200 Gb, thus they remain to be thoroughly investigated.

Based on the available evidence of OXPHOS abnormalities in *Viscum album*, a number of questions relating to the nuclear-encoded gene complement become pertinent. Since complex I genes are lost from the mitochondrial genome (Petersen et al., 2015a; Skippington et al., 2017) and not reported from the transcriptome (Zervas et al., 2021), and since no complex I proteins have been detected (Maclean et al., 2018; Senkler et al., 2018), is it unclear what has happened to the nuclear complex I gene complement. Has the nuclear gene complement also experienced gene loss, or can any genes be found in the transcriptome? Since most mitochondrial complex II–V genes are highly divergent (Petersen et al., 2015a; Skippington et al., 2017) and biochemical studies have shown reduced amounts of proteins from these complexes (Maclean et al., 2018; Senkler et al., 2018), complex I loss may have had a substantial impact on the nuclear complex II–V genes. Are they all present in the genome? Are they transcribed? If so, are they equally divergent as the mitochondrial genes, possibly showing signs of compensatory changes

in order to conserve protein structure and function?

These questions should ideally be answered by examining both the transcriptome and the complete nuclear genome of *Viscum album*. However, due to the large size of the nuclear genome the current study is based on transcriptome data. To address the uncertainty, we identify transcribed nuclear OXPHOS genes from all complexes, to investigate sequence divergence compared to other angiosperm OXPHOS genes, and we use a homology modelling approach to predict protein structure.

2. Materials and methods

2.1. Sampling, RNA extraction and sequencing

Leaves and stems of *Viscum album* were collected in November 2016 in the Botanical Garden of the Natural History Museum of Denmark. Tissue was sampled from the same specimen as previously used for plastome (Petersen et al., 2015b) and mitogenome (Petersen et al., 2015a) sequencing. The sampled tissue was immediately immersed in liquid nitrogen and stored at -80°C . A voucher specimen, C2546, is deposited in the herbarium (C) at the Natural History Museum of Denmark.

RNA extraction using approximately 100 mg of tissue was performed using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, sigmaaldrich.com) according to the manufacturer's protocol. Libraries were prepared using the SENSE mRNA-Seq Library Prep Kit V2 (Lexogen, lexogen.com) and the included Illumina-compatible barcode and adapter sequences. The purified mRNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, thermofisher.com) with the RNA assay kit. Illumina adapters were attached as specified by the manufacturer's protocol, after running a qPCR to determine the optimal number of amplification cycles. The finalized libraries were quantified in a Bioanalyzer (Agilent Technologies, agilent.com) before being submitted for Illumina sequencing at the National Sequencing Centre, University of Copenhagen, Denmark. Sequencing was done using one lane of an Illumina HiSeq 4000 instrument generating single-end 81 bp reads. Sequences are deposited in GenBank (BioProject PRJNA720665).

2.2. Transcriptome assembly and evaluation

Sequencing generated 368,102,614 single-end reads (GenBank BioProject PRJNA720665). To simplify assembly, duplicate reads were removed using Clumpify from the BBMap package (Bushnell, 2014; <https://sourceforge.net/projects/bbmap/>), leaving ~ 55 million reads. Illumina sequencing adapters and regions at the ends of the reads with quality less than Phred Q20 were trimmed using BBDuk from the BBMap package. Trimmed reads of length less than 50 bp were removed, leaving ~ 51 million reads with an average read length of 79.7 bp (90% of the reads were not trimmed).

The transcriptome was assembled using Trinity v. 2.8.2 (Grabherr et al., 2011) with default settings and “-SS_lib_type R” (reverse strand specific single-end reads). Basic features of the assembled transcriptome were assessed using a Perl script provided with the Trinity package. The transcriptome was further evaluated using BUSCO v. 3.1.0 (Simão et al., 2015). BUSCO assesses the completeness of transcriptome assembly based on phylogenetically informed expectations of gene content of near-universal single-copy orthologs, and was run using the eudicotyledon odb10 pre-release (December 2017) and specifying “species tomato”.

2.3. Identifying nuclear OXPHOS genes

To identify transcripts of nuclear OXPHOS genes we used two different approaches based on either RNA or protein similarity. Based on the list of 114 nuclear loci from *Arabidopsis* (Meyer et al., 2019), we downloaded complete mRNA sequences from GenBank. Most loci are complex I–V genes and 12 code for alternative oxidases and

dehydrogenases. *Viscum* Illumina reads were mapped to *Arabidopsis* sequences using the Map to Reference function in Geneious Prime version 2019.0.4 first under default settings (Medium-Low Sensitivity), but if no or very few reads were mapped the allowed mismatches per read were increased from 20% to typically 40% to allow for mapping of more divergent sequences. Higher percentages of allowed mismatches generally resulted in substantial amounts of spuriously mapped sequence reads. When mapping to *Arabidopsis* gave no or only spurious results, we used potentially orthologous sequences from *Vitis vinifera* (GenBank BioProject PRJNA33471) as alternative references for mapping. Since no annotated nuclear genome is available for a close relative to *Viscum*, *Vitis* was chosen since *Viscum* typically had the top blast hit for *Vitis* sequences compared to other taxa with completely sequenced genomes. Due to sequence divergence between *Viscum* and *Arabidopsis*/*Vitis* the mapping approach typically resulted in a patchy coverage of the loci, so consensus sequences calculated from the mapped reads were extracted and used as reference sequences in new rounds of Map to Reference analyses now, using up to 10 iterations and default sensitivity settings. This procedure was continued until complete coding sequences (CDS) with start and stop codons were obtained. To recover potentially more divergent genes we created a database including the transcriptome sequences assembled by Trinity (210,334 sequences). Amino acid sequences for the 114 *Arabidopsis* loci were then blasted against this database using a standard tblastn algorithm implemented in Geneious. For any new potential OXPHOS gene we checked the Trinity assembly by remapping the sequence read, and to validate sequences as OXPHOS genes, we searched for the best matching protein sequences in GenBank using blastp.

2.4. Sequence analyses

To assess nucleotide and amino acid sequence divergence, the nuclear OXPHOS genes identified using the above approach and the previously identified mitochondrial OXPHOS genes (Petersen et al., 2015a; Skippington et al., 2017) were compared to their potential orthologs in *Arabidopsis* and *Vitis* (Table S1, GenBank acc. nos. NC037304, NC012119). The comparisons of nucleotide sequences were based on pairwise alignment using the Translation Align option of Muscle version 3.8.425 as implemented in Geneious Prime and the pairwise nucleotide identity was calculated. Similarly, amino acid identity was calculated based on pairwise alignments of the translated sequences and their orthologs using Muscle version 3.8.425 as implemented in Geneious Prime. To account for RNA editing, the amino acid sequences of mitochondrial genes of *Viscum* were translated from the transcribed genes (GenBank acc. nos. OK415276–80, OK415285–88, OK415296). Since the protein sequences from *Vitis* mitochondrial genes deposited in GenBank (NC012119) are not corrected for RNA editing, we accessed the corrected proteins from the database REDIdb3.0 (Lo Giudice et al., 2018), which includes *Vitis* sequences from a comprehensive investigation of RNA editing (Picardi et al., 2010).

Nucleotide sequence divergence was further explored by phylogenetic analysis including representative sequences from 15 different orders of eudicots (Table S1). Selection of taxa was limited by availability of well-annotated nuclear genomes and transcriptomes, and orthology of loci has usually not been tested but assumed based on similarity. A list of GenBank accession numbers of all included loci is provided in Table S1. Multiple sequence alignment was performed using the Translation Align option of Muscle version 3.8.425 as implemented in Geneious Prime. Phylogenetic analyses were performed using RAxML version 8.2.11 (Stamatakis, 2014) as implemented in Geneious Prime. A GTR + GAMMA + I nucleotide model was used and branch support was assessed using the Rapid Bootstrapping and search for best-scoring ML tree algorithm running 1,000 bootstrap replicates. Sequences from *Nelumbo* were used to root the gene trees. Phylogenetic analyses were performed for all individual genes, for concatenated matrices of genes belonging to each protein complex, and for a concatenated matrix of 30

genes. The genes encoding subunits of AOX, NDA, MPPalpha, and cytochrome *c* were not included since two or more copies of each occur in other taxa making homology assignment too ambiguous.

In order to investigate the structural diversity of the *Viscum album* OXPHOS proteins we modeled the structure of the proteins identified here as belonging to complexes I–V as well as the 11 mitochondrial complex II–V genes previously identified (Petersen et al., 2015a; Skippington et al., 2017; Zervas et al., 2021). Homology modeling was done using the SWISS-MODEL web server (<https://swissmodel.expasy.org/interactive>) (Bienert et al., 2017; Waterhouse et al., 2018). Templates for model building were searched for in SWISS-MODEL and selected based on a combination of maximum sequence coverage and identity, GMQE/QSQE, and/or X-ray structure resolution (less than 3Å). A minimum of three templates were selected and structures were selected based on best/highest QMEAN. Structure visualization and analysis were done using the VMD software (<https://www.ks.uiuc.edu/Research/vmd/>) (Humphrey et al., 1996). Mitochondrial transit peptides (mTP) were predicted using TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) (Almagro Armenteros et al., 2019), and propeptide cleaving sites were predicted using ProP (<http://www.cbs.dtu.dk/services/ProP/>) (Duckert et al., 2004). The potential effect of RNA editing on protein structure was investigated using amino acid translations from both DNA and RNA sequences for modeling. The pattern of changes in the edited sites is visualized using Cytoscape (Shannon, 2003).

3. Results

3.1. Nuclear OXPHOS genes in the *Viscum* transcriptome

The Trinity assembly resulted in ~ 135 k 'genes' (or transcript cluster) from ~ 210 k transcripts with a contig N50 of 494 based on the longest transcript of each transcript cluster. BUSCO analysis found 61% (1298) of the searched groups of orthologs (2121) (see Table S2).

In the *Viscum* transcriptome, we detected 36 nuclear OXPHOS genes: 2 genes for proteins that have been found associated with complex I, 5 complex II genes, 9 complex III genes, 4 complex IV genes, 9 complex V genes, 1 cytochrome *c* gene, 1 alternative oxidase gene, and 5 alternative NAD(P)H dehydrogenase genes (Tables 1, 2). For most OXPHOS genes we found only one copy, but in some cases we observed synonymous nucleotide polymorphism, which could be caused by either heterozygosity or the existence of more than one locus. Due to the short-read sequencing approach, distinction between the two options is not possible when different nucleotides are >81 bp apart, corresponding to the single-end read length. The MPPalpha subunit of complex III is encoded by two distinct gene copies in *Viscum*, which could be resolved by "read walking".

Based on similarity to the five AOX genes in *Arabidopsis* and the classification system proposed by Costa et al. (2017, 2014) the one *Viscum* gene may tentatively be classified as AOX1A. The transcribed sequence recovered here is incomplete lacking a short part of the 5'-end (compared to other potential orthologous sequences) and without a clear start codon.

The five recovered alternative NAD(P)H dehydrogenase genes include two NDA genes, two NDB genes and one NDC gene. While the NDB genes can be classified as NDB1 and NDB2 with reasonable confidence, and thus potentially orthologous to similarly named genes from *Arabidopsis* and other angiosperm taxa, the NDA genes are more problematic. Here we have assigned the names NDA1 and NDA2 to the *Viscum* genes solely based on highest pairwise identity to the *Arabidopsis* NDA1 and NDA2 genes. However, preliminary phylogenetic analysis, including genes annotated as NDA1 and NDA2 from other angiosperm taxa (results not shown), does not support a single duplicating event suggesting instead many recent duplication events.

Table 1Nuclear OXPHOS genes including alternative oxidases and dehydrogenases identified in the transcriptome of *Viscum album*.

Gene name or description (symbol)	<i>Viscum album</i> GenBank ID	<i>Arabidopsis</i> potential homologue ¹	Protein ²	<i>Arabidopsis</i> AA residues	<i>Viscum</i> AA residues
Complex I					
gamma carbonic anhydrase 1 (GAMMA CA1)	MZ318166	At1g19580	γCA1	275	219
L-galactono-1,4-lactone dehydrogenase (GLDH)	MZ318167	At3g47930	GLDH	610	611
Complex II					
Succinate dehydrogenase 1 (SDH1)	MZ289894	At5g66760	SDH1	634	627
Succinate dehydrogenase 2 (SDH2)	MZ318168	At3g27380	SDH2	279	281
Succinate dehydrogenase 5 (SDH5)	MZ318169	At1g47420	SDH5	257	244
Succinate dehydrogenase 6 (SDH6)	MZ318170	At1g08480	SDH6	142	147
Succinate dehydrogenase 7 (SDH7)	MZ318171	At5g62575	SDH7	99	105
Complex III					
Cytochrome C1 family	MZ318179	At5g40810	cytochrome c1	307	367
Ubiquinol-cytochrome C reductase iron-sulfur subunit	MZ318180	At5g13430	FeS	272	254
Insulinase (peptidase family M16) protein	MZ318181	At1g51980	MPPalpha-1	503	497
Insulinase (peptidase family M16) protein	MZ318182	At1g51980	MPPalpha-1	503	505
Insulinase (peptidase family M16) protein (MPPbeta)	MZ318183	At3g02090	MPPbeta	531	532
Ubiquinol-cytochrome C complex reductase hinge protein	MZ318184	At1g15120	QCR6	69	65
Cytochrome bd ubiquinol oxidase, 14 kDa subunit	MZ318185	At4g32470	QCR7	122	124
Cytochrome b-c1 complex, subunit 8 protein	MZ318186	At3g10860	QCR8	72	72
Ubiquinol-cytochrome C reductase UQCRX/QCR9-like family protein	MZ318187	At3g52730	QCR9	72	72
Complex IV					
Rubredoxin-like superfamily protein	MZ318188	At3g15640	COX5b	176	173
Cytochrome C oxidase Vc family protein	MZ318189	At2g47380	COX5c	64	66
Cytochrome C oxidase-like protein	MZ318190	At4g37830	COX6a	102	105
Cytochrome C oxidase 6B (COX6B)	MZ318191	At1g22450	COX6b	191	196
Complex V					
ATP synthase alpha/beta family protein	MZ318194	At5g08670	beta subunit	556	576
ATPase, F1 complex, delta/epsilon subunit	MZ318195	At5g47030	delta subunit	203	201
MALE GAMETOPHYTE DEFECTIVE 1 (MGP1)	MZ318193	At2g21870	FAD	240	249
gamma subunit of Mt ATP synthase (ATP3)	MZ318197	At2g33040	gamma subunit	325	329
ATP synthase	MZ318192	At5g59613	6 kDa subunit	55	61
ATP synthase epsilon chain	MZ318196	At1g51650	epsilon subunit	70	73
delta subunit of Mt ATP synthase (ATP5)	MZ318198	At5g13450	OSCP	238	241
ATP synthase D chain (ATPQ)	MZ318199	At3g52300	subunit d	168	169
Mitochondrial ATP synthase subunit G protein	MZ318200	At2g19680	subunit g	122	122
Cytochrome c					
Cytochrome c-2 (CYTC-2)	MZ318178	At4g10040	Cytc	112	112
Alternative oxidases and dehydrogenases					
alternative oxidase A1 (AOX1A)	MZ318172	At3g22370	AOX1A	354	>316
alternative NAD(P)H dehydrogenase 1 (NDA1); internal	MZ318173	At1g07180	NDA1	510	539
alternative NAD(P)H dehydrogenase 2 (NDA2); internal	MZ318174	At2g29990	NDA2	508	505
NAD(P)H dehydrogenase B1 (NDB1); external	MZ318175	At4g28220	NDB1	571	580
NAD(P)H dehydrogenase B2 (NDB2); external	MZ318176	At4g05020	NDB2	582	583
NAD(P)H dehydrogenase C1 (NDC1); internal	MZ318177	At5g08740	NDC1	519	434

¹For genes with similar isoforms in *Arabidopsis* we list the most similar locus.²Annotations follow Braun (2020).

3.2. Sequence diversity and phylogenetic analyses of OXPHOS genes

Pairwise nucleotide identity between the 29 complex I–V genes in *Viscum* and their assumed orthologs in *Arabidopsis* and *Vitis* varies from ca. 47–77% (Table 2). Since the pairwise nucleotide identity score ignores gaps, it does not reveal the complete sequence divergence. Most, but not all, *Viscum* genes show slightly higher identity to *Vitis* genes (average 65%) than to *Arabidopsis* genes (average 63%), and identity is generally lower than between *Arabidopsis* and *Vitis* genes (average 71%). For the mitochondrial genes, the pairwise identity between *Viscum* and *Arabidopsis* and *Vitis* genes has a much broader range, ca. 21–84%, but the average identities are almost the same as for the nuclear genes: *Viscum* vs. *Vitis* 64% (or 68% excluding the *sdh* genes which are nuclear and highly divergent in *Arabidopsis*), *Viscum* vs. *Arabidopsis* 59% (67% excluding *sdh* genes). However, identity between mitochondrial genes of *Arabidopsis* and *Vitis* is considerably higher ranging from ca. 37–97% with an average of 82% (91% excluding *sdh* genes) (Table 2).

With a few exceptions, individual gene trees were constructed for all

OXPHOS genes (Fig. S1), minus the AOX and NDA genes, which were not used due to difficulties in homology assignment and apparent inconsistent annotation of sequences in GenBank. Since the two copies of MPPalpha recovered from *Viscum* could not easily be homologized with the two *Arabidopsis* gene copies, and since a variable number of potentially homologous genes have been annotated in other eudicot genomes, a gene tree including multiple gene copies was constructed. The distribution of genes suggests a pattern of repeated duplications and loss (Fig. 1).

The constructed gene trees based on nucleotide sequences show topologies more or less consistent with expected eudicot phylogeny. The trees are generally characterized by short internal and long terminal branches, and many branches are very poorly supported (Fig. S1). An attempt to use amino acid sequences for phylogenetic reconstruction did not generally improve branch support (results not shown). The trees derived from analyses of concatenated matrices of nucleotide sequences (Fig. S2) are generally better supported and more consistent with accepted eudicot phylogeny, and the concatenated matrix of 30 nuclear

Table 2Divergence between OXPHOS genes (including the alternative oxidase and dehydrogenases) of *Viscum album* (Vis), *Vitis vinifera* (Vit) and *Arabidopsis thaliana* (Ara).

Gene	Length of coding sequence (nt)			Nucleotide identity (%)			Amino acid identity (%) ¹		
	Vis	Vit	Ara	Vis/Vit	Vis/Ara	Vit/Ara	Vis/Vit	Vis/Ara	Vit/Ara
Mitochondrial genes									
II: <i>sdh3</i> ¹	225	507	642	47.5	20.5	37.2	39.1	17.3	24.6
II: <i>sdh4</i> ²	225	432	456	44.6	26.3	41.7	31.0	16.1	28.8
III: <i>cob</i>	1212	1182	1182	73.7	72.6	97.1	68.9	69.3	97.7
IV: <i>cox1</i>	1668	1584	1584	80.7	79.1	95.8	81.9	83.3	96.8
IV: <i>cox2</i>	759	786	783	72.9	74.1	94.4	67.1	69.5	93.9
IV: <i>cox3</i>	798	798	798	74.2	73.6	96.4	64.3	68.3	94.0
V: <i>atp1</i>	1539	1530	1524	72.4	72.0	91.4	71.5	70.6	92.1
V: <i>atp4</i>	474	597	597	35.9	35.7	91.4	23.0	25.5	80.8
V: <i>atp6</i> ³	915	1167	1158	66.1	56.7/58.2	70.8/70.1	59.3	55.3/56.0	61.4/63.3
V: <i>atp8</i>	495	480	477	51.7	52.3	88.5	37.8	36.1	84.5
V: <i>atp9</i>	225	225	225	84.4	82.7	96.0	84.0	79.8	93.2
Nuclear genes									
I: GAMMA CA1	660	819	828	75.8	71.8	78.1	78.5	74.4	84.9
I: GLDH	1836	1830	1833	72.7	67.6	72.4	71.2	67.4	76.8
II: SDH1	1884	2013	1905	76.6	77.3	75.9	83.7	83.3	85.7
II: SDH2	846	831	840	70.7	64.3	69.4	74.0	72.8	79.2
II: SDH5	735	729	774	64.3	53.4	59.0	56.6	44.6	51.8
II: SDH6	444	441	429	55.7	57.0	68.3	45.3	46.3	65.3
II: SDH7	318	309	303	58.6	60.7	68.9	50.5	56.4	68.3
III: Cytochrome c1	1104	960	783	62.8	74.4	81.2	64.0	81.5	91.2
III: FeS	765	819	825	64.3	61.6	71.0	67.6	63.9	76.2
III: MPPalpha A	1494	1521	–	69.7	–	–	65.7	–	–
III: MPPalpha B	1518	1521	1356B11500B2	71.1	63.863.0	70.770.6	73.2	61.961.0	70.569.4
III: MPPbeta	1599	1566	1443	65.5	71.9	70.3	63.8	76.7	71.3
III: QCR6	198	210	210	66.1	65.6	74.8	65.1	65.1	73.9
III: QCR7	375	369	369	64.2	65.9	72.6	58.5	58.5	78.9
III: QCR8	219	219	219	65.8	66.7	77.2	60.3	57.5	75.3
III: QCR9	219	216	219	59.5	60.0	76.4	51.5	48.0	73.6
IV: COX5b	519	531	513	62.2	56.6	65.0	54.5	50.6	59.9
IV: COX5c	201	192	195	61.8	60.4	72.4	50.8	53.7	76.6
IV: COX6a	318	297	279	53.7	47.4	60.2	45.7	40.4	62.1
IV: COX6b	588	573	573	55.2	54.7	56.0	44.7	42.9	52.5
V: beta	1731	1674	1671	75.9	70.9	77.2	80.7	76.9	85.0
V: delta	606	621	612	68.9	63.2	70.0	69.1	64.5	72.8
V: MGP1	750	723	723	66.1	60.3	74.1	58.7	52.6	73.8
V: ATP3	990	969	978	72.1	66.9	75.2	68.7	63.5	78.9
V: 6 kDa	186	168	168	62.0	61.0	72.0	57.9	60.7	73.2
V: epsilon	222	228	213	68.2	69.2	78.4	68.9	69.0	80.3
V: OSCP	726	756	717	54.0	50.1	60.0	41.0	35.6	52.6
V: subunit d	510	507	507	60.6	57.7	78.9	44.1	43.5	83.4
V: subunit g	369	369	369	71.8	66.9	72.6	63.5	63.5	79.7
CYTC	339	339	339	71.7	69.3	77.9	70.8	72.6	88.5
AOX1	>951	963	1065	64.5	59.5	59.4	64.2	66.1	61.3
NDA1 ⁴	1620	–	1533	–	59.6	–	–	64.1	–
NDA2 ⁵	1518	–	1527	–	67.9	–	–	73.7	–
NDB1	1743	1671	1716	77.2	68.2	70.4	77.6	68.2	70.0
NDB2	1752	759	1749	78.8	71.0	71.9	75.5	72.0	71.5
NDC1	1305	1632	1560	75.9	65.9	67.2	76.0	67.5	67.7

¹Nuclear encoded in *Arabidopsis*.²Nuclear encoded in *Arabidopsis*.³*Arabidopsis* has two mitochondrial copies.⁴Orthology of NDA genes to similar genes in other taxa is dubious. Here NDA1 and NDA2 are assigned simply based on maximized identity to the *Arabidopsis* gene copies.⁵As above.

genes resulted in a tree with high support for most clades, and major well-established groups such as Asterids, Lamiids, Campanulids, and Rosids (excl. Vitales) are all recovered (Fig. 2). While the trees based on individual genes and protein complex concatemers may not represent the phylogeny of eudicots properly, the branch lengths illustrate the level of divergence of *Viscum* sequences in relation to other eudicots. Although individual genes are variably divergent, complex III-V nuclear genes show higher divergence in *Viscum* than in other taxa included in the gene trees, whereas complex II genes, complex I-associated genes as well as the alternative dehydrogenase genes do not.

3.3. RNA editing of mitochondrial OXPHOS genes

A total of 103 edited sites are identified in the 11 OXPHOS genes

encoded in the mitochondria of *Viscum album* (see also Zervas et al., 2021). All are C-to-U edits, the number of editing sites per gene (CDS) vary from zero in *atp8* to 22 in *atp6*, and the average frequency of editing is 1.21 edits per 100 bases. Editing involves 28 different triplet codon positions in 23 codons and the total count includes 37 first positions, 63 second positions and 3 third positions. Ninety-eight changes result in amino acids changes (three are neutral and two produce a stop codon) and in the most common cases editing changes the amino acids P and S to L and/or F (Fig. S3, Table S3). This pattern of RNA editing resembles very closely the pattern in other plants, e.g., *Vitis* (Picardi et al., 2010). Alignments of amino acid sequences of the 11 OXPHOS proteins encoded in the mitochondria of *Viscum* and *Arabidopsis* are shown in supplementary Figs. S5 and S6. As many as 25 editing sites are found in identical positions.

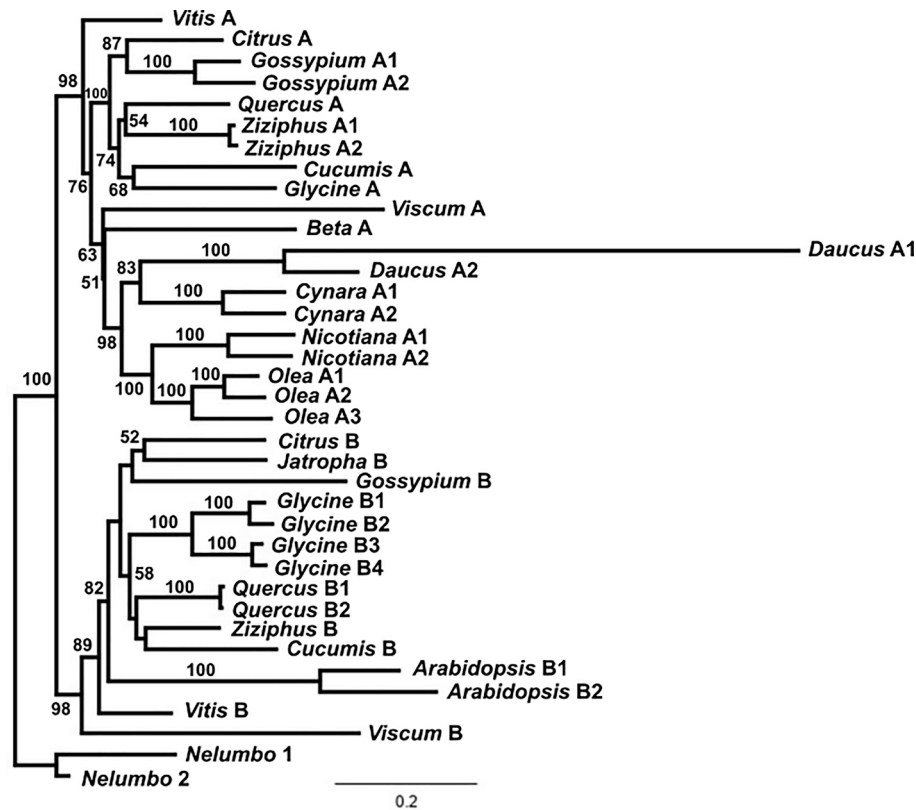


Fig. 1. Gene tree for MPPalpha (complex III) based on nucleotide sequences. Several angiosperms have multiple paralogs of MPPalpha, either originating from recent duplication events (numbered gene copies) or from an ancient duplication event (A and B copies). Clade support is shown as bootstrap values > 50%. The bar shows substitutions per site.

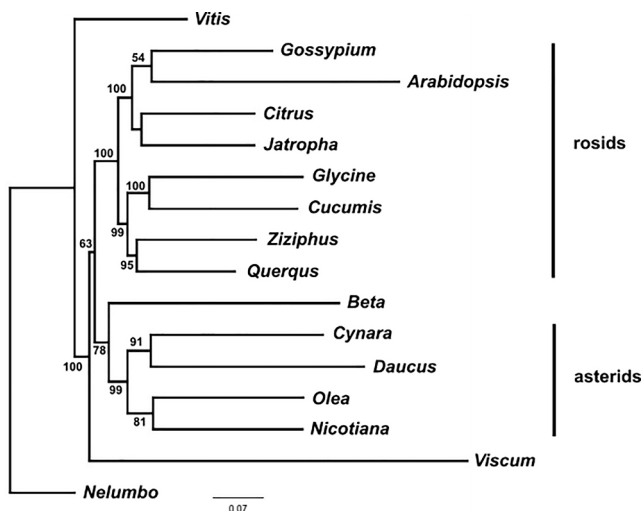


Fig. 2. Phylogenetic tree derived from analysis of a concatenated matrix of 30 nuclear OXPHOS genes including three alternative dehydrogenases. Clade support is shown as bootstrap values > 50%. The bar shows substitutions per site.

3.4. Structure of mitochondrially encoded OXPHOS proteins

The protein-coding OXPHOS genes in *Viscum* mtDNA show an average of 66.7% nucleotide identity with the same genes in *Arabidopsis*, but this number hides a wide diversity – from 82.7% identity for *atp9* to 35.7% identity for *atp4* and even lower for *sdh3* and *sdh4*, which are transferred to the nuclear genome in *Arabidopsis* (Table 2). When we compared the amino acid sequences of mtDNA-encoded proteins to

those of *Arabidopsis*, in all cases except one (*cox1*) we found a trivially, slightly lower degree of identity than for the nucleotide sequences (average 62.0% identity) (Table 2). The protein sequences are 25.5–83.3% identical in *Viscum* and *Arabidopsis* (excluding the *sdh* genes), and the amino acid identity of most of the proteins differ by >30% between the two species. We used these protein sequences for homology modeling of protein structure and obtained backbone structures for all 11 proteins encoded in the mitochondria, although some were only partial (Table S4). In general, there were no changes in the backbone structure of the modeled protein compared to *Arabidopsis* even when the amino acid identity of the aligned proteins was as low as 30% (results only shown for complex III and IV subunits). Modeling of the mitochondrially encoded complex III and IV proteins, *cob*, *cox1*, *cox2* and *cox3*, are shown in Fig. S4. The relative positions of the subunits in the complex III dimer and in complex IV are shown in Fig. 3A,B while the subunits present at the interfaces in the complex III₂IV supercomplex are highlighted in Fig. 3C.

3.5. Structure of nuclear-encoded OXPHOS proteins

Based on transcriptome sequences, the nuclear-encoded OXPHOS genes in *Viscum* show an average of 63.7% nucleotide identity compared to the same genes in *Arabidopsis*, but this number hides a wide diversity: from 77.3% identity for *SDH1* to 47.4% identity for *COX6a* (Table 2). Thus, these nuclear genes exhibit just as high a sequence divergence as the mitochondrial genes. The alternative oxidase and NAD(P)H dehydrogenase genes, encoding OXPHOS proteins that are not part of the respiratory complexes, showed an average nucleotide identity of 65.9% with the *Arabidopsis* genes, or about the same as the other OXPHOS genes.

When we compared the amino acid sequences of nuclear-encoded proteins to those of *Arabidopsis*, we found almost the same degree of

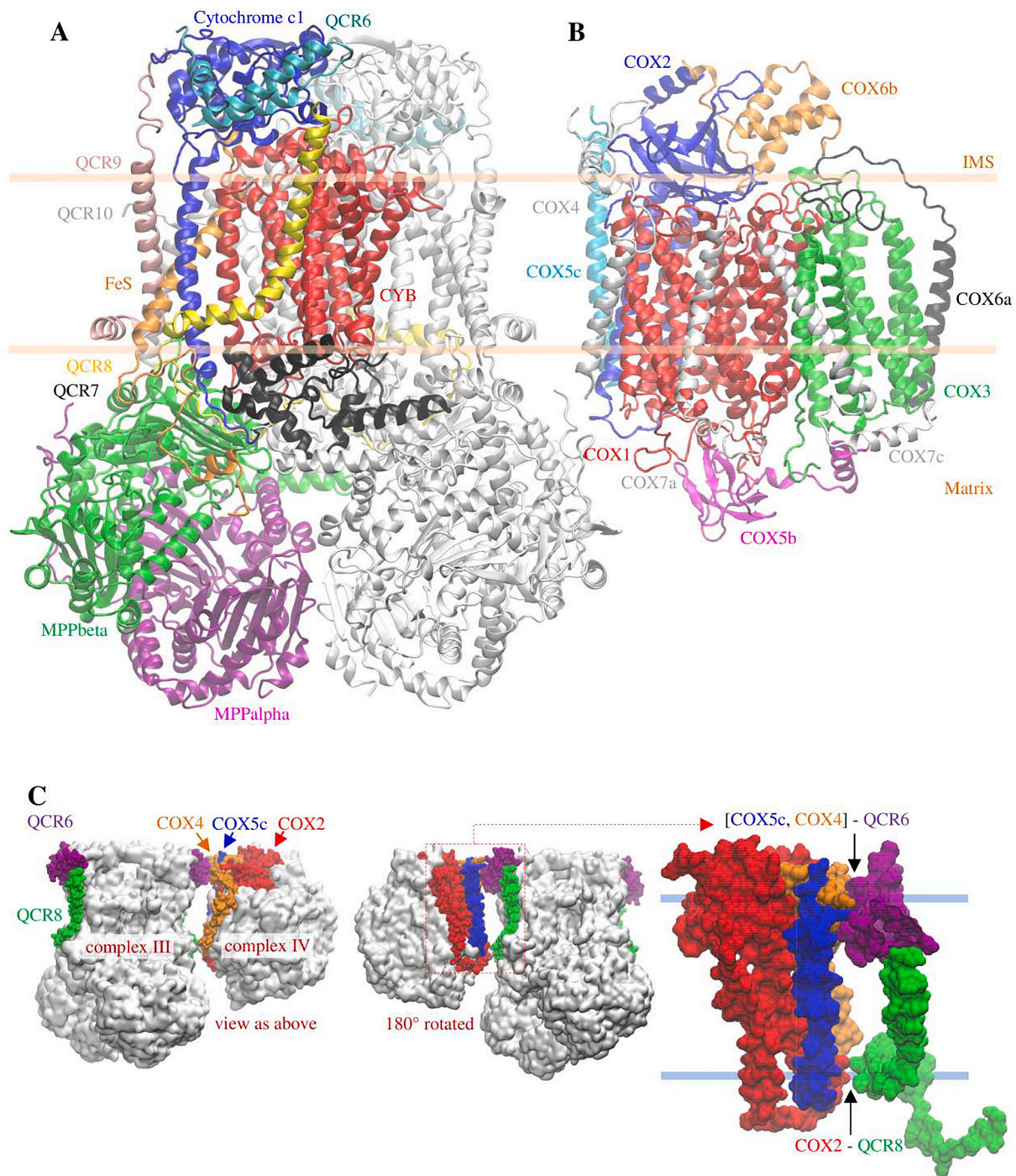


Fig. 3. Structures and relative positions of *Viscum album* (A) complex III₂ and (B) complex IV components (based on *Vigna radiata* templates 7jrg.pdb and 7jro.pdb, respectively). The QCR10 subunit (complex III), and COX4 (=Cox X2), COX7a (=Cox X4), and COX7c (=Cox X3) subunits (complex IV) are present in *Vigna radiata*, but not yet identified in *Viscum album*. (C) Complex III₂ and complex IV are known to form supercomplex III₂-IV in *Vigna radiata* (7jrp.pdb). The components - QCR6 (complex III, near IMS side) with COX5c (and COX4); and QCR8 (complex III, near matrix side) with COX2 form interfaces in the supercomplex. It is not known if COX4 is definitively absent in *Viscum album*; and if so, how the lack of COX4 will affect the formation and/or stability of the supercomplex.

identity (average 61.7% identity) as for the nucleotide sequences, but again there was a wide diversity: from 83.3% identity for SDH1 to 35.6% for OSCP (Table 2). Here the alternative oxidase and NAD(P)H dehydrogenases showed a somewhat higher degree of identity (average 69.2%), but with very little variation (64.1–73.6%) between the genes (Table 2).

When we carried out homology modeling for the 29 nuclear-encoded mitochondrial complex I–V subunits, we succeeded in finding good templates for 23 proteins (Table S4). Although the sequence identity

varied between 43% and 83% there were only minor changes in the backbone structure of the modeled proteins compared to *Arabidopsis* (results not shown). The positions of nuclear-encoded subunits in complex III₂, complex IV and supercomplex III₂-IV are shown in Fig. 3. Several components of complex III and IV (e.g. QCR10, Cox X2–X4) that are found in *Vigna* and *Arabidopsis* are not identified from *Viscum*. If they are definitively missing, it is unknown how this will affect the formation of complexes, and interaction/stability of the supercomplex in *Viscum*.

4. Discussion

Searching the transcriptome of *Viscum album* for nuclear genes involved in oxidative phosphorylation, we managed to recover an almost complete complement compared to the nuclear encoded OXPHOS subunits in *Arabidopsis*. A notable exception is complex I, where we only recovered genes for two of 40 nuclear-encoded subunits; two subunits that are not involved in the NADH dehydrogenase activity of complex I. The details of genes belonging to individual complexes are discussed below.

In order to assess the level of divergence of nuclear OXPHOS genes identified in *Viscum* we compared them to potentially orthologous genes from other eudicot taxa. Ideally, sequence from closely related taxa should be included in the analyses, but no data are yet available for any other species of the Viscaceae than *V. album*, and for the order Santalales only a limited number of genes have been identified and annotated in the *Santalum album* genome (Mahesh et al., 2018). The persistent uncertainty about the phylogenetic position of Santalales within the eudicots (One Thousand Plant Transcriptomes Initiative, 2019; The Angiosperm Phylogeny Group, 2016; Zeng et al., 2017), further adds to the difficulty of selecting appropriate taxa for comparative analyses. The placement of *Viscum* (and the unsupported placement of *Vitis*) in the concatenated tree (Fig. 2) is not consistent with previous phylogenetic hypotheses, but could be biased both by a shallow taxon sampling, alignment ambiguity and the possibility that some of the included genes are not orthologous or misannotated, e.g. due to splicing prediction errors. Annotation of nuclear genes is almost exclusively based on sequence similarity methods, and thorough homology testing will require data from a far larger number of taxa than presently available. A much denser taxon sampling will also improve alignment quality. Our sampling of only 16 species spanning 16 orders of eudicots is too limited to make robust alignments and phylogenetic inferences, not least for individual analyses of short gene sequences with little information content. Thus, we consider it premature to use the current data for more detailed studies of evolution of individual genes including calculations of e.g., substitution rates and changes in the strength of selection.

4.1. Complex I genes and proteins

Complex I is the largest OXPHOS protein complex, and in *Arabidopsis* it was thought to include 49 subunits (Braun, 2020), although the presence of only 47 was confirmed in the recently studied cryoEM structure (Klusich et al., 2021). However, in *Viscum* mitochondria, the protein complex is entirely missing (Maclean et al., 2018; Senkler et al., 2018) and the nine complex I genes usually located in the mitochondrial genome have been lost (Petersen et al., 2015a; Skippington et al., 2015). Accordingly, no transcripts of the mitochondrial genes were found by Zervas et al. (2021), so similar absence of nuclear transcripts was expected. Of the nuclear complex I subunits identified in *Arabidopsis*, we did, however, recover transcripts corresponding to L-galactono-1,4-lactone dehydrogenase (GLDH) and a gamma carbonic anhydrase (γ CA1).

The functional role of GLDH in plant complex I is not entirely clear, as the association with complex I is not conserved in other eukaryotes. Recent evidence from studies of complex I cryoEM structures of *Arabidopsis* and *Brassica* reveals that GLDH is not a part of the mature complex I, but acts as an intermediate assembly factor (Klusich et al., 2021; Soufari et al., 2020). However, GLDH also catalyzes the conversion of L-galactono-1,4-lactone into ascorbate on the outer surface of the inner mitochondrial membrane (Bartoli et al., 2000). This reaction is very important as it is the last step in ascorbate biosynthesis and it is therefore needed even in the absence of complex I. For this reason, the recovery of a transcript of the GLDH gene in *Viscum* is not surprising, and indicates that GLDH can function independently of its association with complex I. The *Viscum* GLDH gene does not exhibit any particular increased divergence compared to the gene in other angiosperms, consistent with

the role of GLDH in an unaltered biochemical process unrelated to complex I structure and function.

A small number of gamma carbonic anhydrase subunits form a well-defined domain of complex I with an elusive function (Braun, 2020; Fromm et al., 2016). In *Arabidopsis*, the protein family consists of three carbonic anhydrases (γ CA1– γ CA3) and two additional proteins termed carbonic anhydrase-like (CAL1, CAL2), but fewer proteins (3–4) have typically been identified in other angiosperms (Braun, 2020; DiMario et al., 2017; Fromm et al., 2016; Maldonado et al., 2020; Soufari et al., 2020). In *Viscum*, we have identified a single transcript which is most similar to the *Arabidopsis* γ CA1 gene. However, the nucleotide identity of the *Viscum* sequence to at least one of the other carbonic anhydrases is also quite high (70.9 % and 65.2 % to γ CA2 and γ CA3, respectively), so without a more thorough study of the evolutionary history of the gene family, the orthology of the *Viscum* gene and genes identified in other species cannot be precisely determined.

Similar to GLDH, the presence of a γ CA transcript may be unrelated to complex I structure or function. The general role of carbonic anhydrases is to catalyze the interconversion between CO_2 and HCO_3^- . Although this activity has not yet been demonstrated for plant γ CAs (DiMario et al., 2018, 2017) the presence of γ CA transcripts in *Viscum* strongly suggests a function not linked to complex I activity. In this respect, it is noteworthy that one of the γ CA proteins, γ CA3, present in *Arabidopsis* has not yet been localized to the γ CA domain of complex I (Fromm et al., 2016). Although the γ CA3 gene has the least similarity to the gene identified in *Viscum*, we tentatively suggest that an alternative function for one γ CA protein may be common to plants. Similar to GLDH, the *Viscum* γ CA1 gene does not exhibit any particular increased divergence compared to other angiosperms. Conservation of γ CA1 in *Viscum* strongly suggests a function in a biochemical process that is unrelated to complex I function.

Although it is highly unlikely, the apparent lack of transcripts for putative nuclear complex I genes in *Viscum*, except the two encoding dual-function proteins, could potentially be explained by low RNA copy numbers or a methodological inefficiency in detecting sequences if they are highly divergent. However, sequencing was made to a great depth, and the lack of transcripts is consistent with previous reports of a complete lack of the complex I genes normally encoded in the mitochondria (Petersen et al., 2015a; Skippington et al., 2017; 2015) and the absence of complex I proteins in the isolated mitochondria (Maclean et al., 2018; Senkler et al., 2018), all suggesting that the deficiency is real. Although the absence of transcripts is not proof of complete gene loss, we further consider it most likely that the nuclear complex I genes, just like the genes encoded in the mitochondria, have been at least functionally lost. However, the actual loss of the genes needs to be confirmed by complete genome sequencing. A loss of complex I genes appears to be shared by other members of Viscaceae (Zervas et al., 2019, 2021), and ongoing research aims to elucidate the evolutionary events leading to this unique situation.

4.2. Complex II genes and proteins

In plants, complex II is composed of eight subunits, SDH1–SDH8 (Braun, 2020; Huang et al., 2019). In addition to the two genes, SDH3 and SDH4, located in the mitochondrial genome of *Viscum* (Skippington et al., 2017) and confirmed in the transcriptome by Zervas et al. (2021), we recovered transcripts of five nuclear genes coding for subunits SDH1, SDH2, and SDH5–SDH7. The SDH1, SDH2, SDH4 and SDH5 proteins were also detected in the isolated *Viscum* mitochondria by Senkler et al. (2018). A transcript corresponding to the last subunit, SDH8, was not detected in the present study. The SDH8 subunit is small (4.9 kDa), its physical location in complex II is still unknown, and so far it has only been characterized in members of Brassicaceae and Poaceae (Braun, 2020; Schikowsky et al., 2017). Thus, it is possible that we have failed to detect a transcript due to its short length, or *Viscum* may lack the subunit entirely.

The mitochondrially encoded subunits, SDH3 and SDH4, are the most divergent of all *Viscum* OXPHOS proteins, and the genes are considerably shorter compared to *Arabidopsis* and other angiosperms (Table 2). Both of these genes have repeatedly been transferred to the nuclear genome in various lineages of angiosperms including *Arabidopsis* (Adams et al., 2002; Huang et al., 2019). In contrast, *Viscum* SDH1 is the most conserved of all OXPHOS proteins, and SDH2 is also among the most conserved proteins. The two subunits SDH1 and SDH2, forming the succinate dehydrogenase domain located in the mitochondrial matrix, are known to be more conserved than SDH3 and SDH4, which together with SDH6 and SDH7 form the membrane anchor domain mostly located in the inner mitochondrial membrane (Huang et al., 2019). In *Viscum*, SDH6 and SDH7 show high levels of divergence (Table 2), although not as much as in SDH3 and SDH4, suggesting that the entire membrane anchor domain is less conserved than the succinate dehydrogenase domain.

In general and despite the large variation in the degree of subunit conservation, our data support complex II functionality as reported previously, although protein abundance is low (Maclean et al., 2018; Senkler et al., 2018).

4.3. Complex III genes and proteins

Complex III in plants is composed of 10 subunits belonging to three categories: three subunits directly involved in electron transport (ubiquinol-cytochrome *c* reductase iron-sulfur subunit (RieskeFeS), cytochrome *c*₁ and cytochrome *b*), two mitochondrial processing peptidases (MPPalpha, MPPbeta), and five small subunits termed QCR6–10 (Braun, 2020). We successfully recovered transcripts of all subunits except for QCR10. Since QCR10 is the smallest subunit (57 amino acids in *Arabidopsis*), we may have failed to detect it due to its small size and sequence divergence. Of the small QCR subunits, Senkler et al. (2018) identified QCR8 and QCR10 proteins in *Viscum*, and Maclean et al. (2018) identified QCR7. Although misidentification of small subunits cannot be excluded, collective evidence suggests that *Viscum* does possess all five QCR genes.

One gene that requires special attention is MPPalpha, two copies of which are found in the *Viscum* transcriptome. Several angiosperms appear to have more than one copy. In *Arabidopsis*, two isoforms have been described (Braun, 2020; Kruff et al., 2001), and in the nuclear genome of *Glycine max* five loci are annotated (NCBI BioProject PRJNA48389). The gene tree suggests that many copies, e.g. the two in *Arabidopsis*, can be explained by repeated recent duplications, but the two copies of *Viscum* likely stem from an ancient gene duplication possibly taking place early in eudicot evolution (Fig. 1). A more precise determination of the timing of the duplication event requires inclusion of more early angiosperm lineages in the phylogenetic reconstruction. In addition to demonstrating the duplication event, the gene tree also shows that either of the two copies have subsequently been lost in some lineages. While *Arabidopsis* and *Jatropha* have retained only the copy here labelled B (Fig. 1), all Asterids and *Beta* have retained only copy A. A deeper taxon sampling will be required for further elucidating the evolutionary history of MPPalpha.

The sequences of the nuclear genes that we recovered generally appear somewhat more divergent compared to other angiosperms (Table 2, Fig. S1). In spite of that, we did not observe any differences in backbone structure that suggest functional changes (Fig. S4). This is consistent with previous biochemical recovery of complex III proteins from *Viscum* albeit with possible low enzymatic activity (Maclean et al., 2018; Senkler et al., 2018).

4.4. Complex IV genes and proteins

The three core units of plant complex IV (Cox I, Cox II, and Cox III) are encoded by genes located in the mitochondrial genome, but a number of additional smaller subunits are encoded by nuclear genes

(Braun, 2020).

In *Viscum*, the mitochondrial genes are present, but unusually divergent (Table 2; see also Petersen et al., 2015a; Skippington et al., 2015). Transcription was confirmed by Zervas et al. (2021). Here we recovered transcripts of four nuclear genes (COX5b, COX5c, COX6a, COX6b), all of which belong to the group of the genes coding for subunits resembling subunits found in animals and fungi (Braun, 2020; Mansilla et al., 2018; Millar et al., 2004). A total of five subunits have been reported from this group, and we found four of them, but failed to find a transcript corresponding to subunit Cox VIc, which was identified in *Arabidopsis* (Millar et al., 2004). The role of this subunit is uncertain, and it does not appear on the most recently published list of OXPHOS proteins (Braun, 2020). Neither did we recover transcripts corresponding to any of the six subunits Cox X1–Cox X6, which were described in *Arabidopsis* and thought to be plant-specific (Braun, 2020; Mansilla et al., 2018; Millar et al., 2004). Recently, three of them (Cox X2–Cox X4) were found to be homologous to subunits (Cox IV, Cox VIIc, Cox VIIa) in mammals and fungi, and they were identified as part of the cryoEM structure of complex IV in *Vigna radiata* (Maldonado et al., 2021).

Complexes III and IV form a III₂IV supercomplex in *Viscum*, which is unusually stable (Senkler et al., 2018) indicating changes at the interface between the component complexes. As shown in Fig. 3, the interface in *Vigna radiata* is formed by Cox II/QCR8 and Cox IV + Cox5c/QCR6 (Maldonado et al., 2021). Therefore, the absence of any of these subunits could change the stability of the supercomplex. Senkler et al. (2018) identified two copies of Cox IV (Cox X2) in *Viscum* mitochondria, and it is possible that we failed detecting it here e.g. due to high sequence divergence. Although both Senkler et al. (2018) and we failed to identify any other Cox X subunits, missing transcripts may be due to missing genes, but lack of transcription, tissue-specific expression or inefficiency in detection due to high sequence divergence cannot be ruled out.

Nucleotide and amino acid sequence divergence is relatively high for the complex IV subunits that we recovered compared to most other nuclear OXPHOS genes (Table 2, Fig. S1). In spite of that modeling suggests only changes to the backbone structure of both mitochondrial and nuclear-encoded proteins (Fig. 3, Fig. S4). Thus, the present recovery of the most essential components of complex IV in the *Viscum* transcriptome is consistent with the biochemical confirmation of complex IV activity, although the relative amount of proteins compared to *Arabidopsis* was low (Maclean et al., 2018; Senkler et al., 2018).

4.5. Complex V genes and proteins

The synthesis of ATP is catalyzed by complex V. The protein complex is composed of two parts, F₀ spanning the inner membrane, and F₁ protruding into the mitochondrial matrix, connected by a central rotating stalk (rotor) and a peripheral stalk (stator) (Braun, 2020; Zancani et al., 2020). The composition of F₁ appears to be very conserved, with five subunits (alpha, beta, gamma, delta, epsilon) described in yeast, animals and plants (Braun, 2020; Zancani et al., 2020). Composition of F₀ together with the peripheral stalk shows more variation, and in *Arabidopsis*, ten subunits (a, b, c, d, g, FAD, OSCP, subunit 8, ATP17, 6 kDa) have been described (Braun, 2020; Zancani et al., 2020). Genes corresponding to the five subunits encoded by the mitochondrial genome (alpha, a, b, c, subunit 8) were previously reported (Petersen et al., 2015a; Skippington et al., 2017; 2015) although two genes (*atp4*, *atp8*) were so divergent that Petersen et al. (2015a) initially failed to identify them. Transcription of the five mitochondrial genes was detected by Zervas et al. (2021), and except for subunit c encoded by *atp9* the proteins have been detected in *Viscum album* mitochondria (Maclean et al., 2018; Senkler et al., 2018). Here we identify transcription of nine additional nuclear genes. Compared to the complex V genes described in *Arabidopsis*, the only gene that we could not detect was the one coding for the ATP17 subunit of F₀. Once again, we may have failed to detect this relatively short gene due to high sequence

divergence, but the protein subunit was not detected by Senkler et al. (2018) and Maclean et al. (2018). However, neither of these studies detected any of the nuclear-encoded F_0 subunits in *V. album* mitochondria.

While some of the mitochondrial genes (*atp4*, *atp8*) are among the most divergent complex V genes, others (*atp1*, *atp9*) are among the most conserved (Table 2). Rather than being dependent on genomic localization, we observe a tendency towards genes coding for F_1 subunits to be more conserved than genes coding for F_0 subunits. This may be correlated with the generally higher level of conservation of F_1 compared to F_0 across major clades of life (Zancani et al., 2020), although it is worth noting that mitochondrial *atp9* coding for subunit c, which makes up the larger part of F_0 , is the most conserved of all complex V genes. For both parts of complex V there is a tendency for the genes coding for protein subunits located in rotor and stator regions (F_0 : b, d, OSCP; F_1 : gamma, delta, epsilon; see Zancani et al., 2020) to be more divergent than the genes coding for head regions (Table 2). This could be due to a higher surface to volume ratio for the subunits in the rotor and stator regions (Zancani et al., 2020), which would potentially impose fewer functional constraints for divergence.

In general, our data confirms the presence of a functional complex V. Whether sequence divergence and potential structural changes in subunit proteins can be directly linked to the low complex V protein abundances previously reported (Maclean et al., 2018; Senkler et al., 2018) remains to be studied.

4.6. Cytochrome c

The cytochrome c protein transfers electrons from complex III to complex IV, but also accepts electrons from other metabolic pathways (Braun, 2020; Welchen and Gonzalez, 2016). In *Arabidopsis*, two genes encoding two cytochrome c isoforms have been described, but in *Viscum* we have identified only one gene, which is most similar to the At4g10040 locus. The level of similarity between the *Viscum* gene and its potential orthologues in *Arabidopsis* and *Vitis* is comparable to other OXPHOS genes (Table 2), but the gene tree depicts the *Viscum* sequence as highly divergent from the rest of the sequences included in the analysis (Fig. S1). However, the topology of the gene tree bears little resemblance to angiosperm phylogeny and the relationships are almost completely unsupported. While this most likely is an effect of the short, divergent sequences and shallow taxon sampling, mistaken orthology assignment is another potential source of error. As in *Arabidopsis*, some angiosperms appear to have two loci, while others seem to have only one; the evolutionary history of the gene requires further study.

4.7. Alternative oxidase (AOX) genes

Alternative oxidases (AOX) are quinol oxidases that can bypass the complex III-IV reactions. The proteins are encoded by a small gene family including up to six members divided into two subfamilies (AOX1 and AOX2) in angiosperms (Costa et al., 2017, 2014; Pu et al., 2015). Since complexes III and IV in *Viscum* are most likely functional, we have no reason to suspect a different composition of AOX in *Viscum* compared to other angiosperms, but we recovered only one AOX gene from the *Viscum* transcriptome. Based on similarity to *Arabidopsis* genes and the classification scheme of Costa et al. (2017), the *Viscum* gene may be tentatively classified as AOX1A. This assignment is consistent with the identification of two AOX proteins (AOX1A and AOX2) in *V. album* by Maclean et al. (2018), but not with Senkler et al. (2018), who identified AOX2 in the *V. album* proteome, but not AOX1. Since several AOX genes are only expressed in specific cell types and under specific conditions (Clifton et al., 2006), it is likely that the *Viscum* genome includes more genes than the one we recovered here.

The classification approach proposed by Costa et al. (2017, 2014) may sometimes be unreliable. Many so-called ‘specific amino acids’ (Costa et al., 2014, p. 175) used for classification have quite a wide

range of variation, and this will most likely increase as more gene sequences become available. Further, identification of the specific sites is based on alignment, which is far from trivial. Finally, neighbor joining is the preferred phylogenetic method proposed by Costa et al. (2017, 2014), but this is a clustering method based on distance scores, and the method is usually considered inferior to character-based phylogenetic methods (e.g., Swofford et al., 1996). Reanalyses of the matrices provided by Costa et al. (2014) using e.g. maximum likelihood show very little support for many clades (results not shown). The classification of groups within the two proposed gene subfamilies appear particularly questionable, and the current annotation of subtypes (AOX1A–E, AOX2A–D) may be based on an underestimation of the true number of gene duplication events. If classification of genes should be based on their evolutionary relationships rather than on similarity, phylogenetic analyses are mandatory, and the work of Costa et al. (2017, 2014) is certainly a step in the right direction. However, elucidating the evolutionary history of this gene family requires a denser sampling of taxa as well as sampling all paralogous genes from any taxon.

4.8. Alternative NAD(P)H dehydrogenase genes

A small group of alternative NAD(P)H dehydrogenases serve as alternative entry points for electrons into the ETC. Whereas the alternative dehydrogenases are a supplement to complex I in other angiosperms, their role appears to be essential in *Viscum* as it lacks complex I.

In *Arabidopsis* seven genes belonging to three subfamilies (NDA, NDB and NDC) have been described (Michalecka et al., 2003; Rasmusson et al., 2008), and whereas Maclean et al. (2018) found evidence for two proteins in *V. album* mitochondria (denoted as NDA1 and NDB2), Senkler et al. (2018) found evidence for five proteins (denoted as NDA1, NDA2, NDB2, NDB3 and NDC1). Differences may stem from both tissue-specific expression and very low levels of expression. Here, we also recovered five genes from the transcriptome, which based on similarity to *Arabidopsis* suggest the same distribution into families (2 NDAs, 2 NDBs and 1 NDC) as found by Senkler et al. (2018). The two NDB genes found in *V. album* also include the EF hand motif domain characteristic of NDB proteins (Michalecka et al., 2003), but similarity to *Arabidopsis* suggest that they are NDB1 and NDB2 rather than NDB2 and NDB3. NDB3 is thought to be co-expressed with AOX1B and most so in embryos and male reproductive organs (Rasmusson et al., 2009). None of these organs were present in the tissue here used for RNA extraction, and may thus explain why none of the genes were found in the transcriptome. Homology assignment of the two NDA genes is more dubious. Preliminary phylogenetic analyses including genes annotated as NDA1 and NDA2 in GenBank suggest that their evolutionary history is far too complicated to stem from a single ancestral duplication event.

The ND genes recovered in *Viscum* do not exhibit increased divergence compared to the genes in other angiosperms (Table 2, Fig. S1). Based on this observation alone, the function and potential efficiency of these genes appears to be unmodified despite their assumed crucial role in electron transfer in the complex I-deficient *Viscum*.

5. Conclusions and perspectives

With the exception of two dual-function genes, the present study confirms complete lack of OXPHOS complex I in *Viscum album*. A number of alternative NAD(P)H dehydrogenase genes are expressed and these may be assumed to substitute for the lack of complex I. The alternative dehydrogenase genes appear to be fairly conserved compared to orthologous genes in other angiosperms suggesting there are no major functional differences. Compared to *Arabidopsis thaliana*, no major differences in the complement of nuclear genes belonging to complexes II–V are observed. The previously reported extreme divergence of most complex II–V genes encoded in the mitochondria (Petersen et al., 2015a; Skippington et al., 2017; 2015) is not matched by the nuclear genes, some of which are only slightly more divergent than

angiosperm OXPHOS genes in general. Structural modeling of OXPHOS proteins using both mitochondrial and nuclear-encoded sequences indicates overall conservation of protein structure.

Viscum album is so far the only multicellular eukaryote that has been shown to be missing complex I completely. However, lack of mitochondrial complex I genes has been shown in other species of *Viscum* and in *Phoradendron*, but not in any other lineages of parasitic plants (Petersen et al., 2020). Why complex I was lost and whether the loss is at all related to the parasitic nature of *Viscum* and *Phoradendron*, remains an open question. There is ongoing research into evolution of OXPHOS genes within Santalales, including pinpointing where in the phylogeny the loss occurred. Further studies of OXPHOS function and efficiency including studies of the seemingly crucial role of alternative NAD(P)H dehydrogenases are anticipated.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mito.2021.10.006>.

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