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Short communication

Functional loss of all *ndh* genes in an otherwise relatively unaltered plastid genome of the holoparasitic flowering plant *Cuscuta reflexa*

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Abstract

We have cloned and sequenced an area of about 9.0 kb of the plastid DNA (ptDNA) from the holoparasitic flowering plant *Cuscuta reflexa* to investigate the evolutionary response of plastid genes to a reduced selective pressure. The region contains genes for the 16S rRNA, a subunit of a plastid NAD(P)H dehydrogenase (*ndhB*), three transfer RNAs (*trnA*, *trnI*, *trnV*) as well as the gene coding for the ribosomal protein S7 (*rps7*). While the other genes are strongly conserved in *C. reflexa*, the *ndhB* gene is a pseudogene due to many frameshift mutations. In addition we used heterologous gene probes to identify the other *ndh* genes encoded by the plastid genome in higher plants. No hybridization signals could be obtained, suggesting that these genes are either lost or strongly altered in the ptDNA of *C. reflexa*. Together with evidence of deleted genes in the ptDNA of *C. reflexa*, the plastid genome can be grouped into four classes reflecting a different evolutionary rate in each case. The phylogenetic position of *Cuscuta* and the significance of *ndh* genes in the plastid genome of higher plants are discussed.

Cuscuta reflexa (Convolvulaceae) is a holoparasitic flowering plant which is fully adapted to parasitic life. This can be seen at the morphological, the cytological and the biochemical level [8]. Since parasitic plants obtain their nutrients directly from the host plants, there is no requirement for photosynthetic assimilation of carbon. Therefore these plants could serve as an excellent tool to investigate the evolution of a plastid genome on the molecular level under the conditions of reduced selective pressure.

Sequence analysis of parts of the plastid DNA (ptDNA) of *C. reflexa* revealed that most of the photosynthetic genes are present in an apparently functional form [6]. Some genes in the ptDNA of *C. reflexa* are strongly conserved compared to corresponding genes from autotrophic plants, whereas other genes differ in their regulatory sequences of either promoter on termination consensus sequences [6, 2]. In addition we found at least one large deletion in the plastid genome of *C. reflexa*. In autotrophic plants the deleted part encodes some ribosomal proteins and tRNAs. As a consequence, this might be pointing to a deficient translation apparatus in *C. reflexa* [2].

Here we report the nucleotide sequence of a 9.0 kb region of *C. reflexa* ptDNA which reflects an accelerated evolution rate in only one gene

(*ndhB*). In *Cuscuta* the *ndhB* gene is a pseudogene due to several frameshift mutations. This situation is comparable to the *ndhB* gene of another parasitic plant, *Epifagus virginiana* (Orobanchaceae), published recently [20]. Although the ptDNA of *C. reflexa* is very different in coding capacity and gene organization, there are some remarkable conformities. One of these, presented in this work, are the functional and putative total loss of all chlororespiratory (*ndh*) genes and the existence of the *ndhB* gene as a pseudogene. Such comparative analyses between the plastid genomes of parasitic plants may serve as a tool to explain still unknown functions of plastid genes.

In higher autotrophic plants eleven plastid-encoded *ndh* genes are known [15] which are homologous to genes coding for subunits of the mitochondrial NADH ubiquinone oxidoreductase (complex I). Most of the plastid *ndh* genes seem to be actively transcribed [9] and recently gene products were identified in thylakoid membranes by immunological methods [16]. These results might suggest the hypothesis of a plastid pathway, termed chlororespiration. Best evidences for the existence of a chlororespiratory chain came from investigations of the unicellular green alga *Chlamydomonas reinhardtii* [1, 5, 12]. Whether a respiratory chain is also present in the chloroplasts of higher land plants cannot as yet be unquestionably answered. However, the putative NAD(P)H dehydrogenase encoded by the plastid *ndh* genes could be involved in reoxidation of reduction equivalents generated during starch breakdown in the dark [4]. In higher autotrophic land plants all *ndh* genes are strongly conserved but the actual function of their products is as yet unknown.

In the present work the sequence of a 8.8 kb region of the ptDNA from the holoparasitic plant *C. reflexa* has been determined. Compared to tobacco ptDNA [14] the area is part of one segment of the inverted repeat (IR) and the gene arrangement is colinear with the gene map of the tobacco chloroplast genome (Fig. 1). The 8.8 kb region contains (in the following order) genes for $tRNA^{Ala}$ (*trnA*), $tRNA^{Ile}$ (*trnI*), 16S rRNA (16S rDNA), the ribosomal protein S7 (*rps7*) and both exons coding for a subunit of a plastid NAD(P)H dehydrogenase (*ndhB*; Fig. 1). Both gene content and nucleotide sequence of individual genes in this area is strongly conserved as compared with autotrophic plants [14, 10, 7], except for the *ndhB* gene of *C. reflexa*. The *ndhB* gene is a pseudogene due to many frameshift mutations (insertions/deletions; Fig. 2). Homologies to corresponding genes of tobacco [14] and rice [7] are in the range of 80% identical residues, whereas the overall degree of nucleotide identity between tobacco and rice is of about 96% (Fig. 2). In order to find out whether the *ndhB* gene is duplicated or translocated in total cellular DNA of *C. reflexa*, hybridization experiments with a homologous *ndhB* gene

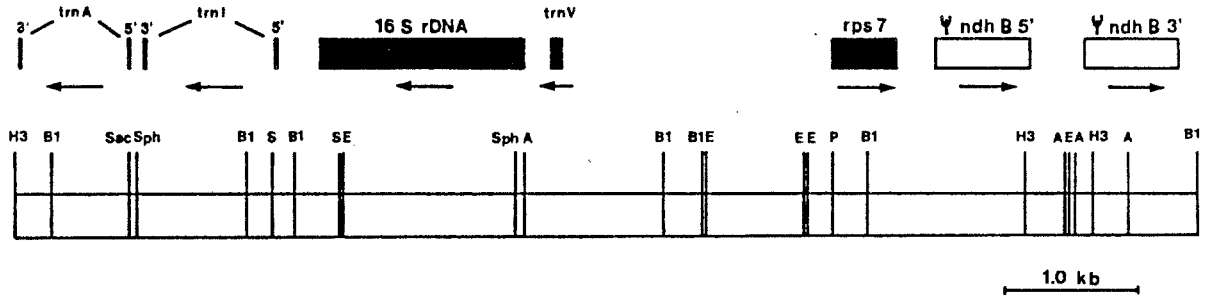


Fig. 1. Gene and restriction site map of a 8.8 kb region from plastid DNA of the holoparasitic plant *Cuscuta reflexa*. Positions and orientations of individual genes were determined by sequencing. Arrows mark the orientation of genes. The *ndhB* gene is present as a pseudogene in both exons. Below the gene map a choice of several restriction sites are indicated which also are present in the polylinker of the plasmid pUC18. Abbreviations: A, *Acc* I; B1, *Bam* HI; E, *Eco* RI; E, *Eco* RI; H3, *Hind* III; P, *Pst* I, S, *Sma* I; Sac, *Sac* I; Sph, *Sph* I.

probe were carried out. Only one signal could be obtained indicating that the *ndhB* gene is unique in *C. reflexa* (data not shown). In northern blot experiments no *ndhB* transcript is detectable indicating that no functional copy of the *ndhB* gene are present elsewhere in the entire genome. In addition we used heterologous gene probes derived from tobacco to identify the other ten *ndh* genes encoded by the plastid genome in autotrophic plants. As in *Epifagus virginiana* [3, 20], all *ndh* gene probes failed to hybridize to *C. reflexa* total cellular DNA (data not shown), suggesting that these *ndh* genes (*ndhA*, *ndhC*...*ndhK*) are completely lost or strongly altered in their nucleotide sequence in *C. reflexa*.

We then analysed genes, adjacent and upstream, the *ndhB* gene in *C. reflexa*. These genes are all components of the genetic apparatus: *trnA*, *trnI*, *trnV*, 16S, *rps7*. The 23S rRNA were identified by heterologous hybridization in *Cuscuta* (data not shown). As shown in Table 1, we computed degrees of sequence homology of genes coding for ribosomal protein S7 (*rps7*) and the 16S rDNA to corresponding genes from other higher autotrophic plants [14, 10, 7, 13] and two parasitic plants [17, 19, 20]. In contrast to *ndhB*, the genes for *rps7* and 16S rDNA are strongly conserved and, hence, appear functional in ptDNA of *C. reflexa*. The gene organization of the ribosomal RNA operon in all land plants investigated has the same order; *trnV*-16S-*trnI*-*trnA*-23S-4.5S-5S [11, 15]. These relations were also found in *C. reflexa* ptDNA.

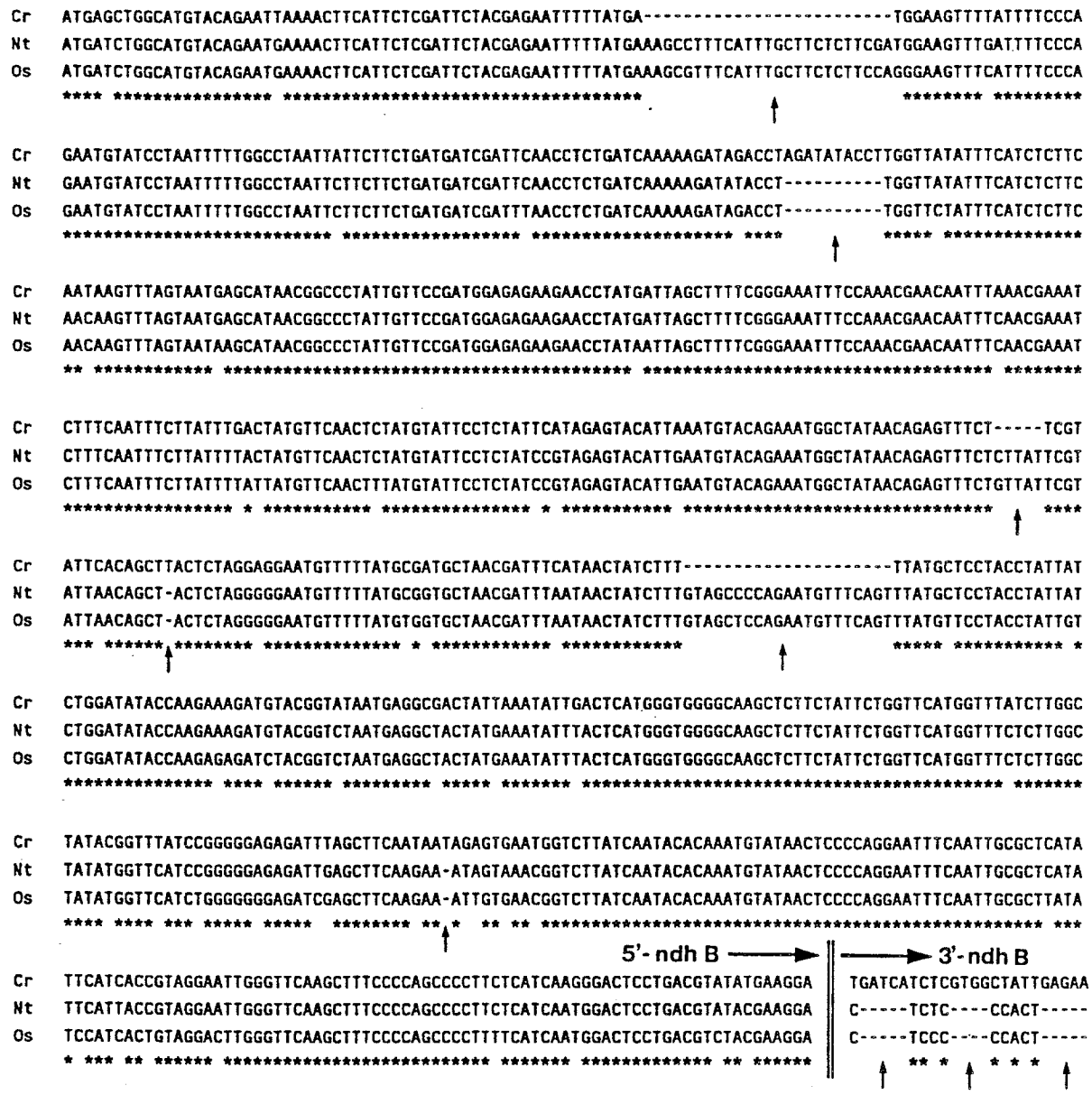
Unlike transfer RNAs described previously [6, 2], the three tRNAs (*trnV*, *trnI*, *trnA*) analysed in the present work are identical to those from tobacco (100% identical residues). Whereas the *trnI* and *trnA* in the 16S–23S spacer are both separated into two exons, the *trnV* upstream the 16S rRNA do not contain an intron. All tRNAs are able to form the typical cloverleaf secondary structure (data not shown). In *Epifagus virginiana* [20] and *Conopholis americana* [18] these genes are either lost or pseudogenes.

Based on the present examination and recently published data [6, 2] the plastid genome of *C. reflexa* reveals that the evolution rates of individual chloroplast genes differ considerably from one another. Therefore the plastid genome of *C. reflexa* can be grouped into four classes of gene reduction: (1) a first class contains genes that vary little from corresponding genes in autotrophic plants; the genes are transcribed at levels similar to autotrophic plants (e.g. *psbA*; [2]); (2) a second group exhibits altered regulatory sequences of either promoter or terminator, which results in a reduced transcription rate (e.g. *rbcL*; [6]); (3) a still further reduction can be obtained in the nucleotide sequence of the *ndhB* gene which is a pseudogene; as a consequence, no transcript was detectable in total cellular RNA of *C. reflexa*; (4) the greatest alteration was found in at least one large deletion in ptDNA of *C. reflexa* concerning two ribosomal proteins and tRNAs [2].

The results are different from *Epifagus virginiana*, a root-parasitic plant, in which a much more uniform reduction of the plastid genome was observed [20]. In *Epifagus* both photosynthesis-related genes and all chlororespiratory genes are either lost or pseudogenes [20]. Therefore,

Table 1. Amino acid sequence homologies between plastid-encoded *rps7* and 16S rDNA genes from *C. reflexa* and corresponding genes from other sources. CR, *Cuscuta reflexa* (this work); NT, *Nicotiana tabacum* [14]; EV, *Epifagus virginiana* [20]; OS, *Oryza sativa* [7]; MP, *Marchantia polymorpha* [10]; CA, *Conopholis americana* [17]; ZM, *Zea mays* [13].

%	*rps7*					%	16S				
	CR	NT	EV	OS	MP		CR	CA	EV	NT	ZM
CR	–					CR	–				
NT	98.1	–				CA	96.2	–			
EV	90.3	91.0	–			EV	96.3	96.0	–		
OS	80.8	82.7	76.3	–		NT	98.8	96.0	96.0	–	
MP	76.1	77.4	75.5	69.2	–	ZM	95.6	93.6	93.0	95.6	–


5'- ndh B
3'- ndh B

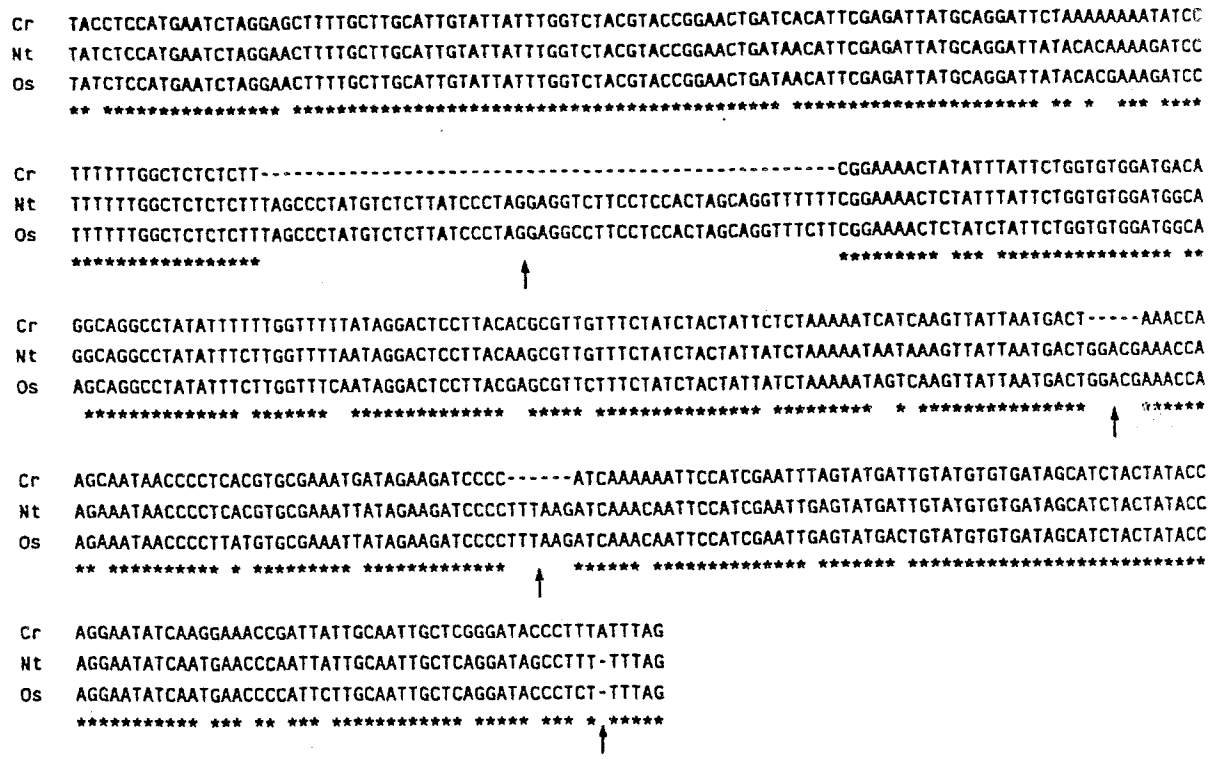


Fig. 2. Nucleotide sequence alignment of both exons of the *ndhB* pseudogene in *C. reflexa* ptDNA. The double vertical line indicates the junction between exon and intron. Both exons (5′ and 3′) are compared to corresponding sequences of tobacco [14] and rice [7]. Arrows mark *C. reflexa*-specific insertions/deletions which are responsible for several frameshift mutations. As a consequence, the reading frame is interrupted by stop codons.

de Pamphilis and Palmer [3] proposed that the *ndh* genes are involved in a metabolism closely associated with photosynthesis in green plants. This is consistent in the case of *Epifagus*, but in *C. reflexa* we are confronted with the phenomenon that all photosynthetic genes investigated are present in an apparently functional form, whereas all *ndh* genes are lost or significantly altered. *Epifagus* may represent a more advanced stage in evolution than *Cuscuta*, but why the *ndh* genes are lost remains a mystery.

A possible explanation might be related to the ecological form of parasitism of both plants. *Epifagus* is a root parasite, whereas *Cuscuta* lives as a stem-parasitic plant. Possibly *Cuscuta* requires photosynthetic genes in the seedling stage. In this case the *ndh* genes may not be directly involved in photosynthetic processes and are therefore unnecessary. On the other hand, the high degree of conservation of *ndh* genes among land plants suggests that the genes must have a function in chloroplasts of higher autotrophic plants. It seems to be a function that can be abandoned first in non-photosynthetic plants.

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