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Loss of photosynthetic and chlororespiratory genes from the plastid genome of a parasitic flowering plant

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PHOTOSYNTHESIS is the hallmark of plant life and is the only plastid metabolic process known to be controlled by plastid genes[1,2]. The complete loss of photosynthetic ability, however, has occurred on several independent occasions in parasitic flowering plants[3,4]. Some of these plants are known to lack chlorophyll and certain photosynthetic enzymes[4], but it is not known to what extent changes have occurred in the genes encoding the photosynthetic apparatus or whether the plants even maintain a plastid genome. Here we report that the nonphotosynthetic root parasite *Epifagus virginiana* has a plastid chromosome only 71 kilobases in size, far smaller than any previously characterized land plant plastid genome[5]. The *Epifagus* plastid genome has lost most, if not all, of the 30 or more chloroplast genes for photosynthesis and most of a large family of plastid genes, the *ndh* genes, whose products may be involved in a plastid respiratory chain[6–9]. The extensive changes in *Epifagus* plastid gene content must have occurred in a relatively short time (5–50 × 10^{6} yr), because *Striga asiatica*, a related photosynthetic parasite, has a typical complement of chloroplast genes for photosynthesis and chlororespiration. The plastid genome of *Epifagus* has retained transcribed ribosomal RNA and ribosomal protein genes, suggesting that it expresses one or more gene products for plastid functions not related to photosynthesis.

Epifagus virginiana (beechdrops) is a member of the *Orobanchaceae*, a family of root-parasitic angiosperms believed to lack chlorophyll and photosynthetic function[4,10], and thus termed 'holoparasitic'. Like most other Orobanchaceae that have been examined[11–13], *Epifagus* lacks detectable levels of chlorophyll and the photosynthetic enzyme ribulose bisphosphate carboxylase/oxygenase, and acquires its carbon entirely from a photosynthetic host (the beech tree, *Fagus grandifolia*) (ref. 14 and C.W.D., J. Seeman, N. Bowlby, J. A. Teeri and J.D.P., unpublished data). Despite this lack of photosynthetic ability, *Epifagus* contains plastids, some of which accumulate starch[15].

Eighty-three cloned fragments representing the entire plastid genome of *Nicotiana tabaccum* were used in filter blot hybridizations to construct a physical and gene map of the *Epifagus* plastid DNA (ptDNA). The genome is a 71-kilobase (kb) circular chromosome with a ribosomal-RNA-encoding inverted repeat (IR) structure characteristic of most ptDNAs[16] and with many other plastid genes colinear with those of *Nicotiana* (Fig. 1). The *Epifagus* genome is the smallest by far of over 1,000 now-examined land plant ptDNAs (range 120–220 kb; ref. 5), and, moreover, has only about one-third the sequence complexity of a typical plastid genome (46 kb versus 130 kb for *Nicotiana* ptDNA; ref. 2).

To illustrate several of the conserved and deleted sequences in *Epifagus* ptDNA, and to examine the distribution of photosynthetic and *ndh* genes in plants related to *Epifagus*, we hybridized various gene probes from *Nicotiana* to DNAs of *Epifagus*, *Nicotiana*, *Antirrhinum majus* and *Striga asiatica*. The latter two species are, respectively, nonparasitic and hemiparasitic (that is, a photosynthetic parasite) members of the Scrophulariaceae family, from which the Orobanchaceae was derived[3]. Each of the gene probes hybridized strongly to the photosynthetic plants,

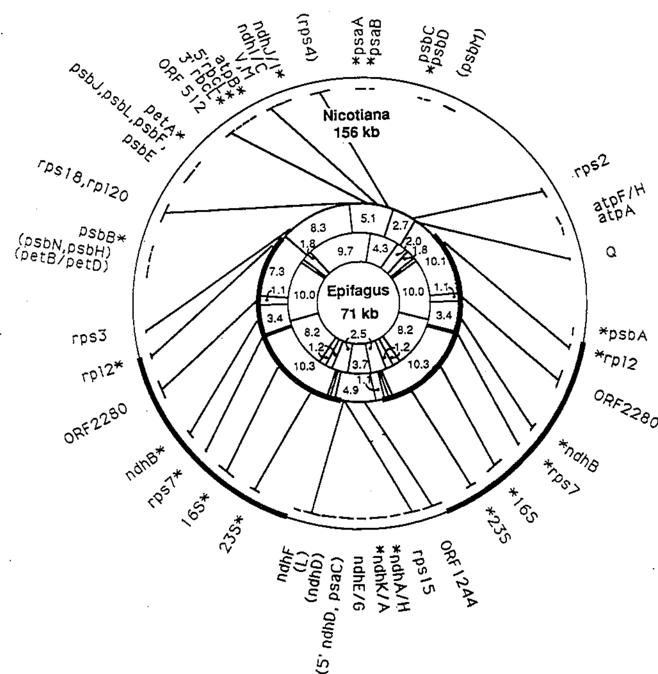

FIG. 1 The 71-kb ptDNA of *Epifagus virginiana* has lost numerous photosynthetic genes and *ndh* genes while retaining rRNA and tRNA genes, ribosomal protein genes, ORFs and an IR. The two inner circles show restriction site maps (*Cla*I, outer; *Hin*dIII, inner), for *Epifagus* ptDNA; outer circle a partial gene map for tobacco ptDNA[2]. Gene nomenclature follows ref. 5. IR segments in both genomes are indicated with thickened lines. Gene probes from *Nicotiana* that hybridized to *Epifagus* ptDNA are shown by lines connecting the two maps; other indicated genes from *Nicotiana* failed to hybridize to *Epifagus* ptDNA. All probes are gene-specific except those given in parentheses. Hybridization results illustrated in Fig. 2 are indicated by an asterisk. Transfer RNA genes are indicated by the single-letter code for their cognate amino acids.

METHODS. Total DNA was extracted from above ground tissue of *Epifagus* (Washtenaw) as described[27] and digested with *Xho*I, *Cla*I, *Hin*dIII, *Sac*I, *Sma*I, *Pst*I and *Sal*I singly and in double digest with *Xho*I. The digested DNAs were separated on 0.8% agarose gels and transferred to nylon filters (Zetabind; AMF Cuno) for initial hybridizations with 60 contiguous subclones of the entire ptDNA of *Nicotiana*. Restriction enzyme digestion, agarose gel electrophoresis, Southern transfer (double-sided dry blots), preparation of ^{32}P-labelled probes and hybridization were as described[28]. Hybridizations were at 65 °C and after hybridization filters were washed once at room temperature and three times at 65 °C in 2 × SSC, 0.5% SDS. Lower stringency hybridizations and washes at 50 °C and 4 × SSC for the genes *rbc*L and *psb*A also failed to detect a signal from *Epifagus*. Construction of the map from the hybridization results was as described[29]. Complete restriction maps for each of seven enzymes showing size and location of each deletion and conserved region will be presented elsewhere. A list of the probes used for the restriction site mapping may be obtained from the authors. After construction of the restriction site map, hybridization results from small probes specific for one or a few genes, were used to identify gene locations on the *Epifagus* map. The probes used for gene mapping are listed below, followed by their coordinates from the *Nicotiana* plastid genome[30]: *psb*A, 419–1731; (Q), 6,652–7,637; *atp*A, 10,611–12,060; *atp*F/H, 12,305–13,913; (*rps*2), 16,000–17,446; (*psb*M), 29,820–31,918; *psb*D, 34,656–35,492; *psb*C, 35,839–36,455; *psa*A, 41,611–43,426; (*rps*4), 45,076–48,602; *ndh*J/I, 51,205–52,330; (*ndh*I/C), 52,330–53,599; V, M, 53,599–54,870; *atp*B, 55,921–56,836; 5′ *rbc*L, 56,836–58,047; 3′ *rbc*L, 58,047–59,305; ORF 512, 59,305–60,654; *pet*A, 64,101–65,302; *psb*J, *psb*L, *psb*F, *psb*E, 65,302–67,129; (*rps*18, *rpl*20), 70,773–74,167; *psb*B, 75,822–76,545; (*psb*N, *psb*H), 76,545–77,375); *pet*B/*pet*D, 77,375–79,188; rps3, 85,250–85,632; *rpl*2, 86,681–87,725; ORF 2280, 91,923–94,562; *ndh*B, 96,981–98,527; *rps*7, 99,726–99,987; 16S RNA, 101,532–104,801; 23S RNA, 107,136–108,816; *ndh*F, 111,924–113,113; (L), 113,119–116,171; (*ndh*D), 116,171–118,604; *psa*C, 118,604–119,520; *ndh*E/G, 119,520–120,809; *ndh*K/A, 120,809–123,676; *ndh*A/H, 123,676–124,907; (*rps*15), 124,907–127,440; ORF 1244, 127,440–130,600. For *psa*B, we used a 1.6-kb *Bam*HI gene-internal fragment from the ptDNA of *Spinacia oleracea*.

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including the hemiparasite *Striga* (Fig. 2). In addition, most of the gene probes derived from the *Nicotiana* IR hybridized strongly to the IR region of *Epifagus* ptDNA. Ribosomal protein genes and various open reading frames (ORFs) hybridized less strongly to the *Epifagus* single-copy regions. By contrast, only one of the 22 photosynthetic genes examined, *psa*A, was detected in *Epifagus* DNA. Because the *psa*A hybridization signal did not map to any location on the *Epifagus* plastid genome (Figs 1, 2), and because its strength appears too great for a single-copy nuclear gene, we suspect that this represents a sequence that has been incorporated into the mitochondrial genome[17,18].

The 10 or more *ndh*-like genes[7,8,19,20] represent the largest family of plastid genes without a clearly defined function. These sequences have significant amino-acid similarity to mitochondrial and nuclear genes encoding components of the mitochondrial respiratory chain NADH dehydrogenase. The products of these genes could be involved in a membrane-bound respiratory process present in the chloroplast (chlororespiration)[7,20], but the only direct evidence for this comes from the alga *Chlamydomonas reinhardtii*[6,9]. That these genes are conserved across a variety of land plants[2] and are expressed[7] supports the view that such a chloroplast respiratory chain also exists in land plants. All *ndh* probes from the tobacco small and large single-copy regions failed to hybridize to *Epifagus* ptDNA; only weak hybridization was detected with the IR gene *ndh*B (Fig. 2). Because mitochondrial respiration is one of the physiological functions that is expected to remain intact in parasitic plants[4], the loss of the *ndh* genes from the *Epifagus* plastid genome makes it unlikely that products of these genes are essential components of a mitochondrial NADH-dehydrogenase complex. Instead, their loss is consistent with their function being tied to photosynthetic metabolism and, hence, to the chloroplast of green plants.

Transcripts from *Epifagus* of plastid 16S rRNA, 23S rRNA and ribosomal protein mRNA were detected on northern blots (Fig. 3 and data not shown). As a proportion of total cellular RNA, *Epifagus* fruits contain about one-fiftieth as much plastid rRNA as do *Nicotiana* leaves, whereas mitochondrial rRNAs are present in nearly equal levels in the two tissues (Fig. 3). As expected, given the evidence from the Southern blots (Figs 1, 2), transcripts of *rbc*L and *psb*A were not detected in *Epifagus*, whereas abundant transcripts of these genes were observed in *Nicotiana*, *Antirrhinum* and *Striga* (data not shown).

We suggest that a rapid loss of photosynthetic and chlororespiratory genes followed the relaxation of selection for photosynthetic capacity in ancestors of *Epifagus*, irreversibly committing the plant to a heterotrophic existence. The range of times since the holoparasite *Epifagus* and the hemiparasite *Striga* shared a common ancestor probably does not extend beyond the limits of 5×10^6 years ago (oldest fossil record of Orobanchaceae) and 50×10^6 years ago (oldest fossil record of Scrophulariales; ref. 21). At either extreme, this implies much more rapid change in gene content than is normally found for ptDNA[2,16,22]. This evolutionary dynamic is also in stark contrast to that of the mitochondrial and nuclear genomes of plants, which are much more prone to the accumulation of foreign sequences, including a variety of chloroplast-derived sequences, and the retention of nonfunctional endogenous sequences[17,18]. The *psa*A sequence of *Epifagus*, which does not presently reside in the plastid genome, may be an example of one such nonfunctional gene that has gained a longer lifespan outside the plastid genome than within it.

An interesting contrast to the photosynthetic gene losses seen in *Epifagus* occurs in *Astasia longa*, a nonphotosynthetic alga morphologically similar to *Euglena gracilis*. A circular 73-kb DNA isolated from *Astasia* has major structural features similar to that of *Euglena* ptDNA, but lacks several chloroplast photosynthetic genes[23]. Unlike *Epifagus*, *Astasia* retains an intact and expressed *rbc*L gene[24]. It is possible that the *Epifagus* plastid genome has gone further in its reduction of unnecessary information. More likely, the *rbc*L gene product could have a function in *Astasia* that is either not found in *Epifagus* or supplied by some other gene product. The presence of transcribed copies of

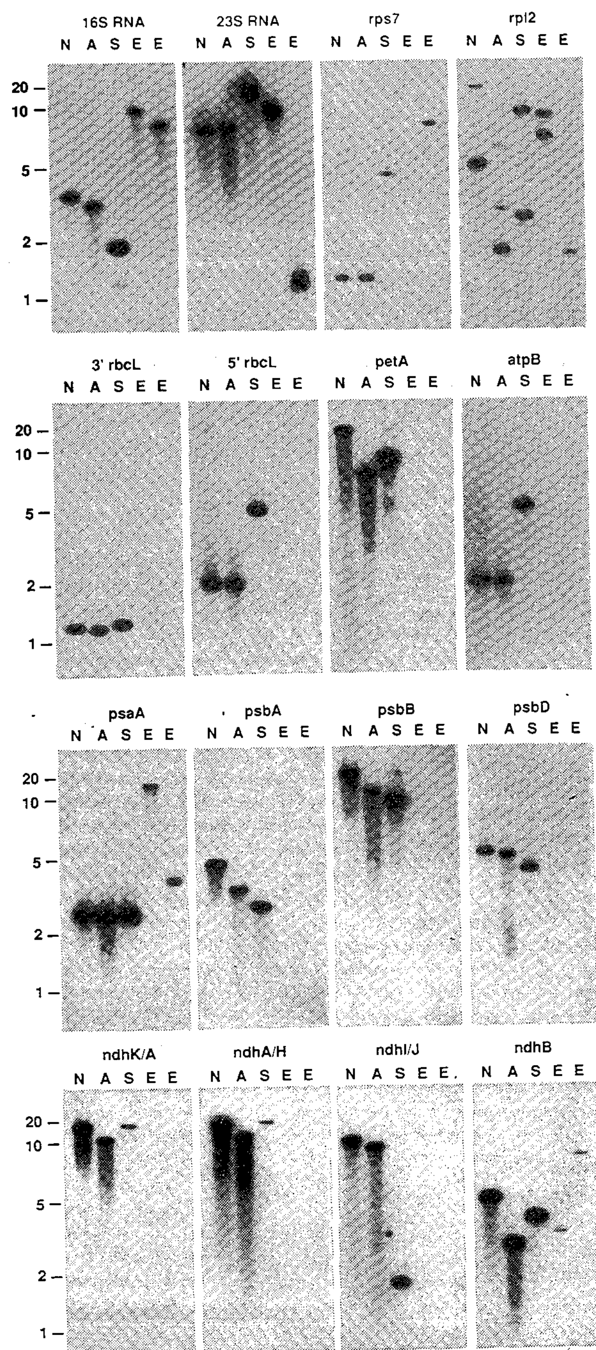

FIG. 2 Southern blots illustrating presence of chloroplast ribosomal RNA and protein genes and absence of numerous chloroplast photosynthetic and *ndh* genes from *Epifagus*. Each of 16 panels shown is a Southern blot of ptDNA of *Nicotiana tabacum* (N), *Antirrhinum majus* (A), and *Striga asiatica* (S), all cut with *Bam*HI, and two lanes (E) of *Epifagus virginiana* total DNA cut with *Cla*I (left) and *Hin*dIII (right), and hybridized to the indicated gene-specific probe (see legend to Fig. 1 for probe coordinates). Fragment sizes are marked at left in kb. A 300-base-pair (bp) *Cla*I fragment hybridizing to *rps*7 cannot be seen here.

METHODS. As in Fig. 1; in addition, ptDNAs were extracted from sucrose gradient-purified chloroplasts as described[29]. Probes for hybridization were isolated from plasmid clones in 1.0% low-gelling-temperature agarose and nick-translated directly in agarose silices or following separation from gel with GeneClean (Bio 101, La Jolla, California).

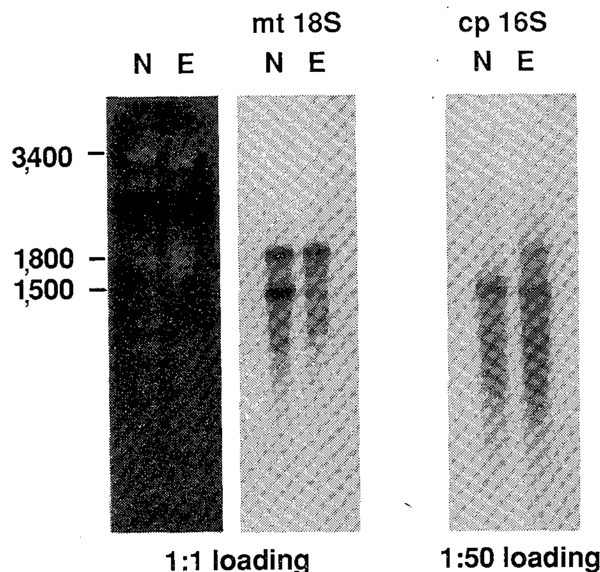

FIG. 3 Northern blot analysis illustrating presence of mitochondrial (mt) and chloroplast (cp) transcripts in *Epifagus*. Total RNA from leaves of *Nicotiana tabacum* (N) and fruits of *Epifagus virginiana* (E) were fractionated on a 1.5% agarose–formaldehyde gel[28]. For 1:1 loadings we adjusted the *Nicotiana* and *Epifagus* RNAs so that the cytoplasmic rRNA bands were of about equal intensity. *Nicotiana* RNA was diluted 50-fold in water for the 1:50 loading. RNAs were transferred to nylon filters and hybridized with clones containing the mitochondrial 18S rRNA gene from *Brassica campestris* and the chloroplast 16S rRNA gene from *Nicotiana*. Sizes of RNA bands in nucleotides are indicated for the 28S and 18S cytoplasmic rRNA and the chloroplast 16S rRNA (1,500 bp). The smaller band in the *Nicotiana* 18S hybridization and the larger band in the *Epifagus* 16S hybridization represent cross-hybridization to the 16S and 18S RNAs, respectively, and reflect the well-established sequence similarities between plant mitochondrial 18S rRNA and chloroplast 16S rRNA[32].
METHODS. Total RNA was extracted from whole green leaves of *Nicotiana* and fruits of *Epifagus*, frozen and powdered in liquid nitrogen, then homogenized in guanidinium isothiocyanate buffer and pelleted through RNase-free 5.66 M CsCl (ref. 31). Prehybridization and hybridization (65 °C) were in 0.5% SDS, 2×SSC, 5% dextran sulphate, 100 μg ml^{-1} carrier DNA[28]. Preparation of ^{32}P-labelled gel-isolated probes and post-hybridization washes were as in Figs 1 and 2.

*rbc*L and *rbs*S in the holoparasite *Cuscuta*[25] might also be explained in either of these ways.

Why does *Epifagus* maintain a plastid genome? The highly selective retention of components of the translational apparatus in the *Epifagus* plastid genome and its transcription suggest that the genome is retained to produce one or more gene products involved in nonphotosynthetic plastid metabolic processes[1,26]. Although these genes may include one or more of the plastid-encoded ORFs with so-far unknown function[2], the *ndh* genes are clearly not among them. □

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