Complete Nucleotide Sequence of the Chloroplast Genome from a Leptosporangiate Fern, *Adiantum capillus-veneris* L.

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Abstract

We determined the complete nucleotide sequence of the chloroplast genome of the leptosporangiate fern, *Adiantum capillus-veneris* L. (Pteridaceae). The circular genome is 150,568 bp, with a large single-copy region (LSC) of 82,282 bp, a small-single copy region (SSC) of 21,392 bp and inverted repeats (IR) of 23,447 bp each. We compared the sequence to other published chloroplast genomes to infer the location of putative genes. When the IR is considered only once, we assigned 118 genes, of which 85 encode proteins, 29 encode tRNAs and 4 encode rRNAs. Four protein-coding genes, all four rRNA genes and six tRNA genes occur in the IR. Most (57) putative protein-coding genes appear to start with an ATG codon, but we also detected five other possible start codons, some of which suggest tRNA editing. We also found 26 apparent stop codons in 18 putative genes, also suggestive of RNA editing. We found all but one of the tRNA genes necessary to encode the complete repertoire required for translation. The missing trnK gene appears to have been disrupted by a large inversion, relative to other published chloroplast genomes. We detected several structural rearrangements that may provide useful information for phylogenetic studies.

Key words: annotation; genome structure; inversion

1. Introduction

In the past decade biologists have witnessed a significant improvement in understanding phylogenetic relationships in most groups of organisms. This progress has resulted from an increase in the use of nucleotide sequence data from one or a few genes, and also from improvements in methods of phylogenetic analysis. However, in some ways, the use of gene sequence data has had diminishing returns especially at deeper (ancient) phylogenetic levels, such that many aspects of relationships among major clades remain unresolved. For example, it is not clear which group is the most basal clade of land plants.¹–³ The problems associated with inferring deep phylogeny using nucleotide data probably relate to the weak historical signal contained in data with a limited number of character states (four) such that homoplasy is likely. These limitations can be overcome to some degree by increasing the number of genes or by incorporating other types of data. One emerging approach is to use complete genome sequences. Such data can be used for traditional phylogenetic analyses of aligned nucleotide regions. However, the data can also be used to infer changes in genome structure, such as inversions, translocations, nucleotide insertions or deletions, gene losses, or expansion and contraction of repeat units. These are complex characters with a large number of potential states and therefore less prone to homoplasy than the four-state nucleotide characters. In fact, structural rearrangements in chloroplast genomes have been used since the 1980s to infer phylogeny.⁴–⁶ For example, a 60-kb inversion in bryophytes and lycopods relative to other vascular plants is strong evidence that lycopods are the basal lineage of vascular plants.⁷ These early studies were done by hybridizing labeled heterologous probes to restriction-digested DNA. Current technology makes it easier to determine complete genome sequences, which leads to simpler data storage and comparison, and eliminates the need for additional cross-taxon probing. Furthermore, structural analysis can be made at any scale, and can therefore detect small rearrangements that would be missed by a large heterologous probe. In addition to phylogenetic studies, complete genome sequence data can be used for many applications, including studies of cellular function, gene function, post-transcriptional modification, and genetic manipulation. This paper is part of a larger effort to sequence and analyze complete
organellar genomes from all major clades of green plants. Here we describe and compare the first complete nucleotide sequence of a leptosporangiate fern chloroplast genome.

2. Materials and Methods

*Adiantum capillus-veneris* L. is in the fern family Pteridaceae within a large clade of recently derived leptosporangiate families: a clade that includes the majority of fern species. The chloroplast genome of this species has been previously cloned and mapped, providing a starting point for our study. *Pst* fragments of *Adiantum capillus-veneris* chloroplast DNA were cloned in pUC18 and introduced into competent JM101 cells. Inserts were gel-purified from the larger *Pst* I clones (more than 10 kb), digested with *Sal*I and subcloned back into pUC18 to provide additional start sequences for primer walking. All inserts (original *Pst* I and a selection of *Sal*I) were then sequenced, and all *Pst* I fragments and bridges were assembled to obtain the complete chloroplast genome sequence, taking into account the large inverted repeat region. All sequencing was performed with the PRISM™ Big Dye™ 2.0 Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA), and reactions were run on an ABI 377 DNA sequencer. We used Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, MI, USA) for sequence analysis and assembly.

To locate putative genes in the *Adiantum* chloroplast genome, we extracted all protein sequences from the published chloroplast genomes of *Psilotum nudum* (NC 003386) and *Nicotiana tabacum* (NC 001879), and used PSI-BLAST 2.2.3 to compare each amino acid sequence to all six reading frames in *Adiantum*. We used BLASTN 2.2.3 to locate ribosomal RNA genes, and tRNAscan-SE v.1.15 to locate putative transfer RNA genes. Any regions in the *Adiantum* sequence longer than 300 bp that did not contain features after this process were compared with the current release of GenBank (26 Aug 2002) using BLASTX to identify additional features. We then compared the gene order in *Adiantum* against its putatively closest relative for which a complete sequence is published (*Psilotum*), as well as to *Nicotiana*. For any discrepancies, we made additional comparisons to *Marchantia polymorpha* (NC 001319), *Zea mays* (NC 001666), and *Pinas thunbergii* (NC 001631). We considered regions to be homologous to annotated regions from other genomes if the e-value of the BLAST hit was less than $10^{-4}$.

3. Results and Discussion

The complete chloroplast genome sequence of *Adiantum capillus-veneris* is deposited in GenBank under accession no. AY178864. The genome assembled according to the map of Hasebe and Iwatsuki with few exceptions. We detected one additional (57 bp) *Pst* I fragment between fragments 6 and 12 that were described as adjacent by Hasebe and Iwatsuki. We also found no evidence of *Pst* I fragments 24 (500 bp) and 25 (200 bp) reported by Hasebe and Iwatsuki. This is not too surprising since the latter study used agarose gel electrophoresis which has much lower resolving power to detect fragments.

3.1. Genome structure

In the fully assembled genome sequence, the large single-copy region (LSC) is 82,282 bp, the small single-copy region (SSC) is 21,392 bp and the inverted repeats (IR) are 23,447 bp for a total of 150,568 bp (Fig. 1). The overall structure of the genome is typical of vascular plants with especially good synteny in the LSC. However, several rearrangements, not detected by restriction fragment analyses, were detected here. For example, the genes *psbM* and *petN* (in the LSC) are included in an ~ 300 bp inversion unique to *Adiantum*, among complete genomes sequenced to date. It should be possible to design PCR primers to screen other ferns to determine if this inversion is unique to a particular clade. Furthermore, *Adiantum* shares an inversion of about 3300 bp (including *psbD, psbC, psbZ*) with *Psilotum*, relative to *Marchantia, Pinus, Nicotiana,* and *Zea*. This inversion may unite all members of the moniliform clade, which includes the horsetails, ferns, and Psilotaceae. Gene order data will be required from *Equisetum, Osmunda,* and euphorbiangiate ferns to test this hypothesis. Both the ~ 300 bp inversion and the ~ 3300 bp inversion are in the same region as the end-point of a large ~ 60 kb inversion in all vascular plants except the lycopsids. This suggests the possibility of a hotspot for inversions in this region. Stein et al. suggest a similar hotspot associated with *psbA*.

We detected a large rearrangement (incorporating most of the inverted repeat region) relative to the other sequenced chloroplast genomes. This structure has been identified previously using mapping studies and appears to be derived in most ferns, but not in the basal lineage represented by *Osmunda*. Relative mapping of *Adiantum* with *Nicotiana* used gene order to interpret this as an expansion of the LSC in *Nicotiana,* and two overlapping inversions in the ferns. This results in expansion of the IR in most ferns. Examination of sequence data confirms the gene order interpretation of Hasebe and
Iwatsuki\textsuperscript{17} with one minor difference that does not affect the “two inversion” hypothesis, above. The \textit{trnK} gene was located by Hasebe and Iwatsuki\textsuperscript{17} using a \textit{Nicotiana} probe that was probably \textit{matK} (based on the nucleotide position in \textit{Nicotiana}), although not annotated as such at the time. In \textit{Adiantum}, the result of the rearrangement is that the first exon of \textit{ndhB} is on the complementary strand at the beginning of the LSC (close to the junction of LSC-IR\textsubscript{A}), the intron spans the LSC-IR\textsubscript{A} junction, and exon 2 continues in the inverted repeat. There is, therefore, an orphan exon 2 at the end of the IR\textsubscript{B} (Fig. 1). In the other genomes compared here, both \textit{ndhB} exons are in the inverted repeat. Additional gene order data from other basal lineages of ferns (such as Gleicheniaceae and Hymenophyllaceae) could be used to test the “two inversion” hypothesis, by finding a lineage with only one of the two inversions.

Table 1 lists all genes that we detected in the chloroplast genome of \textit{Adiantum}. The genes \textit{rps16} and \textit{chlL} are absent from \textit{Psilotum}\textsuperscript{19,20} but are present in \textit{Adiantum}. However, we were unable to locate homologs of \textit{Psilotum orf83} or \textit{orf119} in \textit{Adiantum}. These may be spurious open reading frames (i.e., they are not transcribed) or sequence divergence has lowered the similarity to a level that they are not detectable by BLAST. The gene \textit{psaM} is annotated in \textit{Psilotum}, \textit{Chaetosphaeridium} (NC 004115) and \textit{Pinus}, but not in \textit{Nicotiana}, \textit{Zea}, and \textit{Marchantia}. However, our BLAST analyses located a candidate gene...
Table 1. List of genes annotated for *Adiantum capillus-veneris* chloroplast DNA. Asterisk denotes an intron-containing gene.

| Gene class                  | Genes                                      |
|-----------------------------|--------------------------------------------|
| Ribosomal RNAs              | rrn16 x2, rrn23 x2, rrn5 x2, rrn4.5 x2     |
| Transfer RNAs               | trnQ-UUG, trnG-GCC, trnM-CAU, trnV-UAC*    |
| trnS-GCU                    | trnS-UGA, trnSeC-UCA, trnC-GCA             |
| trnG-UCC*                   | trnT-GGU, trnW-CCA, trnY-GUA               |
| trnR-UCU                    | trnP-GAU, trnP-GGG                         |
| trnD-GUC                    | trnS-GGA, trnL-CAU, trnL-UAG               |
| trnA-TGC* x2                | trnN-GUU x2, trnT-UUG* x2, trn-GAU* x2     |
| trnE-UUC                    | trnF-GAA, trnR-ACG x2, trnL-CAA*           |
| trnH-GUG x2                 |                                            |
| Photosystem I               | psaA, psaB, psaC, psaD                     |
| psaI                        |                                            |
| Photosystem II              | psbA, psbB, psbC, psbD                     |
| psbE                        | psbF, psbH, psbI                           |
| psbI                        | psbK, psbL, psbM                           |
| psbN                        | psbT, psbZ                                 |
| Cytochrome                  | petA, PetB*, PetD*, petG                   |
| petL                         | petN                                      |
| ATP synthase                | apfA, apfB, apfE, apfF*                   |
| apfH                         | apf4                                      |
| Rubisco                     | rbcL                                      |
| Chlorophyll biosynthesis    | chlB, chlN, chlL                          |
| NADH dehydrogenase          | ndhA*, ndhB*, ndhC, ndhD                   |
| ndhE                        | ndhF, ndhG, ndhH                           |
| ndhI                        | ndhJ, ndhK                                |
| Ribosomal proteins          | rpl2*, rpl14, rpl16*, rpl20               |
| rpl21                       | rpl22, rpl23, rpl32                       |
| rpl33                       | rpl36, rps2, rps3                         |
| rps4                        | rps7 x2, rps8, rps11                      |
| rps12 x2                    | rps14, rps15, rps16*                      |
| rps18                       | rps19                                     |
| RNA polymerase              | rpoA, rpoB, rpoC1*, rpoC2                 |
| Miscellaneous proteins      | infA, ccsA, matK, clpP*                   |
| accD                        |                                           |
| Hypothetical proteins       | ycf1, ycf2 x2, ycf3*, ycf10                |
| ycf12                       |                                           |
in Marchantia but not in Nicotiana, Zea, or in Adiantum. The presence of the gene in Pinus suggests that it may have been lost independently in fern and angiosperm clades. Based on gene content, the Adiantum chloroplast genome is typical for that of other vascular plants, lacking only psAM and trnK (see below).

### 3.2. Codon usage and transfer RNA genes

Start positions of most Adiantum chloroplast protein-coding genes could be inferred from comparisons to previously annotated genes. We examined 83 putative genes for which the start position was based on alignments to Nicotiana and Psilotum. We inferred that 57 start at AUG, 16 at ACG, 5 at AUU, 3 at AUC, one at AUA, and one at GUG. We were unable to locate canonical starts nearby upstream or downstream from these putative start positions. RNA editing of Thr (ACG) to Met starts nearby upstream or downstream from these putative genes.

The presence of the gene in Pinus and Marchantia suggests that it may

| Table 2. List of putative tRNA genes located in Adiantum chloroplast genome. |
|---------------------------------|------------------|------------------|------------------|
| First exon (or all) | Second exon | gene-anticodon | tRNA-amino acid |
| start | end | start | end | |
| 1 | 6235 | 6164 | tRNA-UCU | tRNA-Thr |
| 2 | 7290 | 7203 | tRNA-GCU | tRNA-Ser |
| 3 | 6361 | 6383 | 9321 | 9368 | tRNA-UGU | tRNA-Arg |
| 4 | 9578 | 9649 | tRNA-UCC | tRNA-Gly |
| 5 | 22714 | 22787 | tRNA-GUC | tRNA-Asp |
| 6 | 27483 | 27402 | tRNA-GUA | tRNA-Tyr |
| 7 | 27717 | 27645 | tRNA-UCG | tRNA-Glu |
| 8 | 29765 | 29694 | tRNA-GCA | tRNA-Cys |
| 9 | 30146 | 30076 | tRNA-GGC | tRNA-Gly |
| 10 | 30979 | 30107 | tRNA-UGA | tRNA-Ser |
| 11 | 35016 | 34945 | tRNA-GCU | tRNA-Thr |
| 12 | 35274 | 35201 | tRNA-Met |
| 13 | 42897 | 42883 | tRNA-GGA | tRNA-ser |
| 14 | 44775 | 44808 | 45389 | 45435 | tRNA-CAG | tRNA-Leu |
| 15 | 45736 | 45908 | tRNA-UGA | tRNA-Phe |
| 16 | 48841 | 48808 | 48177 | 48198 | tRNA-UCG | tRNA-Val |
| 17 | 49115 | 49187 | tRNA-USC | tRNA-Met-elong |
| 18 | 53449 | 53522 | tRNA-Ser |
| 19 | 61937 | 61864 | tRNA-CAU | tRNA-Cys |
| 20 | 62197 | 62124 | tRNA-GCG | tRNA-Glu |
| 21 | 82234 | 82161 | tRNA-GUA | tRNA-Thr |
| 22 | 83676 | 83709 | 84218 | 84260 | tRNA-UCG | tRNA-Thr |
| 23 | 85246 | 85173 | tRNA-AGG | tRNA-Arg |
| 24 | 89988 | 89952 | 89147 | 89112 | tRNA-TGC | tRNA-Ala |
| 25 | 91123 | 91088 | 90091 | 90056 | tRNA-CAA | tRNA-Asp |
| 26 | 97859 | 97932 | tRNA-Met |
| 27 | 105323 | 105171 | tRNA-CAG | tRNA-Leu |
| 28 | 109673 | 109600 | tRNA-AGG | tRNA-Arg |
| 29 | 109898 | 109977 | tRNA-UAC | tRNA-Thr |
| 30 | 127619 | 127819 | tRNA-GUG | tRNA-His |
| 31 | 134992 | 134919 | tRNA-GGU | tRNA-Ser |
| 32 | 141728 | 141763 | 142760 | 142795 | tRNA-AGG | tRNA-Asp |
| 33 | 142863 | 142899 | 143704 | 143739 | tRNA-UAG | tRNA-Ala |
| 34 | 147605 | 147678 | tRNA-ACG | tRNA-Arg |
| 35 | 149175 | 149142 | 148633 | 148591 | tRNA-UAG | tRNA-Thr |

have similar properties and are often (though not always) interchangeable. Alternatively, tRNA-Lys could be supplied from nuclear origin, as has been proposed to explain the lack of trnR-ACG in the chloroplast genome of Lotus japonicus. Import of nuclear-encoded tRNAs into mitochondria has been documented in several systems and import into chloroplasts has been speculated for a
Table 3. Total numbers of each codon detected in 85 putative genes in the *Adiantum* chloroplast genome, indicated with tRNAs for which genes have been identified in the chloroplast genome.

| Codon   | UUU Phe 920 | UUC Phe 490 | UUA Leu 728 | UUG Leu 670 |
|---------|-------------|-------------|-------------|-------------|
| Ser     | 526         | 365         | 434         | 277         |
| Tyr     | 558         | 346         | 57          | 23          |
| Cys     | 178         | 146         | 34          | 431         |

We detected an additional tRNA gene that encodes a potential tRNA-selenocysteine (Fig. 2), which has been reported in plants but, as far as we know, not in chloroplasts. Although trnSeC was detected by tRNAscan-SE, the sequence does have some problems that brings its function into question. Position 32 is typically a pyrimidine in tRNA molecules but is an ‘A’ in *Adiantum* trnSeC, and the pairing at the base of the TΨC loop is poor (Fig. 2). Thus, it is possible that trnSeC represents a pseudogene of some previously functional tRNA gene. The potential trnSeC that we found has a UCA anticodon, corresponding to one of the common stop codons. We detected 26 stop codons within the otherwise open reading frames of 18 putative *Adiantum* genes: 16 of these are UAA, 8 are UGA and 2 are UAG. Some of these could be RNA editing sites, but it is also possible that the UGA codons are read-through by trnR-SeC. Clearly, transcript sequences are required to distinguish these phenomena.

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nonphotosynthetic angiosperm. However, we have not found any published evidence for this phenomenon in photosynthetic plants.
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