

## Structural evolution and flip-flop recombination of chloroplast DNA in the fern genus *Osmunda*

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**Summary.** The evolution and recombination of chloroplast genome structure in the fern genus *Osmunda* were studied by comparative restriction site mapping and filter hybridization of chloroplast DNAs (cpDNAs) from three species — *O. cinnamomea*, *O. claytoniana* and *O. regalis*. The three 144 kb circular genomes were found to be colinear in organization, indicating that no major inversions or transpositions had occurred during the approximately 70 million years since their radiation from a common ancestor. Although overall size and sequence arrangement are highly conserved in the three genomes, they differ by an extensive series of small deletions and insertions, ranging in size from 50 bp to 350 bp and scattered more or less at random throughout the circular chromosomes. All three chloroplast genomes contain a large inverted repeat of approximately 10 kb in size. However, hybridizations using cloned fragments from the *O. cinnamomea* and *O. regalis* genomes revealed the absence of any dispersed repeats in at least 50% of the genome. Analysis with restriction enzymes that fail to cleave the 10 kb inverted repeat indicated that each of the three fern chloroplast genomes exists as an equimolar population of two isomeric circles differing only in the relative orientation of their two single copy regions. These two inversion isomers are inferred to result from high frequency intramolecular recombination between paired inverted repeat segments. In all aspects of their general organization, recombinational heterogeneity, and extent of structural rearrangement and length mutation, these fern chloroplast genomes resemble very closely the chloroplast genomes of most angiosperms.

**Key words:** Inverted repeat — Colinearity — Flip-flop recombination — Fern chloroplast DNA

### Introduction

Detailed information about evolutionary change in chloroplast genome structure is presently available for only two groups of plants — flowering plants and the green algal genus *Chlamydomonas*. Comparisons among chloroplast DNAs (cpDNAs) from over 200 species of angiosperms indicate that major structural changes occur only very rarely (reviewed in Whitfeld and Bottomley 1983; Gillham et al. 1985; Palmer 1985a, 1985b). Cross-hybridization and gene mapping studies have revealed that the majority of angiosperm cpDNAs have the same gene order and arrangement, and that most of the exceptional genomes differ from the norm by only one or two inversions (Fluhr and Edelman 1981; Palmer and Thompson 1982; Palmer et al. 1983a, 1983b; de Heij et al. 1983; Herrmann et al. 1983; Mubumbila et al. 1984, 1985). Genome size is also highly conserved among angiosperm cpDNAs. Most of these genomes vary in size only from 135 kb to 160 kb (reviewed in Bohnert et al. 1982; Crouse et al. 1985; Gillham et al. 1985) and essentially all the length mutations detected in sequencing and restriction mapping studies of closely related species are short, from one or two bp to 1,000 bp (e.g. Gordon et al. 1982; Takaiwa and Sugiura 1982; Bowman et al. 1983; Palmer et al. 1983b, 1985a; Zurawski et al. 1984; Salts et al. 1984).

Rates of cpDNA structural evolution appear to be higher in *Chlamydomonas* than among flowering plants. The chloroplast genomes of four *Chlamydomonas* species vary in size from 195 kb to 292 kb and in sequence complexity by 78 kb, over twice the range (35 kb) found among all of the more than 200 angiosperm genomes examined (Rochaix 1978; Palmer et al. 1985b; Lemieux et al. 1985a, 1985b). Moreover, small deletions and additions appear to occur at a higher frequency relative to base substitutions in *Chlamydomonas* cpDNA than in

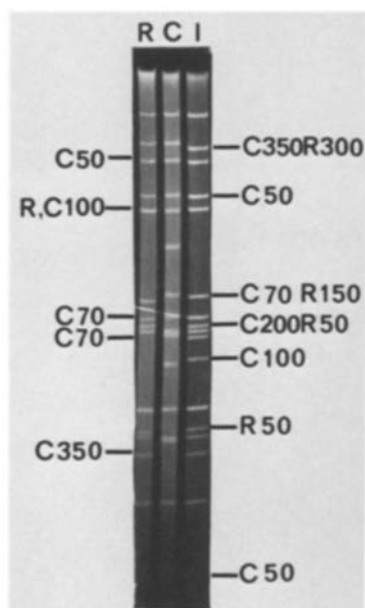


Fig. 1. Comparison of PvuII restriction fragment patterns of *Osmunda* cpDNAs. CpDNAs from *Osmunda regalis* (R), *O. claytoniana* (C), and *O. cinnamomea* (I) were digested with PvuII and the resulting fragments were separated by electrophoresis in a 0.7% agarose gel. Numbers next to various bands refer to size differences (in base pairs) between the *cinnamomea* and either the *claytoniana* (C) and/or *regalis* (R) bands. Assignment of differences in fragment size is based on this gel as well as two other gels containing the same digests

angiosperm cpDNA (Palmer et al. 1985b) Finally, two distantly related *Chlamydomonas* species differ in gene order by an extensive series of sequence rearrangements (Lemieux and Lemieux 1985). This suggests that rates of inversion and transposition may be significantly higher in *Chlamydomonas* cpDNA, although other explanations are possible (Lemieux and Lemieux 1985) and more comparisons desirable.

This disparity in rates of structural evolution among these two groups of chloroplast genomes makes one wonder about genome evolution in other groups of plants. In particular, how do cpDNAs vary in structure and organization among non-angiospermous land plants, such as gymnosperms, ferns and bryophytes, which share a common ancestry with angiosperms but which comprise distinct phyletic lines separated for hundreds of millions of years? Thus far, there have been no comparisons of chloroplast genome evolution within specific groups of non-angiospermous land plants, although the cpDNAs of the few ferns and bryophytes that have been examined do show some general similarities to angiosperm cpDNAs in overall size and certain rudimentary organizational features (Herrmann et al. 1980; Palmer and Stein 1982; Ohyama et al. 1983). In this report, we make the first detailed comparative study of the structure and evolution of cpDNA within a specific group of non-angiosper-

mous land plants. We show that three chloroplast genomes from the fern genus *Osmunda*, which diverged from each other on the order of 70 million years ago (Miller 1967, 1971), are essentially identical in linear order and size, differing in structure only by a series of small deletions and additions.

One structural feature which is common to almost all green algal and angiosperm cpDNAs (reviewed in Crouse et al. 1985; Palmer 1985a, 1985b), as well as those of the fern *Osmunda cinnamomea* (Palmer and Stein 1982) and the bryophyte *Marchantia polymorpha* (Ohyama et al. 1983), is a large (10–76 kb) inverted repeat encoding a complete set of chloroplast ribosomal RNAs. In those few angiosperm and green algal genomes for which restriction enzymes have been found that fail to cleave within this inverted repeat it has been possible to show that the genome exists as a mixture of two inversion isomers differing in the relative polarity of their single copy regions (Palmer 1983; Mubumbila et al. 1983; Palmer et al. 1984, 1985b; Aldrich et al. 1985), presumably as the result of intramolecular recombination between inverted repeat segments. Here we show that all three *Osmunda* species examined contain a 10 kb, rRNA-encoding inverted repeat that confers the same sort of inversional heterogeneity to the chloroplast genome.

## Materials and methods

cpDNA was prepared as described (Palmer and Stein 1982). Plasmid clones containing PstI fragments of the *Osmunda cinnamomea* and *O. regalis* chloroplast genomes were prepared by V. James and G. Bennett. PstI-digested total cpDNA was ligated with PstI-digested pUC8 or pUC9 (Vieira and Messing 1982), transformed into *E. coli* strain JM83, and recombinant white colonies were selected on ampicillin/X-gal plates. The clone banks were characterized by sizing PstI-digested plasmid DNAs isolated from one ml of bacterial culture by the alkaline extraction procedure of Birnboim and Doly (1979). Restriction endonuclease digestions, agarose gel electrophoresis, bidirectional transfers of DNA fragments from agarose gels to nitrocellulose filters, labeling of recombinant plasmids by nick-translation, and filter hybridizations were performed as previously described (Palmer 1982, 1985c). All filters were washed in 2 × SSC (0.3 M NaCl/30 mM trisodium citrate) and 0.5% SDS at 65° prior to autoradiography.

## Results

### Structural comparisons

We compared the structure of the chloroplast genomes of three species of the fern genus *Osmunda*, which are thought to have been separated for at least 70 million years (Miller 1967, 1971; Stein et al. 1979). Initially we compared purified cpDNAs from the three species, *O. cinnamomea*, *O. claytoniana*, and *O. regalis*, by agarose gel electropho-

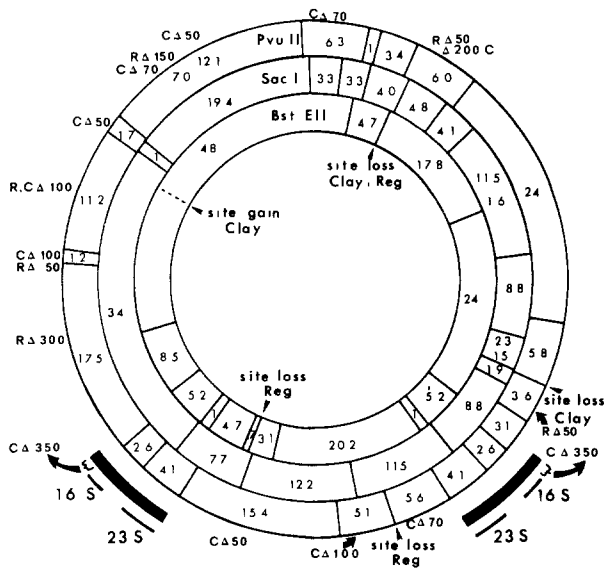


Fig. 2. Comparison of physical maps of cpDNA from three species of *Osmunda*. Locations and sizes of PvuII, SacI and BstEII restriction fragments are indicated for the reference chloroplast genome from *O. cinnamomea* (data taken from Palmer and Stein 1982). Loss or gain of restriction sites in the BstEII and PvuII maps only, is shown for cpDNAs from *O. regalis* (Reg) and *O. claytoniana* (Clay) and is based on hybridization of each of the 19 PvuII fragments from *O. cinnamomea* to filter blots containing BstEII and PvuII fragments of the other two species. Differences in fragment sizes (in base pairs) are given for *O. claytoniana* (C) and *O. regalis* (R) with reference to *O. cinnamomea* PvuII fragments (Fig. 1). Note that only one of the two genome orientations is shown (see text and Fig. 5).

resis of fragments produced by some 25 restriction endonucleases that have 6-bp recognition sites (data not shown). Not surprisingly, given the long period of separation of the three species, their cpDNA restriction patterns showed a large number of differences and relatively few similarities with most of the enzymes surveyed. However, one enzyme in particular, PvuII, produced strikingly similar fragment patterns for all three cpDNAs (Fig. 1).

In order to determine the overall arrangement of homologous sequence elements in the three genomes we hybridized each of the 19 nick-translated, gel-purified (Palmer 1982) PvuII fragments from *O. cinnamomea*, for which a complete restriction map has already been published (Palmer and Stein 1982), to nitrocellulose filters containing restriction fragments of all three genomes produced by PvuII, BstEII, SacI, and PstI. The results of this hybridization study are summarized in Fig. 2 for PvuII and BstEII, the two enzymes whose recognition sites have been least altered among the three cpDNAs. The three genomes were found to be identical in linear sequence order and extremely similar in absolute sequence arrangement, differing only by a series of small deletions and additions of between 50 bp and 350 bp

in size (Fig. 2). These length mutations are best seen by comparing the PvuII patterns (Fig. 1) and maps (Fig. 2), in which *O. claytoniana* and *O. regalis* cpDNAs are seen to differ from that of the reference species, *O. cinnamomea*, by the loss of only a single PvuII site each. The PvuII patterns (Fig. 1) reveal a large number of small mobility differences among fragments that map to the same position (Fig. 2). Assuming a random distribution of base substitutions, statistical considerations suggest that most of these differences must reflect small deletions and additions within fragments, rather than base substitutions that create new restriction sites near the ends of fragments. Note that the length mutations are scattered more or less randomly throughout the genome.

### Repeated sequences

The only repeated element in the three *Osmunda* cpDNAs revealed by the restriction mapping and cross-hybridization studies is a large inverted repeat, approx. 10 kb in size, that encodes a complete set of ribosomal RNA genes (Fig. 2; Palmer and Stein 1982). As in angiosperms, the *Osmunda* inverted repeat is situated asymmetrically, with the same orientation of the rRNA operon relative to the small and large single copy regions. However, as previously noted (Palmer and Stein 1982), the *Osmunda* repeat is substantially smaller than the vast majority of angiosperm repeats, which are generally between 20 kb and 30 kb in size (Bohnert et al. 1982; Crouse et al. 1985). A single large insertion of 350 bp has occurred symmetrically in both segments of the inverted repeat in *O. claytoniana* relative to the other two species (Fig. 2). The symmetric nature of this inverted repeat "insertion" is consistent with a large body of data derived from comparisons of angiosperm and green algal cpDNAs indicating that some sort of copy-correction mechanism maintains sequence identity between the repeat elements present within a given cpDNA chromosome (reviewed in Gillham et al. 1985; Palmer 1985a, 1985b).

Although no other repeats were revealed by the hybridization studies, this conclusion is necessarily limited by the fact that the probes consisted of uncloned PvuII fragments purified from digests of total cpDNA and which always showed a low level of cross-contamination with closely migrating PvuII fragments. In order to detect dispersed repeated sequences with greater sensitivity we hybridized various cloned PstI fragments of *O. regalis* and *O. cinnamomea* cpDNAs to filter blots containing PstI fragments of the parent genomes. This self-hybridization analysis was performed with a single *O. regalis* clone containing a 9.7 kb fragment that is located primarily within the small single copy region, and with seven *O. cinnamomea* clones that cover 57.8 kb of the large single copy region (Figs. 3, 5). None of

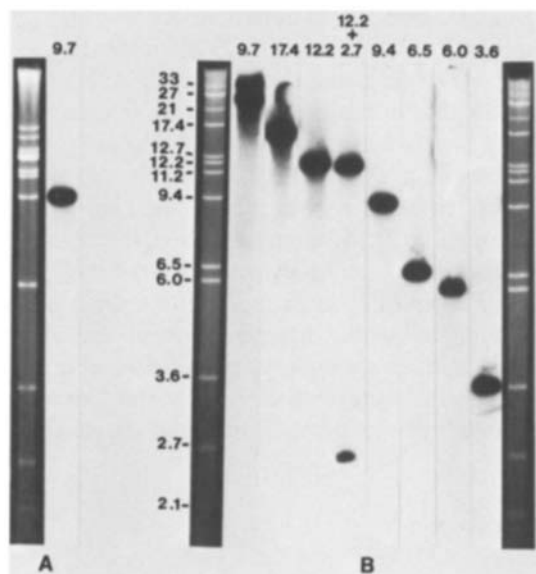


Fig. 3. Absence of dispersed repeated sequences in the *Osmunda* chloroplast genome. *Panel A*: Hybridization of a clone containing a 9.7 kb PstI fragment from *O. regalis* to a filter containing PstI fragments of *O. regalis* cpDNA that had been separated on a 0.7% agarose gel. *Panel B*: Eight identical filter strips containing PstI restriction fragments of *O. cinnamomea* cpDNA that had been separated on a 0.7% agarose gel were hybridized with each of seven plasmid clones containing *O. cinnamomea* PstI restriction fragments (sizes in kb of the PstI inserts are given above each filter strip) and with a clone containing the 9.7 kb PstI fragment from *O. regalis*

these clones hybridized to any PstI fragments except for the one(s) contained within the clone itself (Fig. 3 and in a 5-fold longer autoradiographic exposure of the filters used to produce Fig. 3). This analysis therefore rules out the presence of dispersed repeated sequences within the 50% of the genome (in terms of sequence complexity) covered by the clones used. It assumes any repeats would be of sufficient size and sequence homology as to be detectable under our conditions of hybridization (washes were performed in  $2 \times \text{SSC}$  at  $65^\circ$ ) and autoradiography. It is still possible that short repeated sequences are present, either clustered within an individual PstI fragment or distributed among the PstI fragments not used as hybridization probes.

#### Flip-flop recombination

The cross-hybridization mapping experiments described in the first section revealed that the enzyme PstI does not cleave within the inverted repeat of *O. cinnamomea* and that SacI fails to cleave within the inverted repeats of *O. claytoniana* and *O. regalis* (data not shown). Resolution of these enzyme digests in a 0.5% agarose gel run at low voltage for 24 h revealed the presence of large, approximately half-molar bands for each cpDNA (Fig. 4). For *O. cinnamomea* there are two half-molar PstI fragments, of 21 kb and 33 kb, and the sum of all the PstI fragments is approx. 27 kb larger than the genome size

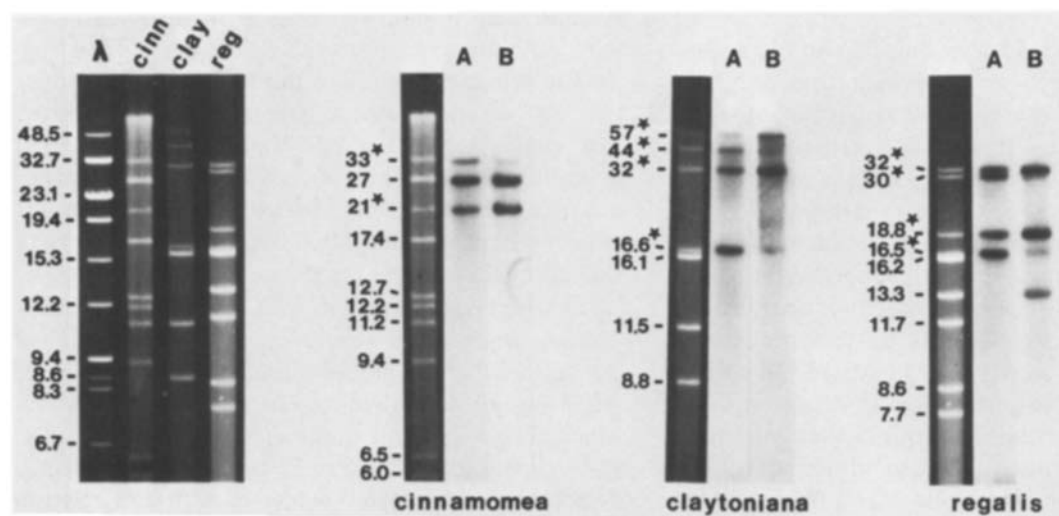


Fig. 4. Physical heterogeneity of *Osmunda* cpDNAs. CpDNAs from *O. cinnamomea*, *O. claytoniana*, and *O. regalis* were digested with PstI, SacI, and SacI, respectively, and the resulting fragments were separated by electrophoresis on a 0.5% agarose gel run for 40 h (until the bromophenol blue tracking dye had run 20 cm). The fragments were transferred to filters and subjected to hybridization with  $^{32}\text{P}$ -labelled clones containing an inverted repeat-specific fragment from mung bean (lanes marked A) and a largely small single copy region-specific fragment from *O. regalis* (lanes marked B). The exact positions in the *Osmunda* genomes of the sequences homologous to these clones are shown in Fig. 5 (also see text). Stars indicate half stoichiometry bands (see text). Size markers (lane marked 2) include intact lambda phage DNA (48.5 kb), lambda SalI fragments (32.7 kb, 15.3 kb), lambda HindIII fragments (23.1 kb, 9.4 kb, 6.7 kb), and lambda SmaI fragments (19.4 kb, 12.2 kb, 8.6 kb, 8.3 kb). Only the portion of the gel above 6 kb is shown

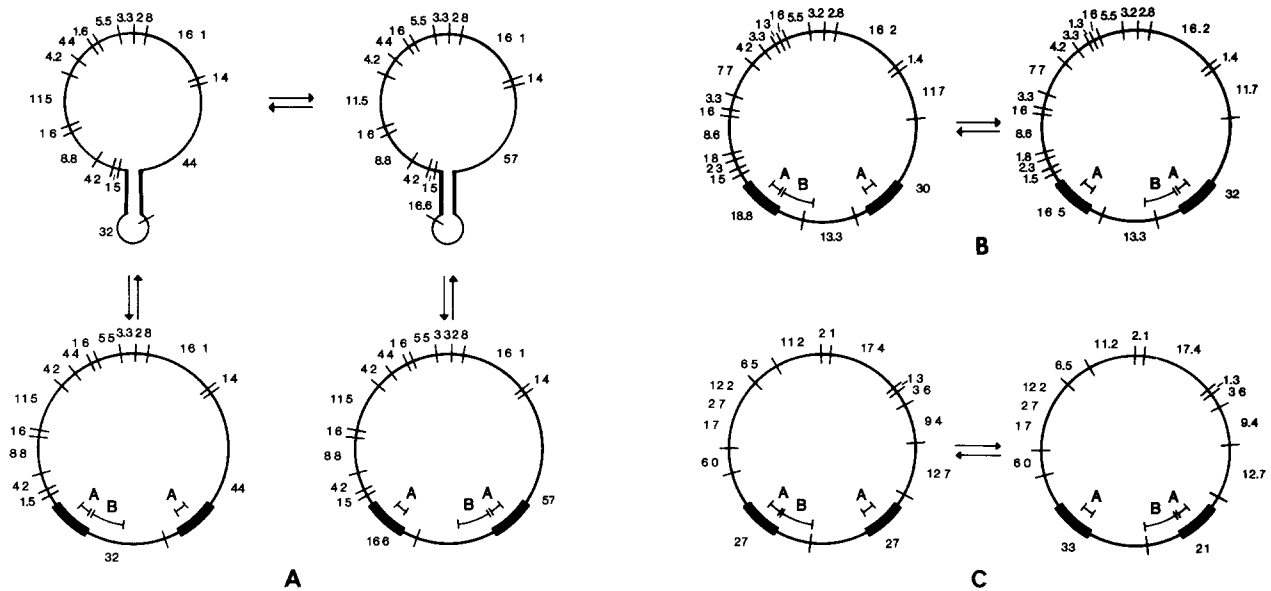


Fig. 5A–C. Model for isomerization of *Osmunda* cpDNAs via intramolecular recombination between segments of the inverted repeat. **A Bottom**, *SacI* restriction maps of the two inversion isomers of cpDNA from *O. claytoniana*. These two molecules differ only with respect to the relative orientation of their single copy DNA regions. **Top**, Dumbbell-shaped molecules postulated to occur as intermediates in the interconversion, via reciprocal recombination within intramolecularly paired inverted repeat segments, of the two inversion isomers shown at bottom. Radial lines on the circles indicate *SacI* sites and numbers give the size of the *SacI* fragments. Heavy black lines indicate the location of the inverted repeat. The brackets under A and B indicate the positions of the corresponding hybridization probes used in Fig. 4 (also see text). **B** Inversion isomers of cpDNA from *O. regalis*. *SacI* sites and fragment sizes are indicated. **C** Inversion isomers of cpDNA from *O. cinnamomea*. *PstI* sites and fragment sizes are indicated

estimate obtained with the other three mapped enzymes (Fig. 2; Palmer and Stein 1982). For each of the other two genomes there are four half-molar *SacI* fragments (Fig. 4). In each case, the smallest and largest half-molar fragments and the two intermediate half-molar fragments sum to the same approximate size (75 kb for *O. claytoniana* and 49 kb for *O. regalis*), a number equal to the genome size obtained with *SacI* minus the genome size obtained with the three other mapped enzymes.

The above observations suggest that each genome might exist in two different forms with opposite polarities of the single copy regions. According to this model, each of the large half-molar fragments would contain an entire inverted repeat segment (Fig. 5). To verify this interpretation, filter hybridizations were performed using two cloned fragments as probes. The first (probe "A" in Fig. 5) is a 3.5 kb *SacI* fragment that contains most of the 23S rRNA gene and part of the transcribed rDNA spacer from mung bean (Palmer and Thompson 1981). This mung bean fragment has homology only to sequences within the inverted repeat in *Osmunda*, for example, the only *Osmunda* *PvuII* fragment to which it hybridizes is the 4.1 kb fragment internal to the inverted repeat (see Fig. 2; hybridizations not shown). The second (probe "B") is the 9.7 kb *PstI* fragment from *O. regalis* that was described in the preceding section and which is

localized primarily within the small single copy region, although containing about one kb of inverted repeat sequences. The inverted repeat-specific probe, probe A, hybridizes to all four of the large half-molar fragments of *O. claytoniana* and *O. regalis* cpDNAs, producing hybridizations signals proportional in strength to the staining intensity of the four half-molar bands (Fig. 4). The simplest interpretation of these data is that each of the four large fragments does indeed contain an entire inverted repeat segment, with one pair of fragments present in one genomic form and the other pair in its inversion isomer (Fig. 5a, b). Consistent with this interpretation, probe B, the largely small single copy-specific fragment, also hybridizes to all four of the half-molar fragments in both genomes, but much more strongly to one of the two half-molar fragments from each of the two predicted inversion forms than to the other (Fig. 4; also see Fig. 5a, b). The weak hybridizations to the two other half-molar fragments can be accounted for by cross-hybridization with the inverted repeat portion of probe B.

The inverted repeat probe hybridizes more or less equally strongly to the two large half-molar fragments of *O. cinnamomea* cpDNA (Fig. 4). In addition, it hybridizes about twice as strongly to the unimolar fragment of 27 kb. We interpret this to mean that the 33 kb and 21 kb fragments contain the inverted repeats of one ge-

nomous form, and that the 27 kb fragment is composed of two different comigrating half-molar fragments that reside on the inversion isomer of the first molecule (Fig. 5c). Consistent with this interpretation, probe B hybridizes approximately equally strongly to the half-molar 21 kb fragment as to the unimolar 27 kb fragment (the putative doublet of half-molar fragments) (Fig. 4). The weak hybridization to the 33 kb half-molar fragment derives from the inverted repeat portion of probe B.

## Discussion

Although separated by at least 70 million years of evolution (Miller 1967, 1971; Stein et al. 1979), these three *Osmunda* species possess chloroplast genomes that are remarkably similar in size and organization. The three fern cpDNAs have the same linear sequence order, i.e. they have not sustained any major inversions and transpositions since their divergence from a common ancestral genome. Moreover, they are almost identical in size, differing only by a number of small deletions/additions of less than 400 bp in size. In both these respects, cpDNA appears to be evolving at least as slowly in the fern genus *Osmunda* as it does among the majority of angiosperms (see Introduction).

Since chloroplast genome structure is so invariant both in *Osmunda* and also among most angiosperms one might predict a high degree of structural similarity between the genomes of the two groups. Indeed, we earlier noted similarities between angiosperm cpDNA and that of *O. cinnamomea* in overall size, presence and position of the rRNA-encoding inverted repeat, and location of four protein genes (Palmer and Stein 1982). In the accompanying paper, we now extend these comparisons and show that at a high level of resolution the *O. cinnamomea* chloroplast genome is essentially colinear with that of the ancestral angiosperm (Palmer and Stein 1986). In other words, the extremely conservative mode of structural evolution of cpDNA in *Osmunda* and in most groups of flowering plants allows one to derive a basic chloroplast genome organization that traces back to the common ancestor of vascular plants, if not all land plants.

The absence of any detectable dispersed repeated sequences in the 50% of the *Osmunda* chloroplast genome assayed by our cpDNA clones is one more feature in which the *Osmunda* genome resembles that of most angiosperms. Self-hybridizations using cloned probes have failed to detect any dispersed repeats in cpDNAs from eight angiosperms (Palmer and Thompson 1981, 1982; Coates and Cullis 1982; Ko et al. 1983; Spielmann et al. 1983; de Heij et al. 1983; Fluhr et al. 1983; J. Palmer, J. Aldrich and W. Thompson, unpublished data).

It is interesting to note, however, that those cpDNAs – from several species of *Chlamydomonas* and from the angiosperms *Trifolium subterraneum* and *Pelargonium hortorum* – that do contain dispersed repeats are also those which show evidence of extensive and perhaps recent rearrangement (Rochaix 1978; Gelvin and Howell 1979; Lemieux and Lemieux 1985; Lemieux et al. 1985a; Palmer et al. 1985b; J. Palmer, B. Osorio and W. Thompson, unpublished data; J. Palmer, J. Nugent and L. Herbon, unpublished data). Whether these rearrangements are in any way directly related to the presence of these dispersed repeats, and conversely, whether the extreme stability of the genome in *Osmunda* and in most angiosperms results from the absence of repeated sequences, await further study.

Our studies add the three species of *Osmunda* to the list of plants for which cpDNA “flip-flip” heterogeneity has been demonstrated. In addition to the three ferns, these include the angiosperms *Phaseolus vulgaris* and *Glycine max* (Palmer 1983; Palmer et al. 1984; Mubumbila et al. 1983), the green alga *Chlamydomonas reinhardtii* (Aldrich et al. 1985; Palmer et al. 1985b), and *Cyanophora paradoxa* (Bohnert and Löffelhardt 1982), an alga of uncertain taxonomic placement. It therefore seems likely that all chloroplast genomes which contain a typical inverted repeat exist as an equimolar mixture of two inversion isomers. Yet, in spite of the presence of this recombinational dualism in chloroplast genomes spanning hundreds of millions of years of evolution, we presently have little idea as to how this recombination occurs (Palmer et al. 1985b), or what, if any, function it may serve.

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