*Short communication*

# Methylation of somatic and sperm DNA in the homosporous fern *Ceratopteris richardii*

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## Abstract

Plants, in general, have a high proportion of their CpG and CpNpG nucleotide motifs modified with 5-methylcytosine (5mC). Developmental changes in the proportion of 5mC are evident in mammals, particularly during gametogenesis and embryogenesis, but little information is available from flowering plants due to the intimate association of gametes with sporophytic tissues. In ferns, sperm are uninucleate and free-swimming and thus are easily isolated. We have examined 5mC in DNA isolated from fern sperm and other tissues with methylation-sensitive and -insensitive restriction enzyme isoschizomers, Southern blots probed with chloroplast and nuclear ribosomal RNA genes and end-labeled restriction fragments. We conclude that fern sperm DNA is methylated to a similar or greater degree than DNA isolated from either sporophytes or gametophytes.

Methylation of cytosines within CpG and CpNpG sequence motifs is nearly ubiquitous in nuclear DNA from vascular plants, including pteridophytes, gymnosperms and flowering plants [2]. A general, but not universal, observation is that actively transcribed sequences are more often hypomethylated and inactive chromatin appears to hypermethylated in plants (reviewed in [4]). Significant differences in the relative levels of 5-methylcytosine (5mC) among various tissue types have been reported in tomato where mature tissues, seeds in particular, appeared to have greater methylation than immature tissues [11]. Within seeds of maize, differential demethylation of tubulin genes in endosperm relative to embryo tissues has been observed, and for some tubulin genes, differential demethylation appears restricted to those contributed by the maternal parent [9]. Parental imprinting mediated by DNA methylation patterns may explain non-Mendelian inheritance of characters in some instances [6].

The difficulty in isolating sperm cells from flowering plants has prevented an examination of methylation in the male gametes (see [8] for review). In the flowering plants, pollen is multinucleate with both sperm and generative nuclei. In maize, evidence suggests that tubulin genes are hypermethylated in sperm cells [9]. Pollen nuclei from tomato show little difference in 5mG content, relative to other tissues [11]. Sperm cells of ferns, however, are uninucleate and free-swimming. In this report, we have examined the DNA methylation status of three tissues (diploid sporophyte, haploid gametophyte and sperm) of the homosporous fern *Ceratopteris richardii*.

Spores of *C. richardii* strain Hn were cultured ([7] and references therein) and DNA from sporophytes and gametophytes was isolated as described [10]. Spermatocyte DNA was isolated from three high-density (>500 spores per 100 mm × 15 mm Petri plate) axenic multispore cultures of *C. richardii* strain Hn gametophytes which were grown on 1% agarose-solidified Parker/Thompson media supplemented with antheridiogen (a maleness promoting factor obtained from previous media used for gametophyte culture and used here at a concentration of 20% v/v, modified from
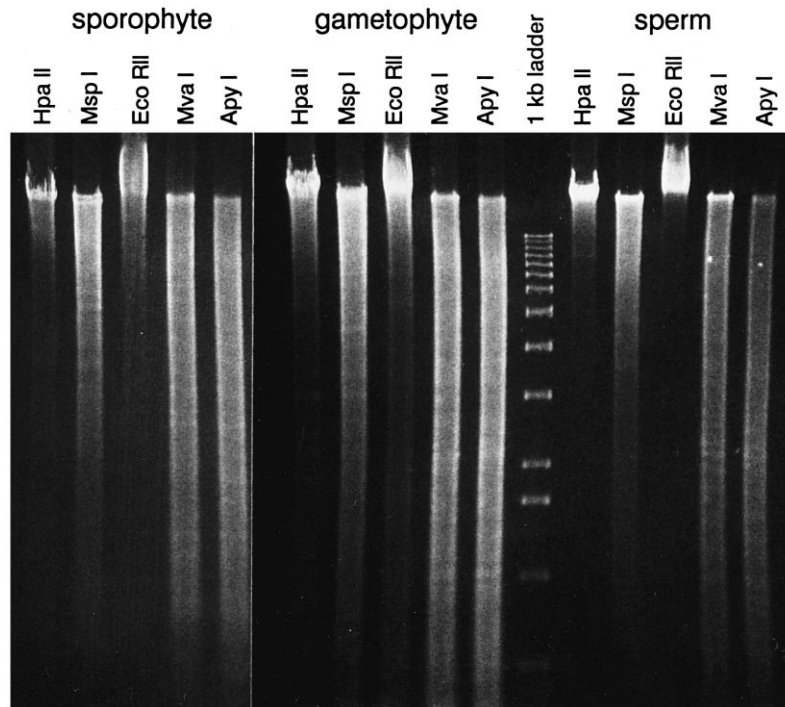
*Figure 1. Ceratopteris richardii* DNA isolated from sporophytic, gametophytic and sperm tissues digested with methylation-sensitive (*Hpa*II and *Eco*RII) and methylation-insensitive (*Msp*I, *Mva*I and *Apy*I) restriction enzyme isoschizomers. The 1 kb ladder (BRL) was used for molecular weight standards.

Scott and Hickok [14]). Most (>95%) gametophytes were male. After culturing gametophytes for 18 days at 28 °C, 5 ml of tap-water was added to each plate and allowed to sit for 15 min, at which time the majority of sperm appeared to have been released. The free liquid was collected and filtered to remove detached gametophytes through one layer of Miracloth (Calbiochem) into a 30 ml Corex tube, and the filter was washed with an additional 5 ml of distilled water. Sperm were pelleted at 10 000 rpm for 20 min and pellets were resuspended in 10 ml of Sorbitol-CTAB-Sarkcosyl lysis buffer as described [10]. After a 20 min incubation at 60 °C, 2 ml of phenol were added, mixed, and then 7 ml of chloroform were added. The aqueous phase was recovered, DNA precipitated with 2 vol of 100% ethanol and the precipitate was collected with a pair of tweezers, washed in 100% ethanol and finally resuspended in 600 $\mu$l of TE. No inhibition of restriction enzyme activity was observed in sperm DNA digested with a battery of restriction enzymes, however internal control DNA digestions were not performed with methylation sensitive enzymes.

Isoschizomers whose activities differ when their substrates are modified by 5mC were used in assessing the methylation state of native DNA. These enzymes included *Hpa*I and *Msp*I (recognition sequence CCGG) that are sensitive and insensitive to 5mC at the internal C, respectively [12]. Isoschizomers that differentially cleave the sequence CC(A/T)GG, *Eco*RII (methylation-sensitive at the internal C) and both *Mva*I and *Apy*I (methylation-insensitive at the internal C, although there are different specificities with regard to modification at other positions; [12]) were also used.

DNA (generally 2.5 $\mu$g) was digested with each enzyme according to manufacturer's recommendations for at least 3 h and size-fractionated in 0.8% agarose gels. Gels were stained with ethidium bromide and photographed. DNA from photosynthetically active diploid tissue of the mature sporophyte and haploid tissue the free-living gametophyte both showed (marginally) greater digestion with methylation-insensitive isoschizomers (e.g. *Msp*I and *Mva*I) than did DNA isolated from photosynthetically inactive free-swimming sperm cells (Figure 1). With methylation-sensitive enzymes such as *Hpa*II and *Eco*RII, the signal intensity of low molecular weight ethidium bromide-stained DNA from sperm was reduced relative to equivalent amounts of sporophyte and gametophyte DNA (Fig-
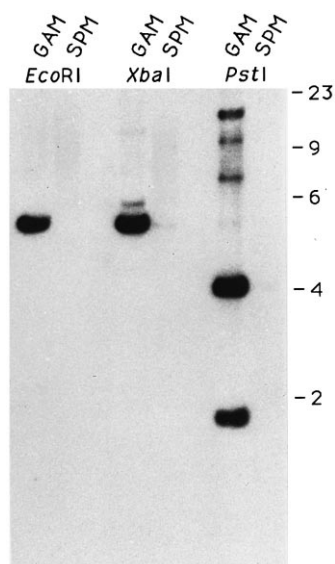
*Figure 2. Ceratopteris richardii* DNA isolated from gametophyte and sperm digested with *Eco*RI, *Xba*I and *Pst*I. DNA was probed with tobacco chloroplast nucleotide sequence T30.

ure 1). It should be noted that the sporophyte DNA was isolated from fronds, which relative to the other tissues examined likely have a greater complexity of cell types. DNA methylation in other tissues (i.e. sporophytic roots) was not examined.

Since chloroplast DNA is to a large extent unmodified, at least part of the difference in signal intensity between somatic and sperm DNA could be due to the exclusion of chloroplast DNA from sperm cells. This was tested by probing gametophyte and sperm DNA with the $^{32}$P-labeled chloroplast probe T30 ([13]; performed as described [10]). Little or no chloroplast signal was evident in DNA isolated from sperm cells (Figure 2). Thus, exclusion of chloroplast DNA from sperm cells appears to account for the difference in signal intensifies. Faint signals in Figure 2 may have been due to contamination of sperm cell preparations with gametophyte DNA (although no green material was observed) or sequences with nucleotide similarity to probe T30 may be present in the mitochondrial or nuclear DNA (e.g. a faint 9.0 kb fragment seen in *Xba*I-digested sperm but not apparent in gametophyte DNA, Figure 2). It should be noted that approximately twice as much sperm DNA relative to gametophyte DNA was loaded on the gel in Figure 2 (see Figure 3A).

The methylation status of nuclear ribosomal RNA genes was assessed on the same blot as above using clone pTA71 from wheat [5], using DNA digested with the methylation-sensitive restriction enzyme *Pst*I versus digestion with methylation-insensitive enzymes *Eco*RI or *Xba*I as detected with a non-radioactive label (Figure 3A). DNA digested with *Pst*I appeared to be marginally digested in gametophyte and little digestion was seen with sperm DNA. When probed with the ribosomal gene probe pTA71, similar patterns were seen between gametophyte and sperm DNA with methylation-insensitive enzymes *Eco*RI or *Xba*I. With *Pst*I, however, the hybridization signal was greatly reduced (gametophyte) or not detectable (sperm) relative to methylation-insensitive enzymes (Figure 3B). This indicates either that the majority of ribosomal genes are methylated in both gametophyte and sperm DNA, or that there is a paucity of *Pst*I sites within *Ceratopteris* ribosomal gene arrays. Compared with gametophyte DNA, the ribosomal genes in *Ceratopteris* sperm DNA appeared completely methylated at *Pst*I sites since no signal was detected other than within the undigested, high-molecular-weight DNA (data not shown and Figure 3B).

Although the bulk of DNA in *Ceratopteris*, particularly in sperm, appeared recalcitrant to digestion with cytosine-methylation sensitive enzymes as visualized on ethidium bromide-stained gels, it was possible that differentially-methylated, smaller-sized fragments such as those expected to be associated with CpG islands [1] were not detected by ethidium bromide staining. Failure to detect CpG islands, if present, may have been due to the relatively large genome of *Ceratopteris richardii* ($5 \times 10^9$ bp; [7]) and the presumably high proportion of repeated DNA sequences in homosporous pteridophytes (e.g. [15, 3]). To examine smaller-sized fragments, DNA from each stage was end-labeled with $^{32}$P and size-fractionated on 6% polyacrylamide gels. Sporophyte and gametophyte DNA showed virtually identical patterns of low molecular weight DNA fragments when *Hpa*II and *Msp*I were used (CCGG motif), although a few bands had lower intensities or were absent in *Hpa*II-digested DNA (e.g. ca. 40 bp, Figure 4). In contrast, the banding patterns of sperm DNA were highly dissimilar relative to sporophyte and gametophyte DNA (Figure 4), perhaps as the result of chloroplast exclusion. However, many bands present in the sperm *Msp*I digest were absent in the *Hpa*II digest, indicating that these sequences were methylated in sperm. Three bands were prominent in *Hpa*II-digested sperm DNA (in the range of 200–260 bp; Figure 4), indicating either that methylation of the low molecular weight fraction is not complete in sperm cells or these sequences are derived from mitochondria.
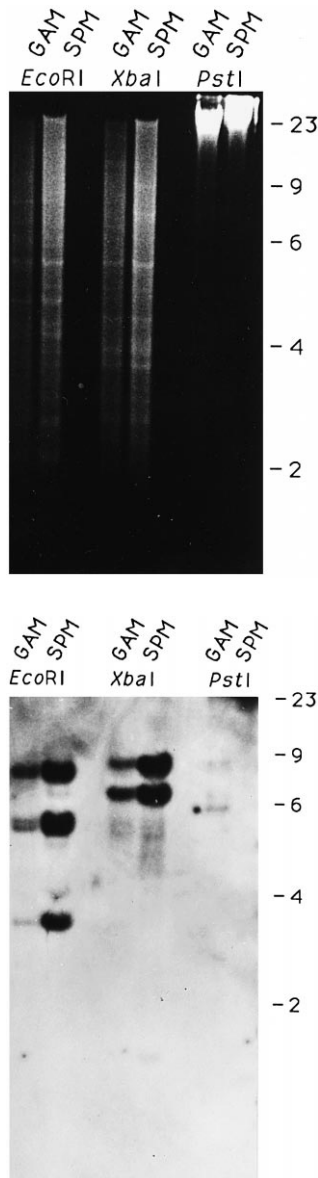
*Figure 3*. A (up). Ethidium bromide-stained gel of digested and electrophoresed *Ceratopteris richardii* DNA isolated from gametophyte and sperm used in Figure 2. B (down). Blot of Figure 2 stripped and reprobed with ribosomal RNA gene probe pTA71.

Digestion of sporophyte, gametophyte and sperm DNA with methylation-insensitive *Mva*I (recognition sequence CCWGG where W = A or T) showed similar patterns of end-labeled small fragments, although there were slight differences in signal intensity in some cases (Figure 5). This result suggests that few if any chloroplast sequences are present in this size range. If so, then the comparison of *Mva*I (methylation-insensitive)
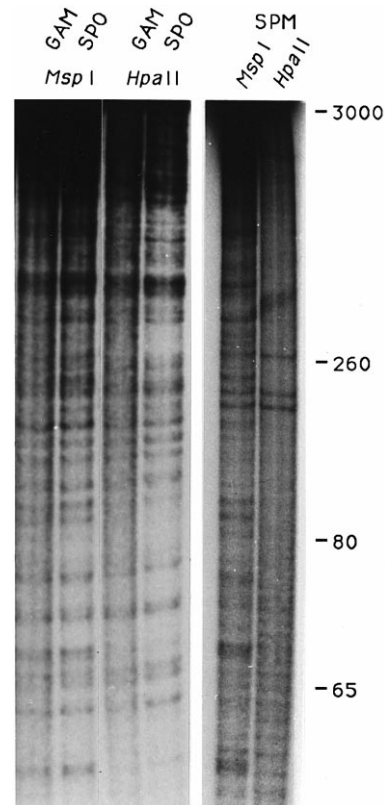


*Figure 4*. Sporophyte, gametophyte and sperm DNA of *Ceratopteris richardii* digested with methylation-sensitive *Hpa*II and methylation-insensitive *Msp*I restriction isoschizomer recognition sequence CCGG end-labeled with $^{32}$P and size-fractionated on a polyacrylamide gel.

and *Eco*RII (methylation-sensitive) digestions should be an accurate assessment of the methylation status at the internal cytosine residue in all tissue types. Few, if any, fragments were the same size between digests, indicating that each of the visible bands is methylated in all three tissue types (Figure 5). *Eco*RII-digested sporophyte and gametophyte DNA were similar, at least for the major bands. In contrast, no fragments were detected in end-labeled sperm DNA digested with *Eco*RII (Figure 5), suggesting complete methylation within this size range of DNA fragments.

We have examined the patterns of DNA methylation from actively-growing sporophyte and gametophyte tissues and compared this with DNA isolated from motile, but non-growing, sperm cells. With each of the methods employed, sporophyte and gametophyte DNA was virtually indistinguishable. However, the presence of unmethylated chloroplast DNA in photosynthetically active tissue may have obscured minor
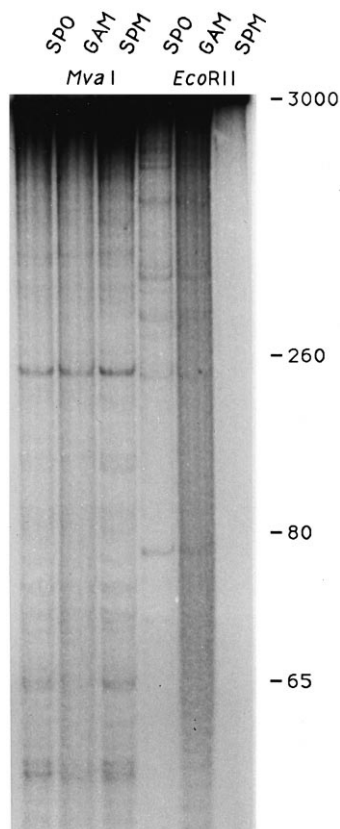
*Figure 5.* Sporophyte, gametophyte and sperm DNA of *Ceratopteris richardii* digested with methylation-sensitive *Eco*RII and methylation-insensitive *Mva*I restriction isoschizomer recognition sequence CCWGG (W = A or T) end-labeled with $^{32}$P and size-fractionated on a polyacrylamide gel.

differences in these tissues. In contrast, DNA from sperm cells was methylated to a higher degree than that of sporophyte and gametophyte. The degree of hypermethylation is difficult to ascertain because of chloroplast DNA contamination in non-sperm DNA. However, where chloroplast contamination did not affect a comparison (i.e. analysis of nuclear ribosomal DNA), sperm methylation was virtually complete.

## References

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