Pl. Syst. Evol. 189: 193–202 (1994)

—Plant—
Systematics
and
Evolution


# Restriction fragment length polymorphisms distinguish among accessions of *Ceratopteris thalictroides* and *C. richardii* (*Parkeriaceae*)

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Received April 24, 1993

**Key words:** Homosporous ferns, *Parkeriaceae, Ceratopteris, C. thalictroides, C. richardii.* – RFLP, cDNA, polyploidy.

**Abstract:** We have used cDNA clones as probes on Southern blots to detect restriction fragment length polymorphisms among seven *Ceratopteris thalictroides* accessions, three *C. richardii* accessions, and one putative interspecific hybrid. We found that the stringency of post-hybridization washes was a critical parameter affecting the quality of our blots; even with homologous cDNA sequences low stringency conditions resulted in a smear of signal, but high stringency washes gave blots with distinct bands. Most probes showed hybridization with four or more genomic fragments. Similarities in the number and size of fragments between and within species indicated that (i) *C. richardii* shows limited polymorphism among accessions tested, (ii) *C. thalictroides* is highly polymorphic, and (iii) Hawaiian accessions of *C. thalictroides* are divergent relative to their continental cohorts and among themselves. The putative interspecific hybrid did not group closely with either of these species.

The genus *Ceratopteris* (*Parkeriaceae*) comprises four recognized species of annual homosporous ferns (LLOYD 1974). These species are found at elevations from sea level to 1000 meters in aquatic and semi-aquatic tropical and subtropical habitats on most continents and on many Pacific and Caribbean islands. However, delineation of individual species is complicated by extensive morphological variability and the occurrence of natural interspecific hybrids with intermediate characteristics (LLOYD 1974, HICKOK & KLEKOWSKI 1974). Fertile interspecific hybrids have been synthesized in all combinations among diploid (n = 39) species *C. richardii, C. pteridoides*, and *C. cornuta* (HICKOK & KLEKOWSKI 1974, HICKOK 1977). The fourth species, *C. thalictroides*, is the most morphologically variable and widely distributed, and is generally considered tetraploid (n = 77, 78; HICKOK 1979).

In the present report, we describe the application of restriction fragment length polymorphism (RFLP) technology using cDNA clones as probes to examine the taxonomic status of *C. thalictroides* and *C. richardii*. We used cDNA clones as probes because they are encoded by the nucleus and thus have the potential to identify interspecific taxa, unlike maternally inherited chloroplast DNA probes.

Using eleven cDNA clones derived from *C. richardii* mRNA as molecular probes, we show that RFLPs exist among eleven diverse *Ceratopteris* accessions and these RFLPs are useful in discriminating between species and interspecific hybrids. Also, RFLPs are present within each species, but to a lesser extent in *Ceratopteris richardii* than *C. thalictroides*. The distribution of RFLPs within *C. thalictroides* suggests that the Hawaiian types form a distinct group. Our results demonstrate the feasibility of applying RFLP technology to fern systematics, and also indicate some limitations of this technology in resolving relationships at higher order taxonomic levels.

## Material and methods

**Plant material.** The accessions tested are listed in Table 1, and were grown from spore stocks maintained at the University of Tennessee. Spores were surface-sterilized, axenically sown and germinated (WARNE & al. 1986) in Petri plates containing 1% agar-solidified Parker/Thompson inorganic nutrient medium (KLEKOWSKI 1969) in a growth chamber maintained at 28 °C under fluorescent and incandescent light (c. 5 W $m^{-2}$) with a 16 h light/8 h dark cycle. After reaching sexual maturity (c. 18 days), gametophyte cultures were flooded with distilled water at 1 week intervals to effect fertilization, and the resulting sporophytes were transferred to a commercial soiless potting mix. Cultured sporophytes were kept moist and humid at all times in a growth chamber (16 h light cycle, 28 °C) by immersing pots in 2 cm of water and sealing the pots with lightly-macerated clear plastic wrap.

**Construction and isolation of a cDNA library.** A cDNA library containing $1 \times 10^6$ independent cDNA clones was constructed by the custom services division of Invitrogen, Co. (San Diego, CA) using poly-A+ RNA isolated with the company's Fast Track RNA isolation system and Librarian II cDNA library construction kit. RNA was isolated from 2 g of unfertilized *Hn-n* gametophytes grown in high-density cultures (> 500 spores/100 mm Petri plate). The gametophytes were harvested at 11, 12, and 16 days, and thus represented a mixture of pre-fertilization developmental stages. The average size of cDNA clones in this library was c. 800 bp, with a range of 500 bp to greater than 2.5 kb. Three cDNA clones encoding non-identical chlorophyll *a*/*b*-binding (CAB) proteins (1-20-16, 3-31-1, 3-31-13;

Table 1. *Ceratopteris* accessions utilized in the present study. Cytological data taken from HICKOK (1977, 1979) and HICKOK & KLEKOWSKI (1974)

| Key | Accession | Location | Species | n= |
|---|---|---|---|---|
| 1 | *IN* | India | *C. thalictroides* (L.) BRONGN. | 77 |
| 2 | *19K1* | Hawaii | *C. thalictroides* | |
| 3 | *185 H* | Surinam | *C. thalictroides* | |
| 4 | *231-4* | New Guinea | *C. thalictroides* | 77 |
| 5 | *JDA 1* | Australia | *C. thalictroides* | |
| 6 | *KN 5* | Japan | *C. thalictroides* | |
| 7 | *NPA 6* | Puerto Rico | *C. richardii* BRONGN. | 39 |
| 8 | *PhiN 8* | Nicaragua | putative *C. richardii* × *C. pteridoides* hybrid | 39 |
| 9 | *RLMN 8* | Hawaii | *C. thalictroides* | 78 |
| 10 | *Hn-n* | Cuba | *C. richardii* | 39 |
| 11 | *D 176* | Guyana | *C. richardii* | 39 |
| 12 | *D 176* × *Hn-n* | $F_1$ hybrid | *C. richardii* | 39 |

McGRATH & al., unpubl.) and eight randomly-isolated anonymous cDNA clones were chosen for this study. Cloned cDNA inserts, ranging from 0.7 to 1.3 kb in size, were recovered after digestion of the plasmids with *Eco* RI, electrophoresis through a 1% agarose gel matrix and electroelution (SAMBROOK & al. 1989), and labelled to high-specific activity with [$^{32}$P] (FEINBERG & VOGELSTEIN 1983). Clones were named for their date and order of isolation.

**Genomic DNA isolation.** The DNA isolation protocol described here was modified from both SAGHAI-MAROOF & al. (1984) and BERNATZKY & TANKSLEY (1986). DNA was isolated by grinding 1 to 5 g of tissue under liquid nitrogen in the presence of sand as an abrasive and solid sodium bisulfite as an antioxidant (final concentration of 20 mM), then thawed in the presence of 10 ml of 350 mM sorbitol, 100 mM Tris-HCl pH 8.2, 5 mM $Na_2EDTA$; 10 ml of 200 mM Tris-HCl pH 8.2, 50 mM $Na_2EDTA$, 2 M NaCl, 2% CTAB (Sigma #H-5882); and 1 ml 10% (w/v) sarkosyl (Sigma #L-5125). The slurry was incubated at 60 °C for 20 min, with occasional mixing by inversion. One volume of chloroform was added to the slurry, and after several gentle inversions, the mix was allowed to cool to 4 °C. The aqueous and organic phases were separated by low-speed centrifugation (full speed setting in a clinical centrifuge) for 15 min. To the aqueous phase was added one volume of 95% ethanol and the DNA-containing precipitate was either removed with tweezers or collected by low-speed centrifugation at room temperature for 5 min. DNA was resuspended in 2 ml of 50 mM Tris-HCl pH 7.6, 10 mM $Na_2EDTA$, and added to 3.45 g solid CsCl. After the CsCl had dissolved, the final volume was adjusted to 4.5 ml with 50 mM Tris-HCl pH 7.6, 10 mM $Na_2EDTA$, to which 100 µl of 10 mg/ml ethidium bromide was added, and the mixture was centrifuged for 12 h in a Dupont TV-865 rotor at 50K rpm at 25 °C. DNA bands were harvested under long-wavelength UV light with a Pasteur pipet, and the ethidium bromide was removed with sequential washes of NaCl-saturated, $H_2O$-saturated isopropanol, until the solution was colorless. Finally, one vol of $H_2O$, then 2 vol ethanol was added to precipitate the DNA, which was resuspended in 0.5 ml of 10 mM Tris-HCl pH 7.6, 1 mM $Na_2EDTA$. DNA concentrations were assessed either photospectrometrically or visually on ehtidium bromide stained gels compared against standards of known DNA concentration. Typically, 60 to 300 µg of DNA per extraction was obtained, with $A_{260}/A_{280}$ ratios between 1.5 and 1.7.

**Restriction digests and Southern blotting of genomic DNA.** DNA (2.3 to 7.4 µg) was digested in a total of 35 µl with the restriction enzyme *Eco* RI (25 units) according to manufacturer's recommendations (Boehringer Mannheim Co.), size-separated in 0.7% agarose and capillary transferred to nylon membrane (Hybond-N, Amersham, Co.) under alkaline conditions as suggested by the manufacturer. Hybridization of radiolabelled probe to filter-bound DNA was done in solution (5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 0.1 mg/ml sonicated fish sperm DNA; SAMBROOK & al. 1989), and subsequent washing steps were performed for 30 min each at 65 °C; first in 2 × SSC, 1% SDS, then in 0.2 × SSC, 0.1% SDS as the final wash. Filters were wrapped in plastic wrap, placed against Kodak XAR-5 film with a Dupont Cronex intensifying screen, and exposed for 4 to 10 days at − 80 °C. Filters were reused after removing the previous probe in 0.1 × SSC at 95 °C for 30 min.

## Results

**Factors affecting the quality of the Southern analysis.** The purity of DNA had a greater effect on detecting single-copy fern sequences than the absolute amount of DNA (which ranged from < 3 to 7 µg per sample). Without DNA purification using CsCl density gradient centrifugation, we found that *Ceratopteris* DNA was cleaved with restriction enzymes but DNA migration in agarose gels was severely retarded (data not shown). The nature of the interfering substance(s) is not known.

However, once purified, fern DNA behaved in a similar manner to DNA from angiosperm sources in the detection of gene sequences on Southern blots.

Hybridization conditions were also critical. Our procedures exclusively used a high criterium for hybridization, i.e., the annealing of the probe DNAs with their target sequences was done at 65 °C. We washed blots under both low and high stringency regimes (2× SSC and 0.2× SSC at 65 °C, respectively). In our experiments, washing at low stringency showed a smear of fragments detected by each probe (data not shown), while high stringency washes resulted in the detection of discrete fragments (Fig. 1). This result suggests that each probe exhibits a low level of sequence similarity with a large number of genomic fern DNA sequences.

**Detection and distribution of RFLPs within *Ceratopteris*.** Multiple genomic blots were prepared using 11 accessions of *Ceratopteris* materials and an $F_1$ hybrid from a cross between *C. richardii* strains *D 176* and *Hn-n* (SCOTT & HICKOK 1987). The seven morphologically-diverse accessions of *C. thalictroides* came from six regions of the world, and three accessions and one putative hybrid form of *C. richardii* were from Central America and the Caribbean islands (Table 1). Each blot was hybridized with one of 11 probes cloned from *C. richardii*. The 11 probes included

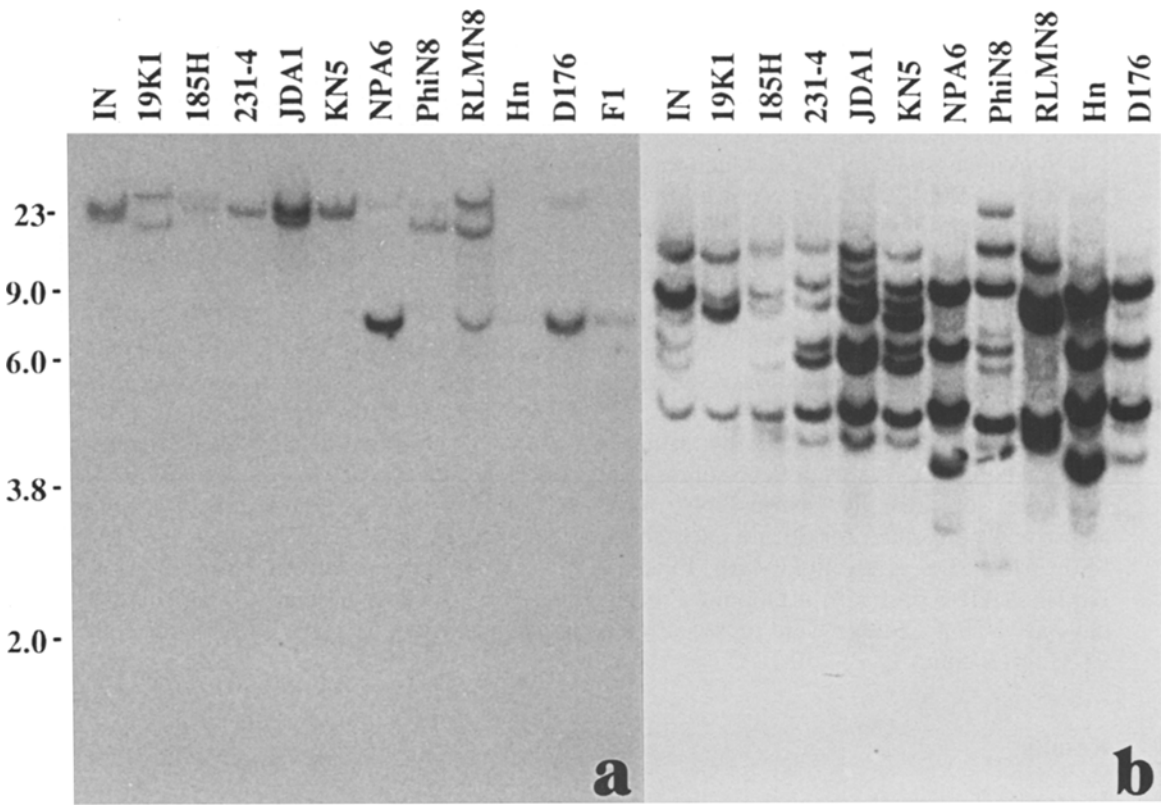


Fig. 1. Southern blots of *Ceratopteris* DNA probed with two cDNA clones. *a* Probe 1-20-2 showing an RFLP pattern of low complexity. $F_1$ indicates a lane of DNA obtained from the hybrid between *D 176* and *Hn-n* (SCOTT & HICKOK 1987). *b* Probe 4-1-7 showing a high complexity RFLP pattern. The majority of probes used were of this type. The accessions tested are indicated above, and fragment size standards are indicated on the left

three different CAB gene cDNA clones (1-20-16, 3-31-1, 3-31-13) and eight other randomly-isolated cDNA clones (1-20-2, 1-20-4, 1-20-11, 1-20-19, 3-31-2, 3-31-17, 4-1-7, 4-1-14). Data for each probe was collected in the form of autoradiographs, which were visually inspected and scored for similarities and differences among accessions.

Most polymorphic fragments observed were not unique to any particular accession, but were shared between two or more accessions, and in some cases even between species. For example, probe 1-20-2 (one of two probes which hybridized to no more than three discrete fragments in all accessions) showed a fragment of 7.5 kb present in each accession of *C. richardii* as well as in *C. thalictroides* strain *RLMN 8* from Hawaii (Fig. 1 A; the low intensity signal in strain *Hn-n* was due to a low DNA content on this particular blot). Most probes showed complex hybridization patterns with four or more fragments of variable intensity (for example probe 4-1-7; Fig. 1 B). The large number of fragments plus the general lack of species-specific fragments complicated the analysis of interspecific relationships. Despite the apparent complexity of banding patterns exhibited by most probes, some features were common between subsets of these accessions and were manifested as a shared suite of bands. Often, only a single polymorphism separated members within each subset.

A visual assessment of all banding patterns for all probes suggested that accessions within *C. richardii* (*Hn-n, D 176*, and *NPA 6*) formed a coherent subset or group. Five different probes (1-20-2, 1-20-4, 3-31-1, 3-31-17, and 4-1-7) each showed different hybridization patterns, but were monomorphic among *C. richardii* accessions and the $F_1$ hybrid between *D 176* and *Hn-n* (e.g., Fig. 1 A and B). Two other probes (1-20-19 and 3-31-2) showed one or two polymorphic fragments within *C. richardii*. The one exception to the general conservation of fragment sizes within this group was shown with probe 1-20-16, where *Hn-n* and *D 176* were identical but *NPA 6* showed a pattern more similar to *C. thalictroides* accessions *KN 5* and *JDA 1*. The three remaining probes (1-20-11, 3-31-13, and 4-1-14) could not be reliably scored in either *D 176* or *Hn-n*.

The Hawaiian *C. thalictroides* accessions *19K1* and *RLMN 8* also appeared to form a coherent group. Between these two types, one probe (4-1-14) showed an identical RFLP pattern and two other probes (3-31-1 and 3-31-13, both were CAB gene sequences) showed markedly dissimilar RFLP patterns. The remaining eight probes showed minor differences in their RFLPs such that only one or a few bands distinguished between them. For example, both types carried two fragments of 20 and 25 kb seen with probe 1-20-2, but *RLMN 8* also carried the 7.5 kb fragment present in *C. richardii* (Fig. 1 A). Both Hawaiian types also showed an identical pattern obtained with probe 4-1-7, although signal intensity differed due to unequal DNA loadings per lane (Fig. 1 B). An unusual pattern of hybridization was seen with probe 1-20-4: both Hawaiian accessions had a single intense band, but all other *Ceratopteris* accessions showed only a barely detectable, smaller-sized monomorphic fragment.

The largest group was composed of *C. thalictroides* accessions *IN, KN 5, JDA 1, 231-4*, and *185 H* from India, Japan, Australia, New Guinea, and Surinam, respectively. This group showed the greatest level of polymorphism, but overall their RFLP patterns were more similar among themselves than they were to other groups.

For instance, probe 1-20-2 identified identical patterns between *IN* and *JDA 1* and different but identical patterns between *KN 5* and *231-4* (Fig. 1 A). Identical RFLP patterns were also observed between accessions *KN 5* and *231-4* for three probes (1-20-2, 1-20-19, and 3-31-1), between *KN 5* and *JDA 1* for two other probes (1-20-16 and 3-31-13), and between *KN 5* and *IN* with probe 1-20-19.

Accession *PhiN 8* (from Nicaragua) was dissimilar to all other accessions in that eight of the 11 probes showed RFLP patterns which had two more polymorphisms compared with each other accession from *C. thalictroides* (or *C. richardii*). For example, probe 4-1-7 (Fig. 1 B) showed a unique 23 kb fragment of high intensity and another of low intensity at 7 kb. Most other fragments shown with this probe were similar in size to fragments seen in other accessions, such as the 9 and 6 kb fragments in *NPA 6* (*C. richardii*) and the 4.5 kb fragment in *RLMN 8* (*C. thalictroides*) (Fig. 1 B). *PhiN 8* also showed similar RFLP patterns with the two Hawaiian accessions for probes 1-20-2 and 3-31-2, and to the larger group of *C. thalictroides* accessions with probe 1-20-19. For example, probe 1-20-2 (Fig. 1 A) showed two fragments in *PhiN 8* at 20 and 25 kb which were similar in size to the Hawaiian *C. thalictroides* types.

For a quantitative measure of the similarities in RFLPs among accessions, the number of shared fragments between each pair of accessions was determined. For example, accessions *KN 5* and *JDA 1* were the most similar and shared 47 of the 99 fragments present in two or more accessions (Table 2, below diagonal). Since data for three probes from accessions *Hn-n* and *D 176* were missing, the number of shared fragments was converted to a similarity score in order to compare among other accessions (Table 2, above diagonal), in the following manner. The observed number of shared fragments between any two accessions, with the exception of *Hn-n* and *D 176*, was divided by the maximum number of shared fragments (e.g.,

Table 2. Number of fragments shared between accessions (below the diagonal) and the proportion of shared fragments (as a percentage) relative to accessions with the highest number of shared fragments (above the diagonal). The highest number of shared fragments (47) was observed between *KN 5* and *JDA 1*. Due to missing data for accessions *Hn-n* and *D 176*, the proportion of shared fragments between these and the other accessions was calculated from their observed maximum shared number of fragments (26, see text)

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *IN* | – | 53 | 74 | 79 | 87 | 83 | 32 | 38 | 55 | 31 | 35 |
| 2 | *19K1* | 25 | – | 61 | 47 | 51 | 43 | 26 | 34 | 98 | 19 | 23 |
| 3 | *185 H* | 35 | 29 | – | 81 | 79 | 74 | 26 | 36 | 60 | 31 | 35 |
| 4 | *231-4* | 37 | 22 | 38 | – | 91 | 91 | 32 | 34 | 51 | 35 | 38 |
| 5 | *JA 1* | 41 | 24 | 37 | 43 | – | 100 | 30 | 45 | 57 | 38 | 38 |
| 6 | *KN 5* | 39 | 20 | 35 | 43 | 47 | – | 34 | 43 | 55 | 42 | 46 |
| 7 | *NPA 6* | 15 | 12 | 12 | 15 | 14 | 16 | – | 30 | 34 | 92 | 88 |
| 8 | *PhiN 8* | 18 | 16 | 17 | 16 | 21 | 20 | 14 | – | 47 | 35 | 38 |
| 9 | *RLMN 8* | 26 | 46 | 28 | 24 | 27 | 26 | 16 | 22 | – | 35 | 38 |
| 10 | *Hn-n* | 8 | 5 | 8 | 9 | 10 | 11 | 24 | 9 | 9 | – | 100 |
| 11 | *D 176* | 9 | 6 | 9 | 10 | 10 | 12 | 23 | 10 | 10 | 26 | – |

by 47). But for comparisons with accessions *Hn-n* and *D 176*, scores corresponding with the missing data points for all accessions were not considered, and the maximum number of shared fragments, in this case between *Hn-n* and *D 176*, was recalculated to be 26 (Table 2).

Comparisons among these normalized scores generally confirmed the interpretations described above. Between 88% and 100% of fragments present in two or more accessions were shared among the three *C. richardii* accessions (Table 2), indicating a close genetic relationship. The two Hawaiian *C. thalictroides* accessions had 98% of their shared fragments in common. Accessions *KN 5*, *JDA 1*, and *231-4* were also very similar in that > 90% of shared fragments were common among them. Other *C. thalictroides* types were less similar, but the proportion of shared fragments between *IN* and *185 H* with the other Asian types ranged from 74% to 87%, as compared with the Hawaiian types where the similarity ranged between 53% to 61%. Accession *185 H* shared more fragments with the Hawaiian types than did other members of *C. thalictroides*. Accession *PhiN 8* was the least related to the other accessions since the proportion of shared fragments ranged from 30% when compared with *C. richardii* accession *NPA 6* to 47% as compared with *C. thalictroides* accession *RLMN 8*.

A cladistic analysis was performed using the shared fragment data set. Using the PAUP algorithm (Phylogenetic Analysis Using Parsimony; SWOFFORD 1990), and setting the *C. richardii* accessions as the outgroup, the dendrogram presented in Fig. 2 was obtained. These results were concordant with the relationships de-

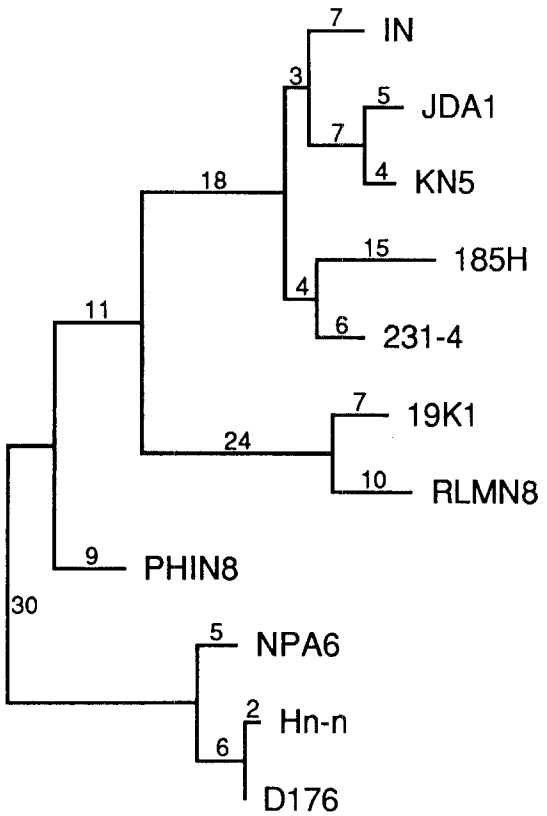


Fig. 2. Cladogram with branch lengths for 11 *Ceratopteris* accessions generated by the PAUP algorithm. This was the single best tree obtained when accessions of *C. richardii* accessions (*NPA 6*, *Hn-n*, and *D 176*) were considered as outgroup taxa. A branch-and-bound bootstrap analysis of this tree gave a consistency index of 0.55 and a homoplasy index of 0.45. The major inconsistency in this tree was contributed by accession *185 H* (see text)

scribed above; namely the Hawaiian types appeared as a single group, *PhiN8* formed a separate clade, and the remaining accessions of *C. thalictroides* (*IN, 231-4, 185 H, JDA 1*, and *KN5*) also formed a group. For the latter group, however, the bootstrap confidence interval indicated a polytomy for all members except *JDA 1* and *KN5*. If *185 H* was excluded from this bootstrap analysis, *IN, 231-4, JDA 1*, and *KN5* formed a single clade. This result may reflect the larger number of shared bands between *185 H* and the Hawaiian accessions relative to other *C. thalictroides* types.

## Discussion

Success in obtaining good quality Southern blots with *Ceratopteris* DNA was dependent on the use of cDNA clones of the same, or closely related, species. Even with such cDNA clones, low stringency washes showed a smear of signal across the blot; however, at high stringency discrete bands were detected. This observation suggests that many restriction fragments in homosporous ferns appear to carry motifs which are similar to most nuclear DNA sequences used as probes. Since extensive cross-hybridization of sequences appears to occur at low stringency, this presents a limitation of RFLP technology to examine relationships between higher order taxa. However, within *Ceratopteris*, RFLP analysis appears to be useful in discriminating among species.

Within this group of eleven *Ceratopteris* accessions, three were previously identified as *C. richardii*, one as a putative inter-specific hybrid (accession *PhiN8*) and the remainder as *C. thalictroides* (HICKOK & KLEKOWSKI 1974, HICKOK 1977, HICKOK 1979, and unpubl. obs.). Our results generally support these taxonomic assignments. The putative hybrid *PhiN8*, a diploid, shared the least number of fragments with both *C. richardii* and *C. thalictroides* and a number of fragments were unique to *PhiN8*, as well. HICKOK & KLEKOWSKI (1974) suggested that *PhiN8* is a putative hybrid between *C. richardii* and *C. pteridoides* on morphological grounds. From our data, we can not confirm the hybridity of this accession since the full range of *Ceratopteris* species was not tested. Other inter-specific hybrid combinations, or even specific classification of *PhiN8*, could also explain our results.

Within *C. thalictroides* two groups could be distinguished which were roughly correlated with their geographic distributions, namely the Asian and Hawaiian groups. The Hawaiian types are morphologically similar to each other (LLOYD 1973) and also showed a high similarity in their RFLP patterns. The Asian clade was more diverse, and included the South American accession, *185 H*. The correct placement of *185 H* in this clade is suspect since it also shares a higher proportion of fragments with the Hawaiian types than with Asian accessions, and a nearly identical RFLP pattern with the Hawaiian types for one probe. In addition, one of the Hawaiian accessions carried an otherwise unique *C. richardii* (Central American) fragment. These results contradict LLOYD's (1973) assertion that *C. thalictroides* was probably introduced by colonists to Hawaii from the Orient and most closely resemble Japanese accessions, since our data suggests a closer affinity between Hawaiian and Meso-American types. However, these observations could also mean that the Hawaiian germplasm base has been expanded with additional Meso-American introductions after an initial population was founded by a single

propagule and subsequently spread to other Hawaiian islands, as LLOYD (1973) suggests.

HAUFLER (1987) has proposed that homosporous fern genomes have evolved through polyploidy followed by extensive gene silencing. This hypothesis makes concrete predictions that can be directly tested through the inheritance of RFLP markers. In the present paper, we have demonstrated the utility of RFLP markers for fern systematics. In a companion paper (MCGRATH & al. 1994, this volume) we discuss the use of RFLP data to examine levels of polyploidy and gene duplication in the evolution of homosporous ferns.

We thank AUGUST SICK of Invitrogen, Co. for his perseverance in constructing the cDNA library and MARINA JANSCO and CORNELIA HUCKING for their help in library screening and nucleotide sequencing. We also thank the University of Michigan Phoenix Foundation for their generous financial support.

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Accepted August 20, 1993 by D. J. CRAWFORD