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12. We acknowledge valuable conversations with Dr. Benjamin E. Cohen.

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DNA Hybridization and Evolutionary Relationships in Three Osmunda Species

Abstract. *Molecular hybridization techniques have been used to estimate the degree of DNA base sequence homology between some members of the fern genus* Osmunda. *Under conditions permitting extensive reassociation, measurements of the extent of interspecific reaction and the thermal stability of the hybrid molecules indicated that* O. claytoniana *L. (interrupted fern) shares more DNA homology with* O. cinnamomea *L. (cinnamon fern) than it does with* O. regalis *L. (royal fern). These findings are in conflict with predictions from a recent analysis of living and fossil specimens by numerical techniques. However, they are consistent with the earlier, more traditional, taxonomic assignments.*

Molecular hybridization techniques have been used to explore evolutionary relationships in both plants (*1*) and animals (*2*). In cases where fossil evidence is available, it has been possible to relate relative rates and patterns of DNA change in different groups of organisms (*2*) to an absolute time scale. We describe here a case in which DNA hybridization experiments provide molecular evidence to support one of two possible phylogenetic schemes and help clarify interpretation of the fossil record.

It has recently been suggested (*3*) that lines leading to *Osmunda claytoniana* and *O. cinnamomea* diverged at least 70 million years ago, but that only 1 million years have elapsed since *O. claytoniana* and *O. regalis* last shared a common ancestor. This newly derived phylogeny is based on the most extensive fossil record found among the ferns (*4*) as well as on anatomical features of living representatives. However, *O. claytoniana* and *O. cinnamomea* are extremely similar morphologically while *O. regalis* has a clearly different leaf morphology. Since previous classifications have considered *O. claytoniana* to be more closely related to *O. cinnamomea* than to *O. regalis* (*5*), we used interspecific DNA hybridization as an independent method of assessing genetic relationships. Our results accord more closely with the earlier system of classification.

We extracted and purified DNA from the three *Osmunda* species native to North America; we used only mature, vegetative fronds collected from fertile plants in order to assure proper identification (*6*). The DNA was sheared to a mean fragment length of approximately 400 to 500 nucleotides, concentrated by lyophilization, and dissolved in the reaction buffer (*7, 8*) to a concentration of about 10 mg/ml.

Families of related DNA sequences are a characteristic feature of eukaryotic DNA (*9*), especially in the many vascular plants with large genomes (*10*). These sequences reassociate rapidly in renaturation experiments, the rate of reassociation being greater for more extensively repeated sequence families. Therefore, we compared the reassociation kinetics of DNA from the three *Osmunda* species to determine whether large differences existed in the distribution of repetitive sequences in various kinetic classes. Reassociation was followed optically as a function of C_0t [the product of DNA concentration and time; (*11*)]. As may be seen in Fig. 1, DNA from all three species reassociates with remarkably similar kinetics. In each case, the DNA is highly repetitious, with most fragments reassociating more rapidly than expected for sequences present only once per haploid genome (*12, 13*). There is an essentially continuous distribution of sequences with different repetition frequencies, with no clear separation between repetitive and single-copy DNA. The similarity of reassociation kinetics indicates that there have been no large changes in degree of sequence repetition which would complicate measurements of DNA homology.

In order to measure relationships among the three species, *O. claytoniana* DNA was labeled in vitro with ^{125}I (*14*) and mixed with a 1000-fold excess of unlabeled DNA from *O. claytoniana*, *O. cinnamomea*, or *O. regalis*. At this concentration ratio, the tracer reacts primarily with the unlabeled DNA (*15*). Portions of these mixtures were then denatured by heat and placed at the appropriate temperature for reassociation. Incubation periods of up to 120 hours and unlabeled DNA concentrations of about 10 mg/ml were used to obtain the indicated C_0t values. Reassociated duplexes, both homologous and heterologous (interspecific), were isolated by hydroxyapatite chromatography (*8*) after dilution with 0.12*M* sodium phosphate buffer. The extent of reassociation of unlabeled DNA was the same for all three species and was about 90 percent in all experiments reported.

In all our experiments, the percentage of interspecific duplexes formed at any C_0t is always higher for reactions between ^{125}I-labeled *O. claytoniana* and *O. cinnamomea* than for reactions between ^{125}I-labeled *O. claytoniana* and *O. regalis*. For example, at a C_0t of 1.6×10^4 the reaction of ^{125}I-labeled *O. claytoniana* DNA with *O. cinnamomea* DNA is 86 percent of that of the homologous control, while the corresponding value with unlabeled *O. regalis* DNA is 73 percent under the same conditions.

Precision of base pairing in interspecific duplexes provides another measure of DNA homology, especially where the interspecific reactions are as extensive as they are in the *Osmunda* species. Thermal stability of DNA is reduced in proportion to the percentage of bases that are unpaired; the T_m (midpoint of the thermal dissociation profile) is depressed on the order of 1°C for each 1 percent of mismatched base pairs (*16*).

Thermal stability profiles for native *Osmunda* DNA and reassociated homologous and heterologous duplexes were obtained by thermal elution from hydroxyapatite (*8*). In all three species, reassociated homologous duplexes showed identical profiles with a $T_{m,i}$ (*17*) nearly 8°C lower than that for native DNA of the same fragment length. Similar reductions in thermal stability, resulting from cross-reactions between similar but nonidentical sequences within families of repetitious DNA, have been observed in many organisms (*9, 15*). In the case of the interspecific duplexes, however, an additional reduction in thermal stability is observed (Fig. 2). When compared with the reassociated duplexes of ^{125}I-labeled *O. claytoniana* with *O. claytoniana*, the $T_{m,i}$ of the DNA hybrids of ^{125}I-labeled *O. claytoniana* with *O. cinnamomea* shows an additional reduction of 1.8°C, while the $T_{m,i}$ of ^{125}I-labeled hybrids of *O. claytoniana* with *O. regalis* is reduced by 3.5°C. This indicates less homology of DNA sequence

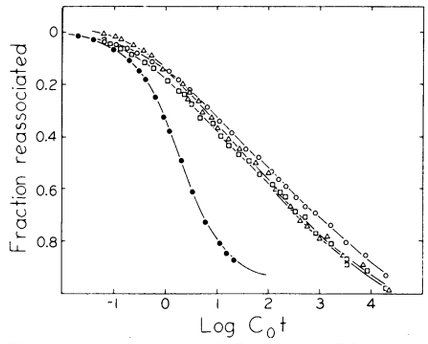

Fig. 1. Reassociation of *B. Subtilis* DNA (●) and DNA from *O. claytoniana* (○), *O. cinnamomea* (△), and *O. regalis* (□). After heat denaturation, the DNA was incubated at 42°C in buffer containing 50 percent formamide, 10 m*M* PIPES buffer, *p*H 6.7, and 1.0*M* NaCl. Reassociation was measured optically (hypochromicity) at 270 nm. Data were obtained from continuous records of absorbance as a function of time, or, for the highest C_0t values, from thermal denaturation measurements made on samples reassociated at high concentration and then diluted in reaction buffer at 42°C. The fraction reassociated was calculated (after correction for collapse hypochromicity which occurs on cooling single-stranded DNA) by assuming that the hypochromicity of fully reassociated DNA is 80 percent of that for native DNA.

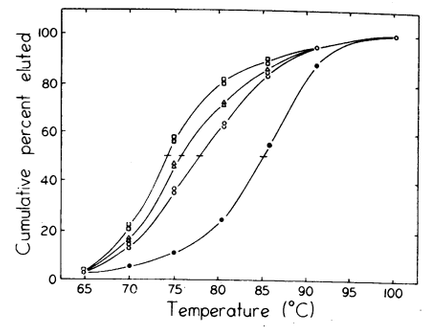

Fig. 2. Thermal elution profiles for sheared, native *O. claytoniana* DNA (●) and duplexes formed between *O. claytoniana* ^{125}I tracer and unlabeled DNA from *O. claytoniana* (○), *O. cinnamomea* (△), or *O. regalis* (□). Except in the case of native DNA, appropriate samples were denatured by heat and incubated under our standard conditions to a C_0t of 1.6×10^4. Duplicate samples for each species were then diluted 200-fold with 0.12*M* sodium phosphate buffer (*p*H 6.8) and passed over hydroxyapatite at 60°C (multiple columns maintained in a single water bath). After the columns were washed to remove single-stranded DNA, thermal elution was performed by raising the temperature of the column in increments of 5°C and eluting with 0.12*M* phosphate buffer at each temperature. Results are presented as the cumulative percentages of the initially bound DNA eluted at each temperature.

and presumably greater evolutionary divergence between *O. claytoniana* and *O. regalis* than between *O. claytoniana* and *O. cinnamonea*. Results of repeated experiments were highly reproducible, and experiments involving reassociation at both higher and lower temperatures have given results consistent with this pattern of sequence relationship.

In early DNA hybridization techniques, DNA was embedded in filters or agar, the concentrations of DNA in solution were low, and the times of incubation were short. Under these conditions only a small fraction of the DNA reacts, usually less than 35 percent, even in the homologous case (*2*). Despite this limitation, such techniques have often given results consistent with other lines of evidence. For example, Goldberg *et al.* have reported that relationships determined by such techniques closely parallel results of more traditional systematic studies in the genus *Cucurbita* (*18*). Although discrepancies have been encountered in cases where distantly related organisms have been compared, the extent of cross-reaction in these cases is often vanishingly small. In more recent methodology (*2, 10*), high concentrations of DNA are allowed to react in solution for long periods of time, thereby permitting larger fractions of the genome to be compared. In our study, approximately 90 percent of the DNA reassociated in the homologous case, and cross-reactions between species involve a large fraction of the total DNA.

Complications in estimating evolutionary divergence time, based on DNA homology measurements, could still arise if significant amounts of DNA were added to (or deleted from) the genome in the time after divergence, since the affected sequences might not be representative of the rest of the genome. Rapid, large-scale multiplication of certain sequences has been observed in rodents (*15*), and difficulties in interpretation which seem likely to be related to large differences in genome size have been encountered in comparisons of cereal grains (*19*).

It appears that the three *Osmunda* species have similar genome sizes, since both chromosome number and size are the same in all three (*20*). In addition, the similarity of reassociation kinetics indicates similarity in the distribution of repetition frequency classes within each genome, and melting profiles of reassociated homologous duplexes reveal no differences in the average divergence of sequences within the repetitious families. Reciprocal experiments with *O. claytoniana* or *O. regalis* as the source of labeled DNA yield essentially the same values for both cross reactivity and hybrid thermal stability, and no distinctive, high thermal stability components have been seen in thermal dissociation profiles of homologous reassociated duplexes (*21*). Thus we have failed to detect any recent, large-scale addition of repeated sequence families containing closely related sequences (*15*), or other large differences either in size (*22*) or intraspecific divergence (*1*) of such families which would affect interpretation of our results.

We conclude that *O. claytoniana* DNA is more closely related to DNA from *O. cinnamomea* than to *O. regalis* DNA, and we support earlier classifications (*5*) which place *O. claytoniana* and *O. cinnamomea* in the same subgenus and *O. regalis* in a separate subgenus. Miller (*3*) has postulated that lines leading to *O. claytoniana* and *O. cinnamomea* diverged over 70 million years ago, while less than 1 million years separate *O. claytoniana* and *O. regalis*. Such a postulate is inconsistent with our data unless it is assumed that *O. regalis* DNA evolved more than 140 times as fast as *O. claytoniana* or *O. cinnamomea* DNA, or, alternatively, that parallel evolution has produced a secondary increase in DNA homology between *O. claytoniana* and *O. cinnamomea* (*23*). Since neither of these alternatives appears at all likely, we disagree with the phylogenetic scheme proposed by Miller. Indeed, Miller's own studies show that when the more general morphological and anatomical characters of fossils and extant species are considered, the distance relationships in two out of three of his numerical analyses would be consistent with our DNA sequence studies. However, his final phylogenetic scheme stresses anatomical characters of aerial parts of living species, plant parts for which fossil evidence is scanty. These characters may be of recent origin and, therefore, of less phylogenetic significance. DNA comparisons, in contrast, provide a means of evaluating overall relatedness with greater objectivity, provided that comparisons are made on species with similar genome size and organization and under conditions in which most of the genome is included in the analysis.

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6. Lyophilized leaf tissue was extracted at 60°C, and the majority of polyphenols and low-molecular-weight contaminants were removed by chromatography on BioGel P-200. Final purification was then accomplished by hydroxyapatite chromatography with buffers containing 8*M* urea. Specimen vouchers for *O. regalis*, *O. claytoniana*, and *O. cinnamomea* are on file in the Herbarium, Department of Botany, University of Massachusetts, Amherst. Voucher numbers are 73830 to 73835.
7. DNA dissolved in a mixture of 0.2*M* sodium acetate and 67 percent glycerol was sheared with a

VirTis 60 homogenizer at 48,000 rev/min for 1 hour while cooling the sample with ice water. Fragment length was determined from measurements of the T_m depression observed upon thermal dissociation of the fragments in 0.12*M* phosphate buffer (*8*). Reassociation buffer contained 50 percent formamide, 10 m*M* piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), *p*H 6.7, and 1.0*M* NaCl. Our standard incubation temperature of 42°C was 21°C below the T_m of native, sheared DNA in this solvent.

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11. C_0t is defined as the concentration of DNA in moles of nucleotides per liter times time in seconds (*8*).
12. The genome size of *O. cinnamomea* is estimated to be 9.6×10^{10} nucleotides (*13*), or about 1.5×10^4 times as large as that for *Bacillus subtilis*. The DNA of *B. subtilis*, when reassociated under our conditions, has a $C_0t_{1/2}$ (C_0t required for 50 percent reassociation) of 2, and it can therefore be predicted that single-copy DNA sequences in the *O. cinnamomea* genome would be half reassociated at a C_0t of approximately 3×10^4.
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23. Evidence that parallel evolution does not produce a parallel change in DNA coding for serum albumin is seen in a recent study of tree frogs [L. R. Maxson and A. C. Wilson, *Science* **185**, 65 (1974)]. Although the two species of tree frogs considered had evolved nearly identical morphologies, they had very dissimilar albumins, an indication of evolutionary separation.
24. We thank D. W. Bierhorst, O. L. Stein, E. J. Klekowski, Jr., for valuable discussions. Supported by NSF grant GB38242 to W.F.T. This is Carnegie Institution of Washington publication 546.

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Chestnut Blight: Biological Control by Transmissible Hypovirulence in *Endothia parasitica*

Abstract. *Hypovirulence in* Endothia parasitica *is caused by a cytoplasmic determinant that is transferred by hyphal anastomosis in host tissue and in culture. Transmission of this determinant affects the virulence of the fungus to the extent that host invasion by previously virulent isolates is limited.*

Chestnut blight, caused by the fungus *Endothia parasitica* (Murr.) Anderson, eliminated the American chestnut as a dominant or codominant species throughout its natural range in North America. Sprouts of this tree have continued to grow from stumps, but before reaching maturity they are attacked, girdled, and killed by the pathogen. The virulent pathogen is still present, surviving and producing its abundant spores on the sprouts. The search for resistant American chestnut trees and attempts to produce resistant hybrids comparable to the original tree have not succeeded. The work reported here offers hope that the virulent strain of *E. parasitica* can be modified in nature so that the American chestnut can once again survive and multiply.

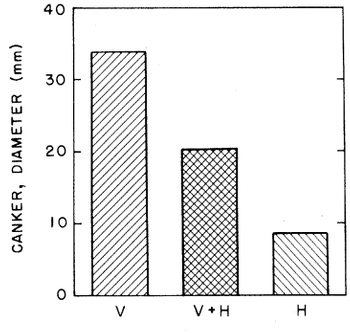

Fig. 1. Cankers formed by virulent (V) and hypovirulent (H) strains of *E. parasitica* inoculated as pairs, or singly, on field-grown American chestnut seedlings. There were 13 inoculations per tree (six V, one H, six V plus H) replicated once on each of six trees. Two French H and six American V isolates were used. The size of cankers was measured 54 days after inoculations.

A variant of *E. parasitica* low in virulence toward chestnut trees was first observed in Italy by Biraghi in stands of European chestnut (*Castanea sativa* Mill.) in which the disease was no longer spreading (*1*). Typical virulent forms of the fungus could not be isolated from these stands. The variant later found in France and in the Pyrenees was isolated and described as hypovirulent by Grente (*2*, *3*). The hypovirulent form (H) is not only weakly virulent itself but prevents more virulent forms (V) from vigorously invading the host tissue when both are together in the same canker (*2*, *3*). Grente (*4*) reported that H survives well in nature. When he artificially inoculated European chestnut plots, H appeared to dominate the *E. parasitica* population after 3 years. Grente and Sauret (*3*) speculated that hypovirulence was due to the presence of a transmissible determinant.

Anagnostakis and Jaynes (*5*) obtained cultures from Grente and demonstrated that H can limit the size of the canker formed by the French V strain on American chestnut trees. In their limited experiments, cankers formed by an American V strain were not restricted by using the French H strain.

We set out to find an H strain that could protect American chestnut trees from being killed by the American V strain, and to examine the nature of hypovirulence. We used hypovirulent strains 2025 and 2043 (a single spore isolate of 2025) from Grente and American virulent strains from infected native American chestnut trees growing in Connecticut. All isolates were grown on potato dextrose agar (PDA, Difco). Seedlings (3 to 8 years old) of American chestnut were used and inoculations, measurements, and isolations were as described (*6*).

Auxotrophic mutants of *E. parasitica* were obtained according to the procedures of Puhalla and Anagnostakis (*7*). Hypovirulent colonies of *E. parasitica* grown on PDA under lights have fewer pycnidia, less pigment, and a more milky appearance than virulent colonies. These cultural differences were expressed at all temperatures tested in the laboratory (10° to 30°C) and they correlated perfectly with virulence in the host (54 V and 56 H cultures were tested).

When trunks of American chestnut trees were inoculated with strains of American V and French H, alone and paired, cankers resulting from the combination were significantly smaller than those of V alone (Fig. 1). However, the mean size of the cankers from the paired inoculations was greater than that of H strains alone, indicating that protection was not complete. Isolations from the edges of the cankers formed by these paired inoculations yield-

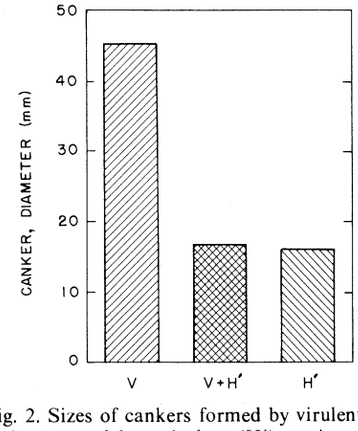

Fig. 2. Sizes of cankers formed by virulent (V) and recovered hypovirulent (H′) strains of *E. parasitica* inoculated as pairs, or singly, on field-grown American chestnut seedlings. There were nine inoculations per tree (three V, three H′, and three V plus H′) replicated once on each of eight trees. The H′ strains were isolated from cankers formed from V plus H paired inoculations. Size of cankers was measured 42 days after inoculations.