

**RECONSTRUCTING *DRYOPTERIS* “SEMICRISTATA”
(DRYOPTERIDACEAE): MOLECULAR PROFILES OF TETRAPLOIDS
VERIFY THEIR UNDISCOVERED DIPLOID ANCESTOR¹**DIANA B. STEIN^{2,7}, CYNTHIA HUTTON³, DAVID S. CONANT⁴, CHRISTOPHER H. HAUFLE⁵,
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- *Premise of the study:* Discovering missing ancestors is essential to understanding the evolutionary history of biodiversity on Earth. Evidence from extinct species can provide links for reconstructing intricate patterns of reticulate relationships among extant descendants. When fossils are unavailable and other evidence yields competing hypotheses to explain species ancestry, data from proteins and DNA can help resolve conflicts and generate novel perspectives. The identity of a parent shared by two tetraploid species in the cosmopolitan fern genus *Dryopteris* has remained elusive for more than 50 years. Based on available data, four hypotheses were developed previously, each providing a different resolution to this uncertainty.
- *Methods:* New molecular evidence from studies of isozymes and restriction site analysis of chloroplast DNA tested the competing hypotheses about the diploid ancestors of these two extant *Dryopteris* polyploids.
- *Key results:* The results falsify two of the hypotheses, resolve the uncertainty in the third, and support the fourth.
- *Conclusions:* Our data validate the prior existence of *Dryopteris* “*semicristata*,” which was proposed 38 years ago as a diploid progenitor of the allotetraploids *D. cristata* and *D. carthusiana* but has never been collected. After developing a phylogeny using the new molecular data, we describe a plausible morphology for *D. “semicristata”* by extrapolating likely character states from related extant species.

Key words: chloroplast DNA; Dryopteridaceae; *Dryopteris carthusiana*; *Dryopteris cristata*; *Dryopteris “semicristata”*; ferns; hybridization; isozymes; reticulate evolution.

Ever since Darwin, evolutionary biologists have sought answers to basic questions about the origin of biodiversity. Although reconstructing species origins has often proven difficult, applications of molecular tools have provided valuable information about both primary speciation (divergent or cladogenetic events) and secondary speciation (usually reticulate, hybrid-derived events) (Haufler, 2008). Secondary species have been considered less evolutionarily significant than primary species (Wagner, 1969); yet more than 50% of plant species, including such important crops as wheat, cotton, and coffee,

originated through hybridization (Stebbins, 1950; Leitch and Bennett, 1997). Most secondary species are allopolyploids, which begin as sterile interspecific hybrids but regain fertility through genome doubling (Stebbins, 1950; Harlan and deWet, 1975; Grant, 1980; Soltis and Soltis, 1993; Leitch and Bennett, 1997). Because allopolyploids are often morphologically and biochemically intermediate between extant progenitors, hypotheses about their origins can be proposed and tested. Complications to reconstructing the ancestry of allopolyploids include (1) formation of reticulate complexes involving several related diploid and/or hybrid species and their derived allopolyploids, (2) recurring origins of hybrids, (3) backcrossing among the members of reticulate complexes (Soltis and Soltis, 1993; Haufler, 2008), and (4) extinction. When the progenitors of extant allopolyploid species are extinct and/or unknown (as in wheat), reconstructing their origins becomes both more interesting and more difficult. In this study, we use molecular fingerprinting (both isozymic and chloroplast DNA) to document such an undiscovered progenitor, analyses of chloroplast DNA sequences to hypothesize its phylogeny, and structural comparisons of two extant allotetraploid species to extrapolate the leaf morphology of this progenitor.

Dryopteris, the wood fern genus, contains over 150 species and is distributed worldwide. The North American taxa, with 13 described fertile species (diploids plus polyploids) and 29 sterile hybrids, illustrate nicely the intricacies of reticulate evolution (Montgomery and Paulton, 1981; Montgomery and Wagner, 1993). Well-supported hypotheses addressing the relationships of most species were developed using morphological,

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This paper reports research by the preeminent pteridologist Dr. Charles R. Werth (1947–2001) before his untimely death. Charlie was passionate and committed to resolving intractable conundrums in the evolution of ferns. He addressed tough questions about challenging groups, and none was more important to him than *Dryopteris*. He campaigned hard to raise the profile and recognize the importance of *D. “semicristata”*, and we hope his work reported here helps to reach that goal. The authors thank G. Yatskievich for providing *Dryopteris* spp. leaves and data for outgroups for the DNA study, K. Perkins for the *D. ludoviciana* leaves; J. T. Mickel for the *D. tokyoensis* leaves; O. L. Stein, P. Wolf, and an anonymous reviewer for excellent editorial comments on the manuscript; and the Faculty Resource Center at Mount Holyoke College for help with figure preparation. This research was supported in part by NSF Grants BSR-881849 and DEB-9207211 to D.B.S. and D.S.C. and NSF BSR 9023787 to D.B.S.

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biochemical, breeding, and cytological analyses (Wagner, 1971; Hickok and Klekowski, 1975; Gibby and Walker, 1977; Widén and Britton, 1985). However, recovering the complete parentage of the tetraploids *D. carthusiana* and *D. cristata* has been contentious (Fig. 1). Analyses of chromosome pairing in natural hybrids and synthetic crosses (Gibby and Walker, 1977) provided evidence for three different genomes A, B, and C (Fig. 1A) of which one, B, was common to both *D. carthusiana* and *D. cristata*. Three additional hypotheses were developed to explicate how these allotetraploid species originated. In all four hypotheses (Fig. 1A–D), the diploid *D. intermedia* was accepted as the source of genome C. However, the models differ as follows: In hypothesis one (Fig. 1A), *D. ludoviciana* was proposed as the second diploid involved, contributing either the A or B genome; the donor of the third genome was not identified (Gibby and Walker, 1977). Hypothesis two (Fig. 1B) was based on a reanalysis of the higher than expected chromosomal pairing between nonidentical genomes. This led the authors to conclude that *D. ludoviciana* had donated the shared B genome, whereas the second parent of *D. cristata* (contributing the A genome) was a divergent version of *D. goldiana* (Hickok and Klekowski, 1975). In developing hypothesis three (Fig. 1C), investigators used phloroglucinol content to propose that the Asian species *D. tokyoensis* (Widén and Britton, 1985) had provided the B genome. Hypothesis four (Fig. 1D) proposed

that *D. ludoviciana* was the source of the A genome and a hypothetical, apparently extinct species called *D. “semicristata”* contributed the shared B genome (Wagner, 1971; Montgomery and Wagner, 1993). To test these competing hypotheses, we examined isozymes as a measure of nuclear gene expression and chloroplast DNA restriction fragments to follow cytoplasmic inheritance.

MATERIALS AND METHODS

Plant material—See Appendix 1 for voucher information and collection sites.

Molecular methods—

Isozyme analysis—Leaf or gametophyte tissue was homogenized and homogenates were subjected to starch gel electrophoresis as reported elsewhere (Werth, 1989). We use the term “isozyme” to refer to the various gene products of different functional loci that may be visualized as band patterns on substrate-containing gels (Crawford, 1990). Isozyme is also used to refer in general to genetic variants of individual enzymes. Interpretation of band patterns as allelic genotypes (allozymes) coded by individual segregating loci in diploids and by two or more loci (isozymes) in the tetraploids followed standard procedures (Wendel and Weeden, 1989; Werth, 1989). Interpretation of the genetic control of allozymic variation was straightforward and was verified through segregation analysis of gametophytes.

Chloroplast DNA analysis—Total (nuclear plus organellar) DNAs were isolated from leaves and subjected to Southern hybridization as described by Stein (1993). DNAs were digested with restriction enzymes (*Bam*HI, *Bcl*II, *Bgl*II, *Eco*RV, *Hind*III, *Pst*I, *Pvu*II, *Sac*I, *Sac*II, *Scal*, *Stu*I, *Sma*I, *Xho*I) singly or in pairwise combinations, separated on 1% agarose gels and blotted. Blots were hybridized to nick-translated probes of cloned chloroplast DNA from lettuce and petunia (Jansen and Palmer, 1987), *Adiantum capillus-veneris* L. (Hasebe and Iwatsuki, 1990), or *Polystichum acrostichoides* (Michx.) Schott. The probes represent overlapping segments of an entire chloroplast genome and were used singly; in some cases, two to three smaller adjacent fragments were combined to reduce the number of hybridizations. Exposed films were scored for mutations. Partial or complete restriction maps were prepared for all 11 enzymes used. Mutations were compiled into a data matrix to generate evolutionary hypotheses.

Phylogenetic analysis—Parsimony analysis of the cpDNA restriction site data was carried out using the program PAUP* 4.0b10 (Swofford, 2002). We employed exhaustive searches in which all characters were treated as unordered and computed a strict consensus tree from the most parsimonious trees stored in memory. The degree of support for monophyletic groups was evaluated via bootstrap analysis (Felsenstein, 1985; Sanderson, 1989). The bootstrapping procedure employed involved 1000 replications and branch-and-bound searches where only minimal trees were saved. We investigated support for monophyletic groups using decay analysis or Bremer support (Bremer, 1988) and calculated decay index values using the program TreeRot (Sorenson and Franzosa, 2007). Recent phylogenetic analyses (Liu et al., 2007; Schuettpelz and Pryer, 2008) suggest that *Arachniodes* is sister to *Dryopteris*; however, another study (D. S. Barrington, University of Vermont, unpublished data) indicates that *Arachniodes* may not be separable from *Dryopteris*. If *Arachniodes* is part of the *Dryopteris* clade, then the sister clade includes *Phanerophlebia*, *Cyrtomium*, and *Polystichum*. To account for this ambiguity, we ran our analyses using four different outgroups: three species of *Phanerophlebia*, three species of *Cyrtomium*, three species of *Polystichum*, and one species each of *Phanerophlebia*, *Cyrtomium*, and *Polystichum* (Yatskievych et al., 1988).

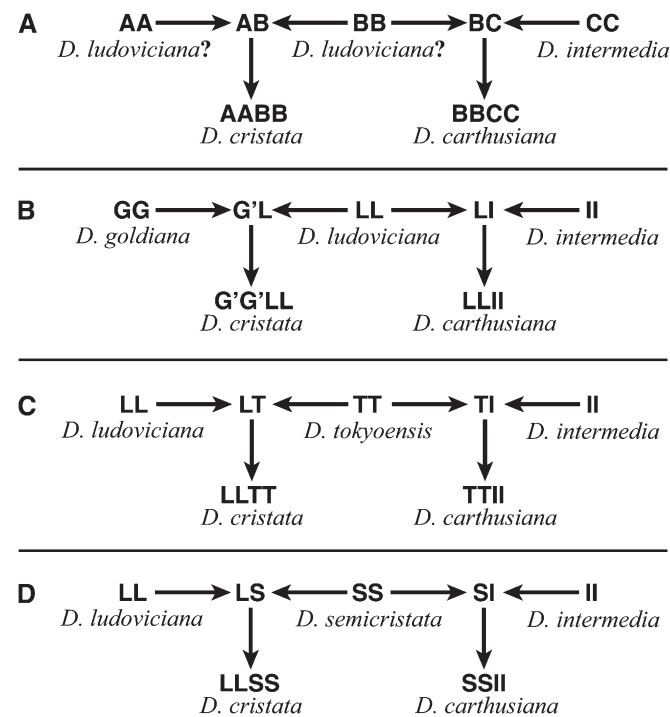


Fig. 1. Hypotheses of the parentage of the allotetraploids *Dryopteris cristata* and *D. carthusiana*. *Dryopteris intermedia* is the provider of a genome to *D. carthusiana* in all cases. In hypothesis (A) three genomes (A, B, and C) are represented among the parents of *D. cristata* and *D. carthusiana*; genome (B) is shared by the allotetraploids. *Dryopteris ludoviciana* was described as the donor of either the A or the B genome (Gibby and Walker, 1977). In hypotheses B–D, the species contributing its genome to both *D. cristata* and *D. carthusiana* was hypothesized to be: *D. ludoviciana* (Hickok and Klekowski, 1975), *D. tokyoensis* (Widén and Britton, 1985), or *D. “semicristata,”* an undiscovered species (Wagner, 1971), respectively. All notation is that used by the original authors.

RESULTS AND DISCUSSION

Data from molecular methods can provide evidence for both extant and extinct ancestors. In the case of allotetraploids, isozyme patterns often yield important data for the identification of diploid ancestors (Wendel and Weeden, 1989; Werth,

TABLE 1. Comparison of the allozyme genotypes of *Dryopteris* species. Alleles are designated as allozymic mobility values relative to the principal allozyme of *D. ludoviciana*, arbitrarily designated 100. Mean allelic frequencies (an average of population frequencies) are given in parentheses where population data were available. Genotype of the unknown was inferred from its polyploid derivatives (see text). Fixed heterozygous genotypes in polyploids are indicated by a pair of alleles separated by a slash mark. The three diploid species possessed polymorphisms at some loci for which alleles are listed in descending frequency. The tetraploids showed variable genotypes at some loci also listed in descending frequency. In case 1, the alleles listed for the inferred diploid ancestor are extrapolated by comparison of the diploid *D. ludoviciana* with the tetraploid *D. cristata*. In case 2, the alleles listed for the inferred diploid ancestor are extrapolated by comparing the diploid *D. intermedia* with the tetraploid *D. carthusiana*. In isozyme group A, there is little or no isozymic variability across all species. In isozyme group B, there is greater isozymic variability, but there are fixed patterns in the allotetraploids. In isozyme group C, the unique isozyme profile of the inferred diploid can be deduced unambiguously.

Locus	Case 1			Case 2			
	Known diploid ancestor <i>ludoviciana</i>	Allotetraploid derivative <i>cristata</i>	Inferred diploid ancestor	Known diploid ancestor <i>intermedia</i>	Allotetraploid derivative <i>carthusiana</i>	Inferred diploid ancestor	Proposed diploid ancestor <i>tokyoensis</i>
A <i>Ald-1</i>	100	100	100	100	100	100	100
<i>Got-2</i>	100	100	100	100 (0.952) 136 (0.021) 115 (0.019) 77 (0.008)	100	100	75
<i>G6pd</i>	100	100	100	100	100	100	100
<i>Mdh2</i>	100	100	100	100 (0.998) 124 (0.001) 90 (0.0004)	100	100	100
<i>Mdh-4</i>	100	100	100	100 (0.982) 180 (0.014) 130 (0.003)	100	100	100
<i>Pgi-1</i>	100	100	100	100	100	100	100
B <i>Mdh-1</i>	100	125/100	125	125 (0.960) 100 (0.028) 150 (0.012)	125	125	100 80
<i>6Pgd-2</i>	100	100/0 (2-banded 100/96)	unknown	92 (0.979) 74 (0.012) 105 (0.005) 88 (0.002) 68 (0.002)	~105/92	~105	100
<i>Pgm-1</i>	100	100/105 100 105	105	105 (0.814) 98 (0.174) 110 (0.012)	105/98 98	98	100
<i>Pgm-2</i>	100	100/67	67	67 (0.956) 78 (0.013) 58 (0.021) 50 (0.007)	67	67	67
<i>Skdh</i>	100 114	100/112 100 100/88	112 88	100 (0.990) 90 (0.007) 114 (0.002)	100/95	95	100
<i>Tpi-1</i>	100 45	100 25 (0.001)	100	63 (0.999)	100/63	100	100
<i>Tpi-2</i>	100	99/0	99	55	100/99	99	100
C <i>Hk</i>	100	100/112 100/107	112 107	108 (0.664) 106 (0.333)	106/112	112	106
<i>Idh-1</i>	100	100/92 92	92	130 (0.889) 145 (0.106) 114 (0.003) 111 (0.0003)	130/92	92	100 114
<i>Lap-1</i>	100	100/83 83	83	103 (0.437) 93 (0.395) 98 (0.128) 105 (0.020) 100 (0.011) 90 (0.008) 95 (0.002) 85 (0.001)	103/83 103/80	83	100 91
<i>6Pgd-1</i>	100	100/88	88	100 (0.980) 125 (0.015) 88 (0.005)	100/88	88	100

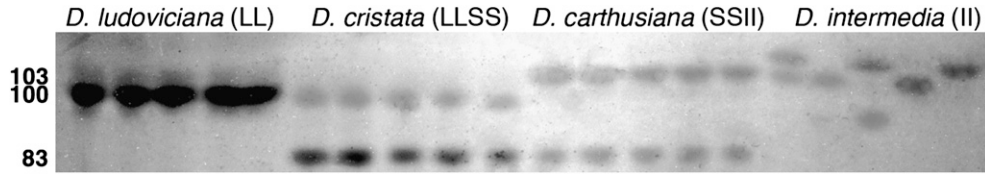


Fig. 2. Starch gel electrophoretic band patterns for the enzyme leucine amino peptidase (*Lap-1*) in *Dryopteris*. Lanes 1–5, *D. ludoviciana*; 6–10, *D. cristata*; 11–15, *D. carthusiana*; 16–20 *D. intermedia*. Both allotetraploids, *D. cristata* and *D. carthusiana*, exhibit a fixed heterozygous phenotype (two-banded because LAP is a monomer) for allozyme *Lap-1*⁸³, which they share, presumably contributed by *D. “semicristata”* and another allozyme contributed by their respective extant ancestors, *D. ludoviciana* and *D. intermedia*. *D. cristata* possesses *Lap-1*¹⁰⁰ of *D. ludoviciana* (more faintly expressed in *D. cristata*), while *D. carthusiana* possesses *Lap-1*¹⁰³ of *D. intermedia*. Polymorphisms for other allozymes (*Lap-1*¹⁰⁵ and *Lap-1*⁹³) are seen in *D. intermedia*, and some faint artifactual bands (ghosts) are visible in lanes 1–5 and are common for this enzyme. The brightness and contrast of the photograph were enhanced for publication.

1989). Species comparisons involving 17 different isozymes are shown for five taxa of *Dryopteris* in Table 1. The first set of isozymes (Table 1A) is identical for all species studied and thus is not informative in distinguishing members of the complex, but it is consistent with their classification as related members of a genus. The exception to uniformity is found in the enzyme *Got-2*, where the Asian *D. tokyoensis* has an isozyme that differs in mobility compared to the members of the North American complex examined.

The second set of enzymes (Table 1B) shows greater variation. However, for both *D. cristata* (case 1) and *D. carthusiana* (case 2), these loci have fixed allozymic heterozygosity (i.e., two bands representing the alleles inherited from each parent). The presence of two bands permits deduction of the allelic state of the unidentified contributor based on the known parent and the respective tetraploid. *Tpi-2* differs slightly in that the inferred diploid ancestor appears to have provided the same allele to both tetraploids; however, one parent (*D. intermedia*) contained an orphan (unique) allele rather than either of the alleles found in the tetraploid. Two loci, *Pgm-1* and *Skdh*, contain different alleles for the unidentified genome in case 1 compared with case 2. Such a result is consistent with polymorphism in populations of *D. intermedia* today and suggests that different alleles could have been involved in the recurring formation of the original hybrids.

The story is unambiguous for the four enzymes summarized in Table 1C. The data show (e.g., Fig. 2) that the deduced contributions of the missing diploid species (the B genome) based

on gene expression patterns are the same in both tetraploids, *D. cristata* and *D. carthusiana* (case 1 and case 2). For all four enzymes shown in Table 1C, the products of the B genome differ from the isozymes present in *D. ludoviciana*, *D. goldiana*, and *D. tokyoensis*. These results provide strong evidence for an unknown species, a taxon whose genes continue to be expressed in the extant tetraploids *D. cristata* and *D. carthusiana*.

We also used restriction enzyme studies of chloroplast DNA to test these hypotheses. All available evidence indicates that chloroplasts are inherited from the maternal parent in ferns (Whatley, 1982; Stein and Barrington, 1990; Gastony and Yatskievych, 1992). To examine all four previous models for the origin of the tetraploids, we included DNA from the two known diploid parents and their allotetraploid progeny as well as *D. tokyoensis*, *D. goldiana*, and another North American diploid, *D. marginalis*. Because each tetraploid might have inherited its chloroplasts from a previously identified parent, there was a one in four chance that both tetraploids would have a chloroplast genome from (1) *D. tokyoensis* or (2) *D. ludoviciana* or (3) an unknown progenitor, three possibilities consistent with the hypotheses of origin in Fig. 1. However, the potential for additional information about the allotetraploids made this an important component of our investigation.

In our analysis of chloroplast DNA restriction sites (Fig. 3A, B), we found 25 phylogenetically informative mutations for the seven *Dryopteris* species and outgroup taxa. Exhaustive Wagner parsimony searches using three species of *Phanerophlebia* as the outgroup taxa yielded two trees 48 steps long. The strict consensus tree is shown in Fig. 4. Searches using three species

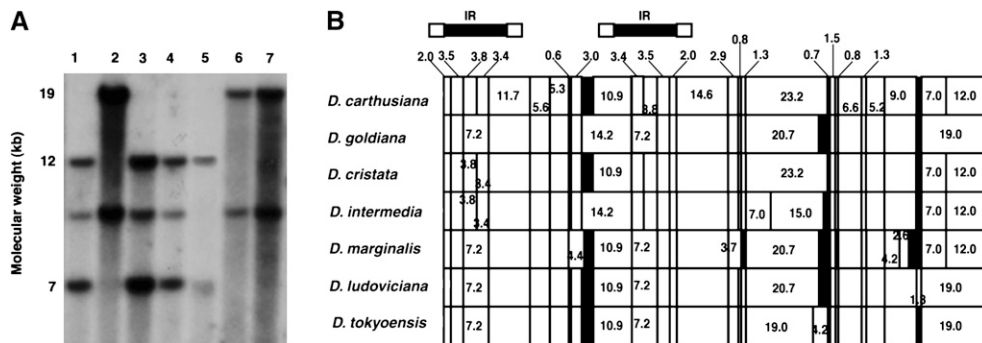


Fig. 3. Chloroplast DNA analysis of seven *Dryopteris* species. (A) Autoradiograph of Southern blot hybridization of ³²P-labeled lettuce chloroplast DNA fragments (4.6 + 5.4 + 6.3 kb) from the large single-copy region of the genome hybridized to total DNA cut with *PvuII*. Lanes 1, *D. carthusiana* (4n); 2, *D. goldiana* (2n); 3, *D. cristata* (4n); 4, *D. intermedia* (2n); 5, *D. marginalis* (2n); 6, *D. ludoviciana* (2n); 7, *D. tokyoensis* (2n). The photograph was computer enhanced for brightness and contrast for publication. (B) Linearized restriction site maps of the chloroplast DNA of seven *Dryopteris* species cut with *PvuII*.

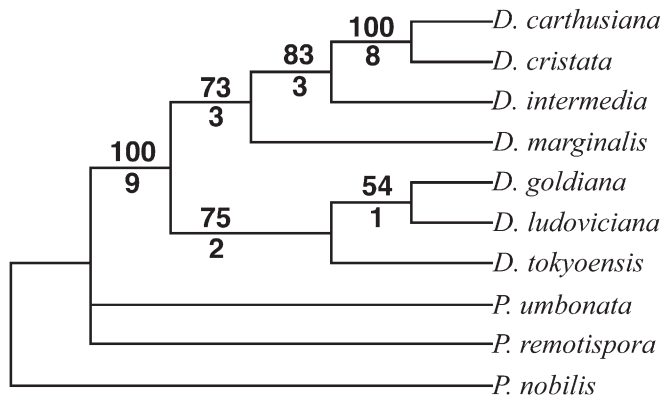


Fig. 4. Consensus tree of two trees 48 steps long from an exhaustive search in a Wagner parsimony analysis of 25 shared mutations, autapomorphies excluded. The consistency index was 0.83. Numbers above lines are bootstrap percentages (1000 replicates); those below lines are decay indices. The outgroup species were three members of *Phanerophlebia*, a genus in the sister group to *Dryopteris* based on *rbcL* sequence data (Little and Barrington, 2003). Restriction site data for the outgroup species were obtained by rescoring films from a previous study of the polystichoid ferns (Yatskievych et al., 1988).

of *Cyrtomium*, three species of *Polystichum*, or one species from each genus as outgroup taxa yielded either the same two most parsimonious trees or a single most parsimonious tree, which was identical to one of the two most parsimonious trees above. Bootstrap analysis for each of the alternative outgroups yielded confidence values that were closely similar to those reported in Fig. 4. The 100% bootstrap value for the clade containing *D. cristata* and *D. carthusiana* (Fig. 4) indicates that these chloroplast DNAs are most similar; decay analysis showed that only in trees eight steps longer would this clade be lost. A distance analysis (data not shown) reveals that among the phylogenetically informative mutations, there is no difference between these two taxa. This is somewhat misleading as a few autapomorphic differences between the chloroplast DNAs of *D. carthusiana* and *D. cristata* were found but not included in the phylogenetic analysis. Nevertheless, the near identity of the restriction sites present in the chloroplast DNAs of these two species supports two likely conclusions. First, both allotetraploid

species received their chloroplast DNA from the same maternal species, even though there was only a one in four chance that this would have occurred. Second, the parental species that contributed the chloroplast genome differs from all of the extant proposed candidates. Therefore, our results strongly support the 38-yr-old hypothesis that postulated the existence of *D. "semicristata,"* a species that has never been found either alive or as a fossil. Moreover, these data are congruent with DNA sequence data from one nuclear and 10 chloroplast genes (E. Sessa, T. Givnish, University of Wisconsin, and E. Zimmer, Smithsonian Institution, unpublished data).

Our conclusions based on molecular data also support earlier analyses based on features of leaf morphology (Werth and Kuhn, 1989), leaf trichomes (Viane, 1986), spore morphology (Britton, 1972), and phloroglucinols (Euw et al., 1980). Because the undiscovered diploid, *Dryopteris "semicristata,"* has been so well characterized through isozymes and chloroplast DNA, Werth and Lellinger (1992) proposed that the *International Code of Botanical Nomenclature* should include rules for naming, describing, and typifying such "genomically preserved plants." Fraser-Jenkins (2001), who prefers the name *Dryopteris stanley-walkerii* to *Dryopteris "semicristata,"* argued against using genetic profiles to typify species and has instead formally described the "missing ancestor" based on morphology alone.

Repeated hybridizations between the same two taxa provide the potential for either species to serve as the maternal donor of its cytoplasm (Stein and Barrington, 1990; Gastony and Yatskievych, 1992; Soltis and Soltis, 1993). Because the above analyses were based on DNA from individual plants, we examined eight individuals of *D. cristata* and 11 of *D. carthusiana* collected from diverse locations in the United States and Canada. These chloroplast DNA comparisons showed that in each case the chloroplast genome came from *D. "semicristata."* Thus, we have no unequivocal DNA evidence (i.e., chloroplast DNA contributed by *D. ludoviciana* or *D. intermedia*) to support multiple origins of these allotetraploid species as has been observed in other hybrid species (Stein and Barrington, 1990; Gastony and Yatskievych, 1992; Soltis and Soltis, 1993). We cannot rule out that different hybridization events involving different plants of *D. "semicristata"* might have occurred.

The small number of autapomorphies distinguishing *D. cristata* from *D. carthusiana* can be explained in two possible ways. The maternal progenitor, *D. "semicristata,"* may have been

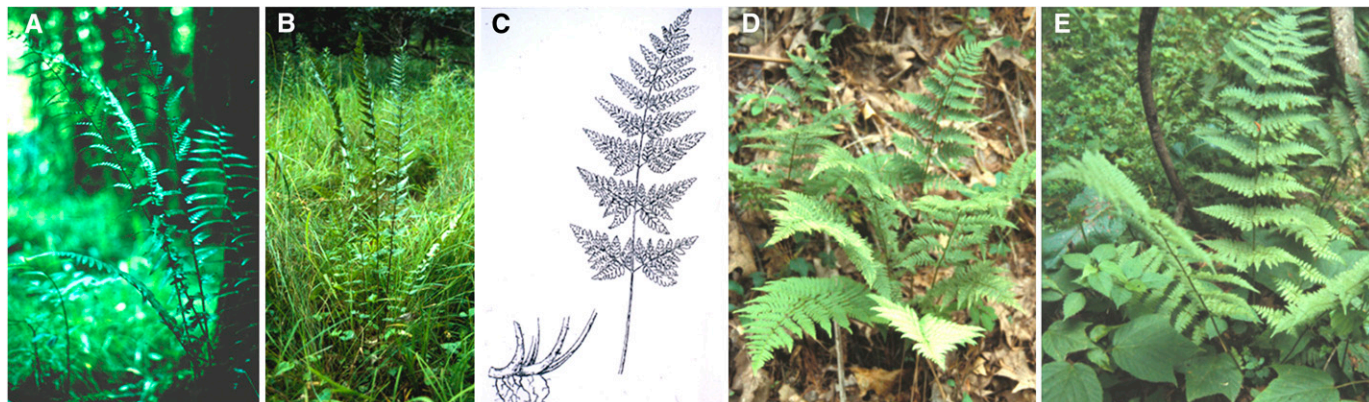


Fig. 5. Two *Dryopteris* allotetraploids and their diploid progenitors. (A) *D. ludoviciana*, (B) *D. cristata*, (C) *D. "semicristata,"* as reconstructed by morphometric study of Werth and Kuhn (1989), (D) *D. carthusiana*, (E) *D. intermedia*. *Dryopteris ludoviciana* and *D. "semicristata"* were the diploid parents of the allotetraploid *D. cristata*; *D. "semicristata"* and *D. intermedia* were the diploid parents of the allotetraploid *D. carthusiana*.

polymorphic and contributed two slightly different chloroplast genomes to the two hybrid offspring, *D. cristata* and *D. carthusiana*. The other possibility is that these differences resulted from mutations that have occurred since formation of the hybrids.

We can only speculate why *D. “semicristata”* has never been found alive or in fossil form. One possibility is indicated by the tree topology (Fig. 4) in which the *D. cristata* and *D. carthusiana* chloroplast genomes derived from *D. “semicristata,”* are shown to be most similar to those of *D. intermedia*. *Dryopteris intermedia* s.l. is variable both morphologically and isozymically (see Table 1, Fig. 2) and may still harbor undetected populations of *D. “semicristata.”* Another possibility is that field biologists searching for *D. “semicristata”* may have been using an ambiguous search image. As seen in Fig. 5A–E, *D. ludoviciana* (A) and *D. cristata* (B) have pinnate-pinnatifid leaves, while those of *D. carthusiana* (D) and *D. intermedia* (E) are 2–3-pinnate-pinnatifid. Morphometric comparisons of each known diploid with its derivative tetraploid species (Werth and Kuhn, 1989; Kuhn and Werth, 1990) predict that the missing diploid had leaves that were more dissected than *D. ludoviciana* and less dissected than *D. intermedia*, the laciest of all North American diploid wood ferns. However, *D. “semicristata”* was named for *D. cristata*, a pinnate-pinnatifid fern, and Wagner (1971) may have assumed they were similar. This assumption is most likely inaccurate, and the artist’s interpretation of *D. “semicristata”* (Fig. 5C), based on morphometric analyses (Werth and Kuhn, 1989; Kuhn and Werth, 1990), therefore depicts a partly dissected fern frond. Fraser-Jenkins (2001) has conducted his own analysis and species description. His drawing shows a still more highly dissected version of the missing parent than the leaf depicted in Fig. 5. No matter which illustration is closer to reality, both analyses could be interpreted to indicate that the reason this diploid has not been collected is that field biologists may have had the wrong search image. However, we think a more likely possibility is that this fern is extinct. Our research demonstrates that although probably no longer surviving as a living lineage, it is possible to discover molecular signatures supporting its previous existence.

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APPENDIX 1. Evidence for the missing diploid ancestor is derived mainly from the analysis of two diploid and two tetraploid species. (A) Geographic ranges of these taxa are provided to show that sampling of these taxa was over a wide part of their respective ranges. (B–D) A listing of the specific plant collections supplying material for (A) isozyme study, (B) chloroplast phylogeny, and (D) chloroplast population survey. Each species name is accompanied by a brief indication of the collection locale(s), collector(s), and the collection number(s) (collections without numbers indicated as s.n.). Acronym of the herbarium housing voucher specimens is at the end of each section or line. Herbarium acronyms: FLAS, Florida Museum of Natural History; IND, Indiana University; TTC, Texas Tech University.

A) **Taxon**—Geographic range

- D. intermedia*—Newfoundland, south to Georgia, west to Minnesota and Arkansas
- D. ludoviciana*—Florida, west to Texas, north to Kentucky and North Carolina
- D. carthusiana*—circumboreal, south to South Carolina, Tennessee, and Arkansas, and in the western US, entering western Montana, northern Idaho, and Washington
- D. cristata*—Newfoundland, west to Saskatchewan and British Columbia, south to North Carolina, Tennessee, Iowa, Nebraska, and Idaho

B) **Taxon**—Collection locale, Collector, *Voucher* (No. individuals sampled from each population). Herbarium.

- Dryopteris ludoviciana* (Kunze) Small: **USA**: North Carolina; Chowan, *Werth 92JP* (60), and Brunswick, *Werth 85W* (5) Counties; South Carolina; Darlington County, *Werth 85X* (9); Florida; Alachua, *Werth 90O* (7), Putnam, *Werth 90P* (64), Sumter, *Werth 85FFF* (25), Dixie, *Werth 90R* (19), Gadsden, *Werth 85Y* (102), Leon, *Werth 85Z* (15), and Escambia, *Werth 85UWF* (44) Counties; Alabama; Crenshaw County, *Werth 92G* (28), 92H (25); Arkansas; Bradley County, *Werth 85AA* (19). TTC.
- D. intermedia* (Muhl.) A. Gray: **USA**: Virginia; Giles County, *Werth 86PD* (135), 90GG (78); West Virginia; Hampshire, *Werth 85HR* (162) and Preston, *Werth 85K* (130) Counties; Ohio; Hocking County, *Werth 83OMC* (63); New York; Green County, *Werth 90JJJ* (74); Vermont; Chittendon County, *Werth 89B* (36); Wisconsin; Waukesha County, *Werth 86WCT* (36); **Canada**: Ontario; Wellington County, *Werth 86V* (24). TTC.
- D. tokyoensis* (Matsum. & Makino) C. Chr.: **Netherlands**: Utrecht, from living collections cultivated by E. Hennipman, *Haufler 86EH*. (2). TTC.
- D. cristata* (L.) A. Gray: **USA**: Minnesota; Pine County, *Werth 89JJ* (4); Wisconsin; Waukesha County, *Werth 86WCT* (36); Vermont; Addison County, *Werth 89C* (5); Michigan; Washtenaw, *Werth 86U* (16) and Kalamazoo, *Werth 85P* (8) Counties; Pennsylvania; Luzerne, *Werth 86L* (23) and Chester, *Werth 86J* (11) Counties; Virginia; Giles County, *Werth 85LM* (38), and *Werth 90KF* (22); Tennessee; Johnson County, *Werth 86Z* (18); **Canada**: Ontario; *Werth 89X* (27). TTC.
- D. carthusiana* (Villars) H.P. Fuchs: **USA**: Minnesota; Cook County, *Werth 89HH* (5); Wisconsin, Waukesha County, *Werth 86WCT* (23); Michigan;

Washtenaw, *Werth 86U* (35) and Kalamazoo, *Werth 85P* (10) Counties; West Virginia; Hampshire County, *Werth 86O* (12); Virginia; Giles County, *Werth 85LM* (14), *86IN* (18), *85HB* (13); North Carolina; Gates County, *Werth 88E* (6); **Canada**: Ontario; Wellington County, *Werth 86V* (24), Muskoka, *Werth 89V* (6) and Sudbury, *Werth 89Z* (5) Districts; Quebec; Riviere Portneuf, *Werth 89H* (3); **Switzerland**: Murgthal, *Werth JSP1* (8), Hagendorf, *Werth JSP2* (2), and Zurich, *Werth JSP3* (9); **Germany**: Berlin, *Werth 87BE* (6). TTC.

C) **Chloroplast phylogeny**

- D. carthusiana* (Villars) H.P. Fuchs: **USA**: Indiana, *Yatskievych 86-118*. IND.
- D. cristata* (L.) A. Gray: **USA**: Indiana, *Yatskievych 86-117*. IND.
- D. goldiana* (Hook.) A. Gray: **USA**: Indiana, *Yatskievych 86-114*. IND.
- D. intermedia* (Muhl.) A. Gray: **USA**: Indiana, *Yatskievych 86-115*. IND.
- D. ludoviciana* (Kunze) Small: **USA**: Florida; Leon, *Orzell s.n.*, and Alachua, Perkins 983 Counties. FLAS.
- D. marginalis* (L.) A. Gray: **USA**: Indiana, *Yatskievych 86-116*. IND.
- D. tokyoensis* (Matsum. & Makino) C. Chr.: **USA**: New York; Living Collection, Mickel. NYBG.
- Phanerophlebia nobilis* (Schlecht. & Cham.) Presl: **Mexico**, *Yatskievych 85-211*. IND.
- P. remotispora* Fourn.: **Mexico**, *Yatskievych 83-158*. IND.
- P. umbonata* Underw.: **Mexico**, *Yatskievych 83-87*. IND.

D) **Chloroplast population survey**

- D. carthusiana* (Villars) H.P. Fuchs: **USA**: Indiana; Brown County, *Werth s.n.*; Quebec, Canada; Duplessis, *Werth 89J*, *89H*; Virginia; Giles County, *Werth 85LM*, *86IN*, *85HB*, Massachusetts; Leverett, *Werth s.n.*; Minnesota; Pine County, *Werth 89II*, Michigan; Washtenaw County, *Werth 86U*; **Canada**: Ontario; Muskoka District, *Werth 89V*. TTC.
- D. cristata* (L.) A. Gray: **USA**: Virginia; Giles County, *Werth 85LM*, *90KF*; Indiana; Owen County, *Werth s.n.*; Minnesota; Pine County, *Werth 89JJ*; Michigan; Washtenaw County, *Werth 86U*; **Canada**: Quebec; Duplessis, *Werth s.n.* TTC.