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Isozyme Studies on the *Dryopteris "spinulosa"* Complex, I: The Origin of the Log Fern *Dryopteris celsa*

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ABSTRACT. Most of the species in the genus *Dryopteris* of eastern North America, as well as several European species, are implicated in an intricate hybrid/polyploid complex referred to here as the *D. "spinulosa"* complex. Conflicting hypotheses have been proposed to account for the ancestry of the allopolyploid species of this complex: 1) the *"semicristata"* scheme invokes a hypothetical diploid, putatatively extinct, as an ancestor of certain alloploids; 2) the reinterpretation scheme interprets all all alloploids as derived from extant diploids. To test these hypotheses, a comparative study of isozymes among the species comprising the *D. "spinulosa"* complex has been undertaken. The present paper reports evidence relative to the origin of the allotetraploid *D. celsa*, a widespread but locally occurring wetland species. The parentage of *D. celsa* has been alternatively hypothesized as either *D. ludoviciana* × *goldiana* (*"semicristata"* scheme) or *D. ludoviciana* × *marginalis* (reinterpretation scheme). Isozyme patterns were compared for 12 enzymes coded by 19 loci resolved and interpreted across the four relevant species. The inferred isozyme genotype of all *D. celsa* individuals examined was identical, with rare exceptions, and was homozygous for 13 loci and fixed heterozygous for seven loci. Under the assumption that *D. ludoviciana* is one ancestor of *D. celsa* (as both hypotheses agree), all loci were consistent with the hypothesis that *D. goldiana* was the other ancestor, whereas only 12 were in any way consistent with ancestry by *D. marginalis*. The assumption of ancestry by *D. ludoviciana* was supported by all loci except *Pgi-2*, where *D. celsa* is fixed for an allele not detected in any of the putative parents. While the origin of this orphan allele is uncertain, its fixation in *D. celsa* suggests that this allopolyploid species has had a unique origin, in contrast to the multiple origins inferred for other allopolyploid species. This study has provided unequivocal evidence that the ancestry of *D. celsa* is *D. goldiana* × *ludoviciana*, a result that substantially strengthens the plausibility of the *"semicristata"* scheme.

The woodfern genus, *Dryopteris*, has posed one of the more challenging evolutionary puzzles among temperate plants. Species of this genus are often conspicuous components of temperate ecosystems owing to their generally large size and their tendency to form large populations in woodlands and swamps. Interspecific hybridization is common, including almost every combination of species pairs with overlapping ranges (Wagner 1971; table 1). Hybridization, coupled with polyploidy, has played a very significant role in speciation within *Dryopteris*. Manton (1950) first discovered that all three British elements of the *D. "spinulosa"* complex [*D. carthusiana* (Vill.) H. P. Fuchs {=*D. spinulosa* (Mueller) Watt}, *D. cristata* (L.) A. Gray, and *D. austriaca* (Jacq.) Schinz & Thell. {=*D. dilatata* (Hoffmann) A. Gray}] were tetraploid ($2n = 164$ based on $\chi = 41$) and that hybrids between these species were sterile due to meiotic irregularities. (*D. "spinulosa"* is used here in the broadest sense to refer to all members of the reticulate species complex diagrammed in fig. 1.)

The numerous hybrids that occur in *Dryopteris* have subsequently provided a wealth of information regarding genome homologies (table 1). For example, studies of chromosome pairing in a number of taxa by Manton and Walker (1953) and Walker (1962, 1969) established that the southeastern U.S. endemic, *D. ludoviciana* (Kunze) Small, was a key element in the evolution of the complex, having contributed a diploid genome to three of the polyploid taxa, tetraploids *D. cristata* and *D. celsa* (W. Palmer) Small and the hexaploid *D. clintoniana* (D. C. Eaton) Dowell. The studies of Walker as well as those of Wagner and his colleagues were synthesized by Wagner (1971) as the hypothetical scheme of reticulate evolution in eastern North American *Dryopteris* presented in figure 1A. Central to this scheme is the postulation of a hypothetical ancestral diploid, *D. "semicristata,"* as a progenitor of both *D. cristata* and *D. carthusiana* based on the occurrence of 41 bivalents in the hybrid between these two tetraploids. This scheme will henceforth be referred to as the *"semicristata"* scheme.

An alternative hypothesis of relationships was

TABLE 1. Chromosome pairing behavior (pairs = II, univalents = I) in interspecific hybrids of *Dryopteris* (χ = 41). Compiled from information in Gibby 1977; Hickok and Klekowski 1975; and Wagner 1971.

	× *campyloptera*	× *intermedia*	× *carthusiana*	× *cristata*	× *ludoviciana*	× *celsa*	× *goldiana*	× *clintoniana*	× *marginalis*
expansa	35–40 II 43–53 I	1–6 II 70–80 I	0–8 II 107–123 I	—	—	—	—	—	0 II 82 I
campyloptera		41–45 II 34–41 I	—	—	—	—	—	—	0–3 II 117–123 I
intermedia			39–41 II 41–46 I	0–5 II 113–123 I	—	0–7 II 109–119 I	0–1 II 80–82 I	1–19 II 126–162 I	0–4 II 4–83 I
carthusiana				40 II 84 I	—	—	—	40–65 II 75–125 I	0–8 II 107–123 I
cristata					41 II 41 I	35–40 II 83–90 I	—	38–83 II 39–129 I	0 II 123 I
ludoviciana						40–44 II 39–44 I	—	—	—
celsa							39–42 II 39–44 I	—	2–17 II 89–123 I
goldiana								36–42 II 80–91 I	0–6 II 70–82 I
clintoniana									0–11 II 142–163 I

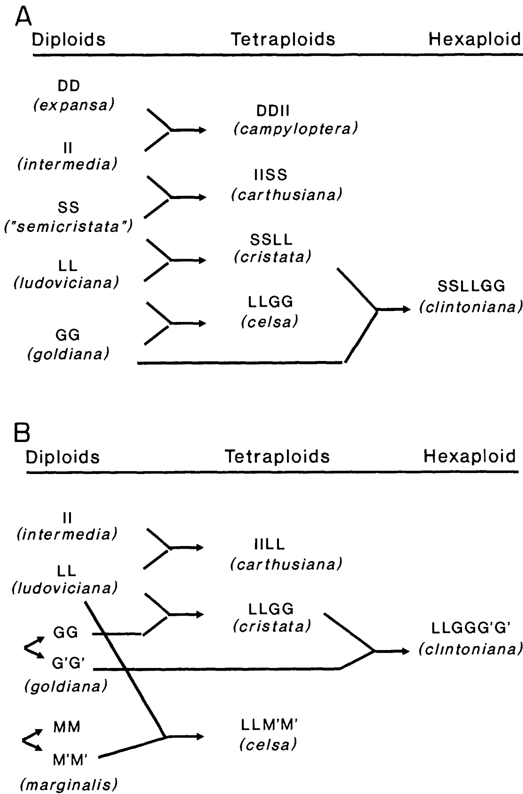

FIG. 1. Diagrams of hypothesized relationships in *Dryopteris* of eastern North America. A. The *"semicristata"* scheme as hypothesized by Walker (1969) and Wagner (1971). B. The reinterpretation scheme of Hickok and Klekowski (1975). Divergence within genomes indicated by ('). (Ancestry of *D. campyloptera* as *D. expansa* × *intermedia* was not contested in the reinterpretation.)

proposed by Hickok and Klekowski (1975; fig. 1B) based on, and consistent with, essentially the same set of cytological observations reviewed by Wagner (1971). This scheme, referred to hereafter as the reinterpretation scheme, proposes that all of the allopolyploids arose from extant diploids, thus eliminating the need to postulate a hypothetical diploid ancestor. However, the reinterpretation scheme cannot be considered more parsimonious than the *"semicristata"* scheme, because infraspecific divergence of two of the diploid genomes (perhaps through acquisition of pairing factors) had to be invoked to account for lack of multivalent formation when these genomes are present in more than two doses (Hickok and Klekowski 1975).

In addition to cytological observations, evidence from gross morphology (Wagner 1971), trichome morphology (Viane 1986), spore morphology (Britton 1972a, 1972b, 1974), and secondary compounds (reviewed by Euw et al. 1980) have been obtained. In general, these sources of evidence provide some support for the *"semicristata"* scheme and relatively little support for the reinterpretation scheme. For example, the mutual possession of the compounds desaspidin and trisdesaspidin by *D. cristata* and *D. carthusiana* support the hypothesis of a common ancestor (*D. "semicristata"*) for these allotetraploids (Widen et al. 1975). However, some aspects of morphology are not consistent with either hypothesis (cf. Wagner 1971), and at present neither hypothesis can be said to be unequivocally supported or discounted.

The use of isozymes to test hypotheses in reticulately evolved complexes has often proved valuable, for example in the angiosperm genus *Tragopogon* (Roose and Gottlieb 1976), in the moss genus *Plagiomnium* (Wyatt et al. 1989), and in various fern genera (see Werth 1989 for review). In these studies, marker alleles were detected that allowed the contribution of the diploid parents to be recognized in their allopolyploid derivatives. Furthermore, in many of these studies an insight beyond parentage was obtained: the allopolyploids were found to vary at those loci that were polymorphic in the diploids, indicating that their origin was polytopic (Haufler et al.; Werth et al. 1985b; Wyatt et al. 1989). As increasing numbers of allopolyploid taxa are scrutinized using isozymes, it appears that multiple origins may be the general case for species derived through this mode.

As a contribution toward clarifying the relationships in the *Dryopteris "spinulosa"* complex, a comparative isozymic study of all species hypothesized to comprise the complex has been initiated. In the present paper I report evidence concerning the ancestry of the North American endemic tetraploid *D. celsa*, a locally occurring wetland species whose greatest abundance is in and near the Dismal Swamp of Virginia and North Carolina (Carlson and Wagner 1982). It ranges widely across the northern part of the southeastern U.S. and occurs as a disjunct in the Great Lakes region (fig. 2A). As first hypothesized by Walker (1962), one of the diploid genomes is widely believed to be derived from *D. ludoviciana*, also a swamp-inhabiting species oc-

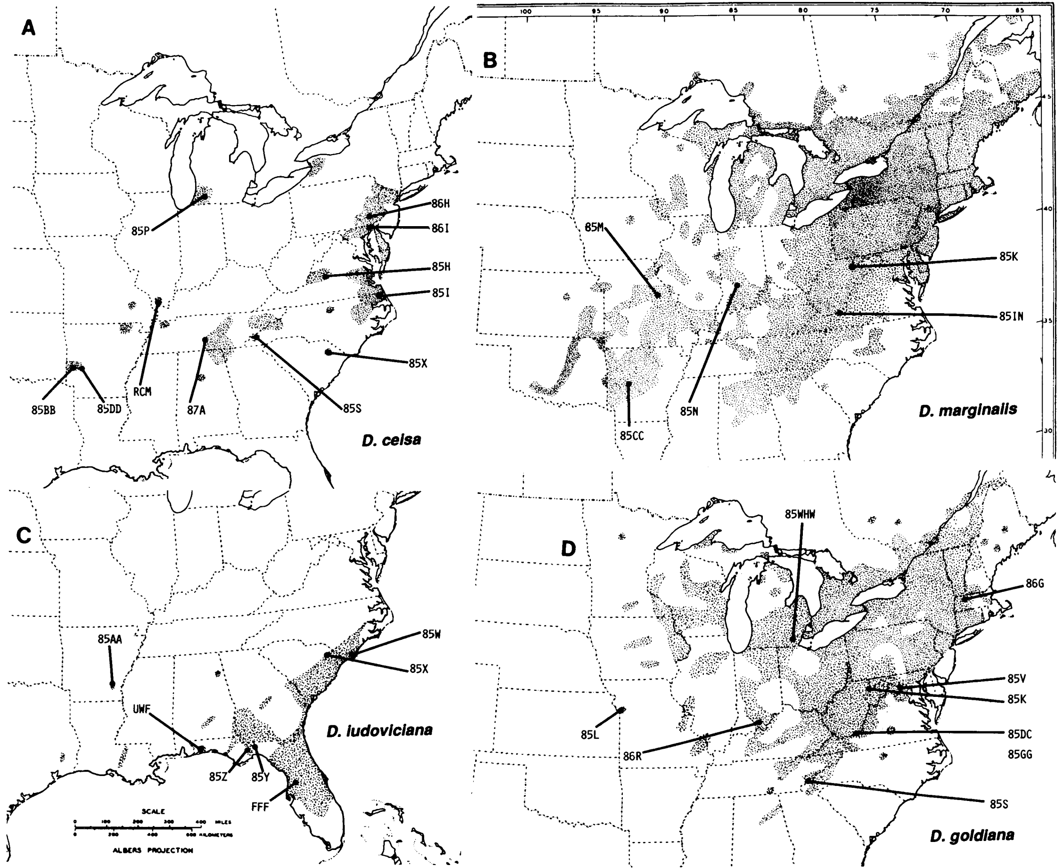

FIG. 2. Distribution of the four *Dryopteris* species studied, with localities sampled indicated (see table 2).

curring principally in the southernmost part of the Atlantic and Gulf coastal plains, with disjunctions in southern Arkansas (Peck et al. 1985) and southeastern Texas (Correll 1972) (fig. 2C). The source of the second diploid genome is disputed as being either *D. goldiana* (Hook.) A. Gray (Wagner 1971), a somewhat uncommon species occurring in rich, moist, wooded ravines and coves over much of eastern North America (fig. 2D), or *D. marginalis* (L.) A. Gray (Hickok and Klekowski 1975), the most abundant *Dryopteris* species in eastern North America (fig. 2B), occurring in a variety of habitats, most commonly talus slopes and cliffs.

MATERIALS AND METHODS

Four species of *Dryopteris* were compared in this study: *D. ludoviciana, D. goldiana, D. marginalis,* and *D. celsa*. Representative populations were sampled over a wide portion of each species' range (table 2; fig. 2). Voucher specimens are at KANU. To insure that the sample was representative, the extent of each population was first assessed, and then samples were taken at regularly paced intervals, usually along a transect. Vigorous vegetative reproduction by horizontal rhizomes in *D. ludoviciana* and *D. celsa* made recognition of individuals belonging to the same vegetative lineage (genets) difficult or impossible. Samples from extensive colonies of these species were taken from evenly spaced crowns (ramets). The lack of genetic variation within and among most populations of these species (see results) made this a consideration of little importance. *Dryopteris goldiana* also has a horizontal rhizome, but vegetative propagation appears to be limited to at most a few meters. To insure sampling of separate genets, only one sample was taken from among clustered

TABLE 2. Collection localities for populations of *Dryopteris* species sampled in this study.

Species and population designation	Locality and habitat (sample sizes in parentheses)
D. ludoviciana	
85W	North Carolina: Brunswick Co., swamp along US 17, 3 mi N of Supply (5)
85X	South Carolina: Darlington Co., Swift Creek swamp, near junction of routes 13 and 151, W of Darlington (9)
85AA	Arkansas: Bradley Co., cypress swamp adjacent to Warren Prairie Scientific Area, between Warren and Monticello 1 mi S of state route 4 (19)
FFF	Florida: Sumter Co., swamp along Withlacoochee River at crossing of state route 50, SW of Tarrytown (25)
85Z	Florida: Leon Co., Mesic wooded slope in Tallahassee at junction of Timberlane Rd. and Timberlane School Rd. (15)
85Y	Florida: Gadsden Co., swamp, tributary of Ochlockonee River, along state route 267 N of junction with US 20 (102)
UWF	Florida: Escambia Co., swamp on the campus of the University of West Florida, Pensacola (45)
D. goldiana	
86G	Massachussetts: Franklin Co., mesic slope of Mt. Toby W of state route 63, S of junction with state route 47 (20)
85WHW	Michigan: Washtenaw Co., Sharon Township, E of state route 52, 6 mi S of Chelsea (36)
85L	Missouri: Clay Co., rich wooded ravine at Hidden Valley Park, 1 mi SW of junction of I-435 and US 69 (49)
86R	Illinois: Williamson Co., ravine near Devil's Kitchen Lake (20)
85K	West Virginia: Preston Co., cove along US 50 near Erwin (50)
85V	Virginia: Loudon Co., north facing mesic ravine S of Bluemont and W of state route 601 (18)
85DC	Virginia: Giles Co., mesic ravine at head of Doe Creek, N of route 613, on Beanfield Mt. near Mountain Lake (50)
85GG	Virginia: Giles Co., "Goldie's Gulch," mesic ravine on northwest slope of Potts Mt. along route 613 (5)
85S	North Carolina: Swain Co., mesic ravine along trail from Big Creek Campground to Newfound Gap, Smoky Mountain National Park (20)
D. marginalis	
85K	West Virginia: Preston Co., rich cove along US 50 between Aurora and Erwin (22)
85N	Indiana: Monroe Co., mesic ravine near Bloomington (57)
85M	Missouri: Montgomery Co., Graham Cave State Park, sandstone ledge near park rd. (47)
85IN	Virginia: Giles Co., mesic ravine opposite park at Interior along state route 635 (22)
85CC	Arkansas: Garland Co., wooded talus slope near entrance to Charleton Campground, N side of US 27 (47)
D. celsa	
85P	Michigan: Kalamazoo Co., swamp along Howerfield Creek 5 mi W of Schoolcraft (9)
86H	Pennsylvania: Berks Co., springy slope 3 mi N of Morgantown, 1 mi W of state route 10 (40)
86I	Maryland: Harford Co., Susquehanna River State Park, wooded slope near Conowingo (12)
85H	Virginia: Albemarle Co., streamside on Greene Mt., 2 mi NW of Keene, near junction of 712 with 627 (14)
RCM	Illinois: Johnson Co., rocky wooded ravine near Heron Pond (7)
85X	South Carolina: Darlington Co., Swift Creek Swamp, near junction of routes 13 and 151, W of Darlington (22)
85BB	Arkansas: Montgomery Co., seep along Caney Creek below forest service rd. S of state route 8 (40)
85DD	Arkansas: Garland Co., along Meyers Creek, 5 mi. S of US 270 (25)
85I	North Carolina: Gates Co., Dismal Swamp along US 158 near Sunbury (45)
85S	North Carolina: Swain Co., swamp at Big Creek Campground, Smoky Mountain National Park (19)
87A	Tennessee: Hickman Co., wooded ravine SE of Centerville (15)

ramets. Rhizomes of *D. marginalis* are erect with only short ramifications, and recognition of individual genets in this species presented no problem.

At some sites (85P, 85X, 86I) collection was complicated by the presence of large numbers of hybrids. An attempt was made to keep collections of hybrids separated from the samples of parental species; however, hybrids were easily recognizable in the field only if they possessed fully developed fertile leaves. Therefore, population samples from these localities became "contaminated" with hybrids. Fortunately, comparison of isozyme patterns of known hybrids with those of parental species revealed that species-specific isozyme markers could be used to recognize inadvertent collections of small hybrid plants or leaves (e.g., Werth et al. 1988). Data from hybrid individuals are omitted from the present paper and will be treated separately.

Samples consisted of whole plants or detached leaves. Where possible, sporulating leaves were obtained for studies on inheritance and reproductive biology. Collections were kept refrigerated in individual Ziploc sandwich bags. Viability of tissues and enzyme activity in detached leaves were maintained for a number of weeks in all species, although considerable variation was observed among species for this capacity, apparently correlated with blade thickness. The leaves of the strongly deciduous *D. goldiana* deteriorated after a few weeks, while those of the strongly wintergreen *D. ludoviciana* and *D. marginalis* lasted for months provided there was air in the bags.

Homogenates were prepared by grinding leaf tissue in the "phosphate extraction buffer" (Soltis et al. 1983) enriched with 5% polyvinylpyrrolidone (mw 40,000) and five drops 2-mercaptoethanol per 10 ml buffer. Homogenates were routinely prepared a day in advance of electrophoresis and frozen overnight at −80°C. Comparison of frozen samples with freshly ground samples showed no appreciable loss of enzyme activity or resolution for most loci. Homogenates were absorbed onto rectangular filter paper wicks (Whatman 3 mm), which were placed in origin slits of horizontal 12% starch gels. Gels were subjected to electrophoresis until a bromphenol blue dye marker had migrated 10 cm, then sliced repeatedly in a horizontal plane, and each slice stained for an enzyme using the recipes in Werth (1985) except for HK in which the stain buffer was modified to pH 7.0 (T. Ranker, pers. comm.) and TPI which was stained using agarose as in Soltis et al. (1983).

The following electrophoretic buffer systems were employed for the enzymes indicated: discontinuous tris-citrate/lithium borate ("lithium hydroxide" of Selander et al. 1971): leucine aminopeptidase (LAP), hexokinase (HK), triosephosphate isomerase (TPI), glutamate-oxaloacetate transaminase (GOT); discontinuous tris-citrate/sodium-borate (no. 6 of Soltis et al. 1983): phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), TPI, LAP, HK; continuous tris-citrate pH 8.0 (Selander et al. 1971): glucose-6-phosphate dehydrogenase (G-6PDH), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME), 6-phosphogluconate dehydrogenase (6-PGD), shikimate dehydrogenase (SKDH); 0.005M histidine/sodium-citrate (no. 11 of Soltis et al. 1983): aldolase (ALD) IDH, MDH, 6-PGD; morpholine-citrate (electrode: 0.04 M citric acid titrated to pH 7.5 using n(3-aminopropyl)morpholine; gel: 1:19 dilution of electrode; M. D. Windham (pers. comm.): IDH, MDH, 6-PGD, SKDH. Staining of the same enzyme on different buffers was often helpful in interpretation, especially for the complex patterns seen in MDH and TPI, and also provided a partial check against hidden heterogeneity.

Interpretation of electrophoretic banding patterns as representing the products of alleles (allozymes) expressed by individual gene loci followed standard practices. Isozyme phenotypes for the loci reported are comparable to those detected in other ferns in appearance, in inferred number of loci and in subunit composition (reviewed by Haufler and Soltis 1986). Due to the large number of alleles detected at a number of loci in *D. marginalis*, numerous comparisons were necessitated to establish identity and relative mobility of allozymes. Where possible, these comparisons were made using more than one electrophoretic buffer system. A few of the rarer alleles occurred in individuals that perished before such comparisons could be carried out. These rare *D. marginalis* alleles were conservatively assumed to be identical to those encoding other similarly migrating allozymes in *D. ludoviciana* or *D. goldiana* unless there was overwhelming evidence to the contrary.

Loci were numbered starting with the most anodal in *D. ludoviciana* being designated locus "1," the next locus "2" and so on. Alleles were

named using relative mobilities of the allozymes they encode. The most common allele of *D. ludoviciana* was assigned a mobility value of 100, and all other alleles were assigned numbers representing the percent anodal migration of the 100 allele. All enzymes studied migrated anodally except a single allozyme of *Pgi-2* in *D. marginalis*, which migrated close to the origin, sometimes slightly anodally, sometimes slightly cathodally. This allele was assigned a relative mobility of 0.

Results

Interpretation of Isozyme Phenotypes. As many as 15 enzymes, apparently encoded by 23 loci, were resolved in an individual species, but of these only 12 enzymes encoded by 19 loci were consistently interpretable across all species studied and could be used for interspecific comparison. Band patterns in *Dryopteris* were comparable to those seen in other leptosporangiate ferns (fig. 3). Information relative to interpretation and variability at each enzyme follows.

ALD: A single well resolved ALD band was observed for all individuals of *D. ludoviciana*, *D. celsa*, and *D. goldiana*. In *D. marginalis* variation was seen for individuals with the same phenotype as the other three species, a slower well resolved band interpreted as representing an allele (Ald^{80}), and wide less-resolved bands overlapping the position of the fast and slow phenotypes. This latter phenotype is interpreted as heterozygous; because ALD is a tetrameric enzyme (Gottlieb 1982), heterozygotes would be expected to exhibit five bands, but there is insufficient separation of the two allozymes for resolution of five bands.

GOT: This enzyme was observed on both the lithium hydroxide and tris-citrate pH 8.0 electrophoretic systems. Activity was superior on tris-citrate, but resolution was considerably greater on lithium hydroxide. Two GOT bands, interpreted as representing separate loci (*Got-1* and *Got-2*), were evident. The more anodal *Got-1* band was always much fainter and often did not appear at all, and, thus, was not considered scorable. Nearly all individuals exhibited a single well resolved *Got-2* band of identical migration. A few individuals of *D. ludoviciana* and *D. marginalis* exhibited rare variant allozymes; these usually combined with the more frequent allozyme to form a three-banded phenotype, interpretable as heterozygous for this dimeric enzyme.

HK: A single HK locus was observed, best resolved on lithium hydroxide electrophoretic buffer but also scorable on system 6. Because of initial difficulties in visualizing this enzyme (corrected by using a stain buffer of pH 7.0—see methods), fewer individuals were scored for HK than for most other enzymes. Both *D. ludoviciana* and *D. goldiana* appeared to be fixed for alleles Hk^{100} and Hk^{106}, respectively. *Dryopteris marginalis* was nearly fixed for allele Hk^{108}; a single individual appeared homozygous for Hk^{106}. *Dryopteris celsa* appeared fixed for a two-banded heterozygous phenotype (HK is a monomer) for alleles Hk^{100} and Hk^{106}.

IDH: Two IDH loci were resolved. Resolution of *Idh-1* was better on electrophoretic system 11 and morpholine than on tris-citrate pH 8.0, but separation of allozymes was greater on the latter. Phenotypes were consistent between both systems. *Dryopteris ludoviciana*, *D. goldiana*, and *D. celsa* shared allele $Idh\text{-}1^{100}$, while *D. marginalis* possesses three other alleles. A few individuals of *D. celsa* from the Gates County, North Carolina site in the Dismal Swamp exhibited an heterozygous IDH phenotype interpreted as $Idh\text{-}1^{100/85}$ (fig. 3).

Idh-2 was consistently resolved only on TC 8.0. The $Idh\text{-}2^{100}$ allele fixed in *D. ludoviciana* was poorly but consistently separated from the $Idh\text{-}2^{95}$ allele shared by *D. goldiana* and *D. marginalis*. The $Idh\text{-}2^{95}$ allele was fixed in *D. goldiana*, while *D. marginalis* possessed two additional alleles in low frequencies. *Dryopteris celsa* exhibited a wide *Idh-2* band overlapping the positions of $Idh\text{-}2^{100}$ and $Idh\text{-}2^{95}$, interpreted as fixed heterozygosity for both alleles.

LAP: As with most ferns studied to date, only one LAP locus, *Lap-1*, could be scored consistently, although a second more cathodal and fainter enzyme (*Lap-2*) was observed on some gels. *Dryopteris ludoviciana* and *D. goldiana* were fixed for alleles $Lap\text{-}1^{100}$ and $Lap\text{-}1^{107}$, respectively. *Dryopteris marginalis* exhibited polymorphism at this locus, and possessed five alleles, including $Lap\text{-}1^{100}$ and $Lap\text{-}1^{107}$ as rare alleles. Heterozygotes in *D. marginalis* were two-banded as expected for this monomeric enzyme. *Dryopteris celsa* was fixed for a two-banded heterozygous phenotype for alleles $Lap\text{-}1^{100}$ and $Lap\text{-}1^{107}$ (fig. 3).

MDH: In ferns, as in flowering plants, MDH

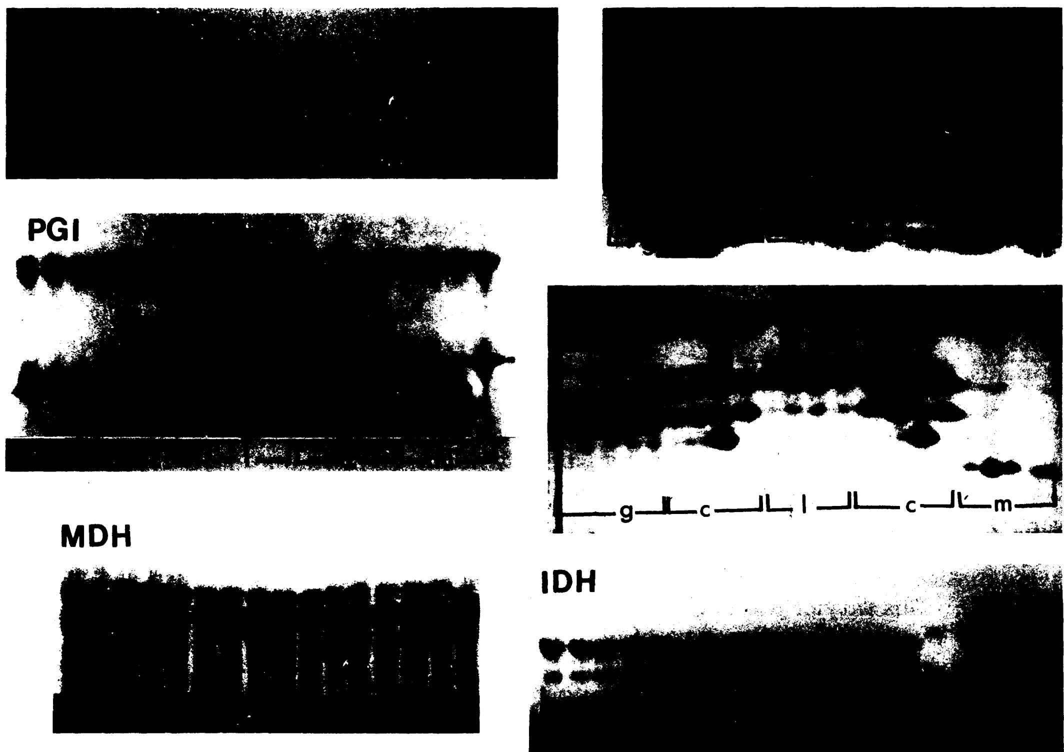

FIG. 3. Photographs of *Dryopteris* isozyme gels. Enzyme illustrated and species run in each lane are indicated for each photograph (see methods for enzyme abbreviations). Species abbreviations: G = *D. goldiana;* L = *D. ludoviciana;* C = *D. celsa;* M = *D. marginalis.* Anode is toward upper side of each photograph. LAP (stained on lithium hydroxide): *D. celsa* shows two-banded fixed heterozygous phenotype combining Lap^{107} and Lap^{100} allozymes of *D. goldiana* and *D. ludoviciana,* respectively. PGI (stained on system 6): *Pgi-1* shows occasionally-seen two-banded phenotype, the upper band apparently shared by all four species; *Pgi-2* of *D. celsa* has fixed heterozygous three-banded phenotype combining $Pgi\text{-}2^{45}$ allozyme of *D. goldiana* and orphan allozyme $Pgi\text{-}2^{115}$ (faster migrating than $Pgi\text{-}2^{100}$ allozyme of *D. ludoviciana*); *D. marginalis* exhibits three allozymes ($Pgi\text{-}2^{75}$, ^{45}, & ^{0}). MDH (stained on tris-citrate): the three fastest bands seen in all three taxa represent *Mdh-1, Mdh-2,* and *Mdh-4; D. marginalis* differs from *D. goldiana* and *D. celsa* in its slower *Mdh-1* allozyme. TPI (stained on system 6): *Tpi-2,* the darkest band about midway across the gel, is monomorphic across all individuals illustrated; *Tpi-1* is represented by a triplet of bands of various migration; *D. celsa* combines allozymes $Tpi\text{-}1^{63}$ of *D. goldiana* and $Tpi\text{-}1^{100}$ of *D. ludoviciana; D. ludoviciana* exhibits segregation for allozymes $Tpi\text{-}1^{100}$ and $Tpi\text{-}1^{45}$ (the third from the left individual of this species is a heterozygote); all *D. marginalis* individuals illustrated are homozygous for $Tpi\text{-}1^{25}$. PGM: *D. celsa* shows a two-banded fixed heterozygous phenotype $Pgm\text{-}1^{100/95}$ combining allozymes of *D. ludoviciana* and *D. goldiana; Pgm-2* phenotype of *D. celsa* also combines allozymes fixed in *D. ludoviciana* and *D. goldiana,* with the exception of an individual from Johnson Co., Illinois (third *D. celsa* from left in each set of four), that possesses orphan allozyme $Pgm\text{-}2^{57}$. IDH (stained on tris-citrate): *D. celsa* is homozygous for $Idh\text{-}1^{100}$, the allele fixed in both *D. ludoviciana* and *D. goldiana* with the exception of three individuals (sixth–eighth from left *D. celsa* lanes) from Gates Co., North Carolina, that are heterozygous for $Idh\text{-}1^{100/85}$; heterozygous phenotype of *D. celsa* for *Idh-2* is not apparent on this gel, but was consistently visualized on runs that separated the two allozymes of *D. goldiana* and *D. ludoviciana.*

is a multilocus enzyme system (fig. 3), and consequently its interpretation is challenging. Proof of genetic interpretation requires study of progeny arrays derived from individuals that are putatively heterozygous for more than one MDH locus. Unfortunately, such individuals were not available due to the low level of polymorphism observed at all of the putative MDH loci. Therefore, the interpretation of MDH in *Dryopteris* is based on consistent patterns ob-

served in interspecific comparisons made on three electrophoretic systems (tris-citrate, histidine-citrate, and morpholine), and on similarities observed in comparison to other fern genera, particularly *Asplenium* (see fig. 3 in Werth et al. 1985a). As in *Asplenium*, MDH in *Dryopteris* appears to be coded by five loci, and locus numbers for *Dryopteris* were assigned so as to be consistent with those in *Asplenium*. This interpretation was supported by the observation of occasional putative heterozygotes for rare alleles in *D. marginalis* and *D. ludoviciana*. In these variants, three-banded patterns were observed for putative loci *Mdh-2* and *Mdh-4*, which were otherwise represented by single bands. Since the remainder of the MDH phenotype was unaffected in these individuals, the independent coding of these putative loci is supported. Three of the MDH loci (*Mdh-1*, *Mdh-2*, and *Mdh-4*) were considered consistently interpretable.

6-PGD: This enzyme was interpreted as encoded by two loci. The phenotype of *6Pgd-2*, best resolved on tris-citrate and morpholine, was monomorphic for a single band of identical mobility (*6Pgd-2*^{100}) in *D. ludoviciana*, *D. goldiana*, and *D. celsa*. In *D. marginalis*, *6Pgd-2* was variable for four allozymes readily interpreted as single-banded homozygotes and three-banded heterozygotes for this dimeric enzyme.

6Pgd-1 was best resolved on the histidine-citrate pH 7.0 buffer and usually appeared as either a two- or three-banded phenotype. The relative intensity of the bands appeared to vary with the age of the tissue, with young croziers showing maximum intensity of the upper two bands, the middle band darkest as in a heterozygote. The slowest band was the most stable, being retained even if the upper two were faint, and was, therefore, used to represent the migration for products of this locus. The complex pattern could result from coding of *6Pgd-1* by two presumably duplicate genes with age dependent expression or by a single gene whose products experience post-translational modification resulting in secondary isozymes. Owing to a lack of intraspecific variation for *6Pgd-1*, it is not possible to decide between these two possibilities. The *6Pgd-1* phenotype of *D. ludoviciana*, *D. goldiana*, and *D. celsa* was identical, while that of *D. marginalis* was slower.

PGI: As with other diploid ferns, two PGI loci were observed. Also consistent with observations on other ferns, resolution of the more anodal (presumably chloroplastic) *Pgi-1* was inferior to that of *Pgi-2*. Moreover, the pattern of *Pgi-1* was somewhat variable: usually a single *Pgi-1* band was observed, but on some gels *Pgi-1* appeared two-banded (e.g., fig. 3). Despite the inferior and somewhat inconsistent resolution, sufficient numbers of gels showed reasonably resolved invariant *Pgi-1* bands to justify interpreting it as a locus that is monomorphic across all four species.

Pgi-2 showed excellent resolution (fig. 3). Homozygotes were single-banded and heterozygotes exhibited the expected three-banded pattern for a dimeric enzyme. Occasionally additional "shadow" bands appeared (especially when enzymes were extracted from older tissue), as is commonly experienced with PGI. *Pgi-2* was fixed in *D. ludoviciana* for allele *Pgi-2*^{100} with the exception of a single individual from Tallahassee, Florida, which had the heterozygous genotype *Pgi-2*$^{100\text{-}75}$. *Dryopteris goldiana* was fixed for allele *Pgi-2*^{45}. *Pgi-2* was quite polymorphic in *D. marginalis*. This species possessed six alleles, of which by far the commonest was *Pgi-2*^{75}, but which also included both *Pgi-2*^{100} and *Pgi-2*^{45} as infrequent alleles. *Dryopteris celsa* was fixed heterozygous for alleles *Pgi-2*^{45} and *Pgi-2*^{115}. The latter allele was unique to *D. celsa*, an interesting circumstance the significance of which is discussed below.

PGM: PGM was represented in all species by two well separated sets of bands, designated *Pgm-1* and *Pgm-2*. Heterozygotes for both loci exhibited a two-banded pattern as expected for this monomeric enzyme. Both loci were monomorphic in *D. ludoviciana* and *D. goldiana* for alleles *Pgm-1*^{100}, *Pgm-2*^{100} and *Pgm-1*^{95}, *Pgm-2*^{78}, respectively. In *D. marginalis*, *Pgm-1* was highly polymorphic, exhibiting principally two alleles, *Pgm-1*^{98} and *Pgm-1*^{93}. *Pgm-2* was also polymorphic in *D. marginalis*. Allele *Pgm-2*^{45} was by far the commonest, with occasional variants possessing alleles *Pgm-2*^{67} or *Pgm-2*^{25} being observed. *Dryopteris celsa* exhibited a two-banded heterozygous pattern at both loci. At *Pgm-1*, *D. celsa* was fixed for *Pgm-1*$^{100/95}$. At *Pgm-2*, nearly all individuals of *D. celsa* possessed the *Pgm-2*$^{100/78}$ phenotype. However all ramets from a small isolated population in southern Illinois (probably a single clone) possessed a *Pgm-2*$^{100/57}$ phenotype (fig. 3).

SKDH: This enzyme was represented by a single locus. Homozygotes were typically one-

banded and heterozygotes two-banded as expected for a monomeric enzyme. However, on occasional gels homozygotes would exhibit an additional fainter band anodal to the principal band, interpreted as a secondary isozyme or "shadow." Heterozygotes on these gels showed three bands, the faster allozyme apparently coinciding with the faster shadow, but also producing its own shadow. Initially *Skdh* was scored on tris-citrate on which allozymes of *D. ludoviciana*, *D. goldiana* (the commoner slow allozyme), and *D. celsa* showed identical migration. Late during the study, electrophoresis on morpholine gels revealed that the slower *D. goldiana* allozyme was encoded by an allele $Skdh^{90}$ distinct from the $Skdh^{100}$ allele fixed in *D. ludoviciana*. *Dryopteris celsa* exhibited a fixed heterozygous phenotype ($Skdh^{100/90}$) on morpholine gels. Although the sample size scored on morpholine was considerably smaller than that on tris-citrate, separation of these allozymes and visualization of the fixed heterozygous phenotype of *D. celsa* was consistent for representatives from several populations, and is assumed to be the pervasive condition.

Most populations of *D. goldiana* exhibited a polymorphism for $Skdh^{90}$ and $Skdh^{114}$ (this was the only polymorphism observed in *D. goldiana*). A low level polymorphism was also observed for *Skdh* in *D. marginalis*, with $Skdh^{85}$ being the most frequent allele in this species, and $Skdh^{100}$ and $Skdh^{80}$ also being present in low frequencies.

TPI: The banding patterns observed for TPI were complex (fig. 3), but were quite comparable to those observed in other leptosporangiate ferns (Haufler and Soltis 1986), thus facilitating their interpretation. All diploid leptosporangiate ferns for which TPI has been studied show two independently varying sets of bands. In homozygous individuals one set is represented by a single dark band (here designated *Tpi-2*) and the other by a fainter three-banded phenotype (*Tpi-1*). Heterozygotes for *Tpi-2* are three-banded, while heterozygotes involving the triplet *Tpi-1* locus show complex patterns with five or more bands. The triplet pattern could alternately be interpreted as a tandemly duplicated enzyme locus or a single locus exhibiting secondary isozymes, i.e., products of post-translational modification (Haufler and Soltis 1986; Gastony 1988; Hickey et al. 1989). In the absence of conclusive evidence of gene duplication, it was conservatively assumed that the triplet is coded by a single locus in *Dryopteris*.

Expression of TPI isozyme patterns in the *Dryopteris* species considered here was quite consistent with those observed in other ferns as discussed above, with the exception that a third, faintly staining set of bands anodal to *Tpi-2* was often observed in *D. goldiana* and *D. marginalis*. They were not seen in either *D. ludoviciana* (except perhaps in a single individual that was homozygous for $Tpi\text{-}1^{45}$) or *D. celsa*, but their appearance may have been occluded by the more anodal position of *Tpi-1* in these species. These bands, believed to represent a third TPI locus, were not consistently scorable and are not included in the data set.

The darkly staining band (*Tpi-2*) showed identical mobility in all individuals with the exception of two *D. marginalis* individuals, which showed a three-banded heterozygous pattern for a slow allozyme. The triplet *Tpi-1* exhibited species-specific allozymes. In most individuals of *D. ludoviciana*, *Tpi-1* isozymes migrated anodal to those of *Tpi-2*. Because *D. ludoviciana* was designated as the "standard" pattern, in the present and subsequent studies the triplet isozyme set is designated *Tpi-1* regardless of its relative position on the gel, and the single-banded isozyme is correspondingly designated *Tpi-2*. This is consistent with notation for these two loci in Gastony and Darrow (1983), but the reverse of that in Haufler (1985). An infrequent allozyme, $Tpi\text{-}1^{45}$, cathodal to *Tpi-2*, was observed in both homozygous and heterozygous states in some populations of *D. ludoviciana*. *Dryopteris goldiana* was fixed for $Tpi\text{-}2^{63}$ the migration of which overlapped with that of *Tpi-1* in this species. The pattern of overlap differed between the lithium hydroxide and system 6 electrophoretic buffers. *Dryopteris marginalis* was nearly fixed for $Tpi\text{-}1^{25}$, but rare variants heterozygous for $Tpi\text{-}1^{0}$ (virtually at the origin) and a faster allozyme were observed. The individual bearing the faster allozyme perished before it could be compared directly to *D. goldiana*; therefore, this allozyme is conservatively assumed identical to $Tpi\text{-}1^{63}$ of *D. goldiana*. The *Tpi-1* pattern of *D. celsa* was invariant, and appeared to be the complex pattern expected for a heterozygote for alleles $Tpi\text{-}1^{100}$ and $Tpi\text{-}1^{63}$.

Comparison of Allozyme Composition and Polymorphism among Species. The alleles

TABLE 3. Allelic composition of four *Dryopteris* species at 19 enzyme loci. Alleles separated by a slash indicate fixed heterozygous genotypes in *D. celsa*. For polymorphic loci, mean frequencies over all populations are indicated in brackets to the right of each allele. Sample sizes for almost all loci equal or exceed 100 individuals from 5 or more populations.

Locus	*ludoviciana*	*celsa*	*goldiana*	*marginalis*
Ald-1	100	100	100	100 [0.809] 80 [0.191]
Got-2	100	100	100	100 [0.997] 77 [0.003]
G6pd	100	100	100	114 [0.008] 100 [0.987] 85 [0.005]
Hk	100	100/106	106	108 [0.984] 106 [0.016]
Idh-1	100	100 [0.976] 100/85 [0.024]	100	130 [0.013] 111 [0.963] 85 [0.006]
Idh-2	100	100/95	95	95 [0.940] 78 [0.060]
Lap-1	100	100/107	107	103 [0.031] 100 [0.255] 96 [0.654] 91 [0.061]
Mdh-1	100	100	100	85
Mdh-2	100 [0.991] 90 [0.009]	100	100	100
Mdh-4	100	100	100	130 [0.010] 120 [0.018] 100 [0.972]
Pgi-1	100	100	100	100
Pgi-2	100 [0.995] 75 [0.005]	115/45	45	130 [0.002] 120 [0.019] 100 [0.095] 75 [0.761] 45 [0.019] 0 [0.105]
Pgm-1	100	100/95	95	105 [0.002] 98 [0.764] 93 [0.234]
Pgm-2	100	100/78 [0.909] 100/57 [0.091]	78	67 [0.009] 45 [0.959] 29 [0.032]
6Pgd-1	100	100	100	88
6Pgd-2	100	100	100	100 [0.195] 88 [0.102] 74 [0.558] 63 [0.144]
Skdh	100	100/90	114 [0.369] 90 [0.631]	100 [0.016] 85 [0.943] 80 [0.041]
Tpi-1	100 [0.842] 45 [0.158]	100/63	63	50 [0.017] 25 [0.977] 0 [0.006]
Tpi-2	100	100	100	100 [0.996] 55 [0.004]

TABLE 4. Morphological, ecological, and phytochemical attributes of four *Dryopteris* species. (Based on Britton 1972a, 1972b, 1974, Montgomery and Paulton 1981, Viane 1986, Widen et al. 1975 and pers. obs.)

Feature	*ludoviciana*	*celsa*	*goldiana*	*marginalis*
Rhizome	long creeping	creeping	short creeping	erect
Leaf dimorphism	pronounced	weak	slight	absent
Leaf outline	narrow	medium	broad	medium
Leaf tip narrowing	gradual	somewhat abrupt	abrupt	gradual
Leaf base	tapering	slightly tapering	truncate	truncate
Leaf dissection	pinnate-pinnatifid	pinnate-pinnatifid	pinnate-pinnatifid	bipinnate-pinnatifid
Pinna incision	¼	½	¾	¾
Soral position	medial	medial	medial	marginal
Scale color	tan	dark brown	very dark brown to black	light brown
Hair types	isocytic	clavate, isocytic, & moniliform	clavate and isocytic to moniliform	glabrous
Spore morphology	prominently winged deep fissures spines lacking	shallowly winged shallow fissures spines present	relatively smooth no fissures spines present	tuberculate surface rough spines present
Phloroglucinol composition	albaspidin 1-3 *p*-aspidin BB flavaspidic acid	albaspidin 1-3 *p*-aspidin BB flavaspidic acid	albaspidin 1-3 ol *p*-aspidin BB flavaspidic acid	Me-bis-aspidin desaspidin BB phloraspidinol margaspidin BB flavaspidic acid
Principal habitat	swamps	swamps	rich wooded ravines	rocky woods and cliffs

detected in each species are listed in table 3. Frequencies of alleles (means over all populations) at polymorphic loci in each species are listed in brackets. It is immediately apparent that levels of polymorphism are quite low in *D. ludoviciana, D. goldiana,* and *D. celsa* (although the tetraploid *D. celsa* is fixed heterozygous for a number of loci), while *D. marginalis* exhibits substantial polymorphism. Detailed analyses of genetic variation within and among populations of these species will be published separately.

DISCUSSION

***Ancestry of* Dryopteris celsa.** One of the advantages of using isozyme data in evaluating parentages of allopolyploids is that each locus provides an independent test of hypothesized parentage (Werth 1989). Here, the isozyme data presented in table 3 are being used to test between two hypotheses as to the ancestry of *D. celsa*: *D. ludoviciana* × *goldiana* (Walker 1962; Wagner 1971) versus *D. ludoviciana* × *marginalis* (Hickok and Klekowski 1975). A summary of previous morphological and biochemical evidence bearing on these hypotheses is presented in table 4.

The isozyme data are most readily analyzed by assuming that *D. ludoviciana* is one parental taxon, as both hypotheses agree. Eighteen of the 19 loci in table 3 are consistent with parentage of *D. celsa* by *D. ludoviciana.* The single locus that is not consistent with this assumption (*Pgi-2*) does not falsify it, because parental taxa may vary for allelic composition in space and time. The significance of this single inconsistency will be discussed below. With the assumption of parentage by *D. ludoviciana,* it can be determined for each locus whether *D. goldiana* or *D. marginalis* could have contributed the correct allele to serve as the second parent (for simplicity, rare variants in *D. celsa* at *Pgm-2* and *Idh-1* are ignored here, but are discussed in detail below). This approach is analogous to that used in testing paternities using allozymes

(Werth 1989). An important difference between considering the parentage of a single individual and that of an allopolyploid species is that the parents of an individual may have at most two alleles at each locus, and the frequency of an allele in a putative parent may only by 0, 0.5, or 1.0. Progenitor species of allopolyploids, however, may have multiple alleles, and each may be present in a frequency ranging continuously from 0 to 1.

Dryopteris goldiana has the correct allele to have served as the second parent at every locus scored (table 3). Furthermore, *D. goldiana* has *only* the correct allele at all loci except *Skdh*, where its frequency for the correct allele (*Skdh*^{90}) is 0.631. In contrast, *D. marginalis* has the correct allele at only 12 loci (*Ald-1, Got-2, G6pd, Hk, Idh-2, Mdh-2, Mdh-4, Pgi-1, Pgi-2, 6Pgd-2, Skdh, Tpi-2*), most of which seem to be conservative loci where the same allele is shared by all three diploids. Furthermore, in all of these except *Mdh-2* and *Pgi-1*, the frequency of the appropriate allele is slightly (*Ald-1, Got-2, G6pd, Idh-2, Mdh-4, Tpi-2*) or substantially (*Hk, Pgi-2, 6Pgd-2, Skdh*) lower than in *D. goldiana*. Significantly, at six of the seven loci at which *D. celsa* is fixed heterozygous (*Hk, Idh-2, Lap, Pgi-2, Pgm-1, Pgm-2, Skdh*), *D. marginalis* has only incorrect alleles. At *Pgi-2* where *D. celsa* is fixed for *Pgi-2*$^{115\text{-}45}$, *D. marginalis* does possess allele *Pgi-2*^{45} in low frequency; however, *D. goldiana* is fixed for this same allele. The conclusion drawn from these electrophoretic data is unequivocal: the hypothesis that *D. marginalis* is ancestral to *D. celsa* is rejected, and the hypothesis of ancestry by *D. goldiana* is robustly supported.

Orphan Alleles. The only inconsistencies in the data with the hypothesis that *D. celsa* is *D. ludoviciana* × *goldiana* are the three orphan alleles: *Pgi-2*^{115} fixed (in combination with *Pgi-2*^{45}) in *D. celsa* but undetected in a large sample of *D. ludoviciana*; *Pgm-2*^{57} fixed (in combination with *Pgm-2*^{100}) in a single population (probably a single genet) in southern Illinois; and *Idh-2*^{85}, which occurs combined with *Idh-2*^{100} in a few individuals from the Dismal Swamp in North Carolina. Three hypotheses may be considered to account for the occurrence of orphan alleles in *D. celsa*: 1) they were contributed by parental taxa that were allied to, but not conspecific with *D. ludoviciana* and *D. goldiana*; 2) they were contributed by unknown genotypes of *D. ludoviciana* and/or *D. goldiana*; 3) they arose in *D. celsa* after its formation.

Of these, hypothesis 1 is certainly the least likely. The presence of the orphan alleles cannot falsify that the ancestry of *D. celsa* is *D. goldiana* × *ludoviciana* in the face of overwhelming supporting evidence from all other loci. The allied taxa called for in hypothesis 1 must either be extinct or geographically remote. Vicariads of both *D. ludoviciana* and *D. goldiana* occur in eastern Asia in the form of *D. tokyoensis* (Mak.) C. Chr. and *D. monticola* C. Chr., respectively (Carlson and Wagner 1982). However, that the range of *D. celsa* is largely included within, and somewhat intermediate between, those of *D. ludoviciana* and *D. goldiana* (fig. 2) argues against a remote origin. Furthermore, preliminary electrophoretic data on *D. tokyoensis* and *D. monticola*, while indicating affinity to *D. ludoviciana* and *D. goldiana*, respectively, do not support either, over their North American counterparts, as parents of *D. celsa* (Werth, unpubl. data).

Given that *D. ludoviciana* and *D. goldiana* are the correct progenitors, the source of the orphan alleles may be considered in light of hypotheses 2 and 3. On the basis of the present evidence, neither hypothesis can be discounted altogether for any of the orphan alleles. However, the distribution of each orphan allele in *D. celsa* provides a strong inference as to its origin. In the case of *Pgi-2*^{115} that is fixed in *D. celsa*, direct contribution from *D. ludoviciana* (hypothesis 2) seems more likely. If the allele was derived from mutation of *Pgi-2*^{100} following hybridization (hypothesis 3), its origin and fixation would have to have taken place early during the initial establishment of the population of fertile allopolyploids, prior to dispersal from the site of origin, as all populations of *D. celsa* sampled are fixed for this allele. Thus there is a very narrow time window for this genotype to become established. However, the possibility that the hybridization itself might have resulted in such a mutation should not be overlooked. The role of transposable elements in inducing mutations in interstrain hybrids of *Drosophila melanogaster* (hybrid dysgenesis) is well documented (Kidwell et al. 1977). In the case of the other two orphan alleles incorporated into *D. celsa*, *Pgm-2*^{57} and *Idh-1*^{85}, post-hybridization mutation (hypothesis 3) seems more likely. Each of these alleles is confined to a single popula-

tion and very probably to single genets. This is consistent with the origin of these rare alleles from post-hybridization mutations in isolated lineages.

If the orphan allele *Pgi-2*^{115} was in fact contributed by a variant genotype of *D. ludoviciana,* the question arises as to why it was not detected in the electrophoretic survey of this species. If the allele is present today in *D. ludoviciana,* its occurrence must be exceedingly rare and/or local, for the sample of *D. ludoviciana* used in the present study was substantial both in number of individuals and geographical representation. Note that rare alleles do occur at several loci, including *Pgi-2,* in the current sample of *D. ludoviciana* (table 3), and increased sampling may eventually reveal the presence of *Pgi-2*^{115} in this species. It is entirely possible that the allele may have represented a substantial polymorphism in *D. ludoviciana* at the time of its incorporation into *D. celsa,* and has since become rare or extinct. A reduction in the level of polymorphism at *Pgi-2* and perhaps other loci in *D. ludoviciana* seems very plausible, given that paleofloristic studies indicate that drastic southward migrations occurred in response to the Wisconsinan glacial advance (Davis 1976; Delcourt and Delcourt 1981). It is likely that the range of *D. ludoviciana* and other plants confined to the southern portion of deciduous forest would have been shifted considerably southward. Available habitat may have been limited, as most of peninsular Florida appears to have been occupied by a xeric-adapted scrub vegetation at the Wisconsinan maximum (Delcourt and Delcourt 1981). It is, therefore, conceivable that the number and size of *D. ludoviciana* populations were reduced drastically, resulting in a depletion of genetic polymorphism as is believed to be an inevitable result of such bottlenecks.

***Evolutionary History of* Dryopteris celsa.** Because the parental taxa *D. ludoviciana* and *D. goldiana* are presently allopatric (fig. 2), it has been hypothesized that *D. celsa* was formed during the time of the glaciation-induced southward migration when substantial range overlap of the two parental taxa might have occurred (Carlson and Wagner 1982). There may have been only a limited period of time during which there was an opportunity for the formation of this allopolyploid species. On the other hand, the ability of spores to travel long distances and form hybrids is well known (Wagner 1943). The apparent success of *D. ludoviciana* to form hybrids beyond its range is evident from the numerous occurrences of its hybrid with *D. celsa* (*D.* × *australis* (Wherry) Small) well north of its present range (F. Wagner and Musselman 1982; Werth et al. 1988).

Was *D. celsa* formed repeatedly as appears to be the case for many other allopolyploid species (Haufler 1990; Roose and Gottlieb 1976; Werth et al. 1985b; Windham 1988; Wyatt et al. 1989) or did it have only a single origin? Because polymorphism, which could mark incorporation of different parental genotypes, is low in the two parental diploids, this question would seem difficult to address. However, the omnipresence of the orphan allele *Pgi-2*^{115} in *D. celsa* may provide an important clue into the evolutionary history of this allotetraploid species. Irrespective of whether *Pgi-2*^{115} arose through mutation or was obtained directly from *D. ludoviciana,* it provides strong evidence that all populations of *D. celsa* have a common origin, very possibly from a single hybrid individual. Even if *Pgi-2*^{115} was once more common in *D. ludoviciana* than at present, it is probable that the surviving *Pgi-2*^{100} was the prevalent allele, or at the very least was frequent. It seems unlikely that a series of recurrent origins of *D. celsa* would have failed to incorporate the *Pgi-2*^{100} allele, and it is virtually certain that *D. celsa* has not been formed very recently.

It could be argued that the variant genotypes in *Pgm-2* and *Idh-1* suggest a multiple origin; however, as indicated above, the isolated occurrences of these orphan alleles are more consistent with post-hybridization mutation. The failure for polymorphisms that do occur in the parental species (*Tpi-1* in *D. ludoviciana* and *Skdh* in *D. goldiana*) to be represented in *D. celsa* provides further evidence against a multiple origin. Thus, *D. celsa* may well represent the most convincing case yet known for a widespread allopolyploid species that has had a unique origin.

Conclusion. While the present study has focused on the origin of *D. celsa,* the conclusion that the ancestry of *D. celsa* is *D. ludoviciana* × *goldiana* and not *D. ludoviciana* × *marginalis* impacts the entirety of both opposing hypotheses of relationships in this intricate complex. In the reinterpretation hypothesis (Hickok and Kle-

kowski 1975), the only role implicit for *D. marginalis* was as an ancestor of *D. celsa*. As it is quite apparent from the isozyme data that this is not the case, it may now be concluded that *D. marginalis* is not an element in the *D. "spinulosa"* complex. While numerous hybrids are known involving *D. marginalis* (table 1), all of these are sterile (Wagner 1971); this species does not appear to have been involved in the formation of any fertile allopolyploids. This conclusion is consistent with the absence of the distinctive morphological features and phloroglucinol arrays of *D. marginalis* (table 4) in *D. celsa* or in any other of the fertile allopolyploids.

The reinterpretation hypothesis also specifies that the ancestry of *D. cristata* is *D. ludoviciana* × *goldiana* (fig. 1B). However, this is the same ancestry that is so strongly implied for *D. celsa* by the isozyme data in the present study. It would, therefore, seem that the isozyme data are not very supportive of the reinterpretation hypothesis as a whole. However, it would be unwise to dismiss all tenets of this hypothesis and by default accept the *"semicristata"* hypothesis until further isozyme data have been obtained from the other species comprising the complex.

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