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The Hybrid *Cystopteris fragilis* × *C. tenuis* (Dryopteridaceae) and the Relationship Between Its Tetraploid Progenitors

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ABSTRACT. Subtle morphological differences between allotetraploid *Cystopteris fragilis* ($2n = 84II$) and allotetraploid *C. tenuis* lead some workers to maintain *C. tenuis* as *C. fragilis* var. *mackayi*. Recent electrophoretic evidence, however, suggests *C. tenuis* shares only one progenitor species with *C. fragilis*. Hybrids between the two allotetraploids encountered in Vermont yielded sporocytes with at least 42 bivalents and at least some univalents, further supporting the single common genome theory. Identification of hybrids and species as collected in the field in Vermont corresponded three times in four with identification based on isozyme markers. Multivariate analysis of variables generated by landmark analysis corroborated traditional field-identification characters, especially the sharper angle of pinna and pinnule departure in *C. tenuis* and the tendency of pinnae in *C. tenuis* to have a concave (vs. convex in *C. fragilis*) acroscopic side. Landmark analysis also indicated that 1) *C. fragilis* is generally smaller in both lamina and pinna size than *C. tenuis* and 2) although largest in both lamina and pinna size, *C. fragilis* × *C. tenuis* is intermediate in lamina and pinna-shape characteristics. In Vermont *C. tenuis* generally inhabits lowland calcareous cliffs and talus below 2,000 ft, *C. fragilis*, upland cliffs above 2,000 ft, and the hybrid, cliffs of intermediate altitude (1,200–2,500 ft). Lowland *C. fragilis* and upland *C. tenuis* populations may occur where microclimate permits.

Subtle and often overlapping morphological variation can obscure the boundaries between reproductively isolated lineages. This generalization is especially true of allopolyploid ferns where morphological differences between progenitors can already be subtle (see for example Gastony 1988; Paris and Windham 1988; Haufler et al. 1995). To circumscribe species with poor morphological differentiation, fern systematists have turned to non-visible ("cryptic") characteristics such as chromosome pairing behavior, isozyme phenotypes, and restriction-enzyme site mapping (Barrington et al. 1989; Paris et al. 1989). The result is that species status may be applied to reproductively isolated units that are difficult to distinguish in the field. Despite this drawback, when species have been defined based on cryptic characters, they can be critically evaluated if treated as hypotheses (Barrington et al. 1989). Such hypotheses should be scrutinized on two levels. First, is the proposed evolutionary relationship among the taxa valid (i.e., do the species represent distinct lineages)? Second, can morphological criteria be discovered that distinguish the taxa reliably? Here we address these questions for problematic species circumscriptions in the fern genus *Cystopteris* Bernh.

Discovering characters that can consistently define species in *Cystopteris* has been a persistent challenge to fern systematists. Without doubt most confusion has centered around the *C. fragilis* (L.) Bernh. complex, a group notorious for confusing morphological plasticity and variability (Haufler 1985; Haufler and Windham 1991). Early treatments circumscribed the complex as a single species, *C. fragilis*, comprising six varieties (Weatherby 1935). Wagner and Hagenah (1956, p. 79) were the first to suggest that many of these varieties "possess phylogenetic significance [and] merit interpretation as species rather than varieties." Using chromosomal and isozymic data, it has been possible to demonstrate that a number of the varieties are allopolyploids and should be recognized as species (Blasdell 1963; Haufler et al. 1990; Haufler and Windham 1991; Haufler et al. 1993).

One of the remaining debates about the *Cystopteris fragilis* complex in North America currently centers around the status of the tetraploid ($2n = 84II$) taxon *C. tenuis* (Michx.) Desv. Traditionally *C. tenuis* has been viewed as a va-

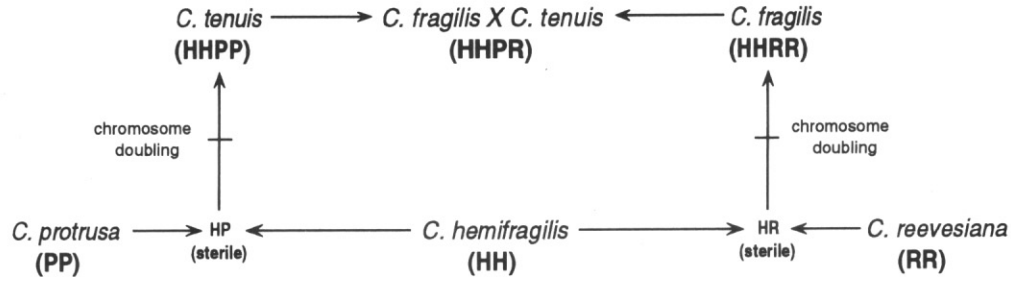


FIG. 1. Diagrammatic representation of reticulate relationships among northeastern members of the *Cystopteris fragilis* complex.

riety (var. *mackayi* Laws.) of *C. fragilis*, but Moran (1983) outlined a suite of morphological characters that distinguished it as a species (*C. tenuis*) from *C. fragilis*. These characters include a sharper angle of pinna departure from the rachis, a tendency for the pinnae to curve towards the apex, a more cuneate (vs. truncate) base of the proximal basioscopic pinnule, and more crenulate (vs. serrate) pinna margins.

Electrophoretic analyses corroborated the morphological findings of Moran (1983) by revealing fixed isozyme phenotypes for *C. tenuis* and *C. fragilis* in northeastern North America (Haufler, pers. comm.). *Cystopteris fragilis* sensu stricto appeared to combine marker bands from a western diploid, *C. reevesiana* Lellinger, and a currently undiscovered or extinct diploid, dubbed "*C. hemifragilis.*" *Cystopteris tenuis* combined marker bands from the same unknown diploid and the southeastern *C. protrusa* (Weatherby) Blasd. This led to the hypothesis that the tetraploids *C. fragilis* and *C. tenuis* are allopolyploids sharing a common progenitor species (Fig. 1; Barrington et al. 1989; Haufler and Windham 1991). The recent treatment of *Cystopteris* in Flora North America (Haufler et al. 1993) maintains species designation for *C. tenuis*. Despite the evidence supporting species status for *C. tenuis*, some workers (A. Tryon, pers. comm.) and at least one recent flora (Gleason and Cronquist 1991) continue to classify it as *C. fragilis* var. *mackayi*.

The goal of the research reported here was to explore the species status of *C. tenuis*. Toward this end, we 1) assessed the ecological differentiation between the taxa; 2) tested the common-progenitor hypothesis, and 3) sought a set of field-recognition characters for distinguishing *C. tenuis* from *C. fragilis*.

To assess the ecological differentiation between the taxa, we tested the working hypothesis that *C. fragilis* is an upland species likely to inhabit shady cliffs above 2,500 ft (at this latitude), whereas *C. tenuis* is a lowland species of moist, calcareous cliffs, the shady talus slopes below them, and (occasionally) steep slopes in soil (Haufler and Windham 1991; Haufler et al. 1993). Field work thus focused on 1) "ordinary" lowland and upland habitats; 2) putative hybrid zones where upland and lowland habitat were in close proximity (such as moist upland notches and steep-faced rock outcrops adjacent to major river valleys), and 3) regionally anomalous lowland habitats with upland microclimate conditions (such as cliffs at the edge of Lake Champlain, deep railroad cuts, and river gorges). Our test of the common-progenitor hypothesis was based on new evidence derived from putative, naturally occurring *C. fragilis* × *C. tenuis* hybrids. The hybrid, discovered at several Vermont localities, provided critical reproductive, cytological, and genetic information relevant to the relationship between its parents. To achieve the final goal, i.e. develop a set of morphological field recognition characters, we employed landmark analysis. Landmark analysis is a recently developed morphometric technique that quantifies differences in shape between different objects by utilizing coordinates of homologous points (landmarks) whose locations are defined by developmental/anatomical criteria (Bookstein 1978; Bookstein et al. 1985; Richtsmeier et al. 1992).

MATERIALS AND METHODS

Fieldwork. During the field season of 1992 a total of 200 separate sporophytes representing all three taxa was collected from 20 sites around Vermont and one from the Gaspé Peninsula,

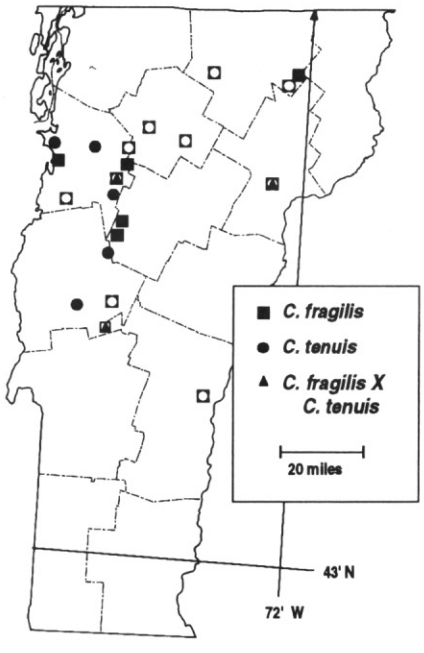


FIG. 2. Map of Vermont illustrating geographic distribution of populations sampled for electrophoretic and morphological analyses. Overlapping symbols denote mixed populations.

Québec (Table 1; Fig. 2). Each sporophyte was assigned in the field to one of three taxa (*C. fragilis*, *C. tenuis*, or hybrid) based on morphological characters. Materials collected for each sporophyte included an herbarium specimen (deposited at VT) with habitat data and field identification, a sample of fresh leaf tissue for isozyme electrophoresis and, when the population included more than 100 individuals, a section of living rhizome for cultivation in the University of Vermont greenhouses. A total of 36 sporophytes was successfully transplanted.

Isozyme Electrophoresis. The enzymes triosephosphate isomerase (TPI) and phosphoglucoisomerase (PGI) were reported by Haufler (pers. comm.) to provide electrophoretic markers for *Cystopteris fragilis* and *C. tenuis*. Hence they were chosen to both corroborate field identifications and reveal hybridization via additivity. Plant tissue collected in the field or from greenhouse-grown individuals was ground in a phosphate extraction buffer (Haufler 1985). Homogenate was absorbed into wicks made from #3 Whatman filter paper and placed in horizontal 12% starch gels. TPI was resolved on

TABLE 1. Collection data for populations of *Cystopteris* sampled. Reported for each sample are collection number (all numbers are M. H. Paler except 190–195, which are M. H. Paler and A. Gilman, and two non-numbered collections by C. Paris from Jericho, Vermont), altitude in ft, field identification, and isozyme identification. B = *C. bulbifera*, F = *C. fragilis*, L = *C. laurentiana*, T = *C. tenuis*. Except for Québec, all localities are in Vermont. Vouchers deposited at VT.

Population locality Collection	Altitude (ft)	Field identification	Isozyme identification
Gaspé Peninsula, Prov. de Québec, Canada			
108	<500	F	F
109	<500	F	F
110	<500	F	F
117	<500	T	T
121	<500	F	F
122	<500	T	T
Burlington Bike Path, Chittenden Co.			
67	100	T	T
Appalachian Gap, Chittenden Co.			
68	3080	F	F
168	2450	F	F
171	2450	F	F
Bolton mountain region, Chittenden Co.			
72	2800	T	F
73	2500	T	F
164	2700	F	F
165	2750	F	F
166	2750	F	F
167	2800	F	F
201	500	T	T
202	500	T	T
203	500	T	T
Bolton cliffs, Chittenden Co.			
198	1600	F	F
199	1600	L	F × T
212	1600	F × T	F × T
213	1600	F × T	F × T
214	1600	F × T	F × T
215	1600	T	T
216	1600	F	F
217	1600	F × T	F × T
Lincoln Gap, Addison Co.			
82	2400	F	T
83	2500	T	T
Mt. Moosalamoo, Addison Co.			
85	1200	F	T
Mt. Philo, Chittenden Co.			
88	780	F	F
89	780	T	T

TABLE 1. Continued.

Population locality Collection	Altitude (ft)	Field identification	Isozyme identification
Mt. Elmore, Lamoille Co.			
95	2450	F	F
96	2450	F	F
97	2450	F	F
98	2450	T	F
99	2000	T	T
Smugglers Notch, Lamoille Co.			
100	2050	F	F
102	2050	F	F
103	2050	T	F
175	2550	F	F
176	2550	F	F
177	2550	F	F
181	2100	T	T
Nebraska Notch, Chittenden Co.			
128	2200	F	F
129	2250	F	F
131	2100	F	F
132	2100	F	F
211	2050	T	F
Preston Brook, Jonesville, Chittenden Co.			
134	700	T	T
Mt. Horrid, Addison Co.			
136	2500	F	F
137	2500	T	F × T
138	2500	T	F × T
139	2500	T	F × T
140	2500	F	F
141	2500	T	F
142	2500	F	F
143	2500	F	F
Mt. Pisgah, Orleans Co.			
144	1900	T	T
145	1900	T	T
146	1900	F	F
Haystack Mtn., Orleans Co.			
150	2600	F	F
152	2600	F	F
153	2600	F	F
154	2600	F	F
Eden Notch, Orleans Co.			
155	1500	F	F
156	1500	L	F
157	1500	T	T
158	1500	F	F
159	1500	F × T	F
160	1500	F × T	F
161	1500	F	F
162	1500	B × T	F
163	1500	B × T	F

TABLE 1. Continued.

Population locality Collection	Altitude (ft)	Field identification	Isozyme identification
Middlebury Gap, Addison Co.			
182	1700	T	T
183	1700	T	F
184	1700	F × T	F
185	1700	F	F
Danville, Caledonia Co. (coll. *Paler & Gilman*)			
190	1000	F × T	F × T
191	1000	F × T	F × T
192	1000	F × T	F × T
193	1000	T	T
194	1000	F	F
195	1000	F	F
Quechee Gorge, Windsor Co.			
204	550	F	F
205	550	F × T	F
206	550	F × T	F
207	550	T	T
208	550	F × T	F
209	550	F	F
Red Rocks County Park, Chittenden Co.			
218	100	F	F
219	100	F	F
220	100	F	F
221	100	T	T
222	100	T	T
Jericho, Chittenden Co. (coll. *C. Paris, s.n.*)			
a	900	T	T
b	900	T	T

buffer system 6 of Soltis et al.(1983) and PGI on system 8 of Haufler (1985). Both enzymes migrated anodally. Each gel was photo-documented and scored by measuring band distance in mm from the origin. The genetic basis for observed banding was inferred from previous isozyme electrophoretic work with *Cystopteris* (Haufler et al. 1990).

Cytology. Greenhouse-grown specimens that were identified as hybrids by isozyme electrophoresis were examined for chromosome number and pairing behavior at meiosis I. Pinnae bearing young sporangia were fixed in Farmer's solution (absolute ethanol and glacial acetic acid, 3:1). Sporocytes were stained in 2% ferric acetocarmine, squashed to spread the chromosomes, and viewed through a Leitz phase-contrast microscope at 1,000×. Sporocytes squashed in diakinesis were scored for

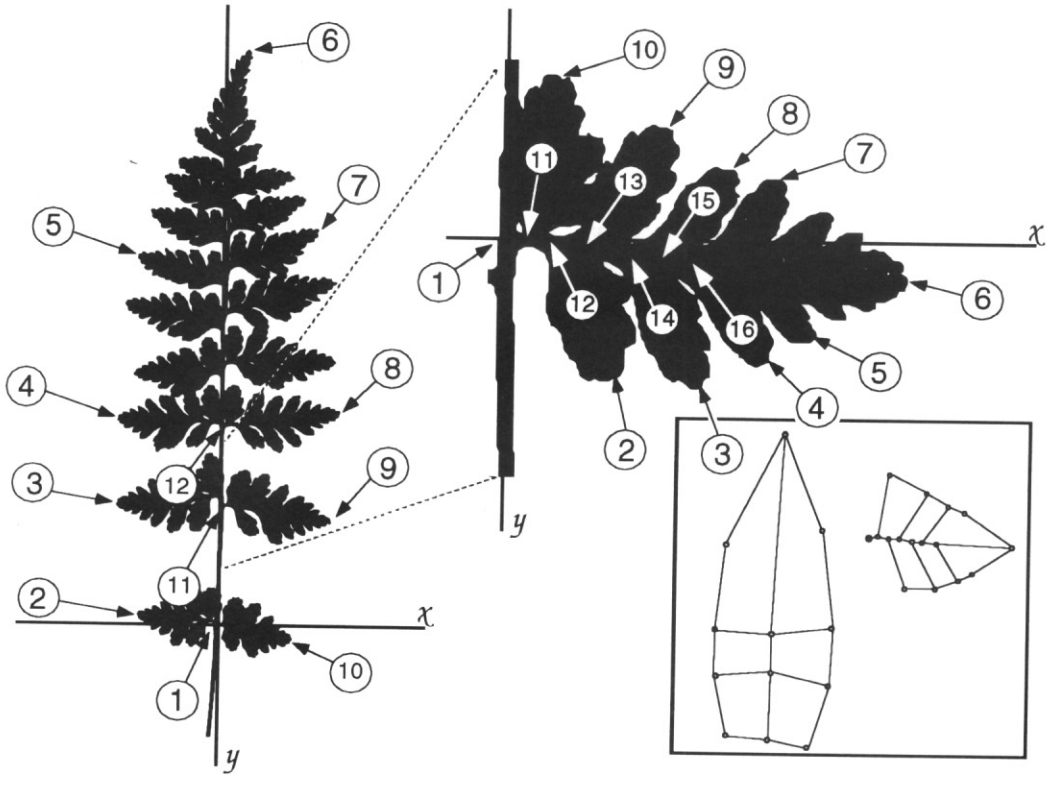


FIG. 3. Locations of landmarks used for analysis of lamina and pinna shape. See Table 2 for corresponding description of landmarks.

number of univalents, bivalents, and trivalents and then photographed with Kodak Technical Pan 2415 film.

Morphology. Landmark analysis was performed using pressed collections divided into three groups (*C. fragilis, C. tenuis,* hybrid) based on their electrophoretic phenotype. For each leaf, we located x,y coordinates for 12 landmarks from the *entire lamina* (not including the petiole) and 16 landmarks from a *single pinna second from the bottom* (Table 2; Fig. 3). Landmarks are all segment tips and segment bases as defined by the location of distal and proximal ends of vascular elements in the leaf; these define the major growth centers of the leaf. Coordinates were acquired by digitizing directly from video images of the leaves using the OPTIMAS (BioScan 1988) digitizing system. OPTIMAS is an interactive computer package that allows the user to extract areas, point-to-point distances, and point coordinates directly from frozen video images. Procedures specific to the lamina and the pinna are detailed next.

For the lamina analysis, leaves were registered for digitizing by aligning the junction of the first pinna pair and the main axis (landmark 1) directly over the origin and orienting the rachis along the y axis (Fig. 3). Leaves were always registered such that any curve of the lamina was to the right. This method ignored whether the abaxial or adaxial surface of the leaf was being viewed, therefore making "left side" and "right side" (as referred to in Table 3) an arbitrary designation applied to each sample subsequent to registration. We required that the tip of a blade not curve more than 2 cm from the y axis and that pinna departure angle (i.e., angle between main central axis and leaflet tips) be consistent for all pinnae. Under these criteria a total of 32 *fragilis,* 19 *tenuis,* and 19 hybrid individuals were admitted to the leaf analysis.

For analysis of pinna morphology, samples were registered by aligning the junction of the main rachis and the second pinna pair directly over the origin, and aligning the main rachis

TABLE 2. List of landmarks and their locations used for shape analysis of leaf and pinna shape. Numbers correspond to Fig. 3.

	Landmarks for entire leaf
1	Junction of rachis and 1st (basal) pinna pair
2	Tip of major vein of 1st pinna on left side
3	Same as 2 for 2nd left side pinna
4	Same as 2 for 3rd left side pinna
5	Same as 2 for 6th left side pinna
6	Tip of lamina
7	Tip of major vein of 6th pinna on right side
8	Same as 7 for third right side pinna
9	Same as 7 for second right side pinna
10	Same as 7 for first right side pinna
11	Junction of rachis and second pinna pair
12	Junction of rachis and third pinna pair
	Landmarks for second pinna from base
1	Junction of pinna base and rachis
2	Tip of major vein in 1st basiscopic pinnule
3	Same as 2 for 2nd basiscopic pinnule
4	Same as 2 for 3rd basiscopic pinnule
5	Same as 2 for 4th basiscopic pinnule
6	Tip of pinna
7	Tip of major vein in 4th acroscopic pinnule from bottom
8	Same as 7 for 3rd acroscopic pinnule
9	Same as 7 for 2nd acroscopic pinnule
10	Same as 7 for 2nd acroscopic pinnule
11	Junction of major vein of 1st acroscopic pinnule and costa
12	Junction of major vein of 1st basiscopic pinnule and costa
13	Junction of major vein of 2nd acroscopic pinnule and costa
14	Junction of major vein of 2nd basiscopic pinnule and costa
15	Junction of major vein of 3rd acroscopic pinnule and costa
16	Junction of major vein of 3rd basiscopic pinnule and costa

with the y axis while keeping the desired pinna pointed in the positive y direction (Fig. 3). Similar to leaf registration, this method ignored whether the abaxial or adaxial surface of the pinna was being viewed during the analysis. We sampled from the second pinna pair from the bottom because it was usually better preserved in the pressing process than the first pinna pair and more dissected than upper pinnae, thus making the landmarks more discernible. Only one pinna was measured for a given leaf; we chose the better preserved and/or more dissected pinna of the pair. For the pinna analysis, we utilized 38 *C. fragilis*, 29 *C. tenuis*, and 33 hybrid pinnae. The greater sample numbers were obtained because broken and/or bent leaves originally excluded from the leaf analysis could still be used for the pinna analysis.

Connecting the landmarks extracted from a single leaf and pinna yields polygons that are a simplification of the shape of that particular leaf and pinna (see inset Fig. 3). We calculated the mean size and shape of this polygon for each taxon and then conducted independent analy-

TABLE 3. Numbers of individuals of *Cystopteris* taxa collected and identified in the field in Vermont (the two collections from Gaspé, Canada are excluded; see Table 1) and percent correct field identification as verified by isozyme phenotypes. Calculations for field identification include one hybrid and one *C. fragilis* misidentified in the field as *C. laurentiana*. *Hybrid* includes both *bulbifera* × *tenuis* and *fragilis* × *tenuis*.

	Field identification			
Isozyme identification	*fragilis*	hybrid	*tenuis*	% correct
58 *fragilis*	42	8	7	72.4
11 *fragilis* × *tenuis*	0	7	3	63.6
22 *tenuis*	2	0	20	90.9
			average	75.6

ses of these parameters. Mean size as area, extracted from the polygons using OPTIMAS, was compared with pairwise Student's t-tests. Mean shape was determined with the morphometrics program Rotational Fit (Rohlf and Slice 1990, 1991). We utilized the "generalized least-squares" option, which first calculates the "consensus configuration" or mean values for the landmarks sampled from a set of objects and then optimally superimposes (translates) each object back onto the consensus configuration according to a least-squares criterion subsequent to *scaling* and *rotation*. Variance of the consensus configuration is visualized with "residuals" or line vectors connecting individuals with the mean for each landmark. A landmark with normal distribution of individuals about the mean will appear as a star with rays of equal length with the center being the mean and the rays being the residuals from the individual samples.

Using the taxa identified by isozyme electrophoresis as a priori groups, we assessed landmarks as a quantitative basis for discerning the two species and their hybrid. Our morphometric analysis included a principal components analysis (PCA) and discriminant function analysis (DFA) of the pinna landmark variables for 100 plants representing the three taxa. The x and y coordinates of each landmark were treated as separate variables, consequently 32 variables entered the multivariate analyses based on the 16 pinna landmarks. For the DFA we used the BMDP stepwise discriminant analysis program 7M (Dixon 1983). Two models were run: The first was at the default f-to-enter of f = 4.03, the second was at a more stringent f-to-enter, f = 13.07, estimated using Bonferoni's inequality (Ranker and Schnabel 1986). The PCA was performed using the BMDP factor analysis program 4M (Dixon 1983).

To allow direct comparison between Rotational-Fit outputs and multivariate statistics, prior to their use in the DFA and PCA models, the raw coordinate data as obtained from Optimas were scaled such that the area within the polygon (inset Fig. 3) was equal to 1. Scaling the raw data simulates the uniform scaling operation in the Rotational-Fit algorithm, thus allowing a more direct comparison between the Rotational-Fit outputs and the multivariate statistics (Rohlf and Slice 1990; see also Kincaid and Schneider 1983). Scaling pinna shape was performed by multiplying the coordinates of each landmark by a scaling factor. This scaling factor is calculated by the formula: X = square root A/B where X is the scaling factor, A is the desired area and B is the area of that shape polygon as utilized in the preceeding size comparison. With this method, all shape polygons have an area equal to one and a common origin of 0,0.

Results

Ecology. The habitat survey indicated that *C. fragilis* in Vermont is occasional on mesic, calcareous outcrops above 2,000 ft, whereas *C. tenuis* is mostly confined to shaded calcareous cliffs and talus below 2,000 ft (Table 1). Neither species, however, is confined altitudinally; both were found to occupy sites at various altitudes with microclimate that met their needs. For example, three lowland populations of *C. fragilis* (Red Rocks, 100 ft; Quechee Gorge, 550 ft; Mt. Philo, 780 ft), were found in shaded clefts proximate to water on large cliffs. Similarly, we found three high altitude populations of *C. tenuis* (Mt. Elmore, 2,200 ft; Smuggler's Notch, 2,300 ft; Lincoln Gap, 2,450 ft) on moist talus.

Of ten mixed populations encountered during the field survey, three were the lowland *C. fragilis* populations just discussed and seven occurred between 1,000 ft and 2,300 ft Two of these sites, a railroad cut at 1,150 ft and the convergence zone of a large cliff and a large talus slope at 1,600 ft, contained hybrids. Curiously, the hybrids at these two sites occupied dry, nonshaded sites, a habitat in which its parent species were never found. The third hybrid population was located in a moist ravine at 2,500 ft that contained only *C. fragilis*.

Isozymes. The putative loci *Pgi-2* and *Tpi* were fixed for the marker alleles identified by Haufler (pers. comm.). In 57 individuals, *Pgi-2* was represented by a single-banded homozygous phenotype (Fig. 4a, lanes f, g). Twenty-two plants showed a three-banded heterozygous phenotype resulting from the interaction of alleles a and b (Fig. 4a, lanes d, e, k, l). The remaining 11 individuals displayed a five-banded phenotype that combined the bands of first two phenotypes plus a new band, presumably a heterodimer composed of subunits encoded by alleles c and b (Fig. 4a, lanes a–c,h–j). These additive hybrid band patterns were found in

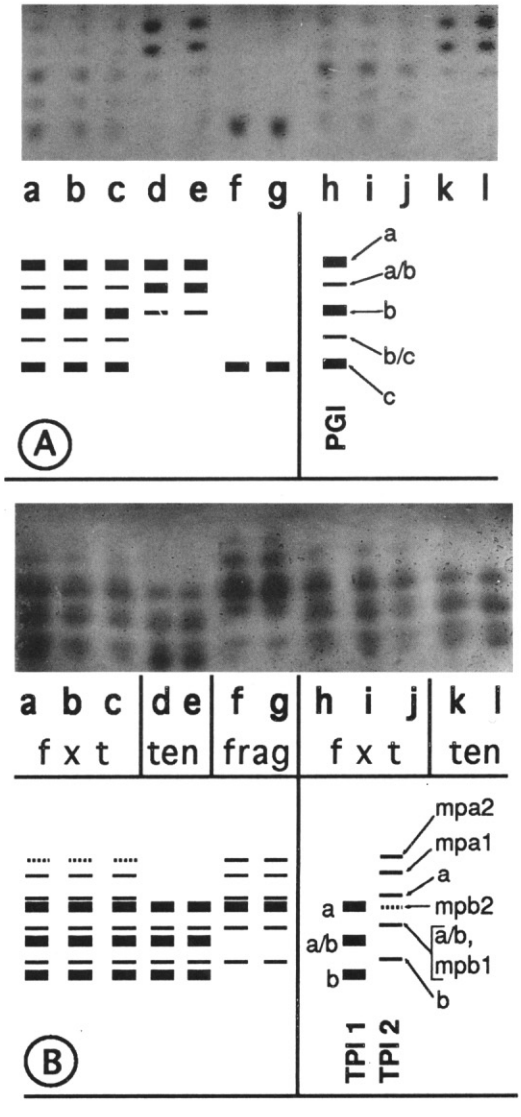


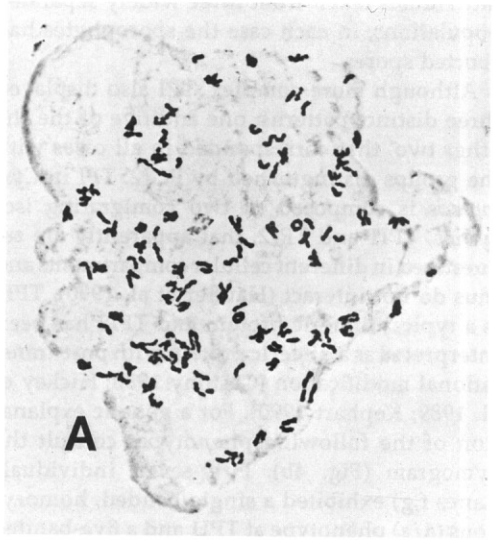
FIG. 4. Representative isozyme profiles with corresponding zymogram. Zymogram depicts first seven lanes plus a locus interpretation. Anode = top of photographs, cathode = bottom. A. PGI2. Three allozymes observed in these taxa. *Cystopteris tenuis* (lanes d, e, k) was fixed heterozygous for allozymes a and b. *Cystopteris fragilis* (lanes f,g) was consistently homozygous for allele c. Hybrid (lanes a–c, h–j) combines a, b, and c alleles. B. TPI1 and TPI2. Loci are separated in corresponding diagram for ease of interpretation. Two allozymes, denoted a and b, were observed at both TPI1 and TPI2. TPI2 shows a six-banded pattern attributed to post-translational modification (Gastony 1988; Hickey et al. 1989). For TPI2, "mp," a letter, and a number correspond to "modified product," the source allozyme, and placement on gel away from source allozyme respectively.

At TPI1, *C. tenuis* was fixed heterozygous for alleles a and b. *Cystopteris fragilis* was consistently homozygous for allele a. At TPI2, *C. tenuis* was homozygous for allele b (three-banded due to modified products). Protein mpb2 is inferred to have comigrated with the a allozyme of TPI1 (represented by dashed line). *Cystopteris fragilis* was heterozygous (five-banded due to modified products). The hybrid combines the banding of *C. tenuis* and *C. fragilis* for both loci, that is, it is heterozygous for both loci. The most anodal modified products of TPI2, not visible in this photograph (but observed in some runs) are designated with a dashed line.

individuals taken from three widely separated populations; in each case the sporophytes had aborted spores.

Although more complex, TPI also displayed three distinct patterns, one additive of the the other two, that corresponded in all cases with the groups distinguished by PGI2. TPI in *Cystopteris* is composed of two comigrating isozymes, TPI1 and TPI2, that apparently are sequestered in different cellular compartments and thus do not interact (Haufler et al. 1990). TPI1 is a typical dimeric protein and TPI2 has been interpreted as a dimeric protein with post-translational modification (Gastony 1988; Hickey et al. 1989; Kephart 1990). For a genetic explanation of the following phenotypes consult the zymogram (Fig. 4b). Fifty-seven individuals (lanes f,g) exhibited a single-banded, homozygous (a/a) phenotype at TPI1 and a five-banded heterozygous (a/b) phenotype at TPI2. Twenty-two plants (lanes d,e,k,l) displayed a three-banded heterozygous (a/b) phenotype at TPI1 and a three-banded homozygous (b/b) phenotype for TPI2. Eleven individuals (lanes a–c,h–j) displayed additive phenotypes for TPI1 and TPI2.

We inferred that the three distinct, multilocus band patterns observed in PGI2 and TPI correspond to *C. fragilis*, *C. tenuis*, and *C. fragilis* × *C. tenuis* and, as such, provide the basis for definitive identification. Preliminary field identifications corresponded just over three times in four with isozyme identifications, indicating a dependable correlation between genetics and morphology (Table 3). Of 58 isozyme-identified individuals of *C. fragilis* col-



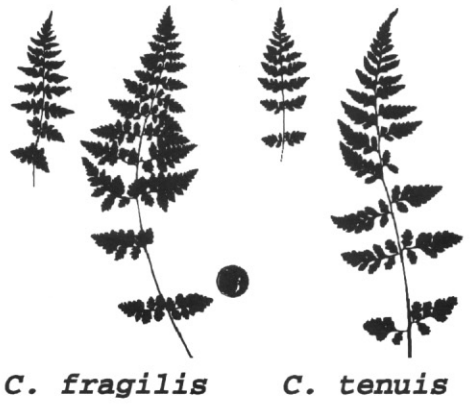


FIG. 6. Size plasticity in fully fertile *Cystopteris.* Laminae sampled from four different populations; all were identified correctly in field. Dime (18 mm diam.) inset for scale. See size analysis, Table 3.

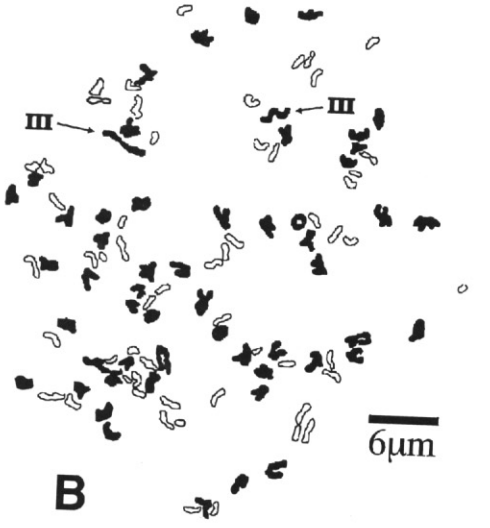


FIG. 5. Photograph (A) and Interpretive drawing (B) of meiosis I at 1,000× in *C. fragilis* × *C. tenuis,* Paler 199 (VT). n = 53 II, 60I, 2III.

lected in Vermont, seven were misidentified in the field as *C. tenuis,* eight as *C. fragilis* × *C. tenuis,* and one as *C. laurentiana* (Weatherby) Blasdell. Similarly, of 22 *C. tenuis* individuals, two were misidentified as *C. fragilis.* Of eleven hybrids, three were misidentified as *C. tenuis* and one as *C. laurentiana.*

Cytology. Chromosome studies were undertaken to test the hypothesis that the sterile hybrids encountered during this study result from crosses between two allotetraploids that share a common ancestor. Tetraploids *C. fragilis* and *C. tenuis* each have $2n = 84$ bivalents, hence the hybrid should show approximately 42 bivalents at meiosis I. In one squash (Fig. 5) we observed 53 bivalents, 56 univalents, and two trivalents. In another squash (not shown) we observed 52 bivalents and 64 univalents. These observations were predicted by Haufler and Windham's (1991) shared-progenitor theory. The additional bivalents indicate partial pairing success between the non-homologous genomes of the parental species.

Morphology. Mean size of the leaf-shape polygons was significantly different between taxa (Table 4). All taxa exhibited a wide range of size variation (Fig. 6); both lamina and pinna were smallest in *Cystopteris fragilis,* intermediate in *C. tenuis,* and largest in the hybrid. With size removed, mean polygon shapes of both lamina and pinna also exhibited notable differences between the taxa (Figs. 7–9). At the level of the leaf (Figs. 7, 9) the three taxa are nearly identical in general outline, yet differ significantly in the departure angle of the first three pinna pairs. *Cystopteris tenuis* has the sharpest pinna departure angle, *C. fragilis* the least, and the hybrid is generally intermediate. The sharper pinna departure angle in *C. tenuis* can be seen most effectively in the pinna shape diagrams (Figs. 8,9). The pinna diagrams also demonstrate that where *C. tenuis* has a slight concavity to the acroscopic side of the pinna, *C. fragilis* and the hybrid are slightly convex. In addition, the first three pinnules depart at increasingly acute an-

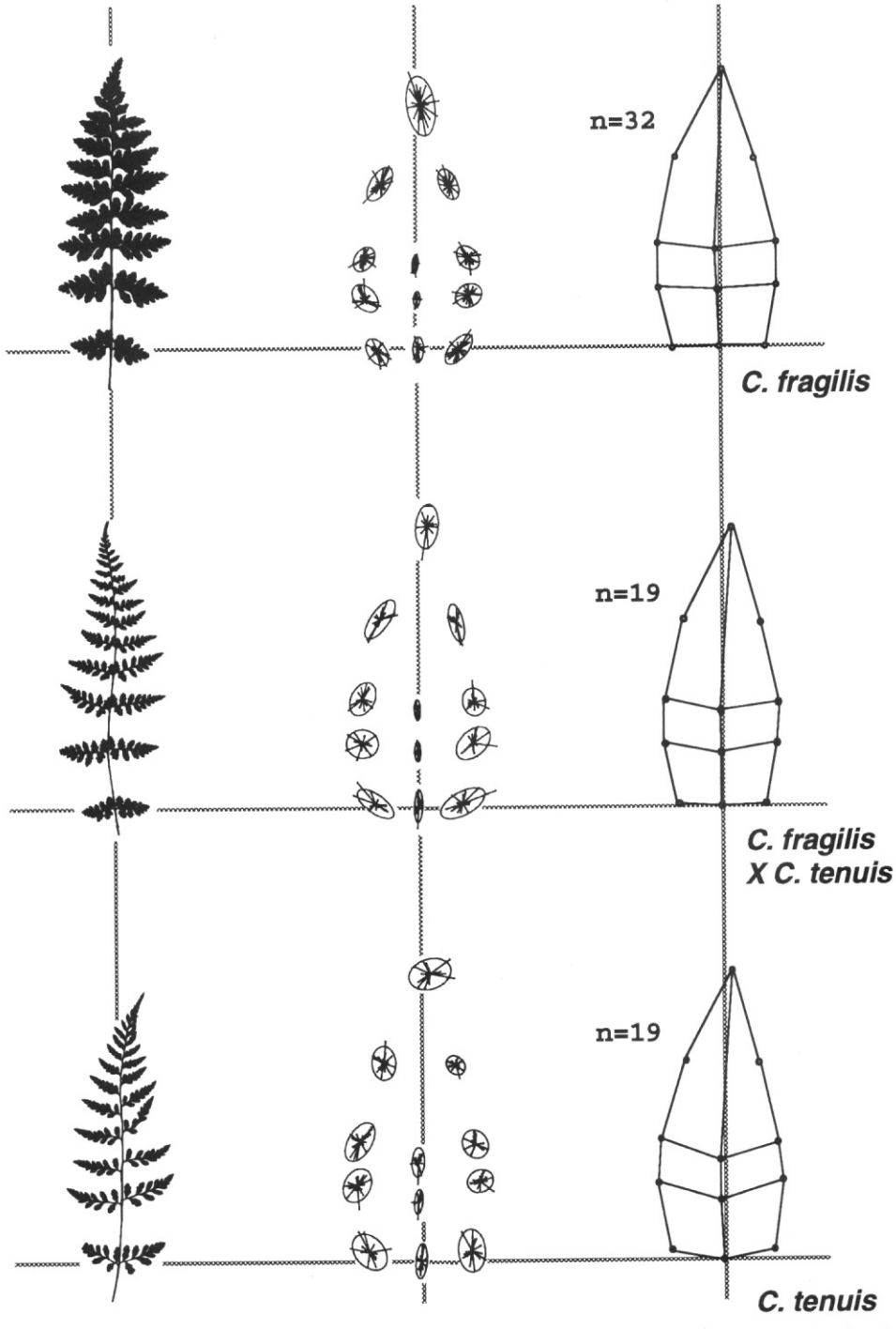


FIG. 7. Results of shape analysis for the laminae of *C. fragilis*, *C. fragilis* × *C. tenuis*, and *C. tenuis*. Depicted for each taxon from left to right are the following. 1) A size-scaled silhouette taken from a representative leaf sample. 2) A diagram depicting the mean landmark positions plus their respective residuals and constant-frequency ellipse. A constant-frequency ellipse summarizes two standard deviations of variance around the mean. Its major and minor axes represent trends in the magnitude and direction of eccentric residuals. 3) A mean shape constructed by connecting the mean landmark positions with lines.

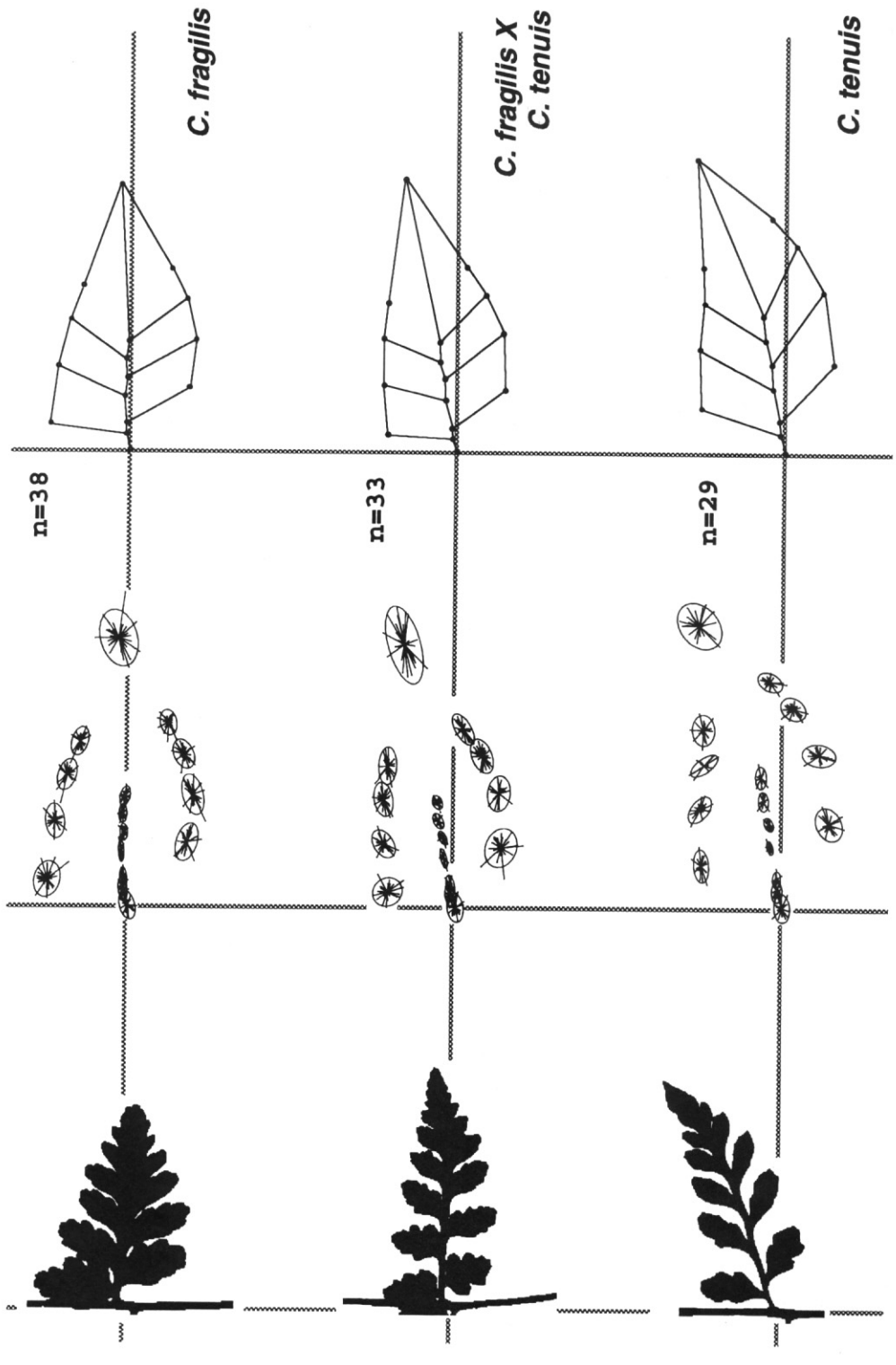


FIG. 8. Results of landmark analysis for the pinnae of *Cystopteris fragilis*, *C. tenuis*, and *C. fragilis* × *C. tenuis*. See Fig. 7 for explanation of features.

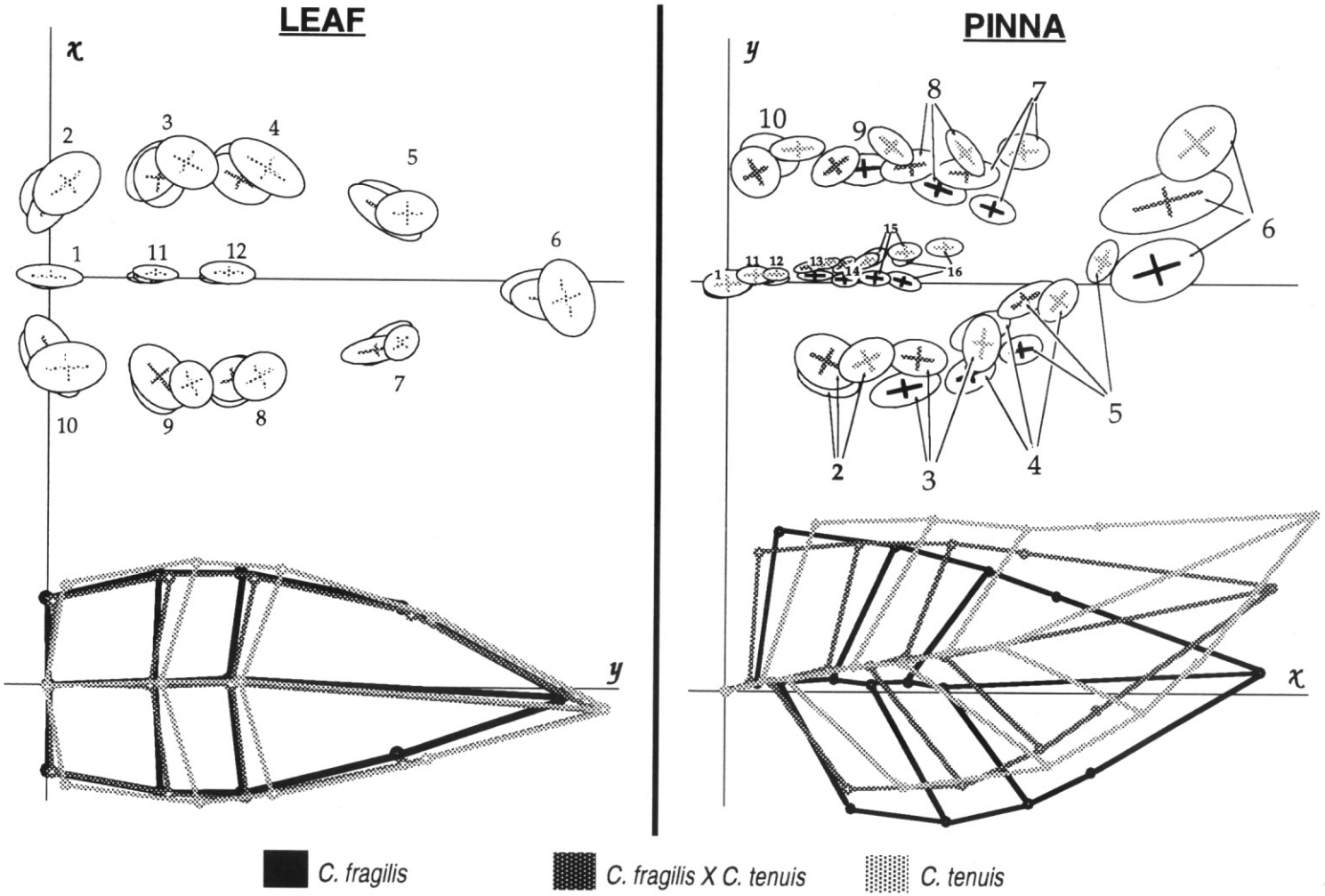


FIG. 9. Overlay of the equal-frequency ellipses and mean shape diagrams from Figs. 6 and 7. Original leaf diagrams are rotated 90 degrees. The crosses in the equal-frequency ellipses, generated with the "PCA" option from Rotational Fit, summarize the residual vectors of a landmark with eigenvector axes scaled one standard deviation in their respective direction (see Rohlf and Slice 1990).

TABLE 4. Size parameters in cm^2 of unscaled lamina and pinna landmark configurations of *Cystopteris fragilis* (frag), *C. tenuis* (ten), and their hybrid (f × t). All means are significantly different (pairwise *t*-tests; $P < 0.05$).

	Lamina			Pinna		
	frag	f × t	ten	frag	f × t	ten
N	32	19	19	38	33	29
Min	7.3	16.2	14.8	0.7	1.4	1.0
Max	71.6	123.5	85.6	4.1	8.8	5.1
Mean	26.2	56.2	34.5	1.9	3.2	2.5
s.d.	14.5	29.7	20.2	0.8	1.7	1.1

gles as one goes from *C. fragilis* to *C. tenuis* (Figs. 8,9) a trait that mirrors the trend seen for pinnae. Finally, *C. tenuis* has a greater distance between pinnule bases than either *C. fragilis* or the hybrid.

Discriminant function analysis of pinna-landmark variables (Fig. 10) separates *C. tenuis* from *C. fragilis* and the hybrid along the first canonical axis and *C. fragilis* from the hybrid along the second canonical axis. Under the default f-to-enter, eight landmark variables were entered into the model (Table 5). At the more stringent f-to-enter using Bonferroni's criterion of significance, only one variable, vertical displacement of the pinna tip (6y), entered the discriminant function model. The jackknifed classification (Table 6) suggests that *C. tenuis* is readily discernable from *C. fragilis* and that morphological confusion involves *C. fragilis* and the hybrid. Both models correctly classified *C. tenuis* in 28 of 29 (96.5% correct classification) cases with the single misclassification being as a hybrid. *Cystopteris fragilis* was misclassified as a hybrid in nine out of 38 cases (76.3% correct classification), and the hybrid was classified as *C. fragilis* in six of 33 cases (81.8% correct classification).

TABLE 6. Percent correct jackknifed classification of cases of *Cystopteris* taxa entered into discriminant function model of pinna landmark coordinates.

		Jackknifed classification			
	cases	*fragilis*	hybrid	*tenuis*	% correct
fragilis	38	29	9	0	76.3
Hybrid	33	6	27	0	81.8
tenuis	29	0	1	28	96.5
Average					84.8

Principal components analysis of the pinna landmark data revealed a similar pattern (Fig. 11). *Cystopteris tenuis* separates as a more distinct group from *C. fragilis* and the hybrid, which are largely unresolved. The variable with heaviest loading on the first principal component axis (PCA) was the vertical displacement of the pinna tip (6y). In all three factors, pinnule-tip landmarks loaded heavily. In factor one, six of eight are on the basiscopic side of the pinna, in factor two, two of three are on the acroscopic side of the pinna, and on the third PCA variable 6x loaded heavily.

DISCUSSION

Evolutionary Implications. The results of this study are consistent with the hypothesis of Haufler et al. (1993; see also Barrington et al. 1989; Haufler and Windham 1991) that the allotetraploids *Cystopteris fragilis* and *C. tenuis* share a common progenitor species (Fig. 1). The common-progenitor hypothesis assumes that an un-

TABLE 5. Standardized discriminant function coefficients of landmark variables (characters) used in the discriminant function analysis of *Cystopteris fragilis, C. tenuis* and their hybrid listed in order of importance. Characters numbered as in Table 2 and Fig. 3. I and II represent the first and second canonical axes of the discriminant function analysis respectively.

Character (number)	I	II
Vertical displacement pinna tip (6y)	−1.001	0.565
Vertical disp. 1st acroscopic pinnule tip (10y)	0.218	1.069
Horizontal disp. 1st acroscopic pinnule tip (10x)	−0.612	0.035
Horizontal disp. junction pinnule rachis and pinna rachis 3rd basioscopic pinnule (16x)	0.081	−1.070
Horizontal disp. junction pinnule rachis and pinna rachis 1st acroscopic pinnule (11x)	0.414	0.489
Vertical disp. junction pinnule rachis and pinna rachis for 2nd acroscopic pinnule (13y)	−0.704	−0.217
Horizontal disp. 2nd acroscopic pinnule tip (9x)	−0.536	0.757
Horizontal disp. 1st basioscopic pinnule tip (2x)	0.483	−0.561

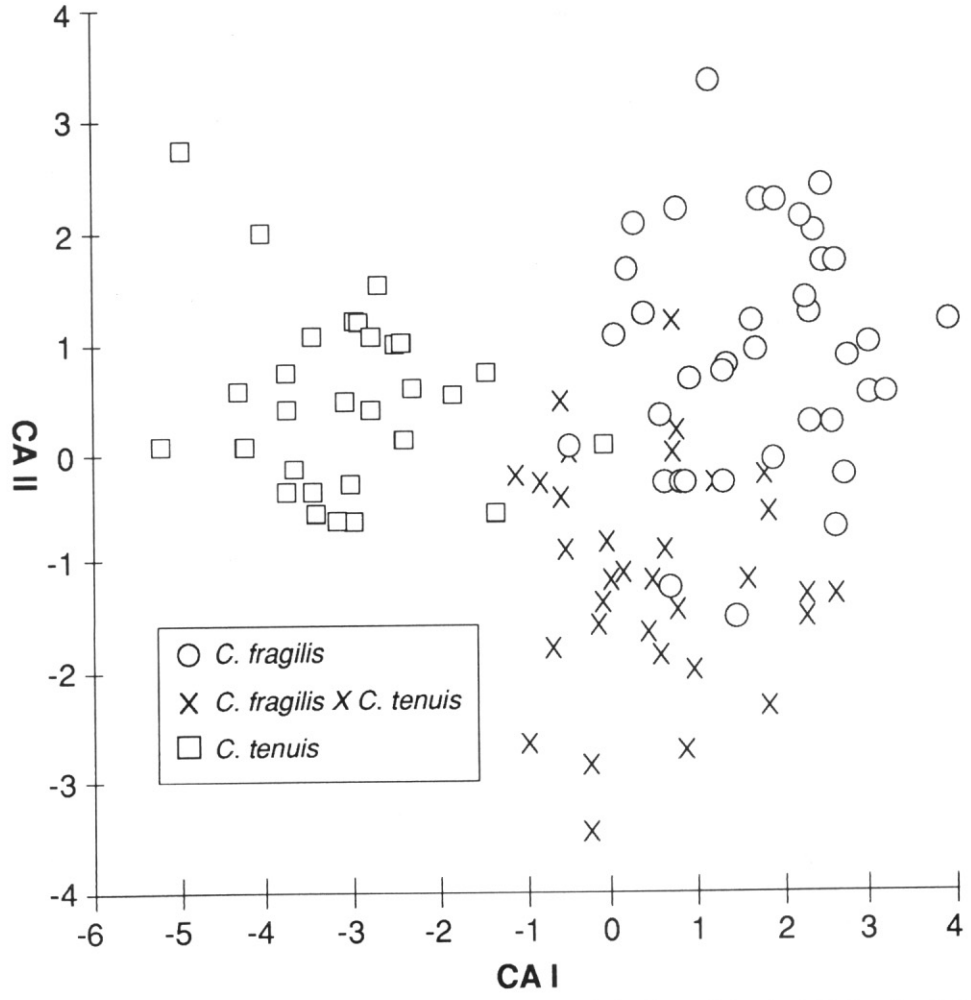


FIG. 10. Individual plants of *Cystopteris fragilis*, *C. tenuis*, and *C. fragilis* × *C. tenuis* plotted on the first and second canonical axes from the discriminant function analysis.

known diploid, "*C. hemifragilis*," hybridized in one event with *C. protrusa* to yield the sterile diploid ($2n = 84$) ancestor of *C. tenuis* and in another event with *C. reevesiana* to yield the sterile ancestor to *C. fragilis*. Under this hypothesis the hybrid between the two fertile allotetraploids *C. fragilis* and *C. tenuis* inherits two sets of chromosomes from "*C. hemifragilis*," one from *C. protrusa*, and one from *C. reevesiana*. Consequently, we expected the minimum number of homologous chromosomes in *C. fragilis* × *C. tenuis*, observable as bivalents at meiosis I, to be 42. We observed 53II and 52II in meiotic cells; the extra bivalents provide evidence for partial complementarity of the diploid genomes of *C. protrusa* and *C. reevesiana* (cf. Wagner 1973; Barrington 1990).

Although isozyme evidence (Fig. 4a, b) suggests *C. tenuis* and *C. fragilis* are allotetraploids, it provides limited support for the common-progenitor hypothesis. Haufler et al. (1990) found the genetic identity of diploids in *Cystopteris* for conservative enzymes to be near zero whereas reports for other ferns (Soltis and Soltis 1989) and angiosperms (Crawford 1983) are considerably higher (0.33 and 0.67 respectively). Based on this information we predicted that PGI2 and TPI for our taxa would demonstrate at least one shared allozyme indicative of the common progenitor. Our system met this ex-

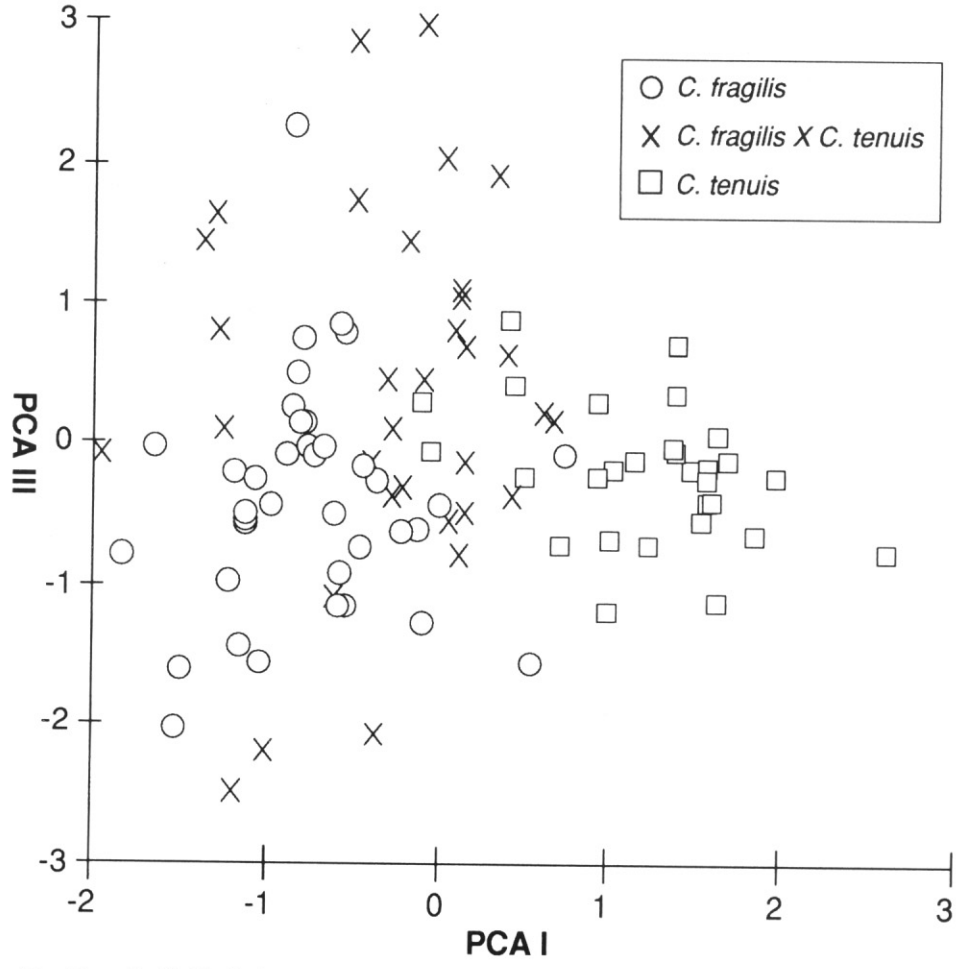


FIG. 11. Plot of individual plants of *Cystopteris fragilis*, *C. tenuis* and *C. fragilis* × *C. tenuis* on first and third principal component axes.

pectation in TPI1 and TPI2 but not in PGI2. At PGI2 the taxa fail to share at least one allozyme; *C. tenuis* is heterozygous for the fast allozymes b and a, whereas *C. fragilis* is a fixed homozygote for slower allozyme c.

Four hypotheses are posed to accommodate this inconsistency: 1) The a or b allele is now silenced in *C. fragilis*; 2) the individuals of "*C. hemifragilis*" involved in forming the hybrids that initiated the two allotetraploids were genetically different at PGI2 (cf. Werth et al. 1985); 3) PGI2 has mutated since initiation of the tetraploid lineages and no longer provides useful phylogenetic information, and 4) *Cystopteris fragilis* is an autotetraploid and does not share a common genome with *C. tenuis*. Under this hypothesis the chromosome pairing behavior observed in *C. fragilis* × *C. tenuis* would be explained as autosyndetic pairing of the two genomes derived from *C. fragilis* (Windham, pers. comm.)

Taxonomic Implications. Two aspects of morphology and ecology that have complicated field identification of *Cystopteris fragilis* and *C. tenuis* in the northeastern United States were uncovered in this study. The first is the presence of hybrids and their confusion with *C. fragilis*. The second is that *C. fragilis* can occupy lowland sites with microclimate that approximates upland sites and, conversely, *C. tenuis* can occupy upland sites with lowland attributes.

The relative success of field identification (Table 3) and jackknifed classifications in the discriminant function model (Table 6) suggest that

there are reliable ecological and structural characters that can be used to discriminate confidently between *C. fragilis* and *C. tenuis*. These characters, summarized next, correspond closely with those described by Moran (1983) and Haufler et al. (1993).

Four major lamina-shape characters, extrapolated from landmark analysis (Figs. 7–9), can be used to discriminate *C. tenuis* from *C. fragilis* reliably: 1) more acute angle of pinna departure; 2) more acute angle of pinnule departure; 3) convex (vs. concave) acroscopic pinna margin, and 4) more open architecture (relative distance between departure points of both pinnae and pinnules). The groups discerned by isozyme electrophoresis also contained a set of notable characters not quantified by landmark analysis. *Cystopteris tenuis* differs from *C. fragilis* in having 1) cuneate (vs. truncate) basal basiscopic pinnules; 2) crenulate (vs. dentate) pinnule margins, and 3) chestnut colored pigmentation extending farther up the petiole.

General Considerations about Landmark Analysis. Literature and computer programs for shape analysis are available, but their application to plants has been spotty at best. Jones (1992, 1993) applied landmark concepts to the study of heteroblasty in *Cucurbita* L., and Niklas (Niklas and Chaloner 1976; Niklas 1977) used finite-element analysis for the interpolation of missing stages in the ontogeny of fossil plants. Kores et al. (1993) used landmarks and thin-plate spline analysis to quantify variation between three orchid species (thin-plate splines graphically model the landmark deformations necessary to transform one object into another). In situations where homologous points are difficult to locate, shape has been analyzed via outline analysis techniques such as eigenshape analysis (Ray 1992; Kores et al. 1993) and Fourier analysis (Kincaid and Schneider 1983; Rohlf and Archie 1984).

Of the various landmark analysis programs available (Richtsmeier et al. 1992), Rotational-Fit was chosen for this study because several characteristics make it an excellent systematic tool. It is effective for comparisons of shapes with subtle differences and it allows for the depiction of a *mean* form plus variance from up to 150 samples of 25 or fewer landmarks. In addition, it provides clear and readily interpretable graphical outputs, and it scales all samples prior to superimposition, a characteristic of primary importance for groups with marked size variation such as *Cystopteris* (Fig. 6).

Landmark analysis is an enticing morphometric approach that bears promise of at least the following three advancements upon traditional approaches.

LANDMARKS ARE OBJECTIVE LOCATIONS. A morphometric analysis is biased if the characters measured are chosen by intuitively discerning the features that best distinguish one taxon from another before measurements are made. Such a priori character selection can lead to the measurement of features that are visibly extreme, such as greatest width or length, deepest dissection, or longest lobe. Such linear measures, although statistically and taxonomically useful, are organism specific, potentially nonhomologous, and often expressed in ambiguous terms such as ratios and transformed values. Homologous landmarks, on the other hand, have an "operational definition in terms of the anatomy and/or ontogenetic history in its vicinity" (Bookstein et al. 1985, p. 7). This characteristic makes the landmark an objective location that exists on all members within (and potentially outside) the study set.

LANDMARKS CAN BE USED TO RECREATE SHAPE. The numerical variables measured in traditional methods are often difficult to relate back to shape, whereas landmark analysis is specifically aimed towards generating simplified two or three dimensional models of an object's shape.

LANDMARKS ARE DEVELOPMENTALLY MEANINGFUL. Based on our current state of knowledge regarding the ontogeny of fern leaves, we contend that landmark deformations reveal real changes in the trajectories of growth centers. In animals, closed (i.e., determinate) development and en masse tissue migration during development complicate position arguments. The fern lamina, on the other hand, has discrete marginal growth centers (meristems) that follow observable trajectories in space and time (Bower 1928). By comparing homologous points on the margins of two differently shaped, fully developed fern laminas, we are comparing forms with primary descriptors that provide insight into the changes in the activity of the marginal meristems yielding the transformations.

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