BIOSYSTEMATIC ANALYSIS OF THE *CYSTOPTERIS TENNESSEENSIS* (DRYOPTERIDACEAE) COMPLEX[1]

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ABSTRACT

The allotetraploid *Cystopteris tennesseensis* and its putative diploid progenitors, *C. bulbifera* and *C. protrusa*, constitute the *C. tennesseensis* complex. Although previous studies provided evidence of morphological, ecological, and chromosomal differences among the members of this complex, puzzling morphological variability precluded consistent identification and treatment of the taxa. The current study combined morphometric analyses with surveys of chromosomal, isozymic, and gametophytic features and supported past treatments of the complex as three separate species. The diploids shared no allozymes for the nine enzymes examined, and meiotic analyses of triploid hybrids with *C. tennesseensis* provided additional evidence that the diploid genomes are nonhomologous. Because *C. tennesseensis* has a relatively narrow range and contains isozymic profiles that are consistently additive of diploid patterns, we suggest that this allopolyploid is a relatively young species. Isozymic variation in the tetraploid parallels that observed in the diploids, implying that genetic variability was introduced through recurrent hybridization. Analyses of isozymic data and gametophytic features indicated that the diploids outcross frequently and thus may form hybrids readily when sympatric with the tetraploid. In part because of these characteristics, precise identification of species and hybrids in this complex is difficult and depends on evaluation of cryptic features.

The cosmopolitan genus *Cystopteris* Bernh. has been called "perhaps the most formidable biosystematic problem in the ferns" (Lovis, 1977, p. 356). Although *Cystopteris* species are primarily north temperate and therefore readily accessible to pteridologists, complex patterns of morphological variation have thwarted satisfactory taxonomic treatments. The most recent monograph (Blasdell, 1963) recognized ten species in two subgenera. Subgenus *Acystopteris* includes the Asian species *C. japonica* and *C. tenuisecta*, which are so distinctive that some have placed them in a separate genus (Nakai, 1933; Pichi-Sermolli, 1977). Blasdell divided the remaining species (all in subg. *Cystopteris*) into two sections based on the pattern of vein termination in the leaves. Species having veins directed into teeth were placed in sect. *Cystopteris*, while those with veins directed into sinuses were assigned to sect. *Emarginatae*. Blasdell encountered variability for this vein termination character in some specimens and suggested that such anomalies could result from introgression. Lovis (1977), on the other hand, considered it more likely that venation features are not as stable in some species as in others and should not be used to define sections.

Our observations suggested that Lovis was correct and, rather than organizing the North American *Cystopteris* species into morphologically based sections, we have chosen to concentrate on interactive species complexes involving allopolyploid species and their putative diploid progenitors. One

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such complex centers on *C. tennesseensis*, a species first recognized by Shaver (1950) who described it as a hybrid between *C. bulbifera* and *C. protrusa*. In 1954 Shaver developed an extended discussion of the morphology and ecology of the complex which still stands as an excellent summary of the natural history of this difficult group.

From the moment it was named, there was disagreement concerning the proper status and disposition of *C. tennesseensis*. McGregor (1950), in collaboration with C. A. Weatherby, reduced this species to a variety of *C. fragilis*. In addition, citing specimens that did not conform to the protologue of *C. fragilis* var. *tennesseensis* and that seemed to be close to Weatherby's *C. fragilis* f. *simulans*, McGregor recognized *C. fragilis* var. *simulans*. This approach seemed justified because some specimens tend to bridge the morphological gap between these two varieties as well as between *C. fragilis* var. *fragilis* and *C. fragilis* var. *tennesseensis*. However, Blasdell (1963) revealed *C. tennesseensis* as a tetraploid with $n = 84$ chromosomes. His analysis showed that hybridization between *C. protrusa* and *C. bulbifera* was the most reasonable explanation for the origin of this tetraploid. Therefore, Blasdell resurrected *C. tennesseensis* as a species, including the former *C. fragilis* var. *simulans*.

The subtlety of morphological features separating the species as well as the continued discovery of intermediates has perpetuated the systematic confusion of the *C. tennesseensis* complex. Even though the accumulated data demonstrate conclusively that *C. tennesseensis* is isolated from its congeners, this tetraploid has not been widely accepted as a distinct species. As recently as 1982, Moran (1982, p. 94) found in a study of *Cystopteris* specimens from Illinois that "all 90 herbarium specimens of *C. tennesseensis* . . . were originally misidentified."

In this paper, we report a series of analyses designed to investigate the patterns and the processes behind the systematic confusion in *C. tennesseensis*. We describe studies of biogeography, morphology, chromosomes, and isozymes that clarify the origin and current status of *C. tennesseensis* and its progenitor diploids.

Materials and Methods

Observations of morphological variability, geographic distribution, and habitat diversity were obtained from surveys of herbarium specimens. If specific locality data were supplied, collection sites were plotted by county and used to obtain distributional information. When the gross morphology of specimens was not sufficient to assign them readily to species, spores were removed and mounted in Permount on glass microscope slides. The longest diameter of the monolete spores (Table 1) and evidence of abortion (e.g., shrunken or malformed spores) was noted. Spore slides were placed in envelopes and attached to herbarium sheets.

Table 1. Mean spore sizes and standard deviations calculated by measuring the long axis of 25 spores. Specimens whose ploidy level has been verified by meiotic chromosome squashes are indicated by asterisks. States listed in parentheses are those from which the specimens were collected.

C. bulbifera (Illinois)	37.48 ± 1.949
*C. bulbifera** (Oklahoma)	35.21 ± 3.408
*C. bulbifera** (Arizona)	36.27 ± 2.560
*C. bulbifera** (Indiana)	35.96 ± 2.325
*C. bulbifera** (Ohio)	39.81 ± 2.944
C. bulbifera (Ohio)	35.71 ± 3.038
C. bulbifera (Kentucky)	34.39 ± 1.764
C. bulbifera (Indiana)	36.78 ± 2.336
	mean = 36.45 ± 1.650
C. protrusa (Illinois)	33.65 ± 1.788
*C. protrusa** (Kansas)	35.22 ± 4.714
C. protrusa (Missouri)	33.33 ± 2.421
*C. protrusa** (Kansas)	30.77 ± 1.868
*C. protrusa** (Kansas)	30.98 ± 2.260
*C. protrusa** (Michigan)	33.21 ± 2.332
*C. protrusa** (North Carolina)	34.22 ± 3.042
	mean = 33.05 ± 1.634
*C. tennesseensis** (Kansas)	40.02 ± 3.250
*C. tennesseensis** (Missouri)	41.40 ± 1.907
*C. tennesseensis** (Illinois)	41.29 ± 2.416
*C. tennesseensis** (Arkansas)	40.65 ± 2.084
	mean = 40.84 ± 0.639

To supplement herbarium collections and supply living material for chromosomal, isozymic, and common garden morphological comparisons, specimens were collected over much of the range of the genus in North America and were donated as noted in footnote 1. Living collections were maintained in the University of Kansas greenhouses. Collection sites for materials used in this study are listed in Table 2.

Ecologically induced morphological variation may obscure genetically based species distinctions. Therefore, we conducted discriminant analyses of morphological variation based on plants cultivated in the greenhouse. All individual plants were identified and assigned to a group on the basis of chromosome number and isozymic composition. Fea-

TABLE 2. Locality data for collections providing material for electrophoretic and cytogenetic analyses. Asterisks indicate localities used for population genetic analyses (see Table 4).

Locality	Collector	Chromosome number verified?
Cystopteris bulbifera		
Arizona: Coconino Co., Rio de Flag	*Windham 194*	Yes, 42II
Arizona: Coconino Co., Oak Creek Canyon	*Windham 314*	Yes, 42II
Arizona: Coconino Co., Lower West Fork	*W. H. Wagner 82113*	Yes, 42II
Indiana: Fountain Co., Portland Arch*	*C. H. Haufler & R. C. Moran s.n.*	No
Indiana: Jefferson Co., Clifty Falls	*C. H. Haufler & R. C. Moran s.n.*	No
Indiana: Monroe Co., Cedar Bluffs*	*C. H. Haufler & R. C. Moran s.n.*	No
Kentucky: Powell Co., Natural Bridge*	*C. H. Haufler & R. C. Moran s.n.*	No
Ohio: Adams Co., near Stout*	*C. H. Haufler & R. C. Moran s.n.*	Yes, 42II
Oklahoma: Ottawa Co., Dripping Springs	*C. H. Haufler & C. K. Teale s.n.*	Yes, 42II
Wisconsin: Door Co., Peninsula State Park	*W. C. Taylor s.n.*	Yes, 42II
C. protrusa		
Iowa: Fremont Co., SW of Sidney	*R. E. Brooks 14929*	Yes, 42II
Illinois: Cook Co., McGinness Slough Conservation Area	*R. C. Moran s.n.*	No
Illinois: Union Co., Shawnee National Forest	*C. H. Haufler s.n.*	Yes, 42II
Indiana: Monroe Co., Cedar Bluffs*	*C. H. Haufler & R. C. Moran s.n.*	No
Indiana: Monroe Co. Cascades Park*	*C. H. Haufler s.n.*	No
Indiana: Monroe Co., Farr Road*	*C. H. Haufler & R. C. Moran s.n.*	No
Indiana: Perry Co., N of Tell City*	*C. H. Haufler s.n.*	No
Kansas: Douglas Co., Breidenthal Woods*	*Windham 579*	Yes, 42II
Kansas: Chautauqua Co., W of Elgin	*R. E. Brooks 16162*	Yes, 42II
Kansas: Miami Co., S of Homewood	*C. H. Haufler s.n.*	Yes, 42II
Michigan: Washtenaw Co., Homer Woods	*C. H. Haufler & W. H. Wagner s.n.*	Yes, 42II
Missouri: Boone Co., SW of Columbia	*R. E. Brooks 15298*	Yes, 42II
Missouri: Cooper Co., Arrow Rock	*R. E. Brooks & C. H. Haufler 15343*	Yes, 42II
Missouri: Franklin Co., Meramec State Park	*R. E. Brooks & C. H. Haufler 15341*	Yes, 42II
Missouri: St. Louis Co., near Allenton*	*R. E. Brooks & C. H. Haufler 15332*	Yes, 42II
North Carolina: Swain Co., Nantahala Gorge	*W. H. Wagner s.n.*	Yes, 42II
C. tennesseensis		
Illinois: Jackson Co., Fountain Bluff	*C. H. Haufler & R. C. Moran s.n.*	Yes, 84II
Kansas: Doniphan Co., N of Wathena	*R. E. Brooks 14898*	Yes, 84II
Missouri: Franklin Co., E of Sullivan	*R. E. Brooks & C. H. Haufler 15334*	Yes, 84II
Missouri: LaClede Co., Big Niangua River	*B. C. Phillips s.n.*	Yes, 84II
Nebraska: Richardson Co., NE of Shubert	*R. E. Brooks 14925*	Yes, 84II
Oklahoma: Ottawa Co., Dripping Springs	*C. H. Haufler & C. K. Teale s.n.*	Yes, 84II
C. tennesseensis × *C. bulbifera*		
Missouri: Boone Co., SW of Columbia	*R. E. Brooks & C. H. Haufler 15302*	Yes, 42II + 42I
Nebraska: Richardson Co., NE of Shubert	*R. E. Brooks 14925a*	Yes, 42II + 42I
C. tennesseensis × *C. protrusa*		
Kansas: Chautauqua Co., W of Elgin	*R. E. Brooks 16163*	Yes, 42II + 42I
Missouri: St. Louis Co., near Allenton	*R. E. Brooks & C. H. Haufler 15331*	Yes, 42II + 42I

tures surveyed are listed in Table 3 and depicted in Figure 1. Because the species are often difficult to discern based solely on leaf features (often the only part preserved on herbarium specimens), we first used only quantitative leaf features to seek species-specific leaf attributes or combinations thereof. Involved in this analysis were 9 plants of *C. bulbifera*, 21 of *C. protrusa*, and 8 of *C. tennesseensis*. Because our first analysis could not completely discriminate the species (see Results),

a second analysis was conducted that included qualitative characters (Table 3) as well as mean spore sizes per plant (Table 1). This second analysis included 8 plants of *C. bulbifera*, 14 of *C. protrusa*, and 4 of *C. tennesseensis*. The data from both sets were analyzed with the BMDP7M stepwise discriminant analysis program (BMDP Statistical Software, 1981, UC Press). For each step in the analyses we allowed an experiment-wise error rate of $\alpha = 0.05$ and judged the significance of each approximate F-ratio as the criterion for entry of a variable into the discriminant models. The critical values of F were estimated using Bonferroni's inequality (Ranker & Schnabel, 1986).

Unlike angiosperms, the gametophyte generation of fern species is independent of the sporophyte. Whereas the sporophyte is important in maintaining long-term survival of individuals, the gametophyte carries out sexual reproduction and initial establishment of populations. Thus, in developing a complete picture of the biology of fern species as well as characterizing evolutionary tendencies, analyses of gametophyte reproductive biology are necessary. In a previous study Haufler & Ranker (1985) determined which *Cystopteris* species produced and/or responded to antheridiogen. In the present study 100 gametophytes of each species were reared individually, employing the culture conditions described in Haufler & Ranker (1985), and assayed for genetic load following the procedures outlined by Lloyd (1974).

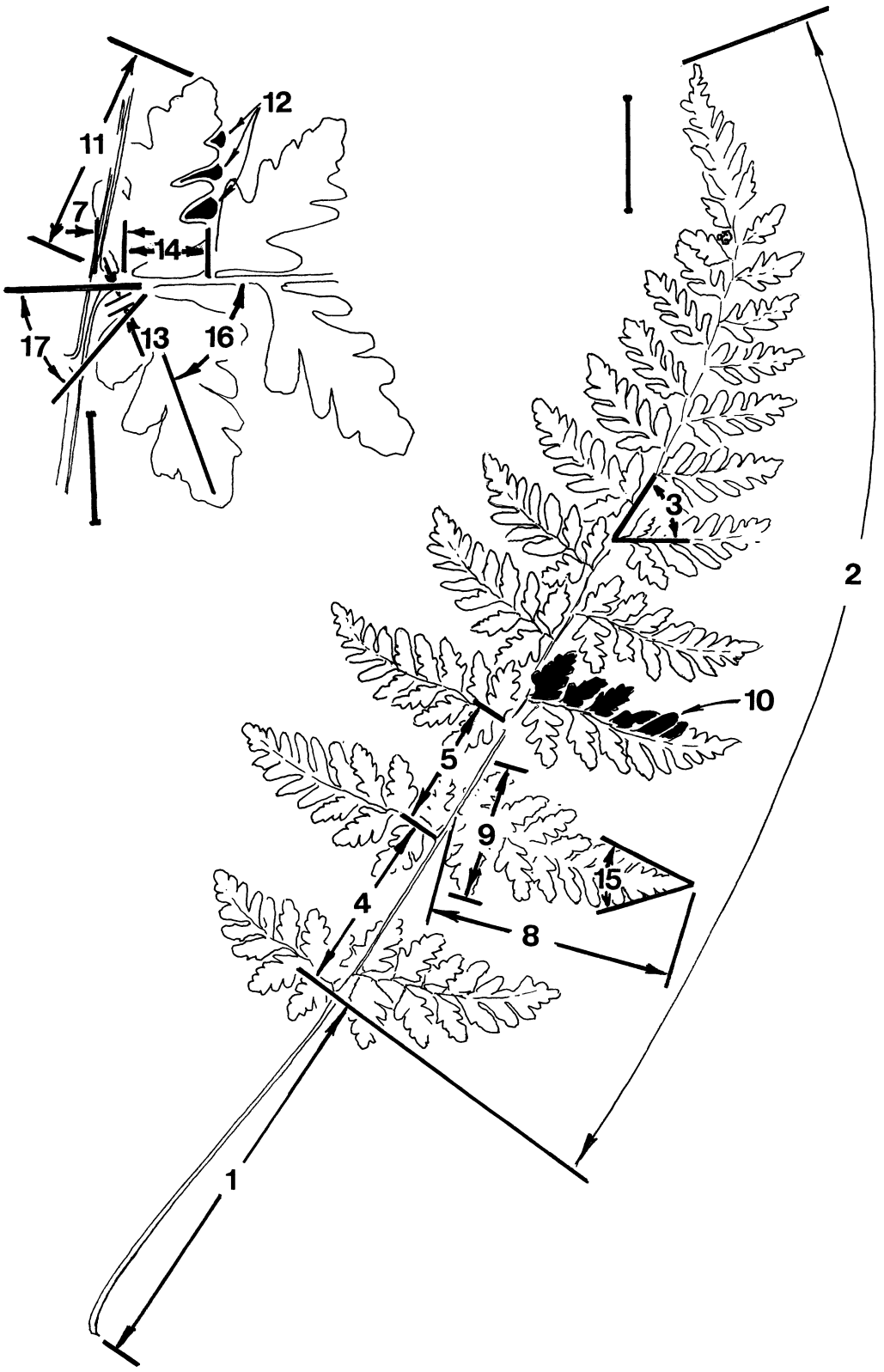
Analyses of chromosomal behavior at meiosis were especially important in characterizing suspected hybrid individuals. The procedures followed in obtaining and photographically documenting stages in sporogenesis were described in Haufler et al. (1985). Voucher specimens for each chromosome count will be deposited at KANU.

Electrophoretic analyses of isozyme variability were performed as described by Haufler (1985). Leaf samples were ground in the phosphate grinding buffer of Soltis et al. (1983) using a mortar and pestle or by placing them in spot plate wells and using a round-bottomed centrifuge tube as a pestle. Each of the 22 enzymes listed in Soltis et al. (1983) was surveyed and was subjected to a variety of gel and electrode buffer conditions (largely those of Soltis et al., 1983). Enzymes that provided consistent, interpretable results were hexokinase (HK), isocitrate dehydrogenase (IDH), leucine amino peptidase (LAP), the more cathodal (presumably cytosolic—see Weeden, 1983) bands of phosphoglucoisomerase (PGI-2), two sets of bands (presumably representing cytosolic and chloroplastic enzymes—see Weeden, 1983) for phosphoglucomutase (PGM-1, the more anodal set, and PGM-2, the more cathodal set), shikimate dehydrogenase (SkDH), and two sets of bands (presumably representing cytosolic and chloroplastic enzymes—see Weeden, 1983) for triosephosphate isomerase (TPI-1, the more anodal set, and TPI-2, the more cathodal set). HK, PGI-2, LAP, TPI-1, and TPI-2 showed the best resolution on either system 6 of Soltis et al. (1983) or the modified system 8 discussed in Haufler (1985). IDH, PGM-1, PGM-2, and SkDH were resolved best on the modified system 11 of Soltis et al. (1983) discussed in Haufler (1985). Identification of bands shared among populations and species was accomplished through co-electrophoresis of the samples on the same gel.

Individual gametophytic progeny were used to assess the genetics of sporophytic banding patterns. We followed the electrophoretic procedures de-

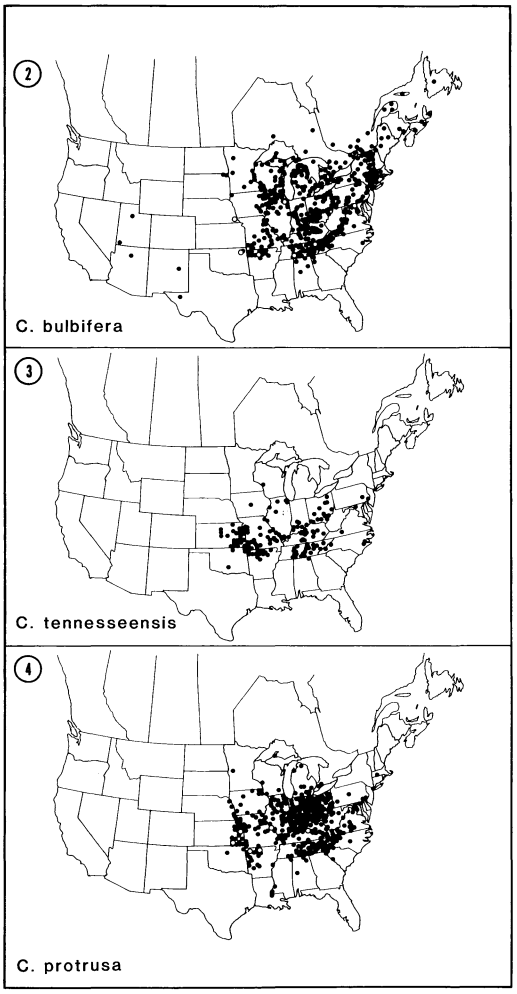
TABLE 3. List of features included in morphometric analysis. Asterisks indicate those illustrated in Figure 1. Plus marks indicate features that were statistically significant in discriminating species.

1–5. FEATURES OF ENTIRE LEAF
*1. Length of petiole (cm)
*2. Length of blade (cm)
*3. Angle of departure of median pinna
*4. Length of rachis between lowest pinna of first pinna pair and lowest pinna of second pinna pair (mm)
*5. Length of rachis between lowest pinna of second pinna pair and lowest pinna of third pinna pair (mm)
6–17. FEATURES OF LONGEST PINNA
6. Number of pinna pairs from base (first one = number 1)
*7. Length of pinna stalk (petiolule) (mm)
*8. Length of pinna (mm)
*9. Width of pinna (mm)
+*10. Number of segments along acroscopic edge of pinna (including pinnatifid ones)
*11. Length of first acroscopic pinnule (mm)
*12. Number of major sinuses along distal edge of first acroscopic pinnule
*13. Length of stalk of basiscopic pinnule (mm)
*14. Length of pinna rachis between lowest two pinnule pairs (mm)
*15. Angle formed by apex of pinna
+*16. Angle formed by basiscopic pinnule axis with pinna axis
*17. Angle of proximal portion of basiscopic pinnule base with pinnule axis
18–20. QUALITATIVE FEATURES
18. Presence or absence of bulblets
+19. Presence or absence of glandular trichomes
20. Color of petiole
+21. Spore size (see Table 1)



scribed by Gastony & Gottlieb (1982) as modified by Haufler & Soltis (1984). For the enzymes surveyed, gametophytes express the same isozymes as do sporophytes. Because gametophytes are multicellular, haploid individuals derived from single meiotic products (spores), gametophytic progeny arrays from single sporophytes can be used to determine directly the genetic constitution of complex sporophytic banding patterns. In this way, segregational analyses of putatively heterozygous banding patterns can be performed without pursuing more time-consuming crossing programs.

After allelic determinations were made for each of the nine putative loci, allozymic data from a set of representative populations (Table 4) were analyzed. The proportion of polymorphic loci (P) and the mean number of alleles per locus (A) were calculated from these populational data. A statistical program (LYNSPROG) written by Marilyn Loveless, The College of Wooster, Wooster, Ohio, was used to calculate levels of heterozygosity, the fixation index (*F*), and Nei's coefficients of genetic identity and distance. Because *C. protrusa* tends to be clonal, separate calculations were made for populations consisting of (1) each leaf sampled (ramets) and (2) only the number of different genotypes (genets). The first should overestimate the number of individual organisms while the second should underestimate it.



FIGURES 2–4. Geographic distributions of members of the *Cystopteris tennesseensis* complex based on data from herbarium specimens. Dots indicate counties where specimens were collected.—2. *C. bulbifera*.—3. *C. tennesseensis*.—4. *C. protrusa*. Open circles on Figures 2 and 4 show locations of putative triploid backcross hybrids.

RESULTS

SPORES

Blasdell (1963) and Moran (1982) noted the value of spore features for identifying hybrids and in determining the ploidy of specimens. Despite Lovis's (1977) caveat that it may not be appropriate in *Cystopteris* to infer ploidy levels from spore measurements, we were able to verify independently through analyses of meiotic chromosome behavior that spore size did correlate with several critical genetic features of species (Table 1). We agree with Lovis that spore size comparisons alone do not substitute for direct chromosome analyses, but once a strong correlation between spore features and genetic condition is established, spores can be extremely valuable in surveying specimens rapidly. Each species differed significantly in mean spore size from the other two (pairwise *t*-tests, $P < 0.002$). Although *C. bulbifera* spores were larger than those of *C. protrusa*, spores of tetraploid *C. tennesseensis* showed the further size increase commonly associated with higher ploidy.

MORPHOLOGICAL ANALYSES OF SPOROPHYTES

The taxa in the *Cystopteris tennesseensis* complex are among the most distinctive in the genus. There are more unique features that characterize

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FIGURE 1. Leaf features included in morphometric analysis. Numbers correspond to descriptions of features in Table 3. Scale bars: for whole leaf = 2.0 cm; for pinna base = 0.5 cm.

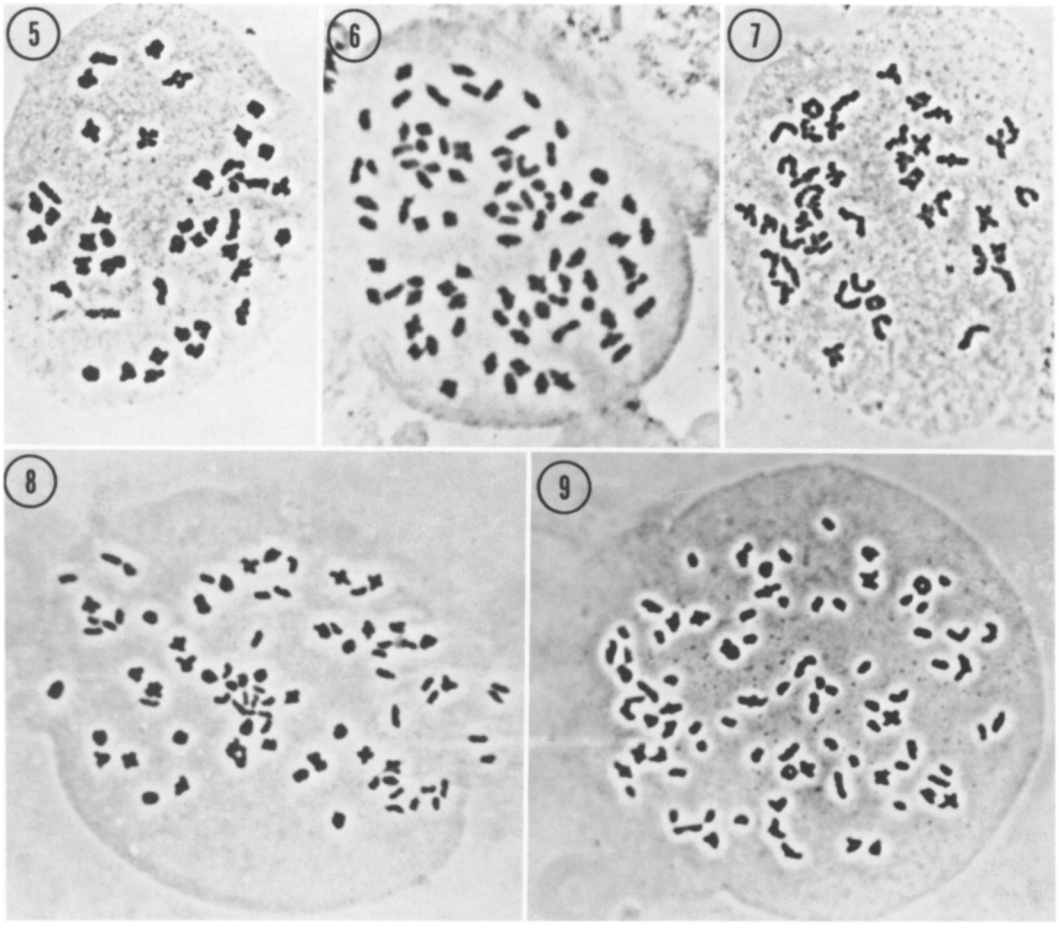
each species than there are in any other group of *Cystopteris* species. *Cystopteris protrusa*, for example, has long internodes and a peculiar, protruding rhizome apex whose growing point extends past the current season's leaves. Given the evidently rapid growth of the rhizome, we were not surprised to find extensive clones of this species in mature woodlands. *Cystopteris bulbifera*, commonly found on moist cliffs, has short internodes and bears asexual reproductive bulblets on its leaves, and most individuals have tack-shaped, glandular trichomes that are particularly prominent on the indusia and along the rachis between pinnae. The allotetraploid *C. tennesseensis* combines the genomes of its progenitor diploids and, perhaps as a result of this genetic amalgamation, is morphologically variable. In many features, the tetraploid is intermediate between the diploids, e.g., it has poorly formed bulblets and a reduced frequency of glandular trichomes. Its rhizome features, however, are not intermediate. Perhaps because it inhabits somewhat disturbed, often dry cliffs rather than forest floors, it has the short internodes typical of *C. bulbifera*.

Even though past workers (Shaver, 1954; Blasdell, 1963) provided well constructed species descriptions and indicated the distinctive features of each species, taxonomists not familiar with the genus continue to encounter problems when identifying members of the *C. tennesseensis* complex. To generate additional discriminating features and in trying to separate environmentally induced variability from that based on genetic differences, we performed a discriminant analysis of leaf morphology on plants grown in a common garden. In the first analysis, employing only quantitative leaf characters, only two features were entered into the model as being statistically significant, the number of acroscopic segments on the longest pinna (#10, Table 3, Fig. 1) and the angle formed by the basiscopic pinnule axis with the pinna axis (#16). The species differed significantly from one another in the space defined by the two canonical axes ($P < 0.01$). The first axis accounted for 85% of the total variance and separated *C. bulbifera* from the other two species. The two variables in the model exhibited similar character loadings (standardized coefficients) on the first axis, 0.608 for #10 and 0.720 for #16, indicating a nearly equal contribution to the canonical variable. Inspection of the original data revealed that *C. bulbifera* is distinguished from the other species by a larger number of acroscopic segments on the longest pinna and a larger angle formed by the basiscopic pinnule. The second axis accounted for the remaining 15% of the variance. The jackknifed classification matrix correctly identified 100% of the *C. bulbifera* individuals, 71.4% of *C. protrusa*, and 87.5% of *C. tennesseensis*. Six individuals of *C. protrusa* were incorrectly identified as *C. tennesseensis*, and one individual of *C. tennesseensis* was incorrectly identified as *C. protrusa*.

A second discriminant analysis was conducted in which qualitative leaf features (listed in Table 3) and spore size were added to the data set in an attempt to identify combinations of characters that would allow a better discrimination of species than was obtained in the first analysis. In this second analysis, three characters were entered into the model as statistically significant: angle of the basiscopic pinnule (#16), presence or absence of glandular trichomes, and mean spore size (Table 1). The first canonical axis accounted for 82% of the total variance, and the character loadings were 0.809 (#16), 0.875 (glandular trichomes), and 0.485 (spore size). The first axis primarily separated the two diploids from one another. As indicated by the relative value of the loadings, the diploids were primarily distinguished by the angle of the basiscopic pinnule and the presence or absence of glandular trichomes. On the second axis, the character loadings were −0.081 (#16), 0.429 (glandular trichomes), and −0.887 (spore size). The large spore size of the tetraploid, therefore, was the most important factor in discriminating it from the diploids. The jackknifed classification matrix produced 100% correct classifications for all individuals in the analysis.

BIOGEOGRAPHY

The distribution of species is shown in Figures 2–4. *Cystopteris bulbifera* is the most northern species, extending well into Canada. *Cystopteris protrusa* is most common in the east-central United States and is rare in southern Canada (Britton et al., 1984; Haufler et al., 1985). The allotetraploid derivative *C. tennesseensis* is found primarily in the region of overlap of the two diploids. Using spore abortion as an indicator of sterility, we identified interspecific hybrids between the tetraploid and its diploid parents. The locations of these hybrids are indicated as open circles on Figures 2 and 4. Because the morphological features are quite plastic, it is not always possible to determine which of the diploids hybridized with *C. tennesseensis* in forming the sterile plants. Using chromosomal and isozymic data, we were able to document that backcrosses to both parents do occur in nature (see below).

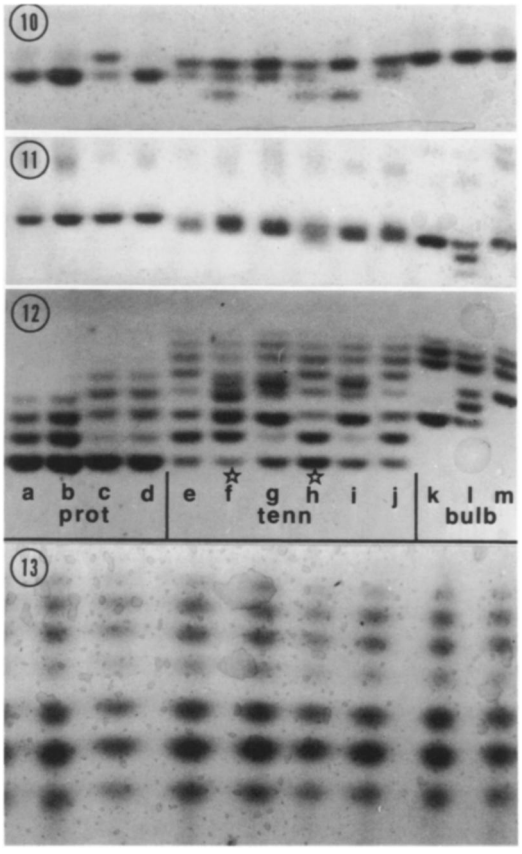


FIGURES 5–9. Representative meiotic chromosome squashes.—5. *C. bulbifera*, $n = 42II$.—6. *C. tennesseensis*, $n = 84II$.—7. *C. protrusa*, $n = 42II$. 8, 9. Backcross hybrids between *C. tennesseensis* and its diploid progenitors. Identification of hybrids was based on morphological and isozymic data.—8. Hybrid between *C. tennesseensis* and *C. bulbifera*, $n = 42II + 42I$.—9. Hybrid between *C. tennesseensis* and *C. protrusa*, $n = 42II + 42I$. All × 1,000.

GAMETOPHYTES

Summarizing previous studies of *Cystopteris* gametophytes (Blasdell, 1963; Profumo, 1969; Auquière & Moens, 1972), Blasdell (1963, p. 6) stated, "All of the taxa examined are similar in the bulk of their gametophytic features." Our study of gametophyte ontogeny confirmed Blasdell's assessment of the level of morphological variability. However, Haufler & Ranker (1985) demonstrated significant interspecific variation in response to the pheromone antheridiogen.

Genetic load studies have been used to look into aspects of reproductive biology (Lloyd, 1974). Because inbreeding brings about the expression of recessive alleles, species with low genetic load should be more inbred, whereas those having high genetic load are presumed to be outcrossers. In homosporous ferns, levels of genetic load can be determined by assessing the ability of isolated gametophytes to produce sporophytes. In the present study, none of the 100 isolated gametophytes of *C. bulbifera* and *C. protrusa* produced sporophytes, even after repeated waterings over a four-month period. However, 25 of the 100 isolated gametophytes of *C. tennesseensis* did form sporophytes. Although we did not examine each isolated gametophyte, we have verified that gametophytes of the diploid species do become hermaphroditic in culture. Thus, genetic load in the tetraploid is significantly lower than that in the diploids (3×2 contingency table of species vs. sporophyte production: $G = 59.63$, $P < 0.001$).



FIGURES 10–13. Representative isozyme profiles. Anode = top of photographs, cathode = bottom of photographs. 10–12. Interspecific comparisons of banding patterns. Lanes a–d = *C. protrusa*; e–j = *C. tennesseensis*; k–m = *C. bulbifera*. Lanes f and h (starred) are from triploid backcross hybrids between *C. tennesseensis* and *C. protrusa*.—10. SkDH (monomeric, single compartment). Compare lanes i and j where the upper band of these fixed heterozygotes are from *C. bulbifera* and the lower bands are from *C. protrusa*. Differences in the *C. protrusa* contribution may have resulted from recurring origin of the tetraploid. Note that the backcross hybrids contain both *C. protrusa* bands.—11. PGI (dimeric; anodal, presumably chloroplastic bands are not well resolved and were not scored; cathodal bands are presumably cytoplasmic). Because the *C. bulbifera* and *C. protrusa* bands have very similar mobilities, the fixed heterozygous pattern in *C. tennesseensis* appears as a broad fuzzy band consisting of the parental bands and an interlocus heterodimeric band.—12. TPI (dimeric; in both diploids, anodal, multiple-banded pattern probably represents a post-translational modification of the enzyme (see discussion in Gastony, 1988, and Hickey et al., 1989)); cathodal bands show typical dimeric enzyme expression in homozygotes and heterozygotes (compare lanes k–m); in tetraploids, anodal and cathodal bands overlap. Lanes a and b vs. lanes c and d show differing mobilities for the multiple-banded enzymes. Lanes i and j probably are from plants that had different *C. bulbifera* parents. Lane i received the more anodal band from *C. bulbifera* while lane j received the more cathodal band.—13. TPI expression in individual gametophytic progeny of a sporophyte having the banding pattern seen in lanes e and j of Figure 12. There is no segregation for the complex pattern, indicating that the sporophyte was a fixed heterozygote.

MEIOTIC CHROMOSOME BEHAVIOR

Analyses of meiosis from plants collected across the species ranges generated consistent counts of $n = 42$ in *C. bulbifera* (Fig. 5) and *C. protrusa* (Fig. 7), and $n = 84$ in *C. tennesseensis* (Fig. 6). Table 2 lists the collection localities of the plants yielding new chromosome counts. In addition to analysis of the sexual species, plants verified via isozyme analysis to be backcross hybrids were also studied cytogenetically. As shown in Figures 8 and 9, all hybrids showed a complement of 42 bivalents plus 42 univalents expected from backcross hybrids of an allotetraploid to its diploid progenitors. These results indicate little or no chromosomal homology between the diploid species *C. protrusa* and *C. bulbifera* and suggest that *C. tennesseensis* contains one genome of each.

ISOZYMES

Isozyme patterns of the tetraploid *C. tennesseensis* are perfectly additive of those from the two diploid species. Gametophytic progeny arrays demonstrated that the complex banding patterns did not segregate during meiosis (Fig. 13), suggesting that the isozymes comprising these patterns were coded by genes situated on nonhomologous chromosomes contributed by the two diploid parents. Given that all tetraploids showed fixed heterozygosity that always combined patterns from each diploid progenitor, no evidence of "orphan alleles" or gene silencing could be detected. All isozymic variability observed in the allotetraploid apparently resulted from the incorporation of various diploid allozymes into the polyploid derivative via recurring allopolyploid events (Figs. 10–12, and see Haufler & Soltis, 1986).

Combining chromosomal and isozymic data made it possible to identify hybrids and determine their parentage. Sporogenesis was studied in plants having aborted spores. Such plants were shown to be triploids having 42 pairs and 42 univalents during meiosis and thus were determined as backcrosses between *C. tennesseensis* and either *C. protrusa* or *C. bulbifera*. Even though the variability of morphological features could preclude precise identification of parents, the triploids always contained unequal enzyme contributions from the two parents. Thus, dosage effects could be used to show

TABLE 4. Locations and sizes (n) of populations of diploid *Cystopteris* species surveyed in generating genetics statistics (see Tables 5–7).

Cystopteris bulbifera

Population #1—Fountain Co., Indiana, Portland Arch, N of Covington—$n = 18$
Population #2—Adams Co., Ohio, limestone cliffs near Stout—$n = 41$
Population #3—Monroe Co., Indiana, Cedar Bluffs, S of Bloomington—$n = 11$
Population #4—Powell Co., Kentucky, along trail in Natural Bridge State Park—$n = 12$

C. protrusa

Population #1—Douglas Co., Kansas, Breidenthal Woods, near Lawrence
Population #2—Monroe Co., Indiana, Cedar Bluffs, S of Bloomington
Population #3—Monroe Co., Indiana, Cascades Park, NNW of Bloomington
Population #4—Monroe Co., Indiana, Farr Rd., N of Bloomington
Population #5—St. Louis Co., Missouri, near Allenton
Population #6—Perry Co., Indiana, N of Tell City

Population sizes for *C. protrusa* calculations

First set of calculations based on ramets	Second set of calculations based on genets
Population #1—$n = 104$	Population #1—$n = 15$
Population #2—$n = 17$	Population #2—$n = 3$
Population #3—$n = 18$	Population #3—$n = 2$
Population #4—$n = 12$	Population #4—$n = 6$
Population #5—$n = 10$	Population #5—$n = 7$
Population #6—$n = 24$	Population #6—$n = 10$

which backcross had formed the hybrids. Hybrids formed between *C. tennesseensis* and *C. protrusa* had the *C. protrusa* bands stained more intensely than the *C. bulbifera* contribution (Figs. 10–12, lanes f and h). Conversely, *C. tennesseensis* × *C. bulbifera* hybrids contained a double dose of *C. bulbifera*.

Although all nine enzymes helped corroborate that *C. tennesseensis* was an amalgam of its progenitor diploids, the most complex and informative enzyme was TPI (Fig. 12). For both diploids, banding patterns indicative of two genetic loci were resolved. The cathodal bands showed banding patterns typical of dimeric enzymes (single-banded in homozygotes and three-banded in heterozygotes). The anodal zone of bands were expressed as fixed, multiple-banded patterns which probably represent post-translational modifications (Gastony, 1988; Hickey et al., 1989). In *C. protrusa* (lanes a–d), the strongly staining, most cathodal band was an invariant marker for the species. The more weakly staining, multiple-band pattern was variable. As shown in Haufler & Soltis (1986), three patterns were detected among the sporophytes surveyed: a slow-migrating triplet (as in lanes a and b of the present paper), a fast-migrating triplet (not illustrated here, but see Haufler & Soltis (1986, fig. 1a)), and the complex, five-banded heterozygote between them (as in lanes c and d). In *C. bulbifera* (lanes k–m), the rapidly migrating triplet pattern was invariant while there were two alleles expressed for the cathodal locus. Lane l is the heterozygote formed from outcrossing between homozygotes such as k and m. When these diploid variants are combined in *C. tennesseensis* (lanes e–j), some remarkably complex patterns result. Lanes e and j appear to be relatively simple additive patterns between plants such as those in lanes a (*C. protrusa*) and k (*C. bulbifera*). Lanes g and i combine the bands found in lanes a and m. Lane f (identified via morphology and meiotic chromosomal behavior as a triploid backcross between *C. protrusa* and *C. tennesseensis*) combines one dose of a profile like lane m with two doses of lane b. Lane h (also a *C. protrusa* × *C. tennesseensis* triploid) appears to combine one dose of a plant like lane k with two doses of one like lane b or c. Note also the banding patterns for the triploids in SkDH (Fig. 10). For lanes f and h, the two cathodal bands are shared with *C. protrusa* while only the most anodal band is found in *C. bulbifera*. These SkDH patterns help confirm that both triploids contain two genomes from *C. protrusa* and only one from *C. bulbifera*. The slowest migrating SkDH band in *C. tennesseensis* (lanes f, h, and i) is from *C. protrusa* even though it is not seen in the representative *C. protrusa* plants on this gel. The rapidly migrating SkDH band in lane c is a rare *C. protrusa* variant

TABLE 5. Population genetics statistics. P = proportion polymorphic loci, A = average number of alleles per locus. P and A were identical for the ramet and genet samples of *C. protrusa*.

Cystopteris bulbifera

Population number	P	A	Mean heterozygosity Observed	Mean heterozygosity Expected
1	0.67	1.89	0.435	0.470
2	0.56	1.78	0.138	0.219
3	0.56	1.78	0.394	0.334
4	0.67	1.78	0.347	0.273
Mean	0.62	1.81	0.329	0.324

Cystopteris protrusa

Population number	P	A	Mean heterozygosity for ramets Observed	Mean heterozygosity for ramets Expected	Mean heterozygosity for genets Observed	Mean heterozygosity for genets Expected
1	0.56	1.67	0.128	0.153	0.152	0.223
2	0.22	1.22	0.042	0.104	0.048	0.134
3	0.56	1.56	0.524	0.302	0.429	0.286
4	0.78	1.88	0.357	0.342	0.286	0.345
5	0.56	1.67	0.157	0.235	0.204	0.273
6	0.44	1.56	0.185	0.176	0.200	0.198
Mean	0.52	1.59	0.232	0.219	0.220	0.243

not incorporated in any of the *C. tennesseensis* plants shown here.

A series of populations was surveyed (Table 4) and used to generate calculations of P, A, and levels of heterozygosity (Table 5), Nei's coefficients of genetic identity and distance (Table 6), and the fixation index (*F*) (Table 7). Results derived from ramet and genet views of individuality in populations of *C. protrusa* did not differ markedly. Observed heterozygosity differed by 0.012 and mean genetic identities were within 0.013 of each other. Although the calculations of *F* derived from the ramet samples appear to have a large number of significant departures from random mating, these values are both positive and negative. A mean of −0.0687 for these significant values suggests that the ramet figures may be influenced by oversampling of certain clonal genotypes. *Cystopteris bulbifera* and *C. protrusa* shared no alleles at the loci examined; thus, based on nine putative loci (most of which code for "conservative" enzymes (Gillespie & Kojima, 1968)), the genetic identity between these congeneric species was zero.

DISCUSSION

BIOGEOGRAPHY AND ECOLOGY

Fieldwork and mapping of species distributions provided new perspectives on the biology and evolution of the *Cystopteris tennesseensis* species complex. Often two and occasionally all three species were sympatric. In such situations, *C. protrusa* was confined to the forest floor, *C. bulbifera* was on moist cliffs and among the talus at the base of cliffs, and *C. tennesseensis* could be found on drier cliffs and/or disturbed sites such as old rock walls. When plants of *C. protrusa* and *C. bulbifera* were intermingled, a special effort was made to locate the primary diploid hybrid between them. However, primary hybrids could not be found. Others have noted that primary hybrids of well established allopolyploids can rarely be discovered (e.g., the Appalachian *Asplenium* complex (Wagner, 1954; Werth et al., 1985a) and the *Dryopteris carthusiana* complex (Wagner, 1971)). Perhaps in these situations, the intermediate niche necessary for establishment of a diploid hybrid is occupied by the allopolyploid species.

In contrast to the primary diploid hybrid situation, when *C. tennesseensis* was sympatric with either parent, triploid backcross hybrids could be identified. Although an equivalent number of stations having putative hybrids has been identified (Figs. 2, 4), fieldwork demonstrated that at any one locality, hybrids with *C. protrusa* were more frequent than those involving *C. bulbifera*. This result may reflect the greater specificity of gametophytic safe sites for the latter species. Alternatively, differences in hybrid frequencies could be related to variation in response to antheridiogen. Haufler & Ranker (1985) showed that *C. protrusa* responded to antheridiogen while *C. bulbifera* did not, and that *C. tennesseensis* had a reduced sensitivity to this antheridia-inducing pheromone. Under these circumstances, it is likely that crosses involving *C. protrusa* would be more frequent. In a given safe site containing spores of *C. protrusa* and *C. tennesseensis*, it is likely that *C. tennesseensis* gametophytes would be mostly female while neighboring *C. protrusa* gametophytes would be primarily male. If this scenario is accurate, one would predict that *C. protrusa* should be the male parent in most crosses.

Extending this argument to the origin of *C. tennesseensis*, it may be predicted that *C. bulbifera* would be the egg parent in most cases. This hypothesis could be tested using information from chloroplast DNA (cpDNA) sequence variability.

TABLE 6. Population genetics statistics. Calculation of Nei's genetic identity and distance between infraspecific populations. Identity measures above the diagonal, distance measures below the diagonal.

Cystopteris bulbifera

Population number	1	2	3	4
1	—	0.7356	0.7716	0.8103
2	0.3071	—	0.9203	0.8734
3	0.2592	0.0830	—	0.8474
4	0.2104	0.1353	0.1656	—

Mean genetic identity = 0.8264

Cystopteris protrusa—RAMET POPULATIONS

Population number	1	2	3	4	5	6
1	—	0.9857	0.9161	0.8518	0.8940	0.7917
2	0.0144	—	0.9219	0.8313	0.9191	0.7937
3	0.0876	0.0813	—	0.8578	0.8475	0.7599
4	0.1604	0.1848	0.1534	—	0.7791	0.8271
5	0.1120	0.0844	0.1655	0.2496	—	0.7280
6	0.2336	0.2310	0.2745	0.1898	0.3174	—

Mean genetic identity = 0.8470

Cystopteris protrusa—GENET POPULATIONS

Population number	1	2	3	4	5	6
1	—	0.9280	0.9449	0.8253	0.8769	0.7670
2	0.0747	—	0.9275	0.7534	0.9364	0.7708
3	0.0567	0.0753	—	0.8840	0.8674	0.7765
4	0.1920	0.2832	0.1233	—	0.7285	0.7886
5	0.1313	0.0657	0.1423	0.3167	—	0.7371
6	0.2652	0.2603	0.2530	0.2375	0.3050	—

Mean genetic identity = 0.8342

Recent analyses (Stein & Barrington, 1990) indicated that cpDNA is inherited uniparentally in ferns (but see Andersson-Kottö, 1930). If cpDNA is carried in the egg, most *C. tennesseensis* plants should contain the chloroplast genome of *C. bulbifera*.

The available data indicate that *C. tennesseensis* is a relatively young species. The range of the derived allotetraploid *C. tennesseensis* extends only slightly beyond that of its diploid progenitors (Figs. 2–4). Further, *C. tennesseensis* shows no evidence of gene silencing. For the enzymes surveyed, all plants displayed fixed heterozygotic banding patterns that were additive for bands found in the diploid progenitors. All variability in the tetraploid could be attributed to recurring hybridization between genetically different diploid ancestors. All of these features are those anticipated for allopolyploids having recent origins (Haufler & Werth, 1986).

GENETICS

The mean levels of polymorphism and allelic variability for *C. protrusa* and *C. bulbifera* (Table 5) are similar to those expected of long-lived perennials (P = 0.66, A = 2.07) having a primarily outcrossed breeding system (P = 0.51, A = 1.85) (Hamrick et al., 1979). Based on calculations of mean observed and expected heterozygosity values (Table 5), neither diploid species appears to have an excess or deficiency of heterozygosity relative to Hardy-Weinberg expectations. The mean genetic identity among populations (Table 6: 0.8359) is at the lower end of that found among other fern species (I = 0.912, range = 0.78–0.99 in Soltis & Soltis, 1989). Table 7 lists the values of F and indicates which of these values represents a significant departure from zero. Those values that do not depart from zero are consistent with random mating for the species. Only the ramet populations

TABLE 7. Population genetics statistics. Calculation of the fixation index by population. Values not calculated for invariant loci. Asterisks after values = chi-square test. No asterisk indicates that the locus did not differ significantly from 0 and thus suggests random mating. * indicates significance at the $P < 0.05$ level; ** indicates $P < 0.01$ level; *** indicates $P < 0.001$ level.

Cystopteris bulbifera

Population number	PGI-2	IDH	SkDH	PGM-2	TPI-2	LAP
1	0.1806	0.0941	−0.0606	−0.2500	0.1923	0.4582
2	0.3836**	0.5220***	−0.0167	0.2458	0.3324*	0.0000
3	−0.3125	−0.3208	0.0000	−0.1053	0.0000	0.3000
4	−0.0952	−0.3450	−0.0952	−0.2778	0.0000	−0.0455

Cystopteris protrusa—RAMET POPULATIONS

Population number	PGI-2	IDH	SkDH	PGM-1	PGM-2	TPI-1	LAP
1	0.0437	−0.0781	0.0000	−0.1228	0.0000	0.5071***	0.6697***
2	0.0000	0.0000	0.0000	0.0000	0.0000	0.3478	1.0000***
3	−0.0606	0.0000	−0.6667**	−0.9444***	0.0000	−0.6667**	−0.6667**
4	−0.5972*	0.0336	0.0000	0.5175	−0.3529	0.0000	0.4202
5	0.5476	−0.0556	0.4328	0.0000	0.0000	0.7467*	−0.1343
6	0.0627	0.0000	0.0784	0.0000	0.0000	−0.2703	0.0000

Cystopteris protrusa—GENET POPULATIONS

Population number	PGI-2	IDH	SkDH	PGM-1	PGM-2	TPI-1	LAP
1	0.0114	0.0000	0.0000	0.1944	0.0000	0.6420*	0.7100**
2	0.0000	0.0000	0.0000	0.0000	0.0000	0.4444	1.0000
3	0.0000	0.0000	0.0000	−0.5000	0.0000	0.0000	0.0000
4	−0.2222	0.5926	0.0000	0.5926	−0.2222	0.0000	0.6857
5	0.3500	−0.0833	0.3953	0.0000	0.0000	0.7111	−0.1143
6	0.2400	0.0000	0.1204	0.0000	0.0000	−0.2667	0.0000

of *C. protrusa* show a large number of significant figures. Two facts suggest that these values should not be used as evidence that *C. protrusa* deviates from random mating. First, the significant values are both positive and negative with a mean of −0.0687, a value that is very close to zero. Second, deviations from zero can be caused by oversampling of cloned genotypes. Examining the *F* values for the genet populations shows that the number of significant figures drops to only two. Thus, the overall *F* values (Table 7) indicate that breeding systems for both diploid species conform to random mating models.

Genetic analyses of sporophytic populations can be correlated with antheridiogen data and measures of genetic load to draw conclusions about the breeding systems of diploid members of the *C. tennesseensis* complex. Antheridiogen response data indicate that *C. protrusa* should be outcrossing while *C. bulbifera* should be inbreeding. Genetic load data suggest that both diploids should be outcrossing. As discussed above, chi-square tests of fixation index values (Table 7), however, indicate that neither diploid species deviates significantly from random mating. Thus it may be that genetic load has a stronger control over fern mating systems than does possession of an antheridiogen system. By combining laboratory study of gametophytes and isozymic analyses of natural sporophytic populations we can obtain a clearer understanding of the factors controlling breeding systems among these *Cystopteris* species (Schneller et al., 1990).

Although the lack of allelic variability in *C. tennesseensis* precluded the application of isozyme data to analysis of breeding systems, genetic load data do provide clues to possible reproductive modes of this tetraploid. Other studies of diploid/polyploid complexes (Masuyama, 1979; Masuyama et al., 1987), have shown that although diploid progenitors may have high genetic loads, polyploids derived from them do not. Our studies of the *C. tennesseensis* complex provided another example of this phenomenon. Isolated gametophytes of both diploids were incapable of forming sporophytes, presumably because of post-zygotic lethal genes. However, 25% of isolated *C. tennesseensis* gametophytes did yield sporophytes. It has been hypothesized that homoeologous chromosome sets may provide buffers against expression of lethals and thereby allow polyploids to inbreed even if their progenitors cannot (Haufler, 1989).

Our data indicate that genetic identity between the diploids is zero. Given that the enzymes we surveyed are considered to be evolutionarily conservative (Gillespie & Kojima, 1968), the lack of similarity between *Cystopteris* congeners is quite remarkable. Angiosperm congeners have much higher mean genetic identity values (0.67 in Crawford, 1983) than those of most ferns that have been examined (0.33 in Soltis & Soltis, 1989). For *Bommeria*, Haufler (1985) suggested that the low genetic identity among congeners could indicate that the morphological characters uniting the species resulted through convergent evolution and, therefore, that this genus was not monophyletic. Few would dispute, however, the clear relatedness of *Cystopteris* species. Apparently, the lack of homology between diploid genomes observed in the cytogenetic study extends to the isozyme data as well. Thus, these data support alternative hypotheses that either (1) speciation in ferns may be accompanied by greater isozymic divergence than is typical for angiosperms (Haufler, 1987), or (2) that most congeneric fern species diverged prior to most angiosperm congeners (Soltis & Soltis, 1989).

The extraordinary genetic divergence of the diploids ensures a fixed heterozygote pattern for all enzymes in the derived allotetraploid. Yet, as introduced above, there is variability among individuals of *C. tennesseensis* (Figs. 10–12). These variants do not segregate among gametophytic progeny (Fig. 13), and are a direct reflection of the variability observed among diploid populations. In *Asplenium*, Werth et al. (1985b) used such information to propose recurring origins of the allopolyploids. It seems clear that *C. tennesseensis* originated more than once; but, in contrast to the situation in *Asplenium*, there does not seem to be any geographic pattern to these variants. The lack of evidence for gene silencing and the fact that no orphan alleles were detected also suggests recent origins.

SYSTEMATICS

The current investigation did not result in revision of the current systematic treatment of members of the *C. tennesseensis* complex. It was possible, however, to clarify the genetics of species boundaries and identify biological factors that have contributed to confusion in species circumscriptions. Perhaps the most significant factor blurring the boundaries between species is the formation of interspecific backcross hybrids. We collected and positively identified hybrids between *C. tennesseensis* and each of its diploid progenitors. These sterile triploids are morphologically intermediate between the sexual species and, if they are not removed from consideration, can bridge the morphological gaps between what are otherwise reasonably distinct taxonomic entities.

When not confounded by hybrids, a suite of qualitative features clearly discriminates *C. protrusa* from *C. bulbifera* (presence or absence of glandular trichomes and bulblets, clear differences in rhizome characteristics). In addition, we have demonstrated genetically regulated differences in quantitative leaf features (Table 3, Fig. 1) and significant differences in spore size between diploids and tetraploids (Table 1). Thus, although the characters are subtle, it is possible to identify each species through awareness of cryptic morphological characteristics and recognition of backcross hybrids. If pteridologists hope to resolve significant evolutionary units it will be necessary to consider suites of qualitative differences, which are sometimes cryptic.

Cryptic features are even more important in the identification of tetraploid *C. tennesseensis* than they are in the diploids. Especially significant are spore characteristics, both in terms of detecting spore abortion in backcross hybrids and calculating size measurements for positive identification of ploidy level (Table 1). Somewhat problematic is our observation that spores of autotriploid *C. protrusa* can appear normal and can be quite large (Haufler et al., 1985). It is therefore important to consider the mean and standard deviation in developing an accurate assessment of the spore size (and thus the ploidy) of individual specimens.

Given the large genetic distance between the diploid congeners in the *C. tennesseensis* complex,

it is probable that they have been phylogenetically isolated from each other for a long time and may represent systematic poles of the genus. The magnitude of the genetic distance between the two diploid species might also lead to the conclusion that hybrids between them should be rare and allopolyploid derivatives unsuccessful. Stebbins (1980) suggested that most successful polyploids are not strict autopolyploids or allopolyploids, but occupy a position somewhere between these extremes. Yet, our evidence indicates that even the highly differentiated *C. protrusa* and *C. bulbifera* have a history of recurring hybridizations and allopolyploid initiation. The derivative allotetraploid *C. tennesseensis* appears to be a successfully fledged and vigorous young species. It is quite widespread, is beginning to extend beyond the region of origin, and appears to be exploiting a niche that is not inhabited by either parent: neither diploid is found in the drier, more disturbed localities characteristic of *C. tennesseensis*.

Conclusions

Morphometric, chromosomal, and isozymic analyses of the *C. tennesseensis* complex support the taxonomic treatment originally proposed by Shaver (1954). The present studies document the genetic distinctness of the diploids *C. protrusa* and *C. bulbifera* and confirm the hybrid origin of the tetraploid *C. tennesseensis*. Considering the limited geographic range of the tetraploid, its sympatry with its parental diploids, and the lack of gene silencing, it appears that this allotetraploid is of relatively recent origin(s). Our studies have also pinpointed why these three species continue to be misidentified and misinterpreted taxonomically. First, the diploid species have a rather plastic morphology that, though genetically determined, is subtle and may be modified by environmental conditions. Second, there are autotriploid individuals of *C. protrusa* that expand the range of variability for that diploid (Haufler et al., 1985). Third, outcrossing breeding systems (driven by antheridiogen systems and/or high genetic loads) may contribute to frequent formation of backcross hybrids. These sterile triploid individuals span the morphological gaps between species, obscuring the boundaries that separate these genetically discrete taxa. Fourth, through recurring allopolyploid events, *C. tennesseensis* has incorporated much of the genetic variability of the diploids into its populations. As a result, positive identification of taxa in this complex depends on observations of cryptic spore and leaf features.

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