Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4389–4393, June 1986
Genetics

Genetic evidence suggests that homosporous ferns with high chromosome numbers are diploid

(Pteridophyta/diploidy/polyploidy/electrophoresis/isozymes)

CHRISTOPHER H. HAUFLER* AND DOUGLAS E. SOLTIS†

*Department of Botany, University of Kansas, Lawrence, KS 66045; and †Department of Botany, Washington State University, Pullman, WA 99163

Communicated by Peter H. Raven, February 10, 1986

ABSTRACT **Homosporous ferns have usually been considered highly polyploid because they have high chromosome numbers (average $n = 57.05$). In angiosperms, species with chromosome numbers higher than $n = 14$ generally have more isozymes than those with lower numbers, consistent with their polyploidy. By extrapolation, homosporous ferns would be expected to have many isozymes. However, ongoing surveys indicate that within fern genera, species having the lowest chromosome numbers have the number of isozymes considered typical of diploid seed plants. Only species above these lowest numbers have additional isozymes. Therefore, homosporous ferns either have gone through repeated cycles of polyploidy and gene silencing or were initiated with relatively high chromosome numbers. The latter possibility represents a radical departure from currently advocated hypotheses of fern evolution and suggests that there may be fundamental differences between the genomes of homosporous ferns and those of higher plants. These hypotheses can be tested by genetic, karyological, and molecular techniques.**

The average haploid chromosome number for homosporous ferns, 57.05 (1), is so high that up to 95% of all fern species have been considered polyploid (2). However, since the essence of polyploidy is the number of component genomes and not the number of chromosomes, the genetic significance of such high polyploidy estimates may be questioned. As reviewed by Wagner and Wagner (3), defining absolute levels of polyploidy in homosporous ferns has never been a simple matter. While accepting that these taxa reached their extant chromosome numbers through ancient polyploid events, Wagner and Wagner also discussed the alternative hypothesis "that the original vascular land plants may have had high chromosome numbers" (p. 201 in ref. 3). To avoid proposing original basic chromosome numbers in ferns, Manton and Vida (4) developed a practical, working definition for haploidy in homosporous ferns as "the lowest actual gametic number for which there is direct evidence in any group" (p. 365 in ref. 4). Vida (5) subsequently used this definition in calculating that only 43.5% of homosporous ferns are polyploid. Because this method of determining ploidal levels simplifies the presentation of data, we have adopted it in the present paper (see Table 1).

Chromosomal data may be open to a wide range of interpretation when estimating ploidy. It is also possible to estimate ploidal levels by counting the number of genes specifying enzymes (6). In the present communication, we introduce an independent data set from electrophoretic analyses for addressing the question: "Are homosporous ferns truly polyploid?"

Electrophoresis of constituent enzymes has been used to identify isozymic forms (those encoded by different loci) as discrete bands on a starch or acrylamide gel. Gottlieb (7) demonstrated that, because the genes in the contributing genomes are usually expressed, polyploid angiosperm species (defined as those having chromosome numbers greater than $n = 14$) generally exhibit isozymic multiplicity—i.e., they have more isozymes per enzyme than do diploids. Such extra isozymes are most easily detected as fixed heterozygote (nonsegregating) bands in progeny arrays. However, care must be exercised when interpreting multiple-banded enzyme patterns because they can result from a variety of sources. For example, extra bands that appear to represent the polyploid accumulation of loci can actually represent the compartmentalization of enzymes within plant cells (6, 8). In seed plants, the glycolytic enzymes typically are found as a pair of isozymes, one in the chloroplasts and the other in the cytosol (8). Gastony and Darrow (9) demonstrated that enzymes in the fern *Athyrium filix-femina* show a similar pattern of compartmentalization.

Determining the genetic basis of enzyme banding patterns is essential, since diploids may appear to have additional isozymes merely because they are heterozygotes. Analysis of meiotic segregation patterns can be used to identify bands that are allelic variants (allozymes) at a single locus. In homosporous ferns, this analysis is relatively straightforward because gametophytes are multicellular, mitotic derivatives of single, meiotically produced spores. These gametophytes can be analyzed individually by electrophoresis similar to the case for pollen of angiosperms (10), and studies have been carried out in several fern species (11, 12). Using these and other (13) established procedures, we examined a representative set of homosporous ferns (Table 1).

RESULTS AND DISCUSSION

As introduced by Gastony and Darrow (9), if ferns with high chromosome numbers are polyploid, they should have many isozymes. However, patterns of isozyme expression in all diploid fern species surveyed are the same as those for diploid angiosperms (8), even though the average number of chromosomes in the two groups is remarkably different (2). We found only a few instances of isozyme multiplicity. Phosphoglucomutase, for example, has a duplicated locus in *Bommeria ehrenbergiana* (14), *Asplenium montanum* (17), and *Polypodium virginianum* (16). Such occasional departures from diploid expression may be considered insignificant; they are also seen, for example, in diploid angiosperms. The complex banding patterns of triose-phosphate isomerase detected in most homosporous ferns stand out as exceptions to the general simplicity of other enzymes. In diploid seed plants, triose-phosphate isomerase typically displays two isozymes, one in the chloroplasts and the other in the cytosol (18). Nearly all diploid species surveyed from the Polypodiaceae (*sensu lato*) have an additional triose-phosphate isomerase isozyme; in *Athyrium filix-femina* (9), *Dryopteris expansa* (D.E.S., P. S. Soltis, and L. H. Rieseberg, unpublished data), and *Pteridium aquilinum* (P. G. Wolf, personal

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Table 1. Fern taxa examined electrophoretically

Species[a]	Chromosome number (*n*)	Loci examined, no.	Loci coding duplicated enzymes[b]	Ref.
Group A				
Asplenium abscissum	36	17	0	G. J. Gastony[c]
A. cristatum	36	17	0	G. J. Gastony[c]
Athyrium filix-femina	40	18	0	9
Bommeria ehrenbergiana	30	13	1[d]	14
B. hispida	30	13	0	14
B. pedata	90[e]	13	5	14
B. subpaleacea	30	13	0	14
Cystopteris reevesiana	42	17	0	C.H.H.[f]
C. fragilis	84[e]	15	4	C.H.H.[f]
C. laurentiana	126[e]	17	10	C.H.H.[f]
C. protrusa	42	17	0	C.H.H.[f]
C. bulbifera	42	17	0	C.H.H.[f]
C. tennesseensis	84[e]	17	11	C.H.H.[f]
C. tenuis	84[e]	17	11	C.H.H.[f]
Pellaea andromedifolia	29	7	0	15
P. ternifolia	29	20	1[d]	M. D. Windham[c]
P. truncata	29	20	1[d]	M. D. Windham[c]
P. wrightiana	58[e]	20	11	M. D. Windham[c]
Polypodium amorphum	37	14	1[d]	16
P. virginianum	37	14	1[d]	16
P. virginianum	74[e]	14	3	16
Pteridium aquilinum	52	23	0	P. G. Wolf[c]
Woodsia mexicana	76[e]	17	10	M. D. Windham[c]
Group B				
Adiantum pedatum	29	11	0	C. A. Paris[c]
Asplenium bradleyi	72[e]	15	13	17
A. ebenoides	72[e]	15	10	17
A. montanum	36	15	1[d]	17
A. pinnatifidum	72[e]	15	10	17
A. platyneuron	36	15	0	17
A. resiliens	108[e]	12	2	C. R. Werth[c]
A. rhizophyllum	36	15	0	17
A. ruta-muraria	72[e]	13	2	C. R. Werth[c]
A. trichomanes	36	17	0	C. R. Werth[c]
A. trichomanes	72[e]	17	3	C. R. Werth[c]
A. viride	36	17	0	C. R. Werth[c]
Dryopteris campyloptera	82[e]	20	5	C. R. Werth[c]; D.E.S.[f]
D. carthusiana	82[e]	20	5	C. R. Werth[c]
D. celsa	82[e]	22	6	C. R. Werth[c]; D.E.S.[f]
D. cristata	82[e]	20	4	C. R. Werth[c]
D. expansa	41	20	0	C. R. Werth[c]
D. goldiana	41	22	0	C. R. Werth[c]; D.E.S.[f]
D. ludoviciana	41	22	0	C. R. Werth[c]; D.E.S.[f]
D. marginalis	41	18	0	C. R. Werth[c]; D.E.S.[f]
Hemionitis elegans	30	12	0	T. A. Ranker[c]
H. palmata	30	15	0	T. A. Ranker[c]
H. pinnatifida	60[e]	14	5	T. A. Ranker[c]
H. rufa	30	8	0	T. A. Ranker[c]
H. subcordata	30	13	0	T. A. Ranker[c]
Notholaena dealbata	27	17	0	M. D. Windham[c]
Onoclea sensibilis	37	23	0	D. Buckley[c]
Pellaea ternifolia	58[e]	20	8	M. D. Windham[c]
Phanerophlebia juglandifolia	41	19	0	G. Yatskievych[c]
P. macrosora	41	19	0	G. Yatskievych[c]
P. nobilis	41	19	0	G. Yatskievych[c]
P. remotispora	41	19	0	G. Yatskievych[c]
P. umbonata	41	19	0	G. Yatskievych[c]

Table 1. Continued

Species[a]	Chromosome number (n)	Loci examined, no.	Loci coding duplicated enzymes[b]	Ref.
Polypodium hesperium	74[e]	11	6	M. D. Windham[c]
Polystichum acrostichoides	41	19	0	G. Yatskievych[c]; D. S. Barrington[c]
P. braunii	82[e]	9	3	D. S. Barrington[c]
P. imbricans ssp. *cortum*	41	19	0	G. Yatskievych[c]
P. munitum	41	19	0	G. Yatskievych[c]; D.E.S., and P. S. Soltis[f]
P. talamancanum	82[e]	11	5	D. S. Barrington[c]
Woodsia obtusa	76[e]	15	4	M. D. Windham[c]
W. oregana	76[e]	15	6	M. D. Windham[c]

[a]Group A comprises taxa for which the genetic basis of band patterns has been confirmed by analysis of gametophytic progeny arrays. Group B comprises taxa for which the genetic basis of band patterns has been inferred from sporophytic population samples.
[b]Duplicated loci are here defined as those that usually exhibit fixed heterozygote phenotypes—i.e., have several bands per locus that fail to segregate in population samples or among gametophytic progeny. Triose-phosphate isomerase appeared to have an extra isozyme in all species tested. Since it seems likely that this complex banding pattern resulted from either a tandem duplication or post-translational modification, it has not been included as evidence of polyploid isozyme expression. See text for more discussion.
[c]Personal communication.
[d]Diploid taxa that appear to have extra isozymes.
[e]Polyploid taxa, following the definition of Manton and Vida (4).
[f]Unpublished data.

communication), it is located in the cytosol. The banding pattern expressed for these cytosolic enzymes appears to reflect a fixed heterozygotic genotype that could have resulted from the polyploid addition of chromosomes. Interestingly, this duplication is not seen in species surveyed from the eusporangiate genus *Botrychium* (19).

Consideration of triose-phosphate isomerase expression in *Cystopteris protrusa* helps to elucidate the genetic constitution of the complex polypodiaceous pattern. Sporophytes of this species have either three rapidly migrating enzymes (Fig. 1*a*, lanes A), three slowly migrating ones (Fig. 1*a*, lanes B), or a five-banded pattern (Fig. 1*a*, lanes C). The five-banded pattern appears additive of the two three-banded patterns, with the lower band of the rapid triad nearly overlapping the upper band of the slower set. If the three-banded patterns resulted from the polyploid addition of a second genome, then the genes should be on separate chromosomes, and four different genotypes should be seen in gametophytic progeny arrays from the five-banded, presumably heterozygous sporophyte (Fig. 1*b*). In the more than 300 sporophytes examined from geographically diverse population samples and more than 150 individual gametophytic progeny, only two of these genotypes are evident (Fig. 1*c*). These findings indicate that the apparent duplications do not segregate independently and are not, therefore, on separate chromosomes. The complex banding patterns may represent either a tightly linked, tandem duplication or post-translational modification of the gene products, but in neither case could the pattern seen in this diploid species be attributed to genome multiplication through polyploidy.

If additional studies demonstrate that this genetic analysis of triose-phosphate isomerase is typical, then there is no consistent evidence that diploid homosporous ferns have additional isozymes resulting from polyploidy. While these data do not fully resolve the "question of whether the high chromosome numbers of homosporous pteridophytes are primitive or derived" (p. 202 in ref. 3), they certainly contradict the current hypothesis that ferns have selectively retained genetic polyploidy to maintain variability (21).

On the other hand, extant polyploid species do exhibit increases in isozyme number. *C. tennesseensis*, for example, is a putative allotetraploid resulting from polyploidy after hybridization between two diploids, *C. protrusa* and *C. bulbifera* (22, 23). Isozymic patterns of the diploids are distinct from each other, and those of the allotetraploid are additive of the two putative parents (Fig. 1*d*). Analysis of gametophytic progeny demonstrates that these complex patterns are fixed and, therefore, have resulted from the allopolyploid amalgamation of the diploid genomes. Similarly, complex isozyme patterns have been found in other polyploid fern species (14, 17).

CONCLUSIONS

In this paper, data from published and on-going analyses of 39 diploid and 25 polyploid species are reported. In the diploids, only 6 of the 652 loci may be interpreted as having duplicated expression that could have arisen through polyploidy. Among the polyploids, 162 of the 394 loci had fixed heterozygotic enzyme phenotypes that probably resulted from the polyploid amalgamation of divergent genomes. Thus, among extant species, the practical determination of ploidal levels by Manton and Vida (4) appears genetically sound. A similar pattern has been reported for the fern ally *Equisetum* (24). Species of this genus have uniformly high chromosome numbers of $2n = 216$ and, like the homosporous ferns, have been considered ancient polyploids, although isozymically they too are diploid.

Three alternative evolutionary hypotheses may be drawn from these observations. First, the high chromosome numbers of extant diploid ferns could have resulted from ancient, repeated episodes of allopolyploidy. The resulting fixed heterozygotic expression typically seen in allopolyploids must then have been reduced to the pattern characteristic of diploid species by silencing nearly all of the duplicated genes. Although no studies have been made to document gene silencing in polyploid ferns, loss of duplicate isozyme expression has occurred in recently originated, polyploid crop plants (25, 26).

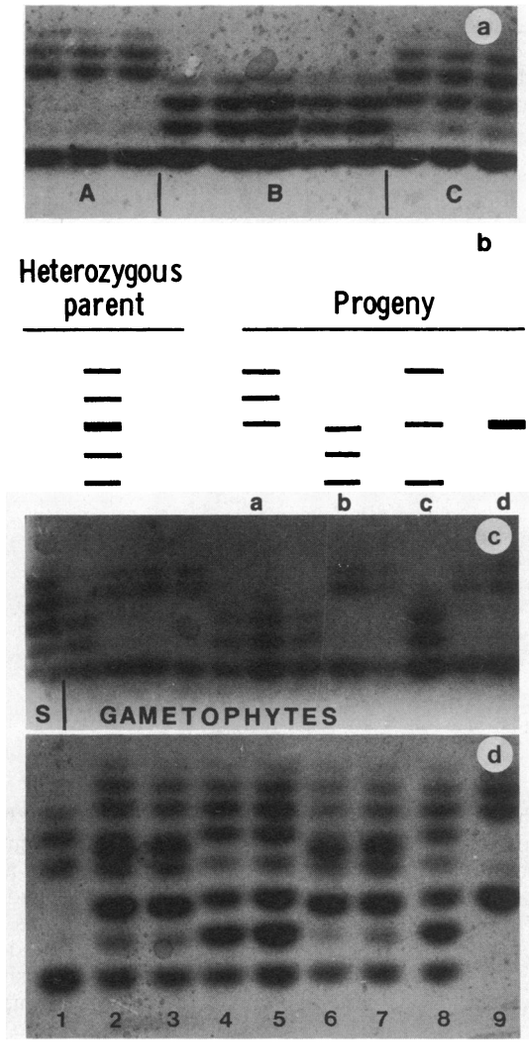

FIG. 1. (*a*) Zymogram showing variability among individuals for triose-phosphate isomerase in a population of *C. protrusa* sporophytes. The lowest (most cathodal) isozyme is invariant for this species. The more anodal bands occur as three rapidly migrating bands (lanes A) or as three slowly migrating bands (lanes B) or as a five-banded pattern (lanes C) that combines the two three-banded patterns. The most anodal bands stain faintly. (*b*) Diagrammatic representation of expected banding patterns among gametophytic progeny of a five-banded *C. protrusa* sporophyte. These hypothetical patterns assume that the sporophyte is heterozygous at two loci that segregate independently. (*c*) Zymogram showing a gametophytic progeny array from a five-banded *C. protrusa* sporophyte. A sporophyte (S) was run in the left-hand lane. All other lanes have individual gametophytes grown from spores of that sporophyte. Only two of the four patterns diagrammed in *b*, progeny a and b, are present. (*d*) Zymogram of triose-phosphate isomerase expression in sporophytes of the allopolyploid *Cystopteris tennesseensis*. Lanes: 1, *C. protrusa*; 2–8, *C. tennesseensis* from a variety of geographical areas in North America (2, Donaphan Co., KS; 3, Richardson Co., NB; 4, Franklin Co., MO; 5, Jackson Co., IL; 6, Ottawa Co., OK; 7, Pike Co., IL; 8, Carter Co., KY); 9, *Cystopteris bulbifera*. The chromosome number of all individuals shown here has been verified by meiotic chromosome squashes. Variability in tetraploid *C. tennesseensis* electromorphs parallels that in the two diploid progenitors and indicates that the tetraploid has multiple origins (cf. ref. 20).

The second hypothesis suggests that the high chromosome numbers in homosporous fern species may actually represent the original diploid condition. Supporting this hypothesis is the improbability that wholesale silencing of all duplicated loci occurred in so many different evolutionary lineages. There certainly is no evidence of such extensive diploidization in the angiosperms (8).

A third, less likely possibility is that autopolyploidy is common in ferns and of recent origin so that isozyme loci were duplicated without divergence. If this were true, the genetic consequences of autopolyploidy, specifically tetrasomic inheritance, should be evident. However, all analyses of segregation in diploid species have revealed only disomic patterns. Thus, there is good evidence that recent autopolyploidy cannot be implicated in explaining diploid isozyme expression in homosporous ferns.

Although data on the biochemical consequences of polyploid evolution in plants are scanty, consideration of the more extensive fish literature can provide insights for interpreting fern isozyme patterns. The catostomid fishes present the best studied example of ancient allopolyploidy (27). In this group, it has been suggested that the event initiating the allopolyploid lineage occurred 50 million years before present. Since that event, an average of 50% of the duplicated genes in different lineages have been silenced (28). Available data indicate that the lineages of extant homosporous fern genera (the level at which diploidy is inferred in this paper) are at least 100 million years old (T. Delevoryas and J. Skog, personal communication). Thus, by analogy with the catostomid fish data, there has been sufficient time to silence most, if not all, of the duplicated genes. However, the catostomid data also suggest that species in allopolyploid lineages should exhibit some pattern in the loss of duplicate gene expression. In such fish lineages, although an average of 50% of the duplicated loci have been silenced during the estimated 50 million years, the level of silencing varies among the species. The less modified (more primitive) species show only about 35% silencing, while the highly derived (more advanced) species have lost expression in 65% of their duplicates. As discussed above, there are occasional instances of duplicate gene expression among the diploid ferns surveyed. However, these duplications are not extensive enough to draw any phylogenetic inferences.

The two most likely hypotheses may be evaluated through a series of genetic, cytological, and molecular studies. First, detailed comparison of isozymic expression in allopolyploids and their diploid progenitors could provide insight into whether gene silencing has occurred. Second, critical information could be obtained through linkage analyses, since the data discussed here predict that ferns have fewer loci per chromosome than do angiosperms. Third, meiotic and karyotypic analyses may help to identify chromosomal homologies within and between genomes. As recently reviewed (3), only univalents or bivalents are typically encountered, even in artificial hybrids between closely related pteridophyte species. However, current studies (29) demonstrate that multivalents can be seen clearly in natural autotriploids. Further work with such taxa may help to establish genomic homologies and/or genetic control of chromosomal pairing in pteridophytes. In addition, karyotypic studies could indicate whether there are multiple (homoeologous) copies of chromosomes with similar morphologies. Although some fern karyotypes have been described, critical evaluation demonstrates that there are no significant data to support the existence of multiple sets of chromosomes in the ferns here considered as diploids (30). In fact, Walker (31) has recently refined standard karyotypic analyses for work with ferns and comments that there are no data to support the proposal of low base chromosome numbers in the ferns. Additional studies using chromosome banding techniques may generate markers that can determine whether there are multiple sets of distinctive chromosomal morphologies. Finally, by using molecular hybridization techniques, it may be possible to identify silenced genes that no longer code enzyme products. Tests of the two alternative hypotheses should lead to an

improved understanding of the genetic and chromosomal systems of homosporous ferns.

We are indebted to David S. Barrington, Donald Buckley, Gerald J. Gastony, Cathy A. Paris, Thomas A. Ranker, Pamela S. Soltis, Charles R. Werth, Michael D. Windham, Paul G. Wolf, and George Yatskievych for supplying us with their unpublished results. We thank Theodore Delevoryas and Judy Skog for offering their opinions of the age of homosporous fern genera. Finally we are grateful to David Barrington, Daniel Crawford, Leslie Gottlieb, Yvonne van der Meer, Cathy Paris, Tom Ranker, Pam Soltis, and Paul Wolf for providing valuable ideas and constructive criticisms of this manuscript. This research was supported in part by National Science Foundation Grant BSR85-15842 to C.H.H.

1. Klekowski, E. J. & Baker, H. G. (1966) *Science* **153,** 305–307.
2. Grant, V. (1981) *Plant Speciation* (Columbia Univ. Press, New York).
3. Wagner, W. H. & Wagner, F. S. (1980) in *Polyploidy: Biological Relevance*, ed. Lewis, W. H. (Plenum, New York), pp. 199–214.
4. Manton, I. & Vida, G. (1968) *Proc. R. Soc. London Ser. B* **170,** 361–379.
5. Vida, G. (1976) in *Evolutionary Biology*, eds. Novák, V. J. A. & Pacltová, B. (Czechoslovak Acad. Sci., Prague), pp. 267–294.
6. Gottlieb, L. D. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3726–3729.
7. Gottlieb, L. D. (1981) *Prog. Phytochem.* **7,** 1–46.
8. Gottlieb, L. D. (1982) *Science* **216,** 373–380.
9. Gastony, G. J. & Darrow, D. C. (1983) *Am. J. Bot.* **70,** 1409–1415.
10. Weeden, N. F. & Gottlieb, L. D. (1979) *Biochem. Genet.* **17,** 287–296.
11. Gastony, G. J. & Gottlieb, L. D. (1982) *Am. J. Bot.* **69,** 634–637.
12. Haufler, C. H. & Soltis, D. E. (1984) *Am. J. Bot.* **71,** 878–881.
13. Soltis, D. E., Haufler, C. H., Darrow, D. C. & Gastony, G. J. (1983) *Am. Fern J.* **73,** 9–27.
14. Haufler, C. H. (1985) *Syst. Bot.* **10,** 92–104.
15. Gastony, G. J. & Gottlieb, L. D. (1985) *Am. J. Bot.* **72,** 257–267.
16. Bryan, F. A. & Soltis, D. E. (1984) *Am. J. Bot.* **71,** 159.
17. Werth, C. R., Guttman, S. I. & Eshbaugh, W. H. (1985) *Syst. Bot.* **10,** 184–192.
18. Pichersky, E. & Gottlieb, L. D. (1984) *Plant Physiol.* **74,** 340–347.
19. Soltis, D. E. & Soltis, P. S. (1986) *Am. J. Bot.* **73,** 588–592.
20. Werth, C. R., Guttman, S. I. & Eshbaugh, W. H. (1985) *Science* **228,** 731–733.
21. Klekowski, E. J. (1979) in *The Experimental Biology of Ferns*, ed. Dyer, A. (Academic, London), pp. 133–170.
22. Haufler, C. H. (1985) *Proc. R. Soc. Edinburgh* **86,** 315–323.
23. Shaver, J. M. (1954) *Ferns of the Southeastern United States with Special Reference to Tennessee* (General Publishing Co., Toronto).
24. Soltis, D. E. (1986) *Am. J. Bot.* **73,** 908–913.
25. Hart, G. E. (1983) in *Isozymes: Current Topics in Biological and Medical Research* (Liss, New York), Vol. 10, pp. 365–380.
26. Wilson, H. D., Barber, S. C. & Walters, T. (1983) *Biochem. Syst. Ecol.* **11,** 7–13.
27. Ferris, S. D. (1984) in *Evolutionary Genetics of Fishes*, ed. Turner, B. J. (Plenum, New York), pp. 55–93.
28. Ferris, S. D. & Whitt, G. S. (1977) *Nature (London)* **265,** 258–260.
29. Haufler, C. H., Windham, M. D., Britton, D. M. & Robinson, S. J. (1985) *Can. J. Bot.* **10,** 1855–1863.
30. Duncan, T. & Smith, A. R. (1978) *Syst. Bot.* **3,** 105–114.
31. Walker, T. G. (1985) *Bull. Br. Mus. (Nat. Hist.) Bot.* **13,** 149–249.