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Hybridization between *Dianthus superbus* var. *longicalycinus* and *D. shinanensis* Evidenced by Resolvable Esterase Isozymes from Herbarium Specimens

By

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加藤辰己*： 腊葉標本を用いたカワラナデシコとシナノナデシコの交雑現象の電気泳動的解析

Tsukuba Botanical Garden, National Science Museum was founded in 1974 in order to promote experimental studies on plant taxonomy and its adjacent fields. Natural vegetations of Central Japan are reproduced in twelve sections in the garden. In the sandy and gravelly section, one of the twelve sections, a total of 52 individuals of *Dianthus superbus* L. var. *longicalycinus* (Maxim.) Williams and seven individuals of *D. shinanensis* (Yatabe) Makino (=*D. barbatus* L. var. *shinanensis* Yatabe) were planted during 1979 to 1981. In 1985, Dr. S. Kurokawa, the director of the garden, noticed that some plants showing morphologically intermediate features between the above two species were growing. More various forms of morphological intermediates, which increased in number and spread over the section, were detected in 1986 (Fig. 1). These intermediates and plants of the two *Dianthus* species formed a morphological complex, which is designated here as the *D. superbus* — *shinanensis* complex. Because of the morphological intermediacy itself, the intermediates were supposed to have been derived through hybridization between *D. superbus* var. *longicalycinus* and *D. shinanensis*. However, to confirm the hybridity of the intermediates and to elucidate the pathway through which the observed variability was induced, more detailed analyses on the genetic basis are needed.

Only fresh or freezed plant tissue and extracts preserved through freezing or lyophilization have been believed to be available for electrophoretic studies of isozyme variation in plants. Ranker & Werth (1986) broke this common belief by demonstrating that materials from pressed herbarium specimens were, in certain cases, also available for electrophoretic investigations. Since their innovative contribution was limited to pteridophytes, it is now of great interest to successive researchers whether or not the utility of pressed herbarium specimens for electrophoretic studies can be extended to plants other than pteridophytes, especially to angiosperms.

Another problem lies in the extraction method. Leaf tissue of some taxa of plants, including *Dianthus*, contains high level of impurities such as phenolic compounds which

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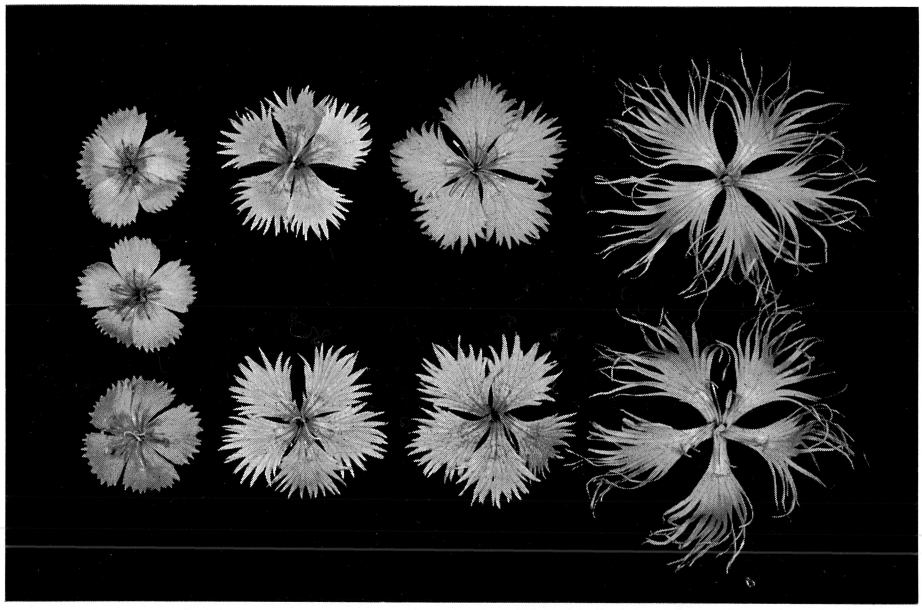

Fig. 1. Representative flowers of the *Dianthus superbus — shinanensis* complex detected in 1986 (vertical view). Left (2 fls.) : *D. superbus* var. *longicalycinus*. Middle (4 fls.) : morphological intermediates. Right (3 fls.) : *D. shinanensis*.

denature enzymes and thus prevent extraction of active enzymes. Although grinding buffers for extraction have been considerably improved (Mitton *et al.* 1979; Soltis *et al.* 1980, 1983; Gottlieb 1981a, 1982, 1984; Werth *et al.* 1982), direct application of crude extracts obtained by grinding leaf tissue in various conditions of buffers does not always yield satisfactory results. In order to remove impurities with small molecular weights from crude extracts and prepare extract samples containing active, electrophoretically resolvable enzymes, the author initiated to utilize an extraction method including gel filtrations with simple procedures.

As a result of preliminary electrophoresis using fresh leaf materials, it had been suggested that the two *Dianthus* species were characterized by different esterase phenotypes and thus the esterase isozymes could be used as genetic markers to solve the above-mentioned problems on the *D. superbus — shinanensis* complex. It is desirable but difficult to complete electrophoretic investigations of the complex using only their living materials, because *D. shinanensis* is biennial. Another preliminary study showed that, in *Dianthus*, preservation of esterase activities in pressed specimens was much better than in materials freezed at −30°C. Under these circumstances, electrophoretic investigations were extended to elucidate the genetic variation of esterase isozymes in a total of 285 individuals of this complex using their pressed herbarium specimens. From the results, hybridization between *D. superbus* var. *longicalycinus* and *D. shinanensis* in Tsukuba Botanical Garden, which has been suggested by morphological features, was genetically evidenced. The results are described, and the processes of the hybridization are discussed from a genetic standpoint in the present paper. As Ranker & Werth (1986)

pointed out, utilization of herbarium specimens for electrophoretic studies potentially enlarges chances to solve many kinds of biosystematic problems on the genetic basis. A practical example of utilizatin of herbarium specimens in this regard was given in the present study. During the course of this study, a simplified gel filtration method was employed to prepare electrophoretic samples. Details of this method is also included in the present paper. Results of morphological and cytological analyses of the plants concerned will be described elsewhere.

Materials and Methods

Plants

A total of 285 plants belonging to the *Dianthus superbus — shinanensis* complex were collected in the garden in late July, 1986. They were held between blotting papers, placed inside a plastic box filled up with granular silica gel and pressed for two weeks at room temperature (air conditioned at 20–25°C). Electrophoresis was conducted from December, 1986 to February 1987, and thus the specimens were about a half year old when studied. Voucher specimens are preserved in the herbarium of National Science Museum, Tokyo (TNS).

Extraction

Crude extracts were obtained by grinding small quantities (10–20 mg) of leaf material removed from pressed specimens in 0.5 m*l* cold extraction buffer; 0.1 M Tris-HCl buffer, pH 8.0, containing 70 mM 2-mercaptoethanol, 26 mM sodium metabisulfite, 11 mM L-ascorbic acid sodium salt and 4% (w/v) soluble polyvinylpyrrolidon with an aver agemolecular weight of 40000 (Sigma, PVP-40). Soon after the grinding, crude extracts were filtrated through the simplified gel filtration method, which was firstly employed here. Procedures of this method consisted of the following two steps.

Step 1. A small pinhole was made in the bottom of disposable 0.5 m*l* microcentrifuge tube (Eppendorf type) and a small bit of glass microfibre filter paper (Whatman GF/D) was stuffed into the bottom to cover the pinhole. Eighty to 90 mg dry gel of Sephadex G-25 Medium (bead size: 50–150 μm) was put into the tube, which was kept at 4°C until use. Crude extracts obtained by the grinding were immediately poured into the prepared tube, and this 0.5 m*l* microcentrifuge tube was placed inside a 1.5 m*l* microcentrifuge tube. These tubes were centrifuged at 1000×G for 5 minutes at 2°C, and 0.05–0.1 m*l* semifiltrated extracts were obtained in the 1.5 m*l* tube. Through this procedure, residuary leaf tissue was also removed from extracts and the extracts were effectively concentrated.

Step 2. A 0.5 m*l* microcentrifuge tube with a pinhole and a bit of glass microfibre filter paper was prepared in the same way as the step 1. Sephadex G-25 suspended in 0.1 M phosphate buffer, pH 7.0, containing 30 mM 2-mercaptoethanol was put into the tube until bed volume reached 0.5 m*l*. Excessive buffer was removed from the tube using a

micropipet. This 0.5 m*l* microcentrifuge tube was set in a 1.5 m*l* microcentrifuge tube, and used as a Sephadex column for gel filtration. The 0.05 m*l* semifiltrated extracts obtained through the step 1 procedure were added to the Sephadex column, and about 0.05 m*l* of the first fraction was deposited in the 1.5 m*l* tube by natural elution. When the first fraction was not eluded naturally, the tubes were gently shaken several times by hands or centrifuged tenderly with a hand-centrifuge. After discarding the first fraction, 0.05 m*l* of the suspending buffer without Sephadex G-25 was added to the Sephadex column. The second fraction was obtained in the same way as the first fraction, and then discarded. This procedure of obtaining 0.05 m*l* fraction was repeated twice again and the fourth fraction was used for electrophoresis as an extract sample. The step 2 procedures were conducted promptly in cool conditions to protect enzymes from denaturation.

This simplified gel filtration method, originally utilized to remove pigmentary impurities from leaf extracts of *Hypericum*, is available not only for *Dianthus* but also for many angiosperm species. A slight modification on the condition of the suspending buffer or an adequate choice of Sephadex (Sephadex G-50 can be substituted for G-25 in certain cases) will give better results according to the particular taxon or enzyme studied. Although consistent utilizations of both the steps 1 and 2 procedures are preferable, single uses of either of them, in certain taxa, often improve resolutions of banding patterns drastically. A modified method with a single use of the step 2 procedure, for example, was applied to *Eupatorium* with a satisfactory result for peroxidase (Yahara & Sullivan 1986).

Electrophoresis

For horizontal electrophoresis, 12.8% (w/v) starch gel made up with the system 7 buffers of Soltis *et al.* (1983) was employed. Extract samples filtrated through the simplified gel filtration method were absorbed onto rectangular wicks of Toyo 51B chromatography paper, which were inserted into a slice made across the gel 5 cm from the cathode. Electrophoresis was conducted at 4°C at 150 volts (c.v.) for 15 minutes, after which the wicks were removed from the gel, and the electrophoresis continued for approximately four to five hours until the bromphenol blue (BPB) marker had migrated 9 cm from the origin. Enzymatic activities of esterase were assayed according to the standard staining schedule of Soltis *et al.* (1983).

Pollen stainability

For the examination of pollen stainability, pollen grains were gathered from mature anthers on the pressed specimens which were electrophoretically resolved, and then stained using aceto-carmine solution. Pollen stainability was calculated as the percentage of stained, normal-shaped pollen grains per 500 grains.

Results

Resolvable enzymatic activities were present in two anodal zones on esterase zymograms (Fig. 2); zone 1 (more anodal) and zone 2 (less anodal). They were easily distinguished each other because bands were stained black in zone 1 and purple in zone 2. This difference in colour is due to substrate specificities of esterase isozymes; that is, isozymes in zone 1 preferentially use α-naphthyl acetate and those in zone 2 preferentially use β-naphthyl acetate (Gottlieb 1974). Two bands, designated here as band 61 and band 51 according to their mobilities in mm from the origin under the present conditions, were detected in zone 1. Based on combinations of these bands, the following three phenotypes were recognized; phenotype I with only band 51, phenotype II with bands 51 and 61 and phenotype III with only band 61.

Table 1 summarizes the results of the electrophoretic investigations of esterase isozymes in the *Dianthus superbus — shinanensis* complex. A total of 269 individuals showed resolvable banding patterns. Among them, all of 210 individuals of *D. superbus* var.

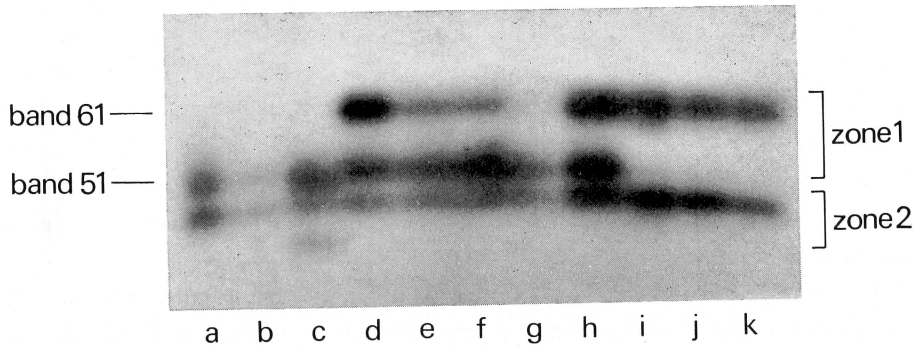

Fig. 2. Photograph of a zymogram showing the three phenotypes of esterase isozymes in the *Dianthus superbus — shinanensis* complex. The anode is toward the top of the figure. Lanes (individuals) a-c: *D. superbus* var. *longicalycinus* expressing phenotype I. Lanes d-h: morphological intermediates expressing phenotypes I (g) and II (d-f and h). Lanes i-k: *D. shinanensis* expressing phenotype III.

Table 1. Electrophoretic expressions of esterase isozymes in two *Dianthus* species and their morphological intermediates

	D. superbus var. *longicalycinus*	morphological intermediates	*D. shinanensis*
phenotype I	210	4	0
phenotype II	0	34	0
phenotype III	0	0	21
activities present but not resolvable	3	1	4
no enzymatic activities	1	1	6
n	214	40	31

Table 2. Distribution of pollen stainabilities in four groups of plants belonging to the *Dianthus superbus — shinanensis* complex classified according to taxa and esterase phenotypes

	Pollen stainability (%) 0—1—5—20—50—80—95—99—100								n	*
D. superbus var. *longicalycinus*										
phenotype I	—	—	—	—	—	—	6	106	112	98
morphological intermediates										
phenotype I	—	—	—	—	1	3	—	—	4	0
phenotype II	20	1	—	—	—	—	—	—	21	13
D. shinanensis										
phenotype III	—	—	—	—	—	—	—	21	21	0

* number of individuals whose pollen stainabilities were uncountable because of completely abortive anthers or lack of mature anther.

longicalycinus expressed phenotype I. No individuals expressed phenotypes II nor III in this species. On the other hand, all of 21 individuals of *D. shinanensis* expressed phenotype III, and no other phenotypes were detected. Among 38 morphological intermediates, a total of 34 individuals expressed phenotype II and 4 individuals expressed phenotype I. Besides the electrophoretically resolved individuals, eight individuals expressed faint or broad enzymatic activities which could not be resolved into discrete banding patterns and another 8 individuals expressed no enzymatic activities. Isozymes in zone 2 were left out of considerations because no specific phenotypes characterizing either of the two species were detected.

The results of examinations of pollen stainability are summarized in Table 2. *Dianthus superbus* var. *longicalycinus* is gynodioecious. Nearly half of the examined plants of this species bore completely abortive anthers, and thus pollen stainabilities of them were not countable. It is notable that the 4 morphological intermediates with phenotype I exhibited comparatively high pollen stainabilities ranging from 63.8 to 94.6% with a mean value of 84.7%. These pollen stainabilities are obviously higher than those of the other intermediates with phenotype II (0-2.4%), but lower than those of both *D. superbus* var. *longicalycinus* and *D. shinanensis* (96.6-100%).

Discussion

Esterase isozymes are, in general, considered to be usually monomeric (Gottlieb 1981b). Since three (or more) banded patterns were not observed in zone 1 throughout the present investigations, it seems safe to conclude that the esterase isozymes in zone 1 are monomeric. This inference on subunit composition of esterase isozymes leads alternative interpretations that can genetically explain the three phenotypes observed in zone 1; the isozymes expressing bands 61 and 51 are coded (1) by alleles on a single

locus, or (2) by genes on two independent loci. In the former case, plants expressing phenotype II are regarded to be heterozygous with two alleles, each of which specifies either phenotype I or III plants. In the latter case, plants expressing phenotypes I and III are regarded to be homozygous with "null" alleles at the loci which specify bands 61 and 51, respectively. Although data of progeny tests that can test these two possibilities are not available bacause the putative F_1 hybrids are almost completely sterile as noted below, the fact that only one resolvable isozymes preferentially using α-naphthyl acetate are usually observed in some other diploid species of *Dianthus* under the present conditions supports the former interpretation. The postulated allozymes and their coding alleles are designated as in the following example (cf. Gastony & Gottlieb 1985): Est-1^{61} (the allozyme), *Est-1*^{61} (the allele).

All of electrophoretically resolvable individuals of *Dianthus superbus* var. *longicalycinus* were fixed in phenotype I, and thus this species is considered to be specified by the *Est-1*^{51} allele. On the other hand, the *Est-1*^{61} allele specifies *D. shinanensis*. These allelic specificities between the two species directly indicate that the phenotype II intermediates, heterozygous with *Est-1*^{61} and *Est-1*^{51}, are derived through hybridizations between *D. superbus* var. *longicalycinus* and *D. shinanensis*. The phenotype II hybrids exhibited considerably low pollen stainabilities, usually less than 1% (Table 2). Seed sets of them in the garden are roughly estimated to be 0.1–1%. Accordingly, high level of hybrid sterility is present between the two *Dianthus* species, but this hybrid sterility is not complete. This means that the phenotype II hybrids can yield progeny, with very low possibilities, through backcrossings to the parental species or inbreedings (cf. Stebbins 1959; Kato 1986). The presence of phenotype I intermediates, which were homozygous with *Est-1*^{51} and exhibited comparatively high pollen stainabilities (Table 2), provides an evidence supporting this idea. These intermediates are considered to be derived through backcrossings of gametes (with *Est-1*^{51}) of the phenotype II hybrids to *D. superbus* var. *longicalycinus* which is specified by *Est-1*^{51}. The latter possibility that the phenotype I intermediates originated through inbreedings of the phenotype II hybrids was tentatively rejected because some thirty bagged plants expressing phenotype II did not bear any seeds at all.

Dianthus superbus var. *longicalycinus* and *D. shinanensis* each possess high morphological identities. They are distinguished each other based on more than twenty morphological characters, and thus it is very easy to discriminate morphological intermediates from the two species. Throughout the examinations of more than one thousand herbarium specimens preserved in TNS, TI, KYO and MAK, no morphological intermediates (putative hybrids, in other words) between the two *Dianthus* species were detected. Consequently, it is doubtless that the hybrids detected in the garden originally arose in Tsukuba Botanical Garden itself. Based on the genetic evidence on esterase isozymes discussed above, process of the concerned hybridizations is presumed as follows.

Plants of the two *Dianthus* species, which do not occur sympatrically in the field by virtue of their different habitat preferences (i.e., the habitat of *D. superbus* var. *longicalycinus* is rather mesic but that of *D. shinanensis* is xeric), were obliged to grow

in close proximity in an artificial habitat of the garden, and then accidentally hybridized. The available evidence does not confirm when the F_1 hybrids firstly emerged. The following three observations, however, seem to support the idea that the hybridization between the two *Dianthus* species and the first emergence of their F_1 hybrids were comparatively recent events; (1) putative hybrids were detected, in 1985, in restricted areas where the two parental species were in contact, (2) putative hybrids detected in 1985 exhibited relatively high morphological affinities as compared with those in 1986, and (3) all of 19 putative hybrids detected in 1985 exhibited very low pollen stainabilities (0–1.6%) or bore completely abortive anthers. In and/or closely before 1985, backcrossings of F_1 hybrids to *Dianthus superbus* var. *longicalycinus* occurred and, as a result, B_1 hybrids and their progeny which expressed phenotype I in 1986 originated. These backcrossed hybrids contributed to increase morphological variabilities of the *D. superbus* — *shinanensis* complex, as observed in floral characters (Fig. 1). No hybrid plants backcrossed to *D. shinanensis* were electrophoretically detected in 1986. This can be explained by the following two possibilities; (1) because of hybrid inviability of the backcrossed B_1 hybrids, or (2) due to small population size of *D. shinanensis*. Cross experiments between putative F_1 hybrids and *D. shinanensis* which are now under practice will favor one possibility than the other.

Since the backcrossed hybrids detected in 1986 exhibited considerably high pollen stainabilities, it is much probable that backcrossings to *Dianthus superbus* var. *longicalycinus* repeatedly occur. This probability brings about further questions; (1) how and to which extent gene exchanges between the two *Dianthus* species proceed, and (2) whether or not "hybrid races" with unique morphological features are established. To answer the above questions, it will be needed to succeed enzymatic and morphological investigations for several years.

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Summary

Morphological intermediates between *Dianthus superbus* var. *longicalycinus* and *D.*

shinanensis, which were regarded as putative hybrids between the two species because of their morphological intermediacy itself, were detected in Tsukuba Botanical Garden. To confirm the hybridity of the intermediates on the genetic basis, genetic variation of esterase isozymes in a total of 285 plants of *Dianthus* was electrophoretically investigated using their pressed herbarium specimens.

As a result, it was revealed that *D. superbus* var. *longicalycinus* was specified by the *Est-1*^{51} allele and *D. shinanensis* by the *Est-1*^{61} allele. Among 38 intermediates electrophoretically resolved, 34 plants were heterozygous with *Est-1*^{51} and *Est-1*^{61} and the other 4 plants were homozygous with *Est-1*^{51}. The allelic specificities of the two *Dianthus* species directly indicated that the heterozygous intermediates were derived through hybridizations between the two parental species. Notable recovery of pollen stainability in the homozygous intermediates supported the idea that they originated through backcrossings of heterozygous hybrids to *D. superbus* var. *longicalycinus*.

During the course of this study, a simplified gel filtration method was employed to prepare extract samples. Details of this method was also described in text.

摘　　要

筑波実験植物園において，カワラナデシコとシナノナデシコの間の中間的な形態的特徴を示す非常に多型的な一群のナデシコ属植物が新たに見出された。これら形態的中間型の実体と由来を明らかにするために，285 個体のナデシコ属植物の腊葉標本を用い，エステラーゼ・アイソザイムを遺伝的マーカーとした電気泳動的解析を行った。

その結果，(1)形態的中間型はカワラナデシコとシナノナデシコの雑種起源のものであること，(2)雑種個体とカワラナデシコとの間に戻し交雑が起きつつあり，これが高い形態的変異性をもたらしていること，が明らかになった。

本研究は同時に，種子植物の腊葉標本からもアイソザイムが検出でき，さまざまな種生物学的問題を遺伝学的見地から解析できる可能性があることを示した。また本研究においては，電気泳動用サンプルを精製するにあたって新たに考案した簡便なゲル濾過法を採用し良好な結果を得たので，その詳細な手順についても記載した。

References

Gastony, G.J. and L.D. Gottlieb, 1985. Genetic variation in the homosporous fern *Pellaea andromedifolia*. Amer. J. Bot. **72**: 257-267.

Gottlieb, L.D., 1974. Genetic confirmation of the origin of *Clarkia lingulata*. Evolution **28**: 244-250.

———, 1981a. Gene numbers in species of Asteraceae that have different chromosome numbers. Proc. Natl. Acad. Sci. USA. **78**: 3726-3729.

———, 1981b. Electrophoretic evidence and plant populations. Prog. Phytochem. **7**: 1-46.

———, 1982. Genetic evidence for the hybrid origin of the diploid plant *Stephanomeria diegensis*. Evolution **36**: 1158-1167.

———, 1984. Electrophoretic analysis of the phylogeny of the self-pollinating populations of *Clarkia xantiana*. Pl. Syst. Evol. **147**: 91-102.

Kato, T., 1986. Taxonomical studies on the *Hypericum pseudopetiolatum* complex. II. Natural hybridizations in Kyushu. Bull. Natn. Sci. Mus., Tokyo, Ser. B. **12**: 139-149.

Mitton, J.B., Y.B. Linhart, K.B. Sturgeon and J.L. Mamrick, 1979. Allozyme polymorphism detected in mature needle tissue of ponderosa pine. J. Hered. **70**: 86-89.

Ranker, T.A. and C.R. Werth, 1986. Active enzymes from herbarium specimens: electrophoresis as an afterthought. Amer. Fern J. **76**: 102-113.

Soltis, D.E., C.H. Haufler and G.J. Gastony, 1980. Detecting enzyme variation in the fern genus *Bommeria*: an analysis of methodology. Syst. Bot. **5**: 30-38.

———, ———, D.C. Darrow and G.J. Gastony, 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. Amer. Fern J. **73**: 9-27.

Stebbins, G.L., 1959. The role of hybridization in evolution. Proc. Amer. Phil. Soc. **103**: 231-251.

Werth, C.R., A.A. Karlin and S.I. Guttman, 1982. Enzyme extraction from phenolic-containing plant tissue using caffeine. Isozyme Bull. **15**: 139.

Yahara, T. and V.I. Sullivan, 1986. Peroxidase phenotypes of *Eupatorium mohrii* and *E. scabridum* (Compositae), widespread all-agamospermous "species" in the southeastern United States. Pl. Sp. Biol. **1**: 27-34.