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(Citation)
American journal of botany,76(8):1247-1253

(Issue Date)
1989-08

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90001248

Amer. J. Bot. 76(8): 1247–1253. 1989.

EXTENSIVE GENE DUPLICATIONS IN DIPLOID EUPATORIUM (ASTERACEAE)[1]

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ABSTRACT

An electrophoretic study of isozyme number for seven soluble enzymes revealed extensive gene duplications in eight diploid species of American *Eupatorium* belonging to three morphological groups. The enzymes isocitrate dehydrogenase, phosphoglucomutase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase, and shikimate dehydrogenase occur as three to six isozymes in all species, whereas the minimal conserved number typical of diploid plants is two isozymes for each. Fructose 1, 6-biphosphate aldolase is expressed as multibanded pattern suggesting fixed heterozygosity in all examined species. It was not possible to document gene duplication for triosephosphate isomerase from the electrophoretic patterns. All species examined have a chromosome number of $2n = 20$, which has been regarded as the basic diploid number for *Eupatorium*. However, the detection of extensive duplications suggests that $2n = 10$ may be the original diploid chromosome number in *Eupatorium* and that plants with $2n = 20$ are of polyploid origin. This hypothesis would mean that extensive duplications at isozyme gene loci have been maintained since the origin of the genus, despite chromosomal diploidization having occurred.

IT HAS BEEN documented that diploid vascular plants have a minimal highly conserved number of isozymes for most of the enzymes routinely examined by electrophoresis (Gottlieb, 1981a, 1982, 1983, 1984). This means that an increase in isozyme number for a given enzyme is the result either of gene duplication at the diploid level or of polyploidy. If a plant is polyploid, then it should have duplications at many isozyme loci, whereas a diploid plant would exhibit much less extensive increase in isozyme number (Gottlieb, 1981a, 1983, 1984). While inferences about whether given plants are diploids or polyploids have often rested primarily on their chromosome numbers, Gottlieb (1981b) employed isozyme number to infer the ploidy level of plants when chromosome data per se were inconclusive. Gottlieb (1981b) argued that an essential feature of polyploid is the number of genomes being expressed, and that chromosome number alone cannot be employed as the sole criterion. Isozyme number has been successfully applied to systematic questions in several plant groups. Recent studies of ferns and fern-allies have shown that plants traditionally viewed as polyploids on the basis of high chromosome numbers are genetically diploids at isozyme loci (Haufler and Soltis, 1986; Haufler, 1987; D. Soltis and P. Soltis, 1987, 1988). Using the same criterion, Soltis et al. (1987) and Chase and Olmstead (1988) rejected the hypothesis that species with high chromosome numbers are polyploids in the Bromeliaceae and the subtribe Oncidiinae of Orchidaceae, respectively. On the other hand, Witter (1988) supported the hypothesis that plants of the Hawaiian silversword alliance, in which $2n = 28$, are polyploid by demonstrating duplicate gene expression for four soluble enzymes.

In this paper we report results of an electrophoretic study of isozyme number for seven enzymes from eight species of *Eupatorium* with a chromosome number of $2n = 20$. This has been regarded widely as the original base number for the tribe Eupatoriae (King and Robinson, 1970a, 1987; King et al., 1976), although a number of $n = 5$ has been reported for the tribe (Turner and Irwin, 1960).

[1] Received for publication 1 November 1988; revision accepted 2 March 1989.

We thank V. I. Sullivan for her kind help in the field. This study was partly supported by grant no. 63041048 from Ministry of Education, Culture and Scientific Reserch, Japan.

MATERIALS AND METHODS—Eight species of *Eupatorium* were examined; the species, number of plants studied from natural populations and localities of the populations are as follows: *E. capillifolium* (Lam.) Small (25, Vernon Parish, Louisiana; 24, Guilford Co., North Carolina); *E. fistulosum* J. Barratt (24, Fairfield Co., Ohio; 24, Hocking Co., Ohio); *E. maculatum* L. (12, Franklin Co., Ohio); *E. mikanioides* Chapman (12, Wakulla Co., Florida); *E. perfoliatum* L. (26, Fairfield Co., Ohio; 25, Hocking Co., Ohio); *E. purpureum* L. (24, Fairfield Co., Ohio); *E. semiserratum* DC. (24, Evans Co., Georgia; 18, Allen Parish, Louisiana) and *E. serotinum* Michx. (12, Franklin Co., Ohio; 27, Marion Co., Indiana). For *E. fistulosum* and *E. perfoliatum,* progenies grown from seeds were also examined (36 from three maternal parents of *E. fistulosum*; and 36 from two plants of *E. perfoliatum*; seeds were collected from Fairfield Co., Ohio).

Flower buds and a small piece of leaf material of individual plants were ground in 1.0 ml of cold extraction buffer consisting of 0.1 M tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 1.0 mM EDTA, 10 mM KCl, 10 mM $MgCl_2$, 0.25% (v/v) triton X-100, and 15 mg PVP (modified from Odryzykoski and Gottlieb, 1984). For *E. capillifolium* and *E. mikanioides,* fresh leaves alone were used.

Enzymes were resolved in 12% starch gels using three buffer systems. System I had a gel buffer of 0.033 M tris, 0.005 M citric acid, 0.004 M lithium hydroxide and 0.3 M boric acid with the pH adjusted to 7.6 and an electrode buffer of 0.039 M tris and 0.263 M boric acid with the pH adjusted to 8.0 (P. Soltis and D. Soltis, 1987). System II had an electrode buffer of 0.5 M tris, 0.016 M EDTA and 0.57 M boric acid with the pH adjusted to 8.0. The gel buffer was a 1:9 dilution of the electrode buffer. System III consisted of an electrode of 0.065 M L-histidine (free base) and 0.007 M citric acid (monohydrate) adjusted to pH 6.5. The gel buffer was a 1:3 dilution of the electrode buffer (Cardy, Sturber, and Goodman, 1981).

System I resolved fructose 1, 6-biphosphate aldolase (ALD, EC 4.2.1.3), phosphoglucoisomerase (PGI, EC 5.3.1.9) and triosephosphate isomerase (TPI, EC 5.3.1.1). System II resolved phosphoglucomutase (PGM, EC 2.7.5.1). System III was employed to resolve isocitrate dehydrogenase (IDH, EC 1.1.1.42), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) and shikimate dehydrogenase (SKDH, EC 1.1.1.25). Staining schedules followed Soltis et al. (1983). Enzymes for study were selected because the minimal conserved number of isozymes in diploid plants has been well established for each (Randall and Givan, 1981 for IDH; Gottlieb, 1982; Mousdale and Coggins, 1985, for SKDH). In all instances, one isozyme occurs in the plastids and the other is cytosolic.

Chloroplastic isozymes were isolated from a single plant of *E. perfoliatum* collected in Columbus, Ohio. The method of extracting intact chloroplast followed Gastony and Darrow (1983) except that the chloroplasts were not centrifuged through Percoll. The chloroplast-enriched pellets were broken with the tris-HCl grinding buffer (above). Activities of IDH, 6-PGD, and SKDH were stronger in flower buds but very weak in leaves and we could not obtain sufficient activity of these enzymes in the chloroplast fraction from leaves.

RESULTS—*PGI*—In all eight species, enzyme activity appeared in two zones in the gels. In the chloroplast-enriched fractions of *E. perfoliatum,* isozymes in the faster migrating (more anodal) zone only were expressed. This result agrees with all the formerly obtained results on electrophoretic mobilities of PGI isozymes from various plants; i.e., the chloroplastic isozyme migrates faster than the cytosolic one.

In the slower migrating (more cathodal) zone, three or five bands were detected for this dimeric enzyme in most individuals of the eight species examined. This suggests gene duplication for cytosolic PGI in diploid *Eupatorium.* Among progenies grown from a single maternal parent of *E. fistulosum,* three phenotypes segregated in the more cathodal zone (1–3 in Fig. 1A and Fig. 2); one pattern with a seemingly single heavily staining band (1 in Fig. 1 and 2), one phenotype having three bands consisting of a fast migrating very faint, a slow migrating very dark, and a band intermediate in both mobility and staining intensity (2 in Fig. 1 and 2), and the last pattern having six bands (3 in Fig. 1 and 2). The first phenotype is interpreted as having three bands that migrate very closely and the most anodally migrating band is very faint (see dots in Fig. 1A and Fig. 2-1). Also, among progenies grown from a single maternal parent of *E. perfoliatum,* three- and five-banded phenotypes segregated in the more cathodal zone (Fig. 1B, Fig. 2-4, 2-5). These patterns suggest that fixed heterozygosity due to duplicated gene loci for cytosolic PGI isozyme occur in *E. fistulosum* and *E. perfoliatum.* In both species, three-banded phenotypes have one faint band, and the same pattern was seen in *E. mikanioides* (Fig. 1E), in which one faint band of the three-banded phenotype migrated near to a band representing a chloroplastic isozyme. By com-

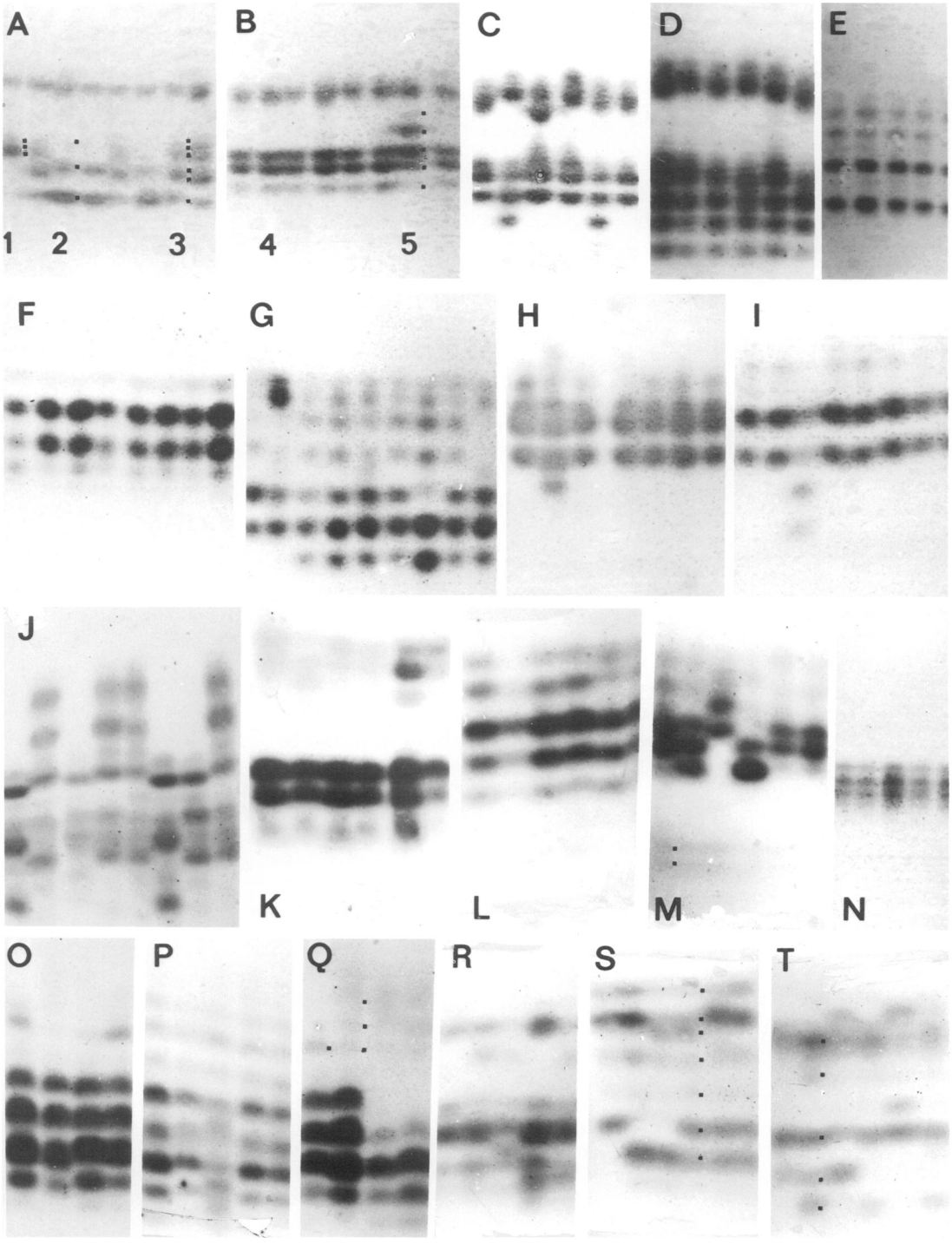

Fig. 1. Electrophoretic patterns of six soluble isozymes in diploid species of *Eupatorium*. (A–E) PGI; A, *E. fistulosum*; B, *E. perfoliatum*; C, *E. semiserratum*; D, *E. capillifolium*; E, *E. mikanioides*. (F–I) PGM; F, *E. fistulosum*; G, *E. perfoliatum*; H, *E. semiserratum*; I, *E. capillifolium*. (J–M) 6PGD; J, *E. fistulosum*; K, *E. perfoliatum*; L, *E. semiserratum*; M, *E. capillifolium*. (N) ALD in *E. capillifolium*. (O–Q) IDH; O, *E. perfoliatum*; P, *E. semiserratum*; Q. *E. capillifolium*. (R–T) SKDH; R, S, *E. fistulosum*; S, *E. perfoliatum*; T, *E. semiserratum*. All isozymes migrate to the anode at the top of the photograph.

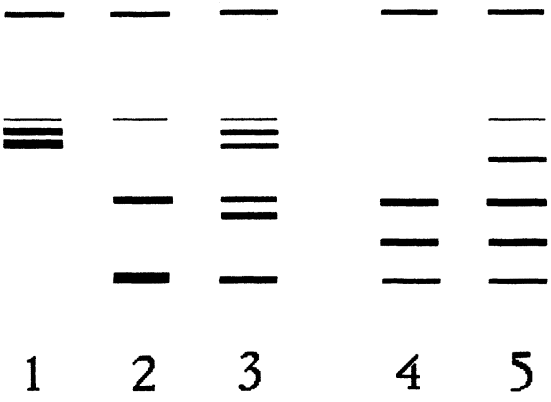

Fig. 2. Diagrammatic scheme of representative patterns of PGI isozymes segregated in progenies of *Eupatorium fistulosum* (1–3) and *E. perfoliatum* (4, 5). Numbers are correspondent to those in Fig. 1A and 1B.

paring with these patterns, seemingly four-banded phenotypes in *E. semiserratum* (Fig. 1C), and *E. capillifolium* (Fig. 1D) can be interpreted as having an additional band that is too faint to be recognized. *Eupatorium serotinum* expressed a pattern similar to *E. semiserratum* while *E. maculatum* and *E. purpureum* displayed easily interpretable three- or five-banded patterns. Clearly, two gene loci are expressed for the more cathodal, cytosolic isozymes. In the more anodal zone, three-banded patterns were seen for most plants of *E. semiserratum* (Fig. 1C) and *E. capillifolium* (Fig. 1D). However, these bands were not well resolved in many instances and it is not clear whether one or two gene loci are specifying chloroplastic isozymes of PGI. Thus, a total of at least three genes encoded PGI in all eight species.

PGM—Six- to seven-banded patterns were observed in 36 progenies grown from two maternal parents of *E. perfoliatum*. Two of these bands, which were the most anodally migrating, were always expressed in 36 progenies (Fig. 1G). The chloroplast-enriched fraction from a single plant of *E. perfoliatum* yielded these two bands. Because PGM is a monomeric enzyme, the data suggest that *E. perfoliatum* has two gene loci encoding chloroplastic PGM. The remaining four to five bands are viewed as cytosolic, and there are at least three gene loci encoding these isozymes. A total of 26 progenies from three maternal parents of *E. fistulosum* always expressed four-banded patterns (Fig. 1F) and this species is considered to have a total of at least four gene loci specifying PGM. In the remaining six species, four- to six-banded patterns were seen (Fig. 1H, I), indicating that at least four loci are being expressed.

6PGD—In *E. fistulosum* (Fig. 1J) and *E. purpureum,* complex multibanded patterns were resolved. In most individuals, more than five bands were seen, and these two species appear to have at least three loci expressed. In *E. perfoliatum* (Fig. 1K) and *E. capillifolium* (Fig. 1M), electromorphs of 6PGD occurred in two electrophoretic zones. In *E. perfoliatum,* a three-banded pattern was always observed in the more cathodal zone. Bands in the anodal region were not well resolved but three-banded patterns were frequently observed; we cannot say with certainty, however, that all plants have three bands. These patterns indicate that at least three loci encode 6PGD in *E. perfoliatum.* In *E. capillifolium,* two faint but clearly observable bands were always present in the more cathodal area (indicated by dots in Fig. 1M), and three or more bands were observed in the more anodal area. Two-banded patterns in the more cathodal area could be due to very faint staining for an additional band, but we could not document this with certainty. This species is interpreted as having at least three loci for 6PGD. In *E. semiserratum* (Fig. 1L) and *E. serotinum,* five bands were always resolved, and faint bands were sometimes detected in the more cathodal area. These species are considered to have at least three loci for 6PGD. In *E. maculatum* and *E. mikanioides,* 6PGD was not resolved well.

ALD—A multibanded pattern was invariably expressed in all eight species examined. In well-resolved gels, five bands with two faint ones can be recognized; however, a three-banded pattern was seen on most gels (Fig. 1N). Because ALD is a tetrameric enzyme, three-banded pattern could be due to low activity of the two additional bands. Those gels containing five well-resolved bands provide good evidence for duplicated loci for ALD in all eight species.

IDH—For *E. perfoliatum* (Fig. 1O), *E. capillifolium* (Fig. 1Q), *E. purpureum* and *E. serotinum,* enzyme activity was found in two distinct zones of the gels. In these species, one- or three-banded patterns were seen in the more anodal zone, suggesting segregation of two alleles at one locus. These bands often stained quite faintly and were not always detectable. The more cathodal zone of activity consisted of a fixed four-banded pattern with the most cathodal of these bands being much fainter

than the other three. It was not possible to ascertain with certainty whether this represents enzyme activity or is some sort of "ghost." These four species are considered to have at least three isozymes, one in the more cathodal and two in the more anodal zone of activity. In *E. semiserratum* (Fig. 1P), a seven-banded pattern was seen in all 42 individuals collected from Georgia and Louisiana. It was not possible to interpret this very complex pattern genetically, but it appears likely that at least four gene loci are expressed. In *E. fistulosum, E. maculatum* and *E. mikanioides,* IDH was not resolved well.

SKDH—Six- or seven-banded patterns were observed in certain individuals of *E. perfoliatum* (Fig. 1S) and *E. mikanioides.* Because SKDH is a monomeric enzyme, these species must have at least four gene loci specifying it. *Eupatorium fistulosum* (Fig. 1R), *E. semiserratum* (Fig. 1T), *E. purpureum* and *E. capillifolium* sometimes had five or more bands, indicating that at least three loci are expressed in these species. In *E. maculatum* and *E. serotinum,* SKDH was not resolved well.

TPI—We were unable to document gene duplications for this enzyme. In progenies of *E. fistulosum* and *E. perfoliatum,* one- or three-banded patterns were segregated with an additional invariable band, indicating that two loci, one monomorphic and the other polymorphic, are expressed in these species. Results from chloroplast isolation for *E. perfoliatum* suggested that the monomorphic isozyme is cytosolic. This isozyme migrated between the fast and slow homodimeric allozymes of the chloroplastic isozyme. One- or three-banded patterns with an additional invariable band were also observed in *E. capillifolium.* In *E. maculatum,* two-, four-, and six-banded patterns were found, indicating that two polymorphic loci were expressed. In *E. mikanioides, E. semiserratum, E. serotinum* and *E. purpureum,* two bands were always seen. These species are regarded as having two monomorphic loci.

DISCUSSION—The eight species included in this study were selected with two criteria in mind. First, seven species (*E. purpureum* is the exception) are all strictly diploid and sexual, and polyploidy and agamospermy are not known. Cytology of American species of *Eupatorium* have been studied extensively (Sullivan, 1972, 1976; Watanabe, 1986; Watanabe et al., in preparation) and only the chromosome number $2n = 20$ has been reported for these seven species. With regard to *E. purpureum,* an exceptional tetraploid was reported from Canada (Grant, 1953) but sexual diploids are widespread in the United States (Watanabe, 1986).

The second reason for choosing these eight species of *Eupatorium* is that they represent the three morphologically distinct groups recognized for the American representatives of the genus (King and Robinson, 1970a, 1987; Sullivan, 1972). *Eupatorium fistulosum, E. maculatum,* and *E. purpureum* are from the *Eutrochium* group, *E. capillifolium* from the *Traganthes* group, and *E. mikanioides, E. perfoliatum, E. semiserratum,* and *E. serotinum* from the *Uncasia* group. Thus, these five species represent a cross section of the variation found in American *Eupatorium.*

Gottlieb (1982) summarized data demonstrating that diploid plants have two isozymes for enzymes of glycolysis and the pentose phosphate pathway, including the enzymes ALD, PGI, PGM, 6PGD and TPI examined in this study. In each case, one of these isozymes is cytosolic and the other is localized in the plastids. Isocitrate dehydrogenase in higher plants typically has two isozymes, one being cytosolic and the other in plastids (Randall and Givan, 1981; Gottlieb, 1987). Shikimate dehydrogenase in higher plants also has two isozymes, one in the plastids and the other possibly being cytosolic (Mousdale and Coggins, 1985). Thus, the minimal conserved number of isozymes in diploid plants of all the enzymes included in this study is two. This means that for the dimeric enzymes (IDH, PGI, 6PGD, and TPI) six is the greatest number of bands that would be expected when two genes are expressed. This banding pattern would occur when a plant is heterozygous at both loci. For the two monomeric enzymes PGM and SKDH, four bands would be the maximum number observed when two gene loci are expressed.

The complex banding patterns resolved for all enzymes except TPI indicate that three or more isozymes occur in all species examined. The multiplicity in these enzymes is also expressed in some Asian species of *Eupatorium* (Kawahara et al., unpublished data). While it would be highly desirable to have more extensive genetic data for the banding patterns (this work is in progress), in certain instances variation in banding patterns among the progenies of individual plants, variation among individual plants within populations (together with knowledge of the active subunit composition of the enzymes), and subcellular localization

of isozymes of several enzymes combine to allow for rather strong inferences about the genetic bases of the banding patterns. In all cases, one must hypothesize gene duplication as the basis of the banding patterns.

The extensive isozyme multiplicity in diploid *Eupatorium* contrasts sharply with many other members of the Asteraceae, such as *Aster* and *Machaeranthera* of Astereae (Gottlieb, 1981b), *Lasthenia* (Crawford, Ornduff, and Vasey, 1985) of Heliantheae, and *Crepis* (Roose and Gottlieb, 1978) and *Stephanomeria* (Gallez and Gottlieb, 1982) of Lactuceae, in which diploid species with haploid chromosome number less than 10 typically have the conserved isozyme number for most enzymes. The reason or reasons for the extent of gene duplication in these taxonomically diverse species of *Eupatorium* are not known. Although $x = 10$ has been regarded as the original basic chromosome number for the tribe Eupatorieae (King et al., 1976; King and Robinson, 1987), a chromosome number of $n = 5$ was reported for *Adenostemma brasiliense* (Pers.) Cass. (Turner and Irwin, 1960), now correctly determined as *A. involucratum* R. King et H. Robinson (King et al., 1976). King et al. (1976) and King and Robinson (1987) suggested that the $n = 5$ number may be derived from $n = 10$ because the latter number is prevalent in many genera of the tribe Eupatorieae, including *Adenostemma* itself. It is also notable that $n = 4$ has been reported for *Eupatorium microstemon* Cass. (Baker, 1967), now treated as *Fleischmannia microstemon* (Cass.) R. King et C. Robinson (King et al., 1976). The same chromosome number was also determined for *Fleischmannia hymenophylla* (Klatt) R. King et C. Robinson (Graschoff, Bierner, and Northington, 1972). Baker (1967) considered $n = 4$ to be a derived number because the chromosomes are much larger than those found in plants with $n = 10$. Additional evidence for this hypothesis came from meiotic behavior of hybrids between plants with the chromosome numbers $n = 4$ and $n = 10$. King et al. (1976) cited Baker's statement with a note on the other species of *Fleischmannia* having $n = 10$ and regarded $n = 4$ as the derived state. Our results, however, cast some doubt on the hypothesis that $x = 10$ is the original base number of the tribe Eupatorieae, and it appears that more extensive cytological surveys of *Adenostemma* and *Fleischmannia* are needed.

Species of *Eupatorium* with a chromosome number of $2n = 20$ are not autotetraploid karyotypically if they were polyploids (Watanabe et al., in preparation), and they form normal bivalents at meiosis (Watanabe, Fukuhara, and Huziwara, 1982; Watanabe, unpublished data). Thus, cytological features seem to indicate an amphidiploid or diploid condition in these "diploid" species of *Eupatorium*. As stated earlier, American species of *Eupatorium* have diversified into three morphologically distinct groups. It seems unlikely, therefore, that evolution and diversification have occurred recently. *Eupatorium* has a disjunct distribution in eastern North American and East Asia, and it is hypothesized to be of Tertiary origin (King and Robinson, 1970b; Watanabe, 1986). If this hypothesis is correct, then it means that extensive gene duplication and fixed heterozygosity have been maintained for a rather long geological period subsequent to the origin of the genus and through extensive chromosomal diploidization.

If, by contrast, $2n = 20$ does represent the original diploid number for *Eupatorium,* then extensive gene duplications have occurred both in eight species belonging to the three morphological groups in North America and in certain species in Asia. If this were the case, then it would mean that the mechanism (or mechanisms) producing these gene duplications in *Eupatorium* is rare in diploid plants because *Clarkia* is the only other genus that shows such extensive duplication of isozyme loci among plants viewed as unquestionably diploid (Gottlieb, 1986). Even in *Clarkia,* duplication has been confirmed on at most four enzymes, ADH, PGI, PGM and 6PGD. We consider it more likely that plants with a chromosome number of $2n = 20$ in *Eupatorium* are of polyploid origin.

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