J. Plant Res. 113: 91-96, 2000

Journal of Plant Research
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Molecular Phylogeny of Eupatorieae (Asteraceae) Estimated from cpDNA RFLP and its Implication for the Polyploid Origin Hypothesis of the Tribe

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The tribe Eupatorieae has a chromosome base number that ranges from 4 to 25. A molecular phylogenetic analysis using cpDNA restriction site mutations was performed. Fifteen species representing 13 subtribes of the tribe Eupatorieae were examined, together with three species from the tribe Heliantheae as outgroups. A total of 103 restriction site mutations were detected, and 31 of these were phylogenetically informative. Parsimony analysis produced a single most parsimonious tree with 117 steps. This tree suggested that two clades diverged early in the evolution of the tribe Eupatorieae. One clade includes *Neomirandea* ($x=17$ and 25), *Ageratina* ($x=17$) and *Sclerolepis* ($x=15$) with the higher chromosome base numbers, and the other includes *Mikania* ($x=17$) and the remaining genera with lower chromosome base numbers ($x=10$-11). However, the monophyly of the former clade is supported with a low bootstrap value. In the latter clade, *Mikania* ($x=17$) diverged first, then *Stevia* ($x=11$), and finally eight genera with $x=10$ diverged in succession. This result supports the hypothesis that the genera in the tribe Eupatorieae with $x=10$ evolved from an ancestor with a higher base number, and the tribe is of polyploid origin.

Key words: Ancient polyploidy — Asteraceae — Chloroplast DNA — Diploidization — Dysploidy — Eupatorieae

It has been well documented that the origin of species through polyploidization is a major mode of speciation in vascular plants (Stebbins 1950, Lewis 1980). However, there is limited evidence that polyploidy has contributed to the evolution of higher taxa, such as genera, tribes, and families. The paucity of evidence may be attributed to difficulty in demonstrating ancient polyploidy, because diploidization of the duplicated karyotypes and genes may mask their polyploid nature very quickly (Haufler 1987).

During the 1980's, Gottlieb and his colleagues made a concentrated effort to demonstrate the highly conservative nature of isozyme number in true diploid plants, with one cytosol isozyme and another plastid isozyme (Gottlieb 1981, 1982, 1983). The extensive gene duplications of glycolytic enzymes in a putative diploid plant with a diploidized karyotype and regular bivalent formation in meiosis were regarded as evidence of paleopolyploids. Ancient polyploidy has been sought by determining the isozyme number for leptosporangiate ferns (Haufler and Soltis 1986), lycopods (Soltis and Soltis 1988), and primitive angiosperms (Soltis and Soltis 1990) with high chromosome numbers, and has been confirmed for primitive angiosperms, but not for leptosporangiate ferns or lycopods.

The tribe Eupatorieae possesses a wide array of chromosome base numbers, ranging from $x=4$ to $x=25$. The predominant chromosome number at the species, genus, and subtribe levels is $x=10$, and most previous workers regarded $x=10$ as the ultimate base number (see King and Robinson 1987 for review). *Eupatorium* species with $2n=20$ have 10 well-diverged pairs of chromosomes and show regular bivalent formation in meiosis (Watanabe *et al.* 1990). The lowest chromosome base number, $x=4$, had been reported only from few species of *Fleischmannia*, in which $x=10$ is also predominant, and thus, $x=4$ is considered to have evolved by reduction of the base number (Watanabe *et al.* 1995). By determining their isozyme number, Yahara *et al.* (1989) estimated the ploidal level of the *Eupatorium* species with $2n=20$. They found that those species shared extensive gene duplications in glycolytic enzymes and suggested that they were polyploid states. Since $n=10$ prevails in the tribe Eupatorieae, they suggested that this tribe was also of polyploid origin. On the other hand, Watanabe *et al.* (1995) suggested that the chromosome base number of the tribe Eupatorieae might be $x=17$ based on evidence

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obtained from karyotypic comparisons between taxa with low and high base numbers.

Recently developed molecular systematic methodologies have enabled us to test various evolutionary hypotheses. Several studies employing RFLP analysis of chloroplast DNA (cpDNA) have demonstrated the utility of this method for estimating the phylogenetic relationships in the Asteraceae (Jansen and Palmer 1987, 1988, Jansen *et al.* 1991, Kim *et al.* 1992a, Ito *et al.* 1998).

In this paper, we estimate the phylogenetic relationships of taxa in the tribe Eupatorieae using cpDNA RFLP data, and test the hypothesis that $x=17$ is ancestral and $x=10$ derivative. In addition, we examine the hypothesis that dysploidal reduction in chromosome number has occurred in the tribe Eupatorieae.

Materials and Methods

The sources of the plant species examined are listed in Table 1. The 15 species of the tribe Eupatorieae examined belong to 14 genera representing 13 subtribes. The chromosome data were largely described in Watanabe *et al.* (1990, 1995). For the genus *Neomirandea*, species with $n=17$ and $n=25$ were examined to confirm the monophyly of the genus. As outgroups, three genera of the tribe Heliantheae were also examined, because recent molecular systematic studies (Jansen *et al.* 1991, Watson *et al.* 1991, Kim *et al.* 1992b, 1995) and cladistic studies based on morphological characters (Karis 1993, Bremer *et al.* 1994) showed that the tribe Eupatorieae was derived from a member of the tribe Heliantheae.

Total DNA was extracted from fresh leaves of 18 species using the CTAB extraction method (Doyle and Doyle 1987). The extracted DNA was further purified in a $CsCl_2$/EtBr density gradient, and then digested with 12 restriction enzymes (*BamHI, BanI, BstXI, EcoRV, HindIII, NcoI, PvuII, PstI, SalI, SmaI, StuI, and XhoI*). The fragments were separated by electrophoresis in 0.7 or 1.0% agarose gels and transferred to nylon filters (Hybond N^+, Amersham). The probes for Southern hybridization were prepared from the clone bank of *Lactuca sativa* cpDNA (Jansen and Palmer 1987). For most of the hybridizations, several contiguous fragments were combined, employing a total of four groups to cover the genome (Table 2). Only one fragment was used to facilitate interpretation of the patterns obtained in terms of mutational change. Detection was performed with the ECL gene labeling and detection system (Amersham) according to the manufacturer's instructions. Hybridization was performed at 40 C for 15 hrs.

A data matrix encoding the presence or absence of each restriction site was prepared. Cladistic analyses were performed using the parsimony method in PAUP 3.1 (Swofford 1993) with the branch and bound option. To test the confidence intervals of each branch, the bootstrap method was employed (Felsenstein 1985) with 1,000 replicates. The basic chromosome number character states were estimated on the most parsimonious tree using MacClade 3.05 with ACCTRAN optimization.

Results

A total of 103 restriction site mutations were detected, of which 31 were phylogenetically informative (Table 3). The parsimony analysis using PAUP 3.1 produced a single most parsimonious tree with 117 steps (Fig. 1). The consistency index with and without uninformative mutations was 0.880 and 0.689, respectively. The number above each branch indicates the estimated number of mutations that occurred on the branch. While larger numbers of apomorphic mutations were found on distal branches, the number of synapomorphic mutations on internodes only varied from one to three. The tree was rooted using *Coreopsis lanceoratum* as the outgroup, because *Coreopsis* is the basal genus in the Heliantheae. Sometimes *Coreopsis* is separated from the Heliantheae *sensu strict* as the tribe Coreopsideae, which is considered to be the sister group to the Heliantheae *sensu strict* and the Eupatorieae (Jansen *et al.* 1991). The resulting tree shows that the genera *Helianthus* and *Clibadium* in the Heliantheae, with chromosome base numbers of $x=17$ and 16, respectively, branched from the base of the tree. The monophyly of the tribe Eupatorieae was supported with a 67% bootstrap value.

The tribe Eupatorieae separated into two basal clades early in its evolution. One clade consists of *Neomirandea* ($x=17$ and 25), *Ageratina* ($x=17$), and *Sclerolepis* ($x=15$), and the other of *Mikania* ($x=17$) and the remaining genera with lower base numbers ($x=10$-11). The monophyly of the former clade was only supported with a 49% bootstrap value. The two species of *Neomirandea* clustered together in spite of their different chromosome numbers. In the later clade, the first branch separates *Mikania* ($x=17$) from the remaining genera. The monophyly of the remaining genera was well supported, with an 86% bootstrap value. Then *Stevia* ($x=11$) diverged from a clade consisting of eight genera with $x=10$, which formed a monophyletic clade with a 95% bootstrap value. The basal part of this clade was not resolved well, although the first branch in this clade was *Carminatia*. Within this clade, *Critonia, Fleischmannia, Ageratum*, and *Conoclinum* formed a clade supported with a high bootstrap value.

Discussion

The most parsimonious tree obtained indicates that the genera with high chromosome numbers diverged first, and then the chromosome base number in the tribe Eupatorieae was successively reduced from $x=17$, to $x=11$, to $x=10$. This is concordant with our hypothesis that $x=17$ is ancestral and $x=10$ derivative in the tribe Eupatorieae (Watanabe *et al.* 1995). Judging from the estimated character states for the chromosome base number on the tree, a dysploidal reduction in chromosome number occurred in the course of the diversification of the tribe Eupatorieae (Fig. 2). In parallel with our study, Schling *et al.* (MS) estimated the phylogenetic relationships of 12 genera in the tribe Eupatorieae, based on cpDNA RFLP data using a different set of restriction enzymes, and their result fundamentally agreed with ours.

Table 1. Materials used for this study. Classification of subtribes in Eupatorieae followed King and Robinson (1987).

Species	Locality	Vouchers	Subtribe (or tribe in outgroup)	Chromosome base number (x)
Eupatorieae				
1. *Carminatia tenuifolia* DC.	U.S.A. Davis Mts., Jeff Davis Co., TX.	*Yahara et al. 4*	Alomiinae	10
2. *Ageratum microcarpum* Hemsley	U.S.A. Chisos Mt., Brewster Co., TX.	*Yahara et al. s.n.*	Ageratinae	10
3. *Stevia ovata* Willd.	U.S.A. Chisos Mt., Brewster Co., TX.	*Yahara et al. s.n.*	Ageratinae	11
4. *Polyanthina nemorosa* (Klatt) R.M. King & H. Robinson	Costa Rica. 0.5 km NE from Orosi.	*Yahara et al. s.n.*	Ayapaninae	10
5. *Critonia morifolia* (Miller) R.M. King & H. Robinson	Costa Rica. Campus of National University of Costa Rica, San Jose.	*Yahara et al. s.n.*	Critoniinae	10
6. *Eupatorium serotinum* Michx.	U.S.A. Campus of Ohio State University, Columbus, OH.	*Yahara et al. s.n.*	Eupatoriinae	10
7. *Fleischmannia sideritides* (Benth. in Orsted) R.M. King & H. Robinson	Costa Rica. Tapanti National Wildlife Refuge, 1,200m alt.	*Yahara et al. 128*	Fleischmaniinae	10
8. *Conoclinum gregii* (A. Gray) Small	U.S.A. Chisos Mt., Brewster Co., TX.	*Yahara et al. 15*	Gyptidinae	10
9. *Liatris punctata* Hook.	U.S.A. Austin, TX.	*Yahara et al. s.n.*	Liatrinae	10
10. *Mikania banisteriae* DC.	Costa Rica. 0.5 km NE from Orosi, Costa Rica	*Yahara et al. s.n.*	Mikaniinae	17
11. *Neomirandia angularis* (B.L. Rob.) R.M. King & H. Robinson	Costa Rica. Tapanti National Wildlife Refuge.	*Yahara et al. 66*	Neomirandinae	25
12. *Neomirandia arthoides* (B.L. Rob.) R.M. King & H. Robinson	Costa Rica. Tapanti National Wildlife Refuge.	*Yahara et al. s.n.*	Neomirandinae	17
13. *Ageratina bustamenta* (DC.) R.M. King & H. Robinson	Costa Rica. Volcan Irazu, 2,100m alt..	*Yahara et al. 39*	Oxylobinae	17
14. *Chromolaena odorata* (L.) R.M. King & H. Robinson	Indonesia. Mt. Gede, Java.	*Kawahara s.n.*	Praxelinae	10
15. *Scleolepis uniflora* (Walter) Britten, Sterns & Poggenb.	U.S.A. Sussex, Delawer.	*King 10235*	Trichocoroninae	15
Outgroup				
16. *Clibadium sp.*	Costa Rica. On the way from Cartago to Tapanti, W limit of Paraiso.	*Yahara et al. s.n.*	Heliantheae	16
17. *Helianthus annus* L.	U.S.A. Campus of Ohio State University, Columbus, OH.	*Yahara et al. s.n.*	Heliantheae	17
18. *Coreopsis lanceolatum* L.	Cultivated, OSU green house, U.S.A.	*Yahara et al. s.n.*	Heliantheae	13

Table 2. Probe group of clones from lettuce cpDNA clone bank used in this study.

Probe group	Fragment size (kbp)	Region
1	18.8, 1.8, 3.5, 9.9	SSC+IR
2	7.5, 7.2, 7.0, 6.7	LSC
3	4.6, 5.4, 6.3, 12.3	LSC+IR
4	3.8, 6.9, 7.7, 10.6	LSC

IR: inverted repeat, LSC: large single copy region, SSC: small single copy region.

Table 3. Chloroplast DNA restriction site mutations which share two or more taxa. Taxa showing derived mutations (comparing with *Coreopsis*) are indicated by the numbers in Table 1.

No.	Enzyme	probe	mutation	Taxa having mutations
1.	*BamH I*	1	3.9=1.5+2.4	2, 8
2.	*BamH I*	1	2.4=1.5+0.9	2, 8
3.	*BamH I*	1	3.9=3.1+0.8	5, 7
4.	*BamH I*	2	5.6=4.9+0.7	2, 5-8, 14
5.	*BamH I*	2	2.2+1.7=3.9	2, 8
6.	*BamH I*	2	1.9+0.7=2.6	2, 3
7.	*BamH I*	3	34.0=17.5+16.5	1-16
8.	*Ban I*	2	4.7=3.7+1.0	11, 12
9.	*Ban I*	3	6.9=4.0+2.9	1-16
10.	*Ban I*	4	2.1=2.0+0.1	1-9, 14
11.	*EcoR V*	1	1.2+7.8=9.0	1-10, 14, 17
12.	*EcoR V*	2	2.8=2.4+0.4	2, 5, 7, 8
13.	*EcoR V*	3	3.7+2.5=6.2	2, 5, 7, 8
14.	*EcoR V*	4	5.6=2.8+2.8	1-3, 5, 7-13, 15-17
15.	*EcoR V*	4	14.0=11.2+2.8	1-15
16.	*Hind III*	1	4.2=4.1+0.1	1-3, 5-17
17.	*Hind III*	2	8.5+6.5=15.0	2, 5-8,14
18.	*Nco I*	1	9.2+7.8=17.0	2, 4-9, 14
19.	*Pvu II*	1	9.6+2.8=12.4	1, 7, 1 4
20.	*Pvu II*	2	24.6=20.0+4.6	1, 2, 4-7, 9, 14
21.	*Pvu II*	3	29.0=18.0+11.0	1, 3-6, 9-12, 14, 16, 17
22.	*Pvu II*	4	27.0=17.0+10.0	1-9, 14
23.	*Pvu II*	4	3.8+10.0=13.8	2, 7, 8
24.	*Sal I*	3	12.0=9.4+2.6	1, 2, 4-9, 14
25.	*Sal I*	3	1.7=1.1+0.6	2, 4-9, 14
26.	*Sma I*	2	13.5+12.6=26.1	2, 5, 7, 8
27.	*Sst I*	2	7.2+4.9=12.1	11-13, 15
28.	*Stu I*	1	18.0=13.6+4.4	2, 8
29.	*Stu I*	2	6.2=3.9+2.3	1-12, 14, 15
30.	*Xho I*	1	24.0=17.3+6.7	9, 16
31.	*Xho I*	2	9.0=8.0+1.0	1, 2, 4-9, 14

In the cladistic tree based primarily on morphological characteristics (Bremer *et al.* 1994), the genera of *Hofmeisteria, Oaxacania, Oxilobus, Ageratina*, and *Mikania* with high base numbers (x=16-18) branch proximally and the genera with low base numbers (x=4-12) branch distally. However, the genera *Decachaeta* and *Neomirandea* with x=16-25 were placed on the most distal branch in their tree. Bremer *et al.* (1994) postulated that an initial reduction in base number in the tribe Eupatorieae was followed by an increase.

Recent molecular and cladistic analyses have revealed that the tribe Eupatorieae originated from the tribe Heliantheae (Jansen *et al.* 1991, Watson *et al.* 1991, Kim *et al.* 1992b, Karis 1993, Bremer *et al.* 1994, Kim and Jansen 1995). Chromosome numbers also vary considerably in the tribe Heliantheae, ranging from x=4 to x=19. However, x=10 is rare in the tribe Heliantheae, while x=8 and 9 are common and higher base numbers of x=17-19 are also prevalent (Stuessy 1977). Robinson *et al.* (1981) proposed that ancient polyploidy played an important role in the early history of the Heliantheae, based on the distribution of chromosome numbers.

Watson *et al.* (1991) focused on the systematic position of the genus *Marshallia*. They found that several genera of Heliantheae with high chromosome numbers (*Helianthus* [x=17], *Wyethia* [x=19], *Perityle* [x=17 and 19], and *Geraea* [x=18]) were more closely related to the tribe Eupatorieae than to the other genera with low chromosome numbers (*Marshallia* [x=9], *Palafoxia* [x=10-12], and *Bahia* [x=8-12]). This result also supports our hypothesis that x=17, not 10, is plesiomorphic in the tribe Eupatorieae. Our hypothesis originally arose from the finding that *Eupatorium* species with $2n$=20 have extensive gene duplications for genes encoding glycolytic enzymes (Yahara *et al.* 1989). Thus, we assumed that the genus *Eupatorium* was in a polyploid state with regard to the number of genes. However, this does not mean that $2n$=20 was derived from polyploidization of an ancestral base number of x=5 or 4, as proposed by Grant (1953) and Turner and King (1964).

The tribe Helenieae possesses chromosome numbers ranging from x=3 to x=20. In molecular systematic studies, the species of this tribe formed a monophyletic clade together with the tribes Heliantheae and Eupatorieae (Jansen *et al.* 1991, Kim *et al.* 1995). Based on non-coding chloroplast sequences, Bayer and Starr (1998) suggested that the tribe Helenieae was paraphyletic or polyphyletic. Some members of this tribe clustered together with the Heliantheae and Eupatorieae. Based on the available data from chromosomal and molecular phylogenetic studies of the tribes Helenieae, Heliantheae and Eupatorieae, we suggest that a polyploidization event occurred during the course of the divergence of the tribes Helenieae and Heliantheae, changing the chromosome base number, and that one of the polyploid progeny was the ancestor of the tribe Eupatorieae. This hypothesis is supported by the fact that the genera *Helianthus* with x=17 and *Heliomeris* with x=8 also have many gene duplications (Rieseberg and Soltis 1989), suggesting polyploid states. Since the tribe Helenieae also has diverse chromosome numbers, the phylogenies of the tribes Helenieae and Heliantheae both must be examined in more detail to determine when polyploidization events occurred during the course of the evolution of these tribes.

The genera in the tribe Eupatorieae with x = 16 and 18

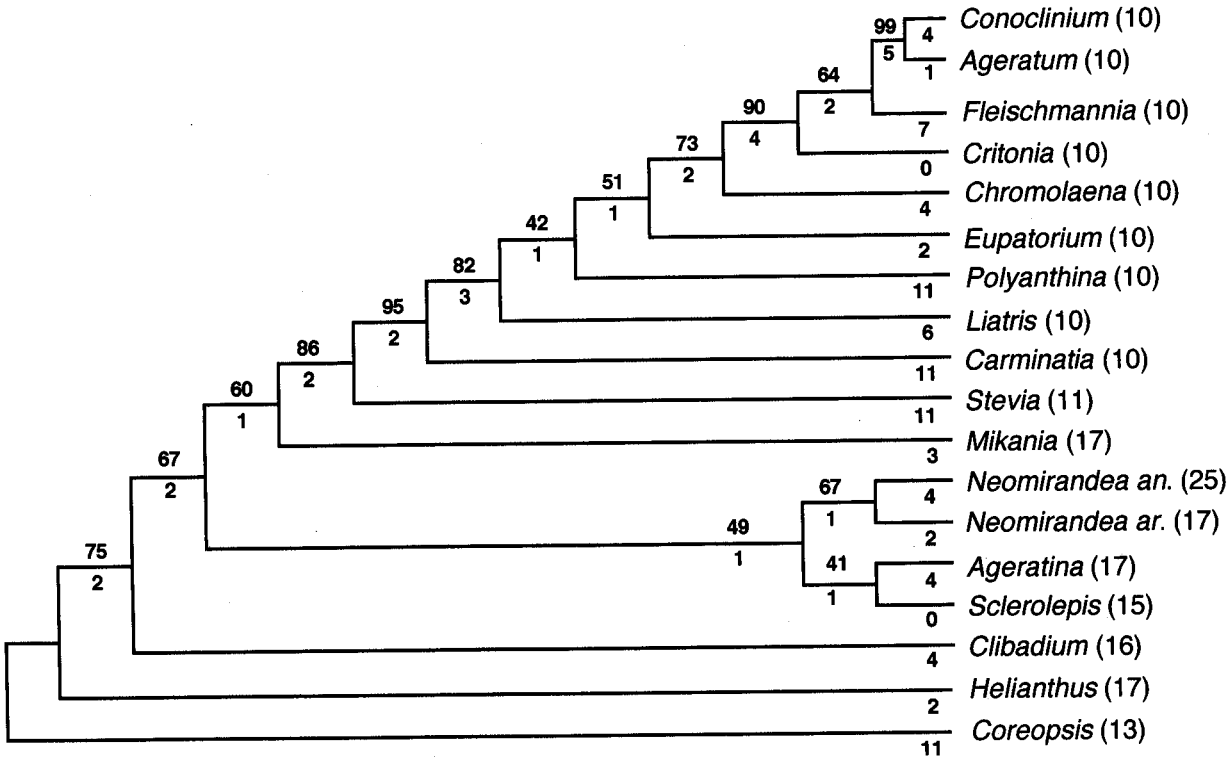

Fig. 1. The single most parsimonious tree obtained using Wagner parsimony. The number above each branch shows the bootstrap support of the branch using 1,000 replicates, and the number below each branch is the estimated number of site changes along the branch. The numbers in parentheses are the basic chromosome numbers for each taxon.

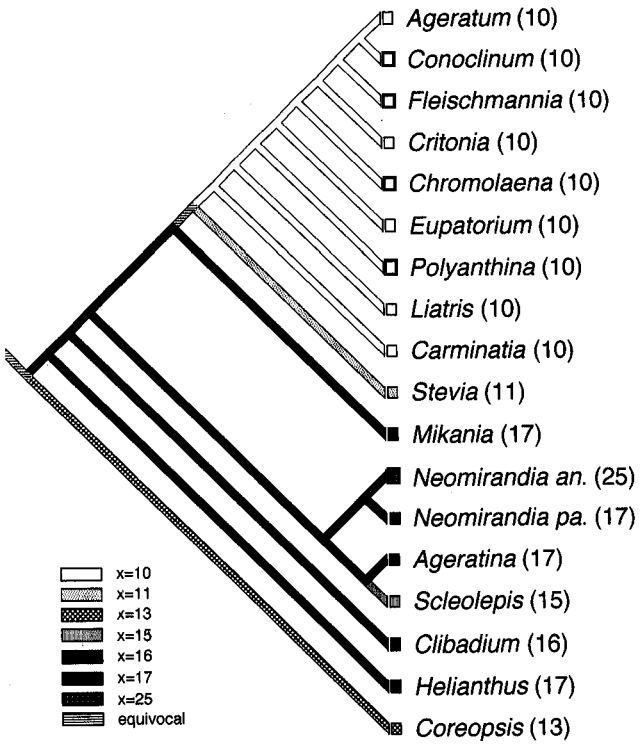

Fig. 2. Possible state assignments of basic chromosome numbers on the most parsimonious tree.

could not be examined in our present study. Since chromosome base numbers of $x=16$ and 18 are also found in the tribes Helenieae and Heliantheae, these numbers are also candidates for the ancestral character state in the tribe Eupatorieae. Using DNA sequence data, further systematic study including these genera is now in progress.

This study adduced the first evidence to support the polyploid origin of a tribe in the Asteraceae. It is notable that many genera of the tribe Eupatorieae have 10 well-diverged pairs of chromosomes karyomorphologically and show regular bivalent formation in meiosis. Thus, they have no sign of a polyploid state at $2n=20$. In addition, new polyploid series ($3x$, $4x$, $5x$, and $6x$) based on $x=10$ have developed extensively in the genera of *Eupatorium* and *Chromolaena* (Watanabe 1986, Watanabe *et al.* 1995). On the other hand, many gene duplications of glycolytic enzyme genes have been retained, even at $2n=20$ (Yahara *et al.* 1989). These facts suggest that karyotypic diploidization through the dysploidal reduction from $x=17$ to $x=10$ may have proceeded much faster than genic diploidization in the tribe Eupatorieae.

Polyploidization and subsequent diploidization may have occurred frequently during the diversification of the angiosperms. The tribe Eupatorieae provides a good opportunity to critically examine the process of diploidization. Further integrated studies of diploidization in the tribes Helenieae, Heliantheae, and Eupatorieae are required.

This research was supported by Monbusho International Scientific Research Program No. 03041028, and Grant-in-Aid No. 03304007 from the Ministry of Education, Science, and Culture, Japan.

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(Received September 13, 1999; accepted January 20, 2000)