Genetic diversity of *Echinacea* species based upon amplified fragment length polymorphism markers

D.-H. Kim, D. Heber, and D.W. Still

Abstract: The taxonomy of *Echinacea* is based on morphological characters and has varied depending on the monographer. The genus consists of either nine species and four varieties or four species and eight varieties. We have used amplified fragment length polymorphisms (AFLP) to assess genetic diversity and phenetic relationships among nine species and three varieties of *Echinacea* (sensu McGregor). A total of 1086 fragments, of which approximately 90% were polymorphic among *Echinacea* taxa, were generated from six primer combinations. Nei and Li's genetic distance coefficient and the neighbor-joining algorithm were employed to construct a phenetic tree. Genetic distance results indicate that all *Echinacea* species are closely related, and the average pairwise distance between populations was approximately three times the intrapopulation distances. The topology of the neighbor-joining tree strongly supports two major clades, one containing *Echinacea purpurea*, *Echinacea sanguinea*, and *Echinacea simulata* and the other containing the remainder of the *Echinacea* taxa (sensu McGregor). The species composition within the clades differs between our AFLP data and the morphometric treatment offered by Binns and colleagues. We also discuss the suitability of AFLP in determining phylogenetic relationships.

Key words: Echinacea, AFLP, genetic distance, phylogeny.

Résumé : La taxonomie du genre *Echinacea* est basée sur des caractères morphologiques et varie d'un auteur à un autre. Le genre comprend soit neuf espèces et quatre variétés ou encore quatre espèces et huit variétés. Les auteurs ont employé le polymorphisme de longueur des fragments amplifiés (AFLP) pour mesurer la diversité génétique et les relations phénétiques parmi les neuf espèces et trois variétés d'*Echinacea* (d'après McGregor). Un total de 1086 fragments, dont environ 90 % s'avéraient polymorphes au sein du genre *Echinacea*, ont été produits à l'aide de six combinaisons d'amorces. Le coefficient de distance génétique de Nei et Li et l'algorithme NJ ont été employés pour produire un arbre phénétique. La mesure des distances génétiques suggère que toutes les espèces du genre *Echinacea* sont très apparentées et la distance moyenne entre paires de populations était environ trois fois plus grande que les distances intrapopulations. La topologie de l'arbre NJ supporte nettement deux clades principaux, l'un regroupant l'*E. purpurea*, l'*E. sanguinea* et l'*E. simulata* tandis que l'autre regrouperait les autres espèces telles que définies par McGregor. Le regroupement d'espèces, au sein de clades, suggéré par les données AFLP diffère de celui obtenu par Binns et al., ce dernier ayant été obtenu en analysant des données morphométriques. Les auteurs discutent également de l'utilité des AFLP pour la détermination des relations phylogénétiques.

Mots clés : Echinacea, AFLP, distance génétique, phylogénie.

[Traduit par la Rédaction]

Introduction

Echinacea species (Asteraceae), known collectively as purple coneflowers, are native to North America and have a distribution ranging from Manitoba to central Texas and eastward to the Appalachians. The greatest diversity of species occurs in Arkansas, Oklahoma, Missouri, and Kansas (McGregor 1968; Urbatsch et al. 2000). Species within this genus are found in diverse habitats including dry and mesic prairies, savannahs, and barren woodlands. The genus includes widespread species such as *Echinacea angustifolia* and *Echinacea purpurea* and narrowly distributed species, *Echinacea tennesseensis* and *Echinacea laevigata*. Purple coneflowers have had a long history of use in North Amer-

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ica first by Native Americans and later by European settlers throughout the nineteenth and twentieth centuries. *Echinacea purpurea* is commonly used as an ornamental with several selections based on flower color. *Echinacea* species are one of the most popular herbal supplements in the United States and Europe, and several hundred products are on the market. *Echinacea* species are mainly cultivated for their phytochemicals, including lipophilic alkamides, caffeic acid derivatives, and polysaccharides, which have been reported to have immunostimulant properties (for review see Percival 2000).

The sunflower tribe (Heliantheae), to which coneflowers (subtribe Rudbeckiinae) belong, consists of the genera *Rudbeckia*, *Ratibida*, *Dracopsis*, and *Echinacea* (Bremer 1994). Relationships among and within the coneflower genera vary by monographer and the characters used in the classification. Previous investigators have based classifications on growth habit, phyllotaxy, and morphological characteristics of the achene, corolla, leaf, and pollen (Bentham 1873; Binns et al. 2002; Cox and Urbatsch 1990; Fernald 1950; Gray 1884; Robinson 1978, 1981), while cytological and biochemical evidence has also been utilized (Binns et al. 2002; Cox and Urbatsch 1990; McGregor 1968; Perdue 1959; Robinson et al. 1981). Recent molecular analyses of these genera have utilized cpDNA restriction site data (Urbatsch and Jansen 1995) and internal transcribed spacer (ITS) sequence homology (Urbatsch et al. 2000). *Echinacea* has traditionally been affiliated with subtribe Rudbeckiinae (Bremer 1994; Cronquist 1980) but has also been assigned to subtribe Ecliptinae, Helianthinae, or Zinniinae (Robinson 1981; Stuessy 1977; Urbatsch et al. 2000). Within *Echinacea*, molecular and biochemical analyses have included isozyme, random amplified polymorphic DNA (RAPD), cpDNA, and ITS sequence data, but not all currently recognized taxa were included in these studies (Baskauf et al. 1994; Cox and Urbatsch 1990; Kapteyn et al. 2002; Urbatsch et al. 2000; Wolf et al. 1999). Confusion has long existed in delimiting taxa within this genus, and the fact that *Echinacea* species hybridize along sympatric distributions has only added to the confusion (McGregor 1968). Recently, it was reported that *Rudbeckia purpurea* L. had variously been identified and published as *Echinacea purpurea* L. Moench, *Echinacea laevigata* F.E. Boynton & Beadle S.F. Blake, *Brauneria laevigata* F.E. Boynton & Beadle in Small, and *Echinacea purpurea* L. Moench var. *laevigata* F.E. Boynton & Beadle Cronq. (Binns et al. 2001*a*, 2001*b*). Similarly, *Echinacea serotina* Nutt. DC has variously been identified and published as *Rudbeckia purpurea* var. *serotina* Nutt., *Rudbeckia serotina* Nutt., *Rudbeckia hispida* Hoffmanns, and *Echinacea purpurea* auct. non L. (sensu McGregor) (Binns et al. 2001*a*, 2001*b*). Circumscription of *Echinacea* species based on morphological characters has resulted in two different views. McGregor (1968), using traditional taxonomic methods, delimited *Echinacea* into nine species and four varieties. The Binns et al. (2002) classification, based on morphometric multivariate statistical analyses, strongly supported two subgenera containing four species and eight varieties (Table 1). Both morphological classifications are based on relatively minor differences among characters, and McGregor (1968) noted that "specific differences are narrowly defined."

Differences in phenotypes can result from single gene mutations, nonallelic mutations, and phenotypic plasticity in response to the environment. Morphological plasticity can obscure assessment of genetic diversity and make classifications based on phenotypes less robust. On the other hand, the linear arrangement of DNA sequences is highly conserved within and among species and is not affected by environmental conditions (Fulton et al. 2002; Ku et al. 2000; Paterson et al. 2000; Rossberg et al. 2001). Therefore, a clearer understanding of phylogenetic relationships might be gained by assessing the genetic divergence among *Echinacea* taxa using comparative genomic methods. Amplified fragment length polymorphisms (AFLP) are ideally suited to assess germplasm because of their ability to generate and detect numerous polymorphisms that are largely distributed throughout the genome and the method is highly reproducible (Hansen et al. 1999; Jones et al. 1997; Vos et al. 1995). The AFLP method largely detects single nucleotide polymorphisms (Vuylsteke et al. 2000), and estimates of nucleotide diversity based on genome-wide AFLP variation are similar to that reported for nuclear genes (Miyashita et al. 1999). The discriminatory power of AFLP has been used to distinguish among closely related inbred species and cultivars and ecotypes, such as *Lactuca* (Hill et al. 1996; Koopman et al. 2001) and *Arabidopsis thaliana* (Alonso-Blanco et al. 1998; Miyashita et al. 1999), and was shown to detect mutations in plants arising from in vitro clonal propagation of *Arabidopsis* (Polanco and Ruiz 2002). Our objective was to provide additional information on genetic diversity and phylogenetic relationships among *Echinacea* taxa (sensu McGregor) using the AFLP technique. Our results support earlier observations based on morphological characters that *Echinacea* taxa are distinct but closely related.

Materials and methods

Plant material

Thirty-nine plants representing nine species and three varieties of *Echinacea* (sensu McGregor) and three plants of *Ratibida columnifera* were included in the study. Based on the phylogeny reported by Urbatsch et al. (2000), the genus *Ratibida* (Rudbeckiinae) is closely related to *Echinacea* and was included as an outgroup for phylogenetic rooting purposes. *Echinacea angustifolia* and *E. purpurea* are represented by two populations each. With the exception of the nursery stock population of *E. purpurea* and the North Dakota populations of *E. angustifolia* and *R. columnifera*, all taxa were obtained as seed from the USDA (ARS National Genetic Resources Program, Ames, Iowa). Seeds were germinated in a glasshouse and seedlings transplanted to a field adjacent to our laboratory at Cal Poly Pomona. Leaves harvested from these plants were used as source DNA. Taxon names reported are consistent with the USDA ARS National Genetic Resources Information Network. Accession, plant identification numbers, and collection sites are given in Table 2. Voucher specimens from all populations used in this study have been deposited at the University of California Los Angeles herbarium.

Table 1. Comparison of McGregor's (1968) and Binns et al.'s (2002) classification of *Echinacea* taxa.

McGregor (1968)	Binns et al. (2002)
E. purpurea	*E. purpurea*
E. paradoxa var. *paradoxa*	*E. atrorubens* var. *paradoxa*
E. paradoxa var. *neglecta*	*E. atrorubens* var. *neglecta*
E. atrorubens	*E. atrorubens* var. *atrorubens*
E. laevigata	*E. laevigata*
E. pallida	*E. pallida* var. *pallida*
E. tennesseensis	*E. pallida* var. *tennesseensis*
E. simulata	*E. pallida* var. *simulata*
E. sanguinea	*E. palliada* var. *sanguinea*
E. angustifolia	*E. pallida* var. *angustifolia*
E. angustifolia var. *angustifolia*	*E. pallida* var. *angustifolia*
E. angusifolia var. *strigosa*	*E. pallida* var. *angustifolia*

AFLP analysis

Genomic DNA was isolated from recently expanded leaves using a modified cetyltrimethylammonium bromide method (Doyle and Doyle 1987). Freshly harvested leaves (200 mg) were homogenized in extraction buffer using a FastPrep® System cell disrupter (Qbiogene, Inc., Carlsbad, Calif.). The quality of the extracted DNA was estimated by measuring the 260 and 280 UV absorbance and the integrity was verified by electrophoresis on a 1.0% agarose gel. Only samples with 260:280 ratios of 1.8–1.9 and no degradation were used for AFLP analysis.

AFLP reactions were performed as described in Vos et al. (1995) with minor modifications optimized for capillary electrophoresis. Adapters were synthesized by Operon Technologies (Alameda, Calif.), *Mse*I primers were from Gibco (Invitrogen Life Technologies, Baltimore, Md.), and phosphoramidite dye linked *Eco*RI primers were synthesized by Research Genetics (Huntsville, Ala.). Preliminary experiments using a single primer pair labeled with different phosphoramidite dyes (black, blue, and green) revealed significant differences in signal intensity. Consequently, all *Eco*RI primers were labeled with a blue phosphoramidite dye, which gave the highest signal strength.

Genomic DNA (500 ng) was incubated at 37 °C for 3 h with 1× T4 ligase buffer (with ATP), 50 mM NaCl, 45 mM bovine serum albumin, 5 U of *Eco*RI, 5 U of *Mse*I, 5 μM *Mse*I adapter, 1 μM *Eco*RI adaptors, and 1 U of T4 DNA ligase. After ligation, the reaction mixture was diluted 10-fold with 1× TE 0.1 buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) and stored at –20 °C.

Preselective primer pairs with a single selective nucleotide extension of the AFLP adapter were used (Table 3). The reaction mix (total volume of 20 μL) contained 5 μL of restricted and ligated template DNA, 1× polymerase chain reaction (PCR) buffer (2 mM Tris–HCl, pH 8.0, 10 mM HCl, 0.2 mM $MgCl_2$), 200 μM each dNTP, 0.3 μM each of the two primers, and 0.5 U of *Taq* polymerase. PCRs were performed as follows: one cycle at 72 °C for 2 min followed by 20 cycles at 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min. The resulting PCR products were diluted 10-fold in 1× TE 0.1 buffer and used as DNA template for selective amplification. An aliquot from these reactions was subjected to electrophoresis on a 1.0% agarose gel stained with ethidium bromide to verify that preselective amplification had occurred.

In a preliminary test to identify suitable selective primer extensions, 25 primer combinations were tested on *E. purpurea*. A subset of six primer combinations (*Eco*RI +3/*Mse*I +3) was selected for use across all taxa based on the high number of fragments and polymorphisms detected (Table 3). Selective PCRs (20 μL total) contained 5 μL of diluted preamplification mixture, 1× PCR buffer (with 20 mM $MgCl_2$), 500 μM each dNTP, 0.3 μM each of the two selective primers, and 1 U of *Taq* polymerase. Selective amplification was performed as follows: an initial denaturation step of 2 min at 94 °C, 20 s at 94 °C, 30 s at 66 °C, and 2 min at 72 °C; then, for the next nine cycles, the annealing temperature was lowered 1 °C in each cycle followed by 25 cycles at 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min. After selective amplification, 0.5 μL of the reaction products were mixed with 30 μL of sample loading solution (Beckman-Coulter Inc., Fullerton, Calif.) and 0.5 μL of fragment size standard was added to each sample (60–600 bp) (Beckman-Coulter Inc.). Samples were separated using capillary electrophoresis on an automated CEQ 8000 DNA fragment analysis/sequencer (Beckman-Coulter, Inc.) with running conditions as follows: denaturation at 90 °C for 120 s, injection for 30 s at 1000 V, and separation at 4800 V for 60 min.

Data analysis

Fragment sizes were automatically calculated by CEQ 8000 software (version 4.2.0) using the local Southern sizing algorithms (Elder and Southern 1987). Each fragment was treated as a separate character and scored as either present (1) or absent (0) across all genotypes by CeqCluster fragment analysis software (Beckman-Coulter Inc.). Inclusion or exclusion of fragments with automated fluorescent dye capillary electrophoresis systems was performed by adjusting threshold levels of signal heights and slopes. The CeqCluster software slope threshold (ST) algorithm specifies the minimum rate of signal increase on the leading edge of a given peak, while the relative peak height threshold (RPHT) specifies the minimum height (relative to the second highest peak) required before being included in the fragment list. In this study, two different RPHT:ST combinations were evaluated, 5:1 and 5:10. A preliminary fragment list was constructed using RPHT:ST = 5:1 to establish a baseline fragment list that was then compared with the 5:10 fragment list. Where discrepancies existed, the electropherograms were visually reviewed to reconcile ambiguities. For each individual, the AFLP product was run in three separate capillaries, and a fragment was included only if its presence was detected in at least two of the three capillaries. Fragment sizes greater than 400 bp were less reproducible and were therefore omitted from the analysis.

For each pairwise comparison between accession *i* and *j*, the 1/0 matrix was used to calculate the Dice (1945) estimate of genetic similarity ($GS_D = 2a/(2a + b + c)$) (equivalent to 1, Nei and Li's (1979) genetic distance), where *a* is the number of bands shared by *i* and *j*, *b* is the number of bands present in *i* and absent in *j*, and *c* is the number of bands present in *j* and absent in *i* for the data pooled over all primer combinations.

The genetic distance matrix was used to construct pheno-

Table 2. List of *Echinacea* species, origin, accessions, and location of populations used in the AFLP study.

Species	Plant No.[a]	Population	Origin	Accession No.	Latitude	Longitude
E. angustifolia	1, 2, 3	1	Oklahoma	PI421331	36°09′00′′	097°07′00′′
E. angustifolia	4, 5, 6	4	North Dakota	ND-*Ea*-007[b]	47°34′51′′	103°26′12′′
E. angustifolia var. *angustifolia*	7, 8, 9	7	Oklahoma	Ames 23906	34°30′00′′	097°21′00′′
E. angustifolia var. *strigosa*	10, 11, 12	10	Oklahoma	Ames 23887	34°30′00′′	096°59′00′′
E. atrorubens	13, 14, 15	13	Kansas	Ames 23869	38°47′00′′	095°12′00′′
E. laevigata	16, 17, 18	16	South Carolina	Ames 23963	34°46′00′′	083°11′00′′
E. pallida	19, 20, 21	19	Oklahoma	Ames 23880	34°00′00′′	095°17′00′′
E. paradoxa var. *neglecta*	22, 23, 24	22	Oklahoma	Ames 23884	34°30′00′′	096°57′00′′
E. purpurea	25, 26, 27	25	Louisiana	Ames 25104	32°00′00′′	092°00′00′′
E. purpurea	28, 29, 30	28	—	Nursery stock	—	—
E. sanguinea	31, 32, 33	31	Louisiana	Ames 23874	30°58′00′′	093°13′00′′
E. simulata	34, 35, 36	34	Missouri	Ames 22782	38°22′30′′	090°45′00′′
E. tennesseensis	37, 38, 39	37	Tennessee	Ames 25162	36°03′54′′	086°24′03′′
Ratibida columnifera	40, 41, 42	40	North Dakota	ND-*Rc*-001[b]	47°1′15′′	103°28′30′′

[a]Numbers refer to individuals labeled in Figs. 1 and 2.
[b]Collected by D.W.S.; specimens deposited in the UCLA herbarium.

Table 3. Oligonucleotide adaptors and primers used for AFLP analysis of *Echinacea*.

	Sequence
Adapters	
*Eco*RI adapters	5′-CTCGTAGACTGCGTACC-3′
	3′-CTGACGCATGGTTAA-5′
*Mse*I adapters	5′-GAC GATGAGTCCTGAG
	3′-TACTCAGGACTCAT-5′
Primers	
*Eco*RI +1	GACTGCGTACCAATTCA
*Mse*I +1	GATGAGTCCTGAGTAAC
*Eco*RI +3	GACTGCGTACCAATTCACA
	GACTGCGTACCAATTCACT
	GACTGCGTACCAATTCAGG
*Mse*I +3	GATGAGTCCTGAGTAACAG
	GATGAGTCCTGAGTAACTG
	GATGAGTCCTGAGTAACTT
Selective primer combinations	
PC14	E-ACT/M-CAG
PC15	E-AGG/M-CAG
PC19	E-ACT/M-CTG
PC20	E-AGG/M-CTG
PC22	E-ACA/M-CTT
PC25	E-AGG/M-CTT

grams using the neighbor-joining (NJ) method (Saitou and Nei 1987). Internal branch support was evaluated by bootstrap analysis (Felsenstein 1985) of 1000 bootstrap replicate data sets with PAUP software (beta version 10) (Swofford 2002). To visualize the dispersion of individual plants in relation to the first two principal axes of variation, principal coordinates analysis (PCA) (Gower 1966) was performed on the genetic similarity data matrix using the DCENTER and EIGEN modules of NTSYSpc (Rohlf 2002).

Results

AFLP polymorphism and genetic similarity

Six primer combinations generated 1105 fragments between 60 and 400 bp in the 42 plants studied (Table 4). A high percentage of fragments were polymorphic between two or more plants, with 1105 (94% polymorphic) and 1086 (90% polymorphic) total fragments present in the *Echinacea* plus outgroup and the *Echinacea* subset, respectively (Table 4). Considering the *Echinacea* plus outgroup population, each primer combination generated an average of 66 fragments per plant, and because of the high percentage of polymorphism observed among plants, an average of 184 fragments were scored per primer combination (Table 4). Results from a preliminary screening of 25 primer combinations revealed substantial variation in the number of fragments generated among species and many combinations produced considerably fewer fragments per primer pair (data not shown).

Genetic distance between and within populations and taxa

Excluding the outgroup *Ratibida*, the genetic distance (Nei and Li 1979) averaged within a population ranged from 0.019 to 0.043 (*E. laevigata* population 16 and *E. angustifolia* population 1, respectively) with an intrapopulation average of 0.029 for all *Echinacea* taxa (Table 5). The average genetic distance between *Echinacea* populations of 0.088 was more than three times greater than the intrapopulation distance, with a low of 0.051 (between *E. angustifolia* population 1 and *E. angustifolia* var. *angustifolia* population 7) and a high of 0.115 (*E. atrorubens* population 13 and *E. purpurea* population 28). The average genetic distance between all *Echinacea* populations and the outgroup *Ratibida* was 0.177 with a range from 0.167 (*E. pallida* population 19 and *R. columnifera* population 40) to 0.185 (*E. laevigata* population 16 and *R. columnifera* population 40).

Genetic divergence based on marker frequency data

Phenograms were constructed using Nei and Li's (1979) genetic distance and NJ methods. Internal branch support was tested by bootstrap analyses with 1000 replications using the software PAUP (beta version 10) (Swofford 2002). The analysis resulted in a phenogram with all individual

Table 4. Characteristics of fragment variation generated by six primer combinations in the AFLP analysis of *Echinacea* (n = 39 individuals) and *Ratibida columnifera* (n = 3 individuals per population).

Characteristic	*Echinacea* plus outgroup							*Echinacea* species						
Primer combinations	PC14	PC15	PC19	PC20	PC22	PC25	Total	Mean	PC14	PC15	PC19	PC20	PC22	PC25	Total	Mean
Total fragments	186	189	181	187	174	188	1105	184	186	186	178	177	171	188	1086	181
Average no. of fragments per plant	72	62	59	61	63	79	396	66	74	63	61	61	65	82	406	68
No. of fragments present in all plants	1	2	2	3	2	2	12	2	7	8	4	7	10	10	46	8
No. of fragments unique to one plant	7	8	13	10	8	6	52	9	8	12	15	11	10	7	63	11
No. of fragments present in two or more plants	178	179	166	174	164	180	1041	174	171	166	159	159	151	171	977	163
Polymorphic fragments (%)	95.7	94.7	91.7	93.0	94.3	95.7		94.2	91.9	89.2	89.3	89.8	88.3	91.0		90.0

Note: Fragments were scored within a range of 60–400 bp.

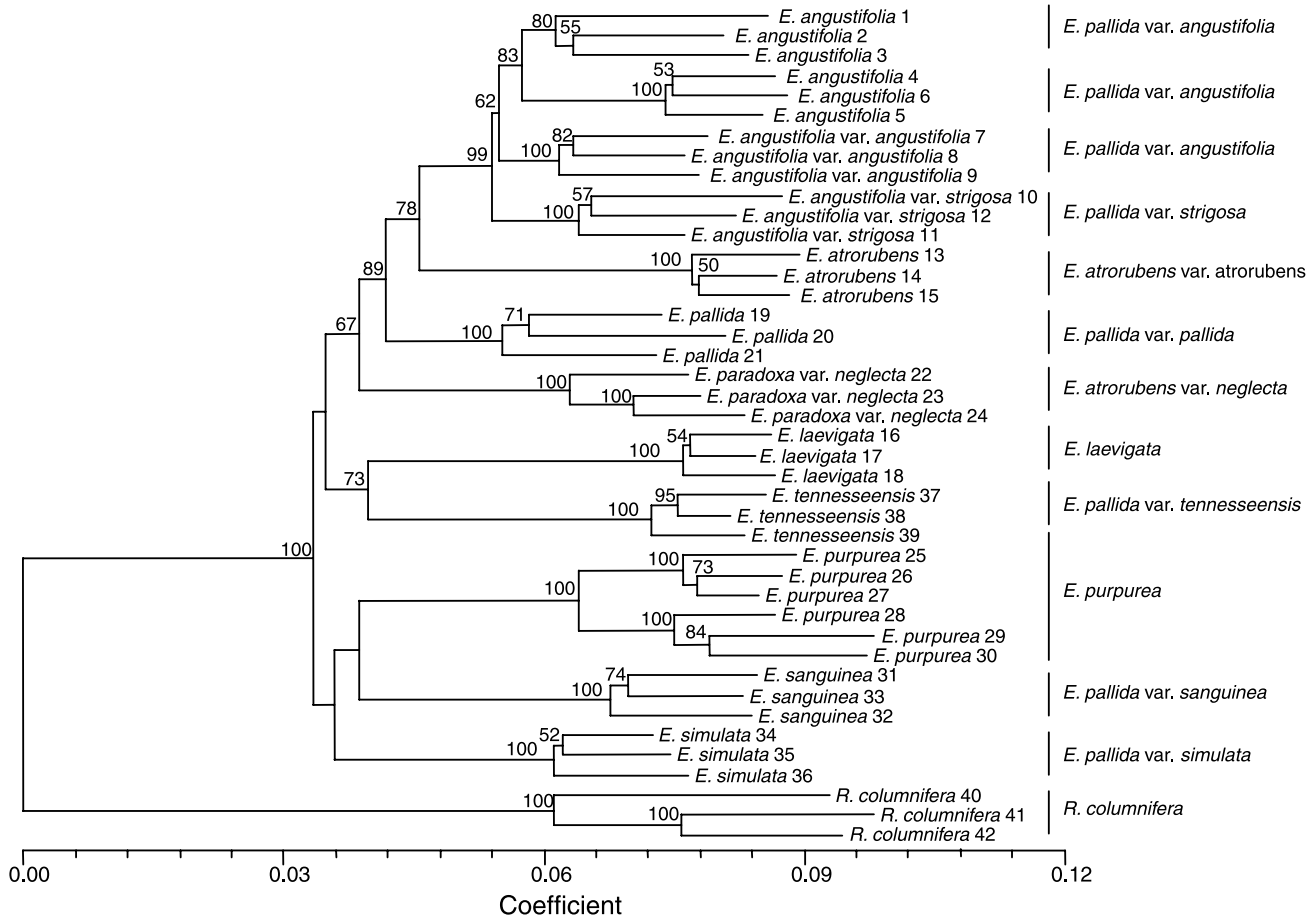
plants from a given population clustering together at the terminal nodes under its respective population. The intrapopulation clustering was supported by relatively high bootstrap values (BV), and the majority of the interior nodes were moderately to well supported (Fig. 1). The AFLP-based analysis was sufficiently sensitive to discriminate among individual plants within a population as well as between populations of the same species, placing the two accessions of *E. purpurea* as sister clades and separately clustering all *E. angustifolia* taxa. The topology of the NJ tree shows that *Echinacea* species are clustered into two separate clades (100% BV). The basal clade consisted of *E. purpurea*, *E. sanguinea*, and *E. simulata* and hereafter is referred to as the group 1 clade. The second clade consisted of *E. atrorubens*, *E. laevigata*, *E. pallida*, *E. paradoxa* var. *neglecta*, *E. tennesseensis*, and the *E. angustifolia* complex (*E. angustifolia*, *E. angustifolia* var. *angustifolia*, and *E. angustifolia* var. *strigosa*), hereafter referred to as the group 2 clade. Within the group 2 clade, a well-supported sister relationship was identified between *E. laevigata* and *E. tennesseensis* (73% BV). Within the group 1 clade, *E. sanguinea* and *E. purpurea* form sister clades, although weakly supported (41% BV).

A PCA of the AFLP-based distance data was performed to examine relationships among *Echinacea* populations. The first and second principal coordinates described approximately 14% and 10% of the total variation, respectively (Fig. 2). In contrast with the NJ tree, the PCA showed that *E. purpurea* clustered apart from the other taxa. All individuals within a population clustered together, and notwithstanding *E. purpurea*, all taxa were approximately equally dispersed along both coordinates (Fig. 2).

Discussion

Our data indicate that *Echinacea* taxa are closely related, in agreement with McGregor's (1968) morphometric assessment that relatively minor differences distinguish *Echinacea* taxa. Excluding *R. columnifera*, the average within-population genetic distance (Nei and Li 1979) was 0.029, while the average pairwise distance between two populations was approximately three times that distance. Using ITS-1, ITS-2, and 5.8s data in an analysis of coneflower genera, Urbatsch et al. (2000) reported only a single substitution and six indel events between two closely related taxa, *E. paradoxa* and *E. tennesseensis*. The genetic distance among the *Echinacea* species examined in their study (*E. atrorubens*, *E. pallida*, *E. paradoxa*, *E. purpurea*, *E. simulata*, and *E. tennesseensis*) ranged from 0.18% to 3.2% and several *Echinacea* species had identical ITS-2 sequences. Based on RAPD markers, Kapteyn et al. (2002) reported a much greater range in genetic distances among *Echinacea* taxa than that reported in either Urbatsch et al. (2000) or that observed in our studies. Pairwise genetic similarities in the Kapteyn et al. (2002) study ranged from 0.185 to 0.978 among *E. purpurea*, *E. angustifolia*, *E. pallida*, and *E. atrorubens*, with the greatest distance between *E. purpurea* and *E. angustifolia*. Our data indicate that the greatest genetic divergence among *Echinacea* taxa is between *E. atrorubens* and the nursery-derived *E. purpurea* (populations 13 and 28, respectively; Table 5). We note that

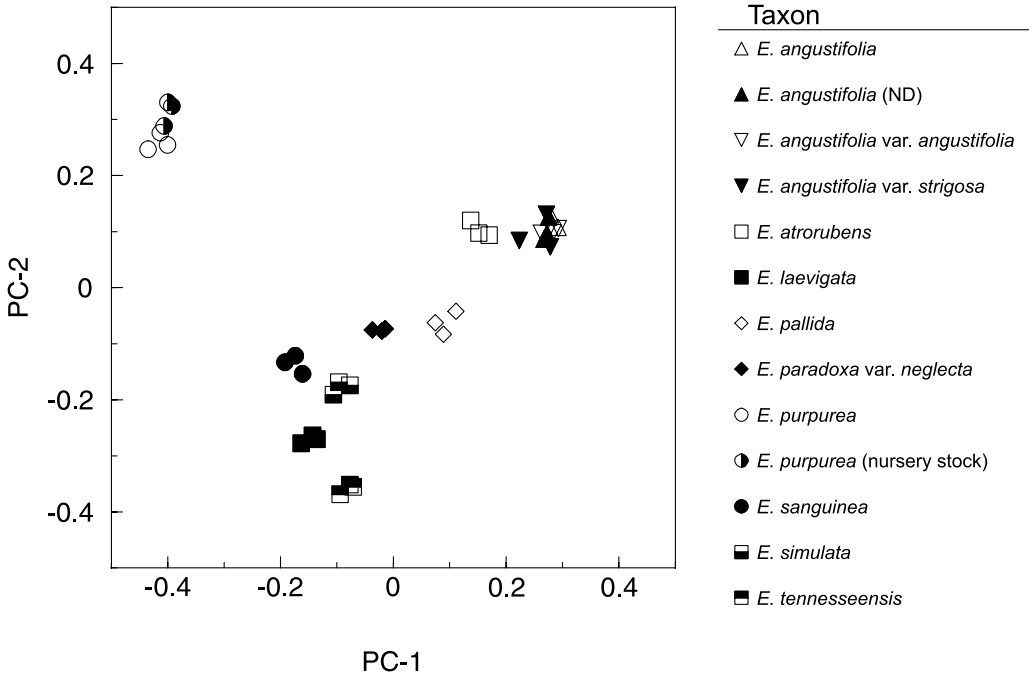
Fig. 1. Neighbor-joining phenogram of *Echinacea* taxa using Nei and Li's (1979) genetic distance based on AFLP markers obtained with six primer combinations and 5:10 relative peak height threshold to slope threshold. Numbers shown at the node represent bootstrap values (as a percentage of 100 replicates).



the majority of their plant material was obtained through nursery sources, whose geographic origin was unknown, and it is possible that some selection, either directed or unintentional, had occurred within these lines once collected and propagated and thus contributed to the greater genetic distances than those found in our study. This possibility is supported by the observation that the genetic distance between our nursery population of *E. purpurea* (population 28) and the wild-collected *E. purpurea* population (population 25) was 0.053, a distance more than 1.8 times greater than the within-population distance averaged across all populations and similar to the pairwise genetic distances measured among the *E. angustifolia* varieties (populations 1, 4, 7, and 10; Table 5). Given that genetic drift occurs in horticultural accessions, the nursery-derived *E. purpurea* population used in our study can be excluded for the purpose of identifying the greatest divergence between taxa. By this measure, the greatest divergence measured was between the *E. purpurea* population, originally collected in Louisiana, and *E. tennesseensis*, originally collected in Tenneessee (populations 25 and 37, respectively).

Other factors are likely to contribute to the discrepancy in genetic distance reported between the Kapteyn et al. (2002) data set and ours. A significant advantage of AFLP relative to other techniques is the greater number of polymorphic fragments detected by single primer pairs. The discriminatory power of automated fluorescent-dye capillary electrophoresis based AFLP appears to be particularly suited to detecting fragments relative to radioactively labeled, manually scored, slab gel based AFLP methods, as the latter technique generally detects and scores far fewer fragments per sample (Alonso-Blanco et al. 1998; Schmidt and Jensen 2000). In our study, an average of 66 fragments were detected per plant, an average of 184 fragments per primer pair, and a total of 1041 polymorphic fragments were scored. In contrast, Kapteyn et al. (2002) reported an average of 4.6 bands per primer, and a total of 101 bands were scored. Because accuracy in the measurement of genetic distance increases with an increase in the number of loci used (Travis et al. 1996), it is likely that the AFLP method is a less-biased assessment of genetic distance. Although no direct confirmation exists in distinguishing bona fide peaks on an electropherogram from background noise, we took several precautions to minimize acceptance of false fragments. We employed a filtering process that used increasingly discriminatory parameters to exclude fragments and compared the two fragment lists and corrected any ambiguities by visual inspection of the electropherograms. To gauge reproducibility of the AFLP fragments, aliquots of each sample were run in triplicate, that is, the same AFLP–PCR product was run in three capillaries. The fact that each individual of the experimental population clustered to the appropriate taxon cluster at the

Fig. 2. Principal coordinates analysis among *Echinacea* species based on their AFLP-based genetic distances (Nei and Li 1979). The first two dimensions accounted for 24% of the variability.



terminal nodes strongly supports the robustness of these precautions. Conversely, each member of a population did not cluster together at the terminal node when *Echinacea* species were analyzed using RAPDs (Kapteyn et al. 2002), suggesting that the AFLP procedure more accurately assesses genetic fingerprints relative to RAPDs.

There is high bootstrap support for two major clades in *Echinacea* species based on our AFLP data (Fig. 1), and Binns et al. (2002) came to a similar conclusion based on a cladistic analysis of morphological features. However, the composition of the two clades differs between that suggested by the AFLP data and the classification put forth by Binns et al. (2002). The latter classification separated the genus into two subgenera, *Echinacea* and *Pallida*, with subgenus *Echinacea* consisting of only *E. purpurea*. Subgenus *Pallida* was further divided into three divisions, containing the remainder of McGregor's (1968) taxa (Table 1). Our NJ tree analysis separates the genus into two major clades (100% BV), with the basal clade consisting of *E. purpurea*, *E. sanguinea*, and *E. simulata*. Although *E. purpurea* clustered separately from all other taxa in the PCA, the first two coordinates accounted for only 24% of the total amount of variation observed (Fig. 2). Based on ITS and cpDNA sequence homology, Urbatsch et al. (2000) placed *E. paradoxa* sister to *E. purpurea* (83% BV) as part of a larger clade that included *E. simulata* and *E. tennesseensis*. The Binns et al. (2002) classification maintains *E. laevigata* as a separate species under subgenus *Pallida*, while there is moderately strong bootstrap support for the sister relationship between *E. laevigata* and *E. tennesseensis* based on our AFLP data (73% BV) (Fig. 1). Based on ITS sequence homology, Urbatsch et al. (2000) placed *E. pallida* sister to *E. atrorubens* (93% BV), while our analysis placed *E. pallida* immediately basal to *E. atrorubens* (89% BV). Based on AFLP data, branch support is relatively high for the clades comprising *E. atrorubens*, *E. pallida*, and the *E. angustifolia* but incongruous to the Binns et al. (2002) classification. Other studies have also shown a lack of correlation between molecular data and morphology-based taxonomic classifications (Knox and Palmer 1995; Small and Wendel 2000; Pelser et al. 2002).

The relatively small genetic distance values and low bootstrap values in several clades suggest a recent divergence among *Echinacea* taxa. Among closely related outcrossing species, a greater genetic distance might be expected between populations separated by greater geographic distances, relative to sympatric species, but this was not observed in our study. The greatest geographic distance between two populations was between *E. angustifolia* (population 4) and *E. laevigata* (population 16) followed by *E. angustifolia* (population 4) and *E. sanguinea* (population 31). The genetic distance was estimated to be 0.098 for both of these pairwise comparisons (Table 5). However, excluding the nursery crop of *E. purpurea* (population 28), greater pairwise genetic distances were observed between several other populations (e.g., 1 and 25, 1 and 31, 4 and 25, 13 and 31, 25 and 37, and others; Table 5). Three taxa, *E. angustifolia* var. *angustifolia* (population 7), *E. angustifolia* var. *strigosa* (population 10), and *E. paradoxa* var. *neglecta* (population 22), were collected within close proximity of each other in Oklahoma (Table 2). The genetic distance between *E. angustifolia* var. *angustifolia* and *E. angustifolia* var. *strigosa* was 0.053, while the genetic distance between these two *E. angustifolia* taxa and *E. paradoxa* var. *neglecta* averaged 0.081 (Table 5). The smallest genetic distances observed among the populations used in this study were among the *E. angustifolia* complex (populations 1, 4, 7, and 10), irrespective of their geographic origin (Tables 2 and 5).

The AFLP technique is well suited to detect polymorphisms in populations with little genetic variation

Table 5. Matrix of average genetic distance (Nei and Li 1979) among 13 *Echinacea* and 1 *Ratibida columnifera* (outgroup) population.

	1	4	7	10	13	16	19	22	25	28	31	34	37	40
1	0.0426													
4	0.0539	0.0249												
7	0.0512	0.0548	0.0309											
10	0.0569	0.0593	0.0528	0.0351										
13	0.0820	0.0877	0.0739	0.0790	0.0216									
16	0.0980	0.0982	0.0951	0.0970	0.1099	0.0189								
19	0.0789	0.0774	0.0668	0.0763	0.0766	0.0973	0.0390							
22	0.0839	0.0899	0.0813	0.0816	0.0948	0.0979	0.0784	0.0278						
25	0.1051	0.1057	0.0987	0.1004	0.1026	0.0983	0.0991	0.0944	0.0210					
28	0.1130	0.1088	0.1093	0.1123	0.1153	0.1045	0.1060	0.1006	0.0530	0.0354				
31	0.1072	0.0976	0.0970	0.0983	0.1061	0.1003	0.0888	0.0925	0.0946	0.1006	0.0309			
34	0.0914	0.0870	0.0881	0.0908	0.1015	0.0935	0.0779	0.0828	0.0973	0.0979	0.0835	0.0262		
37	0.0994	0.0982	0.0923	0.0956	0.1023	0.0895	0.0874	0.0918	0.1081	0.1080	0.1003	0.0917	0.0201	
40	0.1790	0.1740	0.1780	0.1750	0.1782	0.1846	0.1666	0.1784	0.1797	0.1809	0.1761	0.1739	0.1775	0.0582

Note: Corresponding information about each population is given in Table 1. For each population, $n = 3$.

(Soleimani et al. 2002; this study). The distinct advantage of the AFLP technique is that it requires no prior sequence knowledge of the species of interest but, as applied to phylogenetic relationships, has several potential limitations. Because the fragment sequence is not known, AFLP data are usually analyzed phenetically, and only rarely has a cladistic analysis been presented (but see Koopman et al. 2001). The assumption in AFLP analyses is that in closely related species, fragments of the same size are related by descent and therefore have the same sequence and origin within a genome. Sequencing and mapping of AFLP fragments would serve as direct confirmation and was attempted by Rouppe van der Voort et al. (1997), Cervera et al. (2001), and Peters et al. (2001). Rouppe van der Voort et al. (1997) sequenced 20 putative homologous fragments and found that 19 had identical sequences. A single nucleotide deletion and substitution was observed in the other sequenced fragment. Cervera et al. (2001) cloned several fragments of identical length that were presumed to be related and found that each had the same sequence, but in other fragments, they found that several nonvisible bands were present and were coamplified during cloning. Because the complete sequence is available for *A. thaliana*, a different approach to investigate the homology and origin of AFLP fragments could be undertaken by Peters et al. (2001). An in silico AFLP performed on *A. thaliana* (Col-0 ecotype) assigned 1267 markers to unique positions, and another 107 markers could be assigned to more than one position within the genome (Peters et al. 2001). These multiple-origin markers were located either from the same genetic segment (recombinant breakpoint) or within a single bacterial artificial chromosome clone (83 and 24 markers, respectively). Of the genetic segment markers, 90% of the in silico sequences were totally different from each other. Additionally, they sequenced 70 randomly chosen AFLP fragments and the in silico predicted sequence matched the experimentally verified sequence. The *Arabidopsis* genome has high levels of sequence conservation and segmental duplications, and this could partially explain the multiple origins of a given AFLP fragment within this genome (The *Arabidopsis* Genome Initiative 2000). Indirect evidence supporting the orthologous nature of AFLP fragments of the same length is provided by the observation that in *Lactuca* species, the topology of phylogenetic trees using cladistic analysis was similar to that of a phenetic analysis in clades with high bootstrap or jackknife support (Koopman et al. 2001). Thus, although the fragment sequence and origin are unknown in species that have not been sequenced, these results indicate that most fragments are both unique and orthologous and therefore suitable for determining phylogenetic relationships.

We have scored AFLP fragments as a dominant marker, but others have shown that in certain circumstances, these fragments can be scored codominantly by the relative intensity (or relative fluorescent units (RFU)) of the bands (Jansen et al. 2001; Piepho and Koch 2000) or, when the parental genotypes are known, by identification of heterozygous loci as two distinct fragments (Yu and Wise 2000). In the latter case, the impact on the phylogenetic signal of scoring heterozygous loci as two unrelated fragments is not known and can only be determined by controlled crosses. Because of the high number of fragments generated by the

AFLP technique, scoring errors can occur, but with experience and the use of replications, the reproducibility of AFLP is quite high (Jones et al. 1997; Remington et al. 1999). Scoring errors are particularly evident in mapping populations and are identified when marker order is not conserved, whereas when assessing genetic diversity, these errors would not be readily apparent.

We observed low bootstrap values in some internal nodes and the low amount of variation accounted for by the PCA leaves the phenetic relationships somewhat unanswered. Any of the aforementioned shortcomings inherent to AFLP could potentially obscure the phylogenetic signal and contribute to relatively low support values. Alternatively, in a number of studies, it has been suggested that low levels of support and short branch lengths can be a result of rapid radiation (Malcomber 2002; Small and Wendel 2000). The low internal branch support in certain clades, the low level of variation accounted for by the PCA, the relatively minor morphological differences between species, and the fact all taxa form fertile hybrids suggest a relatively recent divergence among *Echinacea* species. The phylogenetic relationships that we have presented can be independently verified by sequencing orthologous gene families. A phylogenetic consensus may be reached if a representative sampling of all taxa is included and the same plant materials are used by each group. In conclusion, our study indicates that all *Echinacea* taxa are closely related and illustrates the discriminatory power of AFLP to distinguish among different taxa as well as between different populations within the same taxon.

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