

Identifying commercially relevant *Echinacea* species by AFLP molecular markers

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Abstract: The rising interest in medicinal plants has brought several species of the genus *Echinacea* to the attention of many scientists. *Echinacea angustifolia*, *E. pallida*, and *E. purpurea* are the most important for their immunological properties, well known and widely used by the native Americans. The three species are easily distinguishable on the basis of their morphological characteristics, but it would be difficult, if not impossible, to distinguish them in commercial preparations of ground, dry plant parts of *E. purpurea* (the most valuable species for chemotherapeutic properties) mixed with the other two species. Species-specific molecular markers could be useful to address this issue. In the present work, using fresh material collected from cultivated *Echinacea* spp., AFLP analysis was used to discriminate the three species and to detect species-specific DNA fragments. By using 14 primer combinations it was possible to detect a total of 994 fragments, of which 565 were polymorphic. Overall, 89 fragments were unique to *E. purpurea*, 32 to *E. angustifolia*, and 26 to *E. pallida*. *E*+CAC/M+AAT or *E*+CAC/M+AGC alone provided 13, 9, and 4 or 7, 5, and 5 specific fragments for *E. purpurea*, *E. angustifolia*, and *E. pallida*, respectively. A validation trial to confirm the results was carried out on bulked samples of 23 accessions covering most of the genetic diversity of the three species. The results are discussed in terms of practical applications in the field of popular medicine, detecting frauds, and implications for the genus *Echinacea*.

Key words: *Echinacea pallida*, *E. purpurea*, *E. angustifolia*, AFLP, species identification, commercial frauds.

Résumé : L'intérêt croissant pour les plantes médicinales a attiré l'attention de plusieurs chercheurs sur le genre *Echinacea*. Les espèces *E. angustifolia*, *E. pallida* et *E. purpurea* sont les plus importantes pour leurs propriétés immunologiques, bien connues et largement utilisées par les amérindiens. Les trois espèces sont faciles à distinguer sur la base de leurs caractéristiques morphologiques, mais il serait difficile, voire impossible, de les distinguer au sein de préparations commerciales composées de parties sèches broyées de l'*E. purpurea* (la plus prisée des espèces pour ses propriétés chimiothérapeutiques), mêlées avec celles provenant des deux autres espèces. Des marqueurs moléculaires permettant de distinguer les espèces seraient utiles dans ce cas. Dans le présent travail, à partir de matériel frais d'échinacées cultivées, des marqueurs AFLP ont été employés pour différencier les trois espèces et pour détecter des amplicons spécifiques de chacune. À l'aide de 14 combinaisons d'amorces, 994 amplicons ont été détectés au total, dont 565 étaient polymorphes. Globalement, 89 amplicons étaient uniques à l'*E. purpurea*, 32 à l'*E. angustifolia* et 26 à l'*E. pallida*. Les combinaisons *E*+CAC/M+AAT et *E*+CAC/M+AGC ont permis, à elles seules, de fournir respectivement 13, 9 et 4 ou 7, 5 et 5 marqueurs spécifiques des espèces *E. purpurea*, *E. angustifolia* et *E. pallida*. Un travail de validation pour confirmer ces résultats a été mené sur des échantillons mélangés de 23 accessions couvrant l'essentiel de la diversité génétique au sein de ces trois espèces. Les résultats sont discutés en fonction des applications pratiques dans le domaine des médecines douces, de la détection des fraudes et des implications pour le genre *Echinacea*.

Mots-clés : *Echinacea pallida*, *E. purpurea*, *E. angustifolia*, AFLP, identification des espèces, fraudes commerciales.

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Introduction

The genus *Echinacea* Moench (Compositae) is native to the prairies of North America, from which it spreads from southern Canada to Texas and Georgia, but the greatest diversity of species is found in Arkansas, Oklahoma, Missouri, and Kansas (McGregor 1968; Urbatsch et al. 2000). In the

past the genus was known as *Brauneria* and *Rudbeckia*, while the name *Echinacea* appeared for the first time in 1762. The classification of the taxa within the genus is controversial. McGregor (1968) distinguished 9 species and 4 varieties, while Binns et al. (2002), on the basis of a morphometric multivariate statistical analysis, supported 2 subgenera containing 4 species and 8 varieties.

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Echinacea angustifolia D.C., *Echinacea purpurea* (L.) Moench, and *Echinacea pallida* Nutt. are the most widespread and most known species because of their commercial importance due to immunological properties. The use of these species as medicinal plants dates back several centuries; *E. angustifolia* was widely used by the indigenous populations of North America for healing injuries, to treat snakebites, or to counter infectious diseases (Gilmore 1919). In recent years many studies have been carried out to better explore the pharmacological activities of the most common species of *Echinacea* and to identify the chemical compounds that confer such properties (Barrett 2003). However, in the past, in popular medicine these species were probably used indifferently for the same medical treatments, either because they have similar properties or because it was difficult to distinguish one from the other. Although the three species have some of the same pharmacological activities owing to the presence of active compounds that act synergistically, each species shows slight variations in the amount of individual active compounds (Percival 2000; Speroni et al. 2002). Efforts are being made to analyze the type and amount of the chemical compounds of each species, to optimize their use for proper therapeutic application (Barrett 2003). Following intense research activity on these species in the medical and pharmacological fields, interest in their genetic characteristics and phylogenesis has greatly increased in recent years. On the contrary, knowledge about their cytology is scant, being limited to the chromosome number: *E. angustifolia* and *E. purpurea* are diploid, with $2n = 22$, while *E. pallida* is tetraploid, with $2n = 44$ (McGregor 1968; Mechanda et al. 2004a; Qu et al. 2004).

In this study, AFLP (amplified fragment length polymorphism) molecular markers were used to characterize the three species, investigate their genetic similarities, and gain insight into their genetic relationships. Cytological investigations were also carried out to confirm their chromosome number. The main objective of the study was to look at the possibility of distinguishing them using AFLP markers, a field with potential practical applications and where literature is scarce.

Materials and methods

Trial 1: plant material, DNA isolation, and marker analysis

The first trial was conducted in 2005 on seed samples of *E. angustifolia*, *E. purpurea*, and *E. pallida* provided by Aboca S.p.A. (Arezzo, Italy), a company growing, processing, and marketing medicinal plants. The seed lots of the three species were originally imported by Aboca in 1999 from the USA in a commercial quantity. Seeds were germinated in Petri dishes, and some of the seedlings were utilized for cytological preparations; the remainder were transplanted in Jiffy pots, transferred to a greenhouse, and grown at a constant temperature of 21 °C and a day length of 16 h. The molecular analysis was carried out on 10 plants per species. Young, newly formed leaves were collected for total genomic DNA extraction based on the protocol described by Dellaporta et al. (1983). AFLP marker analysis was carried out according to Vos et al. (1995), as modified by Cnops et al. (1996). A fluorescently labeled *E+CAC* pri-

mer was used for selective PCR. The AFLP analysis was carried out on single plants, using 14 primer combinations as listed in Table 1.

Trial 2: validation

To validate the results of the first trial, seeds of 23 accessions (4, 11, and 8 for *E. angustifolia*, *E. pallida*, and *E. purpurea*, respectively) received from the USDA, ARS, National Plant Germplasm System (NPGS) were used. The accessions, all natural populations, were chosen randomly from the NPGS germplasm collection list. The information available in the Germplasm Resources Information Network (USDA, ARS, National Genetic Resources Program 2007) in terms of collection sites, source history, and improvement status ensured the sampling of sufficient genetic diversity for the validation trial. Seeds were germinated in Petri dishes, and seedlings were transplanted in pots in the greenhouse and grown in the same conditions described above. In May 2008, similar leaf segments (of approximately 1 cm²) from 6 plants of each accession were sampled and bulked (Table 2). DNA extraction from bulked leaves was carried out using the GenElute Plant Genomic DNA Miniprep Kit (Sigma). Also, an equal amount of DNA from each bulk was used to prepare a single bulk for each *Echinacea* species and, from these, a whole bulk was prepared with all species together. The AFLP analysis was carried out as described above, except that for the validation experiment only one primer combination (*E+CAC/M+AGC*) was used. Amplifications were performed in a 20 µL reaction mix containing 1/100 of the pre-amplified DNA, 50 ng of 6-FAM-labeled *E+CAC* primer, 50 ng of unlabeled *M+AGC* primer, 2 µL of 10× PCR buffer (Invitrogen), 0.2 mmol/L dNTPs, and 0.4 U of *Taq* polymerase (Invitrogen). Samples were denatured and run on an ABI 3130xl genetic analyzer (Applied Biosystems).

Cytological investigation

For determination of the chromosome number, actively growing root tips were excised when they were about 1.5 cm in length, pretreated in a saturated solution of α -bromonaphthalene for 3–4 h, and then fixed overnight in 3:1 ethanol – acetic acid. Somatic chromosomes were stained using the Feulgen procedure. Squashes were performed in 1.5% acetic orcein and attached to a cover slip with glycerine albumen.

Statistical analysis

AFLP fragments were scored as dominant markers, and individual profiles were considered as phenotypes (Mechanda et al. 2004b). In trial 1, fragments that could be unequivocally scored (1 for presence and 0 for absence) across all individuals were included in the analysis; the scores were used to prepare a data matrix based on 30 individuals (10 plants per species). The binary data were analysed by tabulation and frequency procedures using SAS software (SAS Institute Inc. 1999) to inspect for the presence of specific fragments and polymorphism within and between species. The binary data were also used to prepare a matrix of genetic similarity by using the coefficient of Jaccard (1908).

The similarity matrix was used in clustering the individuals by the unweighted pair group method with arithmetic

Table 1. Number of total, polymorphic, and species-specific AFLP fragments, scored over 14 *EcoRI/MseI* primer combinations, in three *Echinacea* species from trial 1.

Primer combination*	Total AFLP fragments	Polymorphic fragments	Species-specific fragments			
			Total	<i>E. angustifolia</i>	<i>E. pallida</i>	<i>E. purpurea</i>
<i>E</i> +CAC/ <i>M</i> +AAA	85	40	5	1	1	3
<i>E</i> +CAC/ <i>M</i> +AAC	76	34	14	6	2	6
<i>E</i> +CAC/ <i>M</i> +AAG	130	89	11	—	4	7
<i>E</i> +CAC/ <i>M</i> +AAT	91	57	26	9	4	13
<i>E</i> +CAC/ <i>M</i> +ACA	64	30	9	—	1	8
<i>E</i> +CAC/ <i>M</i> +ACC	28	16	10	3	—	7
<i>E</i> +CAC/ <i>M</i> +ACG	87	65	14	—	6	8
<i>E</i> +CAC/ <i>M</i> +ACT	41	6	2	2	—	—
<i>E</i> +CAC/ <i>M</i> +AGA	53	33	1	—	1	—
<i>E</i> +CAC/ <i>M</i> +AGC	74	47	17	5	5	7
<i>E</i> +CAC/ <i>M</i> +AGG	75	56	12	2	1	9
<i>E</i> +CAC/ <i>M</i> +AGT	66	37	13	—	1	12
<i>E</i> +CAC/ <i>M</i> +ATA	59	22	11	2	—	9
<i>E</i> +CAC/ <i>M</i> +ATC	65	33	2	2	—	—
Total	994	565	147	32	26	89

**E*+3/*M*+3: 5'-AGACTGCGTACCAATTC+NNN-3'/5'-GACGATGAGTCCTGAGTAG+NNN-3'.

Table 2. Accession number from the USDA, ARS, NPGS catalogue, geographic coordinates, and state of the collection sites of three species of *Echinacea* used in the validation trial.

Accession No.	Origin			State
	Lat	Long	Elevation (m)	
<i>E. angustifolia</i>				
PI 631268	34.500	-97.350	370	Oklahoma
PI 631284	43.417	-95.600	475	Iowa
PI 631288	42.633	-96.317	400	Iowa
PI 631317	39.267	-96.583	385	Kansas
<i>E. pallida</i>				
Ames 26351	37.122	-93.023	440	Missouri
Ames 27324	40.852	-91.137	186	Iowa
PI 597603	34.519	-97.869	360	Oklahoma
PI 597604	37.361	-88.922	106	Kansas
PI 631252	42.249	-88.608	260	Illinois
PI 631253	41.967	-93.467	289	Iowa
PI 631279	37.017	-94.700	400	Kansas
PI 631280	34.083	-93.167	220	Arkansas
PI 631282	42.517	-94.617	375	Iowa
PI 631315	36.150	-78.767	107	North Carolina
PI 631322	31.140	-93.273	68	Louisiana
<i>E. purpurea</i>				
Ames 27338	40.807	-93.664	308	Iowa
Ames 27725	36.695	-93.441	326	Missouri
PI 631307	38.467	-90.783	270	Missouri
PI 631313	35.917	-82.883	490	North Carolina
PI 633665	34.197	-93.314	128	Arkansas
PI 633668	31.089	-93.056	95	Louisiana
PI 633670	32.340	-89.439	141	Mississippi
PI 649040	33.000	-87.000	140	Alabama

mean (UPGMA) (Sneath and Sokal 1973). Cophenetic matrices derived from the dendrograms were compared with the original similarity matrix by the test of Mantel (1967) to estimate the goodness-of-fit of the clustering to the data

matrix. The results of the cluster analysis were also validated by principal coordinate analysis (Gower 1966) by double-centering the similarity matrix, extracting the eigenvalues and eigenvectors, and displaying the relation-

ships among individuals in 3 dimensions. Estimation of genetic distances, cluster analysis, the Mantel test, and principal coordinate analysis were carried out by NTSYS-pc software (Rohlf 1993).

AFLP fragments in the validation trial were scored by GeneMapper 4.0 software (Applied Biosystems). An AFLP locus in the bulked samples was considered to be polymorphic if the amplified band was present in some accessions and absent in others, species-specific if the band was shared between all accessions belonging to one species and absent in all other accessions, and monomorphic if the band was present in all samples. To avoid an underestimation of the genetic similarities, all loci, polymorphic or not, were considered.

Results

Cytological analysis

The analysis confirmed the chromosome number typical of each species: *E. angustifolia* and *E. purpurea* are diploid, with $2n = 22$, while *E. pallida* is tetraploid, with $2n = 44$.

Trial 1: AFLP analysis of cultivated *Echinacea* spp.

The scoring of AFLP gels showed the presence of 994 fragments. Of these, 429 (43%) were monomorphic, found in all plants of all three species, while 565 (57%) were polymorphic (Table 1). Of the latter group, 147 fragments were discriminant, that is, unique to all plants of a single species. The analysis showed that 89 fragments were unique to *E. purpurea*, 32 were unique to *E. angustifolia*, and 26 were unique to *E. pallida*. However, discrimination among species was also possible by using combinations of fragments. For instance, *E. angustifolia* and *E. purpurea* were found to share 28 fragments that were absent in all individuals of *E. pallida*. *Echinacea angustifolia* and *E. purpurea* could, in turn, be distinguished from one another by 103 bands, 69 present in the former and absent in the latter and 34 absent in the former and present in the latter (data not shown).

Discrimination properties shown by each primer combination are of particular interest. For instance, the primer combinations *E+CAC/M+AAG*, *E+CAC/M+ACA*, *E+CAC/M+ACG*, *E+CAC/M+AGA*, and *E+CAC/M+AGT* did not produce any AFLP fragment specific to *E. angustifolia*, while *E+CAC/M+AAT* alone was able to generate 9 specific fragments. In *E. pallida* the best primer combinations able to generate specific fragments were *E+CAC/M+ACG* (6 fragments) and *E+CAC/M+AGC* (5 fragments), while for *E. purpurea* the best primer combinations were *E+CAC/M+AAT* and *E+CAC/M+AGT*, with 13 and 12 fragments, respectively.

The primer combination that gave the highest number of fragments was *E+CAC/M+AAG*, while *E+CAC/M+ACC* gave the lowest. It is interesting that the former also gave the highest number of polymorphic fragments, but was not the most effective in terms of species discrimination. Five primer combinations were able to detect fragments specific to each of the three species, namely *E+CAC/M+AAA*, *E+CAC/M+AAC*, *E+CAC/M+AAT*, *E+CAC/M+AGC*, and *E+CAC/M+AGG*. Of these, the most powerful was *E+CAC/*

M+AAT, able to provide 13, 9, and 4 specific fragments for *E. purpurea*, *E. angustifolia*, and *E. pallida*, respectively.

The UPGMA dendrogram in Fig. 1 indicates a high similarity within each species and clear differences among species. The goodness-of-fit of the analysis was validated by the high and significant correlation coefficient between the similarity and cophenetic matrices ($r = 0.997$, Mantel $t = 20.557$, $P < 0.001$). All individuals of *E. purpurea* are grouped together, apart from the rest of the individuals, at a similarity value of 0.623. The rest of the individuals are then split into 2 other groups at a similarity value of 0.728, clearly distinguishing *E. angustifolia* and *E. pallida* and confirming that they are phylogenetically closer to one another than to *E. purpurea*. Although the three species share a large part of their genome, as shown by the high number of monomorphic fragments, peculiarities do exist, thus allowing distinctiveness. This is confirmed by the pattern of clusters, with no individuals being mis-classified into different species. A further statistical validation of these results is provided by principal coordinate analysis, where the projection of individuals plotted against the axis representing the most significant eigenvectors shows low within-species variability and consistent variability between species (Fig. 2). Individuals of each species were tightly grouped together, confirming the clustering pattern. The first 3 eigenvalues were able to explain as much as 0.82 of the total variation (0.51, 0.28, and 0.02, respectively), with the first 2 able to significantly explain more variation than expected under the broken stick model (Joliffe 1986).

Trial 2: validation

The primer combination *E+CAC/M+AGC*, used for validation analysis, produced a total of 76 amplification products, a value very close to the 74 found on cultivated *Echinacea* spp. in trial 1; 31 of these amplicons (41%) were polymorphic. Of these, 10 amplicons were species-specific (3, 2, and 5 for *E. angustifolia*, *E. pallida*, and *E. purpurea*, respectively), while 4 amplicons were common between *E. angustifolia* and *E. pallida* and absent in *E. purpurea*. The remaining polymorphic fragments were specific to some accessions within the same species rather than to the whole species; in particular, 3 amplicons were found in *E. angustifolia*, 3 in *E. pallida*, and 11 in *E. purpurea*. The species-specific amplicons were also easily found in the bulked samples of all accessions of the same species and in the bulk comprising all 23 accessions. A duplicate lane of one sample confirmed the correctness of the profile.

The numbers of species-specific AFLP fragments detected by the *E+CAC/M+AGC* primer combination in the validation trial were lower than those found in the cultivated samples (3, 2, and 5 vs. 5, 5, and 7 in *E. angustifolia*, *E. pallida*, and *E. purpurea*, respectively), and this could be due to the high number of accessions used in the validation test. Most likely, some of the bands scored as species-specific in trial 1 might belong to those classified as accession-specific in the validation trial (i.e., bands present in only some of the accessions of a given species). The sizes of the species-specific fragments amplified by primer combination *E+CAC/M+AGC* are as follows: 75, 160, and 200 bp for *E. angustifolia*; 127 and 212 bp for *E. pallida*; and 38, 53, 73, 153, and 155 bp for *E. purpurea*.

Fig. 1. Dendrogram of individual plants belonging to *E. angustifolia*, *E. pallida*, and *E. purpurea*, generated by UPGMA cluster analysis of Jaccard's similarity coefficients calculated from AFLP marker data.

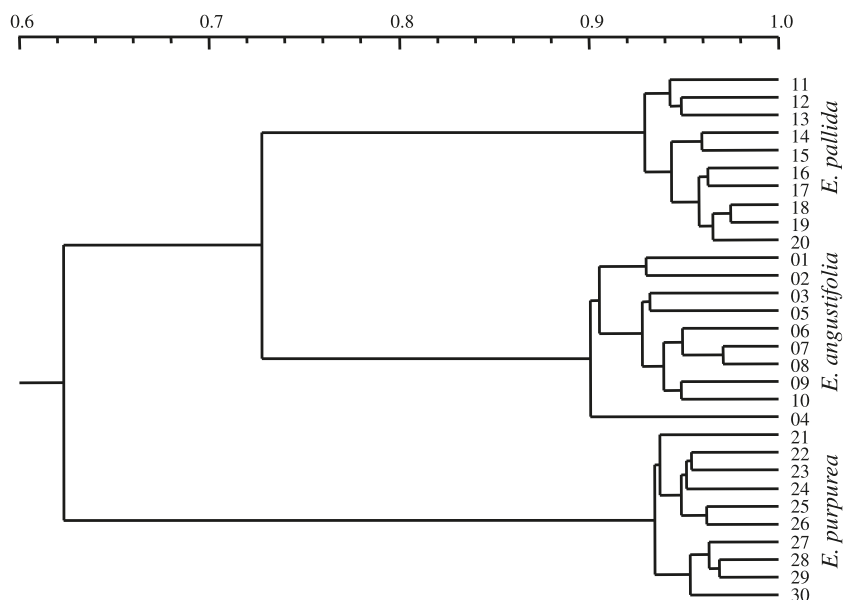
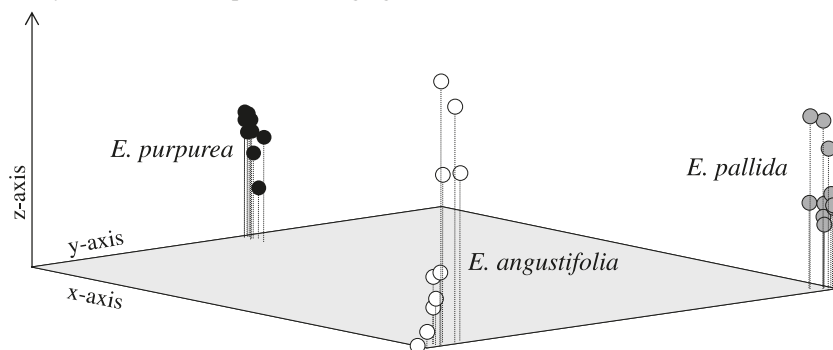


Fig. 2. Principal coordinate analysis of individual plants belonging to *E. angustifolia*, *E. pallida*, and *E. purpurea*, based on AFLP markers.



Discussion

Echinacea purpurea, *E. angustifolia*, and *E. pallida* are the most known species of the whole genus, especially for their immunological properties. *Echinacea purpurea* in particular is the most significant for its medicinal use (Tyler 1997; Percival 2000; Oomah et al. 2006). The objective of the present study was to investigate the genetic similarities of the three species and look at the possibility of distinguishing them using AFLP markers. Characterization of genomes using molecular markers has developed rapidly and a vast literature is available. Useful applications of molecular markers include genetic control of plant reproduction, genetic mapping, marker-assisted selection, cultivar characterization, estimates of genetic distances, analysis of gene expression, and cloning. In the last decade the fields that have benefited the most from extensive use of molecular markers are those connected with phylogenetic studies, cultivar characterization, and marker-assisted selection.

The use of AFLP technology for these purposes has become very popular because of its unique ability to detect polymorphisms within the genome without requiring prior sequencing information (Still et al. 2005). This technology can faithfully generate many fragments per primer combina-

tion and highlight several differences with few primer combinations. Moreover, results from AFLP data sets have proved to be concordant with those from other molecular markers (Powell et al. 1996). Most, but not all, AFLP fragments of a specific size can be considered to represent the same loci (Cervera et al. 2001; Peters et al. 2001), although this likelihood apparently decreases with an increase in genetic distance between species (Still et al. 2005). Large data sets can offset the assumption of orthology simply by increasing the number of independent loci sampled across a genome and establishing "correct" phylogenetic relationships among species (Rokas et al. 2003). Still et al. (2005) reported that AFLP methodology largely fulfills this requirement and any non-orthologous fragments detected among populations should be overcome by the much higher number of orthologous fragments.

AFLP analysis has been found to be useful in predicting the levels of bioactive phytochemicals in cultivated *E. purpurea* germplasm (Baum et al. 2001). Twenty-four AFLP primer combinations were used to construct a genetic map of this species via individual pollen DNA fingerprinting (Aziz and Sauve 2008), with 104 scorable markers in 11 linkage groups. RAPD markers were successfully used by

Kapteyn et al. (2002) to investigate the genetic relationships and diversity of the same three *Echinacea* species studied in the present work; they found 17 diagnostic markers useful for distinguishing among taxa. They did not find any species-specific marker, and postulated as discriminant those found with a frequency of at least 0.95 in a taxon and absent in other taxa, or present with a frequency of less than 0.05. The results of the present research show that in *Echinacea*, AFLP markers seem to be much more effective than RAPD markers in distinguishing among species, firstly because of the higher number of polymorphisms detected (Table 1) and secondly because many of the fragments were found to be specific to a single species. AFLP analysis was also successfully employed by Kim et al. (2004) and Mechanda et al. (2004a) in studying the genetic diversity of the whole *Echinacea* genus, and the former pointed out the superiority of AFLP versus RAPD markers in phylogenetic studies, in terms of the number of polymorphic fragments per primer pair. In the present study the average number of polymorphic fragments per primer pair was 40, significantly lower than the 66 fragments reported by Kim et al. (2004), 62 by Mechanda et al. (2004a), and 82 by Still et al. (2005), but consistently higher than the 4.6 fragments obtained by RAPD analysis. Independently of the type of marker used, the results from cluster analysis are in perfect agreement with those presented by Kapteyn et al. (2002), who analyzed natural and cultivated populations from several sources and found low variability within species. Kim et al. (2004) also reported similarities within species of the same size as those in the present study. The explanation suggested by Kapteyn et al. (2002) is that the high genetic uniformity among populations within species is the result of a continuous gene flow, particularly in *E. purpurea*, the most widespread species, and in *E. pallida*. The differences found among populations of *E. angustifolia* were ascribed to a discontinuous distribution and a consequent difficulty of gene flow. However, it is also possible that the genetic base of the materials used in the above studies was narrow, or that this is the result of using dominant types of markers (such as AFLP and RAPD). Large within-population variation was also found by Mechanda et al. (2004a) and by Still et al. (2005) in *E. angustifolia*.

The results of the present study have a practical application. Single specimens belonging to any of the three species are easily and distinctly classified correctly on the basis of a number of morphological and physiological characters (McGregor 1968; Aiello 1998; McKeown 1999), but with ground, dry plant parts, as in some commercial preparations, it is difficult to detect and (or) quantify the amount of *E. purpurea* (the most valuable species) mixed with the other two species (with poorer chemotherapeutic properties) (Wolf et al. 1999). RAPD analyses have been successful in some cases (Wolf et al. 1999; Nieri et al. 2003), but less so in others (Kapteyn et al. 2002), certainly because of the different primers used. RAPD analyses are simple and inexpensive, but have been shown to be difficult to reproduce from one laboratory to another (Jones et al. 1997). AFLP markers could then be useful in detecting commercial frauds. In the present work, exploring a rather high number of primer combinations, it has been shown that this is achievable by

using only a single combination of primers, as confirmed by the validation trial based on *E*+CAC/*M*+AGC.

A sequence characterized amplified region assay carried out on DNA from the three *Echinacea* species of the same origin as in the present study showed that the expected band (330 bp) was present in *E. purpurea* and not in the other two species (Adinolfi et al. 2007). The same approach applied to our species-specific fragments and verified experimentally would provide a simple tool able to distinguish each of the three species.

The present study focussed on the most commercially important species and has no contribution to make in the dispute on the origin of the tetraploid *E. pallida*. However, the results indicate a closer genetic similarity between *E. pallida* and *E. angustifolia* than between *E. pallida* and *E. purpurea*, in line with most of the published literature based on molecular markers. McGregor (1968) supposed *E. pallida* to derive from a chromosome doubling of the hybrid between *E. simulata* and *E. sanguinea*. On the basis of morphometric analysis based on 74 characters, Binns et al. (2002) revised the genus *Echinacea* and included 4 species rather than 9. According to Binns et al. (2002), 5 botanical varieties (*E. pallida* var. *pallida*, var. *angustifolia*, var. *tennesseensis*, var. *simulata*, and var. *sanguinea*) belong to *E. pallida*, but the tetraploidy of *E. pallida* var. *pallida* and the diploidy of var. *angustifolia* and/or the other 3 varieties would be in contrast with the accepted definition of a species (a group of individuals able to interbreed and give rise to fertile progenies). The work of Binns et al. (2002), while confirming the similarities of *E. pallida* and *E. angustifolia*, also shows high similarities of these species with *E. sanguinea* and *E. simulata*, *sensu* McGregor (1968), thus giving support to McGregor's hypothesis.

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