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THE TAXONOMY, PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITY OF THE GENUS *ECHINACEA* (ASTERACEAE)

© SHANNON E. BINNS

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We are deeply indebted " to those predecessors of ours on the North American continent who,... studied the flora of a new world, learned its secrets, and encouraged the next generations to study closer and to learn more. Their diligence and energy, their insight and creativity, these are the marks of true scientists, dedicated to gaining meaningful and useful knowledge from a complex and confusing world."

D. E. Moerman

Native American Ethnobotany

Timber Press, 1998.

ABSTRACT

Echinacea Moench (Asteraceae) is a native North American plant genus with a long history of use as phytomedicine in both the Indigenous and European traditions (Shemluck 1982; Bauer 1998). A taxonomic revision of the genus *Echinacea* was conducted for the first time using morphometric analysis of natural populations sampled throughout the entire genus range. Individual plants were grouped without *a priori* taxonomic labeling, by morphological similarity using non-hierarchical and hierarchical, agglomerative clustering strategies. Canonical discriminant analysis (CDA) supported the distinction of two new subgenera, four species and eight varieties in the genus according to overall morphological similarity. When the same dataset was labeled *a priori* according to McGregor's taxonomy (1968), his taxa were supported (with the exception of one variety) at the variety level. The new morpho-taxa were sufficiently distinguished by CDA of phytochemical variation (except for two varieties). Also, a class of outliers (including artificial hybrids) was not supported in either dataset as a taxon, and was therefore classified as 'hybrids/introgressants'. In the most parsimonious cladistic solution, one new subgenus was basally divergent to a clade of all others (70% bootstrap value) and all four species were distinguishable by at least one synapomorphy. Phytochemical profiles and average content of each known compound were reported here for the first time from all taxa of *Echinacea*, and revealed that five diene alkamides, one polyyne and two phenolic compounds (cichoric acid and echinacoside) were all chemotaxonomic markers within the genus *Echinacea*. Some of the same chemotaxonomic markers contributed heavily to the quantitative multivariate analysis of 26 phytochemicals, which identified five chemotypes

among nine wild populations of the revised *E. pallida* var. *angustifolia* in a controlled greenhouse study. The alkamides and polyynes were also induced to higher concentrations in young roots of *E. pallida* (Nutt.) Nutt., that were sprayed with methyl jasmonate. Furthermore, plant extracts of the *Echinacea* taxa were variably phototoxic to clinically relevant pathogenic fungi, such as *Candida* spp. The different phototoxic activities between *Echinacea* taxa reflected the observed phytochemical variation, especially in the polyynes. Antiviral activity to *Herpes simplex* was linked to the presence of polyynes and cichoric acid in fractionated *Echinacea* root and inflorescence extracts.

RÉSUMÉ

Echinacea Moench (Asteraceae) est un genre de plantes indigènes en l'Amérique du Nord qui a été une source de phytomédicamments dans les systèmes de medicine traditionelle chez les autochtones et aussi en Europe (Shemluck 1982; Bauer 1998). Une revision systématique du genre *Echinacea* Moench (Asteraceae) a été menée pour la première fois avec l'analyse morphométrique des populations naturelles représentant la variation géographique totale du genre. L'étude de ce genre est significative pour la compréhension et la protection de ses populations naturelles en face de son importance économique et pharmacologique. L'analyse ``Canonical Discriminant Analysis`` (CDA) a vérifié les groupes de plantes classifiés selon des méthodes agglomératives, hiérarchiques et non-hiérarchiques, sans identification *a priori*. Deux sous-genres, quatre espèces et huit variétés ont été délimités par les calculs de similarité morphologique, et vérifiés en utilisant CDA. Une analyse préliminaire avec les données de cette étude a été menée selon la taxonomie de McGregor (1968), le seul à étudier le genre complet; elle a résulté à la prise en compte des taxa de McGregor comme variétés (sauf une de ses variétés). De plus, les taxa morphologiques ont été aussi déterminés sur des variations phytochimiques par CDA (sauf deux variétés). Mais, les spécimens qui restaient hors de la classification morphométrique n'étaient pas soutenus par le test CDA pour former un nouveau taxon, ils ont donc été classés comme hybrides ou introgressants (ce qui inclut les hybrides artificiels). Une analyse cladistique des quatres espèces de la taxonomie présente a démontré qu'elles se distinguaient par au moins une apomorphie (souvent plus) et les deux sous-genres divergaient plus fortement (70% ``bootstrap``). La composition chimique a été identifiée pour la première fois pour tous les taxa du genre *Echinacea* : 26 composés dans lesquels se sont révélés comme marqueurs chimiotaxonomiques, cinq alkamides, une polyyne et deux composés phénoliques (l'acide cichorique et l'échinacoside). Parmi ceux-ci, quelques-uns ont contribué le plus à l'ordination par CDA de la variation quantitative dans une expérience en serre identifiant cinq chimiotypes parmi neuf populations sauvages de la variété *E. pallida* (Nutt.) Nutt. var. *angustifolia* DC. Cronq. (nom révisé). La concentration de certains composés alkamides et polyynes augmente dans les jeunes racines d'*E. pallida* (Nutt.) Nutt. var. pallida par traitement avec l'hormone méthyle jasmonate. Enfin, une activité phototoxique contre des souches pathogènes fongiques comme le *Candida* spp., et contre le virus *Herpes simplex*, a été démontrée dans les extraits d'*Echinacea*. Grâce aux différences dans la composition chimique des extraits, surtout des alkamides et polyynes, les taxa ici-révisés possédent chacun des activités antimicrobiennes uniques.

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I would like to dedicate this work with enthusiasm to the growing medicinal plant community, and to all our ancestors who fostered the wisdom of ecological awareness and relationships. I offer heartfelt thanks to my parents, my brother, my family, my friends and all my grandmothers for their spirit, creativity and encouragement. For unconditional love and understanding, I am grateful to Mark Macfarlane DC.

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SPECIAL NOMENCLATURAL NOTE

According to the typification of names presented in chapter 3 of this work, the name *E. purpurea* (L.) Moench is misapplied in current use. The taxon that is currently called by that name should be called *E. serotina* (Nutt.) DC., which was determined to be its correct name in chapter 3 of this work. However, to avoid confusion, *E. purpurea* (L.) Moench is throughout the thesis in the current sense of the name (also *sensu* McGregor 1968 and Cronquist 1980). Furthermore, this name should be maintained in its current usage, pending approval by the International Botanical Congress of a proposal to conserve it with a new type (chapter 4).

Three of the chapters were either published or already submitted upon completion of the morphological taxonomic revision in chapter 2. Therefore, taxonomic names used in chapters 6, 7 and 8 follow the accepted nomenclature of McGregor (1968), and should not be confused with the revised taxonomy (chapter 2) that is applied in all other chapters.

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GLOSSARY

!	Notation used to indicate that the specimen/photo/illustration was seen by the author.
Achene	The most generalized type of dry, indehiscent fruit, usually one-seeded.
Acute	sharp-pointed tip with margins that form an angle of less than 90 degrees.
Adulterated	A term used in the Botanicals industry to indicate the substitution of another species for the commercial plant material and/or products derived from that material. It may also suggest the addition of an ingredient not indicated on the label.
Attenuate	tapering very gradually to a slender tip, more extreme than acute or acuminate.
Basal	lowest, at the base.
Bulbous	shaped like rounded projections or enlarged.
Caudex	short, more or less vertical, persistent stem at the surface of the ground.
Cauline	on or pertaining to the stem.
CDA	Canonical Discriminant Analysis: a multivariate analysis of overall variation within and between groups of individuals.
Centroid	mean value (center of a cluster in hyperspace).
Chemotype	An infraspecific taxon (single population or series of populations) that may be distinguished from others within the species according to by novel phytochemicals or a consistently distinct (novel) profile of compounds.
Clade	a taxonomic group which shares an hypothetical common ancestor.
DAO	Herbarium of the Department of Agriculture Ottawa (Canada).
Derived	character states that are new or unique in an individual taxon or group of taxa.
Dwt	dry weight.

Eclectic	medical system of the early 1900's based on a mixture of traditions, including Native American and European herbalism.
Ecotypes	Genetically different strains of a population (of a single species) that have adapted to a geographically distinct habitat.
Holotype	One specimen or illustration used by the author or designated by the author as the nomenclatural type. Note: If a new name is based on previously published description and/or diagnosis of the taxon, then the type material designated by the earlier author must be conserved.
Homoplasy	characteristics that show reversals back to "primitive" status; parallelisms.
ICBN	International Congress for Botanical Nomenclature.
IL-1,6,10	Interleukins in the mammalian immune system.
Inflorescence	a group of flowers - in the Asteraceae, it is commonly called a capitulum.
Isotype	Any duplicate of the holotype. (Part of a single gathering of a single species or infraspecific taxon made by a collector at one time).
Laminae	leaf blades.
Lanceolate	leaf shaped like a lance: rounded bottom to acute, pointed tip.
Lectotype	A specimen or illustration designated as the nomenclatural type, when no holotype was indicated at the time of publication, or if it is found to belong to more than one taxon, or as long as it is missing. It must be selected from among the syntypes, if such exist.
MJ	methyl jasmonate (= methyl ester of jasmonic acid), a plant hormone that is naturally produced from free linolenic acid and is linked to growth inhibition and senescence.
Monophyletic	term used to describe a clade (taxonomic group) unified by synapomorphies, and which lacks homoplasies.
Morpho-taxa	taxonomic groups distinguished by morphometric comparison.

OTU	Operational Taxonomic Unit (may be a single specimen or a group, but in this work it was always a single plant).
Pappus	the modified calyx crowning the ovary (and achene) of the Asteraceae.
Paratype	A specimen cited in the protologue that is neither the holotype, nor an isotype, nor one of the syntypes.
Phenotypic plasticity	Variation in phenotypes of a single species (or population) according to changes in environment which do not necessarily have a genetic basis.
Phototoxicity	Near ultra-violet light (300-400 nm) and visible light together induce photosensitization (by one of several methods) of secondary phytochemicals, which are thereby capable of toxicity towards microorganisms.
Polyacetylenes	ketoalkenes = ketoalkynes = polyenes = polyynes = polyyn/enes = ketoalkyn/enes .
Population	A group of individuals of a single species occupying a given area.
Primitive	character states that are not unique within a putative group of taxa (a potential clade) or, which are found in outgroups for cladistic comparisons.
Ray floret	the ligule flower (specialized joined corolla that is longer than that of the disk florets) in the Asteraceae.
Receptacle	the end of a stem (pedicel) to which flower parts are attached; in the Asteraceae this is an enlarged organ subtending the capitulum.
Recurved	curved backward gradually.
Reflexed	bend backward abruptly.
Rhizome	creeping/horizontal underground stem.
SAHN	Sequential Arithmetic Hierarchical Nested – a category of several clustering methods.
sd	standard deviation of the mean.

SEM	Standard error of the mean = mean / square root of n (n is the number of individual measurements).
Sessile	attached directly by the base, without a stalk.
Synapomorphy	shared, "derived" characteristic.
Syntype	Any one of two or more specimens cited in the protologue when no holotype was designated. OR Any one of two or more specimens simultaneously designated as types.
Taxonomy	A study aimed at producing a system of classification of organisms that best reflects the totality of their similarities and differences; a classification produced by such a study.
TNFα	Tumour Necrosis Factor Alpha, a chemical mediator in the mammalian immune system which is capable of sensitizing leucocytes and regulating the rate of phagocytosis and immune responses.
Tomentose	covered with tangled, thick hairs.
Trichomes	hairs.
Vestiture	hairiness.
$\mu M/m^2/s$	units for light intensity (micromoles per metre squared per second) = 5 footcandles = 0.402 Lux.

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

Plants have historically been central to many traditional systems of medicine, including that of the indigenous peoples of North America (Moerman 1998). Furthermore, the science of pharmacy is based on traditional plant medicines, since over 25% of the world's drugs contain ingredients isolated from plants (Farnsworth 1990). It is estimated that higher plants are currently utilized by 64% of the total population of the world as phytomedicines, primarily in the developing countries (Farnsworth 1990). Phytomedicine is a returning trend in the western world, where new technologies allow for scientific evaluation of traditional plant uses combined with the advent of complementary systems of medicine. One of the leading sources of modern medicinal plants are species of *Echinacea* Moench, which provided a universal remedy used historically by Native Americans to treat a variety of ailments from wounds and toothache to colds and systemic infections (Table 1.1)(Foster 1991). *Echinacea* was recently reported to be the best-selling product on the North American botanical market with 10% of total sales (Brevoort 1998), and it is also a component of over 800 herbal products in Germany (Bauer 1998).

Plants of the genus *Echinacea* are endemic and indigenous to North America, and throughout their history of human use, there has been taxonomic and nomenclatural confusion with respect to infrageneric groups (McGregor 1968). This continues to have profound effects on the correct botanical identification of plants for cultivation and trade as phytomedicines, as well as hindering the appropriate conservation of genetic diversity in wild populations. In the last decade, phytochemical methods have been increasingly used to distinguish between *Echinacea* species in the medicinal plant industry, and to detect the common root adulterant, *Parthenium integrifolium* L. (Bauer *et al.* 1986). It is clear from extensive work to date that morphologically-distinct *Echinacea* groups exhibited differences in phytochemical composition, with corresponding differences in pharmacological activity (Bauer 1998).

The overall objective of the present work was to answer the need for a modern taxonomic revision of the genus *Echinacea* (Asteraceae) based on morphometric analysis of variation within and between populations throughout its geographical range. The secondary objective was a large-scale assessment of variation in phytochemical content of single plants and populations for a complete chemotaxonomic treatment of the genus, and potential correlations with the morphometric results. In view of reported prominent pharmacological significance, novel investigations of the antifungal and antiviral activities of *Echinacea* phytochemicals were also undertaken.

The interpretation of population variation through the examination of chemical characteristics has been particularly useful, especially where hybridization is suspected (Briggs and Walters 1984). For example, Alston and Turner (1963) attempted to resolve patterns of hybridization among populations of *Baptisia* (Fabaceae) through observed variations in flavonoid accumulations. With respect to *Echinacea*, there is currently widespread use of morphological and chemical means for plant identification in breeding and horticulture, pharmacology and clinical medicine, but it has been based on the assumption that existing taxonomic groups reflect the most discrete units of interbreeding populations. However, the very nature of species groups and their delimiting characteristics remains an unresolved question in view of the taxonomic confusion and reported extensive hybridization in the only complete taxonomic revision of the genus by McGregor (1968). Furthermore, there is considerable evidence to suggest that more than three putative *Echinacea* taxa were used by the Native Americans to treat a large range of different ailments (Foster 1991). Functional species descriptions and a universal system of consistent nomenclature are critical for the important plant genus *Echinacea*, not simply for botany, but for medical safety.

The present work is presented in three sections, which will be introduced in this chapter by a brief literature review, followed by the corresponding research objectives and hypotheses. The first section includes three chapters that describe a morphometric taxonomic revision of the genus *Echinacea* with a cladistic assessment of revised species' relationships. Nomenclatural treatment of the revised taxa is included, followed by two chapters addressing the novel nomenclatural findings with typification and conservation of some of the taxonomic names. Section two provides the first comprehensive analysis of phytochemical variation in the genus from wild, transplanted and cultivated plant materials, including previously unreported profiles for the more rare *Echinacea* taxa. Furthermore, phytochemical variation between populations of the most widespread *Echinacea* species, and the first report of phytochemical induction in the genus by methyl jasmonate, a naturally-occurring plant growth regulator, are also discussed in section two. The third section is dedicated to the modern validation of pharmacological significance through the investigations of antimicrobial activity in *Echinacea* extracts, including two chapters about novel light-mediated antifungal activities and one chapter about novel antiviral activity.

Botany and Ethnobotany

Echinacea Moench is an endemic North American plant genus in the Asteraceae, one of the largest families of flowering plants with 23,000 species to date (Bremer 1994). Native plant populations are distributed throughout the midwestern United States east of the Rocky Mountains from northern Texas to Manitoba, Canada, and more rarely found in wooded areas from the Ozarks to the Appalachian uplands. Plants of the genus *Echinacea* are characterized by their cone-shaped inflorescence, which lends them the common name "purple coneflowers". In the west, they are drought-resistant perennials with long, thick tap roots and a basal rosette of linear to lanceolate, hairy leaves. Plants with a more eastern range have fibrous roots and smoother, lance-ovate leaves. Stems are solitary or branching and generally range from 50 to 120 cm in height (McGregor 1968).

There are three putative species sold commercially: *E. purpurea* (L.) Moench, *E. angustifolia* DC. and *E. pallida* (Nutt.) Nutt. (McGregor 1968). *E. purpurea* has been cultivated in Europe since the early 1900's for the use of its aerial parts (and roots to a lesser extent) as an extremely popular phytomedicine. Worldwide cultivation is currently estimated to be 16,000 hectares in order to support the mass-market sales posted at $33 million in the U.S. alone, over 52 weeks in 1998 (Brevoort 1998). *E. purpurea* is mostly cultivated material, but the other two mass-market species are still being extensively wild-harvested in spite of increases in their cultivation since 1995. It has been suggested *E. pallida* was often used interchangeably with *E. angustifolia* by Native Americans (Hobbs 1989). Furthermore, although Native Americans recognized only three medicinal taxa, in view of more recent reports of geographical variation, it is likely that *Echinacea* ethnomedicines included many of the recognized species and varieties in McGregor's (1968) treatment of the genus (Bauer 1998; Foster 1991).

Historically, whole and extracted roots in the genus *Echinacea* were traditional medicines of most Native American groups in the central Great Plains and eastern prairies (Hobbs 1989; Kindscher 1989; Shemluck 1982). Ethnobotanical literature cited *Echinacea* roots as primary treatments for a variety of illnesses (Table 1.1). Although medicinal uses of *Echinacea* were explored by Eclectic and allopathic doctors, specific chemical composition and clinical activity were not verified until scientific studies in the latter half of this century (Hobbs 1989). The genus *Echinacea* is a good example of modern attempts to reinforce the value of ethnomedicines through research of biological and pharmacological activity relevant to its traditional medicinal uses.

Table 1.1: Some traditional Native American medicinal uses of commercial *Echinacea* roots. 1 *E. angustifolia* DC., 2 *E. pallida* (Nutt.) Nutt., 3 *E. purpurea* (L.) Moench

Traditional Use	Native American Group	Author
Toothache, Mouth sores	Cheyenne [1,2]	(Hart 1981)
	Comanche [1,2]	(Hobbs 1989)
	Crow [1]	(Toineeta 1970)
	Dakota Sioux [1]	(Gilmore 1911)
	Winnebago [1,2,3]	(Gilmore 1911)
Sore Throat, Coughs, Tonsilitis	Kiowa [1]	(Hobbs 1989)
	Cheyenne [1,2]	(Hart 1981)
	Comanche [1,2]	(Hobbs 1989)
	Crow [1]	(Toineeta 1970)
	Choktaw [3]	(Hobbs 1989)
	Dakota Sioux [1]	(Gilmore 1911)
Colds, Septic diseases	Meskwaki [1]	(Smith 1928)
	Cheyenne [1,2]	(Hart 1981)
	Dakota Sioux [1]	(Gilmore 1911)
	Omaha [1]	(Gilmore 1913)
Arthritis	Cheyenne [1,2]	(Hart 1981)
Anti-microbial: Eye-wash, Snakebite, Burns	Cheyenne [1,2]	(Hart 1981)
	Omaha [1]	(Gilmore 1913)
	Dakota Sioux [1]	(Smith 1928)

Taxonomy

Echinacea has been classified in the tribe Heliantheae, family Asteraceae (Stuessy 1977). It is commonly treated as one of the "coneflower" genera, along with *Rudbeckia*, *Dracopis*, and *Ratibida*, recognized by their conical, widened receptacles (Stuessy 1977). The presence of spiny bracts in the capitulum was one of the features distinguishing *Echinacea* from the other coneflowers (Stuessy 1977). Subtribal classifications of *Echinacea* and the other coneflower genera have been subject to recent revisions (Cox and Urbatsch 1990; Karis 1993; Robinson 1981; Urbatsch and Jansen 1995; Urbatsch *et al.* 2000).

The morphological variability within the genus was often reportedly associated with geographical differences, and it has resulted in much debate about the treatment of infrageneric taxa by DeCandolle (1836), Fernald (1900), Boynton & Beadle (1903), Cronquist (1945; 1955; 1980), and Dress (1961). McGregor (1968) suggested that taxonomic confusion in the past was due to inadequate herbarium materials for study. Fernald (1900) distinguished between the species, *E. angustifolia* and *E. pallida*, and this distinction was upheld by Dress (1961), and McGregor (1968). McGregor's (1968) treatment described nine species and two varieties of *Echinacea*, and remains the most widely-accepted taxonomy in current use. On the other hand, Cronquist (1945) reduced *E. angustifolia* to a variety of *E. pallida* and recognized only four species with distinct morphological varieties (Table 1.2).

Both Cronquist (1945) and McGregor (1968) used intuitive methods of assessing overall morphological similarity. Numerical taxonomy does not completely omit the potential for researcher bias, but it is mostly restricted to the choice of character states in order to reflect the largest degree of observed variation. Also, the taxonomic rank is subject to choice by the researcher, and is often determined by the relative distance between determined taxa (Sneath and Sokal 1973). Furthermore, it was shown that the higher the number of characters used in numerical taxonomic analyses, the more reliable were the resulting taxonomic treatments (Crovello 1970). Finally, any subjectivity with respect to nomenclature bears little consequence since it is not the name that is important, but rather its ability to correctly identify a group of organisms following the International Rules of Botanical Nomenlature (Greuter *et al.* 2000).

Table 1.2. Two current taxonomic treatments of *Echinacea* Moench by McGregor (1968) and Cronquist (1980). Synonyms are indicated in smaller type.

R. L. McGregor (1968)	**A. Cronquist** (1945, 1955, 1980)
1. *E. angustifolia* DC. var. *angustifolia*	**1**. *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist
E. angustifolia DC. var. *strigosa* McGregor	-*E. angustifolia* DC. var. *strigosa* McGregor
2. *E. tennesseensis* (Beadle) Small	-*E. tennesseensis* (Beadle) Small
3. *E. pallida* (Nutt.) Nutt.	*E. pallida* (Nutt.) Nutt. var. *pallida*
4. *E. simulata* McGregor	-*E. simulata* McGregor
5. *E. sanguinea* Nutt.	-*E. sanguinea* Nutt. [informal suggested variety]
6. *E. atrorubens* Nutt.	**2**. *E. atrorubens* Nutt. var. *atrorubens*
7. *E. paradoxa* (Norton) Britton var. *paradoxa*	*E. atrorubens* var. *paradoxa* (Norton) Cronquist
E. paradoxa (Norton) Britton var. *neglecta* McGregor	
8. *E. laevigata* (Boynton & Beadle) Blake	**3**. *E. laevigata* (Boynton & Beadle) Blake
9. *E. purpurea* (L.) Moench	**4**. *E. purpurea* (L.) Moench

McGregor's (1968) taxonomy of the genus from widespread collections throughout its entire geographical range contained morphological and anatomical keys to species and varieties. However, there were inconsistencies in McGregor's descriptions and practical difficulties with his key to the nine species and two varieties that he recognized in *Echinacea* (Baum *et al.* 1999) (Table 1.2). McGregor (1968) reported that his species were narrowly defined and this was linked to considerable hybridization within wild populations as well as between all taxa when they were brought together. Currently, natural hybridization and introgression within wild populations may contribute to difficulties in classification of cultivated germplasm resources. Furthermore, McGregor's classification scheme is of no use for incomplete or powdered commercial *Echinacea* plant materials, and misidentification of *Echinacea* materials is still widespread in agricultural, horticultural, herbal and pharmacological industries.

Varying by region, common names for *Echinacea* include: purple coneflower, snakeroot, black sampson, Indian head, black susans and hedgehog (Kindscher 1989).

Phytochemistry

Plants in the genus *Echinacea* contain several classes of secondary metabolites. In order of increasing polarity, they are: polyynes, alkamides, phenolics including caffeic acid derivatives and phenylpropanoid glycosides, polysaccharides and glycoproteins (Bauer and Wagner 1991; Bauer 1998). Nineteen alkamides have been isolated from six of the nine putative species of *Echinacea* recognized by McGregor (1968) (Bauer *et al.* 1988a; 1988b; Bauer and Remiger 1989; Bauer and Foster 1991; Bauer *et al.* 1990). In 1967, one alkamide was isolated from *Echinacea* roots and reported with the name "Echinacein", an insect toxin with anaesthetic properties (Jacobson 1967). The structure of "Echinacein" was never again obtained from any compounds isolated from *Echinacea*, so it was suggested to be a misidentification of the major alkamides, dodeca-2E, 4Z, 8E/Z, 10E/Z-tetraenoic acid isobutylamides (Fig. 5.1)(Bauer and Wagner 1991). Extracts containing *Echinacea* alkamides have demonstrated pharmacological activity (Bauer and Wagner 1991), which makes them potentially suitable marker compounds for chemotaxonomic purposes and for the standardization of *Echinacea* phytomedicines.

Schulte *et al.* (1967) as well as Bohlmann and Hoffman (1983) identified several polyynes in *E. purpurea*, *E. pallida* and *E. angustifolia*. Polyynes, also called polyacetylenes, are typically found in the Asteraceae (Bohlmann *et al.* 1973), and many possess phototoxic activities (Camm *et al.* 1975; Guillet 1997). Alkamides and polyynes are both synthesized biochemically in plants from the long chain, unsaturated fatty acid, oleic acid (Bohlmann 1973). The pharmacological activity of polyynes from *Echinacea* has not been previously investigated.

Phenolics such as caffeic acid derivatives are synthesized biochemically from the intermediates of the shikimic acid pathway in plants (Dewick 1997). *Echinacea* species were reported to contain 12 caffeic acid derivatives (Cheminat *et al.* 1988). Some of these have a tartaric acid backbone, while others are based on a quinic acid structure. Quinic acid forms directly from phosphoenol pyruvate, a key molecule in glycolysis. Variation in concentration and type of the above was reported within some of the putative *Echinacea* species and the different plant organs (Bauer 1998). However, phenolics have not been used chemotaxonomically, except for the characteristic lack of echinacoside in *E. purpurea* (Cheminat *et al.* 1988). Echinacoside and other phenylpropanoid glycosides, such as rutoside and verbascoside, are synthesized in *Echinacea* by esterification reactions that bind shikimic pathway intermediates to sugar residues such as glucose (Dewick 1997).

Phytochemical characterization of *E. purpurea*, *E. pallida* and *E. angustifolia* has been important for safe and ethical development of authentic commercial medicines. In addition, chemotaxonomic information to distinguish between the three commercial species is useful for the standardization of phytomedicines for clinical trials (Bauer 1998). However, four of the McGregor's (1968) putative taxa remain unreported in the phytochemical and pharmacological literature. To date, there is only one reported multivariate phytochemical analysis of a small sample of select, putative *Echinacea* species, and it was based on an uncommon analytical method (Gas chromatography-Mass spectrometry) (Lienert *et al.* 1998).

Pharmacological and Clinical Evidence

Eclectic medical practitioners, King and Newton (1852) and Lloyd (1897), adopted the use of *Echinacea* as a phytomedicine from the Native Americans, but it never became an acceptable pharmaceutical preparation in North America (Hobbs 1989). In Europe, however, the expressed juice of *E. purpurea* aerial parts was commercialized as Echinacin®, which led to the first clinical trials by Madaus in the 1930's (Hobbs 1989). Echinacin® possessed hyaluronidase-inhibiting activity equivalent to that of 1 mg cortisone (Koch and Haase 1952). The proposed anti-hyaluronidase target site was a direct interaction with hyaluronic acid (Bonadeo *et al.* 1971). Indirect immunomodulatory effects were also first observed with Echinacin® (Von Unruh 1915). Preparations including combinations of *Echinacea* roots and tops, alone and with other plant species, were shown to increase phagocytosis among rodent and human leucocytes (Luettig *et al.* 1989; Proksch and Wagner 1987; Roesler *et al.* 1991a; 1991b). Bauer and associated researchers have conducted most of the phytochemical investigations of the genus to date, and they have found differences between extracts of *Echinacea* species with respect to immunostimulatory activity (Bauer *et al.* 1988c; Bauer and Wagner 1991). The rate of phagocytosis was increased three-fold by *E. purpurea* and two-fold by both *E. pallida* and *E. angustifolia* crude extracts, with more immunostimulant activity from the alkamide fractions than from the hydrophilic fractions. However, one hydrophilic compound, cichoric acid, also demonstrated immunostimulant activities (Bauer 1998) and has been isolated in varying amounts from the roots and inflorescences of all commercial *Echinacea* species. Echinacoside demonstrated very weak antibacterial activity (Beuscher *et al.* 1989).

In search of the active components of commercial *Echinacea* drugs, pure phytochemicals have been studied using cellular and animal models. From aerial parts or tissue culture of *E. purpurea*, polysaccharides have been isolated and shown to increase interferon, interleukin-1, and TNFα, and cellular immunity in mouse macrophages (Proksch and Wagner 1987), as well as phagocytosis of human leucocytes *in vitro* (Roesler *et al.* 1991b). Anti-inflammatory activity was reported for the major *Echinacea* alkamide, tetraene which inhibits cyclooxygenase at 50 mg/mL by 54.7%, and 5-lipooxygenase to a similar extent (Muller-Jakic *et al.* 1994). Similar effects were also reported with isolated polysaccharides from *E. angustifolia* roots and the *E. purpurea* preparation, Echinacin® (Tragni *et al.* 1988).

Now research has moved to investigation of cytokine induction in human and or animal cells or *in vivo*, as well as the use of DNA array technologies. For example, Burger *et al.* (1997) incubated human macrophages with *E. purpurea* fresh-pressed juice, and found the induction of the following cytokines was comparable to that produced by lipopolysaccharides (LPS): TNF-α, IL-1, IL-10 and minimally with IL-6.

Many clinical trials show significant immunostimulatory activity of *E. purpurea* pressed juice and hydroalcoholic *E. pallida* root extracts against viral and bacterial infections, but only a few are controlled, double-blinded and compared to a placebo (Brinkeborn *et al.* 1999; Bukovsky *et al.* 1993a; Bukovsky *et al.* 1993b; Scaglione and Lund 1995). Clinical effectiveness of *Echinacea* preparations has been measured by infection frequency, rate of recovery and reduction of disease symptoms, and more objectively, blood sampling followed by *in vitro* leucocyte counts. Only one clinical study indicated no significant differences in either leucocyte, or cytokine production after oral *Echinacea* treatment (Elsasser-Beile *et al.* 1996). There were several uncontrolled studies of *Echinacea*'s ability to inhibit *Candida albicans* (Coeugniet and Kühnast 1986), arthritis, and skin diseases (Tubaro *et al.* 1987). The clinical literature is plagued by non-standardized preparations used in inconsistent procedures and misidentification of certain *Echinacea* plants.

Rationale for Study

The following proposed revision of the genus *Echinacea* is aimed at correction of the confusion in the literature over taxonomic species identity (Foster 1991; Hobbs 1989; Bauer and Wagner 1991; Bauer 1998). Neither McGregor's (1968), nor Cronquist's (1945; 1980) *Echinacea* taxonomic treatments have been assessed using numerical taxonomic methods. Moreover, concurrent evaluation of phytochemical characters may identify chemotaxonomic markers to improve quality and species assurance in *Echinacea* germplasm. High-yielding chemotypes may be discovered, which would contribute to cultivated germplasm improvement for phytomedicine, and help to discourage unsustainable wild population harvesting. Also, a demonstration of chemotaxonomic marker stability and the capacity to induce phytochemical production through the use of methyl jasmonate has implications in the cultivation of high-yielding *Echinacea* crops. Finally, since *Echinacea* phytomedicines are known to be immunostimulants with some additional topical antimicrobial activity, investigations of phytochemically-distinct extracts from revised *Echinacea* taxa for UV light-mediated antifungal and antiviral activity has pharmacological applications. As one of the top three phytomedicines in North America and Europe (Brevoort 1998), *Echinacea* demands authentication, standardization and conservation of wild germplasm.

Hypotheses and Objectives

Hypothesis 1

Morphological variation within and between populations of *Echinacea* could be measured quantitatively and assessed by numerical taxonomy to classify and describe natural taxa in the genus.

Null Hypothesis 1

There is no difference between the taxonomy of the genus *Echinacea* (Asteraceae) as reported by McGregor (1968) and the results of the current infrageneric morphometric study.

Objective 1

Complete a modern morphological taxonomic revision of the genus *Echinacea* (Asteraceae) using numerical taxonomy, cladistics and nomenclatural investigations, and assess differences among existing taxonomic treatments, including the results of this study.

Hypothesis 2

Average phytochemical profiles of all naturally-occuring species and varieties of *Echinacea* have never been reported, but could be used to determine chemotaxonomic marker compounds for the taxa. As well, multivariate analysis of the quantitative phytochemicals within and between populations of the genus *Echinacea* could be used to support the infrageneric taxonomic groups as determined by Objective 1 (see above), and to elucidate the presence of chemotypes within populations of a single variety.

Null Hypothesis 2.1

The phytochemical profiles of plants from all putative natural taxa of the genus *Echinacea* do not exhibit qualitative differences that would allow the use of chemotaxonomic marker compounds for botanical identification.

Objective 2.1

Describe the average phytochemical content in plants from natural populations of each revised morphological *Echinacea* taxon (see Objective 1) and examine qualitative differences for potential chemotaxonomic marker compounds at the species-level and/or subspecific categories.

Null Hypothesis 2.2

There is no significant difference between each infrageneric and infraspecific taxon in the genus *Echinacea* according to phytochemical variation.

Objective 2.2

Determine whether there is support for the morphometric classification by quantitative phytochemical differences between individuals and populations of *Echinacea* that are each flagged according to revised morpho-taxonomic groupings.

Null Hypothesis 2.3

There is no significant phytochemical resemblance between populations of *E. angustifolia* DC. and the variation of single phytochemicals does not correspond with variations in latitude of the germplasm source.

Objective 2.3

Determine overall phytochemical resemblance between populations of *E. angustifolia* DC. and investigate 1) the potential for chemotypes within and among populations using multivariate analysis, and 2) the corresponding effect of latitude on phytochemical content.

Hypothesis 3

Methyl jasmonate is known to affect the biochemical production of secondary phytochemicals in plants. Spraying *E. pallida* (Nutt.) Nutt. plants with methyl jasmonate might change the concentrations and/or types of phytochemicals in their roots compared to those of untreated plants.

Null Hypothesis 3

There is no significant difference in root phytochemistry between the methyl jasmonate-treated and untreated *E. pallida* plants.

Objective 3

Assess the phytochemical profiles and quantitative differences in root extracts of methyl jasmonate-treated and untreated *E. pallida* plants grown from seed in a greenhouse.

Hypothesis 4

Echinacea extracts and some pure *Echinacea* compounds are known to inhibit the growth of *Candida* spp. If tested against *Candida* spp., as well as other fungal pathogens and the *Herpes simplex* virus Type 1 (HSV-1), *Echinacea* extracts may possess antimicrobial activities which 4.1) are enhanced by the addition of UV light and 4.2) differ among *Echinacea* taxa as a reflection of chemotaxonomic variation.

Null Hypothesis 4.1

The light-mediated antimicrobial activity of *Echinacea* extracts is not significant compared to dark controls and to the positive antifungal and antiviral control compound, alpha-terthienyl.

Null Hypothesis 4.2

There are no significant differences between the antimicrobial activities of different *Echinacea* taxa, or between extracts of roots and inflorescences.

Objective 4

Measure the light-mediated biological activity of *Echinacea* root and inflorescence extracts from most infrageneric taxa against clinical fungal and viral pathogens *in vitro*.

SECTION 1

TAXONOMY AND NOMENCLATURE OF *ECHINACEA*

The three chapters of this section have been prepared as manuscripts and submitted for publication in Systematic Botany (chapter 2) and Taxon (chapters 3&4, 2001). As a result of the taxonomic treatment following the morphometric analysis in chapter 2, the work in the following chapters was carried out to meet the need for typification and conservation of the most widely used name in the genus, *E. purpurea* (L.) Moench. We are very grateful to J. McNeill for his nomenclatural and editorial comments on the manuscripts. Many thanks are also extended to the following helpful people for their efforts to locate type material: Charlie Jarvis (BM) and Roy Vickery (BM), David Hewitt (PH) and Rusty Russell (US).

CHAPTER 2: A TAXONOMIC REVISION OF THE GENUS *ECHINACEA* (HELIANTHEAE: ASTERACEAE)

Introduction

Echinacea Moench (Asteraceae) is a native North American plant genus with a long history of use as phytomedicine in both the Indigenous and European traditions (Shemluck 1982; Bauer 1998). Many natural *Echinacea* populations are reportedly threatened by over-harvesting and anthropogenic modifications over a wide range of micro-habitats (Kindscher 1989; Foster 1991). Severe declines in populations of *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake and *E. tennesseensis* (Beadle) Small has resulted in their protection as endangered species (U. S. Fish and Wildlife Service). *Echinacea* species are cultivated in North America, and globally, for commercial use in leading therapeutic herbal products (Brevoort 1998).

Since DeCandolle's description of four *Echinacea* species in 1836, *Echinacea*'s infrageneric taxonomic history has been unsettled. The only classification of wild-occurring taxa based on repeated, widespread sampling is that of McGregor (1968) who described nine species and four varieties using morphological characters and chromosome numbers. However, McGregor (1968) did not employ any numerical or statistical methodology to evaluate the differences between his taxa, and there are inconsistencies between his descriptions and practical difficulties with his keys. Furthermore, during our recent typification of McGregor's species, we discovered that the holotype of *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake was synonymous with *Rudbeckia purpurea* L.(= *E. purpurea* (L.) Moench), among other nomenclatural inconsistencies (chapter 3; Binns *et al.* 2001 (b)).

As a result of poor taxonomic identification, many wild-harvested samples used for medicine and research prior to 1988 were adulterated with *Parthenium integrifolium* L., and cultivated *E. pallida* (Nutt.) Nutt. was being sold under the name of *E. angustifolia*

DC. in Europe (Bauer and Wagner 1991). However, the phytochemical literature post-1988 includes accurate profiles of the three commercial species (Bauer *et al.* 1990; Bauer and Foster 1991; Bauer and Wagner 1991) as well as preliminary phytochemical reports of some of McGregor's other taxa : *E. simulata* McGregor, *E. paradoxa* J. B. Norton and *E. tennesseensis* (Bauer *et al.* 1990; Bauer and Foster 1991).

Recently, infrageneric taxonomic studies were based on incomplete sampling of variation throughout the genus. For example, Urbatsch and Jansen (1995) performed a cladistic analysis using similarity in chloroplast DNA sequences from a few representative specimens of some (not all) *Echinacea* species. Another example of an incomplete assessment of species' differences is a multivariate chemotaxonomic classification of only three putative species from 10 populations (Lienert *et al.* 1998).

The present study undertook to elucidate the natural taxonomic groups and patterns of variation by morphometric assessment of the genus *Echinacea* using vouchers and live specimens. This study provides simple tools for accurate botanical identification which will help to improve safety and quality in phytomedicines.

Materials and Methods

Specimen Collection

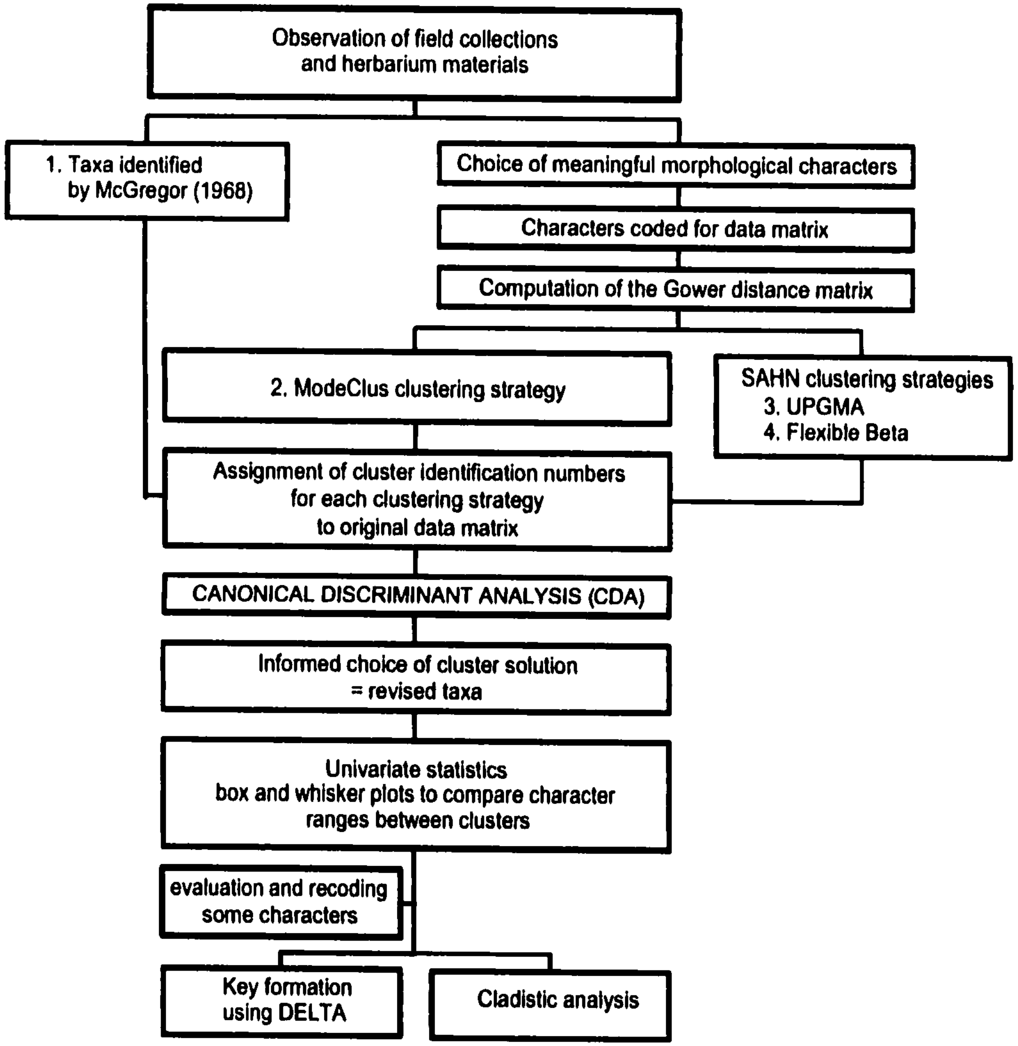
Throughout the geographical range of each putative *Echinacea* species, 95 natural populations were sampled during fall and/or early summer (1997 to 1999) and tentatively identified *sensu* McGregor (1968)(Appendix 1). One to three plants were harvested at each site, as well as a larger number of flowering capitula and seed-heads (in the fall). The aerial parts were pressed and dried, rootstocks were transplanted to a greenhouse, and achenes were harvested from seed-heads and stored (<4°C) until germination at a later date. Voucher specimens were deposited at the Department of Agriculture Herbarium, Ottawa, Canada (acronym DAO in Holmgren *et al.* 1990).

Live specimens were maintained at Agriculture and Agri-Food Canada in Ottawa, Canada. They were grown in a greenhouse at 25-30^{0}C, in a medium of 2 parts soil: 1 part beach sand in large gallon pots, with 16 hr daylight, including natural and cool white lighting (25 $\mu M/m^2/s$). Seeds were sterilized and stratified at 4°C (5 $\mu M/m^2/s$, incandescent) for 14 days, prior to potting in 5:5:2 Promix: vermiculite: quartz sand (Industrial grade #10, 4 mm particles). Developmental variation between accessions required that some plants be induced to flower with a cold treatment at 4°C (20 $\mu M/m^2/s$, incandescent) for 14 days. All plants were fertilized with 20:20:20 N:P:K weekly and watered equal amounts daily, according to age.

Some plant material (transplants and germplasm) was obtained from commercial growers in the U.S. and Canada. In addition, vouchers, including type specimens, were borrowed from contributing herbaria for the analysis. The following herbarium acronyms are referenced in Holmgren *et al.* (1990); The British Museum [BM]; Department of Agriculture Ottawa [DAO]; Harvard University Herbarium [HUH]; University of Kansas Herbarium [KANU]; New York Botanical Garden [NY]; Academy of Natural Sciences of

Philadelphia [PH] and The United States National Herbarium [US].

Figure. 2.1. A step-wise procedure for the morphometric taxonomic revision of *Echinacea* Moench below the level of genus, and cladistic assessment of the relationships between revised taxa.


Observation of field collections and herbarium materials
1. Taxa identified by McGregor (1968)
Choice of meaningful morphological characters
Characters coded for data matrix
Computation of the Gower distance matrix
2. ModeClus clustering strategy
SAHN clustering strategies
3. UPGMA
4. Flexible Beta
Assignment of cluster identification numbers for each clustering strategy to original data matrix
CANONICAL DISCRIMINANT ANALYSIS (CDA)
Informed choice of cluster solution = revised taxa
Univariate statistics
box and whisker plots to compare character ranges between clusters
evaluation and recoding some characters
Key formation using DELTA
Cladistic analysis

Morphological Data and Analyses

An overview of methodological progression to classify and assess groups in the genus *Echinacea* is presented in Figure 2.1. Morphological data for single plants were collected by observation of live transplants and specimens grown from seed accessions in the greenhouse, as well as 103 herbarium specimens. Some dry organs from herbarium specimens were carefully rehydrated in gently boiling water prior to observation. Fresh plant organs were harvested for morphological analyses once inflorescences reached mature bloom stage (>75% anthesis).

After a preliminary survey of a representative sub-sample of fresh and dried specimens, 81 morphological characters (Table 2.1) were chosen as meaningful estimates of the resemblance between individuals. Classic characters were included, such as leaf length and width, and pollen colour, as well as microscopic characters such as stem trichome type and density which were observed with the use of a dissecting microscope (Stereomiscroscope IV Carl Zeiss; 16-40X). I omitted characters that varied within organs on a single plant, as well as those that did not vary measurably among populations at all. Measurements for the 81 quantitative, binary, and multistate characters from 321 observational taxonomic units (OTUs) were recorded numerically in a matrix (available from the author).

Table 2.1:Characters and the scored character states for *Echinacea* Moench specimens. The characters common to the 2-cluster and 4-cluster solutions which were kept for canonical discriminant analysis are here followed by a *. Two additional characters in canonical analysis of the 4-cluster solution are followed by **.

T2.1 Character	Character states (and codes)
FLOWER HEAD	
1*. Height	cm
2*. Width	cm
3. Shape	conical(0), hemispherical(1), flattened (2), elongated(3)
DISCOID BRACTS	
4. Length	cm
5. Habit	straight(0), incurved(1), both(2)
6. Tip colour	orange and red(0), brown-purple(1), green red(2), white or gold(3), two types on one plant(4)
7. Keel	present(0), absent(1), ornamentations only(2)
8. Keel vestiture	acuminate(0), absent(1), sessile/papillate(2), acuminate and sessile(3)
RAY FLORET	
9*. Length	cm
10*. Width	cm
11. Fertility	infertile(0), vestigial style and/or anthers(1), fertile(2)
12. Habit	perpendicular to stem(0), reflexed parallel to stem(1), drooping 45^0(2), recurved with tips touching stem(3), upcurved(4)
13. Colour	pale pink(0), medium pink(1), dark pink(2), white(3), yellow(4)

T2.1 Character	Character states (and codes)
14. Multicellular trichome location	abaxial veins(0), abaxial veins and adaxial base(1), entire abaxial surface(2), glabrous(3), mixture on same capitulum(4), entire abaxial surface with sessile hairs also(5)
RAY PAPPUS	
15. Major tips	three(0), one(1), none(2)
16. Margin	entire(0), undulate(1), uneven undulate(2), toothed(3), uneven toothed(4)
17. Vestiture	glabrous(0), sessile lateral(1), stalked lateral(and on margin)(2), mix(3), dense stalked and sessile(4)
DISK FLORET	
18. Style habit	recurved(0), spreading(1)
19*. Style branch	less than 1/4 total style length(0), 1/4 to ½ total style length(1)
20. Style colour	white and purple(0), brownish-purple(1), yellow(2), brownish black(3)
21. Style length	<bracts(0), =bracts(1), >bracts(2)
22. Corolla vestiture	glabrous(0), base hairy(1), tips/fusedpetals hairy(2), mix(3)
23. Corolla colour	green-pink(0) purple(1) yellow/white tips(2) orange/other(3)
24. Corolla tip length	< 3/4 total corolla length(0) > 3/4 total corolla length (1)
25. Corolla tip habit	straight(0), reflexed(1)
DISK PAPPUS	
26*. Major tips	four(0), one(1), none(2), two on the acute angles(3)
27. Margin	entire(0), evenly lobed(1), unevenly lobed(2), evenly toothed(3), unevenly toothed(4)
28. Vestiture	glabrous(0), sessile lateral hair(1), stalked lateral hair (and on

T2.1 Character	Character states (and codes)
	margin)(2)
ACHENES	
29*. Length	cm
30**. Vertical striation	present(0), absent(1)
31. Horizontal striation	absent(0), dark brown(1), red/orange band(2), brown and red(3)
INVOLUCRAL BRACTS	
32. Habit	reflexed(0), recurved with tips out(1), perpendicular(2)
33. Abaxial stalked trichomes	present(0), absent(1), absent with sessile hair(2), present with sessile hair(3)
34. Adaxial stalked trichomes	present(0), absent(1), absent with sessile hair(2), present with sessile hair(3)
35. Length	cm
36. Width	cm
37. Style base	disk-shaped(0), ball-shaped(1), other(2)
38. Pollen colour	yellow(0), white(1), lemon (2), both on same inflorescence (3)
39. Series in involucre	number
LEAF TRICHOMES	
Stalked Type on Blade	
40*. Type	bicellular(0), multicellular(1), absent(2), both(3)
41. Length	short(0), medium(1), long (2), absent(3)
42*. Surface ornementation	absent (0), sparse/distal(1), dense(2)
43. Stalk cell	cylindrical(0), bulbous(1), absent(2)
44. Basal globules	absent(0), one ring(1), two rings(2), uneven(3)

T2.1 Character	Character states (and codes)
45. Joints	smooth(0), ledge-like(1), knobby(2), absent(3), variable(4)
46. Habit	strigose(0), hirsute(1), straight(2), absent(3)
47*. Density	dense(0), sparse(1), glabrous(2)
48*. Distribution	even(0), more adaxial(1), more abaxial(2)
Stalked Type on Margin	
49. Habit	more appressed than blade type(0) identical to blade type (1) very different from blade type(2)
Sessile Type	
50. Presence	present(0) absent(1)
LEAF LAMINAE	[BL=Basal CL=Cauline]
51*. BL Length	cm
52*. BL Width	cm
53*. BL Petiole	cm
54*. CL Length	cm
55*. CL Width	cm
56*. CL Petiole	cm
57. BL shape	linear(0) linear/lanceolate(1) lanceolate(2) lance-ovate(3), ovate(4) narrowly elliptical(5)
58. CL shape	linear(0) linear/lanceolate(1) lanceolate(2) lance-ovate(3), ovate(4) narrowly elliptical(5)
59. BL margin	entire(0) serrate(1) dentate(2) lobed(3)
60. CL margin	entire(0) serrate(1) dentate(2) lobed(3), lobed+entire(4)
61. Colour	dark green(0), green(1), pale/yellow(2)
62*. Vein type	branching(0) almost parallel(1) intermediate with some

T2.1 Character	Character states (and codes)
	branching(2)
63. Vein number	three(0) five or one(1) mixed (five below, three above)(2)
64. Vein colour	clear(0) white(1) yellow(2) darkened(3)
Table 2.1 continued	
STEM	
65*. Height	cm
66*. Width	even(0) uneven(1)
67. Colour	green(0), brown striations on green(1), white striations on green(2) reddish-green(3)
68*. Habit	unbranched(0), branched(1)
69. Type	furrowed(0), non-furrowed(1), furrowed below involucre(2)
70. Pith	present(0), absent(1), absent in peduncle(2)
71. Peduncle	≤ 1/2 stem height(0), >1/2 stem height (1)
ROOT	
72. Habit	fibrous (0) fusiform taproot with thick laterals (1), one taproot with minimum branching(2)
STEM TRICHOMES	
Stalked Type	
73*. Type	bicellular (0) multicellular (1) absent(2) both(3)
74*. Length	short(0), medium(1), long (2), absent(3)
75*. Surface ornamentation	absent(0), sparse/distal(1), dense(2)
76**. Stalk cell	cylindrical(0), bulbous(1), absent(2)
77. Basal globules	absent(0), one ring(1), two rings(2), uneven(3)
78. Joints	smooth(0), ledge-like(1), knobby(2), absent(3), mixture(4)

T2.1 Character	Character states (and codes)
79. Habit	strigose(0), hirsute(1), straight(2), absent(3),mixture(4)
80*. Distribution	entire stem(0), top half of stem(1), few inches under involucre(2), bottom half of stem(3), glabrous(4)
Sessile Type	
81. Presence	present(0) absent(1)

Character explanations

Characters and their codes are listed in Table 2.1, however certain characters require concise descriptions to clarify their coding in this work. They are as follows:

DISCOID BRACT (HABIT), NO. 5. Habit refers to the awn tip only, not the spinal curvature of body of the bract.

KEEL (PRESENCE), NO. 7. The keel is a fleshy structure on the abaxial side of the vein or spine of the bract.

KEEL (VESTITURE), NO. 8. Keel hairs are uni- or multi-cellular trichomes and/or small glandular ornementations on the spine of bract (which may or may not be a keel).

RAY (HABIT), NO. 12. The ray habit terms refer to the following: reflexed means between 30° and 45° from the vertical stem, drooping is dangling parallel to stem, recurved means an outward arc with only the tip touching the stem, and upcurved is shallowly cuplike.

PAPPUS (MAJOR TIPS), NOS. 15 and 26. The pappus tips are sepal tissues which extend above the top corners of the fruit. Dorsi/ventral flattening in a four-sided disk achene can result in 2 major pappus tips. Pappus is often reported in other genera of the Asteraceae as modified sepals or bristles, but here we report any sepal trichomes or dissections as pappus vestiture (see nos. 17 and 28).

PAPPUS (MARGINS), NOS. 16 and 27. Margin refers to the top edge of all sepals, between the major tips. Smooth-edged lobes form a pattern called undulate, while jagged or pointed lobes were called toothed (the regularity of the margin determined whether it was coded as equal or unequal).

PAPPUS (VESTITURE), NOS. 17 and 28. Vestiture refers to either trichomes or sessile glandular ornaments on major tips or margins, and/or directly from the angled corners of the achenes.

ACHENES, NO. 30. The dried fruits exhibit different coloration than fresh, immature fruits. Striations are a pattern of vertical marks on exocarp (white and black) NO. 31. The horizontal striations refer to a band at crown of calyx/exocarp (brownish-black or reddish-brown).

INVOLUCRE (HABIT), NO. 32. Recurved looks like a sideways letter S. The habit is either spread or reflexed parallel to the stem.

STALKED TRICHOME (LENGTH), NOS. 41 and 74. Short trichomes are up to 1/4 the width of the stem, medium are up to the width of the stem, and long trichomes are greater than the width of the stem.

STALKED TRICHOME (SURFACE ORNAMENTATION), NOS. 42 and 75. The surface of each stalked trichome is often patterned by striations or lumps that vary in density.

Similarity and Clustering

Gower's coefficient of similarity (Gower 1971) was computed between each pair of OTUs in the original matrix, giving a 321 X 321 similarity matrix using SAS ver 8.1 (SAS Institute Inc. 1997). Prior to computation of the Gower similarity matrix, the total percentage of missing values was determined to be 5.3%, so those characters with the majority of missing data were omitted, as well as those demonstrating extensive phenotypic plasticity between members of the same population. As a result, the total amount of missing data was reduced to 4.0% and the following 74 characters were used to compute the Gower similarity matrix; 1-5, 7-14, 18-21, 23-69, 72-81 (Table 2.1). This suite of characters included binary, quantitative, semi-quantitative and qualitative types (Appendix 6), since Gower's coefficient of similarity will account for missing values and simultaneously include weighted or unweighted, binary, alternative, qualitative, quantitative and semi-quantitative characters (Sneath and Sokal 1973). These advantages, while not all acceptable by any other similarity coefficient, allow the use of a very broad range of data to determine the degree of similarity for each pair of OTUs. A complete discussion and evaluation of the Gower coefficient method can be found in St-Laurent *et al.* (2000).

The distance matrix, obtained from the complement of Gower's similarity matrix (1-similarity), was subjected to three types of clustering methods to determine groups of OTUs.

Cluster Method 1

The distance matrix was subjected to a non-parametric, non-hierarchical cluster analysis by the SAS procedure MODECLUS SAS ver 8.1 (SAS Institute Inc. 1997). MODECLUS has the advantage of clustering without assumptions of data distribution, and it eliminates bias found in other clustering procedures such as: equal-sized clusters (i.e. number of OTUs), equal-shaped clusters, equal variance or dispersion. Many other clustering methods are biased in terms of size and shape being equal between clusters (e.g. single linkage, average-linkage, k-means etc.) (St-Laurent *et al.* 2000).

Cluster Methods 2 and 3

Two SAHN clustering techniques were employed: flexible clustering and UPGMA, which are both available in the NT-SYS software package (ver. 2.02, Rohlf 1999). Flexible clustering (Lance and Williams 1967) facilitates intermediate distance clustering by using a smoothing parameter β to overcome the disadvantages of single and complete linkage methods. Also, a clustering solution by average linkages was calculated using a similar SAHN method, UPGMA (Sokal and Michener 1958; also in the NT-SYS package).

Method 4

Each OTU in the original data matrix (not the Gower distance matrix) was assigned a tentative taxonomic identification number which corresponded to McGregor's taxonomic treatment (1968).

Canonical Discriminant Analysis (CDA)

The strength of cluster solutions from each of the four methods described above was assessed in SAS using CDA (Kshirsagar 1972). CDA is one method that provides a rigorous assessment of OTU cluster assignments by describing the variation between clusters relative to the variation within clusters (Pimentel 1979). All possible solutions determined using the above-mentioned clustering strategies (excluding UPGMA, see RESULTS) were appended to the original data set as four separate columns indicating OTU cluster memberships. Missing data and non-quantitative characters in the data matrix were minimized because the CDA method relies on the assumption that characters are quantitative (Appendix 6). Furthermore, the characters exhibiting zero variance within a given cluster were removed to avoid creating singularities in the covariance matrices. However, due to many semi-quantitative characters in the current study, the distances between clusters in CDA were used as guides to assess the validity of different clustering solutions, without statistical inferences.

Choice of cluster solution

Guided by results of CDA, acceptable cluster solutions were chosen based on squared Mahalanobis distances between cluster centroids, and the graphical representation of the canonical vectors on the first three canonical axes (Reyment *et al.* 1984). In addition, the percentage of overall variation between clusters that was explained in the first three canonical axes was a good measure of a robust solution.

Univariate statistics were computed for all characters, and the means and ranges were compared between clusters for each of the solutions in adjacent Box and Whisker plots using SAS ver. 8.0 (SAS Institute 2000). These comparative simple statistics were used to construct dichotomous keys and descriptions by recoding characters for the DELTA program (DEscriptive Language for TAxonomy, Dallwitz 1974).

Cladistic analysis

Cladistic analysis was performed with PAUP* (Swofford 2000) for the *Echinacea* species-level taxa that resulted from the phenetic analysis (described above). Characters for cladistic analysis (Table 2.2) were selected either from the keys generated by the phenetic analysis, or from epi-generic classifications in the tribe Heliantheae (Stuessy 1977; Cox and Urbatsch 1990; Karis 1993), phytochemistry (Bohlmann *et al.* 1973) and floristic descriptions (Torrey and Gray 1841; Gray 1884; Cronquist 1980). Thirty-two binary characters were coded for all taxa (Table 2.3) and the outgroup *Rudbeckia* was coded as three sections or infrageneric groups (previously treated in Cox and Urbatsch 1990). Maximally parsimonious trees were calculated by the branch-and-bound method (Hendy and Penny 1982). Bootstrap values were computed and the resulting tree represented as the 50% majority-rule consensus tree.

Table 2.2. Characters used in the cladistic analysis of the relationships between four species within the genus *Echinacea* Moench, including outgroup comparison.

Characters	Character Codes
0. Basal leaf width	0= 5 cm wide or more, 1= less than 5 cm wide
1. Number of involucral bracts	0= 1 to 3, 1= 4
2. Leaf stalked trichomes <type>	0= bicellular, 1= multicellular
3. Leaf stalked trichomes <joints>	0= ledge-like, 1= knobby
4. Leaf major veins <size>	0= thin, 1= thick
5. Leaf major veins <type>	0= branched faintly, 1= parallel unbranched
6. Leaf adaxial surface <vestiture>	0= scabrous hairs, 1= glabrous
7. Stem stalked trichomes <presence>	0= glabrous, 1= hispid
8. Basal leaf <margin>	0= serrate or dentate, 1= entire
9. Cauline leaf <margin>	0= serrate, 1= entire
10. Stem stalked trichomes <habit>	0= appressed, 1= hirsute or straight pubescent
11. Leaf blade stalked trichomes <density>	0= sparse 1= dense
13. Leaf marginal stalked trichomes <type>	0= more appressed than blade trichomes, 1= identical to blade trichomes
14. Ligule <colour>	0= yellow, 1= white or pale pink, dark pink to purple
15. Ligule <length>	0= up to 4 cm long, 1= 4.01 cm long or more

Characters	Character Codes
16. Stem and peduncle	0= branched, multiple or corymbose, 1= unbranched, solitary or few heads
17. Involucral bract abaxial sessile trichomes	0= present, 1= absent
18. Basal leaf <base>	0= somewhat cordate, 1= decurrent on long petiole
19. Basal leaf <shape>	0= ovate (heart-shape), 1= lanceolate (lance-ovate)
20. Cauline leaf <shape>	0= ovate to lanceolate, 1= linear, reduced, elliptical or spatulate
21. Discoid bract <habit>	0= straight, 1= incurved or both
22. Discoid corolla <base>	0= bulbous, 1= straight
23. Discoid bract awn <tip shape>	0= acuminate, acute or obtuse, 1= cuspidate or mucronate
24. Discoid bract <length>	0= surpassing corolla tips, 1= shorter than corolla tips
25. Alkamide secondary phytochemicals	0= present, 1= absent
26. Anther appendages <abaxial glands>	0= present, 1= absent
27. Disk corolla <petal fusion>	0= more than 3/4 total length, 1= less than 3/4 total length
28. Polyacetylenes with S-containing rings	0= absent, 1= present
39. Sesquiterpene lactones	0= absent, 1= present

Characters	Character Codes
30. Discoid bract awn vestiture	0= distal and tomentose, 1= few proximal or absent
31. UV-light floral absorption pattern	0= whole inflorescence, 1= disk corolla and proximal ligule, 2= mixed pattern
32. Stem surface	0= not glaucous, 1= glaucous

Table 2.3. Data matrix for cladistic analysis of the relationships within the genus *Echinacea* Moench using three sections in the genus *Rudbeckia* L. as the outgroup. Numerical codes for binary, unordered characters in Table 2.2. Nine (9) represents inapplicable or missing values.

	Character numbers from Table 2.2					
	1-5	6-10	11-15	16-20	21-25	26-32
E. pallida	10111	10111	11110&1	0&11111	10000	10001 00&1
E. purpurea	01000	00100	10110&1	00000	0&10000	10001 00&1
E. laevigata	10111	11000	19011	01110	10000	10001 00
E. atrorubens	10111	10111	0000&10	00111	0&10000	11001 00&1
Rudbeckia:						
Sect. *Rudbeckia*	0&10990	0&100&10&10&1	99909	99999	11111	00110 10
Sect. *Macrocline*	10990	0100&10&1	99909	99999	110&111	00010 10
Sect. *Laciniata*	00990	0000&10&1	99909	99999	11111	00110 0&10

Results

Clustering Method 1 (MODECLUS)

The MODECLUS procedure yielded the number of clusters into which the OTUs were segregated at each value of a smoothing parameter called "K" (Fig.2.2)(SAS Institute Inc. 2000). There was stability for either two overall clusters (K=11-22,29-39) or three clusters (K=23-28).

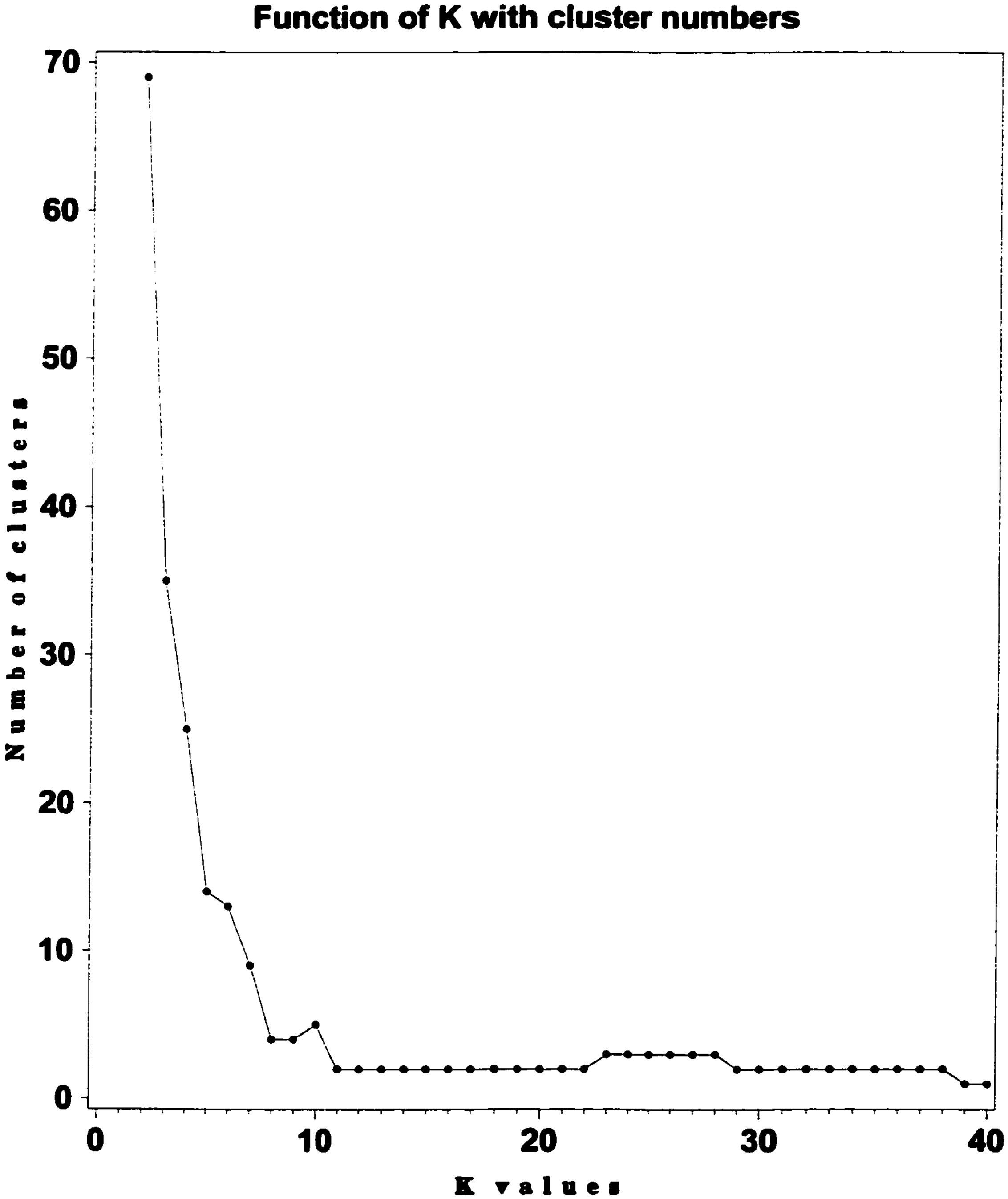
2-CLUSTER SOLUTION

For each value of K in MODECLUS where the outcome was two clusters (Fig. 2.2), one of the two clusters contained exclusively OTUs which were tentatively-labeled *E. purpurea* (sensu McGregor). The other cluster contained all other OTUs in the dataset. An evaluation of the robustness of the MODECLUS 2-cluster solution is included under Clustering Method 2.

3-CLUSTER SOLUTION

At each value of K for which three clusters were identified by MODECLUS (Fig. 2.2), all tentatively-labeled *E. purpurea* occupied cluster 1, and all other OTUs fell mainly into cluster 2, with a subset of 32 specimens in cluster 3. These 32 specimens were separated from their closest relatives (others in their own natural populations). Therefore, the MODECLUS 3-cluster solution was rejected, as guided by CDA where more variation was reported within certain populations than between populations.

Figure 2.2. Plot of the number of clusters into which *Echinacea* Moench specimens were placed according to overall similarity by the MODECLUS procedure (SAS Institute Inc. 2000) for values of the "K" smoothing parameter from 2 to 40.


Function of K with cluster numbers
70
60
50
40
30
20
10
0
0
10
20
30
40
K values
Number of clusters

Clustering Method 2 (flexible beta clustering)

Clustering of the 321 X 321 Gower distance matrix was explored using a range of values (-1 to 1) for the smoothing parameter β. All possible β values yielded a 2-cluster solution with the most distant group comprised entirely of specimens labeled either *E. purpurea* or *E. laevigata*. Distances between clusters which would normally be Euclidean distances (the complement of Gower's similarity coefficients) were represented here as scalar quantities by the Flexible Clustering algorithm and thus sometimes exceeded the value of 1 (Fig. 2.3). The preferred phenogram (β = -0.4)(Fig. 2.3) was determined by the following criteria: the evaluation of cluster assignments so that specimens from the same population clustered together, and agreement with the MODECLUS solution (see above). Frequent observations of hybrid swarms and intermediate plants in wild populations was taken into account, so the presence of some outliers was to be expected. Generally, preferred solutions placed potentially hybrid specimens in a separate cluster, or at least as single outliers.

Three phenon lines were distinguished in Figure 2.3 which identified two, three or four clusters. At the first phenon line, one cluster contained all *E. purpurea* specimens, and the second contained all other specimens. The next two phenon lines showed no change in the *E. purpurea* cluster, but the second cluster was divided into two and three branches, respectively.

From these three possible phenon lines, specimens in the original data matrix (321 X 74) were assigned to clusters in order to evaluate the strength of each cluster solution by CDA, and to measure the amount of support for each.

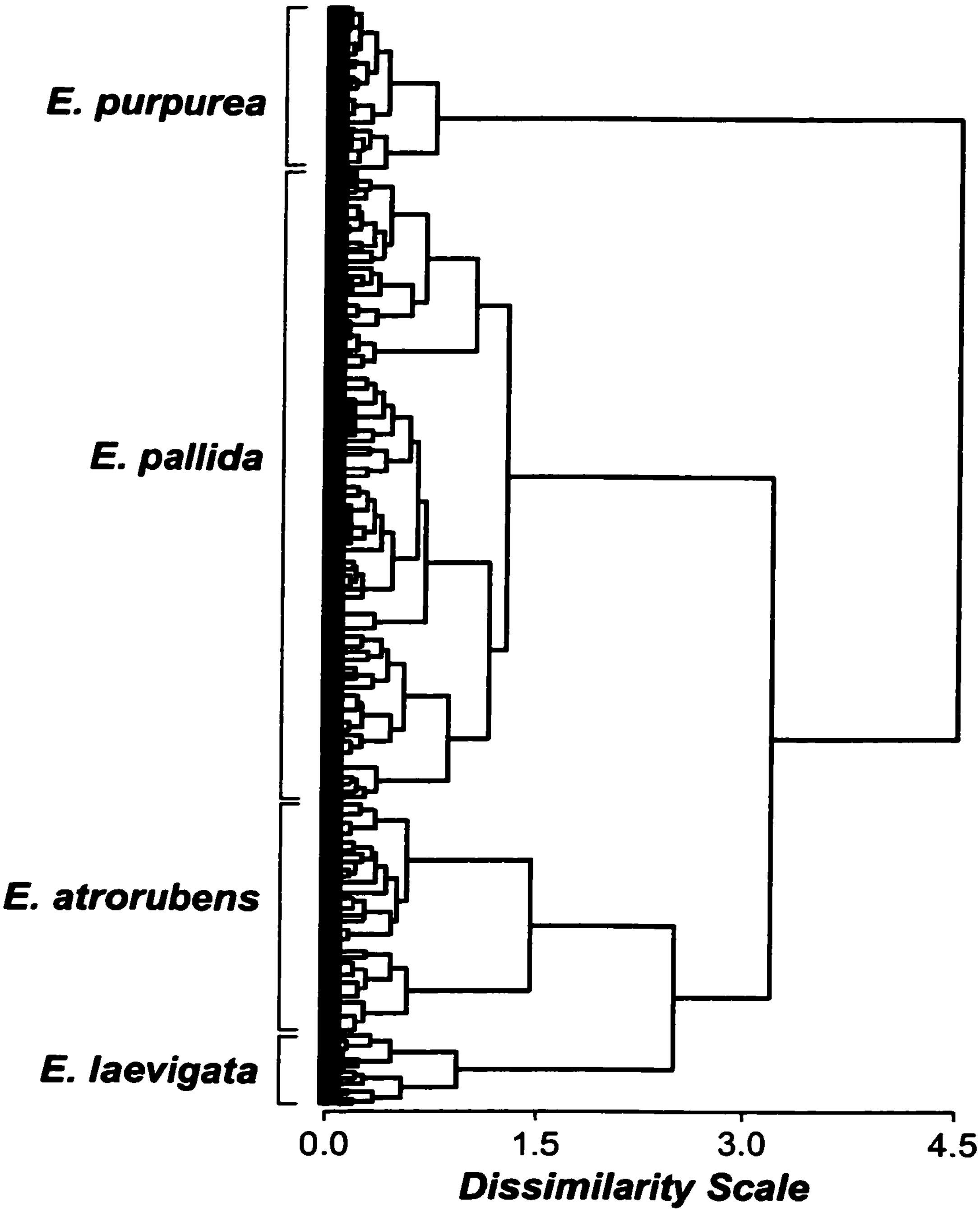
CDA OF THE 2-CLUSTER SOLUTION

The 2-cluster solution (same in MODECLUS and flexible clustering) was extremely well segregated by CDA using 267 OTUs and 25 characters (Table 2.1), as indicated by a squared Mahalanobis distance between the two cluster centroids of 43.15, which was used as a guide. Table 2.4 identifies the 15 characters that contributed significantly ($p<0.05$) to the canonical variables.

CDA OF THE 3-CLUSTER SOLUTION

There was little overlap in the CDA of three clusters determined by flexible clustering, using 267 OTUs and 25 characters (Table 2.1). However, two groups of plants which were morphologically distinct in field observations (here denoted *E. pallida* and *E. atrorubens*) and also easily distinguished in previous taxonomies, were treated as one cluster. Furthermore, at a barely smaller level of dissimilarity in the hierarchy, the third cluster was clearly delimited into those two distinct species groups to form a 4-cluster solution (to follow). Therefore, we accepted the 4-cluster solution over the 3-cluster solution.

Figure 2.3. Phenogram from flexible clustering of 321 *Echinacea* Moench specimens (SAHN technique, $\beta = -0.4$, NT-Sys software) using a dissimilarity matrix calculated from 74 characters (1- Gower's coefficient of similarity). A phenon line at approximately 2.2 units of dissimilarity revealed four major groups.


E. purpurea
E. pallida
E. atrorubens
E. laevigata
0.0
1.5
3.0
4.5
Dissimilarity Scale

CDA OF THE 4-CLUSTER SOLUTION

The canonical discriminant analysis of the 4-cluster solution was carried out using a subset of 267 OTUs and 25 characters (Table 2.1). As a guide to the relative distance between the four clusters, Figure 2.4 represents a dendrogram of the Squared Mahanalobis distances between each pair of clusters. The first canonical axis accounted for 66% of the variation between clusters, the second axis for 20%, and 14% in the third axis. Characters that contributed significantly to the 4-cluster solution are indicated in Table 2.4.

We removed cluster 1 (*E. purpurea*) in the next step (see Fig. 2.3), since it was the most distant from the rest in the 4-cluster solution, and because it rarely displayed character state overlaps with the other clusters (determined by comparison using Box and Whisker plots similar to Fig. 2.6, see also Appendix 2). This was done to ascertain the relative discreteness between the three remaining clusters.

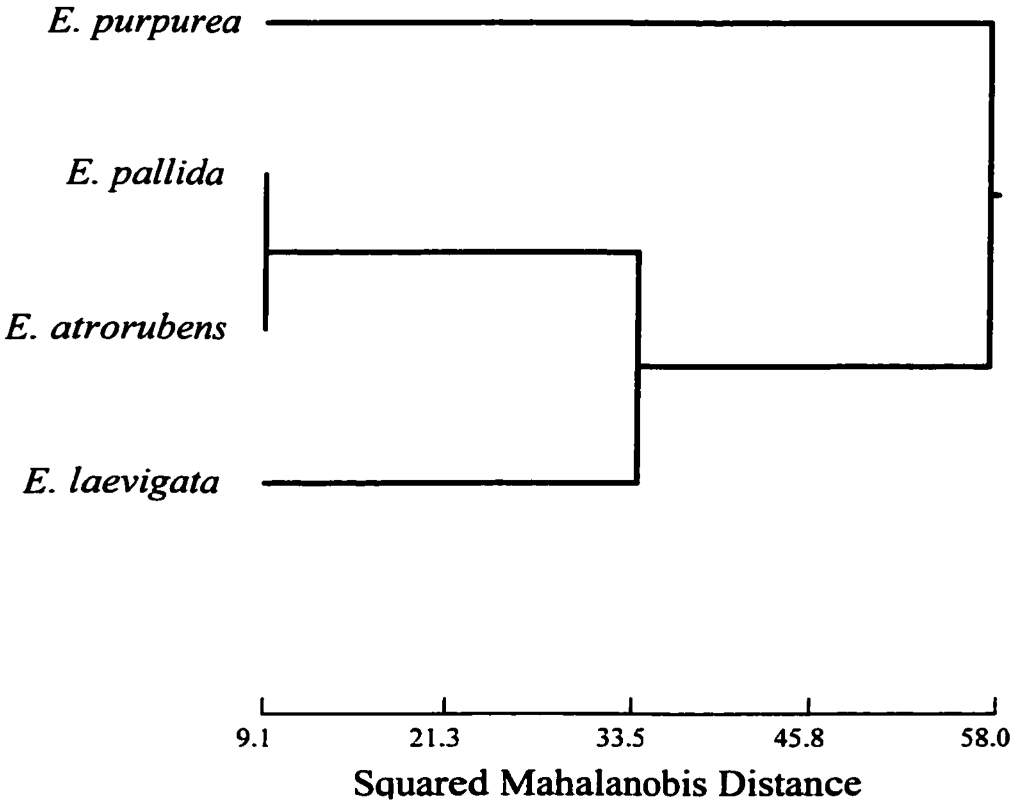
Table 2.4. Univariate F-statistics (F) and related probabilities (p) for each character in the canonical discriminant analyses (CDA) of the 2-cluster and 4-cluster solutions, as well as McGregor's classification. See Table 2.1 for character identification. [1] denotes the five most important characters for the discrimination of the 2-cluster solution. [2] denotes the five most important characters for the discrimination of the 4-cluster solution. [3] denotes the five most important characters for the discrimination of the McGregor classification. (.) denotes character removed from CDA for reasons of missing values or no variance.

T2.4 Character #	F 2-clus	p 2-clus	F 4-clus	p 4-clus	F McGregor	p McGregor
1	2.06	0.1528	11.27	0	4.3	0
2	9.44	0.002	5.22	0.002	.	.
4	.	.	.	.	4.46	0
5	.	.	.	.	5.91	0
9 [3]	1.94	0.1654	6.42	0	14.97	0
10 [1]	83.17	0	28.95	0	0.98	0.4655
12	.	.	.	.	6.55	0
19	2.24	0.136	2.82	0.0396	.	.
21	.	.	.	.	4.04	0
24	.	.	.	.	4.85	0
26	9.31	0.003	4.09	0.007	.	.
28	.	.	.	.	4.47	0

T2.4 Character #	F 2-clus	p 2-clus	F 4-clus	p 4-clus	F McGregor	p McGregor
29	3.4	0.0663	1.18	0.3174	.	.
36	.	.	.	.	3.47	0
38	.	.	.	.	7.16	0
39 [3]	.	.	.	.	13.57	0
40 [1,2]	107.47	0	68.42	0	4.86	0
41		.	.	.	7.85	0
42	42.35	0	25.9	0	.	.
43		.	.	.	7.42	0
47 [2]	3.12	0.0786	49.76	0	4.56	0
48 [3]	0.72	0.3984	43.26	0	16.46	0
49	.	.	.		2.1	0.0246
51	18.69	0	6.33	0	3.49	0
52 [1,2,3]	400.16	0	142.06	0	12.71	0
53 [1]	38.53	0	14.53	0	5.91	0
54	32.4	0	13.57	0	.	.
55 [1,2]	387.2	0	138.03	0	6.69	0
56	0.1322	0.7164	0.5	0.6818	.	.
58		.	.	.	5.43	0
62 [1,2]	288.14	0	99.97	0	.	.
65	7.7	0.006	3.52	0.0156	4.75	0

T2.4 Character #	F 2-clus	p 2-clus	F 4-clus	p 4-clus	F McGregor	p McGregor
66	0.63	0.4275	1.54	0.2039	.	.
68	27.93	0	9.74	0	4.72	0
72 [3]	.	.	.	.	22.05	0
73	46.17	0	25.24	0		
74 [2]	8.69	0.004	49.89	0	8.83	0
75	0.43	0.5107	17.79	0	5.07	0
76	.	.	.	.	7.4	0
79	.	.	.	.	7.41	0
80	1.94	0.1651	33.46	0	10.8	0

Figure 2.4. A dendrogram of the relative squared Mahalanobis distances between the centroids of four *Echinacea* Moench taxonomic clusters, identified according to overall morphological similarity (Fig. 2.3) and determined to be significantly distinct in Canonical Discriminant Analysis, resulting in a taxonomic re-classification to four species.


E. purpurea
E. pallida
E. atrorubens
E. laevigata
9.1
21.3
33.5
45.8
58.0
Squared Mahalanobis Distance

CDA OF THE THREE CLUSTERS IN THE 4-CLUSTER SOLUTION

With the removal of cluster 1, we found that the variation between the clusters 2, 3, and 4 was successfully explained using 227 OTUs and 27 characters (Table 2.1). The first and second canonical axes accounted for 62.3% and 37.7% of the variation between clusters, respectively. All squared Mahalanobis distances between the cluster centroids were slightly increased compared to the 4-cluster solution, which was expected with the removal of *E. purpurea*, the most discrete cluster. The most important character to distinguish between clusters 2, 3 and 4 was ray floret length (no. 9, Table 2.1), which was important (Table 2.4) to effectively cluster the current dataset using McGregor's classification (*Method 4* to follow).

Clustering Method 3 (UPGMA)

The resulting phenogram (results not shown) was very similar to the flexible clustering phenogram (Fig. 2.3). However, it is expected that at the very least, a cluster solution will result in individuals from the same population clustering together, and the most discrete cluster should be homogeneous (containing only specimens that have the same tentative identification). This was not the case in the UPGMA solution. Firstly, the most discrete cluster was not *E. purpurea* (as it was in other methods tested) but *E. laevigata*. Furthermore, the *E. laevigata* cluster, and two others, were unacceptable because they contained some individuals that were separated from their closest populational relatives. As the UPGMA method was therefore determined to be inappropriate here, it was not subjected to canonical discriminant analysis.

Method 4 -Using McGregor's Classification

The CDA of all 321 OTUs, coded according to our morphological characteristics and identified sensu McGregor's 11 taxa, i.e. clusters of the genus *Echinacea*, revealed fair support for his system of classification. In order to best meet the assumptions of CDA (quantitative characters, removal of characters with zero variance), it was performed with a sub-dataset of 138 OTUs and 31 characters (Table 2.4). The first three canonical axes described 63.1% of the total variation (axis 1 = 28%, axis 2 = 18.1%, axis 3 = 17%). The contribution of each of 31 characters to the discriminant analysis was statistically significant at $p<0.0001$, except for four of them (Table 2.4). Table 2.5 demonstrates the relative distances between centroids of clusters achieved by McGregor's classification, and it was used as a guide for discrimination along with the plot of canonical vectors for each OTU (results not shown). According to CDA results (as guides rather than absolute values), the following clusters (names are sensu McGregor) were less discretely delimited from one another; *E. pallida* and *E. simulata*, *E. paradoxa* var. *paradoxa* and *E. paradoxa* var. *neglecta*. There was complete overlap between clusters 2 and 7, which represented the two varieties of *E. angustifolia* DC, sensu McGregor (1968). Overall, CDA provided weak support for McGregor's taxonomy using our characters, compared to the CDA of the 4-cluster solution (flexible clustering). As a result, ten of McGregor's taxa were validated at the rank of varieties, but *E. angustifolia* DC. var. *strigosa* McGregor was reduced to a stable hybrid form of *E. angustifolia* DC. (see Taxonomic Treatment to follow).

In summary, three different cluster solutions were determined to be acceptable with the help of CDA, in order of relative strength: 2-cluster MODECLUS and flexible clustering (no overlaps), 4-cluster flexible clustering (almost no overlaps) and clustering by McGregor's classification (many overlaps).

Cladistic Analysis

Echinacea was a monophyletic clade according to 33 characters (Table 2.2) compared to the outgroup *Rudbeckia* (coded as three taxonomic sections in Table 2.3). Two most parsimonious cladograms were revealed, and one was preferred (Fig. 2.5) because it distinguished *R.* sect. *Macrocline* as the nearest outgroup to *Echinacea*, as did prior cladistic analyses (Cox and Urbatsch 1990). *Echinacea purpurea* was separated from the other three species by eight synapomorphies supported by a bootstrap value of 70%. *E. laevigata* shared two synapomorphies with the other two *Echinacea* species. At the most distal divergent step, *E. pallida* and *E. atrorubens* shared three synapomorphies (basal and cauline leaf shapes and margins).

Table 2.5. Squared Mahalanobis distances between cluster centroids from canonical discriminant analysis using the morphometric dataset with cluster assignments according to McGregor's taxa (1968). Cluster numbers correspond to McGregor's species and varieties: 1. *E. purpurea* (L.) Moench, 2. *E. angustifolia* DC. var. *angustifolia*, 3. *E. pallida* (Nutt.) Nutt., 4. *E. atrorubens* Nutt., 5. *E. paradoxa* (Norton) Britton var. *paradoxa*, 6. *E. paradoxa* (Norton) Britton var. *neglecta* McGregor, 7. *E. angustifolia* DC. var. *strigosa* McGregor, 8. *E. sanguinea* Nutt., 9. *E. tennesseensis* (Beadle) Small, 10. *E. laevigata* (Boyton and Beadle) Blake, 11. *E. simulata* McGregor. As a guide to help assess the validity of McGregor's clusters, inter-cluster distance was significantly greater than intra-cluster distance at $p < 0.0001$, except [a] ($p = 0.1156$) and [b] ($p = 0.0028$).

	1	2	3	4	5	6	7	8	9	10	11
1	0										
2	107	0									
3	84	24	0								
4	102	38	45	0							
5	126	46	51	46	0						
6	111	44	42	65	27[b]	0					
7	115	0.4[a]	35	31	43.3	48	0				
8	89	45	20	31	56.7	62	47	0			
9	161	55	67	71	131	118	62	77	0		
10	109	115	86	126	154	135	139	93	176	0	
11	78	28	11	48	49.5	37	38	30	71	79	0

Figure 2.5. A 40-step most parsimonious cladogram (one of two with consistency indices = 0.825) representing the monophyletic genus *Echinacea* Moench compared to the outgroup *Rudbeckia* L. (including three Sections). Bootstrap values (50% majority-rule consensus method) were as follows; *Echinacea* clade = 98%, *E. laevigata* (Boynton & Beadle) Blake, *E. pallida* (Nutt.) Nutt. and *E. atrorubens* Nutt. = 70%, and the clade of *E. pallida* and *E. atrorubens* = 75%. Dark boxes represent apomorphies and empty boxes represent parallelisms. The numbers refer to characters listed in Table 2.2.

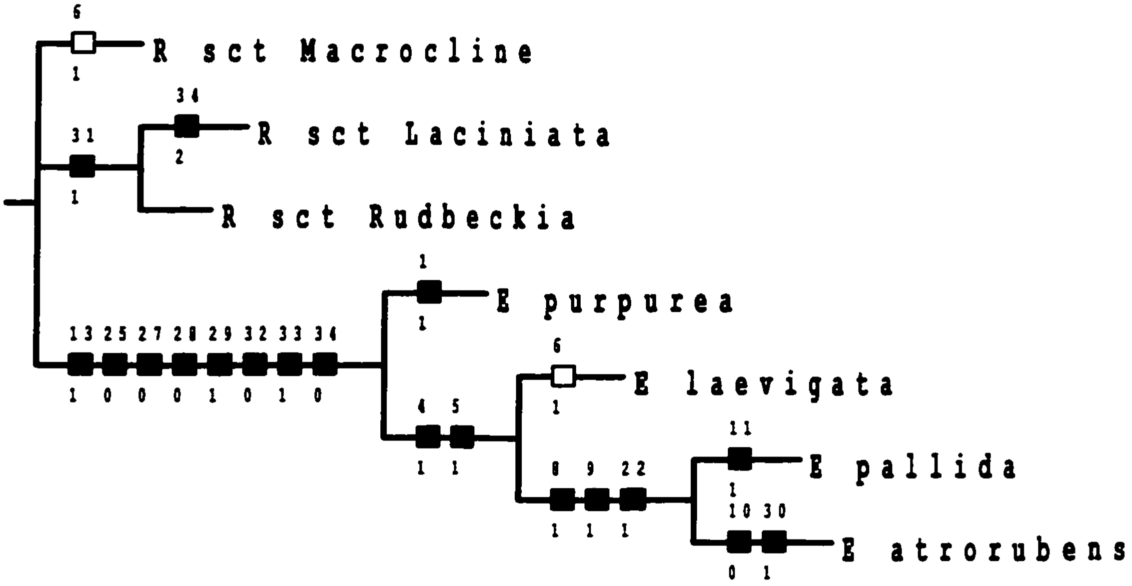
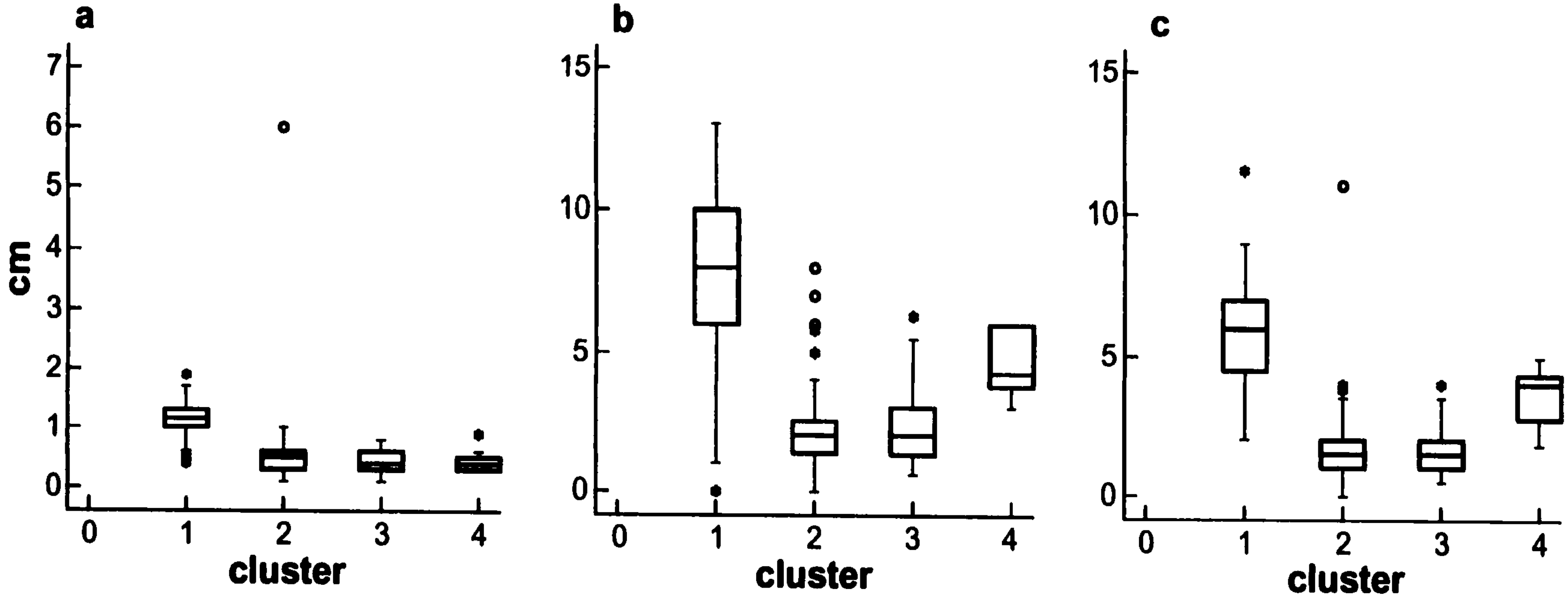

6
1
R sct Macrocline
34
2
R sct Laciniata
31
1
R sct Rudbeckia
1
1
E purpurea
13 25 27 28 29 32 33 34
1 0 0 0 1 0 1 0
6
1
E laevigata
4 5
1 1
11
1
E pallida
8 9 22
1 1 1
10 30
0 1
E atrorubens

Figure 2.6. Box and whiskers plots of three characters among those that contributed the most (F-statistics, $p<0.0001$) in the Canonical Discriminant Analysis between four clusters of *Echinacea* Moench specimens. a. ray floret width in cm (no. 10, Table 2.1), b. basal leaf width in cm (no. 52), c. cauline leaf width in cm (no. 55). All three contributed significantly to separate *Echinacea* OTUs into four clusters (Table 2.4), and a. was also very important in the 2-cluster solution. Boxes represent interquartile (25-75%) range (I.R.) including median, whiskers extend within 1.5 I.R., * = observations outside 1.5 I.R., ^{0} = observations ouside 3 I.R.


a
b
c
cm
0
1
2
3
4
5
6
7
10
15
cluster

Discussion

The results of morphometric analyses supported two nested groupings, which we recognized as two subgenera and four species-level taxa within the genus *Echinacea*. The greatest distance between groups occurs between *E. purpurea* and all other *Echinacea* OTUs, in every clustering method except UPGMA. Therefore, subgenus *Echinacea* contains only *E. purpurea* (see chapter 4; Binns *et al.* 2001 (a)), and subgenus *Pallida* contains all other *Echinacea* taxa. The species are 1) *E. pallida* (Nutt.) Nutt., 2) *E. atrorubens* Nutt., 3) *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake and 4) *E. purpurea* (L.) Moench.

In the current study, all taxa previously described by McGregor (1968) received support in the canonical discriminant ordination at the level of varieties, except *E. angustifolia* var. *strigosa* (which we called a form or group of stable hybrids, an idea put forth by McGregor in 1968).

The four species determined here by morphometrics were previously suggested to some extent by taxonomists working to intuitively assess groups in the genus *Echinacea*. Several publications by Cronquist (1945, 1955, 1980) treated McGregor's 11 taxa as the same four species found here using morphometric methods: *E. pallida*, *E. atrorubens*, *E. laevigata* and *E. purpurea*. Furthermore, Cronquist (1945, 1955, 1980) conserved some of McGregor's species-level taxa as varieties which were upheld in the present treatment; they were *E. pallida* var. *angustifolia* (including an eastern variety, *E. tennesseensis* Beadle), *E. atrorubens* var. *atrorubens* and *E. atrorubens* var. *paradoxa*. Cronquist (1980) also suggested that *E. sanguinea* might be best treated as a variety, and made a similar comment about *E. simulata* (Gleason and Cronquist 1991). In a later taxonomy, McGregor *et al.* (1986) identified three of the same species as Cronquist (1980), although he retained *E. angustifolia* as a species in the Great Plains. In summary, the present taxonomic treatment provided the most support for Cronquist's treatment, although it reinforced some of McGregor's recent work as well.

Current evidence for two subgenera is a novel development in the genus *Echinacea*. According to both overall similarity and cladistic methods, *E. purpurea* clearly occupied its own subgenus, while *E. pallida* and *E. atrorubens* occupied the second. However, despite close morphological resemblance to *E. purpurea* (Table 3.2), the cluster centroid for *E. laevigata* was at a squared Mahalanobis distance of 80.9 units from the *E. purpurea* centroid in CDA, as compared to 33 and 37 units from the other clusters, respectively. Based on closer phenetic similarity to the other clusters, we placed *E. laevigata* in subgenus *Pallida* instead of subgenus *Echinacea*. The cladistic analysis supports the idea that *E. laevigata*, rather than *E. pallida* or *E. atrorubens*, is most closely related to *E. purpurea*, which was suggested historically (see Table 3.2; chapter 3).

In the subgenus *Pallida*, CDA showed that length of ray flowers (character no. 9, Table 2.1) was one of the major contributors to variation between the three species. This finding is justified, since the ray length demonstrated minimal phenotypic plasticity between populations of any one putative species. Other characters, including ray floret colour or habit, often changed drastically upon transplantation.

According to Cox and Urbatsch (1990), *Rudbeckia* sect. *Macrocline* was the closest outgroup taxon to *Echinacea*, which agrees with present cladistic results. *R.* sect. *Macrocline* lacked the thiophenes (sulfur-containing polyacetylene derivatives) found in the other two *Rudbeckia* sections (Cox and Urbatsch 1990), and it displayed parallel evolution of the smooth leaf adaxial surface found also in *E. laevigata*, as well as the leaf width and stem branching habit found in *E. purpurea*.

In the course of this morphometric taxonomic revision, there were factors beyond our control, which affected overall accuracy, such as: phenotypic plasticity, missing characters on herbarium specimens, and inclusion of suspected hybrids which displayed characteristics of intermediate species (*sensu* McGregor) in the field and/or greenhouse. The only other large-scale study of wild *Echinacea* populations (McGregor 1968) reported extensive populational variation within each species and even within each variety of *Echinacea*, which was also observed in this study (data not shown, determined from box plots similar to Fig. 2.6). Therefore, there were few reliable and easily measurable key characters which effectively distinguished between the four revised species; they demonstrated the least overlap of all characters in the present analysis (see Fig. 2.6, Key and Table 2.2). Key characters for identification of varieties of the revised species (five in *E. pallida* and three in *E. atrorubens*, see Taxonomic Treatment) were even more difficult to obtain due to overlapping character ranges.

Specimens that were inappropriately clustered in the flexible clustering solution (suspected hybrids or intermediate forms based on segregation to a cluster far from their population relatives) were carefully keyed out and re-analyzed before final identifications were made. In addition, we used geographical data to help determine the varietal and/or specific cross which we indicated in the hybrid name (see *Additional specimens examined* sections in Taxonomic Treatment).

The present taxonomic revision is based solely on morphology and was designed to complement a systematic DNA study (Mechanda PhD Thesis In Progress) and a phytochemical study (chapter 5) undertaken concurrently with the same field collections of *Echinacea*. The ongoing correlation of all datasets most accurately elucidates infrageneric relationships and provides means to identify potentially high-yielding phytomedicinal lines for cultivation (Baum *et al.* 2001). While molecular systematics is providing new insight into taxonmic relationships, morphological whole plant identification remains a key practical tool, especially for appropriate wild germplasm protection, cultivation and public awareness of one of North America's most important medicinal plants.

Taxonomic Treatment

The following descriptions are based on observations in 321 specimens using the 81 characters from the morphometric analyses and some were re-coded for ease of describing character states. Very rare character states are enclosed in parentheses. Numbers in [square brackets] indicate "Notes" which are found at the end of the Taxonomic Treatment.

ECHINACEA Moench. Meth. Pl. 591. 1794. -TYPE: *Rudbeckia purpurea* L. Sp. Pl. 2: 907. 1753. See note [1]. ***Synonyms***. *Helichroa* Raf. Neogenyton. 3. 1825. ***Brauneria purpurea*** Neck. ex T.C. Porter & N.L. Britton. Mem. Torr. Bot. Club. 5: 333.1894. See note [2]. ***Description***. Herbaceous perennials with short, stout caudices (when present). ROOTS are taproots, fusiform or fibrous. STEMS simple or branched, (7)-45-88-(125)cm tall, hirsute to hispid or appressed, or glabrous. LEAVES simple, alternate or unevenly whorled, lower petiolate and upper sessile, elliptical or lanceolate to ovate, variously hairy or glabrous; margins entire to coarsely dentate, variously hairy; veins three, or three and five (basal) or three and one (upper cauline). CAPITULESCENCE radiate, solitary, long pedunculate with hemispherical or conical or elongated receptacle (rarely flat-topped). INVOLUCRAL BRACTS in 2 to 4 series, lanceolate or linear, attenuated, imbricated, reflexed or spreading or recurved, uneven in length, integrating into discoid bracts, often pubescent abaxially; margins hairy. RAY FLORETS dark purple to pale pink or white or yellow, spreading, reflexed or drooping in one series, with apices 2-3 lobed; mostly sterile or with vestigial style and/or stamens; ray achenes trigonous. DISK FLORETS 5-lobed with green and red, purple or light yellow tips, fertile; tubular corollas with bulbous bases sessile on ovaries; stamens with 5 free filaments; pollen gold, lemon yellow or white; style with bifid, flattened and papillate branches. DISCOID BRACTS firm, with bases clasping achenes, ending in a sharp awn with straight or incurved tip exceeding disk corolla. ACHENES quadrangular, 2-6 mm long, glabrous or sparsely pubescent; pappus of 1 or 4 major teeth, margins entire, toothed or crenate. Chromosome number, $2n = 22, 44$, (rarely 33 in sterile triploids). ***Distribution***. The center of diversity is in Arkansas (AR), Oklahoma (OK), Kansas (KS) and Missouri (MO)(Fig. 2.7)(according to McGregor 1968 and the current study). The genus range is east of the Rocky Mountains; it extends south to the Gulf Coast, north and west across the Great Plains to eastern MT and southern Sask., Canada, as well as east to the Appalachians. Hybrids are found frequently in nature where species ranges overlap and may be distinguished by tetraploidy, triploidy, sterility and/or intermediate forms (McGregor 1968; McKeown pers. comm. 1999). Germplasm is cultivated extensively in Australia, Europe and North America, and also increasingly in Egypt, South Africa and South America.

Close Relatives. *Echinacea* was classified in the tribe Heliantheae (Stuessy 1977), with various sub-tribal classifications ranging from Helianthinae (Stuessy 1977) to Ecliptinae (Robinson 1981). In later cladistic analyses, it was proposed that *Echinacea* be placed in Rudbeckiinae (Cox and Urbatsch 1990; Karis 1993). In their treatment of the Rudbeckiinae, Cox and Urbatsch (1990) found a number of homoplasies supporting the *Echinacea* clade that may be indicative of incorrect sub-tribal relationship. The most recent cladistic analysis of cpDNA moved *Echinacea* back to its position in the Ecliptinae (Urbatsch and Jansen 1995), and then in a new position altogether in subtribe Zinniinae based on recent ITS sequence data (Urbatsch *et al.* 2000). Evidently, there has been no consistent correlation of different datasets for classifications above the genus level. Therefore, *Echinacea* has many closely-related genera, of which the most likely sister group depends on the type of analysis undertaken.

Genera most often sharing the same subtribe as *Echinacea* include *Rudbeckia* L., *Dracopsis* Cass., and *Ratibida* Raf., which all share the synapomorphies: conical, widened receptacles (Stuessy 1977), neuter ray florets, and coroniform pappus (Karis and Ryding 1994). *Ratibida* differs from *Echinacea* in that the disk corolla is narrowed slightly at the base, rather than proceeding from a bulb-like base (Sharp 1935), yet it is similar to *Echinacea* in polyacetylene phytochemistry (Bohlmann *et al.* 1973). Disk achene pappus is generally some form of toothed crown in *Echinacea*, but in relatives is either a shallow cup (*Dracopsis*), quadrangular membrane (*Rudbeckia*), or two awn-like teeth (*Ratibida*). *Echinacea* reportedly has the following unique characteristics: awn of the discoid bract extending beyond tips of disk corolla tube, spiny discoid bracts and the presence of alkamide phytochemicals (Robinson 1981).

***ECHINACEA* SUBGENUS *ECHINACEA*.** Features distinguishing this subgenus from *Echinacea* subgenus *Pallida* Binns B. R. Baum & Arnason (to follow) include fibrous roots, basal leaves 5 cm wide or more and cauline leaves 4.5-9 cm wide. Leaf blade trichomes are bicellular with ledge-like joints. Major leaf veins are branched. Involucre has 4 series of bracts.

ECHINACEA PURPUREA (L.) Moench. *nom. cons. prop.* (see chapters 3 and 4). TYPE: U.S.A. Arkansas: Nuttall "*Echinacea serotina* Arkansa" (lectotype: BM no. 541360! designated in chapter 3 by Binns *et al.* 2001 (b)) [1]. ***Homotypic synonyms.*** ***R. serotina*** (Nutt.) Sweet, Brit. Fl. Gard. 1: pl 4. 1823. ***Echinacea serotina*** (Nutt.) DC., Prodr. 5:554. 1836. ***Brauneria purpurea*** (L.) ex T. C. Porter and N. L. Britton, Mem. Torr. Bot. Club 5:333. 1894. See note [3]. ***Heterotypic synonyms.*** ***R. speciosa*** Link. [4] Enumeratio plantarum Horti regii botanici berolinensis 2:353. 1821. (Type not seen). ***R. hispida*** Hoffmannsegg [5] Verzeichniss der Pflanzenkulturen 2:201. 1824.(Type not seen). ***Echinacea intermedia*** Lindl. [4] Paxt. Mag. Bot. 15:79. 1849. ***Description.*** STEMS branched, 52-88-(120)cm tall, brownish-green (very rarely only green or whitish-green or reddish-green), furrowed or unfurrowed; peduncle less than half total stem height. STEM STALKED TRICHOMES bicellular or tricellular, short or medium length, knobby or smooth or ledge-like, on top half of stem (sometimes absent, very rarely entire stem or top few inches only); with bulbous basal cells (rarely non-bulbous); lacking basal globules (very rarely 1 to many rings of globules subtending trichome); sessile trichomes usually absent but rarely present. ROOTS fibrous. BASAL LEAVES (5)-15.5-22-(30)cm long, (1)-5-10-(13)cm wide, ovate or lanceolate-ovate (rarely lanceolate) with petioles (0)-10-17-(22)cm long; margins serrate or dentate (rarely entire or undulate/crisped). CAULINE LEAVES (4)-11-17-(24)cm long, (2)-4.5-7-(9-11)cm wide, linear-lanceolate or lanceolate-ovate (rarely ovate) with petioles (1)-3-6-(10-18)cm long; margins serrate or dentate or undulate/crisped (rarely entire). LEAVES dark green or bright green, with three clear and branching major veins (very rarely white/yellow with five veins and three on upper cauline, or three and one on upper, or almost parallel with minor branching in between). LEAF STALKED TRICHOMES bicellular (very rarely multicellular), short (very rarely medium), ledge-like (very rarely smooth or knobby), straight (very rarely appressed or hirsute), dense or sparse, even on both surfaces or mostly adaxial (very rarely mostly abaxial); with non-bulbous basal cells (very rarely bulbous); lacking basal globules, or in one ring subtending trichome; marginal trichomes similar and more appressed than blade trichomes (very rarely either identical or entirely different in type and or habit to leaf blade trichomes). CAPITULESCENCE (1.4)-2-3-(3.5)cm tall, (2)-2.5-3.1-(4) cm wide with a conical, hemispherical or flat-topped receptacle (rarely elongated). INVOLUCRAL BRACTS in 4 series (very rarely 3), (0.8)-1-1.5-(2)cm long, (0.1)-0.2-0.4-(0.8)cm wide, usually reflexed parallel to stem or recurved; abaxial stalked trichomes present (very rarely absent); sessile trichomes absent. RAY FLORETS dark pink or purple (rarely pale pink or white), (2.5)-3.5-5-(6)cm long, (0.3-0.6)-1-1.3-(1.7-1.9)cm wide, perpendicular to stem axis or reflexed parallel to stem axis (rarely drooping 45^0 to axis and very rarely recurved tip to stem); achene pappus of three teeth with intermediate margin undulate or equal-toothed (rarely unequal-toothed or very rarely entire); ray stalked trichomes present abaxially or on both surfaces, sparse or dense. DISK FLORETS with green and pink corolla straight tips (rarely purple, very rarely yellowish or orange); petal fusion more than 3/4 total corolla length; pollen yellow; style branches purple (very rarely white or yellow), recurved (very rarely spreading), shorter than discoid bracts (very rarely extending beyond bract tip); style base disk-shaped (very rarely irregular); corolla stalked or sessile trichomes present. DISCOID BRACTS (0.9)-1.1-1.3-(1.5)cm long, awn bodies straight, tips orange and red (very rarely brownish-purple); dorsal keel usually absent or sometimes present; keel stalked trichomes absent (very rarely present); keel sessile trichomes absent. DISK ACHENES (0.3)-0.35-0.5-(0.6)cm long, without vertical or horizontal striation; pappus of four major teeth or sometimes only one major tooth (rarely none) and with equal-toothed (very rarely unequal-toothed, entire or undulate) intermediate margins.

Distribution. Wooded hillsides and banks, especially near waterways. Its natural western limit is IA to AR, LA and east to PA in the north and GA in the south (Fig. 2.8). Very scarce native populations of few plants that prefer part-shade. Some colonies in disturbed habitats probably arose from escaped garden plants. Infrapopulational morphological variation is extensive, especially with respect to hairiness.

Additional specimens examined. USA. AB: Lawrence Co. 2 mi WNW Hatton, 20 Jul 1968, Kral EP-00-8 (KANU). AR: East of Siloam Springs, 20 Sep 1997, Binns and St-Laurent 8, 9, 10, EP-2, EP-5, EP004 (DAO). IL: near Carthage, 14 Sep 1997, Binns and St-Laurent B, EP019 (DAO). MO: Camden Co., 6 Jun 1999, Binns EP 99-0606-1 (DAO). MO: McDonald Co. SW Pineville on open wooded hillside, 7 Aug 1959, McGregor EP-00-7 (KANU). MO: Shannon Co. 22 Sep 1997, Binns and St-Laurent EP010 (DAO). NC:

Durham Co. north of Durham city, 16 Jun 1998, Binns and Campbell EP 98-0616-1 (DAO). OR: Horizon Herbs, cultivated material. WA: Trout Lake Farm LLC, cultivated material.

ECHINACEA **SUBGENUS** ***PALLIDA*** **subg. nov. Binns B. R. Baum & Arnason.** This subgenus contains three species; one with five varieties and one with three varieties. TYPE: *E. pallida* (Nutt.) Nutt. ***Description.*** Features distinguishing the subgenus include taproots (or fusiform, or with few large branch points), basal leaves up to 5 cm wide and cauline leaves 0.5-4.5 cm wide. Leaf blade trichomes are multicellular with knobby joints. Leaf major veins are almost parallel from a common origin at the base. Involucre has 1-3 series of bracts.

ECHINACEA PALLIDA (Nutt.) Nutt., Trans. Am. Phil. Soc. 7:354. 1841. TYPE U.S.A. Arkansas: 1819, Nuttall (BM no. 3521, photo at HUH!). See note [6]. ***Homotypic synonyms.*** *Rudbeckia pallida* Nutt., Journ. Acad. Nat. Sci. Philad. 7:77. 1834. *Brauneria pallida* (Nutt.) Britt., Mem. Torr. Bot. Club. 5:333. 1894. ***Heterotypic synonym.*** *E. angustifolia* Hook., Curtis Botanical Magazine 17:pl. 5281. 1861. (non DC.) ***Description.*** STEMS unbranched or branched, (<45)-45-78-(125)cm tall, green or brownish-green or whitish-green (rarely reddish-green), furrowed or unfurrowed or rarely furrowed just below involucre; peduncle less than half total stem height, sometimes greater than or equal to half of stem height. STEM STALKED TRICHOMES multicellular (very rarely bicellular or mixed), short or medium length (rarely long or mixed lengths), knobby (rarely smooth, very rarely ledge-like, or both smooth and knobby), on top half of stem or on both bottom and top half of stem (rarely top few inches of peduncle only and very rarely bottom half only, very rarely absent); basal cells non-bulbous or bulbous (rarely both); basal globules in one or two rings subtending trichome, or absent (rarely multiple or uneven ring pattern); sessile trichomes present (very rarely absent). ROOTS are branched taproots with few to many hair-like laterals (sometimes fusiform, rarely having fibrous laterals). BASAL LEAVES (<10)-10-17-(27-30)cm long, 1-3-(5)cm wide, linear or linear and lanceolate (rarely elliptical, lanceolate-ovate or ovate); margins entire (very rarely serrate, dentate, lobed or entire and lobed together) and petioles (<6)-6-12-(20-30)cm long. CAULINE LEAVES (<8)-8-13-(20-33)cm long, 1-2-(3.5) cm wide, linear or linear and lanceolate (rarely elliptical, lanceolate-ovate or ovate); margins entire (very rarely some serrate) and petioles (<2)-2-7-(13-18)cm long. LEAVES dark green or bright green or yellowish-green (very rarely reddish) with 3 or 5 major parallel clear or white or yellow veins(rarely darkened; very rarely branching or branching in between; rarely including single-veined leaves). LEAF STALKED TRICHOMES multicellular (very rarely bicellular), short or medium length (very rarely long or both short and medium), knobby (very rarely smooth or ledge-like), hirsute or appressed or straight (sometimes a mix of appressed and hirsute), dense or sparse, even on both surfaces or mostly abaxial (rarely mostly adaxial, very rarely absent); basal cells non bulbous or bulbous; basal globules in one or two rings subtending trichome or absent (very rarely multiple rings); marginal trichomes identical to blade trichomes or entirely different (rarely similar but more appressed). CAPITULESCENCE (1.2)-2-2.5-(3-4.5)cm tall, (1.4)-2.2-3-(4-5)cm wide with a conical or spherical (rarely flat-topped, very rarely elongated) receptacle. INVOLUCRAL BRACTS in 2 or 3 series, (0.3)-0.7-1-(1.4->2)cm long, (<0.2)-0.2-0.3-(0.4-1)cm wide, recurved or perpendicular to stem (rarely reflexed parallel to stem); stalked trichomes present abaxially (or absent) and sessile trichomes sometimes present. RAY FLORETS pale pink or white (rarely dark pink to purple or yellow), (<2.5)-2.5-5.5-(9)cm long, (<0.3)-0.3-0.6-(1)cm wide and reflexed parallel to stem axis or drooping 45^0 to stem axis (rarely perpendicular to stem axis, or recurved with tips touching stem, and very rarely curved upwards); stalked trichomes present abaxially or on both surfaces (rarely absent), and sparse (rarely dense, or dense with sessile trichomes also); ray achenes with toothed or lobed pappus (rarely entire) and intermediate margins unequal or rarely equal. DISK FLORETS with green and purple, or purple corolla tips (rarely yellowish and very rarely orange or other); tips straight, or less often reflexed; petal fusion either more than 3/4 total corolla length or sometimes less than 3/4 total corolla length; pollen yellow or one variety is white (rarely lemon yellow or yellow and white on same capitulum); styles with purple branches (rarely yellowish, or blackish) often spreading or recurved, shorter or equal to discoid bracts (very rarely longer), bases ball-shaped (sometimes disk-shaped, very rarely odd-shaped); corolla trichomes present or absent. DISCOID BRACTS (0.4)-1-1.2-(1.4-1.6)cm long with awn bodies straight to incurved (rarely both), tips orange and red or red or green and red or green to yellowish-white (rarely two types on one plant); dorsal keel present (very rarely absent); keel stalked trichomes present, or absent; keel sessile trichomes absent or rarely present. DISK ACHENES (0.2)-0.3-0.4(0.5-0.6)cm long, with or without vertical striation, and horizontal striation either brown banded, absent or rarely red banded; pappus of four teeth or one major tooth (rarely none and very rarely two on the acute-angled corners) with intermediate margin lobed or toothed, either equal or unequal(rarely entire).

Distribution. See distributions of each variety. Generally found in a range of habitats (depending on the variety) from Canadian prairies south along the high plains to TX, bordered by MT, WY and CO in the west through low plains and pine-oak forests of MN, WI, and IL, east to OH and south to LA, including the cedar glades of TN and adventive eastward (Figs. 2.7, 2.9).

VAR. *PALLIDA*. ***Description***. See description of *E. pallida* (Nutt.) Nutt., with the following unique characteristics of the type variety: STEMS 60-85-(110)cm tall, wider below head. ROOTS usually branched, fusiform taproots with many thick laterals (very rarely unbranched with sparse laterals), with peduncle less than half total stem height (very rarely greater than ½ total stem height). BASAL LEAVES usually 13-20 cm long. CAULINE LEAVES usually 9-15 cm long. Leaf stalked trichomes with non-bulbous basal cell (very rarely bulbous). INVOLUCRAL BRACTS 0.9-1.5 cm long (rarely 0.5 cm or up to 2 cm). RAY FLORETS pale pink to white, drooping 45^0 to stem axis (very rarely perpendicular, reflexed or recurved tip to stem); dense stalked trichomes present abaxially, or on both surfaces (very rarely absent). DISK FLORETS usually without trichomes on corollas (rarely present), pollen white (very rarely lemon). DISCOID BRACTS with incurved awn bodies (very rarely straight).

Distribution. Dry prairies, openings in wooded hillsides and low plains; IL and MN, IA to eastern NE and KS (where it overlaps with *E. pallida* var. *angustifolia*), western MO and south to northeastern TX. Adventive eastward (Fig. 2.9).

Additional specimens examined. USA. AR: Boone Co. near Harrison, 17 Aug 1997, McKeown EPA 23920 (DAO). IL: near Matthieson State Park, 13 Sep 1997, Binns and St-Laurent A, EPA012 (DAO). KS: Allen Co. S of Iola, 17 Jun 1960, McGregor 15658 (KANU). KS: Johnson Co. SW of DeSoto, 20 Jul 1993, Freeman 5080 (KANU). KS: Leavenworth Co. SW of Leavenworth, 22 Jun 1957, Wagenknecht 3804 (KANU). KS: Miami Co. N of Hwy 68, W of MO state line, 31 May 1994, Milburn 656 (KANU). KS: Miami Co. Quivira, 6 Jun 1994, Daugherty 34 (KANU). KS: Neosho Co. E of Chanute, 9 Jun 1957, Lathrop 3728 (KANU). KS: Osage Co. just E of Olivet, 11 Jul 1998, Binns EPA 98-0711-1 (DAO). MO: Wright Co. 3.9 mi W Norwood, 4 Jun 1960, Harms 328 (KANU). MO: Douglas Co. SE of Ava, 21 Sep 1997, Binns and St-Laurent 17, EPA-1, EPA-3, EPA009 (DAO). MO: Benton Co. Hwy 65, 16 Sep 1997, Binns and St-Laurent C, EPA-4, EPA007 (DAO). MO: Benton Co. S of Lincoln, 5 Jun 1999, Binns EPA 99-0605-1 (DAO). MO: Christian Co. S of Ozark, 4 Jun 1998, Binns EPA 98-0604-1 (DAO). MO: Webster Co. E of Rodgersville, 21 Sep 1997, Binns and St-Laurent 18, EPA-12/13, EPA013 (DAO). MO: Wright Co. S Hartville, 9 Jun 1958, McGregor 13864 (KANU). OK: Stephens Co. MLRA:84, 14 Jan 1991, Rice EPA 597603 (DAO). OK: border of Adair Co. S of Kansas near Cemetary, 3 Jun 1998, Binns EPA 98-0603-1 (DAO). OK: Hughes Co. SW Dustin, 9 Jun 1959, McGregor 14353(KANU). OK: Hughes Co. SW Dustin, 31 May 1998, Binns EPA 98-0531-1 (DAO). OK: Osage Co. N of Pawhuska, 1 Jun 1998, Binns EPA 98-0601-1 (DAO). OK: Pushmataha Co. S of Antlers, 20 May 1961, McGregor 16847 (KANU). SD: Lyman Co. S of Lower Brule, 15 Jul 1966, Barker 3117 (KANU). TX: Fannin Co. W of Windom, 21 May 1961, McGregor 16860 (KANU). IA: Webster Co. near Fort Dodge, 28 Sep 1997, Widrlechner EPA 23925 (DAO).

VAR. *ANGUSTIFOLIA* (DC.) Cronq., Vasc. Pls. Pacif. NW. 5:160. 1955. TYPE: USA. TX: Austin, 1692, Berlandier (holotype: G photo! In DC's Herbarium 5:554 p. 940., IDC, DAO). ***Homotypic synonyms***. *E. angustifolia* DC. Prodr. 5:554. 1836. *B. angustifolia* (DC.) Heller. Muhlenbergia. 1:5. 1900. ***Description***. See *E. pallida* (Nutt.) Nutt. except for the following characteristics of the variety; STEMS (15)-30-50-(75)cm tall; pith absent throughout or present below only (rarely present throughout). STEM STALKED TRICHOMES with dense surface ornamentation (usually), hirsute and usually on entire stem; basal globules subtending trichomes in two to many uneven rings. ROOTS usually taproots with sparse, thin laterals and minimal branching (rarely fusiform). BASAL LEAVES usually 8-12 cm long. CAULINE LEAVES usually 7-10 cm long. LEAVES lanceolate, lanceolate-ovate or elliptical with dense stalked trichomes having non-bulbous basal cells (very rarely bulbous); globules usually absent or in one ring subtending trichome (rarely two rings). CAPITULESCENCE 1.5-2.5 cm tall. INVOLUCRAL BRACTS 0.3-0.4 cm wide (rarely to 0.2 cm or up to 1 cm) without sessile abaxial trichomes. RAY FLORETS (1.25)-2.1-3-(4.5)cm long with stalked trichomes dense on both surfaces, and often also with sessile trichomes. DISK FLORETS usually lacking corolla trichomes; style base ball-shaped or irregularly-shaped (rarely disk-shaped). DISCOID BRACTS with awn bodies straighter towards center of capitulum (rarely); tips never yellowish-white.

E. angustifolia DC. included another variety according to McGregor (1968), which was not morphometrically distinct from *E. pallida* var. *angustifolia* at the variety level (in the present study). *E. angustifolia* var. *strigosa* McGregor may be considered a loose geographical group or variant of *E. pallida* var. *angustifolia*. It is a stable introgressant form arising from hybridization between *E. pallida* (Nutt.) var. *angustifolia* (DC.) Cronq. and *E. atrorubens* Nutt. var. *atrorubens* Cronq , as suggested first by McGregor (1968). This form inhabits rocky gypsiferous, sandy-clay prairies along a geographical cline dividing those varieties mentioned above; north-central TX in narrow band to south-central KS and isolated colonies eastward. Distinguishing morphological characteristics of the form are a subset of both *E. pallida* var. *angustifolia* and *E. atrorubens* var. *atrorubens*: STEMS (35)-40-50-(65)cm tall. STEM STALKED TRICHOMES appressed or hirsute (rarely straight). ROOTS are usually taproots with rare branching and few thin laterals or fusiform, branched taproots with many thick laterals. BASAL LEAVES lanceolate (rarely linear or elliptical). CAULINE LEAVES linear, lanceolate or rarely elliptical. LEAF STALKED TRICHOMES appressed (very rarely hirsute or straight).

Distribution. Rocky, dry prairies and limestone outcrops from Man. and Sask., Canada south along the high plains to central TX and west to MT, WY and northwest CO; overlapping with *E. pallida* var. *pallida* to the east where hills meet plains and prairies of OK, KS, IA, NE, SD (Fig. 2.8).

Additional specimens examined. CANADA. BC: Corner Copia Farms, cultivated material, 1999, EA COM-1 (DAO). MB: near Brandon, 1 Aug 1997, Binns, Marles and Arnason EA 98-0717-3,EP001 (DAO). MB: near Souris River, 18 Jul 1998, Binns EA 98-

0718-2 (DAO). MB: near Stockton, 18 Jul 1998, Binns EA 98-0718-2 (DAO). MB: SE of Brandon on gravel esker, 18 Jul 1998, Binns EA 98-0718-1 (DAO).

USA. IA: Clay Co. near Peterson, 23 Sep 1997, Fraley E23930 (DAO). KS: Pottawatomie Co. near Manhattan, 26 Aug 1997, Price-Hurlburt EA 24058 (DAO). KS: Butler Co. dirt road W of Augusta, 17 Sep 1997, Binns and St-Laurent 1,2,EA-5,EA-6,EA003 (DAO). KS: Chase Co. W of Strong City, 8 Jun 1999, Binns EA 99-0608-1 (DAO). KS: Lyon Co. E of Allen, 15 Jun 1960, Harms 399 (KANU). KS: Pottawatomie Co. near Manhattan, 12 Jul 1998, Binns EA 98-0712-1 (DAO). KS: Rooks Co. W of Woodston, 13 Jul 1998, Binns EA 98-0713-1 (DAO). KS: Sumner Co. S of Caldwell, 17 Sep 1997, Binns and St-Laurent 4, EA-1 (DAO). KS: Waubaunsee Co., 19 Jun 1956, McGregor 12380 (KANU). ND: Emmons Co., S of Hazleton, 16 Jul 1998, Binns EA 98-0716-1A (DAO). ND: McClean Co. Fort Stevenson State Park, 17 Jul 1998, Binns EA 98-0717-1 (DAO). ND: McHenry Co., 17 Jul 1998, Binns EA 98-0717-2 (DAO). ND: Oliver Co., 16 Jul 1998, Binns EA 98-0716-2 (DAO). ND: Pierce Co. S and E of Orrin, 27 Jul 1967, Stephens and R. Brooks 15000 (KANU). ND: Slope Co. SW of Amidon, 23 Jul 1971, Stephens 50035 (KANU). NE: 28 Mar 1966, Williams EA 312814 (DAO). NE: Richardson Co., Bernecker EA 421332 (DAO). NE: Custer Co. N of Ansley, 13 Jul 1966, Stephens 6791 (KANU). NE: Custer Co. N of Ansley, 13 Jul 1998, Binns EA 98-0713-3 (DAO). NE: Franklin Co. S of Bloomington, 19 Jun 1966, McGregor 19961 (KANU). NE: Franklin Co. S of Bloomington, 13 Jul 1998, Binns EA98-0713-2 (DAO). OK: Comanche Co., 11 Aug 1997, McKeown EA 23909 (DAO). OK: Logan Co. 1 Jul 1976, Ankle EA 421331 (DAO). OK: Cleveland Co. E of Lexington, 9 Jun 1959, McGregor 14347 (KANU). OK: Comanche Co. near Fort Sill, 30 May 1998, Binns EPA 597603 (DAO). OK: Kiowa Co. 3.5 mi N Saddle Mountain, 8 Jun 1959, McGregor 14336 (KANU). OK: McClain Co. E of Dibble, 9 Jun 1959, McGregor 14345 (KANU). OK: Noble Co. S of

Ceres, 13 Jun 1960, Harms 369 (KANU). OR: Horizon Herbs Farm, cultivated organic seed, 1999, EA COM-2 (DAO). SD: Fall River Co. S Hot Springs in Black Hills, 14 Jul 1998, Binns EA 98-0714-1 (DAO). SD: Hutchinson Co. S of Freeman, 20 Jul 1967, Stephens 14236 (KANU). SD: Hutchinson Co. S of Freeman, 15 Jul 1998, Binns EA 98-0715-1 (DAO). SD: Turner Co. W of Viborg, 20 Jul 1967, Stephens and Brooks 14213 (KANU). TX: Grayson Co. SW of Sherman, 7 Jun 1958, Richards 548 (KANU). WY: Crook Co. S of Devil's Tower, 22 Jul 1966, Stephens 7636 (KANU). WY: Weston Co. N of confluence with E Plum Creek, 2 Aug 1984, Marriott 8330 (KANU).

VAR. *SANGUINEA* (Nutt.) Gandhi and Thomas. Asteraceae of Louisiana. pp. 68-70. 1989. TYPE: U.S.A. OK: "Echinacea * sanguinea Hb. Nuttall, Red River Plains" Nuttall (holotype: BM no. 541359!; isotype: PH! Arkansas, "R. * Nutt." "= *Echinacea angustifolia* Syn. Fl. N. Amer.").[7] ***Homotypic synonym***. *E. sanguinea* Nutt. Trans. Am. Phil. Soc. 7:354. 1841. ***Description***. Similar to *E. pallida* (Nutt.) Nutt. description, except for the following unique states or ranges; STEMS usually branched and 70-90-(125)cm tall (often among the tallest in this species); pith present throughout (very rarely absent); peduncle greater than or equal to ½ stem height. BASAL AND CAULINE LEAVES usually linear-lanceolate, never elliptical; major veins clear. LEAF STALKED TRICHOMES may be shorter than others in the species, always dense. RAY FLORETS usually pale pink to reddish-purple or mottled with darker spots of red (very rarely white), 4-6-(7)cm long. DISK FLORETS with purple corolla tips (very rarely green or yellowish), usually straight and lacking trichomes; style branches usually longer than (or equal to) awn tip of discoid bract; pollen usually yellow. DISCOID BRACTS 0.8-0.95-(1)cm which is shorter than other varieties of the species; awn tips usually brownish-purple; keels usually present without stalked trichomes. DISK ACHENES sometimes have vertical striations (brown flecks in pericarp), but usually lack horizontal striation or may be brown-banded (rarely).

Distribution. Heavy clay-loam soils in pine-oak forest clearings from Texarkana south along eastern TX border and throughout western LA (Fig. 2.8).

Additional specimens examined. USA. GA: Floyd Co, near Cave Springs, 28 Aug 1997, McKeown ES 23962 (DAO). LA: Beauregard Co. just S of DeRidder, 23 May 1998, Binns ESA 98-0523-5 (DAO). LA: Beauregard Parish, 6 Aug 1997, McKeown ESA 23873 (DAO). LA: Bienville Parish, 8 aug 1997, McKeown ESA 23878 (DAO). LA: Vernon Co., 12 May 1960, McGregor 15571 (KANU). LA: Vernon Co., 23 May 1998, Binns ESA 98-0523-3 (DAO). LA: Vernon Co., 23 May 1998, Binns ESA 98-0523-4 (DAO). LA: Vernon Parish., near Leesville, 8 aug 1997, McKeown ESA 23874 (DAO). OK: Choctaw Co., W of Fort Towson, 20 May 1961, McGregor 16850 (KANU). OK: Pushmataha Co. N of Antlers, 27 May 1998, Binns EPA 98-0527-2 (DAO). OK: McCurtain Co. near Idabel, 27 May 1998, Binns EPA 98-0527-3 (DAO). TX: Cass Co. S of Atlanta, 23 May 1998, Binns ESA 98-0523-2A (DAO). TX: Cass Co., 23 May 1998, Binns ESA 98-0523-1(DAO). TX: Jasper Co. E of Jasper, 12 may 1960, McGregor 15569 (KANU). TX: Polk Co. W of Camden, 12 may 1960, McGregor 15565a (KANU). TX: Polk Co. N of Seven Oaks, 24 May 1998, Binns ESA 98-0524-1 (DAO).

VAR. *SIMULATA* (McGregor) Binns B. R. Baum & Arnason. *comb. et stat. nov.* *Echinacea simulata* McGregor, Sida 3:282. 1968. HOLOTYPE: U.S.A. MO: Crawford Co. 4 mi N Dillard, 7 Jun 1960, R.L. McGregor 15636 (KANU!). ***Homotypic synonym***. *E. speciosa* McGregor (*nom. illegit.*)[8], Trans. Kans. Acad. Sci. 70:366-368. 1967. ***Description***. See *E. pallida* (Nutt.) Nutt. var. pallida. except for the following key characteristics: STEM STALKED TRICHOMES with sparse, distal ornamentation (very rarely absent). INVOLUCRAL BRACTS (0.6)-0.8-1-(1.2)cm long. RAY FLORETS often dark pink or purple (also pale pink to white, but usually darker than *E. pallida* var.

pallida), 4-7 cm long (rarely only 2 cm up to 9 cm). DISK FLORETS with corolla trichomes present or absent; style bases usually ball-shaped; pollen yellow (or rarely lemon, or both white and lemon).

Distribution. Clearings in wooded areas and edges of rocky prairies in north-central AR, eastern MO, western IL, KY, TN and rarely to the southeast (Fig. 2.8).

Additional specimens examined. CANADA. BC: Schimpf farm, 25 Aug 1998, T. Schimpf WTS 98-0825-N1 (DAO).

USA. AR: Baxter Co. S of Mountain Home, 21 Sep 1997, Binns and St-Laurent 13, 14, ES-1, ES011 (DAO). AR: Fulton Co. S of Mammoth Spring, 30 May 1959, Marsh 1497-1 (KANU). AR: Fulton Co. near Mammoth Spring, 6 Jun 1998, Binns ES 98-0608-2 (DAO). AR: Marion Co. W of Yellville along Hwy 62,2 Jun 1960, Harms 318-A (KANU). AR: Marion Co. State Park bank along roadside, Jun 6 1956, Marsh 208 (KANU). AR: Stone Co. W Allison, 7 Jun 1957, Marsh 434 (KANU). MO: Baxter Co. E of Almartha, 22 Sep 1997, Binns and St-Laurent 16, EPP-7/8, EPP006 (DAO). MO: Camden Co. WNW Decaturville, 1 Jun 1990, Brooks 19801 (KANU). MO: Camden Co., 6 Jun 1999, Binns ES 99-0606-1 (DAO). MO: Carter Co. N of Van Buren, 14 jun 1962, McGregor 17183 (KANU). MO: Carter Co. near VanBuren, 8 Jun 1998, Binns ES 98-0608-1 (DAO). MO: Dent Co. SW of Sligo, 7 Jun 1960, McGregor 15640 (KANU). MO: Oregon Co. E of Thayer, 12 Sep 1966, Stephens 9250 (KANU). MO: Oregon Co. N of Greer, 3 Jun 1960, Harms 321 (KANU). MO: Oregon Co. S of Winona, 22 Sep 1997, Binns and ST-Laurent 19,20,E-1,E-3,E015 (DAO). MO: Reynolds Co. N of VanBuren, 7 Jun 1998, Binns ES 98-0607-1 (DAO). MO: Shannon Co. W of Eminence, 22 Sep 1997, Binns and ST-Laurent 21,EPA-10/11,EP002 (DAO). MO: Shannon Co. S of Winona on Hwy. 19, 3 Jun 1960, Harms 322 (KANU). MO: Texas Co. W of Cabool, 10 Jun 1958, McGregor 13865 (KANU). MO: Taney Co. W of Gainesville, 21 Sep 1997, Binns and ST-

Laurent 15, EPP016 (DAO). TN: Rutherford Co., 2 Jun 1991, Carol and Steve Baskauf 130553 (KANU). TN: Rutherford Co., 12 Jun 1998, Binns ES 98-0612-1 (DAO).

VAR. *TENNESSEENSIS* (Beadle) Binns B. R. Baum & Arnason *comb. nov.* TYPE: U.S.A. TN: Rutherford Co. near LaVergne, 19 Aug. 1897. H. Eggert (holotype: US; isotypes 4325 HUH! and 162739 NY!) [9]. ***Homotypic synonyms.*** *Brauneria tennesseensis* Beadle. Bot. Gaz. 25:359. 1898. *E. tennesseensis* (Beadle) Small. Man. Southeast Fl. 1421. 1933. ***Description.*** This variety is so similar to *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronq. that the descriptions match, except for the following: STEMS 25-50 cm tall (shortest of all varieties in this species); pith present throughout. BASAL LEAVES usually (6)-7-10-(12)cm, 0.75-1.5 cm wide (thinner than var. *angustifolia*). CAULINE LEAVES (3)-5-8-(12)cm long. LEAVES all linear to linear-lanceolate (very rarely lanceolate); stalked trichomes with basal globules in one, two or many subtending rings (sometimes absent but generally more globules than found around trichomes on leaves of var. *angustifolia*). CAPITULESCENCE 1.8-2 cm tall (rarely more), conical receptacle. INVOLUCRAL BRACTS (<0.15)-0.15-0.2 cm wide. RAY FLORETS dark pink or purple (rarely pale pink to white),usually curved up greater than 90^0 from stem axis. DISK FLORETS with purple tips and corolla trichomes (or rarely absent). DISCOID BRACTS with incurved awn bodies.

Distribution. Cedar glade endemic of central arid regions in TN (Fig. 2.8). Federally protected taxon (U.S. Fish and Wildlife Service, listed 6 June 1979).

Additional specimens examined. USA. TN: Rutherford Co. near LaVergne, 17 Aug. 1897. H. Eggert 6495 (HUH!). TN: Rutherford Co. near LaVergne, 1 Jul. 1897. H. Eggert 162740 (NY!). TN: Davidson Co. 11 Jun 1998, Binns ET98-0611-3 (DAO). TN: Wilson Co. 11 Jun 1998, Binns ET98-0611-1, ET 0911 (DAO). TN: Wilson Co. 11 Jun 1998, Binns ET98-0611-4 (DAO). TN: Wilson Co. 11 Nov 1993, ET22010 (DAO). TN: Wilson Co. on protected private land (with permission), 11 Jun 1998, Binns ET98-0611-2 (DAO).

ECHINACEA ATRORUBENS Nutt., Trans. Am. Phil. Soc. 7:354. 1841. TYPE: U.S.A. Plains of Arkansas. "*Rudbeckia atrorubens* Nutt." 1841, Nuttall (PH!). ***Homotypic synonyms***. *Rudbeckia atrorubens* Nutt. Journ. Acad. Nat. Sci. Phil. 7:80. 1834. *Brauneria atrorubens* (Nutt.) C. L. Boynton & Beadle [10] Bilt. Bot. Stud. 1:11. 1901. ***Description***. STEMS branched (30)-50-77-(100)cm tall, even width throughout or sometimes wider below head, yellowish-green with white streaks or brownish-green (very rarely green or reddish, and usually brown on drying), furrowed or unfurrowed (rarely furrowed just below involucre); pith usually present throughout or absent (rarely absent in peduncle but present below); peduncle less than half total stem height (very rarely greater than or equal to half total height). STEM STALKED TRICHOMES short, or medium length (very rarely mixed or absent), multicellular (very rarely both bi- and multicellular), knobby (very rarely smooth or ledge-like or mixed), appressed (very rarely hirsute), on top half of stem (very rarely bottom half only or top few inches of peduncle only) with sparse, distal papillate or striate surface ornamentation (rarely round and dense); with bulbous basal cells (very rarely non-bulbous) having globules in one to two ring(s) subtending trichome or in an uneven/multiple pattern (rarely absent); sessile trichomes present (very rarely absent). ROOTS either branched fusiform taproots with many varied laterals, or unbranched taproots with sparse, thin laterals. BASAL LEAVES (<10)-10-17.5-(27)cm long, (0.6)-1.25-3-(6)cm wide, linear or linear and lanceolate or lanceolate (rarely ovate); petioles (3)-6-13-(20)cm long; margins entire (very rarely slightly dentate or undulate). CAULINE LEAVES (5)-9-14-20-(23)cm long, (0.5)-1-2-(2.5-4)cm wide, linear or linear and lanceolate (rarely elliptical), petioles (0)-2-7-(13-15)cm long; margins entire (very rarely serrate, dentate or undulate). LEAVES yellowish-green (very rarely deeper green, usually yellow on drying) with 3, or 3 and 5 (basal), or 3 and 1 (upper cauline) almost parallel major veins (very rarely also branching between); veins yellow, rarely clear or white. LEAF STALKED TRICHOMES multicellular (very rarely bicellular or both), short (very rarely medium or long), knobby or smooth (very rarely ledge-like or variable), appressed (very rarely partially hirsute), sparse (rarely dense), mostly abaxial or even on both surfaces (rarely adaxial only, sometimes very scarce or absent); with dense (or sparse and distal) papillate or striate surface ornamentation (very rarely absent); bulbous basal cells (sometimes non-bulbous); basal globules in one ring subtending trichome or lacking (very rarely two, multiple or uneven rings); sessile trichomes usually absent (rarely present); marginal trichomes more appressed than blade trichomes, or different from blade trichomes in type and/or habit (rarely identical). CAPITULESCENCE (1.5)-2-3-(4.5)cm tall, (<2.5)-2.5-3-(3.5)cm wide, conical or hemispherical receptacles (rarely elongated or very rarely flat-topped). INVOLUCRAL BRACTS in 2 series (very rarely 3), (<0.6)-0.6-1-(1.5)cm long, 0.2-0.4-(0.5)cm wide, reflexed parallel to stem or recurved (rarely perpendicular to stem), usually lacking abaxial trichomes (stalked sometimes present and sessile rarely present), and adaxial trichomes (stalked are very rarely present). RAY FLORETS are pale pink or dark pink or purple or yellow (very rarely white), (<2)-2-3.5-(4)cm long, (0.2)-0.3-0.6-(0.8)cm wide, reflexed parallel to stem axis or drooping 45^0 (rarely recurved tip to stem, or spreading perpendicular to stem); with stalked, sparse or dense abaxial trichomes (rarely absent or on both surfaces). RAY ACHENES with pappus of three major teeth (very rarely one major tooth), intermediate margin undulate, or unequal-toothed (very rarely entire or equal-toothed); trichomes present (very rarely absent) laterally on achene edges (rarely margin). DISK FLORETS green with pink tips (or entirely purple or yellowish, rarely white or other); tips usually reflexed, sometimes straight; petal fusion more than 3/4 total corolla length, or less than 3/4 total corolla length; purple style branches recurved or spreading (very rarely darkened), shorter than discoid bracts (rarely equal or longer), bases ball-shaped (very rarely disk-shaped or irregular-shaped); pollen yellow; corolla trichomes absent (when very rarely present, either on the base or tips). DISCOID BRACTS (0.8)-1-1.2-(1.4)cm long; awn bodies straight, or incurved, orange with red tips or brownish-purple or green with red tips (very rarely other colour combinations); awn dorsal keel present, rarely absent; keel stalked trichomes present, or absent; keel sessile trichomes absent. DISK ACHENES (0.2)-0.3-0.4-(0.5)cm long with or without vertical and horizontal brown striations (very rarely red-banded) and lateral trichomes on edges; with a pappus of one or four teeth (very rarely none or two on the acute-angled seams), intermediate margin equal-toothed (rarely undulate or entire).

VAR. *ATRORUBENS* ***Description***. See *E. atrorubens* Nutt. except for the following characteristics that distinguish this variety; STEMS branched (very rarely unbranched). LEAF STALKED TRICHOMES may be completely absent, but usually present sparsely. INVOLUCRAL BRACTS (0.1)-0.2-(0.3)cm wide. DISK FLORETS with petal fusion more than 3/4 total corolla length.

Distribution. Prairies and lowland stony-clay soils of TX and OK north to Topeka KS in narrow band. Hybridizes well with *E. pallida* var. *angustifolia* to form hybrid swarms in southeastern KS and also with *E. atrorubens* var. *neglecta* near Ardmore, OK. A possible parent to the introgressant form strigosa (see discussion under *E. pallida* var. *angustifolia*)(Figs. 2.7, 2.10).

Additional specimens examined. USA. KS: Allen Co. E of Woodson Co., 6 Jul 1999, Binns EAT99-0607-1 (DAO). KS: Coffey Co. NE of Burlington at Jct 50 and 75, 11 Jun 1960, Harms 336 (KANU). KS: Coffey Co. N of Burlington, 9 Jun 1955, McGregor 10429 (KANU). KS: Douglas Co. E of Big Springs, 13 Jun 1956, Lathrop and McGregor 2464 (KANU). KS: Douglas Co. N of Worden near Cemetary, 11 Jul 1998, Binns EAT98-0711-1 (DAO). KS: Geary Co. S of Junction City, 14 Jul 1998, Stephens 57025 (KANU). KS: Greenwood Co. W and S of Quincy, 13 Jun 1956, McGregor 12363 (KANU). KS: Greenwood Co. E and N of Fall River, 12 Jun 1956, McGregor 12360 (KANU). KS: Greenwood Co. NE of Neal, 12 Jul 1998, Binns EAT98-0712-1 (DAO). KS: Lyon Co. E of Admire, 13 Jun 1954, McGregor 9169 (KANU). KS: Osage Co. N of Olivet, 3 Jun 1987, McGregor 38244 (KANU). KS: Osage Co. S of Lyndon, 16 Jun 1994, Russell 150 (KANU). KS: Woodson Co. E and S of Toronto, 18 Jun 1958, Richards 602 (KANU). KS: Woodson Co. in wet roadside ditch, 11 Ju! 1998, Binns EAT98-0711-2 (DAO). OK: 24

Nov 1992, Aljinovic EAT597602 (DAO). OK: Murray Co. near Sulphur, 10 Aug 1997, McKeown EAT23883 (DAO). OK: Bryan Co. E of Durant, 28 May 1998, Binns EAT98-0528-1 (DAO). OK: Bryan Co. near Durant 9 Aug 1997, McKeown EAT23881 (DAO). OK: Garvin Co. S of Garvin, 6 Jun 1959, McGregor 14321 (KANU). OK: McCurtain Co. Idabel, 18 May 1916, Houghton 3642 (HUH). OK: Murray Co. NE of Sulphur on side road, 30 May 1998, Binns EAT98-0530-1(DAO). OK: Murray Co. S of Tishomingo, 29 May 1998, Binns EAT98-0529-1 (DAO). OK: Pushmataha Co. S of Antlers, 20 May 1967, McGregor 16846 (KANU). OK: Roger Mills Co. S of Cheyenne, 16 Jun 1948, Waterfall 7968 (HUH). TX: Wright 260 (HUH). TX: Lindheimer 120 (HUH). TX: Lindheimer 121 (HUH). TX: Brazoria Co. Angleton, 1 Jul 1945, Williams 27 (HUH). TX: Fort Worth, 28 Jun 1912, Ruth 243 (HUH). TX: Polk Co. Livingston, 23 May 1917, Palmer 1215 (HUH). TX: Tarrant Co., 18 Jun 1914, Ruth 243 (HUH). TX: Washington Co. 21 May 1998, Brackett 280V (HUH).

VAR. *PARADOXA* (J. B. Norton) Cronq., Rhodora 47:553:398. 1945. TYPE: MO, Swan. B.F. Bush, 1898. no. 155. (holotype: MO, isotypes: HUH! and NY!) ***Homotypic synonyms***. *Brauneria paradoxa* J. B. Norton, Kans. Acad. Sci. St. Louis. 12:40. 1902. ***Description***. See *E. atrorubens* Nutt. (above) and the following characteristics distinguishing this variety; STEMS are unbranching and 60-95 cm, which is taller than *E. atrorubens* var. *atrorubens*. STEM STALKED TRICHOMES short, joints may also be ledge-like (unlike other varieties); basal cell bulbous and basal globules may be absent (or present as in the other varieties). LEAVES with yellow veins. LEAF STALKED TRICHOMES short, joints ledge-like (or knobby); sessile trichomes present (rarely absent). INVOLUCRAL BRACTS 0.8-1.2 cm, longer than *E. atrorubens* var. *atrorubens* (on average). RAY FLORETS always bright yellow and 3.1-4 cm long.

Distribution. Ozarkian uplands of western MO, dipping into AR; endemic in limestone glades, bald knobs and outcrops, rare. Federal protective status pending (U.S. Fish and Wildlife Service; McKeown, pers. comm. 1999)(Fig. 2.10).

Additional specimens examined. USA. MO: Barry Co. 3 mi SE Jct. 112 and F, 11 Jun 1958, McGregor 13889 (KANU). MO: Barry Co. 3 mi SE of jct of 112 and F in Roaring Rv State Pk on F, 12 Jun 1957, Lathrop 3776 (KANU). MO: Benton Co. just E of Hwy 65, 5 Jun 1998, Binns and Arnason EPP 98-0605-1 (DAO). MO: Benton Co. S of Sedalia, 6 Jun 1998, Binns EPP 98-0606-1 (DAO). MO: Benton Co. S of Lincoln, 5 Jun 1999, Binns EPP 99-0605-1B (DAO). MO: Benton Co. S of Lincoln, 5 Jun and 23 Aug 1998, Binns and Arnason EPP 98-0605-2, EPP 0823 (DAO). MO: Camden Co. SE of jct 42 and 134, 6 Jun 1998, Binns,EPP 98-0606-2 (DAO). MO: Phelps Co. 0.5 mi N Rolla, 8 Jun 1960, McGregor 15642 (KANU). MO: Taney Co. W of Branson, 10 Jun 1960, McGregor 13873 (KANU). MO: Taney Co. Swan, common on Big Baldy Bush 155 (HUH and NY).

VAR. *NEGLECTA* (McGregor) Binns B. R. Baum & Arnason *comb. nov.*

Echinacea paradoxa (Nutt.) var. *neglecta* McGregor Trans. Kans. Acad. Sci. 70: 370. 1967. HOLOTYPE: USA. OK: Murray Co., Platt National Park, 7 Jun 1959, McGregor 14324 (KANU!). ***Description.*** See *E. atrorubens* Nutt. and the following distinguishing characteristics for the variety; STEMS 65-75 cm tall (taller than *E. atrorubens* var. *atrorubens* on average), unbranched. ROOTS fusiform, branching taproots with many thick laterals (very rarely untapered taproot with thin laterals). LEAVES 14-20 cm long (basal ones); usually 5 almost parallel veins in basal leaves and 3 in cauline leaves (rarely only 3 in both). LEAF STALKED TRICHOMES sometimes dense (or sparse) and may be mostly adaxial or mostly abaxial, or even on both surfaces. INVOLUCRAL BRACTS (0.2)-0.3-0.4-(0.5)cm wide. RAY FLORETS pale pink to white, 3-4 cm long, 0.35-0.6 cm wide and reflexed parallel to stem axis; ray stalked trichomes abaxial, or on both surfaces. DISK FLORETS with purple to yellowish, or rarely green corolla tips; petal fusion less than 3/4 total corolla length (rarely more than 3/4 total length); styles shorter, longer or equal to discoid bracts.

Distribution. Endemic to rocky upland prairies and openings of wooded hillsides in

Arbuckle Mountains, OK. Hybridizes frequently with *E. atrorubens* var. *atrorubens* and *E. pallida* var. *angustifolia*. Colonies often heterogenously composed of mixed forms and intermediates with introgressed characteristics; hirsute stems, lemon-yellow pollen, longer purple or white rays or elongated receptacles with minute rays, and glabrous abaxial leaf blades of *E. atrorubens* var. *atrorubens*. (Fig. 2.10) Federal protection pending (U.S. Fish and Wildlife Service).

Additional specimens examined. USA. OK: Johnston Co. SW of Mill Creek, 21 Sep 1997, Binns and St-Laurent 5,6,EPN-2,EPN-3,EPN 98-0529-2 (DAO). OK: Marshall Co. E of Kingston, 7 Jun 1958, Richards 546 (KANU). OK: Marshall Co. E of Kingston on sideroad, 29 May 1998, Binns and Arnason EPN 98-0529-1 (DAO). OK: Murray Co. S of entrance to Turner Falls Park, 8 Aug 1966, Stephens 8433 (KANU). OK: Murray Co. S of Turner Falls road, 7 Jun 1958, Richards 542 (KANU). OK: Murray Co. S of Sulphur, 29 May 1960, McGregor 15606 (KANU). OK: Murray Co. hillside above Turner Falls, 7 Jun 1959, McGregor 13900 (KANU). OK: Murray Co. near Sulphur, 10 Aug. 1997, McKeown EPN 23886 (DAO). OK: Murray Co. S of Sulphur,30 May 1998, Binns EPN 98-0530-1 (DAO). OK: Murray Co. Platt National Park, 13 Jun 1958, McGregor 13900b (KANU). OK: Murray Co. Platt National Park, 7 Jun 1959, McGregor 14323 (KANU).

ECHINACEA LAEVIGATA (C. L. Boynton & Beadle) S. F. Blake, Jour. Wash. Acad. Sci. 19: 273. 1929 [10]. TYPE: USA. SC: Seneca, June 1888, Gerald McCarthy (holotype: US no. 26903, photo!). ***Homotypic synonyms***. *Brauneria laevigata* C. L. Boynton & Beadle in Small, Fl. S.E. United States 1: 1261, 1340. 1903. *Echinacea purpurea* (L.) var. *laevigata* Cronq. Rhodora 47:397. 1945. ***Heterotypic Synonyms***. *Rudbeckia purpurea* L. Sp. Pl. 2: 907. 1753 (before proposed conservation of the name in its current use, see chapters 3 and 4). -TYPE: USA. VA: 1025 no. 7 (lectotype: LINN at IDC, DAO, photo!, designated in McGregor 1968; paralectotypes: Clayton 417, BM, photo!; Plukenet, 1696 alm. t. 21. f. 1., illust.!; Morrison, 1699 hist. vol. 3: sect. 6: t.9: f.1., illust.!; Catesby, 1743 car. t. 59., photo!). *E. purpurea* (L.) Moench (in Darby's Botany of the S. States, 1857. Barnes and Co. Cinn. OH). ***Description***. STEMS usually (35)-60-84-95-(110)cm tall, even width throughout or wider below receptacle, dark green or brownish-green (very rarely whitish or reddish), furrowed or unfurrowed (rarely furrowed just below involucre); pith usually present throughout or only absent in peduncle (rarely absent throughout); peduncle less than half total stem height (very rarely greater than/equal to half stem height). ROOTS are branching, fusiform taproots, with many varied laterals. STEM STALKED TRICHOMES absent [11]; sessile trichomes present or absent. BASAL LEAVES (12)-14-15-(18)cm long, (3)-4.5-6 cm wide, lanceolate or lanceolate-ovate (very rarely ovate or linear-lanceolate), margins serrate (rarely dentate and very rarely undulate); petioles (4)-6-13-16-(26)cm long. CAULINE LEAVES (9)-10.5-12.5-13.5-(15)cm long,(1.5)-2.5-3.75-4.5-(5)cm wide, lanceolate (very rarely lance-ovate), margins serrate (very rarely undulate or mostly entire); with petioles (0)-2-8-(11)cm long. LEAVES dark green or bright green with three, or three and five (basal) or three and one (cauline) major veins which are clear, yellow, or darkened and almost parallel or branching in between. LEAF STALKED TRICHOMES absent (always glabrous abaxially but very rarely present adaxially)[11]; sessile trichomes usually absent, sometimes present; marginal trichomes different from blade trichomes in type and/or habit (always present even if none on blade). CAPITULESCENCE (<2)-2-2.5-3-(3.5)cm tall, 2-3-(4) cm wide, conical or spherical (rarely flat-topped) receptacle. INVOLUCRAL BRACTS in (1)-2-3 series, (0.3)-0.5-1.1-(1.5)cm long, 0.2-0.3-(0.5)cm wide, reflexed parallel or recurved or perpendicular to stem; abaxial and adaxial stalked trichomes absent (very rarely present); sessile trichomes present abaxially only (rarely absent and very rarely present adaxially). RAY FLORETS pale or dark pink to purple, (1.5)-4.0-6.5-(7.5)cm long; (0.2)-0.3-0.5-(0.6-0.9)cm wide, drooping 45^0 to stem or reflexed parallel to stem (very rarely perpendicular or recurved to stem); ray trichomes sparse (very rarely dense) abaxially (rarely absent); ray achenes with pappus of three major teeth, intermediate margin undulate or unequal-toothed (rarely entire, and very rarely equal), trichomes present laterally on achene edges (rarely absent).

DISK FLORETS with purple or green and purple tips straight (rarely reflexed); trichomes absent (very rarely present); petal fusion more than 3/4 total corolla length, rarely less than 3/4 total corolla length; purple styles (very rarely black) with recurved or spreading branches, shorter than discoid bracts (very rarely equal to or longer than bracts), bases ball-shaped (very rarely irregular); pollen yellow. DISCOID BRACTS (0.7)-1.0-1.1-(1.3)cm long, with straight or incurved awn bodies (rarely both); tips orange and red or brownish-purple (very rarely green and red); dorsal keel present (very rarely absent) with stalked trichomes (or without); sessile keel trichomes absent (very rarely present). DISK ACHENES 0.3-0.4 cm long, usually lacking vertical striation but having brown horizontal bands; pappus of one tooth (rarely four, very rarely one) with toothed intermediate margin (rarely equally or unequally undulate); stalked or sessile trichomes rarely present on achene lateral edges.

Distribution. Open wooded hillsides and well-drained rocky, upland soils of VA, NC, SC and GA (Figs. 2.7, 2.11). Federally protected taxon (U.S. Fish and Wildlife Service, listed 8 Oct 1992).

Additional specimens examined. USA. GA: Dry woods on southern slope of Lee Mountain, 13 Jun 1956, Bookout Jr. 34 (HUH). GA: Stephens Co. Currahee Mtn, SW of the city of Toccoa, 21 Jul 1975, Wood and Boufford 2149 (HUH). GA: Stephens Co. slopes N of Currahee Mtn. 29 Jun 1950, Duncan 11209 (HUH). GA: Stephens Co. Currahee Mtn., 14 Jul 1959, McGregor 14541 (KANU). NC: Durham Co. NE of Braggtown, 12 May 1950, Fox and Anderson 50387 (HUH). NC: Durham Co. field near Eno River NE of Braggtown, 16 Jun 1966, Radford 44749 (HUH). NC: Durham Co. N of Durham, 18 Jun 1951, Godfrey and Fox 51165 (HUH). NC: Granville Co. near Butner, 3 Sep 1997, McKeown EL 23969 (DAO). SC: Lancaster Co. Roadside woods N of 40 Acre Rock, 4 Jun 1973, Boufford 9801 (KANU). SC: Lancaster Co. N of Kershaw, near 40 Acre Rock, 5 Jun 1973, Ahles 77404 (KANU). VA: SE of Pulaski, 1 Sep 1946, Wood Jr 6746 (HUH).VA: Franklin Co. Grassy Hill, 24 Sep 1998, Ludwig, Wiebolt and Meyers EL 0924

(DAO). VA: Franklin Co. N of Rocky Mount, 17 Jun 1998, Binns and Campbell EL98-0617-1 (DAO). VA: Montgomery Co. W of Shawsville, 27 Jun 1938, Massey 2015(HUH). VA: Roanoke Co. Fort Lewis Mtn, NNW of Dixie Caverns, 13 Jul 1942, Wood Jr 3921 (HUH). VA: Wytheville, Shriver 453/3(HUH).

Notes.

[1] The recently designated lectotype for *E. purpurea* (L.) Moench, *nom. cons. prop.* was: U.S.A. Arkansas: Nuttall "*Echinacea serotina* Arkansa" (BM no. 541360!)(chapter 4). This specimen was chosen in consideration of Nuttall's first description of *R. purpurea* var. *serotina* (Gen. N. Am. Pl. 178. 1818), and will only become the valid type for *R. purpurea* L., and ipso facto for the generic name *Echinacea*, pending the recommendation of the Committee for Spermatophyta to conserve this name, and subsequent acceptance by an International Botanical Congress. Nuttall collected *R. purpurea* var. *serotina* on lower plains of Louisiana (Nuttall 1818) and Arkansas, which was indicated on the lectotype (for a discussion of lectotype designation, please see chapter 3). DeCandolle (1836) elevated the Nuttall variety to the species *Echinacea serotina* (Nutt.) DC.

[2] *Brauneria* is not considered to be validly published by Necker (1790). Article 20.4 of the *Code* (Greuter *et al.* 2000) stated that Necker's names were to be considered "unitary designations of species, and not generic names." However, *Brauneria* Necker ex T. C. Porter and N. L. Britton was published in 1894, and it has status under the *Code* wo that any species names described first in *Brauneria* must be taken into account for purposes of priority (Art. 55.1, the *Code*).

[3] The new combinations *Brauneria purpurea* (L.) Britton and *Brauneria pallida* Britton are legitimate names according to note [2], since Britton took up what he considered to be the earlier name (from Necker, 1790).

[4] John Lindley (Paxt. Mag. Bot. 15:79. 1849) described a new species, *E. intermedia* which was "closest in habit to *E. speciosa* Link.(= *R. speciosa* DC.)".

Examination of "*R. speciosa*" from DeCandolle's treatment (Prodr. 5:556. 1836) revealed two *R. speciosa* references. One was clearly a true *Rudbeckia* (Wenderoth in Syn. Flora I:30. 1829; Prodr. 5: 556. 1836), while the other, *R. speciosa* Link. (Enum. Pl. Hort. Reg. Bot. Berol. Alt., I: 352. 1821) was a synonym to DC's new species of *Echinacea*, namely *E. serotina* (Nutt.) DC. *E. intermedia* = *E. serotina* (Nutt.) DC. (or *R. speciosa* Link.) and not ≠ *R. speciosa* (Wender.) DC.

[5] *R. hispida* Hoffmansegg was placed in synonymy under *E. serotina* by DC., Prodr. 5: 554. 1836.

[6] The *E. pallida* var. *pallida* type sheet contains two specimens, and the original label reads "*Echinacea pallida* Arkansas Hb. Nuttall" in Nuttall's script. The name applies to the holotype on the right; the specimen on the left is clearly not the type material having the original label "*Gaillardia*, near Souris River, Manitoba" at the top of the page; and representing *E. pallida* var. *angustifolia*.

[7] McGregor (1968) suggested that the "Red River Plains" were in fact found at the confluence of the Kiamichi and Red rivers in South Central Oklahoma.

[8] *E. speciosa* McGregor was used to describe a new species in 1967. However, Lindley (Paxt. Mag. Bot., 1849) had already used the name prior to that time (see Note 3), so it was an illegitimate name on publication and was shortly afterwards renamed *E. simulata* (McGregor, Sida 3:282. 1968).

[9] Two of the isotype specimens cited by McGregor (1968) for *E. pallida* var. *tennessensis:* Eggert 6495 (HUH!) and Eggert 162740 (NY!), were not from the same collection date as the holotype, and therefore cannot be included as isotypes of that name.

[10] We have taken the liberty to distinguish C. L. Boynton from F. E. Boynton and Kenneth Rolande Boynton as listed in Authors of Plant Names.

[11] Some artificial hybrids (*E. laevigata* × *E. simulata sensu* McGregor) have multicellular, medium-long, bulbous hairs.

EXCLUDED TAXA

E. ATRORUBENS VAR. *GRAMINIFOLIA* = ***Rudbeckia graminifolia*** (Torrey & Gray) C. L. Boynton & Beadle (Small's Fl. S.E. U.S. 1257. 1903)

In 1901, C. L. Boynton & Beadle (Bilt. Bot. Stud. 1: 11-12) discovered an error by Torrey & Gray (Fl. N. Amer. 306. 1842) who applied the name *Echinacea atrorubens* var. *graminifolia* to a variety of *Rudbeckia* found in the South Atlantic states. Torrey & Gray had misinterpreted Nuttall's descriptions of a true *Echinacea* (*R. atrorubens* Nutt. in J. Nat. Acad. Sci. Phil. 7:80. 1834 and *E. atrorubens* Nutt. in Trans. Am. Phil. Soc. 7:354. 1841). The error was compounded by Chapman in his Flora of the Southern U.S. (1st Ed. 226. 1860 and 2nd Ed. 1889), and in Darby's Botany of the Southern States (Part 1. 386. 1860). Furthermore, Gray later misapplied the name *Rudbeckia atrorubens* to a true *E. atrorubens* Nutt. (Syn. Flora II:259. 1886), which was also discussed by C. L. Boynton & Beadle (Bilt. Bot. Stud. 1:11-12. 1901).

Figure. 2.7. Distribution of all four revised *Echinacea* Moench species (including populations with hybrid characteristics) from germplasm collections and herbarium specimens analyzed for the present work.

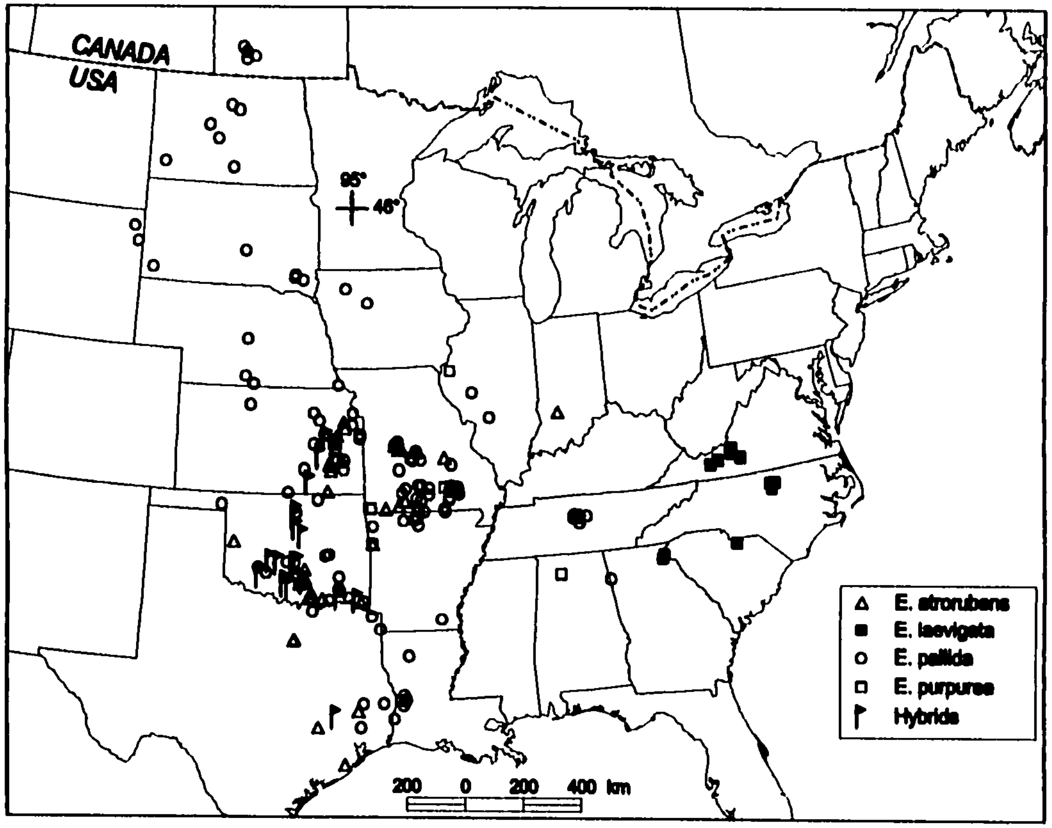

CANADA
USA
95°
46°
E. atrorubens
E. laevigata
E. pallida
E. purpurea
Hybrids
200 0 200 400 km

Figure 2.8. Distribution of revised *Echinacea purpurea* from germplasm collections and herbarium specimens analyzed for the present work.

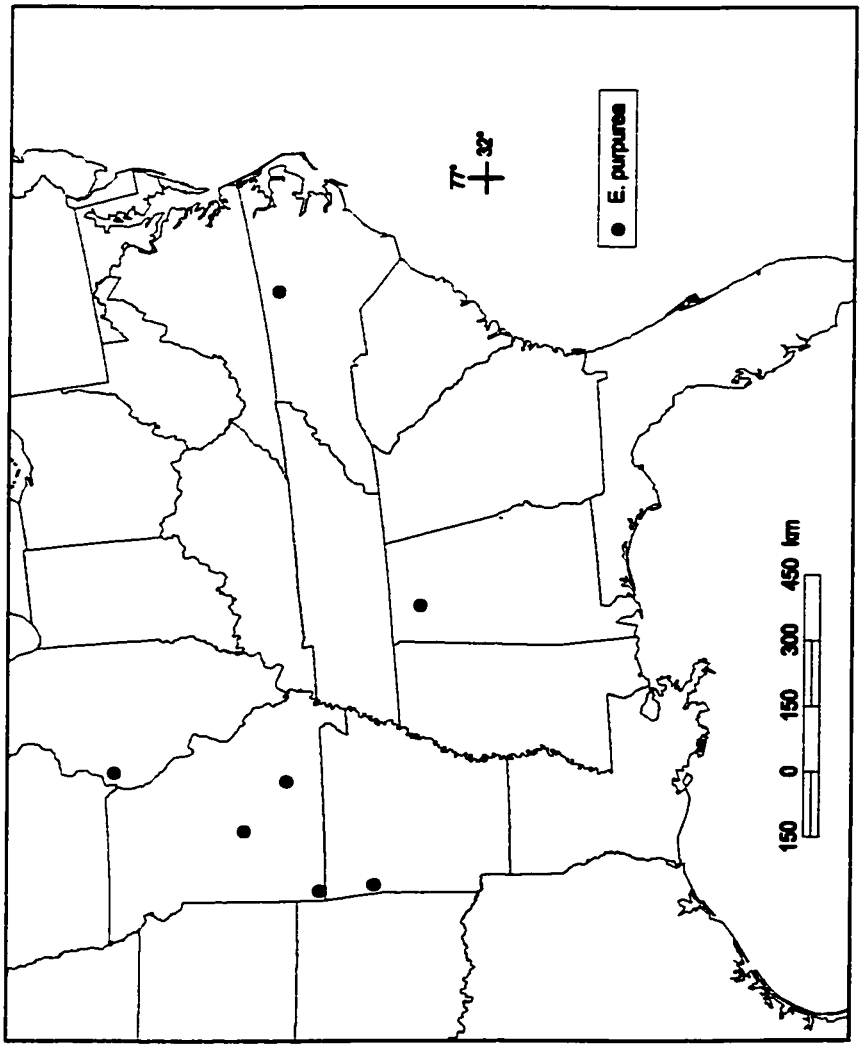

● E. purpurea
77°
32°
150 0 150 300 450 km

Figure 2.9. Distribution of revised *Echinacea pallida*, including five varieties; *E. pallida* var. *angustifolia*, *E. pallida* var. *pallida*, *E. pallida* var. *sanguinea*, *E. pallida* var. *simulata* and *E. pallida* var. *tennesseensis* from germplasm collections and herbarium specimens analyzed for the present work.

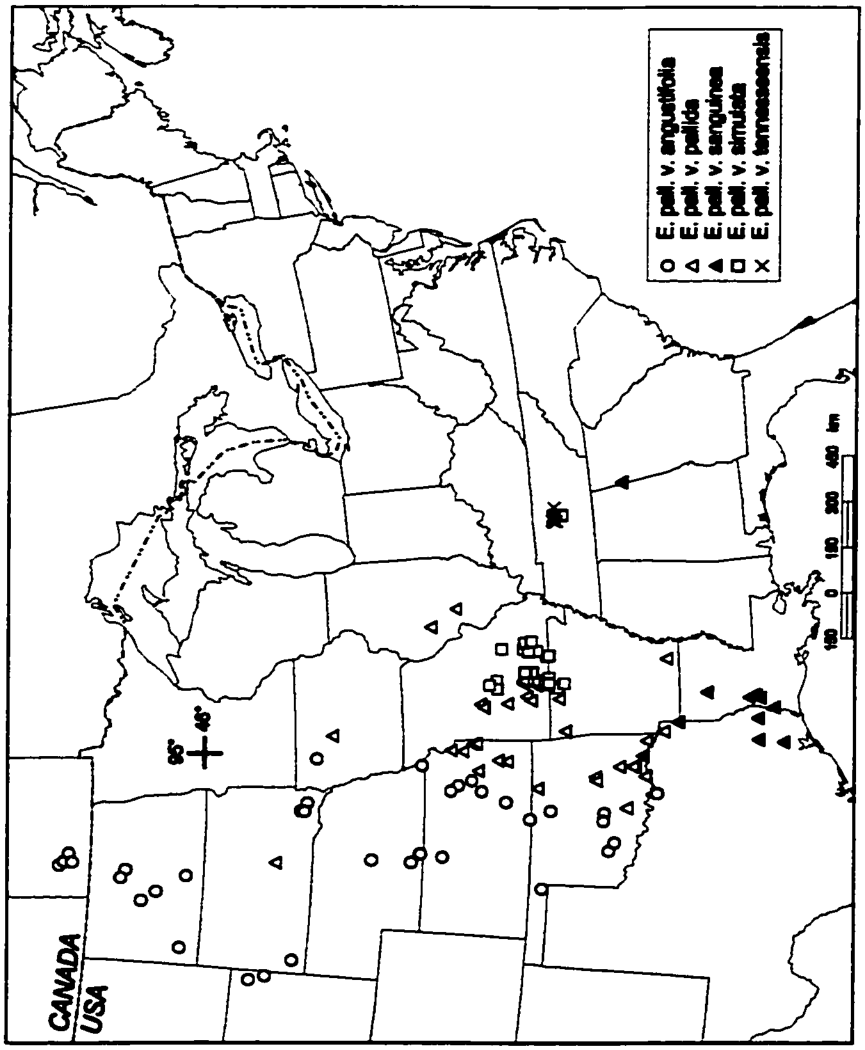

E. pall. v. angustifolia
E. pall. v. pallida
E. pall. v. sanguinea
E. pall. v. simulata
E. pall. v. tennesseensis
160 0 160 320 480 km
CANADA
USA
95°
45°

Figure 2.10. Distribution of revised *Echinacea atrorubens*, including three varieties; *E. atrorubens* var. *atrorubens*, *E. atrorubens* var. *neglecta* and *E. atrorubens* var. *paradoxa* from germplasm collections and herbarium specimens analyzed for the present work.

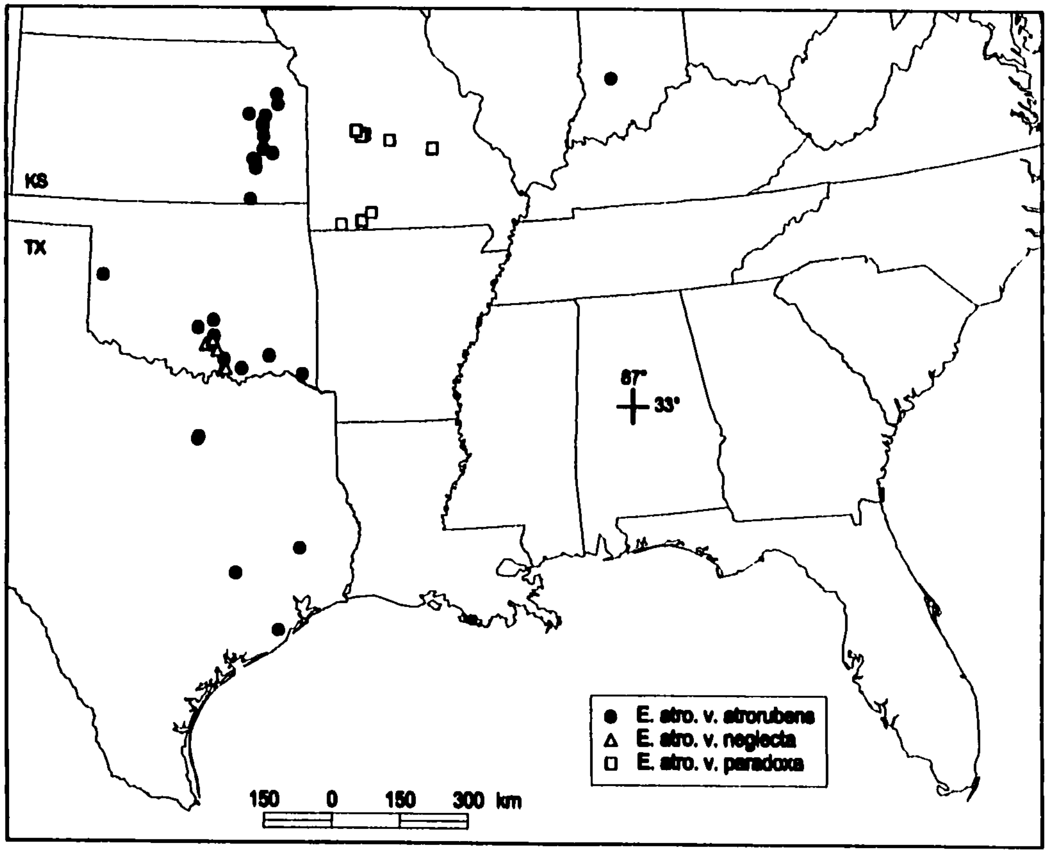
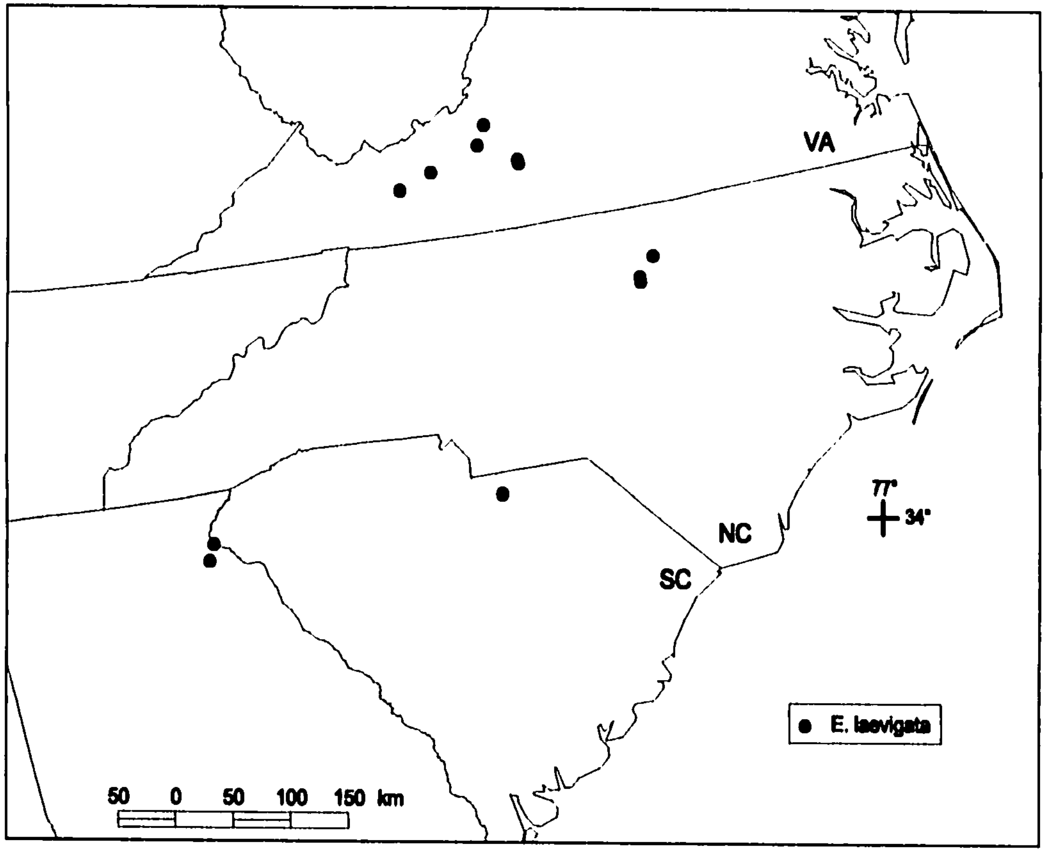

KS
TX
87°
33°
● E. atro. v. atrorubens
△ E. atro. v. neglecta
□ E. atro. v. paradoxa
150
0
150
300
km

Figure 2.11. Distribution of revised *Echinacea laevigata* from germplasm collections and herbarium specimens analyzed for the present work.


VA
NC
SC
77°
34°
E. laevigata
50 0 50 100 150 km

Key to Subgenera, Species and Varieties of *Echinacea*

1. Basal leaf greater than 5 cm wide; cauline leaf 4.5 to 9 cm wide; fibrous roots (from a caudex); leaf blade trichomes bicellular with ledge-like joints; major veins branched; four series of involucral bracts = subg. *Echinacea*, *E.purpurea* (L.) Moench *nom. cons. prop.*

1. Basal leaf up to 5 cm wide; cauline leaves 0.5 to 4.5 cm wide; taproot (may be branching or fusiform); leaf blade trichomes multicellular with knobby joints; major veins almost parallel from a common origin at the base; one to three series of involucral bracts **2.** subg. *Pallida*

2. Basal leaf greater than 3 cm wide; basal leaf margin serrate, or dentate; adaxial leaf blade stalked trichomes absent; stem stalked trichomes absent; cauline leaf margin serrate = *E. laevigata* (C.L. Boynton and Beadle) Blake

2. Basal leaf up to 3 cm wide; basal leaf margin entire; adaxial leaf blade stalked trichomes present; stem stalked trichomes present; cauline leaf margin entire **3**

3. Stem stalked trichomes appressed (strigose); leaf blade stalked trichomes sparse; leaf marginal trichomes different than blade trichomes (more appressed) **4**

4. Ray floret, yellow = *E. atrorubens* Nutt. var. *paradoxa* (J. B. Norton) Cronq.

4. Ray floret, pale pink, or dark pink to purple, or white 5

5. Disk corolla petal fusion more than 3/4 total corolla length; involucral bract up to 0.2 cm wide; stem branched = *E. atrorubens* Nutt. var. *atrorubens* Cronq.

5. Disk corolla petal fusion less than 3/4 total corolla length; involucral bract greater than 0.2 cm wide; stem unbranched = *E. atrorubens* Nutt. var. *neglecta* (McGregor) Binns, B. R. Baum & Arnason

3. Stem stalked trichomes hirsute, or straight pubescent; leaf blade stalked trichomes dense; leaf marginal trichomes identical to leaf trichomes in type and habit 6

6. Ray floret, up to 4 cm long 7

7. Capitulum, up to 2.5 cm wide; involucral bract up to 0.2 cm wide = *E. pallida* (Nutt.) Nutt. var. *tennesseensis* (Beadle) Binns, B. R. Baum & Arnason

7. Capitulum greater than 2.5 cm wide; involucral bract greater than 0.2 cm wide = *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronq.

6. Ray floret, greater than 4.0 cm 8

8. Fresh pollen, white = *E. pallida* (Nutt.) Nutt. var. *pallida* Gandhi and Thomas

8. Fresh pollen, yellow or lemon **9**

  9. Ray achene trichomes present; stem unbranched = *E. pallida* (Nutt.) Nutt. var. *simulata* (McGregor) Binns, B. R. Baum & Arnason

  9. Ray achene trichomes absent; stem branched. *E. pallida* (Nutt.) Nutt. var. *sanguinea* (Nutt.) Gandhi & Thomas

Alternative Key to Species and Varieties of *Echinacea*

The following key was also prepared from the simple statistics generated using the current dataset and OTUs, tentatively identified according to McGregor (1968) and shown using CDA to be distinct clusters. It provides alternative characters and steps to reach the same end taxa as the previous key. McGregor's nomenclature is in square brackets for ease of comparison with the names in current use.

1. Involucral bract abaxial sessile trichomes present 2

2. Basal leaf margins serrate; discoid bract keel sessile trichomes present = *E. laevigata* (C. L. Boynton and Beadle) S. F. Blake
2. Basal leaf margins entire (rarely dentate or undulate); discoid bract keel sessile trichomes absent 3

3. Ray floret, yellow = *E. atrorubens* Nutt. var. *paradoxa* (J. B. Norton) Cronq.
[*E. paradoxa* (J. B. Norton) Britton var. *paradoxa*]

3. Ray floret, pale pink, dark pink, purple, or white = *E. atrorubens* Nutt. var. *neglecta* (McGregor) Binns, B. R. Baum & Arnason [*E. paradoxa* (J. B. Norton) var. neglecta McGregor]

1. Involucral bract abaxial sessile trichomes absent 4

4. Fresh pollen, white = *E. pallida* (Nutt.) Nutt. var. *pallida* [*E. pallida* (Nutt.) Nutt.]

4. Fresh pollen, yellow or lemon 5

5. Cauline leaf greater than 3.5 cm wide = *E. purpurea* (L.) Moench *nom. cons. prop.* [*E. purpurea* (L.) Moench]

5. Cauline leaf up to 3.5 cm wide 6

6. Involucral bract up to 0.2 cm wide 7

7. Stem stalked trichomes appressed; involucral bract abaxial stalked trichomes absent = *E. atrorubens* Nutt. var. *atrorubens* Cronq. [*E. atrorubens* Nutt.]

7. Stem stalked trichomes hirsute, or straight pubescent; involucral bract abaxial stalked trichomes present 8

8. Ray floret, up to 4 cm long; stem stalked trichome ornamentation (markings or papillae) even over entire surface = *E. pallida* (Nutt.) var. *tennesseensis* (Beadle) Binns, B. R. Baum & Arnason [*E. tennesseensis* (Beadle) Small]

8. Ray floret, greater than 4.cm; stem stalked trichome ornamentation (markings or papillae) less dense or absent on basal cell = *E. pallida* (Nutt.) Nutt. var. *simulata* (McGregor) Binns, B. R. Baum & Arnason [*E. simulata* McGregor]

6. Involucral bract greater than 0.2 cm wide **9**

9. Ray achene trichomes, absent = *E. pallida* (Nutt.) Nutt. var. sanguinea (Nutt.) Gandhi & Thomas [*E. sanguinea* Nutt.]

9. Ray achene trichomes, present **10**

10. Ray floret greater than 4 cm long = *E. pallida* (Nutt.) var. *simulata* (McGregor) Binns, B. R. Baum & Arnason [*E. simulata* McGregor]

10. Ray floret, up to 4 cm long **11**

11. Leaf stalked trichome basal cell, non-bulbous = *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronq. [*E. angustifolia* DC. var. *angustifolia* and *E. angustifolia* DC. var. *strigosa* McGregor]

11. Leaf trichome basal cell, bulbous = *E. atrorubens* Nutt. var. *neglecta* (McGregor) Binns, B. R. Baum & Arnason [*E. paradoxa* (J. B. Norton) Britton var. *neglecta*]

Key Characters and Further Notes on Classification

When geographical source data are unavailable, ray floret colour is the only reliable way to morphologically determine specimen identity between *E. atrorubens* var. *neglecta* and *E. atrorubens* var. *paradoxa*. Other characteristics distinguish var. *atrorubens* from the other two varieties. For example, ray floret length (Table 2.2, no. 9) ranges from 1.5 to 2 cm (rarely to 3 cm) in var. *atrorubens* while var. *neglecta* ranges from 3.25 to 4 cm (rarely 2.75 cm) and var. *paradoxa* ranges from 3.25 to 4 cm (rarely 2.25 cm). In addition, basal leaf width (no. 52) is greater than 2 cm in var. *atrorubens* but usually less than 2 cm in the other varieties. Clearly, there is some overlap, but generally the ray florets are shorter and the basal leaves are wider in var. *atrorubens*. Finally, the presence of pith (no. 70) in the upper peduncle indicates either var. *paradoxa* or var. *neglecta*. The above is useful for the purpose of identifying var. *atrorubens* and var. *neglecta* in the Arbuckle mountains of Oklahoma where many populations are in close proximity, and hybrid swarms exist.

The microscopic characters of disk corolla [habit (no. 25), petal fusion (no. 24), and vestiture (no. 22)] also serve nicely to determine var. *atrorubens* from the other two varieties in that var. *atrorubens* corollae are glabrous (very rarely hairy), fused more than 3/4 total corolla length with straight tips, while the other varieties are hairy (rarely glabrous), fused less than 3/4 total corolla length and have reflexed tips.

Colour photographs of *Echinacea*

The first plate illustrates the four revised species: *E. purpurea*, *E. laevigata*, *E. pallida* var. *pallida*, and *E. atrorubens* var. *atrorubens*.

The second plate illustrates selected photos of some of the varieties of *Echinacea* determined in Chapter 2 of this work.

Photo by Shannon Binns

E. purpurea auct. (non L.)
nom. cons. prop.

Photo by Shannon Binns

Photo by Shannon Binns

E. pallida (Nutt.) Nutt.
var. *pallida*

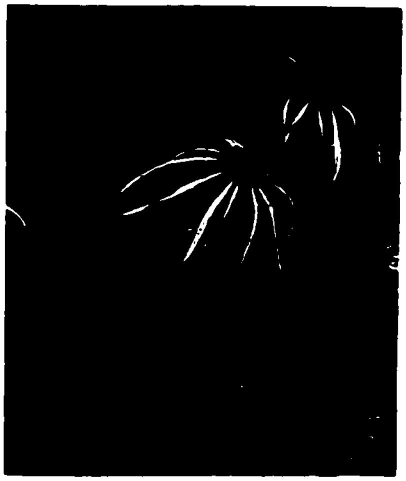


Photo by Steven Foster (with permission)

E. laevigata (Boynton & Beadle)
Blake

Photo by Shannon Binns

E. atrorubens Nutt. var. *atrorubens*

Photo by Steven Foster (with permission)

E. pallida (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist

Photo by Shannon Binns

E. atrorubens Nutt. var. neglecta (McGregor) Binns, Baum & Arnason

Photo by Shannon Binns

E. pallida (Nutt.) Nutt. var. *tennesseensis* (Beadle) Binns, Baum & Arnason

Photo by Steven Foster (with permission)

E. pallida (Nutt.) Nutt. var. *simulata* (McGregor) Binns, Baum & Arnason

Chapter 3: TYPIFICATION OF *ECHINACEA PURPUREA* (L.) MOENCH (HELIANTHEAE: ASTERACEAE) AND ITS IMPLICATIONS ON THE CORRECT NAMING OF TWO *ECHINACEA* TAXA

This chapter was submitted for publication in the journal Taxon, in collaboration with Dr. B.R. Baum and Dr. J.T. Arnason. It was in press at the time of preparation of the current thesis, in which some modifications were made to the original manuscript for formatting purposes.

Introduction and Historical Background

Linnaeus described *Rudbeckia purpurea* L. in 1753, on which Moench based the genus *Echinacea* in 1794. Then, in 1818, Nuttall suggested that *R. purpurea* L. contained a second variety and he described *R. purpurea* L. var. *serotina*. It was DeCandolle, in 1836, who first recognized two distinct species from one: *E. purpurea* (L.) Moench *sensu typo* and *E. serotina* (Nutt.) DC. Ignoring DeCandolle's treatment, many to follow circumscribed *E. purpurea* broadly, suggesting to me that both entities were included therein. For a detailed chronological overview, see Table 3.1. Boynton and Beadle, in Small (1903), again distinguished two species from one, namely *E. laevigata* (as *Brauneria laevigata* C. L. Boynton & Beadle) and *E. purpurea* (as *Brauneria purpurea* (L.) Britton), but DeCandolle's treatment of these two species was ignored. As a result, *E. purpurea*, from 1903 until today, refers to a species matching DeCandolle's description of *E. serotina* (Nutt.) DC., not his *E. purpurea* (L.) Moench (Fig. 3.1). I refer henceforth to DeCandolle's *E. serotina* (Nutt.) DC. as *E. purpurea* auct., and to the taxon *E. purpurea* (L.) Moench *sensu typo* as it is presently called, *E. laevigata* (C. L. Boynton & Beadle) Blake. I will demonstrate that *E. laevigata* was created only when the name *E. purpurea* became incorrectly applied to the taxon *E. purpurea* auct., and that the two recognized species were first legitimately named prior to the application of the type method. The two species were distinguished according to the characteristics in Table 3.2.

There is a need to assess and clarify the typification of the names for the two taxonomic species recognized by DeCandolle (1836), Small (1903), McGregor (1968), and also in my own recent taxonomic revision of the genus (chapter 2). I acknowledge McGregor's (1968) lectotypification, in spite of several difficulties discussed below, including the fact that his designated lectotype of *E. purpurea* (L.) Moench *sensu typo* does not match his description and other examined specimens, which are clearly *E. serotina* (Nutt.) DC. I report several characteristics, which demonstrate the taxonomic differences between *E. purpurea* auct. and *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake; eight of them were clearly observed in the respective lectotype specimens (see Table 3.2). The purpose of this article is to examine the typification of *E. purpurea* (L.) Moench, and thereby present my investigation of all the names and types implicated in the two taxa.

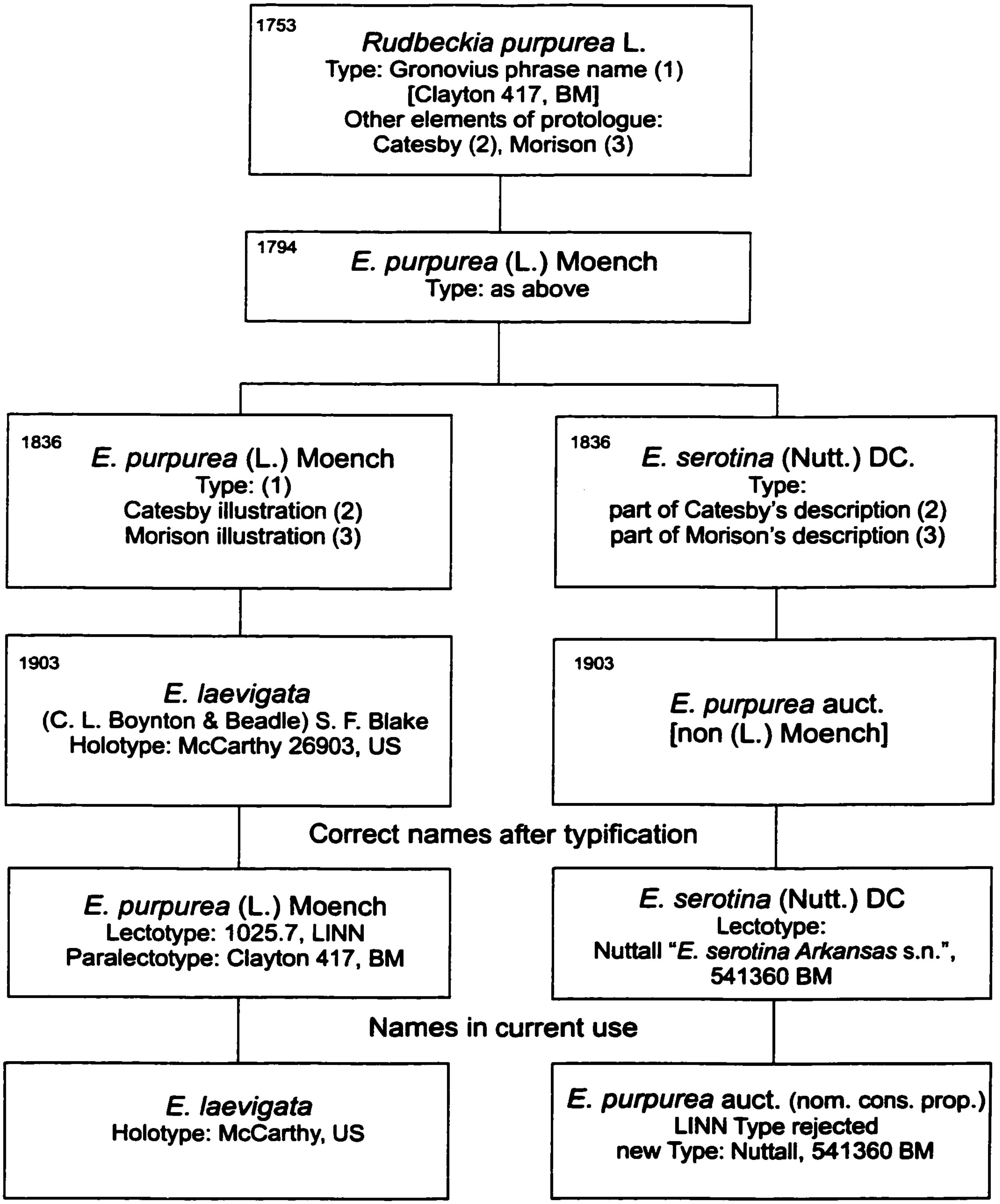
Table 3.1: Summary of the nomenclatural history of *Echinacea purpurea* (L.) Moench *sensu typo* and *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake.

	Pre-Linnean History:
1696	Plukenet described *Chrysanthemum americanum, doronici folio, flore perfici coloris: umbone magno prominente ex atro purpureo viridi & aureo fulgente* from Georgia.
1699	Morison described *Dranunculus virginianus latifolius, petalis florum longissimus purpurascentibus*
1739	Gronovius described *Rudbeckia purpurea foliis lanceolato-ovatis alternis indivisis, petalis radii bifidis* from Virginia and listed specimen no. 417 (now in Clayton Herb., BM)
1743	Catesby described *Chrysanthemum americanum* , *doronici folio, flore perfici coloris: umbone magno prominente ex atro purpureo viridi & aureo fulgente* from Georgia.
1753	Linnaeus described *Rudbeckia purpurea* using the exact phrase name of Gronovius (1739) and citing phrase names by Plukenet (1696), Morison (1699), and Catesby (1743).
	Post-Linnean History:
1790	Necker described the genus *Brauneria* [no species mentioned].
1794	Moench described *Echinacea purpurea* L. with the new combination *E. purpurea* (L.) Moench.
1818	Nuttall described *Rudbeckia purpurea* L. var. *serotina*, designated new taxon (*).
1821	*Rudbeckia speciosa* Link. was described [different from *R. speciosa* Wender or Shrad.].
1823	*Rudbeckia serotina* (Nutt.) Sweet was described by Sweet.
1824	*Rudbeckia hispida* described by J. C. von Hoffmannsegg.

Table 3.1 Continued.

1836	DeCandolle made the combination *Echinacea serotina* (Nutt.) DC. and listed the following synonyms: *R. purpurea* L. var. *serotina* Nutt.; *R. serotina* (Nutt.) Sweet; *R. speciosa* Link.; *R. hispida* Hoffmannsegg. DeCandolle also recognized *E. purpurea* L. Moench as a distinct taxon [description matching Linnaeus].
1836 - 1894	Authors acknowledged only *E. purpurea* (L.) Moench but not *E. serotina* (Nutt.) DC. e.g. Darby (1860) and Chapman (1889).
1894	*Brauneria purpurea* (L.) Necker ex. T. C. Porter & N. L. Britton [includes no description].
1903	Small recognized *Brauneria laevigata* Boynton & Beadle and *Brauneria purpurea* (L.) Britton as two distinct taxa [which we determined to be nomenclaturally misapplied, see text].
	Post-Linnean History:
1929	Blake rejected *Brauneria*, and made the combination *Echinacea laevigata* (C. L. Boynton & Beadle) S. F. Blake.
1945	Cronquist reduced *E. laevigata* to a variety, *E. purpurea* Moench var. *laevigata* (C. L. Boynton & Beadle) Cronquist. He also created the new name *E. purpurea* Moench var. *purpurea* Cronquist which applies to *E. purpurea* (L.) Moench sens. strict.
1959	*Echinacea* was accepted as first validly published name for the genus and *Brauneria* was considered an invalid name (ICBN, Montreal)

Figure 3.1. Flow diagram illustrating the creation of two *Echinacea* taxa which were originally described as *Rudbeckia purpurea* L., their name changes over time and the correct names after typification.


1753
Rudbeckia purpurea L.
Type: Gronovius phrase name (1)
[Clayton 417, BM]
Other elements of protologue:
Catesby (2), Morison (3)
1794
E. purpurea (L.) Moench
Type: as above
1836
E. purpurea (L.) Moench
Type: (1)
Catesby illustration (2)
Morison illustration (3)
1836
E. serotina (Nutt.) DC.
Type:
part of Catesby's description (2)
part of Morison's description (3)
1903
E. laevigata
(C. L. Boynton & Beadle) S. F. Blake
Holotype: McCarthy 26903, US
1903
E. purpurea auct.
[non (L.) Moench]
Correct names after typification
E. purpurea (L.) Moench
Lectotype: 1025.7, LINN
Paralectotype: Clayton 417, BM
E. serotina (Nutt.) DC
Lectotype:
Nuttall "*E. serotina Arkansas* s.n.",
541360 BM
Names in current use
E. laevigata
Holotype: McCarthy, US
E. purpurea auct. (nom. cons. prop.)
LINN Type rejected
new Type: Nuttall, 541360 BM

Typification of *Rudbeckia purpurea* L.

McGregor designated a type for *E. purpurea* (L.) Moench *sensu typo* (≡ *Rudbeckia purpurea* L.) by his citation "Type: In Virginia, Carolina (LINN, photo!)". However, there is significant cause to question his typification because he misapplied the name *E. purpurea* to the taxon *E. purpurea* auct., which is not conspecific with the selected type at LINN. Furthermore, McGregor (1968) did not consider the Linnean protologue in full, even though he had access to a "Guide for the determination of types" in the *Code* (Baehni & al., 1961), which defined the term protologue, and stated that choice of any lectotype should be based on "all aspects of the protologue". Also, at the time of McGregor (1968), it was known according to Stearn (1957, p. 130) that the most important element of the protologue in cases of typification is the phrase name, the publication of which was not necessarily associated with the specimens at LINN. In this case, Linnaeus adopted the full phrase name directly from Gronovius (1739), so the implied lectotype specimen(s) are; *Gron. virg. 104.*, *181*. Of these two, the correct choice would be Gronovius' *104* (USA: Virginia, Clayton 417, BM, photo!) because we determined that Gronovius *181* (USA: Clayton 490, BM, photo!) was *Rudbeckia hirta* L. In addition, McGregor (1968) did not indicate a LINN specimen number or annotations, and his cited type location was "Virginia, Carolina" which can be found written on Clayton 417 (BM), but not on his lectotype, 1025.7 (LINN). In the end, however, the Clayton 417 specimen at BM was taxonomically identical to the lectotype designated by McGregor (1968) according to those characteristics that distinguish these two species (Tables 3.2 and 3.3), so we accept McGregor's lectotype of *E. purpurea* L. Moench (USA: Virginia, 1025 no. 7, LINN, photo!) under Art. 10.5 (Greuter *et al.*, 2000).

I conducted a thorough examination of Linnaeus' protologue for *Rudbeckia purpurea* L., which was not apparently undertaken by McGregor (1968). However, the results of this investigation, which are summarized in Table 3.1 and Figure 3.1, did not change the taxonomic outcome, nor subsequently the lectotypification of McGregor. In short, Linnaeus (1753) included elements in the protologue of *Rudbeckia purpurea* L. which were primarily conspecific with McGregor's lectotype (USA: Virginia, 1025 no. 7, LINN, photo!), except for a few characteristics in the Catesby (1743) and Morison (1699) descriptions which matched the species currently known as *E. purpurea* auct. DeCandolle (1836) was the first to acknowledge two species from the one Linnean taxon (Fig. 3.1). Detailed notes concerning all the elements of the protologue are available from the author.

Table 3.2. Characteristics to distinguish between plants from the two taxa, *E. purpurea* auct. and *E. laevigata* (C.L. Boynton & Beadle) S.F. Blake, from chapter 2 and McGregor (1968). [1-8] Eight features which distinguish between lectotype specimens (see nomenclatural treatment).

Character	*E. purpurea* auct. *nom. cons. prop.* ≡*E. serotina* (Nutt.) DC.	*E. laevigata* (Boynton & Beadle) Blake *nom. cons. prop.* ≡*E. purpurea* (L.) Moench *sensu typo*
Stem:[1]	Branched	Unbranched
Trichomes[2]	Hirsute *	Glabrous
Basal Leaves:[3]	15.5-22 cm long 5-10 cm wide	12-15 cm long 4.5-6 cm wide
Margins[4]	Serrate or dentate	Serrate
Trichomes	Present (bicellular)	Glabrous (rarely adaxial) to glaucous
Blades[5]	Cordate where base joins petiole	Tapering at base to petiole
Veins[6]	Branched major veins*	Unbranched major veins (almost parallel to midvein)*
Cauline Leaves:[7]	Serrate or dentate	Barely dentate to entire
Involucral bract series:	4	2-3
Ray florets:[8]	3.5-5 cm long* 1-1.3 cm wide*	2-6.5(7.5)cm long 0.3-0.5 cm wide
Pappus (disk florets):	4 teeth, even margins*	1 major tooth, even/uneven margins*
Rhizome/caudex:	Hoizontal	Vertical
Roots:	Fibrous	Fusiform tap roots, branching

*new or different information from that found in McGregor (1968)

Table 3.3: Comparison of type specimens and historical descriptions for the two taxa *E. purpurea* (L.) Moench and *E. serotina* (Nutt.) DC.

	***E. purpurea* (L.) Moench** based on lectotype of *R. purpurea* L. Sp. Pl. 2: 907, 1753; see Fig. 3.1 and elements of the protologue	***E. serotina* (Nutt.) DC.** based on lectotype -see typification for designation (this chapter); DC. Prodr. 5: 554, 1836 (with associated specimens); *R. serotina* Sweet. Brit. Fl. Gard. 1: 4.1823
Stem:	glabrous	hispid/scabrous/short hairs
	not branched	branched
Leaves:	glabrous	scabrous (short hairs)
	variously serrate margin	serrate margin
Basal	blades decurrent on long petiole	blades cordate at base
	ovate	ovate (almost heart-shaped)
Cauline	lanceolate	ovate-lanceolate
Inflorescence:	disk strongly convex	disk more hemispherical
Ligules	long and pendulous	spreading (not pendulous) and
	apices bidentate	shorter than *R. purpurea* L.
		apices tridentate (rarely bi-)
Root:	fusiform	fibrous
Geography	Virginia to Florida in boreal mountain regions	Lower plains of Louisiana, Arkansas

Comments on the name and type of *Echinacea laevigata* (C. L. Boynton & Beadle) S. F. Blake

Refer to Table 3.1 for the history of the name *Echinacea laevigata* (C. L. Boynton & Beadle) S. F. Blake.

In the forthcoming taxonomic revision (Binns *et al.*, submitted; chapter 2), it was concluded that *E. laevigata* is distinct from *E. purpurea* auct. (in the current sense) at the species level according to morphological differences, some of which are found here in Tables 3.2 and 3.3. However, the description of *E. laevigata* by C. L. Boynton and Beadle (in Small, 1903) places it clearly within circumscription of *E. purpurea* (L.) Moench *sensu typo* (Linnaeus, 1753) [translated from Latin in square brackets] according to the following features: stem smooth, striate, about 1 m tall [stem simple, striate, 1 foot tall]; basal leaves ovate-lanceolate, upper cauline lanceolate-acuminate [lower leaves ovate-lanceolate, decurrent on petiole and upper leaves thinner]; glabrous, mostly dentate [serrate margins]; long, naked peduncle [solitary peduncle and terminal capitulum]; rays 3-6 cm long, rose-colour, spreading [rays long, pendulous and deep purple].

Therefore, considering the above evidence, *Echinacea laevigata* (C. L. Boynton & Beadle) S. F. Blake is a synonym of *E. purpurea* (L.) Moench *sensu typo*. However, if my proposal to conserve *E. purpurea* auct. with a different type is successful (chapter 4), *Echinacea laevigata* (C. L. Boynton & Beadle) S. F. Blake will be the correct name for the original Linnean taxon.

Implications of the current use of the name *E. purpurea*

and why this species should be correctly named *E. serotina* (Nutt.) DC.

The taxon currently called *E. purpurea* auct. was first recognized in *Echinacea* in 1836 as *E. serotina* (Nutt.) DC. (Table 3.1). Prior to that, Nuttall (1818) accepted this taxon as *R. purpurea* L. var. *serotina*. Then, before DeCandolle (1836), three authors elevated Nuttall's variety to species rank (Table 3.1). Among them, Link's *R. speciosa* (1821) was invalid because it lacked a description, so *R. serotina* (Nutt.) Sweet (1823) became the earliest legitimate name at the species level. Of his new species *R. serotina*, Sweet (1823) declared it "passed under the name *R. speciosa* Link., but without a description, as it is said not to have flowered in that garden; we have no doubt it is the *R. purpurea* var. *serotina* of Nuttall". DeCandolle (1836) subsequently transferred this species to *Echinacea*.

One other author since Nuttall has suggested that one of the two entities in question was a variety of the other. A new combination, *E. purpurea* (L.) Moench (now auct.) var. *laevigata* (C.L. Boynton & Beadle) Cronquist (1945), was made. Therefore, according to Cronquist (1945) C. L. Boynton & Beadle's *E. laevigata*, which "is glabrous throughout", should be a variety of *E. purpurea* auct., which "...has both stem and leaves conspicuously hirsute, but individuals with the stem essentially glabrous are not uncommon".

The characteristics that differentiate *E. serotina* (Nutt.) DC. (≡ *E. purpurea* auct.) from *E. purpurea* (L.) Moench *sensu typo* (≡ *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake) were described in Sweet (1823) and DeCandolle (1836) and are summarized in Table 3.3. I examined original material of *E. serotina* (Nutt.) DC. (see nomenclatural summary to follow) and found it to match the current usage of *E. purpurea* auct., not that of Linnaeus. I concluded that the taxon named *E. purpurea* auct. should be correctly called *E. serotina* (Nutt.) DC. To avoid this disadvantageous nomenclatural change for a plant widely used in popular medicine as well as the horticultural and pharmaceutical industries, I have proposed conservation of *E. purpurea* (L.) Moench in the sense of its current usage (chapter 4).

Nomenclatural summary

Correct names of the two *Echinacea* taxa in question follow in the style of publication for the journal Taxon. Their types are included, as are the synonyms (≡ homotypic, = heterotypic) and the name in current use is in ***bold italic***:

1. *Echinacea purpurea* (L.) Moench Meth. Pl. 591. 1794.

Basionym: *Rudbeckia purpurea* L. Sp. Pl. 2: 907. 1753. – Lectotype: [U. S. A.: Virginia] (LINN 1025 no. 7, photo! Designated by McGregor 1968). – Paralectotype: [U. S. A.: Virginia] *Clayton*, (BM no. 417 Clayton's Herbarium, photo!). – Original elements: *Plukenet*, 1696 (alm. t. 21. f. 1., photo!); *Morison*, 1699 (hist. vol. 3: sect. 6: t.9: f.1., illust.!); *Catesby*, 1743 (car. t. 59., photo!).

≠ *"Brauneria purpurea"* Neck. Elem. Bot. 1:17. 1790 *nom. invalid.* (Art. 32.7 App. V, the Code, Greuter *et al.* 2000).

Heterotypic synonyms – = *Brauneria purpurea* (L.) ex. T. C. Porter & N. L. Britton. Mem. Torr. Bot. Club 5:333. 1894.

= *Brauneria laevigata* C. L. Boynton & Beadle in Small, Fl. S.E. United States 1: 1261.

1903.

= ***Echinacea laevigata*** (C. L. Boynton & Beadle) S. F. Blake Jour. Wash. Acad. Sci. 19: 273. 1929. Holotype: [U. S. A.: South Carolina], *Geraldus McCarthy* (US 26903, photo!)

≡ *Echinacea purpurea* (L.) Moench var. *laevigata* Cronq. Rhodora 47:397. 1945.

2. *Echinacea serotina* (Nutt.) DC. Prodr. 5: 554. 1836. International Documentation Centre, DeCandolle Herbarium. Prodr.: 940. vol. 5: 554. no. 2.2, photo!

≡ *Rudbeckia purpurea* var. *serotina* Nutt. Gen. N. Am. Pl. 178. 1818. – Lectotype: [U. S. A.: Arkansas], *Nuttall* "*Echinacea serotina* Arkansa" (BM no. 541360!) (designated here, see Notes).

≡ *R. serotina* (Nutt.) Sweet Brit. Fl. Gard. 1: pl 4. 1823.

≡ ***Echinacea purpurea*** (auct. non. L., *sensu* McGregor's description (1968), and current use in horticultural, herbal and pharmaceutical commerce)

Heterotypic synonyms. – ≠ *R. speciosa* Link. Enumeratio plantarum Horti regii botanici berolinensis 2:353. 1821. (Type not seen) *nom. invalid.*

= *R. hispida* Hoffmannsegg Verzeichniss der Pflanzenkulturen 2:201. 1824. (Type not seen)

Notes

I designated Nuttall's BM specimen of "*Echinacea serotina* Arkansa" as the lectotype because it clearly shows the characteristic asterisk and the abbreviation "s.n." (species nova), which he has used consistently to designate type specimens. My designated lectotype was collected from "Arkansa" which was known as "Lower Louisiana Territory" prior to 1803, and may correspond to "Lower Louisiana" in Nuttall's description (1818). I investigated several other herbaria for types (PH, GH, NY, BM, MO) and two sheets were located at PH (photo!), but they did not satisfy the requirements to be considered with the BM specimen for type status because they contained multiple specimens from separate collections. All specimens on both sheets were determined to be *E. purpurea* auct. ≡ *E. serotina* (Nutt.) DC. Their annotations were diverse, including: "*R. purpurea* var. *serotina* Nutt.", "*E.* * *laevigata* Nutt.", "*E. purpurea* Moench (*Rudbeckia* L.)", "*E. purpurea* L.", and "*Rudbeckia* Ark. Nutt. T.N.".

Figure 3.2. *Rudbeckia purpurea* L. no. 417 in Clayton's Herbarium at BM. The specimen was collected in Virginia, USA and used by Gronovius for his *Flora Virginica* (1739). It is here designated as paralectotype for *Echinacea purpurea* (L.) Moench (before conservation of the name in current use, see chapter 4).

3. pentaphylla. Dorycnium. Lotus.
4. Polyphylla foliis conjugatis
α. Siliqua [illegible] ... Lathyrus. [illegible] Vicia. Lens. [illegible] Faba. Galega. Orobus. Astragaloides. [illegible] Glycyrrhiza. Onobrychis. Cicer. Colutea. [illegible] Coronilla. [illegible]
β. Siliquis articulatis. [illegible] Ornithopodium. Coronilla. Hedysarum. Scorpioides. [illegible]
γ. Siliquis [illegible] Tragacantha. [illegible] Astragalus.

Rudbeckia foliis lanceolatis ovalis alternis indivisis petalis radii bifidis.
Fl. virg. p. 124. Linn. Syst. gen. 870.

[illegible] Clayton

CHAPTER 4: PROPOSAL TO CONSERVE THE NAME *ECHINACEA PURPUREA* (L.) MOENCH (ASTERACEAE) WITH A DIFFERENT TYPE

Following the results of typification in chapter 3, and in view of the current popularity of this particular infrageneric taxon, conservation of the name for the species in current use was proposed here, according to the Rules of International Botanical Nomenclature. This chapter was prepared as a "Proposal to Conserve" for consideration by the Nomenclatural Committee at the next International Botanical Congress, and for publication in Taxon as a companion article to chapter 3, with the same authors.

Echinacea purpurea (L.) Moench Meth. Pl. 591. 1794. [*Aster.*], *nom. cons. prop.* Type: [U. S. A.: Virginia], **Clayton** *"Rudbeckia purpurea* L." (LINN, 1025 no. 7, photo!), *typ. rej. prop.* (see typification in chapter 3)

(=) *Echinacea serotina* (Nutt.) DC. Prodr. 5: 554. 1836. [*Aster.*], *nom. rej. prop.* Lectotype: [U. S. A.: Arkansas], **Nuttall** "*Echinacea serotina* Arkansa" (BM no. 541360!) (designated in chapter 3), *typ. cons. prop.*

Plants in the genus *Echinacea* Moench are currently among the most sought-after materials for phytomedicines worldwide, which has lead to extirpation of many wild populations and increasing cultivation as alternative crops. I discovered an historical taxonomic error with respect to two taxa in the genus *Echinacea*; which would seriously affect the current accepted nomenclature as well as most literature since 1903 (please see chapter 3 for a full discussion of the nomenclatural background of this proposal). In brief, the correct name for the species currently called *E. purpurea* (L.) Moench (here dubbed "auct.") should be *E. serotina* (Nutt.) DC., and it is not the same taxon as *E. purpurea* (L.) Moench in the Linnean sense. Furthermore, we determined that the name *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake and its holotype were synonymous with the taxon that should be called *E. purpurea* (L.) Moench with its designated type (in the Linnean sense, but not in current use), following Art. 7 of the St. Louis Code (Greuter *et al.* 2000). However, to avoid unnecessary taxonomic confusion implied as a result of typification, we here propose conservation of the name *E. purpurea* in current use.

According to Brevoort (1998), there are over 300 scientific articles about plants in the genus *Echinacea*, and Bauer (1998) refers to over 800 European phytomedicinal products derived from *Echinacea*, mainly from the aerial parts of *E. purpurea* auct. (non L., in current use). Most of the recent taxonomic, agricultural and medicinal literature relies on the taxonomy and nomenclature of *Echinacea* in the treatment by McGregor (1968), in which the historical error we detected went unnoticed. Examples of taxonomic publications in which *E. purpurea* was adopted for plants with the description matching *E. serotina* (Nutt.) DC. include: Small, Fl. se U. S., 1261. 1903; Britton and Brown, Illus. Fl. US & Can. 2: 1913; Cronquist, in *Rhodora* 47: 553: 397. 1945; McGregor, in *Univ. Kans. Sci. Bull.* 48: 113-142.1968; Gleason & Cronquist. Man.Vasc. Pl. ne U. S. & Can. 2: 531. 1991. Clearly, disruption of that nomenclature would be detrimental to the pharmaceutical, herbal and agricultural industries.

Therefore, this proposal is made according to Articles 13 and 14 (Greuter *et al.* 2000), especially Art. 14.9, cf. Ex. 8, in order to "best serve stability of nomenclature" and avoid "disadvantageous changes" which may result by applying the corrected name *E. serotina* (Nutt.) DC. to the popular taxon so widely known as *E. purpurea* auct. Some of the possible disadvantageous changes include future misunderstanding of taxonomic or ethnobotanical literature and phytochemical studies completed to date, as well as undue commercial confusion influencing cultivation, germplasm management, the quality of phytomedicines and clinical studies.

Conservation of the name *E. purpurea* (L.) Moench (in its current usage) for the taxon correctly called *E. serotina* (Nutt.) DC. means that it is no longer applicable as typified to the true Linnean taxon (see chapter 3). Consequently, *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake, a federally- protected species in the dwindling Appalachian uplands of eastern and southeastern U.S.A., remains the correct name for the

Linnean taxon. Nomenclatural stability is especially critical for this rare species to provide correct identification and protection of wild populations, and to facilitate appropriate germplasm conservation and careful cultivation, avoiding hybridization known to occur within the genus (McGregor 1968).

In summary, I propose conservation of the name *E. purpurea* auct. (non L.) in its current usage with its new hereby designated type to avoid confusing name changes which would be detrimental to many individuals and industries. In so doing, the name *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake, typified by its original holotype, becomes the correct name for the taxon *E. purpurea* (L.) Moench (in the Linnean sense) and I reject the type of the latter, which was designated by McGregor (1968).

SECTION 2

PHYTOCHEMISTRY AND CHEMOTAXONOMY OF *ECHINACEA*

Phytochemical profiles in this section were determined concurrently with the morphological taxonomy of section 1, using the same *Echinacea* germplasm. It was hypothesized that the revised morphological taxonomy (chapter 2) would be supported by phytochemical variation measured throughout the genus, and that chemotaxonomic markers would be determined, especially among those *Echinacea* taxa that were not previously reported in the phytochemical literature. This section deals with overall phytochemical variation between *Echinacea* collections (chapter 5) and in two controlled greenhouse studies. In chapter 6, the revised variety *E. pallida* var. *angustifolia* is referred to by the name in current use, *E. angustifolia* DC., and evidence for chemotypes is reported. In chapter 7, I have reported the induction of some phytochemical classes by the plant hormone methyl jasmonate in the revised taxon *E. pallida* var. *pallida* (currently *E. pallida* following McGregor, 1968).

CHAPTER 5: PHYTOCHEMICAL VARIATION IN *ECHINACEA* (HELIANTHEAE: ASTERACEAE) FROM ROOTS AND FLOWERHEADS OF WILD AND CULTIVATED POPULATIONS

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Introduction

The native North American plant genus *Echinacea* has recently been reclassified as four species and eight varieties (and a group of introgressant hybrids), which are associated with specific wild habitats throughout the range (see chapter 2). Ecological amplitude of a taxon is an indicator of environmental constraints leading to speciation, which includes differential expression and evolution of secondary defense phytochemicals (Herms and Mattson, 1992). Today, there is widespread cultivation of *E. purpurea* (L.) Moench, *E. pallida* var. *angustifolia* (DC.) Cronq. and *E. pallida* var. *pallida* (Nutt.) Cronq. for medicinal purposes. These taxa were probably chosen from among all wild species and varieties because of their availability and traditional uses.

Phytochemical variation among wild populations of all *Echinacea* species and varieties has not been reported to date, so current use and research of these plants rely mainly on phytochemical studies of those three cultivated taxa (Bauer *et al.* 1988a, 1988b; Bauer and Remiger 1989; Cheminat *et al.* 1988; Shulte *et al.* 1967). There have also been preliminary phytochemical characterizations of *E. atrorubens* var. *paradoxa*, *E. pallida* var. *tennesseensis*, and *E. pallida* var. *simulata* (Bauer *et al.* 1990; Bauer and Foster 1991). The lipophilic profiles of the latter two were reportedly similar to those of *E. pallida* var. *angustifolia* (Bauer and Foster 1991; Bauer *et al.* 1990). Alternatively, *E. atrorubens* var. *paradoxa* yielded polyacetylenes and their oxidized derivatives similar to those observed in *E. pallida* var. *pallida* (Bauer and Foster 1991). Among the characteristic phytochemical profiles, there were taxonomic errors between the two cultivated *E. pallida* varieties (Bauer *et al.* 1988), as well as adulteration of *E. purpurea* with *Parthenium integrifolium* L., which were both detected and corrected in later publications (Bauer and

Wagner 1991; Foster 1991). Furthermore, *Echinacea* has a confusing taxonomic history, based on primarily morphological studies alone (chapter 2).

Characteristic lipophilic profiles were reported for the three commercial *Echinacea* species. The major alkamides in *E. purpurea* were the 2,4-diene type compounds (Bauer *et al.* 1988a). A quantitative comparison within *E. purpurea* revealed the highest levels of the C_{12} diene-diyne alkamides in the roots, while the C_{11} diene-diynes were highest in vegetative stems (Perry 1997). *E. angustifolia* roots contained tetraene and they were characterized by the presence of monoene alkamides (Bauer *et al.*, 1988b). In *E. pallida* the major compounds were polyyn/enes with only three alkamides reported in the roots, and they did not contain tetraene (Bauer *et al.* 1988b).

Recently, selection of morphologically-superior cultivated *E. purpurea* lines resulted in doubled average phytochemical content in each of the major chemical classes when compared to non-selected lines (Letchamo 1998), and also demonstrated high genetic variability (Baum *et al.* 1999). Clearly, accurate phytochemical distinctions between wild germplasm sources will have a positive impact on cultivation of this medicinal plant. The current study was conducted to determine quantitative phytochemical variation in *Echinacea* from wild populations, transplants and wild germplasm under cultivation. Sampling was carefully conducted throughout the natural range of each putative taxon in the genus (tentatively identified in the field according to McGregor 1968). The large sample sizes and robust analytical methods ensure that this study provides baseline information on phytochemical levels in all taxa, with applications in germplasm improvement and conservation.

Materials and Methods

Collections

Echinacea plants and germplasm from 125 natural populations were sampled from throughout the range of each putative species and variety, and labelled tentatively in the field according to McGregor (1968)(Appendix 1). One root per population was transplanted to a greenhouse at 25-30^{0}C in a medium of 2:1 soil: beach sand in large gallon pots, with 16 hr daylight, including natural and cool white lighting (25 μM/m^2/s). Wild organ samples were coarsely chopped (or left whole when possible), and placed in 95% EtOH in leakproof, inert plastic sample containers *in situ* (Nalgene ^{TM}) as follows: 1) root cutting from one individual plant, 2) bulk leaf sample from 10-20 random plants, and 3) bulk inflorescences (3-10 capitula) randomly sampled. Ripened achenes were collected when available, sterilized and stratified at 4°C (5 μM/m^2/s, incandescent) for 14 days. Germinated seedlings were grown in 5:5:2 Promix: vermiculite: sterile quartz sand (Industrial grade #10, 4 mm particles). Developmental variation between accessions required that some plants be induced to flower with a cold treatment at 4°C (20 μM/m^2/s, incandescent) for 14 days. All plants were fertilized with 20:20:20 weekly and watered equal amounts daily, according to age. Extracts from all greenhouse-cultivated *Echinacea* were made from the organs of individual plants.

Extraction

Method A

Plant material was blended to a homogeneous slurry in EtOH (95% if wild-collected and 60% if cultivated) using an Osterizer blender. The biomass : solvent ratio was approximately 2g fresh :10 mL. Plant-solvent mixtures were mechanically agitated on a shaker (70 rpm) for 24 hours. Solids were removed using a Buchner filter system

(Whatman #1 filter paper) and the residues were dried and weighed to determine final extract concentrations (gram dry weights, dwt/mL). Filtrate was roto-evaporated to dryness and re-dissolved into 60% EtOH to a final concentration of 0.1g/mL and stored at 4^0C in amber glass containers. Other studies in this laboratory (Livesey *et al.* 1999) determined that hydro-alcoholic extractions in 70% EtOH provided optimum recovery of *Echinacea* phytochemicals, such as cichoric acid (a hydrophilic marker compound) and tetraene (lipophilic standard). Ninety-five percent EtOH was used in field work in order to halt enzymatic degradation and ensure sterilization, but all *Echinacea* extracts were brought to final concentrations in 60% EtOH for storage and analyses.

Method B

A second blending method was developed to maximize the extraction of phytochemicals from very small quantities of young, single plants (< 1 year old). Roots, leaves and inflorescences were cut into 1 cm lengths using a razor blade and placed into 20 mL of 60% EtOH in plastic centrifuge tubes (25mL, VWR Toronto, Canada). Fresh biomass varied from approximately 2 to 10 grams per tube. Samples were mixed into a slurry with a high-speed Polytron (Brinkmann Instruments, Westbury, NY) for 30s (repeated three times). After 24 hours on a shaker (70 rpm), samples were vortexed briefly, then centrifuged for 10 min. The supernatant was removed to a clean tube. Fresh 60% EtOH (20 mL) was added to the plant residue, vortexed and then agitated on a shaker (70 rpm) for 24 hours. The sample was again centrifuged for 10 min. and the supernatants were pooled. The entire process was repeated for a final 24-hour extraction period in 10 mL 60% EtOH. Residues were dried and weighed to determine the final extract concentrations.

Extracts were adjusted to 0.1g/mL by rotary evaporation or dilution.

The size of wild populations, rarity of the putative variety, and survival of achenes and live transplants were factors contributing to variation in samples sizes for each revised *Echinacea* variety (see chapter 2) (n= 3 to 157 individual organ extracts).

High Performance Liquid Chromatography

All extracts were filtered (0.2 µm, nylon membrane) prior to HPLC separations using a validated method (Bergeron *et al.* 2000). Hydrophilic chromatography was achieved using a solvent system of acetonitrile : 50mM NaH_2PO_4, pH 2.95, at a flow rate of 1.5 mL/minute following a linear gradient of 5-25% acetonitrile over 7 minutes. Lipophilic chromatography was achieved using a solvent system of acetonitrile : H_20, at a flow rate of 1.0 mL/min following a linear gradient of 40-80% acetonitrile over 15 minutes. In both systems 5 µL of sample was injected on a 7.5 cm x 4.6mm reverse phase C-18 LiChrospher® column (3 µm particle size) (Merck, BDH, Toronto, Canada). Lipophilic compounds were detected at 210 nm and 260 nm and hydrophilic compounds were detected at 326 nm.

Compounds were identified by comparison with reference standards. Reference standards of undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide **1**, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (tetraenes) **8+9**, pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide **18**, 2,3-O-dicaffeoyltartaric acid (**cichoric acid**) and **echinacoside 7** were isolated previously in our laboratory by column chromatography on silica gel and assessed for purity by ^1H and ^{13}C-NMR spectral data (Bergeron *et al.*, 2000). The same method yielded dodeca-2E,4E-dienoic acid isobutylamide **11** (by online UV spectra matching Bauer and Remiger, 1989) and **cynarin** (by ^{1}H and ^{13}C-NMR, Cheminat *et al.*, 1988) in our laboratory by Bergeron *et al.* (Unpubl.). Standards of **caffeic acid** and **chlorogenic acid** (Sigma Aldrich, St. Louis, U.S.A.), as well as **caftaric acid** (Dalton Chemical Laboratories Inc., Toronto, Canada) were purchased. All other alkamide and polyene compounds (Figs. 5.1-5.4) were identified based on relative retention time to the marker compounds (tetraenes) **8+9** and online photodiode array UV spectra (c.f. Bauer and Remiger, 1989). Each previously reported compound (µg/ per mL injected) was quantified using peak area multiplied by the response factor (calculated from the standard curve of tetraenes **8+9**). This figure was then divided by the original concentration of 0.5g extracted dried root/ ml sample and multiplied by 10^3 to reach mg/g dry wt. Peak identification by relative retention time and quantitation by relative response factor was acceptable for the purpose of overall profile comparisons within the present study.

Statistical Analyses

Descriptive statistics (Mean ± standard error of the mean SEM) were calcultated to compare all revised taxa by individual phytochemicals in 358 root extracts (single plants only) and 175 inflorescence extracts (approximately half were bulked wild population samples). Same-aged specimens grown under the same conditions were compared: 1) cultivated; ≤ 1 year-old, 2) cultivated/transplanted; > 1 year old, and 3) all wild-harvested roots and flowers.

Quantitative data collected from HPLC of 327 *Echinacea* root OTUs (operational taxonomic units) were arranged in a matrix as root concentrations (ppm root dry weight) for 26 phytochemical characters (all of which were previously-reported alkamides, phenolics and polyenes). In this matrix, OTUs were grouped according to revised species identity (chapter 2).

Canonical discriminant analysis (CDA) is a multivariate statistical method used to determine whether classes of OTUs (revised taxonomic groups in this case) are distinctly different from one another based on a certain set of interrelated characters (26 quantitative phytochemical characters in this case). The CDA of the phytochemical variation in four revised species and eight varieties of *Echinacea* was performed using SAS ver. 8.0 (SAS Institute 2000), by subsets of the data matrix to accommodate the conditions under which the data were collected. Specifically, CDA was performed for 1) all roots regardless of age or growth conditions, 2) young, cultivated roots, 3) older, transplanted roots in cultivation and 4) wild-harvested roots. Characters which did not vary within a taxonomic group were deleted from the analysis. A complete explanation of the CDA method can be found in Kshirsagar (1972).

Results

HPLC profiles of the phytochemicals typically present in each *Echinacea* species (Figs. 5.7-5.21) show obvious differences between most of the species/varieties, especially in the lipophilic profiles. Differences in the phenolic profiles were less evident, yet species/varieties differences were noted from inflorescence phenolic profiles and the consistent lack of **echinacoside** denoted *E. purpurea* root phenolic profiles (Fig. 5.15). The chromatograms were presented as "typical chemical fingerprints" of each species and variety, and it should be noted that absorbance, or peak size, was only part of the calculation to measure quantitative differences. Quantitative statistics for each compound are found in Tables 5.1-5.6. Lipophilic and hydrophilic constituents were presented separately for both roots and inflorescences.

Root tetraenes

The major alkamides in *Echinacea*, dodeca 2E,4E,8Z,10E/Z tetraenoic acid isobutylamides **8+9**, henceforth called **tetraenes,** were recorded at the highest level in this study from wild *E. pallida* var. *sanguinea* at 1.9% root dwt (Fig. 5.1; Table 5.3). They were high in young, cultivated roots of that variety (but declined in older cultivated roots). **Tetraenes** were second highest in cultivated roots of *E. atrorubens* var. *atrorubens* (0.8-0.96% dwt) (all ages), although they were absent from *E. atrorubens* var. *paradoxa*. **Tetraenes** were present in the roots of all other varieties of *Echinacea*, with the highest mean quantity from older *E. pallida* var. *angustifolia*. *Echinacea* roots generally accumulated more **tetraenes** with age, with the following exceptions: *E. pallida var. tennesseensis, E. laevigata* (insufficient data), and *E. atrorubens* var. *neglecta*, which had relatively small quantities overall, but accumulated the most in younger cultivated roots.

Flower tetraenes

Tetraenes were the predominant lipophilic constituents in flowerheads of all *Echinacea* species and varieties, no matter what age or growth conditions. The highest amounts in this study were measured in seed-grown flowerheads of *E. pallida* var. *tennesseensis* at 1.04% dwt (Table 5.2). *E. atrorubens* var. *neglecta* and *E. pallida* var. *pallida* wild flowerheads both accumulated 0.55% dwt. Flowerheads cultivated from seed generally accumulated more **tetraenes** than wild plants of the same variety, except for *E. pallida* var. *sanguinea* and *E. pallida* var. *pallida.*

Other diagnostic alkamides

The alkamides **1-4** and **7** (Fig. 5.2) have two double bonds in conjugation with the carbonyl group and have been reported as diagnostic "diene" marker compounds for *E. purpurea* that are generally lacking in *E. pallida* var. *angustifolia* and *E. pallida* var. *pallida* (Bauer 1998). *E. purpurea* accumulated the highest amounts of **1**, **2** (0.4% dwt) and **7** in older transplants from wild populations, while **3** and **4** (relatively less) were highest in wild roots of this species. These dienes were also present in all of the other revised species. *E. pallida* var. *sanguinea* displayed all dienes at comparable levels *to E. purpurea*, but with much smaller amounts of **2** and **4** in cultivation. *E. laevigata* contained the next highest amound of **3**, with medium to low levels of **1**, **2**, **4** and **7**. Finally, *E. atrorubens* (vars. *atrorubens* and *neglecta*) showed the same diene pattern as *E. purpurea* (but lower quantities) in wild and older cultivated roots, except **3** which was high in younger roots of var. *neglecta* (0.24% dwt) and in older roots for var. *atrorubens* (see Tables 5.1, 5.3 and 5.4). *E. pallida* vars. *angustifolia*, *tennesseensis* and *simulata* all contained >0.1% dwt root dienes.

Alkamide **10** (Fig. 5.1) was identified in large quantities from young roots of *E. atrorubens* var. *atrorubens* (0.6% dwt), although it was present in *E. pallida* var. *angustifolia* roots with small amounts in other species and varieties. Alkamide **11** (Fig. 5.1) was highest in older roots of *E. pallida* var. *angustifolia* (0.22% dwt), but was present in all species and varieties (sometimes very minimally), except *E. atrorubens* var. *paradoxa*. Alkamides **5** and **15** (Figs. 5.2 and 5.3) were found in highest amounts in *E. atrorubens* var. *atrorubens*, followed by *E. pallida* var. *angustifolia*, despite being previously reported only from the latter. They were also present in *E. purpurea* and *E. atrorubens* var. *neglecta*, along with some other varieties.

Another alkamide pattern is that of the monoene-carbonyl conjugated compounds **12, 13** and **14** (Fig. 5.3). Quantitatively, the *E. pallida* varieties *sanguinea* and *tennesseensis*, as well as the species *E. atrorubens*, were far superior in **12, 13**, and **14** compared to *E. pallida* var. *angustifolia*. Wild roots of *E. pallida* var. *sanguinea* had the highest amount of **14** in the genus (0.36% dwt)(Table 5.3), and young, cultivated *E. atrorubens* var. *atrorubens* roots had the highest level of **13** (3.7% dwt) (Table 5.1). Hybrid populations also displayed the monoene alkamide pattern more than the dienes **1-4** and **7**, which is to be expected since most hybrids likely occurred between *E. pallida* and *E. atrorubens* (see chapter 2; McGregor, 1968).

Alkamide **18**, a longer chain monoene type (Fig. 5.3), was found in the highest amount from the roots of *E. pallida* var. *sanguinea* (2.9% young root dwt). Alkamide **18** was also present in every other variety of *Echinacea* except *E. purpurea*. Alkamide **19** (Fig. 5.3) was rare, especially in young roots, but was found up to 0.05% dwt in the wild roots of *E. atrorubens* var. *neglecta*.

Root ketoalken/ynes

E. pallida var. *simulata*, which is morphologically the most similar to *E. pallida* var. *pallida*, contained twice the amount of **22** (Fig. 5.4), in wild and older roots (Tables 5.3 and 5.4, respectively). Furthermore, young and wild roots of *E. atrorubens* vars. *neglecta* and *paradoxa* contained more **22** than both *E. pallida* vars. *pallida* and *simulata*. Older roots *of E. laevigata* also contained **22** (see Table 5.4). Ketoalkenes **24** and **25** (Fig. 5.4) were major components of *E. pallida* var. *pallida* roots and *E. pallida* var. *simulata*, as previously reported (Bauer and Wagner 1991; Bauer 1998 and Shulte *et al.* 1967). However, these compounds were also accumulated to a large extent in *E. atrorubens* var. *neglecta* (0.3% dwt in young roots to 0.5% dwt in wild roots) and *E. atrorubens* var. *paradoxa* (1% dwt wild roots).

Flowerhead ketoalken/ynes

Ketoalkene **24** was measured in appreciable quantities from the wild flowerheads of *E. pallida* var. *pallida*, *E. pallida* var. *sanguinea* and *E. atrorubens* var. *paradoxa*. *E. pallida* var. *tennesseensis* was the only variety which accumulated **24** in cultivated flowerheads at approximately 0.04% dwt.

Root phenolics

The highest root concentrations of **cichoric acid** (Fig. 5.5) in all *Echinacea* collections were found in cultivated *E. purpurea* at 0.8% dry weight (dwt) of young roots (Table 5.1). This level was over 10 times higher than *E. pallida* var. *angustifolia* roots of the same age and development, 0.063% dwt (Table 5.1). Conversely, levels of the quinic acid derivative **cynarin** (Fig. 5.6) was highest in young, cultivated *E. pallida* var.

angustifolia (0.5% root dwt, Table 5.1) and mostly absent in *E. purpurea* roots of all ages. **Cynarin** was also present in young, cultivated *E. pallida* var. *tennesseensis* roots (0.4%) and hybrid populations (0.5%), but absent from all other *Echinacea* varieties of the same age. Both **cichoric acid** and **cynarin** in the roots decreased with age, with the exception of *E. atrorubens* var. *atrorubens* (Tables 5.1, 5.3 and 5.4).

Echinacoside (Fig. 5.6) is the standard industrial marker for *E. pallida* var. *angustifolia* and it was present in young, cultivated roots of this variety in average amounts of 0.69% dwt (Table 5.1). The levels of **echinacoside** in this variety decreased with age to 0.12-0.2% dwt.(Tables 5.3 and 5.4), similar to the other phenolics mentioned above. Other varieties of *E. pallida* also demonstrated this decreasing trend with age, such as roots of *E. pallida* var. *pallida*, and *E. pallida* var. *tennesseensis*, as well as *E. laevigata* and hybrids (Tables 5.1, 5.3 and 5.4). However, the trend was reversed among *E. pallida* var. *sanguinea* and *E. atrorubens* vars. *atrorubens* and *neglecta*, which all showed increases in **echinacoside** with root age. The highest amount of **echinacoside** was measured from old, wild roots of *E. atrorubens* var. *paradoxa* (3.3% dwt, Table 5.3).

Caftaric acid (Fig. 5.5) in *Echinacea* roots was present in the same species and varieties that contained **cichoric acid**. The age trend was the same (decreasing with age), with the same exception: *E. atrorubens* vars. *atrorubens* and *neglecta* showed increased levels of **caftaric acid** with age (Tables 5.1, 5.3 and 5.4). Finally, **chlorogenic acid** (Fig. 5.6), which is widely distributed throughout the plant kingdom, was highest in wild roots *of E. purpurea* (0.1% dwt) and young cultivated roots of *E. laevigata* (0.1% dwt). The next highest levels were found in the rare varieties: *E. pallida* var. *sanguinea* wild roots (0.05% dwt) and *E. atrorubens* var. *paradoxa* wild roots (0.03% dwt) (Table 5.3). **Chlorogenic**

acid was present in low levels in all other varieties of the genus; it showed an increasing trend with age in *E. pallida* var. *pallida*, *E. pallida* var. *sanguinea*, and a decreasing trend with age in *E. pallida* var. *angustifolia* and *E. pallida* var. *tennesseensis* (Tables 5.1, 5.3 and 5.4).

Flowerhead phenolics

E. purpurea flowerheads from 2 year-old cultivated transplants contained about the same amount of **cichoric acid** as the young roots of that species (0.9% dwt, Table 5.5). Wild flowerheads contained far more **cichoric acid** than cultivated flowerheads or roots. The highest measured value was 3% dwt. in *E. pallida* var. *sanguinea* (Table 5.6). Similar to the root **cichoric acid** pattern, *E. pallida* var. *angustifolia* flowerheads had only 0.017% dwt (Table 5.5). *E. pallida* var. *sanguinea* was the highest accumulator of **cynarin** in flowerheads (0.03% dwt, Table 5.2), not *E. pallida* var. *angustifolia* as would be inferred from root content. In addition, young flowerheads of *E. pallida* var. *tennesseensis*, *E. purpurea*, *E. atrorubens* var. *atrorubens* and *E. pallida* var. *simulata* also contained minute quantities of **cynarin** (Table 5.2).

Echinacoside from *Echinacea* flowerheads was measured in high levels from the wild-collected *E. atrorubens* var. *neglecta* (1.82% dwt, Table 5.6) as well as *E. atrorubens* var. *paradoxa* (0.63% dwt, Table 5.6). *E. purpurea* and *E. laevigata* lacked **echinacoside** in flowerheads at all ages and growth conditions, but all five varieties of *E. pallida* revealed a large range in wild-harvested material (0.17% - 0.27%, Table 5.6) while almost nothing in cultivated flowerheads (Tables 5.2 and 5.5).

Chlorogenic acid was present in considerable quantity in every variety of *Echinacea*, with the highest levels of each variety occurring in wild flowerheads preserved *in situ* (0.17-0.38% dwt, Table 5.6). There were also some unidentified, phenolic derivatives consistently determined by online UV-spectra that resembled **chlorogenic acid** (Figs. 5.15-5.21). The highest level of caftaric acid in flowerheads was 0.17% dwt from seed-grown *E. purpurea* (Table 5.2). Flowerheads from transplanted varieties of *E. pallida* also produced close to that amount of caftaric acid (0.013-0.16%, Table 5.5). For example, cultivated flowerheads from *E. pallida* var. *sanguinea* transplants accumulated caftaric acid at 0.16% dwt, compared to 0.03% in seed-grown flowerheads (Tables 5.2 and 5.5).

Multivariate analysis of quantitative root phytochemistry

Species level Canonical Discriminant Analysis (CDA)

A CDA of all OTUs in the root dataset (n = 327) determined that each of the four revised *Echinacea* species was phytochemically distinct. Overall phytochemical variation between species is represented as distances between group centroids in 3-space on the first three canonical axes (Fig. 5.22). The squared Mahalanobis distance between all group centroids was statistically significant ($p<0.0001$) except between *E. pallida* and the "hybrids" group ($p=0.6546$). All assumptions of CDA were satisfied using 21 of 26 measured phytochemical characters. The first canonical axis explained 60% of the total variation while the second axis included another 32%. Those compounds responsible for the greatest amount of variation in the CDA, according to F-statistic and pooled within canonical structure, were (in order) **2, cichoric acid, 7, 3, 24,** and **1** (Fig. 5.23). Furthermore, for the purpose of species identification, CDA showed that all ages of *E. purpurea* roots were lacking **cynarin, echinacoside, 6, 12, 13, 14, 19, 22, 24** and **25**, and *E. laevigata* roots were lacking **cynarin, 4, 16, 19, 22** and **25.**

CDA of *Echinacea* species was also performed separately for different treatments in the present study (age and growth conditions) to reduce the effect of phenotypic variation on root phytochemical content. Discrimination between species groups was significant in all three root treatments (except for *E. laevigata* and *E. purpurea* older cultivated roots, see below). Those phytochemical variables that were shown to contribute significantly to all the CDA analyses were determined to be the most robust for quantitative phytochemical species identification markers (Fig. 5.23).

Each revised *Echinacea* species was distinctly different according to CDA of the young, cultivated roots (n = 203) (squared Mahalanobis distances between centroids were all $p<0.0001$). The hybrids were not significantly different from *E. pallida* (p = 0.997). Eighty percent of the variation was explained in the first canonical axis, and 15% in the second, for a total of 95% variation explained in the first two axes. In order of importance, the phytochemicals from young, cultivated roots with the most weight in discriminating between *Echinacea* species were **cichoric acid**, **2**, **7**, **3** and **1**.

CDA of older, cultivated roots (n = 54) indicated the following differentiation by phytochemical variation between revised taxa (according to significant Mahalanobis distances between centroids). *E. atrorubens* and *E. laevigata* were significantly different (p= 0.01), *E. pallida* and *E. atrorubens* were significantly different (p= 0.03), and *E. pallida* and *E. laevigata* were significantly different (p= 0.04). *E. purpurea* and *E. laevigata* were not signifcantly different (p= 0.06). Those compounds with the most weight in this analysis were: **2**, **cichoric acid** and **7** (in order of importance by F statistics). The first canonical axis represents 53% of the variation while the second axis adds another 33% for a total of 86% in the first two axes.

CDA of wild (old) roots (n = 75) distinguished each revised *Echinacea* taxon by phytochemical variation ($p<0.0001$ for F-statistics of the squared Mahalanobis distances). However, the hybrids were not significantly distinct from *E. pallida* or from *E. atrorubens*, and this root treatment was least reliable because *E. laevigata* was deleted from the analysis due to missing data, which reduced the number of groups. The first two canonical axes explained 83% of the variation between the remaining taxa. The most important phytochemicals (in order) to differentiate *Echinacea* species using wild root phytochemistry were; **chlorogenic acid, 2, cichoric acid, 7** and **echinacoside.**

Variety level Canonical Discriminant Analysis (CDA)

A CDA of 11 taxonomic classes of *Echinacea* (ten revised varieties and the group of hybrids/introgressants, chapter 2) was performed with 342 OTUs and 13 phytochemical root characters. The probability that each taxon was distinct from the others in this particular CDA was significant ($p<0.0001$) according to squared Mahalanobis distances, except for the following class pairs; *E. pallida* var. *pallida* and *E. pallida* var. *simulata* ($p=0.9841$), *E. pallida* var. *simulata* and "hybrids" ($p=0.2105$), and *E. pallida* var. *angustifolia* and "hybrids" ($p=0.2220$) (Table 5.7). The amount of variation between all *Echinacea* varieties, regardless of growth conditions and age was explained to 42% in the first canonical axis, and up to 83% in the first three canonical axes. The most important root phytochemicals (in order) were **24, cichoric acid, echinacoside, 7, 12, 2, 3.**

Discussion

Implications for chemotaxonomic identification

Accurate knowledge of variation in phytochemical content of different *Echinacea* species and varieties has become increasingly important, especially for quality control and efficacy in phytomedicines. Canonical discriminant analysis (CDA) was chosen as a multivariate approach which allowed differentiation among and between *Echinacea* species and varieties by the overall combination of root phytochemicals instead of single compounds. Lienert *et al.* (1998) successfully discriminated three commercially-identified *Echinacea* species using CDA of phytochemicals measured with gas chromatography-mass spectrometry. In the current study, all four revised *Echinacea* species were distinguished by root CDA, with some exceptions based on different growth conditions and age factors. *E. purpurea* and *E. laevigata* were distinct by CDA of phytochemical content in all root treatments, except older roots grown in identical conditions (either from transplanted or wild germplasm). This finding supported the morphological similarity of those two species (chapter 2). The wild root CDA was inconclusive due to insufficient root phytochemical data for *E. purpurea* and *E. laevigata*.

The phytochemical discrimination of revised *Echinacea* taxa, including all eight varieties as discrete classes in a CDA of 13 phytochemical variables, yielded distinct differences among most taxa (Table 5.7). *E. pallida* var. *pallida* and *E. pallida* var. *simulata* were not significantly distinct by phytochemical multivariate comparison. This finding supported their relative positions in the morphometric CDA (chapter 2). Also, there were very few key morphological characters to distinguish these two varieties, except geographical ranges.

In the variety-level CDA, the hybrid *Echinacea* group of OTUs was not different from both *E. pallida* var. *simulata* and *E. pallida* var. *angustifolia*. Clearly, hybrids/introgressants were not distinguishable here by root phytochemistry at the species or the variety level. Similarly, they did not form a distinct class from *E. pallida* or *E. atrorubens* in morphometric analyses (chapter 2). Finally, although the variety-level CDA showed significant distances between group centroids, the ordination was poor compared to the species-level CDA. The amount of variation explained in the first axis of the all-root variety-level CDA was only 42% compared to 66% in the first axis of the all-root species-level CDA, and this was reflective of inadequate varietal distinctions in 3-space.

In the current study, CDA helped to confirm the best phytochemical marker compounds for species and identification of *Echinacea* root materials. For species delimitation, they were **cichoric acid, 1-3, 7** and **24**. For variety delimitation, they were the same, with the addition of **echinacoside**, and **12**. Accordingly, the mean quantities of the above compounds (n = 358 roots) clearly helped to distinguish between species, and may also be useful at the variety level for chemotaxonomic use (Fig. 5.23; Tables 5.1-5.6). Lack of **echinacoside**, and presence of many diene-type alkamides **1-3**, and **7** was suggested to be diagnostic for *E. purpurea* roots (Bauer 1998).

Most hydrophilic *Echinacea* phytochemicals are caffeic acid derivatives. Recently, high variation in the levels of caffeic acid derivatives present in commercial *E. purpurea* extracts was linked to enzymatic breakdown. This activity can be reduced by adding alcohol and reducing agents. For example, **caffeic** and **caftaric acids** formed from cichoric acid in *Echinacea* preparations with less than 30% EtOH (Nusslein *et al.* 2000). Unfortunately, **caffeic acids** (cis/trans isomers) were not measured in all samples of the current work (shown as "missing data" in Tables 5.1-5.6). However, based on the data available, the *Echinacea* species and varieties containing the most free **caffeic acid** molecules were the same ones that contained the quinic acid derivative **cynarin** (results not shown). Free **caffeic acid** may have been higher in these plants because they lacked **cichoric acid** and **caftaric acid,** the presence of which requires caffeic acid side chains from the pool of "free" molecules for use in side chains.

Cynarin and **cichoric acid**, both major phenolics in *Echinacea* species from different precursors, showed differential distribution temporally and spatially. At the time of first flowering, **cynarin** was concentrated in roots and **cichoric acid** was concentrated in flowerheads, for example cultivated *E. pallida* var. *tennesseensis* (Tables 5.1, 5.2). **Cichoric acid** showed signs of spatial translocation over time from roots to vegetative tissues (root levels decreased), and probably also *in situ* biosynthesis in the flowerheads to achieve significantly higher levels in old, wild flowerheads (especially in *E. pallida* var. *sanguinea*) compared to all other plants and organs studied. Trends in phenolic secondary defense phytochemicals may be explained by distribution and translocation during development, especially in response to phenological pressures such as resource allocation, herbivory and competition. Furthermore, CDA of the phytochemicals in the current study determined that **cichoric acid** is an important discriminator between species, and may prove to be a useful phytochemical marker for identification of the morphologically-revised species.

Current industry practice emphasizes the phenolic glycoside, **echinacoside** as a marker for *E. pallida* var. *angustifolia* vs. *E. purpurea* (absent). Reportedly, this compound is not responsible for the immunostimulant clinical activity of *Echinacea* phytomedicines, and has only minor antimicrobial activity (Bauer 1998). In this study, **echinacoside** was present in the roots of three revised *Echinacea* species (seven of eight varieties, see Tables 5.1-5.6) and this is the first report of the phenolic from the two species *E. atrorubens* and *E. laevigata*. Previously **echinacoside** was reported only from the roots of both *E. pallida* var. *pallida* (0.4-1.7%) and *E. pallida* var. *angustifolia* (0.3-1.3%) (Bauer and Remiger 1989). Comparable levels were noted in the current study for those two market varieties (see Results page 163), but the highest average amount of **echinacoside** was determined from wild *E. atrorubens* var. *paradoxa* roots (3.3% dwt) in this investigation. Among all young, cultivated roots that were studied, it was highest in *E. laevigata*. In addition, **echinacoside** was always at a higher concentration in wild flowerheads compared to those that were cultivated; *E. atrorubens* flowerheads had the highest amounts, followed by moderate levels in all *E. pallida* varieties. *E. purpurea* and *E. laevigata* flowerheads were lacking **echinacoside** in every stage of development, although there were insufficient wild specimen data for these two species. Since **echinacoside** was found in three *Echinacea* species, the present study revealed that it was not a useful "species identification marker" in cultivated market materials, except where the lack of **echinacoside** may be a means to identify the unique presence of *E. purpurea* root material. Species identifications should be reinforced with lipophilic identification markers (see below).

Chlorogenic acid, a quinic acid derivative is widespread outside of the genus *Echinacea* so it is not a diagnostic species marker compound, nor is it thought to be medicinally "active" (Bauer and Wagner 1991; Bauer 1998; Foster 1991). The levels of **chlorogenic acid** in roots and flowerheads of the present study were fairly equal across all the different varieties of *Echinacea*.

The lipophilic alkamides are more often used to identify older commercial roots. For example, *E. pallida* var. *pallida* roots reportedly lack **tetraenes**, which would help to distinguish them from *E. pallida* var. *angustifolia* using phytochemical analyses. However, in the present study, both wild and young cultivated roots of *E. pallida* var. *pallida* accumulated some **tetraenes**, along with their diagnostic ketoalken/ynes. Previously, **tetraenes** were reported as major constituents of *E. pallida* var. *angustifolia* and *E. purpurea* roots as well as the aerial parts of all three major commercial varieties (Bauer 1998), but for the first time, **tetraenes** are here reported in all four revised *Echinacea* species.

Root phytochemical profiles from the species *E. purpurea* are reportedly identified by the series of alkamides **1-4** and **7** (Bauer 1998). These compounds all contain a 2, 4 – diene moiety in conjugation with the carbonyl group (Fig. 5.2) which may be responsible for some biological activity, such as cyclooxygenase and 5-lipoxygenase inhibition (Facino *et al.* 1995). As qualitative (presence/absence) diagnostic markers for *E. purpurea* roots, they are not useful since they were also found in the other species' root profiles in the present study (Tables 5.1-5.6). However, CDA here determined that quantitative differences in **1-3**, and **7** were useful for chemotaxonomic species differentiation.

Alkamide **11** was accumulated at the second highest amount in roots from hybrid populations, which suggests that its presence might be a phytochemical indicator of hybridization. In addition, *E. purpurea* roots may be identified by the lack of alkamide **18**, which is reported here for the first time in the highest amounts from the roots of *E. pallida* var. *sanguinea*. This study is also the first report of alkamides **5** and **15** from *E. atrorubens*.

Finally, the presence of high levels of the monoene-type alkamides **12**, **13** and **14** in root profiles of *E. atrorubens* and other varieties of *E. pallida* (as well as all hybrids) contradicts their current use as industry marker phytochemicals to distinguish *E. pallida* var. *angustifolia* and *E. pallida* var. *tennesseensis* (Bauer and Wagner 1991; Bauer 1998). Generally, both the monoene or diene patterns that diagnose *Echinacea* species roots are not present in appreciable quantities from the flowerheads for identification of market botanicals.

Ketoalken/ynes with a carbonyl group in the 2-position, such as **22**, **24** and **25** (Fig. 5.4) were reportedly predominant in the roots of *E. pallida* var. *pallida* (Bauer and Wagner 1989; Bauer 1998; Shulte 1967). They have been used extensively to identify *E. pallida* var. *pallida* root powders on the market since it was discovered that most "*E. angustifolia*" cultivated in Europe was in fact *E. pallida* var. *pallida* (Bauer *et al.* 1988). However, in the current study, **22** was measured in high levels from *E. pallida* var. *simulata*, *E. atrorubens* and *E. laevigata*, although it was found to be lacking in *E. pallida* var. *angustifolia*. Clearly, the presence of **22** does not serve to identify only *E. pallida* var. *pallida*, although it would eliminate *E. purpurea* from among all possible commercial *Echinacea* plant materials.

Spatial and temporal phytochemical variation

Alkamide variation within and between *Echinacea* varieties with respect to age and distribution in the plant may be explained by biosynthesis of closely-related molecules from a pool of precursor molecules (often termed "phytochemical redundancy"). Also, alkamides may serve as precursors for the longer chain alkenes, which would explain the lack of alkamides in varieties that accumulate high levels of **22**, **24**, and **25**. Alternatively, a reduction in carbon-based secondary metabolites (such as the phenolic caffeic acid derivatives) in cultivated *Echinacea* may result from a shift in the carbon: nutrient balance towards growth with greater nutrient availability (Coley *et al.* 1985). This theory is supported by the results of this study, especially with respect to the **cichoric acid** content of flowerheads from seed-grown compared to wild-grown *Echinacea* (Tables 5.2 and 5.5). Other research by Shalaby *et al.* (1997) found that fertilization with nitrogen increased the biomass of *E. purpurea* aerial parts. This group also studied the accumulation of alkamides as a result of age and fertilization in *E. purpurea* (El-Gengaihi *et al.* 1998). They determined that alkamide content of *E. purpurea* L. roots increased with age and reached a maximum at seed set, while the content in vegetative tissues decreased with maturity. Therefore, they found opposite spatial variation within the plants with respect to alkamide secondary metabolites.

In the present study, **tetraenes** in roots increased with age with some exceptions (see Results) and flowerheads cultivated from seed generally accumulated more **tetraenes** than their wild counterparts, similar to the study by El-Gengaihi *et al.* (1998). These results suggest that more mature aerial tissues (all wild populations were >1 year old) translocated their alkamide defenses to the roots, or stopped alkamide production over time. This supports the observation that alkamide concentrations increase over time and are predominantly spatially-oriented to the roots, while flowerheads increase production of certain phenolics. However, in two *Echinacea* varieties, mature plants accumulated higher mean levels of flowerhead **tetraenes**: *E. pallida* var. *sanguinea* and *E. pallida* var. *pallida*. Biosynthetic changes in response to environmental cues may account for these exceptions;

they were growing in dense stands with higher competition and herbivory than what is found in most other *Echinacea* habitats. There have been many reports of such phenotypic plasticity to buffer the effects of spatial and temporal variation in resource availability, and species with relatively high growth rates may be more plastic than those of slower growth (Bryant *et al.* 1983). In *Echinacea*, the secondary compound classes alkenes, alkamides and caffeic acid derivatives all contain many closely-related compounds whose relative concentrations vary throughout the growth of the plants.

Phytochemical support of taxonomic relationships

Polyploidy is known to promote novel adaptations, such as phytochemical defenses, which may lead to stable introgressants and/or speciation in plants (Lewis 1980). *E. pallida* var. *pallida* is known throughout its range as a tetraploid with chromosome number 2n = 44 (McGregor 1968). All other *Echinacea* taxa were determined to be diploid 2n = 22, except *E. pallida* var. *simulata* (sometimes 2n = 33 triploidy, and sometimes 2n = 22) and triploid hybrids or stable introgressants (McGregor 1968). There are reports of diploidy, triploidy and in the southern ranges, tetraploidy, for the group of stable introgressants which McGregor called *E. pallida* var. *strigosa* McGregor.

In this study, the ability to accumulate ketoalkyn/enes may be associated with polyploidy. High quantities of **24** and **25** were detected in the roots of the *E. pallida* var. *simulata* roots which were diploids and some triploids (according to McGregor 1968), as well as in roots of the putative diploids *E. atrorubens* var. *paradoxa* and *E. atrorubens* var. *neglecta*. The latter two varieties were often collected from sympatric or hybridizing populations in the present work, which raises the possibility of triploids and/or tetraploids among them, although ploidy was not determined. These findings suggest a chemotaxonomic relationship between the putative allotetraploids *E. pallida* var. *pallida* and the mountainous varieties of *E. atrorubens* (vars. *paradoxa* and *neglecta*), as well as the other varieties of *E. pallida* (vars. *sanguinea* and *simulata*). McGregor (1968) suggested that *E. pallida* var. *pallida* (which he treated as a species) arose from hybridization between *E. pallida* var. *simulata* and *E. pallida* var. *sanguinea*. In the current study, **24** was also detected in small amounts in the wild flowerheads of *E. pallida* var. *sanguinea* and of *E. atrorubens* var. *paradoxa* (both diploids according to McGregor 1968). Finally, although the ketoalkynes **24** and **25** were previously reported from the roots and flowerheads of tetraploids *E. pallida* var. *pallida* (Bauer *et al.* 1989) and roots of *E. atrorubens* var. *paradoxa* and *E. pallida* var. *simulata* (Bauer and Foster 1991), they have never been correlated to ploidy or taxonomic inferences. A more thorough examination of ploidy and its relationship to ketoalkyn/ene production is suggested in view of the current results.

There is also phytochemical evidence that *Echinacea* hybrids identified in the current study had recent diploid ancestors from among *E. pallida* and *E. atrorubens* because they were not differentiated by CDA analyses of root phytochemistry (this chapter) or by morphometric CDA (chapter 2). Unfortunately, the ploidy of these hybrids was not determined, but their hybrid status was supported (either interspecific between *E. pallida* and *E. atrorubens*, or intraspecific within *E. pallida* varieties), by overall morphological similarity to McGregor's artificial hybrid specimens (chapter 2). Alloploidy from multiple or recurrent origins exists in plants, for example in *Asplenium* (Werth *et al.* 1985), which may explain the phytochemical observations in *E. pallida* var. *pallida* tetraploids, as well as the putative prevalence of hybridization events along intermediate geographic zones (chapter 2; McGregor 1968).

The phytochemical variation reported in this study from *Echinacea* species and varieties was shown to be invaluable for germplasm improvement and quality control of *Echinacea* phytomedicines, according to multivariate (Fig. 5.22) and descriptive statistics (Fig. 5.23; Tables 5.1-5.6). Specfically, **cichoric acid**, the diene alkamides **1-3** and **7**, as well as ketoalkene **24** were particularly useful markers. Quantitative comparisons of mean phytochemical content indicated the following useful agronomic information for each of 26 phytochemicals measured; 1) varieties with the highest amounts, 2) organs with the highest accumulation and 3) which age and growth conditions were optimal for high-yielding varieties. This study provides baseline data on phytochemicals to be used as tools for the improvement of cultivated *Echinacea* germplasm and the protection of threatened natural populations through correct identification and regulation of wild harvesting.

LEGEND TO TABLES 5.1-5.6: *Echinacea* variety names are shortened to E. pur = *E. purpurea*, E. pal = *E. pallida* varieties ang (*angustifolia*), pal (*pallida*), sang (*sanguinea*), tenn (*tennesseensis*) and sim (*simulata*), E. laev = *E. laevigata*, E. atro = *E. atrorubens* varieties para (*paradoxa*), neg (*neglecta*) and atro (*atrorubens*). The following abbreviations are used for phytochemicals in *Echinacea* spp.: cic = cichoric acid, ech = echinacoside, chl = chlorogenic acid, caf = caffeic acid, ctar = caftaric acid, cyn = cynarin. Compounds indicated with a number correspond to the lipophilic alkamides 1-19 and ketoalkyn/enes 20-25 (see Figs. 5.1-5.6).

Table 5.1: Concentration of previously-reported phenolics, alkamides and ketoalken/ynes in roots of *Echinacea* species and varieties grown from wild germplasm and harvested at the age of one year or less (mean mg/g dwt ± SEM directly below). The n denotes sample sizes for each of the revised species and varieties. See legend p. 177.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
n	11	119	22	11	4	.	3	.	7	12	13
cic	8.06	0.63	0.50	0.08	0.32	.	5.70	.	0.95	0.04	0.37
	1.24	0.10	0.10	0.05	0.08	.	0.66	.	0.29	0.01	0.17
ech	0.00	6.87	1.78	0.01	0.92	.	9.79	.	0.48	0.09	4.89
	0.00	0.54	0.82	0.00	0.32	.	0.76	.	0.22	0.01	1.18
chl	0.09	0.16	0.08	0.06	0.23	.	0.98	.	0.38	0.03	0.18
	0.03	0.02	0.02	0.02	0.03	.	0.98	.	0.21	0.01	0.06
caf	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.
ctar	2.39	0.26	0.12	0.00	1.00	.	2.59	.	0.02	0.00	0.08
	0.22	0.05	0.04	0.00	0.40	.	0.48	.	0.01	0.00	0.01

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
cyn	0.00	5.00	0.00	0.00	4.23	.	0.00	.	0.00	0.04	5.21
	0.00	0.68	0.00	0.00	0.22	.	0.00	.	0.00	0.01	0.98
1	0.92	0.09	0.04	0.91	0.25	.	0.39	.	0.67	0.95	0.10
	0.17	0.01	0.01	0.26	0.05	.	0.05	.	0.54	0.12	0.03
2	3.16	0.09	0.58	0.25	0.82	.	1.03	.	1.28	0.35	0.12
	0.54	0.01	0.09	0.03	0.12	.	0.01	.	0.46	0.10	0.08
3	1.99	0.15	0.09	1.52	0.34	.	3.08	.	2.38	0.89	0.30
	0.15	0.02	0.03	0.30	0.04	.	0.40	.	1.26	0.17	0.19
4	0.23	0.05	0.02	0.00	0.39	.	0.00	.	0.24	0.04	0.13
	0.04	0.01	0.01	0.00	0.14	.	0.00	.	0.21	0.01	0.05
5+15	0.47	1.23	0.01	0.21	0.11	.	0.18	.	0.71	1.87	0.18
	0.12	0.71	0.00	0.07	0.04	.	0.01	.	0.59	0.22	0.07
7	1.99	0.05	0.14	0.00	0.61	.	0.71	.	1.84	0.00	0.11
	0.46	0.01	0.03	0.00	0.17	.	0.06	.	0.59	0.00	0.04
8+9	2.79	5.23	0.00	14.36	6.78	.	1.30	.	1.62	8.00	4.87
	0.64	0.40	0.00	0.86	1.57	.	0.14	.	1.62	1.12	0.91

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
10	0.30	0.46	0.00	0.42	0.58	.	0.08	.	0.00	6.23	0.45
	0.03	0.06	0.00	0.06	0.30	.	0.02	.	0.00	3.84	0.08
11	0.19	0.76	0.00	0.26	0.36	.	0.13	.	0.00	0.33	0.67
	0.04	0.08	0.00	0.05	0.08	.	0.02	.	0.00	0.05	0.14
12	0.00	0.64	0.00	1.87	4.69	.	1.42	.	0.10	2.30	0.57
	0.00	0.10	0.00	0.18	1.60	.	0.28	.	0.10	0.28	0.13
13	0.00	0.62	0.00	1.30	9.14	.	0.30	.	0.12	36.50	0.43
	0.00	0.07	0.00	0.17	4.16	.	0.02	.	0.12	24.55	0.08
14	0.00	0.59	0.03	1.69	1.49	.	0.68	.	0.00	1.41	0.36
	0.00	0.18	0.02	0.21	0.64	.	0.19	.	0.00	0.17	0.05
6	0.00	0.07	0.00	0.30	0.31	.	0.11	.	0.22	0.04	0.09
	0.00	0.01	0.00	0.06	0.04	.	0.01	.	0.22	0.03	0.04
16	0.40	0.16	0.02	0.31	1.13	.	0.00	.	0.03	0.34	0.10
	0.11	0.02	0.01	0.07	0.74	.	0.00	.	0.03	0.04	0.04
17	0.41	0.08	0.00	1.05	0.35	.	0.04	.	0.15	0.11	0.07
	0.16	0.01	0.00	0.34	0.15	.	0.04	.	0.15	0.01	0.01

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	Hybrids
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
18	0.02	0.77	0.38	29.00	1.28	.	0.26	.	0.49	1.40	0.84
	0.01	0.07	0.07	18.26	0.43	.	0.01	.	0.49	0.32	0.20
19	0.00	0.05	0.00	0.00	0.00	.	0.00	.	0.04	0.00	0.08
	0.00	0.01	0.00	0.00	0.00	.	0.00	.	0.03	0.00	0.02
22	0.00	0.02	0.66	0.00	0.00	.	0.00	.	0.80	0.00	0.00
	0.00	0.01	0.07	0.00	0.00	.	0.00	.	0.21	0.00	0.00
24	0.00	0.02	1.12	0.00	0.04	.	0.05	.	3.21	0.00	0.02
	0.00	0.01	0.08	0.00	0.03	.	0.03	.	0.62	0.00	0.01
25	0.00	0.04	1.56	0.00	0.00	.	0.00	.	0.26	0.00	0.14
	0.00	0.03	0.29	0.00	0.00	.	0.00	.	0.08	0.00	0.14

Table 5.2: Concentrations of previously-reported phenolics, alkamides and ketoalken/ynes in inflorescences of *Echinacea* species and varieties grown from wild germplasm and harvested at the age of one year or less (mean mg/g dwt ± SEM directly below). The n denotes sample sizes for each of the revised species and varieties.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
n	4	7	9	6	5	2	2	0	0	4	0
cic	4.17	0.45	1.18	3.30	0.03	0.49	1.78	.	.	0.01	.
	2.40	0.12	0.46	1.12	0.01	0.25	1.56	.	.	0.00	.
ech	0.01	0.11	0.56	0.00	0.00	0.17	0.00	.	.	0.06	.
	0.01	0.03	0.39	0.00	0.00	0.08	0.00	.	.	0.06	.
chl	0.83	1.33	0.46	0.12	0.00	0.52	0.14	.	.	0.06	.
	0.69	0.68	0.15	0.03	0.00	0.05	0.13	.	.	0.01	.
caf	.	.	.	.	0.09	.	.	.	.	.	.
	.	.	.	.	0.05	.	.	.	.	.	.
ctar	1.67	0.08	0.23	0.30	0.04	0.12	0.73	.	.	0.03	.
	1.06	0.04	0.14	0.07	0.01	0.11	0.68	.	.	0.01	.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
cyn	0.00	0.18	0.00	0.32	0.02	0.08	0.00	.	.	0.08	.
	0.00	0.06	0.00	0.05	0.01	0.08	0.00	.	.	0.08	.
1	0.48	1.16	0.30	0.06	0.10	1.04	0.50	.	.	1.51	.
	0.40	0.83	0.16	0.02	0.02	0.85	0.14	.	.	0.49	.
2	0.00	1.46	0.01	0.00	0.03	0.51	0.01	.	.	0.01	.
	0.00	0.83	0.01	0.00	0.01	0.13	0.00	.	.	0.01	.
3	0.03	0.01	0.02	0.01	0.02	0.04	0.03	.	.	0.06	.
	0.02	0.00	0.01	0.01	0.01	0.04	0.01	.	.	0.03	.
4	0.05	0.03	0.02	0.00	0.01	0.10	0.01	.	.	0.14	.
	0.04	0.01	0.01	0.00	0.00	0.07	0.00	.	.	0.03	.
5+15	0.05	0.06	0.08	0.04	0.02	0.18	0.11	.	.	0.10	.
	0.05	0.03	0.05	0.02	0.01	0.18	0.02	.	.	0.03	.
7	0.01	0.02	0.01	0.00	0.07	0.07	0.00	.	.	0.06	.
	0.01	0.01	0.00	0.00	0.02	0.00	0.00	.	.	0.02	.
8+9	3.45	4.53	1.59	1.33	10.35	4.96	1.80	.	.	2.89	.
	2.45	2.81	0.48	0.40	2.03	3.60	0.39	.	.	0.88	.

Species	E. pur	E. pal	E. pal	E. pal	E. pal	E. pal	E. laev	E. atro	E. atro	E. atro	Hybrids
Variety		ang	pal	sang	tenn	sim		para	neg	atro	
10	0.03	0.18	0.07	0.02	0.22	0.25	0.08	.	.	0.07	.
	0.01	0.10	0.03	0.01	0.07	0.12	0.01	.	.	0.03	.
11	0.06	0.11	0.09	0.01	0.15	0.29	0.05	.	.	0.06	.
	0.04	0.07	0.05	0.00	0.05	0.18	0.01	.	.	0.03	.
12	0.68	0.09	0.04	0.02	1.56	0.10	0.28	.	.	0.03	.
	0.40	0.07	0.01	0.01	0.38	0.04	0.16	.	.	0.02	.
13	0.00	0.00	0.03	0.00	0.04	0.00	0.00	.	.	0.09	.
	0.00	0.00	0.02	0.00	0.02	0.00	0.00	.	.	0.07	.
14	0.00	0.01	0.01	0.03	0.04	0.01	0.00	.	.	0.00	.
	0.00	0.01	0.00	0.01	0.01	0.01	0.00	.	.	0.00	.
6	0.08	0.14	0.04	0.00	1.33	0.18	0.00	.	.	0.12	.
	0.07	0.05	0.02	0.00	0.19	0.15	0.00	.	.	0.05	.
16	0.24	0.33	0.08	0.07	0.13	0.06	0.49	.	.	0.17	.
	0.16	0.28	0.03	0.03	0.04	0.06	0.17	.	.	0.08	.
17	0.05	0.02	0.03	0.00	0.02	0.06	0.00	.	.	0.04	.
	0.03	0.01	0.01	0.00	0.01	0.03	0.00	.	.	0.02	.

Species Variety	***E. pur***	***E. pal ang***	***E. pal pal***	***E. pal sang***	***E. pal tenn***	***E. pal sim***	***E. laev***	***E. atro para***	***E. atro neg***	***E. atro atro***	**Hybrids**
18	0.39	0.20	0.52	0.36	0.05	0.19	0.00	.	.	1.41	.
	0.27	0.08	0.20	0.16	0.02	0.07	0.00	.	.	0.45	.
19	0.03	0.00	0.02	0.02	0.00	0.01	0.00	.	.	0.09	.
	0.03	0.00	0.01	0.01	0.00	0.01	0.00	.	.	0.03	.
22	0.00	0.00	0.00	0.07	0.00	0.03	0.00	.	.	0.01	.
	0.00	0.00	0.00	0.07	0.00	0.03	0.00	.	.	0.01	.
24	0.00	0.01	0.07	0.11	0.38	0.05	0.00	.	.	0.00	.
	0.00	0.00	0.05	0.04	0.06	0.02	0.00	.	.	0.00	.
25	0.00	0.00	0.02	0.01	0.41	0.03	0.00	.	.	0.02	.
	0.00	0.00	0.01	0.01	0.19	0.03	0.00	.	.	0.01	.

Table 5.3: Concentrations of previously-reported phenolics, alkamides and ketoalken/ynes in roots of *Echinacea* species and varieties from wild populations (mean mg/g dwt ± SEM directly below).

The n denotes sample sizes for each of the revised species and varieties.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
n	4	20	14	6	4	2	1	4	5	5	11
cic	5.88	0.61	1.19	0.12	0.35	0.46	.	1.75	0.23	0.03	1.20
	3.23	0.70	0.93	0.03	0.08	0.23	.	0.81	0.06	0.01	0.71
ech	0.10	2.03	1.13	0.38	0.38	1.57	.	32.99	8.41	0.18	2.62
	0.10	0.49	0.68	0.13	0.18	0.75	.	9.07	4.28	0.16	1.04
chl	1.92	0.06	0.14	0.45	0.08	0.17	.	0.30	0.17	0.05	0.29
	0.63	0.19	0.07	0.17	0.03	0.10	.	0.07	0.06	0.03	0.19
caf	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.
ctar	0.00	0.04	.	.	.	0.04	.	.	0.10	0.04	0.08
	.	0.01	.	.	.	0.00	.	.	.	0.02	0.04
cyn	0.07	0.42	0.00	.	.	.	.	.	0.00	0.00	0.21
	0.06	0.10	0.00	.	.	.	.	.	.	0.00	0.11

Species	E. pur	E. pal	E. pal	E. pal	E. pal	E. pal	E. laev	E. atro	E. atro	E. atro	Hybrids
Variety		ang	pal	sang	tenn	sim		para	neg	atro	
1	0.25	0.06	0.08	0.52	0.13	0.06	.	0.06	0.68	0.43	0.46
	0.12	0.04	0.05	0.25	0.01	0.03	.	0.01	0.64	0.21	0.12
2	2.13	0.65	0.12	1.26	0.16	0.24	.	0.01	0.11	0.06	0.25
	1.08	0.58	0.07	0.77	0.08	0.13	.	0.01	0.06	0.03	0.15
3	3.88	0.14	0.62	2.71	0.00	0.32	.	0.13	0.25	0.03	0.82
	1.49	0.40	0.30	1.42	0.00	0.09	.	0.06	0.07	0.01	0.48
4	1.94	0.11	0.31	0.36	0.03	0.15	.	0.02	0.26	0.34	0.46
	1.52	0.30	0.11	0.21	0.03	0.06	.	0.02	0.26	0.17	0.25
5+15	0.10	0.13	0.05	0.07	0.11	0.00	.	0.00	0.00	0.00	0.11
	0.10	0.06	0.03	0.05	0.07	0.00	.	0.00	0.00	0.00	0.06
7	1.56	0.07	0.21	0.42	0.12	0.52	.	0.10	0.87	0.60	0.48
	0.67	0.17	0.08	0.09	0.03	0.31	.	0.01	0.62	0.34	0.13
8+9	3.12	4.74	4.83	19.00	0.17	0.55	.	0.00	0.85	1.98	9.23
	0.87	0.87	2.11	7.02	0.14	0.25	.	0.00	0.81	0.78	2.23
10	0.08	0.32	0.50	0.94	0.03	0.03	.	0.00	0.09	0.17	0.80
	0.03	0.07	0.21	0.33	0.03	0.02	.	0.00	0.09	0.09	0.21

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	Hybrids
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
11	0.63	0.58	0.70	0.34	0.03	0.02	.	0.00	0.31	0.11	0.84
	0.58	0.19	0.35	0.09	0.02	0.01	.	0.00	0.30	0.06	0.24
12	0.02	0.55	0.10	1.57	1.36	0.05	.	0.00	0.64	0.17	1.21
	0.02	0.13	0.06	0.78	0.24	0.03	.	0.00	0.63	0.07	0.29
13	0.00	1.38	0.15	1.66	8.42	5.37	.	0.12	0.10	0.41	1.04
	0.00	0.44	0.08	0.47	1.16	5.34	.	0.03	0.05	0.41	0.24
14	0.00	0.87	0.09	3.61	0.58	0.02	.	0.03	0.03	0.03	1.23
	0.00	0.31	0.04	1.66	0.29	0.01	.	0.01	0.02	0.03	0.46
6	0.10	0.15	0.05	0.44	0.42	0.04	.	0.00	0.01	0.00	0.08
	0.08	0.04	0.03	0.35	0.31	0.01	.	0.00	0.01	0.00	0.03
16	0.35	0.29	0.15	0.31	2.23	0.07	.	0.02	0.07	0.12	0.20
	0.21	0.13	0.06	0.09	0.64	0.02	.	0.00	0.05	0.06	0.05
17	0.00	0.28	0.02	1.62	0.20	0.02	.	0.00	0.17	0.11	0.34
	0.00	0.07	0.01	0.39	0.06	0.02	.	0.00	0.10	0.10	0.12
18	0.04	0.33	0.57	1.04	0.19	0.31	.	0.22	0.52	0.31	0.62
	0.02	0.10	0.15	0.34	0.04	0.11	.	0.08	0.22	0.09	0.14

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	Hybrids
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
19	0.02	0.02	0.04	0.08	0.02	0.04	.	0.11	0.47	0.03	0.07
	0.02	0.01	0.01	0.04	0.02	0.04	.	0.03	0.38	0.02	0.02
22	0.02	0.04	0.20	0.00	0.06	0.49	.	0.60	0.99	0.26	0.01
	0.02	0.02	0.08	0.00	0.06	0.18	.	0.30	0.43	0.20	0.01
24	0.13	0.02	0.39	0.19	0.18	0.88	.	10.08	5.29	0.58	0.18
	0.09	0.02	0.16	0.05	0.18	0.28	.	2.23	2.82	0.50	0.07
25	0.02	0.07	0.43	0.20	0.07	0.26	.	0.49	0.41	0.01	0.06
	0.01	0.06	0.15	0.12	0.05	0.08	.	0.23	0.20	0.01	0.02

Table 5.4: Concentrations of previously-reported phenolics, alkamides and ketoalken/ynes in roots of *Echinacea* species and varieties transplanted from wild populations to a greenhouse or grown from seed and harvested after the age of one year (mean mg/g dwt ± SEM directly below). The n denotes sample sizes for each of the revised species and varieties.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
n	5	8	9	7	17	1	3	0	2	6	0
cic	4.81	0.08	0.05	0.06	0.03	.	0.53	.	.	0.07	.
	2.66	0.01	0.02	0.03	0.01	.	0.27	.	.	0.02	.
ech	0.54	1.21	0.18	0.25	0.01	.	0.00	.	.	0.48	.
	0.53	0.54	0.04	0.11	0.01	.	0.00	.	.	0.31	.
chl	0.16	0.04	0.26	0.09	0.02	.	0.07	.	.	0.03	.
	0.10	0.02	0.13	0.05	0.01	.	0.06	.	.	0.01	.
caf	0.07	.	.	.	0.01	.	0.14	.	.	.	.
	0.01	.	.	.	0.00	.	0.04	.	.	.	.
ctar	0.83	0.06	0.04	0.06	0.00	.	0.00	.	.	0.04	.
	0.64	0.01	0.01	0.03	0.00	.	0.00	.	.	0.02	.

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	Hybrids
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
cyn	0.08	0.56	0.00	0.00	0.14	.	0.03	.	.	0.00	.
	0.04	0.36	0.00	0.00	0.03	.	0.03	.	.	0.00	.
1	1.25	0.38	0.08	0.28	0.14	.	0.17	.	.	0.65	.
	0.57	0.12	0.03	0.20	0.02	.	0.07	.	.	0.15	.
2	4.04	0.53	0.61	0.16	0.44	.	0.34	.	.	0.74	.
	1.59	0.19	0.17	0.07	0.14	.	0.16	.	.	0.34	.
3	2.83	0.93	0.16	1.10	0.37	.	1.33	.	.	1.24	.
	1.02	0.36	0.08	0.74	0.07	.	0.63	.	.	0.62	.
4	0.30	0.02	0.02	0.02	0.27	.	0.02	.	.	0.01	.
	0.15	0.01	0.01	0.01	0.08	.	0.01	.	.	0.01	.
5+15	0.53	0.71	0.06	0.11	0.24	.	0.08	.	.	1.70	.
	0.25	0.17	0.03	0.06	0.04	.	0.08	.	.	1.03	.
7	2.30	0.13	0.17	0.13	0.33	.	0.23	.	.	1.22	.
	0.80	0.06	0.06	0.07	0.09	.	0.11	.	.	1.03	.
8+9	4.61	9.94	0.39	6.64	1.62	.	0.06	.	.	9.50	.
	1.81	1.82	0.21	4.15	0.24	.	0.03	.	.	2.61	.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
10	0.39	1.43	0.03	0.32	0.20	.	0.01	.	.	0.97	.
	0.14	0.33	0.02	0.21	0.03	.	0.01	.	.	0.44	.
11	0.20	2.27	0.04	0.18	0.11	.	0.00	.	.	0.68	.
	0.07	0.58	0.02	0.13	0.02	.	0.00	.	.	0.24	.
12	0.06	2.19	0.03	1.30	2.83	.	0.48	.	.	1.78	.
	0.06	0.43	0.02	0.84	0.34	.	0.25	.	.	0.49	.
13	0.00	2.27	0.02	0.87	6.93	.	0.20	.	.	0.49	.
	0.00	0.48	0.02	0.45	0.46	.	0.09	.	.	0.46	.
14	0.02	1.22	0.08	1.57	0.66	.	0.28	.	.	1.68	.
	0.02	0.28	0.04	1.06	0.08	.	0.14	.	.	0.63	.
6	0.00	0.18	0.07	0.17	0.24	.	0.09	.	.	0.17	.
	0.00	0.06	0.03	0.09	0.03	.	0.04	.	.	0.09	.
16	0.74	0.22	0.03	0.09	0.83	.	0.04	.	.	0.25	.
	0.19	0.04	0.02	0.04	0.13	.	0.04	.	.	0.09	.
17	0.06	0.25	0.03	0.45	0.57	.	0.11	.	.	0.07	.
	0.03	0.05	0.02	0.29	0.05	.	0.06	.	.	0.06	.

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	**Hybrids**
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
18	0.08	1.33	0.42	0.80	0.43	.	0.01	.	.	1.28	.
	0.02	0.31	0.13	0.48	0.09	.	0.01	.	.	0.47	.
19	0.00	0.14	0.02	0.12	0.00	.	0.01	.	.	0.13	.
	0.00	0.03	0.01	0.08	0.00	.	0.01	.	.	0.05	.
22	0.00	0.04	0.40	0.14	0.00	.	0.44	.	.	0.01	.
	0.00	0.04	0.14	0.10	0.00	.	0.23	.	.	0.01	.
24	0.02	0.07	0.74	0.22	0.04	.	0.04	.	.	0.13	.
	0.02	0.02	0.19	0.10	0.01	.	0.04	.	.	0.04	.
25	0.00	0.04	0.71	0.08	0.00	.	0.00	.	.	0.03	.
	0.00	0.01	0.23	0.05	0.00	.	0.00	.	.	0.02	.

Table 5.5: Concentrations of previously-reported phenolics, alkamides and ketoalken/ynes in inflorescences of *Echinacea* spp. and varieties that were transplanted from wild and cultivated populations (mean mg/g dwt ± SEM directly below). The n denotes sample sizes for each of the revised species and varieties.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
n	46	3	2	4	0	2	0	0	1	2	0
cic	8.89	0.17	3.24	10.13	.	2.38	.	.	.	0.04	.
	0.92	0.13	3.24	4.62	.	2.30	.	.	.	0.04	.
ech	0.00	0.18	0.34	1.05	.	0.05	.	.	.	0.03	.
	0.00	0.16	0.06	0.76	.	0.05	.	.	.	0.03	.
chl	.	0.33	1.31	0.63	.	0.02	.	.	.	2.66	.
	.	0.19	0.98	0.38	.	0.01	.	.	.	2.64	.
caf	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.
ctar	.	0.13	1.23	1.61	.	0.02	.	.	.	.	.
	.	0.05	1.01	0.25	.	0.01	.	.	.	.	.
cyn	.	0.31	0.00	.	.	0.00	.	.	.	.	.
	.	0.29	0.00	.	.	0.00	.	.	.	.	.

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	**Hybrids**
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
1	.	0.24	0.54	0.42	.	.	.	.	.	1.70	.
	.	0.18	0.48	0.28	.	.	.	.	.	1.61	.
2	.	0.71	0.09	0.00	.	.	.	.	.	0.05	.
	.	0.62	0.09	0.00	.	.	.	.	.	0.05	.
3	.	0.02	0.06	0.14	.	.	.	.	.	0.07	.
	.	0.01	0.06	0.14	.	.	.	.	.	0.06	.
4	.	0.02	0.03	0.01	.	.	.	.	.	0.16	.
	.	0.01	0.03	0.01	.	.	.	.	.	0.15	.
5+15	.	0.03	0.03	0.03	.	0.00	.	.	.	0.28	.
	.	0.01	0.03	0.02	.	0.00	.	.	.	.	.
7	.	0.01	0.14	0.00	.	0.38	.	.	.	0.12	.
	.	0.01	0.08	0.00	.	0.32	.	.	.	0.11	.
8+9	3.13	2.07	1.76	2.17	.	1.84	.	.	.	0.32	.
	0.44	0.81	1.43	0.94	.	.	.	.	.	0.24	.
10	.	0.02	0.27	0.01	.	0.03	.	.	.	0.00	.
	.	0.01	0.13	0.01	.	0.03	.	.	.	0.00	.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
11	.	0.08	0.22	0.00	.	0.03	.	.	.	0.00	.
	.	0.03	0.14	0.00	.	0.01	.	.	.	0.00	.
12	.	0.05	0.05	0.00	.	0.01	.	.	.	0.00	.
	.	0.02	0.03	0.00	.	0.01	.	.	.	0.00	.
13	.	0.00	0.00	0.00	.	0.00	.	.	.	0.00	.
	.	0.00	0.00	0.00	.	0.00	.	.	.	0.00	.
14	.	0.00	0.00	0.00	.	0.00	.	.	.	0.00	.
	.	0.00	0.00	0.00	.	0.00	.	.	.	0.00	.
6	.	0.02	0.00	0.00	.	0.07	.	.	.	0.00	.
	.	0.02	0.00	0.00	.	0.07	.	.	.	0.00	.
16	.	0.05	0.52	0.02	.	0.13	.	.	.	0.32	.
	.	0.04	0.00	0.01	.	0.10	.	.	.	0.31	.
17	.	0.01	0.02	0.01	.	0.00	.	.	.	0.00	.
	.	0.01	0.02	0.01	.	0.00	.	.	.	0.00	.
18	.	0.26	0.00	0.20	.	1.12	.	.	.	0.10	.
	.	0.03	0.00	0.10	.	1.06	.	.	.	0.03	.

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	Hybrids
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
19	.	0.00	0.00	0.00	.	0.04	.	.	.	0.03	.
	.	0.00	0.00	0.00	.	0.04	.	.	.	0.03	.
22	.	0.00	0.00	0.47	.	.	.	.	.	0.00	.
	.	0.00	0.00	0.41	.	.	.	.	.	0.00	.
24	.	0.02	0.03	0.00	.	0.00	.	.	.	0.00	.
	.	0.02	0.03	0.00	.	0.00	.	.	.	0.00	.
25	.	0.00	0.00	0.00	.	0.00	.	.	.	0.05	.
	.	0.00	0.00	0.00	.	0.00	.	.	.	0.05	.

Table 5.6: Concentrations of previously-reported phenolics, alkamides and ketoalken/ynes in inflorescences of *Echinacea* spp. and varieties harvested from wild populations (mean mg/g dwt ± SEM directly below). The n denotes sample sizes for each of the revised species and varieties.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
n	**1**	**16**	**14**	**9**	**5**	**5**	**1**	**4**	**5**	**6**	**10**
cic	.	3.40	12.73	29.75	13.88	6.69	.	0.39	0.54	0.12	3.12
	.	2.12	2.89	5.39	3.37	2.12	.	0.27	0.16	0.05	1.94
ech	.	0.48	0.67	1.56	1.70	0.21	.	6.33	18.20	0.07	4.34
	.	0.11	0.20	0.48	0.56	0.07	.	1.88	5.32	0.03	3.85
chl	.	2.17	2.10	2.69	1.72	2.58	.	3.36	3.80	3.62	3.24
	.	0.52	0.39	0.98	0.53	0.93	.	0.70	0.97	1.62	0.76
caf	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.
ctar	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.
cyn	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
1	.	0.33	0.04	0.04	0.07	0.21	.	0.05	0.28	0.40	0.27
	.	0.05	0.03	0.03	0.01	0.10	.	0.02	0.10	0.08	0.15
2	.	0.25	0.01	0.00	0.01	0.14	.	0.75	0.69	0.02	0.02
	.	0.08	0.00	0.00	0.01	0.11	.	0.41	0.14	0.01	0.01
3	.	0.02	0.01	0.00	0.01	0.03	.	0.04	0.03	0.02	0.05
	.	0.01	0.00	0.00	0.00	0.02	.	0.03	0.00	0.01	0.03
4	.	0.03	0.00	0.00	0.00	0.08	.	0.02	0.07	0.01	0.02
	.	0.02	0.00	0.00	0.00	0.03	.	0.01	0.03	0.01	0.01
5+15	.	0.03	0.00	0.00	0.03	0.02	.	0.63	0.19	0.01	0.00
	.	0.01	0.00	0.00	0.02	0.01	.	0.03	0.03	0.01	0.00
7	.	0.05	0.01	0.00	0.04	0.03	.	0.12	0.05	0.05	0.15
	.	0.01	0.00	0.00	0.02	0.01	.	0.12	0.03	0.05	0.14
8+9	.	3.01	5.55	2.60	4.47	2.46	.	4.77	5.64	2.54	3.94
	.	0.41	4.15	0.74	1.74	0.81	.	0.95	0.52	0.96	1.52
10	.	0.10	0.09	0.33	0.06	0.03	.	0.02	0.00	0.04	0.18
	.	0.02	0.03	0.21	0.03	0.01	.	0.02	0.00	0.03	0.13

Species	*E. pur*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. pal*	*E. laev*	*E. atro*	*E. atro*	*E. atro*	Hybrids
Variety		*ang*	*pal*	*sang*	*tenn*	*sim*		*para*	*neg*	*atro*	
11	.	0.13	0.04	0.14	0.04	0.00	.	0.00	0.03	0.01	0.14
	.	0.02	0.02	0.05	0.01	0.00	.	0.00	0.03	0.01	0.10
12	.	0.02	0.02	0.00	0.81	0.05	.	0.19	0.01	0.09	0.51
	.	0.01	0.01	0.00	0.21	0.02	.	0.11	0.01	0.04	0.37
13	.	0.02	0.01	0.03	0.05	0.02	.	0.01	0.01	0.03	0.02
	.	0.01	0.01	0.03	0.03	0.01	.	0.01	0.01	0.03	0.01
14	.	0.02	0.03	0.00	0.00	0.02	.	0.04	0.02	0.01	0.08
	.	0.01	0.01	0.00	0.00	0.02	.	0.02	0.01	0.01	0.05
6	.	0.01	0.01	0.01	0.34	0.04	.	0.08	0.02	0.03	0.05
	.	0.00	0.00	0.01	0.26	0.02	.	0.01	0.02	0.02	0.04
16	.	0.10	0.03	0.04	0.80	0.23	.	0.02	0.16	0.04	0.09
	.	0.02	0.01	0.03	0.43	0.07	.	0.02	0.04	0.03	0.05
17	.	0.08	0.17	0.00	0.07	0.02	.	1.07	0.00	0.01	0.64
	.	0.07	0.07	0.00	0.02	0.01	.	0.65	0.00	0.01	0.48
18	.	0.21	0.21	0.37	0.14	0.23	.	0.05	0.12	0.68	0.47
	.	0.05	0.05	0.11	0.06	0.05	.	0.02	0.04	0.22	0.14

Species	***E. pur***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. pal***	***E. laev***	***E. atro***	***E. atro***	***E. atro***	**Hybrids**
Variety		***ang***	***pal***	***sang***	***tenn***	***sim***		***para***	***neg***	***atro***	
19	.	0.01	0.01	0.08	0.05	0.00	.	0.00	0.00	0.02	0.03
	.	0.01	0.01	0.04	0.02	0.00	.	0.00	0.00	0.01	0.02
22	.	0.05	0.00	0.00	0.00	0.29	.	0.08	0.00	0.04	0.01
	.	0.05	0.00	0.00	0.00	0.29	.	0.08	0.00	0.04	0.01
24	.	0.27	0.67	1.33	0.06	0.04	.	0.36	0.06	0.02	0.12
	.	0.17	0.29	0.39	0.05	0.04	.	0.33	0.05	0.01	0.04
25	.	0.03	0.06	0.01	0.04	0.01	.	0.06	0.14	0.04	0.06
	.	0.01	0.03	0.01	0.03	0.01	.	0.03	0.06	0.04	0.03

Table 5.7 Squared Mahalanobis distances between *Echinacea* varieties according to a CDA of 13 root phytochemicals. All variety groups were significantly different by the F-statistic for the squared Mahalanobis distances ($p<0.0001$) except those marked by * ($p>0.05$).

	1	2	3	4	5	6	7	8	9	10	H
1	0										
2	31.3	0									
3	23.9	3.3	0								
4	29.3	7.3	6.2	0							
5	159.9	138.7	127.8	138.9	0						
6	39.6	38.5	25.3	29.7	62.7	0					
7	29.7	8.1	7.1	5.2	144.9	33.7	0				
8	49.0	17.4	20.1	15.1	165.6	56.4	17.3	0			
9	16.3	11.2	10.5	14.3	146.9	38.3	9.8	25.2	0		
10	23.2	4.1	0.6	5.8*	126.4	23.1	7.06	19.6	9.42	0	
H	26.3	0.89*	2.2	4.9	136.7	32.4	6.18	17.2	9.05	2.4*	0

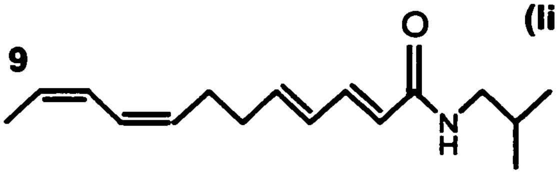
1 = *E. purpurea*; 2= *E. pallida* var. *angustifolia*; 3= *E. pallida* var. *pallida*; 4= *E. atrorubens* var. *atrorubens*; 5= *E. atrorubens* var. *paradoxa*; 6= *E. atrorubens* var. *neglecta*; 7= *E. pallida* var. *sanguinea*; 8= *E. pallida* var. *tennesseensis*; 9= *E. laevigata*; 10= *E. pallida* var. *simulata*; H= hybrids/introgressants.

Figure 5.1: The major alkamide phytochemicals, tetraenes, and two other monoene types in *Echinacea* species.

8

8+9 dodeca-2E,4E, 8Z,10E/Z-tetraenoic acid isobutylamide (lipophilic standard)

9



10

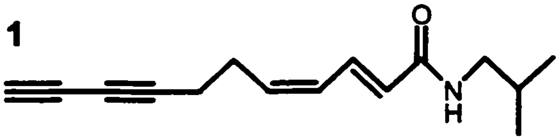
dodeca-2E,4E,8Z-trienoic acid isobutylamide

11

dodeca-2E,4E-dienoic acid isobutylamide

Figure 5.2: Alkamide phytochemicals in *Echinacea* species and their structures (note the common diene in conjugation with the carbonyl group).

1

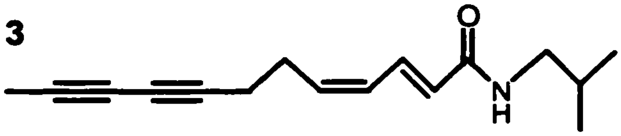


Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide

2

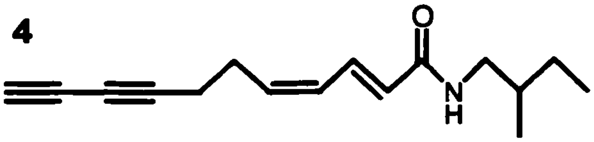
Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide

3



Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide

4



Undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide

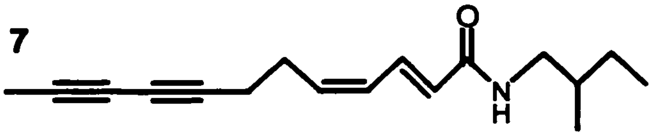
5

Dodeca-2E,4E,10E-triene-8-ynoic acid isobutylamide

6

Trideca-2E,7Z-diene-10,12-diynoic acid isobutylamide

7



Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide

Figure 5.3: Alkamide phytochemicals in *Echinacea* species (predominantly monoenes in conjugation with the carbonyl group).

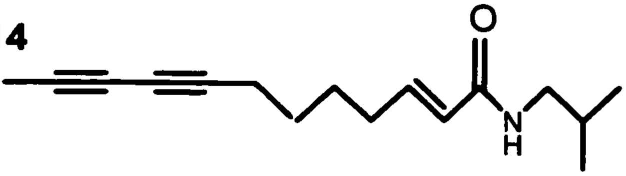
12

undeca-2E-en-8,10-diynoic acid isobutylamide

13

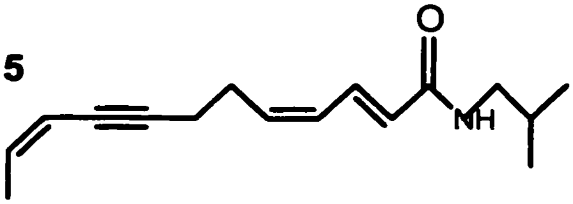
undeca-2Z-en-8,10-diynoic acid isobutylamide

14



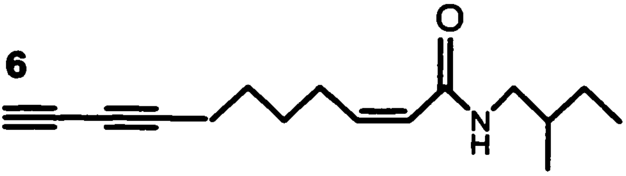
dodeca-2E-en-8,10-diynoic acid isobutylamide

15



undeca-2E,4Z,10Z-trien-8-ynoic acid isobutylamide

16



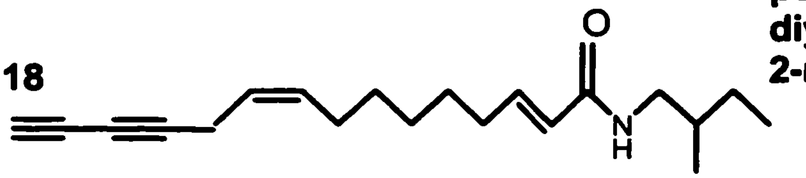
undeca-2Z-en-8,10-diynoic acid 2-methylbutylamide

17

dodeca-2E-en-8,10-diynoic acid 2-methylbutylamide

18

pentadeca-2E,9Z-dien-12,14-diynoic acid 2-methylbutylamide



19

hexadeca-2E,9Z-dien-12,14-diynoic acid 2-methylbutylamide

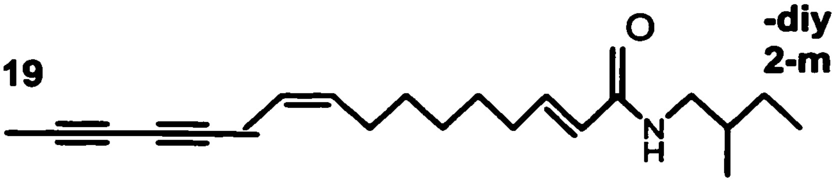
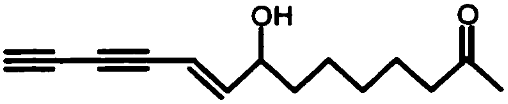


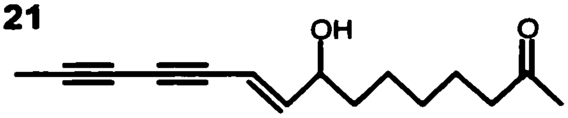
Figure 5.4: Ketoalkyn/ene phytochemicals in *Echinacea* spp. roots and inflorescences (note the lack of amide group).

20



8-hydroxytetradeca-9E-ene-11,13-diy
-one

21

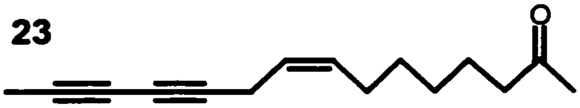


8-hydroxypentadeca-9E-ene-
11,13-diyn-2-one

22

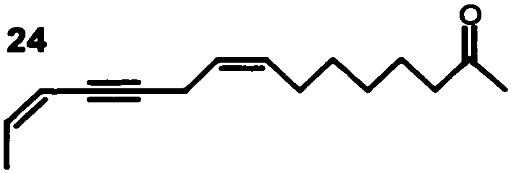
tetradeca-8Z-ene-11,13-
diyn-2-one

23



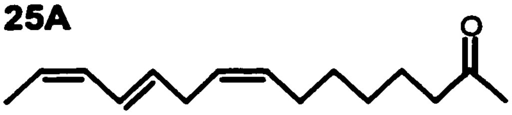
pentadeca-8Z-ene-11,13-
diyn-2-one

24



pentadeca-8Z,13Z-dien-11-
yn-2-one

25A

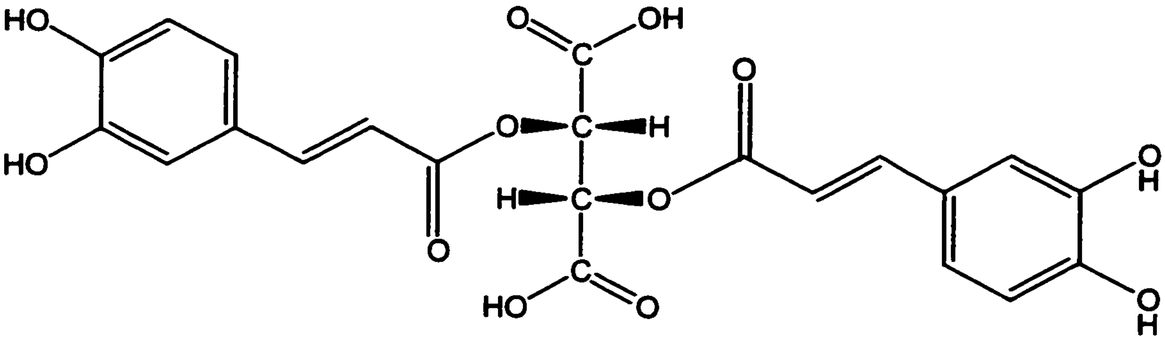


pentadeca-8Z,11E,13Z-
trien-2-one

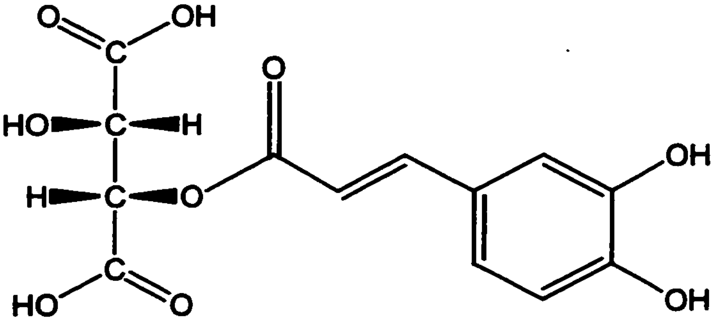
25B

pentadeca-8Z,11Z,13Z-
trien-2-one

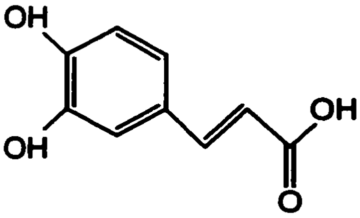
Figure 5.5: Caffeic acid and two of its derivatives from *Echinacea* spp. roots and inflorescences.



Cichoric acid
(2,3-*O*-dicaffeoyl tartaric acid)

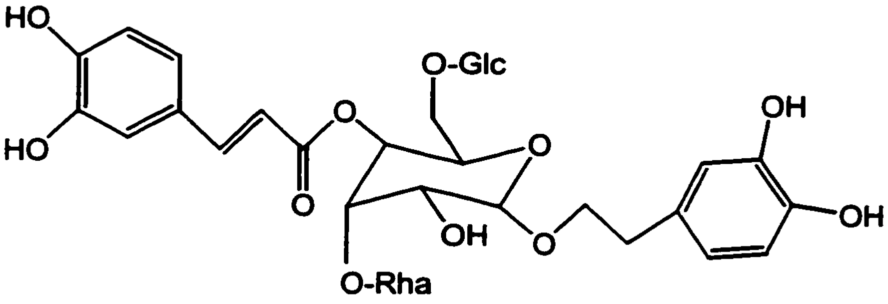


Caftaric acid
(2-*O*-caffeoyl tartaric acid)

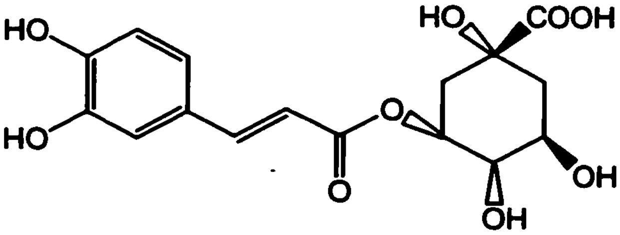


Caffeic acid

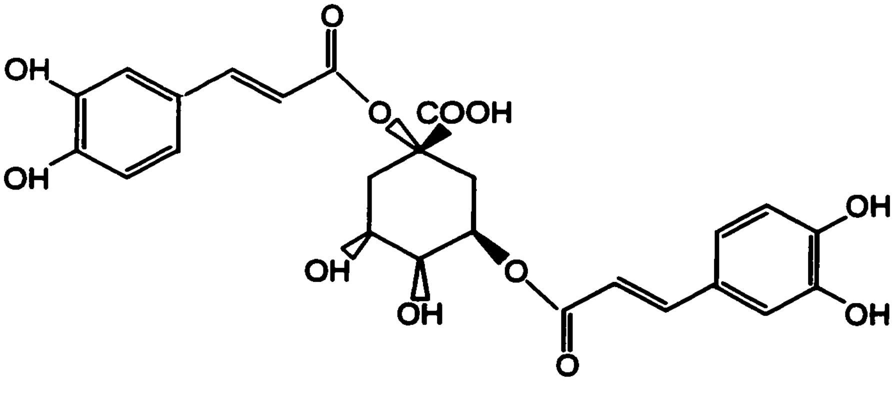
Figure 5.6: The phenolics echinacoside, chlorogenic acid and cynarin found in *Echinacea* spp. roots and inflorescences.



Echinacoside

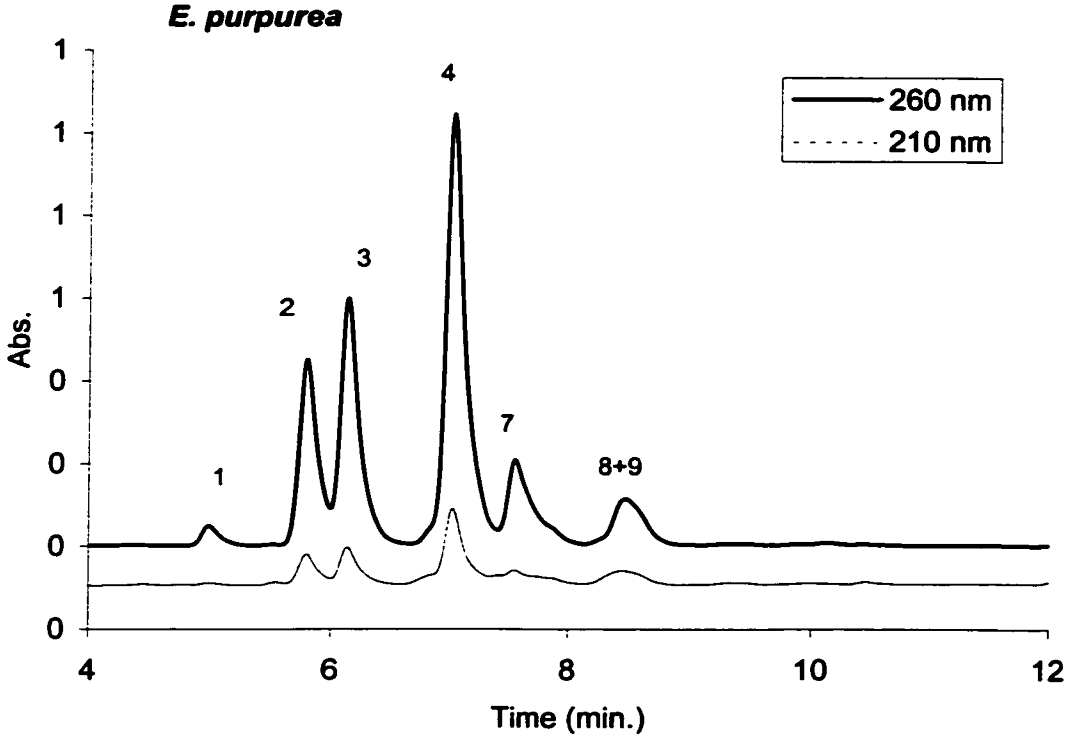


Chlorogenic acid
(3-*O*-caffeoyl quinic acid)

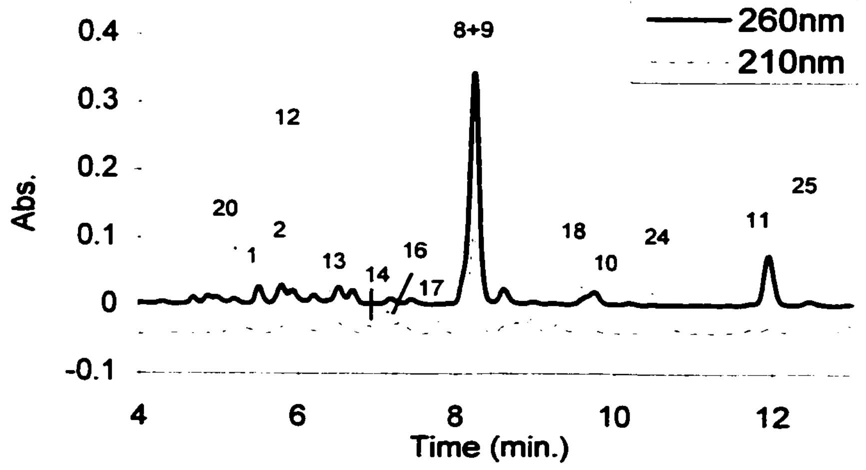


Cynarin
(1,3-dicaffeoyl quinic acid)

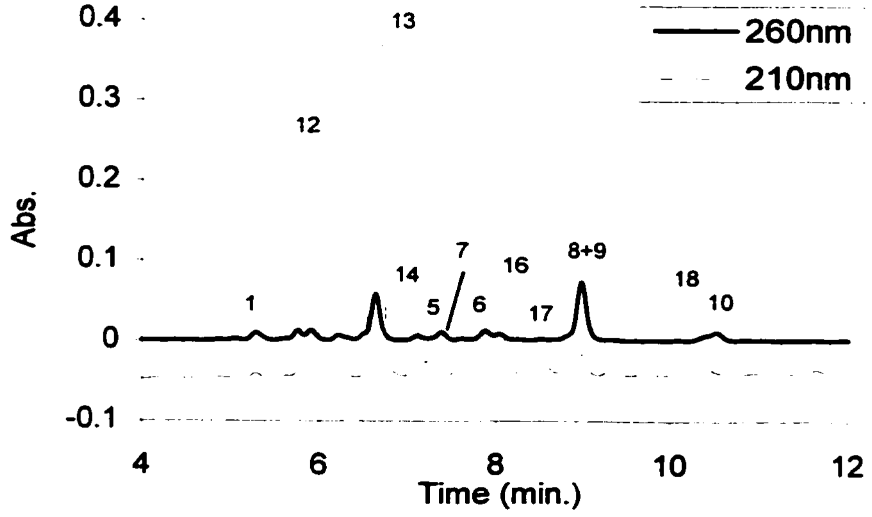
Figures 5.7-5.10: HPLC chromatograms of lipophilic phytochemicals which represent typical root profiles for each revised *Echinacea* taxon. Numbering refers to chemical structure and compound names (Figs. 5.1-5.4). P) unreported polyene confirmed by UV-scan (similar to 22), UA) unreported alkamide confirmed by UV-scan (usually like diene-types).


E. purpurea
1
1
1
1
0
0
0
0
Abs.
4
2
3
1
7
8+9
260 nm
210 nm
4
6
8
10
12
Time (min.)

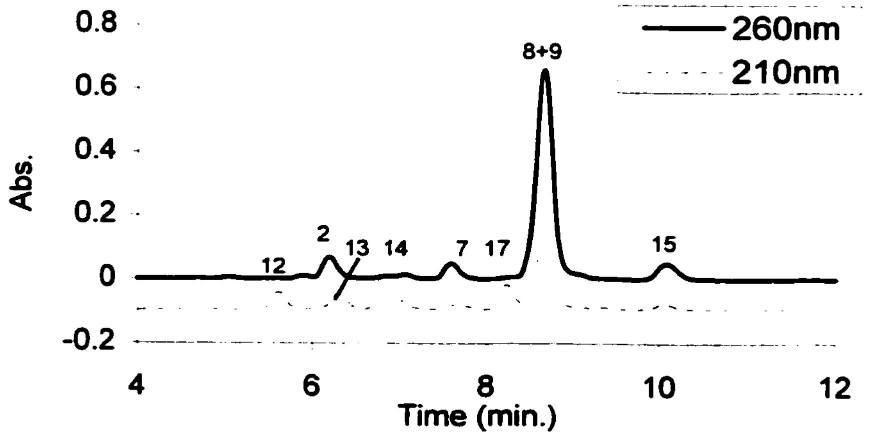
E. pallida* var. *angustifolia

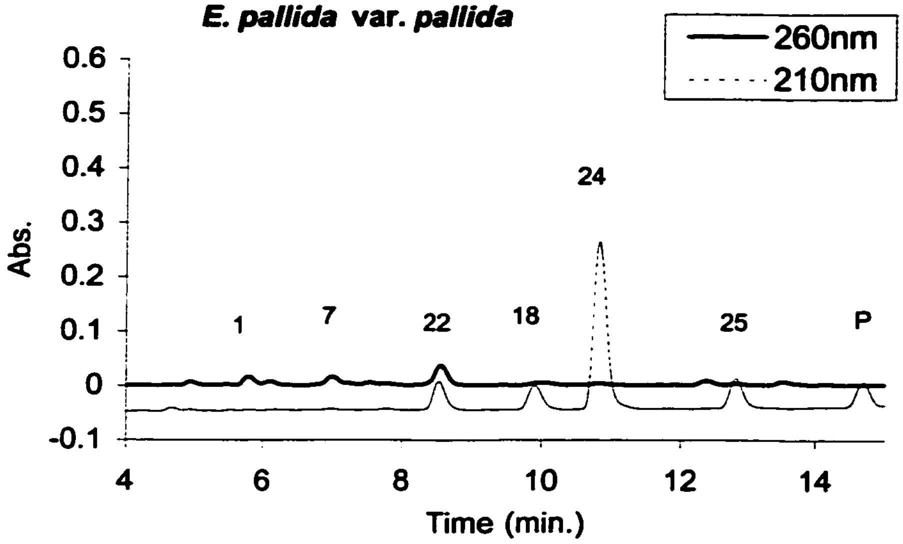


E. pallida* var. *tennesseensis

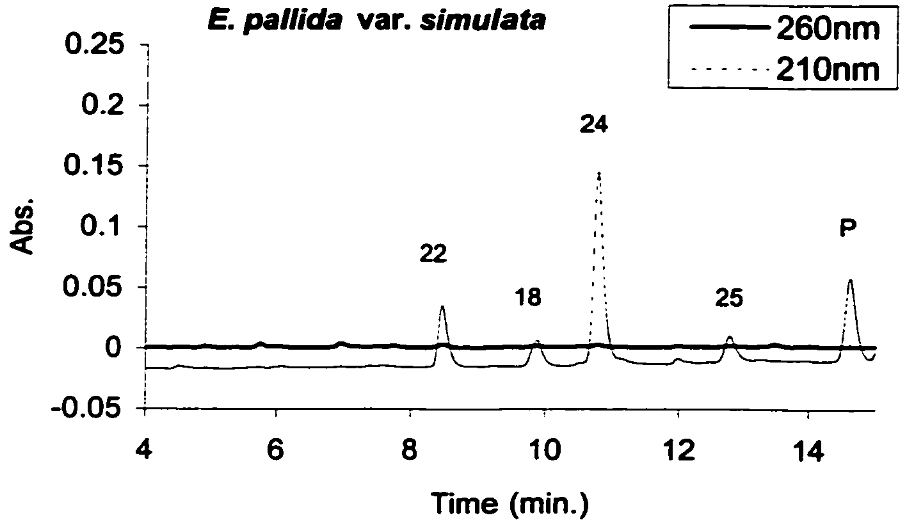


E. pallida* var. *sanguinea

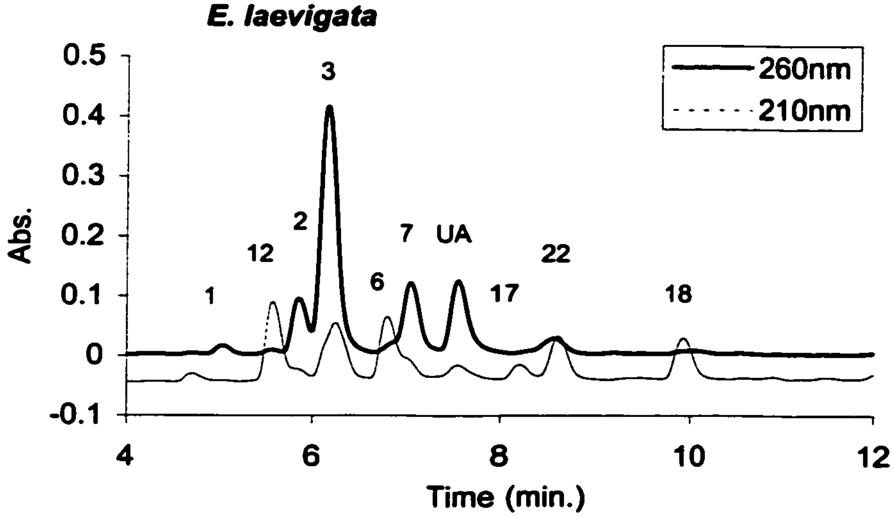




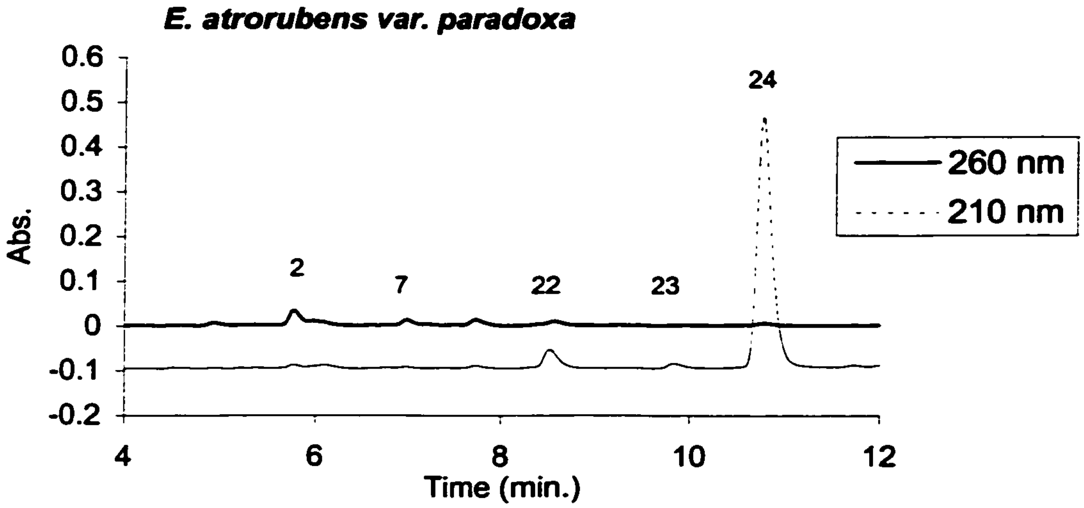
E. pallida var. *pallida*

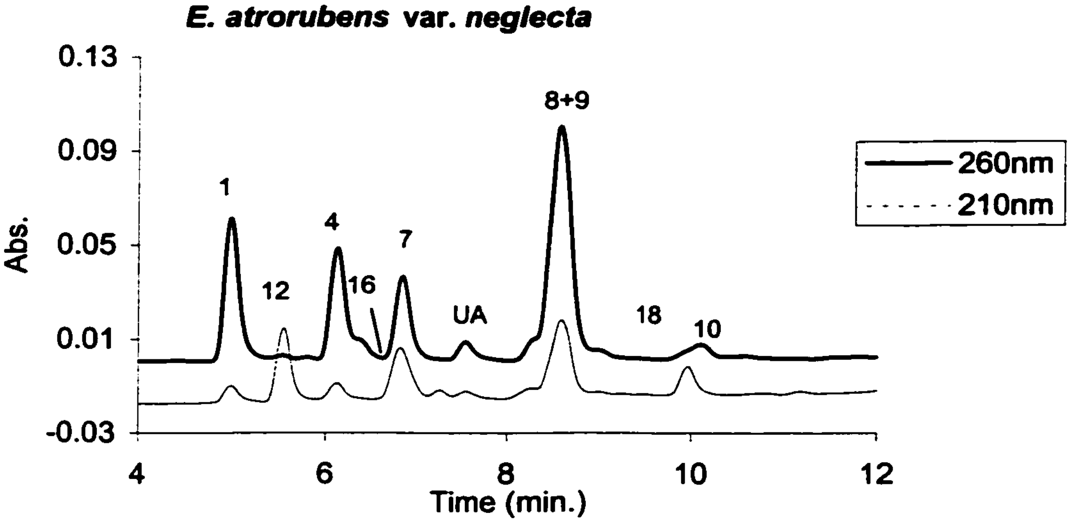


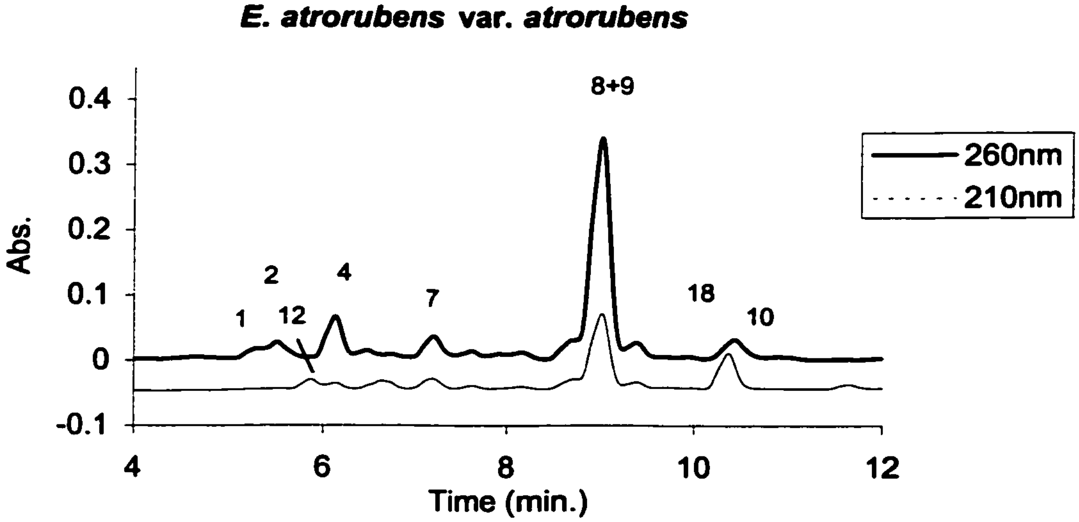
E. pallida var. *simulata*



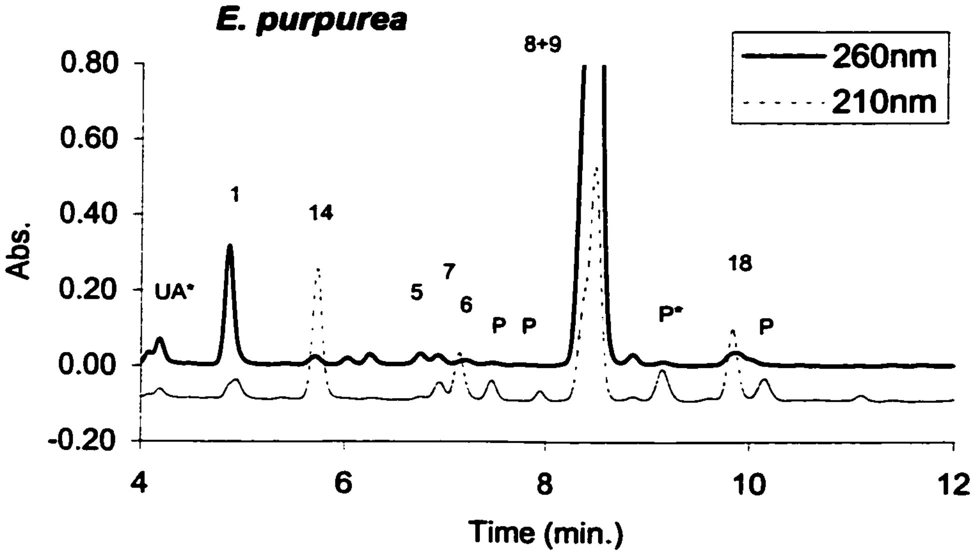
E. laevigata


E. atrorubens var. paradoxa

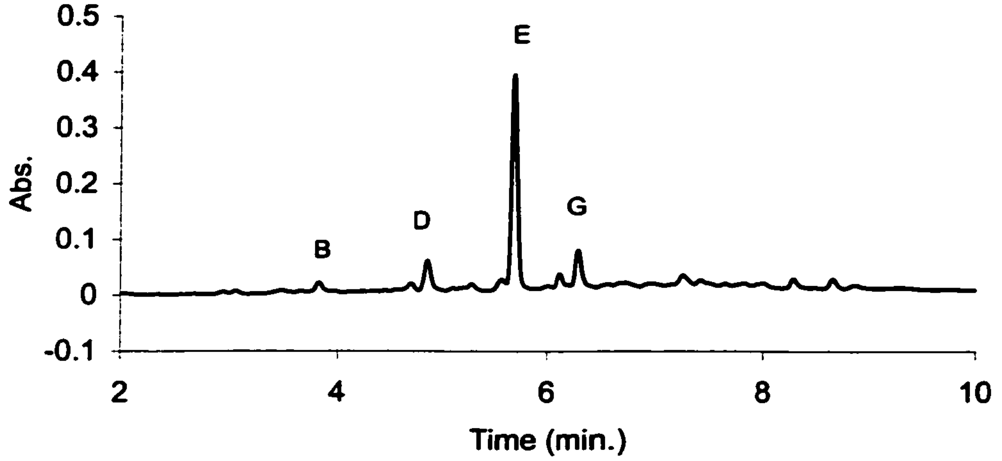

E. atrorubens var. *neglecta*


E. atrorubens var. *atrorubens*

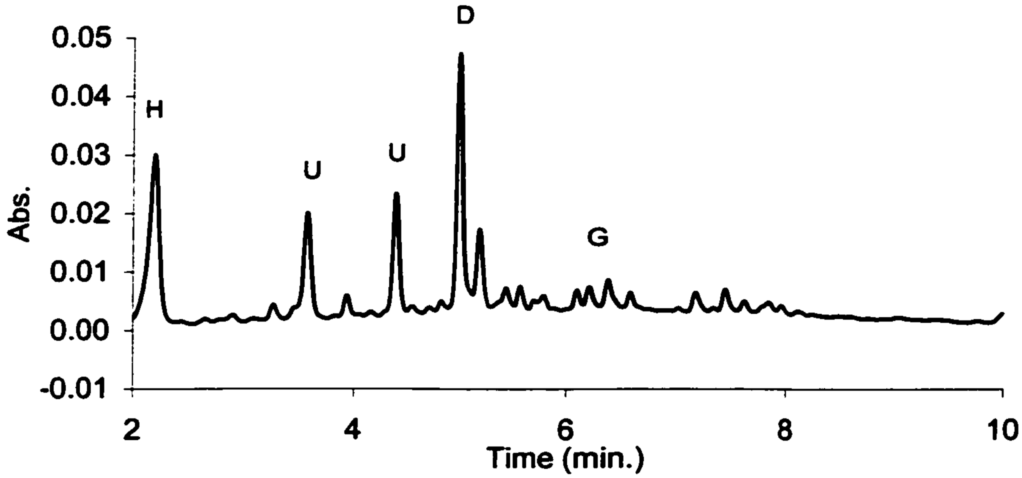
Figures 5.11-5.14: HPLC chromatograms of lipophilic phytochemicals which represent typical inflorescence profiles for each revised *Echinacea* taxon. Numbering refers to chemical structure and compound names (Figs. 5.1-5.4). P) unreported polyene confirmed by UV-scan (similar to 22), P*) unreported polyene confirmed by UV-scan (identical to 24), UA) unreported alkamide confirmed by UV-scan (usually like diene-types), UA*) unreported alkamide confirmed by UV-scan (very similar to **8+9**).


E. purpurea
260nm
210nm
0.80
0.60
0.40
0.20
0.00
-0.20
Abs.
4
6
8
10
12
Time (min.)
UA*
1
14
5
7
6
P
P
8+9
P*
18
P

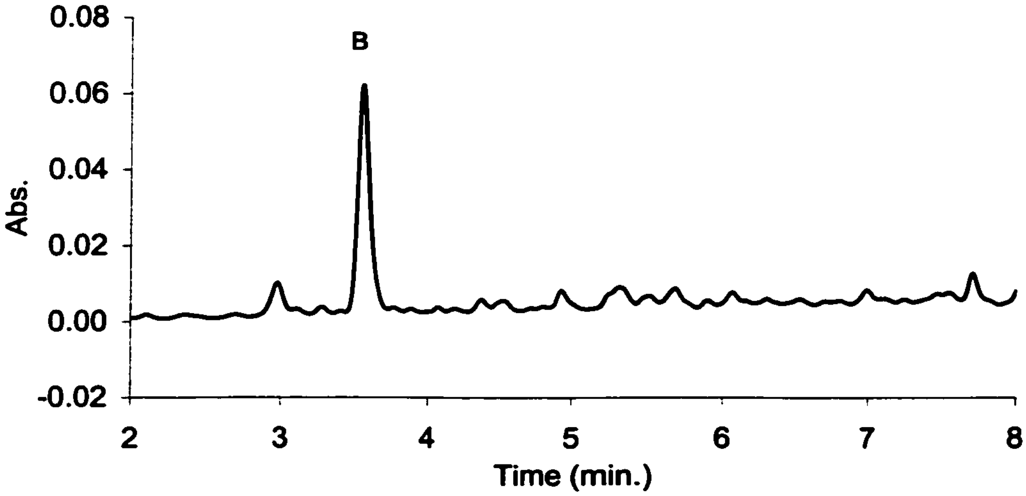
E. pallida* var. *angustifolia

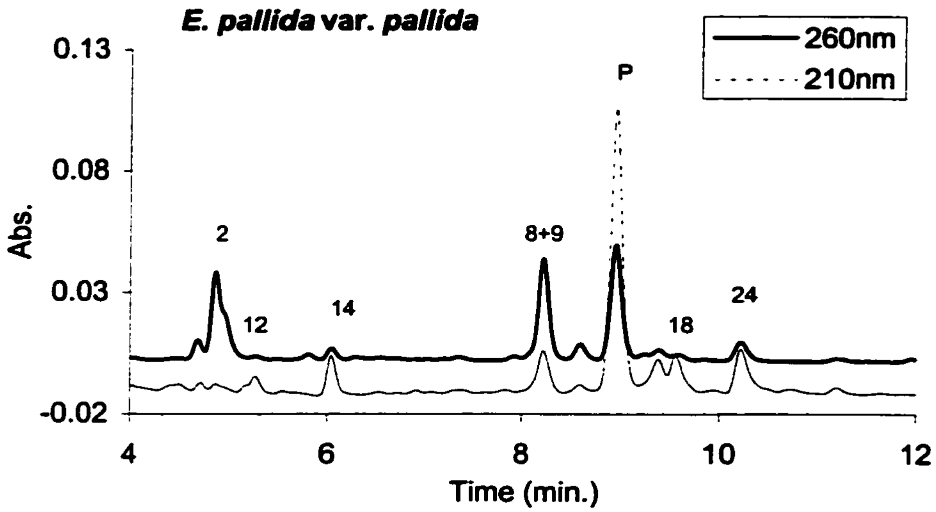


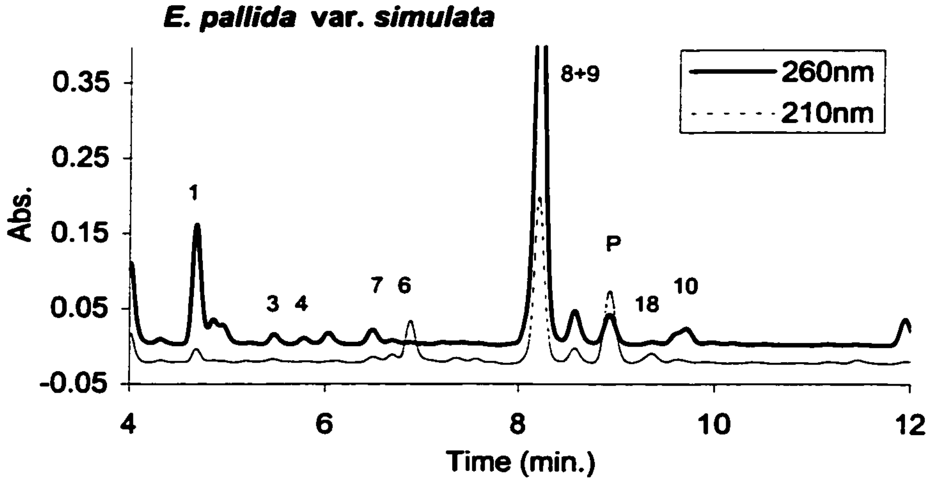
E. pallida* var. *tennesseensis

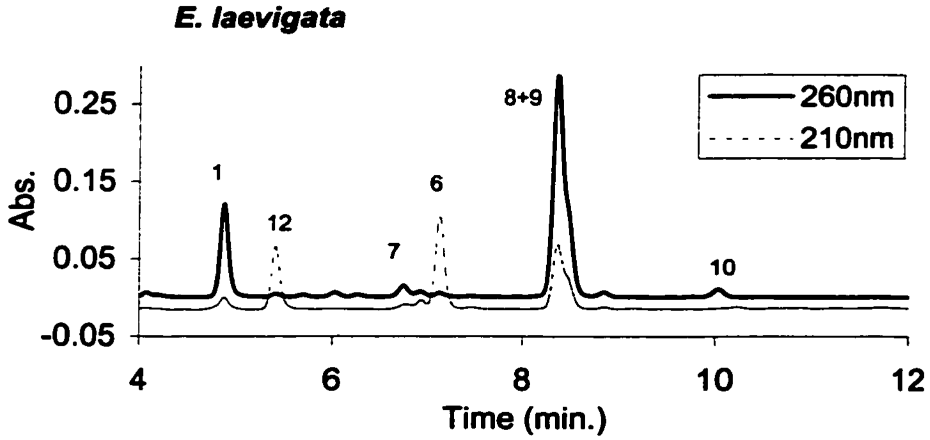


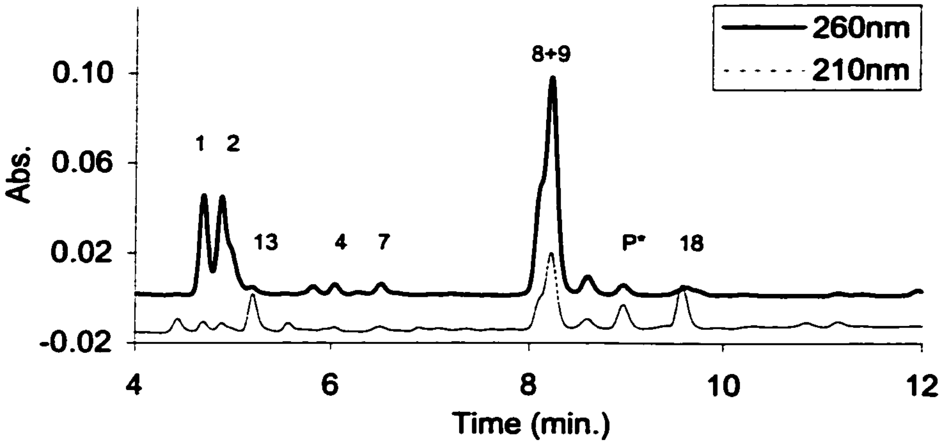
E. pallida* var. *sanguinea

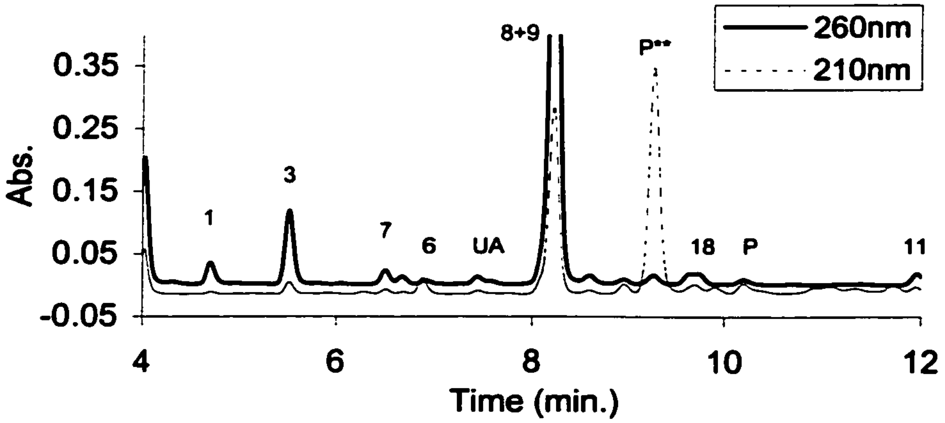


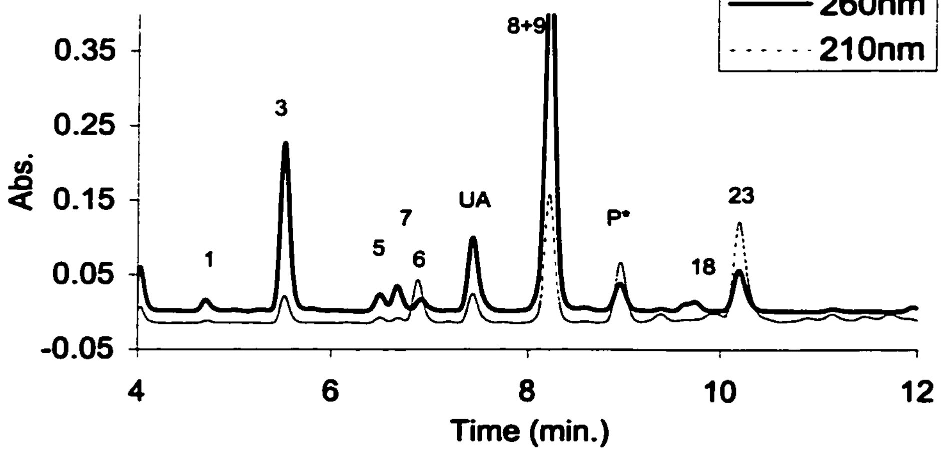

E. pallida var. *pallida*


E. pallida var. *simulata*

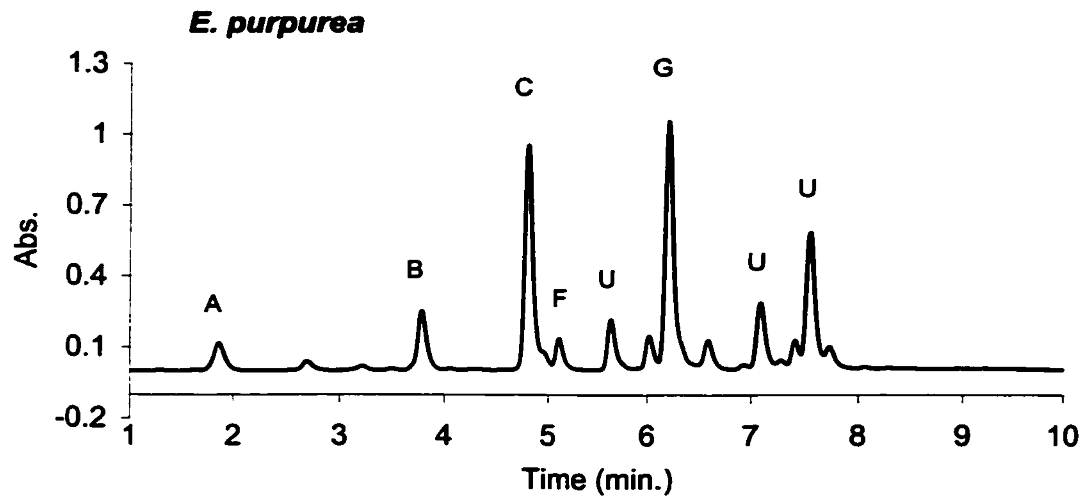

E. laevigata

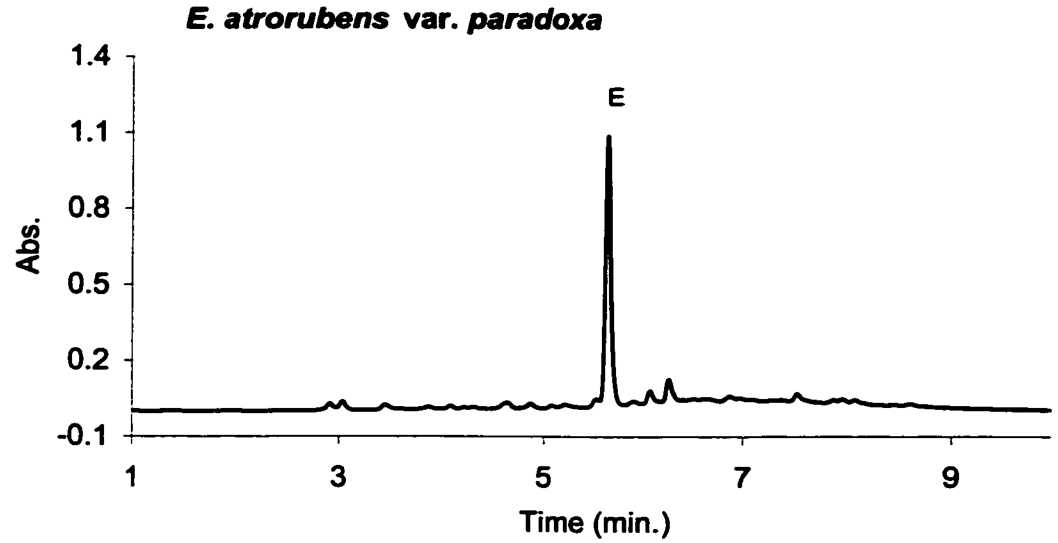

E. atrorubens* var. *atrorubens

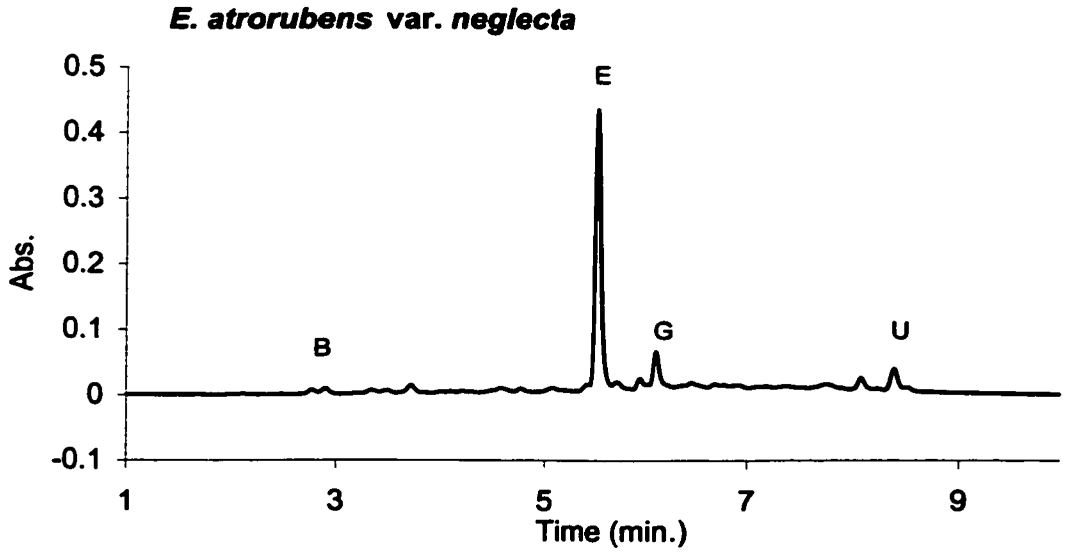

E. atrorubens* var. *neglecta

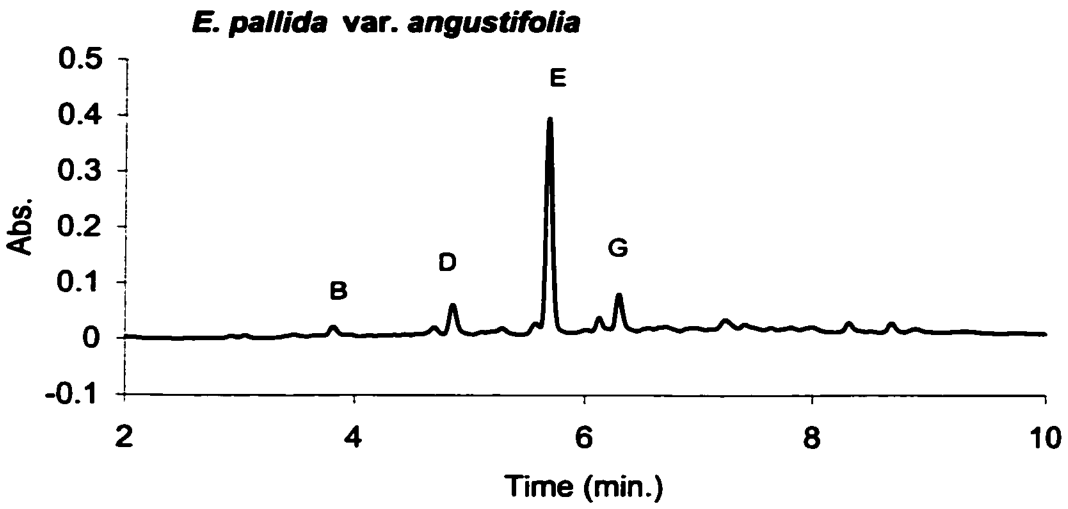

E. atrorubens* var. *paradoxa

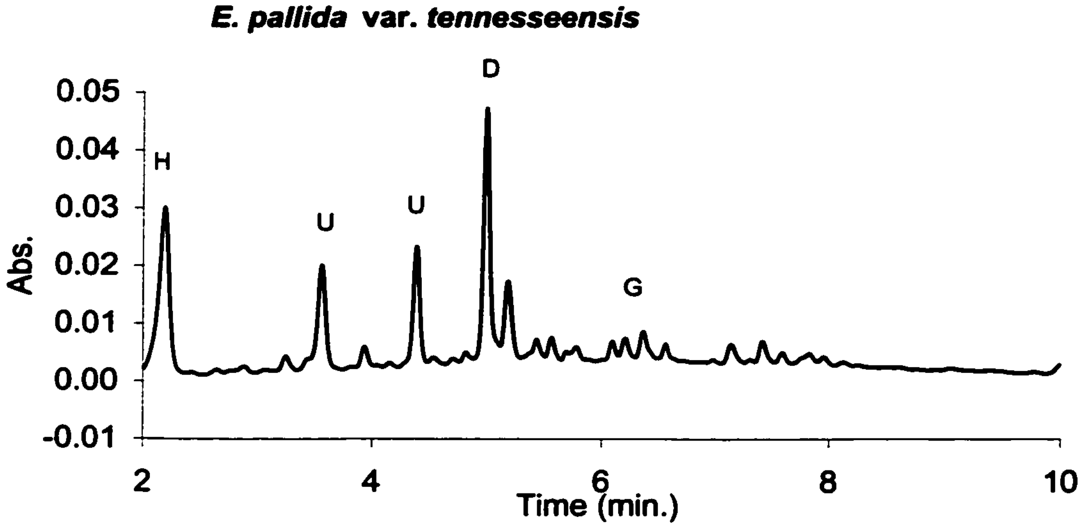
Figures 5.15-5.17: HPLC chromatograms of hydrophilic phytochemicals which represent typical root profiles for each revised *Echinacea* taxon. A) **caftaric acid**, B) **chlorogenic acid**, C) **cichoric acid**, D) **cynarin**, E) **echinacoside**, F) cichoric acid methyl ester, G) rutin, H) **caffeic acid**, U) unconfirmed with UV-scan similar to chlorogenic acid, UC) unconfirmed with UV-scan similar to cichoric acid. Absorbance was detected at 326 nm.

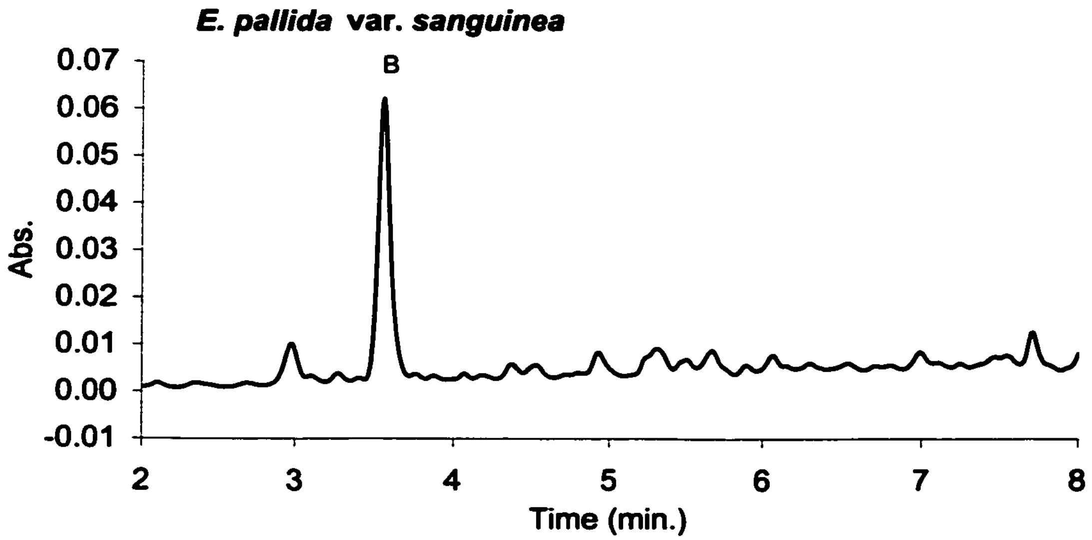

E. purpurea

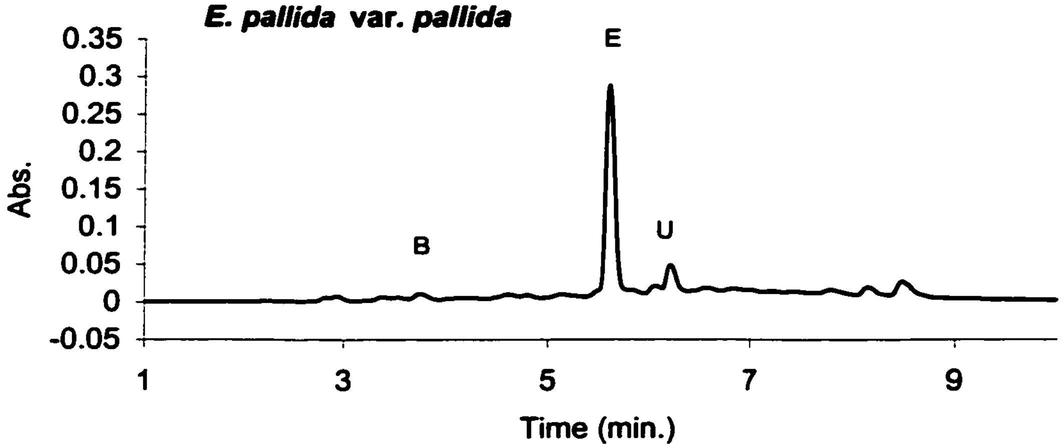

E. atrorubens var. *paradoxa*

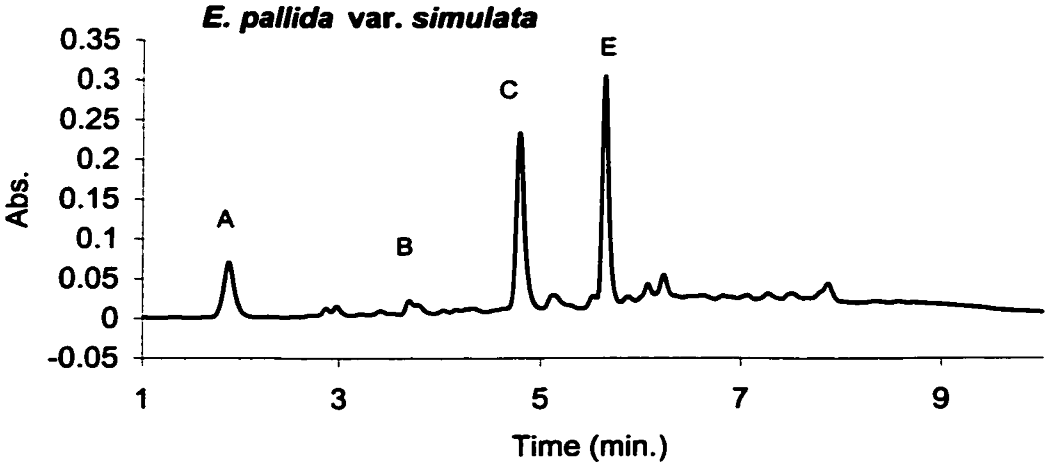

E. atrorubens var. *neglecta*

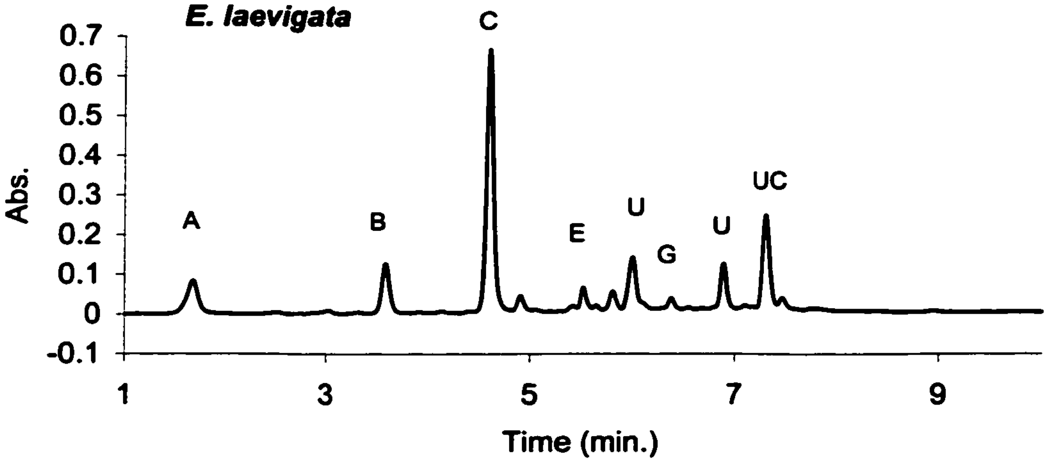

E. pallida var. *angustifolia*


E. pallida var. *tennesseensis*

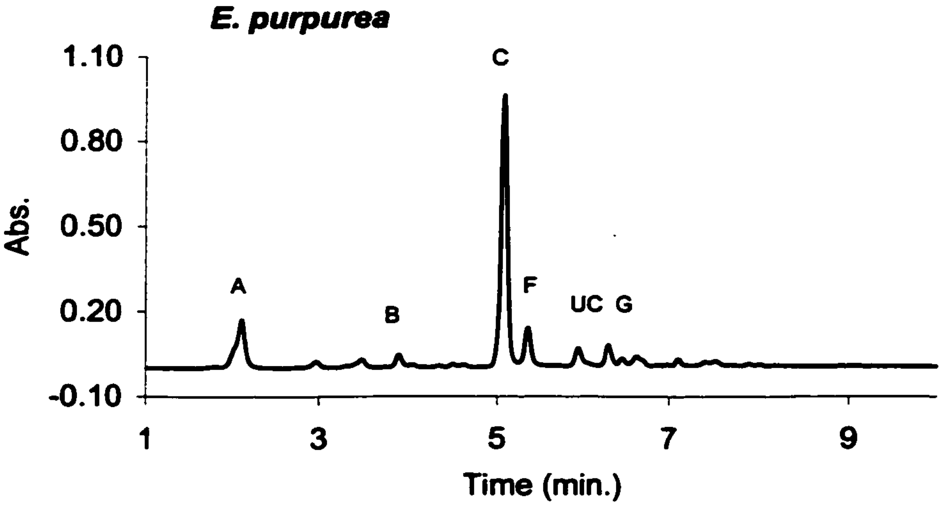

E. pallida var. *sanguinea*


E. pallida var. *pallida*
Abs.
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
B
E
U
1
3
5
7
9
Time (min.)

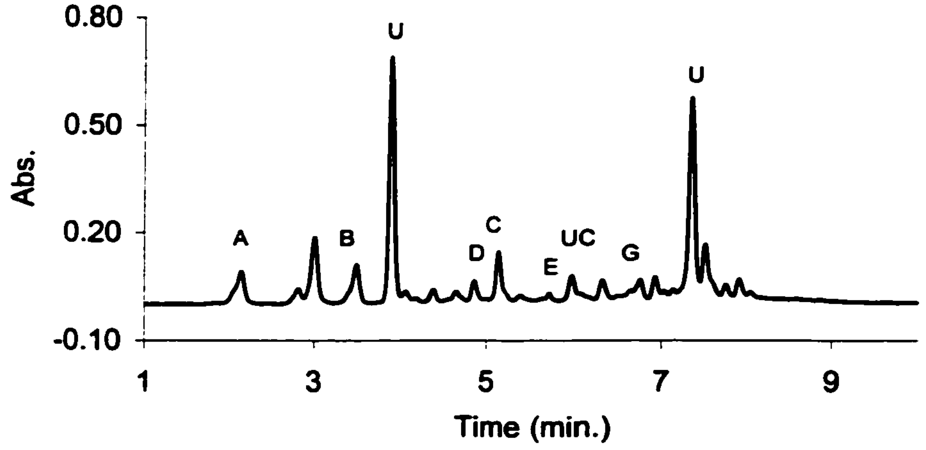

E. pallida var. *simulata*
Abs.
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
A
B
C
E
1
3
5
7
9
Time (min.)


E. laevigata
Abs.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
A
B
C
E
U
G
U
UC
1
3
5
7
9
Time (min.)

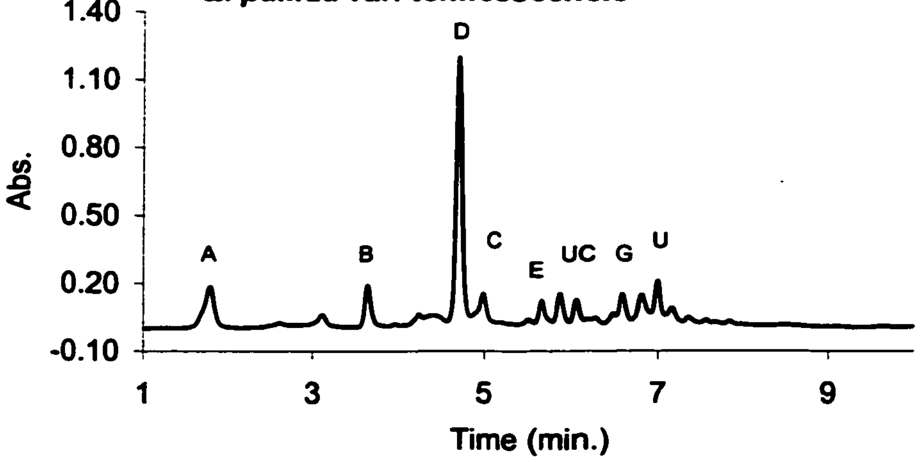
Figures 5.18-5.21: HPLC chromatograms of hydrophilic phytochemicals which represent typical inflorescence profiles for each revised *Echinacea* taxon. A) **caftaric acid,** B) **chlorogenic acid**, C) **cichoric acid**, D) **cynarin**, E) **echinacoside,** F) cichoric acid methyl ester, G) rutin, H) **caffeic acid**, U) unconfirmed with UV-scan similar to chlorogenic acid, UC) unconfirmed with UV-scan similar to cichoric acid. Absorbance was detected at 326 nm.


E. purpurea
1.10
0.80
0.50
0.20
-0.10
Abs.
A
B
C
F
UC
G
1
3
5
7
9
Time (min.)

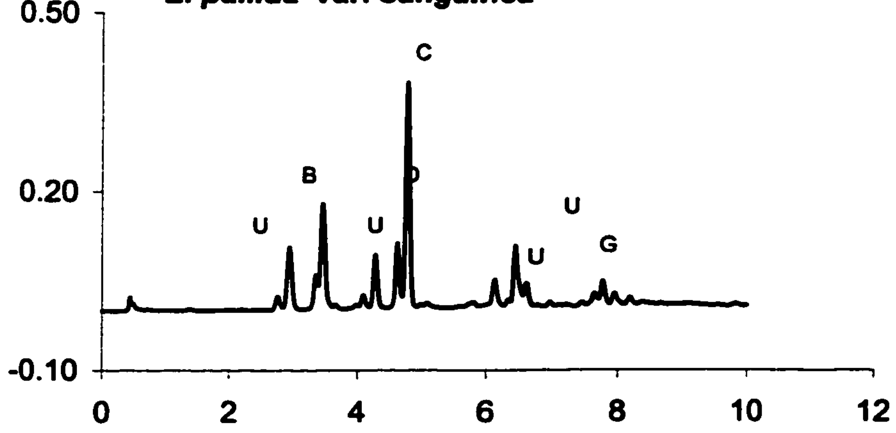
E. pallida* var. *angustifolia

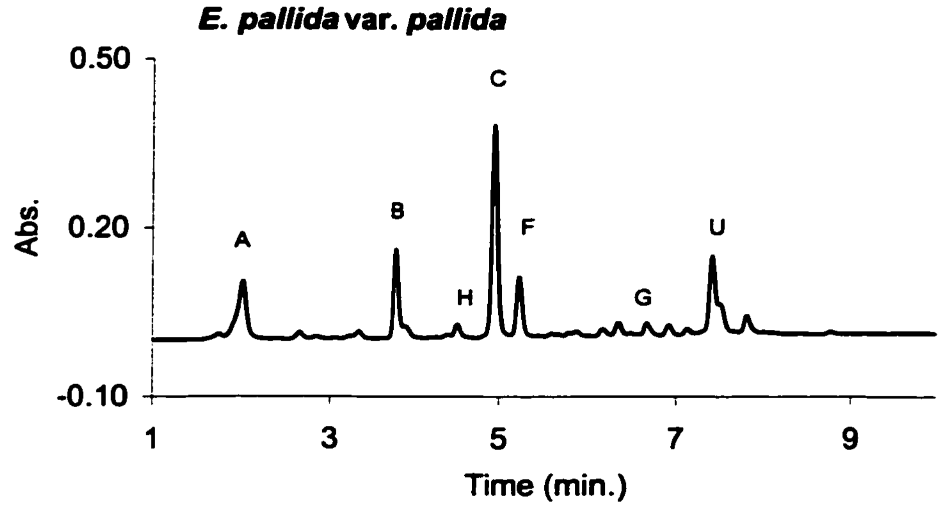


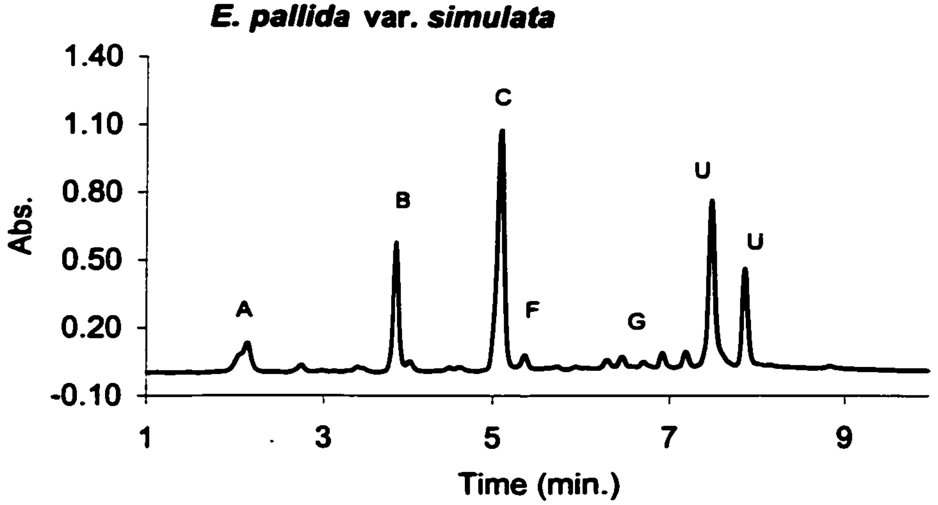
E. pallida* var. *tennesseensis

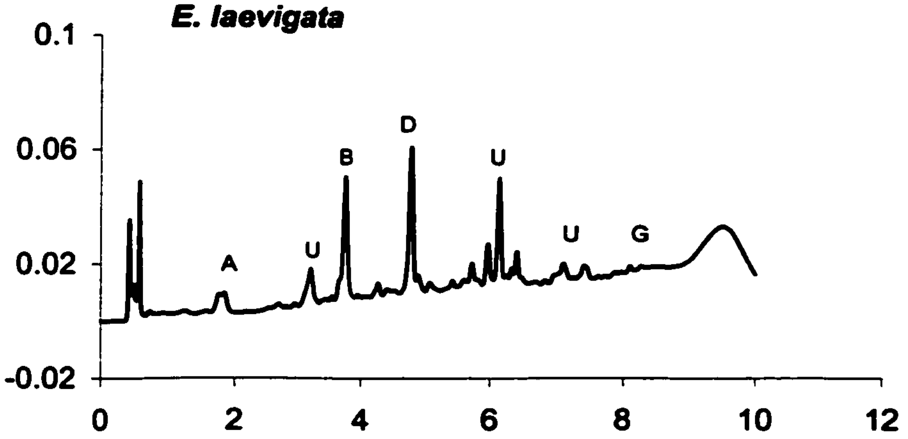


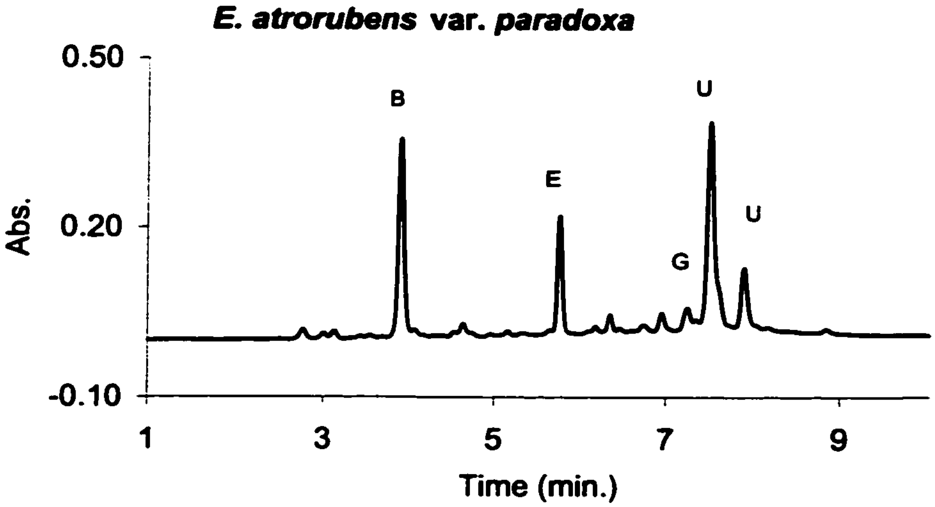
E. pallida* var. *sanguinea



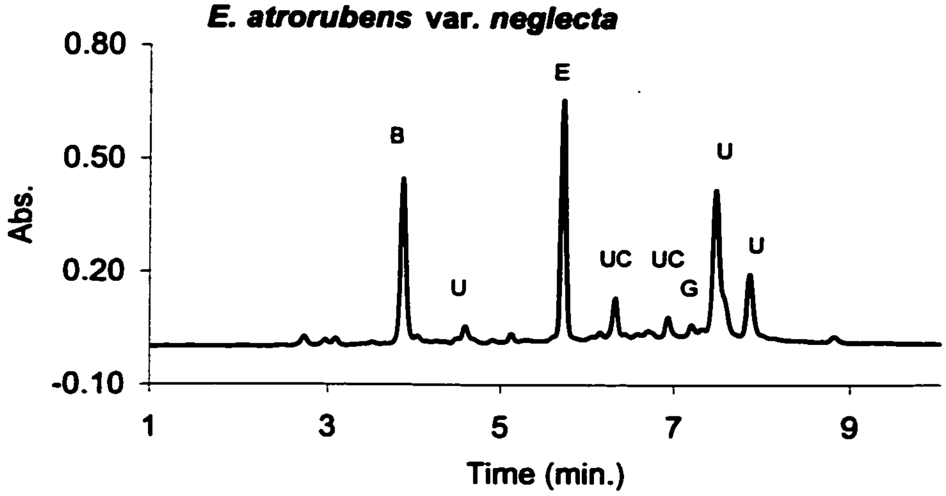

E. pallida var. pallida
0.50
0.20
-0.10
Abs.
A
B
H
C
F
G
U
1
3
5
7
9
Time (min.)


E. pallida var. simulata
1.40
1.10
0.80
0.50
0.20
-0.10
Abs.
A
B
C
F
G
U
U
1
3
5
7
9
Time (min.)

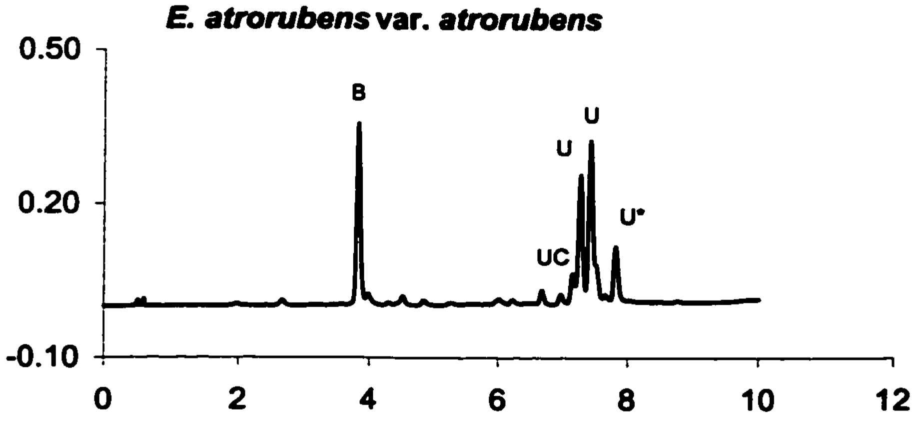

E. laevigata
0.1
0.06
0.02
-0.02
A
U
B
D
U
U
G
0
2
4
6
8
10
12



E. atrorubens var. *paradoxa*



E. atrorubens var. *neglecta*



E. atrorubens var. *atrorubens*

Figure 5.22: The squared Mahalanobis distances between *Echinacea* grouped by revised species, as determined by CDA using variations in quantitative phytochemistry and represented as canonical coordinates of group centroids in 3-space; **1)** *E. purpurea*, **2)** *E. pallida*, **3)** *E. laevigata*, **4)** *E. atrorubens* and **5)** hybrids (*E. pallida* × *E. atrorubens*).

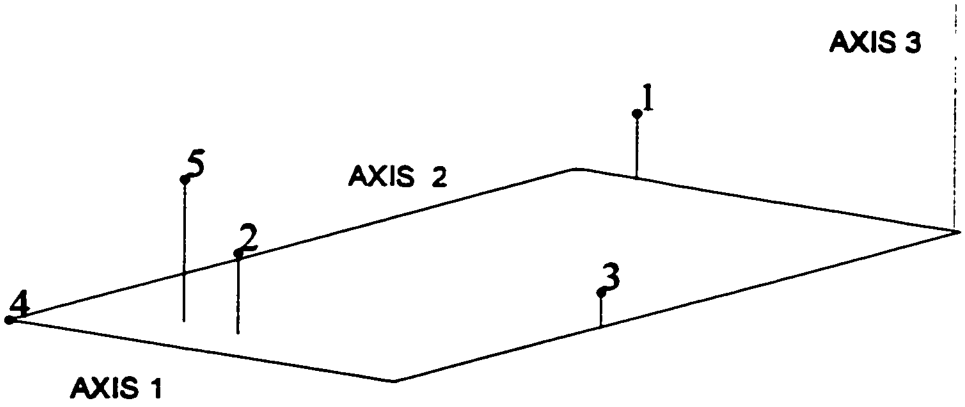
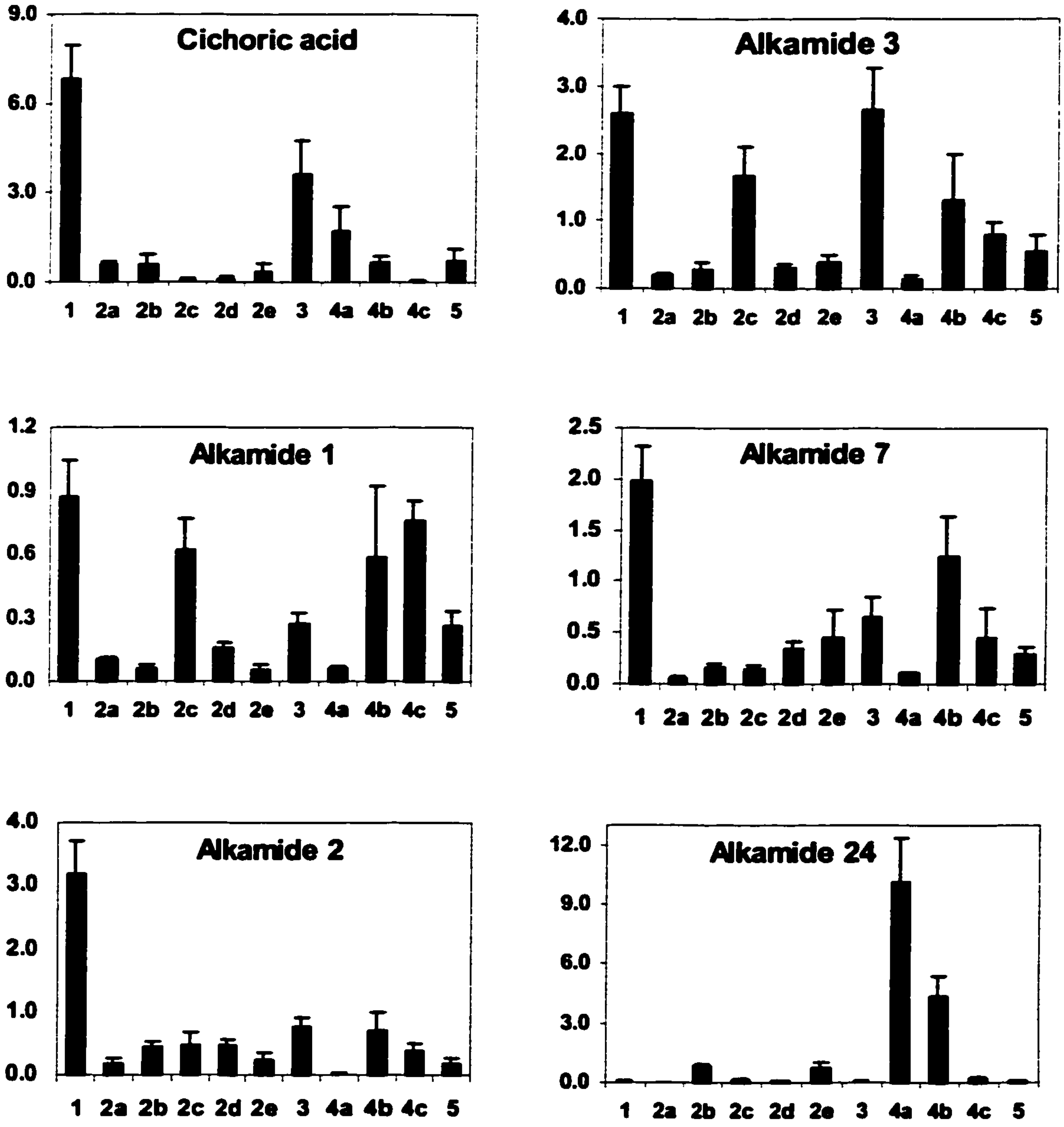

AXIS 3
1
5
AXIS 2
2
3
4
AXIS 1

Figure 5.23: Phytochemicals from roots of all ages (mean ± SEM in mg/g dry weight) which were determined by CDA to be important for discrimination between *Echinacea* taxa: **cichoric acid**, **alkamide 1** (undeca-2E,4Z-dien-8,10-diynoic acid isobutylamide), **alkamide 2** (undeca-2Z,4E-dien-8,10-diynoic acid isobutylamide), **alkamide 3** (dodeca-2E,4Z-dien-8,10-diynoic acid isobutylamide), **alkamide 7** (dodeca-2E,4Z-dien-8,10-diynoic acid 2-methylbutylamide) and **ketoalkene/yne 24** (pentadeca-8Z,13Z-dien-11-yn-2-one).



1= *E. purpurea*; **2**= *E. pallida* varieties *angustifolia* (**2a**), *pallida* (**2b**), *sanguinea* (**2c**), *tennesseensis* (**2d**), and *simulata* (**2e**); **3**= *E. laevigata*; **4**= *E. atrorubens* varieties *paradoxa* (**4a**), *neglecta* (**4b**), *atrorubens* (**4c**); **5**= hybrids (*E. pallida* × ?*E. atrorubens*) (overall n= 337) .

CHAPTER 6: PHYTOCHEMICAL VARIATION IN POPULATIONS OF *ECHINACEA ANGUSTIFOLIA* DC. (ASTERACEAE)

This work was submitted for publication in the journal Biochemical Systematics and Ecology, in collaboration with J.T. Arnason and B.R. Baum. Minor modifications have been made to the manuscript for this chapter.

Introduction

Plants of the genus *Echinacea* are native to North America, with wild populations ranging from the U. S. Great Plains north to the Canadian prairies, east to the Appalachian uplands and the southeastern coastal plains (McGregor, 1968). As one of the most geographically-widespread species in the genus, *E. angustifolia* has adapted naturally to different habitats from Texas to Saskatchewan, but it remains one of the more difficult *Echinacea* species to cultivate successfully. Traditionally, the roots of *E. angustifolia* were the most frequently-used medicine among most First Nations groups of the Great Plains region (Kindscher, 1989; Shemluck, 1982). In the last decade, *Echinacea* species have regained this popularity as the top-selling medicinal market botanical (Brevoort, 1998). Wild-harvested *E. angustifolia* roots have the highest market value of all *Echinacea* material sold as phytomedicine.

Since worldwide economic demand for *E. angustifolia* in particular has far exceeded the capacity for sustainable wild root harvesting, effective cultivation of all *Echinacea* species is imperative. Growers of *E. angustifolia* must take an interest in genetic preservation to protect their native sources of germplasm diversity from the consequences of urban development, herbicide use and overgrazing (Price-Hurlburt, 2000). Morphological races from a range of wild *E. angustifolia* seed sources have been identified in a common garden based on significant variation in aerial yield, height, seed survival, lodging/disease, growth habit, flowering and seed maturity (Little, 1999). Genetic variation within and among natural populations of *E. angustifolia* is reportedly extremely high (Feghahati and Reese, 1994). Viles and Reese (1996) suggested that such genetic variability was responsible in part for reported phytochemical variability (Bauer and

Wagner, 1991). As a measure of phytochemical diversity among wild seed sources, Viles and Reese (1996) studied allelochemical activity in a greenhouse study, with the conclusion that natural selection was acting to differentiate wild populations of *E. angustifolia* genetically, through shifting environmental pressures such as herbivory (Viles and Reese, 1996).

Cultivation of *E. purpurea* (L.) Moench has already enjoyed significant increases through traditional selective breeding practices worldwide. Morphologically superior *E. purpurea* lines from germplasm with demonstrated high genetic variability (Baum *et al.*, 1999) resulted in doubled average phytochemical content in each of the major chemical classes when compared to non-selected lines (Letchamo, 1998). Similar selection of superior cultivars by phytochemical prediction would enhance *E. angustifolia* cultivation.

We demonstrated pathway induction for the *Echinacea* secondary phytochemicals, alkamides and ketoalken/ynes, using a naturally-occurring chemical mediator in *E. pallida* (chapter 7). This phenomenon may be a mechanism under environmental control, which effectively minimizes the energetic costs of defense. However, there are no reports of genetic adaptation leading to differences in plant secondary phytochemical metabolism, although the ability to direct resources to primary metabolism (growth and differentiation) may evolve by through stable genetic polymorphisms in plant species with a broad range of habitats and selection pressures (Herms and Mattson, 1992).

The objective of the current study was quantitative evaluation of the genetic component in phytochemical diversity of *E. angustifolia* populations from different geographic areas. We have evaluated the quantity and type of phytochemicals accumulated in young *E. angustifolia* roots from a range of wild populations, grown under uniform environmental conditions. We also explored the association between the observed phytochemical variability and latitude of germplasm sources.

Table 6.1. Location, latitude, accession labels (corresponding to voucher specimens, DAO) and number of individuals for nine populations of *Echinacea angustifolia*.

Population	**# Plants (n)**	**Label (DAO)**	**Latitude**	**Location**
1	16	EST0822B	34.368 N	OK: Carter Co, Fox
2	10	EST0823	34.419 N	OK: Carter Co, Alma
3	16	EA23909	34.717 N	OK: Comanche Co
4	8	EST0823	35.009 N	OK: Cleveland Co
5	12	EA421331	36.643 N	OK: Logan Co
6*	16	EA014	37.313 N	KS: Cowley Co
7	8	EA24058	39.179 N	KS: Pottawatomie Co
8	17	EA421332	40.065 N	NE: Richardson Co
9	12	EA23930	42.917 N	IA: Clay Co

* Introgressant characteristics (*E. pallida* x *E. angustifolia*) according to morphometric analysis (chapter 2).

Methods

Achene germination

Achenes from nine native populations of *Echinacea angustifolia* were harvested from Aug. to Oct.1997 and 1998, then stored in a freezer at -20°C. Voucher specimens are deposited at the National Herbarium of Canada, DAO (Table 6.1). Under sterile conditions, 50 achenes from each accession were placed in a sealed container with holes (approx. 1 mm diam.) and soaked in Plant Preservation Mixture (PPM™) (10 mL/L in dH_2O) for 24 hours on a rotary shaker. They were rinsed 3 times with dH_2O and placed in petri dishes on filter paper (Whatman#1) soaked with 1 mL/L ethylene (Ethrel™) in distilled H_20. Petri dishes were sealed with parafilm and placed in a growth chamber under constant light (5 $\mu M/m^2/s$, incandescent) at 4°C for 14 days. Gradually, over 5 days, the temperature was raised to 25°C and germination occurred within 10 days following temperature increase.

Mature germlings were potted in a mix of 5:3:1 vermiculite: Promix: quartz sand (large grains) in cell packs for several weeks in the growth chamber (25°C and 16hr. days) and eventually transplanted into individual 3 inch pots in a greenhouse (25°C and 16hr. days). We used a completely randomized design on a single greenhouse bench. The final sample size per population (Table 6.1) varied according to survival, which was 21-78%.

Extraction and isolation

Fresh roots were harvested at the age of six months, washed thoroughly and covered with 60% EtOH. They were coarsely chopped in a blender (Osterizer), followed immediately by 60 seconds on the medium-high setting of a Polytron (Brinkmann

Instruments, Westbury, NY) to completely release cell contents into solution. The root-ethanol slurry was shaken at medium speed (70 rpm) for 20 hours, and filtered by Buchner filter (Whatman #1). Extracted root material was dried in an oven (50°C), then weighed and discarded. The filtrate was rotary evaporated to dryness and re-dissolved in fresh 60% EtOH with the appropriate volume to achieve standardized 0.5g/mL extracts. These were filtered for HPLC using 0.2 μm nylon membranes.

A validated method was used for HPLC phytochemical separations (Bergeron *et al.* 2000). Hydrophilic chromatography was achieved using a solvent system of acetonitrile:50mM NaH_2PO_4 pH 2.95, at a flow rate of 1.5 mL/minute following a linear gradient of 5-25% acetonitrile over 7 minutes. Lipophilic chromatography was achieved using a solvent system of acetonitrile:water, at a flow rate of 1.0 mL/min following a linear gradient of 40-80% acetonitrile over 15 minutes. In both systems, 5 μL of sample was injected on a 7.5 cm x 4.6 mm C-18 column (3 μm particle size) (Lichrospher, Merck BDH Toronto, Canada). Lipophilic compounds were detected at 210 nm and 260 nm and hydrophilic compounds were detected at 326 nm.

Compounds illustrated in figures 5.1-5.6 were identified by comparison with reference standards. Reference standards of undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide **1**, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (tetraenes) **8+9**, pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide **18**, 2,3-O-dicaffeoyltartaric acid (**cichoric acid**) and **echinacoside** were isolated previously in our laboratory by column chromatography on silica gel and assessed for purity by ^1H and ^{13}C-NMR spectral data (Bergeron *et al.* 2000). The same method yielded dodeca-2E,4E-dienoic acid isobutylamide **11** (by online UV spectra matching Bauer and Remiger, 1989) and **cynarin** (by ^1H and ^{13}C-NMR, Cheminat *et al.* 1988) in our laboratory by Bergeron *et al.* (Unpubl.). Standards of **caffeic acid** and **chlorogenic acid** (Sigma Aldrich, St. Louis, U.S.A.), as well as **caftaric acid** (Dalton Chemical Laboratories Inc., Toronto, Canada) were purchased. All other alkamide and polyene compounds (Figs. 5.2-5.6) were identified based on relative retention time to the marker compounds dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (tetraenes) **8+9** and online photodiode array UV spectra (c.f. Bauer and Remiger 1989). Each previously reported compound (μg/ per mL injected) was quantified using peak area multiplied by the response factor (calculated from the standard curve of tetraenes **8+9**). This figure was then divided by the original concentration of 0.5g extracted dried root/ ml sample and multiplied by 10^3 to reach mg/g dry wt. Peak identification by relative retention time and quantitation by relative response factor was acceptable for the purpose of overall profile comparisons within the present study.

Statistical Analyses

The null hypothesis that there was no significant difference in phytochemical quantity between populations (9 locations) was tested for the 26 phytochemicals singly and collectively in 115 individual plants, as follows. The effect of each individual phytochemical on variation between populations was assessed by one-way ANOVA using Systat software version 7.0 (SPSS Inc., Chicago, IL, 1999). Tukey's pairwise differences between the nine populations were also calculated for each individual phytochemical. Then, canonical discriminant analysis of the 26 phytochemicals using SAS CANDISC procedure (SAS Institute Inc., Cary, NC, 1999) assessed squared Mahalanobis distances between populations, measured the variation within and between populations, and ascertained which phytochemical(s) contributed the most towards that variation (Sneath and Sokal 1973). The resulting matrix of Mahalanobis distances was subjected to principal coordinate analysis and single linkage clustering using NT-SYS-pc software (Rohlf Numerical Taxonomy and Multivariate Analysis System, Suny, NY, 1999) to provide several graphical representations of the relative distances between the centroids of each population.

The relationship between geographical latitude and phytochemical content was determined using simple linear regression (Systat v.7) of the population means for each phytochemical (one at a time) on the latitude of the corresponding germplasm source (in Table 6.1). When assumptions of normality and homoscedasticity were violated, we used either the logarithmic transformation or weighting to correct for different variances and sample sizes (n) between populations. The justified weighting procedure, in this particular program, consisted of multiplication of the population means (log-transformed) by the inverse of sample variance so that means with smaller variances received more weight in the analysis.

Results

There was significant quantitative variation in several lipophilic and hydrophilic compounds between nine *E. angustifolia* populations (Table 6.2).

Figure 6.1 illustrates populational variation of the hydrophilic phenolic compounds. The maximum accumulations of the following hydrophilic phenolic compounds were found to be statistically significant ($p<0.05$); **cichoric acid** in #9, **cynarin** in #8, and **caftaric acid** in #6 and #9 (Table 6.2). **Echinacoside** was barely significantly different ($p = 0.041$) among populations, with the highest level in population #3. **Caffeic and chlorogenic acids** were not found to be significantly different, although they were highest in population #5.

Significantly higher levels ($p<0.05$) of the following alkamides were observed in the same two populations that contained more phenolics, the northernmost populations #8 and #9 (Table 6.2): **3, 4, 7, 10, 11, 13, 14, 18, 19**. The major alkamides, **8+9** were found in similar quantity among all populations, except #7 (not significant). Populational differences for the ketoalkene/ynes were not statistically significant, however **24** and **25** were highest in population #7 while compound **22** was unique to #3, #5 and #6.

Table 6.2. Significantly different *E. angustifolia* populations and the most important phytochemical variables contributing to that variation.

Table 6.2: Significantly different mean phytochemical content [± SEM] in *Echinacea angustifolia* populations (two-tailed ANOVA $p<0.05$) from south to north by latitude (Table 6.1). Those followed by the same letter were not significantly different by Tukey's pairwise comparison.

Pop	Phenolics				Alkamides								
	cich^{1}	ech^{1}	caft^{1*}	cyn*	3	4^{1*}	7	10*	11^{1*}	13	14*	18^{1*}	19^{1*}
1	0.18^{a}	9.00^{abc}	0.02^{a}	8.47^{ac}	0.07^{ac}	0.03^{a}	0.05^{abc}	0.23^{a}	0.3^{a}	0.60^{ab}	0.26^{ac}	0.62^{abc}	0.04^{ab}
	[0.02]	[0.98]	[0.01]	[0.92]	[0.02]	[0.01]	[0.01]	[0.05]	[0.07]	[0.16]	[0.06]	[0.16]	[0.01]
2	0.08^{a}	5.14^{abc}	0.003^{a}	6.00^{abc}	0.05^{ac}	0.03^{ac}	0.04^{abc}	0.16^{a}	0.18^{ac}	0.58^{ab}	0.11^{ab}	0.45^{abc}	0.02^{ac}
	[0.02]	[2.15]	[0.002]	[1.23]	[0.02]	[0.00]	[0.02]	[0.06]	[0.04]	[0.18]	[0.03]	[0.12]	[0.01]
3	0.37^{ab}	9.47^{b}	0.12	7.78^{ac}	0.08^{ab}	0.04^{abc}	0.08^{abc}	0.34^{ab}	0.38^{ac}	0.28^{a}	0.15^{ab}	0.16^{a}	0.02^{ac}
	[0.11]	[1.42]	[0.02]	[1.20]	[0.02]	[0.01]	[0.03]	[0.09]	[0.09]	[0.07]	[0.04]	[0.05]	[0.00]
4	0.06^{a}	2.79^{c}	0.003^{a}	2.33^{ab}	0.1^{abc}	0.03^{ac}	0.03^{abc}	0.25^{ab}	0.48^{ab}	0.53^{ab}	0.23^{abc}	0.71^{ab}	0.002^{a}
	[0.03]	[1.04]	[0.002]	[0.67]	[0.03]	[0.01]	[0.01]	[0.11]	[0.15]	[0.06]	[0.06]	[0.26]	[0.00]
5	0.06^{ab}	6.43^{c}	0.01^{a}	7.03^{ac}	0.07^{ab}	0.04^{abc}	0.02^{b}	0.20^{c}	0.35^{a}	0.28^{a}	0.16^{ab}	0.30^{ab}	0.03^{ab}
	[0.03]	[2.43]	[0.00]	[0.98]	[0.01]	[0.01]	[0.005]	[0.01]	[0.05]	[0.03]	[0.01]	[0.03]	[0.01]
6	0.41^{ab}	8.67^{abc}	0.44	0.37^{b}	0.04^{bc}	0.02^{b}	0.06^{abc}	0.40^{ab}	1.00^{bcd}	0.18^{ab}	0.06^{b}	0.27^{ac}	0.02^{ad}
	[0.05]	[0.96]	[0.06]	[0.08]	[0.01]	[0.00]	[0.02]	[0.10]	[0.17]	[0.04]	[0.03]	[0.06]	[0.01]
7	0.10^{ab}	6.61^{abc}	0.01^{a}	6.22^{abc}	0.09^{abc}	0.02^{abc}	0.04^{abc}	0.10^{a}	0.22^{a}	0.22^{a}	0.20^{abc}	0.74^{ac}	0.10^{b}
	[0.01]	[1.15]	[0.005]	[1.16]	[0.03]	[0.01]	[0.01]	[0.03]	[0.05]	[0.08]	[0.06]	[0.21]	[0.03]
8	0.10^{ab}	8.9^{abc}	0.03^{a}	13.36^{c}	0.23^{abc}	0.14	0.10^{abc}	0.67^{b}	1.44c	1.03^{b}	0.59^{c}	0.99^{b}	0.12^{bd}
	[0.01]	[1.39]	[0.01]	3.81	[0.05]	[0.03]	[0.02]	[0.13]	[0.22]	[0.29]	[0.10]	[0.20]	[0.02]
9	0.77^{b}	5.29^{abc}	0.26^{a}	5.03^{a}	0.41^{c}	0.14^{c}	0.15^{c}	0.41^{ab}	0.69^{ade}	0.36^{ab}	0.34^{ac}	0.83^{bc}	0.08^{bc}
	[0.43]	[1.21]	[0.16]	[0.83]	[0.21]	[0.06]	[0.04]	[0.09]	[0.14]	[0.09]	[0.06]	[0.22]	[0.02]

cich = cichoric acid; ech = echinacoside; caft = caftaric acid; cyn = cynarin

* mean population concentrations significantly different by ANOVA $p<0.001$

[1] compounds that contributed most significantly to segregation by canonical discriminant analysis

Table 6.3. Simple or weighted regression of the mean phytochemical quantities per *E. angustifolia* population versus latitude of germplasm source.

Compound	**r^2 (slope)**	**P-value**	**Weighting/Transformation**
3	(-)	(-)	(-)
4	0.477	0.039	none
7	0.375	0.08	none
8+9	0	0.882	none
10	(-)	(-)	(-)
11	0.276	0.146	LOGmean*
13	0.141	0.319	LOGmean
14	0.005	0.858	LOGmean
16	0.107	0.391	LOGmean
18	0.066	0.0504	LOGmean
19	0.695	0.005	LOGmean x LOGVAR^{-1**}
cichoric acid	0.038	0.036	none
caffeic acid	0.017	0.742	none
caftaric acid	0.509	0.031	LOGmean x LOGVAR^{-1**}
cynarin	0.094	0.422	none
echinacoside	0.528	0.027	none

* LOGmean = mean transformed by the logarithm base 10

** LOGVAR^{-1} = inverse variance of the LOGmean

(-) = no results where normality assumption was violated in all trials

Regression

A positive relationship between increasing phytochemical quantity and increasing latitude (of parental populations) was statistically significant for **cichoric acid** and **echinacoside** and alkamide **4** ($p<0.05$, Table 6.3). However, there was a significant inverse relationship between the quantity of **caffeic acid** and alkamide **19** with increasing latitude (Table 6.3).

Canonical Discriminant Analysis

A canonical discriminant analysis is considered acceptable when at least 75% of the variation is explained by the three axes (Sneath and Sokal 1973). Here, the first three canonical axes explain 79% of the phytochemical variation between populations. An F-statistic of the squared Mahalanobis distances between population centroids (Fig.6.6) determined that all were significantly distant from one another at $p<0.001$ or $p<0.01$ except the following pairs of populations; #1 and #2 ($p = 0.7850$), #1 and #5 ($p = 0.1914$), #2 and #4 ($p = 0.4075$) and #2 and #5 ($p = 0.2553$). Population #6 was most easily distinguished from the others. The centroid of population #8 was also significantly segregated from other populations ($p<0.0001$), and even from its closest neighbour, #9 ($p = 0.0026$). Figure 7 is a three dimensional representation of the principle coordinates for the variation of each population along the first 3 canonical axes which facilitates the visualization of quantitative differences between the means.

Figure 6.1. Phenolic compounds (caffeic acid derivatives) in nine *E. angustifolia* populations from Table 6.1 (mean mg/g ± SEM).

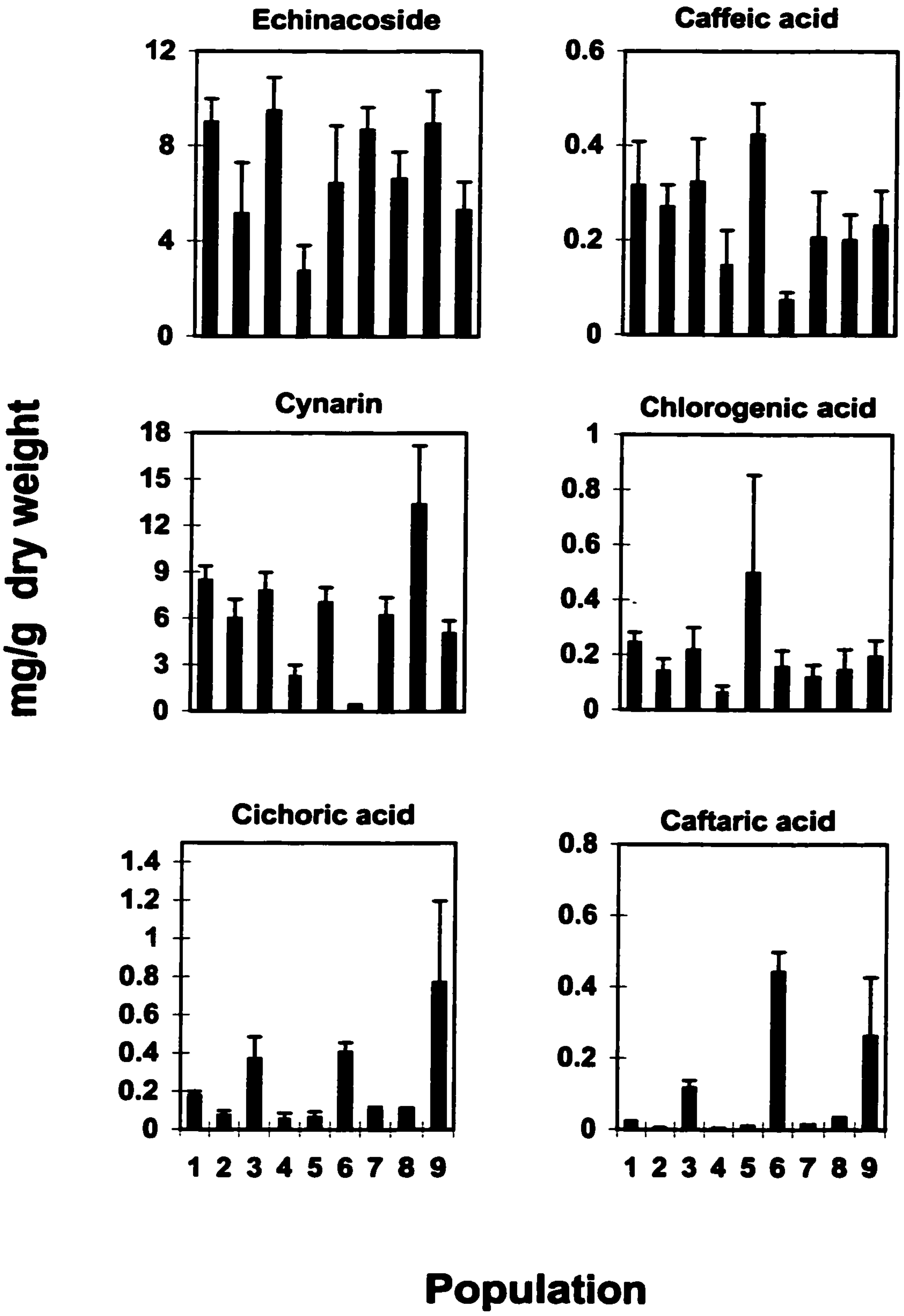

Echinacoside
12
8
4
0
Caffeic acid
0.6
0.4
0.2
0
mg/g dry weight
Cynarin
18
15
12
9
6
3
0
Chlorogenic acid
1
0.8
0.6
0.4
0.2
0
Cichoric acid
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9
Caftaric acid
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9
Population

Figure 6.2. Alkamides **1, 2, 3, 4, 5+15, 6** in nine *E. angustifolia* populations from Table 6.1 (mean mg/g ± SEM).

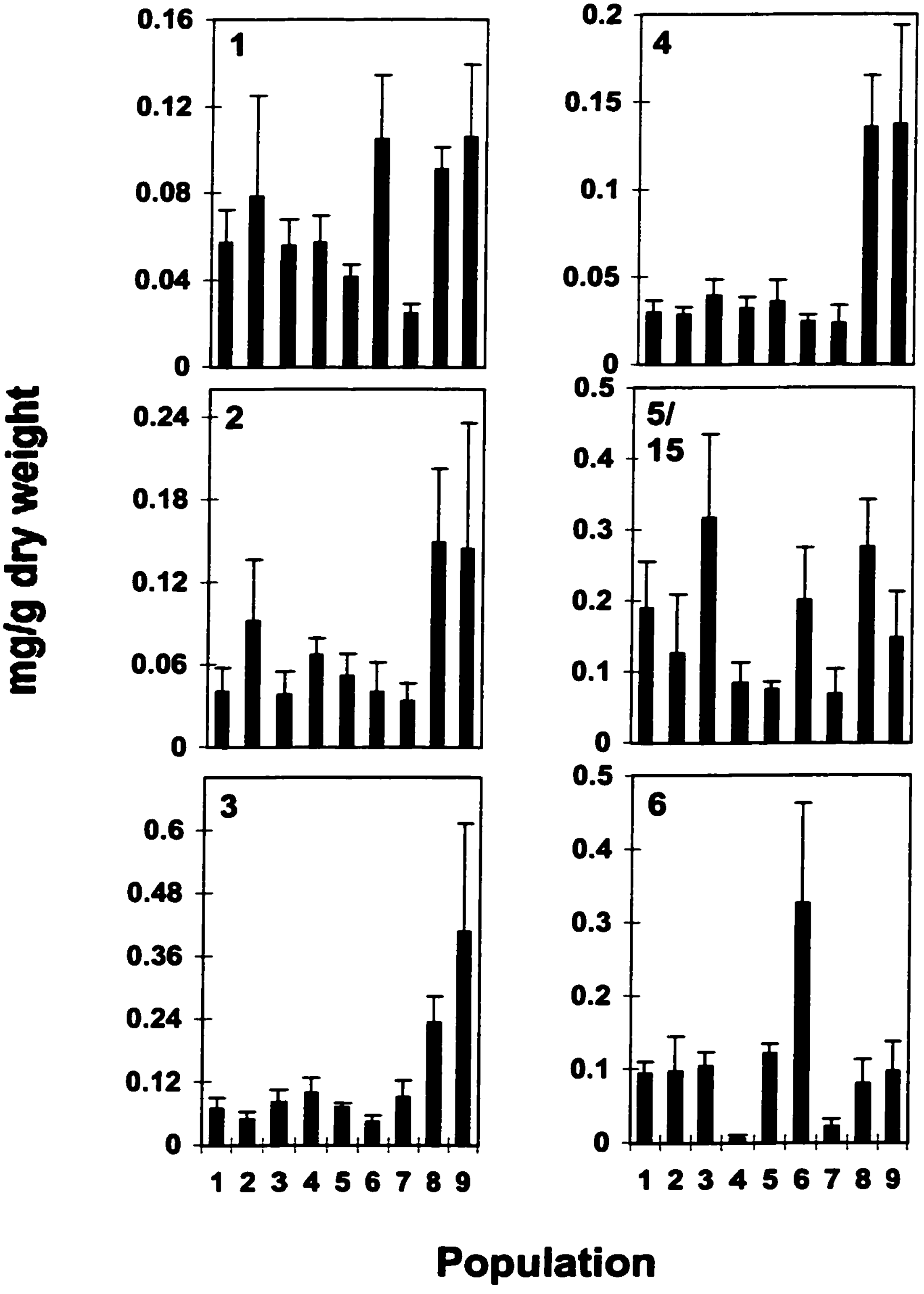

mg/g dry weight
1
0.16
0.12
0.08
0.04
0
4
0.2
0.15
0.1
0.05
0
2
0.24
0.18
0.12
0.06
0
5/
15
0.5
0.4
0.3
0.2
0.1
0
3
0.6
0.48
0.36
0.24
0.12
0
6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
Population

Figure 6.3. Alkamides **7, 8+9, 10, 11, 12, 13** in nine *E. angustifolia* populations from Table 6.1 (mean mg/g ± SEM).

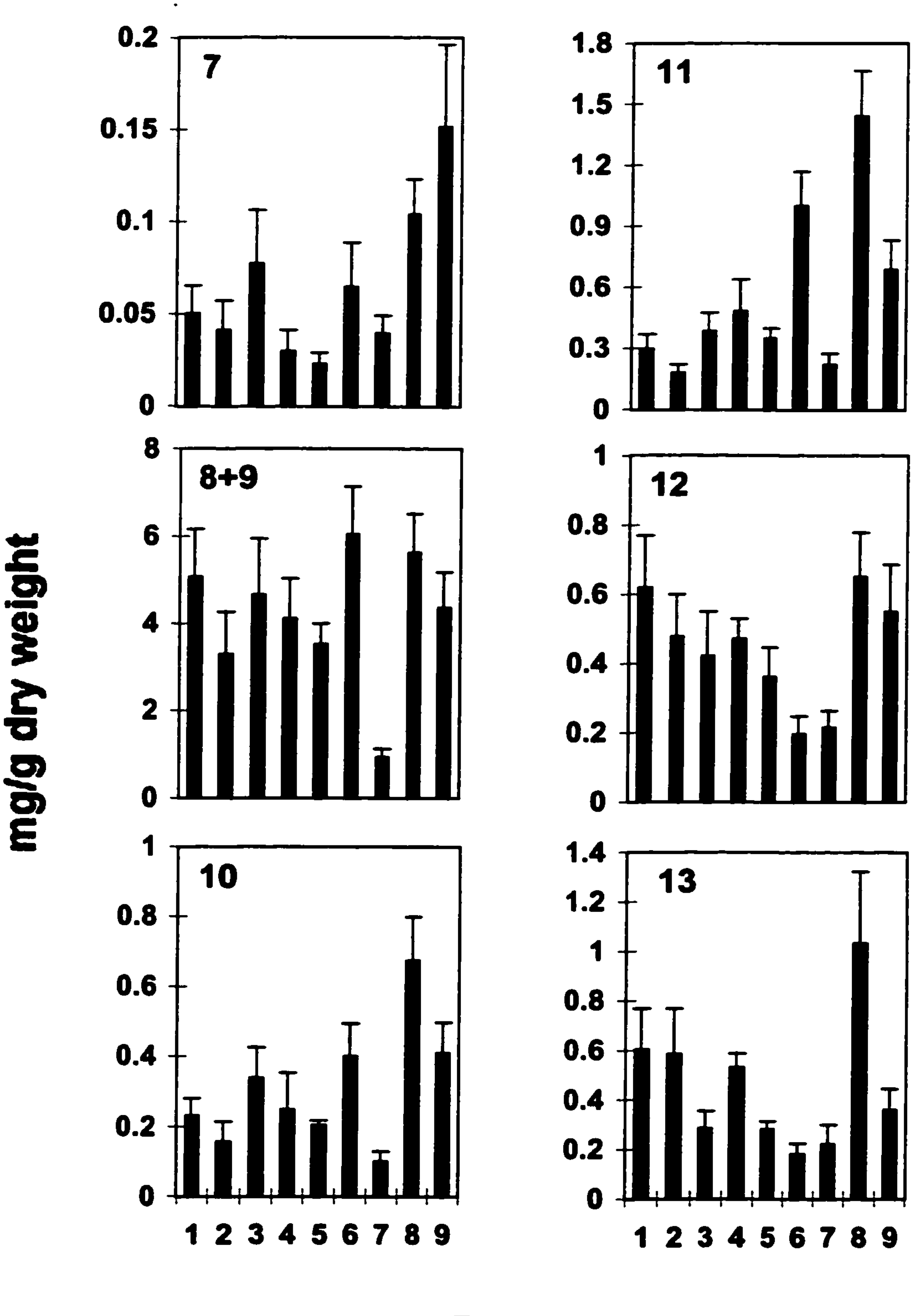

7
11
8+9
12
10
13
0.2
0.15
0.1
0.05
0
1.8
1.5
1.2
0.9
0.6
0.3
0
8
6
4
2
0
1
0.8
0.6
0.4
0.2
0
1.4
1.2
1
mg/g dry weight
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
Population

Figure 6.4. Alkamides **14, 16, 17, 18, 19** in nine *E. angustifolia* populations from Table 6.1 (mean mg/g ± SEM).

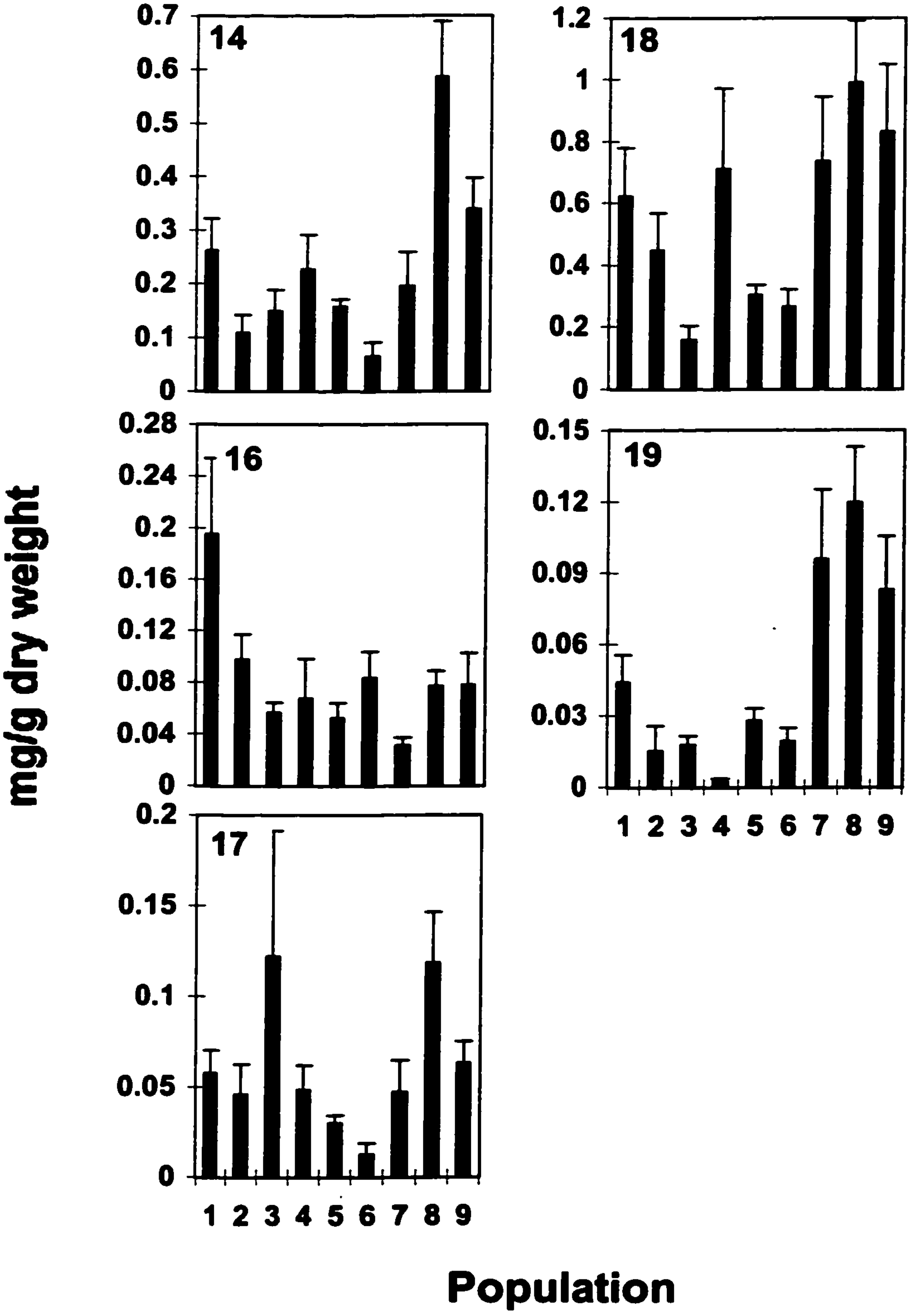

14
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
18
1.2
1
0.8
0.6
0.4
0.2
0
16
0.28
0.24
0.2
0.16
0.12
0.08
0.04
0
19
0.15
0.12
0.09
0.06
0.03
0
1 2 3 4 5 6 7 8 9
17
0.2
0.15
0.1
0.05
0
1 2 3 4 5 6 7 8 9
mg/g dry weight
Population

Figure 6.5. Ketoalkene/ynes **22, 24, 25** in nine *E. angustifolia* populations from Table 6.1 (mean mg/g ± SEM).

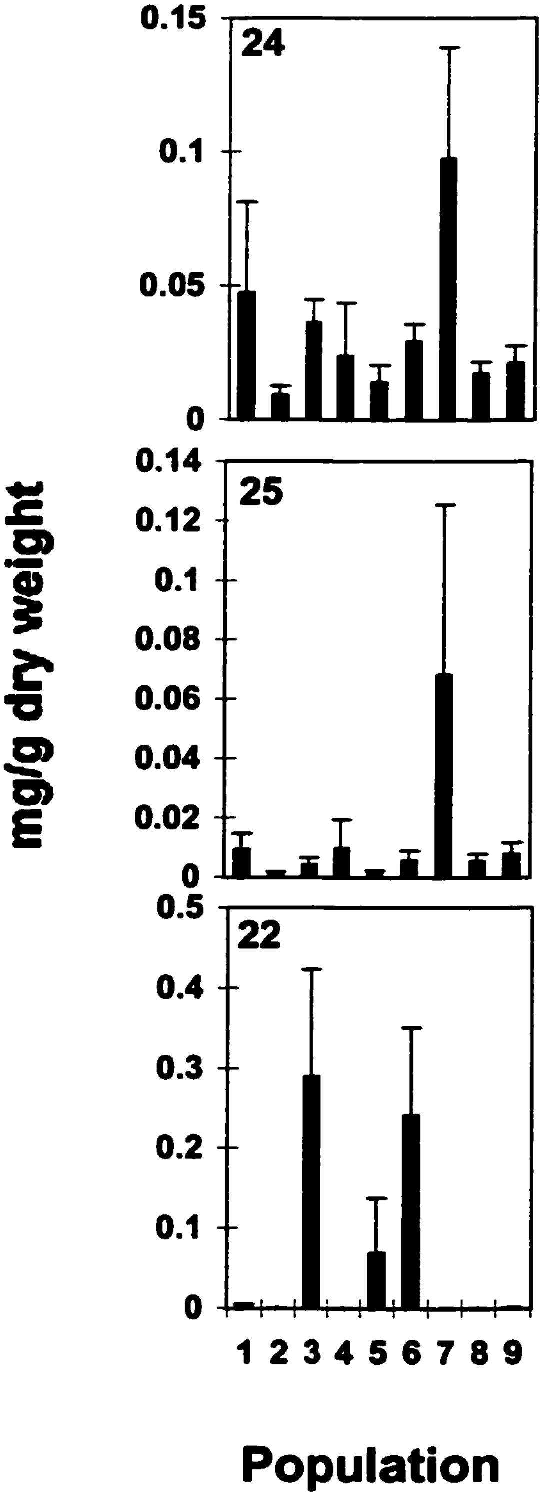

0.15
24
0.1
0.05
0
0.14
25
0.12
0.1
0.08
0.06
0.04
0.02
0
0.5
22
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9
mg/g dry weight
Population

Figure 6.6. Single linkage phenogram of the squared Mahalanobis distances between centroids of *E. angustifolia* populations from Table 6.1.

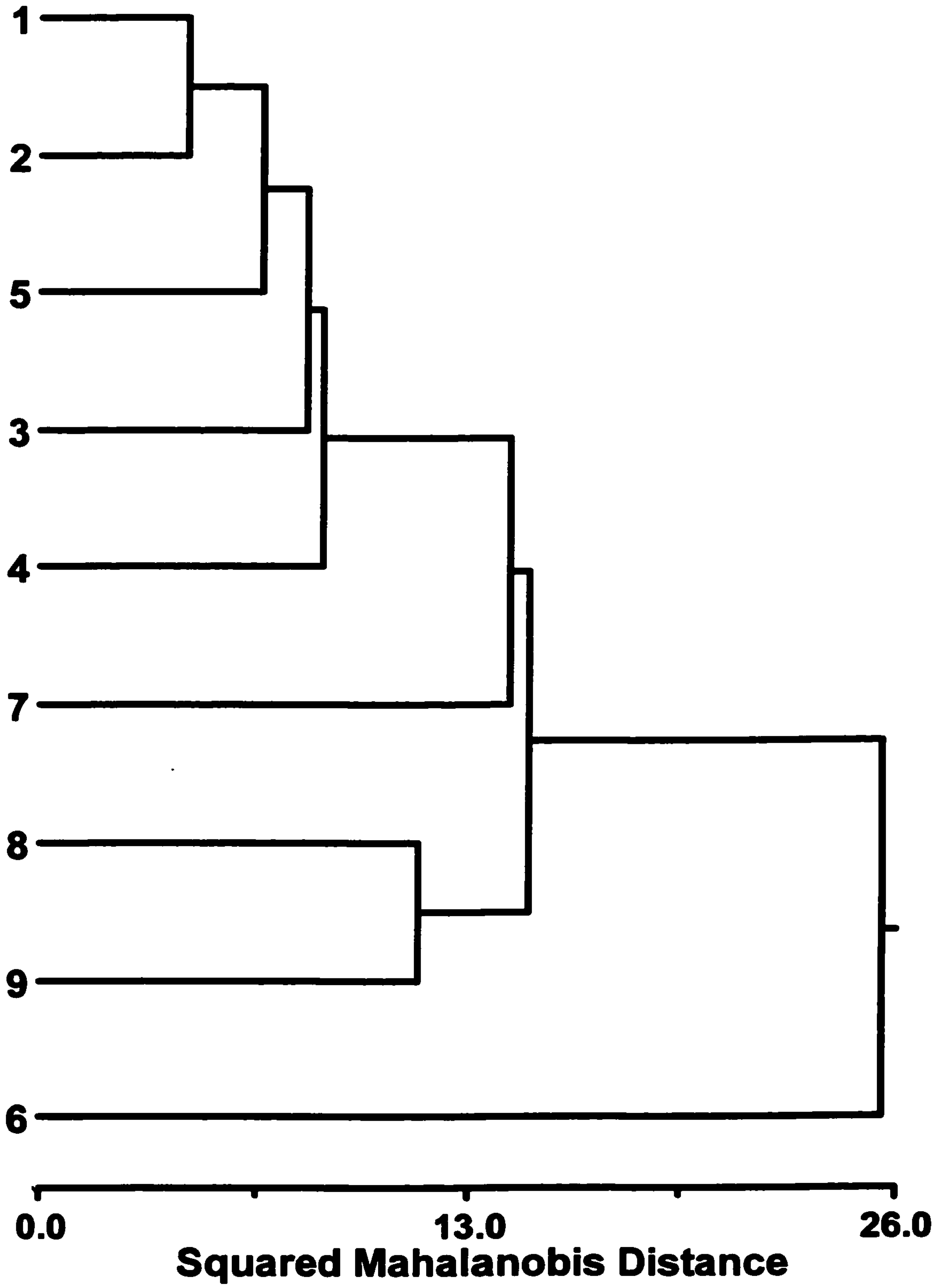
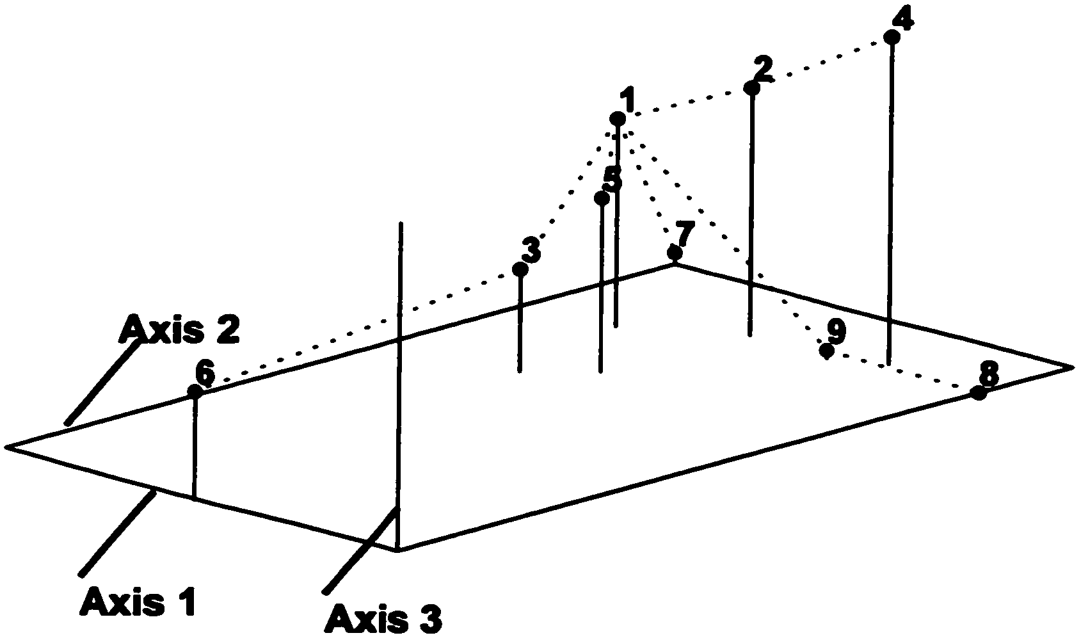

1
2
5
3
4
7
8
9
6
0.0
13.0
26.0
Squared Mahalanobis Distance

Figure 6.7. Principal coordinates of *E. angustifolia* population centroids determined by phytochemical variation with a superimposed minimum spanning tree of the squared Mahalanobis distances (dashed lines).


4
2
1
5
7
3
Axis 2
6
9
8
Axis 1
Axis 3

Discussion

In the current study of wild *E. angustifolia* germplasm, quantitative phytochemical production varied as a function of geographical distance between nine populations. Populations #3, #6, #7, #8 and #9 were the most significantly different according to several statistical analyses. Compared to the above, much smaller geographical distances separated populations #1, #2, #4 and #5 (Table 6.1), and these same populations were the least distinguishable by ordination..

The most distinct chemotype was population #6 according to the multivariate statistical results (Figs. 6 and 7), but considering both its morphological characteristics (chapter 2) and its phytochemical profile, this is likely a result of hybridization/introgression between *E. pallida* (Nutt.) Nutt. and *E. angustifolia* DC. Population #6 contained only trace amounts of *E. angustifolia* species markers, **cynarin** and alkamides **12, 13, 14**, but a large amount of ketone **22** (a commercial *E. pallida* marker) and **echinacoside** (which was reported from both species in Bauer and Wagner 1991; chapter 5). Population #3 also slightly resembled *E. pallida* root profiles (Bauer *et al.* 1988) with an unusual level of **22**, as did #7 with the highest levels of **24** and **25**. However, there was no morphological or ecological indication that populations #3 and #7 were recent hybrids or introgressants, and the phytochemical evidence may be due to the young age of the plants in the current study.

Plant developmental stage influences secondary metabolism; defense compounds are generally more concentrated and diverse when plants are young and more "apparent" to herbivores, but they are known to decrease with age as structural defenses are developed (Feeny 1976; chapter 5). Therefore, as roots in the present study were still young, and they yielded a greater variety of chemical defenses than what is reported for older commercial *E. angustifolia* roots (typically > 2 years) (Bauer *et al.* 1991), this might explain why populations #3 and #7 had intermediate profiles.

It is useful to determine which constituents may be responsible for most of the differences between potential chemotypes in a multivariate analysis. In the present study, phytochemical variables that contributed the most to the squared Mahalanobis distances between populations in the canonical discriminant analysis were alkamides **4**[1], **11**[1], **18**[2], **19**[1], and **cichoric acid**[2], **echinacoside**[2], **caftaric acid**[1] (F-statistic probabilities [1] = $p<0.0001$, [2] = $p<0.05$). Both alkamide **18**, and echinacoside **7** are currently used for commercial standardization of *E. angustifolia*, so their significant variation as chemotype determinants is economically significant. Interestingly, the root concentrations of five of those same compounds, **cichoric acid**, **echinacoside**, **caftaric acid**, alkamides **4**, **19** varied significantly in relation to latitudinal variation (Table 6.3). Populations #8 and #9 are potentially superior chemotypes with significantly increased concentrations of **cichoric acid**, **echinacoside** and alkamide **4** at higher latitudes and including the overall increasing latitudinal trends of most alkamides and phenolics (Figs. 6.1-6.5). As well, we caution that the significant inverse relationship of **caftaric acid** and alkamide **19** with increasing latitude may be an artifact of the regression, because there were no matching trends in the mean population data (Figs. 6.1 and 6.4).

Latitudinal and quantitative variation of **cichoric acid** and **echinacoside** promise to be useful. **Echinacoside** has been reported from all varieties and species of *Echinacea*, except *E. purpurea* (Bauer and Wagner 1991; chapter 5). Here, we indicated that seed source within a single species (and the effects of latitude on that location) influences the overall levels of **echinacoside** in plant materials used for herbal product manufacturing. It is therefore misleading to standardize *Echinacea* products by % **echinacoside** concentration, which is often listed on herbal product labels.

Immunostimulant activity was reported for **cichoric acid**, although it was not measurable with **caftaric acid** in tests of phagocytosis induction (Bauer 1998). **Cichoric acid** is the most abundant constituent in the flowers of all *Echinacea* species (Bauer 1998; chapter 5) with the highest quantity in young *Echinacea purprea* flower buds (Letchamo *et al.* 1999). In addition, *E. purpurea* roots contain **cichoric acid** in large amounts (Bauer and Wagner 1991; Bauer 1998; chapter 5) compared to much smaller trace amounts in *E. pallida* and *E. angustifolia* roots where the major compounds were **echinacoside** and **cynarin** (chapter 5). Therefore, demonstrated latitudinal influence on cichoric acid accumulation in the roots of wild *E. angustifolia* accessions, while not as quantitatively significant for this particular species, has implications for potentially high-yielding *Echinacea* cultivar development, especially if the same latitudinal variation can be determined among cultivated seed sources for *E. purpurea*.

Finally, the significant quantitative variation in *E. angustifolia* populations (Table 6.2) with respect to **caftaric acid** and **cynarin** may impact the *Echinacea* products derived from different seed sources, once the pharmacological activities of these pure compounds are investigated.

Increased phytochemical production in northern plant populations has been reported for the phenolic DIMBOA in corn (*Zea mays* L.) (Levin and York 1978). This research measured higher levels of DIMBOA from temperate germplasm sources compared to the tropical landraces. High levels of DIMBOA were associated with increased insect resistance in temperate varieties of corn (Xie 1991). Similarly, in the present study, ecological gradients that exist across a latitudinal cline helped to create germplasm heterogeneity in wild *E. angustifolia* populations which affected the phytochemical accumulations of both phenolics and alkamides in a controlled environment. These phenomena are best explained by stable or impermanent genetic polymorphisms among populations of the widespread species *E. angustifolia* in response to selection pressures, such as habitat, competition and herbivory.

Those chemotypes which were most distinct in the current study may be identified by their U.S. county names: #3 = Comanche (OK); #6 = Cowley (KS); #7 = Pottawatomie (KS); #8 = Richardson (NE); and #9 = Clay (IA) (Table 6.1). This information is particularly useful when combined with traditional breeding studies (morphology and disease resistance) to improve cultivation of *E. angustifolia* and thereby help to insure germplasm conservation. To provide the tools for the improvement of modern phytomedicines, future investigations should target the effect of genetically-variant wild and cultivated sources of all *Echinacea* species and varieties on biologically-active phytochemical production.

CHAPTER 7: METHYL JASMONATE INCREASES REPORTED ALKAMIDES AND KETOALKENE/YNES IN *ECHINACEA PALLIDA* (ASTERACEAE)

The experiments in this chapter were published in collaboration with I. Inparajah, B.R. Baum, and J.T. Arnason. At the time of thesis preparation, this manuscript was in press in the journal Phytochemistry, vol. 57, pp. XX, 2001. It has been modified here to comply with the phytochemical numbering system of the current thesis.

Introduction

Jasmonic acid (JA) and methyl jasmonate (MJ) are widely distributed regulatory signal molecules derived from linolenic acid in plants in a pathway first described by Vick and Zimmerman (1984). Farmer and Ryan (1992) described the effects of jasmonates on growth, senescence and as a predominant signal in wound-induced defense. MJ induces resistance towards insect herbivory in both tobacco and cabbage crops (Avdiushko *et al.* 1997).

Echinacea, endemic to North America, is a plant genus known for its traditional medicinal qualities (Hobbs 1989). The secondary chemistry of commercial *Echinacea* species (*E. purpurea, E. pallida* and *E. angustifolia*) has been well-characterized (Bohlmann and Hoffman 1983; Bauer *et al.* 1988; Cheminat *et al.* 1988 ; Bauer and Remiger 1989; reviewed in Bauer and Wagner 1991; Bauer 1998; chapter 5) and remains the primary method of botanical identification for marketed *Echinacea* plant materials.

Echinacea possesses a high level of constitutive diversity within each of its numerous classes of defense compounds, particularly the family of alkamides, polyacetylenes and ketoalkene/ynes, some of which are known insecticides (Jacobson 1967). Diversity within phytochemical classes is a common defensive strategy against herbivory (Karban *et al.* 1997). Another strategy is a rapid increase in quantity and/or variability of phytochemical defences in response to wounding or fungal infection, which can be simulated by application of the signal molecule MJ. The induction of *Echinacea* phytochemicals whether naturally or by MJ is important for increased efficacy and efforts to standardize *Echinacea* phytomedicines.

Identified alkamides and polyacetylenes in *Echinacea* spp. exhibit other biological activities *in vitro*, including fungicidal (Binns *et al.* 2000, see chapters 8 and 9) and immunostimulatory activities (Bauer and Wagner 1991). Here we report that the volatile chemical signal MJ induces alkamide and ketoalkene/yne biosynthesis in *Echinacea pallida*.

Experimental

Plant material

E. pallida achenes from a wild population (DAO #EPA013, coll. S.Binns, Arkansas, 1998) were germinated and grown to 34 days and 58 days in 2:1 soil:vermiculite in a controlled environment chamber at 25 ^{0}C under a 16:8 light to dark regime. Voucher specimens are deposited in the Department of Agriculture Ontario Herbarium (DAO), Ottawa, Canada.

Treatment

In the two age categories, the same number of individuals were treated with MJ (n=10 each) with an equal number of untreated control plants (n= 10 each). Treated plants were sprayed with MJ solution and control plants were sprayed with dH_20 and DMSO. MJ (100ppm) was prepared in aqueous solution of 19 μL MJ, 19 μL DMSO and 200 mL distilled water. Initial trials at 10, 100 and 1000 ppm MJ showed the best induction at 100 ppm and this concentration was used in trials reported here. Treated plants were isolated from control plants in different growth chambers, under identical conditions, to avoid inter-plant communication through airborne MJ (Farmer and Ryan 1990).

Extraction and Isolation

Twenty-four hours after MJ treatment, entire roots of all plants were harvested, cleaned, weighed and extracted in 95% ethanol for another 24 hours under moderate mechanical agitation (70 rpm). Extraction time after MJ treatment was based on time course experiments (2 hours to 72 hours) with dill (*Anethum graveolens*) (Belzile 1998), to determine the time required for the MJ signal to activate genes for phytochemical biosynthesis in root tissues. Filtered extracts (#1 Whatman, Buchner funnel) were concentrated *in vacuo* (not to dryness) and root tissue residues dried (50^{0}C). The dry weight of root residues was used to calculate the volume of solvent required to adjust each extract to a standard concentration of 0.01g extracted dried root/ml 95% ethanol. Extracts were then filtered with 0.2 μm nylon membranes prior to separation of lipophilic constituents using HPLC. Chromatography was achieved using a solvent system of acetonitrile:water (6:4) at 1 mL/min and 5 μL injection on a 7.5 cm reversed phase C-18 column (3 mm particle size). Lipophilic compounds were detected at 210 nm and 260 nm and identified by comparison with reference standards. Reference standards **1, 8+9, 11,** and **18** (see Figs. 5.1-5.4) were isolated previously in our laboratory by column chromatography on silica gel (Bergeron *et al.* 1999). Spectra obtained for these by ^{1}H, ^{13}C-NMR, and EI-MS conformed with reported values (Perry *et al.* 1998; Yasuda *et al.* 1981). Other compounds were identified based on relative retention time and online UV spectrum (Bauer and Remiger 1989). The standard **8+9** was used to calculate a response factor for the HPLC detector. Quantitation of each previously reported compound (μg/ per ml injected) was achieved using peak area multiplied by the response factor. This figure was then divided by the original concentration of 0.01g extracted dried root/ ml sample and multiplied by 10^3 to reach mg/g dry wt. of whole root.

Results and Discussion

The alkamide composition of *Echinacea* is known to vary quantitatively and qualitatively throughout growth and development (Letchamo *et al.* 1998) and in our experiments, alkamide levels tended to decline naturally between 34 days and 58 days in *E. pallida* roots of control plants (Figs. 7.1 and 7.2). The only qualitative difference observed was the formation of the ketoalkyne **22*** as the roots aged (compounds denoted by an asterisk are ketoalkene/ynes, while the others are alkamides). Compound **22*** is used in the marketplace to distinguish older *E. pallida* roots from *E. angustifolia*, which has the isomers **8+9** as its major constituents, but lacks **22*** (Bauer *et al.* 1988). In our trials with very young (34 day old) roots, the formation of **8+9** in high concentrations (control and treated) occurs early in development (Fig. 7.1), followed by a remarkable decrease later (58 day old) when **22*** begins to appear (Fig. 7.2).

After treatment with 100 ppm MJ, the majority of compounds identified showed an increase in concentration in the MJ-treated plants over the concentrations in control roots (Figs 7.1 and 7.2) at both plant ages. Statistically significant increases were observed with a number of compounds and ranged from 54%-353% (mg/g dry wt.) (Table 7.1). MJ treated *E. pallida* plants aged 34 days contained significantly higher levels ($p<0.05$) of compounds **6, 10, 11,18, 19** and **25A***, and **25B*** compared to control plants of the same age (Fig. 7.1, Table 7.1). MJ treated *E. pallida* plants aged 58 days contained significantly higher levels of compounds **8+9, 6, 1, 2, 20*** and **22*** compared to control plants of the same age (Fig. 7.2, Table 7.1). The compounds found in treated and control *E. pallida* roots common to both ages were **8+9, 2, 3** and **7**. Younger roots contained approximately five times more of these common constituents than did the older roots (Figs. 7.1 and 7.2).

The statistically significant increases presented here suggest that alkamides and ketoalken/ynes are inducible defense compounds in *Echinacea* plants. Further investigation of this phenomenon in other *Echinacea* species should be undertaken to determine the effect of MJ on other phytochemical classes, especially the pharmacologically-active polysaccharides.

Table 7.1. Alkamides and ketoalkene/ynes in *E. pallida* roots of two age classes (34 days and 58 days) that increased after methyl jasmonate treatment. Compound numbering corresponds to Figures 5.1-5.4. Significantly different means $p<0.05$ by a two-tailed t-test (SYSTAT, v. 7.0, SPSS 1997) using original quantitative data.

	Compound induced		**%[a]**	**increase**	
		34 d	**p =**	**58 d**	**p =**
1	undeca-2E,4Z-dien-8,10-diynoic AIBA	+	0.06	**134**	0
2	undeca-2E,4E-dien-8,10-diynoic AIBA	+	0.27	**156**	0
6	trideca-2E,7Z-dien-10,12-diynoic AIBA	**54**	0.03	**227**	0.035[t]
8+9	dodeca-2E,4E,8Z,10E/Z-tetraenoic AIBA	+	0.09	**63**	0.046[t]
10	dodeca-2E,4E,8Z-trienoic AIBA	**84**	0.03	+	0.128[t]
11	dodeca-2E,4E-dienoic AIBA	**251**	0.003[t]	+	0.539[t]
18	pentadeca-2E,9Z-dien-12,14-diynoic AIBA	**78**	0.019[t]	+	0.1
19	hexadeca-2E,4E-dien-12,14-diynoic acid -2-methylbutylamide	**238**	0.035[t]	+	0.170[t]
20	8-hydroxytetradeca-9E-ene-11,13-diyn-2-one*	+	0.269[t]	**353**	0.001[t]
22	tetradeca-8Z-ene-11,13-diyn-2-one*	-	-	**151**	0.016[t]
25	pentadeca-8Z,11Z/E-13E/Z-trien-2-one*	**151**	0.006[t]	+	0.33

\+ denotes an increasing trend that was not statistically significant

[a] % increase = treatment mean/control mean x 100

[t] original data transformed by LOG_{10} to meet assumptions for statistical analyses

* denotes ketoalkene/ynes without amide group

AIBA = acid isobutylamide; n = 10 each age class

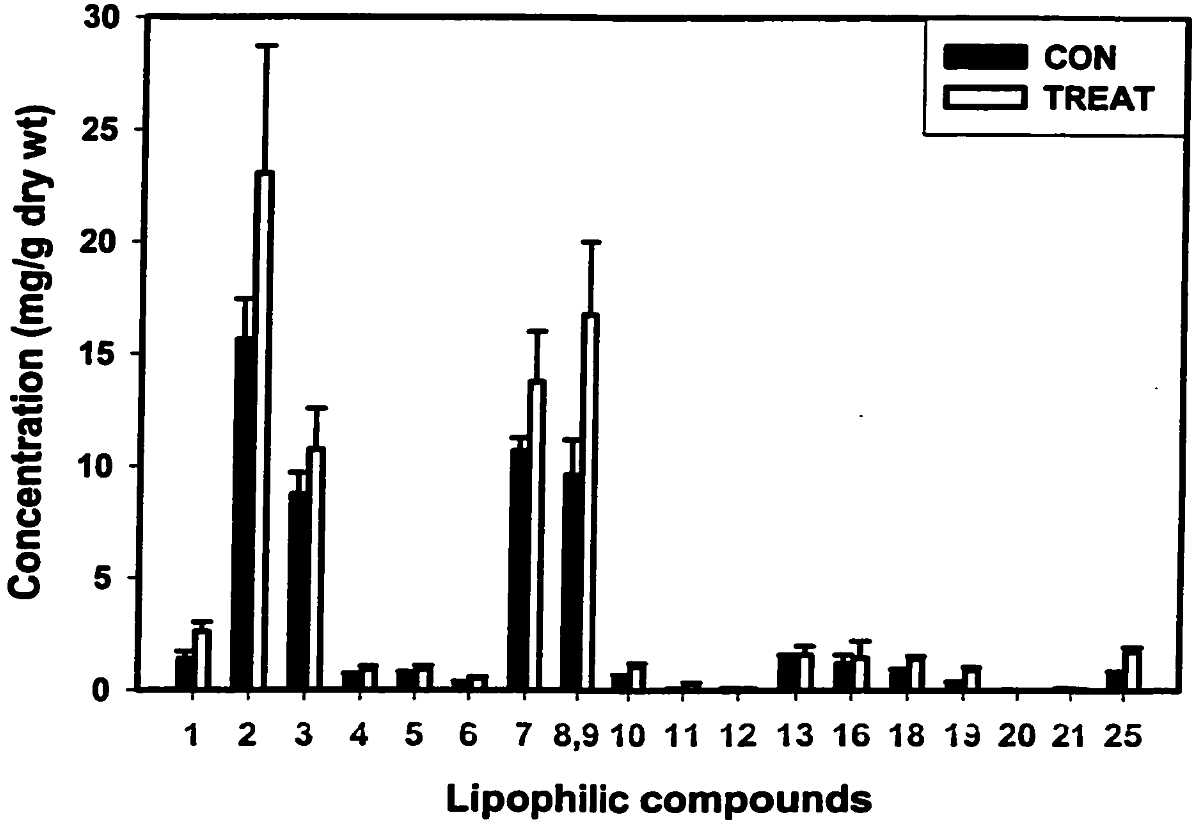


Figure 7.1. Lipophilic compounds (mean + std. error) in *Echinacea pallida* roots aged 34 days (n=10) from methyl jasmonate-treated (TREAT) and control plants (CON).

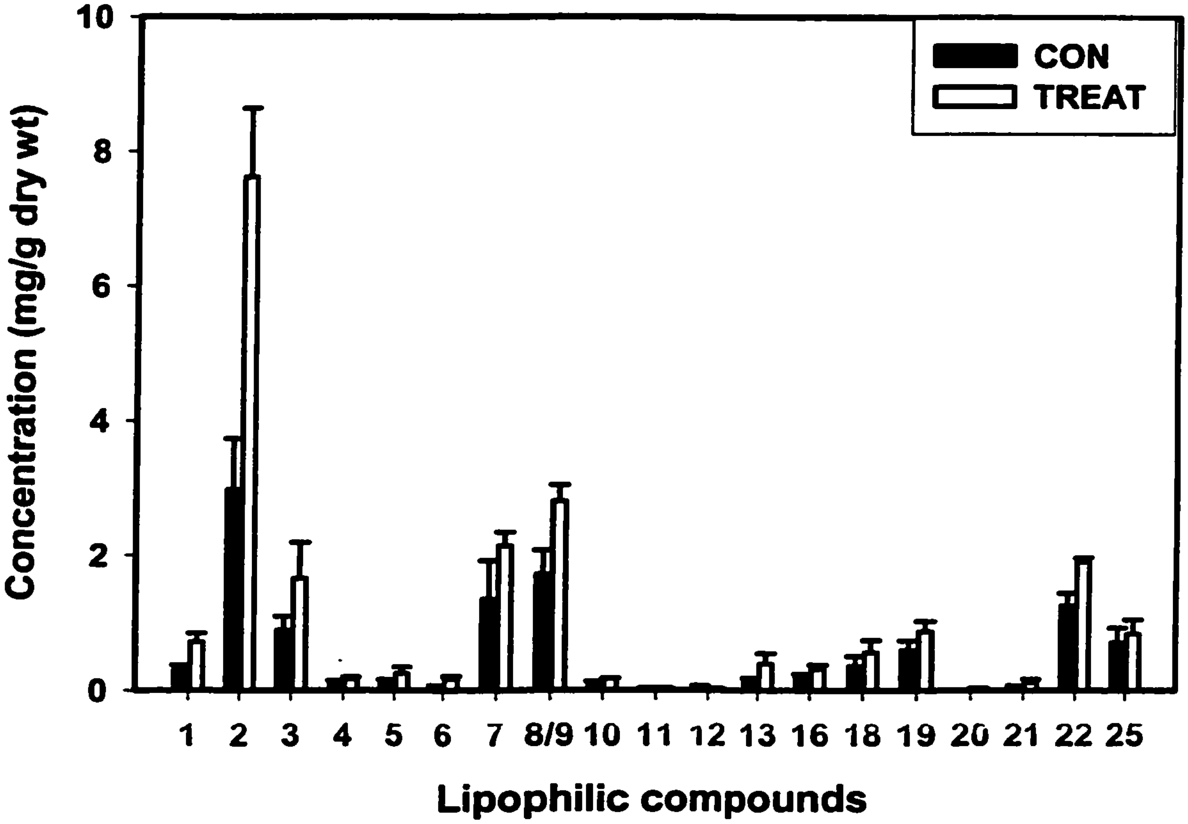


Figure 7.2. Lipophilic compounds (mean + std. error) in *Echinacea pallida* roots aged 58 days (n=10) from methyl jasmonate-treated (TREAT) and control plants (CON).

SECTION 3

BIOLOGICAL ACTIVITY

The phytochemical variation described in Section 2 has a direct impact on the therapeutic efficacy of *Echinacea* phytomedicines. In the following section, antimicrobial activity of *Echinacea* extracts was investigated in chapters 8 and 9 using disc susceptibility tests, and compared with phytochemical profiles.

Taxonomic Note

Chapter 8 reports novel light mediated antifungal activity from *E. purpurea* and *E. pallida* extracts, which were both identified *sensu* McGregor at the time of publication of this chapter in Planta Medica. However, taxonomic identities of *Echinacea* materials studied in chapters 9 and 10 were made according to the revised morphometric taxonomy (see chapter 2). Anti-fungal bioassays were carried out in the laboratory of Dr. Myron Smith at Carleton University, who helpfully provided the clinical fungal isolates for the study. Anti-viral bioassays were carried out by Dr. Jim Hudson and colleagues, in the University of British Columbia Faculty of Medicine.

CHAPTER 8: LIGHT MEDIATED ANTIFUNGAL ACTIVITY OF *ECHINACEA* EXTRACTS

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Introduction

A recent market survey has reported *Echinacea* products to be the best selling over the counter herbal product in North America (Brevoort 1998). In Europe, over 800 different commercial preparations are marketed (Bauer *et al.* 1988b) and 2.5 million prescriptions are filled per year.

Besides a primary use of *Echinacea* preparations for oral treatment of colds and flu, they are also indicated for infections and topical conditions such as candidiasis, strep throat, staphylococcus infections, infected wounds, skin ulcers and burns (Hobbs 1994). At least three clinical trials show positive results using *E. purpurea* juice as a topical antibiotic on skin and in the oral cavity (Hobbs 1994). Traditionally, first nations groups of U.S.A. and Canada, including the Crow, Kiowa, Cheyenne, Sioux and Omaha, used *E. angustifolia* roots for sore gums, mouth and throat as well as septic conditions (Shemluck 1982). Pharmacological research has emphasized the immunomodulatory effects of *Echinacea*, documenting increased phagocytosis by granulocytes which were induced by *Echinacea* extracts *in vitro* and *in vivo* (Bauer *et al.* 1988b). Modes of action of *Echinacea* in topical preparations have not been sufficiently investigated.

Antimicrobial phototoxic effects have been observed with excised plant parts, lipophilic extracts or isolated phytochemicals. Near UV light (300-400nm) is the wavelength range required for phototoxicity observed in members of the Asteraceae. Research by Camm *et al.* (1975), on species of Asteraceae that are closely-related to *Echinacea*, demonstrated that these plants possess light-activated antimicrobial action (phototoxicity) as well as light-independent toxicity (Towers *et al.* 1979). For example, the genus *Rudbeckia*, a close relative of *Echinacea*, contains phototoxic substances which exhibit antimicrobial and insecticidal activities (Guillet 1997).

Echinacea spp. are reported to contain mono, di and tri acetylenes (Schulte *et al.* 1967) which belong to the structural class of compounds having phototoxic activity to microoganisms (Marchant and Cooper 1987; McLachlan *et al.* 1986). They also contain acetylenic isobutyl amides (Bauer *et al.* 1988b; Bauer and Wagner 1991) which remain largely uninvestigated for phototoxic activity. Therefore the purpose of the present study was to assess the potential of *Echinacea* extracts and medicinal preparations for phototoxicity and light-independent toxicity to clinically relevant pathogenic fungi (including clinical isolates of the genus *Candida).*

Materials and Methods

Plant materials and extracts

Plants of *Echinacea purpurea* L. Moench were obtained from commercial culture at Trout Lake Farm, Washington, U.S.A. and from a local market in Ontario, Canada (voucher specimens EP97-10 and EP97-9, coll. S. Binns). The *Echinacea pallida* (Nutt.) Nutt. plants were transplanted to a greenhouse from a natural population in Oklahoma, U.S.A.(voucher EPA97-17, coll. S. Binns). All plants were taxonomically identified according to McGregor (1968) and are deposited at the Department of Agriculture Ottawa Herbarium, Ottawa, Canada (DAO). The commercial tinctures and tea, which were tested and presented here, were purchased locally as typical *Echinacea* products used in North Amercian households. Tincture 1 of *E. purpurea* (lot # 12871) is composed of 95% herb mother tincture and 5% root mother tincture in 55% ethanol. Tincture 2 is composed of root (200mg/ml in 55% ethanol) of equal parts *E. angustifolia* and *E. purpurea* (lot # 027TH). The tea bags contained equal parts of whole plant *E. angustifolia* and *E. purpurea* and "concentrated extract of *E. purpurea* root".

Polyacetylene fractions from fresh *Echinacea* plant organs were extracted using standard methodology (Guillet *et al.* 1997). For each plant, two types of *Echinacea* extracts were prepared; root extract and either a "tops" extract (stems, leaves and inflorescences) or an inflorescence extract. Organs were homogenized in 95% ethanol (1g fresh weight/10mL) and filtered via Buchner filtration. In a separatory funnel, n-hexane was added to the filtrate in a volumetric 1:1 ratio. This mixture was diluted with an equal volume of distilled water and the n-hexane fraction collected: the process was repeated three times for each alcoholic filtrate. In the same way, *Echinacea* tinctures 1 and 2 were fractionated into n-hexane. The *Echinacea* tea was infused in distilled boiling water (1g dry weight/10 mL), then extracted three times into n-hexane (1:1 volumetric ratio). Each hexane fraction was rotary evaporated to 2 g equivalent /mL, based on plant dry weight. The extracts were then analyzed by HPLC, and used for phototoxicity tests.

Phototoxicity testing

Fungal strains used for phototoxicity tests were obtained from the Ottawa General Hospital (Ottawa, ON, Canada), except *Saccharomyces cerevisiae* which was from cultured from commercial dessicated bread yeast. Fungal cells from actively-growing cultures were uniformly streaked by sterile cotton swabs onto sterile yeast nitrogen base agar medium in 10 cm diameter petri plates (6.7 g/L Difco yeast nitrogen base without amino acids, 1% glucose, 1.5% agar). Nine clinical isolates were used: seven of *Candida albicans*, and one each of *C. kefyr* and *C. tropicalis*. In addition, we tested plant extracts on the non-pathogenic strains *C. steatulytica, C. shehata* and *Saccharomyces cerevisiae*. To determine phototoxic activity, the disk susceptibility method was used. The disk susceptibility test, as a rapid screening method, was validated through correlation to minimum inhibitory concentrations obtained for amphotericin B against *Candida* (Law *et al.* 1997). n-Hexane extracts (40 ul) of *Echinacea* spp. were added to Whatman #3 filter disks (0.5 cm diameter) and allowed to air dry. Three identical disks were applied to the surface of each yeast-inoculated petri plate (n=3). All cultures were manipulated using a Biological Containment Hood (BioKlone 2, Microzone, Nepean, ON, Canada).

For each plant extract; a "near-UV" plate (treated), and a "no-UV" plate (dark control) were prepared. Both a positive and vehicle control were also included for each light treatment (10 μL of 1 mg/mL α-terthienyl in 75% methanol and 40 μL of n-hexane, added to three filter disks per plate). The treated plates were irradiated with near-UV light (300-400nm) for two hours from a bank of blacklight blue bulbs (Westinghouse F20T12/BLB, 0.1 W/m^2) and visible light (cool-white, 80 W/m^2). The plates that were not exposed to near-UV light were immediately wrapped in tinfoil at room temperature. All plates were then incubated at 30°C, for 48 hours before examination. After two days, the plates were removed from the incubator and inspected for zones of inhibition of fungal growth. These clear zones were measured in millimeters as the shortest distance from the outer edge of the filter disk to the edge of the living yeast cells.

High Pressure Liquid Chromatography

Freshly prepared n-hexane extracts were analyzed by HPLC using a LiChroCART^R 90 x 4.6mm RP-18 column (3μm particle size) with a 4 x 4.6mm pre-column (5μm). Separation was achieved with a 5 μL injection in the solvent system of acetonitrile:water (7:3) at a flow rate of 1 mL/min. for 18 minutes, graduating over 1 min. to 100% acetonitrile for 7 mins. For peak identification, the on-line diode array UV spectral profile and relative retention time of each compound was compared to the literature (Bauer *et al.* 1988b; Bauer and Remiger 1989).

Compound isolation and testing

The compounds dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (**8+9**) (see Fig. 5.1) and undeca-2E,4Z-diene-8,10-diynoic isobutylamide (**1**) (Fig. 5.2) were isolated from *E. purpurea* tops in our laboratory (Bergeron *et al.* 2000) and identified by comparison of spectral data described by Bauer and Remiger (1989). Trideca-1-en-3,5,7,9,10-pentayne was isolated as described in Guillet *et al.* (1997) and was identified in *E. purpurea* and *E. pallida* roots by online UV spectra. Compound purity was assessed by HPLC as described above. Each compound eluted as a single peak which represented >95% total peak area in the chromatogram, and was detected at 210 and 360 nm. Since the n-hexane extracts of *Echinacea* were approximately as active to *S. cerevisiae* as to *Candida* spp., we chose *S. cerevisiae* as the test organism to eliminate the requirement for biohazard protection.

Results and Discussion

All extracts of *Echinacea* spp. tested, except *E. purpurea* tops and the *Echinacea* tea, exhibited near UV-mediated antimicrobial action. This was measured as the growth inhibition of clinical isolates of *Candida* spp. and *Saccharomyces cerevisiae* (Table 8.1) when subjected to n-hexane extracts of *E. purpurea* roots and inflorescences (Figs. 8.1 and 8.2). Also, *E. purpurea* and *E. pallida* roots and tops, commercial tinctures and tea inhibited growth of *Candida shehata* (Table 8.2). The *E. purpurea* root extracts were phototoxic to all twelve isolates and light-independent activity was seen with eight isolates (Fig. 8.1). The *E. purpurea* inflorescence extract also had phototoxic activity to seven isolates and light-independent activity to two (Fig. 8.2). *E. pallida* roots also demonstrated light-independent inhibition of fungal growth (Table 8.2). Near UV-mediated (+UV) toxicity to *C. shehata* was found to be significantly greater ($p=0.05$) than light-independent

(-UV) toxicity for extracts of *E. purpurea* root and *E. pallida* roots and tops.

Comparison between mean inhibition zones (indicating the degree of phototoxicity) of near UV treated extracts suggests that *E. purpurea* root extracts are significantly more phototoxic than all other extracts tested (Table 8.2). Root extracts were significantly more phototoxic than "tops" extracts for both *E. purpurea* and *E. pallida* ($p<0.05$). This phototoxic variation between organs is consistent with variation in amounts and types of acetylenic compounds between plant organs of *E. purpurea* (Table 8.3) and with acetylenic variation between organs observed previously in other members of Asteraceae (Guillet *et al.* 1997).

Table 8.1. Test organisms including *Saccharomyces cerevisiae* and clinical isolates of *Candida* spp. (maintained at the laboratory of M. Smith, Carleton University, Ottawa, ON, Canada).

Label	**Species**	**Strain #**
al-1	*C. albicans*	308-1329
al-2	*C. albicans*	311-0628
al-3	*C. albicans*	312-0305
al-4	*C. albicans*	316-0504
al-5	*C. albicans*	316-0588
al-6	*C. albicans*	316-433
al-7	*C. albicans*	FR2853
ke-1	*C. kefyr* (pseudotropicalis)	301-0788
sh-1	*C. shehata*	2883
st-1	*C. steatulytica*	2959
tr-1	*C. tropicalis*	301-0810
sacch	*Saccharomyces cervisiae*	-

Table 8.2. Zone of inhibition of growth of *C. shehata* (mean in mm ± sd) by *Echinacea* n-hexane extracts with and without near UV treatment. Means (n=3) followed by the same letter are not significantly different in Tukey's test ($p<0.05$). Significant differences between treatment means for a given extract are denoted by (*), indicating those with photo-activated toxicity ($p<0.05$).

	Extract	**+ UV**	**- UV**	**t-test p value**
Roots	*E. purpurea*	**4.67** ± 0.29 [a]	**2.00** ± 2.59	0.001*
	E. pallida	**2.50** ± 0.50 [b]	**0.73** ± 0.25	0.038*
Tops	*E. purpurea*	**0** [c]	**0**	-
	E. pallida	**0.73** ± 0.25 [c]	**0**	0.014*
Commercial	tincture 1	**0.67** ± 0.30 [c]	**0**	0.057
	tincture 2	**0.67** ± 0.58 [c]	**0**	0.184
	tea	**0** [c]	**0**	-
control	a-terthienyl	**5.33** ± 0.58 [c]	0	0*

The mechanism of phototoxicity is usually oxygen-dependent and is elicited by a type II photosensitization process in which the photo-activated phytochemical transfers its energy to molecular oxygen, forming singlet oxygen (McLachlan *et al.* 1986). Singlet oxygen mediates toxicity through lipid peroxidation in the target cell. Di- and tri-acetylenes as well as acetylenic thiophene derivatives have been shown to be responsible for these effects (Marchant and Cooper 1987; McLachlan *et al.* 1986). In addition to photooxidative mechanisms, polyacetylenes are known to react directly with olefins in the presence of near UV to yield 1:1 cyclobutanyl adducts. The direct action of a polyacetylene on cell membrane integrity was found to be responsible for both light-activated and light-independent toxicity to fungi of the genus *Fusarium* (Arnason *et al.* 1986; Bourque *et al.* 1985). In this study, phytochemical HPLC profiles of E. *purpurea* n-hexane root and inflorescence extracts (Table 8.3) are similar to the reported phytochemical variation in *E. purpurea* plant parts (Bauer 1998). To identify the principles of these n-hexane extracts potentially responsible for antifungal activity, two representative substances were isolated for phototoxicity tests and tested in a preliminary trial at a level of 5 mg/disk against *Saccharomyces cerevisiae* (Table 8.4). Compounds isolated from *E. purpurea* tops were undeca-2E, 4Z-diene-8,10-diynoic acid isobutylamide (1)(Fig. 5.2) and dodeca-2E, 4E, 10E/Z-tetraenoic acid isobutylamide (**8+9**)(Fig. 5.1). The highly unstable trideca-1-en-3,5,7,9,10-pentayne, while present in *E. purpurea* roots, was previously isolated in our laboratory (Guillet *et al.* 1997). Clearly phototoxicity is associated with the polyacetylenic compounds (Table 8.4), especially those with higher numbers of triple bonds as found previously (Arnason *et al.* 1986). Other photoactive compounds are undoubtedly present which will be identified in future work. The roots of *E. pallida* contain primarily ketoalkenes and ketoalkynes, and we suspect the latter are the active phototoxins, but we did not investigate pure preparations of them due to lack of standards.

Previously reported autooxidation of polyacetylenes in dry root powders (Bauer *et al.* 1988a) may explain the lower amount of near UV-mediated antimicrobial toxicity from major commercial tinctures and lack of activity in the tea. Photodegredation of the pure compounds tested here is also a possible source of error which merits further investigation, and has been observed previously (McLachlan *et al.* 1986).

Table 8.3. Concentration of standard compounds dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic AIBA (acid isobutylamide), and relative percentage to the standard for other compounds in n-hexane extracts (0.5g/ml) of *E. purpurea* separated by HPLC. Authentic standards were confirmed by C^{13}-NMR, all others were identified by on-line diode array UV spectra and published relative retention times (Bauer and Remiger 1989). Compound numbers correspond to Figs. 5.1-5.4 in chapter 5 of this work.

T 8.3. Substance	**Root % of (6)**	**Inflorescence % of (6)**
(1) undeca-2E, 4Z-diene-8,10-diynoic AIBA	1.45	7.57
(3) dodeca-2E, 4Z-diene-8,10-diynoic AIBA	13.5	2.7
(4) undeca-2E, 4Z-diene, 8,10-diynoic acid-2-methylbutylamide	0.48	2.84
(5) dodeca-2E, 4E, 10E-triene-8-ynoic AIBA	7.51	-
(7) dodeca-2E, 4Z-diene-8,10-diynoic acid 2-methylbutylamide	10.1	2.35
(8+9) dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic AIBA	**1123ppm**	**356ppm**
(10) dodeca-2E, 4E, 8Z-trienoic AIBA	14.6	-
(19) hexadeca-2E, 9Z-diene-12,14-diynoic AIBA	-	69.6

T 8.3. Substance	**Root % of (6)**	**Inflorescence % of (6)**
(**22**) tetradeca-8Z-ene-11,13-diyn-2-one	26.2	-
(**24**) pentadeca-8Z, 13Z-diene-11-yn-2-one	3.03	197
(**25**) pentadeca-8Z, 11E/Z, 13 E/Z-triene-2-one	-	197
trideca-1-en-3,5,7,9,10-pentayne	n/a	n/a

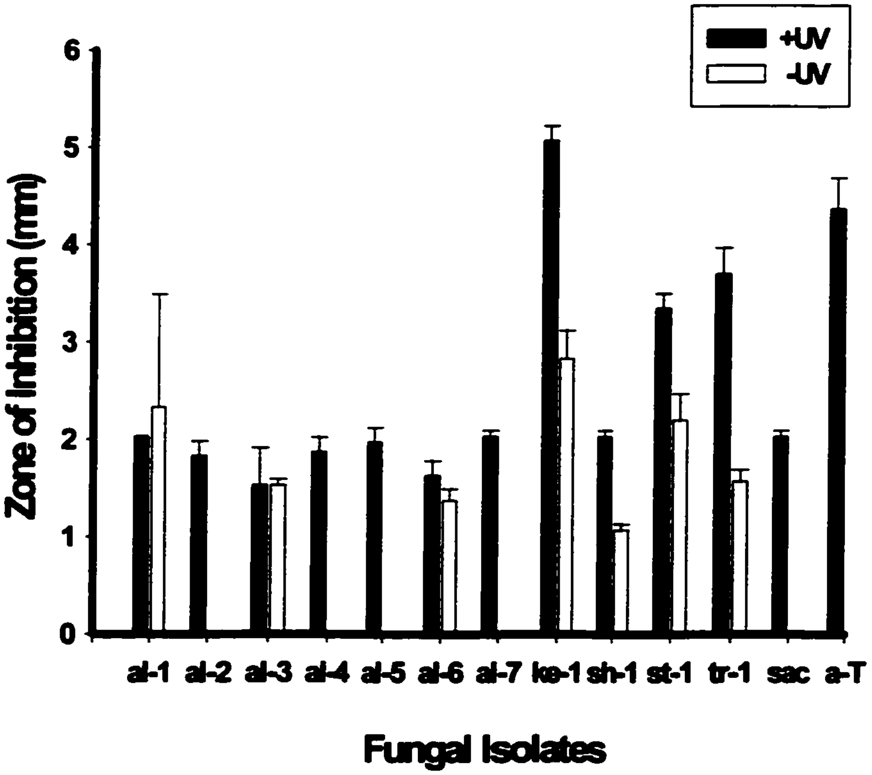
A study by Towers *et al.* (1979) determined the effects of 24 phototoxic polyacetylene and thiophene derivatives on human skin. Only thiophene derivatives such as α-terthienyl were found to cause photodermatitis in human skin. Other polyacetylenes tested had similar phototoxic effects as a-terthienyl on pathogenic microorganisms, including *Candida albicans,* but did not result in any phototoxic effects on human skin.

The present finding suggests a mode of action of *Echinacea* products in the topical treatment of infections, especially in phototherapeutic applications. More attention needs to be paid to the light mediated activity of *Echinacea* products.

Table 8.4. Zone of inhibition of growth of *S. cerevisiae* (mean in mm ± sd) by pure *Echinacea* compounds with and without near-UV treatment. Means (n=3) of compounds in the near-UV group were not significantly different from the alpha-terthienyl control ($p<0.05$).

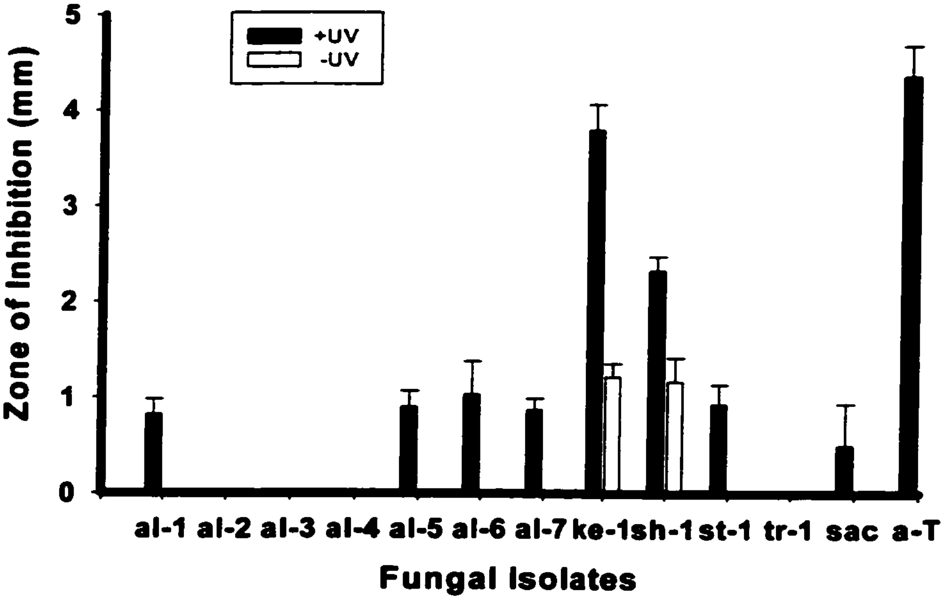
Compound	**+UV**	**-UV**	**p-value**
trideca-1-en-3,5,7,9,10-pentayne	>5	0	N/A
(**1**) undeca-2E,4Z-diene-8,10-diynoic isobutylamide	1±1	0	0.116
(**8+9**) dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide	2±1	0	0.065
α-terthienyl control	4±2	1±0.3	0.034

Fig. 8.1. The toxicity of *Echinacea purpurea* n-hexane root extracts (80mg/disk) towards fungal isolates in near UV (+UV) and non UV (-UV) treatments. Fungal isolates included were; *Candida albicans* (al-1 to al-7), *C. kefyr* (ke-1), *C. shehata* (sh-1) , *C. steatulytica* (st-1), *C. tropicalis* (tr-1), and *Saccharomyces cerevisiae* (sac). Positive alpha-terthienyl control (α-T) tested against *C. albicans* (al-4) was included for reference.



+UV
-UV
Zone of Inhibition (mm)
0
1
2
3
4
5
6
al-1
al-2
al-3
al-4
al-5
al-6
al-7
ke-1
sh-1
st-1
tr-1
sac
a-T
Fungal Isolates

Fig. 8.2. The toxicity of *Echinacea purpurea* n-hexane inflorescence extracts (80mg/disk) towards towards fungal isolates in near-UV (+UV) and non-UV (-UV) treatments. Fungal isolates included were; *Candida albicans* (al-1 to al-7), *C. kefyr* (ke-1), *C. shehata* (sh-1) , *C. steatulytica* (st-1), *C. tropicalis* (tr-1), and *Saccharomyces cerevisiae* (sac). Positive control alpha-terthienyl (α-T) tested against *C. albicans* (al-4) was included for reference.



+UV
-UV
Zone of Inhibition (mm)
0
1
2
3
4
5
al-1
al-2
al-3
al-4
al-5
al-6
al-7
ke-1
sh-1
st-1
tr-1
sac
a-T
Fungal Isolates

CHAPTER 9: LIGHT-MEDIATED ANTIFUNGAL ACTIVITY OF THE GENUS *ECHINACEA* (ASTERACEAE)

The UV light-mediated anti-fungal activity observed with lipophilic extracts from the roots (and inflorescences) of *E. purpurea* and *E. pallida* (*sensu* McGregor) in chapter 8 spurred further anti-fungal investigations using other *Echinacea* species and varieties. Chapter 9 addresses the UV light-mediated anti-fungal activities of root extracts from wild prairie populations of the revised *Echinacea* taxa. The bioassays included *Candida* spp. with amphotericin B resistance, as well as some filamentous fungal pathogens.

Introduction

The predominant *Echinacea* of the North American prairie, *E. pallida* var. *angustifolia* (*E. angustifolia* DC. *sensu* McGregor 1968), has been reported as a systemic and topical anti-microbial, among a host of therapeutic uses from North American Native traditions (Hart 1981; Kindscher 1989; Shemluck 1982) and Eclectic medicine (reviewed in Foster 1991; Hobbs 1989, 1994). Many of these applications suggest therapeutic antifungal appplications of prairie *Echinacea* species roots as phytomedicine. Commercial phytomedicines are predominantly hydroalcoholic preparations from the roots of *E. pallida* var. *angustifolia.* In contrast, the study of antifungal biological activity from *Echinacea* extracts has been limited to *E. purpurea* (L.) Moench until a preliminary investigation (chapter 8) which also included the eastern prairie variety, *E. pallida* var. *pallida* (*E. pallida* (Nutt.) Nutt.). *E. purpurea* exhibited significant clinical reduction of recurrent candidiasis using oral and injectible preparations (Coeugniet and Kuhnhast, 1986; Lasch *et al.* 1983 in Hobbs 1994). Topical effects of *E. purpurea* against fungal skin and urogenital infections were also determined by a few clinical studies of questionable validity according to the current model of double-blinded, controlled trials with standardized phytomedicines (reviewed in Hobbs 1989; 1994).

Candidiasis is a prevalent microbial infection that is especially common in immuno-compromised AIDS patients, diabetics, pregnant women and neonates. The degree of recurrence is very high, and clinical isolates increasingly reveal resistant *Candida* strains. Evidently, alternatives to conventional antifungal drugs, such as amphotericin B, are in demand. Amphotericin B is an amphoteric polyene that alters the permeability of fungal cellular membranes by inhibition of synthesis of ergosterol, the fungal equivalent of cholesterol in animal and bacterial cell membranes (Law *et al.* 1997). *Echinacea* phytomedicines are good candidates for alternative antifungal therapy, especially considering their long history of clinical use.

This study was conducted to investigate the biological activity of prairie *Echinacea* varieties against clinical pathogenic filamentous fungi and yeasts, including resistant strains of *Candida* spp. According to the current taxonomic revision, seven morphological varieties with demonstrated phytochemical differences (chapter 5) were investigated from the two prairie species, *E. pallida* and *E. atrorubens* (chapter 2). These wild accessions reflected the range of plants used traditionally as antimicrobials according to habitat and geographic data in Native and Eclectic medical references to *E. angustifolia* (Foster 1991).

Materials and Methods

Plant materials and extracts

All plants were grown from wild germplasm accessions and voucher specimens are deposited at the Department of Agriculture Ottawa Herbarium, Ottawa, Canada (DAO) (Table 9.1). Entire, fresh *Echinacea* roots were extracted in 95% ethanol (approx. 1g fresh weight/10mL) and filtered via Buchner filtration. In a separatory funnel, n-hexanes were added to the filtrate in a 1:1 ratio, followed by distilled water at half the volume of n-hexanes. The hydrophilic fraction was re-extracted three times and the lipophilic fractions in n-hexanes were pooled. Each n-hexane fraction was rotary evaporated to a standard concentration (usually 0.5 g/mL by dry root weight, with a few exceptions).

Fungal cultures

Fungal strains, including both yeasts and filamentous types, were chosen because they were opportunistic human skin or systemic pathogens (see Table 9.2). Among them are the causative organisms in diseases such as candidiasis (*Candida shehata*, *C. albicans* from the Ottawa General Hospital, Ottawa, Canada and amphotericin B-resistant strains of *C. albicans*, CN1A and D10, from N.D. Lees, Ottawa, Canada), cryptococcosis (*Cryptococcus neoformans* from the Ontario Ministry of Health, Ottawa, Canada), and dermatophytic infections such as ringworm (*Microsporum gypseum* and *Trichophyton mentagrophytes* from the Ontario Ministry of Health, Ottawa, Canada). Each culture was maintained at 4^0C and sub-cultured at 30^0C 24 hours prior to testing, to obtain test colonies in their optimal growth phase.

Disc susceptibility testing

The disk susceptibility test was chosen because it is a validated bioassay for testing antifungal natural products (Hadacek and Greger 2000) and has been used in similar trials with *Candida albicans* (Law *et al.*1997). All yeasts were uniformly streaked by sterile cotton swabs onto sterile Sabouraud's agar medium (DIFCO 10 g/L neopeptones + 20g/L dextrose and 15 g/L agar) in 10 cm diameter petri plates. *C. albicans* strains with resistance to amphotericin B were identified *a priori* by comparison of inhibition zones to control fungi known to be susceptible to this compound, using the method of Law *et al.* (1997). Inocula of filamentous fungal cultures were prepared by excision of a 5 cm^3 block of agar containing actively growing mycelium which was blended in 20mL liquid (19 mL distilled water and 1mL LB broth) for 1.5 mins. in a Waring blender. The fungal cell suspension was micropipetted (100 μL) onto Sabaraud's medium in a petri plate (prepared as above) and spread with a sterile bent glass rod. n-Hexane root extracts were added to sterile filter paper discs (Whatman no. 3, 0.5 cm diameter) and allowed to air-dry (20mg of extract per disc) before application to inoculated petri plates (n=3 discs per plate). Plates were sealed with parafilm and irradiated with near-UV light (300-400 nm, Westinghouse blacklight blue bulbs F20T12/BLB, 0.1 W/m^2) and visible cool-white (80 W/m^2), while a duplicate set of plates (no UV) was immediately wrapped in tinfoil at room temperature. Ten μL of 1 mg/mL α-terthienyl in 75% methanol and 40 μL of n-hexane were applied to three filter discs each, and plated separately in duplicate to be used as positive and vehicle controls in both light treatments. Manipulation of fungal cultures was conducted with a Biological Containment Hood (BioKlone 2, Microzone, Nepean, ON, Canada). All plates were incubated at 30^0C in the dark for 48 hours prior to examination and measurement of inhibition zones. The diameter of each zone of clearing was measured in millimeters, and the 5 mm disc diameter subtracted from the amount.

High Pressure Liquid Chromatography

Freshly prepared n-hexane extracts were filtered (0.2 µm, nylon) prior to HPLC separations using a validated method (Bergeron *et al.* 2000). Lipophilic chromatography was achieved using a solvent system of acetonitrile : H_20, at a flow rate of 1.0 mL/min following a linear gradient of 40-80% acetonitrile over 15 minutes. Samples of 5 µL were injected on a 7.5 cm reverse phase C-18 column (3 µm particle size). Compounds were detected at 210 nm and 260 nm and identified by comparison with reference standards. Standards of alkamides **1**, **8+9**, **11** and **18** were isolated previously in this laboratory by column chromatography on silica gel (Bergeron *et al.* 2000). Standard purity was tested by ^1H and ^{13}C-NMR, EI-MS for all of the above, and their respective spectra conformed with reported values (Perry *et al.* 1997; Yasuda *et al.* 1981). All other compounds were identified through comparison to previously-reported retention times (relative to **8+9**) and online diode array UV spectral profiles (Bauer and Remiger 1989). Quantities were expressed as a percentage of the amount of pure standard (mg/g dwt.) in each root extract.

Statistical analyses

Mean zones of inhibition (including SEM) were calculated for each fungal organism and *Echinacea* root extract combination. Comparison between treatment means (with and without UV irradiation) was conducted using paired t-tests (SPSS 1997). Statistics were limited to comparisons only within the same trial. The variation between trials was evidenced by relative activities of the positive control alpha-terthienyl, which limited the comparison of mean zones measured for the same species and/or variety.

Table 9.1. *Echinacea* accessions identified according to a revised taxonomy (chapter 2). All specimens deposited at DAO.

Echinacea variety	Voucher label
E. pallida var. *angustifolia*	EA312814, EA421331, EA008
E. pallida var. *tennesseensis*	ET22010
E. pallida var. *simulata*	ES017
E. pallida var. *sanguinea*	ESA23873, ESA23878
E. pallida var. *pallida*	EPA23920, EPA013, EPA0823
E. atrorubens var. *atrorubens*	EAT23881
E. atrorubens var. *neglecta*	EPN005, EPN23886

Table 9.2. Fungal pathogens against which *Echinacea* extracts were tested for UV light mediated anti-fungal activity. * denotes amphotericin B-resistant strains, [1] denotes filamentous fungi.

Ca	*Candida albicans*
Cs	*Candida shehata*
Cn	*Cryptococcus neoformans*
Fo	*Fusarium oxysporum* [1]
Pb	*Pseudallescheria boydii* [1]
Tt	*Trichophyton tonsurans* [1]
Tm	*Trichophyton mentagrophytes* [1]
Mg	*Microsporum gypseum* [1]
CN1A *	*Candida albicans*
D10 *	*Candida albicans*

Results and Discussion

Table 9.3 is a summary of antifungal activity from all trials after statistical pairwise t-tests of significance were performed. This assay technique is not quantitative, but useful for comparative purposes between plant and fungal varieties (Hudson *et al.* 1993).

E. pallida var. *sanguinea* and *E. pallida* var. *simulata* demonstrated UV light-mediated toxicity to the majority of fungi tested. *Trichophyton mentagrophytes* was the most susceptible to root extracts of *Echinacea pallida* var. *pallida*, *E. pallida* var. *sanguinea* and *E. pallida* var. *simulata*, as well as *E. atrorubens* var. *atrorubens* in the presence of UV light ($p<0.05$) (Table 9.3). A related fungal species of *Trichophyton*, *T. tonsurans*, was only inhibited by root extracts of the species *E. atrorubens* (var. *atrorubens* and var. *neglecta*) (Table 9.3). *Microsporum gypseum* was significantly inhibited by *E. pallida* var. *simulata* in the presence of UV light ($p<0.05$). Finally, *Pseudallescheria boydii* and *C. neoformans* were also moderately inhibited by extracts of *E. pallida* var. *sanguinea* and *E. pallida* var. *simulata* in the presence of UV light (not significant, $p> 0.05$).

Phototoxicity to strains of *Candida* spp. which are resistant to amphotericin B was significant from root extracts of *E. pallida* var. *sanguinea*, *E. pallida* var. *pallida* and also from *E. atrorubens* var. *atrorubens* (not significant $p> 0.05$). Resistant strains were cultured with difficulty, which limited the number of successful trials of many *Echinacea* extracts (Table 9.3).

Conventional (UV-independent) antifungal activity against *T. mentagrophytes* was observed with extracts of *E. atrorubens* var. *neglecta* ($p<0.05$), and *E. atrorubens* var.

atrorubens (not significant, $p>0.05$) (Table 9.3). *E. pallida* var. *tennesseensis* demonstrated moderate conventional antifungal activity against *Candida albicans* and *E. atrorubens* var. *atrorubens* significantly inhibited the growth of *Microsporum gypseum* in the absence of UV-light ($p<0.05$) (Table 9.3).

Polyynes from the Asteraceae are known photo-activated toxins with biological activity against fungi (Camm *et al.* 1975; Towers, 1997) and other microorganisms (Hudson *et al.* 1991; McLachlan *et al.*1986). The quantity and diversity of polyn/enes in the current *Echinacea* extracts may be partly responsible for increased UV light-mediated antifungal activity (Table 9.4). Mechanisms of phototoxicity determined for similar straight-chain polyynes from the Asteraceae specify interactions with, or energetic modifications of molecular 0_2, causing free radical damage to cell membranes in the target organism (Towers *et al.* 1997).

Among those extracts which demonstrated significant UV light-mediated toxicity in the current bioassays, *E. pallida* var. *pallida*, *E. pallida* var. *simulata*, and *E. atrorubens* var. *neglecta* all contained elevated levels of polyn/enes in relation to the major compounds **8+9** (Table 9.4). Also, the combination of very high **8+9** levels, together with some polyn/enes, in *E. pallida* var. *sanguinea* root extracts showed some of the most effective UV light-mediated antifungal action against the tested clinical pathogens (Table 9.3). Phototoxic activity against *C. albicans*, *C. shehata* and *F. oxysporum* was minimal or absent from the *Echinacea* root extracts tested (Table 9.3).

Root extracts of *E. pallida* var. *angustifolia* and *E. pallida* var. *tennesseensis* were not significantly phototoxic to any strains (Table 9.3). Both of these varieties have very similar phytochemical root profiles with a richness and diversity of alkamides, but very few polyn/enes, if any (Table 9.4; chapter 5). The major *Echinacea* alkamide **8+9** was shown previously to have significant light-mediated toxicity against *Saccharomyces cerevisiae* (chapter 8). High alkamide concentrations in *E. pallida* var. *tennesseensis* extracts may explain its conventional antifungal actions against *C. albicans* in Table 9.3, compared to the light-enhanced phototoxicity discussed above. Also, moderate inhibition of *Cryptotoccocus neoformans* and *Fusarium oxysporum* by both *E. pallida* var. *tennesseensis* and *E. pallida* var. *angustifolia* (Table 9.3) suggested that *Echinacea* root alkamides contributed to light-enhanced antifungal activities. An example of mean zones of fungal growth inhibition from a few representative trials conducted for the current study can be found in the Appendix 3.

Future antifungal bioassays should include: testing purified polyn/enes and alkamides from *Echinacea*, testing standardized phytomedicines, and performing large, comprehensive trials to increase comparability and statistical power.

According to this preliminary study, the most useful varieties for topical and systemic anti-fungal applications in *Echinacea* phytomedicines were *E. pallida* var. *simulata*, *E. pallida* var. *sanguinea*, *E. pallida* var. *pallida*, *E. atrorubens* var. *atrorubens* and *E. atrorubens* var. *neglecta*. Of these varieties, *E. pallida* var. *tennesseensis* and *E. atrorubens* var. *neglecta* are rare and protected, while the only one currently cultivated for commercial use is *E. pallida* var. *pallida*. Antifungal activities from these prairie *Echinacea* species, combined with those of the previous chapter, provide preliminary *in vitro* support for the traditional anti-microbial uses in the Native American Ethnobotanies and Eclectic medical literature. Further investigation with *Echinacea* extracts and phytomedicines against amphotericin B-resistant yeasts should be undertaken, leading to potential clinical applications.

Table 9.3: UV light-activated toxicity of *Echinacea* variety root extracts to fungal strains (see Table 9.2). Nomenclature to indicate fungal inhibition follows Camm *et al.* (1975).

	E. pallida					*E.atrorubens*	
Fungi	var. *angustifolia*	var. *tennesseensis*	var. *pallida*	var. *sanguinea*	var. *simulata*	var. *atrorubens*	var. *neglecta*
Ca	-	anti *	-	-	-	+	+
Cs	+	nt	nt	+	+	+	anti
Cn	+	+	+	+ *	+ *	+	+
Fo	+	+	-	-	+	+	-
Pb	nt	nt	-	+	+ *	-	-
Tt	nt	nt	nt	+	-	+ *	+ *
Tm	nt	nt	+ *	+ *	+ *	+ anti	anti *
Mg	nt	nt	nt	+	+ *	anti *	+
CN1A[1]	-	nt	+	+ *	nt	+	nt
D10[1]	nt	nt	+ *	nt	nt	nt	nt

Notes:

+	phototoxic
anti	conventional antifungal activity (dark)
+ anti	conventional antifungal activity enhanced in UV light
-	no measurable inhibition
nt	not tested or n/a
*	significant differences between treatment means, by paired t-test ($p<0.05$)
[1]	*Candida albicans* amphotericin B-resistant strains

Table 9.4. Alkamides and polyn/enes in n-hexane extracts of *Echinacea* spp. expressed as % of standard **tetraenes 8+9** (mg/g dwt). Compounds numbered as in Bauer and Remiger (1989).

	E. pallida					*E. atrorubens*	
Compound	var. *angustifolia*	var. *tennesseensis*	var. *pallida*	var. *sanguinea*	var. *simulata*	var. *atrorubens*	var. *neglecta*
1	4.29	4.43	20.2	4.18	12.6	5.44	6.86
2	5.99	5.90	167	1.67	219	2.63	58.4
3	13.6	14.8	53.0	16.3	58.9	8.73	20.4
4	0.24	12.2	2.27	0.23	0.00	0.07	0.00
5+15	6.94	10.0	18.39	1.58	0.00	15.95	6.10
6	0.48	16.1	36.5	2.34	0.00	3.31	9.40
7	2.43	12.2	84.1	1.49	0.00	1.74	47.0
8+9	**9.40**	**1.40**	**0.33**	**17.64**	**0.25**	**6.88**	**0.64**
10	13.6	12.1	7.47	4.18	8.51	5.78	6.89
11	23.1	7.04	7.37	2.41	0.00	6.12	8.30
12	20.8	149	11.0	19.1	0.00	18.2	0.00
13	23.7	424	0.72	12.5	0.00	0.46	0.00
14	15.1	54.8	4.46	23.3	47.6	12.6	0.00
16	1.93	49.1	14.7	1.26	0.00	2.94	29.4
17	2.56	20.3	16.3	6.81	0.00	0.32	0.00
18	6.25	3.20	113	11.6	79.0	9.96	54.7
19	1.16	0.00	18.6	1.56	0.00	1.03	0.00
22*	0.00	0.00	98.2	0.00	85.4	0.00	0.00
24*	0.62	0.00	230	0.68	181	1.16	843
25*	0.58	0.00	223	0.18	59.2	0.23	35.3
poly**	2.52	0.00	157	0.83	50.0	2.89	0.00
Total Peak Area	641	197	182	1036	80	401	268

*polyn/enes; ** previously unreported peaks determined as polyn/enes by online UV spectra

CHAPTER 10: ANTIVIRAL ACTIVITY OF *ECHINACEA* SPP. EXTRACTS AGAINST *HERPES SIMPLEX* VIRUS

This chapter was prepared for submission as a note in the journal Planta Medica, in collaboration with Dr. J. Hudson and Dr. J.T. Arnason.

Introduction

Echinacea preparations are common oral treatment for colds and flu, but they are also indicated for infections and topical conditions such as candidiasis, strep throat, staphylococcus infections, infected wounds, skin ulcers and burns (Hobbs 1994). At least three clinical trials in Europe showed positive therapeutic results using pressed juice of *E. purpurea* (L.) Moench aerial parts as a topical antibiotic in the oral cavity and gynaecologically (Hobbs 1994). Hydroalcoholic root extracts of *Echinacea* spp. have not yet been tested clinically for topical antimicrobial activity. Traditionally, roots were used by native healers for treatment of mouth sores, toothaches, sore throats, wounds and boils and upper respiratory tract infections (Hart 1981; Kindscher 1989; Moerman 1998; Shemluck 1982). Mouth sores, possibly caused by *Herpes simplex* virus (HSV), and infections of the oral cavity received the most frequent mention in ethnobotanical reports of *Echinacea* root use (Moerman 1998).

Whole plant extracts of *E. purpurea* (L.) Moench were shown to protect cell lines against herpes, influenza and vesicular stomatitis viruses (VSV), possibly as a result of interferon induction in the experimental cells (Wacker and Hilbig 1978). In a similar bioassay, three phenolic compounds from *Echinacea* were determined to be antiviral agents against VSV (Cheminat *et al.* 1988). Other members of the Asteraceae have been shown to have potent antiviral activities which are activated by light (Hudson and Towers 1999). The current study was therefore undertaken to determine the light-activated antiviral potential of root extracts of *Echinacea* spp. Three different phytochemical fractions were prepared for eight *Echinacea* species or varieties (including rare wild germplasm). For a preliminary comparison to the preparations which use aerial parts of *Echinacea* spp, one crude inflorescence extract (*E. pallida* Nutt. var. *sanguinea* Gandhi and Thomas) was included.

Material and Methods

Plant material

Wild-harvested and cultivated *Echinacea* spp. seeds were germinated (see protocol chapter 5), potted in 5:3:2 vermiculite:Promix:quartz sand (industrial grade #10, 4mm particles) and grown in a controlled environment greenhouse at 25 ^{0}C under a 16-hour light regime. *E. laevigata* (voucher label: EL98-0924), *E. purpurea* (EP-COM2), and *E. pallida* (EPA0823) were 7 months old at harvest. *E. angustifolia* (EA-COM1) and two accessions of *E. tennesseensis* (ET98-0926; ET98-0611) were aged 5.5 months. Voucher specimens were deposited in the Department of Agriculture Ottawa Herbarium, Ottawa, Canada (DAO).

Extraction and Chromatography

10-15 roots from each of the 6 *Echinacea* populations were washed, dried whole at room temperature and ground into bulk samples. They were then extracted in 70% ethanol (1g dry wt./10 mL) for 24 hrs under moderate mechanical agitation (70 rpm). Ethanolic extracts were filtered (#1 Whatman, Buchner funnel) and the entire volume fractionated three times with equal parts hexanes: water. Hexane fractions were pooled and hydroalcoholic fractions were fractionated with an equal volume of ethyl acetate. During the second separation into ethyl acetate (which was subsequently pooled with the first), the mixture was adjusted to pH < 2.5 for maximal separation of cichoric acid from the hydroalcoholic mixture. Therefore, all phytochemicals from the original ethanolic extract were separated into: 1) the lipophilic fraction containing the alkamides and ketoalken/ynes (hexane), 2) the phenolic fraction containing cichoric acid and other caffeic and quinic acid derivatives (ethyl acetate) and 3) a remaining hydroalcoholic remaining fraction which was discarded. All fractions were concentrated *in vacuo* at 30^{0}C in a rotary evaporator (hexanes not to dryness), redissolved in the appropriate fresh solvent to 0.05 g/mL by dry weight and filtered with 0.2 μm nylon membranes prior to HPLC separation.

A second set of 70% ethanolic extracts (0.05 g/mL) were made from the same 6 batched samples of *Echinacea* root powder using a rapid ultrasonic extraction method (Bergeron *et al.* 1999), and filtered prior to HPLC (0.2 μm nylon membranes).

Chromatography by HPLC was achieved using a solvent system of acetonitrile:water (6:4) at 1 mL/min and 5 μL injection on a 7.5 cm reverse phase C-18 column (3 μm particle size). Lipophilic compounds were detected at 210 nm and 260 nm. An internal standard **8+9**, isolated in our laboratory (Bergeron *et al.* 2000), provided a reference by which all previously reported compounds were identified using retention times and online UV spectra (Bauer and Remiger 1989).

Antiviral bio-assay

The following method is a modified version of the procedures described in Marles *et al.* (1992). Vero cells (monkey kidney cell line, American Type Culture collection) were grown in Dulbecco's Modified Eagle Medium (MEM) containing 5% fetal bovine serum (cell culture reagents obtained from GIBCO Life Sciences, Ontario), in 96-well microtest trays (Falcon), with 0.1 mL well volume.

Each *Echinacea* plant extract was diluted 1:20 in MEM and filtered through a sterile syringe of 0.2 nm pore diameter. This filtrate was the starting test material, equivalent to 25 mg/mL of extract in <2% ethanol.

Two-fold serial dilutions of the extracts were made in MEM without serum (100 µL into 100 µL MEM) across a row of wells in an empty 96-well microtest tray (in duplicate), with the aid of a multipipetting device. Then 100 µL of HSV-1 (BC Center for Disease Control, Vancouver, Canada) comprising 100 plaque-forming units (pfu) in MEM without serum was added to each well. Immediately, the tray was transferred to an environmental chamber at 30^0C and exposed to a combination of visible light (5 W/m^2 of incident fluorescent and incandescent radiation) and long-wave ultraviolet light (5 W/m^2 UVA, 300-400nm) for 30 min. with continuous gentle shaking of the tray. Each diluted extract volume was then transferred by multipipettor to aspirated Vero cell monolayers in another 96-well tray, which was returned to the cell culture incubator. There were two types of control cells: those with no virus and no plant extract, and those infected with virus, but without plant extract added.

Cultures were inspected periodically under the microscope for viral characteristic cytopathic effects (cpe). Complete cell destruction (100% viral cpe) required 4 days for infected control cells (those not treated with *Echinacea* extracts). Partial inactivation of the virus was indicated by substantially less than 100% cpe (*i.e.* 50% or fewer cells showing cpe). A complete inactivation of virus by treatment was indicated by total absence of cpe with a given volume of plant extract. This volume, an average of three replicates (n=3), was determined to be the minimum inhibitory concentration (MIC) for that particular plant extract.

Antiviral bio-assays were performed by Dr. J.B. Hudson at the Department of Pathology, University of British Columbia, Vancouver, Canada.

Results and Discussion

The present study determined that *Echinacea* spp. root and inflorescence extracts were capable of inhibiting the growth of Type 1 *Herpes simplex* (HSV) virus *in vitro* when exposed to visible and UVA light. Antiviral activity varied according to plant species and variety, as well as by phytochemical fraction of the crude extracts (Table 10.1).

E. pallida var. *sanguinea* crude (70% ethanol) inflorescence extract (MIC = 0.026 mg/mL) and *Echinacea purpurea* n-hexane root extract (MIC = 0.12 mg/mL) were the most potent inhibitors of HSV. The MIC of *E. pallida* var. *sanguinea* crude inflorescence extract (0.026 mg/mL) was comparable to the MICs (0.002-0.115 mg/mL) of crude aerial ethanolic extracts of the West African phytomedicine, *Momordica charantia* (Cucurbitaceae), determined against HSV using the same method as the current study (Beloin 1998). In the case of *M. charantia*, the pure compound Momordicin, was much less active than the crude extracts (MIC > 0.25 mg/mL) (Beloin 1998). Crude plant extracts are often more potent viral inhibitors than single pure compounds suspected as responsible for the activity (Hudson and Towers 1999).

The *Echinacea* phytochemical families most likely responsible for antiviral activity were implied through comparison of root and inflorescence MICs, in conjunction with the results of HPLC phytochemical profiles for each extract. The superior antiviral activity of the hydroalcoholic inflorescence extract can be associated with phenolics, which were present in significantly higher amounts than found in the root extracts (Table 10.1). For example, cichoric acid, a phenolic shown to have antiviral activity (Cheminat *et al.* 1988) was highest in *E. pallida* var. *sanguinea* inflorescences (Fig. 10.1). Additional activity in the inflorescence extract may be attributed to the high concentration of the ketoalkyne **24**, or the better than average concentration of alkamides **8** and **9**.

Phytochemical profiles of the root extracts included phenolics, alkamides and ketoalken/ynes that varied among *Echinacea* species and fraction tested (Table 10.1). There was no correlation of inhibitory activity of *Echinacea* extracts (MIC against HSV) with higher measured levels of any phytochemical family or single compounds. However, among the seven tested root varieties of *Echinacea* spp., the lowest MICs and therefore highest antiviral activity were found almost consistently from n-hexane fractions containing alkamides and ketoalkyn/enes (with the exception of *E. laevigata*, see Table 10.1).

The mechanism of HSV inhibition by heterogeneous fractions of *Echinacea* spp. extracts presented here may be similar to that of the phototoxic polyacetylene phenylheptatriyne (PHT), which is also known to inactivate membrane-bound herpes viruses at concentrations as low as 0.1 nM, as well as being isolated from *Bidens pilosa*, another member of the Asteraceae (Hudson and Towers 1999). This would explain the lack of antiviral activity observed by May and Willuhn (1978) using *E. pallida* var. *angustifolia* aqueous root extracts. However, the current study exhibited superior antiviral activity from caffeic acid derivatives in the crude (70% ethanol) inflorescence extract containing both hydrophilic and lipophilic components (Fig. 10.1; Table 10.1), which indicates either synergism among phytochemical classes (reported in some cases, Hudson and Towers 1999) or another mechanism of action.

The mode of antiviral action for phenolic compounds, widespread among medicinal plants, may be linked to their reported cytoprotective actions. Studies with pure cichoric acid and 3,5-dicaffeoylquinic acid (related to the *Echinacea* compound 3,5-*O*-

dicaffeoylquinic acid or cynarin) demonstrated inhibition of HIV-1 integrase and HIV-1 replication in tissue culture (Robinson *et al.* 1996). In the current bioassay, cichoric acid inhibited HSV-I at 45 μg/mL, and it is part of an ongoing study of pure *Echinacea* phenolics (Hudson unpubl. results). This may be due to protective action on the cells in question, which was previously shown for both caffeic acid and chlorogenic acid; they were antioxidants of low density lipoprotein in a time-dependent manner (Yamanaka *et al.* 1997) and antimutagenic in a *Salmonella typhimurium* system (Yamada and Tomita 1996). Furthermore, cichoric acid protected against degradation of collagen by free radical scavengers (Facino *et al.* 1995). However, the antiviral effect of caffeic acid derivatives from *Echinacea* may be dose-dependent, since caffeic acid derivatives were reported to be slightly cytotoxic to host cells in a similar system at levels 100 fold greater than antiviral concentrations (Robinson *et al.* 1996). Cheminat *et al.* (1988) suggested cytotoxic effects were due to interaction with cellular DNA metabolism.

We conclude that high phenolic concentrations, particularly cichoric acid in inflorescences, as well as the ketoalken/yne **24** and alkamides **8** and **9**, which were more concentrated in the roots, may be associated with inhibitory actions of *Echinacea* spp. extracts against HSV. The current findings support previously-reported antiviral actions of *Echinacea* spp. phenolics, as well as novel antiviral activity for lipophilic constituents *in vitro*. Pure compounds from *Echinacea* spp. are under investigation as potential inhibitors in the current HSV-1 bioassay, and should be tested in other systems. Topical antiviral clinical activity in *Echinacea* phytomedicines is worthy of further investigation, with special attention to *Echinacea* aerial parts and the phytochemical differences which may be attributed to germplasm, cultivation and processing conditions.

Table 10.1. Minimum inhibitory concentrations (MIC in mg/mL) of three fractions of *Echinacea* spp. root extracts to completely inhibit 100 infectious Type 1 *Herpes simplex* (HSV) virus particles. Fractions (Frc) of crude 70% ethanolic root extracts (et) were separated into lipophilic n-hexanes (hx) and hydrophilic ethyl acetate (ac). "n/a" indicates not active at >25 mg/mL. "."denotes compound class not soluble in that fraction. n = 3 for each extract. * denotes inflorescence extract.

Species and Variety	Frc	MIC	Caffeic acids	Keto-alkenes	Diene amides	Monoene amides
		mg/mL	total % dry wt.			
E. pallida var. *sanguinea**	et	< 0.026	7.68	0.43	0.06	0.05
E. purpurea	hx	0.12	.	0.00	1.89	0.00
E. pallida var. *angustifolia*	hx	0.2	.	0.01	0.22	0.22
E. pallida var. *pallida*	hx	0.2	.	0.29	0.03	0.00
E. pallida var. *tennesseensis*	hx	0.5	.	0.25	0.37	1.25
E. atrorubens var. *atrorubens*	et	0.625	0.01	0.03	1.23	0.73
E. pallida var. *angustifolia*	et	0.78-1.04	1.56	0.01	0.22	0.21
E. pallida var. *tennesseensis*	ac	1.5-25	1.60	.	.	.
E. purpurea	et	1.56	1.90	0.00	0.78	0.00
E. pallida var. *pallida*	et	1.56	1.28	0.24	0.02	0.00
E. purpurea	ac	2.5	5.96	.	.	.
E. pallida var. *angustifolia*	ac	3	12.89	.	.	.
E. laevigata	hx	3	.	0.00	0.43	0.17
E. laevigata	ac	3	5.29	.	.	.
E. atrorubens var. *paradoxa*	et	3	4.32	1.92	0.03	0.03
E. laevigata	et	3.13-4.7	2.99	0.01	0.62	0.34
E. pallida var. *tennesseensis*	et	3.13	1.21	0.01	0.35	1.18
E. pallida var. *pallida*	ac	n/a	10.48	.	.	.

Table 10.2. Predominant phytochemicals in *Echinacea* spp. n-hexane (hx) root extracts and one inflorescence extract (F)(in 70% ethanol) which demonstrated antiviral activity to Type 1 *Herpes simplex* virus. All compounds are previously-reported alkamides (Bauer and Remiger 1989) except ketoalken/ynes denoted by * and a caffeic acid derivative **.

E. purpurea	**2, 3, 7, 8/9, 10, 11**
E. pallida var. *pallida*	**18, 22***
E. pallida var. *angustifolia*	**8/9, 12, 13, 18**
E. pallida var. *tennesseensis*	**12, 13, 18**
E. pallida var. *sanguinea*	**13, 16, 18, 24*** (to 0.4% dwt)
(F)	**cichoric acid**** (to 6.3% dwt)

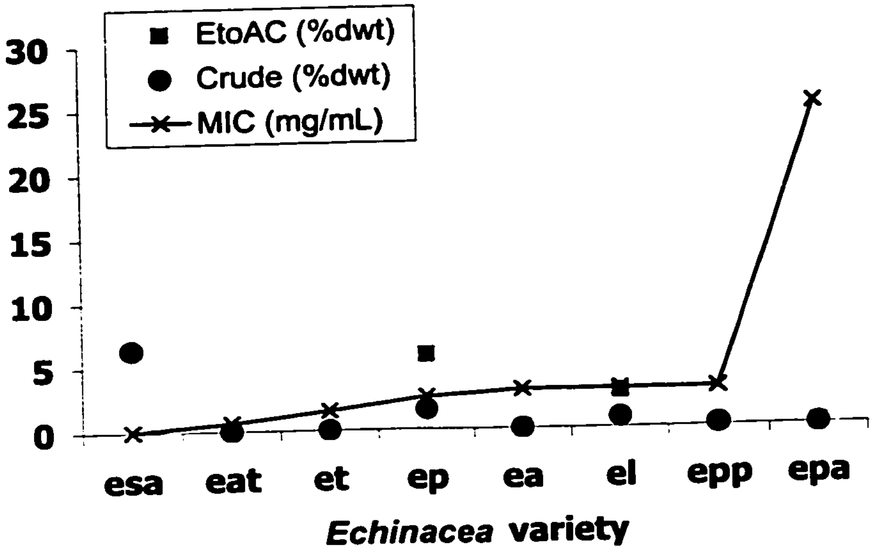


Figure 10.1: Cichoric acid content (% dry weight) and Minimum Inhibitory Concentrations (MIC in mg/mL) of *Echinacea* extracts from different solvents and varieties. EtOAc is the solvent ethyl acetate and crude refers to the original 70% ethanol extracts. The varieties of *Echinacea* were as follows: **et** = *E. pallida* var. *tennesseensis*, **ep** = *E. purpurea*, **ea** = *E. pallida* var. *angustifolia*, **el** = *E. laevigata*, **epa** = *E. pallida* var. *pallida*, **esa** = *E. pallida* var. *sanguinea*, **eat** = *E. atrorubens* var. *atrorubens*, and **epp** = *E. atrorubens* var. *paradoxa*. See chapter 2 for the revised taxonomic nomenclature. MIC should be inversely correlated with % dwt to show that cichoric acid is partly responsible for the antiviral activity of an extract.

CHAPTER 11: GENERAL DISCUSSION AND CONCLUSIONS

This study was the first comprehensive examination of wild plant variation within and between populations throughout the range of the genus *Echinacea*. From 220 different wild populations across the range of each putative *Echinacea* taxon (*sensu* McGregor 1968), fresh and dried plants were assessed by morphometric analysis to determine infrageneric taxa. The resulting morphological keys and descriptions of *Echinacea* presented here were more functional and less ambiguous than McGregor's (1968), which contained several inconsistencies and method bias (Baum *et al.* 1999). The description of functional infrageneric taxa in *Echinacea*, at the species-level and below, has implications in North America for the protection of natural ecosystems, and worldwide for the medicinal plant industry.

A "species" is the only category of organisms that is *consistently* recognized by all peoples of the world (Stuessy 1990). The concept of species, a much-debated topic, can be described as mechanistic, historical or phenetic (Luckow 1995; Levin 2000). Mayr's (1963) Biological Species Concept (BSC), the typical mechanistic concept, was found lacking for the study of sexually-reproducing plant populations in which reticulate evolution is common (Luckow 1995; Levin 2000). *Echinacea* species were delimited in the present study following the Phenetic Species Concept (Michener 1970). In 1990, Stuessy proposed that the Phenetic Species Concept was still the most operational for plants. According to this concept, organisms are assigned to species defined based on overall similarity that is calculated by simultaneous consideration of morphological variables. Overlapping ranges are acceptable for a given variable as long as there is a significant difference between mean similarities overall. Using this concept of morpho-taxa, which supports patterns of punctuational and ecotypic differentiation in plant populations, species may then be further evaluated using molecular, genetic, geographic, reproductive, ecological or chemical variables (Davis and Gilmartin 1985). Morphological identification of plants remains essential to ecology and economy.

In this study, after species were determined, cladistic analyses of the four revised *Echinacea* morpho-taxa indicated potential phylogenetic relationships (chapter 2). In cladistics, the units of investigation are species, which, by definition, share discrete, unique character states or unique combinations of character states. This phylogenetic concept of species (the PSC) supported the distinction of varieties below the level of species for those populations in the current study that differed by continuous variation, not discrete, unique combinations of character states. In a recent review, the PSC was not found to be the most useful to determine flowering plant species because it does not support widespread reticulation (Luckow 1995), but once species are delimited, it provides a guideline for the evaluation of interrelationships. Further support for the newly-determined *Echinacea* morpho-species was found in significant differences among patterns of phytochemical production.

Geographic and environmental traits were considered *a posteriori*, along with phytochemical characters to explain the results of morphometric revision and patterns of hybridization and potential speciation. For example, the species *E. purpurea* was placed in its own subgenus, *Echinacea*, by extensive morphological dissimilarity and this distinction was upheld by the phytochemical data (chapter 5). *E. purpurea* also inhabited a unique habitat in open woods (McGregor 1968) with less rockiness, more shade, and potentially higher soil nitrogen, acidity, and moisture than all other *Echinacea* types classified in subgenus *Pallida*.

Distribution of many plant species of the central Great Plains, U.S.A. is known to vary along a moisture gradient from the semi-arid shortgrass upland prairie to the tallgrass prairie, and to be correlated with soil nutrient variation (Vinton and Burke 1997). At certain points along the moisture gradient, and similar ecological gradations, identification of closed gene pools in *Echinacea* has proven difficult (Baum *et al.* 1999; McKeown 1999; McGregor 1968). Populations of *Echinacea* species and varieties which converge at intersections, such as between prairie types (short- and tallgrass) or upland cretaceous soils and nutrient-rich grasslands, may interbreed to produce hybrids. First generation hybrids between some varieties of *E. pallida* and *E. atrorubens* are known to be fertile and result in varying degrees of introgression.

Ecological zones of intersection between species create the necessary conditions for hybridization, and McGregor (1968; pers. comm.1998) identified one such zone as the "center of diversity in the genus *Echinacea*": the Arbuckle mountains to central and eastern Kansas and the western edge of the Ozarks. In this evolutionary "hotspot", McGregor (1968; pers. comm.1998) frequently reported populations with mixed ploidy levels, sterility and intermediate or introgressed characteristics. The current study provides the only comprehensive and statistical analysis to date, of population diversity that clearly supports McGregor's theory, although McKeown (1999) made similar field observations of hybrid swarms during her collections of the genus for the USDA.

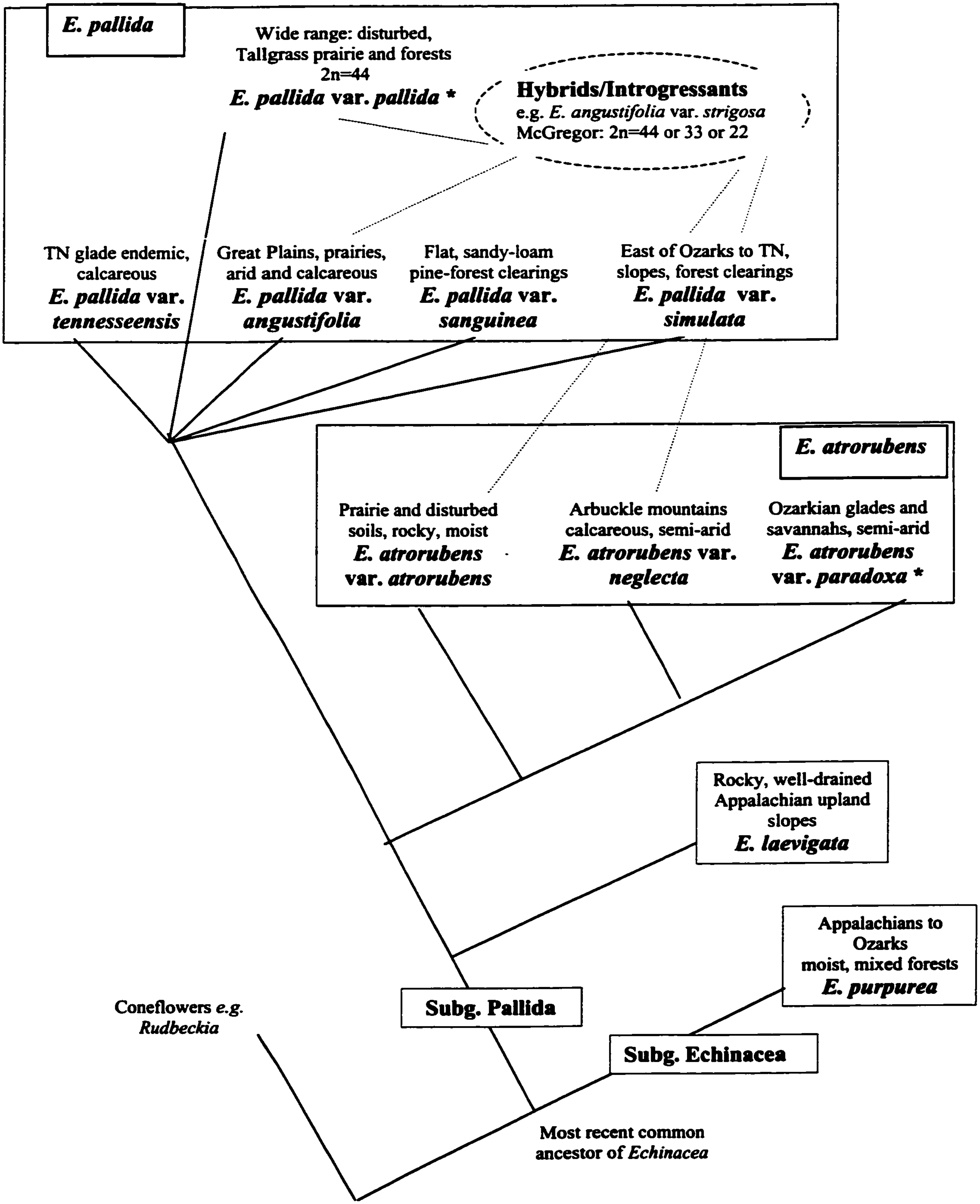
The apparent persistence through environmental changes shown by groups of *Echinacea* populations may be explained by its potential to interbreed (or hybridize) and differentiate into stabilized introgressants. A study in *Iris* (Liliaceae) found that hybridization was usually restricted to populations where flowering individuals of one species were in a quantitative minority (Arnold *et al.* 1991). Since this finding was also determined from an *Echinacea* relative, *Helianthus* (Asteraceae) (Reiseberg 1995), hybridization leading to stabilized introgression in native *Echinacea* populations may follow a similar mechanism. Study of another asteraceous relative, *Senecio*, indicated that stabilized tetraploid introgressants were not likely to result from triploid F_1 hybrids, but rather by backcrossing of F_1 tetraploids with a diploid parent (Lowe and Abbott 2000), which may also be the case for *E. angustifolia* DC. var. *strigosa* McGregor. The latter taxon was not recognized in the current treatment, except as a group of similar, stable introgressants, which may have arisen as tetraploids from the hybridization of *E. pallida* var. *angustifolia* and *E. atrorubens* var. *atrorubens*. Potentially, the extensive back-crossing with *E. pallida* var. *angustifolia* may have occurred until they became morphometrically indistinct from that putative parent (chapter 2). Artificial *Echinacea* hybrids displayed a mosaic of parental and intermediate characteristics and putative wild introgressants were not always distinguishable from either parent using morphological keys due to extensive introgression with either parent (McGregor 1968 and chapter 2).

The stable introgressant that McGregor called *E. angustifolia* DC. var. *strigosa* was restricted to overlapping regions of edaphic, climactic and other ecological characteristics, such as a border region between rocky outcrops in the Flint Hills of Kansas where they meet tallgrass prairie. The populations of putative *E. angustifolia* var. *strigosa* consisted of diploids, sterile triploids, and tetraploids (McGregor 1968), which were not distinguishable from *E. pallida* var. *angustifolia* in the current study (chapter 2). All other *Echinacea* species and varieties were found to be diploid (McGregor 1968), except for *E. pallida* var. *pallida*, which was identified as a vigorous tetraploid of putative allopolyploid origin. Polyploids, which form by chromosome doubling following hybridization events, are known to be common in plants, often from recurring or multiple origins (Werth *et al.* 1985). Tetraploidy of *E. pallida* var. *pallida* may have caused fixed heterozygosity for alleles that facilitated its adaptation to the tallgrass prairie and open woods where different soil nutrition engendered higher growth rates and subsequently distinct morphology and phytochemistry. The assemblage of interbreeding *Echinacea* populations among *E. atrorubens* and *E. pallida* varieties may be referred to as a "syngameon". The latter is "the most inclusive unit of interbreeding in a hybridising species group" or sympatric species (varieties here), which have typically divergent edaphic requirements, yet hybridize extensively and hybrids are partially fertile (Grant 1981). For example, groups of sympatric, hybridising white oaks (*Quercus alba* L.), which form partially fertile progeny in areas of ecological overlap, but maintain their distinction as species beyond the localized zones, have been reported as a syngameon (Levin 2000). A syngameon is therefore an extension of the concept of semispecies, which is a term to describe the introgressant relationship between two or more species, rather than as a category subordinate to species (Willmott 1949). Further cytogenetic research of wild populations is the next logical step for the understanding of evolutionary adaptation in *Echinacea*, especially a close investigation of the apparent syngameon of *E. atrorubens* and *E. pallida* varieties in the center of diversity.

Echinacea in the grassland ecosystem occupies an intermediate prairie plant guild, as a result of its intermediate characteristics between grasses and shade-intolerant perennial forbs and woody species. With a long taproot, the arid-adapted *Echinacea* varieties (*E. atrorubens* and *E. pallida*) have the ability for underground carbon and nitrogen storage that permits compensatory growth in response to herbivory, similar to the adaptive strategy of graminoids in the same habitat (Bryant *et al.* 1983). However, most *Echinacea* varieties also contained elevated levels of defensive nitrogen-containing alkamides in juvenile growth when they were cultivated (chapters 5, 6, 7 and Appendix 4), showing the characteristic strategy of shade-intolerant woody species.

Treeless grasslands are a recent geological development in North America. Inhabitants of this east-west moisture gradient from the tallgrass prairie to shortgrass communities of the high, arid plateau include *E. pallida* and *E. atrorubens*, with their associated varieties. In accordance with the recent geological differentiation of the grasslands, these two species occupied the "most-recently derived" positions on the cladogram in chapter 2. In contrast, the same figure supported *E. purpurea* and *E. laevigata* as more primitive species. They are currently confined to the uplands east of the Alleghenies from Georgia to Pennsylvania, with *E. purpurea* extending west to the Ozarkian upland by way of southern Illinois. Sharp (1935) identified the Appalachian-Ozarkian upland to be the origin of the *Echinacea-Rudbeckia* complex. Expanding on Sharp's hypothesis with the results of the current study, a natural history approach to speciation of the current morphological taxa is discussed and summarized in an hypothesized phylogeny (Fig. 11.1).

Figure 11.1. An hypothesized phylogeny of the genus *Echinacea* Moench which was synthesized from the results of the current study together with ecological, geographical, cytogenetic and historical data. Branch nodes indicate putative ancestors and branch lengths are not indicative of evolutionary distance or dissimilarity indices. * indicates sympatric populations. 2n = indicates the ploidy of regular somatic cells in plants studied by McGregor (1968).


E. pallida
Wide range: disturbed, Tallgrass prairie and forests 2n=44
E. pallida var. pallida *
Hybrids/Introgressants
e.g. E. angustifolia var. strigosa
McGregor: 2n=44 or 33 or 22
TN glade endemic, calcareous
E. pallida var. tennesseensis
Great Plains, prairies, arid and calcareous
E. pallida var. angustifolia
Flat, sandy-loam pine-forest clearings
E. pallida var. sanguinea
East of Ozarks to TN, slopes, forest clearings
E. pallida var. simulata
E. atrorubens
Prairie and disturbed soils, rocky, moist
E. atrorubens var. atrorubens
Arbuckle mountains calcareous, semi-arid
E. atrorubens var. neglecta
Ozarkian glades and savannahs, semi-arid
E. atrorubens var. paradoxa *
Rocky, well-drained Appalachian upland slopes
E. laevigata
Appalachians to Ozarks moist, mixed forests
E. purpurea
Coneflowers e.g. Rudbeckia
Subg. Pallida
Subg. Echinacea
Most recent common ancestor of Echinacea

From a common *Echinacea* ancestor suited to the rocky slopes of the Appalachians, there is evidence that *E. purpurea* was separated by a combination of discrete variables, including fibrous roots that facilitated its invasion of moist, flat forest soils. Present-day *E. laevigata* remains in the ancestral habitat, reduced to rare and dwindling populations, distinct from the progenitor of subgenus *Pallida* which also gave rise to *E. pallida* and *E. atrorubens*. There was sufficient similarity between *E. laevigata* and *E. atrorubens* to support common ancestry, which was also indicated by shared historical and ecological traits. These taxa shared glabrous to sparsely strigose vestiture, similar leaf shape and inflorescence characteristics, and high oncentrations of root phytochemicals, including many common alkamides and polyn/enes. They thrive in soils of similar geological origin: the Appalachians, Arbuckle mountain limestone outcrops and the savannas amid climax oak-hickory forests in the Ozark mountains. The *E. atrorubens* varieties *paradoxa* and *neglecta* are geographically isolated from one another, but their related morphology and phytochemistry indicated that they may both be adapted descendents of a common Ozarkian ancestor. Another potential descendent of such an ancestor is the variety *E. atrorubens* var. *atrorubens* of moist and mixed-grass prairies.

E. pallida, with its five varieties, was putatively derived from shared ancestry with the progenitor of *E. atrorubens*, and adapted significant traits for survival across a range of moisture and edaphic conditions from the glades of central Tennessee through moist grasslands to the arid, harsh limestone outcrops of the high prairies. Morphological adaptations among the western and southern variants included hispid pubescence, longer and narrower taproots for access to deeper water tables, and low growth rates in response to lower nitrogen and other soil nutrients.

E. pallida var. *angustifolia* colonized the most arid habitats throughout the mixed to short-grass prairies through morphological and phytochemical adaptations. For example, deeper tap roots for nutrient storage and drought-resistance, reduced vegetative growth compared to other varieties and the subsequent allocation of nitrogen to alkamide secondary metabolism. *E. pallida* var. *angustifolia* alkamide phytochemical defenses increased as resources or water were likely more limiting, following other examples of cost: benefit balance in plant growth and defense (Bryant *et al.* 1983). Other varieties that preferred potentially nutrient-limiting edaphic conditions included *E. pallida* var. *tennesseensis* and var. *sanguinea*, and they consequently also accumulated higher levels of alkamides, compared to other *Echinacea* varieties. Alkamide diversity among all wild *Echinacea* populations was highest in the three varieties mentioned above (chapter 5). However, root alkamide type and quantity was higher in young cultivated/fertilized accessions, compared to wild roots from the same germplasm (Appendix 4; chapters 5 and 6). Conversely, wild-grown inflorescences from nutrient-poor soils in the current study contained much higher levels of cichoric acid than their cultivated counterparts (Tables 5.2 and 5.5).

In previous reports, concentration and diversity of secondary metabolites were highest during early stages of seedling growth compared to older plants (Cates and Rhoades 1977; Feeny 1976; Herms and Mattson 1992). For example, the highest concentration of cichoric acid in *E. purpurea* was reportedly from the infloresences in the youngest stage of bud development (Letchamo *et al.* 1999) and similar findings were reported with other plant families (Palo 1984; Hatcher 1990 in Herms and Mattson, 1992). All species and varieties in the current work displayed developmental variation in phenolic and alkamide secondary metabolism which may be partially explained by delayed differentiation of structural modifications (e.g. spines, thorns, bark) and cell-wall metabolites in secondary growth (e.g. lignin, tannins and suberin) that reduce the costly requirement for chemical defenses (Herms and Mattson 1992). In general, developmental variation in *Echinacea* phenolic and alkamide phytochemistry should be taken into account for commercial and medicinal purposes.

The most recently-derived *E. pallida* variety, according to Sharp (1935), McGregor (1968) and the current study, was the only consistent tetraploid, *E. pallida* var. *pallida*. Purportedly, *E. pallida* var. *pallida* originated through stabilized introgression of an allopolyploid hybrid from *E. pallida* var. *sanguinea* X *E. pallida* var. *simulata* (McGregor 1968). The question of parental origin remains unclear. Current phytochemical, morphological and geographical evidence suggested that original parents for this allotetraploid may also have included one or more of the *E. atrorubens* varieties. The predominant ketoalkyn/ene phytochemicals from *E. pallida* var. *pallida*, *E. pallida* var. *simulata*, *E. atrorubens* var. *paradoxa* and *E. atrorubens* var. *neglecta* indicate possible evolutionary relationships between these taxa. More evidence is required to elucidate the lineage(s) of the *E. pallida* var. *pallida* teteraploids, and further investigations should take into account the potential chemotaxonomic significance of ketoalkyn/enes, which were also determined to possess significant antimicrobial activities (Section 3).

E. pallida exhibited the largest ecological amplitude of all revised species. Some explanations (among many interacting variables) for its ecological flexibility and segregation into five distinct morpho-varieties include: higher genetic diversity through mechanisms such as backcrossing and introgression, as well as fixed heterozygosity at gene loci in allopolyploids. In a study by Snyder *et al.* (1994), the allopolyploid *E. pallida* var. *pallida* was more vigourously competitive than *E. pallida* var. *tennesseensis* (diploid), and both outcompeted *E. pallida* var. *angustifolia* (diploid). Vigour and competitive superiority in *E. pallida* var. *pallida* may also be explained by its preference for the tallgrass prairie, which is known to be a repository of nutrients and abundant, hardy graminoids. Ecological amplitude is a relatively good predictor of genetic diversity (Bradshaw 1984). For example, the endemic *E. pallida* var. *tennesseensis*, which has little opportunity to outcross with other *Echinacea* species and varieties, had half the amount of genetic variability in isozyme studies compared to *E. pallida* var. *angustifolia* (Baskauf *et al.* 1994). Overall genetic variability can also be used to address taxonomic questions, which is the aim of a concurrent molecular investigation using the same germplasm as the present thesis (Mechanda PhD Thesis In Progress).

In summary, most of McGregor's *Echinacea* taxa were supported with current morphometric evidence, yet four of his species were reduced to varieties. *E. purpurea* was the most distinct group of *Echinacea* according to two multivariate analyses; 78 morphological characters and 21 quantitative phytochemical characters, which each warranted its placement into a new subgenus. A similar four-species taxonomy was presented by Cronquist (1945; 1980), prior to this taxonomic revision, by intuitive morphological comparison of herbarium specimens (Fig. 11.2). For the first time, the phytochemical diversity among all wild *Echinacea* taxa was presented in this report. Furthermore, the lipophilic phytochemical biosynthetic pathway(s) were shown to be inducible in *E. pallida* var. *pallida* by the systemic signal molecule methyl jasmonate, which may be a strategy for defense with a minimum energetic cost to the plant.

Phytochemical variation in *Echinacea* was attributed in part to genetic adaptation. A controlled experiment suggested that populations within the widespread variety, *E. pallida* var. *angustifolia* (*E. angustifolia sensu* McGregor in chapter 6) produced offspring whose phytochemical defenses varied significantly according to geographical source, in the absence of environmental variation. Alkamides **4, 11, 14** and **19**, as well as **cichoric acid**, **echinacoside** and **caftaric acid** were found to be most important in the distinction of chemotypes, and they varied significantly with latitude, except **11** and **14**.

Alkamides and ketoalken/ynes from *Echinacea* demonstrated novel growth inhibition of pathogenic yeasts and certain filamentous fungi in bioassays with UV light-dependent effects. This was the first report attributing antimicrobial activity of *Echinacea* to its ketoalkyn/enes, although other plants in the family Asteraceae contained related photo-activated toxins, especially thiarubrines, thiophenes and other polyenes (Guillet 1997). Many of these phytochemicals had light-mediated inhibitory effects against viruses, microorganisms, cells and insects (Towers *et al.* 1997). *E. purpurea, E. pallida* (especially vars. *simulata* and *sanguinea*) and *E. atrorubens* (especially var. *atrorubens*) exhibited the most light-mediated activity against the yeasts and filamentous fungi tested. Strains were chosen because they were amphotericin B-resistant or clinical skin pathogens. Novel antiviral activity in the presence of UV-light was attributed to phenolic extracts as well as the above-mentioned lipophilic phytochemicals from *Echinacea* inflorescences and roots.

The present evidence for antimicrobial activity from hydroalcoholic *Echinacea* root and inflorescence extracts was a step towards justification of therapeutic antimicrobial use, both traditionally and currently. The mode of antifungal action is not fully understood, yet extracts from *Echinacea* present potential alternatives to the typical azole antifungal drugs for the treatment of clinical infection by drug-resistant strains of yeasts and filamentous fungal pathogens. *E. purpurea* was already shown to reduce clinical symptoms of candidiasis (Hobbs 1989), a prevalent microbial infection that is especially common in immuno-compromised AIDS patients, diabetics, pregnant women and neonates. Additional novel antimicrobial activity of *Echinacea* extracts was here documented against *Herpes simplex* (HSV-1). Varieties from all *Echinacea* species demonstrated biologically-significant HSV- inhibitory actions, especially the phenolic extracts of inflorescences. The relative order of antiviral activity for the lipophilic root extracts was; *E. purpurea*, *E. pallida* var. *angustifolia*, *E. pallida* var. *pallida* and *E. pallida* var. *tennesseensis*. Previously, *E. purpurea* exhibited antiviral actions from whole plant extracts or fresh-pressed juices (Cheminat *et al.* 1988 ; Wacker and Hilbig 1978).

Misidentification of *Echinacea* species in cultivated and wildcrafted medicinal preparations continues to impact the standardization and clinical justification of traditional *Echinacea* phytomedicines. As a whole, the current study provided tools to improve the quality and safety of phytomedicines through accurate identification and phytochemical characterization of *Echinacea* varieties using taxonomic identification keys in this work. Significant contributions were made to the study of *Echinacea* pharmacology, such as novel evidence for antimicrobial biological activity of various *Echinacea* extracts against clinical pathogens (including antibiotic-resistant fungal strains). Additional benefits of the current research include the capacity to distinguish between wild *Echinacea* taxa, and so increase the protection of endangered and dwindling wild populations (US Fish and Wildlife Service), as well as providing important baseline data for comparable molecular, genetic, phytochemical and ecophysiological studies of the various wild and cultivated varieties for crop improvement.

Figure 11.2. Two taxonomic treatments of *Echinacea* Moench by McGregor (1968), Cronquist (1980), and the revised taxonomic treatment by Binns, B.R. Baum & Arnason (chapter 2).

Figure 11.2. Taxonomic treatments of *Echinacea* Moench by McGregor (1968), Cronquist (1945, 1955, 1980), and Binns, Baum & Arnason (Chapter 2). Synonyms are indented in [square brackets].

R. L. McGregor (1968)	A. Cronquist (1945, 1955, 1980)	Binns, B.R. Baum & Arnason (2001)
1. *E. angustifolia* DC. var. *angustifolia*	**1**. *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist	**1**. *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist
E. angustifolia DC. var. *strigosa* McGregor	[*E. angustifolia* DC. var. *strigosa* McGregor]	[*E. angustifolia* DC. var. *strigosa* McGregor]
2. *E. tennesseensis* (Beadle) Small	[*E. tennesseensis* (Beadle) Small]	*E. pallida* (Nutt.) Nutt. var. *tennesseensis*
3. *E. pallida* (Nutt.) Nutt.	*E. pallida* (Nutt.) Nutt. var. *pallida*	*E. pallida* (Nutt.) Nutt. var. *pallida*
4. *E. simulata* McGregor	[*E. simulata* McGregor]	*E. pallida* (Nutt.) Nutt. var. *simulata* (McGregor) Binns, B. R. Baum & Arnason
5. *E. sanguinea* Nutt.	[*E. sanguinea* Nutt.] (suggested variety)	*E. pallida* (Nutt.) Nutt. var. *sanguinea* (Nutt.) Gandhi and Thomas
6. *E. atrorubens* Nutt.	**2**. *E. atrorubens* Nutt. var. *atrorubens*	**2**. *E. atrorubens* Nutt. var. *atrorubens*
7. *E. paradoxa* (Norton) Britton var. *paradoxa*	*E. atrorubens* var. *paradoxa* (Norton) Cronquist	*E. atrorubens* Nutt. var. *paradoxa* (Norton) Cronquist
E. paradoxa (Norton) Britton var. *neglecta* McGregor		*E. atrorubens* Nutt. var. *neglecta* (McGregor) Binns, B. R. Baum & Arnason
8. *E. laevigata* (Boynton & Beadle) Blake	**3**. *E. laevigata* (Boynton & Beadle) Blake *nom. cons. prop.*	**3**. *E. laevigata* (Boynton & Beadle) Blake *nom. cons. prop.*
9. *E. purpurea* (L.) Moench	**4**. *E. purpurea* (L.) Moench *nom. cons. prop.*	**4**. *E. purpurea* (L.) Moench *nom. cons. prop.*

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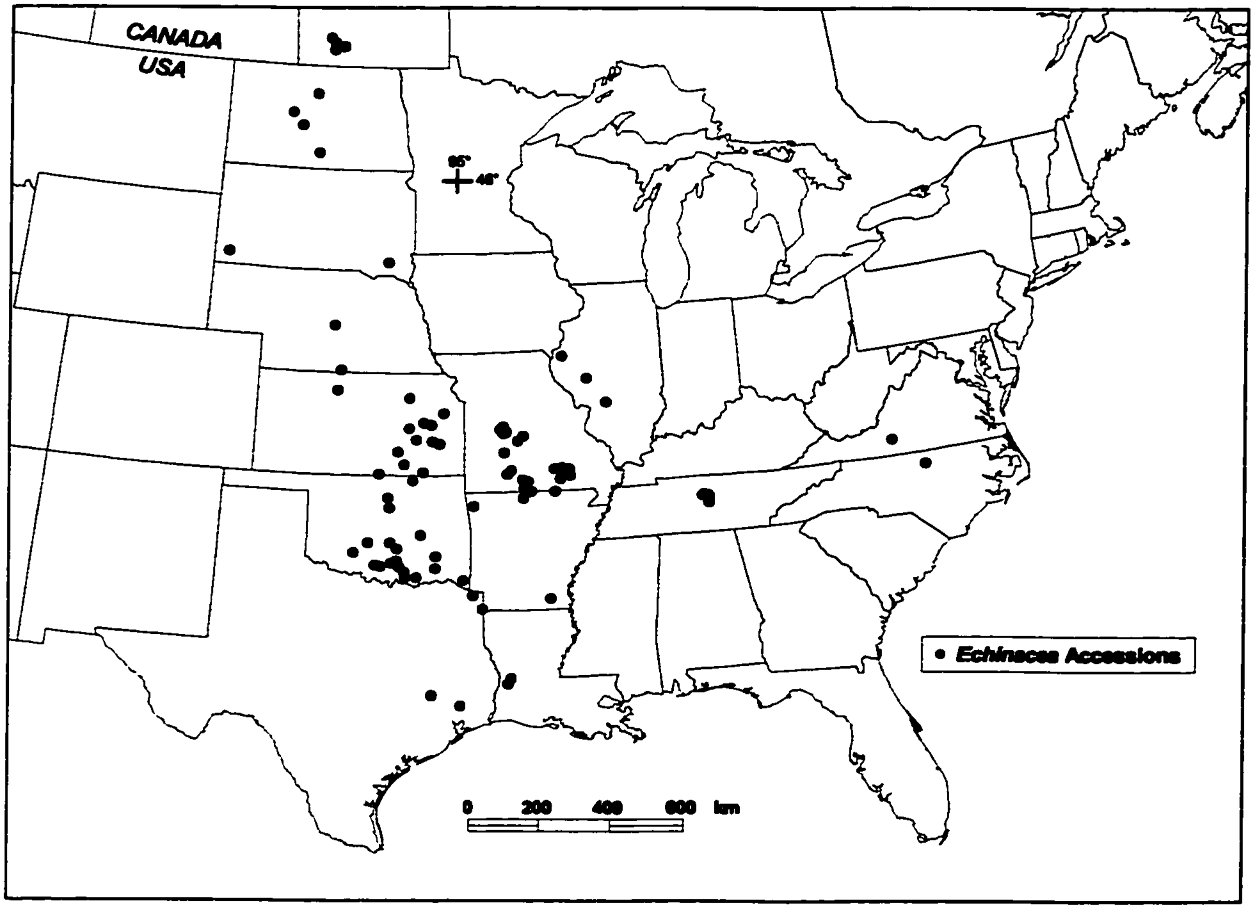
APPENDIX

Appendix 1. Wild populations of *Echinacea* spp. that were collected and examined for the current thesis work. Voucher specimens are deposited at DAO, coll. S. Binns.


CANADA
USA
95°
46°
● Echinacea Accessions
0 200 400 600 km

Appendix 2. Frequency tables for the discontinuous variables which were found to be important discriminators between taxa by CDA in morphometric analyses (chapter 2).

2.1: Frequency of each state of variable 42 in the CDA used to distinguish the four revised-species in rows: 1= *E. purpurea*, 2= *E. pallida*, 3= *E. atrorubens* and 4= *E. laevigata*. The number of specimens for each state of variable 42 "stalked leaf trichome basal globules" are listed in columns: 0= bicellular, 1= multicellular, 2= absent.

	0	1	2	n
1	0	2	45	47
2	14	104	67	185
3	4	30	29	63
4	6	0	6	12

2.2: Frequency of each state of variable 62 in the CDA used to distinguish the four revised-species in rows: 1= *E. purpurea*, 2= *E. pallida*, 3= *E. atrorubens* and 4= *E. laevigata*. The number of specimens for each state of variable 62 "leaf vein type" are listed in columns: 0= branching, 1= almost parallel, 2= intermediate with parallel and branching.

	0	1	2	n
1	46	0	1	47
2	3	173	9	185
3	1	50	12	63
4	11	0	3	14

2.3: Frequency of each state of variable 74 in the CDA used to distinguish the four revised-species in rows: 1= *E. purpurea*, 2= *E. pallida*, 3= *E. atrorubens* and 4= *E. laevigata*. The number of specimens for each state of variable 74 "stem stalked trichome length" are listed in columns: 0= short, 1= medium, 2= long, 3= absent.

	0	1	2	3	n
1	18	26	4	0	48
2	19	103	64	0	187
3	44	20	0	0	65
4	0	0	0	15	15

2.4: Frequency of each state of variable 42 in the CDA used to distinguish the two subgenera in rows: 1= *Purpurea*, 2= *Pallida*. The number of specimens for each state of variable 42 "stalked leaf trichome basal globules" are listed in columns: 0= bicellular, 1= multicellular, 2= absent.

	0	1	2	n
1	0	3	48	51
2	31	133	99	263

2.5: Frequency of each state of variable 62 in the CDA used to distinguish the two subgenera in rows: 1= *Purpurea*, 2= *Pallida*. The number of specimens for each state of variable 62 "leaf vein type" are listed in columns: 0= branching, 1= almost parallel, 2= intermediate with parallel and branching.

	0	1	2	n
1	46	4	1	51
2	15	222	28	265

2.6: Frequency of each state of variable 40 in the CDA used to distinguish three subclusters in subgenus *Pallida* in rows: 2=*E. pallida*, 3= *E. atrorubens*, 4= *E. laevigata*. The number of specimens for each state of variable 40 "leaf stalked trichome cell types" are listed in columns: 0= bicellular, 1= multicellular, 2= absent, 3= both.

	0	1	2	3	n
2	2	176	3	2	183
3	5	50	2	7	64
4	0	5	13	3	21

2.7: Frequency of each state of variable 47 in the CDA used to distinguish three subclusters in subgenus *Pallida* in rows: 2=*E. pallida*, 3= *E. atrorubens*, 4= *E. laevigata*. The number of specimens for each state of variable 47 "leaf stalked trichome density" are listed in columns: 0= dense, 1= sparse, 2= glabrous.

	0	1	2	n
2	149	34	1	184
3	17	46	1	64
4	1	16	4	21

2.8: Frequency of each state of variable 48 in the CDA used to distinguish three subclusters in subgenus *Pallida* in rows: 2=*E. pallida*, 3= *E. atrorubens*, 4= *E. laevigata*. The number of specimens for each state of variable 48 "leaf stalked trichome blade distribution" are listed in columns: 0= even, 1= more adaxial, 2= more abaxial.

	0	1	2	n
2	146	27	11	184
3	20	6	38	64
4	3	7	11	21

2.9: Frequency of each state of variable 73 in the CDA used to distinguish three subclusters in subgenus *Pallida* in rows: 2=*E. pallida*, 3= *E. atrorubens*, 4= *E. laevigata*. The number of specimens for each state of variable 73 "Stem stalked trichome cell type" are listed in columns: 0= bicellular, 1= multicellular, 2= absent, 3= both.

	0	1	2	3	n
2	6	175	1	3	185
3	3	55	1	7	66
4	0	5	17	0	22

2.10: Frequency of each state of variable 74 in the CDA used to distinguish three subclusters in subgenus *Pallida* in rows: 2=*E. pallida*, 3= *E. atrorubens*, 4= *E. laevigata*. The number of specimens for each state of variable 74 "Stem stalked trichome length" are listed in columns: 0= short, 1= medium, 2= long, 3= absent.

	0	1	2	3	n
2	18	102	64	0	185
3	44	21	0	0	66
4	1	3	0	17	22

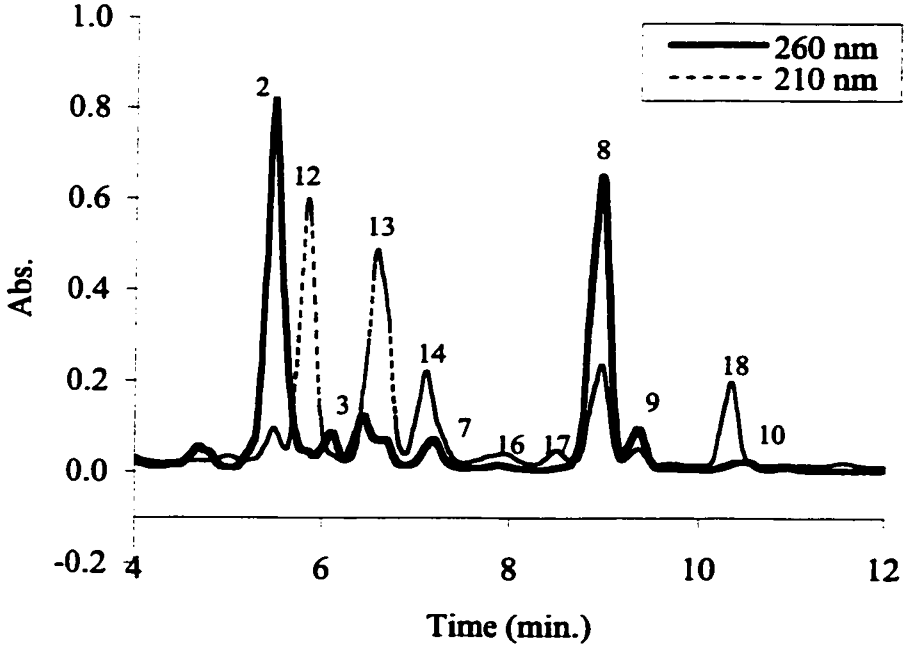
Appendix 3. Mean zones of inhibition of fungal growth (mm) with SEM below, by *Echinacea* root lipophilic extracts in two different light treatments. See Table 9.2 for explanation of fungal strain codes. (n=3)

	Ca		Cs		Cn		Fo		Pb		Tm		Mg		CN1A		D10	
***Echinacea* variety**	**UV**	**dark**	**UV**	**dark**	**UV**	**dark**	**UV**	**dark**	**UV**	**dark**	**UV**	**dark**	**UV**	**dark**	**UV**	**dark**	**UV**	**dark**
E. pallida* var. *angustifolia	0	0	1	0	0.333	0	0.233	0	0	0					0	0		
			0		0.09		0.084											
E. pallida* var. *pallida	0	0			2	0	0	0			9	3	0	0	1.167	1	4	0
					0.577						0	0	0		0.096	0	0.33	0
E. pallida* var. *sanguinea	0	0	0.167	0	1.167	0	0	0	0	1	0.61	0	0.49	0.789	3.667	0		
			0.096		0.096					0	0.04		0.02	0.057	0.192			
E. pallida* var. *tennesseensis	1	4.333			1	0	0.5	0										
	0.12	0.192			0		0											
E. pallida* var. *simulata			0.5	1	2.167	1	0.333	0	2	0			0	8.333	0	0		
			0	0.333	0.096	0	0.096		0					0.385				
E. atrorubens* var. *atrorubens	1	0	0.333	0	0.333	0	0.667	0	0	0	0.56	0.856	0.36	0.533	3	0		
	0		0.192		0.096		0.385				0.05	0.107	0.04	0.04	0			
E. atrorubens* var. *neglecta	0.5	0	0.5	0.133	1	0.667	0	0.333	0		0.3	0.478						
	0		0.167	0.038	0	0.192		0.192			0.03	0.034						
alpha-t *	4.33	0.67	3.667	0.033	3.222	0	4	0	13	0	4	0	5	0	0.667	0	1	0
	0.38	0.087	0.096		0.706		0		0.882				0		0.192		0.58	

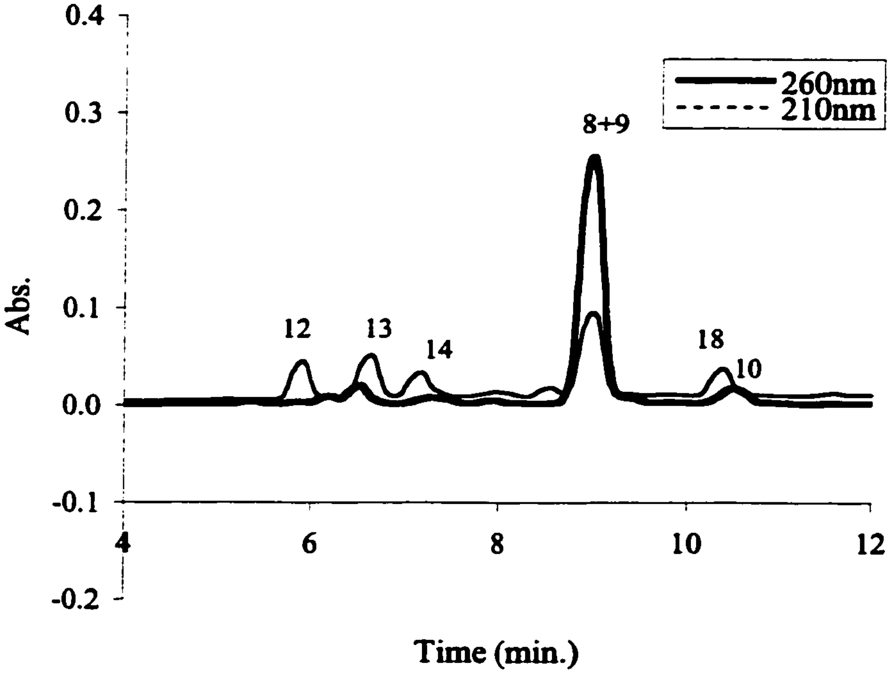
* denotes the phototoxic control compound
blanks = not tested

Appendix 4. A comparison of lipophilic profiles of wild *E.pallida* var. *angustifolia* roots with 1 year-old cultivated roots from the same germplasm in Manitoba, Canada.

Cultivated *E. angustifolia* DC.



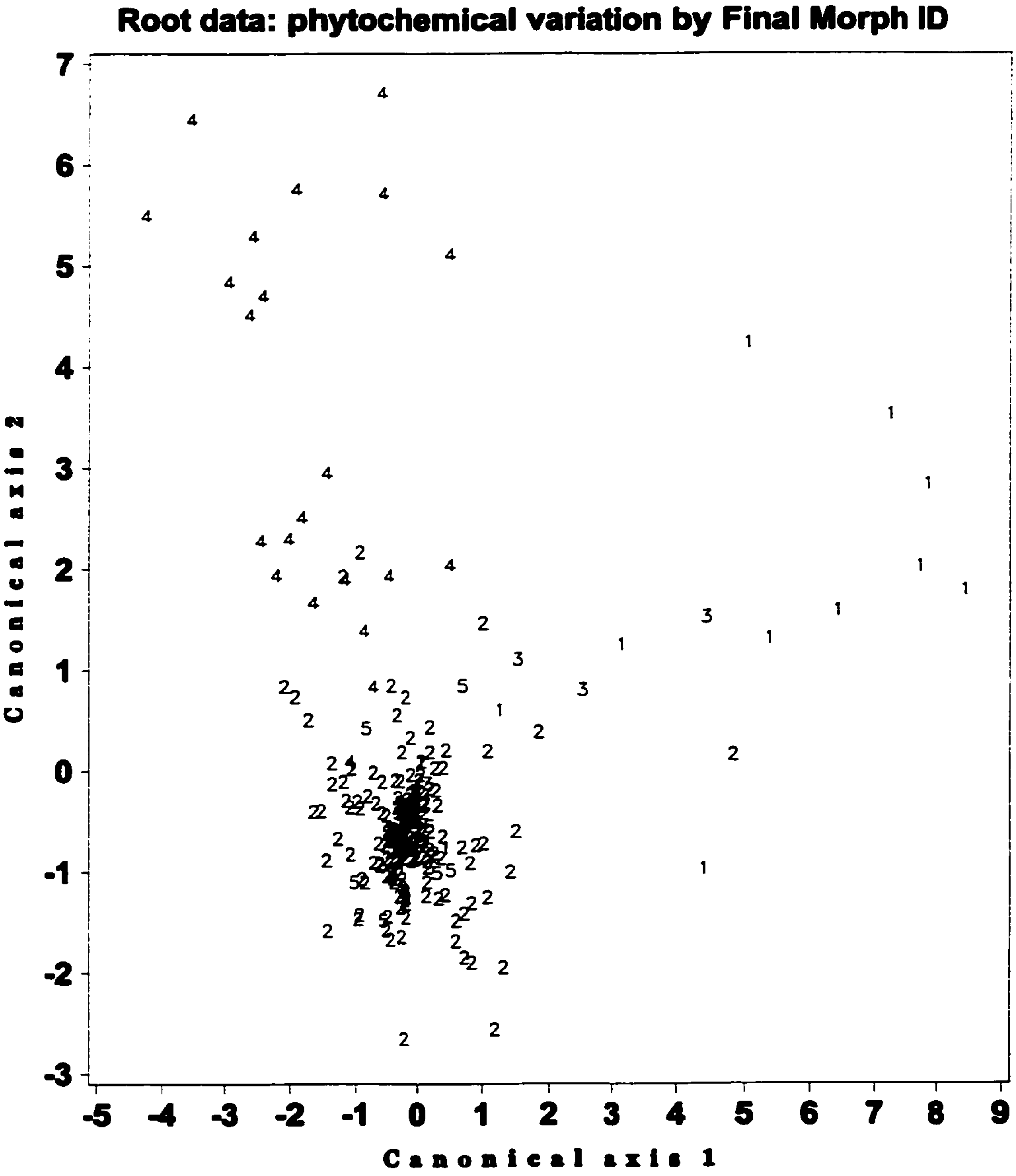
Wild *E. angustifolia* DC.



Appendix 5. The phytochemical variation of *Echinacea* roots in the current study as represented by the first two axes in the canonical discriminant analysis (see chapter 5). Cluster numbers represent single specimens and correspond to the following revised morphometric taxa: 1= *E. purpurea*, 2= *E. pallida*, 3= *E. laevigata*, 4= *E. atrorubens*, and 5= Hybrids (either *E. pallida* × *E. atrorubens* or intraspecific between varieties).

Appendix 6. Characters used in the morphometric analysis (chapter 2, Table 2.1) according to character state category.

Character Type	Suite of characters from Table 2.1
Binary	18, 19, 25, 30, 50, 66, 71, 81
Quantitative	1, 2, 4, 9, 10, 24, 29, 35, 36, 39, 51-56, 63, 65, 72
Semi-quantitative	3, 5, 7, 8, 11, 12, 20-22, 26-28, 31-34, 38, 40-49, 57-61, 67, 69-70, 73-80
Qualitative	6, 13, 15-17, 37, 62, 64


Root data: phytochemical variation by Final Morph ID
Canonical axis 2
7
6
5
4
3
2
1
0
-1
-2
-3
Canonical axis 1
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9