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Metabolic Profiling of *Echinacea* Genotypes and a Test of Alternative Taxonomic Treatments

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Abstract

The genus *Echinacea* is used as an herbal medicine to treat a variety of ailments. To better understand its potential chemical variation, 40 *Echinacea* accessions encompassing broad geographical and morphological diversity were evaluated under controlled conditions. Metabolites of roots from these accessions were analyzed by HPLC-photo diode array (HPLC-PDA), GC-MS, and multivariate statistical methods. In total, 43 lipophilic metabolites, including 24 unknown compounds, were detected. Weighted principal component analysis (WPCA) and clustering analysis of the levels of these metabolites across *Echinacea* accessions, based on Canberra distances, allowed us to test two alternative taxonomic treatments of the genus, with the further goal of facilitating accession identification. A widely used system developed by McGregor based primarily on morphological features was more congruent with the dendrogram generated from the lipophilic metabolite data than the system more recently developed by Binns et al. Our data support the hypothesis that *Echinacea pallida* is a diverse allopolyploid, incorporating the genomes of *Echinacea simulata* and another taxon, possibly *Echinacea sanguinea*. Finally, most recognized taxa of *Echinacea* can be identified by their distinct lipophilic metabolite fingerprints.

Keywords

Echinacea (Asteraceae); alkamides; alkylamides; HPLC; GC-MS; PCA

Introduction

Echinacea extracts, particularly from roots, have historically been used by Native Americans and, more recently, by Western cultures as herbal remedies to treat ailments ranging from snake bites to pain, burns, cough, sore throats, and toothache [1], [2], [3]. *Echinacea* products are promoted for immune system enhancement and are among the best-selling

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herbal preparations in the United States [4]. Many unusual phytochemicals have been found in *Echinacea*, some with reports of bioactivity in animal cells or animals, including alkalamides and ketones, caffeic acid derivatives, glycoproteins, and unusual polysaccharides (as reviewed by Bauer [5]). Despite its popularity as an herbal dietary supplement, and many pertinent pharmacological and clinical studies, little is known regarding the specific compounds primarily responsible for observed bioactivity or whether they are consistently efficacious in humans [6].

Although morphologically distinct *Echinacea* groups exhibit differences in phytochemical composition [5] and bioactivity [7], most studies have focused on three major medicinal species: *Echinacea angustifolia* DC., *E. pallida* (Nutt.) Nutt., and *E. purpurea* (L.) Moench. The most comprehensive study to date was conducted by Binns et al. [8], who reported on phytochemical variation in *Echinacea* from roots and capitula of wild and cultivated populations representing all nine *Echinacea* species recognized by McGregor [9]. However, in that report, most alkalamides and ketones were identified by UV spectra and relative retention times compared with one major pair of alkalamide standards. Baum et al. [10], in a recent review of the status of *Echinacea* systematics and phytochemistry, indicated that *Echinacea* taxa are readily distinguishable on the basis of HPLC profiles and that HPLC profiles for lipophilic compounds contain more information than those based on caffeic acid derivatives. This is noteworthy because of the existence of two alternative taxonomic treatments for *Echinacea*. The older, developed by McGregor [9], was based on field observations, common garden studies, and cytological and anatomical analyses. McGregor's classification has been widely used by botanists and herbalists [11] and serves as the basis for the recent *Flora North America* treatment [12]. Binns et al. [13] proposed a revision, based on morphometric data and phytochemical data from greenhouse-grown and wild plants, using canonical discriminant and cladistic analyses. This revision recognizes all but one of McGregor's taxa, the most significant changes being a reduction in the number of species, an increase in the number of varieties, and, in particular, the incorporation of 5 morphologically diverse clades characterized as species by McGregor [9] into a single species. This Binns et al. [13] revision is controversial in the botanical community [11].

The current DNA-based molecular marker evidence is not yet refined sufficiently to generate accession-level systematics [14], [15]. Even an extensive DNA-sequencing study using these identical accessions and based on multiple loci has thus far been unable to completely evaluate systematic relationships among these accessions [16].

Here we have taken a targeted, metabolite-profiling approach to investigate the accumulation of putatively bioactive alkalamides and ketones in 40 geographically and morphologically diverse *Echinacea* populations, which already had been well characterized morphologically and as to origin. We used as standards authentic alkalamides and ketones that were purchased or synthesized by our group [17], [18], [19], as well as structural information obtained by a combination of HPLC-PDA and GC-MS, for more comprehensive compound identification. Because we lacked reference standards for many of the metabolites reported in this study (43 in all), we used relative instead of absolute metabolite concentrations to compare overall lipophilic-metabolite profiles across the 40 accessions. This approach, coupled with weighted principal component analysis (WPCA) and clustering analysis based on Canberra distances [20], provides an opportunity to test these two taxonomic classifications. Furthermore, these metabolic profiles may help standardize *Echinacea* products, characterize plant material of unknown provenance, and identify genetic sources to select for increased production of desired compounds.

Materials and Methods

Plant materials

We selected 40 well-characterized *Echinacea* accessions (Table 1) representing a broad geographic and morphological sampling of the germplasm conserved by the U.S. National Plant Germplasm System, USDA-ARS North Central Regional Plant Introduction Station, Ames, Iowa [21]. Initially we looked at roots of two ages of plants: 6-month-old and 3-year-old. We found that the relative levels of metabolites vary, but the same identified alkalamides, ketones, and unknown metabolites are present at both ages (Fig. 1S, Supporting Information); therefore, we focused on 6-month-old plants because we are able to grow them under well-controlled conditions. Characterization data for a broad range of (> 40) morphological traits are available at Germplasm Resources Information Network database (http://www.ars-grin.gov/cgi-bin/npgs/html/desc_form.pl?221). Accessions were keyed to species (or subspecies) during initial regeneration on the basis of McGregor [9], and we converted McGregor identifications to the treatment of Binns et al. [13] via Table 2 in [10]. Growth conditions and sampling methods are available in the Supporting Information.

Extraction, HPLC, and GC-MS analysis

Plant extraction, HPLC, and GC-MS analyses were performed as in [17], [18], and [22].

Compound identification and relative abundance

In addition to 19 known alkalamides and ketones, another 24 unknown lipophilic metabolites were detected and grouped according to their retention times and UV spectra (Table 1S, Supporting Information). Methods for determining the relative abundance of metabolites are provided in Supporting Information.

Statistical analysis

Multivariate analyses by WPCA and hierarchical clustering analysis were performed in R software, version 2.2.1 (<http://www.r-project.org/>). Detailed information is provided in the Supporting Information.

Supporting information

Detailed methods and additional data are available as Supporting Information.

Results and Discussion

Most *Echinacea* extractions use dry materials and rigorous methods that last 1–24 hours (e. g., soxhlet extraction [23], ultrasonic extraction [24], and shaking [8]). To minimize possible degradation of unstable metabolites during extraction, we used a quick extraction method by powderizing a small amount of fresh tissue with liquid N₂ and extracting at low temperature [17], [18]. By using authentic synthesized standards, combined with GC-MS and HPLC-PDA, we evaluated 40 accessions (Fig. 1) and detected 43 UV-absorbing lipophilic metabolites. Of these, 19 metabolites were identified, including 16 alkalamides and 2 ketones among those reported so far by the pioneering studies of Bauer and colleagues [5] and another recently reported alkalamide, herein referred to as “Chen alkalamide” [25] (for structures, see Fig. 2S, Supporting Information). In addition, we detected 24 unknown lipophilic metabolites, some of which (e. g., unknown **B5** and unknown **9**) are relatively abundant. Five unknowns (unknowns **A1–A5**) have UV spectra similar to the 2,4-diene alkalamides (**1**, **2**, **3**, **4**, **7**, **10**, and **11**). Six unknowns (**B1–B6**) have UV spectra similar to the monoene alkalamides (**12**, **13**, **14**, **16**, and **17**) (Table 1S, Supporting Information). Interestingly, 10 unknown metabolites have atypical UV spectra. Most of the unknowns (**3–**

10) are highly lipophilic and thus elute at later times. Identification of these unknowns is currently being conducted by HPLC-tandem mass spectrometry (LC-MS/MS), semi-preparative HPLC, and NMR.

Our observation of 43 lipophilic metabolites (Table 1S, Supporting Information) can be contrasted with the findings of Binns et al. [8], who distinguished 15 unique alkamides, 2 pairs of alkamides (alkamide **8/9** and alkamide **5/15**), and 3 ketones. We detected the presence of all but 4 of these, 3 of the alkamides and 1 of the ketones. Our ability to distinguish more than twice the compounds from these samples is likely attributable to two factors: rapid extraction under low temperature, minimizing possible degradation, and a more sensitive HPLC separation method with extended retention times.

To elucidate how *Echinacea* accessions are related in terms of their overall metabolite profiles, two multivariate statistical approaches were used: WPCA and clustering analysis. Traditional PCA assumes that all observations of a particular metabolite have the same variance, although variances may differ between metabolites. However, for our dataset, standard deviations among biological replicates increase with metabolite abundance, i. e., the abundant compounds are more variable (Fig. 2). We used WPCA to account for this pattern. Each element of the data is given a corresponding “weight”, proportional to the inverse of the variance. Thus, smaller peaks with smaller errors are given a larger weight, placing more emphasis on these less abundant compounds.

WPCA reveals large quantitative and qualitative differences in lipophilic metabolites among *Echinacea* populations (Fig. 3). Each composite metabolite profile indicates metabolites that tend to be present together or absent together (Fig. 3A). The accession profiles indicate the relative abundance of corresponding metabolite profiles for each accession (Fig. 3B). Profile 1 (WPC1) focuses on the most abundant compounds, primarily amides **8** and **9**, with smaller amounts of amides **3**, **5**, **11**, and **12**. Profile 1 is most abundant in accessions identified on the basis of McGregor [9] as *E. angustifolia*, *E. atrorubens* (Nutt.) Nutt., *E. purpurea*, *E. pallida*, and *E. sanguinea* Nutt. Profile 2 (WPC2) contains predominantly amides **2** and **3**, the Chen alkamide, ketones **22**, and **24**, and unknown **8**. Profile 2 is most abundant in *E. purpurea* as well as in many other species, except *E. angustifolia*, *E. atrorubens*, and *E. tennesseensis* (Beadle) Small. Profile 3 (WPC3) is almost exclusively composed of amides **12**, **13**, and **14** and is most abundant in *E. angustifolia* and *E. tennesseensis*. The three-profile solution explains 94.8% of the variance of the whole dataset.

The hierarchical dendrogram constructed from Canberra distances [20] for all pairs of 40 accessions (3 plants per accession), based on average linkage (Fig. 4), displays phenetic relationships among accessions, labeled on the basis of McGregor [9] (Fig. 4A) and coded for the four species recognized by Binns et al. [13] (Fig. 4B). The primary area of agreement between the two taxonomic schemes is the recognition of a distinct species pair, *E. purpurea* and *E. laevigata* (C. L. Boynton & Beadle) S. F. Blake, which share a distinct stem anatomy, leaf shape, and phyllary structure, clustered in adjacent groups in Fig. 4.

The major difference between the two treatments centers on the remaining taxa, which McGregor [9] treated as seven species with two additional varieties and Binns et al. [13] treated as only two species with six additional varieties. The dendrogram presented in Fig. 4 lends little support for the circumscription of the two, diverse species as proposed by Binns et al. [13], for two primary reasons. First, the observed degree of differentiation that distinguishes *E. laevigata* from *E. purpurea* in Fig. 4, recognized by Binns et al. [13] as a clear distinction at the subgeneric level, would support the recognition of three additional subgenera, which we do not feel is warranted based on all other relevant data. In addition, the two inclusive species recognized by Binns et al. [13] as *E. pallida* and *E. atrorubens*

cluster in an intercalated fashion within the dendrogram, above and below the cluster containing *E. laevigata* and *E. purpurea*. Thus, lipophilic metabolic profiles do not support the broad species combinations proposed by Binns et al. [13].

In contrast, there is a better correspondence to the species and at least one of the varieties recognized by McGregor [9] (Fig. 4). Of the nine species recognized by McGregor [9], accessions from six cluster together into a single branch in the tree: *E. laevigata*, *E. purpurea*, *E. tennesseensis*, *E. sanguinea*, *E. atrorubens*, and *E. angustifolia*. The three that do not are *E. pallida*, *E. simulata* McGregor, and *E. paradoxa* (Norton) Britton.

For *E. pallida*, three of five accessions cluster together (Fig. 4), but accessions PI 631315 and PI 631275 do not. Interestingly, the three *E. pallida* accessions that are clustered together are adjacent to *E. simulata*, which McGregor [9] considered to be very close to *E. pallida* and a likely progenitor of this tetraploid species. He hypothesized that the other species involved in the parentage of *E. pallida* was *E. sanguinea*; the two “atypical” accessions are located on our dendrogram closer to *E. sanguinea* than to *E. simulata*. Support for a close biochemical relationship between *E. pallida* and *E. sanguinea* was reported recently by Senchina et al. [26], who conducted a phenetic analysis of the immunomodulatory characteristics of seven *Echinacea* taxa. Binns et al. [13] treated both *E. simulata* and *E. sanguinea* as varieties of *E. pallida*.

For *E. paradoxa*, the two varieties recognized by McGregor [9] form clean clusters adjacent to each other (Fig. 4). Taken as a single group, the *E. paradoxa* “cluster” also includes two putative hybrid accessions, both of which were collected from populations where *E. paradoxa* was sympatric with other taxa (Table 1), and *E. angustifolia* var. *strigosa* McGregor PI 631320. Five of six accessions of *E. angustifolia* cluster together (Fig. 4), the outlier being *E. angustifolia* var. *strigosa* PI 631320. We speculate that *E. angustifolia* var. *strigosa* occupies some “hybrid middle ground” between *E. paradoxa* var. *neglecta* McGregor and *E. angustifolia* var. *angustifolia* (supported geographically) and/or that *E. angustifolia* var. *strigosa* is not well differentiated based on lipophilic compounds. Variety *strigosa* has been recognized as problematic by other researchers as well. McGregor [9] considered it to be of hybrid origin, as did Binns et al. [13], and the conversion table presented by Baum et al. [10] does not recognize it as a distinct taxon nor does *Flora North America* [12].

In general, the dendrogram generated on the basis of Canberra distances for lipophilic metabolite profiles among our 40 accessions supports the taxonomic treatment presented by McGregor [9], with the possible exception of *E. angustifolia* var. *strigosa*. The metabolic profiles also indicate that there are diverse chemotypes of *E. pallida*, consistent with its proposed allopolyploid origin.

Although we sampled a broad geographic distribution of accessions representing each taxon, these accessions generally clustered consistently with taxa as identified by morphological, anatomical, and cytological characteristics used by McGregor [9] rather than by geographic or environmental variables gleaned from their provenance data. Thus, our analyses imply that the distribution and types of alkaloid and ketone metabolites in *Echinacea* do not evolve in a convergent manner in response to particular geo/environmental conditions.

Finally, the relative concentrations of the 43 lipophilic compounds appear to be distinctive enough by taxon to allow us to develop “typical” profiles for *Echinacea* fingerprinting, which could be validated by evaluating additional populations. Thus, this research expands the basis for the evaluation, standardization, and identification of plant material of unknown provenance for cultivated *Echinacea* and for commercial *Echinacea* products. In addition, these data contribute to the identification of genetic resources for the production of specific

alkamides and ketones. In the course of this study, we found more than 20 unidentified metabolites, some of which may be alkamides or ketones. Bioactivity-guided fractionation together with compound identification will elucidate these as yet unidentified metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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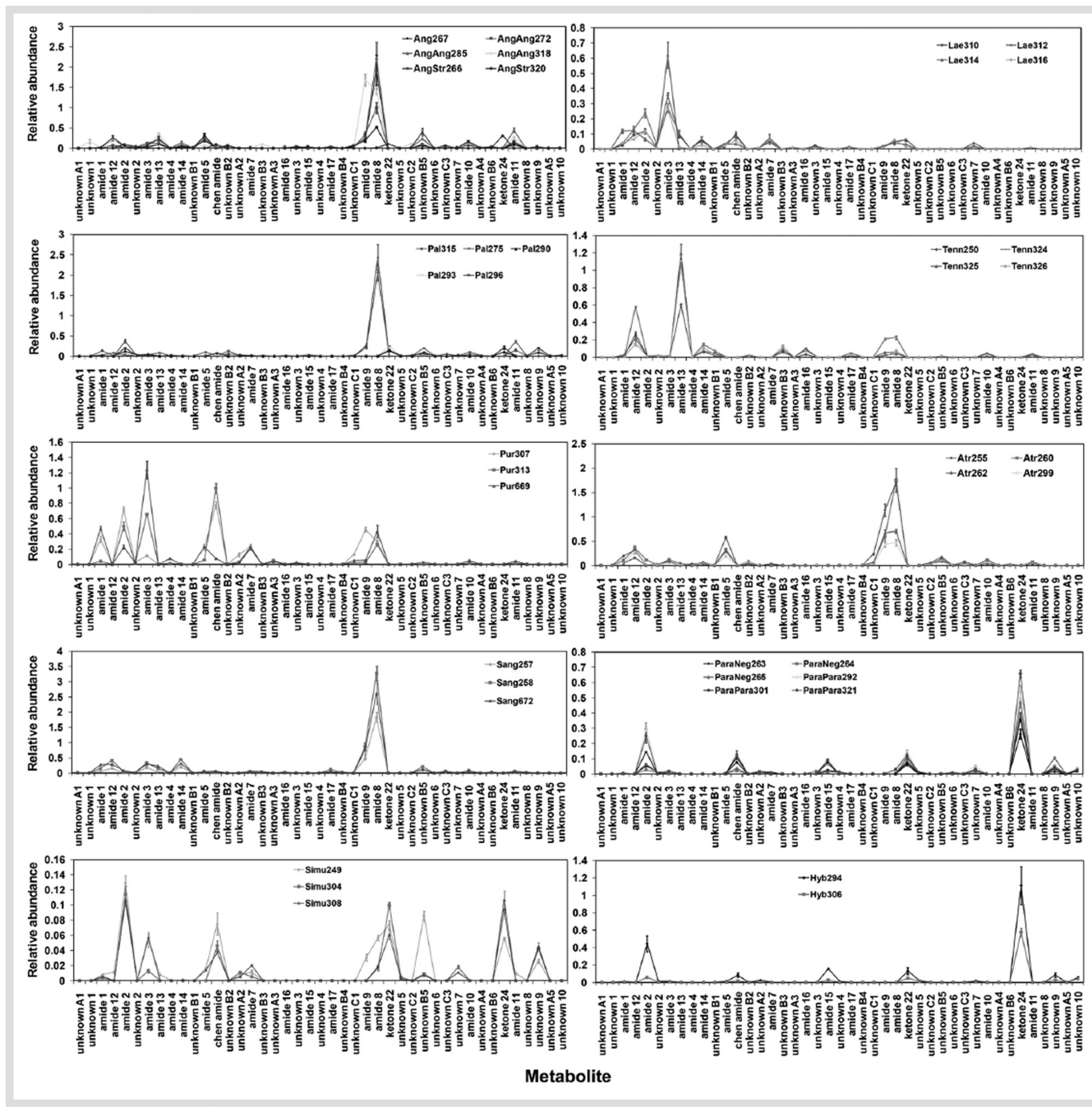


Fig. 1. Relative abundance of lipophilic metabolites in roots from 6-month-old plants of 40 accessions of *Echinacea*. Error bars indicate standard deviations of means of triplicate experiments.

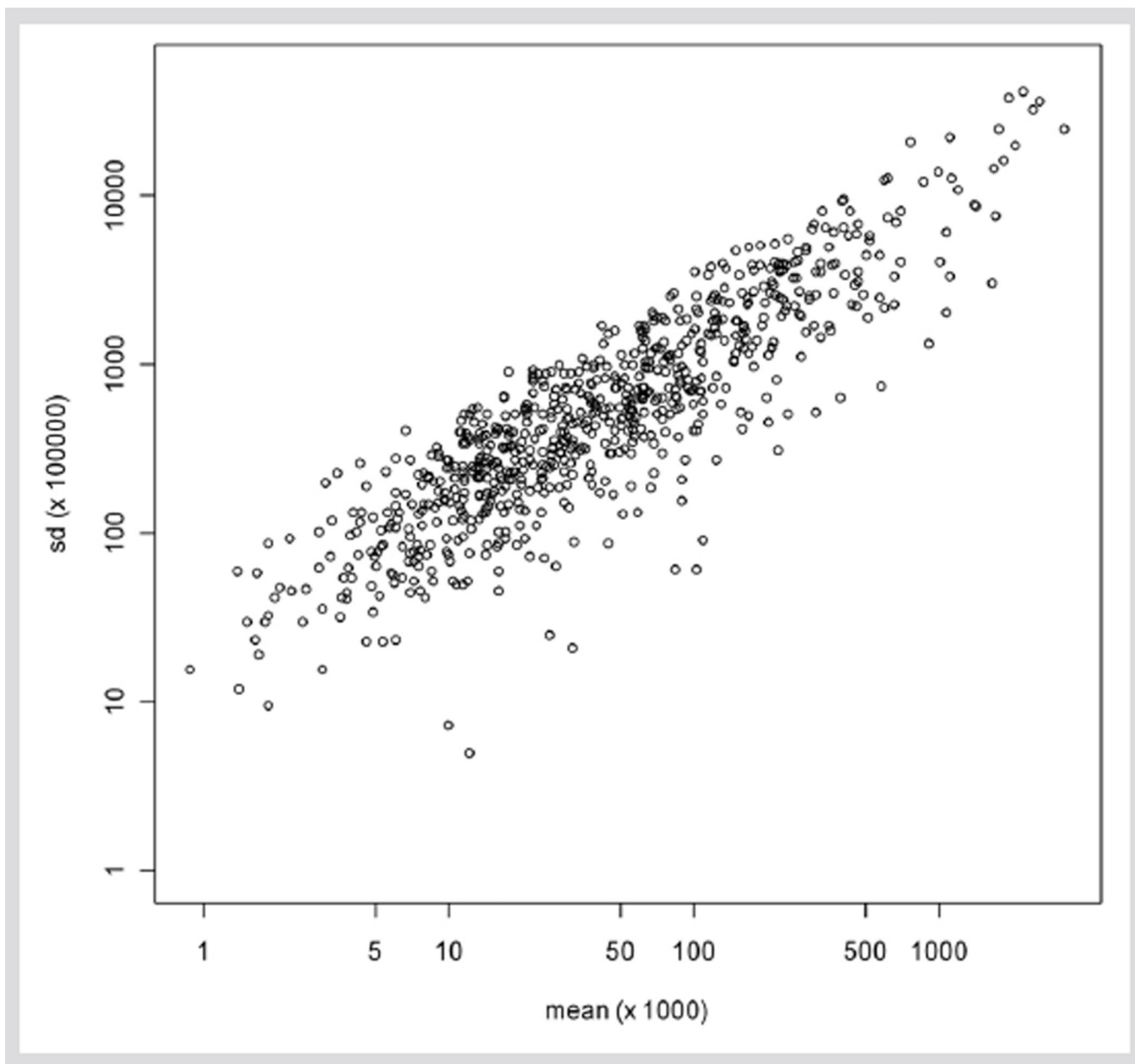


Fig. 2. Relationship between the standard deviation (sd) and mean across all combinations of accessions and metabolites. Both sd and mean are plotted on log scales.

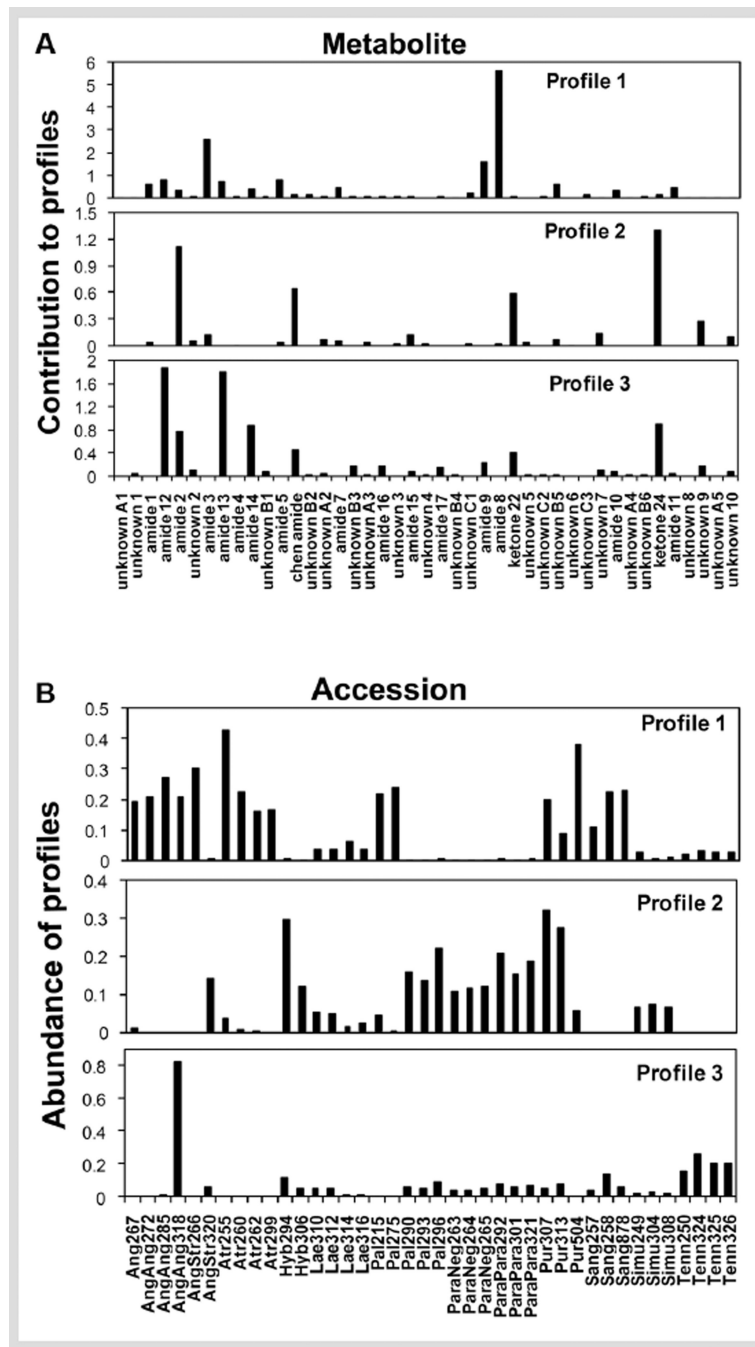


Fig. 3. Weighted principal component analysis (WPCA) of lipophilic metabolite profiles in roots from 6-month-old plants of 40 accessions of *Echinacea*, illustrating the differences among *Echinacea* accessions. **(A)** The relative importance of each metabolite to each of the three WPCs. **(B)** The relative abundance of each component in different accessions.

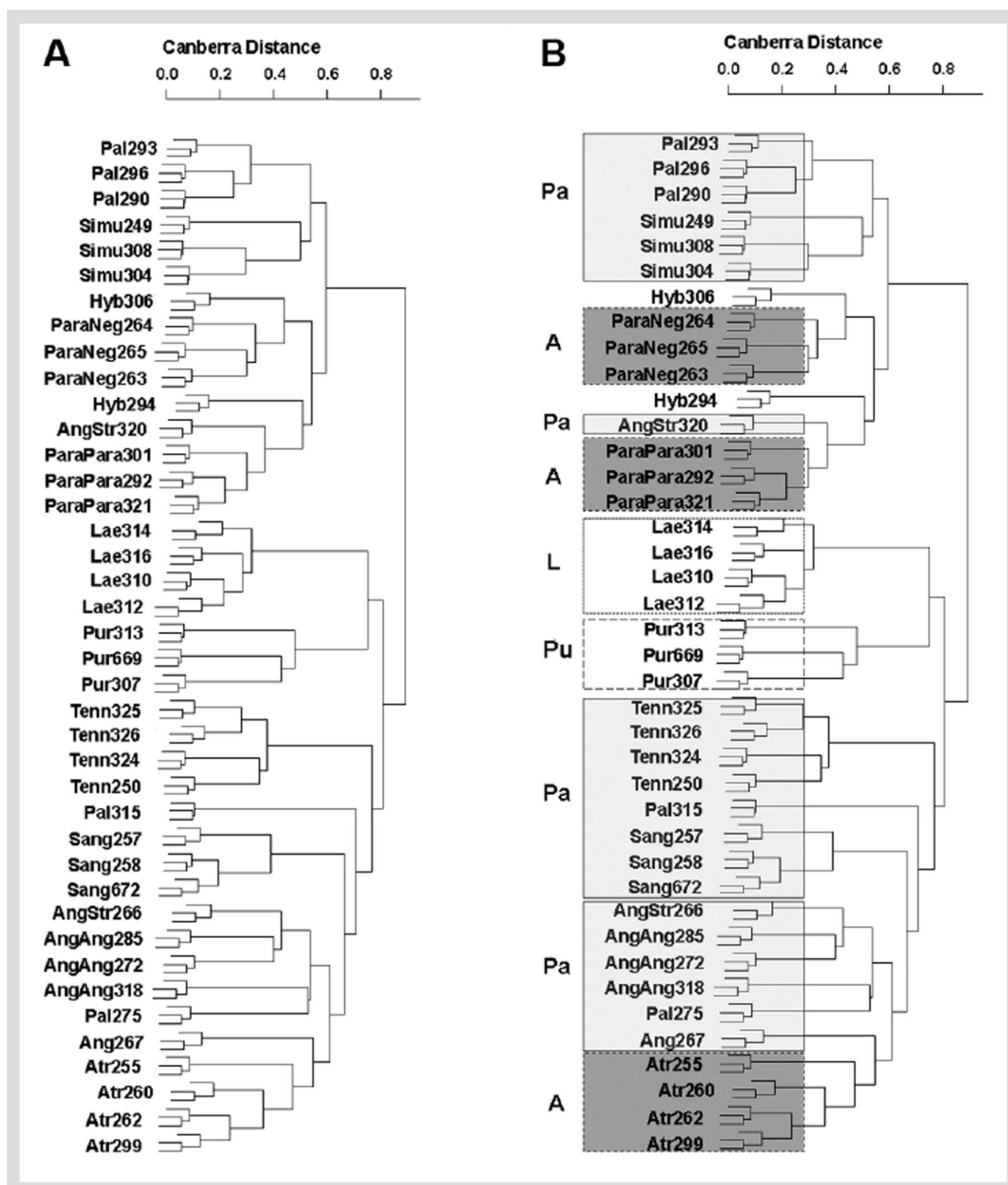


Fig. 4. Hierarchical dendrogram constructed from profiles generated from 43 lipophilic metabolites, using Canberra distance, illustrating the distances between 40 *Echinacea* accessions (3 plants per accession). **Panel A** is marked only with abbreviations of the accessions following McGregor [9] and the final three digits of the accession number. **Panel B** also includes blocking corresponding to the four species recognized by Binns et al. [13], with A = *E. atrorubens*, L = *E. laevigata*, Pa = *E. pallida*, and Pu = *E. purpurea*.

Table 1

Accession information for *Echinacea* evaluated in this study

Taxon (sensu McGregor) [9]	Taxon (sensu Binns et al.) [13]	Accession number	Location of source population
<i>E. angustifolia</i>	<i>E. pallida</i> var. <i>angustifolia</i>	PI631267	Murray County, OK
<i>E. angustifolia</i> var. <i>angustifolia</i>	<i>E. pallida</i> var. <i>angustifolia</i>	PI631272	Comanche County, OK
<i>E. angustifolia</i> var. <i>angustifolia</i>	<i>E. pallida</i> var. <i>angustifolia</i>	PI631285	Lyon County, IA
<i>E. angustifolia</i> var. <i>angustifolia</i>	<i>E. pallida</i> var. <i>angustifolia</i>	PI631318	Rooks County, KS
<i>E. angustifolia</i> var. <i>strigosa</i>	<i>E. pallida</i> var. <i>angustifolia</i>	PI631266	Murray County, OK
<i>E. angustifolia</i> var. <i>strigosa</i>	<i>E. pallida</i> var. <i>angustifolia</i>	PI631320	Pontotoc County, OK
<i>E. atrorubens</i>	<i>E. atrorubens</i> var. <i>atrorubens</i>	PI631255	Douglas County, KS
<i>E. atrorubens</i>	<i>E. atrorubens</i> var. <i>atrorubens</i>	PI631260	Bryan County, OK
<i>E. atrorubens</i>	<i>E. atrorubens</i> var. <i>atrorubens</i>	PI631262	Murray County, OK
<i>E. atrorubens</i>	<i>E. atrorubens</i> var. <i>atrorubens</i>	PI631299	Osage County, KS
<i>E. laevigata</i>	<i>E. laevigata</i>	PI631310	Oconee County, SC
<i>E. laevigata</i>	<i>E. laevigata</i>	PI631312	Oconee County, SC
<i>E. laevigata</i>	<i>E. laevigata</i>	PI631314	Granville County, NC
<i>E. laevigata</i>	<i>E. laevigata</i>	PI631316	Franklin County, VA
<i>E. pallida</i>	<i>E. pallida</i> var. <i>pallida</i>	PI631275	Osage County, OK
<i>E. pallida</i>	<i>E. pallida</i> var. <i>pallida</i>	PI631290	Sac County, IA
<i>E. pallida</i>	<i>E. pallida</i> var. <i>pallida</i>	PI631293	Stone County, AR
<i>E. pallida</i>	<i>E. pallida</i> var. <i>pallida</i>	PI631296	Taney County, MO
<i>E. pallida</i>	<i>E. pallida</i> var. <i>pallida</i>	PI631315	Granville County, NC
<i>E. paradoxa</i> var. <i>neglecta</i>	<i>E. atrorubens</i> var. <i>neglecta</i>	PI631263	Murray County, OK
<i>E. paradoxa</i> var. <i>neglecta</i>	<i>E. atrorubens</i> var. <i>neglecta</i>	PI631264	Murray County, OK
<i>E. paradoxa</i> var. <i>neglecta</i>	<i>E. atrorubens</i> var. <i>neglecta</i>	PI631265	Murray County, OK
<i>E. paradoxa</i> var. <i>paradoxa</i>	<i>E. atrorubens</i> var. <i>paradoxa</i>	PI631292	Stone County, AR
<i>E. paradoxa</i> var. <i>paradoxa</i>	<i>E. atrorubens</i> var. <i>paradoxa</i>	PI631301	Camden County, MO
<i>E. paradoxa</i> var. <i>paradoxa</i>	<i>E. atrorubens</i> var. <i>paradoxa</i>	PI631321	Camden County, MO
<i>E. purpurea</i>	<i>E. purpurea</i>	PI631307	Franklin County, MO
<i>E. purpurea</i>	<i>E. purpurea</i>	PI631313	Madison County, NC
<i>E. purpurea</i>	<i>E. purpurea</i>	PI633669	Caldwell Parish, LA
<i>E. sanguinea</i>	<i>E. pallida</i> var. <i>sanguinea</i>	PI631257	Vernon Parish, LA
<i>E. sanguinea</i>	<i>E. pallida</i> var. <i>sanguinea</i>	PI631258	Vernon Parish, LA
<i>E. sanguinea</i>	<i>E. pallida</i> var. <i>sanguinea</i>	PI633672	Bienville Parish, LA
<i>E. simulata</i>	<i>E. pallida</i> var. <i>simulata</i>	PI631249	Grayson County, KY
<i>E. simulata</i>	<i>E. pallida</i> var. <i>simulata</i>	PI631304	Franklin County, MO
<i>E. simulata</i>	<i>E. pallida</i> var. <i>simulata</i>	PI631308	Rutherford County, TN
<i>E. tennesseensis</i>	<i>E. pallida</i> var. <i>tennesseensis</i>	PI631250	Wilson County, TN
<i>E. tennesseensis</i>	<i>E. pallida</i> var. <i>tennesseensis</i>	PI631324	Wilson County, TN

Taxon (sensu McGregor) [9]	Taxon (sensu Binns et al.) [13]	Accession number	Location of source population
<i>E. tennesseensis</i>	<i>E. pallida</i> var. <i>tennesseensis</i>	PI631325	Wilson County, TN
<i>E. tennesseensis</i>	<i>E. pallida</i> var. <i>tennesseensis</i>	PI631326	Wilson County, TN
Putative hybrid population involving <i>E. paradoxa</i> var. <i>paradoxa</i> and <i>E. pallida</i>	n/a ^b	PI631294	Stone County, AR
Putative hybrid population involving <i>E. paradoxa</i> var. <i>paradoxa</i> and <i>E. simulata</i>	n/a ^b	PI631306	Franklin County, MO

^aThis accession was intermediate in characteristics between McGregor's [9] two described varieties.

^bn/a = Could not be determined through the application of Baum et al. [13].