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BARCODING THE ASTERACEAE OF TENNESSEE, TRIBES HELIANTHEAE AND MILLERIEAE

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ABSTRACT

Results from barcoding studies of tribes Heliantheae and Millerieae for the Tennessee flora using data from the nuclear ribosomal ITS marker region are presented and include first complete reports of this marker for 14 of the 64 species of the two tribes that occur in the state. Sequence data from the ITS region separated almost all genera of the two tribes in Tennessee from one another, with the exception of *Ratibida* and *Rudbeckia*. The ITS data also distinguished almost all species of the smaller genera, including *Ambrosia*, *Echinacea*, *Parthenium*, and *Verbesina* from one another. In contrast, many (though not all) species of the species-rich *Helianthus*, *Rudbeckia*, and *Silphium* were not uniquely distinguished by this marker. ITS sequence data provided support for the recognition of several varieties as distinct species, including *Chrysogonum australe* (vs. *C. virginianum*), *Rudbeckia umbrosa* (vs. *R. fulgida*), and *Silphium reniforme* (vs. *S. compositum*). The results of this study provide further evidence of a heterogeneous time frame for migration of Asteraceae lineages into southeastern North America.

The boundaries of the classically recognized tribe Heliantheae have been significantly altered based on the results of recent molecular studies, and in a narrowed circumscription it is just one of several tribes in a group now referred to as the "Heliantheae alliance" (Baldwin 2009). Millerieae with about 380 species is one of several newly recognized tribes in the Heliantheae alliance; the modified tribe Heliantheae sensu stricto now includes 113 genera and about 1500 species (Anderberg et al. 2007). The current study of these two tribes continues the effort to characterize the levels and patterns of molecular diversity found in species of Asteraceae in Tennessee and southeastern North America (Schilling & Floden 2012, 2013, 2014; Schilling 2013; Schilling et al. 2014, 2015) and to broaden the database and assess the potential of the nuclear ribosomal ITS region as a molecular barcode to identify species.

Heliantheae and Millerieae are most diverse in subtropical and tropical regions, with the largest concentrations of species occurring in Mexico, Central America, and South America (Anderberg et al. 2007). Many of the North American representatives of these tribes represent the northern extensions of their respective genera or lineages. Heliantheae is represented in Tennessee by 15 genera and 61 species (Table 1), of which almost all are native; only three genera, each with a single species in Tennessee (*Eclipta*, *Iva*, and *Xanthium*), are introductions and an additional three species of *Helianthus* are considered to be non-native (Chester et al. 2009). Millerieae in Tennessee includes only three species; the two species of *Galinsoga* are non-native, and a single species of *Smallanthus* is native (Chester et al. 2009).

The goal of this study was to sample the nuclear ribosomal ITS marker for all species of Heliantheae and Millerieae that occur in Tennessee to provide a reference base for future taxonomic studies and particularly to facilitate identification of samples of rare species. Many species of

Heliantheae are considered to be rare in the state, including *Acmella repens*, *Chrysogonum virginianum*, *Echinacea pallida*, *E. simulata*, and *E. tennesseensis*, *Helianthus eggertii*, *H. glaucophyllus*, *H. occidentalis*, and *H. verticillatus*, *Rudbeckia subtomentosa* and *R. triloba* var. *pinnatiloba*, and *Silphium brachiatum*, *S. laciniatum*, and *S. pinnatifidum* (Crabtree 2016).

Materials and methods

DNA was extracted from leaf samples either collected fresh or taken from herbarium specimens (Table 1). For most samples the DNeasy Plant Mini Kit protocol (Qiagen, Valencia CA) was used. PCR amplifications and sequencing of the ITS region followed Schilling et al. (2007). A few samples required the use of the internal primers "5.8S 79 for" and "ITS 5.8SR" for sequencing to obtain clean sequence, either because of fungal contamination or because of length polymorphisms (Schilling et al. 2007). GenBank accession numbers are provided in Table 1. Although this study was not designed to undertake a rigorous phylogenetic analysis, a maximum likelihood tree was generated using GARLI as implemented in the Geneious program package to provide a convenient way to make a comparative visualization of the sequence results. A sample of another member of the "Heliantheae alliance," *Polymnia canadensis* L., was utilized as the outgroup. The analysis also incorporated sequences deposited at GenBank of conspecific samples or closely related species.

Results and discussion

Newly obtained ITS sequences for Heliantheae ranged in length from 628-654 bp. For genera in which multiple species were sampled, there were differences in the amount of length variability: samples of *Echinacea* were uniformly 639 bp; samples of *Silphium* fell into two length classes, with a class all of 630 bp and a second of 639-640 bp; samples of *Helianthus* were mostly 651 bp, with two species at 650 bp and one at 654 bp; species of *Ambrosia*, *Verbesina*, and *Rudbeckia* were most variable, with almost every species having a different ITS sequence length. Sequence length polymorphisms, resolvable by sequencing with multiple primers, were encountered in *Ambrosia bidentata*, *Parthenium auriculatum*, *Silphium perfoliatum*, and *Verbesina virginica*. The samples of the two species of *Galinsoga* each exhibited multiple length polymorphisms, making it impossible to obtain clean sequence across the entire ITS region using direct sequencing approaches; a partial sequence for *G. quadriradiata* was included in the analysis. The sample of *Ratibida pinnata* had an extremely long (14 bp) poly C region that acted in the same manner as a length polymorphism in disrupting the ability to obtain clean sequence downstream from it. Sequence length polymorphisms complicate the rapid interpretation of a region used as a molecular barcode, particularly if they are not observed but can also provide identifying information if interpreted accurately.

The ITS sequences of the sampled genera of Heliantheae were different from one another, with one exception (Fig. 1). Samples of the two species of *Ratibida* were placed as an ingroup to *Rudbeckia* (Fig. 1); this is in contrast to the results reported by Urbatsch et al. (2000), which placed *Ratibida* as sister to *Rudbeckia*, and may represent differences in sampling of both genera.

There were differences in the patterns of distinctiveness of species for ITS sequences among different genera. For *Ambrosia* (4 species) and *Verbesina* (4 species), all of the species in Tennessee were distinct from one another. In *Parthenium*, a genus that has been considered to be represented in Tennessee by only a single species, it appears that the two varieties might represent different species. *Parthenium integrifolium* var. *integrifolium* is very widespread, but *P. integrifolium* var. *hispidum* in Tennessee is distinct in both ITS sequence as well as occupying a distinct habitat in cedar glades; at the species level it has been recognized as *P. auriculatum*, although it might prove to be conspecific with *P. hispidum* from the Ozark region (Weakley 2015). The distinctiveness and relationships among the other named varieties of *P. integrifolium* also needs further assessment. Within *Echinacea*,

Figure 1. Maximum likelihood tree showing relationships of species of Heliantheae and Millerieae based on ITS sequence data, using *Polymnia* (Polymnieae) as the outgroup. Newly obtained sequences designated by DNA number preceding species name (Table 1); GenBank numbers of other sequences follow species name.

the four species were basically identical to one another, differing only by within-sample positional polymorphisms. As has been documented previously, although there was variability within *Helianthus*, many of the 20 species did not have unique ITS sequence (Schilling et al. 1998). For *Rudbeckia* and *Silphium*, there were major groupings of species, but within the groups there were few or no differences. This suggests that there are varied patterns of the place and timing of variation within different genera. For both *Ambrosia* and *Verbesina*, it seems likely that the species that occur in Tennessee are representatives of lineages that have evolved elsewhere. In contrast, differentiation within *Echinacea*, *Helianthus*, *Rudbeckia*, and *Silphium* is probably recent and ongoing in the southeastern USA.

A surprising result was the distinctiveness noted between *Chrysogonum australe* (which does not occur in Tennessee) and *C. virginianum*, because these have consistently been considered to be at most varieties despite being allopatric (Stuessy 1977; Nesom 2001), although Weakley (2015) treats them as distinct. Samples of the two differed by at least 10 changes in ITS sequences. The sequence data are accompanied by morphological differences, and *Chrysogonum australe* is notably distinct based on its prominent rhizomes. Preliminary data from additional samples of *C. virginianum* (not shown) suggest that patterns of differentiation within this species are complex and will require adjustment beyond simply elevating the varieties recognized by Nesom (2001) to species level.

The taxonomically most difficult groups of Heliantheae in the southeastern USA are *Helianthus*, *Rudbeckia*, and *Silphium*, and in each genus there are unresolved taxonomic issues. Perhaps most contentious is the species level taxonomy of *Rudbeckia*, and in the recent floristic guide for Tennessee (Chester et al. 2015) there are actually two different treatments presented, one of which lists 5 species and a total of 12 taxa; the other lists 11 species and 16 total taxa. The major difference in the treatments involves whether to consider *R. fulgida* as a single species with three varieties in Tennessee or separate it into at least 6 species with more outside of the state (e.g., Campbell & Seymour 2013). For the current study it was not possible to undertake the extensive sampling that will be require to resolve the taxonomy. As shown in Figure 1, one of the segregates, *R. umbrosa*, is clearly distinct and is not even placed in the *R. fulgid*a clade, but the remaining ones are not unambiguously different. There are multiple possible explanations for these results, but clearly ITS data will not allow rapid barcoding of variants within *R. fulgida*. Most of the other species of the genus are, however, distinct from one another (Fig. 1). An exception is that samples identified as *R. pinnatilobata* or *R. beadlei* Small (DNA 4606, 4609) from Tennessee were not resolved as distinct from *R. triloba* (DNA 4607); thus there is no support to indicate that the degree of leaf lobing (trilobed vs. pinnately lobed) is taxonomically significant at least within the state. It should be noted that in Claiborne County, where the sample for DNA 4606 originated, plants of the two leaf types both occur in the area. In contrast, the Polk County sample (DNA 4609) had a distinctive overall appearance compared to *R. triloba* as well as a significantly earlier flowering time, so it might still represent a distinct taxon. Also, no sampling was made of *R. pinnatilobata* from Florida, where it was originally described, and it still might represent a distinctive entity.

Within *Silphium*, previous molecular phylogenetic studies have confirmed that the genus is monophyletic (Clevinger & Panero 2000) but have failed to produce a species-level taxonomy that is totally accepted. As found by Clevinger and Panero (2000) the ITS sequence results show that there are several major clades within the genus (Fig. 1), but within each clade there is little differentiation. This result is similar to what was found in *Liatris* (Schilling 2011). The species pair *S. compositum* and *S. reniforme* formed a distinct clade, and the multiple sequence differences between them is in contrast to other clades of the genus, thus supporting morphological differences in suggesting that they be recognized as distinct species rather than varieties of a single species. This clade was not placed with the other clade of sect. *Composita* in the phylogeny, and the two clades differ in the number of ray flowers per head (Clevinger & Panero 2000). In contrast, samples of *S. wasiotense* from its disjunct areas of distribution in Kentucky and Tennessee gave identical ITS sequences, and

the species was placed sister to *S. perfoliatum*, as was reported by Clevinger and Panero (2000). The case of *S. integrifolium* reveals some of the complications of the molecular barcoding approach. A specimen originally used in the current study to represent this species from Tennessee (DNA 5102) proved on closer examination to be a misidentified *S. glabrum*. Although there are several specimens of *S. integrifolium* from Tennessee at TENN, all were collected over 50 years ago, and DNA extracts from them failed to amplify. The sequence used in Figure 1 came from a specimen collected in Missouri, and sequence data from it matched ITS2 sequence data for two recently deposited samples in Genbank. However, in the Genbank records from Clevinger and Panero (2000) for *S. integrifolium* it appears that ITS1 and ITS2 (deposited as separate sequences) did not come from the same original samples; the ITS2 sequences matched the others for *S. integrifolium*, but the ITS1 sequences were significantly different, and two other ITS1 sequences deposited from the Clevinger and Panero (2000) study gave 100% match to the matching region of the ITS sequence from the Missouri specimen. Still another ITS sequence for *S. integrifolium* that was very recently deposited (MH984908) appears to represent an interspecific hybrid, and its source was listed as a botanical garden. As noted further below, sequence data from Genbank should not be accepted uncritically as being accurate.

Although not all species of *Helianthus* had unique ITS sequences, there were some notable patterns of variation within the genus. The ITS sequences of the morphologically distinctive *H. mollis* and *H. occidentalis* were identical; in contrast the sequences of *H. atrorubens* and *H. silphioides*, which have not always been recognized as distinct, were different from one another. We have found some individuals that have ITS sequences that were polymorphic at positions for which *H. atrorubens* and *H. silphioides* differed, suggesting that they were of hybrid origin. Thus, ongoing hybridization may blur the boundaries of these two species. Similarly, hybridization between *H. divaricatus* and *H. microcephalus* has been documented, and one sample collected as *H. microcephalus* was polymorphic in ITS sequence at each of the positions for which these two species differ, suggesting that it was a hybrid. The sample tested of the polyploid species, *H. strumosus*, had numerous sequence polymorphisms, suggesting that it might be a recently formed polyploid in which the ITS region had not yet been homogenized.

The results of BLAST searches in GenBank for members of Heliantheae generally gave a top match, and usually an almost identical sequence, to a conspecific sample if the species had been sampled previously. One exception involved *Heliopsis helianthoides* for which the top hits included identical matches to samples labeled *Helianthus annuus* (MG217894) and *Erigeron* sp. (MF349088) and an almost identical sequence of a sample labeled *Platygyrium leptohymenioides* (AJ288573). Another was a sequence for *Carex radiata* (MG216726) which gave a 100% match to sequences from *Ratibida columnifera*. An ITS sequence purported to be for *Campsis radicans* (MG218111) gave a 100% match to *Helianthus annuus*; one for *Heliopsis helianthoides* (MF348947) was a 100% match for the sequences from *Silphium perfoliatum*. In a somewhat less glaring case, the ITS sequences for two species of *Smallanthus* reported by Rauscher (2002) appear to have been swapped, with that for *S. quitensis* almost identical to our sample of *S. uvedalia*, but the one reported for *S. uvedalia* only 96% similar to ours reported here. The presence of inaccuracies in GenBank shows that caution must be taken in using this database blindly as a reference for species identifications from molecular results.

The results presented here for the nrITS marker show the potential for this region to provide identification of unknown material to genus and in many cases to species for members of Heliantheae in Tennesseee. The results also suggest, however, that broader sampling of both taxa and molecular markers is needed to clarify the species level taxonomy of some genera of the group, notably *Rudbeckia* and *Silphium*.

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Table 1. Plant material used for ITS barcoding studies of Heliantheae and Millerieae. All specimens at TENN unless noted otherwise.

Species	DNA#	Genbank	Voucher info
HELIANTHEAE			
Acmella Rich. Ex Pers.			
A. repens (Walter) Rich.	3099	KX671842	*DeSelm 06-03*, Monroe Co., TN
Ambrosia L.			
A. artemisiifolia L.	2522	KX671843	*Schilling DNA07-2522*, Knox Co., TN
A. bidentata Michx.	3076	KX671844	*Estes 2739*, Giles Co., TN
A. trifida L.	2532	KX671845	*Schilling DNA07-2532*, Knox Co., TN
Chrysogonum L.			
C. virginianum L.	4036	KX671846	*Panero 695*, Scott Co., TN
Non-Tennessee:			
C. australe Alexander ex Small	4029	KX671847	*Rogers 9437A*, Wayne Co., MS
Echinacea Moench			
E. pallida (Nutt.) Nutt.	3101	KX671848	*DeSelm s.n.* 6/29/1992, Coffee Co., TN
E. purpurea (L.) Moench	4490	KX671849	*Floden et al. 2535*, Claiborne Co., TN
E. simulata McGregor	3102	KX671850	*Bailey & Withers* 6/18/2004, Davidson Co., TN
E. tennesseensis (Beadle) Small	3103	KX671851	*Pyne 95-143*, Davidson Co., TN
Eclipta L.			
E. prostrata (L.) L.	2520	KX671852	*Schilling DNA07-2520*, Knox Co., TN
Helianthus L.			
H. angustifolius L.	39	AF047956	*Schilling 89-6*, Morgan Co., TN
H. annuus L.	3242	KX671853	*Schilling 660*, Knox Co., TN
H. atrorubens L.	149	AF047947	*Schilling 140*, Blount Co., TN
H. decapetalus L.	139	AF047940	*Schilling 117*, Knox Co., TN
H. divaricatus L.	154	AF047954	*Schilling OS-98*, Sevier Co., TN
H. eggertii Small	107	AF047962	*Schilling 90-16*, Davidson Co., TN
H. giganteus L.	250	AF047939	*Schilling 171*, Jefferson Co., TN
H. glaucophyllus D.M.Sm.	138	AF047941	*Schilling 138*, Unicoi Co., TN
H. grosseserratus M.Martens	301	AF047951	*Schilling 301*, Knox Co., TN
H. hirsutus Raf.	127	AF047963	*Schilling 90-17*, Davidson Co., TN
H. maximiliani Schrad.	109	AF047949	*Schilling 109*, Knox Co., TN
H. microcephalus Torr.&A.Gray	131	KX671855	*Schilling 131*, Knox Co., TN
H. mollis Willd.	203	AF047946	*Schilling 90-8*, Davidson Co., TN
H. occidentalis Riddell	213	AF047944	*Schilling 303*, Davidson Co., TN
H. silphioides Nutt.	3247	AF047936	*Schilling & Spring Ark-47*, Hardeman Co., TN
H. smithii Heiser	3253	KX671856	*Schilling & Spring Ark-44*, Polk Co., TN
H. strumosus L.	133	KX671857	*Schilling 90-26*, Davidson Co., TN

H. tuberosus L.	146	KX671858	*Schilling 90-3*, Davidson Co., TN
H. verticillatus Small	1188	AF315080	UT Gardens Plant
H. x *laetiflorus* Pers.	216	KX671854	*Schilling 90-9*, Knox Co., TN
Heliopsis Pers.			
H. helianthoides Sweet	2514	KX671859	*Schilling DNA07-2514*, Knox Co., TN
Iva L.			
I. annua L.	2561	KX671860	*Schilling DNA07-2561*, Knox Co., TN
Melanthera Rohr.			
M. nivea (L.) Small	3100	KX671861	*Lincicome & McCoy s.n.*, 9/2002, Perry Co., TN
Parthenium L.			
P. auriculatum Britton	3524	KX671862	*Guthrie 1988*, Decatur Co., TN
P. integrifolium L.	2517	KX671863	*Schilling DNA07-2517*, Knox Co., TN
Ratibida Raf.			
R. columnifera Raf.	3072	KX671864	*DeSelm s.n.* 7/12/97, Campbell Co., TN
R. pinnata Barnh.	3073	KX671865	*McNeilus 99-546*, Knox Co., TN
Rudbeckia L.			
R. fulgida Aiton	3074	KX671866	*Estes & Beck 8360*, Coffee Co., TN
	4006	KX671867	*DeSelm s.n.*, Williamson Co., TN
	4007	KX671868	*Beck 6260*, Marion Co., TN
R. hirta L.	2541	KX671869	*Schilling DNA07-2541*, Knox Co., TN
R. laciniata L.	2571	KX671870	*Schilling DNA07-2571*, Knox Co., TN
R. subtomentosa Pursh	3075	KX671871	*Chester 4202*, Montgomery Co., TN
R. tenax C.L.Boynton & Beadle	4005	KX671872	*Datillo 1015*, Maury Co., TN
R. triloba L.	4607	KX671873	*Estes et al.* Cumberland Co., TN
	4009	KX671874	*Floden et al 2012-198*, Polk Co., TN
	4606	KX671875	*Floden et al 2536*, Claiborne Co., TN
R. truncata Small	3535	KX671876	*Floden & Zale 2279*, Union Co., TN
R. umbrosa C.L.Boynton & Beadle	4008	KX671877	*Estes 1464*, Lewis Co., TN
Silphium L.			
S. asteriscus L.	2589	KX671878	*Schilling DNA07-2589*, Knox Co., TN
	4018	KX671879	*Bailey & Lincicome s.n.*, Franklin Co., TN
S. brachiatum Gatt.	3077	KX671880	*Patrick et al. 1555a*, Franklin Co., TN
S. compositum Michx.	3078	KX671881	*DeSelm s.n.* 8/23/2005, Roane Co., TN
	4012	KX671882	*DeSelm s.n.*, Greene Co., TN
S. glabrum Eggert	3079	KX671883	*Patrick 1564a*, Franklin Co., TN
	3080	KX671884	*DeSelm s.n. 8/29/1997*, Decatur Co., TN
	5102	MK305127	*Bailey & Harden 8/18/2004*, McNairy Co, TN
S. laciniatum L.	4014	KX671886	*McNeilus 96-844*, Haywood Co., TN
S. mohrii Small	4020	KX671887	*Estes 922*, Giles Co., TN
S. perfoliatum L.	4015	KX671888	*DeSelm 04-18*, Hancock Co., TN
S. pinnatifidum Elliott	3104	KX671889	*Pyne 94-262*, Rutherford Co., TN

	4013	KX671890	*Beck 4828*, Hamilton Co., TN
S. reniforme Raf.	3988	KX671891	*Floden & Schilling 8/15/2013*, Greene Co., TN
S. terebinthaceum Jacq.	3105	KX671892	*DeSelm s.n. 8/26/1988*, Sevier Co., TN
S. wasiotense Medley	2937	KX671893	*Floden 565*, Campbell Co., TN
Non-Tennessee:			
S. albiflorum A. Gray	3964	KX671894	UT Gardens Plant
S. glutinosum J.R.Allison	3969	KX671895	UT Gardens Plant
S. integrifolium Michx.	5075	MK305126	*Yatskievych 4257*, MO (MO)
S. wasiotense Medley	2938	KX671896	*Risk 6758*, Knox Co., KY
Verbesina L.			
V. alternifolia (L.) Britton	2572	KX671897	*Schilling DNA07-2572*, Knox Co., TN
V. helianthoides Michx.	3071	KX671898	*Chester 14636*, Gibson Co., TN
V. occidentalis (L.) Walter	2508	KX671899	*Schilling DNA07-2508*, Knox Co., TN
V. virginica L.	2515	KX671900	*Schilling DNA07-2515*, Knox Co., TN
Non-Tennessee:			
V. aristata A.Heller	4021	KX671901	*McNeilus 90-726*, Santa Rosa Co., FL
	4022	KX671902	*Kral 31363*, Geneva Co., AL
V. chapmaii J.R.Coleman	4023	KX671903	*Evans s.n.*, 5/25/1979, Liberty Co., FL
V. microptera J.R.Coleman	4491	KX671904	*Keeney s.n.*, 9/15/1972, Uvalde Co., TX
V. walteri Shinners	4024	KX671905	*Thomas* 152955, Pike Co., MS
Xanthium L.			
X. strumarium L.	2625	KX671906	*Schilling DNA07-2625*, Knox Co., TN
MILLERIEAE			
Galinsoga Ruiz & Pav.			
G. parviflora Cav.	3512	-	*Kelly 1040*, Williamson Co., TN
G. quadriradiata Ruiz & Pav.	3227	KX671907	*Schilling 11-21*, Knox Co., TN
Smallanthus Mack.			
S. uvedalius (L.) Mack.	2586	KX671908	*Schilling DNA07-2586*, Knox Co., TN