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BARCODING THE ASTERACEAE OF TENNESSEE, TRIBES GNAPHALIEAE AND INULEAE

EDWARD E. SCHILLING and **AARON FLODEN**
Herbarium TENN
Department of Ecology & Evolutionary Biology
University of Tennessee
Knoxville, Tennessee 37996
eschilling@utk.edu and afloden@utk.edu

ABSTRACT

Results from barcoding studies of tribes Gnaphalieae and Inuleae for the Tennessee flora using data from the nuclear ribosomal ITS marker region are presented, and include first complete reports of this marker for 10 of the 13 species of these tribes that occur in the state. Sequence data from the ITS region separated all genera and most species of Gnaphalieae from Tennessee. Species pairs that were not distinguished included *Antennaria plantaginifolia/ A. parlinii* and *Pseudognaphalium helleri/ P. micradenium*. The ITS sequence data provided supporting evidence for recent changes in the classification of the group, most notably segregation of *Gamochaeta* and *Pseudognaphalium* from *Gnaphalium*, as well as the species-level taxonomy of *Gamochaeta*. Issues were noted with current GenBank records, including apparent poor quality of some sequence data and possible mistakes in species identifications, which must be taken into account in barcoding efforts and which highlight the need to expand this highly useful database.

KEY WORDS: Asteraceae, Gnaphalieae, Inuleae, molecular barcoding

The Gnaphalieae, generally referred to as "cudweeds", are relatively anonymous yet common and widespread plants. They can be easily be overlooked or passed up by collectors. The major diversification of the tribe has occurred in the southern hemisphere, and the species from southeastern North America have not been well sampled in broad phylogenetic surveys of the tribe (e.g. Ward et al. 2009; Galbany-Casals et al. 2010; Nie et al. 2012). The current study was undertaken as part of a general effort to increase sampling for molecular markers of Asteraceae from southeastern North America in general and the state of Tennessee in particular.

The classification of Gnaphalieae has changed dramatically at several levels in recent years. Gnaphalieae were once included in Inuleae, but more recent studies have shown that the two tribes are clearly distinct (Bremer et al. 1994). Both tribes are characterized by having heads that are often discoid or disciform, but Gnaphalieae is the sister group to Astereae and Anthemidae whereas Inuleae is a lineage at the base of the clade that includes the Heliantheae alliance (Funk et al. 2009). The generic level classification has also changed, with a formerly large *Gnaphalium* retaining the Old World species but the basically New World *Gamochaeta* and *Pseudognaphalium* being segregated (Nesom 1990). Species level recognition has also been sharpened through careful studies that have refined circumscriptions and even described several previously unrecognized species in *Gamochaeta* and *Pseudognaphalium* (Nesom 1990, 2001a, 2004a). The relatively technical features required to recognize accurately species of the genera of Gnaphalieae make them a logical target that would benefit from a molecular barcoding approach (Kress et al. 2005) to verify identifications.

Gnaphalieae are represented in the state of Tennessee by a total of 12 species (Table 1), most of which were traditionally placed (e.g. Cronquist 1980) in two genera, *Gnaphalium* and *Antennaria*, as well as the introduced *Facelis*. Current classification separates *Gnaphalium* in Tennessee into

three different genera, including *Gamochaeta* and *Pseudognaphalium* (Table 1). Inuleae is represented by a single species, *Pluchea camphorata*.

Most of the species of Gnaphalieae in Tennessee are currently considered to be native to the flora, although their ruderal nature makes it difficult to be certain in all cases (Nesom 2004b). A species that appears to be clearly introduced is *Facelis retusa*, a South American weedy species (Cronquist 1980). *Gnaphalium uliginosum* is considered by Cronquist (1980) to be introduced from Europe, although its probable sister relationship to *G. exilifolium* A. Nelson suggests that it is likely also to be native, perhaps in part, to North America (Nesom 2001b). One species, *Pseudognaphalium helleri*, is listed as a species of special concern for Tennessee with a state ranking of S2, based on its rarity within the state, although its global ranking of G4/G5 indicates that it is common in other parts of its range (Crabtree 2012).

Prior to initiation of this study, GenBank records for the complete nuclear ribosomal ITS region were reported for only three of the thirteen species of Gnaphalieae and Inuleae found in Tennessee. The GenBank records for *Antennaria* are relatively old and consist of separate ITS-1 and ITS-2 sequences rather than the entire ITS region as a single entity. The purpose of the study was to make a survey of sequence variation for the ITS region across Gnaphalieae and Inuleae that have been collected in the state of Tennessee. The goals included expanding the database of available samples for both barcoding and phylogenetic studies and assessing the value of this marker in identifying members of these tribes to genus or species level.

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 (Qiagen, Valencia CA) was used, although some freshly collected samples were processed using the CTAB method (Doyle & Doyle 1987). PCR amplifications and sequencing of the ITS region followed protocols outlined by 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, likely because of fungal contamination (Schilling et al. 2007). GenBank accession numbers are provided in Table 1. Although this study was not designed to undertake a rigorous phylogenetic analysis, parsimony analysis using the PAUP* 4.0b10 program (Swofford 2003) was utilized to provide a convenient way to make a comparative visualization of the sequence results and incorporated sequences deposited at GenBank of conspecific or closely related samples. This included a sequence for *Anaphalis margaritacea* (L.) Benth., which though native to North America has not been clearly documented to occur in Tennessee outside of horticultural settings.

RESULTS AND DISCUSSION

The complete ITS sequence region (ITS-1, 5.8S ribosomal DNA, ITS-2) varied in length in the newly reported Gnaphalieae samples from 633–641 bp. Sequences of *Pseudognaphalium* were consistently 633 bp; those of *Gamochaeta* 636–637 bp, and *Antennaria* showed the most length variation, with three of the four sampled species having a different length (636–638 bp). The ITS sequences for *Gnaphalium* (641 bp) and *Pluchea* (642 bp) were the longest of those sampled. There was no evidence in the electropherograms that any sample had multiple polymorphisms, either length or at individual sequence sites, which would provide evidence of recent or fixed interspecific hybridization.

Results of a phylogenetic analysis of the ITS sequence data for samples of Gnaphalieae are shown in Figure 1, with the single member of Inuleae used as the outgroup. Each genus was placed as monophyletic with moderate to strong bootstrap support. *Gamochaeta*, which was once considered to be part of *Gnaphalium*, was placed sister to *Facelis* and further sister to *Antennaria* (Fig. 1).

Pseudognaphalium, also formerly considered part of *Gnaphalium*, was placed as sister to *Anaphalis* with strong support.

Table 1. Plant material used for ITS barcoding studies of Gnaphalieae and Inuleae. All specimens at TENN, collected in Tennessee.

Species	**DNA#**	**Genbank**	**Voucher info**
ANTENNARIA Gaertn.			
A. howellii subsp. *neodioica* (Greene) R. Bayer	3444	JX524604	*Sharp 26822*, Sevier Co.
A. parlinii Fernald	3446	JX524605	*Murrell* 75, Polk Co.
A. plantaginifolia (L.) Hook.	2542	JX524601	*Schilling 07-2542*, Knox Co.
	3445	JX524602	*Sharp 26281*, Sevier Co.
A. solitaria Rydb.	3082	JX524603	*Crabtree FSF-07-034*, Marion Co.
FACELIS Cass.			
F. retusa (Lam.) Sch.Bip.	3083	JX524606	*Beck 4671*, Marion Co.
GAMOCHAETA Wedd.			
G. argyrinea G.L. Nesom	2766	JX524596	*Schilling 08-2766*, Knox Co.
	3084	JX524597	*Phillippe 35455*, DeKalb Co.
G. pensylvanica (Willd.) Cabrera	3085	JX524600	*Browne 78*, Shelby Co.
G. purpurea (L.) Cabrera	3443	JX524598	*Estes 7859*, Moore Co.
	3086	JX524599	*Bresowar 122*, Knox Co.
GNAPHALIUM L.			
G. uliginosum L.	3087	JX524592	*Henry et al. s.n.*, Cocke Co.
PSEUDOGNAPHALIUM Kirp.			
P. helleri (Britton) Anderb.	3089	JX524593	*DeSelm s.n.*, Franklin Co.
P. micradenium (Weath.) G.L. Nesom	3442	JX524594	*Patrick 3813*, Roane Co.
P. obtusifolium (L.) Hilliard & B.L.Burtt.	2566	JX524595	*Schilling CF-4*, Unicoi Co.
PLUCHEA Cass.			
P. camphorata (L.) DC.	3088	JX524607	*McNeilus 00-919*, Fentress Co.

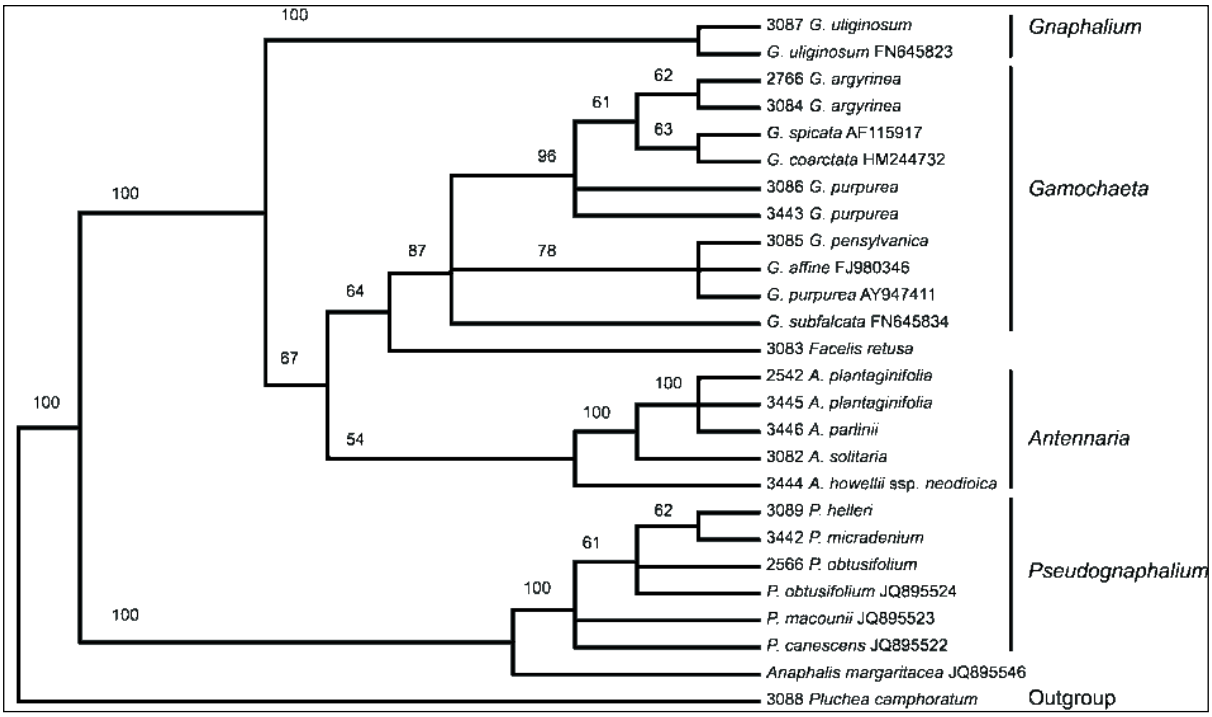

Figure 1. Single shortest tree from parsimony analysis of nuclear ribosomal ITS data showing relationships among species of Gnaphalieae from Tennessee. Bootstrap values shown above branches. Samples labeled by DNA number (Tennessee samples, Table 1) or GenBank accession number. The sample of *Pluchea camphorata* (Inuleae) was used as the outgroup

Antennaria. Four species of *Antennaria* are recorded for Tennessee, of about 45 species total for the genus. The genus is clearly distinguished from other Gnaphalieae by at least 32 bp changes in the ITS sequences. The two samples of *A. plantaginifolia* were identical — the second sample tested was collected at the exact same site as the sample of *A. howellii* subsp. *neodioica* — and also identical to the sample of *A. parlinii*. All of the other species were separated by multiple changes, with 11 differences between *A. solitaria* and *A. plantaginifolia* and 25–27 differences between *A. howellii* subsp. *neodioica* and the other species. Complete ITS sequences (e.g., ITS-1, 5.8S rDNA, ITS-2) have not been previously deposited in GenBank for *A. parlinii*, *A. plantaginifolia*, or *A. solitaria*. Older records include individual ITS-1 and ITS-2 sequences for *A. plantaginifolia* and *A. solitaria*, but these were not retrieved in a BLAST search with standard parameters using newly obtained complete ITS sequences. The older sequences required several small gaps for alignment, which likely reflects the lower accuracy that was achieved using older technology.

Facelis. *Facelis* is represented in Tennessee by a single introduced species, *F. retusa*. The current report is the first record for the genus, which has 3-4 species total, in GenBank.

Gamochaeta. The three species of *Gamochaeta* documented from Tennessee were each distinctive for ITS sequence, with pairwise differences as follows: *G. argyrinea*-*G. pensylvanica*, 9 differences; *G. argyrinea*-*G. purpurea*, 4 differences; *G. pensylvanica*-*G. purpurea*, 5 differences. No intraspecific variation was encountered in the two samples each of *G. argyrinea* and *G. purpurea* (Table 1). Samples from GenBank labeled *G. purpurea* and *G. affine* were identical to our sample of *G. pensylvanica*, raising questions about whether they were accurately identified. Also distinct based on a GenBank sequence deposited for it was *G. coarctata* (Willd.) Kerguélen, which may be expected

to be found naturalized in western Tennessee (Guy Nesom, pers. comm.) The name for another GenBank sample, *G. spicata* (Lam.) Cabrera, is now considered to be a synonym for *G. coarctata* (Pruski & Nesom 2004), and sequences for the two GenBank samples were identical and differed at 3 positions from those of *G. argyrinea*. The distinctiveness of individual species of *Gamochaeta* is notable, especially considering that *G. argyrinea* was only recently recognized to be distinct from *G. purpurea* (Nesom 2004a). Similar results were obtained with a broader sampling of both species and samples of *Gamochaeta* by Cameron (2010), and the molecular results are further supported by detailed morphological analysis (Mac Alford and Kree Cameron, pers. comm.) Thus, a barcoding approach can be utilized to confirm the identity of samples of *Gamochaeta* collected in Tennessee.

Gnaphalium. *Gnaphalium* as currently interpreted is represented by a single recent collection of the apparently non-native *G. uliginosum* from eastern Tennessee. The ITS sequence from this sample was almost identical with a GenBank record for the species. It is clearly distinct from all other Gnaphalieae of Tennessee, differing by at least 51 bp changes.

Pseudognaphalium. The amount of interspecific variation for *Pseudognaphalium* was the least observed for any of the genera of Gnaphalieae in Tennessee. Samples of *P. helleri* and *P. micradenium* were identical to one another and differed by two changes from a sample of *P. obtusifolium*. A GenBank sequence for *P. obtusifolium* differed at a single position from our sample, and two other *Pseudognaphalium* sequences at GenBank (*P. macounii* and *P. canescens*, identification of the latter determined by G. Nesom) differed at three positions. These results suggested that species level diversification in *Pseudognaphalium* might be relatively recent, similar to what has been observed for other Asteraceae genera in eastern North America, such as *Helianthus* and *Solidago*.

Pluchea. A single species of *Pluchea* out of the 40 or more species of the genus is native to Tennessee. The ITS sequence is the first record for the species in GenBank, and it is significantly different (minimum of 45 bp differences and 6 gaps) from other species of the genus represented in GenBank.

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