Schilling, E.E. 2013. Barcoding the Asteraceae of Tennessee, tribes Cardueae and Vernonieae. Phytoneuron 2013-19: 1–7.
Published 20 March 2013. ISSN 2153 733X

BARCODING THE ASTERACEAE OF TENNESSEE, TRIBES CARDUEAE AND VERNONIEAE

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ABSTRACT

Results from barcoding studies of tribes Cardueae and Vernonieae for the Tennessee flora using data from the nuclear ribosomal ITS marker region are presented and include first complete reports of this marker for 5 of the 19 species of these tribes that occur in the state. Sequence data from the ITS region separated all species of Cardueae from Tennessee from one another, although some species of *Cirsium* differed only by positional polymorphisms from other, non-Tennessee species. In contrast, species of *Vernonia*, both from the state and from other parts of the southeastern USA, had basically identical ITS sequences. Interpretation of some of the sequences was complicated by the presence of length polymorphisms. Issues were noted with current GenBank records, including possible mistakes in species labeling or identifications, which must be taken into account in barcoding efforts.

KEY WORDS: *Cirsium*, *Vernonia*, Cardueae, Vernonieae, molecular barcoding

Tribes Cardueae and Vernonieae are similar in having discoid or disciform heads that in the Tennessee species are often purplish in color, and a pappus typically of bristles. The major diversification of both tribes occurred outside of North America, with Cardueae primarily Old World (Susanna et al. 2006) and Vernonieae having an Old World origin and secondary radiation in South America (Keeley et al. 2007). Each tribe, however, has a genus that shows a small burst of diversity in the southeastern USA: *Cirsium* in Cardueae and *Vernonia* in Vernonieae. In these two genera, lack of clearcut morphological differentiation can make species level identification difficult, and part of the rationale for the current study was to assess the potential for the nuclear ribosomal ITS region to serve as a molecular barcode to identify species. The ITS region has continued to receive attention as a potential barcoding region for plants (Yao et al. 2010; Li et al. 2011). In addition, the study continues the effort to characterize the level and patterns of molecular diversity found in species of Asteraceae in Tennessee (Schilling & Floden 2012).

Cardueae is represented in Tennessee by four genera and 13 species (Chester et al. 2009). All of the species of three of the genera, *Arctium*, *Carduus*, and *Centaurea*, are introduced, with several considered to be problematic as invasives. ITS sequences are available in GenBank for all but one of the introduced species. The fourth genus, *Cirsium*, is represented by seven species, five of which are native and not listed as invasives; the other two, *C. arvense* and *C. vulgare*, are considered to be non-native invasives. *Cirsium* in North America has been the focus for two studies (Kelch & Baldwin 2003; Slotta et al. 2012), but ITS sequence data are available in GenBank for only two of the five species native to Tennessee.

Vernonieae is represented in Tennessee by two genera and six species, all of which are considered to be native (Chester et al. 2009). Although common in old fields, none of the species of Vernonieae is considered to be a problematic invasive. Two morphologically distinctive species of the otherwise mostly tropical and subtropical *Elephantopus* occur in Tennessee. The four species of

Vernonia that occur in Tennessee are distinct but individual specimens are sometimes difficult to identify because species boundaries may be blurred by the presence of hybrids (Urbatsch 1972). None of the species is listed as rare, although *V. flaccidifolia* is a southern Appalachian endemic (Burnett et al. 1977) that is known from only a few counties in the state.

The goal of this study was to complete the sampling for the ITS marker for all species of Cardueae and Vernonieae that occur in Tennessee. Particular emphasis was placed on the two genera, *Cirsium* (Cardueae) and *Vernonia* (Vernonieae), which have radiated in southeastern North America. Sampling of additional species of both genera from areas of southeastern North America outside of Tennessee was done to allow evaluation of the overall patterns of diversification and compare them to other speciose Asteraceae genera of the region.

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, 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, neighbor-joining analyses using the PAUP* 4.0b10 program (Swofford 2003) were utilized to provide a way to make a comparative visualization of the sequence results, with the sample of *Arctium minus* utilized as a convenient outgroup for both tribes. The analysis also incorporated sequences deposited at GenBank of conspecific samples or closely related species.

Results and discussion

Newly obtained ITS sequences for Cardueae ranged in length from 636-645 bp. Sequences of *Cirsium* and *Carduus* were uniformly 644-645 bp; the sequence of *Arctium* was 640 bp; those of *Centaurea* were 634 or 635 bp. The sequences of *Cirsium* species had relatively high numbers of positional polymorphisms, with *C. arvense*, *C. discolor*, *C. muticum*, and *C. vulgare* having eight or more polymorphic positions, and only one species, *C. altissimum*, having fewer than three polymorphisms. It was impossible to obtain a complete sequence for *Centaurea stoebe* using our conditions because of the presence of length polymorphisms involving at least three positions; other approaches such as cloning would be required to obtain the individual underlying sequences.

The newly obtained sequences for individual species of Cardueae produced the highest match to conspecific records in GenBank where those were available, although only the sequence for *Arctium minus* was identical to its GenBank counterpart. Most of the differences among conspecific samples involved the presence of a polymorphic vs. fixed position. Sequences for *Cirsium altissimum*, *C. carolinianum*, *C. horridulum*, and *Centaurea nigrescens* were first reports for the respective species.

The ITS sequences of the sampled genera of Cardueae were quite different from one another, and sequences for the individual Tennessee species were also somewhat different from each other (Figure 1). Thus, a barcoding approach using ITS sequence data could be employed to verify identifications of members of Cardueae in Tennessee. However, the sequence for *Cirsium carolinianum* differed from that of *C. altissimum* only by 3 positions that were polymorphic, and the sequence of *C. horridulum* differed by only a single bp from those of the more western *C. pitcheri* and *C. foliosum*. The length polymorphisms in *Centaurea stoebe* would also lead to complications in interpreting ITS sequence from any unknowns of this species.

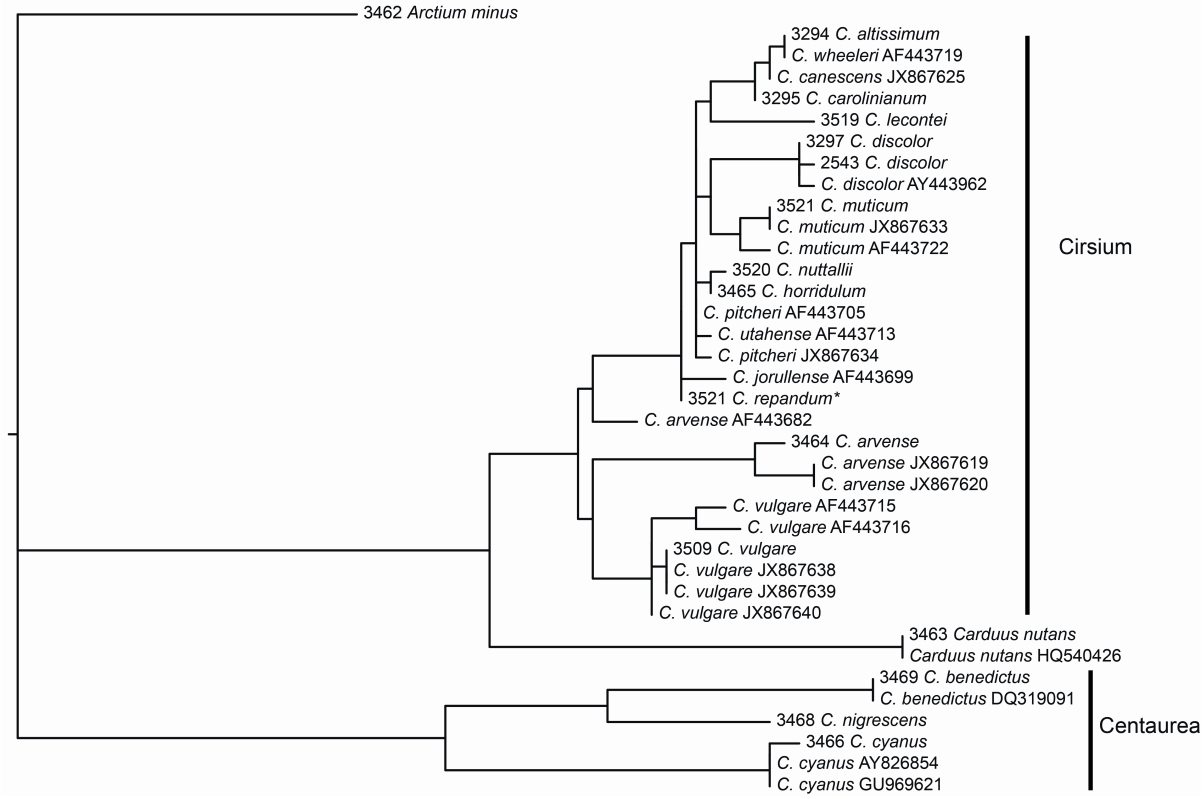

Figure 1. Neighbor-joining tree showing relationships of species of Cardueae based on ITS sequence data, using *Arctium minus* as the outgroup. Newly obtained sequences designated by DNA number preceding species name (Table 1); GenBank numbers for other sequences follow species name. *partial sequence used for *C. repandum*.

The pattern of variability observed for ITS sequences in *Cirsium* is consistent with a recent diversification in eastern North America, but not limited to this region. There was a low number of differences between species for ITS sequences, both within the region and compared to more western species such as *C. foliosum*, *C. pitcheri*, and even the Mexican *C. jorullense* (Figure 1). In contrast to other members of Asteraceae that have diversified in the southeastern USA, the species with the most southeasterly geographic distributions were not placed in a basal position on the tree. These results are in agreement with larger samplings of the genus in North America by Kelch and Baldwin (2003) and Slotta et al. (2012).

The newly obtained ITS sequences for Vernonieae ranged in length from 622 to 643 bp. The sequences for the two Tennessee species of *Elephantopus* both were 633 bp. The sequences for the Tennessee species of *Vernonia* were either 622 or 624 bp in length, with a single indel of 2 bp in the ITS-1 region accounting for the difference. The longer sequence length was reported in GenBank records for *V. noveboracensis*, but there was variability in the Tennessee samples of this species. Three samples were checked because of the discrepancy between the initial sampling and the GenBank records: two samples had an ITS length of 622 bp, but the other one gave results consistent with a length polymorphism at this position and could be inferred to have alleles of both lengths. Evidence of the length polymorphism at the same position was seen in sequences obtained for two species from the Coastal Plain areas outside of Tennessee, *V. blodgettii* and *V. glauca*.

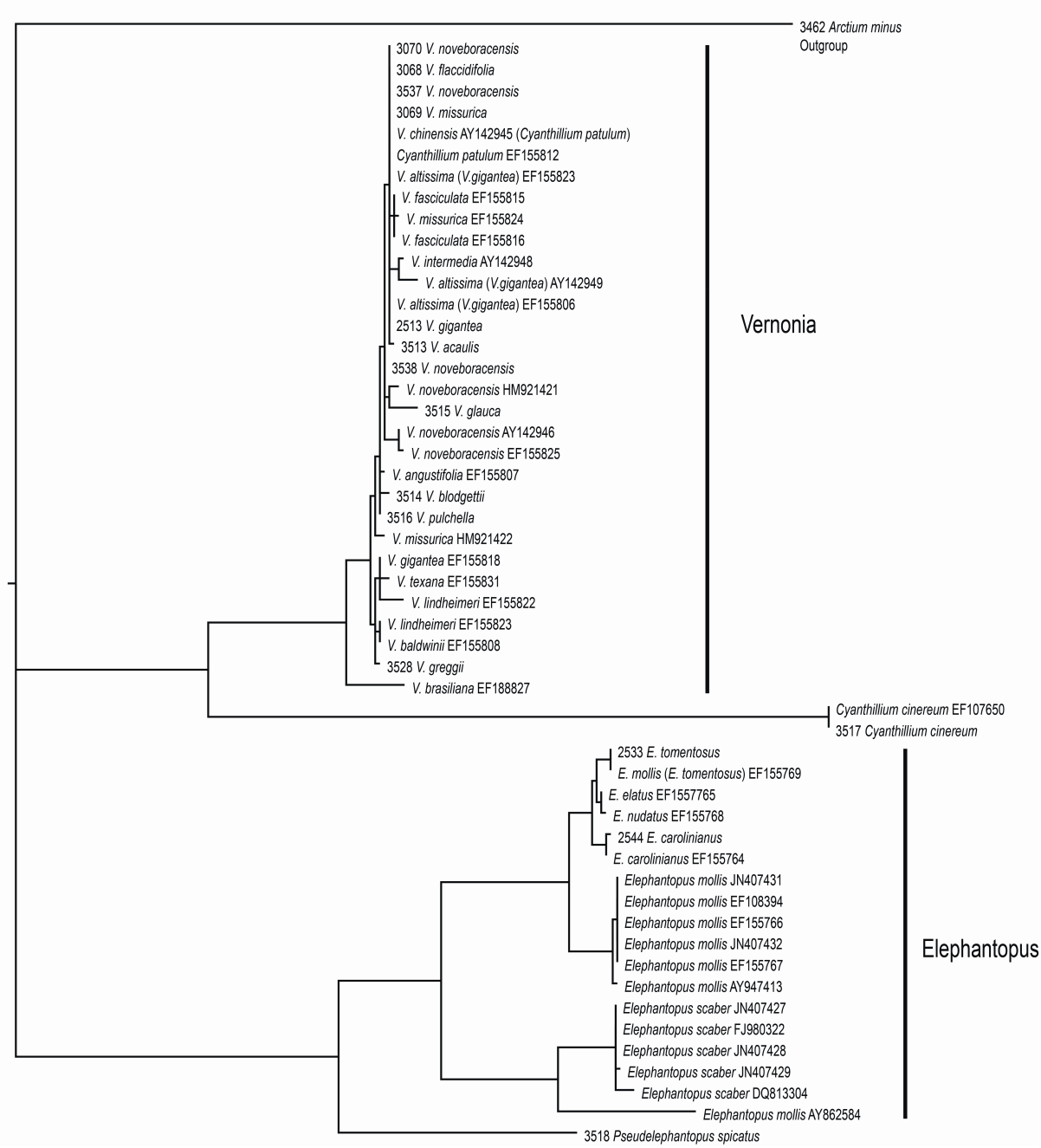

Figure 2. Neighbor-joining tree showing relationships of species of Vernonieae based on ITS sequence data, using *Arctium minus* as the outgroup. Newly obtained sequences designated by DNA number (Table 1) preceding species name; GenBank numbers for other sequences follow species name. Species names in parentheses indicate where the likely identity is different from the GenBank listing for a sample.

Other than the length difference and some positional polymorphisms, the ITS sequences for the Tennessee species of *Vernonia* were basically identical to one another (Figure 2). They were also nearly identical to other species of *Vernonia* both from the southeastern U.S. and also from southwestern North America. Thus, the ITS region would not serve as a barcode to confirm a species-level identification for this genus. In contrast, the two Tennessee species of *Elephantopus*

were separated from each other and from other species of the genus for which data are available (Figure 2).

The contrast between having differentiated, apparently species-specific ITS sequences in *Cirsium* with the lack of divergence in the ITS marker in *Vernonia* may reflect the difference in breeding barriers in the two genera in eastern North America. Natural hybrids in *Cirsium* have been found to be sterile because of chromosomal repatterning (Bloom 1977; Dabydeen 1997). In contrast, several studies have noted that interspecific hybridization is frequent between members of *Vernonia* from eastern North America (Jones 1967, 1968, 1972, 1976; Urbatsch 1972), and no reduction in hybrid fertility was reported (Jones 1967).

The results of BLAST searches in GenBank for some species of Vernonieae led to additional sampling of non-Tennessee samples, and the results highlight some problems in using this database as a reference for species identification. The GenBank sequences identified as coming from *Cyanthillium patulum* and *Vernonia chinensis* (now considered a synonym of *C. patulum*; Robinson 1990) were almost identical to one another and to the sequences for several southeastern USA samples of *Vernonia* (Figure 2). This raised the possibility that *Cyanthillium* may be improperly separated from *Vernonia*, and to check this, a sample of *Cyanthillium* (*C. cinereum*) available at TENN was sequenced. The sequence for *C. cinereum* was identical to a GenBank sequence from this species and was quite divergent from North American *Vernonia* (Figure 2). Thus, either the identities of the GenBank samples of *Cyanthillium patulum*/ *Vernonia chinensis* are incorrect, or this species is incorrectly placed in *Cyanthillium*. The highest BLAST identity for the sample of *Elephantopus tomentosus* was to a GenBank sample identified as *E. mollis*, but both were clearly separated from other samples of *E. mollis* (Figure 2). However, this sample (EF155769) was identified as *E. tomentosus* in a publication (Keeley et al. 2007); the reason for the discrepancy is not clear. Still another sample deposited in GenBank as *E. mollis*, AY862584, appeared to be significantly different from the multiple other samples of this species (Figure 2), but its identity is not clear because its highest BLAST match was only 92%, to samples of *E. scaber*. These results show that GenBank cannot be used uncritically as a reference for comparison of molecular barcoding data.

ACKNOWLEDGEMENTS

Thanks to S. Robertson, D. Schilling, and J. Schilling for laboratory assistance, J. May and V. Brown of the University of Tennessee MBRF for help with DNA sequencing, A. Floden and L. Urbatsch for helpful feedback, and the numerous collectors, notably D. Estes and R. D. Thomas, who have helped to assemble a broad and useful herbarium at TENN. Financial support provided by the Hesler Fund of the University of Tennessee Herbarium.

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Table 1. Plant material used for ITS barcoding studies of Cardueae and Vernonieae. All voucher specimens at TENN.

Species	DNA#	Genbank	Voucher info
CARDUEAE			
ARCTIUM L.			
A. minus (Hill) Bernh.	3462	KC603906	*Estes 6432*, Giles Co., TN
CARDUUS L.			
C. nutans L.	3463	KC603920	*Estes 2041*, Giles Co., TN
CENTAUREA L.			
C. benedicta (L.) L.	3469	KC603917	*Jackson 20432*, Giles Co., TN

C. cyanus L.	3466	KC603919	*Thomas 172731*, Knox Co., TN
C. nigrescens Willd.	3468	KC603918	*Wiggins s.n*, 8/4/2003, Blount Co., TN
C. stoebe L.	3467	-	*DeSelm s.n.*, 7/16/2000 Cocke Co., TN
CIRSIUM Mill.			
C. altissimum (L.) Hill	3294	KC603907	*Estes 4025*, Co., TN
C. arvense (L.) Scop.	3464	KC603911	*Estes & Estes 04818*, Claiborne Co., TN
C. carolinianum (Walt.) Fern.& B.G. Schub.	3295	KC603909	*McNeilus 93-337*, Co., TN
C. discolor (Muhl. ex Willd.) Spreng.	2543	KC603916	*Schilling DNA07-2543*, Knox Co., TN
	3297	KC603908	*Estes 5402*, Co., TN
C. horridulum Michx.	3465	KC603910	*Pyne 93-046*, Decatur Co., TN
C. muticum Michx.	3296	KC603912	*Estes 1236*, Co., TN
C. vulgare (Savi) Ten.	3509	KC603921	*Baskauf s.n.*, 6/8/2003, Cheatham Co., TN
non-Tennessee species			
C. lecontei Torr. & A.Gray	3519	KC603913	*Thomas 119790*, St. Tammany Par., LA
C. nuttallii DC.	3520	KC603914	*McNeilus 98-2*, Charlotte Co, FL
C. repandum Michx.	3521	KC603915	*Morton 7827*, Georgetown Co SC
VERNONIEAE			
ELEPHANTOPUS L.			
E. carolinianus Willd.	2544	KC603934	*Schilling 07-DNA2544*, Knox Co., TN
E. tomentosus L.	2533	KC603933	*Schilling 07-DNA2533*, Knox Co., TN
VERNONIA Schreb.			
V. flaccidifolia Small	3068	KC603923	*Kral 60615*, Franklin Co., TN
V. gigantea (Walt.) Branner & Coville	2513	KC603927	*Schilling 07-DNA2513*, Knox Co., TN
V. missurica Raf.	3069	KC603926	*Pyne 94-283*, Rutherford Co., TN
V. noveboracensis (L.) Willd.	3070	KC603922	*Durr s.n. 9/5/2005*, Campbell Co., TN
	3537	KC603924	*Estes et al. 111*, Polk Co., TN
	3538	KC603925	*Estes & Beck 8493*, Van Buren Co., TN
non-Tennessee species			
V. acaulis (Walt.) Gleason	3513	KC603928	*McNeilus 93-1924*, Colleton Co., SC
V. blodgettii Small	3514	KC603930	*McNeilus 93-1924*, Colleton Co., SC
V. glauca (L.) Willd.	3515	KC603929	*Ahles 4492*, Mecklenburg Co., NC
V. greggii A. Gray	3528	KC603931	*Chapman & Chapman 68*, Hidalgo, Mexico
V. pulchella Small	3516	KC603922	*Jones 1448*, Liberty Co., GA
CYANTHILLIUM Blume			
C. cinereum (L.) H. Rob.	3517	KC603932	*McNeilus 98-1130*, Highlands Co. FL
PSEUDELEPHANTOPUS Rohr			
P. spicatus (Juss. ex Aubl.) C.F. Baker	3518	KC603935	*Panero et al. 660*, Michoacán, Mexico