Schilling, E.E., A. Floden, and D.E. Schilling. 2015. Barcoding the Asteraceae of Tennessee, Tribe Cichorieae. Phytoneuron 2015-19: 1–8. Published 19 March 2015. ISSN 2153 733X

BARCODING THE ASTERACEAE OF TENNESSEE, TRIBE CICHORIEAE

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ABSTRACT

Results from barcoding studies of tribe Cichorieae for the Tennessee flora using data from the nuclear ribosomal ITS marker region are presented and include first complete reports of this marker for 15 of the 24 species of the tribe that are native to the state. Sequence data from the ITS region separated all genera of Cichorieae in Tennessee from one another, and almost all species of the introduced *Cichorium*, *Crepis*, *Hypochaeris*, *Lapsana*, *Leontodon*, and *Hieracium* subgen. *Pilosella* from one another. In contrast, many (though not all) species of the native members of *Hieracium*, *Krigia*, *Lactuca*, and *Nabalus* were not uniquely distinguished by this marker. ITS sequence data provided support for the recognition of *Nabalus* as distinct from *Prenanthes*, and helped to confirm the identification of *N. albus* samples newly reported for Tennessee. ITS sequence data also suggested that *N. cylindricus* may represent a species distinct from *N. roanensis*, with which it has been lumped. The results of this study suggest that further study is needed to clarify patterns of diversification in the four genera of Cichorieae that exhibit radiations in southeastern North America.

Tribe Cichorieae is one of the most morphologically distinctive tribes in Asteraceae and with more than 1600 species one of the larger ones (Kilian et al. 2009). The tribe has traditionally been considered to have two prominent apomorphies in milky sap and homogamous ligulate heads and at earlier times was regarded as a separate subfamily or even family (e.g. Cronquist 1980). Molecular data have been decisive, however, in placing it as just one of several tribes, including Arctotidinae and Vernonieae, that form the subfamily Cichorioideae (Funk & Chan 2009). Like many groups that are highly distinctive, classification of subordinate groups of the tribe remains problematic and generic distinctions are still being refined. Thus, the separation of *Nabalus* as distinct from *Prenanthes* has only recently been accepted (Killian et al. 2009), and the circumscription of *Lactuca* remains problematic. The current study contributes further support for recognition of *Nabalus* as a distinct genus and continues the effort to survey the molecular diversity found in species of Asteraceae in Tennessee (Schilling 2013; Schilling & Floden 2012, 2013, 2014; Schilling et al. 2014), with further documentation of the varied diversification patterns that are found in members of the family.

Cichorieae is most diverse in temperate regions, with the largest concentration of species occurring in the Eastern Hemisphere (Kilian et al. 2009). The tribe is represented in Tennessee by 14 genera and 42 species, of which almost half are introduced; only 5 genera and 24 species are considered to be native (Chester et al. 2009). The larger genera are, however, composed mostly or entirely of native species and include *Hieracium* (7 species, 5 native), *Krigia* (5 species, all native), *Lactuca* (7 species, 5 native), and *Nabalus* (8 species, all native). Several species are considered to be rare in the state, including *Hieracium longipilosum*, *Krigia montana*, *Nabalus albus*, *N. asper*, and *N. barbatus* (Crabtree 2014); of these only *K. montana* and *N. barbatus* are somewhat rare globally (G3 ranking).

The goal of this study was to sample the nuclear ribosomal ITS marker for all species of Cichorieae that occur in Tennessee. Particular emphasis was placed on the four larger native genera, each of which exhibit modest radiations in southeastern North America. Despite the fact that Cichorieae is the most well studied tribe of Asteraceae (Kilian et al. 2009), most species from southeastern North America (with the exception of *Krigia*; Kim & Jansen 1994) have not been included in molecular phylogenetic studies. Sampling of additional species of these genera from areas of southeastern North America outside of Tennessee was done to evaluate overall patterns of diversification and compare them to other 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 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 MEGA 6 (Tamura et al. 2013) to provide a convenient way to make a comparative visualization of the sequence results. The samples of the introduced *Tragopogon* were utilized as the outgroup, based on results of Kilian et al. (2009). The analysis also incorporated sequences deposited at GenBank of conspecific samples or closely related species.

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

Species	DNA#	Genbank	Voucher info
CICHORIUM L.			
C. intybus L.	2644	HQ172904	*Schilling 07-DNA2644*, Knox Co., Tenn.
CREPIS L.			
C. capillaris (L.) Wallr.	3018	HQ161936	*Fleming FCF-533*, Van Buren Co., Tenn.
C. pulchra L.	3019	HQ161937	*Fusiak 525*, Knox Co., Tenn.
C. setosa Haller f.	3020	HQ161938	*Wofford 79-139*, Greene Co., Tenn.
HIERACIUM L.			
H. caespitosum Dumort.	3005	HQ161951	*DeSelm s.n.,* 5/29/2001, Washington Co., Tenn.
H. gronovii L.	2521	HQ161948	*Schilling 2521*, Knox Co., Tenn.
H. longipilum Torr. ex Hook.	3003	HQ161949	*Chester 13138*, Stewart Co., Tenn.
H. paniculatum L.	2568	HQ161947	*Schilling CF-7*, Sullivan Co., Tenn.
H. pilosella L.	3021	HQ161945	*Powers s.n.* 6/6/2008, Cumberland Co., Tenn.
H. scabrum Michx.	3004	HQ161950	*DeSelm 01-222*, Johnson Co., Tenn.
H. venosum L.	2573	HQ161946	*Schilling CF10*, Sullivan Co., Tenn.
	3272	KP828834	*Thomas 173842*, Sevier Co., Tenn.
Non-Tennessee samples			
H. megacephalon Nash	3262	KP828831	*McNeilus 00-33*, DeSoto Co., Fla.
	3263	KP828832	*Kral 64068*, Martin Co., Fla.
H. traillii Greene	3267	KP828833	*Christy F/6*, Bath Co., Va.
	3285	KP828835	*Wieboldt 5215*, Bath Co., Va.
HYPOCHAERIS L.			
H. radicata L.	3022	HQ161932	*McNeilus 01-171*, Knox Co., Tenn.

KRIGIA Schreb.			
K. biflora (Walt.) S.F. Blake	3015	HQ161943	*Schilling DNA3015*, Campbell Co., Tenn.
K. cespitosa (Raf.) Chambers	3023	HQ161942	*McNeilus 99-113*, Jackson Co., Tenn.
K. dandelion (L.) Nutt.	3024	HQ161941	*McNeilus 97250*, Monroe Co., Tenn.
K. montana (Michx.) Nutt.	3050	HQ172903	Greenhouse plant
K. virginiana (L.) Willd.	2772	HQ161940	*Schilling DNA2772*, Knox Co., Tenn.
LACTUCA L.			
L. biennis (Moench) Fernald	3006	HQ161959	*Estes 9543*, Morgan Co., Tenn.
	3160	KP828828	*Schilling LB1*, Knox Co., Tenn.
L. canadensis L.	2587	HQ161956	*Schilling 07-DNA2587*, Knox Co., Tenn.
	3200	KP828829	*Schilling DNA3200*, Knox Co., Tenn.
L. floridana (L.) Gaertn.	2570	HQ161957	*Schilling CF-1*, Sullivan Co., Tenn.
	3159	KP828827	*Schilling LF-1*, Knox Co., Tenn.
L. graminifolia Michx.	3261	KP828830	*Floden 2139*, Polk Co., Tenn.
L. saligna L.	3026	HQ161960	*Beck 6311*, Marion Co., Tenn.
L. serriola L.	3049	HQ172902	*Estes 1084*, Maury Co., Tenn.
Non-Tennessee samples			
L. graminifolia Michx.	3008	HQ161958	*Rogers 5247*, Copiah Co., Miss.
L. hirsuta Muhl. ex Willd.	3048	HQ172901	*Floden 941*, Oregon Co., Ark
LAPSANA L.			
L. communis L.	3027	HQ161939	*DeSelm s.n.*, 6/14/2005, Claiborne Co., Tenn.
LEONTODON L.			
L. saxatilis L.	4251	KP828825	*Floden et al.2789* , Fentress Co., Tenn.
	4250	KP828826	*Floden & Fleming 5/14/2012*, Polk Co., Tenn.
NABALUS Cass.			
N. albus (L.) Hook.	2840	HQ162003	*Floden763*, Campbell Co., Tenn.
	2963	HQ162005	*DeSelm s.n.*, Knox Co., Tenn.
N. altissimus (L.) Hook.	2832	HQ161970	*Estes 1479*, Giles Co., Tenn.
	2948	HQ161971	*Marcum et al. PBM1683*, Blount Co., Tenn.
N. asper (Michx.) Torr. & A. Gray	2955	HQ161988	*McKinney & Jackson 6892*, Coffee Co., Tenn.
	3042	HQ161979	*Horn 98-54*, Franklin Co., Tenn.
N. barbatus Torr. & A. Gray	2834	HQ161979	*Horn 98-60*, Coffee Co., Tenn.
	3041	HQ161981	*Gunn S6-792*, Humphreys Co., Tenn.
N. crepidineus (Michx.) DC.	2835	HQ161984	*Sweetser s.n.*, 9/26/2003, Knox Co., Tenn.
N. roanensis Chickering	2836	HQ161993	*Fusiak 580*, Carter Co., Tenn.
	2950	HQ161994	*Busemeyer et al. 756*, Blount Co., Tenn.
	2981	HQ161997	*DeSelm s.n. 8/31/2000*, Greene Co., Tenn.
	2979	HQ161995	*Fusiak 571*, Polk Co., Tenn.
	2980	HQ161996	*Murrell 953*, Polk Co., Tenn.
	2978	HQ161998	*Fusiak 571*, Unicoi Co., Tenn.
	2956	HQ162000	*Wofford 79-277*, Unicoi Co., Tenn.
N. serpentarius (Pursh) Hook.	2646	HQ161990	*Schilling 07-DNA2646*, Knox Co., Tenn.
	2837	HQ161989	*Estes & Beck 8352*, Grundy Co., Tenn.
	2952	HQ161991	*Rogers 42438*, Sevier Co., Tenn.
N. trifoliolatus Cass.	2838	HQ161976	*Murrell 620*, Polk Co., Tenn.
	2565	HQ161975	*Schilling CF-9*, Sullivan Co., Tenn.

Non-Tennessee samples

N. albus (L.) Hook.	2831	HQ162002	*McNeilus 93-2215*, Wood Co., Wis.
	2962	HQ162004	*Jones s.n.*, 9/11/1976, Erie Co., Ohio
	2964	HQ162001	*Morton 7990*, Hardy Co., W.Va.
N. asper (Michx.) Torr. & A. Gray	2954	HQ161987	*Athey 1470*, McCracken Co., Ky.
N. autumnalis (Walt.) Weakley	2959	HQ162007	*Pringle 31236*, Swain Co., N.C.
N. barbatus Torr. & A. Gray	3040	HQ161980	*Kral 37700*, Etowah Co., Ala.
N. crepidineus (Michx.) DC.	3045	HQ161984	*Athey 2932*, Graves Co., Ky.
N. racemosus (Michx.) Hook.	2960	HQ162009	*McNeilus 90-981*, 9/26/2003, Norman Co., Minn.
N. roanensis Chickering	2951	HQ161992	*Busemeyer et al. 780*, Swain Co., N.C.
N. trifoliolatus Cass.	2949	HQ161974	*Phillippe 31167*, Swain Co., N.C.
PRENANTHES L.			
P. purpurea L.	2965	HQ161931	*Schilling 09-01*, Baden-Württemberg, Germany
PYRRHOPAPPUS DC.			
P. carolinianus (Walt.) DC.	2519	HQ161944	*Schilling 07-DNA2519*, Knox Co., Tenn.
SONCHUS L.			
S. arvensis L.	3029	HQ161952	*DeSelm s.n.*, 7/12/1997, Campbell Co., Tenn.
*S. asper (*L.) Hill	3030	HQ161953	*Thomas 173736*, Sullivan Co., Tenn.
S. oleraceus L.	3031	HQ161955	*Estes 2617*, Bedford Co., Tenn.
TARAXACUM Wiggers			
T. erythrospermum Andrz. ex Besser	3032	HQ161933	*Estes 1974*, Giles Co., Tenn.
T. officinale Wiggers	2531	HQ161934	*Schilling 07-DNA2531*, Knox Co., Tenn.
TRAGOPOGON L.			
T. dubius Scop.	2332	HQ161962	*Schilling2332*, Knox Co., Tenn.
T. porrifolius L.	3033	HQ161961	*Fusiak 523*, Knox Co., Tenn.
YOUNGIA Cass.			
Y. japonica (L.) DC.	3017	HQ161935	*Schilling 10-1*, Knox Co., Tenn.

Results and discussion

Newly obtained ITS sequences for Cichorieae ranged in length from 623-647 bp. The range for species native to Tennessee was narrower, between 638 (*Pyrrhopappus*) and 642 (*Nabalus*, uniform in all species sampled); *Hieracium* was consistently 640, *Krigia* was 640-641, and the widest infrageneric variation among natives was in *Lactuca* (639-641). Among native species, the occurrence of length polymorphisms was limited to the sample of *Krigia montana*, which is known to be an allopolyploid (Kim & Jansen 1994). The number of positional polymorphisms (inferred from a double peak on the sequencing electropherogram) was relatively low for all samples, varying from none to six in individual samples. The relatively low number of polymorphisms in Cichorieae ITS sequences contrasts to frequent polymorphism occurrences in other Asteraceae of the southeastern USA, such as Coreopsideae (Schilling et al. 2014).

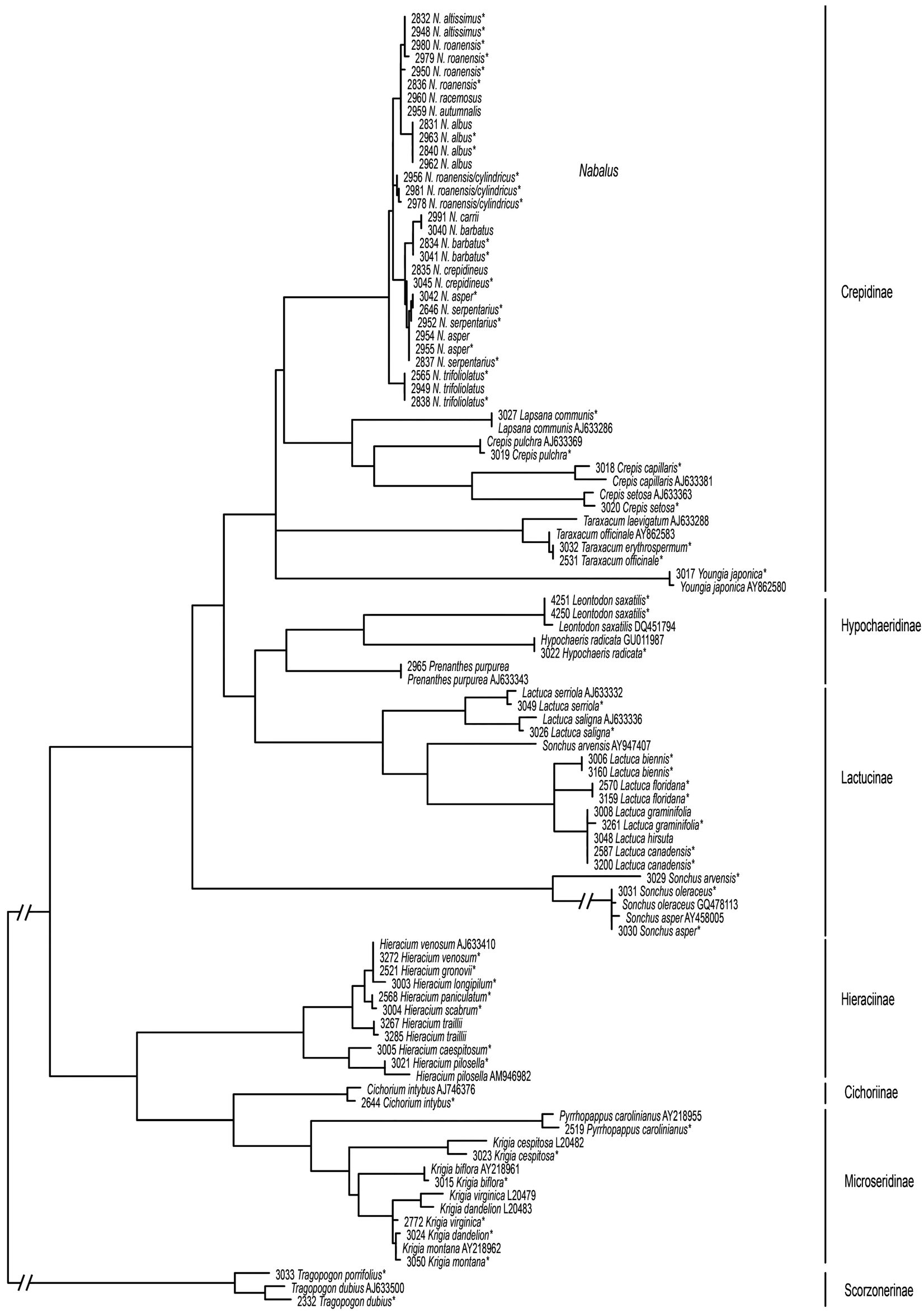

Figure 1. Maximum likelihood bootstrap tree (100 replicates) showing relationships of species of Cichorieae based on ITS sequence data, using *Tragopogon* as the outgroup. Subtribes of Cichorieae shown along right margin. Newly obtained sequences designated by DNA number preceding species name (Table 1); GenBank numbers for other sequences follow species name. *= sample from Tennessee.

The ITS sequences of the sampled genera of Cichorieae were quite different from one another (Figure 1). The overall placement in the ML tree was consistent with the subtribal circumscriptions of Kilian et al. (2009). The four native genera that have radiated in eastern North America are each placed in a different subtribe. This is notable for *Nabalus*, which as a member of Crepidinae is phylogenetically distant from *Prenanthes* (Hypochaeridinae), with which it has been frequently lumped. For Cichorieae of Tennessee, an ITS sequence will clearly identify an unknown sample at least to genus.

For the introduced genera, including *Cichorium*, *Crepis*, *Hypochaeris*, *Lapsana*, *Leontodon*, and *Hieracium* subgen. *Pilosella*, species were distinct from one another based on ITS sequences (Fig. 1), with two exceptions. There was no clear separation between *Sonchus asper* and *S. oleraceus*, although both were distinct from *S. arvensis* (Fig. 1). And within *Taraxacum*, a Tennessee sample identified originally as *T. laevigatum* and annotated later as *T. erythrospermum* did not differ in ITS sequence from samples of *T. officinale*, although a Genbank sample of *T. laevigatum* (and also one of *T. erythrospermum*, not shown) was clearly distinct. Species level identification in *Taraxacum* is complicated by both morphological plasticity and by a mixed apomictic and sexual breeding system (Kirschner et al. 2014), and further study is needed to confirm that more than a single species is actually present in Tennessee.

Differentiation at the species level based on ITS sequences was less well resolved for the native genera. *Krigia* was the best resolved, although the complications introduced by polyploidy and possible hybridization (Kim & Jansen 1994) appear to be reflected in the lack of separation among *K. montana*, *K. dandelion*, and *K. virginica* (Fig.1). Within *Hieracium*, the native species, which are all sexual and diploid, were clearly separated as a group from the Eurasian apomicts *H. pilosella* and *H. caespitosum*, consistent with the results from plastid sequences of Gaskin and Wilson (2007); the latter often are separated as part of a distinct genus *Pilosella* (Kilian et al. 2009). Among the native species, there was little separation with the exception of the shale barren endemic, *H. traillii* (Fig. 1). The native species of *Lactuca* formed a monophyletic group, within which *L. floridana* and *L. biennis* were clearly distinct, but the remaining species (*L. canadensis*, *L. graminifolia*, and *L. hirsuta*) were basically identical to one another for ITS sequences (Fig. 1). The species of *Lactuca* from North America are all allopolyploids, with $2n = 34$ (Whitaker & Thompson 1941), but there was no evidence of polymorphisms in the ITS sequence data that might help to resolve their ancestry.

The most complex native group of Cichorieae was *Nabalus*, in which there was differentiation for ITS sequences among some species and groups of species, but not all. The overall placement of North American *Nabalus* in a clade well removed phylogenetically from *Prenanthes* supports their separation as distinct genera. Within *Nabalus*, samples of *N. trifoliolatus* formed a clade that was sister to the remaining species in the ML tree (Fig. 1). A group of samples of *N. roanensis* representing material that has been identified as *N. cylindricus* was placed in a clade separate from the other samples of this species, suggesting a reevaluation of its status is needed. Although Cronquist (1980) and Fusiak and Schilling (1984) lumped these two species based on overall morphology, the two can be separated based on stem pubescence (glabrous in *N. cylindricus*, pubescent in *N. roanensis*) and leaf morphology (at least some leaves pinnately 3-5 lobed in *N. cylindricus* vs. dentate in *N. roanensis*). Both *N. cylindricus* and *N. roanensis* occur in montane habitats in the Blue Ridge, and it is unclear if there might be geographic or elevational separation of the two. Samples of *N. roanensis* were placed among a group of species including *N. altissimus*, *N. autumnalis*, and *N. racemosus*, within which there was little differentiation (Fig. 1). The sister clade to this group was formed by samples of *N. albus*, which was only recently documented to occur in the state, where it forms the southern extension in the middle of the range of this species. The ITS sequences of the Tennessee samples of *N. albus* were identical to those sampled from northern areas. The other clade in *Nabalus* was formed by samples of *N. asper*, *N. barbatus*, *N. carrii*, *N.*

crepidineus, and *N. serpentarius*, among which there was little differentiation for ITS sequence (Fig. 1).

The results of BLAST searches in GenBank for members of Cichorieae generally gave a top match and usually almost identical sequence to a conspecific sample, if the species had been sampled. The exception was the GenBank sample labeled *Sonchus arvensis* (AY947407), which based on its placement (Fig. 1) is clearly from a member of *Lactuca*, although its sequence did not match that of any of the species sampled for this study.

The results of molecular phylogenetic analysis of the native genera of Cichorieae add to the diversity of biogeographic patterns observed in Asteraceae of southeastern North America. A general pattern of having basal lineages of genera of Asteraceae found in the extreme southeastern part of North America was noted for *Helianthus* by Schilling et al. (1998). None of the genera of Cichorieae appear to exhibit this pattern. *Krigia* is a derived member of a subtribe that is otherwise found in western North America (Lee et al. 2007), suggesting that its origin is from that region, similar to *Vernonia* and some genera of Senecioneae (Schilling 2013; Schilling & Floden 2014). In contrast, the basal branching members (Fig. 1) of both *Nabalus* and *Hieracium* are species from northeastern North America. The case for *Lactuca* is unclear, in part because of the widespread geographic distribution of its native species, including *L. floridana* which is placed sister to the remainder of the North American species (Fig. 1). The results presented here for the single marker ITS suggest that broader sampling of both taxa and molecular markers is needed to clarify the diversification patterns in genera of Asteraceae from southeastern North America.

ACKNOWLEDGEMENTS

Thanks to S. Robertson and J. Schilling for laboratory assistance, J. May and V. Brown of the University of Tennessee MBRF for help with DNA sequencing, T. Wieboldt (VPI) for a sample of *Hieracium traillii*, and the numerous collectors, notably D. Estes and the late V. McNeilus, 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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