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Hybrid genera in Liatrinae (Asteraceae: Eupatorieae)

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ABSTRACT

Liatrinae is a small subtribe of Eupatorieae that occurs in North America with a center of generic-level diversity in the southeastern United States. Molecular phylogenetic data were sought to assess whether two monotypic genera, *Garberia* and *Hartwrightia*, are accurately placed in the subtribe, and to resolve questions of the generic-level classification of *Carphephorus*. Phylogenetic analyses of nuclear ITS/ETS and plastid DNA data indicated that *Garberia* is the basalmost diverging lineage, and that *Hartwrightia* is phylogenetically embedded in the subtribe. There was significant incongruence between the ITS/ETS and plastid DNA datasets in the placement of *Hartwrightia* and another monotypic genus, *Litrisa*, suggesting that both are of original hybrid origin. The results also showed that *Carphephorus* s.l. is not monophyletic, and even after removal of the two species of *Trilisa*, it is still paraphyletic to *Liatris*. The apparent hybrid origin of *Hartwrightia*, which is morphologically transgressive relative to its inferred parental lineages, suggests that reticulation between phylogenetically distinct lineages may be a recurrent problem for phylogenetic estimation in Asteraceae.

1. Introduction

There is growing recognition that interspecific hybridization is both common and has played a significant role in the evolution of plants (Arnold, 2006; Paun et al., 2009). Particularly with the use of molecular data, many examples of hybridization have been verified and new ones revealed. It remains to be shown, however, that hybridization has broad importance in generation of new diversity. A potentially important role was uncovered by Rieseberg et al. (2003) in documenting that hybridization can allow new genetic combinations that alter the ecological amplitude of the daughter lineage relative to the parents and allow it to move into a new habitat or niche.

The recognition that a lineage is of hybrid origin is not, however, always clear. A primary source of evidence is intermediacy in phenotypic traits, but there is abundant evidence that deviations from an intermediate phenotype often occur and in fact may be the rule (Rieseberg, 1995). Intermediacy may also be a reflection of traits retained from an ancestral lineage rather than having been secondarily derived via hybridization. Molecular data have been critical in documentation that hybridization is actually the source for intermediacy (e.g. Chapman and Burke, 2007; Siripun and Schilling, 2006). Where hybridization occurs, however, it can introduce considerable complications for those attempting to estimate phylogenetic relationships. It is already widely recognized that immediate products of hybridization can disrupt phylogenetic estimation, and they are typically removed before data are analyzed. More insidious, however, would be species or lineages that are the products of wide hybridization, and in which there has been subsequent divergence or recombination of marker genes. There is a hint that this process might be occurring in the form of samples that seem to complicate phylogenetic analysis, sometimes referred to informally as "rogue taxa" (e.g. Thines et al., 2006; Thomson and Shaffer, 2010; Wortley et al., 2007), which frustrate obtaining well resolved multigene-based phylogenies. Demonstration that reticulation can involve phylogenetic lineages that have diverged to a level where they have been clearly recognized as genera would provide validation that this phenomenon could be a source of complications for some phylogenetic analyses.

Hybridization has also been a convenient explanation for the occurrence of distributions of character states that are otherwise hard to explain (e.g. King and Robinson, 1987). In groups where the defining features for supraspecific taxa are relatively few and involve functional traits that are subject to homoplasy, this can be particularly the case. One such group is Asteraceae, where features of the cypsela, which appear to be clearly related to propagule dispersal or establishment, have traditionally been emphasized in distinguishing genera. For example, in the tribe Eupatorieae, which contains about 10% of the species-level diversity of the family, the type of pappus and number of major ribs on the cypsela have been utilized as the source of characters to delimit not only genera but also subtribes (King and Robinson, 1987; Bremer, 1994). Subtribe Liatrinae is a group of Eupatorieae that appears to be relatively well defined and consists of only a few genera (King and Robinson, 1987). They are characterized by the presence of a basal rosette of leaves, at least at an early stage of growth,

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leaves with an alternate phyllotaxy, and by the occurrence of a distinctive type of biseriate trichome on the cypselae in which the twin cells are separate nearly to the base (King and Robinson, 1987). Liatrinae are restricted geographically to middle and eastern North America and exhibit their greatest taxonomic diversity in the southeastern part of this area (Fig. 1). The familiar *Liatris* (blazing stars) with 37 species (Nesom, 2006a), most of which have a spiciform to racemiform capitulescence and a corm-like underground stem and root system, makes up the bulk of species of the subtribe and has a broad geographic distribution throughout eastern and central North America (not shown). *Garberia* consists of a single shrub endemic to the sandhill vegetation of the central Florida peninsula. All but one of the remaining species of the subtribe are herbs with a pappus of bristles, and are variously placed in one to three genera (King and Robinson, 1987; Nesom, 2006b). The remaining unispecific genus, *Hartwrightia*, was never traditionally associated with the subtribe because it lacks a pappus and has cypselae which are 5 rather than 8–10 ribbed, but was placed there by Robinson and King (1977) based on its rosulate habit and geographic distribution.

The major generic-level problem within Liatrinae prior to this study involves resolution of whether *Carphephorus* (chaffheads) should be circumscribed broadly to include seven total species, or whether *Trilisa* (two species) and *Litrisa* (one species) should be recognized as distinct. As contrasted with *Liatris*, *Carphephorus* s.l. is characterized by having an elongate underground stem and root system and a corymbose rather than spiciform capitulescence. *Trilisa* is separated from *Carphephorus* based on its lack of leaf glandular punctations, smaller heads which have fewer phyllaries and florets, usual lack of receptacular paleae, uniseriate pappus, and entire rather than notched anther appendages. This separation is made less clear by the distribution of these traits in the single species of *Litrisa*, which has heads with an intermediate number of phyllaries and florets relative to *Carphephorus* and *Trilisa* while sharing the glandular-punctate leaves, multiseriate pappus, and notched anther appendages with the former but having the lack of receptacular bracts of the latter (James, 1958; Hebert, 1968; Correa and Wilbur, 1969). This situation has been handled by variously recognizing *Litrisa* as distinct or including it within either *Carphephorus* or *Trilisa*. The current treatment in Flora North

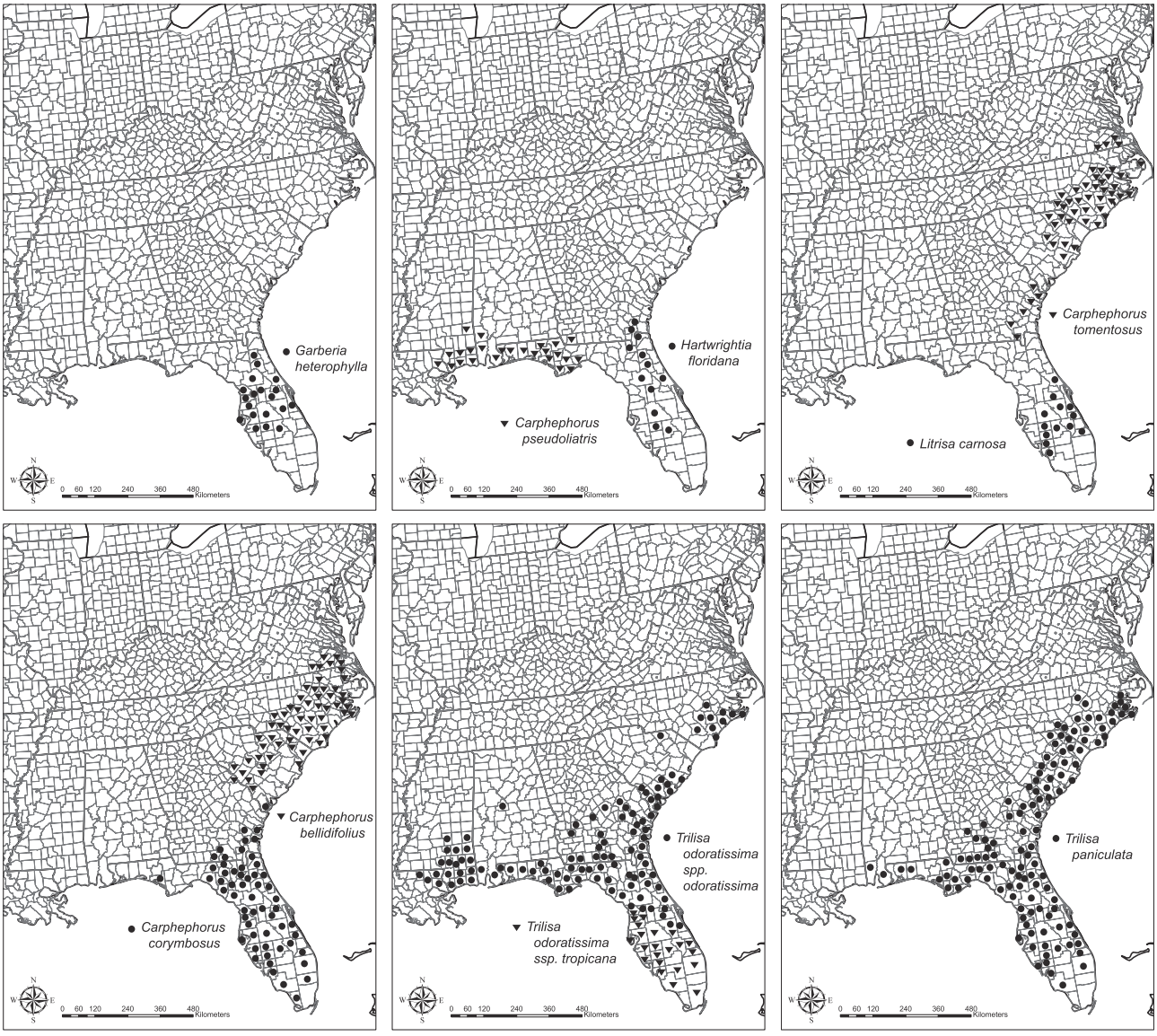

Fig. 1. Maps of southeastern United States showing county-level geographic distributions of Liatrinae (exclusive of *Liatris*, species of which occur across the entire region and beyond).

America (Nesom, 2006b) returns to a broadly circumscribed *Carphephorus*.

This project was initiated to gather molecular phylogenetic data to help to resolve the controversy regarding the generic-level treatment of *Carphephorus* and its potential generic segregates *Trilisa* and *Litrisa*, and to evaluate whether *Garberia* and *Hartwrightia* are accurately placed in Liatrinae. An initial sampling of a single species of each genus revealed the problem of phylogeny estimation to be complex, and sampling was extended to include multiple populations of all of the species of *Carphephorus* s.l. as well as a broad species-level sampling of *Liatris*.

2. Materials and methods

2.1. Sources of plant material

At least one sample each of *Garberia*, *Hartwrightia*, and *Litrisa* and each of the species of *Carphephorus* and *Trilisa* was collected in the field. Additional sampling included a mix of field-collected and herbarium-sampled material (Appendix A). Because of the relative lack of intrageneric variability, no attempt was made to sample every species of *Liatris*, but sampling was extended to include a total of 28 of the 37 species, with at least one species from each of the sections recognized by Nesom (2005). Sequences from one species each of *Ageratina*, *Eupatorium*, and *Eutrochium* were used as outgroups, based on the results of a broad survey of Eupatorieae that indicated that *Ageratina* is relatively basal in the tribe and *Eupatorium* and *Eutrochium* collectively form the sister group to Liatrinae (Robinson et al., 2009; Schilling et al., 1999; Schmidt and Schilling, 2000).

2.2. Molecular methods

Preparations of total DNA were performed primarily with the DNeasy Plant Minikit (Qiagen, Valencia CA) and typically utilized a portion (ca 0.1 g) of a single leaf. The crude DNA extracts of some samples required further purification using the Wizard Kit protocol (Promega, Madison, Wisconsin, USA). ITS amplifications were performed in 20 μl reactions using 10–20 ng of genomic DNA, 10 × PCR buffer (Promega), 1.8–2.25 mM MgCl2, 0.2 mM each dNTP, 1.25 units of Taq polymerase, and 0.2 μM each primer. Primers used were "ITS-4" (5′-TCCTCCGCTTATTGATATGC-3′) and "ITS-5" (5′-GGAAGTAAAAGTCGTAACAAGG-3′; White et al., 1990). PCR was performed with the "ETS" protocol: 95 °C for 2 min; 10 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C initially for 1 min, with 4 s added per cycle; 20 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C initially for 1:40, with 4 s added per cycle; and a final extension of 72 °C for 7 min. PCR products were checked on 1% agarose gels before being cleaned with ExoSAP-IT (USB, Cleveland, Ohio, USA). All DNA sequencing was performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, v. 3.1 (Perkin–Elmer/Applied Biosystems, Foster City, California, USA) and electrophoresed and detected on an ABI Prism 3100 automated sequencer (University of Tennessee Molecular Biology Resource Facility, Knoxville, Tennessee, USA). For some samples, use of the amplification primers as the sequencing primers gave unsatisfactory results (possibly because of fungal contamination) and internal primers located in the 5.8S coding region that are plant specific were used: "5.8S 79 for" (5′-GCAGAATCCCGT GAACCATC-3′; listed at: http://www.science.siu.edu/plant-biology/faculty/nickrent/primer.nuclear.html) and "ITS-5.8SR" (5′-TG ACACCCAGGCAGACGTGC-3′; Small 2004). Amplification and sequencing reactions for the ETS region were performed using the 18-S-ETS (5′-ACTTACACATGCATGGCTTAATCT-3′) primer of Baldwin and Markos (1998) coupled with the Ast-1 primer of Markos and Baldwin (2001) (5′-CGTAAAGGTGTGTGAGTGGTTT-3′). The initial sequence data text files were edited following comparison with the same data displayed in four-color electropherograms before they were analyzed further. Sequence alignment was performed manually. GenBank accession numbers are provided in Appendix A.

2.3. Plastid gene regions

PCR amplification and sequencing utilizing methods and primers outlined in Panero and Crozier (2003) were performed for the following plastid genes or gene regions: *mat*K, *ndh*F, *ndh*I, *rbc*L, *pet*D, *ndh*D, and *trn*H-*psb*A. A complete set of plastid sequence, totaling 8756 bp in the aligned matrix, was obtained for one sample of each species of *Garberia*, *Carphephorus*, *Trilisa*, *Litrisa*, and *Hartwrightia*, and for one sample each of two species of *Liatris*. As a check for variation in plastid DNA sequence, as might occur as a result of lineage sorting or of chloroplast transfer through hybridization, *ndh*F and *ndh*I sequences, the two markers which collectively included at least one synapomorphic site change for each major clade, were obtained for multiple samples of each genus.

2.4. Data analysis

Phylogenetic relationships were analyzed using both maximum parsimony and Bayesian approaches, implemented with the computer programs PAUP* 4.0b10 (Swofford, 2003) and MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). For maximum parsimony, a heuristic search with 1000 random addition replicates and with TBR branch swapping was used, with gaps treated as missing data. Bootstrap analysis (Felsenstein, 1985) was performed with 10,000 replicates using the "Faststep" search strategy. Bayesian analysis was run for a million generations with four separate chains and trees saved every 1000 generations. The number of trees to discard as "burn-in" was assessed by plotting likelihoods of trees sampled throughout the run and discarding all trees prior to the stable likelihood plateau (for these analyses the first 15% were discarded). For each sequence region, an appropriate maximum likelihood model of sequence evolution (GTR + I + G; General Time Reversible model with a proportion of invariant sites and gamma distributed rates) for the Bayesian analysis was chosen using Modeltest (Posada and Crandall, 1998).

3. Results

3.1. ITS and ETS regions

Sequences for the nuclear ITS region of all Liatrinae were consistent with previous reports for the subtribe, with relatively little length variation among genera. Length variation could be explained by a total of three indels. The ITS-1 was 260 bp in most samples, but all samples of *Hartwrightia* had a two bp insertion in this region resulting in a length of 262 bp. The 5.8S rDNA coding region was uniformly 164 bp in length. The ITS-2 was 226 bp in most samples; 225 bp in all samples of *Liatris*, *Carphephorus bellidifolius*, and *Carphephorus tomentosus* because of a 1 bp deletion; and 228 bp in all samples of *Carphephorus corymbosus* because of a 2 bp insertion. No length variation and little sequence variation was observed across multiple samples within any of the species. Samples of *Garberia* had a single bp position which was C in one sample and polymorphic for C/T in the others. Samples of *C. corymbosus* exhibited a single variable position with G in four samples, C in one sample, and a G/C polymorphism in the remaining sample. There was variability at two single bp positions among samples of *C. tomentosus*. Samples of *Carphephorus pseudoliatris*,

C. bellidifolius, *Hartwrightia*, and *Litrisa* showed no variability for ITS sequence. The samples of *Liatris* had few or no polymorphic positions, but a few samples, including one hybrid of known parentage, exhibited polymorphic bp peaks at the five positions that appeared to separate samples of the genus into two groups.

For the ETS region the data were truncated to produce a matrix with an aligned length of 386 positions beyond the 18S rDNA coding region. There was little length variation within Liatrinae, which could be explained by two insertions: a 3 bp insertion in *C. pseudoliatris* and a single base pair insertion in all samples of *Liatris*, *C. bellidifolius*, and *C. tomentosus.*

The results from phylogenetic analysis of the combined ITS and ETS regions are shown in Fig. 2A. Relative to the outgroups of *Ageratina* and *Eupatorium*/*Eutrochium*, Liatrinae was monophyletic with *Garberia* forming the basalmost diverging group (albeit weakly supported). At the next level there was a polytomy, with branches including a clade with samples of *Trilisa*, *Litrisa*, and *Hartwrightia*, another clade with samples of *C. bellidifolius*/*C. tomentosus* and *Liatris*, and individual branches with *C. corymbosus* and *C. pseudoliatris*, respectively. Analysis of a larger data set based only on ITS but with a broader sampling within species showed a similar pattern, albeit with less resolution and lower bootstrap support of some branches (Fig. 3b). The broader sampling revealed a limited amount of variability within *Liatris*. A large group of species were characterized by having four apomorphic site changes. Within this group there was a single apomorphic change that characterized a smaller group, although the most widely sampled species, *L. spicata*, was polymorphic for the presence of this change. A few samples, including the artificially produced hybrid *L.* x *creditonensis*, exhibited bp polymorphisms for each of these four (or five) sites, and were not included in the phylogenetic analysis. There were also a few individual site changes found in single samples or species (not shown).

3.2. Plastid DNA regions

Sequences from cpDNA regions were entirely congruent with one another and were included in a single phylogenetic analysis, results of which are shown in Fig. 2B. As in the ITS/ETS analysis, the Liatrinae were placed in a monophyletic group relative to the *Eupatorium*/*Eutrochium* group with strong statistical support, and *Garberia* was placed as the basalmost diverging group. Above *Garberia* there was a split between a clade with *Trilisa* and *C. bellidifolius* and a second with *Hartwrightia*, *Litrisa*, *Liatris*, and the other species of *Carphephorus*. *Hartwrightia* and *Litrisa* were placed with strong statistical support within the clade with *C. corymbosus* and *C. pseudoliatris* as sister to the former. *C. tomentosus* was placed within the strongly supported clade that included a monophyletic *Liatris*. The broader sampling for the regions *ndh*F and *ndh*I produced a tree with identical topology, albeit lower levels of statistical support (Fig. 4). There was no evidence of variability for chloroplast sequences within any of the Liatrinae species, and there was almost no variability among species of *Liatris* that were sampled.

3.3. Combined analyses

There was obvious incongruence between the biparental ITS/ETS and the plastid DNA data sets in the placement of three species, *C. bellidifolius*, *Hartwrightia floridana*, and *Litrisa carnosa* (Fig. 2). Application of the homogeneity partition analysis in PAUP confirmed the two data sets to be incongruent at a level of $p < 0.001$. Not surprisingly, the results of a combined analysis (not shown) gave a mostly unresolved phylogeny. Removal of the three species gave a data matrix in which there was no incongruence among the ITS/ETS and plastid DNA data sets, and analysis gave a completely resolved phylogeny, with almost complete statistical support of all branches (Fig. 5). *Garberia* was placed as the sister group to the remaining Liatrinae, and *Trilisa* and *Liatris* were both strongly supported as monophyletic. *Carphephorus*, however, was paraphyletic relative to *Liatris* (Fig. 5).

4. Discussion

Results of analysis of molecular phylogenetic data gave strong support for the monophyly of Liatrinae as currently circumscribed (King and Robinson, 1987), with inclusion of *Garberia* and *Hartwrightia*. The results did not, however, provide a simple resolution to problems of generic delimitation within the subtribe. Instead they pointed to the possibility that evolutionary divergence in the subtribe has been complex and may have involved reticulation through hybridization. The strong incongruence between the nuclear ITS/ETS and the plastid DNA trees suggested that *Litrisa* and *Hartwrightia* may both be of hybrid origin, although they displayed contrasting patterns of morphological differentiation relative to their putative parental lineages. The results do not allow classification to be based on a simple overlay of the molecular phylogenetic

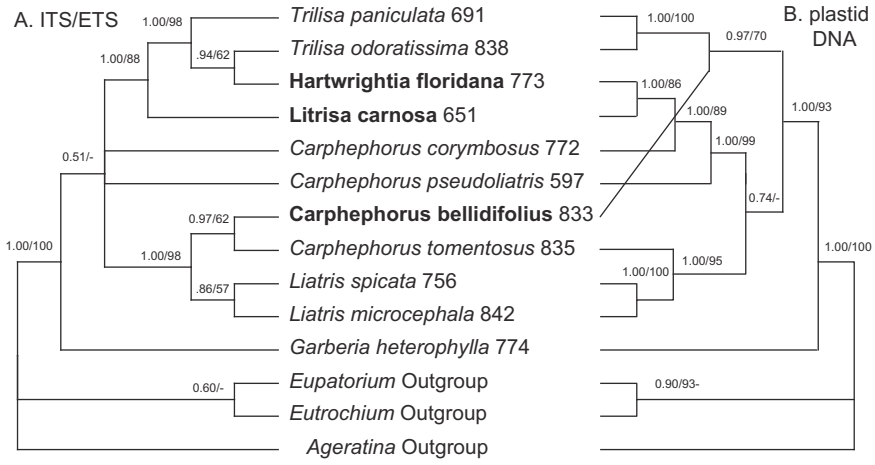

Fig. 2. Phylogenetic estimates of Liatrinae based on: (A) combined ITS/ETS data; and (B) combined plastid DNA data. Majority rule consensus trees from Bayesian analysis are shown with support levels (Bayesian posterior probability/bootstrap %) shown above individual branches. Species with incongruent placement between the two data sets highlighted in bold. Sample numbers from Appendix A.

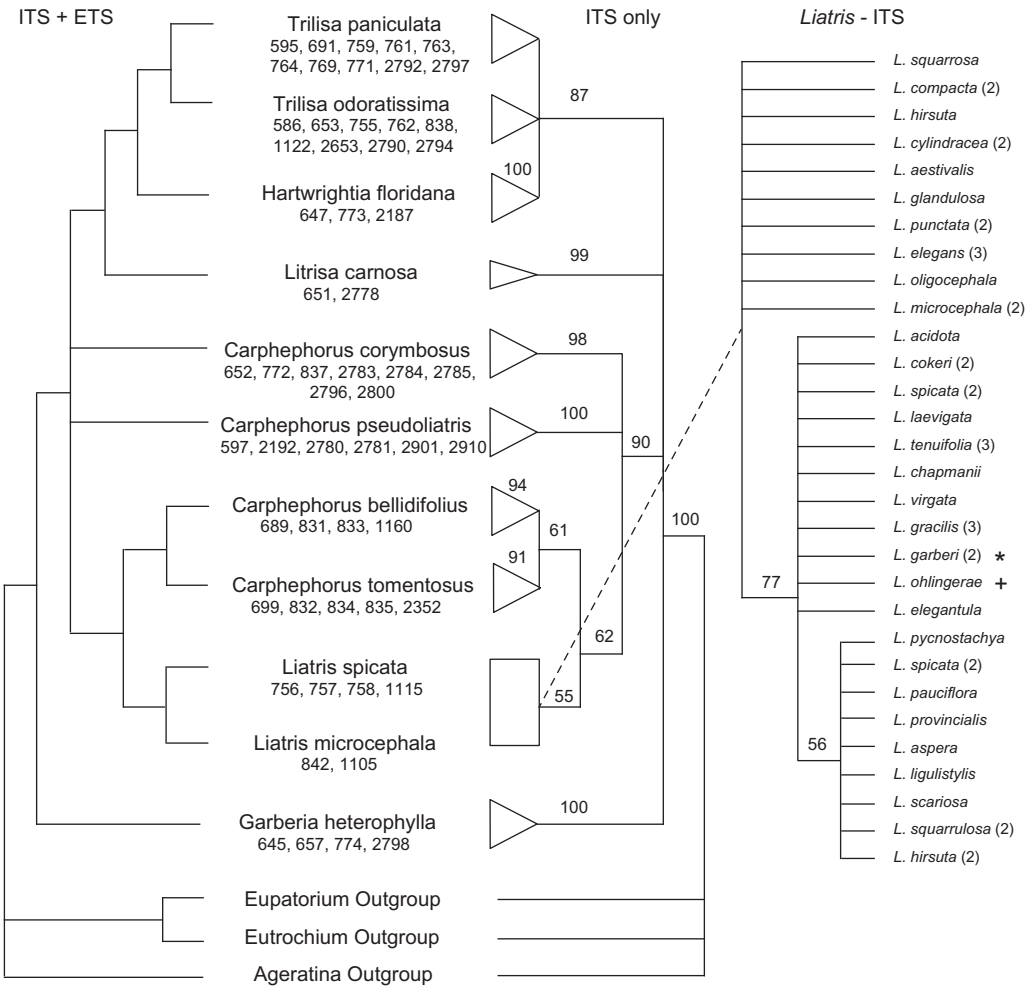

Fig. 3. Majority rule bootstrap trees showing relationships among taxa of Liatrinae based on combined ITS/ETS data (left) and a broader taxonomic sampling based on ITS data alone (middle). Relationships among species of *Liatris* shown in expansion of boxed clade (right); *, elongate rhizome; +, cymiform capitulescence. Bootstrap support values shown above branches. Cones show aggregations of samples, listed by DNA number (Appendix A) under species names.

results, but rather continue to require interpretation of the best manner to portray a somewhat messy phylogeny.

The results of a comprehensive survey suggested that the ITS region was appropriate for phylogenetic estimation in Liatrinae. There was a useful level of variability among species, and no indication of multiple copies within individuals (other than known or possible hybrids) or other intraspecific variability that have been problematic for this marker (Álvarez and Wendel, 2003) in other groups (e.g. *Quercus*, Mayol and Rosselló, 2001). The only caveat is that the variation for this marker within *Liatris* appears to be similar to that of other species-rich Asteraceae genera of eastern North America (e.g. *Helianthus*, Schilling et al., 1998; *Silphium*, Clevinger and Panero, 2000; *Solidago*; Schilling et al., 2008) in having only limited variability among species or groups of species (Fig. 2B). The lack of variability is most likely a reflection of relatively recent divergence, although it could also be the result of gene flow through hybridization.

The inclusion of *Garberia* in Liatrinae, as the basalmost diverging lineage in the subtribe (Figs. 2–5), was not surprising because the single species of *Garberia* was originally described as a member of *Liatris*. *Garberia* is also distinctive in being the only member of the subtribe which is woody. It is tempting to interpret the woody habit as plesiomorphic, because woodiness is not uncommon among the tropical members of Eupatorieae that are phylogenetically basal in the tribe (Robinson et al., 2009). The uniformly herbaceous habit of the sister groups *Eupatorium* and *Eutrochium* indicates instead that woodiness in *Garberia* is probably a derived trait. The habit combined with the geographical location of *Garberia* as part of the sandhill vegetation province in Florida suggests that it evolved at a time when the central portion of the peninsula, now Lake Wales Ridge, was an island. Other members of Asteraceae in herbaceous lineages have developed a woody habit when isolated in an island setting (Baldwin and Sanderson, 1998; Lee et al., 2005; Kim et al., 2007; Andrus et al., 2009).

Molecular phylogenetic results also provided strong support for the placement of the anomalous *Hartwrightia* in Liatrinae. The initial description of the genus placed it in Piquerieae, but shortly thereafter a relationship to *Alomia* was suggested, based on the appendiculate anthers, epappose cypselae, and 2–3 seriate involucre (Holzinger, 1893). The molecular data (Fig. 2–4) strongly supported its placement in Liatrinae, as first suggested by Robinson and King (1977) based primarily on habit and geography. There was, however, strong incongruence between the nuclear ITS/ETS and the plastid DNA markers for its sister group within Liatrinae, which suggests the possibility that *Hartwrightia* is originally of hybrid origin. The presence in *Hartwrightia* of an ITS/ETS pattern otherwise characteristic of *Trilisa* combined with a plastid DNA pattern shared with *C. corymbosus* points to these as the likely parental lineages. Thus the features including lack of a pappus and 5- rather than 10-ribbed cypselae that led to uncertainty about

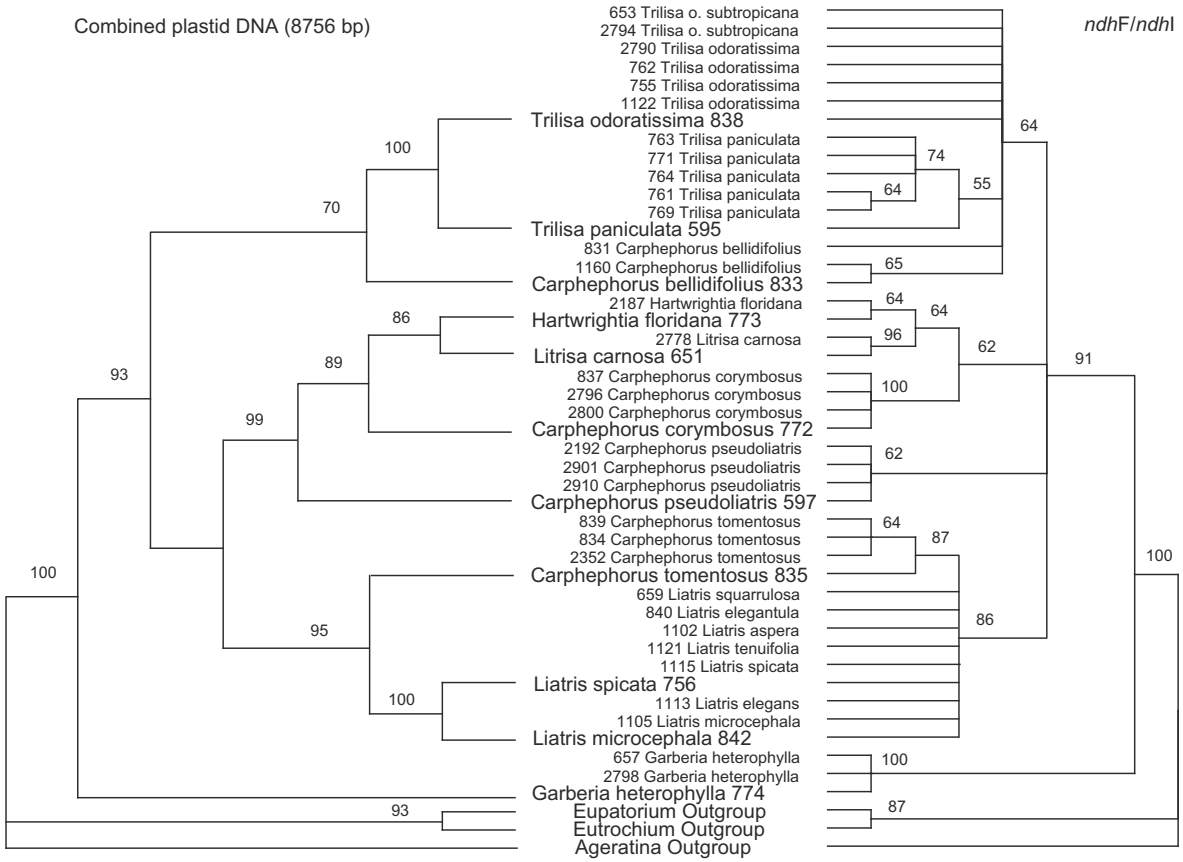

Fig. 4. Majority rule bootstrap trees (support levels shown above branches) showing relationships among species of Liatrinae based on combined plastid DNA data set (left) and a broader taxonomic sampling of a subset of data consisting of *ndh*F and *ndh*I sequences (right). Samples identified by DNA number (Appendix A).

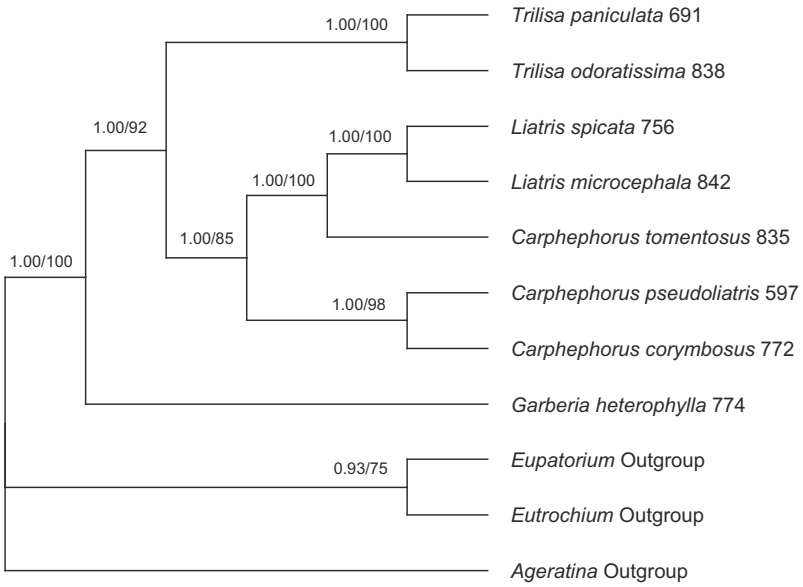

Fig. 5. Phylogenetic estimate showing relationships among Liatrinae, based on combined ITS/ETS and plastid DNA data (9796 bp total), after removal of taxa of inferred hybrid origins. Support levels shown above branches (Bayesian posterior probability/bootstrap percentage). Sample numbers from Appendix A.

the placement of *Hartwrightia* based on morphological data appear to be transgressive relative to the inferred parental lineages. Morphologically *Hartwrightia* is transgressive not only to both *Carphephorus* s.s. and *Trilisa* but in fact to the entire Liatrinae in several characters: almost complete lack of a pappus; 5-ribbed cypselae which lack eglandular trichomes; and broadly campanulate (rather than narrowly funnelform) corolla (Table 1). The habitat of *Hartwrightia* is also distinctive, occurring in dark-colored, peaty muck in wet sloughs (Kral, 1983), rather than in lighter and drier soils typical for most other Liatrinae.

Table 1
Distribution of diagnostic morphological characters in *Litrisa* and *Hartwrightia*, compared to the inferred parental lineages *Trilisa* and *Carphephorus*. Dark shading, intermediate between putative parental lineages; light shading, shared with putative parental lineage.

Character	Trilisa	Litrisa	Carphephorus	Hartwrightia
Phyllary series	2	2–3	3–5	1*
Flowers/head	4–15	5–10	9–30	7–10
Pales	Few or none	Few or none	Usually present	None
Corolla color	Pinkish-purple	Pinkish-purple	Pinkish-purple	Whitish*
Corolla lobe l/w	1.25–1.5	1.5	1.5–2.5	1*
Anther appendage	Unlobed	Notched	Notched	None*
Cypselae ribs	8	8–10	10	5*
Cypselae hairs	Simple + glands	Simple + glands	Simple + glands	Glands only*
Pappus bristles	1 series	2 series	2–3 series	None*
Leaf punctation	Absent	Present	Present	Absent

* Transgressive to either putative parental lineage.

The molecular phylogenetic results gave a remarkably similar placement for *Litrisa* as for *Hartwrightia*, with discordance for its sister group between ITS/ETS and plastid based trees. *Litrisa* was placed, like *Hartwrightia*, near *Trilisa* in the ITS/ETS tree but with *C. corymbosus* (and *Hartwrightia*) in the plastid DNA tree (Fig. 2). In the case of *Litrisa*, the morphology (Table 1) is not inconsistent with a hybrid origin, and is actually reflected in its varied placement within either *Carphephorus* or *Trilisa* (when these are recognized to be distinct). For distinguishing characters, *Litrisa* is either intermediate (number of phyllary series; corolla lobe shape; number of cypsela ribs; number of pappus series) or matches *Carphephorus* s.s. (notched tip of anther appendage; glandular-punctate leaves) or *Trilisa* (number of flowers/head; lack of paleae). Thus, a hybrid origin for *Litrisa* fits with the available data, and it is somewhat surprising that this explanation for the distribution of morphological traits has not been advanced previously.

Detailed analysis of ITS sequences of *Litrisa* and *Hartwrightia* compared to their putative progenitors gave contrasting results (Table 2). The ITS sequences of *C. corymbosus* and *Trilisa* had a total of 19 differences. For these positions, the ITS sequence of *Litrisa* matched that of *Carphephorus* at seven positions and that of *Trilisa* for eleven, and had a unique substitution at one position as well as three other unique changes (Table 2). Thus it appears that in *Litrisa* concerted evolution has resulted in fixation of a mixture of the distinctive sites of the parental lineages. In contrast the ITS sequence of *Hartwrightia* matched that of *Trilisa* at all 19 sites; the *Hartwrightia* sequence also exhibited ten unique substitutions, including a 2 bp insertion (Table 2). The 5.8S ribosomal DNA sequences of both *Litrisa* and *Hartwrightia* were completely identical to that of *Trilisa* (and 1 bp different from *C. corymbosus*), making it unlikely that these are pseudogenes (Bailey et al., 2003).

Both *Hartwrightia* and *Litrisa* are rare species, and even within their limited geographic ranges they are relatively uncommon. This contrasts to *Garberia*, another species considered to be rare, which is nevertheless abundant – and in fact a local ecological dominant – in the areas in which it occurs. It also forms a contrast with many other members of Liatrinae, which are often abundant over relatively widespread areas. As an extreme example of their abundance, it has been documented that the economically important *Trilisa odoratissima*, called "Deer's Tongue" or "Vanilla plant", gave an annual harvest from naturally occurring populations of about two million pounds of cured leaves (which were used primarily as an additive to cigarettes; Krochmal, 1969). With the exception of a few species of *Liatris*, most other Liatrinae are relatively common in the areas within which they are found. The rarity of *Hartwrightia* and *Litrisa*, combined with their phylogenetic distinctiveness, suggest that special efforts be given to their conservation.

The molecular phylogenetic results provided strong support for the distinctiveness of *Trilisa* relative to *Carphephorus* s.s. and *Liatris*, reflected in both ITS/ETS and plastid DNA based trees. Recognition that *Litrisa* is of hybrid origin strengthens the morphological support for *Trilisa* as monophyletic, because there is an explanation for the inconsistency of distribution of character traits between *Trilisa* and *Carphephorus* s.s. Within *Trilisa*, there was a clear separation at the molecular level between the two species, *T. odoratissima* and *Trilisa paniculata*, which are also quite distinct morphologically. In contrast, the limited amount of variation observed within *T. odoratissima* did not correspond to the combination of morphological and chemical variants that were the basis for describing *Carphephorus subtropicanus* (Delaney et al., 1999), supporting instead its treatment as a variety (Wunderlin and Hansen, 2001).

Molecular phylogenetic results also provided strong support for the monophyly of *Liatris* as traditionally defined (Figs. 3 and 5), although there was little resolution of species relationships within the genus. The primary morphological features that have been used to define *Liatris* are that the underground stem is corm-like rather than elongate, and that the capitulescence is spicate or spiciform rather than cymose or paniculate. There are exceptions to both character states within the genus, but their distribution in the ITS based phylogeny indicated that these are reversions rather than plesiomorphies (Fig. 3).

The validity of *Carphephorus* as a phylogenetic unit was not clearly supported by the molecular phylogenetic results, although the incongruence between different data sources makes it complicated to assess. The ITS/ETS based phylogenetic estimations suggested that *Carphephorus* forms a basally paraphyletic assemblage relative to *Liatris* (Fig. 3), with *C. bellidifolius* and *C. tomentosus* sharing both synapomorphic bp changes and indels with *Liatris* relative to *C. corymbosus* and *C. pseudoliatris*. The plastid based results

Table 2
Comparison of ITS sequences of *Litrisa* and *Hartwrightia* for sites that vary between *Carphephorus corymbosus* and *Trilisa*. Light shading, ITS-1; no shading, 5.8S rDNA; dark shading, ITS-2; –, indel.

Taxon	26	34	40	44	60	69	70	76	81	90	100	101	118	126	190	215	233	244	256-7	393	445	453	462	466	468	481	543	588	603	613-4	626	643
Carphephorus	A	C	A	C	A	C	C	T	A	T	C	A	C	T	A	C	T	T	--	T	A	G	T	T	T	T	C	T	A	TA	T	T
Litrisa	C	C	A	A	G	C	G	T	A	C	C	G	T	A	A	T	C	C	--	C	A	G	T	T	T	T	C	C	C	--	T	T
Trilisa	C	T	A	A	G	C	T	T	A	C	C	G	T	T	A	T	C	C	--	C	C	A	T	A	A	G	C	T	A	--	T	G
Hartwrightia	C	T	C	A	G	T	T	A	T	C	T	G	T	T	T	T	C	C	GT	C	C	A	C	A	A	G	T	T	A	--	C	G

showed similar placement as in the ITS/ETS based one for each *Carphephorus* species except for *C. bellidifolius*, which was placed instead in a clade with *Trilisa* (Fig. 2B). This result might be best interpreted as a chloroplast transfer event, because *C. bellidifolius* is almost identical morphologically to *C. tomentosus*, and shares no clearly apparent morphological apomorphies with *Trilisa*.

5. Conclusions

Molecular phylogenetic data do not suggest a simple resolution to the problem of generic delimitation in Liatrinae, beyond the trivial solution of placing all of its members (except perhaps *Garberia*) in a single genus. These data clearly suggest that *Carphephorus* s.l. (e.g. including *Carphephorus*, *Litrisa*, and *Trilisa*) is not monophyletic. Based on a combination of molecular and morphological data both *Liatris* and *Trilisa* appear to be both individually distinctive and monophyletic. *Litrisa* and *Hartwrightia* appear to be of hybrid origin based on the incongruence of nuclear and cpDNA-based trees for their placement, and justification for their recognition as distinct genera can be supported by noting that each has a unique phylogenetic history. *Carphephorus* s.s. remains enigmatic, and not only do the features that appear to be diagnostic for the genus appear to be plesiomorphies (elongate rootstocks; cymose capitulescences; larger heads; and notched tips of the anther appendages), there is no evidence from the molecular results that it represents a monophyletic entity. One potential resolution would be to recognize as distinct genera *C. corymbosus*, *C. pseudoliatris*, and *C. bellidifolius* + *C. tomentosus*. A second and perhaps better approach would be to combine *Carphephorus* s.s. (e.g. excluding *Trilisa* and *Litrisa*) and *Liatris* into a single genus.

The most striking result from this study is to document that the results of relatively wide hybridization between lineages that have diverged to the level of being distinct genera might lead to production of a new and morphologically distinct genus. A recurrent result in molecular phylogenetic studies of various lineages of Asteraceae has been to note that there is incongruence between plastid and nuclear-based phylogenetic trees (Fehrer et al., 2007; Kilian et al., 2009; Pelser et al., 2010), but these usually involve clades in which there has been significant species-level divergence following the inferred hybridization. If *Hartwrightia* had undergone subsequent divergence to produce a multispecies lineage, it could well have been recognized taxonomically as a subtribe, based on its distinction for morphological traits that have traditionally been considered to be significant for higher level taxonomic groupings in Eupatorieae. In that case, it would be more difficult to envision a hybrid origin for it.

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Appendix A

List of samples of Liatrinae sampled for molecular phylogenetic analysis. GenBank sequences listed in following order: ITS, *ndh*F, *ndh*I, ETS, *mat*K, *ndh*D, *pet*D, *psb*A-*trn*H, *rbc*L.

Carphephorus Cass., ***C. bellidifolius*** (Michx.) Torr. & A. Gray, North Carolina: (831) *Schilling 02-08* [HQ416304; HQ416210; HQ416256]; (833) *Schilling 02-10* [HQ416305; HQ416209; HQ416255; HQ416405; HQ416290; HQ416153; HQ416166; HQ416191; HQ416180]; (1160) *Schilling 04-19* [HQ416306; HQ416208; HQ416254]; South Carolina: (688) *Nelson 6960*, USCH [HQ416303]; ***C. corymbosus*** (Nutt.) Torr. & A. Gray, Georgia: (772) *Schilling 2036* [HQ416308; EU337037; HQ416242; HQ416400; EU337049, HQ416148; HQ416161; AY727174; HQ416175]; (1117) *Schilling 03-42* [HQ416307]; (2785) *McNeilus 97-994* [HQ416312]; Florida: (837) *Schmalzer s.n.* [HQ416309; HQ416197; HQ416243]; (2783) *Beck 9145* [HQ416310]; (2784) *Godfrey 83998* [HQ416311]; (2796) *Schilling 08-21* [HQ416313; HQ416199; HQ416245]; (2800) *Schilling 08-25* [HQ416314; HQ416198; HQ416244]; ***C. pseudoliatris*** Cass., Alabama: (2780) *Wofford 10350* [HQ416317]; Florida: (597) *Cox P13 5463* [HQ416315; HQ416204; HQ416250; HQ416401; HQ416286, HQ416149; HQ416162; HQ416187; HQ416176]; (2192) *Schilling 05-27* [HQ416316; HQ416205; HQ416251]; (2901) *Schilling 09-F04* [HQ416319; HQ416206; HQ416252]; (2910) *Schilling 09-F11* [HQ416320; HQ416207; HQ416253]; Mississippi: (2781) *Thomas 152776* [HQ416318]; ***C. tomentosus*** (Michx.) Torr. & A. Gray, Georgia: (2352) *Schilling 06-15* [HQ416301; HQ416214; HQ416260]; North Carolina: (699) *Kral 9/20/77* [HQ416302]; (839) *Siripun 10/4/02* [HQ416298]; South Carolina: (832) *Schilling 02-21* [HQ416299; HQ416211; HQ416257]; (834) *Schilling 02-13* [HQ416297; HQ416213; HQ416259]; (835) *Schilling 02-20* [HQ416300; HQ416212; HQ416258; HQ416404; HQ416289; HQ416152; HQ416165; HQ416190; HQ416179].

Garberia A. Gray, ***G. heterophylla*** (W. Bartram) Merr. & F. Harper, Florida: (657) *Coile 9131* [HQ416293; HQ416195; HQ416240] (645) *Evans et al. 45868* [HQ416295]; (774) *Lickey & Beck s.n.* [HQ416294; HQ416194; HQ416239; HQ416397; HQ416283; HQ416145; HQ416158; HQ416184; HQ416172]; (2798) *Beck 9002* [HQ416296; HQ416196; HQ416241].

Hartwrightia A. Gray, H. ***floridana*** A. Gray, Florida: (647) *Cox s.n.* [HQ416322]; Georgia: (773) *Jensen s.n.*11/29/2001 [HQ416321; HQ416200; HQ416246; HQ416398; HQ416284; HQ416146; HQ416159; HQ416185; HQ416173]; (2187) *Schilling 05-09* [HQ416323; HQ416201; HQ416247].

Liatris Gaertner ex Schreber, ***L. acidota*** Engelm. & A. Gray, Louisiana: (592) *Cox P8* [HQ416345]; ***L. aestivalis*** G. L. Nesom & O'Kennon, Texas: *Nesom & O'Kennon FW56*, KSC [HQ416392]; ***L. aspera*** Michx., Oklahoma: (1102) *Lickey 8/5/03-6* [HQ416346; HQ416219; HQ416265]; ***L. chapmanii*** Torr. & A. Gray, Florida: (2819) *Godfrey 84430* [HQ416347]; ***L. cokeri*** M. Pyne & J. M. Stucky, South Carolina: (765) *Schilling 2030* [HQ416348]; (767) *Schilling 2022* [HQ416349]; ***L. compacta*** (Torr. & A. Gray) Rydb., Arkansas: (2815) *Bates & Pittman 10530* [HQ416350]; (2816) *Bates 10430* [HQ416351]; ***L. cylindracea*** Michx., Alabama: Hardig et al. 2005 [AY804146, AY804145]; ***L. elegans*** (Walt.) Michx., Arkansas: (1182) *Schilling 04-40* [HQ416354]; Georgia: (1113) *Schilling 03-38* [HQ416355; HQ416221; HQ416267]; South Carolina: (770) *Schilling 2031* [HQ416353]; ***L. elegantula*** (Greene) K. Schum., Alabama: (1107) *Schilling 03-05* [HQ416356; HQ416216; HQ416262]; ***L. garberi*** A. Gray, Florida: (2812) *McNeilus 96-868* [HQ416359]; (2813) *Lakela 25349* [HQ416360]; ***L. glandulosa*** G. L. Nesom & O'Kennon, Texas: *Nesom et al. FW62*, KSC [HQ416393]; ***L. gracilis*** Pursh, Florida: (2788) *Schilling 08-13* [HQ416362]; (2793) *Schilling 08-17* [HQ416363]; (2795) *Schilling 08-20* [HQ416364]; ***L. hirsuta*** Rydb., Arkansas: (2817) *Thomas 50140* [HQ416366]; Louisiana: (2818) *Steyermark 24097* [HQ416367]; Missouri: (2810) *Floden s.n.* [HQ416365]; ***L. laevigata*** Nutt., Florida: (2822) *Godfrey 64750* [HQ416368]; ***L. ligulistylis*** (A. Nelson) K. Schum., Minnesota: (2823) *McNeilus 98-747* [HQ416369]; ***L. microcephala*** K. Schum., Alabama: (842) *Schilling s.n.* [HQ416370; HQ416217; HQ416263; HQ416403; HQ416288; HQ416151; HQ416164; HQ416189; HQ416178]; (1105) *Schilling 03-31* [HQ416371; HQ416220; HQ4162664]; ***L. ohlingerae*** (S. F.

Blake) B. L. Rob., Florida: (2824) *McNeilus 96-847* [HQ416372]; ***L. oligocephala*** J. R. Allison, Alabama: Hardig et al. 2005 [AY804147]; ***L. pauciflora*** Pursh, Florida: (2825) *Godfrey 71997* [HQ416373]; ***L. provincialis*** R. K. Godfrey, Florida: (2826) *Godfrey 64627* [HQ416375]; ***L. punctata*** Hook., New Mexico: (726) *Schilling 02-14* [HQ416376]; (727) *Schilling 02-15* [HQ416377]; ***L. pycnostachya*** Michx., Arkansas: (1101) *Lickey 8/6/03-2* [HQ416378]; ***L. scariosa*** (L.) Willd., West Virginia: (2827) *Morton 7989* [HQ416379]; ***L. spicata*** (L.) Willd., Georgia: (1115) *Schilling 03-40* [HQ416383; HQ416222; HQ416268]; South Carolina: (756) *Schilling 2040* [HQ416380; HQ416215; HQ416261; HQ416402; HQ416287; HQ416150; HQ416163; HQ416188; HQ416177]; (757) *Schilling 2044* [HQ416381]; (758) *Schilling 2045* [HQ416382]; ***L. squarrosa*** (L.) Michx., Georgia: (2814) *Moore 1281* [HQ416384]; ***L. squarrulosa*** Michx., Arkansas: (1180) *Schilling 04-36* [HQ416386]; Tennessee: (659) *Cox s.n.* [HQ416385; HQ416218; HQ416264]; ***L. tenuifolia*** Nutt., Georgia: (1121) *Schilling 03-50* [HQ416390; HQ416223; HQ416269]; South Carolina: (752) *Schilling 2033* [HQ416387]; (753) *Schilling 2037* [HQ416388]; ***L. virgata*** Nutt., Georgia: (2828) *Schilling 05-22* [HQ416391].

Litrisa Small, ***L. carnosa*** Small, Florida: (2778) *Kral 64019* [HQ416344; HQ416203; HQ416249]; (651) *Cox s.n.* [HQ416343; HQ416202; HQ416248; HQ416399; HQ416285, HQ416147; HQ416160; HQ416186; HQ416174].

Trilisa (Cass.) Cass., ***T. odoratissima*** (J. F. Gmel.) Cass. var. ***odoratissima***, Florida: (838) *Schmalzer s.n.* [HQ416337; HQ416229; HQ416275; HQ416407; HQ416291; HQ416154; HQ416168; HQ416192; HQ416182]; (1122) *Schilling 03-52* [HQ416338; HQ416230; HQ416276]; Louisiana: (586) *Urbatsch 7013* [HQ416334]; South Carolina: (755) *Schilling 2035* [HQ416335; HQ416231; HQ416277]; (762) *Schilling 2047* [HQ416336; HQ416232; HQ416278]; var. "***subtropicana***", Florida: (2653) *Delaney 4022* [HQ416342]; (2790) *Schilling 08-15* [HQ416339; HQ416233; HQ416279]; (2794) *Schilling 08-19* [HQ416340; HQ416234; HQ416280]; (653) *Cox s.n.* [HQ416341; HQ416235; HQ416281]; ***T. paniculata*** (J. F. Gmel.) Cass., Florida: (595) *Cox 5466* [HQ416324]; (2792) *Schilling 08-16* [HQ416332]; (2797) *Schilling 08-22* [HQ416333]; Georgia: (761) *Schilling 2046* [HQ416327; HQ416225; HQ416271]; South Carolina: (691) *Nelson 21688*, USCH [HQ416325; AF384744; AF383811; HQ416406; HQ416291; AF384491; HQ416167; AY727171; HQ416181]; (763) *Schilling 2021B* [HQ416331; HQ416226; HQ416272]; (764) *Schilling 2027B* [HQ416329; HQ416228; HQ416274]; (769) *Schilling 2029* [HQ416328; HQ416224; HQ416270]; (759) *Schilling 2038* [HQ416326]; (771) *Schilling 2034* [HQ416330; HQ416227; HQ416273].

Outgroups: ***Ageratina luciae-brauniae*** (Fernald) R. M. King & H. Rob., Tennessee, *Schilling 95-15* [AF177781/AF177782; HQ416193; HQ416236; HQ416394; HQ416282; HQ416142; HQ416155; HQ416183; HQ416169]. ***Eupatorium hyssopifolium*** L., Florida, *Siripun 02-EUP-157* [DQ236177; EU337035; HQ416237; HQ416395; EU337047; HQ416143; HQ416156; AY727172; HQ416170]. ***Eutrochium maculatum*** (L.) E. E. Lamont, New York, *Schilling 95-16* [AF177838; EU337036; HQ416238; HQ416396; EU337048; HQ416144; HQ416157; EU337026; HQ416171].

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