USING cpDNA TO ASSESS SPECIES DELIMITATION BETWEEN *Liatris helleri* PORTER (ASTERACEAE) AND *Liatris turgida* GAISER

A Thesis
by
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Abstract

Using cpDNA to assess species delimitation between *Liatris helleri* Porter (Asteraceae) and *Liatris turgida* Gaiser

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Liatris helleri Porter (Heller's Blazing Star) is a threatened (federally-listed) high-elevation rock outcrop perennial species endemic to nine populations in Western North Carolina. Morphological evidence suggests that *L. helleri* is morphologically indistinguishable from *L. turgida* Gaiser (the Shale Barren Blazing Star), and that the two species should be subsumed into a single species under a morphological basis.

However, there are more than 20 species concepts, and conservation agencies are often hesitant to change species designations for protected species without testing several species concepts for species delimitations. This has been the case with *L. helleri*, as ecological and distributional differences between *L. helleri* and *L. turgida* have raised questions about the validity of classifying *L. helleri* strictly on a morphological basis.

We amplified regions of the cpDNA (Chloroplast DNA) genome in order to test the Phylogenetic Species Concept (PSC). Under the PSC, if *L. helleri* and *L. turgida* are different species, then they should form two monophyletic groups. We hypothesized that we would see two distinct monophyletic groups.

Phylogenetic analyses recovered two clades. Neither was monophyletic for *L. helleri* or *L. turgida*, yet both were dominated by one taxa with a single representative of the other taxa in that clade. TCS analysis revealed a central set of populations (mainly *L. helleri* populations), and gave rise to three separate, independent radiations consisting mainly of *L. turgida*. Our results do not support recognition of *L. helleri* and *L. turgida* as two distinct species. These results suggest that *L. helleri* and *L. turgida* are in some phase of a speciation event; however, those lineages have not yet sorted into monophyletic groups. Also, it appears that *L. turgida* is not one cohesive entity, but rather a dustbin group that encompasses several lineages that are derived from the *L. helleri* central group. We suggest a reevaluation of the current distributions of these taxa, as well as continued protection of *L. helleri* sensu USFWS until further nuclear and ecological work can be done, and that conservation efforts focus on this central cluster of populations, as a number of unique radiations appear to be derived from this cluster.

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Dedication

This thesis is dedicated to my graduate committee, Dr. Eva Gonzales, Dr. Zack Murrell, and Dr. Gary Walker. They have all been very influential on my life and my career as a biologist. Dr. Gary Walker taught the first college class I walked into at Appalachian, and has always been there for me when I have needed him. Dr. Zack Murrell has also been very supportive in my times of need, and told a story once that inspired me to apply to graduate school in spite of adversity. Dr. Eva Gonzales opened her lab to me and inspired me in so many ways. She was very accommodating in allowing me to pick a research topic that I was passionate about, and so very patient in helping to develop both my project and my research skills. I could not have completed this project without the help of these three wonderful mentors, and for this reason, I dedicate this thesis to them.

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INTRODUCTION

Since the publication of Darwin's *Origin of Species*, the topic of species concepts has generated tremendous debate and controversy amongst biologists. It has been suggested that more literature pertaining to speciation and species concepts has been produced than any other topic in evolutionary biology (Sites & Marshall 2003). Nevertheless, a consensus among researchers for a definition of a species may be no closer now than it was for Darwin, when he wrote "No one definition has as yet satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species"(Darwin 1859).

The debate for a clear definition of a species is fueled by the necessity for a functional means to classify organisms (De Queiroz 2005). The need for a standardized method of species delimitation is apparent across all branches of biology, due to the use of species as the lowest quantifiable groupings of distinctiveness between organisms in systematics (De Queiroz 2005).There is some debate amongst biologists as to whether or not "species" exist as a natural entity with their own set of important biological properties (Mayr 1963, Mayden 2002) or if the species rank is an arbitrary, manmade designation no different from any other level of taxonomic classification (Nelson 1989). Regardless, systematists use species as the fundamental units of biodiversity, or the lowest degree of difference between two organisms that can be used to accurately distinguish between them. This designation plays a pivotal role in the naming, classifying, and placement into the hierarchy of living organisms that we have developed to date.

It is this degree of difference between species, however, that is usually the cause for controversy. Not only are there controversies concerning the magnitude of differences that may be recognized at the species level, but also concerns surrounding the categorical variables of those differences. If two organisms are morphologically indistinguishable, yet have significant differences in distribution, behavioral, ecological, or genetic characteristic, should they be treated as the same species in lieu of those differences? Also, is there one of these criteria that contributes the most to speciation processes, and thus should be weighted more heavily in species delimitation?

These are questions that have been the subject of the rigorous debate revolving around various species concepts in existence today. To date, there are approximately 26 species concepts in the literature (Frankham et al. 2012). Perhaps the most classical definition of a species comes from the Biological Species Concept (BSC), defined by Ernst Mayr as groups of interbreeding populations which are reproductively isolated from other such groups (Mayr 1940, Mayden 2002). Difficulties with this definition of a species have arisen due to interspecific hybridization and testing for reproductive isolation and has lead to development of other species concepts. The Morphological Species Concept (MSC) is popular among taxonomists, due to its practicality as an operational concept, and defines a species as the smallest groups that are consistently morphologically distinct from other such groups, and are distinguishable by observational criteria (Cronquist 1978, Mayden 2002). Problems arise under this definition as well, including those related to phenotypic plasticity and classification of cryptic species.

With the advent of molecular techniques making sequence data more readily available, one of the most commonly utilized species concepts to date is the Phylogenetic Species Concept (PSC), which recognizes a species as the smallest grouping of organisms with a hereditary pattern of ancestry and descent (Cracraft 1983, Mayden 2002), or speciation on the basis of monophyly. One of the clear advantages of the PSC is recognition of species based on the historical and evolutionary components involved in speciation.

As expected, application of various species concepts yields varying numbers and groupings of species, and the application of different concepts will have an impact on conservation management strategies. In a critique of several species concepts, De Queiroz (2007) notes that each species concept tends to recognize speciation on the basis of one biological property at the expense of all others. While most species concepts have the potential to aid in biological classification, the concepts all focus on a variety of criteria that may be at various levels of importance with regard to speciation processes for a particular organism. As most species concepts have strengths and weaknesses of application, De Quieroz (2007) suggests a Unified Species Concept (USC), taking evidence for or against speciation under the various species concepts together as "operational criteria" for species delimitation.

Over the past decade, phylogenetic relationships have undergone constant revision due to the increased availability of sequence data (Cuénoud et al. 2002, Bateman et al. 2003). However, in concordance with the USC, suggestions for changes to the taxonomic code now often apply several species concepts in defining species (Chan et al. 2002, Cattell & Karl 2004, Friar et al. 2007, Duminil & Di Michele 2009). This multifaceted approach is often particularly useful with species that have a high degree of morphological variability across

their distribution or for organisms with a high degree of taxonomic complexity (Chan et al. 2002).

One species with a unique set of species delimitation issues of particular importance to conservation biologists is in the genus *Liatris* (Asteraceae). Some of the earliest morphological work for the *Liatris* genus by Gaiser (1946) consolidated the *Liatris* name with other synonyms (*Laccinaria, Suprago*, and others), and subdivided the genus into five sections containing 42 species. She also later identified at least ten hybrids within and between those sections using morphological and cytological data (Gaiser 1951). One of the species recognized in Gaiser's 1946 monograph is *Liatris helleri* (Porter 1891).

Liatris helleri Porter (Asteraceae), commonly known as Heller's Blazing Star, is regarded as a high-elevation (above 1000M) rock outcrop endemic, restricted to nine populations in four counties (Avery, Ashe, Burke, and Caldwell) in Western North Carolina (Murdock & Sutter 1989). The species is considered a narrow Southern Appalachian endemic, and is thought to be a relic from a Pleistocene Appalachian alpine flora (Wiser 1994). Due to the narrow distribution and perceived threats (trampling by hikers and recreation seekers, commercial and residential development, and succession due to fire suppression of the natural range) to the species, the long-lived perennial species was listed by the United States Fish and Wildlife Service (USFWS) as "Federally-threatened" on November 19th, 1987 (Murdock & Sutter 1989). The species is one of 37 species currently recognized in the *Liatris* genus (FNA 2006), and is characterized as a grass-like perennial herb, with single-veined leaves arising from a corm-like rootstock. The showy lavender-colored spike inflorescences flower from early July to September and mature from the top to the bottom. The flowering heads each

contain seven to thirteen florets with a reduced pappus, five to seven stamens and a single stigma, and give rise to achenes that are dispersed by wind in mid to late September (Murdock & Sutter 1989).

In a Flora of North America project, Nesom (2005b) revised Gaiser's taxonomic treatments of *Liatris*, using a greater breadth of samples and information about the genus. Gaiser (1946) recognized 42 species in *Liatris,* including two sections, section Euliatris containing four series, and section Suprago containing six series (including section Graminifoliae, which contains *L. helleri* and *L. turgida*, as well as Spicatae, which contains *L. spicata,* an outgroup for this study). In contrast, Nesom (2005b) recognized 37 species within *Liatris*, and placed them into an informal order within an infrageneric classification system adapted from that of Gaiser (1946), on the basis of morphology. Nesom (2005b) recognizes five sections, including section Liatris (further subdivided into three series), section Vorago, section Suprago (which includes the *L. spicata* outgroup), section Pilifilis, and section Graminifoliae. Section Graminifoliae is further divided into five series, including series Virgatae (which contains *L. cokeri,* another outgroup for this study), series Pauciflorae, series Garberae, series Graminifoliae (which contains *L. helleri* and *L. turgida*), and series Scariosae.

Conservation strategies for *L. helleri* have been tailored to what little we know about the species. In the original draft of the recovery document (Murdock & Sutter 1989), criteria for delisting the species are established, along with six necessary actions to meet the goal of delisting. The document estimates the cost of those six actions per year for the first three years after publication (1990-1992) at an average of $48,833.00 per year. The USFWS, along with several other state and local conservation agencies monitor and inventory populations of

L. helleri each year. In addition, prescribed burns are occasionally used to encourage recruitment of *L. helleri* seedlings near populations and reduce competition from other species (Murdock & Sutter 1989). Hiking trails near populations have been either diverted or, in one case (Sutter et al. 1993) have had boardwalks built through populations, to reduce the impact of foot traffic on individuals within those populations. Population augmentation, or introduction of greenhouse grown plants to native populations, has been a controversial strategy used to aid in either the establishment of new populations or augmentation of small populations (Murdock & Sutter 1989). In addition, signs have been posted at several populations warning hikers to stay on trails in order to deter trampling damage (Murdock & Sutter 1989).

Very little ecological, genetic, or morphological work has been done with *L. helleri* to date, despite the recognized need for biological research to guide conservation efforts (Murdock & Sutter 1989). After the species was federally listed, two studies by Godt and Hamrick (1995 and 1996) sought to improve understanding of the species in order to make informed management decisions. In their first study (1995), they sought to understand the reproductive biology of *L. helleri* by isolating allozymes from greenhouse-grown seedlings to detect inbreeding within their populations of origin. They also bagged the inflorescences in a pollinator-exclusion mesh in order to determine if the species was capable of self-fertilization. They detected a "small but significant" amount of biparental inbreeding within populations, as predicted by the small and isolated nature of the populations. They also found that in all but one plant they bagged (N=30) there were no seeds produced, suggesting self-incompatibility (Godt & Hamrick 1995). They suggested that, like many other species in the

Asteraceae, *L. helleri* has a multiallelic sporophytic incompatibility system (Richards 1997) in which plants in a given population may have different alleles encoding the self-incompatibility trait, and fertility of a plant is based on genotype of both the pollen donor and the plant receiving pollen.

In a follow up study (Godt & Hamrick 1996), they again used allozymes to determine genetic diversity and population structure of *L. helleri*. They found that *L. helleri* has a higher genetic diversity than expected for an endemic species. In addition, they also found a correlation between geographic and genetic distance, and low levels of gene flow between populations, suggesting divergence among populations. For this reason, in their recommendations for conservation management they suggested that population distinctiveness be maintained in conservation efforts.

One of the more recent studies involving *L. helleri* came as a result of the Flora of North America treatment of the *Liatris* genus (Nesom 2005b). As the first taxonomist since Gaiser to examine the genus as a whole, Nesom had access to a broader range of samples and new techniques to aid in morphological classification. In his examination of specimens and construction of new keys, he noted that *L. helleri* was so similar to another species, *Liatris turgida*, that he could not recognize one diagnostic character to distinguish the species from one another. He suggested that on the basis of this new morphological evidence, *L. helleri* and *L. turgida* should be grouped into one "broadened concept" of the species (Nesom 2005a).

Prior to Nesom's publication, *L. helleri* was distinguished from *L. turgida* by a few morphological characters, including density of the inflorescence, plant height, and pappus

length (Gaiser 1946, Cronquist 1981, King & Robinson 1987); however, it became apparent that plant height and inflorescence density were variable characters and might be related to resource availability and age of the perennial, and the primary diagnostic character became pappus length. Keys defined *L. helleri* as having a pappus length of one half to two thirds the length of the corolla tube, and *L. turgida* as having a pappus to corolla tube ratio greater than two thirds (Gaiser 1946, Cronquist 1981). Nesom (2005a), however, observed that pappus length was variable in the herbarium material he examined, and thus suggested subsuming the taxa into a single species (*L. helleri* sensu lato) as a way to deal with the species in a "broadened" sense due to this morphological overlap.

In light of Nesom's observation about pappus length variability within *L. helleri*, some clarification of nomenclature within *L. helleri* is necessary. Any populations that have a reduced pappus (pappus length is one half to two-thirds the length of the corolla tube) are designated as *L. helleri* sensu stricto. Populations identified by USFWS as *L. helleri* are designated as *L. helleri* sensu USFWS. Finally, populations that would be defined by a consolidation of *L. helleri* and *L. turgida* (as suggested by morphology) are designated as *L. helleri* sensu lato.

It is interesting to note that although Nesom is the most recent author to compare *L. helleri* with *L. turgida*, he is not the first. In a report to USFWS by Sutter and Murdock (1984), the distinctiveness of *L. helleri* on the basis of pappus length was investigated, with a focus on comparison with *L. graminifolia* (a species that is now split into other entities in the most recent volume of Flora of North America), although the authors mention that they compared these pappus lengths with herbarium vouchers of *L. turgida* as well. However, at any point in

the publication, measurement data from *L. turgida* herbarium vouchers do not appear in text or in figures. The only other mention of *L. turgida* is at the end, when the authors state that the species was included in the study but was "obviously unrelated" to *L. helleri*. It is unclear whether or not this omission of data is intentional.

Liatris turgida Gaiser (1946), the Shale Barren Blazing Star or Appalachian Blazing Star, is considered a rare species but is not protected at the federal or state levels, and is typically found in shale barrens, primarily in Virginia and West Virginia, although disjunct populations in southern North Carolina have been reported. This shale barren habitat is considerably different than the rock outcrops of Western North Carolina (WNC) in soil chemistry and depth, associated flora, temperature and elevation. The rock outcrops of WNC are at the tops of mountains and typically have a soil pH averaging around 4 and are less than 30 cm deep (Wiser et al. 1999); Shale barrens are typically steep southwesterly facing slopes on mountain sides, have low soil pH, and have little to no true soil (Platt 1951). The higher soil temperatures and low soil moisture content, in combination with constraints on root space, eliminate most seedlings, and have allowed the barrens to be inhabited by a unique flora (Keener 1983), with a number of endemic and near endemic species (Platt 1951). It is important to note that shale barrens (Allard & Leonard 1946) have been suggested to be historically lacking successional stages; that is, the vegetation that currently inhabit this ecosystem is both the primary successional species as well as the climax vegetation, and no successional replacements occur to any appreciable degree. This is somewhat the case in high-elevation rock outcrops, although climax vegetation is widely held to be affected by anthropogenic fire suppression in some of those areas.

USFWS has not yet adopted the broadened concept of *L. helleri* without further evidence of speciation, due to the taxonomic and conservation repercussions of such a decision. According to the rules of the International Code of Nomenclature for algae, fungi, and plants (formerly the International Code of Botanical Nomenclature), any time two species are grouped under one name, the specific epithet that is older (named first) is the one that must be kept. In the case of *L. helleri* Porter (1891) and *L. turgida* Gaiser (1946), *L. helleri* is the older name under which the species would be recognized, in accordance with Nesom's broadened concept (as *L. helleri* sensu lato). If adopted by USFWS and other conservation agencies, the expanded distribution resulting from Nesom's taxonomic convention would extend federal protections to the populations previously considered to be *L. turgida*, but could also possibly disqualify *L. helleri* from federal protection if the species is delisted. Classification by a single criterion has been increasingly scrutinized in recent years (De Queiroz 2007, Duminil & Di Michele 2009, Frankham et al. 2012), particularly for species of conservation concern. Here, I assess whether or not *L. helleri* and *L. turgida* should be treated as two separate species by using molecular data (cpDNA sequences) and floral morphology (pappus to corolla tube ratios). I asked these questions: 1) Are *Liatris helleri* sensu USFWS and *L. turgida* the same species? 2) Are the chloroplasts distinguishable using molecular data? 3) How does molecular evidence compare to morphological evidence for these taxa? 4) And finally, how can this information aid in making informed conservation management decisions for the species? This study will provide useful information in order to determine appropriate conservation strategies for the species.

MATERIALS AND METHODS

Population Sampling

I collected leaf tissue from one individual from all known extant populations of *L. helleri* sensu USFWS and seven *L. turgida* populations. I also collected leaf tissue from two other *Liatris* species, *L. spicata* (L.) Willd and *L. cokeri* Pine and Stucky, for comparison as outgroups. *L. cokeri* is within the same section but a different series than *L. helleri* and *L. turgida*, and *L. spicata* is in a different section entirely, according to the infrageneric classification of the genus (Nesom 2005b). I froze the specimens in liquid nitrogen after collection and stored them at -80°C prior to DNA extractions. I obtained voucher specimens from larger populations, which are maintained in the Appalachian State University Herbarium (BOON). I recorded GPS coordinates and altitude for all sampling localities (Figure 1 and Table 1).

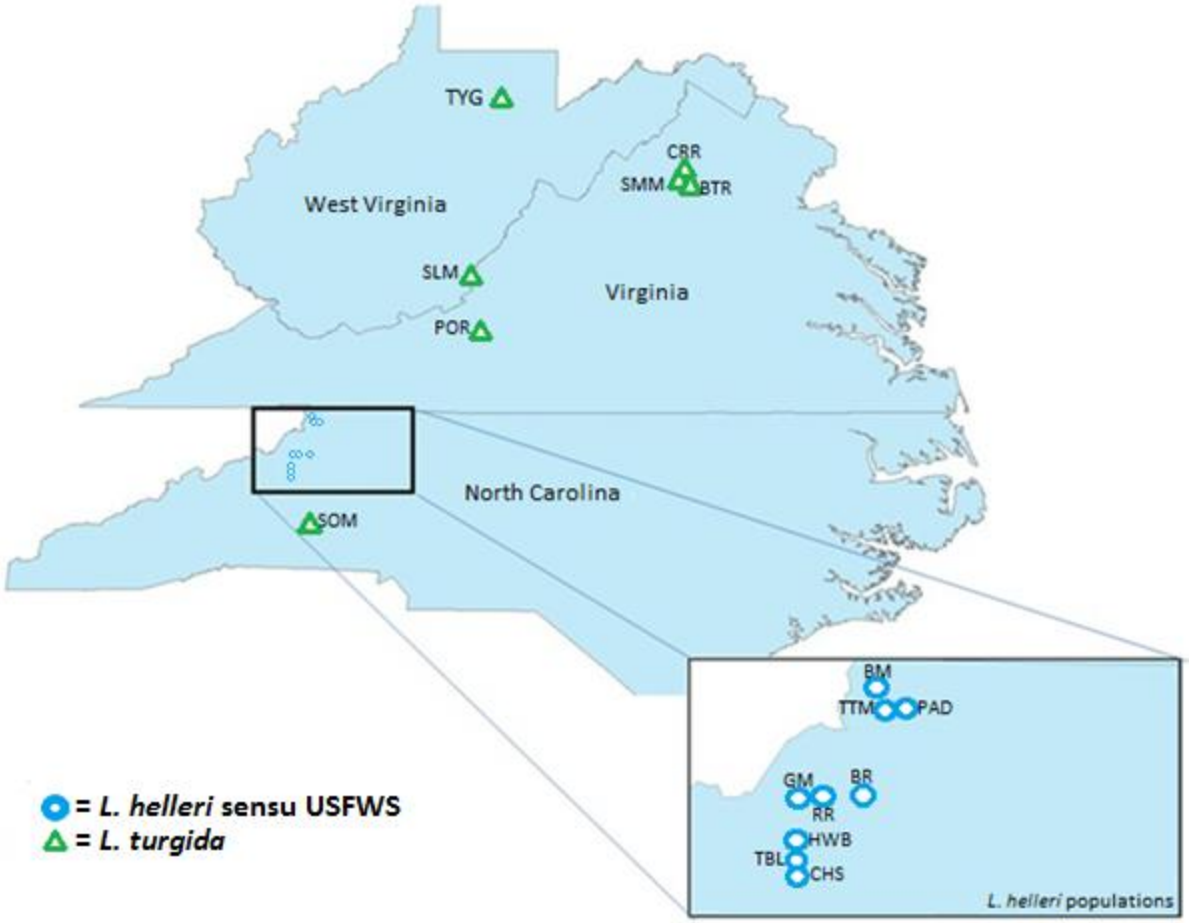

Figure 1. Geographic locations of *Liatris helleri* sensu USFWS and *L. turgida* populations sampled.

Table 1. Species, sampling locality, state, abbreviation, GPS coordinates and altitude for populations sampled.

Species	Location	State	Abbreviation	Latitude	Longitude	Altitude (M)
L. helleri	Blowing Rock	NC	LhBR	N 36^0 06' 49"	W 081^0 39' 41"	1066
L. helleri	Chimneys	NC	LhCHS	N 35^0 52' 43"	W 081^0 53' 15"	1121
L. helleri	Grandfather Mountain	NC	LhGM	N 36^0 05' 43"	W 81^0 49' 54"	1585
L. helleri	Hawksbill	NC	LhHWB	N 35^0 53' 45.7"	W 081^0 53' 14.2"	1180
L. helleri	Bluff Mountain	NC	LhBM	N 36^0 23' 37"	W 081^0 33' 09"	1341
L. helleri	Paddy Mountain	NC	LhPAD	N 36^0 24' 50"	W 081^0 30' 47"	1311
L. helleri	Rough Ridge	NC	LhRR	N 36^0 05' 50"	W 081^0 47' 58"	1333
L. helleri	Table Rock	NC	LhTBL	N 35^0 53' 17.3"	W 081^0 53' 06.6"	1071
L. helleri	Three Top Mountain	NC	LhTTM	N 36^{0}25'08.3"	W 081°35'18.4"	1530
L. turgida	Betty's Ridge	VA	LtBTR	N 38^0 34' 02.7"	W 078^0 22' 02.7"	1125
L. turgida	Crescent Ridge	VA	LtCRR	N 38^0 33' 31.8"	W 078^0 23' 07.7"	1047
L. turgida	Poor Mountain	VA	LtPOR	N 37^0 12' 51.6"	W 080^0 07' 50.0"	618
L. turgida	Slaty Mountain	WV	LtSLM	N 37^0 39' 00.6"	W 080^0 18' 15.5"	728
L. turgida	Stony Man Mountain	VA	LtSMM	N 38^0 36' 09.0"	W 078^0 22' 07.6"	1093
L. turgida	South Mountain State Park	NC	LtSOM	N 35^0 37' 58"	W 081^0 38' 24"	840
L. turgida	Tygart River Valley	WV	LtTYG	N 39^0 12' 23.1"	W 079^0 57' 25.5"	368
L. cokeri	Jones Lake State Park Area	NC	LC	N 34^0 42' 30"	W 078^0 38' 30"	23
L. spicata	Appalachian State University	NC	LS	N 36^0 22' 49"	W 081^0 41' 57"	1016

Lab Analysis

I extracted total genomic DNA from the flash frozen samples, using the DNeasy Mini Kit (Qiagen, Chatsworth, California, USA), following the protocol from the manufacturer, and quantified the DNA concentrations using the Thermo Fisher Nanodrop 2000c (Thermo Fisher Scientific, Waltham, Massachusetts, USA) system prior to amplification. I sequenced non-coding cpDNA regions using published universal primers (Taberlet et al. 1991, Hamilton 1999, Peakall & Smouse 2006) to compare cpDNA sequence similarities between the taxa (Table 2). Non-coding cpDNA sequences are an ideal marker to ask questions at the species or genus level due to the relatively low rate of mutation and the uniparentally inherited nature of plastid DNA. I predicted that these sequences would suggest evidence for the presumed ecological and distributional differences between these taxa.

I amplified the cpDNA using PCR reactions consisting of 12.5μL GoTaq Hot Start Green Master Mix (Promega, Madison, Wisconsin, USA), 9.5μL nuclease-free water, 1μL forward primer (10μM), 1μL of reverse primer(10μM), and 1μL of template DNA. I used the T-gradient thermocycler (Biometra, Goettingen, Germany), with an initial denaturation at 95°C for 5 minutes, followed by 42 cycles of denaturation at 94°C for 30 seconds, annealing at a variable temperature for 30 seconds, and extension at 72°C for 1 minute, followed by a final extension cycle of 7 minutes at 72°C. Annealing temperatures varied between primer pairs, but were within 48°C-56.5°C.

Table 2. Summary of primers, including variability, intergenic region amplified, annotated product size, annealing temperature, and publisher for each primer pair.

Variable regions			
Primer	Non-coding region amplified	Product size (bp)	Annealing temperature (^{0}C)
H5-H6[1]	rpl20 - 5' rps12	690	55.5
E-F[2]	trnL – trnF	315	50
7-8[3]	rps16 exon1 - rps16 exon2	785	50
40-42[3]	psbM - trnD (GUC)	578	50.9
49-50[3]	trnG - rps14	493	50
59-60[3]	trnT (UGU) - trnL (UAA)	513	50
64-66[3]	trnV(UAC2) - trnV (UAC1)	520	50
81-82[3]	petG - trnP (UGG)	288	50.9
83-84[3]	psaJ - rpl33	563	50.9
93-94[3]	clpP exon1 - psbB	399	50.9
97-98[3]	petB exon2 - petD exon2	910	53
99-100[3]	rps11 - rps8	640	56.5
Nonvariable regions			
Primer	Non-coding region amplified	Product size (bp)	Annealing temperature (^{0}C)
61-62[3]	trnF (GAA) - ndhj	241	50.9
85-86[3]	rpl33 - rps18	346	50.9

[1]Hamilton 1999

[2]Taberlet 1991

[3]Ebert and Peakall 2009

Initially, I screened 46 primer pairs to determine if they produced a PCR product, using samples from a single population of each presumed species. I ran PCR products on a 1% agarose gel stained with GelRed (Biotium, Hayward, California, USA) and estimated the fragment length based on a DNA standard. Out of the primer pairs screened, 14 yielded a single band indicating the presence of a PCR product. I amplified the rest of the samples and sent the resulting fragments for DNA sequencing (Retrogen inc., San Diego, California, USA).

In order to examine pappus length in living specimens, I took images of live florets (and herbarium specimens when live tissue was not available) from a single individual of both *Liatris helleri* sensu USFWS and *L. turgida* using the Olympus SZ61 microscope and the Olympus DP21 imaging system (Olympus America, Allentown, PA). I measured three individual pappus bristles as well as the length of the corolla tube (CT), to obtain an average pappus to corolla tube length ratio. The images were then recorded digitally, and the ratios of corolla tube length to pappus length were calculated and averaged for each individual floret.

Data Analysis

I edited DNA sequences manually using Sequencher v. 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). In the case of sequence ambiguity or variation, I obtained both forward and reverse sequences to clear up ambiguities or to confirm differences in sequences among individuals. Insertions and deletions were treated as point mutations, and were weighted equally with other mutations. I confirmed sequence quality by examining the chromatograms for both directions of sequences. Sequences for each primer pair were

aligned in MUSCLE (Edgar 2004) using the default settings with ten iterations and condensed into a sequence contig for each population (which was also realigned using MUSCLE), and the differences in those contigs were used to assign cpDNA haplotypes for each individual. I compared those haplotypes to ascertain whether or not there was any haplotype overlap between individuals belonging to *L. helleri* sensu USFWS and *L. turgida*. I used the program TCS (Clement et al. 2000) to construct a haplotype network resulting from combined cpDNA data, in order to determine the relationships between the haplotypes occurring for each species. The program has been used extensively between organisms with relatively low divergences, and has the fine-scale resolution necessary for this analysis.TCS calculates an absolute distance matrix for pairwise comparisons of the haplotypes. The pairwise distance matrix is then used to calculate the minimum number of "steps" between haplotypes and forms a graphical network of haplotypes. Large indels (more than one continuous mutational difference) were treated as a single stepwise difference.

I also performed phenetic analyses (maximum parsimony, maximum likelihood, and the distance-based neighbor joining method) for a comparison with the results from TCS. I used MEGA version 5.1 to generate consensus trees using a statistical significance cutoff of 50%, in which I reported the bootstrap values on the branch for any branches present in 50% or more of the 10,000 bootstrap replicates. MEGA 5.1 was also used to test which model of DNA evolution to use in all subsequent analyses.

In order to determine if most of the variability in the data set is within or between species I performed an Analysis of Molecular Variance (AMOVA) using GenAlEx v.6.5 (Peakall & Smouse 2006). I also used GenAlEx to construct a genetic distance matrix using Nei's genetic distance (1973) and averages by species of those genetic distances, in order to account for the average level of differences within *L. helleri* sensu USFWS populations, within *L. turgida* populations, and between pairs of populations of both species.

RESULTS

A total of fourteen regions were amplified and DNA sequenced for a total of 7281 bp. There was a high level of cpDNA haplotype variation among populations. Twelve of the fourteen regions sequenced were variable, showing a total of 47 variable nucleotide positions (Table 3). The resulting variation was grouped into thirteen cpDNA haplotypes. Most (69%) populations had unique haplotypes. Seven unique haplotypes occur within *L. helleri* sensu USFWS, with one haplotype (haplotype E) shared by two *L. helleri* sensu USFWS populations (LhPAD and LhHWB). Five unique haplotypes occur within *L. turgida*, with one haplotype (haplotype H) shared by two *L. turgida* populations (LtCRR and LtSMM). Haplotype H was also shared by a *L. helleri* sensu USFWS population (LhTTM).

Table 3. Summary of mutations and their associated species, population, haplotype, and intergenic region of occurrence.

Species	Population	Haplotype	rps16 ex1 - rps16 ex2	psbM - trnD	trnG - rps14
L. helleri	BM	A	...AC...G…----…T…	…C…G…	…C-AA…
L. helleri	BR	B	...AA...G...----...T…	…A…G…	…CAAA…
L. helleri	CHS	C	...AA...G...----...T…	…A…G…	…C-AA…
L. helleri	GM	D	...AA...G...----...T…	…A…G…	…C-AA…
L. helleri	HWB	E	...AC...G…----…T…	…C…G…	…C-AA…
L. helleri	PAD	E	...AC...G…----…T…	…C…G…	…C-AA…
L. helleri	RR	F	...AA...G...---G...T…	…A…G…	…C-AA…
L. helleri	TBL	G	...AC...G…----…T…	…C…G…	…C-AA…
L. helleri	TTM	H	...TA...G...----...T…	…A…T…	…C---…
L. turgida	BTR	I	...TA...G...GGGG...T…	…A…G…	…C--A…
L. turgida	CRR	H	...TA...G...----...T…	…A…T…	…C---…
L. turgida	POR	J	...TA...G...----...T…	…A…T…	…C---…
L. turgida	SLM	K	...AC...G…----…T…	…C…G…	…C--A…
L. turgida	SMM	H	...TA...G...----...T…	…A…T…	…C---…
L. turgida	SOM	L	...AA...G...----...T…	…A…G…	…C-AA…
L. turgida	TYG	M	...TA…C...----...T…	…A…T…	…C-AA…
L. cokeri	LC	----------	...AA...G...----...T…	…A…G…	…A-AA…
L. spicata	LS	----------	...AA...G...----...T…	…A…G…	…C--A…

Table 3 (continued). Summary of mutations and their associated species, population, haplotype, and intergenic region of occurrence.

Species	Population	Haplotype	trnT (UGU) - trnL (UAA)	trnV(UAC2) - trnV (UAC1)
L. helleri	BM	A	…-…TTAATCGTGATAAA…C…	…A…C…C…A…
L. helleri	BR	B	…-…TTAATCGTGATAAA…T…	…A…C…C…A…
L. helleri	CHS	C	…-…TTAATCGTGATAAA…C…	…A…C…C…A…
L. helleri	GM	D	…-…TTAATCGTGATAAA…T…	…A…C…C…A…
L. helleri	HWB	E	…-…TTAATCGTGATAAA…T…	…A…C…C…G…
L. helleri	PAD	E	…-…TTAATCGTGATAAA…T…	…A…C…C…G…
L. helleri	RR	F	…-…TTAATCGTGATAAA…T…	…A…C…C…A…
L. helleri	TBL	G	…-…TTAATCGTGATAAA…T…	…A…C…C…G…
L. helleri	TTM	H	…-…TTAATCGTGATAAA…T…	…A…C…T…G…
L. turgida	BTR	I	…T…TTAATCGTGATAAA…T…	…A…C…C…G…
L. turgida	CRR	H	…-…TTAATCGTGATAAA…T…	…A…C…T…G…
L. turgida	POR	J	…-…----------…T…	…A…C…T…G…
L. turgida	SLM	K	…-…TTAATCGTGATAAA…T…	…A…C…C…G…
L. turgida	SMM	H	…-…TTAATCGTGATAAA…T…	…A…C…T…G…
L. turgida	SOM	L	…-…TTAATCGTGATAAA…T…	…-…C…C…G…
L. turgida	TYG	M	…-…TTAATCGTGATAAA…T…	…A…C…C…G…
L. cokeri	LC	----------	…-…TTAATCGTGATAAA…T…	…A…A…C…A…
L. spicata	LS	----------	…-…TTAATCGTGATAAA…T…	…A…C…C…A…

Table 3 (continued). Summary of mutations and their associated species, population, haplotype, and intergenic region of occurrence.

Species	Population	Haplotype	petG - trnP	psaJ - rpl33	clpP ex1 - psbB	petB ex2 - petD ex2
L. helleri	BM	A	…G…	…C…C…-…-…	…C…	…T…
L. helleri	BR	B	…G…	…C…C…-…-…	…C…	…T…
L. helleri	CHS	C	…G…	…C…C…-…-…	…C…	…T…
L. helleri	GM	D	…G…	…C…C…-…-…	…C…	…T…
L. helleri	HWB	E	…G…	…C…C…-…-…	…C…	…T…
L. helleri	PAD	E	…G…	…C…C…-…-…	…C…	…T…
L. helleri	RR	F	…G…	…C…C…-…-…	…C…	…T…
L. helleri	TBL	G	…G…	…C…C…-…-…	…C…	…T…
L. helleri	TTM	H	…A…	…C…C…A…-…	…C…	…A…
L. turgida	BTR	I	…G…	…C…C…-…-…	…C…	…T…
L. turgida	CRR	H	…A…	…C…C…A…-…	…C…	…A…
L. turgida	POR	J	…A…	…C…C…A…-…	…C…	…A…
L. turgida	SLM	K	…G…	…C…C…-…-…	…C…	…T…
L. turgida	SMM	H	…A…	…C…C…A…-…	…C…	…A…
L. turgida	SOM	L	…G…	…C…C…-…-…	…C…	…T…
L. turgida	TYG	M	…A…	…T…-…-…-…	…C…	…A…
L. cokeri	LC	----------	…G…	…C…C…-…C…	…C…	…T…
L. spicata	LS	----------	…G…	…C…C…-…-…	…A…	…T…

Table 3 (continued). Summary of mutations and their associated species, population, haplotype, and intergenic region of occurrence.

Species	Population	Haplotype	rps11 - rps8	tmL - tmF	rpl20 - 5' rps12
L. helleri	BM	A	…A…A…	…A…A…	…T…A…
L. helleri	BR	B	…A…A…	…A…A…	…T…A…
L. helleri	CHS	C	…A…A…	…A…A…	…T…A…
L. helleri	GM	D	…A…A…	…A…A…	…T…A…
L. helleri	HWB	E	…A…C…	…A…A…	…T…A…
L. helleri	PAD	E	…A…C…	…A…A…	…T…A…
L. helleri	RR	F	…A…A…	…A…A…	…T…A…
L. helleri	TBL	G	…A…A…	…A…A…	…T…A…
L. helleri	TTM	H	…A…A…	…T…A…	…T…C…
L. turgida	BTR	I	…G…A…	…A…A…	…T…C…
L. turgida	CRR	H	…A…A…	…T…A…	…T…C…
L. turgida	POR	J	…A…A…	…T…A…	…T…C…
L. turgida	SLM	K	…A…C…	…A…A…	…T…A…
L. turgida	SMM	H	…A…A…	…T…A…	…T…C…
L. turgida	SOM	L	…A…C…	…A…A…	…T…A…
L. turgida	TYG	M	…A…A…	…A…C…	…A…C…
L. cokeri	LC	----------	…A…A…	…A…A…	…T…A…
L. spicata	LS	----------	…A…A…	…A…A…	…T…A…

Phylogenetic relationships

The TCS haplotype network revealed a paraphyletic relationship between *L. helleri* sensu and *L. turgida* (Figure 2). The *L. helleri* sensu USFWS population at Grandfather Mountain is ancestral (adjacent to both outgroups) and gives rise to several populations of *L. helleri* sensu USFWS. Two *L. turgida* populations are closely related to these L. helleri sensu USFWS populations, while there are three longer branches of *L. turgida* (with one exception in *L. helle*ri) extending from the main cluster of *L. helleri* sensu USFWS populations. From this core, there appears to be three independent radiations of *L. turgida* populations (with the exception of LhTTM).

The *L. turgida* population at Betty's Rock (LtBTR), in the Shenandoah National Park, is the closest peripheral population to the core populations (closest to LhRR) with seven steps difference between the populations. The next closest radiation is eleven steps away from the core (closest to LhGM), and consists of three populations (which represent the haplotype shared between *L. helleri* and *L. turgida*), one *L. helleri* population (LhTTM) and two *L. turgida* populations (LtCRR and LtSMM), which are also from the Shenandoah National Park. The shared haplotype populations gave rise to the furthest *Liatris* population from the core (LtPOR), which is two steps beyond those populations with the shared haplotype. The second furthest radiation (LtTYG) is twelve steps away from the closest *L. helleri* population (LhGM) in the core (Figure 2).

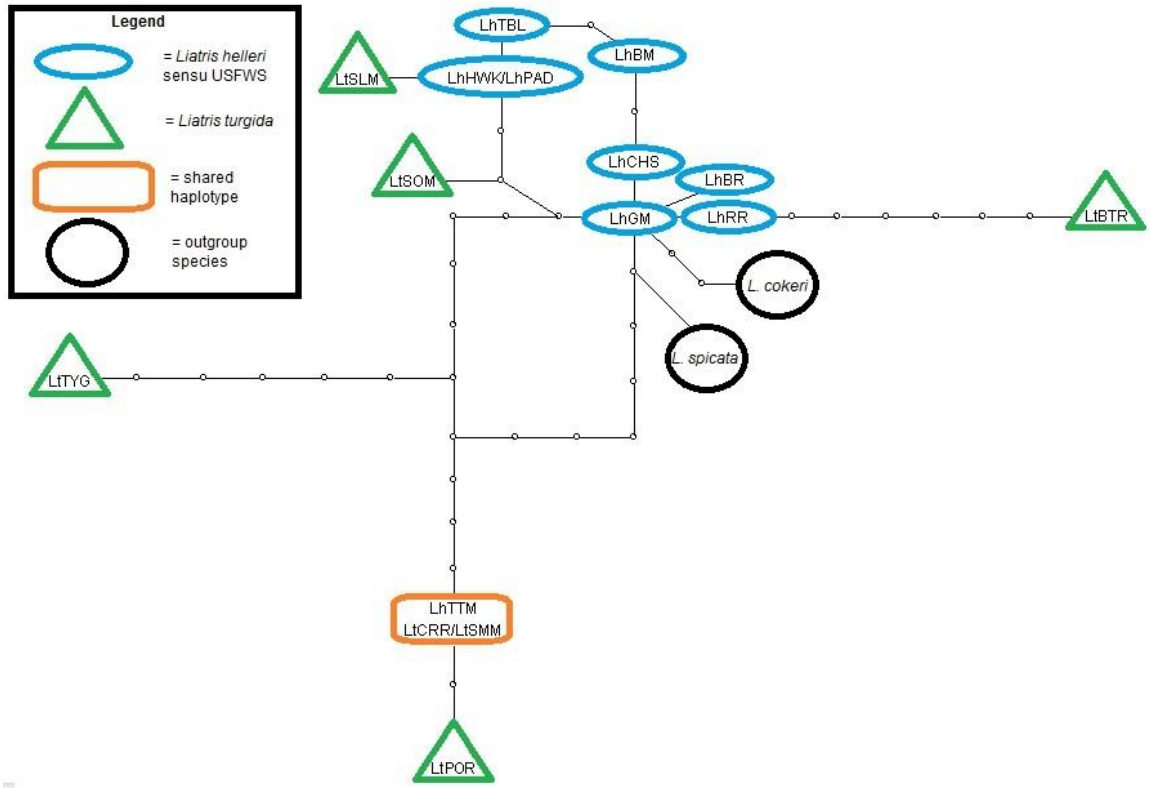

Figure 2. Minimum spanning network of *Liatris helleri*, *L. turgida*, and outgroups (*L. spicata* and *L. cokeri*) constructed from cpDNA sequences generated using TCS. Blue ovals indicate populations identified as *L. helleri* sensu USFWS, green triangles indicate populations of *L. turgida*, and black circles are outgroups.

The distance-based neighbor joining (NJ) consensus tree from the cpDNA concatenated sequences recovered two clades, neither of which were monophyletic (Figure 3). The consensus tree using maximum likelihood (ML) criteria resulted in the same tree as the neighbor joining tree, with differences in bootstrap values (Figure 4). As in the haplotype network, there was no evidence for monophyly of the taxa. There was little support for the phylogeny, except one clade with bootstrap support of 90 or greater (clade A), and another clade with bootstrap support of 50 or greater (clade B). Clade A consists of five individuals of *L. turgida* and a single individual from *L. helleri*. In contrast, clade B consists of three individuals in *L. helleri* and a single individual from *L. turgida*. The consensus tree constructed via the maximum parsimony (MP) methods recognized clade A but did not recognize clade B (Figure 5).

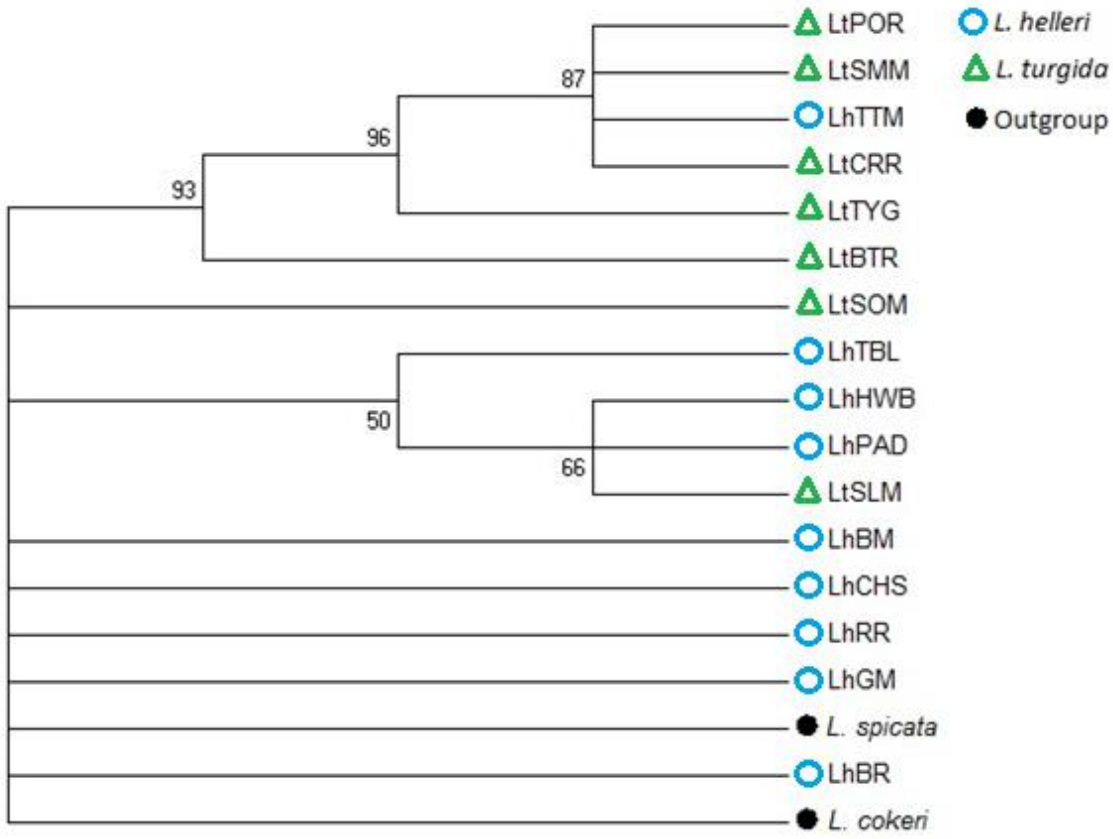

Figure 3. Consensus Neighbor-joining tree, generated in MEGA 5 from cpDNA sequences from *L. helleri*, *L. turgida*, and outgroups, using a 50% significance cutoff of 10,000 bootstrap replicates. Numbers on branches are bootstrap values.

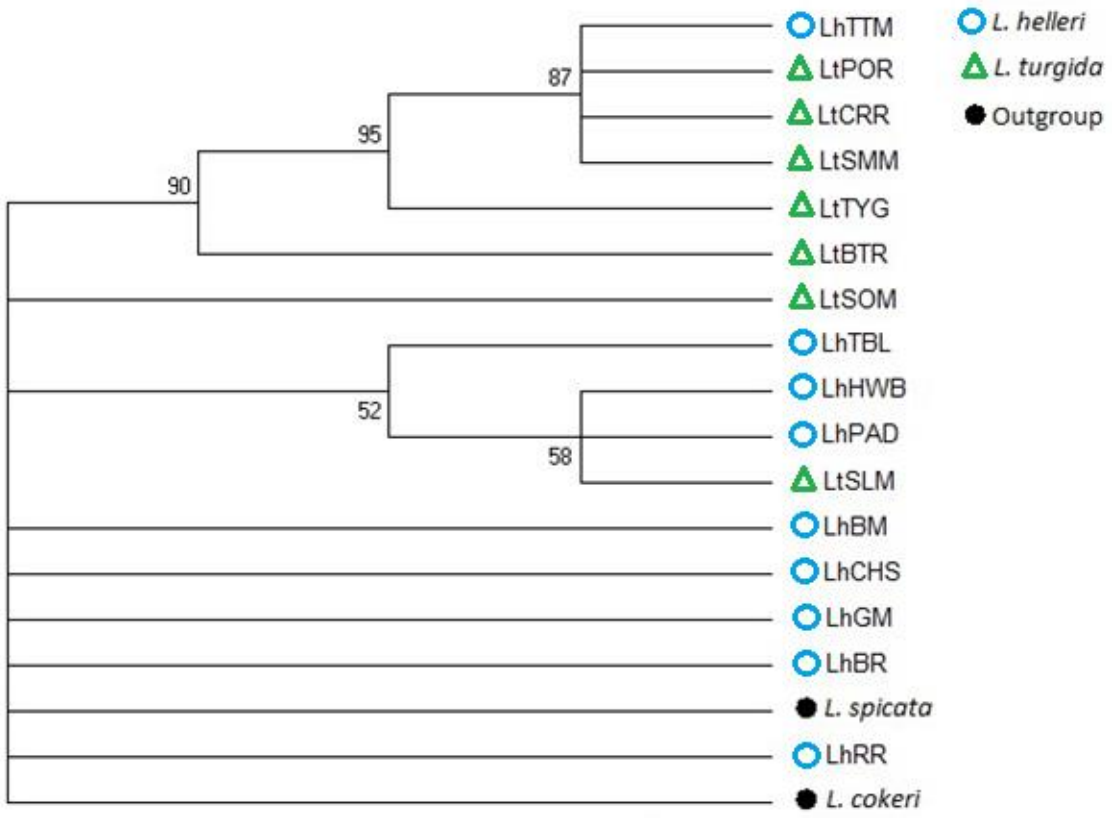

Figure 4. Consensus maximum likelihood tree, generated in MEGA 5 from cpDNA sequences from *L. helleri*, *L. turgida*, and outgroups, using a 50% significance cutoff of 10,000 bootstrap replicates. Numbers on branches are bootstrap values.

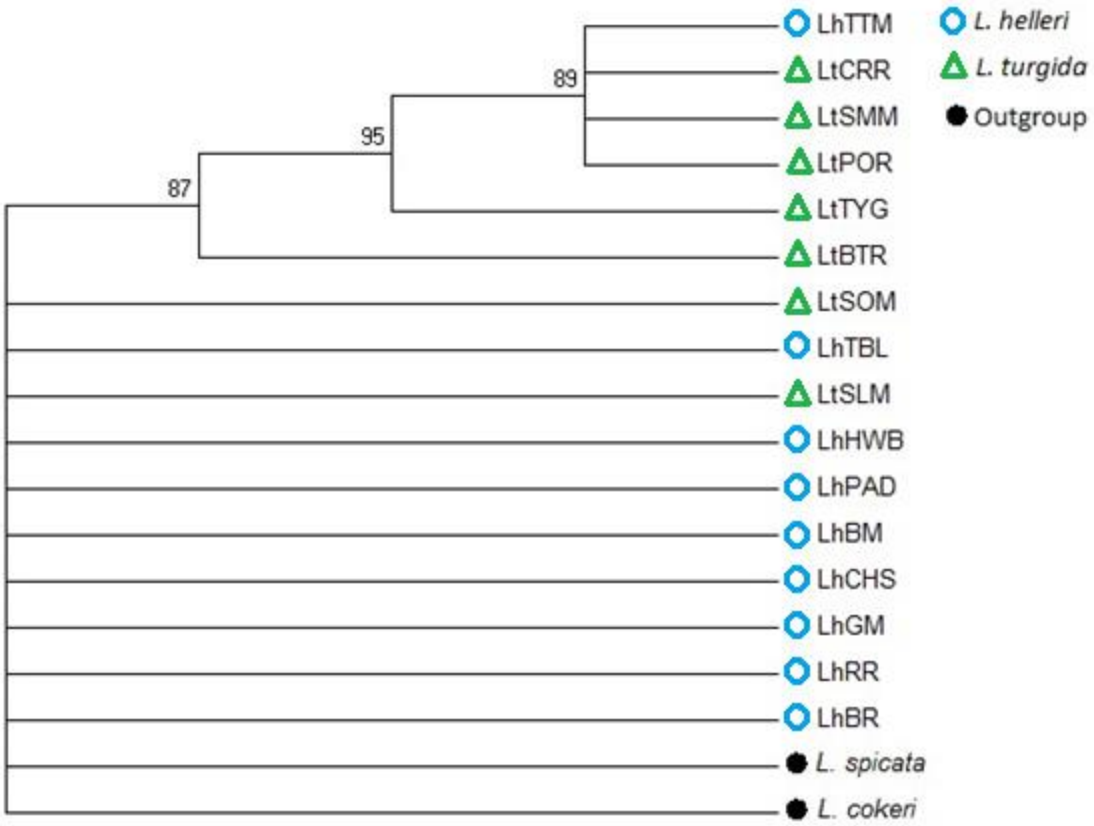

Figure 5. Maximum parsimony tree, generated in MEGA 5 from cpDNA sequences from *L. helleri*, *L. turgida*, and outgroups, using a 50% significance cutoff of 10,000 bootstrap replicates. Numbers on branches are bootstrap values.

Nei's Genetic Distance Matrix

The genetic distance matrix (Table 4) and averages for each species (Table 5) showed a higher Nei's genetic distance between *L. helleri* sensu USFWS and *L. turgida* than observed between the two outgroup species (*L. cokeri* and *L. spicata*). Average Nei's genetic distances between *L. helleri* sensu USFWS populations was 0.120 (SD = 0.106), while the average Nei's genetic distances between *L. turgida* populations was 0.395 (SD = 0.247). The average Nei's genetic distance between populations of *L. helleri* sensu USFWS and *L. turgida* was 0.313 (SD = 0.242). *Liatris cokeri* had an average distance from *L. helleri* sensu USFWS of 0.142 (SD = 0.086) and an average distance from *L. turgida* of 0.377 (SD = 0.271). *Liatris spicata* had an average distance from *L. helleri* sensu USFWS of 0.122 (SD = 0.067) and an average distance from *L. turgida* of 0.308 (SD = 0.236). The Nei's genetic distance between outgroups *L. cokeri* and *L. spicata* was 0.115.

Table 4. Pairwise matrix from comparison of Nei's genetic distances among populations of *L. helleri*, *L. turgida*, and outgroups.

	LhBM	LhBR	LhCHS	LhGM	LhHWB	LhPAD	LhRR	LhTBL	LhTTM	LtBTR	LtCRR	LtPOR	LtSLM	LtSMM	LtSOM	LtTYG	LC	LS
LhBM	0.000																	
LhBR	0.089	0.000																
LhCHS	0.043	0.043	0.000															
LhGM	0.066	0.022	0.022	0.000														
LhHWB	0.066	0.112	0.112	0.089	0.000													
LhPAD	0.066	0.112	0.112	0.089	0.000	0.000												
LhRR	0.089	0.043	0.043	0.022	0.112	0.112	0.000											
LhTBL	0.043	0.089	0.089	0.066	0.022	0.022	0.089	0.000										
LhTTM	0.354	0.295	0.295	0.267	0.324	0.324	0.295	0.295	0.000									
LtBTR	0.324	0.267	0.267	0.239	0.295	0.295	0.213	0.267	0.324	0.000								
LtCRR	0.354	0.295	0.295	0.267	0.324	0.324	0.295	0.295	0.000	0.324	0.000							
LtPOR	0.960	0.854	0.854	0.806	0.906	0.906	0.854	0.854	0.384	0.906	0.384	0.000						
LtSLM	0.089	0.137	0.137	0.112	0.022	0.022	0.137	0.043	0.295	0.267	0.295	0.854	0.000					
LtSMM	0.354	0.295	0.295	0.267	0.324	0.324	0.295	0.295	0.000	0.324	0.000	0.384	0.295	0.000				
LtSOM	0.137	0.089	0.089	0.066	0.066	0.066	0.089	0.089	0.295	0.267	0.295	0.854	0.089	0.295	0.000			
LtTYG	0.384	0.324	0.324	0.295	0.354	0.354	0.324	0.324	0.267	0.416	0.267	0.806	0.384	0.267	0.324	0.000		
LC	0.137	0.089	0.089	0.066	0.161	0.161	0.089	0.137	0.354	0.324	0.354	0.960	0.187	0.354	0.137	0.324	0.000	
LS	0.112	0.066	0.066	0.043	0.137	0.137	0.066	0.112	0.267	0.239	0.267	0.806	0.112	0.267	0.112	0.354	0.112	0.000

Table 5. Average Nei's genetic distances among taxa of interest.	Average	SD
Nei's genetic distance among *L. helleri* sensu USFWS	0.120	0.106
Nei's genetic distance among *L. turgida*	0.395	0.247
Nei's genetic distance between *L. helleri* sensu USFWS and *L. turgida*	0.313	0.242
L. cokeri Nei's genetic distance from *L. helleri* sensu USFWS	0.142	0.086
L. cokeri Nei's genetic distance from *L. turgida*	0.377	0.271
L. spicata Nei's genetic distance from *L. helleri* sensu USFWS	0.112	0.067
L. spicata Nei's genetic distance from *L. turgida*	0.308	0.236
Nei's genetic distance between *L. spicata* and *L. cokeri*	0.112	-------

Analysis of Molecular Variance

An AMOVA (Table 6) revealed that 19% of the genetic variation was between *L. helleri* sensu USFWS and *L. turgida*, and 81% was within species. As only one individual per population was sequenced, within population variation could not be assessed.

Table 6. Summary AMOVA table for variance within and between *L. helleri* sensu USFWS and *L. turgida*.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Estimated Variance	%
Between species	1	12.752	12.752	1.040	19%
Within species	14	63.873	4.562	4.562	81%
Total	15	76.625		5.602	100%

Pappus:Corolla Tube ratios

Pappus:CT ratios revealed interpopulation variability of pappus lengths within *L. helleri* sensu USFWS. Two of the five populations identified as *L. helleri* sensu USFWS (LhBR and LhHWB) with voucher specimens had an average pappus:CT ratio of less than 0.67, congruent with those expected from *L. helleri* sensu stricto (Table 7). None of the *L. turgida* populations had an average pappus:CT ratio below 0.67. Populations of *L. helleri* sensu USFWS had pappus:CT ratios ranging from 0.50 to 1.36, and an average pappus:CT length of 0.78 (SD=0.228), supporting claims of pappus variability among *L. helleri* sensu USFWS. *Liatris turgida* pappus to corolla tube ratios range from 0.76 to 1.24, and average 0.96(SD=0.098). Voucher specimens were not collected from some sites due to small population sizes (LhBM, LhCHS, and LtSMM) or due to permit restrictions (LhPAD and LhRR).

Table 7. Species, population, and average of pappus:CT length ratio and standard deviations for populations sampled.

Species	Population	Average Pappus:CT length ratio	Standard Deviation
L. helleri	BM	NO DATA	NO DATA
L. helleri	BR	0.563	0.047
L. helleri	CHS	NO DATA	NO DATA
L. helleri	GM	0.975	0.160
L. helleri	HWB	0.657	0.093
L. helleri	PAD	NO DATA	NO DATA
L. helleri	RR	NO DATA	NO DATA
L. helleri	TBL	0.923	0.112
L. helleri	TTM	1.136	0.199
L. turgida	BTR	0.820	0.057
L. turgida	CRR	1.010	0.074
L. turgida	POR	0.953	0.039
L. turgida	SLM	0.919	0.037
L. turgida	SMM	NO DATA	NO DATA
L. turgida	SOM	1.007	0.044
L. turgida	TYG	1.157	0.087

DISCUSSION

Liatris helleri sensu USFWS and *L. turgida* do not form two clear, distinct monophyletic lineages. The absence of monophyly of *L. helleri* sensu USFWS is supported by morphological incongruence, as pappus:CT ratio among *L. helleri* sensu USFWS populations appears to be highly variable, with only two populations sampled keying to *L. helleri* sensu stricto. There is a surprisingly high level of cpDNA haplotype diversity within and between *L. helleri* and *L. turgida*, with most populations having a unique haplotype. In the haplotype network, a cluster of populations consisting of eight *L. helleri* populations and two *L. turgida* populations (which could be interpreted as ancestral, but there is a need to understand relationships within the genus clarify this hypothesis) that give rise to three independent radiations, consisting of *L. turgida* populations with one exception from *L. helleri*. Although the data here supports recognition of the broadened concept of *L. helleri*, a number of interesting questions arise from the data regarding the nature of these relationships within *L. helleri*.

Pappus: CT ratio is not a reliable character to distinguish between *L. helleri* sensu USFWS and *L. turgida* (Table 7). Only two *L. helleri* sensu USFWS populations (LhBR and LhHWB) had average pappus to corolla tube ratios less than 0.66 that would define them as *L. helleri* sensu stricto (Gaiser 1946, Cronquist 1981). This lends support to a broadened concept of *L. helleri* due to variability in pappus length. The pappus:CT ratios ranged from 0.5 to 1.36. It is likely that further variability for pappus length exists within and among

populations of *L. helleri* sensu USFWS, as I was only able to take measurements from a few individuals in a few populations. However, these measurements demonstrate that pappus length is variable, and that at least three of the populations identified by USFWS as *L. helleri* are not consistent with the morphological description of the species. If morphological criteria are to be used in delimitation of the species, only the populations at Blowing Rock (the type locality for *L. helleri*) and Hawksbill should be recognized as *L. helleri* (Figure 6). Variability in pappus length across the range of *L. helleri* sensu USFWScould have implications for seed dispersal. Operating under the assumptions that pappus length is a heritable character, it is plausible that populations with a shorter pappus would have a reduced capacity for wind dispersal and thus a reduced range for seed dispersal. This hypothesis might explain why some populations have pappus lengths longer than expected for *L. helleri*, as those populations with a longer pappus are capable of seed dispersal over a larger range, and thus would become more prevalent over time given the competitive advantage they maintain over the populations with reduced pappus length. However, there do not appear to be any obvious trends that effectively correlate pappus length with phylogenetic relationships (Figure 7).

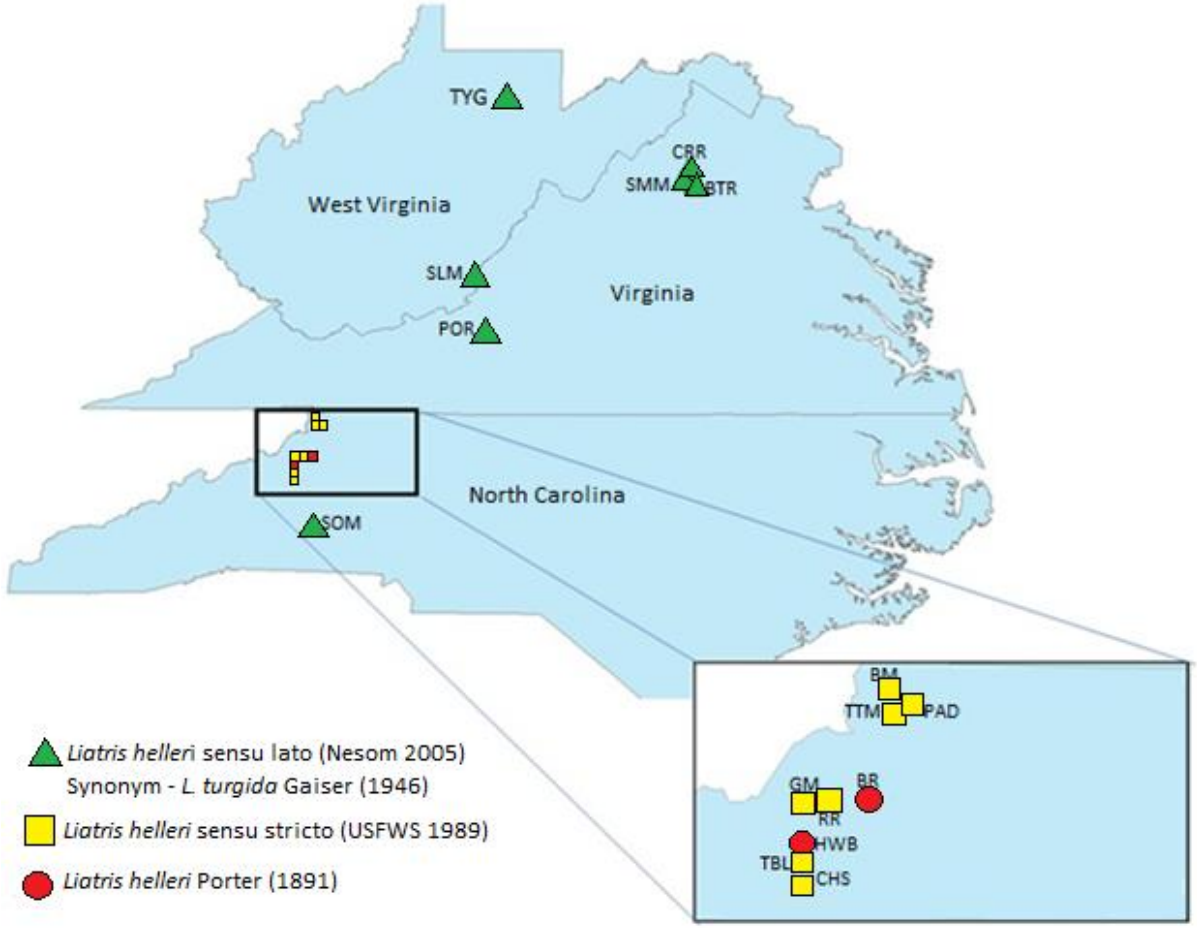

Figure 6. Clarification of historical and contemporary nomenclature within *Liatris helleri*. Populations indicated by red circles are populations that key to *L. helleri* Porter (1891) using pappus:CT ratio. Populations indicated by yellow squares include populations designated as *L. helleri* sensu stricto by the USFWS recovery document (1989). Populations indicated by the green triangles indicate population identified as *L. helleri* sensu lato (Nesom 2005) under the broadened concept of the species.

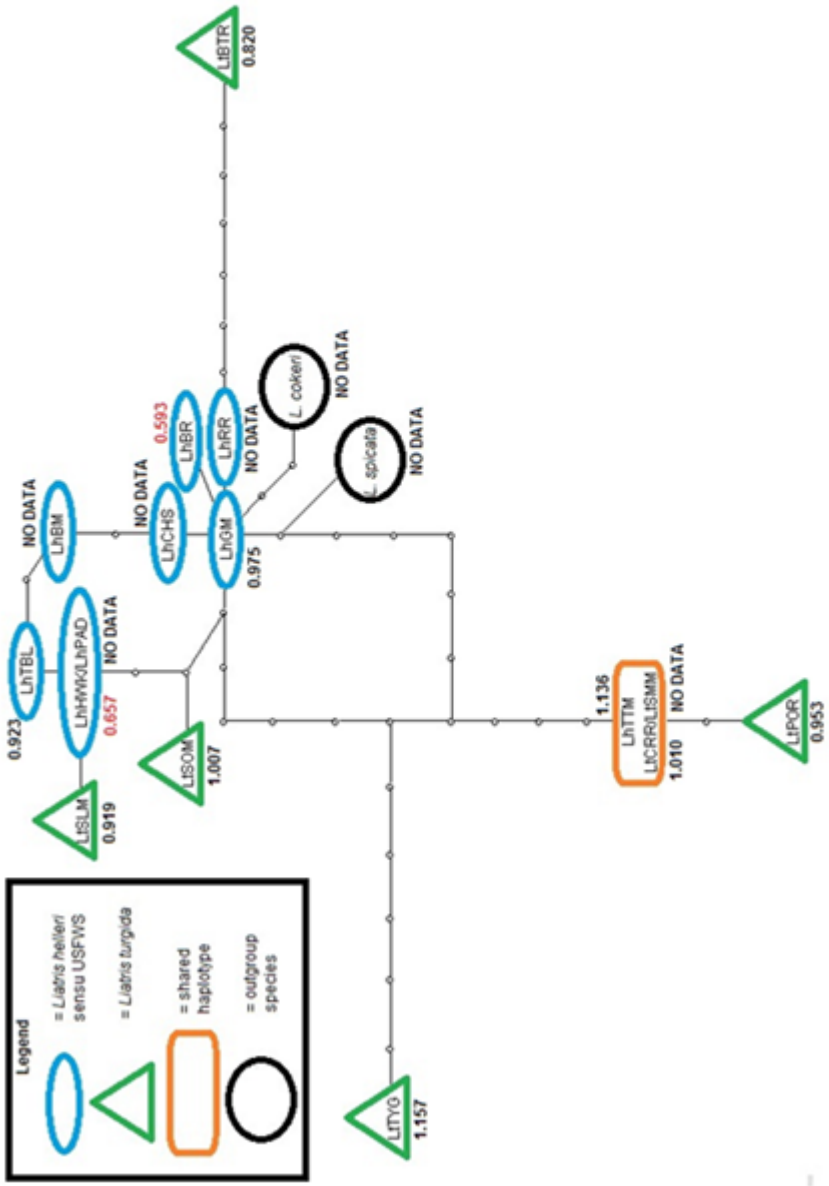

Figure 7. Pappus:CT ratios show no trends with cpDNA haplotype network. Pappus:CT ratios are displayed nearest to the haplotype representing the population of origin for the specimens measured. Red numerals indicate populations that have a pappus:CT ratio less than 0.66 and thus key to *L. helleri* sensu stricto.

It is unclear why populations that do not have pappus:CT ratios less than 0.66 are recognized as *L. helleri* by USFWS. Prior to the broadened concept of *L. helleri* (Nesom 2005), another publication raised questions about pappus length in *L. helleri*, and compared the specimens in *L. helleri* sensu USFWS with *L. turgida* and *L. pilosa*, which at the time was recognized as *L. graminifolia* (Sutter & Murdock 1984). However, the data from the *L. turgida* specimens are not included in the data analysis for that publication, and are only mentioned as "obviously unrelated to *L. helleri*" until Nesom revisited the work in 2005. In the NCNHP EO data (NCNHP EO database, unpublished), there are notes from a number of botanists comparing the morphology of the populations to *L. pilosa* and *L. turgida.* Most of the IDs in the NCNHP database are based on pappus length, with notes that intrapopulation variation exists. It is possible they were classified on the basis of their distribution; however, *L. helleri* is not the only small, single-veined *Liatris* species in the counties in which they occur. *Liatris pilosa* is also distributed in those areas (The NCNHP EO data says that they are "mixed in" with several *L. helleri* sensu USFWS populations), and the species is morphologically similar enough to cause confusion, as *L. pilosa* is in the same series as *L. helleri* and *L. turgida* according to both Nesom (2005) and Gaiser (1946). It is also possible that these populations were classified on an ecological basis, but these populations encompass significant variation in habitat, soil properties, light availability, and associated vegetation.

Regardless of how these populations were initially characterized as *L. helleri* by USFWS, it is interesting that although most populations are lacking the pappus:CT ratios typical of *L. helleri*, most of the *L. helleri* sensu USFWS populations (with the exception of one population, LhTTM) are cluster together in the haplotype network (Figure 2). This cluster is

largely comprised of *L. helleri* sensu USFWS, but also encompasses two *L. turgida* populations (LtSLM and LtSOM). These *L. turgida* populations are two of the three *L. turgida* populations that are geographically closest to the populations identified as *L. helleri* sensu USFWS. From this central cluster of populations, at least three independent radiations of *L. turgida* exist, suggesting that *L. turgida* is not a valid taxonomic group, but rather a dustbin group with multiple origins. If these populations previously held as *L. turgida* cannot be cohesively contained into one group independent of the core populations, then the only two alternatives are to either recognize each radiation as a separate taxonomic entity or to recognize *L. helleri* in the broad sense (*L. helleri* sensu lato).

The populations in the primary cluster of the haplotype network appear to have significant taxonomic, ecological, genetic and geographic similarities to one another, but it is important to note that there are some inconsistencies within the group. The group consists mainly of *L. helleri* sensu USFWS populations, but there are also two *L. turgida* populations in close proximity. Also, most of the populations are geographically close to one another, but the *L. helleri* sensu USFWS population at Three Top Mountain (LhTTM) is also inside this geographic range, yet is several steps away in the haplotype network. The group also consists of populations mainly in high elevation rock outcrops, yet the *L. turgida* populations at Slaty Mountain (LtSLM) and South Mountain (LtSOM) are in ecologically different habitats. Despite these inconsistencies, it is possible that this subset of populations represents a taxonomically important subspecies-level group within *L. helleri*, especially when taken in consideration that at least three independent evolutionary radiations arose from this group. In

conversations with taxonomic and conservation biologists about this subject, the idea of recognition of this group below the species rank has been suggested on several occasions. Further study into the validity of this subspecies group is essential to developing an effective conservation strategy for *L. helleri*.

AMOVA analysis indicates that the majority of the cpDNA variation we found exists within species (Table 6). When this information is cross-referenced with the data in the cpDNA haplotype network (Figure 2), it is apparent that most of the variation exists within *L. turgida*. The ecological variation between the habitats in which *L. helleri* sensu USFWS and *L. turgida* samples originate may contribute to this variation. Also, the geographic range encompassed by *L. turgida* is much greater than the range of *L. helleri* sensu USFWS and thus should be expected to encompass greater genetic variation. However, it is likely that the level of variation within *L. turgida* is primarily due to the independent evolutionary origins of lineages within *L. turgida*, and the observation that the lineages are derived from different ancestral genotypes within *L. helleri*. This is supported by a Nei's genetic diversity among *L. turgida* populations (0.395) that was more than three times the value observed among *L. helleri* sensu USFWS populations (0.120).

The level of cpDNA haplotype variation within and among *L. helleri* sensu USFWS and *L. turgida* is unusual for a species with such a small range. A previous study (Godt & Hamrick 1996) showed a high level of genetic diversity within *L. helleri* sensu USFWS, and attributed this to two possibilities: recent hybridization with another species, or that the species was recently more widely distributed. The latter possibility seems more plausible. Hybridization

is unlikely given the geographic distances and barriers between populations. This hypothesis is also consistent with the "ancient alpine flora" ideas presented by Wiser (1994). *Liatris helleri* is suggested to be a remnant of an ancient alpine flora that colonized areas of the Southern Appalachian high-elevation rock outcrops when the Wisconsin ice sheet moved south about 21,000 years ago (Wiser 1994). The rock outcrops of WNC are believed to have been refugia for a number of rare and threatened herbaceous species in the last glaciation (including the ancestral *Liatris* species that gave rise to *L. helleri*) because they were above the tree line (areas greater than 1200m in elevation) in areas that are not dominated by spruce-fir forests (Wiser 1994). These species became established in these areas as there was little competition for resources (especially light), as the soils were too rocky and shallow to be colonized by tree species. The glaciers moved north and south a number of times, in response to warming and cooling events in the global climate. Eventually the glaciers retreated northward to form the Great Lakes and the climate began to warm, and the distributions of those herbaceous species shifted northward in response, with only a few isolated areas on these outcrops remaining as relict populations from an ancient alpine flora in a new environment (Wiser 1994). Several of these species are uniquely adapted to these rock outcrop habitats, and although they share a number of affinities with their northern relatives, they have become unique Southern Appalachian endemics (Wiser 1994). Many of these ancient alpine floral relicts are considered to be of low competitive ability (at least during interglacial periods), and it is believed this contributes to the reasons they are found only in the rock-outcrop/cliff faces of Western North Carolina. These are extreme habitats

that they have adapted to survive in, but within the habitats, there is a relatively low degree of interspecific competition (Wiser 1994).

This process of glacial expanse and retreat not only gave rise to several independent and isolated populations of *L. helleri*, but also likely gave rise to a number of northward migrations of the species in response to glacial movement. This gives a plausible explanation to the patterns observed in the haplotype network (Figure 2). The number of unique haplotypes in the haplotype network implies that there is little to no seed-mediated gene flow (pollen-mediated gene flow cannot be quantified directly using cpDNA), with the possible exception of the two populations (LhPAD and LhHWB) which have the same haplotype. Given the isolated nature of the areas in which *L. helleri* sensu USFWS populations are found, a low level of seed-mediated gene flow among populations is not surprising. For this reason the areas in which *L. helleri* sensu USFWS populations occur are often considered to be high-elevation interglacial refugia (often referred to as "sky islands"), or mountaintops that are surrounded by lowlands that are ecologically different, including (but not limited to) differences in temperature, moisture, soil chemisty, and light availability. As the habitat for the species that inhabit these areas is no longer continuous across the landscape and are separated by the dominant ecosystem below (chesnut-oak forest), populations in sky islands are thus isolated from other populations, with the dominant ecosystems serving as a geographic barrier to gene flow. Over time these populations may undergo allopatric speciation, whereby they adapt to their unique microenvironment and differentiate not only from the ancestor species but also from one population to the next. The number of unique haplotypes within this narrow geographic range, in conjunction with the genetic diversity

observed by Godt and Hamrick (1996), suggests that these evolutionary forces may be driving the haplotype diversity observed in *L. helleri* sensu USFWS.

It is important to note that, based on the data, it is uncertain if these species are currently in the process of diverging or remerging. We are currently in an interglacial period, and based on climatic data, likely will be for some time. It is likely that the ancestor to *L. helleri* was driven South into the habitats that *L. helleri* sensu USFWS now inhabits several millennia ago, as there is some strong support that the vegetation at high elevation (greater than 1500m) during those times was similar to that of alpine tundra (Wiser et al. 1999), and was likely much more continuous that the habitats are today. As the climate began to warm approximately 16000-12000 years ago, deciduous forests began to occupy the areas surrounding the mountain tops. The tundra disappeared approximately 10000 years ago, and the species that occupied those areas (including *L. helleri* sensu USFWS) remained in several isolated areas. As we believe there was little to no seed-mediated gene flow between populations, it is natural to assume they experienced allopatric speciation (speciation due to restriction of gene flow by geographic/reproductive barriers) and have diverged one from another. However, it is also possible that they had been isolated far longer than we believe, and during the interglacial periods are experiencing some sort of gene flow (possibly pollen mediated), and thus introgression is occurring, and are actually remerging rather than diverging. cpDNA data is essentially a "snapshot in time," as it reflects only the current genetic structure and not the historical record. Also, cpDNA is uniparentally inherited, and cannot directly assess pollen-mediated gene flow. For these reasons, the data here cannot determine if the patterns observed here are due to these populations diverging or remerging

in this current interglacial period. Further research, particularly using nuclear markers, may shed some light on the process by which these patterns are occurring.

Glacial expanse and retreat also provides a mechanism by which the three peripheral radiations from the central populations may have occurred. The peripheral population at Betty's Rock, VA (LtBTR) is the phylogenetically closest radiation, with seven sequence variations to the nearest central population (LhRR). This population is in the Shenandoah National Park, approximately 450 km from the middle of the geographic range of the species. This population is on a rock outcrop, much like those found in Western North Carolina. This population may represent a distant high-elevation intergracial refugia population, with a correlation between the number of sequence variations and the length of time the population has been isolated. This population could also represent a northern migration, in response to a glacier retreat, which became isolated in the rocky outcrops of Shenandoah through a similar process.

The second furthest radiation, derived from a different *L. helleri* population (LhGM) includes two *L. turgida* populations (LtCRR and LtSMM) also in the Shenandoah National Park, as well as a *L. helleri* population (LhTTM) population. There are eleven sequence variations between this haplotype and the nearest population (LhGM) from which it is derived. The appearance of an identical haplotype in both areas and species is a strong indication of a glacial expanse/retreat pattern. It is possible that a migrant derived from the LhGM population went north and became established at the LtCRR and LtSMM populations, and then migrated back south, where it became isolated at the LhTTM population. It is also possible that this happened in the reverse order. Regardless of the order of dispersal, this

haplotype gave rise to another haplotype (LtPOR, the furthest population from the core), one of the shale barren populations of *L. turgida*.

The furthest radiation (LtTYG) was also derived from the Grandfather Mountain (LhGM) *L. helleri* population. This site was unusual in that the plants were not on rock outcrops or shale barrens; they were along a river bed. There were about 200 plants (recognized as *L. turgida* by WVDNR) along riparian zone in the Tygart Valley River. The plants there were considerably larger and more robust than at any of the other *L. turgida* sites I visited, and the elevation, amount of sunlight, and surrounding vegetation were different from the other sites I visited. This serves to make the point that *L. helleri* and *L. turgida* (or *L. helleri* sensu lato) may not be constrained to a habitat as specific as a rock outcrop or a shale barren, but rather to a resource that is abundant to both habitats, light availability. All of the sites I collected from had full sunlight during the majority of the day. This species, like many other ancient alpine floral relict species, are of low competitive ability, and have been able to survive only because they have a unique niche and can occupy areas (rock outcrops and shale barrens) that are not hospitable for many other species, areas which have high insolation (light) and openness.

Much like the rock outcrops of Western North Carolina that harbor a number of rare endemics, shale barrens have also been shown to have a high degree of endemism in their plant assemblages when compared to the limestone and sandstone substrates found in surrounding areas (Platt 1951). Only two of the populations of *L. turgida* I sampled (LtPOR and LtSLM) were in true shale barren habitats. It is interesting that one of those shale barren populations (LtSLM) was in the primary cluster of populations we sampled; the other

(LtPOR) was the most distant population from that cluster. This also supports the suggestion that haplotype may not be dictated by habitat, and also that perhaps the species is not as abundant in shale barrens as suggested by herbarium voucher information.

The degree of variation observed in cpDNA, in conjunction with the high genetic variation observed in a previous study (Godt & Hamrick 1996), gives rise to the idea that each of these high-elevation, rock outcrop populations of *L. helleri* are high elevation interglacial refugia, species-rich areas that are isolated on the tops of mountains. This scenario would strictly limit interbreeding with other populations in the same species, resulting in long-term isolation, which in turn can differentiate populations via allopatric speciation. The variation in both morphology and cpDNA sequences suggest a low level of gene flow between populations and a high level of variability as a species, which may serve to make the case that the populations are at some stage of divergence/introgression, yet the stage at which they are along that scale cannot be ascertained from the data at this time. This concept is supported by the groupings in the clades from the phylogenetic analyses.

Parsimony, likelihood, and distance-based analyses did not reveal distinct monophyletic groupings of *L. helleri* and *L. turgida*, thereby providing further support for recognition of the broadened concept of *L. helleri* using the phylogenetic species concept (Figures 2-5). All three methods recovered clade A with high bootstrap support, which consists of five *L. turgida* populations (LtBTR, LtCRR, LtPOR, LtSMM, and LtTYG) and a single *L. helleri* population (LhTTM). Maximum likelihood (Figure 2) and neighbor-joining (Figure 4) analyses also recovered a second clade (clade B), with lower bootstrap support, consisting of three *L. helleri* populations (LhHWB, LhPAD, and LhTBL) and a single *L. turgida*

population (LtSLM). Although there was not a strict monophyly of *L. helleri* and *L. turgida*, the two clades recovered from phylogenetic analyses are both dominated by one taxa, with only one representative from the other group in each clade. The pattern of lineage sorting observed here also suggests that the populations in these clades are at some stage of divergence or introgression.

Although the clades do not sort into two monophyletic groups, it is essential to remember that the speciation process occurs along a continuum. Organisms involved in the process of speciation can be at various levels of completion, depending on where along that continuum they fall. In addition to progression towards completion of a speciation event, the species concepts and criteria that are used to estimate their progress toward speciation may affect the interpretation of that progress towards speciation (Nosil et al. 2009). Employing different species concepts may result in differences species delimitations, as the criteria used to assess speciation may be at varying levels of importance with regard to the natural and biological processes that affect the speciation of a particular organism. Hence the need for using a unified species concept that considers all relevant evolutionary processes is evident (De Quieroz 2007).

Toward that end, I estimated the level of divergence/introgression between *L. helleri* and *L. turgida* using several criteria (Nosil et al. 2009), based on distributional evidence and data collected here (Figure 9). While it is possible that pollen from a *L. turgida* population could fertilize an individual in a *L. helleri* population (or vice versa), given the geographic distances and barriers between them, it is unlikely that this event could happen. A more

likely form of gene flow is seed-mediated gene flow, in which a seed from one species lands, matures, and fertilizes an individual in another population. There are only two shared haplotypes out of the thirteen haplotypes we recovered (shared haplotypes can be indicative of seed-mediated gene flow), and for that reason I suggest that there is a high degree of reproductive isolation between *L. helleri* and *L. turgida*, as their distributions do not appear to directly overlap. There is, however, direct overlap in the genotypes of the species, but only for a single individual. For that reason, I estimate we have partially bimodal genotypic clustering. Lineage sorting also appears to be intermediate to complete between *L. helleri* and *L. turgida*, as indicated in phylogenetic analyses (Figures 4-6). Finally, although the shale barren and high-elevation rock outcrops appear to be drastically different, I have found no direct comparisons of the soil chemistry, light availability, and climate/mean annual precipitation for these two ecosystems. In addition, they both contain a number of unique endemics and may partially overlap in the constitution of the flora contained therein. For that reason, I conservatively estimated a mild level of ecological difference between the two. While these taxa may not yet be completely diverged or remerged, the biological reality is that they are likely on their way towards such a state. This general trend towards divergence/introgression is occurring both among the populations (particularly those isolated populations) and at the species level. This is supported by the fact that the average genetic distance between *L. helleri* sensu USFWS and *L. turgida* populations was 0.313, more than twice the value (0.112) observed between the two outgroup species, which are considered to be more distantly related than *L. helleri* sensu USFWS and *L. turgida* (Nesom 2005b).

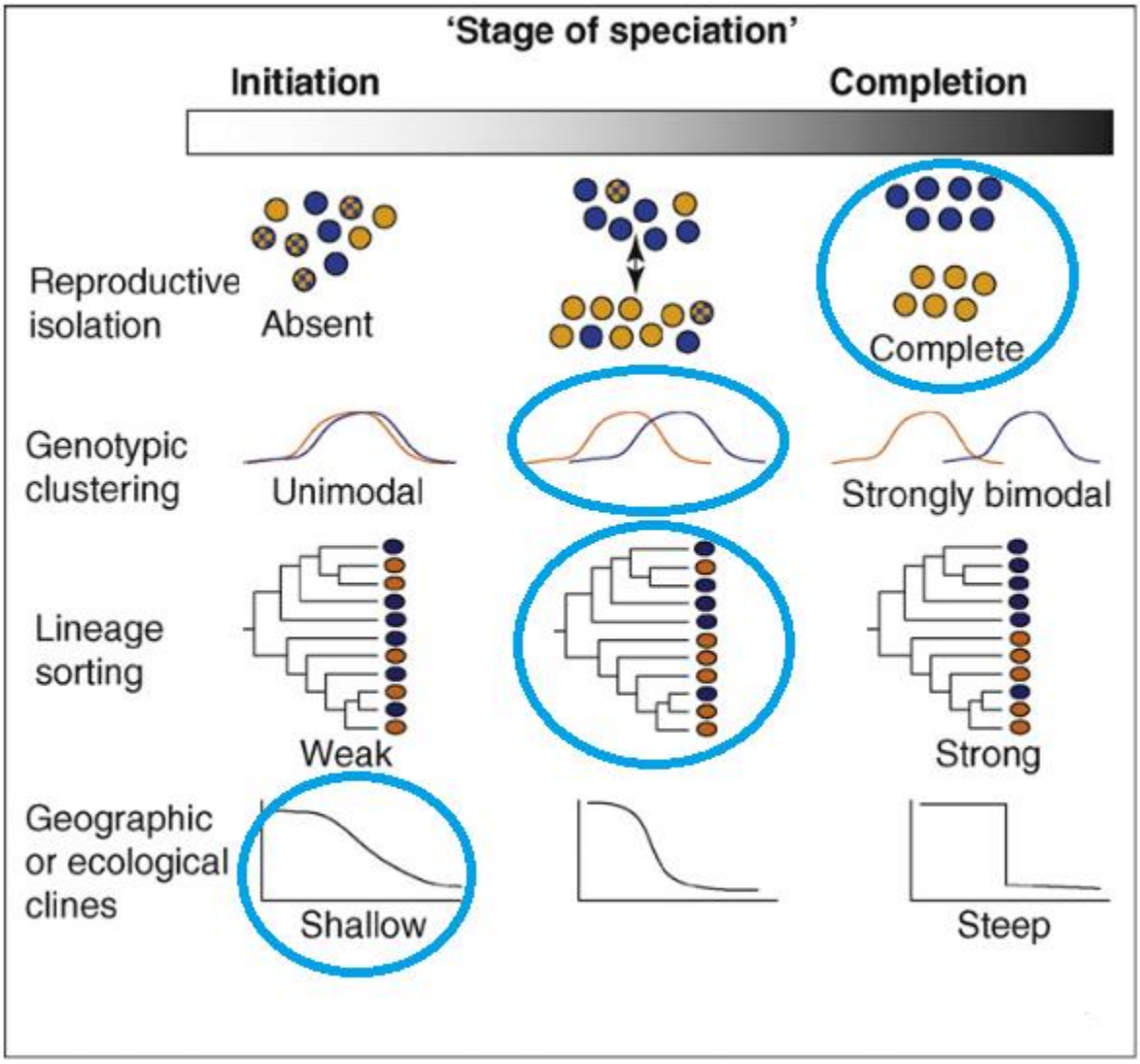

Figure 8. Stages of speciation of *Liatris helleri*. Blue circles indicate the estimated stage of divergence between *L. helleri* and *L. turigida* across four criteria often used to evaluate speciation between closely related organisms. Adapted from Nosil et al. 2009.

Conservation implications and conclusions

Before any new conservation management decisions regarding *L. helleri* are enacted, I recommend a reevaluation of the current distribution of the species. In collecting fresh material and vouchers for the study, I attempted to use populations that had been analyzed by Nesom's 2005 study. I was unable to locate several of the *L. turgida* populations in his analysis. It is important to note that some of these vouchers date back more than 100 years, and some of these populations may have been translocated or extirpated by invasive species, natural succession and human development (particularly by logging of the spruce-fir forests in the early 1900's and by mountain top removal coal mining in the mountains of Virginia and West Virginia). For this reason, a biological inventory and recalculation of the distribution of the species is essential prior to making any changes in recognition of the species.

Due to lack of morphological distinctiveness and lack of monophyletic grouping of *L. helleri* sensu USFWS and *L. turgida*, the data here support recognition using the broadened concept of *L. helleri* (*L. helleri* sensu lato), as suggested previously (Nesom 2005a); however, a number of questions arise from the data presented here. There appears to be a subset of populations with ecological, phylogenetic, geographic and taxonomic similarities (although some discontinuities exist) to one another. This group is potentially of signicant biological importance, as it appears to have given rise to at least three independently derived lineages, and appears to be at some stage of divergence from most of the populations previously identified as *L. turgida*. The presence of unique haplotypes at nearly all populations within

this group and among peripheral populations highlights a conservation concern at each of those unique populations.

The central cluster of populations is of particular interest to conservation. A high degree of cpDNA variation in combination with a high degree of genetic diversity (Godt & Hamrick 1995) indicates that these populations that may contain a high level of adaptive variation. *Liatris helleri* has wind-dispersed seeds, and as most of these populations are at high altitude on rock outcrops and cliff faces, they are in ideal areas for seed dispersal. I suggest placing a conservation priority on this group of populations, as these populations appear to have previously played a major role in the evolution of the species through major climatic change events, events that may be paralleled in the future due to trends associated with global climate change. One means to accomplish this is retention of taxonomic distinctiveness at a level beneath the species rank (subspecies, variety, ecotype, etc.), and thus retain protections at some level, at least until the full role these populations play in the evolution of the species is known.

The more highly supported clade from the phylogenetic analyses may also warrant further investigation. Five *L. turgida* populations and one *L. helleri* population appeared together in the likelihood, parsimony, and distance-based phylogenies with a high degree of bootstrap support; however, these populations formed three separate branches in the haplotype network. It is possible that further sampling and research into the taxonomic validity of this second group could reveal if this is also a biologically and phylogenetically important group or if this is simply a case of long-branch attraction due to sample size.

In addition, with regard to lineage sorting and genotypic clustering, *L. helleri* and *L. turgida* appear to be in some intermediate stage of divergence. The level of that divergence cannot be precisely calculated using the data from this study. It is important to recognize that I sequenced only a small portion of the chloroplast genome, which represents only a small portion of the entire genome. A number of conservation and phylogenetic studies utilize both cpDNA markers and nuclear markers to understand the evolutionary processes best revealed by each markers. Development and application of an appropriate nuclear marker set could serve to increase our understanding of the complex evolutionary history of these species, and would serve as a logical next step in developing a conservation strategy for *L. helleri*.

In addition to the use of nuclear markers, further ecological work could also greatly contribute to understanding the evolutionary processes that have affected the phylogenetic patterns in this study may also help in developing conservation management practices for *L. helleri*. I am unaware of any studies that directly compare the ecological aspects of rock outcrops with those of shale barrens, although a number of coarse similarities exist, namely the high degree of endemism, rates of succession, relative isolation from other similar areas and high levels of insolation accompanied by low levels of competition within those habitats. Any correlations drawn between those habitats could have a major impact on our knowledge and understanding of *L. helleri* and *L.* turgida, as well as other rare and endemic plants that inhabit those areas.

Several *L. helleri* populations within the core range of the species co-occur with other protected species or on land owned/managed by conservation agencies, and may thus fall under protection in some fashion regardless of how this study is interpreted by conservation

management agencies. As most core populations are on federal lands (and in many cases cohabitating with other imperiled species), I suggest maintaining protections until further ecological and nuclear DNA work can be completed. I also recommend a moratorium on population augmentations and reintroductions until the genetic and ecological background of the species can be further understood.

The goal of this study was to provide an answer to a specific question facing conservation biologists. As it turns out, more questions than conclusions have arisen as a result of this study. I sequenced a single individual per population, and recovered unique haplotypes for most populations. How much haplotype variation exists within each population? If nuclear DNA data becomes available, how will that data complement or obscure the story told by the cpDNA data? How will conservation management react to this study, and how will management practices for *L. helleri* change once they process the information provided herein? However the species is to be treated in the wake of this investigation, it is my aspiration that this data be interpreted both cautiously and thoroughly in order to appropriately manage a species with such a rich historical and natural heritage as *Liatris helleri*.

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LIST OF ABBREVIATIONS

AMOVA – Analysis of Molecular Variance

BOON – Appalachian State University Herbarium

bp – Base Pairs

BSC – Biological Species Concept

cpDNA – Chloroplast DNA

CT – Corolla Tube

GenAlEx – Genetic Analysis in Excel

GPS – Global Positioning System

Lc – *Liatris cokeri*

Lh – *Liatris helleri*

LhBM – Bluff Mountain *Liatris helleri* population

LhBR – Blowing Rock *Liatris helleri* population

LhCHS – Chimneys *Liatris helleri* population

LhGM – Grandfather Mountain *Liatris helleri* population

LhHWB – Hawksbill *Liatris helleri* population

LhPAD – Paddy Mountain *Liatris helleri* population

LhRR – Rough Ridge *Liatris helleri* population

LhTBL – Table Rock *Liatris helleri* population

LhTTM – Three Top Mountain *Liatris helleri* population

Ls – *Liatris spicata*

Lt – *Liatris turgida*

LtBTR – Betty's Rock *Liatris turgid*a population

LtCRR – Crescent Ridge Rock *Liatris turgid*a population

LtPOR – Poor Mountain *Liatris turgid*a population

LtSLM – Slaty Mountain *Liatris turgid*a population

LtSMM – Stoney Man Mountain *Liatris turgid*a population

LtSOM – South Mountain *Liatris turgid*a population

LtTYG – Tygart River Valley *Liatris turgid*a population

MEGA – Molecular Evolutionary Genetics Analysis

ML – Maximum Likelihood

MP – Maximim Parsimony

MSC – Morphological Species Concept

N – Sample Size

NC – North Carolina

NJ – Neighbor Joining

PCR – Polymerase Chain Reaction

PSC – Phylogenetic Species Concept

TCS – Templeton, Crandal, and Sing phylogenetic network estimation

USC – Unified Species Concept

USFWS – United States Fish and Wildlife Service

VA - Virginia

WNC – Western North Carolina

WV – West Virginia

Vita

Patrick Cory Sullins was born in 1985 in Spruce Pine, North Carolina, to Richard William Sullins and Nelda Shirlyn Chandler Sullins. Patrick has a younger brother, Mitchell Aaron Sullins, and a younger sister, Kristin Amber Sullins. Patrick was awarded a Bachelor of Arts in Biology with a minor in Spanish in 2008 from Appalachian State University. He worked in public agriculture for a year and a half before returning to Appalachian in 2010 to enter the Master of Science in Biology program. He was awarded the MS degree and is currently serving as Appalachian State University's General Biology Laboratory Manager, and aspires to continue his family's tradition of working as a public educator.