Summer 7-11-2017

Population Genetics and Host Specificity of a Rare Parasitic Plant, Cuscuta harperi

Brandy N. Rogers
Kennesaw State University

Population Genetics and Host Specificity of a Rare Parasitic Plant, *Cuscuta harperi*

Thesis Submitted by Brandy Rogers

In Partial Fulfillment of Requirements for the Master of Science in Integrative Biology

Department of Ecology, Evolution, and Organismal Biology

Kennesaw State University

Summer 2017

Dr. Joel R. McNeal, Thesis Committee Chair

Dr. Thomas C. McElroy, Thesis Committee Member

Dr. Paula C. Jackson, Thesis Committee Member

ABSTRACT

Cuscuta harperi is a rare parasitic plant endemic to a small number of widely disjunct populations in Georgia and Alabama. It is a habitat-specialist, occurring on sandstone and granite outcrops within its limited range; it is also exhibits a high level of host specificity, parasitizing only a few select species. *C. harperi* is of conservation concern due to small population sizes and threats from habitat degradation.

Here we develop genetic markers to address questions regarding population genetics of the species. We discuss the utility of microsatellites and single nucleotide polymorphisms as molecular markers to evaluate genetic variability between individuals and across populations. We also investigate patterns of host selection in populations of *C. harperi* using greenhouse and field-based experiments.

We failed to find variation in putative microsatellite markers and conclude that methods of SNP detection, such as targeted sequence capture, are likely to be superior for identification of polymorphisms in the genome of *C. harperi*. We also show that host selection by *C. harperi* is a result of active choice by seedlings and that there are additional environmental factors contributing to patterns of host use observed in natural populations. Finally, we discuss the design and implementation of a project that combines *in situ* and *ex situ* strategies for the conservation of the species.

Keywords: *Cuscuta harperi*, parasitic plant, host-parasite interaction, microsatellites, high-throughput targeted capture methods, sequence capture

ACKNOWLEDGEMENTS

There are many individuals and organizations to which a debt of gratitude is owed for their contributions to the completion of this project. First, I would like to express my deepest appreciation to my advisor and mentor, Dr. Joel McNeal, for introducing me to the fascinating world of parasitic plants, as well as for his guidance and unwavering support over the last two years. I would also like to thank my thesis committee members, Dr. Paula Jackson and Dr. Thomas McElroy, for their willingness to consider my ideas and for all of their contributions and encouragement.

The incredible amount of field and lab work that went into this project could not have been accomplished without my amazing lab members. To Cory Peay, Turner Stapleton, Sean Caton, Alyx Koval, and Rebecca Webster: thank you for all of your help with field work on the blazing hot outcrops and sheer cliffs, for paddle-boarding down Bear Creek with me in search of an elusive population, for counting literally thousands of flowers, and most of all, for your friendship. You each know who you are, and I owe you more than I can repay. Thank you to Malcolm Hodges of The Nature Conservancy for your help with access to study sites and your collaboration on the Camp Meeting Rock project. It wouldn't have been possible without you, and I look forward to continued collaboration.

Special thanks to Tom Patrick of the GA Department of Natural Resources for providing occurrence records which were instrumental in locating Georgia populations and to Dan Spaulding of the Anniston Museum of Natural History for assistance in locating Alabama populations of *C. harperi*.

Thanks also to Mary Shew of the US National Park Service, Chuck Byrd of The Nature Conservancy, Ray Peacock of Hancock Timber Resource Group, Ken Wills of Friends of Moss Rock Preserve, and Michelle Reynolds for permissions and assistance in accessing study sites. Thanks to Pam Knight, Jennifer Amerson, Jim Martin, and all of the numerous land owners who allowed me to traipse around on rock outcrops on your property looking for dodder and for not chasing me away when I showed up uninvited on your doorsteps and told you I found you using Google satellite maps.

Thank you to Lauren Eserman and Dr. Jim Leebens-Mack of the UGA Department of Plant Biology for your time and advice on targeted sequence capture.

Funding for this project was provided by the Garden Society of America and the Center for Plant Conservation through the Catherine H. Beattie Fellowship for Conservation Horticulture and by the Georgia Native Plant Society through the Jeane Reeves Research Grant. I am grateful for the financial support of these organizations, as well as that of the Kennesaw State University Department of Ecology, Evolution, and Organismal Biology, the College of Science and Mathematics, and the Graduate College.

Finally, I would like to express my deepest gratitude to my family for their support during the completion of my master's program and for always encouraging and believing in me. And to my MSIB cohorts, it's been a pleasure to experience this journey with you.

STATEMENT OF INTEGRATION

The Master of Science in Integrative Biology program at Kennesaw State University is designed to train biologists to think across disciplines, to ask and answer questions spanning organizational levels, and to acquire understanding of biological systems from the fine to the broad scale. For this project, we address questions regarding interactions between *Cuscuta harperi* and its host plant species and investigate a myriad of factors that influence those interactions, from phenological, temporal, and spatial factors to cellular and genetic mechanisms. We also address questions regarding genetic relatedness and variability between individuals and among populations. Many hours were spent in the field making observations and collecting data and tissue samples; numerous hours were also spent in the lab prepping and analyzing samples and developing methods and techniques.

We included *C. harperi* individuals from thirteen separate populations in this study, and location and access of study sites required collaboration with multiple entities, including the US National Park Service, the Georgia Department of Natural Resources, The Nature Conservancy, Hancock Timber Resource Group, and private botanical organizations. Many study populations were located using herbarium specimen records maintained and made available online through the Alabama Herbarium Consortium and the University of West Alabama, and also through the use of GA DNR Elemental Occurrence Reports. Involvement of these organizations was imperative for the success of the project and also served to extend the project's integrative scope.

This thesis project is broadly integrative, encompassing field, greenhouse, and laboratory-based experiments and employing techniques from disciplines ranging from molecular and computational biology to population ecology and conservation biology. Completion of this project has allowed me to develop and broaden my skills and exercise my creativity and problem-solving as a scientist, as well as to make meaningful connections between diverse biological concepts.

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CHAPTER ONE: Introduction

Study Organism

Cuscuta is a genus of parasitic plants comprised of approximately 200 different species. Although traditional taxonomy placed the genus in its own family (Cuscutaceae), molecular phylogenies have used highly constrained chloroplast gene sequences to place *Cuscuta* within the Morning Glory Family (Convolvulaceae) (Stefanovic et al. 2002, 2003). Close relatives of *Cuscuta* include plants in the genus *Ipomoea*, which includes Common Morning Glory (*Ipomoea purpurea*) and Sweet Potato (*Ipomoea batatas*). All *Cuscuta* species are annual stem parasites that never develop roots and whose leaves are reduced to vestigial scales. Almost all species have yellow to orange stems and white to greenish flowers which range from 1 to 6 mm in diameter, and many species are notoriously difficult to distinguish from one another due to lack of differences in vegetative morphology.

Cuscuta species are classified as obligate holoparasites due to their ultimate dependence on their host plants for survival and completion of their life cycle, although some species are considered cryptically photosynthetic (McNeal et al. 2007a). Many photosynthetic genes, including the large subunit of Rubisco (*rbcL*), are present and under strong selection within the chloroplast genome of most *Cuscuta* species (McNeal et al. 2007a/b). However, loss of many other chloroplast genes, lack of functional stomates, and expression of chlorophyll often localized to developing seeds indicates remaining photosynthetic genes may be repurposed for efficient lipid synthesis as in Canola (Schwender et al. 2004), and the parasite likely obtains all required carbohydrates from the host plant (Hibberd et al. 1998). Like other parasitic plant lineages, *Cuscuta* species form epidermal projections known as haustoria that invade host tissue and connect to the vascular system (xylem and phloem) of the host for siphoning water, carbohydrates, and other nutrients.

All of the nutrients and energy available to unattached *Cuscuta* seedlings must be stored in the seeds, because the seedling never exhibits root formation or photosynthetic ability. Seedlings must locate and successfully attach to an appropriate

host within days of germination in order to survive, at which point the parasite tissue between the ground and the host plant connection dies (Kuijt 1969).

Cuscuta species occur worldwide, and many are capable of parasitizing multiple hosts. *C. campestris* is known to cause extensive damage to crop plants, leading to significant agricultural and economic losses (Kaiser et al. 2015); a number of species are classified as noxious weeds (USDA 2012). Multiple species are restricted or prohibited in many US states, and bringing plants or seeds across state lines is illegal in many cases. On the other hand, some *Cuscuta* species are found in much smaller and more restricted ranges and display a higher degree of host specificity. Most of these species are not considered agricultural pests but, instead, are more frequently of conservation concern due to narrow habitat and host preference.

Some *Cuscuta*, especially weedy species that are considered crop pests, are considered generalists; they are able to successfully parasitize a broad range of host plants and will often grow on more than one type of plant concurrently. Species with a narrower host range are considered specialists, sometimes only known to parasitize one or two particular host species. Previous research has shown that even species that are considered generalists do not always use host plants proportionally to availability; although the parasite is capable of utilizing multiple host species in a mixed-host habitat, it may utilize them in a non-random pattern (Kelly et al. 1988, Kelly and Horning 1999). One study suggests that *C. campestris* may exhibit selective foraging by reallocation of resources to ramets that are attached to more beneficial hosts. An example of this reallocation is increased haustorial formation on hosts supplying more nutrient reward and a decreased number of haustoria formed on less rewarding hosts when multiple hosts are being parasitized concurrently (Koch et al. 2004). In another study, *C. europaea* exhibited the ability to distinguish between potential host plants of varying nitrogen content prior to forming attachments and was able to selectively parasitize those with higher nitrogen levels (Kelly 1992).

Although the molecular pathways that are utilized are not well-understood, there is substantial evidence that *Cuscuta* species interact with host plants using mechanisms of chemo-attraction and chemo-repulsion. Specifically, a *Cuscuta* species has been

shown to grow preferentially toward volatile organic compounds emitted by tomato plants (*Lycopersicon esculentum*) and away from volatiles emitted by wheat (*Triticum aestivum*) (Runyon et al. 2006). These findings are particularly significant because *Cuscuta campestris* is an agricultural pest on tomato crops but does not parasitize crop plants in the grass family (Poaceae). Additional research is needed to investigate whether similar patterns of chemotaxis are observed during foraging in other *Cuscuta* species.

Cuscuta harperi, named for renowned southeastern U.S. botanist Roland Harper, is a species that is endemic to a low number of scattered populations in Georgia and Alabama. It is a habitat specialist that occurs only on exposed sandstone and, more rarely, granite outcrops and barrens in Alabama and Georgia while further displaying a high level of host specificity within this limited habitat. The plant is a slender vine, bright orange in color, with small whitish flowers about 1 mm long. It can be differentiated from other similar species in the genus by its primarily four-parted rather than five-parted flowers, its flower size, which is among the smallest of any *Cuscuta* species worldwide, and capsules that only rarely produce more than a single seed (Yuncker 1932). It is also known to be highly capable of self-pollination, as suggested by its reduced flower size, because when grown under greenhouse conditions isolated from any other individuals of *C. harperi* the flowers invariably produce seed.

Populations of *C. harperi* are widely disjunct, and most populations are restricted to small areas. While individuals are plentiful in a few populations within the core range of northeast Alabama, other surveyed populations consisted of between one and ten individuals in 2015 (Table 1.1). The plant is assigned a legal status of endangered in Georgia, with four reported populations in two widely disjunct counties (GA DNR 2010). It has a state rank of S2 in Alabama, where most populations occur, and is assigned a global rank of G2/G3 (NatureServe Explorer 2015); both the state and global rankings indicate the species' vulnerability to extinction based on low number of individuals present and threats due to habitat destruction.

Population Name	**County**	**Number of Individuals**	**% using *Bigelowia* exclusively**	**% using *Liatris* exclusively**	**% using another host exclusively**	**% using mixed hosts**
Allen/Aubrey Flatrock	Heard, GA	8	0.0	87.5	12.5	0.0
Bear Creek	Marion, AL	8	0.0	87.5	12.5	0.0
Chitwood Barrens	Dekalb, AL	1	0.0	100.0	0.0	0.0
DeSoto State Park	Dekalb, AL	10	10.0	90.0	0.0	0.0
Flat Rock	Jackson, AL	9	0.0	100.0	0.0	0.0
Harrison Outcrop	Washington, GA	20	95.0	0.0	0.0	5.0
Hinds Road	Etowah, AL	30+	33.3	20.0	6.7	40.0
Little River Canyon NP	Dekalb, AL	69+	24.6	50.7	21.7	2.9
Moon Rock	Dekalb, AL	13	92.3	0.0	0.0	7.7
Moss Rock Preserve	Jefferson, AL	24	54.2	29.2	4.2	29.2
North Fork Creek	Marion, AL	8	0.0	87.5	0.0	12.5
South Texas Flatrock	Heard, GA	6	0.0	50.0	50.0	0.0
Town Creek	Dekalb, AL	10	100.0	0.0	0.0	0.0

Table 1.1. Host use by population in 2015. Number of individuals refers to number of plants sampled. In the case of larger populations, n+ indicates more than the number of individuals sampled were present.

Another potential threat to *Cuscuta harperi* populations is fire suppression in the surrounding habitat. (NatureServe Explorer 2015). Historically, many of the rock outcrops where *C. harperi* is found would have been surrounded by Longleaf pine savannas and woodlands, ecosystems widely known to be dependent upon frequent burning as a natural disturbance regime (McPherson 1997, Platt 1999). Prior to massive deforestation for timber and agriculture in the early 20th century, Longleaf pine savannas and woodlands would have been a dominant habitat type throughout much of the documented range of *Cuscuta harperi* (Fig. 1.1).

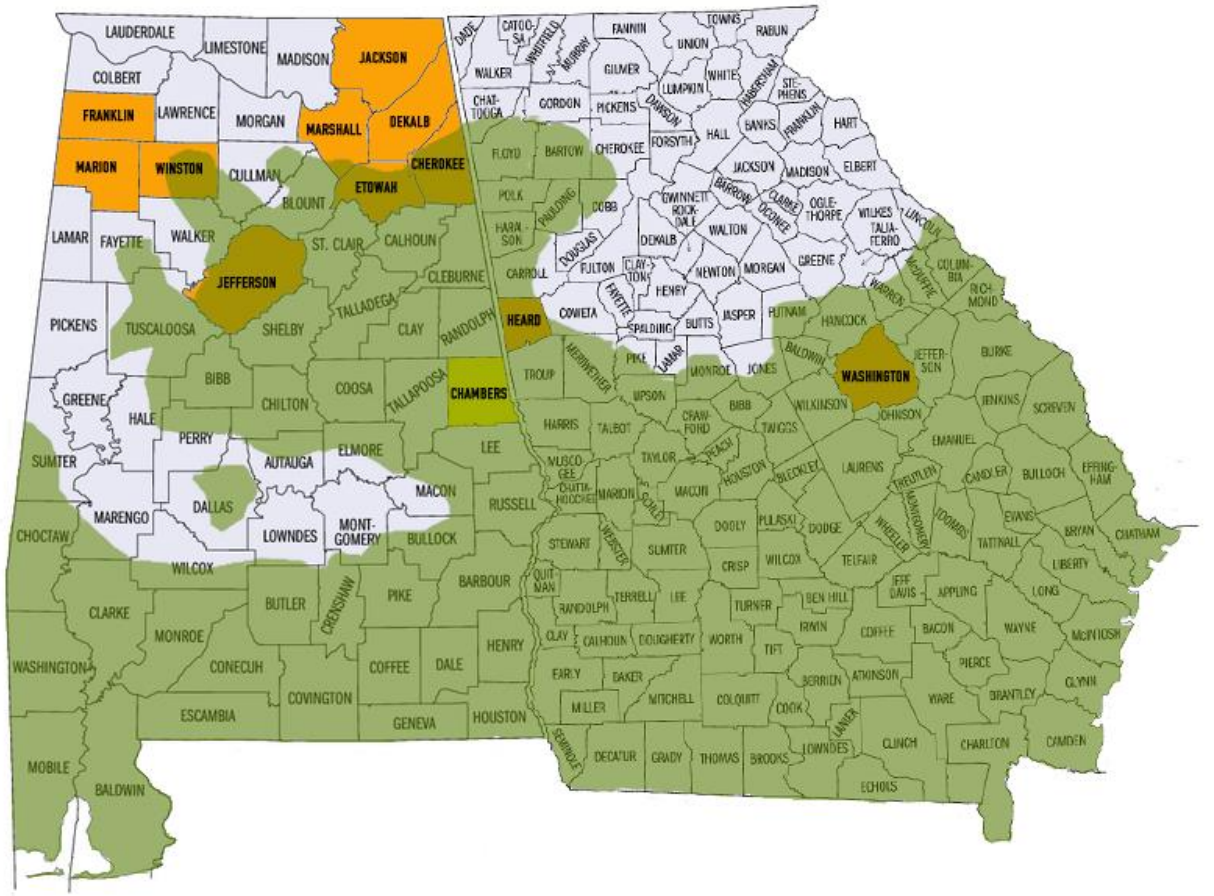


Fig. 1.1. Map of documented range of *C. harperi* and historic range of Longleaf Pine (*Pinus palustris*). Orange-colored counties indicate extant populations of *C. harperi*; population in Chambers County (yellow) is listed as likely extirpated. Green-colored area indicates extent of historic Longleaf pine habitat in Georgia and Alabama. Longleaf pine range map adapted from Little 1971; *C. harperi* county occurrence records taken from www.natureserve.org.

Populations of *C. harperi* outside of the historic range of Longleaf pine habitat would have been embedded within Appalachian Shortleaf Pine-Xeric Oak forest, a habitat type which, similar to Longleaf pine dominated habitat, would have historically been a pyric ecosystem. Multiple plots of this habitat type are protected within Little River Canyon National Preserve where the core range of *C. harperi* occurs. Also within the preserve, *C. harperi* populations occur in close proximity to Low Mountain Seepage Bog habitat where populations of *Sarracenia oreophila* (Green pitcherplant) are found. *Sarracenia oreophila* is a federally endangered species whose decline is known to be caused in large part by anthropogenic fire suppression (Godt and Hamrick 1996). Both the

Appalachian Shortleaf Pine-Xeric Oak forest and the Low Mountain Seepage Bog habitat types are fire-maintained as part of management strategies within Little River Canyon National Preserve (Schotz et al. 2008).

The Physical Chemistry Fire Frequency Model (PC2FM) was developed using fire history data combined with climate data and physical and chemical properties of North American ecosystems to be used as a predictor of mean fire intervals. According to the PC2FM, habitats within the range of *C. harperi* would have historically been subject to burning in intervals of between 2 and 8 years (Guyette et al. 2012). Within the pyric ecosystems that would have encompassed the range of *C. harperi*, the ecotones surrounding rock outcrops would have been the driest and most fire prone habitats.

Without frequent fire disturbance to prevent succession, woodland species encroach on the outcrops until dispersal is limited by edaphic factors (Anderson et al. 1999). For *Cuscuta harperi*, whose preferred hosts often thrive along the edges of the outcrop, this means fewer available hosts in these areas and restriction to more isolated patches of vegetation on the outcrop itself. Within these isolated patches, the soil frequently becomes buried beneath a layer of organic matter composed predominantly of detritus, lichens, and mosses which can be detrimental to the germination and foraging of new *C. harperi* seedlings. Frequent fire would largely eliminate this accumulation of organic material and help to maintain barren, sandy soil in which *C. harperi* seeds could more readily germinate and forage, and fire may also potentially aid in scarification of the tough seed coat.

The most commonly-used host plants of *C*. *harperi* are Nuttall's Rayless Goldenrod (*Bigelowia nuttallii)* and Dwarf Blazing Star (*Liatris microcephala*), both in the family Asteraceae. Pineweed (*Hypericum gentianoides*- Hypericaceae), Longleaf Sunflower (*Helianthus longifolius*- Asteraceae), Outcrop Rushfoil (*Croton willdenowii*- Euphorbiaceae), and Menges' Fameflower (*Phemeranthus mengesii*- Montiaceae) are lesser-used hosts. Although often abundant on the same outcrops, other members of Asteraceae such as Showy Tickseed (*Coreopsis pulchra*) and Confederate Daisy (*Helianthus porteri*) are rarely utilized as hosts. Although four recently-attached *C. harperi* individuals were found on *Coreopsis* at Little River Canyon National Preserve

during field work in May 2016, no blooming individuals of *C*. *harperi* were found on *Coreopsis* at that site or elsewhere in August-October 2015. In Spring 2017, individuals were also found growing abundantly on Blue Toadflax (*Nuttallanthus canadensis*-Plantaginaceae) and rarely on Appalachian Stitchwort (*Minuartia glabra*-*Caryophyllaceae*) at Little River Canyon, although these annual, spring ephemeral hosts typically aren't present on the outcrops during the late summer blooming season of *C. harperi*.

While some of the larger populations of *C*. *harperi* use a mix of the hosts listed above, *C*. *harperi* parasitizes *Bigelowia* almost exclusively on some outcrops even though the other species used as hosts in the core range, including *Liatris*, are present and abundant in close proximity. A well-documented example of this host specificity can be seen at the type locality of *C*. *harperi* in the coastal plain region of Georgia. The species was first discovered by renowned botanist Roland Harper in 1906, when he found it parasitizing *Bigelowia nuttallii* on an outcrop of sandstone conglomerate known as Altamaha Grit in Washington County. In his details of the new *Cuscuta* species, Harper carefully noted that all of the individuals he located were exclusively using *Bigelowia* as a host; "The *Cuscuta* was quite plentiful, but I could not find a trace of it on any other host." (Harper 1906). A Georgia DNR occurrence report from that outcrop in 1999 also mentions the species was observed that year occurring strictly on *Bigelowia*, and another visit to the site in 2012 by Joel McNeal and GA DNR botanist Tom Patrick once again found *Bigelowia* as the sole host at the site despite an abundance of *Liatris microcephala* in close proximity to parasitized individuals (McNeal pers. comm. 2015). Interestingly, while visiting the type locality in September 2015, we also found *Bigelowia* used as the sole host with the exception of a lone individual that was found parasitizing *Liatris*.

In other sites, *Liatris* may be the most common host even where *Bigelowia* is readily available, and in *C. harperi* populations that exist outside of the range of *Bigelowia*, *Liatris* and *Hypericum* become the sole hosts. The host specificity observed at some sites may be indicative of host choice by the parasite at the seedling stage, evolution of resistance to parasitization by local host populations, differential survival of the parasite

seedlings on particular hosts due to environmental conditions, or long-term suitability of the host across multiple seasons since the same patch of these perennial hosts is often parasitized year after year.

Study Sites

Populations were located using Element Occurrence Records provided by GA Department of Natural Resources, herbarium specimens from the Alabama Plant Atlas (Alabama Herbarium Consortium 2016), and identifying appropriate outcrop habitat with Google satellite maps. From August to October 2015, we collected tissue from 208 individuals across 12 separate populations spanning the known range of *Cuscuta harperi*. In September 2016, a 13th population was located, and tissue from 8 additional individuals was collected. We recorded GPS location information for each individual sampled as well as which host species each sampled individual was parasitizing. Current availability of potential host species was documented in the field and corroborated using species range maps (USDA 2012). All maps were generated using Google Maps, 2017.

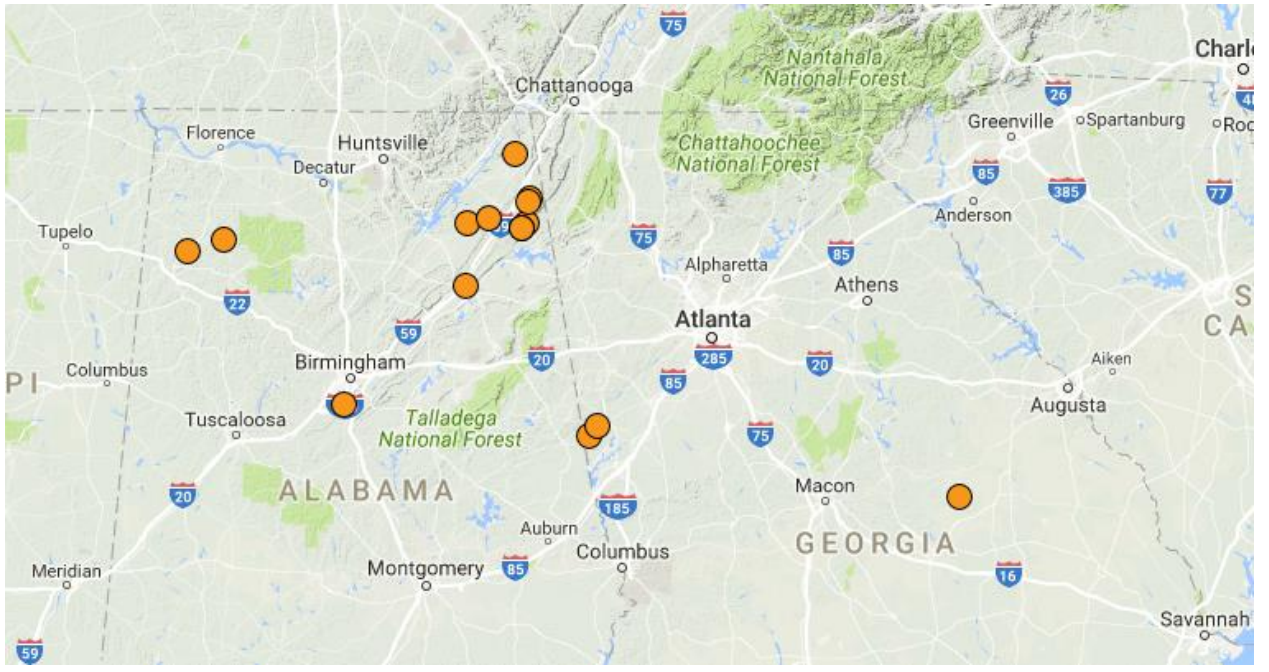


Fig 1.2 Map of *C. harperi* range. Each marker represents one of the thirteen populations located and sampled in this research project. These populations span the entirety of the documented range of *C. harperi*.

List of Study Sites by Population

Site maps showing the location of sampled individuals on each outcrop are color-coded to indicate the host species each sampled *C. harperi* individual was parasitizing (Fig. 1.3). See captions for distance and area information for each study site.

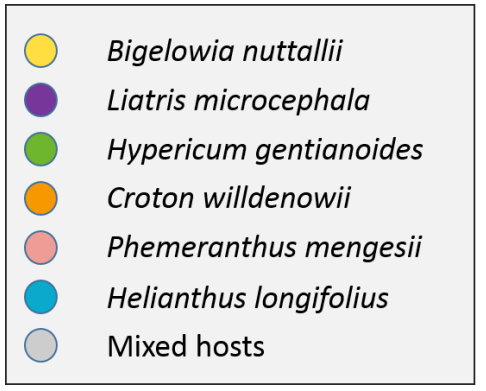


Fig. 1.3 Host Species Key

Allen/Aubrey Flatrock (AA), Heard County, Georgia (33.26608, -85.15032)

Allen/Aubrey Flatrock is a granite outcrop, privately owned and accessed through permission from the land owner in collaboration with The Nature Conservancy, Georgia. Eight individuals were located and sampled at this site on 28 October 2015. All individuals were parasitizing *Liatris microcephala*, with one individual concurrently attached to *Croton willdenowii*. Individuals in this population were widely scattered across the expansive outcrop. Allen/Aubrey Flatrock is outside the documented range of *Bigelowia nuttallii*.

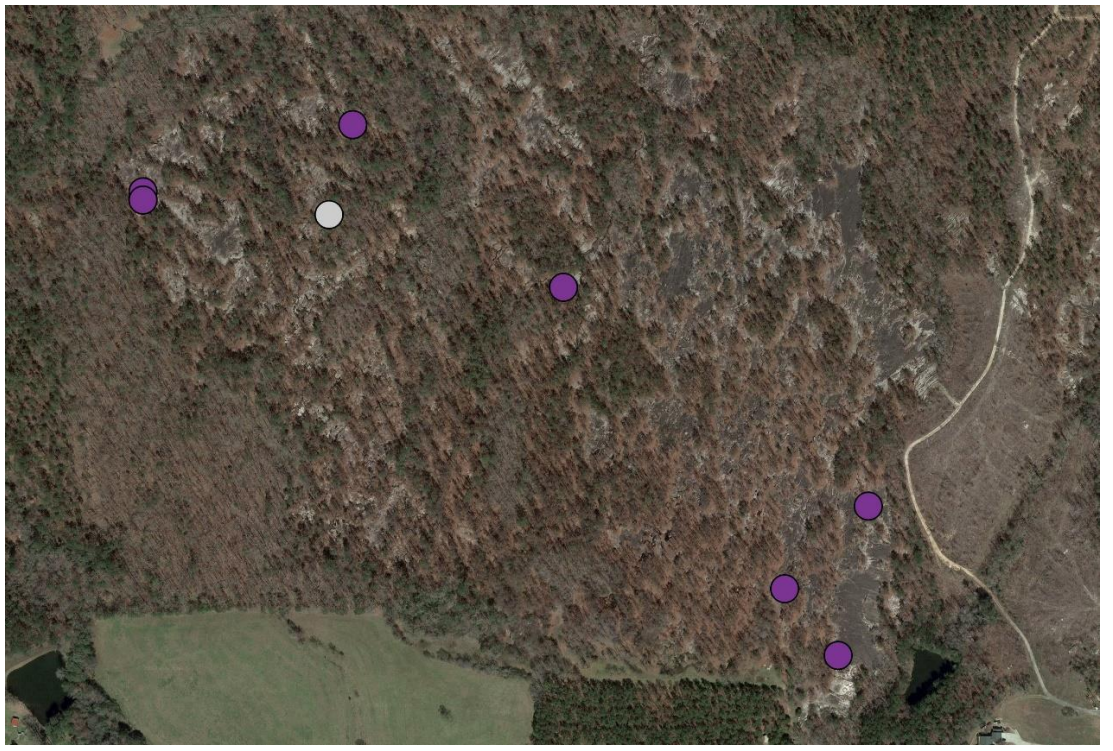


Fig. 1.4. Allen/Aubrey Flatrock. Individuals span a linear distance of 966 meters. Total area of exposed rock outcrop is approximately 117 acres (473,482 sq. meters).

Bear Creek (BC), Marion County, Alabama (34.29372, -87.64619)

The Bear Creek population is located on sandstone along the north bank of Bear Creek, most easily accessible by water. Eight individuals were located and sampled at this site on 17 September 2016. Seven out of eight individuals were parasitizing *Liatris microcephala*, and one individual was parasitizing *Hypericum gentianoides*. Bear Creek is outside the documented range of *Bigelowia nuttallii.*

Fig. 1.5 Bear Creek. Individuals 1-7 are within a linear distance of approximately 122 meters; individual 8 is 483 meters downstream on another area of exposed sandstone.

Chitwood Barrens (CB), Dekalb County, Alabama (34.41808, -85.88087)

Chitwood Barrens Preserve is a grassy sandstone barren that is protected by the Nature Conservancy as a habitat for several rare plant species, including *Sarracenia oreophila* (Green Pitcherplant) and *Allium speculae* (Little River Canyon Onion). Permission to access the preserve was granted through The Nature Conservancy, Alabama. A single individual was located at this site on 11 September 2015, parasitizing *Liatris microcephala*. The individual was found on the opposite side of AL Hwy 44 from the main protected area. Other potential host plants, including *Bigelowia nuttallii* were present in some abundance, though in scattered patches surrounded by unsuitable hosts.

Fig. 1.6 Chitwood Barrens. A single individual was located growing approximately 33.5 meters west of Hwy 44.

DeSoto State Park (DSP), Dekalb County, Alabama (34.50203, -85.61396)

The DeSoto State Park population is located on a sandstone glade within the park boundaries. Ten individuals were located at this site on 9 October 2015. Nine of the ten individuals were parasitizing *Liatris microcephala*; one individual was parasitizing *Bigelowia nuttallii*.

Fig 1.7 DeSoto State Park. C. harperi individuals are spread across a linear distance of 45.7 meters. Total exposed rock outcrop area is approximately 0.5 acres (2023.4 sq. meters).

Flat Rock (FR), Jackson County, Alabama (34.7696, -85.70637)

The Flat Rock population is located on sandstone outcrop along the bank of Flat Rock Creek and is the northernmost population in the range of *Cuscuta harperi*. Nine individuals were located and sampled on 9 October 2015. All individuals were parasitizing *Liatris microcephala*. Flat Rock is outside the documented range of *Bigelowia nuttallii*.

Fig. 1.8 Flat Rock. *C. harperi* individuals span a linear distance of 29 meters along the north bank of Flat Rock Creek. Exposed sandstone continues for approximately 107 meters on the east side of Hwy 117, and another expanse of rock (approximately 4450 sq. meters) is exposed on the south shore of the creek; however, no *C. harperi* individuals were located on either of these areas.

Harrison Outcrop (HO), Washington County, Georgia (32.87133, -82.73328)

Harrison Outcrop, the type locality of *Cuscuta harperi*, is found on outcrops of Altamaha grit, a sandstone conglomerate. Twenty individuals were located and sampled on 26 September 2015. Nineteen of the twenty individuals were parasitizing *Bigelowia nuttallii*; one individual was parasitizing *Liatris microcephala* as its primary host, with secondary connections to *Croton willdenowii* and *Hypericum gentianoides*. This population has historically been reported as exclusively parasitizing *Bigelowia nuttallii*, although other potential hosts, including *Liatris microcephala*, are abundant in proximity to *Cuscuta harperi* individuals. (For further discussion, see chapter 3.)

Fig. 1.9 Harrison Outcrop. All individuals are within a linear distance of approximately 645 meters to the west of Peacock Road. Total area of exposed Altamaha grit outcrop is approximately 81.5 acres (329,819 sq. meters).

Hinds Road (HR), Etowah County, Alabama (34.04753, -86.02973)

The Hinds Road population is found on a sandstone outcrop and has one of the highest densities of *Cuscuta harperi* individuals of all thirteen populations sampled. Thirty individuals were sampled, which represented a fraction of individuals present. Individuals were found parasitizing *Bigelowia nuttallii*, *Liatris microcephala*, *Croton willdenowii*, and *Hypericum gentianoides*, with many individuals parasitizing multiple hosts concurrently.

Fig. 1.10 Hinds Road. All individuals are within a linear distance of 290 meters. Total area of exposed sandstone outcrop is approximately 9.2 acres (37,231 sq. meters).

Little River Canyon (LRC), Dekalb County, Alabama

The Little River Canyon population is divided into four subpopulations, each designated for its location within Little River Canyon National Preserve. Managed by the U.S. National Park Service, this preserve is home to many protected plant species, including *Cuscuta harperi*, *Sarracenia oreophila*, *Allium speculae*, and *Phemeranthus mengesii*. Tissue sampling and plant collections within LRCNP were done under permission of the U.S. National Park Service (Permit# LIRI-2015-SCI-0002). Little River Canyon includes the population with the highest density of individuals of *Cuscuta harperi*, as well as the widest host range of all sampled populations.

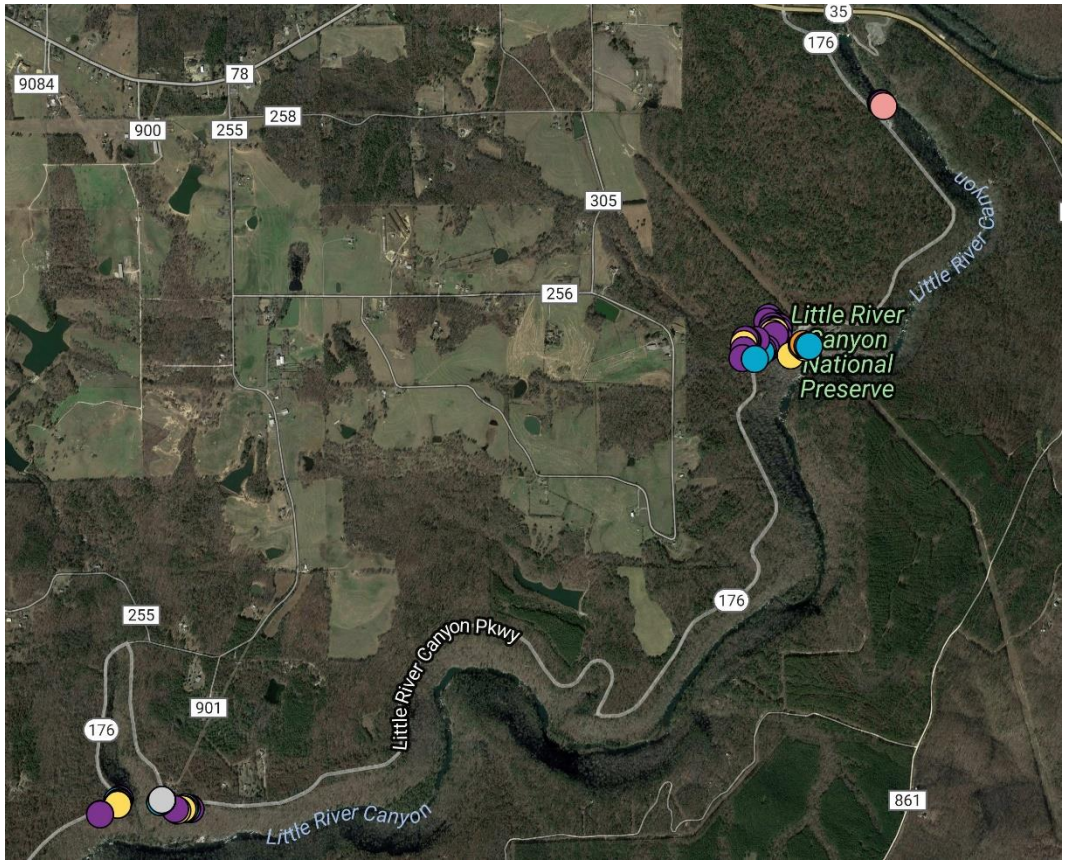


Fig. 1.11 Little River Canyon. The four populations sampled within Little River Canyon National Preserve span 4 miles (6.44 km) along the canyon rim.

Canyon View Overlook (LRC-CVO) (34.36414, -85.65886)

The Canyon View Overlook subpopulation is located on sandstone outcrops along the rim of the canyon. Thirteen individuals were located and sampled on 28 August 2015. Three individuals were parasitizing *Bigelowia nuttallii*, one was parasitizing *Hypericum gentianoides*, three were parasitizing *Liatris microcephala*, five were parasitizing *Helianthus longifolius*, and one individual was parasitizing *Liatris* and *Helianthus* concurrently.

Fig. 1.12 LRC-Canyon View Overlook. Individuals occur within a linear distance of approximately 152 meters along the canyon rim.

Falls Overlook (LRC-FO) (34.39239, -85.62534)

The Falls Overlook subpopulation is located on sandstone outcrops along the rim of the canyon. Four individuals were located and sampled on 28 August 2015. Three individuals were parasitizing *Liatris microcephala*; one individual was parasitizing *Phemeranthus mengesii*.

Fig. 1.13 LRC-Falls Overlook. These four individuals occur within a linear distance of 20 meters along the canyon rim.

Lynn Overlook (LRC-LO) (34.3838, -85.63078)

The Lynn Overlook population is located on exposed sandstone along the rim of the canyon and continues into an extensive glade adjacent to and contiguous with the overlook glade on the north side of Little River Canyon Parkway. This is the population with the highest density, with hundreds of individuals present on multiple host species. The glade to the north of the parkway is adjacent to habitat that is managed for *Sarracenia oreophila*, and, as such, is subject to periodic

prescribed burning. For more on fire dependency of *Cuscuta harperi*, see Introduction: Study Organism. Twenty-seven sampled individuals were parasitizing *Liatris microcephala*, ten were parasitizing *Bigelowia nuttallii*, four were parasitizing *Helianthus longifolius*, and one was parasitizing *Croton willdenowii*. Notably, *Coreopsis pulchra*, a species closely related to *Bigelowia*, *Liatris*, and *Helianthus*, is present and abundant on this glade; however, no individuals were observed successfully parasitizing *Coreopsis* during the 2015 field season.

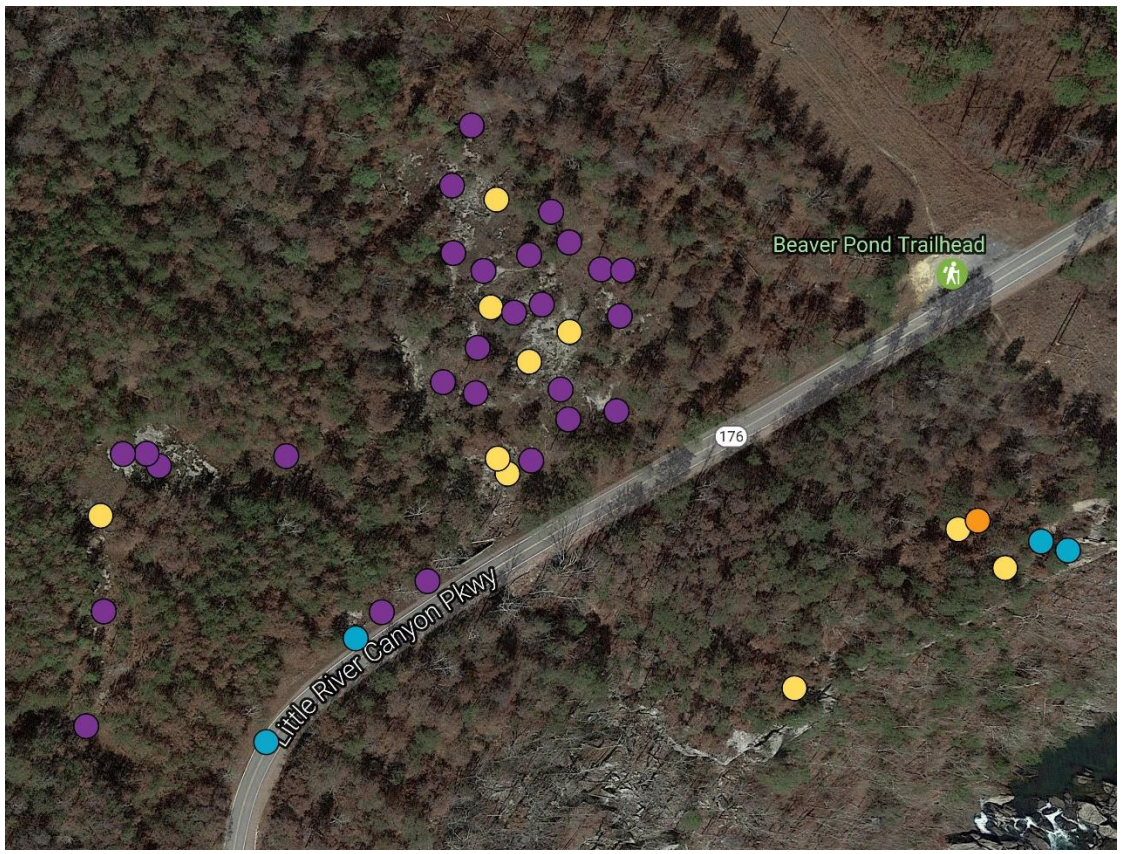


Fig. 1.14 LRC-Lynn Overlook. The majority of individuals at this location are in the glade to the north of Little River Canyon Parkway within an area of approximately 7.34 acres (29,704 sq. meters). Individuals near the canyon rim occur within a linear distance of approximately 96 meters.

Wolf Creek Overlook (LRC-WCO) (34.36452, -85.66228)

The Wolf Creek Overlook subpopulation is located on exposed sandstone along the rim of the canyon. Ten individuals were located and sampled on 28 August 2015. Four individuals were parasitizing *Bigelowia nuttallii*, two individuals were parasitizing *Liatris microcephala*, three individuals were parasitizing *Helianthus longifolius*, and one individual was parasitizing *Bigelowia* and *Liatris* concurrently.

Fig. 1.15 LRC-Wolf Creek Overlook. Individuals occur within a linear distance of 130 meters along the canyon rim.

Moon Rock (MR), Dekalb County, Alabama (34.52125, -85.61079)

The Moon Rock population is located on sandstone outcrops within Comer Scout Reservation (Boy Scouts of America) and was accessed with guidance from Alabama botanist Michelle Reynolds and landowner permission. Thirteen individuals were located and sampled on 21 August 2015. Twelve individuals were parasitizing *Bigelowia nuttallii*, and one was parasitizing *Liatris microcephala* and *Croton willdenowii* concurrently. This population showed a skew toward parasitization of *Bigelowia*, although *Liatris* was abundant in proximity to *Cuscuta* individuals.

Fig. 1.16 Moon Rock. Linear distance between the two groups of clustered individuals is approximately 114 meters. Total area of all exposed sandstone outcrop, which encompasses all individuals in this population, is approximately 2.54 acres (10,279 sq. meters).

Moss Rock Preserve (MRP), Jefferson County, Alabama (33.38174, -86.84912)

The Moss Rock Preserve population is located on three separate sandstone outcrop glades within the preserve, which is owned by the City of Hoover, Alabama and maintained largely by volunteer groups. Permission to access and collect samples at Moss Rock Preserve was granted through Friends of Moss Rock Preserve. Twenty-four individuals were located and collected on 12 September 2015.

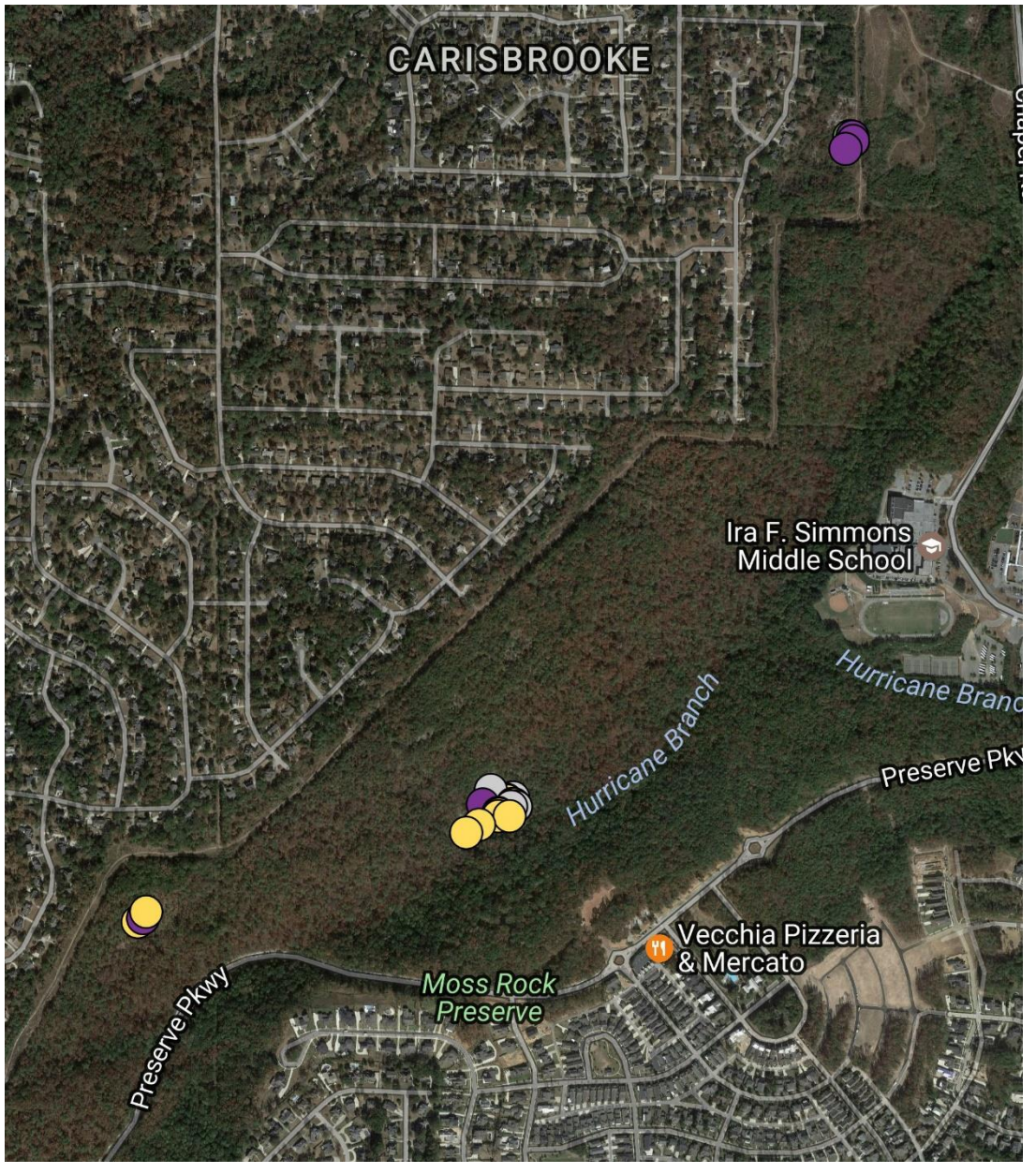


Fig. 1.17 Moss Rock Preserve. The three subpopulations are located on three disjunct outcrops within the preserve. Linear distance between the two farthest glades is 2012 meters.

Boulder Glade

Sixteen individuals were located at Boulder Glade. Eleven individuals were parasitizing *Bigelowia nuttallii*, three were parasitizing *Bigelowia* and *Croton willdenowii* concurrently, one was parasitizing *Bigelowia* and *Hypericum gentianoides* concurrently, and one individual was parasitizing *Liatris microcephala*.

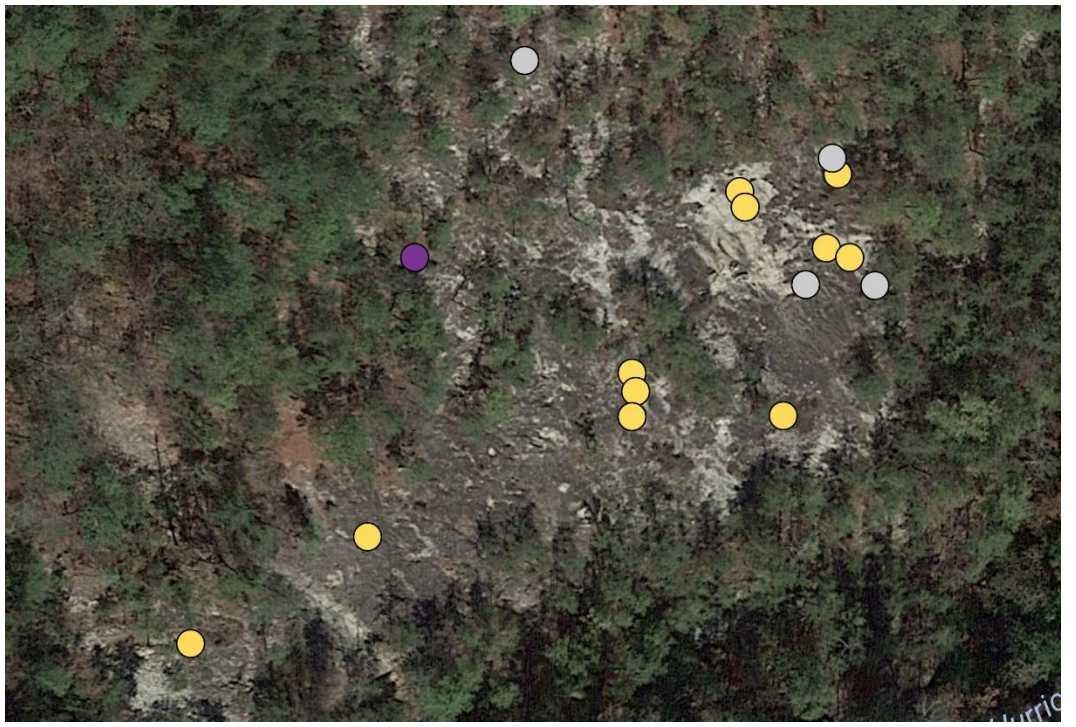


Fig. 1.18 MRP-Boulder Glade. Individuals in this glade occur within a linear distance of approximately 128 meters. Total area of exposed sandstone is approximately 9.85 acres (39,862 sq. meters).

Little Glade

Three individuals were located and sampled at Little Glade. Two individuals were parasitizing *Bigelowia nuttallii*, and one was parasitizing *Liatris microcephala*.

Fig. 1.19 MRP-Little Glade. The three individuals on this glade occur within a linear distance of 23 meters. Total area of outcrop is approximately 0.9 acres (3683 sq. meters).

Moon Rock Glade

Five individuals were located and samples at Moon Rock Glade. Four individuals were parasitizing *Liatris microcephala*, and one individual was parasitizing *Hypericum gentianoides*. No individuals were found parasitizing *Bigelowia*, although it was abundant in proximity to other host plants and *Cuscuta harperi* individuals.

Fig. 1.20 MRP-Moon Rock Glade. Individuals on this glade occur within a linear distance of 29 meters. Total area of exposed outcrop is approximately 1.87 acres (7568 sq. meters).

North Fork Creek (NFC), Marion County, Alabama (34.23639, -87.89535)

The North Fork Creek population is located on exposed sandstone on the east bank of North Fork Creek and is the westernmost of *Cuscuta harperi* populations located. Eight individuals were located and sampled on 4 September 2015. Seven individuals were parasitizing *Liatris microcephala*, and one individual was parasitizing *Liatris* and *Hypericum gentianoides* concurrently.

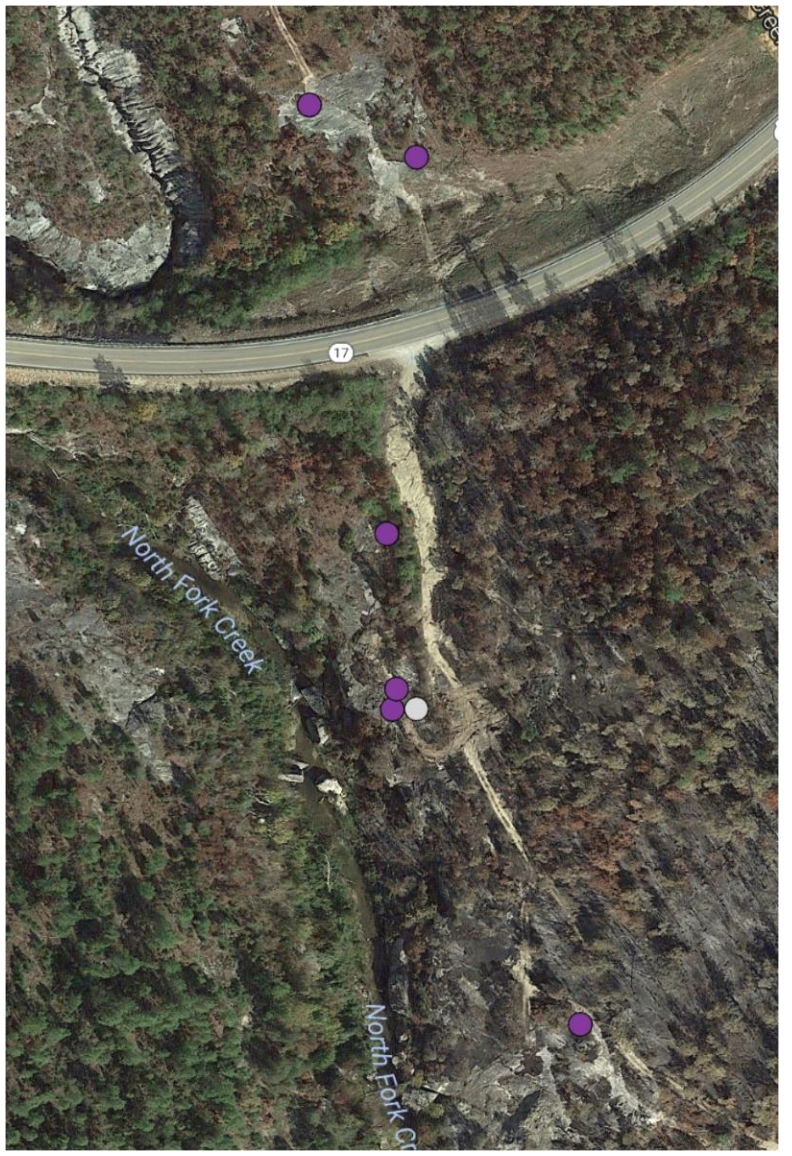


Fig. 1.21 North Fork Creek. All individuals occur within a linear distance of 363 meters along the east bank of North Fork Creek.

South Texas Flatrock (ST), Heard County, Georgia (33.21074, -85.21247)

The South Texas population is located on sandstone outcrops on and adjacent to lands managed by Hancock Forest Management, a division of Hancock Timber Resource Group. Tissue sampling was performed with permission from HTRG (Permit# TAP-2015-20). A total of six individuals were located and sampled on 18 September 2015 and 2 October 2015. Three individuals were parasitizing *Liatris microcephala*, and three were parasitizing *Hypericum gentianoides*. Individuals in this population were widely scattered across the expansive outcrop. South Texas Flatrock is outside of the documented range of *Bigelowia nuttallii*.

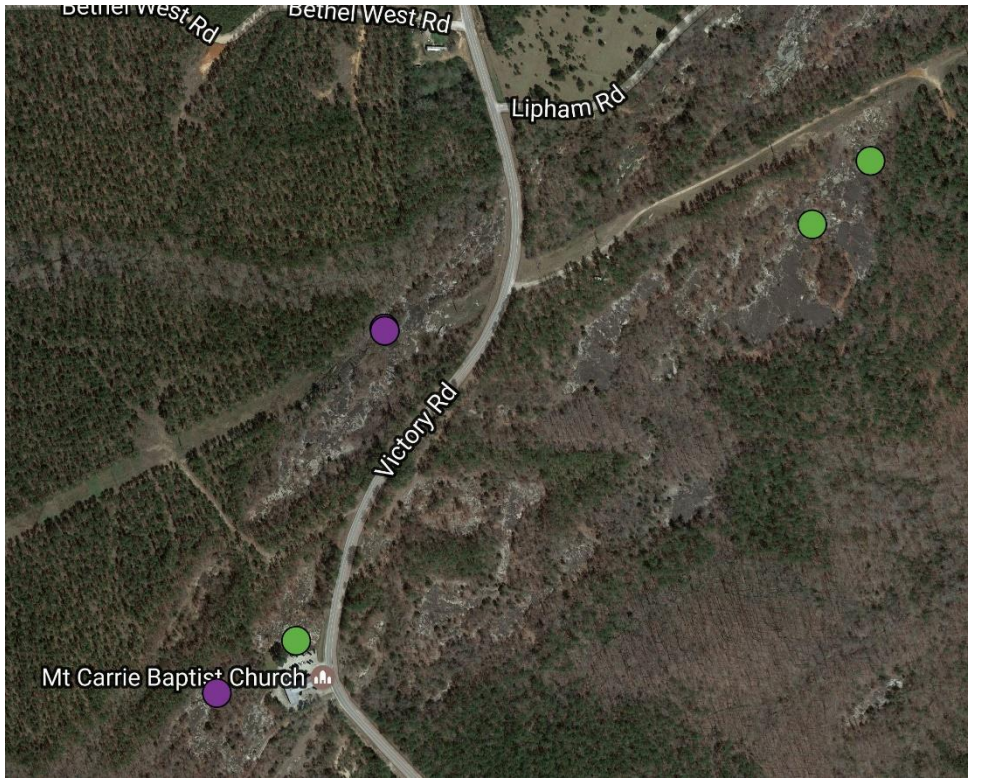


Fig.1.22 South Texas Flatrock. Individuals on this outcrop occur along a linear distance of approximately 900 meters. They are widely distributed across a total area of approximately 94 acres (151,278 sq. meters) of exposed granite.

Town Creek Glade (TC), Dekalb County, Alabama (34.38781, -86.02216)

The Town Creek population is located on exposed sandstone on the north bank of Town Creek. Ten individuals were located and sampled on 11 September 2015. All individuals were parasitizing *Bigelowia nuttallii*, although *Liatris microcephala* is present and abundant in this habitat.

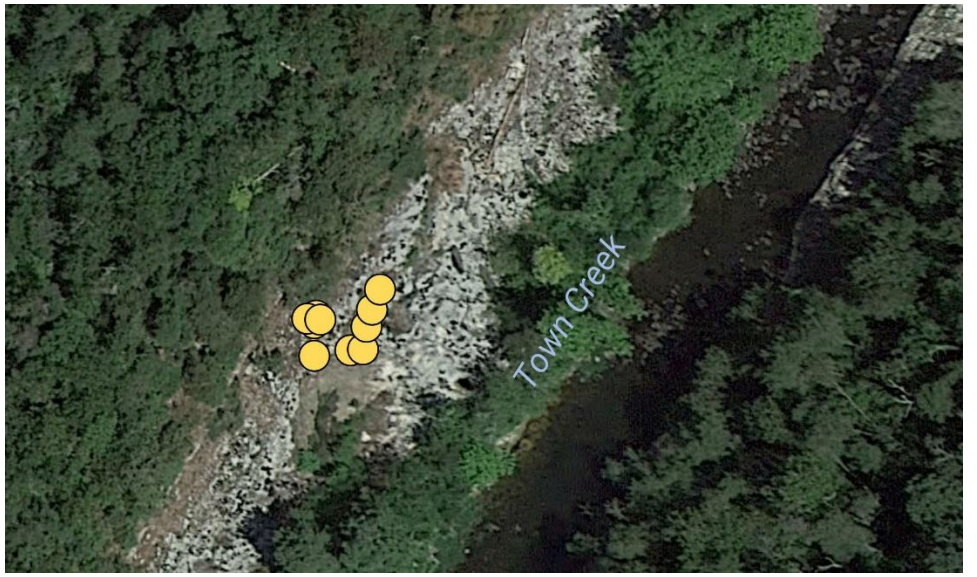


Fig. 1.23 Town Creek Glade. Individuals are clustered within a linear distance of 12 meters, although the outcrop expands for more than 645 meters along the bank of Town Creek.

Research Objectives

Overarching goals of this project include investigation into population genetics and genomics of *Cuscuta harperi* as well as elucidation of mechanisms underlying host selection by individuals of the species. To address these goals, we: 1) develop molecular markers to be used in evaluation of genetic diversity within and among populations of *C. harperi*, 2) use greenhouse-based experiments to examine foraging behavior and host selection by *C. harperi* seedlings in order to test whether observed differences in host use by population are due to active choice by parasite seedlings or, instead, are due to differential establishment success and survival of seedlings after random attachment, and 3) use field-based experiments to evaluate differential survival and reproductive success of *C. harperi* individuals on different host species.

Development of molecular markers for *C. harperi* will allow us to address population-level questions regarding heterozygosity and genetic variation within and among populations. Due to the primarily selfing reproductive strategy of the species, we expect to find a high inbreeding coefficient within all populations. Population genetics studies in other primarily autogamous species have predicted F values as high as 0.986 (Cascante-Marin et al. 2014). Because we expect similar results in *C. harperi*, we are primarily interested in evaluating genetic variability between individuals and among populations. We evaluate the efficacy of different methods to address these and other questions regarding genetic variation and population structure, the results of which can be used to determine conservation priorities for distinct populations of *C. harperi* and contribute to current efforts to protect the plant on public and private lands.

Additionally, we discuss the development and implementation of a conservation project for *in situ* safeguarding of *C. harperi* on property owned and protected by The Nature Conservancy. We evaluate *ex situ* cultivation and propagation methods to conserve the genetics of local parasite populations and host species and determine the best methods of transplantation in order to establish a novel population for long term protection.

Greenhouse-based trials are used to test the hypothesis that seedlings from populations where mature individuals are found growing overwhelmingly on one host over another differ in foraging behavior from seedlings originating from a population with more generalist host usage by mature plants. Field-based studies are used to test the hypothesis that *C. harperi* displays differential, non-random survivorship on different host species in the population sampled. Results of greenhouse and field studies combined help elucidate whether observed patterns of host specificity are due to active choice by parasite seedlings or, instead, are governed by environmental factors that make specific hosts more suitable in particular habitats. These results also have potential to be informative in studies of host use by closely related, weedy *Cuscuta* species, some of which are widespread and destructive agricultural pests.

CHAPTER TWO: Developing Population Genetics Markers: Testing Microsatellite Primers

INTRODUCTION

Microsatellites have long been a popular method of detecting variability within genomes; their heritability, selective neutrality, and high mutation rate make them ideal markers for studies of disease linkage, parentage and breeding, and population genetics. Their ubiquitous use in medical science and forensics helped to make the method convenient and affordable, and applications of the method in ecological genetics and conservation biology have become exceedingly common in the last few decades (Jarne and Lagoda 1996, Selkoe and Toonen 2006).

Microsatellites can be a powerful tool for population biologists because of their high levels of polymorphism. Allelic variability can range from 1-50 alleles per locus, with expected heterozygosity often above 0.5 (Jarne and Lagoda 1996, Peakall et al. 1998). However, some limitations on this variability occur in small or isolated populations, populations subject to recent bottlenecks, and in species that employ an autogamous reproductive strategy (Vaird 1996, DeWoody and Avise 2000).

A multitude of studies has shown that microsatellite regions are often shared among congeners and sometimes even across genera. Some examples of organisms that have been used in cross-amplification studies are fish, turtles, cetaceans, birds, and primates (Rico et al. 1996, Fitzsimmons et al. 1995, Schlötterer et al. 1991, Primmer et al. 1996, Garza et al. 1995). Extensive work has also been done with plants, including gymnosperms and angiosperms (van de Ven and McNicol 1996, Whitton et al. 1997, Brown et al. 1996). This increases the utility of microsatellites as genetic markers, especially in species without available reference genomes.

In this study we assess the utility of microsatellites to answer population genetics questions, including individual relatedness within populations and gene flow between populations, in *Cuscuta harperi*. PCR amplification and identification of variable microsatellite regions has been successfully utilized in the closely related species *Cuscuta rostrata* with similar test sample sizes (McNeal, unpublished data). We also

investigate cross-amplification of *C. harperi* microsatellites in other closely related *Cuscuta* species.

METHODS

Sampling and DNA Isolation

In order to obtain plant tissue for DNA isolation, field methods included minimally destructive sampling of flower buds and stem tips from *Cuscuta harperi* individuals from each population. Samples of plant material were preserved by desiccation using silica gel in labeled specimen bags at the site of collection. DNA isolations were performed following the protocol set forth by Doyle and Doyle (1987), with minor modifications by the McNeal lab.

Primer Testing

In a previous project in the McNeal lab, primer pairs were developed to amplify putative microsatellite loci mined from *Cuscuta harperi* transcriptome data. MSATCOMMANDER 1.0.8 was used to detect microsatellite arrays, identify highly conserved regions of sequence flanking those arrays, and design primers specific to those regions (Faircloth et al. 2008). Parameters were set to search for stretches of at least five consecutive trinucleotide repeats or four consecutive tetranucleotide repeats. Optimal primer pairs were designed via the program to generate amplicons of 150-450 base pairs. Tetranucleotide repeats were chosen preferentially over trinucleotide repeats, and loci containing a higher number of repeat units were given precedence. Using this specific set of design parameters, forty unique primer pairs were generated.

Primer pairs were initially tested on a single test DNA (Population HO) using standard PCR conditions. Thermocycler settings were as follows:

1. 94°C for 2 minutes
2. 94°C for 30 seconds
3. 54°C for 30 seconds
4. 72°C for 1 minute 30 seconds
5. Go to Step 2 30 times
6. 72°C for 4 minutes
7. Hold at 4°C

PCR products were loaded onto 1% agarose gels, and successful amplification of fragments was determined by gel electrophoresis, ethidium bromide staining, and photography under ultraviolet light. Primers that failed to amplify products in initial testing were retested on a different test DNA (Population LRC). For retest, concentration of primers was increased to 50 µM to maximize available primer relative to amount of sample DNA, and annealing temperature (Step 3) was lowered to 50° C to lower binding stringency of primers.

Primer pairs that successfully amplified products of appropriate size were subsequently tested on seven individuals with each representing distinct, widely-scattered populations (Fig. 2.1). Assessment of allelic variation based on fragment size was made by visualization of PCR products run on 3% agarose gels.

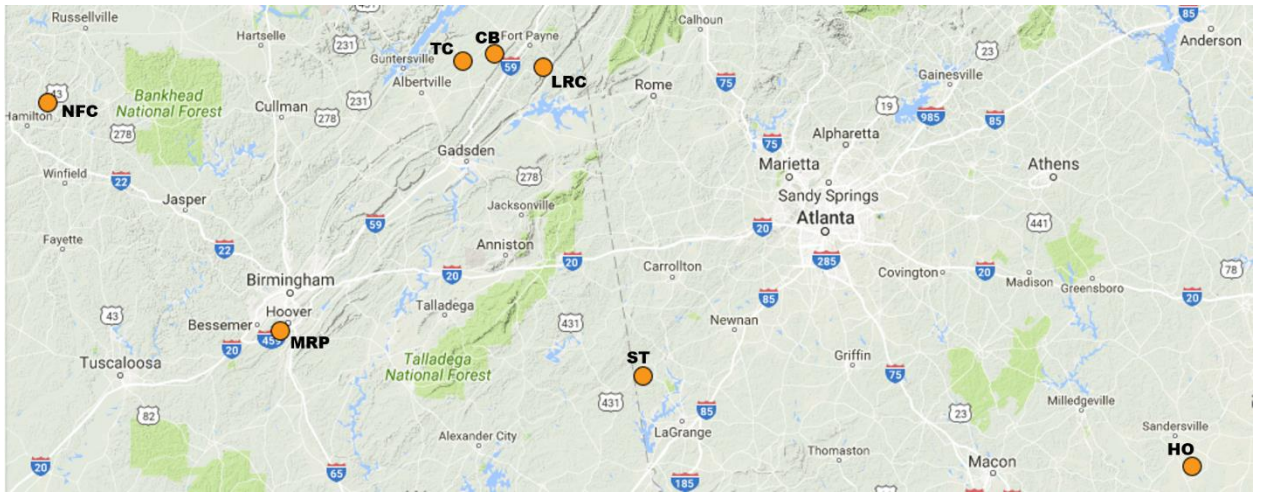


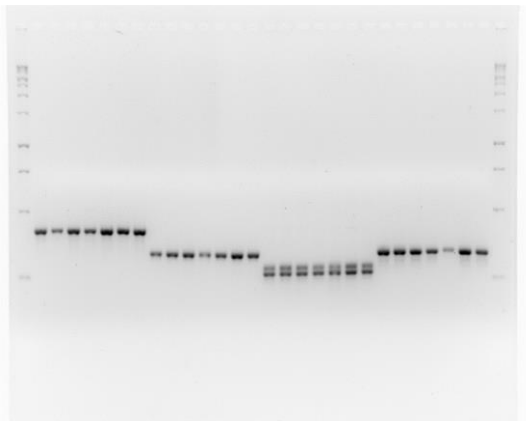
Fig. 2.1 Map of populations represented in microsatellite primer testing. CB=Chitwood Barrens; HO=Harrison Outcrop; LRC=Little River Canyon; MRP=Moss Rock Preserve; NFC=North Fork Creek; ST=South Texas Flatrock; TC=Town Creek Glade.

Primer pairs were also tested in three closely-related *Cuscuta* species (*C. pentagona*, *C. rostrata*, and *C. tasmanica*) to determine whether cross-amplification of fragments would occur. Successful cross-amplification was assessed by visualization of PCR products on 1% agarose gels using PCR products amplified from *C*. *harperi* as a positive control.

RESULTS

Primer Testing on *Cuscuta harperi*

Fourteen of the forty primer pairs successfully amplified products of appropriate size; these loci were subsequently tested across seven populations. (For primer specifications, see Table 2.1). Twelve out of these fourteen primer pairs amplified fragments of uniform size across all seven populations (Fig. 2.2a). Only two of the fourteen primer pairs, (Ch_comp27033; Ch_comp27123), exhibited allelic variation based on variability in amplified fragment length among the seven test individuals (Fig. 2.2b). No heterozygous individuals were identified.



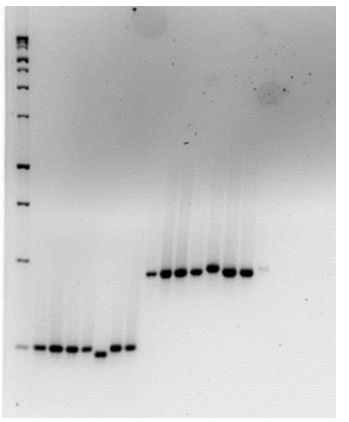


Fig. 2.2a. Invariable loci **2.2b.** Variable loci.

Fig. 2.2a. Gel picture of PCR products from four of the twelve primers. Lanes 1, 30 are 1Kb ladder; 2-8 represent primer pair Ch_comp14089; 9-15 represent primer pair Ch_comp20347; 16-22 represent primer pair Ch_comp25752; 23-29 represent primer pair Ch_comp26430. Uniform fragment sizes indicate invariable loci. **2.2b.** Gel picture of PCR products from two primers that produced fragments of varying size, indicating variation at these loci. Lane 1 is 1Kb ladder; 2-8 represent primer pair Ch_comp27033; 9-15 represent primer pair Ch_comp27123.

Oligonucleotide Sequence	Oligo Name	Repeat Type
CACGACGTTGTAAAACGACCATGTGCAGAGACAAGTGGC	Ch_comp14089_F	tetranucleotide
TAACCCTGCGCACCATCC	Ch_comp14089_R	tetranucleotide
CACGACGTTGTAAAACGACGTTCGCCGTCTCTTTGCTTG	Ch_comp20347_F	tetranucleotide
CCGGACGGCTGTAAGAAGAG	Ch_comp20347_R	tetranucleotide
CACGACGTTGTAAAACGACACACCAAAGCACAAGTACAATC	Ch_comp25752_F	tetranucleotide
AGGAGCTTGGACAGTGAGTG	Ch_comp25752_R	tetranucleotide
CACGACGTTGTAAAACGACTGGTCTTGGTGCTCTCAGTC	Ch_comp26430_F	tetranucleotide
CCACAGGACAAACAATGAAGTC	Ch_comp26430_R	tetranucleotide
CACGACGTTGTAAAACGACACCACACTAAATTCCACCGC	Ch_comp27033_F	tetranucleotide
CATCCAGCAACAGGGTTTCC	Ch_comp27033_R	tetranucleotide
CACGACGTTGTAAAACGACCAAAGAATGGAAATTGGCTCGC	Ch_comp27123_F	tetranucleotide
GTGTTGTGATCACCCGGTTG	Ch_comp27123_R	tetranucleotide
CACGACGTTGTAAAACGACGGCATCACCCTCGAGTATATG	Ch_comp29570_F	tetranucleotide
CTTTCTTCGACCAACCAGCC	Ch_comp29570_R	tetranucleotide
CACGACGTTGTAAAACGACACGATTCCTTCAGAGCCCAC	Ch_comp10301_F	trinucleotide
AGCAACAGTAACAATGGCGG	Ch_comp10301_R	trinucleotide
CACGACGTTGTAAAACGACGCCTCATGAACGTGCTCTTC	Ch_comp11041_F	trinucleotide
GCCTCTTCACTCCCACCC	Ch_comp11041_R	trinucleotide
CACGACGTTGTAAAACGACGGCGTGTCAAGGTCAAGATC	Ch_comp11155_F	trinucleotide
ATAACCTCCAGTGCAGCATC	Ch_comp11155_R	trinucleotide
CACGACGTTGTAAAACGACGAATCCCAATGCCAGGCTTC	Ch_comp16408_F	trinucleotide
TTGGTCATCAGTGGAGGGTC	Ch_comp16408_R	trinucleotide
CACGACGTTGTAAAACGACAACTGGACAGAGAGCCTTCG	Ch_comp16764_F	trinucleotide
GTCGGGCCGGAGATTATAAG	Ch_comp16764_R	trinucleotide
CACGACGTTGTAAAACGACCTTCCTGAAACCGTGGCATC	Ch_comp18019_F	trinucleotide
TCCGCCGTATTACCAGTACC	Ch_comp18019_R	trinucleotide
CACGACGTTGTAAAACGACACTTTCAGGGACAGAGCCAG	Ch_comp22220_F	trinucleotide
GGCATGCAGTAACCAACCAG	Ch_comp22220_R	trinucleotide

Table 2.1 Sequence and identification information for *C. harperi* primer pairs that amplified fragments of target size (150-450 base pairs).

Cross-amplification of PCR products in related *Cuscuta* species

Results of cross-amplification experiments show that all fourteen primer pairs that successfully amplified fragments of appropriate size also amplified PCR products in at least one of three closely related *Cuscuta* species. All primers tested amplified fragments in *C. pentagona*, eleven primers amplified fragments in *C. rostrata*, and eight primers amplified fragments in *C. tasmanica* (Table 2.2).

Oligo Name	*C. harperi*	*C. pentagona*	*C. rostrata*	*C. tasmanica*
Ch_comp14089_F/R	●	●	●	
Ch_comp20347_F/R	●	●	●	●
Ch_comp25752_F/R	●	●	●	
Ch_comp26430_F/R	●	●		
Ch_comp27033_F/R	●	●	●	
Ch_comp27123_F/R	●	●	●	
Ch_comp29570_F/R	●	●		
Ch_comp10301_F/R	●	●	●	●
Ch-comp11041_F/R	●	●		●
Ch_comp11155_F/R	●	●	●	●
Ch_comp16408_F/R	●	●	●	●
Ch_comp16764_F/R	●	●	●	●
Ch_comp18019_F/R	●	●	●	●
Ch_comp22220_F/R	●	●	●	●

Table 2.2 *C. harperi* primers amplify fragments across closely related *Cuscuta* species

DISCUSSION

Microsatellite primer testing resulted in minimal success in identifying loci that had allelic variation across seven individuals representing widely spaced populations of *C. harperi*. The populations tested in these experiments were chosen as representatives from

across the entire range of *C. harperi*; therefore it is unlikely that any variation at these loci would be discovered with further testing across other populations. Not only was an extremely low level of variation found in microsatellite loci between populations, no heterozygosity was detected within populations. Although the third locus sampled on the gel in Fig. 2.2a shows two distinct bands, it is unlikely that each individual from all seven populations is heterozygous at that locus. A more likely explanation for the appearance of double bands on the gel for those seven individuals is that two paralogous loci were amplified concurrently, considering all individuals are homozygous at the rest of the loci sampled. There is not strong enough evidence based on our results to call any individuals heterozygous at any locus sampled. This lack of genetic variability and apparent heterozygote deficiency could be the result of a predominantly autogamous reproductive strategy which eliminates heterozygosity from the population, relatively recent divergence from a single small founder population, or a genome less prone to slip-strand mispairing and generation of new alleles. Regardless of the underlying cause, there is insufficient variability across populations at these microsatellite loci to address population genetics questions in *C. harperi*.

Interestingly, when tested on individuals of closely related *Cuscuta* species, *C. pentagona*, *C*. *rostrata*, and *C*. *tasmanica* specifically, primers successfully amplified fragments of appropriate size. These results indicate potential utility of these loci in future studies of those species, as well as the possibility for use in other *Cuscuta* species not yet tested.

CHAPTER THREE: Developing Population Genetics Markers - Targeted Sequence Capture

INTRODUCTION

The use of high-throughput targeted capture methods has become increasingly widespread as their utility and affordability surpasses other genome sequencing methods such as whole-genome sequencing (WGS) and multiplex PCR. Targeted capture was first developed and utilized in human genomics, with initial applications including identification of genetic variants associated with specific diseases as well as addressing human evolutionary questions (Jones and Good 2016). Since their advent, sequence capture methods have become more technically streamlined, and their use in non-model species without reference genomes has become more popular.

Targeted sequence capture utilizes DNA or RNA baits designed to hybridize to complementary DNA fragments in order to isolate specific regions of the genome for sequencing. The technique allows for pull-down of specific genes of interest or regions containing putative molecular markers, such as single nucleotide polymorphisms (SNPs). Selection of these specific regions allows for greater depth of coverage of each targeted sequence than other less-specific methods of genome partitioning. We will employ sequence capture followed by high-throughput sequencing to identify SNPs from hundreds of captured loci concurrently. This technique should greatly increase the likelihood of finding variable genetic loci within the *C. harperi* genome as compared with using microsatellites, with which we have had minimal success in identifying variable loci in this species. Using sequence capture, we will isolate exon regions and noncoding sequences flanking those regions from approximately seven-hundred single-copy genes from each of the sampled individuals of *C. harperi* and analyze those loci to identify independently assorting SNPs scattered across the genome.

Use of targeted sequence capture in genomic studies of species without *a priori* sequence data is possible in part because RNA baits can work across closely related taxa. Studies have shown that capture sensitivity, (the percentage of targets covered by at least one mapped read), is as high as 90% when sequence divergence is below 9% (Jones and Good 2016). Baits designed from one reference genome have successfully

captured orthologous sequences between numerous related species, including humans and chimpanzees, several species of chipmunks and squirrels, and multiple anurans (Vallender et al. 2011, Bi et al. 2012, Good et al. 2015, Hedtke et al. 2013). The ability to utilize bait sequences across taxa not only simplifies initial study design, it also eliminates cost incurred from obtaining sequence data prior to bait design. In order to potentially take advantage of these benefits, we tested baits pre-designed for use in *Ipomoea*, a closely related genus to *Cuscuta* also in Convolvulaceae, on two *Cuscuta* libraries; however, due to suboptimal results, we proceeded with bait design using sequence data from the recently sequenced and assembled *Cuscuta harperi* genome (unpublished data).

METHODS

DNA Isolation

DNA isolations were performed from silica gel-dried plant material following the protocol set forth by Doyle and Doyle (1987), with minor modifications by the McNeal lab. After initial isolation protocol, DNA samples were resuspended in 50 ul 0.01M Tris and reprecipitated with a solution of 30% (w/v) polyethylene glycol (PEG 8000) and 30 mM $MgCl_2$. At this concentration of PEG 8000, fragments less than 300 bp in length will not precipitate efficiently; this is an initial size-selection step to eliminate DNA fragments that are smaller than our target fragment size of ~300 bp. Following PEG 8000 precipitation, DNA samples were resuspended in 21 ul 0.01M Tris, and concentrations were quantified using a NanoDrop™ spectrophotometer.

Library Preparation

One hundred individual DNA samples were chosen for inclusion in the first sequencing run. 96 *C. harperi* individuals were selected based on population and spatial location within study sites in order to provide a comprehensive cross-section of individuals spanning the species' range which will allow us to maximize genetic variation captured. Samples were also screened for inclusion based on quality of DNA isolation, including concentration and purity. We included DNA samples from four additional *Cuscuta*

species as outgroups for comparison of SNP signatures: *C. campestris*, *C. pentagona*, *C. polygonorum*, and *C. rostrata*.

DNA samples were sheared to appropriate size for Illumina sequencing (~300-700 base pairs in length) using the NEBNext dsDNA Fragmentase® enzyme, which can be used to cut DNA into a desired size-range of fragments based on time-dependent reactions with a fair amount of precision.

After enzymatic shearing, samples were purified and further size-selected using Agencourt® AMPure® XP magnetic beads to capture DNA while leaving contaminants in solution. Once cleaned, library preparation of samples was performed using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina. With this kit, individually-assigned, uniquely barcoded sequencing adapters were ligated onto the total genomic DNA fragments to generate libraries of barcoded fragments ready for sequencing on Illumina platforms. Upon completion of library prep, concentrations of DNA in each library were accurately quantified with a Qubit™ flourometer, and sequencing libraries from ten individuals at a time will be pooled together in equal concentration in preparation for targeted sequence capture reactions.

Bait Design

We targeted genomic regions surrounding exon sequence identified as being under selection to remain in single copy in most plants, including the closest photosynthetic relatives of *Cuscuta*. By selecting loci that are known to be single-copy, we can better ensure that any SNPs detected are polymorphisms at homologous positions and not the result of divergence of paralogous genes or repeat elements. This is particularly important in plants, which often have widespread paralogy and repetitive or complex genomes due to gene duplication and polyploidy events. Targeted sequence capture has been shown to be useful in identifying both orthologous and paralogous genes, both within individual genomes and across species, which makes it especially important to correct for this type of bias during data collection and analysis (Grover et al. 2012).

We provided a dataset to MYcroarray which contained exon sequence from 704 single-copy nuclear genes from the *C. harperi* genome as identified by a collaborator using the

program OrthoMCL (Todd Michael, pers. comm.). After stringent filtering to remove any non-specifically binding baits or baits matching repeat regions or organellar sequences in *Ipomoea nil* (used as a divergent reference genome) a total of 19,188 baits covering 938,513 bp of targeted sequence were selected. Baits were designed at approximately 3X tiling density; each bait is 120 nucleotides (nt) in length, and baits overlap at ~43 nt intervals so that three baits cover each base of unique sequence.

Synthesis of baits is currently in progress. Once baits are received, we will proceed with sequence capture reactions on prepared libraries of *C. harperi* individuals and outgroups.

Sequencing

Products of sequence capture reactions will be amplified using PCR in order to increase concentration of enriched libraries. Ten amplified capture libraries, each containing library fragments from ten uniquely barcoded individuals, will be sent to Georgia Genomics Facility where they will be pooled and sequenced on an Illumina NextSeq. A Mid Output flow cell will be used to generate up to 130 million reads, each 150 bp in length, for a total of approximately 18 gigabases (Gb) of sequence data. Since the initial run will include ten enriched libraries, each comprised of ten pooled individuals, this 18 Gb of data will be shared among 100 individuals; this translates to approximately 180 million bp (Mb) of sequence per individual. We estimate approximately 1 Mb of sequence to be captured by baits, including the 938,513 targeted bases plus captured flanking regions. If capture efficiency is 50%, (an extremely conservative estimate), we would expect ~90X coverage per targeted base for each individual. This ensures that coverage of flanking non-coding intron and untranslated regions, where we expect to find more SNPs but where coverage will decrease rapidly as distance increases from exons targeted by the baits, will be adequate to accurately distinguish homozygotes, heterozygotes, and sequencing errors. Once data from the first sequencing run is analyzed, a second run will be performed in order to sequence additional individuals and supplement sequence data from the first run for individuals with insufficient coverage.

Even with a high level of capture efficiency, we anticipate a portion of reads will be off-target and that the random genomic sequence contained in those reads will include chloroplast genome sequence that can be used for chloroplast haplotyping. For example, if capture efficiency is 80%, ~36 million bp of sequence from each individual will be from the chloroplast genome assuming that roughly 5% of the DNA in the initial libraries is chloroplast DNA. A conservative estimate of 5% chloroplast DNA is appropriate based on returns from previous genomic sequencing of *C. harperi*; 36 million bp of sequence at 5% cpDNA would lead to over 20X coverage of the chloroplast genome for each individual.

DISCUSSION

Variation in SNPs among individuals as well as variability of chloroplast haplotypes can be used to assess genetic diversity within and among populations. We will analyze these data in order to address various questions related to the population genetics of the species. We hope to elucidate patterns of population dispersal, genetic bottlenecks, founder effects, and drift, using this information to inform conservation priorities. We also anticipate the potential to address questions related to phenotypic variation and genome evolution from this large pool of data.

CHAPTER FOUR: Host Specificity: Greenhouse Trials

INTRODUCTION

Host-parasite and predator-prey interactions are important topics of study within ecology and evolutionary biology. Plants that have evolved a parasitic lifestyle are a compelling group of organisms in which to study both, because some parasitic plants display foraging behavior that is similar in many ways to animal foraging behavior (McNamara and Houston 1987). Research has shown that *Cuscuta* species can detect volatile organic compounds (VOCs) emitted by potential host plants and use them as chemical cues during foraging (Runyon et al. 2006); they are also capable of detecting host quality prior to making attachments and selectively attaching to hosts determined to provide the most benefit to the parasite (Kelly 1992). These behaviors are similar to those studied in models of optimal foraging strategies in animals.

Mechanisms of host selection and resource acquisition by parasitic plants are of particular interest when studying an organism that has adapted to a specialist host-selection strategy. When describing predators and parasites, there is a continuum that ranges from broad generalism to narrow specialization; one widely accepted definition of specialization is the discriminant use of available resources (Kelly et al. 1988). This essentially means that an organism does not use resources proportionally to their availability, but rather displays patterns of preference for one resource over another regardless of the abundance of that resource.

Cuscuta harperi can be classified as a specialist species due to its narrow host range. It is most frequently found parasitizing *Bigelowia nuttallii* and *Liatris microcephala* while seemingly ignoring closely related species growing in proximity to these hosts. However, field observations of *C. harperi* have revealed varying levels of host specificity among populations, with some populations displaying a clear preference for one host over another. Other populations utilize a wider host range which includes *Bigelowia* and *Liatris* but show no apparent preference for one over the other. Individuals from the Hinds Road population (HR), located within the species' core range in northeast Alabama, utilize a variety of hosts that includes a relatively even mix of *Bigelowia nuttallii* and *Liatris microcephala*. In contrast, individuals from the Harrison Outcrop

population (HO), located in the coastal plain region of Georgia, 140 miles southeast of the next closest population, have historically used *Bigelowia* as a near-exclusive host even though *Liatris* is also abundant on that outcrop (Fig. 4.1, 4.2a/b).

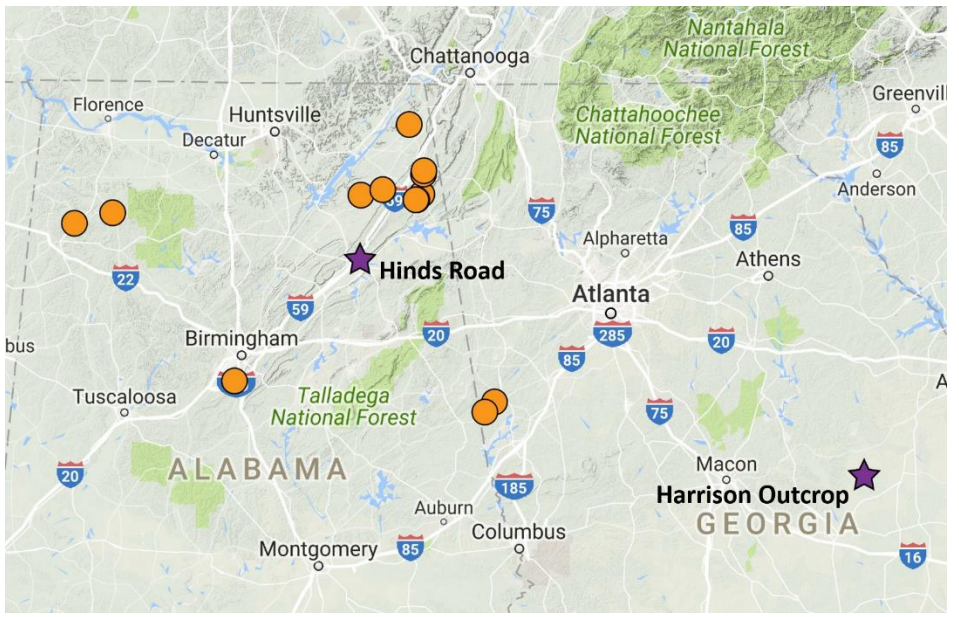


Fig. 4.1 Map of sampled *C. harperi* populations, with Hinds Road and Harrison Outcrop populations indicated by stars.

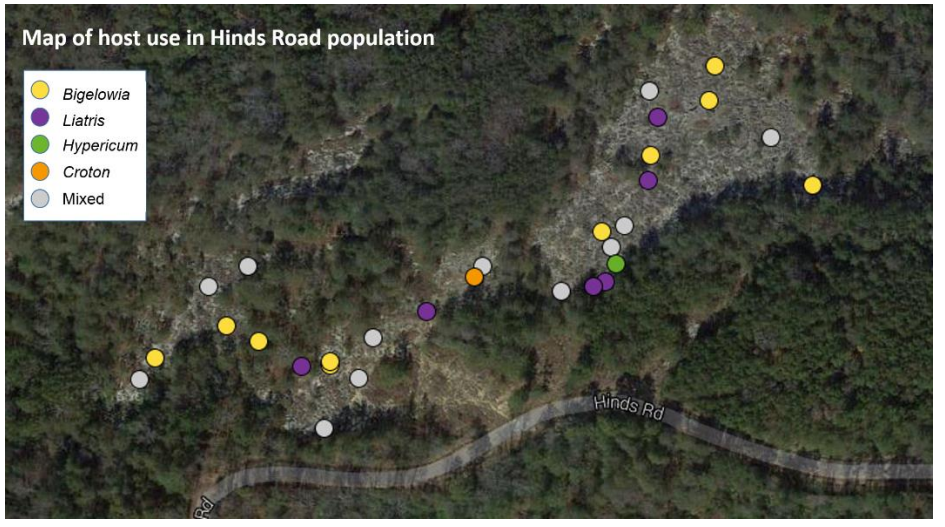


Fig. 4.2a. Hinds Road. Each marker represents a *C. harperi* individual sampled; different colors represent different host species parasitized.

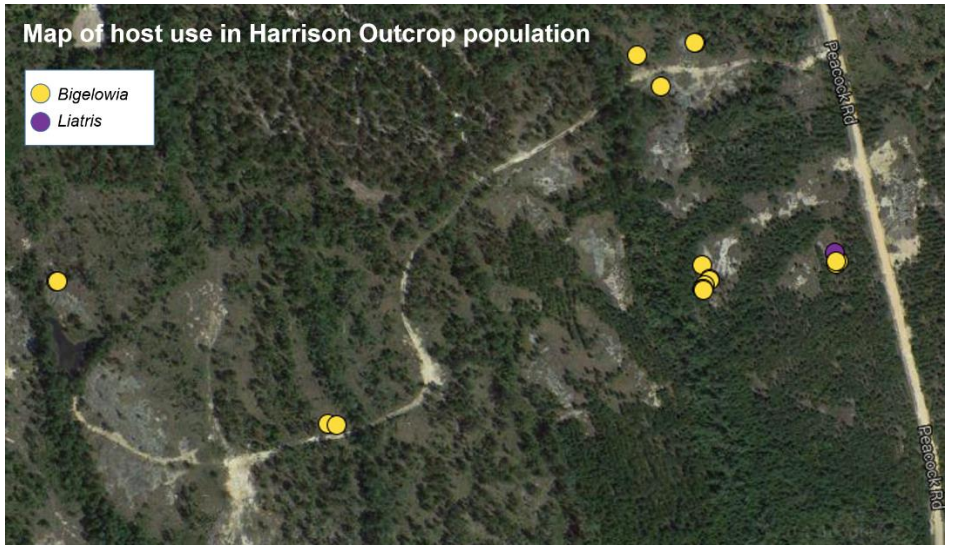


Fig. 4.2b. Harrison Outcrop. Each marker represents a *C. harperi* individual sampled; homogeneity of yellow markers indicates almost exclusive use of *Bigelowia nuttallii* as a primary host.

We utilize greenhouse experiments to address whether observed patterns of host use result from active choice by seedlings or, rather, are due to differential establishment success after random attachment. We also investigate whether patterns of host selection exhibited by seedlings from Hinds Road differ from patterns exhibited by seedlings from Harrison Outcrop.

Greenhouse experiments were designed to test the following hypotheses:

1. *Cuscuta harperi* seedlings will exhibit selective foraging behavior and host selection. We predict seedlings will display patterns of host selection that differ from random host selection and growth.

2. *Cuscuta harperi* seedlings from Harrison Outcrop, where mature individuals are found growing overwhelmingly on *Bigelowia*, will display a different pattern of host choice and establishment success than that of seedlings from Hinds Road, where mature individuals are found parasitizing a mix of hosts.

METHODS

Plant Collections

Cuscuta harperi seeds were collected from Harrison Outcrop in September 2015 and from Hinds Road in November 2015. Host plants were either germinated from seeds or propagated from living plant material collected in summer and fall 2015. *Bigelowia nuttallii* host material used in this study was collected from Harrison Outcrop, Little River Canyon-Canyon View Overlook, Little River Canyon-Wolf Creek Overlook, Hinds Road, and Town Creek Glade. *Liatris microcephala* host propagules were collected from Little River Canyon-Lynn Overlook and South Texas Flatrock. *Coreopsis pulchra* propagules were collected from Little River Canyon-Lynn Overlook.

Study Design

Experiments were conducted in 4-inch plastic pots containing a soil mixture of equal parts potting soil, perlite, and sand. Each pot contained four alternative host choices placed equidistantly from the seedling in the corners of the pot. Potential choices for the seedling were *Bigelowia*, *Liatris*, *Coreopsis*, and a nonliving bamboo skewer. *Bigelowia* and *Liatris* were included as known preferred hosts. *Coreopsis* does not occur on Hinds Road or Harrison Outcrop; it was not found as a host for mature parasites on outcrops where it abundantly co-occurs with *C. harperi* despite being in the same family as the preferred hosts (Asteraceae) and, as such, was included as a non-preferred host. The bamboo skewer was inserted vertically into the soil to serve as an artificial plant stem control that should produce a shadow but a negligible volatile chemical gradient in the air. Hosts and control were arranged within pots using four different configurations to control for directional growth variables, such as light and airflow, within the greenhouse (Fig. 4.3). Host plants and non-living control were arranged within the pots to be equidistant from parasite seedlings while maximizing distance between potential hosts. Each pot contained a 9-cm filter paper disc delineated into quadrants representing each of the potential hosts (Fig. 4.4).

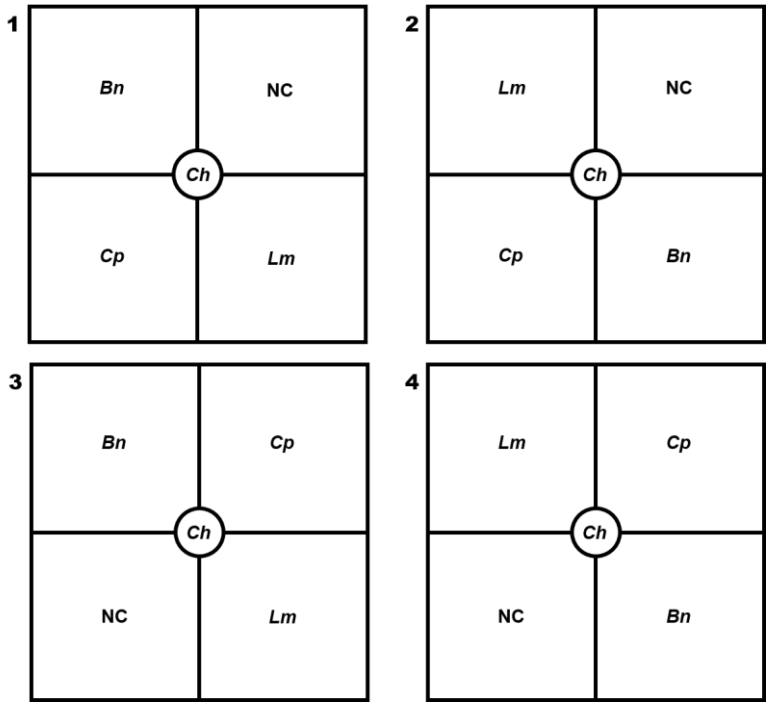


Fig. 4.3. Pot configurations. Potential hosts: *Bigelowia nuttallii* (*Bn*), *Coreopsis pulchra* (*Cp*), *Liatris microcephala* (*Lm*), and Non-living Control (NC). Seedling: *Cuscuta harperi* (*Ch*).

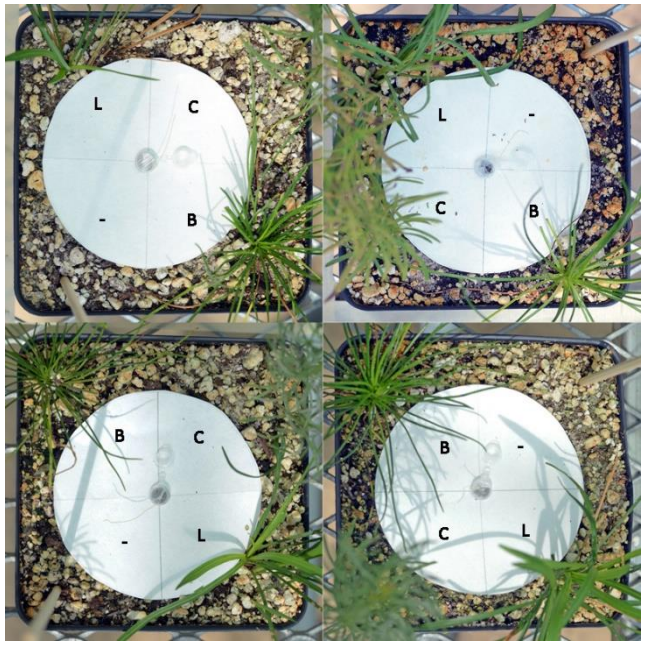


Fig. 4.4. Host pots with filter paper labeled to quadrant. B=Bigelowia C=Coreopsis L=Liatris (-)=Non-living control.

Experiments were conducted using block design; the greenhouse bench was divided into eight blocks with eight pots in each block. Each block was randomly assigned two pots of each configuration (1-4), and pots were randomly assigned a parasite population, (HR or HO), so that one pot of each configuration contained a parasite from each of the populations in every block. Position of pots within blocks was also randomized in order to control for microclimate variables within the greenhouse (Fig. 4.5). Pots were arranged to allow maximum distance between replicates within study blocks. [For complete listing of *C. harperi* parent populations and host populations for each pot ID, see Appendix 1.]

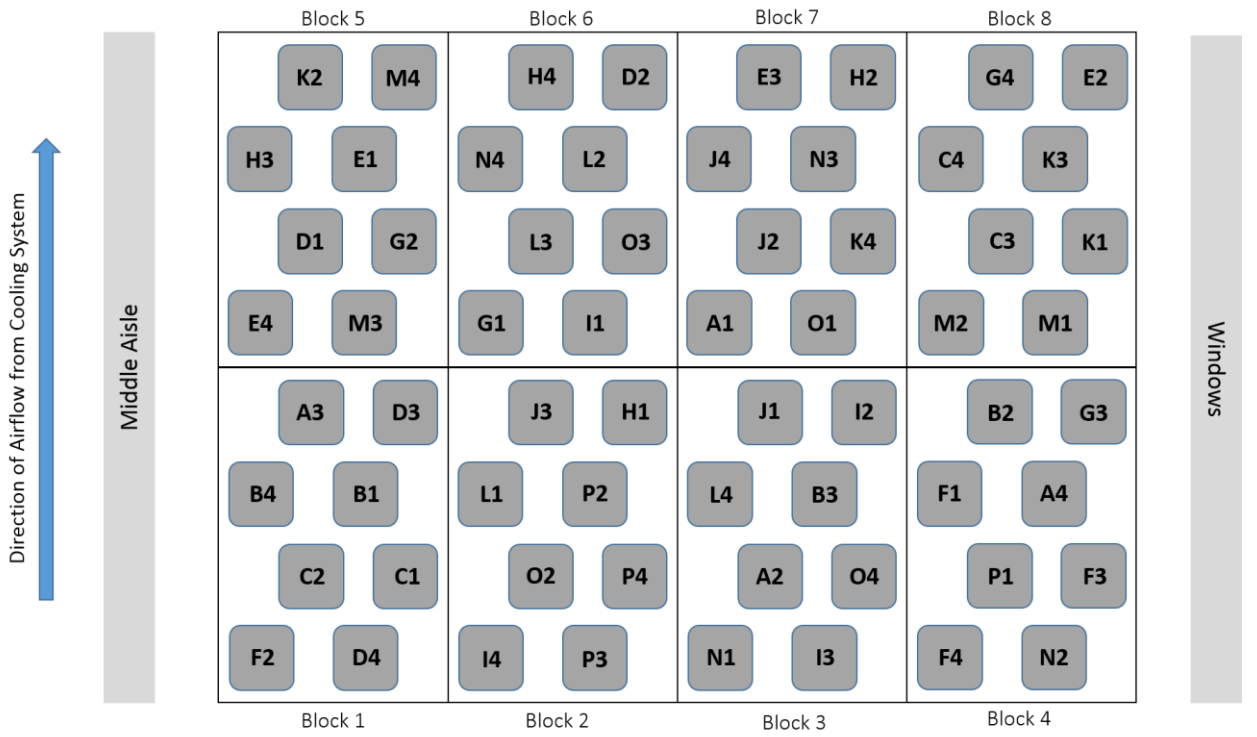


Fig. 4.5. Schematic representation of bench layout for greenhouse trials. Bench dimensions: 208 cm L X 112 cm W; block dimensions: 56 cm L X 52 cm W.

A total of 128 replicates were performed in two separate trials within the Joyce and Ira Pegues Memorial Greenhouse at Kennesaw State University. Trial 1 (replicates 1-64) ran from 20 July 2016 through 04 August 2016. Trial 2 (replicates 65-128) ran from 13

August 2016 through 29 August 2016. Experiments were performed under standard greenhouse conditions. [For temperature data, see Appendix 2].

Seedling Germination

In order to germinate seeds of *Cuscuta harperi*, they were placed in Gooch crucibles for scarification with concentrated sulfuric acid for 30 minutes, rinsed with deionized water, soaked in 10% bleach solution for 2 minutes, and thoroughly rinsed again with deionized water to slough off excess dead chaff from the outer seed coat. The sterile, scarified seeds were placed on damp filter paper in petri dishes which were sealed with wax film strips until germination to prevent fungal contamination. Once the seedlings had grown to approximately 1-2 cm in length, they were transferred to microcentrifuge tubes with the swollen hypocotyl anchor end of the seedling stem immersed in deionized water and the growing tip of the seedling extending out from the mouth of the tube. Seedlings were placed in proximity to a light source and allowed to straighten for approximately 24 hours to bring all seedlings to a relatively uniform shape and length, at which point the seedlings were placed into experimental pots.

Data Collection

In order to determine whether seedlings displayed selective foraging behavior, directionality and quadrant position of apical meristem were monitored and recorded twice daily until seedlings formed an attachment to a host. Host attachment was characterized by a seedling irreversibly coiling around host stem or leaf tissue. If an individual died before an attachment was made, the quadrant into which the apical meristem was growing when the seedling died was recorded as the final quadrant location.

In order to address the question of differential survival by seedlings after host selection, establishment success was measured for each individual that formed an attachment. Successful establishment was qualified as secondary stem growth after formation of haustoria at initial point of attachment.

To address whether seedlings were simply attaching to the most proximal host tissue, straight-line distance from the center of the microcentrifuge tube to the point of

attachment to host was measured as well as straight-line distance from center to two additional potential attachment points on nearby host tissue.

Total seedling growth in length was measured for those individuals that did not form attachments in order to ascertain maximum growth before exhaustion of seedling resources.

Data Analysis

In order to address the question of whether patterns of host use were due to active choice by seedlings or secondary to differential establishment success after random attachment, distribution of attachments and final quadrant distribution data were analyzed using Chi-square Goodness of Fit test to compare results to a random distribution. To address the question of whether seedlings from different populations would display different patterns of host selection, attachment and final quadrant distribution data were analyzed using Chi-square Contingency Analysis.

To evaluate whether seedlings from each population showed different patterns of survival on different hosts, post-attachment establishment success was analyzed using Chi-square Contingency Analysis.

For all analyses a p-value of <0.05 was considered statistically significant.

Distances to attachment points were compared to distances to nearby host tissue in order to determine whether seedlings were attaching to the closest available host tissue.

Average, median, and range values for unattached seedling length were calculated for both trials separately and for all replicates combined. A two-sample unequal variance t-test was performed to determine whether there was a difference in final length of seedlings between populations.

RESULTS

To address the question of random vs. non-random attachment to hosts, all replicates from both populations of seedlings were first considered together. Out of 128 total replicates, 55 seedlings formed an attachment to a host or the non-living control (Table 4.1). 52 of the 55 attachments were made to either *Bigelowia* or *Liatris*; distribution is significantly different from random attachment distribution (p= 5.614e-11). Final quadrant distribution was also significantly different from random distribution (p=3.582e-05). Attachment and final quadrant distribution data from individual populations were also considered separately. Attachment distributions for both Harrison Outcrop and Hinds Road were significantly different from a random distribution; p= 3.219e-6 for both analyses. Final quadrant distribution for each population was also significantly different from a random pattern of distribution when considered independently; p= 0.034 and p= 0.001, respectively.

Population	*Bigelowia*	*Liatris*	*Coreopsis*	Non-living Control
Harrison Outcrop	15	6	0	1
Hinds Road	18	13	1	1
TOTAL	33	19	1	2

Table 4.1. Attachment data by population.

To address the question of whether seedlings from different populations display a significant difference in host choice, the number of successful attachments on each host were compared between HO and HR. Chi-square contingency analysis indicated the difference in host attachments by seedlings between populations is not statistically significant (p= 0.3261). Contingency analysis of final quadrant distribution also showed no significant difference by population (p= 0.5406).

Post-attachment establishment success on different hosts, qualified by secondary stem growth past the initial point of attachment, showed a significant difference between populations (p= 0.04) (Table 4.2). No individuals that attached to *Coreopsis* or the non-living control demonstrated secondary stem growth and were therefore not included in the analysis.

Population	*Bigelowia*	*Liatris*
Harrison Outcrop	8	1
Hinds Road	6	7

Table 4.2 Establishment success by population.

Straight-line distances to the point of attachment on host tissue was measured and compared to straight-line distances to two other potential attachment points closest to the center of the pot (Fig 4.6). In 96 percent of replicates, straight-line distance to attachment was greater than the distance to the closest host tissue, indicating that the parasite is not simply attaching to the closest host available.

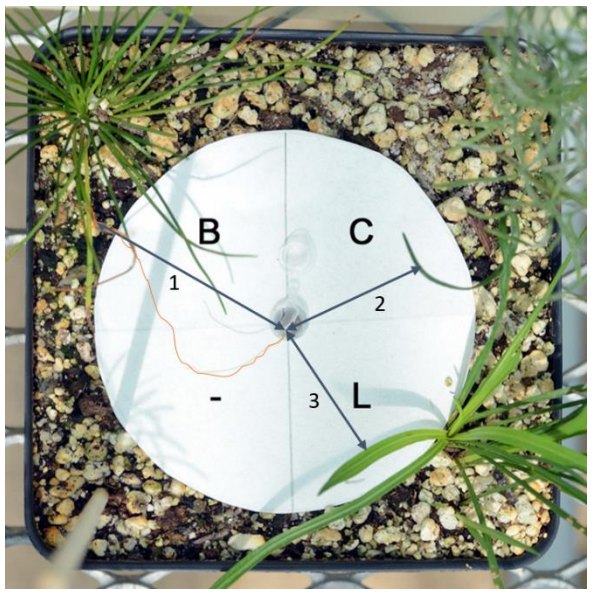


Fig. 4.6. Example of distance measurement method.

Orange line represents *C. harperi* seedling attached to *Bigelowia* leaf. Distance 1 is straight-line to point of attachment. Distances 2 and 3 represent straight-line distances to other host tissue closest to center of experimental pot.

Average total length of unattached seedlings was 62.55 mm overall. Median seedling length was 59 mm. Difference in final seedling length between populations was not statistically significant (p=0.13). The seedling with the most growth in length was 141 mm long at the time of its death, most likely due to resource exhaustion. The seedling with the least growth in length at its death was 9 mm, which was possibly to due to an alternative cause, such as pest damage.

DISCUSSION

Results of this study indicate that *Cuscuta harperi* seedlings are capable of selective foraging and that patterns of host selection are the result of active choice by seedlings rather than due to differential establishment success after random attachment to a host. The data clearly show that host attachment patterns differ from distributions that would be expected if seedlings were attaching at random to any available host, with a significant majority of attachments on *Bigelowia* and *Liatris*, the two most frequently parasitized hosts in natural populations (Fig. 4.7). These findings are consistent with the idea that *C. harperi* seedlings are capable of detecting signals from potential host plants and using those cues to actively choose an appropriate host when presented with multiple options. These results are not surprising considering that other *Cuscuta* species have been shown to display chemotactic responses to volatile organic compounds as well as the ability to choose a host based on perceived nutritional content prior to haustorial connections (Runyon et al. 2006, Kelly 1992).

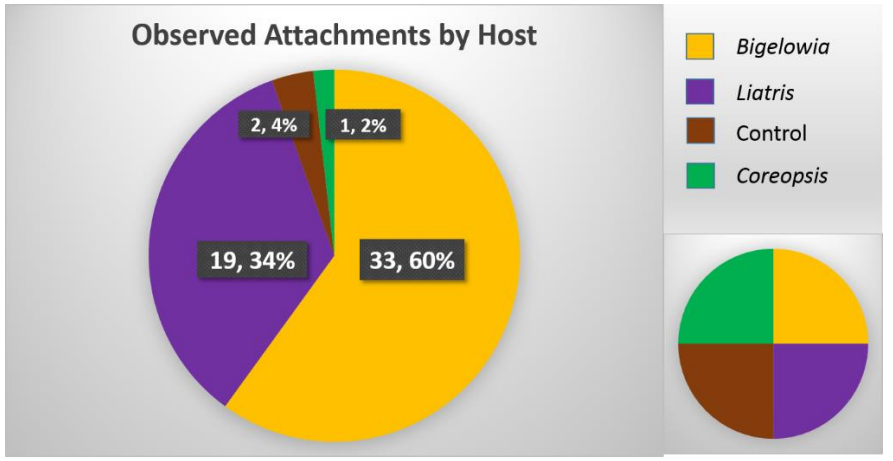


Fig. 4.7. Observed attachments by host plant. Lower right represents a random attachment distribution.

Additionally, we recorded the final quadrant into which unattached seedlings were growing at the time of their deaths, presumably due to exhaustion of resources initially stored in the seed before they were able to successfully find and attach to a host. We evaluated these data in combination with host selection data from attached seedlings in order to evaluate growth directionality. Final quadrant distribution was significantly different from an expected random distribution, which also supports the hypothesis that

C. harperi seedlings display selective foraging behavior (Fig 4.8). It is notable that this behavior is present in seedlings from both populations tested, which supports the hypothesis that selective foraging behavior arose in a common ancestor before these two populations diverged and, possibly, prior to the divergence of the species from the shared common ancestor with other *Cuscuta* species. It is also particularly interesting that seedlings in these trials appear to actively avoid *Coreopsis*, which is also seemingly avoided by *C. harperi* in natural populations. *Coreopsis* is absent at both Hinds Road and Harrison Outcrop; therefore it can be assumed that individuals from parent populations were never exposed to VOCs or other cues from *Coreopsis* and that mechanisms governing the avoidance response likely evolved in *C*. *harperi* prior to the divergence of these two populations.

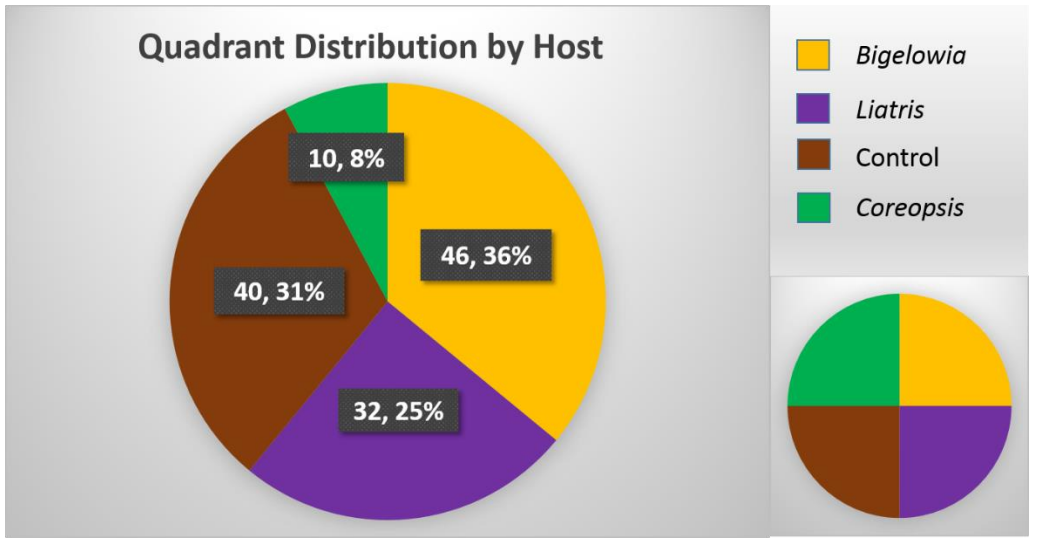


Fig. 4.8. Distribution of final quadrant location of seedling apical meristem. Figure in lower right represents expected quadrant distribution if seedlings were foraging at random.

Patterns of host selection by population were compared to determine whether seedlings from different population displayed significantly different foraging and attachment behavior parallel to that exhibited by individuals in parent populations. Twenty-two HO seedlings successfully attached to hosts in these trials; fifteen out of twenty-two

attachments (68.2%) were on *Bigelowia* and six (27.3%) were on *Liatris*. Of the thirty-three HR individuals that successfully attached to hosts, eighteen (54.5%) were on *Bigelowia* and thirteen (39.3%) were on *Liatris* (Fig. 4.9).

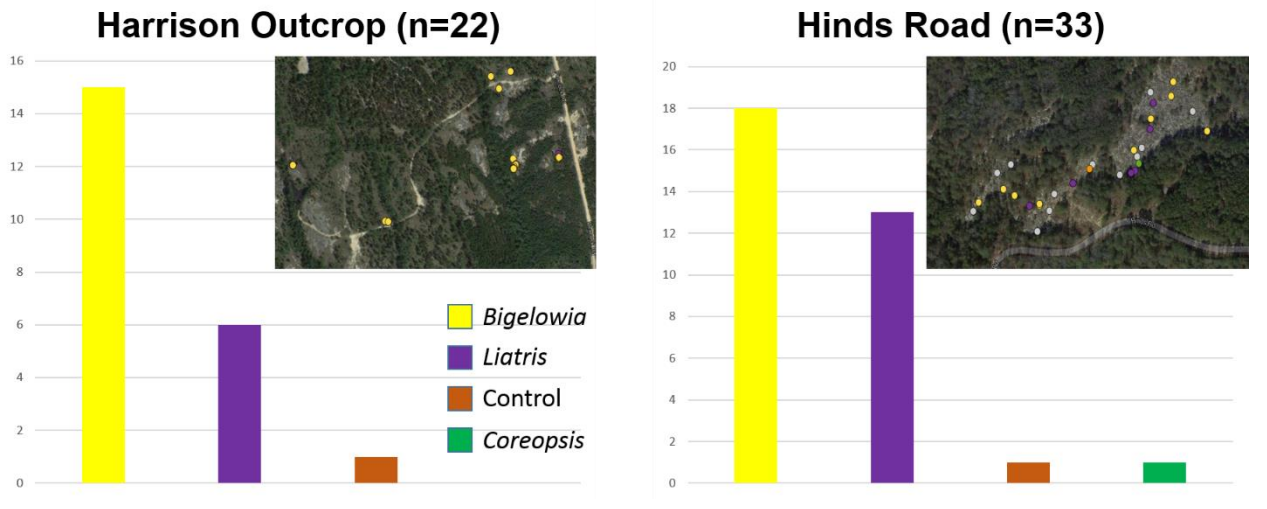


Fig. 4.9. Observed distribution of host selection by population. Different colored markers on maps indicate host species each sample individual was found parasitizing.

Contingency analysis did not find the difference in distribution of host use between populations to be statistically significant; however, HO seedlings showed a more pronounced skew toward parasitization of *Bigelowia* while HR seedlings used a more even mix of both *Bigelowia* and *Liatris*. This trend aligns with patterns of host selection observed in mature parent populations and suggests that foraging behavior governed by innate cellular and molecular mechanisms may evolve rapidly enough to be divergent between populations. Sample size of attachments is relatively small for both Harrison Outcrop and Hinds Road, (n=22 and n=33, respectively), and differences in patterns of host selection might be significant with further replication of these trials.

These results raise another consideration: if a percentage of seedlings from Harrison Outcrop choose and successfully parasitize *Liatris* in a greenhouse setting, then why are individuals found on *Bigelowia* almost exclusively in the natural population? One potential explanation is that seasonal climate differences in the coastal plain physiographic region of Georgia, as compared to the piedmont and ridge and valley

physiographic regions in which other populations are located, could contribute to host availability and, ultimately, to observed host use patterns. Specifically, average temperatures at Harrison Outcrop are typically higher earlier in the spring than at other populations, and that difference could potentially lead to earlier germination of *C. harperi* seedlings. *Bigelowia* and *Liatris* are both perennial species; however, *Bigelowia* is evergreen with leaves present year-round, while *Liatris* dies back to underground corms over winter. If *C. harperi* seedlings germinate earlier at Harrison Outcrop than in populations found farther north, then they may be seeking hosts and making initial attachments while *Bigelowia* is abundant on the outcrop but before new *Liatris* growth is available.

In order to investigate this possibility, we visited Harrison Outcrop on March 11, 2017, almost two months earlier than seedlings have been observed in an early stage of growth post-attachment at Little River Canyon on the Cumberland Plateau of Alabama. During this visit we located multiple newly germinated seedlings; based on size and the retention of the connection to the ground, seedlings had likely germinated within two to three days prior to our visit (Fig. 4.10a-d). All of the seedlings located were already attached to or growing straight toward *Bigelowia*; no above-ground *Liatris* growth was observed. Further study is required to more thoroughly investigate this phenomenon; however, these preliminary observations suggest specialization on *Bigelowia* may result from early *Cuscuta* seed germination at this site.

Fig. 4.10 a/b. Seedlings are attached to *Bigelowia* leaves and forming haustoria. Initial connection to the ground is still intact, indicating age of seedling is approximately 3-5 days.

Fig. 4.10 c/d. 4.10.c Top: Seedling, still unattached, growing toward *Bigelowia*. Bottom: Seedling attached to *Bigelowia* with initial ground connection intact. **4.10.d** Top: Two seedlings, unattached and growing toward *Bigelowia*. Bottom left: Seedling attached to *Bigelowia* with ground connection intact.

Differential Establishment Success

Establishment success after host selection and attachment was evaluated in order to investigate whether seedlings from either population were better able to parasitize one host over another in the longer term and whether differential survival may contribute to patterns of host use observed across populations. Successful establishment was

qualified as secondary growth past the point of initial coiling around host tissue, which required formation of haustoria and extraction of nutrients from the host in order to enable the seedling to send out new stem shoots. Results of this trial showed that Harrison Outcrop seedlings had much greater establishment success on *Bigelowia*, while Hinds Road seedlings had nearly equal success on *Bigelowia* and *Liatris*. These results, paired with results discussed previously that indicate Harrison Outcrop seedlings preferentially parasitize *Bigelowia,* suggest that observed patterns in foraging behavior and host use may be due to a suite both genetic and environmental factors.

Further research is needed to investigate the genetic and ecological factors influencing host use patterns observed in *C. harperi* populations. We have shown that *C. harperi* is capable of selective foraging and active host choice; however, the cellular and molecular mechanisms underlying these behaviors remain to be elucidated. More extensive investigation into environmental factors, such as parasite virulence, host resistance, and nutrient availability and composition, is also necessary to better understand how local adaptations affect patterns of host use in *C. harperi*.

CHAPTER FIVE: Host Specificity: Field Survivorship Study

INTRODUCTION

Lynn Overlook at Little River Canyon National Preserve has the highest *Cuscuta harperi* population density of any of our study sites, and both *Bigelowia nuttallii* and *Liatris microcephala* are present in abundance. Interestingly, *Liatris* was parasitized much more frequently than *Bigelowia* and other potential hosts in late August 2015 when tissue from mature individuals was sampled for DNA isolation from this site (Fig. 5.1). Notably, *Coreopsis pulchra*, a species related to *Bigelowia* and *Liatris* in Asteraceae, is also present and abundant on this glade; however, no *C. harperi* individuals were observed successfully parasitizing *Coreopsis* during the 2015 field season.

In May 2016, recently attached seedlings were observed connected to both primary hosts, *Liatris* and *Bigelowia*, as well as *Coreopsis*. At this point in the season, *Bigelowia* and *Liatris* are relatively comparable in size and biomass available for parasitization, and *C. harperi* seedlings were observed forming attachments to both species with similar frequency. In this study, we assess survival and reproductive fitness of *C. harperi* individuals that formed initial attachments on different hosts at Lynn Overlook.

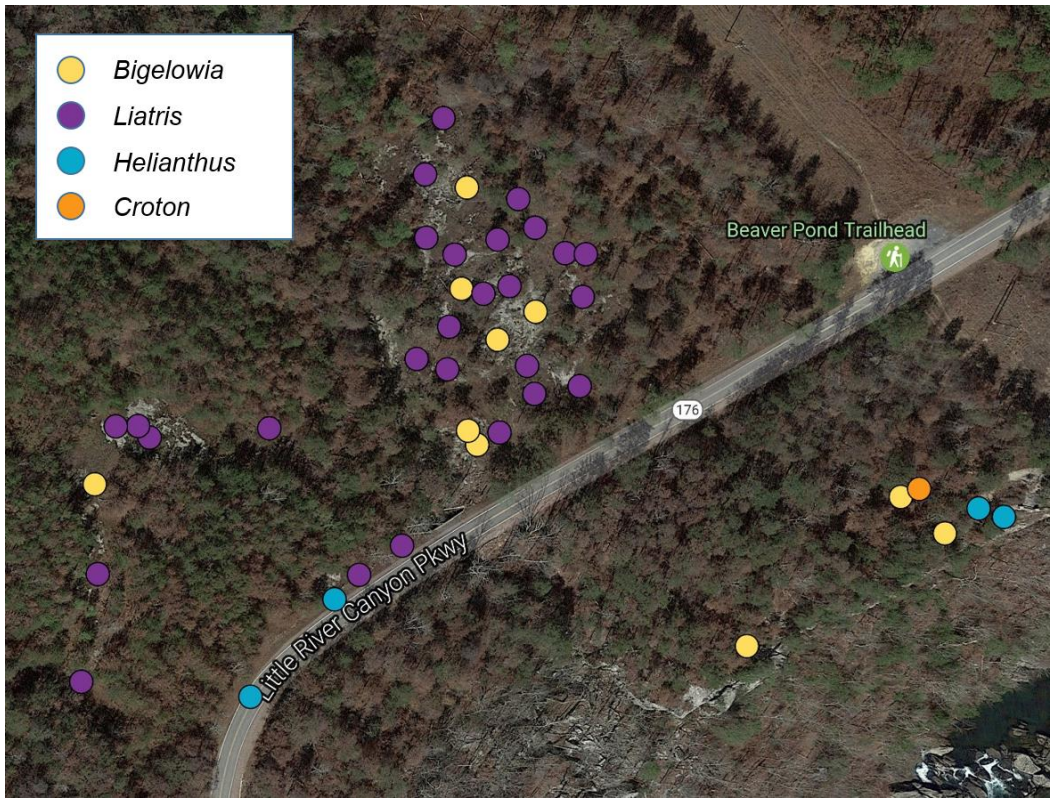


Fig. 5.1. Map of host use at Lynn Overlook (LRC-LO). Different colored markers indicate host species *C. harperi* individuals were parasitizing at the time of collection in August 2015.

METHODS

For our initial census, we marked 65 total host plants with nascent parasite attachment and growth located within four relatively small sample areas where high parasite density was observed. Thirty-one *Liatris*, thirty *Bigelowia*, and four *Coreopsis* were marked at the base of their stems with flagging tape, and GPS coordinates were recorded for each of the four sample areas (Fig. 5.2). Study sites were selected to be out of view from the road in order to avoid drawing public attention to both the research project and to the sensitive habitat itself.

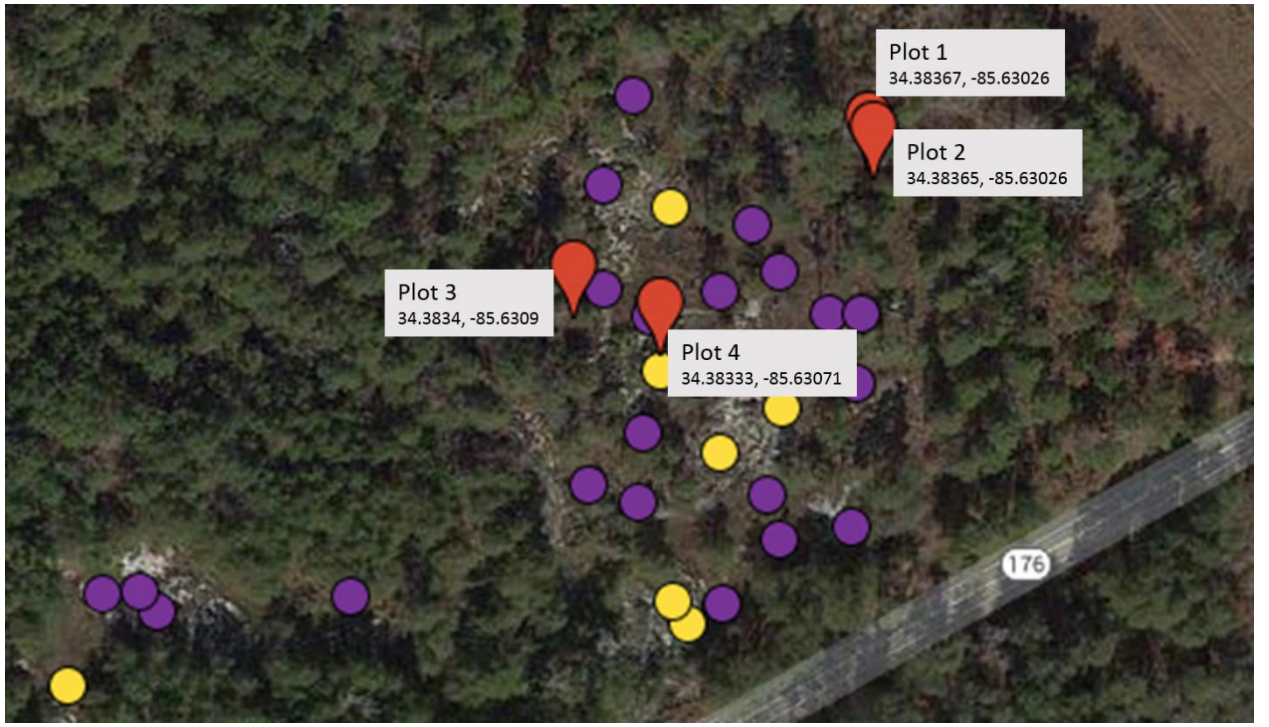


Fig. 5.2. Red markers indicate locations where hosts were marked on 11 May 2016 for survivorship study.

In September 2016, a follow-up census was made to assess survivorship of marked individuals. In order to be counted as a survivor, individuals were required to be present and flowering and/or fruiting at the time of census.

RESULTS

Twenty-one out of thirty-one marked individuals whose initial attachments were made on *Liatris* and seven out of thirty marked individuals whose initial attachments were made on *Bigelowia* were present and flowering at the time of the census. Two of the four individuals marked on *Coreopsis* were also present and flowering; however, these individuals were not included in the analysis.

Chi-square contingency analysis was performed in order to address the question of whether *C. harperi* individuals are more likely to survive to reproductive stage when initial haustorial attachment at the seedling stage is on *Bigelowia* or *Liatris*. Analysis revealed a significantly higher rate of survival to reproduction by individuals initially parasitizing *Liatris* as compared to those initially parasitizing *Bigelowia* ($p=0.0005$).

Because *C. harperi* typically produces a single seed per flower, we counted the number of flowers and/or seed capsules produced by each successful individual as a direct measure of reproductive fitness. A two-sample t-test with assumed unequal variance was performed to address whether there was a difference in reproductive fitness between flowering individuals using *Bigelowia* versus *Liatris* as initial hosts. The difference in fitness of individuals starting on each host, as measured by mean number of flowers/capsules produced, was not statistically significant ($p=0.276$).

DISCUSSION

The population of *C. harperi* at Lynn Overlook has one of the widest host ranges of all observed populations and can be considered to exhibit a relatively generalist pattern of host use. However, observational data has revealed a noticeable trend toward parasitization of *Liatris microcephala* over *Bigelowia nuttallii* and other potential host species in this population by mature plants in late summer. Results of this study indicate that seedlings from the Lynn Overlook population have a significantly higher chance of surviving to reproductive maturity when seedlings establish initial attachment on *Liatris* than when initial attachment is made on *Bigelowia*; this evidence supports the hypothesis that differential establishment success after initial host attachment influences observed patterns of host use by mature *C. harperi* individuals in this population.

Interestingly, results also indicate that once a seedling formed a successful parasitic attachment and was able to reach reproductive status, fitness did not significantly differ between the two primary host species. While the two individuals found flowering on *Coreopsis* were not included in the analysis, they do provide evidence that it is possible for *C. harperi* to survive to maturity on that species. We hypothesize that there are underlying environmental or genetic factors in this population that make *Liatris* an easier host for *C. harperi* to successfully parasitize; differences in host defenses, nutrient or water availability, or some combination of these factors may be contributing to observed patterns of parasite survival to maturity.

Further investigation is clearly needed to elucidate the factors that contribute to host use patterns observed among *C. harperi* populations. In May 2017, thirty-three host stems with nascent parasite attachments were marked for observation. A follow-up census will be performed in September 2017.

CHAPTER SIX: *Cuscuta harperi Ex Situ* Conservation Project

INTRODUCTION

Preservation of biodiversity and the protection of rare and endangered species are some of the foremost goals in conservation biology; development of effective strategies to achieve these goals is a major challenge facing individuals and organizations working in this field. Available funding for conservation projects is limited, and deciding how best to allocate resources can also be a formidable task.

For preservation of species and ecosystems, in situ conservation strategies have long been the standard. Protecting plants and animals within their natural habitats, as well as the habitats themselves, is the overarching goal of conservation biology. However, management of natural habitats and ecosystems is not achievable in some instances due to circumstances beyond general lack of resources, such as uncooperativeness of land owners, local governing bodies, or native citizens. *Ex situ* strategies are often employed to protect individuals of threatened species and to preserve genetic diversity when *in situ* conservation is not practical. Captive breeding programs, seed banking, and germplasm tissue collections are some popular examples of *ex situ* conservation strategies.

Both *in situ* and *ex situ* strategies present specific sets of challenges. *In situ* conservation requires a massive amount of planning, organization, and resource allocation. Specific issues that must be addressed by *in situ* project development teams include determining breadth of focus, from the ecosystem level to the molecular level, as well as consideration of size and surroundings of the protected area and potential impact of biotic and abiotic factors (Wilcox 1984.) *Ex situ* conservation also requires careful allocation of resources as well as cooperation between entities involved in collection, storage, care, and breeding of protected species. *Ex situ* conservation of plants has also traditionally been focused on maintaining genetic diversity of agriculturally valuable species, although more attention has been shifted to forestry applications and the conservation of wild and native flora (Cohen et al. 1991).

Occurring in only four populations- all on private property- in two widely disjunct counties in Georgia, *Cuscuta harperi* is assigned a legal status of endangered in the state. The species is also assigned a global ranking of G2/G3 (NatureServe Explorer 2015), indicating its vulnerability to extinction due to small population sizes and habitat destruction. Here, we describe the development, implementation, and preliminary results of a project designed in collaboration with The Nature Conservancy that combines *ex situ* and *in situ* strategies for the conservation of *C. harperi*. The project objectives include introduction of individuals into the habitat, careful monitoring of introduced individuals for survival and reproductive success, and establishment of a novel population for long-term management and continued research on the species.

Project Site

Camp Meeting Rock, 52 miles southwest of Atlanta in Heard County, Georgia, is home to Flat Rock Methodist Campground, an important local historic site where religious camp meetings were held every summer beginning around 1878. The campground itself is public property owned by the City of Franklin and managed by a board of trustees; however, the 130 acres of adjacent granite outcrop is owned and protected by The Nature Conservancy. Camp Meeting Rock is home to many rare and endangered species, including *Isoetes melanospora* (Black-spored quillwort) and *Pinus palustris* (Longleaf pine), and The Nature Conservancy is working to protect the outcrop habitat from anthropogenic disturbances associated with forestry practices and quarrying (The Nature Conservancy 2017).

In addition to preservation of the habitat, The Nature Conservancy has implemented a program of prescribed burning at the preserve in order to restore a more natural disturbance regime. The region surrounding the xeric habitat of the outcrop would historically have been dominated by longleaf pine forest and pine/oak forest, both of which would have been dependent on frequent fire. Many of the rare species associated with this type of habitat would have thrived in the shallow soil ecotone between the outcrop and the forest, which becomes too dense and shady with the overgrowth of secondary successional understory species in the absence of fire. There is substantial evidence that prescribed burning of habitats that are adapted to frequent, low-intensity

surface fires can maintain biodiversity and protect natural resources (Pausas and Keeley 2009). By reintroducing fire to the ecosystem, a more natural outcrop, ecotone, and pyric forest community can be established.

Camp Meeting Rock houses the ideal habitat for *Cuscuta harperi*, including vegetative sandy soil patches with abundant host plants of *Liatris microcephala* and *Hypericum gentianoides*. Although no *C. harperi* individuals have been documented at Camp Meeting Rock, the property lies in between two of the recorded populations in Heard County; it is located one mile southwest of Allen/Aubrey Flatrock and four miles northeast of South Texas Flatrock. Given the location, habitat, and host availability, it is not only possible, but likely that the species would have occurred historically on and around Camp Meeting Rock. These factors, coupled with long-term protection of the property and prescribed burning by The Nature Conservancy, make Camp Meeting Rock an ideal location for introduction of *C. harperi* for long-term study and conservation.

METHODS

Plant Collection and Propagation

Preserving the genetics of target species is one of the major objectives of most conservation programs. For this project, we used seeds from individuals in the Allen/Aubrey Flatrock (AA) population, the closest naturally occurring population to Camp Meeting Rock (CMR). *C. harperi* seeds were collected from AA individuals during field work on 28 October 2015 and maintained in dry storage.

In order to germinate seedlings of *Cuscuta harperi*, the seeds were placed in Gooch crucibles for scarification with concentrated sulfuric acid for 30 minutes, rinsed with deionized water, soaked in 10% bleach solution for 2 minutes, and thoroughly rinsed again with deionized water to slough off excess dead chaff from the outer seed coat. The sterile, scarified seeds were placed on damp filter paper in petri dishes which were sealed with wax film strips until germination to prevent fungal contamination. Once the seedlings had grown to approximately 1-2 cm in length, they were transferred to

microcentrifuge tubes with the swollen basal anchor end of the seedling stem immersed in deionized water and the growing tip of the seedling extending out from the mouth of the tube. Seedlings were placed in proximity to host plants in the greenhouse and monitored for survival, host attachment, and post-establishment success.

Liatris microcephala was selected as the initial host species for this project. *Liatris* is abundant on the CMR outcrop and, unlike *Hypericum*, is perennial. In order to avoid introduction of genetic material from outside the preserve, *Liatris* individuals were collected from CMR and planted in 4-inch plastic pots in the Joyce and Ira Pegues Memorial Greenhouse at Kennesaw State University. Plants were maintained for several weeks under standard greenhouse conditions, including a regular watering and fertilizer regimen, in order to alleviate any deleterious effects of transplantation prior to the introduction of parasites.

Approximately twenty *C. harperi* seedlings were placed in proximity to *Liatris* hosts in the greenhouse beginning on 20 July 2016. On 22 Aug 2016, eight individuals were selected based on viability criteria, including size, number of stem attachments to host, and observed robustness; both host and parasite were subsequently transported to CMR for transplantation.

Site Selection and Outplanting

On 12 July 2016 we scouted CMR for vegetative patches to serve as potential locations to introduce transplants. Experimental plots were chosen based on abundance of available host species, sparseness of competitive vegetation, and observed patterns of water flow and retention. In collaboration with a Nature Conservancy Ecologist, experimental outplanting plots were designated as Unburned (UB) if plots fell outside of the controlled burn area, Burned (B!) if the plots were located inside the burn area, and Questionable (B?) if the plots fell outside of the burn area but could be included in future burns if needed. Each Plant ID refers to an individual of *C. harperi* growing on a single *Liatris* individual.

Hosts and attached *C. harperi* individuals were planted in experimental plots on 22 August 2016. GPS points were obtained and mapped for all outplanted individuals (Fig. 6.1).

Fig. 6.1. Map of outplanted *C. harperi* individuals. UB indicates plot is outside the prescribed burn area; B! indicates plot is within the burn area; B? indicates plot could potentially be included in future burns.

RESULTS AND DISCUSSION

Plants were initially monitored for survival and reproductive fitness. The first assessment visit was made on 19 Sep 2016. Living *C. harperi* tissue was located for seven of the initial eight individuals, although three of the initial *Liatris* hosts did not survive. Six of the seven surviving *C. harperi* individuals were producing flower buds, and some had open flowers.

We performed a second assessment on 15 Nov 2016 in order to check for successful seed set by individuals found flowering at the first assessment. We located and counted

seed capsules on individuals in four plots, totaling approximately 1335 capsules. [For complete flowering and seed set data, see field notes, Appendix 3].

In order to assess initial success of the project, we compared flowering and seed set data from individuals in the newly established CMR population with similar data from individuals in the Little River Canyon-Lynn Overlook (LRC-LO) population, where the greatest density of *C. harperi* individuals occurs and where reproductive success has been previously quantified (See Chapter 5). Since all individuals at CMR were outplanted on *Liatris* as the initial host, we only compare flower and seed set data from LRC-LO individuals that established initial host connection on *Liatris*. Individuals from LRC-LO were collected from the field at the flowering stage; because each *C. harperi* flower generally produces a single seed, we used flower count as a proxy for reproductive success in this population, assuming that each flower represented one seed that would have been produced by each individual. For the CMR population, we assessed flower/seed data collected at the end of the season (15Nov2016). For individuals with seed capsules present, we counted number of seeds as reproductive success; if no seed capsules were present, number of flowers or buds was counted as a proxy for reproductive success.

In the LRC-LO sample (n=31), 10 individuals (32.2%) did not survive to reproduction, 6 individuals (19.4%) achieved low reproductive success, and 15 individuals (48.4%) achieved high reproductive success. Mean number of flowers was 234.3 with a range from 6 to 803. In the CMR population, 2 individuals (25.0%) did not survive to reproduction, 3 individuals (37.5%) achieved low reproductive success, and 3 (37.5%) achieved high reproductive success. Mean number of flowers/seeds was 225.5 with a range from 10 to 639. Statistical analyses of these data are not informative due to low sample size and high variance; however, comparison of data from these two populations does allow us to make inferences about the success of the CMR population. The distribution of individuals in the novel CMR population into categories of no, low, or high reproductive success is comparable to the categorical distribution in the LRC-LO sample (Fig. 6.2), taken from the most successful of all thirteen populations visited during this thesis project.

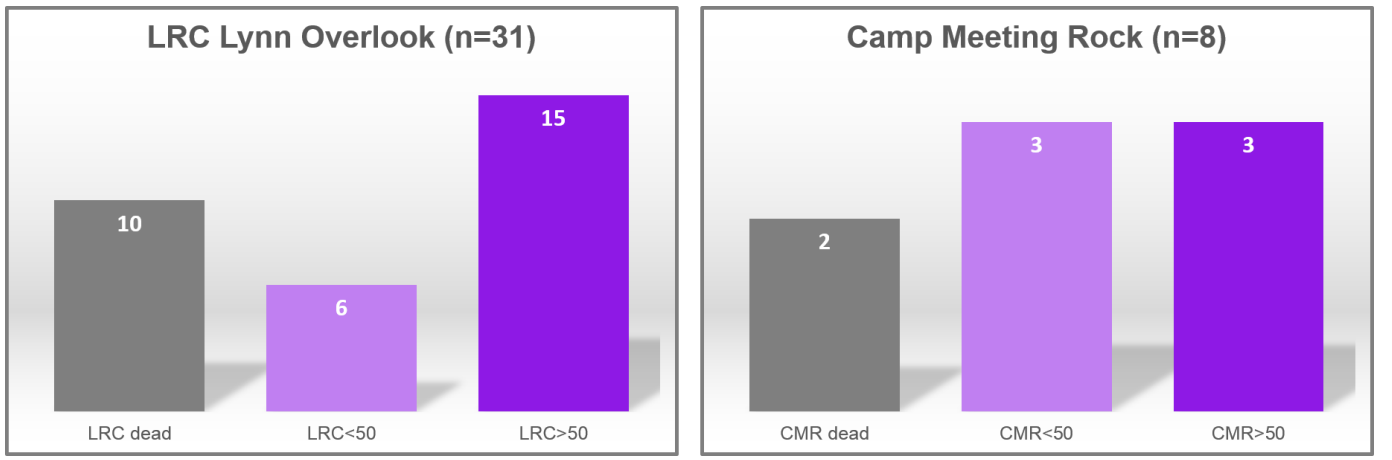


Fig. 6.2 Comparing Distributions of Reproductive Success – Little River Canyon-Lynn Overlook and Camp Meeting Rock**.** Less than 50 flowers/seed capsules is considered Low Reproductive Success; greater than 50 flowers/seed capsules is considered High Reproductive Success.

We surveyed the CMR sites on 20 May 2017 to assess the status of experimental plots after the first winter. At the site of CMR01UB, healthy *C. harperi* stems were located growing on two separate *Liatris* hosts in the plot, with approximately ten connection points. Since *C. harperi* is an annual, this individual is clearly the result of germination of seed from the first round of 2016 outplantings. This preliminary result is encouraging considering the level of scarification required to initiate germination of *C. harperi* seeds as well as the myriad factors that make it challenging for a seedling to locate and attach to a host.

Although new *C. harperi* was only found growing at one of the plots, there are now considerable seed banks present at the locations of the four individuals that produced seeds in the first season, and those seeds should remain viable in the soil to potentially germinate in later years. It is also notable that *Liatris microcephala* within the plots appeared abundant and healthy, clearly benefitting from the reduction in competition from pine trees that did not survive drought conditions in late summer and fall 2016. With a solid seed bank and ample host plants, we anticipate continued germination and success of *C. harperi* at these experimental plots in the future.

The Nature Conservancy plans to burn the section of the property adjacent to our plots in spring 2018. We are in discussions with Nature Conservancy contacts to arrange for inclusion of some of the plots in the burn area. In May 2017 we observed significant build-up of organic plant material, which may present a barrier for newly germinated seedlings to reach host plants, covering the ground in many of the plots. It is likely that fire would clear the organic duff layer, revealing bare sandy soil and improving germination and successful host attachment by removing obstacles impeding the search of the tiny seedlings.

Additionally, we plan to use the same methods described here to introduce a new set of *C. harperi* individuals into experimental plots on the outcrop in August 2017. We will continue monitoring survival and reproductive success of outplanted individuals, as well as assessing the effects of prescribed fire on *C. harperi* and its host plants. Preliminary results presented here are encouraging, and we anticipate this project to continue well into the foreseeable future.

Appendix 1: Host and Parasite Population Data

This table provides information about block number, *C. harperi* parent populations, and host plant populations for each greenhouse trial replicate (chapter 4). *C. harperi* parent populations are abbreviated as follows: HO=Harrison Outcrop HR=Hinds Road. Host populations are abbreviated as follows: CVO=Canyon View Overlook HO=Harrison Outcrop HR=Hinds Road LO=Lynn Overlook ST=South Texas Flatrock TC=Town Creek Glade WCO=Wolf Creek Overlook.

All host plants were used for both trials unless designated with an asterisk (*), in which case the population listed is the source population of the replacement individual.

Trial One – Replicates 1-64					
Pot ID	Block #	Host Population			*C. harperi* Parent Population
		Bigelowia	*Coreopsis*	*Liatris*	
A1	7	HR	LO	LO	HO
B1	1	CVO	LO	LO	HR
C1	1	HO	LO	ST	HO
D1	5	HO	LO	ST	HR
E1	5	HO	LO	LO	HO
F1	4	HO	LO	LO	HO
G1	6	HO	LO	ST	HR
H1	2	HO	LO	LO	HO
I1	6	HO	LO	LO	HO
J1	3	HO	LO	LO	HR
K1	8	HO	LO	ST	HR
L1	2	HO	LO	LO	HR
M1	8	HO	LO	LO	HO
N1	3	TC	LO	LO	HO
O1	7	HO	LO	LO	HR
P1	4	WCO	LO	LO	HR
A2	3	HR	LO	LO	HO
B2	4	CVO	LO	LO	HO
C2	1	HO	LO	ST	HO
D2	6	HO	LO	ST	HO
E2	8	HO	LO	LO	HO
F2	1	HO	LO	ST	HR
G2	5	HO	LO	ST	HO
H2	7	HO	LO	LO	HO
I2	3	WCO	LO	LO	HR
J2	7	HO	LO	LO	HR
K2	5	HR	LO	ST	HR
L2	6	HO	LO	LO	HR
M2	8	HO	LO	LO	HR
N2	4	TC	LO	LO	HR
O2	2	HO	LO	LO	HO
P2	2	WCO	LO	LO	HR
A3	1	HR	LO	LO	HR

B3	3	CVO	LO	LO	HR
C3	8	HO	LO	ST	HO
D3	1	HO	LO	ST	HO
E3	7	HO	LO	LO	HR
F3	4	HO	LO	ST	HO
G3	4	HO	LO	LO	HR
H3	5	HO	LO	LO	HO
I3	3	WCO	LO	LO	HO
J3	2	HO	LO	LO	HR
K3	8	HR	LO	ST	HR
L3	6	HO	LO	LO	HO
M3	5	HO	LO	LO	HR
N3	7	TC	LO	LO	HO
O3	6	WCO	LO	LO	HR
P3	2	HO	LO	LO	HO
A4	4	HR	LO	LO	HR
B4	1	CVO	LO	LO	HR
C4	8	HO	LO	ST	HO
D4	1	HO	LO	LO	HO
E4	5	HO	LO	LO	HO
F4	4	WCO	LO	ST	HO
G4	8	HO	LO	LO	HR
H4	6	HO	LO	LO	HO
I4	2	WCO	LO	LO	HO
J4	7	HO	LO	LO	HR
K4	7	HO	LO	ST	HO
L4	3	HO	LO	LO	HO
M4	5	HO	LO	LO	HR
N4	6	HO	LO	LO	HR
O4	3	HO	LO	ST	HR
P4	2	HR	LO	LO	HR

Trial Two – Replicates 65-128					
Pot ID	Block #	Host Population			*C. harperi* Parent Population
		Bigelowia	*Coreopsis*	*Liatris*	
A1	7	HR	LO	LO	HR
B1	1	CVO	LO	LO	HO
C1	1	HO	LO	ST	HR
D1	5	HO	LO	ST	HO
E1	5	HO	LO	LO	HR
F1	4	HO	LO	LO	HR
G1	6	HO	LO	ST	HO
H1	2	HO	LO*	LO	HR
I1	6	HO	LO	LO	HR
J1	3	HO	LO	LO	HO

K1	8	HO	LO	ST	HO
L1	2	HO	LO*	LO*	HO
M1	8	HO	LO	LO*	HR
N1	3	TC	LO	LO	HR
O1	7	HO	LO	LO	HO
P1	4	WCO	LO	LO	HO
A2	3	HR	LO	LO	HR
B2	4	CVO	LO	LO	HR
C2	1	HO	LO	ST	HR
D2	6	HO	LO	ST	HR
E2	8	HO	LO	LO*	HR
F2	1	HO	LO*	ST	HO
G2	5	HO	LO	ST	HR
H2	7	HO	LO	LO	HR
I2	3	WCO	LO	LO	HO
J2	7	HO	LO	LO	HO
K2	5	HR	LO	ST	HO
L2	6	HO	LO*	LO*	HO
M2	8	HO*	LO	LO	HO
N2	4	TC	LO	LO	HO
O2	2	HO	LO	LO	HR
P2	2	WCO	LO	LO	HO
A3	1	HR	LO	LO	HO
B3	3	CVO	LO	LO	HO
C3	8	HO	LO	ST	HR
D3	1	HO	LO*	LRC*	HR
E3	7	HO	LO*	LO*	HO
F3	4	HO	LO	ST	HR
G3	4	HO	LO	LO	HO
H3	5	HO	LO*	LO*	HR
I3	3	WCO	LO	LO	HR
J3	2	HO	LO	LO	HO
K3	8	HR	LO	ST	HO
L3	6	HO	LO	LO	HR
M3	5	HO	LO	LO	HO
N3	7	TC	LO	LO	HR
O3	6	WCO	LO	LO*	HO
P3	2	HO	LO*	LO	HR
A4	4	HR	LO	LO	HO
B4	1	CVO	LO	LO	HO
C4	8	HO	LO	ST	HR
D4	1	HO	LO*	LO	HR
E4	5	HO	LO	LO	HR
F4	4	WCO	LO	ST	HR
G4	8	HO	LO	LO	HO
H4	6	HO	LO	LO	HR
I4	2	WCO	LO*	LO	HR
J4	7	HO	LO	LO	HO
K4	7	HO	LO	ST	HR

L4	3	HO	LO	LO*	HR
M4	5	HO	LO	LO*	HO
N4	6	HO	LO	LO	HO
O4	3	HO	LO	ST	HO
P4	2	HR	LO	LO	HO

Appendix 2: Greenhouse Temperature Data

Trial 1

Date		Low	High	Average
18 July 2016	Day	73°F	88°F	80.0°F
	Night	73°F	88°F	78.1°F
19 July 2016	Day	73°F	90°F	81.5°F
	Night	73°F	90°F	78.7°F
20 July 2016	Day	73°F	90°F	80.5°F
	Night	73°F	90°F	78.1°F
21 July 2016	Day	74°F	94°F	80.5°F
	Night	73°F	94°F	78.7°F
22 July 2016	Day	72°F	87°F	80.0°F
	Night	72°F	87°F	76.8°F
23 July 2016	Day	73°F	85°F	78.5°F
	Night	73°F	85°F	76.4°F
24 July 2016	Day	73°F	89°F	80.5°F
	Night	73°F	89°F	77.4°F
25 July 2016	Day	73°F	91°F	81.0°F
	Night	73°F	91°F	77.5°F
26 July 2016	Day	73°F	90°F	81.5°F
	Night	72°F	90°F	78.4°F
27 July 2016	Day	74°F	85°F	80.0°F
	Night	74°F	85°F	79.1°F
28 July 2016	Day	73°F	85°F	79.5°F
	Night	73°F	85°F	77.5°F

Trial 2

Date		Low	High	Average
11 Aug 2016	Day	73°F	85°F	79.0°F
	Night	73°F	85°F	77.3°F
12 Aug 2016	Day	74°F	86°F	80.0°F
	Night	74°F	86°F	79.3°F
13 Aug 2016	Day	74°F	86°F	80.5°F
	Night	73°F	86°F	78.3°F
14 Aug 2016	Day	73°F	86°F	80.5°F
	Night	73°F	86°F	77.3°F
15 Aug 2016	Day	73°F	86°F	80.0°F
	Night	73°F	86°F	77.9°F
16 Aug 2016	Day	73°F	86°F	80.0°F
	Night	73°F	86°F	77.5°F
17 Aug 2016	Day	73°F	87°F	81.5°F

	Night	72°F	87°F	78.3°F
18 Aug 2016	Day	73°F	88°F	81.0°F
	Night	73°F	88°F	78.0°F
19 Aug 2016	Day	73°F	86°F	79.5°F
	Night	72°F	86°F	76.4°F
20 Aug 2016	Day	73°F	86°F	79.5°F
	Night	72°F	86°F	76.1°F
21 Aug 2016	Day	72°F	85°F	79.0°F
	Night	72°F	85°F	76.0°F
22 Aug 2016	Day	71°F	84°F	79.0°F
	Night	71°F	84°F	75.8°F
23 Aug 2016	Day	70°F	85°F	79.0°F
	Night	70°F	85°F	75.9°F
24 Aug 2016	Day	73°F	84°F	79.0°F
	Night	73°F	84°F	77.0°F
25 Aug 2016	Day	72°F	86°F	80.0°F
	Night	72°F	87°F	77.2°F
26 Aug 2016	Day	74°F	87°F	80.5°F
	Night	73°F	87°F	78.5°F
27 Aug 2016	Day	72°F	85°F	80.0°F
	Night	72°F	85°F	77.4°F
28 Aug 2016	Day	73°F	85°F	80.0°F
	Night	73°F	85°F	78.9°F
29 Aug 2016	Day	73°F	85°F	79.5°F
	Night	72°F	85°F	78.0°F

Appendix 3: Camp Meeting Rock Field Notes

Plant ID	**Burn Status**	***C. harperi* parent population**	***Liatris* parent population**	**Initial Seedling Viability** (1=good; 2=very good; 3=excellent)	**Project Notes** (*Ch= Cuscuta harperi* *Lm= Liatris microcephala*)
CMR 01 UB	Not routinely burned	Allen/Aubrey	Camp Meeting Rock	2	22 Aug 2016: Outplanted and watered in. 19 Sep 2016: Healthy *Ch* and *Lm*; *Ch* spread to 3 adjacent *Lm*; flowering. 15 Nov 2016: 639 seed capsules 20 May 2017: 1 *Ch* seedling located! Approx. 10 connections on 2 *Lm*. *Lm* in patch is abundant and lush. *Lm* appears to be benefiting from lack of trees 2^{o} to drought.
CMR 02 UB	Not routinely burned	Allen/Aubrey	Camp Meeting Rock	1	22 Aug 2016: Outplanted and watered in. 19 Sep 2016: Initial *Lm* host dead; no *Ch* present. Evidence of animal digging.
CMR 03 UB	Not routinely burned	Allen/Aubrey	Camp Meeting Rock	2	22 Aug 2016: Outplanted and watered in. 19 Sep 2016: Initial *Lm* host dead; appears drought-killed. Minimal living *Ch* tissue on adjacent Lm. No flowers or buds. 15 Nov 2016: Approx. 10 buds present; no capsules. 20 May 2017: No new *Ch* seedlings located. *Lm* appears lush and healty.
CMR 04 UB	Not routinely burned	Allen/Aubrey	Camp Meeting Rock	1	22 Aug 2016: Outplanted and watered in. 19 Sep 2016: *Ch* present and healthy on initial *Lm* host and 10+ adjacent

					Lm. Abundant buds; few open flowers. 15 Nov 2016: Approx. 20-25 capsules. Very difficult to visualize due to drought-stricken *Lm* stems the same color as capsules and dried *Ch* tissue. 20 May 2017: No new *Ch* seedlings located.
CMR 05 B?	Potential to include in burn	Allen/Aubrey	Camp Meeting Rock	2	22 Aug 2016: Outplanted and watered in. 19 Sep 2016: Present and healthy on initial *Lm* host and 6-7 adjacent *Lm*; flowering. 15 Nov 2016: No capsules visible; no sign of flowers; knocked off by animal?? Very dry. 20 May 2017: No *Ch* seedlings located. *Lm* is healthy and lush; lots of leaf litter on the ground- could potentially hinder growth of new *Ch* seedlings. Including this patch in the next burn could be beneficial.
CMR 06 B!	Routinely included in burn	Allen/Aubrey	Camp Meeting Rock	3	22 Aug 2016: Outplanted and watered in. Placed in proximity to other *Lm* and also where water flow could disperse to another *Lm* dense area of habitat in same patch. 19 Sep 2016: *Ch* very healthy on initial *Lm* host; spread to 5-6 adjacent *Lm*; buds and open flowers abundant. 15 Nov 2016: 473 capsules; still numerous open flowers and buds. 20 May 2015: No *Ch* seedlings located. Monitor closely post-burn to evaluate effect on leaf litter and *Ch* germination.

CMR 07 UB	Not routinely burned	Allen/Aubrey	Camp Meeting Rock	3	22 Aug 2016: Outplanted and watered in. 19 Sep 2016: Initial *Lm* dead; appears drought-killed. Living *Ch* tissue on adjacent *Lm* and *Seymeria*. 10+ buds. 15 Nov 2016: No *Ch* tissue relocated. 20 May 2017: No *Ch* seedlings located.
CMR 08 UB	Not routinely burned	Allen/Aubrey	Camp Meeting Rock	2	22 Aug 2016: Outplanted and watered in. 19 Sep 2016: Healthy on initial *Lm*; spread to approx. 10 adjacent *Lm*. Flowers and buds abundant. 15 Nov 2016: 198 swollen capsules; lots of dry flowers that didn't set seed. 20 May 2017: No *Ch* seedlings located. Dense leaf litter could potentially hinder growth of *Ch* seedlings that germinate. Including this patch in the next burn could be beneficial for *Ch*.

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