BIOLOGICAL SCIENCES

CONSERVATION GENETICS OF THE ENDANGERED SUNFLOWER *HELIANTHUS VERTICILLATUS*

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Dissertation under the direction of Professor David E. McCauley

One of the greatest factors contributing to the worldwide decline in biodiversity is habitat destruction leading to the loss and fragmentation of populations of many species. Knowledge of the genetic and demographic factors that are affected by and influence rarity advances our understanding of the consequences of habitat degradation, and this knowledge is crucial for creating management plans for rare or endangered species. In this dissertation, I address these factors in a rare sunflower species, *Helianthus verticillatus*, through studies of its population genetics and taxonomic status, the assessment of population size, and the evaluation of fitness. A population genetic study, employing a novel genetic marker, demonstrated that this species is not the product of recent hybridization and, surprisingly, harbors high levels of genetic diversity despite its small number of populations and disjunct range. A study of the clonal diversity and structure in this species revealed far fewer numbers of individuals than were previously reported; these results led to the upgrading of the species' priority status for the Endangered Species Act. Finally, populations differed with respect to phenotypic fitness related traits; this was not predicted by population genetic data and further highlights the need for comprehensive studies of endangered species in order to fully evaluate the effects of rarity and fragmentation on population viability.

Approved: David E. McCauley

CONSERVATION GENETICS OF THE ENDANGERED SUNFLOWER

HELIANTHUS VERTICILLATUS

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

December, 2008

Nashville, Tennessee

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To my grandparents, Charles and Mary Lipscomb, not only for all the support and love they have given during this research, but for the many desserts and snacks as well.

ACKNOWLEDGEMENTS

It is quite remarkable how many people have contributed to this dissertation research, and I would like to say that this research would not at all have been possible without all of them. Many, many have provided technical knowledge, manuscript support, and assistance in the field, laboratory, and greenhouse. I would like to thank David Wills, Mark Welch, Mark Chapman, Vikram Chhatre, Eric Janson, and Scott Egan for all of the help with equipment, protocols, and general science questions. I appreciate Natasha Sherman for the many hot and buggy hours spent in the field. Jonathan Ertelt was extremely helpful over the years in the greenhouse. Christopher Brown also put in many field hours, created figures for talks and manuscripts, and took the best photos I have of the sunflower. Mark Mandel too trudged away in the field with me (recurring theme: get my friends to help) and proofread numerous versions of manuscripts and this dissertation. I would like to thank my committee: John Burke, Carol Baskauf, Terry Page, Patrick Abbot, Dan Funk, and formerly Manuel Leal for all of their input with writing and really teaching me how to do science. I would like to thank the people who have allowed me to go out into the field and shown me where the populations of the sunflower are: Temple Inland Cooperation, Sam Breyfogle, Jim Allison, Jim Matthews, Andrea Bishop, Claude Bailey, David Lincicome, and Silas Mathes. I would especially like to acknowledge my terrific advisor, David McCauley; he has been patient, laid-back, knowledgeable, and all around awesome. Finally, I would like to thank all of my friends and family, some which have already been mentioned, for all the love, support, and good times. Thanks!!!

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CHAPTER I

INTRODUTION

Conservation Biology and Genetics

The field of conservation biology is a relatively young scientific discipline whose goals are to examine, analyze, and protect the Earth's biodiversity. The field's beginnings were prompted by awareness in the 20th century of issues surrounding habitat loss and fragmentation, pollution, and escalating human population growth. During the first half of the century, conservation was mostly practiced by departments of natural resources and forestry (Primack 2002). In the 1970's, traditional biologists began to discuss the need for a conservation science (Takacs 1996). The First International Conference on Conservation Biology was held at the San Diego Wild Animal Park in 1978 where a variety of people interested in conservation came together to discuss the emerging field (Gibbons 1992). In 1981, Soulé and Wilcox edited a book, *Conservation biology: an evolutionary-ecological perspective*, which is regarded as one of the founding documents of the field. Subsequently, Soulé (1985) published an article entitled 'What is conservation biology?' where he discussed the goals of the field to conserve the Earth's biodiversity. The journal, *Conservation Biology*, introduced its inaugural issue in 1987 to provide an international voice for this growing discipline. The field of conservation biology has developed, over the past 25 years, into an interdisciplinary science drawing from the subjects of ecology, population genetics, evolution, and systematics. Studies of conservation biology often also incorporate a practical application through providing management strategies and suggestions to preserve biodiversity.

While the field's aims are quite broad, a main goal is to conserve endangered species through the maintenance of genetic diversity within species and the preservation of biological communities and habitats, i.e., conservation genetics (Primack 2002). Scientific studies of endangered species involve understanding ecological and evolutionary issues when populations are rare or isolated, as well as identifying populations of conservation concern through measuring population size, levels of genetic diversity and gene flow, and fitness of individuals. Population genetic and inbreeding theory connect the issues of population size, levels and partitioning of genetic diversity, and fitness together to describe and understand the evolutionary processes occurring in rare and endangered species (Figure I-1).

Human-induced habitat destruction has led to the loss and fragmentation of populations of many taxa, including over 6000 plant species, causing reductions in both the number of populations and the size of individual populations (Wilcove et al. 1998) (Figure I-1A). Genetic diversity declines in small populations since heterozygosity is an inverse function of the effective population size, and loss of alleles due to genetic drift is more pronounced in small populations (Lynch et al. 1995; Young et al. 1996; Hedrick 2000) (Figure I-1B). Moreover, small and isolated populations are subject to inbreeding because of reduced numbers of potential mates (Barrett and Kohn 1991; Ellstrand and Elam 1993). Inbreeding leads to a loss of genetic diversity through the redistribution of alleles from the heterozygous to the homozygous state, and often, inbred individuals have lower fitness than outbred individuals owing to inbreeding depression (Keller and Waller 2002; Edmands 2007) (Figure I-1C). This fitness reduction is often due to the expression of deleterious recessive alleles in homozygous inbred individuals (Charlesworth and Charlesworth 1987). The interconnection of these factors suggests that positive feedback loops

exacerbating one another may lead to the demise of a population or species—the so called 'extinction vortex' (Gilpin and Soulé 1986; Lynch et al. 1995).

Conservation geneticists are interested in knowing if there are generalities that can be made with regard to rare species, such as whether or not reduced genetic diversity or restricted gene flow between populations are commonly associated with rarity and whether these aspects can predict population viability. Therefore, we often study various aspects of the feedback loop in an effort to understand why and how a population is declining. Many studies evaluate the effects of rarity and isolation on the genetic diversity of species by quantifying levels of genetic variation within and among populations with genetic markers, e.g., Song and Mitchell-Olds (2007); Neel (2008). Population surveys and estimates of effective or genetic population size are also conducted to aid in identifying populations of immediate concern, especially since population size and fitness are often related, e.g, Newman and Pilson (1997), Winter et al. (2008). Others studies investigate the consequences of habitat loss and fragmentation on endangered species by assessing fitness related phenotypic characteristics in populations through crossing studies either in the field or in controlled environments, e.g., Heiser and Shaw (2006); Bossuyt (2007). These different types of inquiries are often conducted independently due to time and monetary restrictions. However, when conducted in concert, they provide a more complete picture of how rarity and isolation negatively affect population viability, long term evolutionary potential, and how management efforts and resources are best focused.

Population Genetics

Levels of genetic diversity, both within and among populations, are of great interest to conservation geneticists since theoretical and experimental examinations have emphasized the importance of possessing genetic variation in order for populations to adapt to changing

environments (reviewed in Willi et al. 2006). In general terms, there is a trend for rare species to exhibit reduced genetic diversity. Hamrick and Godt (1989) reported limited genetic diversity at allozyme loci as a consequence of rarity in many plant species. In a large literature survey, they found that at the species level, geographic range (endemic, narrow, regional, or widespread) was a good predictor of levels of genetic diversity with endemic species having the lowest and widespread taxa possessing the highest genetic diversity. At the population level, widespread species also had greater genetic diversity than more geographically limited species.

Hamrick and Godt's (1989) study is often used as a compendium of allozyme genetic diversity in plants; such a compendium for dominant DNA markers and anonymous microsatellites exists as well (Nybom 2004). However, a limitation of these compendiums is that they do not account for phylogenetic relatedness. Felsenstein (1985) and Silvertown and Dodd (1996) suggest that comparisons to common congeners may minimize the confounding effects of phylogeny. Therefore, in order to determine if a rare species does indeed exhibit low genetic diversity, it is advantageous to compare genetic diversity measures in the rare species to a more widespread, common congener. Gitzendanner and Soltis (2000) reviewed congeneric species comparisons to determine if geographic distribution was related to genetic diversity measures when controlling for phylogenic effects. They found that, overall, rare plants had lower levels of genetic diversity when compared to their more widespread congeners. However, some rare species exhibit equivalent, and oftentimes, higher levels of diversity when compared to their common congeners.

In the genus, *Agastache*, Vogelmann and Gastony (1987) found that measures of genetic variation for narrowly endemic species were higher than their geographically widespread congeners. Ranker (1994) found that the only known population of a rare *Adenophorus periens*

fern demonstrated high levels of allozyme genetic variability when compared to two widespread congeners. This high level of variability was attributed to its outcrossing mating system and perennial life cycle. In another narrowly endemic fern, *Polystichum otomasui*, genetic variability at 13 allozyme loci was unexpectedly high given its limited distribution in only a few valleys (Maki and Asada 1998). These authors also found that the sites consisted of a relatively large number of individuals. They posited that recent decline in the species, the large effective population size, and outcrossing mating system have contributed to the high levels of genetic diversity. The buffering effects of large population sizes and long generation times on genetic diversity have also been seen in animals (Kuo and Janzen 2004; Lippe et al. 2006). These studies suggest that life history traits and population size may play a major role in determining the amount of genetic variation at the population and species level.

The partitioning of genetic variation between and within populations is also important when considering a conservation strategy for an endangered species. Knowledge of population genetic structure can give insight into population connectivity and identify populations of reduced or unique genetic diversity. This is particularly important if not all populations can be protected. In some rare species, the loss of a single population may have little impact on the species-wide genetic diversity, whereas in another this might significantly reduce total genetic variation and have implications for the survival of the species.

The organization of genetic diversity, or population structure, can be assessed by measuring the proportion of total genetic variation that resides among populations, i.e. F_{ST} (Wright 1951), θ (Weir and Cockerham 1984) or G_{ST} (Nei 1973). Somewhat surprisingly, geographic range does not tend to influence measures of population structure at nuclear loci. Hamrick and Godt (1989) found no significant differences in allozyme measures of G_{ST} between

endemic, narrow, regional, or widespread taxa. Similarly, Gitzendanner and Soltis (2000) observed no differences in how nuclear genetic variation is partitioned within and among populations in their comparisons of rare and widespread plant congeners. However, for maternally inherited DNA markers, Duminil et al. (2007) found that narrowly restricted taxa had higher population structure (higher values of G_{ST}) than those regionally distributed. Other factors that appear to be most influential in determining population structure are mating system, for nuclear markers, and mode of seed dispersal, for maternally inherited markers (Duminil et al. 2007).

Levels and patterns of genetic diversity may also be shaped by natural hybridization, in which hybrids are likely to exhibit high levels of genetic diversity resulting from the mixing of parental genomes (Arnold 1997; Rieseberg and Wendel 1993). Thus, when a history of hybridization is suspected, comparisons made with congeners may not reveal the true loss of genetic variation. For example, if a rare species is of hybrid origin but subsequently loses genetic diversity, the rare hybrid could still maintain higher variation than a common congener, for some time. Thus, for rare or endangered species in which hybrid origin has been suggested, it is important to test for hybridity. In addition, introgression, the permanent incorporation of alleles from one species into another, can increase genetic diversity in a rare species (Arnold 1997). A further complicating issue with regard to hybridization and endangered species is that hybrid ancestry can affect the protection status of an endangered species. The listing of hybrids for the US Endangered Species Act has historically been a difficult undertaking (Allendorf et al. 2001). Hybrid ancestry is most reliably established with molecular data—biparentally and/or uniparentally inherited makers (Rieseberg and Ellstrand 1993), and conservation studies that use appropriate genetic markers may help to characterize genetic relationships between taxa when

instances of hybridization and introgression are in question (e.g. Bruneau et al. 2005). Thus, studies of genetic diversity and its organization should employ both nuclear (biparental) markers and uniparentally inherited DNA markers.

Organellar genomes are largely uniparentally inherited (Sears 1980; Corriveau and Coleman 1988; Zhang et al. 2003). The traditional rule for organellar genomes, mitochondrial and chloroplast, in angiosperms is maternal transmission (paternal transmission in conifers) (Petit et al. 2005). As such, organellar DNA markers are employed in a variety of evolutionary applications including studies of hybridization (Rieseberg and Ellstrand 1993), phylogeography (Dobes et al. 2004), seed dispersal (Petit et al. 2005), and population genetics (McCauley et al. 2003). Biologists typically accept this rule and rarely test this assumption. However, occasional paternal and/or biparental inheritance of organellar DNA in angiosperms has been documented (Hansen et al. 2007; McCauley et al. 2005; 2007). Therefore, in population genetic studies utilizing organellar DNA, the mode of inheritance should be verified since incorrect conclusions may be drawn if the organellar DNA is not strictly maternal.

Population Size

As mentioned previously, population size surveys and inventories are quite useful for identifying populations of immediate conservation concern, especially since these endeavors are relatively inexpensive (Primack 2002). However, this undertaking may be complicated if a species exhibits some level of asexual, or clonal reproduction, as do many plant species (Cook 1983; Sipes and Wolf 1997; Esselman et al. 1999; Brzosko et al. 2002). Plants are able display clonal growth, in part, due to their construction: almost all plants grow through sequential reiteration of a basic module or structural unit (Harper 1981). This modular form of growth means that active meristem can always be available to reproduce these structural units; this type

of reproduction is vegetative since it does not involve meiosis and fertilization. Clonal growth is achieved through a variety of forms including the creation of bulbils or plantlets, stolons, and rhizomes (Moore et al. 1998). A clone is biologically defined by two terms: the genet and the ramet. A genet, or genetic individual, consists of all of the genetically identical members that derive from a single zygote (Sarukhan and Harper 1973). A ramet is an independent physiological individual consisting of its own shoot and root system and which is capable of independent survival and death (Cook 1983).

Determining the extent of clonality, including the spatial structure and clonal diversity in a population, may be achieved through excavating the root system. However this method is not only extremely intrusive, it may incorrectly estimate the true number of individuals if root systems have degenerated or if ramets of the same genet have been disassociated. Therefore, the use of polymorphic genetic markers to distinguish individuals is advantageous given that it is a non-invasive sampling strategy yielding a high probability of distinguishing genets when sufficient markers are surveyed (Ainsworth et al. 2003).

Populations of endangered species often have low numbers of individuals because of habitat reduction, but clonal growth may give the appearance of a large population even if there are far fewer genetic individuals. For example, a study of the rare Bartley's reed bent grass (*Calamagrostis porteri* ssp. *insperata*) using allozymes, Random Amplified Polymorphic DNA (RAPD), and Intersimple Sequence Repeat (ISSR) markers revealed low numbers of genetic individuals indicating extensive clonal reproduction in four populations of this species (Esselman et al. 1999). Another investigation, using both Simple Sequence Repeat (SSR) and RAPD markers, showed that 170 apparently individual trees of the endangered *Elaeocarpus*

williamsianus across seven sites actually represented the same genetic individual (Rossetto et al. 2004)!

In addition to generating estimates of genetic population size, clonal studies aid in understanding the ecological and spatial dynamics of related individuals (Murawski and Hamrick 1990) and how pollinator movement influences gene flow in insect pollinated species (Cook 1983). Identifying the clonal diversity and structure of a plant population is important to understanding population dynamics since the genetic individual is likely the unit of selection (Harper 1985; Eriksson and Jerling 1990). Brzosko et al. (2002) were able to determine the clonal structure and diversity in three populations of a rare and endangered lady's slipper (*Cypripedium calceolus*) using five polymorphic allozymes. They found that clonal reproduction had a significant impact on the genetic structure and diversity in these populations. Spatial structure and clonal diversity are also quite influential in species which exhibit a self-incompatible (SI) mating system since relatedness at SI loci will reduce the number of potential mates (Eriksson and Jerling 1990). Therefore the identification of clonal structure and diversity using genetic markers gives insight into ecological and evolutionary processes in plant populations exhibiting such features.

Population Fitness

Small and isolated populations of endangered species may be subject to inbreeding because of reduced opportunities for mating. Consequently, such inbreeding may lead to a decrease in fitness relative to outcrossed individuals (inbreeding depression) due to decreased heterozygosity or expression of deleterious recessive alleles. Inbreeding depression or lowered population fitness may increase extinction risk (Newman and Pilson 1997; Saccheri et al. 1998; Wright et al. 2008). In addition, inbreeding depression is usually more severe in outcrossing

species. Husband and Schemske (1996) reviewed inbreeding depression in fifty-four plant taxa and found that predominately outcrossing species exhibited higher measures of inbreeding depression than those of predominately selfing species. Additionally, the deleterious effects of inbreeding depression are often more pronounced in stressful versus benign environments (Armbruster and Reed 2005). While small size alone is detrimental to population fitness, even relatively large populations separated by great distances may suffer negative fitness consequences since extreme isolation can inhibit gene flow from mediating the deleterious effects of inbreeding due to genetic drift (Keller and Waller 2004) and hinder the spread of advantageous mutations across populations (Rieseberg and Burke 2001). Isolated populations may also experience differentiation with regard to phenotypic fitness characters; these differences in quantitative traits are important for determining populations of immediate conservation concern or which populations would serve as appropriate sources for *ex situ* conservation reserves.

Many conservation studies have accordingly investigated the fitness effects of crossing within and among populations to understand how rarity and isolation affect population viability (reviewed in Keller and Waller 2002). These studies often address the influence of population size and geographic proximity of populations on fitness. Frequently, studies find that there is increased fitness, or heterosis, in the F_1 generation when gene flow is from a large to small population or in crosses between populations separated by large distances indicating inbreeding depression in those populations. Heterosis is thought to occur if inbred populations are fixed for different sets of deleterious recessive alleles that are masked in the F_1 individuals (dominance) or if the F_1 individuals exhibit higher levels of heterozygosity and thus higher fitness (overdominance). For this reason, a possible conservation strategy for increasing the likelihood

of survival in rare species is to introduce new genetic material into populations suffering from inbreeding depression, i.e., genetic rescue (Ingvarsson 2001; Tallmon et al. 2004).

In the rare perennial, *Scabiosa columbaria*, van Treuren et al. (1993) found that crosses among populations enhanced fitness as compared to within population crosses. Self and intra-site crosses had significantly lower values for seed mass, germination rates, and survivorship rates than inter-site crosses of the extremely rare yellow pitcher plant, *Sarracenia flava* (Sheridan and Karowe 2000). In experimental crosses of the weedy perennial, *Silene alba*, Richards (2000) demonstrated that crosses among isolated sites restored germination rates, highlighting the importance of population connectivity and suggesting possible conservation strategies for rare and endangered species. Finally, Newman and Tallmon (2001) found evidence for the beneficial fitness effects of gene flow into experimentally fragmented populations of *Brassica campestris*. In this study, they simulated gene flow using different numbers of migrants into populations over five generations. In a sixth generation, they planted individuals in a common garden and evaluated individual fitness in the different treatments. Populations with a higher number of migrants per generation fared better than those with a lower number of migrants, providing evidence for the beneficial effects of gene flow.

The immigration of genetically divergent individuals into a population can also lead to a decrease in fitness owing to the dilution of local adaptations or the disruption of favorable gene combinations. This decrease in fitness is termed outbreeding depression (Templeton 1986; Waser and Price 1989; Lynch and Walsh 1998). Waser and Price (1994) found evidence for outbreeding depression in the larkspur, *Delphinium nelsonii*, where progeny from intermediate crossing distances grew larger and survived longer than plants from more distant crosses. Similarly, Fenster and Galloway (2000) found evidence for reduced fitness in inter-population crosses of

the legume, *Chamaecrista fasciculata*, when compared to the parental fitness, indicating outbreeding depression. The negative fitness effects of outbreeding depression, however, may not be manifested until the F_2 generation and beyond. The disruption of positive epistatic interactions among parental alleles will not occur until the F_2 generation (and beyond) when recombination proceeds to break up co-adapted gene complexes (Lynch 1991; Tallmon et al. 2004). Edmands (2007) reviewed evidence for inbreeding and outbreeding depression across a wide variety of taxa and found ample evidence for inbreeding depression. However, evidence for outbreeding depression was much less common in the literature; Edmands (2007) asserts that the experimental design of many studies does not provide for revealing outbreeding depression since many are limited to measuring fitness in a single F_1 generation. Edmands (2007) and others (Tallmon et al. 2004) argue that it is important to study fitness past the F_1 generation to fully investigate the effects of both inbreeding and outbreeding on population fitness.

Comprehensive Studies

The relationships between population size, genetic diversity, and fitness are of great importance to conservation biologists and managers. Positive correlations among these factors may indicate an "extinction vortex." Thus, it is important to investigate whether and how these factors are connected and what role they play in the population viability of endangered species. Often in the case of endangered plant species, time and/or money constraints allow the study of only one or two of these aspects. Population estimates and surveys are relatively inexpensive but may require a large amount of search time, especially for relatively inconspicuous plants. If genetic markers are available, these studies may yield quick results; however, developing the high quality, polymorphic markers needed to study a population that may already be suffering from reduced genetic diversity is often time-consuming and expensive. Studies of the fitness

consequences of genetic isolation are quite informative but can take years depending on the biology of the organism. If there are strong correlations between the three aspects of the feedback loop, it may not be necessary to assess all three. Decisions of which populations are in need of immediate protection (*in situ* conservation strategies) or which populations would serve as a good source for genetic material (*ex situ* conservation strategies) could then be made in a timely manner using, for example, population genetic marker information (recommended by Center for Plant Conservation 1991; Primack 2002).

Positive correlations have been found between population size and heterozygosity (Frankham 1996; Palstra and Ruzzante 2008), measures of genetic diversity and fitness (Reed and Frankham 2003), and population size and fitness (Newman and Pilson 1997; Reed 2005). The negative effects of rarity and habitat fragmentation are highlighted in a recent meta-analysis in which Leimu et al. (2006) reported overall positive relationships between population size, genetic diversity, and fitness. However, a conclusive association is not always clear since the variation in quantitative traits important to fitness may not be accurately revealed by neutral genetic variation (Lynch 1996), and some studies report non-significant or even negative relationships among the factors. Moreover, the sign and magnitude of these correlations may depend on a variety of factors including population history, mating system, life cycle, etc.

In the rare biennial, *Gentianella austriaca*, a negative correlation was found between fitness and genetic diversity, and between population size and genetic diversity, whereas a positive correlation was found between population size and fitness (Greimler and Dobes 2000). By combining the population size with reproductive and genetic traits data, these authors concluded which populations were in most need of conservation. Further, Lammi et al. (1999) found that population size and genetic diversity were not associated with number of seeds,

germination rate, or seedling mass, but population size was correlated with genetic diversity in the regionally endangered, perennial clammy campion, *Lychnis viscaria*. These authors concluded that conservation efforts should include even the small populations with low genetic diversity since they showed no fitness declines—a point that may have been overlooked had only genetic diversity been evaluated. Thus, it is evident that to fully address the effects of habitat loss and fragmentation, studies should combine estimates of accurate population sizes, measurement of genetic diversity, and assessments of the fitness consequences of rarity.

In this dissertation, I address these types of conservation genetic issues, including genetic population size, levels and patterns of genetic diversity, and fitness of populations in an extremely rare sunflower species. The whorled sunflower, *Helianthus verticillatus* Small, is a perennial sunflower restricted to only four locations in the United States: two in western Tennessee (Madison Co. and McNairy Co.), one in northeastern Alabama (Cherokee Co.), and one in northwestern Georgia (Floyd Co.) (Figure I-2). The species is a candidate for federal listing for the Endangered Species Act (ESA) and is listed as endangered by the state natural programs in each of the three states. It was first collected in western Tennessee in 1892 and not found again in the field until 1994 in Georgia (Matthews et al. 2002). In 1996 and 1998, populations of *H. verticillatus* in Alabama and Tennessee were discovered. In the fall of 2006, another population in McNairy Co., Tennessee, about 50 km from the first, was discovered. The Alabama and Georgia populations are about 3.5 km from each other whereas the Tennessee populations are about 350 km from the others. The species has slender rhizomes, a glaucous stem, leaves mostly verticillate in three's or four's, and ranges in height from 0.6-4.2m. *H. verticillatus* is clonal—growing in somewhat distinct clusters of stems in nature (personal observation). It has the diploid number of chromosomes for sunflowers: n=17 (Matthews et al.

2002). There is virtually no information on the historical range of *H. verticillatus*; the species may represent a narrow endemic or a relictual species that was once more extensive through this region.

Since the species was not collected during most of the twentieth century, several authors studying an 1892 herbarium specimen speculated on its origin as an aberrant hybrid of either *H. angustifolius* L. (n=17) X *H. eggertii* Small (n=51) (Beatley 1963) or *H. angustifolius* X *H. grosseserratus* Martens (n=17) (Heiser et al. 1969). The former hybrid combination is not likely since *H. eggertii* is a hexaploid and *H. verticillatus* is a diploid species. However, *H. angustifolius* and *H. grosseserratus* are both diploid species and could represent parents of *H. verticillatus*. Matthews et al. (2002) reported on the current status of the species, concluding, based on morphological characters, that *H. verticillatus* should be considered a distinct diploid species. However, the United States Department of Agriculture Plant Database (http://plants.usda.gov) continues to list the species as a hybrid between *H. angustifolius* and *H. grosseserratus.* Natural hybridization and hybrid speciation is well documented in *Helianthus* with several named hybrids, stable hybrid zones, and three homoploid hybrid species in the genus (Heiser et al. 1969; Rieseberg 1991). Morphological characters can be unpredictable in how they will be expressed in hybrids (Rieseberg and Ellstrand 1993), therefore a genetic study of hybridization using molecular markers with known inheritance patterns should be carried out to either corroborate or reject the findings of Matthews et al. (2002). Since the species is a candidate for federal listing on the ESA, a study of hybrid ancestry may have important implications for the species' listing. Specifically, hybrids have represented a concern for listing on the ESA, and there has been little resolution concerning the issue of whether or not they warrant legal protection (Allendorf et al. 2001).

First, I investigate patterns of genetic diversity, population structure, and hybridization, in *H. verticillatus* using 22 gene based simple sequence repeats (SSRs). Generally, primer development for SSR markers is laborious and time-consuming and may be prohibitively expensive (Zane et al. 2002, Squirrell et al. 2003). An alternative to developing an SSR library de novo is to search EST databases for SSRs (Kantety et al. 2002). There are hundreds of EST libraries for organisms available on GenBank, including a library for *H. annuus* L., the domesticated sunflower. SSRs developed from ESTs of *H. annuus* have proven to be more transferable across *Helianthus* species than traditionally derived anonymous SSRs (Pashley et al. 2005), and EST-SSRs may be more highly conserved than anonymous SSRs since they are in genes. In this study, I will use *H. angustifolius* as a phylogenetic and ecological control with which to compare genetic diversity and population structure. The two species are in the same section of the genus *Helianthus* and grow in similar habitats—they overlap in some parts of their ranges. These genetic markers amplify in both species, therefore when the same loci are evaluated in cross-species comparisons, one can include the inherent differences in the level of variation from one locus to another in statistical analyses.

Next, I study the inheritance patterns of chloroplast DNA (cpDNA) in controlled crosses of *H. verticillatus* to determine if cpDNA is indeed passed on strictly maternally as is thought to occur in most angiosperms. Inheritance of cpDNA can be expressed as a continuous trait ranging from strict maternal to strict paternal inheritance with all values of intermediate levels of DNA being passed from both parents (Welch et al. 2006). When both parents contribute DNA to their offspring, the mode of inheritance is bi-parental. DNA transmitted from the paternal donor in a typically maternally transmitted system constitutes paternal leakage. This can result in an individual who consists of a mixture of parental organellar genomes, i.e., the individuals are

heteroplasmic (McCauley et al. 2007). Utilizing three chloroplast SSRs, I examine 323 offspring haplotypes along with the respective maternal and paternal haplotypes to look for any evidence of non-maternal transmission of cpDNA.

Then, I examine the genetic population size in the four known populations using EST-SSRs. Previous reports made to the US Fish and Wildlife Service indicate that Georgia, the only formally protected site, contains thousands of individuals. However, this estimate was made by counting stalks and may significantly overestimate the number of distinct genetic individuals. Measurements of clonal diversity and spatial structure of clones are carried out to determine the extent of clonality in populations of *H. verticillatus*.

Finally, I evaluate population differentiation with regard to phenotypic fitness characteristics of this rare sunflower and determine the potential consequences of gene flow among populations. The effects of rarity and isolation on the fitness of *H. verticillatus* populations are addressed by conducting controlled intra-population crosses in a common environment and asking 1) do the populations differ in their phenotypic fitness characteristics, and 2) how are these phenotypic fitness characteristics related to population genetic information? The potential for genetic rescue, through gene flow events among disjunct populations of extremely rare species, is examined by conducting inter-population crosses through the F_2 generation. In particular, questions relating to the genetic, or intrinsic, fitness consequences are addressed by asking: 1) is there the potential for genetic rescue as evidenced by the fitness outcomes of F_1 crosses and 2) is there evidence for intrinsic outbreeding depression, especially in the F_2 generation? Given these results, I discuss the implications for combining genetic marker information with that of controlled crosses for the management of extremely rare species. I will relate the results of this crossing study to the population size and genetic diversity studies.

In light of all of these results, I will consider the implications for conservation of this endangered species as well as those of combining population estimates, population genetic studies, and crossing experiments for the study of population viability in rare and endangered plant species.

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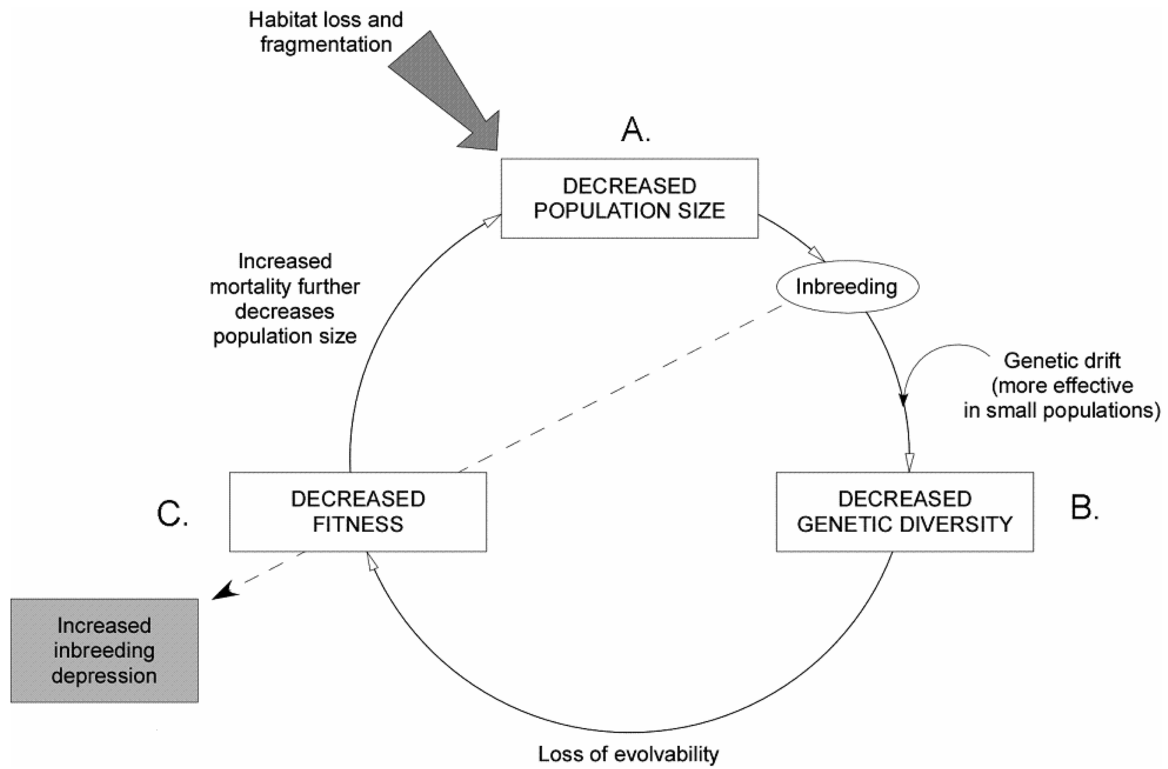

Figure I-1. Extinction vortex schematic of the problems associated with rare and endangered species. Credit: Christopher G. Brown.

Figure I-2. Location of the four known *Helianthus verticillatus* populations.

CHAPTER II

HIGH GENETIC DIVERSITY IN A RARE AND ENDANGERED SUNFLOWER AS COMPARED TO A COMMON CONGENER

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Keywords: chloroplast DNA (cpDNA), conservation, EST-SSRs, genetic diversity, *Helianthus*, hybridization.

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Running title: High genetic diversity in a rare sunflower

This chapter is published in *Molecular Ecology* 2006, 15: 2345-2355.

Abstract

Determining the genetic structure of isolated or fragmented species is of critical importance when planning a suitable conservation strategy. In this study, we use nuclear and chloroplast SSRs to investigate the population genetics of an extremely rare sunflower, *Helianthus verticillatus* Small, which is known from only three locations in North America. We investigated levels of genetic diversity and population structure compared to a more common congener, *H. angustifolius* L. using both nuclear and chloroplast SSRs. We also investigated its proposed hybrid origin from *H. grosseserratus* Martens and *H. angustifolius*. Twenty-two nuclear SSRs originating from the cultivated sunflower (*H. annuus* L.) expressed sequence tag (EST) database, and known to be transferable to *H. verticillatus* and its putative parental taxa, were used in this study thereby allowing for statistical control of locus-specific effects in population genetic analyses. Despite its rarity, *H. verticillatus* possessed significantly higher levels of genetic diversity than *H. angustifolius* at nuclear loci and equivalent levels of chloroplast diversity. Significant levels of population subdivision were observed in *H. verticillatus* but of a magnitude comparable to that of *H. angustifolius.* Inspection of multi-locus genotypes also revealed that clonal spread is highly localized. Finally, we conclude that *H. verticillatus* is not of hybrid origin as it does not exhibit a mixture of parental alleles at nuclear loci, and it does not share a chloroplast DNA haplotype with either of its putative parents.

Introduction

The genetics of endangered species have been of great interest to both evolutionary biologists and conservation managers for some time (Hedrick 2001; e.g., volumes by Falk & Holsinger 1991; Avise & Hamrick 1996; Young & Clarke 2000). Conservation biologists are interested in knowing if there are generalities that can be made with regard to rare species, such as whether or not they typically exhibit reduced genetic diversity or restricted gene flow between populations, as predicted by population genetic theory when populations are small and isolated. While there is indeed a trend for rare species to exhibit reduced genetic diversity, some exhibit equivalent levels of diversity compared to their common congeners (Gitzendanner & Soltis 2000). In order to determine if a rare species does exhibit low diversity, we must have a measure or standard with which to compare. Many studies of rare plants can make comparisons against other plant species that share similar life histories by making use of compendiums of studies of genetic diversity that utilize allozyme (Hamrick & Godt 1989), RAPD (randomly amplified DNA), or anonymous SSR (simple sequence repeat) markers (Nybom 2004). However, when using novel types of markers for which no compendium exists, comparisons against a common congener provide a useful standard against which rare species can be evaluated. Such comparisons minimize the confounding effects of phylogeny and life history on population genetic parameters (Felsenstein 1985; Karron 1987, 1991; Baskauf *et al.* 1994).

In addition to considering species-wide levels of diversity, knowledge of the partitioning of genetic variation within and between populations, or population structure, is important when considering a conservation strategy for an endangered species, especially if not all populations can be protected. With a low level of population structure, the loss of a single population may

have little impact on the species-wide genetic diversity. With a high level of structure, the loss of a single population might significantly reduce overall genetic variation. Further, a species whose distribution has been reduced to small and isolated populations may be at particular risk of extinction due to: (1) the fixation of deleterious alleles within populations as a result of inbreeding due to restricted gene flow, (2) reduced genetic variation, and consequently an inability to adapt to a changing environment (Barrett & Kohn 1991), and/or (3) demographic or environmental stochasticity (Lande 1988, 1993). In fact, Lande (1988) argues that demographic factors may have a more immediate effect on population persistence than genetic factors. Despite this, Reed & Frankham (2003) found a significant positive correlation between heterozygosity and fitness in a meta-analysis of 34 plant and animal data sets, indicating that genetic variability is an important component to consider when formulating management plans.

Levels and patterns of genetic diversity can also be shaped by natural hybridization, wherein hybrids might exhibit elevated levels of genetic diversity resulting from the mixing of parental genomes (Arnold 1997; Rieseberg & Wendel 1993). Given this possibility, comparisons made with a common congener may not accurately reflect the overall effects of rarity on the level of genetic variation found within a rare hybrid derivative. Thus, for rare or endangered species in which a hybrid origin has been suggested, it is important to test for hybridity. Hybrid ancestry is most reliably established with molecular data (Rieseberg & Ellstrand 1993; Chapman & Abbott 2005), and conservation studies that use appropriate genetic markers may help to characterize genetic relationships between taxa when hybridization and introgression might have occurred (e.g. Bruneau *et al*. 2005). Furthermore, hybrid ancestry can impact the status of endangered species that might otherwise be eligible for listing under the U.S. Endangered Species Act (ESA), as the listing of hybrids has sometimes been difficult (Allendorf *et al*. 2001).

Here we report the results of a population genetic survey of a rare and endangered sunflower species, *Helianthus verticillatus* Small, using both EST (expressed sequence tag) nuclear and chloroplast SSRs. Our nuclear markers are derived from the cultivated sunflower (*H. annuus* L.) EST database (http://cgpdb.ucdavis.edu) and have been found to be highly transferable across species within the genus *Helianthus* (Pashley *et al*. 2006). As such they are particularly useful for our purposes. Similarly, the chloroplast markers that we employed have been used successfully in species from across the Compositae (Wills *et al*. 2005). Thus, we were able to include in our survey a more widely distributed congener (*H. angustifolius* L.) as a phylogenetic and life history control whilst statistically controlling for inherent differences in the level of genetic variation from one locus to another. Specifically, we compare the species with regard to the level of standing genetic variation found at these markers and the degree to which that variation is partitioned among populations. Further, since *H. verticillatus* is thought to be clonal, we used EST-SSRs to determine if closely spaced stalks were indeed a single genetic individual or perhaps represented several individuals. This is an important issue when evaluating genetic effective population size from census data. Finally, we looked for a genetic signature of hybridization in *H. verticillatus* through a comparison with its putative parents, *H. angustifolius* and *H. grosseserratus* Martens. If *H. verticillatus* is a hybrid, then its genome should consist of a mixture of alleles from its parents.

Materials and Methods

Study Species

The whorled sunflower, *Helianthus verticillatus*, is an extremely rare, diploid (n = 17), perennial restricted to only three locations in the southeast interior of the United States: one in western Tennessee (35.49N, -88.72W; Madison Co.), one in northeastern Alabama (34.13N, -85.44; Cherokee Co.), and one in northwestern Georgia (34.14N, -85.38W; Floyd Co.). This species is a candidate for federal listing for the Endangered Species Act (ESA) and is listed as endangered in each of the three states. First collected in western Tennessee in 1892, *H. verticillatus* was not found again in the field until 1994 in Georgia (Matthews *et al.* 2002). In 1996 and 1998, populations of *H. verticillatus* in Alabama and Tennessee were also discovered. The Alabama and Georgia populations are about 3.5 km from each other whereas the Tennessee population is about 350 km from the others. The soil type in the Alabama and Georgia habitats is deep, poorly drained soils formed in alluvium and residuum from limestone, and the Tennessee soil type is silt loam from alluvial deposits of Tertiary Porters Creek clay (Matthews *et al*. 2002). *Helianthus verticillatus* is clonal with slender rhizomes, a glaucous stem, leaves mostly verticillate in three's or four's, prefers wet habitats, and flowers August to October. This species ranges in height from 0.6-4.2m, and its clones occur in somewhat distinct clusters in nature.

There is no information available on the historical range of *H. verticillatus*; the species may represent a narrow endemic or a relictual species that was once more extensive throughout this region. Since this species was not collected during most of the twentieth century, several authors studying the 1892 herbarium specimen speculated that it might be of hybrid origin,

having resulted from matings between either *H. angustifolius* (n = 17) × *H. eggertii* Small (n = 51) (Beatley 1963) or *H. angustifolius* × *H. grosseserratus* (n = 17) (Heiser *et al.* 1969). The former hybrid combination seems unlikely since *H. eggertii* is hexaploid and *H. verticillatus* is diploid. However, *H. angustifolius* and *H. grosseserratus* are both diploid species and might reasonably represent the parents of *H. verticillatus*. Matthews *et al.* (2002) reported on the current status of the species and concluded on the basis of several morphological characters that *H. verticillatus* should be considered to be a distinct species. However, the USDA Plant Database (http://plants.usda.gov/) continues to list *H. verticillatus* as a hybrid between *H. angustifolius* and *H. grosseserratus*.

Helianthus angustifolius, a close relative of *H. verticillatus*, is commonly distributed over most of the eastern United States from New York to Florida and west to Texas. The species is perennial with slender or lacking rhizomes, leaves linear to narrowly lanceolate and alternate, usually found in moist, shady areas, and flowers September to October (Heiser *et al*. 1969). In the areas where *H. verticillatus* is located, *H. angustifolius* is the most common sunflower species. *Helianthus grosseserratus* is a perennial with short to medium rhizomes, leaves lanceolate to ovate and mostly opposite, found in dry to moderately wet prairies, and flowers August to October. The species is also commonly distributed across the eastern United States from New England to South Dakota and south to Texas (Heiser *et al*. 1969). All three species are members of the section *Atrorubens* within the genus *Helianthus* (Seiler & Gulya 2004), have overlapping distribution ranges, and are outcrossers pollinated by generalists.

Collection of Plant Material and DNA Extraction

Leaf material of *H. verticillatus* was collected from 22, 22, and 27 clusters of stalks found in the three known locations in Tennessee (TN), Georgia (GA), and Alabama (AL), respectively.

The species grows in clusters of up to five or six stalks, separated from other clusters by at least one meter. In order to determine if each cluster represented a single clone we collected leaves from two to three stalks per cluster for analysis. The total number of clusters varies among the populations, with about 70 in Tennessee and 30 in Georgia. In Alabama, the species is not found in well-defined clusters as in the other two populations—there are about 200-300 stalks. Individuals collected from GA were found in a single field, as was the case for the AL population. In contrast, the TN collection consisted of three subpatches separated by 100 to 200 meters.

Helianthus angustifolius leaf material was collected from two locations: (1) a population located about 10 km from the *H. verticillatus* TN population, and (2) a large continuous population consisting of thousands of plants, which connects the GA and AL *H. verticillatus* populations. We collected 13 individuals from the TN population and 25 individuals from throughout the continuous GA/AL site (hereafter referred to as the AL population). While *H. grosseserratus* is known to occur in Tennessee, difficulties in making collections from TN populations required us to obtain seeds from the North Central Regional Plant Introduction Station (NCRPIS; Ames, IA); 20 individuals of *H. grosseserratus* were assayed. Seeds were nicked with a razor blade, germinated on moist filter paper, and grown in the Vanderbilt University Department of Biological Sciences greenhouse. When the resulting plants were large enough, a leaf was collected for DNA extraction. Sampled accessions were: South Dakota (NCRPIS accession Ames 2742), North Dakota (Ames 22739), Wisconsin (PI 547187), Illinois (PI 547205) and Iowa (PI 613793). In all species, total genomic DNA was isolated from ~200 mg of fresh leaf tissue using the Doyle & Doyle (1987) CTAB method. All DNA samples were quantified using a TKO-100 fluorometer (Hoefer Scientific Instruments, San Francisco).

Selection of Loci, PCR Conditions, and Genotyping

Twenty-two EST-SSR loci developed for *H. annuus* and proven cross-transferable to *H. verticillatus* were chosen as genetic markers for this study (Pashley *et al*. 2006). Nineteen loci amplified in both *H. verticillatus* and *H. angustifolius*, and the remaining three amplified in *H. verticillatus* alone (Table II-1). All but one of the 22 EST-SSRs amplified in *H. grosseserratus*. For the survey of cpDNA (chloroplast DNA) variation and hybrid origin, three polymorphic chloroplast SSRs (cpSSRs: N39 and N30 [Bryan *et al.* 1999] and C7 [Weising & Gardner 1999]) were analyzed in the three species.

SSR genotyping was performed using a modified version of the fluorescent labeling protocol of Schuelke (2000), as detailed in Wills *et al*. (2005). PCR was performed in a total volume of 20 µl containing 2 ng of template DNA for *H. verticillatus*, or 10 ng of DNA in the cases of both *H. angustifolius* and *H. grosseserratus*, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl2, 125 µM of each dNTP, 0.2 µM M13 Forward (-29) sequencing primer labeled with either VIC, 6FAM or TET, 0.2 µM reverse primer, 0.02 µM forward primer and 2 units of *Taq* polymerase. The PCR conditions were as follows: 3 minutes at 95° C; ten cycles of 30 s at 94° C, 30 s at 65° C and 45 s at 72° C, annealing temperature decreasing to 55° C by 1° C per cycle, followed by 30 cycles of 30 s at 94° C, 30 s at 55° C, 45 s at 72° C, followed by 20 m at 72° C.

PCR products were visualized on an MJ Research BaseStation automated DNA sequencer (South San Francisco, CA), and MapMarker® 1000 ROX size standards (BioVentures Inc., Murfreesboro, TN) were run in each lane to allow for accurate determination of fragment size. Cartographer v 1.2.6 (MJ Research) was used to infer individual genotypes according to the fragment sizes of the PCR products.

Analysis of Clonal Structure

Leaves collected from 13 putative clones of *H. verticillatus* were initially genotyped for nine arbitrarily selected polymorphic EST-SSR loci (Table II-1 & see below). The probability that each cluster was a single genet and that identical genotypes were not simply obtained by chance, was calculated using a multilocus probability for codominant genotypes, $P_{cgen} = (\Pi p_i)2^h$, where p_i is the frequency of each allele observed in the multilocus genotype and h is the number of heterozygous loci (Parks & Werth 1993; Sydes & Peakall 1998). The probability of obtaining n-1 more copies of that genotype by chance is given by $(P_{cgen})^{n-1}$, where n is the number of times the genotype was observed.

Analyses of Genetic Diversity and Population Structure

Measures of genetic diversity, including mean number of alleles, observed and expected heterozygosity, and the inbreeding coefficient (*f*, Weir & Cockerham 1984) were calculated for each population of *H. verticillatus* and *H. angustifolius* using GDA v 1.0 (Lewis & Zaykin 2001). Unbiased gene diversities (Nei 1987) were calculated for each locus in the two species using FSTAT (Goudet, 2001). Since *H. grosseserratus* seeds were obtained from NCRPIS and the collections were made without knowledge of specific location, this species was not included in the population genetic calculations but was used in the investigation of hybrid origin.

ANOVAs were performed using JMP v. 4 (SAS Institute Inc.) to test for significant differences in measures of genetic diversity between *H. verticillatus* and *H. angustifolius*, without regard to population. We ran the models with the nineteen markers that amplified in both species (Table II-1). The main effects included in the ANOVA were species and locus, with the dependent variables being either the number of alleles at a locus or the expected heterozygosity. Use of the same genetic markers in both taxa resulted in increased statistical power because

locus-to-locus variation was explicitly included in the model. Differences between the two species in *population* level measures of diversity were also tested for statistical significance. These comparisons were made for number of alleles, observed and expected heterozygosity, and the inbreeding coefficient. We calculated Spearman's nonparametric correlation to test whether there was a significant correlation among gene diversities across loci in *H. verticillatus* and *H. angustifolius*. The effects of species and repeat motif (i.e. tri- or tetra-nucleotide repeat) on gene diversity were investigated via nested ANOVA with loci nested within repeat motifs. All proportions were transformed with an angular transformation prior to analysis (Sokal & Rohlf 1995).

Population genetic structure was estimated in an analysis of molecular variance (AMOVA) framework (Weir & Cockerham 1984; Excoffier *et al.* 1992) using ARLEQUIN v. 2.0 (Schneider *et al.* 2000). This hierarchical analysis of variance partitions the total variance into that found within and among populations. The proportion of total diversity that was found among populations was reported as F_{ST}. F_{ST} estimates were analyzed in a two-factor ANOVA with species and locus as main effects. Once again, this model allows locus effects to be included as a factor in the model instead of being ascribed to error, thereby increasing the power to detect differences between species F_{ST} (Sokal & Rohlf 1995; McCauley *et al*. 1995). Principle Coordinate Analysis (PCO) was conducted on pairwise genetic distances among all three populations of *H. verticillatus* using the covariance standardized method implemented in the program GENALEX (Peakall & Smouse 2002).

Analysis of Genetic Admixture

The Bayesian clustering program STRUCTURE (Pritchard *et al*. 2000) was used to test whether *H. verticillatus* represented a genetic mixture of its putative parents, *H. angustifolius* and

H. grosseserratus, as might be expected if it is a hybrid derivative. We used the admixture model and correlated allele frequencies parameter. In this program, one assumes K populations contribute to the gene pool of the sample population. In this analysis, we set K = 2 and the data input consisted of multilocus genotypes from individuals of all three species. For our analysis, the admixture model considers *H. angustifolius* and *H. grosseserratus* as the two populations and determines what proportion of each 'parent' is present in each of the *H. verticillatus* individuals. The putative parents were used as prior population information by employing the USEPOPINFO feature. We assume that a proportion of a proposed hybrid's genotype is drawn from both population one and two (Beaumont *et al*. 2001; James & Abbott 2005). Results are reported as *q*, the estimated proportion of membership from a given cluster. We used a burn-in period of 50,000 with 10^6 MCMC iterations. GENALEX (Peakall & Smouse 2002) was again used to conduct PCO using the covariance standardized method on pairwise genetic distances amongst all three species in order to evaluate how distinct they are from one another.

Results

Clonal Structure in Helianthus verticillatus

Ample levels of genetic diversity were found at the nine nuclear loci used to detect clonal structure in *H. verticillatus* (Table II-1 & see below). Our investigation of clonal structure revealed that all stalks from the same cluster yielded identical multilocus genotypes, consistent with the hypothesis that they are members of the same genet. In fact, the probabilities that the same EST-SSR multilocus genotype would be encountered a second time in an obligate outcrosser purely by chance ranged from 9.67×10^{-8} to 4.01×10^{-11}, and the probabilities that the

same genotype would be encountered n more times (where n is the number of ramets that we surveyed) ranged from 2.05×10^{-9} to 2.62×10^{-20}. Thus, it is highly unlikely that these genotypes are the result of sexual reproduction. Rather, all stalks from each of the observed clusters most likely represent the same genet, and we never found the same multilocus genotype in disjunct clusters. This result suggests that genets can be identified in the field based solely on the clustering of stalks and that the genetic population size is much smaller than the number of stalks.

Genetic Diversity

In *H. verticillatus*, 18 of 22 EST-SSRs were polymorphic, 13 of 19 were polymorphic in *H. angustifolius*, and 19 of 21 markers were polymorphic in *H. grosseserratus*. Average gene diversities calculated without regard to the population from which samples were drawn for each locus in *H. verticillatus* and *H. angustifolius* are shown in Table II-1. Gene diversity ranged from 0 to 0.82 (0.48 ± 0.06, mean ± SE) in *H. verticillatus* (based on 22 loci) and from 0 to 0.78 (0.34 ± 0.07) in *H. angustifolius* (based on 19 loci). Gene diversity was significantly positively correlated across shared loci (r_s= 0.74; P = 0.0006). A nested two-factor ANOVA on average gene diversities with species and repeat motif as main effects, and loci nested within motifs, yielded significant results for all effects (Table II-2), and revealed that *H. verticillatus* had significantly higher average gene diversity than *H. angustifolius* ($F_{1,17}$ = 6.95, P = 0.017). For *H. verticillatus*, the mean number of alleles per polymorphic locus was 7.7 ± 0.96 (6.3 ± 0.83, all loci), and for *H. angustifolius*, the mean number of alleles per polymorphic locus was 4.9 ± 0.72 (3.3 ± 0.64, all loci). These differences were significant (one-way ANOVA, polymorphic loci $F_{1,29}$ = 5.01, P = 0.033; all loci, P = 0.022). Calculations of number of alleles, observed and expected heterozygosity, and the inbreeding coefficient were also made with regard to

populations (Table II-3). Mean expected heterozygosity within populations was significantly higher in *H. verticillatus* than in *H. angustifolius* (one-way ANOVA H_E, $F_{1,3} = 30.94$, $P = 0.012$). The other measures of diversity (number of alleles, observed heterozygosity, inbreeding coefficient) did not differ significantly between the two species.

Twelve unique chloroplast haplotypes were found in the three populations of *H. verticillatus*, whereas eight were found in the two populations of *H. angustifolius.* Among all the *H. grosseserratus* individuals, six unique chloroplast haplotypes were found. None of these haplotypes were shared between populations or species, and measures of chloroplast genetic diversity did not differ between *H. verticillatus* and *H. angustifolius* (Table II-4).

Population Structure

The *H. verticillatus* populations were moderately differentiated in terms of both nuclear and chloroplast diversity ($F_{STnuc} = 0.118$, $P < 0.0001$; $F_{STcp} = 0.620$, $P < 0.0001$). The two *H. angustifolius* populations were somewhat more differentiated with $F_{STnuc} = 0.207$ ($P < 0.0001$) and $F_{STcp} = 0.700$ ($P < 0.0001$). These nuclear and chloroplast measures of population differentiation are similar to values reported in other studies of plant populations (Petit *et al*. 2005). The two-factor ANOVA conducted on twelve common, polymorphic loci revealed variation among loci, but not among species for F_{STnuc} (locus effect $F_{11,11} = 2.84$, $P = 0.049$; species effect $F_{1,11} = 0.23$, $P = 0.63$). Pairwise values, all of which were significantly different from zero ($P < 0.0001$), were as follows: Georgia and Alabama ($F_{STnuc} = 0.083$ and $F_{STcp} = 0.589$), Tennessee and Georgia ($F_{STnuc} = 0.146$ and $F_{STcp} = 0.389$), Tennessee and Alabama ($F_{STnuc} = 0.128$ and $F_{STcp} = 0.814$). Subdividing the three patches of the TN population revealed slight but significant differentiation in nuclear markers among these patches ($F_{STnuc} = 0.048$, $P < 0.0323$) and greater differentiation for chloroplast markers, ($F_{STcp} = 0.432$, $P < 0.0001$). The

PCO carried out on *H. verticillatus* revealed some overlap between individuals from GA and AL, whereas the TN population formed a distinct cluster, separated along PCO1 (PCO 1: 10.0%, PCO 2: 7.2%; Figure II-1A).

Several loci in each of the *H. verticillatus* and *H. angustifolius* populations were found to be significantly out of Hardy-Weinberg equilibrium. Among the three populations of *H. verticillatus* five loci were consistently out of Hardy-Weinberg: BL0001, BL0008, BL0010, BL0018, and BL0027. Loci significantly out of Hardy-Weinberg equilibrium for *H. angustifolius* were BL0018 and BL0025. We used ARLEQUIN to calculate F_{ST} across loci with and without the assumption of Hardy-Weinberg equilibrium. Values of F_{ST} were only slightly different in both cases (i.e. *H. verticillatus* $F_{\mathrm{STnuc}} = 0.118$ vs. $F_{\mathrm{STnuc}} = 0.113$ respectively), and in no instances did the level of significance change. A test for linkage disequilibrium was not carried out on the data as a rejection of the linkage test could be due to departures from Hardy-Weinberg equilibrium (Excoffier & Slatkin 1998). Furthermore, with the large number of alleles per locus and large number of loci, a likelihood ratio test of linkage disequilibrium may not be valid due to a small number of expected individuals per genotypic class (Schneider *et al.* 2000). However, because the number of loci exceeds the number of chromosomes (17), undoubtedly some markers occur on the same linkage group.

Genetic Admixture in Helianthus verticillatus

The three species under consideration shared equivalent numbers of nuclear SSR alleles with each other. The mean number of shared alleles per locus was 1.79 ± 0.31 between *H. verticillatus* and *H. angustifolius*, 2.09 ± 0.20 between *H. verticillatus* and *H. grosseserratus* and 1.50 ± 0.31 between *H. angustifolius* and *H. grosseserratus*. For one locus, BL0022, *H. verticillatus* and *H. angustifolius* were fixed for different alleles. On the other hand, *H.*

grosseserratus was polymorphic for this locus exhibiting four alleles, including the *H. verticillatus* allele. As noted above, each population of the three species possessed unique sets of chloroplast haplotypes.

Genetic admixture analysis indicated that *H. verticillatus* is not a hybrid derivative of *H. angustifolius* and *H. grosseserratus*. The proportion of population membership of each species assigned by STRUCTURE using K=2 was 99.8% of the *H. angustifolius* individuals in one population, and 99.4% and 99.8% of *H. grosseserratus* and *H. verticillatus* individuals in the other. Therefore using a model with two groups corresponding to the two putative parents, all *H. verticillatus* individuals were assigned into the cluster with *H. grosseserratus*, suggesting that *H. verticillatus* is more closely related to *H. grosseserratus* than to *H. angustifolius*. It is important to note that, when using K = 3, *H. verticillatus* no longer groups with *H. grosseserratus*; rather, it formed a distinct cluster, indicating that *H. verticillatus* is genetically distinct from both *H. grosseserratus* and *H. angustifolius*. A PCO of all individuals from the three species also demonstrated these species are genetically distinct, with no overlap of *H. verticillatus* individuals with either of the putative parental clusters (PCO 1: 26.3%, PCO 2: 6.9%; Figure II-1B).

Discussion

Levels and Patterns of Genetic Diversity

Despite the general expectation of reduced genetic variation in a rare species, *Helianthus verticillatus* does not exhibit a reduction in genetic diversity at either the population or the species level relative to its more common congener, *H. angustifolius*. In fact, for nuclear EST-SSRs, *H. verticillatus* has significantly *higher* levels of gene diversity than does *H. angustifolius*.

While this is not a common result, Gitzendanner and Soltis (2000) demonstrated that endangered species sometimes exhibit levels of diversity as high as, or higher than, a common congener (e.g. in the genera *Agastache* [Vogelmann & Gastony 1987], *Adenophorus* [Ranker 1994] and *Daviesia* [Young & Brown 1996]).

Ellstrand and Elam (1993) proposed that high levels of genetic diversity might be expected in rare species assuming they consist of relatively large populations. While the accurate determination of population sizes can be difficult in clonal species in general, the large number of available EST-SSR markers made the determination of clonal identity straightforward in TN and GA. We genotyped 2-3 stalks from 13 putative genets and found that all stalks within an observed cluster exhibited the same nine-locus genotype indicating that clonal identity can be reliably assessed by eye when the species is found in clusters. The number of distinct clusters in TN is about 70, whereas the GA population contains around 30. Because the clusters are less well-defined in AL, an estimate of the number of genets is more difficult to make; this population is, however, not likely to be large, as there are only a few hundred stalks, and some fraction of these are likely to be ramets of the same genet. Thus, large population size does not seem to be a likely explanation of the relatively high level of diversity present in this species. An alternative explanation of the relatively high diversity in *H. verticillatus* is that the widespread *H. angustifolius* exhibits unexpectedly low diversity. However, gene diversity in *H. verticillatus* does not differ significantly from the extremely widespread common sunflower, *H. annuus*. In this latter species, average gene diversity for the same 19 EST-SSRs, based on data from 13 populations (52 individuals in total), was 0.57 ± 0.02 (range 0.45 to 0.70; CH Pashley and JM Burke, unpublished data), as compared to 0.48 in *H. verticillatus* (two-factor ANOVA with species and locus as effects; $F_{1,18} = 2.65$, $P = 0.12$). While we did not do a formal statistical

comparison with *H. grosseserratus* because of differences in the way the samples were obtained, it is worth noting that gene diversity in *H. verticillatus* did not differ markedly from that of *H. grosseserratus* at the nineteen loci (0.48 vs. 0.44, respectively). Because we have no information concerning the historical distribution or population size of *H. verticillatus*, we have no way of knowing how long it has been since the species became rare. When combined with the presently small population sizes, the relatively high levels of genetic diversity in *H. verticillatus* may indicate that this species has not been rare for a long time, especially when we consider that it is a clonal perennial (see below).

Another explanation for the relatively high levels of genetic variation exhibited by *H. verticillatus* is that it is of hybrid origin, as higher levels of genetic diversity may result from a mixing of parental alleles. Hybridization often plays a significant role in the evolution and speciation of plants (Arnold 1997; Rieseberg 1997), and its role in the evolution of the annual *Helianthus* species has been studied extensively (e.g., Heiser 1947; Rieseberg 1991; Rieseberg *et al.* 1995, 1996). Some *Helianthus* species freely hybridize in the wild, resulting in hybrid swarms, and three homoploid hybrid sunflower species have been reported (Rieseberg 1991). Based on our SSR data, however, *H. verticillatus* does not appear to be the product of hybridization between *H. angustifolius* and *H. grosseserratus*, as was proposed by Heiser *et al.* (1969). The STRUCTURE analysis with K = 2 places *H. angustifolius* into one population, whereas *H. grosseserratus* and *H. verticillatus* correspond to another. This result clearly indicates that *H. verticillatus* does not exhibit mixed ancestry as would be expected in the case of a hybrid swarm, as individuals of hybrid origin would likely consist of a mixture of the genomes of each of its parents. When combined with the findings of Matthews *et al*. (2002), who concluded from morphological evidence that *H. verticillatus* should be considered a distinct

species, our data suggest that *H. verticillatus* represents a good taxonomic species of non-hybrid origin. While introgressive hybridization could account for the high levels of heterozygosity observed in this study, our analysis likewise failed to provide any evidence of introgression.

A final possibility is that the unexpectedly high levels of genetic variation in *H. verticillatus* result from the fact that relatively few generations have passed since it became rare. *Helianthus verticillatus* is a clonal perennial and, because of these life history attributes, populations may have not experienced extensive loss of variation due to the effects of small populations such as genetic drift and inbreeding. While nothing is known of the prior history of the species, F_{STnuc} may give insight into the historical distribution. The number of populations of *H. verticillatus* is very low (only three are known), and one of these is quite disjunct from the other two. Hence, gene flow between TN and GA/AL is probably rare. Despite this, there is only modest genetic differentiation among populations ($F_{STnuc} = 0.118$). It is therefore reasonable that a larger number of populations existed in a more continuous range in the past, but the species has experienced severe reduction in population numbers due to the removal of suitable habitat. In fact, other plant species associated with *H. verticillatus* populations are considered to have strong prairie affinities (e.g. *Hypericum sphaerocarpum* Michaux, *Silphium terebinthinaceum* Jacq., *Andropogon gerardii* Vitman), and fire suppression and conversion of large tracts of land to farmland during European settlement may have significantly reduced the prairie habitat that was once present in this region (Allison 1995). Another possible explanation is that *H. verticillatus* represents a historically narrow endemic, and one of the complexes (TN or GA/AL) has recently been derived from the other. However, we did not see evidence for a recent bottleneck in the form of loss of diversity associated with a founder event. In any case, genetic divergence is not

as high as might be expected for disjunct populations with a long history of isolation, especially when compared to estimates of population structure in *H. angustifolius* (F_{STnuc} = 0.207).

On the Utility of EST-SSRs in Evolutionary Genetics

This study is unique in that it involves a population genetic survey of an endangered plant species based on a large number of EST-SSRs. Compared to traditional methods of SSR development which are laborious and expensive (Zane *et al.* 2002, Squirrell *et al.* 2003), the transfer of SSRs from a species with an existing EST database to an endangered species is far less time consuming and costly. In addition, since EST-SSRs are more transferable across taxonomic boundaries than are anonymous SSRs (Pashley *et al*. 2006; Varshney *et al*. 2005), one can survey two or more taxa with a common set of genetic markers, thereby allowing for the statistical control of locus-specific effects when comparing estimates of genetic diversity and/or population structure. Indeed, if we had conducted a one-way ANOVA (accounting only for the effect of species identity) on genetic diversity in *H. verticillatus* and *H. angustifolius*, the means would not have been statistically different from one another ($F_{1,36}$ = 1.60, P = 0.21). Thus, the higher statistical power afforded by the use of common markers across taxa allowed us to detect real differences between these species that would have otherwise gone undocumented.

A point to consider when using EST-SSRs is that selection on these loci could affect population genetic parameters. However, Woodhead *et al*. (2005) found that population differentiation does not seem to be affected by selection as F_{ST} values based on EST-SSRs were similar to those based on anonymous SSRs and AFLPs (amplified fragment length polymorphisms). Given that EST-SSRs appear, on average, to be neutral, they can be used to study the effects of demography on the standing level of genetic variation, a common goal in conservation genetics. Such insights are a prerequisite for understanding the potential influence

of rarity and/or fragmentation on adaptive variation, which is likely to play a role in population persistence.

Another possible concern with SSRs in general has to do with the occurrence of null alleles (i.e. alleles that fail to amplify because of mutations in the primer sites flanking the SSR repeat; Callen *et al*. 1993). Null alleles could account, at least in part, for the significant heterozygote deficits that were observed at some loci. Other explanations for the deviations may be selfing or biparental inbreeding due to spatial structuring. Sunflowers typically exhibit sporophytic self-incompatibility with rare selfing seen in the annuals but none encountered in the perennials (CB Heiser, personal communication). Furthermore, greenhouse work with all three of these species has resulted in no seed set from selfed flower heads (JR Ellis, unpublished data). It seems more likely that this pattern results from biparental inbreeding due to spatial structuring and non-random mating within populations. Recall that in the Tennessee population, a small but significant amount of micro-population structure was seen.

An additional finding of this study was that gene diversity in *H. verticillatus* and *H. angustifolius* was dependent upon the repeat motif of the locus in question. In the two factor nested ANOVA, repeat motif had a significant effect on genetic diversity, with tri-nucleotide repeats exhibiting higher genetic diversity than tetra-nucleotide repeats (0.74 vs. 0.30, $F_{1,17} = 27.77$, $P < 0.0001$). This point is particularly important in the context of cross-species comparisons, as the use of different loci in different taxa could easily bias estimates of genetic diversity. This possibility highlights the value of being able to use the same genetic markers across related species as different loci are likely to have quite different evolutionary histories.

Conclusions and Conservation Implications

It is clear that the expectation of reduced genetic diversity in rare species is not always borne out, and low genetic diversity does not appear to be an immediate concern for *H. verticillatus* at this time. Furthermore, populations of *H. verticillatus* exhibited moderate levels of population differentiation using presumably neutral markers. Since these populations are geographically distinct and vary somewhat in ecological conditions, they are likely to be at least as differentiated at adaptive loci, if not more. Therefore, to preserve maximum species diversity, all three populations of *H. verticillatus* should be protected. Habitat loss is probably the cause of rarity in *H. verticillatus* as the species seems to be adapted to prairie habitats which have declined since European settlement (Matthews *et al*. 2002). Thus, habitat protection is of great concern and is probably the most immediate action to take at this time to preserve the species. Finally, the species is a candidate for federal listing on the U.S. Endangered Species Act (ESA), and our results have important implications for the species' listing. More specifically, there has been little resolution concerning the issue of whether or not hybrids should warrant legal protection under the ESA (Allendorf *et al*. 2001). However, *H. verticillatus* does not appear to be a hybrid between the two proposed taxa; as such the hybrid issue should not inhibit its listing.

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Acknowledgments

We thank Temple-Inland Corporation, Sam Breyfogle, Jim Allison, Natasha Sherman, and the Tennessee Natural Heritage Program (especially Claude Bailey and David Lincicome) for assistance in locating and collecting samples. We also thank Mark Welch, Mark Chapman, and David Wills for assistance in the lab. This work was supported by funds provided by USDA Grant 2002-02213 to DEM. This work was also supported by grants from the NSF Plant Genome Research Program (DBI-0332411 to JMB) and the USDA Plant Genome program (#03-39210-13958 to JMB). *Helianthus annuus* EST data was obtained from The Compositae Genome Project database (http://compgenomics.ucdavis.edu), which was supported by a grant from the USDA IFAFS program.

Table II-1. Locus descriptions and mean values for Nei's gene diversity (1987) for *H. verticillatus* and *H. angustifolius*.

Locus	Type of Repeat	Location	*H. verticillatus*	*H. angustifolius*
BL0001	tri	UTR	0.711	0.336
BL0002[AB]	tetra	UTR	0.133	N/A
BL0003	tri	Coding	0.689	0.704
BL0004[B]	tri	Coding	0.347	0.090
BL0005[B]	tri	UTR	0.817	0.699
BL0006[AB]	tetra	UTR	0.656	N/A
BL0007[B]	tri	Coding	0.609	0
BL0008[B]	tri	Coding	0.777	0.646
BL0010	tri	Coding	0.703	0.508
BL0011	tetra	UTR	0.586	0
BL0012[B]	tetra	UTR	0.222	0
BL0013[A]	tetra	UTR	0.659	N/A
BL0014	tri	Coding	0	0.213
BL0017[B]	tri	UTR	0.533	0.442
BL0018[BC]	tri	Coding	0.738	0.784
BL0020	tri	Coding	0.493	0.529
BL0022	tri	UTR	0	0
BL0023	tri	UTR	0.381	0.419
BL0025	tetra	Coding	0.651	0.400
BL0027	tri	Coding	0.820	0.767
BL0029	tetra	UTR	0	0
BL0030	tetra	UTR	0	0
Mean			**0.478 ± 0.06***	**0.344 ± 0.07***

*Mean values ± SE are significantly different from each other at the $P < 0.05$ level, two-factor ANOVA, see text for details.
[A]EST-SSRs that amplified in *H. verticillatus* only. [B]Nine EST-SSRs genotyped for analysis of clonal structure.
[C]EST-SSR that did not amplify in *H. grosseserratus.*

Table II-2. Two factor nested ANOVA of Nei's gene diversity (1987).

Source	**Degrees of Freedom**	**Sum of Squares**	***F* Ratio**	***P***
Species	1	0.360	6.95	0.017
Repeat Motif	1	1.42	27.8	<0.0001
Locus[Repeat Motif]	17	3.08	4.42	0.002
Species X Repeat	1	0.079	1.54	0.231
Error	17	0.870		

Table II-3. Expressed sequence tag-simple sequence repeat (EST-SSR) diversity in three populations of *H. verticillatus* and two populations of *H. angustifolius*.

Species	**Population (N)**	**A**	**H_O**	**H_E**	***f***
H. verticillatus	TN (22)	4.3(0.54)	0.35(0.05)	0.46(0.07)	0.23(0.14)
	GA (22)	4.5(0.57)	0.40(0.08)	0.51(0.07)	0.22(0.04)
	AL (27)	3.5(0.43)	0.32(0.01)	0.46(0.07)	0.32(0.10)
H. angustifolius	TN (13)	2.8(0.45)	0.23(0.05)	0.34(0.07)	0.39(0.08)
	AL (25)	3.6(0.43)	0.28(0.04)	0.36(0.06)	0.14(0.06)
Mean *H. verticillatus*		4.1(0.31)*	0.36(0.02)	0.48(0.02)*	0.26(0.03)
Mean *H. angustifolius*		3.2(0.40)*	0.26(0.03)	0.35(0.01)*	0.27(0.13)

Values are averaged over all loci in each population: A, mean number ± SE of alleles per locus; H_O, mean observed heterozygosity ± SE; H_E, mean expected heterozygosity ± SE; *f*, within population coefficient of inbreeding. *Mean values significantly different from one another, $P < 0.05$; two-factor ANOVA, species & locus effects (see text for details).

Table II-4. Chloroplast simple sequence repeat (SSR) diversity in three populations of *H. verticillatus* and two populations of *H. angustifolius*.

Species	**Population (N)**	**Haplotypes**	$\mathbf{H_E}$
H. verticillatus	TN (22)	4 (A,B,C,D)	0.29
	GA (22)	6 (E,F,G,H,I,J)	0.55
	AL (27)	2 (K,L)	0.05
H. angustifolius	TN (13)	5 (M,N,O,P,Q)	0.31
	AL (25)	3(R,S,T)	0.35
Mean *H. verticillatus*		4(1.2)	0.30(0.14)
Mean *H. angustifolius*		4(1.0)	0.33(0.02)

Mean ± SE values in the two species were not significantly different from each other.

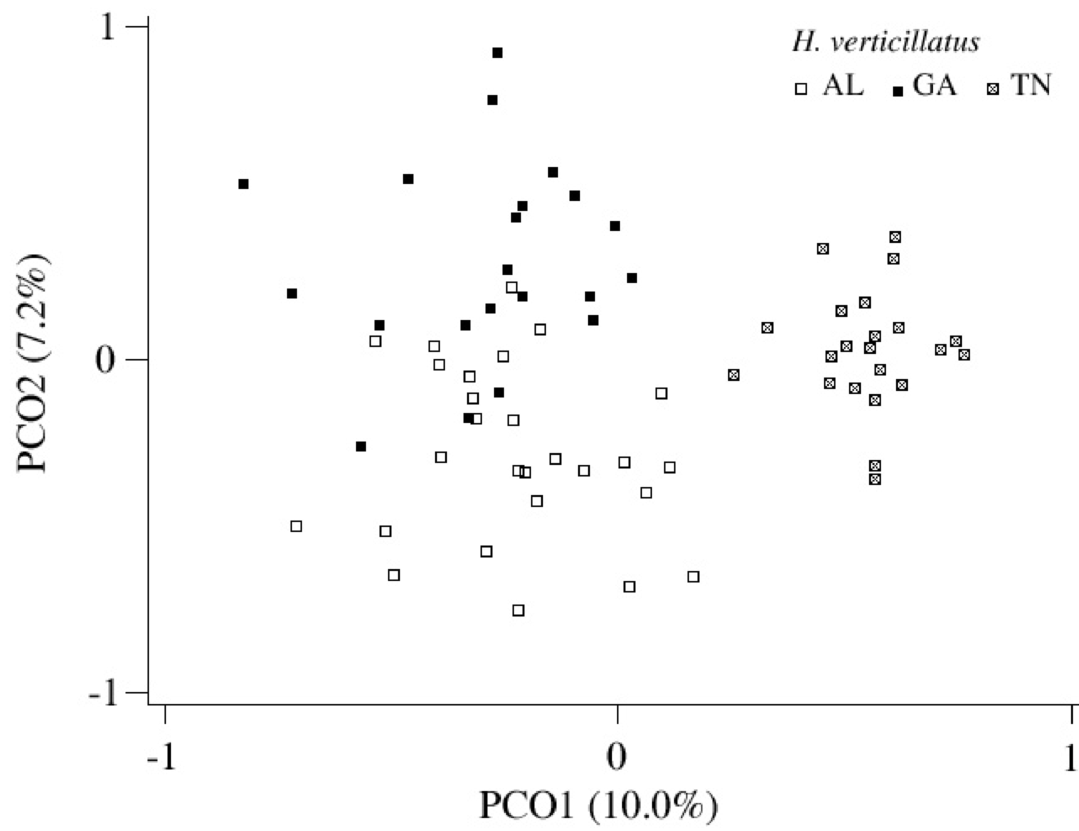

Figure II-1A.

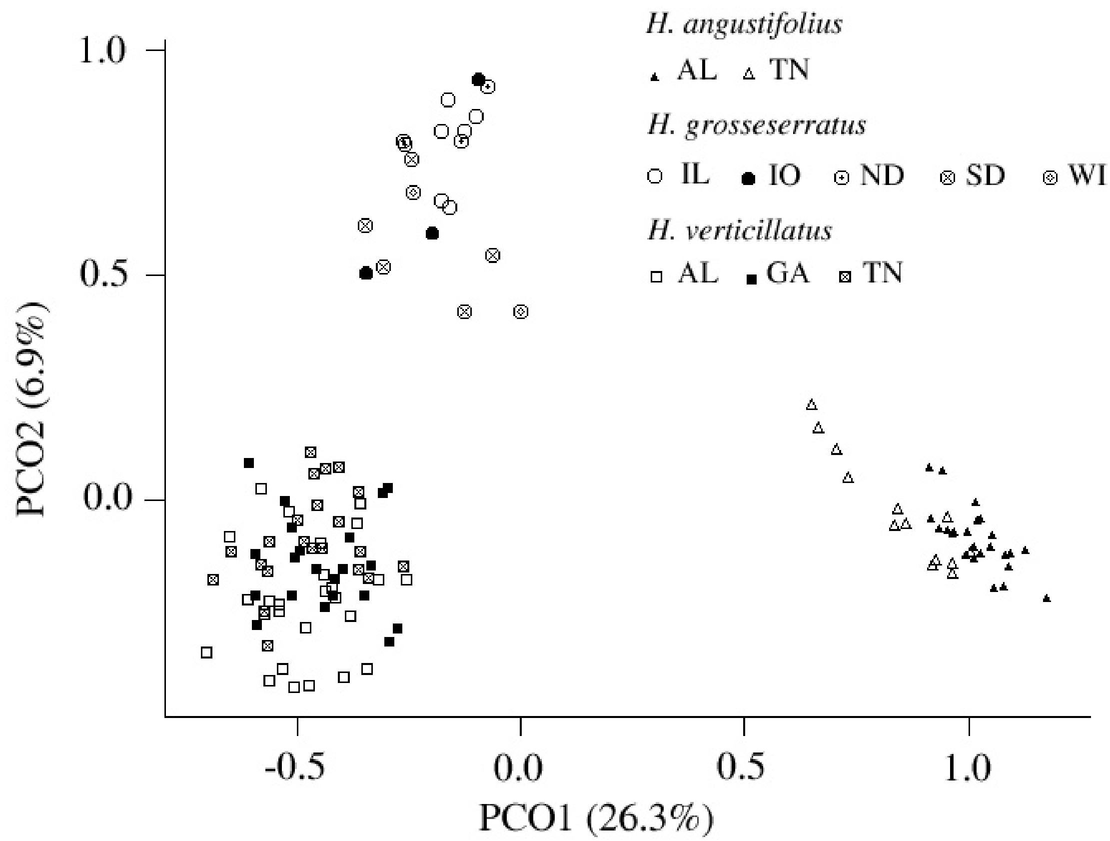

Figure II-1B.

Figure II-1. (A) Principle Coordinate Analysis (PCO) plot based on 22 loci among three *H. verticillatus* populations, (B) PCO plot based on 18 common loci among *H. verticillatus* (squares), *H. grosseserratus* (circles), and *H. angustifolius* (triangles).

CHAPTER III

DETECTION OF RARE PATERNAL CHLOROPLAST INHERITANCE IN CONTROLLED CROSSES OF THE ENDANGERED SUNFLOWER *HELIANTHUS VERTICILLATUS*

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Keywords: chloroplast DNA, endangered species, *Helianthus*, maternal inheritance, paternal leakage, heteroplasmy

Running Title: Chloroplast paternal inheritance in a rare sunflower

Word Count: 3,926

This chapter is published in *Heredity* 2008, 100: 574-580.

Abstract

A variety of questions in population and evolutionary biology are studied using chloroplast DNA (cpDNA). The presumed maternal inheritance in angiosperms allows for certain assumptions and calculations to be made when studying plant hybridization, phylogeography, molecular systematics, and seed dispersal. Further, the placement of transgenes in the chloroplast to lessen the probability of “escape” to weedy relatives has been proposed since such genes would not move through pollen. In many studies, however, strict maternal inheritance is assumed but not tested directly, and some studies may have sample sizes too small to be able to detect rare paternal leakage. Here, we study the inheritance of chloroplast DNA simple sequence repeats in 323 offspring derived from greenhouse crosses of the rare sunflower *Helianthus verticillatus* Small. We found evidence for rare chloroplast paternal leakage and heteroplasmy in 1.86 % of the offspring. We address the question of whether one can extrapolate the mode of chloroplast transmission within a genus by comparing our results to the findings of another sunflower species study. The findings of occasional paternal transmission of the chloroplast genome are discussed in the framework of using these markers in studies of population and evolutionary biology both in *Helianthus* and other angiosperms.

Introduction

Population and evolutionary biologists use chloroplast DNA (cpDNA) in a variety of applications including studies of hybridization and population genetics (Rieseberg and Ellstrand, 1993; Welch and Rieseberg, 2002; Dobeš *et al*., 2004; Van Droogenbroeck *et al*., 2006) and seed movement and dispersal in natural populations (McCauley, 1995; Ouborg *et al*., 1999; Hamilton and Miller, 2002; Petit *et al*., 2005). Since the chloroplast genome is largely maternally transmitted in angiosperms (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al*., 2003), these types of applications assume maternal inheritance (Birky, 1995; 2001), though this assumption is only rarely tested. In fact, occasional paternal or biparental inheritance has been shown in some species (Sears, 1980; Corriveau and Coleman, 1988; Reboud and Zeyl, 1994; Röhr *et al*., 1998; Hansen *et al*., 2007; McCauley *et al*., 2007). Thus, when it occurs, paternal or biparental inheritance of the chloroplast genome could lead to incorrect conclusions in studies involving seed dispersal, hybrid origins, and evolutionary relationships should maternal inheritance be assumed.

Further, researchers have posited that transgenes placed in the chloroplast genome of crops would reduce their probability of "escape" as the genes would not move through pollen if maternally inherited (Gressel, 1999; Grevich and Daniell, 2005; Daniell *et al*., 2005). In crop systems, non-maternal inheritance could lead to the escape of transgenes, for example that may confer herbicide resistance, thus leading to the possibility of creating "superweeds" since many domesticated crops grow in close proximity to their weedy wild relatives (Smith, 1989; Haygood *et al*., 2004; Chapman and Burke, 2006). In fact, Haygood *et al*., (2004) found that even with low levels of paternal transmission, the probability of transgene escape

is considerable. Therefore, studying the transmission of the chloroplast genome can provide valuable information for the likelihood of transgene escape in domesticated crop species as well as accepting or rejecting the assumptions of maternal inheritance in population and evolutionary studies.

In many plant genera, consistency of inheritance has been observed among the several congeners that happen to have been studied (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al*., 2003). These findings suggest that chloroplast inheritance may be conserved within a genus. If so, one could extend information on the mode of inheritance of one species to its congeners. However, there are a few exceptions to this observation in which different members of a genus have conflicting modes of inheritance (Sears, 1980; Zhang *et al*., 2003)—thus raising the question: can one assume that the mode of chloroplast inheritance is identical among congeners? If not, then chloroplast inheritance needs to be assessed directly in each species in question.

Several factors influence whether paternal leakage can occur and what effect it has on the population biology of plant species: whether and how often pollen grains contain cpDNA, how frequently it is transmitted to the zygote, and how intra-individual drift affects copies within cells. Large-scale studies designed to infer organellar inheritance in angiosperms have screened pollen grains for evidence of plastids or plastid DNA in generative or sperm cells (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al*., 2003). Using cytological evidence to determine the mode of plastid DNA inheritance, these studies have designated cpDNA transmission in hundreds of angiosperm species as either maternal or biparental. Due to their nature, these methods can only identify the potential mode of inheritance i.e. whether there is plastid DNA in the cell. Since these studies scan a great number of species, they often use a

small number of individuals per species which may miss rare paternal transmission. It is also not possible to determine the consequences for individual and population biology when the transmission mode is biparental since intra-individual drift of DNA copies during cell divisions will lead to individuals that sort almost completely to one type or the other (i.e. a homoplasmic individual) or to individuals that carry a mixture of paternal and maternal copies (i.e. a heteroplasmic individual) (Birky, 2001). Cell divisions represent founder effects or bottlenecks each generation which should enforce a highly homoplasmic state within the individual. However, occasional biparental inheritance would continue to introduce alternate alleles into the individual generating heteroplasmy. Chloroplast heteroplasmy has been documented in several angiosperms genera: *Passiflora* (Hansen *et al*., 2006; 2007), *Senecio* (Frey *et al*., 2005), *Medicago* (Johnson and Palmer, 1989), and *Turnera* (Shore 1994; 1998).

We have investigated cpDNA inheritance and heteroplasmy in a rare sunflower species, *Helianthus verticillatus* Small, and compared it to the mode of inheritance found in a related economically important species, *H. annuus* (Rieseberg *et al*., 1994; Wills *et al*., 2005). These two prior studies found no evidence for paternal leakage of cpDNA in *H. annuus* crosses and Wills *et al*. (2005) found no evidence for heteroplasmy (personal communication). Ellis *et al*. (2006) investigated population structure in *H. verticillatus* based on F_{ST} calculations for nuclear and cpDNA markers and found the latter to have a much greater F_{ST}. Based on the described cpDNA inheritance in *H. annuus*, the difference in magnitude between nuclear and chloroplast F_{ST} was explained in part by maternal inheritance of cpDNA. However, this assumed mode of inheritance has not been tested directly in *H. verticillatus*. Since we are focusing on just one species, we chose to look at chloroplast inheritance directly by examining progeny from controlled crosses in which the parents had

different cpDNA haplotypes. This approach allows the direct observation of chloroplast transmission and includes much larger sample sizes, thus giving a greater chance of detecting rare paternal leakage and quantifying the rate of leakage accurately.

When designing an experiment to study rare events, one must take into account the power of detection. Milligan (1992) proposed a binomial model of organelle inheritance to determine the power of the analysis to detect paternal leakage at a given rate. He states that many studies that address organelle inheritance use insufficient sample sizes to detect leakage, and since some studies have found leakage rates of 0.01 to 2.5 % (Simmonds, 1969; Tilney-Bassett, 1978; Medgyesy *et al*., 1986), sample sizes in excess of 100 progeny are needed to detect leakage even at the 2.5 % level. Further, since the probability of non-maternal chloroplast inheritance may vary among crosses (Birky, 1995; Mogensen, 1996), individuals will not be completely independent data points if they are from the same family. Consequently, when designing an experiment to evaluate organelle inheritance, one should choose as many families as possible recognizing the trade-off between the number of families and the number of offspring per family. Here we report on a study of cpDNA inheritance using cpSSRs (chloroplast simple sequence repeat; Provan *et al*., 2001) in 323 *H. verticillatus* offspring comprising 53 families and provide evidence for occasional paternal leakage and heteroplasmy of cpDNA in controlled greenhouse crosses.

Materials and Methods

Helianthus verticillatus is an extremely rare, diploid (n = 17) self-incompatible, perennial sunflower restricted to only four locations in the southeast interior of the United

States: two in western Tennessee (Madison Co. and Selmer Co.), one in northeastern Alabama (Cherokee Co.), and one in northwestern Georgia (Floyd Co.). It is a candidate for federal listing for the Endangered Species Act (ESA) and is listed as endangered in each of the three states. First collected in western Tennessee in 1892, it was not found again in the field until 1994 in Georgia (Matthews *et al*., 2002). In 1996 and 1998, the populations in Alabama and Madison Co., Tennessee, respectively, were discovered. In the fall of 2006, the fourth location in Selmer Co., Tennessee was discovered during an annual survey and search for the species. The Alabama and Georgia populations are about 3.5 km from each other whereas the Tennessee populations are about 350 km from the others and about 40 km from one another. In a prior study, the Alabama and Tennessee populations were found to be fixed for different cpDNA haplotypes making it possible to detect paternal leakage easily in crosses between the two populations (Ellis *et al*., 2006).

In order to detect rare paternal leakage, the appropriate sample size must be used. We used Milligan's (1992) equation for calculating the power of analysis for the number of individuals studied and the allowed percentage of non-maternal inheritance:

$$\beta = 1 - (1-P)^N$$

where β is the power of the test to detect leakage, P is the probability of paternal transmission, and N is the number of progeny. We designed our experiment to be able to accept the strict maternal inheritance hypothesis 95 % of the time at a rate of leakage equal to or greater than one percent. To have this statistical power, 300 individuals (i.e. observations of inheritance) were needed according to this calculation.

Achenes from *H. verticillatus* were collected from the Alabama and Tennessee sites and grown in the Vanderbilt Biological Sciences greenhouse to serve as parents for the

crosses. Since the parents need to differ at the markers studied in order to detect paternal leakage, we genotyped the parents for the cpSSRs (method described below) to choose individuals with differing cpDNA haplotypes. Alabama and Tennessee individuals carried different cpDNA haplotypes; therefore, any inter-population crosses would have differing parental genotypes. The Tennessee population was polymorphic; thus, Tennessee by Tennessee intra-population crosses were also conducted with parents that differed in cpDNA haplotype. We used 272 offspring from 45 controlled greenhouse inter-population crosses (24 Alabama X Tennessee and 21 Tennessee X Alabama) and 51 individuals from eight greenhouse intra-population (Tennessee) crosses of *H. verticillatus* for a total of 323 offspring.

The crosses were carried out as follows: inflorescences were bagged prior to anthesis to prevent any unwanted pollinations. Crosses were conducted by brushing pollen with a paintbrush from inflorescences at anthesis into aluminum foil and then brushing pollen onto the stigmas of another inflorescence in which the same pollen removal had been conducted. Pollinations were conducted within one hour of collecting pollen and all pollinations were conducted at mid-morning (~1000 hours). Inflorescences were re-bagged and achenes were allowed to mature. Achenes (i.e. the offspring) were nicked with a razor blade, germinated on moist filter paper, and grown in the greenhouse. When the resulting plants were large enough, a leaf was collected for DNA extraction.

Total genomic DNA was isolated from ~200 mg of fresh leaf tissue using the Applied Biosystems 6100 Nucleic Acid PrepStation DNA extraction machine and associated protocols (Foster City, California). All parents and offspring were genotyped for three polymorphic cpSSRs, [N39 and N30 (Bryan *et al*., 1999) and C7 (Weising & Gardner, 1999);

also used in Wills *et al*., 2005; Ellis *et al*., 2006] using PCR and fragment length analysis. Any individuals that indicated paternal leakage were re-genotyped two additional times to verify the results. Briefly, SSR genotyping was performed using a modified version of the fluorescent labeling protocol of Schuelke (2000), as detailed in Wills *et al*., (2005). PCR was performed in a total volume of 20 µl containing 2 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl2, 125 µM of each dNTP, 0.2 µM M13 Forward (-29) sequencing primer labeled with either HEX, 6FAM or TET, 0.2 µM reverse primer, 0.02 µM forward primer and 2 units of *Taq* polymerase. The PCR conditions were as follows: 3 minutes at 95° C; ten cycles of 30 s at 94° C, 30 s at 65° C and 45 s at 72° C, annealing temperature decreasing to 55° C by 1° C per cycle, followed by 30 cycles of 30 s at 94° C, 30 s at 55° C, 45 s at 72° C, followed by 20 m at 72° C.

PCR products were visualized on an MJ Research BaseStation automated DNA sequencer (South San Francisco, California). MapMarker® 1000 ROX size standards (BioVentures Inc., Murfreesboro, Tennessee) were run in each lane to allow for accurate determination of fragment size. Cartographer v 1.2.6 (MJ Research) was used to infer individual genotypes according to the fragment sizes of the PCR products. The parents used in this study carried one of four haplotypes with Alabama and Tennessee containing different haplotypes. Haplotypes were denoted A1, T1, T2, T3 named by the three-locus SSR sizes (C7-N30-N39: 145-176-174bp = A1; 148-177-181bp = T1; 149-177-184bp = T2; 149-177-185bp = T3). Given that individuals showing paternal leakage would likely still carry some of the maternal haplotype (i.e. heteroplasmy), we scored individuals first for their primary fragment peak and scored any secondary peak when it met two criteria 1) it was the alternate allele that would be expected given the type of cross and 2) it was at least 10 % of the

primary peak fluorescence level. Any individual that indicated heteroplasmy was also genotyped two additional times (from the same DNA extraction). All parents were found to be of one primary haplotype.

Since paternal leakage is most likely a rare occurrence, it is critical that parentage be verified to ensure that mistakes were not made during the handling of individuals throughout cultivation, DNA extractions, and genotyping. In order to detect any possible errors in mother-offspring assignment, parentage was verified in those offspring that did not indicate maternal cpDNA inheritance by genotyping the suspected leakage individuals as well as the maternal and paternal donors for nine previously described highly polymorphic nuclear EST-SSRs BL 1, 2, 3, 4, 5, 8, 10, 13, and 17 (for details see Pashley *et al*., 2006; Ellis *et al*., 2006).

Results

Out of 323 observations of inheritance, we found five cases of non-maternal inheritance equaling a leakage rate of at least 1.55 %. Table III-1 provides the cpDNA haplotypes for the offspring indicating paternal leakage. One offspring each of the AL2 X TN2, TN4 X AL3, TN6 X AL3 families showed the paternal haplotype. Two individuals of the TN3 X AL2 family had the paternal haplotype. Maternity and paternity was confirmed in each case that indicated paternal leakage using EST-SSRs. In each of the 323 offspring scored, a primary haplotype was observed; however, some offspring contained a secondary haplotype (at least 10 % of the primary peak) that represented the alternate allele of the appropriate size for the cross type (see Figure III-1 for a representative example). The highest

ratio observed for primary to secondary haplotype was roughly 2:1. All five individuals that indicated primary non-maternal inheritance also carried at least 10 % of the maternal haplotype, and one additional individual (from family AL2 X TN2) primarily contained the maternal haplotype but contained an observable percentage of the paternal haplotype according to our criteria (Table III-2). Further, a Fisher's Exact Test indicated that the probability of detecting heteroplasmy is not independent of the probability of which parent provides the primary haplotype ($p < 0.001$).

Discussion

In this study we found paternal leakage of the chloroplast at a rate of 1.55 % (5/323 observations) or 1.86 % (6/323) accounting for the individual that showed secondary paternal heteroplasmy. This low level of paternal transmission was observable in our study since we designed the experiment to be able to detect leakage 95 % of the time at a transmission rate of one percent or greater (Milligan, 1992). One caveat we would like to discuss with regard to calculating the detection ability is that since there is sometimes a family association with leakage (Birky, 1995; Mogensen, 1996; McCauley *et al*., 2007), the actual number of *independent* data points may not be the total number of observations, but rather probably lies somewhere between the number of families and the number of individuals. Thus when approaching the trade-off between number of families and offspring, and given a constraint on the total number of samples in a study, it is better to sample as many families as possible. Studies that employ a small number of families to detect non-maternal inheritance may be more likely to miss rare events of paternal leakage. In this study we sampled 53 families—a

number that is much larger than previous studies finding strict maternal chloroplast inheritance in other angiosperms (e.g. Vaillancourt *et al*., 2004; Van Droogenbroeck *et al*., 2005). We only found evidence for paternal leakage in inter-population crosses. Often paternal leakage is reported in interspecific hybrids (Soliman 1987; Cruzan *et al*., 1993; Hansen *et al*., 2007) and thus it may be more likely for paternal leakage to occur in crosses between divergent populations than within population crosses. However, no formal statistical calculations were performed on this conclusion as we had so few within population crosses, thus limiting our statistical power.

We also found evidence for chloroplast heteroplasmy in six individuals. Documentation of chloroplast heteroplasmy is rare (but see Frey *et al*., 2005) perhaps due in part to the dogma of strict maternal inheritance in angiosperms. Intra-individual variation has been observed and quantified for plant mitochondrial genes in several cases, (e.g. Hattori *et al*., 2002; McCauley *et al*., 2005; Welch *et al*., 2006) but such observations remain rare. Perhaps not surprisingly heteroplasmy occurs if paternal leakage takes place given that the mother presumably always transfers organelles to her offspring. Chloroplast heteroplasmy has consequences for the population biology of *H. verticillatus*. Given that founder effects or bottlenecks of chloroplasts occur with each successive cell division, even a small amount of paternal leakage at fertilization could, by chance, lead to a mature offspring with the majority paternal haplotype just as it might often be lost. Genetic drift within the individual will also vary at different life stages of the plant. A young plant will likely have fewer cell divisions and may harbor a greater mixture of chloroplast genes, while an older plant has completed more cell divisions thus allowing genetic drift to create a more homoplasmic state (recall we sampled *H. verticillatus* offspring at the young plant stage).

The findings of this study indicate caution must be used when assuming strict maternal inheritance of the chloroplast genome in angiosperms and in unstudied species of *Helianthus* for several reasons. First, chloroplast DNA is often employed in studies examining hybridization and introgression in plants (Rieseberg and Ellstrand, 1993; Edwards-Burke *et al*., 1997; Welch and Rieseberg, 2002; Van Droogenbroeck *et al*., 2006). When strict maternal inheritance is assumed but paternal leakage occurs, incorrect conclusions regarding parental contributions during hybridization and directionality of introgression may be drawn. The sunflower genus is noted for having significant amounts of hybridization and introgression (Rieseberg, 1991; Rieseberg *et al*., 1995; 1996). Paternal transmission of the chloroplast found in *H. verticillatus* indicates the need to be cautious when studying aspects of hybridization in this genus.

Next, the difference between measures of population structure (F_{ST}) using cpDNA and nuclear DNA is often used to evaluate the relative contributions of seed and pollen movement to total gene flow. In theory, if there is strict maternal inheritance of organellar genes then seeds will carry copies of the nuclear and cytoplasmic genomes while pollen will carry only nuclear genes (Birky *et al*., 1983, 1989; Petit *et al*., 1993). Paternal leakage will decrease values of F_{ST} based on organellar DNA, skewing estimates of the contributions of seeds and pollen to gene flow relative to that when maternal inheritance is assumed.

Finally, it has been proposed that transgenes be placed in the chloroplast genome of crop species to prevent their escape (Gressel, 1999; Grevich and Daniell, 2005; Daniell *et al*., 2005). However, low rates of paternal transmission of the chloroplast have been shown in crops species including tobacco (Medgyesy *et al*., 1986) and potato (Simmonds, 1969). Haygood *et al*., (2004) modeled transgene escape and found that even genes with leakage

rates as low as that found in our study will have an appreciable probability of escape into the wild. The possibility of transgene escape at such low leakage rates further highlights the necessity for studies examining mode of organellar inheritance to have a high statistical power to detect rare leakage.

We were also interested in addressing the question of whether it is necessary to study chloroplast transmission in more than one species within the same genus (i.e. is it possible to extrapolate within the genus if one member species' transmission has already been determined). In order to address this, we considered the results of three surveys of potential chloroplast transmission in angiosperms (Sears, 1980; Corriveau and Coleman, 1988; Zhang *et al*., 2003) and after accounting for overlap among them and assuming equal sampling intensities, we found that six out of 113 genera contained conflicting modes of inheritance. In three of the genera, there was predominantly one type of transmission with a low frequency of the other. In our study, we found *H. verticillatus* to have a low level of paternal leakage. This is in contrast to the observation of strict maternal inheritance found in another species, *H. annuus*, within the genus (Rieseberg *et al*., 1994; Wills *et al*., 2005). Combining their dataset with that of Rieseberg *et al*. (1994), Wills *et al*. (2005) determined they were able to detect paternal leakage at a rate of 1.35 % or greater, 95 % of the time, a study that is comparable in magnitude to ours. The findings of this literature survey and our results indicate the mode of chloroplast inheritance cannot always be extrapolated within a genus. Furthermore, the methods used in these studies may only provide conservative estimates given the limited sample sizes per species and ability to detect only the potential mode of inheritance.

In conclusion, we found evidence for a modest amount of paternal transmission of cpDNA and heteroplasmy in the perennial sunflower, *H. verticillatus*, in greenhouse crosses. We are also interested in the consequences of paternal leakage and heteroplasmy for the biology of natural populations. The next step is to address paternal leakage in the field. If we extrapolate our primary paternal leakage rate (1.55 %) to natural populations and assume every leakage event is detectable (i.e. the parents have different cpDNA haplotypes), at least 200 offspring from the field would be necessary to have a 95 % chance of detecting leakage at the frequency we found in the crosses. However, every leakage event will not be detectable given the high level of chloroplast population structure, $F_{ST} = 0.620$ (Ellis *et al*., 2006). The probability of the parents being different, assuming random mating within populations, can be calculated for n number of haplotypes by

$P = 1 - p^2 - q^2 - r^2 - s^2 \ldots n^2$

where p, q, r and s are cpDNA haplotype frequencies from a given natural population. For example, using the four haplotype frequencies from the Tennessee population of *H. verticillatus* (Ellis *et al*., 2006), $P = 0.48$ $(1 - 0.7^2 - 0.12^2 - 0.12^2 - 0.06^2)$. Roughly, only half of the matings will be between parents that differ at these cpSSRs. Thus, multiplying 200, the number of offspring that must be inspected to observe leakage at a rate of 1.55 when parents differ at marker loci, by $1/P$ yields the expectation that 417 offspring must be examined to have a 95 % chance of detecting leakage at a rate of 1.55 % in natural populations given the population structure. These results indicate large sample sizes are necessary to ensure detection of rare paternal leakage in angiosperms especially in natural populations.

Acknowledgements

The authors thank Charles and Mary Lipscomb and Jonathan Ertelt for their assistance in the greenhouse.

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Tables and Figures

Table III-1. Mother and offspring haplotypes showing primary non-maternal inheritance.

Family	Maternal/Paternal Haplotype	# Offspring/Haplotype
AL2 X TN2	A1/T3	6A1/**1T3**
TN4 X AL3	T1/A1	9T1/**1A1**
TN6 X AL3	T2/A1	3T2/**1A1**
TN3 X AL2	T2/A1	2T2/**2A1**

Notes: Given are mother and offspring haplotypes consisting of alleles at three cpSSRs from controlled greenhouse crosses within and among the Madison Co., Tennessee and Cherokee Co., Alabama populations. Only families that showed evidence for primary non-maternal inheritance are presented. Information in bold type indicates paternal leakage.

Table III-2. Inheritance and heteroplasmy classification for all offspring.

	Paternal Inheritance	Maternal Inheritance
Heteroplasmy	5	1
No Heteroplasmy	0	317

Notes: Offspring from all crosses classified according to the type of inheritance and presence or absence of heteroplasmy (see text for details).

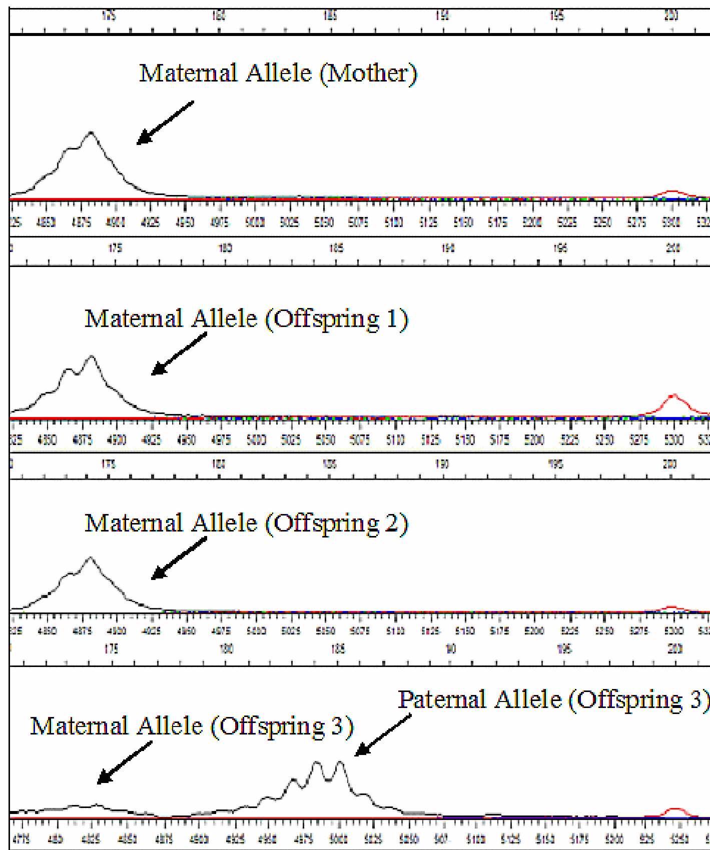

Figure III-1. Electropherogram from cpSSR (chloroplast simple sequence repeat) locus N39 to show an example of chloroplast inheritance. Shown are a mother and three offspring from the AL2 X TN2 family. Note offspring one and two show maternal inheritance while offspring three contains the paternal allele as well as evidence of the maternal allele, indicating paternal leakage and biparental inheritance. There is a size marker at the 200bp position.

CHAPTER IV

ESTIMATION OF CLONAL DIVERSITY IN POPULATIONS OF A RARE SUNFLOWER

Abstract

Populations of rare and endangered species often face many issues detrimental to their fitness and population viability, including the reduction of standing levels of genetic variation, the increased likelihood of inbreeding, and the fixation of deleterious alleles. These problems are exacerbated in small, isolated populations and may also contribute to further reductions in population size. Conservation biologists are thus concerned with active monitoring and management of rare and endangered populations since lowered fitness increases the probability of extinction. Knowledge of the population size is an important first step for identifying populations of immediate concern. However, this task may be difficult in plant species that exhibit clonal growth since a simple "head count" may not be appropriate. The use of simple sequence repeats markers (SSRs) provides a non-invasive sampling strategy for determining genetic individuals with high statistical power; but, the de novo development of such markers is often time consuming and costly. Here, I determine the genetic population size and clonal diversity in a rare sunflower, *Helianthus verticillatus*, using SSRs developed from the Expressed Sequence Tags of the domesticated sunflower *H. annuus*. This approach provides a relatively rapid and inexpensive method for assessing these factors in endangered species. Despite high clonal and genotypic diversity, populations of *H.*

verticillatus consist of far fewer genetic individuals than indicated in previous reports based on head counts. Findings are discussed in the context of the ecological and biological dynamics in clonal plant populations. Finally, the results of this study led to an upgrade in the priority status of this species for the Endangered Species List.

This manuscript will be submitted for publication most likely to the journal *Conservation Genetics.*

Introduction

Conservation biologists and managers are concerned with preserving genetic variation and maintaining fitness in populations of rare or endangered species to promote population viability and evolvability (Primack 2002). Conservation guidelines have also emphasized the significance of possessing genetic variation because of its positive relationship to fitness (Leimu et al. 2006) and its importance for many ecological processes (Hughes et al. 2008). Standing levels of genetic variation may also provide the raw evolutionary material for populations to adapt to changing environments (Willi et al. 2006). However, human induced habitat destruction has led to the loss and fragmentation of many populations, causing reductions in both the number of populations, and the size of individual populations (Wilson 1992; Wilcove et al. 1998). Genetic diversity declines in small populations since heterozygosity is an inverse function of the effective population size and the loss of alleles due to genetic drift is more pronounced in small populations. Moreover, inbreeding is enhanced in small and isolated populations because of the reduced numbers of potential mates. Inbreeding also leads to a loss of genetic diversity through the redistribution

of alleles from the heterozygous to the homozygous state, and often, inbred individuals have lower fitness than outbred individuals owing to inbreeding depression (Charlesworth and Charlesworth 1987).

Active monitoring and management of populations of endangered species are important conservation tasks since lowered population fitness increases extinction risk (Newman and Pilson 1997; Saccheri et al. 1998; Wright et al. 2008). Population censuses and surveys are often a first step to identify populations of conservation concern since population size can determine whether or not a species receives high priority for endangered status and protection (Primack 2002) and is often correlated with fitness (Lemui et al. 2006). However, these tasks may be complicated if species exhibit asexual or clonal reproduction, as do many plant species (Cook 1983), because a simple "head count" may not reveal the true number of genets, or genetic individuals, in a population. A genet consists of all of the genetically identical members that derive from a single zygote (Sarukhan and Harper 1973), while a ramet is an independent physiological individual consisting of its own shoot and root system and capable of independent survival and death (Cook 1983).

The patterning of clones within populations can range from a clumped distribution (ramets of the same genotype always tightly clustered) to one that is uniform (no association). Investigating the extent of clonality, including the spatial structure and clonal diversity, in a population may be achieved through excavating the root system. However, this method is not only extremely intrusive in the case of rare and endangered species, it may incorrectly estimate the true number of individuals if root systems have degenerated between clone-mates or if some ramets have been disassociated and relocated to other areas of the site. Alternatively, the use of polymorphic genetic markers to distinguish individuals is a

non-invasive sampling strategy which yields high probabilities for distinguishing genets (Ainsworth et al. 2003). Often clonal studies employing genetic markers find a significantly lower number of genetic individuals than would have been determined from traditional surveys (Sipes and Wolf 1997; Esselman et al. 1999; Rossetto et al. 2004).

Beyond generating estimates of genetic population size, studies of the clonal diversity and structure of rare and endangered species may aid in understanding the ecological and spatial dynamics of related individuals (Murawski and Hamrick 1990). Such studies also give insight into how pollinator movement influences gene flow in insect pollinated species (Cook 1983). Moreover, these types of investigations are fundamental for understanding population evolutionary dynamics since the genetic individual is likely the unit of selection (Harper 1985; Eriksson and Jerling 1990).

The use of highly polymorphic genetic markers, such simple sequence repeats (SSRs) is advantageous for these types of investigations since they generally provide ample diversity to distinguish individuals with high probability, and their use provides a non-invasive sampling strategy for determining clonal relationships. Moreover, in a recent meta-analysis, Honnay and Jacquemyn (2008) demonstrated that studies which employed highly polymorphic loci, such as SSRs, yielded higher resolution than allozymes for measuring and determining clonal diversity and structure. However, in the case of rare or endangered taxa, obtaining SSRs is sometimes time-consuming and expensive. Employing SSRs developed from publicly available expressed sequence tag (EST) databases is a practical alternative to de novo methods (Bouck and Vision 2007; Ellis and Burke 2007).

Here, I describe a study determining the genetic size and clonal diversity in populations of an extremely rare sunflower species, *Helianthus verticillatus*, using genetic

markers developed from ESTs of the domesticated sunflower, *H. annuus*. This rare sunflower, which is known to only four locations, is native to the southeast United States and exhibits sexual reproduction and clonal growth through rhizomes (Matthews et al. 2002). Management for the species has included population censuses and extensive surveys for any additional populations. Counts of the number of stalks have previously been made and reported to the United States Fish and Wildlife Service (USFWS) to aid in the listing and priority status of *H. verticillatus*. However, these counts may not represent the true number of genetic individuals since the species exhibits clonal growth, e.g., one site was reported to contain thousands of individuals. Moreover, the distribution and arrangement of clones within populations may be particularly important in this species since it is self-incompatible and insect pollinated.

A previous population genetic survey using EST-SSRs revealed surprisingly high levels of genetic diversity in populations of this rare species (Ellis et al. 2006). Despite this, one population exhibited significantly lower fitness values for achene viability and germination, and the fitness differences may be related to the disparities in population sizes (Ellis and McCauley unpublished); however, a study of the clonal structure of these populations is necessary to make accurate estimates of population sizes. Here, I use EST-SSRs to investigate clonal structure in populations of the rare *H. verticillatus* and ask 1) how many genetic individuals are in each population, 2) how are genetic individuals distributed within populations, 3) is this distribution similar in all populations, 4) how does clonality relate to previous findings of high genetic diversity in this species, and 5) are there associations between genetic population size and fitness in this species?

Materials and Methods

Study Species

Helianthus verticillatus is a diploid (n = 17) perennial restricted to only four locations in the southeast interior of the United States: two in western Tennessee in Madison County (Co.) and McNairy Co. (discovered 2006), one in northeastern Alabama in Cherokee Co., and one in northwestern Georgia in Floyd Co. The sunflower, which can grow to greater than four meters (m), has a glaucous stem, leaves mostly verticillate in three's or four's, and flowers August to October. The species exhibits rhizomatous clonal growth and often appears to grow in somewhat distinct clusters or clumps (personal observation). It is a candidate for federal listing for the Endangered Species Act (ESA) and is listed as endangered in each of the three states. The species was first discovered and named in the 1890's in Tennessee near the Madison Co. population. Ellis et al. (2006) demonstrated that *H. verticillatus* harbors high levels of genetic diversity at microsatellite markers as compared to a common congener, perhaps due to its clonal and perennial life history. Habitat loss is a likely cause of rarity in *H. verticillatus* as the species appears to be adapted to prairie habitats which have declined since European settlement (Allison 1995; Matthews et al. 2002).

Ellis et al. (2006) assessed the clonal structure in the Madison Co., Tennessee location and found that clones were clumped, or highly structured, and estimated about 70 genetic individuals in that population. However, the extent to which this species reproduces clonally in the remaining populations is not known. Also, Ellis et al. (2006) only qualitatively

assessed clonal structure in Madison Co., Tennessee; here, I re-evaluate this clonal data to include measures of clonal diversity (see below).

Sample Collections

Leaf samples of *H. verticillatus* were collected from population sites in Cherokee Co., Alabama, Floyd Co., Georgia, and a newly discovered population in McNairy Co., Tennessee. The Alabama population consists of two parts: plants along a small dirt road (AL1) and adjacent to this road (about 5 m away) in a wet field separated by a row of *Rubus* plants (AL2). The Georgia (GA) population contains individuals growing together in a wet prairie, while the McNairy Co., Tennessee population (McTN) consists of two sections of plants growing along Prairie Branch Creek. In all sites except the AL2, plants frequently grow in somewhat distinct clusters. In AL1, GA, and McTN sites, a leaf was collected from three stalks per cluster and the relative locations of individual stalks and distances between stalks were recorded. Note: clusters tended to consist of three to six stalks. In these sites, every cluster that was observed was sampled. Plants in AL2 do not grow in well-defined clumps, rather stems grow in abundance with no definition of clusters. Here, a meter wide transect was set through the largest patch of *H. verticillatus* individuals and the first 100 stalks were collected along the transect (resulting in approximately an 11 m long transect). In total, 243 leaf samples were collected and analyzed (Alabama dirt road, AL1: 15 clusters, 45 stalks; Alabama wet field, AL2: 100 stalks; Georgia, GA: 15 clusters, 45 stalks; McNairy Co., Tennessee site, McTN: 19 clusters, 57 stalks). Figures IV-1 to IV-3 provide rough schematics of the cluster layout in each of the collection areas. Total genomic DNA from each sample was isolated from ~200 mg of fresh leaf tissue using the Doyle and Doyle (1987) CTAB method.

Genotyping

Genotyping for clonal investigations was performed on five previously developed highly polymorphic EST-SSRs (Pashley et al. 2006; Loci BL 2, 4, 6, 7, and 17), known to be in Hardy-Weinberg equilibrium within populations (Ellis et al. 2006), using a modified version of the fluorescent labeling protocol of Schuelke (2000), as detailed in Wills et al. (2005). PCR was performed in a total volume of 20 µl containing 2 ng of template DNA, 30 mM Tricine pH 8.4-KOH, 50 mM KCl, 2 mM MgCl2, 125 µM of each dNTP, 0.2 µM M13 Forward (-29) sequencing primer labeled with either VIC, 6FAM or TET, 0.2 µM reverse primer, 0.02 µM forward primer and 2 units of *Taq* polymerase. The PCR conditions were as follows: 3 minutes at 95° C; ten cycles of 30 s at 94° C, 30 s at 65° C and 45 s at 72° C, annealing temperature decreasing to 55° C by 1° C per cycle, followed by 30 cycles of 30 s at 94° C, 30 s at 55° C, 45 s at 72° C, followed by 20 m at 72° C.

PCR products were visualized on an MJ Research BaseStation automated DNA sequencer (South San Francisco, CA), and MapMarker® 1000 ROX size standards (BioVentures Inc., Murfreesboro, TN) were run in each lane to allow for accurate determination of fragment size. Cartographer v 1.2.6 (MJ Research) was used to infer individual genotypes according to the fragment sizes of the PCR products.

Analysis of Clonal Structure

The probability that each cluster was a single genet and that identical genotypes were not simply obtained by chance, was calculated using a multilocus probability for codominant genotypes, $P_{cgen} = (\Pi p_i)2^h$, where p_i is the local population frequency of each allele observed in the multilocus genotype and h is the number of heterozygous loci (Parks and Werth 1993;

Sydes and Peakall 1998). The probability of obtaining n-1 more copies of that genotype by chance is given by $(P_{cgen})^{n-1}$, where n is the number of times the genotype was observed. Following Ellstrand and Roose (1987), I calculated the proportion of ramets distinguishable by their multilocus genotypes as the number of genotypes divided by the sample size (ramets sampled), G/N ; the inverse of this is the number of ramets per genet. The average distance between stalks of the same genotype was also calculated and compared to the average distance between stalks of different genotypes.

I calculated a measure of clonal diversity using the complement of the Simpson index corrected for finite sample sizes, D, as $D = 1 - \Sigma [n_i(n_i - 1)]/[N(N - 1)]$, for i 1 to G, where n_i is the number of ramets per genet i, N is the total number of individuals sampled, and G is the number of genets (Pielou 1969). For example, if all ramets were different genotypes, i.e., each stalk surveyed was a separate genet, D would equal one. However, if all ramets surveyed were the same genotype, i.e., all the same genet, D would equal zero. Then, I calculated an evenness measure which scales the diversity measure, D, to the minimum and maximum possible values, as $E = (D - D_{min})/(D_{max} - D_{min})$, where $D_{min} = [(G - 1)(2N - G)]/[N(N - 1)]$, and $D_{max} = [N(G - 1)]/[G(N - 1)]$ (Fager 1972). When stalks are collected randomly in a site or patch (as in AL2), values of G/N, D, and E may accurately be compared across other clonal studies which do the same. This was not the sampling strategy in AL1, GA, or McTN; however, since sampling within clusters was equivalent across these sites, clonal diversity values may be compared across these populations in this study.

Results

Ample levels of genetic diversity were detected at the five nuclear loci analyzed to detect clonal diversity patterns in *H. verticillatus*. P_{cgen} values across the all sites ranged from 0.01 to 1.28 x 10^{-12}; $(P_{cgen})^{n-1}$ values range from 4.19 x 10^{-4} to 3.07 x 10^{-33}. The qualitative range of clonal structure from AL1, GA, and McTN varied from all stalks within a cluster consisting of identical genotypes (8 clusters in AL1, 11 in GA, 14 in McTN) to all stalks consisting of different genotypes (2 clusters in AL1, 1 in GA). The number of clusters that consisted of two or more genotypes was 7 in AL1, 3 in GA, and 5 in McTN. There was also one case in which two clusters separated by one meter shared identical genotypes in GA. Tables IV-1 to IV-3 provide information regarding the clonal data for these three sites. In AL2, several genotypes were observed more often than others with two genotypes being surveyed eight times each (Table IV-4).

To a large extent, clusters tended to consist of the same genotype. The average distance between stalks of the same genotype was 0.12 m ± 0.32 (mean ± SE) with the average distance between stalks of different genotypes being 14.98 ± 0.25, indicating a clumped distribution. In AL1, 24 unique multilocus genotypes, or genets, were found out of 45 sampled stalks and 15 clusters, the AL2 sample detected 46 genets out of 100 samples; in GA there were 18 genets out of 45 sampled stalks and 15 clusters, and McTN had 24 genets for 57 sampled stalks and 19 clusters. In the four sites about half of the stalks sampled resulted in unique genetic individuals, i.e., the proportion distinguishable, G/N, was AL1 = 0.53, AL2 =0.46, GA = 0.40, McTN = 0.42. The number of ramets per genet is the inverse of this value: AL1 = 1.9, AL2 = 2.2, GA = 2.5, McTN = 2.4. Table IV-5 provides values for

G/N, D, and E. Note that the pattern of one genotype per two stalks as seen in the AL1, GA, and McTN sites was also observed in AL2 despite the un-ordered spacing of stalks.

The clonal structure identified in these populations was somewhat different from that obtained previously from the Madison Co., Tennessee (MdTN) population, where all stalks from the same cluster yielded identical multilocus genotypes, compared to the finding here of some clusters having multiple genotypes (Ellis et al. 2006). This previous result suggested that genets could be identified in the field based solely on the clustering of stalks and that the genetic population size is much smaller than the number of stalks. However, the AL1, GA, and McTN samples have somewhat different patterns of clonal structure given that some clusters contain more than one genotype and that stalks of two clusters in GA had identical genotypes. Still, the number of genetic individuals in the three populations surveyed here is smaller than the number of stalks—about two times smaller. Note that the difference in clonal structure between the two studies was not due to a lack of statistical power as nine nuclear loci were used in the previous study compared to five here.

Discussion

Genetic Population Size and Clonal Variation

This study revealed that populations of *H. verticillatus* consist of far fewer genetic individuals than previously reported based solely upon counting stalks. The proportion of distinguishable ramets based on their multilocus genotypes ranged from 0.40 to 0.53, and the mean number of ramets per genet ranged from 1.9 to 2.5. In general, most clusters of stalks consisted of the same genotype. This was similar to the pattern found for the previously

studied Madison Co., Tennessee population in which clusters corresponded to genetic individuals (Ellis et al. 2006). Therefore, counting distinct clusters is more appropriate than counting stalks for estimating the number of genetic individuals. All observed clusters were sampled in the Georgia and McNairy Co., Tennessee populations and along the Alabama dirt road, and simply counting clusters would have only slightly underestimated the number of genetic individuals (AL1: 15 clusters, 24 genets; GA: 15 clusters, 18 genets; McTN: 19 clusters, 24 genets). However, given the sensitive nature of endangered species and the extreme rarity both in numbers of individuals and populations, a conservative estimate of counting clusters seems more appropriate in this species. In AL2, 46 distinct genets were identified along the 100 stalk transect. In this entire site, several hundred stalks were present, and given that roughly half are distinguishable, or put differently, on average there were 2.2 ramets per genet, there may be at least 100 but probably no more than 200 genetic individuals present in the wet field.

Clonal diversity was similar and high across all sites surveyed ranging from 0.95 to 0.98. Fager's evenness measures were also high and uniform in all the study sites ranging from 0.93 to 0.96 and indicated that genotypes were evenly distributed among clones. The Madison Co., Tennessee population also had equivalent values for these measures (as calculated from Ellis et al. 2006). Contrasting clonal values in the AL2 site, which was sampled randomly with respect to clusters (see Methods), with means from studies of other clonal species reveals that this population exhibits high levels of clonal diversity and high evenness measures (AL2: $D = 0.97$, $E = 0.95$; self-incompatible species' mean from Honnay and Jacqeumyn' meta-analysis [2008]: $D = 0.75 \pm 0.04$, $E = 0.67 \pm 0.05$).

Consequences of Clonal Reproduction

Knowledge of levels and patterns of clonal diversity is important to understanding biological and ecological dynamics in plant populations. In insect pollinated species, these factors are associated with how pollinator movement influences gene flow (Cook 1983), and a high level of clonal structure can have detrimental effects of plant fitness. For example, a clumped clonal structure in the whortleberry, *Vaccinium myrtillus*, promoted increased selfing and subsequently reduced fitness through geitonogamous self-pollination (the transfer of pollen between flowers of the same genet) as bumblebee pollinators displayed short flight distances between foraging visits (Albert et al. 2008). In self-incompatible species, clonal diversity and structure may be especially important since these factors influence mate availability and influence the likelihood of receiving related, incompatible pollen (Handel 1985; Charpentier et al. 2000).

Some have also suggested that clonal reproduction may lower genotypic diversity (Chung 1995; Sydes and Peakall 1998). Honnay and Jacqeumyn (2008) proposed that there may be negative consequences associated with clonal reproduction in self-incompatible species through reduced mate availability and decreased sexual recruitment given that self-incompatible species have lower genotypic diversities than self-compatible species. The findings of previous work (Ellis et al. 2006), however, indicate that genotypic diversity is high in this extremely rare species. Clonal reproduction does not appear to have negatively affected genotypic diversity; but rather, it may have provided a buffer against the loss of genetic diversity, generally associated with such rare species, by reducing the probability of genet death (Cook 1983). Its perennial life cycle, combined with clonal growth, may mean that relatively few generations have passed since *H. verticillatus* became rare. This

possibility of a relatively recent decline is also suggested by Matthews et al. (2002) based upon historical information from species with similar prairie affinities.

The high level of clonal and genotypic diversity in this species may also indicate ongoing sexual reproduction and recruitment in these populations, facilitating the maintenance of genetic diversity. Soane and Watkinson (1979) demonstrated through modeling and experimentation that even low rates of seedling recruitment can be enough to maintain or increase population genetic diversity. Other clonal species have exhibited similar levels of clonal and genotypic diversity attributed to sexual reproduction, for example, Burke et al. (2000) found high levels of genotypic diversity in clonal populations of Louisiana Iris, indicating ongoing sexual reproduction in spite of substantial clonal growth. This pattern has also been observed in rare species; in the outcrossing endangered lady's slipper, *Cypripedium calceolus*, populations maintained high levels of genotypic diversity despite a low level of sexual recruitment (Brzosko et al. 2002). Finally, in the endemic perennial, *Adenophora grandiflora*, Chung and Epperson (1999) demonstrated high levels of genetic diversity attributed, in part, to predominate outcrossing this species.

Another interesting point to consider with respect to conservation management is the relationship among population size, genetic diversity, and fitness of endangered species. Population genetic and inbreeding theory predict positive associations among these factors (Charlesworth and Charlesworth 1987; Ellstrand and Elam 1993; Lande 1995); and correspondingly so, Leimu et al. (2006) found overall positive correlations among these factors in a meta-analysis considering studies which evaluated these relationships in plants. Previous work indicated high levels of genetic diversity in all populations of this species (Ellis et al. 2006), yet a recent study of fitness related traits demonstrated that the Madison

Co., Tennessee populations exhibited lower levels of achene viability and germination rates than the Alabama population (Ellis and McCauley unpublished data). The lower quantitative fitness values in the Madison Co., Tennessee population may be related to the smaller number of genetic individuals in this site and suggest the potential for higher levels of inbreeding there as compared to Alabama. Moreover, the small Georgia population exhibited extremely poor fitness, with low germination rates and no individuals surviving to flowering. The McNairy Co., Tennessee site had not been discovered when this fitness study was conducted; given the previous results, however, the fitness and viability of this population should be considered since it also contains a low number of genetic individuals.

Conservation Implications

While *H. verticillatus* harbors high levels of genetic and clonal diversity, the number of individuals is alarmingly low. This, combined with the fact that only four populations are known to exist in this species, calls for an immediate conservation management plan of these populations. The only officially protected population is the Georgia site which contains the smallest number of genetic individuals and exhibits poor fitness. This site was originally reported to contain thousands of stalks and photographs from a 1998 survey indeed demonstrate a large number of tall (> 4 m) *H. verticillatus* individuals (Allison 2002; J. Matthews personal communication). However, in recent trips (annually 2004 – 2007) to this population, a drastically lower number of stalks was observed and no plants were taller than 1.5 m (personal observation). The data of this study was reported to the USFWS, and they subsequently changed the priority ranking for this species from a low to high priority. The Alabama wet prairie site and the adjacent dirt road individuals, by far, represent the largest population of *H. verticillatus* individuals known, yet there is not formal protection for it.

Protection of this site is especially warranted since it also harbors significantly different quantitative variation for achene viability and germination rates. If possible, habitat protection for the remaining Tennessee sites is also important given the low number of populations in this species.

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Table IV-1. Alabama (Site 1) Clonal Data

Cluster (No. stalks Sampled)	**No. genotypes within cluster**	**Genotype**	**No. times genotype observed**	**Prob. of genotype (P_{cgen})**	**Prob. being present n times $(P_{cgen})^{n-1}$**
AL 1 (3)	2	AL 1 - A	1	0.04204494	N/A
		AL 1 - B	2	0.00070654	0.00070654
AL 2 (3)	1	AL 2 - A	3	0.00014479	2.09641E-08
AL 3 (3)	3	AL 3 - A	1	0.00011285	N/A
		AL 3 - B	1	6.8406E-05	N/A
		AL 3 - C	1	4.5313E-05	N/A
AL 4 (3)	2	AL 4 - A	2	4.4167E-07	4.4167E-07
		AL 4 - B	1	4.9349E-08	N/A
AL 5 (3)	1	AL 5 - A	3	3.4987E-05	1.22406E-09
AL 6 (3)	3	AL 6 - A	1	5.7933E-05	N/A
		AL 6 - B	1	0.00102266	N/A
		AL 6 - C	1	5.8018E-06	N/A
AL 7 (3)	1	AL 7 - A	3	2.279E-06	5.19384E-12
AL 8 (3)	1	AL 8 - A	3	0.00024296	5.90298E-08
AL 9 (3)	1	AL 9 - A	3	9.7243E-06	9.45629E-11
AL 10 (3)	2	AL 10 - A	2	0.00254701	0.00254701
		AL 10 - B	1	0.00102266	N/A
AL 11 (3)	1	AL 11 - A	3	8.9943E-06	8.08983E-11
AL 12 (3)	1	AL 12 - A	3	4.7908E-05	2.29522E-09
AL 13 (3)	1	AL 13 - A	3	3.7862E-06	1.43352E-11
AL 14 (3)	2	AL 14 - A	2	4.5889E-06	4.58886E-06
		AL 14 - B	1	9.2725E-07	N/A
AL 15 (3)	2	AL 15 - A	2	8.2291E-05	8.22907E-05
		AL 15 - B	1	7.5728E-06	N/A

Cluster (No. stalks sampled) = a priori defined cluster and the number of stalks sampled from that cluster. No. genotypes within cluster = number genotypes present in cluster as detected by genetic data. Genotype = arbitrary multi-locus genotype descriptor. No. times genotype observed = how many time the particular genotype was observed in the total sample. Prob. of genotype (P_{cgen}) = $(\Pi p_i)2^h$. Prob. being present n times = $(P_{cgen})^{n-1}$ (see text for more detail).

Table IV-2. Georgia Clonal Data

Cluster (No. stalks Sampled)	No. genotypes within cluster	Genotype	No. times genotype observed	Prob. of genotype (P_{cgen})	Prob. being present n times $(P_{cgen})^{n-1}$
GA 1 & 2 (6)	1	GA 1 - A	6	0.00014722	6.91611E-20
GA 3 (3)	1	GA 3 - A	3	6.58E-06	4.32786E-11
GA 4 (3)	1	GA 4 - A	3	1.5619E-10	2.43945E-20
GA 5 (3)	1	GA 5 - A	3	1.5334E-08	1.5334E-08
GA 6 (3)	1	GA 6 - A	3	0.00583714	3.40722E-05
GA 7 (3)	1	GA 7 - A	3	1.4606E-05	2.13344E-10
GA 8 (3)	2	GA 8 - A	2	2.9662E-05	2.9662E-05
		GA 8 - B	1	3.2596E-06	N/A
GA 9 (3)	1	GA 9 - A	3	2.7798E-08	7.72712E-16
GA 10 (3)	3	GA 10 - A	1	1.2214E-10	N/A
		GA 10 - B	1	5.4272E-08	N/A
		GA 10 - C	1	2.4926E-12	N/A
GA 11 (3)	1	GA 11 - A	3	1.4111E-08	1.99124E-16
GA 12 (3)	2	GA 12 - A	2	1.3779E-08	1.3779E-08
		GA 12 - B	1	7.3056E-06	N/A
GA 13 (3)	1	GA 13 - A	3	1.3779E-08	1.89871E-16
GA 14 (3)	1	GA 14 - A	3	1.2827E-12	1.64541E-24
GA 15 (3)	1	GA 15 - A	3	7.0206E-05	4.92887E-09

See Table IV-1 for description of columns.

Table IV-3. Tennessee Clonal Data

Cluster (No. stalks Sampled)	No. genotypes within cluster	Genotype	No. times genotype observed	Prob. of genotype (P_{cgen})	Prob. being present n times $(P_{cgen})^{n-1}$
McTN 1 (3)	2	McTN 1 - A	2	7.76932E-05	7.76932E-05
		McTN 1- B	1	0.000197375	N/A
McTN 2 (3)	1	McTN 2 - A	3	0.007146792	5.10766E-05
McTN 3 (3)	1	McTN 3 - A	3	5.90467E-06	3.48651E-11
McTN 4 (3)	1	McTN 4 - A	3	2.7314E-06	7.46056E-12
McTN 5 (3)	1	McTN 5 - A	3	0.02904768	0.000843768
McTN 6 (3)	1	McTN 6 - A	3	4.44787E-05	1.97835E-09
McTN 7 (3)	1	McTN 7 - A	3	7.16164E-06	5.12891E-11
McTN 8 (3)	1	McTN 8 - A	3	2.07024E-06	4.28589E-12
McTN 9 (3)	2	McTN 9 - A	2	7.00322E-07	7.00322E-07
		McTN 9- B	1	2.5625E-07	N/A
McTN 10 (3)	1	McTN 10 - A	3	1.57001E-05	2.46492E-10
McTN 11 (3)	1	McTN 11 - A	3	0.000133436	1.78052E-08
McTN 12 (3)	1	McTN 12 - A	3	2.44915E-05	5.99834E-10
McTN 13 (3)	1	McTN 13 - A	2	5.5084E-06	5.5084E-06
McTN 14 (3)	2	McTN 14 - A	2	6.15797E-06	6.15797E-06
		McTN 14- B	1	0.002778662	N/A
McTN 15 (3)	1	McTN 15 - A	3	2.09002E-07	4.36819E-14
McTN 16 (3)	1	McTN 16 - A	3	0.000538697	2.90194E-07
McTN 17 (3)	2	McTN 17 - A	1	5.59503E-06	N/A
		McTN 17- B	2	0.000601665	0.000601665
McTN 18 (3)	2	McTN 18 - A	2	1.34903E-06	1.34903E-06
		McTN 18- B	1	6.71404E-07	N/A
McTN 19 (3)	1	McTN 19 - A	3	0.000230218	5.30002E-08

See Table IV-1 for description of columns.

Table IV-4. Alabama wet prairie site (AL2) transect of stalks.

Genotype (put. genet)	**No. times genotype observed (put. ramet)**	**Prob. of genotype (P_{cgen})**	**Prob. being present n times $(P_{cgen})^{n-1}$**
AL2 – 1	1	0.00261791	N/A
AL2 – 2	3	0.00012423	1.5433E-08
AL2 – 3	2	4.0148E-05	4.0148E-05
AL2 – 4	1	0.00046984	N/A
AL2 – 5	1	5.7854E-05	N/A
AL2 – 6	2	3.9908E-06	3.9908E-06
AL2 – 7	1	2.288E-06	N/A
AL2 – 8	5	1.7616E-05	9.63E-20
AL2 – 9	3	6.2577E-05	N/A
AL2 – 10	1	1.0032E-05	N/A
AL2 – 11	2	2.6387E-05	2.6387E-05
AL2 – 12	1	3.7748E-07	N/A
AL2 – 13	1	0.08399425	N/A
AL2 – 14	4	0.00018769	6.61147E-12
AL2 – 15	1	0.01421948	N/A
AL2 – 16	1	0.00010162	N/A
AL2 – 17	2	0.00041909	0.00041909
AL2 – 18	1	1.7272E-05	N/A
AL2 – 19	1	1.5296E-05	1.5296E-05
AL2 – 20	1	1.001E-06	1.0087E-05
AL2 – 21	1	1.0087E-05	N/A
AL2 – 22	1	0.00190656	N/A
AL2 – 23	1	3.1442E-06	N/A
AL2 – 24	3	7.2727E-07	5.28924E-13
AL2 – 25	2	5.2654E-06	5.2654E-06
AL2 – 26	1	1.1103E-05	N/A
AL2 – 27	1	0.000806	N/A
AL2 – 28	5	1.4492E-05	6.39229E-25
AL2 – 29	3	1.076E-06	1.15785E-12
AL2 – 30	1	5.4592E-06	N/A
AL2 – 31	8	1.108E-07	2.05076E-49
AL2 – 32	1	7.4821E-08	N/A
AL2 – 33	2	0.00031687	0.000316866
AL2 – 34	8	2.2664E-05	3.07182E-33
AL2 – 35	3	2.5634E-06	6.57088E-12
AL2 – 36	1	2.8723E-07	N/A
AL2 – 37	1	1.0758E-06	N/A
AL2 – 38	1	8.1648E-05	N/A
AL2 – 39	2	3.3368E-05	3.3368E-05
AL2 – 40	1	6.1011E-07	N/A
AL2 – 41	7	0.00041909	5.41812E-21

AL2 – 42	1	9.9748E-05	N/A
AL2 – 43	1	3.8097E-05	N/A
AL2 – 44	4	9.7325E-07	9.21876E-19
AL2 – 45	3	0.00016732	2.79958E-08
AL2 – 46	2	4.4671E-05	4.4671E-05

See Table IV-1 and Results for description of columns.

Table IV-5. Clonal diversity measure for all sites.

Population	**G/N**	**D**	**E**
Alabama 1	0.53	0.98	0.96
Alabama 2	0.46	0.97	0.95
Georgia	0.40	0.95	0.96
McNairy Co, TN	0.42	0.98	0.93
Madison Co, TN*	0.41	0.95	0.99

*Madison Co. data from Ellis et al. (2006).

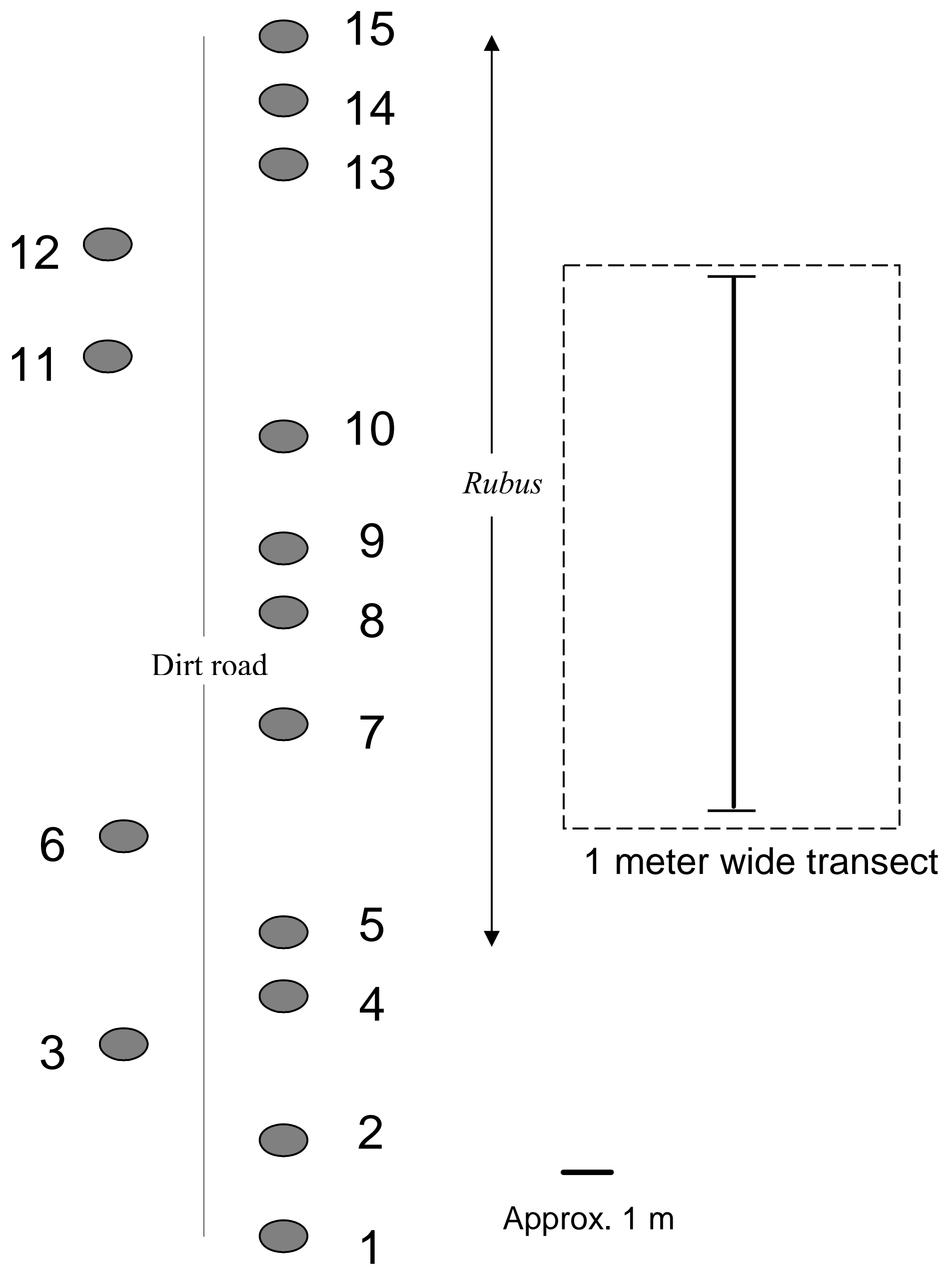

Figure IV-1. Alabama dirt road site (AL1) and adjacent wet field (AL2). Note: width of cluster not to scale. Individuals of AL1 were collected along a small dirt road. Adjacent to the dirt road, separated by a row of *Rhubus*, was a large patch of *H. verticillatus* individuals that did not appear to grow in distinct clusters. Thus, for these individuals (AL2), a one meter-wide transect was set through the middle of the patch of *H. verticillatu*s plants and running the entire length of the patch. The dotted rectangle roughly estimates the boundaries of the patch.

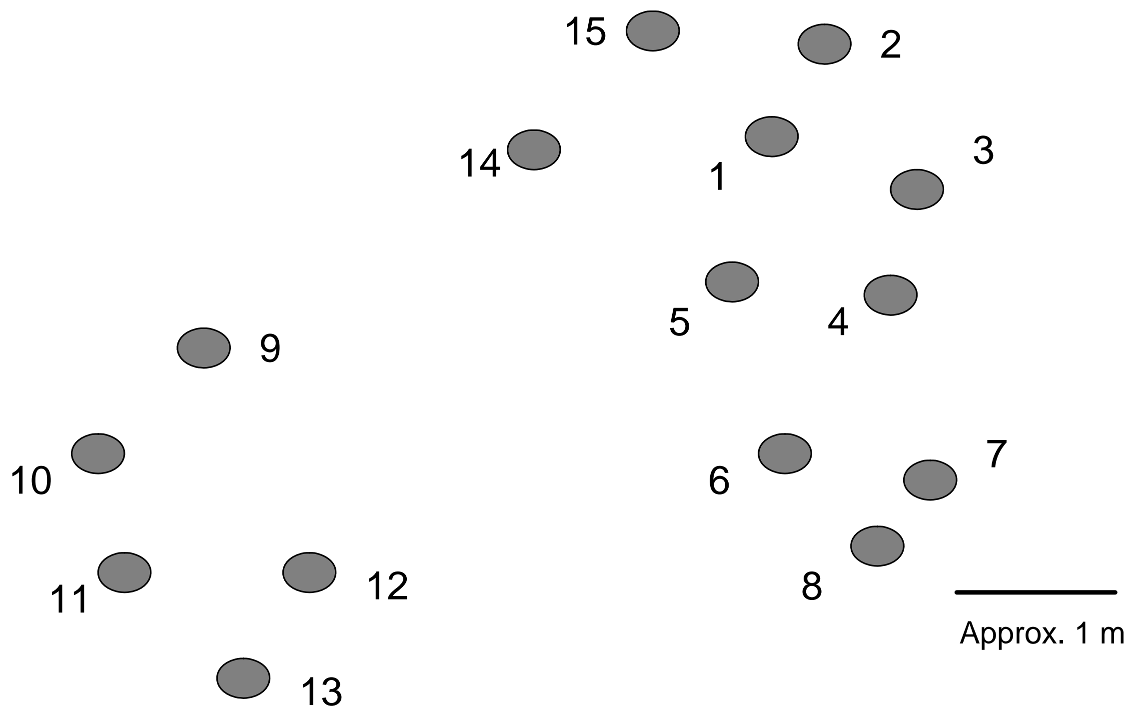

Figure IV-2. Georgia site (GA). Note: width of cluster not to scale.

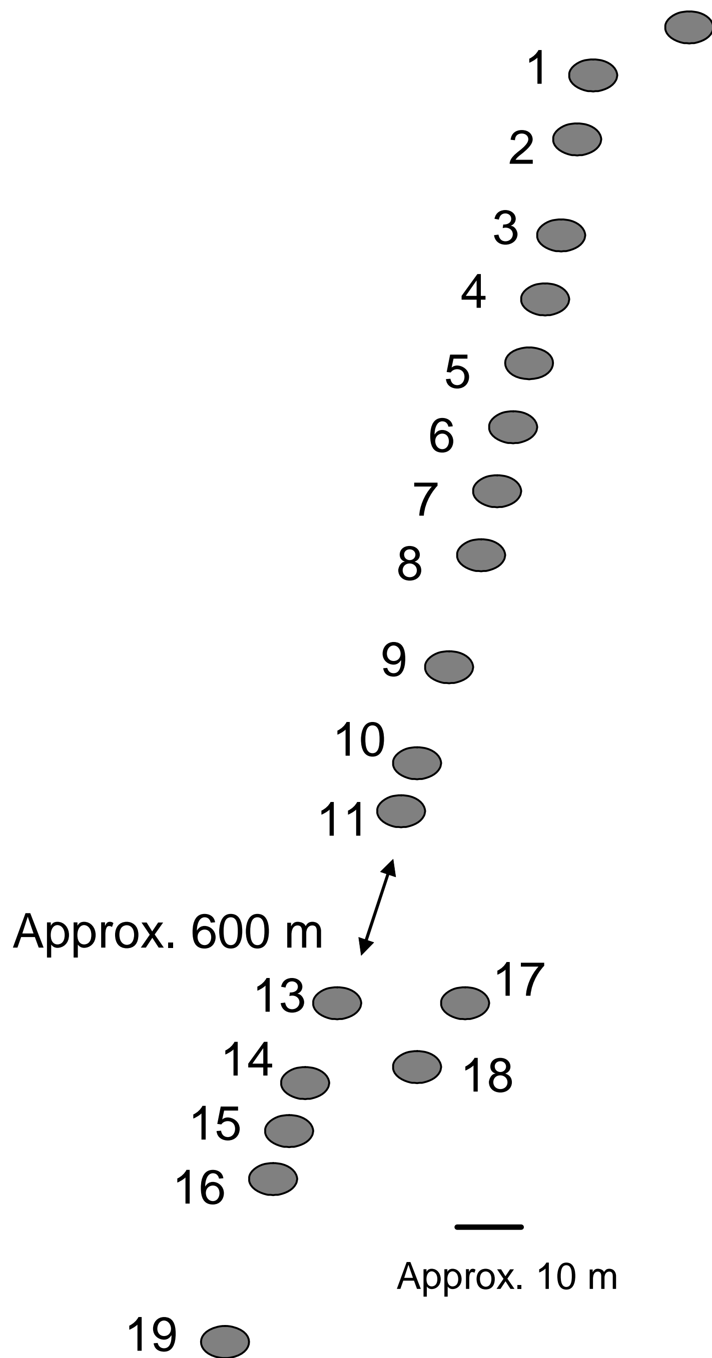

Figure IV-3. McNairy County, Tennessee Site (McTN). Note: width of cluster not to scale. Individuals here, were collected along a small creek.

CHAPTER V

PHENOTYPIC DIFFERENTIATION IN FITNESS RELATED TRAITS BETWEEN POPULATIONS OF AN EXTREMELY RARE SUNFLOWER: CONSERVATION MANAGEMENT OF ISOLATED POPULULATIONS

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This chapter has been submitted to *Biological Conservation*.

Abstract

Knowledge of the genetic and demographic consequences of rarity is crucial when evaluating the effects of habitat loss and fragmentation on population viability, and for creating management plans in rare plant species. Reduction in population size and in the number of populations can lead to decreased genetic diversity and increased inbreeding. Genetic diversity is often correlated with levels of fitness and is frequently used to identify populations of greatest conservation concern, or those that may be good candidates for ex situ conservation programs. However, an association between these factors is not always clear, and crossing studies evaluating whether there is phenotypic differentiation among populations in fitness related traits can inform managers of suffering populations or good sources for ex situ materials. Crossing studies can also evaluate the potential for 'genetic rescue' to boost fitness in suffering populations. To address these questions, we conducted two generations of controlled crosses between populations of the extremely rare and fragmented sunflower, *Helianthus verticillatus*. We measured achene viability, germination, survival, and pollen viability (F_1 only) in 176 F_1 and 159 F_2 families. The populations were differentiated with respect to phenotypic fitness measures with one population having significantly lower achene viability and germination. Also, the potential for genetic rescue was observed as gene flow into the less fit population resulted in higher fitness measures in both the F_1 and F_2. Results are discussed with respect to the importance of combining genetic marker data with crosses and the implications for conservation in disjunct populations of rare species.

Keywords: outbreeding depression, conservation, genetic variation, F_2, genetic rescue, population viability

Introduction

Habitat destruction is one of the greatest factors contributing to the decline of global biodiversity and has led to the fragmentation and loss of whole populations, as well as to reductions in numbers of individuals within populations, in many species worldwide (Wilson, 1992; Wilcove et al., 1998). Many issues affect small, isolated populations, including genetic factors, that make them a crucial concern for conservation biologists and managers. Populations which remain small lose genetic diversity faster than their larger counterparts since genetic drift is stronger in small populations (Lande, 1995). Reductions in standing levels of genetic diversity can limit a species' ability to respond or adapt to changing environmental conditions, affecting long-term viability and important ecological processes (Frankel et al., 1995; Willi et al., 2006; Hughes et al., 2008). Further, the accumulation of deleterious mutations can be significant given that purifying selection is less effective in small populations (Lynch et al., 1995) and subjects such populations to inbreeding depression, which has been shown to increase extinction risk in small populations (Charlesworth and Charlesworth, 1987; Ellstrand and Elam, 1993; Newman and Pilson, 1997; Wright et al., 2008). Moreover, even if populations are relatively large, extreme isolation could prevent populations from realizing the positive effects of gene flow, such as spreading advantageous mutations (Rieseberg and Burke, 2001) and alleviating the

deleterious effects of inbreeding associated with genetic drift (Keller and Waller, 2004; reviewed in Palstra and Ruzzante, 2008).

Conservation geneticists are thus interested in the consequences of rarity and population isolation for the long-term persistence and sustainability of species, including rare or endangered plant species. Data from numerous genetic marker studies demonstrates that rarity and fragmentation tend to erode levels of genetic variation in plants (Hamrick and Godt, 1989; Gitzendanner & Soltis, 2000; Nybom, 2004). These measures of genetic diversity are often positively correlated with levels of fitness in populations (Leimu et al., 2006) and are therefore frequently used to identify populations of the greatest conservation concern (Bonin et al., 2007). Since resources for protecting endangered species are limited and time constraints may be substantial, decisions of which populations are in need of immediate protection (in situ conservation strategies) or which populations would serve as a good source for genetic material (ex situ conservation strategies) are often made using information regarding population genetic information from markers (Center for Plant Conservation, 1991; Primack 2002; McDonald-Madden et al., 2008).

A definitive association between these factors is not always clear, however, given that some studies report non-significant or even negative relationships between fitness and genetic variation, e.g., Lammi et al. (1999), Greimler & Dobes (2000), Jacquemyn et al. (2007). Particularly interesting are the many studies of rare or endangered plant species that report unexpectedly high levels of genetic diversity as revealed by markers (Lewis & Crawford, 1995; Young and Brown, 1996; Maki & Asada, 1998; Song & Mitchell-Olds, 2007). In these cases, we may especially want to ask how the molecular genetic diversity and fitness variation within and among populations are related. Information from neutral genetic

variation may not accurately reflect variation in quantitative traits that are important to fitness (Lynch, 1996; Merila and Crnokrak, 2001). Thus, it may be risky to draw conclusions about the population viability of rare species based solely upon genetic marker information (Oostermeijer et al., 2003; Ouborg et al., 2006). Studies which combine measurements of population fitness, genetic diversity, and population structure may ensure that quantitative variation related to viability as well as neutral genetic variation for future adaptability are preserved, especially when not all populations can be protected (Rader et al., 2005).

Comparing phenotypic fitness measures from controlled intra-population crosses of an endangered species can inform conservation biologists whether there is population differentiation with regard to these fitness related life-history traits. When populations are isolated, their evolutionary trajectories and dynamics can become independent of one another; these populations may exhibit differentiation in fitness characteristics due to differences in susceptibility to inbreeding depression as a result of genetic drift (Keller and Waller, 2002; Glemin et al., 2003; Willi et al., 2005) or due to differential selection and adaptation (Nagy and Rice, 1997; Becker et al., 2006). Information regarding both genetic and phenotypic differentiation will be important when determining which populations may be the most appropriate to protect or the best source candidates for captive breeding programs or seed storage (Primack, 2002).

Evaluation of the fitness consequences of controlled inter-population crosses can give insight into how more active conservation strategies, such as introducing new individuals or gene flow events, will affect the viability of populations, as well as provide a first look at the genetic basis of any differentiation. Frequently, experiments find that there is increased fitness, or heterosis, in the F_1 generation of crosses between small or isolated

populations (e.g. Heschel and Paige, 1995; Richards, 2000). For this reason, a possible conservation strategy for increasing the likelihood of survival in rare species is to introduce new genetic material into populations suffering from reduced fitness, i.e., 'genetic rescue', the increase in population fitness owing to immigration of new alleles (Richards, 2000; Ingvarsson, 2001; Tallmon et al., 2004).

Although genetic rescue can lead to heterosis, the immigration of genetically divergent individuals into a population can also lead to a decrease in fitness (outbreeding depression) owing to the dilution of local adaptations or disruption of co-adapted gene combinations, and the success of genetic rescue may decrease with increasing genetic isolation, especially in the case of highly disjunct rare species (Templeton, 1986; Waser and Price, 1989; Lynch, 1991). Fewer studies have found evidence for outbreeding depression perhaps since many experiments only study fitness of individuals in the F_1 generation (Tallmon et al., 2004; Edmands, 2007). Outbreeding depression is more likely to be manifested in the F_2 generation and beyond since heterozygosity peaks in the F_1 and recombination will not break up any co-adapted gene complexes until later generations (Lynch, 1991; Fenster and Galloway, 2000). Furthermore, outbreeding depression due to the disruption of favorable epistatic interactions would have to be greater than the positive fitness effects on the population fitness due to heterosis in the F_1 generation, in order for outbreeding depression to be detected in the F_1 generation. Studies evaluating the fitness consequences of outbreeding should be carried out to at least the F_2 generation in order to fully investigate the effects of both inbreeding and outbreeding on population fitness, as emphasized by Tallmon et al. (2004) and Edmands (2007).

In this study, we evaluate population differentiation with regard to phenotypic fitness characteristics and consider the potential consequences of gene flow in populations of the rare sunflower, *Helianthus verticillatus*. Despite the general expectation of reduced genetic variation in rare species, *H. verticillatus* does not exhibit low levels of genetic diversity nor are there significant differences in levels of genetic variation among its few and isolated populations (Ellis et al., 2006). If fitness is correlated with genetic diversity, one might expect no significant decline in fitness or differences in fitness between these populations. Moreover, measures of population genetic structure revealed only moderate levels of genetic differentiation despite extreme geographical separation in this species. The high levels of population genetic diversity and relatively low population structure indicated that these populations may be interchangeable with regard to protection and that any population would be a good source for seed storage. However, as mentioned previously, it may be dangerous to rely solely upon information from genetic markers for determining populations of immediate concern, especially in species with unexpected population genetic results. Thus, by conducting controlled intra-population crosses in a common environment, we investigate the effects of rarity and isolation on the fitness of *H. verticillatus* populations and ask: 1) do the populations differ in their phenotypic fitness characteristics or are they interchangeable, and 2) how are these phenotypic fitness characteristics related to population genetic information? We are also interested in the potential for genetic rescue through gene flow events among disjunct populations of extremely rare species. Specifically, we also address questions relating to the genetic, or intrinsic, fitness consequences of isolation by conducting inter-population crosses through the F_2 generation and asking: 1) is there the potential for genetic rescue as evidenced by higher mean fitness of hybrid individuals as compared to any or all

parental populations, and 2) is there evidence for intrinsic outbreeding depression, especially in the F_2 generation? Given these results, we discuss the importance of combining genetic marker information with that of controlled crosses and the implications for management of extremely rare, isolated species.

Materials and Methods

Study Species

Helianthus verticillatus was first collected in western Tennessee in 1892 and was not found in nature again until 1994 when it was discovered in Floyd Co., Georgia. In 1996 and 1998, populations of *H. verticillatus* in Cherokee Co., Alabama and Madison Co., Tennessee, respectively, were discovered (Matthews et al., 2002). In the fall of 2006, another population in McNairy Co., Tennessee about 50 km from the Madison Co. site, was discovered. The species is an extremely rare diploid (n = 17) self-incompatible perennial known only to these four locations in the southeast interior of the United States. The Alabama and Georgia populations are about 3.5 km from each other whereas the Tennessee populations are about 350 km from the others. The species is clonal with slender rhizomes, a glaucous stem, leaves mostly verticillate in three's or four's, prefers wet habitats, and flowers August to October. Mature plants range in height from 0.6-4.2m in the field, and clones occur in somewhat distinct clusters in nature. It is a candidate for Federal listing for the Endangered Species Act (ESA) and is listed as endangered in each of the three states. The Georgia site has formal protection under a conservation easement; however, there is no official protection for the remaining sites. The populations are highly clonally structured and consist of a small number

of genetic individuals (Ellis et al., 2006). Extensive surveys for any additional populations of this species have been carried out since the rediscovery of the species in 1994 and still only four locations are known (pers. comm. Tennessee Natural Heritage Division).

Crossing Experiment and Design

Composite flower heads of *H. verticillatus* from different genetic individuals were collected in the fall of 2004 from the Alabama, Georgia, and Madison Co., Tennessee sites. These are the three populations for which we also have extensive genetic marker information (Ellis et al., 2006). The McNairy Co., Tennessee population had not been discovered when this experiment began and thus, was not included. Two attempts at cultivating Georgia plants failed, as these flower heads contained a very low number of viable achenes which exhibited poor germination rates. No Georgia plants survived to flowering in the greenhouse. Further, many of these Georgia plants do not flower in the field and this may indicate extremely low fitness in this population. Therefore, only the Madison Co., Tennessee and Alabama populations were included in the crossing experiment. As mentioned earlier, these are two of the populations which were studied previously for population genetic diversity and structure (Ellis et al. 2006). Thus, at the time of this crossing study, these two sites represented two-thirds of the known populations of this species and half of the currently known populations.

Heads were allowed to dry for one week, and achenes were then removed and placed in small coin envelopes. These envelopes were placed in sealed plastic jars in the refrigerator (four degrees Celsius) for one month to break seed dormancy (pers. comm. J.F. Matthews). After one month, achenes were nicked with a razor blade, allowed to germinate on moistened filter paper, and then transplanted and grown in the Vanderbilt University Department of Biological Sciences greenhouse. These represented the parental individuals in

the following cross study and are hereafter referred to as ‘parentals’. Plants were grown in a commercially purchased top soil and perlite mixture. Grow lights were kept on a 16:8 light: dark cycle. The temperature ranged from about 25-30 degrees Celsius with roughly 70 percent humidity over the course of the experiment. Since this species can grow over four meters high, we had to build a support system of PVC piping in the greenhouse and crosses were conducted from a three meter ladder. This experiment was the first time a crossing study of this magnitude had been conducted in this species.

Parental plants reached reproductive maturity in the fall of 2005 and four types of crosses were conducted: intra-population F_1 crosses (Tennessee X Tennessee, or TT_1; Alabama X Alabama, or AA_1; seed donor is listed first followed by pollen donor) and inter-population F_1 crosses in both directions (Tennessee X Alabama, or TA_1; Alabama X Tennessee, or AT_1). The parental individuals included 16 Alabama and 16 Tennessee genets (non-clones). The species is self-incompatible and hermaphroditic; thus, parent individuals served as both pollen donor and pollen recipient, e.g., crossing individual TN1 with individual AL2 produced two families: TN1 X AL2 and AL2 X TN1. Our original intended experimental design provided that every individual would receive all treatments; however, due to the asynchronous nature of flowering, our final design was not fully factorial at the family level. The crosses were carried out as follows: inflorescences were bagged prior to anthesis to prevent any unwanted pollinations. Crosses were conducted by brushing pollen with a paintbrush from an inflorescence into aluminum foil and then brushing pollen onto the stigmas of another inflorescence in which the same pollen removal had been conducted. Pollinations were conducted within one hour of collecting pollen and all pollinations were

carried out at mid-morning (~1000 hours) for consistency. Inflorescences were re-bagged and achenes were allowed to mature.

Components of fitness of the F_1 offspring were assayed by determining the proportion of achenes that were viable, the proportion of viable achenes that germinated, the proportion of germinating seedlings that survived to the five true leaf stage, and pollen viability for each cross, i.e., family. Achene viability proportion was measured by counting the number of hard, black achenes (filled or viable) and the number of flat (unfilled or inviable) achenes and dividing the number of filled achenes by the total. Achenes were then put through the same vernalization period as the parentals. After a month, up to 20 achenes per cross, i.e., family, were germinated using the same nicking method as before and proportion of seeds germinated was scored as the number of seeds germinated in seven days out of the total attempted. Then, up to 10 germinated seedlings per family were planted in cone-tainers in the greenhouse to assess survival proportions. Plants were monitored until they died or reached five sets of true leaves. At this stage, three individuals from each of 100 families (25 of each cross type) were randomly selected and repotted into large pots and grown to maturity in order to measure pollen viability and for the next generation of crossing. When plants reached reproductive maturity, pollen was removed from an inflorescence and placed on a glass slide. One drop of a 0.1% solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) was added to each pollen sample, and a cover-slip was used to mix and cover the pollen and MTT mixture. The pollen samples were then examined under a light microscope at 1000X magnification. More than 300 pollen grains per sample were scored as either purple (viable) or clear (inviable), and the viable proportion of

pollen grains was calculated as the ratio of purple-stained pollen grains to the total number of pollen grains scored.

The same crossing methods were used to produce the F_2 generation (designated: AA_1 X AA_1 = AA_2, AT_1 X AT_1 = AT_2, TA_1 X TA_1 = TA_2, TT_1 X TT_1 = TT_2; direct inbreeding of relatives was avoided). Achene viability, germination, and survival to five true leaves were measured. Figure V-1 summarizes the entire crossing design.

Statistical Analysis of Fitness

We used Analysis of Variance (ANOVA) as calculated by JMPIN version 5 software (SAS Institute) to analyze the effect of cross type on the F_1 and F_2 offspring fitness measurements. Family was not included as an effect in the analysis since the design was not fully factorial at the family level due to asynchronous flowering of individuals. All proportion variables were arcsine square root transformed (Sokal and Rohlf, 1995). The main effect was cross type, with the variables achene viability, germination, survival, and pollen viability (F_1 only) initially analyzed separately. Cumulative fitness for the F_1 and F_2 offspring was calculated as the product of achene viability, germination, and survival. Post hoc Tukey-Kramer tests were performed in order to indicate which specific treatment means differed, e.g., do the two parental populations differ from one another with regard to fitness traits, do the offspring of inter-population crosses have higher fitness values than offspring of one or both of the pure population crosses?

One caveat worth mentioning pertains to the various relationships between the intra-population and the F_1 (or F_2) fitness measures possible in a crossing experiment. Generally, when evaluating heterosis, the fitness of hybrid individuals is compared to the mid-parent value, or the mean of the F_1 (or F_2) inter-population families is compared to the mean of the

source population families (Lynch & Walsh 1998). However, this approach may overlook information on possible fitness differences between the individual source populations being studied; thus, we have chosen to evaluate source populations separately. We also identify the potential for genetic rescue when the fitness of the inter-population crosses is higher than that of either set of intra-population crosses.

Results

Overall, 176 F_1 crosses, or families, were produced (39 AA_1, 51 AT_1, 42 TA_1, and 44 TT_1). In total, 4754 F_1 achenes (filled and un-filled) were scored, of those germination was attempted with 2306 filled achenes, and 990 plants were subsequently monitored for survival in the greenhouse. Achene viability means across all F_1 families ranged from zero to one, with an overall mean of 0.71. F_1 family means for germination ranged from 0.20 to one, with an overall mean of 0.76. F_1 family survival means ranged from zero to one, with an overall mean of 0.70. Pollen viability F_1 family means overall means ranged from 0.5 to 0.98, with an overall mean of 0.80. ANOVA indicated that there was a significant effect of cross type on achene viability, germination, and cumulative fitness (achene viability $F_{3,172} = 3.48$, $p = 0.017$; germination $F_{3,129} = 4.34$, $p = 0.006$; cumulative fitness $F_{3,161} = 2.95$, $p = 0.034$). Mean survival and pollen viability did not differ among cross types. For achene viability, a Tukey-Kramer test revealed that TT_1 families had a significantly lower mean than AT_1; the test also revealed that TT_1 had lower germination rates than AA_1 and AT_1. TT_1 crosses had significantly lower cumulative fitness values than AT_1 crosses (Figure V-2).

The F_2 crosses included 159 families (35 AA_2, 53 AT_2, 49 TA_2, 22 TT_2) in which achene viability, germination, and survival were measured. In total for F_2 crosses, 3846 achenes (filled and un-filled) were scored, germination was attempted with 1870 filled achenes, and 1063 plants were monitored for survival in the greenhouse. Achene viability means across all F_2 families ranged from zero to one, with an overall mean of 0.66. Germination F_2 family means ranged from zero to one, with an overall mean of 0.87. Survival F_2 family means ranged from zero to one, with an overall mean of 0.87. ANOVA indicated that there was also a significant effect of cross type on achene viability, germination, and cumulative fitness in the F_2 (achene viability $F_{3,155} = 8.44$, $p < 0.001$; germination $F_{3,116} = 5.31$, $p = 0.002$; cumulative fitness $F_{3,155} = 7.27$, $p < 0.0001$). Mean survival did not differ among cross types. For achene viability, a Tukey-Kramer test revealed that TT_2 families had significantly lower mean than all other families. TA_2 families had the highest germination rates and were significantly higher than AT_2 and TT_2 families; TT_2 families had significantly lower germination rates than AA_2 families. Finally, TT_2 families had significantly lower cumulative fitness values than all other cross types (Figure V-3).

Discussion

Phenotypic Differentiation

In this study, we found evidence for phenotypic differentiation between the Tennessee and Alabama populations with regard to components of fitness. Over two generations of crossing within these populations, Tennessee displayed lower germination rates than Alabama (roughly 25 percent less in the both generations). In the second

generation, Tennessee also exhibited lower achene viability and cumulative fitness values than the Alabama population (nearly 20 percent lower achene viability and almost 40 percent lower cumulative fitness). Given the general positive correlation between genetic marker diversity and fitness measures in the literature (Liemu et al., 2006), these results were somewhat unexpected since a recent investigation of the population genetics of these same *H. verticillatus* populations found no significant differences in heterozygosity between these two populations at 22 SSR loci (Madison Co., Tennessee $H_e = 0.46 \pm 0.07$; Cherokee Co., Alabama $H_e = 0.46 \pm 0.07$, Ellis et al., 2006). Moreover, this highly disjunct species exhibited only moderate levels of nuclear population genetic differentiation ($F_{ST} = 0.12$).

The evolutionary mechanism for the observed phenotypic fitness differences between the two populations is not known. However, two explanations seem likely: 1) more extensive inbreeding within the Tennessee population, or 2) differential adaptation between the two populations—note that these possibilities are not mutually exclusive.

Lower fitness values in the Tennessee population may indicate inbreeding as a result of genetic drift and/or bi-parental inbreeding within this population. Our finding of lower fitness at the early stages of the life cycle corresponds with the general observation that inbreeding depression in outcrossing perennials occurs at this time (Husband and Schemske, 1996). The increase in fitness of Tennessee plants following inter-population crossing to levels comparable to the Alabama population also indicates that this population may have been somewhat inbred since inter-population crosses may mask deleterious recessive alleles and increase heterozygosity (Charlesworth and Charlesworth, 1987). Many studies have also reported similar low offspring fitness in small, isolated populations of plant species often

attributed to inbreeding (Heschel and Paige, 1995; Fischer and Matthies, 1998; Luijten et al., 2002; Paschke et al., 2002; Severns, 2003; Yates et al., 2007).

Two factors may contribute to higher rates of inbreeding and the subsequent lower fitness in the Tennessee populations compared to the Alabama population: 1) population size and 2) minor population sub-structuring. The Tennessee population is smaller than the Alabama population, consisting of about 70 genets (versus several hundred in Alabama) (Ellis et al., 2006). It should be noted that the Georgia population, which exhibited such poor fitness that it could not be studied, consists of less than 20 genets, and the newly discovered Tennessee population, which was not available at the time of this study, contains just 19 genets (Ellis, unpublished data). Other studies have reported positive correlations between population size and fitness measures in fragmented plant populations. For example, Kery et al. (2000) reported strong reductions in fitness measures in smaller populations compared to larger populations in two rare, self-incompatible perennial species, *Primula veris* and *Gentiana lutea*. Leimu et al. (2006) found overall positive correlations between population size and fitness in a meta-analysis considering 45 studies which evaluated this relationship in plants. While the fact that our species has only four populations did not allow us to conduct a replicated study of the relationship between size and fitness, these results indicate a trend towards a similar correlation. Another factor potentially contributing to the observed inbreeding depression in this study is a small level of population structure in the Tennessee population. Ellis et al. (2006) found slight, but significant ($F_{ST} = 0.048$), population sub-structuring among three closely spaced patches which may further exacerbate the effects of inbreeding.

An alternative explanation for the observed population differentiation at fitness traits is differential adaptation between the two populations without inbreeding. For example, a mutation appearing in the Alabama population which provides increased achene viability or higher germination rates may be selected for in this site. This selection could lead to population differentiation at these traits, independent of any inbreeding-associated reduced fitness, if Tennessee has not experienced the same mutation/selection regime or if the adaptation has not spread by gene flow. In a species connected by moderate gene flow, a beneficial mutation is expected to spread across populations relatively rapidly (Slatkin, 1976; Rieseberg and Burke, 2001). However, in this highly disjunct species, where gene flow among populations is currently improbable, advantageous mutations are unlikely to be spread. This possibility emphasizes the importance of population connectivity for long term sustainability. Note, because our experiment was conducted in a controlled greenhouse environment, the differences between populations probably represent intrinsic genetic differences separate from adaptation to local environments.

While differential adaptation in fitness traits is plausible, inbreeding depression may become a more parsimonious explanation for the observed fitness differences with increasing numbers of differentiated traits since inbreeding affects the genome globally. The potential reasons for the underlying phenotypic differentiation require further investigation; however, our results demonstrate that, contrary to genetic marker information, these populations are not interchangeable with regard to quantitative fitness characteristics.

Potential for Genetic Rescue

We were also interested in the potential for genetic rescue as a more active conservation strategy. Thus, we monitored inter-population crosses to the F_2 generation to

increase the likelihood of observing any possible outbreeding depression; fewer studies have followed fitness this far (Tallmon et al., 2004; Edmands, 2007), and outbreeding depression in the F_2 can be as detrimental as inbreeding depression (Edmands, 2007). While some have found outbreeding depression in a single generation of crosses, e.g., Montalvo and Ellstrand (2001), there are theoretical reasons to expect that outbreeding depression might not be observable until the F_2 and later generations as mentioned previously.

We did not, however, find any evidence for outbreeding depression in inter-population crosses in either the first or second generation of this study. Inter-population crosses had equivalent fitness values to the Alabama crosses and were statistically higher than the Tennessee crosses. The potential 'hybrid breakdown', a decline in inter-population offspring as compared to the parents, was not detected. By evaluating populations independently, we were able to show that the fitness of the inter-population crosses was higher than pure Tennessee crosses but equivalent to pure Alabama crosses. This information would have been lost had we averaged the parental fitness values. The F_1 results indicate that some dominant gene action owing to the masking of deleterious recessives in the Tennessee population, instead of additive or overdominant effects, may play a role in the fitness characteristics studied. This finding corresponds with the general understanding that traits underlying fitness often have high dominance components (Crnokrak and Roff, 1995) and that inbreeding depression frequently involves the action of partially recessive harmful alleles (Charlesworth and Charlesworth, 1999; Keller and Waller, 2002). Our results did not mimic pure dominance as we did not observe the decline in fitness that is expected due to segregation in the F_2, but rather the fitness benefit gained by outcrossing Tennessee individuals as compared to the pure Tennessee crosses was exaggerated in the F_2 generation.

Thus, the mode of gene action underlying fitness loci remains to be explained, and our findings indicate it may not be simple.

The increased fitness in the inter-population F_1 and F_2 hybrids suggests the potential for genetic rescue in the Tennessee population. The potential for genetic rescue has been shown in other rare or endangered plant species. Immigrant pollen from regional populations of the rare pitcher plant, *Sarracenia flava*, resulted in more vigorous offspring and inter-site crosses were recommended for conservation management (Sheridan and Karowe 2000). Paschke et al. (2002) found that introducing pollen from outside populations resulted in higher reproductive success and greater offspring size in the self-incompatible, endemic *Cochlearia bavarica*. Finally, Willi et al. (2007) demonstrated that inter-population hybrids of the rare *Ranunculus reptans* maintained high fitness even into the F2 generation, indicating genetic rescue in this species.

Implications for Conservation

Our results indicate that these populations are not interchangeable with regard to phenotypic fitness-related characteristics. While a general positive relationship exists between genetic diversity and population fitness (Leimu et al. 2006), some studies have reported non-significant or negative correlations between these measures, e.g., Bonnin et al., 2002; Leimu and Mutikainen, 2005; Lopez-Pujol et al., 2008). In these studies and the case of *H. verticillatus*, measuring genetic diversity alone would have been insufficient in identifying the Tennessee population as a conservation concern. Many studies of endangered species only include information from genetic marker data as these types of studies may often be conducted in a timely manner (Conner and Hartl 2004). Knowledge of quantitative measures for fitness related traits is important in determining the best source for ex situ

conservation management. When possible, marker and fitness studies should be combined to fully assess the future evolutionary potential and the demographic and fitness consequences of small, isolated populations.

This study also demonstrates a potential for genetic rescue and suggests management strategies to address the population viability of the Tennessee population. Outcrossing Tennessee individuals produced more fit offspring than pure Tennessee offspring and these fitness benefits carried through to the F_2 generation. A potential conservation strategy could be to transplant Tennessee X Alabama seeds or seedlings into the Tennessee population to boost fitness. In a self-incompatible species, like *H. verticillatus*, loss of S alleles is also important to mate availability, e.g., Young et al. (2000), and introducing new genetic individuals into a population can increase S allele diversity thereby increasing the number of potential mates (DeMauro, 1993; Hoebee et al. 2008). Some have proposed that only a few introduced individuals per generation are needed for genetic rescue (Tallmon et al., 2004), and even one individual may be sufficient to increase fitness in a suffering population (Ingvarsson, 2001). However, the possibility for outbreeding depression in later generations cannot be ruled out (Fenster and Galloway, 2000); it is also feasible that transplanted hybrid individuals would be ill fit for surviving in the Tennessee environment. We did not examine the potential for 'hybrid breakdown' due to the dilution of locally adapted alleles (extrinsic outbreeding depression) since our study was conducted in the greenhouse (cf. Fenster and Dudash 1994). We chose a common environment to address intrinsic factors associated with inbreeding and outbreeding and to control external factors such as nutrient availability, precipitation, and temperature. Future studies that monitor survival of reciprocal transplant

individuals and inter-population hybrids planted into each of the different biotic and abiotic environments may give insight into these issues.

The comparison in the greenhouse of Tennessee to Alabama crosses *alone* demonstrates the considerable fitness differences between the two populations, with Tennessee being much lower overall. The assessment of fitness in the greenhouse may also be a relatively conservative measure since the field is likely to exhibit much harsher conditions (Lynch & Walsh, 1999). The statistical increase in fitness owing to immigration of Alabama alleles into the Tennessee population offers great promise and stresses the urgency for a conservation strategy in this population.

Acknowledgements

We would like to thank M. Mandel, C. Brown, M. Chapman, C. and M. Lipscomb, J. Ertelt, N. Sherman, and D. Wills for assistance in the greenhouse and field. K. Bentley gathered data for the F_2 germination and survival data. We would also like to thank D. Funk, M. Welch, M. Bailey, E. Janson, S. Egan, J. Gibbons, and S. Pearl for discussion, ideas, and comments on an earlier version of the manuscript. Temple-Inland Cooperation, Tennessee Natural Heritage Department, and United States Fish and Wildlife Services were extremely helpful in providing access to the property. Funding was provided by United States Fish and Wildlife Services to J.R.E.

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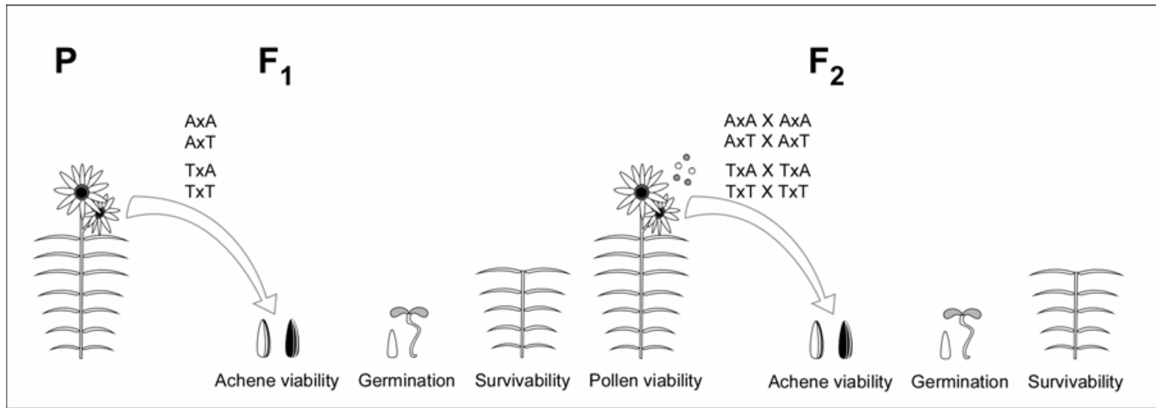
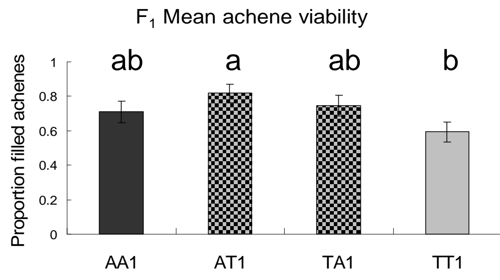


Figure V-1. Schematic of the crossing experiment. Sixteen parental individuals (P) from each population were collected from the field, grown to maturity, and crossed (Alabama X Alabama-AxA, Alabama X Tennessee-AxT, Tennessee X Alabama-TxA, Tennessee X Tennessee-TxT). The offspring (F_1) were assayed for fitness at the achene stage (achene viability), the embryonic stage (germination), the seeding stage up to five sets of true leaves (survivability), and the mature plant stage (pollen viability). The F_1 individuals were then crossed (AxA X AxA, AxT X AxT, TxA X TxA, TxT X TxT), and their offspring (F_2) were also measured for achene viability, germination, and survivability. Graphic credit: Christopher G. Brown.

A.



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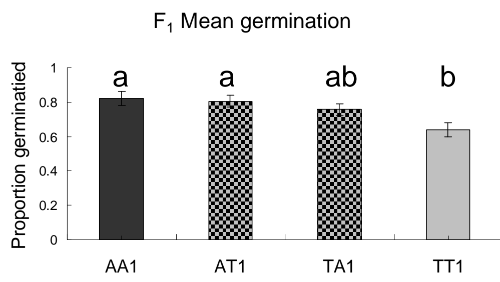

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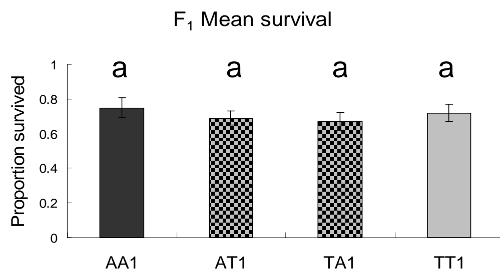

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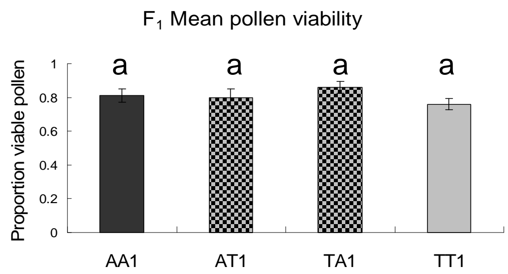

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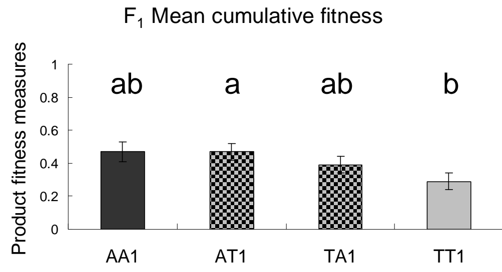
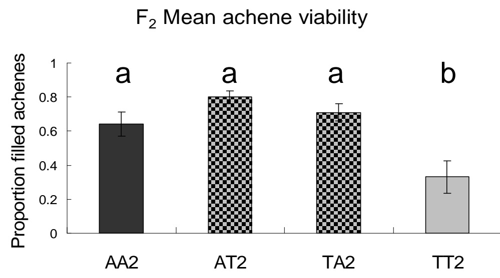
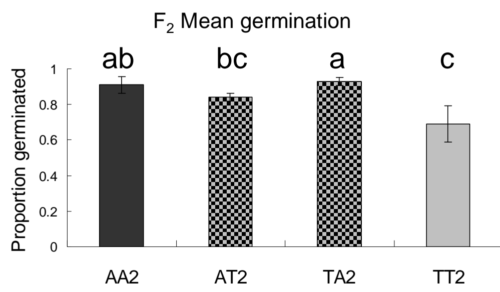


Figure V-2. Mean fitness measurements ± SE for the F_1 families (A.) Achene viability. (B.) Germination. (C.) Survival. (D.) Pollen Viability (E.) Cumulative Fitness. Different letters indicate measurements that showed significant cross treatment effects at the $p \leq 0.05$ level for ANOVA and Tukey-Kramer testing.

A.



B.



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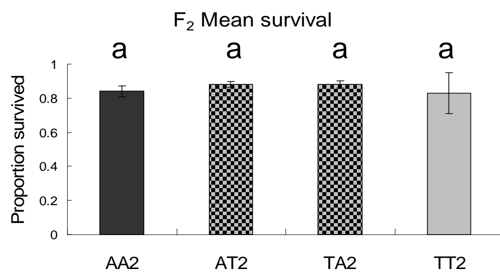

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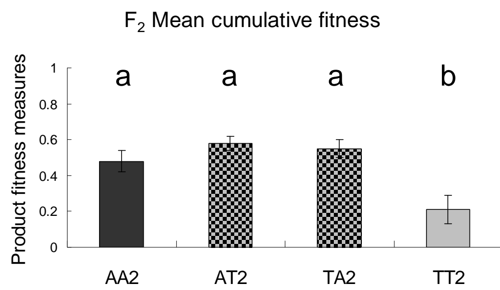

Figure V-3. Mean fitness measurements ± SE for the F_2 families (A.) Achene viability. (B.) Germination. (C.) Survival. (D.) Cumulative Fitness. Different letters indicate measurements that showed significant cross treatment effects at the $p \leq 0.05$ level for ANOVA and Tukey-Kramer testing.

CHAPTER VI

ANCILLARY

The following is a short collection of several small projects I conducted during my dissertation research. This ancillary chapter contains interesting findings that were not included in separate chapters because they were the result of smaller scale studies.

Helianthus verticillatus Herbarium Sample

In addition to the studies based on extant individuals of *Helianthus verticillatus*, I obtained leaves from two herbarium specimens, derived from the original 1892 collections and housed at the New York Botanical Garden (NY Specimen ID: 73465; Collector: S. M. Bain, Aug. 1892). I examined the genetic relationship between the two herbarium specimens and extant individuals employing previously studied EST-SSRs (Ellis et al. 2006; Pashley et al. 2006). Specifically, I was interested in asking 1) are the two herbarium samples from the same or different genets, 2) since the species is clonal, perennial, and may have long generation times, are the herbarium specimens still living clones in the extant population, and 3) how different are the herbarium specimens from extant individuals? Special precautions were made so as not to contaminate the specimen DNA with other sunflower DNA. To avoid contamination issues, the DNA was extracted in a different laboratory located in a different building than where the sunflower research is normally conducted with Qiagen DNeasy Plant Maxi Kit (Qiagen, Valencia, CA). None of the equipment (pipettes, pipette tips, etc.) had

previously been used to study vascular plants. The dried leaf (~ 0.2g) was ground with liquid nitrogen. The herbarium specimens were genotyped for the same EST-SSR loci as the extant material. To examine the relationship between the *H. verticillatus* herbarium specimen and the extant *H. verticillatus* populations, a Principle Coordinates Analysis (PCO) was conducted on the four *H. verticillatus* populations and the herbarium specimen.

Results of the Herbarium DNA

Both herbarium specimens consisted of the same multi-locus genotype, $(P_{cgen})^{n-1}$ = 1.13E-17 (see Chapter IV Materials and Methods for description of multi-locus probability calculation). Most likely then, the two samples were collected from the same genet. The specimens contained six unique alleles out of 24 detected alleles when compared to the extant *H. verticillatus* individuals (Ellis et al. 2006). Two loci that were monomorphic in the extant populations were also monomorphic (having the same alleles) in the herbarium samples, and the herbarium specimens contained the '*H. verticillatus*' allele at a diagnostic locus between the three species (*H. verticillatus*, *H. angustifolius*, *H. grosseserratus*). In a PCO plot of *H. verticillatus* and the herbarium specimen (figure VI-1), the herbarium specimen clustered near individuals of the Tennessee populations. The first and second components explained 29.9 and 19.2 percent of the variation respectively. Genetic marker data indicates that the herbarium specimens represent a single genet of *H. verticillatus* possibly from Tennessee, and it was not an individual that was found in the previous Ellis et al. (2006) study.

Newly Discovered McNairy Co., Tennessee Population

Methods and Analysis

Measures of genetic diversity, including mean number of alleles, observed and expected heterozygosity, and the inbreeding coefficient (*f*, Weir and Cockerham 1984) were calculated in the newly discovered McNairy Co., Tennessee population using the program GDA v 1.0 (Lewis and Zaykin 2001). Data from the McNairy Co., Tennessee population was also analyzed with the existing information on the other three locations of this species for population genetic structure (Ellis et al. 2006). Using an AMOVA framework (Weir and Cockerham 1984; Excoffier et al. 1992), population genetic structure was estimated in the program ARLEQUIN v. 2.0 (Schneider et al. 2000). Also, the new population was included in a PCO which was conducted on pairwise genetic distances among all four populations of *H. verticillatus*.

Results McNairy Co., Tennessee Population Genetics

The McNairy Co. population contained previously un-sampled alleles at several of the EST-SSRs studied and contained new multi-locus genotypes. Mean number of alleles per polymorphic locus measured 3.65 ± 0.44 (mean ± SE). Mean expected heterozygosity was 0.53 ± 0.06 and mean observed heterozygosity was 0.39 ± 0.05. F_{IS}, the inbreeding coefficient was 0.24 ± 0.07. These values are comparable to those of the other three populations (Ellis et al. 2006).

Population structure, as calculated by AMOVA, for the four populations (McNairy Co. and AL, GA, Madison Co., TN studied in Ellis et al. 2006) was $F_{ST} = 0.168$, $P < 0.0001$. This value was slightly higher than the calculation from Ellis et al. (2006), $F_{ST} = 0.118$.

Pairwise values, however, demonstrated that the new McNairy Co., Tennessee population was somewhat more differentiated from the other populations of *H. verticillatus*. All pairwise values of F_{ST} were significantly different from zero ($P < 0.0001$), and were as follows: Georgia and Alabama ($F_{ST} = 0.083$), Madison Co., TN and Georgia ($F_{ST} = 0.146$), Madison Co., TN and Alabama ($F_{ST} = 0.128$), McNairy Co, TN and Madison Co., TN ($F_{ST} = 0.111$), McNairy Co, TN and Georgia ($F_{ST} = 0.168$), and McNairy Co, TN and Alabama ($F_{ST} = 0.185$). Furthermore, the PCO carried out on all four populations of *H. verticillatus* revealed similar relationships to the AMOVA and explained a large portion of the variance, cumulatively 49.38%. The PCO demonstrated overlap between individuals from GA and AL along both PCO1 and PCO2. The Madison Co, TN population formed a somewhat distinct cluster and was only separated along PCO2. The newly discovered McNairy Co., TN formed the most distinct cluster of individuals and was separated along PCO1 (PCO 1: 29.9%, PCO 2: 19.2%; Figure VI-1).

Reproductive barriers in *Helianthus verticillatus*

Insect Observations

I was also interested in identifying whether *H. angustifolius* poses a threat to the taxonomic identity of *H. verticillatus* through natural hybridization by conducting interspecific crosses in the greenhouse (see below) and observing pollinators in the field. The threat for natural hybridization is present given that the two species grow in close proximity with one another and overlap in flowering time. The rare species flowers early in the fall (August to early October), and the common species flowers a little later (September to late

October). I observed insects in the field and collected visitors, herbivores, and pollinators from both *H. verticillatus* and *H. angustifolius* individuals. This was to determine if they overlap in pollinators and thus have an opportunity for natural hybridization. Native bees, *Mellisodes bimaculatus*, were present when only *H. verticillatus* was flowering in August to mid September. However, in late September, when the common species was flowering with the rare, these native bees were no longer observed. Instead, European honeybees, *Apis mellifera*, were common and observed flying between the rare and the common species. Also, bees were observed flying mainly short distances between visits which often resulted in visits to flowers on the same plant.

Interspecific Crosses of Helianthus *species*

Interspecific crosses were conducted among *H. verticillatus*, *H. grosseserratus*, and *H. angustifolius* individuals to investigate crossing barriers among the species once thought to be the progenitors of the putative hybrid, *H. verticillatus* (Heiser et al. 1969). Crosses and fitness measurements were carried out following the methods outline in Chapter V. While some individuals from hybrid crosses were successful (i.e. several individuals survived to five true leaves), reproductive isolation remains strong but not complete in the greenhouse. In total, 17 *H. angustifolius* by *H. grosseserratus*, 88 *H. angustifolius* by *H. verticillatus*, and 6 *H. grosseserratus* by *H. verticillatus* families were evaluated. Intraspecific crosses were more likely to have at least one viable achene ($X^2 = 70.2$, $p < 0.0001$), one germinated seedling ($X^2 = 68.7$, $p < 0.0001$), and one seedling surviving to five true leaves ($X^2 = 90.4$, $p < 0.0001$) than interspecific crosses. See Tables VI-1 and VI-2.

I also genotyped surviving seedlings at a species diagnostic EST-SSR (BL 22) in putative experimental hybrid individuals for which I was able to obtain a leaf for DNA; this

locus is fixed between the species. All methods were carried out for marker BL 22 as in Chapter II. Out of 34 putative hybrids created through interspecific hybridization, only seven were heterozygous at the species specific marker. Thus, the rest of the individuals may have been the result of selfing or the Mentor effect (Richards 1986). Finally, an interesting observation to point out is that when flower heads were bagged, no achenes were viable.

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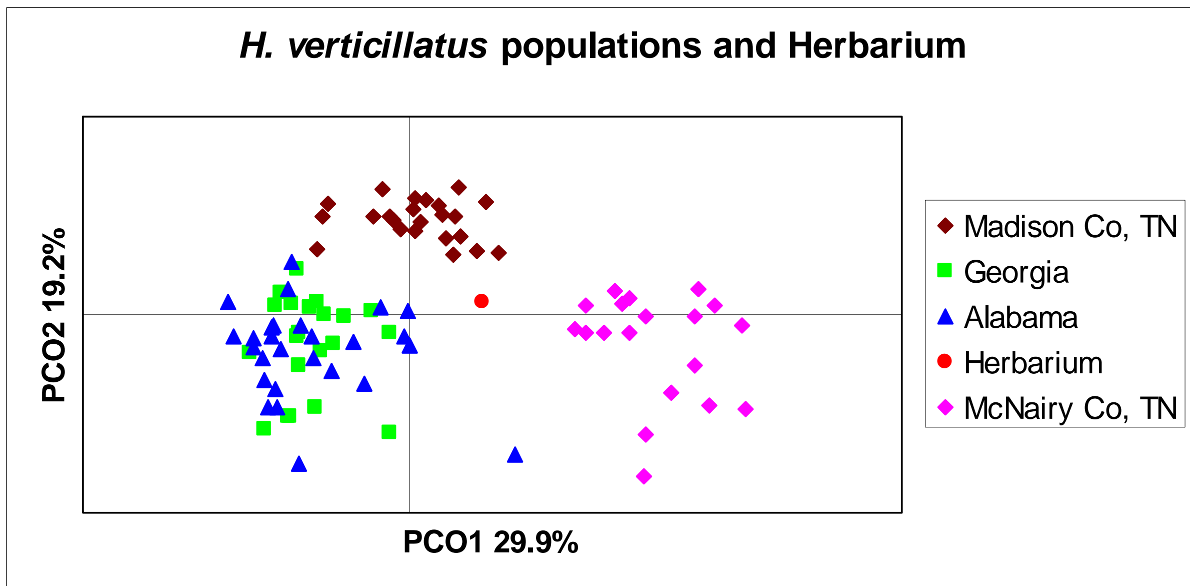

Figure VI-1. Principle coordinates analysis of *Helianthus verticillatus* populations and the herbarium specimen.

Table VI-1. Number of interspecific families with at least one viable achene, one germinated seedling, and one seedling surviving to five true leaves.

Interspecific	No. Crosses	Set >1 achene	Germinated >1 achene	Survival >1 achene
HA x HG	17	7	6	5
HA x HV	88	24	24	11
HG x HV	6	2	2	2
HV x HV	176	156	153	146

Table VI-2. Interspecific families grouped by cross type (Inter- or Intra- specific) with at least one viable achene, one germinated seedling, and one seedling surviving to five true leaves.

Cross Type	No. Crosses	Set>1 achene	Germinated >1 achene	Survival >1 achene
INTER-	111	33	32	18
INTRA-	176	142	141	136

Table VI-3. Interspecific crosses genotyped for a species-diagnostic EST-SSR to determine if seedlings were the product of selfing or hybrid crossing.

Interspecific	No. crosses	Genotyped	Nuclear Heterozygotes
HA x HG	17	6	2
HA x HV	88	25	4
HG x HV	6	3	1

HA = *Helianthus angustifolius*
HG = *H. grosseserratus*
HV = *H. verticillatus*

CHAPTER VII

CONCLUSION

The results of this dissertation demonstrate the need for studies of rare or endangered species to include combined assessments of population genetic parameters, population size, and fitness, particularly since some do not follow general theoretical and experimental expectations. The finding of high genetic diversity in populations of *Helianthus verticillatus* was somewhat surprising given its small number of populations and the considerable geographic isolation among them. However, the species exhibits clonal growth and has a perennial life cycle, potentially affording longer generation times and reducing the probability of genet death (Cook 1983). Moreover, a sufficient amount of time may not have yet passed for the effects of rarity on the population genetic diversity to be manifested: the species is often found with other rare and endangered plant species that exhibit high prairie affinities and have likely become rare since European settlement and the subsequent land conversion in the 19th century (Allison 1995; Matthews et al. 2002). Additionally, there may be more, undiscovered populations which serve to connect the known populations through gene flow further buffering the loss of genetic diversity.

This dissertation also demonstrates the value of utilizing existing genomic resources of related species for the development of highly polymorphic genetic markers. Here, the use of simple sequence repeats (SSRs) developed from Expressed Sequence Tag (EST) databases allowed for the rapid and relatively inexpensive study of the population genetics of *H. verticillatus*. De novo development of SSRs would have required significantly more time and

money—two common and limiting factors for the study of rare or endangered species. The availability of these markers also allowed for the investigation of the hypothesis that *H. verticillatus* was the product of recent hybridization between two diploid, widespread sunflowers, *H. angustifolius* and *H. grosseserratus* (Heiser et al. 1969). Confirming previous morphological findings, genetic marker data revealed that *H. verticillatus* represents a good taxonomic species of non-hybrid origin. Finally, employing these genetic markers, I was able to investigate the genetic population size and the extent of clonality in all four populations of *H. verticillatus*. This work demonstrated that populations consisted of far fewer individuals than previously reported, with some populations consisting of quite low numbers. This finding, combined with that of the taxonomic status of the species, prompted the United States Fish and Wildlife Service (USFWS) to upgrade the priority ranking of *H. verticillatus* from low to high.

Another somewhat surprising result of this dissertation research was the finding of rare paternal inheritance of chloroplast DNA (cpDNA) in inter-population crosses of *H. verticillatus* individuals. Many types of evolutionary investigations employ organellar markers, and these applications typically assume strict maternal inheritance (Birky 2001); this assumption, however, is rarely tested. Paternal inheritance may lead to incorrect conclusions in population genetic studies employing cpDNA, thus it is important to test these assumptions. While there was evidence for paternal inheritance, it was rare and did not appear to significantly decrease the calculations of chloroplast population structure given that values in *H. verticillatus* did not differ from that of the widespread *H. angustifolius* or the average of other angiosperm species.

Populations of *H. verticillatus* exhibited significant differentiation in phenotypic fitness related traits, despite high amounts of genetic variation and no significant differences in levels of variation among populations. In a crossing study between the Alabama and Madison County, Tennessee populations, I found that the Tennessee population had significantly lower achene viability and germination rates as compared to the Alabama population. Crosses among these populations revealed elevated values for these traits in the hybrids and no evidence for outbreeding depression through the second generation. These results indicate genetic rescue as a potential conservation strategy in the considerably smaller Tennessee population. Moreover, the smallest population, Georgia, also exhibited low levels of achene viability and germination rates, and no individuals survived to flowering, making a formal crossing study not possible.

Throughout the duration of this research, recommendations have been made to both the Tennessee Natural Heritage: Rare Plants Division and the USFWS for conservation management guidelines. Greenhouse propagated individuals have also been transplanted to a wet prairie on the campus of Freed-Hardeman University (near the Madison Co., Tennessee site) for a restoration project. Many important insights have been gained during the course of this research; however, several future projects would be valuable. First, surveys for any additional populations of the species using information regarding the soil type, associated species, and historical locations of prairies would be pertinent. Second, the fitness crossing study was conducted in the greenhouse in order to have a controlled environment for measuring fitness traits; thus, extrinsic outbreeding depression due to the dilution of locally adapted alleles was not tested. A transplant study which monitors the survival of inter-population hybrids planted into each of the different biotic and abiotic environments would

be appropriate to further evaluate the potential for genetic rescue. Finally, given the likelihood of pollen transfer from a widespread congener, additional studies of the threat to the taxonomic integrity of *H. verticillatus* are warranted. These studies would be especially important to the conservation of this rare species given the reduced fitness associated with interspecific crossing, either through extreme outbreeding depression in true interspecific hybrids, or extreme inbreeding depression in actual self-fertilized individuals (recall Mentor Effect from ancillary data).

In conclusion, this dissertation research has demonstrated that an appropriate conservation strategy is to protect the only four known populations. Populations of *H. verticillatus* harbor high levels of genetic diversity, exhibit a great deal of geographical distinctness in regard to locality, and show evidence of phenotypic differentiation in fitness traits. Habitat protection is the most immediate and comprehensive action to take at this time to preserve the species.

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