CONSERVING & ASSESSING THE HEALTH OF GRAY'S LILY (*LILIUM GRAYI* S. WATSON)

A Thesis
by
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Abstract

Conserving & Assessing the Health of Gray's Lily (*Lilium grayi* S. Watson)

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Lilium grayi S. Watson (Liliaceae), Gray's Lily, is a threatened perennial herb endemic to high elevations in the Southern Appalachians of Virginia, North Carolina, and Tennessee, and now restricted to 46 extant occurrences in 15 counties. A Federal Species of Concern that is also listed at the state level in the three states where it occurs (NatureServe, 2009), *L. grayi* faces multiple challenges, including a limited and declining geographic distribution, severely limited reproduction due to a disease caused by an invasive fungal phytopathogen, and many small and fragmented populations. Demographic monitoring of *L. grayi* populations is necessary to estimate the reproductive success of populations and the impact of Lily Leaf Spot (LLS) disease. Additionally, the small size and isolation of many populations suggest they are potentially suffering from a lack of gene flow and erosion of genetic diversity, making a population genetics study a critical research need. Such demographic data alongside a genetic diversity analysis would generate a robust evaluation of population health and structure.

Most remaining *L. grayi* populations have fewer than 200 mature plants and are isolated by both distance and topography, making them susceptible to genetic diversity loss. Additionally, the species is experiencing early-season population collapse due Lily Leaf Spot disease (LLS) caused by the invasive ascomycete fungus *Pseudocercosporella inconspicua* (G. Winter) U. Braun (Ingram et al., 2018). LLS results in wilting and premature senescence of aboveground tissues, often preventing individuals from reproducing successfully. LLS has been monitored at Bluff Mountain, Sparta Bog, and Roan Mountain in NC, where it results in premature senescence in most flowering plants in all populations (Bates, 1998; Bates, 1999; Bates, 2000; Ingram et al., 2018), and recently *P. inconspicua* has been confirmed across the range of *L. grayi* (Barrett, 2017).

This study addresses research gaps in *L. grayi* by conducting a health assessment of populations in two primary ways: demographic monitoring of flowering individuals to determine the proportion that reproduce, and a genetic diversity analysis using microsatellite markers to be used as a metric of population health and to investigate population structure and gene flow. Demographic monitoring occurred in 15 and 22 *L. grayi* populations in 2020 and 2021, respectively, where a minority of plants reproduced each year (2020 = 30.85%, 2021 = 12.97%) due to a combination of premature senescence due to LLS and browsing. Genetic diversity was analyzed in 24 populations of *L. grayi*, finding the overall average effective number of alleles was 1.807, average observed and expected heterozygosities were 0.344 and 0.338, respectively, with an average fixation index of -0.021. Analysis of population structure found no natural groupings, indicating a high level of gene flow between populations. Four management units are suggested: Roan Mountain populations, Grandfather Mountain populations, Amphibolite Mountains populations, and hybrid populations.

Author's Note

This thesis uses a two-article format. Chapters 2 and 3 will be submitted for peer review and publication.

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Dedication

This thesis is dedicated to my fiancée, Carson Rose Funk, and to my father, Samuel Todd Brewer. My dad's love of North Carolina, its natural places, and the things that grow there is the inspiration that drove all the efforts described in this study. Carson's strength, love, and support kept this research alive when the struggles of the world felt insurmountable. May they both rest in peace.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Lilium, the type genus of Liliaceae, originated approximately 12-14 million years ago in the Himalayas and Hengduan Mountains alongside the genus *Fritillaria* (Gao et al., 2013; Patterson & Givnish, 2002) and now consists of about 110 species found in temperate areas across the northern hemisphere (Weakley & SF Team, 2022). *Lilium* species are perennial herbs, with showy flowers atop erect stems and many leaves that grow from rhizomatous bulbs (Skinner, 2002). All members of the genus have a haploid chromosome count of 12, and nearly all are exclusively diploid in nature (Du et al., 2017; Givnish et al., 2020; Kostoff, 1939; Pelkonen & Pirttilä, 2012). The genus possesses exceptionally large genomes, ranging from 44.58 pg to 167.58 pg (Du et al., 2017), and has a long history of cytogenetics study (Farmer, 1893; Fogwill, 1957; Sargant, 1896; Stern & Hotta, 1977; Stewart, 1947). Although largely self-incompatible, species readily hybridize in both natural and horticultural settings (Duan et al., 2022; Gao et al., 2013; Skinner, 2002; Weakley & SF Team, 2022). Traditionally split into seven sections based on morphology, the section *Pseudolirium* is endemic to North America, includes all ~21 species on the continent, and has been consistently found to be monophyletic (Du et al., 2017; Duan et al., 2022; Gao et al., 2013; Gong et al., 2017; Nishikawa et al., 1999; Nishikawa et al., 2001; Pelkonen & Pirttilä, 2012; Stewart, 1947). In North America, *Lilium* species are pollinated by butterflies, hawkmoths, and hummingbirds, however phylogenetic analysis suggests that the first colonizers were butterfly pollinated and hawkmoth and hummingbird pollination evolved *in situ* (Skinner, 1988). Hummingbird pollination is unique to the New World lilies, as are the birds themselves (Bochenski & Bochenski, 2008), with six total *Lilium* species pollinated by

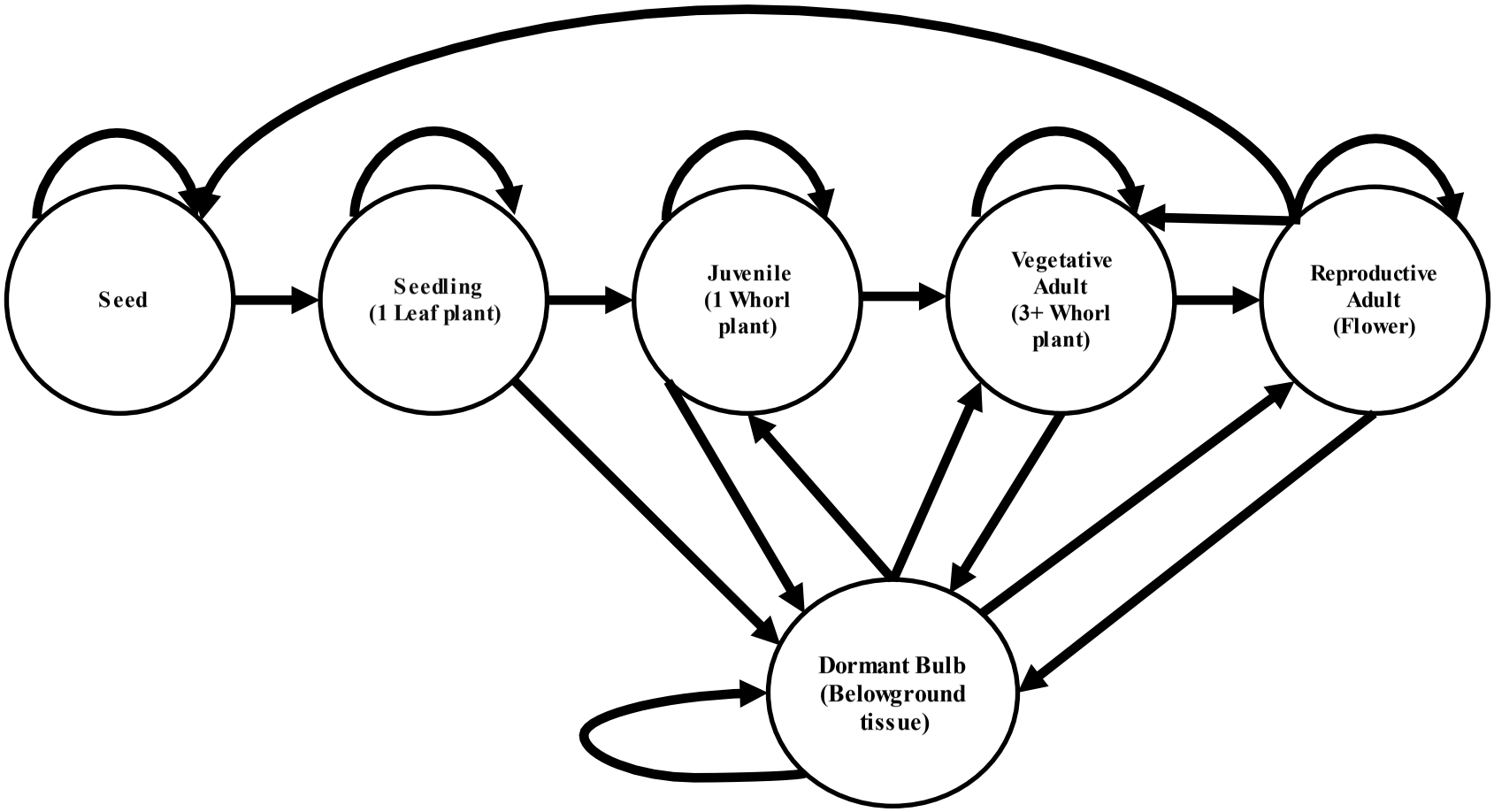
hummingbirds: four in western North America and two in eastern North America (Givnish, 2020). The two eastern species are *Lilium canadense* L. (Canada Lily), a common species with a broad range from Alabama to Canada, and *Lilium grayi* S. Watson (Gray's Lily), a rare narrow endemic found in the Southern Appalachians of North Carolina, Virginia, and Tennessee (Figure 1).

Figure 1. *Lilium grayi*. Photo taken on grassy bald at Roan Mountain 07/09/2020.

L. grayi is a perennial species, reaching reproductive maturity approximately 3-7 years after germination (Ingram et al., 2018). A life diagram for *L. grayi* (Figure 2) was estimated based on data from *L. pyrophilum* (Hohmann & Wall, 2018). Each year plants overwinter as bulbs, emerging aboveground in early spring with aboveground tissues senescing in mid-autumn. Sexual reproduction is conducted via flowers in June and July and capsules from August through October. Asexual reproduction occurs rhizomatously from

bulbs, which are yellow and produce scaleless offshoots that terminate in new bulbs. Stems can reach nearly 2 m in height. Leaves grow primarily in 3-5 whorls with 3-12 leaves per whorl, but alternate or opposite leaves do infrequently occur. Leaves are elliptic to lanceolate, 4.1-12.7 cm long and 1.5-3.6 cm wide, with an acute to barely acuminate apex. Inflorescences grow terminally, either solitary or racemose with 1-12 flowers. The flowers are campanulate with six tepals 3-5.5 cm long and 3-4 cm in diameter, with the distal third of the tepals recurved. The outer tepal surfaces are a deep orange-red, with occasional proximal areas of lighter orange. Inner tepal surfaces are distally orange-red, becoming yellow medially and basally, covered in purple-brown spots that become denser towards the base. Six stamens occur in two whorls, bearing large magenta anthers that are included within the perianth. A single compound pistil includes a three-lobed stigma and a superior ovary with three carpels, which develops into a three-lobed 2.1-3.7 cm long loculicidal capsule (Massey et al., 1983; Skinner, 2002). Healthy capsules produce an average of 201 seeds, which are flat and winged (Ingram et al., 2018). Data on how long dormant seeds stay viable in the seed bank is a research gap. Skinner (2002) states that the ruby-throated hummingbird (*Archilochus colubris* L.) is the only reliable pollinator, describing the red, tubular, horizontal-to-nodding flower as both representing the zenith of pollinator-mediated evolution in eastern lilies and exhibiting floral convergence with independently derived western *Lilium* species.

Figure 2. Proposed *Lilium grayi* life diagram.

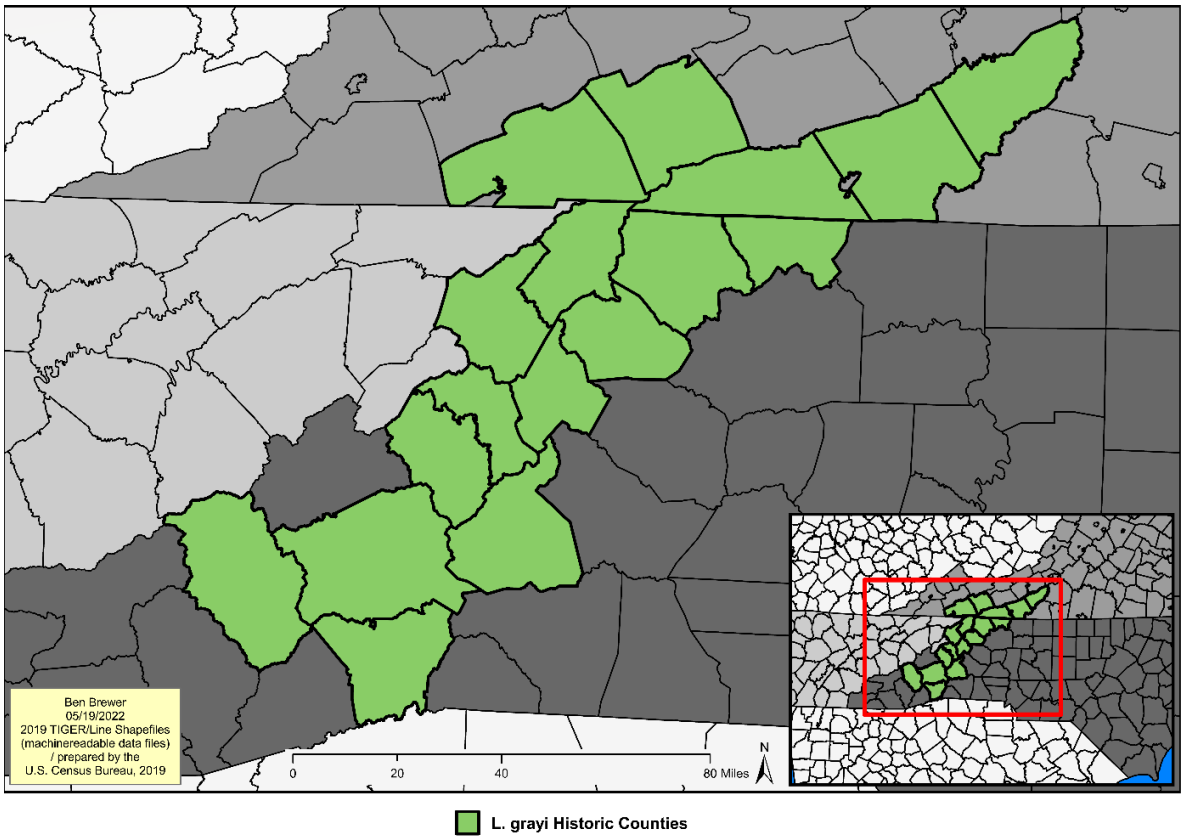


Discovered on Roan Mountain, NC in 1840 by Asa Gray, the species was described by Sereno Watson in 1879 and named in honor of Gray (Lounsberry, 1901, Watson, 1878). Watson characterized *L. grayi* through its erect, horizontal flowers, compared to the nodding flowers of congener *Lilium canadense* L. The morphological plasticity exhibited by *L. canadense* across its range, combined with nodding flowers in a *L. grayi* individual transplanted to Philadelphia, led some contemporaries to conclude that the species was a "brood" of *L. canadense* (Meehan, 1881). *L. canadense* exhibits considerable variation across its range, with the red-flowered *Lilium canadense* var. *editorum* Fernald serving as the primary cause of confusion with *L. grayi*. (Weakley & SF Team, 2022). Wherry (1946) described *L. grayi* as grading imperceptibly into *L. canadense*, with a hybridization zone between the two occurring in southwestern Virginia (Skinner, 2002).

Misidentifications of *L. grayi* has led to erroneous inflations in its historical distribution. For part of the species' history, populations of *L. grayi* were thought to be present in Maryland and West Virginia (Massey et al., 1983), though local studies (Reveal & Broome, 1981; Strausbaugh & Core, 1970) correctly identified these populations as *L. canadense* var. *editorum*. Current or historic populations were found in 17 counties (Figure 2); however, the species now exists in 46 extant populations across 15 counties, most of which occur in NC (NatureServe, 2009). Buncombe County, NC and Floyd County, VA represent the respective Southern and Northern extents of the range. The largest populations are found on the Roan Mountain massif, TN and Long Hope Valley in Watauga and Ashe counties, NC. Capable of growing both under a canopy and in treeless settings, *L. grayi* typically prefers mesic habitats, including bogs, seepages, moist forests, and wet meadows, in addition to the more xeric grassy balds (Weakley & SF Team, 2022). The majority of *L.*

grayi populations occur above 850m elevation (NatureServe, 2009), up to 1880m atop Grassy Ridge Bald at Roan Mountain (Ingram et al., 2018).

Figure 3. Historic *Lilium grayi* counties. Counties in green contain either a historic or current occurrence of *L. grayi.*



L. grayi is a critically imperiled G1 ranked species, a Federal Species of Concern, and listed as threatened, endangered, or imperiled by North Carolina, Tennessee, and Virginia, respectively (Crabtree, 2021; NatureServe, 2009; Townsend, 2022; Wichmann & Wojcik, 2022). The species faces threats used as criteria for the International Union for Conservation of Nature (IUCN) red list including limited geographic extent, loss of remaining habitat, small population sizes with recent declines (International Union for Conservation of Nature

[IUCN], 2001), as well as a widespread early season collapse event resulting from a fungal pathogen (Ingram et al., 2018). *L. grayi* is most successful on grassy balds, a habitat type that is actively being lost due to woody encroachment (Crawford & Kennedy, 2009) and continued land use change (North Carolina Wildlife Resources Commission, 2015). Most remaining *L. grayi* populations have fewer than 200 mature plants and are relatively isolated by both distance and topography (Ingram et al., 2018), putting them at increased risk of extirpation. Limited gene flow between isolated populations is expected, however a genetic diversity survey of the species is a primary research need (NatureServe, 2009). Significant herbivory is also observed in *L. grayi*, as white-tailed deer populations in Eastern North America are at densities exceeding pre-settlement forests (McGraw & Chandler, 2018). Herbivory from ants and rabbits has also been observed (Bates, 1999), in addition to harvesting of the beautiful flowers by humans (Skinner, 2002). In southwestern VA *L. grayi* is experiencing introgression from the more common *L. canadense* (Skinner, 2002; Weakley & SF Team, 2022), raising concerns about genetic swamping.

L. grayi is an example of endemic biodiversity, the kind of species that exemplifies the evolution of local adaptation, that is being lost from our planet at an increasingly rapid rate. Biodiversity is directly related to ecosystem function and services (Cardinale et al., 2012), and thus the species' loss would have dire implications at multiple scales. The extinction of species is both a natural process and a fundamental component of evolution, eliminating unsuccessful strategies to create niches for future life. The rate at which species go extinct has varied significantly through time, peaking in five mass extinction events severe enough to define the boundaries of geologic eras. The fossil record provides a glimpse of the scale of these mass extinctions, with an estimated 40% of genera and 76% of species

lost in the most recent event (Barnosky et al., 2011). In recent centuries anthropogenic impacts have driven extinction rates an order of magnitude higher than background levels (Ceballos et al., 2015; De Vos et al., 2015; Otto, 2018), setting the stage for a sixth mass extinction. Approximately 200 vertebrate species have gone extinct in the past century, a loss that should have occurred over 10,000 years according to background rates (Ceballos et al., 2017). This abrupt loss of biodiversity is both alarming and foreshadowing. The primary factors accelerating biodiversity loss, anthropogenic land use change and resulting habitat fragmentation (Ellis et al., 2012, Mimura et al., 2017), are associated with a time lag known as extinction debt (Helm et al., 2006, Kuussaari et al., 2009). The concept of extinction debt is clearest in critically endangered species with no plausible chance of recovery, with estimates of current extinction debts ranging from 9% to 90% of local species richness (Figueiredo et al., 2019). This extremely broad range is partially due to the lack of data on the health and viability of many threatened and endangered species, making this data critical in developing future management plans in a world with continually decreasing habitat.

It could be argued that biodiversity is worth conserving for its own sake, however the evidence is also clear that conserving biodiversity is essential to maintaining the ecosystem functions and services that power our planet (Cardinale et al., 2012, Dirzo et al., 2014, Tilman et al., 2014, Weisser et al., 2017). Overall productivity in ecosystems is increased when communities are biodiverse, as multiple species can utilize many different sections of natural resources simultaneously (Hardin, 1960; MacArther, 1958). Species richness in primary producers increases the efficiency of inorganic nutrient uptake in both terrestrial and aquatic systems, resulting in more biomass on average compared to monocultures (Cardinale et al., 2011). Tilman et al. (2012) found that in perennial grassland ecosystems increasing

species richness affected annual biomass production at least as much as nitrogen addition, carbon enrichment, herbivory, drought, or fire disturbance. Biodiversity at the community level has a temporally stabilizing effect on ecosystem net primary production due to asynchronous responses of different species to disturbances or environmental variables, akin to the concept of risk-spreading (Hector et al., 2010, Morin et al., 2014). The resilience of ecosystem functions in the presence of environmental stochasticity will become increasingly important as the effects of anthropogenic climate change continue to manifest. Reduction in ecosystem functions accelerates with continued biodiversity loss, a non-linear response that is exaggerated when losses occur across trophic levels (Cardinale et al., 2012; Estes et al., 2011). Biodiversity at the intraspecific level, genetic diversity, is also associated with increased ecosystem function. Reusch et al. (2005) found positive correlations between genotypic diversity and shoot number, dry biomass, and faunal abundance in eelgrass (*Zostera marina*) populations following an extreme heat wave. Similarly, Reynolds et al. (2012) found that areal productivity, shoot density, and invertebrate density increased with allelic richness at all depths tested. The continued functioning of life on Earth is inextricably connected to its diversity, thus the conservation and restoration of threatened species must be among our greatest priorities.

The field of conservation genetics is focused on conserving biodiversity through studying and managing the genetic diversity of rare species and populations. Genetic diversity in populations is a metric of population health, and evaluating the genetics of multiple populations allows us to examine gene flow. Conservation genetics also allows for the identification of populations with unique and important genetic diversity, both adaptive and neutral, including Evolutionarily Significant Units (ESUs) and Management Units

(MUs) (Funk et al., 2012). Although ESUs and MUs might appear morphologically identical to other populations of a species, because of genetic drift they may contain private alleles, or alleles in high frequency that are rare elsewhere, that could serve as valuable relief via genetic rescue for other isolated, inbred populations (Coates et al., 2018). Additionally, ESUs and MUs with unique adaptive genetic diversity represent critical evolutionary potential as climate change rapidly affects ecosystems (Funk et al., 2012, Swarts et al., 2014).

The level of genetic diversity in populations is known to influence susceptibility to pathogens and disease, as well as the ensuing severity of response, as reviewed in King and Lively (2012). Examining the existing literature on the subject, they found evidence of greater disease transmission and severity in genetically depauperate populations of snails, multiple aquatic and terrestrial arthropods, and all classes of vertebrates. They then constructed a model simulating a host population with six genotypes and a parasite with nine genotypes to examine the effect of increasing genetic diversity on a theoretical population affected by a disease. Their model was consistent with the "matching-alleles" hypothesis for infection, which assumes that each host genotype is susceptible to a specific parasite genotype (Frank, 1993), and found that introduction of a novel allele (which increased total genotypes from six to nine) after 100 generations decreased disease prevalence from a consistent 0.6 to near zero. Empirical examples of reduced genotypic diversity leading to increased disease susceptibility have long been observed in agriculture (Browning & Frey, 1969; Wolfe, 1985; Zhu et al., 2000)

Plant pathogens represent a threat to agriculture, human health, and the conservation of biodiversity, and are predicted to become more impactful in a continually warming world (Anderson et al., 2004; Mordecai, 2011; Raza & Bebber, 2022). Examples include the

invasive myrtle rust (*Austropuccinia psidii*) which is a threat to members of the Myrtaceae family worldwide, including various plant communities in New Caledonia (Soewarto et al., 2018), wet sclerophyll forests in southeast Queensland (Pegg et al., 2017), eastern Australian rainforests (Fernandez et al., 2020), and Hawai'i (Stewart et al., 2017). In eastern North America a nonnative plant pathogen, Chestnut blight (*Cryphonectria parasitica*), had dramatic effects on entire ecosystems when it drove the functional removal of the American chestnut (*Castanea dentata*), a foundational species (Ellison et al., 2004). Chestnut blight was likely introduced in the early 1900s and spread approximately 37 km per year, resulting in approximately 3.6 million ha of dead or dying American chestnuts within 50 years (Anagnostakis, 1987). Other invasive pathogens affecting North American forest communities include Dutch elm disease across the continent (Brasier, 2001), beech bark disease in eastern forests (Cale et al., 2017), and sudden oak death in California and Oregon (Rizzo & Garbelotto, 2003).

This study works towards filling research gaps in *L. grayi* including a lack of understanding of the impacts from Lily Leaf Spot disease (LLS) across the range of the species, out-of-date population records, and the absence of population genetic diversity data. To address these critical research needs, demographic monitoring of 15 and 22 Elemental Occurrences (EOs) was conducted in 2020 and 2021, respectively, (Chapter 2) and a genetic diversity analysis of 24 EOs was conducted using microsatellite markers (Chapter 3). All collected data and results from this research work will be submitted to state, federal, and private land conservancy agencies, including the North Carolina Natural Heritage Program, to ensure population records are updated and an accurate knowledge of the status of this species is established.

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Chapter 2

Demographic Monitoring of *Lilium grayi*

Introduction

Lily Leaf Spot Disease

To evaluate the impacts of Lily Leaf Spot disease (LLS), *Pseudocercosporella inconspicua*, on native populations, demographic monitoring of populations is necessary. Monitoring populations involves collecting repeated observations or measurements to evaluate changes in condition of the population (Elzinga et al., 2001), with demographic monitoring focused on changing population dynamics at different stages in an organism's lifecycle. Moni Bates (1998) of the North Carolina Plant Conservation Program was the first to investigate premature senescence and early season population collapse due to LLS in *L. grayi* via demographic monitoring. She studied two subpopulations at Bluff Mountain, NC, one at the summit in a fen and the other at a lower elevation in the floodplain of a small stream. She found that premature senescence was more widespread in the floodplain on all three visits and had a strong influence on reproductive output, as plants wilted before producing capsules. Analysis of senesced stems found three native fungal species, *Botrytis* sp., *Alternaria* sp., and *Collectotricum* sp., with the latter suspected of causing the premature senescence. The following year an additional site was added and experimental plots with cleared woody understory were established, again concluding that premature senescence was more frequent in shady, moist sites than dry, open sites (Bates, 1999). Monitoring continued the next year, when premature senescence occurred significantly less and capsule production was higher in the cleared experimental plots (Bates, 2000). Fungicide treatments were tested at these sites, but they did not have a positive effect on the extent of premature senescence

(Coomans, 2002). Powell (2011) monitored *L. grayi* in plots at Roan Mountain, TN and analyzed 12 plants for fungi, finding the ascomycete *Pseudocercosporella inconspicua* (G. Winter) U. Braun on all four plants with disease symptoms. *P. inconspicua* is a *Lilium*-specific pathogen, described in 1884 from the Eurasian *Lilium martagon* L. (Braun, 1988; Ingram et al., 2018). An extensive search of herbarium records found the earliest records of *P. inconspicua* in North America on *L. canadense* individuals in Maine and Vermont, from 1916 and 1922 respectively, with the first record in *L. grayi* in an individual collected from Roan Mountain in 1947 (Ingram et al., 2017). A two-year monitoring survey conducted at Roan Mountain determined 59 and 70% of mature individuals experienced premature senescence due to LLS in the growing seasons they assessed, in addition to confirming spatial clustering of infection (Ingram et al., 2018). Further work was done to complete Koch's postulates and confirm *P. inconspicua* is the causal agent for LLS (Ingram & Levy, 2020). Congener *L. superbum* is susceptible to LLS and is more common than *L. grayi*, making it a potential disease reservoir (Barrett, 2017; Ingram et al., 2018). The *L. grayi* populations at Bluff Mountain and Roan Mountain are considered some of the most robust (NatureServe, 2009, Weakley & SF Team, 2022), thus the prevalence of LLS at both populations presents a clear risk to the species overall. Many *L. grayi* populations have not been surveyed since the initial LLS work (Bates, 1998), however *P. inconspicua* has since been identified at populations across the range of *L. grayi* (Barrett, 2017).

Pseudocercosporella inconspicua is the pathogen responsible for LLS (Ingram et al., 2018). *Pseudocercosporella* is a genus of ascomycete phytopathogens associated with multiple diseases in agricultural crops. The most well-known member of the genus is likely *P. herpotrichoides*, responsible for eyespot disease in wheat and rye (Crous et al., 2003). *P.*

inconspicua shares many features characteristic of the genus, including internal mycelium, fasciculate conidiophores, and solitary conidia (Crous et al., 2013; Ingram et al., 2018). A polycyclic mode of reproduction is also shared, which allows for many rounds of reproduction in a single season and exponential growth of disease propagules (Ingram, 2013). Hyphae are septate, branched, and hyaline. Conidiophores are arranged in a loose fascicle that emerges from stomatal openings, with 1-10 conidiophores per fascicle. The solitary conidia are generally cylindric and straight to slightly curved, as well as septate and hyaline. The presence of conidia on host tissues results in necrosis of photosynthetic tissues (Burdon, 1993; Ingram, 2013; Ingram & Levy, 2020). Affected species include *L. candidum*, *L. regale* (Zerova, 1940), *L. distichum*, *L. longiflorum* (Kim & Shin, 1998), *L. superbum*, *L. grayi* (Ingram et al., 2018), and *L. martagon*. Originally discovered on *L. martagon* in Europe in 1884 (Braun, 1988), the species has been present in North America since at least 1916 (Ingram et al., 2017) and in Eastern Asia since at least 1994 (Shin & Braun, 1996) and possibly as early as 1925 (Hiura, 1925). The seemingly circumboreal distribution and infectious nature of the pathogen (Ingram et al., 2018) make *P. inconspicua* a potential threat to many *Lilium* species.

In *L. grayi P. inconspicua* affects seedlings, juveniles, and adult plants, and is most prevalent and severe on leaves and maturing capsules which are likely sites of secondary inoculum production. While impacts on germination are unknown, the disease reduces fecundity through reduced capsule production (Bates, 1998; Bates, 1999; Bates, 2000; Ingram et al., 2018) and reduced seed production in infected capsules (Ingram, 2013). Early season collapse has been observed in juvenile, adolescent, and adult life stages, suggesting that recruitment is hindered by a truncated growing season leading to delayed maturity

(Ingram, 2013). The disease appears each season at host emergence (Ingram, 2013). In a cultivated *L. maximowjczii* population Hiura (1925) hypothesized that the fungus overwintered on dead leaf tissues, indirectly supported by Ingram & Levy (2020) who found little to no concentration of *P. inconspicua* conidia on non-host species nearby infected *L. grayi*. Further research on *P. inconspicua* in *L. grayi* populations is needed, both for the conservation of *L. grayi* and to understand how *P. inconspicua* may affect other threatened *Lilium* species.

Deer Browse

Overabundance of white-tailed deer (*Odocoileus virgianus*) in eastern deciduous forests has been observed since the 1930s (Kain et al., 2011), with population densities far exceeding those of pre-settlement forests (McGraw & Chandler, 2018). Negative impacts of deer browse have been seen in understory herbs across eastern North America, including reduced stem height (Anderson, 1994), reduced leaf area (Beauvais et al., 2017), and decreased population growth rate (λ) (Knight et al., 2009; Rooney & Gross, 2003) in *Trillium grandiflorum*. Augustine & Frelich (1998) found reduced leaf area and reproduction in *T. cernuum* and *T. flexipes*. In American ginseng (*Panax quinquefolius*), McGraw and Chandler (2018) found subpopulations with deer browse experienced population size reductions of 4.5% per year, while a subpopulation inaccessible to deer slowly increased in number. Deer overabundance also has a long-term legacy effect; in an 11 year study, Pendergast et al. (2016) found that excluding browsers increased species richness only modestly, concluding substantial recovery of diversity will take much longer than 11 years of protection from browse.

A large amount of deer browse has been seen in *Lilium grayi* and *L. superbum* in eastern North America as well. Fletcher et al. (2001) planted a total of 493 *L. superbum* bulbs, half of which were covered with wired cages to exclude deer, in three different environments. They found white-tailed deer ate the apical meristem of 28% of plants that successfully emerged, which resulted in lower average height and stopped growth and reproduction for that season. Ingram et al. (2018) found that in *L. grayi*, along a transect at Roan Mountain, 50% (47/94) and 69% (83/120) of plants were browsed in 2011 and 2012, respectively.

Objectives

The focus of this study was to continue previous demographic monitoring in *Lilium grayi* and expand the geographic extent, evaluating potential changes in populations previously monitored (Roan Mountain, Bluff Mountain) and collecting new data from as many populations as possible across the range of the species. This was achieved through monitoring of flowering individuals to determine the proportion of successful reproduction. The primary hypothesis for demographic monitoring was that populations on the Roan Mountain massif represent the primary source of reproduction in the species, and it is predicted that these populations will have the highest proportion of reproduction. These data will be provided to state, federal, and private land conservancy agencies to ensure population records are updated, as many populations have not been observed in decades.

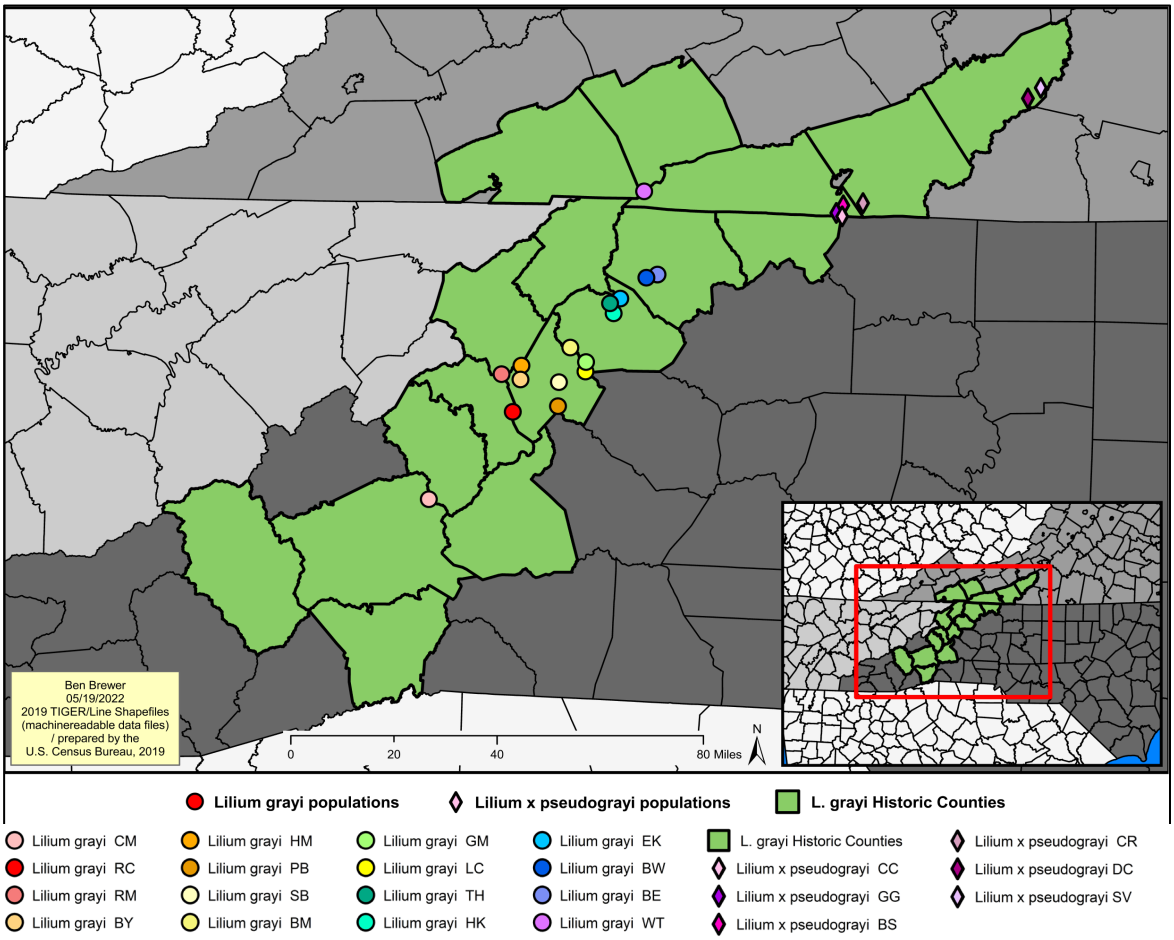
Materials and Methods

Field Work

Demographic monitoring of flowering plants to determine reproductive success was conducted in the growing seasons of 2020 and 2021 in 15 and 22 *L. grayi* and *L.* x *pseudograyi* populations, respectively. Methodology followed a community monitoring protocol developed by National Park Service, North Carolina Department of Agriculture & Consumer Services, and Appalachian State University botanists. Populations were visited at least twice during the growing season: first during anthesis in June and July, then during capsule production in late August through October. First visits to populations occurred between 06/11/2020 and 07/13/2020 as well as 06/11/2021 and 07/13/2021. Second visits to populations occurred between 08/12/2020 and 10/15/2020 as well as 09/02/2021 and 10/06/2021.

Populations were chosen based on Element Occurrence (EO) health rankings and geographic distribution (Figure 1). Only populations with a health ranking of C or higher were visited. Twenty-seven populations were visited in total, representing a majority (59%) of the 46 remaining extant populations (NatureServe, 2009). Of the 22 populations monitored, 15 are in NC and 7 are in VA. All 15 NC populations and 1 VA population are thought to be pure *L. grayi*, while 6 VA populations contain *L.* x *pseudograyi* individuals (Weakley & SF Team, 2022).

Figure 1. Monitored Elemental Occurrences. All visited elemental occurrences where demographic monitoring was conducted. Sites visited between 06/11/2020 and 07/13/2020 or 06/11/2021 and 07/13/2021 and again between 08/12/2020 and 10/15/2020 or 09/02/2021 and 10/06/2021.



During first visits to populations plant location and health data were recorded, plants were affixed with aluminum identification tags, and a small leaf tissue sample was taken for later genetic diversity analysis. Location data were collected with a Garmin Oregon 750t handheld GPS unit, and photos of plants including their backgrounds were taken to assist in rediscovery on second visits. Collected plant health data included flower height from the ground, number of leaf whorls, number of leaf whorls with Lily Leaf Spot (LLS) lesions, number of flowers, and the degree of competitive shading in a $1m^2$ area. Shading at three height strata was recorded: from 0-1m, 1-2m, and canopy cover. Amount of shading was classified into one of 10 cover classes: <0.1%, 0-1%, 1-2%, 2-5%, 5-10%, 10-25%, 25-50%, 50-75%, 75-95%, 95-100%.

On second visits to populations the monitored plants were rediscovered and their status at the end of the season was recorded. Plant status was recorded as one of the following: fruiting, senesced, herbivory but otherwise healthy, herbivory and senesced, or other (either healthy with no capsule or mowed). For fruiting individuals, the quantity and quality of capsules was recorded. In 2020 viable seeds were collected at two populations for *ex situ* conservation in the North Carolina Botanical Gardens seed bank.

All flowering individuals were monitored in an attempted census of 13 populations in 2020 (CM, HM, PB, SB, BM, LC, BW, WT, BS, GG, CR, DC, SV) and 17 populations in 2021 (CM, RC, BY, PB, SB, BM, GM, HK, EK, BE, WT, CC, BS, GG, CR, DC, SV). In five larger populations where a complete census was not possible (RM and TH both years, HM, LC, BW in 2021) at least 32 individuals were chosen for monitoring haphazardly across the population. In one of the five populations where a census was not attempted (TH), plants were also monitored in eight $25m^2$ plots.

In the large population where eight $25m^2$ plots were used for monitoring, the plots were established as four pairs of plots on 06/26/2019. Pairs were established based on elevation and plant community, as the population is found along a grassy bald ridgetop and the *Crataegus* forest located below it. Four plots were located on the grassy bald, two at the highest elevation and two at lower elevation nearer to the transition to the *Crataegus* forest. The remaining four plots were placed in the *Crataegus* forest. To examine the effect of deer browse on the population, deer exclosures were constructed around two plots – one on the grassy bald at lower elevation and one in the *Crataegus* forest – in the winter between the growing seasons of 2019 and 2020. Exclosures were constructed with 6' tall wire fencing wrapped around fenceposts with the assistance of the 2020 Appalachian State University Wildlife Biology class. To determine if deer were being successfully deterred, trail cameras were placed on posts facing both exclosures. Cameras were located approximately 3-4' off the ground, and the entire plot was in the camera's field of vision. Cameras were programmed to take a burst of five photographs when motion was detected, with a 30 s interval between bursts.

To prevent further spread of LLS, footwear and all measuring equipment used was sterilized with 90% ethanol between visits to populations. Clothing worn was washed before being used at another population.

Statistical Analysis

Statistical analysis of demography data was performed in Minitab v. 21.1. Average values for data collected on first visits, as well as capsules produced, was calculated for each population and for the entire dataset. Additionally, the proportion of plants in each condition

on second visits was calculated and averaged for each population, for pure *L. grayi* populations, for *L.* x *pseudograyi* populations, and for the entire dataset. Plants that were not successfully re-discovered were not included in these calculations.

All plant health and shading data collected on first visits, as well as capsules produced, were tested for correlation with each other. Regression analysis was used to evaluate the degree to which flower height, proportion of diseased whorls, number of flowers, and number of capsules were dependent on competitive shading at stratum 1, stratum 2, and canopy. Welch's unequal variance *t*-tests were used to test for significant differences between pure *L. grayi* populations and *L.* x *pseudograyi* populations in flower height, whorl number, diseased whorl number, diseased whorl percentage, flower number, capsule number, and competitive shading in stratum 1, stratum 2, and canopy. Welch's tests were also used to evaluate whether there was a significant difference in diseased whorls on first visits between plants that produced fruit and those that eventually senesced.

Results

First Visits

In 2020 a total of 218 individuals were measured and tagged on first visits, and in 2021 a total of 371 individuals were measured and tagged on first visits. Average values for plant health and competitive shading are summarized for each population and across the dataset in Table 1 and Table 2 for 2020 and 2021, respectively. In 2020 the average flower height was 89.1 cm, plants had an average of 6.2 whorls per plant, of which 24.37% showed signs of Lily Leaf Spot (LLS), and plants had an average of 1.7 flowers per plant. Average

shading cover in stratum 1 was 8.9 (~75-95% shading) and in stratum 2 was 3.7 (~2-5% shading). In 2021 the average flower height was 88.42cm, plants had an average of 5.6 whorls per plant, of which 26.68% showed signs of Lily Leaf Spot (LLS), and plants had an average of 1.4 flowers per plant. Average shading cover in stratum 1 was 8.2 (~50-75% shading), in stratum 2 was 2.9 (~1-2% shading), and canopy cover was 6.3 (10-25% shading).

Table 1. 2020 First Visit Data. Plant Health Metrics and Competitive Shading Data Collected on First Visits to Populations in 2020. Data collected between 06/11/2020 and 07/13/2020.

Site	Total Flowering Plants	Height (cm)	Whorl #	Yellow whorl #	Yellow whorl %	Flower #	Stratum 1 (0-1m)	Stratum 2 (1-2m)
CM	2	96.0	7.0	2.5	31.3%	1.5	8.0	7.5
RM	56	79.2	5.8	1.2	20.9%	2.4	9.5	2.0
HM	11	102.3	7.0	1.7	24.4%	3.0	9.2	4.0
PB	18	120.2	6.6	0.4	6.3%	1.2	8.9	6.6
SB	9	99.2	5.4	0.1	1.6%	1.4	9.3	6.7
BM	3	85.5	6.0	0.0	0.0%	1.7	8.0	6.0
LC	4	87.0	4.8	2.0	40.0%	1.0	8.3	4.0
TH	33	63.0	5.2	3.7	71.9%	1.1	9.8	0.6
BW	8	90.6	5.1	1.4	28.3%	1.0	5.5	4.6
WT	11	72.5	6.3	1.3	21.8%	1.5	8.9	1.5
BS	17	86.2	6.5	0.6	11.8%	1.5	7.1	5.2
GG	7	94.8	6.4	0.3	4.6%	2.0	7.6	4.6
CR	4	120.5	7.3	1.0	14.0%	2.8	10.0	6.0
DC	14	115.3	7.4	0.7	9.8%	1.7	9.2	6.8
SV	21	100.1	7.4	0.9	11.3%	1.4	8.7	4.5
Total	**218**	**89.10**	**6.2**	**1.4**	**24.37%**	**1.7**	**8.9**	**3.7**

Table 2. 2021 First Visit Data. Plant Health Metrics and Competitive Shading Data Collected on First Visits to Populations in 2021. Data collected between 06/11/2021and 07/13/2021.

Site	Total Flowering Plants	Height (cm)	Whorl #	Yellow whorl #	Yellow whorl %	Flower #	Stratum 1 (0-1m)	Stratum 2 (1-2m)	Canopy
CM	30	86.0	5.4	2.0	36.5%	1.5	8.2	3.3	8.4
RC	8	103.7	4.9	1.4	28.3%	1.1	9.4	0.0	9.3
RM	32	68.6	5.5	1.1	18.5%	1.3	9.6	1.2	0.9
BY	5	89.2	5.0	1.2	24.0%	1.0	7.6	0.0	9.0
HM	48	91.4	6.4	2.8	42.6%	2.0	8.2	3.9	2.2
PB	11	114.0	6.5	1.5	30.0%	1.7	7.3	5.5	7.8
SB	7	112.9	5.3	0.1	2.4%	2.4	7.6	7.4	8.1
BM	1	83.0	6.0	0.0	0.0%	1.0	8.0	7.0	7.0
LC	33	79.9	4.3	0.9	20.7%	1.1	7.9	0.6	9.7
GM	6	96.6	5.8	3.3	56.6%	1.3	6.0	6.5	7.7
TH	17	85.8	5.7	4.2	75.6%	1.4	9.6	1.2	4.2
HK	1	84.0	6.0	6.0	100.0%	1.0	8.0	0.0	9.0
EK	30	78.8	4.3	0.3	6.2%	1.1	7.9	3.0	8.1
BW	92	89.3	5.6	1.2	21.1%	1.2	7.7	2.7	8.9
BE	3	100.8	6.3	0.3	5.6%	2.7	8.3	2.3	8.3
WT	3	90.5	5.7	3.0	55.6%	1.3	8.3	4.7	6.3
CC	2	118.8	7.5	0.5	5.0%	3.0	7.0	7.5	6.0
BS	10	99.1	6.1	0.2	4.2%	1.5	9.1	3.4	6.2
GG	4	113.9	6.5	0.0	0.0%	1.3	7.3	7.8	4.0
CR	2	105.8	7.0	1.0	14.3%	1.0	6.0	9.0	0.0
DC	14	104.2	6.6	2.6	43.0%	1.8	7.7	5.6	1.1
SV	12	80.9	6.3	0.3	6.1%	1.5	9.6	1.3	1.2
Total	**371**	**88.42**	**5.6**	**1.5**	**26.68%**	**1.4**	**8.2**	**2.9**	**6.3**

Second Visits

In 2020, 201 of the 218 tagged individuals (92.2%) were successfully rediscovered for monitoring, while in 2021 347 of the 371 tagged individuals (93.5%) were successfully rediscovered for monitoring. The proportion of plants in each status condition are summarized for each population, as well as averages for the year, in Table 3 and Table 4 for 2020 and 2021 respectively. In 2020, 30.85% (62 / 201) of plants successfully produced fruit, 36.32% (73 / 201) were found prematurely senesced to LLS, 11.44% (23 / 201) were found both senesced and browsed, 12.44% (25 / 201) were found browsed but otherwise healthy, and 8.96% (18 / 201) were found either mowed or healthy with no fruit present. The average number of capsules produced per flowering plant was 0.56. In 2021, 12.97% (45 / 347) of plants successfully produced fruit, 66.28% (230 / 347) were found prematurely senesced to LLS, 14.41% (50 / 347) were found both senesced and browsed, 2.31% (8 / 347) were found browsed but otherwise healthy, and 4.03% (14 / 347) were found either mowed or healthy with no fruit present. The average number of capsules produced per flowering plant was 0.22.

Table 3. 2020 Second Visit Data. Plant Status at End of Growing Season Collected on Second Visits to Populations in 2020. Data collected between 08/12/2020-10/15/2020.

Site	Total Plants Rediscovered (%)	Fruiting Plants (%)	Senesced Plants (%)	Senesced & Browsed Plants (%)	Browsed Plants (%)	Other No-Capsule Plants (%)	Average Capsule per Flowering Plant
CM	2 (100%)	0	2 (100%)	0	0	0	0.0
RM	52 (92.86%)	13 (25.00%)	13 (25.00%)	12 (23.08%)	11 (21.15%)	3 (5.77%)	0.8
HM	11 (100%)	5 (45.45%)	4 (36.36%)	2 (18.18%)	0	0	0.8
PB	12 (66.67%)	5 (41.66%)	2 (16.67%)	0	2 (16.67%)	3 (25.00%)	0.4
SB	7 (77.78%)	3 (42.86%)	1 (14.29%)	0	2 (28.57%)	1 (14.29%)	0.7
BM	3 (100%)	1 (33.33%)	0	0	2 (66.67%)	0	0.3
LC	4 (100%)	0	4 (100%)	0	0	0	0.0
TH	32 (96.97%)	2 (6.25%)	22 (68.75%)	5 (15.63%)	3 (9.38%)	0	0.1
BW	8 (100%)	1 (12.5%)	6 (75.0%)	0	1 (12.5%)	0	0.1
WT	11 (100%)	1 (9.09%)	5 (45.45%)	2 (18.18%)	3 (27.27%)	0	0.1
BS	14 (82.35%)	7 (50.00%)	5 (35.71%)	2 (14.29%)	0	0	0.9
GG	6 (100%)	4 (66.67%)	0	0	1 (16.67%)	1 (16.67%)	0.6
CR	4 (100%)	4 (100%)	0	0	0	0	1.8
DC	14 (100%)	4 (28.57%)	1 (7.14%)	0	0	9 (64.29%)	0.6
SV	21 (100%)	12 (57.14%)	8 (38.10%)	0	0	1 (4.76%)	0.8
Total	**201(92.20%)**	**62 (30.85%)**	**73 (36.32%)**	**23 (11.44%)**	**25 (12.44%)**	**18 (8.96%)**	**0.56**

Table 4. 2021 Second Visit Data. Plant Status at End of Growing Season Collected on Second Visits to Populations in 2021. Data collected between 09/02/2020-10/06/2020.

Site	Total Plants Rediscovered (%)	Fruiting Plants (%)	Senesced Plants (%)	Senesced & Browsed Plants (%)	Browsed Plants (%)	Other No-Capsule Plants (%)	Average Capsule per Flowering Plant
CM	30 (100%)	0	28 (93.33%)	0	0	2 (6.67%)	0.0
RC	8 (100%)	0	7 (87.50%)	1 (12.50%)	0	0	0.0
RM	29 (90.63%)	3 (10.34%)	3 (10.34%)	21 (72.41%)	0	2 (6.90%)	0.2
BY	4 (80.00%)	0	4 (100%)	0	0	0	0.0
HM	44 (91.67%)	7 (15.91%)	18 (40.91%)	13 (29.55%)	1 (2.27%)	5 (11.36%)	0.4
PB	9 (90.00%)	1 (11.11%)	5 (55.56%)	0	0	3 (33.33%)	0.4
SB	6 (85.71%)	4 (66.67%)	2 (33.33%)	0	0	0	2.0
BM	1 (100%)	0	0	0	1 (100%)	0	0.0
LC	33 (100%)	0	33 (100%)	0	0	0	0.0
GM	6 (100%)	0	5 (83.33%)	1 (16.67%)	0	0	0.0
TH	17 (100%)	2 (11.76%)	15 (88.24%)	0	0	0	0.2
HK	1 (100%)	0	1 (100%)	0	0	0	0.0
EK	30 (100%)	1 (3.33%)	22 (73.33%)	7 (23.33%)	0	0	0.0
BW	87 (94.57%)	6 (6.90%)	74 (85.06%)	6 (6.90%)	1 (1.15%)	0	0.1
BE	3 (100%)	2 (66.67%)	1 (33.33%)	0	0	0	1.7
WT	3 (100%)	0	3 (100%)	0	0	0	0.0
CC	2 (100%)	0	2 (100%)	0	0	0	0.0
BS	10 (100%)	7 (70.00%)	1 (10.00%)	0	1 (10.00%)	1 (10.00%)	0.8
GG	4 (100%)	3 (75.00%)	0	0	0	1 (25.00%)	0.8
CR	2 (100%)	1 (50.00%)	1 (50.00%)	0	0	0	0.5
DC	6 (42.86%)	1 (16.67%)	3 (50.00%)	1 (16.67%)	1 (16.67%)	0	0.3
SV	12 (100%)	7 (58.33%)	2 (16.67%)	0	3 (25%)	0	0.7
Total	**347 (93.53%)**	**45 (12.97%)**	**230 (66.28%)**	**50 (14.41%)**	**8 (2.31%)**	**14 (4.03%)**	**0.22**

Ex Situ Conservation

A total of 690 viable seeds were collected from 10 individuals for *ex situ* conservation at the North Carolina Botanical Gardens seed bank (Table 5**).** Seeds were collected between 09/07/2020-10/15/2020 and given to NCBG staff. These 10 plants were also genotyped, yielding genetic data on the female parents of these seeds. Individuals 10416 and 10417, as well as 10440 and 10441, were found to have the same multilocus genotype.

Table 5. Seed Collections. Seeds Collected for Ex Situ Storage at North Carolina Botanical Gardens Seed Bank.

Individual #	Location	Date	Seed #
10506	HM	09-07-20	32
10510	HM	09-07-20	29
10512	HM	09-07-20	44
10439	RM	10-11-20	61
10440	RM	10-15-20	144
10441	RM	10-15-20	143
10416	RM	09-27-20	49
10417	RM	09-27-20	56
10425	RM	09-12-20	87
10445	RM	10-04-20	45
Total			690

Herbivory Analysis

Of the eight $25m^2$ plots located at population TH, four (plot 3 on the Grassy Bald and plots 5, 6, and 8 in the *Crataegus* forest) did not contain flowering individuals in either 2020 or 2021. Average values for plots 1, 2, 4, and 7 are summarized in Table 6. Plots 1, 2, and 4 were located on the Grassy Bald, while plot 7 was located in the *Crataegus* forest. Plot 1 contained 9 flowering plants with one capsule produced in 2020, and 3 flowering plants with no capsules produced in 2021. Plot 2 contained 2 flowering plants with no capsules produced in 2020, and no flowering individuals in 2021. Plot 4 was located inside a deer exclosure and contained 9 flowering plants with 1 capsule in 2020 and 5 flowering plants with 4 capsules in 2021. Plot 7, located in the *Crataegus* forest, contained no flowering plants in 2020, and 9 flowering plants with no capsules produced in 2021. Trail camera photos of plots 4 and 7 revealed deer regularly browsing around the exclosure but never inside.

Table 6. Population TH Plots. Average Values for 25m^2 Plots Per Year. Plots 3, 5, 6, and 7 did not Contain Flowering Individuals in 2020 or 2021.

Plot	Year	Habitat	Fenced	N	Height (cm)	Whorl #	Yellow Whorl #	Yellow Whorl %	Flower #	Stratum 1	Stratum 2	Canopy	Capsules Per Plant	Total Capsules
1	**2020**	Grassy Bald	No	9	54.2	4.6	3.8	83.3%	1.0	9.8	0.0	0.0	0.1	1
1	**2021**	Grassy Bald	No	3	75.5	5.7	4.0	79.2%	1.3	10.0	0.0	0.0	0.0	0
2	**2020**	Grassy Bald	No	2	48	4.5	4.5	100.0%	1	10	0	0	0	0
2	**2021**	Grassy Bald	No	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	**2020**	Grassy Bald	Yes	9	72.4	5.7	4.7	0.8	1.0	10.0	0.7	0.0	0.1	1
4	**2021**	Grassy Bald	Yes	5	81.7	6.0	4.4	0.7	1.8	10.0	0.0	0.0	0.8	4
7	**2020**	Forest	Yes	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	**2021**	Forest	Yes	9	91.6	5.6	4.1	0.8	1.1	9.2	2.3	8.0	0.0	0

Statistical Analysis

Average values for end-of-season plant status data were also calculated independently for pure *L. grayi* populations and *L.* x *pseudograyi* populations (Table 7). Ten pure *L. grayi* populations were monitored in 2020, increasing to 16 in 2021. In 2020, 21.83% (31 / 142) of plants successfully produced fruit, 41.55% (59 / 142) were found prematurely senesced to LLS, 14.79% (21 / 142) were found both senesced and browsed, 16.90% (24 / 142) were found browsed but otherwise healthy, and 4.93% (7 / 142) were found either mowed or healthy with no fruit present. The average number of capsules per flowering plant was 0.46. In 2021, 8.36% (26 / 311) of plants successfully produced fruit, 71.06% (221 / 311) were found prematurely senesced to LLS, 15.76% (49 / 311) were found both senesced and browsed, 0.96% (3 / 311) were found browsed but otherwise healthy, and 3.86% (12 / 311) were found either mowed or healthy with no fruit present. The average number of capsules produced per flowering plant was 0.18. Five hybrid *L.* x *pseudograyi* populations were monitored in 2020, increasing to 6 in 2021. In 2020, 52.54% (31 / 59) of plants successfully produced fruit, 23.73% (14 / 59) were found prematurely senesced to LLS, 3.39% (2 / 59) were found both senesced and browsed, 1.69% (1 / 59) were found browsed but otherwise healthy, and 18.64% (11 / 59) were found either mowed or healthy with no fruit present. The average number of capsules produced per flowering plant was 0.81. In 2021, 52.78% (19 / 36) of plants successfully produced fruit, 25.00% (9 / 36) were found prematurely senesced to LLS, 2.78% (1 / 36) were found both senesced and browsed, 13.89% (5 / 36) were found browsed but otherwise healthy, and 5.56% (2 / 36) were found either mowed or healthy with no fruit present. The average number of capsules produced per flowering plant was 0.61.

Table 7. Second Visit Data by Species. Plant Status at End of Growing Seasons Collected on Second Visits to Populations, Separated Based on Hybridization Status.

Year	Sites	Plants Monitored	Fruiting Plants (%)	Senesced Plants (%)	Senesced & Browsed Plants (%)	Browsed Plants (%)	Other No-Capsule Plants (%)	Average Capsule per Flowering Plant
Lilium grayi								
2020	10	142	31 (21.83%)	59 (41.55%)	21 (14.79%)	24 (16.90%)	7 (4.93%)	0.46
2021	16	311	26 (8.36%)	221 (71.06%)	49 (15.76%)	3 (0.96%)	12 (3.86%)	0.18
Lilium* x *pseudograyi								
2020	5	59	31 (52.54%)	14 (23.73%)	2 (3.39%)	1 (1.69%)	11 (18.64%)	0.81
2021	6	36	19 (52.78%)	9 (25.00%)	1 (2.78%)	5 (13.89%)	2 (5.56%)	0.61
Combined								
2020	15	201	62 (30.85%)	73 (36.32%)	23 (11.44%)	25 (12.44%)	18 (8.96%)	0.56
2021	22	347	45 (13.00%)	230 (66.28%)	50 (14.41%)	8 (2.31%)	14 (4.03%)	0.22

Correlation tests run on plant health and shading metrics collected on first visits, as well as fruit produced, yielded many significant correlations (Table 8). However, regression analyses of significant correlations all resulted in low R^2 values, ranging from 0.79% in the Yellow Whorl % versus Stratum 1 comparison to a high of 32.85% in the Fruits versus Flowers comparison.

Table 8. Correlations. Pairwise Pearson Correlations between Plant Health and Shading Metrics across all *L. grayi* and *L.* x *pseudograyi* Individuals. * p<0.05 ** p<0.01 ***P<0.001

	Height (cm)	Whorls	Y Whorls	Yellow %	Flowers	Fruits	Stratum 1	Stratum 2
Whorls	0.556***							
Y Whorls	-0.064	0.076						
Yellow %	-0.139**	-0.066	0.971***					
Flowers	0.502***	0.452***	0.026	-0.043				
Fruits	0.390***	0.318***	-0.106*	-0.135**	0.574***			
Stratum 1	-0.227***	0.019	0.092*	0.099*	-0.047	-0.111*		
Stratum 2	0.491***	0.291***	-0.104*	-0.153***	0.182***	0.239***	-0.410***	
Canopy	0.197***	-0.255***	-0.140**	-0.107*	-0.134**	-0.090	-0.417***	0.109*

Multiple significant differences between *L. grayi* and *L.* x *pseudograyi* populations were discovered via Welch's Tests (Table 9). Plants in *L.* x *pseudograyi* populations were significantly taller (100.6 cm) than pure *L. grayi* populations (85.8 cm) (Figure 2), had more whorls (6.9 to the 5.6 in pure *L. grayi*) (Figure 3), and produced far more fruit (0.737 per plant compared to 0.260 in pure *L. grayi*) (Figure 6). The average proportion of whorls suffering from LLS was significantly higher in pure *L. grayi* populations, 29.2% compared to 11.2% of *L.* x *pseudograyi* (Figure 4). There was not a significant difference in average number of flowers produced between the two groups (Figure 5), or in the average competitive shading in stratum 1. However, average competitive shading in stratum 2 was significantly higher in *L.* x *pseudograyi* at 4.95 (~5-10% shading) to the 2.75 (0-2%) of pure *L. grayi* (Figure 7). Conversely, average canopy cover in *L. grayi* was significantly higher at 5.35 (5-10%) compared to 3.57 (1-2%) of *L.* x *pseudograyi* populations (Figure 8). There was also a significant difference in the average proportion of whorls suffering from LLS on first visits to populations between plants that later produced capsules and those that senesced. Plants that eventually senesced had a higher average proportion of infected whorls (31%) than did plants that successfully produced fruit (13.5%) (Figure 9).

Table 9. Welch's Unequal Variances *t*-tests. Evaluating Whether Differences in Means of Plant Health and Competitive Shading Data between *L. grayi* and *L.* x *pseudograyi* Populations are Significant. Also Whether Differences in Means of Diseased Whorls on the First Visit between Plants that Fruited and Plants that Prematurely Senesced are Significant

Height	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	451	85.8	22.4	-5.47	130	0.000
x *pseudograyi*	95	100.6	24.2			
Whorls	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	453	5.56	1.2	-8.24	122	0.000
x *pseudograyi*	95	6.86	1.44			
Yellow Whorl %	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	453	29.2%	0.326	6.99	212	0.000
x *pseudograyi*	95	11.2%	0.201			
Flowers	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	453	1.52	1.19	-1.28	147	0.203
x *pseudograyi*	95	1.67	1.07			
Fruits	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	453	0.260	1.01	-4.79	157	0.000
x *pseudograyi*	95	0.737	0.841			
Stratum 1	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	453	8.41	1.62	-0.16	138	0.870
x *pseudograyi*	95	8.44	1.58			
Stratum 2	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	453	2.75	3.24	-6.65	149	0.000
x *pseudograyi*	95	4.95	2.86			
Canopy	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
grayi	431	5.35	4.36	4.18	160	0.000
x *pseudograyi*	95	3.57	3.62			
First Visit % Yellow Whorls	**N**	**Mean**	**St. Dev**	**T-Value**	**DF**	**P-Value**
Fruiting	107	13.5%	0.202	-6.67	290	0.000
Senesced	376	31.0%	0.339			

Figure 2. Boxplot of Height Welch's *t*-test. Testing for Significance in Difference of Means between *L. grayi* (85.8 cm) and *L.* x *pseudograyi* (100.6 cm) p <0.000.

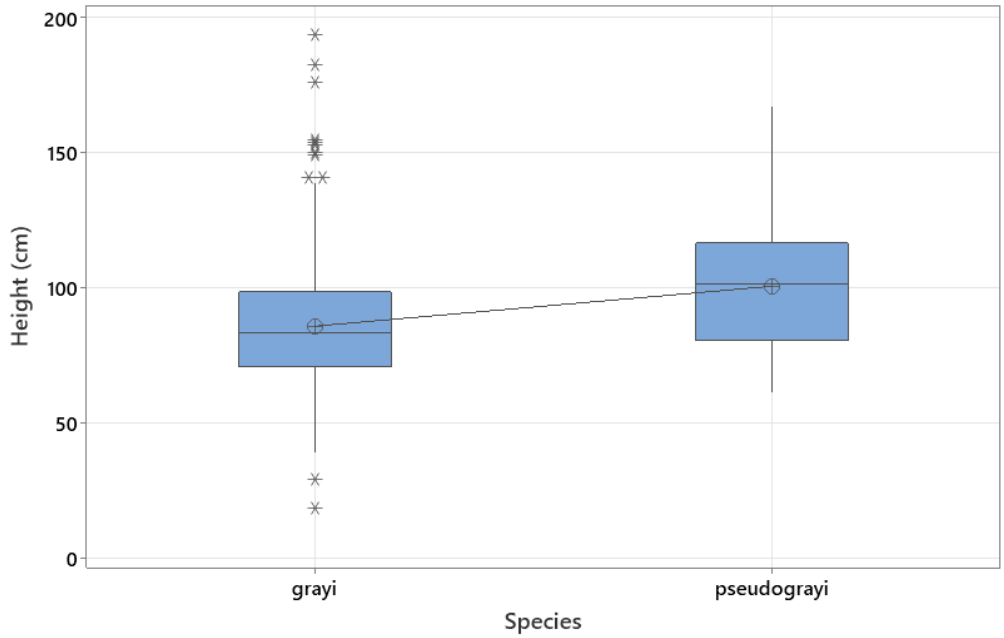


Figure 3. Boxplot of Whorls Welch's *t*-test. Testing for Significance in Difference of Means between *L. grayi* (5.56) and *L.* x *pseudograyi* (6.86 cm) p < 0.000.

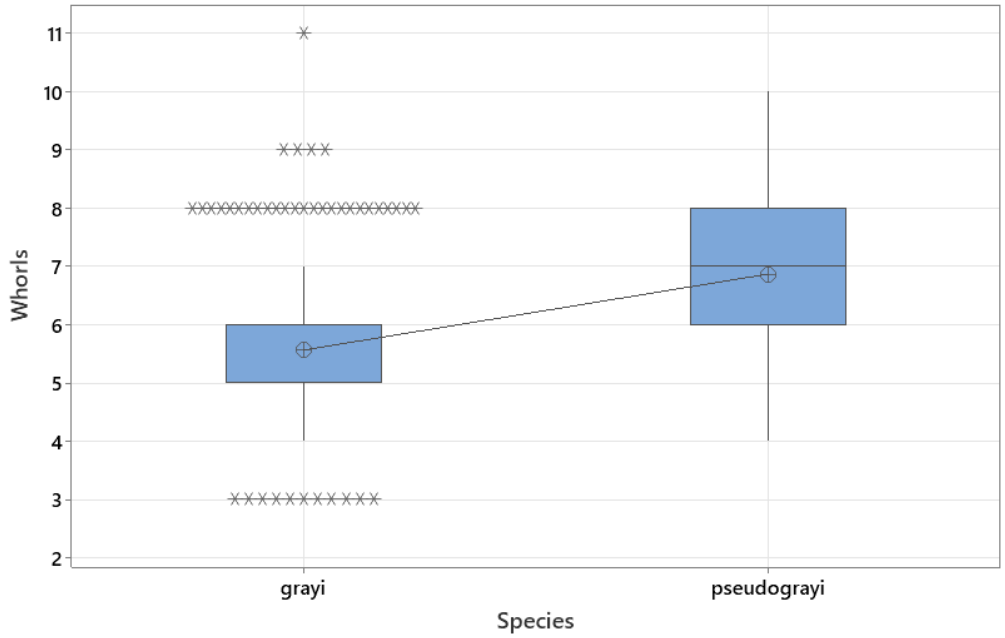


Figure 4. Boxplot of Percent Yellow Whorls Welch's *t*-test. Testing for Significance in Difference of Means between *L. grayi* (29.2%) and *L.* x *pseudograyi* (11.2%) p < 0.000.

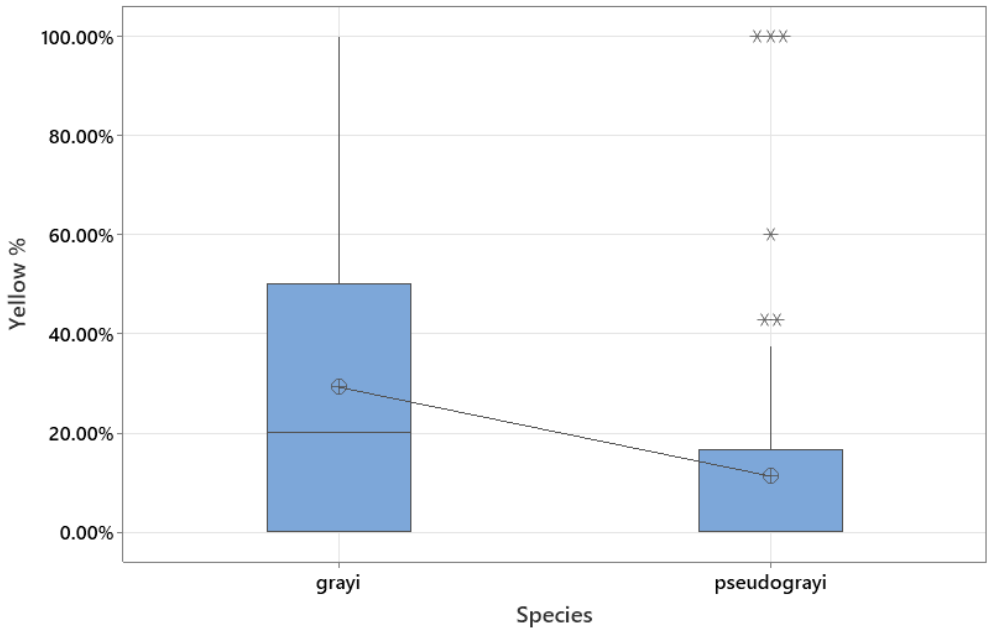


Figure 5. Boxplot of Flowers Welch's *t*-test. Testing for Significance in Difference of Means between *L. grayi* (1.52) and *L.* x *pseudograyi* (1.67) p = 0.203.

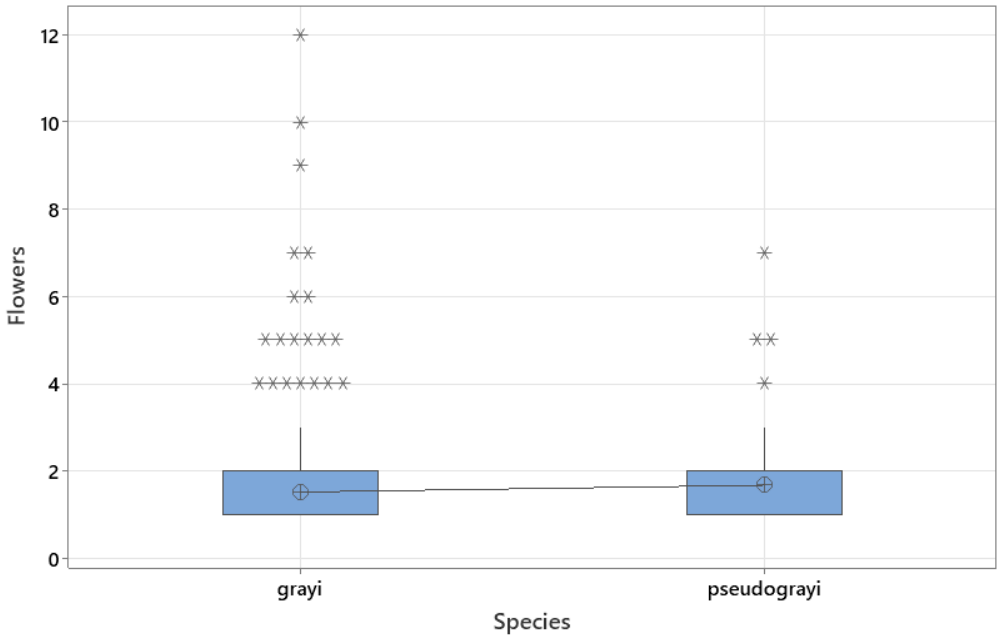


Figure 6. Boxplot of Fruits Welch's *t*-test. Testing for Significance in Difference of Means between *L. grayi* (0.260) and *L.* x *pseudograyi* (0.737) p < 0.000.

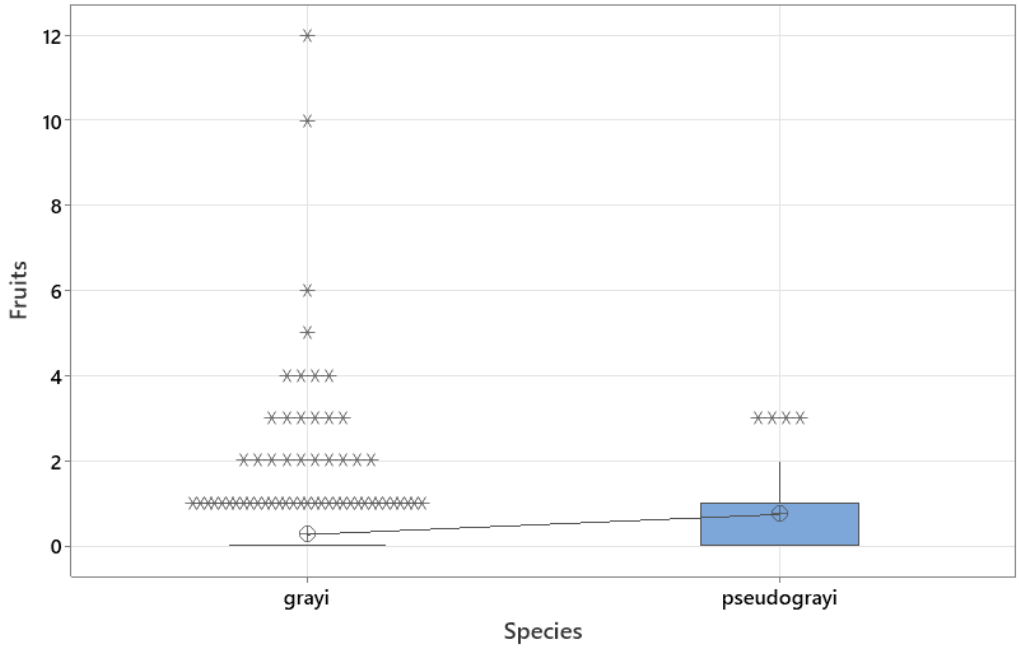


Figure 7. Boxplot of Stratum 2 Welch's *t*-test. Testing for Significance in Difference of Means between *L. grayi* (2.75) and *L.* x *pseudograyi* (4.95) p < 0.000.

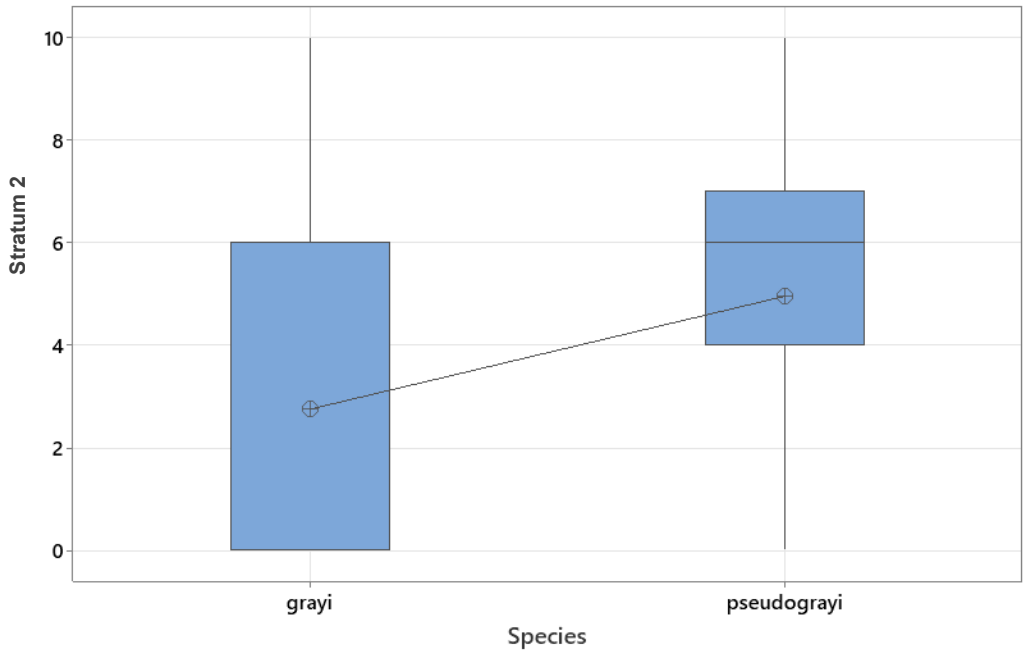


Figure 8. Boxplot of Canopy Welch's *t*-test. Testing for Significance in Difference of Means between *L. grayi* (5.35) and *L.* x *pseudograyi* (3.57) $p < 0.000$.

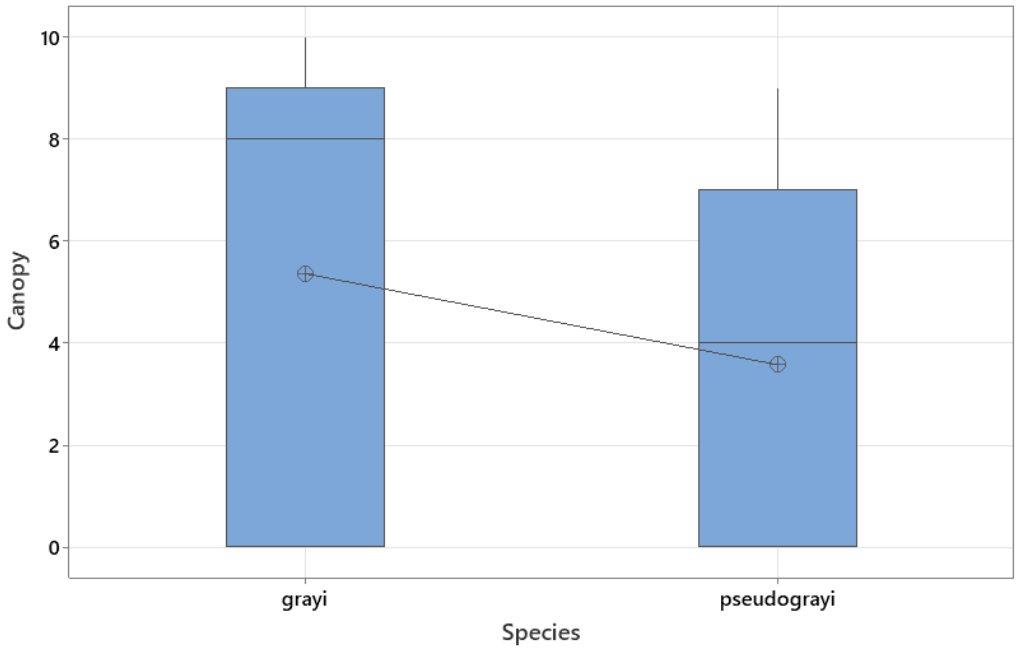
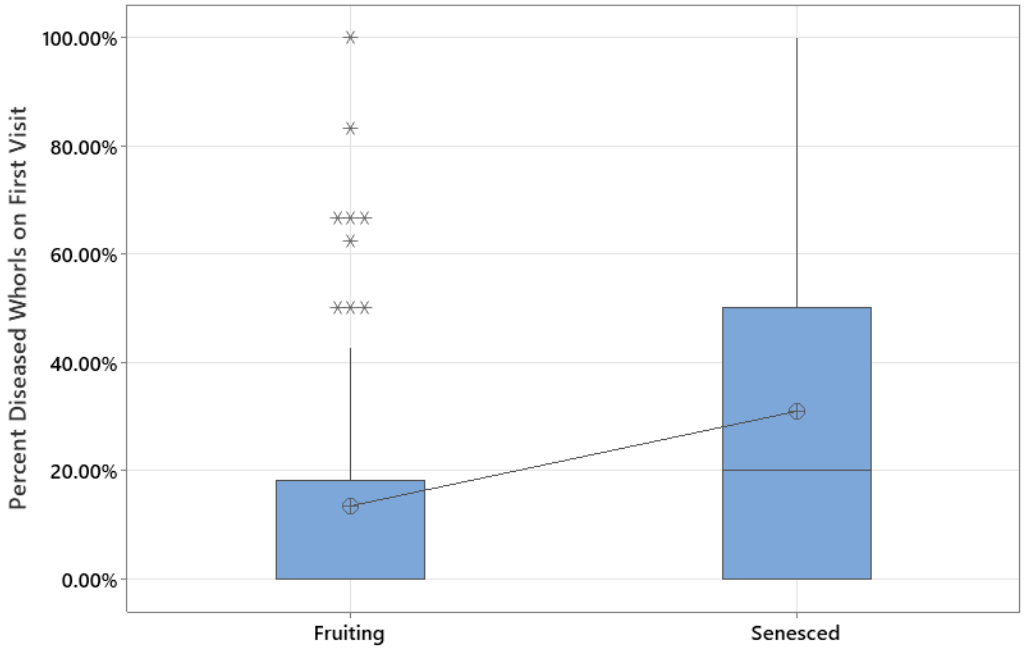


Figure 9. Boxplot of First Visit Percent Diseased Whorls Welch's *t*-test. Testing for Significance in Difference of Means between Plants that Produced Capsules (13.5%) and Plants that Prematurely Senesced (31.0%) $p < 0.000$.



Discussion

Successful capsule production in *Lilium grayi* was alarmingly low across the range of the species, with only 30.85% and 12.97% of plants reproducing in 2020 and 2021, respectively. The majority of plants did not successfully reproduce in any pure *L. grayi* population in 2020, with an average 21.83% producing capsules. In 2021 the majority of pure *L. grayi* plants did not successfully reproduce in any population aside from two very small ones (SB, BE), with an overall average of 8.36% producing capsules. This was primarily due to Lily Leaf Spot (LLS), with 56.34% of plants prematurely senesced in 2020 and 86.82% in 2021. Two populations (CM, LC) did not produce capsules in either year of monitoring, with 93.33-100% of plants prematurely senescing. In 2021 an additional six populations (RC, BY, BM, GM, HK, WT) had no capsule production. The average number of capsules per flowering plant was considerably higher than the proportion of flowering plants, (0.46 to 0.22 in 2020, 0.18 to 0.08 in 2021), indicating that reproduction is driven by a small number of highly productive individuals. The Roan Mountain population (RM) was predicted to have the highest proportion of reproduction, but this was not the case in either year. In 2020 nearby population HM, also on the Roan Mountain massif, was most successful with 45.45% of flowering plants producing capsules, while in 2021 populations SB and BE were both most successful with 66.67% of flowering plants producing capsules each. HM was third most successful in 2021, however successful capsule production dropped to 15.91%

Hybrid *L.* x *pseudograyi* populations were considerably more successful, as a slight majority of 52.54% and 52.78% of plants reproduced in 2020 and 2021, respectively. The population CC is concerning, as no flowering plants were found here in 2020 and both flowering plants in 2021 prematurely senesced. The dramatic difference in capsule

production between *L. grayi* and *L.* x *pseudograyi* is further reason for concern about the long-term threat of genetic swamping to *L. grayi*. Welch's unequal variances *t*-tests reveal both the significance and the extent of this difference: although the number of flowers produced in *L. grayi* and *L.* x *pseudograyi* populations are statistically the same, *L.* x *pseudograyi* populations are producing nearly three times as many capsules on average. This is primarily due to decreased impact from LLS, with only 27.12% and 27.78% of plants prematurely senesced in 2020 and 2021 and a significantly lower number of diseased whorls on first visits (11.2% to the 29.2% of *L. grayi*).

Although non-rediscovered individuals were not used in calculations for second visits, it is believed they likely prematurely senesced. As expected, LLS was present during first visits at all sites visited in all years visited. Comparing this study to previous demographic monitoring work in *L. grayi* reveals 2020 falls in line with prior years, approximately 20-30% of plants reproducing, however 2021 represents a sharp decline in successful reproduction (Table 10). This is likely due to a combination of multiple effects, including a record number of flowering stems in populations along the Blue Ridge Parkway (C. Ulrey, personal comm.) and high population density at all forested populations combined with the spatially clustered LLS (Ingram et al., 2018). Additionally, previous monitoring efforts focused solely on large populations, while 2021 included 10 small ($N < 10$) and isolated populations that are more susceptible to demographic stochasticity and more likely to be genetically depauperate. Continued monitoring efforts are needed to determine if the low level of reproduction in 2021 is an aberration or the beginning of a new trend.

Table 10. *Lilium grayi* Monitoring across Studies.

Year	Sites	Plants Monitored	Fruiting Plants (%)	Flower Mortality from All Causes (%)	Average Capsules per Flowering Plant	Source
2021	22	347	45 (13.0%)	302 (87.0%)	0.22	This Study
2020	15	201	62 (30.9%)	139 (69.2%)	0.56	This Study
2019	2	55	13 (23.6%)	42 (76.4%)	0.27	Brewer 2020
2012	1 (RM)	120	27 (25%)	93 (78%)	0.31	Ingram 2013
2011	1 (RM)	94	30 (32%)	76 (81%)	0.35	Ingram 2013
1997	2 (BW, BE)	105	23 (22%)	82 (78%)	0.46	Bates 1998

Demographic monitoring suggests that LLS is the primary barrier to reproduction and population in *L. grayi*, as a plurality (36.32%) and majority (66.28%) of flowering individuals prematurely senesced to the disease in 2020 and 2021, respectively. The high proportion of flowering individuals prematurely senescing, and the associated reduction in capsule production, dramatically decreases fecundity. Even plants that don't fully prematurely senesce are affected, as diseased capsules contain fewer seeds than healthy ones, 162 compared to 201. The seeds themselves also weigh less, 0.31 g from diseased capsules compared to 0.77 g from healthy capsules (Ingram, 2013). This study found that plants that prematurely senesced had a significantly higher ($p < 0.000$) percentage of diseased leaf whorls on first visits than plants that produced capsules, 31.0% compared to 13.5% (Figure 9), and that fruit produced was significantly negatively correlated ($p < 0.01$) with the percent of diseased leaf whorls on first visits (Table 8). Together these reinforce the hypothesis that the level of LLS inoculum load is associated with severity of disease in reproductive

structures, proposed by Ingram (2013) based on positive correlations between the number of diseased whorls on a plant and reproductive damage from LLS. The disease appears to affect all above-ground life stages in *L. grayi*, with immature plants suffering most severely. Ingram et al. (2018) found 100% of seedlings and 99.8% of juvenile plants prematurely senesced in monitoring plots, resulting in a shortened growing season and less photosynthetically active time. Although this study only monitored flowering individuals, heavy LLS was observed in juvenile and adolescent life stages in all populations, suggesting that the disease is delaying and potentially preventing recruitment of reproductively active individuals across the range of the species.

The reduction in fecundity due to LLS represents a serious threat to the species overall, as sexual reproduction is important for both adapting to environmental challenges and maintaining populations overall. According to the Red Queen hypothesis, the success of LLS should lead to selection for sexual reproduction (Van Valen, 1977; Lively and Dybdahl, 2000), which generates novel genetic diversity that is critical for adapting to the threat of pathogens like LLS. The species is capable of asexual reproduction; however this is rhizomatously and thus adjacent to the original plant. Seeds are flat and winged, adapted for long-distance wind distribution from highly productive populations on grassy bald mountaintops to the less fecund canopied populations on mountain slopes. By hindering sexual reproduction LLS is interfering with the source-sink dynamics that help sustain the canopied populations. Elasticity matrices of natural populations of another eastern North America endemic congener *L. pyrophilum* M.W. Skinner & Sorrie show that reproductive adults contribute far more to population growth than any other stage, however production of seeds (elasticity = 0.11) contributed less than maintaining reproductive adults (elasticity =

0.19) (Hohmann and Wall, 2018). Given the similarities of their life cycles it is reasonable to assume similar trends would be found in *L. grayi*, suggesting that maintaining reproductive adults on the landscape and ensuring seeds are produced will have the greatest effect on population viability.

Ex situ conservation of seeds was successful overall, with a large number of seeds collected from multiple places along the Roan Mountain massif. Seeds were hand-delivered to the North Carolina Botanical Gardens (NCBG), and maternal genotype data associated with each seed was given to them as well. NCBG staff have stored most seeds for long-term saving and are in the process of germinating a small number of seeds to test for viability.

Monitoring of plants and evaluating the effect of herbivory via plots at the Tater Hill Plant Preserve has had mixed success. The trail camera photos have not detected deer inside of the fencing at any point, indicating they are successfully protecting plants inside the plots from browsing. Unfortunately, the plots have had few flowering individuals overall: Plots 3, 5, 6 and 8 had zero flowering individuals in 2020 and 2021, while Plot 7 had none flowering in 2020 and pPlot 2 had none flowering in 2021. The limited dataset collected so far indicates the fencing could be helping capsule production, as the only plot to successfully produce capsules in both years is the fenced grassy bald plot. Additionally, the fenced forested plot was the only forested plot where flowering individuals were found since 2019. Given the number of deer seen around Plot 7 on the trail camera, it is plausible flowering individuals in Plots 5, 6, and 8 were browsed before the first monitoring visit occurred.

Although many highly significant correlations between plant health and shading metrics were found, regression analyses all resulted in uninformative R^2 values. Stratum 2 and canopy were both negatively correlated with the percentage of whorls afflicted with LLS,

echoing previous work that suggested LLS is more severe in shady habitats (Bates, 1999). However, stratum 2 was highly positively correlated with both the number of flowers and fruits produced, while canopy was negatively correlated with flowers.

Moving forward, continued demographic monitoring of populations is needed, both to determine if the sharp decline in reproduction seen in 2021 is the beginning of a new trend and to collect further data on herbivory and the life stages of *L. grayi*. Monitoring of small populations at risk of extirpation is of particular importance, as many of them experienced little to no sexual reproduction during this study (RC, BY, BM, GM, WT, CC). Additional fenced plots at the Tater Hill Plant Preserve will help with data collection on the effects of herbivory, in addition to likely increasing capsule production in the population. The long-term plots at Tater Hill allow for an excellent opportunity to collect data on non-reproductive individuals, including how *L. grayi* transitions from seeds to juvenile plants and finally to reproductive individuals. These data will be critical to conduct population modeling, a valuable tool for estimating extinction probabilities of populations. Additionally, the hypothesis that *P. inconspicua* hibernates on dead *Lilium* tissue and is thus present when plants re-emerge in the spring (Hiura, 1925) should be tested in monitoring plots by examining the effects of removing senesced *L. grayi* tissue at the end of the growing season. If a positive effect is observed, this would be the first known effective LLS treatment (Coomans, 2002) and a relatively easy and cost-effective way to manage populations for the disease.

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Chapter 3

Lilium grayi Genetic Diversity Analysis

Introduction

Population Genetic Diversity

The most fundamental unit of biodiversity is genetic diversity within a species, or intraspecific variation. Genetic diversity is often measured in populations by the number of alleles present and fixation index, and measured in individuals via heterozygosity (Reynolds et al., 2012). The number of alleles present is best observed through one of two statistics: the effective number of alleles (A_e) is a statistic that corrects for the presence of rare alleles with frequencies less than 0.05, while allelic richness (A_R) corrects for differences in sample size and genotyping success. The fixation index, or inbreeding coefficient, is a measure of the relationship between observed and expected heterozygosity (Peakall & Smouse, 2012; Adamack & Gruber, 2014).

Loss of genetic diversity can result from various factors, both through natural processes such as genetic drift and anthropogenic effects including habitat loss and fragmentation (Epps et al., 2005). Habitat fragmentation results in decreased genetic diversity in both rare and common plant species (Honnay & Jacquemyn, 2007), as small, isolated populations receive less gene flow and are more vulnerable to genetic drift (Young et al., 1996). In a comparison between 170 threatened species and taxonomically related nonthreatened species, reduced genetic diversity, measured by heterozygosity, was found in 77% of comparisons with an average 35% lower heterozygosity (Spielman et al., 2004). The maintenance of genetic diversity is one of the UN's Sustainable Development Goals, and conservationists must consider genetic diversity when making management decisions.

Theoretical models and simulations suggest the level of genetic diversity in populations has a direct impact on population fitness and viability through the mechanisms of inbreeding depression and future evolutionary potential (Ellstrand & Elam, 1993), however studies that utilize both genetic and demographic data to analyze population viability are uncommon (Hens et al., 2017). Understanding how population genetic diversity manifests as increased fitness, as well as how inbreeding and outbreeding depression decrease fitness, is critical for managing threatened and endangered species in general (Bell et al., 2019).

Studies that have investigated genetic diversity and demographic traits concurrently have found generally positive effects of genetic diversity on fitness. Bowles et al. (2015) conducted a large restoration of *Asclepias meadii* (Mead's milkweed) using seeds and plants from over 50 source populations. They measured fitness with germination, survivorship, growth, leaf area index (LAI), and flowering, finding that the number of flowering plants in three populations was significantly correlated with number of genotypes, that seed pods were not produced in sites containing only flowering individuals represented by a single genotype, and that populations that did produce seed pods showed significant correlation between reproductive effort and number of genotypes. However, plants that resulted from inter-population crosses showed smaller LAI and persistence, which they described as evidence of outbreeding depression.

Genetic data on a reintroduced population of *Arenaria grandiflora*, collected eight and twelve years following the reintroduction, found more heterozygous individuals produced more flowers regardless of genetic origin or level of admixture between original and reintroduced populations (Zavodna et al., 2015).

A study of *Pulsatilla vulgaris* examined the relationship between genetic diversity and both seed set (proportion of developed seeds per seed head) and seed mass (mean mass of developed seeds per seed head), finding a significant positive linear association between allelic richness and both seed set and mass. Expected heterozygosity had a significant positive linear association with seed set and mass, but not observed heterozygosity, while at the population scale mean seed set was also strongly positively correlated with allelic richness, which remained significant after controlling for population size. They were surprised to find that, after controlling for population size, 64% of variation in seed set was explained by genetic diversity (DiLeo et al., 2017).

Barmentlo et al. (2018) studied offspring of *Primula vulgaris* sourced from the remaining three natural populations in a common garden setting, using four pollination treatments ranging from a highly inbred self-cross to between-population crosses. They used 12 microsatellite loci and measured fitness with fruit set, viable seeds, and mean seed weight, combined into cumulative fitness. Offspring from between-population crosses had higher diversity and performance than within-population crosses, resulting in a positive relationship between cumulative fitness and observed heterozygosity and negative relationship between cumulative fitness and inbreeding coefficient. As a caveat, the authors noted that their study considered only the early stages of the life cycle to the F_1 generation, when heterosis effects are expected to be strongest (Szucs et al., 2014).

Population Structure

Genetic structure develops in species when gene flow is impeded between subpopulations, creating genetically distinct subpopulations in the absence of panmixia

(Putman & Carbone, 2014). Many natural populations of species are now thought to exist as part of a metapopulation, or "a population of populations", first described by Richard Levins (1970). Metapopulations are often defined as a group of local populations that are connected by dispersing individuals or gene flow that occurs infrequently (Hanski & Gilpin, 1991). The presence of metapopulations and other population structure can be identified by analyzing the genetic diversity in populations via multivariate statistical analyses such as principal components analysis (PCA) (Putnam & Carbone, 2014) or discriminant analysis of principal components (DAPC) (Jombart et al., 2010), as well as model-based clustering methods that search for admixture such as STRUCTURE (Pritchard et al., 2000). Identifying this population structure is critical for management of threatened and endangered species, as genetically distinct populations and metapopulations represent evolutionarily significant units (ESUs) and management units (MUs) which are a primary conservation concern (Funk et al., 2012; Swarts et al., 2014)

Microsatellite Loci

Molecular markers are tools that analyze a specific locus in the genome across multiple individuals to determine the variance between them. There are many types of modern molecular markers, but the most useful for population genetics are codominant markers that can identify heterozygotes. One of the most common codominant markers are microsatellite markers, also called simple sequence repeats (SSRs) or short tandem repeats (STRs), which analyze tandem arrays of small repeats DNA to identify unique alleles based on their size in base pairs. Repeat motifs are most commonly two to six nucleotides long (Holderegger & Wagner, 2008). Novel alleles are likely generated by slippage of DNA

polymerase during DNA replication or recombination errors, with mutation rates up to 10 orders of magnitude greater than point mutations (Gemayel et al., 2012). Due to their high degree of polymorphism, microsatellites are powerful enough to detect 95% of alleles in a population through sampling fewer than 35 individuals (Hale et al., 2012). Microsatellites are more concentrated outside of gene regions; however, they are present in both coding and non-coding regions (Viera et al., 2016). When used across multiple populations, microsatellite markers identify allele frequencies in each population and can be used to test if populations are in Hardy-Weinberg Equilibrium, determine the level of inbreeding or outbreeding in the population, and evaluate genetic distance between populations. (Putman & Carbone, 2014).

Objectives

The focus of this study is to fill a critical research gap in *Lilium grayi*: the absence of population genetic diversity data. This was achieved through genotyping individuals in 24 populations using 11 previously developed microsatellite loci. Genetic diversity metrics obtained include the raw number of alleles (A), average number of effective alleles (A_e), allelic richness (A_R), private alleles (P), expected and observed heterozygosities (H_e, H_o), and fixation index (F) for each locus and each population, as well as Hardy-Weinberg Equilibrium (HWE) and evenness for each locus. The primary hypothesis for the genetic diversity analysis is that *L. grayi* consists of many small and isolated populations, thus high genetic differentiation between populations is predicted. It is also hypothesized that populations on the Roan Mountain massif represent the primary source of reproduction in the species, and it is predicted that these populations will have the highest level of genetic

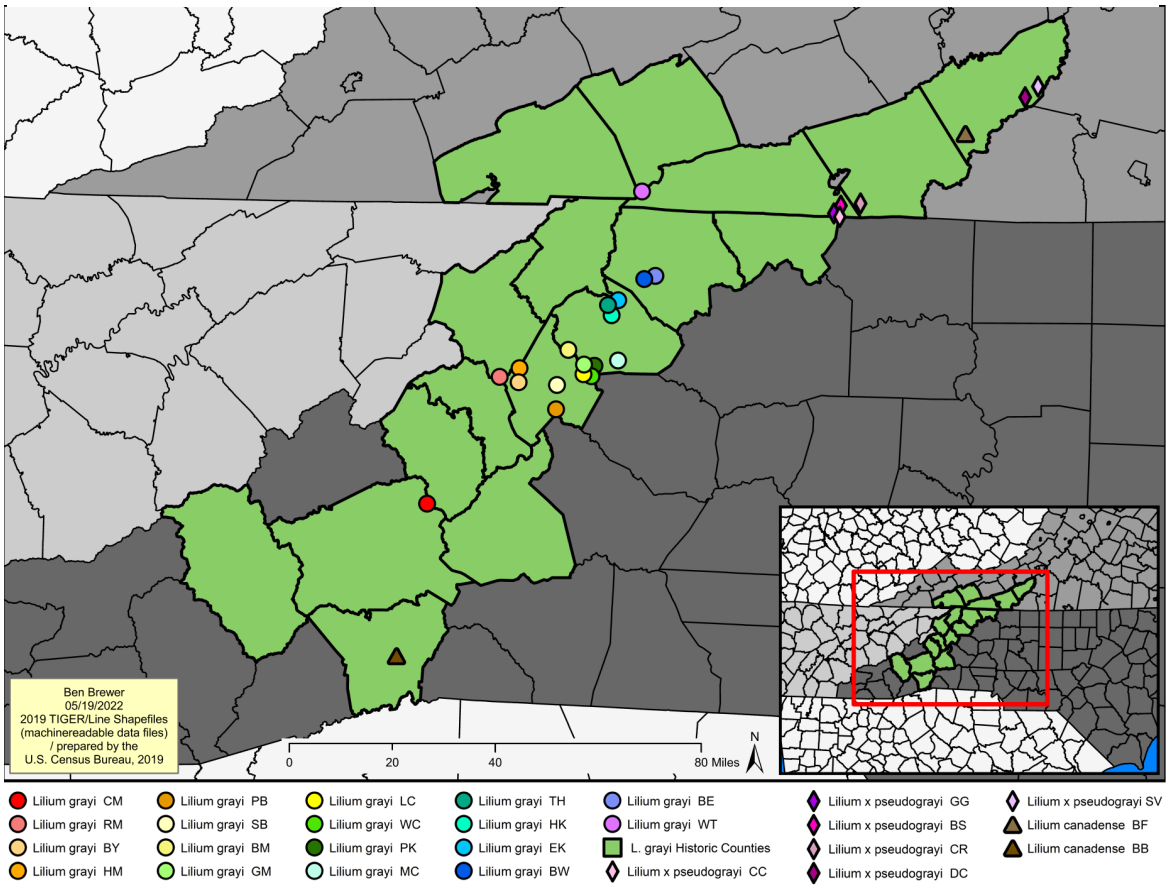
diversity. Additionally, it is predicted that populations will have decreasing genetic diversity as geographic distance from the Roan Mountain massif increases.

Materials & Methods

Tissue Collection

In June and July of 2020 and 2021, leaf tissue for genetic diversity analysis was collected from 614 individuals across 26 populations, representing distinct EOs in NC and VA Heritage Program records, (Figure 1). The 26 include 18 *L. grayi* populations, 6 *L.* x *pseudograyi* populations, and two *L. canadense* populations. In 19 populations all flowering individuals were sampled, ranging from 1-30 individuals. In the 7 remaining populations, between 35-57 plants per population were sampled in a haphazard manner across the population. Approximately $2cm^2$ of leaf tissue was taken from each plant and placed into a 2mL cryovial filled with ~1mL silica gel. Leaf tissue was taken from the lowest whorl on the stem where undiseased tissue was present. GPS location of sampled plants was recorded with a Garmin Oregon 750t handheld GPS unit. Cryovials containing leaf tissue were stored at -80°C until DNA extraction.

Figure 1. Sampled Elemental Occurrences. Sampling Conducted between 06/11/2020 and 07/13/2020 or 06/11/2021 and 07/13/2021.



DNA Extraction and Genotyping

Leaf tissue was ground using a micropestle and sterile sand inside of 1.5mL centrifuge tubes, then a modified CTAB method (Doyle & Doyle, 1987) was used to extract DNA. Extracted DNA was analyzed quantitatively and qualitatively via a Nanodrop 1000 Spectrophotometer (Thermofisher Scientific, Wilmington, DE, USA) and 1% TBE agarose gel, respectively. Extracted DNA was stored at -20°C.

Seven individuals from populations across the range representing *L. grayi, L. canadense,* and *L.* x *pseudograyi* were diluted to 30 ng/µl to evaluate the utility of 25 published *Lilium* microsatellite markers (Horning et al., 2003; Lee et al., 2011), modified to

include a 5' M13 tag (5'-CACGACGTTGTAAAACGAC-3'), for successful cross-species amplification via polymerase chain reaction (PCR). PCR reactions were prepared in 10 µL volumes consisting of 1x GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 800 µM dNTPs, 0.5 µM of reverse primer, 0.25 µM of tagged forward primer, 0.25 µM of a M13 fluorescent labeled primer (FAM, VIC, NED, or PET; Invitrogen, Carlsbad, CA, USA), 0.5 units of GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, Wisconsin, USA), and ~30 ng of DNA. PCR was completed using a touchdown thermal cycling program on an Eppendorf Mastercycler (Eppendorf, Hauppauge, New York, USA) with annealing temperatures ranging from 68°C to 55°C. Initial denaturation was 94°C for 5 min, followed by 13 cycles (45 s at 94°C, 2 min at annealing temperature, and 1 min at 72°C), followed by 24 cycles (45 s at 94°C, 1 min at 55°C, and 1 min at 72°C), followed by 10 min at 72°C. Presence of PCR products was verified via 1% agarose gel, then products were combined with 10µL of HI-DI and a GeneScan Liz 500 size standard (Invitrogen, Carlsbad, CA, USA) and sent to West Virginia University Core Facilities (Morgantown, WV, USA) for genotyping. Resulting chromatograms were scored using Geneious Prime 2022.2.1 (https://www.geneious.com). The 614 extracted DNA samples were arrayed randomly across seven 96 well plates, diluted to 30 ng/ µl, utilizing both positive and negative controls and then amplified at these loci using the same conditions.

Chromatograms were imported into Geneious Prime 2022.2.1 (https://www.geneious.com) for allele scoring and genotyping. A size standard, GeneScan 500 LIZ (Applied Biosystems), was scored for each individual. Locus expected product size, repeat motif, and fluorescent dye information was entered into Geneious for the amplified loci (Flores-Renteria & Krohn, 2013; Galinskaya et al., 2019). The expected product size and

repeat motifs were used to automatically predict allele calls, then individuals were manually checked for alleles outside of the expected size range (Dewoody et al., 2006). If alleles outside of the expected size range were found in at least three individuals they were added as a new bin; otherwise they were regarded as the result of error (Flores-Renteria & Krohn, 2013). Allele peak pattern was evaluated in each locus to ensure consistent scoring, paying close attention to loci L5 and L67 due to their dinucleotide repeat motifs being more susceptible to incorrect scoring when polymerase slippage causes stutter bands (Dewoody et al., 2006; Galinskaya et al., 2019; Pompanon et al., 2005). The two positive controls, individuals #10903 and #10510, included in each plate were genotyped first to ensure consistency across plates (Flores-Renteria & Krohn, 2013). Once consistency in the positive controls was verified, allele calling was automated in the rest of the dataset, then allele calls were verified manually according to the observed peak patterns for each locus (Dewoody et al., 2006). The final dataset was exported and formatted for GenAlEx (Peakall & Smouse, 2006) for further analysis.

Genetics Data Analysis

GenAlex 6.5 (Peakall & Smouse, 2012) and R packages *poppr* (Kamvar et al., 2014) and *PopGenReport* (Adamack & Gruber, 2014; Rstudio, 2020; R Core Team, 2022) were used to calculate basic genetic diversity statistics including: average alleles per locus (A), average effective alleles per locus (A_e), allelic richness (A_R), private alleles (P), expected and observed heterozygosities (H_e, H_o), and fixation index (F) for each locus and each population, as well as Hardy-Weinberg Equilibrium (HWE) and evenness for each locus. GenAlex was used to calculate pairwise F_{ST} values for genetic distance between populations.

A Genotype Accumulation Curve (GAC) and Minimum Spanning Network (MSN) were generated with *poppr* (Kamvar et al., 2014). A Principal Components Analysis (PCA) and Discriminant Analysis of Principal Components (DAPC) were created in R package *adegenet* (Jombart, 2008; Jombart et al., 2010). Clustering was analyzed using STRUCTURE (Pritchard et al., 2000) with initial parameters set to 50,000 burn-ins with 100,000 MCMC reps after burn-in, using an admixture model, correlated allele frequency model, and run on K values 1-27 with 10 iterations for each K value. Results files were compressed and input into CLUMPAK (Kopelman et al., 2015) to determine the optimal K-value via the Evanno method (Evanno et al., 2005) and generate bar plots. The same parameters were then used to analyze clustering in a dataset without the two *L. canadense* populations from K values 1-25 and a dataset without the two *L. canadense* populations and the six *L.* x *pseudograyi* populations from K values 1-19. In all three datasets, K values 1-8 were further examined via new STRUCTURE runs with 100,000 burn-ins and an MCMC chain of 1,000,000.

Results

Locus Descriptive Statistics

Of the 25 screened *Lilium* markers, 11 successfully amplified across the seven test individuals and were reliably scorable: L5, L9, L20, L27, L60, eL16, eL28, eL42, eL65, eL70, and eL75. Six hundred twelve individuals were successfully genotyped; however, due to excessive missing data 60 individuals were removed from the dataset. The resulting 552 individuals contained 551 unique multi-locus genotypes identified via 10 loci (Figure 2). Across all 11 loci a total of 76 alleles were identified, with product sizes ranging from 160-

410 bp (Table 1). This ranged from a low of 2 alleles at L27 to a high of 16 at eL16, averaging 2.678 across all loci and populations. Average effective alleles per locus ranged from 0.915 (L60) to 2.704 (L9) with an average of 1.806. Observed heterozygosity per locus ranged from 0.002 (L27) to 0.578 (L20) with an average of 0.346. Expected heterozygosity per locus ranged from 0.004 (L27) to 0.593 (L9) with an average of 0.338. Loci L60, eL42, eL65, and eL70 did not significantly deviate from Hardy-Weinberg Equilibrium, while the other seven did ($P \leq 0.002$). Fixation indices ranged from -0.236 (L20) to 0.489 (L27) with an average of -0.030. Evenness ranged from 0.29 (eL75) to 0.84 (eL65, eL70) with an average of 0.613.

Figure 2. Genotype Accumulation Curve. In 552 Individuals, 551 Genotypes were Detected via 10 Microsatellite Loci.

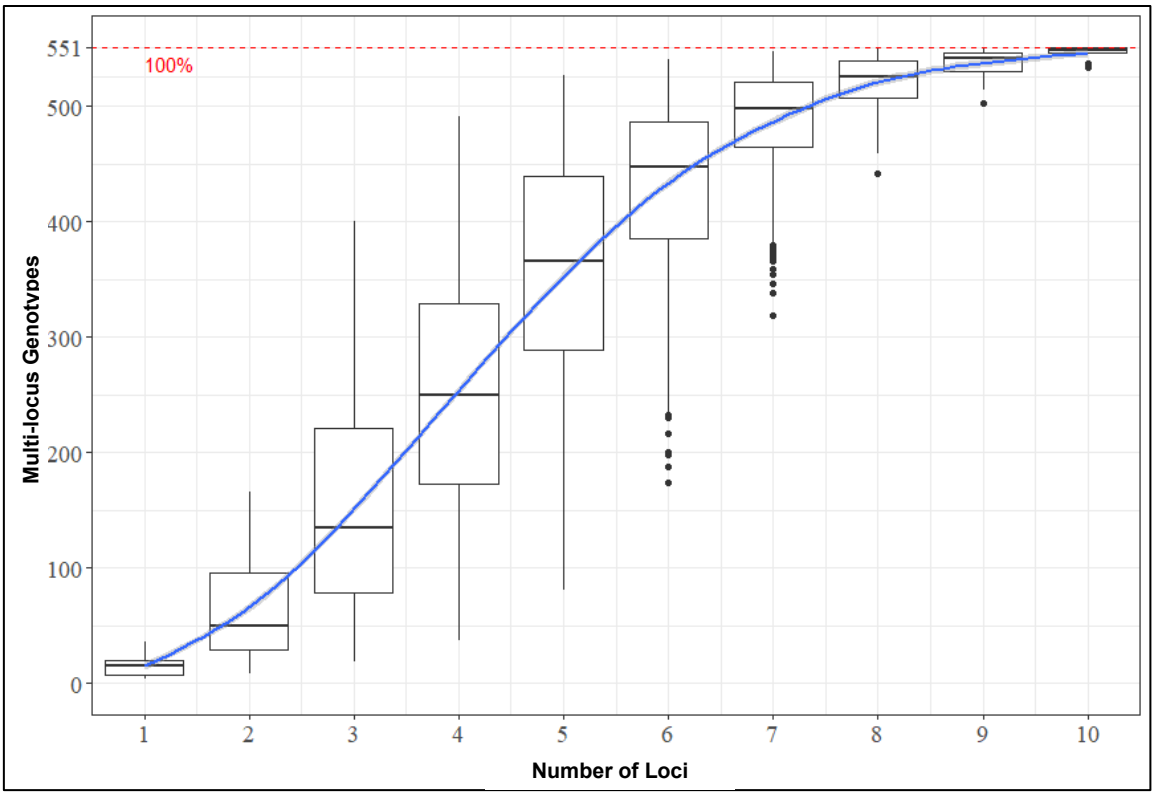


Table 1. Locus Summary Statistics. Size Range = Amplified Product Size Range (bp). A = Number of Alleles. A_e = Number of Effective Alleles. H_o and H_e = Heterozygosity Observed and Heterozygosity Expected.
Significant Deviations from HWE (Key: ns = Not Significant, * p<0.05).
F= Fixation index.

Locus	Size Range	A	A_e	H_o	H_e	HWE	F	Evenness
L5	386-410	9	2.129	0.555	0.439	*	-0.138	0.67
L9	194-214	8	2.704	0.524	0.593	*	0.088	0.68
L20	225-237	5	2.041	0.578	0.459	*	-0.236	0.72
L27	253-259	2	1.004	0.002	0.004	*	0.489	0.4
L60	344-365	5	0.915	0.060	0.059	ns	0.020	0.32
eL16	266-335	16	2.604	0.379	0.554	*	0.316	0.38
eL28	215-236	8	2.280	0.539	0.472	*	-0.162	0.79
eL42	160-178	7	1.922	0.545	0.438	ns	-0.235	0.82
eL65	217-235	6	1.275	0.191	0.171	ns	0.013	0.84
eL70	256-283	3	1.104	0.013	0.013	ns	-0.030	0.84
eL75	195-213	7	1.973	0.420	0.456	*	0.032	0.29
Mean	**-**	**2.678**	**1.806**	**0.346**	**0.338**	**-**	**-0.030**	**0.613**

Population Descriptive Statistics

Individuals sampled per population ranged from 2 (CC) to 52 (RM) with an average of 22.25 (Table 2). The number of individuals genotyped, averaged across all 11 loci, ranged from 1.55 (CC) to 51 (RM). The number of alleles across the 24 *Lilium grayi* and *L.* x *pseudograyi* populations ranged from 13 (CC) to 42 (HM) with an average of 29.9. The effective number of alleles ranged from 1.109 (CC) to 2.315 (TH) with an average of 1.807. Allelic Richness ranged from 1.143 (CC) to 1.440 (PK). Private alleles were found in nine populations (CM, RM, HM, PB, MC, BW, GG, CR, DC). Observed heterozygosity ranged from 0.091 (CC) to 0.499 (LC) with an average of 0.344, while Expected heterozygosity ranged from 0.114 (CC) to 0.436 (PB, PK) with an average of 0.338. Fixation indices ranged from -0.448 (CR) to 0.295 (DC) with an average of -0.021.

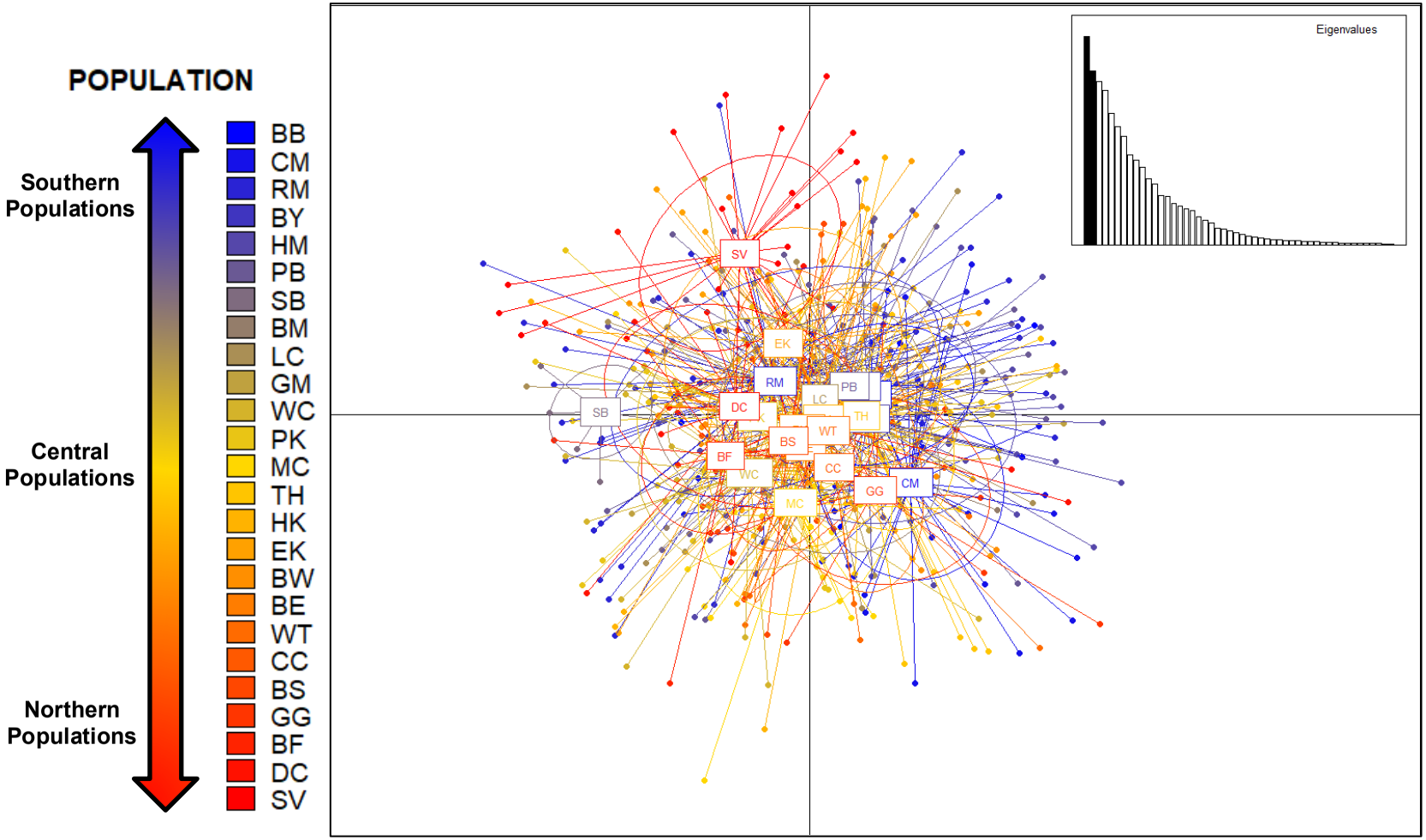
Table 2. Population Summary Statistics. N_S = Total Individuals Sampled. N_G = Total Individuals Genotyped Averaged Across 11 Loci. A = Number of Alleles. A_e = Number of Effective Alleles. A_R = Allelic Richness. P = Private Alleles. H_o and H_e = Heterozygosity Observed and Heterozygosity Expected. F= Fixation Index. * = Population Missing Loci eL28, eL75. ** = Population Missing Locus L60.

Population	N_S	N_G	A	A_e	A_R	P	H_o	H_e	F
Lilium grayi									
CM	34	28.82	35	1.805	1.365	1	0.401	0.361	-0.077
RM	52	51.00	40	2.012	1.388	3	0.349	0.386	0.139
BY*	4	3.27	18	1.395	1.352	0	0.341	0.270	-0.282
HM	40	38.27	42	2.262	1.434	3	0.391	0.431	0.062
PB	34	32.46	36	2.202	1.439	1	0.385	0.436	0.065
SB	6	5.64	21	1.512	1.257	0	0.342	0.246	-0.318
BM	3	2.55	21	1.682	1.329	0	0.394	0.294	-0.360
LC	37	33.27	38	2.212	1.431	0	0.499	0.427	-0.147
GM	11	9.55	34	2.199	1.431	0	0.405	0.420	-0.008
WC	33	29.82	39	1.921	1.385	0	0.429	0.381	-0.114
PK	31	28.18	40	2.284	1.440	0	0.472	0.436	-0.091
MC	23	19.00	34	1.869	1.359	2	0.345	0.354	0.008
TH	39	35.46	37	2.315	1.428	0	0.329	0.424	0.218
HK	32	30.64	40	2.150	1.422	0	0.383	0.419	0.052
EK	25	23.73	35	2.132	1.427	0	0.426	0.423	-0.033
BW*	33	25.09	33	1.579	1.389	2	0.317	0.316	0.079
BE*	3	2.28	16	1.340	1.316	0	0.273	0.237	-0.136
WT*	14	10.64	20	1.478	1.356	0	0.269	0.285	0.024
MEAN	**25.2**	**22.76**	**32.2**	**1.908**	**-**	**0.667**	**0.375**	**0.364**	**-0.037**
Lilium* x *pseudograyi									
CC**	2	1.55	13	1.109	1.143	0	0.091	0.114	0.111
BS	17	12.09	24	1.428	1.252	0	0.244	0.233	0.057
GG	13	10.55	27	1.650	1.329	1	0.334	0.319	0.011
CR**	4	3.36	16	1.281	1.247	1	0.318	0.210	-0.448
DC**	16	12.09	34	1.929	1.427	5	0.277	0.380	0.295
SV**	25	17.91	25	1.634	1.337	0	0.231	0.301	0.169
MEAN	**12.8**	**9.59**	**23.2**	**1.505**	**-**	**1.167**	**0.249**	**0.26**	**0.036**
TOTAL MEAN	**22.25**	**19.47**	**29.9**	**1.807**	**-**	**0.792**	**0.344**	**0.338**	**-0.021**

Population Structure & Genetic Differentiation

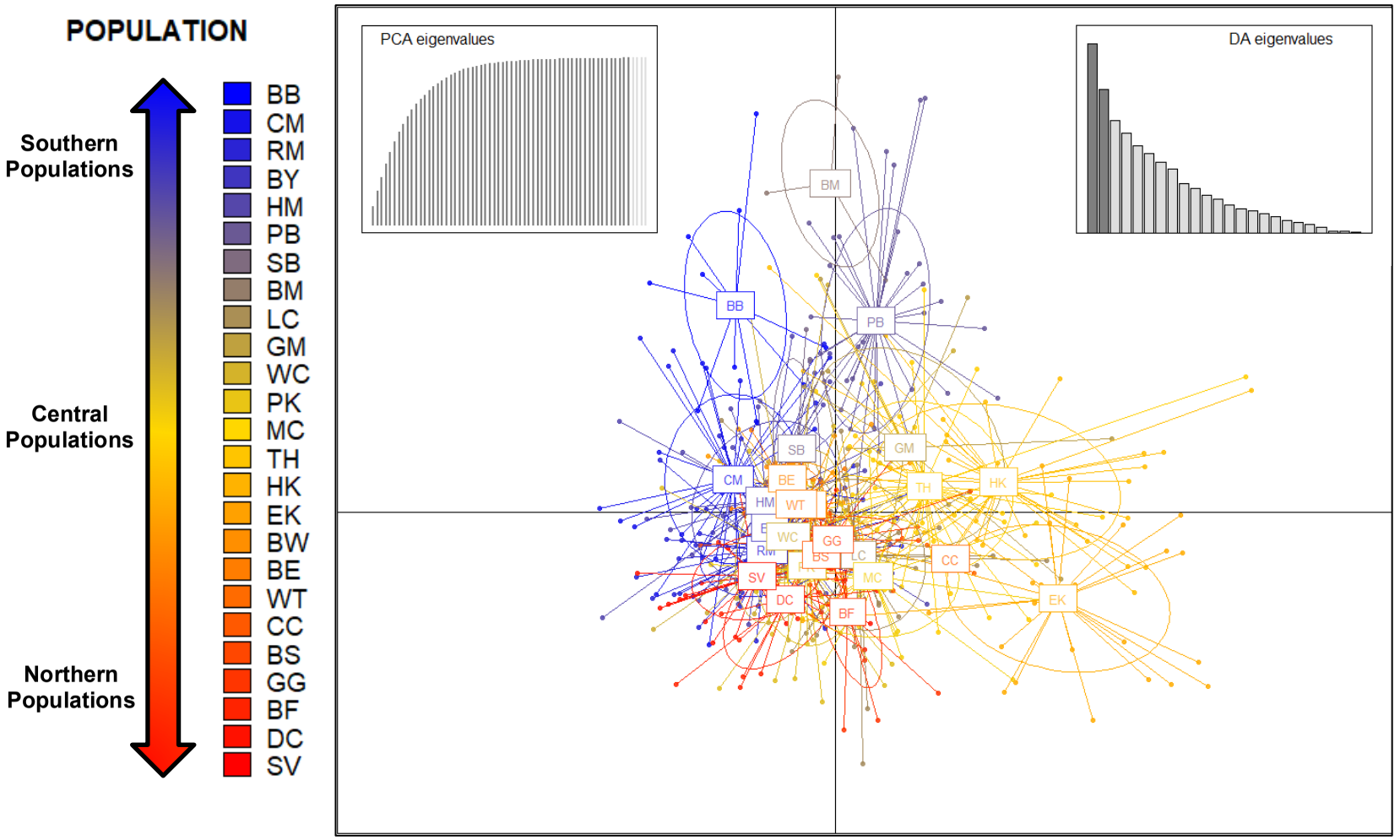
A single small population (CR, N = 4) heavily skewed cluster analyses and was removed from the dataset to analyze population structure. The Principal Components Analysis (PCA) found no discreet groupings among populations and indicated all populations are genetically similar (Figure 3).

Figure 3. Principal Components Analysis (PCA). Constructed in *adegenet*, Each Oval Corresponds to a Population and Each Point Represents an Individual in that Population.



The Discriminant Analysis of Principal Components (DAPC) also found an overall lack of natural groupings (Figure 4). Populations in the center of the range from the Amphibolite Mountains (TH, HK, and EK) are slightly separated from the main cluster, as are a few southern populations (BB, CM, PB).

Figure 4. Discriminant Analysis of Principal Components (DAPC). Constructed in *adegenet*, Each Oval Corresponds to a Population and each Point Represents an Individual in that Population.



The Minimum Spanning Network (MSN) found no well-defined sections (Figure 5). The Evanno K method detected three genetic clusters in the dataset (Figure 6), however STRUCTURE analysis found they did not correspond to any geographic pattern and found high levels of admixture between clusters in all populations (Figure 7).

Figure 5. Minimum Spanning Network (MSN). Constructed in *poppr*, each Point Corresponds to an Individual Multi-Locus Genotype (MLG).

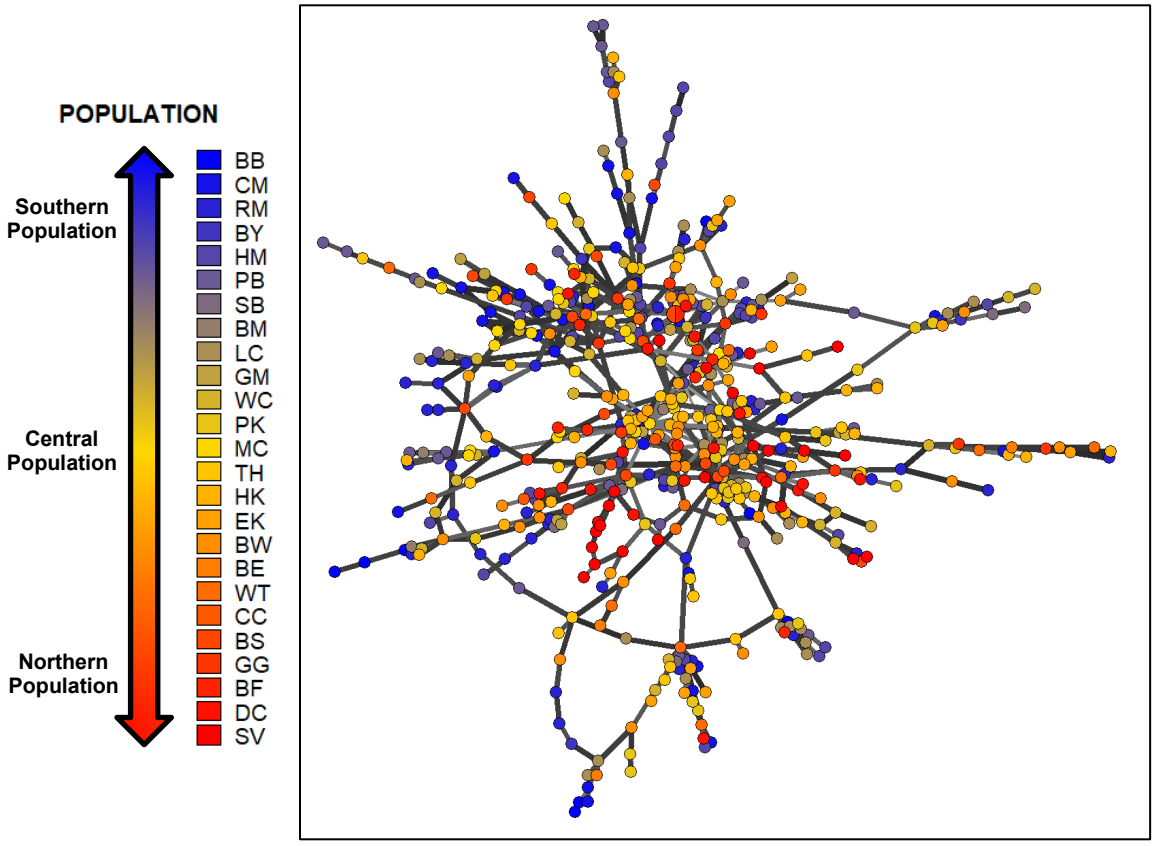


Figure 6. Optimal Delta K by Evanno Method. Plotted with CLUMPAK (Kopelman et al. 2015).

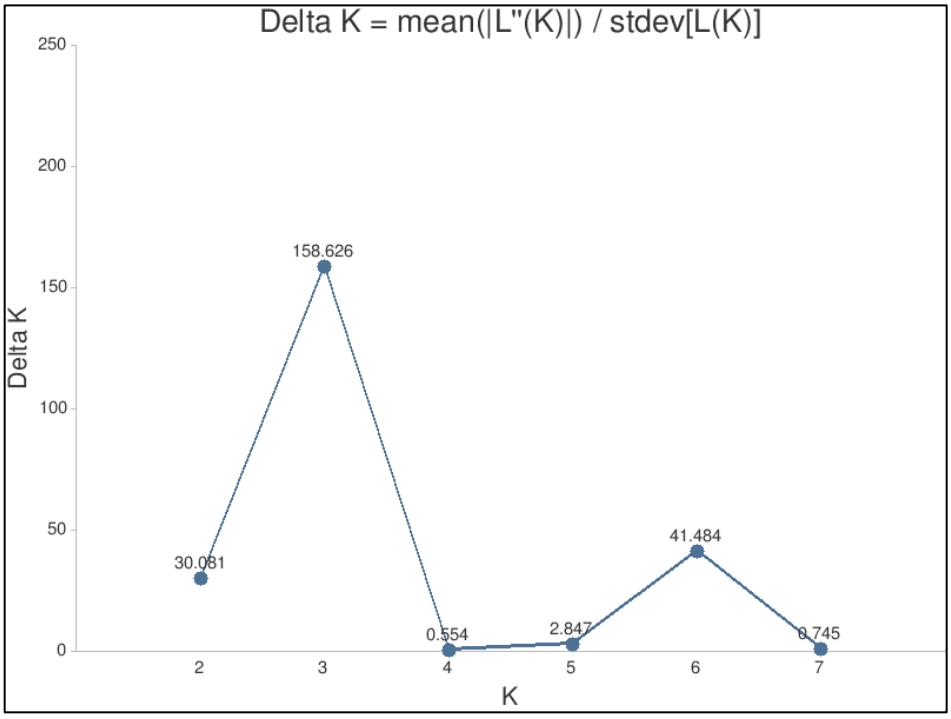
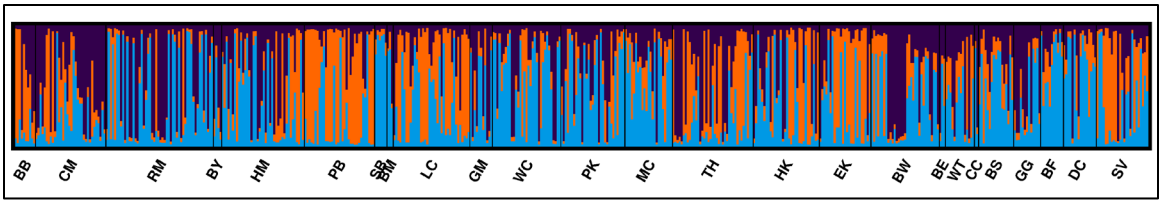


Figure 7. STRUCTURE Plot for K = 3. Plotted with CLUMPAK (Kopelman et al. 2015).



Genetic Distance

F_{ST} values between pairs of populations ranged from 0.019 (LC & HM) to 0.509 (CC & BE) with an average of 0.245 (Table 3). In general, high values were observed between populations at opposite ends of the geographic range. The two populations of *L. canadense* were found to be more related to neighboring *L. grayi* or *L.* x *pseudograyi* populations than each other.

Table 3. Pairwise F_{ST} Values.

	BB	CM	RM	BY	HM	PB	SB	BM	LC	GM	WC	PK	MC	TH	HK	EK	BW	BE	WT	CC	BS	GG	CR	BF	DC	SV	
BB	0.000																										**BB**
CM	0.079	0.000																									**CM**
RM	0.073	0.054	0.000																								**RM**
BY	0.209	0.207	0.169	0.000																							**BY**
HM	0.057	0.034	0.021	0.160	0.000																						**HM**
PB	0.056	0.060	0.046	0.166	0.037	0.000																					**PB**
SB	0.133	0.212	0.096	0.304	0.117	0.120	0.000																				**SB**
BM	0.121	0.177	0.103	0.313	0.117	0.122	0.159	0.000																			**BM**
LC	0.071	0.042	0.028	0.146	0.019	0.023	0.107	0.129	0.000																		**LC**
GM	0.083	0.060	0.038	0.182	0.036	0.049	0.123	0.122	0.052	0.000																	**GM**
WC	0.082	0.043	0.028	0.183	0.040	0.048	0.118	0.152	0.027	0.048	0.000																**WC**
PK	0.061	0.059	0.027	0.179	0.032	0.037	0.096	0.125	0.029	0.034	0.023	0.000															**PK**
MC	0.096	0.041	0.037	0.173	0.031	0.053	0.131	0.158	0.033	0.045	0.020	0.040	0.000														**MC**
TH	0.070	0.052	0.034	0.187	0.036	0.026	0.154	0.139	0.028	0.042	0.038	0.032	0.047	0.000													**TH**
HK	0.063	0.050	0.021	0.168	0.028	0.029	0.117	0.098	0.023	0.036	0.029	0.028	0.036	0.014	0.000												**HK**
EK	0.086	0.074	0.036	0.168	0.039	0.050	0.128	0.141	0.026	0.046	0.043	0.034	0.046	0.039	0.026	0.000											**EK**
BW	0.173	0.169	0.140	0.218	0.139	0.141	0.236	0.250	0.131	0.149	0.137	0.131	0.149	0.145	0.141	0.147	0.000										**BW**
BE	0.243	0.273	0.247	0.352	0.244	0.201	0.355	0.373	0.204	0.222	0.223	0.201	0.227	0.221	0.205	0.222	0.258	0.000									**BE**
WT	0.190	0.198	0.175	0.273	0.170	0.153	0.299	0.304	0.145	0.178	0.167	0.157	0.171	0.166	0.157	0.168	0.214	0.301	0.000								**WT**
CC	0.375	0.301	0.258	0.445	0.261	0.255	0.438	0.469	0.244	0.203	0.268	0.251	0.221	0.275	0.210	0.239	0.333	0.509	0.444	0.000							**CC**
BS	0.206	0.136	0.110	0.239	0.111	0.087	0.273	0.269	0.075	0.126	0.101	0.122	0.088	0.088	0.080	0.108	0.185	0.316	0.244	0.261	0.000						**BS**
GG	0.122	0.053	0.051	0.188	0.038	0.070	0.208	0.189	0.044	0.060	0.060	0.074	0.044	0.054	0.043	0.070	0.156	0.282	0.200	0.230	0.083	0.000					**GG**
CR	0.286	0.264	0.219	0.376	0.224	0.219	0.329	0.391	0.193	0.218	0.197	0.221	0.187	0.229	0.184	0.197	0.290	0.450	0.361	0.408	0.242	0.204	0.000				**CR**
BF	0.168	0.121	0.056	0.250	0.067	0.097	0.162	0.237	0.054	0.083	0.050	0.072	0.035	0.099	0.057	0.055	0.193	0.326	0.258	0.313	0.130	0.086	0.239	0.000			**BF**
DC	0.187	0.180	0.133	0.303	0.144	0.144	0.227	0.271	0.131	0.145	0.130	0.125	0.134	0.149	0.123	0.130	0.238	0.328	0.282	0.289	0.206	0.180	0.242	0.179	0.000		**DC**
SV	0.228	0.237	0.182	0.344	0.205	0.187	0.287	0.333	0.174	0.211	0.189	0.174	0.211	0.212	0.176	0.157	0.273	0.367	0.328	0.376	0.264	0.271	0.357	0.276	0.191	0.000	**SV**
	BB	**CM**	**RM**	**BY**	**HM**	**PB**	**SB**	**BM**	**LC**	**GM**	**WC**	**PK**	**MC**	**TH**	**HK**	**EK**	**BW**	**BE**	**WT**	**CC**	**BS**	**GG**	**CR**	**BF**	**DC**	**SV**	

Discussion

Of the 11 loci used, 3 (L27, L60, and eL70) appear mostly monomorphic while the others are highly polymorphic. One locus (eL16) had possible evidence of null alleles in select populations due to an excess of homozygotes, but was left in the dataset as it has the highest number of total alleles and second highest number of effective alleles. The genotype accumulation curve shows these loci were powerful enough to identify 551 multi-locus genotypes (MLGs) using 10 loci. Two loci (eL28, eL75) are missing from populations BW, BE, BY, and WT, while one locus (L60) is missing from populations CC, CR, DC, and SV.

The results of the genetic diversity analysis were positive overall, an unexpected result, finding the opposite of all original predictions. Due to the small and isolated nature of many populations, they were predicted to have high genetic differentiation between them. Instead, low genetic differentiation between populations was seen, and there is overall more outbreeding than inbreeding. The Roan Mountain massif is believed to be the primary source of reproduction for the species, and thus it was predicted to have the highest level of genetic diversity. Instead, there are seven different populations with a higher number of effective alleles than Roan Mountain and Roan Mountain has the second highest fixation index of all pure *L. grayi* populations. However, the Roan Mountain populations (RM, HM) contain the highest number of total alleles (42 at HM) in any population, including a combined 6 private alleles. Finally, populations were predicted to have decreasing genetic diversity with increasing geographic distance from Roan Mountain. This was somewhat true, as populations furthest from Roan Mountain, *L.* x *pseudograyi* populations in VA, did have the lowest number of effective alleles and overall positive fixation indices. Looking at pure *L. grayi*

populations, populations with high diversity were found across the range, regardless of distance from Roan Mountain.

Pure *L. grayi* populations were more genetically diverse than *L.* x *pseudograyi*, seen through a higher number of effective alleles (1.908 to 1.505) and expected heterozygosity (0.364 to 0.260), as well as a negative fixation index (-0.037 to 0.036). These data, combined with the relatively high number of private alleles in *L.* x *pseudograyi* populations, suggest that the hybrid populations are more isolated and inbred than pure *L. grayi* populations in the core of the range, which appear to be receiving regular gene flow. A high number of effective alleles was observed at populations on the Roan Mountain massif, around Grandfather Mountain (LC, GM, WC, PK), and in the Amphibolite mountains (TH, HK, EK). The Roan Mountain and Amphibolite populations, located on opposite ends of the pure *L. grayi* range, both had an overall positive fixation index, while the centrally located Grandfather Mountain populations had an overall negative fixation index.

In congruence with the findings of high gene flow from the diversity statistics, no distinct population structure could be identified, suggesting pollinators are connecting populations at a distance. The principal components analysis (PCA) and discriminant analysis of principal components (DAPC) resulted in populations grouping near each other, or virtually on top of each other in the case of the PCA, due to overall genetic similarity. The minimum spanning network (MSN) contained many loops and no clearly defined sections. The Evanno K method identified K = 3 as the optimal number of clusters in the dataset, however the STRUCTURE analysis showed all populations were highly admixed between clusters. Despite overall relatively tight clustering, the DAPC did show central populations from the Amphibolites (TH, HK, EK) beginning to separate as a distinct group, as well as far

Southern populations CM and BB. The two *L. canadense* populations (BB, BF) are unexpectedly located on opposite ends of the DAPC, suggesting the little structure seen is based on geography rather than species boundaries, a hypothesis supported by pairwise F_{ST} values. The lack of structure observed may also partially be a result of the expressed sequence tag nature of the microsatellite markers used, as protein-coding loci are more likely to be conserved. The five private alleles in population DC are interesting and unexpected, as the population is relatively close to two others: *L.* x *pseudograyi* population SV is approximately 6 miles to the Northeast and *L. canadense* population BF, which also had a single private allele, is approximately 11 miles to the West. The private alleles in DC were found at 5 different loci, in a total of 4 individuals.

Comparing *L. grayi* to other *Lilium* species reveals the average negative fixation index found in this study is rare and potentially unique (Table 4). Multiple studies on population genetic diversity in Asian lily species, some using the same microsatellite loci, found an average positive fixation index in the Japanese *L. auratum* (Yamamoto et al., 2017), the Chinese *L. cernuum*, and the Korean *L. amabile* (Chung et al., 2014a), *L. distichum*, and *L. tsingtauense* (Chung et al., 2014b). *L. grayi* had relatively high expected heterozygosity as well, second only to *L. auratum*. Horning & Webster (2009) used 6 microsatellite loci to evaluate 8 remnant *L. philadelphacum* populations around Lake Michigan, finding a positive fixation index in 5 of 6 loci. Linhart & Premoli (1994) used 12 polymorphic loci to evaluate 8 populations of *L. parryi*, 5 highly inbred populations in Arizona and 3 larger populations in Southern California, finding a positive fixation index in all populations. Together these are surprising results, especially considering *L. grayi* exists in many small and isolated populations whereas many of these other studies were performed on large populations. The

most readily apparent difference between *L. grayi* and these other *Lilium* species is its migratory hummingbird pollinator rather than a bee, butterfly, or hawkmoth. The Ruby-throated Hummingbird (*Archilochus colubris* L.) migrates from Central America to Eastern North America and back every year, with an estimated flight range of over 2200 km (Zenzal et al., 2016). This allows for pollen dispersal at broad spatial scales and helps explain the high degree of outbreeding and lack of population structure in *L. grayi*.

Table 4. Comparing *Lilium grayi* Genetic Diversity to Other *Lilium* Species.

Species	Loci	Sites	Ns	H_o	H_e	F	Source
Lilium grayi	11	22	22.25	0.344	0.338	-0.021	This study
Lilium auratum* var. *auratum	8	7	14.57	0.49	0.64	NA	Yamamoto et al. 2017
Lilium auratum* var. *platyphyllum	8	6	22.33	0.27	0.51	NA	Yamamoto et al. 2017
Lilium cernuum	14	8	33	0.119	0.159	0.253	Chung et al. 2014
Lilium amabile	14	8	24	0.041	0.048	0.145	Chung et al. 2014
Lilium distichum	14	7	35	0.151	0.190	0.223	Chung et al. 2014
Lilium tsingtauense	14	11	40	0.090	0.113	0.176	Chung et al. 2014

Based on below average numbers of effective alleles and nearly all positive fixation indices, the hybrid populations have a low level of genetic diversity and thus are at higher risk of extirpation. This is likely the result of the increased geographic isolation of the *L.* x *pseudograyi* populations. *L.* x *pseudograyi* populations exist in two geographic clusters: one near the NC/VA border (CC, BS, GG, CR) that is approximately 40 miles Northeast from the nearest pure *L. grayi* cluster in the Amphibolites (TH, HK, EK, BW, BE) and a second cluster (DC, SV) that is approximately 35-40 miles further Northeast of the first cluster. These are much larger distances than the minimum distance between the pure *L. grayi* management units, as the Grandfather Mountain MU is approximately 17 miles from both the Roan Mountain and Amphibolite MUs. In VA *L. canadense* has no conservation status thus populations are not tracked, but based on herbarium records (SERNEC, 2023) *L. canadense* is not common in this area. It is possible that the *L.* x *pseudograyi* populations are far enough from the core ranges of both *L. grayi* and *L. canadense* to receive little gene flow from either, a hypothesis supported by the high fixation index and number of private alleles in *L.* x *pseudograyi* populations.

Based on genetic data, four management units (MUs) are suggested: Roan Mountain populations (RM, BY, HM), Grandfather Mountain populations (LC, GM, WC, PK, MC), Amphibolite populations (TH, HK, EK), and hybrid populations (CC, BS, GG, CR, DC, SV). Additionally, the Amphibolite populations are proposed as an evolutionarily significant unit (ESU). This is based on geographic distance, low F_{ST} values between populations in each unit, and similarities in effective allele numbers and fixation indices between populations in each unit. However, only the Amphibolite populations are supported by genetic structuring analysis, and this support is limited. Amongst the pure *L. grayi* populations there are many

small populations with a low number of effective alleles (BY, SB, BM, BE), some of which also have positive fixation indices (MC, WT). These are the least genetically healthy populations. In *L.* x *pseudograyi* almost all populations had a small number of effective alleles (CC, BS, GG, CR, SV) except for DC, while almost all populations had positive fixation indices (CC, BS, GG, DC, SV) except for CR. Populations CC and CR are the least genetically healthy populations, however DC is the only population that could be considered moderately healthy.

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Chapter 4

Discussion

The demographic monitoring and genetic diversity analysis revealed different conclusions about the health of *Lilium grayi*. Depending on monitoring year, results of the demographic monitoring ranged from expected levels (20-30% reproduction) to far below expected (13% reproduction). Pure *L. grayi* populations were significantly less successful at reproduction than *L.* x *pseudograyi* populations. Results of the genetic diversity analysis were overall better than predicted in pure *L. grayi* populations, with a high level of gene flow and outbreeding seen in most of them. However, *L.* x *pseudograyi* populations were found to be less genetically diverse than the pure *L. grayi* populations, as they contained a below-average number of effective alleles and high degree of inbreeding. Despite a high level of gene flow, most *L. grayi* and *L.* x *pseudograyi* populations are at a moderate to high risk of extirpation due to a combination of some or all of these factors: small population size, little to no reproduction, below-average genetic diversity, or high level of inbreeding.

Presence of these at-risk populations, as well as two populations visited during this study that are believed to be now extirpated, combined with overall low level of reproduction across the species supports a higher federal conservation level for *Lilium grayi*. Published criteria for deciding whether to add species to the Federal List of Endangered and Threatened Plants include "the present or threatened destruction, modification, or curtailment of its habitat or range; disease or predation; other natural or manmade factors affecting its survival." (United States Fish & Wildlife Service [USFWS], 2016). *L. grayi* meets these three of the total five criteria, with disease, predation, and other natural factors affecting its survival in the form of genetic swamping described already in this study. Although multiple

large *L. grayi* populations do occur on federal or state owned property, the lack of federal protection subjects these and all other populations to the continued threat of habitat loss or modification through anthropogenic development. A current example of this kind of threat is the development of the North Peaks State Trail (NPST) in Watauga and Ashe counties, North Carolina. The NPST as proposed traverses the Amphibolite mountain range, home to 11 known elemental occurrences (EO) of *L. grayi* including one of strongholds of the species: Long Hope Valley. Five of the 11 Amphibolite EOs were assessed in this study, resulting in three of them (TH, HK, EK) being proposed as a management unit. These populations were the only ones with any sort of natural grouping from population cluster analyses, and thus represent evolutionarily significant diversity that must be conserved. Federal listing of *L. grayi* would help ensure that conservation of the species will factor into decisions regarding the route and construction of the NPST or any other future development projects.

The "other natural or manmade factors affecting its survival" (USFWS, 2016) criterium could also be triggered through pollen limitation, as the Ruby-Throated Hummingbird (*Archilochus colubris* L.), *L. grayi*'s only known pollinator, is changing migration patterns in a warming climate. Courter et al. (2013) analyzed reported first arrival dates of Ruby-Throated hummingbirds in the US over two time periods, 1880-1969 and 2001-2010, finding that hummingbirds in recent years arrive an average of 12-18 days earlier than historical records. In the latitudes where *L. grayi* is found, hummingbirds arrived approximately 15 days earlier than historical records. As the climate continues to warm hummingbirds will likely continue changing migration phenology, potentially resulting in a future timing mismatch with *L. grayi*. The negative fixation index found in *L. grayi* is potentially a result of long-distance pollen movement via Ruby-Throated Hummingbirds.

However, this pollinator is also the vector of introgression from *L. canadense*, the only other eastern North American *Lilium* species to be pollinated by the bird.

Climate change is likely to affect *L. grayi* directly as well, since it is a narrow endemic found only at high elevations with cool climates (Schwartz et al., 2006). In a study of another narrow Southern Appalachian endemic that shares at least five mountains with *L. grayi*, Ulrey et al. (2016) found that 58-83% of *Geum radiatum* Michx., Spreading Avens, populations will fall below minimum habitat suitability by 2080 under the RCP 4.5 or 8.5 emissions pathways. *L. grayi* already exists at the summits and ridgetops of most of the highest mountains in the regionso cannot retreat to higher elevations, making migrating north the only potential option to find cooler climates.

Another hypothesis for the negative fixation index seen in *L. grayi* is a genetic bottleneck event, which could be associated with an excess of heterozygotes (Bellinger et al., 2003). Based on the search of herbarium records by Ingram et al. (2017), Lily Leaf Spot (LLS) was first introduced to *L. grayi* in the 1940s, a mere 70-80 years ago. If the original introduction resulted in a population crash and genetic bottlenecking, theory suggests this would create an excess of heterozygotes. The remaining individuals would be those most resistant to the disease, as the population crash would function as a selective sweep against vulnerable genotypes.

Future areas of research include analyzing the demography and genetic dataset from this study in concert, as well as a future full project investigating the species boundary between *L. grayi* and *L. canadense*. By examining how demographic and genetic data are related we may discover links between genetic metrics like effective allele numbers or heterozygosity and demographic metrics including population size, amount of capsule

production, or disease susceptibility. The number of flowers and fruit produced, as well as seed set, has been shown to be positively related with genetic diversity in other plant species (Barmentlo et al., 2018; Bowles et al., 2015; DiLeo et al., 2017; Zavodna et al., 2015).

A karyotype analysis of 38 *Lilium* species, including *L. grayi* and *L. canadense*, found *L. grayi* has a unique karyotype and is evidence that *L. grayi* is a unique species (Stewart, 1947). Given this chromosomal evidence, the finding in this study that the two *L. canadense* populations (BB, BF) were overall genetically similar to nearby *L. grayi* or *L.* x *pseudograyi* populations yet genetically distant to each other was surprising. This may be explained by the type of microsatellite markers used, as they were derived from expressed sequence tags. Since such loci are found inside of protein-coding genes, their alleles have a higher likelihood of being conserved across species and thus they are less informative for detecting species boundaries. A larger study focused solely on establishing a species boundary between *L. grayi* and *L. canadense* is needed, sampling from *L. canadense* populations across its broad range and using a next-gen SNP approach, to finally determine the true identity of *L.* x *pseudograyi* populations.

Management Recommendations

Four management units (MUs) are suggested based on genetic data: Roan Mountain populations (RM, BY, HM), Grandfather Mountain populations (LC, GM, WC, PK, MC), Amphibolite populations (TH, HK, EK), and hybrid populations (CC, BS, GG, CR, DC, SV), with the Amphibolite populations constituting an evolutionarily significant unit (ESU). The data collected in this study support the assertion of Ingram et al. (2018) that *Lilium grayi* should be reexamined for Candidate listing under the Endangered Species Act, as the species meets multiple criteria for listing (USFWS, 2016). In addition, identifying a way to manage populations for Lily Leaf Spot disease (LLS) is a critical research need.

Summaries of reproduction and genetic diversity were used to propose primary threats to populations with both demographic and genetic data available (Table 1). The primary proposed threat in 14 populations (CM, RM, BY, HM, PB, LC, GM, TH, HK, EK, BW, WT, CC, SV) is LLS, a large majority of the 21 total populations. The primary proposed threat in 6 populations (SB, BE, BS, GG, CR, DC) is low genetic diversity, with herbivory as the proposed primary threat in the final population (BM). Total reproductive output in capsule number and estimated seed number, based on an average of 201 seeds per capsule in healthy capsules (Ingram, 2013), are provided for each population, as well as a qualitative assessment of relative genetic diversity. Capsule counts represent an attempted census in populations CM, BY, PB, SB, BM, WT, BS, GG, CR, DC, and SV in both 2020 and 2021, population LC in 2020, and populations GM, EK, BE, and CC in 2021. Populations RM, HM, TH, HK, and BW were too large to census in either year, in addition to population LC in 2021, thus overall reproduction is certainly higher than reported in these populations.

The most robust populations included in this study are RM, HM, and TH, based on large population sizes, consistent reproduction, and high genetic diversity. Ensuring *L. grayi* is protected and remains successful at these populations must be a management priority, as these three populations represent two MUs including the only proposed ESU. Although the primary risk in these populations is LLS, managing for herbivory via cages of individual plants or deer fences around dense patches of *L. grayi* would likely be effective in increasing reproduction, as 16.7-72.4% of plants were found browsed in these populations, depending on year. Populations PB, SB, EK, BW, and hybrid populations BS, DC, and SV, are proposed to have low to moderate extirpation risk, based on either high genetic diversity and moderate population size (EK), relatively large population size (BW), or consistent successful reproduction (PB, SB, BS, DC, SV). Populations CM and LC both have moderate population sizes and medium to high genetic diversity, but little to no sexual reproduction appears to be occurring in these populations. Hybrid populations GG and CR both have relatively low genetic diversity and a small number of adult plants, but are consistently reproducing and have some of the lowest LLS rates. Finally, populations BY, BM, GM, BE, WT, and hybrid population CC are proposed to have the highest extirpation risk due to either a very small population size (BE, BM), little to no sexual reproduction (GM), low genetic diversity, or some combination of these three (BY, WT, CC). As these populations all contain relatively few plants in concentrated areas, cages for individual plants or cages around dense clusters to prevent browsing are highly recommended. Additionally, at populations accessible to the public (GM, WT) educational signage should be posted about the species and the need to not touch or pick flowers. Based on the overall lack of genetic structure, managers should consider augmenting these populations with seeds from nearby

productive populations, specifically those with low pairwise F_{ST} values. Based on habitat and genetic similarity, populations RM and HM could be used to augment populations BY, BM, GM, while populations TH and BW could be used to augment BE, WT, and CC.

Table 1. Population Reproductive Output and Extirpation Risk. Total Count of Capsules Observed in Each Population in Each Year, and Estimated Number of Seeds Produced Based on the Average Value of 201 Seeds per Capsule. * = Population was too Large to Census in at Least One Year.

Site	2020 Plants Monitored	2020 Capsules Produced	2020 Estimated Seeds Produced	2021 Plants Monitored	2021 Capsules Produced	2021 Estimated Seeds Produced	Genetic Diversity	Proposed Primary Risk to Population
CM	2	0	0	30	0	0	Medium	Lily Leaf Spot
RM*	52	41	8241	29	5	1005	High	Lily Leaf Spot
BY	0	0	0	4	0	0	Low	Lily Leaf Spot
HM*	11	9	1809	44	16	3216	High	Lily Leaf Spot
PB	12	5	1005	9	4	804	High	Lily Leaf Spot
SB	7	5	1005	6	12	2412	Low	Genetic Diversity
BM	3	1	201	1	0	0	Low	Herbivory
LC*	4	0	0	33	0	0	High	Lily Leaf Spot
GM	NA	NA	NA	6	0	0	High	Lily Leaf Spot
TH*	32	2	402	17	4	804	High	Lily Leaf Spot
HK*	NA	NA	NA	1	0	0	High	Lily Leaf Spot
EK	NA	NA	NA	30	1	201	High	Lily Leaf Spot
BW*	8	1	201	87	8	1608	Low	Lily Leaf Spot
BE	NA	NA	NA	3	5	1005	Low	Genetic Diversity
WT	11	1	201	3	0	0	Low	Lily Leaf Spot
CC	NA	NA	NA	2	0	0	Low	Lily Leaf Spot
BS	14	13	2613	10	8	1608	Low	Genetic Diversity
GG	6	4	804	4	3	603	Low	Genetic Diversity
CR	4	7	1407	2	1	201	Low	Genetic Diversity
DC	14	8	1608	6	2	402	Low	Genetic Diversity
SV	21	16	3216	12	8	1608	Low	Lily Leaf Spot
Total	**201**	**113**	**22713**	**339**	**77**	**15477**	**NA**	**NA**

Conclusion

This study found that the majority of *Lilium grayi* flowering individuals do not reproduce, primarily due to the impacts of an invasive fungal pathogen, however moderate genetic diversity and a high degree of both gene flow and outbreeding between populations is present. Four management units (MUs) are suggested: Roan Mountain populations (RM, HM, BY), Grandfather Mountain populations (LC, GM, WC, PK), Amphibolite populations (TH, HK, EK), and hybrid populations (CC, BS, GG, CR, DC, SV). The populations most at risk of extirpation included in this study are pure *L. grayi* populations BY, BM, GM, BE, and WT, as well as *L.* x *pseudograyi* population CC. The populations least at risk of extirpation included in this study are RM, HM, TH, PB, SB, EK, BW, BS, DC, SV.

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Vita

Ben Brewer was born in Lexington, KY to Todd Brewer and Sandra McClanahan. He graduated from Forsyth Country Day School in North Carolina in 2008. He is a graduate student who took an unconventional education path. He first came to Appalachian State in 2008, then left to pursue interests outside of academia in 2009. Eventually his love of nature and conservation brought him back to Appalachian, where he received a Bachelor of Science in Ecology, Evolution and Environmental Biology in the spring of 2020. He began his graduate research on Gray's Lily at Appalachian the following fall of 2020, working towards a Master of Science in Biology. After receiving his M.S. in Spring 2023 he plans to further his education, continuing study of *Lilium grayi*.