Evaluating Pollination Ecology of the Endangered *Pityopsis ruthii* (Small) Small (Asteraceae)

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Dedication

I dedicate this research to my mother and father, Bette and Maxie Moore. Their guidance, effort, and love are the reasons I am able to succeed. I also dedicate this to John Skinner, for giving me the opportunity to pursue this work and for invigorating me with his jovial spirit and antics. In addition, the love and support from Mackenzie Hodges has been priceless.

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Abstract

Pityopsis ruthii (Small) Small, also known as Ruth's golden aster, is a federally endangered herbaceous perennial, endemic to two river systems, the Hiwassee and the Ocoee, within the Cherokee National Forest, Polk County, Tennessee. There are approximately 13,000 individuals that may be at high risk of short-term extirpation (Thompson and Schwartz, 2006). Little is known of the basic reproduction and life history of *P. ruthii.* Clebesh and Sloan (1993), Cruzan (2001), Park (1998), and Wadl *et al.* (2014) found evidence that seed production and seed viability are highly variable. Clebesh and Sloan (1993) indicated that pollinator visitation was highly temporal variable, while limited genetic diversity may also be preventing cross-compatibility (Sloan, 1994) in this self-incompatible species (Bowers, 1972). This research project identified the pollinators to *P. ruthii* and characterized the impacts of genetic diversity and pollinator abundance to seed set. The objectives were to: 1) collect and identify insect floral visitors to *P. ruthii*, 2) assay pollen carried by insects for qualitative estimates of pollination service, and 3) compare the pollinator communities across the endemic landscape and to an experimental *ex situ* plot to contribute evidence of pollinator dynamics and gene flow to the variability in seed production. Based on this study, the primary pollinators of *P. ruthii* include *Bombus impatiens* Cresson, which is the most important pollinator due to abundance, large pollen load, confirmed *P. ruthii* pollen presence, and behavior and *Apis mellifera* L. that is less widespread, and may be more temporally variable. *Toxomerus geminatus* Say (hover fly) is prevalent, yet its pollen load and behavior is less significant for performing pollination services. Differences in mean seed production by population indicate that floral visitor abundance significantly affects *P. ruthii* seed production (R^2 [R squared] =0.91, P=0.046), while flower density impacts the frequency of Apoidea visitation (R^2 [R squared] =0.219, P=0.007). Genetic deficiencies like in-breeding depression and poor diversity are likely contributing to limited sexual reproductive output, based on seed viability and germination tests. Understanding the vectors of gene

flow and variability in vector abundance is essential to facilitating preservation of *P. ruthii* in its endemic habitat

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Introduction

Synopsis of

Pityopsis ruthii

and

Pollination Ecology

Abstract

An important prerequisite for understanding a species' life history is knowledge of its breeding system (Brown *et al.,* 1975). Because reproductive patterns in a population determine the genetic structure of future generations, information on mating system and fertility in natural populations is critical to plan conservation programs and to manage breeding populations (Muona and Harju, 1989; Morgante *et al*., 1991). In addition, information on mating mode and pattern of distribution of rare and endangered species may help to reveal their evolutionary history and potential, which is the basis of scientific conservation management (He *et al.,* 1998). For insect-pollinated plants, pollination effectiveness may vary by the behavior and morphology of pollinators, the number of open flowers on each plant at a given time, plant density, and breeding habits of the plants. More recently pollinator abundance has become a potential factor in the decline of natural plant populations and pollinator limitation can reduce seed output by 50-60 percent in rare plants (Meffe, 1997). However, habitat loss, fragmentation, and degradation are the primary causes for declines in populations of native plants and their pollinators. Decline in habitat leads to a reduction in biodiversity that further exacerbate problems through inbreeding and genetic drift. The history of decline for *Pityopsis ruthii* on the Hiwassee River may have begun in 1943, when the Apalachia [*sic*] Dam was completed by the Tennessee Valley Authority (TVA). The dam created the Apalachia Reservoir, an over four square kilometer (km^2) lake. Water from the reservoir is diverted into in a pipeline and tunnel system 13.36 km downriver to the Apalachia powerhouse where it is used for hydroelectric energy generation. The Apalachia dam creates $2.5 million of net revenue per year (TVA, n.d.). The damming and diversion of water through the pipeline system prevents the natural water flow and scouring of the riparian areas that would occur during regular heavy rain and flooding events, which would remove built up soil and debris (Farmer, 1977; Bowers, 1972; White, 1977). Removal of the river's flow regime allows for competitive species, which require more soil depth and moisture, to compete for *P. ruthii* habitat (Cruzan, 2001) and forces *P. ruthii* to the driest, most inhospitable habitat

(Bowers, 1972a). In addition, encroachment of the forest canopy and debris diminish the quality of this habitat. Seed distribution from water flow is also potentially eliminated and as a result gene flow may be limited and populations may be in-bred. Little is known about the life history or threats from competitive species, genetic disorders, or climate change to *P. ruthii*. As a result, conservation management is poorly executed. Recent progress in population genetics and reintroduction techniques (Wadl *et al.*, 2014) give hope that the species may be saved, but the risk of extinction is potentially still high (Thompson and Schwartz, 2006). Knowledge on reproductive ecology, seed distribution, and population genetics are needed to make informed decisions.

Historical Perspective

The importance of pollination to the production of seeds and fruit has been recognized since the agricultural revolution. Camerarius (1694) may have been the first to publish the need for pollination in the sexual reproduction of higher plants when he said “pollen is necessary for the fertilization and formation of seeds.” The scientific study of pollination by animals was first initiated by Joseph Gottlieb Kölreuter (1761) who described plant-pollinator interactions as “secrets of nature” and even demonstrated that inter-specific hybridization could occur. The usefulness for the beauty of flowers to attract pollinators was first published by the reverend Christian Conrad Sprengel with a title in reference to Kölreuter, *The Secret of Nature in the Form and Fertilization of Flowers Discovered* (1793). He noted that the bright hues, nectaries, and nectar guides of flowers serve as signals to attract the attention of nectar-loving insects flying nearby. He even noticed the occurrence of cross-pollination. Charles Darwin (1862) recognized the advantage of cross-pollination and theorized the evolutionary connection of plants to bees, in contrast to the intelligent design Sprengel surmised. He identified nearly all the known out-crossing mechanisms in plants and described in-breeding depression, proclaiming, “Nature abhors perpetual self-fertilization.” Hermann Müeller read Darwin’s *Origin of Species* in 1866, leading him to devote his life to the study of the pollination of flowers. He was the first to publish a collection of pollinators on a large scale (843 different anthophilous insect species) and his book *The Fertilization of Flowers* (1883), contained descriptions of floral mechanisms and insect visitors of many plant species. Paul Knuth, Müeller’s successor, then published a monumental record of flower visitation and pollination of 4,028 European flower species and 2,357 flower species in the rest of the world (1898-1804).

Flower and Pollinator Interactions

We now know that the development of flower structure and function is directly related to the most efficient pollinating vector in the region when it evolved. Darwin recognized that evolutionary deviations, which are mutually beneficial to flowers and bees, preserve the individuals that better adapt

themselves to each other. This interaction is due to the sessile nature of plants. For many angiosperms, a pollinator must move pollen from the anther of one flower to the stigma of another flower. The caloric needs and stimulus of the pollen vector and the precision and efficiency of the pollen transfer regulate this mutualistic interaction between flowers and pollinators. The visual and olfactory attractors in flowers are most often a stimulus to floral visitors for food, who inadvertently pick up and transfer pollen while foraging. Flowers may also serve other purposes for visitors such as a refuge or breeding site.

Not all anthophilous animals are effective pollinators and pollination is often not carried out by only one agent. Insects are the primary biotic pollination agents (entomophily). Bees (Apoidea) in general and social bees in particular are the most important pollinators of cultivated plants. Bees depend almost entirely on pollen and nectar from flowers. They have co-evolved with flowering plants and are morphologically adapted to carry large amounts of pollen. Bees, unlike Lepidopteran (butterflies, moths), and Dipteral (flies) floral visitors, provision food for their brood, rather than just selecting a suitable egg-laying site. Bees spend more time foraging and therefore more time interacting with flowers and pollinating.

Honey bee (*Apis mellifera*) pollination (melittophily) is the most important system for cultivated plants (Klein *et al.,* 2007). Due to the large population of a hive (60,000+ individuals), ability to be artificially managed, and remarkable flower consistency, honey bee pollination is very efficient. One honey bee may make 5-10 foraging trips a day and the average hive will make about 4 million trips a year (Frankel and Galun, 1977). In some situations bumble bees (*Bombus* spp.) may be even more efficient since they forage for longer hours, move more quickly, and carry larger loads of pollen. Solitary bees, which often specialize on a host plant, are less widespread and more temporal. Diptera (flies), Lepidoptera (butterflies, moths), Coleoptera (beetles), other Hymenoptera (ants, wasps), Trochilidae (hummingbirds), and Chiroptera (bats) are also important pollinators in some systems.

Recent Declines to Pollinators

Within the last half-century the populations of world-wide honey bee colonies and native pollinators has declined precipitously (Meffe, 1998). Many causes of decline have been identified, including: habitat degradation and fragmentation, increased pesticide prevalence, climate change, and introductions of non-native pests, diseases, and plants (Klein *et al.,* 2007). In general, pollinator loss may lead to plant loss or vice versa. The loss of flowers on which pollinators depend may already be causing food shortages for many pollinating species (Delaplane *et al.,* 2000), which exacerbates environmental decline by reducing ecological diversity.

Climactic Variables Affecting Pollination

The dispersal of pollen to a receptive surface depends on the timing of many variables. Climatic factors like wind, rain, temperature, humidity, and light intensity lead to flowering in plants and pollinator activity. These flowering inducing conditions may only occur once a year, particularly for perennials. Cross-pollination can only be achieved by synchronizing the events of pollen transfer: flowers must be open, anthers dehisced, vectors active, stigma receptive, and pollen viable for germination and fertilization (Frankel and Galun, 1977). Temperature and relative humidity regulate the duration of pollen presentation. Pollen is typically released passively through splits along longitudinal grooves on the side of the anther. The distance between pollen donor and recipient is the most important factor affecting pollen dispersal. During the mobile phase of dispersal, pollen is subject to extreme environmental conditions but only for a short period. The viability of pollen once released varies but is generally only a few hours and is dependent on environmental variables and plant vigor (Frankel and Galun, 1977).

Pollinator activity is regulated by climatic variables and the presence of a stimulus such as nectar. Floral preference and consistency is limited by the vector's ability to discriminate between flowers, the presence of competitive flowers, and the number of flowers present in the foraging area (Free, 1970).

Receptivity of flower stigmas may last from a few hours, up to one month. In most plants the best pollination is achieved on the day of flower opening.

Plant Nectaries

Nectaries serve to attract floral visitors and may be found in any flower part depending on the species. Nectar is a fluid composed of mostly sugar (sucrose, glucose, and fructose) and water in various concentrations and can contain small amounts of protein, vitamins, organic acids, and enzymes. Nectar is a by-product of photosynthesis. It is produced by nectar gland cells then transported to the nectaries through the phloem. The amount of nectar and its production timing varies widely and coincides with the pollinator's preference. Extra-floral nectaries may also be present in plant tissue away from the flower itself and may have nothing to do with attracting insects. The function of extra-floral nectarines is debatable, and is principally an excretory product of a plant's metabolism (Barth, 1985).

The Insect Proboscis

The mouthparts of insects largely determine their ability to access nectaries. A bee proboscis is composed of five individual structures. The glossa or tongue is covered in long hairs and has a spoon shaped end, which attracts nectar up by capillary action. The other structures (galeae, labial and maxillary palpi, and paraglossa) form a suction tube by grouping around the glossa. The tube is connected to a set of muscles in the head which form a pump that pulls the nectar up. As the nectar volume increases, the pressure gradient diminishes and the process starts again (Barth, 1985). The lepidopteron mouthpart is functionally different because it depends solely on nectar. The galeae remain the sole functional part and the others are reduced. The proboscis consists of a central tube that sucks the nectar, surrounded by two crescent shaped halves on both sides that contain tracheae, nerves, and muscles (Barth, 1985). In flies there are three tubes, the innermost tube is the food-sucking tube (a modified labrum), surrounded by a salvia tube (elongated pharynx), and enclosing the tube is the labium. The house fly and hover fly have

the predominant external structure as a massive labial tube with two terminal labella, which are composed of many grooves and channels used to take up liquid food and are covered in sensory organs to provide information about the food. On the inner surface of the labella are little teeth used to scrape pollen from a flower (Barth, 1985).

The length of the sucking proboscis varies by species and is a critical determinate of the flowers an insect will visit. Lepidoptera most commonly have the longest proboscis followed by bees and then flies. Within each order is a great variation of proboscis length ranging from 2-4 millimeter (mm) *Syrphus* flies to the 250 mm *Neococytius cluentis* Hodges hawkmoth. Honey bees (*A. mellifera*) have a modest 6.5 mm proboscis length and bumble bees (*Bombus* spp.) range from 8 to 16 mm length (Barth, 1985).

Cross-Pollination versus Self-Pollination

Cross-pollination (allogamy) occurs when pollen of one individual fertilizes the egg of another. In contrast, self-pollination occurs with the fertilization of gametes within an individual, either from the same flower (autogamy) or a different flower of the same individual (geitonogamy). The primary advantage of cross-pollination is genetic variability in the progeny. A range of genetic variation in a population is advantageous because the population is more able to adapt over time and persist. The advantages of self-pollination are to guarantee seed set when little or no pollinators are present or the plant is a pioneer in a population and has no other intra-specific sexual partners. To promote cross-pollination, the plant may adapt its timing, interaction, or location of reproductive factors. The maturation time of stigma and anthers may be different (dichogamy), their location or presence within a flower may vary, or self-incompatibility mechanisms of pollen and stigma interaction are all common modes of ensuring cross-pollination.

Self-incompatibility (SI) is a genetic mechanism to avoid self-fertilization. It is based on a single locus or multiple loci termed the S locus/loci with many alleles expressed in a population, both of the

pollen grain and the pistil. In essence the diploid pistil has two of these alleles, normally in a heterozygous state and the pollen (and pollen tube) has a corresponding allele or two alleles if from the sporophytic parent (Frankel and Galun, 1977). Successful pollination only takes place when the S allele from the pollen is different from the S allele in the pistil. Otherwise pollen tube growth is prevented on the stigma, halted in the style or fertilization is aborted in the ovule. Based on the variability in the expression of SI, it has apparently evolved independently many times and is present in about 60 percent of angiosperms (Franklin-Tong, 2008). The most important reference in the study of SI was produced by de Nettancourt (1977) and since then the growing understanding and advances in genetic control of SI has allowed mapping of the genes responsible for SI in many species (Franklin-Tong, 2008)

Plant Population Size

Plant population sizes vary in time and space and are the result of a multitude of complex interactions within the environment and historical ecology of a species. Wright (1931, 1938, 1946) first recognized the significance of population size in terms of breeding structure, genetics, and evolution. The genetic variation of a species is organized by the way it is distributed in a region or population. Genetic variation of a species is linked to a plants ability to withstand both changes in the environment and disease and pest pressure. Genetic variation is the single greatest factor in the long-term viability of rare plant populations and is determined by: mutation, natural selection, migration, and random genetic drift, all interacting within an organism's breeding system (Barrett *et al.*, 1991). The effective size of a population is not equivalent to the total number of individuals; instead the number of breeding individuals is needed. Determination of the genetic diversity of individuals is difficult to obtain because it requires many genetic markers (Silander, 1985).

Although many diverse ecological and environmental factors limit population size, the major reasons why rare plant populations are small is summarized by Harper (1977): (1) the availability of sites is limited and separated beyond a species' normal distribution ability; (2) the carrying capacity of the site

is low; (3) the quality of the site is low because of successional displacement; (4) colonization is in its early stages and full exploitation of the site has not occurred.

The consequence of a small population is the reduction of genetic diversity caused by random genetic drift (a reduction in allele frequency resulting from random variations in the distribution of alleles from one generation to the next), in-breeding depression (a loss in fitness resulting from the mating of close relatives), or out-breeding depression, (a fitness decline from hybridization). Each problem leaves a population more vulnerable to local extinction (extirpation). Understanding the vulnerability of a species to genetic problems is important for population viability and a high priority should be given to understanding the breeding system and geographical differentiation across a species range (Huenneke *et al.*, 1991).

Discovery and Description of Ruth's Golden Aster

Pityopsis ruthii, then known as *Chrysopsis ruthii* was first described by John Small (1897) a botanist with the Biltmore estate in Asheville, North Carolina. It was named after the discoverer Albert Ruth (1844 - l932), a botanist from The University of Tennessee, Knoxville who collected the species from the McFarland site in the Hiwassee River valley between 1894 and 1902. It was described as:

> "Perennial slender, silvery-pubescent, stoloniferous. Stems diffusely branched, 1-3 decimeters (dm) long, the branches ascending or decumbent, very leafy, densely so above; leaves linear or some linear lanceolate, 2-5 centimeters (cm) long, acuminate, entire, sessile, the old ones becoming longitudinally ribbed; heads solitary, or corymbosely disposed, about l cm high; peduncles 1.5-2 cm long, densely glandular; involucral bracts linear or linear-lanceolate, in 4-5 series, glandular on the back, the pale edges ciliate, the apex bearded; rays bright yellow, elliptic spatulate, 7-8 mm long, slightly notched at the apex; corolla 5 mm long, yellow, the segments triangular, sparingly ciliate, nearly erect; pappus dirty white, slightly shorter than the corolla; filaments and anthers glabrous; style glabrous, except the very sparingly glandular top; achenes pubescent. Rocks in the Hiawassee Valley, eastern Tennessee. A low stoloniferous species related to *Chryopsis graminifolia* from which it differs conspicuously in being low, diffusely branched and bushy. Besides the very slender habit, the small acuminate leaves, the glandular peduncles and narrower and more acuminate involucral bracts distinguish *Chryopsis ruthii* from *C. graminifolia*."

Taxonomy

The taxonomic treatment of the species shifted many times among genera. Small (1933) transferred *C. ruthii* to the genus *Pityopsis* due to its narrow, grass-like, leaf morphology. Fernald (1942) rejected the concept of *Pityopsis* as a unique genus, since some of the geographic forms of the genus appeared in other sections and maintained the species in *Chrysopsis*. Shinners (1951) merged *Chrysopsis* into *Heterotheca* for morphological and cytogentic reasons which was accepted by Harms (1969), Bowers (1972a), and Cronquist (1980). Dress (1953) in a Doctoral dissertation of eastern *Chrysopsis* maintained Small's (1933) morphological distinction of *Pityopsis.* Semple (1977), Semple *et al.* (1980), and Semple and Bowers (1985) separated *Heterotheca*, *Chrysopsis*, and *Pityopsis* into distinct genera, based on cladistic analysis, which has been widely accepted. Hereon, Ruth's golden aster is referred to as *P. ruthii* (Small) Small.

First Experiments

Bowers (1972a) conducted biosystematic taxonomy of the *Heterotheca* genera section *Pityopsis* using cytoglogical, morphological, chromatographic, and hybridization studies. His Doctoral dissertation identified the **Pinifolia** group: *H. falcata* (Pursh) V.L. Harms (now known as *Pityopsis falcata* (Pursh) Nutt.), *H. flexuosa* (Nash) V.L. Harms (now known as *Pityopsis flexuosa* (Nash) Small), *H. pinifolia* (Elliot) H.E. Ahles (now known as *Pityopsis pinifolia* (Elliot) Nutt.), and *H. ruthii* (Small) V.L. Harms (now known as *Pityopsis ruthii* (Small) Small) and the **Graminifolia** group: *H. adenolepsis* (Fern.) Semple (now known as *Pityopsis aspera* (Shuttlew. ex Small) Small var. *adenolepsis* (Fern.) Semple & Bowers), *H. aspera* (Shuttlw. ex A. Grey) Small (now known as *Pityopsis aspera* (Shuttlw. ex Small) Small var. *aspera*), *H. graminifolia* (Michx.) Nutt. (now known as *P. graminifolia* (Michx.) Nutt. var. *graminifolia* , *H. oligantha* (Chapm. Ex Torr & A. Grey) V.L. Harms (now known as *Pityopsis oligantha* (Chapm. Ex Torr & A. Grey) Small), and *H. microcephala* (Small) Shinners var. *microcephala* (now known as *P. graminifolia* (Michx.) Nutt. var. *tenuifolia* (Torr.) Semple & F.D. Bowers) and *H.*

microcephala (Small) Shinners var. *aquilifolia* (now known as *P. graminifolia* (Michx.) Nutt. var. *aequilifolia* Semple & F.D. Bowers).

Pityopsis ruthii was found to be similar to the Graminifolia group in terms of the pubescent leaf surface, but more similar in the growth habit of the Pinifolia group. *Pityopsis ruthii* was argued to be the most primitive and the ancestor of the Pinifolia group, which may have split off geographically from the Appalachian center of origin. All of these species appeared to have adapted to arid habitat through specialized structures like reduced leaf surfaces and trichomes. *Pityopsis ruthii* was described as barely surviving in a habitat unique for that species; it could not compete with other plants and was forced to survive in the driest of rock crevices.

Hybridization of *P. ruthii* was tested with the other species and was successful with *H. graminifolia, H. pinifolia,* and *H. flexuosa*. Although seeds from the F_1 generation germinated for these crosses, the *H. graminifolia* cross did not mature to flowering and died after three weeks. Self-compatibility was tested with all species and none produced seeds when flowers were bagged with nylon. Pollen from each species' dried herbarium specimen was stained with cotton blue lactophenol. *Pityopsis ruthii* pollen was measured to be between 16.3 and 22.2 microns (µ), an average of 19.22 µ, with 91.8 to 99 percent of pollen able to be stained, an average of 96.47 percent. Significant differences were found between species in pollen size. The four *H. graminifolia* vouchers collected from Polk, Campbell, Morgan, and Pickett Counties in TN (likely now considered to be *P. graminifolia* (Michx.) Nutt. var. *latifolia* (Fernald) Semple & F.D. Bowers) had a mean pollen size of 23.0-24.2 µ, a range of 20.9 – 25.1 µ, with 96.4- 99.3 percent of pollen able to be stained. Pollen size has to do with the rate of self compatibility and style length, with larger pollen sizes in species that are self-incompatible and have longer styles. These larger pollen grains are so because the resources required for successful pollen tube growth and fertilization are greater when the pollen is subject to inter-male competition, rather than self-compatible pollen, which need not compete (Barrett *et al.*, 1996).

Bowers (1972b) made the first collections of *P. ruthii* since Ruth and Dress in 1970 at the McFarland site. The habitat was described as within 15 meters (m) of the Hiwassee River on phyllite outcrops; the river as a fast flowing clear stream, dammed 20 kilometers (km) upstream, with water diverted from the dam to the Apalachia Powerhouse, resulting in a diminished flow of water downstream. Bowers hypothesized the vegetation on exposed river rocks was seldom subject to the inundation seen prior to building of the dam. The rocks were covered in numerous plant and lichen species in the early stages of succession. *Pityopsis ruthii*, he thought, was an early pioneer in areas where sufficient soil was available for growth. On large, flat rocks hundreds of *P. ruthii* plants were observed, but were being displaced by competitors (*Liatris microcephala* (Small) K. Schum. *Andropogon ternarius* Michx. and *Aster linariifolius* (now known as *Ionactis linariifolius* (L.) Greene) as soil built up. He mentioned no evidence of hybrids and found *P. ruthii* to be located separate from the shale soil areas where a sister species, *Pityopsis graminifolia* (Michx.) Nutt. (now known as *Pityopsis graminifolia* var. *latifolia* (Fern.) Semple & Bowers), grew. The chromosome number for *P. ruthii* was listed as diploid, (N=9) based on meiotic counts from buds in greenhouse grown transplants.

Farmer (1977) reported the first successful nursery propagation of *P. ruthii.* Seed was collected from Hiwassee populations on September 25, 1976, dried, and the filled seed separated by visual inspection reporting 5 percent of the total seeds to be filled. In December, filled seed was germinated on moistened filter paper in three scenarios of variable temperature (7 - 16°centigrade (C), 16 - 24°C, and 24 - 29°C) and with either 16 hours of light or continuous darkness in a growth chamber. Germination over 90 percent was observed at 16 - 24°C with 16 hours of light, and at 7 -16°C in complete darkness or with 16 hours light. The temperature range 24 - 29°C reduced seed germination to 63 percent for 16 hours of light and 75 percent for dark treatments over the 20-day period. Seedlings were transplanted into peat:perlite filled pots and supplied with Hoagland's fertilizing solution every other day. Within 3 to 4 months after germination, basal shoots developed. Six months after germination, plants were transplanted

outdoors in silt loam soil and irrigated weekly. Flower buds were observed in late August to early September, which opened on 20 percent of the population and lasted until frost in November. Of the total collected seed from the nursery population, 12 percent was filled and germinated to start a second generation. All initial plants overwintered and began to form a dense mat by tillering. Farmer noted that *P. ruthii* had no special germination requirements and that the dense mat of golden flowers and attractive foliage made it a promising horticultural plant.

White (1977) conducted the first life history experiment for *P. ruthii*. He used aerial photographs and physical inspection by boating the Consauga, Hiwassee, Ocoee, and Tellico Rivers to find new populations. He extending the range of *P. ruthii* from 2.4 km of the Hiwassee River to 4.8 km and found populations on the Ocoee River. Ocoee populations were all on the southern bank expect for one, indicating *P. ruthii* likely existed on more of the northern bank prior to the construction of Highway 64, which runs parallel to that side of the river. White also observed that *P. ruthii* only occurred in locations with greater than 50 percent sunlight. Heavily vegetated boulders were found to not support *P. ruthii*. White speculated that *P. ruthii* is only able to persist on boulders because of seasonal floods which scour the boulders, enabling it to avoid competition. The damming of the Hiwassee River likely allowed *P. ruthii* to expand its range, but the lack of souring enabled cracks to accumulate soil and support competitive vegetation, which require more soil depth.

Water quality of the Ocoee River was discussed by White, based on a report from the Tennessee Stream Pollution Control Board titled “A Study of the Industrial and Domestic use of the Ocoee River and its Relation to Biological Productivity”. A pH reading as low as 1.6 was found and a technician from TVA declared this reading to be not uncommon. Biological samples of the river were found to be nearly devoid of aquatic life and disruption of fish reproduction was documented for many species. The effect of this on *P. ruthii* was not known, but it was expected to have had some impact.

Soil supporting *P. ruthii* was sampled and found to be a sandy loam texture (72 percent sand, 25 percent silt, 3 percent clay). Field capacity of the soil was 24.8 percent at 0.33 bars of pressure and wilting point was 14.3 percent at 15 bars of pressure. The soil had a low pH, and was moderately fertile: low to medium concentration of macronutrients, and high concentration of micronutrients, none in extreme limitation or abundance. Ant colonies were found while soil sampling, but their relationship to *P. ruthii* was not known. Peak flowering was found to be in the last week of September and lasted for two weeks. New *P. ruthii* shoots were recognized as having difficulty becoming established. An average of 50 achenes per inflorescence was produced in October, with an average of 9 seeds per head filled and potentially viable. A four-way factorial greenhouse test with competing species *Solidago arguta* Aiton var. *caroliniana* A. Gray and *Aster dumosus* (now know as *Symphyotrichum dumosum* (L.) Nesom var. *dumosum*) showed competition from *S. arugata* var. *caroliniana* reduced growth more than other factors. Soil moisture, plant density, and light all affected growth. He concluded that *P. ruthii* inhabits dryer parts of boulders than other species because it cannot compete with other taxa in shade or deeper soils.

Endangered Species Recovery Plan

The *Ruth's Golden Aster Recovery Plan* (1992) and the five-year review (2012) are the most comprehensive conspectuses of *P. ruthii* knowledge. Among its' details are the systematics, description, distribution, ecology and life history, threats, current conservation status and efforts, and the proposed recovery objectives, costs, timeline, and involved parties. A vast array of information is covered, much of which is repeated in this review. A complete synopsis is not attempted here and the reader is directed to the original source for more information.

The threats to *P. ruthii* are explicitly different depending on the river system and therefore the recovery objectives are designed specifically for each river. Reduced water flow leading to encroachment from competitive species is listed to be the greatest threat to Hiwassee populations, even though the drop in water level also exposed new habitat to *P. ruthii* such that Collins and Gunn (1986) believe the

Hiwassee populations had reached a historic high. The Ocoee River, also dammed, is managed for white-water recreation much of the year and has regular heavy water flows (1,200 cubic feet per second). The Ocoee River drainage has fewer suitable habitats and Highway 64 construction on the north bank may have removed much of it. Trampling from rafting activities is a threat to Ocoee River populations. Hiwassee River populations are harder to access and have little foot or boating traffic. Water quality of the Ocoee River is also a concern. Copper mining began in 1847 along with smelting of ore, and deforestation of over 130 square km of the Ocoee watershed. The "open roasting" process of smelting released large amounts of sulfur into the air, which eventually settled into the surrounding landscaping killing vegetation by formation of acids. Documented presence of high concentrations of iron salts, zinc, and copper in the Ocoee River has had a negative impact on aquatic life even since mining ceased in 1987 (Maher, 1973).

The need for formal agreements among the many agencies involved in the preservation of *P. ruthii* was announced in this report. Formal recommendations were also made to learn more of the life-history of *P. ruthii*, to maintain permanent research plots, and to determine what is necessary for the long-term reproduction, maintenance, and vigor of each river system population.

Modern Experiments

Clebesh and Sloan (1993) in a report to the Tennessee Department of Environment and Conservation (TDEC) investigated various aspects of *P. ruthii* life history. They searched for populations on the Conasauga/Jack, Hiwassee, Ocoee, and Tellico Rivers. No new populations were identified and some identified by White (1977) were not found. This was not due to the loss of plant populations, but inaccuracy and ambiguity in the reporting of population locations. High variability in the percentage of filled seed per head was observed. Ray florets were observed to be sterile and early opening flowers also did not produce filled seed. September and October was reported to be when pollination and fertilization occurred. Pollinator abundance was found to be temporally and spatially variable, but identification of

collected insect specimen was not included. The germination time of *P. ruthii* was recorded, with seedlings found on December 27, mid-January, and mid-November in Hiwassee River locations. They observed that seeds were not wind dispersed due to their size and weight. The seed pappus, they thought, was adapted for rolling the seed along a substrate and then progressively wedge into a crevice or for water dispersal. The ridges and hairs along achenes may allow for diurnal changes in humidity to drive the seed into the substrate.

An experiment by Clebesh and Sloan was conducted at the Upper Bend site on the Hiwassee River to test if favorable crevices not inhabited by *P. ruthii* were suitable for germination and survival of seeds. Three of the twelve plots had (2 of l5) seedlings germinate. No seedlings survived after four months, likely due to water inundation or drought. Also in the report was a note on a large crew of workers from The University of Tennessee and other state and federal agencies who cleared vine and woody vegetation from the McFarland site in 1991 to increase the available habitat and sunlight. Clearing was also done in the spring of 1993. Two years later, no new *P. ruthii* plants were found to have colonized the cleared area.

Sloan (1994) estimated the genetic variation of *P. ruthii* using two polymorphic allozyme loci and allozymes. Six subpopulations on the Hiwassee Rivier and five subpopulations on the Ocoee River, with 18 to 31 samples per subpopulation were sampled. Based on a model by Perkins *et al.* (1993), he found that 1.05 percent of the genetic variation of *P. ruthii* was attributable to differences between river systems, 15.16 percent was attributable to between subpopulation variation within a river system, and 83.79 percent was attributable to within subpopulation variation. The populations of *P. ruthii* were found to be moderately differentiated (F_{st} =0.163), indicating that the species may be avoiding in-breeding by reproducing clonally. A long held notion that two separate breeding river populations exist was upheld, but new evidence was found that supported the hypothesis that *P. ruthii* individuals within specific rocks within a subpopulation may be genetically different from adjacent habitat. Aerial stem clusters, in close

physical distance, showed genetic variation, rejecting the assumption that individuals who share a crack are identical. More sampling of these rock populations was indicated to be needed to determine how many separate breeding groups exist and to determine what constitutes a breeding population.

Lee (1996) performed an undergraduate research project, presented at the Tennessee Academy of Sciences investigating the competition of *P. ruthii* with *L. microcephala* and *A. ternaries*. Concrete blocks spaced 6.35 mm apart were used to simulate the cracks found in *P. ruthii* native habitat and filled with sand. Six treatments with 3 replicates each were composed of various combinations of each species at a density of one plant every 2.5 cm. Seedlings of each species were grown from seed collected at a Hiwassee River population and transplanted into the simulated cracks within the corresponding treatments. Measurements of plant height, leaf number, and length of longest leaf were recorded over four months. The experiment did not proceed as planned. Seedling growth was arrested and mortality was high. Results were inconclusive because only six blocks survived to the end of the experiment. The limited nutritional benefit or water holding capacity of the sand substrate was speculated to have caused the growth deficiency.

Park (1998), in an undergraduate senior capstone project, looked at seed production and germination of *P. ruthii*. In November 1996, 480 seed heads were collected from a Hiwassee River site, separated into filled, eaten, and unfilled seeds. On average, 26.3 percent of seed from each head were found to be filled, 20.3 percent were predated, and 53 percent were unfilled. Between September 12 and November 7, 1997, 190 seed heads were collected from the same site. An average of 48.8 seeds per flower head was found, with 73.5 percent unfilled, 4.5 percent filled, 15.7 percent predated, 4.5 percent immature, and 1.8 percent uncategorized. It was unknown what would account for the large variation in seed production between years. A larval seed predator found in collected seed was matured to adulthood and identified by Dr. John Brown of the Smithsonian Institute to a member of the Tortricidae family (Lepidoptera).

Cruzan and Beaty (1998) investigated the population biology of *P. ruthii* under contract with TDEC. Germination was examined in 1994, with 160 filled seeds sown into 10 cm rock crevice plots at the Fisherman's Trail site on the Hiwassee River in December. By February, 37 seedlings were observed and considered to be at peak germination. In April, 17 seedlings remained and by October there were only two. The remaining two survived four years. In an attempt to study reintroduction techniques, seed was collected from the Fisherman's Trail site, sorted, germinated, and grown at University of Tennessee greenhouses. This time, seed characteristics were somewhat different and not easily identified as filled or not. Germination of the lighter in color, flat, and soft seeds were found to be viable. When sowing filled and questionable seeds, about half (52 percent) of the filled seed germinated, but had high rates (71 percent) of mortality after a month. The questionable seed had about 40 percent viability with 40 percent mortality. In January 1997, vegetation was removed around the Fisherman's Trail population and *P. ruthii* was located and mapped. Twelve square meter (m^2) areas were marked and crevices were probed for depth and width then divided into 10 cm plots. Filled seeds (n=10) were sown into 10 of the plots that day, randomly distributed. In February seedlings (n=5) were randomly transplanted into 5 of the plots. No germination of the previous seeds was noted. None of the transplanted seedlings survived by March and only three seedlings from the seed plots were observed. The authors indicated that this might be due to the disturbance of the soil from vegetation removal leading to the inability of seedlings to remain within the cracks when heavy storms scoured the area. By October, only one seedling remained. With the intention of reintroducing more seedlings in different seasons, plug flats were maintained at the university greenhouse, but by late summer, these too had died due to lack of water.

Cruzan (1998) used over ten years of data from 1 m^2 plots established in 1986 at three Hiwassee River (n=10) and two Ocoee River (n=10) sites to identify long-term demographic trends in *P. ruthii*. Measurements were taken by an *ad hoc* group of researchers on *P. ruthii* characteristics such as: number of plants, total number of stems, number of flowering and vegetative stems, average stem height, and

percent coverage. Data for each quadrant was gathered on: percent coverage and number of woody stems of all other species, length and width of each crevice, elevation of the plot, and daily light exposure. Data for mean annual rainfall, total annual spill volume, maximum annual spill volume, variation in spill, and number of spill days; with spill being the volume of water released from the upstream dam was provided for each river by TVA. In a multiple regression model, quadrants on the two rivers were relatively similar. Ocoee quadrants had significantly more flowering stems and less herbaceous cover. For both river system populations: the number of stems per quadrant was positively correlated (R^2=0.21) to the amount of precipitation received in the previous year; but negatively correlated (R^2 =-0.35) to the maximum spill volume; the number of *P. ruthii* plants per quadrant was positively correlated to the crevice length (R^2 =0.42) and width (R^2 = 0.24); and negatively correlated (R^2 =-0.28) to other species' percent coverage; conditions of the previous year had no effect. The number of flowering stems was positively correlated to the mean spill volume (R^2 =0.5), crevice width (R^2 =0.49), and negatively correlated to other species' percent coverage (R^2 =-0.4l). According to Cruzan, the primary factors affecting *P. ruthii*'s survival and growth were the availability of suitable habitat, competition from other species, and the amount of precipitation received.

Cruzan (2001) investigated the reproductive ecology of *P. ruthii* to identify the factors that may be limiting seed set, seed viability, and seedling recruitment. Flower heads were collected from five populations each on both the Hiwassee and Ocoee Rivers. The number of florets per head and pollen grains on three florets were quantified. Differences were not found by population (P> 0.75), but were found by collection date (P< 0.001), with later dates having greater pollen deposition. Means were not given, but pollen limitation was not found to be a factor. Seed heads were collected; seeds were separated into filled, unfilled, or predated categories. The mean number of filled seeds per head was greater in the Hiwassee River locations (P= 0.004), but the number of unfilled and predated seeds were similar for both rivers. Hiwassee River populations averaged 11 to 12 filled seeds per head, Ocoee River populations

ranged from 3 at Tablesaw and Bouquet Rock to 8 filled seeds per head at the Powerhouse location. The numbers of filled seeds per head was greater at early and later collection dates for the Hiwassee River populations, but in the middle of the flowering season for the Ocoee River populations ($P< 0.00l$). Seed germination tests were conducted in agar plates with nutrient medium in a growth chamber with a l2 hour light/dark cycle and alternating temperatures of 20° and 10°C. Tetrazolium chloride was used to check any ungerminated seed after three weeks and 38.7 percent of the filled seed proved to be viable. A larger number of total seeds but with fewer viable seeds was seen in Hiwassee River seed heads; Ocoee River seed heads had fewer seeds but with a greater proportion of them viable. Seed viability was shown to be dramatically variable by location, indicating local inbreeding was likely.

Cruzan and Estill (2001) then looked at phylogeography with a survey of chloroplast DNA variation among populations. A nested clade analysis was used to determine the levels of distribution and displacement to infer historical levels of migration and dispersal. Up to ten leaves were used from each of five sites in both the Hiwassee and Ocoee River populations. Four distinct genotypes were detected, one at the Ocoee, three at the Hiwassee. The Hiwassee River was characterized by distinct haplotypes at the upper and lower portions of the drainage, separated by one mutational step. The Ocoee River genotype was separated from the Hiwassee River genotype by two mutational steps. He placed *P. ruthii* into the category paleo-endemies, species with distributions that are relics of once abundant, broader geographic distribution. Cruzan indicated that historically cooler temperatures provided more suitable habitat, based on field and greenhouse observations, leading to the notion that *P. ruthii* is in a precarious position in an inhospitable environment. As climates began to change and glaciers retreated, *P. ruthii* may have been trapped in its local topography. Conclusions of this work are that: (1) gene flow and seed dispersal was very limited; (2) all populations were derived from separate Pleistocene refugia and were present in the Southern Appalachians during colder climates while surviving warm interglacial periods; (3) the divergence between populations likely occurred during the early to mid-Pleistocene era. Management

implications were to collect for *ex situ* preservation from both river drainages, while avoiding hybridization of genetic material between drainages until more is known of the effects of inbreeding, out-breeding, and hybridization. In addition, removal of competitive vegetation should be continued, to increase suitable habitat, because periodic flooding no longer provided that service.

Thompson and Schwartz (2005) used diffusion approximation analysis with 15 years of *P. ruthii* census data and river flow rates to estimate the risk of extinction and impact of flow rate on nine populations' growth. They found that of the measured populations, Tablesaw had a greater than 50 percent short-term (50 years) risk of extinction (defined as a population less than l0 plants) four others (McFarland, Bouquet Rock, Bridge, and Upper Bend) had a risk between 20 percent and 50 percent. Three populations (Double Suck, Cat's Pajamas, Powerhouse) were predicted to be secure from short-term extinction. This is especially important because these three populations represent almost 90 percent of the individuals on the Ocoee River. Sites on the Hiwassee River had larger starting populations but lower growth rates than the Ocoee River populations. Intermediate water flow and high-flow rates were correlated with population growth in a quadratic model (R^2 =0.38 and R^2 =0.31 respectively). Population growth was negative in low-flow years (M = -0.068 ± 0.017, P=0.001) and positive in high-flow years (M= 0.0l 8 ± 0.017, P=0.00l). They summarize by saying that the effects of low flow years were the most important for determining habitat quality and extinction risk for *P. ruthii*.

Wilson (2006) attempted varying population monitoring methods for a Hiwassee River *P. ruthii* population at Big Rock Island. The extent of *P. ruthii* on that end of the river was determined with GPS points. Total census counts in 10 m^2 grids were made between summer and fall for four sub-populations. The number of rosettes and stems on 25 *P. ruthii* plants were counted at three points. Transects of 100 m (laterally and longitudinally) were laid and *P. ruthii* individuals were mapped for one sub-population. An individual was scored as those *P. ruthii* plants within 6 cm apart and contained in the same crevice. Differences between summer and fall population counts of the four subpopulations were nearly

significant ($P = 0.055$). Count of rosettes were significantly greater in October than September ($M = 118$ and 81 respectively, $P = 0.0001$). The results indicate that the time of year censuses are conducted may affect the number of rosettes or the number of plants counted. Positive reasons for fall census counts were more comfortable temperatures, easier to see blooming plants, and fewer incidences of venomous snakes. The transect line for monitoring populations was not permanent and future use of this technique would require at least the end points to permanently marked. Encroachment from inter-specific vegetation was observed to be limiting habitat availability for this population. Debris from drift logs and resulting soil build up and plant litter also affected habitat quality. Trash, campfires, and other evidence of human traffic were indicated as a possible threat to this population.

The US Forest Service (2008) produced an environmental assessment outlining the various options for removal of competitive species at Hiwassee River *P. ruthii* populations. A proposed action was to start a pilot study with four $10\,m^2$ plots at two sites (Loss Creek and The Narrows). Mechanical and chemical (Triclopyr and Glyphosate) removal would be implemented the following spring and again in 4-6 weeks. Annual data collection and potential follow up spring treatments were scheduled to be conducted for 10 years. Plans to cut out invasive species and apply small amounts of herbicide to the cuts was approved in September 2008 and initiated in the following spring and early summer. Data has not been released on the effectiveness of this intervention.

Teoh (2008) performed the first phylogenetic analysis of the *Pityopsis* genus with molecular data. Internal transcribed spacer (ITS) and E26 transformation-specific (ETS) ribosomal DNA markers and four chloroplast DNA (cpDNA) markers were used on all seven species in *Pityopsis* including the four varieties of *P. graminifolia* in maternal, biparental, and combined datasets. For cpDNA and biparental markers, *P. ruthii* fell into a **Ruthii** clade (96 percent Bootstrap support (BS)), which consisted of two other species adapted to xeric-sandy or rocky environments (*P. falcate*, *P. pinifolia*). *P. graminifolía* var. *latifolia* fell into the **Flexuosa** clade, in contrast to morphological analysis, which is likely due to

convergent evolution and hybridization of the **Ruthii** clade with the **Flexuosa** clade. Hybridization may have resulted in the polyploidy evolution of *P. graminifolía* var. *latifolia*. For rDNA markers, *P. graminifolía* var. *latifolia* was shown to be more related to *P. ruthii* than the other members of the *P. graminifolía* species complex forming a weakly supported clade (64 percent BS). Teoh indicated that *P. ruthii* may be an ancestor to *P. graminifolía* var. *latifolia*, especially due to their similar morphology and geographical overlap, although additional studies would be needed.

On a technical note, Wadl *et al*. (2011a) published 12 microsatellite DNA markers for the study of population genetics in *P. ruthii*. Primer pairs amplified loci with allele number per locus ranging from 0.05 to 0.80 and expected heterozygosity ranging from 0.23 to 0.75. These microsatellites are currently being used to assay genetic diversity between and within populations of *P. ruthii* (Wadl, personal communication).

Wadl *et al*. (201lb) also published methods for *in vitro* regeneration of *P. ruthii*. Both flower receptacles and leaf tissue successfully regenerated shoots when cultured on Murashige and Skoog medium with ll.4 µM indole-3-acetic-acid (IAA) and 2.2 µM 6-benzyladenine (BA) along with sucrose, myo-inositol, and vitamins (glycine, nicotinic acid, pryodixine, thiamine HCL) and solidified with phytagar. Shoots were visible within l4-28 days of culture initiation for flower receptacles and 21-35 days for leaf tissue. Rooting of the shoots was difficult. Most shoots were hyperhydric with reduced or hypertrophied surfaces. Three months after culture, three plants were transferred to *ex vitro* conditions. One plant died but the remaining two became fully acclimatized.

Trigiano *et al.* (2011) published a disease report of powdery mildew, *Golovinomyces cichoracearum* (*Erysiphe cichoracearum*) on *in vitro* and stem cutting propagated greenhouse grown *P. ruthii*. Signs of conidia, mycelium, and conidiophores were observed on the adaxial surface of leaves as well as the symptom of curled upward leaf margins. Morphological characters were used to identify the

species and ITS region DNA sequencing confirmed the infectious species (GenBank Accession No. JF779687 99 percent identical to Nos. AB77627 and AB77625). When infected tissue was exposed to healthy tissue it inoculated the leaf with the signs within 7 to 10 days. There was no report of the disease occurring in wild populations, but does impact greenhouse grown plants.

A fiscal year 2012 US Forest Service report for the Cherokee National Forest (2012) indicated the annual total census counts for all Hiwassee River populations at 10,750 and 10,404 for 2010 and 2011 respectively. Previous census data was not comparable, because incomplete census counts were done through random quadrants in select sites. Ocoee populations have had continual complete census counts and the trend is positive (R^2=0.74; Figure 1). Ocoee populations had a total population number of 1,145 individuals in 2011, compared to a baseline 631 counted in 1987 (Baxter *et al.* 2005). The threat from competive species is regarded as being more pronouced with the Hiwassee River populations; whereas the Ocoee River populations are expected to become more secure and possibly growing (Figure 1).

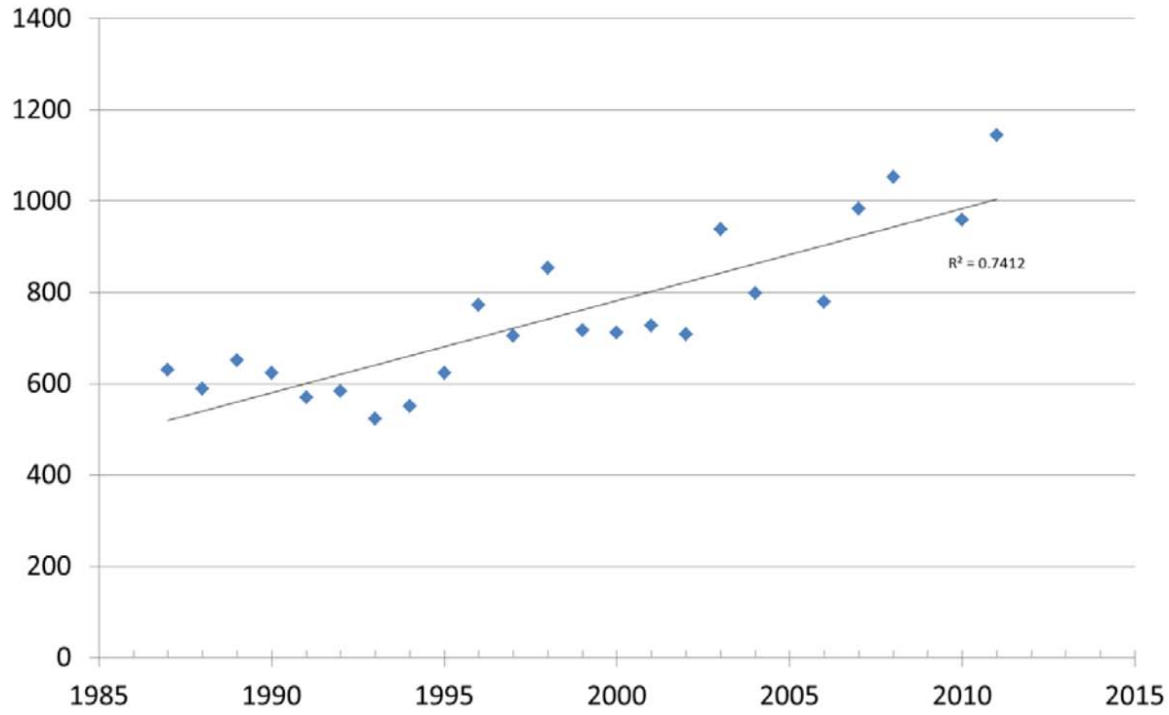

Figure 1: US Forest Service-Cherokee National Forest (2012) annual Ocoee River *Pityopsis ruthii* population census data with trend line; Horizontal axis = year, vertical axis = number of *Pityopsis ruthii* individuals; R^2 =0.74

Modern Recovery Plan and Review

The 5-Year Review (USDA-FS, 2012) of the *Ruth's Golden Aster Recovery Plan* (USDA-FS, 1990) updates a list of recovery objectives with the work that has been completed towards those tasks. This review has summarized much of the completed work, but information on tasks not yet completed is listed here by task number:

(1) maintain formal agreements among concerned agencies on the preservation of *P. ruthii*. This task has not be accomplished, although the Recovery Coordination Working Group (RCWG) has been meeting since the 1990s and formal agreements are not considered necessary

(3.1) study achene dispersal – was partially completed by Clebsch and Sloan (1993)

(3.2) determine the life history, seed germination, and seedling establishment - partially completed by Clebsch and Sloan (1993), Cruzan and Beaty (1998), and Cruzan (2001)

(3.3) determine the role of interspecific and intraspecific competition - has not been investigated

(4) determine what constitutes suitable habitat - was partially completed by Cruzan (1998)

(6.1) determine and compare past and present stream flow regimes- has not been met due to inadequate reporting and analysis by TVA, although Thompson and Schwarz (2006) contributed some information

(6.2) determine the nature and role of natural succession on the phyllite boulders -has not been accomplished

(6.3 and 7.4) determine whether or not the populations are self-sustaining -has been partially met by Cruzan and Beaty (1998) and Thompson and Schwarz (2006)

(6.4 and 7.5) establish *P. ruthii* on unoccupied habitat -has not been met though it was attempted unsuccessfully by Cruzan and Beaty (1998) and a pilot reintroduction was completed by Wadl *et al.* (2014) using a bonded fiber matrix to anchor plants into suitable habitat on the Ocoee River. A larger reintroduction experiment began in 2013, progress and pooled survivorship for six sites was greater than 30 percent by the end of the first growing season. Flowering was observed at two locations indicating success (Wadl *et al.*, Unpublished data)

(6.5 and 7.6) establish a cultivated population of plants descended from each population and provide for long-term seed storage -has been partially met with collections at the University of North Carolina Botanical Garden and Wadl *et al.* (2014)

(6.6) determine feasibility and/or necessity of water releases and hand clearing of phyllite boulders -has not been met, although Thompson and Schwarz (2006) and Clebsch and Sloan (1993) contributed some data and a manual and chemical removal project is ongoing by Pistrang (USDA-FS, 2008)

(7.1) determine the relationship of the Ocoee River to *P. ruthii* population dynamics -has not been accomplished, although Thompson and Schwarz (2006) contributed some information

(7.2) determine the recreational river users and implement required management actions -has been partially met from an environment impact statement (USDA-FS, 1994) prepared prior to the 1996 Summer Olympic Games whitewater events on the Ocoee River

(7.3) ensure highway construction will not damage or destroy plants or suitable habitat -has not been met; proposed improvements to Highway 64 could adversely affect *P. ruthii* populations

Conclusions of the report express the need to maintain *P. ruthii* as an endangered species; indicating the primary threats to be: the altered river flow and vegetative encroachment along the Hiwassee River populations and whitewater recreation related threats to Ocoee River populations. The need to prevent vegetative encroachment and to develop reintroduction methods to establish *P. ruthii* into newly restored habitat is the upmost priority.

Sarah Boggess (2013) conducted a small-scale population structure analysis for *P. ruthii* (*n*=167) and *P. graminifolia* var. *latifolia* (*n*=76) each sampled from two locations on the Hiwassee River and one location on the Ocoee River using 16 microsatellite loci. A possible natural inter-specific hybrid of *P. ruthii* x *P. graminifolia* var. *latifolia* was also tested. Microsatellite markers were developed for *P. graminifolia* var. *latifolia.* Ploidy level of *P. graminifolia* var. *latifolia* was determined to be tetraploid ($2n=4x=36$) and *P. ruthii* was coded as diploid, triploid, or tetraploid based on electropherograms. Because of the differences in ploidy level, four separate datasets were used for analyses. In all of the data sets, a low Shannon's information index (I= 0.13-0.14) and low expected heterozygosity (H_{ex}=0.08-0.09)

indicated low genetic diversity. Bayesian cluster analyses reveled that *P. ruthii* individuals clustered around their respective sampling location, *P. graminifolia* var. *latifolia* resulted in one cluster. When analyzed separately, *P. graminifolia* var. *latifolia* indicated three clusters. All populations contained 90 percent or more of their own genetic identity, indicating low gene flow between populations. Genetic diversity of *P. ruthii* was moderate between populations when analyzed alone or with *P. graminifolia* var. *latifolia*. When analyzed separately, diversity of *P. graminifolia* var. *latifolia* was low. The possible interspecific hybrid clustered halfway between *P. ruthii* and *P. graminifolia* var. *latifolia*, indicating hybridization of the species. Because *P. graminifolia* var. *latifolia* is considered tetraploid and *P. ruthii* diploid, although variation in ploidy of *P. ruthii* was observed (Wadl and Boggess, unpublished data), an inter-specific hybrid would have the potential to produce an odd ploidy, sterile offspring. Management implications were to maintain separate *ex situ* populations from each of the genetically diverse clusters and to supplement low genetic diversity locations by transferring plants between populations, however first studying any negative effect of this intervention. A larger scale project to assess additional populations is also needed and is ongoing by Wadl *et al.* (personal communication).

Wadl *et al.* (2014), refined previously published (Wadl *et al.* 2011b) vegetative and *in vitro* propagation techniques as well as seed germination methods for use in *P. ruthii* reintroduction. Clones of previously propagated wild material using methods from Wadl *et al.* (2011b), and seed from Hiwassee and Ocoee *P. ruthii* populations as well as those growing at The University of Tennessee were used. Seed germination tests were conducted in two experiments. Each experiment used germination with moist filter paper in darkness at 16° to 24° C. The first used filled seeds from four sites on the Hiwassee River, obtained from long-term storage at the North Carolina Botanical Garden that were originally collected in 1994-95. Seeds collected in 1994 had extremely low germination results, between 0 and 1 percent of filled seed, whereas 38 percent of seeds collected in 1995 germinated. Reduced vigor was observed for all

seedlings and only four survived a year. The second experiment used seeds collected from three sites at the Ocoee River and one site at the Hiwassee River in 2010, germinated weeks later. Filled seed percentage of total seed collected for each site was listed as 28 percent of Lone Rock (n=162), 22 percent of the Powerhouse (n=18), 52 percent of Tablesaw (n=322), and 9 percent (n=575) of the Hiwassee River site. None of the filled or unfilled seed from Tablesaw or Lone Rock sites germinated. All of the filled and none of the unfilled powerhouse seeds germinated, 17 percent of the Hiwassee filled seeds germinated. All seedlings exhibited reduced vigor similar to that of the long-term storage seeds.

In vitro seed germination was first done with disinfected seeds by submerging in 70 percent ethanol and briefly flaming with an ethanol burner to remove the pappus, then sanitizing in 20 percent Clorox bleach with three rinses of sterile water. Seeds were then places in Petri dishes with Murashige and Skoog basal medium supplemented with sucrose, vitamins, myo-insitol, and solidified with phytagar. The plates were incubated in darkness between 22° and 25° C for 3 weeks. Refinements had to be made to prevent contamination; only by completely removing the pappus with a razor blade was fungal and bacterial growth prevented. Various levels of concentration of bleach solution were tested and had significant effect (P= 0.0118) when the pappus was intact, but no effect was found once the pappus was removed.

Vegetative propagation was conducted with terminal cuttings of greenhouse grown *P. ruthii*, originally from stored North Carolina Botanical Garden seeds. Two experiments were conducted. The first used terminal cutting (about 6.3 cm) with leaves removed from the (2.5 cm) base of the stem treated with 0.1 percent IBA talc (Indole-3-butyric acid, a commonly used rooting hormone) and then stuck into propagation trays with Pro-Mix BX (Primer Tech Horticulture, Rivière-du-Loup, Quebec, Canada). Cuttings were misted regularly. In the second experiment, cuttings from *P. ruthii* at Hiwassee and Ocoee locations were collected, wrapped in paper towels, and stored in plastic bags. Cuttings were handled as previously described within 12 hours post-collection. The experiment tested the effect of the rooting

hormone, growth stage of the stem, and rooting medium on propagation success. Application of 0.1 percent IBA talc resulting 100 percent success of rooting compared to 87.5 percent for no IBA control. Growth stage effects were significant ($P < 0.0001$), with 84.4 percent rooting when using vegetative stems compared to 5.4 percent rooting from plants in flowering. Success from rooting medium was also significant ($P < 0.0001$), with no rooting when cuttings were placed in pine bark or sand, 72 percent success with a pine bark:Pro-Mix BX, and 93.1 percent success with cuttings in only Pro Mix BX. All those that rooted did so within 2 weeks.

Multiplication of in vitro clones was found to be easily achieved by placing lateral shoots into with Murashige and Skoog basal medium and transferring every two weeks. Rooting readily occurred and plantlets were transferred to greenhouse conditions in Jiffy-7 Peat Pellets (Jiffy International, Kristiansand, Norway). Clones were then introduced into suitable wild habitat with a bonded fiber matrix, applied in a slurry composed of polymers and wood fibers that stabilize the vegetation. After a full growing season, 14 of 19 plants at two sites on the Ocoee River had survived. Ninety-eight plants derived from all these experiments were also tested at field plots in Poplarville, MS and Oak Ridge, TN to initiate *ex situ* conservation. After a full growing season, without irrigation, all plants in Poplarville had survived and flowered, 75 percent of plants in Oak Ridge survived. Results were shown to be congruent with Clebesh and Sloan (1993) and Cruzan (2001) with regard the large variation in the viability of seeds by river system, collection location, and year of collection. Reasons for this variation were hypothesized to be linked to inbreeding depression as expressed by Cruzan (2001), but current genetic diversity studies have been limited, therefore interpretation was avoided, and further studies were indicated to be needed. The success of reintroduction and the ease of propagation was an optimistic note of progress in the long-term conservation and restoration efforts for *P. ruthii*.

Trigiano *et al.* (2014, in press) describe the first report of aerial blight in *P. ruthii* caused by *Rhizoctonia solani*. Propagated plant material in a landscape at Poplarville, Mississippi exhibited

symptoms including desiccation of stems and leaves. Leaf samples were cultured and *Rhizoctonia* spp. was identified from hyphal morphology. Colonies were maintained, measured, DNA extracted, and PCR amplified. The PCR product was sequenced, deposited in GenBank (Accession No. KF843729 and KF843730), and was 96 percent identical to two *R. solani* ITS sequences (Nos. HF678125 and HF678122). *R. solani* was then grown and inoculated greenhouse *P. ruthii* plants. The infected plants exhibited the same signs and symptoms as plants from the field. Resulting morphology, hyphal characteristics, and ITS regions of rDNA was identical to the original isolate. The disease is not known to occur in wild populations, but may affect landscape and greenhouse specimen.

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Part One:

Evaluating Pollination Ecology of the Endangered *Pityopsis ruthii*

Abstract

In September and October of 2013, floral insect visitors to *Pityopsis ruthii* (Small) Small were observed and captured during timed observations at three Hiwassee River populations and at an experimental plot in Oak Ridge TN. Captured visitors were identified and cataloged. Pollen coverage on the visitors was measured for each specimen. Pollen grains within a sample of visitor collection vials were assayed for presence of *P. ruthii* DNA. Seed heads were collected from each location in November 2013 and separated into filled and unfilled seed. A sample of seed from each group and location was tested for viability with a germination test and for living tissue with Tetrazolium chloride (Sigma Aldrich, St. Louis, MO). Analysis of variation (ANOVA) in a completely randomized design (CRD) was conducted in SAS (ver. 9.3, SAS Institute, Cary, NC) to compare differences in percent filled seed set by collection location and pollen coverage by taxon. Simple linear regression (SAS, ver. 9.3) was used to predict bee and syrphid fly visitation by flower density (*P. ruthii* flowers per 2 m^2 plot) and mean visitor abundance was used to predict mean percent filled seed by location. Results give evidence that a narrow range of insects visit *P. ruthii* flowers, including the bees: *Bombus impatiens* Cresson, *Apis mellifera* L., *Augocholora pura* Say, *Megachile mendica* Cresson, and *Ceratina calarata* Robertson and the wasp *Isodontia mexicana* de Saussure. *Toxomerus geminatus* Say hover flies and Hesperiidae and Nymphalidae butterflies were also frequently observed and captured, but carried little to no pollen. Confirmation of *P. ruthii* pollen coverage was made for many taxa. The primary pollinator of *P. ruthii* is *B. impatiens*, based on abundance, pollen coverage, confirmed *P. ruthii* pollen presence, and foraging behavior. Flower density was able to predict the abundance of Apoidea at the Hiwassee River locations (R^2 =0.219, P=0.007, $F_{1,30}$=8.39) and may indicate that smaller populations receive less pollination service. Differences in mean floral visitor abundance between sites was shown to predict the mean filled seed set per site (R^2=0.91, $F_{1,2}$=20.28, P=0.046). Seed germination and viability tests give evidence of potential

genetic limitation to seedling emergence and establishment. Amending populations to supplement genetic variation and density of flowers may increase success of *P. ruthii* sexual reproduction.

Introduction

Studies by Cruzan (2001), and Park (1998), and Wadl *et al.* (2014) on *Pityopsis ruthii* seed production indicate that *P. ruthii* may be reproductively limited. In one experiment, seed heads collected in 1996 by Park from a Hiwassee River site had 26.3 percent filled seed. The following year, seed heads collected from the same site between September and November had 4.3 percent filled seed. Cruzan (2001) found variation between 6 and 18 filled seed per head for Hiwassee River populations depending on the sampling date within a year. Ocoee River flower heads had an average of 3-8 filled seeds per head, while Hiwassee River populations averaged 11 to 12 filled seeds per head (Cruzan, 2001).

Farmer (1977) conducted the first germination tests from seed collected the McFarland site and reported over 90 percent viability from filled seed. Cruzan (2001), in contrast, found variability in the viability of filled seeds depending on the collection site, a range of 14 to 87 percent viability. For Hiwassee River sites, Cruzan (2001) found 33 percent of the filled seed was viable, while 53 percent of the filled seed from the Ocoee River was viable. Wadl *et al.* (2014) reported partial germination of *P. ruthii* filled seed, indicating that some of the seed will pass a germination test (producing a radical), but not mature to produce cotyledons. For long-term stored seed at a botanical reservoir, nearly all the filled seeds collected from three sites in 1994 would not germinate (0 percent, 1 percent, and 0.5 percent), but seeds collected at one site in 1995 germinated at 38 percent (Wadl *et al.* 2014). Seed freshly collected from three sites on the Ocoee River produced varying amounts of filled seed, between 22 and 52 percent of total, but none of the filled seed from two sites and 100 percent of filled seed from one site germinated (Wadl *et al.* 2014). Variation in viable seed production and seed germination between sites within a population, between populations, between river systems, and within sites between years indicates that one or more factors are contributing to poor sexual reproductive output of *P. ruthii*.

P. ruthii is only found within the boundaries of two river systems, bound within the valley of the Unicoi Mountains (part of the Blue Ridge Mountain). Populations of *P. ruthii* within each river system are all contained within 5 km of each other. Pollen may not be capable of travel beyond each sub-population and cross-pollination may not be occurring. Boggess (2013) and Sloan (1993) showed that for the Hiwassee and Ocoee Rivers sampled sites, 90 percent of the genetic material within each site is identical and therefore little gene flow is occurring between populations. Minimal water flows of the Hiwassee River may prevent seeds from dispersing downriver. Geographical isolation is known to induce in-breeding depression by the function of genetic drift (Falk and Holsinger, 1991). Such drift limits the reproduction, vigor, and viability of plant populations. Boggess (2013) showed a low Shannon's information index (I= 0.13-0.14) and low expected heterozygosity (H_{ex} =0.08-0.09) for *P. ruthii* indicated low genetic diversity, which likely occurred from genetic isolation and resulting genetic drift. If this is occurring, *P. ruthii* may have few compatible mates within a site and may also lack ability to adapt to a changing landscape or climate. The availability of pollinators for *P. ruthii* has also been called into question by Clebesh and Sloan (1993), who noted that bees would be abundant one day and absent the next at the same site.

To address the lack of knowledge about factors contributing to *P. ruthii* reproductive population dynamics, the objectives of this Master's thesis were to: (1) identify the arthropod gene vectors and estimate individual contributions to success at local gene dispersal within three of the dominant population locales and to compare their assemblage with an idealized experimental plot; (2) compare pollinators across the landscape to contribute evidence regarding pollination dynamics to the variability of seed production. Knowledge of the vectors for gene dispersal in these populations will help us understand how the plants are genetically differentiated and which sites will need to be amended to improve genetic diversity or plant density. Addressing what insects visit and pollinate *P. ruthii* will lead to more in depth analysis of genetic dispersal and the reproductive variation of sites and populations.

Materials and Methods

Study Sites

Investigation of insect floral visitors was conducted at three of the largest *P. ruthii* populations in the Cherokee National Forest on the Hiwassee River (Table 1) and at an experimental plot at The University of Tennessee, Forest Resources, AgResearch, and Education Center, Oak Ridge Unit, in Oak Ridge, Tennessee. The Hiwassee River populations were chosen based on accessibility, high density of plants, and to represent the geographic range of species on that river system. Ocoee River locations could not be accessed because of high water flow and were therefore excluded from examination. Hiwassee River populations in this study are approximately 3 to 60 m from the river on the north bank and found on exposed phyllite and occasionally greywacke boulders. Cracks and crevices with built up sandy loam soil are the substrate that anchors *P. ruthii* within boulders. Water flow from the river rarely reaches the plants, but strong spring storms, leading to high water releases from the Apalachian Dam were reported to inundate much of the Hiwassee River populations in March, 2013 (Dattilo, personal communication). Direct sun exposure occurs at the sample sites from about 11:00 am until 4:00 pm. Weather during observations was mild and sunny with highs ranging from 24-28° C and lows ranging from 10-15° C.

The similar flowering phenology of competitive species, which share *P. ruthii* habitat, was observed (Appendix). Widespread, profuse blooms of *Solidago arguta* Aiton, *Symphyotrichum patens* (Aiton) G.L. Nesom, *Symphyotrichum dumosum* L., and *Symphyotrichum lanceolatum* (Willd.) G.L.Nesom all competed for floral visitation. *Pityopsis graminifolia* var. *latifolia* (Fern.) Semple & Bowers was also observed to be in flower, and was located nearby, in less exposed areas than *P. ruthii*, often under partial or complete canopy closure. Remnants of *Liatris microcephala* (Small) K. Schum. flowers were found but had peaked weeks earlier.

The University of Tennessee Forest Resources, AgResearch, and Education Center, Oak Ridge Unit, hereafter referred to as the UT Arboretum is located at 901 S. Illinois Avenue, Oak Ridge, TN. It is

a 2,260 acre natural area with 250 acres of a public arboretum and the remaining grounds used for forestry, horticultural, and wildlife research. The UT Arboretum contains 2,500 specimens of native and exotic plant species, while the remaining area consist of native forests, warm-season grasses, herbaceous and woody hedge rows, and experimental research plots of various species including many horticultural cultivars. Weather conditions and sun exposure were similar to Hiwassee River sites. Differences from Hiwassee River locations are primarily in relation to isolation from civilization, affecting the proximity to beekeepers, the biodiversity of plant life, the size and flower abundance of *P. ruthii* plants, and the soil substrate for *P. ruthii*. The soil likely affected flower abundance and growth rate presumably through increased nutrient, water, and root space availability, and greater soil biotic activity.

The experimental population of *P. ruthii* plant material (Figure 1) was grown from seed or propagated from stem cuttings from both Hiwassee and Ocoee River populations (Wadl *et al.* 2014). Plants were matured for 1-3 years at The University of Tennessee, Ornamental Horticulture Nursery in trade gallon containers, with Pro Mix BX (Primer Tech Horticulture, Rivière-du-Loup, Quebec, Canada) medium and periodic watering. Mature *P. ruthii* were transplanted to the experimental population in October 2012. The soil had been recently rotary tilled. *P. ruthii* transplants were spaced on 2 foot centers. Plants were not irrigated after the initial watering at planting time. Immediately after planting the area was mulched with approximately 7.5 cm of shredded hardwood mulch to control weeds and conserve moisture. Weeds were physically removed by hand from the area as necessary.

Table 1: *Pityopsis ruthii* study sites*

Site Name	Total Population Size	Subpopulations
McFarland	675	5
Loss Creek	2,840	7
The Narrows	1,622	4
UT Arboretum	120	1

* Hiwassee river site names from adjacent river features, ordered from upriver to downriver. Population size from Dattilo (2013, unpublished data), who approximated based on rosettes within 6 inches of each other considered one plant. Subpopulations delineated by any break in habitat, typically a separate rock face. UT Arboretum indicates an *ex situ* conservation plot in Oak Ridge TN established by Wadl *et al*. (2014).

Figure 1: UT Arboretum *ex situ Pityopsis ruthii* population with 2 m^2 plot denoted in white PVC pipes, red flags indicate (current or former) *Pityopsis ruthii* locations

Floral Visitor Collection and Processing

Investigation of insect floral visitors was conducted at peak flowering time, September 14-15 and 27-30, 2013 at the Hiwassee River locations, 1 site per day, and October 5, 6, and 9, 2013 at the UT Arboretum. Observations were taken in 2 meter squared (m^2) plots of *P. ruthii*, which were chosen by accessibility and high flower density at Hiwassee River locations due to a clumped pattern of *P. ruthii* distribution and the inability to randomize. UT Arboretum observational plots were chosen randomly using a random number table. Two to three days of observation per site were made during peak insect activity times, between 11:00 am and 4:00 pm. Five to six 30-minute observations per day were made at separate plots across the range of each population.

The number of *P. ruthii* flowers within each 2 m^2 observation plot (flower density) was counted if the number was below 100, which represents all Hiwassee River plots. Flower density greater than 100 per plot was estimated to the nearest 50 flowers and represents many UT Arboretum plots. The number of stems or number of *P. ruthii* plants per plot to correspond to flowers was not counted because cracks which contain the plants could not be accessed and rhizomes did not allow for separation of one plant from another. Previous investigators' method (Dattilo, unpublished data) of an arbitrary distinction that all *P. ruthii* plants within 6 inches are considered a single individual was not satisfactory in this experiment because it does not represent a reliable measurement of *P. ruthii* individuals, or a breeding population.

All visitors which alighted on receptive reproductive floral surfaces were counted based on the lowest taxonomic level of visual identification (Hymenoptera, Syrphidae, *Bombus*, *Apis mellifera*). Attempts were made to capture all counted visitors using an aerial net (Bioquip, Rancho Dominguez CA), swept rapidly at flowers for the Hiwassee plots. At the UT Arboretum, the even surface of the ground allowed for capture by draping the opened net over the flower, which induced the insect to fly upwards

into the net, for easy capture. Insects were contained in individual plastic vials or in glassine envelopes (Bioquip), for Lepidoptera, labeled, and frozen until processing.

Pollen coverage on each captured insect was determined by microscopically examining the insect's integument. Qualitative pollen coverage measurement were made for the: head, mouthparts, thorax, abdomen, wings, and legs, using a modified Likert scale based on Beattie (1971): 0 = No pollen; 1 = Very sparse pollen, fewer than 10 grains; 2 = Patchy pollen coverage, 10-40 grains; 3 = General pollen coverage, 41-99 grains; 4 = Heavy pollen coverage, greater than 100 grains. Scores were summed for all segments of an individual, which gave the total pollen coverage per specimen. Total coverage per specimen was averaged for each insect taxa. Insects were then pinned or pointed, labeled, and stored in Cornell drawers. The completed collection was permanently placed in the University of Tennessee, Department of Entomology and Plant Pathology, Insect Museum.

Identification of insects was made using:

(1) Apoidea: *Discover Life Bee Species Guide and World Checklist* (Asher and Pickering, 2011);

(2) Syrphidae: *Key to the Genera of Nearctic Syrphidae* (Miranda *et al.*, 2013);

(3) Lepidoptera: *A Field Guide to Eastern Butterflies* (Opler and Malikul, 1992);

(4) Bombyliidae : *Bee Flies of the World: The Genera of the Family Bombyidae* (Hull, 1973);

(5) Other Resources: *Bugguide.net* (Bartlet, 2011); *Insects: Their Natural History and Diversity* (Marshall, 2006). Sam Droege of the US Geological Survey confirmed the identification of Apoidea specimens.

Floral Voucher Specimen Collection and Mounting

One floral voucher specimen of all other flowering, herbaceous plants species in the immediate vicinity (50 m) of *P. ruthii* populations, were collected, stored in paper or plastic bags, and kept on ice. The specimen were dried in a standard plant press (Bioquip) within 48 hours after cutting, and remained in the press for 1 week. Specimen were then mounted on herbarium cards (Bioquip) and identified to

species level by Aaron Floden at the UT Herbarium (Appendix). The floral voucher specimens were permanently placed in the University of Tennessee, Department of Ecology and Evolutionary Biology, Herbarium.

Seed Collection and Viability Tests

Seed was collected in early November, from each Hiwassee River population and the UT Arboretum in 3 15-m circumference circles chosen based on high plant density. Twenty seed heads were randomly picked within each circle and stored in individual plastic vials (Bioquip). Achenes from each seed head were visually separated into filled and unfilled seeds based on seed size and depth (Figure 2), with those without depth considered unfilled. Seed heads that were predated were eliminated from the experiment. Separated filled and unfilled seeds from each head were aggregated into airtight plastic vials (Bioquip) for each site. Seed was stored at ambient temperature (15-20° C) for two months, until germination tests were performed.

For germination tests, 40 filled and 40 unfilled seeds from each site were randomly chosen (except for unfilled Loss Creek seed, which totaled 35). Seed germination protocol followed Wadl *et al.* (2014) for *in vitro* germination methods. The seed pappus (Figure 2) was removed with dissection shears. The seed sterilized for 20 minutes with 20 percent Clorox Concentrated Regular Bleach (The Clorox Company, Oakland CA; sodium hypochlorite, sodium chloride, sodium carbonate, sodium chlorate, sodium hydroxide, sodium polyacrylate, and water) in sterile water and rinsed three times with sterile water. The seed was added to sterile water moistened sterile filter paper and sealed in sterile Petri dishes. Petri dishes were stored at ambient temperature (15-20° C) in complete darkness. Germination was monitored every week for three weeks and scored as successful if the seed produced a green cotyledon. Seeds that did not mature beyond radical emergence were considered partially germinated.

A separate group of forty filled seeds from each site were also tested for living tissue using 2, 3, 5 triphyenyl tetrazolium chloride (TZ, Sigma Aldrich, St. Louis, MO) a commonly used oxidation-

reduction indicator. TZ indicates living tissue by enzymatically reducing the white TZ compound to a red compound (1,3,5-triphenylformazan) when there is activity of dehydrogenases. Therefore tissue that are undergoing metabolism and cellular respiration are stained red and considered living, whereas areas where the TZ compound remain white are because of inactivity of these enzymes and thus necrotic. Seeds used in germination tests could not be used in TZ tests because they were no longer solid after soaking in water for three weeks. Unfilled seeds could not be sampled with this method because the seed coat could not be removed without disintegration. Seeds with the seed coat intact could not be used because they would not absorb the stain. Methods for prepping and stain the seeds were modified from Cottrell (1947). First the pappus (Figure 2) was removed from dry seeds and then the seed was soaked in sterile water for 24 hours. The seed coat was then manually removed with forceps. Two milliliters (ml) of 1 percent TZ solution was added to seeds in 2 ml micro-centrifuge tubes and stored in complete darkness at 15-20° C for 4 hours. Seeds were observed in Petri dishes under a 30X stereo microscope and viability was scored if the exposed tissue was stained red.

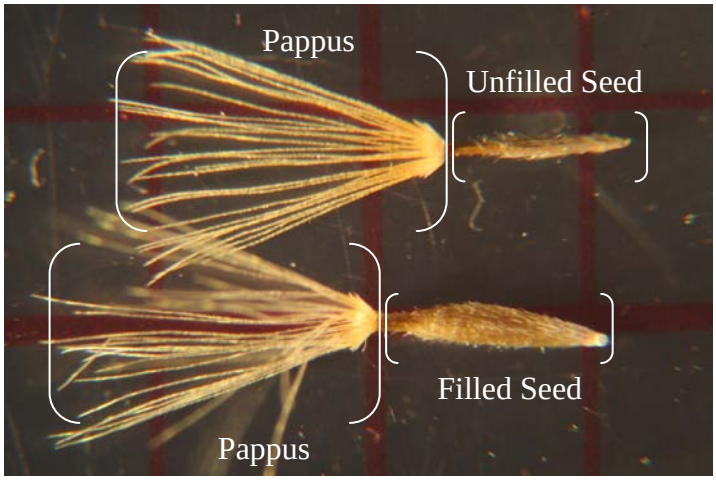

Figure 2: *Pityopsis ruthii* achenes

Assay of Pollen DNA within Insect Collection Vials

Because eight other Asteraceae species (Appendix) were present in the sampling locations, including a sister species (*P. graminifolia* var. *latifolia*), visual identification and isolation of *P. ruthii* pollen was not possible. Instead, presence of *P. ruthii* pollen on insect visitors was confirmed using microsatellite markers (SSR) developed by Wadl *et al.* (2011). Methods were modified from Matsuki *et al.* (2007), who identified parentage of a single pollen grain deposited on a stigma, whereas this experiment used pollen deposited into insect collection vials. The pollen was inadvertently removed by collected insects from either their integument while flying in the collection vials prior to freezing, or from regurgitation of their digestive contents into the vial.

Insect collection vials (*N*=64) were chosen based on visible pollen coverage at 30X microscopic examinations, and to represent the collected species and collection sites: up to five individual *Apis mellifera* vials, up to five individual *Toxomerus* spp. vials, up to five individual *Bombus impatiens* vials, and up to five other individual Apoidea vials (*Melissodes dentriventris* Smith, *Melissodes druriella* Kirby, *Megachile mendica. Megachile brevis* Say, *Halictus ligatus/poeyi* Say/ Lapeletier, *Augocholora pura*, *Augochlorella persimilis* Viereck, *Ceratina calcarata,* or *Isodontia mexicana*) from each site.

DNA was extracted from each floral voucher (Appendix) as a negative control (a sample that should not amplified SSR loci) and from a *P. ruthii* plant growing in the University greenhouses, originally collected from the Hiwassee River, as a positive control (a sample that should amplify SSR loci). Approximately 2 nanograms (ng) of leaf tissue from each dried voucher specimen (Appendix) and *P. ruthii* was placed into 2 ml micro-centrifuge tubes with approximately 15 silicate micro-beads. Tubes were frozen in liquid nitrogen for 5 minutes and then agitated for 2 minutes with the FastPrep 24 (MP Biomedicals, Santa Ana, CA) to homogenize the leaf tissue for DNA extraction. Extraction protocol for both leaf tissue and insect vials followed the manufacturer's instructions and included reagents in the

DNeasy Plant Mini Kit (Qiagen, Venlo Limburg, The Netherlands) with slight modification to increase concentration of DNA.

1. Pollen vials and micro-centrifuge tubes (for leaf tissue) were washed in 400 micro-liters (µl) of AP1 buffer, pipetted into 2 ml micro-centrifuge tubes along with 4 µl RNase. Tubes were incubated in a Durabath water bath (Baxter, Deerfield, IL) at 65° C for 20 minutes with a vortex of each tube every 6-7 minutes
2. 130 µl of P3 buffer was added to each tube, vortexed and incubated for 30-60 minutes on ice
3. Tubes were centrifuged for 5 minutes at 13,200 rotation per minute (rpm), then the lysate pipetted into QIAshredder spin column (Qiagen) in 2 ml collection tubes; centrifuged for 2 minutes at 13,200 rpm
4. Flow-through was transferred to a new collection tube, 1.5 volumes of AW1 buffer added and mixed by pipetting
5. 650 µl of the solution was added to a DNeasy Mini spin column in a 2 ml collection tube (Qiagen); centrifuged for 1 minute at 8,000 rpm. Flow through was discarded and the remaining solution was added to the spin column; centrifuged for 1 minute at 8,000 rpm
6. The spin column was transferred to a new collection tube, and 500 µl of AW2 buffer added and incubated for 5 minutes at room temperature (20° C); centrifuged for 1 minute at 8,000 rpm. Flow through was discarded and another 500 µl of AW2 buffer was added; centrifuged for 2 minutes at 13,200 rpm
7. The spin column was transferred to a new 2 ml micro-centrifuge tube, 50 µl AE elution buffer added and incubated for 5 minutes at room temperature (20° C); centrifuged for 1 minute at 8,000 rpm. Another 50 µl AE elution buffer was added and incubated for 5 minutes at room temperature; centrifuged for 1 minute at 8,000 rpm
8. Resulting DNA in AE buffer solution was pipette into 2 ml micro-centrifuge tubes

The quality and quantity of DNA was measured with the Nanodrop ND-1000 specrophotometer using the NanoDrop 1000 version 3.5.2 software (Thermo Fisher Scientific, Waltham, MA). DNA samples were diluted with 0.1X TE Buffer (Tris-HCl and EDTA (Ethylenediaminetetraacetic acid)), a solution commonly used to solubilize and protect DNA from degradation, to 2.0 ng/ µl, unless DNA concentration was already below that level.

Extracted DNA from each source was PCR amplified using 3 polymorphic microsatellite loci (PR002, PR003, PR005; Wadl *et al.*, 2011; Table 2) for amplification *P. ruthii* DNA. Samples which amplified at least one of the loci but not all three were PCR amplified a second time with 3 additional loci (PR006, PR009, PR020; Wadl *et al.*, 2011; Table 2). 10 µl PCR reactions were prepared in a 96 well plates:

- 2 µl (≤ 2 ng/ µl) DNA
- 1 µl (25 milli-molar) $MgCl_2$ (Applied Biosystems, Foster City, CA)
- 1 µl (10X) GeneAmp PCR Buffer II (Applied Biosystems)
- 1 µl (2 mM) dNTPs
- 1 µl (2.5 mM) PCR primer (forward and reverse)
- 0.5 µl dimethyl sulfate (Fisher Scientific, Pittsburgh, PA)
- 0.08 µl AmpliTaq Gold DNA polymerase (5 U/ µl) (Applied Biosystems)
- 3.5 µl sterile water

The following conditions were performed in a Mastercycler Pro 6321 (Eppendorf, Hamburg, Germany) thermo regulator for all PCR reactions:

- 94° C for 3 minutes
- 35 cycles of:
    - 94° C for 40 seconds
    - 55° C for 40 seconds
    - 72° C for 30 seconds
- 72° C for 4 minutes

Table 2: Microsatellite loci for *Pityopsis ruthii* developed by Wadl *et al*., 2011

Locus	Number of Alleles	Observed Heterozygosity	Expected Heterzygosity	Genebank Accession No.
PR002	3	0.80	0.53	JF261124
PR003	5	0.20	0.56	JF261125
PR005	4	0.70	0.50	JF261126
PR006	4	0.42	0.60	JF261127
PR009	6	0.35	0.68	JF261128
PR020	5	0.25	0.75	JF261129

Electrophoresis through 2 percent agarose gel with ethidium bromide (Sigma Aldrich) was used to separate the PCR fragments. The PCR product was allowed to migrate through the gel for 45 minutes at 100 volts using a Powerpac 300 (BioRad, Hercules, CA) and a FB-SB-131 electrophoresis system (Fisher Biotech, Hampton, NH). The gel was then photographed in Gel Doc 2000 (BioRad) with a UV transilluminator. The software program Quantity One (BioRad) was used to view and save a gel image of the PCR product.

Statistical Analyses

Statistical testing was performed in SAS ver. 9.3 (SAS Institute, Cary, NC) with analysis of variation (ANOVA), (PROC MMAOV; Saxton, 1998) in a completely randomized block design and Fisher's least significant difference (LSD) mean separation at the $\alpha < 0.05$ level of significance to compare pollen coverage by all captured floral visitor families and by the lowest taxonomic identification level. ANOVA comparisons were also made between percentages of filled seed set per head by collection site. ANOVA comparisons of seed germination and seed viability were not performed because the data was not normally distributed, and sample size was low. Simple linear regression, (PROC REG; Saxton, 1998) was performed in SAS (SAS Institute) with respect to measurements of floral visitor abundance (Apoidea, Syrphidae, total) and flower density (*P. ruthii* flowers per 2 m^2 plot). Simple linear regression was also performed to compare the mean number of floral visitors and mean filled seed set by site.

Results and Discussion

Insect Visitation to *Pityopsis ruthii* Flowers

Insect representatives (*N*=240) from 3 orders, 13 families, and 28 genera were collected from flowers of *P. ruthii*; 497 floral visitors were observed (capture success rate 48 percent). Syrphidae flies were the most abundant and rich of the families captured (*n*=134). The most commonly captured insect was the hover fly *Toxomerus geminatus* Say (*n*=89), representing 31.6 percent of all captured floral visitors, and was present in all populations. In addition to the ubiquitous *T. geminatus*, *Toxomerus marginatus* Say was common at the UT Arboretum (*n*=17), but not found at Hiwassee River sites. Syrphids in the genera *Cheilosia* (*n*=1), *Eupeodes* (*n*=8), *Ocyptamus* (*n*=3), *Pelecinobaccha* (*n*=3), and *Trichopsomyia* (*n*=1) were also caught at Hiwassee River locations. *Eupeodes* was the only other syrphid caught at the UT Arboretum (*n*=4). Other captured Diptera were members of the Bombyliidae family, *Lepidophora lepidocera* Wiedemann (*n*=2) and *L. lutea* Painter (*n*=1), and unidentified specimens in the Conopidae (*n*=1) and Phoridae (*n*=1) families (Table 3).

Within Hymenoptera, six families were found; the most common family captured was Apidae (*n*=73). *Apis mellifera* was widespread at two sites: the UT Arboretum (*n*=31) and The Narrows (*n*=5), but only 1 was caught at Loss Creek and none were observed at McFarland. *Bombus impatiens* was frequently caught at all sites (*n*=27). *Ceratina calcarata* was captured twice at McFarland and a single *Xylocopa virginica* L. was captured at Loss Creek. Two *Melissodes* bee species were caught: *M. druriella* Kirby was found at Loss Creek (*n*=1) and the UT Arboretum (*n*=2), *M. dentiventris* Smith (*n*=3) was caught at the UT Arboretum (Table 3). Megachildae species *Megachile mendica* was captured at all Hiwassee River sites (*n*=5), while *M. brevis* Say was only found at the UT Arboretum (*n*=2). No Andrenidae were caught at the Hiwassee sites, but two species, *Andrena bisalicis* Viereck (*n*=1) and *Pseudopanurgus compositarum* Robertson (*n*=3) were caught at the UT Arboretum (Table 3).

Table 3: All captured visitors to flowers of *Pityopsis ruthii* at four sites in East Tennessee

			Insects captured by site*				Mean Pollen Coverage†			
Family	**Genus**	**Species**	**UT**	**MF**	**LC**	**NA**	**UT**	**MF**	**LC**	**NA**
Order: Diptera										
Bombyliidae	*Lepidophora*	*lepidocera*	1		1		3.0±1.8		3.0±1.8	
	L.	*lutea*		1				4.0±1.9		
Conopidae		spp.	1				9.0±3.1			
Phoridae		spp.	1				0			
Syrphidae	*Cheilosia*	spp.		1				3.0±1.9		
	Eupeodes	(*metasyrphus*) spp.	4	1		3	4.0±1.5	9.0±1.9		3.5±1.5
	Ocypatamus	*fuscipennis*			2	1			1.0±1.3	3.0±2.1
	Pelecinobaccha	*costa*			1	1			1.0±1.8	2.0±2.12
	Toxomerus	*geminatus*	4	13	23	36	3.0±1.6	2.4±0.5	2.2±0.4	3.0±2.1
	T.	*marginatus*	17				4.0±1.0			
	Trichopsomyia	spp.			1				2.0±1.8	
Order: Hymenoptera										
Andrenidae	*Andrena*	*bisalicis*	1				13±3.1			
	Pseudopanergus	*compositarum*	3				10.5±2.2			
Apidae	*Apis*	*mellifera*	31		1	5	12.2±0.6		13.0±1.8	11.6±1.0
	Bombus	*impatiens*	11	5	6	5	11.6±1.0	10.4±0.8	7.7±0.8	8.8±1.0
	Ceratina	*calcarata*		2				6.5±1.3		
	Melissodes	*dentiventris*	3				12.3±1.8			
	M.	*druriella*	2		1		13.0±2.2		10.0±1.8	
	Xylocopa	*virginica*			1				5±1.8	
Halicidae	*Augochlorella*	*aurata*	5				9.2±1.4			
	A.	*persimilis*	3				7.7±1.8			
	Augochlora	*pura*	2	2	1		3.5±2.2	6.5±1.3	1.0±1.8	
	Halictus	*ligatus/poeyi*	5				10.6±1.4			
	Lasioglossum	*callidum*	1				1.0±3.1			
	L.	*imitatum*	5				12.6±1.4			
	L.	*trigeminum*	1				3.0±3.1			
	L.	(*dialictus*) spp.	2				9.5±2.2			
Megachilidae	*Megachile*	*brevis*	2				13.5±2.2			
	M.	*mendica*		1	3	1		10.0±1.9	10.3±1.0	12.0±2.1
Sphecidae	*Isodontia*	*mexicana*			1	2			7.0±1.7	9.5±1.5
Vespidae	*Polistes*	*metricus*				1				0
Order: Lepidoptera										
Hesperiidae	*Euphyes*	*vetris*			5				0	
	Hylephila	*phyleus*				1				0
	Prygus	*communis*			1				0	
Nymphalidae	*Phyciodes*	*tharos*		2	2			0	0	
Pieridae	*Abaeis*	*nicippe*			1				0	
Total genera collected			15	9	15	11				
Total insects collected			105	28	50	57				
Total observation hours			5.5	5.0	6.0	5.0				
Average insects caught per hour┼			19.1	5.6	8.3	11.4				

* Site designation for this table: UT – UT Arboretum (*ex situ*); MF – McFarland (*in situ*); LC – Loss Creek (*in situ*); NA – The Narrows (*in situ*)

†Pollen coverage was assessed on each insect integument section: the legs, wings, thorax, head, mouth parts and abdomen on a scale of 0-4: 0 – No pollen; 1 = <10 grains; 2 =10-40 grains; 3 = 41- 100 grains; 4 = >100 grains. Individual integument scores were summed for each specimen and averaged with standard error for each taxa (analyzed by site) in SAS v. 9.3 with ANOVA and Fisher's LSD mean separation; P <0.001, N=240;

┼Average insects caught per hour by site = total insects collected / total observation hours

Few Halictidae were captured at Hiwassee sites. The blue-green sweat bee *Augochlora pura* was the only Halictid species present at McFarland (*n*=2) and Loss Creek (*n*=1). *Augochlorella aurata* Smith (*n*=5), *A. persimilis* Viereck (*n*=3), *Halictus ligatus/poeyi* Say/Lepeletier (*n*=5) and the *Lassioglossum* species *L. callidum* Sandhouse (*n*=1), *L. imitatum* Walker (*n*=5), *L. trigeminum* Gibbs (*n*=1), and two unidentified male species were found at the UT Arboretum. Two wasps were also caught at Hiwassee River sites, *Polistes metricus* Say (n =1; Vespidae) and *Isodontia mexicana* (n =3; Sphecidae) (Table 3).

Lepidopteran families were observed at all Hiwassee River sites. Hesperiidae species were the most common, represented by *Euphyes vestries* Boisduval (*n*=5), *Hylephila phyleus* Drury (*n*=1), and *Prygus communis* Grote (*n*=1). *Phyciodes tharos* Drury (n=4, Nymphalidae) and *Abaeis nicippe* Cramer (*n*=1, Pieridae) were also caught (Table 3). Although none were caught at the UT Arboretum, Lepidopteran visitors were observed in adjacent plots.

The difference in the abundance, diversity, and proportion of bees captured at *P. ruthii* flowers between Hiwassee River sites (Figures 3,4,5; Table 3) and the UT Arboretum (Figure 6; Table 3) may be attributed to the greater abundance and diversity of floral resources and nesting habitat at the UT Arboretum compared to Hiwassee River locations. The unique mix of native and exotic silvicultural and horticultural plant species present at the UT Arboretum likely provided more foraging resources than the nearly contiguous deciduous forest of the Hiwassee River valley. In addition the open meadow and general variation between habitat types of the UT Arboretum likely supported the wider array of bee species habitat preferences better than the homogeneous forest and boulder outcropping habitat found at the Hiwassee River sites. Similar data was published by Rhoades *et al.* (2011), who collected floral visitors to *Cornus florida* L. (Cornaceae) at three locations in east Tennessee: UT Arboretum, a residential subdivision, and an unmanaged forest nature preserve. Greater than double the number of total insects collected each year, which mainly composed of Halictidae and Andrenidae bees were captured at the UT Arboretum compared to the other sites. Holzschuh *et al.* (2012) showed that a highly diverse

habitat within a one 1 km radius increased wild pollinator abundance and the resulting fruit set in sweet cherry (*Prunus avium* L. (Roseaceae) var. Regina and Kordia). This diversity in habitat provided a diversity of nesting sites and foraging resources, which resulted in a significant linear relationship to wild bee abundance. Similar results in mango (Carvalheiro *et al.*, 2010) and sunflower (Carvalheiro *et al.*, 2011) have also been published.

The great abundance of *A. mellifera* at the UT Arboretum is likely a reflection of the number of beekeepers in Oak Ridge, TN, rather than that of wild colonies. The higher occurrence of *A. mellifera* to The Narrows site is also likely due to managed colonies. Feral *A. mellifera* colonies in the US are scarce and ephemeral due in part to the recent introductions of the invasive pests *Varroa desctructor* Anderson & Trueman (Varroidae) and *Acarapis woodi* Rennie (Tarsonemidae) (Meffe, 1998). Other causes for declines in wild *A. mellifera* colonies include increases pesticide prevalence and reduction in forage and habitat (Meffe, 1998). The Narrows location is closest to the residential portion of the Hiwassee River valley than the other sampled sites and local abundance of *A. mellifera* may be related to the isolation of Hiwassee River *P. ruthii* populations from nearby beekeepers. In addition, beekeepers often move their colonies to collect sourwood (*Oxydendrum* spp.) or other varietal honeys and movement of *A. mellifera* colonies to US Forest Service land for this purpose is permitted. It may be that the migration of local beekeepers to the area in late summer through fall induces the abundance of *A. mellifera* at *P. ruthii* populations. Therefore it can be reasonably assumed that captured *A. mellifera* are the result of artificially managed colonies and should be considered highly temporally and spatially variable. Wadl (personal communication) has observed many *A. mellifera* at Loss Creek and the McFarland sites in multiple seasons. Therefore *A. mellifera* floral visitation is expected to occur at many *in situ* locations, but may not be a reliable pollinator unless perennial colonies are found or artificially established and maintained.

In addition to the non-native *A. mellifera*, variation in the abundance of native bees is expected between years. Edens-Meier *et al.* (2011), in a study of the pollination biology of an endemic herb,

Physaria filiformis Rollins (Brassicaceae), found that only 11 percent (four species) of the bees collected were present in all three of the years they sampled. These four species accounted for 39 percent of the total bees collected and although they were the most dependable, a diversity of bees was suggested to hedge pollen flow in the rare plant. Tependino e*t al.* (1999) also observed variation in bee abundance between two sampling years for the endangered *Penstemon penlandii* W.A. Weber (Scrophulariaceae). For example, different species of *Osmia* were observed between years and bees in the genera *Anthophora* and *Hoplitis* were only collected in one year. This recognition was particularly important because *P. penlandii* is only effectively pollinated by *Osmia* and *Hoplitis* bees (Lawson *et al.,* 1989*)*. The most abundant native bee collected as a visitor to *P. ruthii* at Hiwassee River sites was *B. impatiens.* The very common *B. impatiens* has been shown to be growing in population and distribution, which may make it a persistent and reliable pollinator for *P. ruthii*. But the use of *B. impatiens* as a commercial pollination agent in greenhouse tomatoes has spread the species beyond its natural range of the eastern US to California, Western Canada, and Mexico, even becoming a potentially invasive species in its non-native range (Ratti & Colla, 2010). Where commercial *B. impatiens* colonies exist, increased pests and pathogens in wild colonies are reported (Goulson *et al.,* 2007). Therefore, it is unknown if colonies of *B. impatiens* at Hiwassee River populations can be considered secure.

Differences in the behavior and morphology of insect visitors impact the pollination of *P. ruthii.* All captured bees at Hiwassee River sites are pollen generalists and will forage on a variety floral species (Droege, personal communication). The only pollen specialist captured on *P. ruthii* at UT Arboretum was *Pseudopanurgus compositarum* (*n*=3), an Asteraceae specialist (Droege, personal communication). Foraging consistency will impact the success of pollen transfer and *A.mellifera* is highly flower consistent (Butler, 1945). Flower consistency is in part a response of the time required to learn how to access caloric rewards for each flower type and therefore the memory of floral visitors and recognition of different floral types (Waser, 1986). It has been hypothesized that social bees like *A. mellifera*, that forage over a large

area (>1 km) have a large flower memory and forage more consistently (passing over other rewarding flower types in a given foraging bout) because of the time required to understand and manipulate a new flower type (Waser, 1986). Solitary bees, in contrast, with a much more limited foraging range (~100-300 m) cannot afford to skip over rewarding flowers in favor of the efficiency (flower handling time/ caloric reward) found from constancy (Waser, 1986). Long tongued bees (which allow the insect greater access to a variety of flowers) with large bodies (that allow for greater pollen and nectar loads) like *A. mellifera, X. virginica,* and, *B. impatiens* will travel many miles to forage (McGlynn, 2009). From the modest average flight distance of 1 km for *A. mellifera,* to the 10 – 20 km distance of *X. virginica*, the range of each species varies widely (McGlynn, 2009). Smaller, solitary bees like *C. calcarata, A. pura,* and *M. mendica* will only forage in a range of a few hundred meters from their nest and are therefore more restricted in their foraging options and may be more subject to local disturbances in forage availability (McGlynn, 2009). The population size of bee colonies can impact the number of individuals present at plant populations. *Apis mellifera,* the only truly social bee captured, will have colonies with over 50,000 foraging members all produced by a single queen (McGlynn, 2009). *Bombus impatiens* is eusocial and has a queen with up to 400 foraging members in a colony (McGlynn, 2009). However solitary bees like *X. virginica, A. pura, C. calcarata,* and *M. mendica* nest individually and each female may produce less than a dozen individuals (McGlynn, 2009). The abundance of each species at *P. ruthii* populations is reflected in the relative size of their colonies.

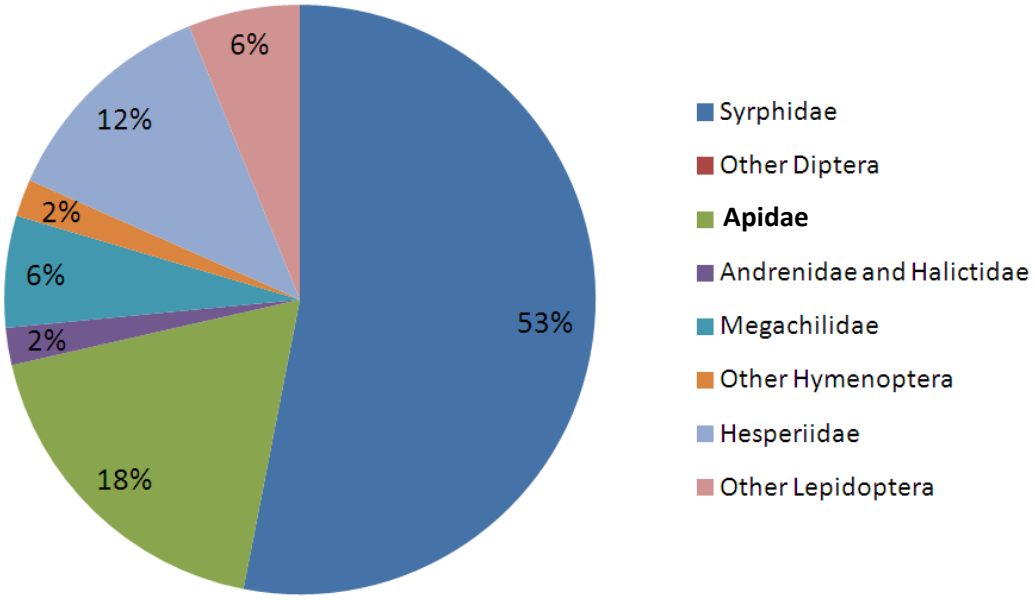

Figure 3: Proportion of captured floral visitor families to *Pityopsis ruthii* at Loss Creek; *N* =49

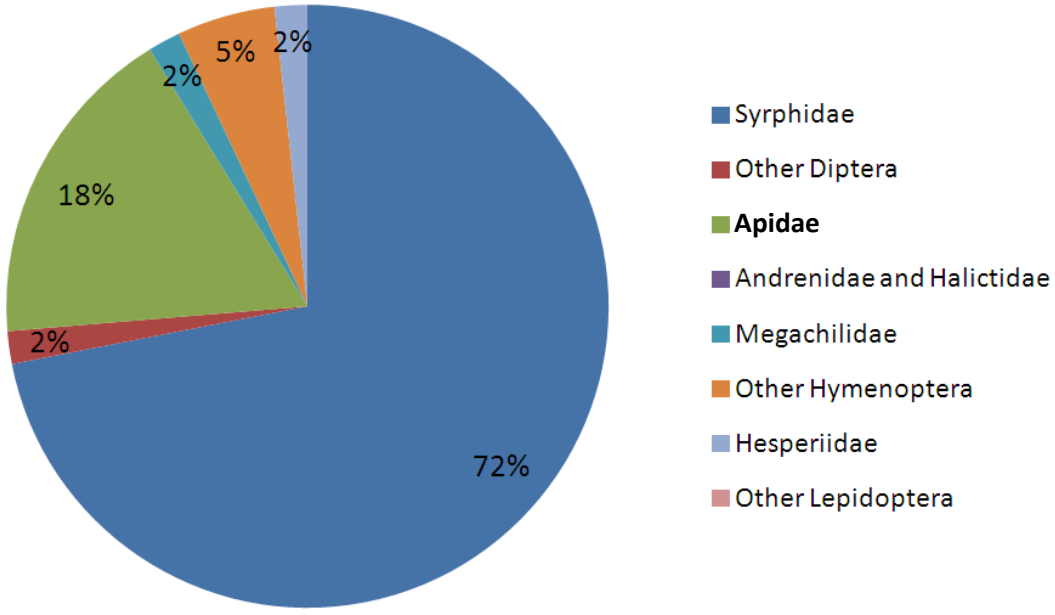

Figure 4: Proportion of captured floral visitor families to *Pityopsis ruthii* at The Narrows; *N* = 57

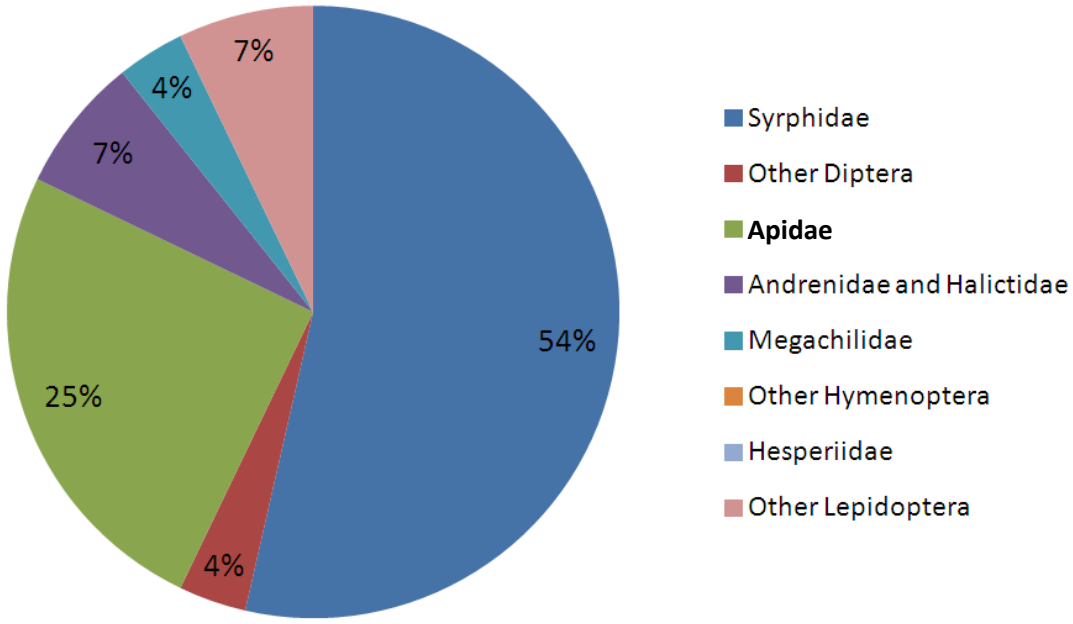

Figure 5: Proportion of captured floral visitor families to *Pityopsis ruthii* at McFarland; *N* = 28

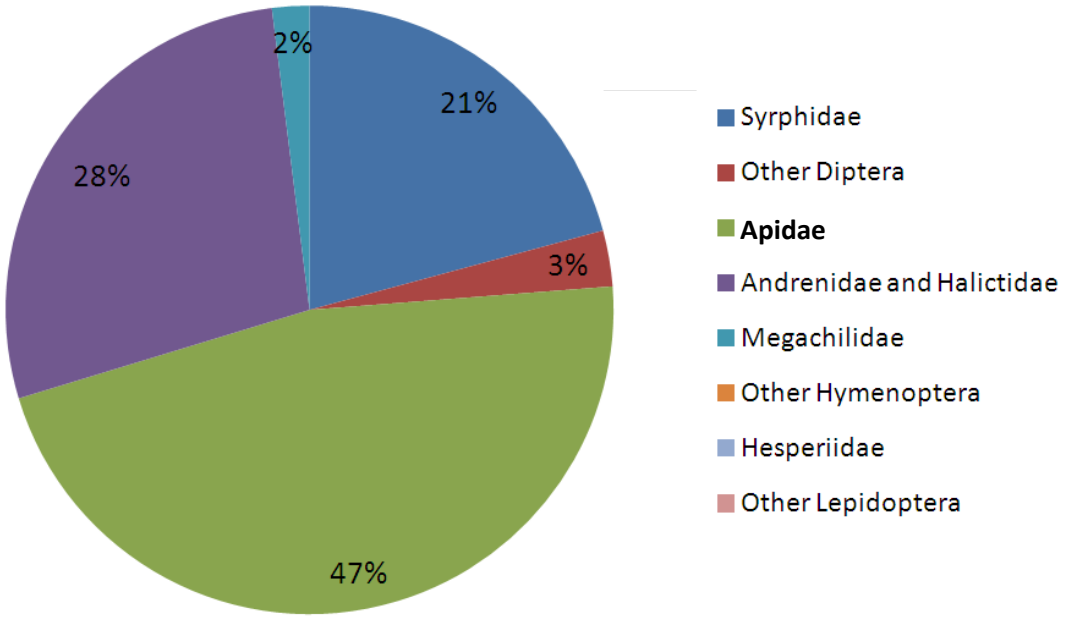

Figure 6: Proportion of captured floral visitor families to *Pityopsis ruthii* at the UT Arboretum; *N* = 101

Analysis of Flower Density Effect on Floral Visitation

The frequency of observed Apoidea visitation at Hiwassee River locations is related to the flower density (number of *P. ruthii* flowers per 2 m^2 plot). In a simple linear regression model (SAS, 2009) the flower density in 32 Hiwassee River *P. ruthii* observations explained 21.9 percent of the variation in bee visitation (R^2 =0.219, $F_{1,30}$= 8.39, P = 0.007, Figure 8). When bee visitation is at zero, flower density is expected to be 29.1 flowers per 2 m^2 (SE =4.20). From that point, to increase bee visitation by 1, the flower density would need to increase by 5.72 flowers per 2 m^2 (SE =1.98). This experiment focused on large *P. ruthii* populations, because they would have the greatest effect on *P. ruthii* reproduction. Small, discontinuous populations may receive far fewer floral visitors than this evidence illustrates and should be further studied.

Other investigators have found the size and continuity of plant populations will affect the abundance of floral visitors. Pavlik *et al.* (1993) demonstrated that the availability of pollinators was the primary limit to the reproductive success of the endangered *Erysimum capitatum* (Douglas ex. Hook) Greene (Brassicaceae), but was only limited by pollinators when plant populations were small. Moody-Weis and Haywood (2001) found pollen limitation in the flowers of *Oenothera macrocarpa* Nutt. (Onagraceae). Pollen limitation was greatest in the smallest populations of *O. macrocarpa* because of diminished pollinator activity. Lennartsson (2002) found habitat fragmentation (from invasive species invasion) quantitatively increased extinction risk in the endangered herb *Gentianella campestris* (L.) Boerner (Gentianaceae). Habitat fragmentation led to reduced pollinator visitation and genetic isolation, which caused inbreeding depression and reduced seed set. An example with similar floral visitors to this experiment was by Scott and Molano-Flores (2007), who found that the presence of insect families to different populations of *Rudbeckia fulgida* Ait.var. *sullivantii* (C. L. Boynt and Beadle) Cronq. (Asteraceae), was highly variable (from 11 to 22 families) and related to *R. fuligida* var. *sullivantii* population size and floral density. The theory that habitat fragmentation and discontinuous plant

populations attract fewer pollinators and may limit seed production has been well established. The limited and highly variable seed production of *P. ruthii* may be attributable to this phenomenon.

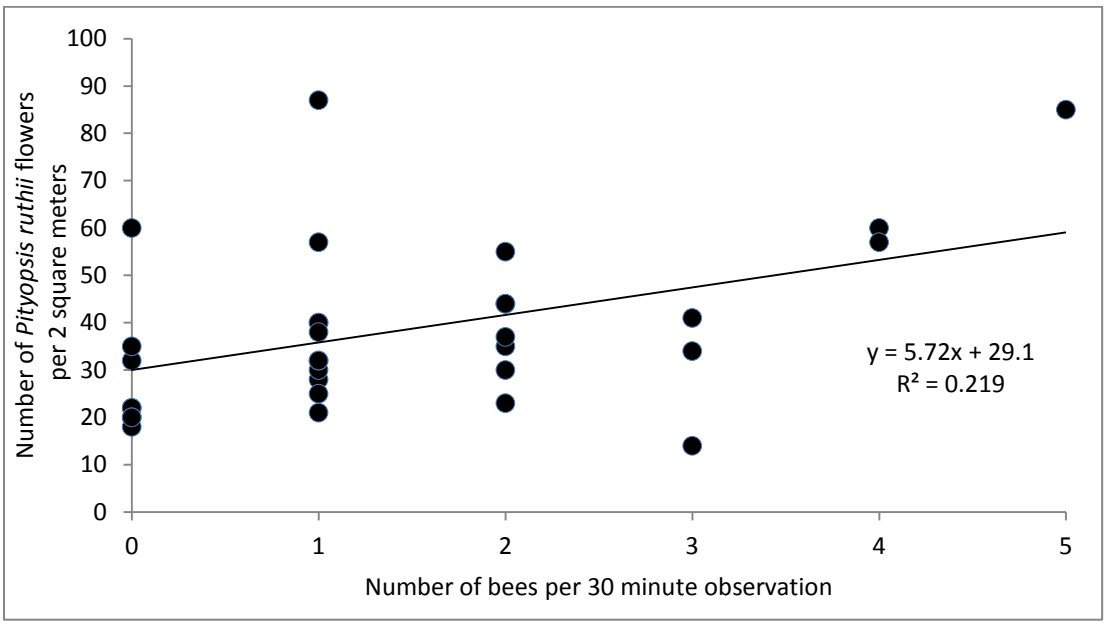

Figure 7: Bees abundance was positively correlated with *Pityopsis ruthii* flower density per plot for three sites on the Hiwassee River in east Tennessee; PROC REG, SAS v. 9.3, *N*=32 plots, *P* =0.007, $F_{1,30}$= 8.39

Syrphidae fly visitation was not affected by flower density at Hiwassee River locations (*P*= 0.265, $F_{1,30}$= 1.29, *N*=32; SAS 2009).The life history of syrphids, in particular the mating and egg laying behavior of adults can help explain the inability of flower density to predict syrphid visitation. Male adult syrphids tend to hover in a single area, without feeding, and dart at competitors or pounce on females in an attempt to mate (Marshall, 2006). This behavior was frequently observed at Hiwassee River and UT

Arboretum plots. Female syrphids consume pollen using small teeth on the inside of their labium to scrape anthers, but rarely consume plant nectar, because their shallow mouthparts lack an extended proboscis and cannot access nectar deep inside of flowers (Marshall, 2006). Syrphid larvae are predators of Aphididae and female adult syrphids will seek aphid infested plant material to lay their eggs (Marshall, 2006). *Pityopsis ruthii* plants at Hiwassee River locations were observed with aphid adults along the underside of leaves by previous investigators (Rhoades and Klingeman, unpublished data). All bee visits were observed to be made for collection of floral caloric rewards, whereas only a fraction of syrphids were observed consuming pollen or nectar during floral visits.

For 16 UT arboretum observations, neither bee nor syrphid fly visitation was affected by flower density in a simple linear model ($F_{1,14}$=0.51, P= 0.556 and $F_{1,14}$=2.65, P = 0.119 respectively; SAS 2009). The significant differences between the Hiwassee and UT Arboretum locations in the ability of flower density to predict bee abundance is likely due the large number of flowers at the UT Arboretum observation plots. The average number of flowers at Hiwassee observation plots was 38, and ranged from 14 to 87 flowers, whereas the average number of flowers at UT Arboretum observation plot was 187, and ranged from 40 to approximately 400 flowers. The floral attraction was much greater at UT Arboretum plots and therefore the variation in flower density at those plots apparently had no effect on bee or syrphid fly abundance, where the small size of flower displays did have a significant effect on bee abundance at Hiwassee River plots.

Analysis of Pollen Coverage on Floral Visitors

Generally bees carry more pollen than other floral insect visitors, because their integument is typically lined with dense, branched, electrostatic hairs that readily attract and hold pollen. Many bees also have specialized areas on the legs (corbiculae) or abdomen (scopa) for carrying large loads of pollen back to the nest, and will scrape pollen from other parts of their bodies into these areas. Bees collected in

this experiment exhibited these characteristics (Figure 9). Aside from an occasional exception, other non-bee insect visitors carried few pollen grains upon examination (Table 3).

The ANOVA (SAS, 2009) analysis of pollen coverage (measured on 0-4 scale per segment, segments summed per specimen) of all collected visitor families found that bees in the families Apidae and Megachilidae had the greatest pollen coverage (M = 11.2 and 11.4, SE= 0.35 and 1.1 respectively, $P < 0.001$, $F_{13,232} = 45.13$, Figure 9). The other bee families captured, Andrenidae and Halictidae, also had large pollen coverage measurements (M = 10.8 and 8.5, SE=1.4 and 0.54 respectively, $P < 0.001$, $F_{13,232} = 45.13$, Figure 9). Syrphidae flies carried little to no pollen (M = 2.6, SE=0.28, $P < 0.001$, $F_{13,232} = 45.13$, Figure 9). Captured Lepidopteran families, Hesperiidae, Nymphalidae, and Pieridae, carried no pollen (Table 3). Other Dipteral and Hymenopteran families carried little to no pollen, expect for a single Conopidae specimen and three Sphecidae wasps, which had substantial pollen coverage (Table 3).The lack of pollen on captured Dipteral and Lepidopteran visitors is consistent with other studies. Bernhardt and Dafni (2000) noted Lepidopteran visitors to *Mandragora officinarum* L. (Solanacaea) perched on petal lobes and failed to contact stamens and stigma while collecting nectar. Herrera (1987) found that Dipteral and Lepidopteran visitors to *Lavandula latifolia* Medikus (Lamiaceae) were not significantly different in pollination effectiveness (measured as pollen coverage and behavior) and scored among the lowest of floral visitors.

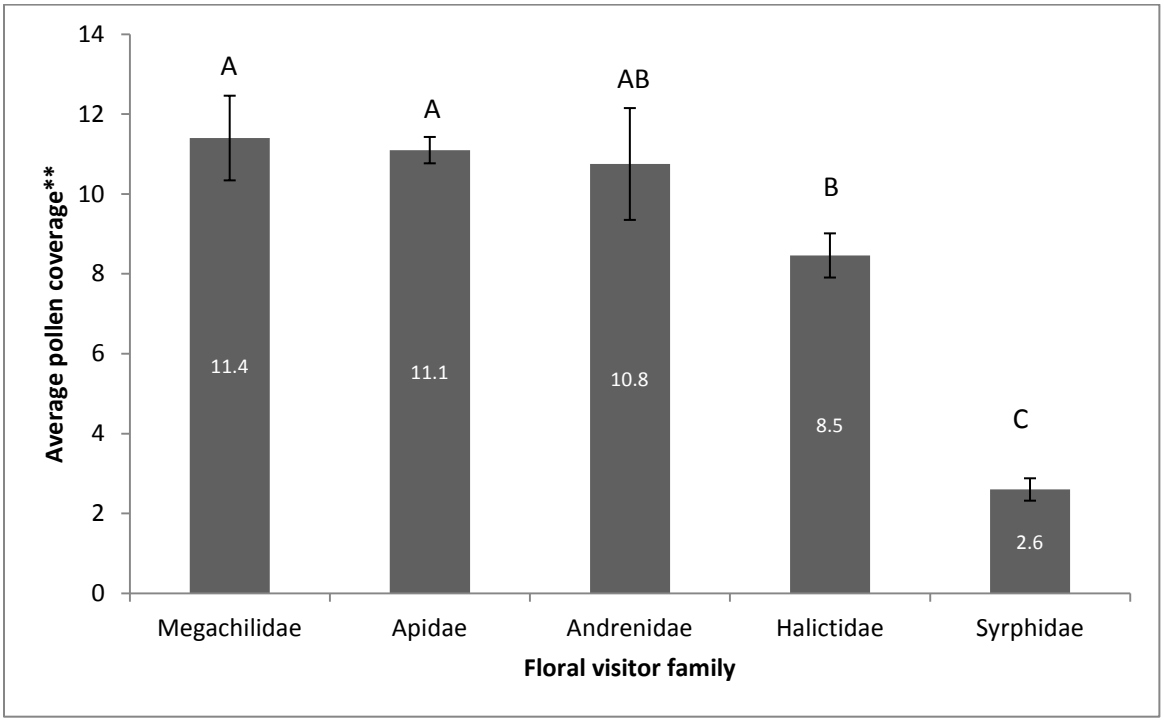

Figure 8: Average pollen coverage of visitors to flowers of *Pityopsis ruthii* at four sites in east Tennessee
**** Pollen coverage was assessed on each insect integument section: the legs, wings, thorax, head, mouth parts and abdomen on a scale of 0-4: 0 – No pollen; 1 = <10 grains; 2 =10-40 grains; 3 = 41- 100 grains; 4 = >100 grains. Individual integument scores were summed for each specimen and averaged with standard error for each taxa by family in SAS v. 9.3 with ANOVA and Fisher's LSD mean separation; P <0.001, N=240, $F_{13,232}$ = 45.13**

Genetic Analysis of Pollen

DNA from 24 samples (out of 64 attempted) amplified *P. ruthii* specific microsatellite loci (Table 4). None of the DNA from the floral voucher plants collected amplified with the primers tested. Although, DNA from *Pityopsis graminifolia* var. *latifolia* (a sympatric species) has been shown to amplify primers for *P. ruthii* (Wadl and Boggess, personal communication), locus PR003 (in bold, Table 4) was shown to not amplify *P. graminifolia* in over 100 samples tested and is therefore the best indicator of *P. ruthii* DNA presence.

Table 4: *Pityopsis ruthii* microsatellite amplification from pollen DNA isolated from captured visitor vials at four sites in east Tennessee*

Species	Site	Locus/ Loci amplified	Species	Site	Locus/ Loci amplified
Apis mellifera	UT Arboretum	002,**003**,020	*Apis mellifera*	The Narrows	002,**003**,020
Apis mellifera	UT Arboretum	005,**003**,009	*Apis mellifera*	The Narrows	**003**,020
Apis mellifera	UT Arboretum	005,009,020	*Apis mellifera*	The Narrows	**003**,020
Apis mellifera	UT Arboretum	002,**003**,005	*Apis mellifera*	The Narrows	002,**003**,005
Apis mellifera	UT Arboretum	002,**003**,005	*Toxomerus geminatus*	The Narrows	005
Apis mellifera	UT Arboretum	**003**,005,009,020	*Augochlora pura*	Loss Creek	005
Bombus impatiens	UT Arboretum	005,009,020	*Bombus impatiens*	Loss Creek	005
Halictus ligatus/poeyi	UT Arboretum	005,**003**,009,020	*Bombus impatiens*	Loss Creek	002,**003**,005
Halictus ligatus/poeyi	UT Arboretum	005,009,020	*Bombus impatiens*	Loss Creek	005
Megachile brevis	UT Arboretum	002,**003**,005	*Bombus impatiens*	Loss Creek	005
Melisodes dentrivitus	UT Arboretum	002	*Toxomerus geminatus*	Loss Creek	**003**,005
Toxomerus geminatus	McFarland	005	*Ceratina calcarata*	McFarland	005

*Locus indicated in bold does not amplify *P. graminifolia* DNA

Many insect species were confirmed to either carry *P. ruthii* pollen on their integument or to have consumed and regurgitated the pollen into the collection vial through analysis with microsatellites. Four of the five *A. mellifera* tested (all that were collected) from The Narrows site were shown to carry *P. ruthii* pollen. Four of the five tested (out of 6 total collected) *B. impatiens* from the Loss Creek site were shown to carry *P. ruthii* pollen. Two of the six non-*Apis*, non-*Bombus* bees (*C. calcarata* and *A. pura*) tested (*n*=12) from Hiwassee sites were shown to carry *P. ruthii* pollen. Four of the five tested (*n*=35) non- *Apis*, non-*Bombus* bees (*Megachile brevis*, *Mellisodes dentrivitus*, and two *H. ligatus/poeyi*) from the UT Arboretum carried *P. ruthii* pollen. Three out of fifteen *T. geminatus* (one from each of the sites: UT Arboretum, McFarland, and The Narrows) tested positive for the presence of *P. ruthii* pollen either externally or internally (regurgitated) (Table 4).

The remaining samples tested from insect vials that did not amplify *P. ruthii* primers even though they contained visible unidentified pollen may be due to an insufficient quantity of *P. ruthii* pollen or poor quality DNA, which could not be observed with the simple qualitative test performed in this experiment. Only 30 percent of the total vials used to collect floral visitors were tested for *P. ruthii* DNA and only those which contained the most visible pollen. But the resulting representative sample was satisfactory to confirm the hypothesis that these species move *P. ruthii* pollen in these populations.

Analysis of Seed Viability

Cruzan (2001) and Wadl *et al.* (2014) hypothesized that in-breeding depression or limitation in compatible mates impacts both the total amount and spatial and temporal variation in filled seeds produced by *P. ruthii*, and the variability in filled seed germination observed by Clebsh and Sloan (1993) and Cruzan and Beaty (1998). To investigate this, a comparison of filled seed production, seed germination, seedling emergence, and seed viability was conducted between the UT Arboretum *ex situ* population and three Hiwassee River *in situ* populations, which are primarily different in their genetic diversity of *P. ruthii*. Filled seed production indicates that floral visitors may be limiting sexual reproduction, especially in an isolated *P. ruthii* population (McFarland, Figure 10), while seed germination, seedling emergence, and viability testing indicate that i*n situ P. ruthii* populations may be genetically in-bred or limited in the number of compatible mates (Figure 11).

On average a range of 47 to 51 total seeds (filled and unfilled) were set per head at each site and less than half of the seeds produced per head were filled (Figure 9). The average percentage of filled seed per head from seed heads collected at Hiwassee River sites Loss Creek (M= 0.42, SE= 0.025, n= 55, Figure 9) and McFarland (M= 0.37, SE= 0.024, n= 58, Figure 9) were not different to those collected at the UT Arboretum (M= 0.43, SE= 0.025, n= 55, Figure 9). The Narrows site had statistically greater average percent filled seed per head (M= 0.49, SE= 0.025, n= 59, P <0.001, $F_{3,223}$ =4.27, Figure 9), but not different from the UT Arboretum. The impact of floral visitors to *P. ruthii* filled seed set was

investigating in two linear models. One regression model was significant (P=0.046, Figure 10) and one was nearly significant (P=0.186), both were essentially identical in the regression equation and indicate that floral visitation is critical to *P. ruthii* filled seed production, resulting in a correlation of 91-92 percent. The first used the average floral visitors per plot in each site to predict the average percent filled seed set in each site, but excluded one visitor collection day at the UT Arboretum site because the number of visitors collected that day was twice the number collected in two previous days. This model predicted that with no floral visitors an average of 15 percent (SE= 6 percent) filled seed would be produced and each additional floral visitor will increase mean filled seed set by 3 percent (SE=0.7 percent) (R^2=0.91, $F_{1,2}$=20.28, P=0.046, Figure 10, SAS 2009). The second model used only visitor observations and percent filled seed set for the three Hiwassee River populations. This model had nearly the same predictions; with no floral visitors an average of 15 percent (SE= 8 percent) filled seed per head would be produced and each additional floral visitor will increase mean filled seed set by 3 percent (SE=0.9 percent) (R^2=0.92, $F_{1,1}$=11.09, P=0.186, SAS 2009). Other investigators found substantiating results in greenhouse analyses. Bowers (1972) showed that when *P. ruthii* flowers were excluded from insect visitation, no filled seed were produced. Wadl (personal communication) noted that when *P. ruthii* flowers were open to visitation, but without a compatible mate (genetic clones, 119 of 121 RAPD amplicons were in common between the two individuals), no filled seed were produced. *Pityopsis ruthii* is recognized as self-incompatible, and the variation in filled seed produced by site is, therefore in part, attributable to the rate of floral visitation. Jennersten (1988) compared two locations of *Dianthus deltoides* L. (Rutaceae), one in an undisturbed large population, and the other in small fragmented populations. The isolated populations produced fewer viable seeds, had lower diversity, and received fewer floral visitors. Reduced pollinator visitation to the isolated populations was regarded to be the cause in reduced seed set.

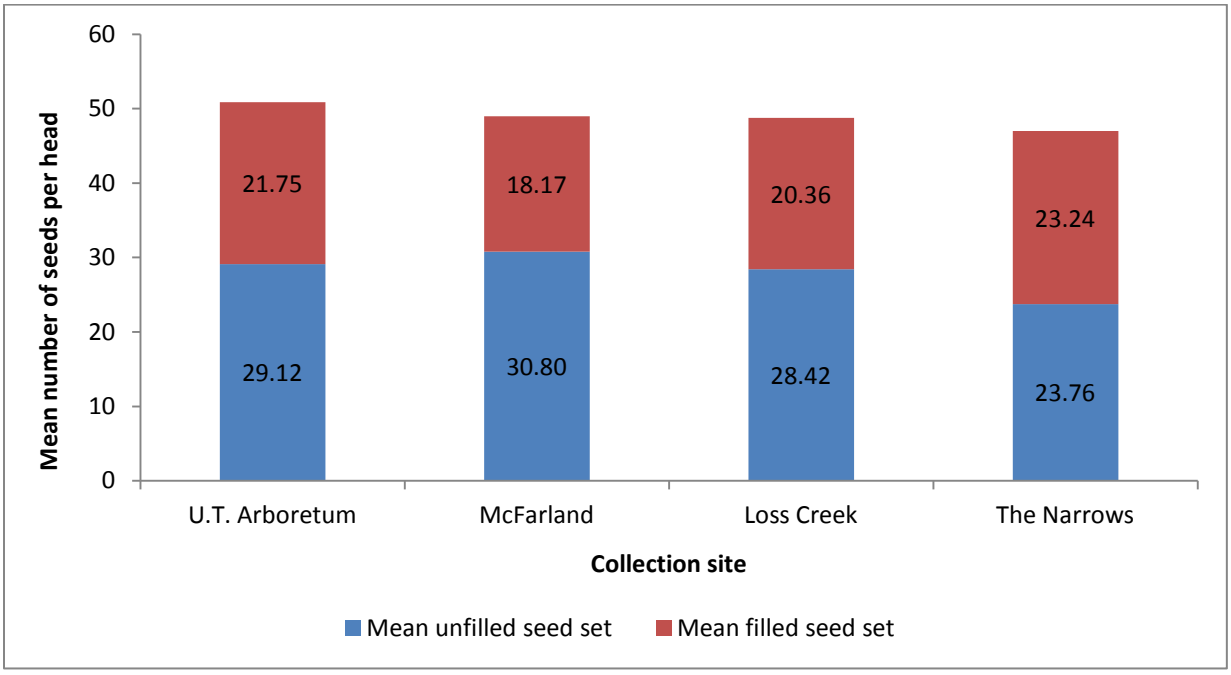

Figure 9: Mean filled and unfilled seeds per head for *Pityopsis ruthii* at four sites in east Tennessee

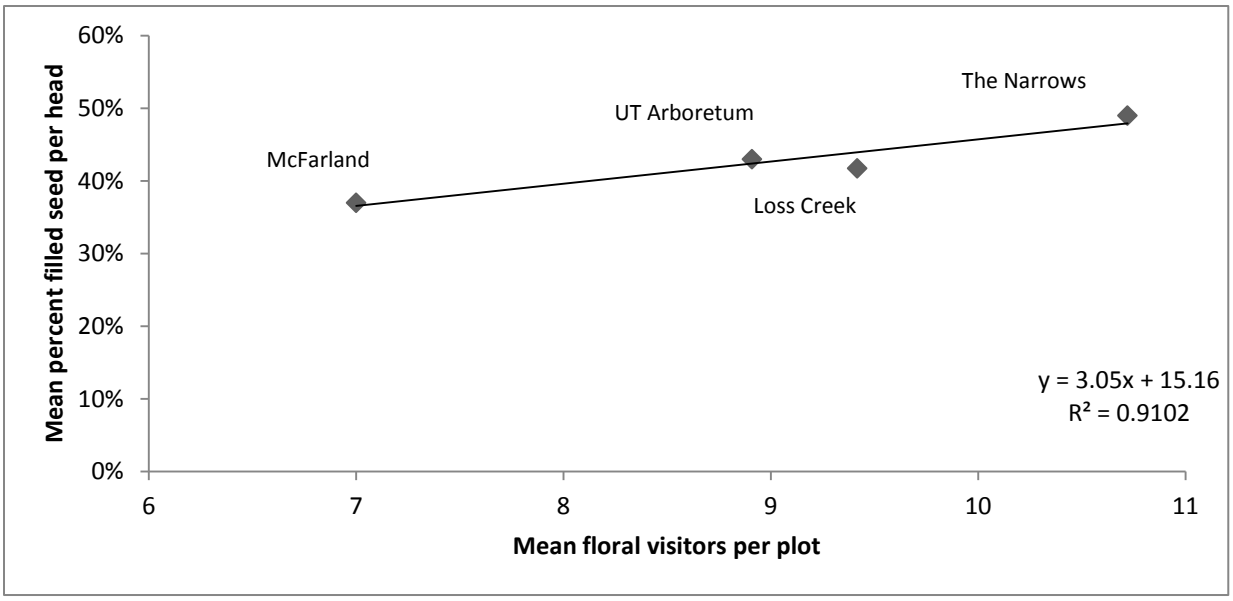

Figure 10: Mean floral visitor abundance was positively correlated with mean percent filled seed per head of *Pityopsis ruthii* for four sites in east Tennessee; R^2=0.91, $F_{1,2}$=20.28, P=0.046, N=4, SAS 2009

For seed germination tests, between 75 and 85 percent of *P. ruthii* filled seeds germinated, depending on the collection site (*n=40,* Figure 11). As expected, unfilled seeds did not germinate (*n*=40 (*n*=35 for Loss Creek)), because unfilled seeds are only a hollow seed coat and pappus that likely lack an embryo or endosperm. Previous investigators found extremely variable germination rates of filled seeds, between 0 and 100 percent depending on the sampling site and year (Wadl *et. al.*, 2014; Cruzan, 2001). Overall germination rates for this experiment were fairly consistent between sites, but differences were seen in the percentage of seeds that were able to produce cotyledons (seedling emergence). Only 50 percent of the filled seeds (*n*=40) sampled from McFarland produced cotyledons, whereas 65 percent of the filled seeds from Loss Creek (*n*=40) and The Narrows (*n*=40) produced cotyledons (Figure 11). All germinated filled seed from the UT Arboretum (*n*=40) produced cotyledons. The limited germination and seedling establishment of filled seeds from Hiwassee River sites may be due in-breeding depression and reduced vigor, caused by genetic isolation as hypothesized by Boggess (2013) and Sloan (1994). Hensen and Oberprieler (2005) measured genetic diversity and seed production of the rare *Dictamnus albus* L. (Rutaceae) in isolated populations of central Germany and found that viable seed production and seed weight were positively correlated with genetic diversity (R^2=0.9). Vergeer *et al.* (2003) showed that small populations of *Succisa pratensis* L. (Dipsacaceae) produced fewer seeds, had lower germination rates, greater seedling mortality, larger in-breeding coefficients, and smaller observed heterozygosity.

Tetrazolium chloride (TZ), which will stain metabolically active tissue red was used to asses the viability of filled seed (*n*=40, *N*=160) from each site. TZ showed that 80 percent of filled seeds (*n*=40) from the UT Arboretum were metabolically active and potentially viable, which is very similar to the 75 percent germination at this site. All of the McFarland sampled filled seeds (*n*=40) were show to be metabolically active. Nearly all of filled seeds from The Narrows (97.5 percent, *n*=40) and Loss Creek (92.5 percent, *n*=40) were show to be metabolically active. Filled seed TZ staining was much higher than the germination rates for Hiwassee River sampled sites and indicates that nearly all of the filled seeds

have living tissue and are viable, but for another reason, failed to germinate. Filled seeds from the UT Arboretum were more consistent with respect to staining viability conforming to the germination rate. The reason for reduced germination of Hiwassee River *P. ruthii* seed may be genetic, which conforms to the hypotheses of Boggess (2013) and Sloan (1993), who predicted in-breeding depression of the endemic populations. Rossetto *et al.* (2004) investigated genetics of the clonally reproducing endangered tree *Elaeocarpus williamsianus* Guymer (Elaeocarpaceae), and found that although none of the seeds collected from two trees responded to germination tests, 100 percent of one tree's seed (n=8) and 40 percent of another tree's seed (n=11) were viable according to TZ tests, inferring that self-incompatibility and inbreeding were likely causes for the discrepancy. Inconsistent seed TZ staining and partial seed germination are both potential evidence of in-breeding depression in *P. ruthii*.

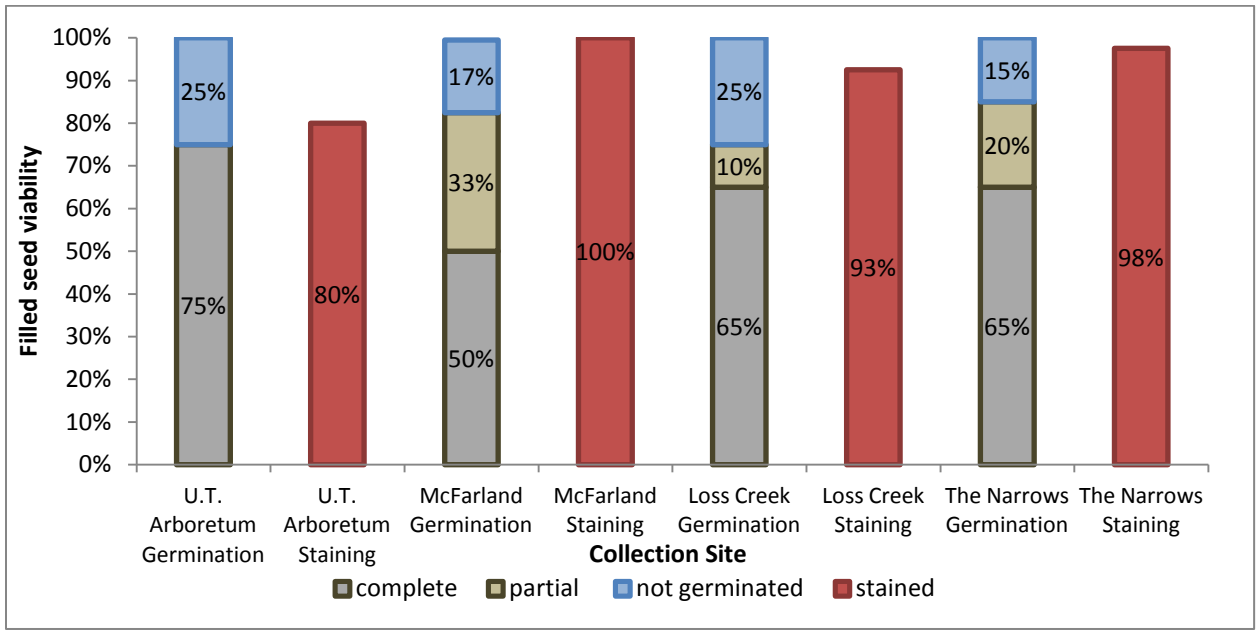

Figure 11: Percentage partial, complete, ungerminated, and stained filled seed germination for *Pityopsis ruthii* at four sites in east Tennessee; n=40, N=320

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Conclusion

Empirical evidence has revealed many challenges to the conservation of *P. ruthii*. The consequences of habitat disruption and fragmentation such as competitive species encroachment and in-breeding depression are beginning to be substantiated, while other life history research projects have been limited in length and scale, and provide for little interpretive value. Defining population locations has been obscured by the use of colloquial names (i.e. McFarland, Bridge, Cats Pajamas, Powerhouse) leading some investigators to not find their locations (Clebesh and Sloan, 1993), while others misinterpret designations, complicating comparisons over time. Evidence of genetic hybridization with *P. graminifolia* has been poorly studied and has not been acknowledged by the US Forest Service and US Fish and Wildlife Service. Limited reporting from TVA has prevented in-depth analysis of water flow impacts and mitigation of water recreation and water flow related threats remain to be articulated (USFWS, 2006). The apparent growth of Ocoee *P. ruthii* populations (USDA-FS, 2012) and recent success in reintroduction and propagation is encouraging (Wadl *et al.* 2014), but threats from human interference in the interest of energy generation, transportation, and recreation persist. The need to conserve existing populations of *P. ruthii* is well understood, but the reasons why *P. ruthii* is unable to expand and establish are still largely unknown.

The primary pollinator for *P. ruthii* is *B. impatiens* due to its abundance, pollen coverage, and confirmed presence of *P. ruthii* pollen and there is also evidence that *A.mellifera* is an important pollinators, although its presence is more spatially and likely temporally variable. Other native bees like, *M. mendia, A. puru, M. druriella,* and *C. calarata* are also likely important pollinators of *P. ruthii* in certain locations. *T. geminatus* is ubiquitous at the sampling locations and although carry little pollen, *P. ruthii* pollen presence was confirmed; therefore these syrphids are expected to contribute to the pollination of *P. ruthii.*

There is evidence that the abundance of bee floral visitors is partially dependent on the density of flowers. Management may be needed to increase the attractiveness of *P. ruthii* to pollinators by increasing

the density of *P. ruthii* individuals, especially in small, isolated populations. Seed production of *P. ruthii* is less than full, and pollinator abundance does appear to impact the proportion of filled seeds produced. However the viability of filled seeds is likely influenced by in-breeding depression, based on partial germination and staining results and the genetic evidence found by Boggess (2013). Management will be needed to increase the genetic diversity of populations by supplementing populations with diverse genotypes, which should increase the viability of seeds and vigor of seedlings. Increasing seeding establishment will grow *P. ruthii* populations, increase flower density and attract more pollinators, which should increase filled seed set.

One limitation of these results is the inability to correlate *P. ruthii* population size with seed set, seed germination, seed viability, or floral visitor abundance. This is because population data to date has counted sites with the rule that all rosettes within 6 inches are considered a single plant. This method does not conform to the genetic or reproductive parameters of a population. Because *P. ruthii* clonally reproduces with rhizomes that can be more than 6 inches long (Wadl, personal communication), what constitutes an individual is obscured. *Pityopsis ruthii* is self incompatible, and knowledge of which how many compatible mates are present in a population affect the sexual reproductive success more than total number of individuals. Population counts, in addition, do not differentiate between sexually mature *P. ruthii* and those which are only vegetative and arbitrarily differentiate sub populations by visual breaks in topography or geography. Knowledge of how many breeding individuals are in a population (an area where gene flow is occurring) is needed for assessment of a population's size impact. This experiment focused on some of the largest populations of *P. ruthii* and analysis of smaller populations is needed to elucidate any effect of population size on sexual reproduction. One example of this type of assessment was by Menges (1991), who measured seed germination in small populations of *Silene regia* Sims (Caryophyllaceae) and found that large populations (>150 individuals) had high germination rates (>85 percent), where small populations had highly variable germination rates. Germination rate was correlated

to the population size in a linear model (R =0.49, P= 0.017), and could be explained by either in-breeding depression or reduced pollinator visitation to the small *S. regia* populations

This project only encompassed one field season, but based on other observations (Wadl, personal communication) and comparisons with a previous year of preliminary data (Rhoades and Klingeman, unpublished data), high variability in pollinator visitation is expected. Replication of the field collections in this experiment should be conducted to help determine the variability between years in pollinator abundance and rate of pollinator visitation for the same and additional sites, especially those of the Ocoee River. Analysis of pollinator movement within and between *P. ruthii* populations was not attempted and should be conducted to determine the amount of gene flow that is occurring and to delineate a subpopulation by the separation of gene flow. Pollinator movement with respect to inter-specific pollen movement and the potential for hybridization with *P. graminifolia* var. *latifolia* should also be measured. Tests using bagged flowers, unbagged open flowers, and bagged flowers with a single pollinator visit will be needed to quantitatively determine the effectiveness of pollen vectors and pollen limitation, beyond the qualitative approach used in this study. Effects of competitive species interference with respect to pollinator visitation may also inform the vegetative removal projects currently underway by Pistrang *et al.* (USDA-FS, 2008).

Appendix

Floral Voucher Specimen

Figure 12: ***Solidago gigantea*** **Vit.**

Figure 13: *Eupatorium rotundifolium L.* or *E. rotundifolium* var. *scabridum* (Elliott) A. Gray

Figure 14: ***Lobelia cardinalis*** **L.**

Figure 15: ***Lobelia puberula*** **Michx.**

Figure 16: ***Symphyotrichum patens*** **(Aiton) G.L. Nesom**

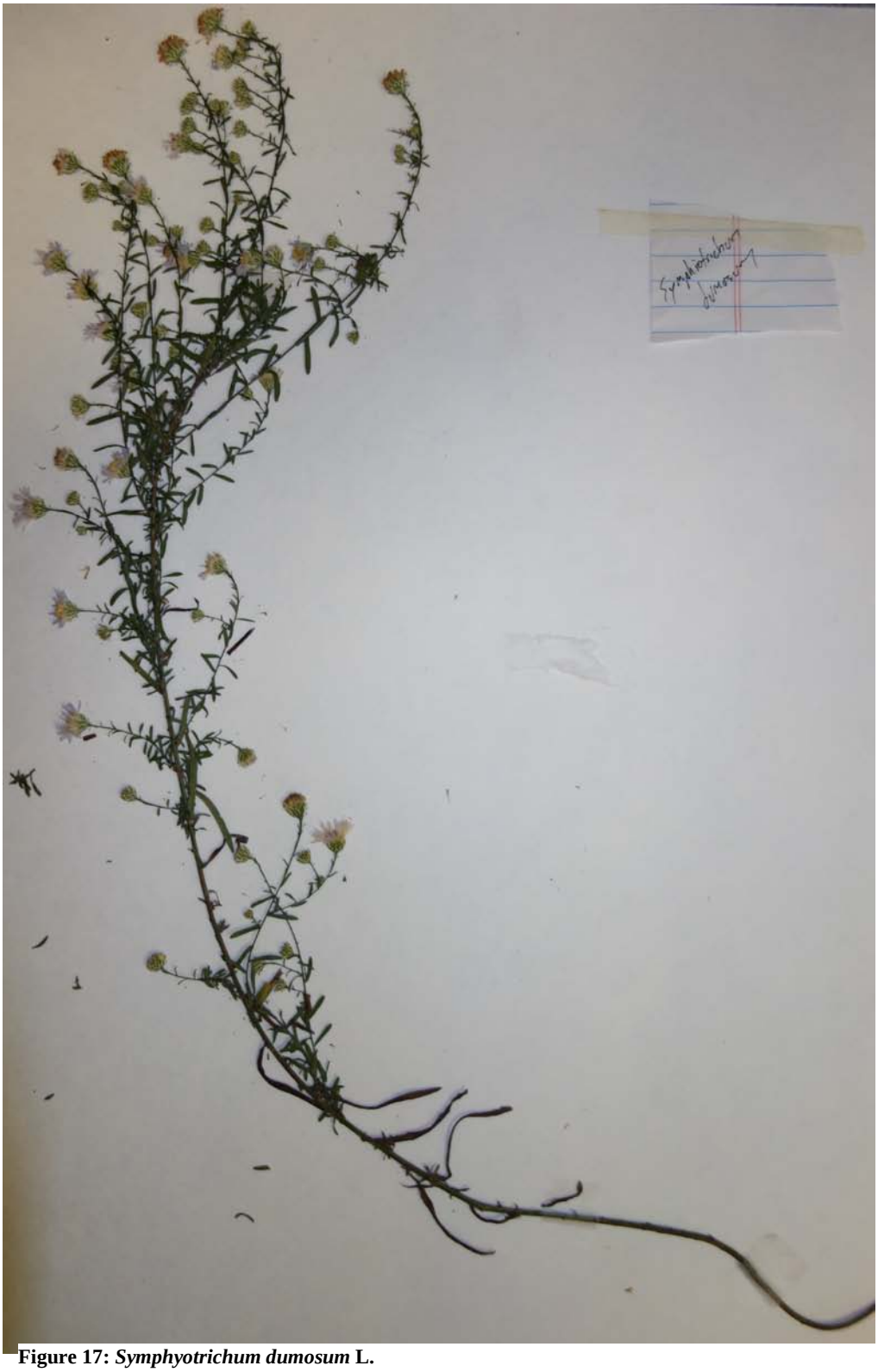

Figure 17: *Symphyotrichum dumosum* L.

Figure 18: ***Solidago arguta*** **Aiton**

Figure 19: ***Eurybia divaricata*** **(L.) G.L. Nesom**

Figure 20: ***Symphyotrichum lanceolatum*** **(Willd.) G.L.Nesom**

Figure 21: ***Pityopsis graminifolia*** **(Michx.) Nutt. var.** ***latifolia*** **(Fernald) Semple & F.D. Bowers**

Figure 22: ***Agalinis tenuifolia*** **(Vahl) Raf.**

Vita

Philip Moore grew up in Hendersonville Tennessee, where he attended Hendersonville High School, graduating in 2006. He pursued a website design and culinary career in Nashville before settling on a Bachelors of Science degree in Agricultural and Natural Resource Economics from The University of Tennessee in Knoxville, where he graduated with honors in 2011.

He believes that an internship with the University of Tennessee, Plant Sciences Department, Organic and Sustainable Crops Farm in 2009 led to his love of science in addition to his already outstanding appreciation for plants, food, and farming. His eventual career with bees began in 2011, when he became a beekeeper and assistant to Dr. John Skinner, who later encouraged him to attend Graduate School. He was accepted into the Master of Entomology program at The University of Tennessee in 2012, where his growing interest in pollination ecology, native wildflowers, and impact of humans on the natural landscape led to this research project.