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Small Scale Population Structure of Pityopsis ruthii and P. graminifolia var. latifolia

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I am submitting herewith a thesis written by Sarah Lynn Boggess entitled "Small Scale Population Structure of Pityopsis ruthii and P. graminifolia var. latifolia." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

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Abstract

Pityopsis graminifolia var. *latifolia* (Michx.) Small var. *latifolia* (Fern.) or narrowleaf silkgrass is an herbaceous perennial native to the southeastern United States and northern Central America. *Pityopsis ruthii* (Small) Small is an endangered plant endemic to the Hiwassee and Ocoee Rivers in Polk County, Tennessee. Little is known about the genetic diversity and population structure of both *Pityopsis* species as well as the phylogenic relationships between the species. In this study, 16 microsatellite loci were used to assess genetic diversity and population structure of 261 individuals of *P. ruthii* (n=167) and *P. graminifolia* var. *latifolia* (n=76). *Pityopsis ruthii* is characterized as a diploid whereas *P. graminifolia* var. *latifolia* is characterized as a tetraploid. Because of unknown ploidy of *P. graminifolia* var. *latifolia,* Polysat, a package in program R, was used to infer ploidy of the individuals. Arlequin and GenAlEx were utilized to calculate genetic diversity measurements. The genetic software STRUCTURE and BAPS used Bayesian cluster analyses to group individuals based on multilocus genotypes, and were used to evaluate the genetic structure and gene flow. Samples of *Pityopsis ruthii* and *P. graminifolia* var. *latifolia* were analyzed as one data set and separated by species to ensure accurate results. Analyses in GenAlEx for all data sets resulted in low Shannon's information index (I=0.13-0.14), and expected heterozygosity ranged from 0.08-0.09. Analysis of molecular variance indicated that the majority of the variation is within populations with moderate to higher genetic differentiation when *P. ruthii* and *P. graminifolia* var. *latifolia* are analyzed together or *P. ruthii* alone ([genetic differentiation] Φ_{PT} =0.13 and 0.18, respectively). Low levels of genetic differentiation (Φ_{PT} =0.02) were found when *P. graminifolia* var. *latifolia* was analyzed independently. STRUCTURE revealed three clusters with *P. ruthii* clustering by watershed and *P. graminifolia* var. *latifolia* resulting in one cluster. When analyzed separately, *P. graminifolia* var. *latifolia* indicated presence of three clusters with considerable admixture in each cluster. Analysis with BAPS provided similar results as found with STRUCTURE when a fixed K was

selected. Understanding the population structure and genetic diversity will aid in the creation of guidelines for conservation programs of *P. ruthii.*

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Chapter 1. Introduction

Pityopsis ruthii and *P. graminifolia* var. *latifolia* are members of Asteraceae (Compositae). The Asteraceae includes annual, biennial, or perennial species, and is commonly known for sunflowers and daisies. Species of the family are located on every continent except of Antarctica. The genus *Pityopsis* Nutt. is classified within the "goldenaster group", which contains common wildflowers and weeds in the mid- and subtropical latitudes of North America and northern Central America (Semple, 2013). Some common genera within the group include *Braduria*, *Chrysopsis*, *Heterotheca*, and *Pityopsis*. *Pityopsis* species are located in the Atlantic coastal plain, southeastern United States (U.S.), and northern Central America and are perennial, rhizomatous, with simple or branched stems, and moderate to dense appressed silky and shiny hairs; the inflorescence contains 15-60 yellow florets (Semple, 2006).

The genus *Pityopsis* has historically been treated as a section of *Chyrsopsis* (Gray, 1884), or a section of *Heterotheca* (Shinners, 1951). However, *Pityopsis* is now considered a separate genus because of unique morphological, anatomical, cytotaxonomical, and habitat differences (Semple, 1977; Semple *et al.,*1980). Distinct leaf anatomy and hair morphology of *Pityopsis* species create a unique and distinctly different appearance when compared to *Heterotheca* and *Chrysopsis* (Semple *et al.,*1980). Leaves of *Pityopsis* are grass-like and have a parallel venation without a substantial petiole, whereas *Heterotheca* and *Chrysopsis* are petiolate (Semple *et al.,*1980). The hairs on the leaves of *Pityopsis* are long, soft, and filamentous; in contrast, *Chyrsopsis* hairs are flagelliform and arranged in rows and *Heterotheca* grow long stiff hairs (Semple *et al.,*1980).

Pityopsis graminifolia is the most common species of the goldenasters and includes five variations. *Pityopsis graminifolia* (Michx.) Small var. *graminifolia* (2n=2x=18) naturally occurs from eastern Louisiana through northern Florida and as far north as southeastern North Carolina. The flower heads are subtended by bracts, and is the common variety of the species (Semple, 2006). *Pityopsis*

graminifolia (Michx.) Small var. *aeqilifolia* Bowers and Semple (2n=2x=18) occurs in central Florida, and the large oblong, mid- to upper- stem leaves, and its petite basal leaves help distinguish it from other varieties (Semple, 2006). *Pityopsis graminifolia* (Michx.) Small var. *tenuifolia* Semple and Bowers (2n=2x=18) occurs through the southeastern U.S. and blooms later than other variations (Semple, 2006). *Pityopisis graminifolia* (Michx.) Small var. *tracyi* (Small) Semple (2n=6x=54) occurs in the peninsula of Florida, and flowers later than other varieties (Semple, 2006). Among the five variations, *P. graminifolia* (Michx.) Small var. *latifolia* (Fern.) Semple is morphologically variable and is mostly tetraploid (2n=4x=36), but hexaploid (2n=6x=54) populations have been reported to occur in northern Alabama and eastern Tennessee (Semple, 2006). *Pityopsis graminifolia* var. *latifolia* has been known previously as *C. graminifolia* (Michx.) Ell and *H. graminifolia* (Michx.) Shinners. The common names for *P. graminifolia* var. *latifolia* are narrowleaf silk grass, grassleaf golden aster, and silk-grass. In addition to being a wildflower, *P. graminifolia* var. *latifolia* has been used in conservation seed mixes and for the purpose of controlling upland erosion (Gonter *et al.,*2007). It prefers dry soils with full sun and blooms in late summer to fall. The species can propagate either asexually via rhizomes or sexually by seed (Brewer, 1995). *Pityopsis graminifolia* var. *latifolia* occurs abundantly along the Hiwassee and Ocoee River systems. This species was used for this study because it is a sympatric with *P. ruthii* and it is the only variation to be placed in the clade with *P. ruthii* based on rDNA sequence similarity (Toeh, 2008). Sympatric species are closely related and grow in close proximity. *Pityopsis ruthii* is hypothesized to be the maternal ancestor of an allopolyploid *P. graminifolia* var. *latifolia*. (Toeh, 2008). Allopolyploid species arise through hybridization and mutations in chromosome numbers, whereas autopolyploid arises only from a mutation in chromosome number (Comai, 2005).

Pityopsis ruthii (Small) Small is an herbaceous plant and is endemic to Polk County, Tennessee (U.S. Forest Service, 2010) and was listed as a federally endangered species in the U.S. in 1985 (49 FR

45766 45769) . It is found growing on exposed phyllite or graywacke boulders along the banks or in the main channel of the Hiwassee and Ocoee Rivers in the Blue Ridge Mountains (U.S. Fish and Wildlife Service, 1990). *Pityopsis ruthii* is commonly known as Ruth's golden aster and prefers full sunlight. The number of plants in the population on the Ocoee River was assessed in 1977 (White, 1977) and 1980 (Wofford and Smith, 1980) and less than 500 individuals were counted. The number of plants in the Hiwassee River population was estimated to be 10,000 to 15,000 plants in 1986 (Collins and Gunn, 1986) and 1992 (U.S. Fish and Wildlife Service, 1992). The Tennessee Department of Environment and Conservation conducted a more detailed survey in 1999 and counted 8,235 plants along the Hiwassee River. However, this survey did not include all known subpopulations or locations of the plants at the time (Major *et al.,* 2000). A comprehensive survey of the Hiwassee River has been conducted annually since 2010. The Ocoee River populations have been counted since 1987 (with exception of 2005 and 2009) and the population consists of six discrete locations (TVA, 2001). The most recent surveys (2013) found a total of 11,968 plants on the Hiwassee River and 1,213 occurring along the Ocoee River (TVA, unpublished data).

Threats that affect *P. ruthii* populations include the following: succession/competition, road repair, competition from other plants, water quality decline, changes in water levels and flow regimes, and getting trampled during recreational activities along the river banks (US FWS, 2012). Conservation efforts have begun for *P. ruthii* and include culturing *P. ruthii in vitro* (tissue culture) using shoot organogenesis and plant regeneration (Wadl *et al.,*2011b), developing *in vitro* seed germination and initiating multiplication and stem cutting protocols for use in reintroduction and augmentation of populations (Wadl *et al.,*2013), conducting seed production, pollination, and germination studies (Clebsche and Sloan, 1993; Cruzan, 2001), and maintaining an *ex situ* collection at the University of

Tennessee, Knoxville, Tennessee, UT Arboretum, Oak Ridge, Tennessee, and USDA-ARS, Poplarville, Mississippi.

The total number of *P. ruthii* plants on both river systems is approximately 13,181 . The Ocoee and Hiwassee Rivers are separated by approximately 15 kilometers of mountainous terrain and the populations are effectively isolated from each other. If genetic diversity of an endangered plant is reduced or lost, the risk of those individuals becoming extinct can be elevated (Frankel, 1974; Frankham, 2005) as evidenced in the following studies. The endangered medicinal plant *Commiphora wightii* (Arn.) Bhandari comb. nov. has been reported to have low genetic diversity due to the geographic isolation and limited gene flow (Haque *et al.,*2010). *Sonchus gandogeri* Pitard is expected to become extinct due to inbreeding depression (Kim *et al.,*2005). *Abies ziyuanensis* L. K. Fu et S. L. Mo is thought to be decreasing in prevalence due to evolutionary forces, such as genetic drift, and historical events to the natural habitat (Tang *et al.,*2008). The Hiwassee and Ocoee River populations were predicted to have a high risk of short-term extinction (within 50 years) due to low and variable population growth rates along the Hiwassee whereas the Ocoee had higher growth rate (Thomson and Schwartz, 2006).

A population can be defined many ways, but for this thesis a population will be defined as a group of plants of the same species that are occupying a specific location at the given time (Roughgarden *et al.,*1989; Krebs, 1994; Waples and Gaggiotti, 2006). Therefore, we represent the *P. ruthii* populations as all the plants occurring on the Hiawassee and Ocoee River systems. The population of *P. ruthii* along the two river systems is not continuous; instead, subpopulations are staggered at various intervals. Gene flow between the two river populations is expected to be low (Cruzan and Estill, 2001) and is at an unknown level between the subpopulations along each river. Sloan found low gene diversity with 15.16% of the genetic diversity being attributed to within the subpopulations tested (Sloan, 1994). Currently, the knowledge regarding genetic diversity and population structure within

Pityopsis populations along the Hiawassee and Ocoee Rivers is limited. My objective was to assess population structure and genetic diversity at a small scale among *P. ruthii* and *P. graminifolia* var. *latifolia* populations.

Microsatellite loci, also known as simple sequence repeats (SSRs), are repetitive sequences of DNA that consist of di-, tri-, tetra-, and pentanucleotides. Microsatellite loci are codominant, polymorphic, and inherited in a Mendelian manner, and widely used for studies of kinship, population structure and classifications (Balloux, 2002; Wan *et al.,*2004). Although mutation rates are unknown, it is assumed that microsatellites have high mutation rates (approximately 10^{-3}) with long alleles containing more variation than shorter alleles (Jarne and Lagoda, 1996; Jin *et al.,*1996; Weber and Wong, 1996; Wierdl *et al.,*1997; Balloux and Lugon-Moulin, 2002). Microsatellite loci have been developed for *P. ruthii* that can be used to estimate population size, population variance, genetic diversity, and gene flow (Wadl *et al.,*2011a).

Pityopsis graminifolia var. *latifolia* occurs more frequently along the rivers than *P. ruthii*, as well as other areas of the southeastern U.S. *Pityopsis graminifolia* var. *latifolia* produces leaves 30-90 x 2-6 mm, the bracts surrounding the inflorescence are 8-12 mm, flowers are 10-16 mm (Semple, 2006). *Pityopsis ruthii* has short, thin leaves ranging from 30-40 ×3-4 mm in size, the bracts surrounding the inflorescence 6-8 mm, and flowers 9-14 mm. Both species reproduce by rhizomes or seed. *P. ruthii* is diploid (2n=2x=18) and *P. graminifolia* var. *latifolia* is mostly tetraploid species (2n=4x=36) with some hexaploid variation (Semple, 2006; Semple, 1977). The size of the seed heads, flowers and leaves in the greenhouse and wild habitat can be an indication of ploidy variations (Osburn *et al.,*2003; Levin, 1983). *P. ruthii* shows the signs that may indicate the ploidy of the plant (and its hybrid with *P. graminifolia* var. *latifolia*) is variable between diploid, tetraploid, and hexaploid (Wadl and Dattilo, personal

communication). The two species are sympatric (occurring together and flowering during the same interval) and appear to be able to hybridize. A natural interspecific hybrid has been tested with microsatellite loci and confirmed (Wadl and Trigiano, unpublished data). In an artificial setting, successful hybridization has occurred between *P. ruthii* and *P. graminifolia* var. *latifolia* producing two seeds (only one germinated, however it died a week after germination) (Bowers, 1972). Hybridization between the two species could lead to an odd-number ploidy and sterilization [triploid (2n=3x=27 or pentaploid 2n = 5x = 45)], which would remove genes from the gene pool because the hybrids will be unable to mate and therefore unable to inherit genes. This could ultimately decrease genetic diversity.

The genetic diversity, allelic richness, and gene flow between and among populations of both species are thought to be limited. Understanding the population dynamics and genetic interactions within and between species is important to assess the overall reproductive health and potential of *P. ruthii*. Therefore, this study was undertaken to (1) develop microsatellite markers for *P. graminifolia* var. *latifolia*; (2) determine the cross-transferability of microsatellite markers for *P. ruthii* and *P. graminifolia* var. *latifolia;* (3) analyze the genetic diversity and structure in three subpopulations in *P. ruthii* and *P. graminifolia* var. *latifolia;* and (4) determine the potential for a natural interspecific hybrid of *P. ruthii* and *P. graminifolia* var. *latifolia*.

Chapter 2. Materials and Methods

Section 1. Microsatellite marker development

An enriched microsatellite library of *P. graminifolia* var. *latifolia* was prepared according to the procedures outlined in Wang *et al.* (2007) and Wadl *et al.* (2011a). Genomic DNA was digested with AluI, HaeIII, StuI and RsaI (New England BioLabs, Beverly, Massachusetts, USA) and SNX linker adaptors (Hamilton *et al.,* 1999) were ligated to the digested DNA. Fragments were then amplified utilizing PCR with SNX linker primers. PCR fragments were hybridized to $(GT)_{12}$ biotinylated oligonucleotides and recovered using MagneSphere® streptavidin-coated paramagnetic particles (Promega, Madison, Wisconsin, USA). Biotinylated PCR fragments were PCR amplified with SNX linker primers and purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, California, USA). The purified PCR products were then ligated into EcoRV (New England Biolabs) digested pBluescript SK II (+)™ vector (Fermentas, Glen Burnie, Maryland, USA) and then transformed into electrocompetent *Escherichia coli* Top-10 cells (Invitrogen, Carlsbad, California, USA). Transformed cells were dispersed onto Luria-Bertani Broth (LB)-Amp_{100} agar (Sambrook *et al.,*1989) with 7µl Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 16µl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated at 37°C overnight. White colonies (presumably from a single cell that contained insert DNA) were transferred to LB Amp_{100} freezing medium. Cells from individual colonies were incubated at 37°C and grown overnight (~16 h). One µl of cells was used as a DNA template source to screen for possible microsatellites. The reaction volume was 10µl consisting of the following: 1X GeneAmp PCR Buffer (Applied Biosystems, Carlsbad, California, USA) , 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5µM T3 Primer, 0.5µM T7 Primer, 0.5µM $(GT)_{12}$ primer, 0.3U AmpliTaq Gold DNA Polymerase (Applied Biosystems), and sterile water. The amplification was completed using the following conditions: one cycle at 95°C for 3 min; 35 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and one cycle at 72°C for 1 min. The PCR products were separated by electrophoresis on a 2% agarose gel run at 100 V and visualized using 1% ethidium bromide. Electrophoresis products

exhibiting a “smear” were considered indicative of microsatellite inclusion in the vector (Wang *et al.,* 2007) and selected to be sequenced on ABI 3730XL (Applied Biosystems).

Sequences were checked for vector or SNX linker contamination using the program VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) to ensure only *P. graminifolia* var. *latifolia* sequences were used for primer design and assembled using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Microsatellites were discovered using imperfect SSR finder (Stieneke and Eujayl, 2007) with default parameters (minimum of 6 repeats for dinucleotides, 4 for trinucleotides, and 3 for pentanucleotides). Primers were designed using Primer3 (Koressaar and Remm, 2007; Untergrasser *et al.,*2012) and ranged from 22-25 base pair (bp) long with an annealing temperature of 60°C and a minimum expected product size of 75 bp. Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA).

Section 2. Marker Optimization

Single leaf samples of *P. graminifolia* var. *latifolia* were collected from 24 plants. These plants were propagated from seeds collected from two plants at site H-2. Samples were homogenized in Bio101 FastPrep Homogenization System FP120 (Thermo Savant, Waltham, Massachusetts, USA). DNA was extracted using a DNeasy Plant Mini Kit (Qiagen). The concentration and purity of DNA samples were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware, USA). PCR amplifications were completed in 10 µl reaction mixtures containing 1µl DNA template (4ng/µl genomic DNA), 2.5mM $MgCl_2$, 1X GeneAmp PCR Buffer II (Applied Biosystems), 0.2mM dNTPs, 0.25µM primer (forward and reverse), 5% dimethyl sulfide (DMSO; Fisher Scientific, Pittsburgh, PA, USA), 0.4U AmpliTaq Gold DNA Polymerase (Applied Biosystems), and sterile water. The PCR reaction was conducted using the following touchdown thermal cycler conditions: 1 cycle at 94°C for 3 min; 15 cycles at 94°C for 40sec, 63°C for 40sec 72°C for 30 sec and decreased by

0.5°C at each subsequent cycle; 15 cycles at 94°C for 40sec, 55°C for 40sec, 72°C for 30sec; 72°C 4min, 4°C for 15min, and held at 23°C until removed from the thermocycler (Don *et al.,*1991). Allelic products were separated via electrophoresis on the QIAxcel Capillary Electrophoresis System (QIAGEN) and sized using an internal 25-bp DNA size marker and electropherograms files for each PCR reaction were visualized using the software BioCalculator (QIAGEN version 3.2). Sequences for primers that yielded amplified products were deposited into National Center for Biotechnology Information GenBank (Table 1) (*all tables located in Appendix 1*) , six of the polymorphic markers that provided consistently strong amplifications were selected to be included in this study (Table 2).

Section 3. Microsatellite analyses

Leaf tissue of *P. ruthii* and *P. graminifolia* var. *latifolia* was collected from three locations along the Hiwassee (H-2 and H-1) and Ocoee (O-1) Rivers in Polk County, Tennessee (Table 3). Plants and seeds were collected under Tennessee Valley Authority Permit # TE117405-2 and U.S. Fish and Wildlife Service Permit # TE134817-1. The locations of *P. ruthii* are not revealed due to the endangered status. DNA was extracted and quantified as described previously. Each individual sample of genomic DNA was amplified with markers for *P. graminifolia* var. *latifolia* using protocols described under Marker Optimization and *P. ruthii* microsatellite loci. Ten *P. ruthii* microsatellite loci were amplified according to the parameters in Wadl *et al.* (2011a) (Table 4). The cycle conditions described in Wadl *et al.* (2011a) were as follows: 94°C for 3 min; 35 cycles at 94°C for 40 sec, 55°C for 40 sec, 72°C for 30 sec; and a final extension finishing at 72°C 4 min, 4°C for 15 min. Electrophoresis of the alleles was carried out on the QIAxcel Capillary Electrophoresis System (QIAGEN) as previously described. The electropherograms were visualized with the software BioCalculator (QIAGEN version 3.2). Although *P. ruthii* is considered a diploid (2n=2x=18) based on limited cytological observations (Semple, 1977), potential ploidy variation has been observed in microsatellite analyses of individuals (Wadl, unpublished data). Due to ploidy

uncertainty in *P. ruthii,* two different data sets were developed and used for all subsequent data analyses. One data set coded *P. ruthii* as diploid, triploid or tetraploid, based on the observed electropherogram file. The second data set was created to show *P. ruthii* designated as diploid, with allelic estimation based on two highest electropherogram peaks for amplification having more than two peaks (Fehlberg, 2012; Esselink *et a*l., 2004) and *P. graminifolia* var. *latifolia* was analyzed as a tetraploid (2n=4x=36). Therefore, four data sets were used for analyses of population structure:

1. Combined *P. ruthii* (2X, 3X, or 4X), *P. graminifolia* var. *latifolia* (4X), and a Test population
2. Combined *P. ruthii* (2X), *P. graminifolia* var. *latifolia* (4X), and Test population
3. *P. ruthii* (2X) only
4. *P. graminifolia* var. *latifolia* (4X) only

The test population contained the following individuals: one *P. ruthii* (2X), one interspecific hybrid [*P. ruthii* (2X) × *P. graminifolia* var. *latifolia* (4X)], three *P. graminifolia* var. *latifolia* (4X) seedlings from H-2 location (Hiwassee), one *P. ruthii* (2X) from Br-1 location (Hiwassee), one *P. graminifolia* var. *latifolia* (4X) from O-1 location (Ocoee), and one *P. falcata* (2X) from Rhode Island. The *P. falcata*, *P. ruthii* from Br-1, and *P. graminifolia* var*. latifolia* in the test population's ploidy was confirmed using cytology (Wadl and Trigiano, unpublished data). This test population is an artificial population of unknown locations and to monitor the microsatellite loci for spurious bands. The data was exported from the Biocalculator software to Excel (Microsoft Excel, 2007) and formatted accordingly based on the software used for data analyses. In polyploid organisms, using microsatellite loci can be problematic due to inability to determine exact allele copy numbers in partially heterozygous genotypes as well as complex inheritance patterns of individuals in mixed ploidy populations (Bruvo *et al,.* 2004; De Silva *et al.,* 2005; Clark and Jasieniuk, 2011). Additionally, determination and scoring of auto- and/or

allopolyploidy of an organism can confound data analyses and underestimate distribution of genotypes in a population. With above-mentioned obstacles in analyzing polyploidy data, interpreting results can be significantly impacted. In the case of endangered species such as *P. ruthii*, consequences of underestimating diversity parameters can be detrimental and conservation efforts could be affected.

To resolve the issue of analyzing polyploid data, microsatellite loci can be coded as binary data (presence/absence) (Andreakis *et al.,*2009; Fehlberg and Ferguson 2012; Sampson and Byrne, 2012). For binary data, the alleles at each microsatellite locus are treated as multiple independent dominant loci (losing the co-dominant benefit of microsatellites), and each allele (or locus) is recorded as present or absent (Rodzen *et al.,*2004; Fehlberg and Ferguson 2012). In analyses, data was scored as followed:

1. Binary Data Analyses:
    a. Combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X)
    b. Combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X)
    c. *P. ruthii* (2X) only
    d. *P. graminifolia* var. *latifolia* (4X) only
2. Allelic Data Analyses:
    a. Combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X)
    b. Combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X)
    c. *P. ruthii* (2X) only
    d. *P. graminifolia* var. *latifolia* (4X) only

Section 4. Data Analysis

Allelic data was binned using FlexiBin (Amos *et al.,*2007). The program converts raw allele lengths into allelic classes by using a simple algorithm coded in Visual Basic as an Excel Macro. Upon finding the best-fit values, raw allelic data are replaced with their repeat unit equivalents and a graphical

output file. The program also incorporates and generates additional summary statistics. Using described algorithm, the errors associated with automated allele-calling were minimized. In this study, binned data (allelic classes) were used for analyses.

To infer the ploidy of *P. ruthii* and *P. graminifolia* var. *latifolia*, POLYSAT, an R package for polyploid microsatellite analysis was used (Clark and Jasieniuk, 2011). POLYSAT was also used to export genotype data into STRUCTURE format (Pritchard and Donnelly 2000; Hubisz *et al.*, 2009). STRUCTURE version 2.3.4 was also used to infer population structure of *Pityopsis* subpopulations using Bayesian cluster analysis data. STRUCTURE groups individuals based on their multilocus genotypes without assumptions about the relationship between sample sites and population structure. Ten independent runs (burn-in period of 100,000 steps and 100,000 Markov chain Monte Carlo) iterations were performed at each value of K from 1 to 8. The program was run using an admixture model with correlated allele frequencies and assuming no prior information of population origin. Parameters of Evanno's method (Evanno *et al.,* 2005) were calculated using the program Structure Harvester version 6.93 (Earl and von Holdt, 2012).

BAPS version 6.0 (Corander *et al.,*2003; Corander *et al.,*2004; Corander *et al.,*2006; Corander *et al.,*2007; Corander *et al.,*2008) was used to complement STRUCTURE since it pregroups data based on clustering of populations instead of individuals. In addition, BAPS can handle combined data of individuals with varying ploidy levels, whereas STRUCTURE is limited to one designated ploidy (Schmickl and Koch, 2011). The relationship among identified clusters was computed and visualized using unweighted pair group method with arithmetic mean (UPGMA) clustering analysis and Nei's standard genetic distance (D's) matrix (Nei 1972). Gene flow networks were used to identify the ancestral admixture of all *Pityopsis* subpopulations with α=0.05 for all analyses.

Genetic diversity statistics were calculated for all binary data sets [combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X); combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X); *P. ruthii* (2X) only; *P. graminifolia* var. *latifolia* (4X) only] using GenAlEx 6.5 (Peakall and Smouse, 2012). Data included sample size, total number of alleles (N_a), range of alleles per locus and population, Shannon's information index (I), expected (H_e) and unbiased expected heterozygosity (uH_e), genetic differentiation (Φ_{PT}) (significance based on 9999 permutations) for all populations across all tested loci. Principal coordinate analysis (PCoA) was performed to visualize clustering of the relationships among *Pityopsis* populations using a covariance matrix with data standardization. Finally, a Mantel test using 9,999 permutations was calculated to evaluate correlation between genetic and geographic distance following Smouse et al. (1986).

GenAlEx 6.5 was also used for analyses of molecular variance (AMOVA) calculation across all binary data sets (analyses obtained using 9,999 permutations), with a 0.05 *p*-value utilized to determine significance. However, Arlequin version 3.5.1.2 (Excoffier and Lischer 2010) was used to compute hierarchical AMOVA in diploid *P. ruthii* (2N) subpopulations (analysis obtained using 99,999 permutations). Data was analyzed by combining all *P. ruthii* subpopulations (H-2, H-1 and O-1) into one hierarchical group as well as partitioning subpopulations into two groups, based on STRUCTURE analysis.

Chapter 3. Results and Discussion

Sixteen microsatellite loci were used in this study for each data set (Table 2 and Table 4). When the combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* binary data set was analyzed, 115 different alleles were detected, and the number of alleles per locus ranged from 0 to 20 (Table 3). The P*. ruthii* (2X, 3X, or 4X) population was separated into three subpopulations [H-2 (Hiwassee), H-1 (Hiwassee), and O-1 (Ocoee)]. For this group (n=167) 47 alleles were detected and the number of alleles per locus ranged from 2 to 16 (Table 5). Tetraploid population of *P. graminifolia* var. *latifolia* was also separated into three subpopulations [O-1 (Ocoee), H-2 (Hiwassee), and H-1 (Hiwassee)]. For *P. graminifolia* var. *latifolia* group (n=86) 56 alleles were detected and ranged from 0 to20 alleles per locus (Table 5). For the test population (n=8), 12 alleles ranging from 3 to 12 alleles per locus were observed (Table 5). The overall Shannon's information index was 0.14. Mean expected heterozygosity was 0.081, whereas the unbiased expected heterozygosity was 0.082.

The combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X) binary data set revealed that in 261 individuals, 109 alleles were detected and the number of alleles per locus ranged from 0 to 20 (Table 6). Diploid *P. ruthii* population was separated into three subpopulations [H-2 (Hiwassee), H-1 (Hiwassee), and O-1 (Ocoee)]. For this group (n=167), 41 alleles ranging from 2 to 15 alleles per locus were detected (Table 6). Tetraploid *P. graminifolia* var. *latifolia* population was also separated into three subpopulations [O-1 (Ocoee), H-2 (Hiwassee), and H-1 (Hiwassee)]. For *P. graminifolia* var. *latifolia* group (n=86) 56 alleles ranging from 0 to 20 alleles per locus were observed (Table 6). For the test population (n=8), 12 alleles ranging from 3 to12 alleles per locus were detected (Table 6). The overall Shannon's information index was 0.14. Mean expected heterozygosity was 0.079, whereas unbiased expected heterozygosity was 0.081.

When the binary *P. ruthii* (2X) data set was analyzed independently of *P. graminifolia* var. *latifolia*, 167 individuals amplified, 47 alleles that ranged from 2 to 16 per locus (Table 7). Diploid *P. ruthii* was separated into three subpopulations [H-2 (Hiwassee), H-1 (Hiwassee), and O-1 (Ocoee)]. The overall Shannon's information index was 0.13. Mean expected was 0.078 and unbiased expected heterozygosity was 0.079. When the *P. graminifolia var. latifolia* (4X) binary data set (n=86) was analyzed independently of *P. ruthii* 65 alleles were detected and ranged from 0 to 20 alleles per locus (Table 8). Tetraploid *P. graminifolia* var. *latifolia* was also separated into three subpopulations [O-1 (Ocoee), H-2 (Hiwassee), and H-1 (Hiwassee)]. The overall Shannon's information index was 0.14. Mean expected heterozygosity was 0.084, whereas unbiased heterozygosity was 0.086.

Low Shannon's information indices values for all data sets analyzed indicate that there is a low genetic diversity within the data sets. Some microsatellites did not amplify across all examined loci and across populations. A single *P. ruthii* microsatellite locus did not amplify in the *P. graminifolia* var. *latifolia,* which could indicate that either the locus is not being present in *P. graminifolia* var. *latifolia* or a possible sequence change in the primer region did not permit amplification (Chapuis and Estoup, 2007). The low expected and unbiased expected heterozygosity also indicated that the populations have very low genetic diversity. Polyploid species are expected to have slightly higher levels of heterozygosity and diversity than diploid plants (Sampson and Byrne, 2011). Low genetic diversity can contribute to population decline (Frankham, 2005). Populations can respond more readily to environmental changes, such as new disease or insect infestations, when a greater level of genetic variation exists. Therefore, *P. ruthii* and *P. graminifolia* var. *latifolia* may not be expected to respond well to changes in the environment as is the case for *Antirrhinum Microphyllum* (Huenneke, 1991; Torres *et al.,*2003). Inbreeding and genetic drift could be occurring due to the small size of the populations. Inbreeding and genetic drift are two consequences of small population size and lack of

genetic diversity in *Cathaya argyrophylla,* endangered conifers of China (Barrett and Kohn, 1991; Ellstrand and Elam 1993; Ge *et al.,* 1998). Inbreeding and genetic drift could affect the populations of *P. ruthii* negatively by decreasing the different alleles currently in the populations. It is thought because of previously F_{IS} values that P. ruthii has avoided inbreeding by reproducing vegetatively (Sloan, 1994).

STRUCTURE and BAPS analyses indicated different genetic structure of populations of *P. ruthii* and *P. graminifolia* var. *latifolia*. Using STRUCTURE harvester, the allelic combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* indicated a clear maximum of three different clusters (Δk at k = 3) (Figure 1A). Multiple runs of ten independent k runs used in all subsequent analyses guaranteed consistent individual assignment probabilities. Three clusters included *P. ruthii* (H-2 and H-1 populations), *P. ruthii* O-1 population, and *P. graminifolia* var. *latifolia* as the third cluster (O-1, H-2 and H-1, and Test populations) (Figure 1A). All individuals from Test population grouped with the *P. graminifolia* var. *latifolia* cluster with the exception of two individuals, *P. ruthii* from O-1 and *P. ruthii* from Br-1. *Pityopsis falcata* indicated the highest probability of clustering with *P. graminifolia* var. *latifolia*. The possible interspecific hybrid of *P. ruthii* x *P. graminifolia* var. *latifolia* probabilities of clustering with both *P. ruthii* and *P. graminifolia* var. *latifolia* possibly indicating a hybrid of the species.

Contrary to STRUCTURE, BAPS indicated the presence of six clusters when the "clustering of groups of individuals" option was selected regardless of the ploidy of *P. ruthii*. With that setting, all examined populations clustered independently of species or location except *P. graminifolia* var. *latifolia* at H-2 and H-1 (both Hiwassee), which clustered together (Figure 2A). When "fixed K option" was selected at 3 in BAPS, clusters were identical to STRUCTURE findings for all data sets. The three clusters identified by STRUCTURE did not correspond to the PCoA scatter plot (Figure 3A).

When allelic data was designated as combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X), STRUCTURE harvester indicated a maximum of two different clusters (Δk at k=2) (Figure 1B). Two groups

were clustered based on species designation (*P. ruthii* and *P. graminifolia* var. *latifolia* cluster). The test population grouped with the *P. graminifolia* var. *latifolia* cluster with the exception of two individuals, *P. ruthii* from O-1 and *P. ruthii* from Br-1. *Pityopsis falcata* indicated the highest probability of clustering with *P. graminifolia* var. *latifolia* cluster. The possible interspecific hybrid of *P. ruthii* x *P. graminifolia* var. *latifolia* clustered with both *P. ruthii* and *P. graminifolia* var. *latifolia* possibly confirming a hybrid of the species. Similarly to previous BAPS results, ploidy level of *P. ruthii* did not change the number of identified clusters. BAPS indicated six clusters with each subpopulation creating a cluster, with the exception of *P. graminifolia* var. *latifolia* at H-2 and H-1 (both Hiwassee River populations) clustering together (Figure 2B). The two clusters identified by STRUCTURE corresponded well to the PCoA scatter plot (Figure 3B).

The allelic *P. ruthii* (2X) data set indicated a clear maximum of two different clusters (Δk at k = 2) (Figure 1C). The two clusters were separated by geographic location of rivers, with H-2 and H-1 as one cluster and O-1 as the second location (Hiwassee and Ocoee Rivers, respectively) (Figure 1C). BAPS analysis indicated three clusters with each subpopulation creating an individual cluster (Figure 2C). The two clusters identified by STRUCTURE were the same when analyzed as a PCoA scatter plot (Figure 3C).

The allelic *P. graminifolia* var. *latifolia* (4X) indicated a two different clusters (Δk at k=2) (Figure 1D). The subpopulations resulted in a mix of clusters at each geographic location (Figure 1D). Ten independent runs of the same k were utilized in all analyses to guarantee dependable individual assignment probabilities. BAPS resulted in three clusters with each subpopulation creating a cluster [H-2, H-1 (both Hiwassee) and O-1 (Ocoee)] (Figure 2D). The clusters identified by STRUCTURE were clearly indicated when analyzed as a PCoA scatter plot with each geographic location containing a mixture of each subpopulation (Figure 3D).

The results of STRUCTURE and BAPS analyses are different. However, STRUCTURE for the combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X) creates the most supported and biologically relevant conclusion. This data was supported by BAPS when "fixed K option" was selected (data not presented). The terrain and geographic distance between the Hiwassee and Ocoee Rivers is unlikely to allow for gene flow via pollen. Within the watersheds the gene flow would be increased due to the seed floating downstream and the distance between the two locations along the Hiwassee. BAPS was chosen due to its ability to analyze data sets of a mixed ploidy, however the results were not conclusive with STRUCTURE when "clustering of groups of individuals options" was selected. BAPS is thought to overestimate the number of clusters (Latch *et al.,* 2006; Rowe and Beebee, 2007; Rodriguez-Ramilo *et al.,* 2009; Frantz *et al.,* 2009). STRUCTURE only allows one ploidy, whereas BAPS will allow a mixture of ploidies. When analyzing polyploidy with codominant markers, STRUCTURE makes the assumption that there is no ambiguity (Pritchard and Donnelly 2000). Both programs assume Hardy-Weinberg equilibrium within clusters (Exocoffier and Heckel, 2006), and are not coded for allopolyploids. Because the data does not fit within the constraints of either of Baysian analysis programs used, the results could be skewed. When species were separated based on ploidy, both PCoA scatter plots resulted in clear maximum of two groups. Furthermore, *P. ruthii*'s clusters are consistent with the two geographic regions (the Ocoee and the Hiwassee Rivers). *Pityopsis graminifolia* var. *latifolia* is thought to be an allopolyploid species. *Pityopsis adenolepsis* and *P. ruthii* are hypothesized as potential maternal ancestors of *P. graminifolia* var. *latifolia* (Teoh, 2008), which can explain complexity of grouping these individuals and estimating their diversity levels. *Pityopsis graminifolia* var. *latifolia* could appear to have each population's genetic identity because it is widespread throughout the area and it could be one large continuous population.

The interspecific hybrid of *P. ruthii* × *P. graminifolia* var. *latifolia* from the Test population contained one-half the genetic makeup of both parents. Hybridization can affect the gene pool by reducing the effecting population size or could lead to genetic assimilation of a species (Hardig *et al.,* 2005; Ellstrand and Elam, 1993). Considering that *P. graminifolia* var. *latifolia* is more abundant than *P. ruthii,* this could be problematic (Ellstrand, 1991; Allendorf *et al.,* 2001). Hybridization can reduce a population by affecting its reproductive effectiveness, competitive status, and alter it interactions with pathogens and herbivores (Levin *et al.,* 1996). An interspecific hybrid of *P. ruthii* x *P. graminifolia* var. *latifolia* has the potential to produce an odd ploidy, which will result in a sterile plant. This process will remove genes from the gene pool. Because of the abundance of *P. graminifolia* var. *latifolia*, the removal of genes will not drastically affect the gene diversity, however because *P. ruthii*'s population is drastically small, a loss of genetic material will affect the gene diversity. The lower gene diversity can ultimately weaken the *P. ruthii* populations.

AMOVA analyses were completed with binary data using GenAlEx 6.5 for all data sets. Additionally, allelic *P. ruthii* (2X) was analyzed using Arlequin version 3.5.1.2 (Excoffier and Lischer 2010). Arlequin was utilized for *P. ruthii* only due to its inability to analyze polyploid data (Excoffier and Heckel, 2006). Binary combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X) and binary combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X) indicated that 87% of the genetic variation is within populations rather than among the two species (13%), (p<0.001) (Table 9). When the data sets were divided by species, binary *P. ruthii* (2X) and binary *P. graminifolia* var. *latifolia* resulted with *P. ruthii* contain 18% of variation among the populations and 82% of variation within populations(p<0.001). Binary *Pityopsis graminifolia* var. *latifolia* (4X) contained only 2% variation among the populations and 98% within populations (p<0.003) (Table 9). When *P. ruthii* (2X) was analyzed using Arlequin, the first analysis included the three subpopulations as one hierarchical group and the second AMOVA analysis

partitioned subpopulations into two different clusters recognized by STRUCTURE. Most of the genetic variation of *P. ruthii* is primarily within individuals (59%, $p<0.001$), followed by among individuals within population (26%, $p<0.001$) when partitioned in two groups as resulted in STRUCTURE (Table 10).

When binary combined *P. ruthii* (2X, 3X, and 4X) and *P. graminifolia* var. *latifolia* (4X) and binary combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X) are analyzed, the Φ_{PT} (analogous to standardized F_{ST} for binary data) are fairly high indicating moderate genetic differentiation among those subpopulations (Table 9)(Wright, 1943; Wright 1951). When *P. ruthii* was analyzed without *P. graminifolia* var. *latifolia* it resulted in high Φ_{PT} values among three subpopulations $\Phi_{PT}=0.18$ (Table 7). Due to self-incompatibility, *Wyethia bolanderi*, a species in the Asteraceae, was hypothesized to have a higher F_{ST} due to biparental inbreeding (Ayres and Ryan, 1999). When *P. graminifolia* var. *latifolia* (4X) was analyzed without *P. ruthii* Φ_{PT} resulted in little differentiation ($\Phi_{PT}=0.025$), which further supports the STRUCTURE and PCoA results showing each location with a mixture of genetic information from each population.

Gene network analyses revealed very little gene flow between the populations (Figure 4). All populations contain 90% or more of their own genetic identity. Limited gene flow in *Abronia macrocarpa*, an endangered species, was suspected to be due to low viability of seeds and pollen (Williamson and Werth, 1999). The small amounts of genetic drift that are occurring between populations could be due to different reasons, including, but not limited to, water flow, seeds being dispersed by wind, animals, or pollinators. The dominant influence on genetic distribution within and between plant populations is the mating system (Duminil *et al.,* 2009; Sampson and Byrne, 2011). *Pityopsis ruthii* and *P. graminifolia* var. *latifolia* propagate sexually (by seed) and asexually by rhizomes. This could influence the genetic distribution to be contained within populations and decrease gene flow.

The Isolation by Distance (IBD) test revealed a positive relationship between genetic and geographic distance for binary *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (r=0.072, p<0.023) (Figure 5A). The Isolation by Distance (IBD) test also revealed a positive relationship between genetic and geographic distance and showed a positive relationship for binary *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (r=0.077, p<0.023) (Figure 5B). Both species are sympatric and this could affect the isolation by distance measurements when analyzed together. When species were analyzed separately each resulted in a higher positive relationship between genetic and geographic distance. *Pityopsis ruthii* (2X) resulted in r=0.486, p<0.001, and *P. graminifolia* var. *latifolia* (4X) resulted in r=0.486, P<0.001 (Figure 5 C and D). When IBD measurements are highly significant the analysis can indicate the genetic exchange is limited within populations (Haque *et al.,* 2010).

CONCLUSION

When analyses of *P. ruthii* and *P. graminifolia* var. *latifolia* are combined, moderate genetic diversity is exhibited among subpopulations, which is the case for *P. ruthii* populations as well. However, when *P. graminifolia* var. *latifolia* is analyzed separately, overall genetic diversity level is low. The AMOVA and IBD both support the hypothesis that gene flow is limited between the populations. When analyzed by species, the genetic structure for *P. ruthii* separates by river, whereas *P. graminifolia* var. *latifolia* has little apparent genetic structure between or within populations. Understanding these population structures can help to provide guidelines for conservation strategies and management (Balloux and Lugon-Moulin, 2001; Rossiter *et al.,*2000; Eizirik *et al.,*2001) of *P. ruthii.* Due to the high genetic differentiation between clusters of *P. ruthii,* plants from each cluster should be maintained in *ex situ* conservation efforts. The low genetic diversity could be increased by transplanting plants into other populations. However, studies should be completed to determine if movement of genetic material

between populations would have any negative effects on the population (Cruzan and Estill, 1998). A large-scale study incorporating the remaining known populations of *P. ruthii* on the Hiwassee and Ocoee Rivers should be completed to gain a full understanding of the population dynamics within the endangered species.

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Appendices

Appendix 1. Tables

Table 1. Characteristics of seven microsatellite loci developed for *Pityopsis graminifolia* var. *latifolia*. Shown for each locus are the forward and reverse primer sequence, repeat motif, annealing temperature and allelic class size range.

Locus	Primer sequence (5'-3')	Repeat motif	Expected size (bp)	T_a (°C)	GenBank Accession No.
PG002	F: ATTCATTCATCCTTTCCACACC	TAA (4)	157	58-60	KF817599
	R: AACTTCAATGTGAGATCTTCTTGG				
PG010	F: CAATCTTCACATACCCACATCC	GT(19)	187	59	KF817600
	R: CACACCATCTTTCCAAACTCC				
PG011	F: CCTTTCCTTTCCATTCATTCC	GT(8)GT(9)GT(7)	243	59-60	KF817601
	R: AAGCCCATAAGCTTCATCTTCC				
PG012	F: CTGAGAACCCCTAGTGTCACG	TA(5)TG(8)	235	59	KF817602
	R: GTAAAAGGGTTGAATGGCTACG				
PG017	F: TACCAAATTAGAGATGGCTTGG	AT(6)	132	58-60	KF817603
	R:AAGTATATGAAAAATAGAAAAATGACC				
PG019	F: TTTACCATGTGGCATGAGTAGC	TG(8)	225	60-61	KF817604
	R: GTCAAGGCTAAGGGGAGTGG				
PG020	R: AATCACAAACAAGCAATTAACG	AC(15)	186	57-60	KF817605
	F: GACACTGCATGTTAGTATCATTTAAGC				
PG022	F: GCAAATCCCAATACCATAATGC	AC(8)	204	60	KF817606
	R: TTCTGGTGAGGTAGCAGAAAGC				
PG023	F: GGGGTATTTTCGTCCTTTGC	CA(20)	230	60	KF817607
	R: GGGTTTGAATGAGGTAATGTGC				
PG024	F: ATGAATCGATGGTCTTGAGTCG	TG(10)	158	60-61	KF817608
	R: CCATCGAATGTACTCATGTTCG				
PG025	F: GGGTTTGAATGAGGTAATGTGC	GT(6)	173	60	KF817609
	R: TTTGCTCCTGTACTCCATACCC				
PG026	F: AATTTGCAATCACACGTACTGG	AC(9)	209	60	KF817610
	R: TTGTTCCGTTGTTGTTAGATGC				
PG029	F: GGTCTTTGGACGTTTTTAACTACC	AC(13)	195	59	KF817611
	R: ATTTTCCCGAATTTTGTATTGG				
PG034	F: TGGTATGAAATTGATTATGAGAATGG	GT(6)	217	60	KF817612
	R: ACTTACCTCAGCCACGATATGC				
PG035	F: TGTAAATGTGTGTTAATGGAGTCG	GT(5)	190	57-59	KF817613
	R: TTTTGTAATGTTCTAAGTGTAGAAAGG				
PG036	F: GCACACACCTCCACAAAGC	ATGA(4)	204	59-60	KF817614
	R: TGACTTCCCTTTGTATTTTCACC				
PG037	F: TGGTGGGTGTGTGTTTGC	TG(7)	233	60-61	KF817615
	R: TCTACCAATTTTTGGCACAACC				

Table 2. Characteristics of six microsatellite loci developed in *Pityopsis graminifolia* var. *latifolia*. Shown for each locus are the forward and reverse primer sequence, repeat motif, and allelic class size range.

Locus	Primer Sequence (5'-3')	Repeat motif	Expected size (bp)	T_a (°C)	GenBank Accession No.
PG002	F: ATTCATTCATCCTTTCCACACC R: AACTTCAATGTGAGATCTTCTTGG	$(CA)_{16}$	157	58-60	KF817599
PG012	F: CTGAGAACCCCTAGTGTCACG R: GTAAAAGGGTTGAATGGCTACG	$(TA)_5(TG)_8$	235	59	KF817602
PG017	F: TACCAAATTAGAGATGGCTTGG R: AAGTATATGAAAAATAGAAAAATGACC	$(AT)_6$	132	58-60	KF817603
PG020	R: AATCACAAACAAGCAATTAACG F: GACACTGCATGTTAGTATCATTTAAGC	$(AC)_{15}$	186	56-59	KF817605
PG023	F: GGGGTATTTTCGTCCTTTGC R: GGGTTTGAATGAGGTAATGTGC	$(CA)_{20}$	230	60	KF817607
PG025	F: GGGTTTGAATGAGGTAATGTGC R: TTTGCTCCTGTACTCCATACCC	$(GT)_6$	173	60	KF817609

Table 3. Sample locations for *Pityopsis ruthii* and *P. graminifolia* var. *latifolia*.

Sample Location	Species	Number of samples
H-2	*P. ruthii*	50
H-2	*P. graminifolia*	30
H-1	*P. ruthii*	50
H-1	*P. graminifolia*	30
O-1	*P. ruthii*	67
O-1	*P. graminifolia*	26
Varies*	*P. ruthii, P. graminifolia,* P. falcata*	8
Total		**261**

*Individuals in test population have designated ploidy levels as follows: *P. ruthii* (2X) (female), Interspecific hybrid [*P. ruthii* (2X) x *P. graminifolia* var. *latifolia* (4X)], *P. graminifolia* var. *latifolia* (4X) seedlings from H-2 location (Hiwassee), *P. ruthii* (2X) from Br-1 (Hiwassee), *P. graminifolia* var. *latifolia* (4X) from O-1 location (Ocoee) and *P. falcata* (2X) from Rhode Island.

Table 4. Characteristics of ten microsatellite loci developed in *Pityopsis ruthii* (Wadl *et al.,* 2011). Shown for each locus are the forward and reverse primer sequence, repeat motif, and allelic class size range, annealing temperature (T_a), and the GenBank accession number.

Locus	Primer Sequence (5'-3')	Repeat motif	Expected Size (bp)	T_a (°C)	GenBank Accession No.
PR002	F: TTTTCTGGACAGCTTTTGG R: CACATAGCTCAAAAGCACAACC	$(TG)_9$	151-161	55	JF261124
PR003	F: TGAGAGRGCTTGGTTCATGC R: TCCAAATTTCCAATCCAACC	$(TG)_{14}$	142-155	55	JF261125
PR005	F: TTTTAGGGAAATGTGTAATTTAGG R: TGTGTGAGTGTGTGTTTGAGC	$(CT)_7$	207-228	55	JF261126
PR006	F: GGTTGATAGGTATAGCATTGATTCG R: TGTTAATTTTGATGGATTCTTGC	$CA)_{16}$	200-215	55	JF261127
Pr020	F: GAGGCCACTTGGGAGAGG R: TGTGAGTCGCCTTCTTTTCC	$(GT)_8$	162-173	55	JF261129
Pr027	F: ATGGAAGATGCCGGTGTTAG R: CGGACTCACATCAAACACTAGAAC	$(GTGTC)_5$	158-176	55	JF261130
Pr028	F: CGTTTGCTGCACGAGGTAT R: TCCTCCAAAGCTTCTCTCCA	$(GT)_{10}$	230-237	55	JF261131
Pr029	F: TTGAAAAGAATGTTTCACCACCT R: TTGAAAAGAATGTTTCACCACCT	$(GT)_3 A(GT)_8$	225-249	55	JF261132
Pr030	F: CCATCTAAACCAATGAAATGAAA R: TCCCAGTAATCCAGCCATTC	$(AC)_{12}$	184-196	55	JF261133
Pr031	F: TGCCTTTGGGCGAATTAGTA R: TCAGCTGACATTGGTTCACA	$(GT)_9 AA(GT)_5$	207-218	55	JF261134

Table 4: Continued.

Locus	Primer Sequence (5'-3')	Repeat motif	Expected Size (bp)	T_a (°C)	GenBank Accession No.
Pr035	F: TGATGGTGTCCGTAAAGTTG R: CAATTAATTTGTAGCATAATACCTCTG	$(GT)_5 A(TG)_7 (AG)_{15}$	104-129	55	JF261135

Table 5. Descriptive statistics for each population of *Pityopsis ruthii* and *P. graminifolia* var. *latifolia* were obtained using GenAlEx 6.5. Genetic diversity measurements were calculated using binary data set where microsatellite alleles were coded as present or absent [combined *P. ruthii* (2X, 3X or 4X) and *P. graminifolia* var. *latifolia* (4X)].

Species	Subpopulation	Ploidy level	Sample size	Total N_a	Range N_a/locus	Shannon's information index	H_e	uH_e
Pityopsis ruthii								
	H-1	2, 3, or 4X	50	15	3-15	0.14	0.080	0.081
	H-2	2, 3, or 4X	50	16	4-16	0.15	0.086	0.087
	O-1	2, 3, or 4X	67	16	2-16	0.12	0.069	0.070
Pityopsis graminifolia var. *latifolia*								
	O-1	4X	30	20	0-20	0.15	0.086	0.087
	H-2	4X	30	17	0-17	0.14	0.083	0.085
	H-1	4X	26	19	0-19	0.12	0.084	0.086
P. ruthii and *P. graminifolia**								
	Test Population	2, 3, or 4X	8	12	3-12	0.13	0.076	0.081
Total			**261**	**115**	**0-20**	**0.14**	**0.081**	**0.082**

*N_a =number of different alleles; H_e= expected heterozygosity; uHe = unbiased expected heterozygosity. Individuals in test population have designated ploidy levels as follows: *P. ruthii* (2X) (female), Interspecific hybrid [*P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X)], *P. graminifolia* var. *latifolia* (4X) seedlings from H-2 location (Hiwassee), *P. ruthii* (2X) from Br-1 location (Hiwassee), *P. graminifolia* var. *latifolia* (4X) from O-1 location (Ocoee) and *P. falcata* (2X) from Rhode Island.

Table 6. Descriptive statistics for each population of *Pityopsis ruthii* and *P. graminifolia* var. *latifolia* were obtained using GenAlEx 6.5. Genetic diversity measurements were calculated using binary data set where microsatellite alleles were coded as present or absent [combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia*(4X)].

Species	Subpopulation	Ploidy level	Sample size	Total N_a	Range N_a/locus	Shannon's information index	H_e	uH_e
Pityopsis ruthii								
	H-1	2X	50	12	3-12	0.12	0.069	0.070
	H-2	2X	50	15	4-15	0.13	0.075	0.076
	O-1	2X	67	14	2-14	0.10	0.060	0.060
Pityopsis graminifolia var. *latifolia*								
	O-1	4X	30	20	0-20	0.15	0.090	0.091
	H-2	4X	30	17	0-17	0.15	0.088	0.090
	H-1	4X	26	19	0-19	0.15	0.089	0.091
P. ruthii and *P. graminifolia**								
	Test Population	2, 3, or 4X	8	12	3-12	0.14	0.080	0.085
Total			**261**	**109**	**0-20**	**0.14**	**0.079**	**0.081**

*Na =number of different alleles; He= expected heterozygosity; uHe = unbiased expected heterozygosity. Individuals in test population have designated ploidy levels as follows: *P. ruthii* (2X) (female), Interspecific hybrid [P*. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X)], *P. graminifolia* var. *latifolia* (4X) seedlings from H-2 location (Hiwassee), *P. ruthii* (2X) from Br-1 location (Hiwassee), *P. graminifolia* var. *latifolia* (4X) from O-1 location (Ocoee) and *P. falcata* (2X) from Rhode Island.

Table 7. Descriptive statistics for each population of *Pityopsis ruthii* were obtained using GenAlEx 6.5. Genetic diversity measurements were calculated using binary data set where microsatellite alleles were coded as present or absent [*P. ruthii* (2X)].

Species	Subpopulation	Ploidy level	Sample size	Total N_a	Range N_a/locus	Shannon's information index	H_e	uH_e
Pityopsis ruthii								
	H-1	2X	50	15	3-15	0.14	0.080	0.081
	H-2	2X	50	16	4-16	0.15	0.086	0.087
	O-1	2X	67	16	2-16	0.12	0.069	0.069
Total			**167**	**47**	**2-16**	**0.13**	**0.078**	**0.079**

*N_a =number of different alleles; H_e= expected heterozygosity; uHe = unbiased expected heterozygosity.

Table 8. Descriptive statistics for each population of *Pityopsis graminifolia* var. *latifolia* were obtained using GenAlEx 6.5. Genetic diversity measurements were calculated using binary data set where microsatellite alleles were coded as present or absent [*P. graminifolia* var. *latifolia* (4X)].

Species	Subpopulation	Ploidy level	Sample size	Total N_a	Range N_a/locus	Shannon's information index	H_e	uH_e
Pityopsis graminifolia var. *latifolia*								
	O-1	4X	30	20	0-20	0.15	0.086	0.087
	H-2	4X	30	17	0-17	0.14	0.083	0.085
	H-1	4X	26	19	0-19	0.15	0.084	0.086
Total			**86**	**65**	**0-20**	**0.14**	**0.084**	**0.086**

*N_a =number of different alleles; H_e= expected heterozygosity; uHe = unbiased expected heterozygosity.

Table 9. Hierarchical analysis of molecular variance (AMOVA) for *Pityopsis ruthii* and *P. graminifolia* var. *latifolia* subpopulations using sixteen microsatellite loci. The analyses included binary data for combined and individual grouping based on ploidy and species across seven subpopulations.

Groups*	Variance partition	df	Sum of Squares	Variance component	Percentage of variation	*p*-value	F-statistics
Combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* (4X)	Among Populations	6	136.75	0.54	0.13	<0.0001	
	Within Populations	254	888.00	3.50	0.87	<0.0001	
Total		**260**	**1024.75**	**4.04**	**1.00**		Φ_{PT}=0.13
Combined *P. ruthii* (2X) and *P. graminifolia* (4X)	Among Populations	6	128.22	0.51	0.13	<0.0001	
	Within Populations	254	837.13	3.30	0.87	<0.0001	
Total		**260**	**965.35**	**3.80**	**1.00**		Φ_{PT}=0.13
P. ruthii (2X)**	Among Populations	2	440.44	3.68	0.18	<0.0001	
	Within Populations	164	2839.41	17.31	0.82	<0.0001	
Total		**166**	**3279.85**	**21.00**	**1.00**		Φ_{PT}=0.18
P. graminifolia (4X)**	Among Populations	2	105.19	0.77	0.02	<0.003	
	Within Populations	83	2531.36	30.50	0.98	<0.003	
Total		**85**	**2636.55**	**31.27**	**1.00**		Φ_{PT}=0.02

* Grouping was based on binary data analyses. ***P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X) groups did not take into account Test population.

Table 10. Analysis of molecular variance (AMOVA) analyzed by Arlequin version 3.5.1.2 for *Pityopsis ruthii* subpopulations using sixteen microsatellite loci. The analyses included three populations as one hierarchical group and partitioning populations into two groups identified by the program STRUCTURE.

Variance partition	d.f.	Sum of squares	Variance component	% of variation	*P*-value
Among populations	2	179.189	0.75792 Va	13.97	<0.0001
Among populations within 1 cluster	164	998.251	1.42069 Vb	26.19	<0.0001
Within populations	167	542.000	3.24551 Vc	59.83	<0.0001
Total	**335**	**1718.440**	**5.42412**		
Fst=0.14					
Among groups	1	129.889	0.44735 Va	8.07	<0.3347
Among populations within 2 clusters	1	49.300	0.43213 Vb	7.79	<0.0001
Among individuals within populations	164	998.251	1.42069 Vc	25.62	<0.0001
Within individuals	167	542.000	3.24551 Vd	58.52	<0.0001
Total	**333**	**1719.440**	**5.54569**		
Fsc=0.085; Fct=0.081					

F_{ST} – the variance among subpopulations relative to the total variance.
F_{SC} – the variance among subpopulations within groups.
F_{CT} – the variance among groups relative to the total variance.

Appendix 2. Figures

Figure 1. STRUCTURE. (A) [combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X) as tetraploid (Δ K=3)]; (B) [combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X) as tetraploid (Δ K=2)]; (C) *P. ruthii* (2X) only; (D) *P. graminifolia* var. *latifolia* (4X) only.

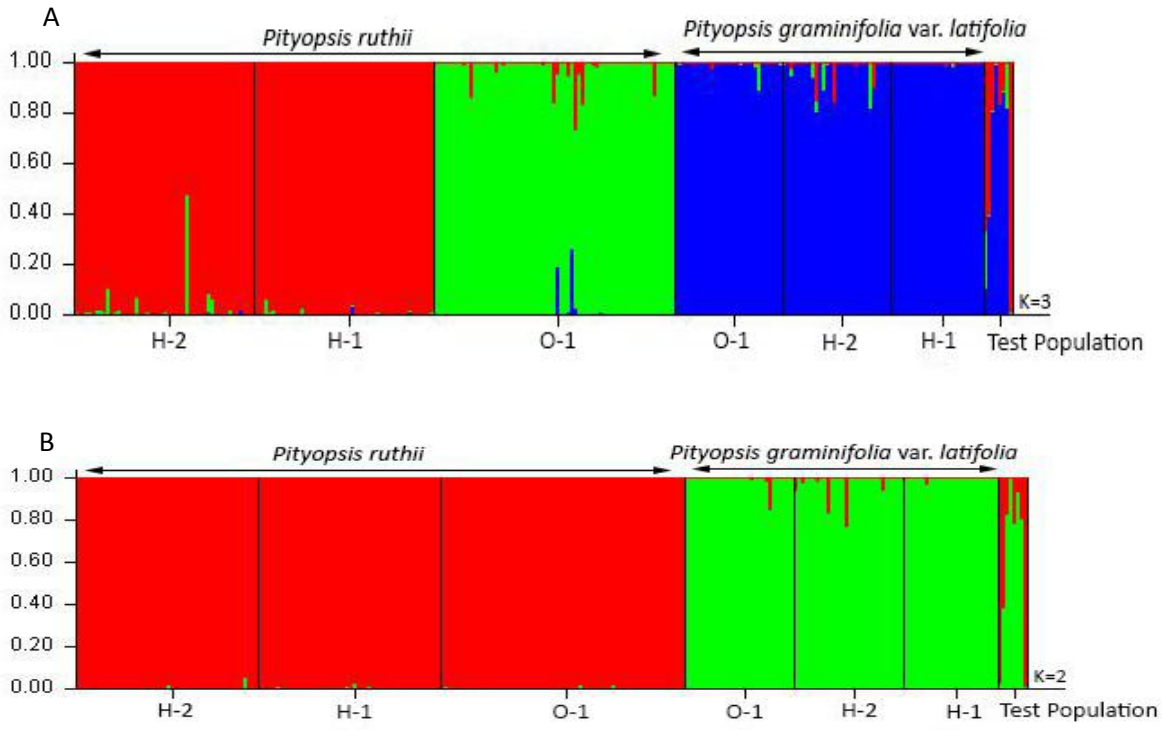
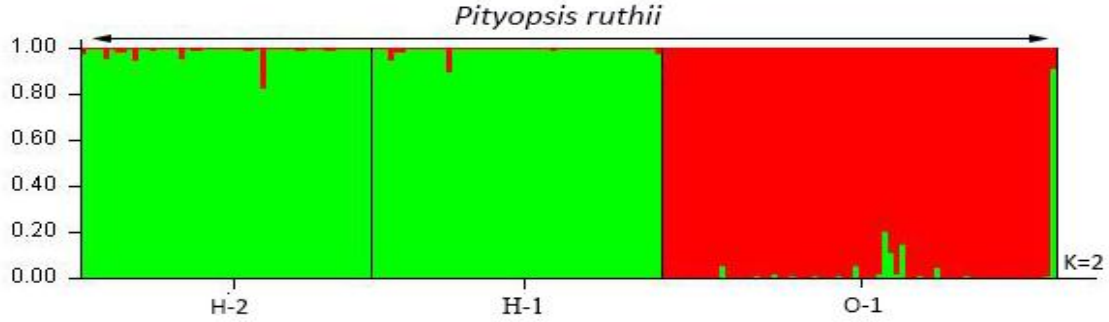


Figure 1. Continued.

C



D

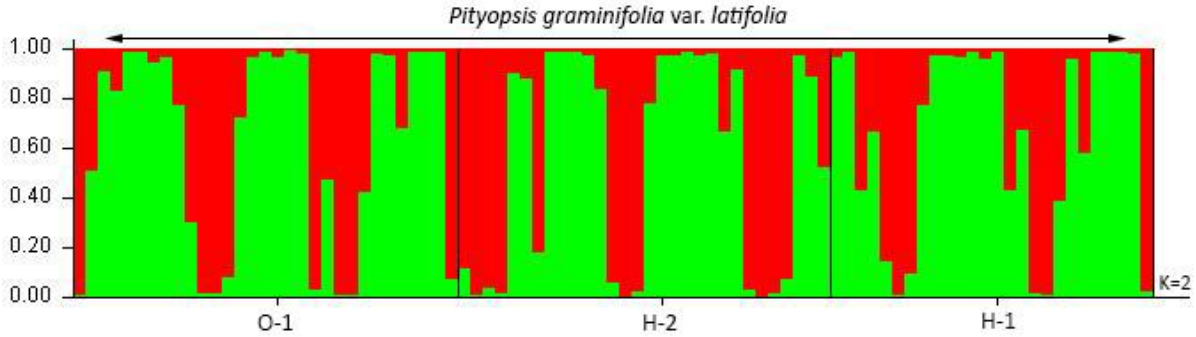


Figure 1. Continued.

Figure 2. BAPS. (A) [combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X); (B) combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X); (C) *P. ruthii* (2X) only; (D) *P. graminifolia* var. *latifolia* (4X) only.

A

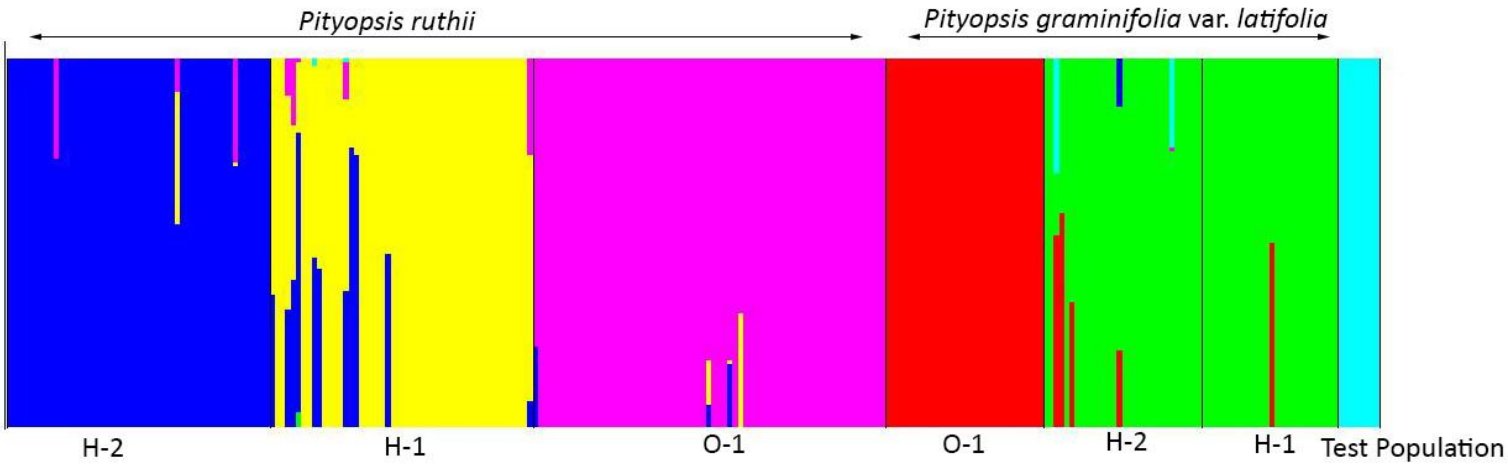


Figure 2. Continued.

B

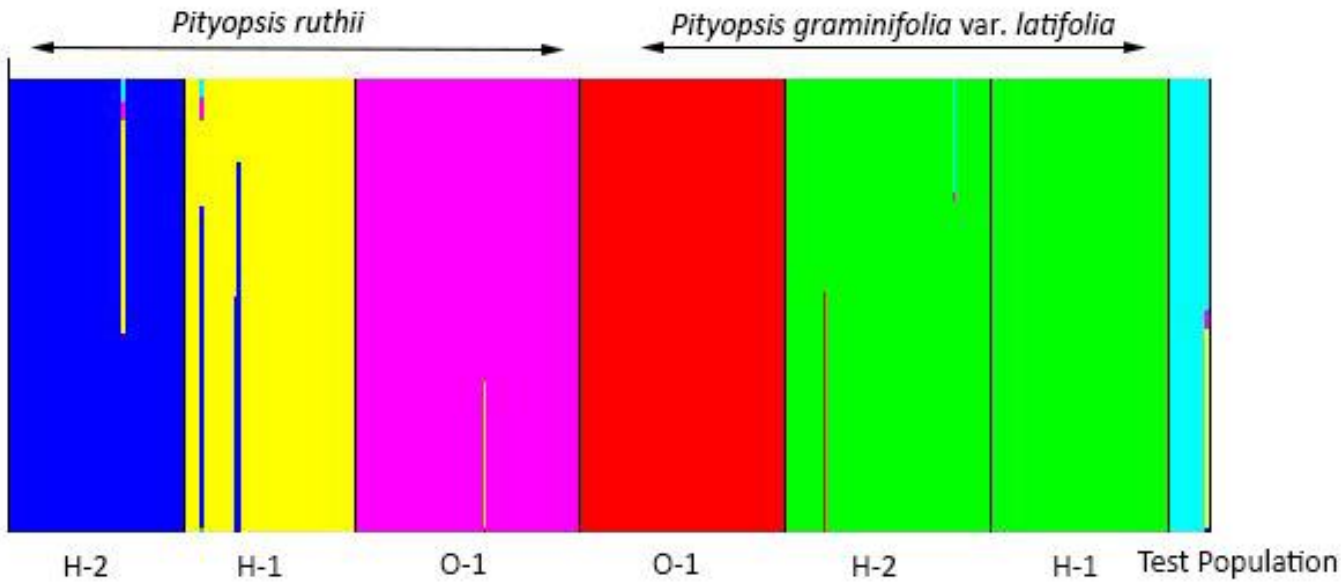


Figure 2. Continued.

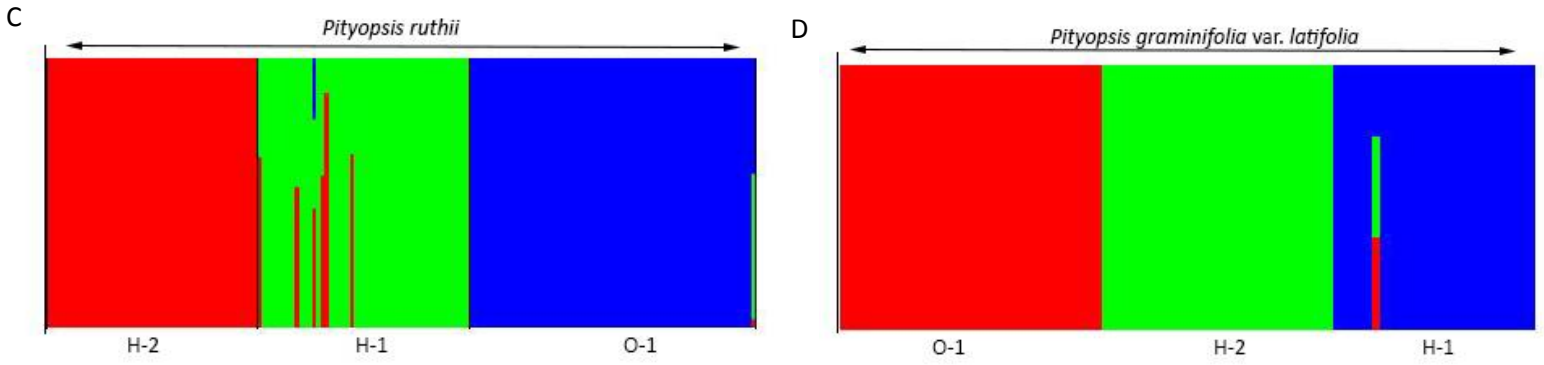


Figure 2.Continued.

Figure 3. Principal coordinate analysis (PCoA). (A) [combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X); (B) combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X); (C) *P. ruthii* (2X) only; (D) *P. graminifolia* var. *latifolia* (4X) only.

A

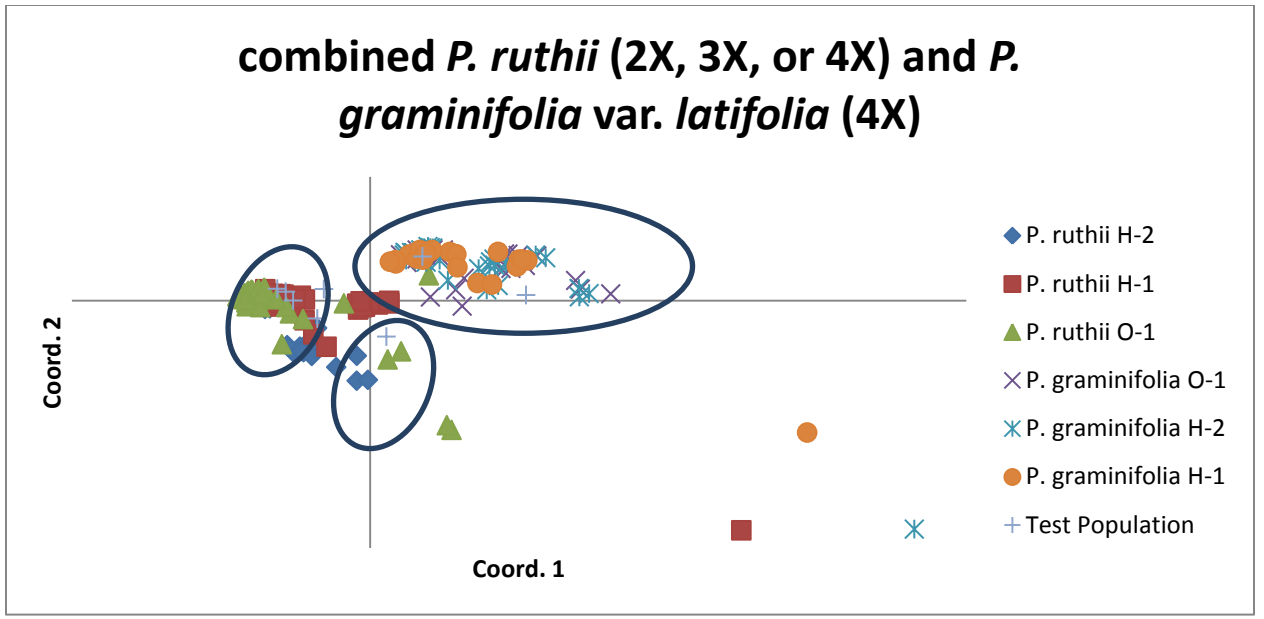


Figure 3. Continued.

B

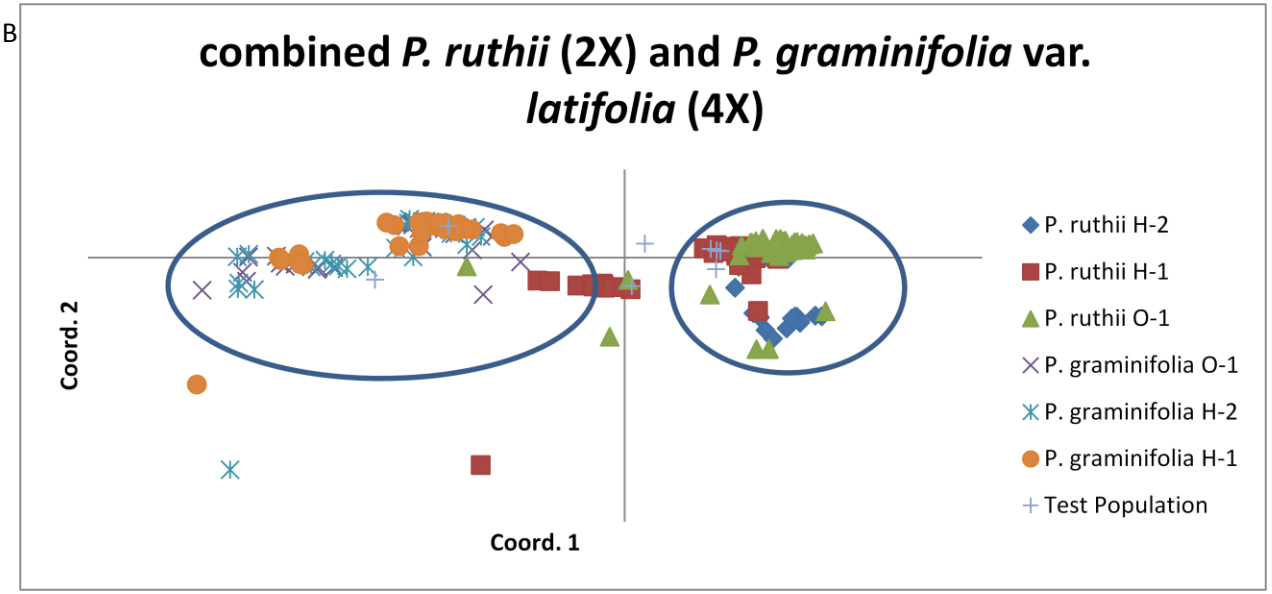


Figure 3. Continued.

C

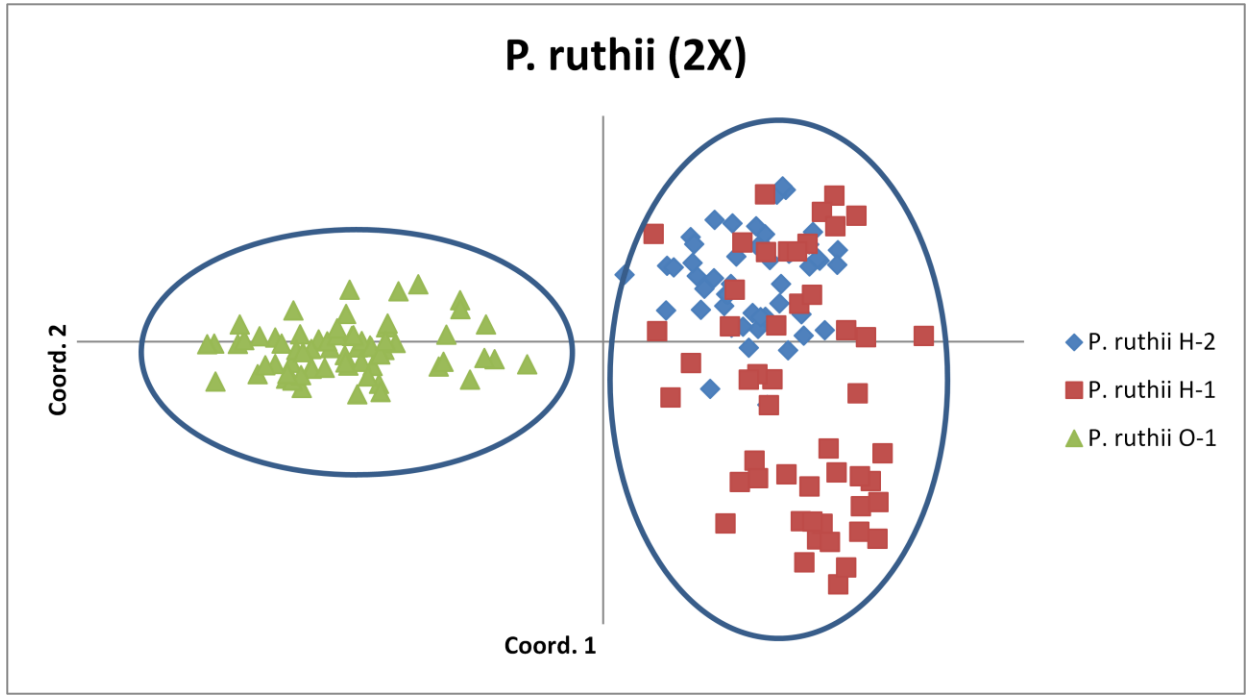


Figure 3. Continued.

D

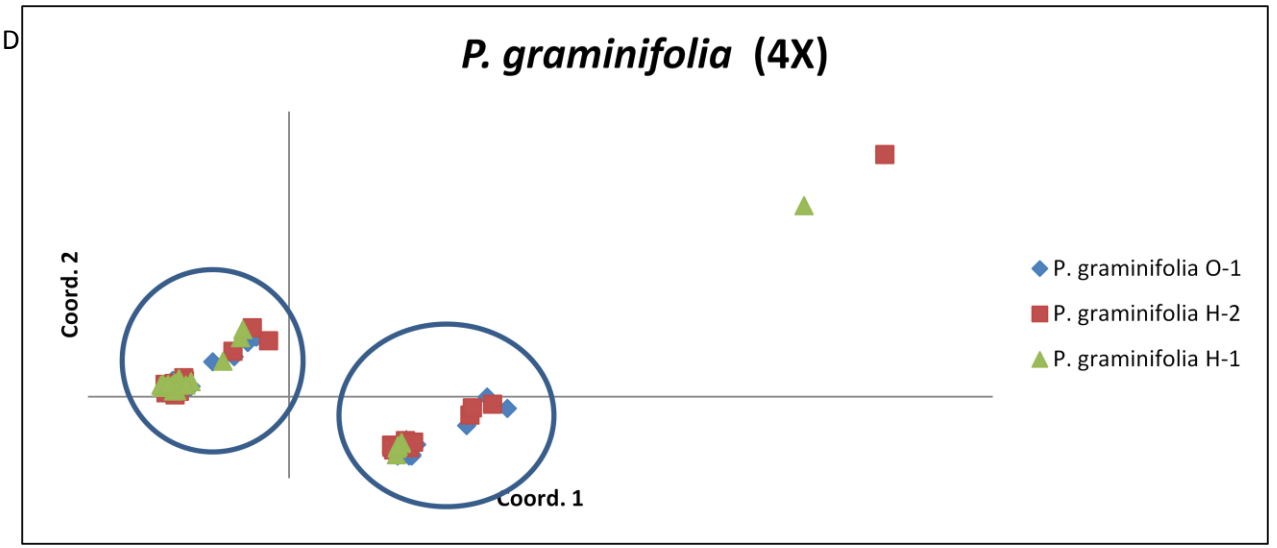


Figure 3. Continued.

Figure 4. Scatterplot of pairwise genetic distances vs. geographical distances were obtained for all four binary data sets for each population of *Pityopsis ruthii* and *P. graminifolia* var. *latifolia* using GenAlEx 6.5. P values were obtained using 9,999 randomizations. Geographic distance values are in log scale. (A) [combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X); (B) combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X); (C) *P. ruthii* (2X) only; (D) *P. graminifolia* var. *latifolia* (4X) only.

A

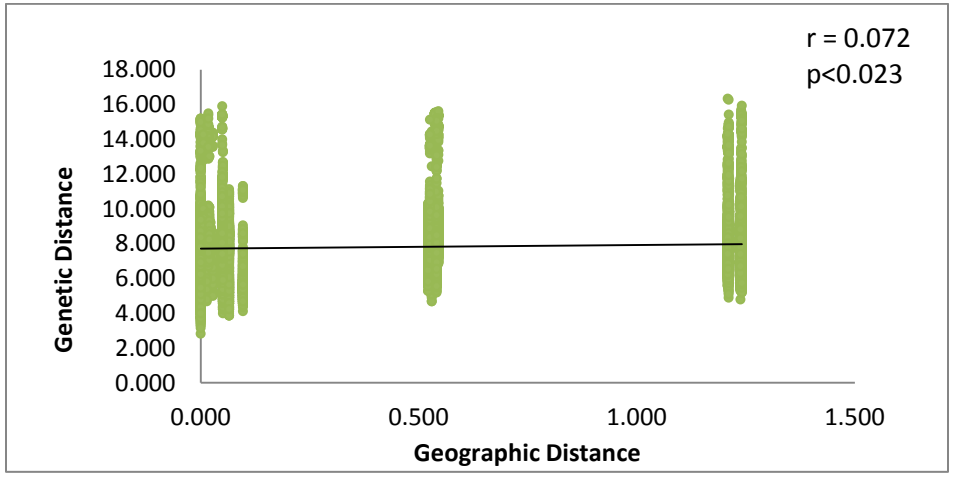


Figure 4.Continued.

B

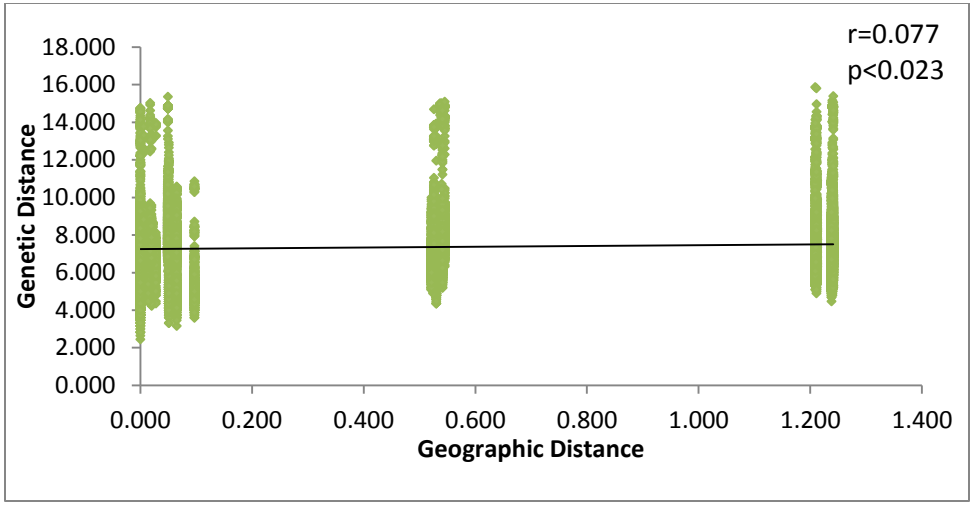


Figure 4.Continued.

C

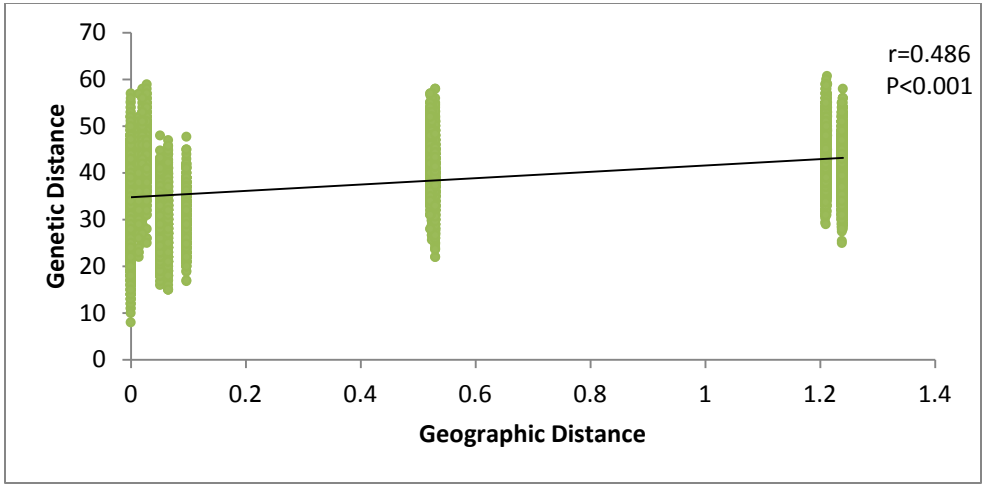


Figure 4.Continued.

D

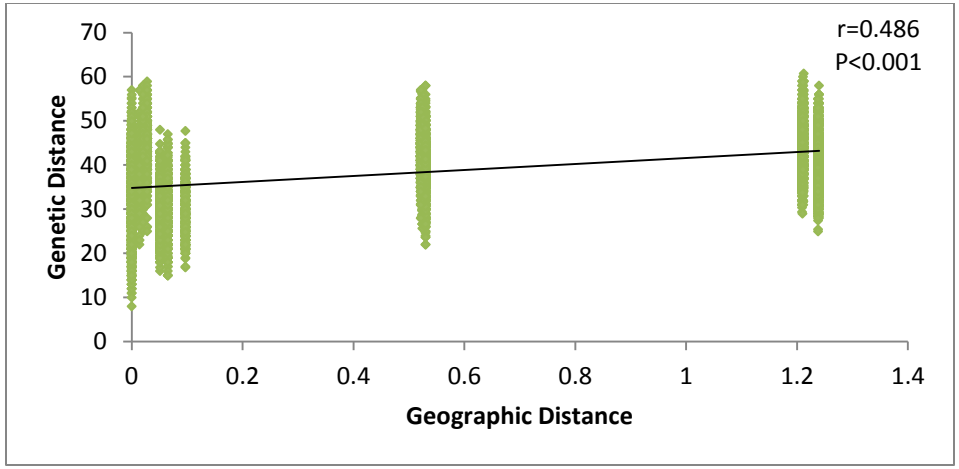


Figure 4.Continued.

Figure 5. Gene flow network identified in *Pityopsis ruthii* and *P. graminifolia* var. *latifolia* populations was computed using program BAPS 6.0. (A) [combined *P. ruthii* (2X, 3X, or 4X) and *P. graminifolia* var. *latifolia* (4X); (B) combined *P. ruthii* (2X) and *P. graminifolia* var. *latifolia* (4X); (C) *P. ruthii* (2X) only; (D) *P. graminifolia* var. *latifolia* (4X) only.

A

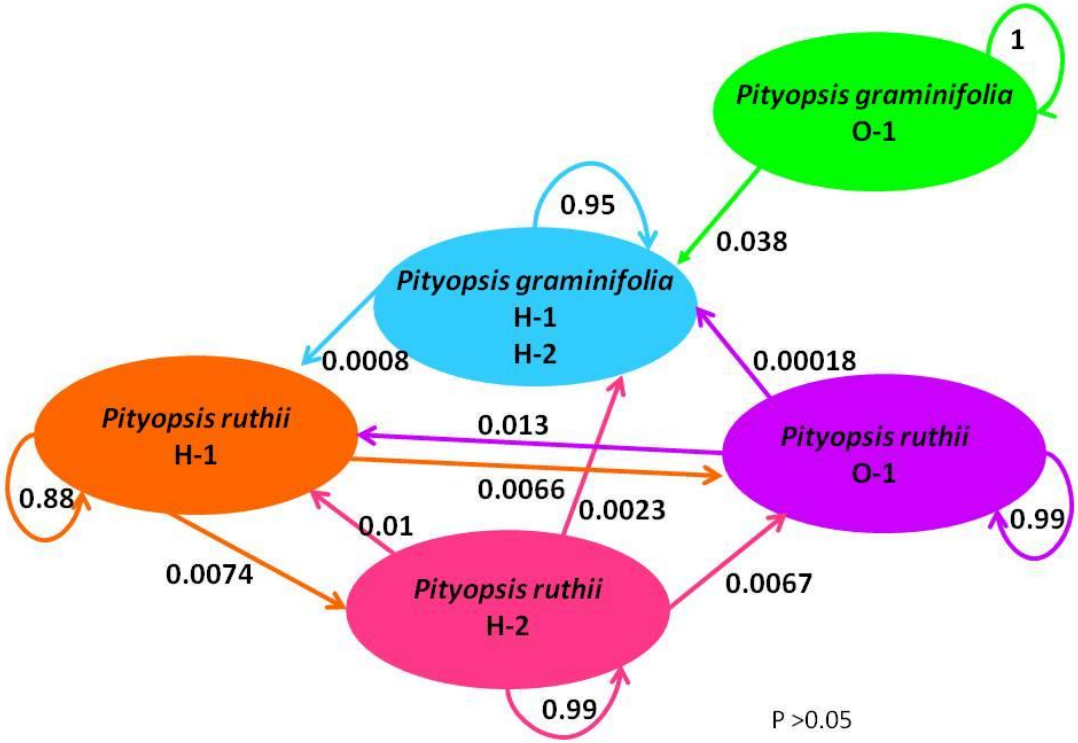


Figure 5. Continued.

B

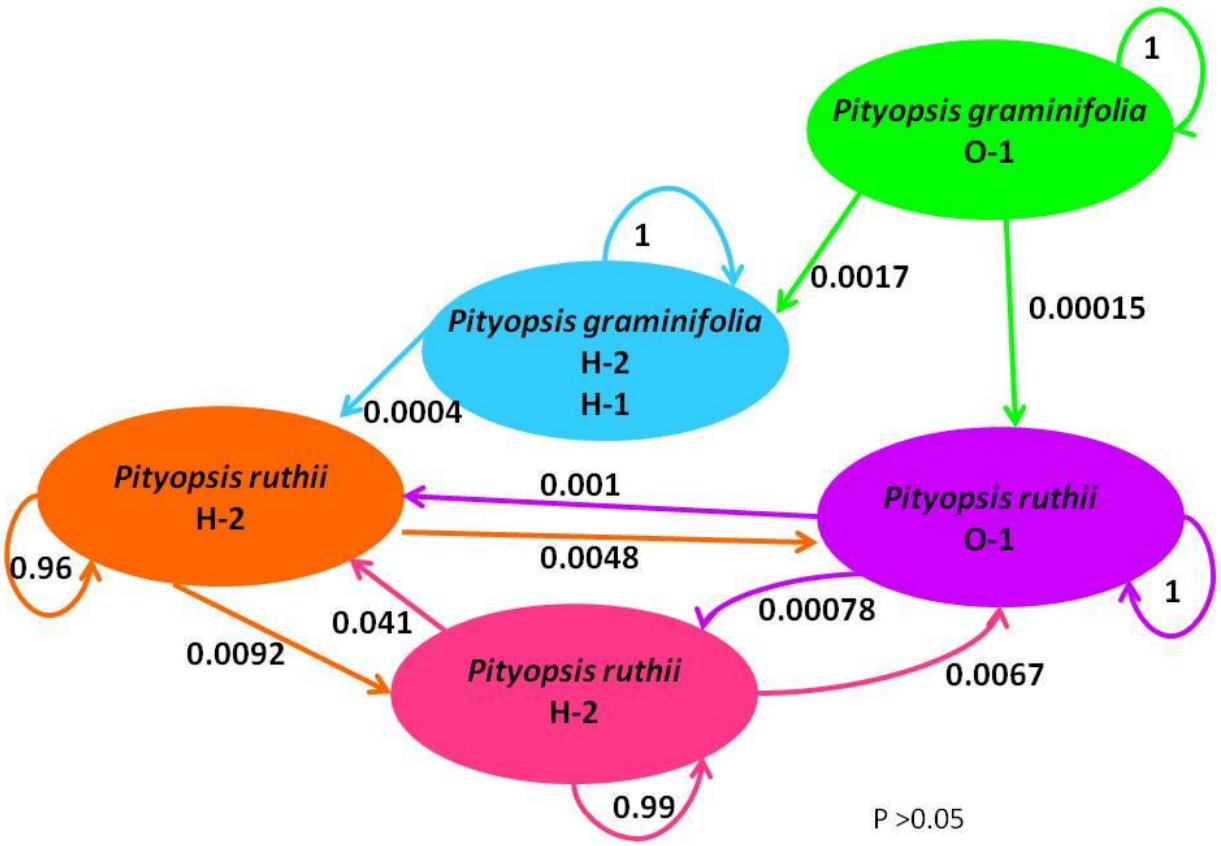


Figure 5. Continued.

C

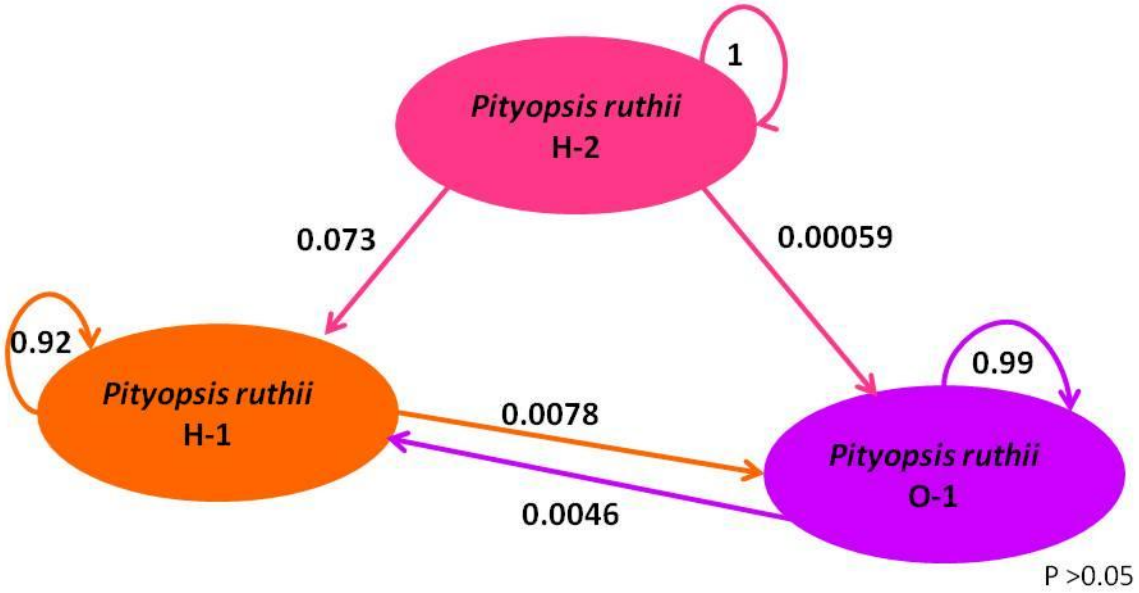


Figure 5. Continued.

D

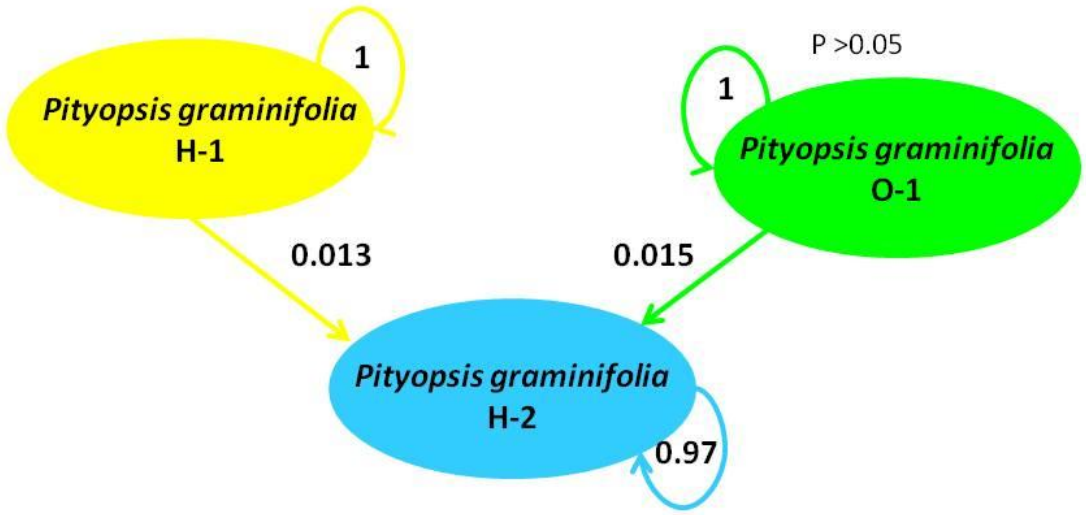


Figure 5. Continued.

VITA

Sarah Boggess was born in Oak Ridge, TN, to parents Toney and Dorri Boggess. She attended Valley View Elementary, Lafollette Middle School, and Campbell County Comprehensive High School; all located in Campbell County, TN. During primary education she was very active in the state FFA organization, as well as state 4-H, where she held various officer positions. After graduating high school in May 2007, she attended the University of Tennessee, Knoxville where she majored in Plant Science with a concentration in Biotechnology. After she was awarded a bachelor's degree in Plant Science in December 2011, she accepted a graduate research assistantship in the laboratory of Dr. R. N. Trigiano to continue her education in plant pathology. Sarah graduated with a Master's of Science in Entomology and Plant Pathology in December 2013.