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A Morphological and Genetic Study of Taxonomy and Evolutionary Divergence in Xanthisma Gracile and Xanthisma Spinulosum

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A morphological and genetic study of taxonomy and evolutionary divergence in *Xanthisma gracile* and *Xanthisma spinulosum*.

By

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Discerning the basis of phenotypic and genotypic differences within and between taxa is crucial for understanding the evolution of species, subspecies or varieties and races. In this dissertation, I have presented three studies, which use morphological characters and genetic Amplified Fragment Length Polymorphisms (AFLPs) to differentiate cytotypes, populations and species of the genus *Xanthisma*. The first study is aimed at clarifying the species status of *Haplopappus ravenii,* which has been considered to be a separate species by some taxonomists and a race of *Xanthisma gracile* by other researchers. Considering the morphological species concept and the genotypic cluster definition of a species, there was insufficient distinction in either dataset to support these taxa as distinct species. It was found that *H. ravenii* is more appropriately classified as a a cytotype or a race of *X. gracile*. In the second study, the genetic structure of *X. gracile* was quantified across populations occupying distinct habitat types (desert, grasslands, and pinyon juniper woodlands) in order to test the hypothesis of local adaptation and to determine the potential for intraspecific divergence. Samples from desert habitats showed higher genetic divergence than samples in the other two habitats. This study is indicative

of local adaptation of populations and that changes in climate and habitat play a very important role in the genetic differentiation of plant systems. The third study evaluated the taxonomy of *Xanthisma spinulosum* and three of its subspecies that co-occur in Arizona. Herbarium specimens representative of the three subspecies were used to test for significant morphological and genetic divergence that would support their recognition. The morphological characters originally utilized by taxonomists who named these taxa were not significantly different among the three taxa. This finding was further supported by the molecular data, suggesting the presence of one contiguous species. This dissertation aims at stressing the importance of taxonomic status and understanding the role that environment can play on shaping differentiation between taxa.

DEDICATION

To my family- for all your support and encouragement without which this would not have been possible!

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CHAPTER I

INTRODUCTION

1.1 Recognition of the species rank in biology:

What are species? How are species formed? What factors affect speciation? These are among the most compelling questions of taxonomy, systematics and evolutionary biology. As species are considered the fundamental unit of biology, understanding the patterns and processes governing speciation is vital. Failure to delineate species accurately can have huge impacts in biological applications, especially in conservation and management of biodiversity (May 1990; Funk et al. 2002; McNeely 2002; Mace 2004; Morrison III et al. 2009).

Understanding and defining the term "species", though crucial, has been a topic of conflict in biology. Many species concepts (approx. 22 in number; Mayden 1997) have been formulated, each definition having its own strengths and weaknesses (Coyne & Orr 2004). Most species names in use today are based on morphological criteria, using what is known as the morphological (MSC) or typological species concept. According to the MSC, species are groups of individuals that are morphologically similar and clearly distinguishable from individuals of other groups (Darwin 1859; Wallace 1865). This species concept relies on type specimens that represent an "ideal" form of the species (Cronquist 1978). Any variation below the species level, for example, across populations, is usually defined at the level of subspecies or varieties.

Morphological variation can be created by local adaptation, phenotypic plasticity, or genetic drift working independently or in tandem (Andersson 1991; Linhart & Grant 1996; Stuessy et al. 2006). It is not always that plants that occur in distinct geographic locations and are morphologically different from each other are considered as different taxa. Common garden experiments, where plants of different taxa are grown together, can demonstrate whether morphological similarity is environmentally or genetically determined (Turesson 1925, Hall 1932). For example, the classic experiments of Clausen et al. (1940, 1947) emphasized the role of ecological genetics and phenotypic plasticity in plants, to understand whether environment plays a key role in controlling differences between morphologically distinct plants. Stebbins (1950) suggested that the continuous variation in widespread species is probably due to ecotypic adaptation, where clines or character gradients occur as responses to changes in habitats. Anderson (1968) suggested that the differences between two species are substantially greater than the differences between two individuals of the same species. When intraspecific differences are influenced by selection or other forces, they can produce new species and would be recognized as such. When populations are morphologically distinct but not actually genetically divergent, a taxonomic classification based solely on morphology is misleading. Alternatively, populations may exhibit morphological similarities despite being genetically distinct. These are known as cryptic species, which would go undetected if only morphology is considered for taxonomy.

Commonly used morphological characters to delimit species are also sometimes ambiguous or overlap among populations, making identification inconclusive. This could potentially be due to hybridization between taxa or habitat variables causing shifts in

morphology. In such a scenario, genetic data can be very helpful to elucidate species boundaries. With the development and widespread use of molecular genetic techniques, taxonomists have increasingly turned to species concepts that incorporate genetic diversity. For example, Mallet (1995) proposed the Genotypic Cluster Criterion (GCC) to distinguish species as "morphologically or genetically distinguishable group of individuals that has few or no intermediates when in contact with other such clusters". This concept accommodates gene flow, selection, mutation and genetic drift and allows for intraspecific hybridization.

The most common species concept in use is the Biological Species Concept (BSC; Mayr 1963). It is defined as groups of interbreeding natural populations that are reproductively isolated from other such groups. However, this definition is not always applicable and, in particular, controversy remains about whether certain groups of individuals are species, subspecies or just different populations of the same species. In particular, when groups of individuals are geographically isolated, it is not always clear whether they fit the definition of species (Price, 2007), i.e., whether they are reproductively isolated and thus would not merge into a homogeneous group upon secondary contact (Coyne and Orr 2004). Whether they can interbreed successfully can sometimes be evaluated if there is a recent hybrid zone between the two species. For instance, human disturbance may bring into contact what turn out to be "true" species. Since reproductive isolation will lead to reduced hybrid fitness, hybrid zonesshould remain stable over time or quickly disappear. In contrast to the BSC, the MSC and GCC do not require that species exhibit reproductive isolation, although it is often assumed that reproductive isolation is likely to lead to genetic and/or morphological divergence.

Species can be delimited only in relation to other species. With the presence of so many species concepts, there are situations when there is no complete agreement among datasets used to test species boundaries (Hey 2006). This recently led to a trend towards "integrative taxonomy", which combines multiple data types and has been recognized as the most objective means to delimit species (Schlick- Steiner et al. 2010; Padial et al. 2010; Barrett and Freudenstein 2011; Cruz-Barraza et al. 2012). Many recent studies have emphasized the importance of combining traditional morphology with molecular data when testing taxonomic hypotheses (Shipunov, Fay and Chase 2005; Kučera, Lihová and Marhold 2006; Reeves and Richards 2011; Lega et al. 2012).

Many different types of molecular markers have been used in taxonomic and systematic studies. These markers include allozymes (Crawford 1985), RAPD's (Random Amplified Polymorphic DNA; Williams et al. 1990), RFLP's (Restriction Fragment Length Polymorphism; Botstein et al. 1980), AFLP's (Amplified Fragment Length Polymorphism; Vos et al. 1995), SSR's (Single Sequence Repeats; Tautz 1989) and DNA sequences(Tautz et al. 2003). Each of these markers has advantages and disadvantages for studying the nature of species (Arif et al. 2010). In this study, I used AFLP as a marker of choice. Collection of AFLP data does not require *a priori* sequence information and has the potential to generate a large number of polymorphic loci for species lacking extensive genetic resources (Powell et al. 1996; Guo et al. 2002, Das et al. 1999; Steiger et al. 2002; Liu et al. 2005). As a genomic survey, AFLP's have the capacity to survey a much greater number of loci for polymorphism than many other commonly employed molecular genetic techniques in taxonomy, hence resulting in the detection of high levels of genetic variation within species (Thomas et al. 1995; Yee et al.

1999). AFLP's have been used to infer phylogenetic relationships (Després et al. 2003; Dasmahapatra et al.2009), to analyze intraspecificgenetic variation (e.g., investigations of population structure, estimation of F_{ST} analogs, inferences about sexual vs. asexual modes of reproduction; Mueller and Wolfenbarger 1999; Meudt and Clark 2007), and to study genetic diversity in endangered species (e.g., Coart et al. 2003; Gobert et al. 2002; Brienholt et al. 2009; Tribsch et al. 2002; Wilding et al. 2001; Skøt et al. 2002; Savolainen et al. 2006; Bonin et al. 2006). Han and Ely (2002) suggest that the AFLP band patterns are species-specific and can be used to identify closely related species since it is not based on the presence of one particular allele. AFLP has been used extensively in taxonomy to address species rank questions (Dodd et al. 2002; Mukherjee et al. 2003; Meudt and Clark 2007; Gaudel et al. 2012).

In this study, I address taxonomic hypotheses in *Xanthisma* section *Sideranthus* using a combination of morphological and genetic data and application of the MSC and GCC. Specifically, I aim to determine if *Haplopappus ravenii* R. C. Jackson is distinct from *Xanthisma gracile* (Nutt) D.R. Morgan and R.L. Hartman (Chapter II), test for the presence of genetically differentiated geographic races in *X. gracile* (Chapter III), and evaluate the genetic and morphological distinctiveness of the varieties of *Xanthisma spinulosum* (Pursh) D.R. Morgan and R.L. Hartman (Chapter IV).

1.2 Study System:

1.2.1 Taxonomic History

Xanthisma has a notable past, with the currently recognized species placed in several different genera over the last two centuries. This genus of the family Asteraceae (Compositae) currently includes nine species (Morgan and Hartman, Flora of North

America, vol 20, 2003). Species of *Xanthisma* are annual or perennial herbs that can be distinguished from other genera based on yellow rays, glandular or pubescent stems which are erect or spreading, leaves that are moderately to densely pubescent with bristles 1-4 mm long, and phyllaries that range from 2-8 mm in length (Morgan and Hartman, 2003). Members of *Xanthisma* were first recognized by De candolle (1836) who established section *Blepharodon* in the genus *Aplopappus* (= *Haplopappus*). Hall (1928) revised the section to consist of ten species, which were distinguished by the presence of yellow and purplish to red rays. Shinners (1950) moved *Haplopappus* sect. *Blepharodon* to the genus *Machaeranthera* and included species with white, purple and yellow rays. Cronquist and Keck (1957) later moved sect. *Blepharodon* back to *Haplopappus* and included only the yellow ray species. Hartman (1976) placed most of the yellow ray taxa treated by Hall in *Machaeranthera* subgenus *Sideranthus* and later (Hartman 1990) included 36 species with cyanic (white, blue, pink, purple) and yellow rays. In the most recent treatment of the genus, Morgan and Hartman (2003) divided *Machaeranthera* into four genera based on nrDNA and cpDNA evidence (Morgan 2003). Members of sect. *Sideranthus* are now referred to as species of *Xanthisma*.

In all taxonomic treatments of this group, *X. gracile* and *X. spinulosum* were always placed in the same section and considered to be closely related (Hall 1928; Hartman 1990). Genetic data have supported this assessment (Morgan 1993, 2003; Morgan et al. 2009). Both *X. gracile* and *X. spinulosum* are characterized by yellow ray flowers and they occur throughout the southwestern United States and northern Mexico. Morgan (2007) suggested these species may have hybridized to give rise to a new species known as *Xanthisma stenolobum* (Greene) D.R.Morgan & R.L.Hartman. Given the close

relationship and similar geographic distributions and common attributes it is advantageous to compare and contrast intraspecific variation in these closely related species to understand evolutionary patterns within *Xanthisma* and to better inform the taxonomic treatment of this group.

1.2.2 *Xanthisma gracile*

Xanthisma gracile, also known as the Yellow Spiny Daisy, is a small annual species (5-45 cm in height) with erect pubescent stems, basal and cauline pinnatifid leaves, and yellow radiate heads that are borne singly on leafy peduncles (Morgan and Hartman, Flora of North America, vol 20, 2003). This species flowers from late Summer through Fall and is widely distributed in the southwestern U.S. in Arizona, California, Colorado, Nevada, New Mexico, Texas, and Utah (USDA NRCS 2012) as well as northern Mexico (Jackson 1960). It is a chromosomally polymorphic species with 2n numbers of 4, 5, and 6, but populations are predominantly 2n=4 (Jackson 1964). Populations with 2n=6 (Jackson 1964) are limited to some areas of New Mexico. Plants with 2n=5 appear to be hybrids because they are found only where n=2 and n=3 plants come into contact in some parts of south central Arizona (Jackson 1960). *Xanthisma gracile* has also been found across a varied climatic range from xeric to mesic habitats, and intraspecific cytological variation may be associated with these different habitats (Jackson 1965).

According to Jackson (1962), *X. gracile* evolved through reciprocal translocations and loss of centromeres from a higher karyotype, which he named *Haplopappus ravenii* (n=4). The distribution of this ancestral karyotype is limited to areas of southern California, southwestern Utah and north-central Arizona. Jackson (1962) described *H.*

ravenii to have shorter and fewer pappus bristles and shorter achenes than *X. gracile*. The pubescence of the involucre is suggested to be hirsute and appressed in *X. gracile* with a more short and stiff pubescence in *H. ravenii*. Within *H. ravenii*, two races have been recognized, one from the arid mountains of California and the other with a more mesic habitat of Utah and Arizona (Jackson and Crovello 1971). In a study conducted by Matos (1979), *H. ravenii* is shown to have undergone morphological and genetic variation under different climatic conditions.

Based on the morphological and karyotypic differences, Jackson and Crovello (1971) suggested *H. ravenii* was a distinct species. However, Cronquist (1971) and other researchers have considered *H. ravenii* to be a race or simply reflective of variation within *X. gracile*. This conflict lays the foundation for Chapter II, which re-evaluates the distinctness of *H. ravenii* relative to *X. gracile* using morphological and genetic data sets. This study utilizes herbarium specimens to determine if there are consistent morphological differences in samples identified as *H. ravenii* (or collected within the geographic range of this species) compared to *X. gracile*, based on the taxonomic characters as described by Jackson and Crovello (1971) and genetic differentiation between the taxa using Amplified Fragment Length Polymorphisms (AFLP).

Given that *X. gracile* has a distribution across varied habitat ranges and genetic diversity affects the survival and fitness of a species, measures of genetic diversity can provide insights into the way species have reacted and will react in the future to environmental change (Bush and Barrett 1993; Bagley et al. 2002). Chapter III addresses genetic differentiation among geographically isolated populations and ecological races of *X. gracile* within Arizona utilizing AFLP's.

1.2.3 *Xanthisma spinulosum*:

The *X. spinulosum* complex is composed primarily of diploids (2n = 2x = 8) with a number of scattered tetraploid populations (2n = 4x = 16; Hauber 1986). This weedy, perennial herb is found throughout the western Great Plains of North America from central Mexico to southern Canada (Hall 1928). It often occurs in association with disturbed areas, such as along road grades and in old pastures. Throughout its distribution, *X. spinulosum* is morphologically highly variable, and this has led to taxonomic treatments of the group that categorized the different morphological types as subspecies or varieties (Hall 1928; Turner and Hartman 1976). Turner and Hartman (1976) recognized two subspecies with seven varietal ranks, including *spinulosum, glaberrima, chihuahuanum, goodingii, scabrella, paradoxa* and *incisifola*. In a recent revision, Nesom and Turner (2007) raised the ranks of vars. *glaberrima, scabrella, paradoxa* and *incisifola* to species. The remaining three taxa, *spinulosum, chihuahuanum* and *goodingii,* are still considered as varieties of *Xanthisma spinulosum*. *Xanthisma spinulosum var. spinulosum* occurs from Montana to Texas, New Mexico and eastern Arizona, whereas var. *chihuahuanum* is localized to southern Arizona. *Xanthisma spinulosum* var. *goodingii* occurs across Western Arizona, South Central Nevada and Baja California. The morphological characters traditionally used to differentiate subspecific ranks overlap among the taxa. For example, involucre width is 8-12 mm in var. *spinulosum,* 12-16 mm in var. chihuahuanum, and 12-22 mm in var. goodingii (Nesom and Turner 2007). Turner and Hartman (1976) described *X. spinulosum* as "... an exceedingly complex, variable taxon" and suggested that it was unlikely that an 'absolute' key could be developed to distinguish recognize intraspecific taxa. The

presence of intraspecific variation, which underlies the extensive taxonomic revision of this system, and the continuity of populations considered to be different taxa across Arizona lays the foundation for Chapter IV. In this chapter, I test the taxonomy of Nesom and Turner (2007) using morphological characters and genetic data from AFLP's.

1.3 Goals and significance of research

This dissertation aims at using both morphological and genetic data to sufficiently differentiate the different taxonomic questions and clarify the taxonomic status of the study organisms. It is important to provide a stable taxonomy to a set of organisms as it helps in understanding the evolutionary relationship between such groups. Traditionally, taxonomic status has been justified by referring to differences between named taxa. All three studies conducted in this dissertation are addressing these questions from different taxonomic ranks. Chapter II addresses the criteria required for being designated the species rank. Chapter III uses a population genetic approach to demarcate geographic races and determine how habitat plays a key role in shaping the genetic differentiation. Lastly, Chapter IV addresses infraspecific rank of variety.

Taxonomists are often called "lumpers" or "splitters" based on their preference to recognize intraspecific variation. Lumping of taxa usually occurs when intraspecific variation is recognized and allows for differences between individuals from a single species. On the other hand, when low levels of intraspecific variability are assumed, taxa are split into separate species based upon the smallest of differences between individuals. This puts into perspective that species definition is to some extent dependent on the person evaluating it or the methods being utilized (O' Higgins 1989). Every time a taxonomic revision occurs, and a previously "lumped" species is split or vice versa, it

impacts the species conservation status (Frankham et al. 2002). However, change in taxonomic status does not always have an adverse effect. Morrison et al. (2009) provide a comprehensive study of how changes in taxonomic status have affected species status. Conservation implications are not the only consequence of understanding taxonomy. Better understanding of taxonomic status helps us evaluate the evolutionary relationships within taxa and to develop hypotheses about the underlying causes of diversification. Study of patterns of genetic diversity and differentiation also provides us with an understanding of the requirements for species survival and the effect of the environment and changing habitats.

Finally, studies of *X. gracile* may have other applications outside of taxonomy. This species has the smallest known number of chromosomes among land plants and could serve as a simple model for evaluating how cytological changes affect molecular and phenotypic evolution. For example, *X. gracile* contains supernumerary (B) chromosomes, which are considered as genomic parasites exploiting the host genome because of their transmissional advantage. B chromsomes are frequently deleterious to the organism carrying them (Östergren 1945, Camacho et al. 2000, Burt and Trivers 2006), but they are ubiquitous in eukaryotes. This research provides a basic understanding of genetic structure within *X. gracile* that could be coupled with future studies of B chromosome variation to determine if or how environment influences the evolution of extra genomic components.

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CHAPTER II

A REVALUATION OF THE TAXONOMIC STATUS OF HAPLOPAPPUS RAVENII

2.1 Abstract

Slender goldenweed (*Xanthisma gracile*), previously known as *Haplopappus gracilis,* is an annual, highly polymorphic and taxonomically controversial species occurring in the Southwestern United States and Northern Mexico. Two cytotypes are known to occur, 2n=4 and 2n=8, which have been traditionally called two species, *Xanthisma gracile* and *Haplopappus ravenii,* respectively. Various researchers have questioned the distinctiveness of *H ravenii*, but no study has quantified differences in morphology between the species or tested for genetic differentiation between these taxa. The difference in chromosome number suggests that these taxa could be reproductively isolated, which would support recognition of *H. ravenii* as a unique taxon relative to *X. gracile*. To test this taxonomic hypothesis, flower and leaf characters were measured on herbarium specimens and genetic diversity of herbarium specimens was determined using AFLP's. Morphological measurements show small differences across all the samples studied, but these differences were not significant. Genetic variation between the two taxa was significant in an analysis of molecular variance, but the level of divergence is quite low ($\phi_{ST} = 0.015$, $p < 0.05$) in comparison to values for other clearly demarcated species. Clustering analysis revealed intermediates throughout the genetic and morphological

characters, suggesting that *X. gracile* is more likely a polymorphic species with a varied distribution of cytotypes.

2.2 Introduction

Darwin (1859) stated, "… look at the term species as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other and that it does not essentially differ from the term variety which is given to less distinct and more fluctuating forms." This summarizes the important questions asked in evolutionary biology: What is species and how do we define species and how do we differentiate between a variety, subspecies or a race? The speciation process involves formation of spatial or geographic barriers and/or ecological and reproductive isolating mechanisms that allow new and different gene combinations to arise in separate populations. These novel gene combinations are responsible for genetic divergence, eventually resulting in speciation (Grant 1981; Coyne 1992; Levin 2003).

Karyotypic diversity in number, size and organization of chromosomes between and within taxa has been discovered in a multitude of cytological analyses since the discovery of chromosomes (Levin 2002). Polyploidy, defined as a genome duplication event, which causes differences in ploidy level (Grant 1981; Soltis et al. 2004), has been suggested to be a key feature responsible for plant diversification in most of the vascular plants (Wood et al. 2009) and specifically angiosperms (Soltis et al. 2009). Polyploidy can occur in two ways, autopolyploidy (i.e., genome duplication within a single species) and allopolyploidy (i.e., genome expansion occurs as a result of hybridization between species and retention of both genomes). Aneuploidy, on the other hand, occurs as a result of irregular segregation of chromosomes during cell division leading to difference in

chromosome numbers between taxa. It involves changes in one or a few chromosomes but not the entire genome. Other karyotypic differences, such as gene duplications, chromosomal rearrangements and the presence of mobile elements (San Miguel et al. 1996), have also contributed to quantitative cytological diversity.

The theory of chromosomal speciation proposes that changes in chromosomal structure or arrangement may cause reproductive isolation between sister taxa and lead to subsequent speciation (White 1968). Cytotaxonomists often give more credence to cytological characters, such as change in chromosome numbers or rearrangements, than traditional morphological characters, because cytological differences are strong barriers to reproduction for many taxa. According to Löve (1960)," the chromosomes determined the characters and not the other way around". Changes in cytological characters may be overemphasized in the delimitation of some species given that many species are suspected of having an allopolyploid history and triploid bridges often allow different cytotypes within a species to successfully interbreed (reviewed in Ramsey and Schemske 1998). Historically, all taxa differing in karyotype were considered as distinct taxa and species status was attributed to populations, even though they were morphologically similar. Even when plant evolution associated with karyotypic changes has been addressed, a lot more studies have been undertaken to understand the role of polyploidy in speciation, much less than aneuploidy. The use of multiple data sets, including genetic markers, morphology, and ecology, in addition to cytological data, helps in providing robust support of taxonomic conclusions and avoids potential bias associated with a single data point. This approach has been termed as integrative taxonomy (Schlick-

Steiner et al. 2010; Padial et al. 2010; Barrett and Freudenstein 2011; Cruz-Barraza et al. 2012) and is rapidly gaining credence in addressing such hypotheses.

An excellent example where difference in karyotype has been attributed to a separate taxonomic status is in the genus *Xanthisma*, specifically, *Xanthisma gracile* (Nutt.) D.R. Morgan & R.L. Hartman and *Haplopappus ravenii* R. C. Jackson. *Xanthisma gracile* is a small annual species (5-100 cm in height) with erect pubescent stems, basal and cauline pinnatifid leaves, and yellow radiate heads that are borne singly on leafy peduncles. This species occurs principally in the southwestern United States and some regions of northern Mexico. *Xanthisma gracile* is a chromosomally polymorphic species with 2n numbers of 4, 5, and 6, but populations are predominantly 2n=4 (Jackson 1965). Populations with 2n=6 are limited to some areas of New Mexico, and plants with 2n=5 appear to be hybrids because they are found only where n=2 and n=3 plants come into contact in some parts of south central Arizona (Jackson 1960). Plants identified as *X. gracile* in southwestern California, southern Utah and portions of western Arizona have eight chromosomes. Jackson (1962) referred to these populations as a distinct species, known as *Haplopappus ravenii*, and suggested that *X. gracile* was derived from this species by an aneuploid reduction. Cytological data from F1 hybrids between the two species show that the two chromosomes of *X. gracile* completely synapse with the four of *H. ravenii* (Tanaka 1967).

Jackson and Crovello (1971) stated that *X. gracile* has at least three morphological races, each existing in a different habitat range. One of these occurs in the desert grasslands of Arizona and Sonora, Mexico, the second in the dry foothills surrounding areas where the first race occurs in Arizona, and the third race in mesic

habitats, such as the pinyon juniper woodlands of Arizona, northern New Mexico and southern Colorado and in the arid grasslands and savannas in southeastern Arizona, southern New Mexico, northwestern Texas and western Sonora, Mexico. Two races are also proposed for *H. ravenii:* the arid California race occurring in the mountains of San Bernardino County and the mesic race of Utah and Arizona. A biosystematic study (Jackson and Crovello 1971) summarized that along with the different chromosome numbers, morphological characters such as the phyllary pubescence, achene and pappus characters, and aspects of leaves supported the recognition of *H. ravenii* as distinct from *X. gracile.* However, these inferences were based on data from only two populations of *H. ravenii* from Utah and California. Consequently, the separation of *H. ravenii* as a species was questioned by researchers (Cronquist 1971), who suggested that it be treated as part of one species with a varied cytology and that this difference in cytology was not "taxonomically controlling".

This discrepancy in the taxonomic status of *X. gracile* and *H. ravenii* forms the premise for this study, wherein I evaluate morphological and genetic distinctiveness of the two species and evaluate whether or not *H. ravenii* should be recognized as a separate species. I consider the morphological species concept (MSC) and the genotypic cluster definition (GCC) (Mallet 1995) as the criteria for defining a species. Briefly, MSC defines species as groups of individuals that are morphologically similar and clearly distinguishable from individuals of other groups. The genotypic-cluster definition (GCC) of species classifies organisms based upon overall genetic similarity. It emphasizes genetic distinctiveness and defines species as: “a morphologically and genetically identifiable clusters of individuals that can co-exist with other similar clusters with a few

or no intermediates"(Mallet 1995). Herbarium specimens were utilized to measure the morphological differences and for genetic analyses. Specific expectations from the criteria are that if *H. ravenii* is indeed a distinct species, there should be no continuity in characters that are used to delimit it from *Xanthisma gracile* and samples of these species should be genetically divergent.

2.3 Materials and Methods

2.3.1 Plant Material and Morphological Measurements:

A total of 201 specimens, 108 from Rancho Santa Ana Botanical Garden, 77 accessions from the Arizona State University herbarium and 16 voucher specimens which were collected on a field trip to Arizona in 2008 (to be housed at the herbarium at Mississippi State University) were utilized in this study. The geographic areas covered by these samples represent much of the range of *X. gracile* in the southwestern United States. The data set also included a few specimens from the Sonora and Chihuahua regions of northern Mexico. The accession numbers and locations are listed in Appendix A.1. Individuals were assigned to either *H. ravenii* or *X. gracile* based on the characters described by Jackson (1962), Jackson and Crovello (1971) and the Flora of North America (Morgan and Hartman, 2003 vol. 20, 2003) and the annotations by R. C. Jackson on the herbarium sheets. Samples from Arizona were treated as *H. ravenii* only if they were sampled from Yavapi County, where Jackson (1962) identified the type specimen. Samples from Utah and California were also considered to be *H. ravenii*. This was done to test for the presence of distinct morphologies in the purest groups. Morphological variation was quantified in eight traits, which correspond to the characters Jackson and Crovello (1971) used to differentiate *H. ravenii* from *X. gracile*. These traits

include phyllary number, phyllary pubescence, leaf length, leaf width, number of teeth on leaf, pubescence of stem, achene length and pappus length. All measurements were made manually under an Olympus dissecting microscope using a miniscale. The measurements were made across three different flowers, leaves and stems on each individual, and the average of these readings for each sample was used in analyses. Mean values were used to account for maximum variation possible within a specimen. Phyllary number was estimated by counting the rows of bracts. Phyllary pubescence was characterized as a binary character as appressed or stiff. Lengths were measured from point of attachment to the tip and the longest leaves were measured at the bottom of the plant to rule out variation related to incomplete development. Leaf width was measured at the point of maximum width. Number of teeth on the leaf edge was counted. The achene and pappus lengths were measured from the tip to the point of attachment and an effort was made to choose mature seeds. The pubescence of the stem was coded as a binary character as dense or sparse. Ratio of length and width for the leaves was also used as an alternate character for the analyses.

2.3.2 DNA Extraction

Leaf tissue from 85 herbarium specimens of Rancho Santa Ana botanical garden and the Arizona State University was used to extract genomic DNA following a modified CTAB DNA extraction protocol (Doyle and Doyle 1987). After extraction, DNA was dissolved in TE Buffer, treated with RNase A (10mg/ml, ABgene) to remove any residual RNA, and run on a 1.5% agarose gel to check for quality.

2.3.3 AFLP Analysis

AFLP analysis was performed using a protocol modified from Vos et al. (1995) and incorporating the recommendations made by Trybush et al. (2006). Individual genomic DNA was digested in 30 µl reactions incubated at 37 °C for 1 hour in a thermal cycler, followed immediately by ligation of the linkers. Restriction digest enzymes and reagents utilized per reaction were: 0.25 µl of *EcoRI* (20,000 U/ml, New England BioLabs, Ipswich, MA, USA), 0.5 µl of *MseI* (10,000 U/ml, New England BioLabs,), 3 µl 10X NEBuffer2, 0.15 µl of 100 µg/ml BSA, 5 µl of individually purified genomic DNA, and 21.1 µl of sterile water. Eco AFLP linkers were annealed in a thermal cycler by heating to 65 °C for 10 minutes and cooled to room temperature using the following reagents: 10 µl of Eco Linker 1 (100 µM, 5'-CTC GTA GAC TGC CC) and 10 µl of Eco Linker 2 (100 µM, 5'-AAT TGG TAC GCA GTC TAC). Mse AFLP linkers were also prepared using the above procedure utilizing 10 µl of Mse Linker 1(100 µM, 5'-GAC GAT GAG TCC TGA G) and10 µl of Mse Linker 2 (100 µM, 5'-TAC TCA GGA CTC AT). Annealed linkers were stored frozen at -20 °C until use.

Ligation of Eco and Mse linkers was conducted in 40 µl reactions, including 0.1 µl Eco and 1 µl Mse Linker (as annealed above), 0.15 µl T4 DNA Ligase enzyme and 4 µl 10X T4 DNA Ligase Reaction Buffer (New England BioLabs), 30 µl digested DNA, and 4.75 µl sterile water. Ligation reactions were incubated in a thermal cycler at 37 °C for 4 hours followed by storage at -80 °C to prevent degradation. Pre-selective amplifications were conducted in 10 µl reactions using 0.5 µl each Eco+A (10 µM, 5'-GAC TGC GTA CCA ATT CA) and Mse+C (10 µM, 5'-GAT GAG TCC TGA GTA AC) primers, 1.25 µl dNTPs (2 mM dATP, dCTP, dGTP, and dTTP; New England

BioLabs), 2 µl 5X *GoTaq*™ FlexiBuffer (Promega Corp Madison, WI, USA), 1.25 µl $MgCl_2$ (Promega Corp.), 2.5 µl individually ligated DNA, 2.55 µl sterile water, and 0.1 µl *GoTaq*™ DNA polymerase (5u/µl, Promega Corp.). Pre-selective amplifications consisted of an initial denaturing step of 65 °C for 5 minutes, 30-cylces of 30 seconds at 94 °C, 30 seconds at 56 °C, and 1 minute at 72°C.

Pre-selective amplification products were individually diluted 1:20 with sterile water. Selective amplifications consisted of a single Mse primer with three fluorescent-labeled Eco primers per reaction. Selective amplification for all individuals was conducted in 10 µl volume consisting of 0.7 µl each Mse-CAG (5µM), Eco-ACT FAM, Eco-ACC NED and Eco-AGG VIC (1 µM) selective primers, 0.5 µl dNTPs (2 mM dATP, dCTP, dGTP, and dTTP; New England BioLabs), 2.5 µl LongAmp™ Buffer (New England Biolabs), 1 µl of diluted pre-selective amplification product, 2.7 µl sterile water, and 0.5 µl *LongAmp Taq*™ DNA polymerase (5u/µl, New England Biolabs). Selective amplifications consisted of an initial denaturing step of 95 °C for 15 minutes, 13-cylces of 30 seconds at 94 °C, 1 minute at 65 °C, and 1 minute at 72°C (reducing annealing temperature by 0.7 °C/cycle), 25-cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 1 minute at 72°C, and finished with 10 minutes at 72 °C. The selective amplification fragments were diluted 1:10 with distilled water and one µl of the diluted fragments was transferred to a 96 well plate and allowed to air dry before being sent to Arizona State University for capillary electrophoresis with 0.3 µl LIZ-600 size standard (Life Technologies) per sample. Peaks were visualized using GeneMarker® (Softgenetics, LLC, State College, PA, USA) and polymorphic bands were scored as

present (1) or absent (0). Only bands in size ranges of 75 - 600 were used for all the primer combinations.

2.3.4 Data Analyses:

For the morphological data set, a one way Analysis of variance (ANOVA) was run in R using the function *aov* (R Development Core Team 2012) to determine if there were significant differences among the taxa for each continuous trait. Additionally, clustering analysis was used to look for natural breaks in the morphological data set that would correspond to each of the species. A non-hierarchical method for clustering, using *kmeans* function in R (R Development Core Team 2012) was conducted. The data were standardized by standard deviation such that each trait was equally weighted. K-means uses an a priori identified number of groups and utilizes an optimality criterion to fit the data within those groups. The sum of squares within each group is calculated and assigned to the predefined number of clusters to assess the best fit (Everitt 2005; Knaus 2008). As the number of groups increases, the sum of squares should decrease and the optimal group number is identified by a sudden reduction in the sum of squares. To choose the most probable number of clusters represented by the data, an iterative method using the “calinski” criterion was conducted using the *cascadeKM* function in the vegan package of R software (R Development Core Team 2012). The "calinski" criterion is an analysis of variance statistic that compares the sum of squares among groups relative to the within group sum of squares. The value is plotted with respect to the cluster solutions, and the maximum value defines the number of groups, thereby providing the best *k means* solution. The null hypothesis is that *k* clusters are not significantly different and the larger the value of the calinski criterion, the better a group solution. I chose the

"calinski" criterion from the other available criteria as it has been suggested to be able to provide the best solution after comparing it with 30 other such criteria (Milligan and Cooper 1985; R manual for Vegan library function cascadeKM; Oksanen et al. 2009). Principal components analysis was also performed using the *princomp* function in R (R Development Core Team 2012) to visualize the groups as defined by the calinski criterion.

To estimate genetic differentiation and genetic variation between the two taxa, 85 specimens from the 201 were used (to reduce sample size bias). Only 15 specimens were assigned as *H. ravenii* as described by Jackson (1962), whereas 70 were assigned to *X gracile*, and these represented different geographic locations. GenAlEx 6.0 (Peakall and Smouse 2001) was used to generate diversity statistics (i.e., percentage polymorphic loci, number of private bands, heterozygosity and Shannon's Index) and to conduct a hierarchical Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) to understand the partitioning of the genetic variation between groups. Polymorphic bands that occur in a single group are referred to as private bands. To understand genetic relatedness among samples, a NJ tree was constructed using the AFLP data binary matrix based on Nei-Li distances (Nei and Lei 1979) in PAUP* v 4.0b10 (Swofford 2002), and a bootstrap (Felsenstein 1985) analysis was also conducted in PAUP* with 500 replicates to assess support of clusters. The consensus tree was visualized in FigTree v1.3.1 (Rambaut 2012).

2.4 Results

Results from ANOVA suggested that out of the four quantitative traits, none showed significant differences between the two groups (Figure 2.2). Both *H. ravenii* and

X. gracile had similar leaf lengths (10.13 mm and 10.45 mm, respectively). The number of teeth on the leaf surface was higher in *X. gracile* than *H. ravenii* (11.97 vs. 9.33), but this was not significant. Similar trends were observed in phyllary number and leaf width (Table 2.2). The stiff/ appressed phyllary pubescence and moderate/ sparse density of stem pubescence were present in both taxa, not providing a clear differentiation between the two based on these characters. Lack of seeds in all the herbarium specimens limited the use of pappus and achene lengths as diagnostic characters. K-means analysis revealed a smooth curve, which does not have a natural break point, suggesting a single cluster (Figure 2.3). This was further confirmed using the "calinski" criterion, which suggested a single cluster best, fits the data set (Figure 2.4). The PCA plot also lacks clear distinction of *X. gracile* and *H. ravenii* points (Figure 2.5). The blue dots represent the individuals, which occur in Yavapai county, where Jackson (1960) had described the type specimen for *H. ravenii.* When ratio of leaf length and width was used instead of actual values, the inferences from the data set were not different.

The AFLP primer combinations resulted in the generation of 856 loci scored between 75 and 500 bp. The number (frequency) of private bands detected in *X. gracile* and *H. ravenii* was 400 (0.47) and 22 (0.04) private bands, respectively. The percentage of polymorphic loci was 97.43% in *X. gracile* and 53.27% in *H. ravenii.* Shannon's Information Index (I) was 0.158 and 0.133 in *X. gracile* and *H. ravenii,* respectively. These data are summarized in Table 2.2. The Analysis of Molecular Variance (AMOVA) revealed that most of the variation was contained within groups (98%, $p<0.05$) rather than between groups (2%, $p<0.05$; Table 2.5). The NJ tree revealed no distinct clustering of the two species (Figure 2.5).

2.5 Discussion

Jackson (1960, 1962, 1965) conducted different studies to characterize the genetics of *X. gracile* and suggested that *H. ravenii* was an ancestral race from which *X. gracile* was derived, hence suggesting a progenitor-derivative relationship between the two. The taxonomic placement of *H. ravenii* as a separate species has been questioned (e.g., Cronquist 1971) ever since Jackson and Crovello (1971) initially proposed the independent species recognition. *Haplopappus ravenii* has not been recognized in the recent treatments of this group (Morgan and Hartman 2003).

The data and criteria used in this study do not support recognition of *H. ravenii* at the species level. According to the morphological species concept, a species is described as a group of individuals that differ from other groups by possessing constant diagnostic characters. In their taxonomic evaluation of the two taxa, Jackson and Crovello (1971) used an extensive array of morphological characters but had a limited number of samples, including one population sampled each from Utah and California. According to the criteria of the MSC, I expected to see distinct clusters corresponding to the individuals from Yavapai county AZ, Utah and California, which were described as the range of *H. ravenii* by Jackson and Crovello (1971). However, no such support for distinct clusters was found based on the morphological characters. Jackson (1962) defined phyllary pubescence as one of the important distinguishing factors, with *X. gracile* exhibiting long appressed hair in comparison to *H. ravenii* with shorter, stiffer hairs. According to Jackson and Crovello (1971), "Phyllary pubescence is a qualitative character always associated with the *H. ravenii* karyotype, this one morphological character is a better discriminator than the 28 other characters in separating the races of *H. ravenii* from those

of *H. gracilis*". However, in the current study no such clear demarcation was found. Instead, multiple individuals in both taxa had this character state.

Because morphology can be misleading of underlying genetic diversity or evolutionary history, I also tested for genetic differentiation in the data set. If *H. ravenii* samples exhibit unique genetic markers, then they could be considered at the rank of species according to the genotypic cluster criterion (Mallet, 1995). There is little evidence in the genetic data set to support that *H. ravenii* is distinct from *X. gracile.* No distinct clusters were identified in the NJ analysis (Fig. 2.5), the two groups exhibited similar levels of genetic diversity (Table 2.4), and AMOVA indicated a much greater amount of genetic diversity within (98%, $p<0.05$) than among groups (2%, $p<0.05$).

Species complexes exhibiting phenotypic, genetic, and/or cytological variation can be problematic for a stable taxonomy. Several similar studies have been conducted in other plant taxa, which address questions raised in this study. For example, studies of the wild potato complex (Alvarez et. al. 2008 and Fajardo et al. 2008), which consist of morphologically similar species, resulted in a collapse of a number of taxa at the species level for lack of support in morphological and AFLP datasets. In a study conducted by Pelser and Houchin (2004), morphological and genetic analyses of two species of *Senecio* indicated major overlap across characters leading to the suggestion that the varietal rank was the best taxonomic solution for this complex.

In light of the lack of support for sufficient divergence across both morphology and genetic data in this study, the only major difference between the two taxa appears to be the difference in chromosome number and the hypothesized aneuploid reduction from *H. ravenii*. Jackson (1962) in his experimental crosses in the green house found that

hybrids were as robust as the parents and "were intermediate in characters separating the species", however the pollen fertility was on an average only 6.9%. Taxonomists frequently consider cytological variants to be distinct taxa, especially if they are able to maintain cytologically stable populations, reflecting reproductive isolation. In order for any taxonomic consideration, it is important to have strong support in terms of divergence in more than one type of data. The presence of the varied chromosome number in this case lends only support to the taxa being called cytotypes or karyotypic races that occur in their own geographic range. However, if these chromosomal differences lead to possible reproductive isolation between the two races, under the definition of biological species concept, they may be defined as separate species (sibling species; Mayr 1942). Chromosomal rearrangements and the process of apparent aneuploid reduction of the chromosome number from the higher karyotype can lead to translocations which can create barriers to reproduction, producing sterility in the offspring (Rieseberg 2001).

2.6 Conclusion

Both the morphological data, which were based on the same characters used by Jackson and Crovello (1971), and the genetic data fail to provide support for the separate species status of *H. ravenii*. The presence of different chromosome numbers along with the highly sterile hybrids with intermediacy in characters separating the two suggests that they are cytotypes of *X. gracile*. Although the genetic differentiation between the two taxa is statistically significant in an AMOVA, I do not consider this sufficient to warrant their recognition as separate species.

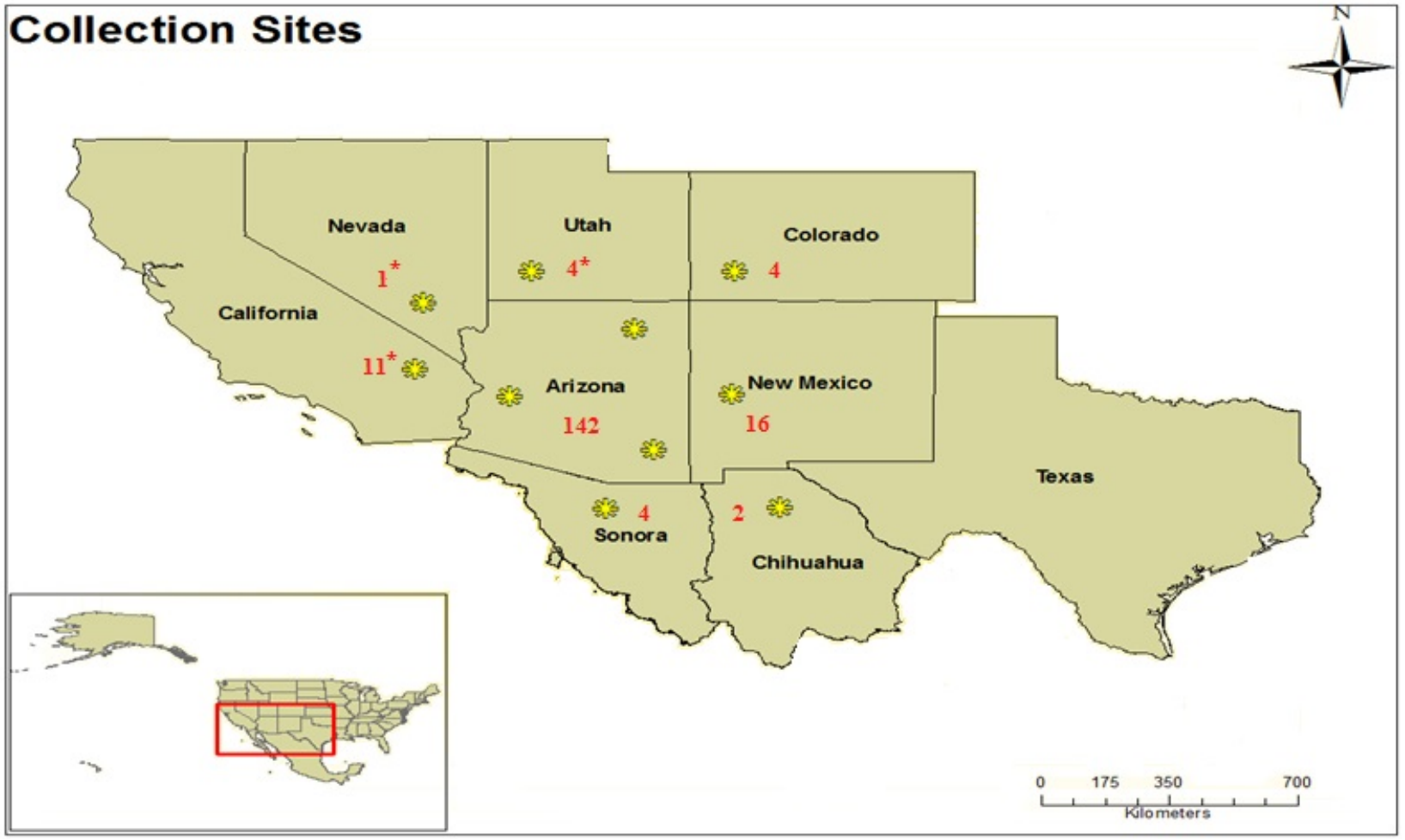

Figure 2.1 Sample distribution of *Haplopappus ravenii* and *Xanthisma gracile*.

Table 2.1 Number of samples of *Haplopappus ravenii* and *Xanthisma gracile* from their geographic range.

Location	***H. ravenii***	***X. gracile***
Arizona, US	-	142
California, US	11	-
Colorado, US	-	4
Mexico	-	6
New Mexico, US	-	16
Utah, US	4	-

Table 2.2 Means of Morphological measurements measured across herbarium specimens of *H. ravenii* and *X. gracile*

Character	***H. ravenii***	***X. gracile***
Phyllary Number	3	3.5
Leaf length (mm)	10.1	10.4
Leaf Width (mm)	0.9	1.0
Number of teeth	9.3	11.9

Table 2.3 Estimates of genetic diversity between *Xanthisma gracile* and *Haplopappus ravenii*

Variety	**Sample Size**	**Percentage Polymorphism**	**Number of Private Alleles**	**Nei's gene diversity**	**Shannon Index**
Gracile	70	97.43%	400	0.079	0.158
Ravenii	15	53.27%	22	0.075	0.133
Mean	13.33	75.35%	211	0.077	0.146

Table 2.4 Analysis of Molecular Variance (AMOVA) using the herbarium specimens of *Xanthisma gracile* and *Haplopappus ravenii*.

Source	Df	SS	MS	Estimated Variance	% Of total variation	Phi	P-value
Among taxa	1	81.245	81.245	0.899	2%	ϕ_{ST} =0.015	<0.05
Within taxa	84	4858.488	57.839	57.839	98%		
Total	85	4939.733		58.738	100%		

Note: P-value estimate based on 9999 permutations. df= degrees of freedom, SS= sum of squares, and MS= mean squared deviations

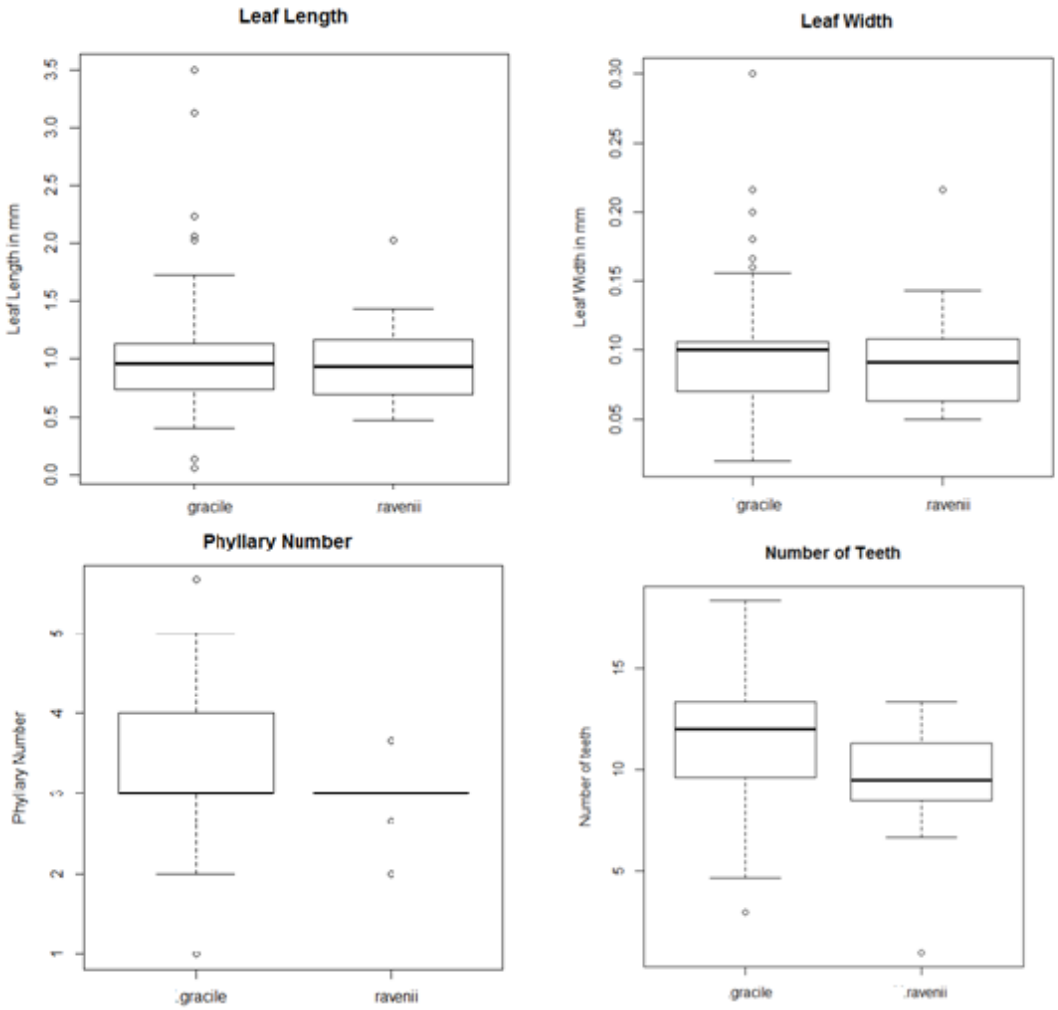

Figure 2.2 Boxplots of quantitative morphological characters between *Xanthisma gracile* (168) and *Haplopappus ravenii* (15) using ANOVA. None of these characters showed significant difference between the two taxa.

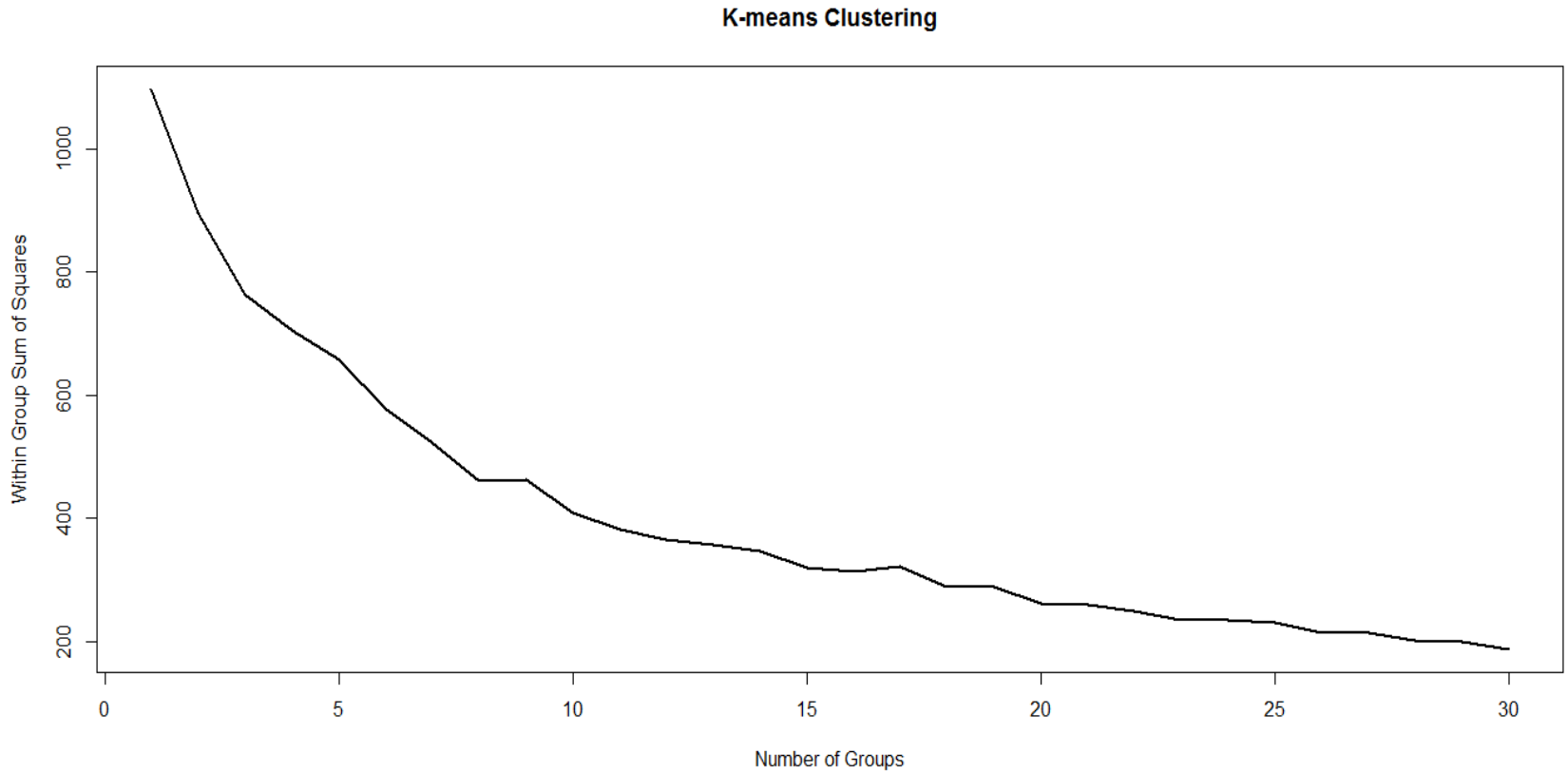

Figure 2.3 K-means clustering method depicting the number of groups based on within group sum of squares.

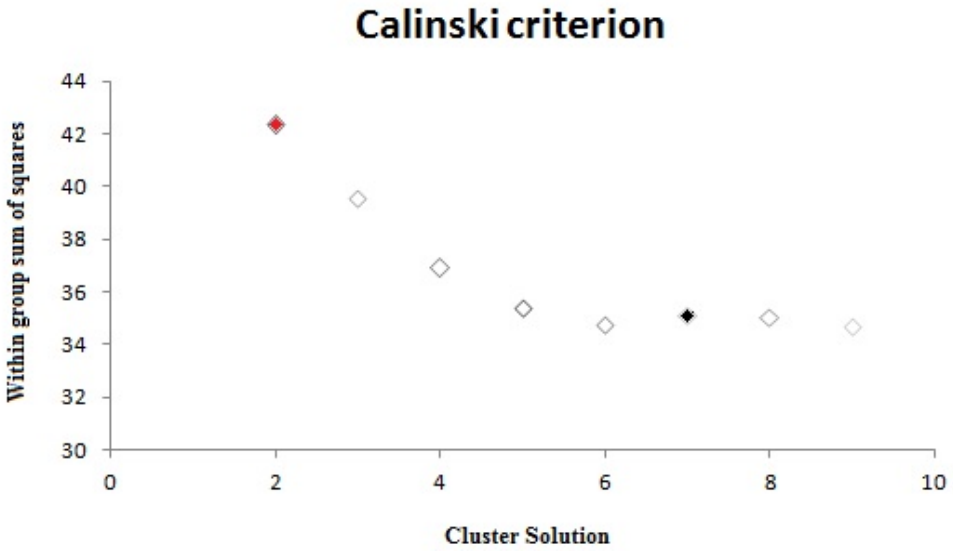

Figure 2.4 K-means clustering method utilizing the stopping rule of calinski and Harabasz ("calinski criterion") using the function cascadeKM.

Note: The red dot represents the optimum cluster solution , where the within group sum of square value is maximum. The black dot represents partitions that can increase the value of the criterion.

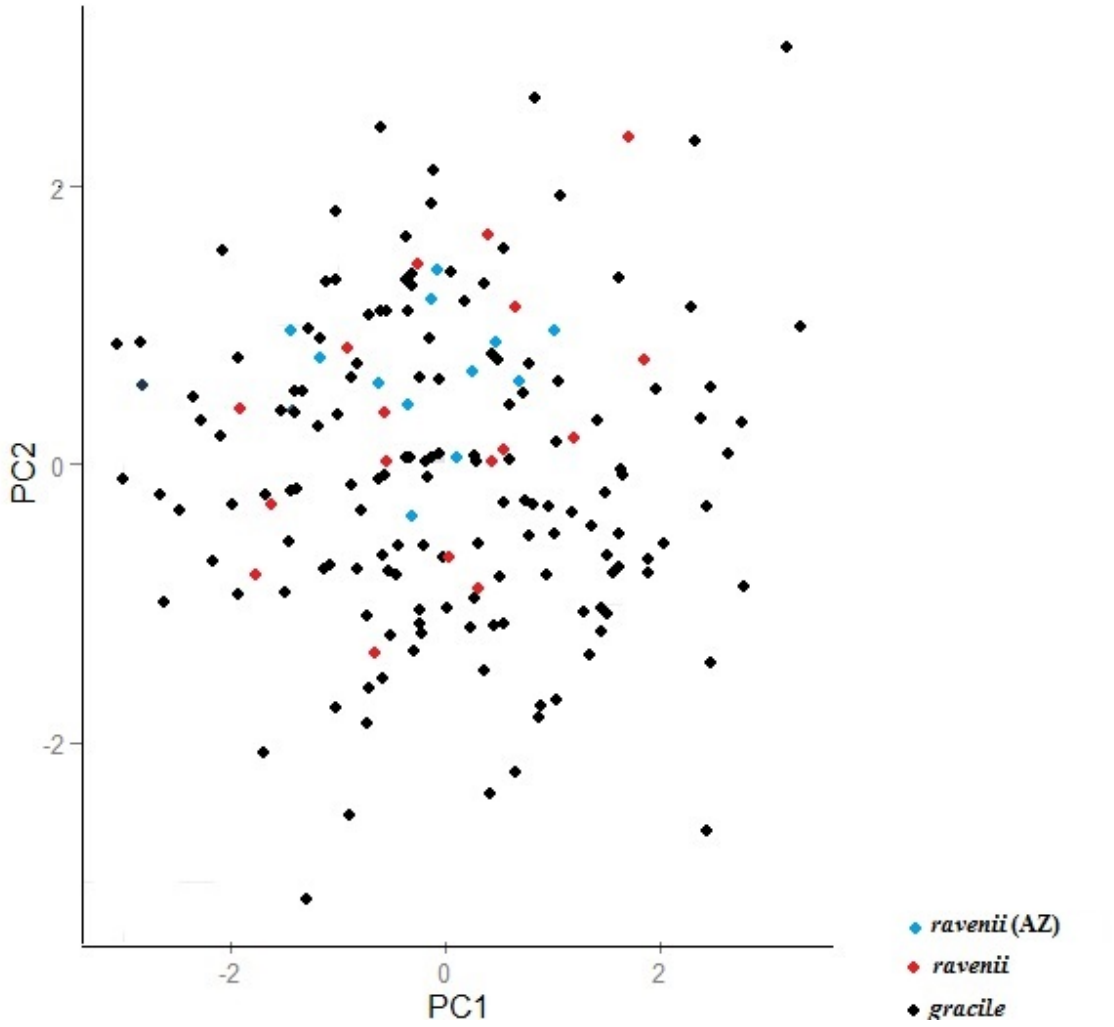

Figure 2.5 Scatter plot of Principal components #1 and #2 (PC1 and PC2). Combined these two components explain 47.84% of the morphological variation.

Note:- Blue points refer to the individuals occuring in Yavapai county of AZ, where the type specimen for *H. ravenii* was identified by Jackson (1960)

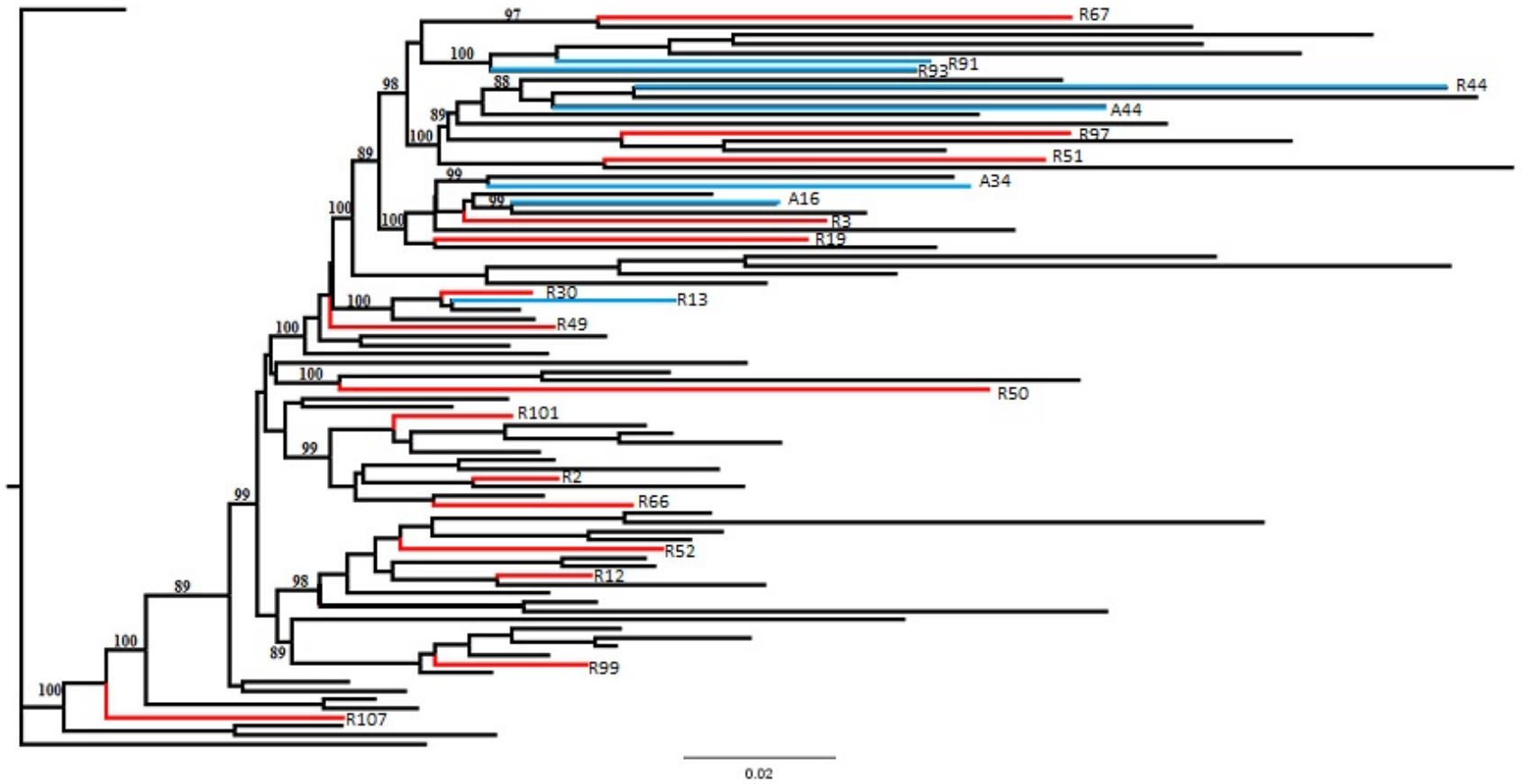

Figure 2.6 Neighbor joining tree based on the AFLP presence/absence binary matrix depicting the genetic relationship of *X. gracile* and *H. ravenii*. Color-coding is the same as that used for principal components analysis. The numbers on branches represent bootstrap values >80% and the tip labels indicate the specimen description as outlined in tables A.1 and A.2.

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CHAPTER III

PATTERNS OF GENETIC DIVERGENCE ACROSS GEOGRAPHICALLY VARIABLE POPULATIONS OF *XANTHISMA GRACILE*

3.1 Abstract

Numerous biotic (e.g., grazing pressure, breeding system) and abiotic factors (e.g., soil chemistry, water availability, light conditions, temperature differences) can contribute to local adaptation in plants. This often leads to geographic structure and genetic divergence between populations, and with continued isolation, new evolutionary lineages can arise. The southwestern U.S. contains many distinctive plant communities, ranging from woodlands to desert scrub, that are shaped by species adapting to environmental variation in elevation, precipitation, seasonality, and soils. Given this environmental variation, I expect that species that have achieved wide distributions will exhibit evidence of local adaptation to different habitats. Here, I test whether there is significant genetic divergence within *Xanthisma gracile* (Asteraceae) across Arizona. Populations occur in a variety of habitats, including desert grasslands at low altitudes to open pine forests at intermediate altitudes, and exhibit phenotypic variation in plant height, leaf shape and pubescence, and floral traits. These variants have been previously named races, varieties and even distinct species by some authors, but the nature of these differences has not previously been quantified. I sampled 16 populations across Arizona and collected data from 18 additional herbarium specimens to augment samples from the

desert region. Using Amplified Fragment Length Polymorphisms (AFLP's), data were collected to test for evidence of genetic structure corresponding to geographic and environmental variation. Analysis of Molecular Variance revealed a moderate amount of genetic variation (ϕ_{RT}= 0.122, p<0.01) among regions, which were defined as northern, central, and southern. Most of this differentiation is due to genetic distinctiveness of the central samples, which occur in desert habitats. Average genetic distance was 0.28 between the central samples and those in the north, 0.20 between central and southern, and 0.01 between northern and southern. The higher degree of differentiation of the desert samples may indicate differential selection for surviving in a dry habitat versus the more mesic areas of the northern and southern regions.

3.2 Introduction

Plants tend to locally adapt and modify their phenotypes to divergent climates more readily than animals because of their sessile nature (Schlichting 1986). Plants with wide distributions may be expected to have increased levels of genotypic and phenotypic variation compared to more restricted species as a result of local adaptation (Bradshaw 1984). Leimu and Fischer (2008), in a metaanalysis of local adaptation in plants, concluded that large populations are more susceptible to the effects of local adaptation than those with a limited distribution.

Genetic variation allows for population survival and reproduction in new and changing environments (Huenekke 1991, Hamrick et al. 1991, Holsinger and Gottleib 1991, Barrett et al. 1991). Comparisons of a species' genetic diversity and divergence patterns across large connected populations, rather than isolated populations, may provide insight into understanding the distributional responses of species to changes in climate

and other biotic and abiotic factors (Petit et al. 2003; Vandewoestijne et al. 2008; Kunin et al. 2009; Sepulveda-Villet & Stepien 2012). Changes in geography and climate affecting the landscape and habitat A wide range of disturbances in the landscape and habitat created by changes in the geography and climate provide an excellent opportunity to study the effect of these changes on the genetic structure and variability within and among populations. Linhart and Grant (1996) have reviewed how natural forces, including soil characteristics, grazing, temperature, and parasites, play key roles in shaping patterns of phenotypic differentiation and underlying physiological and biochemical diversity associated with life history and breeding system variation. Specifically, that natural selection is primarily involved in shaping the genetic variability of plant populations. Investigating the genetic basis of these adaptations of the species to their environments continues to be an important focus in evolutionary biology (Nielsen 2005). As populations adapt to novel environments, ecotypes are formed that are suited for the new conditions but are not reproductively isolated from each other (McNair 1992; Gibson and Pollard 1988; Clausen et al. 1948). Population genetic diversity is vital for the survival and sustainability of species and the rate of evolutionary change (Wimp et al. 2004; Bagley et al. 2002; Falk and Holsinger 1991) and is essential for adaptation to occur (Reed et al. 2003; Clausen 1951; Clausen et al. 1948).

In the environment there is a balance between forces that increase genetic diversity (e.g., mutation and gene flow) and those that reduce it (e.g., selection and drift) (Leimu and Fischer 2008; Eckhart et al. 2004; Knight and Miller 2004; Clausen 1951). When local adaptation resulting from natural selection is stronger than gene flow or genetic drift, it can affect the patterns of genetic differentiation in accordance with

environmental variables (Wright 1951). The importance of local adaptation in populations has been documented in a number of studies (Knight and Miller 2004; Kawecki and Ebert 2004; Bonin et al. 2006; Raabová et al. 2007; Kronholm et al. 2012).

Since genetic diversity affects survival and fitness of a species, measuring such diversity provides insights into the way a species will respond to environmental change (Bagley et al. 2002; Bush and Barrett 1993). The presence of geographic barriers tends to restrict gene flow leading to limited adaptation of a taxon to its environment. When combined with differential selection pressures, the restricted gene flow may lead to formation of new species, following genetic divergence of the taxa into polymorphic species (Schultz and Soltis 2001; Schluter 2001; Funk et al. 2006).

Xanthisma gracile (Nutt.) D. R. Morgan and R. L. Hartman, also known as the Yellow Spiny Daisy or slender goldenweed, is a member of the tribe Astereae in the family Asteraceae. This species flowers from late summer through fall and is widely distributed in the southwestern U.S. in Arizona, California, Colorado, Nevada, New Mexico, Texas, and Utah (USDA NRCS 2012) as well as northern Mexico (Jackson 1960). The southwest and specifically, Arizona, is currently defined by a number of vegetation types (Akin 1991). These vegetation types are extremely diverse, and their distribution can generally be attributed to changes in elevation, rather than to changes in latitude and longitude (Bahre 1991; Akin 1991). Vegetation types include grasslands, desert scrub, evergreen or pinyon juniper woodlands, ponderosa pine and mixed conifer forest, and riparian wetlands (Bahre 1991). Within Arizona, *X. gracile* occurs in at least three different habitats (Fig. 3.1). The semi desert grasslands of southern Arizona, which are found at elevations of between 3000 and 5500 ft, are characterized by the presence of

shrub like flora. The desert scrub of central Arizona is further divided into the Sonoran desert scrub and the Chihuahuan desert scrub. The former is mostly found in the upland sites of Arizona and characterized by the presence of a wide variety of succulents. The Chihuahuan desert scrub occurs above elevations of about 3500 ft on calcareous soils through southern Arizona. The third habitat is the pinyon- juniper woodlands found at elevations between 4000 - 7000 ft in Arizona and the density of these woodlands increases with elevation. Given this wide range of habitat variation, this study aims at understanding the extent of genetic diversity of *Xanthisma gracile* within Arizona and tests for genetic structure associated with its occurrence in diverse habitats (Jackson 1962, 1973; Jackson and Crovello 1971). Comparison of population genetic diversity across these divergent habitats provides important data for understanding its response to changes in habitats and other biotic and abiotic factors. In this study, I test the hypothesis that given the widely different habitats (pinyon-juniper woodlands, desert, grasslands) in which *X. gracile* occurs in Arizona, it is expected that there will be strong differences among populations. Furthermore, it is expected that gene flow between populations is limited by distance such that populations within a given area will be more similar to one another than populations from different areas. Finally, in comparison to other plant species with similar life history traits, it is expected that *X. gracile* will also exhibit high genetic diversity reflective of its need to adapt to these variable habitats.

3.3 Materials and Methods

3.3.1 Sample Collection

Seeds and leaf tissue from 16 populations of *X. gracile* were sampled across Arizona in 2008 and were used for assessing genetic variation in this study. The seeds

were maintained in dry conditions at 4˚C until germination, and the leaf tissue was preserved at -80˚C. Seed viability reduced over time leading to reduced germination rates. Thus, sample sizes are not even across populations. Samples were obtained from three geographic regions: eight populations from the north (woodland region), seven populations from the south (grassland) and one central population (desert scrub). Voucher specimens from each population are deposited at the Mississippi State University Herbarium. To supplement samples from the central desert scrub region, leaf tissue was also collected from 21 herbarium specimens of *X gracile* from Arizona State University Herbarium. Populations and their geographic coordinates are listed in Appendix B1 and Figure 3.1.

3.3.2 DNA Extraction

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA.) or a modified CTAB DNA extraction protocol (Doyle and Doyle 1987) from frozen leaf tissue, herbarium specimens, or 10- day old germinated seedlings. After extraction, DNA was dissolved in TE Buffer and run on a 1.5% agarose gel to check quality.

3.3.3 AFLP Analysis

AFLP analysis was performed using a protocol modified from Vos et al. (1995) and incorporating the recommendations made by Trybush et al. (2006). Individual genomic DNA was digested in 30µl reactions incubated at 37 °C for 1 hour in a thermal cycler, followed immediately by ligation of the linkers. Restriction digest enzymes and reagents utilized per reaction were: 0.25 µl of EcoRI (20,000 U/ml, New England BioLabs, Ipswich, MA, USA), 0.5 µl of MseI (10,000 U/ml, New England BioLabs,), 3

µl 10X NEBuffer2, 0.15 µl of 100 µg/ml BSA, 5 µl of individually purified genomic DNA, and 21.1 µl of sterile water. Eco AFLP linkers were annealed in a thermal cycler by heating to 65 °C for 10 minutes and cooled to room temperature using the following reagents: 10 µl of Eco Linker 1 (100 µM, 5'-CTC GTA GAC TGC CC) and 10 µl of Eco Linker 2 (100 µM, 5'-AAT TGG TAC GCA GTC TAC). Mse AFLP linkers were also prepared using the above procedure utilizing 10 µl of Mse Linker 1(100 µM, 5'-GAC GAT GAG TCC TGA G) and10 µl of Mse Linker 2 (100 µM, 5'-TAC TCA GGA CTC AT). Annealed linkers were stored frozen at -20 °C until use.

Ligation of Eco and Mse linkers was conducted in 40 µl reactions, including 0.1 µl Eco and 1 µl Mse Linker (as annealed above), 0.15 µl T4 DNA Ligase enzyme and 4 µl 10X T4 DNA Ligase Reaction Buffer (New England BioLabs), 30 µl digested DNA, and 4.75 µl sterile water. Ligation reactions were incubated in a thermal cycler at 37 °C for 4 hours followed by storage at -80 °C to prevent degradation. Pre-selective amplifications were conducted in 10 µl reactions using 0.5 µl each Eco+A (10 µM, 5'-GAC TGC GTA CCA ATT CA) and Mse+C (10 µM, 5'-GAT GAG TCC TGA GTA AC) primers, 1.25 µl dNTPs (2 mM dATP, dCTP, dGTP, and dTTP; New England BioLabs), 2 µl 5X *GoTaq*™ FlexiBuffer (Promega Corp Madison, WI, USA), 1.25 µl MgCl2 (Promega Corp.), 2.5 µl individually ligated DNA, 2.55 µl sterile water, and 0.1 µl *GoTaq*™ DNA polymerase (5u/µl, Promega Corp.). Pre-selective amplifications consisted of an initial denaturing step of 65 °C for 5 minutes, 30-cylces of 30 seconds at 94 °C, 30 seconds at 56 °C, and 1 minute at 72°C.

Pre-selective amplification products were individually diluted 1:20 with sterile water. Selective amplifications consisted of a single Mse primer with three fluorescent-

labeled Eco primers per reaction. Selective amplification for all individuals was conducted in 10 µl volume consisting of 0.7 µl each Mse-CAG (5µM), Eco-ACT FAM, Eco-ACC NED and Eco-AGG VIC (1 µM) selective primers, 0.5 µl dNTPs (2 mM dATP, dCTP, dGTP, and dTTP; New England BioLabs), 2.5 µl LongAmp™ Buffer (New England Biolabs), 1 µl of diluted pre-selective amplification product, 2.7 µl sterile water, and 0.5 µl *LongAmp Taq*™ DNA polymerase (5u/µl, New England Biolabs). Selective amplifications consisted of an initial denaturing step of 95 °C for 15 minutes, 13-cylces of 30 seconds at 94 °C, 1 minute at 65 °C, and 1 minute at 72°C (reducing annealing temperature by 0.7 °C/cycle), 25-cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 1 minute at 72°C, and finished with 10 minutes at 72 °C. Each sample was diluted 1:10 with sterile water and then 1 ul was mixed with 8.7 µl of formamide (Hi-Di™, Life Technologies, Carslbad, CA, USA) and 0.3 ul LIZ-600 size standard (Life Technologies) before being sent to Arizona State University for capillary electrophoresis. Peaks were visualized using GeneMarker® (Softgenetics, LLC, State College, PA, USA) and polymorphic bands were scored as present (1) or absent (0). Only bands in size ranges of 75 – 600 base pairs were used in data analyses. Four samples were consistently run on all plates to test for repeatability of banding patterns.

3.3.4 Data Analysis

Genetic diversity for 16 populations of *X. gracile* was quantified by determining the percentage polymorphic loci, number of private bands, Nei's gene diversity and Shannon Index using GenAlEx ver.6.0 (Peakall and Smouse 2006). Polymorphic bands that occur in only one population are referred to as private bands. Heterozygosity, a measure of genetic diversity, was averaged over each population. To estimate the

frequency of rare bands, which accumulate over time and indicate old divergence (Schönswetter & Tribsch 2005), the Rarity Index or DW (frequency- down weighted marker values) was calculated using the R- script AFLPdat (Ehrich 2006, last modified 23 January 2008) in R ver. 2.15.0. For each individual each AFLP band is divided by the total number of occurrences of this band in the dataset. These relative values are then summed up to the rarity index for this individual. Population values are then estimated as the average of these individual values (Ehrich et al. 2008).

For ascertaining genetic variation among regions and genetic distance between regions, Nei's pairwise unbiased genetic distances (Nei 1972; GenAlEx v.6.3) and pairwise F_{ST} in Arlequin v.3.5 (Excoffier and Lischer 2010) were calculated. This intraspecific examination also evaluated among-population genetic variation and structure. Genetic differentiation among regions and populations was assessed by analysis of molecular variance (AMOVA; Excoffier et al. 1992) using GenAlEx ver. 6.0 (Peakall and Smouse 2006). Two different AMOVA's were performed; the first three level hierarchical analysis utilized population data to assess genetic differentiation among the three regions (pinyon juniper woodlands, desert, grasslands), populations within each region and individuals within the populations. The second two level AMOVA included data from the herbarium specimens and was conducted to analyze the partitioning of molecular variance among and within the regions. Relationships among populations were visualized by constructing a dendogram based on the neighbor joining (NJ) clustering algorithm on the original presence/absence matrix based on Nei–Li distances (Nei & Li 1979) in PAUP* 4.0b10 (Swofford 2002).

3.4 Results

3.4.1 Genetic Diversity within populations of *Xanthisma gracile*

To reduce genotyping error and ensure repeatability of samples four individuals were run along with each 96 well plate for the entire data set. The repeatability of the bands for the multiple runs ranged from 96%- 98.5%. A total of 754 polymorphic loci from 3 primer combinations were scored in 264 individuals. The percentage of polymorphic loci within a population ranged from 2.12% to 55.31% with an average of 26.49% (Table 3.1). Population X_01 from the central desert region exhibited the highest gene diversity (h= 0.126), which was followed by X_11 in the southern grasslands region. The southern region also contained the population (X_12) with the lowest gene diversity (h= 0.007). The number of private alleles per population ranged from 0 to 29 (Table 3.1). Population X_08 from the northern-forested region had the highest number of private bands (29) in comparison to the X_01 (28) even though it had a lower genetic diversity than the latter population. Population X_01 also had the maximum Shannon index (0.201) than all the other populations (Table 3.1).

The addition of the herbarium specimens lends more support to the high gene diversity maintained within the central region (h = 0.127), followed by the northern (h =0.074) and southern (h = 0.045) region, although the northern region had the maximum number of private bands. The Shannon index was also higher for the central population (0.202) compared to the other two regions.

3.4.2 Genetic differentiation and structure among the three regions.

Populations of the northern and southern region were found to be much more closely related (F_{ST}=0.17) than the central and northern populations (F_{ST} =0.31) or central

and southern populations (F_{ST} =0.31). Furthermore, the genetic rarity index DW was much higher in the central population (0.3636) than an average of either the northern (0.1071) or southern (0.0519) populations. The three level AMOVA revealed that variation between regions (ϕ_{RT}= 0.073; Table 3.2), is higher than between populations within regions (ϕ_{PR}= 0.045) even though most of the variation occurs within populations (ϕ_{PT}= 0.115). With the addition of the herbarium samples from the central desert area within-region variation is higher than variation among the regions (ϕ_{PT}= 0.122, Table 3.3). All of these differences are significant with a p-value <0.01. Also, when pairwise F_{ST} was compared between the regions, the central and the northern region were the least similar with F_{ST} = 0.28251 and the northern and southern region much more similar to each other (F_{ST} = 0.01704, Table 3.4) than either of them were to the central samples. The NJ dendogram also shows that the desert population was much differentiated in comparison to the northern and southern, which were interspersed throughout (Figure 3.3). The central population is distinct from the cluster that contains the northern and southern populations. From the principal components analysis the central X_01 population separates out from the other two in the bottom right quadrant. The populations from the northern and southern region cluster together towards the left of the plot (Figure 3.4).

3.5 Discussion

The distribution of genetic variation within and among populations is influenced by the characteristics of the species (Hamrick 1989). Each plant species has a unique combination of life-history traits, historical factors, and habitat preferences that can influence patterns of genetic variation within and among populations (Vellend and

Waterway 2000). For example, the mating system of the species determines the extent of gene flow in the population, which in turn influences the amount of genetic variation within and between populations. Marked differences in genetic variation can therefore occur over short distances in populations, resulting in a non-random distribution of genetic variation (Hamrick 1989). These forces all interact to influence the amount of genetic variation in a population. In *X. gracile*, the average among population differentiation value (ϕ_{PT} = 0.112) was closer to the number reported for outcrossing species (mean ϕ_{PT} =0.27) than to values associated with mixed mating or inbreeding (mean ϕ_{PT} = 0.40 and 0.65 respectively) (Nybom 2004).

In species having a large range, the interaction of gene flow and exposure to varied environmental selection pressures creates a multiscale structure. Limited gene flow, combined with large distances between populations, tends to increase genetic differentiation among populations (Slatkin 1977; Hutchinson and Templeton 1999). Within its geographic range *X. gracile* occurs in a variety of habitat and soil types and experiences variation in precipitation levels (Figures 3.1, 3.2) and amounts of disturbance conducive to reduced gene flow. This suggests that genetic drift may be driving the genetic differentiation of populations. Population X_01 occurs in a region that experiences higher mean temperature (>70˚F) in comparison to the north (50-51˚F) and south populations (60-65˚F). It also has lower precipitation levels (~12 inches) when compared to the precipitation levels of the other populations (30-45 inches). Monson and Szarek (1981) compared the life cycle characteristics of two cytotypes of *X. gracile,* the desert race and the mesic foothills race and found several differences in their response to varied environmental pressures. For example, in the field study, they found that the mesic

race had a lower mortality than the desert race. When grown in a common garden experiment setting, the desert race seemed to exhibit better life cycle characteristics in comparison to the mesic race. Thus, variable selection pressures may contribute to the maintenance of genetic variation and enables this species to exploit a broad spectrum of environmental conditions.

The effect of geographical variation has been studied previously in a cytotype of *X. gracile*, across four geographically isolated populations (Matos 1979). Based on the limited sampling and geographic range the study concluded that both morphological and genetic divergence had taken place in those four isolated populations, to adapt to the different selection pressures imposed by the environment. The present study has expanded population level sampling and provides a better understanding of the effect of climatic changes and habitat pressure on genetic variation between the populations of *X. gracile*.

From the analysis of diversity statistics (Table 3.2) the central region exhibited higher diversity when compared to the northern and southern regions. The central populations of Arizona occur in an extremely xeric habitat with the primary vegetation being upland desert scrub. Species in these harsh environments are typically experiencing continuous selective pressures, especially for drought tolerance, to ensure their sustainability, which could lead to the observed high levels of diversity. The strong level of differentiation between the desert samples and other samples in Arizona suggests that gene flow between regions is not very likely. This inference is supported by the maintenance of private alleles within the central region (Table 3.2).

The level of polymorphism found in *X. gracile* is similar to that detected in other plants when the AFLP technique was used for measuring genetic variability. Kraus (2000) suggested that outcrossing species generally have > 75% polymorphic loci, whereas selfing or clonal species have <50% and very often it is <10% (Haldimann et al. 2003, Ramakrishna et al. 2004). The frequency of rare bands (DW) was higher in population X_01 (0.36367; Table 3.1), which occupies the desert habitat, than the other populations. This suggests that it may have a long history of being separated from other sampled populations and is consistent with the progressive aridification of Arizona from pinyon juniper woodlands (Schönswetter & Tribsch 2005). The central population might have originated in the woodland region but due to the continuous selective pressures exerted by the extreme habitat shift was able to significantly differentiate from the rest of the sampled populations. Hence, the combination of habitat change and isolation of the populations has likely contributed to the differentiation of the populations and generation and maintenance of genetic variability between them.

This conclusion can be supported by the abundant literature describing the changes modern day Arizona has undergone since the Pleistocene era. The impact of Pleistocene climatic fluctuations in the arctic and temperate mountain ranges has been investigated in both plants and animals (Hewitt 1996; Hewitt 2001; Schönswetter et al. 2005; Rebernig et al. 2010). During the wetter and cooler pluvial period, the desert vegetation, as suggested by paleoclimatic and vegetation data, was strongly restricted to the lower Colorado River basin and the plains of Sonora and southern Chihuahua Deserts (van Devender & Spaulding 1979; Thompson and Anderson 2000; Van Devender 1990; Hunter et al. 2001). Aridification began at the end of the last glacial maxima (McClaran

and Van Devender 1995; Musgrove et al. 2001; Holmgren et al. 2007). Progressive drying resulted in the conversion of historical woodlands (Van Devender 1977) to semi-desert grassland to present day desert scrub (Nielson 1986). Based on the amount of diversity described and genetic differentiation of the desert region populations as suggested by the AFLP data, lends support to the overall genetic divergence encountered in *X. gracile*. There has been increasing evidence that suggests post-Pleistocene several plant populations have had to undergo variation leading to genetic differentiation within populations to survive the glaciation cycle (Rebernig et al. 2010; Slovák et al. 2010; Allen et al. 2012).

3.6 Conclusions

Xanthisma gracile was found to have a high within population genetic diversity, which is consistent with other such out crossing species. The populations are spread across a variety of climate and habitat ranges, which include the pinyon- juniper woodlands, semi-desert grasslands and desert scrub. The populations are genetically differentiated with the central region being highly separated from the northern and the southern region populations. The extreme local environmental selection pressures in the central region (desert scrub) might have contributed to the genetic differentiation. There was also support for the early divergence of the central population from the rest, following the progressive aridification of Arizona post Pleistocene.

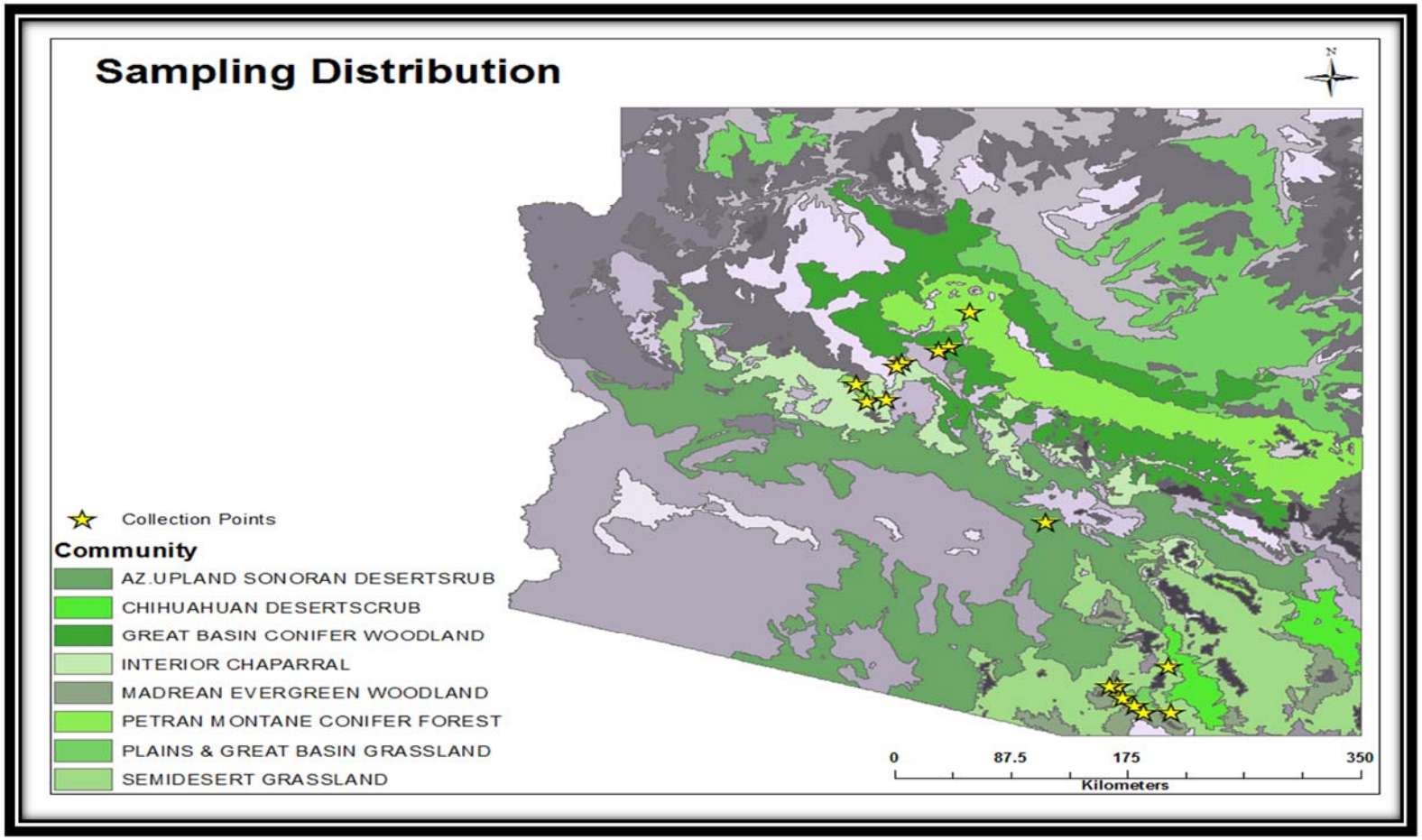

Figure 3.1 Sampling locations of 16 *Xanthisma gracile* populations along with their corresponding habitat community in Arizona are shown in the map. The three broad communities between which the samples are distributed are the desert scrub, conifer woodland and semi desert grassland.

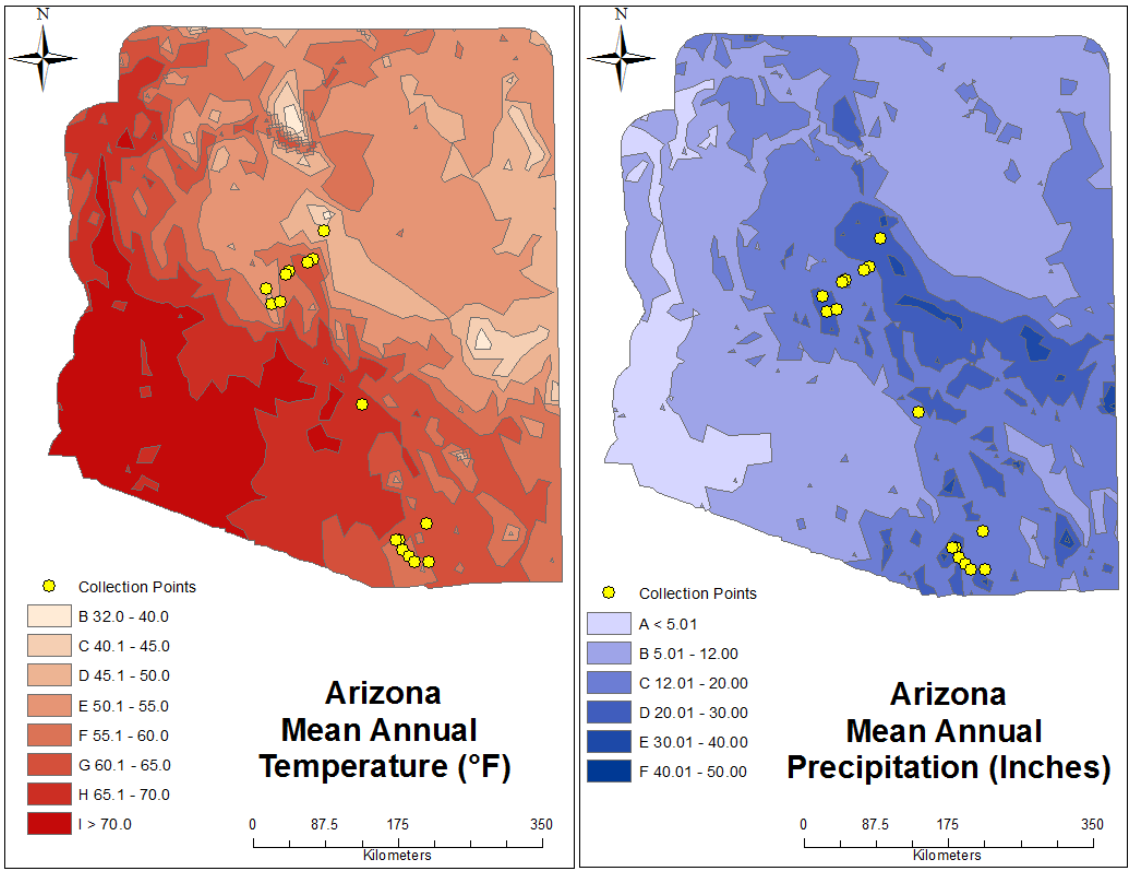

Figure 3.2 Maps depicting the varied climatic condition, specifically, temperature and precipitation differences across Arizona. Populations are indicated by yellow dots

Table 3.1 Estimates of genetic diversity for all the populations and regions of *X. gracile.*

Population/Region	N	P	PA	H	DW	I
X_01	13	47.08%	28	0.126	0.36367	0.201
X_03	14	16.31%	2	0.029	0.04940	0.052
X_04	23	53.18%	12	0.094	0.16618	0.164
X_05	19	43.77%	13	0.084	0.14266	0.144
X_06	11	13.13%	5	0.032	0.03685	0.052
X_07	13	20.16%	7	0.039	0.05960	0.068
X_08	21	55.31%	29	0.092	0.18932	0.164
X_09	14	18.17%	14	0.032	0.07091	0.057
X_10	19	44.43%	14	0.083	0.14149	0.145
X_11	20	48.41%	21	0.096	0.19177	0.163
X_12	13	2.12%	0	0.007	0.00267	0.011
X_13	15	17.24%	3	0.029	0.04073	0.051
X_14	15	14.85%	1	0.027	0.02759	0.047
X_15	12	13.79%	3	0.045	0.05810	0.069
X_16	10	10.61%	1	0.028	0.03352	0.045
X_17	12	5.31%	0	0.013	0.00949	0.022
Mean	15.25	26.49%	9.56	0.0535	0.09899	0.0909
Central	34*	48.14%	28	0.127	NA	0.202
North	133	91.51%	181	0.074	NA	0.147
South	97	60.21%	30	0.045	NA	0.092

*Sample populations which contain individuals from herbarium specimens that were not sampled at population level.

Table 3.2 Analysis of Molecular Variance (AMOVA) without the herbarium populations. P-value estimates are based on 9999 permutations. df= degrees of freedom, SS= sum of squares, and MS= mean squared deviations

Source	df	SS	MS	Estimated Variance	% of total variation	Phi	P-value
Among Regions	2	340.242	170.121	1.983	7%	ϕ_{RT} =0.073	<0.01
Among Pops	13	532.039	40.926	1.114	4%	ϕ_{PR} = 0.045	<0.01
Within Pops	228	5448.198	23.896	23.896	89%	ϕ_{PT} = 0.115	<0.01
Total	243	6320.480		26.993	100%		

Table 3.3 Analysis of Molecular Variance (AMOVA) with the herbarium populations included. P-value estimate based on 9999 permutations. df= degrees of freedom, SS= sum of squares, and MS= mean squared deviations.

Source	df	SS	MS	Estimated Variance	% of total variation	Phi	P-value
Among Regions	2	635.481	317.740	3.707	12%	ϕ_{PT} =0.122	<0.01
Within Regions	261	6981.716	26.750	26.750	88%		
Total	263	7617.197		30.457	100%		

Table 3.4 Pairwise F_{ST} between regions including the herbarium specimens.

	Central	North	South
Central	0		
South	0.20865	0.01704	0

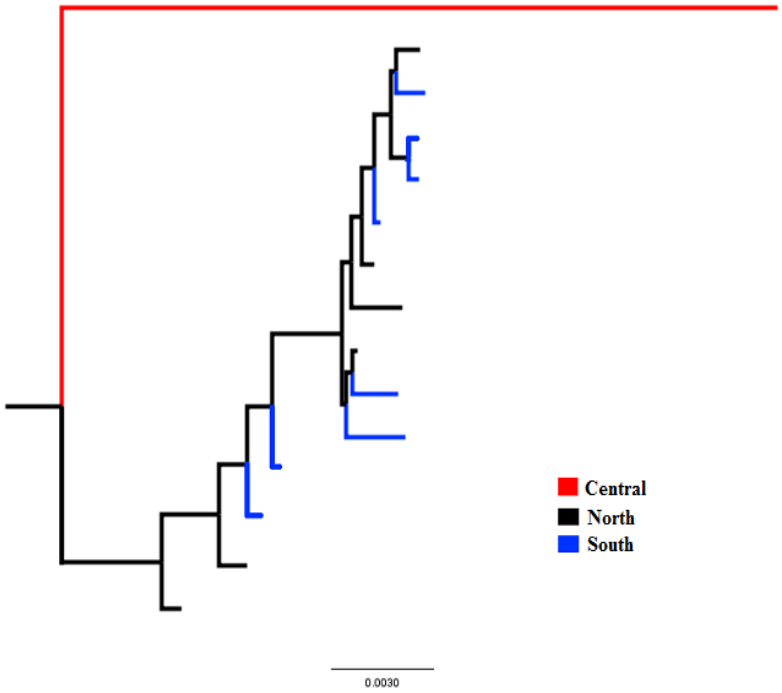

Figure 3.3 Neighbor-joining tree based on the AFLP presence/absence binary matrix depicting the genetic relationship

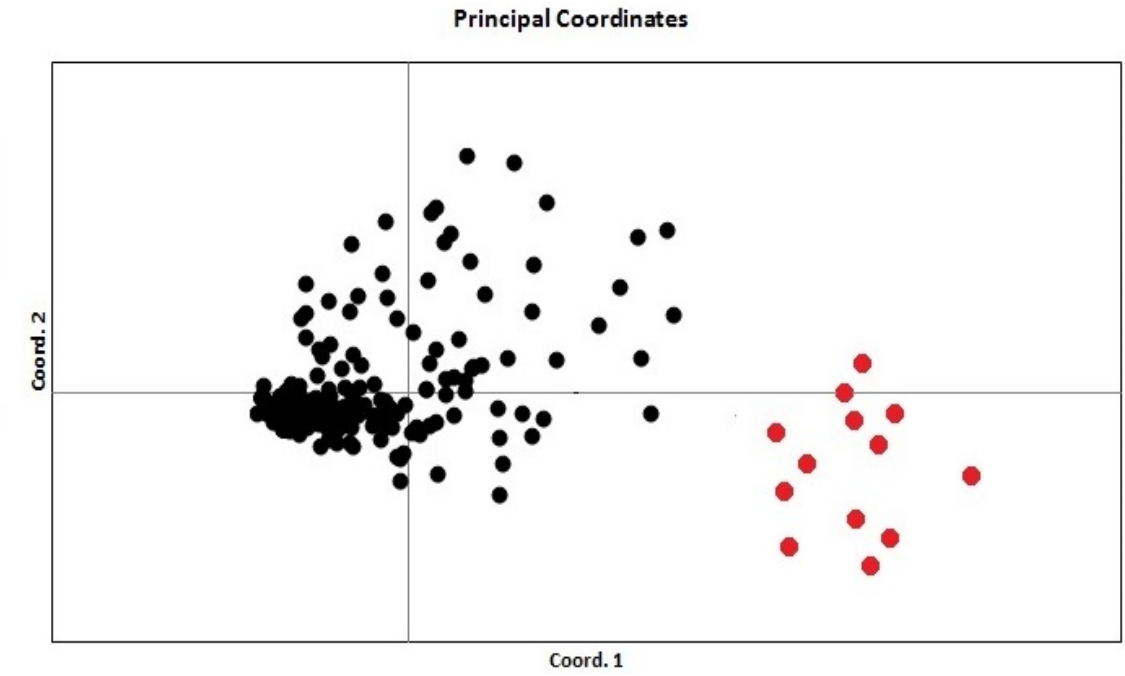

Figure 3.4 Principal coordinates analysis (PCA) of genetic covariance with standardized data between individuals of *Xanthisma gracile*.

Note: Individuals in red represent the central region. Black dots represent Individuals from northern and southern regions

3.7 References

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CHAPTER IV

EVALUATION OF INTRASPECIFIC TAXONOMY IN *XANTHISMA SPINULOSUM* IN ARIZONA BASED ON MORPHOLOGICAL AND GENETIC ANALYSES

4.1 Abstract

Xanthisma spinulosum encompasses five varieties, three of which occur in Arizona. Despite this recognition of varieties, there is considerable variation within and across populations, which makes identification of these taxa difficult in the field. This suggests that recognition of varieties may not be warranted. In this study, I test the hypothesis that there are three varieties of *X. spinulosum* occurring in Arizona by quantifying morphological and genetic variation on herbarium collections. Morphological traits were measured on stems, leaves, inflorescences and flowers and genetic variation was estimated using Amplified Fragment Length Polymorphism (AFLP). Neither K-means analysis nor principal components analysis of the morphological dataset indicated the presence of distinct groups, and no diagnostic characters separating the three taxa could be identified. The genetic analysis utilized AFLP markers for 50 specimens representing the three varieties. Partitioning of genetic variation among the three varieties was very small ($\phi_{ST} = 0.024$, $p < 0.05$). A neighbor-joining analysis based on the presence/absence matrix of AFLP bands also suggests lack of three distinct varieties, which is consistent with the morphological dataset. These results support the presence of a highly variable species, *Xanthisma spinulosum* without discernible intraspecific ranks.

4.2 Introduction

Phenotypic diversity among individuals of a population can be a result of phenotypic plasticity, differential selection or genetic drift (Clausen et al. 1948; Stebbins 1950; Lesica and Allendorf 1999). Species are considered the fundamental units of taxonomy, and there are many definitions and criteria used to diagnose species. However, variation in groups of populations within species is taxonomically more ambiguous. The International Code of Botanical Nomenclature (Greuter et al. 2000) has suggested guidelines for taxonomic ranks below the level of species (i.e., species, subspecies, variety and form) (Knaus 2008), but these are not requirements and are inconsistently followed by taxonomists, who frequently use subspecies and varieties interchangeably (Haig et al.2006; Mallet 2007). For example, Haig et al. (2006) did not find a universally accepted subspecies definition. More subspecies were described in vertebrates or plants than invertebrates or fungi (Haig et al. 2006). In an assessment of taxonomic practices among botanists, McDade (1995) found that authors referred to intraspecific ranks as either varieties or subspecies, but rarely used both categories.

In one of the earliest attempts to quantify exactly what variation constitutes a subspecies, Amadon (1949) proposed the "75% rule", which states that a subspecies should be considered valid if 75% or more of a certain sample of individuals (i.e., a potential subspecies or operational taxonomic unit) can be distinguished from 99% of all other individuals of the same species according to the characters examined (Amadon 1949, Mayr et. al., 1953, Patten and Unitt 2002). However, this approach was not widely accepted because of lack of consensus on the number of characters that should be used for comparing populations and how the 75% threshold should be determined (Patten and

Unitt 2002, Haig 2006). Geography has also been used to define a subspecies as an "aggregate of local populations which differ taxonomically from other such subdivisions of the species." (Mayr and Ashlock 1991) More recent definitions of subspecies have focused on the evolutionary processes that might produce subspecies. For example, Patten and Unitt (2002) suggest reproductive isolation in their definition: "collection of populations occupying a distinct breeding range and diagnosably distinct from other such populations". Many taxonomists consider subspecific categories as incipient species potentially evolving to full species (Darwin 1896, Mayr 1942 Stebbins 1950; Clausen 1951; Grant 1981). For example, Frankham et al. (2002) defined subspecies as "populations partway through the evolutionary process of divergence towards full speciation". To ensure a stable taxonomy and accurate assessments of biodiversity, it is important to determine the naturalness and distinctiveness of intraspecific ranks, especially in highly variable species.

The presence of infraspecific variation is responsible for the extensive taxonomic revisions of the *Xanthisma spinulosum* (Pursh) D.R. Morgan and R.L. Hartman complex (Asteraceae). This species has been previously treated as *Haplopappus spinulosus* (Hall 1928) a nd *Machaeranthera pinnatifida* (Hartman 1976; Turner and Hartman 1976), but I will use *X. spinulosum* throughout the remainder of this paper because it follows the most recent nomenclature (Morgan and Hartman 2003, Flora of North America, vol.20, 2003). The *X. spinulosum* complex is primarily composed of diploids (2n = 2x = 8) with a number of scattered tetraploid populations (2n = 4x = 16; Hauber 1986). This weedy, perennial herb is found throughout the western Great Plains of North America from central Mexico to southern Canada (Hall 1928). It often occurs in association with

disturbed areas, such as along road grades and in old pastures. Throughout its distribution, *X. spinulosum* is morphologically highly variable. For example, the involucre width varies from 8 to 22 mm, and this has led to taxonomic treatments of the group that attempted to categorize the morphological variation into taxonomic ranks (Hall 1928; Turner and Hartman 1976). Turner and Hartman (1976) recognized two subspecies - subsp. *goodingii* and subsp. *spinulosum* with seven varietal ranks, including vars. *spinulosum, glaberrima, chihuahuanum, goodingii, scabrell, paradoxa* and *incisifola.* A recent revision by Nesom and Turner (2007) raised vars. *glaberrima*, *scabrella*, *paradoxa* and *incisifola* to species. The other three taxa, vars. *spinulosum, chihuahuanum* and *goodingii,* are still considered within *X. spinulosum* along with two newly identified varieties, *austrotexanum* (endemic to Texas; Turner 2007) and *hartmanii* (endemic to Mexico; Nesom and Turner 2007). The distributions of *X. spinulosum* var. *spinulosum* and var. *chihuahuanum* within the USA are described in Nesom and Turner (2007). The former variety occurs from Montana to Texas, New Mexico and eastern Arizona, whereas the latter variety is localized to southern Arizona. *Xanthisma spinulosum* var. *spinulosum* shows a gradual change in morphology across its distribution from Canada to northern Mexico (Turner and Hartman 1976). The low- growing, pubescent plants with small heads are gradually replaced by pubescent or partially glabrous to completely glandular-pubescent plants. In Texas and towards Mexico the plants show larger heads and more basal leavestypical of var. *chihuahuanum.* The latter variety differs from the var. *spinulosum* only in having "stiffly ascending stems, larger, fewer heads, longer peduncles and basally clustered leaves" (Morgan and Hartman 2003).

Xanthisma spinulosum var. *goodingii* occurs across western Arizona, south-central Nevada and Baja California (Figure 4.3). Initial placement of *goodingii* as a subspecies of *Haplopappus spinulosus* in the section Blephadron was by Hall (1928). This was later modified as Hall frequently grouped many morphologically and cytologically diverse plants into the same species (Stambak 1994). It was then classified as a subspecies of *Machaeranthera pinnatifida* by Turner and Hartman (1976). Ramon (1968) found *goodingii* to be related to *Haplopappus arenarius* subsp. *arenarius*, subsp. *incisifolius* and *Haplopappus texenis*. The latter was ignored as a formal taxon until recently when Turner (2007) classified it as another variety of *X. spinulosum*, *Xanthisma spinulosum* var. *austrotexanum*, which is endemic to Texas. Within Arizona, the distribution of the *spinulosum* complex begins with var. *goodingii* in the western part moving further south where it intergrades into var. *chihuahuanum* and var. *spinulosum*.

Alternative classifications have treated regional variations in the *spinulosum* complex, including hybrids, as a single species without infraspecific categories (Hartman 1976). Additionally, despite publishing subspecific ranks, Nesom and Turner (2007) suggested that because of the possibility of intergradation of var. *goodingii* with var. *spinulosum* and var. *chihuahuanum*, there should be no longer any formal recognition of subspecies in the *X. spinulosum* complex. In this study, I test the distinctiveness of the three varieties occurring in Arizona by quantifying morphological and genetic variation from herbarium specimens. These data are used to address the identification of diagnostic morphological characters and determine the extent that differences warrant taxonomic recognition. Specifically, I expect no overlap in the characters used for morphology and

the genetic data. Any overlap in the data set would suggest the presence of continuous variation across taxa not enough for the delimitation of the taxa as distinct.

4.3 Materials and Methods:

4.3.1 Plant Material and Morphological Measurements:

A total of 76 herbarium specimens of *X. spinulosum* collected in Arizona were examined. All specimens were loaned from the Arizona State University herbarium (Appendix). The specimens were divided into the three varieties based on the geographical distribution of the varieties as described in the taxonomic keys (Turner & Hartman 1976; Nesom 2003). The var. *goodingii* is distributed across western Arizona, var. *spinulosum* occurs in the eastern part of the state, specifically the counties of Navajo, Apache, Pima, Pinal and Graham, and var. *chihuahuanum* is restricted mostly to Cochise county but overlaps with var. *spinulosum* in Graham county. In this study, 50 accessions were defined as var. *goodingii,* 14 as var. *spinulosum* and 12 as var. *chihuahuanum* (Figure 4.3). Measurements were made for eight morphological characters selected from the keys of Turner and Hartman (1976) and Nesom and Turner (2007) and which are considered to reflect the taxonomic differences recognized by these authors. These characters included phyllary number, arrangement of flower head as pedunculate or not, involucre width, leaf length, leaf width, leaf surface, stem glandularity, and distribution of leaves on stems. All measurements were made manually under an Olympus dissecting microscope using a miniscale. The measurement was made across three different flowers, leaves and stems on an individual, and the average of these values was used in data analyses. This was done to account for variation possible in each specimen. Phyllary number was determined by counting the rows of bracts. Lengths were measured from

point of attachment to the tip. Leaf and involucre width were measured at the point of maximum width. Other measured characters include presence or absence of a peduncle, leaf surface coded as dull, shiny or fuzzy depending on the presence of a waxy layer or degree of pubescence, presence or absence of gland-like structures on the stem, and distribution of leaves on the stem as dense at top or bottom (1 or 0) respectively. Ratios of leaf length and width were calculated and used as an alternate character for the analyses.

4.3.2 DNA Extraction

Genomic DNA was extracted from leaf tissue of the herbarium specimens following a modified CTAB DNA extraction protocol (Doyle and Doyle 1987). After extraction, DNA was dissolved in TE Buffer, treated with RNase A (10mg/ml, ABgene) to remove any residual RNA, and run on a 1.5% agarose gel to check for quality.

4.3.3 AFLP Analysis

AFLP analysis was performed using a protocol modified from Vos et al. (1995) and incorporating the recommendations made by Trybush et al. (2006). Genomic DNA was digested in 30µl reactions incubated at 37 °C for 1 hour in a thermal cycler, followed immediately by ligation of the linkers. Restriction digest enzymes and reagents utilized per reaction were: 0.25 µl of EcoRI (20,000 U/ml, New England BioLabs, Ipswich, MA, USA), 0.5 µl of MseI (10,000 U/ml, New England BioLabs,), 3 µl 10X NEBuffer2, 0.15 µl of 100 µg/ml BSA, 5 µl of individually purified genomic DNA, and 21.1 µl of sterile water. Eco AFLP linkers were annealed in a thermal cycler by heating to 65 °C for 10 minutes and cooled to room temperature using the following reagents: 10 µl of Eco

Linker 1 (100 µM, 5'-CTC GTA GAC TGC CC) and 10 µl of Eco Linker 2 (100 µM, 5'-AAT TGG TAC GCA GTC TAC). Mse AFLP linkers were also prepared using the above procedure utilizing 10 µl of Mse Linker 1(100 µM, 5'-GAC GAT GAG TCC TGA G) and10 µl of Mse Linker 2 (100 µM, 5'-TAC TCA GGA CTC AT). Annealed linkers were stored frozen at -20 °C until use. Ligation of Eco and Mse linkers was conducted in 40 µl reactions which comprised of 0.1 µl of Eco and 1 µl Mse Linker (as annealed above), 0.15 µl T4 DNA Ligase enzyme and 4 µl of included 10X T4 DNA Ligase Reaction Buffer (New England BioLabs), 30 µl of individually digested DNA from above, and 4.75 µl sterile water. Ligation reactions were incubated in a thermal cycler at 37 °C for 4 hours followed by storage at -80 °C to prevent degradation. Pre-selective amplifications were conducted in 10 µl reactions using 0.5 µl each Eco+A (10 µM, 5'-GAC TGC GTA CCA ATT CA) and Mse+C (10 µM, 5'-GAT GAG TCC TGA GTA AC) primers, 1.25 µl dNTPs (2 mM dATP, dCTP, dGTP, and dTTP; New England BioLabs), 2 µl 5X *GoTaq*™ FlexiBuffer (Promega Corp., Madison, WI, USA), 1.25 µl MgCl2 (Promega Corp.), 2.5 µl individually ligated DNA, 2.55 µl sterile water, and 0.1 µl *GoTaq*™ DNA polymerase (5u/µl, Promega Corp.). Pre-selective amplifications consisted of an initial denaturing step of 65 °C for 5 minutes, 30-cylces of 30 seconds at 94 °C, 30 seconds at 56 °C, and 1 minute at 72°C.

Pre-selective amplification products were individually diluted 1:20 with sterile water. Selective amplifications consisted of a single Mse primer with three differently fluorescent labeled Eco primers per reaction. Selective amplification for all individuals was conducted in 10 µl volume consisting of 0.7 µl each Mse-CAG (5µM), Eco-ACT FAM, Eco-ACC NED and Eco-AGG VIC (1 µM) selective primers, 0.5 µl dNTPs (2 mM

dATP, dCTP, dGTP, and dTTP; New England BioLabs), 2.5 µl LongAmp™ Buffer (New England Biolabs), 1 µl of diluted pre-selective amplification product, 2.7 µl sterile water, and 0.5 µl *LongAmp Taq*™ DNA polymerase (5u/µl, New England Biolabs). Selective amplifications consisted of an initial denaturing step of 95 °C for 15 minutes, 13-cylces of 30 seconds at 94 °C, 1 minute at 65 °C, and 1 minute at 72°C (reducing annealing temperature by 0.7 °C/cycle), 25-cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 1 minute at 72°C, and finished with 10 minutes at 72 °C. The selective amplification fragments were diluted 1:10 with distilled water and one µl of the diluted fragments was transferred to a 96 well plate and allowed to air dry before being sent to Arizona State University for capillary electrophoresis with 0.3 µl LIZ-600 size standard (Life Technologies) per sample. Peaks were visualized using GeneMarker® (Softgenetics, LLC, State College, PA, USA) and polymorphic bands were scored as present (1) or absent (0). Only bands in size ranges of 75-600 base pairs were used for all the primer combinations.

4.3.4 Data Analyses:

For the morphological data set, a one-way Analysis of variance (ANOVA) was conducted in R using the function *aov* (R Development Core Team 2012) to determine if there were significant differences among the taxa in each of the continuous traits. Additionally, k-means clustering analysis was used to look for natural breaks in the morphological data set that would correspond to each of the varieties irrespective of my initial assignment of samples to a group. A non-hierarchical method of clustering, using the *kmeans* function in R (R Development Core Team 2012) was conducted. The data were standardized by standard deviation such that each trait was equally weighted. K-

means uses an a priori identified number of groups and utilizes an optimality criterion to fit the data within those groups. The sum of squares within each group is calculated and assigned to the predefined number of clusters to assess the best fit (Everitt 2005; Knaus 2008). As the number of groups increases, the sum of squares should decrease, and the optimal group number is identified by a sudden reduction in the sum of squares. To choose the most probable number of clusters represented by the data, an iterative method using the “calinski” criterion was conducted using the *cascadeKM* function in the vegan package of R software (R Development Core Team 2012). The "calinski" criterion is an analysis of variance statistic that compares the sum of squares among groups relative to the within group sum of squares. The value is plotted with respect to the cluster solutions, and the maximum value defines the number of groups, thereby providing the selected *kmeans* solution. I chose the "calinski" criterion from the other available criteria as it has been suggested to be able to provide the best solution (Milligan and Morgan 1985; R manual for Vegan library function cascadeKM; Oksanen et al. 2009). Principal components analysis was also performed using the *princomp* function in R (R Development Core Team 2012) to visualize the groups as defined by the calinski criterion. This analysis utilized a standardized covariance matrix based on the morphological characters.

To estimate genetic variation and genetic differentiation among the three taxa, GenAlEx 6.0 (Peakall and Smouse 2001) was used to determine the percentage polymorphic loci, number of private bands, heterozygosity and Shannon's Index and to conduct a hierarchical Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992). Polymorphic bands that occur in only one population are referred to as private bands. To

understand genetic relatedness among samples, a NJ tree was constructed in PAUP* 4.0b10 (Swofford 2002) using Nei–Li distances (Nei & Li 1979) calculated from the AFLP data (presence/absence) matrix. Support for nodes in the NJ tree was determined using the bootstrap method (Felsenstein 1985) with 500 replicates in PAUP* version 4.0b10**.** The consensus tree was visualized in FigTree v1.3.1 (Rambaut 2012).

4.4 Results

Results from ANOVA suggested that out of the four quantitative characters, none showed significant differences among the varieties. The qualitative characters (pedunculate head, leaf surface, stem glandularity, and distribution of leaves on stems) exhibited overlap in character states among the three taxa. K-means analysis revealed a smooth curve, which does not have a natural break point, suggesting a lack of structure within the data set (Figure 4.4). This was further confirmed using the *cascadeKM* stopping rule with the "calinski" criterion, which also confirmed the lack of any clear number of clusters beyond one (Figure 4.4). The principle component analysis did not reveal distinct clusters corresponding to the three varieties and clustered together towards the center (Figure 4.5). However, there were a few individuals from each of the varieties that clustered together separate from the main cluster into the bottom right quadrant. The use of the ratio of leaf length to width did not affect the inferences from the data

From the AFLP analysis, the primer combinations resulted in the generation of 699 loci scored between 75 to 600 bp. The number (frequency) of private bands (PA) detected was 345 (0.540), 20 (0.066) and 12 (0.0631) in *var. goodingii, var. spinulosum, and var. chihuahuanum,* respectively. The percentage of polymorphic loci (P) among the

three taxa was 92.99%, 43.06% and 26.04% respectively, with a mean of 54.03%. Shannon's Information Index (I) was 0.311, 0.172 and 0.141, with an overall mean of 0.208. These data are summarized in Table 4.2. The Analysis of Molecular Variance (AMOVA; Table 4.1) revealed significant structure as most of the variation was contained within groups (98%, $p<0.05$). The remaining variation was distributed among the three groups (2%, $p<0.05$). The NJ tree indicated no distinct clustering of samples of the three varieties (Figure 4.6).

4.5 Discussion

Morphological diversity of *X. spinulosum* is reflected in its taxonomic history, where many authors have variously described varieties and subspecies (Hall 1928; Jackson 1961; Hartman 1976; Turner and Hartman 1976). Results from this study suggest that there are not distinct clusters corresponding to the three varieties of *X. spinulosum.* The eight morphological characters measured, which have been identified as the diagnostic characters of this complex in the Flora of North America (Morgan and Hartman 2003 vol.20), show overlap in the specimens examined here (Figure 4.2). Additionally, from the K-means clustering, there does not appear to be a 'natural' breakpoint in the data. This indicates that no clear optimal number of groups exists within the exceptional amount of diversity contained within this species. A similar morphometric approach was also used by Knaus (2008) to identify infra-taxa in *Astragalus lentiginosus* wherein the statistical clustering (K-means) also failed to provide support for the presence of varieties.

From the keys described in Turner and Hartman (1976) and Morgan and Hartman (2003), the morphological characters chosen for the morphological analysis should have

differentiated into three different varieties, but instead I find intermediacy in all these characters across the taxa. *Xanthisma spinulosum* occurs throughout Arizona across a variety of different climate and habitat ranges, which have an affect on the overall morphology of the plants. For example, var. *chihuahuanum* occurs in the southeastern part of Arizona (Cochise county), which has a semi-arid climate, and exhibits intermediate characters. The smaller heads are typically attributed to the var. *spinulosum* but the specimens from the current dataset that key out to *spinulosum* are present across the entire geographic range within Arizona, with overlapping values. Consequently, it is difficult to ascertain the variety unless its geographic location is known. For example, the involucre widths range from 8-12 mm in *spinulosum*; 12-16 mm in *chihuahuanum* and 12-22 mm wide in *goodingii* (Figure 4.2).

AFLP data also do not indicate the presence of three genetically divergent groups. From the neighbor-joining tree, the presence of three distinct varieties was expected to correspond to at least three different clusters, with individuals from each variety much closely related to each other than between the varieties. However, I was not able to find such distinct clusters.. A similar study conducted by Manoko et al. (2007) aimed at using AFLP for examining relationships of *Solanum* species. In their study, even though they found sufficient differentiation between two taxa to call them separate species, they were not able to justify the presence of any subspecies within either species.

Along with varying morphological characters, *X. spinulosum* also exhibits different karyotypes among its varieties. *X. spinulosum* var. *goodingii* has been described to have 2n=8, whereas, both var. *spinulosum* and var. *chihuahuanum* contain 2n=8, 16. The presence of 2n=8 individuals in all the three taxa might be responsible for similar

morphology, whereas tetraploid populations could exhibit differences. Also, to my knowledge, no data has been published about possible hybrids between the possible hybrids between the 8 and 16 individuals I was not able to quantify karyotype variation in the samples used in this study, but future comparisons of natural populations with different karyotypes will contribute to the understanding of the basis of variation and whether this can lead to the classification of the taxa based on the chromosomal differences supported by morphology and genetic data.

Although the phenotypic and genetic differences between the taxa are not significant enough to grant separate taxonomic status, it is important to acknowledge them. Taxa occupying heterogeneous environments are often subjected to spatially variable natural selection, which has important consequences for how phenotypic variation is partitioned within and among populations and genetic differentiation (Hedrick 2006; Nosil et al. 2009; Temunović et al. 2012). In this study, clinal variation may more likely explain the observed morphological variation. Stebbins (1950) suggests that the continuous variation in widespread species is probably due to ecotypic adaptation, where clines or character gradients occur as responses to changes in habitats.

4.6 Conclusions

Based on this study it can be concluded that *X. spinulosum* in Arizona is a polymorphic species. There were no clear distinctions based on the morphological characters across the three taxa even though they occupy different geographic areas. Although genetic variation was detected among the three groups tested, it is not nearly sufficient for the taxonomic rank of varieties, thus lending support to the suggestion by Nesom and Turner (2007) to not formally recognize the taxonomic level of varieties of

Xanthisma spinulosum. However, it is important to keep in mind that the present dataset used to address these questions is based on a limited number of herbarium specimens from one location. A more extensive study is essential to lend more support to the above conclusion along with a population level genetic study to ascertain if there are any significant differences between the populations, which might contribute to the presence of three subspecies.

Table 4.1 Analysis of Molecular Variance (AMOVA) using the herbarium specimens. P-value estimate based on 9999 permutations. df= degrees of freedom, SS= sum of squares, and MS= mean squared deviations

Source	df	SS	MS	Estimated Variance	% of total variation	Phi	P-value
Among Varieties	2	142.856	71.428	1.357	2%	ϕ_{ST} =0.024	<0.05
Within Varieties	37	2076.819	56.130	56.130	98%		
Total	39	2219.675		57.487	100%		

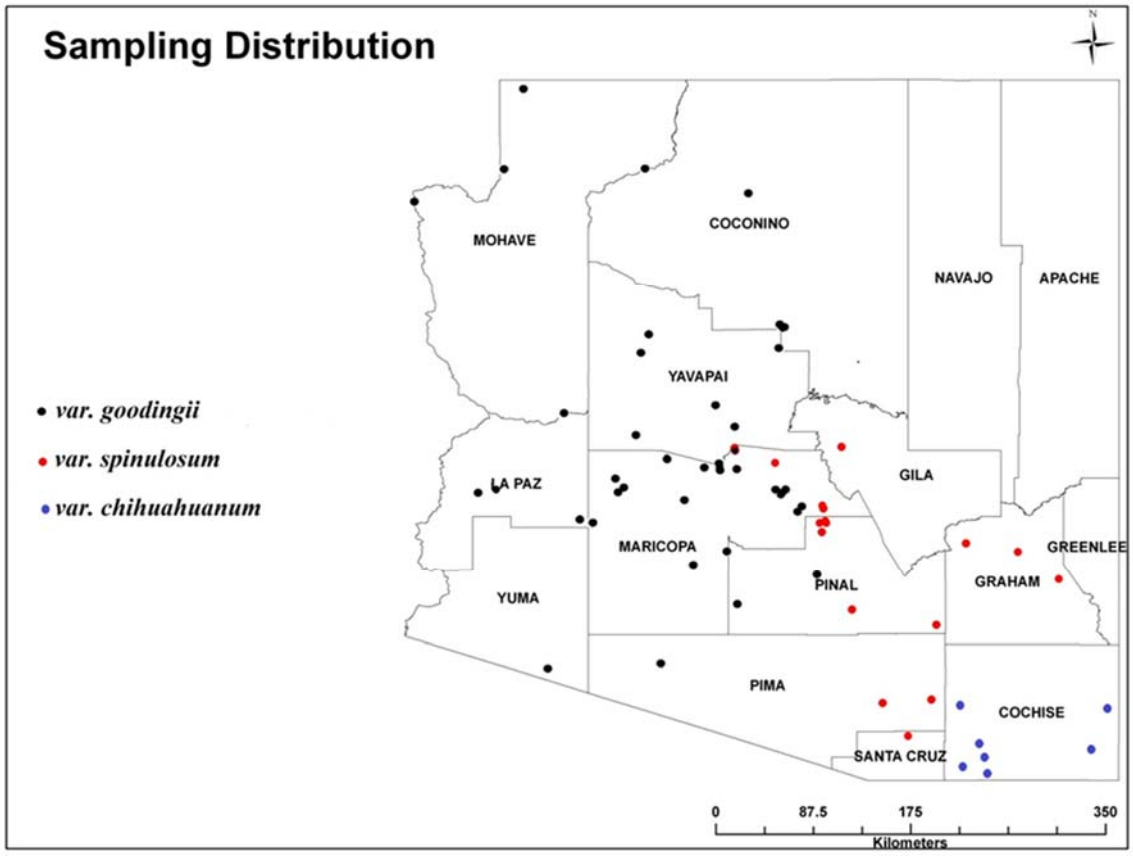

Figure 4.1 Specimen distribution across Arizona of all three varieties of *Xanthisma spinulosum.*

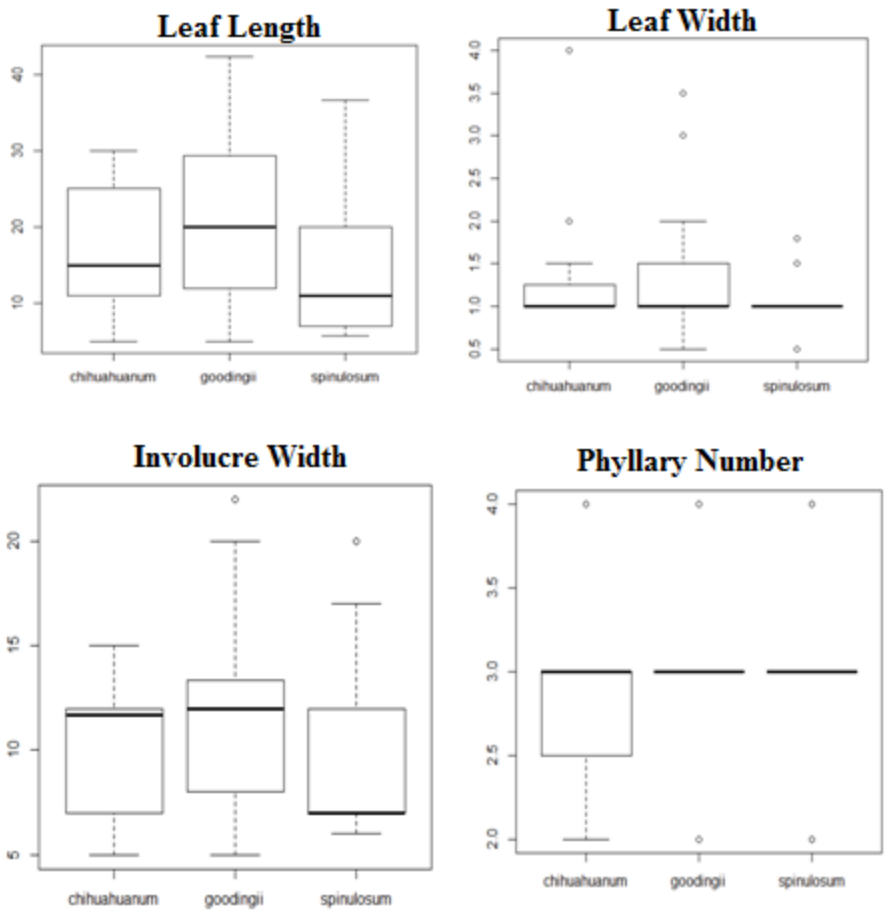

Figure 4.2 Boxplots of quantitative morphological characters between *Xanthisma spinulosum* var. *goodingii* (52), var. *spinulosum* (16) and var. *chihuahuanum* (8) using ANOVA. None of these characters showed significant difference among the three taxa.

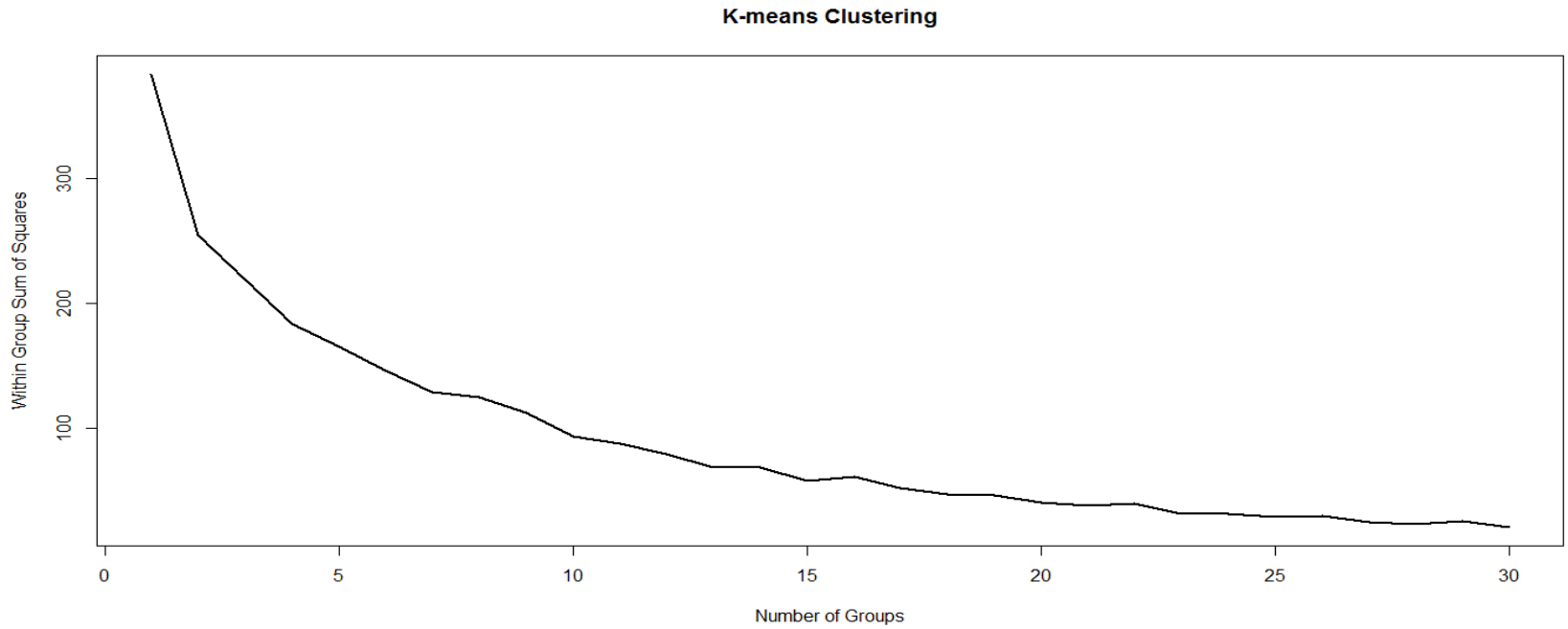

Figure 4.3 K-means clustering method depicting the number of groups based on within group sum of squares.

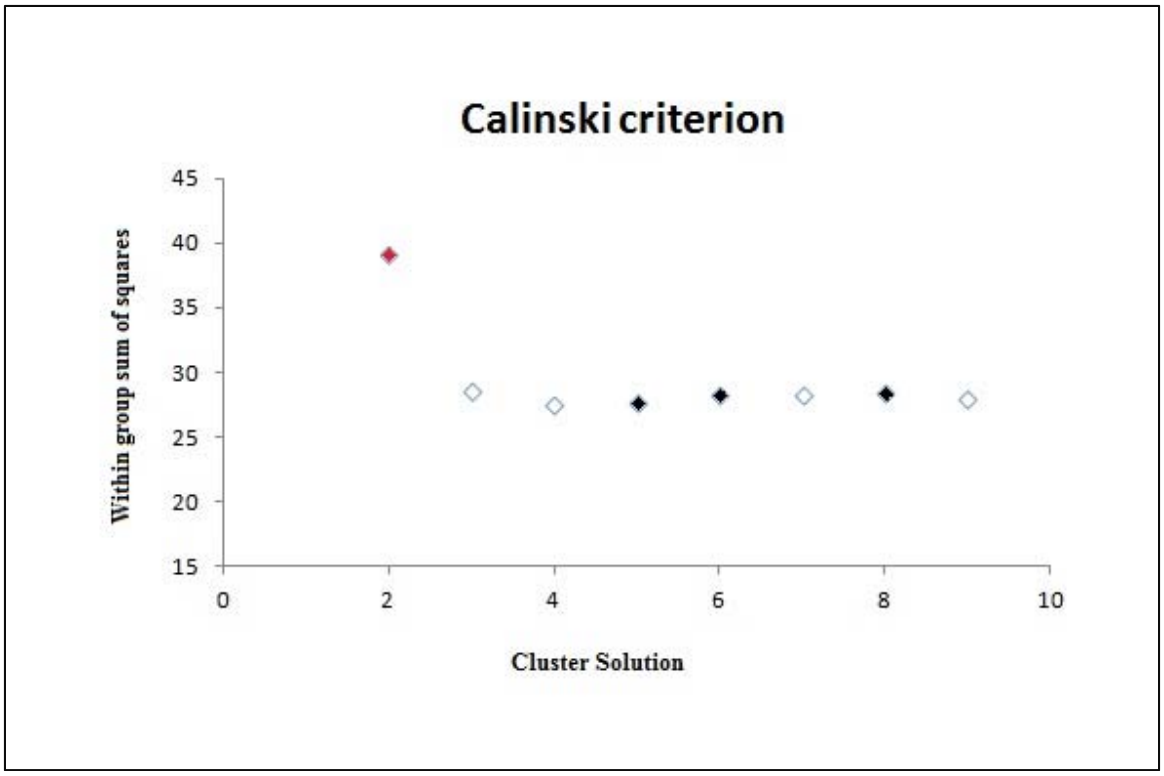

Figure 4.4 K-means clustering method utilizing the stopping rule of calinski and Harabasz ("calinski criterion") using the function cascadeKM.

Note: The red dot represents the optimum cluster solution, where the within group sum of square value is maximum. The black dots represent partitions that can increase the value of the criterion.

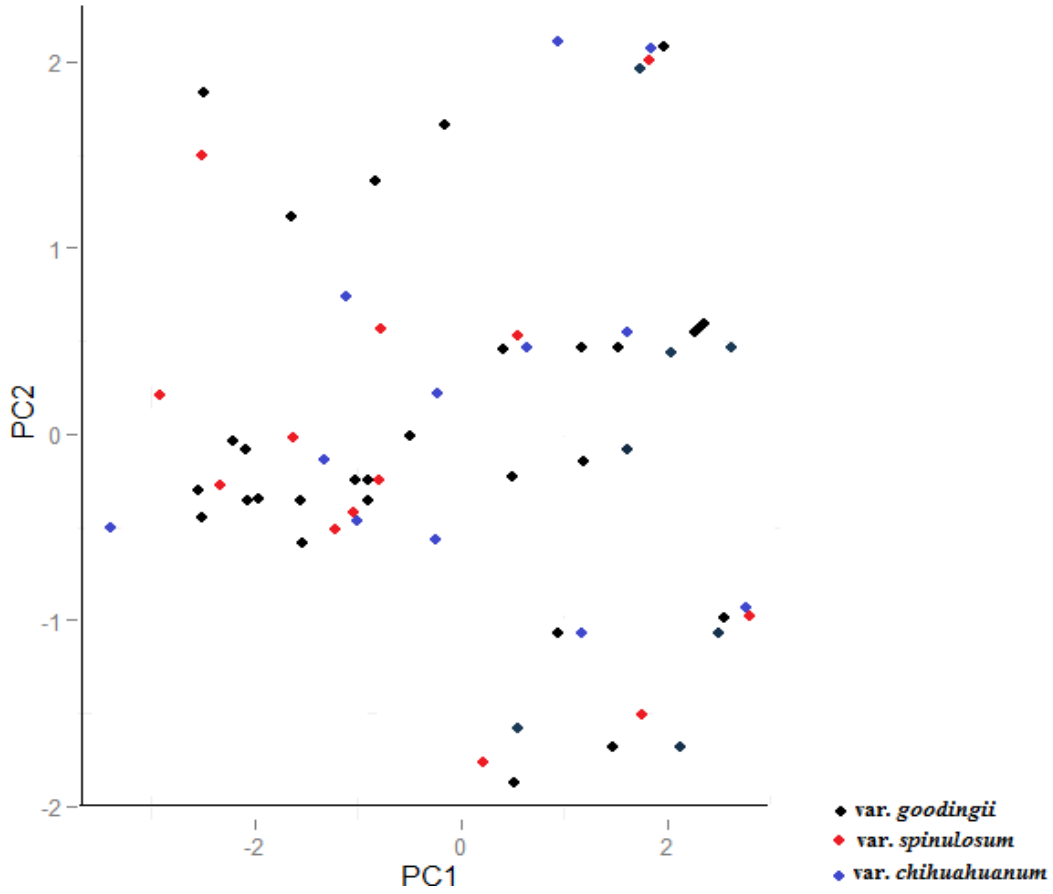

Figure 4.5 Scatter plot of Principal components #1 and #2 (PC1 and PC2). Both these components explain 60.46% of the observed variation.

Table 4.2 Estimates of genetic diversity for all the three varieties of *Xanthisma spinulosum.* Note: Population size –N, Percentage polymorphic loci –P, Number of private alleles –PA, Nei's gene diversity –H, Shannon Index – I

Variety	N	P	PA	H	I
Goodingii	24	92.99%	345	0.191	0.311
Spinulosum	9	43.06%	20	0.115	0.172
Chihuahuanum	7	26.04%	12	0.117	0.141
Mean	40	54.03%	125.67	0.141	0.208

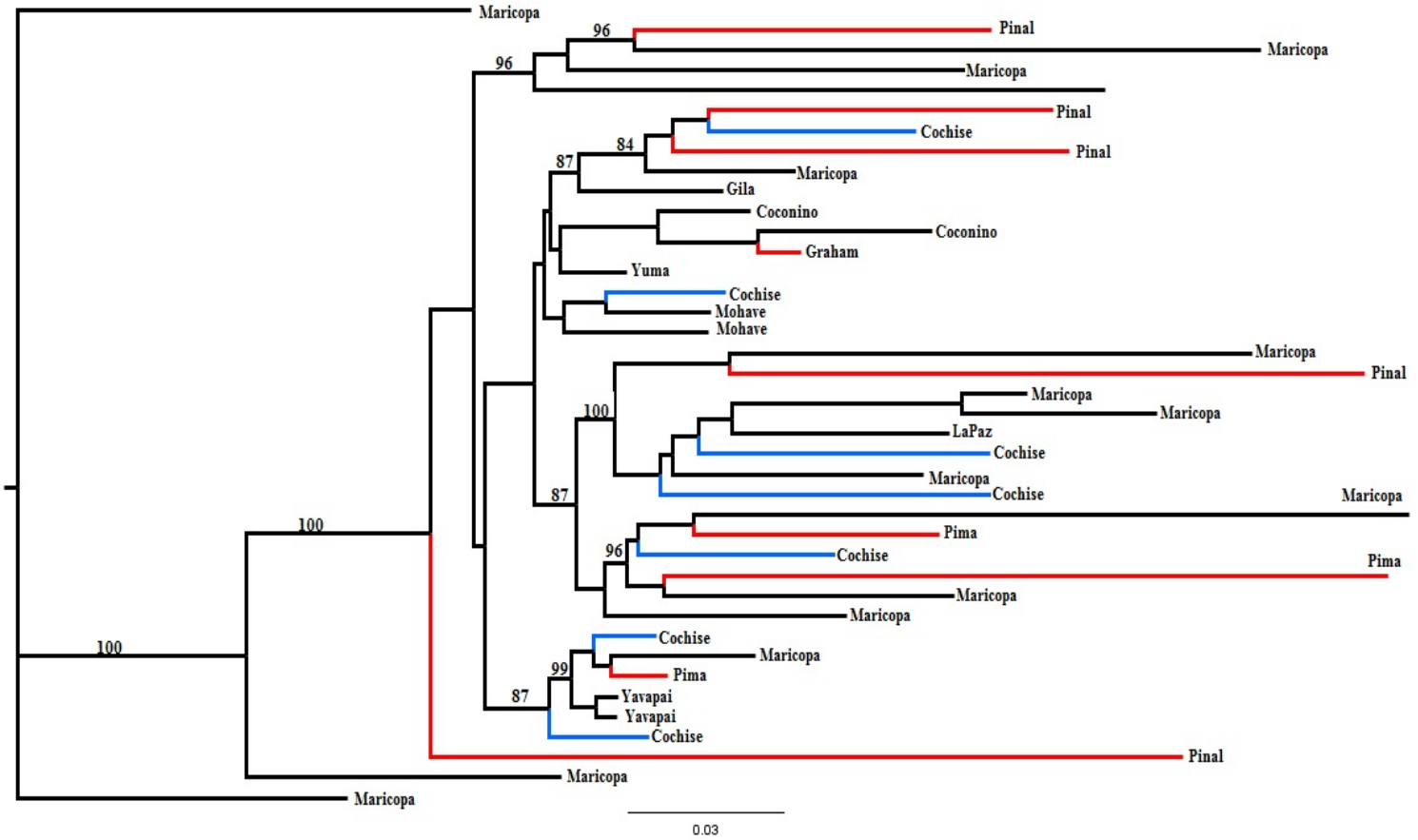

Figure 4.6 Neighbor-joining tree based on the AFLP presence/absence binary matrix depicting the genetic relationship of *X. spinulosum* var. *goodingii,* var. *spinulosum* and var. *chihuahuanum*. Color-coding is the same as that used in Fig. 4.5. The numbers on branches indicate bootstrap values >80% and the tip labels refer to county of origin.

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APPENDIX A

LIST OF ACCESIONS FROM RANCHO SANTA ANA BOTANICAL GARDEN, ARIZONA STATE UNIVERSITY HERBARIUM AND MISSISSIPPI STATE UNIVERSITY HERBARIUM.

Table A.1 Rancho Santa Ana Botanical Garden, Accession Numbers, Counties, States and specimen labels of *Xanthisma gracile* (*Haplopappus ravenii*)

Voucher Accessions	**County**	**State**	**Specimen Labels**	**Taxon**
9897	Coconino	AZ	R80	*X. gracile*
9929	Yavapai	AZ	R91	*X. gracile*
32088	Washington	UT	R50	*H. ravenii*
36502	La Plata	CO	R7	*X. gracile*
36693	Mesa	CO	R85	*X. gracile*
39572	Sierra	NM	R35	*X. gracile*
39728	Dona Ana	NM	R38	*X. gracile*
39725	Dona Ana	NM	R55	*X. gracile*
39735	Yavapai	AZ	R93	*X. gracile*
39802	Grant	AZ	R37	*X. gracile*
39804	Pima/Santa Cruz	AZ	R24	*X. gracile*
39805	Pima/Santa Cruz	AZ	R25	*X. gracile*
39806	Chihuahua	Mexico	R11	*X. gracile*
39807	Chihuahua	Mexico	R63	*X. gracile*
39808	Pima/Santa Cruz	AZ	R95	*X. gracile*
39809	Pinos Altos	NM	R39	*X. gracile*
39810	Cochise	AZ	R96	*X. gracile*
39811	Pima	AZ	R75	*X. gracile*
40042	Grant	AZ	R36	*X. gracile*
40246	Coconino	AZ	R86	*X. gracile*
40252	Springdale	UT	R97	*H. ravenii*
40253	Kanab	UT	R51	*H. ravenii*
41537	Pima	AZ	R23	*X. gracile*
45862	near Santa fe, NM	NM	R34	*X. gracile*
47479	San Bernardino	CA	R67	*H. ravenii*
57272	Durango	NM	R6	*X. gracile*
80872	Dona Ana	NM	R57	*X. gracile*
81857	Santa Cruz	AZ	R29	*X. gracile*
93257	Mohave	AZ	R92	*X. gracile*
102869	Yavapai/Maricopa	AZ	R98	*X. gracile*
102871	Beaver Dam, AZ	AZ	R5	*X. gracile*
117951	Mt Meadows UT	UT	R99	*H. ravenii*
119099	Sonora	Mexico	R89	*X. gracile*

Table A.1 (Continued)

125239	San Bernardino	CA	R12	*H. ravenii*
125814	Dona Ana	NM	R53	*X. gracile*
159647	Hurricane rd, N.W AZ	AZ	R84	*X. gracile*
168770	Sonora	Mexico	R28	*X. gracile*
190478	Coconino	AZ	R100	*X. gracile*
192553	Mohave	AZ	R8	*X. gracile*
193882	Cochise	AZ	R26	*X. gracile*
200398	Gila bend	AZ	R31	*X. gracile*
202052	Coconino	Az	R76	*X. gracile*
214540	Pima	AZ	R77	*X. gracile*
222726	Nye	NV	R49	*H. ravenii*
235189	Pima	AZ	R83	*X. gracile*
247797	San Bernardino	CA	R19	*H. ravenii*
250578	AZ	AZ	R78	*X. gracile*
265191	Deming Luna	NM	R40	*X. gracile*
268286	Mohave	AZ	R74	*X. gracile*
270345	San Bernardino	CA	R3	*H. ravenii*
270353	San Bernardino	CA	R30	*H. ravenii*
270357	San Bernardino	CA	R101	*H. ravenii*
270360	San Bernardino	CA	R2	*H. ravenii*
272039	Cochise	AZ	R42	*X. gracile*
285676	Coconino	AZ	R17	*X. gracile*
288765	Coconino	AZ	R102	*X. gracile*
288766	Cochise	AZ	R81	*X. gracile*
288767	Pima	AZ	R90	*X. gracile*
299176	San Bernardino	CA	R66	*H. ravenii*
342080	Coconino	AZ	R43	*X. gracile*
353021	Coconino	AZ	R73	*X. gracile*
355688	Sierra	NM	R56	*X. gracile*
359560	Cochise	AZ	R9	*X. gracile*
359561	Cochise	AZ	R60	*X. gracile*
359562	Santa Cruz	AZ	R79	*X. gracile*
359563	Cochise	AZ	R15	*X. gracile*
359564	coyote mountain	AZ	R71	*X. gracile*
359565	showlow springerville	AZ	R14	*X. gracile*
359566	Santa Cruz	AZ	R58	*X. gracile*
359567	blue river	AZ	R27	*X. gracile*

Table A.1 (Continued)

359568	Sheldon	AZ	R62	*X. gracile*
359570	Coronado	AZ	R103	*X. gracile*
359571	Archuleta	CO	R16	*X. gracile*
359572	Dona Ana	NM	R33	*X. gracile*
359573	Otero	NM	R104	*X. gracile*
359631	NM	NM	R105	*X. gracile*
370105	Santa Cruz	AZ	R59	*X. gracile*
370321	Santa Cruz	AZ	R72	*X. gracile*
297773	NA	NA	R4	*X. gracile*
444487	San Bernardino	CA	R1	*H. ravenii*
488278	Santa Cruz	AZ	R106	*X. gracile*
495325	Sonora	Mexico	R64	*X. gracile*
498868	San Antonio, lower CA	CA	R107	*H. ravenii*
503011	El salto	NA	R88	*X. gracile*
505006	NA	NA	R70	*X. gracile*
510508	Yavapai	AZ	R44	*X. gracile*
515595	Pima	AZ	R45	*X. gracile*
522145	Pima	AZ	R46	*X. gracile*
524147	Yavapai county, AZ	AZ	R47	*X. gracile*
538160	Washington	UT	R52	*H. ravenii*
538244	Coconino	AZ	R94	*X. gracile*
543569	Maricopa	AZ	R48	*X. gracile*
575452	Hidalgo	NM	R108	*X. gracile*
579187	Santa Cruz	AZ	R18	*X. gracile*
584749	San Bernardino	CA	R65	*X. gracile*
605531	Santa Cruz	AZ	R54	*X. gracile*
625998	NM-AZ state line	NM_AZ	R32	*X. gracile*
628159	Cochise	AZ	R61	*X. gracile*
644737	Coconino	AZ	R10	*X. gracile*
655901	Sonora	Mexico	R82	*X. gracile*
662358	Pima	AZ	R41	*X. gracile*
680843	Mohave	AZ	R69	*X. gracile*
685521	Yavapai	AZ	R13	*X. gracile*
698688	Coconino	AZ	R68	*X. gracile*
743870	Pinal	AZ	R22	*X. gracile*
750467	Cochise	AZ	R21	*X. gracile*
763349	San Bernardino	CA	R20	*H. ravenii*

Table A.2 Arizona State University Herbarium Accessions, Counties, Sample labels and Geographic Coordinates of *Xanthisma gracile* (*Haplopappus ravenii*)

Voucher Accessions	County	State	Latitude	Longitude	Specimen Labels	Taxon
12202	Coconino	AZ	35.1866	-111.618	A1	*X. gracile*
12203	Coconino	AZ	35.1981	-111.651	A2	*X. gracile*
12204	Gila	AZ	33.74	-110.93	A3	*X. gracile*
12205	Navajo	AZ	34.2542	-110.029	A4	*X. gracile*
12206	Maricopa	AZ	33.9686	-112.729	A5	*X. gracile*
12208	Pima	AZ	31.8078	-110.594	A6	*X. gracile*
12209	Coconino	AZ	35.8333	-112.083	A7	*X. gracile*
12210	Pinal	AZ	33.3722	-111.201	A8	*X. gracile*
12213	Gila	AZ	33.6485	-111.114	A9	*X. gracile*
12214	Gila	AZ	33.6008	-110.517	A10	*X. gracile*
12215	Maricopa	AZ	33.8642	-111.467	A11	*X. gracile*
12217	Maricopa	AZ	33.5323	-111.369	A12	*X. gracile*
12218	Cochise	AZ	31.4481	-109.928	A13	*X. gracile*
12219	Maricopa	AZ	33.7916	-111.467	A14	*X. gracile*
12221	Gila	AZ	34.3152	-111.016	A15	*X. gracile*
12224	Yavapai	AZ	34.46	-112.43	A16	*X. gracile*
12228	Maricopa	AZ	33.5454	-111.452	A17	*X. gracile*
12229	Pima	AZ	31.7639	-110.749	A18	*X. gracile*
12250	Coconino	AZ	35.1866	-111.618	A19	*X. gracile*
12323	Pima	AZ	31.5747	-111.332	A20	*X. gracile*
12330	Coconino	AZ	35.1981	-111.651	A21	*X. gracile*
12331	Gila	AZ	34.2308	-111.324	A22	*X. gracile*
12332	Santa Cruz	AZ	31.4092	-111.127	A23	*X. gracile*
12333	Pima	AZ	32.5	-110.921	A24	*X. gracile*
12337	Santa Cruz	AZ	31.5394	-110.756	A25	*X. gracile*
12340	Cochise	AZ	31.9341	-109.117	A26	*X. gracile*
12342	Navajo	AZ	33.7906	-109.988	A27	*X. gracile*
12348	Gila	AZ	34.3086	-111.343	A28	*X. gracile*
12355	Graham	AZ	32.67	-109.88	A29	*X. gracile*
12379	Gila	AZ	33.6478	-111.114	A30	*X. gracile*
12380	Navajo	AZ	34.4314	-110.593	A31	*X. gracile*
12381	Pinal	AZ	33.2794	-111.157	A32	*X. gracile*
12382	Yavapai	AZ	34.2017	-112.763	A33	*X. gracile*
12383	Yavapai	AZ	34.5833	-112.6	A34	*X. gracile*
12386	Gila	AZ	33.6891	-111.241	A35	*X. gracile*
12389	Gila	AZ	34.1014	-110.963	A36	*X. gracile*

Table A.2 (Continued)

15917	Cochise	AZ	31.4269	-110.455	A37	*X. gracile*
15935	Maricopa	AZ	33.8984	-111.823	A38	*X. gracile*
15965	Graham	AZ	33.1786	-109.863	A39	*X. gracile*
15966	Greenlee	AZ	33.4	-109.333	A40	*X. gracile*
15971	Yavapai	AZ	34.4247	-113.241	A41	*X. gracile*
15972	Santa Cruz	AZ	31.5148	-110.727	A42	*X. gracile*
16024	Gila	AZ	33.2822	-110.821	A43	*X. gracile*
16025	Yavapai	AZ	34.5833	-112.6	A44	*X. gracile*
20059	Santa Cruz	AZ	31.4092	-111.085	A45	*X. gracile*
39433	Apache	AZ	NA	NA	A4	*X. gracile*
78098	Gila	AZ	33.6941	-110.587	A47	*X. gracile*
78354	Santa Cruz	AZ	31.4611	-111.331	A48	*X. gracile*
78362	Navajo	AZ	34.0482	-110.222	A49	*X. gracile*
81357	Pinal	AZ	33.28	-111.17	A50	*X. gracile*
83050	Maricopa	AZ	33.5975	-111.205	A51	*X. gracile*
83899	Maricopa	AZ	33.9686	-112.729	A52	*X. gracile*
85188	La Paz	AZ	33.8224	-113.384	A53	*X. gracile*
88116	Graham	AZ	32.65	-109.82	A54	*X. gracile*
88380	Coconino	AZ	34.8262	-111.76	A55	*X. gracile*
101360	Maricopa	AZ	33.8642	-111.467	A56	*X. gracile*
113414	Mohave	AZ	34.9567	-113.678	A57	*X. gracile*
146889	Apache	AZ	34.5341	-109.307	A58	*X. gracile*
160847	Gila	AZ	34.2859	-111.664	A59	*X. gracile*
160881	Cochise	AZ	31.8825	-109.203	A60	*X. gracile*
167286	La Paz	AZ	33.91	-113.63	A61	*X. gracile*
184895	Maricopa	AZ	33.5333	-111.333	A62	*X. gracile*
190241	Pinal	AZ			A63	*X. gracile*
200251	Mohave	AZ	36.7849	-113.232	A64	*X. gracile*
211369	Pima	AZ	32.4303	-110.705	A65	*X. gracile*
229082	Graham	AZ	32.9251	-110.167	A66	*X. gracile*
238214	Mohave	AZ	36.8983	-112.918	A67	*X. gracile*
240927	Pinal	AZ	32.9765	-110.777	A68	*X. gracile*
245401	Cochise	AZ	31.7197	-110.163	A69	*X. gracile*
246109	Yavapai	AZ	34.659	-111.749	A70	*X. gracile*
263703	Maricopa	AZ	33.982	-111.71	A71	*X. gracile*
263830	Santa Cruz	AZ	31.6184	-110.496	A72	*X. gracile*
264708	Maricopa	AZ	33.97	-111.861	A73	*X. gracile*
264788	Maricopa	AZ	33.9662	-111.863	A74	*X. gracile*
266649	Yavapai	AZ	34.495	-112.545	A75	*X. gracile*

Table A.2 (Continued)

268506	Pima	AZ	31.7769	-110.722	A76	*X. gracile*
270622	Gila	AZ	33.72517	-110.99	A77	*X. gracile*

Table A.3 Mississippi State University Herbarium Accessions of *Xanthisma gracile*, Specimen labels, Geographic Coordinates.

Population	**Voucher Accession**	**Latitude**	**Longitude**
X_01	LC1	33.26482	-111.17558
X_03	LC3	35.1562	-111.68711
X_04	LC4	34.8498	-111.82837
X_05	LC5	34.81525	-111.90518
X_06	LC6	34.70784	-112.1489
X_07	LC7	34.67791	-112.187
X_08	LC8	34.51982	-112.45229
X_09	LC9	34.35759	-112.38261
X_10	LC10	34.37462	-112.25768
X_11	LC11	31.7841	-110.6965
X_12	LC12	31.78249	-110.74254
X_13	LC13	31.67921	-110.65564

Table A.3 (Continued)

X_14	LC14	31.60082	-110.57849
X_15	LC15	31.54105	-110.51155
X_16	LC16	31.54361	-110.33031
X_17	LC17	31.96148	-110.34653

APPENDIX B

LIST OF POPULATION NAMES AND COORDINATES OF *XANTHISMA GRACILE*

Table B.1 Geographic coordinates with sample names of herbarium specimens of *Xanthisma gracile* from central Arizona used to supplement the dataset for chapter 3.

Sample	Voucher Accession	Latitude	Longitude
X_18	ASU81357	33.288	-111.17
X_19	ASU263703	33.982	-111.71
X_20	ASU12379	33.6478	-111.114
X_21	ASU12228	33.5454	-111.452
X_22	ASU12217	33.5323	-111.369
X_23	ASU12213	33.6485	-111.114
X_24	ASU83899	33.9686	-112.729
X_25	ASU240927	32.9765	-110.777
X_26	ASU12381	33.2794	-111.157
X_27	ASU167286	33.91	-113.63
X_28	ASU85188	33.8244	-113.384
X_29	ASU12206	33.9686	-112.729
X_30	ASU15971	34.4247	-113.241

Table B.1 (Continued)

X_31	ASU246109	34.659	-111.749
X_32	ASU245401	31.7197	-110.163
X_33	ASU12340	31.9341	-109.117
X_34	ASU12204	33.74	-110.93
X_35	ASU12210	33.3722	-111.201

APPENDIX C

LIST OF ACCESSIONS OF *XANTHISMA SPINULOSUM* AND ITS VARIETIES FROM ARIZONA STATE UNIVERSITY

Table C.1 Arizona State University Herbarium Accessions, Counties, Sample labels and Geographic Coordinates of *Xanthisma spinulosum*.

Accession Number	County	Latitude	Longitude	Specimen Label	Variety
12236	Maricopa	33.5536	-111.442	P1	*goodingii*
12240	Pinal	33.4286	-111.418	P2	*spinulosum*
12244	Yavapai	34.3592	-112.311	P3	*goodingii*
12245	Maricopa	33.4992	-111.641	P4	*goodingii*
12246	Maricopa	33.85	-112.14	P5	*goodingii*
12247	Maricopa	34.02	-112.15	P6	*goodingii*
12249	Pinal	32.9778	-111.479	P7	*spinulosum*
12251	La Paz	33.6639	-114.229	P8	*goodingii*
12253	Maricopa	33.686	-111.739	P9	*goodingii*
12327	Pinal	33.341	-111.446	P10	*spinulosum*
12329	Maricopa	33.4149	-111.41	P11	*goodingii*
12376	Pinal	33.415	-111.462	P12	*spinulosum*
12377	Yavapai	34.825	-111.788	P13	*goodingii*
12387	Maricopa	33.5453	-111.606	P14	*goodingii*
15934	Maricopa	33.8984	-111.823	P15	*goodingii*
15936	Mohave	36.0161	-114.737	P16	*goodingii*
15939	Yavapai	34.1229	-112.951	P17	*goodingii*
15985	Mohave	34.3106	-113.526	P18	*goodingii*
16042	Gila	34.0282	-111.286	P19	*goodingii*
20052	Maricopa	33.8533	-112.269	P20	*goodingii*
20060	Pinal	32.7067	-111.202	P21	*spinulosum*
20061	Maricopa	33.8776	-112.277	P22	*goodingii*
20062	Pima	31.9422	-112.003	P23	*goodingii*

Table C.1 (Continued)

20239	Maricopa	33.5319	-112.573	P24	*goodingii*
23074	Maricopa	33.5965	-112.557	P25	*goodingii*
77366	Cochise	31.9136	-109.141	P26	*chihuahuanum*
78349	La Paz	33.68	-114.08	P27	*goodingii*
78804	Pima	32.2686	-112.738	P28	*spinulosum*
80692	Maricopa	33.6466	-111.779	P29	*goodingii*
83304	Pima	31.6913	-110.749	P30	*spinulosum*
84071	Yuma	32.2261	-113.663	P31	*goodingii*
85011	Yavapai	34.1909	-112.141	P32	*goodingii*
85041	Maricopa	33.1842	-112.217	P33	*goodingii*
102701	Graham	33.1797	-109.861	P34	*spinulosum*
103073	Graham	33.25	-110.28	P35	*spinulosum*
103099	Graham	32.9549	-109.531	P36	*spinulosum*
144281	Coconino	36.0822	-112.037	P37	*goodingii*
164406	Maricopa	33.935	-112.698	P38	*goodingii*
173374	Cochise	31.6262	-110.174	P39	*chihuahuanum*
190324	Maricopa	33.531	-111.433	P40	*goodingii*
190991	Coconino	36.1372	-111.816	P41	*goodingii*
190995	Mohave	36.2797	-112.875	P42	*goodingii*
191790	Cochise	31.5802	-109.268	P43	*chihuahuanum*
194221	Yavapai	34.938	-112.844	P44	*goodingii*
212390	Yuma	32.3089	-114.05	P45	*goodingii*
212463	Yavapai	34.49	-113.07	P46	*goodingii*
213920	Yavapai	34.7833	-112.907	P47	*goodingii*
219127	Cochise	31.3874	-110.11	P48	*chihuahuanum*

Table C.1 (Continued)

219416	Pima	31.9575	-110.955	P49	*spinulosum*
226781	Apache	NA	NA	P50	*spinulosum*
233635	Cochise	31.4479	-110.308	P51	*chihuahuanum*
238470	Mohave	36.2725	-114.013	P52	*goodingii*
240268	Pima	31.9842	-110.561	P53	*spinulosum*
240915	Cochise	31.9401	-110.329	P54	*goodingii*
243742	Mohave	36.925	-113.857	P55	*goodingii*
244850	Coconino	35.0069	-111.772	P56	*goodingii*
245184	Coconino	34.9891	-111.747	P57	*goodingii*
248202	Maricopa	33.9979	-112.147	P58	*goodingii*
249272	Maricopa	33.0681	-112.486	P59	*goodingii*
251146	Maricopa	33.8604	-112.393	P60	*goodingii*
253097	Cochise	31.5183	-110.13	P61	*chihuahuanum*
260715	Cochise	34.26	-111.31	P62	*goodingii*
265230	La Paz	33.4439	-113.397	P63	*goodingii*
267191	Pinal	32.7516	-112.125	P64	*spinulosum*
270715	Yavapai	34.1094	-112.112	P65	*goodingii*
271532	Maricopa	33.4085	-113.302	P66	*goodingii*
271629	Pinal	32.585	-110.52	P67	*spinulosum*
272162	Maricopa	33.7716	-113.119	P68	*goodingii*
272268	Maricopa	33.6926	-113.055	P69	*goodingii*
272389	Maricopa	33.6591	-113.084	P70	*goodingii*
276010	Pima	NA	NA	P71	*spinulosum*
276421	La Paz	NA	NA	P72	*goodingii*
279274	Maricopa	NA	NA	P73	*goodingii*

Table C.1 (Continued)

282264	Maricopa	33.67832	-111.809	P74	*goodingii*
282284	Maricopa	33.67812	-111.811	P75	*goodingii*
283733	Cochise			P76	*chihuahuanum*