KNOWLEDGE OF GENETIC DIVERSITY INFORMS CONSERVATION OF A RARE, CLONAL WETLAND PLANT *LILAEOPSIS SCHAFFNERIANA* SUBSP. *RECURVA* (APIACEAE)

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ABSTRACT

Ciénegas and other marshland habitats distributed throughout mid-elevations in the American Southwest have declined dramatically over the past century. This decline has likely affected natural processes for many plants and animals dependent on these unique habitats. The primary goal of this study is to describe genetic diversity within and among populations of the U.S. federally endangered Huachuca water umbel, *Lilaeopsis schaffneriana* subsp. *recurva*, an herbaceous aquatic perennial endemic to ciénegas and river edges of southeastern Arizona and northern Sonora, Mexico, to learn about its basic biology and inform conservation management. Population samples were collected from 13 sites across the range (287 total samples), and genetic diversity data were gathered for 14 microsatellite loci (six of which were discovered to be monomorphic). Results of data analyses of eight variable microsatellite loci revealed that most populations are dominated by a single multilocus genetic clone; only two populations have more than one multilocus genetic clone present (represented by more than one individual). Genetic diversity is low within populations, but genetic differences do exist among most populations. Those populations that are very similar to one another likely experienced recent or historical gene flow. Conservation considerations should include preserving multiple, genetically distinct populations as well as maintaining local population connectivity and quality of suitable habitat for the establishment of new clones.

Key Words: Clonality, Ciénegas, Conservation genetics, Geographic structure, Microsatellites, Multilocus genotypes

RESUMEN

Las ciénagas y otros hábitats pantanosos distribuidos por las elevaciones medias del suroeste americano han disminuido dramáticamente a lo largo del siglo pasado. Esta declinación ha afectado probablemente los procesos de muchas plantas y animales que dependen de estos hábitats únicos. El principal objetivo de este estudio es describir la diversidad genética en y entre poblaciones de la planta en peligro del listado federal de U.S. "Huachuca water umbel", *Lilaeopsis schaffneriana* subsp. *recurva*, una herbácea acuática perenne endémica de ciénagas y riberas del sureste de Arizona y norte de Sonora, México, para conocer su biología básica e informar a los gestores de conservación. Se colectaron muestras poblacionales de 13 lugares a lo ancho de su rango (287 muestras totales), y los datos de diversidad genética se obtuvieron de 14 loci de microsatélites (seis de los cuales fueron monomórficos). Los resultados del análisis de datos de ocho loci variables de microsatélites revelaron que la mayoría de las poblaciones están dominadas por un clon genético simple multilocus; solo dos poblaciones tienen más de un clon genético simple multilocus presente (representados por más de un individuo). La diversidad genética es baja en las poblaciones, pero existen diferencias genéticas entre la mayoría de las poblaciones. Esas poblaciones que son muy parecidas unas a otras probablemente experimentaron deriva genética reciente o histórica. Las consideraciones de conservación deben incluir preservar poblaciones múltiples, genéticamente diferentes, así como mantener la conectividad local de las poblaciones y el hábitat apropiado para el establecimiento de nuevos clones.

INTRODUCTION

Ciénegas, mid-elevation wetlands with permanently saturated, organic soils, were once a widespread feature of the American Southwest (Hendrickson & Minckley 1985; Minckley et al. 2013). However, in the last 100 years, ciénegas have declined precipitously, primarily because of intensive human activities such as groundwater withdrawal, streambed modifications, and grazing, as well as shifts in climate (Hendrickson & Minckley 1985; Stromberg et al. 1996). Shallow streams meandering through alluvial fills surrounded by large marshes and wetlands have become deeply incised arroyos with scattered marshy remnants. Perennial water bodies have become intermittent, ephemeral, or dry. Intact ciénegas perform many ecosystem services such as water purification, flood control, nutrient cycling, and provision of habitat for a wide variety of plants and animals,

including 15 U.S. federally-listed as Endangered, Threatened, or Candidate species (Minckley et al. 2013). Despite the high conservation value of ciénegas, little progress has been made toward preventing or reversing the loss of these unique areas. Over 20% of known ciénegas have lost their ecological function, and over 50% of remaining ciénegas have no formal protection (Minckley et al. 2013). This continuing decline has no doubt affected natural processes throughout the region and has significant implications for all living communities dependent on this distinctive habitat.

Huachuca water umbel, *Lilaeopsis schaffneriana* (Schltdl.) J.M. Coult. & Rose subsp. *recurva* (A.W. Hill) Affolter, is an herbaceous semi-aquatic to fully aquatic perennial plant closely associated with the ciénegas of southeastern Arizona and adjacent northern Sonora, Mexico (Titus & Titus 2008a; Minckley et al. 2013; U.S. Fish & Wildlife Service 2016). The U.S. Fish and Wildlife Service determined endangered status for *L. schaffneriana* subsp. *recurva* in 1997 and designated critical habitat for this species in 1999. Because of the loss of ciénegas and other associated wetland habitats throughout the region, *L. schaffneriana* subsp. *recurva* has likely experienced declines in the number, size, and connectivity of populations (U.S. Fish & Wildlife Service 2016), potentially leading to decreased genetic diversity and threatening long-term survival (Booy et al. 2000; Amos & Balmford 2001; Unmack & Minckley 2008). Recently verified, extant populations of *L. schaffneriana* subsp. *recurva* number around 20, and these are primarily distributed in the San Pedro and Santa Cruz watersheds along the San Pedro River, in the Western Huachuca Mountains, and along Cienega Creek (U.S. Fish & Wildlife Service 2016: fig. 2). Fewer populations are known from the Rio Yaqui, Rio Concepcion, and Rio Sonora watersheds, though surveys there have been limited and infrequent. It is difficult to assess the abundance of plants dispersed across these populations due to the diminutive size of the plant, clumped rhizomatous growth of leaves, rapid expansion and contraction of patch sizes across seasons and years, and variation in monitoring approaches used. Most extant populations are composed of several dispersed patches of plants, though more than one quarter of extant populations are composed of a single patch of plants (U.S. Fish & Wildlife Service 2016).

Populations are primarily maintained through clonal growth facilitated by rhizomes (Affolter 1985); however, *L. schaffneriana* subsp. *recurva* also produces flowers, fruits, and seeds under a variety of environmental conditions (Titus & Titus 2008a, b). Although controlled studies have not been performed, field observations of *L. schaffneriana* subsp. *recurva* and greenhouse studies of seed production in isolated plants in a related species *L. carolinensis* (Affolter 1985) suggest that plants are self-compatible and self-pollinating. Other potential pollinators for this species are unknown, although ants have been observed visiting flowers (U.S. Fish & Wildlife Service 2016). Seeds are persistent in the seed bank (Titus & Titus 2008a, b, c) and have high germination rates (Titus & Titus 2008b). *Lilaeopsis schaffneriana* subsp. *recurva* plants are dispersed into new habitats, both locally and more distantly, through large buoyant fruits or dislodged vegetative fragments moved by wind, water, or the action of birds (birds may consume seeds or transport vegetative fragments; Affolter 1985; Titus & Titus 2008a). Regardless of the dispersal mechanism, new populations are likely to be established by one or a few genetic individuals (Affolter 1985).

These three factors—clonal growth, selfing, and colonization by one or a few genotypes—are expected to result in genetically uniform populations. While some research investigating higher-order genetic relationships within the genus *Lilaeopsis* has been completed (Spalik et al. 2010; Bone et al. 2011), research addressing genetic diversity within and among populations of *L. schaffneriana* subsp. *recurva* is lacking. An investigation of population genetic diversity can provide baseline information about this species, including how much genetic variation is found within populations, how populations and watersheds compare to one another, how much genetic exchange takes place among populations, and how prevalent clonal growth and sexual reproduction are within populations.

It is generally acknowledged that genetic information is a valuable resource for designing management strategies for rare plants, and such information may be especially valuable when managing for the conservation and long-term persistence of rare plants capable of clonal growth. Without a detailed understanding of the prevalence of asexual and sexual reproduction, the number of individuals and populations may be

overestimated. Although a species may seemingly be represented by a reasonable number of individuals and populations, if those individuals and populations consist of just one or a few clones, then the effective population size is significantly reduced (Gitzendanner et al. 2011; Martin et al. 2013; Sampson & Byrne 2016). Additionally, knowledge of the extent of genetic differentiation among individuals and populations can be used to carefully plan augmentations and introductions (Sampson & Byrne 2016).

Here, I report on an analysis of genetic diversity within and among populations of *L. schaffneriana* subsp. *recurva* distributed across five watersheds. The specific objectives were to infer the relative importance of sexual and asexual reproduction and generate baseline data regarding levels of genetic diversity within populations and extent of genetic differentiation among populations. I discuss the implications of these findings for the continued management of *Lilaeopsis schaffneriana* subsp. *recurva*, including the setting of conservation priorities and planning of reintroduction efforts.

MATERIALS AND METHODS

Sampling and DNA extraction

Defining populations and individuals in plants that grow clonally can be difficult. Here, the term "population" is used to denote concentrations of this species within a distinct locality, geographically distant from other such concentrations. Within populations, the term "patch" is used to denote continuous growth forms composed of numerous, upright leaves arising from underground rhizomes. Within patches, individual plants cannot be distinguished; therefore, the term "individual" is used to denote a single leaf, separated from another leaf by at least 10 cm.

A total of 287 individual samples from 13 geographically distinct populations were collected between September 2010 and August 2014 (Fig. 1; Table 1; exact geographical coordinates are not provided due to the sensitive nature of this species). Populations were small, and samples were collected evenly across the plant growth at each site. Some populations consisted of several dispersed patches, while other populations consisted of only a single patch (Table 1). At each population, between 14 and 24 individual leaves were collected across patches, when possible, or within a single patch, when necessary. The location of each sampled individual within the population was recorded using meter tape and a coordinate system. Populations were assigned to a watershed (hydrologic unit) based on the 8-digit Watershed Boundary Maps publically available from USGS and USDA-NRCS National Cartographic and Geospatial Center. Following collection, genomic DNA was extracted using a Qiagen DNeasy Plant Miniprep Kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol.

Data acquisition

To examine genetic diversity within and among populations of *L. schaffneriana* subsp. *recurva*, species-specific microsatellite loci were developed in conjunction with the University of Georgia's Savannah River Ecology Laboratory (SREL) in two rounds. In the first round of development, based on shotgun sequencing on the 454 platform (Tew et al. 2012), 62 microsatellite loci were exhaustively screened for use as consistent, high quality markers, and eight potentially variable microsatellite loci were identified (Tew et al. 2012, 2017). In the second round of development, based on shotgun sequencing on the Illumina platform (Lance et al. 2013), 35 microsatellite loci were selected based on microsatellite repeat type, flanking region quality, and number of times the sequence occurred throughout the genome and screened for amplification and variability in up to 144 individual samples. Following this screening, six potentially variable microsatellite loci were identified.

PCR amplifications of microsatellite loci were performed in 12.5 μL volume reactions containing 3.7 μL nuclease free water, 2.5 μL Promega 5X PCR buffer, 1.5 μL 25 mM $MgCl_2$, 1.0 μL 10 mM dNTPs, 1.25 μL 10X bovine serum albumin, 0.45 μL 10μ M 5'-GTTT-3' tagged primer, 0.05 μL 10 μM 5'-CAGTCGGGCGTCATCA-3' tagged primer, 0.45 μL 10μM 5'-CAGTCGGGCGTCATCA-3' FAM-labeled primer, 0.10 μL GoTaq DNA Polymerase (5U/μL; Promega, Madison, Wisconsin, USA), and 1.5 μL DNA template (5 ng/μL). Thermocycling conditions consisted of a touchdown protocol with an initial denaturation step of 2 min at 94°C followed by 20

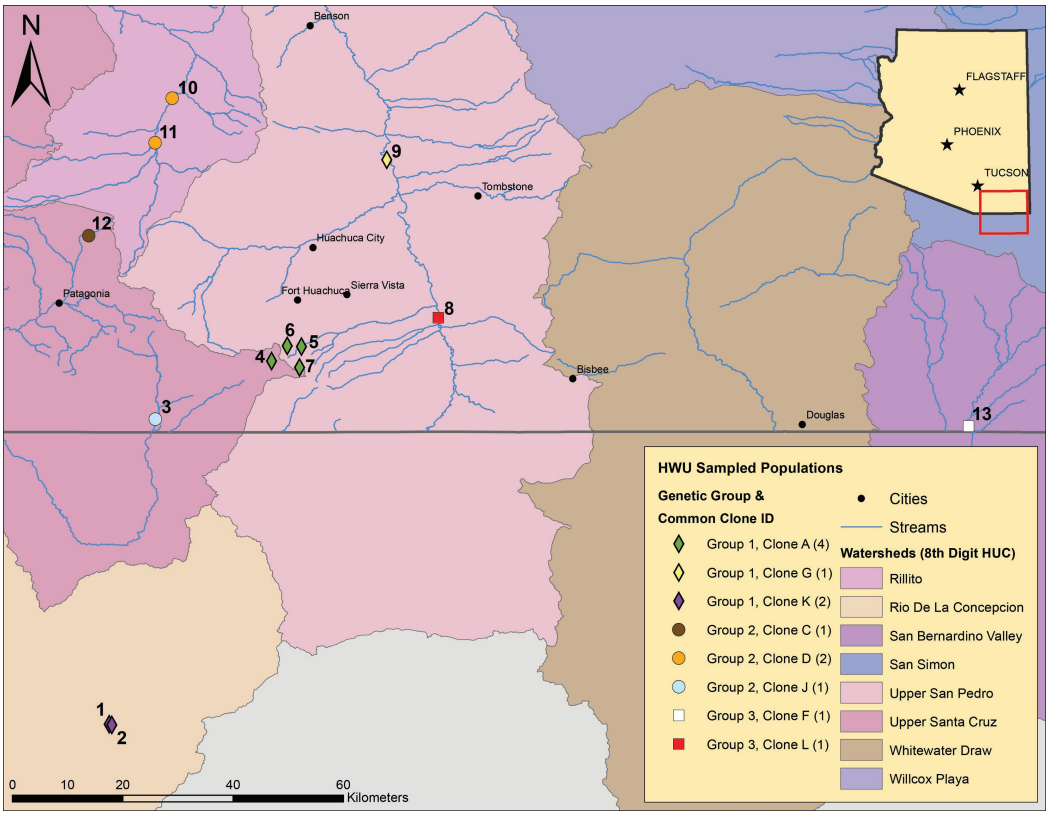

Fig. 1. Sampling locations for 13 populations of *Lilaeopsis schaffneriana* subsp. *recurva* included in the genetic study. Explanations for population numbers and other sampling details are included in Table 1. Populations are coded according to genetic grouping (symbol shape), as indicated in Fig. 2, and multilocus genotype (symbol color), as listed in Table 1. For populations with more than one multilocus genotype, the color for the most common clone is shown.

cycles of 96°C for 30 s, 60°C for 30 s (decreased 0.5°C per cycle), and 72°C for 30 s; 20 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and a final elongation step of 10 min at 72°C. Amplification products were screened by gel electrophoresis to determine if the reaction was successful. The sizes of microsatellite alleles were determined using a LIZ 600 (Life Technologies, Grand Island, New York, USA) internal size standard and capillary sequencer (model 3730; Applied Biosystems) at the Arizona State University DNA sequencing facility. The raw output from the capillary sequencer was read using the program GENEMARKER version 2.6 (SoftGenetics, State College, Pennsylvania, USA), and allele sizes were manually determined and recorded for each locus for each individual sample.

Throughout data collection, allele sizes for individual samples were selected for verification in two cases: (1) for individual samples where the internal size standard had failed and (2) for individual samples possessing a questionable allele (i.e., the allele differed by only one base pair as compared to a common allele, or the allele was extremely rare across the data set). For individual samples selected for verification, the data for the locus in question were re-acquired at least two additional times. In most cases, once this verification was performed, the allele with the failed internal size standard or the questionable allele was shown to be in error, and the actual allele was shown to be a common one. Following the verification process, four loci from the first and two loci from the second round of microsatellite development were found to be invariable across all samples and were excluded from further analysis. Problems with DNA quality for individuals from population 2 were also

TABLE 1. Sampling localities for genetic study of *Lilaeopsis schaffneriana* subsp. *recurva*. Shown are population number and name, occurrence ID, number of patches within each population, number of individual leaves sampled, locality and watershed information, approximate size of the sampling area, and number and type of multilocus genetic clones recovered based on analysis of eight microsatellite loci. Occurrence ID is based on photo vouchers deposited at the Southwest Environmental Information Network (SEINet) at http://swbiodiversity.org/seinet/index.php. NCA = National Conservation Area. *MLG clone "M" is found in only a single individual, which differs from all other individuals in the population by a single allele.

Pop. no	Population name	Occurrence ID	No. of patches	No. of samples	Nearest City, State, Country	USGS Watershed (8-HUC)	Approx. sampling area (m^2)	MLG clones present
1	Rancho El Aribabi - North	2540943	1	24	Magdalena, Sonora, MX	Rio de la Concepcion	230	E, H, I, K, N
2	Rancho El Aribabi - South	2540944	7	16	Magdalena, Sonora, MX	Rio de la Concepcion	344	K
3	San Rafael State Natural Area	2999708	4	24	Patagonia, AZ, USA	Upper Santa Cruz	95	B, J
4	Scotia Canyon	2999709	4	24	Sierra Vista, AZ, USA	Upper Santa Cruz	399	A
5	Garden Canyon	2999710	7	24	Ft. Huachuca, AZ, USA	Upper San Pedro	71	A
6	McClure Canyon	2540945	1	24	Ft. Huachuca, AZ, USA	Upper San Pedro	13	A
7	Sawmill Canyon	2999711	2	24	Ft. Huachuca, AZ, USA	Upper San Pedro	8	A
8	San Pedro Riparian NCA - South	2999712	1	24	Sierra Vista, AZ, USA	Upper San Pedro	6	L
9	San Pedro Riparian NCA - North	2999713	3	24	Sierra Vista, AZ, USA	Upper San Pedro	11	G
10	Pump Canyon - Las Cienegas NCA	2999714	8	23	Sonoita, AZ, USA	Rillito	56	D, M*
11	Empire Gulch - Las Cienegas NCA	14440090	1	18	Sonoita, AZ, USA	Rillito	1	D
12	Sonoita Creek	14440149	3	24	Sonoita, AZ, USA	Upper Santa Cruz	26	C
13	San Bernardino National Wildlife Refuge (sourced from Leslie Canyon)	14423641	2	14	Douglas, AZ, USA	San Bernardino Valley	Not recorded	F

discovered, and these individuals were excluded from further analysis. Results from the first round of development (Tew et al. 2012) found to be erroneous as a result of the verification process described here, have been corrected (Tew et al. 2017). Ultimately, data were acquired from four loci from the first round and four loci from the second round of microsatellite development (Table 2). There were no missing data. This final data set is available upon request from the author.

Data analysis

To determine the ability of our microsatellite loci to distinguish among different genetic individuals, the probability of identity (PI) for all individuals in each population was calculated in GENALEX version 6.5 (Peakall & Smouse 2006, 2012). PI is the average probability that two unrelated individuals, drawn from the same randomly mating population, will by chance have the same multilocus genotype. Higher statistical power of genetic markers is reflected in lower PI values.

The complete data set was examined using the program GENALEX to determine multilocus genotypes (MLGs) for all individual samples. MLGs were considered different if individuals differed by at least one allele. Standard population genetic diversity statistics were calculated in GENALEX (Table 3). For these calculations, identical MLGs in each population were removed to avoid biasing the results, as is typically done in studies of clonal plants (e.g., Brzyski & Culley 2011); however, results from analyses of the full data set did not differ substantially. Genetic diversity statistics included percentage of variable microsatellite loci (P; polymorphic loci), mean number of alleles per microsatellite locus (N_a), total number of private alleles (P_a; alleles found exclusively in that population); observed and expected heterozygosity (H_o and H_e), and inbreeding coefficient (f).

Table 2. Characteristics of eight variable microsatellite loci developed in *Lilaeopsis schaffneriana* subsp. *recurva* and used in this study. [a] = Previously reported in Tew et al. 2012, 2017. [b] = GTTT tag added to 5′ end. [c] = CAG tag (CAGTCGGGCGTCATCA) added to 5′ end.

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	Alleles
LISC4[a]	F: [b]CCACTCAAGCCACACAGATAG R: [c]TCACCAACTCCTCATCCGTG	AG	201–213	3
LISC9[a]	F: [b]TCATTTCTGCCGACTGATCAC R: [c]CAGAGTAATGCAACAAACACCC	ACAT	216–224	2
LISC15[a]	F: [b]ACGAAGTCCGATTACACACG R: [c]GCTCTTGTCTCCTTGTAAGCC	ACAT	266–278	2
LISC25[a]	F: [c]CTCATTTGAACCATGCCTTCTC R: [b]AAGCGTTTCTGGAAGGAAGC	ACAT	202–214	3
LS1	F: [c]TTTCATGCATTCCACTTTCG R: [b]CAACCAATGCAACTGACTGC	TGCTGG	286–298	3
LS15	F: [c]GAGAATCTGCACCGAAGTCC R: [b]AAATCAGGAGGGACAGGTCG	ATC	207–219	3
LS22	F: [c]GGAGTTGGTTCAAATAGAGCTGC R: [b]GCTCCACAAATAACATTCACTGC	ATC	215–224	2
LS25	F: [c]TGTTTCTTCAAATGCCCTGC R: [b]GTTGCTGCTGGGATAGTTGC	TGC	287–293	2

Table 3. Descriptive population genetic diversity statistics in *Lilaeopsis schaffneriana* subsp. *recurva*. Shown are number of individuals sampled (N), estimated number of MLG clones present (G), Percentage of polymorphic loci (P), mean number of alleles per locus (N_a), total number of private alleles (P_a); observed and expected heterozygosity (H_o and H_e), inbreeding coefficient (f), and clonal richness (R) = (G-1)/(N-1).

Population	N	G	P (%)	N_a	P_a	H_o	H_e	f	R
1	24	5	75.0	1.750	2	0.550	0.360	-0.496	0.17
2	16	1	37.5	1.375	0	0.375	0.188	-1.000	0.00
3	24	2	50.0	1.500	1	0.483	0.234	-0.833	0.04
4	24	1	37.5	1.375	0	0.375	0.188	-1.000	0.00
5	24	1	37.5	1.375	0	0.375	0.188	-1.000	0.00
6	24	1	37.5	1.375	0	0.375	0.188	-1.000	0.00
7	24	1	37.5	1.375	0	0.375	0.188	-1.000	0.00
8	24	1	50.0	1.500	0	0.500	0.250	-1.000	0.00
9	24	1	50.0	1.500	0	0.500	0.250	-1.000	0.00
10	23	2	62.5	1.625	0	0.563	0.297	-0.867	0.05
11	18	1	50.0	1.500	0	0.500	0.250	-1.000	0.00
12	24	1	50.0	1.500	1	0.500	0.250	-1.000	0.00
13	14	1	50.0	1.500	0	0.500	0.250	-1.000	0.00
Mean	22	1.46	48.08	1.481	0.31	0.456	0.237	-0.913	0.02

Clonal richness (R) was calculated by hand as R = (G – 1)/(N – 1) where G is the estimated number of MLG clones and N is the number of individuals sampled.

Genetic distances between all possible pairs of populations, with all samples included regardless of MLG, were calculated in GENALEX as the average number of allele differences between individuals from each population. Major patterns of genetic relationships among populations based on these genetic distances were visualized using principle coordinate analysis (PCoA) using a covariance matrix with data standardization in GENALEX. The relationship between genetic distances and the natural log of geographic distances between populations was evaluated using a Mantel test in GENALEX (isolation by distance). The partitioning of genetic diversity among individuals, populations, and watersheds was calculated using an analysis of molecular variance (AMOVA) based on genetic differentiation (Φ_{PT}) in GENALEX. Departures from Hardy-Weinberg

equilibrium (HWE) were calculated for each locus for each population in GENALEX. Sequential Bonferroni corrections (Rice 1989) were used when testing for statistical significance of these departures.

RESULTS

Among the 13 sites, the median PI for an eight-locus genotype was 0.01. This minimum PI value was achieved after combining six of the eight loci. Individual population values indicated that PI was sufficiently low to distinguish clones at seven of the populations (PI<0.01; populations 1, 8–13)), but that clonal diversity was potentially underestimated at six of the populations (PI<0.05; populations 2–7).

A total of 14 unique MLGs were found across 287 samples from 13 sites. MLGs differed from each other by one to 10 alleles across loci. The total number of different MLGs in each population ranged from 1 to 5, and most populations had only one MLG present (Table 1). One individual in population 10 differed from all other individuals in the population by a single allele, resulting in a unique MLG not found in any other individuals surveyed. When more than one MLG was found in more than one individual in the population (this occurred in populations 1 and 3), individuals with different MLGs appeared to intermingle spatially throughout the sampling area. Most populations were characterized by a unique MLG not shared with any other populations. However, identical MLGs were shared across populations in three cases: (1) all individuals in population 2 were characterized by MLG "K", which was also found in population 1; (2) all individuals in populations 4, 5, 6, and 7 were characterized by MLG "A"; (3) all individuals (but one) in population 10 were characterized by MLG "D", which was also found in all individuals in population 11.

A summary of genetic diversity statistics is shown in Table 2. The percentage of polymorphic loci was low, ranging from 37.5% to 75% for each population. A total of 20 alleles were found across all microsatellite loci and populations. Mean number of alleles per microsatellite locus was also low, ranging from 1.375 to 1.750. Most alleles were shared widely across all populations, and only three populations had private alleles: populations 1, 3, and 12. Mean observed heterozygosity was 0.456 with a range of 0.375 to 0.563, which was much higher than mean expected heterozygosity (0.237) with a range of 0.188 to 0.297. Mean inbreeding coefficient was highly negative (-0.913) with a range of -0.496 to -1.000. Clonal richness was low, ranging from 0.00 to 0.17.

PCoA of genetic distances among populations revealed three genetic clusters (Fig. 2): group 1 = populations 1 and 2 (MX), populations 4, 5, 6, 7 (Ft. Huachuca), and population 9 (SPRNCA - north); group 2 = population 3 (San Rafael) and populations 10, 11, 12 (Las Cienegas/Sonoita); and group 3 = population 8 (SPRNCA - south) and population 13 (San Bernardino). There was a significant positive correlation between genetic and geographic distances (rxy = 0.283, P = 0.001). Results from the AMOVA indicated that much of the observed genetic variation was attributable to differences among populations (57%) rather than differences within populations (3%), and 40% of the observed genetic variation was attributable to differences among watersheds. Departures from HWE were significant for all polymorphic loci in all populations except for two cases: locus LS1 in populations 1 and 10.

DISCUSSION

Clonal growth is clearly important for maintaining populations of *Lilaeopsis schaffneriana* subsp. *recurva*. Clonal growth is expected to result in certain population genetic patterns: identical MLGs within populations, an excess of heterozygotes relative to HWE (fixed heterozygotes due to a lack of sexual recombination), high negative values for inbreeding, and departures from HWE (Wright 1965; Balloux et al. 2003). *Lilaeopsis schaffneriana* subsp. *recurva* strongly exhibits each of these patterns, with a single MLG primarily dominating each population, mean H_o = 0.456 much greater than mean H_e = 0.237, a high negative mean f = -0.913, and all but two instances of departure from HWE. Although clonal growth is the dominant pattern detected here, sexual reproduction may also generate diversity among clones in *L. schaffneriana* subsp. *recurva* on rare occasions. More than one MLG in more than one individual was detected in two populations (1 and 3), and it appears that this diversity resulted from local sexual reproduction, due to the observation of recombined alleles at particular microsatellite loci (the single allele difference found in one individual in population 10 is more likely the

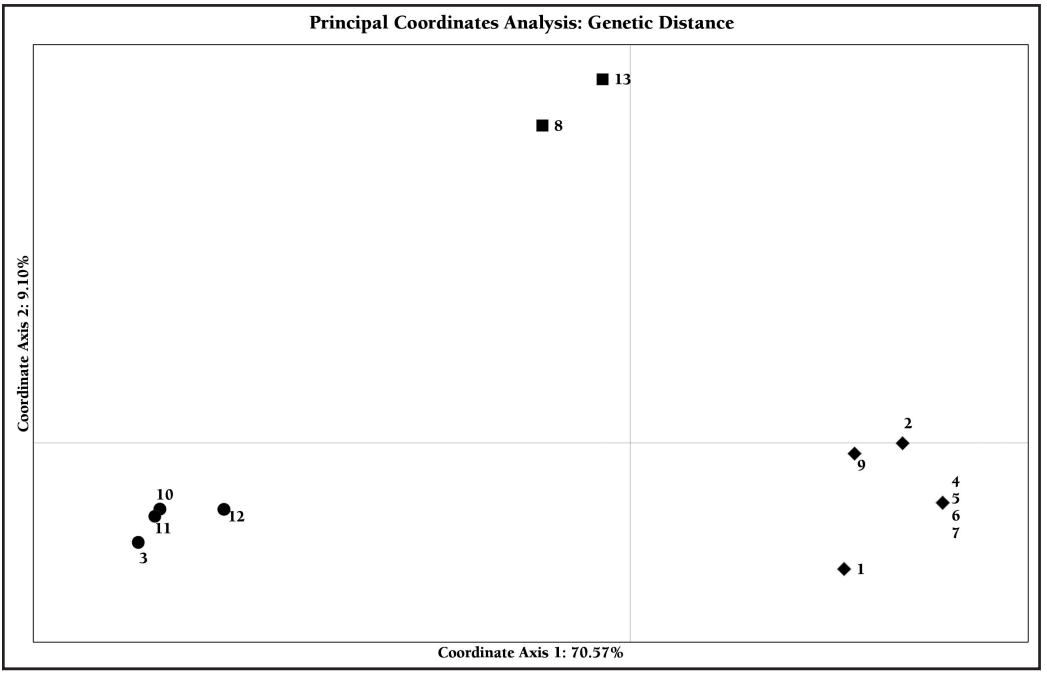

Fig. 2. Principal coordinates analysis of genetic distances calculated between populations of *Lilaeopsis schaffneriana* subsp. *recurva* based on variation at eight microsatellite loci. Genetic grouping is indicated by shape of the markers and corresponds to Fig. 1. Principle coordinate axis 1 explains 70.57% of the variation, and axis 2 explains 9.10%.

result of somatic mutation within a clone). Furthermore, the frequent production of viable seed and the presence of heterozygosity observed across loci, suggests that sexual reproduction may occur when populations harboring different clones come into contact (physically or through dispersal), as might have been possible when populations were more widespread and connected in the past (Hendrickson & Minckley 1985; Stromberg et al. 1996; Minckley et al. 2013). In fact, if all unique MLGs are treated as a single population, mean H_o = 0.509 is much closer to mean H_e = 0.507, and f = 0.017 fits expectations for outcrossing. The lack of detection of clonal diversity in most other populations appears to be independent of the size of the population or the number of patches sampled in the population (Table 1). Instead, detection of clonal diversity might be influenced by the history of the population at the precise sampling location (Malcom et al. 2017), i.e., whether sampling occurred in an area that was recently established or has long been stable, or in an area that is rarely or frequently disturbed. The number of MLGs detected in some populations could also have been underestimated because of the low diversity found in these microsatellite loci.

The presence of different MLGs in populations 1, 3, and 10 indicates that establishment of new genotypes (resulting from sexual reproduction, somatic mutation, dispersal, or the seed bank) must play a role in the maintenance of populations and clonal diversity within populations. For example, intermediate disturbance events, such as seasonal flooding or moderate grazing, could create suitable habitat for the establishment of new and unique clones (Titus & Titus 2008c; Malcom et al. 2017), potentially contributing to the preservation of genetic diversity in this species (Reusch 2006). Although it is not entirely clear why population 1 exhibits higher clonal diversity relative to other populations, the presence of habitat use by cattle was noted during collection of both populations 1 and 2, with population 2 being highly disturbed and population 1 being adjacent to a stream bed and somewhat less disturbed. Perhaps, the combination of recent, intermediate disturbance

followed by establishment of clones dispersed by stream waters or from the seedbank resulted in the higher clonal diversity observed in population 1. In contrast to intermediate disturbance, if large disturbance events remove all plants and most seeds from the seed bank in any given area, unlikely long-distance dispersal would have to occur for a population of *L. schaffneriana* subsp. *recurva* to become re-established. Even if such unlikely long-distance dispersal occurred, re-established populations would likely be limited to a single or very few clonal types, reducing overall genetic diversity (Affolter 1985; Evans et al. 2014). Further work is needed to better understand the relationship between various management and disturbance regimes and clonal diversity. In general, it appears MLGs can occupy spaces that are scattered throughout the population area. This is evidenced by the dispersed location of identical MLGs in populations 1 and 3, as well as the widely-dispersed presence of a single MLG across a large sampling area in populations 2 and 4 (i.e. >300 m^2).

Genetic diversity within populations of *L. schaffneriana* subsp. *recurva* is very low. Only two or three possible alleles for each microsatellite locus were detected, and the mean number of alleles per microsatellite locus is lower than expected for clonal plants (Balloux et al. 2003) and similar to that observed in other threatened wetland plants with clonal growth (Brzyski & Culley 2011; Gitzendanner et al. 2011). Mean expected heterozygosity in *L. schaffneriana* subsp. *recurva* (0.237), which is based on allele frequencies independent of fixed heterozygosity, is much lower than the average values observed for many different plant groups with various life histories for which data is available (Nybom 2004), including short-lived perennials (0.55), endemics (0.42), selfing plants (0.41), and plants with water dispersed seeds (0.61). Conservation efforts should therefore include actions that promote the preservation of existing genetic diversity in *L. schaffneriana* subsp. *recurva* populations. All of the U.S. populations surveyed here, as well as most known, extant populations from the U.S., occur in protected areas. In contrast, most populations from Mexico are not well-known, either in terms of current status or genetic diversity, and occur on private lands. More work is needed to document the status, protection, and genetic diversity of *L. schaffneriana* subsp. *recurva* populations from Mexico, as this represents a significant, potential opportunity to preserve genetic diversity and improve long-term survival of this species. Whether or not populations occur in protected areas, habitat quality is likely critical to population success. Recent research suggests that the presence of a perennial water source may play a critical role in the establishment and maintenance of populations (Malcom et al. 2017); however, additional research regarding the environmental and ecological factors that contribute to population persistence is needed.

Despite the generally low levels of genetic diversity observed in *L. schaffneriana* subsp. *recurva*, some patterns of shared genetic variation are evident across sampled populations. First, there is a positive correlation between genetic and geographic distance – populations in close geographic proximity are also the most similar genetically, even sharing identical MLGs. This includes populations 1 and 2 from Mexico, populations 4–7 from Scotia, Garden, McClure, and Sawmill Canyons, and populations 10 and 11 from Las Cienegas NCA. Local movement of clones or recent population connectivity is most likely responsible for the observed pattern of sharing, and conservation efforts should seek to preserve local dispersal pathways (Johansson & Nilsson 1993), which might include intervening areas of suitable habitat with a sufficient water source. Second, some distantly located populations are genetically similar to one another, such as populations 8 and 13 or populations 2 and 9. It seems unlikely that long distance dispersal could account for this pattern, especially since MLGs are not identical between populations. Conceivably, these similarities could have arisen because these populations have a shared history and were connected through additional, intervening populations in the recent past (e.g., Gitzendanner et al. 2011), as indicated by the documented loss of ciénegas throughout the American Southwest (Hendrickson & Minckley 1985; Stromberg et al. 1996; Minckley et al. 2013). However, the geographic distance between each pair of noted populations is large, and historical records do not support extensive connectivity between these particular regions (especially between populations 8 and 13). Management activity in Leslie Canyon and San Bernardino National Wildlife Refuges (near population 13) has been high over the last two decades (U.S. Fish & Wildlife Service 2016), and it is possible that similarities between populations 8 and 13 resulted from intentional (or even unintentional) movement by humans. Moreover, movement associated with human activity anytime in the past could account for some of the observed genetic

diversity and structure across populations, including these similarities between populations or clonal diversity within populations. Nonetheless, perhaps the most likely explanation is that with so few alleles observed for each microsatellite locus, the similarities seen between MLGs of distantly located populations could have arisen purely by chance. Eventually, with enough genetic markers, more of which could be developed from genomic resources generated in other ongoing projects (Christy Edwards, pers. comm.), the possibility of past connectivity could be more convincingly supported or rejected.

Although genetic diversity within populations is low, differences do exist among populations and watersheds. Conservation of large numbers of presumably genetically distinct populations, regardless of their size, may contribute to the preservation of genetic diversity in this species. Almost every population possesses a unique MLG, indicating that populations are not genetically homogeneous across the range, and care should be taken when introducing new populations to new areas to avoid the introduction of foreign alleles and potential effects of outbreeding (Booy et al. 2000). On the other hand, knowledge of genetic diversity could be used to guide reintroductions so that the genetic diversity of plants selected for reintroductions is similar but not identical to local populations. In this way, local or regional genetic diversity could be increased leading to a greater capacity for adaptation to diverse environments or recovery from disturbance events (Reusch 2006; Evans et al. 2014; Sampson & Byrne 2016).

CONCLUSIONS

Results of the genetic analyses presented here provide fundamental information about *L. schaffneriana* subsp. *recurva*: (1) clonal growth is the primary mode of reproduction, (2) genetic diversity within populations is very low, (3) genetic differences among populations and watersheds exist and should be considered when planning reintroductions, (4) some populations share specific clonal genetic types (MLGs) likely due to local movement of genes, and (5) some populations show genetic similarities that may have arisen by chance, or possibly through historical population connectivity. Numerous questions remain regarding fine-scale patterns of clonal growth and the long-term persistence of patches and populations. What are the patterns of clonal diversity within and among populations in Sonora, MX, and how do these compare to those observed thus far? How do the number and diversity of clones compare across various types and sizes of occupied areas? Is there variation at a local scale in where and when clonal growth, sexual reproduction, and establishment of new clones occur? How long-lived are clones? How are local vegetative and seed dispersal mediated and to what extent does each type of dispersal serve to connect populations? What levels and types of disturbance serve to promote the establishment of new genetic clones? In the future, the collection of fine-scale genetic diversity information with additional high-resolution markers, for example, intensive sampling within a single creek or watershed, could provide answers to questions that have arisen because of the present study and more.

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