



# Spatially explicit and multi-sourced genetic information is critical for conservation of an endangered plant species, San Diego thornmint (*Acanthomintha ilicifolia*)

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Received: 18 June 2017 / Accepted: 14 March 2018

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## Abstract

San Diego thornmint *Acanthomintha ilicifolia* (Gray) Gray (Lamiaceae) is a winter herb restricted to San Diego county in the United States and Baja California Norte in Mexico. Historic records document 80 occurrences of this species, with 55 extant occurrences in San Diego County currently known. We compared three measures of genetic variation to inform ongoing conservation efforts: putatively neutral genetic structure revealed from isozyme markers, apparent cytogenetic variation confirmed using flow cytometry, and potentially adaptive morphological variation quantified in a common-garden study. Together, these data indicated that this rare endemic is genetically complex, revealing significant differentiation of neutral and potentially adaptive genetic variation among populations, and possessing at least two cytotypes, sometimes even within the same population. While additional study is required to resolve the extent of potential local adaptation in this species, conservation plans should limit the movement of germplasm among occurrences and monitor populations in order to limit potential long-term impacts to population viability. Given that these findings challenge the canonical model of genetic structure in rare plants (low genetic variation and limited genetic structure), we recommend guidelines to apply genetic information to conservation strategies.

**Keywords** Local adaptation · Ploidy · Population genetic structure

## Introduction

The importance of genetic diversity for long-term persistence of native species is well established within conservation genetics (Schoenwald-Cox et al. 1983; Spielman et al. 2004; Aguilar et al. 2008). However, while technological advances have hastened the rate of development of genetic

information, the incorporation of such information into management actions is still relatively rare. The reasons for lack of practical application of genetic information are diverse and may include failure to frame research objectives within a management context (Ottewell et al. 2016). Moreover, the choice of analytical method has consequences for conservation-directed interpretation (Leimu et al. 2006). It is particularly critical that genetic information be used in decisions regarding rare and endangered species where there is little buffer for mistakes and opportunities to understand the natural patterns of diversity and linkages with environment are rapidly eroding.

San Diego thornmint *Acanthomintha ilicifolia* (Gray) Gray (Lamiaceae) is a winter herb restricted to San Diego county in the United States and Baja California Norte in Mexico. Historic records document 80 occurrences of this species, with approximately 55 known extant occurrences in San Diego County currently (i.e., not all mapped occurrences had been recently confirmed). An additional 13 occurrences in Baja California Norte are of uncertain status. The narrow range of this endemic, the decline in numbers

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**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s10592-018-1062-y>) contains supplementary material, which is available to authorized users.

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of the populations, and the susceptibility of the species to habitat degradation have all been cited in its protection as a threatened species under federal regulations, as an endangered species by the state of California, and as a species of concern by several municipalities.

There have been no genetic studies of this species prior to our undertaking, though a handful of previous studies have examined the ecology of San Diego thornmint to inform conservation strategies. Bauder and Sakrison (1997) demonstrated that seed required cool nighttime temperatures and light to germinate, and that manual removal of weeds (i.e., non-native species) increased seed production in experimental plots. Plants flower indeterminately at nodes, and while each flower produces a maximum of only four seeds, each plant can produce somewhere between 70 and over 200 seed. However, only limited and indirect evidence is available describing the mating system in this species—information that may be critical to guide appropriate conservation efforts. Examination of other species in the genus *Acanthomintha* revealed one, *A. duttonii*, to be self-compatible and autogamous with low frequency of insect visitors, while a second species, *A. obovata* ssp. *cordata*, was also self-compatible but less likely to self-fertilize (Steeck 1995). These results indicate mating systems are likely variable in this genus. Field observations of San Diego thornmint identified several species of insects, including checkered beetles, bee-flies, and a variety of bees, visiting flowers (Bauder and Sakrison 1997). Bee-flies were observed to visit flowers without alighting, and were thus unlikely to serve as pollinators. Checkered beetles moved among flowers on a single plant, possibly serving as pollinators for self-fertilization events. A variety of bees were observed moving among flowers on different plants, indicating within-population outcrossing could be possible, though the likelihood of bees transferring pollen over long distances (e.g. among populations) is low. Together, the annual life cycle, the potential for a self-compatible mating system, and the limited dispersal range of pollinators indicate that gene flow among occurrences (e.g. populations) of San Diego thornmint is likely limited.

The pollination biology and genetic structure of San Diego thornmint is further complicated by the possibility that the species may be a polyploid. The role of polyploidy, or the increase of chromosome number to anything greater than two copies per individual, is well established in the evolution of plant species (Stebbins 1947; Ramsey and Schemske 1998) and genome organization (Blanc and Wolfe 2004; Tuskan et al. 2006). The evolutionary and conservation implications for a species having a complex ploidy pattern (either variable ploidy levels or high levels such as tetraploid, hexaploid or even octoploid) differ remarkably based on whether the higher ploidy arise through chromosome doubling (autopolyploidy) or hybridization events (allopolyploidy, frequently followed by genome doubling).

In addition, polyploidy may be complicated by aneuploidy, which can result in isozyme pattern variation consistent with euploidy (Grant et al. 1984). Both autopolyploid (e.g. López-Pujol et al. 2004) and allopolyploid species (e.g. Huck and Chambers 1997) have been reported within the Lamiaceae. Gaining evidence as to whether polyploids are present in San Diego thornmint, and whether they are autopolyploid or allopolyploid in nature, would benefit ongoing restoration and conservation efforts.

This first genetic study of San Diego thornmint examined the genetic variation and differentiation among a robust representation of populations across its California range. A resolution to the limitations of single-source genetic assessments for conservation purposes is to approach discovery with multiple tools that will provide insights into these different and important aspects of genetic diversity and structure. We used a suite of isozyme markers, flow cytometry, and measurements of genetic variation in phenotypic traits through a common-garden study. Notwithstanding the substantial displacement of isozyme analysis in recent years with more direct measures of DNA polymorphisms such as microsatellite variation, the former provides still a well-established means of measuring biparentally inherited, selectively neutral diversity, with often minimal investment in a species-specific protocol. The consistent responsiveness of this approach is particularly valuable for previously unqueried species that might otherwise require significant investment in marker development, especially where the time and funds required may not justify the potential returns for conservation in practice (Kohn et al. 2006). In addition to the rich literature of hundreds of previous studies and well-established theoretical models with which to interpret results (Hamrick and Godt 1996; Cole 2003), isozymes can provide partial evidence of higher ploidy levels. As the enzyme activity levels are correlated with the number of copies of each dimer, the relative weights of each visualized band (e.g., AAAB vs. AABB) can be used to infer copy number variants indicative of higher level polyploids (Grant et al. 1984).

The relative frequency of these complex banding patterns differs between autopolyploid and allopolyploid species, following Ramsey and Schemske's (1998) definition of each. Allotetraploid species contain two copies of each allele from two parental species, and the two genomes fail to form synapses at meiosis, resulting in a typical "fixed heterozygote" with balanced heterozygous banding patterns. Alternatively, autotetraploid species arise through intra-population or intraspecific crosses, allowing the segregation of up to four alleles (in theory) in one individual at one locus. This complexity results in every combination of unbalanced heterozygotes, though the frequency of each is predicted to occur in fixed ratios due to the independent assortment of alleles (Soltis and Rieseberg 1986). By examining banding

patterns in San Diego thornmint for evidence of more than two alleles at a locus, and comparing the frequency of “fixed heterozygote” and unbalanced heterozygous banding patterns, isozyme data may provide evidence of polyploid levels and inheritance mechanisms.

In order to determine whether complex isozyme banding patterns were due to aneuploidy or euploidy, flow cytometry was used to compare relative DNA content of leaf tissue from a subset of populations. Finally, variation in several phenotypic traits were examined in a common-garden study as a first investigation of potential adaptive differentiation among populations spanning the geographic and environmental range of our collections.

This study reports on the use of three complementary analytical tools to address the following questions: (1) What is the nature (amount and structure) of selectively neutral genetic diversity in this species? (2) Is there evidence of differentiation in potentially adaptive traits? (3) Is there evidence of polyploidy within and/or among populations and, if so, are patterns consistent with certain cytotype identities and more indicative of either autopolyploid or allopolyploid origin? (4) In general, how do these methods compare in their results and implications for conservation of this endangered plant species?

## Methods

### Study site selections and sample collections

From the approximately 55 natural (“element”) occurrences (EOs) extant in California, a subset was selected for inclusion in the study based on representation of the latitudinal, longitudinal, and elevational range of the species. Occurrences were disqualified from consideration if they were known to have been restored (for this species) and thus potentially contain non-local genotypes. For example, EO 36, Sabre Springs West, near the City of Poway, was disqualified for this reason. Six occurrences—representing sites with potentially significant within-site differences in elevation or distance—were selected for sampling as two subpopulations. In total, the collections were made from 15 EOs and six additional ‘subpopulations’, resulting in 21 collection sites (Table 1; Fig. 1). From this total array, all sites were included in the isozyme analysis and subsets were selected for inclusion in the common-garden study and flow cytometry analysis. For all collection sites, permission was obtained from landowners in addition to required regulatory agency approvals. Details of permits are provided in the Supplementary Materials.

For the isozyme analysis, foliar tissue collections were made from 14 EOs (and an additional subpopulation from each of six of those occurrences) in March and April, 2013.

The range in collection dates represented phenological differences among the sites and the objective to collect fresh, green material when plants were large so that loss of some photosynthetic capacity would have a minimal impact on donor plants. It was not possible to sample leaf material from EO75 because of a small occurrence that year. As a resolution, plants were grown out from seeds collected from EO75 in 2012, resulting in 21 locations being included in the isozyme analysis.

For the study of genetically-based phenotypic variation using a common-garden design, seeds were collected from a subset of sites (due to the relatively large expense of this study) representing the range of elevational and latitudinal sources examined for isozyme variation (Fig. 1). Seeds were collected from seven EOs in June and July 2012. Collections were made from two subpopulations of the occurrences EO70 to provide preliminary insights into genetic variation at the more local level.

Based on the isozyme study results that provided evidence of ploidy variation both among and within some occurrences, a subset of sites was selected to resample foliage for examination using flow cytometry. Two sites were selected from each of the putative cytotype locations (i.e., 2x, 4x, 6x), selecting those sites that resembled, as closely as possible, a single cytotype. Foliage tissue was collected in April, 2016. Upon arriving at the one of the two putative hexaploid collection sites, no plants could be detected, resulting in only one collection for the putative 6x ploidy level.

In order to compare genetic, phenotypic, and climate variables among population, four weather variables were calculated for each population location using 36-years (1980–2015) of daily weather data obtained from Daymet (Thornton et al. 2014). Weather variables included the average total annual precipitation (mm; MeanPpt) and coefficient of variation (CVPpt), the average wet-season (November–June\*) maximum temperature [ $^{\circ}\text{C}$ , MaxTemp (W)] and its coefficient of variation [CV (temp)].

### Isozyme analyses

Details of the isozyme electrophoresis are provided in the supplementary information. Sample preparation and starch–gel electrophoresis followed well established laboratory standard operating protocols (USDA Forest Service 2012). The final isozyme data for each sample consisted of an 18-locus isozyme genotype with each allele contributing to the banding pattern coded by a number. As isozymes are co-dominant markers, the relative weight of bands was scored in addition to the band position on the gel in order to infer differences in dosage that may indicate polyploidy. Matching genotypes—indicative of possible inbreeding, autogamy, or low levels of allelic variation—were identified

**Table 1** Sampling details of 21 collections (some occurrences sampled at two subpopulations) of San Diego thornmint examined for isozyme and morphological variation

Occurrence	Abbrev.	Latitude	Longitude	Elevation (m)	C <sup>a</sup>	N <sup>b</sup>	G <sup>c</sup>	M <sup>d</sup>
Los Penasquitos Canyon	EO19	32.92721	-117.17955	26		16	15	
McGinty Mountain (southwest slope)	EO21	32.73829	-116.86717	420	10	30	29	
McGinty Mountain (summit and ridgeline)	EO22	32.75339	-116.86012	612		30	30	
Lux Canyon (east)/Manchester A	EO28A <sup>e</sup>	33.13129	-117.24972	95		21	20	
Lux Canyon (east)/Manchester B	EO28B <sup>e</sup>	33.03090	-117.24978	95		21	21	
Sycamore Canyon 1	EO32-1	32.92979	-116.96805	317 <sup>f</sup>		20	20	
Sycamore Canyon 2	EO32-2	32.93472	-116.97651	317 <sup>f</sup>		19	19	55
Mission Trails Regional Park	EO33	32.82753	-117.07378	204		21	20	55
Los Penasquitos Canyon, Sabre Springs west	EO36	32.94246	-117.09327	86		14	12	
Emerald Point	EO58	33.12097	-117.29922	62		11	9	
Wright's Field N	EO67N <sup>g</sup>	32.81704	-116.76555	564		30	29	
Wright's Field S	EO67S <sup>g</sup>	32.82143	-116.76563	567		30	19	
Carlsbad Oaks North A	EO70A	33.13698	-117.26333	84		21	21	55
Carlsbad Oaks North B	EO70B	33.13254	-117.26460	48	9+2	21	21	55
La Costa Greens	EO82	33.11818	-117.26533	73		17	16	
Rancho Jamul Ecological Reserve, Hollenbeck Canyon	EO85	32.69991	-116.81348	483		30	30	
South Crest 1	EO72-1	32.78789	-116.86620	398		20	18	
South Crest 2	EO72-2	32.78896	-116.86527			20	20	
San Diego National Wildlife Refuge lower	EO87l	32.75008	-116.87154	377	10	30	30	55
San Diego National Wildlife Refuge upper	EO87u	32.74997	-116.87027			30	29	
Cleveland National Forest	EO75	32.85634	-116.74304	827	4	30 <sup>h</sup>	28	55

<sup>a</sup>Number of plants examined using flow cytometry

<sup>b</sup>Number of plants examined for isozyme variation

<sup>c</sup>Number of distinct genotypes observed with isozyme data

<sup>d</sup>Number of plants examined for phenotypic variation in the common-garden study

<sup>e</sup>The Lux Canyon occurrence is also referred to as EO42

<sup>f</sup>Average of min and max reported

<sup>g</sup>Wright's Field North is also referred to as EO63

<sup>h</sup>EO75 assessed as 30 seedlings germinated from a bulk seed lot collected in 2012

using the multilocus matching function of GenAlEx v. 6.5 (Peakall and Smouse 2006). When matching genotypes were identified, the population of origin was assessed for each sample.

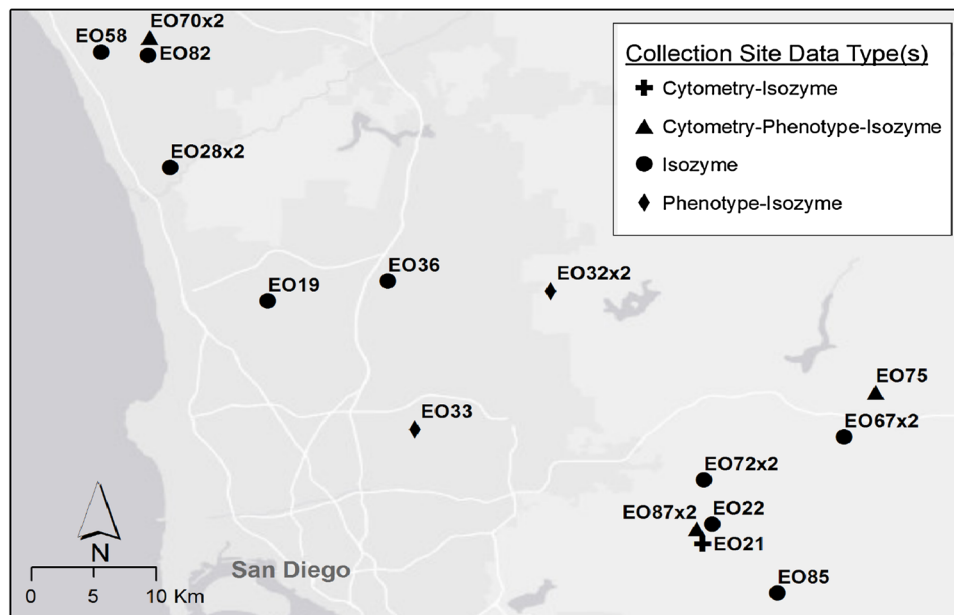
Four standard measures of allelic diversity were estimated for each subpopulation: the effective alleles per locus (accounting for differences in sample size) ( $A_e$ ), number of alleles expected in two gene copies (a measure of allelic richness) ( $A_r$ ), gene diversity corrected for sample size; (Nei 1978) ( $H_e$ ), and the individual inbreeding coefficient (or fixation index) ( $F$ ). The probability that  $F$  differed from the null hypothesis ( $H_0: F=0$ ) was determined by bootstrap replicates.

To further examine possible inbreeding or autogamous seed production within populations, Nason's kinship coefficient, an estimate of the correlation of homologous alleles in two individuals (as described in Loiselle et al. 1995), was estimated among all pairs of individuals in the complete data

set, and among all pairs of individuals within each subpopulation. All measures were estimated using SPAGeDi v. 1.4 (Hardy and Vekemans 2002).

Three measures of genetic differences were estimated: (1) a hierarchical test of differentiation first among all subpopulations, and then among populations separated by three geographic distances (up to 20, 20–50 km, and over 50 km); (2) Nei's (1978) genetic distance among all collections; and (3) pairwise differentiation ( $F_{ST}$ ), all implemented in SPAGeDi v. 1.4 (Hardy and Vekemans 2002). Two landscape-level analyses were conducted to examine patterns of genetic differentiation across the sampling area. First, isolation by distance (IBD) was examined following Rousset (1997, implemented in SPAGeDi v. 1.4, Hardy and Vekemans 2002) using a paired Mantel test (GenAlEx v. 6.5, Peakall and Smouse 2006). Second, a principal coordinate analysis (as implemented in GenAlEx v. 6.5) was conducted over Nei's genetic distance among all subpopulations

**Fig. 1** Locations of San Diego thornmint populations assessed for variation in neutral genetic markers (isozymes), ploidy level (flow cytometry), and/or putative adaptive morphological traits (phenotype). Six sites were sampled at two subpopulations (“x2”). Figure created using ArcGIS



(as estimated using SPAGeDi v. 1.4). In addition, the first principle coordinate was compared to the Datamet data for each collection site to examine concordance between genetic structure and climate.

### Flow cytometry

Fresh leaf tissue for flow cytometry analysis was collected from five sites in April 2016. Leaves collected from single plants were placed in individually-labeled plastic baggies and kept cool (on ice or in a refrigerator, not frozen) until shipment to NFGEL. Fresh leaf samples were analyzed on a Sysmex Partec CyFlow® Ploidy Analyser dual laser flow cytometer (Sysmex-Partec GmbH, Gortitz, Germany) using the CyStain UV Ploidy buffer. For analysis, approximately a dime-sized piece of leaf tissue was diced in 0.5 mL of cold buffer using a double-edged blade for approximately one minute, until the buffer was green, 1.5 mL of cold buffer was added, and the samples were filtered through a 30 µm nylon Partec CellTrics® filter. Filtered samples were immediately analyzed using a threshold of 0.0122 and gain of 575. In a few cases, to confirm whether two samples were displaying the same peak value, the samples were prepared and analyzed together.

### Phenotypic study

Details of seed collection and preparation methods are provided in the supplementary information. The five EO locations included in the common-garden study represent much of the latitudinal, longitudinal, and elevational range of the populations included in the isozyme analyses

(Fig. 1—diamond and triangle locations; Table 1), plus two (differing by approximately 35 m in elevation) subpopulations from one of those locations, resulting in six (6) distinct seedlots.

In February, mimicking the approximate timing of germination in the field, seeds were sown at the Institute for Conservation Research, San Diego Zoo Global at Escondido, CA, within the species' natural range. Fifty-five pots were sown per population, with five seeds per pot to allow for variation in germination. Anderson Plant Bands AB58 (five in width × eight in depth) were used with a substrate of 3:1 potting mix and washed sand. Pots received 500 mL of water after planting, and were misted twice per week during germination. When germination had plateaued, plants were thinned to one plant per pot. Pots were randomly arranged on benches in an outdoor area and received full sun. Established plants received 1000 mL of water every two weeks. When signs of water stress were apparent, frequency was increased to once per week. Irrigation continued until all plants senesced.

Individual plants were measured for three traits related to fitness: days to flowering, number of inflorescences, and total above-ground biomass. To determine the number of days to first flower, we monitored plants 5 days per week once we observed the initial development of inflorescences (April 16 to June 7, at which time all but one plant had flowered) and we recorded the date of first flowering. Plants were checked for senescence 5 days per week starting in late April. The first plants senesced on May 2 and continued through July. After senescence, the number of inflorescence whorls per plant was recorded. The plants were then harvested, dried, and above-ground biomass weighed.



Total biomass was not measured because the roots were so fine that separating them from the potting mix proved too difficult.

Variation in phenotype was assessed using general linear models, with seedlot ( $n=6$ ) as a fixed factor, as implemented by the *stats* package in R v. 3.0.2 (The R Foundation for Statistical Computing). Correlations between phenotype and the four climate measures were assessed using Pearson's product moment correlation as implemented in R v. 3.0.2 (The R Foundation for Statistical Computing), with sequential Bonferroni corrections for multiple testing implemented to control for multiple testing error.

## Results

### Isozyme analyses

The 18 isozyme loci distinguished 456 multilocus genotypes in the collection of 483 plants. In total, 20 genotypes were observed in more than one sample, with between two and five plants having the same genotype (Supplementary Information). One population, EO58, had high levels of missing data, reducing the power to distinguish between genotypes, and likely contributing to the classification of three samples as the same genotype. Another population, EO67s, showed low levels of genotypic diversity (19 genotypes in 30 samples), and all genotypes were inferred to be hexaploid (see next paragraph), indicating a non-random mating system may effectively exist at this site. Most pairs of plants with matching genotypes occurred in the same population ( $n=14$  genotypes), or two subpopulations at the same site ( $n=2$  genotypes), though four genotypes were observed at different sites. Of the four genotypes observed in more than one sampling site ( $N=11$  plants), the two sites were separated by variable distances: populations EO58 and EO82 (genotype "B") are separated by 3.2 km; EO28 and EO70 (genotype "C") by 11.4 km; EO32 and EO67n (genotype "D") by 23.4 km; and EO32 and EO67s (genotype "L") by 23.7 km. The genotype probabilities indicate that these matches may be due to chance rather than long-distance vegetative dispersal (genotype probabilities for matching genotypes range from  $1.6 \times 10^{-4}$  to 0.013; genotype probabilities for the entire collection had a median of  $5.9 \times 10^{-4}$  and range of  $7.7 \times 10^{-11}$  to 1.0).

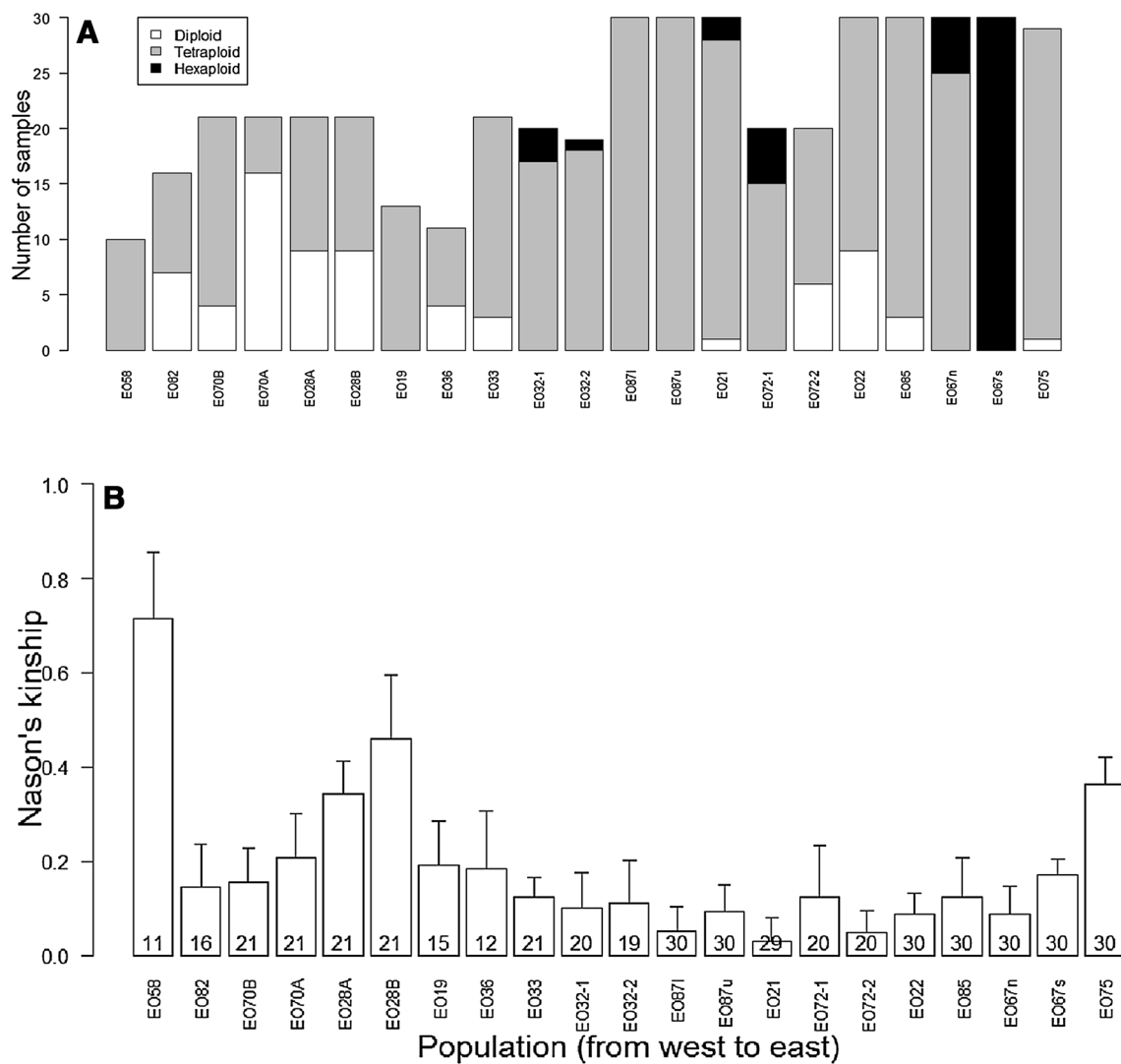
Seven of the 18 isozyme loci (38%) displayed banding patterns more complex than could be explained by diploid inheritance. The frequency of these putative polyploid banding patterns ranged from very low (one sample at PGM1, one at TPI2, two samples at UGPP1) to moderate (3% of samples at FEST, 6% of samples at DIA2, and 10% of samples at IDH), to high (80% of samples at PGI2). The minimum number of alleles required to explain the

banding pattern was inferred for each individual, either two (diploid), four (tetraploid) or six (hexaploid). Up to six alleles at one locus were required to fully explain the patterning observed at PGI2. When a sample displayed a polyploid banding pattern at even one locus it was inferred to be polyploid at all loci. Most populations contained individuals of different ploidy levels, and the frequency of higher ploidy (e.g. hexaploids) tended to increase from west to east (Fig. 2). No evidence of fixed heterozygotes, as is frequent in allopolyploid systems, was observed, indicating San Diego thornmint is likely autopolyploid.

The mean kinship value estimated over the entire collection was moderate and significantly greater than the null expectation ( $\rho=0.14$ , 95% distribution of permutations:  $-0.005$ ,  $0.003$ ), indicating that some inbreeding or non-random mating occurs. Mean values for each population or subpopulation (Fig. 2) ranged from 0.03 in population EO21 to 0.71 in population EO58. Only 11 samples were collected from population EO58, and many of these suffered from high rates of missing data, so the value for this site is likely inflated. Samples from site EO75 were seedlings grown from a bulk seed collection remaining from the 2012 collections, and it is possible that half- or full-sibs were sampled instead of putatively unrelated individuals. However, no such mitigating circumstances are known for the subpopulations at EO28, indicating this site might experience higher levels of inbreeding, self-fertilization, or autogamy. Together, these values are not consistent with high levels expected in a strictly self-fertilizing or apomictic reproductive system, and indicate some level of outcrossing or a mixed mating system to be present.

Moderate levels of allelic variation were observed in the entire collection (Table 2, Supplementary Information). The effective alleles per locus, which accounts for variation in sample sizes across populations, was lower ( $A_e=1.39$ ). Gene diversity was moderate ( $H_e=0.216$ ), and significant fixation was observed overall ( $F=0.32$ ,  $P<0.001$ ), consistent with the moderate kinship values and possible inbreeding. Values varied across populations, with some sites displaying near negligible fixation (EO72-2,  $F=0.07$ ,  $P=0.047$ ) and others far exceeding the mean (EO28B,  $F=0.51$ ,  $P<0.0001$ ).

The one-level hierarchical model comparing all populations and subpopulations revealed significant fixation within and differentiation among all collections of thornmint (Table 3). Significant fixation within individuals ( $F_{IS}=0.198$ , 95% distribution of permutations:  $-0.015$ ,  $0.011$ ) is consistent with the inbreeding coefficients and the kinship coefficients reported above. Allele frequencies varied significantly among sampling sites ( $F_{ST}=0.154$ , 95% distribution of permutations:  $-0.004$ ,  $0.005$ ), indicating gene flow is restricted among populations and potentially subpopulations.



**Fig. 2** **A** Three putative ploidy levels (diploid, tetraploid, and hexaploid) were identified in San Diego thornmint from isozyme banding patterns. Ploidy tended to increase with longitude (from west to east) across the sampling range, roughly corresponding with increasing elevation. **B** Mean pairwise estimates of Nason's kinship coefficient

(Loiselle et al. 1995) varied among collections. The number of samples is noted in each bar. Error bars represent standard error of the mean. EO75 was assessed as seedlings from a bulk seed lot and may include related individuals. Figure created in R

The three-level hierarchical model built from the distribution of pairwise distances between sampling sites provided greater insight into the pattern of differentiation. For populations or subpopulations separated by less than 20 km, genetic differentiation was lower than that predicted by the random permutation of data ( $F_{ST(<20)} = 0.139$ , 95% distribution of permutations: 0.146, 0.208), indicating that gene flow is greater than expected at shorter distances. For populations separated by 20–50 km, the differentiation was no different than predicted by the permutation analyses ( $F_{ST(20-50)} = 0.167$ , 95% distribution of permutations: 0.146, 0.219). At longer distances, populations separated by more than 50 km displayed differentiation greater than predicted by the permutation tests ( $F_{ST(>50)} = 0.259$ , 95% distribution

of permutations: 0.122, 0.241). These patterns indicate gene flow is sufficient to reduce differentiation at small scales, but is limited across the sampling range.

Tests for isolation by distance (IBD) further confirm the pattern of gene flow decreasing as a function of distance. Rousset's (1997) estimate of differentiation increased with the log of geographic distance, and Mantel tests confirmed the pattern to be significantly different from the null model of no relationship between genetic and geographic distance ( $R_{XY} = 0.379$ ,  $P < 0.001$ ).

The principal coordinates analysis (PCoA) identified additional geographic structure in the genetic differences among populations. The first coordinate explained 57.3% of the variance among populations, and indicated the

**Table 2** Genetic variation observed in 21 collections of thornmint

Population	N	$A_e$	$A_r$	$H_e$	$F$	$\Pr(F < > 0)$
EO19	12.6	1.42	1.21	0.208	0.15	0.0030
EO21	28.9	1.37	1.20	0.202	0.21	<0.0001
EO22	28.4	1.32	1.17	0.172	0.22	<0.0001
EO28A	19.5	1.25	1.16	0.159	0.25	<0.0001
EO28B	18.9	1.21	1.12	0.127	0.51	<0.0001
EO32-1	19.6	1.52	1.25	0.253	0.24	<0.0001
EO32-2	18.9	1.48	1.24	0.239	0.21	<0.0001
EO33	19.8	1.27	1.15	0.152	0.11	0.0050
EO36	11.0	1.40	1.20	0.204	0.16	0.0070
EO58 <sup>a</sup>	5.9	1.19	1.25	0.104	-0.02	n/a
EO67n	28.2	1.38	1.18	0.180	0.15	<0.0001
EO67s	29.2	1.30	1.15	0.151	0.09	<0.0001
EO70A	17.5	1.28	1.18	0.162	0.33	<0.0001
EO70B	20.0	1.24	1.15	0.149	0.25	<0.0001
EO72-1	19.5	1.38	1.19	0.190	0.36	<0.0001
EO72-2	19.8	1.41	1.19	0.195	0.07	0.0470
EO75	23.7	1.29	1.14	0.140	0.18	<0.0001
EO82	13.6	1.35	1.2	0.190	0.25	<0.0001
EO85	28.4	1.43	1.23	0.224	0.29	<0.0001
EO87lower	29.1	1.34	1.20	0.201	0.24	<0.0001
EO87upper	29.4	1.42	1.22	0.219	0.13	<0.0001
Overall	441.8	1.39	1.22	0.216	0.32	<0.0001

The mean number of samples genotyped (reflects missing data) (N), effective alleles per locus ( $A_e$ ), expected alleles among 2 gene copies ( $A_r$ ), and gene diversity ( $H_e$ ) were estimated for full genotypes (polyploid where observed). The fixation indices ( $F$ ) represent individual inbreeding coefficients, with significance determined from bootstrap tests

<sup>a</sup>Due to missing data and low allelic variation, mean values for EO58 were calculated by hand

seedlings from EO75 to be distinct from the 2013 collections (Fig. 3). This population displayed private alleles at two loci: allele 7 at PGI2 and allele 2 and GDH2. The second coordinate explained 28.0% of the variance among populations, and separated the population with high rates of missing data (EO58) and other collections from the northern part of the range (EO28, EO70, and EO82). As population EO75 was analyzed with seed of unknown parentage collected the previous year (i.e., previous generation), the PCoA was repeated omitting those samples. When restricted to the 2013 foliage collections, the first coordinate (explaining 68% of the variation) distinguished the northern occurrences, and the second coordinate (explaining 11% of the variation) provided greater separation of the bulk of the collection (Fig. 3).

Correlation tests revealed potentially marginal correspondence between genetic structure (quantified as the first principle coordinate) and two estimates of climate [MeanPpt and CV (temp)], but neither correlation was significantly different from random after Bonferroni corrections (Table 4).

## Flow cytometry

The mean peak values (DNA content) varied over all samples, over collection sites, and over putative ploidy classes based on the isozyme data (Fig. 4). Three peak patterns were observed in the flow cytometry histograms. Only three samples displayed the large genome content pattern. The underlying ploidy levels or genetic mechanisms of this variation cannot be determined from these data alone, although the mean peak values do provide a quantitative measure of relative DNA content.

## Phenotypic variation

Populations differed in days to flowering ( $F_{5,251} = 13.07$ ,  $P < 0.0001$ ), the number of inflorescences ( $F_{5,261} = 8.55$ ,  $P < 0.0001$ ), and total above-ground biomass ( $F_{5,261} = 16.67$ ,  $P < 0.0001$ ) of individual plants (Fig. 5). Two of the measures of phenotypic variation varied with the local climate of the collection site (days to flowering and the number of inflorescences), while correlations between above ground



**Table 3** Significant fixation ( $F_{IS}$  and  $F_{IT}$ ) and differentiation ( $F_{ST}$ ) observed from isozyme data assessed in San Diego thornmint

Model	$F_{IS}$	$F_{IT}$	$F_{ST}$
One-level			
Observed	0.198*	0.322*	0.154*
Permutation	-0.002	1.0E-05	-9.7E-05
95% CI	(-0.015, 0.011)	(-0.011, 0.011)	(-0.004, 0.005)
Three-level			
< 20 km			
Observed			0.139*
Permutation			0.178
95% CI			(0.146, 0.208)
20–50 km			
Observed			0.167
Permutation			0.177
95% CI			(0.145, 0.219)
> 50 km			
Observed			0.259*
Permutation			0.179
95% CI			(0.122, 0.241)

The one-level hierarchical model assessed all individuals with each population and all populations over the total. The three-level hierarchical model assessed populations separated by three geographic distances: up to 20, 20–50 km, and over 50 km. Significance (\*) was determined by 999 permutations of the data, producing the 95% confidence interval (CI) of the permutation estimate

biomass and climate were non-significant (Table 4). After Bonferroni corrections, the days to flowering varied with MeanPpt, MaxTemp (W), and CV (temp), while the number of inflorescences on a plant varied with MeanPpt and CV (temp). Longitude and temperature extremes are likely confounded factors given the coastal geography of these sites.

## Discussion

San Diego thornmint (*Acanthomintha ilicifolia*), an endangered species protected by multiple levels of government and managed by many agencies and organizations, is recently and currently the subject of diverse efforts aimed at conservation and recovery. Each of these projects may be improved by including information on the amount and structure of genetic variation in this species. In the absence of genetic information, management activities may cause further threats or undermine conservation objectives. In addition, any insight into the mating system or ploidy levels in San Diego thornmint may help future restoration and recovery actions that involve enhancing current or re-establishing historic populations in previously established locations.

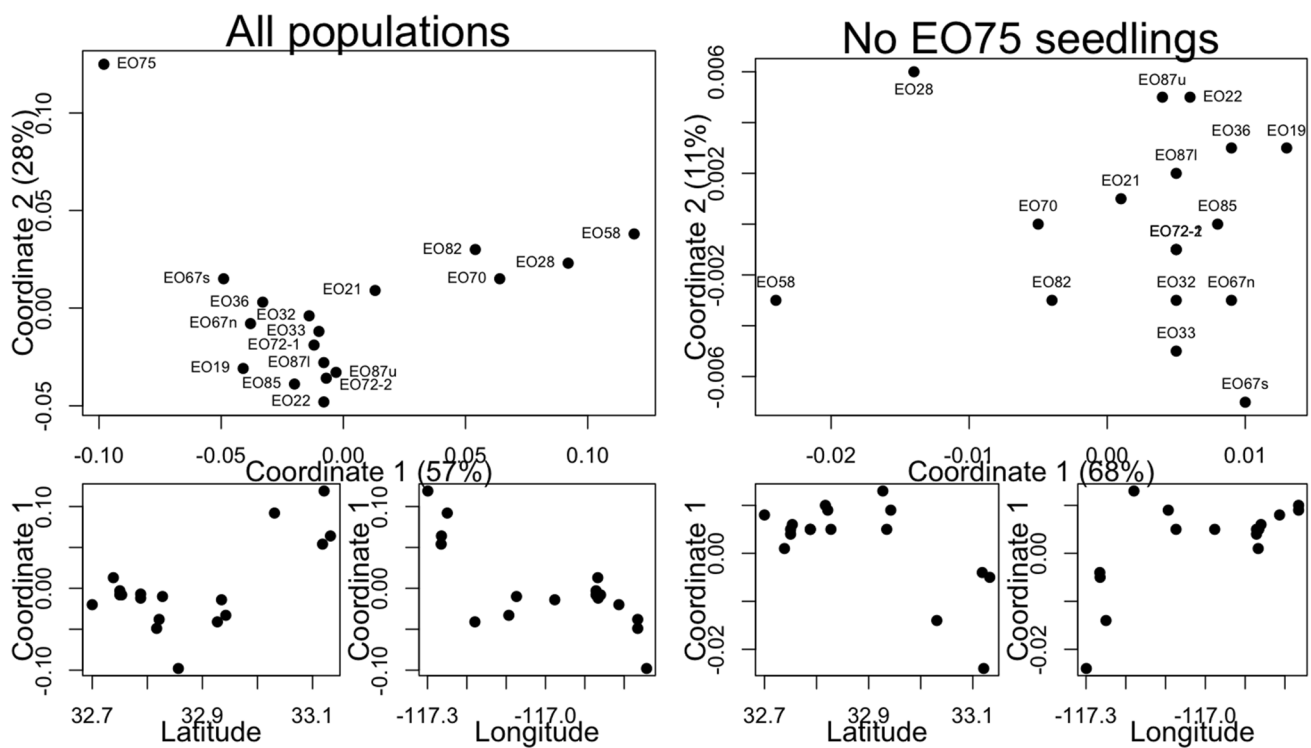
Analysis of isozyme markers in 21 collections of San Diego thornmint revealed moderate levels of genetic

variation within and significant differentiation among populations. The percentage of polymorphic loci (88%) and gene diversity ( $H_e=0.216$ ) were greater in San Diego thornmint than mean levels observed in annual endemic species [ $P=50$ ,  $H_{es}$  (genetic diversity within the species)=0.149] (Hamrick and Godt 1996). Genetic diversity in thornmint was also higher than that reported in other threatened Lamiaceae, such as *Macbridea alba* [ $P=50$ ,  $H_e$  (gene diversity within the species)=0.121]; Godt et al. 2004) and *Stachys maritima* ( $P=14$ , expected heterozygosity  $H_e=0.066$ ; López-Pujol et al. 2003).

The plants sampled for this study represent not only a sample of the spatial range but of the diversity that was the result of germination in the sampling years. Seed longevity and the role of the seed bank has not been explored in San Diego thornmint. The large fluctuation of population sizes across years indicates that the seed bank may play a significant role in population persistence, with seed remaining dormant until appropriate or ideal weather conditions occur (Godfrey and Spiegelberg 2015). If confirmed, a persistent seed bank may serve as a genetic reserve, buffering populations from genetic drift and maintaining genetic variation.

Due to the extensive sampling conducted for the isozyme study, the putatively neutral genetic markers provided evidence of landscape-scale differentiation in San Diego thornmint, indicating gene flow is restricted to within a local geographic region. Populations within this neighborhood (ca. 20 km) were more genetically similar than populations separated by greater distances. Plants with limited gene flow capabilities often maintain high levels of differentiation among populations (Hamrick and Godt 1996; Ægisdóttir et al. 2009). Alternatively, these populations may have only recently become genetically isolated, and allele frequencies have not yet differentiated (López-Pujol et al. 2003). Given that this species' life history traits are consistent with spatially limited gene flow, we attribute this landscape-level differentiation to historic processes and not recent changes in population connectivity. The level of genetic structure in San Diego thornmint ( $F_{ST}=0.198$ ) was similar to that reported in other annual endemic species ( $G_{ST}=0.223$ ; Hamrick and Godt 1996).

Limited dispersal within populations, the occurrence of self-pollination, or cryptic genetic populations within a sampling site (e.g. a Wahlund effect) may all result in fine-scale genetic structure (Loiselle et al. 1995; Kalisz et al. 2001). In San Diego thornmint, isozyme markers revealed structure within sampling locations as well as across the landscape. When comparing levels of fixation (Table 3), ploidy classification, and kinship (Fig. 2), three occurrences show putative differences among subpopulations: EO28, EO63, and EO72. Additional examination of fine-scale spatial structure within sites is warranted to determine the potential roles of evolutionary processes such as limited seed or pollen



**Fig. 3** Principal coordinates analysis based on genetic distances between populations revealed geographic structure among San Diego thornmint. Each point represents one population or subpopulation. When all populations were analyzed (plots on left) the first two coordinates explained over 85% of the variation among populations and

the first coordinate varied with latitude and longitude of population location. When seedlings from EO75 were omitted (plots on right), similar trends were observed though slightly less variation was explained. Figure created in R

neighborhoods or microclimate adaptation in maintaining genetic variation. Cytotypic variation, for instance, may represent an adaptive strategy providing greater standing variation for selection to act on (at the cost of potential reduced

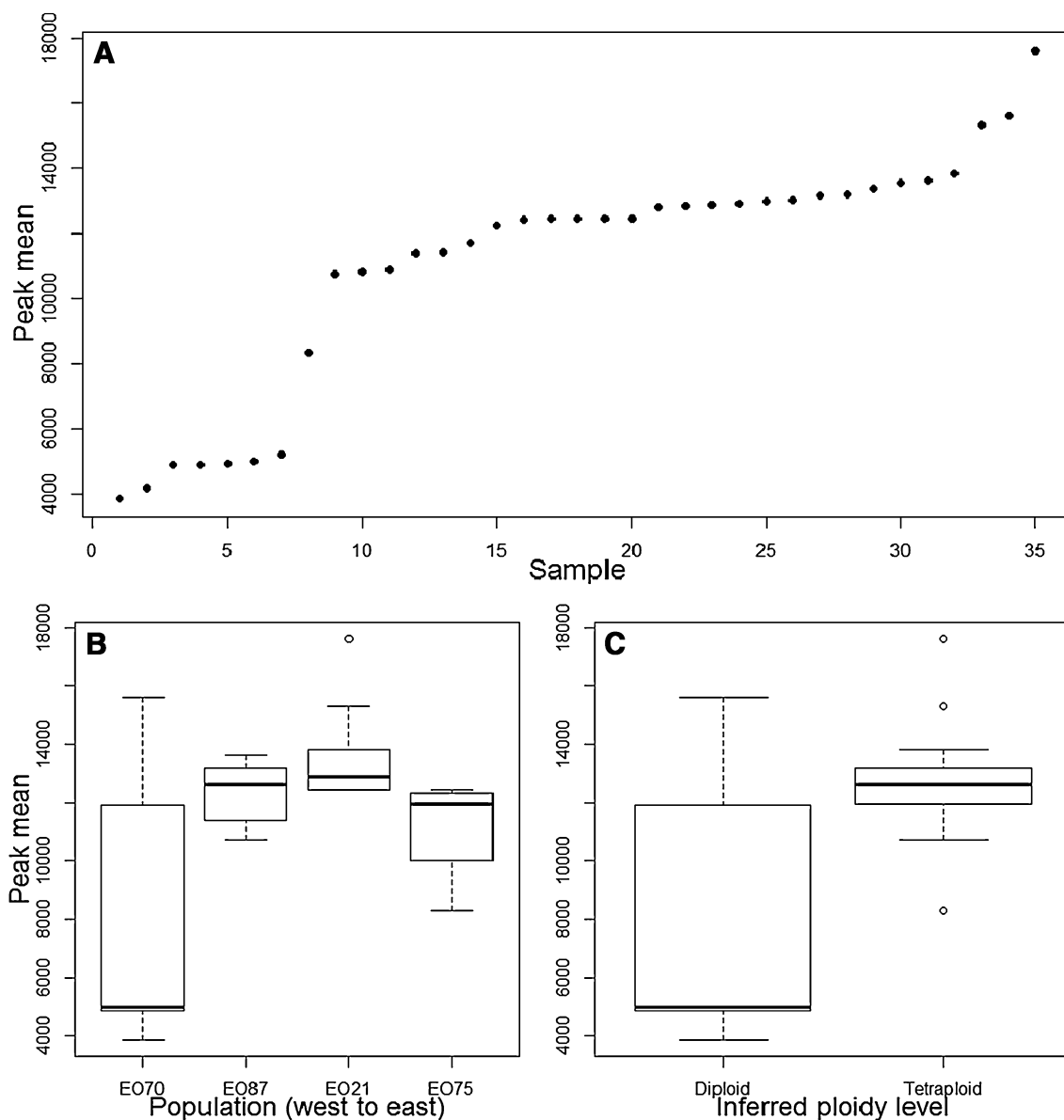
fecundity), and may result in two (or more) sympatric yet effectively genetically isolated populations (Soltis et al. 2014).

**Table 4** Putative adaptive variation in San Diego thornmint was observed as the correlation between climate, morphology, and genetic structure

Population mean	MeanPpt	CV (Ppt)	MaxTemp (W)	CV (temp)
Days to flower	Rho=0.47** P<0.0001	Rho=-0.02 P=0.62	Rho=-0.45** P<0.0001	Rho=0.47** P<0.0001
No. inflorescence	Rho=-0.20** P<0.0001	Rho=0.08 P=0.06	Rho=0.08 P=0.06	Rho=-0.20** P<0.0001
Biomass	Rho=0.03 P=0.48	Rho=-0.04 P=0.40	Rho=0.03 P=0.48	Rho=0.03 P=0.48
PC1 (isozymes)	Rho=-0.51 P<0.05	Rho=0.29 P=0.25	Rho=0.13 P=0.61	Rho=-0.59 P=0.01

Historic climatic variables included average annual precipitation, MeanPpt; coefficient of variation of monthly precipitation, CVPpt; average maximum temperature of the wet season, MaxTemp (W); and coefficient of variance for the wet season maximum temperature, CV (temp) at collection sites. Morphological variation was measured in a common-garden experiment (days to first flower, number of inflorescences at senescence, aboveground biomass at senescence), and genetic structure was estimated from isozyme data (PC1). Correlation was estimated using Spearman’s rank order test (rho)

\*\*P<0.01 after Bonferroni correction



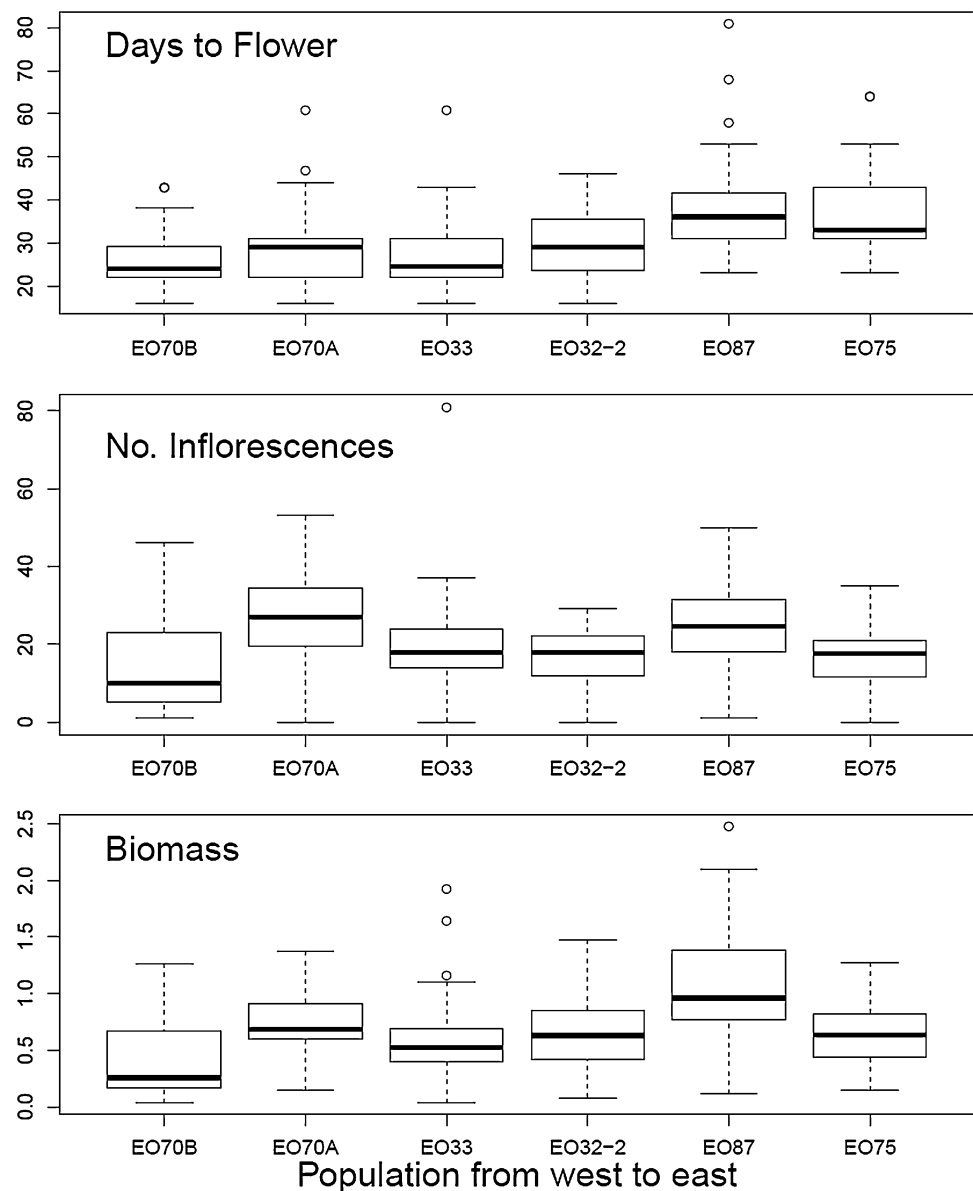
**Fig. 4** DNA content (as assessed by flow cytometry) varied **a** among samples of San Diego thornmint, **b** among collection sites, and **c** among a priori classifications based on isozyme banding patterns, although the frequency of putative tetraploids was underestimated.

Each boxplot represents the distribution of observations, with the median shown as the thick line, and outliers shown as points. Figure created in R

Isozyme banding patterns also identified remarkable cytotypic variation both within and among populations of San Diego thornmint, which were confirmed using flow cytometry. Understanding the origin of polyploidization may reveal the evolutionary history of a species and provide additional guidance for conservation. Polyploidy can provide a variety of adaptive benefits (Warner and Edwards 1989; Casler et al. 2004), has been a recurring event in angiosperm evolution (Comai 2005; Soltis et al. 2014) and likely occurs through multiple independent origins even within populations (Hegarty and Hiscock 2008; Soltis et al.

2014). Autopolyploidy may be a mechanism contributing to ecological speciation across diverse environments (Ramsey et al. 2008), but is not always so (Visser and Molofski 2015), and the effect of ploidy level is confounded with phylogeny (Martin and Husband 2009). The lack of fixed heterozygotes and the occurrence of several isozyme banding patterns consistent with unbalanced heterozygotes observed are consistent with an autopolyploid (rather than allopolyploid) origin of variation in San Diego thornmint. In addition, all populations displayed significant positive values of fixation (e.g.

**Fig. 5** Significant variation in putatively adaptive phenotypes was observed among six seed lots of San Diego thornmint grown in a common-garden experiment. Each boxplot represents the distribution of observations, with the median shown as the thick line, and outliers shown as points. Figure created in R



$F_{IS}$ ), and many showed moderate kinship, indicating some level of self-fertilization or inbreeding may occur.

The benefit of maintaining cytotypic variation in rare species is not as well established, however. While cytotypic variation is often considered a barrier to allogamy due to the production of incompatible or sterile offspring (Comai 2005), and does not necessarily present a barrier to seed production (Hardion et al. 2015), differences in ploidy level may correspond to differences in breeding system (Hörandl and Greilhuber 2002). The cytotypic structure in San Diego thornmint adds to the growing evidence that cytotypic variation may be maintained (i.e., not be an evolutionary dead-end) in plant species. Here, variation in ploidy levels was confirmed with flow cytometry (Fig. 4), and although insufficient tissue was available from the plants at the only

putative majority hexaploid population (EO67s), results confirmed two genome sizes, with variation observed within as well as among populations. Cytotypic variation has been described among populations of other narrowly distributed plants (e.g. Fehlberg and Ferguson 2012), but the level and potential intrapopulation distribution of variation observed in San Diego thornmint is remarkable and may help maintain standing genetic variation and intrapopulation structure (Hegarty and Hiscock 2008).

While useful to understand demographic processes and evolutionary relationships, genetic markers do not provide evidence of adaptive differences among populations. Common-garden experiments assessing putatively adaptive traits relative to fitness can provide indirect evidence of adaptive divergence, another piece of information critical

to successfully managing threatened species. For annual herbs, differences in plant size (biomass) and potential reproductive output (number of inflorescences) may be significant indicators of fitness (Farris and Lechowicz 1990; Aronson et al. 1993). In addition, comparing morphological variation with the climatic conditions at sampling sites can indicate whether the patterns may be evolutionarily important (Kawecki and Ebert 2004; DeWoody et al. 2015). The assessment of putatively adaptive morphological variation (days to flower, number of inflorescences, and biomass) in six collections of San Diego thornmint revealed significant phenotypic differences among sites. While our study design did not allow for direct inference of localized adaptation (we lacked reciprocal transplant experiments), correlations between phenotypic traits and climatic variables provided evidence of potential adaptive divergence in San Diego thornmint. Specifically, two morphological traits (days to flower and the number of inflorescences) varied with mean annual precipitation and the coefficient of variance of wet season maximum temperature at the collection site. Despite the narrow geographic distribution of San Diego thornmint, these results show there may be sufficient climatic variation among populations to drive local adaptation. Climate measures for the six sites sampled for the common-garden study differed by a maximum of 192 mm for annual rainfall, 2 °C for the average maximum temperature of the wet season, and 0.104 for the coefficient of variation of the average maximum temperature. Plants from wetter, cooler sites that have greater variance in temperature during the rainy season took longer to begin flowering, potentially due to the longer duration of the wet season. Similarly, plants from sites with lower rainfall and less variable winter temperatures produced a greater number of inflorescences, possibly reflecting a longer growing season at the collection sites.

In addition, the correlation of flowering traits, but not biomass, with climate variables indicates that reproductive strategy may be a key adaptation in this annual herb. Correlation between reproductive success and temperature variation likely results from natural selection, not solely from demographic processes, and may result in this species being susceptible to the changes in temperature predicted for the coming decades (Messner et al. 2009). While warmer, longer growing seasons could result in greater seed production, a greater variance in temperature and precipitation would likely result in inconsistent reproductive success among years, increasing the stochasticity of population sizes. Future analyses of the common-garden study data may provide the first insight into the adaptive potential of this species (per Etterson 2004).

The ongoing conservation efforts for San Diego thornmint have five overarching goals: maintaining large populations, enhancing small populations, establishing new populations, maintaining genetic diversity, and promoting connectivity

(San Diego County 2017). The patterns of genetic variation observed here may serve to evaluate and prioritize those efforts: putatively neutral genetic structure revealed from isozyme markers, apparent cytogenetic variation confirmed using flow cytometry, and potentially adaptive morphological variation quantified in a common-garden study indicate this rare endemic is genetically complex. At least two cytotypes were observed, sometimes occurring within the same population, and populations were differentiated for neutral and potentially adaptive genetic variation. The observed landscape-level structure and phenotypic differentiation among populations indicate that increased connectivity may not be appropriate across the species range. Further, enhancing small populations should be directed not only by demographic but by genetic considerations.

The complexity of the differentiation in San Diego thornmint was only resolved through a combination of data types, demonstrating the utility of combining neutral and adaptive data (here, marker and quantitative genetic measures) to best inform conservation strategies (Kohn et al. 2006). Had our study been limited to any one of the three measures of genetic variation we examined the results would have failed to reveal the scale and scope of differentiation, and would not provide as meaningful information for conservation plans. For example, while the potentially adaptive variation revealed by the common-garden experiment would have provided evidence for limiting the movement of germplasm outside of a local area, such information would not have revealed that multiple ploidy levels exist within populations. Similarly, although the isozyme data revealed significant differentiation across the landscape (as did the morphological variation), potential local adaptation would not have been inferred from these neutral markers.

Conservation practitioners often work under the canonical expectation that rare species such as endemic plants typically maintain low level of genetic variation with little differentiation among populations (Cole 2003; Ouborg et al. 2006). Following this expectation, maintaining the highest level of genetic variation within and genetic connectivity among populations is prioritized in conservation plans to maintain the long-term survival of the species (Bowen 1999). Our data present a challenge to these assumptions about rare plant species and motivated us to recommend the following management principles to incorporate genetic information into conservation decisions.

First, all species have a *genetic neighborhood*, and the movement of germplasm between neighborhoods should only be done thoughtfully and with a monitoring strategy in place. Given limited historic gene flow even within a small geographic area, increased connectivity may not be appropriate and population enhancement, if done via nonlocal material, may result in undesirable outbreeding. If germplasm has been moved or is proposed to be



moved among genetic neighborhoods, population monitoring would be important to identify any long-term consequences (e.g. outbreeding depression).

Second, *polyploidy happens*, and cytotypic variation should be considered as another form of genetic structure. Screening seed collections or germplasm for cytotype variation can be quickly and relatively economically assessed using flow cytometry, and could provide valuable information to managers to prevent the co-location of potentially incompatible cytotypes. This principle is especially important for rare species in families known to have high frequencies of polyploid taxa.

Third, populations may display *local adaptation* within relatively slight environmental clines and even within genetic neighborhoods. Geographic or climatic factors such as elevation should be considered when developing seed collection or population augmentation plans, even when data on adaptive differentiation (quantitative genetics) is not available. In sum, within a genetic neighborhood, conservation plans should be designed to maximize the collection and maintenance of genetic variation without combining incompatible cytotypes or poorly adapted germplasm. With this study of San Diego thornmint, we have demonstrated that these principles can be as important for rare as for more widespread species. For species that are also endangered, their appropriate application can be critical.

**Acknowledgements** The authors acknowledge and appreciate funding provided by the San Diego Association of Governments' Environmental Mitigation Program (Grant Agreement #5001964), by the Center for Natural Lands Management, and by a donation from Dr. Hans and Elisabeth Spiegelberg. We thank Markus Spiegelberg, Sarah Godfrey, Patrick McConnell, and Jessie Vinje for their leadership in thornmint conservation and assistance in collecting foliage and seed samples from natural populations; Courtney Owens, Rosanna Hanson, Jody Mello, Randy Meyer, and James Boom for their assistance with laboratory technical work; and Laurie Lippitt and Stacy Anderson for managing the nursery common-garden activities. We thank Erin Conlisk, Kelly Anderson, and Burak Pekin for their for their assistance with data management and analyses of the common-garden research. We thank Michelle Labbé and Benjamin Janes for technical support in acquiring permits and property access permissions and other documents and data. We appreciate the conservation managers and regulatory agencies for protecting the wild populations of thornmint and for allowing us access and permission to sample them for this study. This paper is more robust in its content and eloquent in its presentation as a result of comments from the anonymous reviewers.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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