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RESEARCH ARTICLE

High-throughput sequencing reveals distinct regional genetic structure among remaining populations of an endangered salt marsh plant in California

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Abstract
Conservation of rare species requires careful consideration to both preserve locally adapted traits and maintain genetic diversity, as species' ranges fluctuate in response to a changing climate and habitat loss. Salt marsh systems in California have been highly modified and many salt marsh obligate species have undergone range reductions and habitat loss with concomitant losses of genetic diversity and connectivity. Remaining salt marshes are threatened by rising sea levels, and so these habitats will likely require active restoration and re-establishment efforts. This study aims to provide a reference point for the current status of genetic diversity and range-wide population structure of a federally and state listed endangered plant, salt marsh bird's-beak (*Chloropyron maritimum* subsp. *maritimum*) that can inform future preservation and restoration efforts. We used historical data and current monitoring information to locate and sample all known occurrences throughout the range of this subspecies in southern California, and three additional occurrences from Baja California, Mexico. We used flow cytometry and single nucleotide polymorphic markers (SNPs), generated by double-digest restriction-site associated DNA sequencing (ddRAD), to assess relative ploidy, and estimate genetic diversity and population structure across the region. Overall, we found five distinct genetic clusters that coincide with geographic regions. Genetic diversity was greatest in the southern part of the range including Baja California and San Diego. These findings can bolster management and restoration efforts by identifying potentially isolated occurrences and areas that are the richest sources of allelic diversity, and by providing insight into the amount of genetic differentiation across the taxon's range.

Keywords Salt marsh bird's-beak · *Chloropyron maritimum* subsp. *maritimum* · Population structure · Genetic diversity · Rare plants

Introduction

Coastal ecosystems are among the most imperiled on earth in an era of human habitat modification, climate change, and rising sea levels (McGranahan et al. 2007; Gedan et al. 2009; Parris et al. 2012; Thorne et al. 2016, 2018; U.S. Global Change Research Program 2017). Preserving species within these systems will rely not only on protecting existing populations, but will require active habitat restoration efforts, including transplantation and establishment of new populations (Zedler and Kercher 2005; Zhao et al. 2016). Genetic data can provide information on genetic connectivity and diversity, which are useful when determining impacts to species and in planning conservation management strategies (Ellstrand and Elam 1993; Frankham et al. 2002). Genetic factors, including ploidy and levels of genetic relatedness among individuals, can influence compatibility and survivorship of transplanted individuals or outcrossed offspring (McKay et al. 2005; Gibson et al. 2017). A key genetic concern when faced with restoration choices is determining the appropriate balance between retaining local adaptation and maximizing overall diversity to avoid negative inbreeding

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effects and retain the ability to respond to future environmental change (Weeks et al. 2011; Harrisson et al. 2014). Understanding population genetic structure can aid in this assessment by estimating the scale of genetic connectivity and by providing information on the distribution of genetic diversity across individuals and occurrences (Hughes et al. 2008; Engelhardt et al. 2014).

Chloropyron maritimum subsp. *maritimum* (Orobanchaceae; salt marsh bird's-beak) is an endangered salt marsh halophytic herb (U.S. Fish and Wildlife Service 1978). Salt marsh bird's-beak is restricted primarily to coastal salt flats and elevated salt marsh habitat, and it does not overlap in range with other *C. maritimum* subspecies. Though once described as a common taxon found in salt marshes along the southern California coast (Purer 1942), *C. maritimum* subsp. *maritimum* is now restricted to just a few salt marsh systems from Morro Bay, California, to Punta Azufre in northern Baja California, Mexico (Fig. 1a), and is at risk of losing much of its remaining habitat to sea level rise (U.S. Global Change Research Program 2017). Taxa within *Chloropyron* are hemiparasitic and several have been shown to form haustorial connections with multiple host species (Chuang and Heckard 1971). *Chloropyron maritimum* subsp. *maritimum* is not host specific, and hosts may be variable depending on location. Salt marsh bird's-beak is a predominately out-crossing annual, pollinated by a number of solitary bee species that nest in the ground in upland habitat adjacent to salt marshes (Purer 1942; Lincoln 1985). Seed dispersal range has not been directly measured in *C. maritimum* subsp. *maritimum* but seeds can reportedly float for 50 days (Newman 1981; U.S. Fish and Wildlife Service 1984), and thereby dispersal may be facilitated by tidal flows.

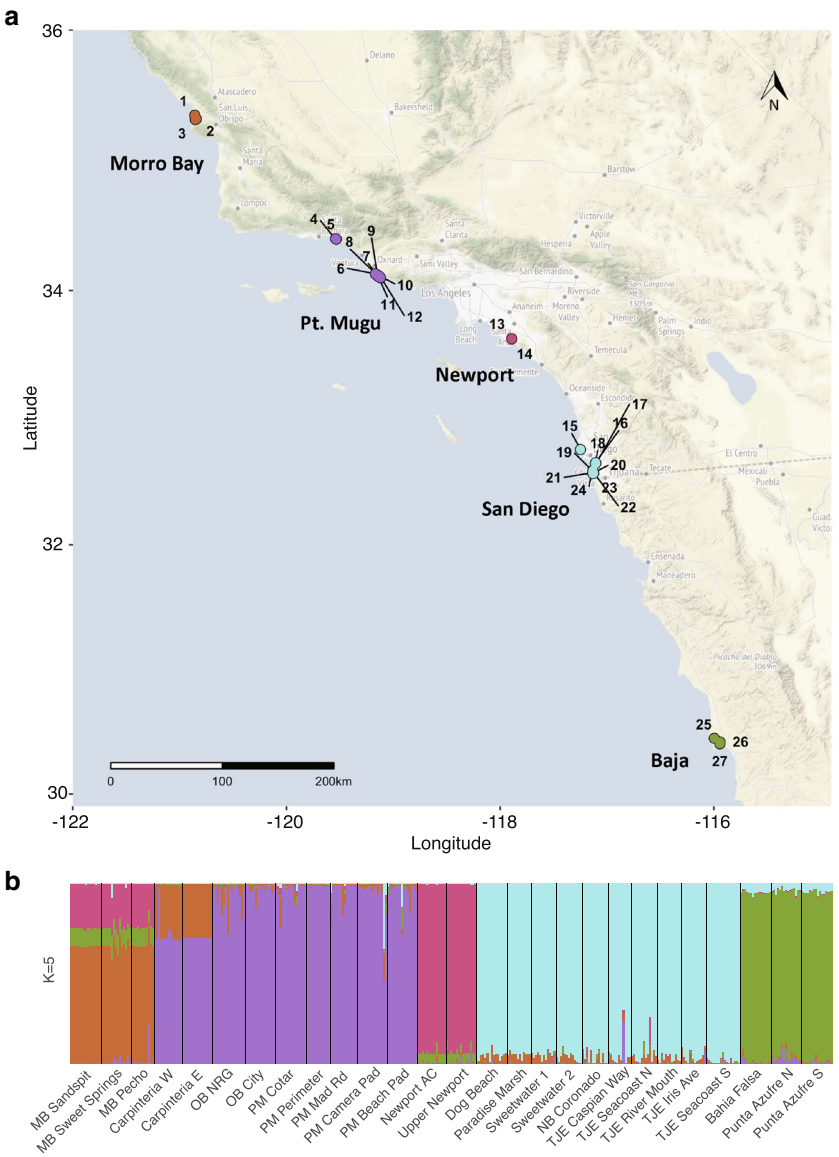

Fig. 1 Map of sampled occurrences (**a**). Colors indicate geographic region associated with genetic clusters, numbers correspond to occurrence names in Table 1. STRUCTURE plot of K = 5 genetic clusters (**b**). Each vertical bar represents an individual; occurrences are partitioned by black vertical bars and ordered from north (right) to south (left)

Ongoing long-term monitoring has shown that populations undergo booms and busts in abundance that are associated with climatic conditions including tidal amplitude, rainfall and temperature (Noe et al. 2019).

These life history characteristics coupled with the extirpation of several occurrences throughout the range have likely reduced connectivity and diversity from historical levels among remaining disjunct occurrences. A previous range-wide genetic assessment was unable to determine regional population structure due to a lack of variation in allozyme markers, but concluded that genetic diversity was low across the range, and that populations could be highly susceptible to genetic drift (Helenurm and Parsons 1997).

Reintroduction projects have been attempted in the subspecies, including one at Sweetwater Marsh in San Diego County that was planted with seeds from multiple occurrences around the Tijuana Estuary (Helenurm and Parsons 1997). Ongoing monitoring of this restored site over the past 26 years has suggested that successful restoration and reintroduction is possible in this taxon (Noe et al. 2019). Anecdotally, a more recent planting of seeds occurred at Dog Beach in San Diego, resulting in one of the largest populations in the county. However, little is known about the establishment methods that were used in this case (San Diego Natural History Museum, Botany Dept. 2018). There have also been unsuccessful attempts at reintroduction, including several attempts at Seal Beach National Wildlife Refuge between 1982 and 1986 where the population steadily declined and is now considered extirpated (Parsons and Zedler 1997). Historically, reintroduction events in rare plants have a low success rate (Godefroid et al. 2011). Traditional approaches require resources and long-term monitoring for successful outcomes of self-sustaining and genetically diverse populations. Incorporating a genetic component into the reintroduction plan can bolster genetic diversity and inform source material decisions and thereby potentially improve the likelihood of success (Maschinski and Albrecht 2017).

Our study aims to describe the current status of genetic diversity and population structure across the range of *C. maritimum* subsp. *maritimum*. To do so, we first surveyed and sampled most known occurrences throughout the subspecies range along the southern California coast and through Baja California, Mexico. Flow cytometry was used to first assess genome size and possible size variation across the range. Double-digest restriction-site associated DNA sequencing (ddRAD) was used to develop genomic resources, including a panel of single nucleotide polymorphic (SNP) markers. We used these markers to estimate genetic diversity and population structure for all sampled locations to provide a comprehensive survey with both site-specific information and regional context that will inform future preservation and restoration efforts.

Materials and methods

Sample collection

Samples were collected in the spring and fall of 2016 and the spring of 2017. Sampling locations were chosen based on current and historical location information obtained from sources including the San Diego Management and Monitoring Program (SDMMP; https://sdmmp.com/), the Consortium of California Herbaria (CCH; http://ucjeps.berkeley.edu/consortium/), the U.S. Fish and Wildlife Service (2009), Naval Base Coronado, and Naval Base Ventura. Collections were made from 20 randomly selected plants at each location (which we refer to as occurrences). Five plants from each occurrence were further sampled for flow cytometry analysis. Leaf tissue for DNA extraction was collected into coin envelopes and assigned a unique identifier. Envelopes were stored with color-indicating silica gel desiccant in plastic bins at room temperature. Containers were gently shaken daily to expedite moisture extraction from the leaf tissue. Silica gel beads were changed regularly when fully saturated. Occurrence size and other site characteristics were estimated and recorded according to a standardized field survey protocol (San Diego Management and Monitoring Program 2019). Voucher specimens were deposited in the San Diego Natural History Museum Herbarium (SD) collection.

Flow cytometry

Flow cytometry was performed to assess genome size and ascertain whether there are ploidy differences among occurrences. Fresh leaf tissue was shipped on ice overnight to the U.S. Department of Agriculture (USDA) Forest Service Shrub Science Laboratory in Provo, Utah, for flow cytometric analysis. All samples were run on a Cyflow Space cytometer (Sysmex Corporation) with *Atriplex canescens* (2C-value = 3.28 pg) 2x as an internal standard. The 2C-value of each sample was determined by a ratio of the sample peak position to the internal standard peak position, where peak position was indicated by the mean-x value of the designated peaks on the histogram output from the flow cytometer, multiplied by the 2C-value of the internal standard (Dolezel et al. 2007). The mean 2C-value and standard deviation of individual samples within occurrences were calculated to determine relative levels of ploidy. Multiple levels of ploidy would be suspected if twofold differences in genome size were detected. A microscopy analysis was not conducted but Chuang and Heckard (1973) found a chromosome count of n = 15.

Genomic library preparation

Genomic DNA was extracted from 15 samples per occurrence. Additional collected samples were used as reserve in case of tissue damage or low yield of DNA following extraction. In total, 390 samples were prepared for genetic analysis across 27 occurrences. Extractions were performed using the E-Z 96® Plant DNA Kit (Omega Bio-tek Inc., Norcross, Georgia) according to manufacturer's guidelines, with the modification of a 1-hour room temperature incubation and single 100 µl elution into nuclease-free water. Reduced representation genomic libraries were prepared using a modified ddRAD scheme (Peterson et al. 2012). In brief, 300 ng of DNA were cut with EcoRI and SbfI restriction enzymes. In-line barcodes were ligated onto the SbfI cut site, fragments were size-selected on a Pippin Prep (Sage Science, Inc., Beverly, Massachusetts) at a 350 ± 50 bp range, and Illumina sequencing adaptors were ligated onto the EcoRI cut site. Each library, containing 16 multiplexed samples, was polymerase chain reaction (PCR) amplified for 14 cycles with the addition of a unique library index sequence and quantified with quantitative PCR (qPCR). This dual index scheme allowed for multiple libraries to be sequenced in a single sequencing lane (8–12 libraries per lane; U.S. Geological Survey data release https://doi.org/10.5066/P9OWNCG8). Libraries were quantified, pooled, and sequenced on 3–100 bp paired-end HiSeq 4000 lanes (Illumina, Inc., San Diego, California) at the Vincent J. Coates Genomic Sequencing Laboratory at University of California, Berkeley (Berkeley, California).

Genotyping and analysis

Raw sequence demultiplexing, quality filtering, and genotyping was performed using the software Stacks v2.41 (Catchen et al. 2011) on the USGS Yeti High Performance Computing platform. Clustering, assembly, and filtering parameters were optimized using a subset of individuals, comprised of 22 percent of all samples, evenly distributed across collection locations, following the *r80* method by Paris et al. (2017). Individuals were only chosen for this subset if read coverage fell within 2 standard deviations of overall mean coverage. This sample subset was also used to create a locus catalog (*cstacks*) for the full Stacks genotyping pipeline. The following parameters were used to call the final dataset: maximum number of mismatches between stacks within individuals, $M = 3$; maximum number of mismatches between stacks between individuals, $n = 3$; minimum percentage of individuals across populations required to process a locus, $R = 0.65$ (Catchen et al. 2011). From this we created two datasets, one 'single SNP' with a single SNP per locus (using the *–write-random-snp* flag in Stacks *populations*) and one 'all SNP' with all variable sites included. The single SNP dataset was used to calculate genetic diversity (H_e), inbreeding (F_{IS}), F_{ST}, perform a PCA, and run STRUCTURE. The all SNP dataset was used to calculate haplotype diversity (π) and coancestry assignments in fineRADstructure. Genetic diversity statistics (H_e, F_{IS}, π) were calculated using the *populations* program in Stacks (detailed in Hohenlohe et al. 2010). Specifically, H_e, a measure of genetic diversity within site, was calculated as the mean expected frequency of heterozygotes under Hardy-Weinberg conditions (Catchen et al. 2013). F_{IS}, a measure of genetic variation within an individual, was calculated as the degree of difference between observed and expected heterozygotes within occurrences (Wright 1931, 1978; Catchen et al. 2013). Haplotype diversity (π), a measure of locus haplotype richness, is equivalent to nucleotide-level π. To account for missing data, sample size is presented as an average of the number of individuals per occurrence with an allele call at each locus. GenoDive v3.03 (Meirmans and Tienderen 2004) was used to calculate a nested analysis of molecular variance (AMOVA) and pairwise genetic differentiation (F_{ST}; Excoffier et al. 1992; Michalakis and Excoffier 1996). The AMOVA nested individual sample within site, and site within genetic cluster, as determined by population structure analyses. The F_{ST} confidence interval was generated over 9999 permutations.

Genetic clustering was used to explore the pattern of genetic structure across the taxon's range. An optimal number of clusters (K) was chosen using both the Bayesian information criterion (BIC) and the Evanno (2005) method. Bayesian STRUCTURE (Pritchard et al. 2000) and a principal component analysis (PCA) were used to identify differences in allele frequencies. A mantel test was used to test for a significant pattern of isolation by distance (IBD); latitude and longitude coordinates were transformed in ArcMap 10.4.1 using the North America Albers Equal Area Conic projection. Genetic clustering and spatial analyses were implemented in the R package *adegenet* v2.1.1 (Jombart 2008). Additionally, we used fineRADstructure v0.3.2 to infer recent population structure by calculating a coancestry matrix that summarizes nearest-neighbor haplotypes across loci (Malinsky et al. 2018). This method, adapted for RAD data, builds on the fineSTRUCTURE package that uses a Markov chain Monte Carlo (MCMC) clustering algorithm (Lawson et al. 2012), and allows for the use of all SNPs from each locus rather than a single bi-allelic SNP that is required for standard population structure analysis. Briefly, RAD loci are ordered according to linkage disequilibrium (LD), then a coancestry matrix is calculated to identify populations by MCMC clustering (burn-in and sampling iterations = 100,000, thinning interval = 1000). Data were summarized in a heatmap and maximum a posteriori (MAP) tree using R scripts provided by Malinsky et al. (2018).

Raw sequence data are available at http://www.ncbi.nlm.nih.gov/bioproject/484956, and final genotypes are provided as a USGS data release at https://doi.org/10.5066/P9OWNCG8.

Results

Field sampling

We visited 31 occurrences, which comprised all documented occurrences throughout the subspecies range at the time of sampling (though, recently one new occurrence was found in Punta Banda in Ensenada, MX). No plants were observed at three previously documented occurrences in San Diego (NB Coronado Radar Receiving Facility, Tijuana Estuary Boundary Monument, and Tijuana Estuary North Border Field). At a fourth occurrence, Morro Bay Marina Peninsula, only three plants were observed and so no plants were sampled. Population size estimates in the remaining 27 occurrences ranged from 30 to 3000 (Table 1). Site descriptions, including dominant plant species, aspect, soil texture, and disturbance are reported in Supplementary Table S1.

Flow cytometry

In total, 22 occurrences were sampled for flow cytometry (excluding: MB Sandspit, Pt. Mugu Perimeter Rd. and all

Table 1 Occurrence field size estimates and genetic diversity summary statistics. Occurrences are organized by genetic cluster

	Occurrence	Occurrence size field estimate	Ave. sample size/ locus	F_{IS}	F_{IS} st. err	H_e	H_est. err	Haplotype diversity (π)	Haplotype diversity st. err
Morro Bay									
1	MB Sandspit	1000	13.296	0.024	0.119	0.014	0.006	0.016	0.003
2	MB Sweet Sp.	1500	11.998	0.009	0.110	0.030	0.009	0.023	0.004
3	MB Pecho Rd	200	5.964	0.004	0.167	0.014	0.005	0.014	0.003
Pt. Mugu									
4	Carpinteria West	3000	8.543	0.024	0.166	0.027	0.008	0.027	0.005
5	Carpinteria East	1000	12.990	0.017	0.115	0.021	0.009	0.021	0.004
6	OB NRG	300	11.464	0.007	0.170	0.023	0.007	0.015	0.003
7	OB City	100	13.143	0.028	0.091	0.034	0.011	0.020	0.004
8	PM Cotar	140	12.455	0.022	0.095	0.032	0.010	0.024	0.004
9	PM Perimeter	70	9.181	0.003	0.105	0.032	0.010	0.021	0.004
10	PM Mad Rd	200	11.116	0.020	0.075	0.030	0.009	0.020	0.004
11	PM CameraPad	30	11.008	0.016	0.110	0.033	0.009	0.024	0.003
12	PM Beach Pad	100	11.990	0.022	0.078	0.039	0.011	0.032	0.005
Newport									
13	Newport AC	1500	9.431	0.029	0.149	0.026	0.009	0.035	0.005
14	Upper Newport	1500	12.387	0.024	0.125	0.036	0.010	0.032	0.005
	San Diego								
15	Dog Beach	3000	11.400	0.029	0.138	0.024	0.008	0.042	0.005
16	Paradise Marsh	70	9.370	0.020	0.105	0.004	0.003	0.022	0.004
17	Sweetwater 1	201	9.597	0.019	0.105	0.025	0.009	0.029	0.004
18	Sweetwater 2	293	10.803	0.043	0.086	0.018	0.006	0.029	0.004
19	NB Coronado	200	10.411	0.061	0.102	0.016	0.006	0.043	0.006
20	TJE Caspian Way	80	7.025	0.040	0.123	0.026	0.007	0.044	0.005
21	TJE Seacoast N	1200	7.457	0.052	0.129	0.029	0.008	0.046	0.005
22	TJE River Mouth	1500	7.590	0.041	0.142	0.025	0.008	0.045	0.006
23	TJE Iris Ave	1000	9.410	0.020	0.083	0.018	0.005	0.038	0.005
24	TJE Seacoast S	3000	10.603	0.039	0.198	0.028	0.008	0.039	0.005
Baja California									
25	Bahia Falsa	300	11.779	0.039	0.137	0.041	0.011	0.065	0.007
26	Punta Azufre N	500	11.057	0.034	0.150	0.059	0.013	0.058	0.006
27	Punta Azufre S	150	9.355	0.034	0.188	0.055	0.013	0.039	0.005

three Baja California locations). The mean 2C-value across all occurrences was 4.91 pg (4.8 GB) with a standard deviation of 0.81 pg. This does not suggest evidence of multiple levels of ploidy, as we did not observe a twofold difference between samples that would indicate doubled (or greater) genome size. It does, however, indicate that some occurrences are more variable in genome size than others. In San Diego, four of the five occurrences from the Tijuana Estuary have standard deviations greater than 1.0. Ormond Beach City is the only occurrence outside San Diego that fits this criterion with a 1.10 standard deviation of the 2C-value (Table 2).

Genotype dataset and genetic diversity

We sequenced 375 individuals from all 27 occurrences across the region (Fig. 1a). Raw sequence reads totaled 1,007,336,500 with a mean of 3,249,472.6 (s.d. = 2,446,563.2) reads per individual. Mean read coverage per locus, after *ustacks* filters, was 46.65x. The final genetic marker dataset consisted of 122 independent loci with 481 SNPs and 25% missing data.

Table 2 Flow Cytometry mean peak ratio, standard deviation, and number of observations

Occurrence	Mean 2C-value	St. dev. 2C-value	n
Morro Bay Sandspit	4.72	0.34	5
Morro Bay Pecho Rd	4.55	0.22	5
Carpinteria West	4.39	0.26	5
Carpinteria East	4.41	0.25	5
Ormond Beach NRG	4.49	0.29	5
Ormond Beach City	5.31	1.10	5
Pt. Mugu Cotar	4.89	0.24	5
Pt. Mugu Mad Rd	4.40	0.09	5
Pt. Mugu Camera Pad	5.18	0.52	4
Pt. Mugu Beach Pad	4.22	0.05	5
Newport Aquatic Center	4.61	0.13	5
Upper Newport Bay	4.76	0.27	5
Dog Beach	4.68	0.22	5
Paradise Marsh	5.55	0.95	5
Sweetwater Marsh 1	4.42	0.34	5
Sweetwater Marsh 2	4.51	0.07	5
Naval Base Coronado	4.85	0.65	5
TJE Caspian Way	4.76	0.12	4
TJE Seacoast Dr N	5.89	1.21	5
TJE River Mouth	6.04	1.05	5
TJE Iris Ave	5.32	1.34	5
TJE Seacoast Dr S	6.05	1.33	5
All samples	4.91	0.81	108

Inbreeding did not differ from zero across occurrences. Haplotype diversity (π) ranged from 0.014 ($\pm$0.005 SE) at Morro Bay Pecho Rd. to 0.065 ($\pm$0.007 SE) at Punta Azufre North (Fig. 2; Table 1). Latitude was significantly negatively correlated with haplotype diversity (Pearson's correlation coefficient $r = -0.84$, $p < 0.05$; Fig. 3), whereas genome size was positively correlated with π ($r = 0.44$, $p < 0.05$; Fig. 3). Combined, these associations indicate a reduction in genome size from south to north may be a contributing factor to reduced genetic diversity in northern occurrences. H_e, based on a single SNP per locus, showed the same pattern with low diversity at northern occurrences and greater diversity at occurrences in San Diego and Baja California (Table 1). Interestingly, the sites with the largest population size estimates (Carpinteria West, Dog Beach, TJE Seacoast S) did not have the greatest diversity. Both Sweetwater Marsh sites and Paradise Marsh have the lowest genetic diversity in San Diego (Fig. 2). Lower genetic diversity is likely due to a bottleneck event, or founder's effect in the case of the Sweetwater Marsh reintroduction project.

Population structure

Bayesian structure analysis and multivariate and MCMC genetic clustering methods all indicated groupings that correspond to the geographically disparate marsh systems along the coast. Five distinct genetic groups were supported by K means clustering and BIC. STRUCTURE results clearly delineate these regions (Fig. 1b) with some evidence of admixture or shared ancestry between individuals in the Morro Bay occurrences and both the Carpinteria occurrences (within the Point Mugu cluster) and Newport occurrences. The multivariate methods recovered more nuanced substructure between regions. The first axis of the PCA plot which accounts for almost 40% of the variation distinguishes the San Diego and Baja California occurrences from those of Newport, and the Pt. Mugu/Ormond Beach/Carpinteria and Morro Bay occurrences (Fig. 4). Latitude appears to account for much of the variation along this axis. The Baja California samples were closest to the origin of the plot and share some overlap with the San Diego sites. The second axis of the PC plot (describing 10% of the variation) further distinguishes two smaller clusters: Newport and Morro Bay (Fig. 4). Finally, FineRADstructure analysis of haplotypes showed a similar geographic clustering pattern to the PCA and more detailed substructure within the larger San Diego and Pt. Mugu regions (Fig. 5). For visualization purposes, we created a reduced dataset using five average coverage samples from each occurrence, totaling 135 individuals, but the full dataset revealed the same results. Broadly, San Diego and Baja California clustered together, separate from all the other clusters (Pt. Mugu, Morro Bay and

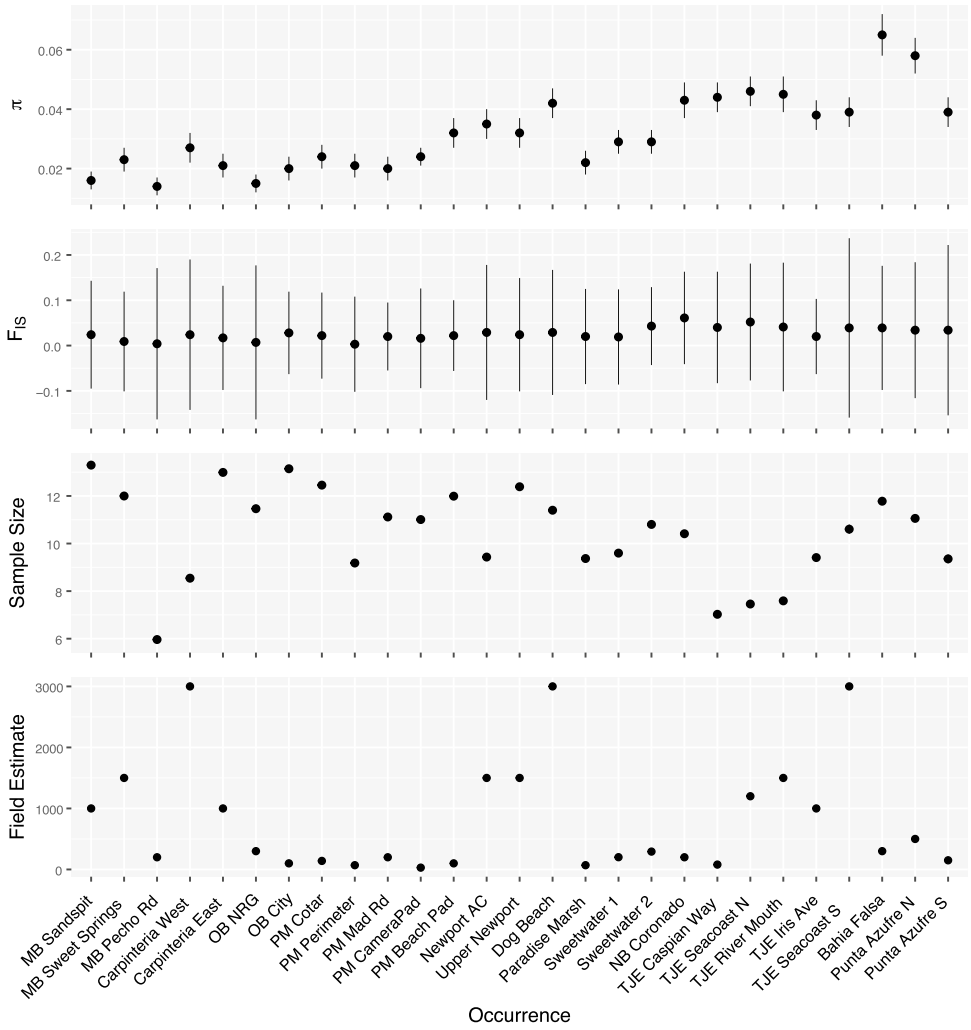

Fig. 2 Haplotype diversity (π), inbreeding (F_{IS}), sample size, and field population size estimates reported as mean values and standard errors for each occurrence

Newport). Minor substructure within the Pt. Mugu cluster distinguished Carpinteria occurrences. Finally, Morro Bay and Newport appear to cluster together. The Newport occurrences also had the highest coancestry values (Fig. 5), indicating individuals within these groups had higher genetic similarity within this cluster than within any other cluster, which could indicate a past bottleneck.

Genetic differentiation (pairwise F_{ST}) between occurrences ranged from 0.056 to 0.86, where the lowest values were among pairs within the San Diego and Pt. Mugu regions. The highest F_{ST} estimates occurred across regions, notably between the Sweetwater Marsh sites and several Pt. Mugu locations (Supplementary Table S2). The largest differences between occurrences coincided with large geographic distance, and we found isolation by distance to be significant (Mantel test $R^2 = 0.696$; $p < 0.001$; 999 permutations), consistent with genetic clustering among geographic regions. A nested AMOVA partitioned 17.4% of genetic variation within individuals, and 70.3% among genetic clusters. While we observed low but significant substructure within clusters (6.4%), the majority of genetic variance between occurrences was partitioned among clusters (Table 3).

Discussion

Overall, we found that remaining occurrences of *C. maritimum* subsp. *maritimum* are structured into five distinct genetic clusters that correspond with geographic regions: Morro Bay, Pt. Mugu, Newport, San Diego, and Baja California. We found a latitudinal trend in both structure and diversity and in genome size. Most genetic variation was partitioned among the five genetic clusters, with large F_{ST} estimates among clusters, indicating little or no genetic connectivity at this spatial scale. We found less differentiation between neighboring occurrences within the same cluster. Our results build upon those found in a previous study using enzymatic loci, which found low genetic diversity in *C. maritimum* subsp. *maritimum* range-wide, and that the Tijuana Estuary in San Diego, followed by

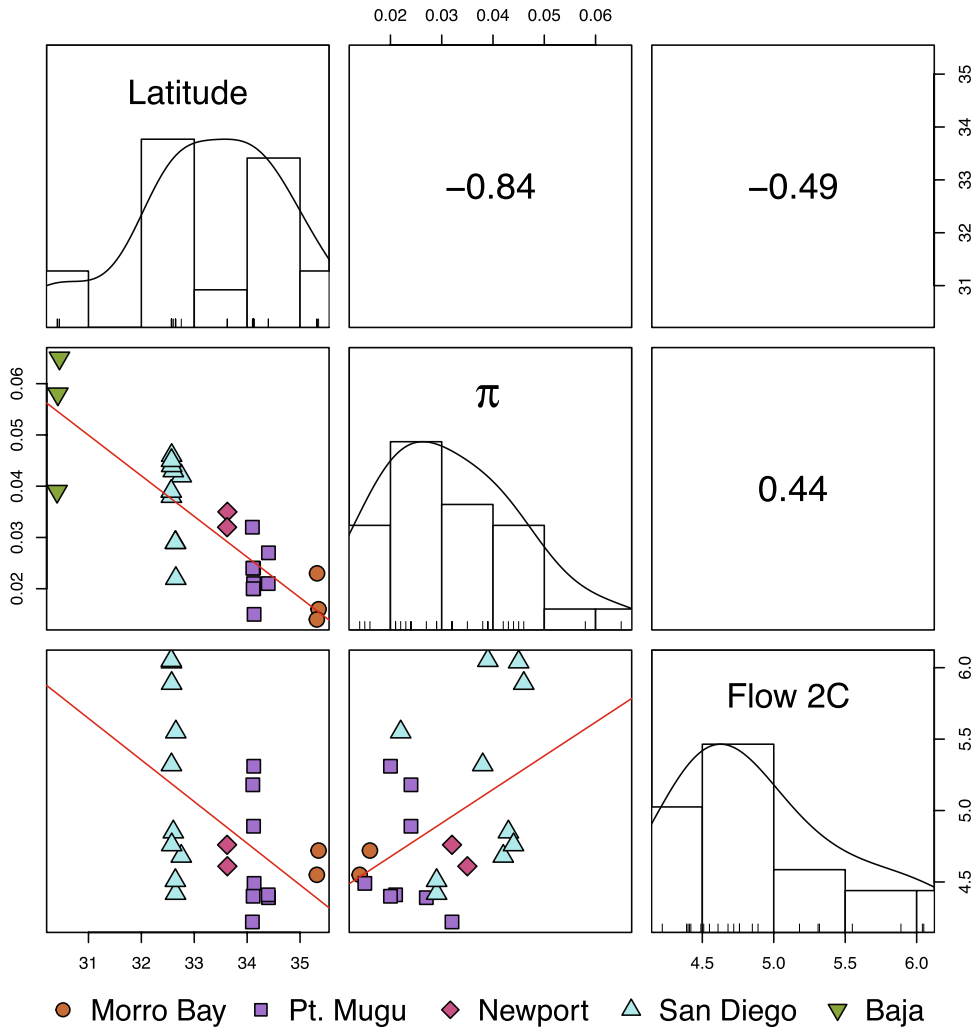

Fig. 3 Scatterplot matrix of correlations for Latitude, haplotype diversity (π), and genome size, by occurrence. Pearson's r above the diagonal, all values significant at $p < 0.05$. Correlation plots below diagonal, color coded by genetic cluster

Point Mugu, had the largest population sizes and number of rare alleles (Helenurm and Parsons 1997). Although Helenurm and Parsons (1997) reported a low global F_{ST} estimate, they also acknowledged that low differentiation was a function of the extremely low diversity in the reported enzyme loci (17 of 21 loci were fixed invariant, with the remaining four loci showing limited variation in just a few sampled sites). We were able to expand upon these previous results, using ddRAD generated SNPs, to gain a more detailed model of substructure across the region, revealing high levels of differentiation. This pattern may be indicative of genetic structure in other rare taxa in the genus. For example, a recent study of an endangered congener *Chloropyron palmatum*, found in the California Central Valley, also reported significant population structure among populations (Ayres et al. 2015).

Genetic diversity

Genetic diversity was greatest in the southern part of the range including San Diego (specifically the Tijuana Estuary occurrences) and Baja California. We found elevated variation in genome size in the Tijuana Estuary and Paradise Marsh; another potential indicator of genetic diversity in the San Diego region. San Diego occurrences also supported the greatest number of plants in total. We postulate that a combination of land area and colonization (evolutionary) history are the main drivers of this diversity pattern. The Tijuana Estuary, Sweetwater/Paradise Marshes, and Pt. Mugu/ Ormond Beach sites are large networks of subpopulations in close proximity that fluctuate in size over time. In some years, these sites can merge into one large population. We have less historical data on population size and fluctuation events in Baja California, but predict they

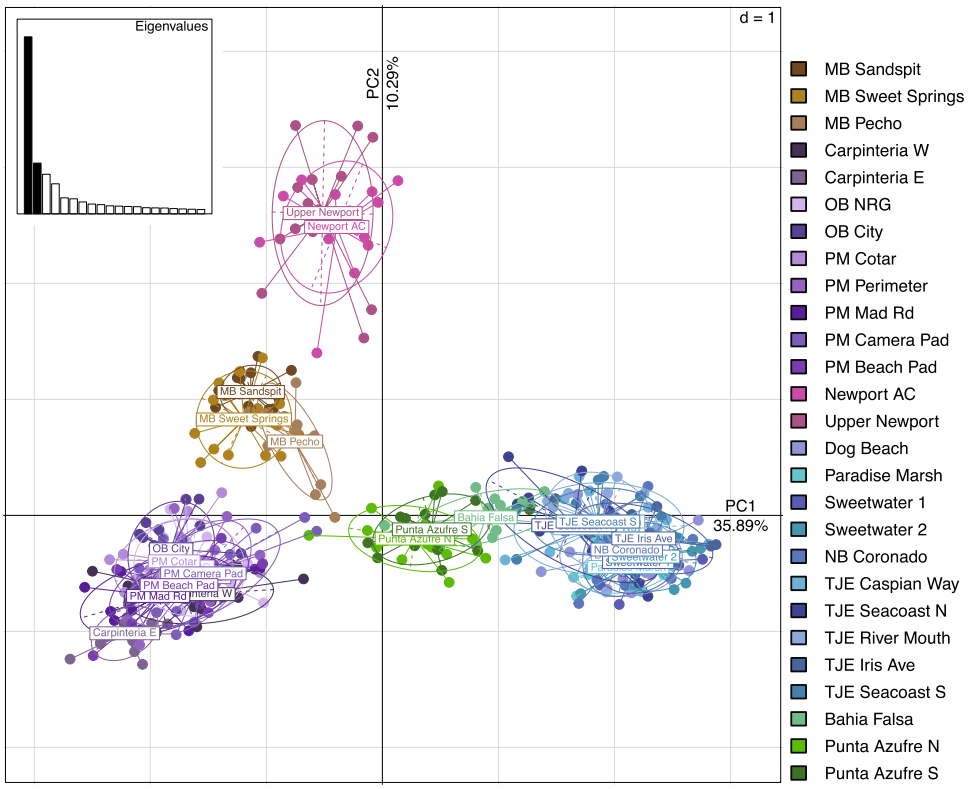

Fig. 4 Principal component (PC) plot with PC1 on the horizontal axis and PC2 on the vertical axis. Each point represents an individual sample, and each color and ellipse represents an occurrence. Inset scree plot shows relative values of retained PCs, dimensional grid scale indicated in the upper right corner of main plot

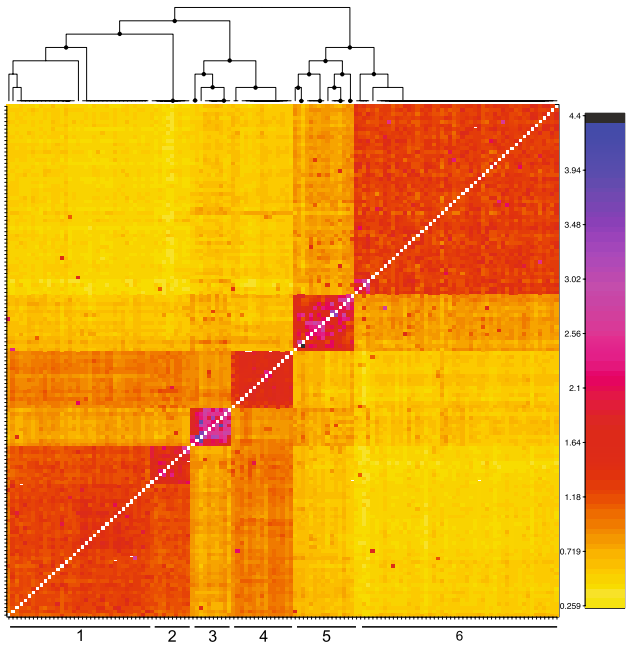

Fig. 5 FineRADstructure coancestry matrix and maximum a posteriori (MAP) tree. Color scale bar indicates estimated coancestry from yellow (0) to black (4.4). Black circles indicate nodes with posterior probabilities greater than 0.9. Genetic clusters are numbered as follows: 1—Pt. Mugu and Ormond Beach, 2—Carpinteria, 3—Newport, 4—Morro Bay, 5—Baja California, 6—San Diego

Table 3 Analysis of molecular variance

Source of variation	Proportion of variation	*F*-statistic (95% CI)
Within individual	0.174	F_{IT}=0.826 (0.743–0.877)
Among individual within site	0.059	F_{IS}=0.254 (0.063–0.411)
Among site within cluster	0.064	F_{SC}=0.215 (0.147–0.287)
Among cluster	0.703	F_{CT}=0.703 (0.606–0.773)

follow a similar pattern. The pattern of decreasing genetic diversity with increasing latitude may also point to a northward colonization or range expansion pattern (reviewed in Excoffier et al. 2009). This is a common phylogeographic pattern found in southern California, also observed in intertidal copepods (Edmands 2001), ground squirrels (Whorley et al. 2004), wrentits (Burns and Barhoum 2006) and other species (reviewed in Calsbeek et al. 2003). In addition to genetic diversity, average genome size also decreased with increasing latitude. The presence of some individuals with larger genome sizes in the southern part of the range could be indicative of supernumerary chromosomes, which can contribute to diversity (Jones et al. 2008). Future microscopy work could help confirm this.

Developing genomic markers for rare taxa with little to no previous genomic data has many challenges. We

acknowledge the number of loci recovered in this study is low for the amount of sequencing effort put forth, and we postulate it may be due to a combination of factors in the laboratory and in nature. Increased sequencing depth is one obvious way to retrieve more loci, however our average read depth was over 40x. We explored parameter space in the Stacks bioinformatics pipeline with several subsets of samples but found that the final loci count was most affected by the missing data filter (−R). We did find some bias in sequence coverage by population but not enough to yield a noticeable increase in loci recovery when select populations were removed from the analysis. If future RADseq studies are warranted, it may be beneficial to test additional combinations of restriction enzymes and size selection to increase the number of cut sites and unique fragments for analysis. Another contributing factor may be inherently low genetic diversity in this taxon. A phylogenetic study using ddRAD markers in the *Chloropyron* genus only recovered an average of 96 variable loci per sample (Gilman and Tank 2018), and a previous isozyme study, in *C. maritimum* subsp. *maritimum* specifically, recovered only 4 variable loci of 21 enzyme systems tested (Helenurm and Parsons 1997).

Genetic connectivity

The distributions of more common marsh plant species that occur along the California coast, are thought to be influenced by tidal movement (Hopkins and Parker 1984; U.S. Fish and Wildlife Service 2009) and it has been proposed that seeds dispersed and deposited by tides may be the major dispersal mechanism in salt marsh bird's beak (Newman 1981; U.S. Fish and Wildlife Service 1984), although no direct dispersal studies have been conducted. Historical occurrences of salt marsh bird's-beak in Los Angeles County, San Bernardino County, and Orange County, including three inland sites, are now extirpated. This has most likely isolated clusters of remaining occurrences from each other. We found an overall signature of isolation by distance, suggesting that gene flow is limited spatially, and very low across regions, likely reflecting the loss of intervening habitat and populations. Loss of genetic diversity within sites can also increase divergence between them due to genetic drift. Signatures of high genetic divergence and low genetic diversity often occur together (Allendorf 1986; Slatkin 1987; Young et al. 1996). Genetic drift may contribute to the regional genetic distinctiveness we found across the species range. Although genetic structure has not been characterized in other rare salt marsh plant species in the region, genetic studies of other, co-occurring marsh obligate vertebrates throughout California have found high levels of genetic structure consistent with patchy distributions of remaining habitat coupled with low population sizes and low genetic diversity within marshes (Walsh et al. 2012; Statham et al. 2016; Wood et al. 2017). In contrast to the high levels of differentiation found among clusters, we found much more genetic similarity among occurrences in close proximity to each other within the clusters, supporting higher levels of genetic exchange at this smaller spatial scale. Future landscape analyses at finer spatial scales could help elucidate the factors that influence genetic connectivity among local occurrences.

Incorporating genetic information into conservation efforts

Understanding levels of differentiation across extant populations of *C. maritimum* subsp. *maritimum* is useful both in developing conservation strategies for existing populations and in selecting sets of compatible occurrences for seed or translocation sources for future restoration. Recovery criteria for *C. maritimum* subsp. *maritimum* include protecting, securing and managing sufficient salt marsh bird's-beak colonies within major marshes within the historic range of the plant within the United States (U.S. Fish and Wildlife Service 1984). The regional genetic structure detected here suggests that each region represents a unique portion of the overall genetic diversity within the subspecies and thus it is important to preserve sites within each of these clusters to protect existing genetic diversity.

Even though habitat loss from development or clearing of land has slowed or ceased since listing in 1983 (U.S. Fish and Wildlife Service 2009), *C. maritimum* subsp. *maritimum* still faces many threats in its remaining habitat. Competition with non-natives such as *Limonium durisuculum* and *L. ramosissimum*, declines in pollinators, hydrologic changes, and in particular, habitat loss due to sea level rise, remain potential threats. Sea level rise has accelerated in the last 25 years and existing tidal wetlands in southern California are projected to be entirely converted to bare mud flats and small patches of low marsh habitat by 2100 (Williams 2013; Thorne et al. 2018; Nerem et al. 2018). With an elevation range of 1–5 m, *C. maritimum* subsp. *maritimum* populations will be vulnerable to loss of habitat, particularly in salt marshes adjacent to developed land or steep slopes where upland marsh migration is largely precluded. Early season influxes of fresh water are needed for germination success, whereas restricted or contaminated freshwater sources and/or more salt water inundation, could decrease plant establishment (Noe and Zedler 2001). Recent work suggests that cyclical variation in tidal amplitude along with early spring rainfall and maximum spring temperatures are linked to occurrence size in San Diego (Noe et al. 2019). Therefore, the combination of rising sea levels and rising temperatures could interact negatively to reduce population sizes and genetic diversity over time or lead to local extirpation. Given the apparent lack of genetic connectivity among remaining regional clusters, it may be less likely for extirpated sites

to be recolonized on their own if there are no other occurrences nearby.

Due to the aforementioned circumstances, habitat restoration, population re-establishment and augmentation will likely be ongoing management strategies utilized to protect this species in the long term. In order to retain regional genetic structure, restoration efforts that include seeding could source seeds from within the same geographic region. If multiple donor sites exist within a region, then utilizing seed from more genetically diverse and larger occurrences or a composite of local occurrences may help increase the potential genetic variation at receiver sites. Decisions about seed sources may be less straight forward when restoration sites are far from extant occurrences, such as between the San Diego and Newport Bay clusters, and between Newport Bay and Point Mugu clusters. In such cases, restoration could rely on the closest regional source, or a "regional admixture provenancing" strategy from multiple sources (Bucharova et al. 2019). Genetic factors that might influence establishment success include overall levels of genetic diversity as well as whether local adaptive traits are present, and composite seed sources may allow for both (Broadhurst et al. 2008).

Efforts to maintain large numbers of individuals at restored sites and habitat for appropriate pollinators to promote outcrossing should also help retain more genetic variation. Small occurrences, those founded from just a few individuals or those that undergo frequent or extended genetic bottlenecks, tend to have lower genetic diversity than those that are large or well-connected via gene flow to other occurrences. Low genetic diversity can be correlated with reduced fitness, increased extinction risks and low adaptive potential (Spielman et al. 2004; Frankham 2005; Markert et al. 2010). Although anecdotal, it is possible that higher genetic diversity in the southern portion of the species range has contributed to relative success of restoration efforts in San Diego. Sweetwater Marsh was re-established in the 1990s and has supported plants since then (Noe et al. 2019). The Dog Beach occurrence is the result of a seeding event in 2009, with material sourced from the Tijuana Estuary near Seacoast Drive. Seeds were collected from the initial reintroduction and redistributed on site for the next two years (San Diego Natural History Museum, Botany Dept. 2018). This occurrence is now one of the largest occurrences in San Diego County. Both of these restoration efforts sourced starting material from the Tijuana Estuary which we found to have the highest diversity within the U.S. In contrast, re-establishment efforts in Seal Beach, California, conducted with seed from the closest extant occurrence, Newport Bay, were unsuccessful after several attempts (Parsons and Zedler 1997).

In addition to maximizing potential genetic diversity and geographic proximity, matching similar habitat conditions, host plant availability and morphological similarities between donor and restoration sites may be important (Maschinski et al. 2012). Despite that salt marsh bird's-beak is not host specific, Chuang and Heckard (1971) found that some species are poor hosts even if haustorial connections are present, and Fellows and Zedler (2005) found differences in flower production when native and non-native hosts were present, so trial and error to find an appropriate host plant or composition of host plants might be needed. In the field, we noted morphological variability among regions and populations, specifically differences in bract shape/size, flower color, and branching angles, consistent with previous reports (U.S. Fish and Wildlife Service 1984). Several of these morphological characteristics overlap with sister taxa *C. maritimum*. subsp. *palustre* and *C. maritimum*. subsp. *canescens*, and it may be warranted to perform further phylogenetic investigation into the *C. maritimum* species complex. It is also interesting to note that both the MAP tree and PCA suggest that Morro Bay and Newport clusters share more genetic similarity than expected by geographic distance. Whether this is a spurious pattern caused by random drift, or reflects more recent common ancestry, recent gene flow, or maintenance of adaptive alleles in response to similar selective pressures, requires more detailed investigation. These points illustrate the need for careful and deliberate reintroduction events and experiments. For example, prior to mixing seeds from multiple sources, test crosses between regional groups could be performed in a greenhouse to ensure compatibility and test for links between fitness and diversity. Field trials could be designated in areas that will not disrupt current native populations if failure should occur.

Conclusions

We found five distinct genetic clusters across the range of *C. maritimum* subsp. *maritimum* that coincide with geographic regions and highlight that each geographic region contains unique genetic diversity worthy of protection. Genetic diversity was greatest in the southern part of the range including San Diego and Baja California. Our study also illustrates that newer genetic analysis techniques such as ddRAD can generate higher resolution datasets to further resolve patterns of population-level genetic structure even in a low diversity plant species. Our data provide a reference point for the current status of genetic diversity and range-wide population structure that can be applied to inform future preservation and restoration efforts across jurisdictions. Such restoration efforts are likely to become increasingly important as the range of *Chloropyron maritimum* subsp. *maritimum* is predicted to fluctuate and contract in response to changing climate and habitat loss.

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