Conservation Genetics (2022) 23:123–137
https://doi.org/10.1007/s10592-021-01406-6

RESEARCH ARTICLE

Historic DNA reveals genetic consequences of fragmentation in an endangered, endemic mustard

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Received: 3 February 2021 / Accepted: 19 September 2021 / Published online: 12 October 2021

Abstract

Understanding how anthropogenic disturbance affects genetic diversity is essential to appropriately incorporating genetic considerations into conservation plans. Unfortunately, we rarely have information about a population's genetic diversity before it becomes imperiled. Here we reconstruct the historic range of the naturally rare annual mustard *Streptanthus glandulosus* subsp. *niger* (*Sgn*) and use herbarium specimens to quantify pre-disturbance genetic diversity. We compare this to the genetic diversity in the contemporary plant populations and to plants in the seed bank. We conclude that *Sgn* was recently a single, panmictic population composed of orders of magnitude more plants than exist today but experienced recent and abrupt declines following housing development. Today *Sgn* persists as two disjunct populations, the larger of which has retained historic levels of diversity although there is a downward trend in all measures. The smaller population has lost 21–28% of the diversity that was present only 50 years ago with an $N_e \sim 5$–16. The contemporary populations have differentiated from each other due to drift. The seed bank contained no novel alleles and had high levels of homozygosity, indicating that it is incapable of providing genetic rescue. This novel combination of *h*DNA, the aboveground plant population and the seed bank can be used to design high impact conservation plans that appropriately incorporate genetic diversity for this and other imperiled species.

Keywords Historic DNA · Seed bank · Fragmentation · Endemic · Genetic diversity · *Streptanthus glandulosus* subsp. *niger*

Introduction

Endemic species found on islands and in insular continental settings contribute to biodiversity in unique ways by virtue of the fact that they are found nowhere else. They are also of particular conservation concern because many exist as small and/or isolated populations, making them inherently more vulnerable to demographic and environmental stochasticity that could lead to extinction (Soulé et al. 1988; Frankham 1996). Low genetic diversity is also frequently correlated with small population size, and this can reduce fitness in the short-term and restrict the capacity for adaptive evolution in the long-term, thereby further increasing the risk of extinction. Selection may have favored traits that mitigate against the loss of genetic diversity, e.g. obligate outcrossing via self-incompatibility alleles or dioecy in plants, leading to larger effective population sizes than would be expected based on census population size alone. Alternatively, there are examples of endemic species that have persisted over long timespans with very low genetic diversity and little evidence of inbreeding depression (e.g., Hadly et al. 2003; Robinson et al. 2016). These populations may maintain fitness through purging (Barrett and Charlesworth 1991; Templeton and Read 1984) and there is evidence of this in some wild populations (e.g., Robinson et al. 2018). Whether populations have evolved strategies that maximize genetic diversity or persist despite low diversity will influence how they respond to anthropogenic fragmentation and the subsequent population decline.

Dormant seeds in the soil may also contribute to the genetic diversity of plant populations. Many plants that live in temporally variable or unpredictable environments produce seeds that are capable of remaining viable in the soil for long periods of time (Leck et al. 1989). These seed banks

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are capable of reducing the risk of extinction in small and fragmented populations (Stöcklin and Fischer 1999) because recruitment from the seed bank can increase the size of the aboveground population after a period of poor growing conditions (Kalisz and McPeek 1992). Because these seeds are produced over many years under different environmental conditions by a diversity of parents, they may also harbor genetic diversity absent from the aboveground population, thereby increasing the effective population size (Nunney 2002; Vitalis et al. 2004).

Increasingly, conservationists are attempting to include genetic diversity in plans to protect rare species. To do so effectively requires quantifying present-day levels of diversity, including the seed bank, and determining whether it is the result of historical processes operating over long time spans or recent anthropogenic disturbance. Unfortunately, we rarely have information about a population's genetic diversity before it is threatened by human activity. The use of historic DNA (*h*DNA) from museum and herbarium specimens allows us to assess diversity prior to decline to inform conservation practices. In some cases, a conservation intervention that increases genetic diversity may reverse a population decline, as was the case with the greater prairie chicken (Bouzat et al. 1998), the Swedish adder (Madsen et al. 1999, 2004) and a grassland daisy (Pickup and Young 2008). In other cases, low levels of genetic diversity may be natural, as appears to be the case for the Chanel Island fox (Robinson et al. 2016) and the tuco-tuco (Hadly et al. 2003). In such cases, increasing genetic diversity via translocations or artificial gene flow might be unnecessary and even harm the recipient population, as was the case for the Isle Royal wolf population (e.g., Hedrick et al. 2014).

*h*DNA has been used in inventive ways to study changes in plant populations through time. For example, Saltonstall (2002) used *h*DNA to show that an introduced genotype of *Phragmites australis* replaced native genotypes in North America, providing compelling evidence of the underappreciated phenomenon of cryptic invasion. Vandepitte et al. (2014) documented genetically based changes in flowering time in invasive populations of a mustard compared to their native populations, a shift that likely accounts for the species' success invading novel environments. Surprisingly few studies have used *h*DNA for the conservation of rare plant species. One exception is Cozzolino et al. (2007) who used *h*DNA to document the effects of habitat loss and fragmentation on genetic diversity in the orchid *Anacamptis palustris*. They found that alleles that were widespread in herbarium samples are now restricted to small remnant populations or have gone extinct, and that population differentiation has increased since fragmentation.

We explore these ideas using DNA extracted from contemporary plants and plants in the seed bank to assess the current level of genetic diversity of a rare endemic plant and compare it to DNA extracted from herbarium specimens to determine whether it is the product of historic processes or recent anthropogenic fragmentation and population contraction. *Streptanthus glandulosus* Hook. subsp. *niger* (Greene) Al-Shehbaz, M.S. Mayer & D.W. Taylor (Brassicaceae) (hereafter *Sgn*) is a member of the Streptanthoid adaptive radiation (Mayer et al. 1994; Cacho et al. 2014). *Sgn* is confined to the 4.5 km long Tiburon Peninsula, Marin County, California (USA) (Fig. 1). This highly restricted geographic range appears to be natural rather than an artifact of anthropogenic disturbance as none of the extensive botanical surveys in Marin County, which began in the mid-1800s, ever recorded it outside of the peninsula. *Sgn* is also a habitat specialist within this small geographic range. It occurs only in outcrops of serpentine soil in grasslands along south- and west-facing slopes (Kruckeberg 1954, 1957). Serpentine soils are characterized by toxic concentrations of heavy metals (Ni, Cr, Co) and low concentrations of essential nutrients (N, P, K, Ca) and plants must evolve to tolerate these conditions (Anacker 2014; Cacho and Strauss 2014). As a result, these soils tend to be hotspots of endemism (Skinner and Pavlik 1994).

How widespread *Sgn* was on the on the Tiburon Peninsula prior to European-American settlement is unknown and our first goal was to determine its historic range. We wanted to use this information to determine if the historic population has been fragmented or reduced in size, as is presumed, or if *Sgn* has always existed as small, disjunct populations as it does today. Our second goal was to quantify the genetic diversity and effective population size of the historic population and compare this to the two contemporary populations to determine if there have been changes. We also sampled seedlings that emerged from the seed bank to determine if the seed bank is capable of maintaining or restoring genetic diversity in the aboveground population. Finally, we wanted to determine how genetically similar the two remaining populations are to one another. We use the results to make recommendations for the effective conservation of this endangered endemic.

Materials and methods

Study species

Sgn has an annual life cycle and germination, survival to flowering, and population growth rate are sensitive to variation in the amount and timing of precipitation (Swope, *in preparation*). As a consequence, the size of the contemporary populations fluctuates dramatically from year to year. In years with favorable growing conditions, total population size may exceed 5000 plants. In years of unfavorable growing conditions, the number of plants may fall to several

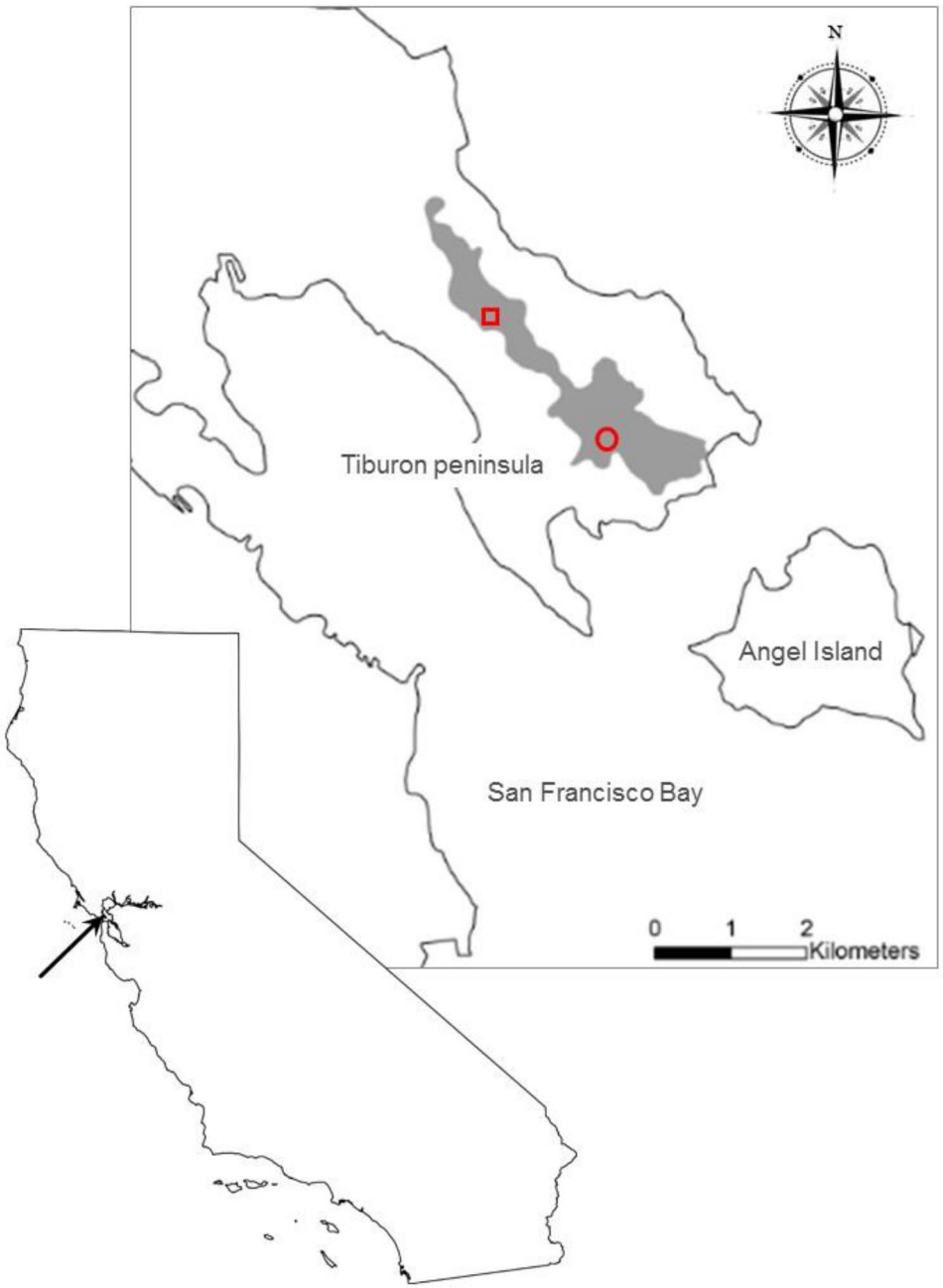

Fig. 1 The Tiburon Peninsula, Marin County, California (USA), to which *Streptanthus glandulosus* subsp. *niger* is confined. The arrow points to the location of the peninsula in the state of California. The grey polygon represents the band of serpentine soil as delineated by the Natural Resources Conservation Service. The red circle shows the location of the Old St. Hilary's preserve and the red box shows the location of the Middle Ridge Preserve

hundred. *Sgn* is primarily outcrossing the field and flowers are visited by native bees (*Bombus* spp.) but it is also capable of geitonogamy. The small, lightweight seeds have no obvious adaptations that promote dispersal.

Sgn plants are small and sparsely distributed, and have narrow habitat requirements within a very small geographic range, making it rare by all three of Rabinowitz's (1981) criteria, even prior to anthropogenic disturbance. *Sgn* is listed as endangered by the State (California Department of Fish and Wildlife 2014) and Federal governments (U.S. Fish and Wildlife Service 2019) and has a California Rare Plant ranking of 1B.1, the highest possible ranking for a species not presumed extinct in the wild (California Native Plant Society 2020).

Study sites

Only two *Sgn* populations persist in the wild (Mayer et al. 1994). The larger of the two occurs at the Old St. Hilary's preserve, a site named for the church built in 1888 at what is now the preserve's southern boundary. This preserve is 50 ha in size, but only 16 ha is serpentine grassland, i.e., suitable habitat for *Sgn*. The preserve is actively managed by Marin County Parks for the protection of this and other rare

plants that occur there. The plant community is composed primarily of native perennial grasses; exotic annual grasses are sparse in the grassland and largely confined to the edge of the fire road that bisects the preserve.

The smaller of the two contemporary populations occurs at a site that we dubbed Middle Ridge although it has no official name. The Middle Ridge site is much smaller (20 ha, of which 3 ha is serpentine grassland) and supports a population of *Sgn* that is ~ 25–30% the size of the population at Old St. Hilary's. The *Sgn* plants at the Middle Ridge site persist in the highly disturbed habitat along the edge of a fire road and on a steep, unstable scree slope that is inaccessible to people and dogs. The serpentine grassland is heavily invaded by exotic annual grasses and *Sgn* is largely absent from there. This site is not actively managed for the protection of this or other endangered plants that occur there (with the exception of a volunteer from the California Native Plant Society who hand pulls invasive grasses along the edge of the fire road where they co-occur with *Sgn*). Both preserves are surrounded by housing and are moderately trafficked by day hikers.

Seed bank

To assess the seed bank, we collected 150 soil cores from Old St. Hilary's and 75 from Middle Ridge in mid-April of 2016, 2017 and 2018, months after germination for the year had ceased and weeks before the aboveground plants had begun producing seed. Soil cores measured 10 cm in diameter and 5 cm deep. We randomly located 100 m long transects (five at Old St. Hilary's and three at Middle Ridge) and collected a soil sample every 3 m. Each sample was sieved to remove large rocks. Pots (15 × 15 cm) were filled with a mix of vermiculite, sand and potting soil and the sieved field soil was spread in a thin layer (~ 1 cm deep) on top of this mixture. Although *Sgn* is restricted to serpentine soils in the field, it grows readily in potting soil. Pots were set outside in a common garden at Mills College in Oakland, California, 25 km from the field site and in the same climatic zone. Pots were protected from herbivores and field soil was added in October. *Sgn* may germinate in the field as early as mid- to late October if there is sufficient rainfall, and germination is largely complete by late December (Swope, *in preparation*). Pots were watered every other day and monitored for emergence by all species.

Reconstructing the historic range and the timing of fragmentation

There is no documentation describing the extent of *Sgn*'s distribution on the Tiburon Peninsula at the time of European-American settlement. One of our goals was to determine its distribution, which would in turn determine whether the historic specimens could reasonably be treated as a single population or if *Sgn* has always been restricted to small, isolated populations and thus the historic specimens should not be pooled. Of the 46 herbarium specimens in existence, 31 list the locality as "Tiburon," possibly referring the slopes immediately above the small cluster of buildings that would become the town of Tiburon, or possibly referring more broadly to the whole peninsula. The remaining 15 specimens cite proximity to the Old St. Hilary's Church as the locality. We attempted to reconstruct the historic range on the peninsula using historic photographs (housed at The Belvedere-Tiburon Landmarks Society) and soil maps (https://websoilsurvey.nrcs.usda.gov) to identify suitable habitat and barriers to dispersal. We also used the historic photographs to determine when and how rapidly the population was fragmented by housing development.

Sampling

Contemporary plants: aboveground plants and the seed bank

Sgn plants were sampled in 2017 from the two remaining wild populations. Whole fresh leaves (~ 2 cm long) were collected in mid-April when plants were large but had not yet flowered. Leaf tissue was taken from 50 individuals at Old St. Hilary's and 30 individuals at Middle Ridge. Leaves from *Sgn* plants that emerged from the seed bank were also collected in mid-April of each year. Of the plants that emerged from the seed bank, we genotyped ten from each of the two sites. Leaf tissue was stored in separate envelopes, in the dark, at room temperature with silica beads (PolyLam-Products, Williamsville, NY) and extracted one week after collection.

Historic plants

We examined all 47 herbarium specimens of *Sgn* listed in the California Consortium of Herbaria. One specimen was collected 330 km south of the species' range and is likely misidentified (mostly likely *S. glandulosus* subsp. *glandulosus*) and so was excluded from this study. The remaining 46 plants were collected from the Tiburon Peninsula between 1886 and 1974. We were given permission to sample from 22 of these specimens, collected between 1902 and 1974. Whole dried leaves were removed from 21 of these specimens; one plant had no leaves so we used a pedicle. We used new gloves and sterilized forceps to remove one leaf (or pedicle) from each plant and forceps were sterilized with bleach between each use. Each leaf was stored in its own sterilized tube until DNA extraction.

DNA extraction

We placed < 0.25 g of dried leaf tissue in 300 µL of a 10% Chelex solution (Chelex 100, Bio-Rad Laboratories, Hercules, California). We vortexed samples for 10 s and spun them for 10 s to ensure that plant material was in the solution, and then incubated them at 65° C for 10 min. After incubation, we vortexed the samples again for 10 s and centrifuged them for 10 s to separate contaminants and Chelex beads from the DNA in the supernatant. We diluted the supernatant from the Chelex extraction 1:1 with diH_2O.

The risk of contamination of historic samples is high (Pääbo et al. 2004) so we took precautions to reduce this risk and ensure that our results were accurate (Wandeler et al. 2007). Historic DNA was extracted in a lab in which no *Streptanthus* species had entered. We used new pipettes and filter tips for the extractions. All equipment, instrumentation and working surfaces were sterilized with UV radiation and clean lab coats were donned between each sample. Additionally, we extracted a maximum of two samples each session. Replicate extractions were performed for the nine historic samples for which we had enough tissue for additional extractions. We performed one negative control for every third extraction.

Microsatellite amplification and scoring

We used 14 microsatellite loci developed for *Sgn* (Swope et al. 2019), all of which yielded PCR products < 220 bp in size and thus were more likely than longer sequences to yield useable results from potentially degraded *h*DNA (Särkinen et al. 2012). All of these loci are known to be polymorphic in contemporary populations and so were deemed suitable for a population genetics study.

PCR conditions were optimized using a using a Bio-Rad T100 thermocycler. Amplification reactions were performed in a final volume of 25.5µL containing approximately 2 ng of DNA, 12.5 µL of Q5 High-Fidelity DNA Polymerase (New England BioLabs), 1 µL of miliQ water, and 0.5 µM of each forward and reverse primer. The PCR program consisted of one cycle of denaturation at 98 °C for 30 s; followed by 34 cycles at 98 °C for 40 s; 60 °C for 30 s, 72 °C for 20 s, and an extension phase at 72 °C for one min. Forward primers were fluorescently labeled at the 5′ end with HEX or FAM (Eurofins). PCR product was diluted (1:30–1:75) and run with 0.2 µL of GeneScan LIZ-labeled internal size standard and 9 µL of HiDi formamide (Applied Biosystems) and analyzed using an Applied Biosystems 3730XL DNA Analyzer.

Peaks were scored manually using PeakScanner 2.0 (Thermo-Fisher Scientific). If peaks could not be clearly scored due to stutter, small peaks, or other ambiguities, PCR was re-run for that individual × primer pair. If peaks were still ambiguous, we did not record a value for that individual at that locus. We duplicated PCR and fragment analysis for 20% of the contemporary samples (randomly selected) for quality control and for all 22 historic samples to check for inconsistent results due to DNA degradation. Microsatellites from both contemporary populations and the historic samples were checked for null alleles and large allele drop out using Micro-Checker 2.2.3 (van Oosterhout et al. 2004).

Genetic diversity

Historic photographs and soil maps indicate that *Sgn* likely formed a single, large population (see "Results": *Reconstructing the historic range*), so we treated all of the herbarium specimens as a single population. We treated the two contemporary populations as separate populations given their discrete geographic boundaries. We quantified genetic diversity for each population using the number of alleles per locus (Na), the number of private alleles (Np), the number of effective alleles (Ne), observed and expected heterozygosities (H_o and H_e), fixation indices (F_{IS}), and deviations from Hardy–Weinberg equilibrium (GenAlEx 6.5, Peakall and Smouse 2006, 2012) and Nei's unbiased estimated of diversity (uH) (Fstat 2.9.3.2, Goudet 1995). Because the number of alleles per locus is sensitive to sample size, we also calculated allelic richness (AR) rarified to the smallest sample size (Fstat 2.9.3.2, Goudet 1995). We used a Wilcoxon signed rank test to look for differences between the populations. We used the same measures of genetic diversity to compare the plants from the seed bank to the aboveground plants at the site from which the soil was collected.

Effective population size

We conducted a single-sample estimate of effective population sizes (N_e) for both contemporary populations (excluding the plants that emerged from the seed bank) and the historic population using a bias-corrected linkage disequilibrium method (Waples and Do 2008) and a molecular co-ancestry method (Nomura 2008; Do et al. 2014). For the linkage disequilibrium model, we set the threshold for rare alleles (P_{crit}) to 0.05. Ninety-five percent confidence intervals were generated using the jackknife procedure. The estimates of N_e employed here assume discrete generations. Given that *Sgn* is an annual species with a minimal seed bank (see "Results"), the two contemporary populations likely comply with this assumption. The historic population, which consists of individuals collected over a 65-year timespan, does not. The violation of this assumption should result in an N_e value that is higher than the true value (Jorde and Ryman 1995).

We calculated the *M* statistic (Garza and Williamson 2001) to determine if any observed bottlenecks were due to recent or historic population declines. The *M* statistic is

calculated by dividing the number of alleles (k) by the range in allele sizes (r). The logic underlying this calculation is that immediately following a population contraction, alleles will be lost (k will decline), but that the range of alleles sizes (r) will decline only when the smallest or largest alleles are lost. Given that rare alleles are lost before common alleles, and that the smallest and largest alleles are not often rare, r will decline more slowly than k, leading to a low M ratio immediately following population contraction.

Interpopulation differences and gene flow between contemporary populations

We assessed population structure in two ways. First, we measured the degree of genetic differentiation (F_{ST}) between the historic population and each contemporary population as well as between the two contemporary populations using Genepop (Weir and Cockerham 1984; Rousset 2008). Additionally, we used STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003), a non-spatial Bayesian clustering algorithm, to infer the likely number of populations and to assign individuals to a source population. For this analysis, we did not assign individuals to a population a priori, assumed correlated allele frequencies, used individual α values for each population, and set the initial value of α to 0.33, or $1/K$ where K is the assumed number of populations (Wang 2017). The initial burn-in period was 100,000 followed by 200,000 Markov Chain Monte Carlo (MCMC) replicates. We performed 10 replicate runs for each value of K from one to five. The likely number of populations was determined using the ad hoc ΔK method (Evanno et al. 2005) as implemented by STRUCTURE HARVESTER (Earl and vonHoldt 2012).

We used two methods to estimate the effective number of migrants per generation between the two contemporary populations, the F_{ST} method in which N_m is indirectly estimated ($N_m = [(1/F_{ST}) - 1]/4$) (Slatkin 1985, 1987) and the private allele method (GENEPOP 4.7.5; Rousset 2008).

Results

Reconstructing the historic range and timing of fragmentation

Photographs show that prior to housing development, the peninsula was continuous grassland along the south- and west-facing slopes of the peninsula and included both contemporary populations (Fig. 2). There is no evidence of natural or human-made features that would act as a physical barrier and prevent *Sgn* from occupying the length of the peninsula. Further, a band of serpentine soil runs the length of the peninsula (https://websoilsurvey.nrcs.usda.gov; Fig. 1). Based on photographs and soil maps, we conclude

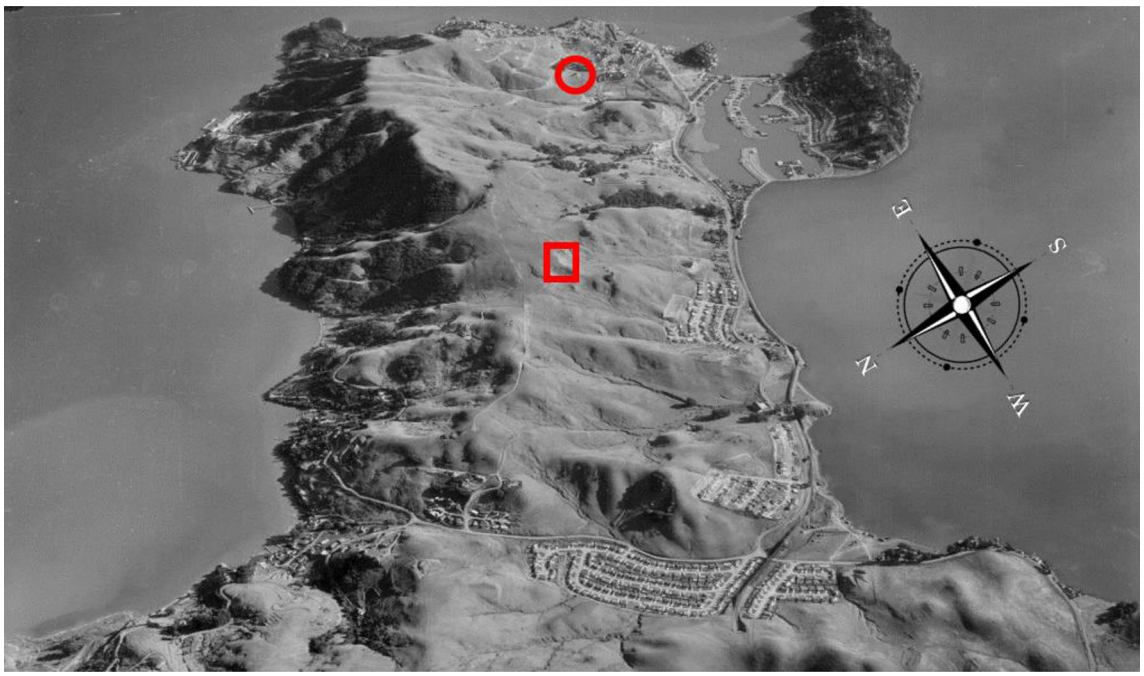

Fig. 2 Aerial photograph of the Tiburon Peninsula taken in 1954 showing continuous grassland on southwest-facing slopes. The red circle near the tip of the peninsula is the location of the Old St. Hilary's church, built in 1888, which marks the southern edge of modern-day Old St. Hilary's preserve (curtesy of the Belvedere-Tiburon Landmarks Society). The red box marks the location of the present-day Middle Ridge Preserve. The two preserves are 1.5 km apart

that *Sgn* almost certainly formed a single, large, continuous population throughout the serpentine band and that contained within its boundaries both of the populations that persist today (Fig. 1). This conclusion is corroborated by anecdotal reports from landowners who recall *Sgn* growing on their property at the time they built their houses in the late 1960s.

Photographs show that as of 1962 housing development was confined to the shoreline and no buildings had yet encroached up the hillsides to the band of serpentine soil where *Sgn* grows (Fig. 3a). A photograph taken in 1964 shows two houses in the band of serpentine. One decade later (1974), dense housing is present from the shoreline to the ridgeline and flanks both sides of the Old St. Hilary's preserve (Fig. 3b). By 2005, housing is even denser and spans the entire peninsula (Fig. 3c). From this, we conclude that fragmentation of the once-continuous population began in 1964 and was largely complete within a single decade.

Amplification

Nine of the 14 loci amplified consistently from the historical samples and so were retained for analysis. Replicate extractions and PCR combined with negative controls indicate there was no contamination during extraction or amplification. Twenty of the 22 historic samples (collected between 1909 and 1974) extracted successfully (ESM Table 1). Of those 20, we were able to genotype 13 individuals at all nine loci. Of the seven historic samples for which amplification was not fully successful even after additional rounds of PCR, we were able to score eight of the nine loci for five of the individuals and seven of the nine loci for two individuals. All 20 individuals were retained for analysis. Forty-seven of the 50 contemporary plants sampled at Old St. Hilary's and 29 of the 30 plants sampled from Middle Ridge extracted and amplified successfully at all loci. All 20 plants that we sampled from the seed bank were successfully genotyped at all loci. All samples chosen for replicate extraction and PCR were identical to the original. There was no evidence of null alleles, large allele drop out or linkage disequilibrium.

Genetic diversity

The larger Old St. Hilary's population has retained the genetic diversity that was present historically, but genetic diversity in the smaller Middle Ridge population was significantly lower than the historic population by all measures. There were no significant differences in allelic richness (AR), the number of effective alleles (Ne), H_E and uh between the historic population and the Old St. Hilary's population (all p values > 0.37; Table 1). In contrast, AR was 27% lower ($Z = -2.380$, $p = 0.017$), Ne was 8% lower ($Z = -2.429$, $p = 0.015$) and H_e was 21% lower ($Z = -2.429$, $p = 0.015$) in the Middle Ridge population compared to the historic population. Fixation indices (F_{IS}) ranged from 0.087 in the Middle Ridge population to 0.186 in the historic population (Table 1). All populations showed significant deviations from Hardy–Weinberg equilibrium in some loci with a deficiency of H_o relative to H_E in almost every case (ESM Table 2). Pairwise F_{ST} values indicate that both the Old St. Hilary's population ($F_{ST} = 0.155$, $p = 0.001$) and the Middle Ridge population ($F_{ST} = 0.146$, $p = 0.001$) have diverged from the historic population.

The seed bank had lower diversity and much higher inbreeding coefficients than the aboveground population at each site. Over three years of collecting soil samples, > 4000 grass and 200 forb seedlings (excluding *Sgn*) emerged from our soil samples but only 12 *Sgn* plants emerged from the soil collected at Old St. Hilary's and 13 emerged from the soil collected at Middle Ridge (Table 2). The seed bank harbored no novel alleles and AR (Old St. Hilary's: $Z = -2.667$, $p = 0.008$, Middle Ridge: $Z = -2.1$, $p = 0.03$), Ne (Old St. Hilary's: $Z = -2.55$, $p = 0.01$, Middle Ridge: $Z = -2.073$, $p = 0.038$) and H_e (Old St. Hilary's: $Z = -2.547$, $p = 0.01$, Middle Ridge: $Z = -2.429$, $p = 0.015$) were all lower in the seed bank than they were in the aboveground plant population at both sites (Table 3). The fixation indices were higher for both seed banks (Old St. Hilary's: $F_{IS} = 0.681$, Middle Ridge $F_{IS} = 0.313$) than they were for the aboveground plant populations at the same site (Table 3).

Effective population size

We estimated effective size of the aboveground plant populations using two different single-sample methods. Both the linkage disequilibrium (LD) method and the molecular coancestry (MC) method estimated the effective population size for the historic population and the Old St. Hilary's population to be infinitely large. The estimated N_e for the Middle Ridge population was considerably lower. The LD method estimated N_e for the Middle Ridge population to be 16.3 (± 95% CI 2.7–infinite) and the MC method estimated it to be even lower (4.8 ± 95% CI 1.2–10.9). The *M* ratios indicate that all populations, including the historic one, have experienced recent and severe bottlenecks. The Old St. Hilary's population had an *M* ratio of 0.28 (95% CI 0.154–0.406) and the Middle Ridge population had an *M* ratio of 0.33 (95% CI 0.224–0.438). Interestingly, the historic population had an *M* ratio of 0.29 (95% CI 0.169–0.403), well below the 0.68 threshold identified by Garza and Williamson (2001) as indicating a recent bottleneck.

Interpopulation differences and gene flow between contemporary populations

There was a trend in which mean values for all measures of genetic diversity were lower in the Middle Ridge population

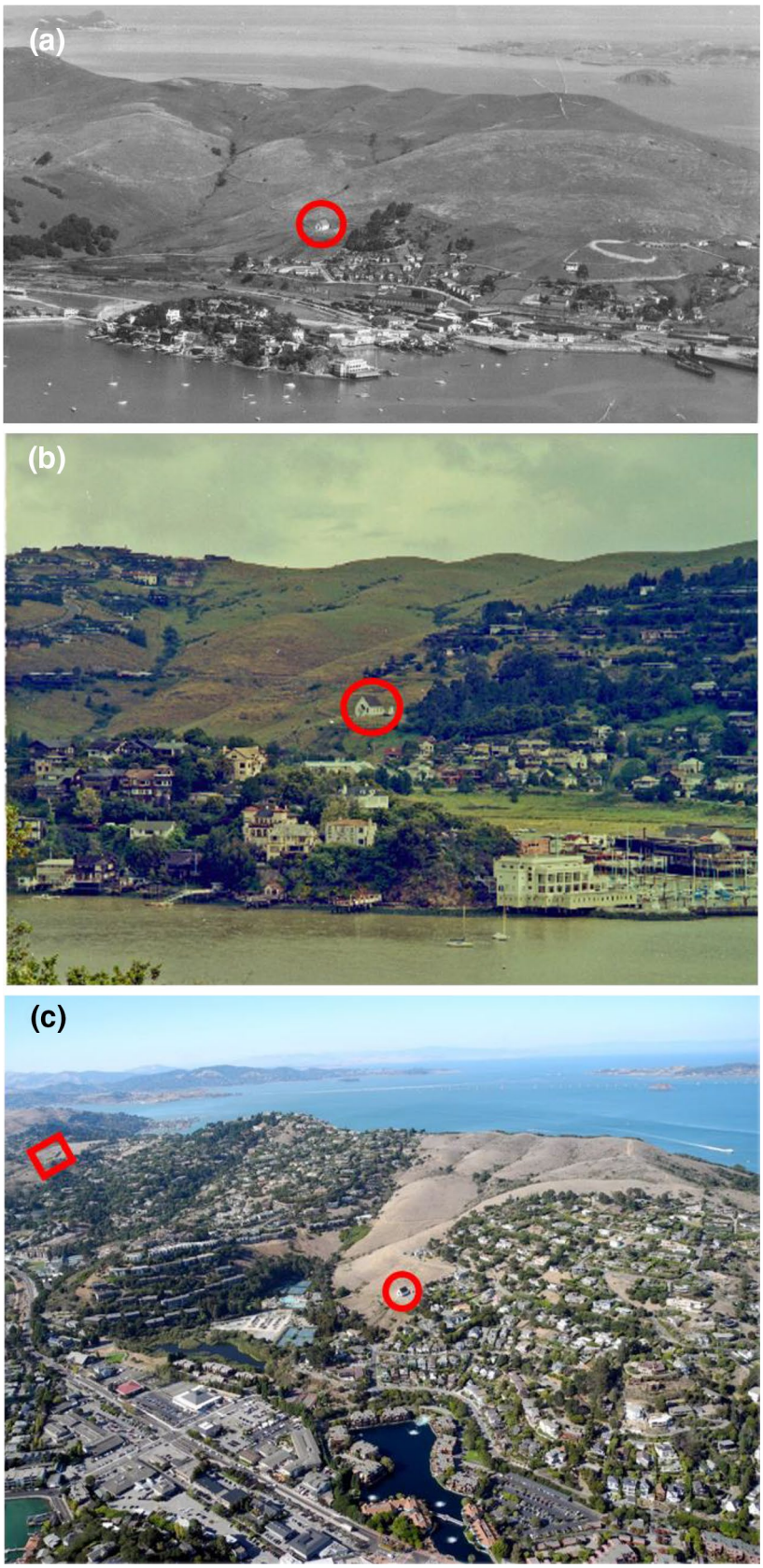

Fig. 3 Aerial photographs taken prior to and following housing development that fragmented the *Streptanthus glandulosus* subsp. *niger* population: (**a**) Taken in 1947 and shows that none of the development had yet encroached into the band of serpentine soil that runs the length of the peninsula (visible as a band of lighter-colored soil mid-slope), (**b**) Taken in 1974 and shows extensive housing development from the coast to the ridgeline. The housing also marks the boundaries of the wedge-shaped Old St. Hilary's preserve, home to the larger of the two extant populations, (**c**) Taken in 2005 and shows even denser housing development. The Old St. Hilary's church is circled in red in all three plates for geographic reference; a red box notes to the Middle Ridge population in (**c**) (the Middle Ridge population is out of frame in (**a**) and (**b**). Photos curtesy of the Belvedere-Tiburon Landmarks Society

Table 1 Summary of genetic diversity in the two contemporary populations of *Streptanthus glandulosus* subsp. *niger* and herbarium specimens over all nine loci

Population	N	Na	Ne	Uh	AR	H_o	H_E	F_{IS}
Historic	20	5.111 (0.512)	2.923[a] (0.404)	0.621[a] (0.053)	4.994[a] (0.499)	0.476 (0.066)	0.604[a] (0.052)	0.186 (0.106)
Old St. Hilary's	47	5.89 (0.564)	2.392[a,b] (0.357)	0.531[a,b] (0.050)	4.61[a(b)] (0.412)	0.437 (0.065)	0.525[a,b] (0.050)	0.184 (0.079)
Middle Ridge	29	4.222 (0.465)	2.108[b] (0.230)	0.488[b] (0.055)	3.660[b] (0.303)	0.441 (0.089)	0.480[b] (0.054)	0.08 (0.131)

Mean (±1 SE)

Different lower case letters indicate significant differences based on Wilcoxon signed rank test. a(b) = the difference between the two contemporary populations was marginally significant (p = 0.05)

N number of individuals sampled, *Na* alleles per locus, *Ne* effective alleles per locus, *uh* unbiased estimator of diversity, *AR* allelic richness, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} Fixation index

Table 2 Number of seedlings that emerged from soil samples collected from the Old St. Hilary's and Middle Ridge sites

Site	Year collected	No. of soil samples	No. of grass seedlings emerged	No. of forb (not *Sgn*) seedlings emerged	No. of *Sgn* seedlings emerged
OSH	2016	150	756 (96)	80 (47)	11 (4)
OSH	2017	150	1887 (110)	31 (23)	1 (1)
OSH	2018	150	691 (90)	23 (20)	0 (n.a.)
MR	2016	75	51 (18)	34 (13)	13 (11)
MR	2017	75	177 (51)	13 (9)	0 (n.a.)
MR	2018	75	441 (33)	21 (14)	0 (n.a.)

The number in parentheses following the number of seedlings is the number of soil samples that contained seedlings for each life form (grass, non-*Sgn* forb, *Sgn*). Fewer soil cores were collected at the Middle Ridge site because the area where *Sgn* grows there is a fraction of the size of the area where *Sgn* grows at the Old St. Hilary's site

Site: *OSH* Old St. Hilary's preserve, *MR* Middle Ridge preserve

Table 3 Summary of genetic diversity in the two contemporary populations of *Streptanthus glandulosus* subsp. *niger* and plants grown from the seed bank at each site. Mean (±1 SE)

Population	N	Na	Ne	uh	AR	H_o	H_E	F_{IS}
Old St. Hilary's	47	5.89 (0.564)	2.392[a] (0.357)	0.531 (0.050)	3.99[a] (0.356)	0.437 (0.065)	0.526[a] (0.050)	0.184[a] (0.079)
Old St. Hilary's seed bank	10	2.667 (0.236)	1.964[b] (0.204)	0.468 (0.059)	2.667[b] (0.236)	0.289 (0.063)	0.468[b] (0.059)	0.681[a] (0.138)
Middle Ridge	29	4.222 (0.465)	2.108[a] (0.230)	0.488 (0.055)	3.21[a] (0.228)	0.441 (0.089)	0.480[a] (0.054)	0.08[a] (0.131)
Middle Ridge seed bank	10	2.333 (0.333)	1.715[b] (0.26)	0.348 (0.083)	2.33[b] (0.33)	0.078 (0.036)	0.331[b] (0.079)	0.313[a] (0.15)

Different lower case letters indicate significant differences (p < 0.001) based on Wilcoxon signed rank test between the aboveground population and the seed bank plants; sites analyzed separately

N number of individuals sampled, *Na* alleles per locus, *Ne* effective alleles per locus, *uh* unbiased estimator of diversity, *AR* allelic richness, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} fixation index

compared to the Old St. Hilary's population but the differences were not significant (Table 1). The pairwise F_{ST} value for the two contemporary populations revealed a moderate degree of differentiation (F_{ST} = 0.119, p = 0.001). After ten replicate runs of STRUCTURE at each value of *K* from one to five, the Ln(likelihood) method identified three distinct clusters (*K* = 3), in which the historic population formed its own genetic cluster as did each of the contemporary populations (along with their respective seed banks) (Fig. 4a, c). The Δ*K* method identified two clusters (*K* = 2), in which the historic population and the Middle Ridge population (and its seed bank) formed one genetic cluster and the Old St. Hilary's population (and its seed bank) formed another (Fig. 4b, d).

The larger Old St. Hilary's population had 25 private alleles across all nine loci while the smaller Middle Ridge population had 10 private alleles across four loci (ESM Table 3). All 10 private alleles in the Middle Ridge population were rare or uncommon (1.7–5.2% frequency) but several of the private alleles in the Old St. Hilary's

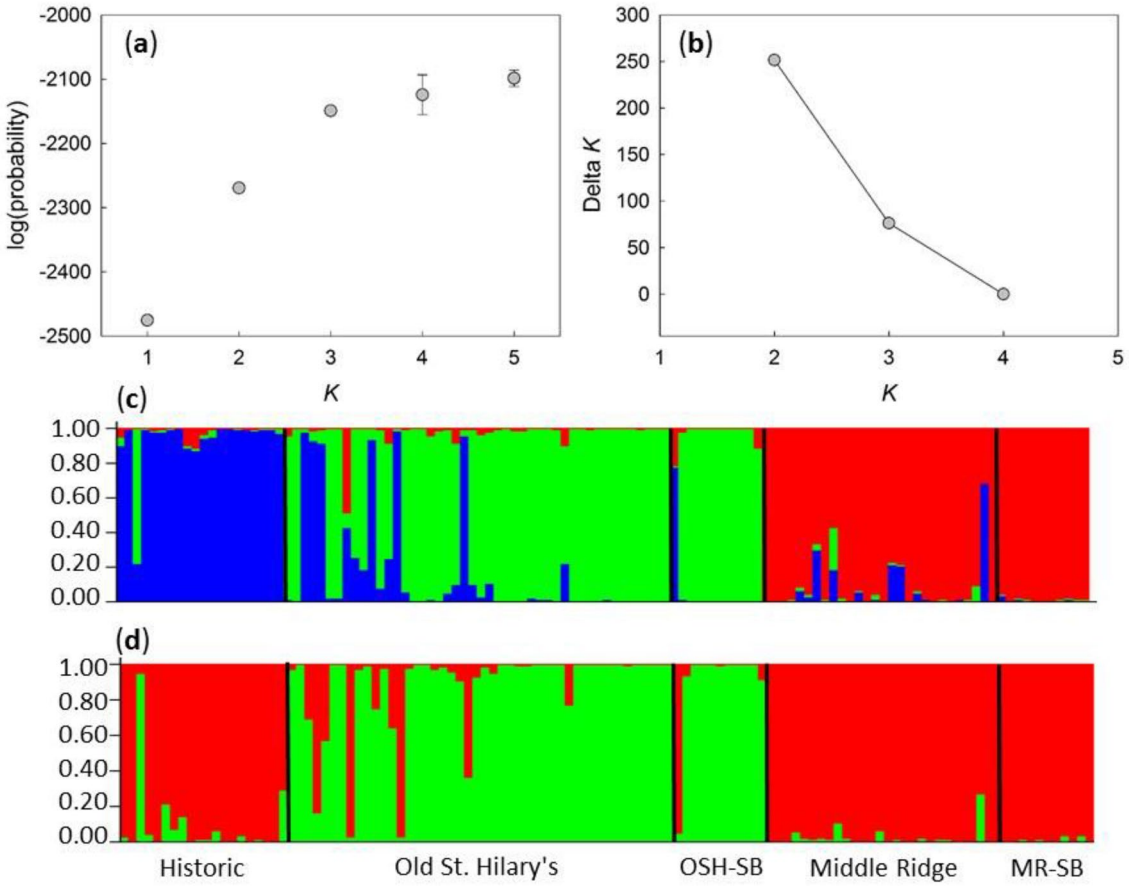

Fig. 4 Estimation of the number of genetic populations: (**a**) means (± standard deviation) of $L(K)$ for $K = 1–5$ based on 10 replicate estimates, (**b**) plot of ΔK for $K = 1–5$ (Evanno et al. 2005), (**c**) bar-plot showing genetically defined populations as determined by $L(K)$ for $K = 3$. (**d**) bar-plot showing genetically defined populations as determined by ΔK for $K = 2$. Bars represent cluster membership coefficient (Q) for individual plants. Labels below the bar plot indicate the source for each sample: historic plants from the herbarium collected between 1909 and 1974, and each of the two contemporary populations (Old St. Hilary's and Middle Ridge). OSH-SB = plants grown from the seed bank at Old St. Hilary's; MR-SB = plants grown from the seed bank at Middle Ridge

population were present in high frequencies (10.6–35.1%; ESM Table 3). Using the F_{ST} method, the mean number of migrants between the two contemporary populations was 3.65. Using the private alleles method, the number of effective migrants was 1.58.

Discussion

Reconstructing the historic range and the timing of fragmentation

Of the many written records and botanical collections from Marin County prior to development of this area, none has documented *Sgn* outside of the Tiburon Peninsula, strongly suggesting that this highly restricted geographic range is natural and not the result of anthropogenic range contraction. Ours is the first attempt to describe its distribution within the peninsula. Historic photos and soil maps lead us to conclude that *Sgn* once formed a single large, continuous, and presumably panmictic population spanning the entire 4.5 km length of the peninsula as recently as 50 years ago (Figs. 1, 2). Assuming that plant density in the past was similar to what it is today, the historic population contained orders of magnitude more plants than the two extant populations combined. The STRUCTURE analysis reveals that the historic samples, which were collected over a 65-year period (1909–1974), belong to a single genetic cluster (Fig. 4c, d), consistent with a large, panmictic population as we hypothesize existed prior to housing development.

The once-continuous historic population has been fragmented into two contemporary populations with discrete geographic boundaries that are separated from one another by 1.5 km of dense housing. Habitat loss and fragmentation and the subsequent population declines were both recent (beginning in 1964) and abrupt (largely complete

by 1974; Fig. 3a, c). Our highly unbalanced sample sizes from the putative populations reduces our ability to infer the actual population structure using these ad hoc methods (Wang 2017) thus, caution is warranted when interpreting these results. Nevertheless, the two contemporary populations, along with their seed banks, form genetic clusters that are distinct from each other (regardless of which statistic one relies on; Fig. 4c, d) and this structure appears to have emerged over just half a century.

Changes in genetic diversity since fragmentation

Even in the historic population, allelic richness (AR) was low relative to other plants with populations of similar size, an annual life cycle and a mixed mating system (cf. Moeller et al. 2011). We suspect that low AR and the low *M* ratio in the historic population are the result of frequent droughts over long periods of time that drive population crashes and genetic bottlenecks. The population at Old St. Hilary's appears to be large enough to have retained the genetic diversity that was present historically but the downward trend in all measures may suggest that genetic diversity is declining, albeit slowly, and the consequences of fragmentation have yet to be fully expressed (Aguilar et al. 2008). Nevertheless this population has differentiated substantially (Frankham et al. 2002) from the historic population ($F_{ST}=0.155$, Fig. 4c, d), largely due to changes in allele frequencies.

The smaller Middle Ridge population has suffered significant losses across all measures of genetic diversity. Allelic richness has declined by 27% and heterozygosity by nearly 21% compared to the historic population. This magnitude of decline is greater than that seen in the Seychelles warbler, which was reduced to a single population of fewer than 30 birds (Spurgin et al. 2014), and in New Zealand's mohua which has experienced precipitous declines in both the number and size of populations (Tracy and Jamieson 2011). The Middle Ridge population showed a moderate (bordering on substantial) degree of differentiation from the historic population ($F_{ST}=0.146$). Perhaps more worrisome than the declines in AR and H_e are the estimates of effective population size (N_e), which were very small (4.8 or 16.3 depending on the method used), especially relative to its census population size, which can be ~ 1000 plants in years of favorable growing conditions. Surprisingly, the N_e for the Middle Ridge population is similar to the N_e for *Caulanthus amplexicaulis* var. *barbarae* (mean $N_e=3.8$–10.1), a closely related, annual mustard with mean census population size of fewer than two dozen individuals (Burrell et al. 2019).

Genetic diversity (AR, Ne) was low in the historic population but N_e was infinite, consistent with our conclusion that *Sgn* once formed a single, panmictic population comprised of substantially more plants than exist today. Even though the overlapping generations in our historic sample means that our estimate of N_e is inflated (Jorde and Ryman 1995), it is likely that the true historic N_e is very high and almost certainly higher than the traditionally accepted threshold of 500 (Franklin 1980; Lande and Barrowclough 1987) or the revised threshold of 1000 (Frankham et al. 2014) required to maintain adaptive potential in the long-term. The high N_e in the historic population also suggests that inbreeding is unlikely to have purged the population of deleterious alleles in the past and as a consequence, the very small N_e in the Middle Ridge population may be cause for concern.

Both contemporary populations have suffered substantial bottlenecks in the recent past (Old St. Hilary's *M* ratio = 0.28, Middle Ridge *M* ratio = 0.33). This is consistent with the sudden and severe population contraction that began with housing development. It is also consistent with frequent drought-driven population crashes. Interestingly, the *M* ratio for the historic population was also very low (*M* ratio = 0.29). This might indicate that the population was already in steep decline when the historic specimens were collected (1909–1974), well before housing development resulted in habitat loss. However, in the absence of any evidence of anthropogenic disturbance before 1964, it seems more plausible that it is the result of repeated bottlenecks following frequent droughts, which have been feature of California's climate for more than a millennium (Griffin and Anchukaitis 2014).

Seed bank

Studies that consider the aboveground population alone risk missing genetic diversity harbored in dormant seeds in the soil. Large and persistent seed banks can buffer a population against extinction in the short-term by increasing recruitment when favorable growing conditions return after a period of poor performance (e.g., Kalisz and McPeek 1992; Stöcklin and Fischer 1999). Seed banks can also increase effective population size, sometimes substantially. Hanin et al. (2013) found that the seed bank increased N_e of *Eruca sativa* populations by 32% and Lundemo et al. (2009) found that the N_e of *Arabidopsis thaliana* populations were on average four times higher when the seed bank was included. McCue and Holtsford (1998) found that genetic diversity was higher in the seed bank than in the aboveground plant population, and that population differentiation (F_{ST}) was lower. The role of the seed bank in maintaining genetic diversity might be especially important for plants facing the dual threats of fragmentation, which can reduce genetic diversity, and climate change, which requires genetic diversity to adapt.

Nothing is known about whether *Sgn* seeds are capable of dormancy and thus accumulating as a seed bank. However, given that seed banks are common (Leck et al. 1989) and that *Sgn* is has an annual life cycle and grows in an

area with a variable climate, two traits that are often associated with seed banks (Cohen, 1966; Kalisz and McPeek 1993), it is reasonable to suspect that it might. The seed bank for *Sgn* appears to be very small and patchily distributed across the landscape. It is possible that more *Sgn* seed persists from year to year in the field but we failed to detect it because plants occasionally grow in rock crevices and other microsites that are too rocky to be cored. However, is also possible that repeated droughts have diminished the seed bank. Droughts in coastal California often follow a pattern in which most of the precipitation occurs in the autumn (http://ipm.ucanr.edu/WEATHER/wxmap.html), leading to high rates of germination and seedling establishment (Swope, *in preparation*). The wet autumn months are then followed by dry winter and spring months, leading to high mortality of those seedlings. This precipitation pattern can rapidly deplete the seed bank even if seeds are physiologically capable of remaining dormant for years.

Any conclusions about the genetic diversity of the seed bank should be drawn with appropriate caution given that we were able to sample only ten plants from the seed bank at each site (Hale et al. 2012) at nine loci (Landguth et al. 2012). Nevertheless, the *Sgn* seed bank appears to be less genetically diverse than the aboveground plant population and showed homozygote excess at almost every locus. The inbreeding coefficients for the seed bank at both sites were very high (Old St. Hilary's $F_{IS} = 0.681$, Middle Ridge $F_{IS} = 0.313$). If the higher level of homozygosity in the seeds that remain in the soil is the result of inbreeding (either selfing or mating between relatives), those seeds may have failed to germinate due to inbreeding depression (Vitalis et al. 2004), a possibility we are currently testing. While it is possible that these results are an artifact of our very small sample size, we have yet to uncover evidence that *Sgn*'s seed bank is large enough or diverse enough to reduce the risk of extinction through demographic or genetic rescue.

Interpopulation differences

Both the STRUCTURE analysis (Fig. 3c, d) and our estimate of F_{ST} ($F_{ST} = 0.119$) reveal that the extant populations have differentiated from one another, perhaps to a greater degree than we might expect given that they have been separated from one another for fewer than 50 generations. Although *Sgn*'s primary pollinators (*Bombus* spp.) are capable of traversing the 1.5 km that separates the two populations, these results suggest that migration between them is insufficient to balance drift. The erosion of genetic diversity in the Middle Ridge population is pronounced. In addition to reductions in AR and H_E, there was a large number of private alleles in the Old St. Hilary's population (25 over all nine loci) and not all of these alleles were rare. In fact, some of the alleles that are missing from the Middle Ridge population have frequencies that range from 10 to 35% in the Old St. Hilary's population (ESM Table 3).

Two (non-mutually exclusive) processes could drive the observed genetic structure in the extant populations. One is random genetic drift, which is likely to be neutral with respect to fitness or may even be maladaptive. The other is adaptation to different environmental conditions. Distinguishing between the two is crucially important for conservation as the former scenario might argue for artificial gene flow while in the latter scenario, population structure ought to be conserved.

Conservation implications

Arguably, the two greatest threats to species today are habitat loss and climate change. *Sgn* has experienced precipitous declines both in total population size and the amount of suitable habitat that remains. The southwest-facing serpentine grasslands to which *Sgn* is restricted covered 170 ha of the peninsula (https://websoilsurvey.nrcs.usda.gov) prior to housing development but today only two patches, 16 and 3 ha in size, remain. Together these patches represent ~ 11% of the suitable habitat that was present only half a century ago. *Sgn* excels at tolerating stressful serpentine soil but is a poor competitor (Cacho and Strauss 2014; Sianta and Kay 2019) and as a result, invasion by exotic species further contributes to habitat loss at Middle Ridge. The loss of habitat due to housing development and invasion means that population size will be permanently constrained. A pressing question for *Sgn* is whether these small populations retain enough genetic diversity to adapt to a rapidly changing climate.

Drought has an adverse effect on both individual- and population- level performance in *Sgn*. *Sgn* is an annual and both germination and survival to flowering are sensitive to annual precipitation. As a result, flowering plant density and population growth rate vary dramatically (> five-fold) from dry to wet years (Swope, *in preparation*). Climate change models predict an increase in the frequency of inter-annual precipitation extremes as anthropogenic climate change accelerates (Pierce et al. 2012; Kirtman et al. 2013; Deser et al. 2014; Berg and Hall 2015). Deeper and/or more frequent droughts could lead to more frequent and/or more severe bottlenecks. To the degree that declines in neutral diversity reflect declines in diversity of traits under selection, bottlenecks may limit the populations' capacity to adapt to a changing climate, a possibility we are currently testing.

Small geographic ranges and narrow habitat breadths, like that exhibited by *Sgn*, greatly increase the risk of extinction even in the absence of anthropogenic disturbance (Harnik et al. 2012). Fortunately, it appears that much of the genetic diversity that was present prior to fragmentation is still present across the taxon but is subdivided between the two contemporary populations. Low genetic diversity probably does

not currently contribute to the risk of extinction for the larger population at Old St. Hilary's, but it may for the smaller population at Middle Ridge. An N_e of 50–100 is required to avoid inbreeding depression (Soulé 1980, Frankham et al. 2014) and an N_e of at least 500 (Franklin 1980, Lande and Barrowclough 1987) but possibly as high as 1000 (Frankham et al. 2014) is generally accepted to be the minimum required to maintain evolutionary potential over the long term. The N_e for the population at Middle Ridge is well below even the lowest of these values. Schwartz et al. (2007) argue that declines in genetic diversity may serve as an early warning signal that a population is nearing (or has crossed) a critical threshold and this ought to elicit a management intervention. We currently do not have any evidence that the present-day population structure is the result of local adaptation, making the risk of outbreeding depression low (Frankham et al. 2011) relative to the risk of inbreeding depression. Given this, intervening to counteract the loss of genetic diversity by moving seed (or pollen) from the larger, more diverse population to the smaller, less diverse population may carry few risks and offer potentially large benefits (Frankham 2015). However, we offer one word of caution before any such intervention is implemented. Recruitment of new plants is strongly seed limited at both sites (Swope, *in preparation*) and removing enough seed from Old St. Hilary's to bolster the population at Middle Ridge must be done with care so as not have the unintended consequence of destabilizing the larger, better protected population.

Genetic diversity is one of the three components of biodiversity (Noss 1990) and its conservation is one of the pillars of the IUCN's global conservation plan (McNeely et al. 1990). To our knowledge, ours is the first study to combine data from herbarium specimens, contemporary plants, and plants from the seed bank to describe genetic diversity in a rare plant. As more studies make use of herbarium specimens, especially in combination with plants from the seed bank, we will be better able to infer whether the genetic diversity we see today is the product of evolutionary forces acting over long time spans or human activity. This, in turn, will guide us towards more effective conservation plans for rare plants that appropriately incorporate population genetics.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10592-021-01406-6.

Acknowledgements We are especially grateful to the following herbaria for allowing us to destructively sample specimens: University and Jepson Herbaria (University of California, Berkeley), Hoover Herbarium (California Polytechnic State University, San Luis Obispo), J.M. Tucker and Beecher Crampton Herbaria (University of California, Davis), Southwest Environmental Information Network (Arizona), Harvard University Herbaria (Harvard University, Cambridge), Rancho Santa Ana Botanic Garden (Claremont, California), and the California Academy of Sciences Herbarium (San Francisco). Two anonymous reviewers offered thoughtful comments that improved this manuscript. Hannah Horten and Brittany Burnett assisted with lab work. We thank Dave Gotz and the Tiburon Landmark Society for access to historic photos. We thank Sam Abercrombie for creating the maps. This work was supported by a grant from Marin County Parks (14-6300-62) to SMS and Jill Barrett Foundation fellowships to TYS and NRKA. This work was conducted under a permit from the California Department of Fish and Wildlife (permit SEMP 2081(a)-14-004-RP) issued to SMS.

Author contributions SMS conceived of the study, collected leaf tissue in the field, acquired herbarium specimens and historical photographs, scored PCR results, analyzed data, conducted the literature search, wrote the manuscript. TYS and NRKA conducted the lab work, provided comments on drafts of the manuscripts, assisted with the literature search.

Funding This work was supported by a grant from Marin County Parks (CA, USA) (award #: 14-6300-62) to SMS and Jill Barrett Foundation fellowships (through the Mills College Biology Dept.) to TYS and NRKA.

Data Availability Data for this study will be available in the Dryad Digital Repository upon final manuscript acceptance. Prior to that, data are available from the corresponding author upon reasonable request.

Code availability Not applicable. All data were analyzed using widely available and clearly cited freeware (GelAlEx, FSTAT, GENEPOP).

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

Ethics approval Ethics approval not required. This work was conducted under a permit from the California Department of Fish and Wildlife (permit SEMP 2081(a)-14-004-RP) issued to SMS.

Consent to participate Not applicable.

Consent for publication All authors and relevant institutions (Mills College, Marin County Parks) give consent for publication of this manuscript.

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