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RESEARCH ARTICLE

Population genetics, demographic and evolutionary history of the Dudley's lousewort (*Pedicularis dudleyi*), a rare redwood forest specialist

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Abstract
Pedicularis dudleyi (Dudley's Lousewort, Orobanchaceae) is an extremely rare plant endemic to the redwood forests of Central California. Until recently, the species was known only from three extant natural populations. However, in 2019, one of those populations was described as a novel species (*P. rigginsiae* D.J. Keil) based on morphological and ecological data leaving only two populations described as *P. dudleyi*. While little is known about the past distribution of the species, historical records have led to speculation that the species was once more widespread and may have suffered from habitat destruction as a result of widespread logging during the early twentieth century. We utilized a combination of ddRAD SNP and Sanger sequencing data to: (1) Test the morphological hypothesis that *P. rigginsiae* is distinct from *P. dudleyi*; (2) Describe the genetic diversity and population structure of *P. dudleyi* and; (3) Test the hypothesis that the species underwent a bottleneck corresponding with increased logging of redwood forests in the early twentieth century. Our results support the recognition of *P. rigginsiae* as distinct from *P. dudleyi*, increasing the conservation priority of both species. Genetic diversity statistics and analyses of genetic structure suggest that both populations of *P. dudleyi* are highly differentiated from each other with one population exhibiting unexpected substructure. Finally, demographic modeling supports a scenario where the contemporary rarity of the species is explained by a recent bottleneck.

Keywords *Pedicularis dudleyi* · Rare plants · Redwood specialist · ddRAD seq · Bottleneck · Demographic history

Introduction

Rare species with small population sizes are at high risk for extinction due to environmental and demographic stochasticity and genetic drift. These forces can lead to a loss of allelic diversity and increased homozygosity (Barrett and Kohn 1991; Ellstrand and Elam 1993), which can increase the risk of inbreeding depression, reduce survival and fecundity, and limit the ability of species to adapt to changing environmental conditions (Booy et al. 2000; Oostermeijer et al. 1994, 1996; Spielman et al. 2004; Reed and Frankham 2003). Still, rarity can arise through a variety of evolutionary, ecological, and demographic processes, each of which are likely to influence the extent to which species are at risk for negative outcomes associated with low genetic diversity (Habel and Schmitt 2012).

Previously widespread species can become rare due to environmental change. For example, anthropogenic factors such as habitat loss, introduction of novel pathogens, and competition with invasive species have all contributed to recent species declines (Anagnostakis 1987; Tilman et al 1994; Rizzo and Garbelotto 2003; Bellard et al. 2016). In many of these cases, initial differentiation among populations is low and genetic diversity within is high. Upon reduction of population size, these species are expected to suffer from inbreeding depression and stochastic shifts leading to the loss of adaptive alleles (Frankham 2005; Habel and Schmitt 2012).

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Alternatively, species may be innately or historically rare, maintaining small effective population sizes for much of their existence. This may be due to specialization on restricted or patchy habitats (Brown 1984; Gaston and Lawton 1990), a combination of metapopulation dynamics and life history traits (i.e., low dispersal ability or breeding system) that may increase the probability of local extinction and reduce recolonization (Hanski 1982; Gaston and Lawton 1990; Hanski and Gyllenberg 1993), or evolutionary history, where recently evolved species may not have had time to expand their range (Stebbins 1979). These species often exhibit high differentiation among, and low genetic diversity within populations and are able to persist over time as such. Over successive generations, small populations are expected to purge the majority of their deleterious alleles such that further reductions in population size may not lead to inbreeding depression (Crnokrak and Barrett 2002; Taylor and Jamieson 2008; Habel et al. 2009). As a result, rare species with historically low levels of genetic diversity may be less susceptible to negative effects associated with loss of genetic diversity than rare species that were more recently widespread (Frankham et al. 2001; Crnokrak and Barrett 2002; Habel and Schmitt 2012). While many conservation genetic studies of rare or threatened plant species focus on quantifying the distribution of genetic diversity and gene flow within and among populations, insight into the ecological, demographic and evolutionary processes that underlie rarity can complement traditional conservation genetic studies and inform effective management and restoration programs (Frankham 2005; Maschinski and Albrecht 2017).

Pedicularis dudleyi Elmer (Dudley's Lousewort, Orobanchaceae) is endemic to the redwood forests of Central California (Fig. 1A and B) and is state listed as "rare" (CDFW 2021) under the California Endangered Species Act (CESA) (California Fish and Game Code §§2050, et seq.) with a California Native Plant Society Rare Plant Rank of 1B.1 (seriously threatened) (CNPS 2021). Until recently, the species was known from three extant populations: one occurring in and around Portola Redwoods State Park in San Mateo County, the second along the North Fork Little Sur River in Monterey County and the third in San Luis Obispo County (Fig. 1A). However, in 2019, the southern-most population was described as a novel species, *P. rigginsiae* D.J. Keil (Arroyo de la Cruz lousewort), based on morphological and ecological data (Keil 2019). While little is known about the past distribution of the species, the existence of herbarium specimens from locations between the two extant populations has led to speculation that it was once more widespread and may have suffered from habitat destruction and fragmentation in the early twentieth century when redwood forests were heavily logged (Gujral et al. In Press).

Pedicularis dudleyi is hemiparasitic and is thought to form haustorial connections with multiple host species including *Vaccinium ovatum*, *Notholithocarpus densiflorus* and *Ceanothus thyrsiflorus* (Sprague 1959, 1962). The species is primarily outcrossing and is chiefly pollinated by two native bees, *Bombus sitkensis* and *Bombus edwardsii* (Gujral et al. In Press). Seeds are relatively large (ca. 1–1.5 mm × 2–3 mm) and experience passive dispersal in areas immediately surrounding the maternal plants. However, the wasp *Vespula alascensi* is a frequent visitor to *P. dudleyi* fruiting stalks and may be an important vector for longer distance seed dispersal (Gujral et al. In Press).

Population sizes at the two known populations, Portola Redwoods State Park and the North Fork of the Little Sur River, are small. A 2019 census of the species at Portola Redwoods found 468 reproductive adults (Gujral et al. In Press) scattered around the campgrounds and visitor's center with up to a few hundred more potentially existing in satellite populations on surrounding private land. A 2020 census of the species along the Little Sur River counted approximately 200 reproductive adults (Hauser unpublished data) distributed linearly along the river and likely represent the full extent of the population in that area. The species is perennial and individuals reach reproductive maturity after 5–6 years and can live for up to 50 years. Long-term surveys suggest

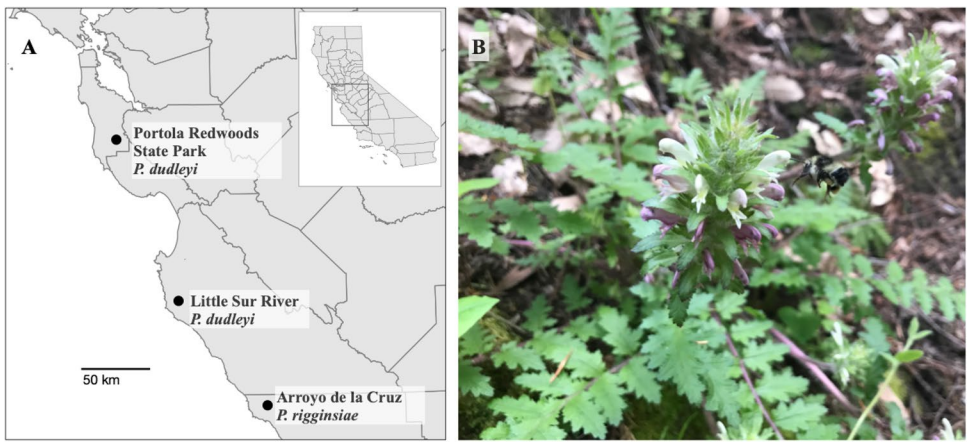

Fig. 1 **A** Map with county delimitations for localities of the two known *P. dudleyi* populations and one known *P. rigginsiae* population. **B** Photo of *P. dudleyi*. Photo credit: Benjamin Carter

that populations may be declining, and recent research has found that low germination and seedling establishment may be the biggest barriers to population growth (Gujral et al. In Press). To date there have been no studies investigating population genetic diversity or differentiation within the species.

We utilized a combination of ddRAD SNP and Sanger sequencing data to: (1) Test the morphological hypothesis that *P. rigginsiae* is distinct from *P. dudleyi*; (2) Describe the genetic diversity and population structure of *P. dudleyi* and; (3) Test the hypothesis that the species underwent a bottleneck corresponding with increased logging of redwood forests in the early twentieth century.

Methods

Sample collection

We sampled 146 *Pedicularis dudleyi* individuals from the two known populations ($n = 75$, Portola Redwoods; and $n = 71$, Little Sur River, North Branch; Figs. 1 and 2) in the summer of 2018 under California Department of Fish and Wildlife permit 2081(a)-19–004-RP. We sampled individuals that were at least 2 m apart to avoid obtaining multiple samples from the same genet. Approximately 2 cm^2 of leaf material was collected per plant and dried and stored on silica gel. Voucher specimens were deposited in the Carl W. Sharsmith Herbarium, San Jose State University (SJSU) and the Robert F. Hoover Herbarium at California Polytechnic State University, San Luis Obispo (OBI). *Pedicularis rigginsiae* leaf material was obtained from two herbarium specimens at the Hoover Herbarium, including one from an Isotype. Due to the rarity of the species we have omitted coordinates for individual plants.

Phylogenetic inference

Genomic DNA was extracted from the leaf material using a Qiagen DNeasy Plant Mini Kit (Valencia, CA, USA). To test the morphological and ecological hypothesis that *P. rigginsiae* is distinct from *P. dudleyi* we generated nuclear ribosomal internal transcribed spacer (ITS) and matK-5'trnK spacer sequences for *P. dudleyi* ($N = 5$) and *P. rigginsiae* ($N = 2$) to reconstruct the phylogeny. ITS was sequenced in two parts, ITS1 and ITS2 using the primer pairs described in Daly and Fine (2011). The matK-5'trnK spacer was amplified using the matK5 and matK6 primers described in Shaw et al. (2005). Polymerase chain reactions were carried out in 25 µl reactions containing approximately 25 ng genomic DNA, 1.5 µM each of forward and reverse primer, 12.5 µl Kapa Hifi HotStart ReadyMix (Roche Sequencing Solutions, Indianapolis, IN, USA) and water. PCR thermocycling conditions for both ITS 1 and 2 consisted of an initial denaturation step at 95 °C for 2:30 m followed by 34 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s and a final extension step of 72 °C for 10 m. Thermocycling conditions for matK-5'trnK spacer consisted of an initial denaturation at 94 °C for 3 m followed by 40 cycles of 94 °C for 30 s, 48 °C for 40 s, and 72 °C for 1 m with a final extension at 72 °C for 10 min. PCR products were visualized on a 1% TBE agarose gel and purified using SPRI beads prior to sequencing. PCR products were cycle sequenced in both directions using the original PCR primers on an ABI 3730 automated DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the UC Berkeley DNA Sequencing Facility. Geneious Pro 5.3.3 (Drummond et al. 2010) was used to analyze and edit chromatograms, and to assemble double-stranded consensus sequences. Only high quality, unambiguous reads were assembled. ITS and matK-5'trnK spacer sequences for closely related taxa as determined by previous phylogenetic analysis of the genus (Robart et al. 2015) were downloaded from GenBank and are listed in Table 1. All sequences for each locus were aligned with MAFFT 7 (Katoh and Standley 2013) using the L-INS-I strategy. Trees were constructed for each locus and the concatenated dataset, using RAxML v8.2.9 (Stamatakis 2014) with the "-f a" option, GTRGAMMA model and 100 bootstrap replicates. Alignment summary statistics were calculated using AMAS (Borowiec 2016).

Library preparation and sequencing

Libraries were constructed following a modified version of the protocol described in Peterson et al. (2012), where amplification was performed prior to size selection. Briefly, 500 – 150 ng of genomic DNA were digested using the restriction enzymes EcoRI and SphI-HF (New England Biolabs, Ipswich, MA, USA) following NEB guidelines. P1 and P2 "flex" adapters were ligated to the digested DNA and ligation products were combined into pools containing 10–24 samples. Each pool was dual indexed using PCR primers as described in Meyer and Kircher (2010). After amplification, indexed libraries were quantified using qPCR and then combined in equimolar ratios. The final library was size selected for 750 bp (including internal adapters) using tight mode on a Pippin Prep System (Sage Science, Beverly, MA, USA) at the Functional Genomics Laboratory at the University of California, Berkeley. The final Illumina library was sequenced by Novogene at the UC Davis Sequencing Center on a single lane of Illumina HiSeq X Ten (150 bp, paired end) (Illumina, Inc., San Diego, CA, USA).

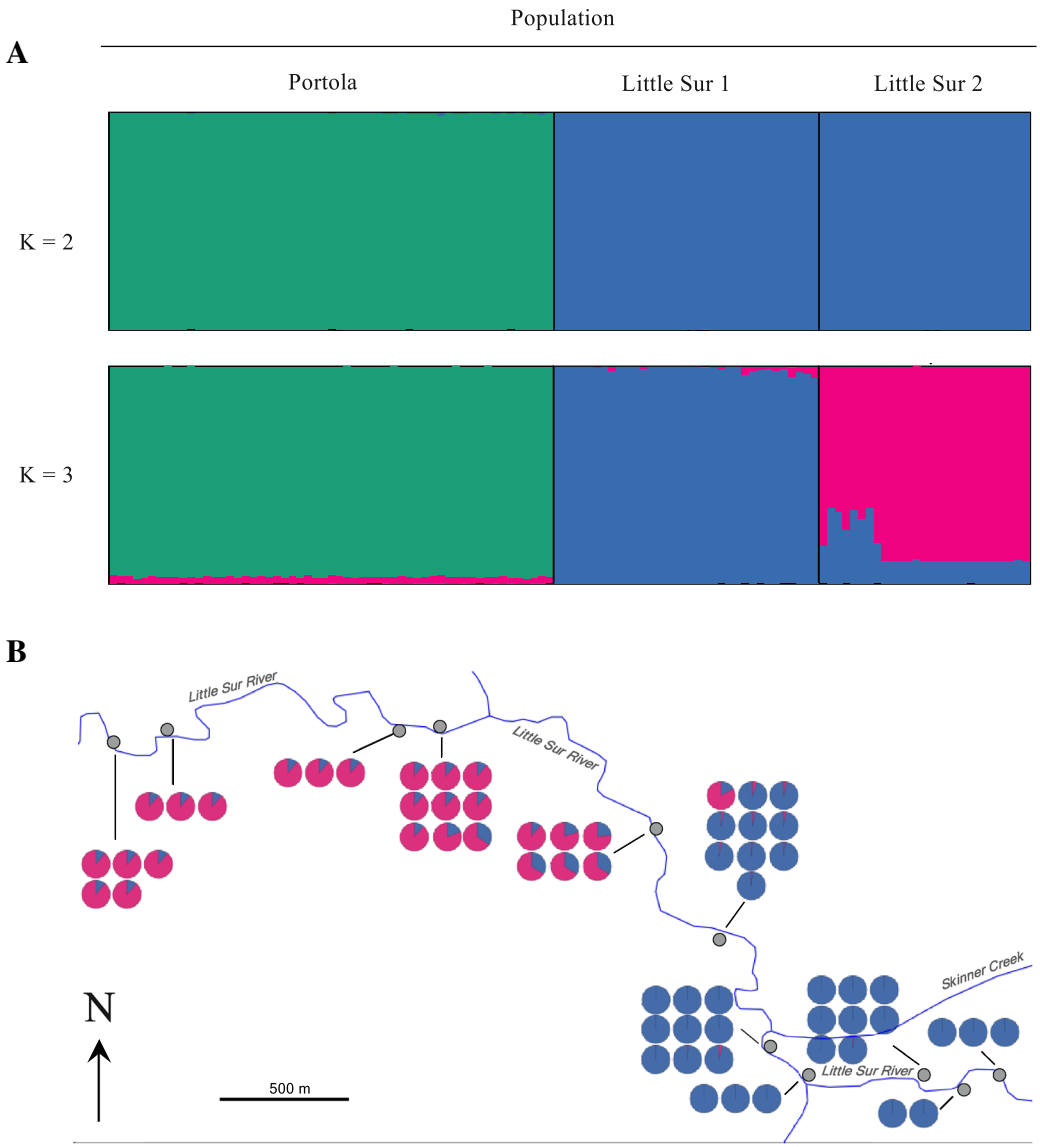

Fig. 2 **A** Evolutionary clusters (K = 2 and 3) inferred from STRUCTURE analysis of 118 *P. dudleyi* individuals from the two known populations. Each color represents an inferred character, and each individual is represented by a vertical line colored according to the probability of its assignment to a given population. **B** Map of individuals from the Little Sur Location. Gray points represent locations at which multiple individuals were sampled along the Little Sur River. Large circles represent individual plants collected at each location and are colored according to the proportion admixture inferred by STRUCTURE (K = 3). The Little Sur River flows from East to West

Data processing and SNP calling

Raw sequences were demultiplexed, quality filtered, and assembled using Stacks v2.54 (Rochette et al. 2019) at the University of Oklahoma Supercomputing Center for Education and Research (OSCER). Sequences were sorted and filtered using the *process_radtags* script. Clustering, assembly, and filtering parameters were optimized with a subset of 24 individuals from both study sites using the *r80* method as described in Paris et al. (2017). The following parameters were used to call the final dataset: minimum number of raw reads required to form a stack, m = 3; maximum number of mismatches between stacks within individuals, M = 1; maximum number of mismatches allowed between stacks among individuals *n* = 2; minimum percentage of individuals across populations required to process a locus, R = 0.75; minimum minor allele frequency, min-maf = 0.01; and maximum observed heterozygosity, max-obs-het = 0.7. All 146 individuals were used to create the locus catalog (*cstacks*) for the final Stacks analysis. Because SNPs found on the same

Table 1 Taxa and loci included in maximum likelihood phylogeny

Taxon	ID/Population	ITS GenBank Accession	matK -5'trnK GenBank Accession	ITS1	ITS2	matK-5'trnK
Pedicularis rigginsiae	Rig	MZ132210*	MZ442360*	Yes	No	Yes
P. rigginsiae	Rigi	MZ132211*	NA	Yes	No	No
P. dudleyi	60/Little Sur 2	MZ132216*	MZ442355*	No	Yes	Yes
P. dudleyi	11/Little Sur 1	MZ132215*	MZ442357*	Yes	No	Yes
P. dudleyi	E2/Portola	MZ132212*	MZ442359*	Yes	Yes	Yes
P. dudleyi	B5/Portola	MZ132213*	MZ442358*	Yes	Yes	Yes
P. dudleyi	M2/Portola	MZ132214*	MZ442356*	Yes	No	Yes
P. densiflora	NA	HG424112.1	HG423931.2	Yes	Yes	Yes
P. densiflora	NA	KJ735491.1	KJ749802.1	Yes	Yes	Yes
P. capitata	NA	KJ735487.1	HG423910.1	Yes	Yes	Yes
P. rainierensis	NA	KJ735505.1	KJ749816.1	Yes	Yes	Yes
P. bracteosa var. siifolia	NA	HM561872.1	HG423907.1	Yes	Yes	Yes
P. bracteosa var. canbyi	NA	HM561870.1	HG423904.1	Yes	Yes	Yes
P. howellii	NA	KJ735495.1	KJ749806.1	Yes	Yes	Yes
P. centranthera	NA	KJ735488.1	KJ749799.1	Yes	Yes	Yes
P. semibarbata	NA	HG424209.1	HG424027.1	Yes	Yes	Yes
P. furbishiae	NA	KJ735493.1	KJ749804.1	Yes	Yes	Yes
P. sceptrum-carolinum	NA	KR699635.1	AB280528.1	Yes	Yes	Yes
P. gloriosa	NA	AY949647.1	AB280946.1	Yes	Yes	Yes
P. iwatensis	NA	AY949654.1	AB280514.1	Yes	Yes	Yes
P. nipponica	NA	AY949663.1	AB280520.1	Yes	Yes	Yes
P. cystopteridifolia	NA	KJ735490.1	KJ749801.1	Yes	Yes	Yes
P. procera	NA	HM561877.1	KJ749819.1	Yes	Yes	Yes

*Indicates sequences generated as part of this study

locus are expected to be tightly linked (Rochette and Catchen 2017) we filtered our dataset to one SNP per locus using the *–write-random-snp* flag in the STACKS script *populations*. Our data was further refined using VCFtools v0.1.17 (Danecek et al. 2011) to remove individuals with > 30% missing data and loci sequenced at > 2 × the SD of coverage as they may represent paralogous loci. Finally, we used BayeScan v.2.1, which measures the dissonance between global and population level allele frequencies, to identify loci that may be under selection in each population. Prior odds for this analysis was set to 100. We used a burn-in of 50,000, thinning interval of 10, sample size of 50,000 and 20 pilot runs. Pilot run length was set to 50,000 and a false discovery rate of 0.05 was used (Foll and Gaggiotti 2008).

Population structure and differentiation

We sought to understand how populations are genetically structured using a combination of Bayesian clustering analysis and descriptive statistics. Population genetic structure was assessed using Bayesian Markov chain Monte Carlo clustering implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000). STRUCTURE was run using the admixture model and assuming correlated allele frequencies with a burn-in period of 500,000 generations followed by 10^5 Markov chain Monte Carlo generations for each value of K = 1–4. Simulations were repeated twenty times for each value of K. Structure Harvester (Earl and vonHoldt 2012) was used to interpret the output as described by Evanno et al. (2005) and Pritchard et al. (2000). Admixture proportions were averaged over all runs using CLUMPP (Jakobsson and Rosenberg 2007), and DISTRUCT 1.1 (Rosenberg 2004) was used to visualize the final matrix. Principal components analysis (PCA) was carried out using the R package adegenet v2.1.1 (Jombart 2008) to identify differences in allele frequencies among populations and confirm our STRUCTURE results. Finally, genetic differentiation among all three populations was assessed using population-level pairwise comparisons of F_{ST} calculated in GenoDive v3.03 (Meirmans and Tienderen 2004) with confidence intervals being generated over 10^5 permutations.

Genetic diversity

Initial analysis of population structure suggested the presence of fine scale structuring in the Little Sur population

(Fig. 2). As a result, estimates of genetic diversity were carried out with individuals being assigned to one of three populations (Portola, Little Sur 1, and Little Sur 2). To characterize genetic diversity within populations, we calculated summary statistics including the number of private alleles, percentage of polymorphic loci, the average frequency of the major allele (P), the average observed heterozygosity (H_O), expected heterozygosity (H_E), and the average Wright's inbreeding coefficient (F_{IS}) for each population using the *populations* script in STACKS.

Population history

To test the hypothesis that *P. dudleyi* populations underwent a bottleneck in the early twentieth century we utilized an Approximate Bayesian Computation (ABC) approach implemented in DIYABC Random Forest v1.0 (Collin et al. 2021). Briefly, ABC implements model-based inference in a Bayesian setting to simulate coalescence of the observed populations given different demographic scenarios. Parameter values are drawn from prior distributions and calculated summary statistics. Simulated datasets are then compared to the observed dataset using the machine learning tool Random Forest (RF) (Breiman 2001) to determine the best supported model and estimate its posterior probability. Because *P. dudleyi* is monoecious, sex ratio was set to 1. Minimum allele frequency was set to 0.01. Three scenarios were considered to investigate whether populations of *P. dudleyi* underwent a bottleneck that corresponds with early twentieth century logging of the redwood forests. We chose the Little Sur 2 population to represent the ancestral population because it has significantly greater levels of genetic diversity than the Portola population (Table 2, Fig. 3). All demographic models included three populations (Portola, Little Sur 1, and Little Sur 2) where Portola and Little Sur initially diverge from the ancestral population followed by the subsequent divergence of Little Sur 1 and 2 (Fig. 3). In scenario 1 population sizes remain stable over time. In scenario 2 all populations undergo a bottleneck after the divergence of Little Sur 1 and 2. In scenario 3 a bottleneck occurs in all populations when Portola diverges from Little Sur but prior to the divergence of Little Sur 1 and 2 (Fig. 3).

Table 2 Summary statistics for each population averaged across loci that are polymorphic in at least one population

Population	*N*	*n*	Private	% Poly	P	H_O	H_E	F_{IS}
Portola	57	54.38	709	28.74	0.987	0.021	0.017	0.013
Little Sur 1	33	25.26	242	48.06	0.922	0.050	0.110	0.229
Little Sur 2	28	30.44	248	46.84	0.915	0.044	0.121	0.254

Statistics include the number of individuals from each population that were included in the final dataset (*N*), the average number of individuals genotyped at each locus (*n*), the number of private alleles (Private), the percentage of polymorphic loci (% Poly), the average frequency of the major allele (P), the average observed heterozygosity per locus (H_O), the average expected heterozygosity per locus (H_E), and the average Wright's inbreeding coefficient (F_{IS})

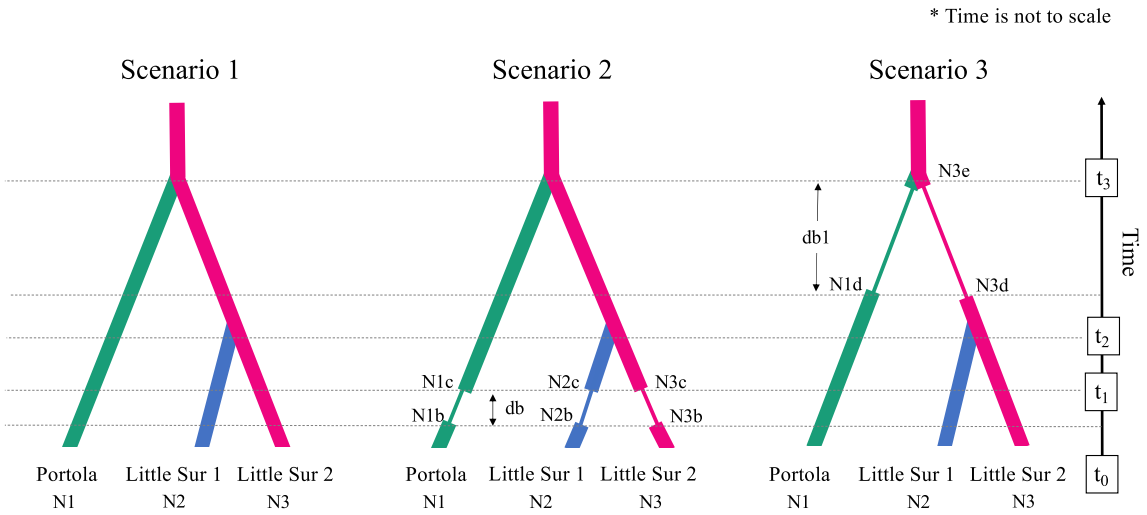

Fig. 3 Schematic representations of models tested for *P. dudleyi* demographic history using approximate Bayesian computation. N1, N2 and N3 represent the effective population sizes for Portola, Little Sur 1 and Little Sur 2 respectively, t represents time, and db represents bottleneck duration. Prior distributions and descriptions are listed in supplementary Table S2

Demographic and historical parameters are illustrated in Fig. 3 and include effective population sizes (N_1, N_2, and N_3 for populations Portola 1, Little Sur 1 and Little Sur 2 respectively) and time events at which divergence and/or bottleneck events occur (t_1, t_2, t_3). For scenarios that included a bottleneck, parameter db represents the duration of the bottleneck in generations. For time events DIYABC RF utilizes generation time as opposed to years. Thus, all priors for time events are in generations where one generation for *P. dudleyi* is equivalent to approximately 5 years (Gujral et al. In Press). All prior values were drawn from uniform distributions and are listed in Supplementary Table S2. Additional conditions were used to specify that $t_3 > t_2 > t_1$ and that N1c > N1b, N2c > N2b, N3c > N3b, N3e > N3d and N3e > N1d.

In our Random Forest analysis each scenario was considered separately. Prior values were drawn from the distributions described above and from a reference table computed using 1964 SNPs and 30^4 simulated data sets per scenario. All summary statistics as well as the optional axes of a linear discriminant analysis (LDA) were used in the RF analyses. Model choice is a two-step process where the best scenario is first chosen based on the number of classification votes and then followed by the calculation of the posterior probability for that scenario as well as global and local error rates. For model choice we utilized 5000 RF trees.

Independent RF analyses were conducted for each parameter of interest. Parameter values were estimated for all time events, bottleneck duration, and effective population sizes in our best supported scenario, scenario 3. The training set included 10^5 datasets and utilized 2000 RF trees. For each parameter, we inferred point estimates and computed global and local accuracy indices corresponding to global and local normalized mean absolute error (NMAE), which is the absolute difference between the point estimate and the true simulated value divided by the true simulated value, using out-of-bag estimators from 50,000 data randomly chosen from the training set.

Results

Phylogenetic inference

The complete datasets for the matK-5'trnK spacer gene contained sequences from 22 individuals and 849 characters of which 5.9% were variable and 3.3% were parsimony informative. The ITS data set (ITS 1 and 2 combined) contained sequences from 22 individuals and 618 characters, of which 27% were variable and 12.6% were parsimony-informative. Results of our ML analysis support the morphological and ecological hypothesis that *P. rigginsiae* is distinct from *P. dudleyi.* The maximum likelihood analyses for individual genes as well as the concatenated dataset all yielded similar topologies with the clade formed by *P. dudleyi*, *P. rigginsiae*, and *P. densiflora* highly supported as monophyletic. *Pedicularis dudleyi* and *P. rigginsiae* each formed their own clades with *P. rigginsiae* sister to *P. densiflora* (Fig. 4 and Figs. S1 and S2). No nucleotide variation was observed in either the ITS or matK-5'trnK spacer when comparing *P. dudleyi* individuals from the Little Sur and Portola populations.

Data processing

After all quality filters were implemented in the Stacks program *process_radtags*, 6,324,404 reads that were missing a

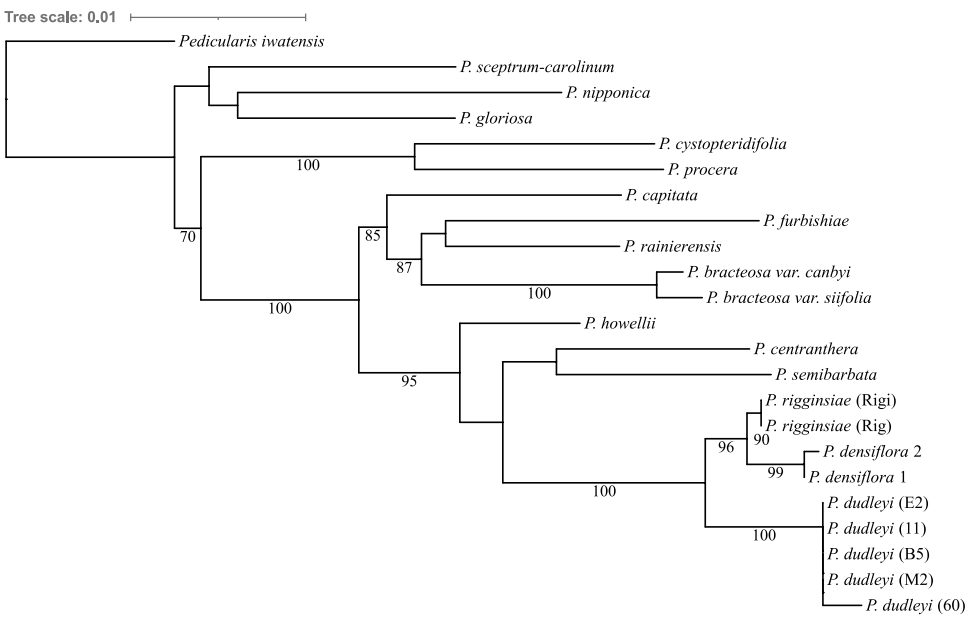

Fig. 4 Phylogeny of *Pedicularis* inferred from Maximum Likelihood (ML) methods using the concatenated nuclear ribosomal internal transcribed spacer (ITS) and the matK-5'trnK spacer. Sample ID for individuals sequenced as part of this study are included in parentheses following the taxon name. Values below the branches indicate ML bootstrap values ≥ 70

barcode, 7,396,631 reads which had no rad tag and 244,114 reads identified as low quality were discarded. A total of 1,030,346,369 reads from the initial 1,044,311,518 raw reads were retained, with an average of 6,291,033 reads per sample. Mean average coverage per locus after ustacks filters, was 52.6 × (± 49.3x). Twenty-eight individuals and 116 sites were subsequently dropped due to missing data and potential paralogy. Average coverage per locus for individuals included in the final dataset can be found in Supplementary Table S1. No loci were identified as being under selection. The final genetic marker dataset consisted 2,026 variant sites with no more than 25% missing data for 118 individuals.

Population structure and differentiation

Analysis of population structure uncovered unexpected structure within the Little Sur population and revealed near complete genetic differentiation between the Portola and Little Sur populations. Using the method of Pritchard et al. (2000), we found K = 3 to be best supported model where LnP(D) begins to asymptote (results not shown). Clustering for K = 2 separated the Portola and Little Sur populations while the identified clusters for K = 3 distinguish additional structure between upstream and downstream portions of the Little Sur population (Fig. 2A and B). Results from the PCA are also consistent with the result of K = 3 with PC1 representing 72% of the variation and accounting primarily for differentiation between Portola and the two Little Sur populations while PC2 accounts for 6% of the variation, primarily differentiating the two Little Sur populations from each other (Fig. 5). Pairwise F_{ST} values between Portola and the two genetic groups in the Little Sur population (Little Sur 1 and 2) were 0.903 and 0.891 respectively suggesting that the Portola and Little Sur populations share very few alleles. F_{ST} for the Little Sur populations was 0.348 which, while lower than F_{ST} values observed for the Portola population, still suggests significant population differentiation.

Genetic diversity

Genetic diversity across the two Little Sur populations were comparable for all indices and they exhibited greater overall genetic diversity compared to the Portola population (Table 2). The proportion of polymorphic loci ranged from 29% to 48%. Average major allele frequency (P) ranged from 0.987 for Portola to 0.915 for Little Sur 2. Expected heterozygosity, a measure of gene diversity, ranged from 0.017 to 0.121 and was significantly greater in both the Little Sur populations than the Portola population. The inbreeding coefficient, which measures the reduction in observed heterozygosity compared to expected heterozygosity, did not significantly differ from zero in Portola (F_{IS} = 0.013) but

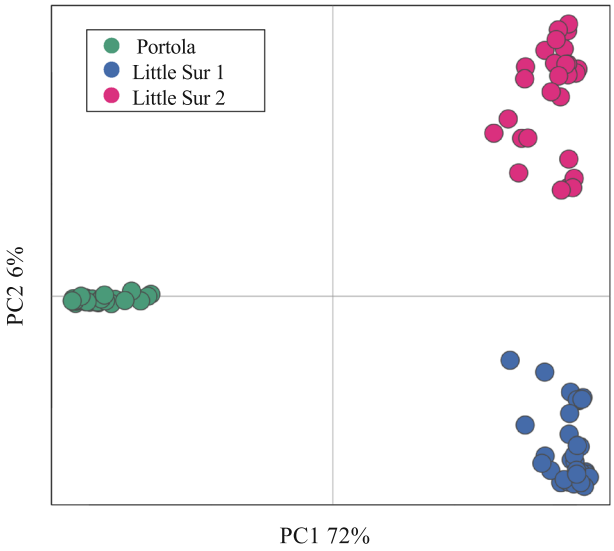

Fig. 5 Principal component (PC) plot of SNP data for PC1 and PC2. Each point represents a single individual from one of the three populations (Portola, Little Sur 1, or Little Sur 2)

was significantly greater than zero in the Little Sur populations (Little Sur 1 F_{IS} = 0.229 and Little Sur 2 F_{IS} = 0.254). Inbreeding values greater than zero indicate higher homozygosity often attributable to inbreeding, assortative mating or cryptic population structure (Nei 1987; Holsinger and Weir 2009).

Population history

Three demographic scenarios were tested. In scenario 1, the three population sizes remain constant over time; in scenario 2, bottlenecks occur in all three populations after Little Sur 1 and 2 have diverged and in scenario 3, bottlenecks occur in all populations beginning when Portola and the ancestral Little Sur population diverge (Fig. 3). Our observed data were located within the simulated dataset of the LDA projection suggesting that our priors and scenarios were compatible with the observed dataset (Fig. S3A). The best supported demographic scenario was scenario 2, which received the highest number of classification votes (99.9%) and greatest posterior probability (PP = 1.00; Table 3). Scenarios 1 and scenario 3 were not supported, with each receiving less than 0.1% of the classification votes (Table 3). Table 4 shows point estimates with 95% confidence intervals of posterior distributions as well as NMAE accuracy measures for parameters of interest corresponding to onset of the bottleneck (t_1), duration of bottleneck (db) and effective population size for the Portola, Little Sur 1 and Little Sur 2 populations (N1c, N2c and N3c) for scenario 2. The median estimate for the onset of the bottleneck was 39 generations

Table 3 Results for scenario choice which includes the number of votes for each of the three tested scenarios as well as the global error rate, local error rate and posterior probability for scenario three, the best supported scenario

Vote scenario 1	Vote scenario 2	Vote scenario 3	Posterior probability	Global error rate	Local error rate
3	4995	2	1.0	0.329	0.0

ago (95% CI 5–117 generations ago) and the median duration was estimated to be 100 generations (95% CI 8–192 generations). The median pre-bottleneck population sizes for Portola, Little Sur 1 and Little Sur 2 were 1747, 10,574, and 14,743 respectively (95% CI 940–5197; 2986–18,708 and 7586–19,672). Estimation for parameter db was less supported than other parameters as reflected by higher local and global NMAE values (Table 4). Additional output from our demographic analysis can be found in Figs. S2 and S3.

Discussion

Pedicularis dudleyi is a rare wildflower endemic to the redwood forests of Central California and is currently known from only two naturally occurring populations. Phylogenetic analysis strongly supports the morphological hypothesis that *P. rigginsiae* is distinct from *P. dudleyi* and is instead sister to the common, widespread, and closely related *P. densiflora*.

Our results provide evidence of strong genetic structuring between Portola and Little Sur but also unexpected population structure within Little Sur. Overall, populations exhibited low genetic diversity. The Portola population was significantly less genetically diverse than the populations from Little Sur while the Little Sur populations were found to have higher levels of inbreeding than Portola. Demographic analyses suggest that the rarity of *P. dudleyi* is likely to be the result of a recent bottleneck and could be the result of habitat destruction during the early twentieth century.

Results from our phylogenetic analysis support the classification of *P. rigginsiae* as distinct from *P. dudleyi. Pedicularis rigginsiae* is known from only one population on private land. While once considered a population of *P. dudleyi*, Keil (2019) recently described it as a separate species and hypothesized that it was more closely related to the common and widespread *P. densiflora. Pedicularis rigginsiae* differs from *P. dudleyi* in both habitat and morphology. While *P. dudleyi* occupies the Southern Coastal Redwood forests, P. *rigginsiae* is found in dwarfed maritime chaparral on coastal terraces. *Pedicularis rigginsiae* is morphologically distinguished from *P. dudleyi* by its narrower and more densely lobed leaves as well as differences in corolla shape and length (Keil 2019). Whether *P. dudleyi's* rarity is due to a young evolutionary age is equivocal. While the fact that *P. dudleyi* is sister to both the extremely rare *P. rigginsiae* and the very common and widespread *P. densiflora* suggests that the observed rarity is unlikely to be the result of young evolutionary age with insufficient time for range expansion, we cannot rule out a scenario where *P. dudleyi* evolved recently from an extinct sister species.

We found evidence of three distinct genetic clusters in *P. dudleyi.* One genetic cluster corresponds with individuals collected from Portola Redwoods. Individuals from this population showed no evidence of admixture with either Little Sur population (Fig. 2A) and F_{ST} values between Portola and Little Sur 1 and 2 were exceptionally high, suggesting that these populations have long been isolated and share very few alleles. Portola Redwoods State Park is separated from the Little Sur populations by nearly 110 km. Given that *P. dudleyi* is pollinated by native bumblebees and seeds are likely dispersed by a combination of gravity and wasps (Sprague 1959, 1962; Gujral et al. In Press) the lack of gene flow among these geographically distant populations is unsurprising. Moreover, small population sizes coupled with low genetic diversity and little to no gene flow makes these populations subject to high levels of genetic drift which is likely driving the genetic differentiation observed in this study (Allendorf 1986; Slatkin 1987).

More surprising was the genetic structure uncovered at Little Sur. The Little Sur population is distributed linearly along the North Fork of the Little Sur River, and the break

Table 4 Results for estimation of parameters of interest under scenario 2. Time estimates (t_1 and db) are reported in generations where one generation is equal to 5 years

Parameter	Posterior point estimates of			Global NMAE computed from		Local NMAE computed from	
	Mean	Median	95% credibility interval	Mean	Median	Mean	Median
t_1	46.4	39	5–117	0.64	0.58	1.21	0.93
db	100.2	100	8–192	2.04	2.06	2.29	2.16
N1c	2254	1747	940–5197	0.44	0.35	0.49	0.41
N2c	10,574	10,055	2986–18,708	0.89	0.91	0.60	0.59
N3c	14,743	15,573	7586–19,672	0.34	0.30	0.29	0.33

in genetic structure occurs about halfway along the population's distribution and shortly after the confluence of Skinner Creek with the Little Sur River. Geneflow appears to be directional to some extent with individuals immediately downstream from the genetic break exhibiting increased admixture with genetic contributions originating from the upstream population (Fig. 2B). F_{ST} between Little Sur 1 and Little Sur 2 was considerable (0.348) especially given that none of the plants sampled in this study are found more than 1 km from their nearest neighbor and bumblebees are known to forage over 1 km and up to 2.5 km from their nesting sites (Kreyer et al. 2004; Osborne et al. 2008; Hagen et al. 2011). One explanation could be that the two populations have been historically isolated along different parts of the river and are now coming into contact. Alternatively, there could be some indiscernible barrier to gene flow, although this seems unlikely given the close ecological and geographic proximity and lack of observable morphological differences between the two populations.

Overall estimates of genetic diversity for *P. dudleyi* populations were comparable to those found in other studies of rare and endangered plants employing ddRAD data. Estimations of H_E for populations of the California endangered salt marsh plant *Chloropyron maritimum ssp. maritimum,* which is also a hemiparasitic plant in the Orobanchaceae, ranged from 0.004 to 0.059 (Milano et al. 2020). H_E estimates for the Hawaiian lobelioids *Clermontia* and *Cyania* ranged from 0.127 to 0.138 (Jennings et al. 2016) and estimates for *Rhododendron cyanocarpum* ranged from 0.060 to 0.65 (Liu et al. 2020). While *P. dudleyi* populations from Portola and Little Sur both exhibit low levels of genetic diversity (0.017–0.121), the Portola population is significantly less genetically diverse than the Little Sur populations in spite of being larger. These differences could be explained by a combination of demography and life history. For example, a small number of progenitors may have given rise to the modern-day Portola population via a founder event. Given the long lifespan and low recruitment observed in the species today, a population arising from only a small number of individuals would be likely to remain small for a long period of time with little accumulation of new genetic diversity. The Little Sur populations on the other hand have substantially fewer individuals than the Portola population but significantly higher genetic diversity, suggesting that they may be the remnants of a once larger and more genetically diverse population. This hypothesis is supported by our demographic modeling results, where the estimate of pre-bottleneck effective population size for the Portola population is significantly smaller than the pre-bottleneck effective population size estimations for the Little Sur populations (Table 4).

Like all model-based methods, our demographic inferences cannot reveal the 'true' history of the species. Instead it allows us to choose the best scenario from among a necessarily limited set of options. Our results suggest that the current-day rarity of *P. dudleyi* can be attributed to a recent bottleneck that may correspond with the heavy logging of redwood forests in the early twentieth century. Given that heavy logging of the Central California redwood forests was most intense between 1850 and 1920 a bottleneck onset driven by logging would likely have begun between 101 and 171 years ago and lasted for approximately 70 years. These times fall well within the confidence intervals for our parameter estimations of the onset and duration of a bottleneck in our best supported scenario. Regardless of the exact timing or cause of the population decline, our data fail to support a hypothesis that *P. dudleyi* has simply persisted in small, naturally isolated populations over time and instead suggests that the observed rarity of the species is likely the result of a relatively recent bottleneck.

Conservation implications

Pedicularis dudleyi is at high risk for extinction from stochastic environmental and demographic events due to its extremely small population sizes. Currently, two potential threats are of concern. First, trampling and routine road and trail maintenance are potential issues as a large proportion of individuals are found along trails, roads and in campgrounds. Second, leaf litter buildup (presumably a result of fire suppression) may be negatively impacting seedling establishment, with the litter layer often exceeding 10 cm around established plants. Herbivory by deer has also been documented several times, including removal of flowering stalks, most leaves from a rosette or, in at least one case, removal of an entire plant with roots. Furthermore, *P. dudleyi* may not be able to withstand a changing climate and shifting habitats given the apparent low capacity for seed dispersal and establishment. This scenario is particularly concerning given predictions of near-term range contractions for redwood forests in Central CA as a result of climate change (Fernández et al. 2015). Both populations of *P. dudleyi* are small, strongly genetically differentiated from one another and likely to have their own site-specific adaptations. Conservation initiatives should not mix individuals from the Portola and Little Sur populations and should source seeds for seed banking initiatives from all three populations. The majority of *P. dudleyi* plants in the Portola region are found on public property at Portola Redwoods State Park and approximately one third of plants in the Little Sur region occur in Los Padres National Forest. While current landowners have been amenable to conservation efforts on their property, we recommend prioritizing permanent protection of private land along the upstream portion of the Little Sur River which houses the bulk of the Little Sur 1 population. Finally, given the extreme rarity of both *P. dudleyi* and *P.*

rigginsiae, we recommend that both species be considered for recognition under the Endangered Species Act.

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Author contributions TMM, CH and BEC designed the study. TMM gathered and analyzed the data and wrote the manuscript. CH and BEC provided guidance on the study and commented on all drafts of the manuscript.

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Declarations

Conflict of interest The authors have no conflict of interest.

Data availability Raw sequence data were deposited at the National Center for Biotechnology Information Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) database under the accession number BioProject ID PRJNA742774. Processed SNP data (vcf, genepop, STRUCTURE and DIYABC-RF formats) and the concatenated ITS and matK-5'trnK intron sequence alignment are available from DataDryad (https://doi.org/10.6078/D1S415). Genbank accession numbers for the ITS and matK-5'trnK intron are listed in Table 1.

Code availability Not Applicable.

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