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Genomic signatures of demographic declines in an imperiled amphibian inform conservation action

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Abstract

Catastrophic population declines due to disease often lead to fragmented remnant populations and loss of gene flow. Managers are left to determine appropriate conservation actions to recover and maintain population persistence. The recent utilization of genomic data to assist in species recovery now allows us to combine genome-wide surveys of differentiation and diversity with the identification of potentially adaptive regions to develop conservation plans that incorporate ecological and evolutionary processes. The unprecedented global decline of amphibian populations due to the pathogen *Batrachochytrium dendrobatidis* has increased the need to apply genomic tools to amphibian conservation practices. We show here how understanding genetic characteristics of remnant frog populations affected by disease can be applied directly to restoration efforts. Cascades frogs (*Rana cascadae*) occur in the mountainous regions of the Pacific northwestern United States and have declined dramatically at the southern edge of their range in California. We conducted genome-wide surveys within this region to inform conservation and reintroduction efforts. We found strong north-south genetic differentiation between Oregon and California and novel spatial structure within California. Genetic diversity was lower in California than Oregon and genetic drift was the most important driver of genetic diversity and population structure in California, making conservation efforts aimed at boosting overall genetic diversity most urgent. Spatial genetic structure of populations within California suggests that reintroductions to Lassen Volcanic National Park, where they were recently extirpated, should use remaining source populations south of the park. Our findings support the treatment of California's *R. cascadae* populations separately from the rest of their range and highlight the importance of conservation genomics in applied species conservation.

Introduction

Infectious diseases can catalyze population declines, accelerate extinctions, and threaten global biodiversity (Daszak, Cunningham, & Hyatt, 2000; Smith, Acevedo-Whitehouse, & Pedersen, 2009; Smith, Sax, & Lafferty, 2006). Pathogens have been implicated in declines of native Hawaiian birds (van Riper et al., 1986), Tasmanian devils (McCallum et al., 2007), and perhaps the greatest loss of biodiversity attributed to a disease—the global decline of amphibian species (Skerratt et al., 2007; Wake & Vredenburg, 2008; Scheele et al., 2019a; but see Lambert et al., 2020). While many species persist following disease epidemics, catastrophic population declines often leave affected populations vulnerable to extinction due to other forces (De Castro & Bolker, 2005; Gerber et al., 2005; Ginsberg, Mace, & Albon, 1995). Conservation biologists are then left to determine which management actions are necessary and how to implement them to maintain long-term population persistence (Gerber et al., 2018; Langwig et al., 2015).

Population declines often lead to fragmentation of remnant populations and loss of connectivity (i.e., gene flow), ultimately increasing probabilities of inbreeding and loss of genetic variation due to genetic drift (Gilpin & Soule, 1986). The loss of genetic diversity can play an additive role in the continuation of declines (Allentoft & O'Brien, 2010) and exacerbate the deterministic effects driving population extinction (e.g., disease; Fagan & Holmes, 2006; Spielman, Brook, & Frankham, 2004). Therefore, understanding the genetic parameters of residual populations post-decline provides

crucial information about extinction risk (Schwartz et al., 2007) and can improve the success of management actions that benefit from considering the genetic ancestries of populations, such as assisted migration/gene-flow efforts (i.e., translocation and reintroduction; Armstrong & Seddon, 2008; Rhodes & Latch, 2010). Conservation actions are now benefiting from the recent advances in genomic methods that have helped to reveal patterns of genome-wide variation and allowed managers to incorporate both neutral and adaptive diversity into conservation planning and decision-making (Allendorf, Hohenlohe, & Luikart, 2010; Flanagan et al., 2018; Funk et al., 2019).

While these advances in sequencing technology have been a boon for the management of some species (e.g., salmonid fisheries; Hohenlohe et al., 2011, 2013; Prince et al., 2017), researchers studying nongame aquatic taxa, such as amphibians, have underutilized genomic approaches compared to other taxa (Funk, Zamudio, & Crawford, 2018). With the unprecedented global decline of amphibian populations due to a fungal disease caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Monastersky, 2014; Wake & Vredenburg, 2008) integrating genomic datasets into amphibian conservation practices will be vital to species persistence. We show here how understanding genetic characteristics of remnant populations following declines due to disease can be applied directly to restoration efforts.

The Cascades frog (*Rana cascadae*) in northern California likely experienced peak declines due to Bd in the late 1970s and 1980s when enigmatic die-offs were observed in Lassen Volcanic National Park (LVNP; DeLeon, Vredenburg, & Piovia-scott, 2017; Fellers & Drost, 1993; Fellers et al., 2008). The species is now extirpated from the Park, which had been the species' core range within the southern Cascade mountains of California (Grinnell, Dixon, & Linsdale, 1930; Pope et al., 2014). Disjunct populations to the north and south of LVNP are also declining and the California Department of Fish and Wildlife is currently reviewing the species' status for listing on the state's endangered species list. Understanding the genetic parameters of these remaining populations will inform the urgency of conservation actions and the best source populations for reintroductions to LVNP.

To understand the extinction risks to residual populations within California and to evaluate reintroduction options for LVNP, we assessed genome-wide diversity and population structure of Cascades frog populations across California and southern Oregon. We based our hypotheses on preliminary research suggesting that *R. cascadae* in California are genetically differentiated from the rest of their range in Oregon and Washington (Monsen & Blouin, 2003) and that there may be differentiation within California (Case, 1978). Previous research in montane amphibian systems has also shown that populations can be structured by elevation, decreasing gene flow between populations in mountainous habitat and eroding genetic diversity (Funk et al., 2005; Giordano, Ridenhour, & Storfer, 2007). Therefore, we predicted that (1) population structure and differentiation exists between Oregon and California and, to a lesser degree, within California between the Klamath and southern Cascades ecoregions; (2) genetic diversity is lower in California than in Oregon where population trends are more stable (Pearl et al., 2009), and also lower at higher elevation sites compared to lower elevations; (3) the southern Cascades region within California has lower genetic diversity than the adjacent but disjunct Klamath Mountains, where declines have been less severe (Pope et al., 2014); and (4) populations in California exhibit adaptive differentiation associated with environmental variables due to increased selective pressures (e.g., disease, climate).

Materials and methods

Sample collection

We collected genetic samples of *R. cascadae* from 12 populations throughout the range in California and 4 populations in southern Oregon. All sites had at least 10 individuals remaining in the population. We selected Oregon populations by sampling known sites from previous studies (Monsen & Blouin, 2003, 2004). We selected sites in California where *R. cascadae* were known to be present within the last 10 years, prioritizing the sampling of sites in the southern Cascades where significant declines have occurred and sites throughout the Klamath Mountains to maximize the distribution of sites within the region. Sampling sites used in this study are referred to in text with three-letter abbreviations for the protection of sensitive locations. We conducted visual encounter surveys of water bodies at each site (Crump & Scott, 1994) and caught frogs via net or gloved hands. We took a nonlethal tissue sample from each animal caught at a site (up to 30 individuals per site) via toe-clipping for genetic analysis. Toe-clipping is a safe, simple, sanitary, and humane method that has been used successfully for amphibians for decades (Donnelly et al., 1994). We used a pair of flame-sterilized scissors to clip 2 full toe bones (phalanges) off the fourth digit of a rear foot and stored in 95% ethanol. At sites where few post-metamorphic frogs were found and tadpoles were observed (TOD, SCR, and COW), small portions of tadpole tails were clipped with a sterile pair of scissors to cut a 1–2 mm amount of tissue off the tail.

Laboratory methods

DNA was extracted using a Qiagen DNEasy Blood and Tissue kit following the manufacturer's protocol. DNA yields were quantified using a Qubit Fluorometric Quantitation assay. Only samples containing >300 ng of total DNA were considered acceptable for sequencing. We used a double-digest Restriction-Site-Associated DNA sequencing method (ddRAD; Peterson et al., 2012) to identify a panel of variable SNP loci. Libraries were prepared and sequenced at the Texas A&M AgriLife Genomics Facility. We tested a suite of restriction enzyme combinations and selected the pair that produced the most fragments of appropriate size, MseI and HindIII. We prepared individually barcoded libraries, selected fragments with insert sizes of 250–400 bp using a PippinPrep (Sage Sciences) and sequenced the libraries across two 150 bp paired end Illumina HiSeq 2500 lanes.

Bioinformatic processing and filtering

We used the bioinformatic pipeline *Stacks-v1.48* (Catchen et al., 2013) to build a dataset of SNP loci using a denovo assembly approach. *Xenopus laevis* and *Nanorana parkerii* were tested as reference genomes for sequence alignment but resulted in few loci being called, likely due to the extreme phylogenetic distance between species. Optimum values for the main *Stacks* assembly parameters (m = 3, M = 4, n = 4) were tested following the methods outlined in Rochette and Catchen (2017) and Paris, Stevens, and Catchen (2017). The '*populations*' unit of *Stacks* was used to filter SNPs. Where multiple SNPs per contig were identified, we retained only the first (most 5′) SNP. We retained SNPs (1) with a minimum minor allele frequency (MAF) of ≥0.03, (2) present in at least 75% of individuals in a population, and (3) that occurred in at least 73% of populations. We used *VCFTools* (Danecek et al., 2011) to remove individuals with large amounts of missing data (>50%) and then reran *populations* with the same filters to improve SNP recovery. We did not filter for loci out of Hardy-Weinberg equilibrium because our system violates the assumptions and such filtering would cede valuable genetic information (Wittke-Thompson, Pluzhnikov, & Cox, 2005). Prior to downstream analyses, we used several approaches to identify putatively adaptive outlier loci and removed them to create a dataset of neutral loci.

Outlier detection

We first explored the dataset for outlier loci—those loci that are closer to or farther from fixation (F_{ST}) than expected from a neutral distribution, patterns characteristic of loci in or near traits under selection. We did this to (1) remove potentially adaptive loci from the total dataset to create a putatively neutral dataset for population analyses and (2) identify outliers that could be responsible for adaptive differentiation across the study area, thus informing conservation priorities. We used three approaches to detect outliers potentially under selection: the R package *PCAdapt* (Luu, Bazin, & Blum, 2017), redundancy analysis (RDA; Forester et al., 2016), and a nonmodel-based approach that is simply the set of loci in the top 5% of F_{ST} values as outliers. Any locus identified in any of the three outlier detection approaches were subsequently removed, leaving a conservative dataset of presumably neutral loci for remaining population analyses.

Because RDA is a multivariate ordination method to detect genotype-environment associations (GEA), we selected 4 environmental predictor variables obtained from ClimateWNA (Hamann et al., 2013) that had the potential to explain adaptive differences across populations: elevation, average fall precipitation, the number of frost-free days, and climatic moisture deficit (a metric of aridity). We selected elevation, average fall precipitation, and frost-free days because they are thought to drive Bd infections in our study area where cool, moist falls could increase Bd (Hardy et al., 2015). Moisture deficit was selected for its potential to mediate Bd on the landscape via increased aridity and breeding pool dry-down, thus decreasing moisture availability for Bd persistence. Predictors were tested for multicollinearity and were uncorrelated (Figure S1). We retained the constrained axes of the RDA that were significant to examine outlier loci using the anova.cca function in the R package *Vegan* (Oksanen et al., 2015). Outlier loci were then correlated with environmental predictors to assess which predictor was most strongly correlated with each SNP.

Spatial genetic structure and differentiation

Population structure and admixture analyses were conducted in *Structure* 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). We ran *Structure* independently five times (Markov-Chain-Monte-Carlo [MCMC] burn-in of 100,000 steps, 100,000 permutations) at each number of hypothesized genetic clusters (K = 1 to 15, the number of sampled sites) under the admixture model with correlated allele-frequencies and location prior (population ID). The optimal value of K among tested values (K = 1–15) was determined by comparing the value of Delta K (Evanno, Reggaut, & Goudet, 2005) and the mean likelihood of K estimate (Ln Pr($X|K$)) (Pritchard, Wen, & Falush, 2003) using *Structure Harvester* (Earl & vonHoldt, 2012). Average q-values (proportion of an individual's genome that belongs to each cluster) were calculated in *CLUMPP* (Jakobsson & Rosenberg, 2007) and individuals were assigned to a cluster based on majority assignment (highest q). *Structure* was run hierarchically, such that each inferred cluster was run again, to clarify patterns of hidden hierarchical structure (Evanno, Reggaut, & Goudet, 2005; Janes et al., 2017; Pritchard et al., 2000).

The outlier loci from the three approaches described above were compared with each other to assess overlap in identified outliers between each method. We then created two sets of outliers; a conservative set that only included loci identified as outliers that were shared across at least 2 of the 3 methods; and a liberal set that included any locus identified as an outlier in at least one method. The neutral and outlier datasets were then used to compare patterns of population genetic structure using principal components analysis (PCA) in the R package *Adegenet* (Jombart, Lyon, & Biome, 2008).

To quantify genetic differentiation among populations and regions, measures of population-pairwise allele fixation (F_{ST}) and allele differentiation (Jost's D; Jost, 2008) were estimated using the *R* packages *Hierfstat* (Goudet, 2005) and *FinePop* (Kitada, Nakamichi, & Kishino, 2017) respectively. While F_{ST} and D are fundamentally distinct measures of differentiation, they are expected to provide similar insight into differentiation in pairwise comparisons of biallelic SNPs (Jost et al., 2018). We tested for isolation-by-distance (IBD) across the dataset by correlating pairwise geographic distance matrices (km) with genetic distance matrices (pairwise F_{ST}) for all sites across the study area and only for sites in California using simple Mantel Tests implemented in the R package *Ecodist* (Goslee & Urban 2007).

Genetic diversity and effective population size

Population-level diversity statistics were estimated for each sampling location directly from the *Stacks* '*populations*' unit (π, H_e, H_o, F_{IS}) and from the R package *hierfstat* (A_R; Goudet 2005). We predicted that genetic diversity would be lower at higher elevation populations with less opportunity for gene flow. We tested this prediction by correlating genetic diversity (H_e and A_R) with elevation using a Pearson's correlation. Effective population size (N_e) was estimated using the linkage-disequilibrium (LD) model in *Nestimator 2.01* with a critical P value of 0.05 (Do et al., 2014).

Results

Sample collection, bioinformatic processing, and filtering

We sequenced 270 individual tissue samples taken from 12 populations in California (n = 192) and four populations in Oregon (n = 78). After cleaning, assembling, and matching reads, genotypes were recovered for 257 of 270 individuals. Filtering steps (as described in Section 2) removed another 47 individuals, including all individuals at one of the four Oregon sites. The final genomic dataset contained 1672 loci across 210 individuals from 15 sites (12 from California, 3 from Oregon) with an average mean read depth per individual of 7.5× (median 7.4, range 6.3–11.1). Tadpole tissues were used from COW = 3, TOD = 4, and SCR = 9.

Outlier detection

A total of 168 out of 1672 loci were identified as outliers by at least one of the three outlier detection methods employed. Thirty-two loci were shared across two methods, and one locus was identified across all three methods (Figure S2). Eighty-four loci were in the top 5% of F_{ST} values. *PCAdapt* identified 111 loci and RDA identified seven loci. Of the seven loci identified in the RDA, four were correlated with the number of frost-free days, two were correlated with moisture deficit, and one was correlated with elevation (Figure S3).

Spatial genetic structure and differentiation

Spatial genetic structure was supported by *Structure* and PCA. *Structure* identified three main clusters (K = 3) that we

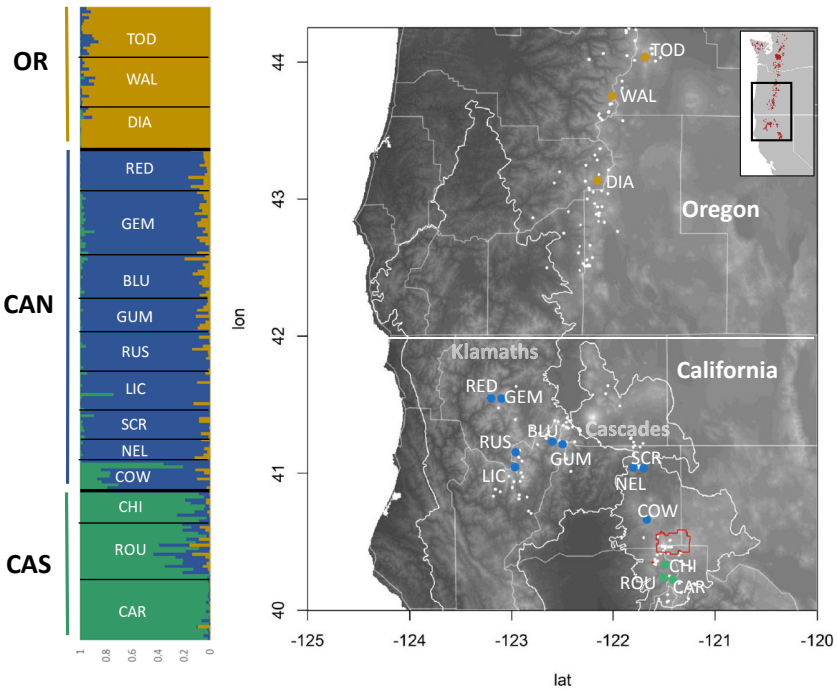

Figure 1 Locations of sampled sites with the spatial relationship of *R. cascadae* genetic clusters. White points are current or historically occupied *R. cascadae* sites and white polygons indicate boundaries of the Klamath and southern Cascade ecoregions. Colored points with three letter abbreviations are sites sampled in this study. Site colors used on the map identify the majority assignment (>50%) of all individuals sampled at that site and correspond to colors on the vertical *Structure* plot. The red polygon between the northern and southern sites in California represents the location of Lassen Volcanic National Park. Inset shows the distribution of *R. cascadae* along the West coast of the United States with observations marked in dark red. To the left is a *Structure* histogram of *R. cascadae* for K = 3 genetic clusters. Individual colored bars represent a single individual and are grouped by site separated by thin black bars. The proportional assignment of an individual's genome to each genetic cluster is displayed from 0 to 1 in green (CAS, California Southern), blue (CAN, California Northern), or gold (OR, Oregon) with each cluster separated by thick black bars [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com.]

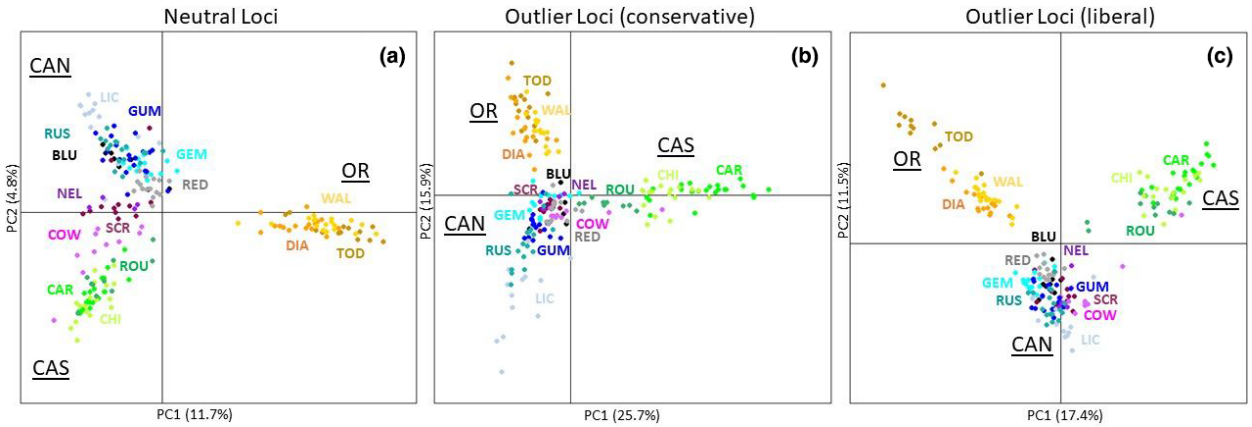

Figure 2 Principal components analyses (PCAs) of (a) all 1504 *R. cascadae* neutral loci, (b) 33 conservative outliers shared across at least two of three methods (*PCAdapt*, RDA, top 5% F_{ST}), and (c) 168 liberal outliers identified in at least one method. OR, Oregon; CAN, California Northern; CAS, California Southern. Color groups correspond to the three genetic clusters as identified by *Structure* ($K = 3$; OR = yellows, CAN = blues/reds, CAS = greens) and variations of color within each group correspond to individual sampling sites as noted with their three-letter abbreviation [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com.]

named Oregon (OR), California Northern (CAN), and California Southern (CAS) (Fig. 1). The CAS cluster included all sites sampled south of Lassen Volcanic National Park (CAR, CHI, and ROU), while CAN included all remaining California sites north and west of the Park, with no regard to ecoregion boundaries. COW, located north of the Park, revealed considerable admixture between the CAS and CAN clusters and was notably positioned between the CAS and CAN groups in our PCA. Iterative runs of *Structure* revealed further subdivision within CAS and CAN (Figure S4).

Patterns of genetic structure when comparing neutral and outlier loci were broadly similar, yet not identical across datasets (Fig. 2). The neutral set of 1504 loci was concordant with our *Structure* analysis, positioning Oregon sites away from both California groups and highlighting the ambiguous positions of COW, NEL, and SCR between the CAN and CAS groups (Fig. 2a). The conservative set of 33 outliers notably shifted Oregon populations much closer to those in California; highlighted strong dissimilarity of LIC from the rest of the CAN group; moved COW, SCR, and NEL closer to the rest of the CAN group; and placed ROU between the CAN group and the remaining CAS populations (Fig. 2b). Conversely, the liberal set of 168 outliers highlighted the dissimilarity among all three genetic groups (OR, CAN, and CAS) and positioned TOD furthest from the remaining Oregon populations (Fig. 2c).

Genetic differentiation (pairwise F_{ST}) between California and Oregon populations ranged from 0.12 to 0.25 (median = 0.20; Table 1). Pairwise values of Jost's D were generally smaller but mirrored F_{ST} estimates (range = 0.001–0.07, median = 0.02; Table 1). Regionally, differentiation was high and similar between CAN and OR ($F_{ST} = 0.22$; $D = 0.03$) and CAS and OR ($F_{ST} = 0.29$; $D = 0.05$). Among the three Oregon sites (Waldo Lake, WAL; Todd Lake, TOD; Diamond Lake,

Table 1 Pairwise estimates of F_{ST} (below the diagonal) and Jost's D (above the diagonal) for Oregon and California populations of *Rana cascadae*. F_{ST}, population-pairwise allele fixation; Jost's D, allele differentiation

	TOD	WAL	DIA	RED	GEM	BLU	GUM	RUS	LIC	SCR	NEL	COW	CHI	ROU	CAR
TOD	0	0.007	0.021	0.029	0.03	0.031	0.031	0.035	0.045	0.034	0.039	0.043	0.049	0.043	0.062
WAL	0.032	0	0.019	0.031	0.031	0.033	0.034	0.038	0.047	0.035	0.039	0.042	0.053	0.045	0.066
DIA	0.076	0.059	0	0.044	0.041	0.042	0.043	0.047	0.055	0.048	0.052	0.056	0.061	0.056	0.073
RED	0.13	0.117	0.128	0	0.001	0.008	0.008	0.007	0.015	0.014	0.018	0.017	0.021	0.019	0.025
GEM	0.163	0.143	0.146	0.005	0	0.009	0.009	0.009	0.017	0.014	0.02	0.02	0.025	0.023	0.028
BLU	0.162	0.142	0.142	0.048	0.072	0	0.001	0.003	0.01	0.01	0.017	0.017	0.019	0.018	0.023
GUM	0.172	0.156	0.165	0.052	0.071	0.009	0	0.005	0.012	0.01	0.017	0.017	0.022	0.02	0.027
RUS	0.187	0.166	0.174	0.043	0.069	0.027	0.04	0	0.01	0.013	0.02	0.017	0.023	0.021	0.029
LIC	0.249	0.222	0.221	0.099	0.141	0.089	0.1	0.08	0	0.019	0.026	0.03	0.028	0.027	0.029
SCR	0.185	0.156	0.184	0.083	0.111	0.074	0.075	0.099	0.155	0	0.004	0.013	0.022	0.02	0.026
NEL	0.204	0.176	0.189	0.119	0.165	0.132	0.136	0.147	0.218	0.034	0	0.016	0.025	0.025	0.031
COW	0.236	0.188	0.209	0.11	0.165	0.131	0.13	0.134	0.199	0.106	0.151	0	0.017	0.019	0.024
CHI	0.228	0.2	0.196	0.11	0.169	0.131	0.151	0.154	0.192	0.149	0.183	0.129	0	0.008	0.009
ROU	0.207	0.178	0.18	0.108	0.161	0.122	0.135	0.141	0.186	0.137	0.179	0.137	0.052	0	0.01
CAR	0.244	0.215	0.207	0.135	0.177	0.148	0.155	0.169	0.18	0.156	0.202	0.167	0.058	0.062	0

Table 2 *Rana cascadae* allelic richness (A_R), expected heterozygosity (H_E), observed heterozygosity (H_O), F_{IS}, nucleotide diversity (π), and effective population size estimates (N_e with 95% confidence intervals) for individuals (n) sampled at sites in Oregon and California

Region	Cluster	Site	n	A_R	H_E	H_O	F_{IS}	π	N_e (95% CI)
Oregon	OR	TOD	16	1.25	0.11	0.14	−0.07	0.0004	146 (88.9–380.4)
Oregon	OR	WAL	18	1.26	0.11	0.15	−0.07	0.0004	285 (146.5–3698.2)
Oregon	OR	DIA	13	1.28	0.13	0.17	−0.07	0.0005	*Inf* (*Inf*–*Inf*)
Klamath	CAN	RED	15	1.18	0.07	0.09	−0.04	0.0003	128 (63.4–4011.8)
Klamath	CAN	GEM	15	1.17	0.07	0.09	−0.04	0.0003	137 (73.9–742.1)
Klamath	CAN	BLU	8	1.21	0.08	0.10	−0.03	0.0004	*Inf* (*Inf*–*Inf*)
Klamath	CAN	GUM	20	1.22	0.09	0.10	−0.04	0.0004	*Inf* (605.2–*Inf*)
Klamath	CAN	RUS	17	1.20	0.08	0.10	−0.04	0.0003	102 (70.4–179.1)
Klamath	CAN	LIC	13	1.17	0.07	0.09	−0.03	0.0003	76 (51–142.9)
Cascade	CAN	SCR	12	1.21	0.09	0.11	−0.03	0.0004	18(16.1–20)
Cascade	CAN	NEL	4	1.20	0.08	0.11	−0.04	0.0004	*Inf* (*Inf*–*Inf*)
Cascade	CAN	COW	10	1.18	0.07	0.09	−0.04	0.0003	16 (14.6–18.6)
Cascade	CAS	CHI	17	1.21	0.09	0.12	−0.05	0.0004	96 (68.5–156.9)
Cascade	CAS	ROU	10	1.21	0.09	0.12	−0.05	0.0004	*Inf* (*Inf*–*Inf*)
Cascade	CAS	CAR	22	1.16	0.07	0.09	−0.04	0.0003	28 (23.8–33.4)

Note: Genetic cluster as inferred by Structure indicated. *Inf* N_e estimates refer to 'infinite'.

DIA), differentiation was low (F_{ST} range = 0.03–0.08, median = 0.06; Jost's D range = 0.01–0.02, median = 0.02). Isolation-by-distance was strong across the entire study-area (Mantel coefficient r = 0.75, P = <0.0001).

Among California populations, overall genetic differentiation was moderate (F_{ST} = 0.15; D = 0.02) and pairwise values varied between populations (F_{ST} range = 0.005–0.21, median = 0.12; Table 1). Pairwise values of Jost's D were generally smaller but mirrored F_{ST} estimates as expected, ranging from 0.001 to 0.06 (median = 0.03; Table 1). Genetic differentiation was also correlated with geographic distance (IBD) within California (Mantel coefficient r = 0.65, P < 0.0001).

Genetic diversity and effective population size

California populations of *R. cascadae* had lower within-population genetic variation than populations in Oregon across three of the four metrics of diversity (A_R, $t_{3.99}$ = 6.63, P = 0.003; H_E, $t_{2.61}$ = 6.22, P = 0.01; H_O, $t_{2.73}$ = 6.82, P = 0.008; π, $t_{2.88}$ = 2.28, P = 0.11; Table 2). F_{IS} values were lower in California than in Oregon (F_{IS}, $t_{8.80}$ = 12.54, P < 0.001; Table 2). Within California, there was no difference in genetic diversity between CAN and CAS (A_R, $t_{2.58}$ = 0, P = 1.00; H_O, $t_{2.41}$ = −1.05, P = 0.39; H_E, $t_{2.42}$ = −0.84, P = 0.47; π, $t_{3.2}$ = −0.59, P = 0.59). We also found no support for a relationship between elevation and genetic diversity (A_R, r = −0.496, P = 0.06; H_E, r = −0.463, P = 0.08; Fig. 3).

Estimates of effective population size (N_e) from *$N_e$estimator* were obtained for 10 of the 15 sites (Table 2). Point estimates ranged from 16 to 285 and were higher in Oregon (mean = 215.5) than in any site in California (mean = 75.1; Table 2). There was not enough data to assess the differences between groups with a formal statistical hypothesis test. Effective population size estimates for the southern

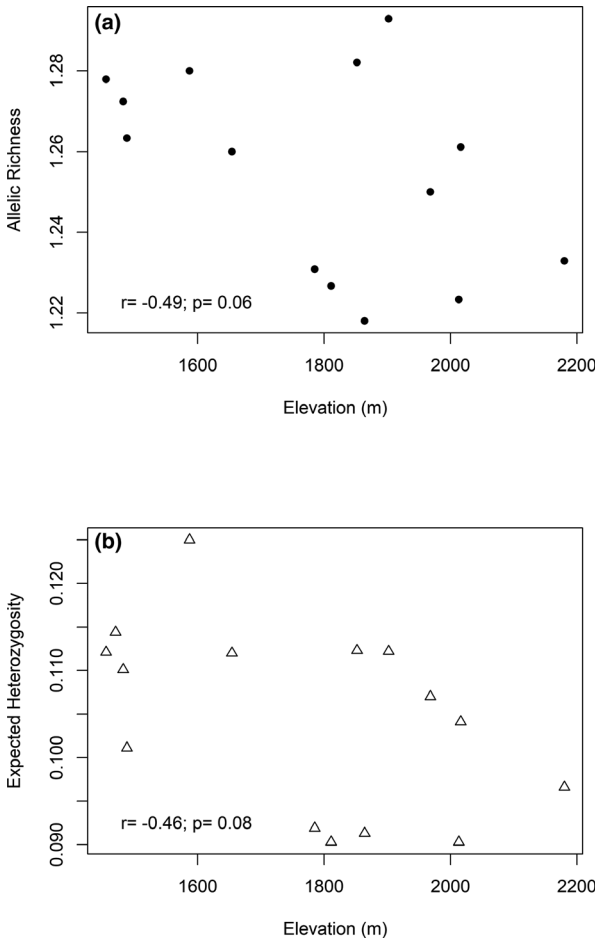

Figure 3 Plot of (a) allelic richness (black circles) and (b) expected heterozygosity (open triangles) vs. elevation by sampling site. Correlation coefficient and P value are provided for each.

group (mean = 61.95) were on average lower than those for the north (mean = 79.53; Table 2).

Discussion

Conservation genomics is a transformative approach that improves species conservation by harnessing the power of large sets of loci from across the genome to answer applied conservation questions (Allendorf, Hohenlohe, & Luikart, 2010). These tools are increasingly necessary to help mitigate species extinctions as rapid population declines continue to isolate and fragment remaining populations, especially at the range edges (Sexton et al., 2009). Our findings highlight the low genetic diversity exhibited in remnant populations of an imperiled amphibian at the southern extent of its range and reveal novel patterns of population structure that suggest conservation measures are warranted to prevent further extinctions. Assessing populations post-decline was especially informative for prioritizing which populations are most threatened and determining appropriate management actions that are population-specific and informed by evolutionary principles.

Neutral genetic structure and differentiation

We found that *R. cascadae* in California are genetically distinct and strongly differentiated from Oregon, verifying Monsen & Blouin's (2003) preliminary hypothesis of divergence between Cascades frog populations in the two states. The California-Oregon border has been identified as a biogeographic barrier for a suite of taxa including many herpetofauna (Bury & Pearl, 1999; Janzen et al., 2002). Remington also denoted it as one of 13 suture zones in North America (Remington, 1968), and Swenson & Howard (2005) validated its importance as a phylogeographic faunal break. For *R. cascadae*, genetic divergence between Oregon and California populations corresponds to a ~80 km natural gap in distribution, making gene flow between these regions extremely unlikely. The differentiation identified between states is important because it may influence how we manage the species across state borders given the significant declines observed in California (Fellers et al., 2008), but not in Oregon (Pearl et al., 2009).

Within California, we had initially hypothesized that populations would be structured across two ecoregions—the Klamath ecoregion and the southern Cascades ecoregion—based on differences in currently occupied habitats, physiography, historical geologic formation, and molecular data (Case, 1978). High resolution sampling, both across the landscape and across the genome, revealed little support for evolutionary differentiation by ecoregion. Volcanic activity and glaciation within and around the Park are suggested as potential barriers to gene flow in other herpetofauna (Jackman & Wake, 1994; Rodríguez-Robles et al., 2001) and are possible explanations of the pattern of differentiation we observe here between the CAN and CAS groups of *R. cascadae*.

The strong signal of admixture between the CAN and CAS groups at COW, which is the southernmost sampled population in the CAN group, was surprising. COW is isolated; both historical and contemporary records document an absence of *R. cascadae* in the ~30 km to the north between COW and the other two populations from the CAN group in the southern Cascades mountains (NEL, SCR). Admixture has also been identified in this same region between Spotted Owl subspecies (Barrowclough et al., 2011; Funk et al., 2008), an organism with arguably much larger dispersal capability. To the south of COW, the nearest occupied *R. cascadae* site (CHI) belongs to the southern group and is also ~30 km away. Before severe population declines and extirpations throughout LVNP, it is likely that COW was better connected to other *R. cascadae* populations to the south. Thus, the pattern of admixture we observed is consistent with historical gene flow in combination with more recent isolation and genetic drift.

Outlier loci and adaptive differentiation

While the overall patterns of genetic structure derived from several outlier datasets and neutral loci were broadly similar, several exceptions suggest some of these loci may be under divergent selection. However, discordance between outlier datasets limit our ability to clarify these processes. The set of conservative outliers highlight an increased similarity among all populations, most notably between those in Oregon and California (Fig. 2b). This could suggest a potential shared suite of selective pressures are influencing apparent adaptive similarities between regions (e.g., climate or habitat factors). In fact, our RDA found correlations between seven outlier loci across all populations and elevation, moisture deficit, and the number of frost-free days, indicating that these variables, or other unmeasured but correlated variables, are potentially contributing to adaptive similarity in *R. cascadae* across our study area.

In contrast, the set of liberal outliers highlight the dissimilarities between genetic groups (Fig. 2c) and more closely align with our prediction that the 40-year presence of Bd in California populations of *R. cascadae* (DeLeon et al., 2017) would act as a strong selective force promoting adaptive divergence between California and Oregon populations. However, the liberal dataset is more likely to contain false positives, including loci identified by selecting the top 5% of F_{ST} outliers (Bierne, Roze, & Welch, 2013). Differences between the conservative and liberal sets of outlier loci emphasize the need for more detailed investigation of these candidate loci to interpret their potential role in adaptation.

We must also acknowledge that drastic demographic declines and widespread extirpations across affected populations likely magnified the effects of genetic drift relative to local adaptation (Wright, 1931; Gaggiotti et al., 2009), leaving any adaptive differentiation difficult to detect. Whole genome sequencing or targeted enrichment approaches could shed light on the role of climatic, environmental, and disease-related variables on adaptive differentiation. Further, the incorporation of ecological niche modeling with these targeted genomic methods could enhance our understanding of adaptive differentiation in the face of current and future selection (Razgour et al., 2017). Yet, it is important to keep our ultimate goals in mind—while the conservation of

adaptive potential may be important for long-term population sustainability, short-term efforts to boost population sizes and overall levels of genetic variation are perhaps more immediately relevant to limiting declines and promoting species persistence (Kardos & Shafer, 2018).

Genetic diversity

Genetic diversity metrics indicated that populations in California are less diverse than those in Oregon, as predicted. California populations of *R. cascadae* have experienced severe declines due to Bd that have not been documented in other portions of their range (Pearl et al., 2009). Demographic declines have increased isolation among populations (Fellers et al., 2008) and have potentially contributed to the erosion of genetic variation. Although the southernmost group in California has experienced the brunt of declines, we found comparable genetic diversity across both groups in California. It is possible that demographic declines in the southern group were not accompanied by a detectable loss of genetic variation, or that diversity was restored through gene flow (Keller et al., 2001; Jangjoo et al., 2016). Our estimates of genetic differentiation between populations in the southern group are among the lowest values in California, providing some support for this hypothesis. Alternatively, our results could indicate cryptic declines in the CAN group that have gone undetected. While several large die-offs have been observed in the Klamath Mountains (Piovia-Scott et al., 2011), many core sites that are intensely monitored appear demographically more stable than those to the south.

While our data are clear that genetic diversity is lower in California, our study does not include samples collected prior to detection of Bd in our study area, thus limiting our ability to directly connect the low diversity observed with recent declines. It is possible that genetic diversity was naturally low prior to Bd-induced population declines, as has been observed in *Leptodactylus fallax* populations in the Lesser Antilles (Hudson et al., 2016). However, *L. fallax* inhabit two small islands, and island populations are expected to inherently display low genetic diversity with severely reduced geneflow (Eldridge et al., 1999). Historical accounts indicate that *R. cascadae* was previously abundant across northern California (Grinnell et al., 1930) prior to the documentation of severe declines and increased isolation of populations (Fellers et al., 2008). There have been no documented declines of *R. cascadae* in Oregon however, and the higher genetic diversity we observed there matches expectations for populations with higher connectivity. Therefore, we argue that our observation of lower genetic diversity of *R. cascadae* in California reflects the pattern of severe, Bd-induced declines in the region. Although not the goal of the present study, recent molecular techniques have advanced enough to be able to attain high-resolution population genomic datasets from birds (Linck et al., 2017), mammals (McDonough et al., 2018), and even formalin-preserved reptiles and amphibians (Ruane & Austin, 2017). These techniques will undoubtedly enhance our ability to distinguish between correlation and causation for temporally important topics such as disease-induced declines across a variety of taxa and are worth further investigation in *R. cascadae* in the future.

We also found no evidence of a relationship between genetic diversity and elevation, in contrast with other montane frogs (Funk et al., 2005) and salamanders (Giordano, Ridenhour, & Storfer, 2007) in the western United States. It is likely that the variation in elevation in our study was not large enough to capture the expected relationship. Funk et al. (2005) recorded a relationship between genetic diversity and elevation across a 1919-m elevational range compared to our 723 m (1458–2181 m). While the historical range-wide elevational distribution of *R. cascadae* is fairly large (230–2740 m; 2510 m difference; Pope et al., 2014), our study area has experienced extirpations of the species at both high and low elevations. The elevational distribution of remnant populations is thus restricted to middle elevations (Figure S5) and limits our ability to gain insight into this previously documented relationship.

Effective population size

Effective population size estimates mirrored genetic diversity results, showing that Oregon populations tended to have larger N_e than California populations. Our findings of low N_e for California populations corroborate low N_e estimates from demographic data (Pope et al., 2014) and offers additional support for strong declines of *R. cascadae* in California. Estimates from the southern group in California, where the brunt of Bd-induced declines occurred, were particularly small compared to the north and especially to Oregon. These findings highlight the link between strong demographic declines, population fragmentation, and reduced genetic variation (Gilpin & Soule, 1986).

Single-sample N_e estimation can be challenging however (Waples, Antao, & Luikart, 2014), especially when populations are small and declining (i.e., nonequilibrium; Luikart et al., 2010). Our N_e estimates were highly variable and included several wide confidence intervals (Table 2). The LD approach we used is generally robust (Gilbert & Whitlock, 2015; Luikart et al., 2010) and yielded estimates that are clearly below any recommended threshold for avoiding inbreeding and maintaining evolutionary potential (Franklin & Frankham, 1998; Lynch & Lande, 1998). When taken together, our estimates of genetic diversity and N_e both ultimately point to an elevated risk of extinction for *R. cascadae* in California in the long term, likely through fixation of deleterious alleles and loss of adaptive variation by drift (Hare et al., 2011; Newman & Pilson, 1997; Phifer-rixey et al., 2012).

We also acknowledge that the inclusion of tadpole tissues collected in California (COW; three tadpoles, SCR; nine tadpoles) and Oregon (TOD; four tadpoles) could increase the probability of sampling siblings and bias our N_e estimates. However, removing putative or suspected siblings from genetic datasets can be problematic and siblings are ultimately recommended to not be purged (Waples & Anderson, 2017). If we sampled many siblings, we would expect the resulting N_e estimates to be biased small. Conversely, Todd Lake's (TOD) N_e estimate was large, and SCR and COW's low estimates are representative of the status of those small populations (Pope et al., 2014). Therefore, we believe the

relatively few tadpoles included in our analyses likely had little influence on estimating N_e.

Conservation implications

Fine-scale genomic data revealed patterns of diversity in remnant populations of an imperiled amphibian that will inform source selection for reintroductions into Lassen Volcanic National Park and identified critical target populations for intensive disease mitigation efforts. Reintroductions are an important step towards reestablishing connectivity of *R. cascadae* populations throughout the southern Cascades. Like other recovery efforts, the goal is to expand the number of populations while preserving the genetic structure of the region to the extent possible. Given the strong geographic structure with isolation-by-distance, translocations to neighboring geographic locations would be preferred over long-distance translocations. If managers are to use the few remaining, genetically unique, southernmost populations of *R. cascadae* as source populations for reintroductions in the region, it seems that focused conservation efforts for those populations are warranted. For example, actively treating juvenile frogs in these populations with an anti-fungal bath reduces the amount of Bd on their skin and increases survival (Hardy et al., 2015), possibly extending the timeframe in which eco-evolutionary rescue could occur (DiRenzo et al., 2018).

Genetic diversity, while low in both groups in California, did not appear so low as to preclude strategic captive rearing and reintroductions. Historical populations south of Lassen Peak within LVNP were likely more closely related to the southern sites than to the sites north of LVNP due to the lack of a physical barrier, their geographic proximity, and the presence of southern-group variation north of LVNP. For reintroductions at the northern edge of the park, incorporating animals from the region of admixture at COW may be warranted, especially if the southern populations continue to decline. While it is unclear whether historically occupied sites within LVNP also carried this mixed ancestry, the supplemental genetic diversity from this population could be beneficial as genetic mixing has shown to aid genetic rescue of amphibians in some cases (Beauclerc, Johnson, & White, 2010).

More generally, demographic declines associated with disease can erode genome-wide diversity of hosts (McKnight et al., 2017) and leave affected populations vulnerable to extinction. Disease-mediated population declines have impacted a broad range of species, reducing viability and increasing extinction risk (Cunningham et al., 2017). Conservation biologists that aim to mitigate declines and restore populations face challenges when prioritizing management actions, and planning is hindered by a lack of data on diversity and structure of residual, post-decline populations (Berger-Tal, Blumstein, & Swaisgood, 2020; Ewen, Soorae, & Canessa, 2014). These challenges are exacerbated when pathogen eradication is not possible, for instance, where there are multiple host species or environmental reservoirs (Scheele et al., 2019). In these instances, integrating genomic data into conservation bridges the gap between researchers and practitioners (Britt et al., 2018; Shafer et al., 2015) and facilitates genetic management of host populations (Glassock et al., 2021). We show here how incorporating genomic data on the structure and diversity of the target species following declines significantly informs prioritization of management actions and the development of effective conservation plans that incorporate ecological and evolutionary processes (Pabijan et al., 2020) to benefit species recovery.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Correlation matrix for the four environmental predictor variables used in the RDA analysis.

Figure S2. Visual representation of the number of outlier loci detected in each of three methods, and those that are shared between them.

Figure S3. RDA results showing identified outliers.

Figure S4. Hierarchical genetic structure of *Rana cascadae* across the study area from program *Structure*.

Figure S5. Density estimates of the distribution of elevations of known extant populations (solid line) and historical populations (dashed line) for Cascades frogs (*Rana cascadae)* in California.