

# The role of neutral and adaptive genomic variation in population diversification and speciation in two ground squirrel species of conservation concern

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## Abstract

Understanding the neutral (demographic) and adaptive processes leading to the differentiation of species and populations is a critical component of evolutionary and conservation biology. In this context, recently diverged taxa represent a unique opportunity to study the process of genetic differentiation. Northern and southern Idaho ground squirrels (*Urocitellus brunneus*–NIDGS, and *U. endemicus*–SIDGS, respectively) are a recently diverged pair of sister species that have undergone dramatic declines in the last 50 years and are currently found in metapopulations across restricted spatial areas with distinct environmental pressures. Here we genotyped single-nucleotide polymorphisms (SNPs) from buccal swabs with restriction site-associated DNA sequencing (RADseq). With these data we evaluated neutral genetic structure at both the inter- and intraspecific level, and identified putatively adaptive SNPs using population structure outlier detection and genotype–environment association (GEA) analyses. At the interspecific level, we detected a clear separation between NIDGS and SIDGS, and evidence for adaptive differentiation putatively linked to torpor patterns. At the intraspecific level, we found evidence of both neutral and adaptive differentiation. For NIDGS, elevation appears to be the main driver of adaptive differentiation, while neutral variation patterns match and expand information on the low connectivity between some populations identified in previous studies using microsatellite markers. For SIDGS, neutral substructure generally reflected natural geographical barriers, while adaptive variation reflected differences in land cover and temperature, as well as elevation. These results clearly highlight the roles of neutral and adaptive processes for understanding the complexity of the processes leading to species and population differentiation, which can have important conservation implications in susceptible and threatened species.

## KEYWORDS

adaptation, conservation genetics, genomics, mammals, speciation, wildlife management

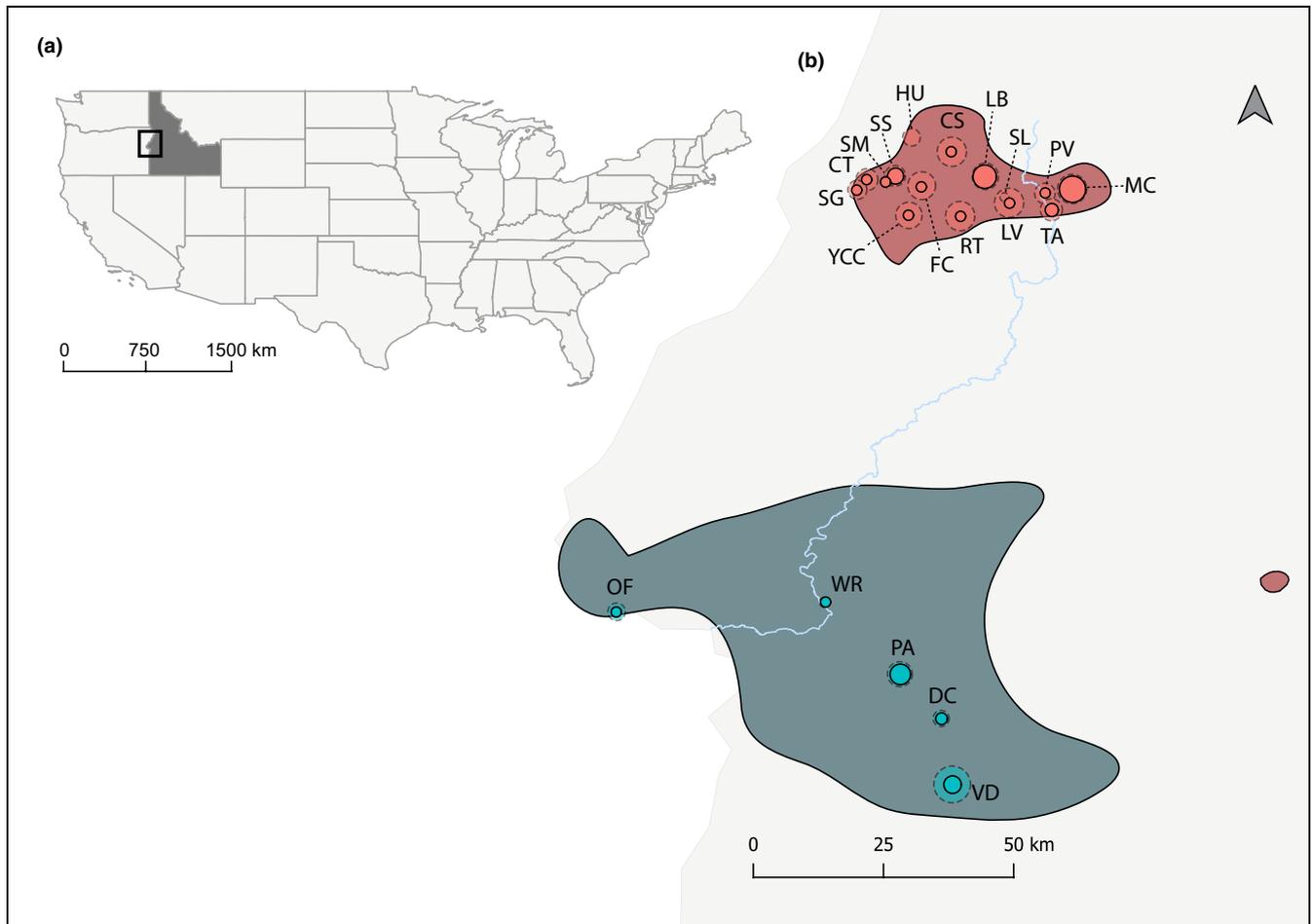
## 1 | INTRODUCTION

Isolation and subsequent local adaptation of populations are considered common processes that lead to speciation. Changes in habitat, barriers to dispersal or stochastic demographic events can cause population isolation and diversification (Slatkin, 1987; Steinberg et al., 2000). In this context, environmental variation plays an important role in facilitating or hindering connectivity, and thus in promoting the persistence of populations, their vicariance or even extinction (Waits et al., 2016). On the one hand, increased structural and functional connectivity facilitates the persistence of small populations that are highly susceptible to demographic stochasticity, genetic drift and density-dependent effects (Hanski, 1998; Lopes & de Freitas, 2012; Wittmann et al., 2018). On the other, high levels of connectivity result in more genetically homogeneous populations, with less propensity for local adaptation (Kawecki & Ebert, 2004). With loss of connectivity between populations, allele frequencies tend to diverge due to genetic drift, ultimately leading to neutral genetic differentiation (Rundell & Price, 2009). Divergence and speciation between allopatric populations occurs more rapidly in the context of divergent selection, leading to a faster accumulation of differences at the adaptive than at the neutral level (Kautt et al., 2020). Spatial variation in local selection pressures within a species' range, particularly when there is habitat fragmentation, can lead to changes in allele frequencies and fixation of new adaptive mutations, resulting in the emergence of adaptive differences (Orsini et al., 2013). This is especially important for small and isolated populations that are restricted to increasingly unfavourable habitat, for which studies have shown that local adaptation tends to occur less frequently than in larger populations (Hoffmann et al., 2017; Wood et al., 2016). Recently diverged populations represent a great opportunity to study the process of genetic differentiation (Fišer et al., 2018; Fletcher et al., 2019; Marques et al., 2019). The comparison of neutral and adaptive variation should provide evidence for distinguishing which processes are contributing most to differentiation, and what can be done to circumvent or sustain that diversification, depending on specific conservation goals (Friis et al., 2018; Orsini et al., 2013).

Northern and southern Idaho ground squirrels (*Urocyonellus brunneus* and *U. endemicus*, respectively; hereafter NIDGS and SIDGS) are a recently diverged pair of sister species, just over 30,000 years ago, which currently have an allopatric distribution (Figure 1) (Hoisington-Lopez et al., 2012; Yensen, 1991). NIDGS and SIDGS were formerly considered two subspecies of *Spermophilus brunneus* and have been distinguished as separate species on the basis of ecological niche modelling, morphology and genetics (Gill & Yensen, 1992; Helgen et al., 2009; Hoisington-Lopez et al., 2012; USFWS, 2015; Yensen & Sherman, 1997). Both species are rare (2,000–3,000 individuals for NIDGS; Wagner & Evans Mack, 2020, and 2000–4500 individuals for SIDGS; Yensen, 2001), endemic to Idaho, and are of high conservation concern (IUCN, 2000, 2018). Ecologically, both species are semicolonial and patchily distributed, representing classic examples of metapopulation structure whereby dispersal among populations is uncommon and tends to occur in a "stepping stone" manner (Gavin

et al., 1999; USFWS, 2003; Yensen, 1991; Yensen & Sherman, 1997). NIDGS live in open meadows, grassy scabs and small rocky outcroppings at an elevation of 1100–2300 m within coniferous forests of central Idaho (Burak, 2011; Goldberg et al., 2020), and they persist within only a small fraction of their former range probably due to habitat loss and reduced population connectivity, mostly as a result of forest encroachment (Gavin et al., 1999; Helmstetter et al., 2021; Sherman & Runge, 2002; Suronen & Newingham, 2013; Yensen & Dyni, 2020). SIDGS live in sagebrush steppe and rolling hill slopes at an elevation of 630–1400 m in southwestern Idaho, and are currently threatened by urban and agricultural development, as well as the spreading of invasive annual plants (Lohr et al., 2013; USFWS, 2000). Morphological differences between the two species include coat colour, which tends to mimic differences in soil colour between the species' geographical ranges (Yensen, 1991), pelage (longer in SIDGS) and baculum characteristics (longer with more spines in SIDGS) (Yensen, 1991). Previous genetic work on NIDGS and SIDGS estimated that the divergence between NIDGS and SIDGS occurred about 32.5 (18.3–63.5) thousand years ago, during the Quaternary climate cycles, and found no subsequent gene flow between the two species (Hoisington-Lopez et al., 2012). Vicariant events of this magnitude have been found to be sufficient for distinct evolutionary lineages to become different species, a pattern frequently found in several North American small mammals (Hope et al., 2014, 2016, 2020).

Local adaptation is likely to be an important factor for ground-dwelling, small mammals like NIDGS and SIDGS with limited dispersal abilities. Both ground squirrel species undergo seasonal torpor, and while both species hibernate, only SIDGS are known to estivate starting around July (USFWS, 2014). There are also differences in the timing of hibernation between the two species, which are probably due to differences in elevation and climate (Goldberg & Conway, 2021; Yensen, 1991). This difference in emergence timing between NIDGS and SIDGS could have a genomic basis, or may simply result from a plastic response (Hut & Beersma, 2011; Santos et al., 2017; Yensen, 1991). Typically, adaptations are associated with the habitat variables that affect fitness the most, which in the case of the Idaho ground squirrels (hereafter IDGS) are probably variables associated with energy consumption, timing of food availability, soil temperature, forage quality and risk of predation, which may vary between the active season and torpor (Goldberg, Conway, Evans Mack, et al., 2020; Goldberg, Conway, Tank, et al., 2020). Variation in the timing of ground squirrel hibernation emergence has been associated with food availability and snowpack in NIDGS and, thus, site productivity appears to dictate differences within and possibly between species differentiation (French, 1982; Goldberg & Conway, 2021). These differences may be determinant for species divergence, but may also lead to intraspecific local adaptation if environmental differences are found across the species range (Kawecki & Ebert, 2004; Savolainen et al., 2013). Previous intraspecific genetic studies on Idaho ground squirrels have found that genetic differentiation was low to moderate among NIDGS populations, with one disjunct population (Round Valley) being completely isolated (Garner et al., 2005; Hoisington, 2007; Hoisington-Lopez et al., 2012; Yensen & Sherman,



**FIGURE 1** Location of the study area, Idaho ground squirrel (IDGS) distributions and sampling sites in the state of Idaho. (a) Map of the USA with state borders represented by dark grey lines, the state of Idaho highlighted in dark grey and a black square indicating the study area detailed in (b). (b) Detail of the study area, where circles represent sampled populations of northern Idaho ground squirrel (NIDGS, red) and southern Idaho ground squirrel (SIDGS, blue). Population codes correspond to those in Table 1. Size of the circles is proportional to the number of samples (from one to 25 samples), where outer dashed circles represent the number of samples analysed, and inner full circles the number of samples successfully RAD-sequenced and used in genomic analyses. Locations with fewer than six samples have the same circle size (see Table 1 for further details on sample numbers). Background red and blue areas represent the distribution of NIDGS and SIDGS, respectively, as estimated using the “Concave Hull” tool from qgis 3.16.1 from presence data (unpublished data, Idaho Fish and Game). The light blue line represents the Weiser River [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

1997). In the late 1990s, translocations of NIDGS were performed from Squirrel Manor and other nearby populations to locations such as Summit Gulch and Cottonwood Corrals (close to the YCC population from Figure 1) (Gavin et al., 1998; Sherman & Gavin, 1999). Survival of translocated individuals was low, varying from 14% to 18% considering the combined success of the 2 years of translocations (Sherman & Gavin, 1999). In SIDGS, the Weiser River was documented as a barrier to dispersal between populations, but connectivity among populations on either side of the river was relatively high (Garner et al., 2005; Hoisington, 2007). Additionally, there are reports of human-mediated translocations, particularly from southern localities close to Van Deusen to multiple populations east of the Weiser River (Yensen & Tarifa, 2012; Yensen et al., 2010). However, translocation success in SIDGS has been very limited, especially into areas without established populations, for which the majority of the

translocated individuals did not survive the first winter (Busscher, 2009; Yensen et al., 2010). Thus, to better understand the probability of population persistence under habitat and climate change, it is essential to determine the role of neutral processes in maintaining population connectivity and overall genetic diversity, and the role of adaptive processes in improving population resilience through local adaptation (Macdonald et al., 2018).

In this study, we aimed to develop and use genomic tools to provide novel information on neutral and adaptive genetic diversity and differentiation within and among NIDGS and SIDGS to address the following five questions: (i) How do genetic patterns of adaptive and neutral variation compare between and within species? (ii) What landscape variables are associated with loci under selection? (iii) Are there any populations that have elevated levels of adaptive differentiation? (iv) Can we identify specific genes under selection

Population	NTotal	NRAD	IDGS	NIDGS/SIDGS
<b>NIDGS</b>				
Cap Gun/Tree Farm (CT)	15	5	0	1
Cold Springs (CS)	20	8	0	1
Fawn Creek (FC)	19	9	0	4
Huckleberry (HU)	12	2	0	0
Lost Valley (LV)	18	2	1	1
Lower Butter (LB)	20	17	11	16
Mud Creek (MC)	20	20	17	18
Price Valley (PV)	14	6	3	3
Rocky Top (RT)	20	8	6	7
Slaughter Gulch (SL)	6	0	0	0
Squirrel Manor (SM)	8	7	2	5
Steve's Creek/Squirrel Valley (SS)	14	13	11	11
Summit Gulch (SG)	13	6	1	2
Tamarack (TA)	15	11	8	9
YCC	18	4	1	2
<b>SIDGS</b>				
Dry Creek (DC)	11	11	3	8
Olds Ferry (OF)	12	12	3	6
Paddock (PA)	17	15	9	14
Van Deusen (VD)	25	24	4	12
Weiser River (WR)	7	6	0	1
<b>Total</b>	<b>304</b>	<b>186</b>	<b>80</b>	<b>121</b>

**TABLE 1** Number of Idaho ground squirrel samples per population used in this study (NTotal) for both northern and southern Idaho ground squirrels (NIDGS and SIDGS, respectively), number of samples that were successfully RAD-sequenced (NRAD), and retained after applying the filters of the interspecific (IDGS) and intraspecific (NIDGS/SIDGS) analyses

and their putative functions? (v) Can we identify patterns of neutral and adaptive variation useful for management of these rare species? To address these questions, we tested the following hypotheses: (a) geographical distance will be the main driver of differentiation for neutral loci in NIDGS and SIDGS, possibly exacerbated by previously identified geographical barriers to gene flow (Hoisington, 2007; Zero et al., 2017); (b) adaptive differences will be mainly associated with timing of torpor, production and storage of fat, and increased metabolism at higher elevation both inter- and intraspecifically (Faherty et al., 2018; Garcia-Elfring et al., 2019); and (c) local adaptation between populations within species will be highest in NIDGS because this species occupies a more topographically diverse area (Yensen & Sherman, 1997). Finally, we synthesize all of this information to identify demographic and adaptive groups that could be in need of special protection due to genetic isolation or evidence of local adaptation.

## 2 | METHODS

### 2.1 | Sampling and genotyping

We analysed 304 Idaho ground squirrel samples. We trapped squirrels from early April to late July 2016 for NIDGS and from mid-March to early May 2016 for SIDGS. At each sampling location, we baited each open Tomahawk live trap (Tomahawk Live Trap Co;

13 × 13 × 41 cm and 15 × 15 × 50 cm) with an oat, peanut butter and imitation vanilla mixture along transects and placed traps near burrows or logs at relatively evenly spaced intervals. We trapped and handled all NIDGS following protocols developed by Idaho Department of Fish & Game (D. Evans Mack, unpublished data). We marked each trapped squirrel with a metal ear tag in each ear (National Band and Tag Co.) and collected DNA samples through buccal swabbing. For DNA collection, we used sterile cotton swabs (Lakewood Biomedical) to collect epithelial cells by swabbing the inside of the squirrels' cheek, and replicated this sampling five times per individual. We conducted all sampling following standard biological and ethical requirements under the auspices of University of Idaho Animal Care and Use Committee Protocol #2015-53. We preserved all replicates from the same individual in the same sample tube containing Qiagen ATL buffer and stored them at room temperature or 4°C until DNA extraction. We extracted all replicates from each DNA sample collected using the Qiagen Blood and Tissue DNA extraction kit (Qiagen, Inc.), and quantified DNA using fluorometric quantitation with a Qubit double-stranded DNA High Sensitivity Assay Kit. For NIDGS, we obtained 232 samples from 15 populations in Adams County in central Idaho (Figure 1b), which represents ~30% of the known populations. For SIDGS, we collected 72 samples from five populations in Washington, Payette and Gem Counties (Figure 1b). Although representing a small proportion of the >300 sites identified during surveys from 1999 to 2013, it is important to note that

sites are operationally defined as sightings of the species separated by more than 160 meters, and thus in many cases multiple sites can represent a single population than can extend over several kilometers (E. Jensen, pers. comm.). Additionally, activity was low or not detected at most sites across multiple surveys from 1999 to 2013, and no recent surveys have been performed to determine presence or abundance across the entire species range (USFWS, 2014). Thus the true number of SIDGS populations is currently unknown. Details on the location and number of samples collected per population are shown in Table 1. We used all DNA samples for restriction site associated DNA sequencing (RADseq) (Andrews et al., 2016; Baird et al., 2008; Murphy et al., 2007). We prepared libraries following Ali et al., (2016) using the *sbfl* restriction enzyme, but excluding the last part of that protocol relating to targeted bait capture, and instead only using the new RADseq protocol with biotinylated adapters. We built four libraries, each consisting of ~80 individually barcoded samples, and sequenced each library on one lane of an Illumina HiSeq 4000 at the University of Oregon Genomics & Cell Characterization Core Facility (GC3F), with 150-bp paired-end reads. We conducted all bioinformatic analyses using the Institute for Bioinformatics and Evolutionary Studies (IBEST) Computational Resources Core servers at the University of Idaho. For single-nucleotide polymorphism (SNP) calling, we used the software STACKS version 2.2 (Catchen et al., 2013) to demultiplex and remove polymerase chain rection (PCR) duplicates. We used the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) genome (GCA\_000236235.1) as a reference to align our data using the software BWA version 0.7.17 (Li, 2013). We conducted our analyses at both the interspecific and intraspecific levels in order to determine putative adaptive differences between the two sister species, but also searching for patterns of population structure and local adaptation among populations within species. To do so, we defined a first data set consisting of all 304 IDGS, and then separated the samples by species, NIDGS (232 samples) and SIDGS (72 samples) data sets. We called SNPs for the IDGS data set using the multisample SNP caller (mpileup) implemented in SAMTOOLS (Li et al., 2009), and subsequently used VCFTOOLS version 0.1.14 (Danecek et al., 2011) to exclude individuals with  $\geq 50\%$  missing data, keeping only biallelic SNPs that had  $< 50\%$  missing data, were located  $> 10,000$  bp apart, had  $> 2\%$  minor allele frequency and  $> 3$  reads. To produce the species-specific data sets, we separated the samples by species (232 NIDGS and 72 SIDGS) and repeated the above filtering with a minimum allele frequency (MAF) value of 3%. We then used part of the R script available from Wright et al., (2019) to exclude loci with heterozygosity  $> 70\%$  and with a  $> 3:1$  ratio of mean read depth for the reference allele vs. the alternative allele, across individuals with these alleles, to avoid loci with significant reference bias. Finally, we used the functions from the WHOA R package to estimate heterozygote miscall rates and excluded loci deviating more than one standard deviation from the mean (<http://150.185.130.98/rcran/web/packages/whoa/index.html>). For all data sets, we compared the percentage of missing data per individual (--missing-indv) with mean read depth (--depth) and heterozygosity (--het) obtained using VCFTOOLS version 0.1.14 (Danecek et al., 2011).

## 2.2 | Identification of adaptive variation

To detect putative loci under selection for the interspecific data set, we used four methods that differ in their assumptions regarding how putatively adaptive loci are detected, their sensitivities to the sampling strategy, neutral population structure, and the extent to which they allow for correlations among environmental variables (Ahrens et al., 2018; Capblancq et al., 2018). Specifically, we used a population structure outlier detection method (PCADAPT), which does not incorporate environmental variables, and three different methods to estimate genotype-environment associations (GEAs): a latent factor mixed model (LFMM), which is a univariate regression model that includes unobserved variables (latent factors) that correct the model for confounding effects such as population structure; a redundancy analysis (RDA), which is a multivariate approach that simultaneously analyses a multivariate response and multivariate predictors; and a partial redundancy analysis (pRDA), which is an RDA that accounts for (excludes) population structure (Capblancq et al., 2018; Forester et al., 2018). For the details on each of these analysis methods see online Supporting Information.

For the interspecific analysis (IDGS data set), we compared the SNPs identified as candidates in the four analyses using the *venn.diagram* function from the VENNDIAGRAM R package (Chen & Boutros, 2011). For the intraspecific analyses (NIDGS and SIDGS data sets), we only conducted the pRDA, given that our main focus was the detection of local adaptation, while controlling for population structure at the interspecific level. For each analysis data set, we performed principal components analysis (PCA) to obtain a visual inspection of population structure without underlying models of evolution. We used the *snpgdsPCA* function from the SNPRELATE R package (Zheng et al., 2012). We used the *ggplot* function from the GGLOT2 R package to construct the plots (Kassambara, 2018).

## 2.3 | Gene Ontology and species-specific adaptations

To gain insight into the ecological and biological functions of putative adaptive loci, we identified candidate loci found within genes and the Gene Ontology (GO) terms associated with such genes (Primmer et al., 2013). We used the Ensembl variant effect predictor (VEP) to perform annotation of the candidate loci (McLaren et al., 2016) and the software SNP2GO (Szkiba et al., 2014) to identify cellular component, biological process and molecular function GO terms associated with the candidate loci using a false discovery rate (FDR) of 0.05, and following the annotations of the thirteen-lined ground squirrel genome. We tested for enrichment considering all the candidates identified by the four analyses combined, as well as for each analysis individually. To evaluate the impact of the different method assumptions on GO term enrichment, we further tested for enrichment considering the identified candidate loci divided into several categories: population structure outlier approach (PCADAPT), representing those candidates uniquely identified for the PCADAPT analysis;

genotype environment associations ("GEA"), considering the candidates uniquely identified for the LFMM, RDA and pRDA analyses; candidates identified including population structure ("POP"), considering those loci uniquely identified for the PCADAPT and RDA analyses; and candidates identified when excluding population structure ("noPOP"), considering those loci uniquely identified for the LFMM and pRDA analyses. For all three data sets (NIDGS, SIDGS and the combined IDGS), we further considered the candidate SNPs resulting in nonsynonymous substitutions and used the online databases Ensembl and UniProt to identify the genes and proteins involved (Bateman, 2019; Yates et al., 2020).

## 2.4 | Population structure

Population structure analyses were performed separately for each species. To obtain a set of "neutral markers" for each species for these analyses, we first removed from each data set the loci identified as candidates for adaptation by the pRDA. Next, we identified loci that deviated from Hardy-Weinberg equilibrium (HWE) proportions for all samples combined using the function `--hardy` from VCFTOOLS, and we removed loci with  $p < .05$  from the data set.

We first estimated isolation-by-distance (IBD) using the *mantel* function from the VEGAN R package (Oksanen et al., 2015) for both the neutral and adaptive data sets. We used the function *dist.genpop* from the ADEGENET R package (Jombart & Ahmed, 2011) to calculate Nei's genetic distances (Nei, 1972, 1978) between all pairs of populations, and plotted those genetic distances against pairwise Euclidean geographical distances (average geographical location of each population). We performed this for both NIDGS and SIDGS data sets and for neutral and adaptive loci separately.

As before, we performed a PCA for the complete data set (all loci), the neutral data set and the adaptive data set of each species. For the neutral data, we also evaluated population structure for each species using an approach with an underlying Bayesian model to estimate the number of populations ( $K$ ), using the program STRUCTURE (Pritchard et al., 2000) and the PARALLELSTRUCTURE R package (Besnier & Glover, 2013). We applied an admixture model run for  $1 \times 10^6$  generations, and five replicates per  $K$ , with  $K = 1$  to  $K = 13$  in NIDGS and to  $K = 5$  in SIDGS, which correspond to the total number of populations sampled for each species. We then determined the optimal number of populations ( $K$ ) according to the Evanno method and the rate of change in the likelihood values (Evanno et al., 2005).

We estimated interspecific population differentiation using the IDGS total data set, and intraspecific population differentiation for NIDGS and SIDGS "neutral" and "adaptive" data sets, using the functions *genet.dist* and *boot.ppfst* from the R package HIERFSTAT to calculate genetic divergence ( $F_{ST}$ ) with 999 bootstraps between all geographical sites (Goudet, 2005). We then built correlation matrices of pairwise  $F_{ST}$  between all populations using the function *corrplot* from the CORRPLOT R package (Wei et al., 2017). Using the same data sets, we determined genetic diversity by estimating observed and expected heterozygosity ( $H_O$  and  $H_E$ , respectively) per

population. To estimate  $H_O$  and  $H_E$ , we used the function *basic.stats* from the HIERFSTAT R package (Goudet, 2005), averaging locus heterozygosity per population. We then performed a paired Wilcoxon test to identify significant differences between  $H_O$  and  $H_E$  for both neutral and adaptive loci among populations of each species, between  $H_E$  of both neutral and adaptive loci among populations of each species, and  $H_E$  between neutral and adaptive data sets for each species, using the function *t.test* from the STATS R package. For all tests of genetic diversity, we included all populations with  $N \geq 2$ , given the potential for accurate population structure metrics for  $N \geq 2$  when large numbers of loci are used (Nazareno et al., 2017). We also report  $H_O$  for populations with a single individual genotyped, given that the genetic variation of a single nonmigrant individual can provide an approximate representation of the population (Lemopoulos et al., 2019), and given the generally low migration rates between populations observed in this species (Hoisington, 2007).

## 3 | RESULTS

### 3.1 | Sampling and SNP calling

We extracted DNA from 232 NIDGS and 72 SIDGS buccal swabs and DNA yield varied from 0.1 to 51.0 ng  $\mu\text{l}^{-1}$ . Of 304 samples for which we conducted RADseq, for the IDGS data set we kept 80 individuals (26% of samples retained) and 4,227 SNPs after filtering the sequence data, with an average mean read depth of coverage of 9.3 reads per individual (min. 3.6 and max. 20.6) (Figure S1a). For the NIDGS data set, we kept a total of 80 individuals (34% of samples retained) and 3,575 SNPs, resulting in an average mean read depth of 7.5 (min. 0.9 and max. 18.8) (Figure S1b). For the SIDGS data set, we kept 41 individuals (57% of samples retained) and 2,348 SNPs, resulting in an average mean read depth of 4.9 (min. 1.0 and max. 16.1) (Figure S1c). Heterozygosity tended to decrease with increasing missing data and decreasing mean read depth, but no single population showed a particular bias towards low read depths across all individuals (Figure S1b,c).

### 3.2 | Interspecific adaptive variation

For the PCADAPT analysis, we considered the first two PCs that explained a total of ~16% of the genetic variance (Figure S2a). The first PC explained ~10% of the variance and clearly separated NIDGS from SIDGS, and some variation within SIDGS, while the remaining axes mostly reflected intraspecific variation within NIDGS (Figure S2b,c). Using a value of  $\alpha = 0.01$ , we detected 48 outlier loci using an adjustment of the  $p$ -values following the Benjamini-Hochberg procedure.

For the GEAs, most environmental variables were not normally distributed, and  $p$ -values for the Shapiro-Wilk normality were all  $< .001$  for all variables. Based on the results of the Kendall correlation analysis, we kept 18 variables for the GEAs, given that these

showed low correlation with one another (Figure S3a). The PCA performed with the environmental variables showed that only PC1 explained more variance than the random broken stick criterion, and thus was kept as a summary of the environmental data to compare in the LFMM univariate analysis (Figure S4a). The PCA performed for the genomic data showed that only PC1 explained more variance than the random broken stick criterion, which suggests that the number of populations was two (Figure S3b). We ran the LFMM using  $K = 2$ , which resulted in a distribution of unadjusted  $p$ -values with a genomic inflation factor of 2.42, which was then adjusted to 1.4 (Figure S5). Using an FDR of 10%, the LFMM analysis identified 52 candidate loci.

We then performed the RDA and pRDA for the IDGS data set. For both analyses, from the 18 environmental variables used, we excluded three (BIO1, BIO2 and LF\_VA) due to high variance inflation factor (VIF). We obtained an adjusted  $r^2$  of .08 and .06 for the RDA and pRDA, respectively, across 15 axes, which correspond to the proportion of genetic variance explained by the environmental predictors kept in each analysis. Analysis of variance (ANOVA) showed that both analyses were significant at  $p < .001$ , and that the first four axes of both analyses were significant at  $p < .001$  (Figure S6). The first RDA axis explained 4.8% of the variance, while RDA2, RDA3 and RDA4 explained 2.8%, 2.5% and 2.2%, respectively. The remaining axes combined (RDA5–15) explained the remaining 13.5% of the total variance (Figure 2a,b). In this analysis, there was a clear separation of NIDGS and SIDGS individuals in RDA1, while RDA2–4 mostly separated populations within species. The environmental variables that loaded the most strongly with RDA1, and thus the separation between NIDGS and SIDGS, were “Steep Slopes” (LF\_SS, associated with 20 SNPs), “Grassland/Herbaceous” (LC\_GH, associated with 32 SNPs) and “Soil Temperature” (soilPartSize, associated with 20 SNPs) (Table 2 and Figure 2a). When accounting for population structure with the pRDA, we no longer find a clear distinction between NIDGS and SIDGS, and instead variation appears to mimic that of the RDA without RDA1 (Figure 2). The first pRDA axis explained 2.9% of the variance (similarly to RDA2), while pRDA2, pRDA3 and pRDA4 explained 2.6%, 2.3% and 2.1%, respectively. The remaining axes combined (RDA5–15) explained the remaining 12.7% of the total variance (Figure 2c,d). We also found a total of 340 SNPs associated with 17 environmental variables, with 38.6% (122 SNPs) also associated with the land formation variable “Ridges and Peaks” (LF\_RP) (Table 2).

Considering all analyses combined, we identified a total of 490 candidate loci, none of which were found by all four analyses (Figure 3). Comparing the population structure outlier approach (PCADAPT) to the GEAs (LFMM, RDA and pRDA), there are 29 loci found by all GEAs vs 18 found only by the population structure outlier approach (Figure 3). Methods that do not account (i.e., correct) for population structure (PCADAPT and RDA) identified 21 loci in common, while methods that account for population structure (LFMM and pRDA) identified one locus in common. The largest overlap between two analyses was 168 loci found by both multivariate GEAs (RDA and pRDA), which also showed very similar results in terms

of population differentiation. The outlier detection analysis (PCADAPT) found a clear distinction between NIDGS and SIDGS, while both multivariate GEAs supported Rocky Top (NIDGS) and Olds Ferry (SIDGS) as the most distinct populations at the adaptive level. The univariate GEA (LFMM) only identified Olds Ferry as distinct.

### 3.3 | Intraspecific adaptive variation

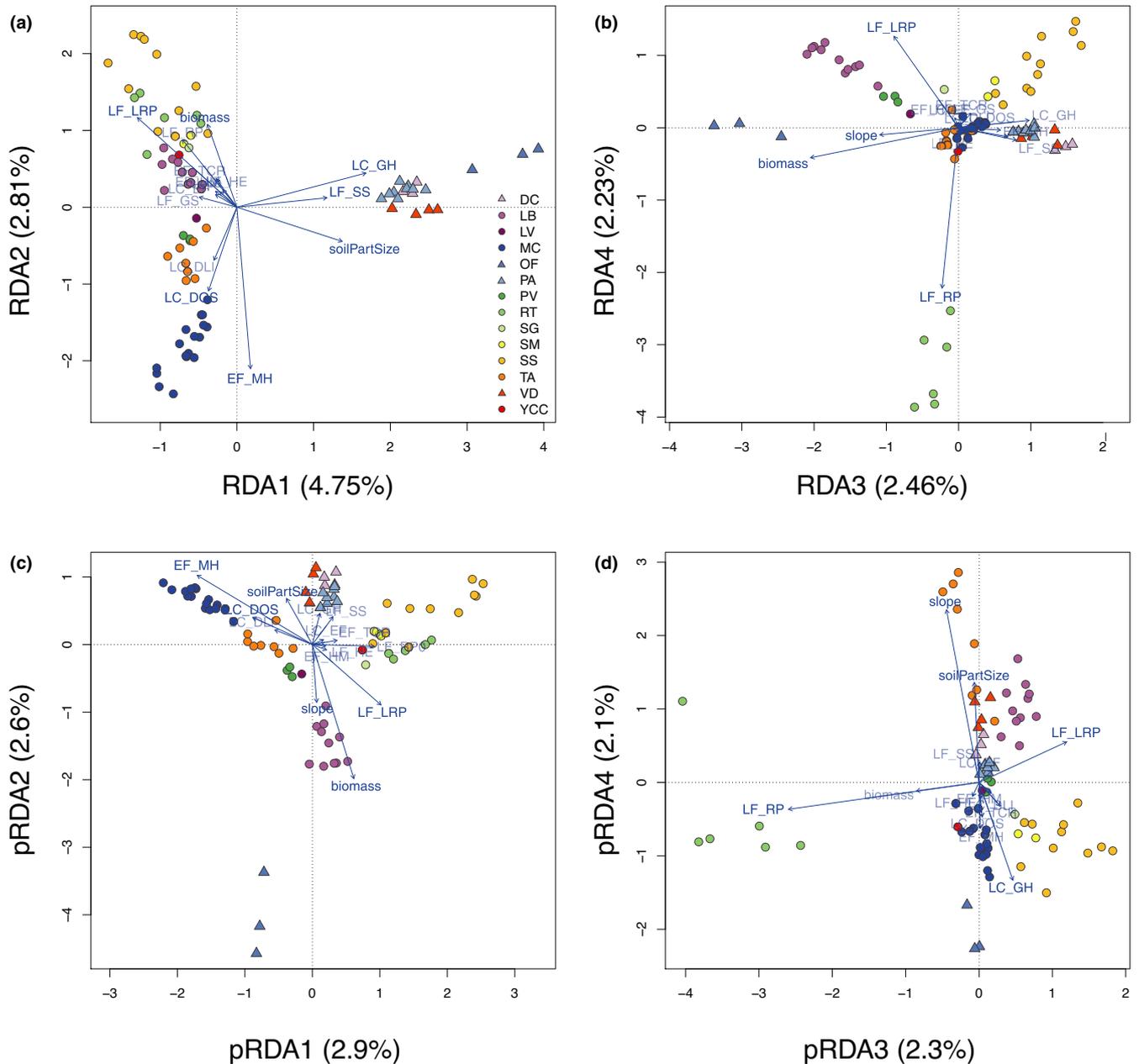
To test for adaptive differences among populations of NIDGS and SIDGS, we performed pRDA on each data set separately. The environmental variables that we used differed among data sets, resulting in 19 variables for NIDGS, while only four variables were kept for SIDGS due to a large number of correlations (Figure S3b,c).

For NIDGS, PCA of the genomic data showed that none of the PCs have eigenvalues greater than random, suggesting  $K = 1$  (Figure S7a). However, this result does not mean there is no genetic structure in the data, but rather that it might not be particularly strong. Thus, because we have evidence from previous studies that NIDGS are divided into at least two groups (Garner et al., 2005; Hoisington-Lopez et al., 2012; Zero et al., 2017), we decided to condition the pRDA using PC1 (i.e., assuming  $K = 2$ ). From the 19 environmental variables used, we excluded four (BIO\_1, BIO\_12, BIO\_15 and LF\_VA) due to high VIF. We then obtained an adjusted  $r^2 = .06$  across 15 axes. ANOVA showed that the pRDA was significant at  $p < .001$ , and that the first three axes were significant at  $p < .001$ . The first pRDA axis explained 3.3% of the variance, while pRDA2 and pRDA3 explained 3.0% and 2.7%, respectively. The remaining axes combined (pRDA4–15) explained 14.0% of the remaining variance. From this analysis, three populations were the most distinct in association with particular environmental variables: Lower Butter (LB) mostly associated with Local Ridge in Plain (LF\_LRP) and slope, Rocky Top (RT) mostly associated with Ridges and Peaks (LF\_RP), and Tamarack (TA) mostly associated with soil particle size (soilPartSize) (Figure 4a,b).

For SIDGS, the PCA on the genomic data showed that PC1 had eigenvalues greater than random, suggesting  $K = 2$  (Figure S7b). From the four environmental variables used, all were kept for the final pRDA, which had an adjusted  $r^2 = .01$ . ANOVA showed that the pRDA was significant at  $p < .001$ , and that only the first axis was significant at  $p < .001$ , explaining 3.7% of the variance. The remaining three axes combined (pRDA2–4) explained 6.7% of the remaining variance. From this analysis, Paddock (PA) was the most distinct population, correlating mostly with grassland areas (LC\_GH), as opposed to the other populations which are more associated with shrub/scrubland, and Annual Mean Temperature and Isothermality (Figure 4c).

### 3.4 | Gene ontology

For the IDGS data set, we obtained a total of 490 candidate SNPs, of which only four were found in coding genes and produced nonsynonymous substitutions (Table 3). Of these four, two were assigned



**FIGURE 2** Genotype-environment association (GEA) analyses for the IDGS imputed data set, consisting of 80 individuals genotyped at 4,227 SNPs. (a,b) Redundancy analysis (RDA) results and (c,d) partial RDA (pRDA) results. Coloured circles (NIDGS) and triangles (SIDGS) represent ground squirrel samples and how they load in the RDA space, coloured by population. Locality codes refer to those listed in Table 1. Only the four significant ( $p < .001$ ) *rda* axes of each analysis are shown. Arrows represent the environmental variables considered and their loadings in the *rda* space. Full description of the codes for environmental variables can be found in Figure S2. For simplicity, environmental variables loading between  $-1$  and  $1$  for both axes shown were de-emphasized [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

to uncharacterized proteins and both were found to be fixed for the reference allele in NIDGS and variable in SIDGS. Considering the other two candidate SNPs, one was found on the B3GNT8 gene, which codes for the hexosyltransferase protein. This SNP was identified as a candidate by the RDA, and its strongest association was with land cover type “Grassland/Herbaceous” (0.384). The second missense candidate SNP was found on the NPR1 gene, which codes for the guanylate cyclase eef. It was identified as a candidate

by both the PCADAPT and RDA analyses, and in the latter it was most strongly associated with “soil particle size” (0.397). For details regarding the assignment of the remaining candidate loci to additional variant classes, refer to Table 3. Considering the results for each analysis separately, the PCADAPT, LFMM, RDA and pRDA identified 48, 52, 316 and 340 candidate loci, respectively, and with 18, 13, 89 and 139 candidate SNPs uniquely identified, respectively (Figure 3). Considering the different categories considered, we considered 18,

**TABLE 2** Environmental variables used in the GEA analyses, with respective codes and number of SNPs found in association for the RDA and pRDA analyses using a 2.5 standard deviation cutoff (two-tailed  $p$ -value = .012).

Environmental variable	Code	IDGS		SIDGS	
		RDA	pRDA	pRDA	pRDA
Annual Mean Temperature	BIO_1	–	–	–	12
Isothermality	BIO_3	–	–	2	4
Biomass	biomass	35	82	–	–
Ecological Factors					
Moderate Hills	EF_MH	37	26	1	–
Tablelands of Considerable Relief	EF_TCR	1	4	0	–
High Mountains	EF_HM	1	4	0	–
Land Cover					
Development, Open Space	LC_DOS	14	6	1	–
Development, Low Intensity	LC_DLI	11	5	0	–
Evergreen Forest	LC_EF	1	1	0	–
Grassland/Herbaceous	LC_GH	32	7	0	2
Land Formation					
Headwaters	LF_HE	2	5	1	–
Ridges and Peaks	LF_RP	120	122	68	–
Local Ridge in Plain	LF_LRP	17	17	25	–
Gentle Slopes	LF_GS	1	–	0	0
Steep Slopes	LF_SS	20	1	–	–
Slope	slope	4	59	23	–
Soil Particle Size	soilPartSize	20	1	29	–
Total		316	340	150	18

Note: Dashes represent environmental variables that were not considered for a given data set and zeroes indicate variables that were considered but no SNPs were found to be significantly associated.

472, 337 and 362 candidate loci for the population structure outlier approach (PCADAPT), genotype environment associations (“GEA”), and including (“POP”) and excluding (“noPOP”) population structure, respectively, of which 18, 26, 21 and one were unique to each analysis type, respectively (Figure 3). GO enrichment analysis of candidate SNPs found no evidence for enrichment of particular cellular components, biological processes or molecular functions for any of the individual or combined analyses considered.

For the NIDGS and SIDGS data sets, we found 150 and 18 candidate SNPs, respectively. For NIDGS, two candidate SNPs were found in coding regions and resulted in nonsynonymous substitutions, although both were assigned to uncharacterized proteins. For SIDGS, no SNPs were found to result in nonsynonymous substitutions (Table 3). For details regarding the assignment of the remaining candidate loci to additional categories, refer to Table 3. For these two data sets, we also found no significant GO term enrichment for all identified candidate SNPs.

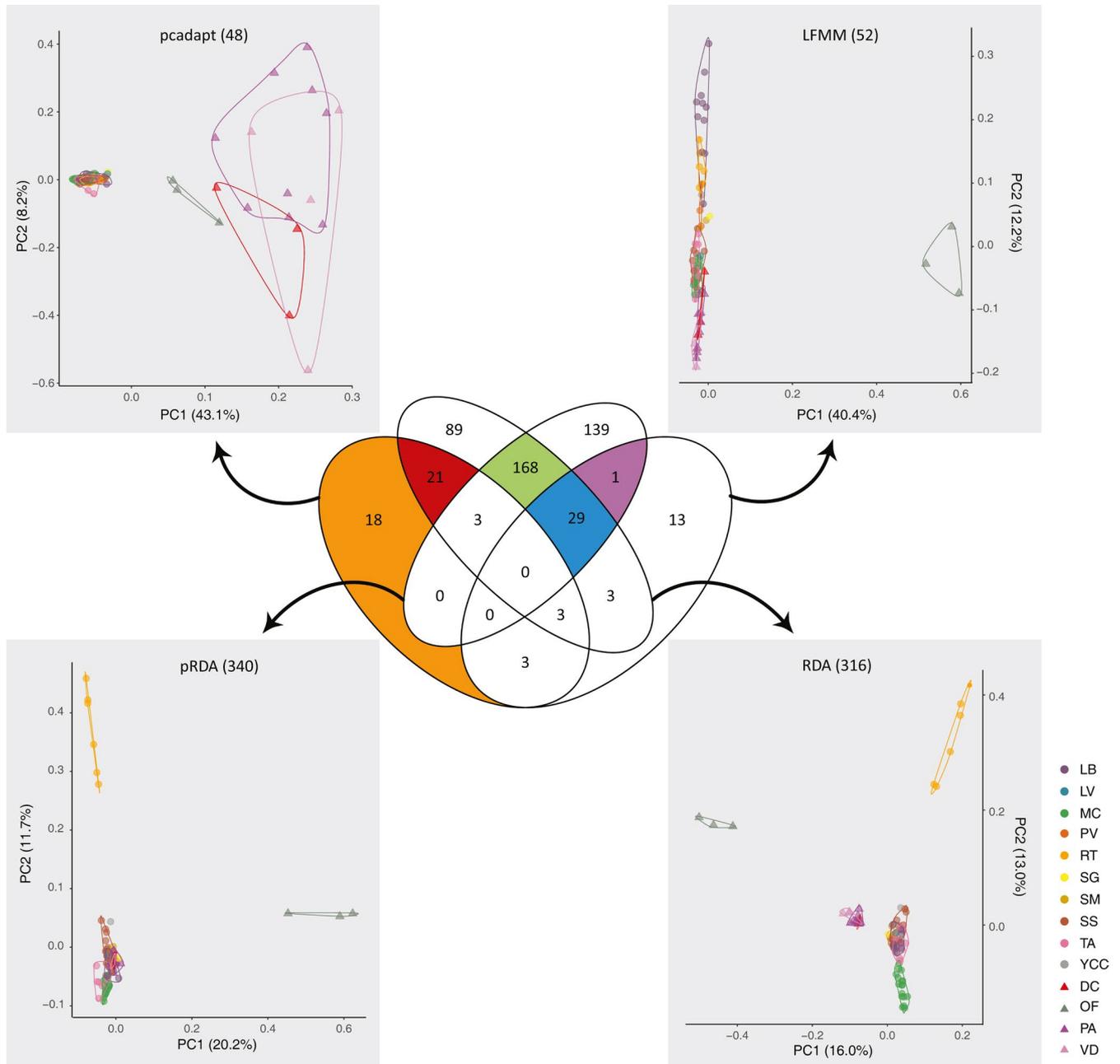
### 3.5 | Population structure and genetic diversity

After excluding the candidate adaptive loci and loci deviating from HWE, we kept 2,663 and 1,711 SNPs for the NIDGS and SIDGS,

respectively. With these sets of neutral loci, we determined that there is a significant pattern of IBD for NIDGS ( $r = .136$ ,  $p = .039$ ) but not for SIDGS ( $r = .556$ ,  $p = .090$ ). IBD was not significant for the NIDGS adaptive data set ( $r = .018$ ,  $p = .396$ ), or for the SIDGS adaptive data set ( $r = -.378$ ,  $p = .950$ ).

Principal components analysis results for the total and neutral data sets in both species were identical (Figure S8). From the NIDGS PCA, there were four axes for the total and neutral data sets and three axes for the adaptive data set explaining a significant proportion of the variance (Figures 5a,b; S8). Both the neutral and adaptive data sets identified similar populations as the most distinct, particularly Lower Butter, Rocky Top and Tamarack (Figure S8). Mud Creek was only distinctive at the neutral level. Considering potential groups, at the first two PCs, Lower Butter was clearly separated from an eastern group (Mud Creek, Tamarack, Price Valley, Lost Valley) and a western group (all other populations). From the SIDGS PCA, we considered one axis to be significant for the total, neutral and adaptive data sets: the total and neutral data sets identified Olds Ferry as the most distinctive populations, while the adaptive data set showed a clear separation of Paddock (Figures 5c,d; S8).

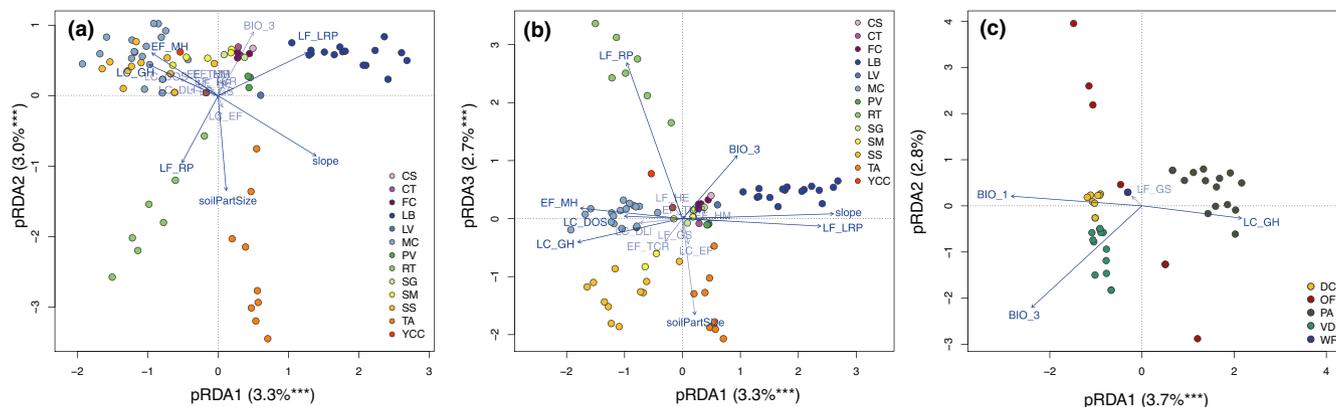
The PCA neutral results for both species agree with the STRUCTURE analyses (Figure 6). For NIDGS, STRUCTURE suggested separation of NIDGS into two main groups corresponding to a western group and



**FIGURE 3** Venn diagram and principal components analyses (PCA) for each of the four individual adaptive loci detection analyses for the IDGS data set. The Venn diagram shows the overlap of the 490 candidate loci identified by the four analyses performed; numbers in parentheses next to the analysis name represent the total number of candidates identified; colours represent the loci associated with different analysis types: population structure outlier approach (orange), genotype environment associations (GEAs, blue), including and excluding population structure (red and purple, respectively), and multivariate vs. univariate GEA (green). For the PCA plots, circles (NIDGS) and triangles (SIDGS) represent ground squirrel samples, coloured by population (locality codes refer to those listed in Table 1), and with a line of the same colour circling the extent of the variation of each population; samples are positioned considering how they load in the PCA space for each analysis, with variance in parentheses for each axis [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

also an eastern group (Figure 6a,  $K = 2$ ). Here, Lower Butter was included in the western group, although there was a high proportion of admixture with the eastern group as well. Lower Butter was differentiated from all other populations at all  $K$  values starting from  $K = 3$  (Figure S10a). The *STRUCTURE* analysis indicated a second peak in the marginal likelihood for  $K = 6$  using the Evanno et al., (2005) method, suggesting the presence of fine-scale population structure

within NIDGS (Figures 6a; S9a,b and S10a). These results are also reflected in the pairwise  $F_{ST}$  estimates, where there is no significant differentiation among seven of the eight westernmost populations (hereafter *west* NIDGS), even at the adaptive level (Figures S11a,b). All other populations are hereafter referred to as *east* NIDGS. Rocky Top, Lower Butter and Mud Creek were the NIDGS populations with the highest neutral average pairwise  $F_{ST}$ , with 0.09, 0.08 and



**FIGURE 4** Partial redundancy analyses (pRDA) for the NIDGS data set (a, b) and the SIDGS data set (c). Individuals are coloured by population represented on the legend of each figure, for which locality codes refer to those listed in Table 1. All significant axes of both analysis are shown ( $p < .001$ , \*\*\*). Arrows represent the environmental variables considered and their loadings in the *rda* space. Full description of the codes for environmental variables can be found in Figure S3. For clarity, environmental variables loading between  $-1$  and  $1$  for both axes shown in each plot were de-emphasized [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**TABLE 3** Distribution of the candidate loci identified from the IDGS, NIDGS and SIDGS data sets along variant classes of the variant effect predictor (VEP) analysis considering the most severe consequences of each locus

	Nsyn	Syn	Splice	3'	Intron	Upstr	Downstr	Interg
IDGS	4	8	3	4	172	18	21	260
PCADAPT	1	—	—	—	18	2	—	27
<i>LFMM</i>	—	2	—	—	22	—	2	26
<i>RDA</i>	3	5	2	5	108	11	13	172
<i>pRDA</i>	2	6	3	3	119	14	15	178
NIDGS	2	2	1	2	47	11	4	81
SIDGS	—	—	—	—	8	3	—	7

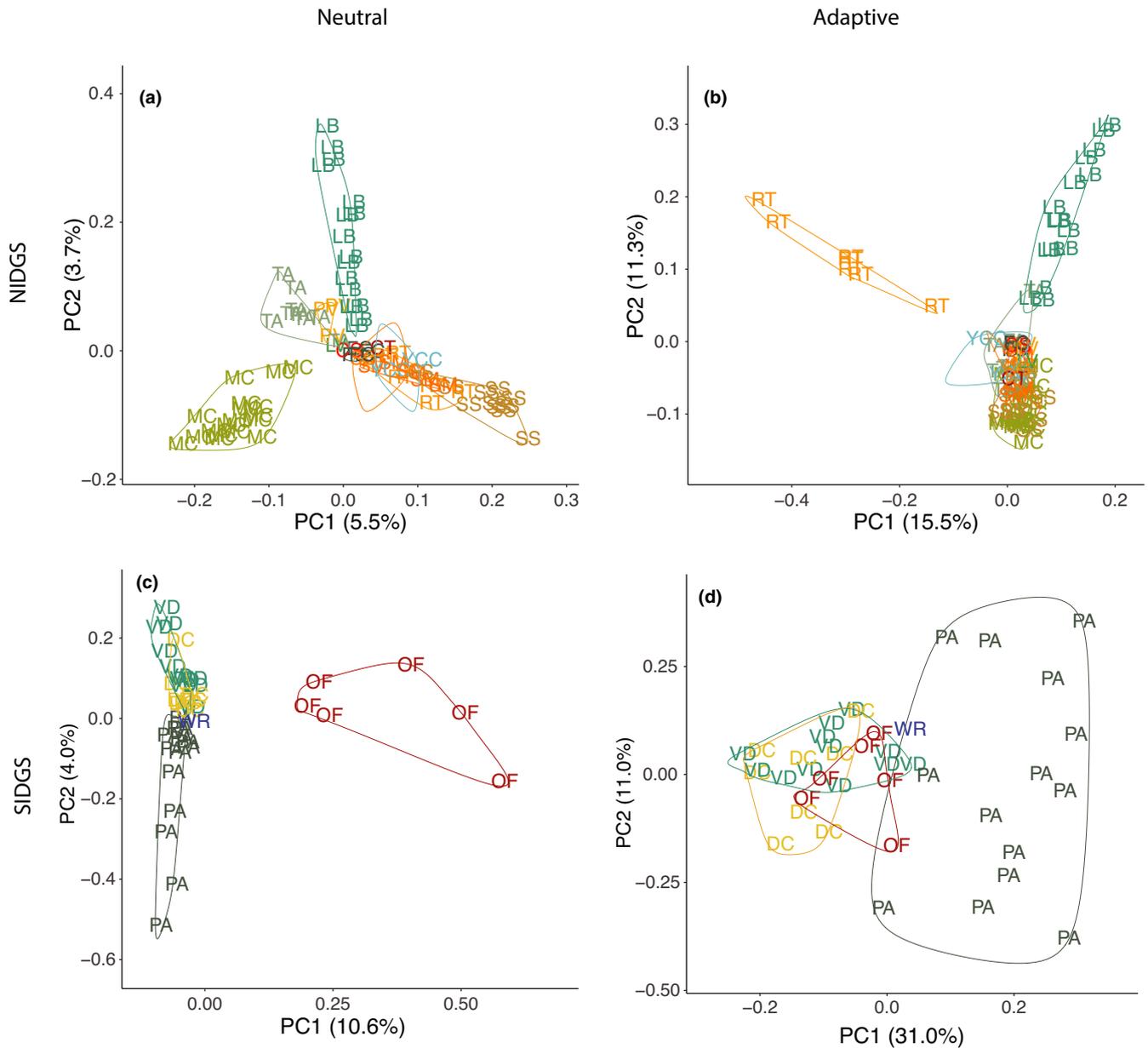
Note: "IDGS" considers all four analyses performed, which are described below in italics individually. NIDGS and SIDGS candidate SNPs were identified using a pRDA approach only. Candidate loci are classified in the following classes: nonsynonymous variants ("nsyn"), synonymous variants ("syn"), splice region variants ("splice"), 3-prime utr variants ("3'"), intron variants ("intron"), upstream gene variants ("upstr"), downstream gene variants ("downstr"), and intergenic variants ("interg").

0.08, respectively, considering an overall average of 0.06, while the highest adaptive average pairwise  $F_{ST}$  was found for Rocky Top and Lower Butter, with 0.26 and 0.20, respectively, considering an average of 0.10. The SIDGS *STRUCTURE* results also mimicked those from the neutral PCA where individuals were found to be divided into two groups, with a separation of Olds Ferry from all other SIDGS localities at  $K = 2$  (Figure 6b).

In terms of population differentiation, at the interspecific level, average  $F_{ST}$  between NIDGS and SIDGS was 0.143, compared to 0.05 and 0.100 within NIDGS and SIDGS for the same data set, respectively. At the intraspecific level and considering the SIDGS data set separately, average neutral pairwise  $F_{ST}$  was highest for Olds Ferry (0.13), considerably higher than the average value of 0.04 (Figure S11c). However, as seen in the SIDGS adaptive PCA, Paddock was the most distinct population and also had the highest average pairwise  $F_{ST}$  (0.24), considerably higher than the average value of 0.15 (Figure S11d).

Considering the levels of genetic diversity, for the IDGS data set, no NIDGS population showed significantly decreased  $H_O$  compared

to  $H_E$ , but Lower Butter did show significantly higher  $H_O$  than  $H_E$  (Table 4). By contrast, for the same data set, two out of four SIDGS populations showed significantly lower  $H_O$  than  $H_E$ . SIDGS populations tended to have significantly lower  $H_E$  than NIDGS populations (Table S1). At the intraspecific level, for NIDGS we observed an average of neutral  $H_O$  (and  $H_E$ ) of 0.279 (0.300) and 0.270 (0.301) for west and east metapopulations, respectively (Table 4). At the adaptive level, we observed an average  $H_O$  (and  $H_E$ ) of 0.170 (0.178) and 0.244 (0.313) for west and east metapopulations, respectively (Table 4). Genetic diversity was not significantly different between metapopulations at the neutral level, but was significantly lower in west at the adaptive level. Additionally, neutral diversity was significantly higher than adaptive diversity in both metapopulations. For the SIDGS, we observed an average of neutral  $H_O$  (and  $H_E$ ) of 0.288 (0.287) and 0.219 (0.277) for west and east metapopulations, respectively (Table 4). The east metapopulation showed significantly lower genetic diversity at both the neutral (only  $H_O$ ) and adaptive (only  $H_E$ ) levels, as well as significantly lower genetic diversity at the neutral compared to the adaptive level (both  $H_O$  and  $H_E$ ).



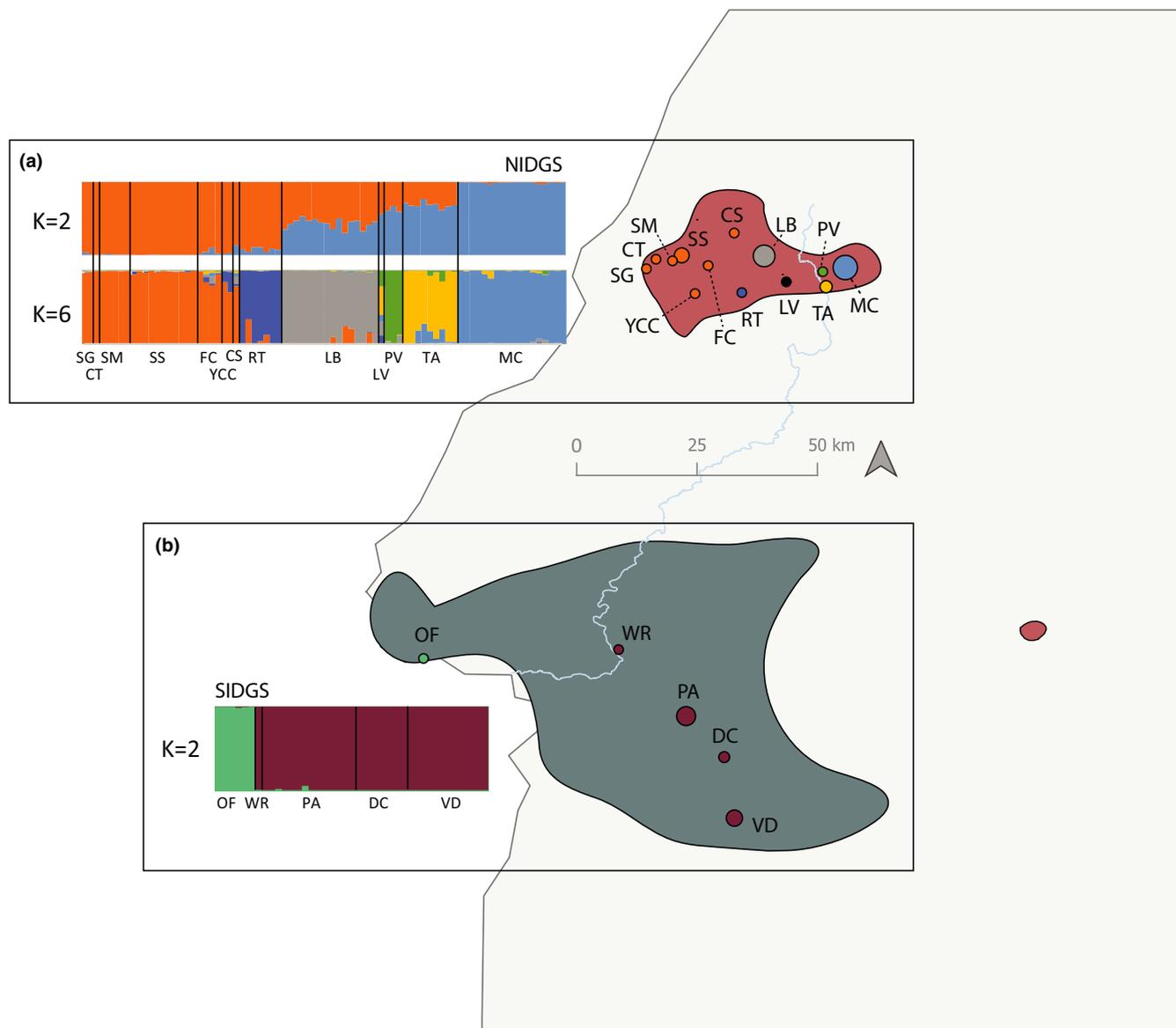
**FIGURE 5** Principal component analysis for the NIDGS neutral (a) and adaptive (b) data sets and the SIDGS neutral (c) and adaptive (d) data sets. Each letter code represents one individual of the respective population name (codes as in Table 1), which are coloured by population. All individuals of the same population are encircled with a line of the same colour. For NIDGS, only PC1 and PC2 are represented, although additional axes were significant (see Figure S10). For SIDGS, only PC1 is significant [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

## 4 | DISCUSSION

We examined the genetic basis of adaptive divergence between two endemic and vulnerable sister species of Idaho ground squirrels and assessed the diversity and differentiation at both neutral and adaptive loci among populations of each species. We found that combining different methods to identify adaptive variation is useful to address adaptive differentiation across the speciation continuum, particularly in recently diverged species, which can be subject to strong and heterogeneous local adaptation among populations within species (Luikart et al., 2019). Additionally, we found that

environmental association analyses (EAAs) generally led to the detection of a larger number of candidate loci. EAAs have more power to detect loci associated with polygenic adaptation, and are fairly robust to the confounding effects of demography, compared to outlier detection methods (Ahrens et al., 2018; Dalongeville et al., 2018).

Comparing the analyses at the inter- and intraspecific level, we found that there were more differences between than within species regarding the association with specific environmental conditions; these differences were consistent both before and after controlling for collinearity with demographic history. At the intraspecific level, more heterogeneous environments promoted increased adaptive



**FIGURE 6** Population structure analyses and conservation unit delimitation for the NIDGS (a) and SIDGS (b). *STRUCTURE* barplots represent the most supported  $K$  ( $K = 2$ ) for the neutral data sets using the Evanno method, after excluding loci deviating from HWE. For NIDGS, we further included  $K = 6$ , as the second most supported  $K$ , as it also provided biologically meaningful results. Populations on the map are coloured according to the largest proportion of assignment to a given  $K$  population based on  $K = 6$  in NIDGS and  $K = 2$  in SIDGS. For NIDGS, population LV is coloured black due to multiple mixed ancestry. Within each species, different colours match the *STRUCTURE* plot population symbols represent. Locality codes refer to localities listed in Table 1. Background red and blue areas represent the distribution of NIDGS and SIDGS, respectively. The light blue and grey lines represent the Weiser River and the Idaho state border, respectively [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

differentiation between populations. This was true both in cases where populations were distinct at the neutral (demographic) level, and in cases where they were not. Understanding patterns of both neutral and adaptive genetic diversity across a species range is important for conservation and management, particularly in the current context of increasing habitat and environmental change (DeWoody et al., 2021; Teixeira & Huber, 2021).

Our work highlights the complex dynamics of inter- and intraspecific differentiation of two closely related small mammal species that are highly susceptible to environmental and habitat change.

#### 4.1 | (dis)Agreement between adaptive loci detection methods

In this study we used multiple methods to identify putatively adaptive loci at the interspecific level. Considering sets of loci detected by multiple methods is a conservative approach for identifying loci associated with local adaptation, while avoiding being overly conservative (Garcia-Elfring et al., 2019). The low overlap between loci identified across detection methods is probably related to the different assumptions and algorithms underlying the different

TABLE 4 Genetic diversity per population, estimated as observed and expected heterozygosity ( $H_O$  and  $H_E$ , respectively) for the total data set of the IDGS ("Interspecific") and for the neutral and adaptive data sets of the NIDGS and SIDGS ("Intraspecific"), with indication of the number of samples per population ( $N$ )

Population	Interspecific					Intraspecific				
	$N$	$\pi$	PA	$H_O$	$H_E$	$N$	Neutral		Adaptive	
							$H_O$	$H_E$	$H_O$	$H_E$
NIDGS										
West										
Cap Gun/Tree Farm (CT)	0	–	–	–	–	1	0.162	–	0.161	–
Cold Springs (CS)	0	–	–	–	–	1	0.210	–	0.167	–
Fawn Creek (FC)	0	–	–	–	–	4	0.244	0.273	0.228	0.220
Huckleberry (HU)	0	–	–	–	–	0	–	–	–	–
Summit Gulch (SG)	1	0.200	0	0.128	–	2	<b>0.188</b>	<b>0.278</b>	0.140	0.182
Squirrel Manor (SM)	2	0.226	1	0.141	0.140	5	<b>0.253</b>	<b>0.290</b>	<b>0.145</b>	<b>0.214</b>
Steve's Creek/Squirrel Valley (SS)	11	0.243	66	0.152	0.148	11	0.309	0.305	0.177	0.168
YCC	1	0.209	0	0.134	–	2	<b>0.226</b>	<b>0.275</b>	0.194	0.200
Total						26	<b>0.279</b>	<b>0.300</b>	0.170	0.178
East										
Lost Valley (LV)	1	0.187	1	0.106	–	1	0.211	–	0.193	–
Lower Butter (LB)	11	0.227	37	<b>0.147</b>	<b>0.138</b>	16	0.269	0.278	0.272	0.263
Mud Creek (MC)	17	0.225	60	0.136	0.130	18	0.280	0.286	0.190	0.175
Price Valley (PV)	3	0.189	2	0.115	0.125	3	<b>0.197</b>	<b>0.259</b>	<b>0.126</b>	<b>0.207</b>
Rocky Top (RT)	6	0.221	23	0.140	0.132	7	0.253	0.258	0.324	0.294
Slaughter Gulch (SL)	0	–	–	–	–	0	–	–	–	–
Tamarack (TA)	8	0.236	23	0.141	0.140	9	0.285	0.296	0.264	0.270
Total						54	<b>0.270</b>	<b>0.301</b>	<b>0.244</b>	<b>0.313</b>
SIDGS										
West										
Olds Ferry (OF)	3	0.188	76	0.133	0.124	6	0.288	0.287	0.265	0.251
East										
Dry Creek (DC)	3	0.156	11	<b>0.100</b>	<b>0.136</b>	8	<b>0.167</b>	<b>0.268</b>	0.197	0.210
Paddock (PA)	9	0.196	83	<b>0.123</b>	<b>0.133</b>	14	<b>0.237</b>	<b>0.283</b>	0.382	0.410
Van Deusen (VD)	4	0.187	17	0.122	0.127	12	<b>0.225</b>	<b>0.263</b>	0.183	0.226
Weiser River (WR)	0	–	–	–	–	1	0.235	–	0.133	–
Total						35	<b>0.219</b>	<b>0.277</b>	<b>0.281</b>	<b>0.403</b>

Note: We additionally report neutral and adaptive  $H_O/H_E$  for IDGS metapopulations (east and west) at the intraspecific level. Bold  $H_O/H_E$  pairs represent comparisons that were significantly different at  $p < .05$ . Dashes indicate that no estimate was made due to sample size. Estimates of  $H_O$  with  $N = 1$  represent individual heterozygosity as a proxy for population heterozygosity.

methods (Ahrens et al., 2018; Capblancq et al., 2018; Dalongeville et al., 2018).

While the outlier approach provides an overview regarding overall single SNP differentiation above what is expected from neutrality, the different GEAs identify loci associated with environmental variables, and are thus more likely to be informative on patterns of local adaptation (Forester et al., 2018; Hoban et al., 2016). With the outlier detection method (PCADAPT), we found a clear separation between species at the adaptive level, which might be related to

ecological differentiation of the two IDGS species (McEwen et al., 2013; Nosil et al., 2009). Still, the observed adaptive differentiation could partially result from collinearity with demographic processes and associated genetic drift, given that outlier detection methods do not explicitly exclude population structure from the analysis, and different GEAs account for it in different ways and based on different assumptions (Ahrens et al., 2018; Forester, Lasky, et al., 2018; Seehausen et al., 2014). For the LFMM and RDA analyses, the causal factors driving differentiation between species (e.g., neutral

or adaptive processes or some combination) also cannot be easily disentangled due to collinearity between environment and demography at the interspecific level (Ahrens et al., 2018). However, these two analyses perform better at identifying adaptive variation when selection gradients are weakly correlated with population structure (Capblancq et al., 2018). The adaptive patterns found by the two multivariate GEAs (RDA and pRDA) were very similar, even though the pRDA explicitly excluded the effect of population structure. This congruence might be related to the fact that, in RDAs, the rates of false positives tend to be lower when allele frequencies show high correlations with ecological variables (Frichot et al., 2015). Thus, despite a clear effect of population structure in the data, the identified SNPs seem to overlap strongly with analyses that control for demographic patterns.

Although we did not find particular GO terms associated with putative functions of adaptive genes in our analysis, we found two nonsynonymous substitutions in known genes identified by the interspecific RDA, one of which was also identified by *PCADAPT*, *NPR1*. This gene has been found to be highly expressed in brown adipose tissue of thirteen-lined ground squirrels during hibernation, relating to heat production during periodic arousals from hypothermic torpor (Hampton et al., 2013). Adaptations relating to adipose tissue are common in high-altitude adapted rodents (Gossmann et al., 2019), and these associations suggest that there are adaptive differences between IDGS species relating to their torpor patterns. In fact, one study on Columbian ground squirrels (*Urocitellus columbianus*) found that emergence timing was heritable and hence, squirrels' emergence date may also reflect local adaptations (Lane et al., 2011). Altitude differences might have resulted in adaptive differences between NIDGS and SIDGS in production and storage of fat, and increased metabolism and oxygen transport at higher elevations, as seen in other mammals (Faherty et al., 2018; Garcia-Elfring et al., 2019; Waterhouse et al., 2018; Werhahn et al., 2018).

## 4.2 | Differentiation of recently diverged species vs populations

As in previous studies (Garner et al., 2005; Hoisington, 2007; Hoisington-Lopez et al., 2012), we observed a clear genetic separation between NIDGS and SIDGS. This separation was clear at both the neutral and adaptive levels, which is a common pattern observed in ecologically divergent groups that are evolving in allopatry (Nosil et al., 2009). We found more loci associated with environmental variables among the two species of IDGS (340 SNPs) than within NIDGS (150 SNPs) and SIDGS (18 SNPs). These results show that adaptive differences between species are more numerous and probably more scattered across the genome compared to adaptive differences within species (Nosil & Feder, 2013). Additionally, the initial adaptive or nonadaptive vicariance of the two species has evolved into what can now be considered ecological speciation (Rundell & Price, 2009). These patterns point to a process of speciation originated from the vicariance of the two IDGS groups with gradually reduced

gene flow, increased neutral genetic drift, and subsequent ecological divergence (and potential reproductive isolation) as a result of local adaptation (Nosil et al., 2009; Rundell & Price, 2009). While the outlier detection analyses identified a clear separation between the two species, the GEAs pointed to a distinction of particular populations within species, which might also relate to an inflated rate of false negatives that is common when environmental distance is correlated with genetic distance (Ahrens et al., 2018). Thus, although GEAs account for and correct analyses for population structure, we found that hierarchical approaches, such as the one used here, are essential in the presence of multiple levels of genetic structure.

Within species, we identified different clustering of populations when considering neutral and adaptive variation separately. This suggests that local adaptation and demographic processes are both important and act independently in shaping genetic variation in IDGS. We found evidence for six different demographic units based on PCA and *STRUCTURE* analyses of neutral loci in NIDGS. As seen in other small mammals with historically reduced ranges across altitudinal gradients (Bi et al., 2019; Waterhouse et al., 2018), the higher differentiation observed among *east* NIDGS populations may result from loss of suitable habitat and dispersal corridors between populations, currently leading to the isolation of populations (Barrett, 2005; Gavin et al., 1999; Sherman & Runge, 2002; Yensen, 1999). In contrast, the higher homogeneity seen in *west* NIDGS populations might reflect both higher connectivity among populations, and assisted gene flow from translocations in the late 1990s (Gavin et al., 1998; Sherman & Gavin, 1999). This result is similar to that of previous microsatellite analyses for the eastern part of the range although this study did not analyse the exact same populations (Garner et al., 2005). However, it somewhat contrasts with our hypothesis of IBD and the results of an allozyme study, which found significant IBD within the western region (Gavin et al., 1999), and with unpublished results from Hoisington (2007) which found evidence for additional substructure and restricted gene flow within both the eastern and western groups. For SIDGS, the neutral data set identified two genetic groups with the highest differentiation between populations on different sides of the Weiser River, which corroborates previous studies (Garner et al., 2005; Hoisington, 2007; Zero et al., 2017). Interestingly, although Olds Ferry was the most distinct population at the neutral level, it was not substantially differentiated from other populations on the eastern side of the Weiser River at the adaptive level, suggesting that its distinction is mostly due to demographic (i.e., neutral) processes (Garner et al., 2005; Hoisington-Lopez et al., 2012; Zero et al., 2017).

Overall, we saw that some populations that were differentiated at the neutral level were also distinct at the adaptive level (e.g., Lower Butter), others that were differentiated at the neutral level were not found to be distinct at the adaptive level (e.g., Mud Creek, Olds Ferry), and others were more distinct at the adaptive than the neutral level (Rocky Top and Paddock). In SIDGS, no populations were distinct at both the neutral and adaptive level. This suggests that population isolation in SIDGS might not be as strong as in NIDGS and thus only populations undergoing strong selective

pressure are currently locally adapted in SIDGS (Funk et al., 2012; Tigano & Friesen, 2016). Although neutral genetic diversity is regarded as an important proxy for population health (DeWoody et al., 2021), recent studies argue for increased consideration of ecologically meaningful diversity, which might be essential for effective conservation strategies (Ralls et al., 2018; Teixeira & Huber, 2021). These results highlight the importance of considering different types of loci for conservation management and also to guide additional research on this species, as populations that are distinct at only the neutral or adaptive level, or that are distinct at both levels, will probably represent different types of conservation units, and contribute differently to the species' adaptive potential (Funk et al., 2012; Razgour et al., 2019).

### 4.3 | High landscape heterogeneity leads to clearer patterns of intraspecific local adaptation

Intraspecific adaptive differences can quickly arise in populations that become isolated and/or that are distributed across highly variable environmental gradients (Doebeli & Dieckmann, 2003; Smith et al., 2019). Small populations can naturally become locally adapted during the process of isolation, resulting in a gradual increase in adaptive differentiation from other populations, compared to neutral differentiation (Doebeli & Dieckmann, 2003; Holderegger et al., 2006; Wood et al., 2016). Thus, there is an important role of adaptive variants and their specific adaptations in the maintenance of genetic diversity and the long-term persistence of threatened species (Rubidge et al., 2012). From our results, most SIDGS populations were associated with a similar set of environmental variables, while there were no generalizable associations common to most NIDGS populations. These differences are within our expectations of adaptive differentiation between the two species, given that NIDGS occur at more heterogeneous landscapes than SIDGS, mainly in relation to elevation (Hoisington-Lopez et al., 2012; Yensen, 1991).

NIDGS showed a higher number of distinct populations at both the neutral and adaptive level, with Rocky Top and Lower Butter as the most distinct populations. Interestingly, these are the highest elevation populations sampled in this study but not the highest documented populations for the species. No population showed particularly low genetic diversity, indicating that NIDGS populations are unique but are not being affected by inbreeding or extensive drift, despite local bottlenecks (Assis et al., 2013). The emergence of particular adaptations to elevation in NIDGS populations was potentially promoted by a decrease in gene flow, a pattern also observed in other North American ground squirrels (Eastman et al., 2012; Hodgson et al., 2011). Genomic signatures of adaptive differences within NIDGS might reflect associations with timing of snowmelt and site productivity, respectively, which are known to influence torpor timing (Goldberg, Conway, Evans Mack, et al., 2020; Hoisington-Lopez et al., 2012; Zero et al., 2017). The highest adaptive differentiation in SIDGS was observed for Paddock, although it was not distinct at the neutral level from other populations on the

same side of the Weiser River, which is indicative of strong selection in this population despite generalized gene flow among *east* SIDGS populations (Tigano & Friesen, 2016).

The results of the intraspecific GEAs appear to highlight particular adaptive responses as a result of local adaptation facilitated by demographic isolation in NIDGS, while in SIDGS the patterns appear to match local adaptation despite low levels of demographic differentiation. In the face of gene flow, local adaptation depends upon various factors including the strength of selection on the trait and the migration rate, which need to be considered for effective conservation (Razgour et al., 2018; Tigano & Friesen, 2016).

### 4.4 | Conservation recommendations

Both IDGS species have undergone accentuated population declines in the last 50 years (Evans Mack, 2003; Gavin et al., 1999; Yensen, 1999), suggesting high susceptibility to environmental and landscape changes. As a result, NIDGS were listed as threatened under the federal Endangered Species Act (ESA) in 2000 (USFWS, 2000), and as Critically Endangered by the International Union for Conservation of Nature (Hafner et al., 1998). SIDGS are listed as Vulnerable by the IUCN (IUCN, 2018) and were a former candidate for listing under the ESA, although they were ultimately not listed. The decline of these two species has resulted in metapopulations approaching a state of nonequilibrium, where many populations became small and isolated, and thus especially prone to both diversification and extinction (Hanski & Gaggiotti, 2004; Harrison & Taylor, 1997; Pironon et al., 2017). Metapopulations can retain genetic variation more readily than simply isolated subpopulations from a once panmictic population, and can be more resilient to extinction due to their intrinsic colonization-extinction-recolonization dynamics (Gavrilets et al., 2000; Levin, 1995; Nee & May, 1992). However, this resilience is dependent on how many individuals and how much genetic diversity each subpopulation maintains (Fahrig & Merriam, 1994; Furlan et al., 2020). Our results showed that west NIDGS populations are genetically more homogeneous based on low and mostly nonsignificant levels of pairwise  $F_{ST}$ , but *east* NIDGS form a more fragmented patch network. Interestingly, levels of neutral genetic diversity in NIDGS were not different between west and east metapopulations, but were significantly lower in the west at the adaptive level. These results suggest that the habitat and environmental conditions are more homogeneous in the west region, but could also suggest a decrease in the signal of local adaptation due to translocations (Pacioni et al., 2019). For SIDGS, *east* populations were significantly less diverse at the neutral level than in the west (for which we only sampled one population), suggesting that *east* populations have been subjected to stronger declines (Weeks et al., 2011). Translocations among west NIDGS populations (Gavin et al., 1999; Sherman & Gavin, 1999) and among *east* SIDGS populations (Yensen & Tarifa, 2012; Yensen et al., 2010) were performed to supplement small and isolated populations, and might have led to an increased level of genetic homogeneity at the neutral level (Landguth & Balkenhol, 2012; Weeks et al., 2011).

Translocations generally lead to an overall maintenance of genetic diversity with consequent improved survival for the species, by spreading the extinction risk across multiple populations; however, there is a trade-off regarding the maintenance of local adaptation (Furlan et al., 2020). Most conservation-oriented translocations have resulted in beneficial intended consequences, namely in preventing extinction (Novak et al., 2021), but simulations suggest that translocations are most effective if performed multiple times, particularly for the establishment of new populations (Pacioni et al., 2019). Successful translocations would result in increased genetic diversity and low neutral and adaptive differentiation (White et al., 2018), but previous studies have found low rates of translocation success, particularly in SIDGS (Busscher, 2009; Panek, 2005; Smith et al., 2019; Jensen et al., 2010). Significant differences in the timing of torpor emergence have been seen in Columbian ground squirrels translocated to populations with different phenology, potentially leading to changes in fitness of translocated individuals (Lane et al., 2019). This could be related to an effect of strong local adaptation in source populations, resulting in low success in the establishment of new populations in environmentally distinct areas (Hedrick, 1995). In cases where closely located populations are not available, and thus local adaptation could be compromised by translocations, it is still advisable to consider translocations if the impact of low effective population size and genetic drift will result in population extinction (Weeks et al., 2011). Thus, although there is some indication that translocations have maintained neutral genetic diversity in west NIDGS populations, further work is necessary to verify the impact of translocation in *east* SIDGS by sampling source populations. This is especially important as, in line with previous microsatellite work (Garner et al., 2005; Hoisington-Lopez et al., 2012), SIDGS populations were generally less genetically diverse than NIDGS, which could be the result of effects of past bottlenecks and genetic drift (Rousset & Raymond, 1995), and points to the need of increased protection of this species (Garner et al., 2005).

Our results provide an important baseline for further work that aims to develop management strategies in response to land use and habitat changes (Henry & Russello, 2013). Further sampling is required to validate the results of those populations with lower number of samples, given that adaptive variation is less likely to be detected with low sample sizes (Lotterhos & Whitlock, 2015). The inclusion of additional populations would also be ideal, not only for confirming the patterns found in this study, but also for identifying other conservation units that might warrant special protection.

#### 4.5 | High-throughput sequencing of minimally invasive samples

We made use of a minimally invasive sampling method, in order to minimize the impact of sampling on populations (Carroll et al., 2018). Buccal swabs, as used in this study, have been specifically used in high-throughput sequencing (HTS) genotyping using targeted sequencing (Chang et al., 2007; McMichael et al., 2009), and have been

shown to allow for nontargeted HTS genotyping, such as RADseq, in amphibians (Peek et al., 2019). To our knowledge, this is the first study genotyping buccal swabs from a mammal with a nontargeted HTS approach, adding to the growing literature aiming to use less invasive sampling strategies to examine genomic variation in species of conservation concern (Andrews et al., 2018; Carroll et al., 2018). Buccal swabbing may provide an ideal data collection method for numerous species that require noninvasive techniques (i.e., species of conservation concern) but where more traditionally used methods such as hair snares are not ideal. For instance, there are 330 rodent species in the world (besides IDGS) currently listed as vulnerable, endangered or critically endangered according to the IUCN Red List (IUCN, 2012). Most of these species exist in small isolated populations where further information regarding adaptive differences could potentially provide useful information with regard to recovery efforts and metapopulation dynamics. However, our sample genotyping success was low due to low DNA yield, typical of minimally invasive sampling, and thus studies considering using buccal swabs should conduct pilot studies to determine DNA yield and be aware of this limitation.

## 5 | CONCLUSIONS

In this study, we examined two rare and recently diverged species of ground squirrels, identifying multiple sets of new loci with around 3,000 SNPs each using minimally invasive sampling methods. Our results corroborate previous studies regarding the clear differentiation between NIDGS and SIDGS and provide further details regarding the differentiation of these two sister species at the adaptive level. We additionally analysed the demographic and adaptive variation of each species independently and determined that local adaptation played a more prominent role in differentiation among NIDGS populations, while geographical barriers appear to be the largest determinant of genetic differentiation in SIDGS. Identifying appropriate conservation units within both species will require further investigation with more samples and including additional populations, in particular the geographically disjunct Round Valley population at the southern extent of the range (Figure 1) that was previously identified as genetically distinct (Hoisington, 2007; Hoisington-Lopez et al., 2012). However, we are the first to identify adaptive loci that distinguish not only the two species, but also distinguish among populations within each species, and the associations of these adaptive loci with environmental variables. The differences we detected will potentially help inform management plans aiming to protect the evolutionary and adaptive capacity of populations. Our results suggest clear metapopulation structure in both species with strong IBD (and potentially isolation-by-barriers), as well as evidence of recent local adaptation within some of these small and isolated metapopulations. Furthermore, these new collection techniques for mammals combined with our analysis provide a road map for future studies that aim to collect similar information from other small mammal populations.

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## AUTHOR CONTRIBUTIONS

C.C. and L.P.W. designed the study; A.R.G. and L.P.W. participated in sample collection; S.B., K.R.A. and D.S.G. prepared the DNA for RADseq, S.B. processed the RADseq data and performed all analyses. S.B. wrote the manuscript and all authors contributed to editing and revising the manuscript.

## OPEN RESEARCH BADGES



This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at <https://doi.org/10.5061/dryad.sj3tx965c>.

## DATA AVAILABILITY STATEMENT

Raw reads and mapped bam files for the total 304 ground squirrels are available on NCBI's BioProject PRJNA747996. Additional metadata for each individual ground squirrel, including respective scores for all environmental variables tested, as well as the final three VCF files used for all analyses with respective R code employed can be found in DRYAD (<https://doi.org/10.5061/dryad.sj3tx965c>).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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