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Assessing the Evolutionary Potential of Endangered Populations Using Genomic Analyses of Selection in Ecologically Important Gene Regions

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ABSTRACT

Evolutionary potential, the capacity to evolve in response to environmental change, is important for the persistence of threatened species. This potential is diminished by genetic drift in small populations, causing the loss of genetic diversity and reduced efficiency of selection. We introduce an approach to evaluate evolutionary potential that integrates across these components by conducting genome-scale selection analyses of genes of ecological importance. We inferred selection in small populations of the endangered eastern massasauga rattlesnake (*Sistrurus catenatus*) by comparing summary statistics between background regions and focal adaptive gene families underlying the Major Histocompatibility Complex and venom protein phenotypes. We applied tests sensitive to selection at multiple timescales to populations that have experienced differential declines and compared results with an outbred population of the sister species, *S. tergeminus*. We detected signatures of selection in focal regions in the recent and distant past, suggesting balancing selection as a dominant force shaping genetic diversity underlying these traits. Recently declined *S. catenatus* populations exhibited weaker signals of recent selection, consistent with reduced efficiency of selection when effective population size is small. Drift has reduced genome-wide genetic diversity both in relatively large and recently declined *S. catenatus* populations compared to outbred *S. tergeminus*, but these reductions were less pronounced in focal adaptive regions. These findings suggest that selection has buffered the loss of adaptive variation, but that small populations of these snakes may be approaching a critical loss of evolutionary potential limiting their capacity to respond to human-induced environmental change.

1 | Introduction

A central goal of conservation genetics is to conserve variation in small populations of threatened species that contributes to fitness in present or future environments (Avise 2010; Allendorf et al. 2022). Yet, small population sizes can lead to reduced evolutionary potential, defined as a diminished capacity to evolve in response to future environmental changes (Frankham 2005; Willi et al. 2006; Forester et al. 2022). This is because evolutionary responses are the product of two components: levels of genetic variation in traits under selection and the strength of selection acting on the traits (Falconer and Mackay 1996). Specifically, a trait will respond to selection when the product of heritable variation (h^2) and selection differential (S) is >0. Yet drift and inbreeding effects prevail in small populations, causing loss of variation and reduced efficacy of natural selection (Wright 1931; Ohta 1973; Frankham 1996). The fate of an allele is determined by drift when the product $N_e s < 1$, where N_e is the effective population size and s the selection coefficient (Wright 1931; Kimura and Ohta 1969). Therefore, providing some variation is retained,

sufficiently strong selection can produce a change in allele frequency/phenotypic response even when populations are small. In this simplified model, evolutionary potential requires an understanding of both the levels of fitness-relevant genetic variation and the strength of selection that acts on this variation.

Previous work assessing evolutionary potential in small populations has largely focused on assessing levels of heritable or additive genetic variation (Wood et al. 2016), or used neutral genetic diversity as a proxy (Kardos et al. 2021; Willi et al. 2022). Empirical tests of whether small populations respond to selection have largely been limited to experimental evolution studies (Willi et al. 2006; Hoffmann et al. 2017; Forester et al. 2022), with few examples in natural populations of threatened species (Grueber et al. 2013; Funk et al. 2016; Benazzo et al. 2017; Ochoa et al. 2020). Given that measuring the strength of selection is difficult in the wild, we suggest that observing a response to selection on a given trait presents a way to measure retained evolutionary potential. A challenge to this approach is that identifying genes responsible for adaptive traits is difficult and often consists of many loci of small effect (Barrett and Hoekstra 2011). However, a tractable alternative is to measure response to selection on known adaptive traits with relatively simple genetic architectures.

Conservation geneticists have long used an a priori approach to assess adaptive diversity by focusing on genes with known genetic architectures and ecological relevance. One such example is The Major Histocompatibility Complex (MHC) immune gene family that has been argued is an important component of adaptive diversity (Hughes 1991; Ujvari and Belov 2011; Teixeira and Huber 2021). Due to the fundamental function of MHC in immunity, there is evidence for strong selection on these genes ($s > 0.2$) (Radwan et al. 2020), with evidence for a predominating role of balancing selection leading to elevated genetic diversity (Garrigan and Hedrick 2003; Charlesworth 2006; Radwan et al. 2020; Bitarello et al. 2023). Previous work, mostly using amplicon sequencing, has found mixed evidence for the role of balancing selection maintaining MHC diversity in the face of drift in small populations (Aguilar et al. 2004; Sutton et al. 2011; Eimes et al. 2011; Oliver and Piertney 2012; Stervander et al. 2020). A sole focus on MHC and other immune system markers has been criticized because conservation efforts focused on this small fraction of adaptive traits risk depleting variation in other important traits (Vrjijenhoek and Leberg 1991; Kardos and Shafer 2018), and a direct link between MHC variation and population viability in nature is lacking (Radwan et al. 2010). MHC genes do, however, provide a useful test case based on their ecological relevance, selective history and support from good genomic resources.

In venomous snakes, venom provides a useful additional comparison for evaluating levels of adaptive genetic diversity in small populations because, like MHC, the genes that underlie venom proteins are thought to evolve under strong selection (Barlow et al. 2009; Casewell et al. 2013). Venom is key to effectively capturing prey, yet it is comparatively underexplored as a marker of evolutionary potential in species where it represents a significant trophic adaptation (Gibbs and Chiucchi 2011; Ochoa et al. 2020; Hirst et al. 2025). A significant advantage of venoms as a test case of adaptive diversity is that they have well-characterized genetic architectures that can be traced to phenotypes (Rokyta et al. 2015; Schield, Card, et al. 2019; Mason et al. 2022, 2026; Hogan et al. 2024). Moreover, venom genes generally show high levels of intraspecific variation (Chippaux et al. 1991; Gibbs and Chiucchi 2011), with local adaptation contributing to this variation (Smiley-Walters et al. 2017, 2019). Previous work suggests a role for positive directional selection in venom evolution (Aird et al. 2017; Margres et al. 2019). In addition, Schield et al. (2022) provided population genomic evidence that balancing selection also plays a significant role in maintaining venom gene variation at different evolutionary timescales. The potential parallels between venom and MHC as targets of selection make the genes underlying these traits promising a priori targets for analyses of adaptive variation, enabling comparative analysis to assess whether response to selection is detectable in small populations.

The eastern massasauga rattlesnake (*Sistrurus catenatus*) is a threatened species that has been the subject of extensive conservation genetic analyses (Gibbs et al. 1997; Chiucchi and Gibbs 2010; DiLeo et al. 2013; Bradke et al. 2018; Clark et al. 2025), providing a model for genomic analysis of functional variation in the context of population declines. The species currently exists as a series of small, isolated populations across most of its range in the US and Canada (US Fish and Wildlife Service 2021). Populations have declined due to habitat fragmentation and destruction, leading to the listing of the species as 'Threatened' under the United States Endangered Species Act 1973 (United States Congress 1973; US Fish and Wildlife Service 2016), and 'Endangered' under Species at Risk Act 2002 in Canada (Government of Canada 2002, 2018). Recent work on functional variation has shown high frequencies of deleterious mutations in these populations and a pervasive influence of long-term drift on adaptive diversity (Ochoa and Gibbs 2021; Mathur et al. 2023). Another study of small populations in Illinois found signal of both selection and drift in MHC Class IIβ based on variation detected using amplicon sequencing (Jaeger et al. 2016; Baker et al. 2018; Sovic et al. 2019). Finally, Ochoa et al. (2020) surveyed venom genes using target capture-based data across the species' range, finding a pronounced effect of drift on venom gene variation within populations, but also evidence for selection potentially related to local adaptation.

Here, we use a comparative approach to infer the role of drift on adaptive genetic diversity and the detectability of selection on specific traits at different timescales in populations of *Sistrurus* rattlesnakes. We compare the effects of drift in a relatively large population of *S. catenatus* to those from two exceptionally small populations that have declined dramatically in the past few hundred years (Figure 1A). Using selection tests relevant to different timescales, we then partition the effects of historical and recent demography on the response to selection (Figure 1B,C). For perspective, we contrast these results with the sister species, the Western Massasauga rattlesnake (*S. tergeminus*; Figure 1A) (Kubatko et al. 2011). *S. tergeminus* has a larger effective population size and shows little evidence of genetic structure, characteristics predicted to facilitate more efficient selection (McCluskey and Bender 2015; Ochoa and Gibbs 2021; Bylsma et al. 2022; Bolton et al. 2026) (Figure 1A).

We focus our selection analyses on MHC and venom genes for several reasons. First, these gene families underlie ecologically

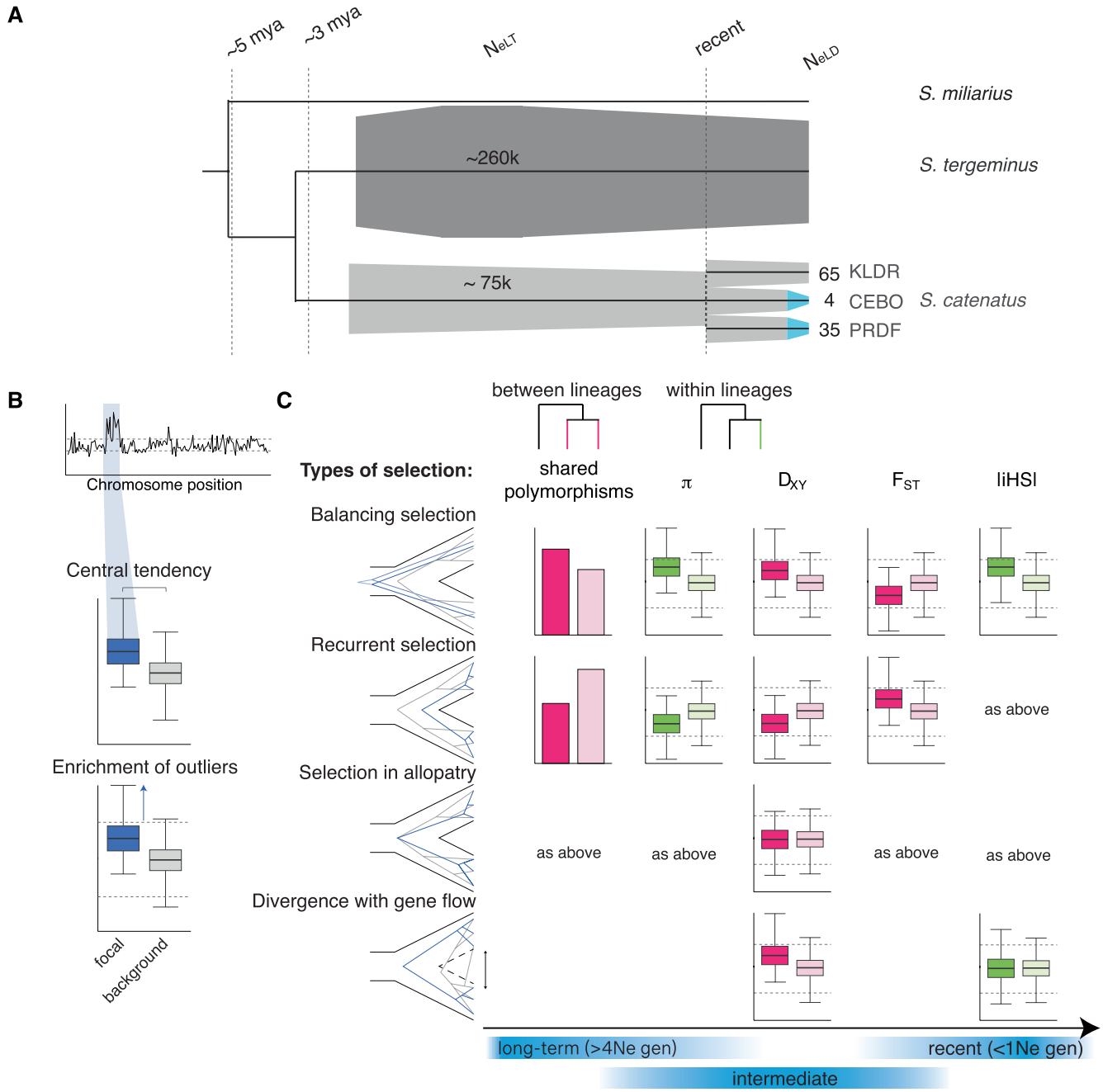

FIGURE 1 | The demographic histories for studied rattlesnakes and the predicted effects of selection on summary statistics within annotated venom and MHC gene regions. (A) Schematic of the evolutionary history of rattlesnake genus *Sistrurus*, each species is indicated by a different shade of grey. The following abbreviations will be used throughout: *S. miliarius* = SMIL, *S. tergeminus* = STER, and KLDR is a large population of *S. catenatus*, and PRDF and CEBO are two substantially smaller populations in Ohio. The grey bars indicate relative approximate population sizes based on demographic modelling and annotated with estimates of long-term size (Ne_{LT}) from θ_W (Ochoa and Gibbs 2021). The endangered *Sistrurus catenatus* population in Ohio is characterized by recent fragmentation and very recent population declines, indicated by blue tips and estimates of recent effective population size for sampled populations (Ne_{LD}, estimated in Mathur et al. (2023)). (B) Overview of selection summary statistics workflow. Manhattan plot demonstrates an example genome scan, where blue highlight is the focal region, the rest is the chromosomal background, and dotted lines indicate quantiles for chromosomal background. When represented as a boxplot, focal regions are inferred under selection when there is a difference in central tendency or the proportion of focal windows outside the background quantiles. (C) Predictions for the distributions of summary statistics for focal and background regions, when under different forms of selection (vertical). Colour indicates whether the summary statistic compares within or between lineages. Statistic plots arranged (horizontal, arrow) to indicate the time period at which these statistics are most sensitive for detecting selection (Fijarczyk and Babik 2015).

important phenotypes in snakes: immunocompetency and venom diversity. Thus, they represent "best case" scenarios for detecting selection in small populations; a failure to detect selection on these genes therefore implies overwhelming impacts of drift in these populations. In contrast, signals of selection would suggest that these populations have been able to

respond to selection at a given timescale. Second, these traits are rare among potentially adaptive genotypes in that these gene families are well-characterized at the genome level in this species which is a requirement for the application of the techniques that we employ for analysing selection (Schield et al. 2022). Our goal is to illustrate the use of this approach for assessing evolutionary potential in small populations of endangered species, which has not to our knowledge been previously used, rather than to comprehensively assess the evolutionary potential of these populations.

2 | Materials and Methods

2.1 | Samples and Sequencing

We performed whole genome sequencing of individuals from four rattlesnake populations: three Ohio populations of *S. catenatus* and one population of *S. tergeminus* (Figure 1A). Within *S. catenatus*, we sequenced individuals from the relatively large Killdeer Plains Wildlife Area (KLDR; $N_e \sim 65$; Mathur et al. 2023) ($n = 30$ individuals) and from two smaller populations: Cedar Bog Preserve (CEBO; $N_e \sim 4$; $n = 21$ individuals) and Prairie Road Nature Preserve (PRDF; $N_e \sim 34$; $n = 20$ individuals). Locations of these populations are described in Mathur et al. (2023). We compared these populations to a population of the outbred sister species *S. tergeminus* collected from Cheyenne Bottoms Nature Preserve, Kansas, which has much higher N_e (Ochoa and Gibbs 2021) (Figure 1A). For *S. catenatus* populations, we selected individuals for sequencing from within a single generation to minimize the effects of overlapping generations and temporal changes in N_e on downstream analyses. We also analysed sequences from four individuals of *S. miliarius* (sister to *S. tergeminus* and *catenatus*—Kubatko et al. 2011) to identify ancestral alleles in *S. catenatus* and *S. tergeminus* for specific analyses.

To obtain sufficient coverage to detect copy number variation, we also generated additional sequencing data for individuals from previous studies with lower depth of coverage (Ochoa and Gibbs 2021; Mathur et al. 2023; Mathur and Gibbs 2025). All sequencing was completed on Illumina NovaSeq 6000 or NovaSeq X Plus platforms, with a targeted depth of > 20× per individual. Sampling and sequencing details are provided in Table S1.

2.2 | Read Processing, SNP Calling and Filtering

Raw reads were trimmed with *fastp* v. 0.22.0 (Chen et al. 2018) and mapped with *bwa* v. 0.7.17 (Li and Durbin 2009), using the latest *S. catenatus* assembly as the reference genome (GCA_039880765.1). We annotated the genome for Sauropsida odb10 BUSCO genes using *compleasm* v. 0.2.5 (Huang and Li 2023), and used *RepeatMasker* v4.1.2 (Smit et al. 2013) to identify repetitive regions using squamate-specific reference databases (Pasquesi et al. 2018). We manually annotated venom and MHC genes using available sequences (see Supplementary Methods for details and Table S2 for a list of queries and their sources; Roseman et al. 2024, Mason et al. 2026). We called variants and generated an all-sites VCF using GATK v. 4.1.2.0 *HaplotypeCaller* and *GenotypeGVCFs* (McKenna et al. 2010) and conducted site-level filtering according to GATK recommendations (Caetano-Anolles 2024).

To minimize the effects of technical artefacts which could be impacted by paralogous mappings in these duplicated regions, we conducted additional filtering following Schield et al. (2022). We removed sites with mean read depth below four and above the 97th percentile of mean read depth on each chromosome (25–27×) to exclude variants in collapsed paralogous regions. We masked individual genotypes supported by read depths below five and genotype quality (GQ) scores of less than 20. We removed sites within annotated repetitive regions unless they were within MHC or venom exons (see Supplementary Methods). We masked individual genotypes supported by read depths below five and genotype quality (GQ) scores of less than 20. To further address inflated heterozygosity artefacts from collapsed paralogs (Hartasánchez et al. 2018), we masked individual genotypes within CNVs detected using *CNV-seq* (Xie and Tammi 2009) and removed sites with a significant excess of heterozygotes (see Supplementary Methods; Schield et al. 2022). The resulting output was used for all subsequent analyses except for haplotype-based analysis (Table S3).

To facilitate haplotype-based analysis, we used *SHAPEIT* v2.12 *extractPIRs* to perform read-aware phasing of genotypes (Delaneau et al. 2012, 2013). Prior to phasing, we filtered the above dataset to include only biallelic sites and to exclude singletons and sites with > 50% missing data. We allowed 1000 states in *SHAPEIT* analysis, pruning every 210 SNPs and using a burn-in of 200 and main analysis of 2000 cycles.

2.3 | Tests of Selection—Overview

We assessed signatures of selection on MHC and venom genes in *S. catenatus* and *S. tergeminus* using within- and between-population summary statistics (Figure 1B,C). We differentiate among these based on their power to detect selection operating at different approximate timescales (Fijarczyk and Babik 2015; Ebert and Fields 2020). Here, long-term selection occurred approximately $> 4N_e$ generations ago, intermediate selection occurred $> 1N_e$ but $< 4N_e$ generations ago, and recent selection is detectable within the past $< 1N_e$ generations, and perhaps as little as 0.02–0.4 N_e generations (Figure 1C; (Fijarczyk and Babik 2015, Soni and Jensen 2024)). The long-term Ne of 75,000 calculated for *S. catenatus* is based on previously estimated θ_W, a generalized squamate mutation rate (2.4×10^{-9}), and a generation time of three years (Green et al. 2014; Ochoa and Gibbs 2021). Based on this calculation, long-term selection is detectable over a timeframe of $\gtrsim$ 300,000 ybp, given estimated speciation times (Figure 1A); selection at intermediate timeframes is detectable between 300,000 and 30,000 ybp, and recent selection has a timeframe of approximately 30,000 to 1500 ybp.

Following previous work (Ochoa et al. 2020; Schield et al. 2022, 2025), we compared summary statistics between focal and background regions derived from windowed genome scans (Figure 1B). Analysis of background regions allows us to account for genome-wide effects of drift, demography, and chromosome-specific recombination rates, and therefore isolate the signal of selection

on focal regions. Selection scans often assume that selected sites lie at the tails of the distribution, but this may not always be true under different modes and strengths of selection, or different demographic histories (Charlesworth and Jensen 2021). We also expect evidence of selection to come from signals at multiple genes (Schield et al. 2022). For these reasons, we inferred selection based on both differences in central tendency (median) and excess numbers of outliers in focal regions relative to background distributions (Figure 1B). We used a resampling approach to test for differences in central tendency between focal and background distributions. To generate background distributions, we performed 1000 random samples from chromosomes containing focal regions, each time sampling the number of continuous windows equal to the length of the focal region. The background excludes focal regions, BUSCO genes, and non-venom homologues (see Supporting Information). We calculated median values of each statistic for each sample, then compared distributions with observed median values in the focal region using two-tailed tests (unless otherwise stated) with a Benjamini-Hochberg correction for multiple testing. We defined outliers as focal windows falling outside the upper 97.5% and lower 2.5% quantiles of the empirical chromosomal background distribution. We tested for enrichment of focal windows using a Fisher's exact test by comparing the number of windows in focal and background regions above and below our threshold. After correcting for multiple testing, significant enrichment was considered as suggestive of selection. All statistical analyses were conducted using *R* v 4.5.0 (R Core Team 2025).

2.3.1 | Long-Term Selection

Signal of long-term balancing selection has been previously detected for MHC and venom genes (Charlesworth 2006; Fijarczyk and Babik 2015; Radwan et al. 2020; Schield et al. 2022; Bitarello et al. 2023). To examine whether MHC and venom have been under long-term balancing selection in *Sistrurus*, we estimated rates of shared polymorphism between *S. catenatus*, *S. tergeminus*, and *S. miliarius*, following Schield et al. (2022), and compared proportions of shared polymorphisms in focal and background regions using the *prop.test* function in R with Benjamini-Hochberg correction.

2.3.2 | Selection at Intermediate Timescales

Analyses based on nucleotide diversity within populations and divergence among populations are appropriate for detecting selection at intermediate timescales (Charlesworth 2006; Fijarczyk and Babik 2015; Ebert and Fields 2020). As described above, balancing selection may be particularly relevant in MHC and venom gene regions. Balancing selection is expected to produce high nucleotide diversity (π) within populations and high absolute divergence (D_{XY}) and low relative divergence (F_{ST}) among populations (Charlesworth 2006; Radwan et al. 2020; Schield et al. 2022) (Figure 1C). We used *pixy* v2 (Korunes and Samuk 2021; Bailey et al. 2025) to calculate π, D_{XY}, and F_{ST} in 10 kb windows, except for in the PLA2 venom gene region, where we used 1 kb windows. We also compared focal region diversity to homologous sequences and BUSCO genes across the genome. For simplicity, and because interpretations do not change based on choice of background, we report these additional analyses in the Supporting Information.

2.3.3 | Recent Selection

Recent positive or balancing selection will generate similar signatures of partial selective sweeps, where selected variants not yet fixed will have longer stretches of homozygous haplotypes than for a neutral variant undergoing normal recombination (Voight et al. 2006; Sabeti et al. 2007). Comparing haplotype homozygosity therefore allows the detection of selection at recent timescales, ranging from 0.02 to <1 N_e, because recombination has yet to break down linkage between targets of selection and neutral variation present on the selected haplotype(s) (Voight et al. 2006; Sabeti et al. 2007; Soni and Jensen 2024). We tested for such signatures using the integrated haplotype score (iHS) (Voight et al. 2006), calculated with the *rehh* R package (Gautier and Vitalis 2012; Gautier et al. 2017). We used data from the outgroup *S. miliarius* to polarize variants in *S. catenatus* and *S. tergeminus* for this analysis, where alleles present in 75% of *S. miliarius* genotypes were considered ancestral. Positive iHS values indicate extended haplotypes around the ancestral allele and negative values indicate extended haplotypes carrying the derived allele, but either could be found in the case of balancing selection (Voight et al. 2006). Following Voight et al. (2006), we used |iHS| to identify extended haplotypes, agnostic to the form of selection. Polarized data allows more power to detect extended haplotypes than unpolarized data (Klassmann and Gautier 2022), but polarization also reduced our SNP density. Given this tradeoff, we compare iHS scores between polarized and unpolarized data in focal regions. Because |iHS| scores are expected to be higher than the background, either due to positive or balancing selection (Voight et al. 2006), we used one-tailed resampling tests to compare values in focal and background distributions.

2.4 | Impacts of Drift and Selection at Different Time Scales on the Reduction of Adaptive Diversity

We compared the relative loss of genetic diversity in focal regions to the genome background among populations of different sizes to examine how recent demographic changes influence loss of adaptive diversity. Specifically, for each comparison, we contrasted patterns in a large and small population to approximate effects of diversity loss at different timescales (Lucena-Perez et al. 2021; Bolton et al. 2026). First, we compared *S. tergeminus* (STER) with the largest *S. catenatus* population (KLDR) to assess long-term effects of drift in *S. catenatus*. We then then compared variation between each of the smaller *S. catenatus* populations (CEBO and PRDF) to the KLDR population to assess how recent demographic declines over the period of significant anthropogenic impacts ($\leq$100 ybp) affect adaptive variation (Sovic et al. 2019). Drift effects can be measured by comparing the relative loss of nucleotide diversity in focal and background regions between populations, which we tested using Wilcoxon tests. Patterns of loss in focal regions that differ from the genome background suggest the action of selection at the timescales of demographic decline (ancient and recent). In contrast, F_{ST} summarizes how variation is partitioned within and among populations and is sensitive to variation in Ne among populations (Holsinger and Weir 2009; Waples 2025). Because of this sensitivity, similar F_{ST} between focal and background regions may reflect equivalent drift effects in both regions since the recent isolation of *S. catenatus* populations. We present

these results in combination with the diversity and differentiation tests for selection.

3 | Results

3.1 | Genome Sequencing and Annotation

Whole genome sequencing yielded between 163 and 492 million mapped reads across 91 *S. catenatus* and *S. tergeminus* individuals. This resulted in average per-individual genotype depths between 10 and 33×, with an even spread of low- and high-coverage individuals across populations and an average depth of ~20× in all populations. In the haplotype-free all-sites data, we called up to 10,360,507 biallelic and multi-allelic SNPs across the focal chromosomes. We generated a total of three alternate filtered datasets for downstream analyses (Table S3).

Our annotation of the *S. catenatus* (GCA_039880765.1) genome assembly revealed similar numbers of MHC and venom genes to those identified in previous analyses (Figure S1; Table S4) (Roseman et al. 2024; Mason et al. 2026). For more details see Supporting Information. Our analyses also indicated that most individuals contained one or more CNVs in focal regions that were filtered from final analyses (Figures S2–S8) and suggest structural variation within and between populations.

3.2 | Long-Term Selection Occurred Among Species

Most focal regions harbour significantly more shared polymorphisms among *Sistrurus* species than the chromosomal background (Figure 2). Among sites in all focal regions, an average of 60% of alleles are shared between species (trans-species polymorphisms), whereas background variation was consistently lower (averaging 50%). PLA2 was the sole exception to this pattern, showing fewer shared polymorphisms than the background between *S. tergeminus* and *S. miliarius* (STER vs. SMIL: $\chi^2 = 7.4$, df = 1, two-tailed $p = 0.008$) or no differences among species (Figure 2C; $\chi^2 = 1.5$, df = 1, $p = 0.2$). Overall, there were fewer shared polymorphisms, focal or background, between *S. catenatus* and *S. miliarius* (KLDR vs. SMIL) than between *S. tergeminus* and *S. miliarius*. This could be due to drift effects leading to increased rates of fixation or loss in *S. catenatus*, gene flow between *S. tergeminus* and *S. miliarius*, or both. These results suggest that MHC loci and several major venom gene loci have experienced long-term balancing selection, leading to the persistence of shared ancestral polymorphism retained during speciation, or trans-species polymorphisms, between these closely related rattlesnake species.

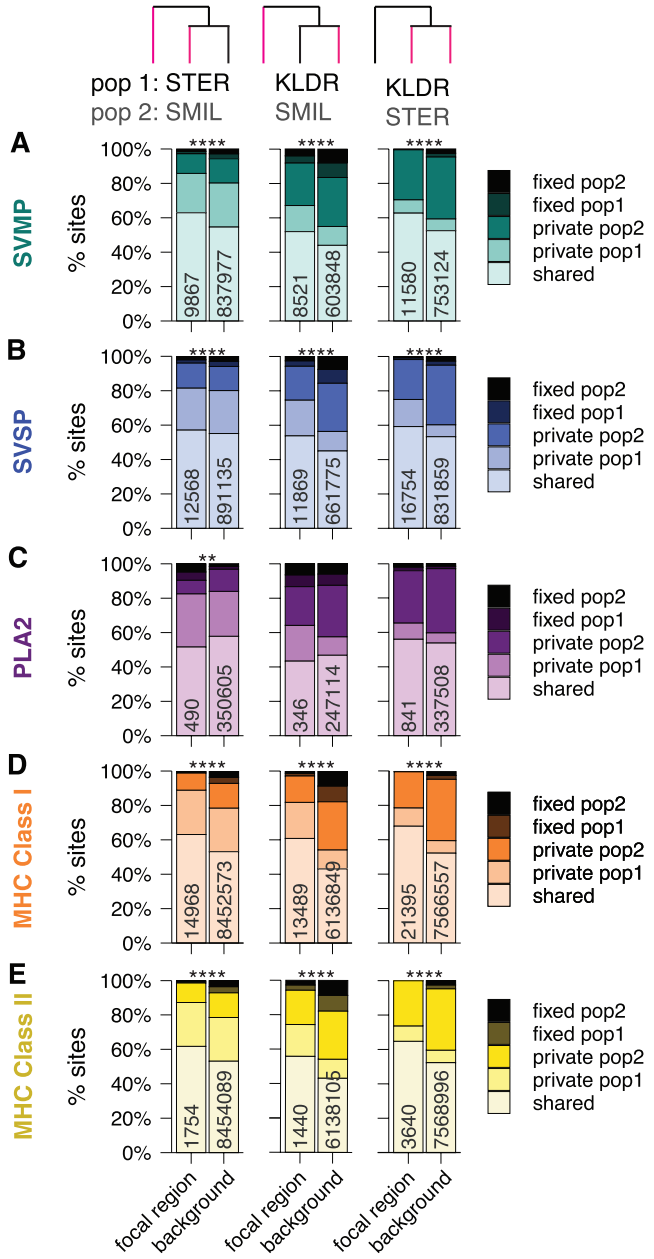

FIGURE 2 | More trans-species polymorphisms in venom and MHC regions than background. Trans-species polymorphism in focal venom and MHC sites among the *S. miliarius* (SMIL), *S. tergeminus* (STER), and *S. catenatus*. In these comparisons, *S. catenatus* is represented by the larger KLDR population. Polymorphisms shared between species are indicated by the lightest colour bar, where darker colours indicate polymorphisms that are private to one of the two species, and the darkest colours indicate variants that are fixed.

3.3 | Nucleotide Diversity Reveals Mixed Patterns of Drift and Selection Across Timescales

Comparisons of nucleotide diversity (π) between focal and background regions showed a range of patterns among species, including signatures consistent with balancing or positive selection in particular focal gene regions (Figure 3A). First, we compared variation between species by contrasting *S. tergeminus* with the large (KLDR) population of *S. catenatus*. At this level, π in SVMP and MHC Class I focal regions was substantially higher than background, with median diversity ≥1.3× the median of observed background diversity (Figure 3A). However, no resampling test was significant in *S. tergeminus* in these regions, and only MHC Class I in the large KLDR population was significantly more diverse (3.6×) than background (two-tailed resampling test, $p \cong 0$). The overall pattern weakly supports balancing selection maintaining functional diversity in SVMP and MHC Class I in both species (Figure 1C). In contrast, SVSP and MHC Class II showed non-significantly lower diversity

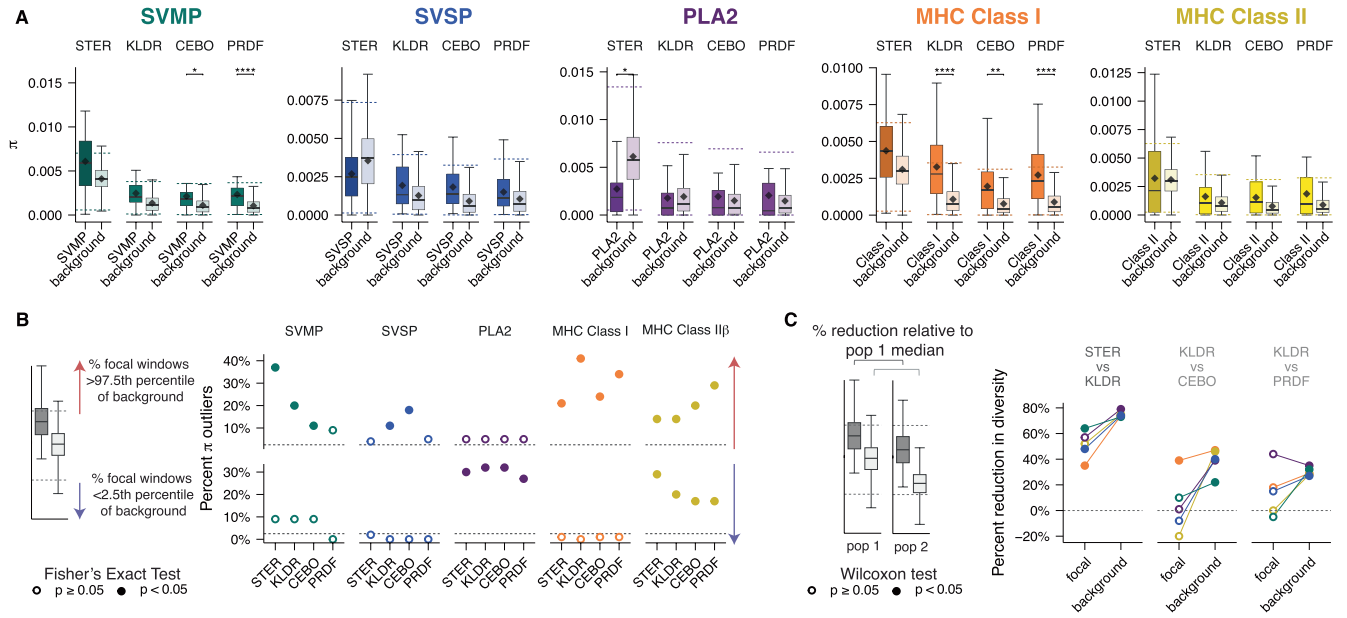

FIGURE 3 | Focal gene regions show patterns of diversity consistently different from those in background. (A) Boxplot showing empirical medians (lines), means (diamonds), and variation in windowed nucleotide diversity (π) for focal (darker fill) and background regions of the same chromosome. The dotted lines indicate the 97.5th and 2.5th percentiles of the chromosomal background for each population. Populations are examined such that the largest and outbred *S. tergeminus* (STER) is leftmost with darker fill, followed by the largest population of *S. catenatus* (KLDR), and followed by the two smallest Ohio populations (CEBO and PRDF). (B) Enrichment of outliers is when there was significantly more than the 2.5% of the focal data in the upper or lower quartiles of the background distribution (left). The percent of focal region windows outside the background quantiles, indicated by the dotted line, is plotted right, where filled circles indicate significantly more windows than the 2.5% observed in the background based on Fisher's exact test. Red arrow indicates outliers above the 97.5th percentile cut-off, while blue arrow indicates outliers below the 2.5% cut-off. (C) Loss of focal or background diversity was calculated as the percent reduction in the median value relative to the larger population in the comparison (STER and KLDR). The percent reduction in diversity for focal and background regions is plotted with an interconnected line as a visual aid to showing steeper slopes mean greater differences in the loss of diversity, where subplot title colour indicates a between species comparison (dark grey) or within species comparison (light grey). Filled circles are significant comparisons from a Wilcoxon test, and the horizontal dotted line indicates a 0% loss in diversity.

(both ~0.7×) than observed background in *S. tergeminus* (both regions: resampling test, $p = 0.3$), but non-significantly higher diversity than background in the KLDR population of *S. catenatus* (Figure 3A; both medians are at least 1.3× greater than observed background; resampling test $p > 0.7$). A third pattern was evident in PLA2, showing a significantly lower diversity (0.3×) relative to observed background in *S. tergeminus* (resampling test, $p = 0.03$), potentially resulting from positive selection in this species. The PLA2 region in KLDR *S. catenatus* showed non-significantly lower diversity (0.7 ×) than the observed background (resampling test, $p = 0.5$). These results are broadly consistent when comparing the focal regions to other coding sequences (Figure S9).

The two smaller *S. catenatus* populations, where we expect stronger recent drift effects, also showed variable patterns across focal regions. (Figure 3A). The strongest evidence for selection came from comparisons for SVMP and MHC Class I where significantly elevated levels of π relative to background were observed across each population, with median diversity at least 2.2× greater than background diversity (two-tailed resampling test, $p < 0.01$). MHC II showed a similar but nonsignificant pattern where focal diversity was at least 1.8× greater than the observed background (resampling test, $p > 0.07$). Median diversity in PLA2 was equivalent (~1.1 ×) to background in both populations (resampling test, $p > 0.5$).

We find significant enrichment for π outliers in all regions when comparing patterns between KLDR *S. catenatus* and *S. tergeminus*. Specifically, the SVMP venom gene region, and both MHC class I and II all showed evidence for significant enrichment in high diversity windows in both *Sistrurus* species where > 14% of windows outside 97.5% percentile of background (Figure 3B; Fisher's exact tests; one-tailed $p < 0.008$). By contrast, there was no significant enrichment of high or low diversity outlier windows in SVSP in *S. tergeminus*, but SVSP showed significant enrichment for high diversity outliers in *S. catenatus* (KLDR = 10.5%, Fisher's exact test, $p = 8 \times 10^{-3}$). MHC Class II also had significant enrichment for low diversity windows in both species (STER = 29%, KLDR = 20%, Fisher's exact tests; $p < 5 \times 10^{-5}$), while PLA2 only showed significant enrichment for low diversity windows (30%–32%, Fisher's exact test, $p < 2 \times 10^{-9}$). Except for SVMP and SVSP in the smaller PRDF population, these populations had significant enrichment for outlier windows mirroring the larger populations (Figure 3B; Fisher's exact tests, $p < 0.02$). These results support that selection is detectable in small *S. catenatus* populations, at least in some focal regions, despite strong drift effects.

Nucleotide diversity results also revealed that smaller populations have disproportionately more focal diversity than background diversity (Figure 3C), suggesting that selection has retained diversity in focal regions during population contractions. This is

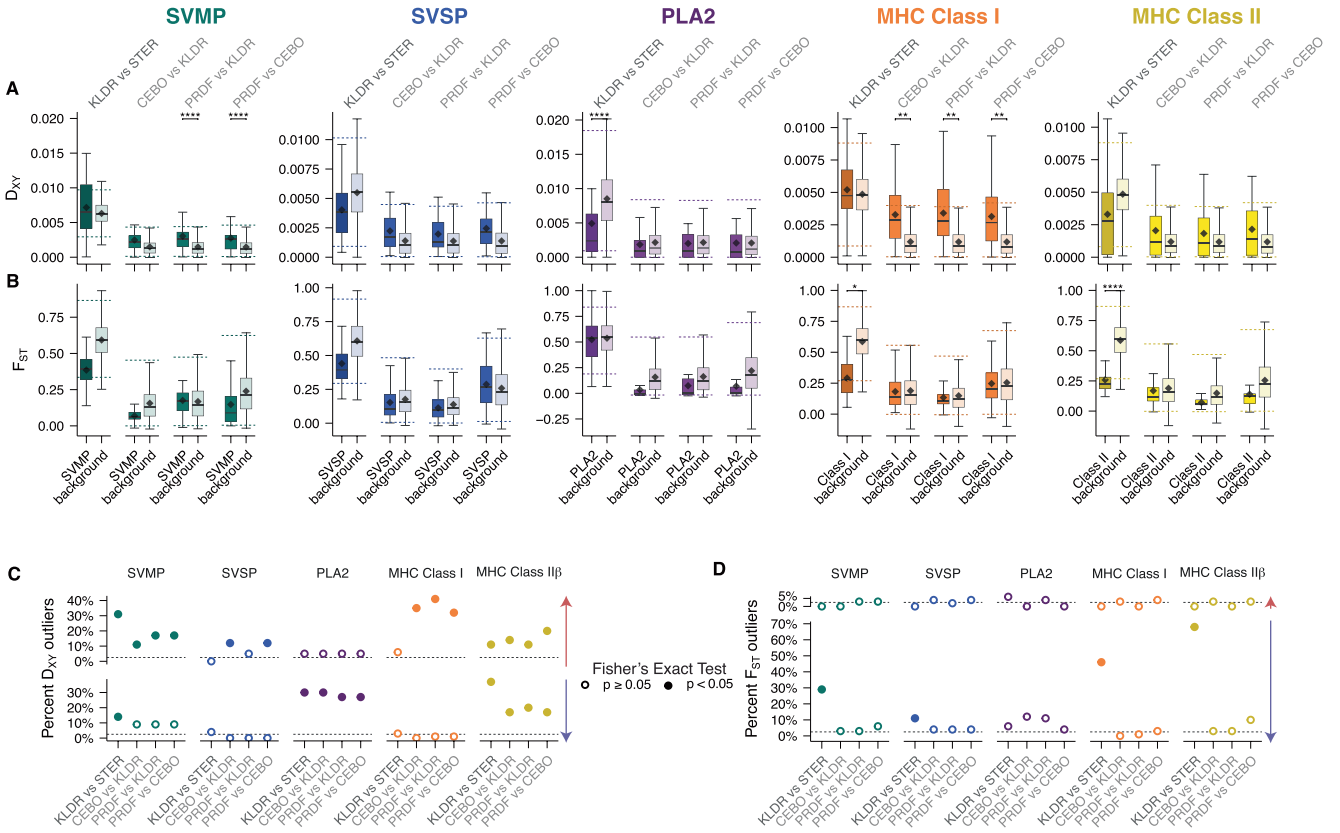

FIGURE 4 | Focal gene regions show consistently different patterns of divergence (D_{XY}), but not differentiation (F_{ST}), relative to background. (A) Boxplots of windowed absolute divergence (D_{XY}) between *S. catenatus* and *S. tergeminus* (represented by KLDR vs. STER comparison) with darker fill, and among Ohio *S. catenatus* populations. Explanation of boxplot otherwise as per Figure 3A. (B) Boxplots of relative differentiation (F_{ST}) between species and among *S. catenatus* populations. (C) Outlier enrichment plots for D_{XY} of focal comparisons, explanation as per Figure 3B. (D) Outlier enrichment for F_{ST} in focal comparisons. Throughout: Between species population comparisons are indicated with dark grey axis or titles, and within species comparisons are indicated with light grey.

true across time scales—differences in long-term effective size between *S. catenatus* (KLDR population) and *S. tergeminus* have resulted in significantly lower genetic diversity in both focal and background regions, but the effect size is greater in background. Here, the median diversity of background regions in KLDR is approximately 74% less than *S. tergeminus* (Wilcoxon test, $p < 4 \times 10^{-293}$), while the greatest reduction in median diversity is in SVMP at 64% (Wilcoxon test, $p < 0.02$). Similarly, the small populations that experienced recent demographic declines lost a median of 22%–47% background genetic diversity relative to KLDR (Figure 3C; Wilcoxon tests, $p < 2 \times 10^{-12}$). Most focal regions lost little to no diversity in the small populations (Wilcoxon tests, $p > 0.08$), except for MHC Class I in CEBO, which lost a significant 39% of median diversity (Wilcoxon test; $p = 2 \times 10^{-4}$). These patterns suggest that the differences in levels of diversity lost in focal versus background regions among populations are the result of selection maintaining functional diversity in the face of recent drift effects that have reduced background diversity to different degrees in smaller populations.

3.4 | Divergence and Differentiation at Intermediate to Recent Timescales

Next, we measured absolute divergence (D_{XY}) between the two *Sistrurus* species and among *S. catenatus* populations. Focal regions had mixed patterns in median divergence relative to background (Figure 4A; Figure S10), but in *S. catenatus* there was a tendency for focal regions to have accumulated more nucleotide differences than background regions, which is consistent with balancing selection or divergence with gene flow (Figure 1C) (Cruickshank and Hahn 2014). Between-species comparisons showed no significant differences between focal and background divergence (resampling tests, two-sided $p > 0.05$), except in PLA2 where divergence in focal regions was significantly less (0.7×) than the background (resampling test, $p = 0.007$). By contrast, two focal regions had significantly higher divergence than background divergence among *S. catenatus* populations. For example, MHC Class I had a significantly greater median divergence (> 3.3 ×) than the median background among *S. catenatus* populations (two-tailed resampling test, $p \cong 0$). Only among the small *S. catenatus* populations was SVMP significantly more diverged (3.3 ×) than background (resampling test, $p \cong 0$).

Following the pattern of the D_{XY} central tendency results, most focal regions showed significant enrichment highly diverged windows (Figure 4C). For example, a significant percentage (15%–31%) of SVMP windows were outliers in all within- and between-species comparisons (Fisher's exact tests, $p < 0.01$). By contrast, the MHC Class I region showed no significant enrichment for outlier windows between species, where 6%

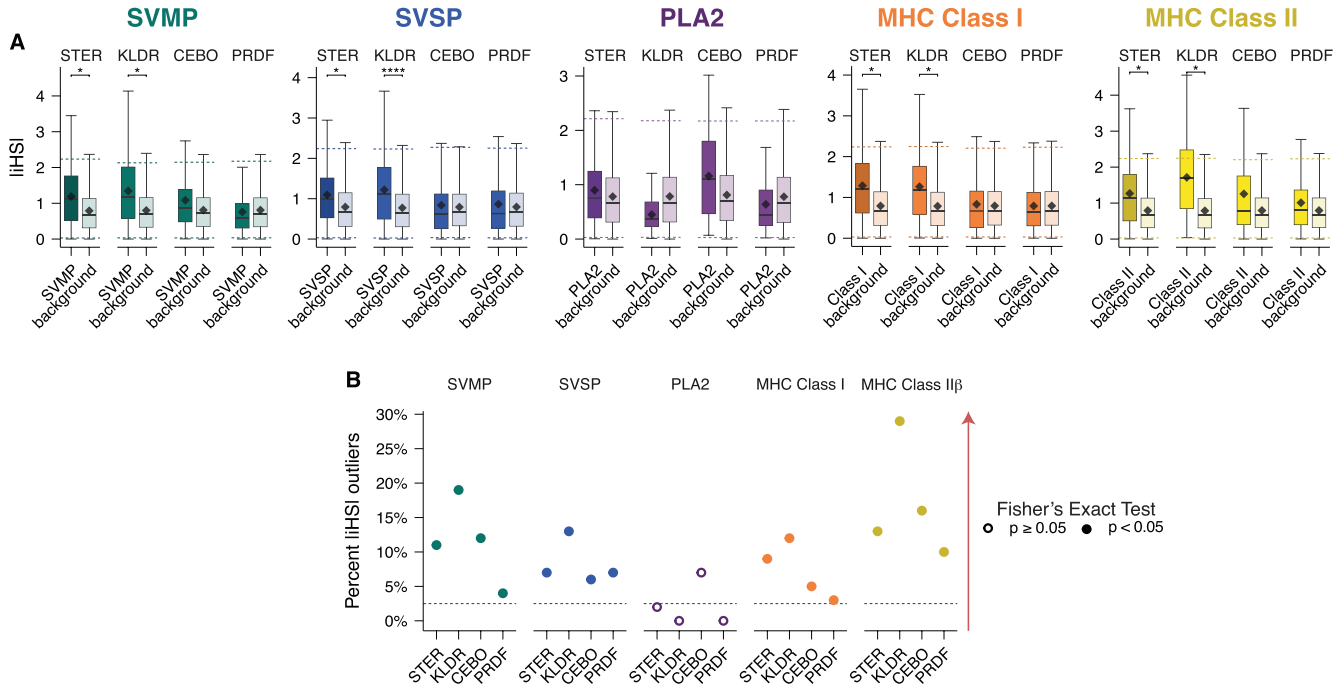

FIGURE 5 | SNPs in focal gene regions show consistently longer haplotypes than background only in large populations of *Sistrurus*. (A) Integrated Haplotype Score (iHS) for SNPs focal and background regions. Absolute value of score indicates longer haplotypes of either ancestral or derived allele, following Voight et al. (2006). Explanation of the boxplot otherwise as per Figure 3A. (B) Enrichment of focal |iHS| outliers above the 97.5th percentile of background for each population, explanation as per Figure 3B.

of windows were outliers in the between species comparison (KLDR vs. STER: Fishers exact test, $p = 0.09$). In two comparisons within *S. catenatus*, there was a significant enrichment with more than 12% of outlier windows in the SVSP region (Fisher's exact test, $p = 0.002$). All MHC Class II comparisons showed significant excess of both high divergence (a mean of 14% of focal windows; Fisher's exact tests, $p < 0.01$), and low divergence outliers (mean of 23%; Fisher's exact test, $p < 2 \times 10^{-4}$). However, all PLA2 comparisons showed significant enrichment for regions that were much less diverged than the genomic background, a mean of 28% of windows across all comparisons (Fisher's exact tests, $p < 2 \times 10^{-8}$). Together the patterns of high divergence in most focal regions revealed by outlier and central tendency analyses suggest balancing selection has operated in both species, while recurrent positive selection may shape PLA2s and with no clear pattern of selection in SVSP.

Focal regions generally have lower differentiation (F_{ST}) than background regions between species, but not among *S. catenatus* populations (Figure 4B). However, only MHC I and II loci showed significantly lower F_{ST} in focal regions compared to background between species (MHC Class I: 0.6× lower F_{ST} than background; resampling test, two-sided $p = 0.01$; MHC Class II: 0.4× lower than background; resampling test, $p \cong 0$) (Figure 4B). Other focal regions showed non-significant trends of lower F_{ST} than background between species, except PLA2, which showed no difference (Figure 4B; full results Figure S11). Meanwhile, focal region F_{ST} was sometimes slightly lower than background in specific *S. catenatus* comparisons.

Patterns of F_{ST} outliers reflected central tendency patterns (Figure 4D), where all focal regions except for PLA2 showed significant enrichment of low differentiation outliers between *Sistrurus* species only; at least 11% of focal windows were outliers between species (KLDR vs. STER: Fisher's exact test, $p < 0.007$). There was a non-significant maximum of 4% of regions with low F_{ST} outliers among *S. catenatus* populations (Fisher's exact test, $p > 0.5$). There was no enrichment for high differentiation outliers in any comparison. Together, F_{ST} results further suggest the action of balancing selection at longer time scales among species based on predictions in Figure 1C. Differentiation in focal regions more closely resembles background within *S. catenatus*, potentially due to stronger drift effects over the species history.

3.5 | Signatures of Recent Selection Are Strongest in Large Populations

Haplotype homozygosity-based selection tests (iHS) revealed signatures of recent selection (~1500–30,000 ybp) in *S. tergeminus* and the large KLDR population of *S. catenatus* (Figure 5A; (Voight et al. 2006)). The median |iHS| for SNPs in focal regions (except PLA2) was between 1.3 and 2.6× greater than in background (one-tailed resampling tests, $p < 0.02$). This trend is supported by the unpolarized results and smaller effect sizes (less than 2×), with only MHC Class II and SVMP showing significant differences (Figure S12; resampling tests, $p < 0.05$). In contrast to the results from the large KLDR population, the two small *S. catenatus* populations showed no evidence of selection in focal regions based on iHS tests (Figure 5A; resampling tests, $p > 0.2$). This pattern was consistent when iHS was calculated based on

polarized or unpolarized SNPs (Figure S12). Patterns of central tendency therefore support the detectability of selection on these regions in large but not small *S. catenatus* populations.

In contrast with the central tendency-based tests, most focal regions for all populations showed significant enrichment for SNPs with outlier |iHS| scores (Figure 5B). Aside from PLA2, all populations sampled showed between 3% and 29% of windows with |iHS| larger than the 97.5th percentile of background scores (Fisher's exact tests, one-sided $p < 0.02$). By contrast, PLA2 showed no enrichment with a maximum of 7% of windows considered outliers in the small CEBO population (Fisher's exact tests, $p > 0.4$). Contrary to our expectations, for regions enriched for iHS outlier SNPs, there tended to be more outliers in the large KLDR population of *S. catenatus* (12%–29%) than the larger outbred *S. tergeminus* (7%–13%), and for SVMP and MHC Class II regions, the small CEBO population contained more outlier regions than *S. tergeminus*. Overall, these results suggest that selection has acted on this recent time scale in all populations, but subsequent demographic declines and small population size have increased length of LD blocks that has erased the weaker signatures of selection in the central tendency-based tests.

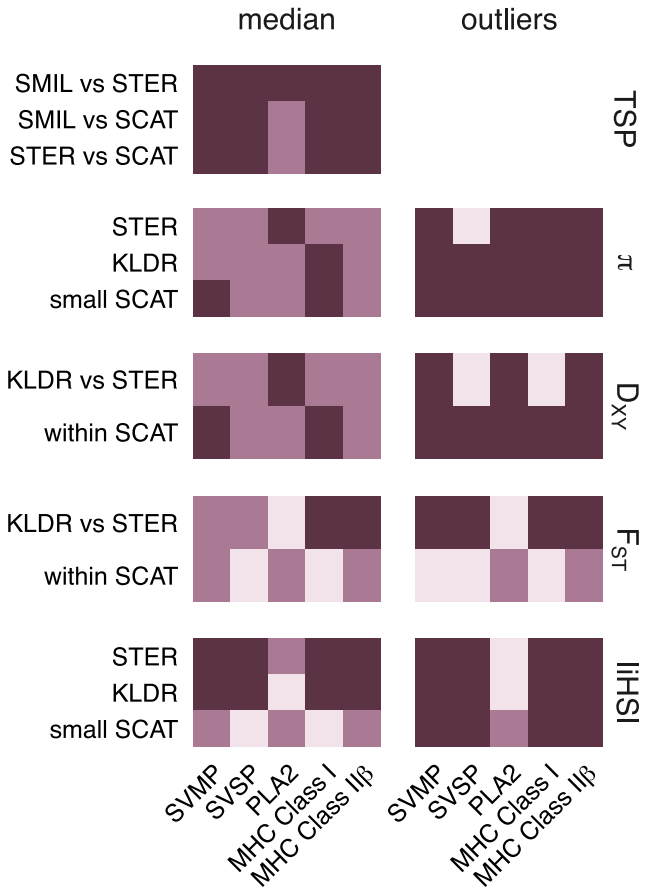

FIGURE 6 | Summary of the strength of selection inference based on median and outlier tests across different metrics. Darkest squares indicate at least one comparison was significant, mid-tones indicate at least one comparison showed a non-significant but strong tendency, while lightest indicates no trend.

4 | Discussion

Our goal in this study was to survey whether a response to selection is detectable for a small number of ecologically relevant gene families in small populations of an endangered species as a tool for evaluating their evolutionary potential. The results of the selection analyses are summarized in Figure 6. The overall pattern is that signatures of selection are stronger at longer timescales, but patterns measured via the central tendency become weaker as tests measure selection at more recent timescales. By contrast, outlier analyses generally provide support for selection across timescales. We also find that populations with smaller recent Ne have lost more variation in the background regions than in adaptive focal regions, suggesting a role for selection in maintaining functional variation. Below, we discuss the mode of selection, the combined effects of drift and selection on adaptive variation, and issues with assessing evolutionary potential in endangered populations.

4.1 | Modes of Selection

Together, our results reveal patterns of the form of selection operating on these regions in *Sistrurus* species. For example, increased shared polymorphisms among most focal regions (except PLA2) among species are consistent with long-term balancing selection maintaining trans-species polymorphism in both venom and MHC genes in these snakes. Signatures of long-term balancing selection in MHC genes are widely documented (Radwan et al. 2020); while for venom genes, our findings are consistent with previous observations of trans-species polymorphism on SVMP and SVSP loci among *Crotalus* rattlesnakes (Schield et al. 2022).

At intermediate-to-recent timescales there is also consistent support for balancing selection in some regions. Balancing selection is detected for SVMP and MHC Class I in both *Sistrurus* species because these regions tend to show high diversity in both species, higher divergence indicating deeply diverged alleles (D_{XY}), and lower F_{ST} between species. However, SVSP and MHC Class II showed more shared polymorphisms, but lower D_{XY} between species, and lower or similar-to-background diversity in *S. tergeminus*. This pattern may be explained by two non-mutually exclusive processes: local selection in *S. tergeminus* after species divergence, or introgression of alleles from *S. tergeminus* into *S. catenatus* thus increasing *S. catenatus* diversity. Introgression is increasingly documented in rattlesnakes overall (Schield, Perry, et al. 2019; Holding et al. 2021; Myers 2021; Myers et al. 2024), and previous work has identified geographically restricted populations consisting of individuals that are admixed between *S. catenatus* and *S. tergeminus* (Sovic et al. 2016). Indeed, alleles under balancing selection may be more prone to differential introgression due to rare allele advantage, as has been shown for MHC genes (Abi-Rached et al. 2011; Fijarczyk et al. 2018). Finally, we note that reference bias and higher masking rates may influence the lower diversity estimates we detected in *S. tergeminus* (Figure S2) (Akopyan et al. 2025), but are insufficient to explain the surprisingly strong differences in diversity between background and focal regions in small populations of *S. catenatus* (Figure 3). In contrast to evidence for balancing selection, patterns in PLA2 provide weak support for divergent selection between species. This is shown by a reduction in shared polymorphisms, low D_{XY} between species, strongly reduced

polymorphism in *S. tergeminus*, and an enrichment of low diversity windows in *S. catenatus*.

Tests relevant to the most recent time scale (i.e., iHS) support the presence of partial selective sweeps which could reflect either directional or balancing selection (Voight et al. 2006; Soni and Jensen 2024). Surprisingly, *S. tergeminus* had weaker effect sizes of central tendency and outlier tests for recent selection, which may indicate different strengths of selection among species, or reference bias effects. However, local iHS peaks co-occur with local signatures of selection from other summary statistics, suggesting genuine signals of recent selection (e.g., Figure S6).

A strength of our windowed approach is that it accommodates the fact that paralogs in these multi-copy families may evolve differently. Gene duplication in multi-copy families enables the neo- and sub-functionalization of paralogs, meaning that some copies evolve neutrally, with others under different forms of selection (Ohno 1970). Indeed, the patterns of selection we find are not uniform across windows/paralogs as some windows are outliers on both ends of the distribution, such as in MHC Class II and SVMP in D_{XY} between species. This implies independent evolution of paralogs, which may be enabled by locally high recombination rates in these regions, as observed in *Crotalus* rattlesnakes (Schield et al. 2022). However, this evolutionary mosaic likely results in wide variances and little difference in the overall central tendency of these regions. Indeed, central tendency tests for many of these statistics are conservative, likely requiring approximately uniform selection across paralogs to produce significant departures from background regions. Therefore, our results are consistent with previous work that shows multi-copy paralogs can be subject to different forms of selective pressures (Teshima and Innan 2008; Kloch et al. 2018; Fortier and Pritchard 2025; Mason et al. 2026). Moreover, Manhattan plots (e.g., Figure S3) indicate that local peaks and troughs are not necessarily shared among species, suggesting that selection does not necessarily act on the same local targets within these regions across species.

Among *S. catenatus* populations, variation among windows in selection regime could contribute to focal regions showing similar differentiation as background regions. Estimates of F_{ST} can capture more recent selection, with its directionality indicating either shared polymorphism or fixed differences (Fijarczyk and Babik 2015). Among species, lower F_{ST} in focal regions than background indicates similar allele frequencies and heterozygosity is consistent with balancing selection. But this pattern weakens in comparisons among *S. catenatus* populations where patterns of variation in focal regions resemble the genetic background, with no outlier windows. This contrasts with previous work that found high differentiation outliers among range-wide *S. catenatus* populations in a small number of non-synonymous sites in MHC Class I and venom paralogs, which was interpreted as evidence for local adaptation (Ochoa et al. 2020; Bolton et al. 2026). There are four non-mutually exclusive explanations for the differences in findings: (1) Technical differences owing to site-based vs. window-based approaches; (2) The spatial scale of our sampled populations is much reduced, thus local adaptation may be relevant for populations not investigated here; (3) Local adaptation in closely related populations may cause different selection coefficients against homozygote genotypes in focal regions, which can increase differentiation estimates and obscure our signal relative to background (Brandt et al. 2018); (4) Given F_{ST} is sensitive to recent drift, finding no F_{ST} patterns indicative of selection suggests the breakdown of selection in these adaptive regions for small populations, or that F_{ST} is unable to detect past selection due to subsequent drift. Without additional data we are uncertain as to the relative importance of each of these explanations.

4.2 | Drift Versus Selection and the Retention of Adaptive Variation

The retention of variation in small populations at these MHC and venom genes, relative to background, may be a consequence of the dominant effects of balancing selection. However, there are indications that recent and extreme drift in the smallest populations has reduced the efficacy of selection. Early theoretical work suggests that equilibrium gene frequencies close to unity from balancing selection will minimize the impact of drift in small populations (Robertson 1962). This "drift resistance" is often invoked when small or bottlenecked populations contain surprisingly high diversity at MHC loci (Aguilar et al. 2004; Oliver and Piertney 2012; Stervander et al. 2020). The retention of variation under balancing selection could contribute to increases in additive variance that can occur after bottlenecks (Taft and Roff 2012; Hoffmann et al. 2017). Some authors have even suggested that patterns of high diversity typical of balancing might be easier to detect in small populations (Gos et al. 2012; Koenig et al. 2019), and may not suffer from the same challenges as detecting positive selection in bottlenecked populations (Poh et al. 2014; Kardos and Shafer 2018).

However, other theoretical and empirical work suggest that loci under balancing selection can lose diversity in small and bottlenecked populations at a level equivalent to, or greater than, that predicted by genetic drift (Alcaide 2010; Sutton et al. 2011; Ejsmond and Radwan 2011; Eimes et al. 2011). Therefore our findings of retained variation could result from three non-mutually exclusive explanations: (1) *S. catenatus* population declines have not been as severe as those in other species studied, (2) Heterozygote advantage is the dominant mode of selection in most of our focal regions, effectively buffering loss (Robertson 1962; Sutton et al. 2011; Ejsmond and Radwan 2011), (3) Loci under balancing selection carry more drift debt due to recent population declines, and will continue to lose variation regardless of opposing selection coefficients (Gilroy et al. 2017; Sovic et al. 2019; Ochoa et al. 2020; Gargiulo et al. 2025). However, if we rearrange $N_e s > 1$ considering the smallest contemporary N_e of 4, the minimum selection coefficient required to counter drift is 0.2 (Wright 1931; Kimura and Ohta 1969). This is congruent with selection coefficients estimated for MHC genes (Radwan et al. 2020), though we are not aware of any studies that have independently estimated selection coefficients on venom. Therefore, theoretically, if selection is sufficiently strong, its action is not precluded in the future.

We also find empirical evidence that drift is beginning to dominate evolutionary processes that influence these ecologically relevant gene regions in small *S. catenatus* populations. These signals are that the small population of CEBO appears to have

lost a small but significant amount of variation in MHC Class I and signals of recent selection are diminished in the central tendency analyses for |iHS| and all tests of differentiation (F_{ST}). The latter tests rely on patterns of linkage disequilibrium which become longer and noisier in small populations (|iHS|), and allele frequencies (F_{ST}) which vary substantially under prolonged drift. However, we cannot distinguish between the erasure of the signal of past selection from an absence of selection (Black et al. 2024), or weak selection (Kimura and Ohta 1969). However, the finding of the increasing importance of drift shaping adaptive variation in smaller populations is in qualitative agreement with previous work on *S. catenatus* (Ochoa et al. 2020, Bolton et al. 2026), and other rare species (Strand et al. 2012). Thus, while adaptive diversity is retained in small non-equilibrium populations at present (Figure 3C) (Sovic et al. 2019), some portion likely reflects drift debt, the time-lag between a demographic decline and a reduction in genetic diversity to equilibrium levels (Gilroy et al. 2017; Clark et al. 2024; Gargiulo et al. 2025; Exposito-Alonso 2025).

The question of drift debt is also relevant to the time scale encompassed by different summary statistics and selection tests used for conservation genetic studies, including this study (Allendorf 1986; Ebert and Fields 2020; Mathur et al. 2023; Clark et al. 2024). Our tests detect selection over a diversity of timescales, some of which predate recent anthropogenic impacts, and raise the possibility of a potential lag in the detection of signatures of drift overwhelming selection and reducing adaptive variation in present day populations (Gargiulo et al. 2025). For example, the most recent formal test of selections provided by haplotype homozygosity tests (iHS) suggests effective selection at the timescale preceding recent anthropogenic impacts (0.02 and <1Ne or ~1500–30,000 ybp), but there may be a lag in the breakdown of this signal for small populations (see above). Only our analysis comparing diversity at focal and background sites in large and recently declined *S. catenatus* populations (Figure 3C) provides suggestive evidence that selection has been acting following recent significant human impacts. However, these results cannot be used to assess selection in contemporary populations over timescales of a few generations that are most relevant to contemporary management actions. Only detailed studies of allele frequency change in serially sampled contemporary populations will confirm whether selection is currently operating on the remaining adaptive diversity in these small populations (Stervander et al. 2020; Davies et al. 2021; Funk et al. 2026) or not (Grueber et al. 2013; Hoffmann et al. 2017).

5 | Conservation Implications

Our results have several conservation implications. First, our results suggest that small populations of *S. catenatus* retain genetic variation in ecologically important gene regions and show indications that they have responded to selection, and so retain some evolutionary potential, at least under scenarios of strong selection. The focal regions examined here also likely represent a best-case scenario for assessing evolutionary potential because of balancing selection enabling the retention of diversity over longer timespans than for other ecologically important genes. Therefore, a more comprehensive assessment of evolutionary potential across additional gene regions using multiple methods is needed for a complete assessment of the potential of *S. catenatus* populations.

Second, the mixed evidence for selection operating over recent timescales in the smallest populations implies that strong drift effects may be on the verge of overwhelming the selection currently operating on functional variation in small populations. Thus, these populations may be on the cusp of losing their ability to evolutionarily respond to environmental change. If this is the case then it raises the issue that some of the functional diversity observed in ecologically relevant loci in these small populations may represent drift-debt (Gilroy et al. 2017; Ochoa et al. 2020). Indeed, long lifespans and overlapping generations in these rattlesnakes argue that these populations are not at genetic equilibrium and that drift debt will remain a significant issue in assessing genetic issues in small populations of these snakes (Sovic et al. 2019; Gargiulo et al. 2025).

Finally, from our results we argue that the most effective management actions to maintain the evolutionary potential of small populations of these rattlesnakes are those that will increase population size and minimize drift effects. This may include genetic augmentation of small populations (Fitzpatrick et al. 2020) but this should be done with caution due to the potential to introduce deleterious variation (Mathur and Gibbs 2025).

Author Contributions

Peri E. Bolton: conceptualization, investigation, methodology, formal analysis, data curation, validation, visualization, supervision, writing – original draft and writing – reviewing and editing; **Drew R. Schield:** conceptualization, methodology, writing – review and editing; **Daphne Kaur:** data curation, software, investigation, writing – review and editing; **H. Lisle Gibbs:** conceptualization, methodology, writing – original draft and writing – reviewing and editing, supervision, resources, project administration, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The sequence data used are archived in NCBI BioProject PRJNA1397008, and code is available here: https://github.com/peripheripatus/Sistrurus_multicopy_evolution/.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Annotations for MHC and venom sequences in *Sistrurus catenatus*. **Figure S2:** CNV content of focal and background regions. **Figure S3:** Windowed data for snake venom metalloproteinases (SVMP) gene region. **Figure S4:** Windowed data for snake venom serine proteases (SVSP) gene region. **Figure S5:** Windowed data for snake venom phospholipase A2 (PLA2) gene region. **Figure S6:** Windowed data for major histocompatibility complex (MHC) class I on Chromosome 2. **Figure S7:** Windowed data for major histocompatibility complex (MHC) class IIβ on Chromosome 2. **Figure S8:** Windowed data for major histocompatibility complex (MHC) class IIβ on Unplaced Scaffold 21. **Figure S9:** Boxplots of nucleotide diversity (π) for additional sequences examined. **Figure S10:** Boxplots of absolute divergence (DXY) for additional sequences examined. **Figure S11:** Boxplots of relative differentiation (F_{ST}) for additional sequences examined. **Figure S12:** Boxplots of |iHS| scores of unpolarized SNPs. Supplemental References. Supplementary Methods. **Table S1:** Details of samples used in this study. ncluding the sample code; Year collected; species code: SCAT = *S. catenatus*, STER = *S. tergeminus*, SMIL = *S. miliarius*; Anonymyzed population code; State collected; Number of reads that are new to this study that mapped to the genome; total reads mapped to the genome; SRR columns indicates the contributions of reads from previous studies and their accession numbers. **Table S2:** Sequences used to query *Sistrurus catenatus* genome (GCA_039880765.1) using BLAST and Fgenesh+. **Table S3:** Number of sites in analyses and descriptions of main filters applied to analysis. Where N focal region for iHS analyses shows number of sites, remainder are window sizes. **Table S4:** Comparison of numbers of genes annotated in multi-copy gene families in *S. catenatus* genome versions.