Pronounced genetic structure associated with differences in a reproductive trait and climatic barriers in Canadian populations of western toads (*Anaxyrus boreas*)

Jayna Bergman
Bachelor of Science, University of Lethbridge, 2022

A thesis submitted to the University of Ottawa
in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGICAL SCIENCES

Department of Biological
Faculty of Science
University of Ottawa

Pronounced genetic structure associated with differences in a reproductive trait and climatic barriers in Canadian populations of western toads (*Anaxyrus boreas*)

Jayna C. Bergman

Dr. J. A. Lee-Yaw Thesis Supervisor	Assistant Professor	PhD
Dr. C.I. Cullingham Carleton University Thesis Examination Committee Member	Associate Professor	PhD
Dr. J.G.A. Martin University of Ottawa Thesis Examination Committee Member	Associate Professor	PhD

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Julie Lee-Yaw for her guidance and unwavering support throughout my academic journey. Her mentorship has been pivotal in shaping my abilities, building my confidence, and fostering my growth as a scientist. Julie's enthusiasm for every achievement, big or small, has been a constant source of motivation. I am profoundly thankful for her dedication as a supervisor and the time that she has invested in my academic development.

Thank you to my thesis advisory committee members, Dr. Catherine Cullingham and Dr. Julien Martin, for their valuable input, support, and time. Thank you to Catherine for her expertise and advice with handling genomic data and to Julien for his statistical expertise and his commitment to student advocacy within the graduate program.

I would like to thank Melissa Todd and Roseanna Gamlen-Greene for tissue samples from otherwise inaccessible locations, and for sharing tips and tricks for locating tadpoles in the field. Roseanna also provided valuable advice during the initial stages of the bioinformatics work, which I greatly appreciate. A huge thank you to Greg Pauly for the data he collected during his dissertation and the insightful conversations we have had. This project would not have been possible without the inspiration from his dissertation.

I would like to thank the members of the Lee-Yaw lab, past and present. Juan Enciso has been instrumental in helping me develop my bioinformatics skills and I am deeply thankful for his guidance, knowledge and patience. Thank you to Alice Cang and Madeleine Robitaille for their encouragement and support through the final, extremely challenging weeks of my thesis. I am appreciative for all the lab members — including Ashley Smith, Christie Crews, Juliet Cruz,

Natalie Fleece, and Yisa Ginath — for their feedback and support. A special thanks to Kaegan Finn for his encouragement and support, and for checking in during the particularly tough early months of my thesis. I appreciate his mentorship, contagious positivity, and friendship.

My field seasons were some of my best experiences in graduate school, thanks to invaluable field assistants. Thank you to my dear friend, Shay Marks, for I would not have survived my first field season without her expertise and her positivity on even the longest field days. Thank you for spending two months in the bush with me, and making it extremely fun along the way. I'm also immensely grateful for my field assistant Hannah Meikle, who has since become one of my closest friends. The second field season was just as memorable because of her patience and kindness, even when I was hangry. I am thankful for her continued encouragement and for showing up with pep talks when I needed them most.

I'm grateful to the many Parks Canada employees who assisted with site access, including Cathy Gill, Kimberly Pearson, Tony Einfeldt, and Colin Bergeron. Additionally, thank you to Geoff Skinner and Griffin Dare for joining us in Jasper National park to help collect samples over several days. Thank you as well to Alberta Biodiversity Monitor Institute staff, Brandon Allen and Jenet Dooley, for their support with field logistics and information. A big thank you to Mark Thompson for his invaluable insight into locating toads throughout BC.

I am grateful to Dr. Carole Yauk for allowing me to use her lab space. Special thanks to David Eickmeyer for help navigating university systems, learning the ins and outs of lab work, and for offering advice and support through many helpful chats. I am deeply appreciative to David Schuster for the time he spent training me in the lab, optimizing protocols, and for the company and support through long lab days.

Thank you to the members of the Coltman lab and Dr. Catherine Cullingham's lab for their insightful conversations and the invaluable advice on genomic filtering. I would also like to thank Rebbeca Taylor for answering my many questions about genomic analyses and for her excellent advice and support.

Thank you to the agencies that funded my work, including NSERC, the Alberta Conservation Association, the province of Ontario and the University of Ottawa.

I would not have made it to this point without the love and support of my family. Thank you to my big sister, Kayla, who inspires me daily and has spent countless hours on the phone listening, offering advice, and keeping me sane. Thank you to Mom and Eli for always being a phone call away with loving support, and to my Dad and grandparents for encouraging me to purse academia. And thank you to Aurora, for being the most loving cat and providing endless emotional support every single day.

Finally, I want to thank the toads for being unique, charismatic creatures that I Toad-ally adore. I deeply appreciate the organisms whose vital contributions and ultimate sacrifices were made for the benefit of science and conservation.

Abstract

Identifying genetic groups within species is essential for understanding biodiversity, characterizing species' distributions, and for delineating conservation units. I aimed to characterize genetic structure for western toads (*Anaxyrus boreas*) in Canada where they are currently recognized as two legal conservation units (i.e. designatable units): a Calling population, which has a pronounced breeding call and vocal sac, and a Non-calling population, which lacks both these traits. I assess whether genetic differences exist between these populations and correspond to known phenotypic differences in calls. I additionally explore ecological differences between Calling and Non-Calling populations using ecological niche models. Genetic analyses revealed the Calling and Non-calling populations are genetically distinct, with a high concordance with the call phenotypes. The observed genetic boundary between these groups also aligns with ecological breaks in habitat suitability. In addition to differences between Calling and Non-Calling western toads, I found evidence of a third genetic group in southwestern Alberta and southeastern British Columbia, in what is known as the Crown of the Continent Ecosystem. Overall, my thesis suggests the continued recognition of the Calling and Non-calling populations as separate conservation units is warranted, and highlights the potential for northern latitudes to harbor biologically significant genetic structure.

Resumé

Identifier les groupes génétiques au sein des espèces est essentiel pour comprendre la biodiversité, caractériser la répartition des espèces et délimiter les unités de conservation. J'ai cherché à caractériser la structure génétique des crapauds de l'Ouest (Anaxyrus boreas) au Canada, où ils sont actuellement reconnus comme deux unités de conservation légales (c'est-à-dire des unités désignables) : une population « appelante », qui présente un chant nuptial marqué et un sac vocal, et une population « non-appelante », qui ne possède ni l'un ni l'autre. J'évalue si des différences génétiques existent entre ces populations et si elles correspondent aux différences phénotypiques connues dans les chants. J'explore également les différences écologiques entre les populations appelante et non-appelante à l'aide de modèles de niche écologique. Les analyses génétiques ont révélé que ces deux populations sont génétiquement distinctes, avec une forte concordance avec les phénotypes d'appel. La frontière génétique observée entre ces groupes correspond aussi à des ruptures écologiques dans la qualité de l'habitat. En plus des différences entre les crapauds appelants et non-appelants, j'ai trouvé des preuves d'un troisième groupe génétique dans le sud-ouest de l'Alberta et le sud-est de la Colombie-Britannique, dans ce que l'on appelle l'écosystème de la Couronne du Continent. Dans l'ensemble, ma thèse suggère que le maintien de la reconnaissance des populations appelante et non-appelante comme unités de conservation distinctes est justifié, et met en lumière le potentiel des latitudes nordiques à abriter une structure génétique biologiquement significative.

Preface

There will be several co-authors on this manuscript when it is submitted. I collected the majority of the tissue samples, developed most of the code, ran all the analyses, and wrote the final version of this thesis. Juan Enciso helped develop some of the bioinformatics coding and assisted with trouble shooting the bioinformatics. Melissa Todd and Roseanna Gamlen-Greene contributed tissue samples from twelve sites across British Columbia. Greg Pauly contributed the phenotypic dataset on the scored call morphology.

Table of Contents

List of Figures

List of Acronyms

DU	Designatable Unit
ENM	Ecological niche models
IBD	Isolation-by-distance
COSEWIC	Committee on the Status of Endangered Wildlife in Canada

Introduction

Characterizing genetic structure within species' geographic ranges is an integral part of understanding species' distributions. In particular, genetic structure provides insight into the history of species' ranges and the factors influencing colonization (Hewit, 2004; Cairns et al., 2021), as well as the extent to which populations are currently connected. Genetic breaks in the range inform our understanding of geographic and ecological barriers that influence dispersal (Waters et al., 2020; Suarez et al., 2022) and thus inform our understanding of the ability of species to track suitable conditions as the climate changes. Finally, assessments of genetic structure within species' ranges have become important from the standpoint of inventorying biodiversity, allowing for the identification of cryptic taxa (Bickford et al., 2007; Fiser et al., 2017) and the delineation of conservation units for wildlife management (Funk et al., 2012; Coates et al., 2018; Forester et al., 2022).

Genetic substructure is expected to be particularly pronounced in the northern hemisphere, where species experienced repeated range expansions and contractions into isolated refugia during the Pleistocene glaciations (Hewitt, 2004). Indeed, numerous studies have used molecular markers and methods to assess substructure within species' ranges in areas impacted by the Pleistocene glaciations, revealing distinct genetic groups within what are otherwise widespread species (see review by: Soltis et al., 1997; Hewitt, 2000; Brunsfelt et al., 2001; Sommer and Zachos, 2009; Shafer et al., 2010; Lyman and Edwards, 2022; Fu and Wen, 2023; Garcia-Rodriguez et al., 2024). However, gaps remain in our understanding of structure in some regions. For example, in North America, genetic studies are often biased towards populations in the United States, limiting our understanding of genetic structure in Canada, which represents the northern portion of many species' ranges (Lesbarrères et al., 2014). What is known about genetic

structure across Canada for many species comes from earlier studies using mitochondrial markers or a limited number of nuclear markers, which may reflect incomplete lineage sorting (e.g. Miller et al., 2012) or introgression following divergence (e.g. Ballard and Whitlock, 2004), thereby limiting our understanding of variation across the entire genome (Funk et al., 2019; Gallego-García et al., 2021).

Next generation sequencing has provided an opportunity to revisit questions about genetic structure within species' ranges using information from across the genome and has provided new insight into the number and distribution of genetic groups within the ranges of several species (e.g. Barbosa et al., 2018; Dinca et al., 2019; Cairns et al., 2021; Gallego-García et al., 2021). However, care is needed when using genomic data to delineate meaningful genetic groups. For instance, recent studies have noted that when there are gaps in the sampling of the geographic range, what is continuous genetic variation due to patterns of isolation-by-distance (IBD) may be misinterpreted as distinct genetic substructure (Chambers and Hillis, 2020; Turbek et al., 2023). Ensuring samples are representative across suspected contact zones is thus important for identifying true genetic breaks within species' ranges. Additionally, the high resolution of genomic data is likely to reveal fine-scale structure which may lead to issues with the over splitting of groups (Sukumaran and Knowles, 2017; Coates et al., 2018). Thus, pairing genomic assessments of structure with phenotypic (e.g. Gordon et al., 2020; Waples and Lindley, 2018) and/or ecological (e.g. Campbell et al., 2022) data that can provide insight into the biological significance of any observed groups is ideal.

Amphibians are expected to exhibit strong genetic structure within their ranges because they have low dispersal ability, are often philopatric, and show evidence of limited gene flow over small spatial scales (Beebee, 2005; Zeisset and Beebee, 2008). Indeed, amphibians in North

America have been shown to consist of distinct genetic groups, with continentally distributed species often consisting of eastern and western clades (e.g. Lee-Yaw et al., 2008; Everson et al., 2021). Within the eastern United States, intraspecific genetic structure is often aligned with major geographic features such as the Atlantic Coast of the Florida peninsula, Apalachicola and Mississippi Rivers, and the Appalachian Mountains (Soltis et al., 2006; Lyman and Edwards, 2022). In the west, sharp genetic breaks also tend to line up with major geographic features, including the Cascade/Coast mountains range, the southern Rocky Mountains, and the Columbia River Basin (Zeisset and Beebee, 2008; Shafer et al., 2010). However, most phylogeographic studies of amphibians in North America show a heavy bias towards sampling of populations in the United States (Lesbarrères et al., 2014; but see Lee-Yaw et al., 2008; Wilson et al., 2008; Lee-Yaw and Irwin, 2012; Cairns et al., 2021). This limits our understanding of the features that shape genetic structure in the north, as well as our ability to delineate appropriate conservation units and implement conservation strategies to protect intraspecific genetic diversity (Lesbarreres et al., 2014).

Western toads (*Anaxyrus boreas*) are one of the most widely distributed amphibians in western North America. They are unique in that they are one of just two amphibian species in Canada to maintain an extensive range on either side of the Rocky Mountains, a barrier that shapes genetic diversity in wide-ranging species in other groups (Shafer et al., 2010; Jensen et al., 2024). Furthermore, western toads on either side of the Rocky Mountains exhibit differences in a key reproductive trait, with individuals in Alberta having a pronounced breeding call and a vocal sac, and those in British Columbia (BC) lacking both traits (Pauly, 2008). To the best of our knowledge, this is the only documented case in anurans where there is polymorphism in terms of the presence or absence of male breeding calls. Based on these differences, “Calling”

and “Non-Calling” populations are recognized as distinct conservation units in Canada (referred to as Designatable Units or DUs) by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2012). Yet, despite attention to genetic structure elsewhere in the species’ range (Goebel et al., 2009; Switzer et al., 2009; Addis et al., 2015; Gordon, 2017; Gordon et al., 2020; US Fish and Wildlife, 2017; Gamlen-Greene, 2022; Trumbo et al., 2023; Fig. 1A), the extent to which Calling and Non-calling populations of western toads are genetically distinct remains unclear. Given conservation concern for western toads across much of their range (e.g. Trumbo et al., 2023), including in Canada (COSEWIC, 2012), understanding genetic structure is essential for informing future management decisions such as developing recovery strategies or identifying suitable source populations for translocations.

Objectives

In this study, I set out to clarify genetic structure across the Canadian range of western toads. My goal was to specifically evaluate whether genomic data support the recognition of the Calling and Non-calling populations as distinct groups and to assess concordance of any observed genetic boundaries with the previously-described variation in call morphology (Pauly 2008). To understand the potential role of climate in shaping structure in this group, I used ecological niche models to assess the amount of niche overlap between the two populations and to explore the extent to which the Rocky Mountains serve as a potential barrier to gene flow. This work is timely as the legal status of this species in Canada, including whether the two DUs should continue to be recognized as separate units, is scheduled for re-evaluation by COSEWIC in the near future and results from my thesis have the potential to inform these discussions.

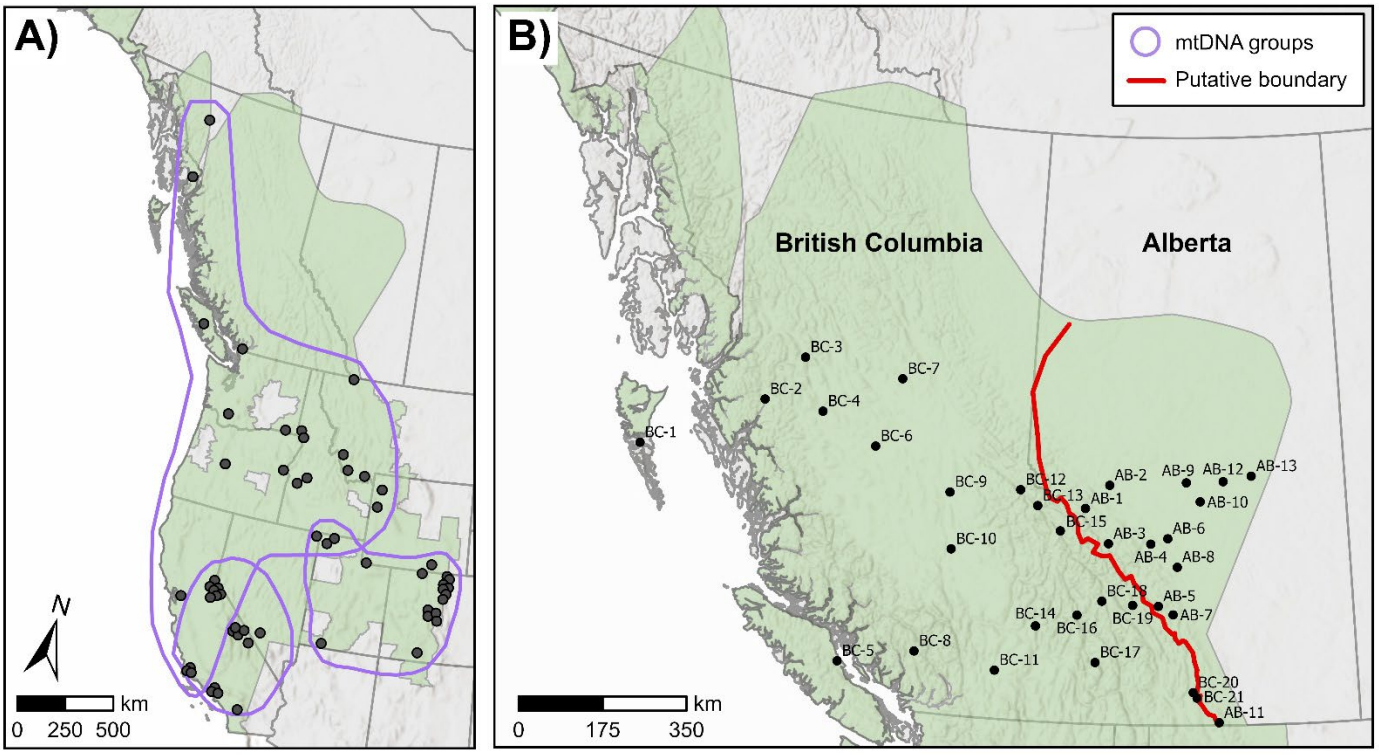

Figure 1. (A) Geographic range of western toads (*Anaxyrus boreas*) and previously identified mitochondrial DNA clades (Goebel et al., 2009) and (B) locations of the genetic sampling sites used in the present study to assess genetic structure across the Canadian portion of the species' range. In both figures, green shading indicates the geographic range of *Anaxyrus boreas*. The putative boundary between the Calling and Non-calling populations is shown as a red line (redrawn from: Environment and Climate Change Canada, 2020). Points in (A) represent approximate locations of the sampling sites used to identify mtDNA groups (purple polygons) in previous studies (recreated from Goebel et al., 2009).

Methods

Tissue sampling

Tissue samples were collected between June and August of 2018 to 2023 from coastal BC to the eastern edge of the species' range in Alberta (Fig. 1B; Table S1), including across the putative boundary between the Calling and Non-calling populations. Sites near the putative contact zone of the two populations were selected to be as close as possible to sites from which individuals phenotyped for the presence or absence of vocal sacs were sampled (as per Pauly, 2008). Between one and five individuals were collected from each site, resulting in a total of 46 tissue samples from 39 sites. Whole larvae (or young of the year when larvae were not available) were captured by hand or by dipnet from each site. Individuals were humanly euthanized on site using a 3% buffered MS-222 solution. Samples were preserved in 95% ethanol before being brought to the lab for long-term storage at -80°C.

DNA extraction and sequencing

Total genomic DNA was extracted from 25 mg of larval tail tissue or metamorphic leg muscle using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA). The manufacturer's protocol was followed with the following modifications: I added a physical homogenization step, using a VWR bead mill with 2.8 mm ceramic beads to improve tissue digestion. Tissues were then digested for three hours in an incubator set at 56°C, after which, I added 6 µl of RNAase A solution (10 mg/ml). Samples were eluted in two steps using 80 µl and 60 µl of buffer AE respectively. DNA concentration and quality were assessed using a Thermo Fisher Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and NanoDrop 1000 spectrophotometer (Nanodrop Technologies Inc. Wilmington, DE, USA) . Samples were diluted and normalized to a concentration of 20 ng/µl in 20 µl (400 ng total) before library preparation.

I obtained sequence data for each individual using double digest restriction site-associated DNA sequencing (ddRADseq; Petersen et al., 2012). Library preparation was done at the Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (IBIS, Université Laval, Quebec, Canada) and was based on the genotyping-by-sequencing procedures described by Poland et al., (2012). Double-stranded DNA was digested using the restriction enzymes *PstI* (5-CTGCAG-3) and *MspI* (5'-CCGG-3'). A set of corresponding Illumina adapters were ligated to the ends of the fragmented DNA and the inline barcodes were ligated to the adapters. Prior to PCR amplification, a Blue Pippin (SAGE Sciences Inc. Beverly, MA, USA) was used to select fragment sizes between 100 and 300 bp using an elution time set between 50 and 65 minutes on a 2% gel. Individuals were sequenced in a single library that included both a technical replicate (replicated from DNA extraction) and two negative controls. A single library was sent to the Centre d'expertise et de service Génome Québec at McGill University in Montreal Quebec for paired-end sequencing on an Illumina NovaSeq 6000. There, multiple libraries were normalized, pooled, and then denatured in 0.02N NaOH and neutralized using HT1 buffer. Using the Xp protocol as per the manufacturer's recommendations, pools were loaded at 200 pM on an Illumina NovaSeq S4 lane and run for 2 x 100 cycles in paired-end mode. Base calling was done with RTA v3 and bcl2fastq2 v. 2.20 (Illumina inc. 2019) was used to demultiplex the libraries 6000 system with S4 PE150 flow cells. A total of 1,506,075,128 reads were generated across all individuals.

Loci assembly and filtering

Raw reads were demultiplexed using *process_radtags* in Stacks (V2.66; Catchen et al., 2013). Removing reads with uncalled bases or adaptor contamination left 971,781,833 reads

across all individuals. Fastp (version 0.23.4; Chen, 2023) was ran without any filters set and then Multiqc (version 1.21; Ewels et al., 2016) was used to generate plots showing the GC content along the reads for all samples (Fig. S1). These plots were visually inspected to identify base pairs at the start of the reads with excessive GC content, which are indicative of barcode and adapter contamination. Following visually inspection, Fastp was then used to trim barcodes and adapters by removing five bases from the forward reads and three bases from the reverse reads. I also used Fastp to remove reads with an average Phred quality score less than 20, resulting in 929,487,304 reads (1,853,538 to 56,194,548 reads per individual). Following these initial data processing steps, multiqc was used again to verify sequencing quality and ensure GC content along the reads was not excessive prior to alignment.

Reads were aligned to the western toad reference genome (Jackson County, Colorado, USA; Trumbo et al., 2023) using the Burrows-Wheeler algorithm in bwa-mem v. 0.7.18 (Li and Durbin, 2009), which is appropriate for aligning short-sequenced pair-end Illumina reads to large-reference genomes. The resulting SAM files were sorted and converted to BAM format using Samtools (Li, 2011). I called single nucleotide polymorphisms (SNPs) from across mapped regions of the reference genome using the *gstacks* module in Stacks (version 2.66) and filtered out stacks of reads with a quality score less than 20, resulting in 1,772,249 loci and 212,670,599 matching paired-end reads with a mean coverage of 12.0X (range: 3.0X to 31.3X, SD=6.2X). SNPs with high heterozygosity often result from paralogs or alignment errors. Thus, I removed SNPs with excess heterozygosity by setting the maximum observed heterozygosity (--max-obs-het) to 0.5 using the *populations* module in Stacks (version 2.66) with all individuals treated as a single population. This resulted in 4,606,447 SNPs across all individuals.

Further filtering was conducted in R (version 4.3.3, R core team, 2024), using the package SNPfiltR (DeRaad, 2023). To ensure accurate genotype calls, I removed SNPs with a genotype depth of less than five or with more than 22 reads resulting in 4,410,477 SNPs. I additionally removed genotype calls with quality of less than 20. The ratio of reads with reference versus alternative bases in heterozygous individuals is expected to be balanced, with large imbalances potentially signaling issues with coverage, multilocus contigs or other artifacts (O'Leary et al., 2018). Thus, I filtered loci with an allele balance less than 0.25 or greater than 0.75 (i.e. minimum number of reads to call a heterozygote was two). I then applied a minor allele count of three across all samples retaining 2,171,753 SNPs (mac = 3, meaning there must be at least one homozygote and one heterozygote for the alternative allele across all individuals to keep a SNP). I iteratively filtered for both missingness of individuals and SNPs (O'Leary et al. 2018; Table S2). As most sites in my study are represented by a single individual, I prioritized the retention of individuals over SNPs during this process. Six individuals were removed due to high amounts of missing data, resulting in 40 samples and 22,118 SNPs being retained. Finally, I removed SNPs that are likely in linkage disequilibrium using the function *--indep-pairwise* in PLINK v.1.90b (Chang et al., 2015). This function calculates the correlation coefficient between pairs of SNPs within a specified window and removes SNPs until all the correlations within the window are below a given threshold. Following sensitivity tests to assess the impact of different thresholds on population structure (see supplementary material), the window size was set to 50 base pairs with a step size of 5 base pairs, and the R^2 threshold was set to 0.8. The full filtering workflow can be found in the supplementary methods (Table S2), with corresponding code available online (https://github.com/Jaynabergman/WETO_ddRAD).

Characterizing genetic structure across the range

I used four approaches to assess genetic structure across the Canadian range of western toads. First, I visualized the distribution of individuals in multivariate genetic space using principal component analysis (PCA) and discriminant analysis of principal components (DAPC). PCA summarizes the variation in the data and is a parameter-free method (Elhaik, 2022), whereas DAPC attempts to maximize among group variation while minimizing the variance within groups using prior group assignments (Jombart et al., 2010). PCA was run using the program PLINK v.1.90b (--pca; Chang et al., 2015). Prior to running DAPC, I used the *find.clusters* function to determine the optimal number of genetic clusters based on the Bayesian Information Criterion (BIC) and to assign individuals to these clusters. DAPC was then run retaining four principal components and two discriminant functions using the R package Adegenet (Jombart, 2008).

In addition to ordination analyses, I assessed the phylogenetic relationships between individuals by generating a maximum likelihood tree in IQTree v.2.2.2.7 (Minh et al., 2020). SNP datasets consist solely of variant sites, and this was accounted for using the ascertainment bias correction (ASC). Models with ASC require the removal of partially consistent sites (SNPs for which one variant is ambiguously called). Removing these sites left 9,648 SNPs for this analysis. IQTree was run using the best-fit model (TMV+F+ASC+G4) as determined by ModelFinder (Kalyaanamoorthy et al., 2017), with 1000 Ultrafast bootstrap replicates.

As a final approach to identifying genetic structure, I ran the program STRUCTURE v.2.3.4 using the admixture model (Prichard et al., 2000). This program estimates proportions of individual ancestry to infer genetic clusters and assess levels of admixture among clusters (Prichard et al., 2000). Structure requires the user to specify the number of groups (K) *a priori*

and I tested values of K from 1 to 10. I used a burnin period of 300,000 steps, 1,000,000 Markov Chain Monte Carlo (MCMC) steps, and 10 replicate runs for each value of K. CLUMPAK (Kopelman et al., 2015) was used to average the runs for each value of K, and ΔK (Evanno et al., 2005) was used to determine the optimal value of K.

All analyses revealed the same clustering of individuals (see Results), and I used the R package dartR (Mijangos et al., 2022) to estimate F_{ST} between genetic groups. To understand the role of IBD in structuring genetic diversity within the species' range, I used Mantel tests to determine if there was a significant association between pairwise geographic and genetic distance. As most sites in my dataset were represented by a single individual, I used the proportion of shared alleles between pairs of individuals as an estimate of genetic distance (rather than differences in allele frequencies). To avoid issues of non-independence associated with multiple individuals at some sites, for the few sites with multiple individuals, I randomly selected a single individual for this analysis. Genetic distances between individuals were calculated using the *gl.dist.pop* function from the dartR package and pairwise Euclidean geographic distances between individuals was calculated using the *distm* function in the R package geosphere (Hijmans, 2024). Separate Mantel tests were run for: 1) all samples, 2) all samples excluding three individuals from southeastern BC and southwestern Alberta which formed a separate genetic group (see Results), and separately for the 3) calling and 4) non-calling parts of the species' range. Mantel tests were run in R using the *gl.ibd* function from the dartR package.

Although patterns of IBD are expected in widespread species with continuous distributions (Turbek et al., 2023), if there is a sharp genetic break in the range we expect estimates of pairwise genetic distances between groups to be higher than estimates within groups at all

distance classes, including at the smallest distance class—that is we expect differences in the y-intercept in a plot of geographic distance versus genetic distances when pairs of samples are separated by comparison type. To test for a genetic break in IBD associated with the boundary between the Calling and Non-Calling populations, I calculated pairwise genetic distances between pairs of individuals that were no further apart than 150 km, noting the comparison type for each value (calling to non-calling: CN, calling to calling: CC, non-calling to non-calling: NN). I then calculated the difference in mean pairwise genetic distances between the different comparison types (i.e. mean CC versus mean CN; mean NN versus mean CN). To test the statistical significance of differences between groups, I compared the observed mean difference in each case to a null distribution of differences in means generated by randomizing comparison type assignment across the pairwise genetic distance values 1000 times. Mean differences greater than the 95th percentile of this distribution were considered to be statistically significant (i.e. one-sided test of significance).

Concordance between genetic boundaries and calling differences

Pauly (2008) previously examined 201 museum specimens of sexually mature, male, western toads from 84 sites across the Canadian portion of the range. Individuals were scored for the presence of vocal slits (the site from which vocal sacs originate). Specimens with one or both vocal slits were scored as calling whereas those without vocal slits were scored as non-calling (Pauly, 2008). To test for an association between genetic and phenotypic differences across the range, I assigned each phenotyped individual to a genetic clade based on Euclidean distance to the nearest genetic sample including in the phylogenetic tree. Focusing on the 39 phenotyped individuals that were within 50 km of a genetic sample, I tested if the association between

morphological group and genetic clade membership was statistically significant using a Fisher's exact test in base R.

Evaluating niche differences and environmental barriers

Ecological Niche Models (ENMs) have emerged as an important tool in molecular ecology for assessing ecological differences between observed genetic groups (e.g. Campbell et al., 2022; Gallego-Garcia et al., 2023; Macdonald et al., 2024) and the potential role of ecological barriers in maintaining groups (e.g. Glor and Warren, 2011; Lee-Yaw and Irwin, 2015). I generated separate ENMs for the Calling and Non-Calling populations to explore the variables that best predict presence and the distribution of suitable habitat for each population. Input locality records for the models were downloaded from the Global Biodiversity Information Facility (GBIF; December 26, 2024). Localities were filtered to include records from 1990 onward, to have an accuracy of <1 km to match the environmental variables (below), and to have one occurrence per grid cell of the environmental variables. Localities were classified as belonging to the Calling (N = 215) or Non-calling (N = 1523) population based on the morphological boundaries described by the species' Management Plan (Environment and Climate Change Canada, 2020). Thirty-one climate variables representing climate normals for the period between 1990 and 2020 were downloaded from Climate NA (Wang et al., 2016; Mahony et al., 2022) at a resolution of 30 arc seconds (1 km by 1 km) and cropped to the boundaries of Alberta and BC. To avoid issues with collinearity in the models, I removed variables with a high variance inflation factor (VIF >10; calculated in R: usdm Naimi et al. 2014), retaining eight variables for the models: Julian date on which the frost-free period begins, degree-days below 18 degrees Celsius, mean temperature of the coldest month, precipitation as snow (mm), summer (June to

August) precipitation (mm), spring (March to May) precipitation (mm), mean annual relative humidity (%), and summer heat moisture index.

Niche models were generated using Maxent version 3.4.3 (Phillips et al., 2006) in the R package predicts (Hijmans, 2024b). Separate models were generated for the Calling and Non-calling population using the current recognized range of each population as the background extent (Environment and Climate Change Canada, 2020). Random background points (5000) were sampled from within the respective range of each population. I used a tuning step to determine the optimal regularization multiplier (values tested: 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4) and feature classes (L= linear, Q = quadratic, H = hinge, P= product and T = threshold; combinations tested: L, LQ, H, LQH, LQP, LQT, LQHP, LQPT, and LQHPT) based on the combination that produced the lowest Akaike information criterion correction (AICc) and conducted in the ENMeval package (Kass et al., 2021) in R. The convergence threshold was set to 0.001% (Phillips et al., 2006) and the number of maximum iterations was set to 5000 to allow the models to converge (Young et al., 2011). All other settings were kept as default values, including the default prevalence ($\tau = 0.5$), which has produced accurate models for this species in previous studies (e.g. Bergman et al., 2024). For each model, I determined the area under the receiver operating characteristic curve (AUC) based on average AUC from spatial block (4-fold) cross validation.

Models were projected across BC and Alberta using Maxent's cloglog output, generating a continuous prediction surface for each population. A Multivariate Environmental Similarity Surfaces (MESS; Elith et al., 2010) was generated using the *mess* function in the R package predicts and was used to mask out areas where extrapolation to conditions outside those used to calibrate the models would be required. Conditions in these areas can be viewed as

environmental space that is "untested" by the population in question. To facilitate interpretation, I converted continuous model predictions into binary surfaces of suitable habitat using the 10% omission rate of the input locality records as a threshold for calling a cell suitable or not (Vale et al., 2014; Rosner-Katz et al., 2020; Bergman et al., 2024).

I further explored the amount of niche overlap between Calling and Non-Calling populations using an ordination approach (Broennimann et al., 2014). A PCA was used to reduce the dimensionality of the eight environmental variables. The background environmental space from within each of the calling and non-calling ranges was projected along PC1 and PC2. Using the input locality records for each population, a smoothed density of occurrences grid was plotted in the environmental space. The plot of niche overlap was then visualized using the function *ecospat.plot.niche.dyn*. All analyses were done using the R package ecospat (Broennimann et al., 2025).

Results

Sequencing and filtering

I sequenced 46 individuals from 39 breeding sites across BC and Alberta. Six individuals were removed during SNP filtering due to large amounts of missing data. After filtering, the dataset consisted of 40 individuals from 34 sites (one to five individuals per site) and 11,950 SNPs. The mean depth of coverage was 19.0X and with no more than 5% missing data per SNP and less than 33% missing data per individual.

Pronounced genetic structure across the Canadian range of western toads

All analyses assessing genetic structure clearly identified two groups of western toads corresponding to what are referred to as the Calling and the Non-calling populations (hereafter I will use Calling and Non-Calling populations to refer to both the current DUs and the genetic groups interchangeably). Specifically, the Calling and Non-calling populations separated along PC1, which explained 27.6% of the variation in the principal components analysis (Fig. 2A). Similar separation between the two groups was confirmed by the DAPC analysis (Fig. S6). Calling and non-calling individuals also formed distinct clades in the maximum likelihood tree (bootstrap support of 100 and 96 respectively; Fig. 2B). Finally, K of 2 in the STRUCTURE analysis showed the Calling and Non-calling populations forming separate groups (Fig. 2C) and received the highest support with a ΔK value of 5,146 (Fig. S7). Individuals in the Non-calling population showed a high average ancestry proportion to cluster one (average Q = 0.94), while individuals in the Calling population were assigned to cluster two (average Q = 0.98).

In addition to the differences between the Calling and Non-calling populations, my results revealed a third genetic group in Canada. Specifically, individuals from three sites in southwestern Alberta and southeastern BC separated from the Calling and Non-Calling populations along PC2, which explained 13.2% of the variation in the principal components analysis (Fig. 2A). This "Crown of the Continent" group (CotC) was also observed in the DAPC analysis (Fig. S6), with K of 3 having the lowest BIC value (231). The CotC group also formed a separate clade in the phylogenetic tree (bootstrap support of 100; Fig. 2B). Although these individuals appear admixed in the STRUCTURE analysis when K = 2, they form a separate group at K = 4 (K=3 pulled out the calling and non-calling populations and a group for which the biological significance was unclear; Fig S8). Genetic differentiation between all three genetic groups was high, with F_{ST} between the CotC group and the Calling (F_{ST} = 0.52) and Non-calling

populations (F_{ST} = 0.32) as high or higher than between the Calling and Non-calling populations (F_{ST} = 0.43).

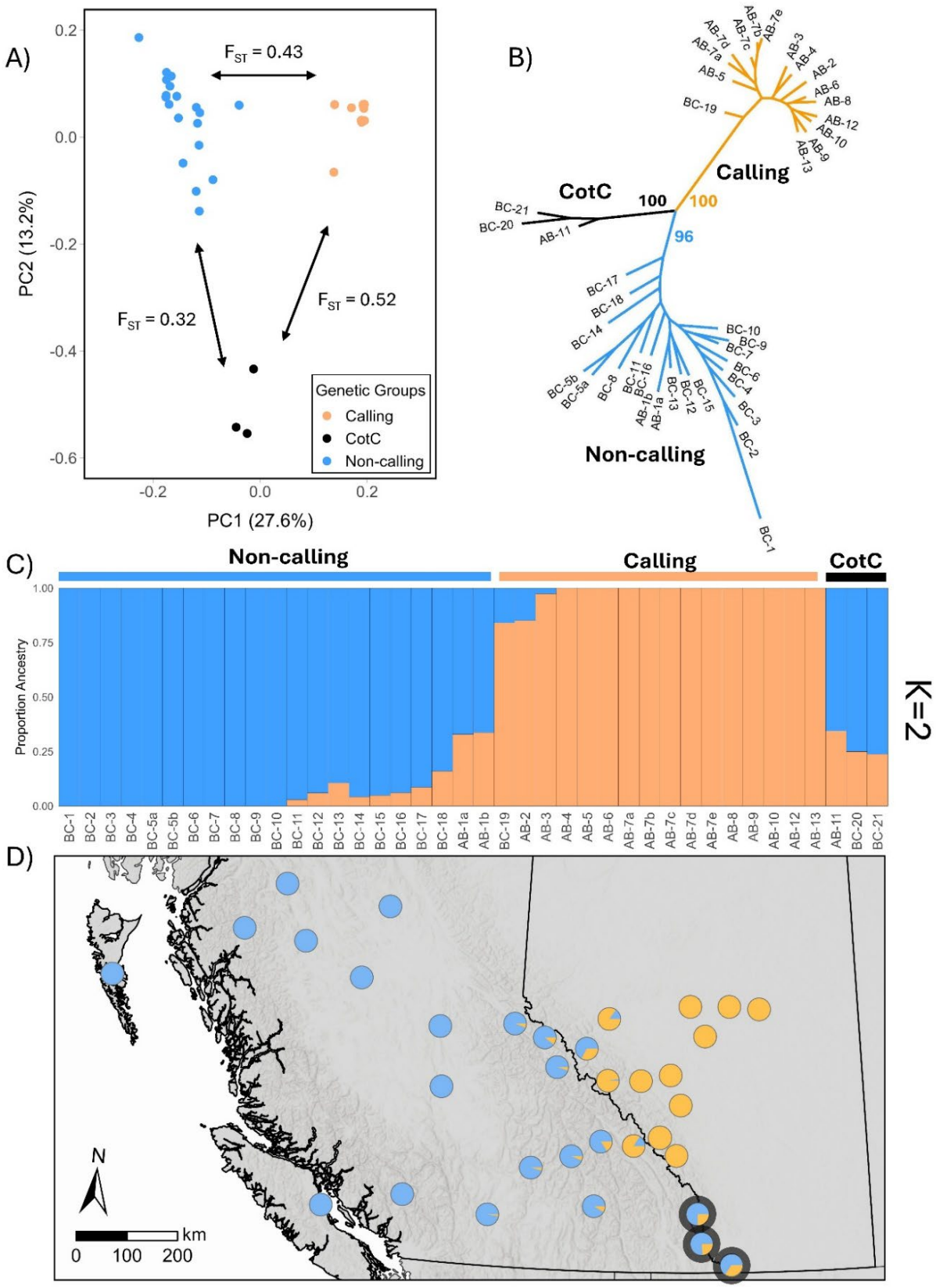
A)
F_{ST} = 0.43
F_{ST} = 0.32
F_{ST} = 0.52
PC2 (13.2%)
PC1 (27.6%)
Genetic Groups
Calling
CotC
Non-calling
B)
Calling
CotC
Non-calling
100
100
96
C)
Non-calling
Calling
CotC
Proportion Ancestry
K=2
D)
N
km
0
100
200

Figure 2. Population structure within western toads (*Anaxyrus boreas)* across the Canadian portion of the species' range. (A) Principal components analysis based on 11,950 SNPs reveals the three discrete genetic clusters corresponding to what are currently considered the Calling (orange) and Non-calling (blue) populations, and well as an additional group associated with the Crown of the Continent (CotC: black). (B) The unrooted maximum likelihood tree based on 9,648 SNPs generated with the TVM+F+ASC+G4 model in IQTree. Three groups are identified in the tree topology as the Calling population (orange branches), Non-calling population (blue branches), and the "Crown of the Continent" group (black branches). Bootstrap support values for the three main groups are shown. (C) STRUCTURE plot based on 11,950 SNPs at the optimal K=2 as determined using the ΔK method. The two groups show a split between the Calling (orange) and Non-calling (blue) individuals with low levels of admixture between the two groups and (D) a geographic representation of the STRUCTURE results. Locations in D represent individuals and show the proportion of each individuals ancestry to the two groups identified by the STRUCTURE analysis for K=2 (orange: calling genetic clade; blue: non-calling genetic clade). Points with dark black borders are individuals from a third genetic group (CotC).

Across the 34 individuals included in the Mantel test there was a pattern of IBD (34 individuals: Mantel $R^2 = 0.60$, $P < 1.0e\text{-}4$; Fig. 3A; Excluding CotC individuals: Mantel $R^2 = 0.61$, $P < 1.0e\text{-}4$). I also found patterns of IBD within each of the Calling and Non-calling populations (Calling: Mantel $R^2 = 0.36$, $P < 0.05$; Non-calling: Mantel R 0.69, $P < 1.0e\text{-}4$; Fig. 3B). However, IBD alone is unlikely to explain the strong structure observed in this system. Across all geographic distances, pairwise genetic distance when comparing calling to non-calling individuals was higher than when comparing calling to calling individuals or non-calling to non-

calling individuals (Fig. 3B). Notably, even over relatively small distances (up to 150 km), including sites spanning the boundary between genetic groups, the mean genetic difference between calling and non-calling individuals was significantly greater than the mean genetic differences between individuals from the same clade (mean proportion of shared alleles CC vs CN = 0.137, p-value <0.05; mean proportion of shared alleles NN vs CN = 0.074, p-value <0.05; Fig S9; Fig 3C). There were two few individuals in the third CotC group to test for IBD within this group. However, I note that pairwise genetic distances between the three individuals in the CotC group were all smaller than pairwise genetic distances between each of these individuals and individuals in either the Calling or Non-calling populations (Fig. 3A).

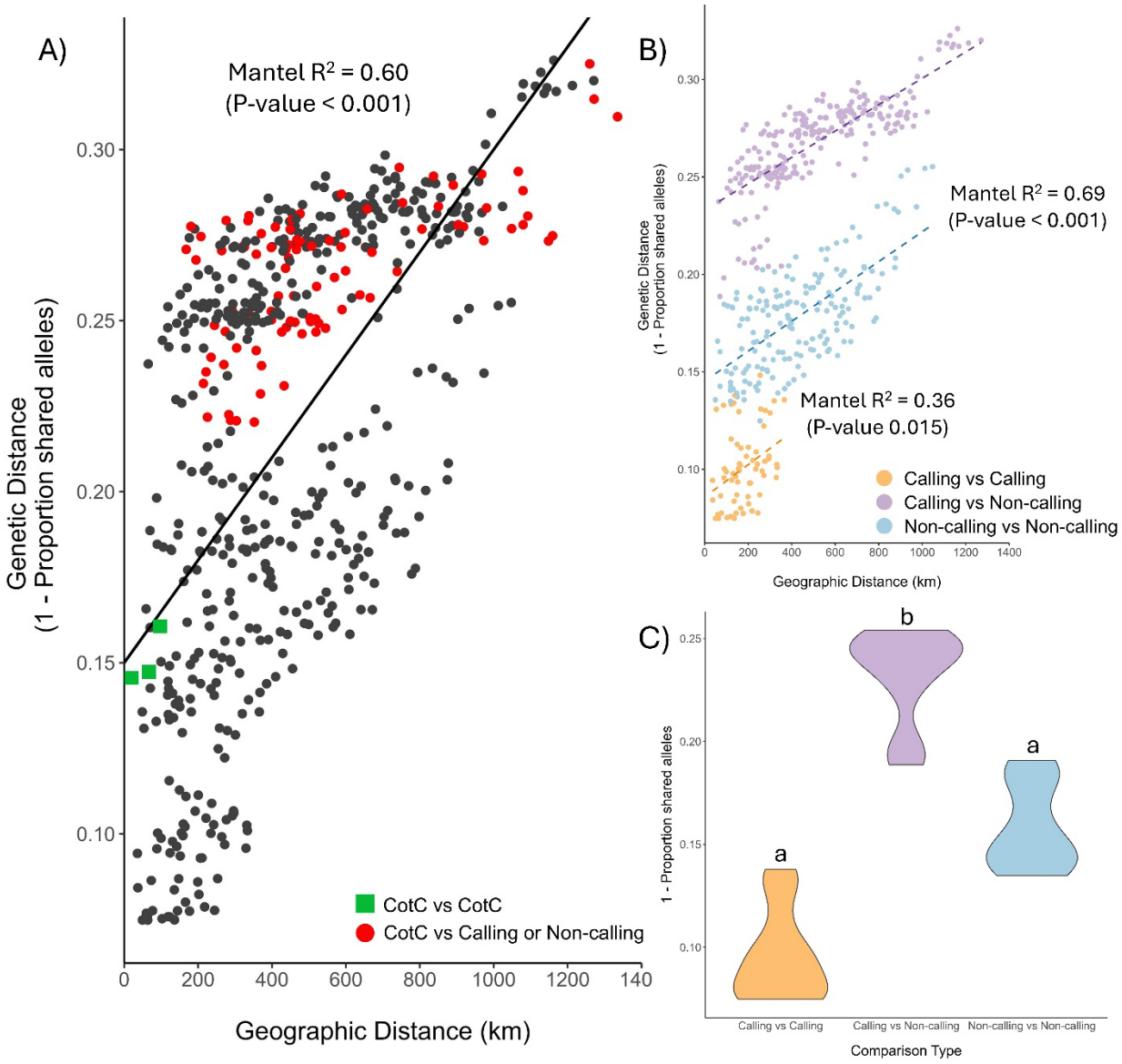

Figure 3. Pairwise comparisons of geographic distance (km) versus genetic distance (1 – proportion of shared alleles) for (A) all individuals, with the solid black line showing the Mantel test regression line for all individuals. Green squares are pairwise comparisons of "CotC" to "CotC" individuals, and red points are pairwise comparisons between "CotC" individuals and either calling or non-calling individuals. (B) The plot from A with comparisons involving the CotC group removed, and points color-coded by comparison type (purple: pairs of individuals from different populations; blue: pairs of individuals within the Non-calling population; orange:

pairs of individuals within the Calling population). The orange and blue dashed lines show the Mantel test regression lines from the IBD analyses within the Calling and Non-calling populations respectively. The calling vs non-calling regression line (purple dashed line) is for illustration purposes only. (C) The mean pairwise genetic distances for different comparison types for those individuals within 150 km of each other.

Agreement between genetic boundaries and call morphology

Assigning individuals that had previously been phenotyped for call morphology to a genetic group based on phylogenetic clade membership of the nearest genetic sample and focusing only on those phenotyped individuals that were no more than 50 km away from a genetic site, I found a significant association between call phenotype and genetic assignment (Fisher's exact test, p-value = 2.5e-07). Specially, all 14 individuals that were morphologically calling (i.e. presence of one or more vocal slits) were geographically closest to sites that fell into the Calling genetic clade in the phylogenetic tree (Fig. 4). Of the 25 individuals that were morphologically non-calling (i.e. absence of vocal slits), the majority (17) were geographically closest to individuals belonging to the Non-calling genetic clade. Of the remaining morphologically non-calling individuals, four were from the same site. This site was geographically closest to an individual in the Calling genetic clade (i.e. mismatch between call morphology and genetic assignment). However notably, this site also included a morphologically calling individual and was the only mixed phenotype site included in the analysis. This site falls along the genetic boundary between the groups and is an area of genetic admixture based on the STRUCTURE analysis. Finally, four morphologically non-calling individuals were geographically closest to the CotC genetic group (Fig. 4).

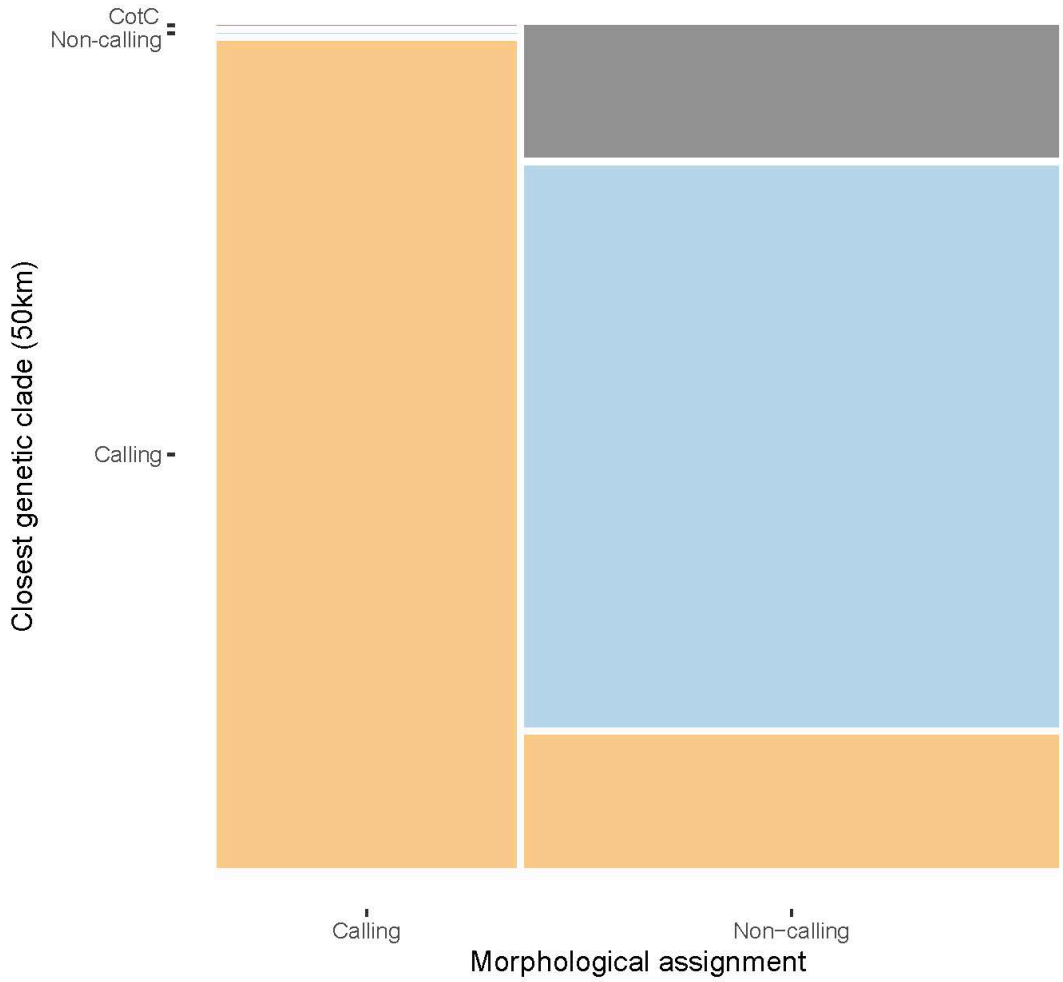

Figure 4. A mosaic plot showing the association between call morphology (presence or absence of vocal sacs from Pauly 2008) and genetic group membership of the geographically closest genetic site (based on the phylogenetic analysis). Only phenotyped specimens within 50 km of a genetic site are shown. Colours are based on genetic group: orange = calling; blue = non-calling; grey = "Crown of the Continent" (CotC).

Climate barriers may play a role in structuring western toad populations

The Maxent models performed well at predicting presence based on spatial block cross-validation (Non-calling: AUC = 0.89, Calling: AUC = 0.82; Table S4). Areas of high suitability for the Non-calling population were predicted from the southern border of BC throughout the interior plateau of BC, on Vancouver Island, and in the southwestern corner of Alberta (Fig. 5A). Areas of high suitability for the Calling population were predicted in central Alberta, along the eastern side of the Rocky Mountains, and in the center of BC (Fig. 5B). The variables deemed to be important for predicting presence based on the Maxent models were different for the two populations (Table S4). For example, the variable with the highest percent contribution for the non-calling model was the number of degree days below 18 degrees Celsius (68.8%) whereas mean temperature of the coldest month made the highest percent contribution to the model for the calling population (42.3%).

The niche overlap plot revealed that both the available climatic niche space and the occupied climatic niche space for the Calling population was smaller and completely nested within the available climatic niche space for the Non-calling population, indicating a high amount of niche overlap between the two populations (Fig. 5C). However, the geographic space predicted to be highly suitable for both populations was limited and geographically disjunct (Fig. 5, Fig. 6). Specifically, the summed binary surfaces based on the 10% omission threshold revealed that only 4% of the cells across BC and Alberta are predicted to be suitable for both populations and this area was highly concentrated within central BC and in southeastern BC and southwestern Alberta (Fig. 6).

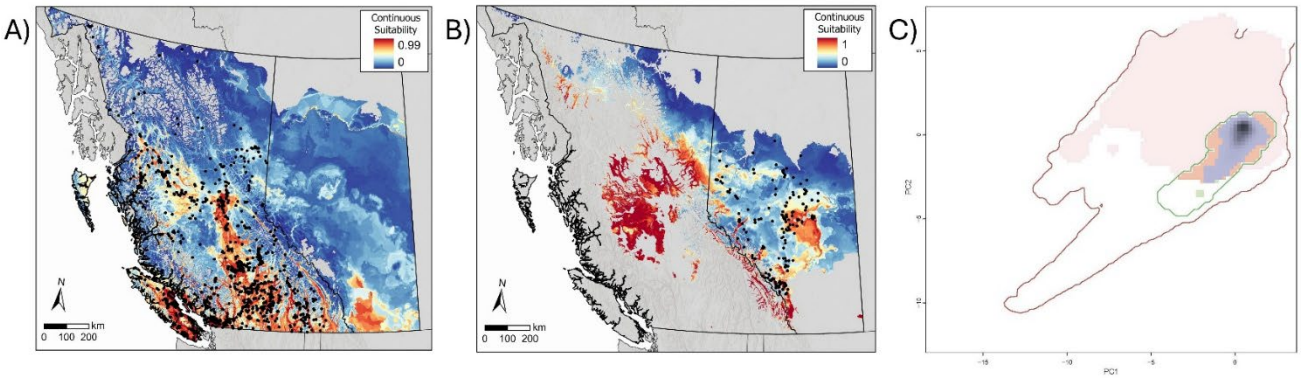

Figure 5. Continuous prediction surfaces generated by Maxent ecological niche models for the (A) Non-calling population and the (B) Calling population. The black points represent the presence records of western toads used to generate each of the models. The warmer colors represent areas of higher predicted suitability, and the cooler colors are areas of lower predicted suitability. Gray areas are areas that were outside the range of conditions used to calibrate the models as identified through Multivariate Environmental Similarity Surface (MESS) analysis. (C) Visualization of niche overlap between Calling and Non-Calling populations. The polygons show available climatic niche space in the calling range (green outline) and non-calling range (red outline). Shaded areas show environmental conditions occupied by the presence records of western toads within the calling (green pixels) and non-calling (red pixels) ranges, with blue pixels showing specific climatic conditions occupied by both populations.

Along the Rocky Mountains between BC and Alberta there is a contact zone where both the genetic and morphological boundaries of the Calling and Non-calling populations of western toads meet (Fig. 6). Focusing on a 400 km by 650 km area surrounding the genetic and phenotypic transition between the Calling and Non-calling populations, there is a very small proportion of cells that are predicted to be suitable for both populations (Fig. 6). Additionally, the contact zone has areas on both sides of the Rocky Mountains that are completely outside of the

available climatic range for either population. However, a large amount of geographic space is predicted to be unsuitable but is within the range of available climatic conditions for at least one of the populations. The geographic areas that were predicted to be suitable for either the Calling population or the Non-calling population aligned well with where the morphological and genetic individuals are found for each respective population (Fig. 6). Interestingly, the one site with both calling and non-calling phenotypes was located in an area largely predicted to be suitable habitat for the Calling population, yet surrounded by habitat predicted to be unsuitable for both populations. The CotC genetic group was found in an area of mixed suitability for the Calling and Non-Calling population.

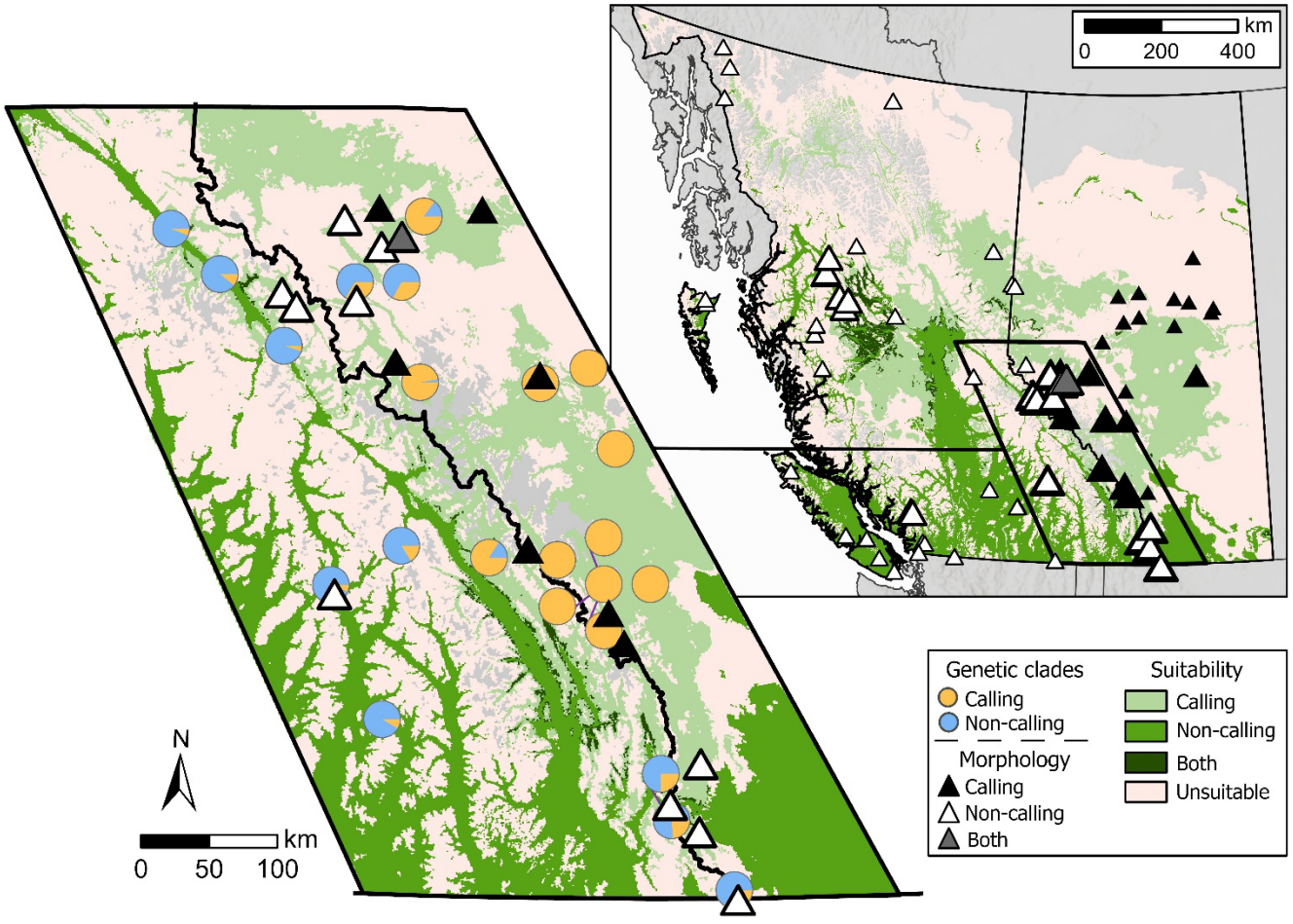

Figure 6. Summary of the distribution of climatic suitable habitat for Calling and Non-calling populations of western toads in Canada based on ecological niche models (ENMs) generated in Maxent. The zoomed in region shows the contact zone between populations delineated by a 400 km by 650 km bounding box. Green shading shows cells that are suitable for one or both populations based on binary prediction surfaces generated using the 10% omission threshold for each population based on the input locality data (see Methods). The light pink areas indicate unsuitable habitat that is within the available climatic conditions for at least one of the populations. Gray areas indicate places where climatic conditions were outside of those used to the calibrate the models for both species. Call phenotypes are shown as the triangles (black: calling morphology; white: non-calling morphology; gray: both calling and non-calling morphology; Data from Pauly 2008). Larger triangles are individuals that were within 50km of a genetic site. Circles represent individuals that were genotyped, with pie charts showing the proportion of each individuals ancestry to the two groups identified by the STRUCTURE analysis (orange: calling genetic clade; blue: non-calling genetic clade).

Discussion

I aimed to understand genetic structure across the Canadian range of western toads in Canada. I specifically paired genomic data with phenotypic data and ENMs to evaluate differences between Calling and Non-calling populations of western toads. My results suggest that the Calling and Non-calling populations are distinct genetic groups. Concordance between phenotypic and genetic boundaries lends additional support to this recognition. Furthermore, although there is some niche overlap between these two populations, my ENMs highlight the potential for climatic barriers to be limiting the extent to which these groups encounter each

other. In addition to the Calling and Non-Calling populations, my results revealed a third, previously unidentified genetic group of western toads in Canada located in the northern extent of the Crown of the Continent Ecosystem. Below I discuss the implications of these results for the conservation status of the Calling and Non-calling populations and how these findings add to our understanding of the biogeographical factors that shape genetic diversity in western Canada.

Support for the continued recognition of two populations of western toads in Canada

Identifying conservation units within species is essential for implementing effective conservation management to protect vulnerable species and preserve genetic diversity (Coates et al., 2018; Funk et al., 2012; Lehnert et al., 2023). In Canada, Designatable Units ("DUs") are established by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) using two main criteria. The first criterion focusses on the "discreteness" of populations (COSEWIC, 2023). Populations are considered to be discrete if there are heritable traits or markers that distinguish them and/or there are natural barriers to the transmission of heritable traits (i.e. barriers to gene flow: COSEWIC, 2023). Several of the results presented here speak to the discreteness of the Calling population and the Non-calling population. My genomic analyses suggest that Calling and Non-calling populations are distinct across much of their genome, with levels of genetic differentiation similar to, or even higher than, those observed between populations of other species that have been recommended for elevation as separate conservation units in other areas (e.g. Jorge et al., 2022; Gallego-Garcia et al., 2023; MacDonald et al., 2025). Additionally, results from the ENMs along with the limited amount of genetic admixture observed, suggest that the Rocky Mountains are a barrier to gene flow between the two

populations. Together, these results support the continued recognition of the Calling and Non-calling populations of western toads as discrete groups.

Isolation by distance (IBD) is expected in widespread species that are continuously distributed across the landscape (Wright, 1943; Turbek et al., 2023) and one challenge when assessing the discreteness of potential groups in widespread species is to ensure that observed genetic groups are not an artifact of IBD (Chambers and Hillis, 2020; MacGuigan et al., 2022; Turbek et al., 2023). For this reason, I sampled individuals along a transect from the west coast, through to the eastern edge of the species' range and was careful to include individuals on either side of the putative boundary between the groups. Although I observed strong IBD across the full extent of the study area and within the ranges of each of the populations, IBD alone is unlikely to account for the observed genetic structure. Specifically, genetic distances between calling and non-calling individuals are larger on average than between individuals within either population at each distance class. Importantly, at relatively small geographic distances (<150 km), including adjacent samples in the contact zone between groups, I found that mean pairwise genetic distance was significantly higher than expected when comparing samples across populations than when comparing samples within populations. Thus, while IBD plays a role in shaping the distribution of genetic variation in western toads, Calling and Non-Calling populations show genetic differences above and beyond what is expected to arise from IBD alone.

The second criteria used to delineate DUs in Canada focuses on the evolutionary significance of populations (COSEWIC, 2023). This criterion often emphasizes the distinct evolutionary histories of populations (COSEWIC, 2023). My focus on the Canadian portion of the species' range means our understanding of the biogeographic history of western toads remains incomplete. However, it is likely that western toads, like other species (e.g. northern

leopard frogs: Hoffman and Blouin, 2004; woodland caribou: Klütsch et al., 2012; snowshoe hare: Cheng et al., 2014), have undergone periods of isolation in separate refugia during the Pleistocene glaciations. Goebel et al. (2009) previously suggested that western toads within their Northwestern mtDNA Clade (see Fig. 1 in Goebel et al., 2009) likely had a southern glacial refugia, possibly in the Klamath-Siskiyou Mountains near the border of Oregon and California, with northern post-glacial expansion for individuals along coastal BC and Alaska. More recently, Gamlen-Greene (2022) hypothesized that Haida Gwaii may have also been a potential glacial refugia for the species (Gamlen-Greene, 2022). However, these previous studies did not include sites from further east in Canada, where other species overlapping the range of western toads in eastern BC and Alberta are thought to have persisted in more eastern refugia. Commonly suggested refugial areas for species in this region include the northern Rocky Mountains (e.g. Brunsfeld et al., 2001; Shafer et al., 2010), cryptic refugia within the ice-sheets (e.g. Shafer et al., 2010), and the interior of Idaho (e.g. Lait et al., 2012). Thus, identifying potential glacial refugia for the more eastern groups of western toads is important to fully understand the species' evolutionary history.

Differences in adaptive traits are also considered by COSEWIC when evaluating the evolutionary significance of different groups (i.e. "DU-wide differences in adaptive traits not found elsewhere in Canada": COSEWIC, 2023). The presence of vocal sacs and a pronounced breeding call in one part of the western toad's range and absence of these traits elsewhere is highly unique amongst anurans (Pauly, 2008). The adaptive significance of these differences is unclear, but variation in the amount of calling that males do and the characteristics of their calls (e.g. frequency) are known to be adaptive in other anuran species in relation to predation pressure (e.g. Dapper et al., 2010; Bonachea and Ryan, 2011) and habitat conditions (e.g. Bosch

and De la Riva, 2004; Goutte et al., 2018; Zhao et al., 2021). Furthermore, differences in call behaviour and characteristics promote divergence between populations (Pröhl, 2003; Funk et al., 2009) and in some cases are thought to have driven speciation (Boul et al., 2007) in some anuran systems. Future work is needed to assess whether the differences in call behaviour of Calling and Non-calling populations of western toads are adaptive and whether they are contributing to reproductive isolation between the populations. However, DU-wide differences in a key reproductive trait make it likely that Calling and Non-calling populations are on separate evolutionary trajectories.

Broader implications for the suturing of genetic diversity in western Canada

A long-standing interest in evolutionary ecology is the locations of where different species, or populations within species, meet and interact (Remington, 1968; Swenson and Howard, 2004; Wait and Peñalba, 2025). In North America, the Rocky Mountains have been identified as a major geographic barrier that has partitioned biodiversity at multiple scales (Malanson et al., 2018). In the Canadian Rocky Mountains, a transition zone exists between three ecozones, the Montane Cordillera in BC and the Boreal foothills and Prairies in Alberta, which also corresponds with several faunal province boundaries (COSEWIC, 2023). Additionally, this is an important area where the western range limits of some species meet with the eastern range limits of others. For species that span the Rocky Mountains, this has been identified as a zone of overlapping genetic breaks across several taxa (e.g. silky pocket mouse, North American red squirrel, little brown myotis: Jensen et al., 2024; Canadian Lynx: Watt et al., 2021; American goldfinch: Cloutier et al., 2024; Black-capped chickadees, Boreal chickadees: Lait et al., 2024).

My study adds an amphibian to this list, further highlighting the Rocky Mountains as an important suture zone in Canada.

My results revealed a third genetic group located in southeastern BC and southwestern Alberta. A recent survey of genetic differences across the range of the western toads in the United States, also revealed a distinct genetic group associated with the Rocky Mountains in Glacier National Park, Montana (Colorado US Fish and Wildlife, 2017). Although that study did not include samples in Canada, the close geographic proximity of that group to the samples included in this study, makes it likely that these patterns are reflective of the same genetic substructure. Furthermore, toads in this part of the Rocky Mountains may be unique with respect to their calling behaviour. Although the specimens examined by Pauly (2008) lacked vocal sacs (both those in Canada and in his broader survey which included sites in Montana), there are reports of toads in southeastern BC and southwestern Alberta producing loud, consistent calls (per. communication L-A. Issac and Monitoring records from Waterton Lakes National Parks) and Pauly (2008) reported males producing low frequency, long-pulsed calls at a single location in northern Montana. Formal analysis of the recorded calls suggested these vocalizations differed from the calls of individuals in Alberta and there was no inflation of vocal sacs during vocalization (i.e. vocal sacs thought to be absent: Pauly 2008). Although the vocalizing individuals were in breeding aggregations, Pauly (2008) did not consider these calls to be true advertisement calls but instead referred to them as “intermediate” or an “extra-long release call” based on their characteristics (Pauly, 2008). These observations raise questions as to the relationship between the CotC toads and the Calling and Non-calling populations. Evaluation of range-wide phylogeographic structure paired with acoustic analyses would help clarify these relationships and shed additional light on breeding call variation in this species.

The CotC group highlights the potential for the Crown of the Continent to harbour unique genotypes. The Crown of the Continent region, spanning mountainous regions of the southwest corner of Alberta, southeast corner of BC, and northern Montana, is characterized by large elevational differences, includes a range of different ecosystems from alpine forests to prairie grasslands, and is one of the most biologically diverse places in North America (Prato and Fagre, 2010). This region is thought to have harbored ice-free areas during the last glacial maximum and may have been an important northern refugia for western species (Brunsfeld et al., 2001; Shafer et al., 2010). Although studies specifically testing the phylogeographic importance of this region are lacking, genetic differences between populations in the CotC and elsewhere have been reported in other groups (e.g. Long-toed salamanders (*Ambystoma macrodactylum*): Lee-Yaw and Irwin, 2012; Half-moon hairstreak (*Satyrium semiluna*): Macdonald et al., 2025). Additional studies focusing on this region would enhance our understanding of whether this region is a hotspot of distinct genetic diversity across species.

Conclusions and Future Directions

This is one of the first studies to evaluate genomic structure in a wide-spread amphibian species in western Canada. Using a combination of genomic data, phenotypic data, and ecological modelling, I found evidence to support distinct genetic groups associated with Calling and Non-calling populations of western toads in Canada. Paired with phenotypic and ecological differences between these populations, the continued recognition of these populations as separate conservation units (DUs) seems warranted. At the same time, I note that I did not have samples from very northern parts of the species range (e.g. Northwest Territories). Additional transects across the species range in these areas are necessary to fully clarify the boundaries between the

Calling and Non-calling populations. More generally, an outstanding question at this point is the extent to which these populations are reproductively isolated. Fine-scale sampling across the contact zone to quantify levels of introgression and hybridization, paired with testing the role of differences in calling behaviour in mate recognition would shed light on this question. Results from my thesis highlight the potential for northern latitudes to harbor biologically significant genetic structure and thus underscore the need to fill gaps in our assessments of intraspecific genetic variation in these areas.

References

Addis, B.R., Lowe, W.H., Hossack, B.R., Allendorf, F.W., 2015. Population genetic structure and disease in montane boreal toads: more heterozygous individuals are more likely to be infected with amphibian chytrid. Conserv Genet 16, 833–844. https://doi.org/10.1007/s10592-015-0704-6

Ballard, J.W.O., Whitlock, M.C., 2004. The incomplete natural history of mitochondria. Mol Ecol 13, 729–744. https://doi.org/10.1046/j.1365-294X.2003.02063.x

Barbosa, S., Mestre, F., White, T.A., Paupério, J., Alves, P.C., Searle, J.B., 2018. Integrative approaches to guide conservation decisions: Using genomics to define conservation units and functional corridors. Mol Ecol 27, 3452–3465. https://doi.org/10.1111/mec.14806

Beebee, T.J.C., 2005. Conservation genetics of amphibians. Heredity 95, 423–427. https://doi.org/10.1038/sj.hdy.6800736

Bergman, J.C., Finn, K.J., Lee-Yaw, J.A., 2024. Study extent influences the predictions and performance of species distribution models: a case study of six amphibian species at the edge of their geographic distributions in western Canada. Biodiversity and Conservation.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology & Evolution 22, 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Bonachea, L.A., Ryan, M.J., 2011. Simulated Predation Risk Influences Female Choice in Túngara Frogs, Physalaemus pustulosus: Bonachea and Ryan: Predation Risk and Mate Choice. Ethology 117, 400–407. https://doi.org/10.1111/j.1439-0310.2011.01889.x

Bosch, J., De la Riva, I., 2004. Are frog calls modulated by the environment? An analysis with anuran species from Bolivia. Can. J. Zool. 82, 880–888. https://doi.org/10.1139/z04-060

Boul, K.E., Chris Funk, W., Darst, C.R., Cannatella, D.C., Ryan, M.J., 2007. Sexual selection drives speciation in an Amazonian frog. Proc. R. Soc. B. 274, 399–406. https://doi.org/10.1098/rspb.2006.3736

Broennimann, O., Fitzpatrick, M.C., Pearman, P.B., Petitpierre, B., Pellissier, L., Yoccoz, N.G., Thuiller, W., Fortin, M.-J., Randin, C., Zimmermann, N.E., Graham, C.H., Guisan, A., 2012. Measuring ecological niche overlap from occurrence and spatial environmental data: Measuring niche overlap. Global Ecology and Biogeography 21, 481–497. https://doi.org/10.1111/j.1466-8238.2011.00698.x

Broennimann O, Di Cola V, Guisan A., 2025._ecospat: Spatial Ecology Miscellaneous Methods_. R package version 4.1.2, <https://CRAN.R-project.org/package=ecospat>

Brunsfeld, S.J., Sullivan, J., Soltis, D.E., Soltis, P.S., 2001. Chapter 15 Comparative phylogeography of north- western North America: a synthesis.

Cairns, N.A., Cicchino, A.S., Stewart, K.A., Austin, J.D., Lougheed, S.C., 2021. Cytonuclear discordance, reticulation and cryptic diversity in one of North America's most common frogs. Molecular Phylogenetics and Evolution 156, 107042. https://doi.org/10.1016/j.ympev.2020.107042

Campbell, E.O., MacDonald, Z.G., Gage, E.V., Gage, R.V., Sperling, F.A.H., 2022. Genomics and ecological modelling clarify species integrity in a confusing group of butterflies. Molecular Ecology 31, 2400–2417. https://doi.org/10.1111/mec.16407

Canada, Environment and Climate Change Canada, 2020. Management plan for the Western Toad (Anaxyrus boreas), calling and non-calling populations, in Canada.

Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A., Cresko, W.A., 2013. Stacks: an analysis tool set for population genomics. Molecular Ecology 22, 3124–3140. https://doi.org/10.1111/mec.12354

Chambers, E., Hillis, D., 2020. The Multispecies Coalescent Over-Splits Species in the Case of Geographically Widespread Taxa. SYSTEMATIC BIOLOGY 69, 184–193. https://doi.org/10.1093/sysbio/syz042

Chambers, E.A., Marshall, T.L., Hillis, D.M., 2022. The Importance of Contact Zones for Distinguishing Interspecific from Intraspecific Geographic Variation. Systematic Biology syac056. https://doi.org/10.1093/sysbio/syac056

Chang, C.C., Chow, C.C., Tellier, L.C., Vattikuti, S., Purcell, S.M., Lee, J.J., 2015. Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience 4, s13742-015-0047–8. https://doi.org/10.1186/s13742-015-0047-8

Chen, S., 2023. Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. iMeta 2. https://doi.org/10.1002/imt2.107

Cheng, E., Hodges, K.E., Melo-Ferreira, J., Alves, P.C., Mills, L.S., 2014. Conservation implications of the evolutionary history and genetic diversity hotspots of the snowshoe hare. Molecular Ecology 23, 2929–2942. https://doi.org/10.1111/mec.12790

Cloutier, A., Chan, D.T.C., Poon, E.S.K., Sin, S.Y.W., 2024. The genetic consequences of historic climate change on the contemporary population structure of a widespread temperate North American songbird. Molecular Phylogenetics and Evolution 201, 108216. https://doi.org/10.1016/j.ympev.2024.108216

Coates, D.J., Byrne, M., Moritz, C., 2018. Genetic Diversity and Conservation Units: Dealing With the Species-Population Continuum in the Age of Genomics. Front. Ecol. Evol. 6, 165. https://doi.org/10.3389/fevo.2018.00165

COSEWIC, 2012. Western toad (Anaxyrus boreas): COSEWIC assessment and status report 2012.

COSEWIC., 2023. COSEWIC guidelines for recognizing designatable units. https://cosewic.ca/index.php/en/reports/preparing-status-reports/guidelines-recognizing-designatable-units.html

Dapper, A.L., Baugh, A.T., Ryan, M.J., 2011. The Sounds of Silence as an Alarm Cue in Túngara Frogs, Physalaemus pustulosus: Silence as an Alarm Cue. Biotropica 43, 380–385. https://doi.org/10.1111/j.1744-7429.2010.00707.x

DeRaad D, 2023. _SNPfiltR: Interactively Filter SNP Datasets_. R package version 1.0.1, <https://CRAN.R-project.org/package=SNPfiltR>.

Dincă, V., Lee, K.M., Vila, R., Mutanen, M., 2019. The conundrum of species delimitation: a genomic perspective on a mitogenetically super-variable butterfly. Proc. R. Soc. B. 286, 20191311. https://doi.org/10.1098/rspb.2019.1311

Elhaik, E., 2022. Principal Component Analyses (PCA)-based findings in population genetic studies are highly biased and must be reevaluated. Sci Rep 12, 14683. https://doi.org/10.1038/s41598-022-14395-4

Elith, J., Kearney, M.R., Phillips, S.J., 2010. The art of modelling range-shifting species. Methods in Ecology and Evolution 1, 330–342.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE : a simulation study. Molecular Ecology 14, 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Everson, K.M., Gray, L.N., Jones, A.G., Lawrence, N.M., Foley, M.E., Sovacool, K.L., Kratovil, J.D., Hotaling, S., Hime, P.M., Storfer, A., Parra-Olea, G., Percino-Daniel, R., Aguilar-Miguel, X., O'Neill, E.M., Zambrano, L., Shaffer, H.B., Weisrock, D.W., 2021. Geography is more important than life history in the recent diversification of the tiger salamander complex. Proc. Natl. Acad. Sci. U.S.A. 118, e2014719118. https://doi.org/10.1073/pnas.2014719118

Ewels, P., Magnusson, M., Lundin, S., Kaller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32, 3047–3048.

Fišer, C., Robinson, C.T., Malard, F., 2017. Cryptic species as a window into the paradigm shift of the species concept. Mol Ecol 27, 613–635. https://doi.org/10.1111/mec.14486

Fu, J., Wen, L., 2023. Impacts of Quaternary glaciation, geological history and geography on animal species history in continental East Asia: A phylogeographic review. Molecular Ecology 32, 4497–4514. https://doi.org/10.1111/mec.17053

Funk, W.C., Cannatella, D.C., Ryan, M.J., 2009. Genetic divergence is more tightly related to call variation than landscape features in the Amazonian frogs *Physalaemus petersi* and *P. freibergi*. J of Evolutionary Biology 22, 1839–1853. https://doi.org/10.1111/j.1420-9101.2009.01795.x

Funk, W.C., McKay, J.K., Hohenlohe, P.A., Allendorf, F.W., 2012. Harnessing genomics for delineating conservation units. Trends in Ecology & Evolution 27, 489–496. https://doi.org/10.1016/j.tree.2012.05.012

Gallego-García, N., Caballero, S., Shaffer, H.B., 2021. Are Genomic Updates of Well-Studied Species Worth the Investment for Conservation? A Case Study of the Critically Endangered Magdalena River Turtle. Journal of Heredity 112, 575–589. https://doi.org/10.1093/jhered/esab063

Gallego-García, N., Vargas-Ramírez, M., Shaffer, H.B., 2023. The importance of cryptic diversity in the conservation of wide-ranging species: The red-footed tortoise Chelonoidis carbonarius in Colombia. Molecular Ecology 32, 4531–4545. https://doi.org/10.1111/mec.17052

Gamlen-Greene, R., 2022. The ecology, distribution and population genetics of amphibians on Haida Gwaii, British Columbia (PhD). The University of British Columbia, Vancouver.

García-Rodríguez, O., Hardouin, E.A., Pedreschi, D., Richards, M.B., Stafford, R., Searle, J.B., Stewart, J.R., 2024. Contrasting Patterns of Genetic Diversity in European Mammals in the Context of Glacial Refugia. Diversity 16, 611. https://doi.org/10.3390/d16100611

Glor, R.E., Warren, D., 2011. Testing ecological explanations for biogeographic boundaries. Evolution 65, 673–683. https://doi.org/10.1111/j.1558-5646.2010.01177.x

Goebel, A.M., Ranker, T.A., Corn, P.S., Olmstead, R.G., 2009. Mitochondrial DNA evolution in the Anaxyrus boreas species group. Molecular Phylogenetics and Evolution 50, 209–225. https://doi.org/10.1016/j.ympev.2008.06.019

Gordon, M.R., 2017. Three new bufonid (Bufo (Anaxyrus)) species discovered within the Great Basin and the consequences of taxonomic crypsis (MSc). University of Nevada, Reno.

Gordon, M.R., Simandle, E.T., Sandmeier, F.C., Tracy, C.R., 2020. Two New Cryptic Endemic Toads of Bufo Discovered in Central Nevada, Western United States (Amphibia: Bufonidae: Bufo [Anaxyrus]). Copeia 108, 166. https://doi.org/10.1643/CH-18-086

Goutte, S., Dubois, A., Howard, S.D., Márquez, R., Rowley, J.J.L., Dehling, J.M., Grandcolas, P., Xiong, R.C., Legendre, F., 2018. How the environment shapes animal signals: a test of the acoustic adaptation hypothesis in frogs. J of Evolutionary Biology 31, 148–158. https://doi.org/10.1111/jeb.13210

Hewitt, G., 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907–913. https://doi.org/10.1038/35016000

Hewitt, G.M., 2004. Genetic consequences of climatic oscillations in the Quaternary. Phil. Trans. R. Soc. Lond. B 359, 183–195. https://doi.org/10.1098/rstb.2003.1388

Hijmans R, 2024. _geosphere: Spherical Trigonometry_. R package version 1.5-20, <https://CRAN.R-project.org/package=geosphere>.

Hijmans R, 2024b. _predicts: Spatial Prediction Tools_. R package version 0.1-17, <https://CRAN.R-project.org/package=predicts>.

Hoffman, E.A., Blouin, M.S., 2004. Evolutionary history of the northern leopard frog: Reconstruction of phylogeny, phylogeography, and historical changes in population demography from mitochondrial DNA. Evolution 58, 145–159. https://doi.org/10.1111/j.0014-3820.2004.tb01581.x

Jensen, A.J., Cove, M.V., Goldstein, B.R., Kays, R., McShea, W., Pacifici, K., Rooney, B., Kierepka, E., 2024. Geographic barriers but not life history traits shape the phylogeography of North American mammals. Global Ecol Biogeogr 33, e13875. https://doi.org/10.1111/geb.13875

Jombart, T., 2008. adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24, 1403–1405.

Jombart, T., Devillard, S., Balloux, F., 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet 11, 94. https://doi.org/10.1186/1471-2156-11-94

Jorge, R.F., Lima, A.P., Stow, A.J., 2022. Selection and localised genetic structure in the threatened Manauense Harlequin Frog (Bufonidae: Atelopus manauensis). Conservation Genetics 23, 559–574. https://doi.org/10.1007/s10592-022-01436-8

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., Von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods 14, 587–589. https://doi.org/10.1038/nmeth.4285

Kass, J.M., Muscarella, R., Galante, P.J., Bohl, C.L., Pinilla-Buitrago, G.E., Boria, R.A., Soley-Guardia, M., Anderson, R.P., 2021. ENMeval 2.0: Redesigned for customizable and reproducible modeling of species' niches and distributions. Methods Ecol Evol 12, 1602–1608. https://doi.org/10.1111/2041-210X.13628

Klütsch, C.F.C., Manseau, M., Wilson, P.J., 2012. Phylogeographical Analysis of mtDNA Data Indicates Postglacial Expansion from Multiple Glacial Refugia in Woodland Caribou (Rangifer tarandus caribou). PLoS ONE 7, e52661. https://doi.org/10.1371/journal.pone.0052661

Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A., Mayrose, I., 2015. CLUMPAK : a program for identifying clustering modes and packaging population structure inferences across *K*. Molecular Ecology Resources 15, 1179–1191. https://doi.org/10.1111/1755-0998.12387

Lait, L.A., Enciso-Romero, J., Lekamlage, T.T.M., Veale, A., Abeyrama, D.K., Burg, T.M., 2024. RADseq data reveal widespread historical introgression in four familiar North American songbirds. Evolution 78, 860–878. https://doi.org/10.1093/evolut/qpae014

Lait, L.A., Friesen, Vicki.L., Gaston, A.J., Burg, T.M., 2012. The post-Pleistocene population genetic structure of a western North American passerine: the chestnut-backed chickadee *Poecile rufescens*. Journal of Avian Biology 43, 541–552.

Lee-Yaw, J.A., Irwin, D.E., 2015. The importance (or lack thereof) of niche divergence to the maintenance of a northern species complex: the case of the long-toed salamander (*Ambystoma macrodactylum* Baird). J. Evol. Biol. 28, 917–930. https://doi.org/10.1111/jeb.12619

Lee-Yaw, J.A., Irwin, D.E., 2012. Large geographic range size reflects a patchwork of divergent lineages in the long-toed salamander (*Ambystoma macrodactylum*). J. Evol. Biol. 25, 2276–2287. https://doi.org/10.1111/j.1420-9101.2012.02604.x

Lee-Yaw, J.A., Irwin, J.T., Green, D.M., 2008. Postglacial range expansion from northern refugia by the wood frog, *Rana sylvatica*. Molecular Ecology 17, 867–884. https://doi.org/10.1111/j.1365-294X.2007.03611.x

Lehnert, S.J., Bradbury, I.R., Wringe, B.F., Van Wyngaarden, M., Bentzen, P., 2023. Multifaceted framework for defining conservation units: An example from Atlantic salmon

(*Salmo salar*) in Canada. Evolutionary Applications 16, 1568–1585. https://doi.org/10.1111/eva.13587

Lesbarrères, D., Ashpole, S.L., Bishop, C.A., Blouin-Demers, G., Brooks, R.J., Echaubard, P., Govindarajulu, P., Green, D.M., Hecnar, S.J., Herman, T., Houlahan, J., Litzgus, J.D., Mazerolle, M.J., Paszkowski, C.A., Rutherford, P., Schock, D.M., Storey, K.B., Lougheed, S.C., 2014. Conservation of herpetofauna in northern landscapes: Threats and challenges from a Canadian perspective. Biological Conservation 170, 48–55. https://doi.org/10.1016/j.biocon.2013.12.030

Li, H., 2011. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27, 2987–2993. https://doi.org/10.1093/bioinformatics/btr509

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25, 1754–1760. https://doi.org/10.1093/bioinformatics/btp324

Lyman, R.A., Edwards, C.E., 2022. Revisiting the comparative phylogeography of unglaciated eastern North America: 15 years of patterns and progress. Ecology and Evolution 12. https://doi.org/10.1002/ece3.8827

MacDonald, Z.G., Dupuis, J.R., Glasier, J.R.N., Sissons, R., Moehrenschlager, A., Shaffer, H.B., Sperling, F.A.H., 2025. Whole-Genome Evaluation of Genetic Rescue: The Case of a Curiously Isolated and Endangered Butterfly. Molecular Ecology 34.

MacGuigan, D.J., Mount, G.G., Watkins-Colwell, G.J., Near, T.J., Lambert, M.R., 2022. Genomic Data Clarify Aquarana Systematics and Reveal Isolation-by-Distance Dominates Phylogeography of the Wide-Ranging Frog Rana clamitans. Ichthyology & Herpetology 110. https://doi.org/10.1643/h2021129

Mahony, C.R., Wang, T., Hamann, A., Cannon, A.J., 2022. A global climate model ensemble for downscaled monthly climate normals over North America. Intl Journal of Climatology 42, 5871–5891. https://doi.org/10.1002/joc.7566

Malanson, G.P., Fagre, D.B., Zimmerman, D.L., 2018. Scale dependence of diversity in alpine tundra, Rocky Mountains, USA. Plant Ecol 219, 999–1008. https://doi.org/10.1007/s11258-018-0852-0

Mijangos, J.L., Gruber, B., Berry, O., Pacioni, C., Georges, A., 2022. *DARTR* v2: An accessible genetic analysis platform for conservation, ecology and agriculture. Methods Ecol Evol 13, 2150–2158. https://doi.org/10.1111/2041-210X.13918

Miller, W., Schuster, S.C., Welch, A.J., Ratan, A., Bedoya-Reina, O.C., Zhao, F., Kim, H.L., Burhans, R.C., Drautz, D.I., Wittekindt, N.E., Tomsho, L.P., Ibarra-Laclette, E., Herrera-Estrella, L., Peacock, E., Farley, S., Sage, G.K., Rode, K., Obbard, M., Montiel, R., Bachmann, L., Ingólfsson, Ó., Aars, J., Mailund, T., Wiig, Ø., Talbot, S.L., Lindqvist, C., 2012. Polar and brown bear genomes reveal ancient admixture and demographic footprints of past climate change. Proc. Natl. Acad. Sci. U.S.A. 109. https://doi.org/10.1073/pnas.1210506109

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., Von Haeseler, A., Lanfear, R., 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Molecular Biology and Evolution 37, 1530–1534. https://doi.org/10.1093/molbev/msaa015

Naimi, B., Hamm, N.A.S., Groen, T.A., Skidmore, A.K. and Toxopeus, A.G., 2014. Where is positional uncertainty a problem for species distribution modelling?. Ecography, 37: 191-203. https://doi.org/10.1111/j.1600-0587.2013.00205.x

O'Leary, S.J., Puritz, J.B., Willis, S.C., Hollenbeck, C.M., Portnoy, D.S., 2018. These aren't the loci you'e looking for: Principles of effective SNP filtering for molecular ecologists. Molecular Ecology 27, 3193–3206. https://doi.org/10.1111/mec.14792

Pauly, G., 2008. Phylogenetic systematics, historical biogeography, and the evolution of vocalization in Nearctic Toads (Bufo) (Dissertation). The University of Texas at Austin.

Peterson, B.K., Weber, J.N., Kay, E.H., Fisher, H.S., Hoekstra, H.E., 2012. Double Digest RADseq: An Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species. PLoS ONE 7, e37135. https://doi.org/10.1371/journal.pone.0037135

Phillips, S.J., Anderson, R.P., Schapire, R.E., 2006. Maximum entropy modeling of species geographic distributions. Ecological Modelling 29.

Poland, J.A., Brown, P.J., Sorrells, M.E., Jannink, J.-L., 2012. Development of High-Density Genetic Maps for Barley and Wheat Using a Novel Two-Enzyme Genotyping-by-Sequencing Approach. PLoS ONE 7, e32253. https://doi.org/10.1371/journal.pone.0032253

Prato, T., Fagre, D.B., 2010. Sustainable Management of the Crown of the Continent Ecosystem. The George Wright Forum 27, 77–93.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of Population Structure Using Multilocus Genotype Data. Genetics 155, 945–959. https://doi.org/10.1093/genetics/155.2.945

Pröhl, H., 2003. Variation in Male Calling Behaviour and Relation to Male Mating Success in the Strawberry Poison Frog (*Dendrobates pumilio*). Ethology 109, 273–290. https://doi.org/10.1046/j.1439-0310.2003.00863.x

R Core Team, 2024. R: A language and environment for statistical computing. R Foundation for Statistical Computing https://www.R-project.org/

Remington, C. L, 1968. Suture-zones of hybrid interaction between recently joined biotas. Pp. 321–428. in T. Dobzhansky, M. K. Hecht, and W. C. Steere, eds. Evolutionary biology. Plenum Press, New York.

Rosner-Katz, H., McCune, J.L., Bennett, J.R., 2020. Using stacked SDMs with accuracy and rarity weighting to optimize surveys for rare plant species. Biodivers Conserv 29, 3209–3225. https://doi.org/10.1007/s10531-020-02018-1

Shafer, A.B.A., Cullingham, C.I., Côté, S.D., Coltman, D.W., 2010. Of glaciers and refugia: a decade of study sheds new light on the phylogeography of northwestern North America. Molecular Ecology 19, 4589–4621. https://doi.org/10.1111/j.1365-294X.2010.04828.x

Soltis, D.E., Gitzendanner, M.A., Strenge, D.D., Soltis, P.S., 1997. Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. Pl Syst Evol 206, 353–373. https://doi.org/10.1007/BF00987957

Soltis, D.E., Morris, A.B., McLACHLAN, J.S., Manos, P.S., Soltis, P.S., 2006. Comparative phylogeography of unglaciated eastern North America: PHYLOGEOGRAPHY OF UNGLACIATED EASTERN NORTH AMERICA. Molecular Ecology 15, 4261–4293. https://doi.org/10.1111/j.1365-294X.2006.03061.x

Sommer, R.S., Zachos, F.E., 2009. Fossil evidence and phylogeography of temperate species: 'glacial refugia' and post-glacial recolonization. Journal of Biogeography 36, 2013–2020. https://doi.org/10.1111/j.1365-2699.2009.02187.x

Suárez, D., Arribas, P., Jiménez-García, E., Emerson, B.C., 2022. Dispersal ability and its consequences for population genetic differentiation and diversification. Proceedings of the Royal Society B: Biological Sciences 289.

Sukumaran, J., Knowles, L.L., 2017. Multispecies coalescent delimits structure, not species. Proc. Natl. Acad. Sci. U.S.A. 114, 1607–1612. https://doi.org/10.1073/pnas.1607921114

Swenson, N.G., Howard, D.J., 2004. DO SUTURE ZONES EXIST? Evolution 58, 2391–2397. https://doi.org/10.1111/j.0014-3820.2004.tb00869.x

Switzer, J.F., Johnson, R., Lubinski, B.A., King, T.L., 2009. Genetic structure in the Anazyrus boreas species group (Anura, Bufonidae): an evaluation of the Southern Rocky Mountain population (U.S. Fish and Wildlife Service). Mountain-Prairie Region.

Trumbo, D.R., Hardy, B.M., Crockett, H.J., Muths, E., Forester, B.R., Cheek, R.G., Zimmerman, S.J., Corey-Rivas, S., Bailey, L.L., Funk, W.C., 2023. Conservation genomics of an endangered montane amphibian reveals low population structure, low genomic diversity and selection pressure from disease. Molecular Ecology n/a. https://doi.org/10.1111/mec.17175

Turbek, S.P., Funk, W.C., Ruegg, K.C., 2023. Where to draw the line? Expanding the delineation of conservation units to highly mobile taxa. Journal of Heredity 114, 300–311. https://doi.org/10.1093/jhered/esad011

U.S. Fish and Wildlife Service, 2017. Species Status Assessment Report for the Eastern Population of the Boreal Toad (Anaxyrus boreas boreas).

Vale, C.G., Tarroso, P., Brito, J.C., 2014. Predicting species distribution at range margins: testing the effects of study area extent, resolution and threshold selection in the Sahara-Sahel transition zone. Diversity Distrib. 20, 20–33. https://doi.org/10.1111/ddi.12115

Wait, D.R., Peñalba, J.V., 2025. Suture zones, speciation, and evolution. Evolution 79, 329–341. https://doi.org/10.1093/evolut/qpae184

Wang, T., Hamann, A., Spittlehouse, D., Carroll, C., 2016. Locally Downscaled and Spatially Customizable Climate Data for Historical and Future Periods for North America. PLoS ONE 11, e0156720. https://doi.org/10.1371/journal.pone.0156720

Waples, R.S., Lindley, S.T., 2018. Genomics and conservation units: The genetic basis of adult migration timing in Pacific salmonids. Evol Appl 11, 1518–1526. https://doi.org/10.1111/eva.12687

Waters, J.M., Emerson, B.C., Arribas, P., McCulloch, G.A., 2020. Dispersal Reduction: Causes, Genomic Mechanisms, and Evolutionary Consequences. Trends in Ecology & Evolution 35, 512–522.

Watt, C.M., Kierepka, E.M., Ferreira, C.C., Koen, E.L., Row, J.R., Bowman, J., Wilson, P.J., Murray, D.L., 2021. Canada lynx (*Lynx canadensis*) gene flow across a mountain transition zone in western North America. Can. J. Zool. 99, 131–140. https://doi.org/10.1139/cjz-2019-0247

Wilson, G.A., Fulton, T.L., Kendell, K., Scrimgeour, G., Paszkowski, C.A., Coltman, D.W., 2008. Genetic diversity and structure in Canadian northern leopard frog (*Rana pipiens*) populations: implications for reintroduction programs. Can. J. Zool. 86, 863–874. https://doi.org/10.1139/Z08-062

Wright S, 1943. Isolation by distance. Genetics, 28(2), 114–138.

Young, N., Carter, L., Evangelista, P., Jarnevich, C.S., 2011. A Maxent Model v3.3.3e Tutorial.

Zeisset, I., Beebee, T.J.C., 2008. Amphibian phylogeography: a model for understanding historical aspects of species distributions. Heredity 101, 109–119. https://doi.org/10.1038/hdy.2008.30

Zhao, L., Santos, J.C., Wang, J., Ran, J., Tang, Y., Cui, J., 2021. Noise constrains the evolution of call frequency contours in flowing water frogs: a comparative analysis in two clades. Front Zool 18, 37. https://doi.org/10.1186/s12983-021-00423-y

Supplementary Materials

Tissue samples

- **Number of individuals included in the study (after bioinformatic filtering):** 40 individuals
- **Number of sites:** 34 breeding sites
- **Range of the number of individuals per site:** 1 to 5 individuals per site (two sites with 2 individuals, one site with 5 individuals, 31 sites with 1 individual)

Table S1. Tissue sample information for the 40 individuals and the corresponding sites that are included in this study.

Sample ID	Site ID	Longitude	Latitude	Genetic Clade	Area
BC-1	BC-1	Not available	Not available	Non-calling	Haida Gwaii
BC-2	BC-2	-128.54	54.36	Non-calling	BC
BC-3	BC-3	-127.51	55.26	Non-calling	BC
BC-4	BC-4	-126.66	54.33	Non-calling	BC
BC-5a	BC-5	-125.11	49.77	Non-calling	Vancouver Island
BC-5b	BC-5	-125.11	49.77	Non-calling	Vancouver Island
BC-6	BC-6	-124.84	53.84	Non-calling	BC
BC-7	BC-7	-124.28	55.15	Non-calling	BC
BC-8	BC-8	-122.94	50.13	Non-calling	Lower mainland
BC-9	BC-9	-122.36	53.15	Non-calling	BC
BC-10	BC-10	-122.15	52.10	Non-calling	BC
BC-11	BC-11	-120.56	49.91	Non-calling	BC
BC-12	BC-12	-120.17	53.31	Non-calling	BC
BC-13	BC-13	-119.61	53.03	Non-calling	BC
BC-14	BC-14	-119.45	50.79	Non-calling	BC
BC-15	BC-15	-118.86	52.59	Non-calling	BC
BC-16	BC-16	-118.24	51.03	Non-calling	BC
AB-1a	AB-1	-118.12	53.03	Non-calling	Jasper National Park
AB-1b	AB-1	-118.12	53.03	Non-calling	Jasper National Park
BC-17	BC-17	-117.65	50.16	Non-calling	BC
BC-18	BC-18	-117.52	51.30	Non-calling	Glacier National Park (BC)
AB-2	AB-2	-117.39	53.48	Calling	Hinton
AB-3	AB-3	-117.38	52.39	Calling	Alberta
BC-19	BC-19	-116.59	51.24	Calling	Yoho National Park
AB-4	AB-4	-116.08	52.40	Calling	Alberta
AB-5	AB-5	-115.83	51.23	Calling	Banff National Park
AB-6	AB-6	-115.56	52.50	Calling	Alberta
AB-7a	AB-7	-115.38	51.08	Calling	Canmore

AB-7b	AB-7	-115.38	51.08	Calling	Canmore
AB-7c	AB-7	-115.38	51.08	Calling	Canmore
AB-7d	AB-7	-115.38	51.08	Calling	Canmore
AB-7e	AB-7	-115.38	51.08	Calling	Canmore
AB-8	AB-8	-115.26	51.97	Calling	Clearwater County
AB-9	AB-9	-114.99	53.55	Calling	Evansburg
BC-20	BC-20	-114.79	49.62	Crown of the Continent	Corbin mines
BC-21	BC-21	-114.68	49.52	Crown of the Continent	Corbin mines
AB-10	AB-10	-114.55	53.19	Calling	Alberta
AB-11	AB-11	-114.05	49.05	Crown of the Continent	Waterton Lakes National Park
AB-12	AB-12	-113.83	53.57	Calling	Alberta
AB-13	AB-13	-112.94	53.66	Calling	Alberta

Sequencing information

- **Number of individuals sequenced:** 46 (inclusive of one positive control - i.e. replicate) (2 negative controls included on the half plate with 48 wells).
- **Sequencing Type:** Double digest restriction-site associated DNA sequencing (ddRADseq)
- **Enzymes used:** PstI / MspI
    - Tested enzyme pair SbfI/MspI – (this did not work for this species) enzyme cut along a repetitive element and PCR amplification resulted in this sequence to be overrepresented in the total reads, washing out other signals within the DNA sequences.
- **Library prep:** Sent to Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (IBIS, Universite´Laval, Quebec, Canada)
- **Sequencing Info:** Paired-end sequencing on an Illumina NovaSeq 6000 at Centre d'expertise et de service Génome Québec at McGill University in Montreal, QC

Bioinformatics

All code can be found on the GitHub repository:
https://github.com/Jaynabergman/WETO_ddRAD

Demultiplexing

Raw reads were first demultiplexed using *process_radtags* in Stacks (V2.66; Catchen et al., 2013). When we first ran *process_radtags* we tested defining the adapter sequences and not defining them. We had 34.9% of the reads containing adapter sequences and decided to remove these in order to ensure cleaner reads downstream. After demultiplexing 971,781,833 reads were retained.

Fastp (and multiqc)

We first ran Fastp (version 0.23.4; Chen, 2023) as a diagnostic tool to determine how many bases need to be trimmed off the forward (read1) and reverse (read2) reads (using flags -f and -F respectively). We brought the json files from Fastp into Multiqc (version 1.21; Ewels et al., 2016) to aggregate the separate files into a single report for easy interpretation. We looked at the "GC content plot" to determine these values (Figure S1). From these plots we decided to set the flags to -f 5 and -F 3. We also used FastP to filter for read quality by setting the Phred score to 20 (-q 20). This resulted in retaining 929,487,304 reads.

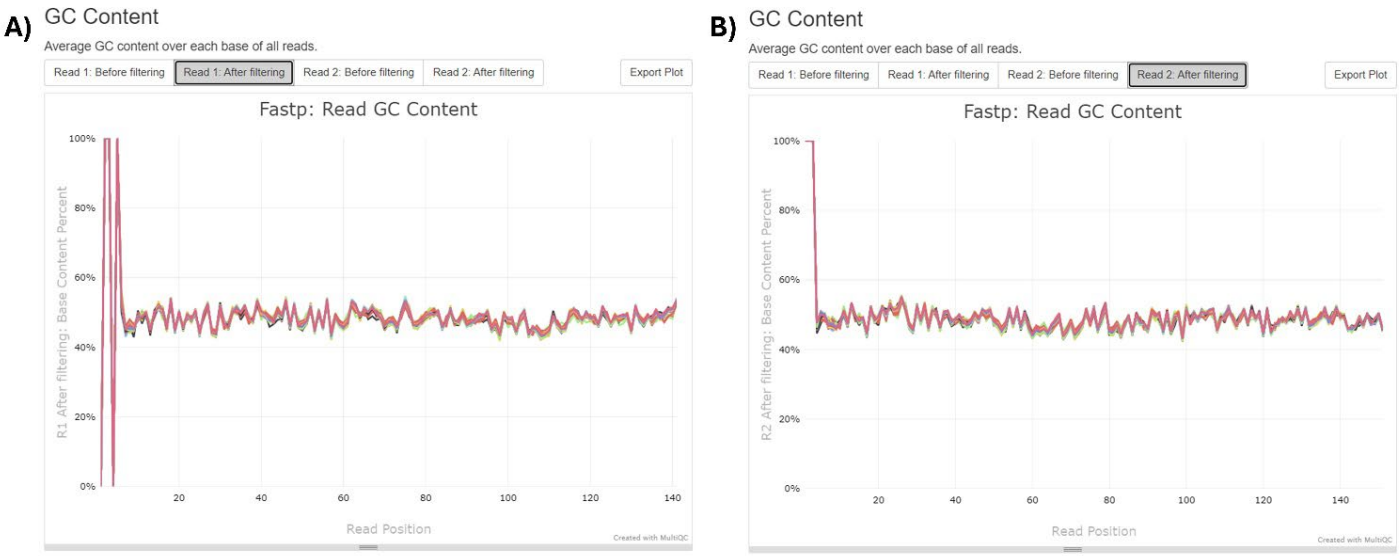

Figure S1. The GC content plot over each based average for all reads for the 46 individuals generated by Fastp and aggregated into a single report by Multiqc. These plots are used to determine how many base pairs need to be trimmed off the start of the reads for both the (A) forward and (B) reverse reads. Reads were demultiplexed previous to this step, but no other filtering has been done.

Align to the reference genome using BWA

The western toad reference genome is approximately 5.2GB and was from an individual collected in Jackson County, Colorado, USA (Trumbo et al., 2023).

We first indexed the reference genome using the program BWA (version 0.7.18). We then used the bwa-mem algorithm to align the reads to the reference genome. SAMtools was used to sort the SAM files and convert them into BAM format (BAM is the compressed binary form of SAM). No additional flags were set for the alignment of the reads to the reference genome. We had a total of 917,788,446 reads uniquely align to the genome (1,835,041 to 55,541,803 reads per individual).

Build loci in gstacks (STACKS)

After aligning to the reference genome, we used the *gstacks* module in STACKS to assemble the loci. *gstacks* identifies SNPs for each locus and then genotypes each individual at the identified SNPs. We set the minimum mapping quality to be at least a Phred score of 20 (--minmapq 20) for reads to be considered in this step.

The gstacks module output was the following:

> Built 1772249 loci comprising 264622451 forward reads and 212670599 matching paired-end reads; mean insert length was 206.1 (sd: 46.3).
>
> Genotyped 1772249 loci:
> Effective per-sample coverage: mean=12.0x, stdev=6.2x, min=3.0x, max=31.3x
> Mean number of sites per locus: 182.3
> A consistent phasing was found for 2496692 of out 2771018 (90.1%) diploid loci needing phasing

Filtering and converting genotypes to VCF format.

The *populations* module in STACKS was used to apply a first round of filters and convert individual genotypes to vcf format. Specifically we filtered for maximum observed heterozygosity (--max-obs-het). We tested the maximum observed heterozygosity settings of 0.5, 0.6, and 0.8. After going through a mock filtering scheme using each of these settings, there was no difference in the number of SNPs retained. We also generated principal component analyses (PCAs) with he different filtered vcf files and all the PCAs produced identical clusters of individuals. Figure S2 shows the PCA for the setting of 0.5 and 0.8. We went with the most stringent setting of 0.5 (--max-obs-het 0.5).

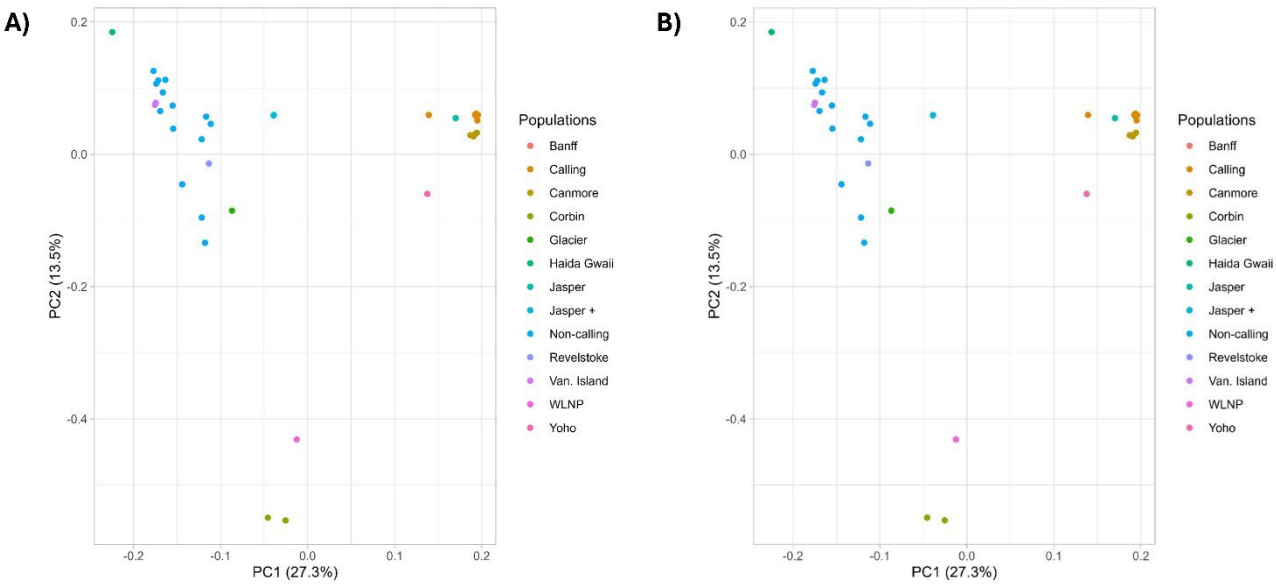

Figure S2. Principal Component Analysis (PCA) for a sampling scheme that tests the setting of maximum observed heterozygosity (--max-obs-het) in the *Populations* module in STACKS. The PCAs were generated with a maximum observed heterozygosity setting of (A) 0.5 and (B) 0.8. Both PCAs had identical filter steps following the maximum observed heterozygosity setting and both were ran with 11,185 SNPs.

Filtering SNPs (using the R package *SNPfiltR*)

After the SNPs were in a vcf file format, the majority of the filtering took place in R (version 4.3.3, R core team, 2024) using the package SNPfiltR (DeRaad, 2023). See Table S2 below for the filtering decisions that were used in the final SNP dataset.

Removing sites in Linkage Disequilibrium

The last filtering setting we conducted we did outside of R, and it was to filter out SNPs that are likely in Linkage Disequilibrium (LD). We used the function *--indep-pairwise* in the program PLINK (v.1.90b Chang et al., 2015). There are three values that need to be set for this function which are the window, the step and the correlation value (r^2). The window is the number of base pairs that are assessed at one time, the step is the number of base pairs that the window is shifted over, and the correlation value is the threshold that a SNP must be under when compared to all over SNPs in the window in order to be kept. Values that are commonly used in the literature are 50, 5, 0.8 and 50, 5, 0.5.

When we tested these values with a mock set of filtering steps we saw that the lower correlation value threshold (0.5) was very stringent (i.e. all SNPs kept have a correlation value of less than 0.5 when compared to all other SNPs in the window). In a filtering scheme that started with 20,369 SNPs only 7,865 SNPs were kept. When the 0.8 correlation threshold was used we kept

11,158 SNPs. We again looked at a PCA to see if the combination of values and the number of SNPs kept influenced how the individuals were clustering, and we saw that there was no impact (Figure S3). We therefore used the 50 5 0.8 because we were able to retain a higher number of SNPs.

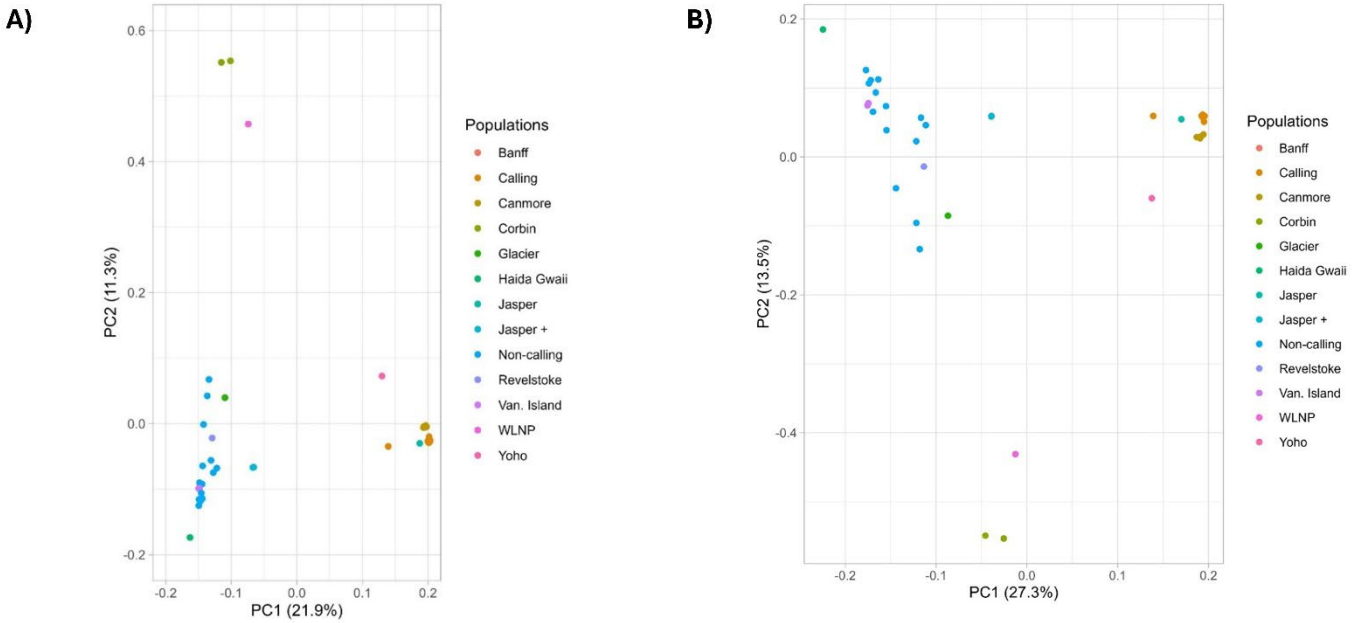

Figure S3. Principal Component Analysis (PCA) to test the differences between correlation thresholds to determine whether a SNP is in linkage disequilibrium with other SNPs. The correlation was set in the program PLINK (v.1.90b Chang et al., 2015) using the function --*indep-pairwise*. For both PCAs the window was set to 50 base pairs and the step between windows was set to 5 base pairs. The correlation thresholds that were tested were set to (A) 0.5 which resulted in 7,865 SNPs retained, and (B) 0.8 which resulted in 11,158 SNPs retained. We see that the positive and negative values on PC2 flipped, but the actual clustering of the individuals stays constant regardless of the threshold and the number of SNPs retained.

Testing amount of missing data per individual and per SNP

In order to see if the amount of missing data retained per individual and the amount of missing data per SNP influenced how the individuals were clustering together we tested multiple missingness schemes and looked at the corresponding PCAs. We found that regardless how the individuals and SNPs were filtered for missing data, there was similar clustering pattern of individuals in the PCAs (Figure S4). The largest differences was seen in the percent of variation explained by PC1 and PC2, but this only changed by about 3%. Thus the filtering scheme was chosen based on balancing the number of SNPs retained and keeping as many individuals as possible (while only keeping individuals with a reasonable amount of missing data).

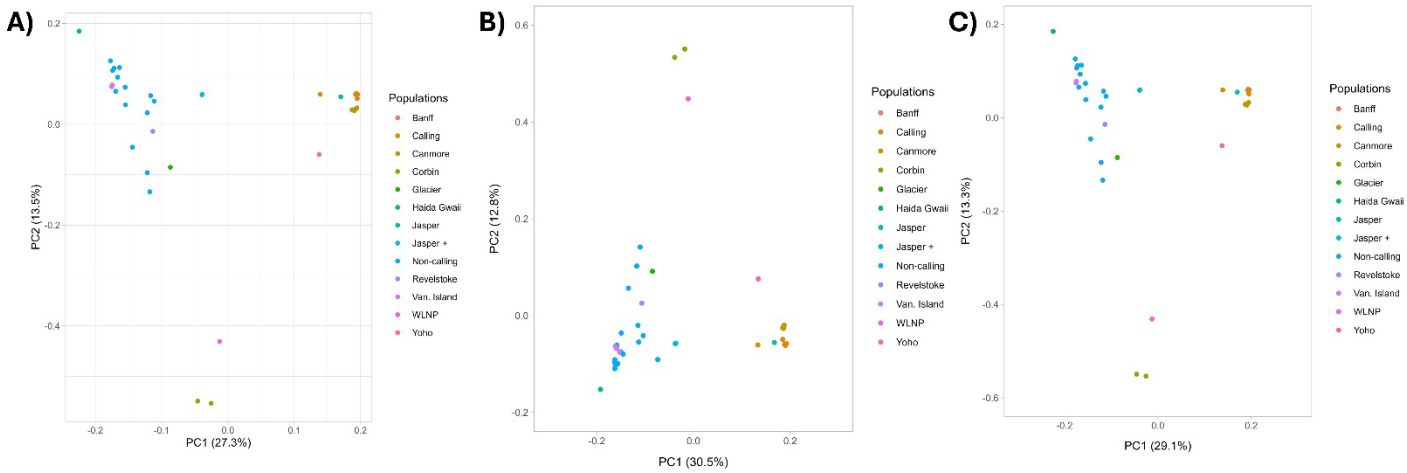

Figure S4. Principal Component Analysis (PCA) generated for three different amounts of missing data allowed per individual and missing data allowed per SNP. All filtering steps prior to the missingness were identical. The amount of missing data tested were (A) individuals have up to 33% missing data and SNPs have up to 5% missing data resulting in 11,950 SNPs for 40 individuals; (B) individuals have up to 55% missing data and SNPs have up to 10% missing data, resulting in 10,342 SNPs for 44 individuals; (C) individuals have up to 20% missing data and SNPs have up to 4% missing data, resulting in 5,031 SNPs for 40 individuals. The dataset with missingness corresponding to A was used for the final analyses conducted in this study.

Final set of SNP filtering decisions

Table S2. The decisions, justification, and settings for the entire filtering scheme (post generating a vcf file) to get the final set of 11,950 SNPs that were used in the downstream analyses.

Step	Filter/Program	What the filter does	How it was decided	Setting	Reported values
1.	Maximum observed heterozygosity (--max-obs-het) In *Populations* (STACKS)	Gets rid of potential false heterozygotes by specifying a maximum observed heterozygosity required to process a	Tested multiple settings and assessed if values impacted individuals on a PCA (*see above section; Figure S2*).	0.5	71,000 sites filtered out – starting with 4,606,447 variant sites

		nucleotide site at a locus.			
2.	Genotype depth In SNPfiltR (R package)	Gives support for the confidence of a genotype call	Based on the histogram by SNPfiltR (Figure S5A) and recommended values in the literature (e.g. O'Leary et al., 2018)	5	28.98% of genotypes fall below a read depth of 5 (converted to NA)
2.	Genotype quality In SNPfiltR (R package)	Support for genotype errors	Based on recommended values in the literature.	20	0.8% of genotypes fall below a genotype quality of 20 (converted to NA)
3.	Allele balance In SNPfiltR (R package)	This compares the number of reads for the reference allele to the number of reads for the alternative allele across heterozygotes. Expected is 0.5.	This range requires heterozygotes to be supported by at least two reads based on the minimum genotype depth that was set.	0.25 and 0.75 (min and max)	20.85% of het genotypes [2.2% of all genotypes] fall outside of 0.25-0.75 allele balance ratio (and converted to NA)
4.	Maximum depth per SNP In SNPfiltR (R package)	Excessively high depth could be indicative of multilocus contigs	Used the max depth histogram and made the cutoff when the frequency dropped (Figure S5B)	22	4.19% of SNPs removed - 4,410,477 SNPs retained
5.	Minor allele count (MAC) In SNPfiltR (R package)	Removes potentially artifactual called SNPs. The number of gene copies carrying the minor allele at a locus.	Based on recommended values in the literature (mac of 3 means there must be at least one homozygote and one heterozygote)	3	50.76% of SNPs fell below a mac of 3 – 2,171,753 SNPs retained
6.	Missing by sample (missing_by_sample()) in SNPfiltR (R package)	Removes samples that have high amounts of missing data.	Looked at missingness by SNP and by sample before setting any values – two individuals have lots of missing data (>90%) (Figure S5C)	0.9	Two individuals removed (DR4 and WETO23-107)
7.	Missing by SNP (missing_by_snp()) in SNPfiltR (R package)	Removes SNPs that have high amounts of missing data across individuals.	Looked at missingness by SNP after removing the two individuals. Used the table that shows how many SNPs are left after removing SNPs with a given percent of missing data.	0.85	96.93% of SNPs fell below a completeness cutoff of 0.85 – 66,625 SNPs retained

8.	Missing by sample		Four individuals are missing >50% of the data.	0.5	Four individuals removed (AS-3, WETO22-086, WETO23-239, RA-04)
9.	Missing by SNP		Tried to remove SNPs with more missing data in order to keep more individuals	0.95	66.8% of SNPs fell below a completeness cutoff of 0.95 - **22,118** SNPs retained
10.	Missing by sample		Checked to see the amount of missing data per individual in the final dataset (Figure S5D)	0.33	All individuals have less than 33% missing data (38 individuals have less than 15% missing data)
11.	Linkage disequilibrium	Removes SNPs that are highly correlated with one another because these SNPs are likely linked and considered to be in linkage disequilibrium.	Tested values from the literature (*see above; Figure S3*)	50, 5, 0.8	Removed 10,168 SNPs – 11,950 SNPs retained
Final number of SNPs retained:					**11,950**
Final number of individuals included:					**40**

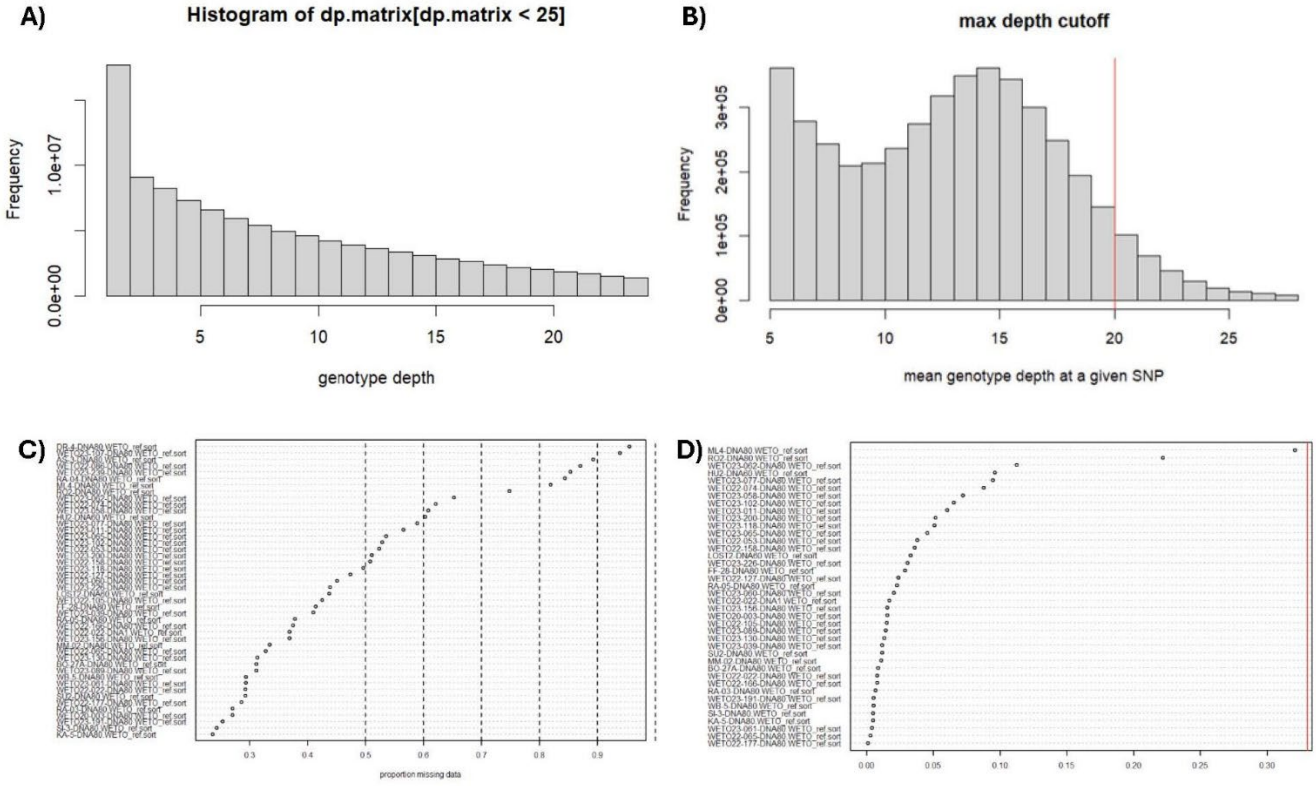

Figure S5. Graphs generated from functions in the R package SNPfiltR (DeRaad, 2023) to help make informed decisions when filtering SNPs. (A) Histogram that shows the frequency of SNPs with a given genotype depth generated by the function *hard_filter()* (corresponds to step 2 in Table S2). (B) Histogram to help determine how to set the maximum depth cutoff by showing the frequency of SNPs with a mean genotype depth at a given SNP. Generated by the function *max_depth()* (corresponds to step 4 in Table S2). (C) Using the function *missing_by_sample(),* this shows the amount of missing data per individual for all 46 individuals before any missing data (for individuals or SNPs) are filtered for. (D) The final amount of missing data per individual for all 40 individuals kept in the final dataset (all individuals have less than 33% missing data).

Population genomic analyses

Discriminant Analysis of Principal Component Analysis (DAPC)

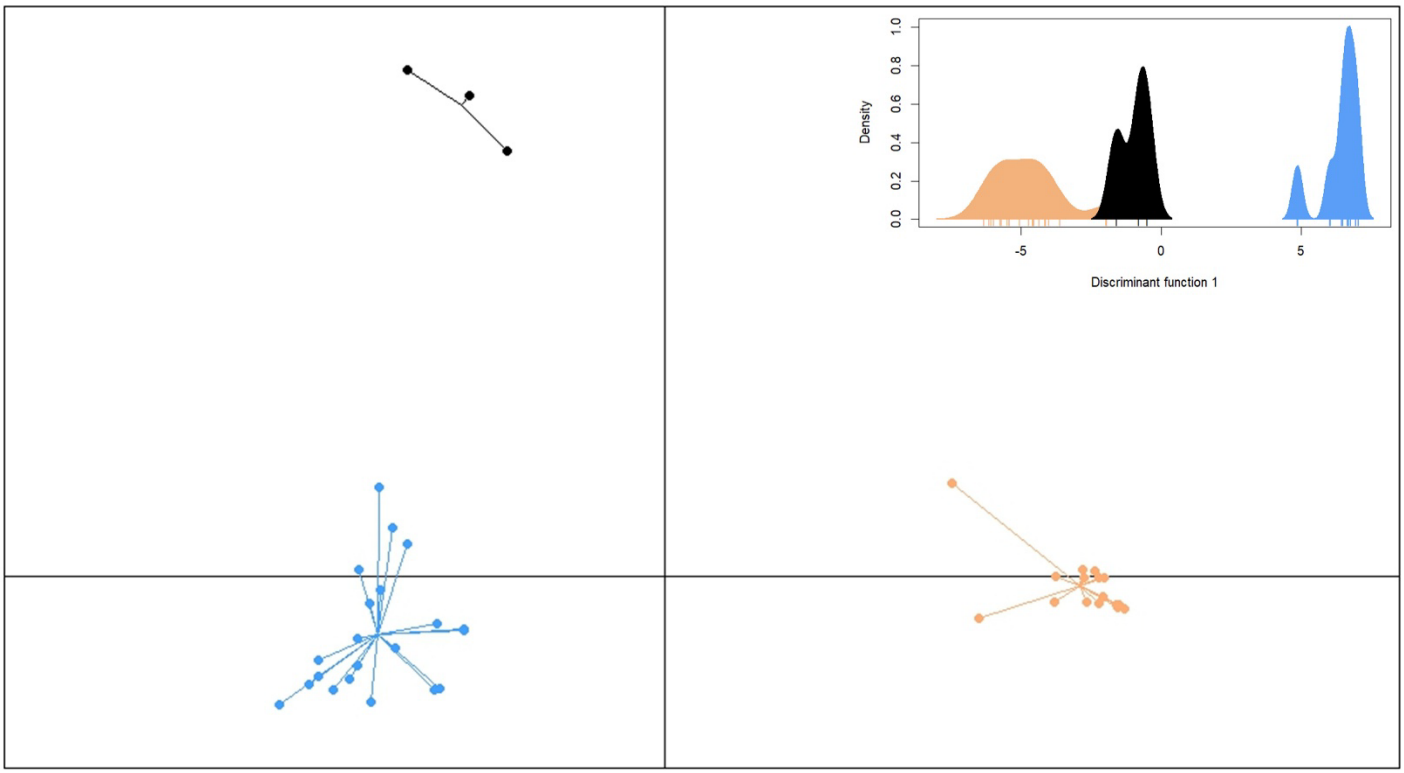

Figure S6. The Discriminant Analysis of Principal Components (DAPC) using 11,950 SNPs generated from 40 individuals across the Canadian range of western toads. Four principal components were retained for two discriminant functions. The top right inset shows the analysis contained within the first discriminant analysis as most of the information was within the first discriminant function. The colors are blue for the Non-calling group, orange for the Calling group, and black for the Crown of the Continent group.

STRUCTURE

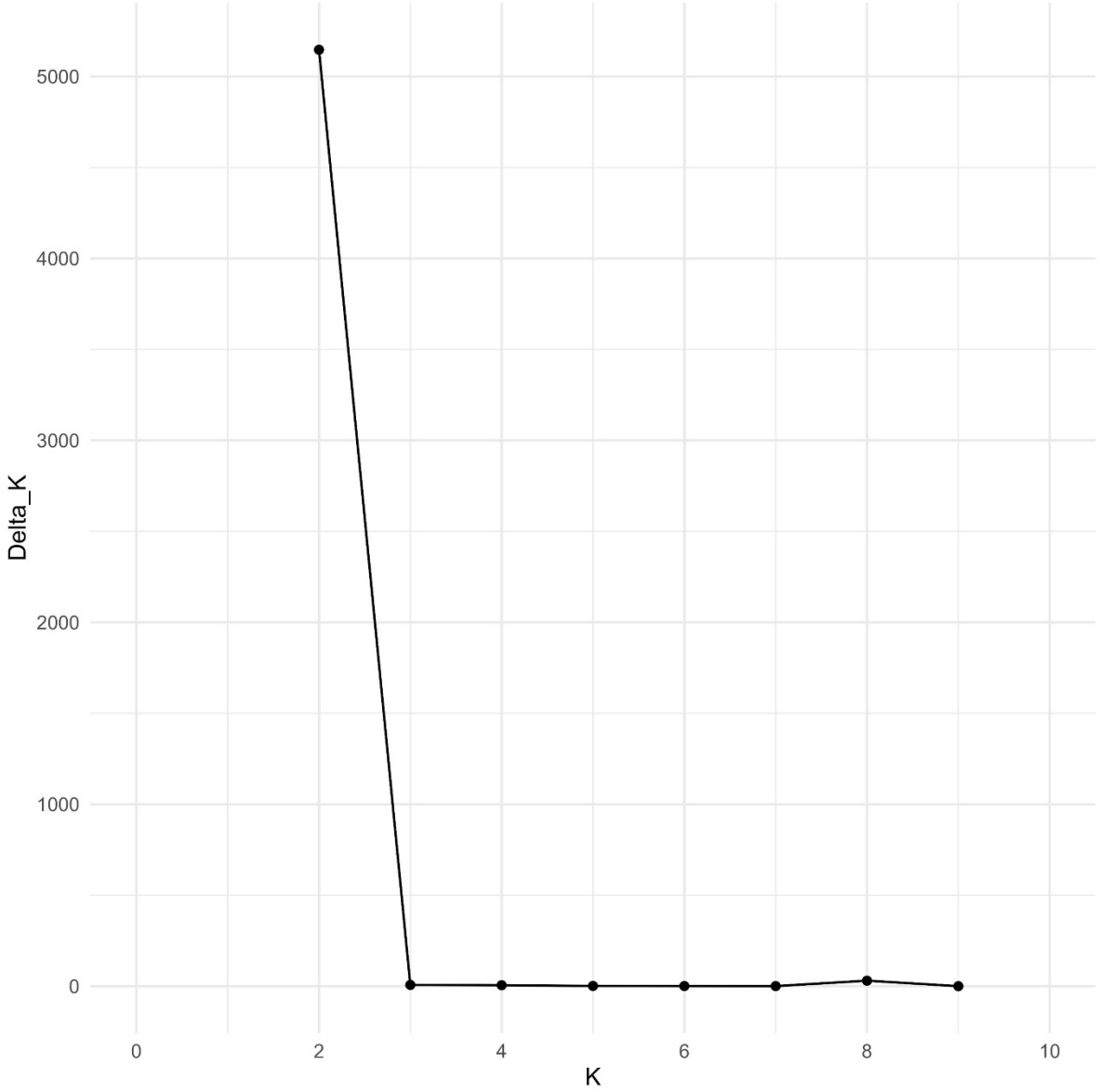

Figure S7. The STRUCTURE Harvester ΔK plot to determine the optimal number of populations (K) using the Evanno method (Evanno et al., 2005). The ΔK plot indicates that K=2 is the optimal K value.

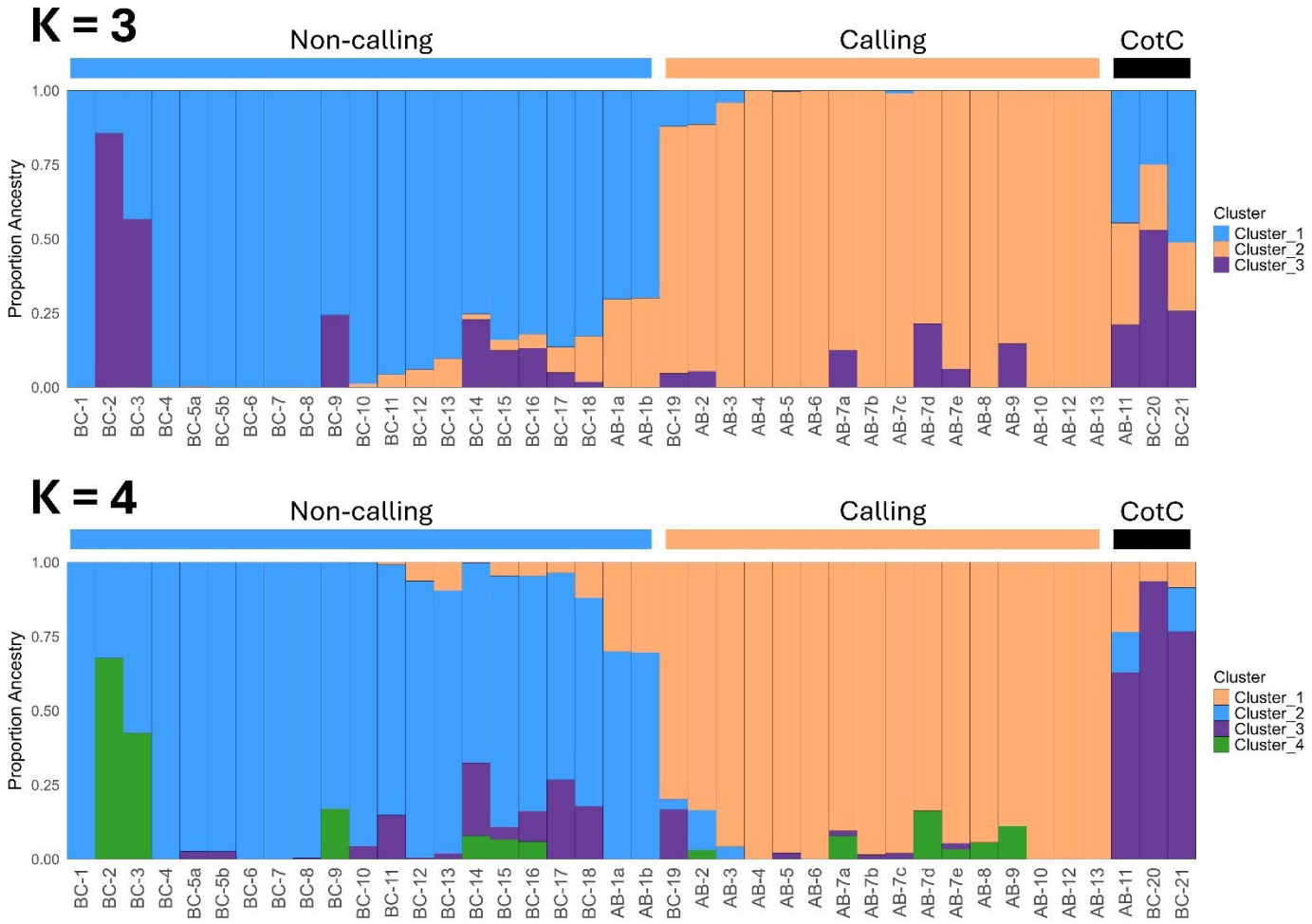

Figure S8. STRUCTURE results for (A) K=3 and (B) K=4 generated using 11,950 SNPs. The colored bars along the top of each plot represent the clustering of individuals found in the PCA in the main text (blue: non-calling, orange: calling, black: “Crown of the Continent” or CotC).

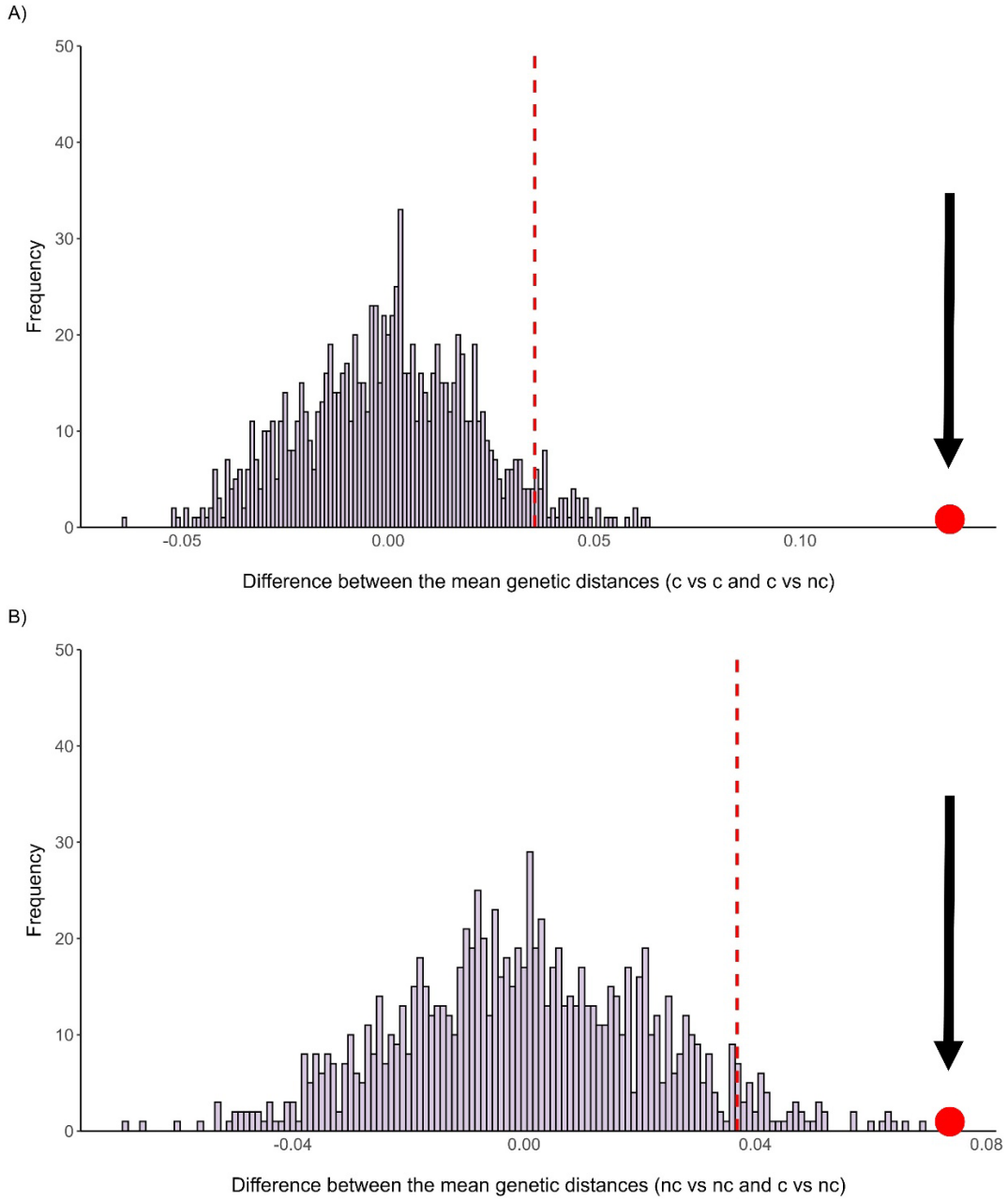

Figure S9. A random distribution of the differences in the means of pairwise genetic distances (1 – proportion of shared alleles) between comparison types calling to non-calling individuals and within population comparison types for (A) calling to calling individuals, and (B) non-calling to non-calling individuals. These distributions are for individuals that are within 150 km of one another. The red dashed lines show the 95% quantile, with all values to the right being significant. In each plot the value of the real data is indicated by the red point and black arrow. In both cases the real data is significantly larger than the random distribution of differences between the mean genetic distances. This supports that there is significant genetic structure in addition to observed patterns of IBD across the range of western toads.

Ecological Niche Models

Table S3. Details of model parameterization for Maxent models developed for the Calling population and the Non-calling population. The range boundaries were based on the morphological boundaries as described by Pauly (2008) and are the same boundaries that are currently used for defining the separate Designatable Units (DUs: COSEWIC, 2012).

Population	**Calling**	**Non-calling**
No. input localities	304	2852
No. random background points	5000	5000
Feature classes*	LQT	LQHPT
Regularization parameter	1.5	0.5
Maximum iterations	5000	5000
Training AUC	0.89	0.82
10% omission threshold value to consider a cell as suitable	0.245	0.196

*L = linear, Q = quadratic, H = hinge, P = product, T = threshold

Table S4. Variable contribution of the eight environmental variables for the Maxent models for the calling population and the non-calling population of western toads, shown as the percent contribution and the permutation importance.

Variable	**Importance**	**Calling model**	**Non-calling model**
mean temperature of the coldest month (MCMT)	Percent contribution	42.3	8.5
	Permutation importance	17.4	22.3
summer (June to August) precipitation (mm) (PPT_sm)	Percent contribution	31.1	4.9
	Permutation importance	11.6	5.7
Julian date on which the frost-free period begins (bFFP)	Percent contribution	14.1	1.5
	Permutation importance	25.3	5.7
summer heat moisture index (SHM)	Percent contribution	6.2	6
	Permutation importance	23.7	26.7
precipitation as snow (mm) (PAS)	Percent contribution	1.6	5.1
	Permutation importance	1.5	4.4
spring (March to May) precipitation (mm) (PPT_sp)	Percent contribution	2.4	1.1
	Permutation importance	2.7	5.3
degree-days below 18 degrees Celsius (DD_18)	Percent contribution	1.4	68.8
	Permutation importance	14.5	25
mean annual relative humidity (%) (RH)	Percent contribution	0.9	4.1
	Permutation importance	3.3	5