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Conservation Genomics of Cascades Frogs (Rana cascadae) at the Southern Edge of Their Range

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CONSERVATION GENOMICS OF CASCADES FROGS (*RANA CASCADAE*) AT THE SOUTHERN EDGE OF THEIR RANGE

by

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ABSTRACT
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Under the Supervision of Dr. Emily K. Latch

Cascades frogs (*Rana cascadae*) in the southern Cascades Range of California have been declining over the last 30 years, primarily due to the fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). In the Lassen Region of the southern Cascades, at least six of the eleven remaining localities face extirpation within 50 years. These small and isolated populations are prone to negative genetic effects including reduced diversity and increased inbreeding which could potentially exacerbate declines. I used a large dataset of SNP loci generated from high-throughput sequencing to characterize patterns of genetic structure and diversity in twelve *R. cascadae* populations in California to prioritize populations for conservation and compared these populations with three in Oregon to determine differences in diversity and population divergence. I also detected outlier loci using genome-scan methods and compared patterns of differentiation between these loci and presumably neutral loci. I found evidence of genetic structure in California creating two main groups of ancestry despite a strong pattern of isolation-by-distance (IBD), with Oregon populations forming a third group. Populations in California were highly differentiated from those in Oregon and had lower estimates of genetic diversity that support documented demographic declines. *Rana cascadae* was also moderately differentiated between the two main regions within California but genetic diversity was similar. Patterns of

genetic differentiation were overall similar between outlier and neutral loci. These findings indicate that Cascades frogs in California should be managed by genetic ancestry and not by ecoregion, as they are currently. Source populations should be selected by choosing the nearest and demographically largest site to the donor population within the same major genetic ancestry group to maximize genetic diversity and minimize both outbreeding and inbreeding depression. This study provides the beginnings for understanding the spatial genetic structuring of Cascades frogs in California and provides managers a way forward for active conservation in the face of ongoing declines.

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Introduction

Conservation Background

Amphibian species are experiencing global declines at an alarming rate. Over the past 40 years, 9 species have been declared extinct and 40% of described species are considered threatened with extinction (Stuart et al. 2004, Wake and Vredenburg 2008, Monastersky 2014). This global extinction crisis is primarily attributed to habitat loss and fragmentation, overexploitation, and novel diseases (Collins and Storfer 2003, Skerratt et al. 2007). In North America, the western United States in particular has experienced dramatic declines of many amphibian species (Lanoo et al. 2005) including the Cascades frog (*Rana cascadae*; Pope et al. 2014).

The Cascades frog (*Rana cascadae*, Slater 1939), endemic to the Pacific Northwest, has significantly declined throughout its range in northern California (Fellers and Drost 1993, Welsh et al. 2006, Fellers et al. 2008, Pope et al. 2014). Once common near Lassen Peak, naturalist Joseph Grinnell wrote of Cascades frogs in 1925 that there was, "…one frog for nearly every meter around the lake [Helen]" (Grinnell et al. 1930). Today, in what is now Lassen Volcanic National Park, Cascades frogs are believed to be extirpated with only one individual found in 2007 and none found during surveys from 2008-2010 (Pope et al. 2014). It is estimated that frogs from 95% of historic localities in the Lassen region of the southern Cascade Range have disappeared since the 1970s and 6 of the 11 remaining populations in the region are facing extinction (Fellers and Drost 1993, Jennings and Hayes 1994, Fellers et al. 2008, Pope et al. 2011, Pope et al. 2014). In the Klamath Mountains of California, about 60km West of the California Cascades, Cascades frogs are also experiencing declines albeit at a slower rate and dozens of sites are still occupied (Piovia-Scott et al. 2011).

The declines of Cascades frogs in California are primarily attributed to novel diseases and introduced aquatic predators (Pope et al. 2014). The amphibian fungal disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been documented at all 11 sites in the southern Cascades and is present at most sites in the Klamath Mountains (Piovia-Scott et al. 2011, Pope et al. 2011, Pope et al. 2014). This disease has been proposed as the primary factor contributing to declines of California Cascades populations (Pope et al. 2014). Predation by non-native trout is responsible for declines of Cascades frogs in the Klamath Mountains, in addition to chytridiomycosis (Pope 2008, Piovia-Scott et al. 2011, Pope et al. 2014). More recently, extreme drought has been hypothesized to negatively impact amphibians in California by altering normal hydrologic regimes such as reducing hydro-periods and mountain snowpack, resulting in loss of breeding and summer habitats for Cascades frogs (CDFW 2016). Because of these declines, Cascades frogs have been listed as a California "Species of Special Concern" by the California Department of Fish and Wildlife (Jennings and Hayes 1994) and a "sensitive species" by the United States Forest Service (USDA Forest Service 1998). The Cascades frog has also recently been classified as a candidate for federal listing under the California Endangered Species Act and as a Level 1 Drought Priority Species by the California Department of Fish and Wildlife (CDFW 2016).

Managers are continuing to monitor Cascades frog populations and have attempted to address some of the major threats by implementing meadow and hydrologic restorations (Pope et al. 2011), developing experimental disease treatments (Hardy et al. 2015), and removing introduced fish (Welsh et al. 2006, Pope 2008). Preliminary results from these targeted actions are

promising; however large-scale manipulative conservation efforts (translocation, re-introduction, captive breeding) may be necessary to ensure the persistence of Cascades frogs across the landscape in California (Pope et al. 2014). Managers are currently planning to reintroduce Cascades frogs back into Lassen Volcanic National Park (K. Pope pers. comm., M. Magnuson pers. comm.). In addition to considering the ecological, logistical, and animal welfare implications of such actions, understanding and mitigating the genetic consequences of wildlife translocations is an often overlooked but important aspect of these efforts that can greatly affect their long term success (Rhodes and Latch 2010). For example, it might be desirable to select source individuals from populations that are genetically similar to those at the translocation site, or to select individuals from multiple source populations to maximize genetic variation in the translocated population.

It remains unclear how California populations of Cascades frogs are connected presently and historically and how much genetic variation remains. Dramatic declines in population sizes, such as those exhibited by Cascades frogs, can lead to fragmentation and loss of connectivity (i.e., gene flow) at local and regional scales, ultimately leading to higher probabilities of inbreeding (and associated negative effects) and loss of genetic variation due to genetic drift (Gilpin and Soule 1986). Loss of genetic diversity plays an additive role in the continuation of declines (Allentoft and O'Brien 2010) and exacerbates the deterministic effects driving population extinction (Spielman et al. 2004, Fagan and Holmes 2006). Assessing the genetic structure and diversity of residual populations, especially after or during large declines, is imperative to the success of future management actions to limit loss of genetic variation and accumulation of inbreeding, thereby maximizing long-term population persistence (Schwartz et al. 2007).

Monsen and Blouin (2003) suggested that Cascades frogs in California form a Distinct Population Segment (DPS), exhibiting significant genetic divergence from populations in Oregon and Washington. Only two populations from California were used in the analysis however and both were from the California Cascades Region, excluding the geographically disjunct Klamath Mountains. Case (1978) also found differentiation in Cascades frog proteins between the Klamath Mountains and California Cascades, indicating there could be divergence between regions within California. The range-wide scale and number of markers used by Monsen and Blouin (2003) did not allow for description of population genetic structure and diversity at local and regional scales in California, which are important for conservation managers. An accurate understanding of population genetic structure must be established in order to focus management and conservation efforts effectively (Avise 1989, Moritz 1999, 2002).

Recently, genomic data sets of non-model organisms have been easier and more cost-effective to attain for conservation-based research using highly-variable nuclear gene markers, such as single-nucleotide polymorphisms- SNPs (Steiner et al. 2013). Next-generation sequencing methods for DNA marker discovery have enabled both basic and applied research on wildlife management to answer previously intractable questions (Haynes and Latch 2012, Santure et al. 2010, vonHoldt et al. 2013). Restriction site associated DNA (RAD) sequencing, in particular, has proven to be a reliable, taxonomically flexible method for characterizing high density suites of single-nucleotide polymorphisms, or SNPs, that can be used to reliably estimate ancestry (Hoffman et al. 2014). Not only can genomic techniques elucidate neutral evolutionary processes

of conservation concern, but are paramount for the inference of selective processes and adaptation of populations for conservation (Flanagan et al. 2017). For these reasons, genomic methods are well-suited for fine-scale population genetic research and delineation of conservation units in wild, non-model organisms (Funk et al. 2012).

Hypotheses

1. If the physiographic and geological differences between the Klamath Mountains and California Cascades Range have shaped the historical biogeography of Cascades frogs, then their spatial genetic structuring will align according to these ecoregions.
2. If Cascades frogs in California have declined more than in Oregon, then California Cascades frogs will have less genetic diversity than those in Oregon.
3. If Cascades frog populations in the California Cascades are more isolated and have declined more significantly than those in the Klamath Mountains, then Cascades frogs in the California Cascades will have less genetic diversity than in the Klamath Mountains.

Study Aims

In this study I used high-throughput reduced-representation genomic sequencing to produce a dataset of single-nucleotide-polymorphism markers to elucidate population genetic processes of declining Cascades frog populations at the southern range edge in California and compare them with central populations in Oregon to inform conservation efforts. I approached this aim in three ways: 1) I described the spatial genetic structure and differentiation of Cascades frog populations and tested for influences of landscape features on differentiation, 2) I estimated parameters of

within-population genetic variation, and 3) I tested for outlier loci and compared population divergence with neutral loci.

Study species

The Cascades frog is a montane frog in the family Ranidae that occurs along the Cascade Mountains axis of Washington, Oregon, and northern California, with disjunct populations occurring in the Olympic Mountains of Washington and the Klamath Mountains of California (Figure 1). *Rana cascadae* was first described by Slater in 1939, previously known as a subspecies of the Oregon spotted frog (*Rana pretiosa*) and was then known as Slater's frog or Cascades frog. From 1939 to 1961 there was doubt that Cascades frogs were in fact a species with Schmidt (1953) and Stebbins (1951) treating them as Northern Red-legged frog (*Rana aurora*) subspecies (*Rana aurora cascadae*). However Dunlap (1955) found no morphological similarities between the two types and Zweifel (1955) reported abnormal embryos after a single cross of a California Red-legged frog (*Rana draytonii*) and a Cascades frog but with successful hatching of hybrids between the two. Porter (1961) then experimentally tested crosses between *Rana cascadae* and *Rana aurora* and found that hybrid larvae became deformed and died before metamorphosing, effectively resolving the Cascades frog species debate.

In 1978, Case used biochemical assays to compare five western frog species from the genus *Rana* and placed Cascades frogs as the sister species of *Rana pretiosa* (Case 1978). Macey et al. (2001) used mitochondrial DNA sequences to place Cascades frogs as sister species to Mountain Yellow-legged frogs (*Rana muscosa*) within the Amerana species group (*Rana boylii* group; Foothill Yellow-legged frog) that includes *R. cascadae*, *R. pretiosa* (and *Rana luteventris*;

Columbia Spotted frog formerly single species as *R. pretiosa*), *R. muscosa*, *R. boylii*, and *R. aurora*. Hillis et al. (2005) also supported this Amerana group but placed *R. cascadae* as sister species with *R. aurora* using different mitochondrial sequences than Macey et al. (2001). Most recently, Yuan et al. (2016) also found support for *R. aurora* as sister to *R. cascadae* using both nuclear and mitochondrial sequences.

Cascades frogs normally display sharply defined black spots on a brown, olive, or bronze dorsum with a cream or yellow venter and a long cream-colored upper jaw stripe of "lipstick" that extends beyond the jaw (Jones et al. 2005). Cascades frogs in the southern Cascades in California are predominantly dark brown with few or poorly recognized spots whereas those in the Klamath Mountains of California are more often olive or bronze with many spots. Adult frogs are sexually dimorphic in size with female frogs weighing up to 56 g (compared to 28 g for males) and up to 81 mm in snout-vent length (SVL) (compared to 56 mm for males) (Garwood 2009).

Cascades frogs are diurnally active from mid-May through mid-October, dependent on snowfall from the previous winter. During the winter when terrestrial and aquatic habitats are covered in deep ice and snow, Cascades frogs "overwinter" up to seven months and remain relatively inactive on lake bottoms deep in silt or in deep springs that don't freeze (K. Pope pers. comm.) Cascades frogs explosively breed over 3-14 days annually after the spring thaw when sections of breeding areas become free of snow (Sype 1975, Briggs 1976, Briggs 1987). Egg masses totaling 300-500 ova are typically oviposited in shallow areas of lentic habitats (Briggs 1976, Nussbaum et al. 1983). Tadpoles hatch in mid-summer and metamorphose into "froglets" by

September of that year (Sype 1975, Briggs 1976, Pope and Larson 2011). Cascades frogs can live more than 10 years (Garwood unpubl. data) and reach sexual maturity relatively late (3-4 years for males and 4-5 years for females) (Garwood and Larson unpubl. data).

Within the sub-alpine and montane habitats of California where Cascades frogs occur, they are patchily distributed and occupy lakes, ephemeral ponds, streams, and wet meadows at altitudes of greater than 2000m (Welsh et al. 2006, Fellers et al. 2008, Pope et al. 2014). In the Klamath Mountains, major aquatic features are usually fragmented between high mountain passes that separate drainage basins, potentially limiting movement and gene flow between aquatic features. However, during a five year multi-basin mark-recapture and VHF telemetry study, Garwood (2009) documented 1% (19 out of 1,980) of marked Cascades frogs moved between basins, with these inter-basin dispersal events occurring over steep ridges with rocky talus and no aquatic features, likely moving along low points in saddles of mountain passes. These long distance dispersers traveled between 736m-1886m (Garwood 2009). For frogs that didn't move between basins, 71 of 1,669 moved over 1km within their own basin (Garwood and Welsh 2007). Garwood (2009) is often miss-cited in the literature claiming individual Cascades frogs can move up to 5km. In fact, Garwood (2009) shows *effective* connectivity between sites 5km apart due to several smaller independent movements of several individuals between sites in a stepping-stone pattern, thereby *effectively* providing evidence for potential gene-flow across a distance of 5km.

These findings indicate that between adjacent divided basins long-distance migrants may disperse between "sub-populations" at a rate that would at least equal the "one migrant per

generation" rule to effectively maintain gene flow and genetic diversity at a local scale (<5km) as long as population sizes remain stable (Mills and Allendorf 1996, Wang 2004). At larger scales, it is still conceivable that occupied sites may be connected by gene flow up to 10km. Monsen and Blouin (2004) found that populations of Cascades frogs have "genetic neighborhoods", where the exchange of frogs between sites drops sharply at distances of greater than 10km. Many other amphibian species exhibit this relationship as well and it has been suggested that most differentiation usually occurs at distances greater than 10km (Smith and Green 2005).

Methods

Study Area

This study was conducted in California in Tehama, Siskiyou, Shasta, and Trinity Counties. I also sampled in Lane, Douglas, and Deschutes Counties in Oregon. I chose to survey sites in California where Cascades frogs were known to be present within the last 10 years (Pope pers. comm), prioritizing the sampling of sites in the California Cascades Region where significant declines have occurred and sites in the eastern, central, and northern portions of the Klamath Mountains to maximize the distribution of sites within their range in that region. Oregon sites were chosen based on how recent Cascades frogs were observed (Monsen and Blouin 2003, Chris Pearl and Mike Adams pers. comm.), and the ease of access to those sites.

Sites in California were previously defined in Pope unpubl. 2008. Because Cascades frogs are typically found within a few meters of water, meadow sites were generally defined by their active hydrology at time of survey (extent of flowing or non-flowing surface water), or previous

knowledge of an average active area across a survey season (May-Sep). Lake sites in California consisted of the discrete boundary of the lake itself, and portions of inlets, outlets, or connected backwaters and ponds if present and accessible. Oregon sites were identified from coordinates used in previous studies (Monsen and Blouin 2003, Chris Pearl and Mike Adams pers. comm.), or via query from species distribution databases (Global Biodiversity Information Facility at GBIF.org). Using these coordinates, sites were then defined using satellite imagery to determine the survey-able area surrounding a coordinate. Once at the site, adjustments were made where to survey based on available surface water, further inspection of satellite imagery, and my prior knowledge of focal species habitat preferences.

Amphibian diversity within the study area is moderate with 19 native species present (Bury and Pearl 1999). However, only six are anurans and three of those are ranid frogs, and none are readily confused with *R. cascadae*. American Bullfrogs (*Rana catesbeina*) are non-native to California and have also been found within the study area, though not at sites surveyed for this study. The other native ranid frog that occurs in our immediate study area in California, the Foothill yellow-legged frog (*Rana boylii*), occurs in high-gradient and high-flow riverine habitats along banks and cobble bars (Stebbins 2003) which are too fast for Cascades frogs. The identifiable pattern of bright yellow coloration under *R. boylii* typically makes them very conspicuous and easy to identify. *R. boylii* has never been observed at *R. cascadae* sites previously and none were observed during this sampling effort. In Oregon, there is potential for overlap between Cascades frogs and Oregon Spotted frogs (*Rana pretiosa*) within a site. *R. pretiosa* are often covered in red splotchy coloring along their venter or dorsum, has upturned eyes, and has a "broken" pattern of their dorso-lateral fold behind the eye. Cascades frogs do not

have red patterning, have side-turned eyes, and have an intact dorso-lateral fold. Oregon Spotted frogs were encountered at one Oregon site where Cascades frogs were not found and identification characteristics were confirmed with various field guides (Stebbins 2003, Jones et al. 2005, Corkran and Thoms 1996). I did not find *R. cascadae* and *R. pretiosa* in the same location during the course of this study.

Cascades frog tadpoles can be distinguished from other co-occurring species by their general size, coloration, and eye position. Pacific treefrog (*Pseudacris regilla*) tadpoles commonly co-occur and breed in the same habitats as Cascades frogs but are usually smaller, more variable in color, and have eyes that protrude from the sides of the head. Cascades frog tadpoles are larger than treefrog tadpoles and are darker in color, have larger tails, and have eyes that are predominantly on the top of their heads. Western toad (*Anaxyrus boreas*) tadpoles may also co-occur with Cascades frogs but are much smaller than treefrog tadpoles, have small tails, are conspicuously dark black in color, and most often occur in large groups of over ~500 conspecifics.

Cascades frog tadpoles can also be differentiated from congenerics in the study area based on physical characteristics and breeding site differences. Tadpoles of *Rana boylii* are lightly mottled in color compared to *R. cascadae* and are found in small pools adjacent to rivers or streams or in stream shallows. These habitats are unsuitable for *R. cascadae* oviposition and would not be found together. *Rana pretiosa* tadpoles have heads that come to a blunt end at the mouth whereas *R. cascadae* have somewhat pointed heads. There is also more patterning visible in *R. cascadae* tadpoles than *R. pretiosa* which are more homogenous in color. Lastly, *R. catesbeiana* tadpoles

are significantly larger than any of the tadpoles mentioned above and often have a mottled green color on top with a cream venter.

Sample Collection

Diurnal visual encounter surveys were conducted at all water bodies within a site (Crump and Scott 1994, Thoms 1997). These surveys consisted of one to four observers walking the perimeter of all water bodies and took place between 0900 and 1800 h (Crump and Scott 1994, Thoms et al. 1997). Surveys were not conducted when temperatures were below 50 F or when raining. This work was completed in California under California Department of Fish and Wildlife SCP # SC-3905. STP # 107-16 was issued for collections in Oregon by the Oregon Department of Fish and Wildlife. Institutional Animal Care and Use Committee Permit 15-16 #41 was approved by the University of Wisconsin-Milwaukee IACUC.

When a Cascades frog was found, it was caught via net or directly captured with gloved hands (non-Latex examination gloves) and placed in a zip-lock bag and processed immediately (weighed, measured, marked (if at a mark-recapture site), swabbed, tissue sample). I recorded weight by attaching a Pesola scale attached to the top of the bag (taking into account the weight of the bag) and snout-urostyle length (SUL) by measuring the tip of the snout to the end of the urostyle with handheld calipers. Frogs were visually assessed for secondary sexual characteristics of enlarged nuptial pads on the first digit of the front limbs if an adult to determine sex. GPS coordinates were recorded, along with life stage of the animal. Life stages used were metamorph, young of previous year (YOPY), sub-adult, and adult. Metamorphs are frogs that have just metamorphosed from the tadpole stage this year and are only encountered in

August surveys. Young-of-previous-year frogs are those that were born and metamorphosed the previous year and usually <38mm SUL. Sub-adults are those born at least two years prior and are between 38-50mm depending on sex. Adults are those that are generally larger than 50mm SUL depending on sex (Pope and Larson 2013). Habitat characteristics were only recorded for individuals captured at sites used in ongoing mark-recapture studies. These characteristics include % cover within a 1m radius around the individual, substrate type (silt, sand, gravel, pebble, cobble, boulder, bedrock), water flow (none, slow, moderate, fast), onshore vegetation type (riparian, meadow, coniferous, mixed coniferous), maximum water depth within a 1m radius, % emergent vegetation within a 1m radius, and water temperature. All frogs were released within 10 minutes of capture. New gloves were used for each individual. To prevent the spread of chytrid fungus, all field gear was disinfected using a 0.1% solution of quaternary ammonia between sites.

If frogs were captured at sites where capture-mark-recapture efforts were on-going for long-term monitoring by the Forest Service, then individuals were checked for previous marks. If the individual was not a recapture with a previous mark then it received a new mark with either a passive integrated transponder (PIT) or visual implant elastomer dye (VIE) depending on the size of the animal. All frogs with a snout-urostyle length (SUL) > 38mm were tagged with a PIT tag (TX1400L, Biomark Inc., Boise, Idaho, USA) if not previously tagged using this method. Following routine procedures, a small incision was made in the skin above the sacral hump between the dorsal lateral folds using sterile surgical scissors. The tags measure <2mm x 8mm, therefore each side of the "v" shaped incision is approximately 1.5-2mm so the tag can be easily inserted (Pope and Matthews 2001). Once inserted, the tag was slid down the back (under the

skin) to a position below the sacral hump; this position is considered most safe for the frogs and does not impair movements (Pope pers. comm.). If the animal was previously tagged using this method, the tag number was recorded. Scissors were dipped in 95% ethanol and flamed and allowed to air dry between individuals. All frogs with a SUL of <38mm were injected with a visual implant elastomer (VIE; Northwest Marine Technologies Shaw Island, WA). A 0.5-1.0 cc sterile syringe attached to a small needle was used to inject a green elastomer dye into the right rear foot of the individual. All needles were flame sterilized in the manner described above.

All *R. cascadae* caught were swabbed for chytrid fungus infection as a part of ongoing disease monitoring. Frogs were swabbed with a sterile cotton swab along the ventral surface of the abdomen, left and right inner thighs, and webbing of the hind feet (each area five times). When finished, the swab was allowed to air dry before being placed in a sterile vial with individual information codes attached. These disease samples were sent to Dr. Jonah Piovia-Scott's lab at the University of Washington- Vancouver for DNA extraction and polymerase chain reaction (PCR) to detect the chytrid infection-causing fungus *Batrachochytrium dendrobatidis* (*Bd*).

A non-lethal tissue sample was taken from each animal caught at a site (up to 30 individuals per site) for population genetic analyses. Toe-clipping was primarily used to collect genetic material and is a safe, simple, sanitary and humane method that has been used successfully for amphibians for decades (Donnelly et al.1994). Using a pair of flame-sterilized scissors, 2 full toe bones (phalanges) were clipped off of the fourth digit of a rear foot. Bactine® spray was used on the wound after to provide an analgesic effect. Toes were then placed in a vial of 85-99% ethanol and marked according to individual. For less than 1% of samples, a tissue sample was taken

using a 4mm biopsy punch of webbing between the 2nd and 3rd digits of a hind foot. Wound disinfection, tissue storage, and punch sterilization were performed as described previously. At sites where few frogs were found, small portions of tadpole tails were clipped with a sterile pair of scissors to cut a 1-2mm amount of tissue off of the tail.

Laboratory Methods

I conducted DNA extractions using a Qiagen DNEasy Blood and Tissue spin-column method following manufacturer's protocol (Qiagen). DNA yields were quantified using a Qubit Fluorometric Quantitation assay (Qubit). Only samples containing >3ng/ul of DNA were considered acceptable for sequencing.

A double-digest Restriction-Site-Associated DNA sequencing method was used (Peterson et al. 2012; ddRAD) by the Texas A&M AgriLife Research facility (TAMU). A suite of enzymes were tested prior to library preparation and ultimately MseI and HindIII were selected based on their performance. Libraries were prepared in equimolar concentrations and size-selected using a Pippin Prep (Sage Science, Beverly, MA). Resulting libraries were pooled and run on a 150bp paired-end Illumina HiSeq 2500 machine across two lanes to provide optimal coverage.

Bioinformatic Processing and Filtering

I received FASTQ files that contained de-multiplexed (demuxed) reads with Illumina barcodes removed for each individual. I used the bioinformatic pipeline *Stacks* (Catchen et al. 2013, v1.48) to build a dataset of SNP loci using a denovo assembly approach in a cluster computing environment at UWM (Mortimer-1 node, 24 cores, 64g). Distant relatives were tested as

reference genomes for sequence alignment but performed poorly. Optimum values for the main *Stacks* assembly parameters (-m, -M, -n) were tested and plotted following the methods outlined in Rochette and Catchen (2017) (Figure 2). The –m parameter controls determines the minimum stack depth or minimum depth of coverage. The –M parameter is the distance allowed between stacks, while –n is the distance allowed between loci in the catalog (Catchen et al. 2013). It is recommended to keep –M = -n in most cases (Paris et al. 2017). The optimal parameter values used were –m 3, -M 4, and –n 4. The '*populations*' unit of *Stacks* was used to filter erroneous SNP calls and provide a dataset with loci shared by 75% of individuals which is close to the recommended 80% threshold by Paris et al. (2017). I lowered this threshold to allow for the inclusion of more loci in the final dataset while still at controlling for errors at a similar level. Previous studies have used thresholds as low as 50% (Funk et al. 2016). *VCFTools* was then used to determine which individuals had large amounts of missing data (>50%) and were removed from the dataset. Specific file formats were either output directly from the *Stacks* '*populations*' unit for downstream analyses (i.e., Vcf, Structure, Genepop) or were converted to software-specific file formats using the *PGD Spider* program (Lischer and Excoffier 2012). The *Stacks* pipeline was run twice to create two SNP datasets- one that contained samples from Oregon and California (ORCA dataset), and another that only contained California (CA dataset). The ORCA dataset was used in comparisons between Oregon and California, while the CA dataset was used to make inferences only within California. Running California sites without Oregon allowed more SNPs to be called for comparisons within California.

Analyses were conducted on datasets that contained all filtered SNPs (ORCA and CA), except for those comparing outlier (ORCA-out, CA-out) and neutral loci (ORCA-neu, CA-neu) when

specified. Analyses that compared neutral and outlier loci used a non-model based approach by identifying the top 5% of F_{ST} outliers. While this approach is likely removing some neutral SNPs from the dataset as false-positives, it is a conservative way of attempting to assure neutrality within the set of SNPs for analysis (Schoville et al. 2012). I also used a Bayesian method implemented in *BayeScan* and an ordination approach to outlier detection in *PCAdapt* to compare patterns between neutral and outlier loci using different methods (detailed below).

Analyses

Genetic structure and differentiation

Measures of population-pairwise allele fixation (F_{ST}) and allele sharing (Jost's D; Jost 2008) were estimated using the R package *Hierfstat* (Goudet 2005) and *FinePop* (Kitada et al. 2017) respectively. DAPCs, PCoAs, and PCAs were conducted using the R package *Adegenet* (Jombart 2008). For PCA and PCoA, the number of PCs and clusters were determined using a K-means clustering approach and BIC. For the DAPCs, I used both a spline-interpretation of alpha values approach (Figure 3) and a cross-validation of PCs approach (Figure 4) to test which method provided more informative results.

Population structure and admixture analyses were conducted in *Structure* 2.3.4 (Pritchard 2000) and *BAPS* 6 (Corander et al. 2003). With many sites containing low pairwise F_{ST} values (< 0.05), these programs are well suited to correctly infer population genetic structure under these conditions (Latch et al. 2006). I ran *Structure* with a Markov-Chain-Monte-Carlo (MCMC) burn-in of 100,000 steps with 100,000 additional iterations to determine clustering under the

admixture model with correlated allele-frequencies and location prior (population ID). These steps were repeated five times for each K, where K is defined as the number of clusters. The maximum value of K for each analysis was equal to the number of sampling sites in that analysis. If genetic clusters were found (optimal K > 1), *Structure* was then run under the same conditions above for each subsequent clustering group until no further sub-clustering was detected (optimal K=1). This hierarchical approach helps to clarify patterns of hierarchical structure, which can be common especially when *Structure* reports K=2 in the initial runs of the full dataset (Janes et al 2017, Evanno et al. 2005, Pritchard et al. 2000). For all *Structure* runs, the optimal K was determined by comparing the value of Delta K (Evanno et al. 2005) and the mean likelihood of K estimate (Ln Pr(X|K)) (Pritchard et al. 2003), while taking into consideration the biological feasibility of the inferred clusters using *Structure Harvester* (Earl and VonHoldt 2012).Average q-values (proportion of an individual's genome that belongs to each cluster) were calculated in *CLUMPP* (Jakobsson and Rosenberg 2007) and individuals were assigned to a cluster based on majority assignment (>50%).

BAPS has the ability to incorporate a spatial model by supplying spatial coordinates for individuals or groups, thereby providing a biologically-relevant non-uniform prior to increase power to correctly detect population genetic structure (Corander et al. 2008). I used the "spatial mixture of groups" model with a max K= 20 to return the highest supported K. Results from the mixture analysis were then input into the admixture analysis with 50 iterations, ten reference individuals from each population, and 50 iterations for references.

Landscape influence on spatial genetic patterns (genetic clusters as determined from *BAPS* and *Structure*) was first tested under an Isolation-by-Distance (IBD) framework as a null-model. IBD

refers to the correlation of lower genetic distances when individuals/populations are close together in geographic distance across a landscape and higher genetic distances when further apart (Wright 1943). In contrast, Isolation-by-Landscape-Resistance (IBR) refers to the correlation of landscape features on genetic distance regardless of geographic distance. Distance matrices were constructed of pairwise genetic distances (pairwise F_{ST}), geographic distances (km), elevation (m), putative genetic barrier (binary with 0 = same side of the barrier and 1 = different sides of the barrier), and other environmental factors as gathered from ClimateWNA (Hamann et al. 2013) to assess landscape effects.

To test these landscape models, I first used simple and partial Mantel tests implemented in the R package *Ecodist* (Goslee and Urban 2007) with 10^4 permutations and 10^5 iterations for the bootstrapped confidence limits. Partial Mantel tests can have high error rates and spurious correlations however, especially when attempting to identify IBR (Kierepka and Latch 2015, Balkenhol et al. 2009). It is recommended to use multiple approaches to identify IBR in the face of IBD and therefore I also used multiple regression of distance matrices (MRDM or MRM; Legendre et al. 1994) to assist in the interpretation of the Mantel test results. MRDM analyses were performed in the package *Ecodist* (Goslee and Urban 2007) in R using 10^4 permutations.

Genetic diversity and demography

Population level diversity statistics were estimated directly from the *Stacks* '*populations*' unit (π, H_e, H_o) and from the R package *hierfstat* (A_R; Goudet 2005). Effective population size (Ne) was estimated using the linkage-disequilibrium (LD) model in *Nestimator 2.01* (Do et al. 2014).

Outlier detection and comparative population differentiation

Outlier loci were identified using quantitative-based approaches in *BayeScan 2.1* (Foll and Gaggiotti 2008) and the R package *PCAdapt* (Luu et al. 2017) in addition to the top 5% of F_{ST} outliers. These loci were used to compare patterns of differentiation with the remaining, presumably neutral loci using PCAs and PCoAs. *BayeScan* identifies loci that are potentially under natural selection by comparing differences in allele frequencies between populations via F_{ST} using a multinomial-Dirichlet model (Foll and Gaggiotti 2008). *BayeScan* was implemented with a burn in of 50,000, a thinning interval of 10, and a sample size of 5000, for a total of 100,000 iterations after 20 pilot runs of 5000 iterations each. Outlier loci were identified by comparing the probability, log probability, and q-value of the estimated F_{ST} for each locus and plotted in R using the *BayeScan*-provided scripts. Direction of selection was determined by the alpha value (negative for balancing or purifying selection, positive for directional selection).

PCAdapt uses a principal components approach to identify population structure without requiring individuals to be grouped into populations. Outlier SNPs are determined using a Mahanalobis distance test statistic to identify outlier loci by how they are related to population structure in each PC. I chose how many PCs to include by generating a scree plot and using Cattel's rule as recommended by Luu et al. (2017). These three sets of outlier loci were compared with each other to determine any overlap in outliers. Regardless of overlap, all three methods were combined into a single outlier dataset for each data group (ORCA-out and CA-out). The remaining loci were used as presumably neutral (ORCA-neu and CA-neu).

Results

Sample Collection, Bioinformatic Processing, and Filtering

In 2016 a total of 675 Cascades frog tissue samples were collected from 31 sites in California and Oregon. 561 samples were collected across 26 sites in California and 107 samples were collected across seven sites in Oregon. Of the 675 total samples, 268 were sequenced, spanning four sites from Oregon (78 samples) and 12 sites from California (192 samples) (Table 1). After cleaning, assembling, and matching reads, loci were recovered across 257 of the 270 individuals (Table 2). The average mean read depth per individual was 7.6x (median 7.4, range 6.3-11.1). After filtering with the *populations* unit and *VCFTools*, 1672 loci/SNPs (1 SNP per locus) were retained across 210 individuals from 15 sites (ORCA dataset, Figure 5). To include more loci for fine-scale analyses within California, we analyzed the 192 California samples separately, recovering loci across 179 individuals (Table 2). The average mean read depth per individual was 7.5x (median 7.3, range 6.3-11.1). After filtering with the *populations* unit and *VCFTools*, 2291 loci/SNPs (1 SNP per locus) were retained across 163 individuals from 12 sites (CA dataset, Figure 5).

Spatial genetic structure and differentiation of populations

Spatial genetic structure was supported by both *Structure* and *BAPS*. Structure identified three main clusters (K=3) in the ORCA dataset- Oregon (OR), California Northern (CAN), and California Southern (CAS, Figure 6 and 7). Further substructure was detected in OR (K= 2), CAN (K= 6), and CAS (K= 3) groups. Substructure detected in Oregon divided the three sites

into two groups, one containing Waldo Lake (WAL) and Todd Lake (TOD) and the other group with Diamond Lake (DIA). Further hierarchy split WAL and TOD. For the CA dataset, *Structure* identified two main clusters (K=2; Figures 7-8), identical to those identified in the California portion of the ORCA dataset (Figures 6-7). Substructure within CAN (K= 6; Figures 12-15) and CAS (K=3; Figures 10-11) was also identical to the ORCA dataset. For both ORCA and CA datasets, the CAN cluster contains all sites from the Klamath Mountains (Red Rock Lake-RED, Gem Lake-GEM, Rush Lake-RUS, Little Caribou Lake-LIC, Blue Divide Lake-BLU, and Gumboot Lake-GUM) as well as two sites in the California Cascades just north of the Pit River (Screwdriver Creek-SCR and Nelson Creek-NEL). The CAS group consists of the three sites south of Lassen Peak (Carter Meadows-CAR, Round Valley Meadows-ROU, Childs Meadows-CHI). Old Cow Meadows (COW), located south of the Pit River but north of Lassen Peak, is admixed between CAS and CAN, with majority assignment (>50%) to CAN (Figure 9). *BAPS* identified main genetic structure in the ORCA dataset for four groups- Oregon (OR), Klamath Mountains (KLA), northern Lassen (NLN), and southern Lassen (SLN) (Figure 16). Differentiation between CAN and CAS was moderate (F_{ST} = 0.15) and higher between CAN and OR (F_{ST}= 0.22) and CAS and OR (F_{ST}= 0.29).

PCAs and PCoAs for both datasets were largely concordant with *BAPS* clusters (Figures 17-20). The ORCA dataset contained four main groups displayed along the first two PCs and the CA dataset was in three groups along the first two PCs. Individuals from Old Cow Meadows were notably in between the California Southern and California Northern clusters. The DAPCs returned quite different patterns using either the spline-interpolation or cross-validation method of PC retention for both datasets (Figure 21; 22). Though most clusters were congruent with

population expectations, several appeared to be overestimated using BIC, with some clusters containing only a few individuals, often from different sites.

IBD was strong in California (Figure 23; Pearson's correlation coefficient= 0.83, p= <0.0001). Simple Mantel tests identified a significant correlation between genetic differentiation (CA dataset) and geographic distance, elevation, and presence of a putative barrier between the Klamath/North Lassen populations and South Lassen populations (Table 5). Partial-Mantel tests were not significant for any of the predictor variables when controlling for distance after a correction for multiple tests was applied using a false discovery rate (FDR) of 0.015 (Benjamini and Yekutieli 2001). Other ecological variables I tested (Level III Ecoregion and average winter temperature) were not significant influences on genetic differentiation (Table 7). However the MRDM analysis did indicate support for a relationship between genetic differentiation and the presence of a genetic boundary between the Klamath/North Lassen populations and South Lassen populations when accounting for geographic distance (r^2= 0.42, p= 0.0001).

Genetic differentiation (pairwise F_{ST}; Table 4) between California and Oregon populations (ORCA dataset) was moderate-high, ranging from 0.117 - 0.248 (median = 0.197). Pairwise values of Jost's D (Table 4) were generally smaller but mirrored F_{ST} estimates ranging from 0.001–0.073 (median = 0.023). Among the three Oregon sites, differentiation was low (F_{ST} range = 0.032-0.076; median=0.0592). Jost's D values ranged from 0.007 - 0.021 (median= 0.019).

Genetic differentiation was low-moderate among Cascades frog populations in California (CA dataset). Pairwise F_{ST} values within California (Table 3) ranged from 0.005–0.205 (median = 0.1214). Pairwise values of Jost's D (Table 3) were generally smaller but mirrored F_{ST} estimates ranging from 0.001–0.063 (median = 0.025). While F_{ST} and D are fundamentally distinct measures of differentiation, they are expected to provide similar insight into differentiation in pairwise comparisons of biallelic SNPs (Jost et al. 2018).

Genetic diversity and population demography

California Cascades frog populations had lower within-population genetic variation than those in Oregon (ORCA dataset; Table 8) across three of the four metrics of diversity (t-test A_R, p = 0.002; t-test H_E, p = 0.012 ; t-test H_O, p = 0.008; t-test π, p = 0.110). When comparing populations within California (CA dataset), they did not significantly differ between the two regions (Table 9) (t-test A_R, p = 1.00; t-test H_O, p = 0.386; t-test H_E, p = 0.474; t-test π, p = 0.594). F_{IS} were just below zero for all sites in both datasets and were all relatively similar. A linear regression of H_O against site elevation in California was significant (Figure 22, p = 0.01), but regressions of H_E, A_R, and π were not significantly related to elevation (Figure 22).

Estimates of effective population size from *$N_e$estimator* were only reliably calculated for 10 of the 15 sites in the ORCA dataset (Table 8; 9). Of the ten sites that calculated N_e, estimates ranged from 16.4 to 285.3.The two defined Oregon sites had N_e estimates larger than any site in California, but the differences were not statistically significant (Table 9; t-test; p = 0.279), likely due to the large variances surrounding N_e estimates. South Lassen sites also did not have lower

N_e estimates than those in the Klamath/North Lassen region (Table 8; t-test; p = 0.707) using the CA dataset.

Identification of outlier loci and comparative patterns of differentiation

Eighty-four of the ORCA dataset loci were in the top 5% of F_{ST} values. *BayeScan* identified fifteen outliers, one of those was a high F_{ST} outlier and fourteen were low F_{ST} outliers. *PCAdapt* identified 112 loci, for a total of 165 unique loci identified by at least one method in the ORCA dataset. Thirty-two were shared across two methods and none were shared among all three (Table 8). In the CA dataset, 115 loci were top 5% F_{ST} values. Ten outliers were detected in *BayeScan*, three of those were high and seven were low. *PCAdapt* identified 154 outlier loci. A total of 219 unique loci detected by at least one method for CA, 66 were shared by two methods and 2 were identified as outliers in all three approaches (Table 9).

PCAs across both datasets comparing neutral and adaptive SNPs showed nearly identical patterns of similarity. Only Diamond Lake (DIA) appeared to change its distribution along the PC axes, moving farther away from the other Oregon sites in the analysis of outlier loci (ORCA-out and CA-out; Figure 15). PCoAs were also mostly similar across both datasets between neutral and outlier loci. The position of the Old Cow Meadows (COW) population shifted closer to Nelson Creek (NEL) and Screwdriver Creek (SCR) in both PCoAs (ORCA and CA datasets) using only outlier loci (Figure 16 and 18).

Discussion

Cascades frogs are declining and geographically isolated at the southern edge of their range in the California Cascades. My findings indicate that genetic diversity is lower at the southern edge than in central populations in Oregon. Cascades frogs in California are also split into two, moderately differentiated groups with differing genetic ancestries. These groups do not correspond to ecoregion as previously thought, but possibly reflect a combination of historical processes, genetic resistance across the Pit River, and genetic drift due to increased isolation. Comparisons between presumably neutral and outlier loci were broadly similar, likely indicating lack of power to detect signals of directional selection with the limited number of loci. These findings have implications for impending management actions in the near future.

Regional Population Genetic Structure

An overall strong pattern of IBD was detected across the study area. This pattern supports previous identification of IBD for *R. cascadae* in Oregon and Washington (Monsen and Blouin 2004) and meets expectations for amphibian populations in general. Despite strong IBD, three to four clusters were identified at the highest level of spatial genetic structure for Cascades frogs across the study area. *Structure* and *BAPS* identified genetic structure that was largely concordant, designating an Oregon cluster and a South Lassen cluster. *BAPS* further divided the northern California cluster between the Klamath Mountains and North Lassen. This distinction was also supported in *Structure*, but in the subsequent hierarchical analysis. Ordination methods also confirmed these patterns of genetic structure, more closely aligning to the results from BAPS. DAPC however distributed individuals from several populations into individual clusters, likely due to overestimated K.

Cascades frogs displayed moderate differentiation across the study area. Differentiation was greatest between California and Oregon and low within Oregon. Previous estimates of differentiation between Cascades frog populations from Oregon and Washington were higher across a slightly larger spatial scale using six microsatellites (Monsen and Blouin 2004), though directly comparing F_{ST} estimates across studies and marker types is generally not advisable. Monsen and Blouin (2004) also did not sample any California sites, so the ranges of differentiation are not directly comparable. A recent RADseq analysis of the closely related species *Rana boylii*, which overlap with Cascades frogs in portions of their range, estimated F_{ST} of 0.31 between two *R. boylii* clusters over a geographic area somewhat similar to my study (Northwest California/Oregon and Northeast California; McCartney-Melstad et al. 2018). Their differentiation estimate is generally in accordance with my pairwise comparison for *R. cascadae* (F_{ST} = 0.29) between the California South Group and Oregon and between the California Northern Group and Oregon (F_{ST} = 0.23). The differentiation of *R. boylii* clusters reported by McCartney-Melstad et al. (2018) that overlap my study area was the lowest in their study, and they consistently identified extreme differentiation between regions that exceeds what I found for *R. cascadae*. In addition to different study designs (e.g. SNP selection criteria), differences in life history between these two species limit direct comparison of F_{ST} estimates.

Rana boylii and *R. cascadae* are closely related species that partially co-occur in range but are ecologically distinct from each other. *Rana boylii* occur in or near rocky streams and rivers, whereas Cascades frogs are found in spring-fed wet meadows and sub-alpine lake habitats (Stebbins 2003). These ecological differences complicate cross-species comparisons of genetic

differentiation. River ecosystems are often structured in dendritic networks, forming tree-like branching patterns along an elevational gradient (Cambell-Grant et al. 2007). The uni-directional flow of water limits dispersal for riverine species between branches, in turn limiting gene flow, and contributes to the distinct spatial structuring of genetic diversity in these environments (Ronce 2007, Paz-Vinas et al. 2015). While some species like stream salamanders (Cambell Grant et al. 2010) can overcome these apparent limits by occasionally moving overland, *R. boylii* are restricted to movements within the stream network, generally between 2-50m from the watercourse and have not been recorded dispersing through upland habitats (Bourque 2008). This is in contrast to many lentic-breeding amphibians (Bartlett 2000, Pope and Matthews 2001), including Cascades frogs (Garwood 2009), that use upland corridors for dispersal, thereby expanding the potential for gene flow between lentic patches. Such fundamental differences in life history are likely central to the disparate estimates of genetic differentiation found between *R. cascadae* and *R. boylii*. These life history differences also change our expectations for responses to historical or contemporary environmental change. For example, the evolutionary consequences of historical stochastic influences like the Sacramento-San Joaquin River Delta, or contemporary processes like altered hydrologic flows due to in-stream damming, will undoubtedly affect the genetic structuring of *R. boylii*, whereas *R. cascadae* may not be affected at all.

At the regional scale, the low-moderate differentiation found in this study provides molecular support for field research that demonstrated Cascades frogs are capable of inter-basin movement, travelling over unsuitable habitats, and making movements of over 1 km (Garwood 2009). These findings together could indicate that Cascades frog populations are more connected than

previously thought at local and regional scales. Connectivity could be maintained at least when there are sufficient densities of nearby sites, such as in the central and eastern portions of the Klamath Mountains and even between some sites in the southern Lassen region. It is also possible that occupied sites remain undetected, helping to increase connectivity. Although thorough surveys of hundreds of suitable sites in California have been conducted (Fellers et al. 2008), the most recently discovered occupied sites have occurred on private lands, and at least half of the sites in the California Cascades region known to have Cascades frogs are on private land.

Oregon-California Boundary

Cascades frog populations sampled in Oregon formed a genetic cluster distinct from all California locations sampled. Both *Structure* and *BAPS* supported this separation (Figure 6 and 14). This finding lends support to previous work that identified 3.2% sequence divergence between a California population and several in Oregon using mitochondrial DNA sequences (Monsen and Blouin 2003). The divergence between Oregon and California populations is likely attributed to the large historical gap in distribution between the northern edge of the Klamath Mountains in California and the southern tip of the Cascades Range in Oregon (c.a. 80 km gap) where no historical or contemporary records of Cascades frogs occur. My data support Monsen and Blouin's (2003) hypothesis that populations of Cascades frogs that remained in the Klamath Mountains during Pleistocene glaciation (2.5mya-10kya) did not migrate north to reconnect with populations in Oregon following glacial retreat.

Patterns of diversification and diversity of the flora and fauna of the Pacific Northwest have been heavily influenced by the Pleistocene glaciation (Brunsfield et al. 2001, Shafer et al. 2011). During this period, ice-free regions provided a variety of organisms refuge from uninhabitable terrain (Hewitt 2000). These fragmented refugial populations were isolated, thereby limiting gene flow among refugia and resulting in genetic differentiation over time (Shafer et al. 2011). As glaciers retreated, refugial species expanded into newly available suitable habitats, primarily reflecting current distributions. There is strong evidence that the Klamath-Siskiyou Mountains were an important glacial refuge for a variety of plant (Soltis et al.1997) and amphibian species (Kuchta and Tan 2005, Steele and Storfer 2006, 2007, Nielson et al. 2006). Cascades frogs may have also found refuge in the Klamath Mountains during the Pleistocene and then expanded north, south, or east after glacial retreat. With the presence of a large and consistent gap in distribution in the areas immediately surrounding the Klamath Mountains, it is not possible to test any of these hypotheses.

This region in northern California and southern Oregon has also been identified as a biogeographic barrier for a large number of species; Remington denoted it as one of 13 suture zones in North America (Remington 1968) and Swenson and Howard (2005) validated its importance as a phylogeographic break. Several trends have emerged from studies focused on the historical biogeography and phylogeography of the Oregon-California border region. The main hydrologic feature in this region, the Klamath River, has been shown to be a barrier for a moth (*Greya politella*; Rich et al. 2008) and a flower (*Collinsia linearis*; Baldwin et al. 2011) with the Siskiyou Mountain salamander (*Plethodon stormi*; Mahoney et al. 2004) also split along each side of the river. While Cascades frogs are not distributed close to either side of the river, it

is possible the Klamath River has historically limited gene flow. Other herpetofauna also display genetic breaks or boundaries near the Oregon and California border (Bury and Pearl 1999, Janzen et al. 2001).

Genetic diversity and population demography

Genetic variation was significantly lower in California than in Oregon based on several measures of diversity. Cascades frogs in California have been declining for years whereas populations in the central part of the range in Oregon and Washington are assumed to be stable (K. Pope pers. comm.). This finding matches the expectation that small and isolated populations will experience a loss of genetic diversity (Wright 1931). Although both Monsen and Blouin (2004) and Phillipsen (2010) found moderate levels of genetic diversity in Cascades frogs in Oregon compared to other frog species, neither sampled California populations adequately to compare (i.e., zero and 1 California populations sampled in the two studies, respectively).

Estimates of effective population sizes (N_e) for Cascades frogs in the present study were only able to be calculated for two-thirds of the sites due to the presence of "infinite" estimates. Infinite estimates occur due to sampling error, either due to extreme population sizes (very large or very small) or insufficient information contained in the population sample (Waples and Do 2010). Generally, Oregon populations tended to have larger estimates of N_e than California. Phillipsen et al. (2010) compared N_e estimation techniques for several western ranid frogs, including Cascades frogs, and determined that Cascades frogs across Oregon and Washington had effective population sizes of less than 50. Most estimates in the present study made

biological sense for what would be reasonable at a given site based on knowledge of recent site demographics, census sizes, and physical size of the site.

Outlier detection and comparison with neutral loci

Overall, patterns across both ordination methods comparing neutral and outlier loci were concordant with genetic structure results using all loci, with Oregon, California Northern, and California Southern groups.. This suggests that either divergent selection is not a strong force across Cascades frog populations in my study area, or more likely, that a signal of selection was not accurately detected in the regions of the genome sampled or by the outlier methods used. Diamond Lake in Oregon (DIA) was the only site that showed evidence of adaptive divergence from the other Oregon sites. Diamond Lake had the highest levels of genetic diversity in the study, suggesting it is unlikely this pattern is due to genetic drift, although N_e was incalculable. Cascades frogs have a large genome size (~ 7800 mb, 26 chromosomes; Vinogradov 1998) which make detecting signatures of selection in a broad, but small fraction of the genome difficult. Additionally, the ORCA dataset contained less than 2,000 loci, and because F_{ST} is influenced by genome-wide and locus-specific effects, the ability to distinguish between the two is difficult without very large numbers of loci (Gaggiotti 2009). Population structure and demography also plays a large role in outlier detection, especially when populations have undergone severe bottlenecks (Schoville et al. 2012). Alternatively, considering that *BayeScan* identified significantly more low-F_{ST} outliers, this could indicate that purifying selection is a stronger force than directional selection in the populations studied. If directional selection is occurring in Cascades frog populations, more loci or a targeted enrichment approach would be needed to detect this signature, as it is not directly apparent in the data available in this study.

While the conservation of adaptive potential may be important for long-term population sustainability, short-term efforts of boosting population sizes are likely more important to population demography and persistence.

California

Population genetic structure and differentiation

Genetic structure was also largely characterized by IBD in California, but clearly separated into two main groups, California North and California South. Differentiation between populations within California was low-moderate despite extreme isolation in the California Southern group. Although direct comparisons between this study and others that have used different genetic markers (e.g., microsatellites) are not possible, the level of differentiation between populations in this study is generally similar to those found in *Rana luteventris*, a related Western ranid frog, in Idaho and Montana over a similar spatial scale (Funk et al. 2005).

Lassen Boundary

Despite IBD, MRDM analysis supported the existence of a genetic boundary between the Klamath/North Lassen Region and the Southern Lassen Region, creating higher amounts of genetic differentiation across these regions than what would be expected based on geographic distance. This boundary occurs between the Pit River and Lassen Peak, separating Screwdriver and Nelson Creeks in the Northern Lassen Region from Carter, Childs, and Round Valley meadows in the Southern Lassen Region. Previous electrophoretic analysis indicated divergence

between Cascades frogs in the California Cascades and those in the Klamath Mountains (Case 1978). These results, along with the differences in site characteristics and physiography between the two mountain ranges implied a boundary along the Klamath and Cascades Level III Ecoregion border, across the Sacramento River Valley. The assignment of individuals from Screwdriver and Nelson Creeks to the northern cluster is therefore somewhat surprising, as they occur firmly in the Cascades Mountains Level III Ecoregion, and have been treated as such by researchers (Pope et al. 2014). Looking to other taxa with similar dispersal capabilities in the Lassen region helps to shed some light on our unexpected findings for Cascades frogs.

The *Ensatina* salamander complex (*Ensatina escholtzii*) and rubber boas (*Charina bottae*) both show north-south patterns of divergence in areas of northern California similar to, but not identical to, Cascades frogs found here (Jackman and Wake 1994, Rodriguez-Robles et. al 2001). *Ensatina escholtzii platensis* and *E.e. oregonensis* have a contact zone c.a. 20 km west of Lassen Peak where intergrades are found between *E. e. platensis* in the south and east, and *E. e. oregonensis* in the north (Jackman and Wake 1994). Rubber boas also have a purported boundary in Lassen Volcanic National Park, dividing two northern sub-clades between the Sierra Nevada Mountains to the south and northwestern California to the north (Rodriguez-Robles et al. 2001). The divergence patterns observed for these species are likely influenced by Pleistocene glaciation and volcanism (Rodriguez-Robles et al. 2001) which may have affected Cascades frogs as well.

Ice sheets during the glaciation reached lower elevations down to 1500 m and perennial snow lines were 2000 m lower than present (Kane 1982, Rogers et al. 1991) and could have prevented Cascades frogs and other amphibians from dispersing even at low elevations. If Cascades frogs were impacted by glaciers in the Lassen Region, they could potentially have found refuge to the northwest in the ice-free Klamath Mountains, which were only glaciated at the highest elevations during the Pleistocene (Davis 1988). Or frogs in the Lassen Region could have existed in known unglaciated regions between Mt. Shasta and Lassen Peak and south of Lassen Peak (Gillespie and Clark 2011). Eruptions of Lassen Peak may also have repeatedly influenced extinction and re-colonization dynamics of Cascades frogs directly through extirpation and indirectly by creating patches of unsuitable habitat that affect dispersal and gene flow. Direct extirpation from volcanic events is an unlikely explanation, because Lassen Peak is not predicted to erupt for another 6,900 years (Clynne and Muffler 2010) and at that scale, any stochastic impacts from volcanic activity in the region are likely due to decreased connectivity in destroyed or altered habitats. Cascades frogs in Washington took seven years to colonize new habitats only 3.7 km from source populations that survived the 1980 Mount St. Helens eruption (Crisafulli et al. 2005), suggesting that volcanic activity can result in profoundly reduced connectivity for Cascades frogs.

My data indicates some uncertainty as to the permeability and placement of the boundary between the two genetic groups in California. Old Cow Meadows, which is practically equidistant between its nearest southern site (Childs Meadow, 40 km) and nearest northern site (Screwdriver Creek, 42km), is admixed according to the *Structure* analysis (Figure 6). Individuals sampled at Old Cow Meadows on average had 66% of their genome assigned to the

northern cluster and 34% assigned to the southern cluster (Figure 9). This apparent admixture could be due to hybridizing of both northern and southern individuals at the site. Alternatively, a signature of admixture could be generated by genetic drift. Old Cow Meadows could have originated from one group, and through isolation and small population size, effects of genetic drift caused the site to more closely resemble the other group (a pattern of alike-in-state, not identical-by-descent).

Although possible, it is unlikely that Old Cow Meadows represents ongoing admixture of Cascades frogs from both genetic groups. A strikingly similar pattern of admixture in this region was described in California Spotted Owls and Northern Spotted Owls (Figure 1. Barrowclough et al. 2011). In Spotted Owls, the Pit River Valley was identified as a narrow hybrid zone connecting two distinct lineages (Shasta and Lassen; Barrowclough et al. 2011, Funk et al. 2008). Cascades frogs can disperse up to over 1 km when moving between patches of suitable habitat (Garwood 2009), but Spotted Owls can undoubtedly disperse much farther. With less dispersal, there would be less gene flow between patches of suitable habitat for Cascades frogs in contrast to Spotted Owls. There are no historical records of Cascades frogs occurring between Old Cow Meadows and Screwdriver Creek however, creating a long-standing c.a. 30 km gap in distribution. This may be for good reason, as the intervening habitat has low predictability for occupancy according to a recent Maxent model developed by US Forest Service biologists (G. Hodgson unpubl. data 2018). The presence of a major river and unsuitable habitat presumably prohibits any contemporary gene flow south from Screwdriver Creek to Old Cow Meadows. Alternatively, considering 66% of individuals' genomes assigned to the northern group on average, one could argue it is more likely that frogs from southern Lassen migrated northward

and integrated into an Old Cow Meadows population with historically northern ancestry. This northern ancestry would have likely been attained at a time prior to the formation of the Pit River however and when suitable habitat existed for migrants from the ancestral northern group to expand south to the vicinity of Old Cow Meadows. If admixture is occurring at Old Cow Meadows, it is impossible to determine the direction of admixture with the data currently available in this study.

Old Cow Meadows could also have been connected historically to southern Lassen populations, but through decreased connectivity and population bottlenecks, allele frequencies drifted such that Old Cow Meadows now looks a bit more like Klamath populations, though not directly by descent. The nearest historical sites to Old Cow Meadows lie 14 – 16km south and southeast, indicating connectivity to the south was possible. Between 2008-2010 however, Old Cow Meadows had the highest prevalence of chytrid compared with other sites in the California Cascades and was in the midst of a decline as documented by repeated surveys and population models (Pope and Larson 2011). Extreme droughts in California followed in 2014 and 2015 prompting an analysis of drought-susceptible species by the California Department of Fish and Wildlife, which included Cascades frogs (CDFW 2016). Old Cow Meadows, as referenced by its name, is also actively grazed with cattle, which could negatively impact Cascades frog survival (Cole et al. 2016). Lastly, this meadow is located on private property owned by timber companies and is surrounded by a heterogeneous landscape of dozens of clear-cut forest patches. These stressors likely contributed to the observed demographic decline and potential a population bottleneck at Old Cow Meadows. Evidence of recent demographic declines and decreased connectivity to historical and contemporary sites in the south points towards genetic

drift as the primary cause of the admixed signal observed, in contrast to ongoing admixture at Old Cow Meadows.

Samples from Cutter Meadows, an occupied site 5 km to the west of Old Cow Meadows and the only other known site in the area, were collected for this study but were not sequenced. Comparing the genetic structure of these two sites, and others in and around this north-south boundary, could help resolve the question of admixture at Old Cow Meadows and potentially identify the most likely mechanism(s) of divergence.

Genetic diversity and population demography

Within California, widespread declines have occurred primarily in the Southern Lassen Region, with only a few die-off events documented in the Klamath Mountains (Piovia-Scott et al. 2011). Genetic diversity between these two regions within California did not appear to differ however, contrary to expectations. Phillipsen et al. (2010) estimated genetic diversity from one site in California, again, making it difficult to compare between studies. They used samples collected between 1997-1998 from Colby Creek (elev. 1496 m), which is located 12.6 km southwest and downslope of Carter Meadows (CAR) in the Southern Lassen Region sampled here. Genetic diversity measured at Colby Creek was higher than what was determined at any site in California in this study. Cascades frogs at Colby Creek were abundant in 1990 (Jennings and Hayes 1994), likely contributing to high diversity in samples from 1997, however severe declines at other sites in the region had already been documented (Fellers and Drost 1993). In the almost 30 years since Jennings and Hayes (1994) surveyed and 20 years since Phillipsen et al. (2010) collected

samples, the Colby Creek population has crashed significantly and presently contains only a single confirmed Cascades frog as of 2016 (Pope et al. unpubl. data) and is projected to have at most, six individuals (Pope and Larson 2013). Colby Creek also has the highest probability of extinction out of the three adjacent Southern Lassen sites sampled in the present study (Pope and Larson 2013). While the high estimates of diversity at Colby Creek may have been accurate at the time of collection 20 years ago, population instability in the region since, and innate differences in marker resolution between microsatellites and RAD tags (Hohenlohe et al. 2013) do not allow robust comparisons of diversity between studies.

Overall, there was no relationship between amounts of genetic diversity and elevation in populations of Cascades frogs in California (Figure 22). This finding is contrary to research documenting a negative relationship of genetic diversity as elevation increases for montane frogs (Funk et al. 2005) and salamanders (Giordano et al. 2007) in the western United States, but supports Phillipsen et al. (2010) who found no correlation between genetic diversity and elevation for Cascades frogs.. This relationship is thought to be due to decreased connectivity among high elevation sites and between low and high elevation sites (Giordano et al. 2007, Funk et al. 2005). Due to the innate distribution of occupied Cascades frog sites in the study area and the sampling scheme employed in this study, it is difficult to robustly test the validity of elevation as a mechanism influencing connectivity in this system. Low-and-high-elevation sites are not evenly distributed between the two regions and there are not enough low and high-elevation sites within each region, thereby creating too much noise for such an analysis. In addition, while the range of pair-wise distances between populations sampled is large (1.5-206.1 km), the distribution of elevations is not (1458-2181m; 723m difference), when compared to the

elevational distribution of Cascades frogs range-wide (230-2740m; 2510m difference; Pope et al. 2014). If I were to explicitly test whether higher elevation populations are less diverse than lower elevation populations, I would attempt to sample paired high-and-low-elevation sites within and between genetic clusters at a range of distances and elevations. This pattern may indeed by present in the Cascades frog system but the sampling scheme used in this study is not suited to test this hypothesis.

Estimates of effective population sizes (N_e) for Cascades frogs in California were defined for only eight of the twelve sites (CA). Most estimates in the present study were higher than found by Phillipsen et al. (2010) in Cascades frogs from Oregon and Washington, however current estimates generally made biological sense for what would be reasonable at a given site based on knowledge of recent site demographics, census sizes, and physical size of the site. Although some in the CA dataset, like RED and GUM, were clearly overestimated as having thousands of frogs, meaning the census size would be even larger. Single sample estimation of N_e is difficult however, especially in iteroparous species with overlapping generations (Waples et al. 2014). This becomes more difficult when sampling from small and declining (i.e., non-equilibrium) populations (Luikart et al. 2010) and estimation in RADseq studies with low coverage (Arnold et al. 2013, Nunziata and Weisrock 2017) that can increase accuracy but lower precision of N_e estimates (Waples et al. 2016).

Outlier detection and comparison with neutral loci

Within California, patterns of Cascades frog population structure between neutral and outlier loci were broadly similar overall, with Klamath and North Lassen sites grouping together and Southern Lassen sites grouping together. Again, this is indicative of either not capturing signals of selection in the genomic regions sequenced or outlier tests used, or that divergent selection does not play a large role in this system. Old Cow Meadows was the only site that appears to be closer to Nelson and Screwdriver Creeks when using only outlier loci compared to neutral. Old Cow Meadows has a low N_e and low genetic diversity however, which could be due to strong genetic drift, giving rise to a false signal of selection (Schoville et al. 2012). Overall, tests for outlier loci are difficult, especially when population structure is high and population demographics are drastically changing, leading to many false-positive loci (Narum and Hess 2011). The number of loci in the present study likely limit the ability to detect outlier loci and either additional anonymous loci or a set of targeted loci could be useful for further exploration of adaptive responses to environmental pressures in Cascades frogs.

Conservation Implications

Several conservation strategies should be altered or implemented given the findings of genetic structure and diversity in California Cascades frog populations in this study. Cascades frogs are currently managed in two groups that are divided by ecoregion (Klamath and Cascades) (Pope et al. 2014). This strategy should be altered to encompass Screwdriver Creek and Nelson Creek with populations in the Klamath Mountains together as the northern cluster. Populations south of Lassen Peak should continue to be managed as is. With the data available in this study, it may be best to manage Old Cow Meadows at a micro-regional scale (including nearest neighbor Cutter

Meadows) to prevent potential outbreeding depression from translocations from either the north or south group until the mechanism of apparent admixture can be confirmed.

Translocations of individuals to bolster other sites should be considered in a hierarchical decision-making fashion based on the results of this study. First, translocations should take place within the same genetic group (north or south) to preserve genetic ancestry and potentially adaptive alleles in each region. Then, translocations should be considered between nearest neighbors, as populations display a pattern of strong IBD. Finally, large census size populations should be selected as donors for translocations to maximize genetic diversity. This last condition is not necessarily a discrete choice as it will likely be rare when the largest population is also the closest population. Other factors to include in a cost-benefit analysis are site habitat characteristics, elevation, and temperature differences between potential donor sites and the receiving site.

Genetic diversity was low in both groups in California and no single site or region stood out as harboring more genetic diversity than another. Although not statistically significant, there was a trend observed of higher diversity estimates at sites that were less isolated from other known sites not sampled in this study than sites that were more isolated. This trend could also help guide decisions.

Lastly, reintroductions to Lassen Volcanic National Park should use sites to the south as donor populations if possible. With Old Cow Meadows showing a signal of apparent admixture

however, it is unclear whether historical sites occupied within the park also had this signature. For a more accurate reconstruction of historical genetic structure in the park, managers should pursue the genetic sampling of historical museum specimens collected from within the park. Genomic techniques have advanced enough to be able to attain high-resolution datasets from formalin-preserved museum samples (McCartney-Melstad et al. 2018, Ruane and Austin 2017) and could provide useful insight into the historical genetic identity of frogs near Lassen Peak.

Other strategies could also play a role in Cascades frog recovery across California. Captive breeding Cascades frogs could be a valuable opportunity to both learn more about the breeding biology of the species in a controlled environment and re-populate declining or extirpated sites. Captive breeding strategies can turn out to be detrimental to natural populations however (Araki 2007, 2009, MciGinnity et al. 2009) and should be well studied for Cascades frogs before being implemented. Additionally, managers could establish entirely new populations in suitable habitats near extant sites. This strategy has been successful for *Rana pretiosa* using several constructed ponds and moving 20 adults and nine egg masses to the site (Chelgren et al. 2008).

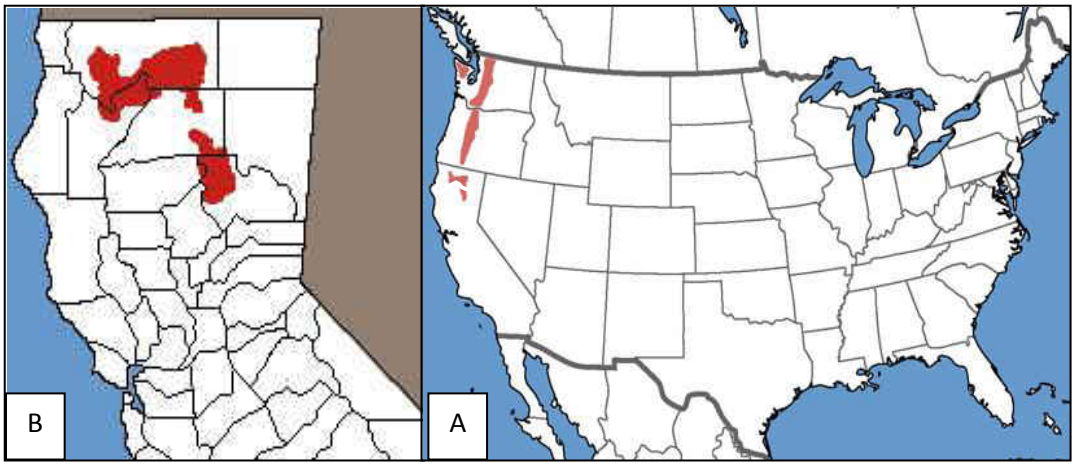

Figure 1. A). Historical range of Cascades frogs (*Rana cascadae*) within the United States. B). Historical range of Cascades frogs within California. Note the two disjunct regions. Figures edited from californiaherps.com.

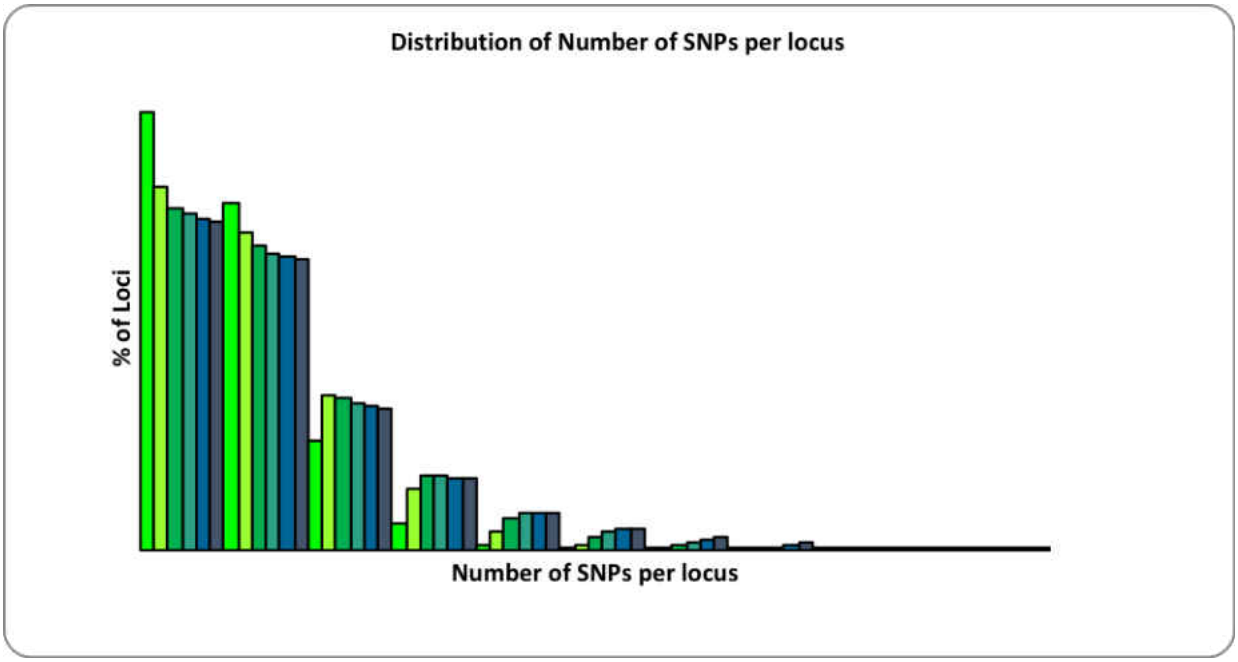

Figure 2. Histogram of the percentage of loci found to have (x) number of SNPs per locus for each ustacks M = n parameter value from 1-6 while holding m constant at 3 as recommended by Rochette and Catchen (2017) to optimize Stacks assembly parameter choice. Optimization is determined at the point of stabilization. M (n) = 4 and m = 3 was ultimately chosen as the optimal parameter combination.

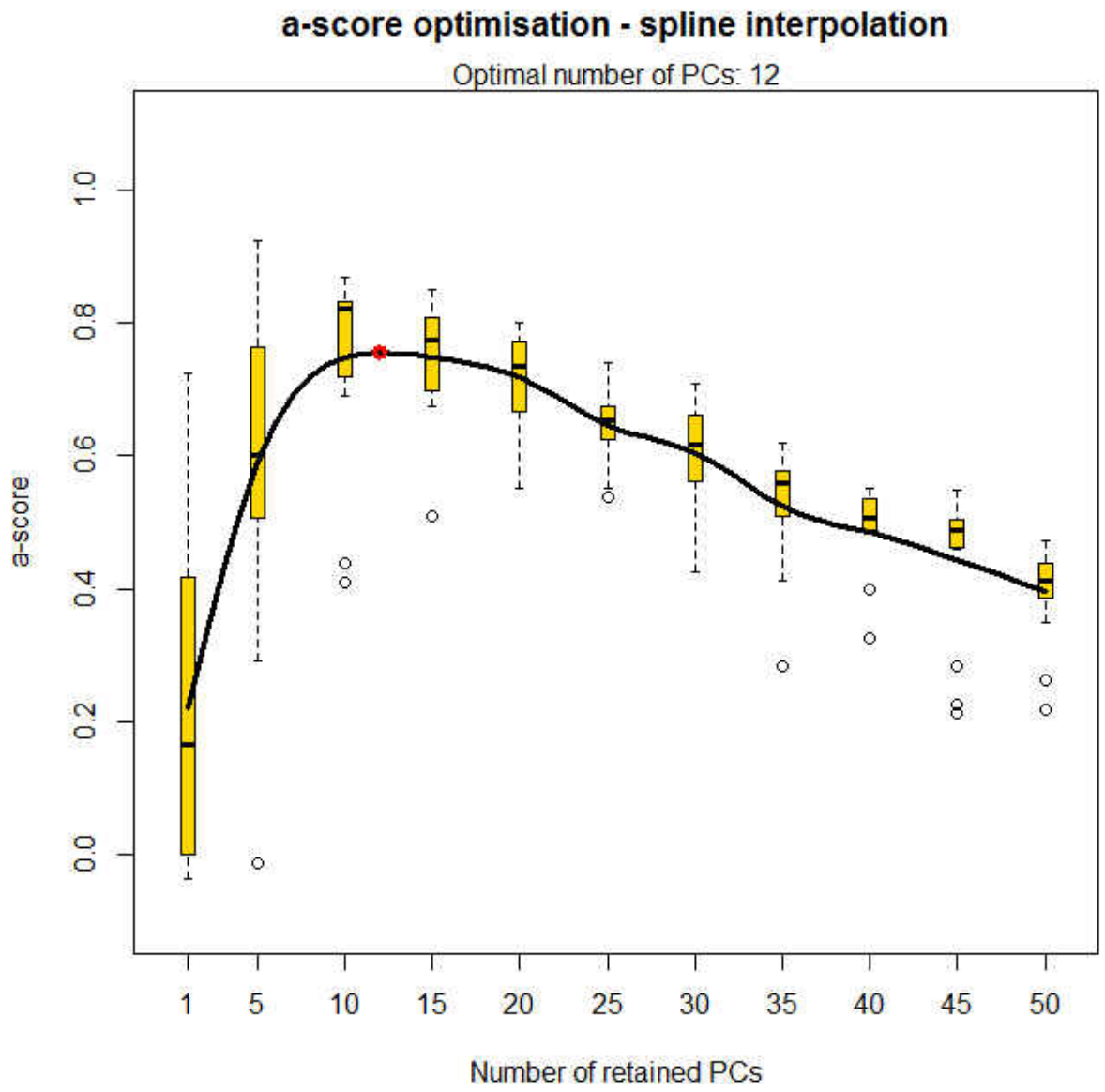

Figure 3. Example of a-score optimization using spline interpolation plot to determine the optimal number of principal components (PCs) retained for a Discriminant Analysis of Principal Components (DAPC) analysis. Here, the a-score is optimized at 12 principal components.

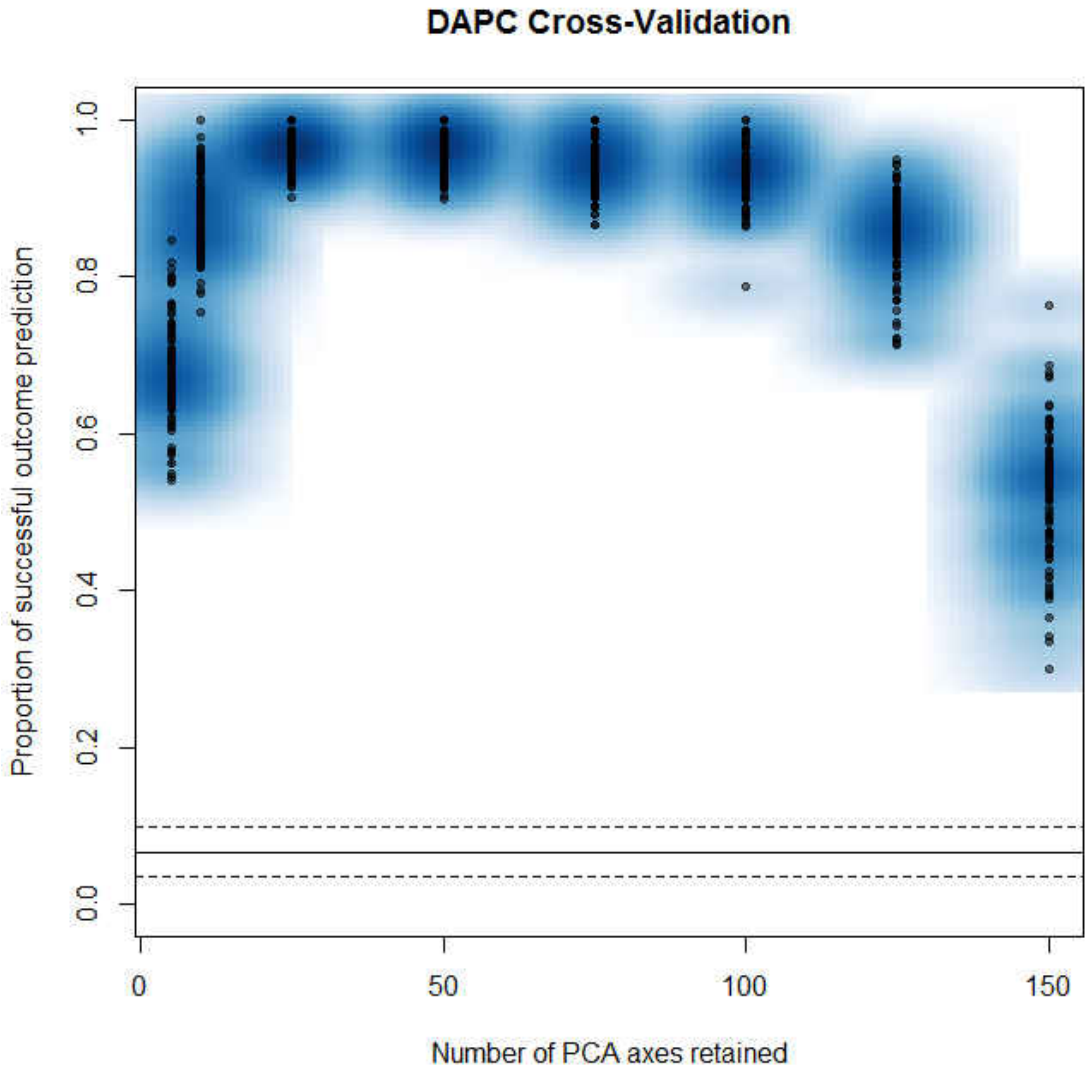

Figure 4. Example of a cross-validation plot to guide selection of the number of Principal Component axes (PC) to retain for a Discriminant Analysis of Principal Components Analysis. The PC value that maximizes the proportion of successful outcomes and minimizes the mean square error (MSE) is 25 in this example.

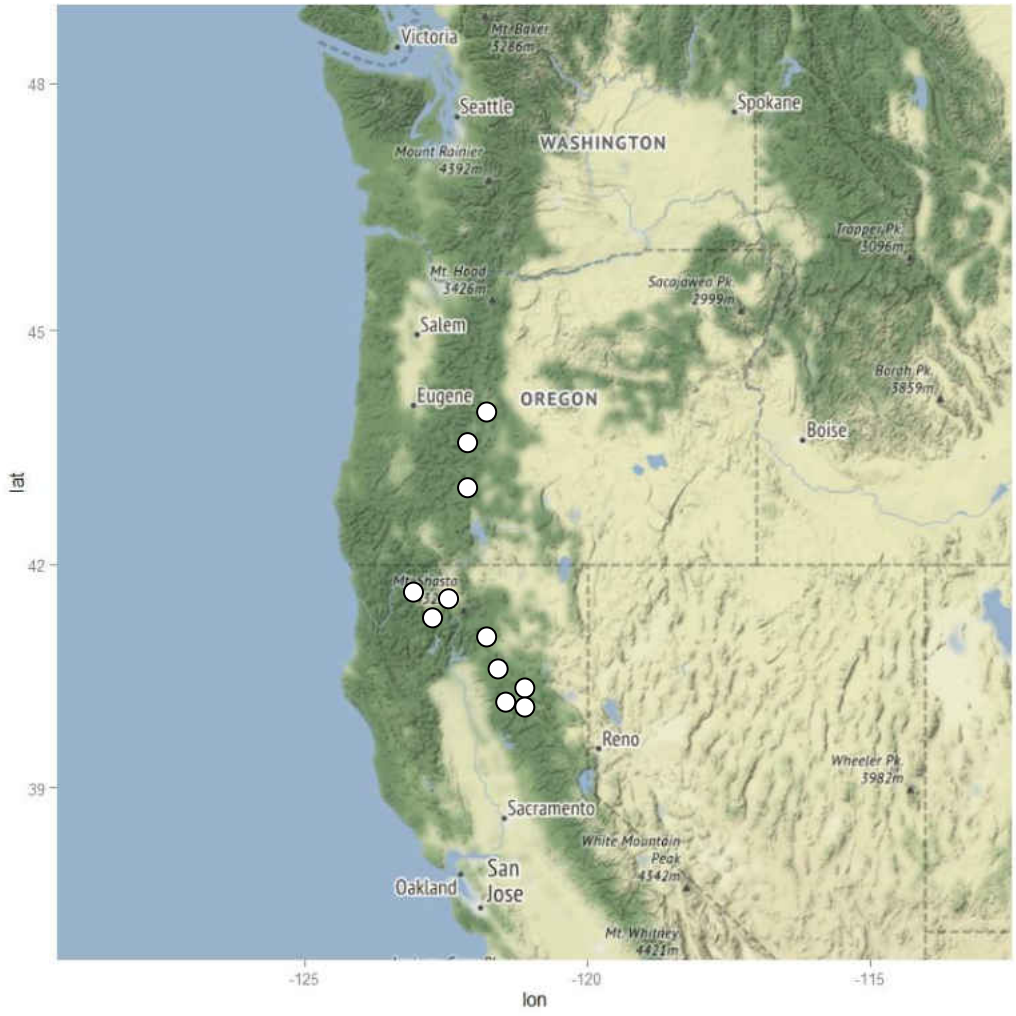

Figure 5. Locations of the 15 sites sampled for Cascades frogs used for analyses. Some points are overlapping.

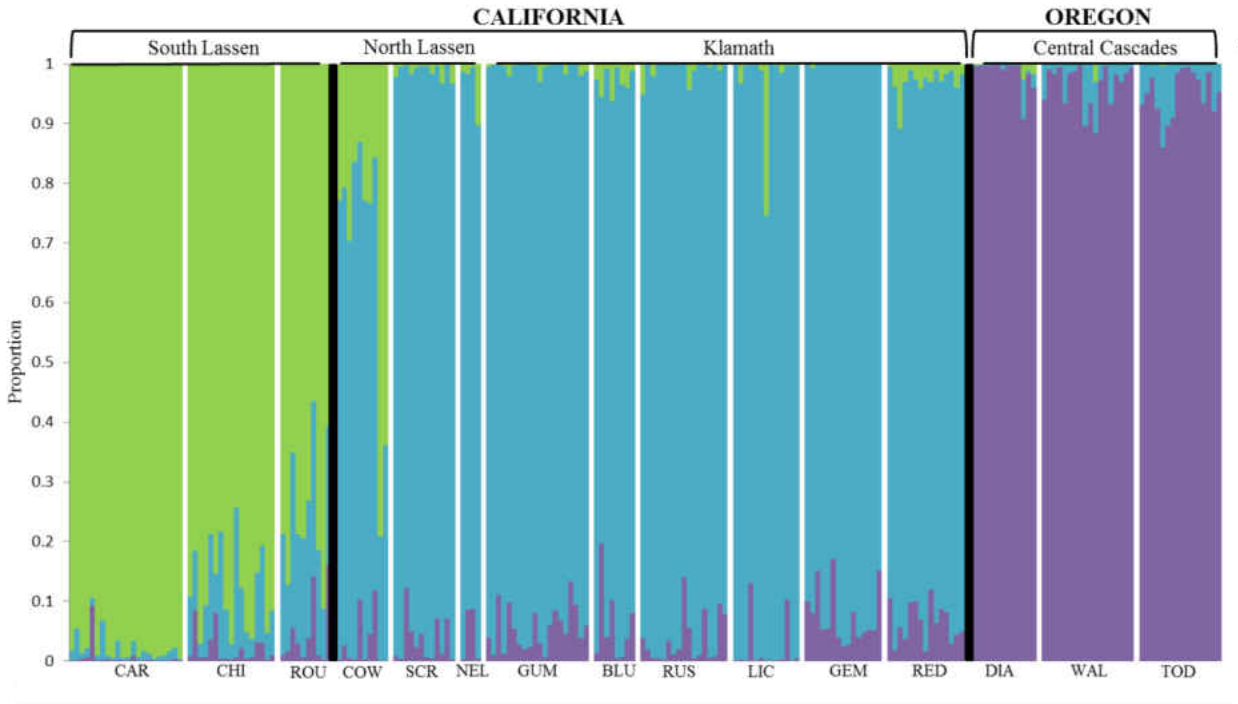

Figure 6. *Structure* histogram of K = 3 for Cascades frog populations throughout the study area (ORCA dataset). Individual bars represent a single individual. The proportional assignment of their genome to each genetic cluster is displayed in either green (southern), blue (northern) or purple (Oregon) ancestries. Site abbreviations are located at the bottom. White bars separate populations and black bars separate genetic clusters according to majority assignment.

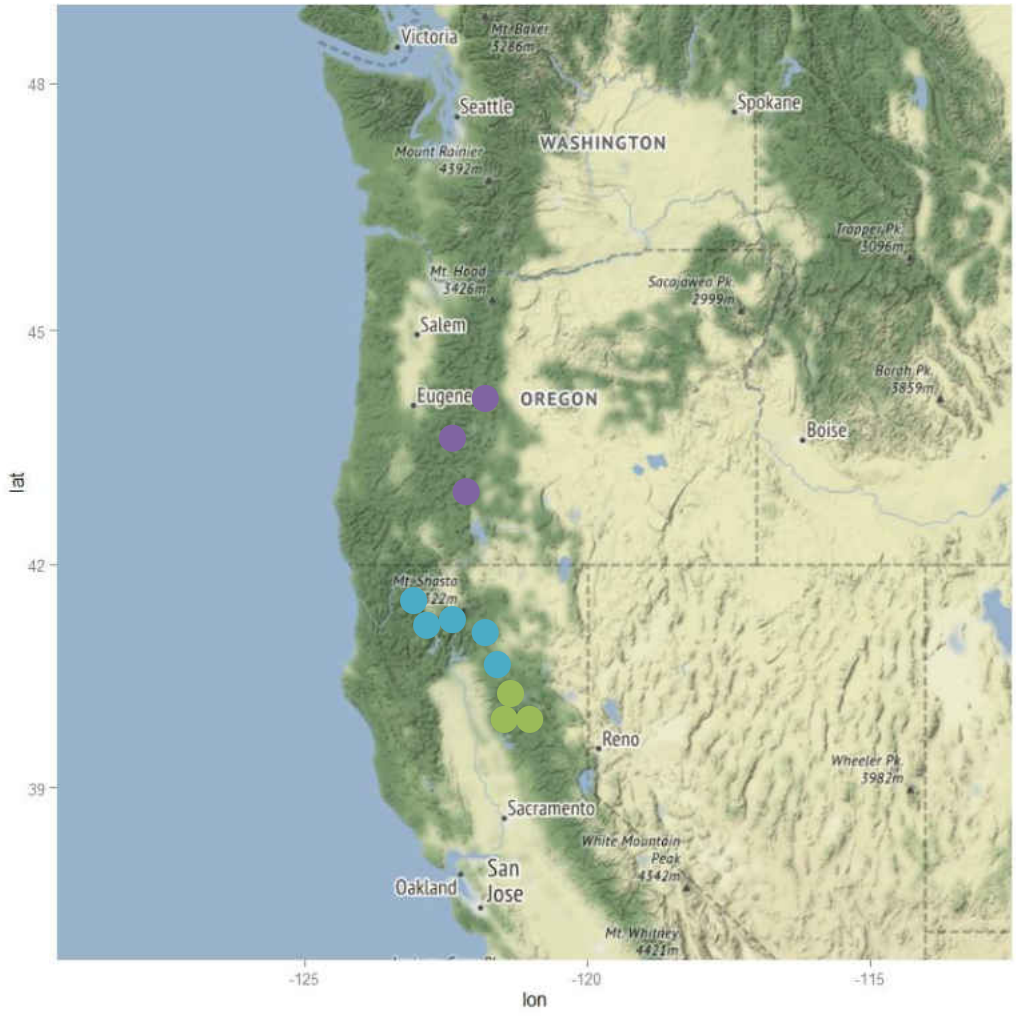

Figure 7. Locations of three main genetic clusters of Cascades frogs as identified by *Structure*.

Green = southern group; Blue = northern group; and Purple = Oregon group.

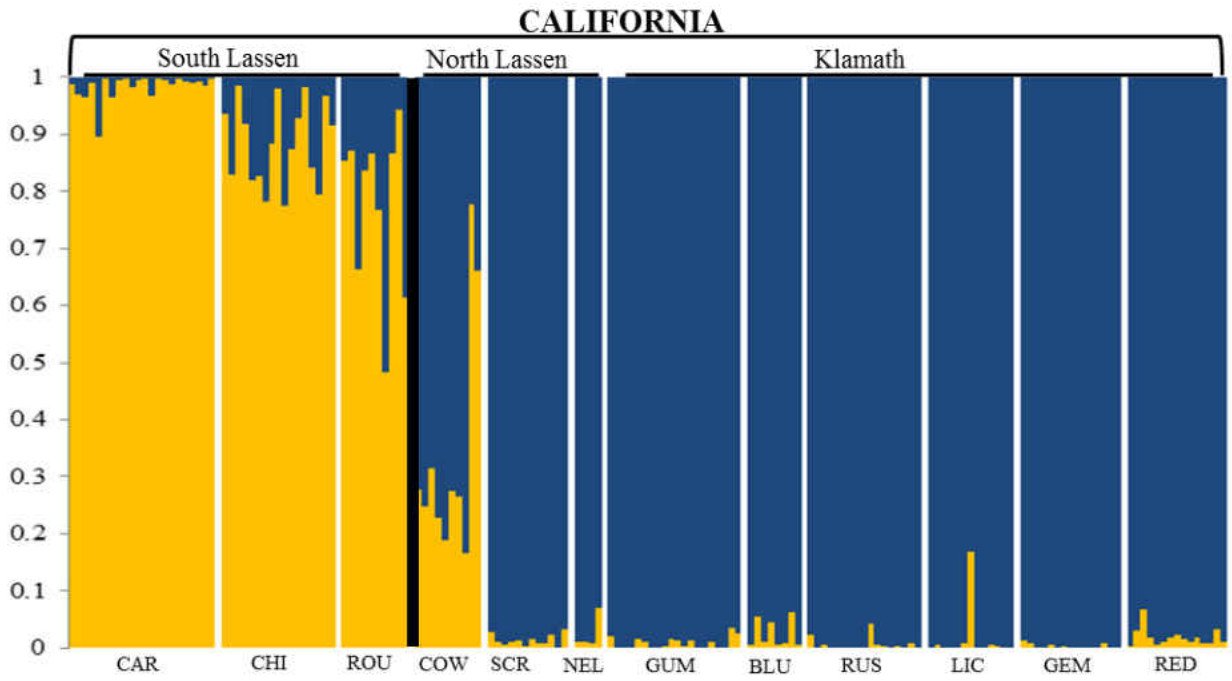

Figure 8. *Structure* histogram of K = 2 for Cascades frog populations in California (CA dataset). Individual bars represent a single individual. The proportional assignment of their genome to each genetic cluster is displayed in either gold (southern) or blue (northern) ancestries. Site abbreviations are located at the bottom. White bars separate populations and black bars separate genetic clusters according to majority assignment.

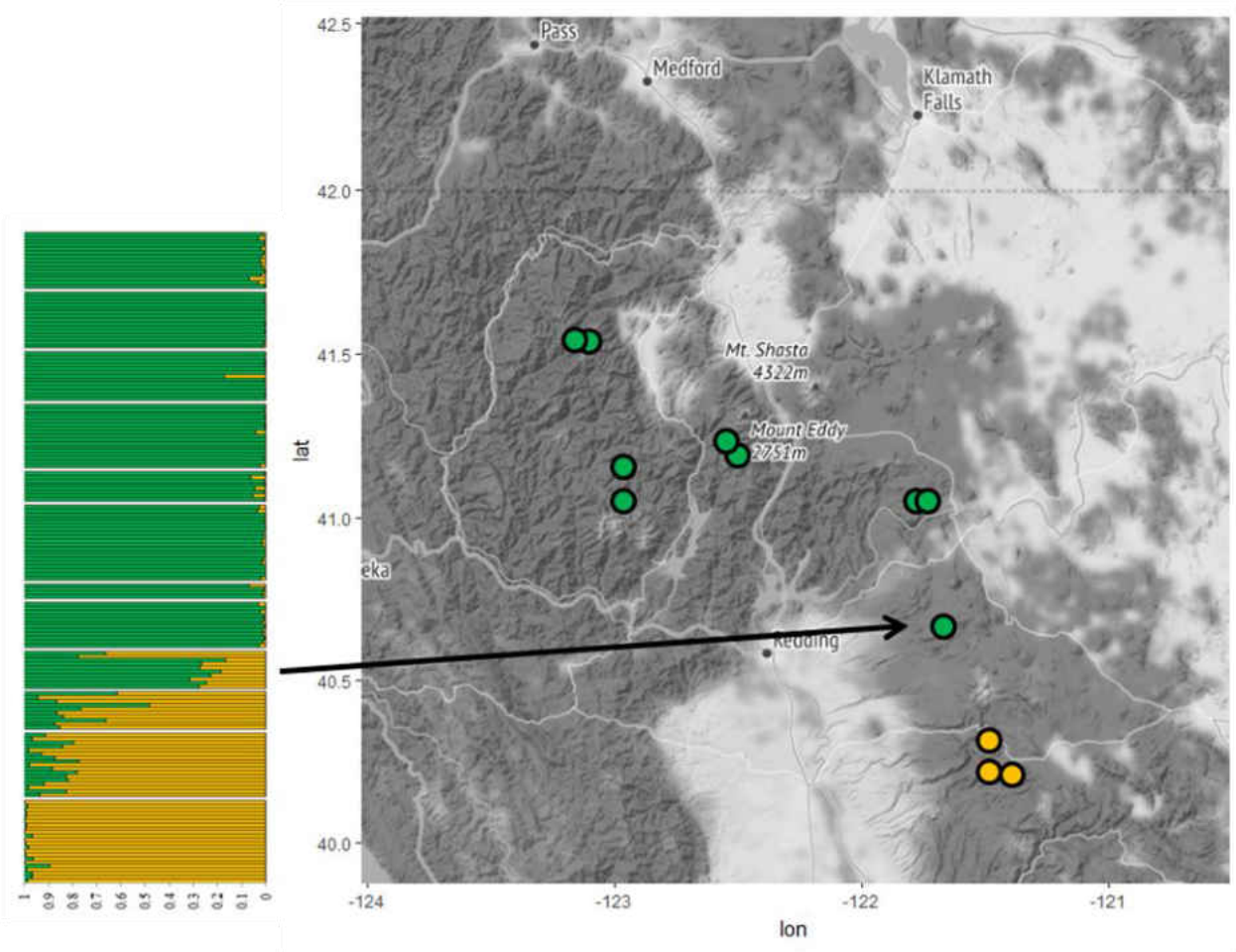

Figure 9. Locations of northern (Green) and southern (Gold) clusters in California Cascades frog populations (CA dataset). Admixture is shown in the Old Cow Meadows site indicated by the arrow.

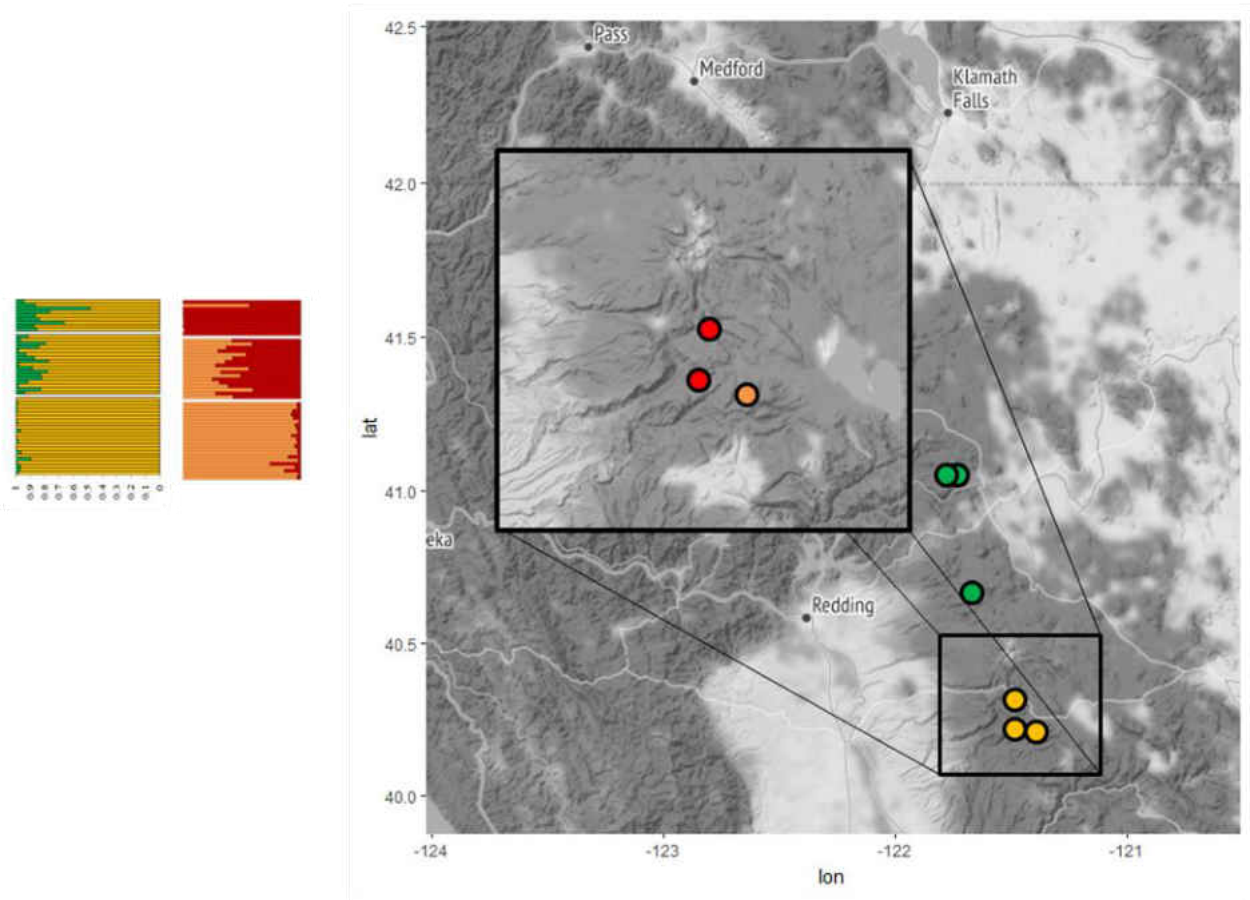

Figure 10. Pattern of hierarchical substructure according to *Structure* in the southern California Cascades frog populations (CA dataset). Gold points represent the California southern group and green points represent the California northern group. Substructure is inset in the southern group with Round Valley and Child's Meadows in red and Carter Meadows in orange.

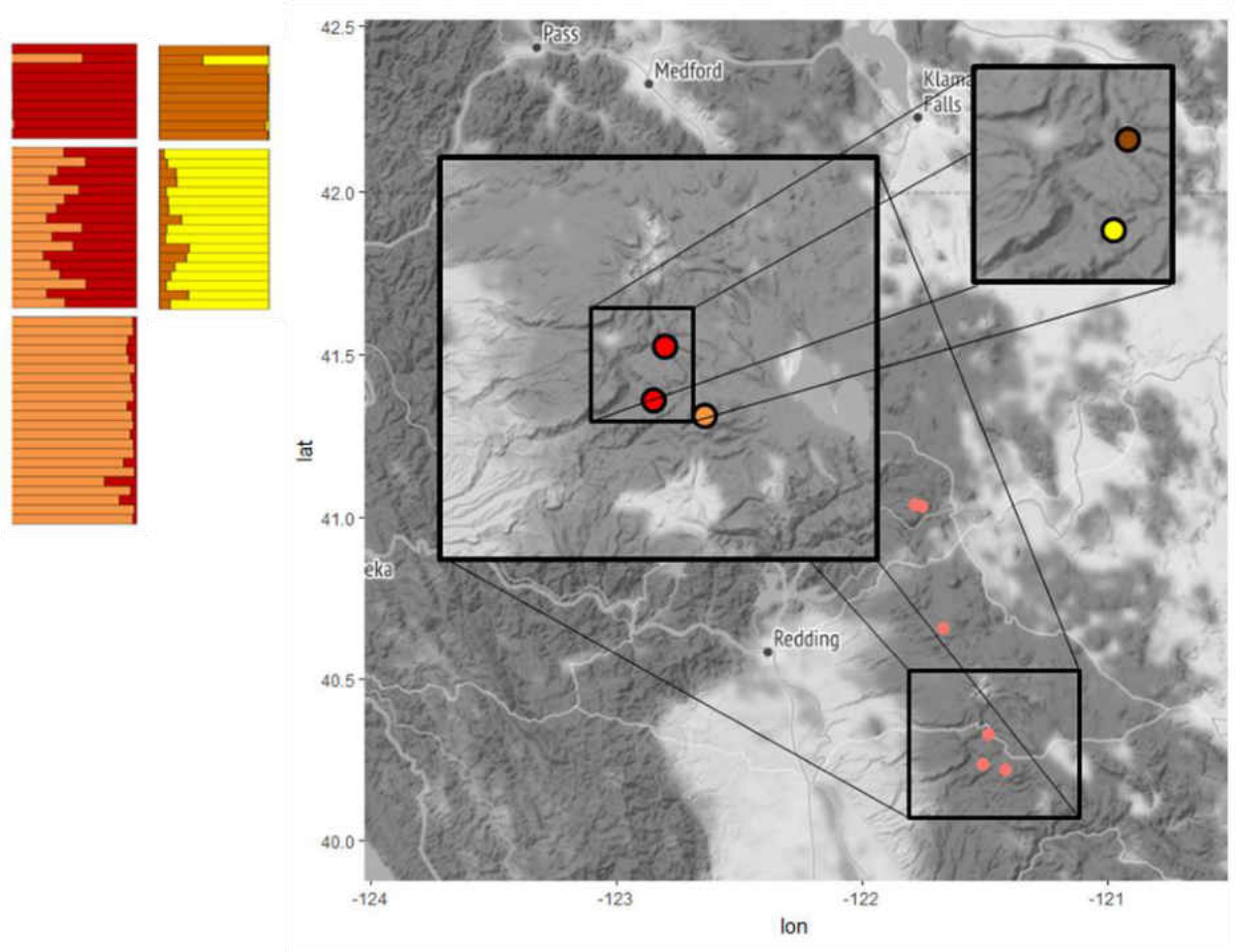

Figure 11. Pattern of hierarchical substructure according to *Structure* in the southern group of California Cascades frogs. Main inset contains Carter Meadow (orange) and Round Valley and Child's Meadows (red). Second inset contains both Round Valley (yellow) and Child's Meadow (brown).

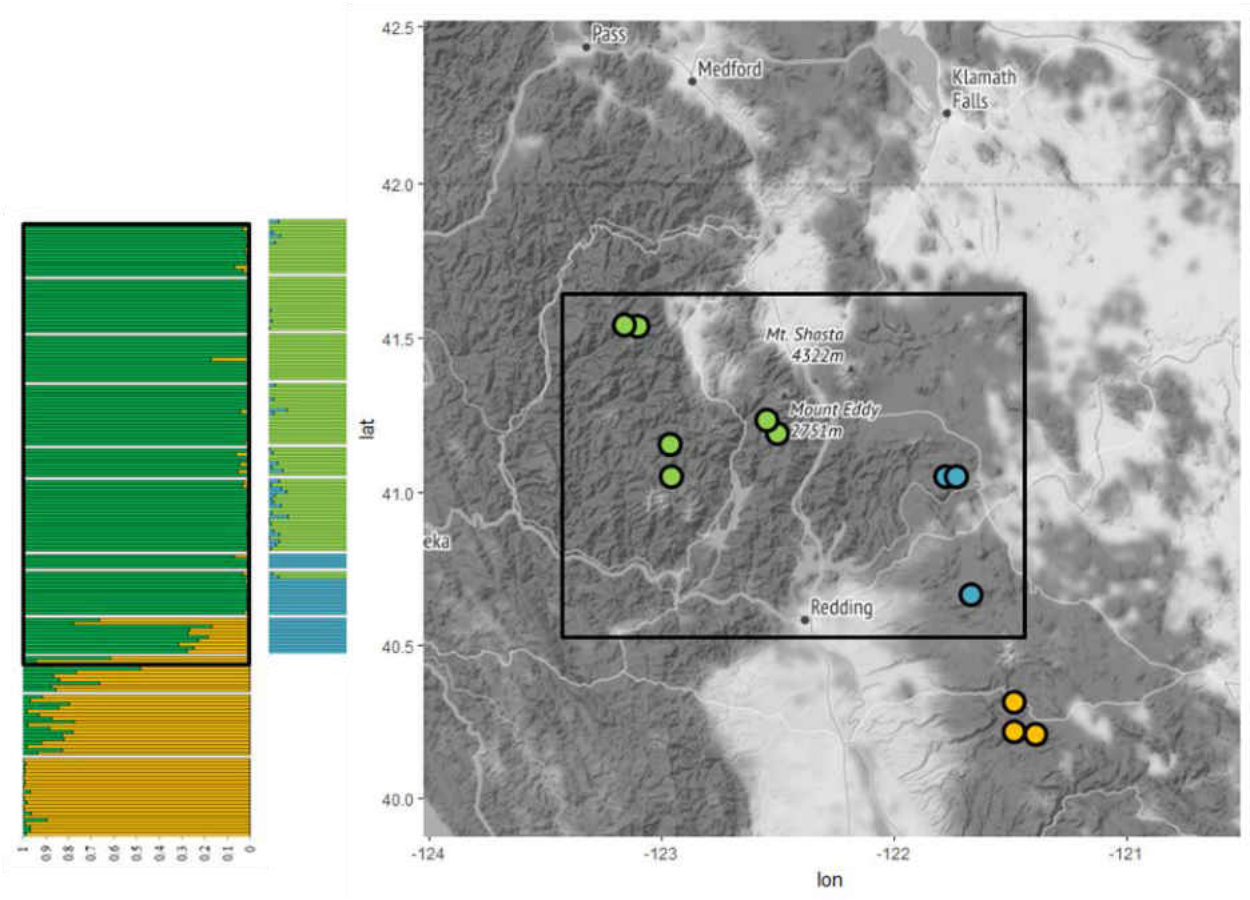

Figure 12. Hierarchical pattern of genetic substructure from *Structure* for Cascades frog populations in California (CA dataset) with focus on the California northern group. Gold indicates California southern group sites, blue represents the north Lassen portion of the northern group sites (dark green on histogram), and light green represents the Klamath sites within the northern group.

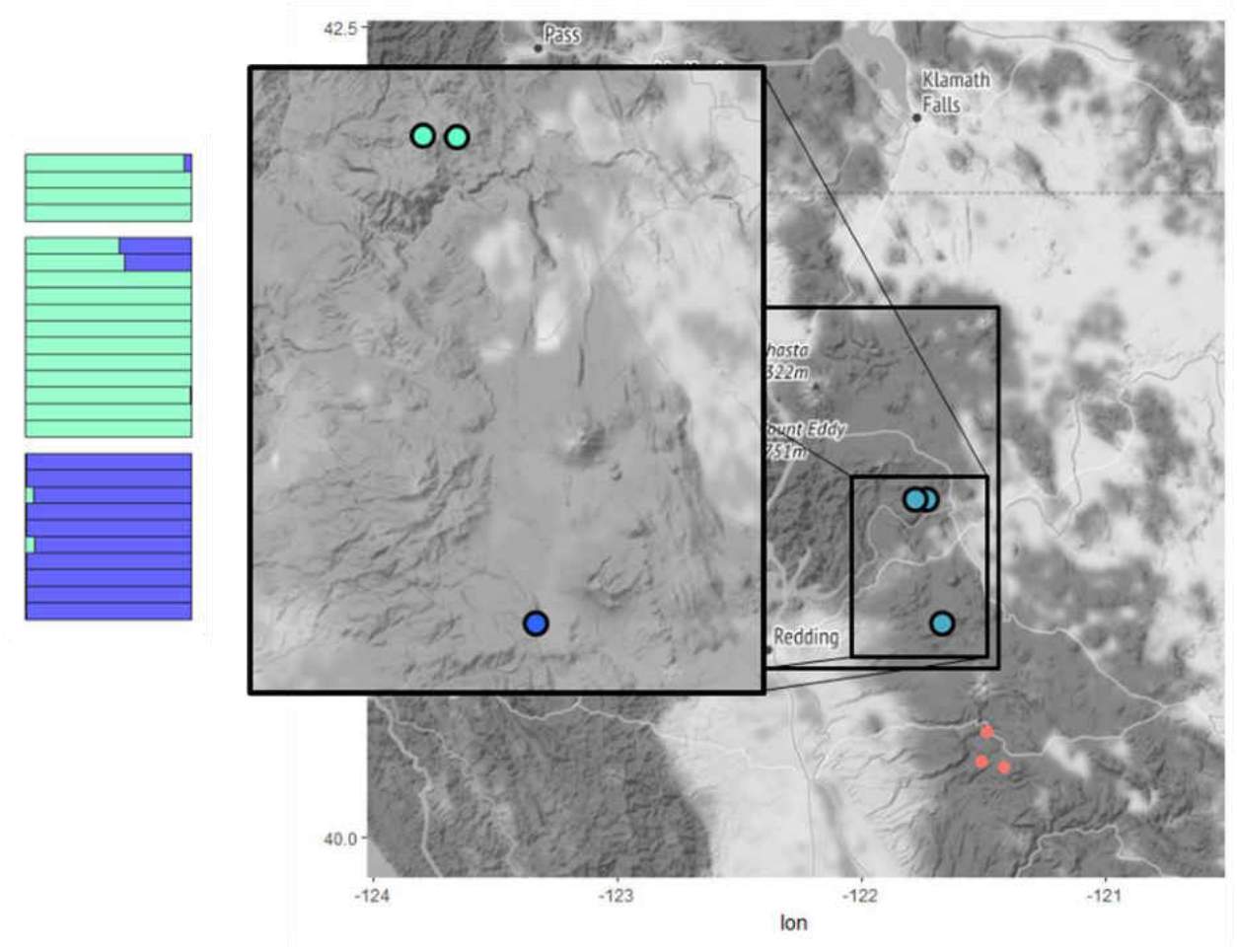

Figure 13. Locations of hierarchical substructure according to *Structure* in California Cascades frog populations in the north Lassen portion of the northern group (CA dataset). Blue represents the Lassen sites of the northern group. The inset shows substructure within the Lassen sites of the northern group with Old Cow Meadows (dark blue) to the south and Screwdriver and Nelson Creeks (bright teal) to the north.

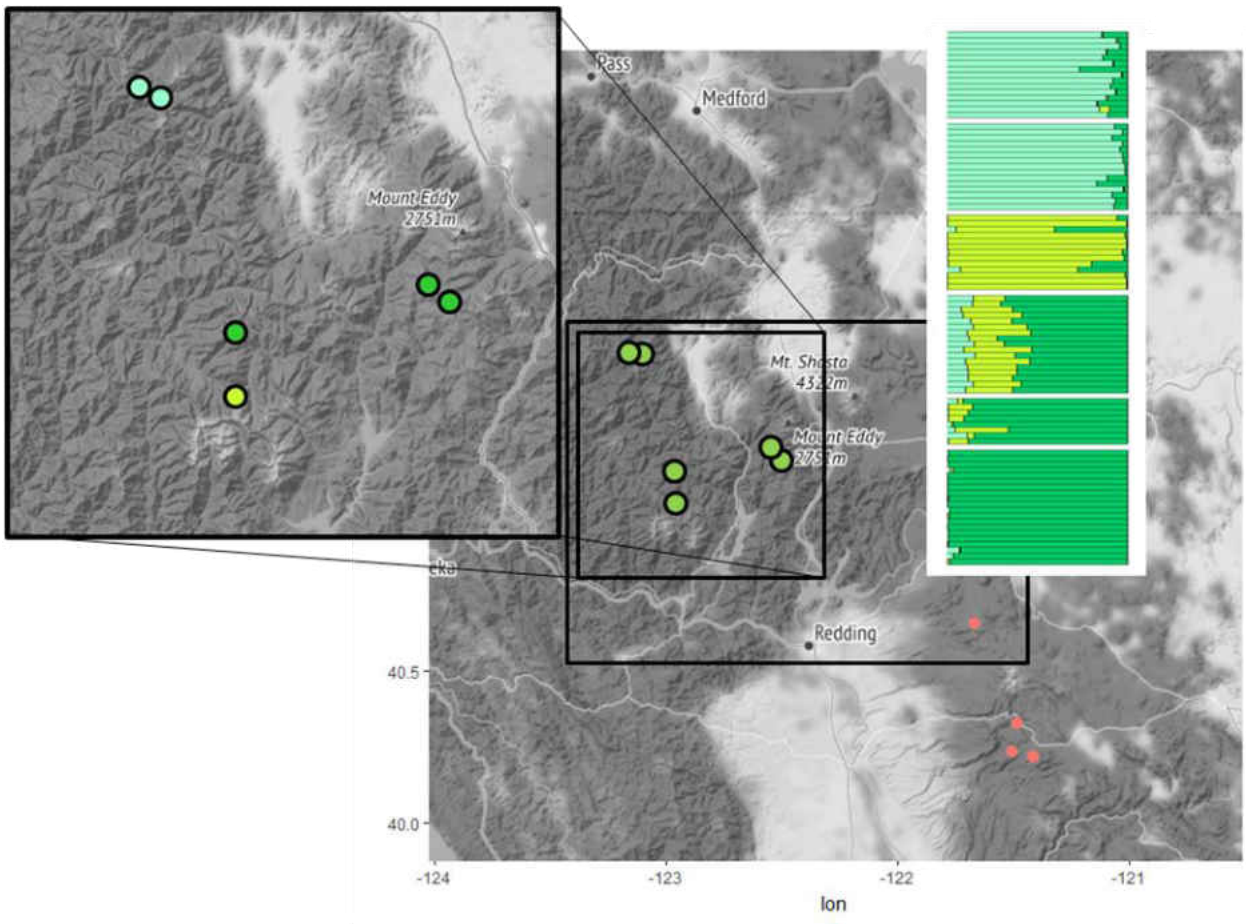

Figure 14. Locations of hierarchical substructure according to *Structure* in California Cascades frog populations in the Klamath portion of the northern group (main inset- green; CA dataset). In the second inset, light teal represents Red Rock Creek Lake and Gem Lake, yellow-green indicates Little Caribou Lake, and medium green represents Blue Divide Lake, Gumboot Lake, and Rush Creek Lake.

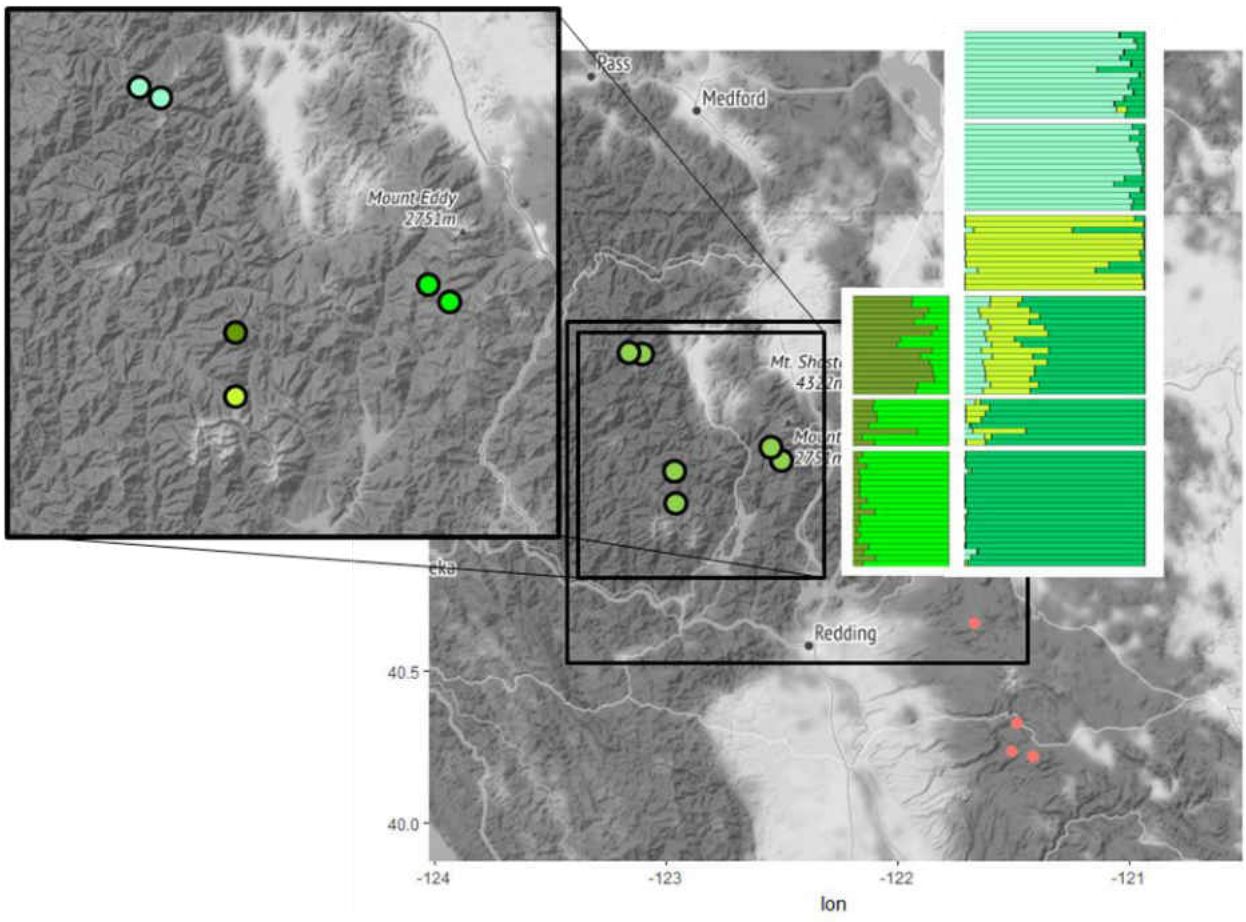

Figure 15. Locations of hierarchical substructure according to *Structure* in California Cascades frog populations in the Klamath portion of the northern group (main inset- green; CA dataset). In the second inset, light teal represents Red Rock Creek Lake and Gem Lake, yellow-green indicates Little Caribou Lake, lime green represents Blue Divide and Gumboot Lakes, and Rush Creek Lake is shown in dark olive green.

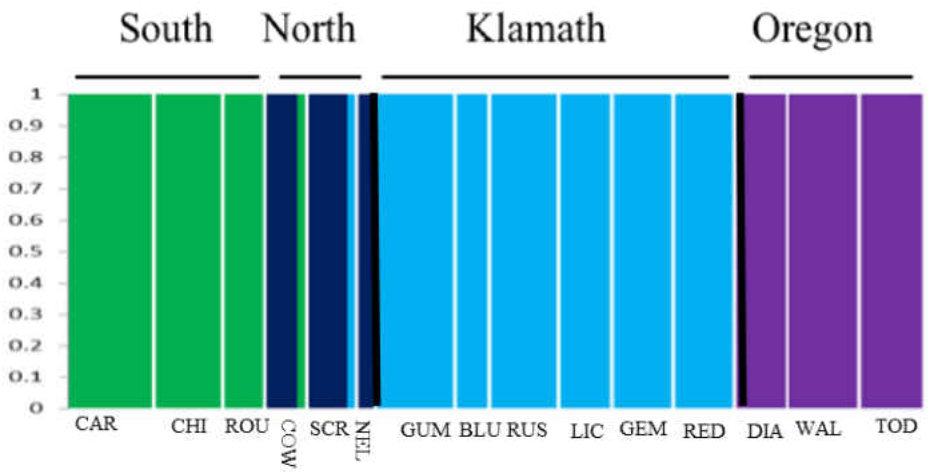

Figure 16. *BAPS* histogram of K = 4 for Cascades frog populations in California and Oregon (ORCA dataset). Individual bars represent a single individual. The proportional assignment of their genome to each genetic cluster is displayed in either green (South Lassen), dark blue (North Lassen), light blue (Klamath) or purple (Oregon) ancestries. Site abbreviations are located at the bottom. White bars separate populations and black bars separate genetic clusters according to majority assignment.

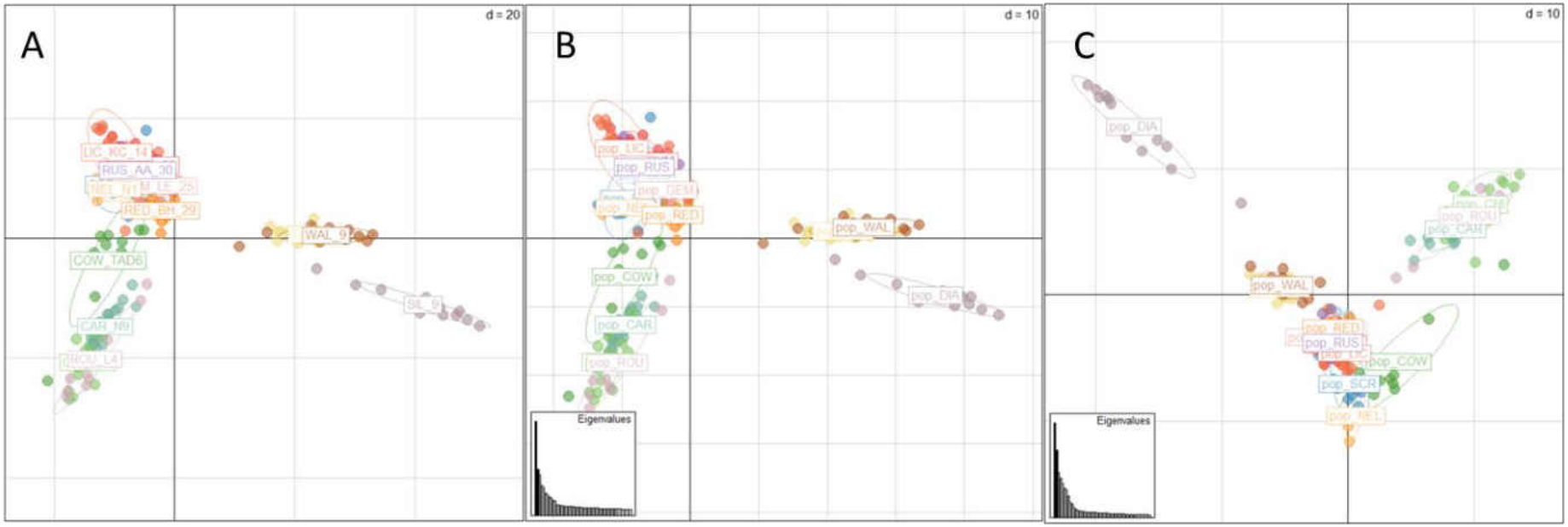

Figure 17. Principal Components Analysis (PCA) of all 15 sites from Oregon and California (ORCA dataset) using all loci (A), neutral loci (B), and outlier loci (C).

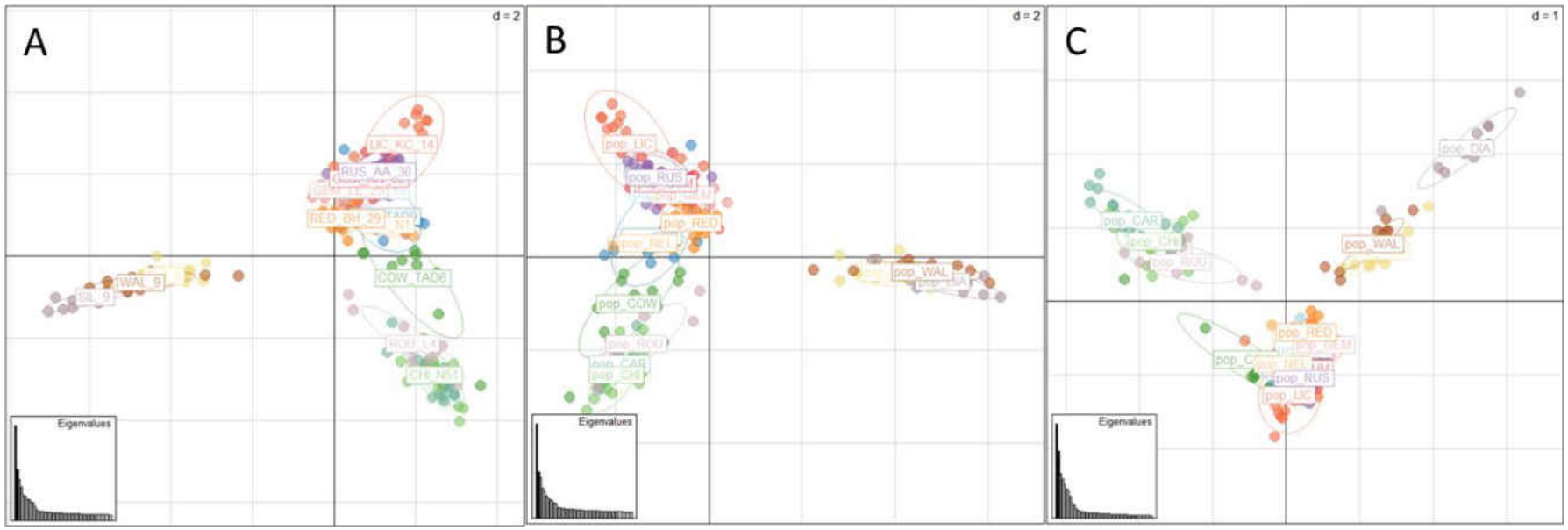

Figure 18. Principal Coordinates Analysis (PCoA) of all 15 sites in Oregon and California (ORCA dataset) using all loci (A), neutral loci (B), and outlier loci (C).

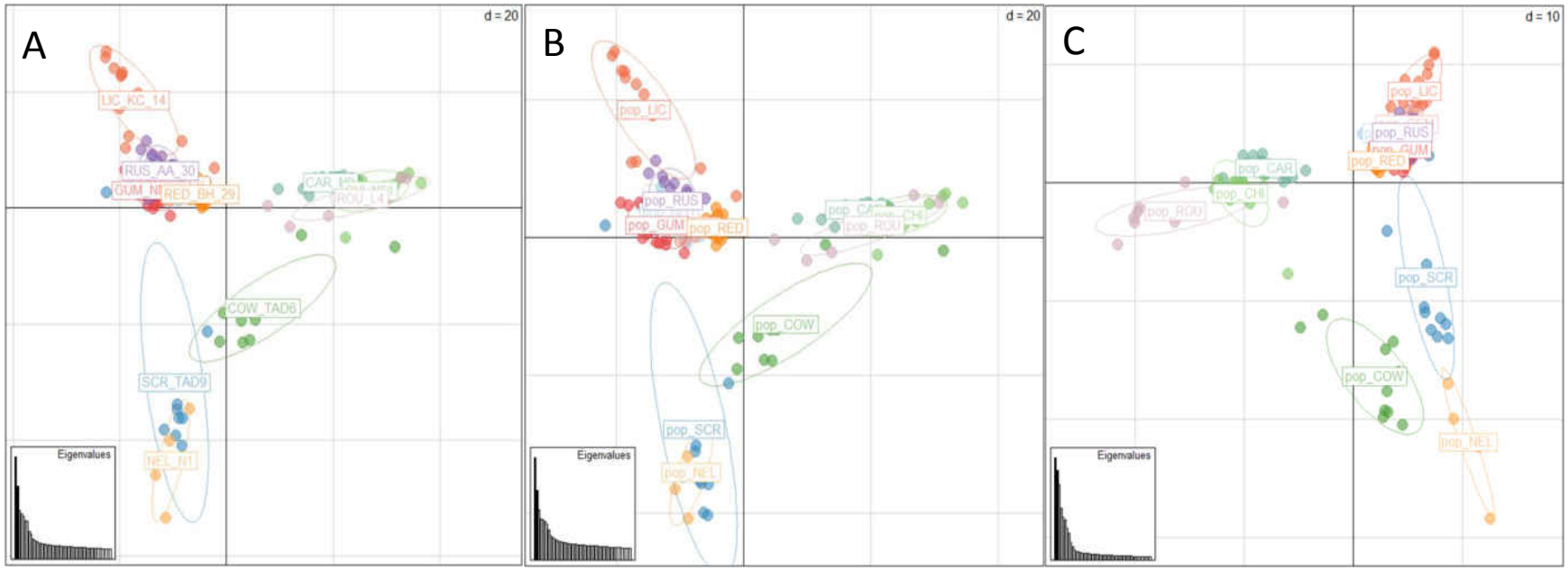

Figure 19. Principal Components Analysis (PCA) of all 12 sites from California (CA dataset) using all loci (A), neutral loci (B), and outlierloci (C).

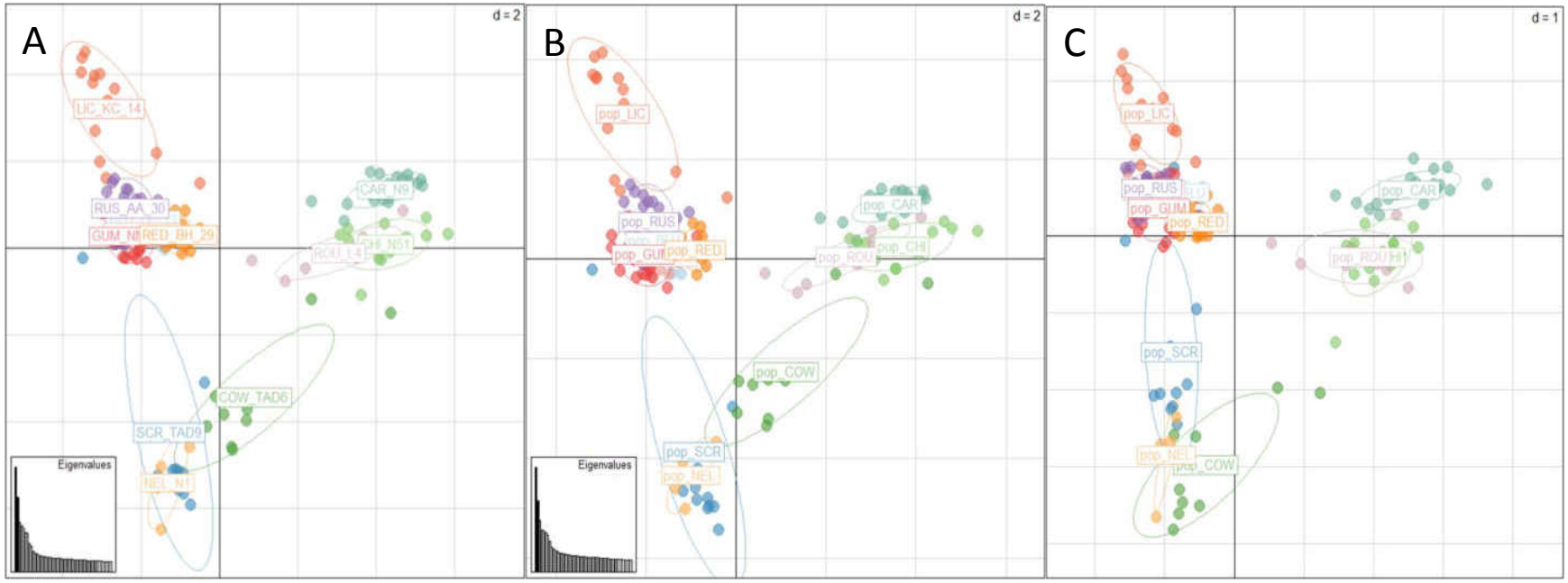

Figure 20. Principal Coordinates Analysis (PCoA) of all 12 sites in California (CA dataset) using all loci (A), neutral loci (B), and outlier loci (C).

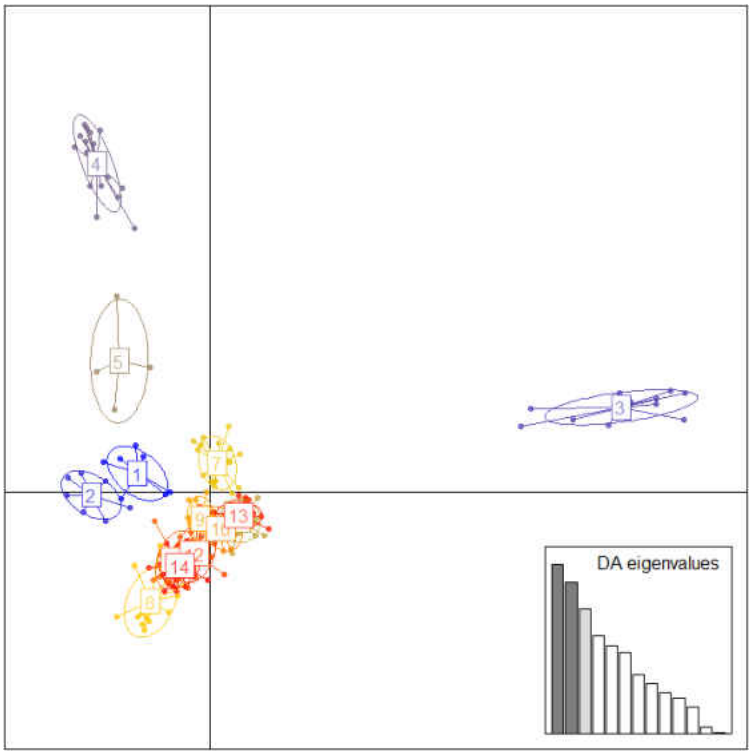

Figure 21. Discriminant Analysis of Principal Components (DAPC) for all 15 sites from Oregon and California (ORCA dataset). Twenty-five principal components (PCs) were retained using the cross-validation method. Fourteen clusters were chosen using K-means clustering approach and BIC. Three discriminant functions were chosen. Points represent individuals, colors and centroid numbers represent clusters. 95% inertia elipses are shown for each cluster.

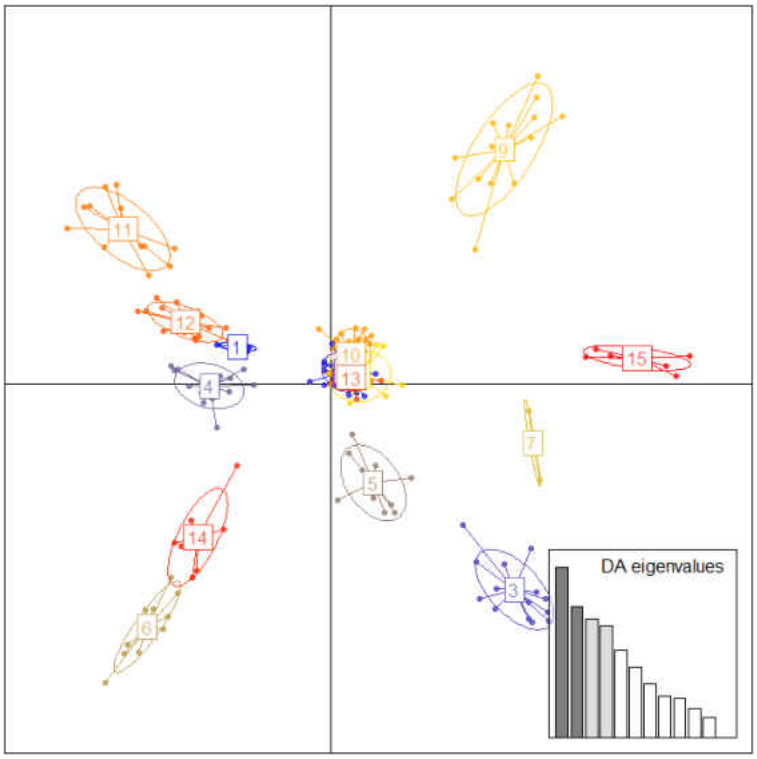

Figure 22. Discriminant Analysis of Principal Components (DAPC) for all 15 sites from Oregon and California (ORCA dataset). Twelve PCs were retained using the a-score and spline interpolation method. Fifteen clusters were chosen using K-means clustering approach and BIC. Four discriminant functions were chosen. Points represent individuals, colors and centroid numbers represent clusters. 95% inertia elipses are shown for each cluster.

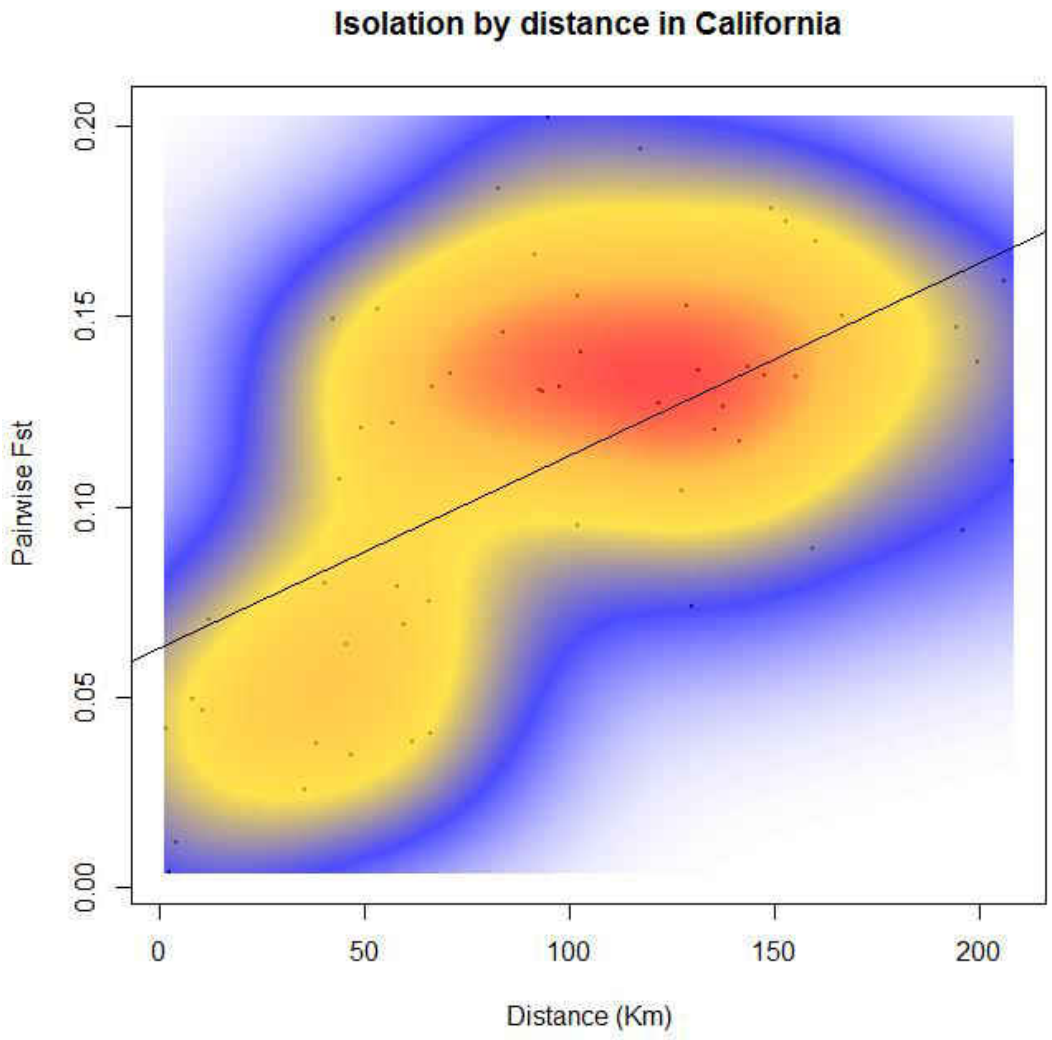

Figure 23. Isolation by Distance (IBD) density plot for Cascades frogs sites in California This plot shows the distribution of pairwise values between sampled sites (black dots) by geographic distance (in kilometers, km). Colors indicate density of points with warmer colors (red and yellow) indicating higher density of sites and cooler colors (blue and white) indicating low densities.

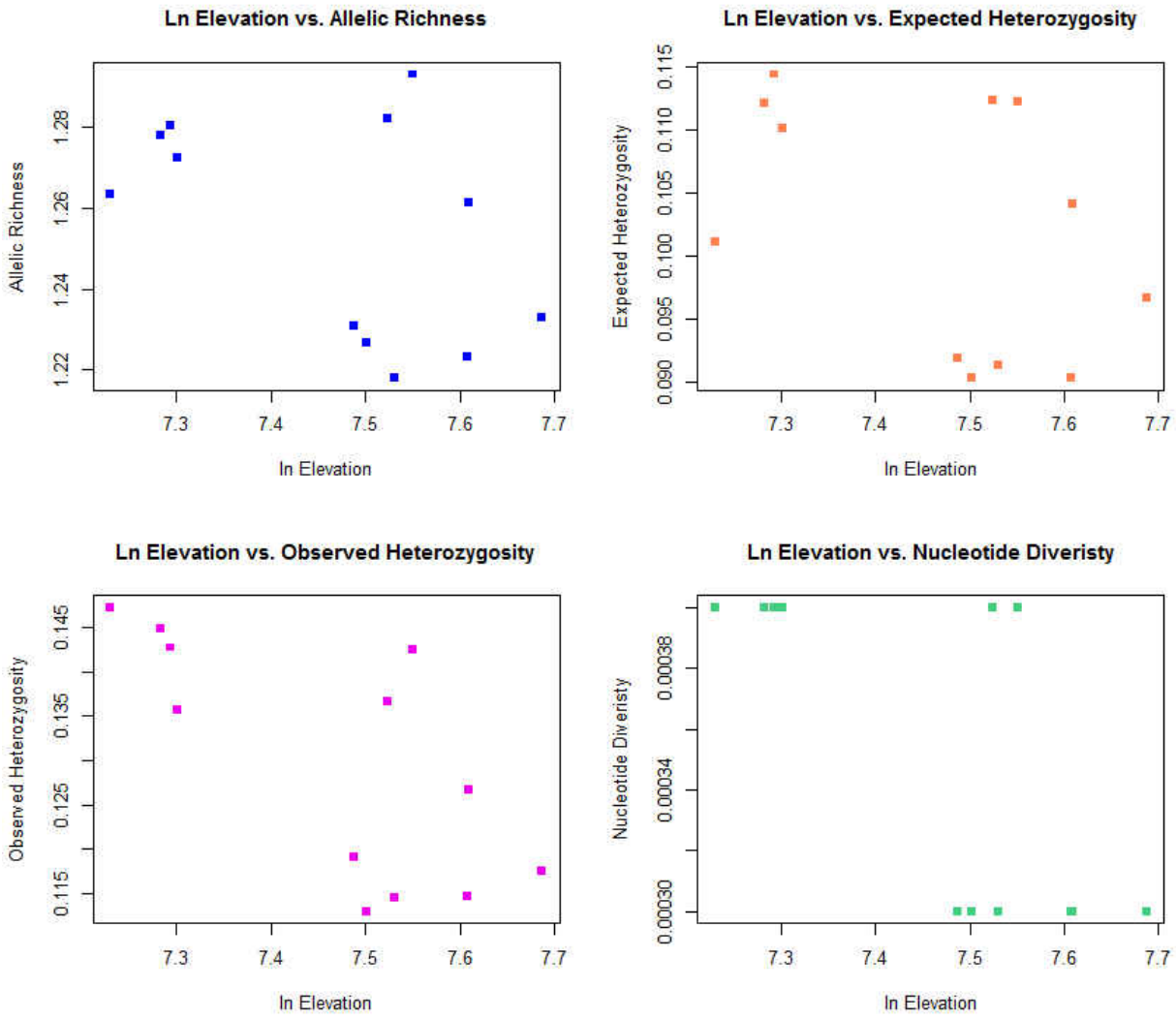

Figure 24. Linear regressions of natural log of elevation (ln Elevation) and allelic richness, expected heterozygosity, observed heterozygosity, and nucleotide diversity for California Cascades frog sites (CA dataset).

Table 1. Cascades frog sampling sites with number of samples sequenced and retained after bioinformatics filtering.

Site Abbreviation	Site Name	State	Region	County	Samples Sequenced	Samples post-filtering
TOD	Todd Lake	Oregon	Cen. Cascades	Deschutes	20	16
SPA	Sparks Lake	Oregon	Cen. Cascades	Deschutes	20	**0**
WAL	Waldo Lake	Oregon	Cen. Cascades	Lane	20	18
DIA	Diamond Lake	Oregon	So. Central	Douglas	18	13
GEM	Gem Lake	California	N. Klamath	Siskiyou	20	15
RED	Red Rock Lake	California	N. Klamath	Siskiyou	20	15
RUS	Rush Creek Lake	California	Klamath	Siskiyou	20	17
LIC	Little Caribou Lake	California	Klamath	Siskiyou	14	13
BLU	Blue Divide	California	E. Klamath	Trinity	12	8
GUM	Gumboot Lake	California	E. Klamath	Shasta	20	20
SCR	Screwdriver Creek	California	No. Lassen	Shasta	12	12
NEL	Nelson Creek	California	No. Lassen	Shasta	4	4
COW	Old Cow Meadow	California	No. Lassen	Shasta	12	10
CHI	Childs Meadow	California	So. Lassen	Tehama	23	17
ROU	Round Valley	California	So. Lassen	Tehama	10	10
CAR	Carter Meadow	California	So. Lassen	Tehama	23	22
					268	**210**

Table 2. Counts of SNP loci after filtering steps for Cascades frogs in the CA dataset.

Filtering step	Count
Pre-filtering	116805
SNPs w/genotypes for >75% of inds. & 1 SNP/locus	68559
SNPs w/genotypes for >75% of pops.	5468
Max heterozygosity/locus 0.70	5414
Min. Minor Allele Frequency (MAF) > 0.03	2291

Table 3. Pairwise F_{ST} (below diagonal) and D (above diagonal) for Cascades frog sites in California (CA dataset). Klamath sites are denoted with a K and Lassen sites with an L.

	K-BLU	L-SCR	L-CAR	L-CHI	L-COW	K-GEM	K-GUM	K-LIC	L-NEL	K-RED	L-ROU	K-RUS
K-BLU	0	0.017	0.035	0.029	0.025	0.014	0.002	0.015	0.025	0.010	0.027	0.005
L-SCR	0.085	0	0.040	0.033	0.019	0.021	0.014	0.028	0.006	0.020	0.028	0.019
L-CAR	0.134	0.149	0	0.009	0.034	0.044	0.038	0.042	0.049	0.036	0.013	0.041
L-CHI	0.120	0.146	0.043	0	0.025	0.036	0.031	0.040	0.038	0.030	0.011	0.033
L-COW	0.132	0.107	0.152	0.120	0	0.028	0.025	0.036	0.025	0.023	0.025	0.026
K-GEM	0.069	0.104	0.159	0.147	0.144	0	0.013	0.023	0.028	0.001	0.031	0.012
K-GUM	0.012	0.075	0.137	0.136	0.130	0.064	0	0.016	0.024	0.010	0.028	0.007
K-LIC	0.080	0.156	0.170	0.179	0.194	0.122	0.091	0	0.036	0.020	0.038	0.013
L-NEL	0.135	0.041	0.203	0.184	0.149	0.153	0.131	0.205	0	0.027	0.034	0.027
K-RED	0.038	0.074	0.112	0.094	0.089	0.004	0.040	0.079	0.111	0	0.025	0.009
L-ROU	0.117	0.131	0.049	0.046	0.121	0.138	0.126	0.175	0.166	0.085	0	0.030
K-RUS	0.026	0.095	0.150	0.134	0.127	0.064	0.038	0.070	0.141	0.034	0.127	0

Table 4. Pairwise F_{ST} (below the diagonal) and D (above the diagonal) for Oregon and California populations of Cascades frogs (ORCA dataset). Klamath sites are denoted with a K, Lassen sites with an L, and Oregon with an O.

	K-BLU	L-SCR	L-CAR	L-CHI	L-COW	K-GEM	K-GUM	K-LIC	L-NEL	K-RED	L-ROU	K-RUS	O-DIA	O-TOD	O-WAL
K-BLU	0	0.010	0.023	0.019	0.017	0.009	0.001	0.010	0.017	0.008	0.018	0.003	0.042	0.031	0.033
L-SCR	0.074	0	0.026	0.022	0.013	0.014	0.010	0.019	0.004	0.014	0.020	0.013	0.048	0.034	0.035
L-CAR	0.148	0.156	0	0.009	0.024	0.028	0.027	0.029	0.031	0.025	0.010	0.029	0.073	0.062	0.066
L-CHI	0.131	0.149	0.058	0	0.017	0.025	0.022	0.028	0.025	0.021	0.008	0.023	0.061	0.049	0.053
L-COW	0.131	0.106	0.167	0.129	0	0.020	0.017	0.025	0.016	0.017	0.019	0.017	0.056	0.043	0.042
K-GEM	0.072	0.111	0.177	0.169	0.165	0	0.009	0.017	0.020	0.000	0.023	0.009	0.041	0.030	0.031
K-GUM	0.009	0.075	0.155	0.151	0.13	0.071	0	0.012	0.017	0.008	0.020	0.005	0.043	0.031	0.034
K-LIC	0.089	0.155	0.18	0.192	0.199	0.141	0.1	0	0.026	0.015	0.027	0.010	0.055	0.045	0.047
L-NEL	0.132	0.034	0.202	0.183	0.151	0.165	0.136	0.218	0	0.018	0.025	0.020	0.052	0.039	0.039
K-RED	0.048	0.083	0.135	0.11	0.11	0.005	0.052	0.099	0.119	0	0.019	0.007	0.044	0.029	0.031
L-ROU	0.122	0.137	0.062	0.052	0.137	0.161	0.135	0.186	0.179	0.108	0	0.021	0.056	0.043	0.045
K-RUS	0.027	0.099	0.169	0.154	0.134	0.069	0.04	0.08	0.147	0.043	0.141	0	0.047	0.035	0.038
O-DIA	0.142	0.184	0.207	0.196	0.209	0.146	0.165	0.221	0.189	0.128	0.18	0.174	0	0.021	0.019
O-TOD	0.162	0.185	0.244	0.228	0.236	0.163	0.172	0.249	0.204	0.13	0.207	0.187	0.076	0	0.007
O-WAL	0.142	0.156	0.215	0.2	0.188	0.143	0.156	0.222	0.176	0.117	0.178	0.166	0.059	0.032	0

Table 5. Mantel test results comparing matrices of genetic distance, geographic distance, elevation, average winter temperature, presence of a genetic barrier, and ecotype among pairs of populations within California. Tests that were significant after false discovery rate correction at an experiment-wide alpha of 0.015 are bolded.

Correlation	Partialled out	Mantel r	P
FST x Distance		**0.5894**	**<0.001**
FST x Elevation		**0.3923**	**0.003**
FST x Avg. Winter Temp.		-0.1551	0.328
FST x Putative boundary		**-0.5789**	**<0.001**
FST x Ecotype		**-0.4633**	**<0.001**
FST x Elevation	Distance	0.2767	0.047
FST x Putative boundary	Distance	-0.3332	0.033
FST x Ecotype	Distance	0.0051	0.9693
FST x Putative boundary	**Ecotype**	**-0.5374**	**<0.001**
FST x Putative boundary	**Elevation**	**-0.6044**	**<0.001**
FST x Ecotype	**Putative boundary**	**-0.3999**	**0.002**
FST x Ecotype	**Elevation**	**-0.33919**	**0.008**
FST x Elevation	**Putative boundary**	**0.4385**	**0.001**
FST x Elevation	Ecotype	0.2158	0.137

Table 6. Allelic richness (A_R), expected heterozygosity (H_E), observed heterozygosity (H_O), F_{IS} , nucleotide diversity (π), and effective population size estimates (N_e with 95% confidence intervals) for individuals (n) sampled at Cascades frog sites in Oregon and California (ORCA dataset). *Inf* in N_e estimates refer to "infinite".

State	Region	Site	n	A_R	H_E	H_O	F_{IS}	π	N_e (95% confidence intervals)
OR	Central	TOD	16	1.25	0.107	0.143	-0.0686	0.0004	145.6 (88.9-380.4)
OR	Central	WAL	18	1.26	0.112	0.148	-0.0686	0.0004	285.3 (146.5-3698.2)
OR	So. Central	DIA	13	1.28	0.125	0.166	-0.0733	0.0005	*Inf* (*Inf*-*Inf*)
CA	No. Klamath	GEM	15	1.17	0.070	0.088	-0.0362	0.0003	136.9 (73.9-742.1)
CA	No. Klamath	RED	15	1.18	0.073	0.092	-0.038	0.0003	128.3 (63.4-4011.8)
CA	Klamath	RUS	17	1.20	0.080	0.098	-0.0373	0.0003	101.8 (70.4-179.1)
CA	Klamath	LIC	13	1.17	0.074	0.091	-0.0318	0.0003	75.9 (51-142.9)
CA	Ea. Klamath	BLU	8	1.21	0.081	0.102	-0.0323	0.0004	*Inf* (*Inf*-*Inf*)
CA	Ea. Klamath	GUM	20	1.22	0.086	0.104	-0.0424	0.0004	*Inf* (605.2-*Inf*)
CA	No. Lassen	SCR	12	1.21	0.085	0.105	-0.0342	0.0004	17.9 (16.1-20)
CA	No. Lassen	NEL	4	1.20	0.078	0.112	-0.0371	0.0004	*Inf* (*Inf*-*Inf*)
CA	No. Lassen	COW	10	1.18	0.072	0.094	-0.0356	0.0003	16.4 (14.6-18.6)
CA	So. Lassen	CHI	17	1.21	0.090	0.115	-0.0515	0.0004	96 (68.5-156.9)
CA	So. Lassen	ROU	10	1.21	0.088	0.117	-0.0512	0.0004	*Inf* (*Inf*-*Inf*)
CA	So. Lassen	CAR	22	1.16	0.071	0.091	-0.0431	0.0003	27.9 (23.8-33.4)

Table 7. Allelic richness (A_R), expected heterozygosity (H_E), observed heterozygosity (H_O), F_{IS} , nucleotide diversity (π), and effective population size estimates (N_e with 95% confidence intervals) for individuals (n) sampled at Cascades frog sites in California (CA dataset). *Inf* in N_e estimates refer to "infinite".

State	Region	Site	n	A_R	H_E	H_O	F_{IS}	π	N_e (95% confidence intervals)
CA	No. Klamath	GEM	15	1.223295788	0.0903	0.1147	-0.048	0.0003	*Inf* (632.2-*Inf*)
CA	No. Klamath	RED	15	1.22669126	0.0903	0.113	-0.046	0.0003	4154.5 (178.6-*Inf*)
CA	Klamath	RUS	17	1.261136115	0.1041	0.1266	-0.0472	0.0003	93.6 (74.8-124.2)
CA	Klamath	LIC	13	1.232907028	0.0966	0.1175	-0.0368	0.0003	99.8 (72.7-156.7)
CA	Ea. Klamath	BLU	8	1.292926945	0.1122	0.1425	-0.0462	0.0004	*Inf (Inf-Inf)*
CA	Ea. Klamath	GUM	20	1.282071658	0.1123	0.1366	-0.0566	0.0004	2089 (482.1-*Inf*)
CA	No. Lassen	SCR	12	1.272405605	0.1101	0.1357	-0.0444	0.0004	19.5 (18.1-21.0)
CA	No. Lassen	NEL	4	1.263351311	0.1011	0.1472	-0.0527	0.0004	*Inf (Inf-Inf)*
CA	No. Lassen	COW	10	1.230847503	0.0919	0.1191	-0.0455	0.0003	21.5 (19.6-23.6)
CA	So. Lassen	CHI	17	1.280201942	0.1144	0.1427	-0.0577	0.0004	60.5 (52.4-71.4)
CA	So. Lassen	ROU	10	1.277937833	0.1121	0.1448	-0.0561	0.0004	*Inf (Inf-Inf)*
CA	So. Lassen	CAR	22	1.218041444	0.0913	0.1145	-0.0481	0.0003	35.9 (32.0-40.6)

Table 8. Outlier loci in the ORCA dataset as identified by the top 5% F_{ST} outliers, *BayeScan*, and *PCAdapt*.

Number	SNP	5% F_{ST}	*PCAdapt*	*BayeScan*
1	39_89		X	
10	448_138	X		
18	824_57		X	
24	951_116	X		
34	1474_93	X		
36	1491_106		X	
66	2699_22	X	X	
80	3102_109		X	
86	3445_84		X	
90	3704_93		X	
117	4764_128	X		
129	5216_46		X	
163	6457_92	X		
166	6615_53	X		
167	6646_117		X	
169	6792_11	X	X	
189	7811_102	X		
221	9340_78	X	X	
233	9865_31		X	
246	10488_104	X		
251	10598_132	X	X	
253	10647_147	X		
257	10755_88	X		
258	10813_20	X		
279	11592_21		X	
280	11642_6		X	
296	12161_144	X	X	
297	12251_0		X	
305	12491_146		X	X
320	12943_51		X	
326	13067_98	X		
328	13119_96		X	
338	13391_34	X		
350	14280_8		X	
359	14594_42	X		
389	15553_137		X	

393	15772_62	X		
396	15855_98		X	
402	16004_146	X		
412	16147_78		X	
413	16177_131		X	
416	16239_88	X	X	
417	16251_129	X		
427	16526_11	X	X	
456	17317_88	X	X	
470	17657_36		X	
480	17972_67	X		
490	18199_89	X		
527	19151_79	X		
530	19239_120		X	
535	19346_143		X	
554	19994_52	X		
559	20101_48	X	X	
573	20648_50		X	
583	20953_27		X	
589	21118_10	X		
604	21584_56	X		
616	21868_113	X	X	
623	22061_119	X	X	
637	22680_74		X	
644	22854_61		X	
651	23317_91		X	
652	23328_85	X	X	
657	23533_33		X	
662	23647_34	X	X	
706	25324_60		X	
710	25421_7		X	
714	25477_35	X		
732	25956_75		X	
736	26050_42	X		
769	26866_61		X	
779	27061_53	X		
780	27069_38	X		
786	27272_46		X	
796	27685_10	X		
799	27776_56	X		
812	28287_27		X	

821	28632_140		X	
822	28699_48	X		
829	28956_112		X	
844	29468_0		X	
849	29695_25		X	
851	29708_36		X	
852	29757_74	X		
856	29867_47		X	
858	29953_52	X	X	
861	30011_36	X	X	
871	30268_13	X		
878	30417_13		X	
882	30541_121	X		
886	30698_21		X	
899	31227_126	X	X	
901	31329_75		X	
913	31583_66	X		
915	31620_51	X	X	
920	31725_145	X	X	
922	31906_19		X	
951	33136_125	X	X	
959	33723_29	X	X	
962	33784_45	X		
975	34332_86		X	
982	34484_95		X	
999	34900_139	X	X	
1016	35750_4		X	
1023	36057_20		X	
1029	36324_109		X	
1042	36786_71	X		
1059	37455_67		X	
1104	39205_112	X		
1120	40196_43		X	
1121	40227_22		X	
1128	40468_3		X	
1132	40586_2		X	
1140	40898_108	X		
1165	41921_43	X		
1173	42123_130		X	
1198	42968_134		X	
1208	43271_143		X	

1212	43404_22	X		
1222	43667_76	X	X	
1224	43736_95	X		
1256	44829_79		X	
1278	45561_43	X		
1283	45927_23	X		
1284	46039_121		X	
1304	46585_117		X	
1306	46686_101		X	
1308	46739_11		X	
1312	46802_114		X	
1350	48569_58		X	
1359	49061_100		X	
1368	49423_78	X	X	
1376	49627_36	X		
1380	49769_40		X	
1408	50493_117		X	
1414	50693_87	X		
1423	51019_10		X	
1424	51031_108		X	
1456	66223_4		X	
1475	76591_91	X	X	
1485	77766_132	X		
1489	78692_129		X	
1493	79343_137	X		
1497	80200_51		X	
1502	82601_71		X	
1504	83552_74	X		
1510	84868_32		X	
1512	86523_50	X	X	
1515	87060_3	X		
1534	97120_132	X	X	
1539	99056_104	X		
1540	99579_91		X	
1546	100935_23	X		
1552	103293_40	X		
1553	103356_1		X	
1556	103608_76		X	
1576	119384_14		X	
1577	119446_29	X	X	
1589	123149_43	X	X	

1618	136075_20	X		
1641	149529_68	X	X	
1647	154710_19	X	X	
1654	159367_60		X	
1664	161872_7	X		
1671	181131_79		X	

Table 9. Outlier loci in the CA dataset as identified by the top 5% F_{ST} outliers, *BayeScan*, and *PCAdapt*.

Number	SNP	5% F_{ST}	*PCAdapt*	*BayeScan*
6	78_96		X	
24	832_26		X	
32	1132_103	X	X	
34	1201_43	X	X	
57	1779_109		X	
75	2245_71	X	X	
92	2751_69	X	X	
94	2797_1		X	
119	3653_91		X	
120	3664_85	X	X	
129	3870_33	X		
133	3985_34	X	X	
137	4128_102		X	
146	4355_63		X	
161	4826_149		X	
164	4879_95	X	X	
177	5226_120	X		
213	6445_40		X	
227	6792_40	X		
251	7331_91	X	X	X
263	7700_10		X	
279	8087_147	X		
288	8290_84	X	X	
302	8618_127		X	
330	9193_78		X	
332	9224_131		X	
336	9286_88	X	X	
340	9364_83		X	
347	9573_134		X	
348	9574_11	X		
360	9830_27		X	
384	10410_107	X	X	
392	10569_143		X	
395	10662_80		X	
397	10700_22	X		
412	11034_95	X		

436	11790_97		X	
437	11793_73		X	
456	12258_87	X	X	
462	12356_31		X	
466	12447_98	X	X	
479	12808_46		X	
504	13409_68	X	X	
512	13593_93	X		
514	13679_67		X	
522	13896_36	X	X	
531	14054_92	X		
556	14646_148		X	
566	14906_112	X	X	
571	15071_56		X	
575	15218_126	X	X	
580	15463_121		X	
587	15721_114	X	X	
604	16120_101		X	
605	16132_85		X	
606	16172_11		X	
612	16261_96		X	
617	16421_79	X		
622	16509_120		X	
627	16617_143	X		
629	16702_149		X	
637	16820_89		X	
639	16910_91	X	X	
651	17299_1	X		
656	17370_48	X	X	
662	17454_10		X	
673	17546_23	X		
698	18200_66		X	
700	18223_27		X	
709	18366_25		X	
711	18379_36		X	
720	18624_52	X	X	
724	18683_36	X	X	
726	18755_35	X		
733	18940_13	X		
748	19266_2		X	
753	19370_21		X	

762	19734_91	X		
781	20169_17		X	
787	20292_51	X	X	
794	20397_145	X	X	
830	21309_6		X	
834	21468_133	X	X	
851	21828_144	X	X	
853	21918_0		X	
858	22045_148		X	
862	22155_143		X	X
874	22398_68		X	
877	22545_120	X	X	
881	22607_51		X	
902	23140_95		X	
908	23355_36		X	
922	23562_139	X	X	
930	23805_145		X	
937	24046_70	X		
953	24416_4		X	
960	24580_130	X		
964	24629_83		X	
965	24707_28	X	X	
967	24720_20		X	
970	24746_30	X	X	
974	24950_68	X	X	
989	25359_146		X	
1005	25729_107		X	
1010	25814_44	X		
1011	25837_119	X	X	
1079	27676_75		X	
1083	27770_42	X		
1133	28672_73	X	X	
1137	28784_105	X		
1144	28907_149	X	X	
1146	28939_44	X		
1148	28991_46		X	
1174	29573_39	X	X	
1177	29692_95	X	X	
1182	29752_88	X	X	
1188	29865_137	X	X	
1212	30407_67	X		

1223	30635_89	X	X	
1252	31219_45	X	X	
1259	31408_111	X		
1260	31437_108	X	X	
1295	32589_31		X	
1299	32871_74		X	
1319	33329_132	X	X	
1329	33594_114		X	
1330	33619_132		X	
1347	34006_10	X		
1373	34826_117	X	X	
1379	35002_17	X		
1389	35308_16	X	X	
1391	35360_58		X	
1409	35911_3		X	
1422	36339_108	X		
1427	36531_71		X	
1428	36541_39	X		
1439	36895_59		X	
1444	37174_110	X	X	
1458	37504_47		X	
1465	37717_90	X		
1476	37951_47		X	
1477	38019_89		X	
1500	38804_57		X	
1517	39270_83		X	
1552	40424_1	X		
1563	40676_22	X		
1572	40841_91	X		
1577	40912_109	X		
1590	41315_14	X	X	
1591	41410_25	X	X	
1593	41427_84		X	
1595	41464_16	X	X	
1596	41471_134	X	X	
1605	41764_143	X	X	
1618	42326_35		X	
1626	42617_56	X	X	
1643	43129_27		X	
1656	43545_48	X		
1664	43801_112		X	

1692	44701_117		X	
1700	45031_61	X		
1714	45729_115		X	
1715	45730_11		X	
1721	45871_51	X	X	
1724	45983_42	X	X	X
1732	46490_37	X		
1756	47546_131		X	
1763	47780_56		X	
1764	47781_112	X		
1784	48773_43	X	X	
1789	48894_32	X		
1802	49272_114	X	X	
1827	50047_136	X	X	
1830	50082_85		X	
1832	50138_29	X	X	
1835	50180_80		X	
1837	50202_125	X	X	
1840	50408_84	X	X	
1848	50768_139		X	
1850	50791_29	X	X	
1859	50945_13		X	
1864	51105_63	X	X	
1866	53840_16	X	X	
1874	55675_134		X	
1876	56235_35	X		
1886	58587_20	X		
1891	59879_20	X	X	
1894	60730_6		X	
1939	72527_35		X	
1968	80194_91	X		
1970	80456_51		X	
1997	83805_51		X	
2024	89826_60		X	
2036	93289_95		X	
2059	97592_104	X		
2063	98259_14		X	
2064	98321_29	X		
2074	100599_89		X	
2081	102029_43	X		
2092	103930_16	X		

2097	104541_65		X	
2100	105698_58	X	X	
2110	107809_4		X	
2113	108203_98		X	
2143	114726_101		X	
2152	116578_98	X	X	
2159	118540_51		X	
2167	120041_74	X		
2175	121361_32		X	
2180	122677_34		X	
2182	123075_68	X	X	
2202	129967_90		X	
2221	138703_2		X	
2243	148098_137	X	X	
2245	149125_79		X	
2254	153512_19	X	X	
2264	158692_5		X	
2265	158722_122		X	
2281	166396_87	X		

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