Hybridization Between *Anaxyrus boreas* × *canorus* within Eldorado National Forest: The First Genomic Assessment

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Cover design and Yosemite Toad photo by Paul Maier (Gene by Gene), Western Toad photo by Ivan Phillipson (Wild Latitudes) with permission.

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Contents

Genetic Glossary

Admixture: Genetic mixture between individuals with distinct ancestry from two or more populations
Ancestry Informative Marker (AIM): Marker with (nearly) fixed difference between species
Allele: One unique version of a genetic locus; a genotype with two different alleles is heterozygous
Ancestry composition: The proportion of one or more ancestral populations that an individual inherits
Backcross: Hybrid resulting from cross between a hybrid individual (e.g., F1) and a pure individual
Contact zone: Geographic area where two different species or lineages overlap, or possibly hybridize
ddRADseq: A method for obtaining thousands of homologous genomic loci across individuals
Diploid: Referring to both of DNA's two copies (i.e., mother's and father's)
Dominance: Phenotype that resembles one (dominant) allele more than the other (recessive) allele
Epistasis: Phenotype resulting from two or more genotypes, which influence each other's expression
F1: Hybrid resulting from cross between two pure individuals of different species or lineages
F2: Hybrid resulting from cross between two F1 individuals; F3s result from two F2s, etc.
Genetic: Pertaining to DNA at one or few loci; sometimes used synonymously with 'genomic'
Genomic: Pertaining to DNA across the entire genome, or many loci spread across the genome
Genotype: For one locus, the set of DNA (alleles) inherited from both mother and father
Haploid: Referring to one of DNA's two copies (e.g., mother's), or single-copy DNA (e.g., mtDNA)
Haplotype: Sequence of DNA along a haploid chromosome (e.g., 'ATTCAGGCT')
Heterozygous: Possessing two different alleles at a locus; more such loci confer higher heterozygosity
Homologous: DNA in the same location for multiple individuals; differences are thus due to change
Homomorphic: (Referring to sex chromosomes) similar in size and shape; unlike X/Y in mammals
Hybrid: An individual resulting from a cross between two species (e.g., F1, F2, backcross)
Introgression: Repeated backcrossing that spreads limited DNA from one species into another
Lineage fusion: Repeated hybridization followed by isolation of the hybrids from both parent species
Linkage disequilibrium (LD): Correlation of alleles along a haplotype due to physical proximity
Locus: Genetic location where DNA is sequenced and used for analysis (plural: 'loci')
Marker: Genetic locus with DNA variation that allows individuals or species to be distinguished
Mitochondria: Organelle with DNA (mtDNA) that is haploid, multi-copy, and inherited matrilineally
Phenotype: Observable trait, behavior, or appearance expressed by underlying genotype(s)
Recombination: Mixing mother's and father's DNA into a mosaic while producing sperm/eggs
Sex chromosomes: XX females, XY males in mammals; ZZ males, ZW females in toads
Single Nucleotide Polymorphism (SNP): The smallest possible marker, e.g., C/T

Bioinformatic Glossary

Adapter: A short (e.g., 30 bp) DNA sequence used to cap both broken ends of digested DNA

Alignment: Process of digitally comparing unknown DNA sequence to a reference to find its position

Assembly: Process of merging short DNA sequence reads to reconstruct the longer original sequence

Barcode: A short (e.g., 5 bp), unique DNA sequence inserted into an adapter to distinguish samples

Base pair (bp): Two complimentary nucleotides that pair together on a DNA strand

Contig: Longer DNA segment assembled from shorter, partially overlapping sequence reads

Demultiplex: Use barcodes and indices to separate pooled DNA into sample-specific DNA

Depth: The number of sequencing reads covering the same genomic position in one sample

Digestion: Using enzymes to cut DNA into small fragments as designated locations

Hybrid index (S_i): Proportion of ancestry from each of two species

Index: A short (e.g., 6 bp), unique DNA sequence inserted into a primer to distinguish samples

Inter-species heterozygosity (H_i): Proportion of genotypes with one allele from each of two species

Library: Collection of DNA fragments from multiple samples and loci that is ready to sequence

Ligation: Enzymatic attachment of DNA adapters to the broken ends of digested DNA

Minor Allele Frequency (MAF): The frequency of the less common allele in the population

Nucleotide (nt): A single A, C, G, or T; i.e., the smallest unit of a DNA sequence

Paired-end sequencing: Sequencing both ends of a DNA fragment; potentially meeting in the middle

Polymerase Chain Reaction (PCR): Technique for amplifying one DNA sequence into many copies

Primer: Short single-stranded DNA sequence essential for binding the target DNA during PCR

Read 1 (R1): For paired-end sequencing, the first read, which is on the 5’ end

Read 2 (R2): For paired-end sequencing, the second read, which is on the 3’ end

Reference genome: DNA database representing the complete chromosome sequences for a species

Size selection: Filtering DNA library to a particular size range to increase homology across samples

Transcriptome: DNA database representing the complete RNA (incl. gene sequences) for a species

Executive Summary

- The first genomic assessment of hybridization between *Anaxyrus boreas* × *canorus* was conducted, and a hybrid panel was developed with future utility for hybrid identification.

- Hybridization between *Anaxyrus boreas* × *canorus* has been confirmed in Eldorado National Forest within the Blue Lakes, Twin Lake, and Deer Valley regions.

- Those hybrids are mostly *A. boreas* with up to 20% *A. canorus* ancestry, and up to 30% heterozygosity from both species, suggesting an origin within ten generations.

- Trace hybridization was also unexpectedly found in several other places, including a mostly *A. boreas* population near Lake Tahoe, and lower elevation *A. canorus* populations in Yosemite National Park.

Introduction

Overview of Toad Hybridization

Natural hybridization between different species of North American toads is widespread. Examples have been reported for decades, for example amongst the *Anaxyrus americanus* group: *A. americanus* × *fowleri* in the eastern United States [1–5], *A. woodhousii* × *houstonensis* in Texas [6], and *A. woodhousii* × *microscaphus* in Arizona [7–11]. More distant hybrids have been observed, for example between *A. woodhousii* × *cognatus* [12], *A. woodhousii* × *punctatus* [13,14], and even between distinct genera *Anaxyrus* (North American) and *Incilius* (Central American) [6,12,15,16]. Approximately 20 million years has elapsed since these two hybridizing genera shared a common ancestor [17], yet they retain some reproductive compatibility. Mature species are often captable of hybridizing without eroding the integrity of either lineage, although some taxa are more predisposed than others. Toad hybrids display high rates of fertility and viability, probably due to the nature of their homomorphic sex chromosomes, and slowly evolving incompatibility [18–21].

In western North America, the *Anaxyrus boreas* group is geographically and phylogenetically separated from other members of the genus [20,22–25]. The group primarily consists of the widespread Western Toad (*A. boreas*) which occurs throughout the Pacific Northwest, from Alaska into Baja California, and inland to the eastern slope of the Rocky Mountains. Excluding the Yosemite Toad (*Anaxyrus canorus*), the other members are single-locality endemics limited to spring-fed habitat in the Great Basin Desert of Nevada and eastern California [26–29]. The Yosemite Toad [30] is a montane and subalpine endemic of the Sierra Nevada in eastern California, where repeated glacial advances during the Pleistocene adapted it to high elevation meadow habitat and isolated it from the lowland Western Toad [31–33]. The species is distributed across 200 km between southern Eldorado National Forest (near Lake Tahoe) and southern Sierra National Forest (near the middle fork of the Kings River), and between 1,980 m (6,500 ft) and 3,414 m (11,200 ft) [34]. With few exceptions [35], all wild hybridization suspected in this group is between the generalist Western Toad and the mountainous Yosemite Toad on federal lands in California.

A putative hybrid *Anaxyrus boreas* × *canorus* female was first described in 1956 at Faith Valley near Blue Lakes, Eldorado National Forest, 30 miles north of the Yosemite Toad's known range limit [36]. E. L. Karlstrom initially disagreed with the categorization [31], which was based upon a primarily *A. boreas* morphology mixed with *A. canorus* coloration. However, a 1978 reanalysis based on parotoid width and web length showed that individual to be intermediate between species [37]. Later USDA Forest Service surveys in 1992 and 2002–2009 of the Blue Lakes region corroborated suspicions by finding other morphologically intermediate toads [34,38] (Fig. 1). This northern contact zone at 2,438 m (8,000 ft) represents the biggest elevational overlap between the two species [39], raising the possibility of higher ecological compatibility. Extensive artificial hybridization experiments by W. F. Blair and others [18,21,31,40,41] have shown that the two species can successfully produce F1 hybrid tadpoles that metamorphose at a high rate (78.2%), and that F1 hybrids can even successfully backcross with *A. boreas* (32.3% metamorphosis compared to 45.0% in control group). Genetically, there is evidence of ancient mitochondrial introgression

between species [23,42], and extensive evidence that hybridization, adaptive introgression partially related to climate change, and "lineage fusion" have occurred intraspecifically in Yosemite Toads [33,43]. Although it seems quite plausible based on the available evidence and relatively short separation time of 2 million years [33] that natural hybridization occurs between the species, there is still no direct genomic evidence to bear on the existence, frequency, or directionality of such events.

Figure 1. Potential hybrid Ab×c individuals from the Blue Lakes region of Eldorado National National Forest. Photos contributed by Rob Grasso (Yosemite National Park) and Jeffrey Mabe (Eldorado National Forest).

Scope of Study

This report describes the first genomic hybrid panel for detecting *Anaxyrus boreas* × *canorus* ("*Ab*×*c*") individuals from the northern contact zone ("NCZ") within Eldorado National Forest (Fig. 2). We chose this largest of putative contact zones due to its unique management concerns, its long record of suspected hybrid detections, and as a pilot study for future work. The NCZ likely extends southward onto adjacent Humboldt-Toiyabe National Forest, where presumed Yosemite Toads may contain Western Toad ancestry. The symmetry of hybridization thus cannot be resolved by the current study. Other regions contain likely *Ab*×*c* hybridization too. With further sampling, the panel developed here could also elucidate the eastern contact zone (Frog Lakes, Inyo National Forest) [37], western contact zone (Huntington Lake, Sierra National Forest; S. Barnes, pers. comm.), and high elevation toads tentatively identified as Western Toads south of the Yosemite Toad distribution, in Sequoia National Park (R. A. Knapp, pers. comm.).

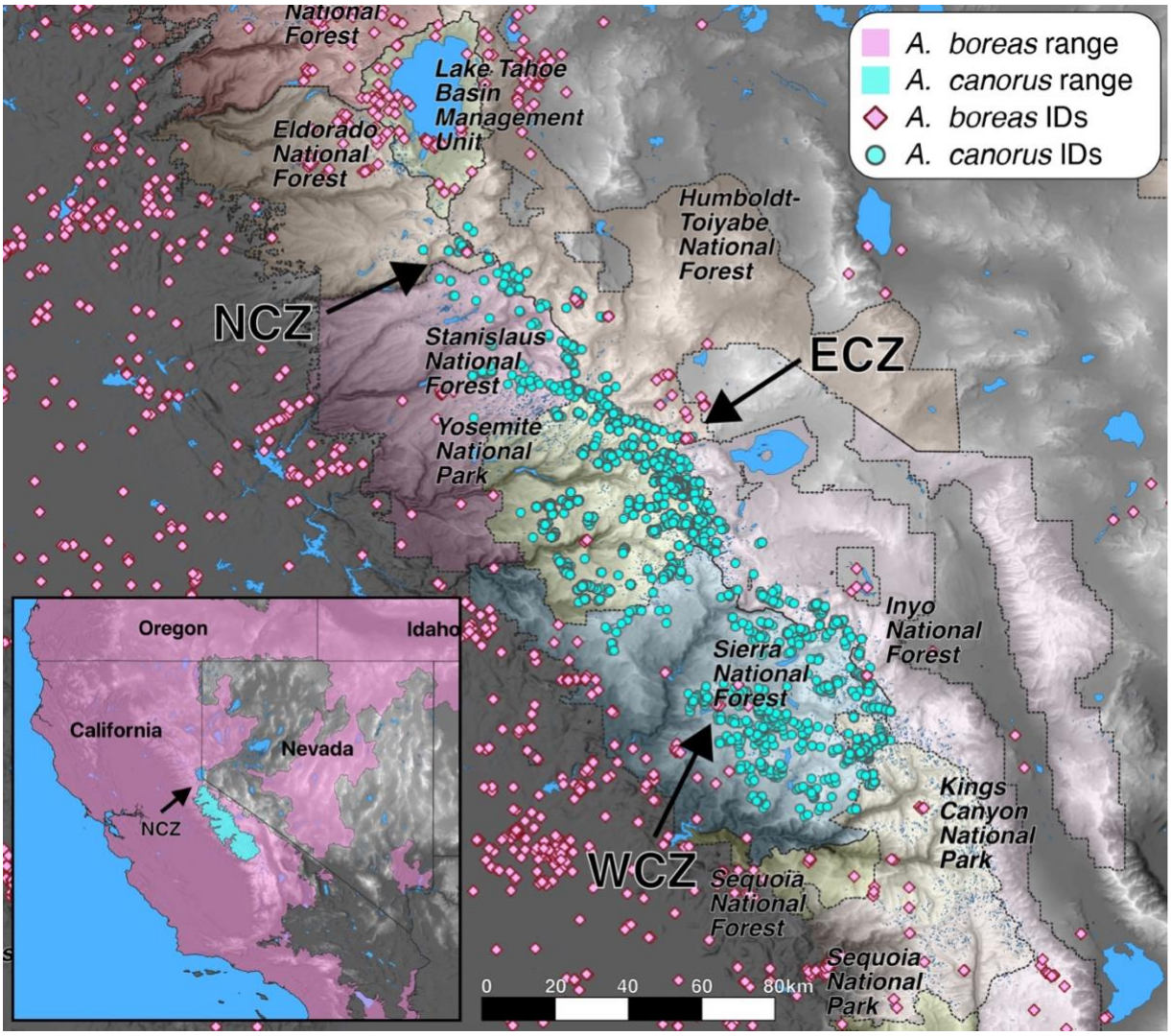


Figure 2. Study area in California showing the distributions of Western and Yosemite Toads, and coordinates of toad identifications ("IDs"). The single arrow inside the inset map shows the northern contact zone (NCZ) which is the specific study area. Two other contact zones have previously been described: eastern contact zone (ECZ), and western contact zone (WCZ). The NCZ in Humboldt-Toiyabe National Forest also contains overlapping species identifications, meriting further study. Sources: U.S. Fish and Wildlife Service (A. canorus), iNaturalist (A. boreas).

The Yosemite Toad is a federally threatened species [44] that faces extreme selection pressure from both climate change [45,46] and disease [47,48]. Its northern contact zone with the Western Toad lies partially within Eldorado National Forest, which includes 82,740 acres of suitable habitat and 14,266 acres of designated Critical Habitat. Several popular OHV routes such as the Deer Valley Trail (19E01), in addition to Meadow Lake Road (09N01), intersect with this toad habitat. Seasonal closures help mitigate the impacts of human disturbance during the early spring breeding season; however, crushed toads are frequently found along these roads.

Due to the location of these road mortalities, it is unclear which species are being affected. Owing to the sensitive nature of one species, there are important conservation ramifications if Yosemite Toads or *Ab*×*c* toads are being threatened by local extirpation. *Ab*×*c* toads may inherit the same protection under the Endangered Species Act (ESA) as Yosemite Toads, although hybrids currently have ambiguous legal status [49,50]. US Forest Service crews have classified toads at nearby Blue Lakes into all three categories (i.e., Western, Yosemite, and *Ab*×*c* toads), which increases the uncertainty of the OHV-affected population. Morphological diagnosis of putative hybrids is notoriously unreliable [51–53]. Its accuracy is unpredictable because (1) traits are not necessarily intermediate between species due to dominance and epistatic effects [51,54], (2) no two hybrids necessarily share the same traits due to recombination [55], and (3) an unknown number of generations has lapsed since hybridization occurred, possibly masking a hybrid's true ancestry [56]. Further complicating identification, catastrophic OHV injury often flattens the toad specimens. Superficially, many toads being killed on Dear Valley Trail resemble Western Toads, but only genomic methods can unambiguously identify the ancestry composition such individuals.

Hundreds or thousands of genetic loci spread throughout the genome are often needed to accurately distinguish hybrids from pure species (Fig. 3). First generation backcrossed (BC-1) individuals only require 5 loci to be identified with 95% confidence, however this number increases dramatically to 95 loci for BC-5 individuals, and 3,067 for BC-10 individuals [57]. Although hybrids initially inherit one allele from each species, successive generations lose this heterozygosity in a random and probabilistic fashion. This means any one genomic marker may resemble a "pure" species, and only the combination of markers can resolve the full picture. Closely related taxa such as Western and Yosemite Toads (which diverged in the Pleistocene [33]) are not only more likely to hybridize, but also less likely to have completely diagnostic species-specific markers, increasing the required number of genomic markers [58,59]. Therefore, we utilized an existing Yosemite Toad genomic dataset which included thousands of loci generated from double digest restriction-site associated DNA sequencing (ddRADseq). The ddRADseq protocol not only targets thousands of single nucleotide polymorphisms (SNPs) across the genome, but consistently targets those same SNPs in each additional sample [60]. Such a large and complete dataset can easily be combined with new Western Toad and putative *Ab*×*c* samples, with enough resolution to investigate population history, hybridization, and even hybrid zone dynamics.

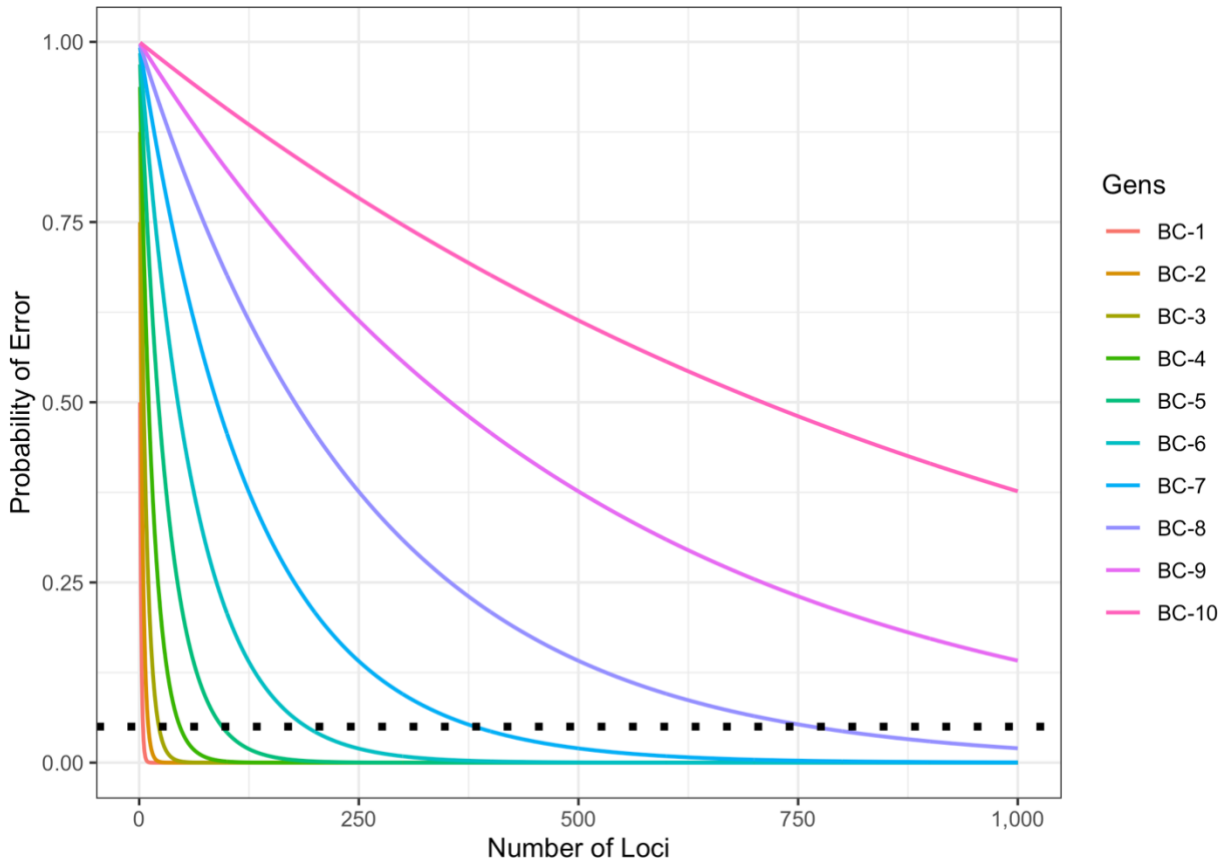


Figure 3. Power to distinguish backcrossed hybrids from pure species. Estimated based on the binomial probability that a hybrid of generation N will possess zero heterozygosity, and thus appear to be a pure individual [57].

In this study, we focused solely on identification and age assessment of putative hybrid *Ab×c* toads in Eldorado National Forest, rather than a complete analysis of any hybrid zone. However, in the future, it may be beneficial to distinguish between alternate hybridization scenarios, by sampling the entire northern contact zone in Humboldt-Toiyabe, and Stanislaus National Forests. Natural hybridization can be beneficial, neutral, or detrimental depending upon the ecological conditions, and adaptive value of novel hybrid genotypes [61–63]. Tension zones are self-limiting because hybrids are unfit regardless of environment, whereas in the other extreme, selection and asymmetric dispersal of highly fit hybrids leads to genetic swamping of one species [64]. Within Yosemite Toads, there is evidence of an upward range shift and introgression of hybrid material across lineage boundaries, as toads adapt to accelerating climate change [43,45,46]. Such a process can often be beneficial if species boundaries remain intact; however, hybrid zone dynamics can only be studied after hybrids are first identified, which is the subject of the current study.

Study Goals

Our goals were:

1. Develop an *Ab*×*c* genomic hybrid panel that can identify both the ancestry proportions and time since hybridization for toad samples collected from Eldorado National Forest.
    a. Sample putative hybrids from Deer Valley, Indian Valley, and Blue Lakes areas.
    b. Sample Western Toads in a transect across the Sierra Nevada in the same proximity as suspected hybrid localities, but far away enough to be "pure."
    c. Merge Yosemite Toad samples from Yosemite and Kings Canyon National Parks published in [33] with newly collected samples.
    d. Construct ddRADseq libraries following the protocol of [33], to maximize the number of homologous loci between datasets.
2. Generate de novo or reference guided assemblies, identify alleles, identify homologues, and call Single Nucleotide Polymorphisms (SNPs).
    a. Write scripts to encode sequence data into any necessary genotype format, including RAD haplotype format.
    b. Identify ancestry-informative markers (AIMs) that distinguish the two species.
3. Use maximum likelihood or Bayesian approaches to infer hybrid index, inter-species heterozygosity, and time since hybridization for samples suspected to be hybrid.
    a. Estimate accuracy and precision of the hybrid panel using simulations.

Methods

Sample Selection

Ideally, multiple unadmixed ("pure") populations of the two species should be sampled nearby the potential hybrid zone of interest. This is important to capture a diversity of genetic profile(s) potentially donated to hybrids, which may vary across geographic space. Anchoring a potential hybrid sample with known allele frequencies of the two parent species makes the hybrid status least ambiguous. Extensive and sufficient spatial sampling already exists for the Yosemite Toad, spanning Yosemite and Kings Canyon National Parks and approximately 33% of all localities [33].

That study only included eight genetic samples of Western Toad spread across California, however, in contrast to the 644 Yosemite Toad samples from 109 populations. Therefore, we focused our sampling on potential hybrids, and a transect of Western Toads from Sacramento to Lake Tahoe, spanning a range of allele frequencies that might contribute to hybrids (Fig. 4). Four putative hybrid locations were sampled (Upper Blue Lake, Lower Blue Lake, Twin Lake, and Deer Valley), and 10 Western Toad locations were sampled, for a total of 225 new samples.

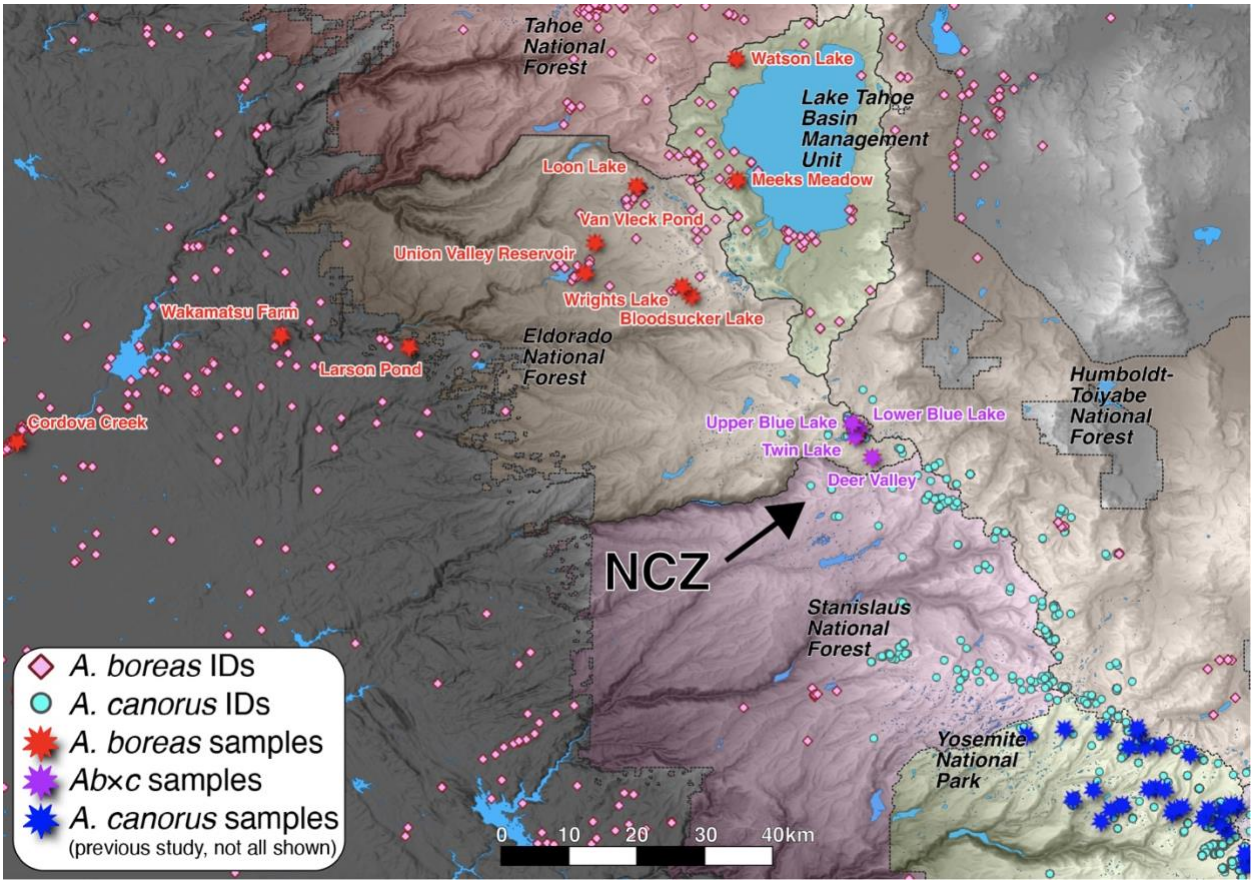


Figure 4. Sampling locations from this study, and a previous study [33], centered on the northern contact zone (NCZ). Not all A. canorus locations are shown, because they span both Yosemite and Kings Canyon National Parks. Coordinates of toad identifications ("IDs") show all potential locations for sampling, and presumed species status.

Tissue was collected from tadpoles by clipping the tips of the tails. Tadpoles were laid on a disinfected surface and a 2 mm section of the tail tip was removed with a sterilized razor blade. Each tail tip was stored in 95% molecular grade ethanol in 1.5 mL Eppendorf tubes. Buccal cells from adult toads (40 mm SVL) were collected with swabs using methods described in [65]. Toads were gently restrained by gripping the outstretched hind legs and supporting the upper body, which was covered with a clean dry cloth. The front of the toad's mouth was gently pried open using a sterile, flat, blunt, and rounded laboratory spatula or sterile swab [66]. The buccal cavity was then gently brushed with a sterile swab for 1 min. Swab tips were cut off and placed in sterilized 1.5 mL Eppendorf tubes. All tubes were placed in an insulted cooler or bag with an ice pack and transferred to a -20 C freezer upon return from the field. Adults and tadpoles of both species were released at the site of capture after sampling.

Molecular Methods

We constructed ddRADseq libraries for newly collected samples to be compatible with previously data [33]. Genomic DNA was extracted using a DNeasy blood and tissue spin column (Qiagen) protocol. Library preparation followed the protocol of [60] and was performed by AUGenomics (San Diego, CA). A starting quantity of 200–500 ng DNA was digested with 5U SbfI-HF and MspI (New England Biolabs), for 3 hr at 37°C and cleaned with 1.5x SparQ PureMag Beads (QuantaBio). Digested DNA was quantified by Qubit 2.0 Fluorometer (Life Technologies) and ligated to oligo-nucleotide adapters with one of 8 unique 5 bp MID barcode sequences at 25°C for 30 min, followed by a 10 min heat kill at 65°C. Ligated DNA was cleaned with 1x SparQ beads and pooled by adapter, then size selected between 424 and 525 bp using a 1.5% gel cassette (Pippin Prep; Sage Science). This optimal choice of two digestion enzymes (SbfI and MspI) and fragment size (424–525 bp) were originally chosen to balance number of loci with projected coverage per locus [33,67], and reproducing these parameters was essential to ensure compatibility between samples and datasets.

Size-selected DNA was amplified with Illumina primers containing one of 24 unique indices using a Phusion PCR kit (New England Biolabs). The following cycle profile was used: 98°C for 30 s, [98°C for 10 s, 72°C for 20 s @ 16% ramp], 72°C for 10 min, 4°C hold. Finally, amplicons were bead-cleaned, quantified by BioAnalyzer (Agilent Technologies), and pooled in equimolar amounts for sequencing. This combinatorial approach allowed 8×24 unique samples to be sequenced in parallel on a single flowcell, and use of double-restricted fragments dramatically increased locus recovery across samples. All ddRADseq libraries were 2×150 bp sequenced on an Element AVITI. This sequencing platform uses Rolling Circular Amplification (RCA), which copies DNA fragments only from the original template, hence greatly reducing error compared to traditional PCR.

Bioinformatics

FastQC [68] was initially used to assess the quality of each sample's FASTQ file. We checked total read length, GC content, percent of duplicated reads, percent of correct overhang sequence (leftover from SbfI and MspI cut sites bounding each R1 and R2), and percent contamination by adapters and primers. The newly sequenced data contained reads 50 bp longer than the original dataset (i.e., 2×150 bp versus 2×100 bp), so we used FASTX-Trimmer from the package FASTX-Toolkit v0.0.14 [69] to shorten all reads to identical lengths (96 nt for R1, 101 nt for R2).

Raw data were filtered and processed using STACKS v2.66 [70,71]. Several scripts from this software package were used to convert raw reads into genotype data. Sequences were demultiplexed using PROCESS_RADTAGS using a threshold of 1 nt error in barcodes. Reads with an average Phred quality score of less than 10 (i.e., 90% probability of being correct) across a sliding window of 15% sequence length were discarded. Remaining reads were trimmed of any adapter sequence or short segments of low quality. The filtered subset of trimmed reads was used in the next step.

Next, USTACKS was used to identify alleles ("stacks") and subsequently call SNPs within samples, using a multinomial likelihood algorithm [72]. Loci needed a depth of at least 3 reads to seed a stack. Secondary stacks with 1–2 sequencing errors were retained to increase power for SNP likelihood ratio tests. A maximum of 3 nt distance (out of approximately 100 nt) was allowed between stacks in one individual toad. We assumed that sequence divergence higher than this threshold was indicative of alleles from different loci, or from paralogs.

Using CSTACKS, we then built a catalog of consensus loci across samples, providing a de novo assembly in lieu of a closely related toad genome. We chose samples from 20 distinct populations and sampling years to represent each pure species (Yosemite and Western Toad), and five based on limited availability for putative *Ab*×*c* hybrids. Meadow neighborhood and lineage [73] were used to classify Yosemite Toads. For all three groups, the sample with highest available read count was chosen. When merging loci between samples, a maximum of 3 nt distance was allowed.

The SSTACKS script was used to align each individual toad's loci (USTACKS) against the reference assembly of loci (CSTACKS). Using TSV2BAM, the aligned data were transposed into per-locus format instead of per-sample. Thus far only R1s were used to represent loci, so R2s were now associated with each R1 read pair. Then GSTACKS was used to examine each locus across samples, assemble R2s into contigs, merge with the R1s, and align reads from each sample to the locus. SNPs were identified across the entire dataset, and genotypes called for each sample using a Marukilow model [74] with an α threshold of 0.05. Multiple SNPs in one locus were phased into RAD haplotypes using a graph-based approach [75].

Finally, we used the populations script to output genotypes with a minor allele frequency (MAF) of 0.005, and maximum heterozygosity of 0.5. Loci were removed if absent from >50% individuals or >50% of a species. Once exported from STACKS, we further filtered loci that were absent from >25% of individuals, and entirely removed samples missing >90% of loci, using the POPPR v2.6.1 [76] and ADEGENET v2.1.5 [77] packages in R v4.1.2 [78].

Hybrid Detection

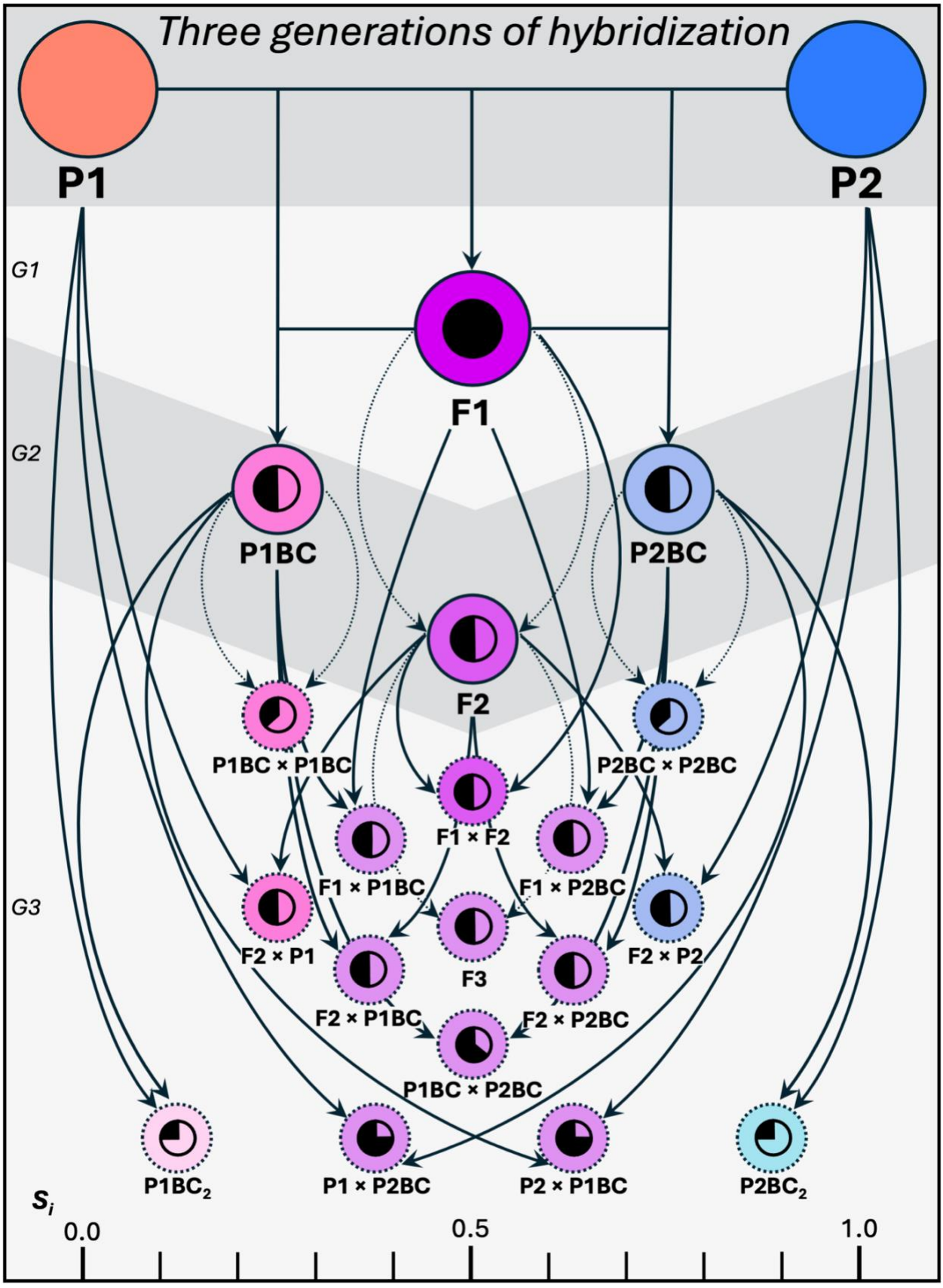
Principal component analysis (PCA) was first used to ordinate genotypes from all three classes (i.e., Western Toad, Yosemite Toad, *Ab*×*c*) into multidimensional space. PCA assumes that markers are independent, so we first used PLINK v1.9 [79] to prune a subset of markers in approximate linkage equilibrium, with pairwise R^2 no greater than 0.5. We also removed closely related samples with kinship coefficient >0.25 and (1st degree relatives) if they both had enough comparable markers (>0.25 total markers) using KING v2.2.2 [80]. We randomly sampled groups to produce even sample sizes, in accordance with PCA best practices [81,82]. Missing genotypes were replaced with the mean allelic value, and loci were zeroed and normalized by their variance. PCA was performed using the PRCOMP function in R.

We estimated proportion of ancestry from each species using an unsupervised ADMIXTURE v1.3 [83,84] analysis with K=2 ancestral components, and all unlinked markers. Although the ADMIXTURE model considers relative contributions of parent species P1 and P2 to hybrids, it does not explicitly consider successive hybrid classes. We next used NEWHYBRIDS v2.0 [85] to assign each putative *Ab*×*c* sample to simple hybrid classes: parental species (P1 or P2), first- or second-generation hybrids (F1 or F2), or first-generation backcrosses (P1-BC or P2-BC). The model uses genotype frequencies to calculate class probabilities using up to 200 markers. We calculated allele frequencies per species using the DARTR v2.9.7 package [86] and randomly selected 200 markers within 1% of fixation between species (i.e., <0.01 Western Toad and >0.99 Yosemite Toad, or vice versa). ADMIXTURE and NEWHYBRIDS were repeated 10× with random starting seeds, and results combined using 10 hillclimbing runs with CRIMP v1.1 [87].

Detecting hybrid classes beyond two generations is difficult due to an exponentially increasing number of possibilities, and their similarity to each other (Fig. 5). Two dimensions encapsulate successive hybrids: hybrid index (S_i), and inter-species heterozygosity (H_i). Hybrid index is simply the proportion of ancestry from each species, whereas H_i is an indicator of how recently hybridization occurred. The first generation contains only F1 hybrids, the sole hybrid class derived from parental species P1 and P2. At fixed loci (0.00/1.00 frequency in P1/P2), F1s are necessarily 1.00 H_i, i.e., heterozygous for 100% of loci. In the second generation, F2s are one possible hybrid class. They are the offspring of two F1s, with a reduction in H_i from 1.00 to 0.50 due to 50% of loci forming from two P1 or two P2 alleles. Third and subsequent generations could hypothetically produce additional symmetric hybrids (i.e., two F2s form an F3, two F3s form an F4, etc.), in which case H_i would remain stable at 0.50. However, this becomes increasingly unlikely because hybrids also continue to mate with pure parental species, which is termed "backcrossing." This asymmetric hybridization has several effects. First, H_i quickly declines; the pace depends upon how asymmetric the ancestry is, which is accelerated by genetic drift (smaller populations randomly lose symmetric hybrids faster). Second, the number of hybrid class possibilities becomes enormous, demonstrated by the first three generations (Fig. 5). Therefore, estimating more advanced hybrid classes is intractable with Bayesian classifiers such as NEWHYBRIDS and more easily done by evaluating S_i and H_i with simulations.

We estimated more specific hybrid ancestry (S_i and H_i) using HIEST v2.0 [88]. HIEST finds the joint maximum likelihood values of S_i and H_i for each sample, by optimizing the correspondence of the observed genotypes with proposed values of S_i and H_i. The SANN optimizer was used for 100 iterations with a starting grid of 10×10. The analysis was repeated 3× to check for convergence, and the estimate with highest log-likelihood score used. Potential hybrid classes for the observed data were simulated via 100 generations of random mating, i.e., a "hybrid swarm" scenario. For the initial generation, Western and Yosemite Toads were pooled in proportions ranging from 0.01 to 0.99, increments of 0.01. During each generation, a population size of 100 was used to simulate genetic drift. We simulated the same number of unlinked and fixed markers from HIEST analyses and introduced a missing proportion of markers for each individual by randomly sampling the missingness rates in the empirical data.

Figure 5. Three generations of hybrid classes, with expected values of hybrid index S_i (horizontal axis, colors) and inter-species heterozygosity H_i (pie charts). Backcrossing (mating with P1 or P2) reduces H_i within 1–2 generations. Dotted arrows indicate mating with the same class of hybrid. The 15 classes unique to the 3rd generation are dotted circles for distinction.



Maier and Mabe 2024

Simulated toad pedigrees were tracked in summarized form as the average number of generations since P1 and P2 ancestors. For example, a P1BC (mating between a male F1 and female P1) is 1.5 generations old, because its paternal ancestors were pure 2.0 generations ago, and its maternal ancestors were pure 1.0 generations ago. Simulated toads were binned by the nearest whole integer of generations. Since F1s are restricted to one possible point, generations 2–100 were considered. The likelihood for each bin was represented by a kernel density estimation (KDE), using the KDE2D function from the MASS v7.3.54 package [89]. A 500×500 grid spanning [0–1] and [0–1] for simulated S_i and H_i was used to capture 2-dimensional point density, choosing an optimal bandwidth based on the Sheather and Jones algorithm.

We then matched empirical toad S_i and H_i to simulated KDEs of S_i and H_i for each generation. Matching was only performed by population, to alleviate the difficulty of assigning single points to multiple overlapping groups. Point density values for each KDE were normalized by first dividing by the maximum value, to make them comparable. Each population's average KDE score was taken for each generation grouping and then ranked from highest to lowest. The ranked curve was smoothed using a loess polynomial regression with smoothing parameter 0.2. Significant matches were approximated by finding the inflection in the smoothed curve. The "elbow" of the curve was identified using the PATHVIEWR v1.1.7 package [90]. From these remaining matches, a weighted mean of hybrid ages (generations) was taken, using KDE scores as weights.

Results

Molecular and Bioinformatic Results

Our initial sample count included 877 individuals, excluding one *Anaxyrus puncatus* individual from the previous study (Table 1). Previous study samples included 535 *A. canorus* individuals from Yosemite National Park (YOSE; n = 90 meadows) and 109 individuals from Kings Canyon National Park (KICA; n = 12 meadows), with a median sample size of 5. The previous study also offered 8 singleton *A. boreas* samples (Museum of Vertebrate Zoology) from 8 locations across California, which we included. In the present study, *A. boreas* and putative *Ab*×*c* sample counts per location varied from 1 (Larson Pond) to 72 (Upper Blue Lake), with a median of 12.

Table 1. List of sampling locations and sizes used in this study.

Location	A Priori Species	Study	N
Bloodsucker Lake	ANBO	This study (*)	3
Cordova Creek	ANBO	This study (*)	13
Larson Pond	ANBO	This study (*)	1
Loon Lake	ANBO †	This study (*)	20
Meeks Meadow	ANBO	This study (*)	10
Union Valley Reservoir	ANBO	This study (*)	12
Van Vleck Pond	ANBO	This study (*)	11
Wakamatsu Farm	ANBO	This study (*)	18
Watson Lake	ANBO	This study (*)	11
Wrights Lake	ANBO	This study (*)	10
<Throughout California>	ANBO	Maier et al. 2019	8
Deer Valley	ANBOxCA	This study (*)	15
Lower Blue Lake	ANBOxCA	This study (*)	5
Twin Lake	ANBOxCA	This study (*)	24
Upper Blue Lake	ANBOxCA	This study (*)	72
Yosemite National Park	ANCA ‡	Maier et al. 2019	535
Kings Canyon National Park	ANCA ‡	Maier et al. 2019	109
Total			877

** Samples from the present study included A. boreas and putative hybrid samples only.*
† Five samples at Loon Lake, a priori assumed to contain A. boreas, were found to have some hybrid ancestry.
‡ 26 samples from the 644-sample A. canorus dataset were found to have trace amounts of hybrid ancestry.

During initial bioinformatic screening, our dataset included 7,441 loci across the 877 individuals, with a mean of 4.40 SNPs/locus (95% CI: 1–13). Once we applied the 25% per-locus cutoff and per-individual 90% missingness cutoff, 12 individuals were removed (Fig. 6). The final dataset contained 3,814 loci across 866 individuals, with mean of 4.42 SNPs/locus (95% CI: 1–12).

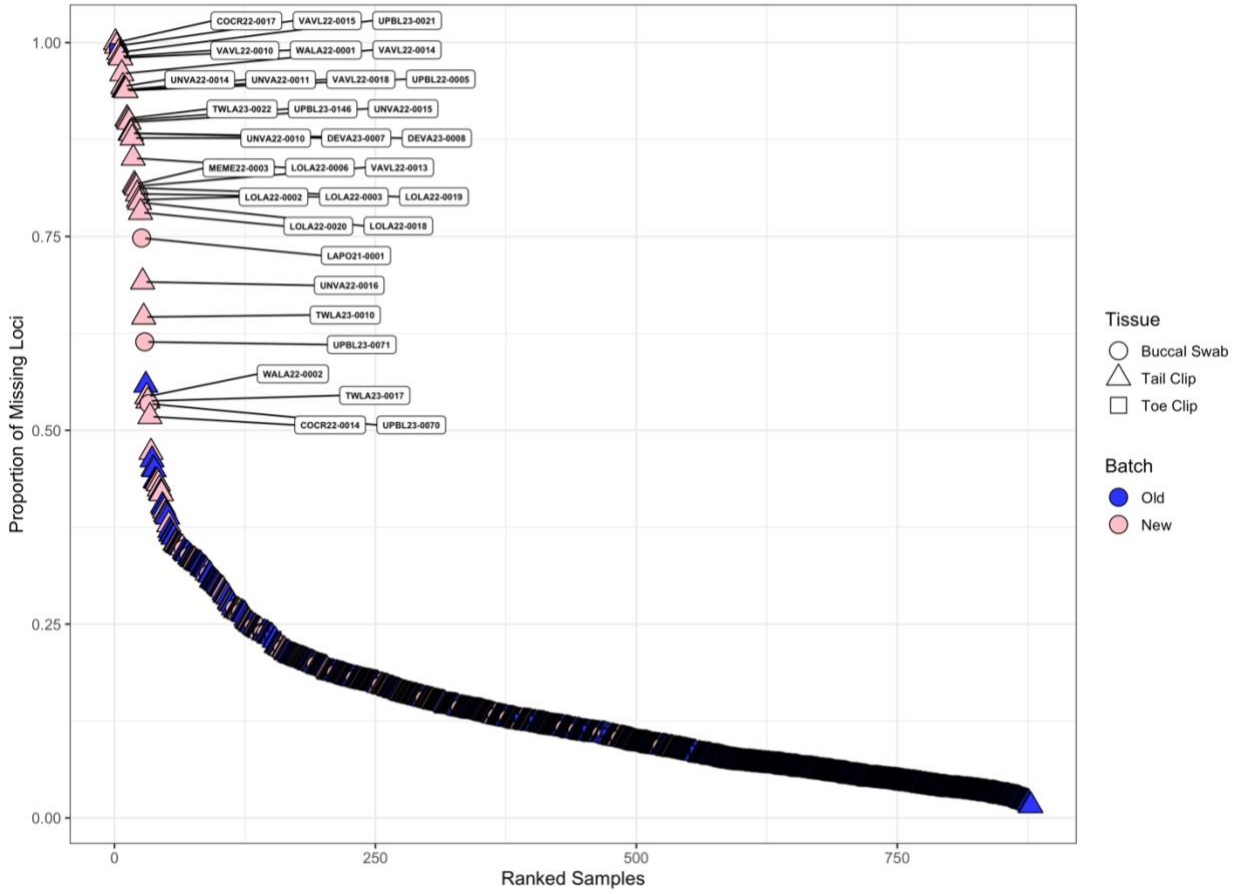


Figure 6. Proportion of missing genetic loci following ddRAD sequencing and bioinformatic processing. Samples are categorized by tissue type (buccal, tail, toe) and whether from the current study ("new") or the previous one ("old"). Sample IDs are shown for those with greater than 50% missing loci. Samples with greater than 90% missing loci were removed from analysis.

Loci were subset depending upon the analysis. For PCA and ADMIXTURE analyses, 2,189 loci were retained after pruning for linkage disequilibrium (LD). We also removed 38 first degree relatives using KING, including two *A. boreas* (Wakamatsu Farm), one putative hybrid (Upper Blue Lakes), three *A. canorus* in Kings Canyon, and thirty-two *A. canorus* in Yosemite. For NEWHYBRIDS and HIEST analyses, 1,127 SNPs were found to be fixed on different ddRAD loci. A random 200 locus subset was used for NEWHYBRIDS analyses.

Population Genetic and PCA Results

In aggregate, samples from suspected *Ab*×*c* locations were found to have much higher diversity than either pure species (Table 2). *A. boreas* is by most metrics 1.5–2× more diverse than its Pleistocene isolate *A. canorus*, whereas *Ab*×*c* hybrids showed higher diversity than *A. boreas*: 35% higher observed heterozygosity (H_O), 15% higher expected heterozygosity (H_E) and gene diversity (π), 14% higher minor allele frequency (MAF; complement of P), 38% lower fixation index (F_{IS}), and nearly 20× fewer private alleles, given that most alleles derived recently from parent species.

Table 2. Population genetic summary of each species and their hybrids, based on a priori groupings.

Species	PA	N	P	H_O	H_E	π	F_{IS}
ANBO	3063	82	0.905	0.097	0.138	0.138	0.157
ANBOxCA	165	87	0.892	0.131	0.158	0.159	0.114
ANCA	5718	495	0.939	0.049	0.089	0.089	0.183

PA = private alleles, N = effective sample size, P = frequency of most frequent allele, H_O = observed heterozygosity, H_E = expected heterozygosity, π = average gene diversity, F_{IS} = fixation index.

PCA results showed a cluster of *Ab×c* samples intermediate between species along the primary dimension (PC1; 9.18% of variance), but more extreme than either species in the second one (PC2; 4.12% of variance) (Fig. 7). This suggested that PC1 might represent hybrid ancestry, and PC2 might represent novel inter-species diversity not present in either parent species.

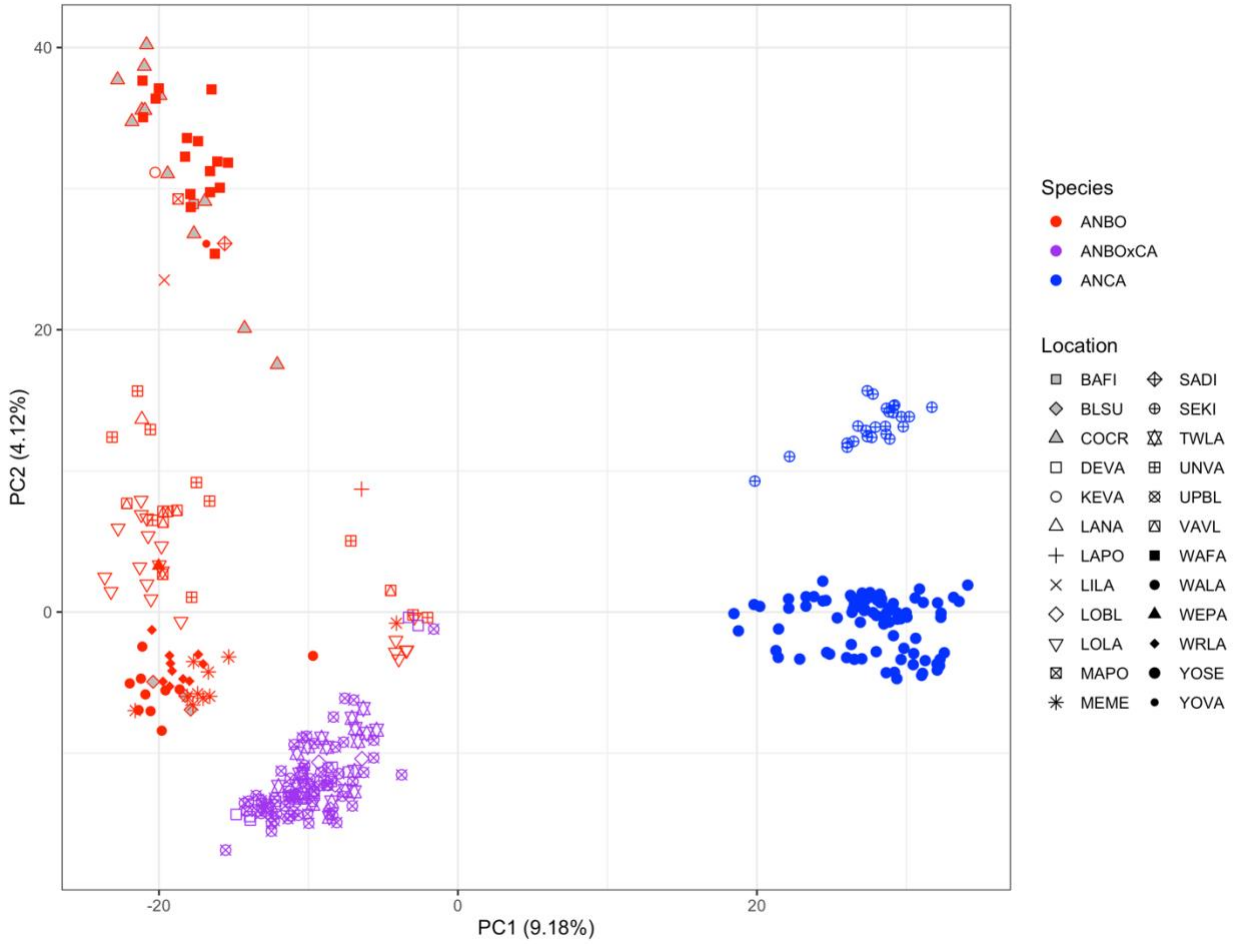


Figure 7. Principal component analysis of all unrelated samples Species codes: ANBO = Anaxyrus boreas, ANBOxCA = hybrid Anaxyrus boreas × canorus, ANCA = Anaxyrus canorus.. Sample codes: BAFI = Bakersfield, BLSU = Bloodsucker Lake, COCR = Cordova Creek, DEVA = Deer Valley, KEVA = Kelso Valley, LANA = Lassen National Park, LAPO = Larson Pond, LILA = Little Lake, LOBL = Lower Blue Lake, LOLA = Loon Lake, MAPO = Mariposa, MEME = Meeks Meadow, SADI = San Diego, SEKI = Sequoia Kings National Park, TWLA = Twin Lake, UNVA = Union Valley Reservoir, UPBL = Upper Blue Lake, VAVL = Van Vleck Pond, WAFA = Wakamatsu Farm, WALA = Watson Lake, WEPA = Westguard Pass, WRLA = Wrights Lake, YOSE = Yosemite National Park, YOVA = Yosemite Valley.

Spearman's rank correlation was computed to estimate a possible relationship between PC2 and inter-species H_O, specifically the ratio between *Ab×c* H_O and the mean H_O of parent species *A. boreas* and *A. canorus*. There was a positive correlation between the absolute PC2 loading of each locus and this H_O ratio (ρ=0.24, $p<0.001$). In contrast, PC1 showed a negative correlation (ρ=-0.23, $p<0.001$). This pattern is consistent with hybridization and suggests that PC1 and PC2 correspond to S_i and H_i as defined earlier. Notably, hybrids were intermediate between species on PC1, but much closer to *A. boreas* than *A. canorus*.

Recent and Advanced Hybrid Detection

ADMIXTURE reinforced the PCA results by showing putative *Ab×c* individuals to have asymmetric hybrid ancestry (Fig. 8; top panel). Across the four sampled locations, *A. canorus* ancestry was on average 6.63%, with a range of 0.11–20.39%. There were also 14 individuals (12%) in this region estimated to be pure *A. boreas*. Surprisingly, we also found trace levels of hybrid ancestry outside of Upper/Lower Blue Lakes, Deer Valley, and Twin Lake. Five (25%) of the Loon Lake samples, west of Lake Tahoe, had *A. canorus* ancestry between 0.69 – 6.64%, with an average of 3.47%. Two previously tested specimens from the Museum of Vertebrate Zoology (locations in Bakersfield and Mariposa, CA) also had 0.70% and 0.42% trace amounts. Curiously, the one Yosemite Valley sample was a pure *A. boreas* individual. Twenty-six (4%) of the *A. canorus* samples had *A. boreas* ancestry between 0.14–3.79%, with an average of 0.91%. Locations with highest admixture were atop Ribbon Falls (nearby N Yosemite Valley), and above Bridalveil Falls (nearby S Yosemite Valley). See Tables 3–4 for a summary of hybrid ADMIXTURE percentages by location.

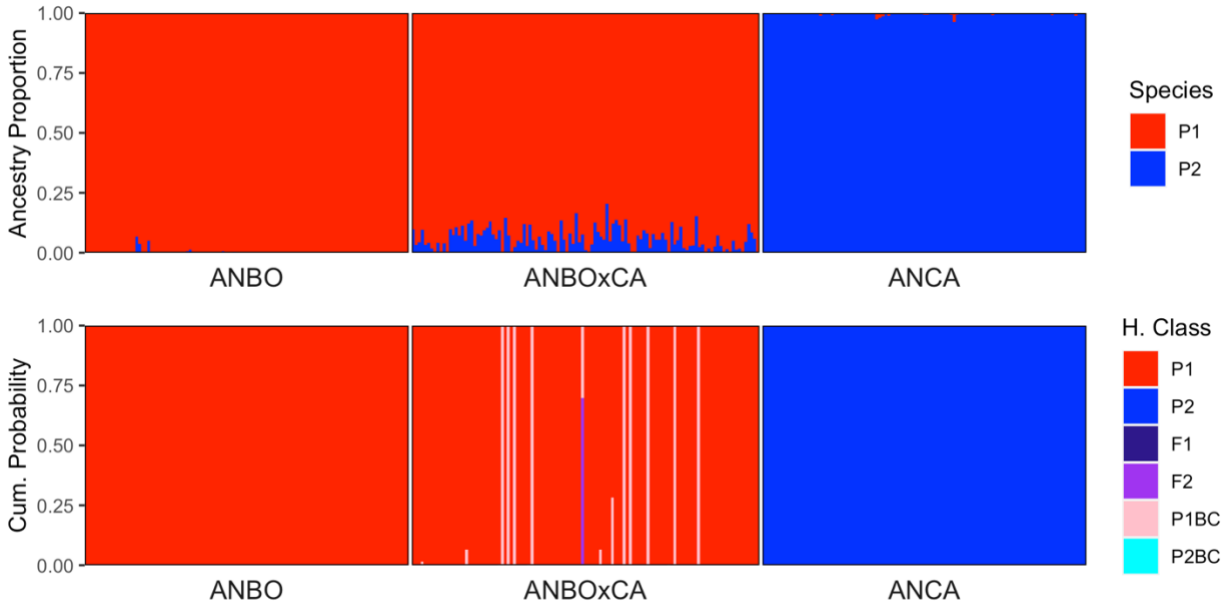


Figure 8. Results of ADMIXTURE (top) and NEWHYBRIDS (bottom) analyses. The top panel estimates ancestry proportion from two ancestral species, whereas the bottom panel estimates probability of specific hybrid classes within two generations of hybridization. A priori species groupings are identical to previous results and map figures, which did not presume hybrid ancestry at the Loon Lake (A. boreas "ANBO") location that shows some hybrid ancestry (see red bars with P2 > 0), or in any A. canorus "ANCA" samples (see blue bars with P1 > 0). Note: approximately 85% of ANCA samples are not shown to achieve equal group sizes and save plotting space.

NEWHYBRIDS classified just 20 of the putative *Ab×c* samples as possible 2nd generation hybrids (Fig. 8; bottom panel). The remaining 93 individuals (82%) in this region were estimated to be pure *A. boreas*, meaning that they are likely more advanced hybrid classes. Out of the 20, one was estimated to be an F2, nine were more than half likely to be P1BC, and ten were less than half likely to be P1BC. Their ADMIXTURE coefficients of *A. canorus* ancestry ranged from 4.10–20.39%, with an average of 11.79%. Altogether, these results suggest that most putative *Ab×c* individuals are advanced *A. boreas* backcrosses more than two generations old, but several samples may be close to two generations old.

Table 3. Estimated hybrid ancestry by location for all sampled A. boreas and anticipated hybrid locations.

Location	A Priori Species	N (Hybrid)	N (Total)	ANCA (%)	S_i (%)	H_i (%)	Age (Gens)
Upper Blue Lake	ANBOxCA	61	70	0.11 – 20.39	4 – 27	6 – 30	5 (2–11)
Lower Blue Lake	ANBOxCA	5	5	4.89 – 13.45	12 – 21	17 – 28	7 (2–36)
Deer Valley	ANBOxCA	12	15	1.64 – 10.73	3 – 18	6 – 25	5 (2–11)
Twin Lake	ANBOxCA	21	23	1.22 – 14.56	5 – 21	8 – 28	8 (2–71)
Loon Lake	ANBO	5	20	0.69 – 6.64	3 – 7	6 – 14	8 (2–36)
Bakersfield	ANBO	1	1	0.70	0	0	.
Mariposa	ANBO	1	1	0.42	0	0	.

Table 4.Estimated trace hybrid ancestry by location for all previously sampled A. canorus locations.

Park	Lineage	Neighborhood	Meadow	N (Hybrid)	N (Total)	ANBO (%)	S_i (%)	H_i (%)	Age (Gens)
YOSE	North	Tilden	4370	1	10	0.54	0	0	44 (2–94)
YOSE	North	Kerrick	4146	1	5	1.17	0	0	44 (2–94)
YOSE	North	Kerrick	4136	1	10	0.84	0	0	44 (2–94)
YOSE	North	Kerrick	4164	1	5	0.36	0	0	44 (2–94)
YOSE	West	Ribbon 1	1841	2	5	2.04 – 3.79	2 – 2.5	2 – 3	44 (2–94)
YOSE	West	Ribbon 1	1779	2	5	0.48 – 0.74	0	0	44 (2–94)
YOSE	West	Ribbon 1	2030	1	5	0.14	2	2	44 (2–94)
YOSE	West	Bald	2369	1	10	0.62	0	0	44 (2–94)
YOSE	West	White Wolf	2385	1	5	0.35	0	0	44 (2–94)
YOSE	East	Tioga	2256	1	2	0.35	0	0	44 (2–94)
YOSE	ES-Adm	Isberg	1097	1	11	0.51	0	0	44 (2–94)
YOSE	South	Bridalveil	1040	3	5	1.07 – 2.59	0 – 1	0 – 2	44 (2–94)
YOSE	South	Bridalveil	1070	1	2	0.6	0	0	44 (2–94)
YOSE	South	Bridalveil	1171	1	5	0.53	2	1	44 (2–94)
YOSE	South	Bridalveil	359	1	5	0.74	0	0	44 (2–94)
YOSE	South	Chilnualna	377	1	5	0.98	0	0	44 (2–94)
YOSE	South	Chilnualna	780	1	5	0.53	0	0	44 (2–94)
YOSE	South	Chilnualna	719	1	5	0.53	0	0	44 (2–94)
KICA	Goddard	Emerald	916	3	22	0.27 – 1.12	0	0	44 (2–94)
KICA	Evolution	Sapphire	958	1	10	0.46	0	0	44 (2–94)

HIEST and comparative simulations found evidence of hybridization within several generations. S_i values were nearly identical to ADMIXTURE coefficients (ρ=0.999, p<0.001), and H_i values ranged from 4–30%, with an average of 16.81% (Fig. 9; left panel). Simulations showed that different hybrid classes may have highly overlapping values of S_i and H_i, particularly when backcrossed (Fig. 9; right panel).

Out of the four a priori *Ab×c* populations, we estimated the following ages of hybridization: Upper Blue Lake, 5 (2–11) generations old; Lower Blue Lake, 7 (2–36) generations old; Deer Valley, 5 (2–11) generations old; and Twin Lake, 8 (2–71) generations old (Table 3). For the five hybrid individuals at Loon Lake, we estimated an age of 8 (2–36) generations old. The aggregated twenty-six samples of *A. canorus* with trace *A. boreas* ancestry had a much older estimated age of 44 (2–94) generations (Table 4). There is a caveat that the previously mentioned Ribbon and Bridalveil Falls samples near Yosemite Valley contained substantially more admixture than the others and could be somewhat younger than 44 generations.

Notably, our age estimates are averaged across the toad's pedigree. This means a 5-gen-old hybrid toad might have hybrid ancestors from more than 5 gens ago, yet more recent backcrossing to pure toads reduced the average. For example, a 5th gen Upper Blue Lakes hybrid with 12.5% *A. canorus* ancestry likely hybridized 6 gens ago, but then backcrossed to *A. boreas* every 2 gens thereafter. Similarly, an 8th gen Loon Lake hybrid with 3% *A. canorus* ancestry likely hybridized 10 gens ago, but then backcrossed to *A. boreas* every 2 gens thereafter. Most hybrids detected in the NCZ are consistent with original hybridization times 1–2 generations older than the reported average, and then backcrossing every 2–4 generations.

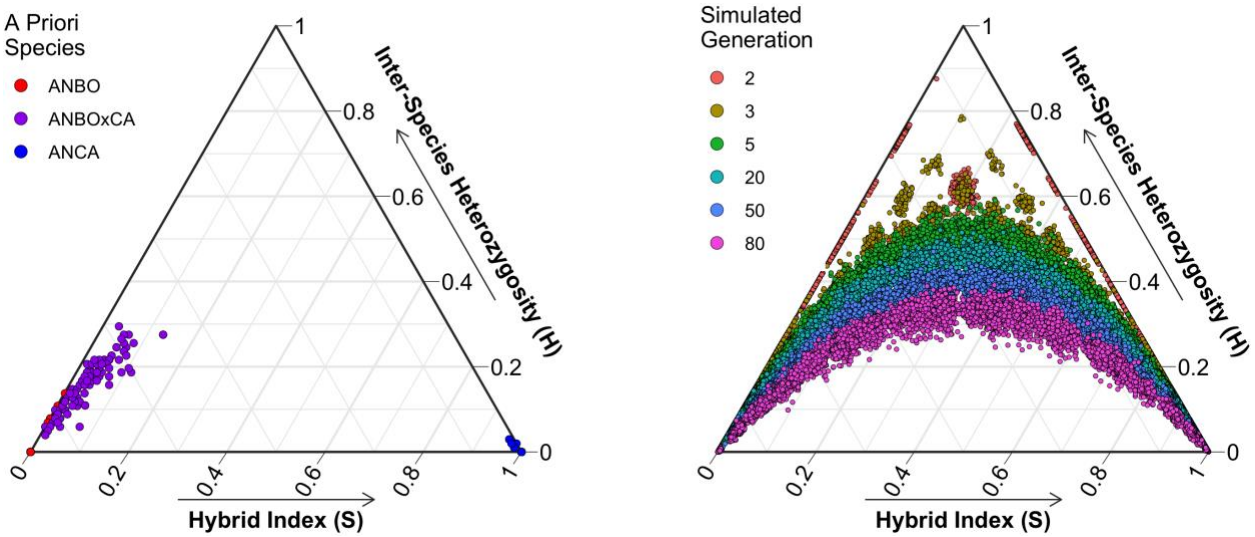


Figure 9. Results of HIest analysis of empirical toad data (left) and comparative simulations (right). A priori species groupings are identical to previous results and map figures, which did not presume hybrid ancestry at the Loon Lake (A. boreas "ANBO") location that shows some hybrid ancestry (see red points with $H_i > 0$), or in any A. canorus "ANCA" samples (see blue points with $H_i > 0$). Simulated hybrids are grouped by the mean number of generations since pure ANBO or ANCA individuals comprised their pedigrees. Note: many points are overlapping.

Discussion

Implications for Conservation

For nearly seven decades there has been intrigue and disagreement over whether Yosemite Toads hybridize with their more widespread sister species [31,36]. We have finally settled that old question with clear and unambiguous evidence that Yosemite Toads and Western Toads have hybridized in the NCZ near Blue Lakes and continue to do so. Based on our estimate of 5–6 generations since the most recent hybrids were spawned, and assuming D. Mullally's observations in 1956 were correct, new hybrids may have been produced 3 or 4 times since his initial observation. Asymmetric backcrossing with Western Toads every several generations might indicate their numerical advantage in the region, or suggest more symmetrical hybridization farther south.

Of course, the next question becomes whether this natural process is somehow deleterious for either species, given the long history of negative connotation toward hybrids in conservation [91,92]. For example, the Yosemite Toad conservation assessment [34] asked whether genetic swamping is occurring, and whether this will be more problematic if climate change causes upward range shifts and more niche overlap. Certainly, climate change has already been shown to be driving upward range shifts within the Yosemite Toad [45], so it is reasonable to assume both species may experience new distributions in the century ahead.

Hybrids should not be perceived in terms of universal benefit or harm; context is essential. Although there is the potential for genomic incompatibilities to cause outbreeding depression, there is a countervailing opportunity for novel genetic variation to quell inbreeding depression suffered by a depauperate species such as the Yosemite Toad [49]. Whether short-term heterosis or relief from inbreeding depression outweighs potential disruption of important local adaptations (e.g. drought or disease resistance) depends upon how genetically and ecologically divergent the species are. Hybrids are not universally unfit, but rather have variable fitness, and on average tend to have equal fitness to parents or higher fitness than at least one parent [62]. Studies are needed to measure multiple fitness components, including genetic transect sampling to infer natural selection from the shape of a hybrid zone, and reciprocal transplant experiments to evaluate fitness directly [93].

The NCZ near Blue Lakes contains hybrids that are primarily Western Toad in ancestry, which opens the possibility of a broader contact zone, with backcrossed Yosemite Toads south of Eldorado National Forest. Sampling that 55 km transect between the NCZ and Yosemite National Park could reveal the width and directional movement of the complete hybrid zone, not to mention infer hybrid fitness. Hybrid zones are really a spectrum [91], between sterile F1s and zero gene flow [94,95], to wider but stable zones [61], to complete erasure of species boundaries [96,97]. Sometimes hybrids may even isolate from both parents to fuse into a third lineage, a phenomenon that has been observed intraspecifically for Yosemite Toads [33]. Our results suggest that the NCZ is a stable zone, although its width and hybrid fitness remain unknown. Species integrity appears intact given the relatively sharp boundary between primarily Western Toads at Deer Valley and presumably Yosemite Toads at the well-studied Highland Lakes, only 13 km away. Our observation of trace hybrid ancestry outside any contact zone (e.g., Loon Lake, Yosemite National Park), suggests that adaptive introgression could be at play, a process that filters only beneficial genetic material from hybrids into one or both genetic backgrounds [43].

Our detection of trace Western Toad ancestry among both northern and southern Yosemite Toad populations (e.g., Ribbon and Bridalveil Falls in Yosemite, Emerald Peak in Kings Canyon) corroborates earlier work showing mitochondrial introgression between species [23,42,98]. Locally, the two species are more closely related to each other than to their own conspecifics overall. This pattern likely emerged as female Western Toads locally interbred with male Yosemite Toads, leaving behind a polyphyetic pattern in their shared mitochondrial tree. We estimated these events to be quite old on average (i.e., 44 generations or c. 150 years), although the low elevation locations in Yosemite have higher admixture and are probably more recent. Notably, these locations have experienced greatest rates of Yosemite Toad extirpation [99], which might reduce future introgression between species.

Stable contact zones exist along a spectrum based on which environment (if any) favors hybrids. Tension zones emerge when hybrids are unfit everywhere regardless of environment, resulting in balance between selection against hybrids and incoming migration [61,64]. Since they are environmentally neutral, they can move around, and persist in equilibrium for long periods of time. However, decades of survey work and morphological classification do not indicate a turnover in species or hybrid composition [31,34,36,38]. Other stable contact zones involve hybrids with superior fitness in particular environments, such as the zone itself ("bounded hybrid superiority"), sporadic patches of landscape ("mosaic"), or for special genotype-environment associations ("evolutionary novelty") [64]. If future genetic work reveals a spatial patchwork of different hybrid proportions without any clear gradient, local adaptation could be responsible.

Regardless of which hybrid zone model best explains these two toads, conservation discussions should focus on demographic and evolutionary impacts to populations rather than semantics of species bounds [100]. Given that *Ab×c* hybridization is a natural evolutionary process (barring any increase from climate change), it has potential to be a conservation threat if hybrid populations have higher extinction risk overall, or if they have deleterious effects on pure species [50]. For example, hybrid *Ambystoma* salamanders in California threaten natives by growing faster and consuming native prey [101]. Similarly, if Western Toads encroach southward and begin to cause genomic extinction of Yosemite Toads, they may constitute a threat. However, in most cases we should expect the best combination of locally adapted and hybrid genotypes to thrive, resulting in the best possible combination of native and novel genes. Therefore, even if future migration were to hypothetically occur, introgressed Yosemite Toads may be a valuable taxon to protect.

Legal treatment of hybrids can have direct biological effect via recovery efforts. In 1990 the USFWS reversed their earlier policy to discourage protection of hybrids; however, they also did not enact their 1996 draft "intercross" policy to formally protect introgressed populations [92]. The ESA today gives no official guidance on how to treat natural or anthropogenic hybrids. Failure to protect introgressed Yosemite Toads based on introgressed status could potentially shrink an already small, threatened population to catastrophic levels, and have the opposite of the intended consequence [102]. Science cannot tell us when we ought to conserve hybrids and protecting them could be vital if they occupy vacant niche space left behind by extirpated parent species [103].

Possible Causes of Hybridization

Mature species are often capable of hybridizing without eroding the integrity of either lineage. Hybridization is much more commonplace than once thought, comprising at least 10% of animal species [104]. Toads might be more predisposed to hybridization for several reasons. First, amphibian reproductive isolation appears to be a slow process that involves hundreds of small

genetic loci, rather than one or several big incompatibilities [19]. Second, unlike mammals and birds, most frogs and toads have homomorphic (indistinguishable) sex chromosomes, protecting XY males and ZW females from rapid evolution of hybrid dysfunction [19,105]. Toad species require exceptionally long periods of separation before they develop postzygotic isolation mechanisms, such as infertility and inviability [18]. Western and Yosemite Toads only diverged two million years ago during the Pleistocene [33] which puts them well within the realm of genomic compatibility for toads.

Sullivan [106] has argued that premating courtship behaviors such as advertisement calls need not diverge for speciation to proceed. However, acoustic experiments have shown that female Yosemite Toads have a clear preference for conspecifics (Western Toads lack an advertisement call, or acoustic preference); therefore, similar vocalizations likely do not promote hybridization [107]. Time of breeding might play a role in either isolating species or promoting hybridization [41], because phenological separation is often observed in other pairs of *Anaxyrus* species, yet toads in the Sierra Nevada are forced to adapt to the same foreshortened snow-free season. Another factor in the NCZ for Western and Yosemite Toads is their similar ecologies. Although the two species normally differ in their selection of natal pond depth and tadpole aggregation behavior [108], the NCZ is a boreal zone of high latitude which may encourage more compatible local adaptations and reduce postzygotic isolation. This would also explain why the NCZ is broader than other putative contact zones, which include starker ecological gradients. Altogether, the two species in the NCZ may be predisposed to hybridize for genomic and ecological reasons.

Conclusions

We have described the first genomic hybrid panel for *Anaxyrus boreas* × *canorus*, and answered a decades-old question about the nature of unidentified toads in the Blue Lakes region of Eldorado National Forest. We found that 88% of samples tested were backcrossed hybrids, containing an average of 93.5% ancestry from Western Toads, and 6.5% from Yosemite Toads. Moreover, these hybrids have an estimated origin less than ten generations ago, which taken with the initial observations from the 1950s, suggests hybridization occurs every 5–6 generations, followed by backcrossing to the Western Toad every 2–4 generations. The conservation implications of these individuals are uncertain without more densely sampling a transect across the entire contact zone to infer hybrid fitness. However, the novel genetic diversity introduced into the genetically depauperate Yosemite Toad may fuel future adaptation to stressors such as climate change and disease and outweigh any potential risk of disturbing existing adaptations. There is no indication from the available data of range shifts or genetic swamping. Interestingly, more ancient introgression is evident within several Yosemite Toad populations in Yosemite and Kings Canyon National Parks. Future work should first fill in sampling gaps to perform hybrid cline analysis, and ideally assess hybrid fitness directly using common garden experiments.

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Author Contributions

Both authors designed the spatial sampling scheme. JAM secured funding, coordinated lab work, collected new field samples, and extracted DNA. PAM advised the selection of lab contracts, performed bioinformatics, developed the hybrid panel, wrote all scripts, and wrote this report.

Data accessibility

Raw sequencing data (FASTQs) and R scripts used to conduct the analysis can be made available upon reasonable request to the authors.

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Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Bloodsucker Lake	BLSU	2022	BLSU22-0001	ANBO	Tail Clip	Tadpole	26.2	500.0	8.5	4,796,660	4,773,304	2,509	12%	130	TRUE
Bloodsucker Lake	BLSU	2022	BLSU22-0002	ANBO	Tail Clip	Tadpole	11.8	418.9	.	4,710,761	4,700,110	2,287	20%	106	TRUE
Bloodsucker Lake	BLSU	2022	BLSU22-0003	ANBO	Tail Clip	Tadpole	22.8	500.0	8.3	3,356,847	3,348,842	2,241	21%	97	TRUE
Cordova Creek	COCR	2022	COCR22-0001	ANBO	Tail Clip	Tadpole	13.7	300.0	6.9	1,630,916	1,627,862	1,995	30%	78	TRUE
Cordova Creek	COCR	2022	COCR22-0002	ANBO	Buccal Swab	Adult	153.0	500.0	6.3	1,206,891	1,200,941	1,951	31%	48	TRUE
Cordova Creek	COCR	2022	COCR22-0003	ANBO	Buccal Swab	Adult	69.7	500.0	6.6	1,197,090	1,190,777	2,165	24%	46	TRUE
Cordova Creek	COCR	2022	COCR22-0004	ANBO	Buccal Swab	Adult	152.0	500.0	6.7	2,298,465	2,286,259	2,337	18%	70	TRUE
Cordova Creek	COCR	2022	COCR22-0006	ANBO	Tail Clip	Tadpole	80.4	500.0	7	3,757,385	3,734,910	2,489	13%	118	TRUE
Cordova Creek	COCR	2022	COCR22-0010	ANBO	Tail Clip	Tadpole	23.4	500.0	8.3	4,123,427	4,112,830	2,087	27%	114	TRUE
Cordova Creek	COCR	2022	COCR22-0011	ANBO	Tail Clip	Tadpole	12.5	443.8	9.2	4,944,497	4,922,015	2,390	16%	134	TRUE
Cordova Creek	COCR	2022	COCR22-0012	ANBO	Tail Clip	Tadpole	11.6	411.8	9.4	3,568,191	3,550,325	2,322	18%	99	TRUE
Cordova Creek	COCR	2022	COCR22-0013	ANBO	Tail Clip	Tadpole	9.5	338.7	9.4	4,227,963	4,203,754	2,386	16%	124	TRUE
Cordova Creek	COCR	2022	COCR22-0014	ANBO	Tail Clip	Tadpole	6.3	222.9	9.3	635,551	633,145	1,373	52%	38	TRUE
Cordova Creek	COCR	2022	COCR22-0015	ANBO	Tail Clip	Tadpole	10.7	379.9	9.3	3,435,663	3,418,292	2,346	18%	103	TRUE
Cordova Creek	COCR	2022	COCR22-0016	ANBO	Tail Clip	Tadpole	4.1	145.6	9.3	2,021,559	2,012,737	2,085	27%	73	TRUE
Cordova Creek	COCR	2022	COCR22-0017	ANBO	Tail Clip	Tadpole	4.1	145.6	9	2,072	1,757	1	100%	0	FALSE
Deer Valley	DEVA	2023	DEVA23-0001	ANBOxCA	Tail Clip	Tadpole	10.1	300.0	9	3,220,845	3,213,166	2,367	17%	93	TRUE
Deer Valley	DEVA	2023	DEVA23-0002	ANBOxCA	Tail Clip	Tadpole	11.0	300.0	8.6	3,839,795	3,829,364	2,435	14%	109	TRUE
Deer Valley	DEVA	2023	DEVA23-0003	ANBOxCA	Tail Clip	Tadpole	10.7	300.0	6.4	2,431,940	2,425,648	2,344	18%	97	TRUE
Deer Valley	DEVA	2023	DEVA23-0004	ANBOxCA	Tail Clip	Tadpole	13.2	300.0	9.5	2,982,105	2,974,723	2,239	21%	105	TRUE
Deer Valley	DEVA	2023	DEVA23-0005	ANBOxCA	Tail Clip	Tadpole	7.1	300.0	9.4	6,492,105	6,475,428	2,259	21%	151	TRUE
Deer Valley	DEVA	2023	DEVA23-0006	ANBOxCA	Tail Clip	Tadpole	10.7	300.0	9.4	3,592,405	3,581,953	2,422	15%	133	TRUE
Deer Valley	DEVA	2023	DEVA23-0007	ANBOxCA	Tail Clip	Tadpole	7.9	300.0	9.3	63,716	61,989	334	88%	5	TRUE
Deer Valley	DEVA	2023	DEVA23-0008	ANBOxCA	Tail Clip	Tadpole	6.9	300.0	9.3	70,152	69,526	333	88%	5	TRUE
Deer Valley	DEVA	2023	DEVA23-0009	ANBOxCA	Buccal Swab	Adult	49.9	300.0	5.6	3,393,449	3,382,835	2,518	12%	78	TRUE
Deer Valley	DEVA	2023	DEVA23-0010	ANBOxCA	Buccal Swab	Adult	48.9	300.0	5.3	2,221,528	2,213,197	2,474	13%	48	TRUE
Deer Valley	DEVA	2023	DEVA23-0011	ANBOxCA	Buccal Swab	Adult	14.5	300.0	6.9	4,037,483	4,024,729	2,542	11%	96	TRUE
Deer Valley	DEVA	2023	DEVA23-0012	ANBOxCA	Buccal Swab	Adult	50.3	300.0	5.5	2,143,751	2,136,731	2,556	10%	53	TRUE
Deer Valley	DEVA	2023	DEVA23-0013	ANBOxCA	Buccal Swab	Adult	52.8	300.0	6.2	5,952,700	5,932,201	2,444	14%	119	TRUE
Deer Valley	DEVA	2023	DEVA23-0014	ANBOxCA	Buccal Swab	Adult	36.4	300.0	6.5	3,394,547	3,378,703	2,586	9%	86	TRUE

Appendix

Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Deer Valley	DEVA	2023	DEVA23-0015	ANBOxCA	Buccal Swab	Adult	43.7	300.0	5.9	3,044,938	3,035,802	2,541	11%	67	TRUE
Larson Pond	LAPO	2021	LAPO21-0001	ANBO	Buccal Swab	Adult	11.2	397.6	7	315,871	314,960	718	75%	23	TRUE
Loon Lake	LOLA	2022	LOLA22-0001	ANBO	Tail Clip	Tadpole	6.4	228.6	9.2	1,930,297	1,920,733	2,435	14%	83	TRUE
Loon Lake	LOLA	2022	LOLA22-0002	ANBO (*)	Tail Clip	Tadpole	12.4	440.2	8.4	137,371	134,407	578	80%	8	TRUE
Loon Lake	LOLA	2022	LOLA22-0003	ANBO (*)	Tail Clip	Tadpole	24.0	500.0	8.5	223,239	218,607	556	80%	7	TRUE
Loon Lake	LOLA	2022	LOLA22-0004	ANBO	Tail Clip	Tadpole	13.1	465.1	9.4	9,762,632	9,694,815	2,295	19%	287	TRUE
Loon Lake	LOLA	2022	LOLA22-0005	ANBO	Tail Clip	Tadpole	8.2	290.0	8.4	10,841,764	10,781,974	2,123	25%	227	TRUE
Loon Lake	LOLA	2022	LOLA22-0006	ANBO (*)	Tail Clip	Tadpole	10.1	358.6	9	116,893	113,644	425	85%	6	TRUE
Loon Lake	LOLA	2022	LOLA22-0007	ANBO	Tail Clip	Tadpole	7.6	270.9	9.3	3,064,223	3,047,886	2,538	11%	123	TRUE
Loon Lake	LOLA	2022	LOLA22-0008	ANBO	Tail Clip	Tadpole	9.7	345.4	9.4	3,990,896	3,964,183	2,515	12%	142	TRUE
Loon Lake	LOLA	2022	LOLA22-0009	ANBO	Tail Clip	Tadpole	14.5	514.8	9.3	4,820,101	4,786,325	2,427	15%	149	TRUE
Loon Lake	LOLA	2022	LOLA22-0010	ANBO	Tail Clip	Tadpole	10.1	358.6	9	3,141,516	3,115,602	2,514	12%	117	TRUE
Loon Lake	LOLA	2022	LOLA22-0011	ANBO	Tail Clip	Tadpole	11.5	408.3	9.2	2,914,779	2,899,451	2,519	11%	111	TRUE
Loon Lake	LOLA	2022	LOLA22-0012	ANBO	Tail Clip	Tadpole	18.1	500.0	9.5	2,327,436	2,310,804	2,566	10%	88	TRUE
Loon Lake	LOLA	2022	LOLA22-0013	ANBO	Tail Clip	Tadpole	10.6	376.3	9	3,757,264	3,732,036	2,448	14%	132	TRUE
Loon Lake	LOLA	2022	LOLA22-0014	ANBO	Tail Clip	Tadpole	9.1	323.1	9.4	3,098,380	3,080,914	2,539	11%	122	TRUE
Loon Lake	LOLA	2022	LOLA22-0015	ANBO	Tail Clip	Tadpole	13.6	482.8	8.7	5,659,752	5,632,218	2,496	12%	156	TRUE
Loon Lake	LOLA	2022	LOLA22-0016	ANBO	Tail Clip	Tadpole	9.3	330.5	9.5	5,944,746	5,910,733	2,400	16%	174	TRUE
Loon Lake	LOLA	2022	LOLA22-0017	ANBO	Tail Clip	Tadpole	14.0	497.0	9.4	7,049,592	7,009,043	2,261	21%	167	TRUE
Loon Lake	LOLA	2022	LOLA22-0018	ANBO	Tail Clip	Tadpole	17.2	500.0	8.9	219,478	216,279	587	79%	8	TRUE
Loon Lake	LOLA	2022	LOLA22-0019	ANBO	Tail Clip	Tadpole	15.0	500.0	9.1	105,977	103,042	534	81%	7	TRUE
Loon Lake	LOLA	2022	LOLA22-0020	ANBO (*)	Tail Clip	Tadpole	10.2	362.1	9.4	267,022	262,572	624	78%	8	TRUE
Lower Blue Lake	LOBL	2023	LOBL23-0001	ANBOxCA	Tail Clip	Tadpole	6.8	300.0	9.2	3,297,501	3,289,829	2,354	17%	96	TRUE
Lower Blue Lake	LOBL	2023	LOBL23-0002	ANBOxCA	Tail Clip	Tadpole	10.7	300.0	9.6	4,291,327	4,280,072	2,402	16%	104	TRUE
Lower Blue Lake	LOBL	2023	LOBL23-0003	ANBOxCA	Tail Clip	Tadpole	9.5	300.0	9.2	3,394,783	3,375,120	2,406	15%	97	TRUE
Lower Blue Lake	LOBL	2023	LOBL23-0004	ANBOxCA	Tail Clip	Tadpole	12.3	300.0	9.1	2,936,326	2,929,346	2,091	27%	74	TRUE
Lower Blue Lake	LOBL	2023	LOBL23-0005	ANBOxCA	Tail Clip	Tadpole	10.3	300.0	9.6	4,846,478	4,833,844	2,468	13%	142	TRUE
Meeks Meadow	MEME	2022	MEME22-0001	ANBO	Tail Clip	Tadpole	13.4	475.7	8.9	8,923,893	8,877,275	2,219	22%	193	TRUE
Meeks Meadow	MEME	2022	MEME22-0002	ANBO	Tail Clip	Tadpole	10.7	379.9	9.4	5,087,784	5,056,208	2,568	10%	164	TRUE

Maier and Mabe 2024

Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Meeks Meadow	MEME	2022	MEME22-0003	ANBO	Tail Clip	Tadpole	19.9	500.0	9	183,293	100,497	522	82%	7	TRUE
Meeks Meadow	MEME	2022	MEME22-0004	ANBO	Tail Clip	Tadpole	14.2	504.1	9.1	17,754,525	17,707,519	1,947	32%	174	TRUE
Meeks Meadow	MEME	2022	MEME22-0005	ANBO	Tail Clip	Tadpole	16.9	500.0	6.7	3,805,060	3,766,345	1,850	35%	63	TRUE
Meeks Meadow	MEME	2022	MEME22-0006	ANBO	Tail Clip	Tadpole	15.8	500.0	9.1	3,767,845	3,715,566	1,877	34%	71	TRUE
Meeks Meadow	MEME	2022	MEME22-0007	ANBO	Tail Clip	Tadpole	11.2	397.6	9.3	3,794,228	3,747,763	1,912	33%	72	TRUE
Meeks Meadow	MEME	2022	MEME22-0008	ANBO	Tail Clip	Tadpole	10.4	369.2	9	7,025,625	7,009,939	2,224	22%	109	TRUE
Meeks Meadow	MEME	2022	MEME22-0009	ANBO	Tail Clip	Tadpole	19.0	500.0	5.9	3,412,569	3,374,255	1,838	35%	52	TRUE
Meeks Meadow	MEME	2022	MEME22-0010	ANBO	Tail Clip	Tadpole	21.1	500.0	8.2	4,278,929	4,221,862	1,913	33%	76	TRUE
Twin Lake	TWLA	2023	TWLA23-0001	ANBOxCA	Tail Clip	Tadpole	9.4	300.0	9.5	4,823,418	4,811,521	2,347	18%	138	TRUE
Twin Lake	TWLA	2023	TWLA23-0002	ANBOxCA	Tail Clip	Tadpole	1.1	210.0	8.5	662,014	660,047	1,770	38%	30	TRUE
Twin Lake	TWLA	2023	TWLA23-0003	ANBOxCA	Tail Clip	Tadpole	1.1	222.0	8.5	572,807	571,225	1,565	45%	30	TRUE
Twin Lake	TWLA	2023	TWLA23-0004	ANBOxCA	Tail Clip	Tadpole	1.1	218.0	9	858,411	856,159	1,855	35%	42	TRUE
Twin Lake	TWLA	2023	TWLA23-0005	ANBOxCA	Tail Clip	Tadpole	2.6	300.0	9.5	3,495,235	3,485,786	2,329	18%	114	TRUE
Twin Lake	TWLA	2023	TWLA23-0006	ANBOxCA	Tail Clip	Tadpole	2.6	300.0	9.1	3,116,940	3,107,013	2,440	14%	112	TRUE
Twin Lake	TWLA	2023	TWLA23-0007	ANBOxCA	Tail Clip	Tadpole	7.6	300.0	9.5	2,501,362	2,494,811	2,310	19%	91	TRUE
Twin Lake	TWLA	2023	TWLA23-0008	ANBOxCA	Tail Clip	Tadpole	12.0	300.0	9.3	8,993,940	8,973,132	2,303	19%	184	TRUE
Twin Lake	TWLA	2023	TWLA23-0009	ANBOxCA	Tail Clip	Tadpole	2.0	300.0	9	1,879,867	1,876,543	1,737	39%	53	TRUE
Twin Lake	TWLA	2023	TWLA23-0010	ANBOxCA	Tail Clip	Tadpole	2.0	300.0	9.4	581,171	579,654	1,008	65%	36	TRUE
Twin Lake	TWLA	2023	TWLA23-0011	ANBOxCA	Tail Clip	Tadpole	6.7	300.0	7.5	3,917,592	3,908,177	2,296	19%	104	TRUE
Twin Lake	TWLA	2023	TWLA23-0012	ANBOxCA	Tail Clip	Tadpole	12.8	300.0	9.4	4,929,711	4,917,422	2,312	19%	120	TRUE
Twin Lake	TWLA	2023	TWLA23-0014	ANBOxCA	Tail Clip	Tadpole	8.0	300.0	8	2,324,244	2,318,351	2,146	25%	77	TRUE
Twin Lake	TWLA	2023	TWLA23-0015	ANBOxCA	Tail Clip	Tadpole	6.9	300.0	9.4	1,070,677	1,067,019	1,656	42%	46	TRUE
Twin Lake	TWLA	2023	TWLA23-0016	ANBOxCA	Tail Clip	Tadpole	7.8	300.0	8.5	7,651,525	7,634,118	2,335	18%	171	TRUE
Twin Lake	TWLA	2023	TWLA23-0017	ANBOxCA	Tail Clip	Tadpole	2.3	300.0	9.5	1,233,108	1,230,361	1,316	54%	33	TRUE
Twin Lake	TWLA	2023	TWLA23-0018	ANBOxCA	Tail Clip	Tadpole	2.8	300.0	8.7	5,016,732	5,004,667	2,353	17%	90	TRUE
Twin Lake	TWLA	2023	TWLA23-0019	ANBOxCA	Tail Clip	Tadpole	2.3	300.0	9.1	5,432,763	5,417,819	2,394	16%	124	TRUE
Twin Lake	TWLA	2023	TWLA23-0020	ANBOxCA	Tail Clip	Tadpole	2.7	300.0	9.4	1,449,344	1,445,660	1,612	43%	54	TRUE
Twin Lake	TWLA	2023	TWLA23-0021	ANBOxCA	Tail Clip	Tadpole	9.2	300.0	9.4	4,297,735	4,286,995	2,436	14%	115	TRUE
Twin Lake	TWLA	2023	TWLA23-0022	ANBOxCA	Tail Clip	Tadpole	2.2	300.0	7.2	52,873	52,223	280	90%	7	FALSE
Twin Lake	TWLA	2023	TWLA23-0101	ANBOxCA	Buccal Swab	Adult	40.0	300.0	2.3	1,101,473	1,095,031	1,859	35%	31	TRUE

Appendix

Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Twin Lake	TWLA	2023	TWLA23-0102	ANBOxCA	Buccal Swab	Adult	30.5	300.0	5.5	1,759,466	1,754,907	2,145	25%	51	TRUE
Twin Lake	TWLA	2023	TWLA23-0118	ANBOxCA	Tail Clip	Tadpole	2.2	300.0	9	1,157,781	1,155,160	1,899	33%	46	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0002	ANBO	Buccal Swab	Adult	254.0	500.0	6.6	5,219,220	5,208,450	2,227	22%	130	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0005	ANBO	Buccal Swab	Adult	66.1	500.0	6.4	4,024,146	4,013,892	2,079	27%	96	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0010	ANBO	Tail Clip	Tadpole	24.3	500.0	8.5	77,028	76,662	351	88%	10	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0011	ANBO	Tail Clip	Tadpole	17.5	500.0	9.5	62,636	62,333	174	94%	5	FALSE
Union Valley Reservoir	UNVA	2022	UNVA22-0012	ANBO	Tail Clip	Tadpole	8.1	289.0	9.1	3,281,502	3,264,304	2,425	15%	129	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0013	ANBO	Tail Clip	Tadpole	18.5	500.0	9.1	2,257,912	2,243,263	2,472	13%	101	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0014	ANBO	Tail Clip	Tadpole	14.6	518.3	8.6	55,119	54,845	162	94%	4	FALSE
Union Valley Reservoir	UNVA	2022	UNVA22-0015	ANBO	Tail Clip	Tadpole	8.5	302.5	8.8	59,038	58,717	293	90%	7	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0016	ANBO	Tail Clip	Tadpole	19.9	500.0	8.4	311,946	307,408	879	69%	19	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0017	ANBO	Tail Clip	Tadpole	7.8	276.9	9.1	10,529,729	10,509,090	2,272	20%	183	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0018	ANBO	Tail Clip	Tadpole	10.5	372.8	9.5	10,013,291	9,969,484	2,127	25%	296	TRUE
Union Valley Reservoir	UNVA	2022	UNVA22-0019	ANBO	Tail Clip	Tadpole	12.6	447.3	9.5	3,923,013	3,895,657	2,530	11%	141	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0001	ANBOxCA	Tail Clip	Tadpole	12.7	300.0	8.9	2,220,116	2,216,012	2,405	16%	78	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0002	ANBOxCA	Tail Clip	Tadpole	27.8	300.0	8.4	2,911,339	2,905,579	2,516	12%	92	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0003	ANBOxCA	Tail Clip	Tadpole	7.9	300.0	9.2	2,544,114	2,538,237	2,499	12%	84	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0004	ANBOxCA	Tail Clip	Tadpole	9.8	300.0	8.7	2,057,163	2,053,146	2,408	15%	70	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0005	ANBOxCA	Tail Clip	Tadpole	10.7	300.0	9.4	39,390	39,095	177	94%	4	FALSE
Upper Blue Lake	UPBL	2022	UPBL22-0006	ANBOxCA	Tail Clip	Tadpole	12.5	300.0	9.4	1,521,389	1,518,373	1,930	32%	49	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0008	ANBOxCA	Tail Clip	Tadpole	7.8	300.0	9	3,343,950	3,335,590	2,526	11%	107	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0009	ANBOxCA	Tail Clip	Tadpole	7.2	300.0	9.5	9,775,277	9,755,607	2,291	20%	165	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0010	ANBOxCA	Tail Clip	Tadpole	10.1	300.0	8.9	877,759	875,260	1,719	40%	37	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0011	ANBOxCA	Tail Clip	Tadpole	7.7	300.0	9.2	3,208,544	3,202,312	2,512	12%	104	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0012	ANBOxCA	Tail Clip	Tadpole	7.8	300.0	8.4	3,299,055	3,290,550	2,563	10%	97	TRUE
Upper Blue Lake	UPBL	2022	UPBL22-0013	ANBOxCA	Tail Clip	Tadpole	18.2	300.0	7.9	3,545,017	3,538,925	2,447	14%	115	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0014	ANBOxCA	Tail Clip	Tadpole	9.6	300.0	9.6	2,147,089	2,142,368	2,450	14%	58	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0015	ANBOxCA	Tail Clip	Tadpole	7.8	300.0	9.5	2,951,282	2,944,659	2,476	13%	80	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0016	ANBOxCA	Tail Clip	Tadpole	7.3	300.0	9.3	2,937,526	2,929,854	2,466	13%	78	TRUE

Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Upper Blue Lake	UPBL	2023	UPBL23-0019	ANBOxCA	Tail Clip	Tadpole	3.6	300.0	9.4	2,789,505	2,783,288	2,507	12%	77	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0020	ANBOxCA	Tail Clip	Tadpole	2.3	231.0	8	563,812	562,525	1,504	47%	21	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0021	ANBOxCA	Tail Clip	Tadpole	2.4	236.0	8	4,490,872	4,478,355	37	99%	3	FALSE
Upper Blue Lake	UPBL	2023	UPBL23-0022	ANBOxCA	Tail Clip	Tadpole	1.2	248.0	8	697,302	695,122	1,639	42%	22	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0023	ANBOxCA	Tail Clip	Tadpole	1.5	296.0	8	2,968,951	2,963,025	2,371	17%	76	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0024	ANBOxCA	Tail Clip	Tadpole	9.1	300.0	9.3	2,475,859	2,470,951	2,460	14%	97	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0027	ANBOxCA	Tail Clip	Tadpole	10.7	300.0	8.6	1,997,152	1,993,335	2,353	17%	81	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0028	ANBOxCA	Tail Clip	Tadpole	7.1	300.0	8.9	8,294,233	8,275,591	2,215	22%	150	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0029	ANBOxCA	Tail Clip	Tadpole	10.5	300.0	8.8	2,903,332	2,898,042	2,445	14%	109	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0030	ANBOxCA	Tail Clip	Tadpole	6.8	300.0	9.1	3,401,749	3,395,113	2,558	10%	124	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0031	ANBOxCA	Tail Clip	Tadpole	2.7	266.0	8.7	2,623,063	2,617,762	2,487	13%	97	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0032	ANBOxCA	Tail Clip	Tadpole	7.0	300.0	9	3,129,359	3,121,949	2,537	11%	115	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0034	ANBOxCA	Buccal Swab	Adult	25.0	300.0	5.7	3,426,878	3,419,511	2,091	27%	95	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0035	ANBOxCA	Tail Clip	Tadpole	2.4	300.0	7.6	1,439,125	1,436,104	1,839	35%	50	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0036	ANBOxCA	Tail Clip	Tadpole	6.7	300.0	9.5	2,745,374	2,740,225	2,248	21%	80	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0037	ANBOxCA	Tail Clip	Tadpole	7.1	300.0	9.2	2,639,443	2,633,100	2,415	15%	81	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0038	ANBOxCA	Tail Clip	Tadpole	8.2	300.0	8.3	2,405,523	2,400,949	2,341	18%	76	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0039	ANBOxCA	Tail Clip	Tadpole	6.7	300.0	9.2	2,575,470	2,570,413	2,320	18%	79	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0040	ANBOxCA	Tail Clip	Tadpole	9.1	300.0	9	3,150,299	3,144,475	2,412	15%	92	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0041	ANBOxCA	Tail Clip	Tadpole	2.7	274.0	8.8	1,751,050	1,747,046	2,056	28%	57	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0042	ANBOxCA	Tail Clip	Tadpole	7.1	300.0	9.5	1,831,771	1,828,210	2,159	24%	62	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0043	ANBOxCA	Tail Clip	Tadpole	4.1	300.0	9.5	2,861,878	2,856,039	2,503	12%	87	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0044	ANBOxCA	Tail Clip	Tadpole	3.9	294.0	9.7	3,100,082	3,094,183	2,396	16%	93	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0045	ANBOxCA	Tail Clip	Tadpole	8.0	300.0	9.6	3,228,843	3,221,031	2,595	9%	112	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0046	ANBOxCA	Tail Clip	Tadpole	11.7	25.7	9.6	3,168,686	3,162,324	2,627	8%	107	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0047	ANBOxCA	Tail Clip	Tadpole	4.4	300.0	8.3	2,010,275	2,006,207	2,400	16%	61	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0048	ANBOxCA	Tail Clip	Tadpole	1.4	274.0	9	984,226	982,190	1,897	33%	39	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0049	ANBOxCA	Tail Clip	Tadpole	2.3	300.0	9	2,360,368	2,353,209	2,389	16%	72	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0050	ANBOxCA	Tail Clip	Tadpole	2.0	300.0	8.8	3,328,231	3,321,805	2,592	9%	104	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0051	ANBOxCA	Tail Clip	Tadpole	6.7	300.0	8.9	1,947,240	1,943,647	2,380	16%	86	TRUE

Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Upper Blue Lake	UPBL	2023	UPBL23-0052	ANBOxCA	Tail Clip	Tadpole	12.8	300.0	9.4	2,444,146	2,439,370	2,262	21%	105	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0053	ANBOxCA	Tail Clip	Tadpole	8.0	300.0	8.4	2,034,955	2,030,432	2,527	11%	98	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0054	ANBOxCA	Tail Clip	Tadpole	6.9	300.0	9.2	2,212,355	2,208,062	2,539	11%	102	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0055	ANBOxCA	Tail Clip	Tadpole	7.8	300.0	9.2	2,204,402	2,200,045	2,570	10%	101	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0056	ANBOxCA	Tail Clip	Tadpole	2.3	300.0	8.7	2,114,817	2,110,713	2,416	15%	82	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0057	ANBOxCA	Tail Clip	Tadpole	2.8	283.0	8.7	2,169,445	2,164,721	2,584	9%	97	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0058	ANBOxCA	Tail Clip	Tadpole	2.3	300.0	8.7	1,867,007	1,863,606	2,447	14%	81	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0059	ANBOxCA	Tail Clip	Tadpole	2.7	268.0	8.5	2,703,308	2,697,513	2,396	16%	90	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0060	ANBOxCA	Tail Clip	Tadpole	1.5	294.0	9	1,075,166	1,073,087	1,620	43%	43	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0070	ANBOxCA	Buccal Swab	Adult	30.1	300.0	2.1	515,880	513,214	1,326	53%	17	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0071	ANBOxCA	Buccal Swab	Adult	17.6	300.0	1.5	275,363	274,478	1,099	61%	11	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0101	ANBOxCA	Tail Clip	Tadpole	10.9	300.0	9.5	2,971,502	2,965,918	2,519	11%	97	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0102	ANBOxCA	Tail Clip	Tadpole	9.5	300.0	9.5	4,039,028	4,030,516	2,330	18%	113	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0103	ANBOxCA	Tail Clip	Tadpole	1.8	356.0	9	2,254,027	2,248,519	2,230	22%	72	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0104	ANBOxCA	Tail Clip	Tadpole	12.3	300.0	9	3,940,434	3,932,192	2,556	10%	117	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0105	ANBOxCA	Tail Clip	Tadpole	1.9	300.0	8.7	1,181,086	1,178,317	1,901	33%	54	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0106	ANBOxCA	Tail Clip	Tadpole	9.7	300.0	8.9	2,768,231	2,763,089	2,312	19%	100	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0108	ANBOxCA	Tail Clip	Tadpole	9.6	300.0	9.4	2,190,380	2,185,591	2,424	15%	87	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0111	ANBOxCA	Tail Clip	Tadpole	8.2	300.0	9.6	1,928,438	1,924,707	2,167	24%	74	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0129	ANBOxCA	Tail Clip	Tadpole	7.5	300.0	9.7	3,297,669	3,291,309	2,555	10%	123	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0135	ANBOxCA	Tail Clip	Tadpole	12.6	300.0	9.5	2,874,983	2,869,394	2,178	23%	99	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0137	ANBOxCA	Tail Clip	Tadpole	12.8	300.0	9	2,505,722	2,500,315	2,030	29%	86	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0138	ANBOxCA	Tail Clip	Tadpole	12.8	300.0	9.2	2,137,184	2,132,966	2,179	23%	81	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0141	ANBOxCA	Tail Clip	Tadpole	9.8	300.0	9.3	3,503,861	3,495,292	2,494	12%	90	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0145	ANBOxCA	Tail Clip	Tadpole	2.9	300.0	9.3	4,517,597	4,506,582	2,398	16%	98	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0146	ANBOxCA	Tail Clip	Tadpole	1.5	298.0	8.5	100,269	94,626	286	90%	9	TRUE
Upper Blue Lake	UPBL	2023	UPBL23-0153	ANBOxCA	Tail Clip	Tadpole	2.1	300.0	7	1,307,922	1,303,204	1,816	36%	43	TRUE
Van Vleck Pond	VAVL	2022	VAVL22-0010	ANBO	Tail Clip	Tadpole	8.3	294.7	7.1	46,200	45,976	52	98%	4	FALSE
Van Vleck Pond	VAVL	2022	VAVL22-0011	ANBO	Tail Clip	Tadpole	15.8	500.0	7.3	2,260,354	2,252,256	2,008	29%	69	TRUE

Maier and Mabe 2024

Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Van Vleck Pond	VAVL	2022	VAVL22-0012	ANBO	Tail Clip	Tadpole	10.2	362.1	9.1	3,450,968	3,443,076	2,321	18%	92	TRUE
Van Vleck Pond	VAVL	2022	VAVL22-0013	ANBO	Tail Clip	Tadpole	11.2	397.6	9	145,593	145,264	528	81%	14	TRUE
Van Vleck Pond	VAVL	2022	VAVL22-0014	ANBO	Tail Clip	Tadpole	11.6	411.8	9.3	103,842	103,550	115	96%	5	FALSE
Van Vleck Pond	VAVL	2022	VAVL22-0015	ANBO	Tail Clip	Tadpole	8.6	304.6	9.5	14,137	14,031	14	100%	4	FALSE
Van Vleck Pond	VAVL	2022	VAVL22-0016	ANBO	Tail Clip	Tadpole	10.4	369.2	9.7	4,232,229	4,223,045	2,313	19%	128	TRUE
Van Vleck Pond	VAVL	2022	VAVL22-0017	ANBO	Tail Clip	Tadpole	11.8	418.9	9.4	5,983,914	5,971,340	2,411	15%	153	TRUE
Van Vleck Pond	VAVL	2022	VAVL22-0018	ANBO	Tail Clip	Tadpole	9.5	335.5	9.4	45,252	45,078	176	94%	7	FALSE
Van Vleck Pond	VAVL	2022	VAVL22-0019	ANBO	Tail Clip	Tadpole	7.5	266.3	9.6	4,318,834	4,307,695	2,470	13%	142	TRUE
Van Vleck Pond	VAVL	2022	VAVL22-0020	ANBO	Tail Clip	Tadpole	8.6	306.7	9.1	3,547,066	3,539,793	2,310	19%	100	TRUE
Wakamatsu Farm	WAFA	2022	WAFA22-0009	ANBO	Buccal Swab	Adult	48.8	300.0	7	2,308,560	2,304,410	2,284	20%	95	TRUE
Wakamatsu Farm	WAFA	2022	WAFA22-0010	ANBO	Buccal Swab	Adult	22.7	300.0	7.8	2,435,866	2,430,099	2,361	17%	102	TRUE
Wakamatsu Farm	WAFA	2022	WAFA22-0011	ANBO	Buccal Swab	Adult	45.9	300.0	5.5	1,187,435	1,185,062	2,167	24%	53	TRUE
Wakamatsu Farm	WAFA	2022	WAFA22-0012	ANBO	Buccal Swab	Adult	37.5	300.0	6.5	1,860,826	1,857,300	2,316	19%	84	TRUE
Wakamatsu Farm	WAFA	2022	WAFA22-0013	ANBO	Buccal Swab	Adult	16.7	300.0	6.8	2,590,818	2,585,171	2,410	15%	98	TRUE
Wakamatsu Farm	WAFA	2022	WAFA22-0014	ANBO	Buccal Swab	Adult	43.9	500.0	6.3	5,136,810	5,075,973	1,885	34%	89	TRUE
Wakamatsu Farm	WAFA	2022	WAFA22-0016	ANBO	Buccal Swab	Adult	31.0	300.0	6	1,587,316	1,582,588	2,168	24%	70	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0001	ANBO	Tail Clip	Tadpole	11.8	300.0	9.8	2,830,454	2,825,740	2,264	20%	117	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0002	ANBO	Tail Clip	Tadpole	6.9	300.0	9.7	2,289,575	2,284,765	2,327	18%	103	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0003	ANBO	Tail Clip	Tadpole	8.6	300.0	9.6	2,121,118	2,117,364	2,279	20%	102	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0004	ANBO	Tail Clip	Tadpole	8.2	300.0	9.7	2,425,522	2,421,375	2,341	18%	112	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0005	ANBO	Tail Clip	Tadpole	8.7	300.0	9.7	2,680,698	2,676,207	2,366	17%	118	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0006	ANBO	Tail Clip	Tadpole	5.9	265.5	9	2,387,473	2,382,321	2,411	15%	102	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0007	ANBO	Tail Clip	Tadpole	7.7	300.0	9.1	2,899,156	2,892,932	2,326	18%	103	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0008	ANBO	Tail Clip	Tadpole	8.6	300.0	9.7	1,833,330	1,830,201	2,267	20%	92	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0017	ANBO	Tail Clip	Tadpole	6.8	300.0	9.8	2,777,000	2,772,081	2,460	14%	121	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0018	ANBO	Tail Clip	Tadpole	10.7	300.0	9.5	1,601,800	1,598,623	2,193	23%	79	TRUE
Wakamatsu Farm	WAFA	2023	WAFA23-0019	ANBO	Tail Clip	Tadpole	7.1	300.0	9.4	2,516,835	2,511,666	2,386	16%	99	TRUE
Watson Lake	WALA	2021	WALA21-0011	ANBO	Buccal Swab	Adult	12.0	426.0	7.7	5,099,993	5,072,715	2,482	13%	134	TRUE
Watson Lake	WALA	2021	WALA21-0021	ANBO	Buccal Swab	Adult	7.3	259.5	8.3	4,689,883	4,656,236	2,527	11%	130	TRUE
Watson Lake	WALA	2022	WALA22-0001	ANBO	Tail Clip	Tadpole	21.6	500.0	8.8	19,555	19,428	57	98%	4	FALSE

Appendix

Location	Location Code	Year	Sample Code	A Priori Species	Tissue	Life Stage	DNA Conc. (ng/uL)	Input DNA Quant (ng)	DNA Integrity (1–10)	Reads	Filtered Reads	Covered Loci	Missing Percent	Mean Depth	Used
Watson Lake	WALA	2022	WALA22-0002	ANBO	Tail Clip	Tadpole	29.6	500.0	9.1	858,149	856,234	1,300	54%	36	TRUE
Watson Lake	WALA	2022	WALA22-0003	ANBO	Tail Clip	Tadpole	25.2	500.0	9.1	3,964,329	3,941,775	2,493	12%	128	TRUE
Watson Lake	WALA	2022	WALA22-0004	ANBO	Tail Clip	Tadpole	13.5	479.3	9.3	3,363,915	3,342,832	2,572	10%	105	TRUE
Watson Lake	WALA	2022	WALA22-0005	ANBO	Tail Clip	Tadpole	14.4	511.2	9.4	3,332,290	3,315,235	2,576	9%	108	TRUE
Watson Lake	WALA	2022	WALA22-0006	ANBO	Tail Clip	Tadpole	19.3	500.0	9.1	4,819,326	4,796,473	2,362	17%	147	TRUE
Watson Lake	WALA	2022	WALA22-0007	ANBO	Tail Clip	Tadpole	12.6	447.3	9.5	5,001,928	4,972,735	2,582	9%	159	TRUE
Watson Lake	WALA	2022	WALA22-0008	ANBO	Tail Clip	Tadpole	19.6	500.0	9.1	5,330,978	5,304,608	2,533	11%	163	TRUE
Watson Lake	WALA	2022	WALA22-0009	ANBO	Tail Clip	Tadpole	47.4	500.0	8.7	4,081,137	4,055,837	2,435	14%	122	TRUE
Wrights Lake	WRLA	2023	WRLA23-0004	ANBO	Tail Clip	Tadpole	9.3	300.0	8.3	2,077,513	2,071,864	2,449	14%	86	TRUE
Wrights Lake	WRLA	2023	WRLA23-0005	ANBO	Tail Clip	Tadpole	11.3	300.0	9.1	1,737,659	1,733,943	2,391	16%	81	TRUE
Wrights Lake	WRLA	2023	WRLA23-0006	ANBO	Tail Clip	Tadpole	7.9	300.0	8.8	1,692,870	1,689,309	2,320	18%	80	TRUE
Wrights Lake	WRLA	2023	WRLA23-0007	ANBO	Tail Clip	Tadpole	6.9	300.0	8.9	1,840,913	1,836,862	2,416	15%	83	TRUE
Wrights Lake	WRLA	2023	WRLA23-0008	ANBO	Tail Clip	Tadpole	12.1	300.0	8.9	1,856,021	1,851,544	2,455	14%	82	TRUE
Wrights Lake	WRLA	2023	WRLA23-0009	ANBO	Tail Clip	Tadpole	8.3	300.0	9.2	2,145,542	2,140,621	2,494	12%	94	TRUE
Wrights Lake	WRLA	2023	WRLA23-0010	ANBO	Tail Clip	Tadpole	7.8	300.0	9.1	1,743,604	1,740,398	2,265	20%	66	TRUE
Wrights Lake	WRLA	2023	WRLA23-0011	ANBO	Tail Clip	Tadpole	8.5	300.0	8.8	1,908,847	1,905,274	2,349	17%	66	TRUE
Wrights Lake	WRLA	2023	WRLA23-0012	ANBO	Tail Clip	Tadpole	10.5	300.0	9.4	1,840,979	1,836,673	2,360	17%	67	TRUE
Wrights Lake	WRLA	2023	WRLA23-0013	ANBO	Tail Clip	Tadpole	11.1	300.0	9.1	1,932,253	1,928,403	2,413	15%	70	TRUE