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Hybridization between two parapatric ranid frog species in the northern Sierra Nevada, California, USA

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Abstract

Contact zones between species provide a unique opportunity to test whether taxa can hybridize or not. Cross-breeding or hybridization between closely related taxa can promote gene flow (introgression) between species, adaptation, or even speciation. Though hybridization events may be short-lived and difficult to detect in the field, genetic data can provide information about the level of introgression between closely related taxa. Hybridization can promote introgression between species, which may be an important evolutionary mechanism for either homogenization (reversing initial divergence between species) or reproductive isolation (potentially leading to speciation). Here, we used thousands of genetic markers from nuclear DNA to detect hybridization between two parapatric frog species (*Rana boylii* and *Rana sierrae*) in the Sierra Nevada of California. Based on principal components analysis, admixture, and analysis of heterozygosity at species diagnostic SNPs, we detected two F1 hybrid individuals in the Feather River basin, as well as a weak signal of introgression and gene flow between the frog species compared with frog populations from two other adjacent watersheds. This study provides the first documentation of hybridization and introgression between these two species, which are of conservation concern.

KEYWORDS
frogs, hybridization, introgression, parapatry, *Rana sierrae*, *Rana boylii*

1 | INTRODUCTION

Landscape changes can influence species demography and dispersal patterns (Li et al., 2017), which can change rates of gene flow within species. Changing migration rates and population sizes can influence population structure; thus, over time, landscape changes can cause significant changes in genetic diversity within a species. Furthermore, cross-breeding or hybridization between closely related taxa can promote gene flow (introgression) between species, which may be an important evolutionary mechanism for either homogenization (reversing initial divergence between species), speciation (from reproductive isolation of hybrid populations), or adaptation (transfer of adaptive alleles; Abbott et al., 2013; Barrera-Guzmán, Aleixo, Shawkey, & Weir, 2018; Jiggins & Mallet, 2000; Mallet, 2007).

While there has been an abundance of research on hybridization contact zones between species (Dowling et al., 2016; Hewitt, 1988; Jiggins & Mallet, 2000; Medina, Wang, Salazar, & Amézquita, 2013; Shaffer, Fellers, Voss, Oliver, & Pauly, 2004; Zhang et al., 2018), less is known regarding the role of introgressive hybridization events in "bimodal hybridization zones" (Harrison & Bogdanowicz, 1997), where hybrids are rare and contact between species may lead to inviability of embryos (Gibeaux et al., 2018; Jiggins & Mallet, 2000). Furthermore, identifying these potential hybridization zones is not

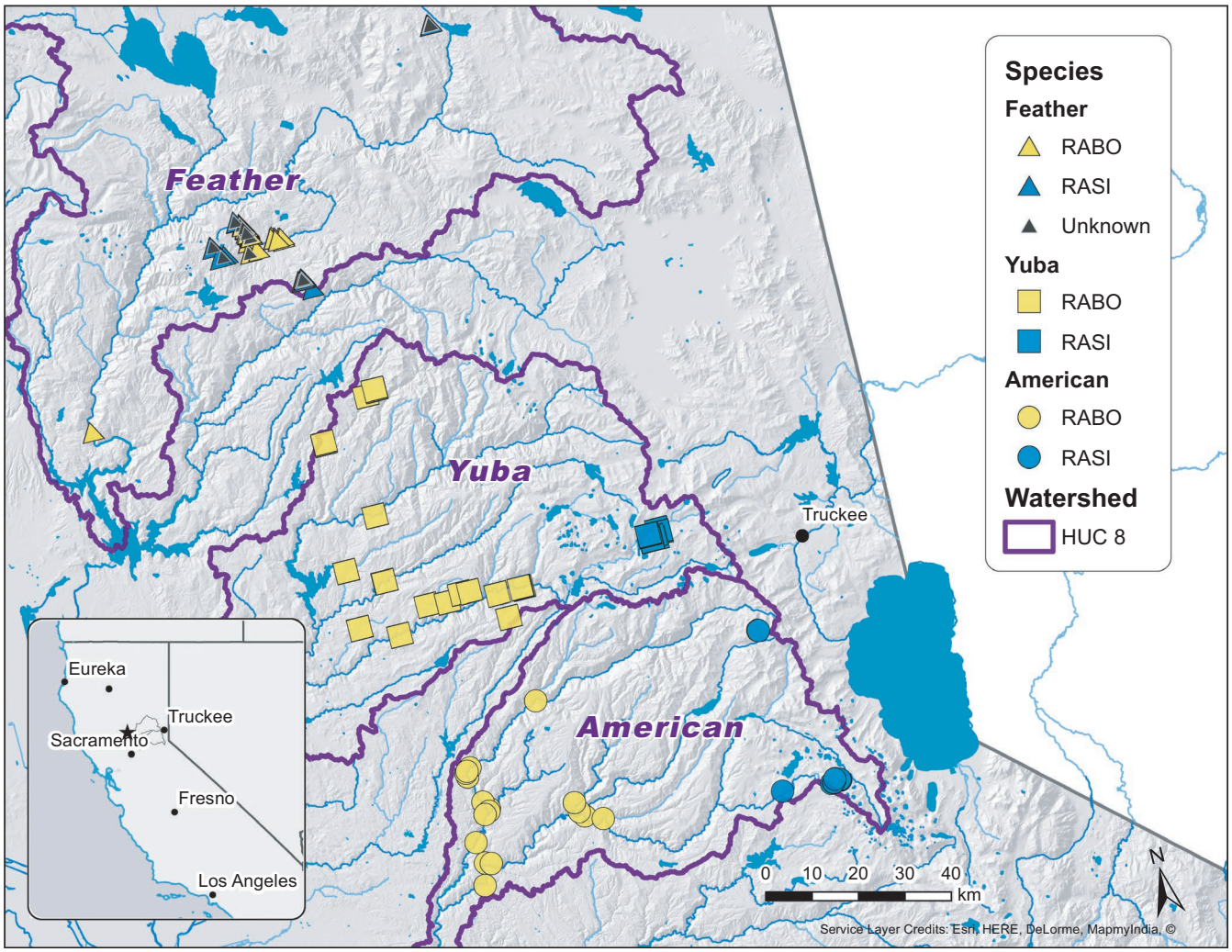

FIGURE 1 Map of sampling locations in the Feather, Yuba, and American watersheds. RABO, *Rana boylii*; RASI, *Rana sierrae*

typically possible in the field with cryptic species, and can be affected by sampling design, timing, and resolution of genetic markers (Zhang et al., 2018). Assessing population admixture or detecting potential hybridization has previously been challenging; however, modern genetic methods provide a powerful approach to assess populations at fine geographic and evolutionary scales (Ali et al., 2016; Prince et al., 2017).

We investigated the potential for hybridization in two parapatrically occurring endemic frog species in the Sierra Nevada of California, USA. Foothill yellow-legged frogs, *Rana boylii*, (Baird, 1856) historically occurred in lower and mid-elevation (<1,500 m) streams and rivers from Southern Oregon to northern Baja California west of the Sierra-Cascade crest (Stebbins, 2003), whereas Sierra Nevada yellow-legged frogs, *Rana sierrae*, (Camp, 1917) typically occurred from 1,500 m to over 3,600 m in lakes and streams (Stebbins, 2003). Population declines have been documented across the former range of both of these species; *R. sierrae* has been extirpated from over 90% of its historical range (Drost & Fellers, 1996; Vredenburg, 2004) while *R. boylii* has been extirpated from 50% of its historical range (Davidson, Shaffer, & Jennings, 2002; Jennings & Hayes, 1994). Both species are of conservation concern; in 2014, the U.S. Fish and Wildlife Service (USFWS) listed *R. sierrae* as endangered under the U.S. Endangered Species Act (ESA) (USFWS, 2014), and *R. boylii* is listed as a species of special concern in California and is a candidate for listing under the California and federal ESAs.

Unlike other ranid frog species with broad areas of potential intergradation (Shaffer et al., 2004), *R. boylii* and *R. sierrae* do not occur sympatrically, and can largely be considered a parapatric species. Zweifel (1955) described one historical location where these two species co-occurred, in Butte County near DeSabla, California. Currently the only known location where both species are found encompasses a tributary to the North Fork Feather River in the northern Sierra Nevada, California (Figure 1).

Interspecific amplexus has been observed in *R. boylii* and similar ranid species (D'Amore, Kirby, & Hemingway, 2009; Lind, Bettaso, & Yarnell, 2003; Pearl, Hayes, Haycock, Engler, & Bowerman, 2005), however, no hybridization between *R. sierrae* and *R. boylii* has been documented. Furthermore, breeding experiments by Zweifel (1955) between *R. sierrae* (formerly known as

Rana muscosa) and *R. boylii* yielded very low viability in fertilization and high incidences of embryological abnormalities—indicating a post-zygotic barrier between the species. However, these experiments only crossed female *R. sierrae* with male *R. boylii*. Also, the individuals were from very different California regions (e.g., Butte and Nevada County vs. Contra Costa County). Historically there may have been other zones of overlap between the species, but current ranges for these species have little potential for additional overlapping zones.

Rana boylii and *R. sierrae* have very similar morphology and habitat preferences in areas where they co-occur (Zweifel, 1955). There are no major differences in adult size between the species; *R. boylii* typically range in size from 38 to 81 mm (snout-vent length) and *R. sierrae* from 41 to 89 mm. These species exhibit similar methods of axillary amplexus (Duellman & Trueb, 1986; Stebbins, 2003). Thus, detecting hybridization and assigning individuals to species is difficult and imprecise using field identification methods. This presents a challenge for management because these species have different conservation status and management objectives. Here we employed modern genetic methodology to better understand *R. boylii* and *R. sierrae* where their ranges overlap. We investigated three primary questions:

1. Can hybridization be detected between two parapatrically occurring frog species in the Sierra Nevada using data generated from genome-wide single nucleotide polymorphisms (SNPs)?
2. If hybrids can be detected, do genetic signatures suggest introgression (i.e., can hybrids reproduce successfully, leading to introgression between species)?
3. Are genetic migration rates between species in parapatrically occurring populations greater than in allopatrically occurring populations in adjacent watersheds?

This study focused on the Yuba and American watersheds, which share a similar Mediterranean climate, underlying geology, watershed aspect (west-slope), and vegetative communities. The Feather watershed shares a similar climate but has a slightly different underlying geology and aspect than that of other watersheds in the Sierra Nevada. The Feather watershed lies in the transition zone of the northern Sierra Nevada and the Cascades/Basin and Range Province, and thus the landscape in the northern portion of the watershed is comprised largely of volcanic bedrock while the southern portion is largely granitic (Durrell, 1988).

2 | MATERIALS AND METHODS

2.1 | Sampling and DNA extraction

To investigate potential hybridization between *Rana boylii* and *Rana sierrae*, a total of 458 tadpole tail clips, buccal swabs, and tissue samples were compiled. Samples were identified to species in the field as either *R. boylii*, *R. sierrae*, or "unknown", which were individuals that could not be visually confirmed as either species. The samples were collected between 1992 and 2016, from three watersheds in the Sierra Nevada (the Feather, Yuba, and American; Table S1). All unknown individuals were from Feather watershed localities.

Field sampling was conducted following methods in Heyer, Donnelly, McDiarmid, Hayek, and Foster (1994) under CDFW SCP Permit #0006881, #0007303, and Federal permit TE-40087B-0 with IACUC protocol #19327 (UC Davis) and #04718-001 (Washington State University). Individual post-metamorphic frogs were buccal-swabbed following established protocols (Broquet, Berset-Braendli, Emaresi, & Fumagalli, 2007; Goldberg, Kaplan, & Schwalbe, 2003; Pidancier, Miquel, & Miaud, 2003). Each post-metamorphic individual was comprehensively swabbed underneath tongue and inside of both cheeks for approximately 30 s to one minute. Swabs were air dried for approximately 5 min and placed in 1.5 ml microcentrifuge tubes while in the field or placed in lysis buffer (Goldberg et al., 2003). Dried samples were stored in the laboratory at −80°C until DNA extraction. Where possible, tail clips from tadpole larvae were collected, and tadpoles greater than 15 mm total length were targeted (Parris et al., 2010; Wilbur & Semlitsch, 1990). One clip was taken per individual tadpole and dried on Whatman filter paper (grade 1) or placed in 95% ethanol and stored at room temperature. DNA was extracted from samples in ethanol and lysis buffer using Qiagen DNeasy Blood & Tissue kits following manufacturer protocol and stored at −20°C. DNA was extracted from dried buccal swabs and tail clips using an Ampure magnetic bead-based protocol (Ali et al., 2016) and stored at −20°C.

2.2 | RAD sequencing and RAD-capture (Rapture) bait design

To produce a genomic resource for frog species with large genome sizes, we interrogated a significant fraction of the *R. boylii* genome using RAD sequencing with *SbfI* (Ali et al., 2016; Baird et al., 2008; Miller, Dunham, Amores, Cresko, & Johnson, 2007). Paired-end Illumina sequence data were generated using 24 *R. boylii* individuals (Table S2). De novo locus discovery and contig extension were carried out as previously described (Miller et al., 2012; Sağlam et al., 2016) using the alignment program Novoalign and the genome assembler PRICE (Ruby, Bellare, & Derisi, 2013). This resulted in a set of 77,544 RAD contigs ranging from 300 to 800 bp which served as a de novo partial reference alignment for all subsequent downstream analyses (Table S3). We next removed loci with five or more SNPs to reduce potential paralogs and chimeras, and randomly selected 10,000 loci from the remaining subset. Of these 10,000 loci, 8,533 were successfully designed into 120 bp RAD capture baits by Arbor Biosciences (Table S4).

2.3 | Rapture sequencing and probabilistic genomic analysis

A total of 458 individual frog samples were prepared for sequencing following the RAD Capture (Rapture) methods outlined in Ali et al. (2016) and Komoroske et al. (2019). We generated RAD libraries from the samples, quantified the libraries using a Fragment Analyzer

(Agilent Technologies, Santa Clara, CA) and pooled them, performed capture on the pooled library with the 120 bp baits described above, and sequenced the resulting Rapture library with paired-end Illumina sequencing.

Sampled individuals were aligned against the de novo RAD reference using the BWA-MEM algorithm (Li, 2013; Li & Durbin, 2010), and converted to BAM format and filtered for properly paired alignments using SAMTOOLS (Li et al., 2009). Next, alignments from different sequencing lanes were merged together and duplicates were removed. For all downstream analysis, we selected individuals that had greater than 25,000 alignments (n = 311), which provided sufficient data to investigate population genetic attributes at broad and fine geographic scales (Table S5). Sequence coverage depth was measured at the mid-point (i.e., base-pair 60) of each 120 bp Rapture bait locus using the depth function in SAMTOOLS.

The probabilistic framework implemented in Analysis of Next Generation Sequencing Data (ANGSD; Korneliussen, Albrechtsen, & Nielsen, 2014) was used for all population genetic analyses as it does not require calling genotypes and is suitable for low-coverage sequencing data (Fumagalli et al., 2013; Korneliussen, Moltke, Albrechtsen, & Nielsen, 2013). ANGSD analyses were conducted following methods from Prince et al. (2017), with a minimum mapping quality score (minMapQ) of 10, a minimum base quality score (minQ) of 20, SAMTOOLS genotype likelihood model (GL 1), estimating allele frequencies (doMaf 2) (Kim et al., 2011), estimation of genotype posterior probabilities using a uniform prior (doPost 2), and specifying the Rapture bait loci (-sites). Only sites represented in at least 50% of the included samples (minInd) were used. Furthermore, genomic sites were designated as polymorphic only if MAFs were greater than 0.05 and the probability of the site not being polymorphic was $<1 \times 10^{-6}$. We also summarized patterns of genetic diversity using two estimators of θ (4 Nμ): Tajima's θ (θ_π) is based on the average number of pairwise differences (Tajima, 1983), and Watterson's θ (θ_S) is based on the number of segregating sites (Watterson, 1975). Estimates of θ statistics were calculated using the empirical Bayes method in ANGSD (Korneliussen et al., 2014).

2.4 | PCA and admixture to assess structure and introgression

To assign samples to species and assess population structure and coancestry, ANGSD was used to generate PCA, and NGSadmix was used to estimate admixture proportions. Settings used in ANGSD for PCA included the -doIBS 1 and -doCov 1 options, in addition to those described above. Principal components (PC) summarizing species identification and population structure were derived from classic eigenvalue decomposition and were visualized using the DPLYR and GGPLOT2 packages in R (R Core Team, 2017; Wickham, 2016; Wickham, François, Henry, & Müller, 2018).

To assess admixture between *R. boylii* and *R. sierrae*, genotype likelihood data was generated in ANGSD (doGLF 2) with the same settings as above. We then used NGSadmix (Skotte, Korneliussen, & Albrechtsen, 2013) to infer ancestry proportions in *R. boylii* and *R. sierrae* individuals assuming two ancestral populations. NGSadmix is a robust admixture method that can be applied to low-depth NGS data, and does not require called genotypes, thus reducing error associated with potential ascertainment and uncertainty in the data (Skotte et al., 2013). We considered any putative hybrids to be individual samples that did not clearly resolve as either species in both the PCA and admixture analyses and were genetically comprised of half of each species in the admixture analysis.

2.5 | F1 vs. F2 test with species diagnostic SNPs

Individuals that have an intermediate position on a PCA and approximately 50% ancestry of each species in the admixture analysis could hypothetically be first generation filial (F1) hybrids or progeny from F1 hybrids from subsequent generations (e.g., F2, F3, etc). To test if intermediate individuals were F1 hybrids, we identified differentially fixed homozygous (i.e., species-specific) SNPs from the Rapture data using only individuals that that were confirmed as either *R. boylii* and *R. sierrae* in the PCA and admixture analyses. We then assessed heterozygosity at these species-specific loci in putative hybrid individuals, as F1 vs. F2 hybrid individuals will have different degrees of heterozygosity in species-diagnostic SNPs. F1 hybrids should be exclusively heterozygous at species diagnostic SNPs. In contrast, F2 hybrids should be heterozygous for 50% of the species diagnostic SNPs, and homozygous at the remaining 50% with 25% allotted to each species. We called genotypes in ANGSD using a uniform prior (-doPost 2) and the following settings: -GL 1, -doGeno 13, -postCutoff 0.95, -doMaf 1, -doMajorMinor 1, -minInd 2, -SNP_pval 1×10^{-6}, -minMapQ 20, -minQ 20, and specifying the Rapture bait locations using the -sites. The subsequent output (*.geno.gz) was then processed in the program R (R Core Team, 2017) using the DPLYR package (Wickham et al., 2018) to manipulate and filter to homozygous diagnostic SNPs. Data were filtered to include only SNPs with called genotypes in 50 or more individuals from each species.

2.6 | Demographic modelling with fastsimcoal2

To quantify divergence times and migration rates between *R. boylii* and *R. sierrae*, we parameterized the best-fit coalescent simulation in fastsimcoal2 (Excoffier, Dupanloup, Huerta-Sánchez, Sousa, & Foll, 2013; Excoffier & Foll, 2011). This maximum-likelihood modelling approach uses simulations to estimate the expected site-frequency spectra (SFS) for a demographic model of interest to calculate a composite likelihood, and then utilizes a maximization procedure to find the maximum-likelihood parameter estimates.

We calculated folded joint SFS for each species in each watershed from SNP data generated from ANGSD because the ancestral condition is unknown. For all models, we assumed the potential for bidirectional gene flow, that extant genetic clusters emerged simultaneously from a common ancestry, and a typical vertebrate mutation rate of 1.0×10^{-8} m s^{-1} g^{-1} (Jaillon et al., 2004; Lynch, 2010; Sağlam, Baumsteiger, & Miller, 2017). We used two main conservative model scenarios to estimate divergence times and migration

rates between species in each watershed (Table S6). To estimate migration probabilities per generation between species within each watershed, we chose to constrain the model estimates in a conservative migration-only model, and thus set model parameters for time since divergence to between 1 and 1.1 billion generations ago to create simplified migration-only models. To estimate divergence time between species, we selected the watershed that had the lowest migration rate from the previous migration-only models, and generated divergence estimates assuming no migration between species, bounded between ten thousand and four million generations, which spans previous estimates for these species (Macey et al., 2001). For each divergence and migration model, we tested models that allowed for population growth, and models with no growth, for a total of four separate independent demographic models for each of the three watersheds (American, Yuba, and Feather).

The basic steps taken to obtain final model estimates from fastsimcoal2 followed methods from (Hotaling et al., 2018), where comparison of maximum observed and expected likelihoods were used to select the best-fit model (Akaike, 1973), then simulation of new SFSs using the best-fit model for parametric bootstrapping. Following Excoffier and Foll (2011), we generate 100,000 coalescent simulations for each model replicate, and performed 25 replicate runs for likelihood calculations (estimation of the expected SFS) with a maximum of 40 cycles for the conditional maximization algorithm. A stop criteria of a 1.0×10^{-4} difference between likelihoods was used to identify convergence. The best fit model was selected by identifying the run that minimized the difference between maximum expected likelihood and maximum observed likelihood. Parametric bootstrapping was then used to generate 95% credible intervals for each best-fit model.

3 | RESULTS

3.1 | Rapture produced high quality genomic data for both *Rana boylii* and *Rana sierrae*

Individual samples were collected across 56 different sampling localities in three different watersheds for each species (Figure 1, Table S1). For downstream analysis, we filtered and retained 311 samples from the original sequencing data that contained a minimum of 25,000 mapped alignments (Table S5). These frog species are cryptic, and often occur in low densities, so we retained all localities in our analysis, regardless of the number of samples (Table S1). On average, 95% (*SD* = 0.953%) of all reads were mapped to the de novo reference (Table S5), the mean mapped alignments per sample for the final merged data set was 229,485 (Table S5), the mean coverage depth across the Rapture bait loci was over 7×, and over 44,000 polymorphic sites were identified across all samples (see Section 2). Genetic diversity (θ) was highest in *R. sierrae* across all three watersheds, with the Feather basin containing the highest diversity of either species or watershed (Table 1). For *Rana boylii*, diversity was lower than *Rana sierrae* across the three watersheds, but in the Feather watershed, the estimates for θ_S were highest, and θ_π was lowest, which indicates more rare variants are present.

TABLE 1 Genetic diversity statistics generated from ANGSD for each frog species by watershed

Watershed	Species	θ_S/kb	θ_π/kb
Feather	*Rana boylii*	6.8172	6.5768
Feather	*R. sierrae*	14.0057	13.5551
American	*R. boylii*	6.3313	6.7248
American	*R. sierrae*	11.4953	11.9821
Yuba	*R. boylii*	6.7098	6.6264
Yuba	*R. sierrae*	11.7446	12.1734

Note: θ_π, Tajima's θ, and θ_S, Watterson's θ, given per kilobase (kb).

3.2 | PCA shows strong separation between species and identifies putative hybrids

To answer the first research question, can hybridization be detected between species, and to assess within-basin population structure; principal components analysis (PCA) was used to provide a dimensionless comparison of putative diagnostic SNPs across species and watersheds (Figure 2). Strong differentiation was observed between species (*R. boylii* and *R. sierrae*) on the PC1 axis, which accounted for approximately 55% of the variation. PC2 showed differentiation in *R. sierrae* among watersheds, particularly

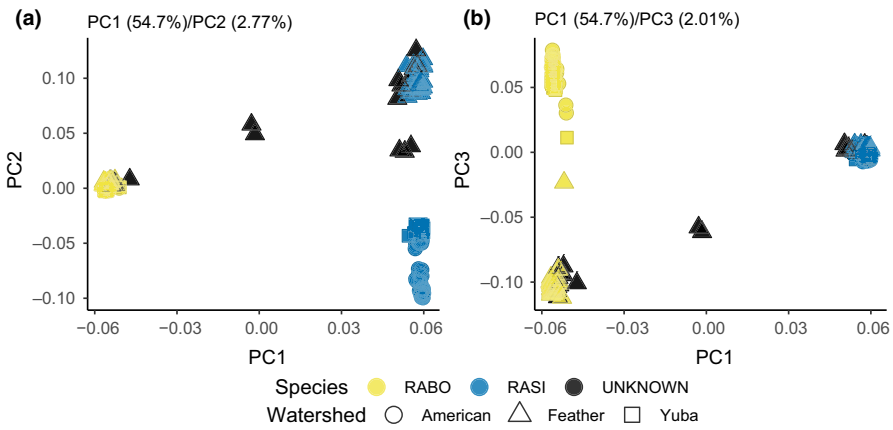

FIGURE 2 Principal component analysis of Rapture sequencing data, RABO, *Rana boylii*; RASI, *Rana sierrae*. (a) PC1 vs. PC2; (b) PC1 vs. PC3

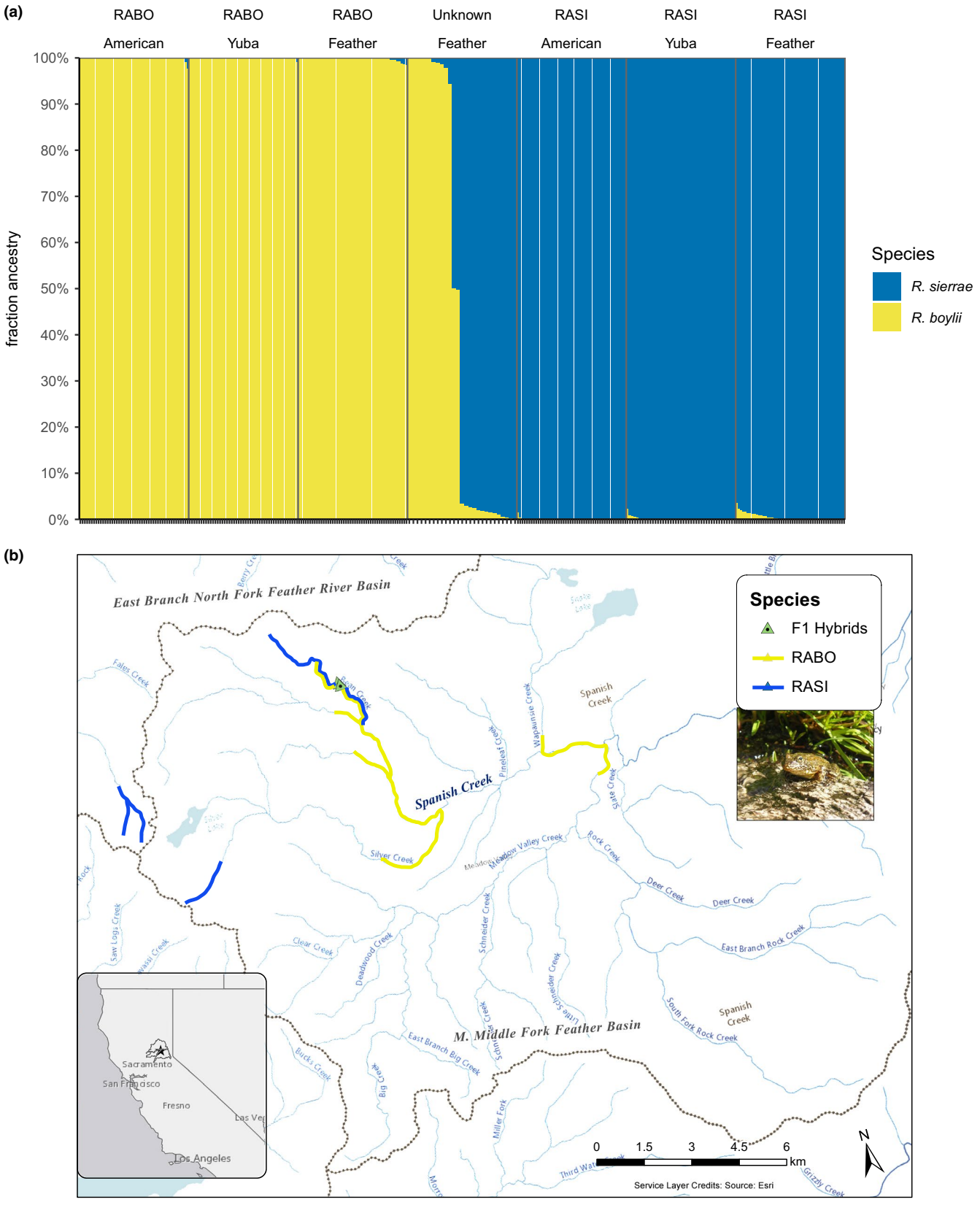

FIGURE 3 (a) Admixture ($k = 2$) of *Rana boylii*, *Rana sierrae*, and unknown *Rana* samples from the Feather, American, and Yuba watersheds; (b) map of sample locations in Bean Creek/Spanish Creek in the Feather watershed where hybrids were identified. RABO, *R. boylii*, RASI, *R. sierrae*

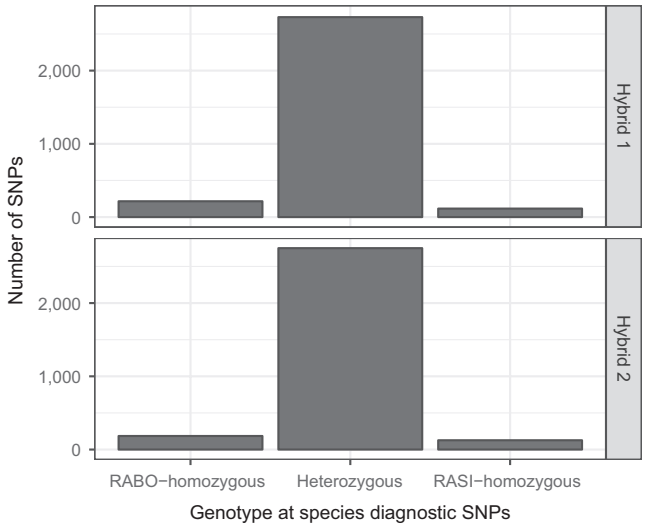

FIGURE 4 The F1 vs. F2 test using species diagnostic SNPs to assess heterozygosity in hybrid individuals. RABO, *Rana boylii*, RASI, *Rana sierrae*

individuals from the Feather with those from the American and Yuba (Figure 2a), while PC3 differentiated *R. boylii* samples from the Feather with the other two watersheds (Figure 2b). Little sign of admixture between the two species appeared in the PCA, however, two samples—collected in the Feather Watershed and designated as "unknown" in the field—clustered halfway between the *R. boylii* and *R. sierrae* groups along PC1, suggesting these individuals were hybrids.

3.3 | Assessing introgression with admixture shows two unknown individuals with equal species ancestry

To further investigate introgression between species and assess if the two unknown individuals identified in the PCA were potential hybrids of *R. boylii* and *R. sierrae*, we used NGSAdmix to assess population structure and individual ancestry from genome-wide SNPs (Skotte et al., 2013), using $k = 2$ to evaluate the fraction of ancestry derived from each species. Admixture showed the same two unknown samples from the Feather basin had approximately 50% ancestry from each species (*R. boylii* and *R. sierrae*), confirming their hybrid ancestry (Figure 3a). Furthermore, ancestry in the individuals designated as "unknown" in the field (only from the Feather watershed) showed greater levels of introgression between *R. boylii* and *R. sierrae* when compared to very low or nearly nonexistent levels of mixed-ancestry in the American and Yuba watersheds. In the Feather watershed for "unknown" samples, the mean proportion for the non-dominant cluster was 0.013, (excluding putative hybrids), while the mean proportions in the American and Yuba were 0.0005 and 0.0007, respectively. The putative hybrid individuals were sampled in Bean Creek, a tributary to Spanish Creek. Bean Creek was one of the only tributaries where both *R. boylii* and *R. sierrae* co-occur; therefore, we conclude there is strong evidence for recent hybridization between *R. boylii* and *R. sierrae* in this drainage (Figure 3b).

3.4 | Assessing introgression with a F1 vs. F2 test on hybrid individuals

To test whether the hybrids were F1 (first-generation) or F2 (progeny of two F1s), we identified species diagnostic SNPs using all nonhybrid individuals identified from PCA and admixture analyses. Our filtering process (see Section 2) yielded 3,062 putative diagnostic SNPs that were homozygous for different alleles in *R. boylii* and *R. sierrae* samples and also had successfully called genotypes at the same base-pair locations in the two hybrid individuals. F1 hybrids should be exclusively heterozygous at species diagnostic SNPs. In contrast, F2 hybrids should be heterozygous for 50% of the species diagnostic SNPs, and homozygous at the remaining 50% with 25% allotted to each species (Fitzpatrick, 2012). Using only the hybrid individuals, the species diagnostic SNPs showed extremely high heterozygosity and very low homozygosity (6% genotyped as *R. boylii*, 4% *R. sierrae*, and 89% were heterozygous) (Figure 4). This level of heterozygosity is far greater than expected for F2 individuals, and the presence of homozygous genotype calls in the hybrid individuals at species diagnostic SNPs is expected due to low coverage sequencing data. Genotyping from low coverage sequencing will cause a low frequency of erroneous homozygous calls, because only one of the two alleles is sampled, causing heterozygotes to be called as homozygotes. We conclude these hybrid individuals are F1 instead of F2 individuals. Furthermore, the hybrid individuals were found to have *R. sierrae* mitochondrial DNA (Bedwell, 2018), indicating the female was from a *R. sierrae* individual and the male was from *R. boylii* in both cases.

3.5 | Estimating divergence times and migration rates between species

To assess migration rates between *R. boylii* and *R. sierrae* in the Feather watershed compared to the Yuba and American, we used fastsimcoal2 (Excoffier & Foll, 2011) coalescent simulations. Using all individuals except the two hybrid individuals, we found migration probability (or the per generation likelihood that any gene from one population transfers to another) between *R. boylii* and *R. sierrae* was highest in the Feather, with a mean of 2.946×10^{-6} (95% CI 2.941×10^{-6}–2.951×10^{-6}) and lowest in the American watershed, 4.313×10^{-7} (4.306×10^{-7}–4.319×10^{-7}). The migration probabilities in the Yuba watershed were lower than estimates from the Feather, but were closer in magnitude, 2.109×10^{-6} (2.104×10^{-6}–2.113×10^{-6}) (Figure 5). We conclude migration probability rates were highest in the Feather watershed, indicating there is probably a greater level of introgression occurring between *R. boylii* and *R. sierrae* in the Feather compared with the other watersheds; which provides evidence that hybridization has successfully transferred genetic information between these species through reproductively viable eggs.

To estimate divergence time between species, we used the American watershed samples because migration estimates between *R. boylii* and *R. sierrae* were lowest, and this approach provided

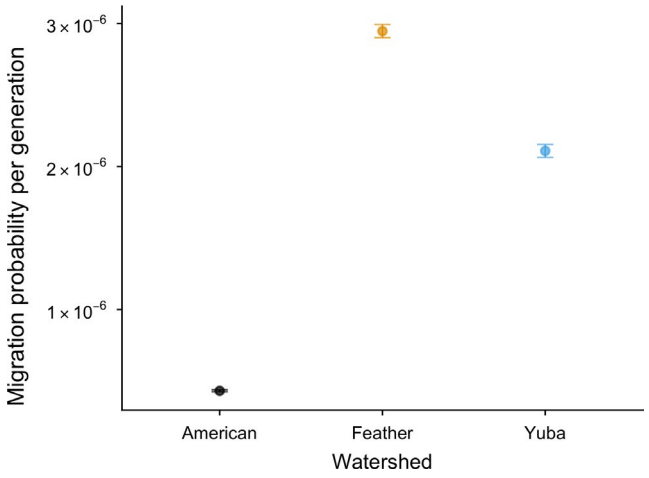

FIGURE 5 Estimates of migration probabilities from fastsimcoal2 models between the two species within the Feather, Yuba, and American watersheds. RABO, *Rana boylii*; RASI, *Rana sierrae*, with 95% credible intervals from 100 bootstrapped estimates (small horizontal lines)

a conservative estimate of divergence by minimizing inaccuracy caused by migration. We then ran fastsimcoal2 models with no migration and divergence bounded between 10 thousand and 4 million generations ago. The best model based on maximum likelihood estimated the time since divergence between *R. boylii* and *R. sierrae* was 370,856 (370,041–371,670) generations. Typically, *R. boylii* have a generation time of 2–3 years, depending on the region (Kupferberg, Lind, Mount, & Yarnell, 2009; Railsback et al., 2016), while *R. sierrae* can have a greater range of generation times, between 3 and 6 years because tadpoles may overwinter as many as 3 years (Knapp et al., 2016; Knapp, Matthews, Preisler, & Jellison, 2003). We may assume the ancestral condition was derived from *R. boylii* (Macey et al., 2001; Vredenburg et al., 2007; Yuan et al., 2016), therefore we suggest a generation time of three years, which means *R. sierrae* probably diverged from *R. boylii* over 1.1 mya. This time period corresponds to the mid-Pleistocene and an era of glaciation and interglaciation ("the Great Ice Age"), where distributions contracted and expanded and lineages became isolated (Birkeland, 1964; Gillespie & Zehfuss, 2004).

4 | DISCUSSION

Although massive parallel sequencing (MPS) technologies have the potential to facilitate collection of high-quality genetic data in virtually any species, a number of challenges still remain for many species including low quality or nonexistent reference genomes, large/complex/repetitive genomes, and high cost of processing/sequencing in studies with many samples. Amphibians are particularly challenging as many species have very large genome sizes (McCartney-Melstad & Shaffer, 2015; Shaffer et al., 2015; Weisrock et al., 2018).

Our results demonstrate that the application of Rapture (Ali et al., 2016) is a robust method to rapidly discover a large number of loci suitable for population genetic analyses in multiple frog species, including assessment of population structure, genetic diversity, and detecting potential introgression between species. We identified strong divergence between *Rana boylii* and *Rana sierrae* across all three watersheds, evidence of two F1 hybrids, and evidence of low levels of introgression (especially in the Feather basin). Hybridization between *R. boylii* and *R. sierrae* has not been previously documented based on field observations and breeding experiments (Zweifel, 1955).

Based on our data, it is unlikely that there is currently major introgression between *R. boylii* and *R. sierrae*, particularly as hybridization initially may not be adaptive and is often selected against (Abbott et al., 2013; Streicher et al., 2014). Although hybridization may be common between some amphibian species (Malone & Fontenot, 2008) and can even occur between highly divergent taxa—up to 21 million years divergent (Prager & Wilson, 1975)—our data show there is strong pattern of divergence between *R. boylii* and *R. sierrae* with limited hybridization and introgression between the species. Furthermore, Bean Creek in the Feather watershed is currently the only known locality where *R. sierrae* and *R. boylii* co-occur, and populations of either species are typically sparse in the Sierra (Catenazzi & Kupferberg, 2013; Knapp, Boiano, & Vredenburg, 2007; Kupferberg et al., 2012). Additionally, these two species may be strongly influenced by elevation due to life history differences (*R. sierrae* are typically found in higher elevations and are capable of overwintering as tadpoles while *R. boylii* are not). Previous work suggests that elevation strongly influences genetic structure in frogs (Dudaniec, Spear, Richardson, & Storfer, 2012; Funk et al., 2005; Metzger, Espindola, Waits, & Sullivan, 2015; Monsen & Blouin, 2004)—further reinforced by the patterns of strong divergence between species within watersheds that we observe in our data.

Populations in the Feather form the northern extent of both species' range in the Sierra Nevada, and our results provide additional support for greater within-species genetic differentiation near the boundary of a species' range, where there is less connectivity and potential gene flow with populations from different watersheds. Richmond, Barr, Backlin, Vandergast, and Fisher (2013) found populations of *Rana draytonii* in Southern California that occurred further away from the center of the range had lower genetic diversity and greater differentiation, even after accounting for genetic isolation by distance. Additional studies of related ranid frogs have found limited gene flow between populations of the same species with high levels of structuring among drainage basins, which has been linked with limited dispersal and gene flow across ridgelines (Funk et al., 2005; Lind, Spinks, Fellers, & Bradley Shaffer, 2011; McCartney-Melstad, Gidiş, & Shaffer, 2018). As far as we are aware, there have been no molecular analyses across deeply divergent ranid species which occur parapatrically. Thus the high level of genetic differentiation between *R. boylii* and *R. sierrae*, as well as within species across biogeographic breaks (Macey et al., 2001; McCartney-Melstad et al., 2018) largely supports these previous studies, with the exception of a novel identification of two hybrid individuals.

There remains the potential for low-levels of naturally occurring hybridization and introgression between *R. boylii* and *R. sierrae*, but currently both species appear to have clear genotypic divergence even in an area of co-occurrence. Thus, our data suggest this is unlikely to be a major concern for current conservation management, at least currently (see below). While there is a potential for misclassification of individuals in intermediate locations, genetic testing and/or monitoring could be a useful tool for clarifying species and population boundaries as well as estimating genetic population sizes.

Investigating the timing of divergence events between species (as well as better understanding bottlenecks and population expansion within species) in relation to landscape history can be informative in understanding what events may have driven divergence. The landscape of the Sierra Nevada during the Pleistocene epoch was one of repeated glaciation (Moore & Moring, 2013). Rivers flowing into the present-day Central Valley were being alternately eroded by west-flowing streams during interglaciation or covered in glaciers. It is therefore likely that adaptation to colder climates (e.g., freezing lakes and streams) may have provided an advantage to individuals or populations occurring in localities where the effects of glaciation were most prominent. Both species have been found to use very similar hydrologic habitat for breeding and rearing in streams and rivers (Bondi, Yarnell, & Lind, 2013; Yarnell et al., 2019), but *Rana sierrae* are uniquely adapted to persist in short-growing periods common in the high Sierras (Bradford, 1983; Knapp et al., 2003). *Rana sierrae* tadpoles may overwinter multiple years before metamorphosing, thus *R. sierrae* may have diverged from *R. boylii* because of their ability to persist in colder climates, common during periods of glaciation during the Pleistocene.

In rare species with small population sizes, hybridization outcomes that fail to produce successful offspring (sterile F1 hybrids) may have a greater cost on the species with low numbers of effective breeders, affecting both locally adapted populations and negatively impacting the probability of population persistence in a given region (Pagano, Dubois, Lesbarrères, & Lodé, 2003). For small populations of *R. sierrae* near the northern extent of the species range, under future scenarios (e.g., warming climate, range contraction, population crashes) the loss of even several breeding individuals (via reproduction with *R. boylii*) may have a significant impact in declining populations (Bradford, 1983, 1991; Joseph & Knapp, 2018; Knapp et al., 2016). With climate change, the future range extent of *R. boylii* may retract from lower elevations and expand into higher elevations, because habitats may become too hot or dry for the species to persist over time. This could increase the range overlap between *R. sierrae* and *R. boylii*, thus increasing the probability of admixture between the species. In the region of the Feather watershed where *R. boylii* and *R. sierrae* currently only occur parapatrically, fewer *R. sierrae* have been observed than *R. boylii*, and very few *R. sierrae* egg masses have been observed (C. Dillingham, personal communication, 25 April 2016). This difference could lead to greater competition for *R. sierrae* females, reducing male *R. sierrae* reproductive success through the loss of mating opportunities. This could potentially lead to reduced fecundity in *R. sierrae* because the female frogs only deposit one egg clutch per year (Bradford, 1983).

Assessing the impacts of current landscape and watershed change on the genetic variation of organisms, particularly sensitive and endangered species, may be a crucial tool for monitoring and more robust restoration, translocation, and conservation efforts. Future conservation of these species requires several key components, including establishing higher resolution population boundaries across the species' ranges, particularly in the northern Sierra Nevada, delineation of distinct population segments that can be utilized in conservation management, and quantification of relative genomic health of these groups. Identification of hybridization is a key step towards better delineating management units and further understanding what conservation steps may be taken.

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AUTHOR CONTRIBUTIONS

R.A.P. designed and performed research, analyzed data, and wrote the manuscript. M.R.M. analyzed data and helped write and revise the manuscript. S.M.O'R. helped perform research and analyze data. M.B. designed research, collected data, and revised the manuscript. C.G. designed research, helped write and revise the manuscript. G.M.W. collected data.

DATA AVAILABILITY STATEMENT

Data analyses scripts, documentation, Rapture probe sequences, and merged RAD-Seq Rapture bams are available available at osf.io: https://osf.io/34ntu/ (Identifier: https://doi.org/10.17605/OSF.IO/34NTU). Illumina raw reads for initial RAD-Seq of *Rana boylii* used to design custom myBaits kit and generate Rapture data are deposited in NCBI Sequence Read Archive (Bioproject PRJNA560634).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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