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Single-locus species delimitation and ecological niche modelling provide insights into the evolution, historical distribution and taxonomy of the Pacific chorus frogs

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The Pacific chorus frogs are a complex of three wide-ranging species (i.e. *Hyliola hypochondriaca*, *Hyliola regilla*, *Hyliola sierra*) whose current taxonomy remains unresolved. We conducted species delimitation analyses of these taxa using fragments of the cytochrome *b* and 12S–16S mtDNA genes to assess the species diversity. Importantly, we included samples from new locations throughout the range to better understand species distributions and identify potential contact zones among clades. Our analyses revealed three slightly parapatric but distinct species-level clades. Molecular dating revealed that these species began diverging in the Pleistocene *c*. 1.4 Mya with *H. hypochondriaca* and *H. sierra* diverging more recently *c*. 0.8 Mya. We found that populations from western Montana and Idaho originated recently from populations to the southwest that belong to *H. sierra*, rather than from *H. regilla* populations directly to the west. Population sizes of each species expanded *c*. 130–80 Kya with *H. hypochondriaca* exhibiting a more pronounced expansion beginning *c*. 100 Kya than the more gradual expansion of the other two species. The climatic niche models suggest that distributions of the three species were similar during the last interglacial (LIG) as they are today. During the Last Glacial Maximum (LGM), *H. hypochondriaca* and *H. sierra* occupied a larger range than they do today whereas *H. regilla* occupied a smaller refugium, shifted south from the current distribution. This study highlights the continued effectiveness of utilizing single-locus data sets for species delimitation and biogeographic analyses.

ADDITIONAL KEYWORDS: Anura – BEAST – *Hyliola* – MAXENT – phylogeny – *Pseudacris*.

INTRODUCTION

Accurately assessing alpha diversity is critical for understanding patterns and processes in ecology and evolution as well as contributing to broader conservation goals. The delineation and recognition of species diversity continues to increase across taxonomic groups (Padial & De la Riva, 2006; Uetz, 2010; Mora *et al.*, 2011) through the discovery of novel taxa (Köhler *et al.*, 2005) and recognition of cryptic diversity that was previously aggregated under a broader species designation (Isaac *et al.*, 2004; Costello *et al.*, 2012). Of particular importance when revising taxa is the evaluation of populations throughout their ranges, in particular contact zones of potentially unrecognized taxa (Hillis, 2019). Recently, the combination of niche modelling and genetic species delimitation has been an integral part of discovering and confirming novel species diversity (Rissler & Apodaca, 2007; Leachè *et al.*, 2009; Alvarado-Serrano & Knowles, 2014). The combination of genomic data sets and multilocus coalescent methods can provide powerful insights

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into species limits and evolutionary history (Edwards, 2009; Crawford *et al.*, 2012; McCormack *et al.*, 2012; McCormack & Faircloth, 2013; McCormack *et al.*, 2013; Edwards *et al.*, 2016). However, single locus (e.g. mtDNA, cpDNA) species delimitation methods also remain popular, particularly given the worldwide adoption of the DNA barcoding paradigm (Hebert *et al.*, 2003). Although single locus methods ignore the stochasticity of the coalescent process, relatively large sample sizes can be rapidly typed from typical biodiversity surveys, including microbiome assays. These large single-locus data sets can then be combined with both distance- (Puillandre *et al.*, 2012) and tree-based methods (Pons *et al.*, 2006; Kapli *et al.*, 2017) of species delimitation to help form initial species boundary hypotheses that can be subsequently tested with additional data [e.g. nDNA, morphology, ecology, behaviour (Carstens *et al.*, 2013; Blair & Bryson, 2017)].

The North American chorus frogs are a clade of 18 small, hylid species in the subfamily Acridinae. Until recently, these species were lumped in the genus *Pseudacris* but Duellman *et al.* (2016) described the genus *Hyliola* for the western taxa (i.e. the Pacific and California treefrogs). Although many studies have explored species diversity and relationships among clades of *Pseudacris* from central and eastern North America (e.g. Moriarty & Cannatella, 2004; Lemmon *et al.*, 2007; Degner *et al.*, 2010; Barrow *et al.*, 2014), systematic conclusions of *Hyliola* remain less resolved. Of particular interest is the Pacific chorus frog, *Hyliola regilla* (Baird and Girard, 1852) [formerly in *Hyla*], that at one point contained up to ten subspecies across its range (Jameson *et al.*, 1966; Duellman, 1970) spanning the west coast of North America across numerous biogeographic boundaries from British Columbia, Canada to Baja California, Mexico. Although morphological variation among populations of *H. regilla* has been detected (Snyder & Jameson, 1965; Jameson *et al.*, 1966, 1970), individual populations have also shown considerable variation (Vogt & Jameson, 1970), further complicating the identity of any diagnostic characters for particular lineages. Most recently, Ripplinger & Wagner (2004) and Recuero *et al.* (2006) utilized the cytochrome oxidase *b* (cyt *b*) gene to assess the evolutionary history of *H. regilla* across its distribution. Although these studies focused on the phylogeographic components of their research, Recuero *et al.* (2006) concluded that *H. regilla* should likely be divided into three species (i.e. *Hyliola hypochondriaca*, *H. regilla* and *Hyliola sierra*). However, these studies lacked broad enough sampling to include populations at boundaries between species' ranges and this gap in knowledge leaves species distribution and taxonomic conclusions questionable (Barrow *et al.*, 2014; Duellman *et al.*, 2016; Banker *et al.*, 2020).

In this study we use phylogenetic and single-locus species delimitation analyses to clarify the evolutionary history and assess the current taxonomy debated of the Pacific chorus frogs, the three taxa we refer to collectively as the *Hyliola regilla* complex. In order to incorporate data from previous phylogeography studies, we employed cyt *b* and 12S-16S sequence data from novel populations, filling gaps at the boundaries of the suggested species to identify potential contact zones or biogeographic barriers separating clades. Furthermore, we include divergence dating to assess timing of diversification and climate-based niche modelling of species' ranges to infer historical distributions, niche distinction and correlation of speciation with climatic/ecological shifts. We then compare our phylogeographic and ecological patterns to the literature in order to better understand the historical biogeography of the Pacific chorus frogs and place it in a broader context. Finally, we use these sequence data to infer the demographic history of *Hyliola* species throughout western North America to assess if population size changes correlate with historical niche expansion or reduction during glaciation events. This study helps to clarify the evolutionary history and species limits within *Hyliola* and further emphasizes the continued utility of single locus studies in systematics and evolutionary biology.

MATERIAL AND METHODS

Specimen sampling

We obtained tissue samples of the *H. regilla* species complex from 47 sites in California, Montana, Oregon, Washington and British Columbia (Fig. 1; Table 1). In particular, we sought individuals from areas devoid of sampling from previous studies (e.g. Ripplinger & Wagner, 2004; Recuero *et al.*, 2006) to examine potential contact zones at the edges of species distributions, currently considered a key aspect of species delimitation (Hillis, 2019). Frogs were transported to the laboratory alive and humanely euthanized with MS-222. After necropsy for parasites, small samples of thigh tissue were removed from the frogs and placed in 95% ethanol and then stored at -80 °C.

DNA extraction, sequencing and alignment

DNA was extracted from sampled tissues using a Qiagen DNeasy Blood and Tissue Kit. To maintain consistency with previous phylogenetic studies (e.g. Ripplinger & Wagner, 2004; Recuero *et al.*, 2006; Lemmon *et al.*, 2007) of *Pseudacris sensu lato*, in particular the *H. regilla* complex, we amplified partial regions of mitochondrial cytochrome *b* (cyt *b*, 600 bp) and 12S-16S (12S-16S,

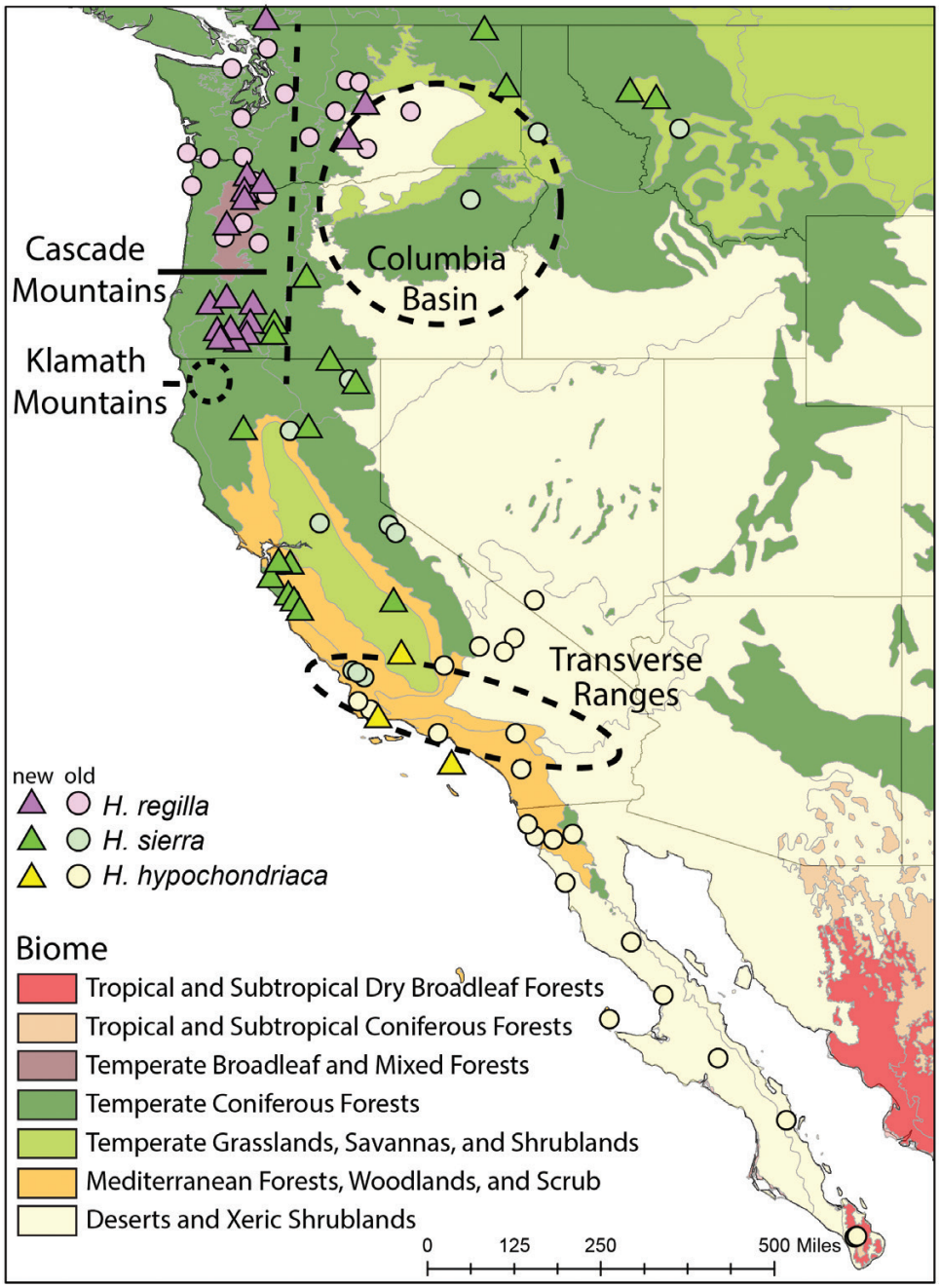

Figure 1. Map of populations sampled across the distribution with new sites labelled vs. previously published sites. Original sampling sites of tissues used in Ripplinger and Wagner (2004), Recuero *et al.* (2006) and this study. Dots/triangles correspond to *H. regilla* (purple), *H. sierra* (green) and *H. hypochondriaca* (yellow). The four physiographic features discussed in the text are indicate by dashed shapes.

726 bp). We used the primer pairs (MVZ 15+MVZ 16) from Moritz *et al.* (1992) and (12L1 + 16Sh) from Hillis & Wilcox (2005) following PCR temperature protocols in Ripplinger & Wagner (2004) and Hillis & Wilcox (2005), respectively. PCR products were sequenced in both forward and reverse directions using the PCR primers on a Beckman Coulter automated capillary sequencer. Sequence chromatographs were edited using Sequencher v.4.2, Gene Codes Corporation, Ann Arbor, MI, USA and all finished sequences were deposited in GenBank (Accession nos. MW308218–MW308304, Table 1). Our novel sequences generated from this study were combined with sequences previously deposited in GenBank (see Supporting Information, Appendices S1,

Table 1. Localities and generated sequences of individuals from the *Hyliola regilla* complex used in this study

Species	Latitude	Longitude	cyt *b*	12-16S	Locality description
H. regilla	49.5079	-122.5542	— — —	MW308259	Hr1 – Isabel Lake, British Columbia
H. regilla	47.284413	-120.340035	MW308218	MW308260	Hr2 – Milo Wood Pond, WA
H. regilla	46.585615	-120.632222	MW308219	MW308261	Hr3 – Cottonwood Elementary, WA
H. regilla	45.8034879	-122.7601343	MW308220	MW308262	Hr4 – Ridgefield, WA
H. regilla	45.541252	-122.844509	MW308221	MW308263	Hr5 – Spyglass, OR
H. regilla	45.51297	-122.39772	MW308222	MW308264	Hr6 – MHCC, OR
H. regilla	45.48922	-122.8394	— — —	MW308265	Hr7 – Alaho, OR
H. regilla	44.70635	-123.209653	MW308223	MW308266	Hr8 – Andy's Pond, OR
H. regilla	43.21082	-123.211825	MW308224	MW308267	Hr9 – Dixonville 1, OR
H. regilla	43.116869	-122.586125	MW308225	MW308268	Hr10 – Carmine, OR
H. regilla	43.077777	-123.604552	MW308226	MW308269	Hr11 – Moony BSA Pond, OR
H. regilla	43.07722	-123.60406	MW308227	MW308270	Hr12 – Moony Next Door, OR
H. regilla	42.687984	-122.733472	MW308228	MW308271	Hr13 – Cattle Pond, OR
H. regilla	42.5296	-123.1475	MW308229	MW308272	Hr14 – Paddle Boat, OR
H. regilla	42.52907	-123.14541	MW308230	MW308273	Hr15 – Dog Pond, OR
H. regilla	42.52897	-123.14169	MW308231	MW308274	Hr16 – New Pond, OR
H. regilla	42.490253	-123.368082	MW308232	MW308275	Hr17 – Barry's Pond, OR
H. regilla	42.450211	-122.8705	MW308233	MW308276	Hr18 – DWA-alt, OR
H. regilla	42.45001	-122.869838	MW308234	MW308277	Hr19 – DWA – Main, OR
H. regilla	42.4287	-123.2816	MW308235	MW308278	Hr20 – Tom Pearce Pond, OR
H. regilla	42.324157	-122.984172	MW308236	MW308279	Hr21 – Jacksonville Res (aka Heins Res), OR
H. sierra	48.90894	-117.77848	MW308237	MW308280	Hs1 – Silver Crown Lake, WA
H. sierra	48.897	-117.781272	MW308238	MW308281	Hs2 – South American Pond, WA
H. sierra	47.65	-117.29	MW308239	MW308282	Hs3 – East Pond Dishman Hills, WA
H. sierra	47.53547	-114.72515	MW308240	MW308283	Hs4 – Toolman Marsh, MT
H. sierra	47.3076	-114.19273	MW308241	MW308284	Hs5 – NBR 8/16/10, MT
H. sierra	43.675	-121.529167	MW308242	MW308285	Hs6 – La Pine 1B, OR
H. sierra	42.661316	-122.225496	MW308243	MW308286	Hs7 – North Lake, OR
H. sierra	42.520708	-122.243843	MW308244	MW308287	Hs8 – Dee lake, OR
H. sierra	41.89933	-121.04413	MW308245	MW308288	Hs9 – Clear Lake NWR, CA
H. sierra	41.44352	-120.49656	MW308246	MW308289	Hs10 – Modoc NWR, CA
H. sierra	40.50429	-121.4347	— — —	MW308290	Hs11 – BW2, CA
H. sierra	40.493011	-122.857846	MW308247	MW308291	Hs12 – Lowden Pond, CA
H. sierra	37.685451	-122.035217	MW308248	MW308292	Hs13 – 5 Canyon 3, CA
H. sierra	37.66135714	-121.931507	MW308249	MW308293	Hs14 – Quick Pond, CA
H. sierra	37.658097	-121.928906	MW308250	MW308294	Hs15 – Sheep Pond, CA
H. sierra	37.64720844	-121.919292	MW308251	MW308295	Hs17 – Kevin's Pond, CA
H. sierra	37.61994388	-121.891402	MW308252	MW308296	Hs16 – GDPND008, CA

Table 1. Continued

Species	Latitude	Longitude	cyt *b*	12-16S	Locality description
H. sierra	37.343307	-122.283436	MW308253	MW308297	Hs18 – DR04, CA
H. sierra	37.334949	-122.275175	MW308254	MW308298	Hs19 – DR02, CA
H. sierra	36.9729	-121.8825	MW308255	— — —	Hs20 – Valencia Lagoon, CA
H. sierra	36.9553	-121.869165	MW308256	MW308299	Hs21 – Santa Cruz Seascape, CA
H. sierra	36.92462	-121.8394	MW308257	MW308300	Hs22 – Ellicot Slough, CA
H. sierra	36.896	-119.511	— — —	MW308301	Hs23 – Sierra Foothills, CA
H. hypochondriaca	35.76181	-119.58373	MW308258	MW308302	Hh1 – Kern NWR, CA
H. hypochondriaca	34.41694	-119.8447	— — —	MW308303	Hh2 – Santa Barbara, CA
H. hypochondriaca	33.339917	-118.33394	— — —	MW308304	Hh3 – Catalina Island, CA

S2). Sequence data were aligned automatically with default settings using MUSCLE sequence alignment (Edgar, 2004) and then manually using Se-Al v.2.0a11 (Rambaut, 2002). No internal stop codons were found within the cyt *b* fragment.

Phylogenetic and species delimitation analysis

We used IQ-TREE v.1.6.12 (Nguyen *et al.*, 2015) to infer a maximum likelihood (ML) gene tree of the concatenated data. Initial runs were used to remove duplicate sequences from the alignment, leaving a total of 193 sequences for subsequent analysis. ModelFinder (Kalyaanamoorthy *et al.*, 2017) was used to select the best-fit substitution model using BIC. Support for nodes was assessed using both SH-aLRT with 1000 replicates (Guindon *et al.*, 2010) and UFBoot with 10 000 replicates (Hoang *et al.*, 2018), with values > 80% and 95% indicating strong support, respectively. All phylogenetic analyses in this study were rooted using the outgroup *Acris gryllus*.

We inferred phylogenetic relationships using Bayesian inference in MrBayes v.3.0b4 (Ronquist & Huelsenbeck, 2003). Prior to our analysis we used Akaike information criterion (AIC) to identify the best-fit models of nucleotide substitution for 12S-16S and each codon of cyt *b* implementing the program MrModeltest v.2.2 (Nylander, 2004), run in PAUP* v.4.0b10 (Swofford, 2002). Two simultaneous runs were conducted [with the default Markov chain Monte Carlo (MCMC) settings], for a total of 8×10^6 generations per run, sampling trees and parameters every 100 generations. We used Tracer v.1.6.0 (Rambaut *et al.*, 2014) to confirm stationarity and convergence of MCMC runs and therefore discarded the first 2.0×10^6 generations from each run as burn-in.

To help determine the number of species in the data set, we utilized the recently developed mPTP algorithm (Kapli *et al.*, 2017). Like the popular GMYC method (Pons *et al.*, 2006; Fujisawa & Barraclough, 2013), mPTP uses a gene tree to determine branching patterns consistent with speciation vs. those likely to represent gene coalescence within species. mPTP is a recent change to the PTP method (Zhang *et al.*, 2013) and explicitly accommodates differences in sampling intensity and/or genetic diversity within species by fitting multiple exponential branch length distributions to the data. Recent research showed that mPTP may be more conservative vs. other tree-based single locus species delimitation methods, though results tend to be more consistent with traditional taxonomy and general levels of genetic structure (Blair & Bryson, 2017). We used the IQ-TREE ML gene tree as input for mPTP. Four separate analyses were performed to asses congruence. First, we performed ML inference using both the single- and multi-rate method. We then ran MCMC analyses using both techniques to quantify support for

interspecific branches. MCMC analyses were run for 100 000 000 generations, sampling parameters every 10 000 generations. The first 10 000 000 generations were used as burn-in. We implemented ten independent MCMC runs to determine concordance to make sure that convergence was reached. We used the default value for the *minbr* parameter (0.0001), which is used to exclude very short branches that are introduced into ML phylogenetic inference to accommodate identical sequences. Although inclusion of a large proportion of identical sequence may introduce bias, this was less of an issue in our analysis because we removed the majority of identical sequences prior to gene tree inference.

We used BEAST v.2.6.2 (Bouckaert *et al.*, 2019) to estimate a gene tree using Bayesian inference using the same 193 sequences as the IQ-TREE analysis. Analyses used a strict clock, bModelTest for model selection (Bouckaert & Drummond, 2017) and a constant size coalescent tree prior. The analysis was run for 20 000 000 generations, sampling every 2000. Tracer v.1.6.0 (Rambaut *et al.*, 2014) was used to monitor ESS values to make sure that analyses were run for a sufficient length of time (target Effective Sample Size [ESS] > 200). TreeAnnotator was used to create a maximum clade credibility (MCC) tree following a burn-in of 10%. Node heights were annotated using mean values. The resulting MCC tree was used to perform GMYC analysis using the R-package Splits to compare results to mPTP.

Species tree and divergence time estimation

We used the multispecies coalescent model in StarBEAST2 (Ogilvie *et al.*, 2017) to simultaneously estimate a species tree with divergence times. Although StarBEAST2 is primarily geared towards multilocus analyses, it can also be used with single locus data. By explicitly accommodating the stochasticity of the coalescent process, species tree analyses can complement more traditional gene tree-based analyses and address issues of node overconfidence. We pruned the full alignment to a total of 46 sequences to reduce computational burden. Individuals were assigned to species based on a combination of current taxonomy (e.g. Recuero *et al.*, 2006; Barrow *et al.*, 2014; Duellman *et al.*, 2016) and the results of our other phylogenetic analyses. Multiple individuals were included for each species to account for intraspecific diversity. A total of 17 species were defined, including the outgroup. Some species were merged into a single taxonomic unit due to non-monophyly in gene trees, likely reflective of mtDNA introgression. Further, although some of our species delimitation analyses suggested that *H. hypochondriaca* should be split (see *Results*), we took a conservative approach and maintained a single taxon for species tree estimation. Analyses used an uncorrelated, lognormal species tree model, analytical population size integration, a GTR+I+G substitution model and a Calibrated Yule tree prior (Heled & Drummond, 2012). The remaining priors were set to their default values. Two independent chains were run for 80 000 000 generations each, sampling every 8000. Tracer v.1.6.0 (Rambaut *et al.*, 2014) was used to monitor ESS values to make sure that analyses were run for a sufficient length of time (target ESS > 200). LogCombiner was used to combine the tree files from both runs, following a burn-in of 10% of trees. TreeAnnotator was then used to construct a MCC tree.

For temporal calibration we followed Smith *et al.* (2005), who used fossil information from *Acris* to constrain the divergence of *Acris* and *Pseudacris sensu lato*. Thus, we calibrated the root node using a lognormal distribution with a mean (in real space) of 7, standard deviation of 0.5 and an offset of 13. This calibration scheme resulted in a mean divergence time of 20 Mya. We chose these values to accommodate both the age of the fossil (15–19 Mya) and the posterior estimate of the divergence time for the split between the two genera [30 Mya (Smith *et al.*, 2005)]. We also created a second constraint by forcing monophyly of the ingroup. However, no temporal calibration was used for this node.

Demographic changes

We examined demographic trends through time within the *H. regilla* complex using Extended Bayesian Skyline plots [EBSPs (Heled & Drummond, 2008)] in BEAST v.2.6.3. Although the EBSP method was developed in part to accommodate multilocus data sets, the method can also be used to examine demographic trends in single locus data. We created three separate alignments (i.e. *H. hypochondriaca*, *H. regilla* and *H. sierra*) that were each used to examine population trends through time. Each species was classified as a single 'population' due to the very short branch lengths in the gene tree, suggestive of panmixia. Each analysis used a strict clock, bModelTest and an EBSP tree prior. All samples (including redundant haplotypes) were used in analyses. For temporal calibration we used the estimated mtDNA substitution rate from our fossil calibrated divergence time analysis. To accommodate uncertainty in this value, we placed a normal prior on the rate using mean = 0.0224, sigma = 0.0025 and offset = 0. We also followed the recommendation of the program authors and changed the prior for populationMean.alltrees to a normal distribution with mean = 1, sigma = 0.1 and offset = 0. All analyses were run for 20 000 000 generations, sampling every 2000. Tracer was used to monitor convergence and ESS values and TreeAnnotator was used to create MCC trees following a burn-in of 10% with nodes annotated with mean heights. Demographic trends were visualized using the plotEBSP in R.

Ecological niche modelling

We reconstructed suitable climatic niche of *H. hypochondriaca*, *H. regilla* and *H. sierra* for current climatic conditions, climatic conditions of the Last Glacial Maximum (LGM; ~22 000 years ago), and those of the Last Interglacial (LIG; ~120 000–140 000 years ago) across the range of each species using ecological niche modelling. This methodology uses environmental data associated with occurrence records to estimate habitat suitability across the landscape by means of various program-specific algorithms (summarized in Elith *et al.*, 2006). For occurrence data, we used sampling localities for which the identification of the three species have been confirmed using genetic data, given that identifications of these frogs using morphological characters are problematic. We were able to obtain 26 records for *H. hypochondriaca*, 41 records for *H. regilla* and 35 records for *H. sierra* (Supporting Information, Appendix S2; Table 1). We then filtered the occurrence records using the R package spThin (Aiello-Lammens *et al.*, 2015) to only include one occurrence record per 10 km. This filtering alleviated potential bias caused by unequal sampling effort (Merow *et al.*, 2013) and yielded 24, 34 and 26 occurrence records for the three species, respectively.

We estimated the spatial distribution of suitable climatic conditions for the three *Hyliola* species using 19 bioclimatic variables with a resolution of 2.5 min (~5 km) from the WorldClim data set v.1.4 (Hijmans *et al.*, 2005). The climatic data for current conditions are derived from interpolations of observed data, representative of the years 1960–1990 (Hijmans *et al.*, 2005). For the climatic conditions of the LGM, we used three simulation models of the LGM climate: community-climate-system-model [CCSM4 (Collins *et al.*, 2006; Otto-Bliesner *et al.*, 2006)], the model-for-interdisciplinary-research-on-climate [MIROC-ESM (Sugiyama *et al.*, 2010)], and the model of the Max Planck Institute for Meteorology [MPI-ESM-P (Stevens *et al.*, 2013)]. The LIG conditions were simulated using the climatic model of Otto-Bliesner *et al.* (2006). These original climatic variables have been downscaled to the spatial resolution of 2.5 min (under the assumption of high spatial autocorrelation) and converted to bioclimatic variables (Hijmans *et al.*, 2005; Peterson & Nyári, 2008).

Because many climatic variables in the WorldClim data sets are highly correlated, we removed highly correlated variables by selecting a subset of variables with all pairwise correlation coefficients less than 0.9. The variables were selected in a way that maximized the number of variables and minimized pairwise correlation coefficients. When one of two variables needed to be removed, we selected seasonal averages over monthly and yearly averages. The resulting models comprised ten variables: mean diurnal range (Bio2), isothermality (Bio3), temperature annual range (Bio7), mean temperature of driest quarter (Bio9), mean temperature of driest quarter (Bio10), mean temperature of coldest quarter (Bio11), annual precipitation (Bio12), precipitation of wettest month (Bio13), precipitation of driest month (Bio14) and precipitation of coldest quarter (Bio19). We constructed climatic niche models for each climatic data set in the program MAXENT v.3.3.3k (Phillips *et al.*, 2006) using the R packages ENMeval (Muscarella *et al.*, 2014) and dismo (Hijmans *et al.*, 2015). MAXENT estimates relative probabilities of the presence of species within defined geographic spaces, with high probabilities indicating suitable environmental conditions (Phillips *et al.*, 2006; Phillips & Dudík, 2008). We used 1000 background points randomly extracted from a polygon drawn around the combined occurrence records of the three species and expanded by 3 °C in all directions. This selection of background points was chosen to exclude distant areas with very different environmental conditions, following recommendations by Merow *et al.* (2013). We explored values for the regularization multiplier (rm) between 0.5 and 4 (by increments of 0.5) and all combinations of available features (i.e. linear, quadratic, product, threshold and hinge). We ran random three-fold cross-validation replicates to choose a model with the best fit, as assessed by the lowest AICc value. The best-fitting models for each climatic data set were projected onto present climatic conditions and the three reconstructions of the LGM climate and the LIG reconstruction (with the extrapolation beyond training conditions enabled). All models were visualized using logistic probability values (Merow *et al.*, 2013). As a performance measure, we used the area under the curve (AUC). The AUC is the probability that a randomly chosen presence site will be ranked above a randomly chosen absence site. Models with AUC values above 0.75 are considered informative (Phillips & Dudík, 2008).

RESULTS

Phylogenetic analysis

The concatenated data consisted of 193 individuals and 1326 bp. Of the 1326 sites, 862 were constant and 364 were parsimony informative. ModelFinder in IQ-TREE selected TIM2+F+I+G4 as the best substitution model for ML. MrModelTest recovered the GTR+Γ model for 12S-16S, K80+I+Γ model for position 1, F81+I model for position 2, and GTR+Γ for position 3. Our ML and MrBayes analyses resulted in highly congruent gene trees with strong support for the majority of nodes (Fig. 2). Two primary clades were recovered: a central/eastern *Pseudacris* clade and a

western *Hyliola* clade consisting of *Hyliola cadaverina*, *H. hypochondriaca*, *H. regilla* and *H. sierra*. Monophyly of each of the western species was strongly supported. *H. hypochondriaca* was strongly supported as sister to *H. sierra*, both of which were sister to *H. regilla*.

Bayesian analysis in BEAST recovered a genealogy similar to IQ-TREE and MrBayes (Fig. 3). The MCMC chain was run for an adequate duration based on all ESS values > 200. Again, there was strong support for the monophyly of all western species, with *H. hypochondriaca* placed sister to *H. sierra* within the *H. regilla* complex. One major difference with the BEAST tree was the placement of the *Pseudacris crucifer+Pseudacris ocularis* clade as sister to the western clade, resulting in a paraphyletic *Pseudacris*. However, this relationship received weak support (posterior probability = 0.7).

Species delimitation

We first performed ML analysis in mPTP using both the single- and multi-rate options. The multi-rate results suggested a total of 13 species. Each western

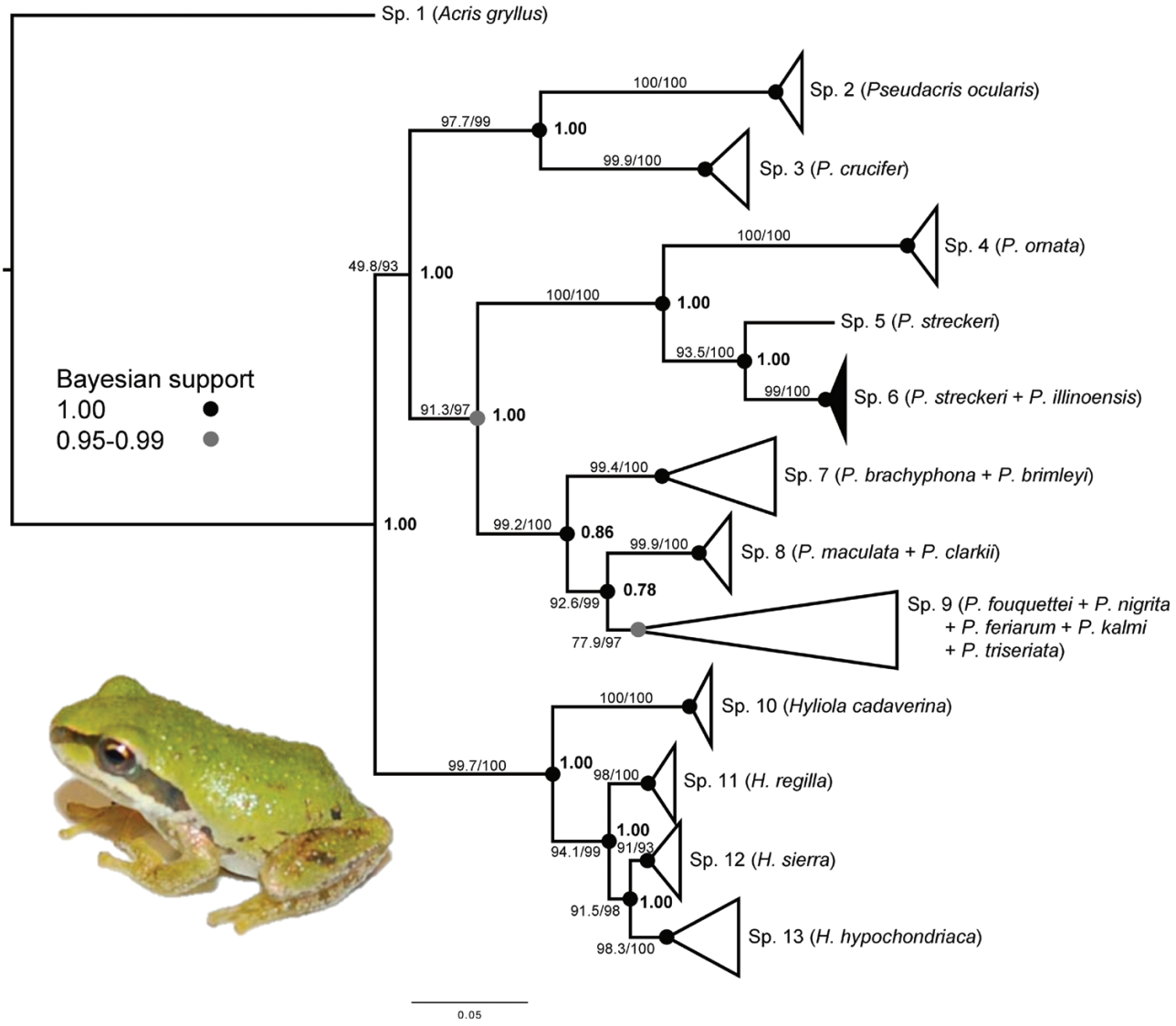

Figure 2. Maximum likelihood (ML) gene tree for cytochrome *b* and 12S-16S sequences from all *Pseudacris* haplotypes sampled in this study. Values above branches represent support values from IQ-TREE (SH-aLRT/ultrafast bootstrap). Values to the right of nodes (in bold) indicate the probability that the branch is involved in the speciation vs. coalescent process. All branches assigned to speciation or coalescence were determined through MCMC analyses in mPTP. Additional nodal support provided by posterior probability distributions from Bayesian inference in MrBayes. Metamorph of *H. regilla* from MHCC, OR (insert).

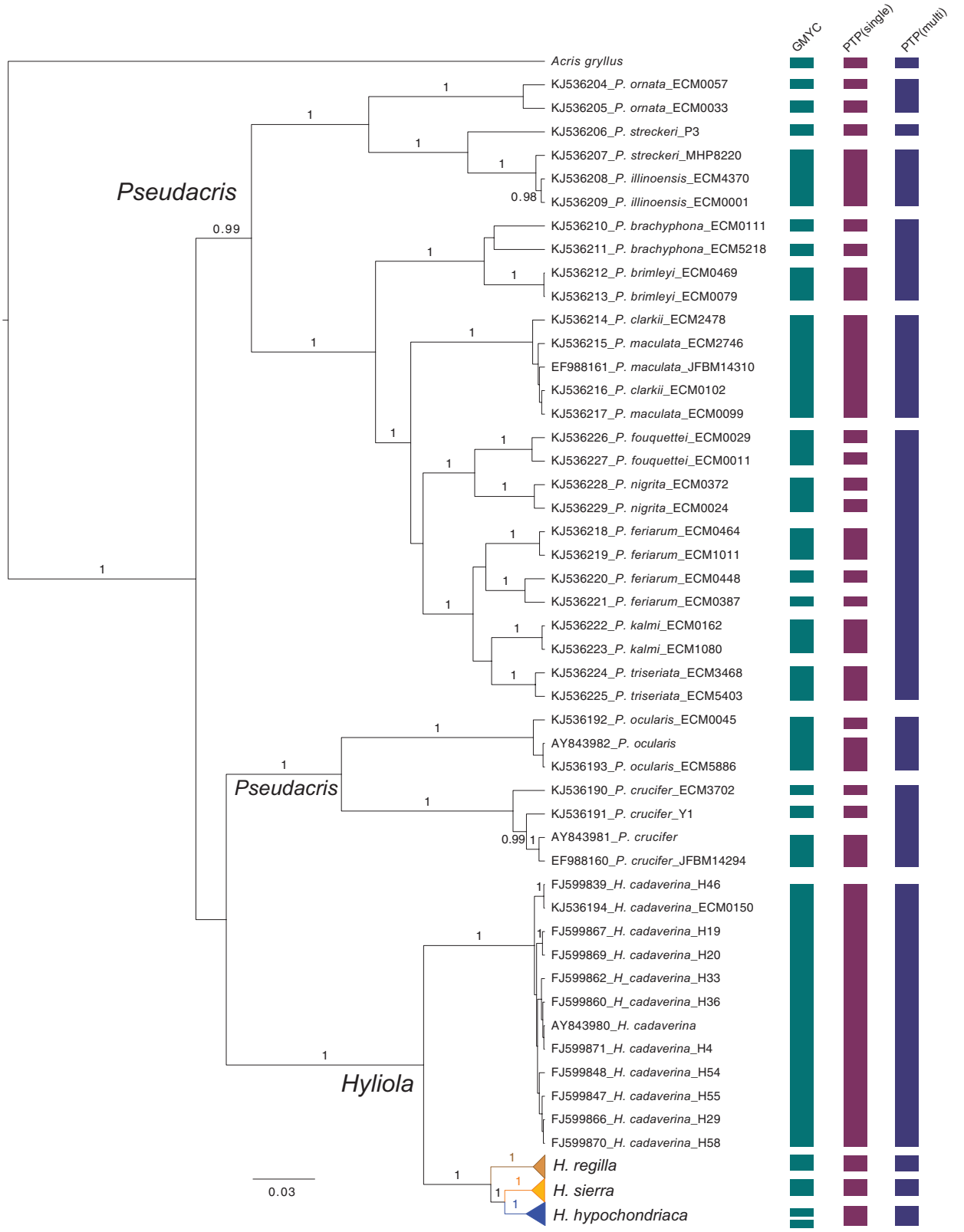

Figure 3. BEAST maximum clade credibility tree inferred from the concatenated mtDNA data. Values on branches indicate posterior probability support (> 0.95). The three target clades of *Hyliola* were collapsed for ease of viewing. Coloured bars to the right of the tree show species delimitation results from the three methods used in this study.

species was designated as distinct. However, many species of *Pseudacris* were merged into a single taxonomic unit. The single-rate results suggested a total of 27 species. Again, each western species was distinct. The single-rate analysis split the *Pseudacris* clade into many different species, many of which were composed of singletons (Fig. 3).

To provide statistical support for branches representing speciation events, we performed MCMC analyses in mPTP under both the single-rate and multi-rate model. Independent runs for both analyses appeared to sample the same parameter space. However, the single-rate analyses seemed to sample two distinct likelihood optima. For the multi-rate analysis there was full support for species-level diversification among all western taxa (Fig. 2). There was considerably more lumping in the *Pseudacris* clade, with results suggesting that branching within *Pseudacris feriarum*, *Pseudacris fouquettei*, *Pseudacris kalmi*, *Pseudacris triseriata* and *Pseudacris nigrita* all belong to the coalescent process. The single-rate analysis also provided full support for all speciation nodes in western taxa. The results within the *Pseudacris* clade were substantially different with this model. Here, the vast majority of nodes belonged to the speciation process with strong support (0.95–1.0), indicating a much larger number of species.

We also wanted to compare our PTP results to GMYC analysis. Using the ultrametric BEAST genealogy as input, GMYC recovered 15 ML clusters (CI = 14–17) and 25 ML entities (CI = 21–27), the latter of which is an indication of the total number of species. The likelihood ratio tests strongly rejected the null model in favour of the GMYC model ($P < 0.001$). Each western taxon was classified as a distinct species. In fact, GMYC suggested that *H. hypochondriaca* contains two distinct species. The GMYC results were broadly congruent with the single-rate PTP analyses, splitting many species into singletons (Fig. 3). These results appear counter to levels of genetic differentiation based on ML branch lengths and are likely a result of the GYMC model (and single-rate PTP) failing to account for different effective population sizes and levels of diversity within species.

Species tree and divergence times

Using results from our phylogenetic and species delimitation analyses, we created a pruned data set to simultaneously estimate a species tree and divergence times. Due to the non-monophyly of some of our species (*Pseudacris clarkii* and *Pseudacris maculata*, *Pseudacris illinoensis* and *Pseudacris streckeri*) we indicated composite species for StarBEAST2. All ESS values were > 100 (all but one over 200) indicating adequate sampling of the posterior. The mean estimated rate of substitution was 0.0224 substitutions per site per million years (95% HPD = 0.0082404–0.0432) based on calibrating the root node with fossil information. The *branchRatesStdev* parameter had a mean value of 0.3972 (95% HPD = 0.0986–0.7778, indicating some substitution rate heterogeneity among branches. The tree topology was identical to the BEAST tree consisting of all 193 sequences estimated under a strict clock. Again, *Pseudacris crucifer* and *Pseudacris ocularis* were placed as sister to the western clade, but with low support (posterior probability = 0.44). Divergence of the ingroup began in the Miocene *c*. 10 Mya (Fig. 4). Divergence within the western clade began in the Pliocene *c*. 3 Mya (Table 2). The three lineages within the *H. regilla* clade diverged during the Pleistocene.

Historical demography

We constructed EBSPs to determine if the three '*H. regilla*' species experienced similar demographic shifts in response to Pleistocene climate change. Although our demographic inferences are based on a single locus, the large sample sizes within each lineage can partly overcome this limitation (Heled & Drummond, 2008). To add a temporal component to these analyses we calibrated runs using our estimated rate of mtDNA substitution. Recovered ESS values indicated adequate sampling of the posterior. All three species appeared to have experienced a population expansion *c*. 100–70 Kya (Fig. 5). *H. regilla* and *H. sierra* appeared to experience a more gradual increase, whereas *H. hypochondriaca* exhibited a more pronounced expansion beginning *c*. 100 Kya. In addition to a qualitative assessment of plots, examination of parameter estimates in Tracer were used to determine demographic shifts. For *H. regilla*, the mean number of shifts was 1.46 (95% HPD = 1–3). The mean number of shifts for *H. sierra* was 1.49 (95% HPD = 1–3). Finally, the mean number of shifts for *H. hypochondriaca* was 1.28 (95% HPD = 0–3). As the latter included zero shifts within the confidence interval we were unable to reject the hypothesis of a constant population size in this species. This is most likely due to the wide confidence interval depicting population stability throughout most of the history of this species (note differences in the scale axes in Fig. 5). However, results showed that the posterior had the highest frequency at one population size change, congruent with the other two species.

Ecological niche modelling

The climatic niche model with the best fit used linear and quadratic features, with rm values of 1.5, 1 and 0.5 for *H. hypochondriaca*, *H. regilla* and *H. sierra*, respectively.

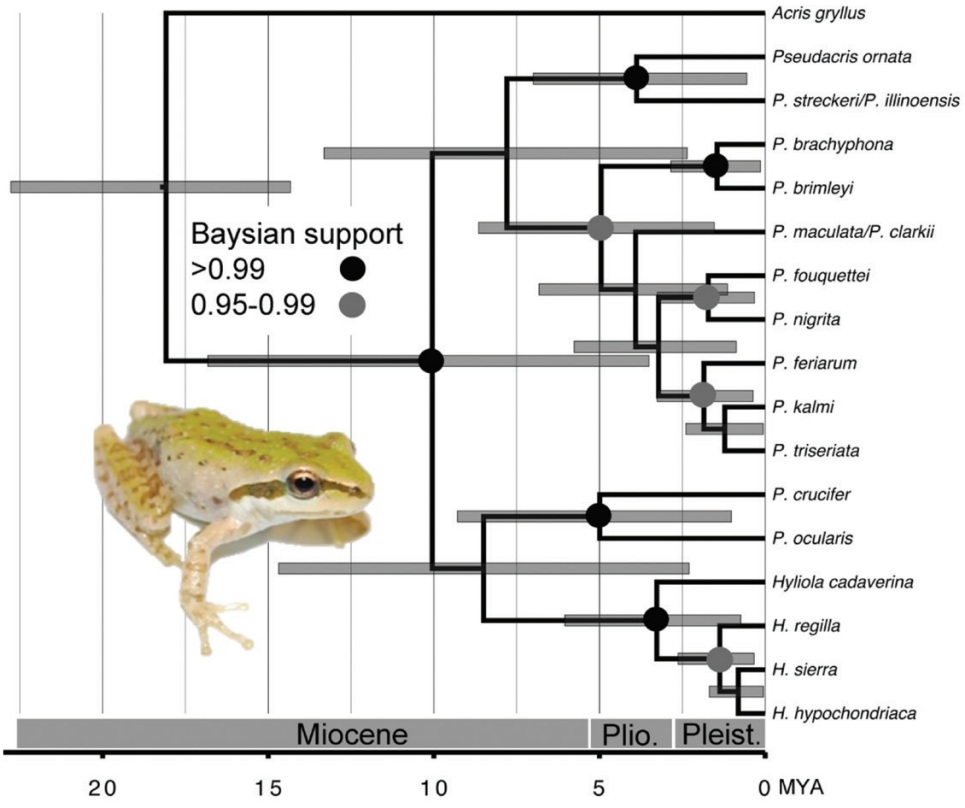

Figure 4. StarBEAST2 results showing divergence times within the *H. regilla* complex and related taxa. Node heights were annotated using mean values (in millions of years). Nodes missing grey or black circles received relatively low posterior probability support (< 0.95). Metamorph of *H. sierra* from Pleasanton, CA (insert).

Table 2. Estimates of divergence times for major splits among the Pseudacrinae found in Figure 4

Node	Mean	Lower	Upper
Outgroup split	18.087	14.327	22.771
Two major clades within the Pseudacrinae	10.049	3.506	16.825
Origin of *Hyliola*	8.499	2.296	14.685
H. cadaverina – *H. regilla* complex	3.271	0.739	6.044
Initial split within the *H. regilla* complex	1.369	0.336	2.634
H. hypochondriaca – *H. sierra*	0.813	0.053	1.686

The AUC values for the best fit model are 0.91, 0.89 and 0.86, same sequence as above. The climatic niche models approximating the distribution of each species under the assumption of niche conservatism suggest that the distributions of all three taxa during the warm LIG were similar as they are today (Fig. 6). The distributions during the cold LGM appear quite different. *H. hypochondriaca* and *H. sierra* appear to have generally persisted within the area they inhabit today; however, their distribution within these areas was broader. This is likely due to the colder and wetter climatic conditions in this part of the world during the LGM (Otto-Bliesner *et al.*, 2006) that allowed these species to occupy areas in southern California that are currently too hot and dry. Conversely, *H. regilla* seems to have been displaced from its current distribution and occupied a smaller refugium, shifted south from the current distribution (Fig. 6). The models further indicate that the three taxa have somewhat overlapping climatic niches as the models of all three species overpredict into the ranges of the other species. This indicates that these taxa likely did not originate due to variations in climatic niches.

DISCUSSION

Systematics of the Pacific chorus frogs and the utility of single locus delimitation methods

H. regilla was classified as a species with up to ten subspecies (Jameson *et al.*, 1966). Several of these

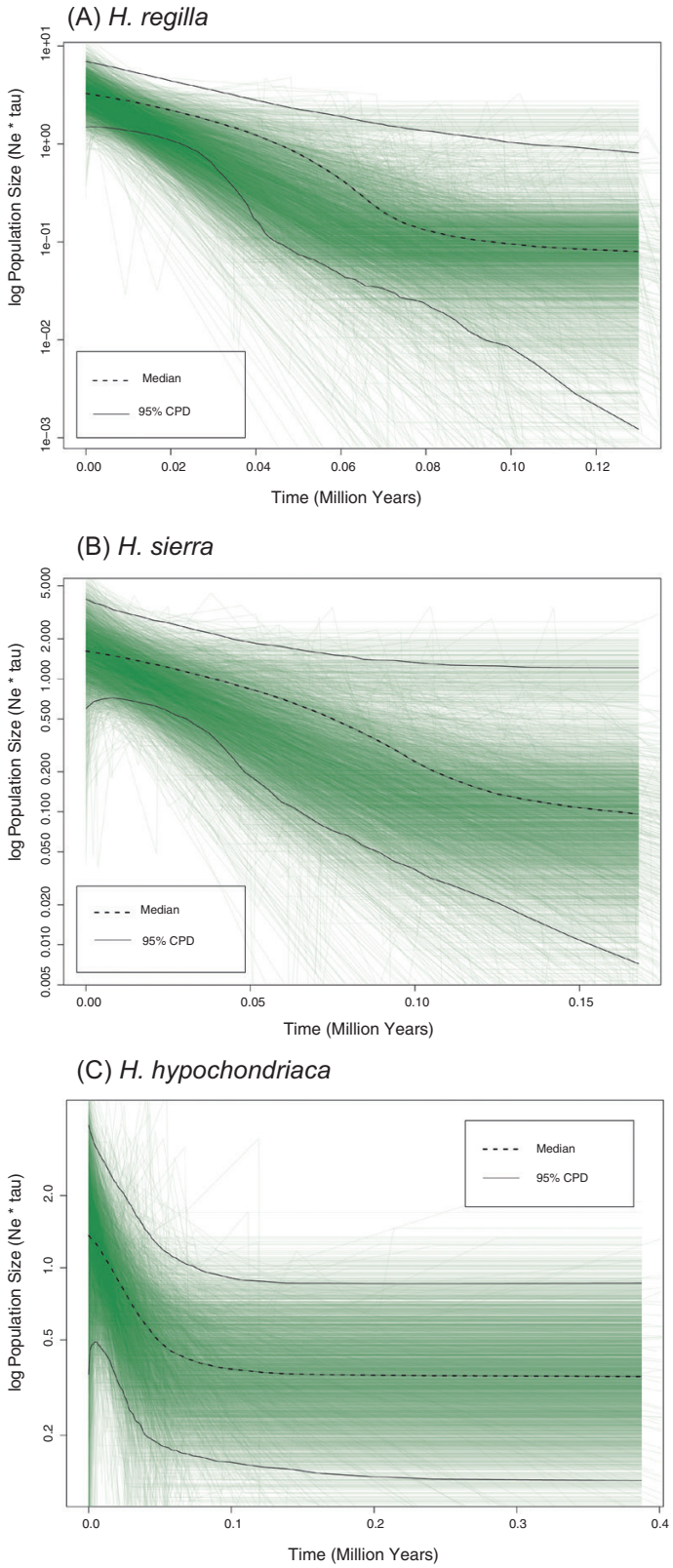

Figure 5. Extended Bayesian skyline plots (EBSPs) for the three *Hyliola* species distributed throughout western North America. A, *H. regilla*. B, *H. sierra*. C, *H. hypochondriaca*. N_e = the effective number of females; tau = generation time; CPD = central posterior density.

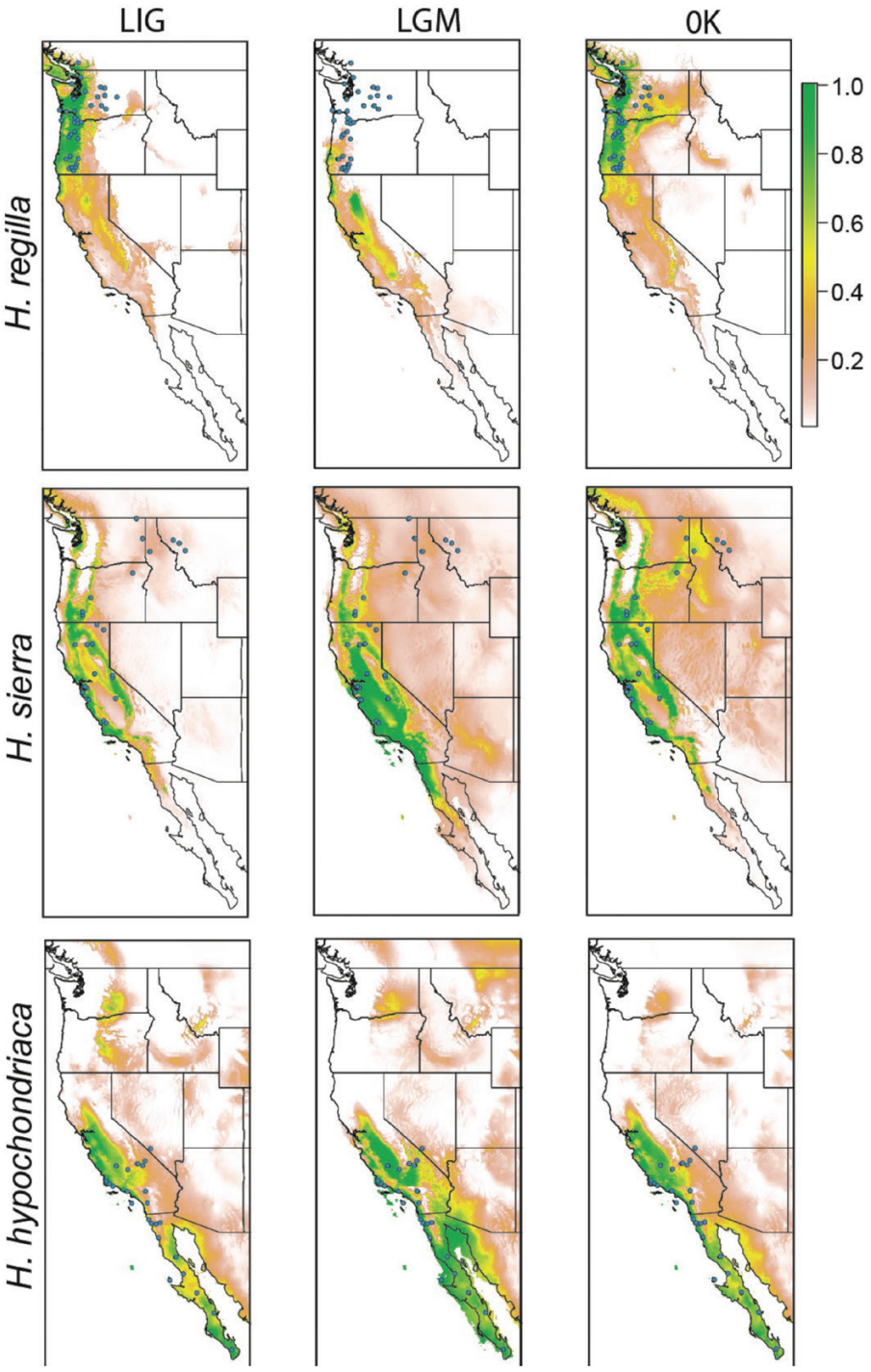

Figure 6. Climatic niche models for *H. hypochondriaca*, *H. regilla* and *H. sierra* for the climatic conditions of the last interglacial (LIG), Last Glacial Maximum (LGM) and the current time (0K). The model for LGM is a consensus of the three reconstructions (CCSM4, MIROC-ESM and MPI-ESM). Blue dots indicate localities used to build the models. The models are visualized using logistic probability values.

subspecies were elevated or reallocated to other species leading to the recognition of seven subspecies (Crother *et al.*, 2000; Duellman, 2001) with intraspecific distinction of morphology and physiology (Jameson *et al.*, 1970). Most recently, phylogeographic analyses have shown evidence for three distinct species

(Recuero *et al.*, 2006) and this taxonomy is currently accepted (Crother *et al.*, 2012; but see Barrow *et al.*, 2014; Duellman *et al.*, 2016; Banker *et al.*, 2020). Our species delimitation analyses support the conclusion by Recuero *et al.* (2006) that the Pacific chorus frogs are three distinct species (i.e. *H. hypochondriaca*, *H. regilla* and *H. sierra*) and this is corroborated by previous studies using allozymes (Case *et al.*, 1975), mating calls (Snyder & Jameson, 1965) and physiological responses (Jameson, 1966; Jameson *et al.*, 1970). Therefore, we recommend treating the taxonomy of the Pacific chorus frogs to include all three of these taxa. Finally, we did not detect strong evidence for the presence of additional lineages that may warrant subspecies or species status.

Our phylogenetic results are mostly congruent with previous studies of evolutionary relationships among the Acridinae (e.g. Moriarty & Cannatella, 2004; Lemmon *et al.*, 2007; Barrow *et al.*, 2014; Duellman *et al.*, 2016; Banker *et al.*, 2020). Similar to these studies we detected a monophyletic *Hyliola* that is sister to *Pseudacris*. These two clades appear to have diverged *c*. 10 Mya (Table 2). Within *Pseudacris* we found support for the monophyly of fat frogs, trilling chorus frogs and crucifer frogs in our BI and ML analyses (Fig. 2) but not in our species tree analysis (Figs 3, 4) due to an inconsistent placement of the *P. crucifer-P. ocularis* clade. Within *Hyliola* we found *H. cadaverina* sister to the Pacific chorus frogs, separating *c*. 3.3 Mya, and found *H. hypochondriaca* sister to *H. sierra*, to the exclusion of *H. regilla*.

Although multilocus species delimitation is now common (Fujita *et al.*, 2012; Grummer *et al.*, 2014; Leaché *et al.*, 2014; Jackson *et al.*, 2017; Blair *et al.*, 2019; Flouri *et al.*, 2018), single locus methods have broad applicability as a rapid and relatively inexpensive means to form initial species hypotheses and test previous hypotheses based on other lines of evidence. However, there is still no consensus regarding which method(s) should be used in which types of data sets. The mPTP method was developed to explicitly account for differences in coalescent histories among species, which may be related to sampling effort, genetic diversity and effective population size (Kapli *et al.*, 2017). Recent results suggest that mPTP may be one of the more conservative and stable single locus methods currently available (Blair & Bryson, 2017), though the degree of conservatism is likely data set-dependent. An additional benefit of the mPTP method lies in its ability to quantify support for speciation nodes in the gene tree (via MCMC). The discordance observed with our species delimitation results suggest that researchers interested in tree-based single locus species delimitation methods explore their data fully to adequately capture levels of uncertainty in the results. Although additional simulation and empirical studies are needed to compare the performance of methods, both distance-based [e.g. ABGD (Puillandre *et al.*, 2012)] and tree-based methods like mPTP and GMYC remain useful as part of an integrative taxonomic framework. However, we urge researchers not to make formal taxonomic changes based solely from the results of these analyses, as the stochasticity of the coalescent process and/or introgression can result in shared polymorphisms and non-monophyly in gene trees.

Biogeography of the Pacific chorus frogs

The range of the *H. regilla* complex in the western USA overlaps with several regions that have been the subject of extensive biogeographic studies including the California Floristic Province and the Pacific northwest (Calsbeek *et al.*, 2003; Lapointe & Rissler, 2005; Steele *et al.*, 2005; Feldman & Spicer, 2006; Pelletier & Carstens, 2016). These areas have a complex geological and climatic history such as mountain uplift, drainage of inland seas and repeated glaciation (Ripplinger & Wagner, 2004; Spinks *et al.*, 2010). Surprisingly, we did not find any biogeographic breaks within any of the three taxa, suggesting high dispersal abilities of these frogs. However, we did examine phylogeographic patterns among the clades of Pacific chorus frog species to assess if significant breaks in distribution match physical (e.g. mountain ranges) or ecological boundaries (e.g. biomes, watersheds) in common with some other co-occurring species (Calsbeek *et al.*, 2003; Feldman & Spicer, 2006; Martínez-Solano *et al.*, 2007; Phillipsen & Metcalf, 2009; Myers *et al.*, 2013; Reilly & Wake, 2015).

Previously, Ripplinger & Wagner (2004) found the Pacific chorus frogs of the Pacific northwest separated as coastal and inland clades by the Columbia Basin. Using the cytochrome *b* gene fragment, they hypothesized a separation of the clades in the Pliocene (~3 Mya) into coastal and inland populations due to High Cascade orogeny and xerification of the Columbia Basin (Ripplinger & Wagner, 2004). Likewise, Recuero *et al.* (2006) used the same gene fragment and detected three distinct groups corresponding to “northwestern”, “central” and “southern” clades also consistent with an earlier allozyme study by Case *et al.* (1975). The “central” clade included the samples from Idaho and eastern Oregon that were added as part of the “inland clade” from Ripplinger & Wagner (2004). With our auxiliary sampling and additional gene fragment, we also found eastern populations of *H. sierra* in Idaho and Montana to be more closely related to populations from central California and Oregon than to those of *H. regilla* in the Pacific northwest. At the southern end of the range, *H. sierra* and *H. hypochondriaca*

were split northwest of the Sierra Pelona Mountains (Recuero *et al.*, 2006; Phillipsen & Metcalf, 2009). Furthermore, our estimate of 3.2 Mya (0.8–6.0 Mya) divergence is consistent with the 2.6–6.6 Mya split estimate between the clades within the *H. regilla* complex and *H. cadaverina* reported by Phillipsen & Metcalf (2009) who utilized cytochrome *b*.

Building on extensive previous research on a diversity of co-occurring taxa, we can place our results into a comparative phylogeographic context. Beginning in the northwest, the boundary between *H. regilla* and *H. sierra* corresponds with other phylogeographical breaks that occur along the Oregon—California border (Swenson & Howard, 2005; Pelletier & Carstens, 2016). For example, the southwestern edge of *H. regilla* is associated with the Klamath Mountains, which were a geologically stable formation throughout the Pliocene and Pleistocene (Reilly & Wake, 2015). In comparison, Reilly & Wake (2015) employed a multilocus data set and found that the northernmost clade of *Aneides flavipunctaus* reaches the Klamath Mountains and is separated from the central core clade of *Aneides* that spans the Coastal Range in an area known as the 'Humboldt Basin,' which is associated with tectonic plate boundaries. However, the boundary between the two clades of *Aneides* is further south compared with our range estimates of *H. regilla* and *H. sierra* (Reilly & Wake, 2015). The boundary between *H. regilla* and *H. sierra* is associated with the Cascade Mountains in the south and to the east is separated by the Columbia Basin (Fig. 1), similar to separations between *Dicamptodon aterrimus* and other *Dicamptodon* species found by Steele *et al.* (2005) utilizing a fragment of cytochrome *b*. This phylogeographic break is thought to be associated with the origin of the Cascades resulting in a rain shadow forming the Columbia Basin approximately 2 Mya (Ripplinger & Wagner, 2004; Steele *et al.*, 2005). This timing falls within the range of our divergence estimate of the *H. sierra* and *H. regilla* clades. A similar pattern was also detected between of *Ascaphus montanus* and *Ascaphus truei* using mtDNA fragments cytochrome *b* and ND2 (Nielson *et al.*, 2006; Carstens & Richards, 2007). However, in both *Ascaphus* and *Dicamptodon* the species ranges are isolated to only Idaho, Montana and far eastern Washington and Oregon, rather than extending south and west to the coast of California as we observe for *H. sierra* (Steele *et al.*, 2005; Nielson *et al.*, 2006; Carstens & Richards, 2007). Furthermore, the range of *H. regilla* extends further east with some of our sampled populations occurring within the Columbia Basin (Fig. 1).

At the southern edge of the distribution, we can examine the boundaries between the *H. regilla* complex and *H. cadaverina* as well as between *H. sierra* and *H. hypochondriaca* and the comparison with other co-occurring taxa. The Transverse Ranges represent a phylogeographic break within *H. cadaverina* and are believed to be the centre of origin for the species with estimates of establishment during the Pleistocene (Phillipsen & Metcalf, 2009). Unlike the other species within the *H. regilla* complex, *H. cadaverina* is a stream specialist that appears to be limited to disjunct populations corresponding to major watersheds and mountain ranges (Phillipsen & Metcalf, 2009). Many other co-distributed taxa show lineage breaks corresponding to the Transverse Ranges including the *Charia bottae* complex, *Diadophis punctatus*, *Elgaria multicarinata*, *Lampropeltis zonata* and *Sepedophilus castaneus* with divergence estimates of these various groups between 5.31–3.33 Mya and 1.63–1.49 Mya, suggesting at least two distinct historical events influenced the evolutionary history of these taxa (Feldman & Spicer, 2006; Chatzimanolis & Caterino, 2007; Phillipsen & Metcalf, 2009). These results also corresponded with an earlier comparative study by Calsbeek *et al.* (2003) that found most animal taxa investigated possessed a significant genetic split to the north and south of the Transverse Ranges. Importantly, breaks in this region have been detected in both aquatic and terrestrial animals (Phillipsen & Metcalf, 2009). The drivers of this species break could be a combination of ecological and geological factors, including mountain uplift and barriers resulting from marine incursions of inland valleys (Phillipsen & Metcalf, 2009). The geographic split between *H. hypochondriaca* and *H. sierra* along the coast appears to be the same break found in trapdoor spiders of the genus *Aliatypus* using a multilocus data set (Satler *et al.*, 2013). However, not all breaks associated with the Transverse Ranges occur in the same location. For example, the lineage break found for *H. sierra* differs from the break that Spinks & Shaffer (2005) found for the western pond turtle (*Actimemys marmorata*) utilizing a multilocus data set.

One important phylogeographic boundary that is lacking in *H. sierra* and *H. hypochondriaca* but found in many species, especially amphibians and reptiles, is the Central Valley that separates the Sierra Nevada and Coast Range mountains (Calsbeek *et al.*, 2003; Feldman & Spicer, 2006; Rissler *et al.*, 2006; Martínez-Solano *et al.*, 2007). This break could be based on ecological factors because the valley habitat is unsuitable for forest dependent taxa, which also explains the ring-like distribution for some species along the surrounding mountains (Calsbeek *et al.*, 2003; Feldman & Spicer, 2006; Hedin *et al.*, 2013). Perhaps surprisingly, the eastern clade of *Batrachoseps attenuatus* also spans the Central Valley as does *Aneides lugubris* and the northern clade of *Actinemys marmorata* (Lapointe & Rissler, 2005; Spinks & Shaffer, 2005; Martínez-Solano *et al.*, 2007;

Spinks *et al.*, 2010). This so called "transvalley leak" was also supported by large-scale comparative analysis (Lapointe & Rissler, 2005). Other notable exceptions to this pattern were birds, which may suggest that highly mobile or migratory species would not reflect this break due to continued gene flow (Calsbeek *et al.*, 2003). Perhaps this break has been overcome by dispersal of *H. hypochondriaca* and *H. sierra*. Specific dispersal data for these species are lacking; however, for *H. regilla* at the northern part of its range dispersal has been estimated at 0.9 km/y (range 1.1–2.5 km/y) for low elevation terrain with lots of ponds (Reimchen, 1991).

Members of the *H. regilla* complex lack other significant breaks found in co-occurring taxa. For example, Martínez-Solano *et al.* (2007), using a multilocus data set, found the San Andreas Fault and the Sacramento–San Joaquin Delta to be important drivers in the diversification of *Batrachoseps attenuatus* lineages, which we did not find for members of the *H. regilla* complex. Likewise, the Monterey Bay region is one historical barrier where several taxa show concordant phylogeographical breaks (Calsbeek *et al.*, 2003; Lapointe & Rissler, 2005; Feldman & Spicer, 2006). It is important to note that as new methods and analyses have been incorporated for some taxa, revisions to previously identified breaks have occurred. In contrast to Feldman & Spicer (2006), who reported a break between lineages of *L. zonata* at the Transverse Ranges using mtDNA, Myers *et al.* (2013) utilized a multilocus data set and revised the divergence to have occurred ~2.07 Mya in association with the Monterey Bay inundation of central California. This revision also occurred based on two different studies of the western pond turtle (*Actinemys marmorata*) (Spinks & Shaffer, 2005; Spinks *et al.*, 2010). These studies highlight two points—the importance of the Monterey Bay barrier and also that future analyses of the *H. regilla* complex, particularly those including multiple loci, may reveal additional or revised phylogeographic breaks (Spinks *et al.*, 2010; Myers *et al.*, 2013).

Although our results contribute a more complete understanding of the biogeography of the *H. regilla* complex, there are important caveats and outstanding questions for future research. Specifically, *H. sierra* is not constrained from contact with its congeners to the north and south and appears to be sympatric at species borders. Therefore, based on our current analysis, definitive conclusions on the original speciation event are still needed. Although our study includes the largest geographic sampling of the Pacific chorus frogs to date, it is still too limited to ensure that we have recovered all major clades or determined the exact location of contact zones between them.

Demography and niche modelling of the Pacific chorus frogs

The Pacific chorus frogs have been and continue to be considered one of if not the most abundant amphibian throughout western North America (Brattstrom & Warren, 1955; Leonard *et al.*, 1993). Our demographic analyses suggest that the three species all experienced population expansions during the sometime during the Eemian/Last Glacial Period transition *c*. 130-80 Kya (Fig. 5). These results are consistent with the changes in distribution inferred by niche models for *H. hypochondriaca* and *H. sierra* as models suggest that the two species have experienced a geographic expansion during the last glacial period. Historical demographic analyses conducted by Recuero *et al.* (2006) also supported past population expansions, especially in *H. hypochondriaca*. Conversely, the demographic assessment of *H. regilla* is inconsistent with the shifts in distribution inferred by the niche models. The genetic data suggest demographic expansion timed during the last interglacial whereas the niche models suggest displacement into a southern refugium during this time period. The placement of the refugium, however, is within the range of *H. sierra,* which would mean that the two species may have been sympatric during the last glacial period. We do not believe this was the case as there is no evidence that the two species were until recently in contact. Alternatively, *H. regilla* might have persisted within its current range, occupying a different climatic niche today than it did during the LGM (Jezkova *et al.*, 2011). The niche model, that is based on the assumption of niche conservatism, has therefore failed to correctly infer the past distribution of the species. In comparison, other co-occurring taxa showed different patterns between northern and southern clades. Rapid population expansion was observed for northern clades of *Contia tenuis*, *D. punctatus*, *E. multicarinata* and *L. zonata*, whereas southern clades showed no or limited population growth (Feldman & Spicer, 2006). More recently, different demographic patterns were found in two northwestern salamanders, *Plethodon dunni* and *Plethodon vehiculum* (Pelletier & Carstens, 2016). Although both species showed evidence of exponential population growth, it was slightly higher in *P. dunni* and the expansion and divergence of *P. dunni* occurred earlier (Pelletier & Carstens, 2016).

Pacific chorus frogs as a clade appear to be restricted to western North America by climatic factors (Fig. 6). However, during the Last Glacial Maximum (LGM; *c*. 21 Kya) the Cascade Mountains from Washington (WA) to southern Oregon (OR) were covered by ice sheets, which altered temperature, water availability and species habitat (Dyke *et al.*,

2003; Steele *et al.*, 2005; Swenson & Howard, 2005; Pelletier & Carstens, 2016). These climatic events have been implicated as an important factor that influenced the phylogeographic history of many species (Pelletier & Carstens, 2016). Likewise, southern California became increasingly arid approximately 10 000 years ago, which reduced the extent and connections among aquatic habitats potentially resulting in habitat isolation and genetic differentiation of associated taxa (Phillipsen & Metcalf, 2009). Ripplinger & Wagner (2004) found low haplotype variation within the coastal and inland clades of *H. regilla* and suggested that this was a result of repeated isolation in small refugial populations and gene flow among expanding populations during Pleistocene glacial cycles. Our niche modelling found that during interglacial and glacial periods, the range of *H. sierra* retracted, making habitat in the northeastern part of its distribution (i.e. Idaho and Montana) unsuitable. This matches our divergence dating analysis showing recent divergence (< 100 Kya) from populations in California and Oregon. The climatic niches of *H. hypochondriaca* and *H. sierra* are not well delimited as suitable conditions of one species seem to extend into the range of the other. Competitive exclusion might prevent *H. sierra* from expanding further south and *H. hypochondriaca* expanding further north. However, direct competition between *H. sierra* and *H. hypochondriaca* may not entirely explain different geographic ranges because we would have observed more similar ranges in all time periods, supporting environmental factors instead (Pelletier & Carstens, 2016). Conversely, the climatic niche of *H. regilla* is most distinct and does not seem to overlap with the niche of the other two taxa and therefore, might be restricted to its current range by climatic factors. These species may differ in their distributions because of physiological tolerances and dispersal ability or a combination of factors (Pelletier & Carstens, 2016). One proposed barrier to dispersal and isolation in the Pacific northwest is changes in elevation; however, this does not appear to isolate these frogs that have an extensive elevational range (Reilly & Wake, 2015). As mentioned earlier, these frogs are habitat generalists and have potentially high dispersal abilities (Reimchen, 1991; Ripplinger & Wagner, 2004). Other co-occurring amphibians in the Pacific northwest are probably more limited in their ability to disperse long distances overland because of stream breeding (*Ascaphus*, *Dicamptodon*) or otherwise being closely associated with seeps and streams (*Plethodon vandykei*/*Plethodon idahoensis*) resulting in isolation even when habitat corridors formed during glacial maxima (Steele *et al.*, 2005).

Conservation implications

The use of molecular methods to accurately characterize biodiversity has important implications for conservation (Rissler *et al.*, 2006; Bickford *et al.*, 2007; Bernardo, 2011). Examples of species previously considered "widespread", which are in reality a complex of distinct species with smaller ranges each warranting unique conservation action, are found across taxonomic groups from turtles and amphipods to birds and pitvipers (Spinks & Shaffer, 2005; Witt *et al.*, 2006; Lohman *et al.*, 2010; Russello *et al.*, 2010; Jadin *et al.*, 2012). Among taxonomic groups, amphibians have been documented with significant declines and extinctions as well as high cryptic diversity (Wake & Vredenburg, 2008; Funk *et al.*, 2012; Crawford *et al.*, 2013; Alroy, 2015). Although common in regional amphibian assemblages, *H. regilla sensu lato* is among the species most severely affected by *Riberioia ondatrae,* the trematode parasite that causes significant malformations that impact fitness related traits (Goodman & Johnson, 2011; Johnson *et al.*, 2012). In addition, these frogs show complex effects from land use change, contaminants, invasive species and combinations of these factors (Pearl *et al.*, 2005; Riley *et al.*, 2005; King & Wagner, 2010; Cole & North, 2014; Dimitrie & Sparling, 2014; Thomassen *et al.*, 2017). A better understanding of genetic diversity, dispersal patterns and barriers to gene flow are important for identifying local threats and genetically significant populations for implementing conservation measures in the western United States (Spinks & Shaffer, 2005; Stöck *et al.*, 2012).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Sequences used as outgroup and to assess the evolutionary history of the Hyliolinae and Pseudacrinae.

Appendix S2. Sequences and localities of individuals from the *H. regilla* complex used in this study.