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Concordant molecular and phenotypic data delineate new taxonomy and conservation priorities for the endangered mountain yellow-legged frog

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Abstract

The mountain yellow-legged frog *Rana muscosa sensu lato*, once abundant in the Sierra Nevada of California and Nevada, and the disjunct Transverse Ranges of southern California, has declined precipitously throughout its range, even though most of its habitat is protected. The species is now extinct in Nevada and reduced to tiny remnants in southern California, where as a distinct population segment, it is classified as Endangered. Introduced predators (trout), air pollution and an infectious disease (chytridiomycosis) threaten remaining populations. A Bayesian analysis of 1901 base pairs of mitochondrial DNA confirms the presence of two deeply divergent clades that come into near contact in the Sierra Nevada. Morphological studies of museum specimens and analysis of acoustic data show that the two major mtDNA clades are readily differentiated phenotypically. Accordingly, we recognize two species, *Rana sierrae*, in the northern and central Sierra Nevada, and *R. muscosa,* in the southern Sierra Nevada and southern California. Existing data indicate no range overlap. These results have important implications for the conservation of these two species as they illuminate a profound mismatch between the current delineation of the distinct population segments (southern California vs. Sierra Nevada) and actual species boundaries. For example, our study finds that remnant populations of *R. muscosa* exist in both the southern Sierra Nevada and the mountains of southern California, which may broaden options for management. In addition, despite the fact that only the southern California populations are listed as Endangered, surveys conducted since 1995 at 225 historic (1899–1994) localities from museum collections show that 93.3% ($n = 146$) of *R. sierrae* populations and 95.2% ($n = 79$) of *R. muscosa* populations are extinct. Evidence presented here underscores the need for revision of protected population status to include both species throughout their ranges.

Introduction

Amphibians are declining worldwide, and 32.5% of the world's recognized amphibian species are classified as threatened (Stuart *et al*., 2004). These declines forecast the impending extinction of many species in the coming decades. Many of these declines occur in protected areas and involve idiosyncratic causal agents and remain enigmatic. Causative factors include increased ultraviolet radiation (Blaustein *et al*., 1994), climate change (Pounds, Fogden & Campbell, 1999), introduction of non-native species (Kats & Ferrer, 2003), chemical contaminants (Boone & Bridges, 2003), emerging diseases (Daszak, Cunningham & Hyatt, 2003) and synergistic interactions among factors (Blaustein & Kiesecker, 2002; Pounds *et al*., 2006). With amphibian fauna in such peril, there is a new urgency to reverse declines. Both here and generally, for effective conservation, it is imperative that species and population boundaries are drawn correctly (Avise, 1989; Moritz, 1999, 2002).

The mountain yellow-legged frog *Rana muscosa* was once abundant in the Sierra Nevada (California and Nevada, USA) and the disjunct Transverse Ranges in southern California (Grinnell & Storer, 1924; Stebbins & Cohen, 1995). Despite the fact that it occurs almost entirely on protected land, *R. muscosa* has declined precipitously, especially in the last three decades (Vredenburg, Fellers & Davidson, 2005). The geographically disjunct southern California populations have been delineated as a 'distinct population segment' and are federally listed as Endangered (Fish & Wildlife Service, 2002). In contrast, despite precipitous declines, Sierran populations are currently not classified as protected. Factors implicated in these declines include introduced predators such as trout (Knapp & Matthews, 2000), disease (Rachowicz & Vredenburg, 2004)

and air pollution (Davidson, Shaffer & Jennings, 2002; Davidson, 2004). In the absence of disease, extirpation of introduced trout (which prey on frogs) leads to rapid recovery of *R. muscosa* populations (Vredenburg, 2004). Five species of trout (Salmonidae) have been widely introduced into historically fishless habitats throughout the entire range of the frog (Pister, 2001), and as a result >90% of historically fishless high Sierra habitats now contain introduced, self-sustaining trout populations (Knapp & Matthews, 2000). Although demographic effects on population viability are already apparent, sound conservation efforts must also include an understanding of species and population boundaries, as well as population dynamics (Moritz *et al*., 2001; Moritz, 2002).

Rana muscosa is one of several western North American frogs in the family Ranidae, subgenus *Amerana* (Dubois, 1992; Hillis & Wilcox, 2005; Frost *et al*., 2006). It is closely related to several other species, known as the *Rana boylii* group (*R. boylii*, *Rana cascadae*, *Rana luteiventris*, *Rana pretiosa*, *Rana aurora*, *Rana draytonii*; Hillis & Wilcox, 2005). A wide range of data has been used to explore relationships of this group, including morphometrics (Camp, 1917; Zweifel, 1955), albumin immunology (Case, 1978), allozyme electrophoresis (Case, 1978; Green, 1986), chromosomes (Green, 1986), restriction-enzyme cleavage analysis of nuclear rRNA genes (Hillis & Davis, 1986), and more recently mitochondrial DNA (Macey *et al*., 2001; Hillis & Wilcox, 2005) and nuclear DNA (Frost *et al*., 2006). *Rana muscosa*, the focus of this study, was originally described as two subspecies of *R. boylii* based on morphology (*Rana boylii muscosa* in the Transverse Ranges and *Rana boylii sierrae* in the Sierra Nevada; Camp, 1917). On the basis of additional morphological data, the two subspecies were separated from *R. boylii* and raised to the species level (*R. muscosa*; Zweifel, 1955), and the subspecies were no longer recognized.

Evolutionary biologists typically use multiple datasets to explore the evolutionary relationships and boundaries of closely related species. In general, the case for delimiting separate species is strongest when results from multiple sets of independent classes of characters are concordant (Sites & Marshall, 2003). Gene flow is expected to counter the diversifying effects of genetic drift and local adaptation. Thus, species containing non-contiguous ranges, such as *R. muscosa*, are predicted to exhibit a higher degree of population structure between non-contiguous areas than within contiguous areas. In contrast to the current boundary between southern California and Sierran populations, both recent molecular (Macey *et al*., 2001) and largely ignored morphological data (Camp, 1917; Zweifel, 1955) from *R. muscosa* suggest discontinuity within the contiguous range of the Sierra Nevada. To better understand the evolutionary relationships of the remaining populations, we (1) sequenced 1901 bp of mitochondrial DNA from 91 specimens (each representing a separate population) collected throughout the entire range and used these data to construct a molecular phylogenetic hypothesis for *R. muscosa* across its historic range, (2) compared 16 morphological characteristics and four habitat-associated characteristics from 232 museum specimens and (3) compared male advertisement calls recorded in the Transverse Ranges and the Sierra Nevada. On the basis of concordant molecular, morphologic and acoustic results, we recognize two diagnosable species-*R. muscosa* and *Rana sierrae*-and for clarity we refer to them as such throughout this paper. To ascertain the conservation status of these newly delineated taxa, we quantify the extent of the decline in each species by comparing recent (1995–2005) site occupancy to historic site occupancy (1899–1994) at 225 sites distributed throughout the Sierra Nevada and Transverse Ranges.

Materials and methods

Molecular phylogenetic analysis

For the molecular analysis, we obtained tissue samples from one individual (adults and tadpoles) from each of 91 populations in California (Fig. 1; Table 1). Samples included seven individuals from the Museum of Vertebrate Zoology (MVZ) tissue bank (University of California Berkeley), eight from the California Academy of Sciences (CAS) tissue bank and 76 collected as toes from live individuals that were released after tissue collection; small population sizes prevented voucher collections at all sites, but tissues have been accessioned (MVZ accession #14139). Of the 91 tissue samples used, sequences were obtained for 26 *R. muscosa* and 65 *R. sierrae* specimens from throughout their historic ranges. In addition, sequences from single specimens of three outgroup taxa were obtained from Genbank (*R. boylii*, MVZ 148941, Genbank AF314019; *R. draytonii,* MVZ 227645, Genbank AF314021; *R. cascadae,* MVZ 230719; Genbank AF314022).

Genomic DNA was extracted from frozen or ethanol-preserved tissues (muscle, liver and tail fin from tadpoles) using Qiagen DNeasy extraction kits (Qiagen Inc., Valencia, CA, USA). The targeted mitochondrial DNA fragment included the protein coding genes ND1 and ND2, intervening tRNA genes and the tRNA genes flanking ND2 (Macey *et al*., 2001). The entire DNA segment was amplified using polymerase chain reaction (PCR) using primers designed for this study (Table 2). PCRs were performed in a total volume of 15 μL, including 0.38 μL *Taq* polymerase (5 U μL^{-1}), 0.75 μL of each primer (10 μmol L^{-1}), 0.28 μL dNTPs (40 mM), 1.5 μL of 10× buffer and 3.75 μL of sample DNA (3 ng μL^{-1}). PCRs consisted of 29 cycles with a denaturing temperature at 95 °C (30 s), annealing at 62 °C (30 s) and extension at 72 °C (1 min), and a final extension at 72 °C (10 min). PCR products were purified using ExoSAP-IT (USB Inc., Cleveland, OH, USA), and sequenced on an ABI 3730 automated DNA sequencer with four overlapping sets of internal primer pairs (Table 2). DNA sequences were edited and aligned unambiguously using Sequencher^{TM} version 4.2 (Gene Codes Corp., Ann Arbor, MI, USA).

Bayesian phylogenetic analyses were implemented using MrBayes version 3.04b (Huelsenbeck & Ronquist, 2001). Both partitioned and unpartitioned analyses were run

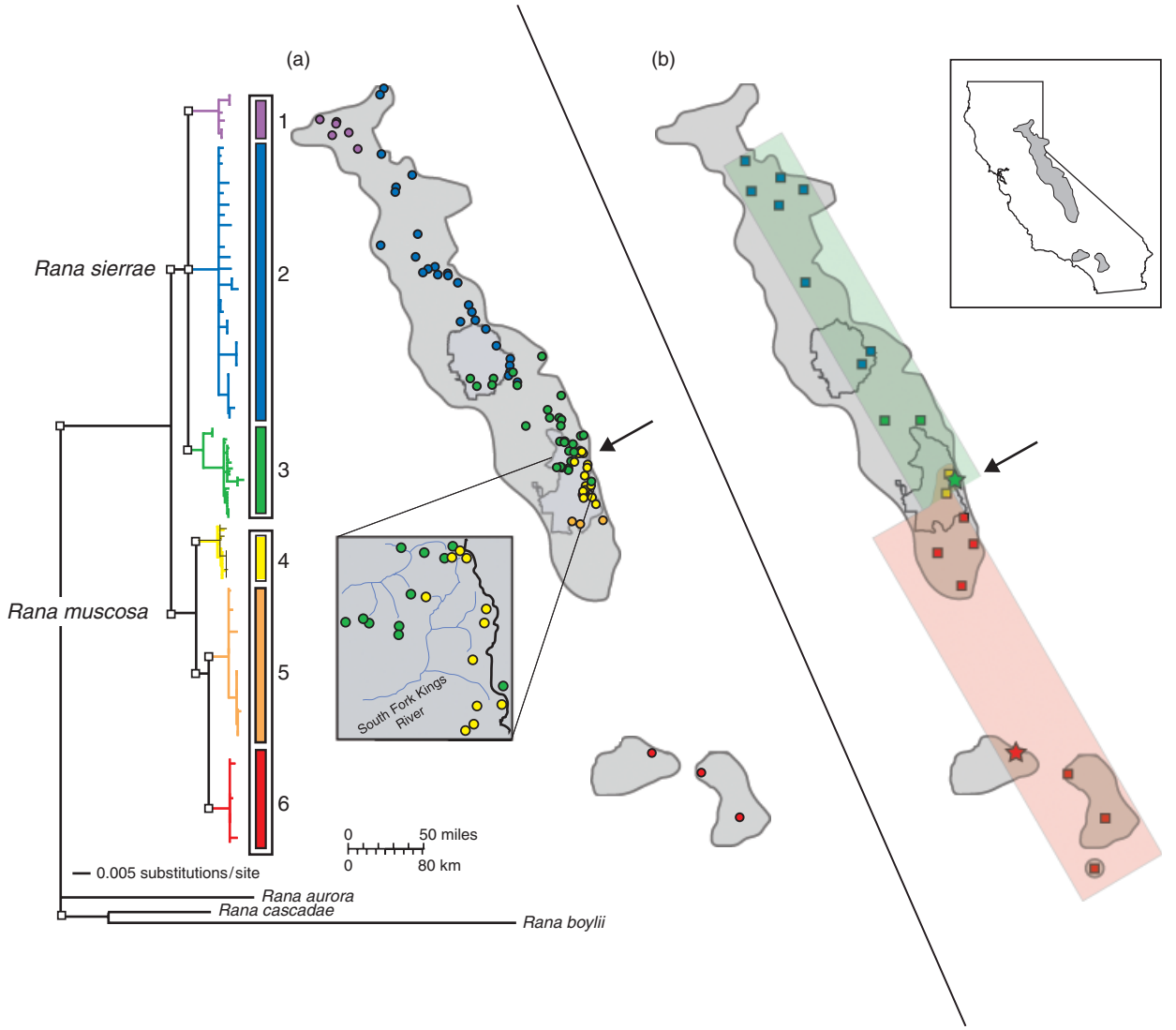

Figure 1 Collection locations of *Rana muscosa* and *Rana sierrae* used for genetic (a) and morphological (b) analyses. Bayes mtDNA phylogram, nodes with □ represent >95% posterior probabilities. Two major clades (*R. muscosa, R. sierrae*) and six minor clades (1–6) were identified from the mtDNA analysis. The mtDNA contact zone (arrow) between the species is located between the Middle and South Forks of the Kings River (inset). Morphological analysis identified two groups (b, green and red polygons) that are concordant with the major mtDNA clades, *R. muscosa* and *R. sierrae*. Localities of type specimens are shown as stars and were included in the morphological analysis. The type for *R. sierrae* occurs farther south than the others, and is separated from nearby *R. muscosa* by the crest of the Sierra Nevada (black line; inset).

because a single model of nucleotide substitution may not be appropriate for the ND1 and ND2 protein-coding genes and flanking and intervening tRNA genes (Brandley, Schmitz & Reeder, 2005). The appropriate model of sequence evolution for each partition was selected using the Akaike information criterion (AIC) implemented in MrModelTest (version 2.2) [modified from Modeltest (Posada & Crandall, 1998) by J. A. A. Nylander]. We ran the Bayesian analysis for 20 million generations and sampled every 1000 generations, using four chains and default priors. Stationarity was determined by plotting the likelihood values against generation time (Leache & Reeder, 2002), and a conservative 10 million generations were discarded as burn-in. The remaining 10 million generations (10 000 samples) were analyzed in PAUP* version 4.0b10 (Swofford, 1996) to reconstruct the topology and calculate posterior probabilities for each node. We used MEGA version 3.1 (Kumar, Tamura & Nei, 2004) to calculate corrected pairwise mtDNA sequence divergence.

To determine whether genetic variation among samples was attributable to isolation by distance (IBD), among-clade variation or a combination of the two, we estimated the correlation between mtDNA sequence divergence and geographical distance between samples with the program IBD version 1.5 (Bohonak, 2002) using full and partial Mantel tests (20 000 randomizations). We used uncorrected

Table 1 Populations sampled for the molecular phylogeny in this study

Specimen ID	mtDNA clade	Locality	Latitude N	Longitude W
VTV 075	1	Plumas County, Oliver Lake	39.98044	121.33
CAS 209386	1	Plumas County, Silver Lake	39.95894	121.1359
CAS 209404	1	Plumas County, Rock Lake	39.94134	121.1425
CAS 206093	1	Plumas County, Rock Creek	39.86452	121.0006
CAS 209370	1	Plumas County, Faggs Reservoir	39.84125	121.1868
CAS 209668	1	Plumas County, Pine Grove Cemetery	39.71904	120.8992
CAS 227640	2	Plumas County, Boulder Creek	40.25435	120.6028
CAS 227639	2	Plumas County, Lone Rock Creek	40.20039	120.6475
VTV 086	2	Sierra County, Haven Lake	39.6703	120.6329
CAS 203394	2	Sierra County, Independence Creek	39.48775	120.2842
VTV 062	2	Nevada County, Mossy Pond	39.3786	120.4692
VTV 999	2	Nevada County, Rattlesnake Creek	39.33535	120.4795
VTV 107	2	El Dorado County, Lake Zitella	38.9605	120.2254
VTV 139	2	El Dorado County, E of Pyramid Lake	38.8489	120.1390
VTV 152	2	El Dorado County, Middle Creek	38.7581	120.2496
VTV 061	2	El Dorado County, SF American River	38.6708	120.0337
VTV 060	2	El Dorado County, N of Granite Lake	38.6519	120.1103
VTV 110	2	Amador County, Tragedy Creek	38.6193	120.1674
VTV 065	2	Tuolumne County, near Tamarack Lake	38.6123	119.8913
VTV 114	2	Alpine County, Deadwood Creek	38.6004	119.9993
LJR 095	2	Alpine County, Little Indian Valley	38.59368	119.8854
MVZ 180163	2	Alpine County, Ebbetts Pass	38.52834	119.7757
MVZ 227662	2	Tuolumne County, Sonora Pass	38.33104	119.6547
MVZ 149008	2	Mono County, Leavitt Lake	38.26977	119.6172
VTV 1547	2	Mono County, Cascade Creek	38.19589	119.5798
VTV 1022	2	Tuolumne County, NE Long Lake	38.18231	119.7454
VTV 1550	2	Mono County, above Peeler Lake	38.1173	119.4614
LJR 1048	2	Tuolumne County, Conness Pond	37.9689	119.344
LJR 260	2	Tuolumne County, Mono Pass	37.8523	119.2195
Y-638	2	Tuolumne County, Kuna Basin	37.79426	119.2262
JAM 70	2	Mono County, Rodgers Lake	37.73335	119.2263
VTV 1552	2	Madera County, Thousand Island Lake	37.70423	119.2395
VTV 1560	2	Madera County, Minaret Creek	37.64667	119.1454
VTV 1938	3	Mono County, Dry Creek	37.882	118.8854
VTV 1101	3	Mono County, Thousand Island Lake Basin	37.7335	119.1931
RAK 4074	3	Madera County, Gray Peak Fork	37.68014	119.3994
RAK 294	3	Mariposa County, Lower Summit Meadow	37.6793	119.6477
Y-258	3	Madera County, Merced Pass	37.62278	119.4158
VTV 1100	3	Madera County, Gertrude Lake	37.6192	119.1468
RAK 4066	3	Tuolumne County, Saddle Horse Lake	37.61139	119.5767
VTV 1555	3	Inyo County, Birch Creek	37.52787	118.6765
VTV 1575	3	Fresno County, Mills Creek	37.40222	118.8235
RAK 2128	3	Fresno County, Big Bear Lake	37.33054	118.8014
RAK 1235	3	Inyo County, Gable Lakes	37.33035	118.7
VTV 1559	3	Inyo County, Horton Creek	37.31209	118.6745
VTV 979	3	Fresno County, Lake Camp Lake	37.25855	119.0544
RAK 123	3	Fresno County, Marmot Lake	37.25831	118.6829
VTV 987	3	Inyo County, Cow Creek	37.1759	118.4398
VTV 997	3	Inyo County, Baker Creek	37.1698	118.4691
VTV 2000	3	Fresno County, below Helen Lake	37.12197	118.642
VTV 2001	3	Fresno County, Wanda Lake	37.11941	118.6915
VTV 2002	3	Fresno County, Upper LeConte Lake	37.11428	118.6437
VTV 2003	3	Fresno County, Dusy Basin	37.09616	118.5535
RAK 2393	3	Fresno County, Upper Palisade Lake	37.03668	118.4728
RAK 100	3	Fresno County, below Upper Mills Creek Lake	37.03225	118.5957
RAK 2378	3	Fresno County, Observation Basin	37.02406	118.5426
RAK 3006	3	Fresno County, Amphitheater Basin	37.02305	118.5029
VTV 2004	3	Fresno County, Amphitheatre Basin	37.01347	118.4951

Table 1 Continued

Specimen ID	mtDNA clade	Locality	Latitude N	Longitude W
RAK 2695	3	Fresno County, Horseshoe Lakes	36.94396	118.572
RAK 3321	3	Fresno County, Slide Lakes	36.89487	118.6829
RAK 2737	3	Fresno County, Swamp Lakes	36.88851	118.7251
RAK 3489	3	Fresno County, above W Kennedy Lake	36.88753	118.6692
RAK 2727	3	Fresno County, near Granite Pass	36.8803	118.5997
RAK 2638	3	Fresno County, Granite Basin	36.86386	118.5989
VTV 1554	3	Inyo County, near Matlock Lake	36.76334	118.3551
RAK 2989	4	Fresno County, Upper Basin	37.02323	118.4561
RAK 2962	4	Fresno County, Upper Basin	37.02271	118.4589
RAK 1311	4	Fresno County, Upper Basin	37.01546	118.4729
RAK 1727	4	Fresno County, Upper Basin	37.0142	118.44
RAK 2162	4	Fresno County, N of Muro Blanco	36.93756	118.536
VTV 874	4	Fresno County, Pinchot Basin	36.9143	118.3964
RAK 671	4	Fresno County, Woods Lake Basin	36.8867	118.4002
MVZ 226112	4	Fresno County, Sixty Lake Basin	36.81409	118.4253
S 508	4	Tulare County, Golden Bear Lake	36.72808	118.36
RAK 606	4	Tulare County, Vidette Lakes	36.7244	118.4161
VTV 2005	4	Tulare County, NE of Mt Genevra	36.68959	118.4237
RAK 1776	4	Tulare County, SE of Mt Jordan	36.6759	118.4428
RAK 559	4	Tulare County, Wright Lakes Basin	36.62267	118.3473
RAK 3552	4	Tulare County, S of Milestone Basin	36.61524	118.4389
RAK 299	4	Tulare County, Hitchcock Lake Basin	36.56159	118.307
S 387	5	Tulare County, SE of Mt Genevra	36.67802	118.4244
S 376	5	Tulare County, SW of Lake South America	36.6627	118.4206
RAK 3924	5	Tulare County, Tyndall Creek	36.65296	118.3834
RAK 3584	5	Tulare County, Milestone Basin	36.64649	118.4501
VTV 1578	5	Tulare County, Mulkey Meadow	36.4024	118.2114
VTV 055	5	Tulare County, Bullfrog Lake	36.3982	118.5536
LJR 089	5	Tulare County, Laurel Basin	36.36813	118.4811
RAK 3713	5	Tulare County, Coyote Creek Basin	36.35824	118.4711
MVZ 230140	6	Los Angeles County, San Gabriel Mountains	34.3514	117.7101
MVZ 230142	6	San Bernardino County, San Bernardino Mountains	34.17718	117.1818
MVZ 230141	6	Riverside County, San Jacinto Mountains	33.77959	116.7746

Table 2 Primers used to amplify and sequence the mtDNA ND1, ND2, tRNA1 and tRNA2 region in this study (see also Macey *et al.*, 2001)

Primer name	Primer sequence (5′ → 3′)
RanaF1	ATT TGT CTC CAC CCT CGC CGA
RanaF2	AAG CTA AAT AAG CTT TTG GGC C
RanaF3	CCC CAA TAA CAC TGC TTC TCC AA
RanaF4	CCC TTG AAT TAA TTA AAC AAA ACG C
RanaF1[a]	GAA TCA GCG GGT GAA TAT CAC AG
RanaF2[a]	GAG GGT TAT GGT AAT AAT GTA TGT
RanaF3[a]	AAT TTT TCG AAG YTG TGT TTG GC
RanaF4[a]	AGT AAA GGA AGG ATT TTA ACC AAC

[a]Reverse primers.

pairwise genetic distances between samples (P), straightline geographical distances between samples (G) and an indicator (0,1) matrix (I) that identifies the minor genetic clade of each sample (i.e. Spinks & Shaffer, 2005). To test for the effect of IBD alone, we corrected the geographic genetic matrix using the clade of origin ($PG|I$). To explore the differentiation among drainages without the effects of IBD, we corrected the genetic and indicator matrix by geographic distance ($PI|G$).

Morphology

We made morphological measurements on 123 and 109 fluid-preserved specimens of *R. muscosa* and *R. sierrae*, respectively, obtained from the MVZ collection (Supplementary Material Appendix S1). All specimens were sexually mature (determination based on gonadal inspection and/or development of secondary sexual characteristics). The type specimens for *R. muscosa* (MVZ 771) and *R. sierrae* (MVZ 3734) were included (Fig. 1b, stars). We measured 16 morphometric characters for multivariate analysis: snout-to-vent length (SVL), head length (HL), head diameter (HD), snout length (SNL), internarial distance (IND), shortest eye-to-eye distance (IUE), longest eye-to-eye distance (UEW), upper arm length (UAL), forearm length (FAL), hand length (HaL), femur length (FL), tibia length (TL), tarsus length (TaL), foot length (FoL), fourth toe length (4thToe) and first toe length (1stToe). All measurements were made to the nearest 0.1 mm (with a digital

calipers and, when necessary, a microscope) and were scored by V. T. V. Symmetrical characters were scored on each specimen's left side.

To reduce the dimensionality of the set of morphological measurements, we conducted principal components analysis (PCA; JMP version 6, SAS Institute Inc.). Before analysis, we evaluated assumptions of normality and homoscedasticity by examining the frequency distributions of each variable (Sokal & Rohlf, 1981). To reduce the effects of body size, we ran a regression for each morphological measurement by SVL and then used the residuals in the PCA analysis. We used multivariate discriminant function analysis (JMP version 6, SAS Institute Inc.) to determine whether morphological characteristics alone accurately differentiated between specimens from the two major mtDNA clades (as defined from the mtDNA phylogeny). As an alternate comparison, we ran the same analysis using a priori groupings based on mountain range (Sierra Nevada vs. southern California). All analyses were conducted separately for males and females.

Morphological variation is often a function of latitude (Bergman's rule: Walters & Hassall, 2006), but can also be affected by other factors (e.g. sex, altitude). Therefore, we described the change in morphological characters with latitude after accounting for the effects of other significant predictor variables. To do this we developed a generalized additive model (GAM; i.e. Cleveland & Devlin, 1988; Hastie & Tibshirani, 1991). On the basis of the results from the PCA of morphological measurements, TL residuals had the strongest effect on PCA eigenvalues. To make comparison to previous work easier, we used the tibia: SVL ratio (TL/SLV) used as the sole response variable to represent morphology. This variable was also recognized by Camp (1917) and Zweifel (1955) as important in distinguishing *R. muscosa* populations from each other. The predictor variables included latitude, elevation, habitat type (lotic or lentic) and sex. We first built a full model containing all four predictor variables, and then one by one removed variables that did not significantly contribute to the model ($P > 0.05$, based on a likelihood ratio test; Hosmer & Lemeshow, 1989) to derive a final model containing only significant variables. GAM analyses were conducted using S-Plus version 7.0 (Insightful Corp.).

Acoustics

Advertisement calls of *R. muscosa* and *R. sierrae* were recorded in the field using a Sony WM-D6C stereo cassette recorder (Sony., New York, NY, USA). Recordings were made above and below water using a Deep Sea Power and Light SM-1000 hydrophone (SM-1000., San Diego, CA, USA). All frog calls were recorded in lentic habitats and while calling frogs were either completely or partially submerged. Calls were recorded from *R. muscosa* populations in Hall Canyon, Riverside County, CA (33.81°N, 116.778°W; recordings made on 23 May 1994). Calls for *R. sierrae* were recorded at Summit Meadow, Yosemite National Park, Mariposa County, CA (37.68°N, 119.65°W; recordings made on 22 June 1993) and Ebbetts Pass, Alpine County, CA (38.55°N, 119.82°W; recordings made on 1 July 1993). To choose individual calls for analysis, we selected a segment of each recording session that contained the entire call repertoire present within that session. Calls were digitized using a GWI-AMP analog-to-digital converter and analyzed with MacSpeech Lab II (GW Instruments Inc., Cambridge, MA, USA).

Fourteen acoustic properties were measured: call rise time, call duration, formant interval, loudest dominant frequency, lowest dominant frequency, calculated pulse rate, loudest five pulse rate, pulse rise time, total number of pulses, tuning, note duration, note rise time, note rate and pulses per note (as defined in MacTague & Northern, 1993; Ziesmer, 1997). On the basis of spectrographic appearance, we classified calls as having wave repetition patterns that were 'unpulsed,' 'semi-pulsed,' 'pulsed only' or 'noted.' Unpulsed calls have a uniform waveform, while pulsed calls contain several deamplified waves between the maximal ones, and noted calls have pulses grouped into 'notes' (MacTague & Northern, 1993). Comparisons were made between mean values of the acoustic properties for *R. muscosa* and *R. sierrae* using a multivariate analysis of variance (MANOVA; JMP version 5.1, SAS Institute). All frog recordings and preliminary analysis were conducted as part of a master's thesis (Ziesmer, 1997) and were reanalyzed with the permission of the author.

Population decline

To describe the decline across the historic range of *R. muscosa*, *R. sierrae* and the six minor clades discovered in the mitochondrial phylogeny, we assessed the current status of populations at historic localities identified using museum collections (Shaffer, Fisher & Davidson, 1998). We first mapped the collection locations for all specimens stored at the MVZ and the CAS that were collected before 1995 ($n = 293$ locations, collection year = 1899–1994, median = 1942). We then eliminated all historic localities that represented the same water body or were separated from other historic localities by < 200 m. This parsing procedure reduced the number of historic locations to 274. Locality information associated with each specimen was occasionally insufficiently precise to identify the exact collection location. Therefore, to ensure that each historic location included the actual collection location, we took a conservative approach and defined each historic locality as a circle centered on the point representing our interpretation of the locality description and with a radius of 1 km.

For each historic locality, we determined whether frogs were currently extant or extinct using data from extensive surveys conducted between 1995 and 2004 at more than 14 000 water bodies within the historic range of *R. muscosa* and *R. sierrae*. The surveys were coordinated by R. Knapp, S. Lehr [California Department of Fish and Game (CDFG) – region 2], J. Kleinfelter (CDFG – region 4), C. Milliron (CDFG – region 6) and R. Fisher (US Geological Survey). In order to make our extinction estimates conservative, we

defined an extinct population as one where no individual frog, tadpole or egg mass could be found within 1 km of the actual historic locality. All suitable habitat at historic localities (i.e. including all water bodies located within the 1 km radius circle) was searched for all life stages using visual encounter surveys (Crump & Scott, 1994). Surveys were conducted during the period of peak activity. Status at a site was categorized as 'extant' if one or more egg masses, tadpoles, subadults or adults were detected, and as 'extinct' if no life stages were found. We identified historic localities for *R. muscosa* and *R. sierrae* within all six minor phylogenetic clades; however, due to lack of recent resurveys, we did not include 22 sites representing Clade 1 and an additional 27 sites scattered throughout the rest of the range of both species. Surveys of all suitable habitat within the 1 km radius circle were completed for 225 historic localities, and these 225 localities (Supplementary Material Appendix S2) form the basis of all subsequent analyses.

The majority of localities were surveyed only once, but because *R. muscosa* and *R. sierrae* are easily detected using visual encounter surveys, this survey effort was likely sufficient to determine accurately presence/absence (Knapp & Matthews, 2000). Detectability is high for at least two reasons. First, during the day adults spend the majority of their time on shore immediately adjacent to water and tadpoles are found primarily in near-shore shallows (Storer, 1925; Vredenburg *et al.*, 2005), making both life stages highly visible during visual encounter surveys. Second, larvae are present all year due to the unusual longevity (1–4 years) of this life stage (Vredenburg *et al.*, 2005).

Results

Molecular phylogeny

The final alignment of sequence data included 364 nucleotide positions of the ND1 protein-coding gene, the entire set of 1031 nucleotides of the ND2 protein-coding gene and 506 nucleotides of the concatenated tRNA genes for a total of 1901 nucleotides. Of the 94 specimens analyzed, a subset of 14 specimens was sequenced for the ND2 gene only. Three tRNA indels were observed to be unique to the outgroup taxa, and were removed from subsequent analyses. The model of evolution selected based on AIC in MrModelTest (Nylander, 2004) varied by codon position in ND1 and ND2 and by flanking and intervening blocks of tRNA (ND1-first position = K80; ND1-second position = F81; ND1-third position = GTR; ND2-first position = HKY + I; ND2-second position = GTR + I; ND2-third position = GTR + G; tRNA block between ND1 and ND2 = HKY; tRNA block following ND2 = GTR + I). All partitioned Bayesian analyses resulted in increased model likelihood scores relative to the unpartitioned analysis, with the strategy partitioning the protein-coding genes separately, and by codon, and concatenating tRNA genes together providing the best fit to the data.

Two major (*R. muscosa* and *R. sierrae*) and six minor clades (clades 1–6) were identified with >95% posterior probabilities (Fig. 1a). The Kimura-corrected mean pairwise genetic distance between the two species was 4.6%, and the mean distance within the species was 1.5% for *R. muscosa* and 1.9% for *R. sierrae*. The between minor clade comparisons are shown in Table 3.

Table 3 Mean pairwise genetic distance (mtDNA) between the six clades in the mountain yellow-legged frog *Rana sierrae* and *Rana muscosa* complex (mean distance between species = 0.046)

		R. sierrae			*R. muscosa*		
		1	2	3	4	5	6
R. sierrae	1						
	2	0.018					
	3	0.028	0.030				
R. muscosa	4	0.023	0.033	0.048			
	5	0.038	0.048	0.060	0.024		
	6	0.036	0.049	0.064	0.023	0.030	

We assigned numbers to clades from north to south, with *R. sierrae* being comprised of clades 1–3 and *R. muscosa* being comprised of clades 4–6 (Fig. 1), with clades 1–5 occurring in the Sierra Nevada and clade 6 in southern California. Clade 1 is restricted to the Feather River (Plumas County) in the northern part of the Sierra Nevada. Clade 2 has the broadest distribution and ranges from the Diamond Mountains (Plumas County), south through the northern and central Sierra Nevada (including the Yuba, American, Mokelumne, Stanislaus and Tuolumne Rivers) to the Ritter Range (Madera County), just south-east of Yosemite National Park where it overlaps with clade 3. Clade 3 ranges from the Merced River (Mariposa County) south through the San Joaquin River and east over the Sierra Nevada crest to the Glass Mountains, south of Mono Lake (Mono County). The southern part of clade 3 is bounded by the Monarch Divide east to Mather Pass in the Middle Fork of the Kings River (Fresno County). From there south, the clade is restricted east of the Sierra crest south to the type locality (*R. sierrae*, MVZ 3734; Inyo County). Within *R. muscosa*, clade 4 ranges from south of Mather Pass and west of the Sierra crest in the South Fork of the Kings River, south through the headwaters of the Kern River to Mount Whitney (Tulare County). Clade 5 is restricted to the Kern River watershed. It overlaps with clade 4 in the headwaters of the Kern River near Lake South America and ranges south to Laurel Basin on the western side of the Kern River (Tulare County). Clade 6 occurs in the Transverse Ranges of southern California which are disjunct from the Sierra Nevada, and includes the type specimen for the species (*R. muscosa,* MVZ 771; Los Angeles County).

Our isolation by distance analyses revealed a significant association of genetic and geographical distance (Table 4), although when corrected by indicator clade (GG/I), the pattern was not significant for either species. In other words, within clades, geographic distance and molecular variation were correlated, but when multiple clades were included in the analyses (and corrected for, i.e. GG/I) the relationship was no longer significant.

Table 4 Partial Mantel test results for isolation by distance

	Correlation type	*Z*	*r*	*P*
Rana sierrae	*GG*	3843.29	0.565	<0.00005
	GI	17.59	0.93	<0.00005
	GG/I		0.067	0.069
	GI/G		0.896	<0.00005
Rana muscosa	*GG*	370.66	0.427	<0.00005
	GI	2.579	0.980	<0.00005
	GG/I		−0.203	0.995
	GI/G		0.977	<0.00005

Analyses performed include correlation of genetic and geographic distance (*GG*), correlation of genetic distance and indicator clade variable (*GI*), partial correlation of genetic and geographic distance correcting for indicator clade variable (*GG/I*), and partial correlation of genetic distance and indicator clade variable correcting for geography (*GI/G*).

Morphology

In females the first three principal components accounted for 79.7% of the total variation, and in males they accounted for 75.9% of the variation (Tables 5 and 6). The most important factors, for both sexes, were those measurements associated with limb lengths, especially femur and tibia lengths (FL, TL), and these, when corrected for body size, indicate two distinct groups of frogs (Fig. 2a and b). *Rana muscosa* specimens had longer limbs relative to body size than *R. sierrae* (Supplementary Material Appendix S3). The discriminant function analysis correctly classified 94.8% of female specimens and 92.2% of male specimens with a priori species grouping (major mtDNA groups), indicating that the two morphological groups are concordant with the two major mitochondrial groups that we recognize as species (Fig. 1a and b). The alternative analysis using a priori groupings by mountain range (Sierra Nevada vs. Transverse Ranges) was not as accurate and correctly classified 76 and 73% of female and male specimens, respectively. Using this grouping, most specimens from mtDNA clades 4 and 5 (Fig. 1) in the southern Sierra Nevada were misclassified (22/25 and 30/34 for females and males, respectively).

Results from the GAM indicated that latitude and sex had significant influences on the TL/SVL ($P<10^{-10}$ and $P=8.5\times10^{-4}$, respectively). Effects of elevation and habitat type were not significant ($P>0.05$). The shape of the response curve describing the effect of latitude on TL/SVL was not changed when sex was dropped from the model. To visualize the relationship between TL/SVL and latitude, we fit a loess line through the points describing this relationship (Fig. 3). The two species had markedly different TL/SVL results. The TL/SVL was highest for *R. muscosa* from southern California (latitude = 33–34.5°) and slightly lower for *R. muscosa* from the southern Sierra Nevada (latitude = 35.5–36.8°). At the contact zone between the two species (between latitudes of 36.8 and 37.8°), the TL/SVL declined sharply and then increased slightly through the northern Sierra (latitude = 37.8–39.6°).

Table 5 Results from principal component analysis of 15 morphologic characters (female specimens), showing the first four principal components (PC I–IV)

	PC I	PC II	PC III	PC IV
Eigenvalue	20.2450	4.7452	2.4591	1.3400
Per cent	58.8444	13.7925	7.1476	3.8948
Cum per cent	58.8444	72.6368	79.7844	83.6792
Eigenvectors				
Residuals HL	0.07423	−0.00735	−0.08441	0.10388
Residuals HD	0.18986	−0.05296	0.00052	0.36270
Residuals SNL	0.05007	−0.01376	−0.10821	−0.03093
Residuals IND	0.02166	−0.00697	0.00239	−0.07577
Residuals IUE	0.10441	−0.01646	−0.00159	0.03144
Residuals UEW	0.11277	−0.00560	−0.08554	−0.00487
Residuals HL 2	0.20521	0.11719	−0.00998	0.35313
Residuals RL	0.21387	0.07108	0.53179	0.18550
Residuals HaL	0.12939	0.16284	−0.12478	−0.20769
Residuals FL	0.59736	−0.51848	−0.27184	−0.42964
Residuals TL	0.47424	−0.06241	−0.17881	0.53627
Residuals TaL	0.27982	−0.13243	0.74659	−0.21910
Residuals FoL	0.29372	0.59765	−0.02149	−0.19352
Residuals 4thToe	0.25906	0.53456	−0.10663	−0.23978
Residuals 1stToe	0.11756	0.13454	−0.01688	0.18049

HL, head length; HD, head diameter; SNL, snout length; IND, internarial distance; IUE, shortest eye-to-eye distance; UEW, longest eye-to-eye distance; HL2, humerus length; RL, radius length; HaL, hand length; FL, femur length; TL, tibia length; TaL, tarsus length; FoL, foot length; 4thToe, fourth toe length; 1stToe, first toe length.

Table 6 Results from principal component analysis of 15 morphologic characters (male specimens), showing the first four principal components (PC I–IV)

	PC I	PC II	PC III	PC IV
Eigenvalue	19.0478	3.7697	2.0191	1.7403
Per cent	58.2480	11.5278	6.1743	5.3217
Cum per cent	58.2480	69.7758	75.9501	81.2719
Eigenvectors				
Residuals HL	0.08417	−0.08133	0.01633	−0.07729
Residuals HD	0.16889	−0.04768	−0.25434	−0.21466
Residuals SNL	0.05681	−0.01329	−0.00770	−0.02489
Residuals IND	0.01415	0.00348	−0.05301	0.01150
Residuals IUE	0.07849	−0.04696	−0.01519	−0.05147
Residuals UEW	0.09764	−0.04108	−0.07830	−0.10065
Residuals HL 2	0.23553	−0.10188	−0.15337	0.34569
Residuals RL	0.22448	−0.20186	0.24877	0.47037
Residuals HaL	0.10875	0.15410	−0.10563	0.17508
Residuals FL	0.52773	−0.28633	−0.62815	0.08002
Residuals TL	0.52150	−0.09979	0.34343	−0.66055
Residuals TaL	0.26091	−0.37104	0.54374	0.28602
Residuals FoL	0.33675	0.57059	0.07444	0.17630
Residuals 4thToe	0.28090	0.56688	0.05004	0.08255
Residuals 1stToe	0.12939	0.18872	0.11416	−0.02399

Acoustics

We found significant differences between the mean values of acoustic properties of 34 calls from *R. sierrae* in the Sierra

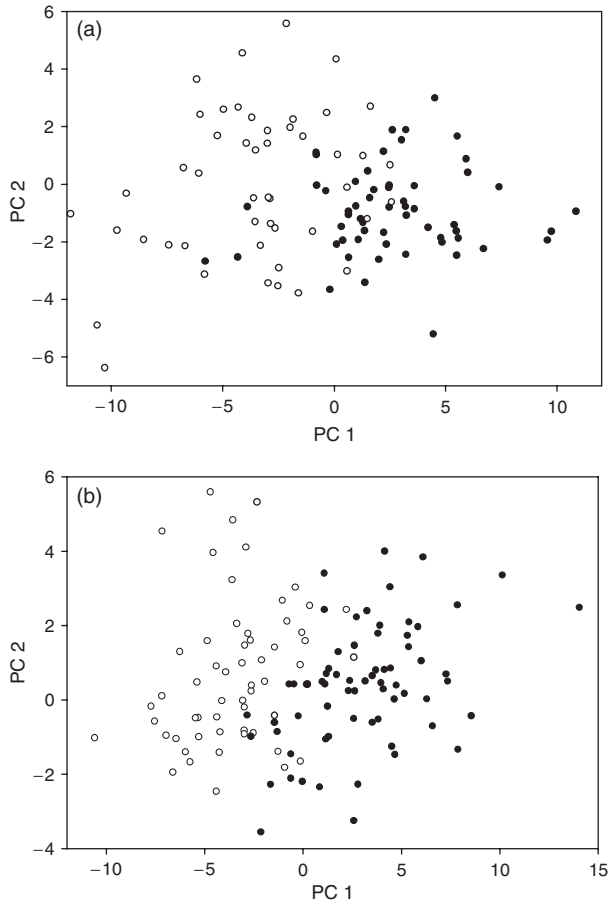

Figure 2 Bivariate orientation of the first two principal components (PC) of the morphological analysis plotted separately for females (a) and males (b); ●, *R. muscosa*, and ○, *R. sierrae*.

Nevada compared with 23 calls from *R. muscosa* in southern California (MANOVA, $F = 40.1$, d.f. = 13, $P < 0.0001$; Fig. 4). Eight of 14 acoustic properties tested were significantly different between the species (Table 7). Water temperature recorded at the calling sites did not differ between the two groups. Recordings of *R. sierrae* contained intergradation between pulsed and noted call types, whereas *R. muscosa* recordings did not.

Population decline

Of the 225 historic sites used in this analysis (79 for *R. muscosa* and 146 for *R. sierrae*), only three *R. muscosa* sites and 11 *R. sierrae* sites contained frogs when revisited between 1995 and 2005 (Fig. 5). On the basis of these sites, the extinction rate is 96.2% for *R. muscosa* and 92.5% for *R. sierrae*. A contingency table analysis (JMP version 6, SAS Institute Inc.) shows that the decline values do not differ statistically between species ($\chi^2 = 2.0$, $P = 0.16$). Calculations for extinction rates in the major geographic areas and for the minor mtDNA clades are presented in Table 8. There are additional sites where populations of both species are

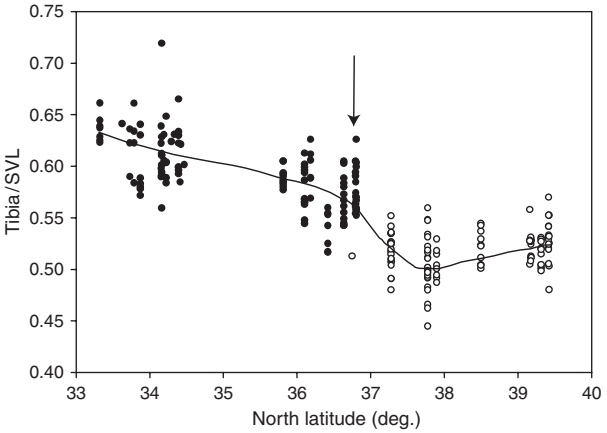

Figure 3 Relationship between the ratio of tibia length/snout-vent-length (SVL) and latitude for ●, *Rana muscosa*, and ○, *Rana sierrae*, as described by a loess line. The arrow shows the latitude of the contact zone between the two species.

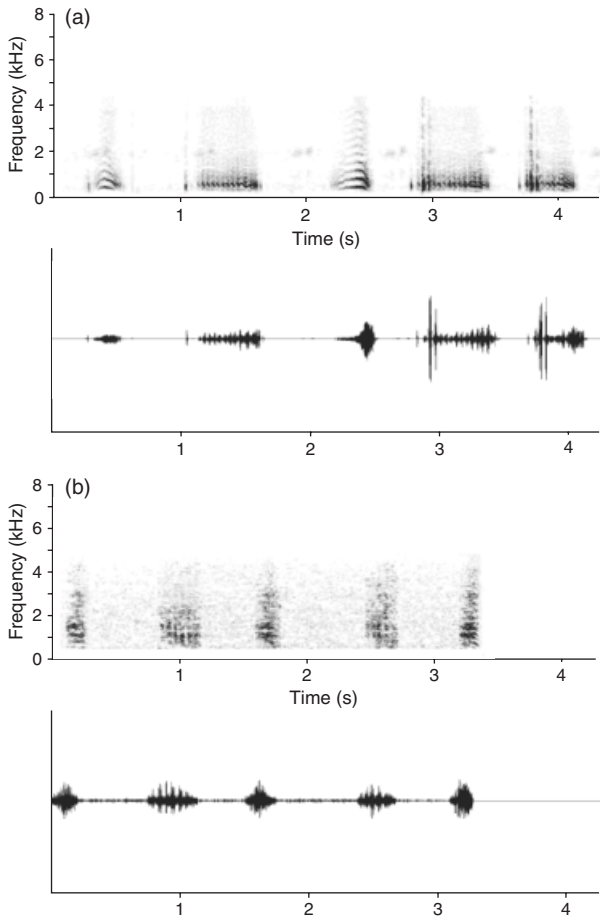

Figure 4 Oscillogram and spectrogram for (a) *Rana sierrae* (Sierra Nevada; Summit Meadow, Yosemite National Park, Mariposa County, CA, USA) and (b) *Rana muscosa* (San Jacinto Mountains, Transverse Ranges; Hall Canyon, Riverside County, CA, USA).

Table 7 Acoustic properties and MANOVA test results between *Rana muscosa* and *Rana sierrae*

Acoustic property	Definition	*F*	*P*
Whole model	All variables tested	109.3	<0.0001
WaTemp	Water temperature	0.40	0.52
CallRist	Call rise time	0.38	0.54
Duration	Duration of call	0.39	0.53
FrmntInt	Formant interval	93.22	<0.0001
LdDF	Loudest dominant Frequency	30.46	<0.0001
LwDF	Lowest dominant frequency	7.74	0.0074
PlsRtCalcd	Pulse rate, calculated	13.25	0.0006
PlsRtLd5	Pulse rate, loudest five	86.84	<0.0001
PlsRisT	Pulse rise time	0.39	0.53
TotNrPls	Total number of pulses	48.55	<0.0001
Tuning	Tuning	10.03	0.0025
NtDur	Note duration	0.40	0.53
NtRisT	Note rise time	0.5	0.45
NtRt	Note rate	15.39	0.009
PlsPerNt	Pulse rate	1.73	0.19

known to exist; however, these were not included in this analysis because no historical information is available at those sites.

Taxonomy

Rana muscosa complex
Mountain yellow-legged frogs

Rana sierrae Camp (1917), new status.
Sierra Nevada yellow-legged frog
Rana boylii sierrae Camp (1917). Original description. Type – MVZ 3734, an adult female collected by H.S. Swarth on 26 July 1912. Type locality – Matlock Lake, 3200 m elevation, Inyo County, California (36.76°N, 118.36°W).

Rana muscosa Zweifel (1955). This author considered the then recognized two subspecies of *R. boylii* to constitute separate species, and he selected the name *muscosa* for the mountain yellow-legged frogs thereby reducing *sierrae* to synonymy.

Diagnosis. *Rana sierrae* differs from *R. muscosa* in having relatively shorter legs. When a leg is folded against the body the tibio-tarsal joint typically falls short of the external nares. The mating call of *R. sierrae* is significantly different from that of *R. muscosa* in having transitions between pulsed and noted sounds. In addition, the two species differ in mitochondrial DNA. These datasets are geographically concordant.

Range. *Rana sierrae* once ranged from the Diamond Mountains north-east of the Sierra Nevada in Plumas County, California, south through the Sierra Nevada to the type locality, the southern-most locality (Inyo County). In the extreme north-west region of the Sierra Nevada, several populations occur just north of the Feather River, and to the east, there was a population on Mt Rose, north-east of Lake Tahoe in Washoe County, Nevada, but it is now extinct. West of the Sierra Nevada crest, the southern part of the

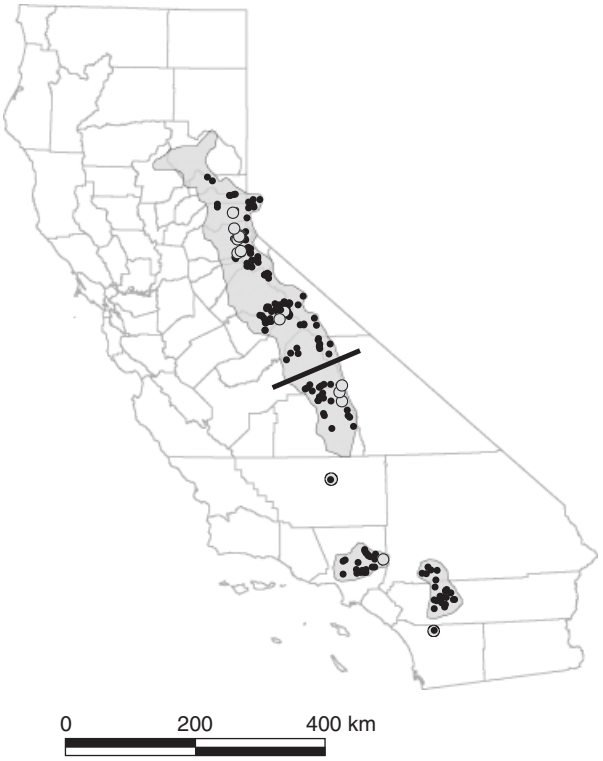

Figure 5 Range (grey area) of *Rana muscosa* and *Rana sierrae* based on all museum specimens at California Academy of Sciences and Museum of Vetebrate Zoolgy. The boundary between the two species is shown with a line. Two disjunct isolated localities are shown separated from the range because they are isolated from all other sites (Breckenridge Mountain, Kern County, and Palomar Mountain, San Diego County). The circles show 225 historic collection sites (1899–1994) that were resurveyed between 1995 and 2005 (●, extinct populations, $n=211$; ○, extant populations, $n=14$).

R. sierrae range is bordered by ridges that divide the Middle and South Fork of the Kings River, ranging from Mather Pass to the Monarch Divide. East of the Sierra Nevada crest, *R. sierrae* occurs in the Glass Mountains just south of Mono Lake (Mono County) and along the east slope of the Sierra Nevada south to the type locality at Matlock Lake (Inyo County).

Comment. This species is a member of the *R. muscosa* complex, which in turn is part of the clade *Amerana*, which also includes *R. aurora, R. boylii*, *R. cascadae, R. draytonii, R. luteiventris* and *R. pretiosa* (Hillis & Wilcox, 2005). Relationships within this clade are not fully resolved, but the following does not conflict with analyses of Hillis & Wilcox (2005) and Macey *et al.* (2001): *(((pretiosa, luteiventris)boylii)(((aurora, cascadae)muscosa, sierrae)draytonii))*. See further comments under *R. muscosa* account (below).

Rana muscosa Camp (1917).
Sierra Madre yellow-legged frog
Rana boylii muscosa Camp (1917). Original description. Type – MVZ 771, adult female collected by J. Grinnell on 3 August 1903. Type locality – Arroyo Seco Canyon,

Table 8 Site occupancy: historic (1899–1994) versus current (1995–2005)

Species	mtDNA clade	Mountain range	No. of historic	No. of current	% extinct
Rana sierrae	**1–3**	**Sierra Nevada**	**146**	**11**	**92.5**
	1	Sierra Nevada			
	2	Sierra Nevada	104	9	91.3
	3	Sierra Nevada	42	2	95.2
Rana muscosa	**4–6**	**Sierra Nevada + Transverse Ranges**	**79**	**3**	**96.2**
	4–5[a]	Sierra Nevada	26	2	92.3
	6	Transverse Ranges	53	1	98.1
R. muscosa + *R. sierrae*	1–5	Sierra Nevada	172	13	92.4
R. muscosa + *R. sierrae*	1–6	Sierra Nevada + Transverse Ranges	225	14	93.8

[a]Clades combined because of geographic overlap of mtDNA clades. Results from combined clades for each species in bold.

366 m elevation, 10 km north of Pasadena, Los Angeles County, California (34.21°N, 118.17°W).

Rana muscosa Zweifel (1955). Raised to species status.

Diagnosis. *Rana muscosa* differs from *R. sierrae* in having relatively longer legs, with the tibio-tarsal joint of the folded leg reaching or exceeding the external nares. The mating call of *R. muscosa* is significantly different from that of *R. sierrae* in having discrete pulsed and noted sounds, with no transitions. In addition, the two species differ in mitochondrial DNA.

Range. *Rana muscosa* once ranged from Palomar Mountain in San Diego County through the San Jacinto, San Bernardino and San Gabriel Mountains of Riverside, San Bernardino and Los Angeles counties in southern California. These formed four isolated clusters of montane populations. In addition, the species occurred as an isolated cluster of populations on Breckenridge Mountain, south of the Kern River in Kern County, and in the Sierra Nevada in Tulare, Inyo and Fresno counties, extending north to Mather Pass. The distribution of *R. muscosa* in the Sierra Nevada is bordered by the crest of Sierra Nevada. No populations occur east of the crest. The mountain ridges that separate the headwaters of the South Fork Kings River from the Middle Fork Kings River, from Mather Pass to the Monarch Divide, form the northern border of the range (Fig. 1, inset). *Rana muscosa* is extinct on Palomar and Breckenridge mountains.

Comment. We revert to the vernacular name originally coined by Camp (1917) for this taxon, in order to stabilize names. Frogs belonging to our *R. muscosa* and *R. sierrae* have long been called mountain yellow-legged frogs, and to assign that name to one or the other of the two sister taxa would be arbitrary and lead to confusion. Accordingly, we recommend that collectively the frogs be called the *R. muscosa* complex, the mountain yellow-legged frogs.

Discussion

Our concordant molecular, morphological and acoustic data reveal the presence of two species and suggest that conservation efforts inappropriately consider remaining populations of frogs in the Sierra Nevada as a single taxon. The contribution of mitochondrial DNA and multivariate statistical approaches used here confirms that there are two distinguishable species, but with different geographical limits than supposed by Camp (1917) and assumed in the current designation of southern California populations. Despite strong statements pointing out large morphological differences between *R. muscosa* in the Sierra Nevada and southern California (Myers, 1952), Zweifel (1955) synonymized the geographically distinct subspecific taxa. Zweifel synonymized the taxa despite reporting significant morphological variation in frogs between the Sierra Nevada and Transverse Ranges (in Zweifel, 1955, fig. 21) because he found extensive overlap in morphology between the frogs from the two ranges. We identified two morphologically distinct groups of frogs without using a priori geographic groupings. Surprisingly, the range disjunction (between the Sierra Nevada and the Transverse Ranges) does not correlate with the morphological or molecular groupings (Fig. 1b). The abrupt change from long-legged to shorter legged frogs occurs in the southern Sierra Nevada over a short distance (<4 km) and cannot be explained by elevation or habitat type (Fig. 3; GAM results). The GAM shows that the morphological characters did not change clinally, but instead changed abruptly near 36.7°N latitude. Moreover, the zone of morphological change aligns with a mitochondrial break. Finally, our reanalysis of previously recorded acoustic data also shows that the acoustic properties of mating calls in *R. muscosa* and *R. sierrae* are distinct.

The biogeographic history of vertebrates in the Sierra Nevada is complex, and evidence for a mid-Sierran break in mitochondrial DNA is an increasingly common phenomenon (Calsbeek, Thompson & Richardson, 2003; Lapointe & Rissler, 2005; Rissler *et al*., 2006). In accordance with a previous molecular study on *R. muscosa* and *R. sierrae* (Macey *et al*., 2001), our results show a distinct mitochondrial break within the southern Sierra Nevada. This pattern of fragmentation between northern and southern populations in the Sierra Nevada is observed among codistributed amphibian and reptilian species (i.e. *Ambystoma californiense*, Shaffer *et al*., 2004; *Ensatina eschscholtzii*, Moritz, Schneider & Wake, 1992; *Taricha torosa,* Tan & Wake, 1995; Kuchta & Tan, 2005; *Pseudacris regilla,* Recuero *et al*., 2006; *Bufo canorus*, Shaffer *et al*., 2000; *Lampropeltis zonata,* Rodriguez-Robles, Denardo & Staub, 1999; Feldman & Spicer, 2006; *Charina bottae*, *Contia*

tenuis and *Elgaria multicarinata*, Feldman & Spicer, 2006; *Emys marmorata*, Spinks & Shaffer, 2005). Although the breaks between phylogeographic units do not always align, the similar pattern of biogeographic fragmentation suggests that these species were influenced by one or more vicariant events. Although glaciation is often suggested as the vicariant event, geologic models for the Sierra Nevada and the Transverse Ranges are controversial (Gillespie & Zehfuss, 2004); thus, reconstructing the evolutionary history of lineages in this region continues to be challenging.

Extensive biological surveys conducted over a century ago concluded that *R. sierrae* and *R. muscosa* were the most abundant vertebrates in the high-elevation habitats of the Sierra Nevada (Grinnell & Storer, 1924) and the Transverse Ranges (Storer, 1925). In this study we documented high rates of extinction for both *R. sierrae* and *R. muscosa*. We believe our extinction estimates are conservative because, if even a single frog or tadpole was recently encountered within a 1 km radius around a historic site, we would consider the population to be extant in this study. In addition, while there is always a possibility of a false negative, both frog species are diurnal, occur mostly in simple habitats, and are closely associated with lentic and lotic shorelines, and thus are easily discovered in surveys (Knapp & Matthews, 2000; Vredenburg, 2004). It is possible that there is some colonization of previously unoccupied sites to offset local extinctions (metapopulations: Andrewertha & Birch, 1954; Hanski, 1999), but recent and repeated large-scale surveys suggest that this is a rare process and not sufficient to compensate for the high extinction rate observed here. In the Sierra Nevada, recent surveys report a number of widely scattered, mostly very small populations (<20 adults) of *R. muscosa* and *R. sierrae* remaining (Knapp & Matthews, 2000; R. A. Knapp, unpubl. data). In southern California only eight small populations (<20 adults) are known (Fish & Wildlife Service, 2002). Clearly, the genetically distinct *R. muscosa* populations in southern California should continue to be a target for conservation; however, our study, concluding that populations of *R. muscosa* exist in the southern Sierra Nevada, may broaden options for management. For example, if the remaining eight southern California populations become extinct, then *R. muscosa* from the southern Sierra Nevada could be used as source populations for future reintroductions.

Correct delineation of species boundaries is essential for conservation (Avise, 1989; Moritz, 2002). Recognition of *R. sierrae* and *R. muscosa*, together with evidence for high extinction rates (93–95%), reveals both species to be endangered under current IUCN criteria (Stuart *et al.*, 2004) and underscores the importance of managing frogs in the Sierra Nevada as two distinct species. Fortunately, nearly all the remaining populations of *R. sierrae* and *R. muscosa* occur on public lands, and previous work showed that in the absence of disease, recovery of populations is possible (Vredenburg, 2004). Several agencies have begun and/or planned recovery efforts based on these results (National Park Service, CDFG and US Forest Service), and a number of both *R. muscosa* and *R. sierrae* populations have recovered (Vredenburg, 2004). However, the discovery of an infectious disease (chytridiomycosis: Fellers, Green & Longcore, 2001; Rachowicz & Vredenburg, 2004), and its association with collapsing *R. muscosa* and *R. sierrae* populations (Rachowicz *et al.*, 2006), adds a new urgency to recovery efforts.

Acknowledgements

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Supplementary material

The following material is available for this article online:

Appendix S1 Specimens examined in morphological analyses [MVZ specimens, plus VTV specimens currently being added to the MVZ collection: MVZ accession #14139]. Highlighted specimens represent type specimens.

Appendix S2 List of historic localities used in the decline analysis.

Appendix S3 Morphometric variation (in mm) in adult members of the *R. muscosa* and *R. sierrae*. Table entries include mean ± SD, range and sample size (*n*). See text for character abbreviations.

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