Molecular Ecology (2001) **10**, 1499–1513

Phylogeography and conservation genetics of the Columbia spotted frog (*Rana luteiventris*; Amphibia, Ranidae)

DAVID H. BOS* and JACK W. SITES JR
Department of Zoology and M. L. Bean Life Science Museum, Brigham Young University, Provo, UT 84602, USA

Abstract

The Columbia spotted frog (*Rana luteiventris*) has a widespread distribution in western Canada and the western US, although the southern reach of its range is highly fragmented into several isolated populations. Threats from various factors have raised concerns regarding the long-term survival of many small, isolated populations. Here, we report a study designed to determine the phylogeographic and conservation genetic parameters of *R. luteiventris* in the western US. Mitochondrial DNA (mtDNA) sequences were examined for phylogeographic structuring using phylogenetic reconstruction methods, coupled with networking and nested clade analyses. These methods permitted a distinction to be made between historic and demographic forces acting to generate geographical patterning of genetic variation. Phylogenetic analysis revealed four geographically correlated monophyletic clades. Three of these clades correspond to well-defined, nonoverlapping geographical locations in the fragmented portion of the range. The other is comprised of all samples collected from the contiguous range and includes one isolate from northern Wyoming. Networking and nested clade analyses confirmed these results and revealed that historical processes, such as range expansion and vicariance, rather than recurrent gene flow are likely responsible for observed patterns of genetic variation. A measure of genetic variation ($\theta = 4N_e\mu$) revealed that *R. luteiventris* populations in Utah have a relatively low amount of genetic variation compared with populations in the continuous portion of the range.

Keywords: conservation genetics, ESU, mtDNA, nested clade analysis, phylogeography, *Rana luteiventris*

Received 3 September 2000; revision received 4 January 2001; accepted 10 January 2001

Introduction

The western spotted frog (*Rana pretiosa*) was first described in 1853 (Baird & Girard, 1853), and in 1913 was subdivided into two subspecies, *R. p. pretiosa* and *R. p. luteiventris*, primarily on the basis of differences in ventral coloration and the absence or presence of tubercles on digits of the forelimbs (Morris & Tanner 1969). This arrangement was later abandoned, but recent genetic (allozyme) and morphological evidence indicates that the western spotted frog is comprised of at least two distinct species, *R. pretiosa* (Oregon spotted frog) and *R. luteiventris* (Columbia spotted frog), whose distributions are not concordant with those of the original subspecies (Green *et al*. 1996, 1997).

Correspondence: David H. Bos. *Present address: Department of Zoology, University of Canterbury, Private Bag 4800, Christchurch, New Zealand. E-mail: dhbos@hotmail.com

The range of *R. pretiosa* is restricted to western and central Oregon and near the Puget Sound in western Washington and south-west British Columbia. Green *et al*. (1997) represent the range of *R. luteiventris* as extending continuously from the Alaska panhandle and north-west British Columbia to south-central Idaho, and from eastern Oregon to western Wyoming (Fig. 1). Isolated populations of *R. luteiventris* are known from the periphery of the continuous distribution in south-east Oregon, south-west Idaho, Utah, Nevada and Wyoming [Hovingh 1993a, 1997; Utah Division of Wildlife Resources (UDWR) 1993, 1994; Green *et al*. 1996; see Fig. 1]. Allozyme evidence of *R. luteiventris* population structure appears consistent with predicted patterns of recent range expansion to the north and contraction in the south following glacial retreat, implying that peripheral populations confined to desert valley spring systems and lakes in isolated mountain ranges are relicts of more widespread populations that have since shifted northward (Green *et al*. 1996).

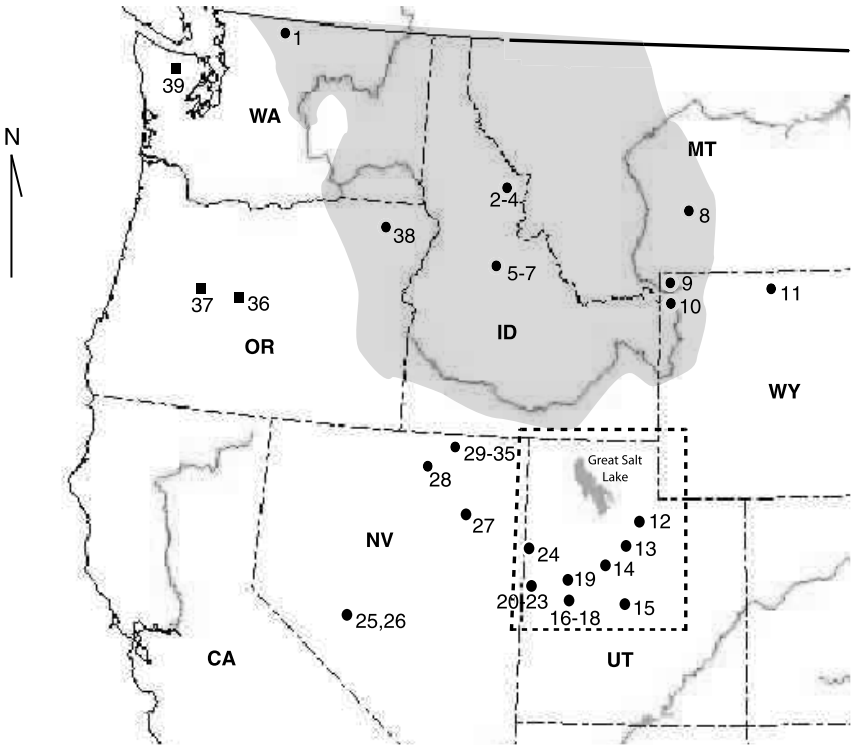

Fig. 1 Map of *Rana luteiventris* and outgroup collection sites in the western US with the continuous range of *R. luteiventris* shaded. Numbers correspond to localities summarized in Table 1; outlined area refers to Fig. 2; state acronyms are: CA = California, OR = Oregon, WA = Washington, ID = Idaho, MT = Montana, WY = Wyoming, UT = Utah, NV = Nevada.

An allozyme study investigating the population structure of *R. luteiventris* (Green *et al*. 1996) showed patterns of genetic variation strongly correlated with geography, and Green *et al*. (1997) emphasized that additional genetic research on *R. luteiventris* was warranted to more fully resolve unanswered taxonomic issues and to clarify its phylogeographic structure. Further, many southern populations of *R. luteiventris* are threatened with extinction due to urbanization, pollution by heavy metals (Lefcort *et al*. 1998), water development and non-native predators and competitors (UDWR 1993, 1994), therefore several populations in Utah have been included in a binding conservation agreement between the state and the US Fish and Wildlife Service (UDWR 1998). The inclusion of genetic information in conservation recovery efforts for threatened species facilitates planning for population augmentation and translocation, if necessary, by way of estimating population structure and distinctiveness (Avise 1989; Moritz 1994a, 1995). This is especially significant for metapopulations of amphibians, which may have small effective population sizes, strongly structured populations (Merrell 1968; Kimberling *et al*. 1996; Hitchings & Beebee 1998), and show different structures in different phylogeographic regions (Barber 1999a,b). This latter point may be crucial in conservation assessments of declining amphibians because the dynamics of distinct metapopulations may differ across small geographical scales (Shaffer *et al*. 2000). Further, peripatric populations of a wide array of organisms have been recognized as having retained or acquired types of variation not found elsewhere in the species complex (Frey 1993), rendering these populations important sources of evolutionary novelty or speciation potential (Lesica & Allendorf 1995).

Toline & Seitz (1999) assessed the genetic structure of Utah populations of *R. luteiventris* using mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) data. Their study revealed significant subdivision among populations but no correlation between patterns of population structure and geographical distribution, a surprising finding given the patterns reported by Green *et al*. (1996). Concerns with RFLP data are that: (i) fragments are not independent, therefore mutational changes between haplotypes may generate phenotypes on a gel in which no fragments are shared, even though the majority of restriction sites are shared (Swofford *et al*. 1996); (ii) fragments may show convergence in size, relative to the resolving power of the gel; and (iii) the presence of insertion–deletion events may obscure band homology assignments (Hillis *et al*. 1994a). RAPDs, like other multilocus data, are technically convenient but imprecise, and have some major technical and/or analytical drawbacks (Sunnucks 2000). These issues may be minimal in comparisons of conspecific populations or closely related species, but collectively they raise irresolvable issues over sources of error that would generally be negligible with mapped restriction site or sequence data.

The geographical isolation and small size of some populations of *R. luteiventris*, and its aquatic lifestyle (Turner

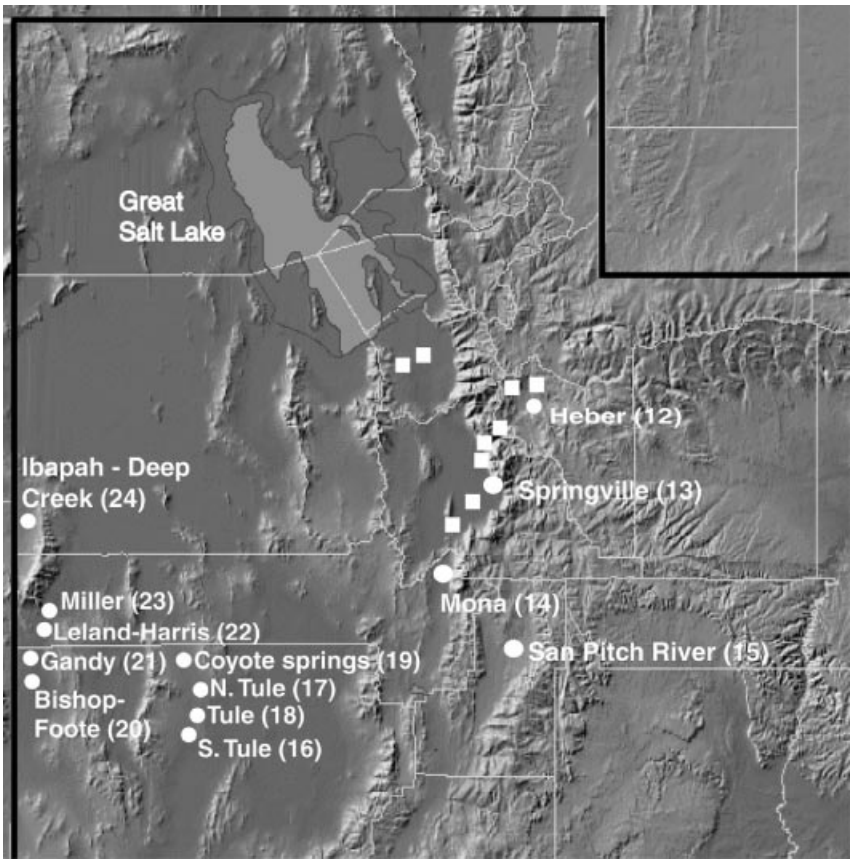

Fig. 2 Detailed topological map of collection sites for all known isolated populations of *Rana luteiventris* in Utah (circles). Numbers are given in Table 1, and squares mark extirpated populations (UDWR 1993, 1994). The Heber, Springville, Mona and San Pete county populations comprise the 'Wasatch Front' unit that is covered by the Utah Division of Wildlife Resources (1998).

1960; Morris & Tanner 1969; Licht 1975, 1986; Cuellar 1994) make genetically structured populations very likely. The inconsistency between the Green *et al*. (1996) and the Toline & Seitz (1999) studies may reflect the difference in sampling scales between the two studies (broad sampling across the entire range, vs. fine-scale sampling only in Utah), but the absence of clear phylogeographic structure in the Toline and Seitz study may also be an artefact of the low resolving power of the RFLP and RAPD data. Here we report on a mtDNA sequence-based phylogeographic study designed to assess the population structure of *R. luteiventris* across a large part of its range. Geographic sampling includes all known peripheral isolates of the species, and resolution is sufficient to distinguish between demographic vs. historical factors responsible for the observed phylogeographic structure, under a null model of no geographical association of haplotypes with geography. We also identify candidate lineages of relevance to further development of a conservation plan.

Materials and methods

Population sampling and outgroup choice

Tissue samples in the form of toe clips or DNA extracts of *Rana luteiventris* were obtained from 121 individuals representing 39 localities from across most of the species' known range (Figs 1 and 2) in the contiguous western United States. Character states were polarized using three outgroup taxa (*R. pretiosa*, *R. aurora* and *R. cascadae*) sampled from within the closely related *R. boylii* species group (Wallace *et al*. 1973; Case 1978; Farris *et al*. 1979; Green 1986a,b; Hillis 1986). Localities are plotted in Figs 1 and 2, and sample sizes are summarized by locality in Table 1.

Laboratory procedures

Total genomic DNA was obtained using extraction methods described by Fetzner (1999). Four microlitres of extraction product were electrophoresed on 1% agarose gel at 140 V for 15 min to estimate the quality and amount of genomic DNA. Sample dilutions (1 : 5) were performed on samples where necessary prior to polymerase chain reaction (PCR) amplification. A fragment of the cytochrome *b* gene region was PCR amplified from 15 samples using the tRNAglu light strand primer and the cyt-b AR heavy strand primer (Goebel *et al*. 1999). These sequences were then used to modify the MVZ-15 light primer to match *R. luteiventris* sequence (5'-AACCTTATGACCCCA-ACAATACG-3') and renamed ralu1, which amplified a 902-bp segment. Two internal sequencing primers were

Table 1 Summary by state of localities from which samples of *Rana luteiventris* and outgroup taxa (*) have been obtained

State	Locality	12s	Cyt *b*	Source	Coordinates
Idaho	(2) North Short Creek	2	2	CP/PM	46°31′ N 114°36′ W
	(3) South Walton Lake	2	5	CP/PM	46°29′ N 114°37′ W
	(4) Grouse Lake	2	4	CP/PM	46°28′ N 114°37′ W
	(5) In and Out Lake	1	1	CP/DP	45°01′ N 114°32′ W
	(6) Cache Lake	2	4	CP/DP	45°05′ N 114°36′ W
	(7) Fawn Lake	2	3	CP/DP	45°07′ N 114°35′ W
Montana	(8) Sweetgrass River	1	1	DB	46°06′ N 109°57′ W
Nevada	(25) Farrington Ranch	1	3	JR	38°25′ N 117°30′ W
	(26) Upper Corral Pond	1	1	JR	38°26′ N 117°31′ W
	(27) Green Mtn. Creek	4	1	JR	40°22′ N 115°30′ W
	(28) Maggie Creek	1	1	JR	41°06′ N 116°00′ W
	(31) North Fork Humbolt	2	1	JR	41°33′ N 115°48′ W
	(29) Sheep Creek Springs	1	1	JR	41°22′ N 115°49′ W
	(35) Electric Fence Pond	1	1	JR	41°35′ N 115°55′ W
	(32) Telephone Creek	1	1	JR	41°55′ N 115°42′ W
	(33) Sand Creek	1	1	JR	41°52′ N 115°46′ W
	(34) Winter Creek Pond	1	1	JR	41°47′ N 116°15′ W
	(30) Chicken Creek	1	1	JR	41°30′ N 116°40′ W
Oregon	(38) Blue Mtns	1	1	MB	44°30′ N 118°00′ W
	(37) Waldo Lake*	2	1	MB	43°54′ N 122°30′ W
	(36) Sunriver*	2	1	MB	43°52′ N 121°26′ W
Utah	(12) Heber/Provo River	0	5	UDWR	40°35′ N 111°22′ W
	(13) Springville hatchery	3	4	UDWR	40°10′ N 111°35′ W
	(14) Mona	3	3	UDWR	39°50′ N 111°59′ W
	(15) Sanpete county	2	3	UDWR	39°40′ N 111°30′ W
	(17) N. Tule valley	2	4	UDWR	39°19′ N 113°30′ W
	(16) S. Tule valley	3	6	UDWR	39°17′ N 113°30′ W
	(18) Tule Valley	0	5	UDWR	39°18′ N 113°30′ W
	(19) Coyote Springs	3	4	UDWR	39°25′ N 113°29′ W
	(20) Bishop-Foote	3	5	UDWR	39°23′ N 113°54′ W
	(21) Gandy	2	5	UDWR	39°27′ N 113°54′ W
	(22) Leland-Harris	2	5	UDWR	39°32′ N 113°51′ W
	(23) Miller Springs	2	5	UDWR	39°35′ N 113°49′ W
	(24) Deep Creek Mtns	7	10	UDWR	39°58′ N 113°57′ W
Washington	(1) N. Cascades NP	3	3	MB	48°30′ N 121°00′ W
	(39) Olympic NP*	1	1	MB	48°16′ N 124°40′ W
Wyoming	(9) Yellowstone NP	3	4	CP/DB	44°45′ N 111°06′ W
	(10) Teton NP	3	5	CP/DB	43°45′ N 110°30′ W
	(11) Bighorn Mtns	3	8	DB	44°45′ N 107°30′ W

Numbers for each location are plotted in Figs 1 and 2 and columns below each gene region indicate the number of individuals sequenced per locality. Outgroup taxa are: *R. aurora* (39), *R. cascadae* (37), *R. pretiosa* (36); acronyms for source of tissue samples are: (CP = Charles Peterson, PM = Patrick Murphy, DP = David Pilliod, DB = David Bos, JR = Jamie Reaser, MB = Mike Blouin, UDWR = Utah Division of Wildlife Resources).

designed and named ralu2 (light strand; 5′-TCTGCAT-CTACTTCCACATCGGC-3′) and ralu3 (heavy strand; 5′-GGGTTGGCGGGCGTAAAGTTATCAGGG-3′). This cytochrome *b* region was sequenced for a total of 121 frogs from all localities sampled (Table 1). Seven hundred and eighty-five base pairs of the *12s* gene were amplified using the 12sz and 12sk primers (Goebel *et al*. 1999) via PCR. This gene region was sequenced for 77 individuals from most sampling localities (Table 1).

For both *12s* and cytochrome *b* PCR amplifications, template DNA was initially denatured at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. A final extension of 5 min at 72 °C ended the PCR reaction. Eight microlitres of PCR product was electrophoresed at 140 V for 10 min on 1% agarose gel that was stained with ethidium bromide to assess product quality. PCR product of the *12s* gene was purified for cycle sequencing using the Gene-clean III kit (Bio 101), and PCR product from the cytochrome *b* gene region was purified using Centricon centrifugal filters (Millipore). Both strands of all PCR product were cycle sequenced for complementary strands using Amplitaq polymerase and FS terminators from Perkin-Elmer. Following cycle sequencing,

unincorporated dye labels were removed by passing the sample through Sephadex columns and subsequently sequenced using an ABI Prism 377 automated sequencer (ABI Biosystems).

Phylogenetic and statistical analysis

Sequences (GenBank Accession nos AY016649–AY016727) were aligned and edited using the SEQUENCHER program. Sequence ambiguities were resolved by comparing complementary strands. Sequence alignment was straightforward for both gene regions because of the low levels of genetic divergence among samples. The *12s* gene sequences contained no indels, and the cytochrome *b* region was translated into amino acid sequence to assess the sequence for stop codons.

One major assumption of methods using molecular variation for investigating evolutionary history is the neutrality of the markers, but as noted by Rand (1996), different kinds of selection may influence the topology of a gene tree. Therefore, the hypothesis that all mutations in the data set are selectively neutral was tested using Tajima's *D*-test statistic (1989) as implemented in DNASP (version 3.0; Rozas & Rozas 1999). Because the data set consists of two separate genes that have different functions (Bull *et al*. 1993), we performed the incongruence length difference test (Mickevich & Farris 1981) as implemented in PAUP* 4.04a (Swofford 1998) to assess the possibility of partition heterogeneity before combining the data. Significance testing that the topologies of two trees are different was accomplished using Templeton's (1983) nonparametric ranking procedure.

Both maximum parsimony and maximum likelihood optimality criteria were used for phylogenetic reconstruction (as implemented in PAUP* 4.04a; Swofford 1998) using all unique haplotypes as terminals, and considering all characters unordered and equally weighted (under MP). Both methods were used in order to assess the sensitivity of the resulting genealogies to different assumptions of character evolution, and to maximize the trade-off between consistency and power of phylogenetic estimators (Hillis *et al*. 1994a,b). Under both optimality criteria, a heuristic search option employing random stepwise addition, 10 random additions and TBR branch swapping was performed. Each of the three outgroup taxa were used as single alternatives (Donoghue & Cantino 1984) while the other two were allowed to 'float' among *R. luteiventris* terminals. This sequential substitution of alternative outgroups provides an assessment of support for monophyly of the ingroup (Nixon & Carpenter 1993). Nodal support was measured utilizing the nonparametric bootstrap method (Felsenstein 1985; 1000 pseudoreplications for the maximum parsimony criterion, and 100 pseudoreplications for the maximum likelihood criterion).

We employed MODELTEST (Posada & Crandall 1998) to select the most likely model of character evolution for ML analysis. We used the default option of a neighbour-joining tree based on distances estimated from a Jukes & Cantor (1969) substitution model as our input tree. This initial tree was used to initiate a hierarchical test that successively compares a relatively simple null model with a nested alternative model which includes one additional parameter of molecular evolution. The model selected as the better fitting model by the LRT is then used as the null model in the next iteration, and this process is repeated with progressively more parameter-rich models until the best fitting model is found (reviewed in Huelsenbeck & Crandall 1997). Retention of the nesting structure of models of evolution requires that likelihood scores be estimated from the same input tree, and tree topology appears to have little influence on likelihood estimates (Yang *et al*. 1995). LRT model comparison used ML estimated base frequencies to more closely approximate the χ^2 distribution used to determine the significance of differences between nested models (Whelan & Goldman 1999).

To compliment conventional phylogenetic methods, the nested pattern of relationships among closely related haplotypes was estimated, because under conditions in which DNA sequences differ by few substitutions, traditional methods generally perform poorly (Huelsenbeck & Hillis 1993; Crandall 1994). Therefore, we used the networking algorithm developed by Templeton *et al*. (1992; program available in TCS; Clement *et al*. 2000) which estimates the probability that a site difference between two randomly drawn haplotypes results from more than one mutational event. The intraspecific maximum parsimony network defines nested sets of haplotypes in which sequences differing by a single mutation are first grouped together in a one-step clade, and these are then nested within two-step clades (which include all haplotypes differing by two mutations; see Templeton & Sing 1993; Crandall 1996; for nesting rules). Clade distances (D_c) and nested clade distances (D_n) can then be defined based on the geographical locations of samples in the nesting cladogram, and were estimated as described in Templeton *et al*. (1995). The difference between interior (ancestral) and tip clade D_c and D_n distances were calculated to yield D_cI – D_cT and D_nI – D_nT values, where I and T are interior and tip clades, respectively.

The null hypothesis of no geographical associations of clades and nested clades was tested by considering that the dispersion distance of clades was not greater or less than expected at random, and comparing observed D_c and D_n values to a distribution of such values calculated for each 1000 random permutations of clades against sampling locations (Templeton & Sing 1993; Templeton *et al*. 1995; Templeton 1998). Permutation tests were conducted separately for each level of the nested cladogram using

Table 2 Geographic locations (Loc.) from Fig. 1 and number of individuals in each haplotype (Num.) from each population included the nested clade and GEODIS analyses

	Num.	Loc.		Num.	Loc.
		Utah Network			
Haplotype 1			**Haplotype 8**		
Bishop-Foote	4	20	Leland-Harris	1	22
Coyote Spring	2	19	Miller	4	23
Heber	3	12	Mona	2	14
Mona	1	14	**Haplotype 9**		
Mona South	1	14	Leland-Harris	1	22
San Pete County	3	15	**Haplotype 10**		
Tule Valley	4	18	South Tule Valley	3	16
South Tule Valley	3	16	North Tule Valley	3	17
Haplotype 2			**Haplotype 11**		
Heber	2	12	Gandy	5	21
Haplotype 3			**Haplotype 12**		
Coyote	1	19	North Tule Valley	1	17
Haplotype 4			**Haplotype 13**		
Springville	3	13	Deep Creek	5	24
Haplotype 5			**Haplotype 14**		
Springville	1	13	Deep Creek	2	24
Haplotype 6			**Haplotype 15**		
Tule Valley	1	18	Deep Creek	2	24
Haplotype 7			**Haplotype 16**		
Bishop-Foote	1	20	Deep Creek	1	24
Coyote Spring	1	19			
Leland-Harris	4	22			
Miller 1	1	23			
		Nevada Network			
Haplotype 17			**Haplotype 19**		
Chicken Spring	1	30	Green Mtn. Creek	1	27
Telephone Creek	1	32	**Haplotype 20**		
Haplotype 18			Upper Corral Pond	1	26
Maggie Creek	1	28	Farrington Ranch	3	25
Haplotype 21			**Haplotype 24**		
Green Mtn. Creek	1	27	Sand Creek	1	33
Haplotype 22			**Haplotype 25**		
North Fork Humbolt	1	31	Sheep Creek Spring	1	29
Haplotype 23			**Haplotype 26**		
Electric Fence Pond	1	35	Winter Creek	1	34
		Rocky Mtn. Network			
Haplotype 27			**Haplotype 33**		
Cache Lake	1	6	North Short Creek	1	2
Fawn Lake	3	7	Grouse Lake	1	4
Grouse Lake	3	4	**Haplotype 34**		
In and Out Lake	1	5	Sweetgrass R.	1	8
North Short Creek	1	2	**Haplotype 35**		
South Walton Lake	5	3	N. Cascades NP	3	1
Teton NP	3	10	**Haplotype 36**		
Yellowstone NP	1	9	Blue Mountains	1	38
Haplotype 28			**Haplotype 37**		
Teton NP	1	10	Cache Lake	1	6
Haplotype 29			**Haplotype 38**		
Teton NP	1	10	Cache Lake	2	6
Haplotype 30			**Haplotype 39**		
Yellowstone NP	1	9	Bighorn Mtns	6	11
Haplotype 31			**Haplotype 40**		
Yellowstone NP	1	9	Bighorn Mtns	1	11
Haplotype 32			**Haplotype 41**		
Yellowstone NP	1	9	Bighorn Mtns	1	11
		Outgroups			
Rana aurora	1	39			
Rana cascadae	1	37			
Rana pretiosa	1	36			

GEODIS 2.0 (Posada *et al*. 2000). Once significance levels for D_c and D_n were determined, inferences about processes likely responsible for observed patterns of clade structure can be made using the inference key provided in Templeton *et al*. (1995).

Genetic polymorphism (represented by $\theta = 4N_e\mu$) was estimated for the entire data set and within each clade using a maximum likelihood estimator, implemented in FLUCTUATE (Kuhner *et al*. 1998). This program relaxes the assumption of constant population size made by most other θ estimation programs and was deemed appropriate here because many *R. luteiventris* populations have undergone significant population size changes (such as recent reductions due to habitat loss) in parts of the range (UDWR 1993).

Results

Sequence variation

Sequencing of the cytochrome *b* gene region revealed 44 unique haplotypes (including the three outgroup taxa; see Table 2) based on 77 parsimony informative sites. The average number of nucleotide differences within *Rana luteiventris* is 6.87, and the same measure for the interspecific comparisons is 27.389. A test of neutrality (Tajima 1989) indicated that all mutations in this DNA segment are selectively neutral ($D = 0.45451$; $P > 0.10$). The *12s* gene was less variable, with 20 haplotypes based on 32 polymorphic sites. Tajima's test indicated that mutations in the *12s* gene also conformed to neutral expectations ($D = 0.11042$; $P > 0.10$). Nonparametric testing of data set partition homogeneity using 1000 replicates revealed no evidence for heterogeneity ($P = 0.33$). Phylogenetic analyses were therefore performed on the combined data set, because larger number of characters will likely improve the phylogenetic estimate (Bull *et al*. 1993; Hillis *et al*. 1994a,b).

Phylogenetic analyses

Equally weighted maximum parsimony (MP) analysis of haplotypes of the combined data set revealed four most parsimonious trees consisting of 349 steps (CI = 0.79; RI = 0.926). All trees recovered *R. luteiventris* as a monophyletic group, and alternate rooting with either *R. aurora* or *R. cascadae* recovered *R. pretiosa* as the sister taxon to *R. lutieventris*. This topology is consistent with Green *et al*. (1996), so most subsequent rooting was performed with *R. pretiosa* as the sole outgroup. All trees recovered strongly supported clades which correspond to distinct geographical regions across the species range. All Nevada haplotypes were recovered as a strongly supported clade (100% bootstrap), as were all haplotypes spanning the northern portion of the study area from Wyoming west through Washington and Oregon (Fig. 3a). This 'Rocky Mountain' clade was recovered with 100% bootstrap support, and two strongly supported clades were identified in Utah. One consisted entirely of haplotypes from the Deep Creek site (location 24 in Fig. 2), with 98% bootstrap support, and the second clade consisted of all other localities in Utah (96% bootstrap support; Fig. 3a). The Utah and Rocky Mountain clades were weakly recovered (56% bootstrap support) as sister clades.

The model selected for the maximum likelihood (ML) analysis of the combined as well as both individual data sets is HKY + I + Γ (Hasegawa *et al*. 1985), which accommodates differing transition–transversion mutation rates, uneven base frequencies and among site rate heterogeneity (Table 3). The ML tree estimated with the HKY + I + Γ model of evolution recovered the same four monophyletic clades as did all of the MP analyses, and all were strongly supported by bootstrap values > 90% (Fig. 3b), suggesting that this component of the tree topology is relatively insensitive to different assumptions of character evolution. The ML bootstrap values also provide only weak support for a basal position of the Nevada clade and Utah and Rocky Mountain clades as sister groups.

These results do not corroborate the results of Green *et al*. (1996), which placed a haplotype from the San Pitch River (locality 15 in Fig. 1) with samples from the Rocky Mountain region (localities 1–10 in Fig. 1) in a UPGMA dendrogram of genetic distances (Nei 1978). We tested this alternative by constraining the San Pitch haplotype to a basal position in the Rocky Mountain clade, and this single constraint resulted in 36 equally parsimonious trees that required 85 extra steps (TL = 394 steps), relative to the most parsimonious trees (Fig. 3a). The Wilcoxon signed-rank test revealed that the shortest unconstrained trees recovered in this study were statistically more parsimonious ($z = -6.7082$; $P < 0.0001$) relative to the shortest constraint trees.

Minimum parsimony networks and nested clade analysis

Three networks were resolved using the cytochrome *b* data set (Fig. 4). Parsimonious connections were statistically

Table 3 Molecular evolution parameter values estimated by MODELTEST for the combined data set

Parameter	Estimate
ti/tv	7.4247
Base frequencies	
A	0.2751
C	0.2956
T	0.1670
G	0.2623
Proportion invariable sites	0.8212
Gamma shape parameter	50.0206
Model selected	HKY + I + Γ

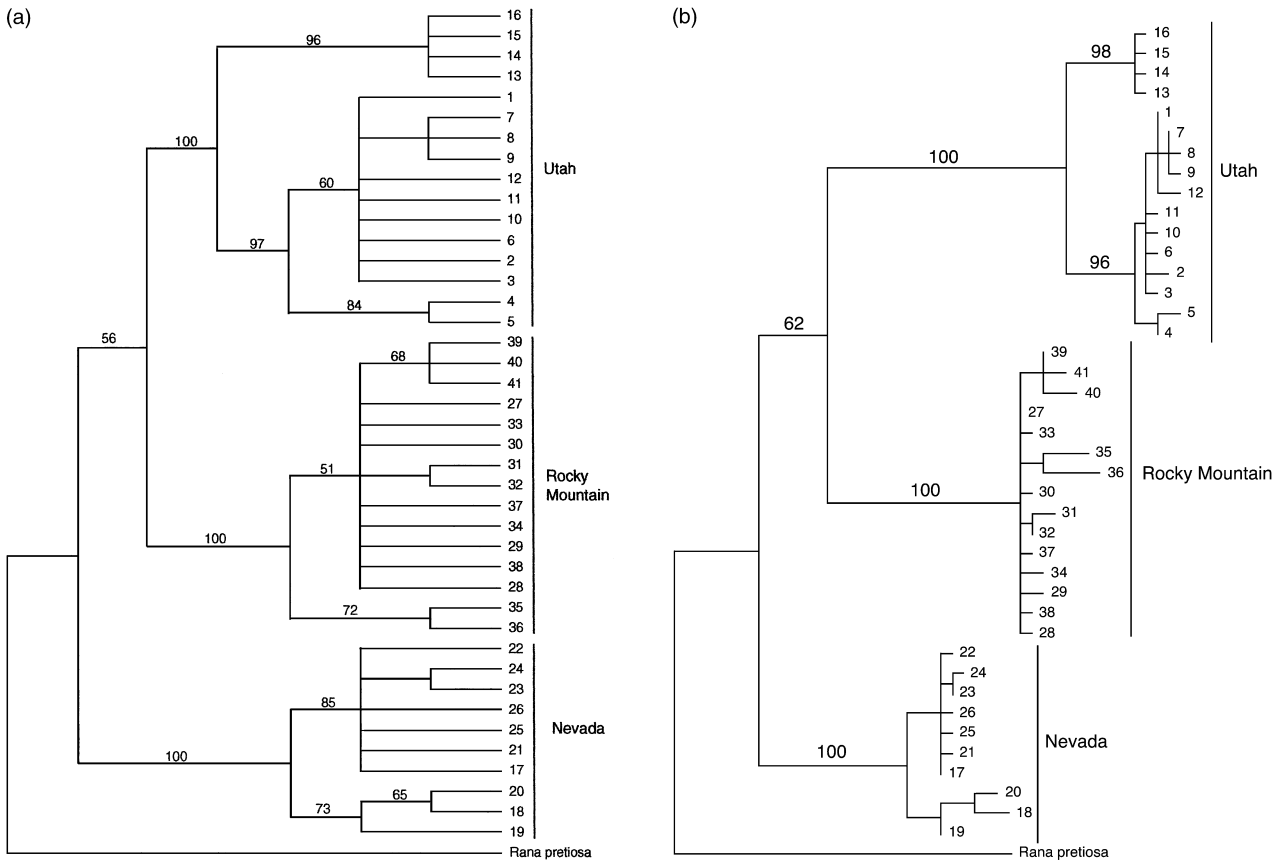

Fig. 3 (a) Strict consensus phylogram of four equally weighted MP trees; bootstrap values (1000 pseudoreplicates) are shown if > 50%. Phylogram drawn with sister group only as outgroup. Numbers correspond to haplotypes from Table 2. (b) Maximum likelihood tree resulting from estimation using HKY + I + Γ model of evolution; bootstrap values (100 psuedoreplications) are shown if > 50%. Numbers correspond to haplotypes from Table 2.

justified ($P_j \geq 0.95$) for between haplotype divergences of up to 13 mutational differences. Individual networks are not joined because divergence between the networks exceeded the 95% confidence limits of parsimonious connections derived from the estimation procedure (Templeton *et al*. 1992), indicating that these connections are beyond the 95% confidence limits of parsimonious connections. Conventional ML and MP analyses were therefore most appropriate for reconstructing these deeper relationships.

The nesting design (Fig. 4) revealed that networks are strongly clustered in geographical regions: two major networks are found in the Great Basin, one consisting of populations found exclusively within the Bonneville basin in Utah (hereafter termed the Bonneville network, clade 4-1, Fig. 4), and another consisting of populations confined to the Lahontan basin in Nevada (Lahontan network, clade 3-5, Fig. 4). The third network (clade 4-2, Fig. 4) is referred to here as the Rocky Mountain network, and is comprised of populations from the northern contiguous range of *R. luteiventris* in Idaho, Montana, Oregon, Washington and Wyoming. Also

Table 4 Nested contingency analysis of geographical associations. Clades with no geographical or genetic variation not shown

Clade	Permutation χ^2	*P*-value
1-1*	89.457	0.000
1-2	9.428	0.319
1-9	1.875	1.000
2-1	36.838	0.000
2-4	1.111	1.000
3-1*	66.673	0.000
Bonneville network*	63.000	0.000
1-12	24.000	0.704
1-13	2.000	1.000
1-16	5.000	0.223
2-6	8.000	1.000
2-7	6.000	0.171
Lahontan network*	11.958	0.193
1-18	73.857	0.017
3-3*	36.000	0.000
3-4	2.000	1.000
Rocky Mtn. Network*	38.000	0.005

*Clades with significant D_n, D_c or I-T-values

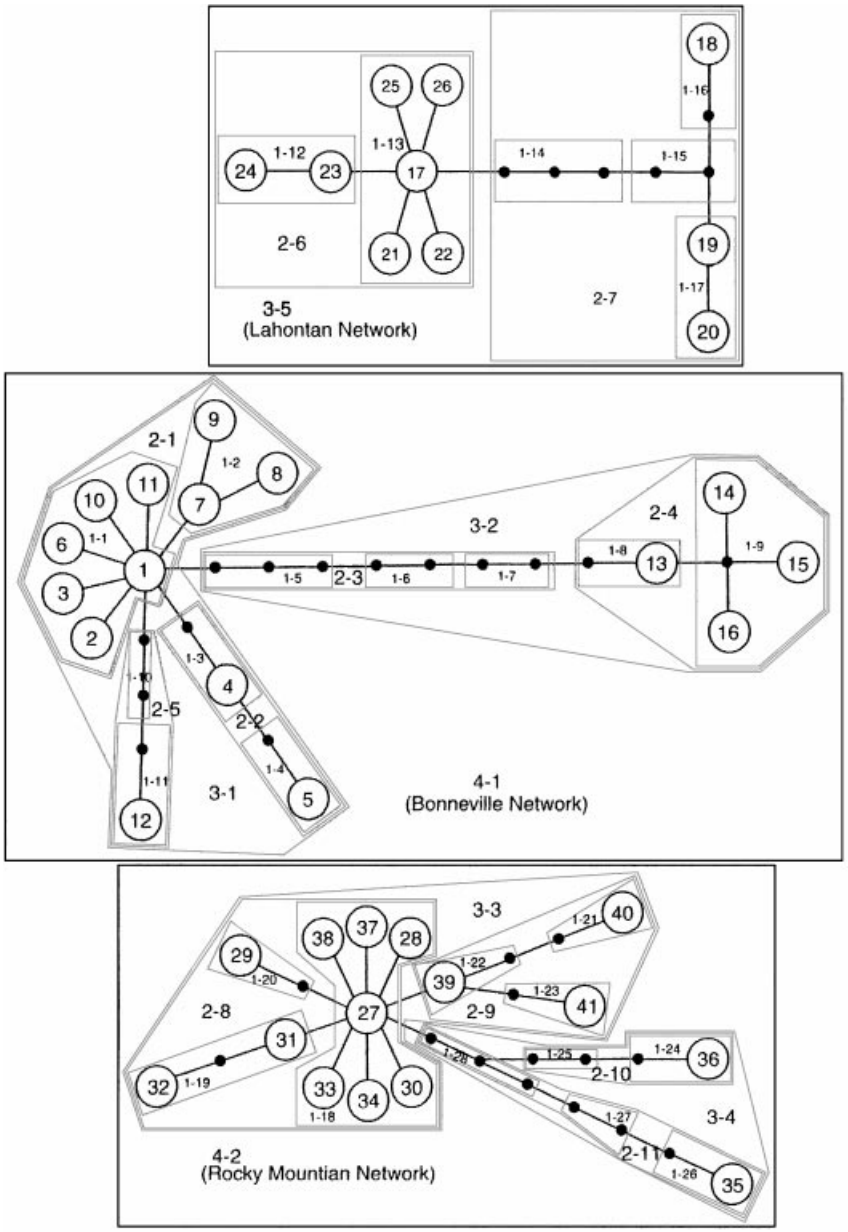

Fig. 4 Haplotype networks and nested clade design for cytochrome *b* sequences of all unique *Rana luteiventris* haplotypes (numbered 1–41; see Table 3). Numbers inside circles indicate haplotype number (see Table 2 for information on each haplotype) and closed circles are extinct or unsampled haplotypes. The first number of each clade and nesting clade corresponds to the number of mutational steps in that clade based on protein coding sequence data.

included in this network is one isolated population from the Bighorn Mountains of Wyoming, which has three haplotypes, including one unique fixed mutation (clade 2-9, Fig. 4). Both the Bonneville and Rocky Mountain networks are characterized by one major haplotype (haplotypes 1 and 27, respectively; see Fig. 4; Table 2) made up of multiple individuals from several locations, and from which many other haplotypes can be derived by single mutational events. There is significant genetic divergence within all three networks, as they are also typified by several extinct or unsampled haplotypes.

GEODIS analysis (Posada *et al*. 2000) revealed significant nonrandom associations of clades and sampling locations, indicating deep phylogeographic structure at higher clade levels, and absence of geographical structuring within most of the lower clade levels (Table 4, Fig. 5). Significantly small geographical associations across the highest nesting clades (three- and four-step clades) led to the unambiguous rejection of the null hypothesis that the Lahontan, Bonneville and Rocky Mountain networks are derived from a single evolutionary lineage. Further, significantly smaller geographical associations are detected within each of the three major networks, and the inference chain (Table 5) reveals a history of allopatric fragmentation across the entire study region, and also among populations within the Bonneville and Lahontan networks. No firm conclusion can be drawn for some parts of the Rocky Mountain network due to insufficient sampling.

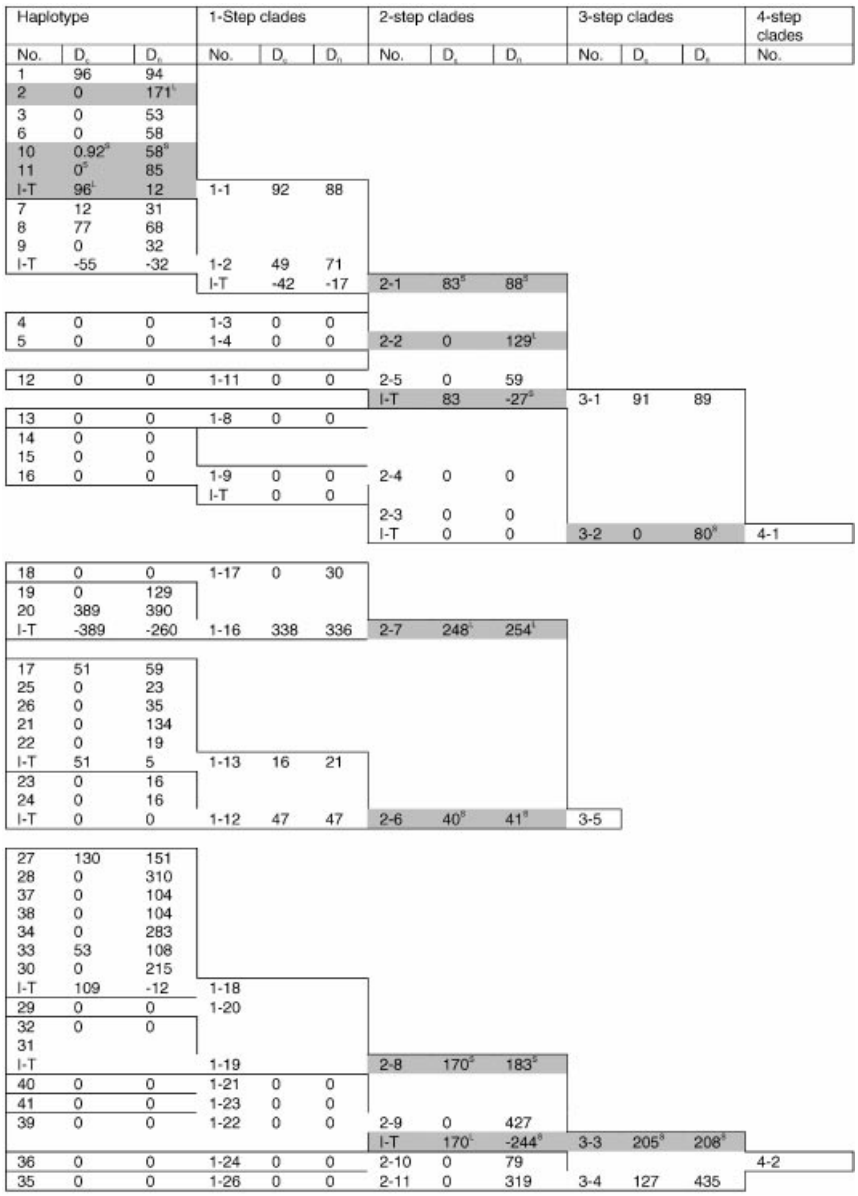

Fig. 5 Results of nested clade analysis of cytochrome *b* networks for *Rana luteiventris*. Clades at one level that are enclosed within a box are nested together, but connected to the nested clade at the right. Average clade (D_c) and nested clade (D_n) distances were calculated and tested for significant deviations from the null hypothesis of no association between nested clades and geographical location within a nested clade. Superscripts 'L' and 'S' identify significantly large and small distances ($P < 0.05$ under the null hypothesis of no geographical association). Clades were also classified as tips or interiors. Shading indicates clades with significantly large or small values. When significant deviations from the null hypothesis were detected, the inference key of Templeton *et al*. (1995) was used for biological interpretation of the patterns. The inference chains for all significant associations are summarized in Table 5.

Table 5 Inference chain based on results of geographical dispersion analysis (GEODIS)

Clade	Chain of inference	Inference
1-1	1,2,3,5,6,7	Insufficient genetic resolution to distinguish range expansion and gene flow
3-1	1,2,11,12	Continuous range expansion
4-1 (Bonneville network)	1,2,3,4,9,10	Allopatric fragmentation
3-5 (Lahontan network)	1,2,3,5,15,16	Allopatric fragmentation
3-3	1,2,3,5,15,16	Allopatric fragmentation
4-2 (Rocky Mountain network)	1,2,3,4,9,10	Insufficient sampling to distinguish fragmentation and isolation by distance

Interior to these, significantly small and large associations are evident in some two-step clades (Fig. 5), but the inference chain reveals different interpretations among these. For example, significantly large and small associations of clades 2-1, 2-2 and 2-5 nested in clade 3-1 of the Bonneville network are best explained by a continuous range expansion (Table 5), yet significant associations among clades 2-8 and 2-9 nested in clade 3-3 of the Rocky Mountain network are best explained by allopatric fragmentation. Further, insufficient genetic variation in the

data set precludes unambiguous explanation of the associations of haplotypes within clade 1-1 of the Bonneville network (Table 5).

The parameter $\theta = 4N_e\mu$ is used as a measure of DNA polymorphism and is estimated for the entire data set to be 0.04181. This summary of variation was also estimated for each individual network; the Rocky Mountain has the highest measure of variation ($\theta = 0.03306$), which is not surprising, considering the perceived larger size and health of these populations. The other two networks have considerably less variation (Lahontan $\theta = 0.01465$, and Bonneville $\theta = 0.00681$) indicating a loss of genetic variation and/or much smaller effective population sizes with respect to the Rocky Mountain group.

Discussion

Phylogeography

The inability to unambiguously distinguish the order of vicariant events separating the three major clades may be due either to a lack of resolution in the data set, or because of the relatively rapid order of events isolating the Great Basin from northern populations and the Lahontan and Bonneville Basins within the Great Basin. The latter scenario can be explained by the nearly equal amount of genetic variation between the Lahontan and Bonneville networks, and between either the Bonneville, or Lahontan network and the Rocky Mountain network. The vicariance responsible for the initial isolation of Great Basin populations, may have occurred during the Miocene epoch after Great Basin uplift and hydrologic isolation (Hovingh 1997). Early during this epoch, the climatic conditions were more mild and wet and *Rana luteiventris* likely had a more widespread, continuous distribution (Hovingh 1997). In the mid-Miocene, orogenic events in the Sierra Nevada and Wasatch ranges caused the climate to become more cold and dry (see refs in Hovingh 1997; Zamudio *et al*. 1997) restricting spotted frog populations and movements, and continuing hydrologic isolation of and within the Great Basin may have geographically separated *R. luteiventris* populations in Utah and Nevada. The vicariance events around the Great Basin during this epoch are also proposed to be responsible for similar phylogeographic structure and levels of polymorphism in short-horned lizards (*Phyronsoma douglasi*; Zamudio *et al*. 1997) and for small mammals (Riddle 1995).

Results also indicate that the more recent subdivision between populations occurred between sub-basins within each of the Bonneville and Lahontan Basins. For example, in the Bonneville network these subdivisions are evidenced by the genetic structuring between samples from the Deep Creek Mountains and all other samples of this network. This relationship is also born out in the results of Toline & Seitz (1999) which indicate that the Deep Creek populations have haplotypes unique to those locations (see Table 2). These samples form a monophyletic group and GEODIS analysis indicates that genetic structuring between clade 3-2 (the Deep Creek population) and clade 3-1 (comprising the remaining samples from Utah; see Figs 4 and 5) is significant ($\chi^2 = 63.00$, $P = 0.000$; Table 4) and due to historical allopatry (Table 5).

In the Lahontan network, genetic structuring between locations does not appear to be complete; haplotype 21 (Table 2) from Green Mountain (locality 27, Fig. 1) is more closely related to samples from northern Nevada than to the other haplotype from Green Mountain and other samples from within the Great Basin, such as Farrington Ranch and Maggie Creek (localities 25 and 28, Fig. 1). The sorting of individuals from this population with different areas probably contributes to the lack of significant geographical structuring within the Lahontan network (see Table 4). This may be due to incomplete lineage sorting, or to introgression between these two areas during hydrologic reunification of these two sub-basins after initial vicariance.

The *R. luteiventris* population that is geographically isolated in the Bighorn Mountains (locality 11, Fig. 1) lay outside the Great Basin and is genetically distinct; however, the Bighorn haplotypes are separated by few mutational steps from the nearest populations (Teton-Yellowstone, localities 9 and 10, Fig. 1), which are in the continuous range. This likely reflects a more recent vicariant event than that separating populations from the Deep Creek Mountain range and remaining populations in Utah and Nevada.

Hovingh (1993b) suggested that most of the current *R. luteiventris* habitat in Utah was submerged during the high shoreline of Lake Bonneville, with two exceptions. First, frog populations restricted to the Deep Creek Mountains (locality 24, Fig. 2) inhabit a complex of springs on a 'bench' above the high-water shoreline of Lake Bonneville, and hence have not recently colonized their current habitat after drying of the lake (Hovingh 1993b). Other populations of *R. luteiventris* in Utah are proposed to have been confined to a late-Pleistocene refugium in the Snake Valley (localities 20–23, Fig. 2) during the Lake Bonneville high shoreline. The general lack of haplotype diversity (only 3 of 11 one-step clades in the Bonneville network has any geographical variation, and only 1 of those 11 clades contains significant geographical structuring; see Table 4), and the presence of a widespread interior allele in the Bonneville network (aside from Deep Creek populations; see Table 2 and Fig. 4) is consistent with the hypothesis of a population contraction during the high shoreline of Lake Bonneville. During Lake Bonneville recession, *R. luteiventris* presumably spread eastward into other sub-basins in Utah from the Snake Valley refugium, and eventually to the Wasatch Front (see model 2 in Hovingh 1997); however, no

migrants were shared between the Deep Creek and Snake Valley refugia. This model can explain the widespread sharing of alleles among many of these locations and the overall lack of genetic structuring in the majority of clades in the Bonneville Basin and the distinctiveness of the Deep Creek population. This interpretation of the data differs from that of Toline & Seitz (1999) who found similar patterns of genetic variation along the Wasatch Front, West Desert and Deep Creek populations, and postulated that this pattern reflects recent (≈ 18 000 ya) migration of *R. luteiventris* into Utah. The existing lack of genetic structure among these populations is likely an artefact of lowered genetic polymorphism during a population decline in the shared ancestral refugium population in the Snake Valley. However, this hypothesis rests upon the general lack of polymorphism in this data set; further study with more polymorphic markers (Sunnucks 2000) may require refinement of this hypothesis.

Conservation genetics

The quantity $\theta = 4N_e\mu$ is the most important parameter for studying the evolution of a DNA region in a population (Fu & Li 1999), and this value conveys more information about population polymorphism than K (number of segregating sites) or π (mean number of nucleotide differences; Fu & Li 1999). The estimates of θ reported here suggest that the Bonneville network has the lowest level of polymorphism among the three networks. The values of θ (often used as a measure of 'genetic health') reported for this network are lower than those reported for the endangered eastern salamander *Plethodon shenandoah* ($\theta = 0.0114$; Carpenter *et al*., in press), but are similar to those calculated for the widespread *P. cinereus* ($\theta = 0.00299$). The smaller sizes of some *R. luteiventris* populations in the Bonneville network (UDWR 1993), combined with their lowered genetic diversity, may put these populations at increased risk of extinction due to the effects of drift, genetic load and possibly inbreeding depression (Frankham 1995; Saccheri *et al*. 1998; Kirkpatrick & Jarne 2000).

Overall genetic diversity for the Lahontan and Northern networks is higher, possibly indicating that past reduction in population size has not been as severe relative to the Bonneville network, and that these populations are less likely to suffer from the effects of inbreeding or genetic load. These results are based on a single genetic marker, however, and must therefore be interpreted with caution because of the effects of evolutionary stochastisity and sampling. Additional information regarding adaptive evolution, and ecological distinctiveness and genetic polymorphism among and within these clades is warranted to confirm these results.

The partitioning of genetic variation into historical and demographic components can be used to identify evolutionarily significant units (ESUs), so that the long-term evolutionary potential inherent in the entire set of ESUs can be preserved (Moritz 1994b; Ryder 1986). There have been many proposed definitions of an ESU (Dizon *et al*. 1992; Moritz 1994b; Vogler & DeSalle 1994), but only some are presented in the form of a testable hypothesis. Moritz (1994a,b, 1995) operationally defined an ESU as a population (or set of populations) that has been isolated from other populations long enough for the effects of lineage sorting to create reciprocally monophyletic groups of mtDNA alleles (see also Neigel & Avise 1986). These monophyletic groups should also differ significantly from each other in frequencies of alleles segregating at Mendelian loci. The mtDNA data presented here indicate significant geographical structuring of genetic variation across the range of *R. luteiventris* in the western United States, and the topologies of all phylogenetic trees derived from these sequences (Fig. 3) show that reciprocal monophyly criterion for ESU designation has been met for the Nevada populations, the Rocky Mountain populations, the Deep Creek population and all other Utah populations.

One limitation of the Moritz (1994b) definition of ESUs is that if a founder population is extremely small (in extreme cases one gravid female), reciprocal monophyly of populations will be virtually instant for the daughter and the parent populations. If both the ancestral and founder populations are large, the ancestral population will be paraphyletic to the daughter population, and in this case, the daughter population would be considered an ESU and the ancestral population would not (Paetkau 1999). Another limitation is that Ryder's (1986) original idea of ESUs advocated the use of many sources of information, while Moritz's definition relies only on lines of evidence from presumably neutral molecular markers. Bowen (1999) and Crandall *et al*. (2000) recently called for incorporation of ecological and/or genetic data of adaptive significance, a proposal closer to the original intent of Ryder (1986). These authors argue that these types of data are more relevant for conservation issues, but collection of corroborating ecological, behavioural or life history data for natural populations of *R. luteiventris* is beyond the original intent of this study.

Given these limitations, we prefer not to designate formal ESUs, but to refer to four distinct lineages in this data set as the Deep Creek, Bonneville, Lahontan and Rocky Mountain clades. These can be considered candidate ESUs that require confirmation by additional criteria of genealogical concordance as suggested by Avise (2000, see Table 5.1).

Conservation recommendations

The results of this study suggest some issues of immediate concern for *R. luteiventris*, especially in Utah (the Bonneville

clade; Fig. 4). The most obvious is the distinctiveness of the Deep Creek population, which has attained reciprocal monophyly with all other populations in the Bonneville clade. This population currently inhabits an extensive complex of springs and marshes, and is judged to be very large and stable, on the basis of egg mass counts (UDWR 1994), and its relatively large number of segregating mtDNA haplotypes (Table 2). An obvious conservation objective would be to manage this population independently of all others, and to work to maintain its current demographic and genetic health.

The remaining 12 populations that comprise the Bonneville clade appear to have attained their present distributions by a recent range expansion, and given that *R. luteiventris* is rarely found far from water (Svihla 1935; Carpenter 1954; Turner 1960; Dumas 1966), these populations are almost certainly not now interconnected by gene flow. Some west desert populations may comprise metapopulations that could be interconnected in wet years, such as those in the Tule and Snake Valleys (localities 16–19 and 20–23, respectively, in Fig. 2), but this needs to be documented using high-resolution nuclear markers. Similarly, some Wasatch Front populations may have been interconnected prior to extensive habitat loss (localities 12–14 in Fig. 2), and might still share similar allele frequencies and life history traits. However, it is likely that the Wasatch Front, Tule Valley and Snake Valley populations are ecologically and demographically distinct from each other, given their current isolation, and the fact that they inhabit springs and wetlands with very different biological, chemical and thermal properties (Hovingh 1993a; UDWR 1993). Thus, artificial mixing of distant populations through translocations should be avoided, even though no ESU boundaries are evident.

Declining populations of *R. luteiventris* along the Wasatch Front should be augmented (once causes of local declines have been ameliorated) by translocation from other Wasatch Front populations. Further research is needed to define metapopulation structure within the Bonneville clade, and to describe the ecological and physiological traits of local populations across the full gradient of wetland habitats. Only then can conservation efforts be successful in maintaining the full range of adaptive potential for *R. luteiventris*.

Acknowledgements

We would like to thank Chuck Peterson, Patrick Murphy, David Pilliod, Mike Blouin, Jim Munger, UDWR and Todd Hogrefe, Harold Golden, Peter Hovingh and Tomy Sikes for assistance in obtaining samples. James Harris, Alison Whiting, Alan Arndt, Liz Sinclair, David Posada, Jim Fetzner, Jason Cryan, Taylor Maxwell, Trevor Beebee and an anonymous referee provided technical assistance and/or useful comments on this manuscript. This project was supported by research grants from Utah Division of Wildlife Resources, Salt Lake City and Boise field offices of the U.S. Fish and Wildlife Service, and scholarships from Brigham Young University. Joanne Sites and Janae Bos deserve appreciation for their help and patience.

References

Avise JC (1989) A role for molecular genetics in the recognition and conservation of endangered species. *Trends in Ecology and Evolution*, **4**, 279–281.

Avise JC (2000) The history of formation of species. In: *Phylogeography*. Harvard University Press, Cambridge, MA.

Baird SF, Girard C (1853) Communication. *Proceedings of the Academy of Natural Sciences Philadelphia*, **6**, 378–379.

Barber PH (1999a) Patterns of gene flow and population genetic structure in the canyon treefrog, *Hyla arenicolor* (Cope). *Molecular Ecology*, **8**, 563–576.

Barber PH (1999b) Phylogeography of the canyon treefrog, *Hyla arenicolor* (Cope) based on mitochondrial DNA sequence data. *Molecular Ecology*, **8**, 547–562.

Bowen BW (1999) Preserving genes, species, or ecosystems? Healing the fractured foundations of conservation policy. *Molecular Ecology*, **8**, S5–S10.

Bull JJ, Huelsenbeck JP, Cunningham CW, Swofford DL, Waddell PJ (1993) Partitioning and combining data in phylogenetic analysis. *Systematic Biology*, **42**, 384–397.

Carpenter CC (1954) A study of amphibian movement in the Jackson Hole wildlife park. *Copeia*, **1954**, 197–200.

Carpenter DW, Jung RE, Sites JW (2001) Conservation genetics of the endangered shenandoah Salamander (*Plethodon shenandoah*, Plethodontidae). *Animal Conservation*, **4**, in press.

Case SM (1978) Biochemical systematics of members of the genus *Rana* native to western North America. *Systematic Zoology*, **27**, 299–311.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1659.

Crandall KA (1994) Intraspecific cladogram estimation: accuracy at higher levels of divergence. *Systematic Biology*, **43**, 222–235.

Crandall KA (1996) Multiple interspecies transmissions of human and simian T-cell leukemia/lymphoma virus type I sequences. *Molecular Biology and Evolution*, **13**, 115–131.

Crandall KA, Bininda-Edmonds ORP, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology: an alternative to 'evolutionary significant units'. *Trends in Ecology and Evolution*, **15**, 290–295.

Cuellar O (1994) Ecological observations on *Rana pretiosa* in western Utah. *Alytes*, **12**, 109–121.

Dizon AE, Lockyer C, Perrin WF, Demaster DP, Sisson J (1992) Rethinking the stock concept: a phylogenetic approach. *Conservation Biology*, **6**, 24–36.

Donoghue MJ, Cantino PD (1984) The logic and limitations of the outgroup substitution approach to cladistic analysis. *Systematic Botany*, **9**, 192–202.

Dumas PC (1966) Studies of the *Rana* species complex in the Pacific Northwest. *Copeia*, **1966**, 60–74.

Farris JS, Kluge A, Mickevich MF (1979) Paraphyly of the *Rana boylii* species group. *Systematic Zoology*, **28**, 627–634.

Felsenstein J (1995) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Fetzner JW (1999) Extracting high quality DNA from shed reptile skins: a simplified method. *Biotechniques*, **26**, 1052–1054.

Frankham R (1995) Conservation genetics. *Annual Review of Genetics*, **29**, 305–327.

Frey JK (1993) Modes of peripheral isolate formation and speciation. *Systematic Biology*, **42**, 373–381.

Fu Y-X, Li W-H (1999) Coalescing into the 21st century: an overview and prospects of coalescent theory. *Theoretical Population Biology*, **56**, 1–10.

Goebel A, Donnelly JM, Atz ME (1999) PCR primers and amplification methods for *12s* ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome *b* in Bufonids and other frogs, and an overview of PCR primers which have amplified DNA in Amphibians successfully. *Molecular Phylogenetics and Evolution*, **11**, 163–199.

Green DM (1986a) Systematics and evolution of western North American frogs allied to *Rana aurora* and *Rana boylii*: electrophoretic evidence. *Systematic Zoology*, **35**, 283–296.

Green DM (1986b) Systematics and evolution of western North American frogs allied to *Rana aurora* and *Rana boylii*: karyological evidence. *Systematic Zoology*, **35**, 273–282.

Green DM, Kaiser H, Sharbel TF, Kearsley J, McAllister KR (1997) Cryptic species of spotted frogs, *Rana pretiosa* complex, in western North America. *Copeia*, **1**, 1–8.

Green DM, Sharbel TF, Kearsley J, Kaiser H (1996) Postglacial range fluctuation, genetic subdivision and speciation in the western North American spotted frog complex, *Rana pretiosa*. *Evolution*, **50**, 374–390.

Hasegawa M, Kisino H, Yano T (1985) Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **21**, 160–174.

Hillis DM (1986) Evolution of ribosomal DNA: fifty million years of recorded history in the frog genus *Rana*. *Evolution*, **40**, 1275–1288.

Hillis DM, Huelsenbeck JP, Cunningham CW (1994a) Application and accuracy of molecular phylogenies. *Science*, **264**, 671–676.

Hillis DM, Huelsenbeck JP, Swofford DL (1994b) Hobgoblin of phylogenetics. *Nature*, **369**, 363–364.

Hitchings SP, Beebee TJC (1998) Loss of genetic diversity and fitness in Common Toad (*Bufo bufo*) populations isolated by inimical habitat. *Journal of Evolutionary Biology*, **11**, 269–283.

Hovingh P (1993a) Aquatic habits, life history observations and zoogeographic considerations of the spotted frog *Rana pretiosa* in Tule valley, Utah. *Great Basin Naturalist*, **53**, 168–179.

Hovingh P (1993b) Zoogeography and paleozoology of leeches, molluscs, and amphibians in Western Bonneville Basin, Utah, USA. *Journal of Paleolimnology*, **9**, 41–54.

Hovingh P (1997) Amphibians in the eastern Great Basin (Nevada and Utah USA): a geographical study with paleozoological models and conservation implications. *Herpetological Natural History*, **5**, 97–134.

Huelsenbeck JP, Crandall KA (1997) Phylogeny estimation and hypothesis testing using maximum likelihood. *Annual Review of Ecology and Systematics*, **28**, 437–466.

Huelsenbeck JP, Hillis DM (1993) Success of phylogenetic methods in the four-taxon case. *Systematic Biology*, **42**, 247–264.

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: *Mammalian Protein Metabolism* (ed. Munro HN), pp. 21–132. Academic Press, New York.

Kimberling DN, Ferreira AR, Shuster SM, Keim P (1996) RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the south-western USA. *Molecular Ecology*, **5**, 521–529.

Kirkpatrick M, Jarne P (2000) The effects of a bottleneck on inbreeding depression and the genetic load. *American Naturalist*, **155**, 154–167.

Kuhner MK, Yamato J, Felsenstein J (1998) Maximum likelihood estimation of population growth rates based on the coalescent. *Genetics*, **149**, 429–434.

Lefcort H, Meguire RA, Wilson LH, Ettinger WF (1998) Heavy metals alter the survival, growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (*Rana luteiventris*) tadpoles. *Archives of Environmental Contamination and Toxicology*, **35**, 447–456.

Lesica P, Allendorf FW (1995) When are peripheral populations valuable for conservation. *Conservation Biology*, **9**, 753–760.

Licht LE (1975) Comparative life history features of the western spotted frog, *Rana pretiosa*, from low- and high-elevation populations. *Canadian Journal of Zoology*, **53**, 1254–1257.

Licht LE (1986) Comparative escape behavior of sympatric *Rana aurora* and *Rana pretiosa*. *American Midland Naturalist*, **115**, 239–247.

Merrell DJ (1968) A comparison of the estimated size and the 'effective size' of breeding populations of the leopard frog, *Rana pipiens*. *Evolution*, **22**, 274–283.

Mickevich MF, Farris SJ (1981) The implications of congruence in *Menidia*. *Systematic Zoology*, **30**, 351–370.

Moritz C (1994a) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, **3**, 401–411.

Moritz C (1994b) Defining 'evolutonary significant units' for conservation. *Trends in Ecology and Systematics*, **9**, 373–375.

Moritz C (1995) Uses of molecular phylogenies for conservation. *Philosophical Transcripts of the Royal Society of London*, **349**, 113–118.

Morris RL, Tanner WW (1969) The ecology of the western spotted frog, *Rana pretiosa pretiosa* Baird and Girard, a life history study. *Great Basin Naturalist*, **29**, 45–81.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Neigel JE, Avise JC (1986) Phylogentic relationships of mitochondrial DNA under various demographic models of speciation. In: *Evolutionary Processes and Theory* (eds Karlin S, Nevo E), pp. 515–534. Academic Press, New York.

Nixon KC, Carpenter JM (1993) On outgroups. *Cladistics*, **9**, 413–426.

Paetkau D (1999) Using genetics to identify intraspecific conservation units: a critique of current methods. *Conservation Biology*, **13**, 1507–1509.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Rand DM (1996) Neutrality tests of molecular markers and the connection between DNA polymorphism, demography, and conservation biology. *Conservation Biology*, **10**, 665–671.

Riddle BR (1995) Molecular biogeography in the pocket mice (*Perognathus* and *Chaetodipus*) and grasshopper mice (*Onychomys*): the late Cenozoic development of a North American aridlands rodent guild. *Journal of Mammalogy*, **76**, 283–301.

Rozas J, Rozas R (1999) DNASP, Version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174–175.

Ryder OA (1986) Species conservation and systematics: the dilemma of subspecies. *Trends in Ecology and Evolution*, **1**, 9–10.

Saccheri I, Kuussaari M, Kankare M *et al.* (1998) Inbreeding and extinction in a butterfly metapopulation. *Science*, **392**, 491–494.

Shaffer HB, Fellers GM, Magee A, Voss SR (2000) The genetics of amphibian declines: population substructure and molecular differentiation in the Yosemite toad *Bufo canorus* (Anura, Bufonidae) based on single-strand conformation polymorphism analysis (SSCP) and mitochondrial DNA sequence data. *Molecular Ecology*, **9**, 245–258.

Sunnucks P (2000) Efficient genetic markers for population biology. *Trends in Ecology and Evolution*, **15**, 199–203.

Svihla A (1935) Notes on the western spotted frog, *Rana pretiosa pretiosa*. *Copeia*, **1935**, 119–122.

Swofford DL (1998) *PAUP* Pylogenetic Analysis Using Parsimony (*And Other Methods), Version 4.0*. Sinauer, Sunderland, MA.

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM (1996) Phylogenetic inference. In: *Molecular Systematics*, Vol. 2 (eds Hillis DM, Moritz C, Mable BK), pp. 407–425. Sinauer, Sunderland, MA.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.

Templeton AR (1983) Convergent evolution and nonparametric inferences from restriction fragment and dna sequence data. In: *Statistical Analysis of DNA Sequence Data* (ed. Weir B), pp. 151–179. Marcel Dekker, New York.

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619–633.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659–699.

Toline CA, Seitz AM (1999) *Mitochondrial and nuclear DNA variation within and among populations of Columbia spotted frog (*Rana luteiventris*) in Utah*. Master's Thesis, Utah State University, Logan.

Turner FB (1960) Population structure and dynamics of the western spotted frog *Rana pretiosa pretiosa* Baird and Girard in Yellowstone National Park, Wyoming. *Ecological Monographs*, **30**, 251–278.

Utah Division of Wildlife Resources (1993) *Distribution of the Spotted Frog Along the Wasatch Front in Utah 1991–1992*. April 13, 1993, Salt Lake City, pp. 1–25.

Utah Division of Wildlife Resources (1994) *Distribution, Habitat Use, and Relative Abundance Indices of Spotted Frogs in the West Desert, Utah, 1993*. January 24, 1994, Salt Lake City, pp. 1–49.

Utah Division of Wildlife Resources (1998) *Conservation Agreement for Spotted Frog*. January 22, 1998, Salt Lake City, pp. 1–18.

Vogler AP, DeSalle R (1994) Diagnosing units of conservation management. *Conservation Biology*, **8**, 354–363.

Wallace DG, King M-C, Wilson AC (1973) Albumin differences among ranid frogs: taxonomic and phylogenetic implications. *Systematic Zoology*, **28**, 627–634.

Whelan S, Goldman N (1999) Distributions of statistics used for the comparison of models of sequence evolution in phylogenetics. *Molecular Biology and Evolution*, **16**, 1292–1299.

Yang Z, Goldman N, Friday A (1995) Maximum likelihood trees from DNA sequences: a peculiar estimation problem. *Systematic Biology*, **44**, 384–399.

Zamudio KR, Jones KB, Ward RH (1997) Molecular systematics of short-horned lizards: biogeography and taxonomy of a widespread species complex. *Systematic Biology*, **46**, 284–305.

David Bos is currently a PhD fellow at the University of Canterbury in Christchurch, New Zealand, where he is researching the dynamics of mating systems and the molecular evolution of the major histocompatibility complex in anurans. His major research interests include evolutionary genetics, behaviour and conservation. JW Sites Jr is interested in evolutionary genetics and conservation biology, and uses various classes of molecular markers to assess phylogenetic relationships, population structure, hybrid zone dynamics and speciation potential; virtually all of his work centres on amphibian and reptile groups in North, Central and South America.
