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Range-wide Phylogeographic Analysis of the Spotted Frog Complex (*Rana luteiventris* and *Rana pretiosa*) in Northwestern North America

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Range-wide phylogeographic analysis of the spotted frog complex (*Rana luteiventris* and *Rana pretiosa*) in northwestern North America

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ABSTRACT

The dynamic geological and climatic history of northwestern North America has made it a focal region for phylogeography. We conducted a range-wide phylogeographic analysis of the spotted frog complex (*Rana luteiventris* and *Rana pretiosa*) across its range in northwestern North America to understand its evolutionary history and the distribution of clades to inform conservation of *R. pretiosa* and Great Basin *R. luteiventris*, candidates for listing under the US Endangered Species Act. Mitochondrial DNA sequence data from a segment of the cytochrome *b* gene were obtained from 308 *R. luteiventris* and *R. pretiosa* from 96 sites. Phylogenetic analysis revealed one main *R. pretiosa* clade and three main *R. luteiventris* clades, two of which overlapped in southeastern Oregon. The three *R. luteiventris* clades were separated from each other by high levels of sequence divergence (average of 4.75–4.97%). Two divergent clades were also uncovered within the Great Basin. Low genetic variation in *R. pretiosa* and the southeastern Oregon clade of *R. luteiventris* suggests concern about their vulnerability to extinction.

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1. Introduction

A fundamental premise of phylogeography is that geological events, climatic history, and environmental heterogeneity play an important role in cladogenesis (Avise, 2000). The dramatic geological and climatic history and striking habitat diversity of northwestern North America, ranging from temperate rainforest to high desert, have made it a geographic focus of many phylogeographic studies (Brunsfeld et al., 2001; Carstens et al., 2005; Soltis et al., 1997). Since the beginning of the Pliocene (5 mya), two main events have dominated the geological and climatic history of the region. First, major uplift of the Cascade/Sierra chain (in southern British Columbia, western Washington and Oregon, and eastern California) in the Pliocene (5–2 mya) produced a rain shadow that caused xerification of the Columbia Plateau (between the Cascade and Rocky Mountain chains; Graham, 1999). This resulted in isolation of mesic coniferous forest in the Cascade Range and northern Rocky Mountains by intervening dry, steppe vegetation in the Columbia Plateau. Subsequently, Pleistocene glaciation occurring in approximately 100,000-year cycles (1.8–20,000 mya) had enormous impacts on the geographic distributions of organisms in the region (Brunsfeld et al., 2001). During these cycles, much of the region was buried under cordilleran and alpine ice for 90,000 years each cycle, splitting species' ranges into isolated refugia.

Several phylogeographic breaks have been uncovered in northwestern North America that have been attributed to Cascade uplift, isolation in habitat refugia during Pleistocene glaciation, and geographic barriers. Many species (and species complexes) exhibit a deep, east–west phylogeographic break between the coastal/Cascade region and areas to the east (*Plethodon idahoensis* and *Plethodon vandykei*, Carstens et al., 2004; *Microtus longicaudus*, Conroy and Cook, 2000; *Sorex monticolus*, Demboski and Cook, 2001; *Thamnophis sirtalis*, Janzen et al., 2002; *Ascaphus truei* and *A. montanus*, Nielson et al., 2001; *Poecile gambeli*, Spellman et al., 2007; *Salvelinus confluentus*, Spruell et al., 2003; *Dicamptodon aterrimus* and *D. copei*, Steele et al., 2005; *Phrynosoma douglasi*, Zamudio et al., 1997). The timing of this split appears to be linked to Cascade orogeny (Carstens et al., 2004, 2005). Coastal and Cascade species also show north–south breaks that have been attributed to isolation in Pleistocene refugia (Brunsfeld et al., 2001; Steele and Storfer, 2006) and by rivers (Miller et al., 2006b; Monsen and Blouin, 2003). Further to the east, a phylogeographic break has been found between the Great Basin (in southeastern Oregon, southern Idaho, Nevada, and northern Utah) and northern Rockies (to the north of the Great Basin; Swenson and Howard, 2005).

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Identifying phylogeographic breaks and the distributions of clades is not only important for understanding the effects of geographic and climatic events on diversification, but also for identifying cryptic species (Bickford et al., 2007) and evolutionary significant units (ESUs; Moritz, 1994) for conservation. An ESU "can be defined broadly as a population or group of populations that merit separate management or priority for conservation because of high distinctiveness (both genetic and ecological)" (Allendorf and Luikart, 2007). Phylogeographic approaches have been particularly important for identifying and defining species and ESUs of declining and threatened frogs in the genus *Rana* in the western US (Monsen and Blouin, 2003; Shaffer et al., 2004). The western US has experienced pronounced amphibian declines (Stuart et al., 2004), and *Rana* frogs and *Bufo* toads in particular have experienced significant declines (Corn, 1994; Drost and Fellers, 1996; Hayes and Jennings, 1986). The California red-legged frog (*R. draytonii*) is listed under the US Endangered Species Act (ESA) as threatened (USFWS, 1996) and the southern Distinct Population Segment (DPS) of the mountain yellow-legged frog (*R. muscosa*) is listed as endangered (USFWS, 1999). Moreover, six ranids are species of concern or sensitive in Oregon, four in Washington, five in British Columbia, and one in Montana (Corkran and Thoms, 1996; Werner et al., 2004).

The Oregon spotted frog (*R. pretiosa*) and the Great Basin DPS of the Columbia spotted frog (*R. luteiventris*) have experienced severe declines and are candidates for listing under the ESA (USFWS, 1993, 1997). Surveys of historically occupied sites indicate that *R. pretiosa* is extirpated from 70% to 90% of its historic range (Hayes et al., 1997; McAllister et al., 1993), and most remaining populations are small, geographically isolated, or restricted to high elevation sites (Hayes et al., 1997; C. A. Pearl, unpublished data). Causes of decline include habitat loss and modification, introduced predators, and water quality degradation (Pearl and Hayes, 2005). Great Basin *R. luteiventris* have also declined significantly in recent years (Reaser, 1997; USFWS, 2004). Surveys in 1994–1996 revealed that *R. luteiventris* has disappeared from 54% of surveyed sites in Nevada known to have populations before 1993. In Idaho, 61% of the 49 known populations have 10 or fewer frogs; in Oregon, 81% of the 16 known populations appear to support fewer than 10 frogs (USFWS, 2004). Threats to the Great Basin DPS likely include habitat loss, modification, and fragmentation; introduced predators; and emerging infectious diseases such as the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) which has been implicated in global amphibian declines (Berger et al., 1998; Pounds et al., 2006; Reaser and Pilliod, 2005; USFWS, 2004). Populations of *R. luteiventris* along the Wasatch Front and Western Desert of Utah are also of conservation concern (Reaser and Pilliod, 2005).

Previous genetic and morphological analyses of the spotted frog complex (*R. luteiventris* and *R. pretiosa*) suggest that there may be significant cryptic diversity in this widespread complex. Based on alloyzme and morphological analysis, Green et al. (1996, 1997) split *R. pretiosa* into two separate species: *R. pretiosa* and *R. luteiventris*. In this same analysis, Green et al. (1996, 1997) suggested that *R. luteiventris* may actually consist of up to four different species. Subsequently, Bos and Sites (2001) analyzed mitochondrial DNA (mtDNA) sequences to investigate phylogeographic patterns in US populations of *R. luteiventris*, focusing on populations in Utah. They found three well-supported major clades—northern, Great Basin, and Utah—as well as two smaller clades nested within the Utah clade. A limitation of this study, however, was that large portions of the range of *R. luteiventris* were not included in the analysis, including the southern Yukon Territory, British Columbia, southeastern Oregon, southwestern Idaho, and much of western Montana. Landscape genetic analysis of *R. luteiventris* in Montana and Idaho has also shown that gene flow is restricted by mountain ridges and elevation (Funk et al., 2005a). Allozyme analyses grouped a population from eastern Oregon (Anthony Lake) with the Great Basin clade (Green et al., 1997), whereas mtDNA analysis grouped a different population from eastern Oregon ("Blue Mountains") with the northern clade (Bos and Sites, 2001). Thus inclusion of additional populations from eastern Oregon and southwestern Idaho is particularly important for resolving the distribution of the Great Basin clade, especially given that the Great Basin DPS is a candidate for ESA-listing.

The goal of this study was to conduct a range-wide phylogeographic analysis of the spotted frog complex to understand its evolutionary history and uncover the distribution of phylogeographic breaks and clades to inform conservation and management. In particular, our main questions were: (1) are there any north–south genetic breaks in the range of *R. pretiosa* as seen in many other taxa in the Pacific Northwest (western Oregon and Washington)?; (2) how many *R. luteiventris* clades are there and what are their distributions (and in particular, what is the distribution of the Great Basin clade)?; and (3) is there a genetic signature of population expansion (particularly in the northern *R. luteiventris* clade, as predicted by postglacial colonization) or population decline (especially in the Great Basin *R. luteiventris* clade and *R. pretiosa* which have experienced recent declines based on field surveys)?

2. Materials and methods

2.1. Sampling for molecular analysis

We analyzed tissue samples (tail or toe clips) from 126 *Rana luteiventris* representing 44 sites and from 60 *R. pretiosa* representing 15 sites (Fig. 1; Appendix A). One to 10 individuals were sampled per locality. We also used Bos and Sites' (2001) sequence data from another 121 *R. luteiventris* from 36 sites and 1 *R. pretiosa*. Thus combined, our analyses included 247 *R. luteiventris* from 80 sites and 61 *R. pretiosa* from 16 sites. This sampling spans the entire extant range of these two species, from the southern Yukon to Nevada and from western Oregon to Wyoming (Fig. 1). We also included one *R. aurora* and one *R. cascadae* which were designated as outgroups from the closely related *R. boylii* species group (= *Amerana* clade in Hillis and Wilcox, 2005).

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from tissue samples using DNeasy Tissue Kits (Qiagen, Inc., Valencia, CA). Overlapping sets of primers were used to amplify a 902 bp segment of the mitochondrial cytochrome *b* gene using the polymerase chain reaction (PCR). Primers, PCR conditions, and sequencing protocol were described in Bos and Sites (2001). Editing and assembly of contigs was completed using BioEdit version 7.0.9.0 (Hall, 1999).

2.3. Alignment and phylogenetic analyses

Sequences were aligned manually with BioEdit so as to minimize the number of changes required across taxa. Autapomorphies were verified by examining the original chromatograms. For phylogenetic analyses, Collapse version 1.2 (D. Posada, http://darwin.uvigo.es) was used to reduce the dataset to unique haplotypes. Phylogenetic inference was based on maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. Parsimony analyses were conducted in Paup* version 4.0b10 (Swofford, 2000) using a heuristic search with 1000 random addition-sequence replicates and tree-bisection-reconnection (TBR) branch swapping. Nodal support was assessed through nonparametric bootstrap analysis of 1000 bootstrap replicates with 10 random addition-sequence replicates per bootstrap replicate.

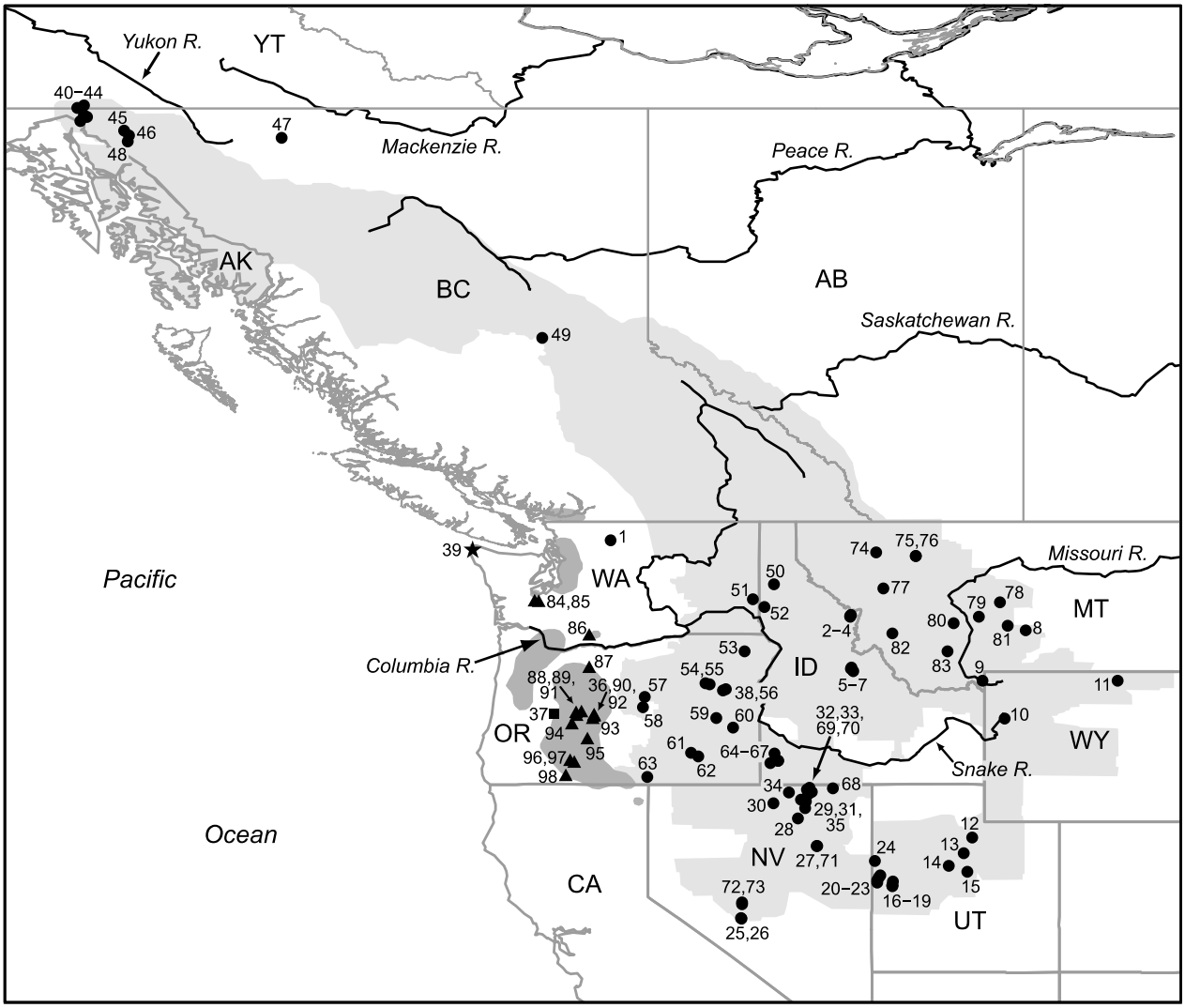

Fig. 1. Distribution of sampling sites for *Rana luteiventris* (circles and light grey shading), *R. pretiosa* (triangles and dark grey), *R. cascadae* (square; outgroup), and *R. aurora* (star; outgroup). Site numbers correspond to those in Fig. 2 and Appendices A and B. Sites 1–39 are the same as in Bos and Sites (2001). Species' ranges are from the IUCN (2006) Global Amphibian Assessment (sites 1 and 47 are *R. luteiventris*, although they are not included in the current IUCN range map for this species). Abbreviations are provided for United States and Canadian provinces where *R. luteiventris* or *R. pretiosa* are found: AB = Alberta, AK = Alaska, BC = British Columbia, CA = California, ID = Idaho, MT = Montana, NV = Nevada, OR = Oregon, UT = Utah, WA = Washington, WY = Wyoming, YT = Yukon Territory.

The most appropriate model of sequence evolution for the likelihood analysis was selected using Akaike's information criterion (AIC; Akaike, 1974) using Modeltest version 3.7 (Posada and Crandall, 1998). Likelihood analysis was then conducted in Paup* using successive iterations with starting parameters based on estimates from the previous tree, a method shown to perform well (Sullivan et al., 2005). Parameters for the first iteration were estimated from the most-parsimonious tree with the best likelihood score. Iterations were continued until successive searches yielded identical trees. Bayesian analyses were conducted in MrBayes version 3.1.1 (Ronquist and Huelsenbeck, 2003), with two runs of four Markov chains each. The chain was sampled once every 1000 generations, and each ran for two million generations. We used a conservative burn-in that was determined by examining stationarity of the likelihood scores and convergence of posterior probabilities between the two runs using the standard deviation of split frequencies.

2.4. Population genetic analyses

We used all 308 *Rana luteiventris* and *Rana. pretiosa* sequences for population genetic analyses, but did not use outgroup sequences (*R. aurora* and *R. cascadae*). All population genetic analyses were performed using Arlequin version 3.01 (Excoffier et al., 2005). Genetic variation within sites was estimated using a variety of diversity statistics, including haplotype diversity (h), number of polymorphic sites (s), and nucleotide diversity (π_n). Historic population expansion and decline were assessed using three different methods. The first method was Harpending's (1994) raggedness index of mismatch distributions. Rapid population expansion results in smooth, unimodal mismatch distributions. A smaller raggedness index indicates a smoother mismatch distribution. One thousand bootstrap replicates were used to test the probability of a raggedness index as large as observed under a null hypothesis of a sudden population expansion. The second and third methods were Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997). Negative values of D and F_s are predicted under population expansion. Positive values of D, on the other hand, indicate population decline. The significance of D and F_s were tested using 10,000 bootstrap simulations.

We also used analysis of molecular variance (AMOVA; Excoffier et al., 1992) to determine the proportion of variation explained by clades identified in the phylogenetic analysis. Only sites with more than one individual were included in this analysis (72 out of 96 *R. luteiventirs* and *R. pretiosa* sites). The five different groupings

analyzed were species (*R. luteiventris* versus *R. pretiosa*); major clades (northern, Great Basin, Utah, and *R. pretiosa*); northern clades (Blue Mountain versus the rest of the northern clade); Great Basin clades (southwestern Idaho and Nevada versus southeastern Oregon); and *R. pretiosa* clades (Columbia, southern Oregon, and the rest of *R. pretiosa*).

3. Results

3.1. Phylogenetic analyses

The final alignment was 902 bp long with 210 variable characters of which 158 were parsimony informative. A total of 62 unique haplotypes was found for the 247 *Rana luteiventris* individuals, of which 21 were new (not found by Bos and Sites, 2001). Six unique haplotypes were found for the 61 *R. pretiosa*, five of which were new.

All three phylogenetic analyses (MP, ML, and Bayesian) recovered the same four main, statistically well-supported clades: one *Rana pretiosa* clade and three *Rana luteiventris* clades (Figs. 2 and 3). The *R. pretiosa* clade was found in the currently recognized range of this species, from the southern Puget Trough (sites 84 and 85; refer to Fig. 1) to southern Oregon (site 98). The northern *R. luteiventris* clade is the largest of the three *R. luteiventris* clades, extending from the southern Yukon Territory (sites 40 and 41) to southeastern Oregon (site 61) and east to Wyoming (site 11). The

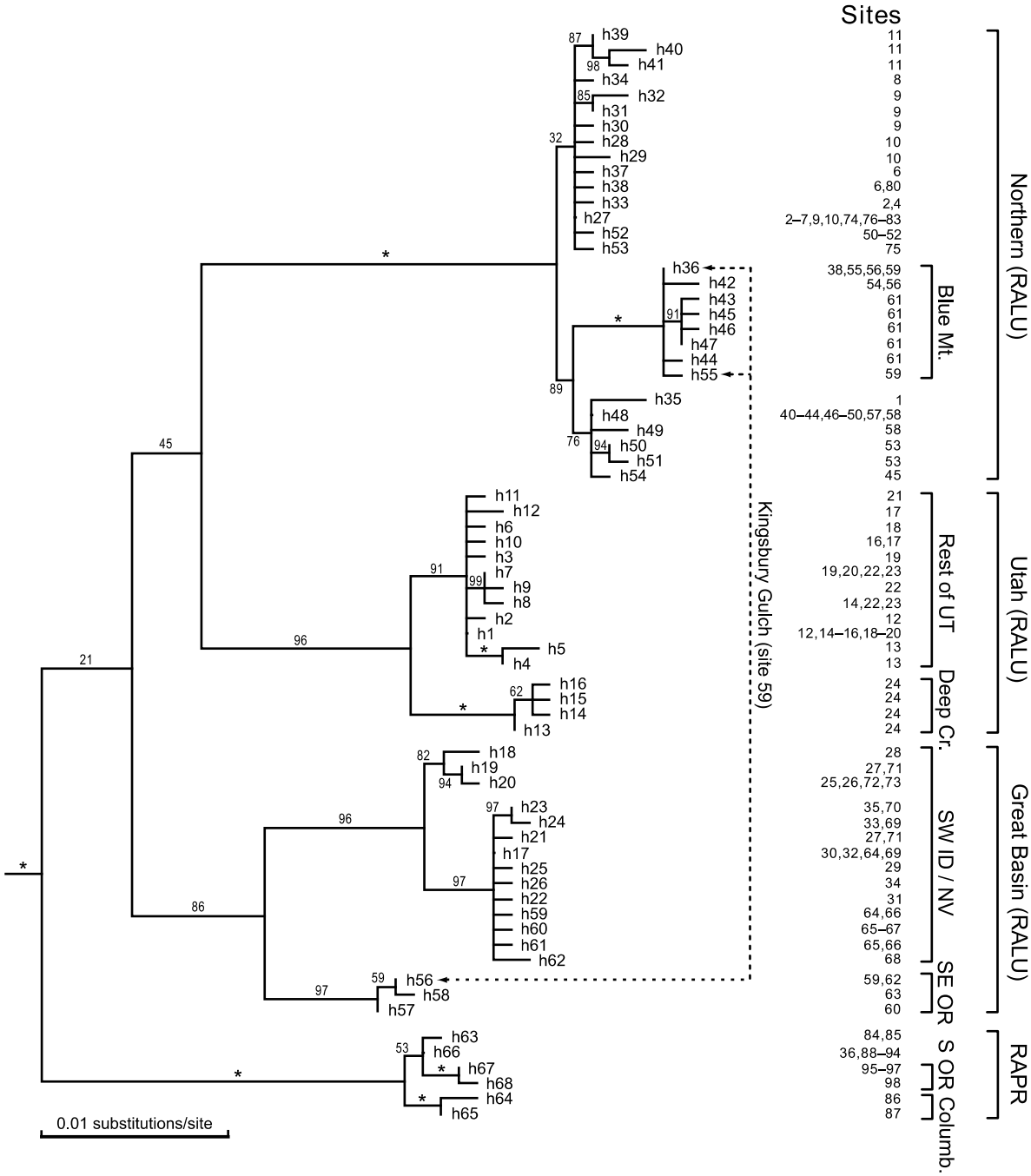

Fig. 2. Maximum likelihood topology. Numbers on branches are Bayesian posterior probabilities; asterisks indicate posterior probabilities of 100%. Site numbers correspond to those in Fig. 1 and Appendices A and B. Haplotype numbers are shown by terminal nodes and correspond to those in Appendix B. Haplotypes 1–41 are the same as in Bos and Sites (2001). Outgroup taxa are not shown.

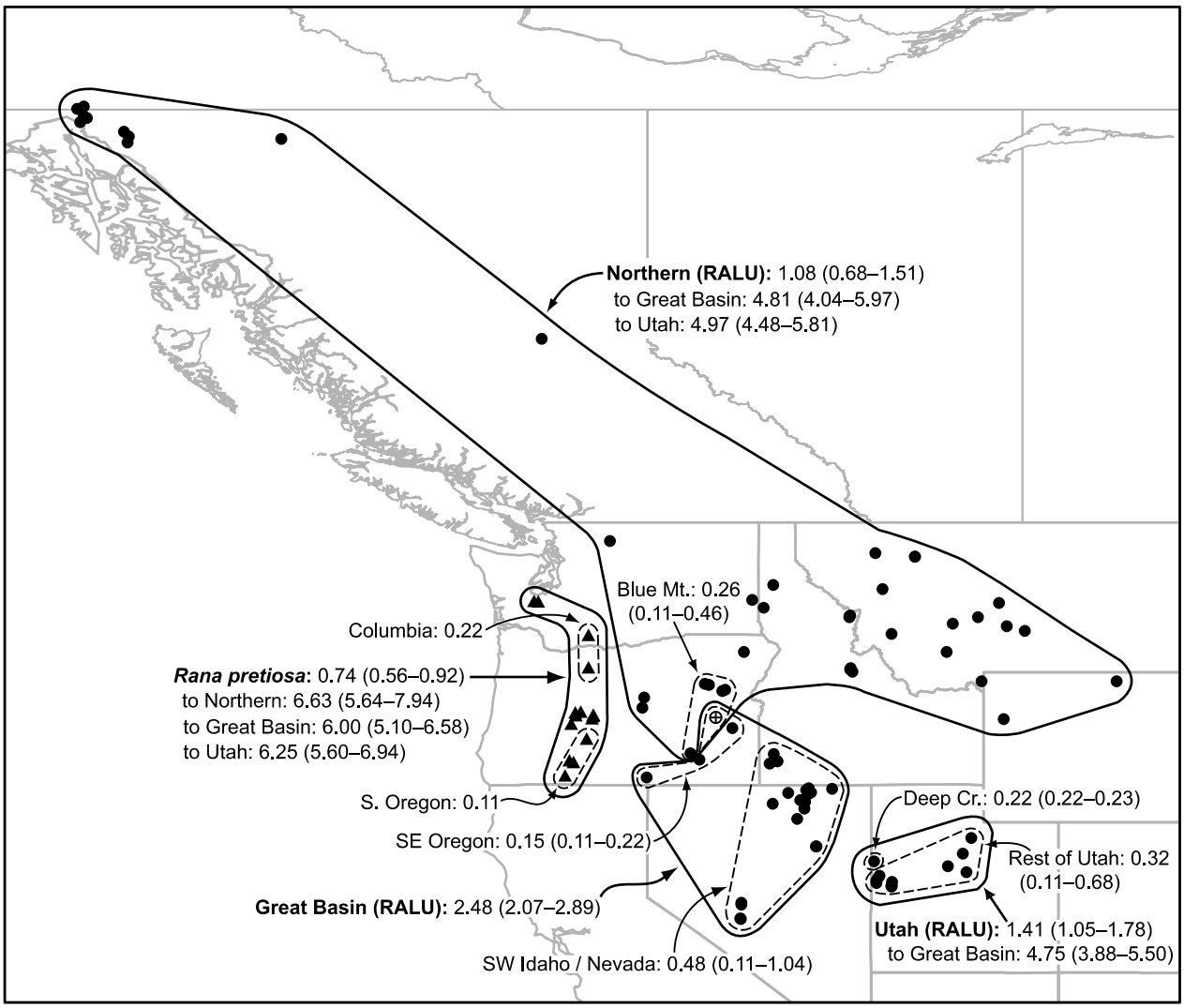

Fig. 3. Geographic distribution of major clades (solid black lines) and nested clades (dashed lines) identified in the phylogenetic analyses, with mean percent corrected sequence divergence (and ranges in parentheses) shown within and among clades. Clade names correspond to those used in Fig. 2 and Tables 1–3. Triangles = *R. pretiosa*; circles = *R. luteiventris*. The open circle with a cross is Kingsbury Gulch (site 59), where haplotypes from the northern clade and Great Basin clade were found.

Great Basin *R. luteiventris* clade abuts the northern clade in southeastern Oregon and extends south to central Nevada (sites 25 and 26). Haplotypes from the northern and Great Basin clades were found together at one site, Kingsbury Gulch (site 59), in southeastern Oregon (Figs. 2 and 3). The Utah clade is restricted to western and central Utah. Mean ML-corrected sequence divergence between *R. pretiosa* and the three *R. luteiventris* clades ranged from 6.00% to 6.63% (Fig. 3). Mean sequence divergence between the three *R. luteiventris* clades ranged from 4.75% to 4.97%.

Well-supported clades were also found within each of the four main clades described above. Within *R. pretiosa*, two well-supported clades were found: the Columbia clade consisting of sites 86 and 87 on either side of the Columbia River and the southern Oregon clade including sites 95–98 (Figs. 2 and 3). Although these clades were well-supported, mean sequence divergence between them was only 0.74%. In the northern clade, the Blue Mountain clade was found in eastern Oregon and included sites 38, 54–56, 59, and 61. Mean sequence divergence between this clade and the rest of the northern clade was 1.08%. Within the Great Basin clade, we found two divergent clades, the southeastern Oregon clade (sites 59, 60, 62, and 63) and the southwestern Idaho/Nevada clade (all other sites in the Great Basin clade), separated by a mean sequence divergence of 2.48%. In the Utah clade, two clades were also recovered, the Deep Creek clade (site 24) and another clade consisting of all other sites in Utah, separated by a mean sequence divergence of 1.41%.

The monophyly of *R. luteiventris*, however, was poorly supported (Bayesian posterior probability, BPP = 21%; Fig. 2). Other phylogenetic arrangements of the four main clades with similar (low) levels of support included ((Great Basin + RAPR), (Utah + Northern)) with BPP = 38%; ((Northern + Utah + RAPR), Great Basin) with BPP = 30%; ((Northern + Great Basin + RAPR), Utah) with BPP = 23%; and ((Utah + Great Basin + RAPR), Northern) with BPP = 18%. Because none of these arrangements was well-supported, we show *R. luteiventris* to be monophyletic (Fig. 2).

3.2. Population genetic analyses

Population genetic analyses revealed substantial variation among clades in the level of within population genetic variation as measured by haplotype and nucleotide diversity (Table 1). For the four main clades, haplotype and nucleotide diversity were lowest for *R. pretiosa* and highest for the Great Basin. For the smaller clades nested within the main clades, haplotype and nucleotide

Table 1
Mitochondrial DNA diversity statistics for *Rana luteiventris* and *R. pretiosa* by clade

Clade	No. of sites	No. of individs.	h	s	π_n
Northern (RALU)	43	114	0.81	41	0.0041 ± 0.0023
Blue Mt.	6	17	0.86	8	0.0021 ± 0.0014
Utah (RALU)	13	66	0.86	27	0.0044 ± 0.0025
Deep Cr.	1	10	0.73	4	0.0016 ± 0.0012
Rest of Utah	12	56	0.82	14	0.0017 ± 0.0012
Great Basin (RALU)	25	67	0.93	33	0.0106 ± 0.0055
SW Idaho/Nevada	21	52	0.91	20	0.0048 ± 0.0027
Southeastern Oregon	4	15	0.64	2	0.0008 ± 0.0007
Rana pretiosa	16	61	0.73	9	0.0023 ± 0.0015
Columbia	2	8	0.57	2	0.0013 ± 0.0010
Southern Oregon	4	18	0.29	1	0.0003 ± 0.0004

Clades correspond to those defined in Figs. 2 and 3. RALU is an abbreviation for *Rana luteiventris*; h is haplotype diversity; s is the number of polymorphic sites; and π_n is nucleotide diversity.

diversity were very low for southeastern Oregon (nested within the Great Basin) and the Columbia and southern Oregon clades (nested within *R. pretiosa*).

Tests of population expansion revealed a consistent signature of expansion only in the northern *R. luteiventris* clade (Table 2). In this clade, results of all three tests (raggedness of mismatch distributions, Tajima's D, and Fu's F_s) were consistent with the predictions of an expanding population. Specifically, the mismatch distribution was smooth as indicated by a low raggedness value (0.031) and a large probability of observing a raggedness value this large or larger under the null hypothesis of expansion ($P = 0.708$); Tajima's D was negative ($D = -1.61$) and significant ($P = 0.025$); and Fu's F_s was large ($F_s = -26.02$) and significant ($P < 0.0001$). Tajima's D was not significant in the other three clades. In fact, in two clades, the Great Basin and *R. pretiosa* clades, Tajima's D was positive, consistent with population decline rather than expansion.

Results of the AMOVAs are summarized in Table 3. Of the two grouping methods that included all *R. luteiventris* and *R. pretiosa* sites, 87.8% of the variation was ascribed to differences among major clades compared to 58.8% explained by currently recognized species. A large percentage of the variation was also accounted for by differences among the smaller, nested clades, ranging from 73.9% in the northern clade, to 80.7% in the Great Basin, to 83.8% in *R. pretiosa*.

4. Discussion

4.1. Phylogeographic breaks

Our phylogenetic and AMOVA analyses support three main *Rana luteiventris* clades plus one main *R. pretiosa* clade (Figs. 2 and 3; Table 3). These clades form four primary phylogenetic breaks between: (1) the Cascade Range (*R. pretiosa*) and inland *R. luteiventris* clades; (2) northern and Utah clades; (3) northern and Great Basin clades; and (4) Great Basin and Utah clades. The first three of these correspond with previously documented phylogeographic breaks or contact zones, but we are unaware of other examples of a major break between Utah and Nevada.

Table 3
Analysis of molecular variance (AMOVA) results for *Rana luteiventris* and *R. pretiosa*

Groups	No. of groups	Variance components	% of variation	*P*-value
Species	2	Among groups	58.75	<0.0001
		Among sites	40.06	<0.0001
		Within sites	1.20	<0.0001
Major clades	4	Among groups	87.83	<0.0001
		Among sites	10.65	<0.0001
		Within sites	1.52	<0.0001
Northern clades	2	Among groups	73.94	<0.0001
		Among sites	16.94	<0.0001
		Within sites	9.13	<0.0001
Great Basin clades	2	Among groups	80.68	0.0025
		Among sites	15.17	<0.0001
		Within sites	4.14	<0.0001
Rana pretiosa clades	3	Among groups	83.76	<0.0001
		Among sites	16.24	<0.0001
		Within sites	0.00	<0.0001

Groupings are species (*Rana luteiventris* versus *R. pretiosa*); major clades (Northern, Great Basin, Utah, and *R. pretiosa*); northern clades (Blue Mt. versus the rest of the northern clade); Great Basin clades (southwestern Idaho and Nevada versus southeastern Oregon); and *Rana pretiosa* clades (Columbia, southern Oregon, and the rest of *R. pretiosa*). *P*-values were calculated using 10,000 simulations.

The deepest split in the spotted frog complex phylogeny is between *R. pretiosa* in the Cascade Range (and lower Puget Trough) and the interior *R. luteiventris* clades. This Cascade Range/coastal vs. inland phylogenetic break has been a focus of attention in species associated with mesic, coniferous forests in disjunct populations in the coastal Pacific Northwest (primarily in western Washington, western Oregon, and northwestern California) and the inland northwest (in northern Idaho, northwestern Montana, and southeastern British Columbia; Brunsfeld et al., 2001; Carstens et al., 2004, 2005; Nielson et al., 2001). *Rana pretiosa* and *R. luteiventris*, however, are not mesic forest species, but instead inhabit lentic water bodies and streams embedded in a variety of terrestrial habitat types ranging from shrub-steppe to subalpine forest to mixed coniferous forests (Reaser and Pilliod, 2005). There are also several other species not tied to mesic forests that exhibit a deep phylogenetic break between the Cascade Range/coast and inland regions, including Pacific chorus frogs (*Pseudacris regilla* complex; Recuero et al., 2006), common garter snakes (*Thamnophis sirtalis*; Janzen et al., 2002), short-horned lizards (*Phrynosoma douglasi*; Zamudio et al., 1997), bull trout (*Salvelinus confluentus*; Spruell et al., 2003), mountain whitefish (*Prosopium williamsoni*; Whiteley et al., 2006), and mountain chickadees (*Poecile gambeli*; Spellman et al., 2007). Thus a Cascade/coastal vs. inland break appears to be the rule for most species (or species complexes), mesic forest or not, although there are some species in which this split is not found (water vole,

Table 2
Results of tests of historical population expansion for *Rana luteiventris* and *R. pretiosa*

Clade	n	Raggedness	P(Raggedness)	Tajima's D	P(Tajima's D)	Fu's F_s	P (Fu's F_s)
Northern (RALU)	114	0.031	0.708	−1.61	0.025	−26.02	<0.0001
Utah (RALU)	66	0.038	0.322	−0.93	0.193	−25.91	<0.0001
Great Basin (RALU)	67	0.036	0.165	1.25	0.914	−24.63	<0.0001
Rana pretiosa	61	0.057	0.560	0.27	0.656	−27.12	<0.0001

Clades correspond to those defined in Figs. 2 and 3. RALU is an abbreviation for *Rana luteiventris*; n is the number of individuals. Raggedness values are measures of the smoothness of mismatch distributions, with lower raggedness values indicating smoother distributions. Smooth Poisson mismatch distributions are characteristic of rapid population expansion. P (Raggedness) is the probability of observing a distribution with higher raggedness under a null hypothesis of population expansion based on 1000 bootstrap replicates. Negative Tajima's D and Fu's F_s values also indicate population expansion. P (Tajima's D) and P (Fu's F_s) were calculated using 10,000 simulations.

Microtus richardsoni; dusky willow, *Salix melanopsis*; whitebark pine, *Pinus albicaulis*; Carstens et al., 2005).

It is not particularly surprising that disjunct, mesic forest species in the Pacific Northwest and Inland Northwest have diverged in allopatry after uplift of the Cascade Mountains and formation of the dry, Columbia Plateau in the Pliocene. The question remains, however, as to why more broadly distributed species not restricted to mesic forests, such as *R. luteiventris* and *R. pretiosa*, have diverged along this same east–west axis. There are several potential explanations for divergence of *R. pretiosa* from *R. luteiventris* clades to the east. One possible reason for this split is that the Cascade Mountains are a barrier to gene flow, causing allopatric speciation. Another possibility is that xerification of the Columbia Plateau east of the Cascade Range resulted in divergent selection pressures, causing parapatric, ecological speciation in the face of ongoing gene flow (Endler, 1977). Lastly, this mtDNA break may have occurred without any barrier to gene flow (e.g., Irwin, 2002), although this seems unlikely since many different species have a similar phylogeographic break in this same area.

Two of the other four phylogeographic breaks observed in this study also match previously observed breaks or contact zones. In particular, the break between the Utah and northern clades corresponds to one of the most significant phylogeographic breaks in North America (Swenson and Howard, 2005). Swenson and Howard's (2005) analysis of phylogeographic breaks did not include aquatic species, but the observation that this same break is seen in *R. luteiventris*, a highly aquatic frog, suggests that this break may also hold for aquatic species (although *R. luteiventris* can travel substantial distances overland; Pilliod et al., 2002). In addition, the break between the northern and Great Basin clades in *R. luteiventris* corresponds closely with one of Remington's (1968) "suture zones" (spatial clusters of hybrid-zones) which passes through southeastern Oregon. This area, however, was not identified by Swenson and Howard (2005) as a hotspot for phylogeographic breaks.

Within the four main clades, we also found support for several significant smaller clades ("nested clades"; Figs. 2 and 3). The most divergent nested clades were found within the Great Basin: the southeastern Oregon and the southwestern Idaho/Nevada clades. Mean sequence divergence between these two clades was 2.48%, much higher than between any other nested clades. Within the northern clade, we also found the Blue Mountain clade in eastern Oregon with a mean sequence divergence of 1.08% from other northern clade haplotypes. Finally, within *R. pretiosa*, we found two nested clades, the Columbia and southern Oregon clades. Although these two clades were well-supported, mean sequence divergence between them was only 0.74%, suggesting relatively recent divergence. These clades formed north–south phylogeographic breaks (albeit shallow breaks) as seen in many other species in the Cascade Range (Miller et al., 2005, 2006a; Nielson et al., 2006; Steele and Storfer, 2006), although exact locations of these breaks vary. Interestingly, the Columbia River does not act as a barrier in *R. pretiosa*. In fact, the Columbia clade crosses the Columbia River and includes one population from Washington on the north side and one from Oregon on the south side (Fig. 3). The effect of the Columbia River as a barrier appears to vary among species. In some, it corresponds with a genetic break (Monsen and Blouin, 2003), but in many it does not (Funk et al., 2008; Nielson et al., 2006; Recuero et al., 2006).

Although our analyses supported four main clades, the monophyly of *R. luteiventris* was not well-supported (BPP = 21%). Five other phylogenetic arrangements of the four main clades had similar, low levels of support (BPP = 18–38%). Resolving the relationships of these clades will require additional sequence data, ideally from multiple nuclear genes.

4.2. Overlap of clades in southeastern Oregon

In southeastern Oregon, the northern and Great Basin clades overlap in Kingsbury Gulch (site 59; Figs. 2 and 3). Also further to the southwest, these two clades are adjacent to each other, separated by only 19 km between Mud Creek (site 61) in the northern clade and Lily Lake (site 62) in the Great Basin clade. In Kingsbury Gulch, two out of ten individuals had northern haplotypes (h36 and h55; Fig. 2) and the remaining eight individuals all had the same Great Basin haplotype (h56). Kingsbury Gulch is an isolated series of small ponds and pools connected by an ephemeral stream situated in dry, shrub-steppe habitat.

It is not possible to determine from the mtDNA data alone whether frogs in Kingsbury Gulch with Great Basin versus northern haplotypes interbreed freely and produce viable offspring, or whether they are reproductively isolated and therefore distinct species. Mean sequence divergence between these two clades is 4.81%, only 1.2% lower than between *R. pretiosa* and Great Basin *R. luteiventris* (6.00%; Fig. 3). Thus it is possible that the Great Basin and northern clades (and perhaps the Utah clade as well) are also different species. Fortunately, the presence of frogs with northern and Great Basin haplotypes at the same site provides an excellent opportunity to test the hypothesis that these two clades represent reproductively isolated species using nuclear markers such as microsatellite loci. If frogs with different haplotypes form distinct genetic groups at nuclear loci, it would provide strong evidence that they are different species. In contrast, if they do not form different genetic groups, then they should be considered members of the same species. Assessment of the taxonomic status of these different clades should also include phenotypic data on their morphology, calls, ecology, and natural history.

4.3. Population expansion and declines

Some evidence was found for population expansion in all four main clades, but only in the northern *R. luteiventris* clade did all three tests consistently indicate expansion (Table 2). Moreover, in the northern clade, one haplotype (h48) was found over a huge area: central Oregon (sites 57 and 58), northern Idaho (50), central British Columbia (49), and extreme northwestern British Columbia and the southern Yukon Territory (sites 40–44, 46–48; Figs. 1 and 2). These observations suggest a recent and rapid population expansion of the northern clade, likely following Pleistocene glacial recession. Similar patterns have been recovered in other phylogeographic studies in the Northwest (Carstens et al., 2004; Matocq, 2002; Spinks and Shaffer, 2005; Steele and Storfer, 2006).

In two clades, the Great Basin clade and *R. pretiosa*, Tajima's *D* was positive which is consistent with population declines, although these values were not statistically significant. In the Great Basin, however, the probability of a *D* value as large as observed by chance was only 0.086 (calculated by subtracting the *P*-value shown in Table 2 which is the probability of a *D* value as small as observed by chance from one). Even though not statistically significant, these positive Tajima's *D* values are of concern given that field surveys have shown severe declines both in Great Basin *R. luteiventris* (Reaser, 1997) and *R. pretiosa* (Hayes et al., 1997; McAllister et al., 1993). Testing for bottlenecks with nuclear markers, larger sample sizes, and more sophisticated bottleneck tests (e.g., program Bottleneck; Piry et al., 1999) will provide a better

understanding of the severity and significance of bottlenecks in these clades.

4.4. Conservation implications

Our results have important implications for *R. pretiosa* and *R. luteiventris* conservation and management. First, by sampling extensively (96 sites) throughout the range of *R. pretiosa* and *R. luteiventris*, we were able to clearly define the boundaries of the four main clades. In particular, the range of the Great Basin clade, a group that is currently a candidate for listing under the US Endangered Species Act (ESA), was previously in question. Based on our analysis, this clade includes populations in Nevada, southwestern Idaho, and southeastern Oregon. Moreover, the high levels of sequence divergence among the *R. luteiventris* clades suggest that they may represent different species, but as explained above, additional genetic (in particular, nuclear markers) and phenotypic data are needed to test this hypothesis.

We also found two well-supported and highly divergent clades within the Great Basin clade, a southeastern Oregon clade and a separate southwestern Idaho/Nevada clade. The southeastern Oregon clade consists of only four known populations: Parsnip Creek (site 63), Lily Lake (62), Dry Creek (60), and Kingsbury Gulch (59). All of these populations appear to be small and highly isolated, separated from each other by 46–236 km straight-line distance, well beyond the maximum known dispersal distance for *R. luteiventris* of 5.8 km (Funk et al., 2005b). Monitoring at Kingsbury Gulch revealed a recent population decline from 211 estimated frogs in 2003 to 18 frogs in 2007 (M. J. Adams, unpublished data). Population estimates at Dry Creek have ranged between 62 and 255 from 2001 to 2006 (J. C. Engle, pers. comm.). No formal surveys have been conducted at Lily Lake or Parsnip Creek, but during sampling in the summer of 2006, only one adult frog was found at Lily Lake and only recently metamorphosed frogs (no adults) were found at Parsnip Creek (W. C. Funk, unpublished data). Given apparently small sizes of populations in the southeastern Oregon clade and their isolation, this very distinct clade (which may represent an incipient species) appears to be highly vulnerable to extinction.

Our population genetic analyses also revealed low levels of within population genetic variation in the southeastern Oregon *R. luteiventris* clade and in *R. pretiosa* (Table 1). Only three haplotypes were found in the southeastern Oregon clade, and only 6 were found across the entire range of *R. pretiosa*. Low genetic variation in these clades likely reflects small effective population sizes, historic or current genetic bottlenecks, and/or low among population gene flow, all of which can reduce population viability via negative inbreeding effects (Crow and Kimura, 1970) and loss of adaptive genetic variation (Bürger and Lynch, 1995). Although loss of genetic variation at nuclear loci may be more likely to reduce fitness, low genetic variation in the mitochondrial genome should mirror low levels of nuclear genetic variation.

5. Conclusions

This study represents one of the largest phylogeographic studies (both in terms of numbers of sites and individuals) for northwestern North America, a focal region of interest in phylogeography. We found one well-supported *Rana pretiosa* clade and three highly divergent *R. luteiventris* clades that represent distinct evolutionary significant units at the very least, but possibly different species. Within the *R. luteiventris* Great Basin clade, we also found two well supported, divergent clades, the southeastern Oregon clade and the southwestern Idaho / Nevada clade, which have not previously been reported. In addition, two *R. luteiventris* clades, the Great Basin and northern clades, overlap in southeastern Oregon. Future genetic analysis using nuclear markers and phenotypic data will be essential for determining whether these clades are different species. Landscape genetic analysis will also be important for understanding demographic history, connectivity, and current population trends of small and declining populations of *R. pretiosa* throughout its range and *R. luteiventris*, particularly in the Great Basin.

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Appendix A

Site information and coordinates for samples used in phylogeographic analyses of *Rana luteiventris* and *R. pretiosa*

State/province	Site no.	Site name/ description	No.	Species	UTM coordinates		
					Zone	Easting	Northing
British Columbia	42	Pond between Bare Loon Lake and Bennett Lake	1	RALU	8	499063	6630791
	43	Main Pond, Log Cabin	1	RALU	8	503747	6625224
	44	Summit Creek wetlands, White Pass	1	RALU	8	493425	6614092
	45	Pond on Torres Channel, Atlin Lake	1	RALU	8	559624	6584856
	46	Pond on east shore of Atlin Lake	1	RALU	8	567424	6571989
	47	Pond near Lang Lake	1	RALU	9	457181	6564215
	48	Pond on Sloko Inlet, Atlin Lake	1	RALU	8	565847	6553406
	49	Pond 1 FFTW	2	RALU	10	511626	5970665

(*continued on next page*)

Appendix A (*continued*)

State/province	Site no.	Site name/ description	No.	Species	UTM coordinates		
					Zone	Easting	Northing
Idaho	2	N Short Creek	2	RALU	11	684103	5154039
	3	S Walton Lake	5	RALU	11	682934	5150289
	4	Grouse Lake	4	RALU	11	682990	5148445
	5	In and Out Lake	1	RALU	11	694363	4987549
	6	Cache Lake	4	RALU	11	688888	4994789
	7	Fawn Lake	3	RALU	11	690091	4998539
	50	Benewah County	2	RALU	11	526874	5241926
	52	Latah County	2	RALU	11	507972	5174736
	64	Meadow Creek	3	RALU	11	530479	4741610
	65	Sam Noble Springs A	3	RALU	11	538610	4719289
	66	Sam Noble Springs B	3	RALU	11	538685	4719055
	67	Stoneman	5	RALU	11	521500	4713163
Montana	8	Sweetgrass River	1	RALU	12	581160	5105476
	9	Yellowstone National Park	4	RALU	12	492084	4954967
	74	Blackfoot Lake, 1 mile S of Tongue Mountain	4	RALU	12	282954	5340530
	75	East Front B	1	RALU	12	360238	5326462
	76	East Front A	1	RALU	12	360476	5326225
	77	Pond, 5.8 miles NW of Fawn Peak	3	RALU	12	293121	5233861
	78	Little Belts	5	RALU	12	527967	5188993
	79	Big Belts	4	RALU	12	484870	5145610
	80	Elkhorns	5	RALU	12	434055	5126965
	81	Crazys	3	RALU	12	544668	5118856
	82	4.9 miles WNW of Beaverhead Mountain	3	RALU	12	307138	5099978
	83	Bow Basin	2	RALU	12	419805	5043783
Nevada	25	Farrington Ranch	3	RALU	11	456350	4251962
	26	Upper Corral Pond	1	RALU	11	454902	4253812
	27	Green Mountain Creek	2	RALU	11	627358	4469328
	28	Maggie Creek	1	RALU	11	583977	4550128
	29	Sheep Creek Springs	1	RALU	11	598967	4579929
	30	Chicken Creek	1	RALU	11	527819	4594105
	31	N Fork Humbolt	1	RALU	11	600082	4600298
	32	Telephone Creek	1	RALU	11	607807	4641131
	33	Sand Creek	1	RALU	11	602356	4635498
	34	Winter Creek Pond	1	RALU	11	562325	4625775
	35	Electric Fence Pond	1	RALU	11	590302	4603866
	68	Pole Creek Big Pond	5	RALU	11	659485	4640193
	69	Tennessee Gulch	4	RALU	11	612846	4628119
	70	Coleman Creek Ponds	4	RALU	11	605337	4620405
	71	South Fork Green Mountain Creek	5	RALU	11	627741	4467650
	72	Warners	3	RALU	11	456847	4300828
	73	Pasture A transect	3	RALU	11	456694	4294723
Oregon	36	Sun River	1	RAPR	10	625894	4858045
	37	Waldo Lake	1	RACA	10	540156	4860672
	38	Blue Mountains	1	RALU	11	420500	4927679
	53	Janet's Pond	4	RALU	11	466176	5042676
	54	Little Greenhorn	2	RALU	11	383862	4948877
	55	N Fork Burnt River	2	RALU	11	392792	4946872
	56	Pine Creek Pond	3	RALU	11	427045	4932116
	57	North Fork Crooked River	2	RALU	10	732898	4911477
	58	Camp Creek	3	RALU	10	729715	4882105
	59	Kingsbury Gulch	10	RALU	11	405774	4847084
	60	Dry Creek	3	RALU	11	440995	4816975
	61	Mud Creek	7	RALU	11	348319	4743846
	62	Lily Lake	1	RALU	11	363840	4733677
	63	Parsnip Creek	3	RALU	10	745866	4676573
	87	Camas	4	RAPR	10	613043	4999106
	88	Hosmer Lake	6	RAPR	10	597653	4868236
	89	Unnamed Marsh, Mud Lake	3	RAPR	10	586790	4865165

Appendix A (*continued*)

State/province	Site no.	Site name/ description	No.	Species	UTM coordinates		
					Zone	Easting	Northing
	90	Lake Aspen	3	RAPR	10	624825	4859984
	91	Muskrat Lake	2	RAPR	10	588352	4857034
	92	CRBF	3	RAPR	10	624846	4856193
	93	Casey Tract North	6	RAPR	10	622965	4847791
	94	Gold Lake Pond	3	RAPR	10	577652	4832065
	95	Jack Creek	3	RAPR	10	612882	4787487
	96	Crane Creek	8	RAPR	10	575443	4723125
	97	Wood	4	RAPR	10	584663	4718912
	98	Buck	3	RAPR	10	566192	4679710
Utah	12	Heber Provo River	5	RALU	12	468966	4492355
	13	Springville Hatchery	4	RALU	12	450330	4446212
	14	Mona	4	RALU	12	415859	4409508
	15	Sanpete County	3	RALU	12	457112	4390675
	16	S Tule Valley	6	RALU	12	284367	4350987
	17	N Tule Valley	4	RALU	12	284470	4354694
	18	Tule Valley	5	RALU	12	284418	4352840
	19	Coyote Springs	4	RALU	12	286215	4365754
	20	Bishop-Foote	5	RALU	12	250217	4363117
	21	Gandy	5	RALU	12	250455	4370521
	22	Leland-Harris	6	RALU	12	255051	4379631
	23	Miller Springs	5	RALU	12	258087	4385091
	24	Deep Creek Mountains	10	RALU	12	248040	4428020
Washington	1	North Cascades National Park	3	RALU	10	647745	5373588
	39	Olympic National Park	1	RAAU	10	376312	5347067
	51	Eden Valley	2	RALU	11	484769	5197827
	84	Kiser Prop	4	RAPR	10	498451	5195473
	85	Beaver Creek	4	RAPR	10	507241	5193011
	86	Trout Lake	4	RAPR	10	611349	5096231
Wyoming	10	Teton National Park	5	RALU	12	540256	4844012
	11	Bighorn Mountains	8	RALU	13	302100	4958003
Yukon Territory	40	Birch Pond on N shore, W Arm, Bennett Lake	1	RALU	8	499071	6660482
	41	Pond on Partridge River tributary	1	RALU	8	488850	6653081

No. is the number of individuals included in the analysis from the given site; RALU = *Rana luteiventris*; RAPR = *R. pretiosa*; RAAU = *R. aurora*; RACA = *R. cascadae*. The map datum was NAD27 for all coordinates except sites 49–52, 65, 72, and 73 for which it was NAD83. Site numbers correspond to those used in Figs. 1 and 2.

Appendix B

Haplotypes, sites where observed, number of individuals with each haplotype, and GenBank accession numbers for *Rana luteiventris*, *R. pretiosa*, and outgroups

Haplotype	Species	Sites (no. individs. with haplotype)	Accession no.
h1	RALU	12(3), 14 (2), 15(3), 16(3), 18(4), 19(2), 20,(4)	AY016650
h2	RALU	12(2)	AY016680
h3	RALU	19(1)	AY016684
h4	RALU	13(3)	AY016663
h5	RALU	13(1)	AY016655
h6	RALU	18(1)	AY016668
h7	RALU	19(1), 20(1), 22(4), 23(1)	AY016653
h8	RALU	14(2), 22(1), 23(4)	AY016656
h9	RALU	22(1)	AY016667
h10	RALU	16(3), 17(3)	AY016666
h11	RALU	21(5)	AY016662
h12	RALU	17(1)	AY016689
h13	RALU	24(5)	AY016654
h14	RALU	24(2)	AY016652

(*continued on next page*)

Appendix B (*continued*)

Haplotype	Species	Sites (no. individs. with haplotype)	Accession no.
h15	RALU	24(2)	AY016649
h16	RALU	24(1)	AY016673
h17	RALU	30(1), 32(1), 64(1), 69(2)	AY016683
h18	RALU	28(1)	AY016688
h19	RALU	27(1), 71(3)	AY016675
h20	RALU	25(3), 26(1), 72(3), 73(3)	AY016674
h21	RALU	27(1), 71(2)	AY016682
h22	RALU	31(1)	AY016671
h23	RALU	35(1), 70(4)	AY016685
h24	RALU	33(1), 69(2)	AY016677
h25	RALU	29(1)	AY016679
h26	RALU	34(1)	AY016678
h27	RALU	2(1), 3(5), 4(3), 5(1), 6(1), 7(3), 9(1), 10(3), 74(4), 76(1), 77(3), 78(5), 79(4), 80(3), 81,(3), 82(3), 83(2)	AY016658
h28	RALU	10(1)	AY016687
h29	RALU	10(1)	AY016676
h30	RALU	9(1)	AY016661
h31	RALU	9(1)	AY016669
h32	RALU	9(1)	AY016664
h33	RALU	2(1), 4(1)	AY016659
h34	RALU	8(1)	AY016672
h35	RALU	1(3)	AY016660
h36	RALU	38(1), 55(2), 56(1), 59(1)	AY016670
h37	RALU	6(1)	AY016665
h38	RALU	6(2), 80(2)	AY016686
h39	RALU	11(6)	AY016651
h40	RALU	11(1)	AY016681
h41	RALU	11(1)	AY016657
h42	RALU	54(2), 56(2)	EU708851
h43	RALU	61(1)	EU708852
h44	RALU	61(3)	EU708853
h45	RALU	61(1)	EU708854
h46	RALU	61(1)	EU708855
h47	RALU	61(1)	EU708856
h48	RALU	40(1), 41(1), 42(1), 43(1), 44(1), 46(1), 47,(1), 48(1), 49(2), 50(1), 57(2), 58(2)	EU708857
h49	RALU	58(1)	EU708858
h50	RALU	53(2)	EU708859
h51	RALU	53(2)	EU708860
h52	RALU	50(1), 51(2), 52(2)	EU708861
h53	RALU	75(1)	EU708862
h54	RALU	45(1)	EU708863
h55	RALU	59(1)	EU708864
h56	RALU	59(8), 62(1)	EU708865
h57	RALU	60(3)	EU708866
h58	RALU	63(3)	EU708867
h59	RALU	64(2), 66(1)	EU708868
h60	RALU	65(2), 66(1), 67(5)	EU708869
h61	RALU	65(1), 66(1)	EU708870
h62	RALU	68(5)	EU708871
h63	RAPR	84(4), 85(4)	EU708872
h64	RAPR	86(4)	EU708873
h65	RAPR	87(4)	EU708874
h66	RAPR	36(1), 88(6), 89(3), 90(3), 91(2), 92(3), 93,(6), 94(3)	EU708875
h67	RAPR	95(3), 96(8), 97(4)	EU708876
h68	RAPR	98(3)	EU708877
h69	RACA	37(1)	EU708878
h70	RAAU	39(1)	EU708879

RALU = *Rana luteiventris*; RAPR = *R. pretiosa*; RAAU = *R. aurora*; RACA = *R. cascadae*. Haplotype numbers correspond to those used in Fig. 2. Haplotypes 1–41 are the same as in Bos and Sites (2001).

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