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ORIGINAL ARTICLE

A test of the vicariance hypothesis of western North American freshwater biogeography

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ABSTRACT

Aim The biogeography of western North American freshwater molluscs has traditionally been attributed to vicariance associated with late Tertiary rearrangement of landscape based on distributional evidence and the putatively limited dispersal ability of these organisms. We examined the phylogeography of a widely ranging western springsnail (*Pyrgulopsis wongi* Hershler, 1989) to test this hypothesis and evaluate the relative importance of vicariance and dispersal in structuring the distribution of this species.

Location Southwestern Great Basin (California and Nevada), United States of America.

Method Two mitochondrial genes (COI, NDI) were sequenced for 28 populations of *P. wongi* spanning its entire geographic range, which consists of 10 topographically closed drainage basins. We also sequenced eight closely related congeners, as well as the type species of the closely related eastern North American genus *Floridobia* Hershler & Thompson, 2002, which was used as the outgroup. Phylogenies based on the combined data set were obtained using several methods, and networks for each gene were generated as an additional means of examining relationships among haplotypes. Partitioning of haplotype variation was studied using AMOVA, migration between populations was estimated using a coalescent-based method (MDIV), and divergence times were inferred using a locally calibrated molecular clock and MDIV.

Results *Pyrgulopsis wongi* is subdivided into narrowly localized and widely distributed lineages that diverged in the Pleistocene, well after the inception of the contemporary regional landscape. While large Φ_{ST} values and the localized geographic distributions of most haplotypes imply absence or negligible contemporary dispersal of this spring-dwelling snail, the pattern of phylogeographic structuring, presence of a few widespread haplotypes, and results of the MDIV analyses suggest geologically recent dispersal across drainage divides.

Main conclusions Phylogeography of *P. wongi* conflicts with the traditional vicariance model as it is not structured by the contemporary landscape and is instead indicative of geologically recent dispersal. In the absence of evidence that dispersal of this species occurred through surface water connections during the relevant (Quaternary) time frame, we conjecture that spread may have instead been mediated by transport on waterfowl or via upland stream capture. The non-concordance between phylogeography and landscape reported in this and other recent studies of *Pyrgulopsis* suggests that members of this diverse and imperiled genus should not be managed using an *a priori*, 'watershed as conservation unit' approach.

Keywords
Conservation biogeography, dispersal, Gastropoda, Great Basin, mtDNA, phylogeography, springsnails, vicariance, western North America.

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INTRODUCTION

The evolutionary history of western North America biota has been influenced by a potentially broad spectrum of events ranging from Neogene rearrangement of topography (Christiansen & Yeats, 1992) to late Quaternary climate changes (Porter, 1983). The relative contribution of these aspects of physical history to the structuring of regional biota has long been a compelling focus of inquiry (e.g. Harper & Reveal, 1978; Hershler *et al.*, 2002) and is now being vigorously studied with the aid of molecular methods for generating well corroborated, temporally constrained phylogenies. However, while many individual taxa have been investigated in this manner and comparative or ecosystem focused analyses of western biogeography are starting to appear in the literature (e.g. Brunsfeld *et al.*, 2001; Calsbeek *et al.*, 2003; Carstens *et al.*, 2005), little attention has been paid to invertebrates other than arthropods. The diverse freshwater molluscan fauna of the region has traditionally been considered to have evolved in association with Neogene tectonics, with species distributions changing but little during the subsequent Quaternary period (Taylor, 1966, 1985, 1987; Taylor & Bright, 1987). This vicariance hypothesis was suggested by the concentration of endemic taxa in tectonically active regions and the close similarity between fossil and contemporary distributions, but has been little tested within the context of rigorously proposed phylogenies. Toward that end we recently initiated molecular studies of the large gastropod genus *Pyrgulopsis* Call & Pilsbry, 1886 (Rissooidea: Hydrobiidae), an apparently poor disperser (constrained by aquatic respiration and high fidelity to isolated or low connectivity spring habitats) which has diversified over much the West. Our results to date have been mixed with respect to the vicariance hypothesis. A molecular phylogeny of *Pyrgulopsis*, based on sampling of 62 of its 126 currently recognized congeners, was generally consistent with a late Neogene origin of extant species, but also implied surprisingly widespread dispersal of multiple lineages early in the evolution of the genus (Liu & Hershler, 2005; also see Hershler *et al.*, 2003a). Phylogeographic investigation of two of the 25 widely ranging members of the genus documented divergent, locally distributed subunits consistent with ancient vicariance, but also delineated shallowly structured lineages suggestive of recent dispersal across western landscapes (Liu *et al.*, 2003; Hershler & Liu, 2004; also see Hurt, 2004).

Herein, we build from our previous studies by analyzing the genetic structure of *Pyrgulopsis wongi* Hershler, 1989, which is a highly suitable subject for testing the vicariance hypothesis. This species ranges among 10 hydrographically closed basins in southeastern California and southwestern Nevada that formed through tectonic break up of an ancestral, erosional landscape of subdued relief beginning in the Miocene (Gilbert & Reynolds, 1973; Robinson & Stewart, 1984; Jayko, 2004). During the Quaternary there were temporary periods of integration of some of these basins along the eastern flank of the Sierra Nevada. At various times between *c.* 1.0 and 0.01 Ma (mid-Pleistocene to Holocene), four of these basins (Mono Lake, Adobe, Owens and Rose) were linked by a chain of spilling lakes which formed the upper segment of an ancestral Owens River basin (Benson *et al.*, 1990; Jannik *et al.*, 1991; Reheis *et al.*, 2002). Prior to this, in the early Pleistocene, Mono Lake instead spilled into the Walker River drainage, establishing a connection between these two basins (Reheis *et al.*, 2002). If *P. wongi* evolved in association with tectonic development of the regional landscape and subsequently dispersed little in accordance with the vicariance hypothesis, then relatively deep, divergent evolutionary subunits should be defined by contemporary topographic basins. If, on the other hand, this species subsequently spread widely within Quaternary drainages, then a layer of shallow population structuring is expected among those basins that were then integrated. If shallow structuring is observed among basins which have not been hydrographically connected subsequent to their Neogene inception, then dispersal by means other than spread through continuous aquatic habitat may be inferred.

Our primary goals are to evaluate genetic variation of *P. wongi* in relation to the scenarios described above and to assess whether the inter-basinal distribution of this species is best explained by the vicariance hypothesis. This analysis is based on two mitochondrial genes. We also briefly discuss the implications of our results for the conservation and management of *Pyrgulopsis* and its fragile habitats, which are imperiled by water development throughout the West (Melhop, 1996; Sada & Vinyard, 2002).

METHODS

Sampling

We sampled 28 populations of *P. wongi* across its entire geographic range (Fig. 1). Twelve sites (including the type locality, W7) were sampled in Owens Valley, which contains most of the known populations of *P. wongi*; while one to three samples were obtained from each of the other nine basins in which this species lives (Fig. 1). Samples were collected with a fine hand sieve and preserved in concentrated (90%) ethanol. All but one sample was collected during 1998–2004 specifically for this project. Because we were unable to find living specimens of *P. wongi* at its single known locality in Huntoon Valley when visiting this site in 2004, we used previously collected (1989) material that had been ethanol preserved and dried. Two to eight specimens from each sample were sequenced for each gene. Single sequences from eight other congeners that are geographically proximal and/or have been shown to be closely related to *P. wongi* (Liu *et al.*, 2003; Liu & Hershler, 2005; H.-P. Liu & R. Hershler, unpublished data) were also included in our analyses in order to explore thoroughly the relationships of populations of the latter. The type species of the closely

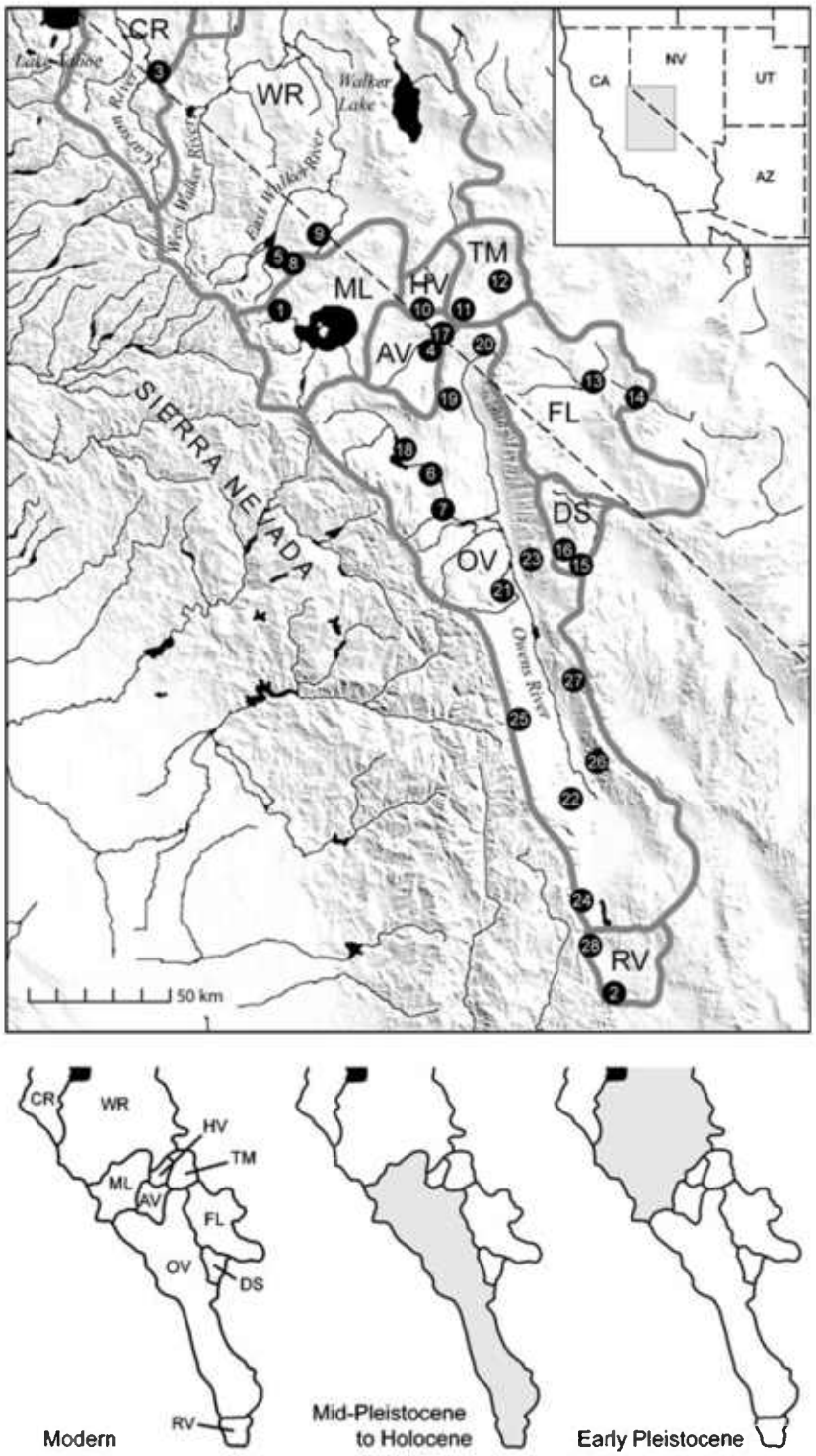

Figure 1 Map showing sampling localities (1–28) for *Pyrgulopsis wongi* Hershler, 1989. Contemporary basinal boundaries identified by a thick grey line. Diagrams below depict contemporary (left), mid-Pleistocene to Holocene (middle), and early Pleistocene (right) watersheds, with grey areas showing (prior) integration of contemporary basins (mid-Pleistocene to Holocene, AV + ML + OV + RV; early Pleistocene, ML + WR). AV, Adobe Valley; CR, Carson River Valley; DS, Deep Springs Valley; FL, Fish Lake Valley; HV, Huntoon Valley; ML, Mono Lake basin; OV, Owens Valley; RV, Rose Valley; TM, Teels Marsh; WR, Walker River basin.

related (*fide* Liu & Hershler, 2005) eastern North American genus *Floridobia* Thompson & Hershler, 2002 was used to root all trees. Voucher material for all samples was reposited in the collections of the National Museum of Natural History, Smithsonian Institution (USNM). Collection localities and sample sizes are summarized in Table 1.

Table 1 Samples used for genetic analysis with codes, localities, USNM voucher numbers, drainage, sample sizes, and GenBank accession numbers for outgroup sequences. Asterisked localities were part of the mid-Pleistocene to Holocene Owens River drainage.

Species	Code	Locality, UTM coordinates (11S), vouchers	Drainage	Sample size	
				COI	NDI
P. wongi Hershler, 1989	W01	Spring near Conway Summit, Mono Co., CA, 4215069N, 308688E, USNM 1022577	Mono Lake basin*	8	5
	W02	Springs at Little Lake, Inyo CO., CA, 3978523N, 418650E, USNM 899091	Rose Valley*	7	6
	W03	Doud Springs, Douglas Co., NV, 4297618N, 269716E, USNM 1023454	Carson River	5	5
	W04	River Spring, Mono Co., NV, 4199999N, 358301E, USNM 1023804	Adobe Valley*	5	5
	W05	Spring in Clark Canyon, Mono Co., CA, 4234078N, 310373E, USNM 1024091	Walker River	4	4
	W06	Springs in Owens River gorge, Mono Co., CA, 4157285N, 358532E, USNM 1009544	Owens Valley*	5	4
	W07	Spring along Pine Creek, Inyo Co., CA, 4144840N, 361388E, USNM 894692	Owens Valley*	7	5
	W08	Spring along Clearwater Creek, Mono Co., CA, 4227580N, 315059E, USNM 1023903	Walker River	4	4
	W09	Spring along Rough Creek, Mono Co., CA, 4241805N, 321822E, USNM 1024090	Walker River	3	3
	W10	Huntoon Spring, Mineral Co., NV, 4215441N, 360552E, USNM 869033	Huntoon Valley	4	2
	W11	Jacks Spring, Mineral Co., NV, 4213539N, 369302E, USNM 1023833	Teels Marsh	4	4
	W12	Company Spring, Mineral Co., NV, 4223768N, 381956E, USNM 1023834	Teels Marsh	4	3
	W13	Spring east of McNett Ranch, Esmeralda Co., NV, 4189470N, 412736E, USNM 1023824	Fish Lake Valley	4	3
	W14	Cave Spring, Esmeralda Co., NV, 4184080N, 425692E, USNM 1023825	Fish Lake Valley	4	4
	W15	Corral Springs, Inyo Co., CA, 4125959N, 409313E, USNM 1021973	Deep Springs Valley	8	6
	W16	Antelope Springs, Inyo Co., CA, 4132061N, 403436E, USNM 1026504	Deep Springs Valley	6	6
	W17	Springs at Pizona, Mono Co., CA, 4203568N, 362746E, USNM 1026513	Adobe Valley*	6	6
	W18	Layton Springs, Mono Co., CA, 4166999N, 348100E, USNM 1021969	Owens Valley*	4	4
	W19	Springs along northeast side of Blind Spring Hill, Mono Co., CA, 4186632N, 367596E, USNM 1026511	Owens Valley*	4	6
	W20	Spring in West Queen Canyon, Mineral Co., NV, 4200412N, 375183E, USNM 1026514	Owens Valley*	6	6
	W21	Spring south of Warren Lake, Inyo Co., CA, 4116652N, 381915E, USNM 1026510	Owens Valley*	6	6
	W22	Spring along Lubken Creek, Inyo Co., CA, 4044765N, 404772E, USNM 1026515	Owens Valley*	6	6
	W23	Spring in Marble Canyon, Inyo Co., CA, 4128000N, 392000E, USNM 1006930	Owens Valley*	4	4
	W24	Spring south of Summit Creek, Inyo Co., CA, 4009532N, 409005E, USNM 1026516	Owens Valley*	5	5
	W25	Boron Springs, Inyo Co., CA, 4072920N, 386613E, USNM 1026509	Owens Valley*	5	5
	W26	French Spring, Inyo Co., CA, 4058774N, 410847E, USNM 1026506	Owens Valley*	5	5
	W27	Barrel Springs, Inyo Co., CA, 4083369N, 404243E, USNM 1026508	Owens Valley*	5	4
	W28	Spring north of Johnson Canyon, Inyo Co., CA, 3995092N, 408104E, USNM 1026512	Rose Valley*	5	5
P. breviloba Hershler, 1998	–	Flag Springs (middle), Nye Co., NV, USNM 894708	White River (Colorado River basin)	AY627928[5]	AY628050[5]
P. deserta (Pilsbry, 1916)	–	Springs along Virgin River, Mohave Co., AZ, USNM 894879	Colorado River	DQ251077	DQ251106
P. gibba Hershler, 1995	–	Spring west of Fee Reservoir, Lassen Co., CA, USNM 1002892	Surprise Valley (Great Basin)	AY197603[2]	AY426413[4]
P. greggi Hershler, 1995	–	Grapevine Creek, Kern Co., CA, USNM 903984	San Joaquin Valley	AF520943[1]	AY367546[3]
P. kolobensis (Taylor, 1987)	–	Toquerville Springs, Washington Co., UT, USNM 894877	Virgin River (Colorado River basin)	AY627939[5]	AY628063[5]

Table 1 *continued*

Species	Code	Locality, UTM coordinates (11S), vouchers	Drainage	Sample size		
				COI	NDI	
P. lata Hershler, 1998	–	Butterfield Springs, Nye Co., NV, USNM 897710	White River (Colorado River basin)	AY627927[5]	AY628049[5]	
P. trivialis (Taylor, 1987)	–	Spring at Three Forks, Apache Co., AZ, USNM 894881	Gila River (Colorado River basin)	AY627941[5]	AY628065[5]	
P. turbatrix Hershler, 1998	–	Horseshutem Springs, Nye Co., NV, USNM 903989	Pahrump Valley (Great Basin)	AF520936[1]	AY367555[3]	
F. winkleyi (Pilsbry, 1912)	–	Salt marsh at Scarborough, Cumberland Co., ME, USNM 883964	Saco River (Atlantic coastal)	AF520917[1]	AY628036[5]	

Sequence reported by [1]Hershler *et al.* (2003a), [2]Hershler *et al.* (2003b), [3]Liu *et al.* (2003), [4]Hershler & Liu (2004), [5]Liu & Hershler (2005).

DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was isolated from individual snails using a CTAB protocol (Bucklin, 1992). For the COI gene, COIL1490 and COIH2198 (Folmer *et al.*, 1994) were used to amplify a 710 base pair (bp) fragment via polymerase chain reaction (PCR). For NDI, ND43F and RND592F were used to amplify an approximately 570 bp fragment by PCR, following protocols described by Liu *et al.* (2003). The amplified PCR product was cleaned and sequenced using a dye terminator cycle sequencing reaction (Beckman Coulter CEQ 8000, Fullerton, CA, USA) as described by Liu *et al.* (2003). These two regions were used because of their proven utility for population and species level studies of *Pyrgulopsis* (Hershler *et al.*, 2003b; Liu *et al.*, 2003; Hurt, 2004; Liu & Hershler, 2005).

Data analysis

All sequences were determined for both strands and were edited and aligned using SEQUENCHER™ (version 3.1.1; Gene Codes Corporation, Ann Arbor, MI, USA) The partition homogeneity/incongruence-length difference test (ILD) (Farris *et al.*, 1994) implemented in PAUP* 4.0b10 (Swofford, 2002) was used to determine whether the two data sets were consistent and could be combined for phylogenetic analysis. For each of the pairwise data partitions, the test was implemented using parsimony-informative sites only and 100 replicates.

Phylogenetic analyses based on distance, parsimony and maximum-likelihood methods were generated using PAUP*. Bayesian inference was performed using MRBAYES 3.04 (Huelsenbeck & Ronquist, 2001). MODELTEST 3.7 (Posada & Crandall, 1998) was used to obtain an appropriate substitution model (using the Akaike Information Criterion) and parameter values for the distance, maximum-likelihood and Bayesian analyses. Appropriate genetic distance was used to generate a neighbour-joining (NJ) tree (Saitou & Nei, 1987). Maximum-parsimony (MP) analyses were conducted with equal weighting, using the heuristic search option with tree bisection reconnection branch-swapping and 100 random additions. The appropriate model was applied for the maximum likelihood analyses (ML). A NJ tree with appropriate genetic distance model was used as the initial topology for branch-swapping. Node support was evaluated by 10,000 bootstrap pseudo-replicates in all but the ML analysis, in which support was based on 100 replications. In the Bayesian approaches, three short runs were first conducted using the default random tree option to determine when the log likelihood sum reached a stable value (by plotting the log-likelihood scores of sample points against generation time). The ln likelihood scores started at around −9000 and quickly converged upon a stable value of about −4500 after approximately 30,000 generations. Metropolis-coupled Markov chain Monte Carlo simulations were then run with four chains (using the model selected by MODELTEST) for 1,000,000 generations, and Markov chains were sampled at intervals of 10 generations to obtain 100,000

sample points. The sampled trees with branch lengths were used to generate a 50% majority rule consensus topology with the first 3000 trees, equal to 30,000 generations, removed to ensure that the chain sampled a stationary portion. In order to provide a readable tree and reduce computation time, only one sequence of each haplotype was used in all phylogenetic analyses. As an additional means of examining relationships among haplotypes of *P. wongi*, unrooted statistical parsimony networks were generated for the separate COI and NDI data sets using the program TCS 1.18 (Clement *et al.*, 2000).

To assess congruence between phylogeny and hydrography, groupings by contemporary and mid-Pleistocene to Holocene drainage basins were forced using the CONSTRAINT function in PAUP* and then compared with unconstrained most parsimonious trees using the Kishino-Hasegawa test. ARLEQUIN 2.0 (Excoffier *et al.*, 1992; Schneider *et al.*, 2000) was used to analyze partitioning of genetic variation (for the separate data sets) among contemporary drainage basins, mid-Pleistocene to Holocene drainage basins, and phylogenetic groupings revealed by our study. The AMOVA was based on a user-defined genetic distance matrix (using the best fit model selected by MODELTEST). We did not utilize the tools of nested clade analysis (Templeton *et al.*, 1987, 1992) in our study owing to the uncertain mode and history of dispersal of *P. wongi*, which prevented confident determination of appropriate geographic distances between populations, especially those living in different basins.

Sequence divergences (based on the best fit model selected by MODELTEST) within and between phylogenetic groupings were calculated using PAUP*. To estimate divergence times of clades within *P. wongi* (whose fossil record is unknown), we calibrated a molecular clock for the COI gene based on another local springsnail, *P. perturbata* Hershler, 1989. This species is endemic to a volcanic tableland in northern Owens Valley that is composed of a thick blanket of ash (Bishop Tuff) produced by a major eruption of the Long Valley caldera in the mid-Pleistocene (Bailey *et al.*, 1976). If *P. perturbata* originated coincident with or subsequent to the 0.7589 Ma (Sarna-Wojcicki *et al.*, 2000) emplacement of this ash flow, then the 1.23% COI divergence of this species relative to its sister, *P. owensensis* Hershler, 1989 (*fide* Liu *et al.*, 2003), implies a (minimum) clock rate of 1.62% per million years. (Note that any pre-existing aquatic biota in this area would have been destroyed by the thick ash flow.) This calibration is similar to the 1.83 ± 0.21% rate for COI previously derived for European hydrobiid snails (Wilke, 2003). A molecular clock hypothesis for the COI data set was tested using the likelihood ratio test (Felsenstein, 1981), based on the ML topology under the best model selected with and without the constraint of a molecular clock. We acknowledge that the application of a molecular clock is laden with difficulties (Arbogast *et al.*, 2002; Heads, 2005), but nonetheless it provides a method of roughly estimating divergence times that is especially useful for studies of taxa such as *Pyrgulopsis* which have a phylogenetically uninformative fossil record (Taylor, 1987).

The coalescent-based program MDIV (Nielsen & Wakeley, 2001) was used to estimate population divergence times and migration rates between pairs of basins. For this analysis, samples were pooled by watershed, with populations living in drainages that were recently integrated with Owens Valley (Mono Lake basin, Adobe and Rose Valleys) grouped with those of this basin (following Table 1). MDIV uses Markov-chain Monte Carlo simulation to estimate the maximum likelihood value for theta (θ, $2N_{ef}\mu$), scaled migration rate ($M = N_{ef}m$), and scaled time of divergence ($T = t/N_{ef}$). (N_{ef} = effective female population size, m = migration rate, and μ = mutation rate.) MDIV assumes that N_{ef} and m are the same for the pairs of populations being analyzed. We ran 2,000,000 chains for each simulation with a burn-in of 500,000 chains, set T_{MAX} and M_{MAX} to 10, and used the finite sites model of sequence evolution (HKY model). The values of theta, scaled migration rate, and scaled time of divergence with the highest posterior probability were considered as the best estimate. Ranges for these values were defined as within two Akaike Information Criterion (AIC) units of the best estimate (AIC: Burnham & Anderson, 1998). N_{ef} was calculated from the best estimate of theta using a mutation rate (μ) of 1.066×10^{-5} over 658 bp of COI sequence (1.62×10^{-8} substitutions per nucleotide per year [see above] × 658 bp), assuming a generation time of one year for *P. wongi* (*fide* Smith, 2001). Estimates of migration rate (m) and population divergence (t) were calculated from multiple scaled migration rate (M) and scaled time of divergence (T) by N_{ef}.

RESULTS

Sequence data

The alignment of COI sequences yielded 658 bp, of which 145 sites were variable (22.0%) and 94 were parsimony informative

Table 2 COI sequence divergence within and among subunits of *Pyrgulopsis wongi*. Mean values are followed by range (in parenthesis)

	Clade A	Clade B	Clade C	Clade D	W22	W21
Clade A	0.31% (0–0.77)					
Clade B	1.53% (1.23–1.86)	0.10% (0–0.31)				
Clade C	0.85% (0.77–1.07)	1.76% (1.70–1.85)	0.00% (0–0.00)			
Clade D	0.89% (0.76–1.23)	1.64% (1.54–1.85)	0.96% (0.92–1.07)	0.06% (0–0.31)		
W22	0.72% (0.61–1.07)	1.63% (1.54–1.85)	0.79% (0.77–0.92)	0.83% (0.77–1.07)	0.05% (0–0.15)	
W21	0.54% (0.46–0.77)	1.14% (1.07–1.23)	0.61% (0.61)	0.50% (0.46–0.61)	0.48% (0.46–0.61)	0.00% (0–0.00)

(14.3%). Overall nucleotide composition was biased towards thymine (T) (37.5%) and adenine (A) (24.8%), followed by cytosine (C) (19.1%) and guanine (G) (18.6%). A total of 530 bp of NDI was analyzed, of which 162 sites were variable (30.6%) and 111 were parsimony informative (20.9%). Average base frequencies for this gene were 28.1% A, 36.8% T, 18.8% C and 16.4% G. New sequences were deposited in GenBank under accession numbers DQ251052–DQ251106. Likelihood ratio tests could not reject clocklike behaviour of COI sequences ($P = 0.85$), indicating that the application of a molecular clock is appropriate for these data.

Phylogenetic reconstruction

The ILD tests did not reveal significant incongruence between COI and NDI ($P = 0.35$) and thus we performed phylogenetic analyses using the combined data set of 1188 bp. MODELTEST selected the General Time Reversible (GTR) model (Tavare, 1986), with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution (e.g. GTR + I + G), as the best fit for the combined data set using the AIC. The optimized parameters were base frequencies of A = 0.2749, C = 0.1897, G = 0.1616, T = 0.3738; Rmat = {2.369327.15440.53240.858819.4976}; shape of gamma distribution = 2.1913; and proportion of invariant sites = 0.6624. The K81uf + I + G (a variation of the K3P model; Posada & Crandall, 1998) and GTR + I + G models were selected for the COI and NDI data sets, respectively.

All analyses congruently resolved combined haplotypes of *P. wongi* into two strongly supported lineages that are not sister to each other (Fig. 2, a Bayesian tree). One of these lineages is composed of haplotypes from the single population (W3) living in the Carson River basin, which represents the northernmost record for *P. wongi* and is broadly disjunct relative to the rest of this species' range (Fig. 1). The other consists of haplotypes from the remaining populations of *P. wongi* (W1–W2, W4–W28), including that from the type locality (W7). The genetic distance between these two lineages was substantial, 6.97% (6.57–7.78%, K3P distance) for COI and 10.09% (9.08–10.99%, GTR distance) for NDI. Based on the range of sequence divergences among 62 congeners documented in a previous molecular phylogenetic study (1.1–13.1% for COI; 1.7–15.8% for NDI; Liu & Hershler, 2005), we consider it unlikely that the Carson River lineage is

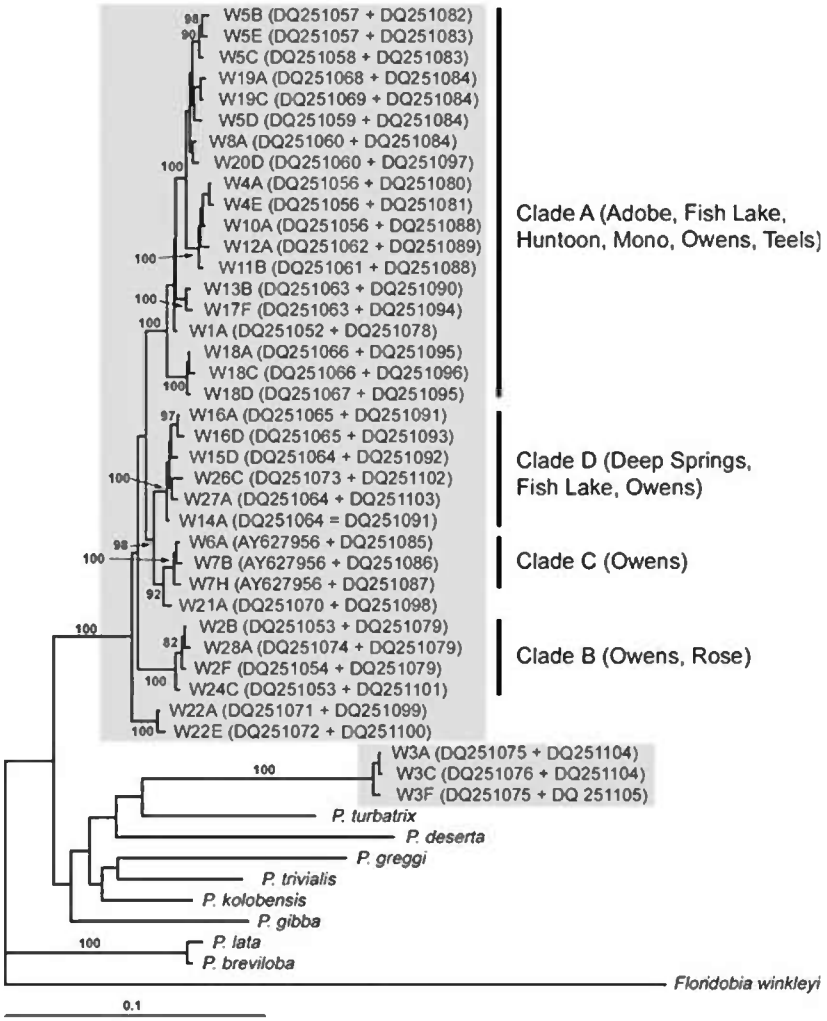

Figure 2 Bayesian tree based on the combined (COI and NDI) data set. The light grey tone is applied to portions of the tree composed of *Pyrgulopsis wongi* haplotypes. Posterior probabilities for nodes are provided when > 70%. Terminals are combined haplotypes (labelled as in Table 1), with only single examples of each included in the analysis.

conspecific with *P. wongi* and accordingly excluded it from further analysis. The MP, ML and NJ topologies were closely congruent with the Bayesian tree, differing only slightly in the positioning of terminals within the *P. wongi* lineage. The sister relationships of both lineages were not well resolved in our analyses.

Within the *P. wongi* lineage, four clades received high (100%) posterior probabilities in the Bayesian tree (A–D, Fig. 2) and strong bootstrap support (> 90%) in the MP, ML, and NJ topologies. One of these is distributed in the middle portion of Owens Valley (clade C, Fig. 3). Another is distributed in southern Owens Valley and adjacent Rose Valley (clade B, Fig. 3). A third (clade A) ranges among northern Owens Valley and six closely proximal basins, while the fourth (clade D) is distributed in southern Owens Valley and two basins to the east of this trough (Fig. 3). Two populations from Owens Valley were not positioned in any of these clades. One (W22) was consistently depicted as sister to all other *P. wongi* haplotypes, while the other (W21) was consistently depicted as sister to clade C, albeit with weak (92%) posterior probability (92%) and bootstrap support (54–68%).

Haplotypes from populations living in basins integrated by the late Quaternary Owens River (Adobe, Mono, Owens and Rose) did not form a shallowly structured clade, but instead were spread among all six of the *P. wongi* lineages (clades A–D, populations W21, W22). Although haplotypes from the two basins which were integrated during the early Pleistocene (W1A, Mono Lake; W5B–E, W8A, Walker River) were jointly positioned in clade A, they did not form a monophyletic subunit within this group. Analyses in which groupings by contemporary drainage basins were forced yielded shortest trees (TL = 297) that were significantly different from the unconstrained, most parsimonious topologies (TL = 277) ($P = 0.001$). When groupings by mid-Pleistocene to Holocene hydrography were instead forced, the shortest trees (TL = 297) were also significantly different from unconstrained topologies.

The sequence divergence of *P. wongi* relative to the seven members of its sister clade (Fig. 2) ranged from 4.16–8.97% for COI. Based on these data and the locally derived clock rate, we infer that the *P. wongi* lineage diverged from its sister clade 2.57–5.54 Ma (Pliocene to latest Miocene). The mean sequence

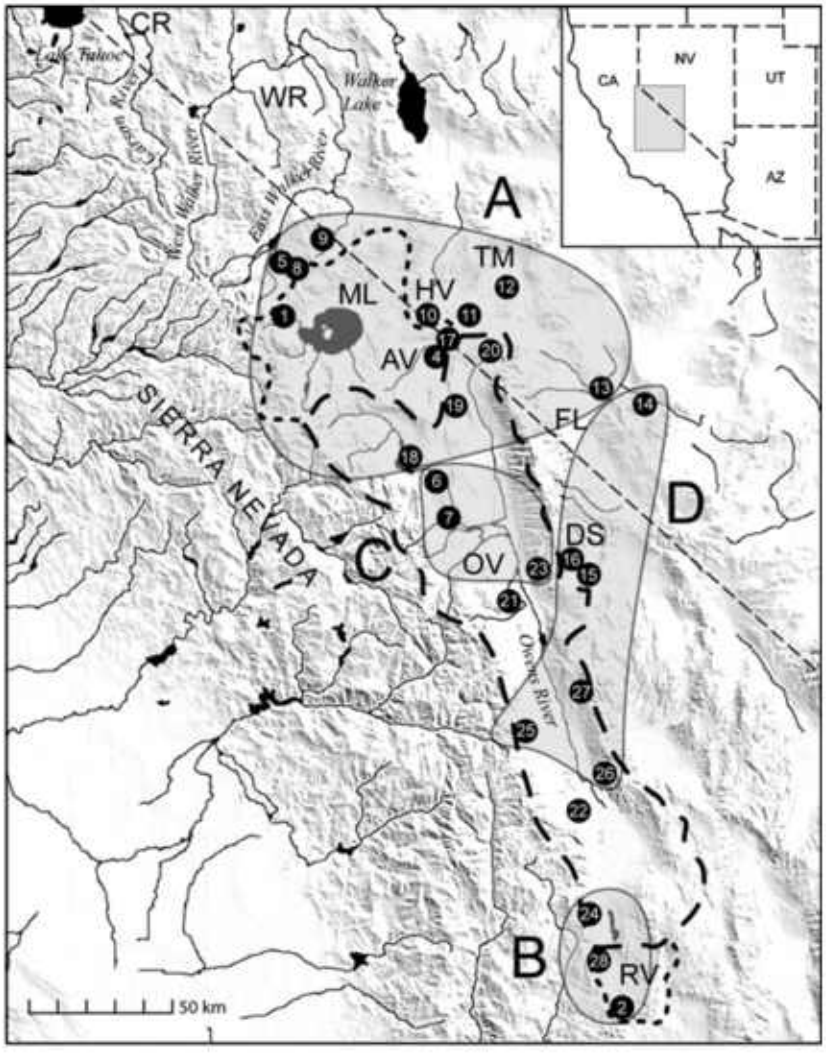

Figure 3 Map showing geographic distributions (shaded ellipses) of the four major clades (A–D) of *Pyrgulopsis wongi*. Contemporary Owens Valley (OV) drainage boundary identified by a thick dashed line; dotted lines indicate hydrographic boundaries of other components of the mid-Pleistocene to Holocene Owens River basin. Geographic labels are as in Fig. 1.

divergence among the six lineages of *P. wongi* ranged from 0.48–1.76% for COI (Table 2), suggesting that they diverged 0.30–1.09 Ma (mid- to late Pleistocene).

Population structure

For the COI gene, 33 variable sites were observed among 138 sequenced specimens of *P. wongi*, resulting in 24 haplotypes (I–XXIV, Table 3); while for NDI, 41 variable sites were observed among 126 specimens, resulting in 26 haplotypes (I–XXVI, Table 4). The haplotype networks generated for these separate data sets depicted clusters congruent with the groupings within *P. wongi* described above (Fig. 4). Most haplotypes are restricted to single populations (COI, 18; NDI, 21), with many (COI, 9; NDI, 11) confined to single specimens. However for both genes, a small number (COI, 5; NDI, 4) of haplotypes is shared among populations of multiple basins. For both genes, all but one of these widespread haplotypes is distributed among basins which have remained hydrographically isolated from each other since their inception. No haplotypes were shared between Walker River and Mono Lake basins, which were integrated during the early Pleistocene. There was also little sharing of haplotypes within the confines of the mid-Pleistocene to Holocene Owens River basin – one haplotype for each gene is narrowly distributed in southern Owens Valley and Rose Valley (COI, XXI; NDI, XXV) while three others are shared by two to three closely proximal populations in Owens Valley (COI, VI, VII, XI; NDI, IV, VII, XIII).

AMOVA

Analysis of the separate molecular data sets based on user-defined matrices (K3P distance for COI, GTR for NDI) revealed significant genetic structuring in relation to the contemporary landscape ($P < 0.001$ at all levels, Table 5). Less than half of the variation was explained by differences among basins (37.6%, COI; 33.5%, NDI). Differences within populations explained only a small amount of the total variation (3.8%, COI, 3.2%, NDI). Although our sampling was modest, there is no correlation between sample size and number of haplotypes observed. When these data were partitioned according to mid-Pleistocene to Holocene hydrography, differences among basins accounted for an even smaller amount of the total variation (11.3%, COI, 15.3%, NDI) and were not significant ($P > 0.05$ for both data sets). When populations were arranged instead in accordance with the six lineages described above, structuring was highly significant ($P < 0.001$ at all levels) and most of the variation (80.5%, COI, 84.6%, NDI) was explained by differences among these groups

Table 3 Frequency distribution of COI haplotypes. Shaded rows identify haplotypes shared among (contemporary) basins. Basin abbreviations are from Fig. 1.

	Drainage basin and locality (W)																												
	WR			HV	TM		FL		DS		ML	AV		OV														RV	
Haplotype	05	08	09	10	11	12	13	14	15	16	01	04	17	06	07	18	19	20	21	22	23	24	25	26	27	02	28
I											8																
II				4	3	1						5	5														
III	2																										
IV	1																										
V	1																										
VI														5	7						4						
VII		4	3														2	6									
VIII					1																						
IX						3																					
X							4						1														
XI								4	8														5	3	5		
XII										6																	
XIII																3											
XIV																1											
XV																	1										
XVI																	1										
XVII																			6								
XVIII																				5							
XIX																				1							
XX																								2			
XXI																						5				5	
XXII																										1	
XXIII																										1	
XXIV																											5

Table 4 Frequency distribution of NDI haplotypes. Shaded rows identify haplotypes shared among (contemporary) basins. Basin abbreviations are from Fig. 1.

	Drainage basin and locality (W)																										
	WR			HV	TM		FL		DS		ML	AV		OV												RV	
Haplotype	05	08	09	10	11	12	13	14	15	16	01	04	17	06	07	18	19	20	21	22	23	24	25	26	27	02	28
I											5																
II												4															
III												1															
IV	1	4	3														6	5									
V	1																										
VI	2																										
VII														4	3						4						
VIII															1												
IX															1												
X				2	4	1							5														
XI						2																					
XII							3																				
XIII								4		5	5												5	3			
XIV									1																		
XV										1																	
XVI													1														
XVII																3											
XVIII																1											
XIX																		1									
XX																			6								
XXI																				5							
XXII																				1							
XXIII																								2			
XXIV																									4		
XXV																						4				6	5
XXVI																						1					

(Table 5). Values of Φ_{ST} were estimated as 0.96 for COI and 0.97 for NDI.

Coalescent analysis

Migration estimates were extremely low (10^{-5}–10^{-7} migrant per generation; Table 6), with the range including zero in 10 of 15 cases (all comparisons among the Walker River basin, Teels Marsh, and Huntoon, Fish Lake, and Deep Springs Valleys). Estimated divergence times among basins ranged from 0.274–0.069 Ma (Table 6), suggesting that all of these events occurred during the mid- to late Pleistocene.

DISCUSSION

Evolutionary diversification of the western North American freshwater molluscan fauna traditionally has been attributed to vicariance associated with ancient tectonic events. We tested this hypothesis by analyzing the genetic structure of a widely ranging member of this fauna in relation to contemporary hydrographic basins that formed as a result of late Neogene rearrangement of landscape. Our findings indicate that although the *P. wongi* lineage may have evolved during the late Miocene to Pliocene when contemporary regional basins were being formed, the contemporary phylogeography of this species does not bear an obvious imprint of this component of landform development. Inter-basinal divergence of *P. wongi* populations occurred during the mid- to late Pleistocene, well after the development of contemporary regional landscape (Table 6), and haplotypes do not form monophyletic groups corresponding to the nine inhabited basins as predicted by the vicariance hypothesis (Figs 2 & 3). Although the larger drainage basins inhabited by this snail have had a complex geologic history (e.g. Fish Lake Valley, Reheis & Sawyer, 1997; Owens Valley, Hollett *et al.*, 1991) congruent with some of our findings, this does not negate the striking overall pattern of population structuring of *P. wongi*, which bears little relation to major regional landscape features.

We also used our data to test another facet of the vicariance hypothesis, which postulates that the western aquatic molluscan fauna was little impacted by the expansion and contraction of drainage that have occurred during the Quaternary period. Our results did not reveal a shallow layer of genetic structuring among the Mono and Walker Lake basins, which were connected in the early Pleistocene, nor among the four basins that were integrated by the late

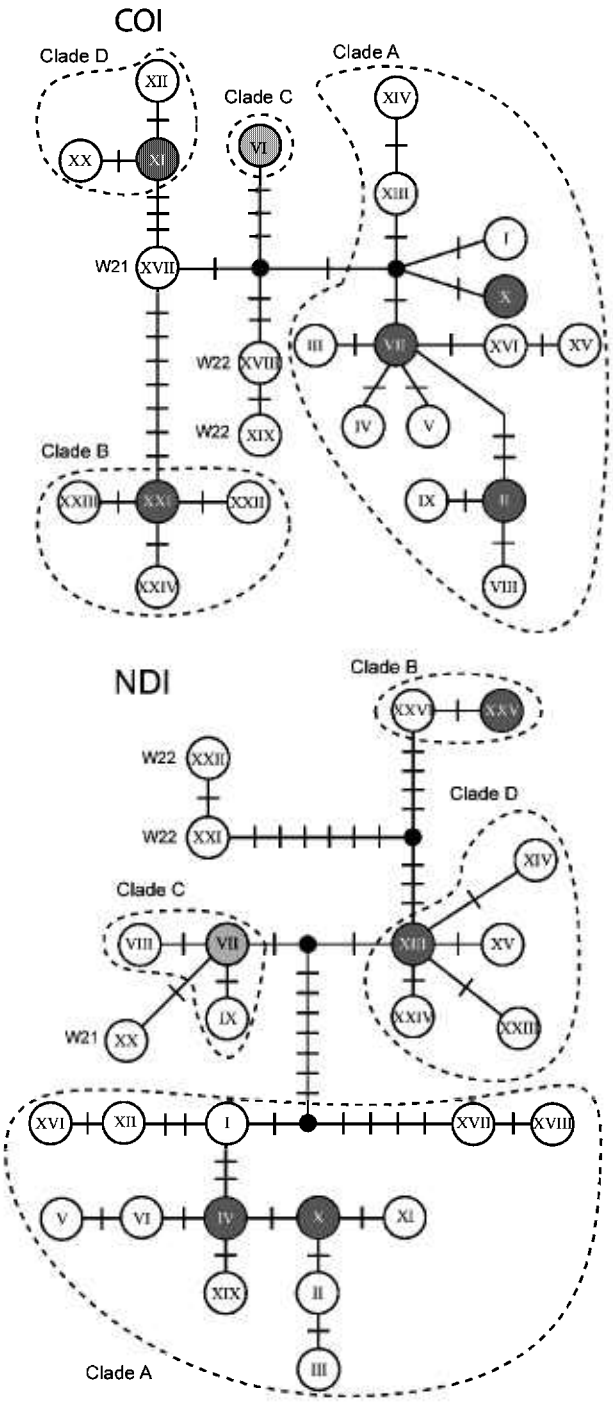

Figure 4 Minimum spanning haplotype networks based on the COI and NDI data sets, with major clades delineated by dashed lines. Haplotypes are numbered as in Table 3 (COI) and 4 (NDI). Bars indicate base substitutions between adjacent haplotypes. Haplotypes having dark grey shading (and white lettering) are found in more than one basin, while those shaded light grey occur in more than one site within a single basin. Small black circles indicate haplotypes which are inferred but were not observed.

Quaternary Owens River, thus confirming that gene flow (of this species) was not greatly enhanced by this recent interval of aquatic connectivity.

The very large Φ_{ST} values (0.96, COI; 0.97, NDI), confinement of many haplotypes to single localities, and estimated migration rates suggest that contemporary dispersal of *P. wongi* is absent or negligible, which is consistent with the restriction of this species to insular spring habitats scattered over an extremely arid landscape. However, the broad geographic distribution of other haplotypes, phylogeographic structure, and estimated divergence times suggest geologically recent connectivity of populations both within watersheds and among hydrographically closed basins. These findings are supported by an unpublished allozyme survey of *P. wongi*, which revealed both extensive differentiation among populations ($F_{ST} = 0.846$) and sharing of alleles among isolated basins (Hamlin, 1996), and are paralleled by recent studies of aquatic biota living in other arid regions (e.g. Hughes & Hillyer, 2003; Carini & Hughes, 2004, 2006; Huey *et al.*, 2006).

Although the broad distributions of multiple *P. wongi* haplotypes may logically be attributed to prior dispersal within integrated drainage systems, this scenario is not supported by available geological evidence. Most of the drainage basins inhabited by this snail were hydrographically isolated from one another throughout the Quaternary time period when divergence of *P. wongi* lineages occurred. Physical evidence of prior drainage connections is surely incomplete in this region and consequently it may be possible that those basins separated by low divides (e.g. Huntoon Valley and Teels Marsh) experienced (as yet undetected) intervals of recent integration that enabled dispersal. However, this scenario is difficult to envision for other portions of the study area such as Owens Valley and basins to the east (Deep Springs and Fish Lake Valleys), which share haplotypes yet are separated by the imposing, ancient White-Inyo Mountains front (Lueddecke *et al.*, 1998; Stockli *et al.*, 2003). Furthermore, our finding that Quaternary drainage patterns have had little impact on phylogeography suggests that *P. wongi*, which today is restricted to the special habitats (Hynes, 1970) provided by headsprings and upper reaches of spring runs, may not be able to disperse widely within stream systems.

In our previous study of a regional congener, *P. micrococcus* (Pilsbry, 1893), whose phylogeography was also enigmatic with respect to drainage history, we concluded that geologically recent dispersal probably did not occur via aquatic connections, but instead may have been facilitated by overland transport on waterfowl (Liu *et al.*, 2003). This hypothesis is supported by experimental evidence that *Pyrgulopsis* can tolerate several hours of desiccation (Mladenka, 1992; United States Fish and Wildlife Service: USFWS, 2005), and may be applicable to *P. wongi* as this species frequently lives in settings which contain open water and thus attract birds (*fide* Ponder *et al.*, 1994). Alternatively, one may postulate that *P. wongi* has spread among basins as a result of stream capture, which is supported by the common occurrence of this species in upland settings and a growing appreciation that springs frequently serve as focal points of headwater retreat and stream piracy across divides (Pederson, 2001).

Table 5 AMOVA results for testing subdivision of *Pyrgulopsis wongi* populations. The contemporary hydrography consisting of nine basins was reduced to six during the wetter periods of the mid-Pleistocene to Holocene (Table 1. Lineages consist of the four major clades (A–D) and two divergent populations from Owens Valley (W21, W22) (Fig. 2). Asterisked Φ values are highly significant ($P < 0.001$).

	COI			NDI		
Source of variation	Variance	% of total	Φ	Variance	% of total	Φ
Among contemporary drainages	0.002	37.6	$\Phi_{CT} = 0.38^*$	0.003	33.5	$\Phi_{CT} = 0.34^*$
Among populations within contemporary drainages	0.003	58.6	$\Phi_{SC} = 0.94^*$	0.006	63.3	$\Phi_{SC} = 0.95^*$
Within populations	0.0002	3.8	$\Phi_{ST} = 0.96^*$	0.0003	3.2	$\Phi_{ST} = 0.97^*$
Among mid-Pleistocene to Holocene drainages	0.0005	11.3	$\Phi_{CT} = 0.11$	0.001	15.3	$\Phi_{CT} = 0.15$
Among populations within mid-Pleistocene to Holocene drainages	0.004	84.8	$\Phi_{SC} = 0.96^*$	0.007	81.5	$\Phi_{SC} = 0.96^*$
Within populations	0.0002	3.9	$\Phi_{ST} = 0.96^*$	0.0003	3.2	$\Phi_{ST} = 0.97^*$
Among major lineages	0.004	80.5	$\Phi_{CT} = 0.81^*$	0.009	84.6	$\Phi_{CT} = 0.85^*$
Among populations within major lineages	0.0009	16.2	$\Phi_{SC} = 0.83^*$	0.001	12.6	$\Phi_{SC} = 0.82^*$
Within populations	0.0002	3.3	$\Phi_{ST} = 0.97^*$	0.0003	2.74	$\Phi_{ST} = 0.97^*$

Table 6 Coalescent-based estimates of population genetic parameters: maximum likelihood value for theta ($\theta = 2N_{ef}\mu$), effective population size (N_{ef}), scaled migration rate ($M = N_{ef}m$), migration rate ($m = MN_{ef}$), scaled time of divergence (T), and population divergence in years ($t = TN_{ef}$). Question marks indicate parameter values for which the likelihood surface was too flat to enable inference. Bold lettering indicates cases in which the preset limit was reached.

Comparison	Theta	N_{ef}	M	m	T	t
Walker River vs. Owens	4.80 (2.60–6.97)	200,751	0.74 (0.08–2.66)	3.69×10^{-6}	0.70 (0.08–**10**)	1.41×10^5
Walker River vs. Huntoon	0.87 (0.20–2.49)	40,807	0.04 (**0**–0.72)	9.80×10^{-7}	2.20 (0.12–**10**)	8.98×10^4
Walker River vs. Teels Marsh	0.86 (0.30–2.69)	40,338	0.02 (**0**–0.46)	4.96×10^{-7}	1.72 (0.46–**10**)	6.94×10^4
Walker River vs. Fish Lake	1.08 (0.40–2.80)	50,657	0.04 (**0**–0.70)	7.90×10^{-7}	1.68 (0.32–**10**)	8.51×10^4
Walker River vs. Deep Springs	0.78 (0.33–2.10)	36,585	0.02 (**0**–0.26)	5.47×10^{-7}	4.94 (0.72–**10**)	1.81×10^5
Owens vs. Huntoon	3.51 (2.12–6.06)	164,634	3.80 (0.62–**10**)	2.31×10^{-5}	0.12 (**0**–**10**)	?
Owens vs. Teels Marsh	4.19 (2.40–6.79)	196,529	1.24 (0.06–3.50)	6.31×10^{-6}	0.56 (0.06–**10**)	1.10×10^5
Owens vs. Fish Lake	3.51 (2.05–5.99)	164,634	1.38 (0.38–8.88)	8.38×10^{-6}	0.34 (0.08–**10**)	5.60×10^4
Owens vs. Deep Springs	3.71 (2.22–6.09)	174,015	0.48 (0.06–1.94)	2.76×10^{-6}	0.96 (0.12–**10**)	1.67×10^5
Huntoon vs. Teels Marsh	0.37 (0.22–1.53)	17,355	2.84 (**0**–**10**)	?	1.2 (**0**–**10**)	?
Huntoon vs. Fish Lake	0.91 (0.29–2.71)	42,683	0.06 (**0**–1.18)	1.41×10^{-6}	2.36 (0.10–**10**)	1.01×10^5
Huntoon vs. Deep Springs	0.56 (0.22–1.74)	26,266	0.02 (**0**–0.26)	7.61×10^{-7}	9.36 (0.64–**10**)	2.46×10^5
Teels Marsh vs. Fish Lake	1.17 (0.38–2.88)	54,878	0.06 (**0**–0.70)	1.09×10^{-6}	1.84 (0.30–**10**)	1.01×10^5
Teels Marsh vs. Deep Springs	0.83 (0.31–1.95)	38,931	0.02 (**0**–0.22)	5.14×10^{-7}	7.04 (0.78–**10**)	2.74×10^5
Fish Lake vs. Deep Springs	0.64 (0.18–1.66)	30,019	0.14 (**0**–2.48)	4.66×10^{-6}	8.76 (0.04–**10**)	2.63×10^5

The phylogeographic pattern of *P. wongi* strongly contrasts with those of other freshwater organisms that readily spread through integrated drainages and whose structuring closely follows drainage boundaries (e.g. Cook *et al.*, 2002; Pfrender *et al.*, 2004), therefore enabling the application of a 'watershed as conservation unit' approach (Wishart, 2000). The results of this investigation and other recent studies (Liu *et al.*, 2003; Hurt, 2004) suggest that *Pyrgulopsis*, which is seemingly unable to disperse widely through aquatic systems (but see Hershler & Liu, 2004), should not be managed based on *a priori* assumptions of population divergence in relation to landscape. Instead, morphological and genetic variation must be surveyed across the entire geographic range of these snails to identify evolutionary distinct units with confidence and thus to determine conservation priorities. Current strategies for conserving and managing this biodiversity jewel and its imperiled habitats rightly emphasize habitat preservation (Sada *et al.*, 2001), but need to be expanded to include this critical element of scientific research.

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