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Mitochondrial DNA sequences reveal extensive cryptic diversity within a western American springsnail

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Abstract

We analysed cytochrome *c* oxidase subunit I and NADH dehydrogenase subunit I sequence variation among 29 populations of a widely ranging southwestern springsnail (*Pyrgulopsis micrococcus*) and 18 regional congeners. Cladistic analyses of these sequences depict *P. micrococcus* as a polyphyletic composite of five well-supported clades. Sequence divergences among these clades and subclades imply the possible occurrence of as many as seven or eight cryptic species in addition to *P. micrococcus*. Our finding that *P. micrococcus* contains multiple, genetically distinct and geographically restricted lineages suggests that diversification within this highly speciose aquatic genus has been structured in large part by the operation of terrestrial barriers to gene flow. However, these sequence data also indicate that recent dispersal among hydrographically separated areas has occurred within one of these lineages, which we attribute to passive transport on migratory waterbirds.

Keywords: cryptic species, freshwater, Gastropoda, mtDNA, phylogenetics, western North America

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Introduction

The tiny gastropods of the subfamily Nymphophilinae (family Hydrobiidae) are ubiquitous members of North American freshwater ecosystems. Nine of the 10 nymphophiline genera have relatively few species (one to 14) and are distributed east of the continental divide. The tenth genus, *Pyrgulopsis* (> 120 species), may be the most diverse element of the western aquatic fauna. *Pyrgulopsis* lives in springs and other permanent waters that are poorly integrated on arid western landscapes. Although the dispersal ability of *Pyrgulopsis* has never been studied, these gill-breathing animals have an entirely aquatic life cycle and thus are presumably unable to move among hydrographically separated habitats (Taylor & Bright 1987). The spread of *Pyrgulopsis* within aquatic systems may also be constrained by its direct mode of development (bereft of a free-swimming dispersal phase) and frequent restriction to (upflow) headsprings, which may reflect physiological specialization for these unique environments (e.g. O'Brien & Blinn 1999).

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The entirely aquatic life cycle and limited vagility of *Pyrgulopsis* suggest that it should be prone to differentiation on a fine geographical scale. The restriction of most species of this genus to a single spring, spring complex, or local watershed is consistent with this premise. However, more than two dozen other congeners range across one or more drainage divides. Given that contemporary gene flow among hydrographically separated populations is unlikely, these widely ranging snails may be expected to have pronounced geographical structuring and to possibly include morphologically cryptic species.

A resolution of this hypothesis will help clarify the biogeographical processes that have shaped the diversification of *Pyrgulopsis*. A finding that widely ranging congeners consist of multiple, geographically localized species, or incipient species would provide additional evidence of the limited vagility of these animals and the importance of topographic and other dry land barriers in structuring their biogeographic patterns. If, instead, these snails are shown to be little differentiated across their disjunct ranges, then their dispersal mechanisms and sensitivity to putative geographical barriers must be re-evaluated.

The only pertinent genetic evidence is an unpublished allozyme survey of *P. wongi*, a congener that is widely

distributed in the western Great Basin, which revealed fixed allele differences among populations consistent with limited gene flow (Hamlin 1996). The paucity of such studies has prompted us to survey the genetic structure of another widely distributed member of the genus, *P. micrococcus*, which ranges among five isolated drainage basins in southeastern California and southwestern Nevada (Hershler & Pratt 1990). The primary objectives of this study were to use mitochondrial DNA (mtDNA) markers to infer the phylogenetic relationships of populations assigned to *P. micrococcus*, and to test the morphology-based classification of this snail.

Recent molecular studies of hydrobiid snails have utilized only one mitochondrial protein-coding gene, cytochrome *c* oxidase subunit I (COI), which has provided useful phylogenetic resolution at or above the species level (Hershler *et al.* 1999; Wilke & Davis 2000; Liu *et al.* 2001; Wilke *et al.* 2001). For this study we developed primers for a second mitochondrial gene, NADH dehydrogenase subunit I (NDI), which served as a useful complement to COI.

Materials and methods

The 29 populations of *Pyrgulopsis micrococcus* that were sampled encompassed the entire range of this species (Fig. 1). The only deliberately excluded sites were in Ash Meadows, where we sampled only four of the 15 closely proximal populations that live in this large spring oasis (Hershler & Sada 1987). All collection localities were small springs. Specimens were collected from benthic vegetation (usually watercress) and fixed in 90% ethanol. In the few springs in which *P. micrococcus* is sympatric with other congeners (in Ash Meadows), its identification was facilitated by the obvious morphological differences that distinguish it from these other species (Hershler & Sada 1987).

To explore thoroughly the relationships of *P. micrococcus* populations in the absence of a robust phylogenetic hypothesis for the genus, we extensively sampled other regional congeners. We sampled two undescribed and five of eight previously described congeners that live in the same drainages as *P. micrococcus*. Each of the excluded species (*P. aardahli*, *P. fairbanksensis*, *P. pisteri*) is closely similar morphologically to one or more sampled congeners. We also sampled 10 species that live in other regional drainages. In addition, we sampled *P. stearnsiana*, a Pacific Coastal species that is closely similar, morphologically, to *P. micrococcus* (Hershler & Pratt 1990; Hershler 1994). *Marstonia agarhecta*, a member of the eastern North American fauna that was depicted as sister to the clade containing *Pyrgulopsis* in an analysis of COI variation among 35 nymphophiline species (Hershler *et al.* 2003), was used to root all trees. Specimens were collected between 1998 and 2002. Collection localities and sample sizes are summarized in Table 1. Vouchers for each sample were reposited in the National Museum of Natural History, Smithsonian Institution (USNM).

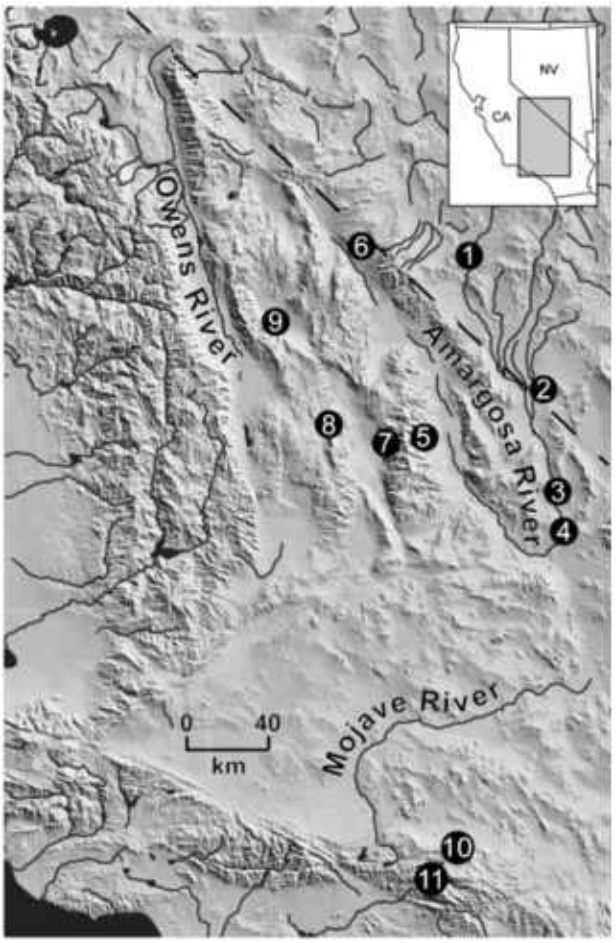

Fig. 1 Map showing sampling areas for *Pyrgulopsis micrococcus* (also see Table 1). 1 = Amargosa River headwaters (populations M1, M31); 2 = Ash Meadows (M7, M8, M29, M30); 3 = Shoshone (M26); 4 = Tecopa (M25); 5 = central Death Valley (M12, M13); 6 = northern Death Valley (M2, M3, M4, M5); 7 = eastern Panamint Valley (M14, M15, M17, M27, M28); 8 = western Panamint Valley (M9, M10, M11, M16, M22, M24); 9 = Saline Marsh (M18); 10 = Cushenbury Spring (southern Mojave drainage) (M19); 11 = upper Santa Ana River basin (M20, M21).

We designed two sets of primers for NDI. The first set (NDIL/NDIH) was based on conserved regions of NDI in an alignment produced with CLUSTALW (Thompson *et al.* 1994) using NDI sequences from three gastropods: *Littorina saxatilis* (GenBank-4165511), *Plicopurpura columellaris* (GenBank-U29705) and *Pupa strigosa* (GenBank-7335676).

NDIL and NDIH were used to amplify a 570-base-pair (bp) fragment from several nymphophiline species. This primer pair did not work consistently so we designed a second set of oligonucleotide primers, ND43F and

Table 1 Details of samples and localities

Code	Sample size COI	NDI	Locality
P. micrococcus			
[1]M1 (1)	16	2	Spring, Fleur de Lis Ranch, Oasis Valley, Nye Co., NV
M2 (6)	3	4	Grapevine Springs, Grapevine Mountains, Death Valley, Inyo Co., CA
M3 (6)	2	4	Spring east of Scottys Castle, Grapevine Canyon, Death Valley, Inyo Co., CA
M4 (6)	2	4	Spring west of Scottys Castle, Grapevine Canyon, Death Valley, Inyo Co., CA
M5 (6)	1	3	Surprise Spring, Grapevine Mountains, Death Valley, Inyo Co., CA
M7 (2)	2	—	Last Chance Spring, Ash Meadows, Nye Co., NV
M8 (2)	1	—	Purgatory Spring, Ash Meadows, Nye Co., NV
M9 (8)	2	—	Stream below Darwin Falls, Darwin Canyon, Argus Range, Panamint Valley, Inyo Co., CA
M10 (8)	2	1	China Garden Spring, Darwin Canyon, Argus Range, Panamint Valley, Inyo Co., CA
M11 (8)	1	1	Unnamed spring above Darwin Falls, Darwin Canyon, Argus Range, Panamint Valley, Inyo Co., CA
M12 (5)	2	2	Lower spring, Johnson Canyon, Panamint Mountains, Death Valley, Inyo Co., CA
M13 (5)	6	5	Stream, Hanaupah Canyon, Panamint Mountains, Death Valley, Inyo Co., CA
M14 (7)	2	1	Stream, Jail Canyon, Panamint Mountains, Panamint Valley, Inyo Co., CA
M15 (7)	2	1	Stream, Hall Canyon, Panamint Mountains, Panamint Valley, Inyo Co., CA
M16 (8)	1	1	Spring, Snow Canyon, Argus Range, Panamint Mountains, Inyo Co., CA
M17 (7)	1	1	'Cottonwood Spring', Wildrose Canyon, Panamint Mountains, Panamint Valley, Inyo Co., CA
M18 (9)	1	1	Saline Marsh, Saline Valley, Inyo Co., CA
M19 (10)	1	1	Cushenbury Springs, San Bernardino Mountains, San Bernardino Co., CA
M20 (11)	1	1	Springs at Big Bear Ranger Station, San Bernardino Mountains, San Bernardino Co., CA
M21 (11)	2	1	Springs, Thurman Flats, Mill Creek Canyon, San Bernardino Mountains, San Bernardino Co., CA
M22 (8)	3	1	Spring, Knight Canyon, Argus Range, Panamint Valley, Inyo Co., CA
M24 (8)	1	2	Tennessee Spring, Argus Range, Panamint Valley, Inyo Co., CA
M25 (4)	3	2	Spring north of Tecopa Hot Springs, Inyo Co., CA
M26 (3)	2	3	Shoshone Spring, Inyo Co., CA
M27 (7)	1	1	Stream below Limekiln Spring, Surprise Canyon, Panamint Mountains, Panamint Valley, Inyo Co., CA
M28 (7)	1	1	Brewery Spring, Surprise Canyon, Panamint Mountains, Panamint Valley, Inyo Co., CA
M29 (2)	1	—	Spring east of Crystal Reservoir, Ash Meadows, Nye Co., NV
M30 (2)	1	1	Spring south of Clay Pits, Ash Meadows, Nye Co., NV
M31 (1)	1	—	Goss Springs, Oasis Valley, Nye Co., NV
Outgroup taxa			
[1]*Marstonia agarhecta*	1	1	Bluff Creek, Lower Ocmulgee River basin (Southern Atlantic drainage), Pulaski Co., GA
P. amargosae	1	1	Saratoga Spring, Death Valley, San Bernardino Co., CA
P. californiensis	1	1	Spring tributary to Snow Creek, Whitewater River basin (Salton Sea Basin), Riverside Co., CA
P. crystalis	1	1	Crystal Pool, Ash Meadows, Nye Co., NV
P. deaconi	1	1	Red Spring, Spring Mountains, Las Vegas Wash (Lower Colorado River basin), Clark Co., NV
P. erythropoma	1	1	Kings Pool (outflow), Point of Rocks, Ash Meadows, Nye Co., NV
P. fausta	1	1	Corn Creek Springs, Las Vegas Wash (Lower Colorado River basin), Clark Co., NV
[1]*P. giulianii*	1	1	Stream, Sand Canyon, Indian Wells Valley (Northern Mojave basin), Kern Co., CA
[1]*P. greggi*	1	1	Grapevine Creek, Fort Tejon Historical State Park (Middle Kern-Tehachapi basin), Kern Co., CA
P. isolatus	1	1	Spring south of Clay Pits, Ash Meadows, Nye Co., NV
P. nanus	1	1	Five Springs, Ash Meadows, Nye Co., NV
[1]*P. owensensis*	1	1	Stream in canyon south of Piute Creek, Owens River basin, Mono Co., CA
P. perturbata	1	1	Northeast Spring, Fish Slough, Owens River basin, Mono Co., CA
[1]*P. stearnsiana*	1	1	Springs, Wildcat Canyon, El Sobrante, San Francisco Bay drainage, Contra Costa Co., CA(WC)
P. stearnsiana	1	1	Partington Creek (Central California coastal drainage), Monterey Co., CA(PC)
P. stearnsiana	1	1	Stream, Colson Canyon, Santa Maria River basin (Central California coastal drainage), Santa Barbara Co., CA (CC)
P. sterilis	1	1	Spring, Hunts Canyon Ranch, Ralston Valley (Great Basin), Nye Co., NV
[1]*P. turbatrix*	1	1	Horseshutem Springs, Pahrump Valley (Great Basin), Nye Co., NV
[1]*P. wongi*	1	1	Spring, lower Pine Creek Canyon, Owens River basin, Inyo Mountains, Inyo Co., CA
P. n. sp. 1	1	1	Spring, Amargosa Gorge, Inyo Co., CA
P. n. sp. 2	1	1	Saratoga Spring, Death Valley, San Bernardino Co., CA

Numbers in parentheses after locality codes refer to sampling areas shown in Fig. 1.
[1]COI sequence reported by Hershler *et al.* (2003).

RND592F, based on nymphophiline sequences to amplify and sequence the remaining specimens. These primer sequences are listed below. The numbers in parentheses refer to the position of the 5′ base in the *Littorina saxatilis* sequence. Degenerate positions are represented by the following ambiguity codes: Y = C/T; R = A/R. To verify the amplified products as NDI, sequences were compared with GenBank data using a BLAST analysis. NDIL (5892): 5′ GTA TTA TTA GCA GTC GCT T 3′; NDIH (6460): 5′ ATA CTA ATT CTG ATT CTC CTT 3′; ND43F (5890): 5′ GCA TCY TAY TAG CAG TCG CTT 3′; RND592F (6441): 5′ TCT GCA AAR TCR AAA GGT GC 3′.

Genomic DNA was extracted from individual snails using a CTAB protocol (Bucklin 1992). Amplifications were conducted in a 25-μL total volume, containing 5 μL of Invitrogen optimizer buffer F (10 mM $MgCl_2$, pH 9.0) (Invitrogen, Inc.) for COI and buffer D (17.5 mM $MgCl_2$, pH 8.5) for NDI, 2.5 μL dNTPs (2.5 mM each), 1.25 μL of each primer (10 μM), 1 unit *Taq* polymerase, 1 μL template (≈ 100 ng double-stranded DNA), and 13.8 μL sterile water. For the COI gene, COIL1490 and COIH2198 (Folmer *et al.* 1994) or COIL1492 and COIH2390 (Liu *et al.* 2001) was used to amplify a ≈ 710-bp or 900-bp fragment. For the NDI gene, NDIL and NDIH or ND43F and RND592F were used to amplify a ≈ 570-bp or 550-bp fragment. The temperature profile for the polymerase chain reaction (PCR) consisted of an initial 2-min denaturation step at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 45–50 °C for COI or 55 °C for NDI, 2 min at 72 °C, and a final extension step at 72 °C for 7 min. Amplified DNA was resolved by electrophoresis on 1.5% agarose gel that was stained with ethidium bromide to check for length, quality and quantity. The amplified PCR product was incubated at 37 °C for 30 min and then at 85 °C for another 15 min with five units of Exonuclease I (*Exo*I, Amersham) and 0.5 unit Shrimp Alkaline Phosphatase (SAP, Amersham) to cleave nucleotides one at a time from an end of excess primers and to inactivate single nucleotides. Approximately 10–30 ng of cleaned PCR product was used as a template in a cycle sequencing reaction using the CEQ DTCS Quick Start Kit (Beckman Coulter, Inc.). The following cycling conditions were used: 96 °C for 2 min, then 30 cycles of 96 °C for 20 s, 45 °C for 20 s, and 60 °C for 4 min. The cycle-sequenced product was cleaned using the Beckman Coulter protocol. Fluorescent dye-labelled DNA was combined with 4 μL stop solution (equal volume of 100 mM ethylenediamine tetraacetic acid and 3 M NaOAc pH 5.2), 1 μL glycogen (20 mg/mL), and 10 μL milli-Q H_2O, mixed well, and precipitated with 60 μL cold 95% (v/v) ethanol/water. Fluorescent dye-labelled DNA was recovered by centrifuging at 12 600 *g* for 20 min at 4 °C. Pellets were washed with 100 μL 70% (v/v) ethanol/water, air-dried and resuspended in 40 μL of dimethylformamide. Resuspended samples were added to the appropriate wells of the CEQ sample plate, overlayed with mineral oil, and run on the Beckman Coulter CEQ8000. Sequences were determined for both strands and were edited and aligned using SEQUENCHER™.

Extensive COI sampling within one of the more morphologically variable populations revealed only slight haplotype variation (see Results) and thus we generally sequenced only one to three snails per population. Owing to the small number of specimens collected from most sites, COI (which was sequenced first) was generally sampled more extensively than NDI. Although the same specimens were usually used for sequencing of both COI and NDI, DNA was sometimes depleted after multiple attempts to amplify one of these genes, and therefore additional snails had to be used for sequencing of the other region. All new sequences have been deposited into GenBank (Accession Numbers AY367415–AY367557).

Since somewhat different sets of specimens were used to sequence the two genes, we decided not to combine the COI and NDI data sets for analysis. Phylogenetic trees were estimated by distance, parsimony and maximum-likelihood methods using PAUP* 4.0b10 (Swofford 2002). The Hasegawa Kishino–Yano (HKY) model with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution (e.g. HKY + I + Γ; Hasegawa *et al.* 1985) was selected as the best fit to both the COI and NDI data (MODEL-TEST 3.06; Posada & Crandall 1998). HKY distances corrected for invariable sites and rate variation were used to generate neighbour-joining trees based on the clustering method of Saitou & Nei (1987). Node support was assessed by completion of 1000 bootstrap replications (Felsenstein 1985) in PAUP, using the fast-search option.

Mutational saturation of COI and NDI for each codon was examined by plotting the absolute number of transitions and transversions against inferred genetic distance (HKY distance), and by plotting *p*-distance against inferred distance (HKY distance) (Berbee *et al.* 1995; Griffiths 1997; Siemer *et al.* 1998). No mutational saturation was evident for any of the three codon positions. Maximum-parsimony analyses were conducted with equal weighting, using the heuristic search option with tree bisection reconnection branch-swapping and 100 random additions. Bootstrapping with 1000 replications (as implemented in PAUP) was used to evaluate node support.

Maximum-likelihood analyses were based on the HKY + I + Γ model with empirical base frequencies using the heuristic search algorithm. A neighbour-joining tree (uncorrected '*p*' distances) was used as the initial topology for branch-swapping. Node support was evaluated by 100 bootstrap pseudoreplicates.

Bayesian phylogenetic analyses were conducted using MRBAYES 2.0 (Huelsenbeck & Ronquist 2001). Again, the HKY + I + Γ model was used in all analyses. All Bayesian analyses were initiated with random starting trees and were run for 2×10^7 generations. Sampling the Markov

chains at intervals of 100 generations thinned the data to 200 000 sample points. Since 35 000 generations were required for likelihood convergence, we discarded the first 350 trees as the 'burn-in.' After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis and data processing. Each sample included a tree topology that contained branch lengths. The topologies were used to generate a 50% majority rule consensus tree, with the percentage of samples recovering any particular clade representing that clade's posterior probability (Huelsenbeck & Ronquist 2001).

Maximum parsimony, maximum likelihood, and Bayesian methods yielded results that differed from neighbour-joining topologies only in the positioning of major clades. Since this paper focuses on the composition of these clades, rather than their weakly supported placements, we decided to only present the results of the neighbour-joining analyses.

Results

Sixty-five specimens of *Pyrgulopsis micrococcus* were sequenced for COI, while NDI sequences were obtained from 45 specimens. Empirical nucleotide base frequencies were biased towards adenine and thymine. Average base frequencies (25.01% A, 36.30% T, 20.19% C and 18.49% G for COI sequences and 27.42% A, 36.45% T, 19.91% C and 16.22% G for NDI sequences) were typical of gastropod mitochondrial genes (e.g. Collins *et al.* 1996; Hershler *et al.* 1999; Liu *et al.* 2001). The alignment of COI sequences yielded 623 bp, of which 214 sites were variable (34.4%) and 152 were parsimony informative (24.4%). Substitutions at the third position, which were predominantly silent, accounted for 83.2% of the variation, while the first and second positions accounted for 14.95% and 1.86%, respectively. For NDI, 507 bp were sequenced, of which 213 were variable (42.1%) and 158 were parsimony informative (31.2%).

Thirty-one COI and 17 NDI haplotypes were observed for *P. micrococcus*. Neighbour-joining trees based on COI (Fig. 2) and NDI (Fig. 3) sequences agreed in their depiction of a polyphyletic *P. micrococcus* and resolved its haplotypes among five well-supported clades (A–E, bootstrap values 89–100%). Clade E had a well-supported (97–99%) sister relationship with an undescribed species from the Amargosa River basin while Clade A was depicted, with moderate bootstrap support (56–65%), as sister to another undescribed species from this drainage. The sister relationships of clades B–D were not well resolved. Uncorrected pairwise sequence divergences among these clades were 3.69–10.59% for COI and 4.35–12.06% for NDI. These are similar to values among other congeners that were included in this study (1.1–11.6%, COI; 1.98–14.42%, NDI) and also conform to sequence divergences among species of *Tryonia*, another western American snail belonging to the superfamily Rissooidea (1.3–14.8%, COI; Hershler *et al.* 1999). The depth of these separations, together with the sister relationships depicted between monophyletic groups of *P. micrococcus* populations and other congeners, suggests that none of the five clades which contain *P. micrococcus* populations are conspecific.

Clades A–C each consisted of weakly divergent ($P <$ 0.8% for both genes) populations that are locally distributed within a single watershed (Amargosa headwaters, northern Death Valley, Ash Meadows, respectively) and presumably represent single species. However, the structuring of the other two clades suggests that both of these may consist of multiple species. Clade E consisted of four *P. micrococcus* populations from northern Death Valley and the upper Amargosa River basin. Although these were not extensively sampled [and NDI was not sequenced for one of them (M8)], they nonetheless belong to separate mtDNA lineages and differed from each other by $P > 1.3\%$ (for both genes), suggesting that they may be distinct species. Clade D contained three weak to moderately supported subclades consisting of *P. turbatrix*, which lives in southwestern Nevada (Hershler 1998), and groups of *P. micrococcus* populations from the Death Valley area, and the San Bernardino Mountains (southern Mojave and Santa Ana drainages). *Pyrgulopsis turbatrix* is morphologically distinctive (Hershler 1998) and differs from other members of clade D by $P =$ 1.3–2.1% (COI) and 0.99–1.78% (NDI), which suggests to us that it is a distinct species. The two broadly disjunct groups of *P. micrococcus* populations in this clade differ from each other by 0.64–1.77% (COI) and 0.7–1.78% (NDI) and may be interpreted as either distinct species or incipient species. The sequence divergences among the clades and subclades discussed above are summarized in Table 2.

Although we generally surveyed no more than three individuals per population, the haplotypes observed within populations of *P. micrococcus* were closely similar in all cases ($P < 0.6\%$ for both genes). An extensive (16 specimens) survey of COI from one sample (M1) provided more convincing evidence of limited intrapopulation variation. The three haplotypes observed differed by only 2–4 bp, and two of these were shared by the two distinctive shell morphs (which do not represent sexual dimorphism) present in this population (Fig. 4).

Thirty-nine of 48 haplotypes were restricted to single populations. Clades A, B and C each contained one or two haplotypes that were shared among two closely proximal populations (within a single watershed). However, clade D contained three haplotypes that were shared among three to five more widely separated populations, e.g. those living in the ranges which flank Panamint Valley, and those distributed on either side of the divide formed by the Panamint Range.

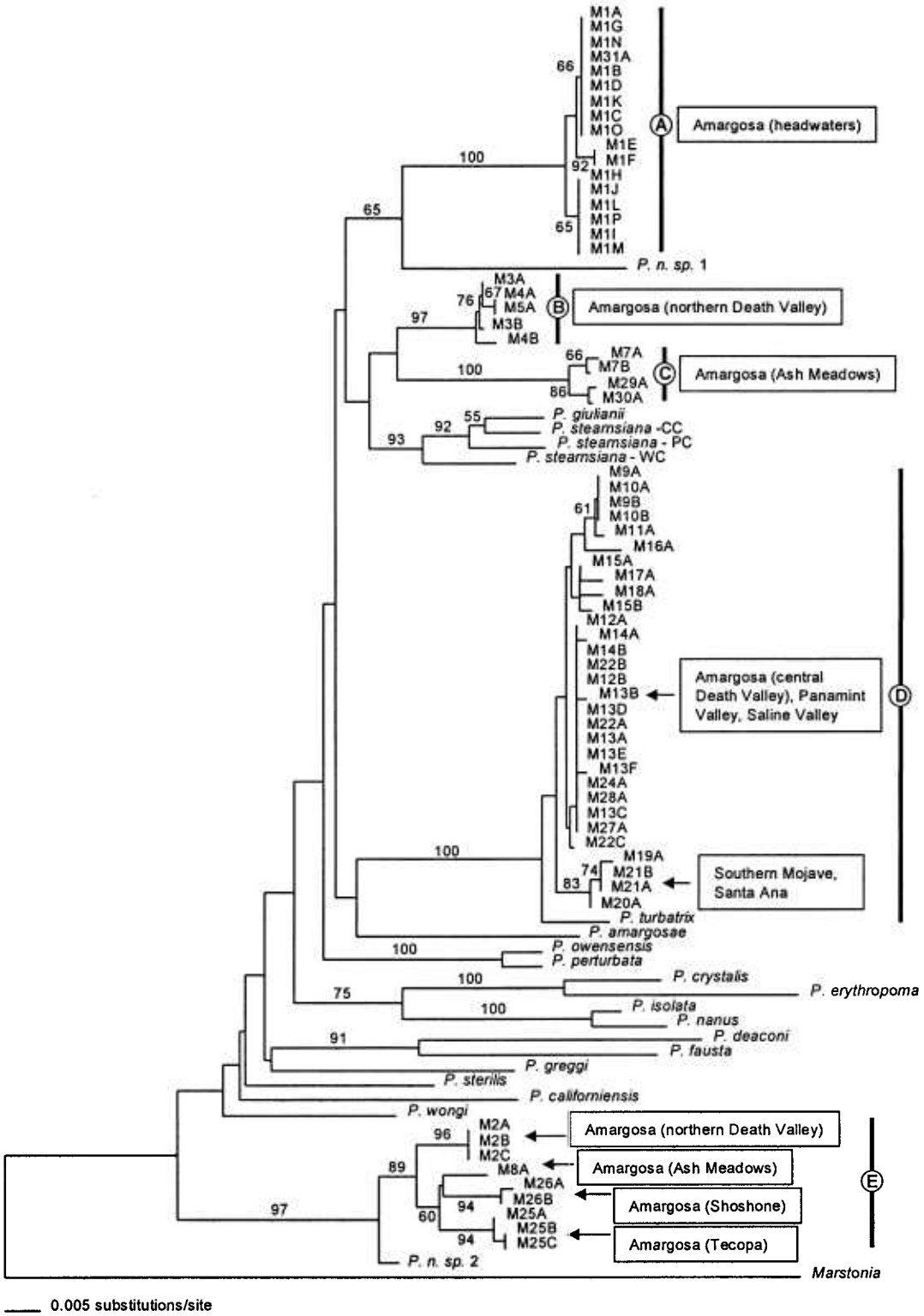

Fig. 2 Neighbour-joining phylogram inferred from partial COI sequences using an HKY substitution model. Major clades containing *Pyrgulopsis micrococcus* populations are labelled A–E and subclades discussed in the text are indicated by arrows.

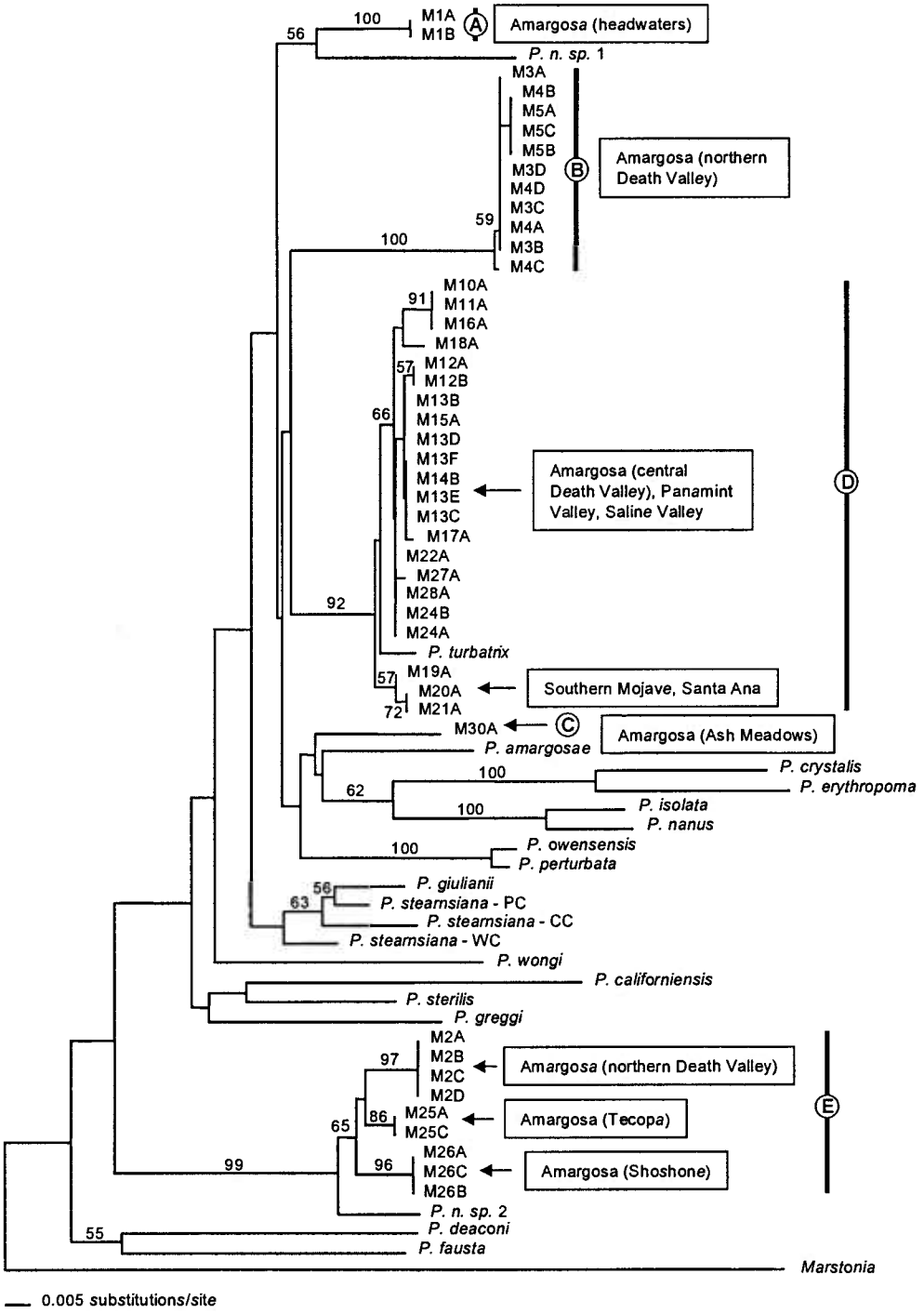

Fig. 3 Neighbour-joining phylogram inferred from partial NDI sequences using a HKY substitution model. Major clades containing *Pyrgulopsis micrococcus* populations are labelled A–E and subclades discussed in the text are indicated by arrows.

Discussion

Phylogenetic analyses of COI and NDI sequences depict *Pyrgulopsis micrococcus* as a polyphyletic composite of five well-supported clades. Note that congruent results were obtained in a recent (unpublished) phylogenetic study of 13 populations of this species based on mitochondrial 16S and nuclear internal transcribed spacer I (ITS-1) molecular markers (Kepes 2003). Our results imply that *P. micrococcus* should be restricted to clade A, which contains two populations from the type locality area of this species (Oasis Valley; Pilsbry in Stearns 1893). The other 27 populations

Table 2 Uncorrected *p* genetic distances (NDI above diagonal, COI below diagonal) for all pairwise comparisons among major clades and subclades of *Pyrgulopsis micrococcus* populations

Clade Subclade	A	B	C	D Death Valley area	D San Bernardino Mts	E Northern Death Valley	E Ash Meadows	E Shoshone	E Tecopa
Population	1,31	3,4,5	7,29,30	9,10,11,12,13, 14,15,16,17,18, 22,24,27,28	19,20,21	2	8	26	25
A	0 0–0.64	7.12–7.31	5.34	4.35–5.14	4.55–4.74	10.28	—	10.47	10.28
B	3.85–4.49	0–0.4 0–0.48	6.92–7.12	5.73–6.32	5.73–6.13	10.28–10.47	—	10.08–10.28	9.88–10.08
C	5.78–6.42	3.69–4.33	0 0.16–0.8	4.94–5.73	4.94–5.14	10.28	—	12.06	10.87
D (Death Valley area)	6.42–7.22	5.14–6.10	6.42–7.38	0–1.19 0–1.12	0.7–1.78	9.68–9.88	—	9.68–10.47	9.49–10.47
D (San Bernardino)	6.58–7.06	5.62–6.10	7.06–7.54	0.64–1.77	0–0.2 0–0.32	9.68–9.88	—	9.49–9.68	9.09–9.29
E (N. Death Valley)	8.99–9.30	8.19–8.35	9.31–9.63	8.99–9.63	9.47–9.79	0 0	—	1.58	1.98
E (Ash Meadows)	9.43–9.60	8.77–8.95	9.63–9.98	9.47–9.79	9.46–9.63	1.54	— 0	—	—
E (Shoshone)	9.31–9.47	8.19–8.51	9.31–9.79	9.63–10.43	9.63–10.11	1.76–1.93	1.37–1.54	0 0.16	1.98
E (Tecopa)	9.79–10.11	8.03–8.35	9.79–10.27	9.15–9.79	10.11–10.59	1.93–2.09	1.39–1.56	1.77–2.09	0 0–0.16

NDI data are missing for clade E (Ash Meadows).

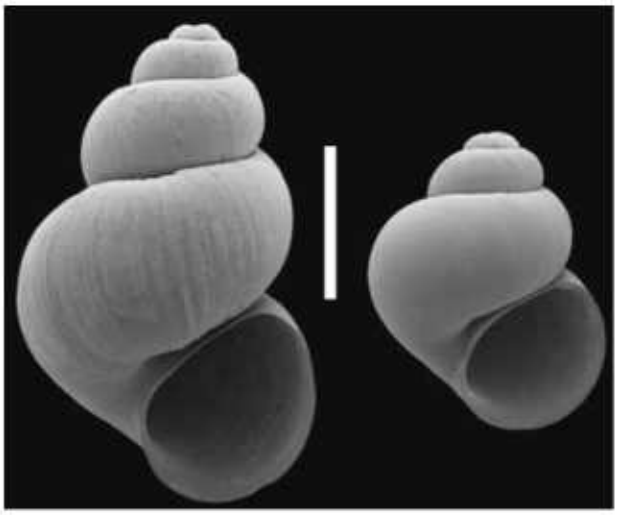

Fig. 4 Shell variation within *Pyrgulopsis micrococcus* population M1. Scale bar = 1.0 mm.

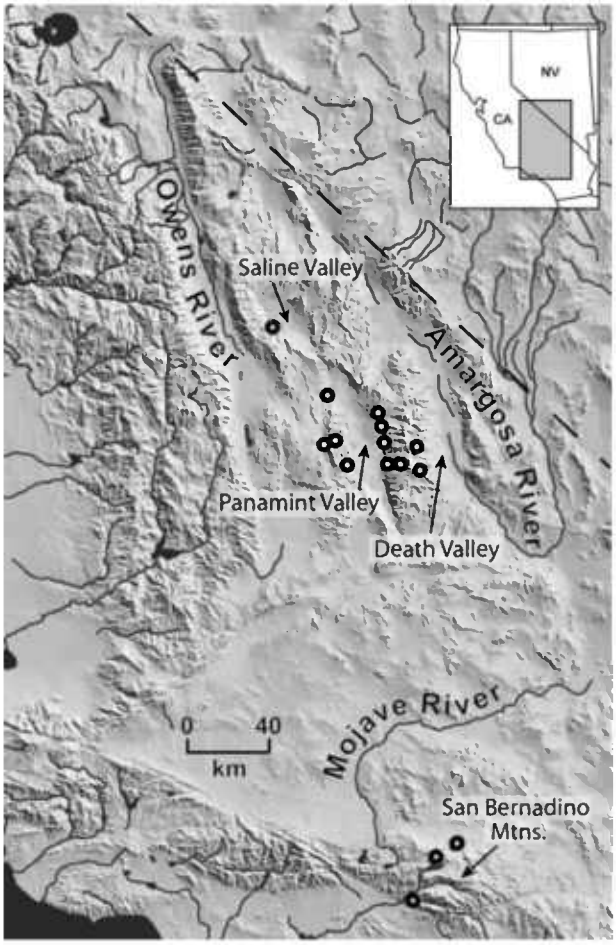

Fig. 5 Map showing distribution of *Pyrgulopsis micrococcus* populations belonging to clade D. Some symbols represent more than one locality.

of *P. micrococcus* that we sampled may represent as many as seven or eight previously undescribed species. None of these distinctive lineages co-occur in the same spring and thus we have no evidence that their molecular differentiation is accompanied by reproductive isolation. Note, however, that sympatry of *Pyrgulopsis* species is rare and usually occurs only in larger, more complex habitats than those inhabited by *P. micrococcus*. We also have no evidence of obvious ecological divergence of these lineages, although most species of *Pyrgulopsis* occupy closely similar niches. The minor morphological variation that has been documented among populations of *P. micrococcus* (Hershler & Sada 1987; Hershler 1989) also provides little clue of the underlying phylogenetic diversity revealed by molecular data. However, it has been previously shown that characters used to describe and diagnose species in this genus provide little phylogenetic resolution (Hershler 1994). Note that while the diagnostic penial features of *P. micrococcus* are locally distinctive, they are shared by various congeners from other western regions (Hershler 1994; Hershler & Sada 2002). The phylogenetic diversity of populations assigned to *P. micrococcus*, and the absence of a close relationship between any of these and *P. stearnsiana* (see Figs 2, 3), a geographically proximal congener which has an almost identical penial morphology, provides additional evidence that these characters are either primitive or subject to homoplasy and do not well delineate monophyletic groups even within a relatively small western area.

Given the absence of a fossil record informative of branching events within *Pyrgulopsis* (Hershler & Sada 2002), or a geological event that can readily be correlated with a phylogenetic node, we cannot directly calibrate the divergence times of these putative species. However, if we assume an evolutionary rate of 1.8% per million years for COI based on sequences of other hydrobiid snails (Wilke 2003), then divergence of these lineages occurred 1.17–19.4 million years ago (Ma). Although these divergence times must be treated cautiously for various reasons (e.g. possible differences in substitution rates among these taxa), they nonetheless are consistent with fossil evidence that *Pyrgulopsis* originated during the Miocene (Hershler & Sada 2002) and suggest that the distributions of *P. micrococcus* lineages could have been shaped by vicariant events ranging from tectonic development of the modern landscape in the mid-Tertiary to drainage rearrangements during the Pleistocene.

Our findings indicate that widely ranging *P. micrococcus* contains multiple, genetically distinctive and geographically restricted subunits consistent with the presumably limited dispersal ability of these animals. However, our data also suggest that recent dispersal may have influenced the biogeography of one of these lineages. In contrast with the other clades, clade D contains weakly divergent lineages that are widely scattered within a large, topographically complex region (Fig. 5). This

biogeographic pattern is not congruent with pertinent drainage history. For example, the only well-documented episode of late Cenozoic integration between the widely separated northern and southern areas occupied by these snails occurred 18 000–12 000 years ago (late Pleistocene), when the Mojave River was briefly connected to the Amargosa–Death Valley drainage via a series of overflowing pluvial lakes (Meek 1989; Anderson & Wells 1996; Anderson 1999). However this interval of drainage connectivity occurred well after the hydrographic isolation of (other) inhabited basins within both of these areas. In the northern area, Panamint Valley became isolated after its pluvial lake last overflowed into Death Valley 0.6–0.7 Ma (Jannik *et al.* 1991). Saline Valley was not integrated with either the Death or Panamint Valleys during the Pleistocene and may have been hydrographically isolated since its tectonic inception, which occurred 3.0–1.8 Ma (Burchfiel *et al.* 1987; Oswald & Wesnousky 2002). Within the southern area, the drainages which flank the southern slope of the San Bernardino Mountains became isolated (from the modern Mojave River basin) ≈ 2.0–1.5 Ma, coincident with uplift of this range (Sadler & Reeder 1983; Cox *et al.* 1998; Albright 1999). (Note that our phylogenetic hypotheses paradoxically imply that separation of the northern and southern areas preceded isolation of individual basins within these areas; Figs 2, 3.) Although integration of all of the basins occupied by members of clade D may have been tenable prior to the development of modern regional topography, the necessarily ancient fragmentation of such a drainage conflicts with the remarkably limited divergence among the populations in this clade (Table 2).

The absence of physical evidence of geologically recent integration of inhabited drainages consistent with the shallow structuring of *P. micrococcus* populations of clade D implies that this biogeographic pattern may not have been solely determined by the history of surface-water connections. The distribution of clade D along an inland sector of the Pacific Flyway, which is a major migratory route (Small 1994), suggests to us that passive dispersal on (the feet and plumage of) waterfowl and wading birds may have played a role in the biogeographical history of these snails. (Similar hypotheses have been previously proposed to explain the distributions of other hydrobiid snails; e.g. Ponder *et al.* 1994; Wesselingh *et al.* 1999.) The scattered bodies of open water and wetlands in this desert area currently serve as important resting places for migratory waterbirds (e.g. Shuford *et al.* 2002) and the abundant fossil record of these animals in regional Pleistocene lake deposits (e.g. Jefferson 1985) testifies to a similar use of such habitats during the recent past. During the wetter or pluvial periods of the late Pleistocene and early Holocene, when the region contained many more perennial and seasonal lakes and marshes than at present (Smith & Street-Perrott 1983), the periodic stopping of migratory birds could have effected the transfer of snails among habitats that did not have a direct aquatic connection, resulting in a broad distribution within the flyway region. Populations founded in this manner may have been isolated for less than 10 000 years, which is consistent with their limited genetic differentiation and the extensive sharing of haplotypes among hydrographically separated localities (see above). The feasibility of such a dispersal mode is supported by experimental evidence (e.g. Malone 1965; Boag 1986), and anecdotal records of small aquatic snails recovered from the feet and plumage of captured birds (e.g. Darwin 1859; Roscoe 1955; Rees 1965). We do not have a ready explanation for why birds may have served as agents of dispersal for clade D but not for other lineages of *P. micrococcus* (which also live along the Pacific Flyway). Perhaps the phylogenetic diversity of these morphologically and ecologically similar snails is mirrored by biological differences which had an impact upon the probability of successful 'aerial dispersal' (e.g. tolerance to desiccation or salinity). This hypothesis can be tested by a comparative physiological study of these clades. Additional sampling of snail populations is also desirable as it would enable a robust estimation of migration rate (gene flow) pertinent to our passive dispersal hypothesis.

Despite its importance as a regional biodiversity jewel that is imperilled by the various threats to its fragile habitats (Hershler & Williams 1996; Sada & Vinyard 2002), *Pyrgulopsis* has been little studied aside from the original descriptions of its species. The limited attention that has been paid to *Pyrgulopsis* may be attributed to its tiny size and relatively simple morphology, and to the relatively recent (post-1970) discovery and taxonomic description of most of its species. The DNA sequence data presented herein indicate that the limited morphological variation of widely ranging *P. micrococcus* masks striking phylogenetic diversity and suggest that a similar re-examination of other congeners will probably result in the detection of additional previously unrecognized species. The number of cryptic species awaiting discovery may be very large given that some congeners range over huge geographical areas which encompass many isolated drainage basins (e.g. *P. gibba*, *P. kolobensis*; Hershler 1998; Fig. 54). This revisionary process, which should include new morphological studies that seek to identify phylogenetically robust characters, together with the continuing discovery of new species at a high rate in association with field surveys (e.g. Hershler 1998), will further substantiate *Pyrgulopsis* as a remarkably speciose element of North American biodiversity.

This paper has also provided an initial framework for robust biogeographical analysis of *Pyrgulopsis*. Continued studies of *Pyrgulopsis* using molecular markers, coupled with investigations of relevant aspects of its biology, life history and palaeoecology, will undoubtedly yield additional insights into the temporal interplay of dispersal and vicariance in shaping the diversification of this huge genus.

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