ZOOLOGICAL Journal of the Linnean Society

Zoological Journal of the Linnean Society, 2013, **167**, 227–242. With 8 figures

Cryptic diversity within and amongst spring-associated *Stygobromus* amphipods (Amphipoda: Crangonyctidae)

JOSHUA Z. ETHRIDGE[1]*, J. RANDY GIBSON[2] and CHRIS C. NICE[1]

[1]*Department of Biology, Texas State University, 601 University Drive, San Marcos, TX 78666, USA*
[2]*San Marcos US Fish and Wildlife Service, San Marcos, TX, USA*

Received 3 May 2012; revised 14 September 2012; accepted for publication 24 September 2012

Multiple species of troglomorphic, spring-associated *Stygobromus* amphipods, including the endangered, narrow-range endemic *Stygobromus pecki*, occupy sites in the Edwards Plateau region of North America. Given the prevalence of cryptic diversity observed in disparate subterranean, animal taxa, we evaluated geographical genetic variation and tested whether *Stygobromus* contained undetected biodiversity. Nominal *Stygobromus* taxa were treated as hypotheses and tested with mitochondrial sequence cytochrome oxidase C subunit 1, nuclear sequence (internal transcribed spacer region 1), and AFLP data. *Stygobromus pecki* population structure and diversity was characterized and compared with congeners. For several *Stygobromus* species, the nominal taxonomy conflicted with molecular genetic data and there was strong evidence of significant cryptic diversity. Whereas *S. pecki* genetic diversity was similar to that of congeners, mitochondrial data identified two significantly diverged but sympatric clades. AFLP data for *S. pecki* indicated relatively recent and ongoing gene flow in the nuclear genome. These data for *S. pecki* suggest either a substantial history of isolation followed by current sympatry and ongoing admixture, or a protracted period of extremely large effective population size. This study demonstrates that Edwards Plateau *Stygobromus* are a complex, genetically diverse group with substantially more diversity than currently recognized.

© 2013 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2013, **167**, 227–242.
doi: 10.1111/j.1096-3642.2012.00877.x

ADDITIONAL KEYWORDS: conservation – karst habitat – phylogenetics – population genetics – spring endemic.

INTRODUCTION

Freshwater crustaceans of the genus *Stygobromus* (Amphipoda: Crangonyctidae), commonly called cave amphipods, are distributed in subterranean aquatic ecosystems across North America and eastern Eurasia. Representatives are found in the Edwards Plateau region of North America (Fig. 1), including the federal and state endangered Peck's cave amphipod, *Stygobromus pecki* (Holsinger, 1967), a short-range spring-endemic species that was the focus of this study. Holsinger (1966, 1967, 1974, 1978) initially studied *Stygobromus* systematics using morphological data to define species and species-groups. Holsinger intended species-groups (an informal category below genus) to reflect evolutionary relationships amongst species and proposed a phylogenetic hypothesis for each (Holsinger, 1967). We examined species in the *flagellatus* (including the endangered *S. pecki*), *hadenoecus*, and *tenuis* species-groups. The *flagellatus* and *hadenoecus* species-groups are endemic to the Edwards Plateau, whereas the *tenuis* species-group is found throughout the eastern half of the USA. Holsinger's (1967) phylogenetic proposal for the *flagellatus* species-group is presented in Figure 2A. Phylogenetic proposals are not presented for the *hadenoecus* and *tenuis* species-groups because the former is monotypic and the latter has limited representation in the Edwards Plateau.

All *Stygobromus* species exhibit cave adaptation or troglomorphic convergence, a suite of adaptations

*Corresponding author. E-mail: josh.zeke@gmail.com

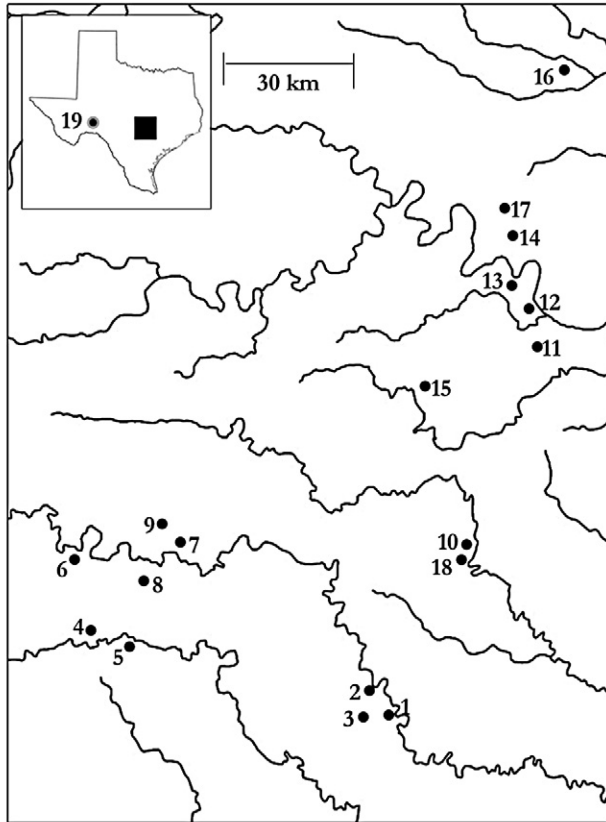

Figure 1. Sampling sites. Map showing *Stygobromus* sampling sites in Texas and their associated river drainages. (1) Landa Lake, (2) Hueco Springs, (3) Mission Valley Bowling Well, (4) Cascade Caverns, (5) Stealth Cave, (6) Cave-Without-A-Name, (7) CM Cave, (8) Honey Creek Cave, (9) Magic Springs, (10) Diversion Spring, (11) Blowing Sink Cave, (12) Cold Spring, (13) Barton Creek Well, (14) Kretchmarr Salamander Cave, (15) Sky Ranch Well, (16) San Gabriel Springs, (17) Adobe Springs, (18) Texas State Artesian Well, (19) upper Devils River springs. Straight-line distance between Devils River and central Texas is approximately 275 km.

relevant to fitness in cave environments that includes loss or reduction of eyes, loss or reduction of pigmentation, elongated appendages, decreased metabolic rates, and increased developmental periods (Porter, 2007). For taxa that already exhibit troglomorphic features, continued morphological diversification is constrained because the selective pressures in cave environments that caused troglomorphic convergence in the first place continue to demand that narrow range of adaptive responses. As troglomorphic convergence inhibits morphological diversification, there may be an increased probability of detecting cryptic species diversity in subterranean fauna. Cryptic diversity is recognizable when a significant molecular divergence between samples has no correlate(s) in morphology, physiology, or behaviour (Porter, 2007). In a practical sense, the presence of cryptic diversity prevents recognition of evolutionary lineages using morphology. As the current study utilizes molecular data, we have the opportunity to test for the presence of cryptic diversity within this genus.

The current study had three objectives relevant to the conservation genetics of *S. pecki* and the detection of hypothesized cryptic diversity within and amongst *Stygobromus* species. The first objective was to place *S. pecki* in an evolutionary context by examining its phylogenetic relationships with its non-endangered congeners. This objective allowed us to assess whether *S. pecki* is a clearly differentiated taxon, and which taxa are sister to it. The second objective was to describe the geographical distribution of genetic variation within *S. pecki* to inform captive propagation efforts through identification of genetic structure that may lead to the recognition of evolutionarily significant units (Crandall *et al*., 2000). The third objective was to assess *S. pecki* genetic diversity and compare it with genetic diversity of its non-endangered congeners and the federally endangered Comal Springs riffle beetle, *Heterelmis comalensis* Bosse, Tuff, & Brown, 1988 (Coleoptera: Elmidae), which co-occurs with *S. pecki* and was studied previously by Gonzales (2008). Gonzales (2008) found that *H. comalensis* populations at higher-elevation sites were genetically impoverished relative to populations at lower-elevation sites (elevation differences are on the order of several metres only, but appear to be significant). She hypothesized that the pattern was caused by cessation of baseflow to the higher-elevation sites during the record drought of the 1950s, which caused bottlenecks or extirpations followed by founder recolonization within *H. comalensis*. Here, we compared measures of *S. pecki* genetic diversity with the findings of Gonzales (2008) for *H. comalensis*.

To address these objectives, we asked four questions. (1) Do nominal species and species-groups (Holsinger, 1967) comprise monophyletic clades with molecular data? (2) Are molecular data congruent with the proposed *flagellatus* species-group phylogeny (Holsinger, 1967) that contains the endangered *S. pecki*? (3) Is there evidence of population structure within *S. pecki*? (4) How do levels of genetic variation within *S. pecki* compare with its non-endangered congeners and the endangered *H. comalensis*?

MATERIAL AND METHODS

Sampling and molecular methods

Between 2004 and 2011, *Stygobromus* individuals were sampled from 19 Edwards Plateau sites [Table 1, Fig. 1, sites are reported as '(site #) site

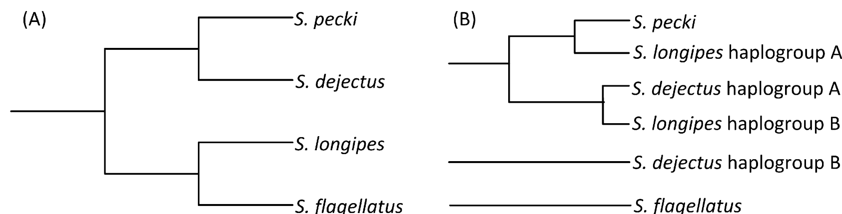

Figure 2. Phylogenetics of the *flagellatus* species-group. A, proposed phylogeny for the *flagellatus* species-group (Holsinger, 1967). B, summary of relationships between these taxa suggested by molecular data (see Results). Relationships for *Stygobromus dejectus* haplogroup B and *Stygobromus flagellatus* are uncertain owing to small sample size and conflicting cytochrome oxidase C subunit 1 (COI) and internal transcribed spacer (ITS1) signals.

name' throughout this text] using several methods: (1) drift nets and a cloth-capture technique (Gibson, Harden & Fries, 2008) at surface springs, (2) bottle traps in wells, and (3) hand collection with dip nets in caves. Specimens were typically stored in 95% ethanol immediately after collection. Sampling efforts focused on the endangered *S. pecki* at sites within (1) Landa Lake (Fig. 3), collected under permits from the United States Fish and Wildlife Service (permit TE876811) and the Texas Parks and Wildlife Department (permit SPR-0390-045). Sampling of non-endangered *Stygobromus* taxa was designed to represent species-groups.

Species identifications were carried out from combinations of morphological characters using Holsinger's (1967) keys and descriptions. Some individuals lacked species-level identification because they were not yet mature adults (required for species-level identification based on morphology). Some of these individuals had sufficient characters to rule out other closely related species but lacked sufficient characters to positively key to a particular species – these are labelled using the conformis convention (e.g. *Stygobromus* cf. *russelli*). Other individuals had sufficient characters to key to a species-group, but lacked sufficient characters to key to a particular species – these individuals are labelled with the term *unknown* and their species group (e.g. *unknown flagellatus*). In all analyses, conformis and unknown specimens were treated as their own species, separate and distinct from any species to which they were morphologically near.

For specimens with a body length greater than 4 mm, tissue samples were dissected from the middle of the organism to preserve the taxonomically relevant head and tail regions. Smaller specimens were vouchered by photograph and digested whole during extraction. DNA extractions used the Gentra Systems Purgene DNA Isolation Kit (Minneapolis, MN) followed by rehydration with 100 μl double-distilled H_2O.

Mitochondrial sequence data from the cytochrome oxidase C subunit 1 (COI) were initially collected using primers designed by E. Sotka (pers. comm., Table 2). Using preliminary sequence results, species-group-specific internal primers were designed (Table 2) and used to amplify COI for the remaining individuals. Initial PCR reactions used standard protocols with annealing temperatures of 50–58 °C. Reactions with internal primers followed a touchdown protocol: the initial annealing temperature was 56–61 °C and reduced by 1 °C for each of the next five PCR cycles, after which the annealing temperature was held at 51–56 °C for 35 cycles.

Noncoding, single-copy nuclear sequence data from the internal transcribed spacer region 1 (ITS1) were collected using primers developed by Carlini *et al.* (2009). PCR reactions used standard protocols with an annealing temperature of 66.5 °C. Some reactions contained multiple amplicons so the desired PCR product was isolated from 2% agarose gels. Gel extraction and PCR clean-up used Promega Wizard SV Gel Kits (Madison, WI). Sequencing was performed in both directions for each individual and gene at the Nevada Genomics Center (Reno, NV) using Applied Biosystems Prism 3730 Analyzers (Carlsbad, CA). GENEIOUS v. 5.3 (Drummond *et al.*, 2011) was used to edit and align sequences.

Phylogenetic analysis

Using an alignment of all haplotypes for each gene, MODELTEST v. 3.7 (Posada & Crandall, 1998; Guindon & Gascuel, 2003; Felsenstein, 2005) within PAUP v. 4.0b (Swofford, 2002) was used to calculate likelihood scores for 56 models of evolution. To select the best-fit model, MRMODELTEST v. 2.3 (Nylander, 2004) was used to calculate Akaike information criterion (AIC) scores. As mitochondrial sequences were protein coding and contained large divergences, a model of evolution was selected for each codon position. MRBAYES v. 3.1.2 (Ronquist & Huelsenbeck, 2003) was used to generate Bayesian phylogenies for COI (Fig. 4) and ITS1 (Fig. 5) with confidence assessed by posterior probabilities. Phylogenies were examined for monophyly of species and species-groups, and topology

Table 1. Sample information and within-group cytochrome oxidase C subunit 1 (COI) diversity

Species (species-group)	Sampling Site	N_{COI}	N_{ITS}	N_{AFLP}	COI h ± SE	COI π_{within} ± SE
Stygobromus pecki (*flagellatus*)	Pooled	71	8	69	0.86 ± 0.04	0.011 ± 0.006
	S. pecki COI haplogroup A	[49]	[4]	[47]	0.74 ± 0.07	0.003 ± 0.002
	S. pecki COI haplogroup B	[22]	[4]	[22]	0.88 ± 0.07	0.005 ± 0.003
	(1a) Panther Canyon Well	(5)	(1)	(5)	0.70 ± 0.22	0.011 ± 0.008
	(1b) Spring Run 1	(11)	(2)	(11)	0.80 ± 0.11	0.010 ± 0.006
	(1c) Kiddy Pool	(3)		(2)	1.00 ± 0.27	0.013 ± 0.011
	(1d) Spring Run 3	(15)	(1)	(16)	0.78 ± 0.10	0.007 ± 0.004
	(1e) Upwelling	(9)		(9)	0.97 ± 0.06	0.015 ± 0.009
	(1f) West shore	(3)		(2)	1.00 ± 0.27	0.013 ± 0.011
	(1g) Spring Island	(15)	(3)	(14)	0.92 ± 0.05	0.011 ± 0.006
	(1h) Spring Run 5	(6)		(6)	1.00 ± 0.10	0.014 ± 0.009
	(1i) Spring Run 4	(3)		(3)	1.00 ± 0.27	0.017 ± 0.014
	(2) Hueco Springs	(1)		(1)	n/a	n/a
Stygobromus dejectus (*flagellatus*)	Pooled	12	6		0.86 ± 0.07	0.074 ± 0.039
	S. dejectus COI haplogroup A	[9]	[4]		0.78 ± 0.11	0.004 ± 0.003
	S. dejectus COI haplogroup B	[3]	[2]		0.67 ± 0.31	0.028 ± 0.022
	(4) Cascade Caverns	(10)	(6)		0.89 ± 0.08	0.085 ± 0.046
	(5) Stealth Cave	(2)			1.00 ± 0.50	0.002 ± 0.003
S. cf. *dejectus* (*flagellatus*)	(3) Mission Valley Bowling Well	1			n/a	n/a
Stygobromus longipes (*flagellatus*)	Pooled	8	4		0.96 ± 0.08	0.046 ± 0.026
	S. longipes COI haplogroup A	[7]	[4]		0.95 ± 0.10	0.028 ± 0.016
	S. longipes COI haplogroup B	[1]			n/a	n/a
	(6) Cave-Without-A-Name	(4)	(2)		1.00 ± 0.18	0.012 ± 0.008
	(7) CM Cave	(2)	(2)		1.00 ± 0.50	0.008 ± 0.009
	(8) Honey Creek Cave	(1)			n/a	n/a
	(9) Magic Springs	(1)			n/a	n/a
Stygobromus flagellatus (*flagellatus*)	Pooled	4	2			
	S. flagellatus COI haplogroup A	[1]			n/a	n/a
	S. flagellatus COI haplogroup B	[1]	[1]		n/a	n/a
	S. flagellatus COI haplogroup C	[2]	[1]		1.00 ± 0.50	0.004 ± 0.005
	(10) Diversion Spring	(3)	(2)		1.00 ± 0.27	0.182 ± 0.136
	(18) Artesian Well	(1)			n/a	n/a
Stygobromus russelli (*tenuis*)	Pooled	14	4		0.88 ± 0.08	0.049 ± 0.026
	(11) Blowing Sink Cave	(7)	(1)		0.52 ± 0.21	0.035 ± 0.020
	(12) Cold Spring	(4)	(2)		0.81 ± 0.13	0.033 ± 0.019
	(13) Barton Creek Well	(1)			n/a	n/a
	(14) Kretchmarr Salamander Cave	(1)	(1)		n/a	n/a
	(15) Sky Ranch Well	(1)			n/a	n/a
S. cf. *russelli* (*tenuis*)	(16) San Gabriel Springs	5	2		1.00 ± 0.13	0.095 ± 0.059
	S. cf. *russelli* COI haplogroup A	[3]	[1]		1.00 ± 0.27	0.017 ± 0.014
	S. cf. *russelli* COI haplogroup B	[2]	[1]		1.00 ± 0.50	0.010 ± 0.011
Stygobromus bifurcatus (*tenuis*)	Pooled	2	2		1.00 ± 0.50	0.116 ± 0.117
	(15) Sky Ranch Well	(1)	(1)		n/a	n/a
	(17) Adobe Springs	(1)	(1)		n/a	n/a
Stygobromus cf. *hadenoecus* (*hadenoecus*)	(19) upper Devils River springs	4	4		0.50 ± 0.27	0.008 ± 0.006
Unknown species (*tenuis*)	(12) Cold Spring	3			1.00 ± 0.27	0.053 ± 0.040
Unknown species (*flagellatus*)	(18) Texas State Artesian Well	4	2		1.00 ± 0.13	0.003 ± 0.003
	Total	128	32	69	0.96 ± 0.01	0.120 ± 0.060

Locations within Landa Lake are numbered 1 and given letter designations a to i, corresponding with letters on Figures 3 and 6.
Samples within species that exhibited polyphyletic disparity are designated 'haplogroups' with measures reported for each.
Within each species, sample sizes are reported for each of the three data sets (COI, ITS1, AFLP) with sample sizes of haplogroups and sites reported in brackets and parentheses, respectively.
Within-group haplotype diversity (h) and nucleotide diversity (π_{within}) with their standard errors (SE) are reported.
ITS1, internal transcribed spacer region 1; n/a, sites for which h and π_{within} could not be calculated because $N = 1$.

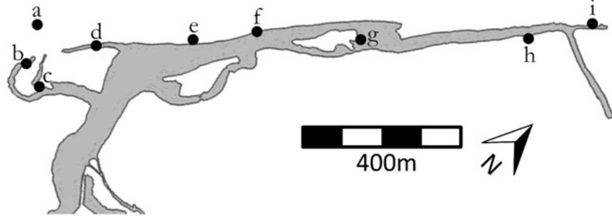

Figure 3. *Stygobromus pecki* sampling sites at Landa Lake, New Braunfels, TX. (a) Panther Canyon Well, (b) Spring Run 1, (c) Kiddy pool, (d) Spring Run 3, (e) Upwelling, (f) West Shore, (g) Spring Island, (h) Spring Run 5, (i) Spring Run 4. Landa Lake is indicated by (1) on Figure 1. West Shore and Spring Island are slightly more elevated than Spring Runs 1 and 3, which spatially correlated with *H. comalensis* genetic diversity.

compared with Holsinger's *flagellatus* species-group phylogeny.

Congeneric outgroup sequence for COI was obtained from the Barcode of Life Database (http://www.boldsystems.org) for *Stygobromus emarginatus* (Hubricht, 1943) (Maryland, USA). Outgroups representing putatively more distant relations were obtained from GenBank (locations and accessions reported in parentheses): *Crangonyx floridanus* Bousfield, 1963 (Florida, USA, AB513835), *Crangonyx pseudogracilis* Bousfield, 1958 (Ontario, Canada, AJ968896), *Crangonyx islandicus* Svavarsson & Kristjansson, 2006 (Iceland, HM015190), and *Gammarus minus* Say, 1818 (West Virginia, USA, EU285912). Outgroups were not used for ITS1 because candidate outgroups contained insertion/deletion variation that caused loss of informative ingroup variation when removed.

Polyphyletic COI diversity was detected in *Stygobromus dejectus* (Holsinger, 1967), *Stygobromus longipes* (Holsinger, 1966), *Stygobromus flagellatus* (Benedict, 1896), and *S.* cf. *russelli* (Holsinger, 1967) (see Results). To test if cryptic species might explain this polyphyletic diversity better than nominal species alone, ARLEQUIN v. 3.5 (Excoffier, Lavel & Schneider, 2005) was used to conduct two analyses of molecular variance (AMOVA, Excoffier, Smouse & Quattro, 1992): (1) a null AMOVA grouped individuals by nominal species only (Table 3A), and (2) an alternative AMOVA grouped individuals by nominal species and when present, distinct haplogroups that cause polyphyly within nominal species, treating each haplogroup as a separate, cryptic species (Table 3B).

Population genetics analysis

To examine population structure within *S. pecki*, three approaches were used in ARLEQUIN v. 3.5: (1) AMOVA (Table 3C), (2) pairwise Φ statistics (F_{ST}-based genetic distances), and (3) pairwise exact test probabilities (Raymond & Rousset, 1995). To examine genetic diversity, ARLEQUIN v. 3.5 was used to estimate unbiased haplotype diversity (h, Table 1, Nei, 1987) and within-group nucleotide diversity (π_{within}, Table 1, Tajima, 1983; Tajima, 1993), and DNASP v. 5.10 (Librado & Rozas, 2009) was used to estimate between-group nucleotide diversity ($\pi_{between}$, Table 4). To compare *S. pecki* genetic diversity with measures for the Comal Springs riffle beetle, estimates of *H. comalensis* genetic diversity with standard errors were estimated (Table 5) using the alignment of *H. comalensis* haplotypes and frequencies reported by Gonzales (2008). To visualize the genealogical relationships amongst *S. pecki* COI haplotypes, TCS v. 1.21 (Templeton, Crandall & Sing, 1992; Clement, Posada & Crandall, 2000) was used to produce a parsimony network of those haplotypes (Fig. 6).

Two monophyletic but significantly diverged COI clades within *S. pecki* (see Results) led to additional analysis of *S. pecki* data: (1) to test for geographical structure within clades, all pairwise Φ statistics and pairwise exact test probabilities were calculated again with clades within sites considered separately; (2) to describe the depth of the COI haplotype divergence, ARLEQUIN v. 3.5 was used to conduct an AMOVA with COI haplogroup as a factor (Table 3D), which partitioned molecular variance into a nested hierarchy between haplogroups, amongst sites within haplogroups, and within sites; (3) to rule out a *Wolbachia* endosymbiont as the cause of the COI divergence within *S. pecki*, the presence of *Wolbachia* was tested using the methods of Nice *et al*. (2009) on three individuals from each COI haplogroup (regarding small sample size, see Hilgenboeker *et al*., 2008); (4) to test for possible spurious amplification of nuclear integration of mitochondrial sequence, or nonhomologous nuclear copies of a mitochondrial locus (Numts), we examined amino acid translations across specimens for conservation of translation and the presence of stop codons.

AFLP markers

To examine genome-wide population structure, AFLP data (Vos *et al*., 1995; Meudt & Clarke, 2006) were collected for 69 *S. pecki* individuals following the methods of Gompert *et al*. 2006, 2008). Two selective primer pairs, mCAGCA (5′ GAT GAG TCC TGA GTA ACA GCA 3′) and mCAGAT (5′ GAT GAG TCC TGA GTA ACA GAT 3′), were each paired with *Eco*R1. Size fragment analysis of selective PCR products was conducted at the Nevada Genomics Center (Reno, NV) using the Applied Biosystems Prism 3730 Analyzer (Carlsbad, CA).

Table 2. Primers used in this study. Primers from Carlini *et al.* (2009) and E. Sotka (unpubl. data) yielded an approximately 650 bp sequence result, whereas the remaining internal primers designed in the current study yielded an approximately 515-bp sequence result

Gene	Primer name	Sequence (5′–3′)	Citation
ITS1	CarliniF	TCC GTA GGT GAA CCT GCG G	Carlini *et al.* (2009)
	CarliniR	AGT GAT CCA CCG CTC AGA G	Carlini *et al.* (2009)
COI	SotkaF	GGT CWA CAA AYC ATA AGA YAT TGG	E. Sotka (unpubl. data)
	SotkaR	TAA ACY TCA GGR TGA CCR AAR AAY CA	E. Sotka (unpubl. data)
	flagellatusF	TCA TCC GAT CCG AAC TAT CCT G	Current study
	flagellatusR	TCG GTA AGT AAT ATA GTA ATA GCA CC	Current study
	tenuisF	TTA TCC GCT CTG AGT TAT CTT G	Current study
	tenuisR	TCA GAA CGT AGT ATT GTA ATA GCT CC	Current study

COI, *cytochrome oxidase C subunit 1*; *ITS1*, *internal transcribed spacer region 1.*

PEAKSCANNER v. 1.0 (Applied Biosystems) was used to format raw AFLP data. RAWGENO v. 2.0 (Arrigo *et al.*, 2009), a package that operates within R v. 2.12.0 (R Development Core Team, 2011), was used to automatically bin AFLP data based on user-defined parameters. STRUCTURE v. 2.3 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2003, 2007) was used to analyse binned AFLP data, assigning individuals probabilistically to clusters using a Bayesian clustering algorithm under an admixture model (because gene flow between sites was a possibility). Runs used a Markov Chain Monte Carlo algorithm of 250 000 generations with a 25 000 generation burn-in. The number of clusters (K) was evaluated from 1 to 11 (number of sampling sites plus one) for ten iterations each. Two approaches were used to select K: (1) the mean log likelihood of K over K was plotted (Fig. 7A), with the asymptote of the plot corresponding to the K that best explained the data (Pritchard *et al.*, 2000). (2) The ad hoc statistic ΔK (Evanno, Regnaut & Goudet, 2005), based on the rate of change in the log probability of data between successive K-values, was calculated for each K and plotted (Fig. 7B), with the best value of K corresponding to the highest value in the plot.

RESULTS

COI reactions yielded a 501-bp product with 73 unique haplotypes from 128 individuals. Of 501 bases in the alignment, 247 (49%) were variable. The protein translation was 166 amino acids and largely conserved with 37 variable amino acid sites (21 of 37 variable sites were caused by two *S. flagellatus* sequences, see below) and no interruption by stop codons. ITS1 reactions yielded a 212-bp product with 11 haplotypes from 32 individuals. Of 212 bases, 34 (16%) were variable. AFLP procedures yielded 296 informative loci from 69 *S. pecki* individuals.

Significant molecular divergences were detected in five nominal species, all indicative of the presence of cryptic diversity. *Stygobromus pecki* contained monophyletic clades, and *S. dejectus*, *S. longipes*, *S. flagellatus*, and *S.* cf. *russelli* each contained polyphyletic clades. To facilitate discussion, these clades are referred to as haplogroups with letter designations and their species name (e.g. *S. pecki* haplogroup A).

PHYLOGENETICS

Species-groups were not generally supported by sequence data. The *flagellatus* and *tenuis* species-groups were not monophyletic for COI (Fig. 4) or ITS1 (Fig. 5). Sequence results for the *hadenoecus* species-group were obtained from one site only, making monophyly an inappropriate test, but it is nonetheless problematic that the *hadenoecus* species-group is nested within a portion of the COI phylogeny (Fig. 4) otherwise polyphyletic for species-groups.

The proposed *flagellatus* species-group phylogeny (Holsinger, 1967, Fig. 2A) conflicted with molecular data (Fig. 2B). *Stygobromus pecki*, *S. dejectus* haplogroup A, and *S. longipes* showed close phylogenetic relationships for both genes, but the placement of *S. dejectus* haplogroup B and *S. flagellatus* within this phylogeny were uncertain because of the greater molecular distance. Holsinger (1967; Fig. 2A) proposed that the sister taxon to *S. pecki* was *S. dejectus*, but our molecular results suggest it to be *S. longipes* haplogroup A (Fig. 2B).

Stygobromus longipes and *S. flagellatus* both exhibited polyphyly for COI and monophyly for ITS1. *Stygobromus longipes* COI haplogroup A was monophyletic with strong posterior probability support (1.0), whereas *S. longipes* haplogroup B (a single, disparate sequence) grouped with *S. dejectus* haplogroup A (Fig. 4). Amongst-group sequence divergence (π) values for *S. longipes* and *S. dejectus*

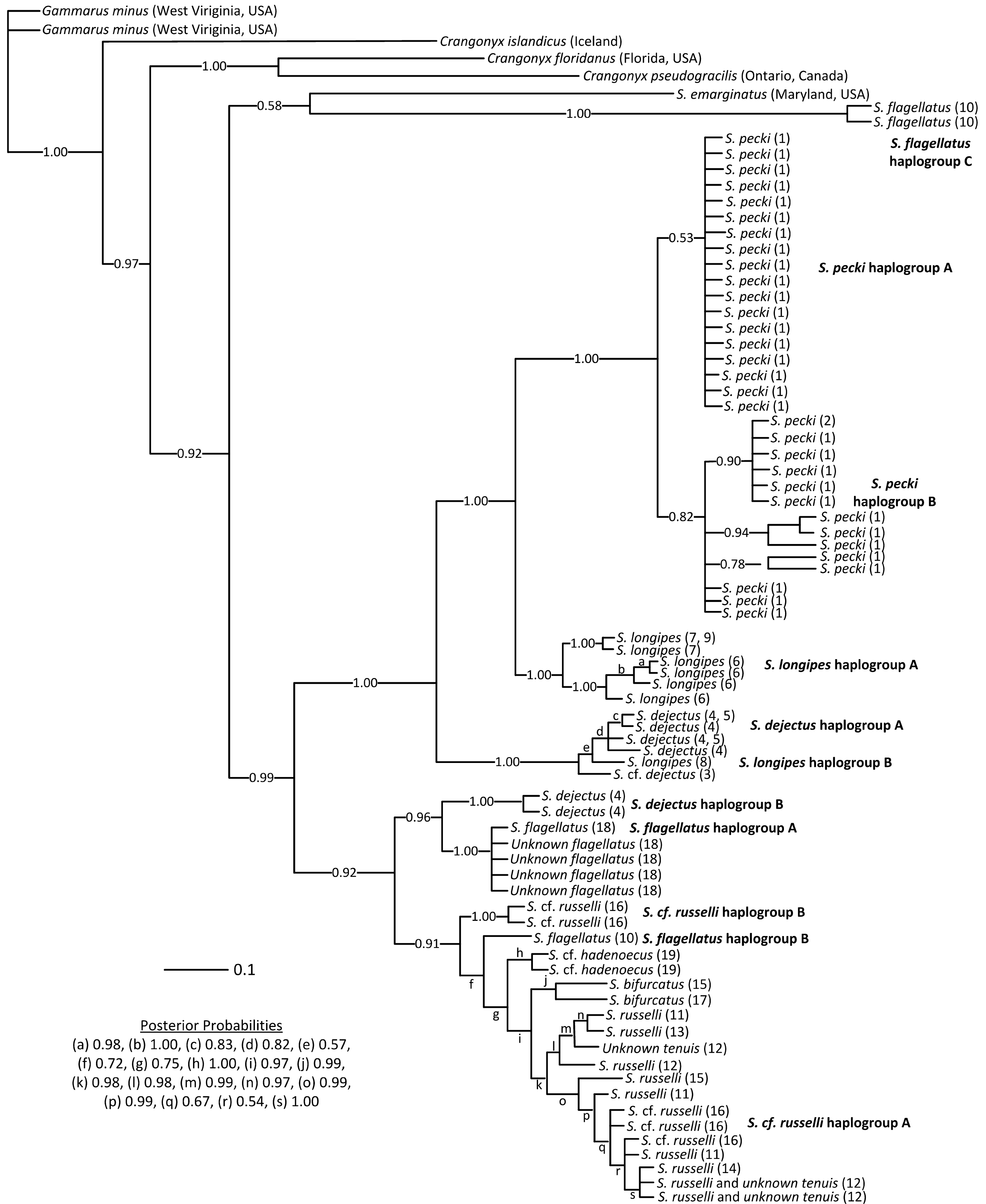

Figure 4. Bayesian phylogeny based on 501 bp region of cytochrome oxidase C subunit 1. Terminal nodes represent unique haplotypes. For each haplotype, species possessing it and sampling site(s) are reported. Haplogroups within *Stygobromus* species are labelled. Outgroups are *Gammarus minus* (West Virginia, USA), *Crangonyx islandicus* (Iceland), *Crangonyx floridanus* (Florida, USA), *Crangonyx pseudogracilis* (Ontario, Canada), and *Stygobromus emarginatus* (Maryland, USA).

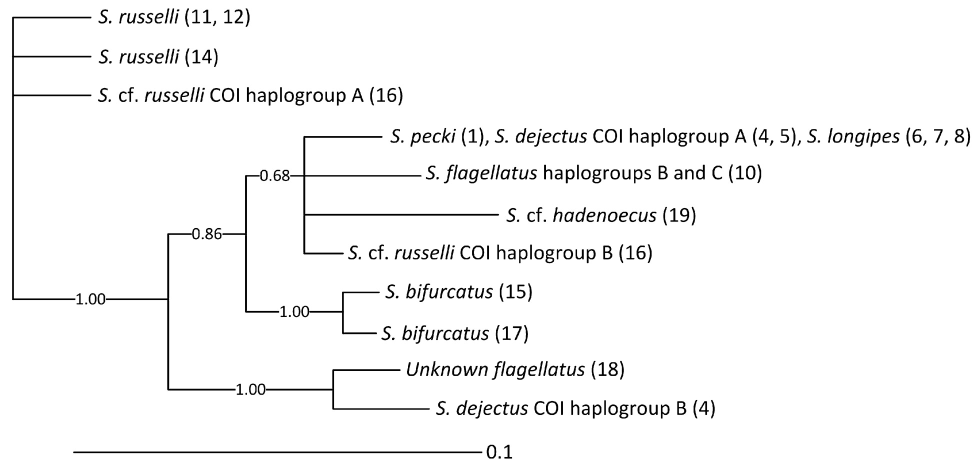

Figure 5. Bayesian phylogeny based on 212 bp region of internal transcribed spacer region 1 (ITS1). Terminal nodes represent unique haplotypes. For each haplotype, species possessing it and sampling site(s) are reported. ITS1 data for *Stygobromus pecki*, *Stygobromus dejectus*, and *Stygobromus* cf. *russelli* haplogroups supported cytochrome oxidase C subunit 1 (COI) findings. *Stygobromus flagellatus* signal conflicted with that found for COI. No outgroups were used in this phylogeny.

Table 3. Cytochrome oxidase C subunit 1 (COI) analysis of molecular variance. For all *Stygobromus* haplotype data: A, results grouping *Stygobromus* haplotype data by the nominal taxonomy; B, results grouping data by the nominal taxonomy and when present, polyphyletic haplogroups within nominal species (i.e. each polyphyletic haplogroup is treated as its own cryptic species). For *Stygobromus pecki* haplotype data: C, results that test for population structure by considering sampling site only; D, results that consider *S. pecki* haplogroups to illustrate the depth of their divergence

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value
A, all *Stygobromus* grouped by nominal species: $\Phi_{ST} = 0.687$ ($P < 0.001$)					
Amongst groups	8	2772.26	28.28	68.72	<0.001
Amongst sites within groups	12	264.27	6.49	15.77	0.035
Within sites	107	682.69	6.38	15.51	<0.001
B, all *Stygobromus* grouped by species + polyphyletic haplogroups: $\Phi_{ST} = 0.807$ ($P < 0.001$)					
Amongst groups	13	3191.24	33.31	80.68	<0.001
Amongst sites within groups	11	314.91	5.91	14.31	<0.001
Within sites	103	213.06	2.07	5.01	<0.001
C, *S. pecki* grouped by site: $\Phi_{ST} = 0.015$ ($P = 0.329$)					
Amongst sites	9	26.40	0.04	1.5	0.329
Within sites	61	162.35	2.66	98.5	
D, *S. pecki* grouped by COI haplogroup: $\Phi_{ST} = 0.826$ ($P < 0.001$)					
Amongst groups	1	128.73	4.21	82.57	<0.001
Amongst sites within groups	17	16.65	0.04	0.72	0.539
Within sites	52	44.25	0.85	16.71	<0.001

haplogroup A contradicted the nominal taxonomy: sequence divergence was about 10% between *S. longipes* haplogroups, but was only about 1% between *S. longipes* haplogroup B and *S. dejectus* haplogroup A (Table 4). *Stygobromus flagellatus* produced three polyphyletic COI haplogroups. *Stygobromus flagellatus* haplogroup A was sister taxon to *S. dejectus* haplogroup B for COI and ITS1. Haplotypes for the unknown *flagellatus* individuals exhibited a close relationship with *S. flagellatus* haplogroup A, and both groups were sampled from (18) Artesian Well at the same time, suggesting they are all *S. flagellatus*. *Stygobromus flagellatus* haplogroup C, exhibiting a distant relationship from all other individuals in this study, was sister taxon to the outgroup *S. emarginatus* from Maryland (albeit with little posterior probability support at 0.58). ITS1 results contradicted COI: the two *S. flagellatus* specimens sequenced (one

Table 4. Cytochrome oxidase C subunit 1 (COI) sequence divergences between groups

	Stygobromus pecki	*S. pecki* A	*S. pecki* B	*Stygobromus longipes*	*S. longipes* A	*S. longipes* B	*Stygobromus dejectus* A	*S. dejectus* B	*Stygobromus russelli*
S. pecki	–								
S. pecki A	n/a	–							
S. pecki B	n/a	0.023	–						
S. longipes	0.117	0.112	0.119	–					
S. longipes A	0.114	0.108	0.117	n/a	–				
S. longipes B	0.142	0.137	0.136	n/a	0.100	–			
S. dejectus A	0.141	0.136	0.136	0.087	0.098	0.011	–		
S. dejectus B	0.184	0.186	0.186	0.170	0.170	0.174	0.174	–	
S. russelli	0.188	0.190	0.183	0.178	0.179	0.168	0.168	0.180	–

n/a, inappropriate comparisons.

from haplogroup B and one from haplogroup C) shared a single ITS1 haplotype.

Stygobromus dejectus and *S.* cf. *russelli* were each polyphyletic for both genes. Within *S. dejectus*, the monophyly of haplogroup A had weak posterior probability support (0.82), whereas support for haplogroup B was strong (1.0). Without posterior probability support, *S. dejectus* haplogroup A collapses into a polytomy with *S. longipes* haplogroup B and *S.* cf. *dejectus*. The closest relative of *S. dejectus* haplogroup B was *S. flagellatus* haplogroup A for COI and ITS1, with strong posterior probability support (0.96 and 1.0, respectively). *Stygobromus* cf. *russelli* haplogroup A exhibited a close relationship with *S. russelli* and is discussed in the next paragraph.

Stygobromus russelli was paraphyletic for COI with respect to *unknown tenuis* from (12) Cold Spring and *S.* cf. *russelli* haplogroup A. However, all may be *S. russelli*. Two of the three *unknown tenuis* haplotypes were shared with positively identified *S. russelli* specimens, and the third was nested amongst *S. russelli* haplotypes. *Stygobromus* cf. *russelli* haplogroup A was also nested amongst *S. russelli* COI haplotypes, and was monophyletic for ITS1 with positively identified *S. russelli*. Furthermore, both ambiguous groups were collected from sites for which *S. russelli* is described. If both groups are accepted as *S. russelli*, the group exhibits monophyly with strong posterior probability support (0.98).

Stygobromus bifurcatus (Holsinger, 1967) and *S.* cf. *hadenoecus* (Holsinger, 1966) were monophyletic for both genes but are not considered further because of small sample sizes.

Stygobromus pecki was monophyletic for COI and monomorphic for ITS1. The distinction between the two *S. pecki* COI haplogroups was dramatic: they were separated by eight nucleotide differences in the parsimony network (Fig. 6) and exhibited a mean sequence divergence of 2.3% (Table 4).

Cryptic species diversity explained polyphyletic clades better than nominal species alone. The AMOVA that treated polyphyletic COI haplogroups as separate units (i.e. treated haplogroups as species, Table 3B, $\Phi_{ST} = 0.807$, $P < 0.001$) provided a better explanation of the distribution of COI variation than did nominal species alone (Table 3A, $\Phi_{ST} = 0.687$, $P < 0.001$).

Population genetics

The *S. pecki* COI haplogroups had 2.3% sequence divergence between them, and the AMOVA that used COI haplogroup as a factor (Table 3D) estimated that 82.6% of COI genetic variation is attributable to differences between haplogroups ($\Phi_{ST} = 0.826$, $P < 0.001$). Despite such divergence, geographical population

Table 5. Comparison of genetic diversity for *Stygobromus pecki* and *Heterelmis comalensis*. Within-group haplotype diversity (h) and nucleotide diversity (π_{within}) with standard errors (SE) are reported. In all interspecies comparisons except h for West shore, *S. pecki* exhibited significantly greater molecular diversity than *H. comalensis*. Within species, *S. pecki* did not show the pattern of relatively impoverished diversity for spring runs 1 and 3 that *H. comalensis* did, suggesting that whatever caused the impoverished diversity for *H. comalensis* did not similarly affect *S. pecki*

	Haplotype diversity (h ± SE)		Nucleotide diversity (π_{within} ± SE)	
Sampling Site	*S. pecki*	*H. comalensis*	*S. pecki*	*H. comalensis*
(1b) Spring Run 1	0.80 ± 0.11	0.000	0.010 ± 0.006	0.000
(1d) Spring Run 3	0.78 ± 0.10	0.000	0.007 ± 0.004	0.000
(1f) West shore	(1.00 ± 0.27)	(0.75 ± 0.04)	0.013 ± 0.011	0.002 ± 0.001
(1g) Spring Island	0.92 ± 0.05	0.53 ± 0.08	0.011 ± 0.006	0.001 ± 0.001
All specimens	0.86 ± 0.04	0.43 ± 0.05	0.011 ± 0.006	0.001 ± 0.001

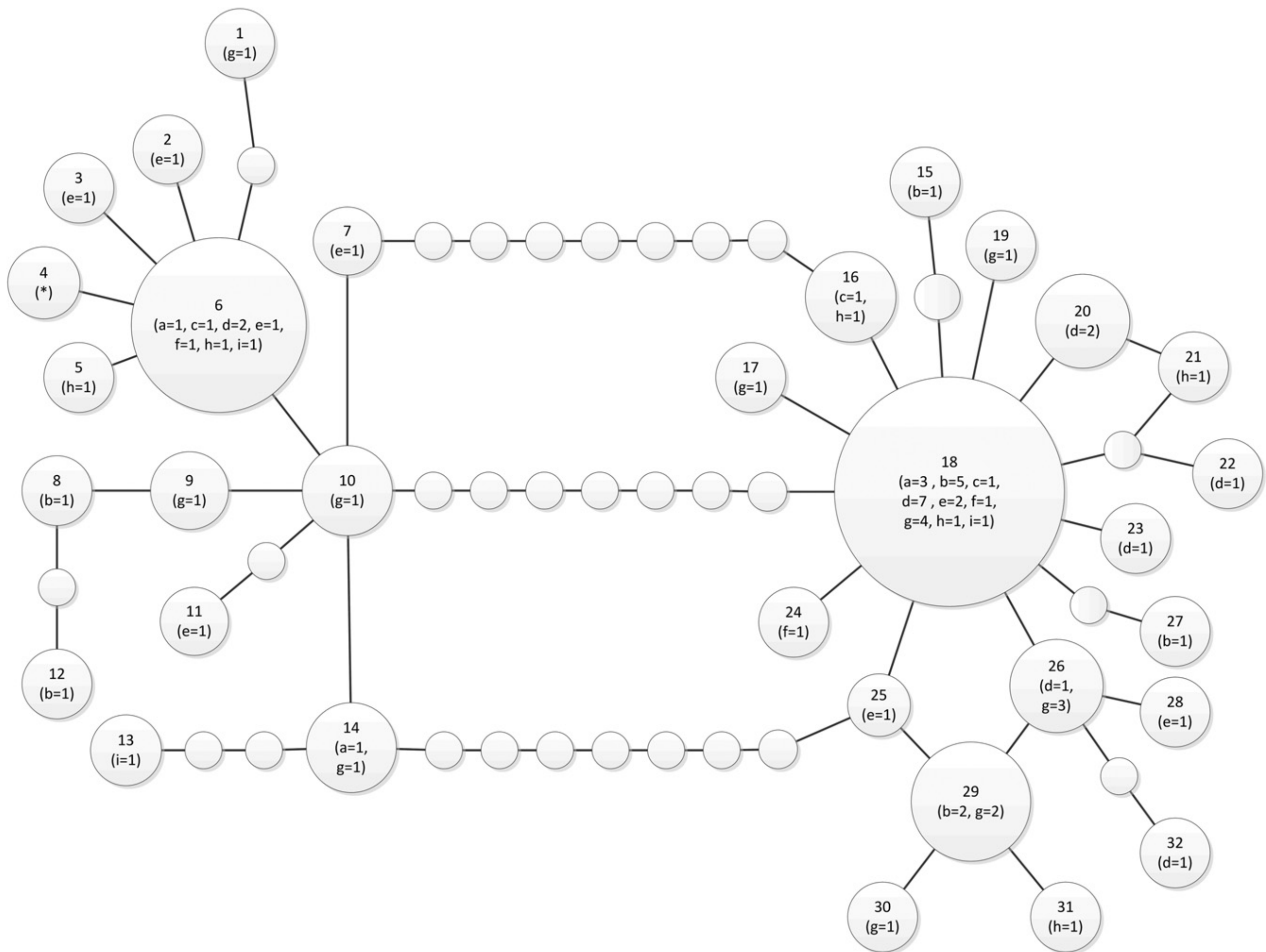

Figure 6. *Stygobromus pecki* cytochrome oxidase C subunit 1 haplotype network. Each circle containing numbers represents a unique haplotype. Within the parentheses, letters are sampling sites within Landa Lake (Fig. 3) and numbers are their sample sizes. Two haplotypes connected by a single line have one nucleotide difference (mutation) between them. Each circle that does not contain numbers represents one additional nucleotide difference. *Haplotype 4 came from Hueco Springs.

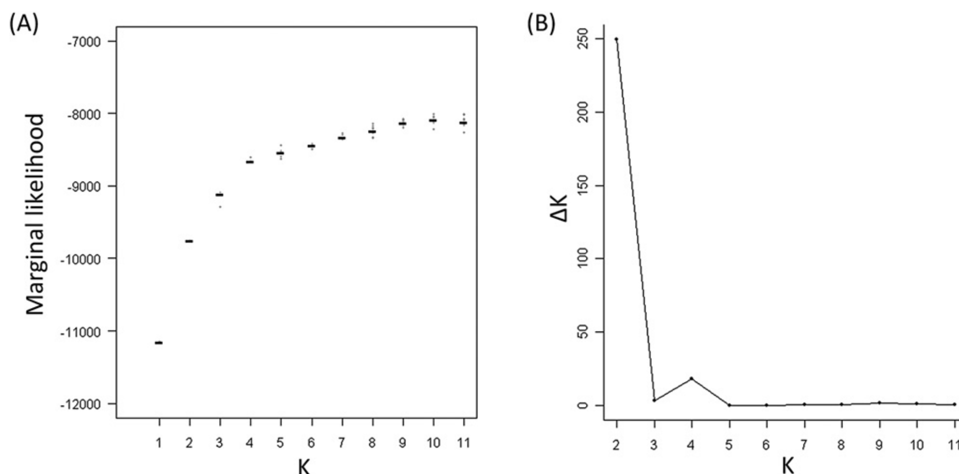

Figure 7. Estimation of the number of clusters (*K*). Two methods were used to estimate the *K* that best explains the data. A, results of the mean marginal likelihood approach (Pritchard *et al.*, 2000), which plots the mean marginal likelihood value of ten replicate STRUCTURE analyses over *K*. B, results of the Δ*K* method (Evanno *et al.*, 2005), which plots the ad-hoc statistic Δ*K*, derived from the mean marginal likelihood, over *K*.

structure was not detected because the haplogroups are nearly equally abundant at each locality. Consequently, AMOVA analysis revealed no significant structure amongst *S. pecki* sites (Table 3C, $\Phi_{ST} = 0.015$, $P = 0.329$). All pairwise Φ statistics and exact tests were nonsignificant except for the Φ statistic between (1d) Spring run 3 and (1e) Upwelling ($\Phi_{ST} = 0.200$, $P = 0.045$). Relative to each other, (1d) Spring run 3 had a disproportionate number of haplogroup A (13 of 15) and (1e) Upwelling had a disproportionate number of haplogroup B (five of seven). Regarding possible structure within each haplogroup, when pairwise comparisons were calculated again with haplogroups within sites considered separately, all within-haplogroup pairwise tests were nonsignificant.

Stygobromus pecki haplotype diversity (*h*) was not significantly different from estimates for its non-endangered regional congeners (Table 1). Nucleotide diversity (π_{within}) for *S. pecki* was not significantly different from *S. dejectus* haplogroup A, and was significantly less than those for *S. dejectus* haplogroup B, *S. longipes* haplogroup A, and *S. russelli* (Table 1). *Stygobromus pecki h* and π_{within} were significantly greater than the same measures for *H. comalensis* (Table 5), and did not follow the *H. comalensis* spatial pattern of relative genetic impoverishment at higher-elevation sites.

Wolbachia endosymbionts were ruled out as the cause of the COI divergence because no *S. pecki* tested positive for *Wolbachia* infection. Numts were judged an unlikely cause of the COI divergence because amino acid translations were highly conserved across all individuals and contained no stop codons.

AFLP markers

The plot of mean marginal likelihoods over *K* (Pritchard *et al.*, 2000) indicated that $K = 4$ best explains AFLP data (Fig. 7A), whereas the ad-hoc statistic Δ*K* (Evanno *et al.*, 2005) indicated that $K = 2$ was the best clustering solution (Fig. 7B). For $K = 4$, clustering was not associated with sampling site, mitochondrial clade, or any other obvious feature of the system, and most individuals were assigned to multiple clusters. Given such ambiguity, we interpret $K = 2$ as the best clustering solution following the Δ*K* statistic. Barplots for $K = 2$–4 are presented (Fig. 8) for completeness.

For $K = 2$, *S. pecki* COI haplogroup B individuals had predominantly high assignment probabilities to one cluster, whereas *S. pecki* haplogroup A individuals had mixed assignment probabilities to both clusters (Fig. 8). Under the admixture model, an individual's cluster assignment probability can be interpreted as the proportion of that individual's genome originating in that cluster (Pritchard *et al.*, 2000). Using this interpretation, differences in assignment probabilities between haplogroups suggest that *S. pecki* consists of two previously isolated genomes that have experienced recent and ongoing gene flow. This gene flow also appears to be asymmetrical, with greater gene flow from haplogroup B to A.

DISCUSSION

Phylogenetic relationships and patterns of genetic variation observed in Edwards Plateau *Stygobromus* amphipods are complex. The species-group taxonomic framework and *flagellatus* species-group phylogeny

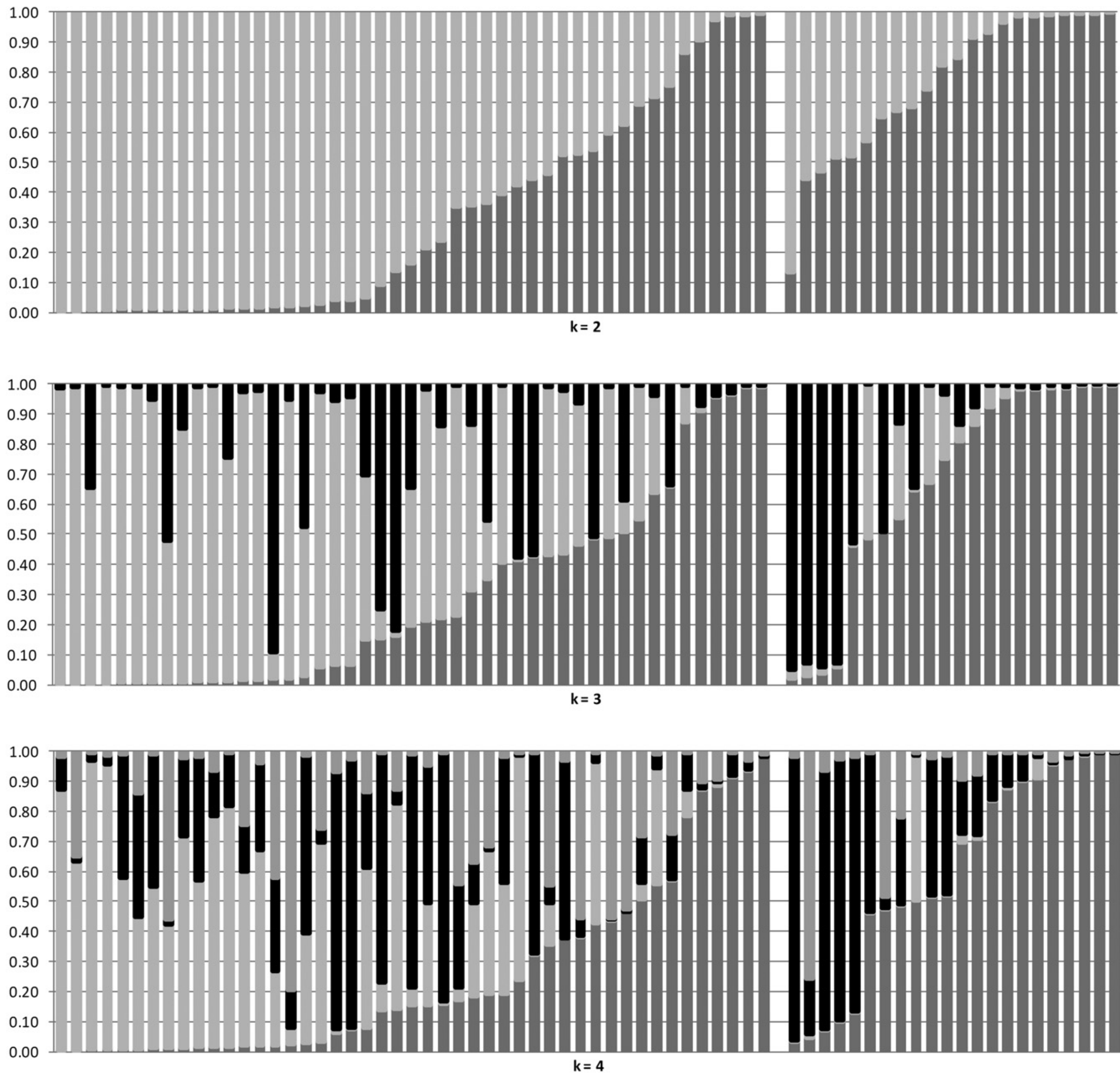

Figure 8. Amplified fragment length polymorphism bar plots for number of clusters (K) = 2–4. Each column represents an individual in the analysis, and the area of each colour equals that individual's probabilistic assignment to that cluster. For K = 2, *Stygobromus pecki* haplogroup B has predominantly high assignment probabilities to a single cluster, whereas *S. pecki* haplogroup A has mixed assignment probabilities to both clusters.

(Holsinger, 1967; Fig. 2A) are largely unsupported by current results. Nominal species have varying levels of support, and there are strong indications that there are more *Stygobromus* species in the Edwards Plateau region than is currently recognized.

As troglomorphic adaptations may confound phylogenetic assumptions of homology, it is not surprising that the morphology-based species-group taxonomy and *flagellatus* species-group phylogeny proposed by Holsinger (1967; Fig. 2A) were largely contradicted by current results. The *flagellatus* and *tenuis* species-groups were polyphyletic for both COI and ITS1, and the *hadenoecus* species-group was nested within a polyphyletic portion of the COI phylogeny (Fig. 4). *Stygobromus dejectus* haplogroup B, *S. flagellatus*, and *unknown flagellatus* from (18) Artesian Well (unknown because they lacked positive identification to a particular species within the *flagellatus* species-group) were distantly related to other *flagellatus* species-group members at both genes. Of these, the

most distantly related haplotypes were for *S. flagellatus* COI haplogroup C, which paired with the outgroup *S. emarginatus* (although sample sizes were small and this finding was not corroborated by ITS1 results).

Regarding proposed phylogenetic relationships within the *flagellatus* species-group (Fig. 2A), the hypothesis of sister-species relationship between *S. pecki* and *S. dejectus* was contradicted by two molecular findings: (1) *S. dejectus* is a polyphyletic group with strong evidence of cryptic species diversity (Table 4, Figs 4, 5); (2) the COI phylogeny suggests that *S. longipes* haplogroup A is the sister taxon to *S. pecki*. The placement of *S. flagellatus* on a revised phylogeny is uncertain (Fig. 2B) because of polyphyletic diversity, small sample sizes, and contradicting signals from COI and ITS1.

Nominal species had varying levels of support. *Stygobromus pecki* was monophyletic for both genes. If *S.* cf. *russelli* haplogroup A and the unknown *tenuis* specimens from (12) Cold Spring are accepted as *S. russelli* given molecular evidence (see phylogenetic results), *S. russelli* was monophyletic as well. *Stygobromus dejectus* and *S.* cf. *russelli* exhibited the strongest evidence of cryptic species diversity, with support from COI and ITS1. *Stygobromus longipes* and *S. flagellatus* were polyphyletic for COI but monophyletic for ITS1, and although they lacked sufficient sample sizes for in-depth explorations, each produced a curious finding: (1) *S. longipes* haplogroup B and *S. dejectus* haplogroup A were more closely related (1.1% sequence divergence) than the haplogroups within *S. pecki* (2.3% sequence divergence); (2) *Stygobromus flagellatus* haplogroup C showed a distant relationship to all other *Stygobromus* sampled.

The *S. pecki* COI haplogroups (Figs 4, 6) appear sympatric, even though the depth of the COI divergence between these haplogroups suggests substantial isolation. AFLP results provided evidence of admixture between two clusters that roughly correspond with the COI haplogroups. To explain these findings, a historical biogeographical hypothesis is offered. This hypothesis posits that an ancestral species split into two groups that underwent a substantial period of allopatric isolation. It was during this period that the COI divergence accumulated, and it has been preserved to the present day because of lack of recombination in mitochondrial DNA. This period of isolation was followed by secondary contact with ongoing, asymmetric admixture indicated by AFLP results. The admixture appears to be ongoing because if it were complete, we would expect either (1) equal assignment probabilities to both clusters for most or all individuals, or (2) the K selection approach of Pritchard *et al*. (2000) to indicate $K = 1$ as the best value to explain the data in the first place (the ΔK approach cannot evaluate $K = 1$).

COI and AFLP data for *S. pecki* contain cryptic variation that, although not suggestive of cryptic species, is still unusual. Niemiller, Near & Fitzpatrick (2012) detected cryptic variation within an endangered species, but the cryptic variation observed in the endangered *S. pecki* may be unprecedented because of its sympatry. However, alternatives to the hypothesis of a period of allopatry might be considered. A protracted period of very large effective population size could have produced stochastic sorting of mitochondrial lineages, essentially producing what appear as two distinct lineages for this nonrecombining marker. Furthermore, endosymbionts could produce the discordance observed for COI and ITS1. We found no evidence of *Wolbachia*, but other endosymbionts, such as *Rickettsia*, *Cardinium*, and *Spiroplasma*, could be important agents (Moran, McCutcheon & Nakabachi, 2008). Another alternative is that Numts (nonhomologous copies of the COI locus) were amplified in some or all individuals, but this seems unlikely given the highly conserved translation without stop codons.

COI genetic diversity estimates for the endangered *S. pecki* are similar to estimates for its regional congeners and significantly higher than estimates for the endangered Comal Springs riffle beetle, *H. comalensis*. The *H. comalensis* pattern of differential genetic diversity based on elevation was not observed for *S. pecki*, suggesting that the cause of reduced diversity in *H. comalensis* did not similarly affect *S. pecki*. Assuming the reduced diversity at the higher-elevation sites (Spring Runs 1 and 3) was caused by the record drought of the 1950s as Gonzales (2008) hypothesized, we may infer that *S. pecki* is better adapted than *H. comalensis* to survival in deeper habitats when water levels drop, or that *S. pecki* collected at the surface derives from deeper source populations in the first place.

Revision of *Stygobromus* taxonomy is not our objective, but several findings can be used for future taxonomic investigations: (1) An alternative to Holsinger's species-groups in the Edwards Plateau is suggested by current results. Figure 4 contains two major clades encompassing all study individuals except the two comprising *S. flagellatus* haplogroup C. The clade at the bottom of Figure 4 is geographically widespread and includes representatives of all nominal species-groups, whereas the upper clade contains only *flagellatus* species-group specimens with a limited geographical range (sites 1–9 on Fig. 1). (2) We have taken those species containing polyphyletic clades as indicative of cryptic species diversity, but it is possible that taxonomically informative morphological differences between haplogroups are present but unde-

scribed. Thought of this way, if a future taxonomic investigation of *Stygobromus* sought to detect undescribed morphological diversity, a good place to start might be with those species containing polyphyletic haplotype diversity.

All aspects of the current study would benefit from expanded geographical and taxonomic sampling. Although the type locality for *S. pecki* is Landa Lake, the species has also been described from Hueco Springs (Gibson *et al*., 2008), a site for which the current study obtained only one COI sequence. That sequence belonged to *S. pecki* haplogroup B (all other Hueco Springs specimens failed to produce PCR amplicons). Increased sampling from Hueco Springs could potentially alter any conclusions we have made about *S. pecki*, which in turn could alter conservation management plans for this endangered species. Another area where increased sampling is needed is for *Stygobromus* species at sites 3–9, which appear to comprise the immediate phylogenetic context of the endangered *S. pecki*. This is particularly true for *S. longipes*, which included very small sample sizes. Increased sampling of *S. flagellatus* at (18) Artesian Well and (10) Diversion Spring could yield interesting insights because *S. flagellatus* haplogroup C was dramatically divergent for COI but not nearly so divergent for ITS1, suggesting mitochondrial introgression.

A complete picture of *Stygobromus* molecular biodiversity and evolutionary history will require broad sampling across North America and Eastern Europe. We detected cryptic species diversity in several Edwards Plateau *Stygobromus* species, suggesting the possibility of more unrecognized cryptic species within morphological species across the range of the genus. The likelihood of this seems particularly high for those *Stygobromus* species that have relatively large ranges (including *S. russelli*), ranges that seem unlikely given that *Stygobromus* species are typically endemic and narrow-ranged. Such efforts would be of potential benefit to conservation efforts for the genus as well: there is one other endangered *Stygobromus* species, *Stygobromus hayi* Hubricht & Mackin, 1940, endemic to a tributary of the Potomac River near Washington DC, and 36 species in North America classified by the International Union for the Conservation of Nature as vulnerable to becoming endangered if threats to their survival and reproduction do not improve.

ACKNOWLEDGEMENTS

For their ongoing feedback on the development of this research and manuscript, the authors thank N. Martin, B. Schwartz, and Z. Gompert. For their specimen collection efforts, the authors thank A. Glusenkamp of Texas Parks and Wildlife who guided all cave sampling and collected most well specimens, Jean Krejca of Zara Environmental LLC, and personnel from Texas Parks and Wildlife, the United States Fish and Wildlife Service, and the city of Austin. For allowing access to *Stygobromus* habitat, the authors thank Cascade Caverns (S. Kyle), Cave-Without-A-Name (T. Summers & M. Burrell), Magic Springs (W. McAllister), CM Cave (J. Eisenhauer), Comal County Water Recreation District #1, the Nature Conservancy (S. McWilliams & J. Karges), the Balcones Canyonlands Preserve (M. Sanders), the Devils River State Natural Area (J. Ranzau & D. Riskind), the Edwards Aquifer Authority (G. Schindel), the Camp Bullis Military Training Reservation (L. Cooksey), Texas State University (G. Longley & V. Castillo), Southwestern University (B. Pierce), and the cities of Austin, Georgetown, New Braunfels, and San Marcos. Funding was provided by a State Wildlife Grant (SWiG) from Texas Parks and Wildlife and a Research Enhancement Grant from Texas State University to C. Nice. Finally, we thank J. Holsinger for leading the way. The authors declare no conflict of interests pertaining to this study. Views presented do not necessarily reflect those of the United States Fish and Wildlife Service.

REFERENCES

Arrigo N, Tuszynski JW, Ehrich D, Gerdes T, Alvarez N. 2009. Evaluating the impact of scoring parameters on the structure of intra-specific genetic variation using RawGeno, an R package for automating AFLP scoring. *BMC Bioinformatics* **10:** 33.

Benedict JE. 1896. Preliminary descriptions of a new genus and three new species of crustaceans from an artesian well at San Marcos, Texas. *Proceedings of the United States Natural History Museum* **18:** 615–617.

Bosse LS, Tuff DW, Brown HP. 1988. A new Species of Heterelmis from Texas (Coleoptera: Elmidae). *The Southwestern Naturalist* **33:** 199–203.

Bousfield EL. 1958. Fresh-water amphipod crustaceans of glaciated North America. *Canadian Field Naturalist* **72:** 55–113.

Bousfield EL. 1963. New fresh-water amphipod crustaceans from Florida. *National Museum of Canada Natural History Papers* **18:** 1–9.

Carlini DB, Manning J, Sullivan PG, Fong DW. 2009. Molecular variation and population structure in morphologically differentiated cave and surface populations of the freshwater amphipod *Gammarus minus*. *Molecular Ecology* **18:** 1932–1945.

Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9:** 1657–1660.

Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK. 2000. Considering evolutionary processes in conservation biology. *Trends in Ecology & Evolution* **15:** 290–295.

Drummond AJ, Ashton B, Heled J, Kearse M, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A. 2011. Geneious v5.1. Available at: http://www.geneious.com

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14:** 2611–2620.

Excoffier L, Lavel G, Schneider S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1:** 47–50.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of Molecular Variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479–491.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164:** 1567–1587.

Falush D, Stephens M, Pritchard JK. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes* **7:** 574–578.

Felsenstein J. 2005. Phylip (phylogeny inference package) v3.6. Distributed by the author. Department of Genome Sciences, University of Washington at Seattle.

Gibson JR, Harden SJ, Fries JN. 2008. Survey and distribution of invertebrates from selected springs of the Edwards aquifer in Hays and Comal counties, Texas. *The Southwestern Naturalist* **53:** 74–84.

Gompert Z, Forister ML, Fordyce JA, Nice CC. 2008. Widespread mito-nuclear discordance with evidence for introressive hybridization and selective sweeps in *Lycaeides*. *Molecular Ecology* **17:** 5231–5244.

Gompert Z, Nice CC, Fordyce JA, Forister ML, Shapiro AM. 2006. Identifying units for conservation using molecular systematics: the cautionary tale of the Karner blue butterfly. *Molecular Ecology* **15:** 1759–1768.

Gonzales T. 2008. Conservation genetics of the Comal Springs riffle beetle (*Heterelmis comalensis*) populations in central Texas, with examination of molecular and morphological variation in *Heterelmis* sp. throughout Texas. Master of Science Thesis, Texas State University-San Marcos.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52:** 696–704.

Hilgenboeker KP, Hammerstein P, Schlattmann P, Telschow A, Werren JH. 2008. How many species are infected with *Wolbachia*? – a statistical analysis of current data. *FEMS Microbiology Letters* **281:** 215–220.

Holsinger JR. 1966. Subterranean amphipods of the genus *Stygonectes* (Gammaridae) from Texas. *American Midland Naturalist* **76:** 100–124.

Holsinger JR. 1967. Systematics, speciation, and distribution of the subterranean amphipod genus Stygonectes (Gammaridae). *Bulletin of the U.S. National Museum* **259:** 1–176.

Holsinger JR. 1974. Systematics of the North American amphipod genus Stygobromus (Gammaridae), part I: species of the western United States. *Smithsonian Contributions to Zoology* **166:** 1–63.

Holsinger JR. 1978. Systematics of the North American amphipod genus Stygobromus, part II: species of the eastern United States. *Smithsonian Contributions to Zoology* **266:** 1–144.

Hubricht L. 1943. Studies on the Nearctic freshwater Amphipoda, III – notes on the freshwater Amphipoda of the Eastern United States with description of ten new species. *American Midland Naturalist* **29:** 683–712.

Hubricht L, Mackin JG. 1940. Description of nine new species of fresh-water crustaceans with notes and new localities for other species. *American Midland Naturalist* **23:** 187–218.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25:** 1451–1452.

Meudt HM, Clarke AC. 2006. Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends in Plant Sciences* **12:** 106–117.

Moran NA, McCutcheon JP, Nakabachi A. 2008. Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics* **42:** 165–190.

Nei M. 1987. *Molecular evolutionary genetics*. New York: Columbia University Press.

Nice CC, Gompert Z, Forister ML, Fordyce JA. 2009. An unseen foe in arthropod conservation efforts: The case of *Wolbachia* infections in the Karner blue butterfly. *Biological Conservation* **142:** 3137–3146.

Niemiller ML, Near TJ, Fitzpatrick BM. 2012. Delimiting species using multilocus data: diagnosing cryptic diversity in the southern cavefish, *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). *Evolution* **66:** 846–866.

Nylander JAA. 2004. MRMODELTEST v2. Distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

Porter ML. 2007. Subterranean biogeography: what have we learned from molecular techniques? *Journal of Cave and Karst Studies* **69:** 179–186.

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

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155:** 945–959.

R Development Core Team. 2011. *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.r-project.org

Raymond M, Rousset F. 1995. An exact test for population differentiation. *Evolution* **49:** 1280–1283.

Ronquist F, Huelsenbeck JP. 2003. MrBayes3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Svavarsson J, Kristjansson BK. 2006. Crangonyx islandicus sp nov., a subterranean freshwater amphipod (Crustacea, Amphipoda, Crangonyctidae) from springs in lava fields in Iceland. *Zootaxa* **1365:** 1–17.

Swofford DL. 2002. *PAUP* phylogenetic analysis using parsimony (*and other methods) v4.0b10*. Sunderland, MA: Sinauer Associates.

Tajima F. 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics* **105:** 437–460.

Tajima F. 1993. Measurement of DNA polymorphism. In: Takahata N, Clark AG, eds. *Mechanisms of molecular evolution. Introduction to molecular paleopopulation biology*. Tokyo and Sunderland, MA: Japan Scientific Societies Press and Sinauer Associates, 37–59.

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

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23:** 4407–4414.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Geographical coordinates of sampling sites. Latitude and longitude are reported in decimal degrees. Coordinates are not reported for some sites (denoted by asterisk) because these sites are private property, or environmentally sensitive sites that are threatened by recreational use, or sites within the confines of a military reservation.