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Subterranean phylogeography of freshwater crayfishes shows extensive gene flow and surprisingly large population sizes

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Abstract

Subterranean animals are currently viewed as highly imperiled, precariously avoiding extinction in an extreme environment of darkness. This assumption is based on a hypothesis that the reduction in visual systems and morphology common in cave faunas reflects a genetic inability to adapt and persist coupled with the perception of a habitat that is limited, disconnected, and fragile. Accordingly, 95% of cave fauna in the United States are presumed endangered due to surface environmental degradation and limited geographic distributions. Our study explores the subterranean phylogeography of stygobitic crayfishes in the southeastern United States, a global hotspot of groundwater biodiversity, using extensive geographic sampling and molecular data. Despite their endangered status, our results show that subterranean crayfish species have attained moderate to high levels of genetic diversity over their evolutionary histories with large population sizes and extensive gene flow among karst systems. We then compare the subterranean population histories to those of common surface stream-dwelling crayfishes. Our results show recent drastic declines in genetic variability in the surface crayfish and suggest that these species also warrant conservation attention.

Keywords: cave fauna, conservation genetics, crustaceans, endangered species, phylogeography, stygobite

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Introduction

According to the Nature Conservancy, 95% of subterranean fauna in North America is considered vulnerable or imperiled using criteria similar to the IUCN-World Conservation Union Red List (Master 1991; Culver *et al*. 2000). The listings are based mostly on surface threats to groundwater systems (Danielopol *et al*. 2003), small geographic ranges (Culver *et al*. 2000), and habitat destruction, not in-depth species-specific biological studies. In fact, current scientific information on subterranean fauna is scarce, leaving the field of biospeleology and the unique biome in the dark. The convergent nature of cave life obscures species' relationships and geographic boundaries, while the inaccessibility of the underground microhabitat makes physical counts of census sizes almost impossible to confidently assess. Molecular genetic approaches are best employed in these situations to accurately estimate biodiversity and critically evaluate the conservation status of elusive organisms (DeSalle & Amato 2004).

Two hypotheses (as reviewed by Kane 1982) have been proposed concerning the genetic diversity, and hence the conservation status and extinction risk (Spielman *et al*. 2004), of subterranean fauna. Barr (1968) suggested that a genetic bottleneck initially occurs during the separation of the surface ancestor from its obligate cave-dwelling descendent. Barr suggested that this bottleneck is short in duration and that cave populations recover from the break in gene flow by range expansion and population growth into new uninhabited subterranean areas. In contrast, Poulson & White (1969) proposed that older fauna show

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low genetic variability due to the long isolation and adaptation to the stable underground environment. They also suggested that the decrease in phenotypic variance in visual structures and morphological traits reflects a decreased genetic variability. Poulson & White (1969) also stressed the probable relationship between reduced genetic variability with the reduction of population size, reduced rate of population growth, longer maturation times, and longer lifespans. Previous studies (Avise & Selander 1972; Swofford *et al*. 1980; Koppelman & Figg 1995) on aquatic obligate cave species (stygobites) were consistent with the Poulson and White hypothesis, but each of the studies had sparse sampling across small geographic areas within the species' ranges and these studies were conducted using allozymes, which can underestimate genetic diversity. Our study tests these two alternative hypotheses for the first time using exceptional sampling and high-resolution genetic data from a group of subterranean crayfishes. We also compare our cave crayfish findings to those of two common surface stream-dwelling crayfish species for broader understanding of subsurface and surface freshwater habitats and conservation.

Materials and methods

Study organisms

One of the largest animals in caves are blind crayfish, which are found in all kinds of subterranean aquatic areas, including deep rivers and lakes, small seeps, rimstone pools, and mudholes. A group of stygobitic crayfishes in the genus *Orconectes* inhabits the karst groundwaters of the western escarpment of the Cumberland Plateau, ranging from eastern Kentucky south to northern Alabama (Hobbs & Barr 1972; Hobbs *et al*. 1977). As currently recognized, there are three obligate cave-dwelling *Orconectes* species along the plateau: *Orconectes incomptus*, *Orconectes australis* (with two subspecies, *australis* and *packardi*), and *Orconectes sheltae*, which was only known from one Mississippian Age cave in Alabama (Cooper 1975; Cooper & Cooper 1997) and is currently presumed extinct, with the last sighting by Hobbs & Bagley (1989). *O. incomptus* is found only in Ordovician Age limestone in an area just west of the escarpment. *O. australis* is found in Mississippian Age limestone along the escarpment, which was formed by the recession and erosion of the Cumberland Plateau in an eastward direction, allowing for cave development on the western side. The conservation categories for these species are: *Orconectes australis australis* (IUCN stable), *O. a. packardi* (IUCN vulnerable), *O. incomptus* (IUCN vulnerable), *O. sheltae* (unlisted).

To thoroughly investigate the genetic diversity and phylogeographic patterning of this unique assemblage, we collected mostly tissue samples (a claw or leg which are regenerated) from 421 individuals from 67 caves spanning the entire geographic range (Table 1). Nondestructive sampling involved returning the captured individual to the capture site immediately after removal of claw or leg. In a few cases, one or two voucher male specimens (preserved in 90% ethanol at the Monte L. Bean Museum at Brigham Young University) were taken from caves discovered after Hobbs *et al*.'s (1977) distribution list of cave crayfish localities to serve as voucher specimens for these caves.

For comparison to surface species, we chose two common surface stream-dwelling *Orconectes* species for which we have substantial molecular data and thoroughly sampled distributions as part of other research investigations. *Orconectes luteus* is a wide-ranging surface species throughout Missouri, while *Orconectes juvenilis* has a restricted range in the Upper Cumberland River and Kentucky River basins of Kentucky. Both *O. luteus* and *O. juvenilis* are assigned to the subgenus *Procericambarus* of the genus *Orconectes* and are IUCN stable species.

Table 1 List of cave *Orconectes* taxa, sampled caves, mtDNA 16S haplotype with number of individuals sequenced in parentheses, 3-step nested clade groupings, geographic information, and geologic age of cave sites used in this study

Species	Cave name	16S Haplotype (# of individuals)	3-step clade	State: county	Geologic age
incomptus	Cherry†	19(2)	3-3	TN: Jackson	Ordovician
incomptus	Flynn Creek	17(1)	3-3	TN: Jackson	Ordovician
incomptus	North Fork	18(2), 20(3)	3-3	TN: Jackson	Ordovician
a. packardi	Teamers	1(1), 2(2)	3-1	KY: Rockcastle	Mississippian
a. packardi	Duvalts	2(1)	3-1	KY: Rockcastle	Mississippian
a. packardi	Pine Hill	2(1)	3-1	KY: Rockcastle	Mississippian
a. packardi	Fletcher Spring	7(2)	3-2	KY: Rockcastle	Mississippian
a. packardi	Cedar Creek	7(14)	3-2	KY: Pulaski	Mississippian
a. packardi	Dykes Bridge	7(3)	3-2	KY: Pulaski	Mississippian
a. packardi	Dave's	6(8), 7(2)	3-1, 3-2	KY: Pulaski	Mississippian
a. packardi	Big Sink	7 (20)	3-2	KY: Pulaski	Mississippian
a. packardi	Hail	3(4), 4(3), 5(1)	3-1	KY: Pulaski	Mississippian

Table 1 *Continued*

Species	Cave name	16S Haplotype (# of individuals)	3-step clade	State: county	Geologic age
a. packardi	Wells	6(3), 7(1)	3-1, 3-2	KY: Pulaski	Mississippian
a. packardi	Jugornot	8(3), 12(13), 13(1), 14(2), 15(1), 16(2)	3-2	KY: Pulaski	Mississippian
a. packardi	Coral	3(1)	3-1	KY: Pulaski	Mississippian
a. packardi	Sloans Valley†	9(1), 10(2), 11(1)	3-2	KY: Pulaski	Mississippian
sp. nov.	Redmond Creek	24(9)	3-4	KY: Wayne	Mississippian
sp. nov.	Grayson Gunner	23(1)	3-4	KY: Wayne	Mississippian
sp. nov.	Stream	24(2), 25(2)	3-4	KY: Wayne	Mississippian
sp. nov.	Tonya's	23(7)	3-4	KY: Wayne	Mississippian
sp. nov.	Buffalo Saltpeter	23(3)	3-4	KY: Clinton	Mississippian
sp. nov.	Clinton	21(5), 22(1)	3-4	TN: Pickett	Mississippian
sp. nov.	Cornstarch	21(9)	3-4	TN: Fentress	Mississippian
sp. nov.	Redbud	21(1)	3-4	TN: Fentress	Mississippian
a. australis	Fallen Entrance	27(6)	3-6	TN: Fentress	Mississippian
a. australis	Skillmans Mark	27(3), 30(1)	3-6	TN: Fentress	Mississippian
a. australis	Mountain Eye	27(4)	3-6	TN: Fentress	Mississippian
a. australis	Mill Hollow	27(16), 28(1), 50(1), 51(3)	3-6, 3-8	TN: Overton	Mississippian
a. australis	Raven Bluff	37(1)	3-6	TN: Overton	Mississippian
a. australis	Bailey's Webb	27(5)	3-6	TN: Overton	Mississippian
a. australis	Capshaw	27(12), 29(1)	3-6	TN: Putnam	Mississippian
a. australis	Knieps Spring	27(4)	3-6	TN: Putnam	Mississippian
a. australis	Blindfish	26(1), 27(2), 31(3), 32(1), 33(1)	3-6	TN: Putnam	Mississippian
a. australis	Virgin Falls	40(4)	3-7	TN: White	Mississippian
a. australis	Merrybranch	34(1), 35(7), 36(1), 40(22), 41(1), 42(1), 43(1), 44(4), 45(1)	3-6, 3-7	TN: White	Mississippian
a. australis	Lost Creek Resurgence	40(1)	3-7	TN: White	Mississippian
a. australis	Rumbling Falls	40(6)	3-7	TN: VanBuren	Mississippian
a. australis	Winching Hollow Water	35(9), 40(3)	3-6, 3-7	TN: VanBuren	Mississippian
a. australis	Glencora Spring	27(1), 40(4)	3-6, 3-7	TN: VanBuren	Mississippian
a. australis	Waterfall Hollow	54(7)	3-8	TN: VanBuren	Mississippian
a. australis	Lost Cove	51(10), 53(1)	3-8	TN: VanBuren	Mississippian
a. australis	Camps Gulf	40(2), 54(1)	3-7, 3-8	TN: VanBuren	Mississippian
a. australis	Laurel Creek	40(1), 51(17)	3-7, 3-8	TN: VanBuren	Mississippian
a. australis	Lower Norton Spring	49(1), 51(3)	3-8	TN: VanBuren	Mississippian
a. australis	Rocky River	46(5), 47(2)	3-8	TN: Warren	Mississippian
a. australis	Jaco Spring	48(4)	3-8	TN: Warren	Mississippian
a. australis	Cumberland Caverns*	46(1), 51(4)	3-8	TN: Warren	Mississippian
a. australis	Blowing	38(5)	3-7	TN: Warren	Mississippian
a. australis	Woodlee	39(1)	3-7	TN: Grundy	Mississippian
a. australis	Dry	39(1)	3-5	TN: Grundy	Mississippian
a. australis	Red Trillium	61(2)	3-5	TN: Grundy	Mississippian
a. australis	Big Mouth	61(4)	3-5	TN: Grundy	Mississippian
a. australis	Crystal	61(5)	3-5	TN: Grundy	Mississippian
a. australis	Smith Hollow NR1	61(4), 63(1)	3-5	TN: Grundy	Mississippian
a. australis	Lusk	51(1), 61(7), 64(1)	3-5, 3-8	TN: Coffee	Mississippian
a. australis	Pearson	61(26), 62(1)	3-5	TN: Franklin	Mississippian
a. australis	Wet	61(2)	3-5	TN: Franklin	Mississippian
a. australis	Dripping Spring	59(1)	3-5	TN: Franklin	Mississippian
a. australis	Witherspoon	51(7)	3-8	TN: Franklin	Mississippian
a. australis	Floorless	51(1), 52(1)	3-8	TN: Franklin	Mississippian
a. australis	Larkin Spring	65(2)	3-5	AL: Jackson	Mississippian
a. australis	Limrock Blowing	65(28), 67(1), 69(1)	3-5	AL: Jackson	Mississippian
a. australis	Doug Green	56(1)	3-5	AL: Jackson	Mississippian
a. australis	Langston	55(1)	3-5	AL: Jackson	Mississippian
a. australis	Scott	65(3)	3-5	AL: Madison	Mississippian
a. australis	Hering	57(1), 65(12), 66(1)	3-5	AL: Madison	Mississippian
a. australis	Shelta†	58(4), 60(1), 65(1), 68(1)	3-5	AL: Madison	Mississippian

*Represents a known introduced population from a nearby cave; †represents type locality.

Data collection

Genomic DNA was extracted using standard methods and the 16S mtDNA gene was amplified during polymerase chain reaction (PCR) with primers 16sf-cray: GACCGTGCKAAGGTAGCATAATC and 16s-1492r: GGTTACCTTGTTACGACTT (Crandall & Fitzpatrick 1996). The 16S mtDNA is the most variable gene for freshwater crayfishes (Crandall 1997; Fetzner & Crandall 2003). Cycle-sequencing reactions were run with purified PCR products and the BigDye Ready-Reaction kit on a PerkinElmer Thermocycler. Reactions were cleaned using Millipore plates and then sequenced using an ABI377 automated DNA sequencer. Sequences were edited and aligned by eye using BIOEDIT (Hall 1999). GenBank (www.ncbi.nlm.nih.gov) Accession nos of the 16S mtDNA haplotypes used for this study are: *Orconectes a. packardi* AY853595–AY853610; *O. incomptus* AY853611–AY853614; *O.* sp. nov. AY853615–AY853619; *O. a. australis* AY853620–AY853663; *Cambarus gentryi* AY853664; and *Cambarus graysoni* AY853665. R. Ziemba collected samples of *O. juvenilis* (n = 100 individuals), which we sequenced for 16S (unpublished data, available upon request from R. Ziemba). The *O. luteus* (n = 393 individuals) aligned 16S data set (Fetzner & Crandall 2003; GenBank AF376483–AF376521) was provided by J. Fetzner. Both surface species were amplified in PCR and sequenced using primers 16s-1492r and 16s-17sub: ATASRGTCTRACCTGCCC (Fetzner & Crandall 2003).

Phylogenetic analyses

Phylogenetic analyses included 69 unique haplotypes (485 base pairs) from the 421 cave individuals and two outgroup sequences from the closest relatives *C. gentryi* and *C. graysoni* (Sinclair *et al*. 2004; Buhay *et al*., unpublished). The Bayesian analysis (Ronquist & Huelsenbeck 2003) was run for 10 million generations using four chains, sampling 1/1000 trees with parameters nst = 6 and rates = adgamma. We discarded the burn-in (first 1001 trees of 10 001 total determined by Tracer (http://evolve.zoo.ox.ac.uk/software.html), checked for convergence using Tracer, and constructed a 50% majority rule consensus tree. Five independent runs of the same data set with random start trees resulted in nearly identical results. Posterior probabilities (PP) greater than 95% are considered significant support for a clade (Huelsenbeck & Ronquist 2001). The maximum-likelihood analysis was run in PAUP* (Swofford 2001) by heuristic search (fast-stepwise addition with random seed) with 500 replicates using the TrN + I + G model of evolution selected by MODELTEST (Posada & Crandall 1998). Nodal support was assessed using 100 bootstrap (BS) replicates (Felsenstein 1985) with strong clade support of 70% (Hillis & Bull 1993).

Genetic diversity and effective population sizes

To address current and recent historical levels of variation, genetic diversity and effective population sizes within each surface and cave lineage were determined using several methods. We used different estimators of the parameter $\theta = 2N_{e(f)}\mu$ for maternally inherited mitochondrial DNA, to determine effective population size (N_e) with a mutation rate μ (2.2×10^{-8} substitutions per site per year; based on Cunningham *et al*. 1992 estimate for crabs) with generation times of 2 years for surface-dwelling species (Hobbs 1991) and 10 years for stygobitic species (Cooper 1975), and an equal sex ratio (Cooper 1975).

Current genetic diversity (θ_π; Nei 1987 equations 10.5 or 10.6, and the standard error, equation 10.7) was assessed using DNASP 4.0 (Rozas *et al*. 2003). Watterson's (1975) historical genetic diversity estimates (θ_W) were determined using LAMARC (http://evolution.genetics.washington.edu/lamarc.html; Kuhner *et al*. 2004). Current genetic diversity estimates (θ_π) are based on pairwise differences between sequences, while historical diversity estimates (θ_W) are based on the number of segregating sites among the sequences. These two methods used together provide insight into population dynamics over recent evolutionary history (Templeton 1993; Crandall *et al*. 1999; Pearse & Crandall 2004). Differences between current diversity and recent historical diversity are indicative of recent bottlenecks (if $\theta_\pi < \theta_W$) or recent population growth (if $\theta_\pi > \theta_W$) (Templeton 1993; Sinclair *et al*. 2002; Roman & Palumbi 2003; Yu *et al*. 2003).

Pairwise comparisons were used for genealogical estimates of diversity (B_1, θ_2, $\theta_{Ancestor}$) and divergence times using the program IM (Isolation–Migration Model: Nielsen & Wakeley 2001; Hey 2005; Won & Hey 2005; http://lifesci.rutgers.edu/~heylab/heylabsoftware.htm#IM). The HKY (Hasegawa–Kishino–Yano) model with an inheritance scalar of 0.25 for mitochondrial DNA was used with a random seed to initiate the run. A burn-in of 200 000 steps was discarded before recording genealogical steps, and each comparison was run until the effective sample sizes (ESS) were larger than 1000, and in most cases, over 1 million. Multiple independent runs with random start seeds were performed to ensure values were converging on similar estimates. Maximum-likelihood estimates of diversity were used to determine bottleneck (< 1) or growth trends (> 1) between descendent pairs and their ancestors (Descendents : Ancestor ratio) to test the two competing hypotheses about subterranean genetic diversity (Poulson & White 1969 and Barr 1968). Descendent : Ancestor ratios were computed by $(\theta_1 + \theta_2)/\theta_{Ancestor}$ for each pair.

Phylogeographic analyses

Nested clade analysis (NCA: Templeton *et al*. 1995; Templeton 1998) was used to test the null hypothesis of no genetic

differentiation between sampled sites and provide insight into historical processes. The program TCS (Clement *et al*. 2000) was used to construct the haplotype network and GEODIS (Posada *et al*. 2000) was used to test for significant associations between geographic cave locations and genetic distances over 5000 random permutations. Latitude and longitude coordinates of cave localities (at the entrance) were used for the geographic analysis. Haplotypes with the most connections and the highest frequencies are thought to be older, while haplotypes on the tips are more recently evolved. Clade distances (D_c) represent geographic ranges of the clades at each step level. Nested clade distances (D_n) represent the average distances of samples with a particular haplotype with respect to the geographic centre of the clade. Inferences about the historical processes that gave rise to the current genetic patterns were made using the 2004 inference key from A. R. Templeton (http://darwin.uvigo/es/software/geodis.html).

Results

Phylogenetic analysis of 16S mtDNA haplotypes

There are several operational methods available to delineate species boundaries using statistically testable frameworks, as reviewed by Sites & Crandall (1997) and Sites & Marshall (2003). The Genealogical Concordance Species concept (Avise & Ball 1990; Baum & Shaw 1995) is a lineage-based extension of the phylogenetic species concept, in which there is concordance among multiple characters (genetic, environmental, geographic, etc.). A genealogical species is a group of organisms whose members are more closely related to each other ('exclusivity') than to any other organisms outside the group (Baum & Shaw 1995).

We determined the phylogenetic relationships among the two extant species (*Orconectes incomptus* and *Orconectes australis*) using sequence data from the mitochondrial 16S gene (485 base pairs) and identified four distinct lineages: *O. a. packardi*, *O. incomptus*, *O. a. australis*, and *O.* sp. nov. (Fig. 1 and Table 1), each with significant posterior probability support. The cave-dwelling *Orconectes* members are most closely related to burrowing members of the genus *Cambarus* (Crandall & Fitzpatrick 1996; Fetzner 1996; Sinclair *et al*. 2004), rather than to the surface-dwelling members of *Orconectes*, as was previously thought based on similar (convergent) male morphology (Hobbs & Barr 1972), and accordingly, *Cambarus gentryi* and *Cambarus graysoni* were used as the closest outgroup taxa (Sinclair *et al*. 2004; Buhay & Crandall, unpublished).

The most basal member, *O. a. packardi*, was represented by 16 unique mtDNA 16S haplotypes from 13 Mississippian Age caves and 93 individuals, and is distributed from Rockcastle County, Kentucky, south to Pulaski County, Kentucky (Fig. 1: range shown as blue circles, haplotypes 1–16). *O. incomptus* was represented by four unique haplotypes from three Ordovician Age caves in Jackson County, Tennessee (Fig. 1: range shown as pink triangles, haplotypes 17–20). A new species, *O.* sp. nov., found along the Kentucky–Tennessee border (Wayne and Clinton counties, Kentucky, south to northern Fentress County, Tennessee), included five unique haplotypes from eight Mississippian Age caves and 40 individuals (Fig. 1: range shown as green pentagons, haplotypes 21–25). *O. a. australis* was represented by 321 individuals from southern Fentress County, Tennessee south to Madison County, Alabama and included 44 unique haplotypes from 43 Mississippian Age caves (Fig. 1: range shown as orange squares, haplotypes 26–69). Genetic data were acquired from type locality specimens: *O. a. packardi* (Sloans Valley Cave, Pulaski County, Kentucky), *O. incomptus* (Cherry Cave, Jackson County, Tennessee) and *O. a. australis* (Shelta Cave, Madison County, Alabama), and this information was used to clarify species boundaries and their geographic distributions.

Each of these lineages will be considered distinct species based on genetic and geographic concordance (Avise & Ball 1990; Baum & Shaw 1995). Rather than two species (*O. australis* and *O. incomptus*), there are five stygobitic cave *Orconectes* species on the Cumberland Plateau, including the unsampled, possibly extinct *Orconectes sheltae*.

Genetic variation, effective population sizes, and divergence times

Estimates of current (θ_π) and historical (θ_W) genetic diversity were moderate to high (Nei 1987) for the cave dwellers, with the exception of *O.* sp. nov. (Table 2). Similarly, current

Table 2 Current ($\theta_\pi \pm$ SE) and Historical-based (θ_W) estimates of genetic diversity and corresponding effective population sizes for obligate cave-dwelling *Orconectes* species and surface-dwelling *Orconectes* species

	Current		Historical	
	θ_π	N_e	θ_W	N_e
Cave species				
O. a. packardi	0.00455 ± 0.00043	41 364	0.00606	55 082
O. incomptus	0.00508 ± 0.00092	46 182	0.00477	43 375
O. sp. nov.	0.00238 ± 0.00027	21 636	0.00242	22 034
O. a. australis	0.00894 ± 0.00020	81 273	0.01593	144 777
Surface species				
O. juvenilis	0.00394 ± 0.00024	179 091	0.03179	1 445 182
O. luteus	0.02501 ± 0.00015	1 136 818	0.06076	2 761 955

$\mu = 2.2 * 10^{-8}$ substitutions per site per year. Surface-dweller generation time = 2 years, cave-dweller generation time = 10 years.

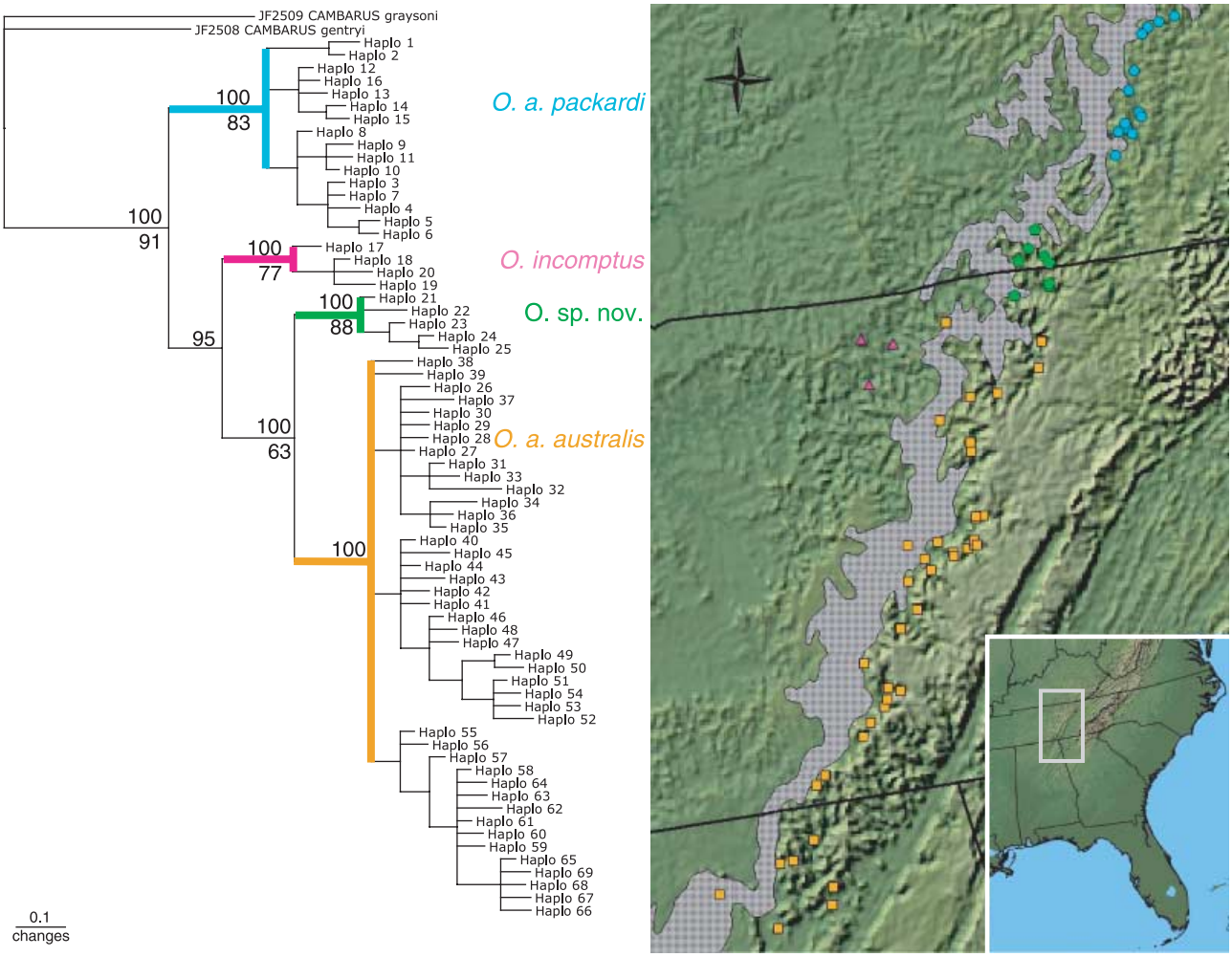

Fig. 1 Geographic distribution (on right) represented by sampled localities for *Orconectes australis packardi* (blue circles), *Orconectes* sp. nov. (green pentagons), and *Orconectes australis australis* (orange squares) along the western escarpment (dark grey shading) of the Cumberland Plateau in Mississippian Age caves at elevations between 180 and 450 m. *Orconectes incomptus* (pink triangles) is found in the area just west of the escarpment in Ordovician Age caves at 150–180 m in elevation. Phylogenetic relationships (on left) are based on 69 haplotypes of 16S mtDNA sequence data using similar results from maximum-likelihood and Bayesian methods. Colours marked on tree match cave species colours from distribution map. *Cambarus graysoni* and *Cambarus gentryi* were used as outgroup taxa. Numbers below branches indicate bootstrap support and numbers above branches indicate posterior probabilities.

effective population sizes (N_e) were also higher than expected, suggesting the occurrence of a vast groundwater network unknown to humans, but as accessible habitat to the stygobitic crayfish. Surprisingly, current (θ_π) and historical (θ_W) estimates for the stygobites were similar (Table 2, with exception of *O. a. australis* which exhibited decline), whereas both surface species estimates show serious recent declines ($\theta_\pi < \theta_W$).

We used a coalescent-based method (Nielsen & Wakeley 2001) to determine genetic diversity over the genealogical histories of each cave species to test the two competing hypotheses regarding genetic diversity of ancestors vs. descendents. Using pairwise species comparisons, we determined genealogical diversity (θ_1 and θ_2) for each crayfish species and $\theta_{Ancestor}$ for their common ancestor, along with their times since divergence (Table 3). These results show a growth trend (descendents/ancestor ratio > 1) after the initial split from the ancestors in cave species comparisons (Fig. 2).

The estimated divergence times for the cave crayfish species are much older than previous speculation (Hobbs *et al*. 1977). Given the broad credibility intervals (90% highest posterior probability densities; HPD) for the *O. a. packardi*–*O. incomptus* and *O. incomptus*–*O.* sp. nov. comparisons, it appears that more loci are needed to resolve divergence times for these species. It is also possible that

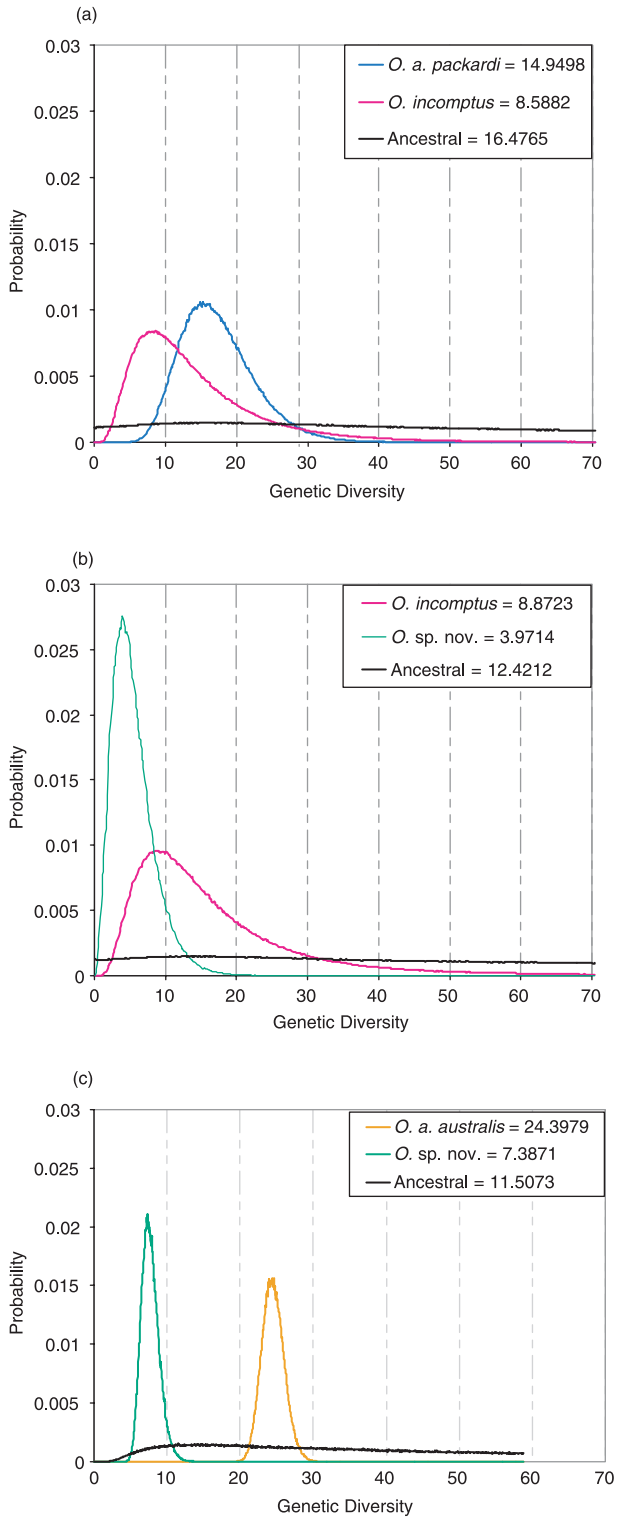

Fig. 2 The marginal posterior probability distributions for the IM model parameter of cave genetic diversity scaled by the neutral mutation rate. Curves are shown for the pairwise analyses of (a) *Orconectes australis packardi* (in blue) vs. *Orconectes incomptus* (in pink) (b) *O. incomptus* (in pink) vs. *O.* sp. nov. (in green), and (c) *O.* sp. nov. (in green) vs. *Orconectes australis australis* (in orange) with their corresponding ancestral (in black) diversities.

more individuals of *O. incomptus* are needed for the IM pairwise analyses, since only eight individuals from three caves of the 10 known sites were sampled for this study. *O. incomptus* is listed in Tennessee as a 'management concern species' and as a 'vulnerable species' by the International Union for Conservation of Nature and Natural Resources (IUCN) which required that sampling restrictions be placed on the collection permit. Interestingly, the split between *O.* sp. nov. and *O. a. australis* was estimated to be 110 million years ago (Ma) (90% HPD interval: 105–116 Ma), in the mid-Cretaceous, which was speculated to be the beginnings of cave invasion for the genus *Cambarus* (Hobbs & Barr 1960). The lower bounds of the 90% HPD intervals for the other two comparisons (*O. a. packardi*–*O. incomptus* at 125 Ma; *O. incomptus*–*O.* sp. nov. at 102 Ma) are similar to that of the *O.* sp. nov.–*O. a. australis* split. Such calculations necessarily make a number of simplifying assumptions and the resulting dates should be taken with caution; however, as outlined below, these divergence times nicely correspond to geological events that might cause such divergences.

Nested clade analysis of cave crayfish

To explain how the cave species attained high levels of genetic variation, we used NCA to uncover the major historical processes and patterns (Templeton 2001). A statistical parsimony network was constructed using a 95% confidence interval, which resulted in 69 unique haplotypes, thirty-four 1-step clades, fourteen 2-step clades, eight 3-step clades, and three 4-step clades in the total cladogram (Table 4, Fig. 3). The statistical parsimony analysis revealed two haplotypes as ancestral, *O. a. packardi* haplotype 7 and *O. a. australis* haplotype 27, and these are shown as rectangles on Fig. 4. *O. a. packardi* haplotype 8 is connected to *O. incomptus* haplotype 17 by 10 mutational steps (the significant 95% level was nine steps). *Cambarus gentryi* and *C. graysoni* were outside the 95% level, at 21 and 25 mutational steps, respectively, from haplotype 2 of *O. a. packardi*.

To geographically illustrate the historical speciation routes, we used the eight 3-step clades because they mostly resulted in significant inferences of 'contiguous range expansion' or 'isolation by distance' and they show 'big picture' historical biogeographic patterns (Table 5). On Fig. 4, *O. a. packardi* is shown as clades 3-1 (light blue) and clade 3-2 (dark blue) in the network and as circles on the corresponding map, and *O. incomptus* is clade 3-3 (pink) and is represented on the map as pink triangles. Clade 3-4 (green) is *O.* sp. nov. and is marked as green pentagons on the map, while four 3-step clades (3-5 through 3-8) comprise *O. a. australis* (marked as squares on the map of Fig. 4). The 3-step clades of *O. a. australis* geographically overlap extensively in central Tennessee, with several *australis* caves containing haplotypes from different 3-step clades (Table 1).

Table 3 Genealogical estimates of genetic diversity, descendents/ancestor ratio, and divergence time of four stygobitic *Orconectes* species and the ancestral species for each pairwise comparison estimated by IM

	θ	$\theta_{Ancestor}$	Descendents/Ancestor ratio	Time since divergence (in millions of years)
Cave species				
O. a. packardi	14.9498			
	8.82–25.51	16.4765	1.43	282.5
O. incomptus	8.5882	0.06–108.21		125.5–454.5
	2.10–27.29			
O. incomptus	8.8723			
	1.94–34.39	12.4212	1.03	356.1
O. sp. nov.	3.9714	0.08–146.94		102.7–454.4
	0.92–10.05			
O. sp. nov.	7.3871			
	5.56–9.74	11.5073	2.76	110.2
O. a. australis	24.3979	5.56–52.71		105.5–116.4
	21.87–27.11			

Upper values are the maximum-likelihood estimates and the lower values represent the confidence interval range for the 90% highest posterior density. Descendents/Ancestor Ratio = $(\theta_1 + \theta_2)/\theta_{Ancestor}$. A mutation rate of 2.2% per million years was used to determine time since divergence.

Table 4 Results of the nested clade analysis of *Orconectes* 16S mtDNA haplotypes based on 5000 permutations in GEODIS

0-step clades			1-step clades			2-step clades			3-step clades			4-step clades		
Haplotype	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n
1	0	5.6	1-1			2-1	3.7	21.1	3-1	19.2L	18.9	4-1	16.2S	148.7L
2	3.3	3.5												
I-T	3.3	–2.1												
3	3.4	3.8S	1-2	3.8L	3.9L	2-2	3.7S	17.7S						
4	0	4.1												
I-T	3.4	–0.3S												
5	0	4.0	1-3	3.5	3.5S									
6	3.5	3.5												
						I-T	–0.1	–3.4						
7	9.9	9.8	1-4	10.1S	10.3S	2-3	12.5	12.7	3-2	12.6S	13.6S			
8	0	18.8L												
9			1-5	0.0S	22.5L									
10														
11														
			I-T	10.1	–12.2S									
12			1-6			2-4	0.0S	12.1						
13														
14														
15			1-7											
16														
						I-T	12.5L	0.6	I-T	–6.6S	–5.3S			
17			1-8	0.0	10.7	2-5			3-3	9.0S	43.5L	4-2	33.9S	50.4S
19			1-9	0.0	8.3									
18			1-10	0.0S	8.1S									
20														
			I-T	0.0S	–1.0									
22			1-11	0.0	13.6	2-6			3-4	8.4S	34.3			
21	5.6S	10.1	1-12	9.4L	9.5L									
23	4.7S	8.8												
24	1.4	1.6	1-13	1.6S	4.8S									
25	0.0	1.6												
I-T	1.4	0.0	I-T	7.9L	4.1L									
26	0.0	23.1	1-19	21.5	21.6	2-10	20.7S	21.1S	3-6	23.9S	30.7S			
27	18.7	18.7												

Table 4 *Continued*

0-step clades			1-step clades			2-step clades			3-step clades			4-step clades		
Haplotype	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n
28	0.0	0.5												
29	0.0	23.4												
30	0.0	25.4												
I-T	18.4	2.4												
31			1-20	0.0S	27.4									
33														
37			1-24	0.0	24.2									
			I-T	20.1S	–2.2									
32			1-21			2-11	0.0	18.0						
35	5.2	5.4S	1-22	5.6S	5.8	2-12	6.0S	50.5L						
36	0.0	13.0												
I-T	5.2	–7.6S												
34			1-23	0.0	12.7L									
						I-T	15.1L	–27.7S	I-T	9.7	–11.7S			
38			1-32	0.0S	6.2L	2-9	4.2	26.9L	3-7	19.2S	49.1	4-3	47.8S	86.3
39			1-33	0.12	3.1S									
40	6.2	6.2	1-25	6.2	6.2	2-13	6.2S	15.0S						
41	0.0	5.0												
42	0.0	5.0												
44	0.0	5.0												
I-T	6.1	1.2												
43			1-26	0.0	4.9									
45			1-27	0.0	4.9									
			I-T	6.2	1.3									
46	3.5	5.1	1-29	4.6S	4.6S	2-14	6.1S	17.9S	3-8	33.6S	48.2			
47	0.0	3.7												
48	0.0	4.5												
I-T	3.5	0.9												
49	0.0	11.1S	1-28	18.6	17.0									
50	0.0	58.0												
I-T	0.0	–46.9												
51	37.8	35.19	1-31	34.2S	36.5S	2-15	38.6L	36.5L						
53	0.0	22.8												
54	0.8S	31.0												
I-T	37.1L	5.1												
52			1-30	0.0	66.2									
			I-T	34.2S	–29.7S	I-T	–32.5S	–18.5S						
61	8.1	8.1	1-14	8.1S	13.6S	2-7	20.5S	35.4	3-5	35.7S	46.5			
63	0.0	2.8												
64	0.0	15.2												
I-T	8.1	–0.9												
62			1-15	0.0	3.7									
58	0.0	31.2	1-16	26.0	48.5L									
59	0.0	22.2S												
60	0.0	31.2												
I-T	0.0	4.5L	I-T	9.9S	13.5									
57			1-34	0.0	19.1 L	2-8	14.1S	36.1						
55	0.0S	5.5	1-18	5.5	12.1									
56	0.0S	5.5												
I-T	0.0	0.0												
65	13.7	13.9	1-17	14.5	15.0									
66	0.0	14.5L												
67	0.0	12.4												
68	0.0	30.0L												
69	0.0	12.4												
I-T	13.8	–3.4S	I-T	–10.8	–0.65	I-T	–6.4	0.7	I-T	–15.7S	2.0	I-T	–5.1	–53.3S

Clade (D_c) and nested clade (D_n) distances are given. An 'S' indicates the distance is significantly small at the 5% level and an 'L' indicates the distance is significantly large. In clades with both tip and interior nested clades, the average distance I-T is given. Shaded regions indicate interior groupings.

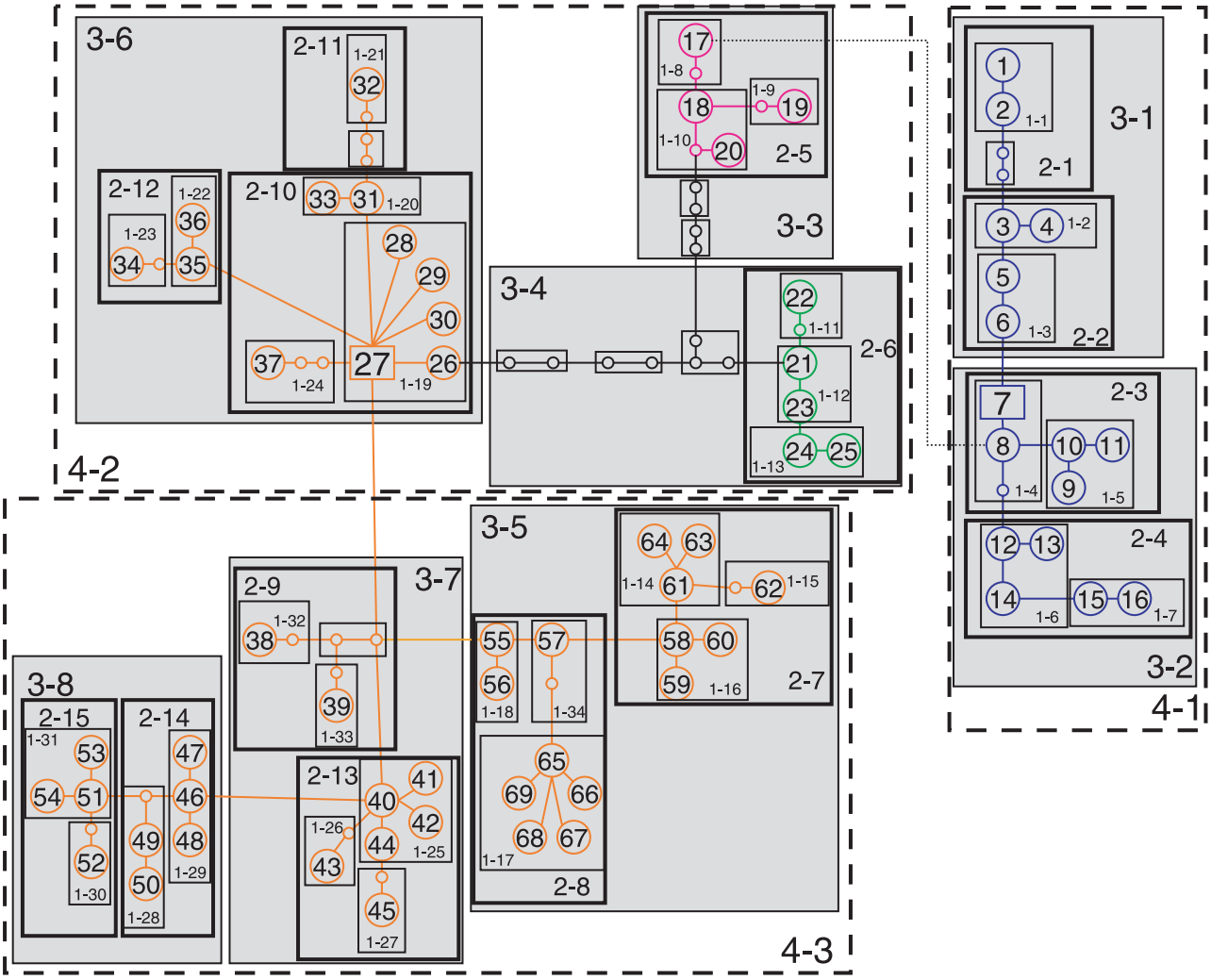

Fig. 3 Haplotype network showing the nesting levels used to infer historical processes. Haplotype circles are coloured to represent four distinct lineages: *Orconectes australis packardi* (blue), *Orconectes incomptus* (pink), *O.* sp. nov. (green), and *Orconectes australis australis* (orange). Empty circles in the network represent unsampled, possibly extinct haplotypes. The total cladogram includes clades 4-1, 4-2, and 4-3.

Discussion

It was hypothesized that the surface ancestor to the cave *Orconectes* originally expanded in a northeast direction from the Mississippi embayment, spawning obligate cave-dwelling species along the Cumberland Plateau en route to the northern Appalachian Mountains (Hobbs & Barr 1972). On the contrary, our phylogenetic and NCA results show that *Orconectes australis packardi*, which is distributed across the northern end of the Cumberland Plateau, is the most basal member of the cave assemblage. This suggests that the surface ancestor (a member of the burrowing genus *Cambarus*) ranged somewhere in eastern Kentucky and gave rise to the stygobitic species *O. a. packardi*. The other stygobitic species then diverged from a common ancestor with *O. a. packardi*. The southern limit of the cave *Orconectes* distribution is the area just north of the Fall Line in Alabama, the prehistoric Atlantic Ocean coastline.

Our estimates of divergence times, although based on one mtDNA region, place the oldest cave *Orconectes* species on the plateau present during the Cretaceous period, which was the suggested time period for cave invasion by surface members of the genus *Cambarus* (Hobbs & Barr 1960). This time frame also correlates with the age estimates of the oldest passages in plateau caves and the beginnings of the eastward recession of the Cumberland Plateau (Barr 1961). It appears that the long evolutionary histories of crayfishes in the stable underground environment have allowed them to persist and accumulate genetic diversity, despite environmental changes on the surface, long generation times, and isolation over the past millions of years. Poulson & White (1969) speculated that older cave

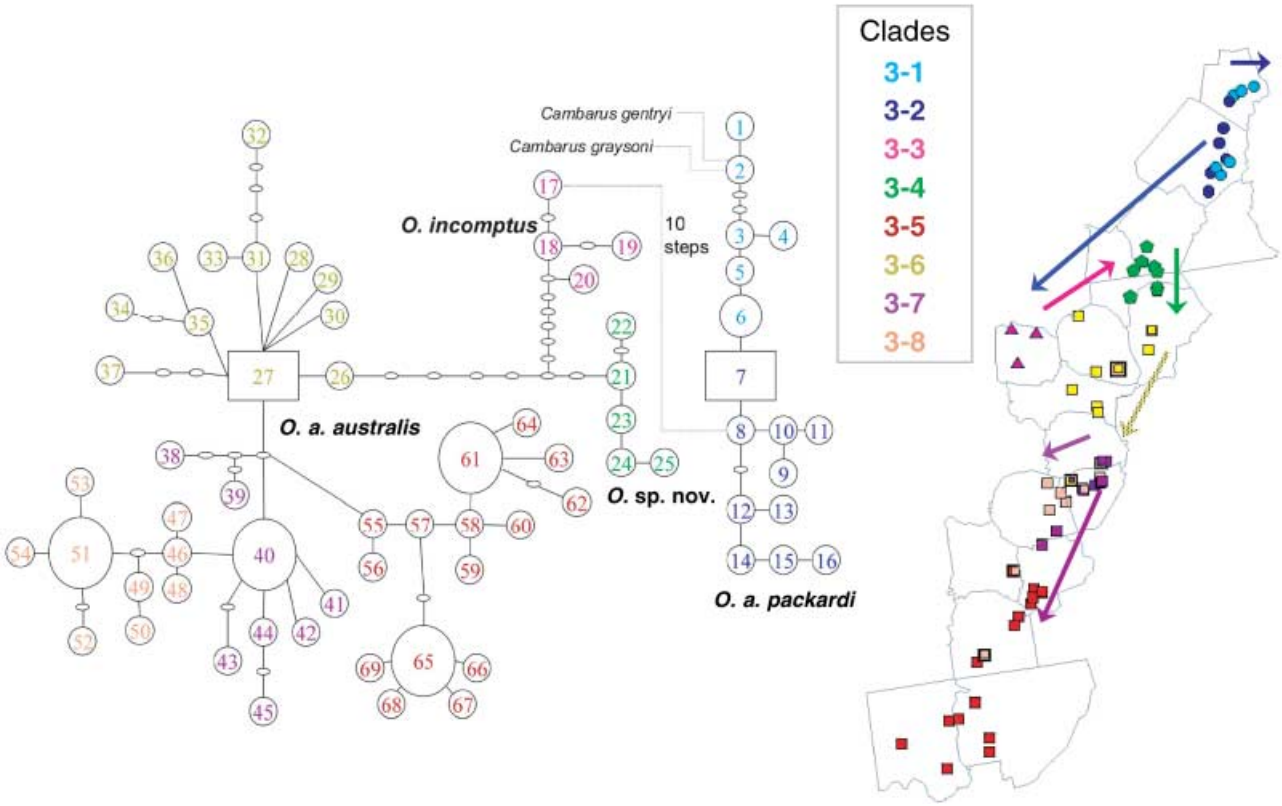

Fig. 4 Haplotype network on left is geographically illustrated using the eight 3-step nested clades, which are corresponding marked by the same colours on the map with grey county outlines to the right. *Orconectes australis packardi* (haplotypes 1–16 in network; circles on map) was outside the 95% confidence limit, while *Orconectes incomptus* (haplotypes 17–20 in network; triangles on map), *Orconectes* sp. nov. (haplotypes 21–25 in network; pentagons on map), and *Orconectes australis australis* (haplotypes 26–69; squares on map) were connected within the 95% confidence level. Coloured arrows on the dot map of sampled caves show routes of contiguous range expansion by the leading-edge expanding clade. Empty circles in the network represent unsampled, possibly extinct haplotypes. The outgroups *Cambarus gentryi* and *Cambarus graysoni* were outside the 95% limit and connected to haplotype 2 of *O. a. packardi*.

species would show low levels of diversity due to the long period of isolation underground, but it appears that levels of diversity for the cave crayfish species are not related to their estimated old divergence times.

One of the arguments made by Culver *et al*. (2000) for the endangered status of cave fauna was restricted geographic ranges, as most United States cave-adapted fauna (61%) are limited to caves in a single county. Although this is a common and practical approach for identifying possible conservation concerns for endemics and rare species as well as habitat types, species-specific information, particularly thorough geographic surveys (Van Jaarsveld *et al*. 1998) and demographic and genetic studies (Lande 1988) are critical pieces of information in assessing the requirements needed for species survival. In this study, *O. a. australis*, with the largest range of the stygobitic *Orconectes*, is now currently known from 11 counties and has the highest genetic diversity of the cave crayfish species; but *O. incomptus*, with the smallest geographic range, and currently only known from nine caves in Jackson County and one cave in Putnam County, Tennessee, has the second highest diversity of the assemblage. *O. a. packardi* is currently known from three Kentucky counties, and *O.* sp. nov. is distributed across four counties in Kentucky and Tennessee, with moderate and low levels of genetic diversity, respectively. In our case, geographic range is not reflective of genetic diversity or conservation status for these cave species. Rather, the decline in genetic diversity over recent history ($\theta_\pi < \theta_W$; Templeton 1993; Sinclair *et al*. 2002; Roman & Palumbi 2003) is a better indicator for conservation concern with *O. a. australis* (currently 0.00894 from historically 0.01593), along with the low levels of diversity for *O.* sp. nov. (currently and historically, 0.00238 and 0.00242). It is interesting that the IUCN stable cave crayfish species, *O. a. australis*, shows a recent loss of diversity, whereas, the two IUCN vulnerable cave species, *O. incomptus* and *O. a. packardi*, show little difference between historical and current diversity estimates.

We show in Fig. 4 a series of colonizations beginning in Kentucky with *O. a. packardi* and progressing down the

Table 5 Nested Contingency Results based on 5000 permutations in GEODIS

Clade	Chi-squared	Probability	Inference chain	Inferred pattern
1-2	0.6857	1.0	1-2-11-17 — No	Inconclusive Outcome
1-4	45.00	0.0002*	1-19-20-2-11-17-4 — No	RGF w/IBD
1-12	26.00	0.0*	1-19-20-2-11-12 — Yes	CRE
1-16	6.00	0.33	1-2-11-17-4 — No	RGF w/IBD
1-17	28.6037	0.17	1-2-11-17 — No	Inconclusive Outcome
1-18	2.000	1.0	1-19-20 — No	Inadequate Geographic Sampling
1-22	1.1953	0.46	1-2-11-17 — No	Inconclusive Outcome
1-28	2.00	1.0	1-19-20-2-11-17 — No	Inconclusive Outcome
1-31	58.9	0.04*	1-2-3-4 — No	RGF w/IBD
2-2	16.354	0.0002*	1-2-11-17–4 — No	RGF w/IBD
2-3	49.0	0.0*	1-19-20-2-11-12 — No	CRE
2-5	16.0	0.0066*	1-19-20 — No	Inadequate Geographic Sampling
2-6	45.385	0.030*	1-2-3-4 — No	RGF w/IBD
2-7	60.093	0.0178*	1-2-3-4 — No	RGF w/IBD
2-8	55.801	0.0136*	1-19-20-2-11-17-4 — No	RGF w/IBD
2-9	7.00	0.05*	1-19-20 — No	Inadequate Sampling
2-10	52.60	0.0174*	1-2-3-4 — No	RGF w/IBD
2-12	1.0588	1.0	1-2-11-17 — No	Inconclusive Outcome
2-14	14.00	0.0238*	1-19-20-2-11-17-4 — No	RGF w/IBD
2-15	27.4909	0.0736	1-2-11-12 — No	CRE
3-1	25.00	0.0*	1-19-20-2-11-12 — No	CRE
3-2	55.13	0.0*	1-2-3-4 — No	RGF w/IBD
3-5	106.27	0.0*	1-2-11-12 — No	CRE
3-6	89.98	0.012*	1-2-3-5-6-7 — Yes	RGF w/some LDD
3-7	57.00	0.0*	1-19-20-2-11-17-4 — No	RGF w/IBD
3-8	55.63	0.0*	1-2-11-12 — No	CRE
4-1	81.044	0.0*	1-19-20-2-11-12 — No	CRE
4-2	257.00	0.0*	1-19-20-2-11-12 — No	CRE
4-3	442.20	0.0*	1-2-11-12 — No	CRE
Total	851.25	0.0*	1-19-20-2-11-12 — No	CRE

*indicates significance at the $P < 0.05$ level. Inferences were made using with Templeton's (2004) revised key. Abbreviations for the inferences are: CRE, contiguous range expansion; RGF, restricted gene flow; IBD, isolation by distance; LDD, long-distance dispersal.

Cumberland Plateau in a leading-edge small-stepwise manner, following the flow of prehistoric waters. This colonization pattern is consistent for animal groups limited by mountain landscapes and by dispersal ability, particularly in response to glacial advance and retreat cycles (Hewitt 1996, 2000). Stygobitic crayfishes are severely limited in dispersal abilities by both subterranean and surface barriers, except during high water levels when they can migrate (or wash) out of caves into a limestone-based surface stream across short distances, and into a nearby underground system via a spring resurgence or cave entrance. These findings suggest that prehistoric groundwater levels were much higher, and allowed for subterranean fauna to disperse over the surface landscape in small distances. Phreatic caves form below the water table, and as karst dissolves and creates voids, the water table lowers to fill in the spaces, which increases groundwater habitat for stygobites (White 1988). Although the major surface rivers along the Cumberland Plateau historically and currently flow in a southern direction, ongoing cave development and subsequent groundwater lowering have probably lead to isolation by distance and the prevention of further stepwise range expansion of the species and clades.

Contiguous range expansion followed by periods of isolation appears to be the main mechanism for the increased variation within the cave crayfish species. A similar trend has been reported for invasive and introduced species (Tsutsui *et al*. 2000; Kolbe *et al*. 2004) in which genetic diversity and population size accumulates and recovers, rather than resulting in a series of bottlenecks leading to lower diversity and extirpation. One example (Sbordoni 1982) has also been documented for a troglobitic beetle species in Italy in which 50 individuals were introduced into an isolated cave with no beetles. After 30 years, the estimated census size was 15 000 individuals with a greater genetic diversity than the original 'founder' population.

Clearly, pre-adaptation and continued expansion into suitable habitat of the subterranean environment allowed cave crayfish to successfully and repeatedly colonize new areas, regardless of population size or genetic diversity of the founder populations.

Orconectes australis packardi, *O. incomptus*, and *O.* sp. nov. are currently distributed across small geographic ranges (four counties or less), possibly due to the hydrologic impacts of the prehistoric watercourses of the Cumberland River. Caves in the path of the Cumberland River during its formation would have been completely submerged by surface waters. The missing haplotypes in the parsimony network may be evidence of past drainage evolution events between the ancestors of *O. a. packardi* and *O. incomptus*, and *O. incomptus* and *O.* sp. nov. leading to local extirpations, range restrictions, and lower diversity in those species compared to *O. a. australis*.

Orconectes luteus and *O. juvenilis* are currently listed as IUCN stable species in conservation status based on the fact that they are widespread throughout their ranges (Taylor *et al*. 1996), but it appears that they are in need of some protection and study (based on the large discrepancy between θ_π and θ_W for both common surface dwellers). The stable underground environment may provide enough suitable 'habitat pockets and hideouts' to buffer the subterranean biota from the direct impacts of ongoing surface pressures, but it appears that the surface species are not so fortunate. It is surprising that species considered to be common stream inhabitants show a reduction in population sizes whereas most of the cave species show consistent population sizes over evolutionary time.

We also hope that these findings shed light on the conservation status of other subterranean taxa and propel biospeleogists to test their assumptions concerning biodiversity. We suggest that management strategies be redirected toward molecular genetic assessments of effective population sizes and diversity (Thorpe *et al*. 1995) for cave species and other elusive fauna considered to be on the brink of extinction because of a lack of scientific information (Holmes 2001). Current cave conservation activities focus on general efforts to protect subterranean habitat by purchasing karstlands, avoiding pollution catastrophes, and gating highly visible entrances. Although these are important defences for the protection of the biome, the ultimate goal of cave conservation is the sustainability of each unique obligate cave-dwelling species. Stochastic factors are well-known causes of biodiversity losses, yet, current research shows that the genetic factors, specifically loss of heterozygosity and inbreeding, can play major roles in driving endangered and threatened species to extinction (Brook *et al*. 2002; Spielman *et al*. 2004). We hope this research will turn the efforts of conservation agencies toward protecting gene flow routes and areas of connectivity to prevent future imperilment of the amazing fauna under our feet and the common inhabitants in our backyards.

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This study represents part of Jen Buhay's doctoral research on the evolutionary history of cave crayfishes with Keith Crandall. Buhay's research interests focus on population genetics and phylogeography of surface and subterranean freshwater fauna. Keith Crandall is a Professor in the Integrative Biology and Molecular Biology Departments at Brigham Young University. Crandall works on a wide variety of organisms in an evolutionary context.
