January 2005

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Subterranean Biology 3: 13-27, 2005

Conservation of subterranean biodiversity in Western Australia: using molecular genetics to define spatial and temporal relationships in two species of cave-dwelling Amphipoda

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ABSTRACT

The Jewel Cave karst system in southwestern Australia contains a community of aquatic invertebrates that is listed as critically endangered owing to lowering water levels in cave lakes. A key question for conservation of biodiversity patterns was to determine the most appropriate spatial scale; viz. lake microscale, cave mesoscale, catchment macroscale, or regional megascale). A second key question was to understand the historical relationship between the community and cave water levels, necessary to evaluate the risk of extinction related to predictions of drying climate in southwestern Australia. This study used molecular genetic techniques (allozyme electrophoresis and mitochondrial DNA sequencing) to assess spatial and temporal relationships in two species of crangonyctoid amphipods (*Uroctena* sp. and *Perthia* cf. *acutitelson* Straškraba) from the Jewel Cave system and other karst drainage systems, springs and surface waters in the Leeuwin-Naturaliste karst region. The molecular data indicated the existence of two cryptic species within *Perthia* cf. *acutitelson*. Within the Jewel Cave karst system, populations sampled from separate groundwater pools both within and between caves were largely panmictic, with no evidence of population sub-structuring in *Uroctena* sp. and only a subtle suggestion of heterogeneity in *Perthia* sp. 1. Beyond the Jewel Cave system, populations of *Perthia* sp. 1 occurring in separate karst drainage systems and surface catchments are strongly differentiated at the population genetic level. For conservation of biodiversity patterns in *Perthia* sp. 1 and *Uroctena* sp., the most appropriate spatial scale is the macrohabitat represented by the karst aquifer or hydrogeologic system. The minimum age estimates, based on molecular clock methods, for divergence of the cave populations from nearby surface populations are > 250 ka. The molecular data support the likelihood that the amphipods in Jewel Cave survived *in situ* lower watertable levels experienced 11 to 13 ka, that coincided with the phase of regional aridity in southwestern Australia near the end of the Pleistocene. However, if the present trend of declining rainfall in southwestern Australia continues, and if water levels in the Jewel Cave system decline below 22.7 m above sea level (ASL), then most of the lakes will become dry and the aquatic invertebrate community will be more vulnerable to extinction because of reduced habitat and consequent reduced population size.

Key words: allozymes, amphipods, caves, conservation, genetics, groundwater, mitochondrial DNA

INTRODUCTION

Groundwater-dependent subterranean ecosystems (GDEs), especially those found in carbonate rocks that have been karstified, and which tend to be hydrogeologically discontinuous at varying spatial scales, exemplify the process of habitat fragmentation along geo-evolutionary time scales. Caves in karst areas frequently host relict populations at different steps of isolation, originating from more or less continuously distributed populations (Culver et al 1995; Culver 1982; Holsinger 2000; Humphreys 2000; Kane et al 1994; Sbordoni et al 2000). Geographically disjunct, small and island-like, cave populations in many parts of the world are imperilled by declining water quantity and water quality resulting from human activities such as groundwater pumping, pollution and nutrient enrichment (for examples, see Drew and Hotzl 1999; Elliot 2000; Juberthie 2000; Katz 2005; Notenboom et al 1994). Cave populations in some Australian karsts are similarly threatened (Boulton et al. 2003; Hamilton-Smith and Eberhard 2000). In southwest Western Australia (Yanchep karst and Leeuwin-Naturaliste karst) for example, groundwater pumping, increased evapotranspiration, and lower rainfall have been implicated in the recent drying-up of cave waters and the near extinction of their dependent aquatic communities (Eberhard 2004; Jasinska 1997; Jasinska and Knott 2000). These aquatic communities (comprising Amphipoda, Copepoda, Ostracoda, Turbellaria, Oligochaeta) occur in pools and streams in caves and, are most diverse in pools and streams that contain submerged tree roots which provide a rich food resource (Jasinska et al 1996). These *aquatic root-mat communities in caves* were listed as critically endangered under the Commonwealth *Environmental Protection and Biodiversity Conservation Act (1999)* (English and Blyth 2000; English et al 2003).

The Leeuwin-Naturaliste karst region contains approximately 100 known caves (Mathews 1985). The

caves provide discrete 'windows' of access to groundwater habitats in numerous karst drainage systems distributed across several major catchment basins. One of the most extensive karst drainage systems is the Jewel Cave karst system which contains more than 10 km of mapped underground passages. The system includes two main caves (Jewel Cave and Easter Cave), with each cave containing a number of lakes in which root-mat communities occur. A key question for conservation of biodiversity patterns was to determine the most appropriate spatial scale, because groundwater ecosystems and processes may be considered as a nested series of spatial and temporal configurations: the microhabitat or microscale (10^{-1} -10^{0} years; 10^{-2} -10^{0} m^3); the habitat (aquifer sector) or mesoscale (10^{0} -10^{2} years; 10^{0} -10^{2} m^3), the aquifer or macroscale (10^{2} -10^{4} years; 10^{2} -10^{5} m^3), and the regional/continental or megascale (>10^{4} years; >10^{5} m^3) (Gibert 2001). Adopting this scheme to the Jewel Cave karst system and the Leeuwin-Naturaliste karst area, the appropriate bio-spatial management units might be, either: (1) individual lake or stream within a cave (microscale); (2) individual cave (mesoscale) which may contain several lakes or streams; (3) karst drainage system/catchment (macroscale) which may include several caves; or (4), whole karst region comprising several karst drainage systems/catchments (megascale).

The lakes both within, and between, Jewel Cave and Easter Cave share groundwater connections via matrix porosity in the limestone, but the extent of hydrologic connectivity via conduit porosity (sensu Ford and Williams 1989; White 1988) has differed in the past. This is a consequence of the narrow vertical range in conduit development (22.5 - 27.5 m above sea level, ASL), and annual-decadal to millennial frequency oscillations in water levels (±5 m) dating from the Early Pleistocene (750-180 ka) to present (Eberhard 2004). In the more recent past (1958 – 1974) when detailed hydrograph records commenced, the water level was relatively high (24.5 – 25.2 m ASL) and many of the lakes were joined together. However, the period 1975 to 1987 encompassed a dramatic drop in the level of the watertable, which overall declined 1.3 m in 13 years. From 1988 to 2003, water levels remained low but comparatively stable at 23.4 – 23.8 m ASL. Radiometric dating of a submerged stalagmite that originally grew in subaerial conditions, indicated that the watertable was lower than 23.2 m ASL during 11 to 13 ka (Eberhard 2004). Presumably, faunal exchange between lakes within each cave, and between both caves, would be facilitated during times of high water levels, whereas populations might become fragmented by hydro-geologic barriers during low watertable periods. This is pertinent to conservation biology in terms of understanding the resilience of the fauna to climatic drying known to have been experienced during the Last Glacial Maximum, and by inference from this, in evaluating the risk of extinction related to predictions of drying climate in southwestern Australia (see Bates et al 2001; Smith et al 2001; Smith et al 2000; Timbal 2004). Accordingly, the second key question for this study was to understand the historical relationship between the community and cave water levels.

The aim of this study was to investigate the spatial and temporal dimensions relating to effective conservation management of the endangered root-mat communities, through a focus on the following questions: (1) Spatial dimension; To what extent are populations in the Jewel Cave karst system genetically isolated from populations dwelling in nearby surface waters, and other cave drainage systems in the Leeuwin-Naturaliste region?, and (2) Historical dimension; Is there genetic evidence that the amphipods survived *in situ* in the Jewel Cave karst system 11 - 13 ka, when water levels were lower than 23.2 m ASL?

To investigate these questions, a species of stygophilic amphipod, *Perthia* cf. *acutitelson* (Crangonyctoidea: Perthiidae), and an undescribed species of stygobitic amphipod, *Uroctena* sp. (Crangonyctoidea: Paramelitidae), were each subjected to a molecular genetic assessment of species and population boundaries using allozyme electrophoresis and mitochondrial DNA sequencing.

Perthia cf. *acutitelson*, which shows clear affinities with *Perthia acutitelson* Straškraba, is a stygophile that is common in cave water bodies, springs and surface streams throughout the Leeuwin-Naturaliste region (Fig. 1). The Jewel Cave karst system populations exhibit weak morphological modification to subterranean existence (reduced body pigment and phenotypic variability in the degree of pigment loss in eyes), but no other troglomorphies, suggesting they have colonised caves relatively recently.

Uroctena sp. is a stygobite exhibiting pronounced morphological modifications to subterranean existence, including loss of eyes and pigment and attenuation of body and appendages. This stygobite appears endemic to the Jewel Cave karst system, although other congeners occur in surface waters of the Darling Range > 100 km to the northeast (Barnard and Williams 1995; Williams and Barnard 1988) (Fig. 1). The cave species exhibits a strong degree of troglomorphy, suggesting that it has been isolated in groundwaters for a comparatively long time.

Two molecular marker systems were selected for this study on the basis of their relative strengths in systematic resolution. Allozymes as nuclear markers have proven utility for detecting cryptic species, hybridization, introgression, and broad population structure (Richardson et al 1986). Mitochondrial DNA was chosen for its ability to provide systematic insight at various taxonomic levels from species right up to family level, identify phylogeographic structure within species, and provide a temporal dimension to evolutionary time via a 'molecular clock' approach (Avise 2004).

MATERIALS AND METHODS

Specimen collection

In common with many groundwater species (Culver 1994; Poulson and Lavoie 2000), both species of amphipod (and indeed all other species present) are relatively rare and cryptic at most localities, and hence it was generally not possible to collect more than a few specimens from each cave without compromising these potentially-threatened populations. Clearly this limitation precludes a detailed analysis of population structure in either species. Nevertheless, the total lack of existing molecular genetic data for any groundwater species in the Jewel

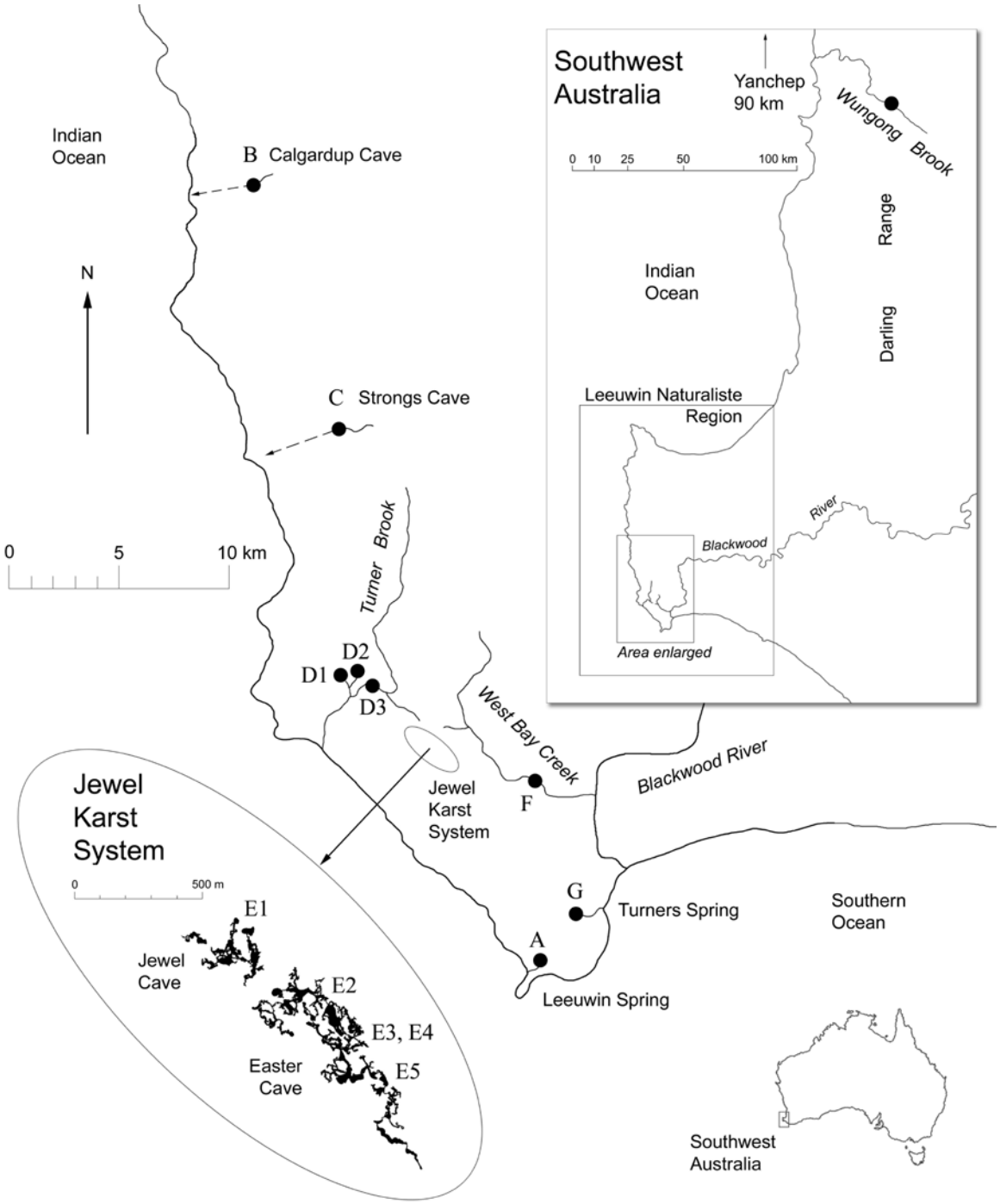

Fig. 1 - Southwest Australia showing sample sites (dots with alpha-numeric site code as in Table 1) and drainage relationships. Enlargement of the Jewel Cave karst system depicts the outline of mapped conduits in Jewel Cave and Easter Cave. The Yanchep karst area (not shown) is located 90 km north of Wungong Brook.

karst system, plus the urgent need to formulate a conservation management plan for this endangered community together justified the use of such small sample sizes, provided caution is exercised when interpreting the data. The statistical limitations of small sample sizes were offset somewhat by the use of two different molecular datasets (allozymes, mtDNA), and by focusing only on broad-brush aspects of the systematic framework.

Samples of *Perthia* cf. *acutitelson* and *Uroctena* sp. were collected from five geographically dispersed sites within the Jewel Cave karst system. The sample sites were groundwater pools within Jewel Cave (one site) and Easter Cave (four sites), with pools being separated from each other by linear distances ranging from 20 m to 850 m (Fig. 1, Table 1).

Representative samples of *Perthia* cf. *acutitelson* were also collected from streams and springs encompassing all contiguous surface catchments (West Bay Creek, Turner Brook) and nearby karst springs (Turners Spring, Leeuwin Spring). Additional samples were collected from two other karst drainage systems in non-contiguous catchments (Strongs Cave and Calgardup Cave), located 16 and 28 km respectively from Jewel Cave (Fig. 1, Table 1). Specimens of the *Uroctena* cf. *whadjukia-yellandia* group (Barnard and Williams 1995) were collected from the Wungong Brook catchment (Curtis Rd) in the Darling Range, 250 km north of Jewel Cave, for use as an outgroup.

Specimens were collected live then frozen in liquid nitrogen or preserved in 100 % ethanol. Larger individuals of *Perthia* cf. *acutitelson* were cut in half, with the posterior half frozen and the anterior half preserved in 100 % ethanol. All frozen material was subsequently maintained at –80°C in the laboratory until required.

Allozyme electrophoresis

Allozyme electrophoresis was undertaken on cellulose acetate gels following the procedures of (Richardson et al 1986). The following enzymes displayed allozymically-interpretable patterns in one or both of the amphipods under study:- *ACON, ACP, ACYC, ADH, ARGK, CA, ENOL, EST, FUM, G6PD, GAPD, GDA, GOT, GPI, GPT, IDH, LDH, MDH, ME, MPI, NDPK, PEPA, PEPB, PEPC, PEPD, PGAM, PGK, PGM, PK, SORDH*, AND *TPI*. Details of enzyme abbreviations, electrophoretic conditions, and stain recipes are found in Richardson (1986) or Lanser (1990). The nomenclature used to refer to loci and allozymes follows Adams (1987).

The allozyme data were analysed using the computer program GENEPOP version 3.3 (Raymond and Rousset 1995) to test for deviation from Hardy-Weinberg expectations, within-population linkage disequilibrium, and between-population differences in allele frequency. All estimates of Fishers exact test thus derived were adjusted using the sequential Bonferonni correction factor to correct for multiple tests (Rice 1989). Although the use of exact probability estimates permits valid statistical analyses to be undertaken on small sample sizes, it is important to recognise the resultant limitations for any analysis of popula-

Table 1 - Details of specimens used in this study.

Location				*Perthia* sp. individuals screened		[a]*Uroctena* sp. individuals screened	
Major Catchment	Sub-catchment	Sample site	Site Code	Alloz.	Mt DNA	Alloz.	Mt DNA
Separate catchments drain to coast	B = Calgardup Cave	stream	B	9	4		
	C = Strongs Cave	stream	C	3	4		
	D = Turner Brook	D1 = resurgence ‘a’	D1	1	1		
		D2 = resurgence ‘b’	D2	4	1		
		D3= Caves Rd	D3	9	4		
Blackwood River	E = Jewel Cave karst system	E1 = Flat Roof I and II	E1	7	2	7	2
		E2 = The Beach	E2	7		2	1
		E3 = Mouse Hole ‘a’	E3	12	1	4	1
		E4 = Mouse Hole ‘b’	E4	6		2	
		E5 = Tiffany’s Lake	E5	14	1	6	2
	F = West Bay Creek	Bussell Highway	F	8	5		
	G = Turners Spring	resurgence	G	8	2		
	A = Leeuwin Spring	resurgence	A	9	4		
		TOTALS		97	29	21	6

[a] Not listed are two outgroup specimens of *Uroctena* cf. *whadjukia-yellandi* group collected from the Wungong Brook catchment in the Darling Range (Fig. 1).

tion structure. The most critical shortcoming here is that the probability of a type II error (ie. falsely accepting the null hypothesis of panmixia when it should be rejected) will always be considerably higher than for large sample sizes (Richardson et al 1986). Fortunately however, this limitation does not affect the validity of any statistically-significant differences obtained using small sample sizes, particularly where these are present at multiple allozyme loci for the same comparison and are supported by a second molecular dataset (as is the case in this study).

F-statistics were also used herein to provide an alternate perspective on population differentiation. These were calculated using the program FSTAT (Goudet 1995,1999), which also determines 95 % confidence intervals through bootstrapping. Genetic distances were calculated as either percent fixed differences (%FD Richardson et al 1986) or Nei's genetic distance (Nei 1978). The UPGMA dendrogram was constructed using TREEVIEW (Page 1996).

MtDNA sequencing and analyses

DNA extraction, PCR amplification and sequencing were performed as described in Cooper (2002). DNA was extracted either from the anterior body portion stored in 100 % ethanol, entire frozen bodies, or from re-frozen homogenate extracts left over after allozyme electrophoresis. A 710 bp region of the mitochondrial genome at the 5 prime end of the cytochrome *c* oxidase subunit 1 (CO1) gene was amplified using primers M414 (forward, LCO1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-'3) and M423 (reverse, HCO2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-'3) (Folmer et al. 1994). Sequence data from both primers were edited using SeqEd version 1.0.3 (Applied Biosystems, Scoresby, Victoria , Australia) and aligned by eye. Distance analyses were carried out using the program PAUP* version 4.0b8 (Swofford 2001) using uncorrected pairwise distances (because of the recent divergences between the lineages it was not necessary to correct for saturation). Maximum likelihood analyses were carried out using MrBayes version 3.0B4 (Huelsenbeck and Ronquist 2001). The GTR model of sequence evolution (Rodríguez et al 1990) was used with specific model parameters for the first, second and third codon positions. Bayesian analyses were performed using one cold and three heated chains that were run for 10^6 generations. Trees were sampled every 10 generations, and summarization of tree topologies and parameter values was done with the first 10000 trees discarded. Sequences have been deposited in GenBank (accession numbers: DQ230097-DQ230130).

Divergence time estimates

For the allozyme data Nei's distance (Nei's D) and the formula, time (T) in 10^3 years (ka) = Nei's D x 5000 (Nei 1987) was used to calculate divergence times between two lineages. For the mtDNA, data the uncorrected pairwise distances were expressed as proportions (pnd) and the formula T (ka) = (pnd/2)/r based on Culver et al (1995), where r is the substitution rate per site per million years. Because there were no fossils or other means to calibrate substitution rates specific for the taxa and the gene CO1, the preliminary mtDNA clock of Brower (1994) was used. This clock uses 0.0115 substitutions per site per million years, and is based on a range of independent estimates of different invertebrates and genes.

RESULTS

Allozyme analyses

(a) *Perthia* cf. *acutitelson*

The allozyme study of *Perthia* cf. *acutitelson* was conducted in two stages. Initially, up to four animals per site were screened for the full component of enzyme markers available. This resulted in 50 individuals from 13 sites being successfully screened for 30 putative allozyme loci, 10 of which were invariant (*Argk, Fum, G6pd, Gpt, Idh2, Ldh, Mdh2, Me, Pgk,* and *Tpi*). Thereafter, all remaining individuals (*N*=47) were screened only for the 20 loci found to be polymorphic in the initial overview screen. The allele frequencies for the 13 sites at these 20 loci are presented in Table 2.

One striking outcome of the overview study was that a single individual from West Bay Creek (hereafter designated F*-1) displayed fixed differences at a number of loci (eg. *Acon2, Mdh1, Ndpk, PepD,* and *Pk*; see Table 2) when compared to the other individuals from that site and elsewhere. Given the occurrence of two such distinctive groups in sympatry, this genetically-unique individual presumably represents a second species (hereafter designated *Perthia* sp. 2, with the remaining populations hereafter referred to as *Perthia* sp. 1), and as such was not included in any assessment of population structure.

As a starting point for these analyses, the broad genetic affinities among populations are displayed diagrammatically in the dendrogram of Fig. 2, constructed using the Nei's D values of Table 3. Five populations or population groupings present themselves as genetically distinctive at Nei's D above 0.1; these correspond to *Perthia* sp. 2 (F*-1), Calgardup Cave (B), Strongs Cave (C), the Jewel Cave karst system sites (E1-E5), and the remaining surface streams and springs (D1-D3, F, G, and A). The same pattern is obtained when %FDs (Table 3) are used as the genetic distance measure (analysis not shown). Interestingly, the Calgardup Cave population in Fig. 2 appears almost as distinctive from all other populations of *Perthia* sp. 1 as is the single individual of *Perthia* sp. 2 (average Nei's D = 0.345 for B and 0.391 for F*-1; Table 3), although this distinction is less pronounced when %FDs are considered (average %FD = 14.9 for B and 29.3 for F*-1; Table 3).

Table 2 - Allozyme profiles at all variable loci for the 13 sites of *Perthia* cf. *acutitelson*. Where a locus is polymorphic, the frequency of the more common alleles are expressed as percentages and shown as superscripts (the frequency of the rarest allozyme can be then be calculated by subtraction). The number in brackets refers to the maximum number of haploid genomes sampled per population. F*-1 = single specimen with a distinctive allozyme profile from the other seven individuals sampled at this site. H_O = observed heterozygosity count; *S.E.* = standard error.

Locus	B (18)	C (6)	D1 (2)	D2 (8)	D3 (18)	E1 (14)	E2 (14)	E3 (24)	E4 (12)	E5 (28)	F (14)	F*-1 (2)	G (16)	A (18)
Acon1	b^{50},c	b	b	b	b^{71},d^{22}, a	c	c	c^{96},e	c	c^{92},e	b	b	b	b
Acon2	b^{89},c	d	a^{50},b	b^{62},a	b^{56},a^{33}, d	a	a^{71},b	a^{75},b	a	a^{29},b	a^{43},d^{36}, b	c	b^{94},a	b^{83},d^{11}, a
Adh	a^{63},b	b	-	b	a	b	b	b	b	b	b^{83},a	a	a^{75},b^{25}	a^{63},b
Enol	a	a	a	a	a	a	a	a	a	a	a	a	a	a^{88},b
Gapd	a	b	b	b	b	b	b	b	b	b	b	b	b	b
Gda	a	b	b	a^{50},b^{38}, c	c^{61},b	a	-	-	-	-	b^{93},a	a	c^{62},a^{19}, b	c^{83},b
Got1	d	c	c	c	c^{89},b^{6}, e	c	c	c	c	c	c^{86},a^{7}, b	d	c^{81},b	c^{94},b
Got2	d^{61},b	b	b	d^{25},b	d^{56},b^{39}, e	b	b	b	b	b	b^{50},d^{43}, a	b	b^{94},d	b^{50},d^{44}, c
Gpi	e^{44},c^{28}, b^{22},d	b	c	c^{87},b	b^{50},c^{33}, e	c^{93},f	c	c	c	c	c^{50},b^{43}, a	e	c^{81},b^{13}, e	c^{44},e^{33}, b^{17},d
Idh1	b	b	b	b	b^{67},a	b	b	b	b	b	b	b	b^{75},a^{13}, c	b
Mdh1	a	a	a	a	a	a	a	a	a	a	a	b	a	a^{94},c
Mpi	a	a	a^{50},b	b^{75},a	a^{61},b	a	a	a	a	a	a^{71},b	a	a^{56},b	b^{78},a^{11}, c
Ndpk	a	a	a	a	a^{94},b	b^{50},a^{43}, c	b^{64},a^{21}, c	a^{88},c	a	a^{89},c	a	c	a	a
PepA	a	c	b	b^{50},c	b^{61},c^{28}, a^{6},d	b	b	b	b	b	c^{64},b	b	c^{62},b^{25}, a	c^{89},d
PepB	b^{94},d	b	b	b^{87},a	b^{79},a	a	a^{93},c	a	a^{92},b	a	b^{50},a^{25},c	c	b^{75},a	a^{67},b^{28}, c
PepD	e	a^{83},b	d	d^{75},b^{13}, e	b^{50},d^{33}, a^{11},e	d	d	d	d	d	b^{64},a^{21},e	c	c^{38},b^{25}, d^{25},a^{6},e	d^{45},a^{33}, b
Pgam	d^{62},b	b	b	b^{75},a	b^{88},c	b	b	b	b	b	b	b	b	b
Pgm	c^{66},b^{28}, a	c	b^{50},c	b^{63},c	c^{83},b	c	c	c	c	c	c^{86},b	c	c^{81},b	c^{67},d^{27}, b
Pk	a^{89},b	b	b	b	b	b	b	b	b	b	b^{86},a	c	b	b
Sordh	a^{63},b	b	b	b	b	b	b	b	b	b	b	b	b	b
H_O (± *S.E.*)	0.173 ± 0.056	0.011 ± 0.011	0.103 ± 0.058	0.158 ± 0.046	0.200 ± 0.048	0.024 ± 0.019	0.034 ± 0.022	0.029 ± 0.019	0.006 ± 0.006	0.027 ± 0.017	0.170 ± 0.045	0.000 ± 0.000	0.183 ± 0.048	0.181 ± 0.046

Before determining whether the qualitative observations above are supported by an focused analysis of population structure in *Perthia* sp. 1, a crucial first step is to ensure that the geographic scale at which sampling was conducted is appropriate for this species (Richardson et al 1986). All within-site tests assessing genotype frequencies against Hardy-Weinberg expectations and searching for linkage disequilibrium proved non-significant, indicating that the null hypothesis of panmixia at individual sites could not be refuted. Further support was obtained from the F_{IS} values obtained from *F*-statistics, which, with one exception (discussed later), were not significantly different from zero (Table 4). Together these outcomes indicate that individual sites can reasonably be regarded as the appropriate unit of analysis for an assessment of population differences between caves.

Table 5 summarizes the results of pairwise comparisons of allele frequencies amongst sites. As indicated by the dendrogram, population B is the most distinctive, displaying between 7-10 statistically-significant differences from all other sites. Nevertheless, 41 of 45 pairwise comparisons amongst the sites outside of the Jewel Cave karst system provide at least one statistically-significant outcome, with 36 of 45 stemming from differences at a minimum of two loci (Table 5). Clearly there is considerable population substructuring amongst *Perthia* sp. 1 in the Leeuwin-Naturaliste region, to the point where nearly every site (excluding those of the Jewel Cave karst system, which was sub-sampled at a smaller geographic scale) behaves as a distinct subpopulation. This conclusion is reinforced by the large, highly-significant F_{ST} values obtained from *F*-statistics (Table 4).

Turning to the Jewel Cave karst system sites, Fig. 2 implies that (a) all five sites are genetically similar to one another, (b) together they constitute a distinctive genetic grouping when compared to all other sites, regardless of geographic proximity, and (c) a subtle dichotomy may exist between sites E1/E2 versus sites E3/E4/E5.

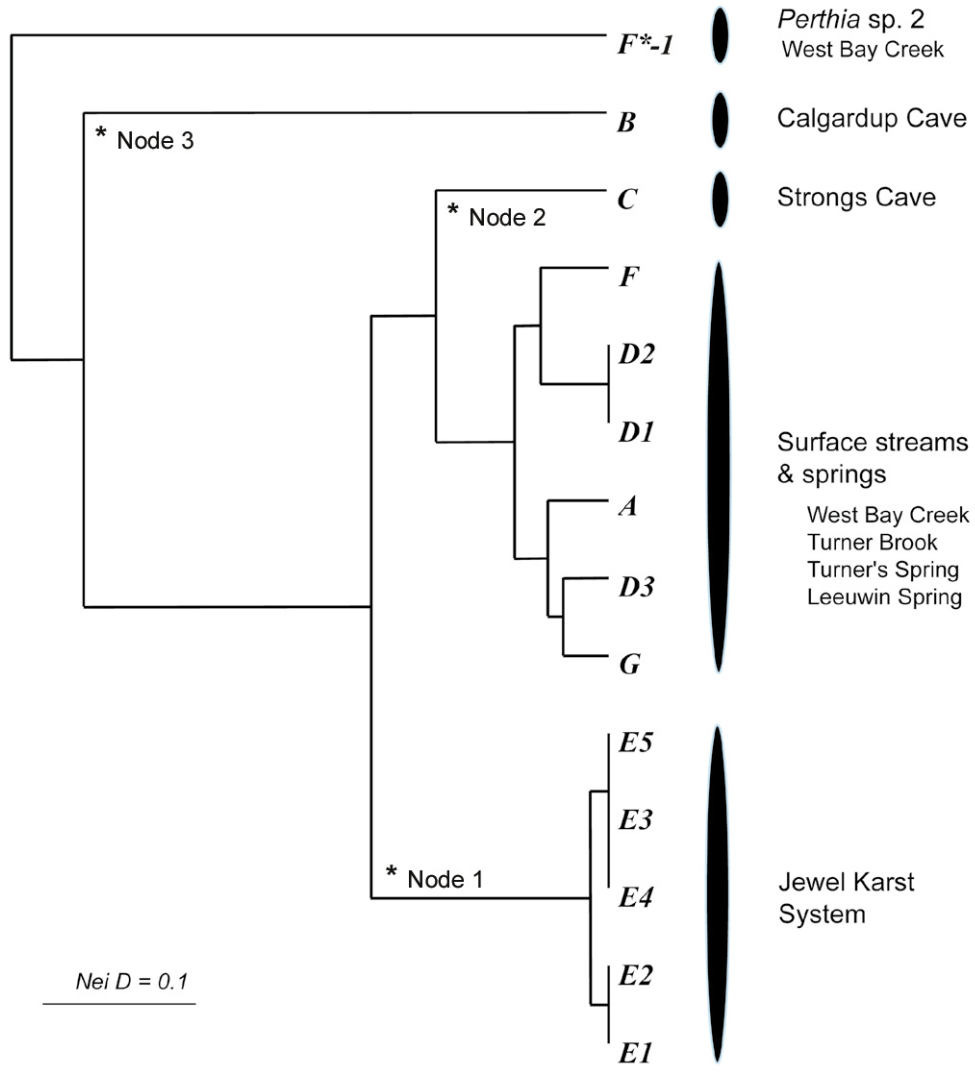

Fig. 2 - UPGMA dendrogram based on Nei's genetic distances calculated using the allozyme data for *Perthia* sp. 1 and *Perthia* sp. 2 (F*-1).

Table 3 - Pairwise genetic distances amongst the 14 populations of *Perthia*. Lower triangle = %FDs; upper triangle = Nei's D.

Site	B	C	D1	D2	D3	E1	E2	E3	E4	E5	F	G	A	F*-1
B	-	0.38	0.33	0.3	0.28	0.4	0.4	0.38	0.39	0.38	0.29	0.27	0.34	0.41
C	20	-	0.13	0.13	0.13	0.27	0.24	0.22	0.22	0.22	0.05	0.13	0.15	0.45
D1	17	14	-	0.01	0.04	0.13	0.09	0.07	0.07	0.07	0.04	0.04	0.1	0.37
D2	10	3	0	-	0.08	0.14	0.14	0.12	0.12	0.12	0.05	0.05	0.07	0.42
D3	10	3	0	3	-	0.2	0.17	0.15	0.16	0.15	0.05	0.03	0.05	0.35
E1	20	23	10	3	10	-	0	0.01	0.01	0.01	0.17	0.19	0.23	0.39
E2	17	21	7	3	7	0	-	0.01	0.02	0.01	0.14	0.17	0.19	0.39
E3	17	21	7	3	7	0	0	-	0	0	0.12	0.15	0.17	0.4
E4	17	17	4	3	7	0	0	0	-	0	0.12	0.16	0.18	0.42
E5	17	21	7	3	7	0	0	0	0	-	0.12	0.15	0.17	0.4
F	10	0	3	0	0	7	7	7	7	7	-	0.06	0.07	0.36
G	7	3	0	0	0	3	3	3	3	3	0	-	0.03	0.33
A	17	0	3	0	0	10	7	7	7	7	0	0	-	0.41
F*-1	23	37	31	30	27	30	28	31	34	31	23	20	30	-

Table 4 - Summary of *F*-statistics on the allozyme data for *Perthia* sp. 1. The 95 % confidence intervals for each value are shown in brackets (* = significant at $P = 0.05$; ** = significant at $P = 0.01$).

Population hierarchy	F_{ST}	F_{IS}
all 13 sites [a]	0.567** (0.483 to 0.646)	-0.040 (-0.105 to 0.024)
all except B	0.498** (0.403 to 0.593)	-0.030 (-0.088 to 0.030)
excluding B and E1-E5	0.238** (0.170 to 0.310)	-0.012 (-0.084 to 0.061)
E1-E5 (Jewel Karst) sites only	0.217 (-0.003 to 0.352)	-0.056** (-0.094 to -0.014)

[a] Site D1 was pooled with site D2 to avoid sample sizes less than 3.

Table 5 - Summary of pairwise comparisons amongst *Perthia* sp. 1 sites for significant differences in allele frequency. Lower left matrix = number of significant differences; upper right matrix = locus involved where $N=1$ (** $P < 0.01$; *** $P < 0.001$). Site D1 was pooled with site D2 to avoid sample sizes less than 3. Comparisons between the five Jewel Cave karst system sites are shown within the box.

Site	B	C	D1/D2	D3	E1	E2	E3	E4	E5	F	G	A
B	-											
C	7	-		Acon2								
D1/D2	7	3	-	*Gda*						Gda		
D3	6	1***	1*	-						PepD		*PepA*
E1	10	7	5	9	-		Ndpk		*Ndpk*			
E2	10	6	4	7	0	-	*Ndpk*	*Ndpk*	*Ndpk*			
E3	9	6	3	8	1**	1***	-					
E4	9	6	3	7	0	1**	0	-				
E5	9	6	4	8	1***	1***	0	0	-			
F	7	0	1**	1***	6	5	6	3	6	-		
G	7	3	0	0	7	6	6	5	6	2	-	
A	8	3	2	1***	7	6	7	4	7	2	0	-

Detailed population structure analysis strongly supports the first two inferences. Thus the allele frequency comparisons of Table 5 indicate that only a single locus shows any genetic heterogeneity amongst the five sites, whereas there are on average 5.9 statistically-significant differences (range 3-9) between the Jewel Cave karst system sites and all others (excluding the distinctive B population). Moreover, the non-cave sites closest to the Jewel Cave karst system (D1/D2/D3 and F) show similar levels of genetic divergence as do those further away, demonstrating that the Jewel Cave karst system also behaves as a distinctive subpopulation.

The suggestion of subtle genetic substructuring within the Jewel Cave karst system is based on the presence of a statistically-significant difference (corrected $P < 0.01$) between some sites but not others at the *Ndpk* locus (Table 5). An examination of the actual allele frequencies for the five sites (Table 2) reveals that these pairwise differences are due to the high frequency of the *Ndpk*b allele at sites E1 and E2 and its absence from the other three sites. No other locus reveals any indication of within-cave substructuring, although this observation must be tempered by the fact that only one other locus (*Acon2*) is sufficiently polymorphic (as defined by Richardson et al. 1986) to be suitable as a genetic marker of population structure within Jewel Cave karst system. Thus a finding of genetic heterogeneity amongst the Jewel Cave karst system sites remains tentative, as it is based on only a single locus. The F-statistics for the Jewel Cave karst system sites offer general support for this conclusion, since they reveal a large but non-significant F_{ST} value plus the only instance of an non-zero F_{IS} value, suggesting that within-site panmixia may not apply at all sites (Table 4).

A comparison of heterozygosity estimates for all *Perthia* sp. 1 sites (Table 2) reveals that, with the exception of site C (H_O = 0.011), the Jewel Cave karst system sites are considerably less variable (H_O range 0.006 – 0.034) than the other sites sampled (H_O range 0.103 – 0.200). The allozyme data indicate that populations of *Perthia* sp. 1 in the Jewel Cave karst system display significantly reduced levels of genetic variability when compared to non-cave dwelling populations in nearby surface streams and springs. Similar reductions in genetic variability are also evident in the Strongs Cave population. Thus in these two cave systems, gene flow between cave and surface populations appears to be reduced.

(b) *Uroctena* sp.

A total of 37 presumptive allozyme loci were considered scorable for the five populations of *Uroctena* sp. surveyed. Of these, 11 loci were polymorphic in one or more populations, leaving the following 26 loci invariant amongst the 21 individuals analysed:- *Acon*, *Acp*, *Acyc*, *Adh*, *Argk*, *Ca*, *Enol*, *Est1*, *Est2*, *Est3*, *Fum*, *Gapd*, *Got1*, *Idh1*, *Idh2*, *Ldh1*, *Ldh2*, *Me*, *PepB1*, *PepB2*, *Pgam*, *Pgk*, *Pk1*, *Pk2*, *Sordh*, and *Tpi*. The allele frequencies for each population at these 11 polymorphic loci are presented in Table 6.

Pairwise comparisons of allele frequency amongst the five sites revealed no instances of statistically-significant heterogeneity and thus provide no evidence of population substructuring for the *Uroctena* metapopulation in Jewel and Easter Caves. The values obtained for both F_{ST} (0.039, 95% CI –0.034 to 0.107) and F_{IS} (0.113, 95% CI –0.093 to 0.314) were not significantly different from zero, indicating that the null hypothesis of panmixia within and between sites cannot be refuted.

Although the observed heterozygosities (H_0) of *Uroctena* populations are slightly higher than those recorded for sympatric *Perthia* populations, there is con-

Table 6 - Allele frequencies at all polymorphic loci for five populations of *Uroctena* sp. Details as for Table 2.

Locus	*E1* *(14)*	*E2* *(4)*	*E3* *(8)*	*E4* *(4)*	*E5* *(12)*
Got2	b^{86},a^{7},c	b	b	b	b
Gpi	b^{83},c	b	b^{50},c	b^{50},c	b^{58},c
Gpt	a^{93},b	a	a	a	a
Mdh	b^{93},a	b	b	b	b^{83},a
Mpi	b^{79},a	b^{75},a	b	b	b
Ndpk	a	a^{50},b	a	a^{75},b	a^{75},b
PepA	a	a	a	a^{75},b	a^{83},b
PepC	a^{86},b	a	a	a	a
PepD1	b^{93},a	b	b^{75},a	a^{50},b	b^{75},a
PepD2	b^{93},a	b	b	b	b
Pgm	a	a	a^{63},b	a	a
H_O (± *S.E.*)	0.042 ± 0.015	0.041 ± 0.030	0.034 ± 0.020	0.027 ± 0.019	0.059 ± 0.028

siderable overlap in the standard errors for both sets of estimates (Tables 2 and 6). Thus the available allozyme data are unable to demonstrate any significant differences in the levels of genetic diversity displayed by the stygobite *Uroctena* and the stygophile *Perthia* in the Jewel Cave karst system.

MtDNA analyses

(a) *Perthia* sp. 1

Phylogenetic analysis using maximum likelihood models in a Bayesian framework resulted in the phylogenetic tree presented in Fig. 3. This phylogenetic tree shows good support (posterior probabilities > 0.75) for population subdivision according to sample localities, an outcome also apparent from the allozyme data. The topology of this tree did not differ from the distance tree estimated using PAUP*. In the Bayesian tree, the populations of Strongs Cave, Calgardup Cave, and Jewel Cave karst system clearly form separated units, while some sub-structuring in the Jewel Cave karst system is suggested by the well supported (posterior probabilities 0.97) clustering of the two specimens from Jewel Cave as distinct from the two specimens from Easter Cave. Nevertheless, this latter finding does not unequivocally indicate the presence of sub-structuring within the Jewel Cave karst system, given the small sample sizes involved (*N*=2 for each site, with only two sites examined).

Phylogenetic analysis using distance methods showed 0.46 % to 3.54 % uncorrected sequence divergence between specimens of *Perthia* sp. from different

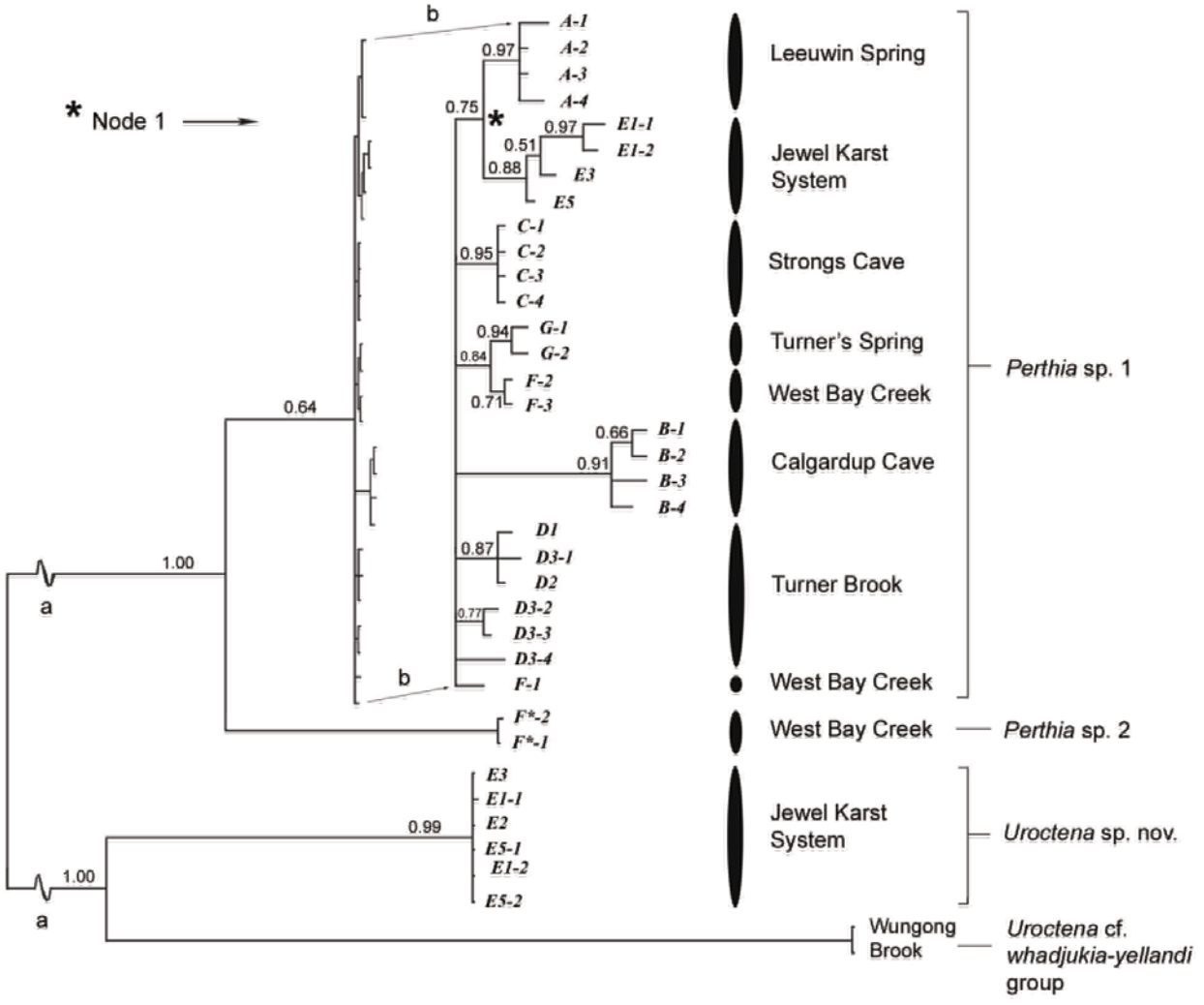

Fig. 3 - A maximum likelihood phylogram based on Bayesian analysis on mtDNA sequence data. Branch support values (as posterior probabilities) are plotted above the branches. The branch lengths have been modified to fit it in the Fig.; (a) the branch leading from the two *Uroctena* species to the root is nearly as long as the branch leading to the two *Uroctena* cf. *whadjukia-yellandi* group specimens, (b). The branches for *Perthia* sp. 1 have been stretched to show more of the internal resolution.

localities. The sequence divergence between specimens within localities did not exceed 0.76 %, with the exception of specimens collected from West Bay Creek (sequence divergences of 1.70 –1.83 %), which reflected the presence of two distinct mtDNA sub-lineages. Sequence divergence between *Perthia* sp. 1 and *Perthia* sp. 2 (F*-1 and F*-2) ranged from 13.2% to 14.5 %. Such a level of divergence confirms that *Perthia* sp. 2 is a different species, as already suggested based on the number of fixed allozyme differences.

(b) *Uroctena* sp.

Phylogenetic analysis using distance methods showed 19.8 - 20.4 % uncorrected sequence divergence between specimens of *Uroctena* sp. from the Jewel Cave karst system and specimens of *Uroctena* cf. *whadjukia-yellandi* from Wungong Brook, evidence that the *Uroctena* from the Jewel Cave karst system warrant recognition as a distinctive species. We sequenced five specimens of *Uroctena* sp. from different sites in the Jewel Cave karst system (Table 1). Sequence divergence among these specimens appeared to be very low (0 – 0.6 % sequence divergence), too low to provide any information as to population structure within the Jewel Cave karst system.

Time estimates of population divergences

The time estimates of populations divergences obtained from the two different molecular techniques were broadly consistent, as the calculated values were within the same order of magnitude and there was considerable range overlap between the respective minimum - maximum values (Table 7). The divergence time estimates between the Jewel Cave karst system sites and those genetically most similar to them were 350 – 1,150 ka for the allozyme data (E1/E2/E3/E4/E5 versus all *Perthia* sp. 1 sites except B; genetic distances in Table 3) and 270 – 1,270ka for the mtDNA sequence data (E1/E3/E5 versus site A). The minimum time estimate for any cave population versus any surface lineage was 250 ka (allozyme data, Strongs Cave versus sites A/D/F/G) or 270 ka (mtDNA data, Jewel Cave karst system versus site A).

DISCUSSION

This study has been constrained by the small sample sizes available for the rare and endangered cave dwelling populations, thus limiting the degree of interpretation possible in relation to some population genetic parameters. Nevertheless, the data provide useful insight into several aspects of the population structure and genetic history of the amphipods under scrutiny, which should aid the future conservation of these threatened cave communities.

Jewel Cave karst system

For both *Uroctena* sp. and *Perthia* sp.1, the genetic data obtained from both allozymes and mtDNA support the hypothesis that each genus is represented by a single species within the Jewel Cave karst system. This outcome is consistent with a detailed morphological exami-

Table 7 - *Perthia* sp. 1 estimated divergence times (ma) based on the data for allozymes (Table 3) and mtDNA (Fig. 3).

	Allozymes[a] Min. – Max. (x 10^3 years)	mtDNA[a,b] Min. – Max. (x 10^3 years)	Nodes as in Fig.s
Jewel Karst System (E1 to E5) *vs*. all other sites (except B, F*-1)	350 – 1,150		Fig. 2 Node 1
Jewel Karst System (E1 to E5) *vs*. A		270 – 1,270	Fig. 3 Node 1
Strongs Cave (C) *vs*. A, D, F, G	250 – 750		Fig. 2 Node 2
Strongs Cave (C) *vs*. A, B, D, E, F, G (except F-4, F*-1)		460 – 790	Fig. 3 Node unresolved
Calgardup Cave (B) *vs*. all other sites (except F*-1)	1,350 – 2,050		Fig.2 Node 3
Calgardup Cave (B) *vs*. A, C, D, E, F-1 to F-3, G (except F-4, F*-1)		1000 – 1,550	Fig. 3 Node unresolved

[a]Allozyme molecular clock used Nei's D, where time (T) in 10^3 years (ka) = Nei's D x 5000 (Nei 1978; 1987). [b]MtDNA used preliminary mitochondrial clock of Brower (1994) 0.0115 substitutions per site per million years, and the formula based on Culver *et al*. (1995) where T(ka)=5 x 10^3 x *pnd,* where *pnd* is the uncorrected pairwise distance expressed as proportion of nucleotide divergence.

nation of both taxa, which noted some minor differences between specimens from Jewel Cave and Easter Cave but concluded that only one morphotype of each species was present (J. Bradbury in Eberhard 2004).

The genetic data also suggested that the Jewel Cave system populations of both species were largely panmictic, with no evidence of population substructuring in *Uroctena* and only a subtle suggestion of heterogeneity in *Perthia* sp. 1. This outcome was consistent with the geomorphic and hydrologic evidence, which indicated that although Jewel Cave and Easter Cave were physically separated from each other to the extent reached by human exploration, both caves were still contiguous parts within the same aquifer system and shared close hydrologic connections (Eberhard 2004). Nonetheless, the suggestion of panmixia must be treated with considerable caution, given the small sample sizes available. Especially relevant in this respect is the allozyme data for *Perthia* sp. 1, which showed that all hypogean populations displayed markedly lower levels of allozyme variability compared with those sampled from epigean sites, suggesting the involvement of population bottlenecks and/or selection (Avise 2004; Richardson et al 1986). Furthermore, both the allozyme and mtDNA datasets suggested that subtle population sub-structuring may have occurred in the Jewel Cave karst system (between the E1/E2 populations, and the E3/E4/E5 populations). Since our preliminary data revealed no specific evidence of inbreeding or fixed allele differences (the genetic signatures of long-term isolation in small populations), additional and preferably more informative (i.e. microsatellites) genetic data are required to determine whether the population substructuring alluded to herein is genuine or simply an artefact of limited sampling.

Leeuwin-Naturaliste region

For *Perthia* sp. 1, the contemporary populations in Calgardup Cave, Strongs Cave, and the Jewel Cave karst system are geographically, hydrologically and genetically isolated from each other. The population in the Jewel Cave karst system is also isolated from populations in nearby springs and surface waters in adjacent catchments. The populations in the Jewel Cave system and the Strongs Cave system displayed significantly reduced levels of genetic variability when compared to non-cave dwelling populations in nearby surface streams and springs. Thus the populations in these two separate karst drainage systems appear to have lost genetic variability, presumably due to the combined effects of inbreeding, small population size, and/or lack of gene flow from surface populations. In contrast, the population in Calgardup Cave did not display reduced levels of genetic variability, probably because this cave drainage system is less isolated from surface waters. Unlike the Jewel and Strongs Cave systems, the Calgardup Cave system has open conduit connections to surface waters via sinking streams and a spring.

The strong phylogeographic structure in the Leeuwin-Naturaliste region has implications for conservation management of the cave communities that are listed as threatened, because each karst system population (which may itself comprise several individual caves, connected in a single hydrogeologic system) of *Perthia* sp. 1 may be regarded as a discrete 'Evolutionarily Significant Unit', i.e. a population that has been historically isolated, is genetically-definable using both nuclear and mitochondrial markers, and has a distinct evolutionary potential (Cooper et al 2000). Thus for conservation of biodiversity patterns in *Perthia* sp. 1, the most appropriate spatial unit is the karst hydrogeologic system. Three karst hydrogeologic systems were represented in this study, associated with Calgardup Cave, Strongs Cave, and Jewel-Easter Caves respectively. This is consistent with genetic studies of a gammarid amphipod, *Gammarus minus* Say 1818 in the eastern United States (Culver et al 1995), which found that populations were separated by karst catchment basins. Additionally, within each basin, dispersal between cave and spring populations was limited by sections of inhospitable habitat in deep flooded conduits. However, in the Leeuwin-Naturaliste karst deep flooded conduits do not occur due to the shallow thickness of limestone that rests on impermeable basement rocks at about 22.5 m ASL (Bastian 1964; Eberhard 2004).

Time estimates of population divergences

Estimates for the time since isolation in caves have also been studied in *Gammarus minus* (Culver et al 1995). *G. minus* comprises a suite of cave and spring dwelling populations occurring in multiple karst drainage basins, a situation that is broadly comparable to *Perthia* sp. 1 in the Leeeuwin-Naturaliste region. The time estimates of isolation of cave populations in *G. minus* (100 to 670 ka using Nei's electrophoretic distance; 345 to 1,900 ka for16S rDNA using formula in Table 7) are broadly comparable to *Perthia* sp. 1. In comparison to their respective surface counterparts, the cave populations of *G. minus* display a slightly greater degree of troglomorphy than cave populations of *Perthia* sp. 1. However, both amphipod taxa are broadly similar to each other in respect of being relatively weakly cave-adapted.

The time estimates obtained from the molecular data suggest separation of the Jewel Cave system populations of *Perthia* sp. 1 from the Leeuwin Spring population, sometime between about 270 and 1,270 ka. The younger divergence estimates are well within the minimum age of the Jewel Cave karst system (> 627 ka), whilst the older estimates approach the Early Pleistocene (750 to 1,800 ka) or Late Pliocene maximum ages inferred for the caves and limestone respectively (Eberhard 2004). These molecular divergence estimates suggest that sepa-

ration of the studied populations occurred sometime prior to Marine Isotope Stage 7 or > 250 ka (see Williams et al 1998).

The minimum age estimates for divergence of the Jewel karst system populations are more than an order of magnitude greater (> 270 ka) than the dated low watertable episode in the karst aquifer (11 to 13 ka, Eberhard 2004) that coincided with the phase of regional aridity in southwestern Australia near the end of the Pleistocene (ca. 12 ka, Harrison 1993; Zheng 1999). While acknowledging that population divergences and evolution of troglomorphism may have occurred well before actual cave colonization took place, the molecular data do not reject the null hypothesis that both species of amphipod were present in groundwaters within the Jewel Cave karst system before 13,000 years ago. This implies the amphipods had survived *in situ* watertable levels that were 23.2 m ASL or lower. However, if the present trend of declining rainfall in southwestern Australia continues as predicted by Timbal (2004), and if water levels in the Jewel Cave system decline below 22.7 m ASL, then most of the lakes will become dry and the aquatic invertebrate community will be more vulnerable to extinction because of reduced habitat and consequent reduced population size. Presumably the aquatic communities in other karst drainage systems will also become more vulnerable if the drying climate trend in southwestern Australia continues as predicted (see Bates et al 2001; Smith et al 2001; Smith et al 2000; Timbal 2004).

ACKNOWLEDGEMENTS

Sincere thanks and acknowledgement are extended to the following people and organisations that assisted with this project: Steve Cooper and the Evolutionary Biology Unit, South Australian Museum for supporting this project; Terry Reardon for technical assistance; John Bradbury for specimen identification; Robyn McBeath and CaveWorks (Augusta-Margaret River Tourism Association) for laboratory facilities and other support; Mike Scanlon, Robyn Luu (nee Phillimore) and John Blyth (Department of Conservation and Land Management, Perth) for equipment loan and funding assistance; Karin Strehlow (Environmental Science Department, Murdoch University) for technical assistance. The manuscript benefited from comments by Stuart Halse and David Culver, and several other anonymous referees whose detailed criticisms greatly helped to further improve the manuscript. This work was completed whilst the first author was in receipt of a Murdoch University Research Scholarship.

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