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Phylogeography of the ancient Parabathynellidae (Crustacea: Bathynellacea) from the Yilgarn region of Western Australia

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Abstract. The crustacean order Bathynellacea is a primitive group of subterranean aquatic (stygobitic) invertebrates that typically inhabits freshwater interstitial spaces in alluvia. A striking diversity of species from the bathynellacean family Parabathynellidae have been found in the calcretes of the Yilgarn palaeodrainage system in Western Australia. Taxonomic studies show that most species are restricted in their distribution to a single calcrete, which is consistent with the findings of other phylogeographic studies of stygofauna. In this, the first molecular phylogenetic and phylogeographic study of interspecific relationships among parabathynellids, we aimed to explore the hypothesis that species are short-range endemics and restricted to single calcretes, and to investigate whether there were previously unidentified cryptic species. Analyses of sequence data based on a region of the mitochondrial (mt) DNA cytochrome *c* oxidase 1 gene showed the existence of divergent mtDNA lineages and species restricted in their distribution to a single calcrete, in support of the broader hypothesis that these calcretes are equivalent to closed island habitats comprising endemic taxa. Divergent mtDNA lineages were also observed to comprise four new and 12 recognised morphospecies. These results reflect the findings of previous studies of stygobitic arthropods (beetles, amphipods and isopods) from the Yilgarn region and reinforce the usefulness of using DNA-sequence data to investigate species boundaries and the presence of cryptic species.

Additional keywords: cryptic species, cytochrome *c* oxidase 1, mitochondrial DNA, phylogeny, stygofauna.

Introduction

In the arid environment of the Western Australian desert, a system of calcrete aquifers has been found to contain a diverse and unique subterranean aquatic invertebrate fauna (herein referred to as stygofauna). Calcrete aquifers are elongate carbonate deposits formed by precipitation following the evaporation of groundwater (Mann and Horwitz 1979). The groundwater in calcretes is characteristically neutral to mildly alkaline and salinity varies from saline to hyper-saline (Arakel *et al*. 1990). In the Yilgarn craton of Western Australia, the calcrete aquifers are thought to be physically isolated and discrete entities, which contrasts strikingly to other karstic regions in Australia, particularly the Pilbara, where the calcrete aquifers are connected by deep groundwater systems (Finston and Johnson 2004; Finston *et al*. 2004). The Yilgarn calcretes have been described as 'islands under the desert' because of the high numbers of short-range endemic taxa, in particular dytiscid diving beetles (Cooper *et al*. 2002; Watts and Humphreys 1999, 2000, 2001, 2003, 2004, 2006), isopods (Taiti and Humphreys 2001) and copepods (Karanovic 2004). Phylogeographic studies of dytiscid diving beetles (Cooper *et al*. 2002; Leys *et al*. 2003), amphipods (Cooper *et al*. 2007) and isopods (Cooper *et al*. 2008) have confirmed the presence of monophyletic groups restricted to single calcretes. Of interest here is whether the 'subterranean island' hypothesis also applies to the diverse and ancient Parabathynellidae.

The Bathynellacea is a primitive group of poorly known crustaceans that are mostly restricted to groundwaters. Of the two families within this order, the Parabathynellidae are better known largely owing to a greater diversity of taxonomic characters (Schminke 1974). Worldwide, the family contains 143 species in 42 genera of which 19 are monospecific (Camacho 2006). Most species are found in groundwater with a few known from oligohaline to polyhaline estuaries (Camacho 2006). A striking diversity of species has been found in the calcretes of the Yilgarn palaeodrainage systems in Western Australia (Cho *et al*. 2006*a*). Of the seven parabathynellid genera known from Australia, three new genera (*Billibathynella* Cho, 2005; *Brevisomabathynella* Cho *et al.,* 2006*b*; and *Kimberleybathynella*

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Cho *et al.,* 2005) and 22 new species have been described from the Yilgarn and northern Australia in the last two years, and another genus and 5–10 morphospecies require formal description (J.-L. Cho, unpubl. data.). The Yilgarn is rich in free-swimming, 1–3-mm long parabathynellids (Cho *et al*. 2006*a*, 2006*b*), some inhabiting waters of high salinity (indicated by the calcrete salinity measurements of Watts and Humphreys (2006)) in contrast to the tiny (<1 mm) interstitial freshwater species characteristic of the family. The Yilgarn is also the locality of the very large (6.3 mm) and primitive *Billibathynella humphreysi* Cho, 2005.

Taxonomic classification of stygobitic organisms is difficult owing to the convergent evolution of morphological characters that are associated with adaptations to the subterranean environment (Jones *et al*. 1992; Kane *et al*. 1994). This convergence confounds the true phyletic descent through the retention of primitive traits and loss of complex features. For instance, reduction and loss of appendages (regularly observed in parabathynellids (Cho *et al*. 2006*a*)), pigmentation, and changes in life-history strategies are common to most stygofauna. This morphological simplification is compounded in the Bathynellacea by extreme progenetic development (Schminke 1981), an evolutionary direction seemingly common in meiofauna (Westheide 1987). In stygobitic crustaceans, the underestimation of species diversity based on morphological characters has been recently highlighted by Proudlove and Wood (2003) and demonstrated in several studies using DNA-sequence data in which cryptic species have been identified (Jarman and Elliot 2000; Finston and Johnson 2004; Lefébure *et al*. 2006*a*, 2006*b*). To address this issue, a combined morphological and genetic approach is generally recommended to assess correctly levels of diversity and identify generic and species boundaries, and to formulate a better natural classification (Proudlove and Wood 2003; Lefébure *et al*. 2006*b*).

Prediction of species diversity and boundaries using DNA-sequence data are being increasingly investigated, particularly with the advent of DNA barcoding. For instance, Lefébure *et al*. (2006*a*) have investigated the relationship between morphospecies and genetically diverse lineages of crustaceans and identified an average of 16% genetic divergence (i.e. patristic distance) to be a consistent indicator of distinct species among the crustacean groups using cytochrome *c* oxidase 1 (*cox1*) mitochondrial DNA (mtDNA). Clear definitions and demarcation of species using genetic divergences requires further research (Hajibabaei *et al*. 2006; Costa *et al*. 2007), but the use of sequence data to combat issues associated with cryptic species and convergent evolution is important.

To date, only one other study has used a molecular systematics approach to investigate parabathynellid taxonomy. Camacho *et al*. (2002) used 16S ribosomal DNA to investigate the systematic position of Syncarida, including a bathynellid (*Iberobathynella* (*Espanobathynella*) *magna* Camacho and Serban, 1998, Parabathynellidae) within the crustacean class Malacostraca. In this study, we present the first comprehensive molecular phylogeny of a diverse array of Bathynellacea. Individuals representing 12 calcrete aquifers were sampled from throughout the Yilgarn region. The mtDNA gene *cox1* was used to evaluate whether mtDNA lineages were phylogeographically restricted in their distribution to single calcretes, and to identify divergent mtDNA clades that may represent cryptic species. We classified individuals according to their 'calcrete population', a term first used by Cooper *et al*. (2007) to identify individuals by their geographical locality. Subsequent morphological evaluation of specimens from genetically divergent lineages identified 12 recognised morphospecies and four new cryptic species.

Methods

Sampling design and isolation and amplification of mitochondrial DNA

Bathynellaceans were collected from calcrete aquifers in the Yilgarn region of Western Australia over six years (2000–06) and stored in 70–100% ethanol. Sampling of bathynellaceans followed the same regime as that used by Cooper *et al*. (2007) for amphipods. All efforts were made to collect multiple individuals from each calcrete but this was not always possible. Difficulties associated with sampling parabathynellids and other stygofauna include their small size and the biases involved in relying on pre-existing bores and wells to access calcrete aquifers. Calcrete locations and the sampled individuals are listed in Table 1. This information directly corresponds with registration numbers (BES numbers) for collection and voucher specimens lodged at the Western Australian Museum (see below). Localities are also shown in Fig. 1. The outgroup taxon, an unidentified species of Psammaspididae, was collected in the Hunter Valley, New South Wales (latitude –32.0100, longitude 150.8611; specimen number PH2HD20 (private collection); GenBank Accession number EU350257). Psammaspids belong to the Anaspidacea, the only other extant order within the Syncarida. The monophyly of the Parabathynellidae and Bathynellidae remains unconfirmed, therefore a member of the Anaspidacea was chosen as an outgroup.

For all DNA extractions, every effort was made to remove one or two appendages from each individual on a single side so that the full set of appendages on the opposing side were retained for morphological examination. Individuals were then lodged at the Western Australian Museum Biospeleology Unit under the designated BES numbers for future work. The formal taxonomy of the Australian Parabathynellidae is presently incomplete and many undescribed species are in collections. Individuals (identified by collection number in Table 1; prefix BES) for each lineage were examined for the morphological characters typically used for Parabathynellidae (Cho 1996, 1997, 2001, 2005; Cho and Schminke 2001, 2006; Ranga Reddy 2002, 2006; Camacho 2003, 2004; Cho *et al*. 2005, 2006*a*, 2006*b*). Morphological examination of specimens from genetically divergent lineages identified 12 known morphospecies and four new morphospecies. Individuals that possessed characters distinct from those previously described were identified as new morphospecies and these will subsequently be formally described. Two lineages were not examined morphologically (Unidentified 1 and 2), but vouchers have been retained for future morphological and molecular examination.

To examine phylogenetic relationships among parabathynellids, we used partial DNA sequences of the mtDNA gene *cox1*. DNA was extracted using the Gentra Systems PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis,

Table 1. Details of calcrete populations and their locations, identified species, number of individuals (*n*) sampled, collection and voucher numbers (BES #) and unique sequence information for parabathynellids (GenBank accession numbers)

Patristic distances within species among haplotypes are also listed

Palaeodrainage and calcrete	Species names	*n*	Haplotype name	BES #	Decimal latitude (°)	Decimal longitude (°)	Accession numbers	Patristic distance
Carey								
1. Paroo	Gen. A sp. 4	1		5620.1[B]	–26.4004	119.7630	EU350221	
2. Hinkler Well	*Atopobathynella wattsi* Cho *et al*., 2006*a*	2	HW1	6609.1[B], 6616.1	–26.6845	120.2151	EU350222	0–0.002
		1	HW2	8147.1	–26.6845	120.2151	EU350223	
3. Lake Violet	Gen. A sp. 13[A]	1	LVA1	14273	–26.6876	120.2977	EU350224	0–0.033
		1	LVA2	14288A	–26.6876	120.2977	EU350225	
		2	LVA3	14288B[B], 13457A	–26.6876	120.2977	EU350226	
		1	LVA4	14288C	–26.6876	120.2977	EU350227	
		2	LVA5	14288E, 13457D	–26.6876	120.2977	EU350228	
		1	LVA6	13457B	–26.6876	120.2977	EU350229	
		1	LVA7	13457C	–26.6876	120.2977	EU350230	
	Unidentified 1	1		13457E	–26.6876	120.2977	EU350231	
	Unidentified 2	1	LVB1	13454	–26.6774	120.2280	EU350232	0
		1	LVB1	14323	–26.6828	120.2213		
	Gen. A sp. 14[A]	1	LVC1	13385[B]	–26.6828	120.2210	EU350233	0–0.013
		1	LVC2	13389A	–26.7090	120.2347	EU350234	
		1	LVC3	13389B	–26.7090	120.2347	EU350235	
4. Uramurdah Lake	Gen. A sp. 9	1		14277[B]	–26.6876	120.3027	EU350240	
	Gen. A sp. 2	1	UL1	6452.1	–26.6878	120.3383	EU350236	0–0.010
		1	UL2	13383.1A	–26.6878	120.3275	EU350237	
		1	UL3	13383.1B	–26.6878	120.3275	EU350238	
		2	UL3	13392.1A, 13392.1B	–26.6876	120.3382		
		1	UL4	14293[B]	–26.6879	120.3274	EU350239	
5. Millbillillie	Gen. A sp. 5	1		13479[B]	–26.5450	119.9855	EU350241	
Naberru								
6. Cunyu	Gen. A sp. 1	1	CU1	8158[B]	–25.5938	120.3724	EU350242	0–0.008
		1	CU2	13331A[B]	–25.5938	120.3724	EU350243	
		3	CU3	13331B, 13331D, 13331E	–25.5938	120.3724	EU350244	
Raeside								
7. Depot Springs	*At. hinzeae* Cho *et al*., 2006*a*	1		8370[B]	–27.9670	120.0578	EU350245	
Gascoyne								
8. Milgun	*Billibathynella* sp. 1	1		8673[B]	–25.1759	118.0614	EU350246	
Murchison								
9. Austin Downs	*Bi.* sp. 2[A]	2	AD1	14239A, 14245[B]	–27.41337	117.71122	EU350247	0–0.020
		2	AD2	14239B, 14239G	–27.41337	117.71122	EU350248	
		1	AD3	14239C	–27.41337	117.71122	EU350249	
		1	AD4	14239D	–27.41337	117.71122	EU350250	
		1	AD5	14239F	–27.41337	117.71122	EU350251	
10. Yarrabubba	*At*. sp. 1[A]	1		13493[B]	–27.21470	118.91860	EU350252	
Carnegie								
11. Jundee	Gen. A sp. 3	1		14301A[B]	–26.2827	120.6757	EU350253	
	Brevisomabathynella cooperi Cho *et al*., 2006*b*	2	JU1	14301B[B], 6579.1	–26.2827	120.6757	EU350254	0
		1	JU2	14301I	–26.2827	120.6757	EU350255	
Burnside								
12. Carnegie Downs	*At. glenayleensis* Cho *et al*., 2006*a*	1		9961[B]	–25.66853	122.36865	EU350256	

[A]Genetically divergent lineages that were subsequently identified as morphospecies.

[B]Specimens used for morphological identification.

MN, USA) according to the manufacturer's protocol. A 633 base pair (bp) region of the *cox1* gene was amplified with the universal oligonucleotide primers C1-J-1718 (5′-GGAGGATTTGGAAATTGATTAGTTCC-3′) and C1-J-2329 (alias K525) (5′-ACTGTAAATATATGATGAGCTCA-3′) (Simon *et al*. 1994) for all except four individuals. The primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*. 1994) were used for the remaining four individuals: Psammaspididae sp., *Billibathynella* sp. 1, Genus A sp. 3 and Genus A sp. 9. PCR amplification of all sequences involved an initial cycle of denaturation at 95°C for 2 min, and 35 subsequent cycles of 94°C for 30s, 47°C for 30s and 72°C for 1 min. PCR was carried out in 25μL reactions containing 10× Eppendorf Hotmaster *Taq* Buffer (Eppendorf, Westbury, NY, USA) containing 2.5 mM Mg^{2+}, 2.5 mM of each deoxyribonucleotide triphosphate (dNTP), 5.0 μM of each primer, 0.1 units of Eppendorf Hotmaster *Taq* Polymerase and ~1 ng of DNA.

Each PCR was purified using AMPure magnetic bead cleanup (Agencourt Bioscience, Beverley, MA, USA) and sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). DNA sequences were analysed commercially on an ABI PRISM 3700 (Applied Biosystems). All sequences were edited with reference to chromatograms using BioEdit version 7.0.1 (Hall 1999) and aligned using Clustal W (Thompson *et al*. 1994).

Mitochondrial DNA sequence analysis

Nucleotide sequence composition statistics were estimated using MEGA 3.0 (Kumar *et al*. 2004) and PAUP* 4.0b10 (Swofford 2002). Patristic distances that provide an estimate of molecular divergence were calculated using the most probable Bayesian

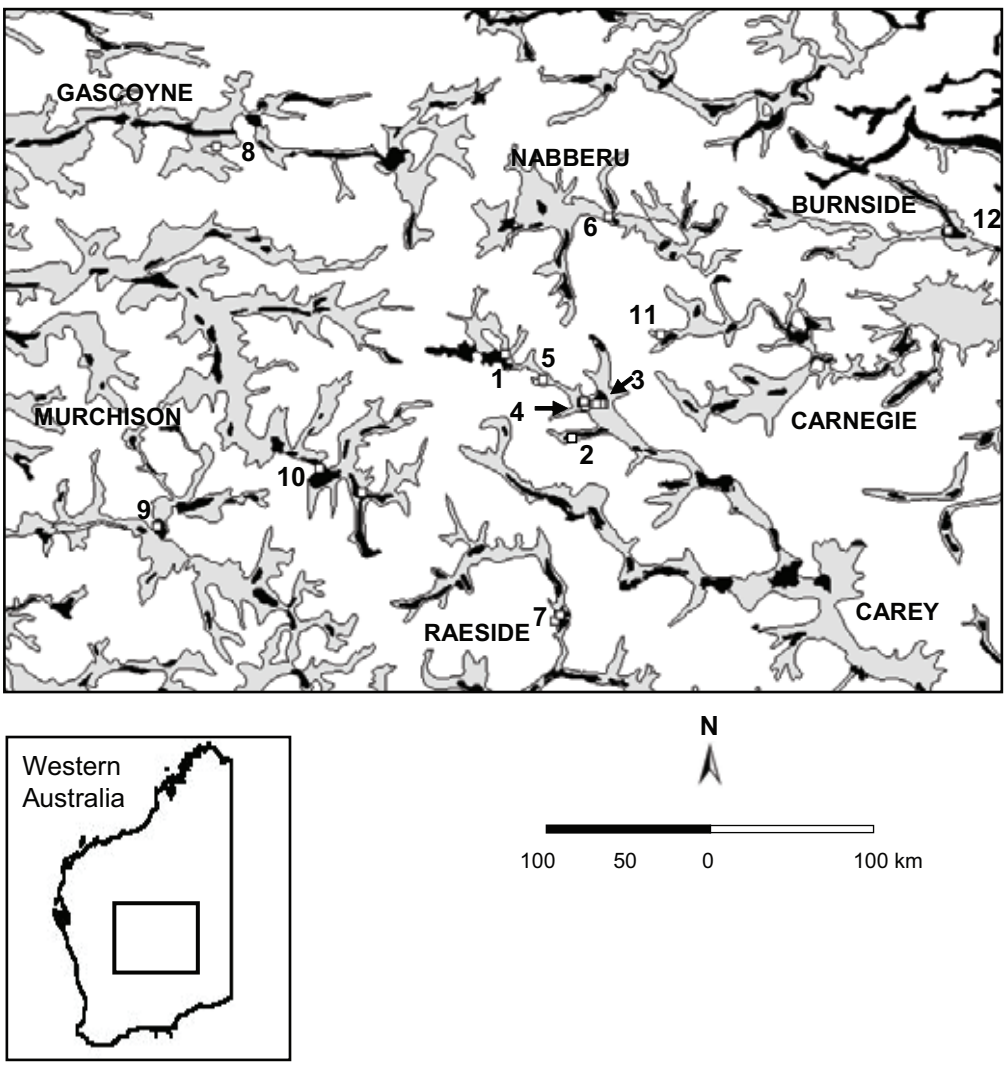

Fig. 1. Calcrete map of the Yilgarn region in Western Australia. Palaeodrainage channels are coloured grey, and calcrete aquifers black. Relevant palaeodrainages are named and calcretes are identified by white squares and are numbered as in Table 1. *Carey Drainage*: 1, Paroo; 2, Hinkler Well; 3, Lake Violet; 4, Uramurdah Lake; 5, Millbillillie; *Naberru Drainage*: 6, Cunyu; *Raeside Drainage*: 7, Depot Springs; *Gascoyne Drainage*: 8, Milgun; *Murchison Drainage*: 9, Austin Downs; 10, Yarrabubba; *Carnegie Drainage*: 11, Jundee; *Burnside Drainage*: 12, Carnegie Downs.

tree reconstruction (see below). Parameters and branch lengths were re-estimated by maximum likelihood with PAUP* using the GTR (generalised time reversible) +I (proportion of invariable sites) +G (gamma) model of substitution (Lanave *et al*. 1984), and the re-estimated branch lengths were represented in the final tree (Fig. 2). Patristic distances were then estimated with PATRISTIC 1.0 (Fourment and Gibbs 2006).

To examine relationships among species, mtDNA *cox1* sequences were analysed using a phylogenetic approach. Phylogeny reconstruction using both maximum parsimony (MP) and Bayesian approaches were implemented with PAUP* 4.0b10 (Swofford 2002) and MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) respectively. The model that best fitted the data was estimated with ModelTest 3.7 (Posada and Crandall 1998) for nucleotide data and ProtTest 1.3 (Abascal *et al*. 2005) for amino acid data under an Akaike information criterion framework. Models were tested for all three codon positions, the TrNef (Tamura–Nei equal base frequencies) +G model was favoured for first, F81 (Felsenstein 1981 model) +G for the second, and HKY (Hasegawa–Kishino–Yano) +G for the third position. The MtREV (mitochondrial reversible model) +I+G model was most suitable for the amino acid sequences. The nucleotide sequence data was partitioned by codon position and each partition was started independently with a different model (listed above). All parameters were unlinked and the rates were allowed to vary over the partitions. This approach circumvents

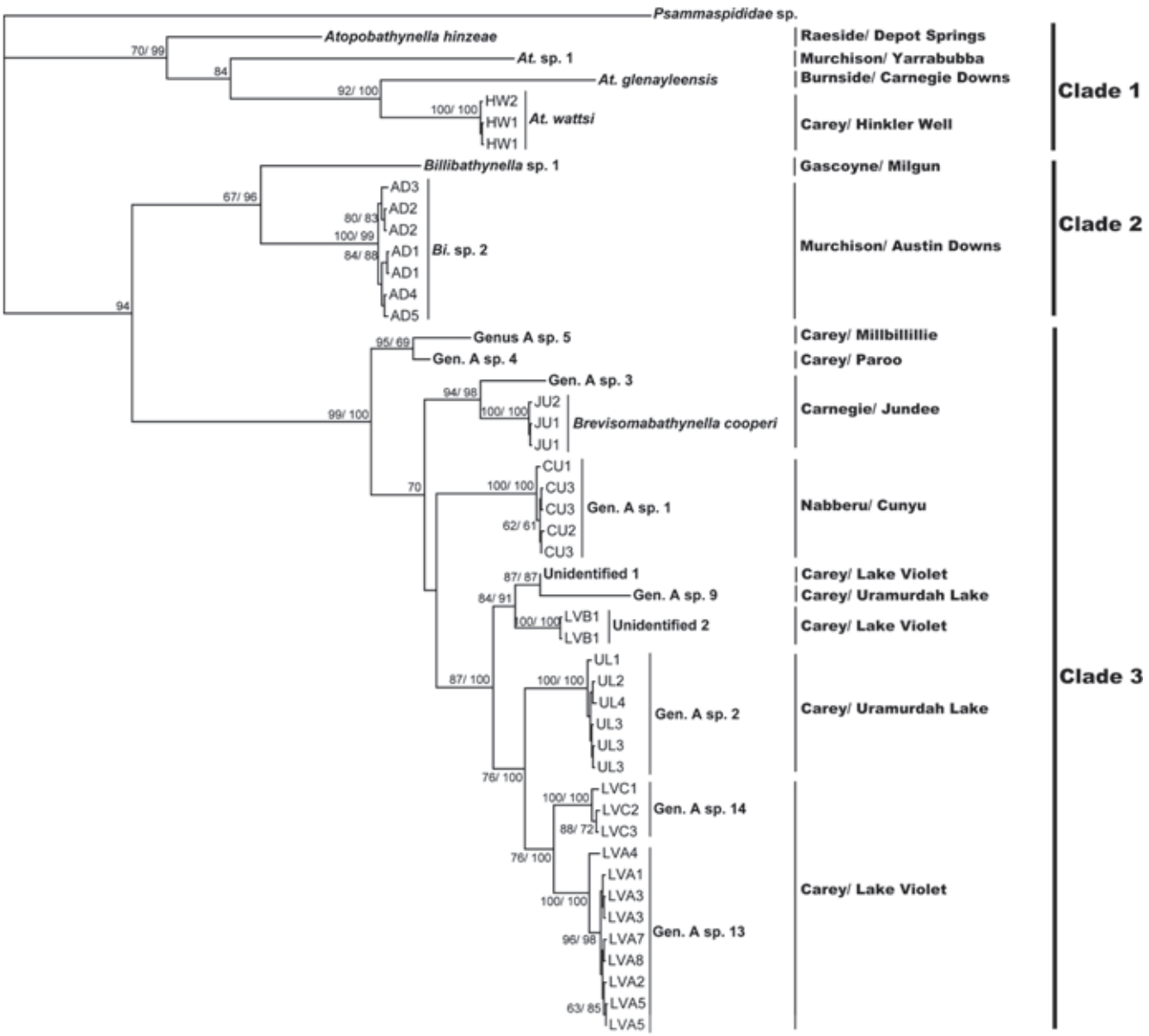

Fig. 2. Posterior probability (50%) Bayesian consensus tree with re-estimated branch lengths using the GTR+I+G model of substitution. Bayesian posterior probabilities are listed on corresponding nodes preceded by maximum parsimony bootstrap support. Sample codes are listed in Table 1 and their palaeodrainage and calcrete localities are nested within the major clades that are listed to the right of the tree.

part of the problem of saturation at the third codon position because each position is treated independently and not assumed to be evolving at the same rate. Four chains were run simultaneously for 2 000 000 generations in two independent runs, sampling trees every 100 generations. To evaluate convergence to the stationary distribution the program Tracer 1.4 (Rambaut and Drummond 2003) was used. The likelihood values converged to relative stationarity after ~75 000 generations. A burnin of 750 was chosen and a 50% consensus tree was constructed from the remaining 19 251 trees.

Maximum parsimony (MP) analysis was carried out using a heuristic unweighted parsimony search that involved tree-bisection-reconnection branch swapping and 10 multiple random addition sequence replicates. The DELTRAN method for character state optimisation was used because the ACCTRAN option is known to give erroneous branch lengths. The bootstrap analysis comprised 1000 replicates of the aforementioned heuristic search.

To evaluate associations between geography and population genetic structure among individuals we used an hierarchical analysis of molecular variance (AMOVA) with ARLEQUIN 3.1 (Excoffier *et al*. 2005). These analyses assessed the proportion of genetic variation partitioned within calcrete populations, among calcrete populations, and among palaeodrainages using genetic distances between haploytpes. The genetic distances were estimated using the GTR+I+G model of substitution (Lanave *et al*. 1984) from the re-estimated maximum likelihood parameters (described above) and then imported into ARLEQUIN 3.1 for the AMOVA. All available individuals and calcrete populations were used within the analyses. Unfortunately, many of the palaeodrainages were only represented by a single calcrete population and there were six calcrete populations represented by a single individual. Therefore, the findings of the test are inconclusive, but they provide some insight into the structure of parabathynellid populations in the Yilgarn.

Results

A 633-bp fragment of the mtDNA gene *cox1* was sequenced for 47 parabathynellid individuals from 12 calcrete populations in the Yilgarn region. In total, 17 genetically distinct lineages were observed among these calcrete populations and morphological examination revealed that these lineages represent 12 known and four new morphospecies (two lineages remain unidentified; see below). Each morphospecies was represented by up to seven sequenced individuals and multiple haplotypes were consistently observed within species. There were no haplotypes shared between calcrete populations, suggesting that genetic lineages were restricted to their individual calcrete populations. Much of this information is summarised in Table 1 and, for ease of interpretation, the genetic lineages we observed are represented by their morphospecies names, which we also use below for description and discussion of our findings. In particular, we discuss a new genus, Genus A, which is recognised but formally undescribed.

Nucleotide analysis

All unique sequences were submitted to GenBank (see Table 1). No stop codons or gaps were observed in any of the translated amino acid sequences suggesting that the genuine mtDNA *cox1* gene was sequenced. On average, a bias towards A–T (70%) was observed in the parabathynellid sequences, which is consistent with results from other crustaceans such as brine shrimp (Valverde *et al*. 1994), giant tiger prawns (Wilson *et al*. 2000) and copepods (Machida *et al*. 2002). The *cox1* sequences comprised 45% variable sites and 37% parsimony informative sites, which was higher than for amino acid sequences (26% and 19% respectively). The observed proportion of transitions to transversions (Ts/Tv ratio) at first codon positions was high (Ts/Tv = 1.4), equal at the second codon position (Ts/Tv = 1), and low at third codon position (Ts/Tv = 0.6). The latter observation is indicative of multiple substitutions (homoplasy) where transversions have probably accumulated in the most variable positions and the true number of changes may be obscured (Simon *et al*. 1994).

Genetic divergences

Pairwise genetic distances (patristic) are shown in Table 2 where each species is represented by a single haplotype. Between morphospecies, genetic divergences varied between 0.12 and 2.24 patristic substitutions per site (subst./site). Multiple haplotypes were found within most morphospecies and showed low sequence divergences (0–0.033 subst./site; Table 1). High intra-calcrete genetic variation was observed among individuals from each of Lake Violet, Uramurdah Lake and Jundee (Table 2). Four genetically distinct lineages with substantial genetic divergences (0.12–0.28 subst./site) were identified in Lake Violet, whereas Uramurdah Lake and Jundee each contained two distinct lineages, divergent by 0.30 and 0.22 subst./site respectively. This magnitude of observed divergences within calcretes was equivalent to that observed between some morphospecies from different calcrete populations (e.g. Gen. A sp. 1 (Cunyu) – Gen. A sp. 4 (Paroo), 0.31 subst./site; Table 2). Morphological examination of the Lake Violet individuals identified two of these lineages as distinct morphospecies from the new Genus A, named here Gen. A sp. 13–14, whereas the other two lineages (Unidentified 1 and 2) are yet to be examined morphologically. The Uramurdah Lake lineages represented Gen. A sp. 2 and Gen. A sp. 9, whereas Jundee lineages were identified as Gen. A sp. 3 and *Brevisomabathynella cooperi* Cho *et al*., 2006*b*.

Phylogenetic analysis

Four individuals (Psammaspididae sp., *Billibathynella.* sp. 1, Gen. A sp. 3 and Gen. A sp. 9) were not sequenced for the complete length of 633 bp owing to amplification with an alternate primer pair. To ensure that these shorter sequences did not unduly influence the results, an analysis was performed where all sequences in the alignment were trimmed to match the shorter fragment length (480 bp) and the resulting tree was compared with one where the lengths varied. The result (not shown) revealed no significant difference in terminal node placement on the phylogeny so differential sequence length was maintained in all subsequent analyses. Additionally, analyses of amino acid sequences supported all major groupings of the Bayesian phylogeny (data not shown).

The mtDNA sequence data for the calcrete-dwelling parabathynellids separated into three distinct clades (1, 2 and 3; Fig. 2), each of which was well supported in all Bayesian and MP analyses (>96% Bayesian posterior probability and >67%

Table 2. Pairwise maximum likelihood patristic distances estimated using the GTR+I+G model of nucleotide substitution between species (that represented the genera: Genus A, *Atopobathynella*, *Billibathynella* and *Brevisomabathynella*), lineages and calcretes (haplotypes used are listed in brackets; comparisons among all haplotypes are not shown because intraspecific divergences are summarised in Table 1)

Bold values are intra-calcrete divergences among individuals. Abbreviations for calcretes (in the order they appear): PA, Paroo; HW, Hinkler Well; LV, Lake Violet; UL, Uramurdah Lake; MI, Millbillillie; CU, Cunyu; DS, Depot Springs; MU, Milgun; AD, Austin Downs; YA, Yarabubba; JU, Jundee; and CD, Carnegie Downs

Calcrete		PA	HW	LV				UL		MI	CU	DS	MU	AD	YA	JU		CD
	Species	Gen. A sp. 4	*At. wattsi* (HW2)	Gen. A sp. 13[A]	Unidentified 1	Unidentified 2 (LVB1)	Gen. A sp. 14 (LVC1)[A]	Gen. A sp. 2 (UL1)	Gen. A sp. 9	Gen. A sp. 5	Gen. A sp. 1	*At. hinzeae*	*Bi.* sp. 1	*Bi.* sp. 2[A]	*At* sp. 1[A]	Gen. A sp. 3	*Br. cooperi* (JU1)	*At. glenayleensis*
PA	Gen. A sp. 4	–																
HW	*At. wattsi* (HW2)	1.83	–															
LV	Gen. A sp. 13[A]	0.32	2.08	–														
	Unidentified 1	0.33	2.10	**0.28**	–													
	Unidentified 2 (LVB1)	0.29	2.06	**0.24**	**0.21**	–												
	Gen. A sp. 14 (LVC1)[A]	0.27	2.04	**0.12**	**0.24**	**0.20**	–											
UL	Gen. A sp. 2 (UL1)	0.30	2.07	0.19	0.27	0.22	0.16	–										
	Gen. A sp. 9	0.37	2.13	0.31	0.25	0.24	0.27	**0.30**	–									
MI	Gen. A sp. 5	0.12	1.88	0.37	0.38	0.34	0.33	0.36	0.42	–								
CU	Gen. A sp. 1	0.31	2.07	0.38	0.40	0.36	0.35	0.37	0.43	0.36	–							
DS	*At. hinzeae*	1.38	1.03	1.63	1.64	1.60	1.59	1.61	1.68	1.43	1.62	–						
MU	*Bi.* sp. 1	1.80	1.18	2.05	2.06	2.02	2.01	2.03	2.10	1.85	2.04	0.99	–					
AD	*Bi.* sp. 2[A]	1.24	2.06	1.49	1.51	1.46	1.45	1.48	1.54	1.30	1.48	1.61	2.03	–				
YA	*At.* sp. 1[A]	0.89	1.72	1.41	1.60	1.12	1.10	1.13	1.19	0.94	1.13	1.26	1.68	0.61	–			
JU	Gen. A sp. 3	0.34	2.11	0.43	0.44	0.40	0.39	0.41	0.48	0.40	0.42	1.66	2.08	1.52	1.18	–		
	Br. cooperi (JU1)	0.31	2.07	0.38	0.40	0.36	0.34	0.37	0.43	0.36	0.37	1.62	2.04	1.48	1.13	**0.22**	–	
CD	*At. glenayleensis*	1.90	0.68	2.19	2.21	2.17	2.15	2.18	2.24	1.99	2.18	1.14	1.29	2.17	1.83	2.22	2.17	–

[A]Genetically distinct lineages identified as morphospecies.

MP bootstrap values; see Fig. 2). Clade 1 contained *Atopobathynella wattsi* Cho *et al*., 2006*a* (Hinkler Well), *At. hinzeae* Cho *et al*., 2006*a* (Depot Springs), *At. glenayleensis* Cho *et al*., 2006*a* (Carnegie Downs) and a new morphospecies from the genus *Atopobathynella* Schminke, 1973, named here *At.* sp. 1 (Yarrabubba). Clade 2 contained morphospecies from the genus *Billibathynella* Cho, 2005: *Bi.* sp. 1 (a recognised but undescribed species (J.-L. Cho, unpubl. data)) and a new morphospecies to the genus, *Bi.* sp. 2, from the Milgun and Austin Downs calcretes respectively.

Overall, Clade 3 contained individuals representing 11 genetically distinct lineages from six calcrete populations. Individuals from Gen. A sp. 5 (Millbillillie), Gen. A sp. 4 (Paroo) and Gen. A sp. 1 (Cunyu) formed monophyletic lineages. The position of these lineages varied between trees (also indicated by low node support) but consistently grouped within Clade 3. The Jundee calcrete clade comprised two distinct genera represented by the species Gen. A sp. 3 and *Brevisomabathynella cooperi*. These lineages formed a well supported monophyletic clade (98% Bayesian posterior probability and 94% MP bootstrap; Fig. 2) to the exclusion of species and genera from other calcretes. The placement of *Br. cooperi* within the broader Clade 3, renders Genus A paraphyletic.

In contrast to the Jundee calcrete clade where species from two genera were monophyletic with regard to their calcrete of origin, six lineages were observed from Uramurdah Lake and Lake Violet calcrete populations that showed a higher than expected level of phylogenetic complexity and structure among individuals. Morphospecies from these calcretes were not reciprocally monophyletic. Instead these morphospecies formed two well supported clades (>91% Bayesian posterior probability and >76% MP bootstrap); one containing Gen. A sp. 2 from Uramurdah Lake and Gen. A sp. 14 and 13 from Lake Violet; the other clade comprised Unidentified 1 and 2 from Lake Violet and Gen. A sp. 9 from Uramurdah Lake. Within the latter clade, the two morphospecies from Lake Violet were not monophyletic (Fig. 2).

Phylogenetic reconstruction revealed that the three major clades (Clades 1–3; Fig. 2) clearly grouped species into their respective genera. Clade 1 represented morphospecies examined from *Atopobathynella*, Clade 2 consisted of the two *Billibathynella* morphospecies and Clade 3 represented the paraphyletic Genus A and *Brevisomabathynella*. Interestingly, all the palaeodrainages represented in Clade 2 (Murchison and Gascoyne) drain west to the Indian Ocean. In contrast, the palaeodrainages represented in Clade 3 (Carey, Carnegie and Nabberu) drain water inland (i.e. to the east). Clade 1 showed a mixture of drainages whereby the Murchison palaeodrainage drains west, and Burnside, Carey, and Raeside drain east. These observations were examined further using AMOVA.

AMOVA

Results of the hierarchical AMOVA showed that most of the genetic variation (69%) could be attributed to variation among calcrete populations within palaeodrainage channels (permutation test $P = 0.0001$). Variation among palaeodrainages contributed 27% of the total variation observed between individuals (permutation test $P = 0.00001$).

Discussion

Inter-calcrete relationships among parabathynellids – 'islands under the desert'

Phylogenetic reconstruction of parabathynellid species and genera from calcrete aquifers in the Yilgarn region showed that, where multiple individuals from a single species and calcrete were sampled, they generally formed monophyletic lineages to the exclusion of lineages representing different calcretes. In an extreme example of such monophyly, the two putative genera from the Jundee calcrete formed a monophyletic lineage (Gen. A sp. 3 + *Br. cooperi*) to the exclusion of all other taxa, including those from the same genus. These results suggest a pattern of phylogeographic structure among parabathynellids where each calcrete contains one or more endemic species or divergent mtDNA lineages and provides support for the 'islands under the desert hypothesis' proposed by Cooper *et al*. (2002) and demonstrated in other studies of stygobitic invertebrates (Leys *et al*. 2003; Cooper *et al*. 2007, 2008). These findings are in partial contrast to some taxonomic (Cho *et al*. 2006*a*) and biological (Schminke 1974) observations of these particular crustaceans that predict some parabathynellid species may be able to move through the interstitial regions between calcretes to enter new aquifers.

The two key factors predicted to influence movement of stygofauna between calcrete aquifers are their general biology and the structure of their environment. First, parabathynellids are known to be strongly adapted to interstitial life with morphological modifications including elongated shape, reduced appendages and life-history traits such as externally reared and large eggs and multiple non-resting larval stages (Cho *et al*. 2006*b*). These traits generally help parabathynellids survive life in alluvium and are thought potentially to facilitate dispersal between calcretes where single species are described from multiple calcretes (Cho *et al*. 2006*b*). Alternatively, the observations and hypotheses of Cooper *et al*. (2002, 2007) suggest that the interstitial voids in the alluvia between calcretes may be too small to permit passage for dispersing obligate groundwater invertebrates. The interstitial environment between Yilgarn calcretes was formed when Tertiary valley-fills within major palaeovalleys were incised into the Precambrian basement of the Yilgarn craton and disrupted by multiple layers of clays (Arakel *et al*. 1990). It is these geological clay formations that are thought to form an inhospitable barrier to stygofauna that are dependent on an aquatic environment. Our current phylogeographic observations of parabathynellids indicate that they may not be as effective at dispersing through the substrate between the Yilgarn calcretes as they are in other interstitial alluvium (Schminke 1974; Cooper *et al*. 2007). However, future molecular phylogenetic studies will require inclusion of single species described from multiple calcretes to verify this observation.

Some discrepancy to the broader hypothesis of island-like biogeography was observed in the clade representing morphospecies from the Uramurdah Lake and Lake Violet calcrete populations. The mtDNA lineages from these calcretes did not form reciprocally monophyletic clades. This may be considered contrary to the island hypothesis but is more likely to indicate a complex history of parabathynellid speciation or calcrete invasion, or both, because there was no evidence of shared intraspe-

cific haplotypes between calcretes. This complex of lineages poses interesting questions regarding the evolution of parabathynellids and other invertebrate taxa in arid zone calcrete aquifers and we propose three possible scenarios to explain these observations. First, considering the close proximity of the Uramurdah Lake and Lake Violet calcretes (~800 m), it is plausible that historically they were connected and have subsequently been geographically separated. This scenario explains the genetic divergence between Gen. A sp. 2 (Uramurdah Lake) and Gen. A sp. 14 and 13 (Lake Violet) by fragmentation. The closest relatives to these sister clades are also from Uramurdah Lake and Lake Violet, and one of these lineages may indeed be similar to the most recent common ancestor to this derived clade (Gen. A sp. 2 + Gen. A sp. 14 + Gen. A sp. 13).

The second scenario assumes that these calcretes may still be joined, whereby the species within them are in the process of diversification within the calcrete. This scenario was first described for stygofauna by Leys *et al*. (2003) for dysticid diving beetles. Under this scenario, the organisms may be occupying different niches or inhabiting disparate locations within the heterogeneous substrate, as suggested by Coineau (2000) to explain the common occurrence of co-existing bathynellids. There is no evidence that these calcretes are presently joined, but niche partitioning may have also played an important role in past speciation if the calcretes were joined historically as suggested above.

The final scenario postulates multiple colonisation events from multiple ancestors, as is possibly the case in the Jundee calcrete where representatives of multiple genera co-exist within a single calcrete. Under this scenario shared ancestors to the two main clades of Lake Violet and Uramurdah Lake species invaded both calcretes and subsequently evolved within the calcretes. This scenario may be less likely than the other scenarios because all species from both Lake Violet and Uramurdah Lake appear to have shared a common ancestor such as that found at Cunyu calcrete, which represents the most morphologically similar and basal clade to the Lake Violet–Uramurdah Lake complex. However, this hypothesis has been proposed as a primary scenario under which multiple co-existing species have come to occur within single caclretes in the Yilgarn and must be considered (Leys *et al*. 2003; Cooper *et al*. 2007, 2008).

Phylogenetic reconstruction of dytiscid sequence data by Leys *et al*. (2003) has indicated no apparent gene flow between calcretes since the Pliocene (5–10 Mya). This timing coincides with a period of aridity (Stein and Robert 1986; Clarke 2001) during which species are hypothesised to have been forced into subterranean environments to survive (Leys *et al*. 2003). It is likely that bathynellaceans have a distinctive, but not exclusive, biogeographic history compared with other fauna (i.e. dytiscids) in this region because their ancestors were not epigean. It is hypothesised that the main mode of dispersal for ancient bathynellaceans is vicariance through geological movement, and such movements may have introduced them into new aquifer systems, particularly those close together. As calcretes have probably been remobilised and redeposited with changing climatic conditions through the late Tertiary (Morgan 1993), in addition to the strong potential for niche partitioning within the aquifers, each of the above hypotheses are plausible and perhaps not mutually exclusive. More intensive sampling of individuals, and additional geological information, are required to discriminate between each of these scenarios to estimate the true history of the parabathynellids in these two calcretes (Lake Violet and Uramurdah Lake).

Phylogenetic relationships among parabathynellids

Here we present the first molecular phylogenetic study of interspecific relationships among parabathynellids. We observed that species clustered into three major clades (1, 2 and 3) that grouped species into their respective genera and largely confirmed their monophyly. Clade 1 contained species from the genus *Atopobathynella – At. wattsi* (Hinkler Well), *At. hinzeae* (Depot Springs), *At. glenayleensis* (Carnegie Downs) and the new morphospecies, *At*. sp. 1 (Yarrabubba). Clade 2 contained a monophyletic group containing the two morphospecies *Bi.* sp. 1 (Milgun) and *Bi*. sp. 2 (Austin Downs), from the 'primitive' genus *Billibathynella* (Cho, 2005). The basal position of *Atopobathynella* to *Billibathynella* was surprising as the latter is thought to show highly primitive morphological characteristics (Cho 2005). Additional sampling of other genera is required to verify and test these relationships. In contrast to the monophyly observed in *Atopobathynella* and *Billibathynella*, Clade 3 revealed a paraphyletic relationship among members of two genera. The species *Br. cooperi* was embedded within Clade 3, which was otherwise dominated by species from the undescribed Genus A, and formed a monophyletic clade with Gen. A sp. 3, a species found in the same calcrete aquifer. The inclusion of *Br. cooperi* within this clade undermines the taxonomic hypothesis that Genus A is monophyletic. Future taxonomic revision of these genera is likely to be required.

Cryptic species

Before the present study, parabathynellid morphospecies had not been identified from Lake Violet. We now recognise two new morphospecies after initially observing four divergent mtDNA lineages from Lake Violet that were evaluated morphologically and have distinct features. Bearing strong morphological similarity to Gen. A sp. 1 from Cunyu calcrete, the level of divergence between Lake Violet lineages was generally at or above the 16% distance threshold for interspecific relationships among crustaceans based on *cox1* identified by Lefébure *et al*. (2006*a*). However, there were divergences between species from both Lake Violet and other calcrete populations that were lower than this threshold (Gen. A sp. 4 – Gen. A sp. 5, 0.12 subst./site; and Gen. A sp. 14 – Gen. A sp. 13, 0.12 subst./site). Indicative of a more recent radiation of species, this finding suggests that evaluation of cryptic species based solely on generalised DNA divergence thresholds can underestimate species diversity if divergences between taxa are recent. As suggested by Proudlove and Wood (2003), morphological evaluation is also required but DNA data can provide an insight into signatures of divergence.

Currently, we present data from a single mtDNA gene, *cox1*. A known difficulty associated with use of a single mitochondrial marker is the possibility of incomplete lineage sorting or introgression, or both, which can lead to a mtDNA gene not reflecting the history of the species (Avise 1989; Moore 1995). Future work on parabathynellids will aim to include additional data from nuclear gene markers. However, the current work provides the first hypothesis of molecular phylogeny for the phylo-

geography of parabathynellids and provides new insights into the evolution of the group.

Biogeography of parabathynellids

Palaeodrainage channels are ancient river systems, and calcrete aquifers are influenced by these systems because they act as water repositories into which water drains and as a course along which water travels. The Western Shield, on which the Yilgarn is found, is divided north to south by a drainage divide that separates those catchments draining to the Indian Ocean and those draining inland to the east (Beard 1998; Humphreys 2000). Interestingly, the three major clades observed in the reconstructed phylogeny suggest there has been some genetic structuring of taxa from calcretes either side of the drainage divide. Both Austin Downs and Milgun (Clade 2) lie in drainages flowing west to the Indian Ocean, whereas calcretes from Clade 3 lie in drainages that flow to the interior of the continent. Conversely, Clade 1 contained individuals from calcretes from both sides of the palaeodrainage divide.

Most of the genetic variation in parabathynellid individuals could be attributed to differences among calcrete populations and within palaeodrainages, whereas 27% of the genetic variation could be attributed to differences among palaeodrainages. This result is consistent with our observation of strong phylogenetic clades and indicates that calcrete populations within palaeodrainage channels are highly divergent but are more similar to each other than to calcrete populations in other palaeodrainage systems. The results of this population genetic analysis are likely to have been influenced by the close relationships among individuals from the Uramurdah Lake and Lake Violet calcrete populations and the low sample numbers from palaeodrainages and calcrete populations. However, they do provide an indicator of an association between the calcrete aquifers that parabathynellids inhabit and their respective palaeodrainages based on their genetic structure.

The present study demonstrates that parabathynellids show a pattern consistent with the 'subterranean island' hypothesis that each calcrete has short-range endemic taxa that are genetically distinct from other calcretes. Studies based on morphology are known to underestimate genetic diversity in stygobitic crustaceans (Jarman and Elliot 2000; Lefébure *et al*. 2006*a*; Finston *et al*. 2007) and significantly high levels of intra-calcrete diversity were observed here. We corroborate the observations of other studies that the calcrete aquifers of the Yilgarn region harbour high numbers of short-range endemic taxa from a broad range of invertebrate groups and future work will aim to investigate the possibility of individual species inhabiting multiple calcrete aquifers.

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