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Subterranean archipelago in the Australian arid zone: mitochondrial DNA phylogeography of amphipods from central Western Australia

STEVEN J. B. COOPER,*‡ JOHN H. BRADBURY,*† KATHLEEN M. SAINT,* REMKO LEYS,†‡ ANDREW D. AUSTIN†‡ and WILLIAM F. HUMPHREYS§

*Evolutionary Biology Unit, South Australian Museum, North Terrace, Adelaide, SA 5000, Australia, †School of Earth and Environmental Sciences, The University of Adelaide, SA 5005, Australia, ‡Australian Centre for Evolutionary Biology and Biodiversity, The University of Adelaide, SA 5005, Australia, §Western Australian Museum, Collections and Research Centre, Locked Bag 49, Welshpool DC, WA 6986, Australia

Abstract

In 1998, a unique subterranean ecosystem was discovered in numerous isolated calcrete (carbonate) aquifers in the arid Yilgarn region of Western Australia. Previous morphological and genetic analyses of a subterranean water beetle fauna suggest that calcrete aquifers are equivalent to closed island habitats that have been isolated for millions of years. We tested this hypothesis further by phylogeographic analyses of subterranean amphipods (Crangonyctoidea: Paramelitidae and Hyalidae) using mitochondrial DNA sequence data derived from the cytochrome oxidase I gene. Phylogenetic analyses and population genetic analyses (samova) provided strong evidence for the existence of at least 16 crangonyctoid and six hyalid divergent mitochondrial lineages, each restricted in their distribution to a single calcrete aquifer, in support of the 'subterranean island (archipelago) hypothesis' and extending its scope to include entirely water respiring invertebrates. Sequence divergence estimates between proximate calcrete populations suggest that calcretes have been isolated at least since the Pliocene, coinciding with a major aridity phase that led to the intermittent drying of surface water. The distribution of calcretes along palaeodrainage channels and on either side of drainage divides, have had less influence on the overall phylogeographic structure of populations, with evidence that ancestral crangonyctoid and hyalid species moved between catchments multiple times prior to their isolation within calcretes. At least two potential modes of evolution may account for the diversity of subterranean amphipod populations: dispersal/vicariance of stygobitic species or colonization of calcretes by surface species and independent evolution of stygobitic characteristics.

Keywords: amphipod, cytochrome oxidase I, mitochondrial DNA, phylogeography, stygofauna

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Introduction

There are many aspects of the evolution of subterranean animals that are still the subject of considerable debate, despite a long research history that dates back to the time of Darwin (1859). The process of regressive evolution, or loss of phenotypic characters that become functionless, is particularly controversial, with some researchers proposing natural selection as the driving force of regressive evolution (Yamamoto *et al*. 2000; Jeffery 2005; Romero & Green 2005), while others propose a neutral process, involving loss of gene function by random mutations (Kosswig 1960; Culver & Wilkens 2000; Leys *et al*. 2005; Wilkens in press). Recent molecular genetic analyses are also challenging some long-held views, such as the notion that subterranean animals are genetically depauperate, because of the spatially restricted and extreme nature of their environment (Buhay & Crandall 2005), or that they have a weak dispersal ability (Lefébure *et al*. 2006a). To further improve our understanding of the

Correspondence: Steven J. B. Cooper, Fax: 61 8 82077222; E-mail: cooper.steve@saugov.sa.gov.au

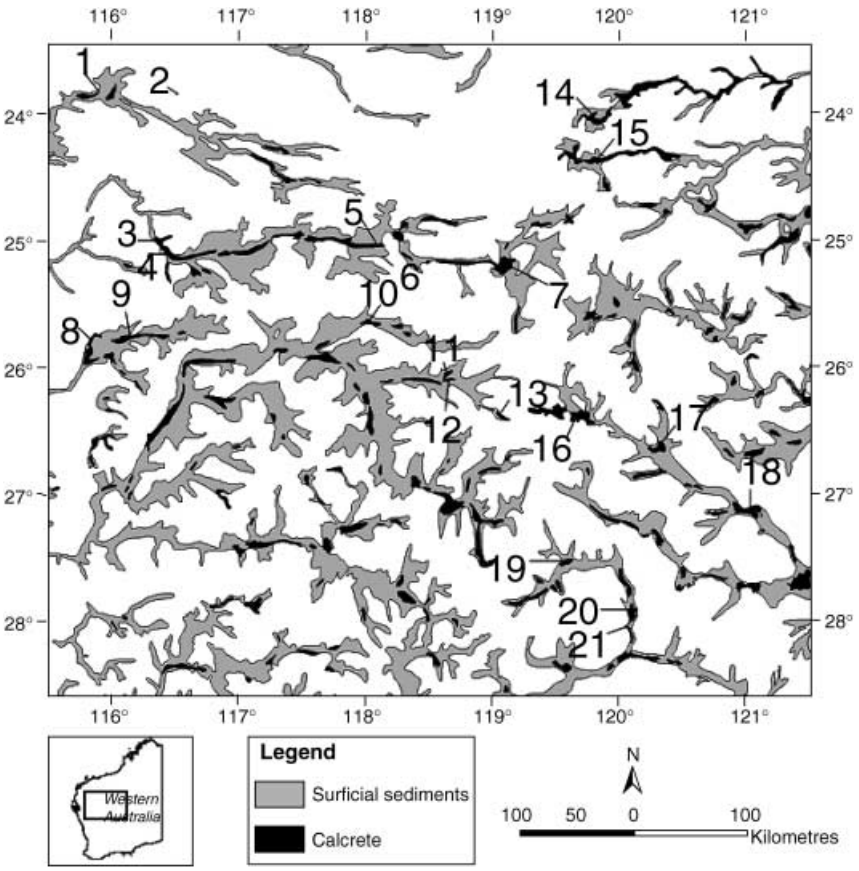

Fig. 1 Map of the northern Yilgarn Region of central Western Australia showing the location of calcrete (black) populations used in the amphipod analyses. Further details of each population are given in Table 1. Grey shaded regions represent surficial sediments in the palaeodrainage systems and these are separated by exposures largely of Precambrian geology. The east–west drainage divide passes between 13 and 16.

evolution of subterranean animals there is a need for additional model systems, particularly ones that provide novel attributes.

In 1998 a unique and diverse subterranean aquatic invertebrate fauna was discovered from numerous isolated groundwater calcretes (henceforth termed 'calcretes') of the arid Yilgarn region of central Western Australia (Fig. 1). The calcretes, thin (generally *c.* 10 m) carbonate formations, were originally deposited by precipitation from groundwater along ancient palaeodrainage channels (rivers that largely stopped flowing in the Palaeocene, Bowler 1976). The entire northern Yilgarn region of Western Australia resembles a subterranean archipelago containing more than 200 major isolated calcrete bodies, many of which have an area greater than 100 km^2 while there are hundreds of smaller calcrete bodies, some less than a few square kilometres in size (Fig. 1).

The calcrete fauna comprises subterranean aquatic animals (known collectively as stygofauna) of largely unknown species in diverse invertebrate groups including water beetles (Dytiscidae), and crustaceans such as Bathynellacea (Cho 2005; Cho *et al*. 2006a, 2006b), Oniscidea (Taiti & Humphreys 2001), Amphipoda (J. Bradbury, unpublished data), Copepoda (Karanovic 2004) and Ostracoda (Karanovic & Marmonier 2002). We have carried out detailed taxonomic (Watts & Humphreys 1999, 2000, 2001, 2003, 2004, 2006) and molecular genetic studies (Cooper *et al*. 2002; Leys *et al*. 2003) of the water beetle fauna and identified over 100 new species from 47 major isolated calcretes that together comprise the world's largest and most diverse collection (by a factor of 10) of subterranean water beetles (Balke *et al*. 2004). Significantly, each species is restricted in its distribution to a single calcrete and molecular-clock analyses (Leys *et al*. 2003) suggest there has been no apparent geneflow between calcretes since the Pliocene [5–10 million years ago (Ma)], coinciding with a major period of aridity of the Australian continent (Bowler 1976; Stein & Robert 1986).

The above findings suggest that calcrete aquifers are equivalent to closed island habitats and, further, that these closed habitats have persisted over long periods of time, of the order of millions of years. If so, it might be expected that stygobitic species of other invertebrate groups also could be highly restricted in their distribution to single calcretes and show similar patterns of diversification within calcrete bodies. Alternatively, the presumed requirement of stygobitic dytiscids for air breathing, in contrast to other stygofauna, or other physiological characteristics may have limited their

dispersal ability leading to their isolation within discrete calcrete bodies. The unknown potential for many of the stygobitic taxa to migrate along palaeodrainage channels between calcretes, either in groundwater connections or in occasional surface (flood) water, needs further investigation to assess the generality of the island hypothesis. In the following study, we consider the stygobitic amphipod fauna of the Yilgarn region.

Two main groups (families) of stygobitic amphipods are present in calcretes of the Yilgarn region; Hyalidae (referred to hereafter as hyalids), found principally in the more saline, southerly water bodies, and Paramelitidae, of the superfamily Crangonyctoidea (referred to hereafter as crangonyctoids), an ancient freshwater lineage, distributed among less saline waters to the north of the Yilgarn region (Williams & Barnard 1988; J. Bradbury, unpublished data). As in other amphipods (Bradbury & Williams 1999; Finston *et al*. 2007), species from each of the two groups are morphologically cryptic and the Yilgarn species are yet to be formally described (J. Bradbury *et al*., in preparation). This uncertainty in species boundaries, however, does not preclude an assessment of the phylogeographic structure of amphipod populations. The prediction from the island hypothesis is that calcretes should each contain populations showing long-term isolation, which would be supported by finding monophyletic groups of mitochondrial DNA (mtDNA) or nuclear haplotypes associated with a specific calcrete body (Avise 1994).

There are additional factors that also may have influenced the overall phylogeographic structure of amphipods from the Yilgarn Region. It is likely that amphipods within calcretes of the same palaeodrainage system will be more closely related than amphipods from different palaeodrainages, because of either historical or recent groundwater or surface water connections. Different palaeodrainages are separated by exposures largely of Precambrian rock and there is a distinct drainage divide, a region of elevated Precambrian rock that separates drainages flowing west to the Indian Ocean and those flowing east towards central Australia and the Nullabor Plain (Beard 1998; Fig. 1). Surface or groundwater flow between palaeodrainages, particularly across the central divide, might be unlikely, restricting the dispersal of aquatic animals, and leading to distinct patterns of phylogeographic structure associated with palaeodrainages.

Here we present the first phylogeographic analysis of the amphipod fauna of the Yilgarn region using the mtDNA marker, cytochrome oxidase I (CO1). We have focused these analyses on the crangonyctoid amphipods, as part of a parallel morphological study, but also have included a number of populations of hyalid amphipods for comparison. Our results show that amphipod populations are highly structured, with calcretes containing distinct populations showing long-term isolation, in support of the 'island hypothesis'. In light of these findings, we further examine the likely history and mode of evolution of these subterranean amphipods.

Methods

Sampling methods

Amphipods were selected from ethanol-preserved samples collected between 1998 and 2005 from calcrete aquifers in 420 000 km^2 of the Yilgarn Region (Table 1, Fig. 1). Access to calcrete aquifers relied entirely on the availability of existing boreholes and pastoral wells, as drilling new boreholes was beyond the financial scope of the project. When possible, multiple boreholes and wells were sampled from each calcrete. However, in many cases, access to the calcrete was limited and only a single hole could be sampled (see Table 1). Despite considerable effort, we have identified no cases of amphipods being present in any well or borehole that is located outside a calcrete body in this region.

Samples were collected by hauling plankton nets of 200 μm or 350-μm mesh through the water column of boreholes or wells, a method that concentrates the macro-invertebrates into a collection tube at the bottom of the net. Macro-invertebrates were sorted under a light microscope and samples were stored in either 100% or 75% ethanol at room temperature, the latter being the more favourable for morphological analyses. One hundred and twenty-six amphipod specimens from 26 distinct calcretes were sorted into two groups, hyalids (n = 17 from six calcretes) and crangonyctoids (n = 109 from 21 calcretes, with one calcrete containing both crangonyctoids and hyalids), on the bases of their morphology (Bradbury & Williams 1999; Lowry & Stoddart 2003). No other amphipod groups were present in the samples. The hyalid amphipod group was used as an outgroup to root the phylogeny of the crangonyctoid group and vice versa.

Mitochondrial DNA sequencing

DNA was extracted from dissected legs or whole animals using a Gentra Puregene Extraction Kit according to the manufacturer's instructions. We polymerase chain reaction (PCR)-amplified a 708-bp region of the CO1 gene using the primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al*. 1994) for 77 of the 126 samples. A further 12 samples were PCR-amplified using the amphipod-specific primers M479 (TTTATTTTAGGDGCMTGATC) and M480 (AATGADGTRTTTARRTTTCG), which span a similar region to LCO1490 and HCO2198. The remaining 37 crangonyctoid samples, of which 23 were from six additional calcrete populations, failed to PCR-amplify with the above primer combinations and additional CO1

Table 1 Locations of calcrete populations and amphipod samples, sample numbers (biospeleology (BES) field numbers, Western Australian Museum), GenBank Accession nos, pairwise sequence divergence, based on patristic distances using a maximum-likelihood model GTR + I + G (ML_p), and nucleotide diversity (π) indices within calcrete populations

Calcrete Population*	Gp.†	*n*	BES numbers	GenBank Accession	ML_p‡ (percentage)§	π (percentage)§
Lyons drainage (west)						
1. Gifford (S23.9324 E115.9569)	c	3	8828, 8834	EF118242–3, 245	0	0
2. Wanna (S23.9220 E116.5573)	c	4	8850	EF118217, 230, 232–233	0–1.3	0.73
Gascoyne drainage (west)						
3. Dalgety Downs (site 1: S25.1228 E116.4764)	c	2	8821	EF118255, 263	0–6.2	2.85
4. Dalgety Downs (site 2: S25. 1810 E116.5609)	c	3	8824	EF118259-61		
5. Milgun Sth_Earrie (S25.2786 E118.0956)	c	7	8664–5	EF118186–8, 209, 214–6	0–39.9	6.32
6. Milgun Sth_Outcamp (S25.2362 E118.1341)	c	2	8697–8	EF118191, 204	0.5	0.46
7. 3 Rivers Plutonic (site 1: S25.2675 E119.164)	c	4	8614–5	EF118181–2, 185, 210	0–0.6	0.19
(site 2: S25.2786 E119. 1834)	c	5	8607–8	EF118183, 207, 218, 222–3		
Murchison drainage (west)						
8. Byro Central (S25.8755 E115.8953)	c	5	9348–9	EF118192-4, 225, 228	0–0.6	0.31
9. Innouendy (S25.822 E116. 1915)	c	6	8811, 9336–7, 9344	EF118197-200, 227, 257	0–1.3	0.80
10. Mt Padbury (site1: S25.6983 E118.0913)	c	4	9331–2	EF118201–2, 244, 254	0–1.3	0.80
(site2: S25.695 E118.0796)	c	3	9309–10	EF118195, 239, 264		
(site 2: S25.695 E118.0796)	h	2	9309	EF118196, 238	0.2	0
11. Killara north (S26.0652 E118.6994)	c	2	9278	EF118237, 258	1.5	1.29
12. Karalundi (site 1:S26.1278 E118.6843)	c	2	9283–4	EF118221, 262	1.1–3.0	1.46
(site 2:S26.1264 E118.6827)	c	1	9272	EF118224		
13. Killara (S26.3419 E118.9607)	c	3	5596, 8130	EF118184, 205, 212	0–0.5	0.36
Lake Disappointment drainage (west)						
14. Savory (S24.0964 E119.7519)	c	1	8480	EF118206	—	—
15. Igarari (S24.4397 E119.7575)	c	2	8493, 6	EF118189–90	0.2	0.15
Carey drainage (east)						
16. Paroo (site 1: S26.4003 E119.763)	c	2	5626	EF118175, 203	0–2.1	2.44
(site 2: S26.4004 E119.763)	c	1	7270	EF118176		
(site 3: S26.4252 E119.7294)	c	2	5609	EF118177, 211		
(site 4: S26.4339 E119.7772)	c	5	8094, 8096, 8142	EF118178–9, 180, 248–9		
(site 5: S26.4339 E119.7766)	c	3	5632–3	EF118208, 219–20		
17. Lake Violet (S26.6749 E120.232)	h	3	6425, 6434	EF118246–7, 250	0–0.3	0.21
18. Barwidgee (S27.1375 E120.9494)	h	2	10377	EF118234–5	1.2	1.09
Raeside drainage (east)						
19. Lake Mason (S27.5400 E119.6243)	h	5	8363	EF118226, 229, 231, 236, 256	0–0.9	0.39
20. Depot Springs nth. (site 1: S27.9308 E120.0792)	h	1	8382.1	EF118253	—	—
21. Depot Springs sth. (site 1: S28.0499 E120.0392)	h	2	8407	EF118251-2	0–0.2	0.08
(site 2: S28.0601 E120.0674)	h	2	8408	EF118240–1		

*Numbers represent distinct calcrete populations with the exceptions of numbers 3 and 4, which represent separate sites in the calcrete Dalgety Downs. †Gp., crangonyctoid (c) or hyalid (h) amphipod groups. ‡ML_p was calculated using the phylogeny shown in Fig. 3, with parameter settings for the GTR + I + G model as given in the figure caption. §Intra-calcrete diversity levels were calculated by pooling data from all sites in the calcrete. For Dalgety Downs data from populations 3 and 4 were pooled.

primer combinations. PCR amplifications were carried out in 25-μL volumes with approximately 100 ng genomic DNA, 4 mM $MgCl_2$, 0.20 mM dNTPs, 1× PCR buffer (Applied Biosystems), 6 pmol of each primer (Geneworks) and 0.5 U of Ampli*Taq* Gold (Applied Biosystems). PCR amplification was performed under the following conditions: 94 °C 9 min, then 34 cycles of 94 °C 45 s; annealing 48 °C 45 s; 72 °C, 60 s; with a final elongation step at 72 °C for 6 min. PCR product was purified using Ultraclean PCR cleanup columns (MoBio Laboratories) and sequenced in both directions using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing was carried out on an ABI PRISM 3700 DNA analyser and raw sequences were edited and aligned using SEQED version 1.0.3 (Applied Biosystems). Sequences have been submitted to GenBank (see Table 1 for accession nos).

Mitochondrial DNA analyses

Phylogenetic analyses of the CO1 sequence data were conducted using maximum parsimony (MP) in PAUP* version 4.0b10 (Swofford 2002), and a Bayesian approach using MRBAYES version 3.1.1 (Huelsenbeck & Ronquist 2001). Concordance of trees from each of the different methods, bootstrap proportions and posterior probability estimates were used to examine the robustness of nodes. MP analyses were conducted using a heuristic search option and default options [TBR (tree-bisection–reconnection) branch swapping], with the exception of using random stepwise addition repeated 100 times. Character-state optimization for MP trees used the DELTRAN option, as there is a bug in PAUP* version 4.0b10 in the default ACCTRAN option that leads to erroneous branch lengths in output trees. MP bootstrap analyses (Felsenstein 1985) were carried out using 500 bootstrap pseudoreplicates, employing a heuristic search option with random input of taxa.

A general time-reversible model (Rodríguez *et al*. 1990), with a proportion of invariant sites and unequal rates among sites (Yang 1996), modelled with a gamma distribution (GTR + I + G) in MODELTEST (Posada & Crandall 1998), was found to be the most appropriate model to use in the Bayesian analyses. The MRBAYES analysis was carried out applying different models to first, second and third codon positions in an unlinked analysis, using default uninformative priors. Four chains were run simultaneously for 2.5 million generations in two independent runs, sampling trees every 100 generations. After this number of generations the standard deviation of split frequencies had reduced to less than 1%, and the potential scale reduction parameter was approximately one for all parameters, indicating Bayesian runs had converged and that a sufficient sample of the posterior distribution had been obtained. The likelihood values converged to relatively stationary values after about 5000 generations. A burn-in of 500 trees (equivalent to 500 000 generations) was chosen for each independent run of MRBAYES, with a > 50% posterior probability consensus tree constructed from the remaining 19 002 trees (9501 trees each run).

The program ARLEQUIN version 3.01 (Excoffier *et al*. 2005) was used to estimate nucleotide diversity levels within calcrete populations. Patristic distances (Lefébure *et al*. 2006b) between sequences were estimated using the GTR + I + G model of evolution and branch lengths and parameters estimated for the MRBAYES consensus tree using PAUP*, with the optimality criterion set to maximum likelihood (ML). An AMOVA was performed using ARLEQUIN to test the hypothesis that genetic divergence between each calcrete population differed from zero (Excoffier *et al*. 1992). We focused these analyses on the crangonycoid amphipod data only as this data set was much more comprehensive in sampling across the Yilgarn region than that obtained from the hyalid amphipods. Fifteen separate calcrete populations of crangonyctoids from five palaeodrainages (Table 1) were defined *a priori* and the proportion of genetic variation apportioned among and within populations, and among and within palaeodrainages was estimated. F_{ST} estimates among pairs of populations was conducted using the distance method as implemented in ARLEQUIN (Excoffier *et al*. 1992).

In order to explore the population structure of the amphipods without *a priori* hypotheses of the expected structure (as with AMOVA) we used the program SAMOVA (spatial analysis of molecular variation, Dupanloup *et al*. 2002). This method uses a simulated annealing procedure to define groups of populations/sampling sites with populations being assigned to groups on the basis that they are geographically adjacent and genetically homogeneous. The method requires the *a priori* definition of the number of groups (K) of populations that exist, and generates F statistics (F_{SC}, F_{ST} and F_{CT}) using an AMOVA approach (Excoffier *et al*. 1992). By exploring the behaviour of the indices F_{CT} and F_{SC} for different values of K, it is possible (with caution, Dupanloup *et al*. 2002) to identify the optimum number of population groups for a set of sample populations. For our analyses, we considered the crangonyctoid amphipod data set only, which contained 22 discrete samples from 15 distinct calcretes. We used 100 simulated annealing processes for each value of K from $K = 2$ to $K = 20$.

Results

A 649-bp fragment of the CO1 gene was sequenced from 89 amphipod samples from 20 calcretes, with between 1 and 13 samples per calcrete (Table 1, Fig. 1). These calcretes were distributed along six palaeodrainage channels with four (13 calcretes) draining to the Indian Ocean, and two (seven calcretes) draining inland. Fifteen of the calcrete

Table 2 Estimated divergence times for crangonyctoid and hyalid calcrete populations from pairs of geographically proximate sister lineages, based on a calibration for CO1 of 0.0125 substitutions per site per million years from Ketmaier *et al.* (2003)

Palaeodrainage/Calcrete populations	Divergence (percentage)*	Date (million years)
Carey/Barwidgee vs. Lake Violet	33.4	13.4
Murchison/Killara Nth vs. Carey/Paroo	18.8	7.5
Murchison/Killara North vs. Karalundi	27.6	11.0
Murchison/Byro Central and Innouendy	10.2	4.1
Lyons/Wanna and Gifford	36.4	14.6
Raeside/Depot Springs north vs. south	21.3	8.5

*Divergences were calculated using minimum patristic distances between haplotypes and maximum likelihood estimated branch lengths using a GTR + I + G model and the Bayesian consensus phylogeny (Fig. 3). The values shown are the minimum divergences found among all calcrete populations.

Table 3 Results from a SAMOVA analysis of mtDNA sequence data from calcrete populations. Group composition numbers are populations as given in Table 1 and Fig. 1. *K* refers to the number of predefined groups used in the analyses

K	F_{SC}	F_{ST}	F_{CT}	Group composition
2	0.930	0.949	0.275	(16.1,16.2,16.3,11,13,16.4,12.1,12.2,6,10.1,10.2,15,14) (7.1,7.2,5,9,2,1,3,4,8)
3	0.922	0.948	0.335	(16.1,16.2,16.3,11,13,16.4,12.1,12.2,6,10.1,10.2,15,14) (7.1,7.2) (5,9,2,1,3,4,8)
4	0.915	0.948	0.390	(16.1,16.2,16.3,11,13,16.4,12.1,12.2,6,10.1,10.2,15,14) (7.1,7.2) (5,9,3,4,8) (2,1)
5	0.906	0.946	0.427	(16.1,16.2,16.3,11,16.4,12.1,12.2) (13,6,10.1,10.2,15,14) (7.1,7.2) (5,9,3,4,8) (2,1)
6	0.899	0.946	0.467	(16.1,16.2,16.3,11,13,16.4,12.1,12.2) (6,15,14) (10.1,10.2) (7.1,7.2) (5,9,3,4,8) (2,1)
7	0.888	0.945	0.509	(16.1,16.2,16.3,11,16.4) (13,12.1,12.2,15,14) (6,10.1,10.2) (7.1,7.2) (5,3,4) (2,1) (9,8)
8	0.870	0.944	0.569	(16.1,16.2,16.3,11, 13, 16.4,12.1,12.2) (6,15,14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2,1) (9,8)
9	0.850	0.944	0.627	(16.1,16.2,16.3,11,16.4) (13,12.1,12.2) (6,15,14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2,1) (9,8)
10	0.830	0.944	0.669	(16.1,16.2,16.3,16.4) (11,13, 12.1,12.2) (6) (15,14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2,1) (9,8)
11	0.801	0.944	0.717	(16.1,16.2,16.3,16.4) (13) (11,12.1,12.2) (6) (15,14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2,1) (9,8)
12	0.760	0.944	0.765	(16.1,16.2,16.3,16.4) (13) (11,12.1,12.2) (6) (15,14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2) (1) (9,8)
13	0.697	0.943	0.812	(16.1,16.2,16.3,16.4) (13) (11,12.1,12.2) (6) (15,14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2) (1) (9) (8)
14	0.577	0.943	0.866	(16.1,16.2,16.3,16.4) (13) (11) (12.1,12.2) (6) (15,14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2) (1) (9) (8)
15	0.278	0.943	0.921	(16.1,16.2,16.3,16.4) (13) (11) (12.1,12.2) (6) (15) (14) (10.1,10.2) (7.1,7.2) (5) (3,4) (2) (1) (9) (8)
16	−0.113	0.943	0.949	(16.1,16.2,16.3,16.4) (13) (11) (12.1,12.2) (6) (15) (14) (10.1,10.2) (7.1,7.2) (5) (3) (4) (2) (1) (9) (8)
17	−0.277	0.942	0.955	(16.1,16.2,16.4) (16.3) (13) (11) (12.1,12.2) (6) (15) (14) (10.1,10.2) (7.1,7.2) (5) (3) (4) (2) (1) (9) (8)
18	−0.409	0.942	0.959	(16.1,16.2,16.4) (16.3) (13) (11) (12.1) (12.2) (6) (15) (14) (10.1,10.2) (7.1,7.2) (5) (3) (4) (2) (1) (9) (8)
19	−0.583	0.942	0.963	(16.1,16.2,16.4) (16.3) (13) (11) (12.1) (12.2) (6) (15) (14) (10.1,10.2) (7.1) (7.2) (5) (3) (4) (2) (1) (9) (8)
20	−1.459	0.942	0.976	(16.1,16.2,16.4) (16.3) (13) (11) (12.1) (12.2) (6) (15) (14) (10.1) (10.2) (7.1) (7.2) (5) (3) (4) (2) (1) (9) (8)

populations were found to contain crangonyctoid amphipods and six contained hyalids, with one calcrete (Mount Padbury) containing both hyalid and crangonyctoid populations. The CO1 sequence data showed high levels of nucleotide sequence divergence (ML patristic distance) among calcrete populations (> 10.2%, see Table 2), but lower levels of variation within a calcrete (usually < 6.2%, see Table 1). The exception to the latter was the calcrete Milgun South (Earrie) (< 39.9%), which contained two distinct mtDNA lineages (see below). Excluding this calcrete, nucleotide diversity estimates within calcrete populations ranged between 0% and 2.85% (Table 1).

AMOVA analyses of the crangonyctoid populations indicated that 92.7% of the total genetic variation was distributed among calcrete populations and only 7.3% distributed within calcretes, with permutation tests highly significant ($P < 0.00001$). When calcrete populations were grouped into palaeodrainages, 9.9% of the genetic variation could be explained by differences among palaeodrainages, 82.8% of variation was distributed among calcrete populations within palaeodrainages, and 7.3% of variation was distributed within calcrete populations (permutation tests were again highly significant $P < 0.00001$). Pairwise F_{ST} estimates among calcrete populations were all greater than 0.76 and were generally highly significant, with the exception of comparisons with a number of populations of low sample size, such as Milgun South (Outcamp), Killara North, Igarari and Savory (data not shown).

Results from the SAMOVA analyses showed, as expected (Dupanloup *et al.* 2002), that when the number of groups of populations (*K*) increased, the value of F_{CT} increased while F_{SC} decreased (Table 3, Fig. 2). However, a plateau in the

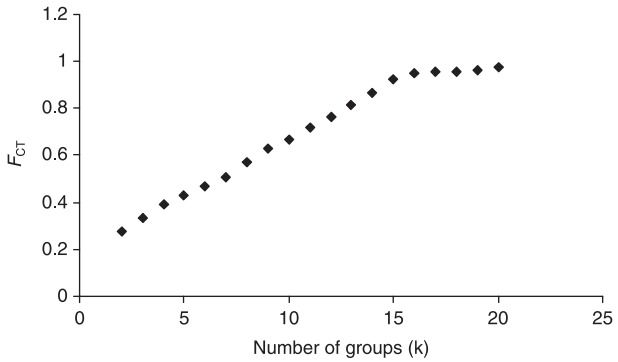

Fig. 2 A plot of the F_{CT} parameter for different values of *K*, the number of population groups, generated using SAMOVA (Dupanloup *et al*. 2002). *F*-statistic values are shown in Table 3.

F_{CT} curve occurred after *K* = 15 to *K* = 16 groups, with F_{CT} only increasing in small increments for *K* = 17 to *K* = 20, suggesting that adding extra groups only moderately improved the model of population structure (Fig. 2). The group membership at *K* = 15 exactly matched the *a priori* group structure proposed in the AMOVA analyses above, with all sites grouped into 15 discrete calcrete populations (Table 3). The *K* = 16 group structure was similar to *K* = 15 with the exception that the two divergent mtDNA lineages in the Dalgety Downs calcrete were separated into two different groups.

MP and Bayesian phylogenetic analyses of the CO1 data provided strong evidence for the existence of 16 crangonyctoid and six hyalid divergent (> 10.2%) mtDNA clades, with haplotypes within each clade grouping with 100% bootstrap and 100% posterior probability support (Fig. 3). Each clade is restricted in its distribution to a single calcrete aquifer. The structure of the Bayesian and MP trees differed with respect to the inter-relationships of a number of calcrete lineages, but both trees provide evidence for two major groups of crangonyctoid amphipods, with members of each group showing an overlapping distribution within the Gascoyne and Murchison palaeodrainages (Fig. 3). One of the groups (A) contains calcrete populations that lie on

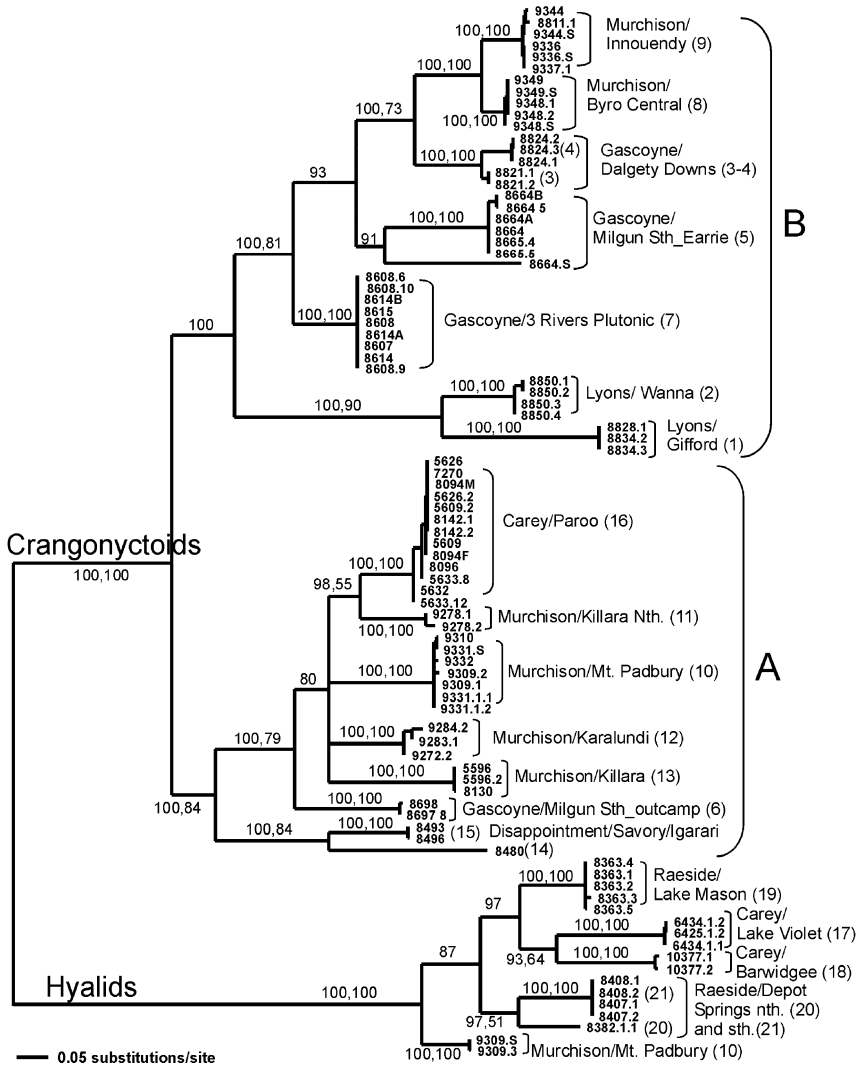

Fig. 3 50% posterior probability Bayesian consensus tree generated from 19 002 trees sampled in two independent runs of MRBAYES (version 3.1.1). Branch lengths were estimated using PAUP*, with the optimality criterion set to likelihood and a GTR + I + G model of evolution, with empirical nucleotide frequencies and number of rate categories = 4. Parameters estimated for this model were as follows: shape parameter (alpha) = 0.713, proportion of invariant sites = 0.425, rate matrix (A-C, 0.360; A-G, 4.668; A-T, 0.678; G-C, 1.412; T-C, 5.847; T-G, 1.000). Sample codes for each specimen are given in Table 1 and palaeodrainage, followed by calcrete population name, are given for each group. Numbers in parentheses are calcrete population numbers shown in Fig. 1. Numbers adjacent to branches refer to Bayesian posterior probabilities (left or single numbers) and MP bootstrap values obtained from 500 pseudoreplicates generated in PAUP* (right), with only numbers > 50% shown.

both sides of the drainage divide (Carey drainage flowing to the east and Gascoyne and Murchison drainages flowing to the west). The second group (B) only comprises calcretes from western flowing drainages.

Two calcretes [Mount Padbury and Milgun South (Earrie)] were found to contain more than one divergent mtDNA lineage. The two lineages from Mount Padbury are clearly distinctive species, with one being a hyalid amphipod and the other a crangonyctoid. The Milgun South (Earrie) calcrete appears to contain two sister lineages according to the Bayesian tree. The monophyly of the Milgun South (Earrie) sister lineages, however, is only moderately supported (posterior probability = 91%, MP bootstrap value < 50%). It is also worth noting that the Depot Springs north calcrete contains both hyalid and crangonyctoid amphipods, although efforts to PCR-amplify samples of the latter were unsuccessful.

The phylogenetic analyses showed no clear grouping of calcretes according to their associated palaeodrainage. However, a number of calcrete populations of the same palaeodrainage were found to be monophyletic to the exclusion of other calcrete populations with high to moderate levels of support: Innouendy and Byro Central of the Murchison palaeodrainage (MP bootstrap value (MP_b) = 99% and posterior probability P_p = 100%); Wanna and Gifford calcretes of the Lyons palaeodrainage (MP_b = 90%, P_p = 100%); Lake Violet and Barwidgee of the Carey palaeodrainage ($MP_b \leq 50\%$, P_p = 91%); Savory and Igarari of the Lake Disappointment palaeodrainage (MP_b = 84%, P_p = 100%); Depot Springs north and south, of the Raeside palaeodrainage ($MP_b < 50\%$, P_p = 97%).

Discussion

Amphipod phylogeography

The population genetic and phylogenetic analyses of the crangonyctoid and hyalid amphipod fauna indicate that there is significant phylogeographic structuring of populations, with evidence for at least 16 crangonyctoid and 6 hyalid divergent mitochondrial lineages, each restricted in their distribution to a single calcrete aquifer. A very high proportion (93%) of the total genetic variation of the crangonyctoid amphipods was accounted for by differences between calcrete populations. Furthermore, SAMOVA analyses, which assumed no *a priori* structure of populations, provided additional support for a group structure comprising individual calcretes as the optimum model by which to partition the genetic variation. This classic phylogeographic pattern is indicative of long-term isolation of populations within each calcrete aquifer and provides further support for the hypothesis that calcrete bodies are equivalent to closed 'subterranean islands' with an absence of any recent gene flow between them.

The 'subterranean island' hypothesis was previously strongly supported by taxonomic (Watts & Humphreys 1999, 2000, 2001, 2003, 2004, 2006) and phylogenetic analyses (Cooper *et al*. 2002; Leys *et al*. 2003) of subterranean dytiscid water beetles. Of the 20 calcretes represented here for amphipods, 14 also have unique species of water beetle, each showing a similar pattern of long-term isolation within a calcrete body, according to mtDNA sequence data (Leys *et al*. 2003; Leys *et al*. submitted). We have previously suggested (Cooper *et al*. 2002) that the isolation of water beetle species within calcretes is most likely due to the structure of the matrix between calcretes, which consists of fine alluvial deposits, containing layers of clay, with limited access to air-filled voids required for respiration by both adult and larval dytiscids (Spangler & Decu 1999). The current study significantly extends the scope of the 'subterranean island' hypothesis to include entirely water respiring invertebrates, with no larval phase, suggesting that the fine structure of alluvial deposits between calcretes is a major barrier to geneflow, at least for taxa of similar size dimensions to these amphipods (length ~1.0–6.5 mm). It may therefore be expected that additional components of the stygofauna, for example, isopods of the genus *Haloniscus* (Taiti & Humphreys 2001) and bathynellids (Cho 2005; Cho *et al*. 2006a,b), which are a similar size to the amphipods, also would show a phylogeographic pattern indicative of long-term isolation within a calcrete body.

With respect to the role of palaeodrainages in influencing the phylogeographic structure of populations, AMOVA analyses suggest only a relatively low proportion of the total genetic variation (9.9%) was explained by differences among palaeodrainages. Phylogenetic analyses also showed an absence of clear groupings of populations by palaeodrainage. For example, the Dalgety Downs calcrete population in the Gascoyne palaeodrainage is more closely related to the Byro Central/Innouendy populations in the Murchison, than it is to other populations within the Gascoyne (Fig. 3). Similarly, the Lake Mason calcrete population in the Raeside palaeodrainage is more closely related to Barwidgee/Lake Violet calcretes in the Carey palaeodrainage than to the Depot Springs population in the same palaeodrainage (Fig. 3). These patterns suggest that ancestral gene flow or range expansion between palaeodrainages occurred at least twice prior to the isolation of populations within calcrete bodies. However, a number of adjacent calcretes of the same palaeodrainage grouped closely together (e.g. Byro Central and Innouendy of the Murchison drainage; Gifford and Wanna of the Lyons drainage), suggesting that groundwater or surface water flow along palaeodrainages has played some role in structuring amphipod populations prior to their isolation within calcretes.

The role of the east–west drainage divide in structuring crangonyctoid populations is uncertain as we have limited samples east of the drainage divide. The one population

(Paroo) from the eastern draining Carey palaeodrainage formed a monophyletic group with a population from Murchison/Killara North (western draining) in both Bayesian and MP phylogenies, within a clade containing additional Murchison calcrete populations. This grouping suggests historical geneflow across the drainage divide between the Murchison and the Carey palaeodrainages prior to the isolation of calrete populations. Close examination of the structure of the central divide shows little or no elevation of land between the Carey and Murchison (specifically between Paroo and Killara calcretes, see Fig. 1). It is likely that some interdigitating surface drainage channels may have merged in the past, possibly through major flooding events, or their headwaters may have been captured, perhaps through groundwater sapping (Pederson 2001), allowing range expansion (Burridge *et al*. 2006). A similar scenario also may explain the apparent historical connection between the Gascoyne and Murchison palaeodrainages, and the Raeside and Carey palaeodrainages.

Timing of diversification

Molecular-clock analyses of the subterranean water beetle fauna suggested that species evolved independently within calcrete bodies following aridification of the Yilgarn region between 10 and 4 Ma (Leys *et al*. 2003). These times were obtained by estimating the date of divergence of sympatric sister species, which most likely speciated within a calcrete aquifer soon after colonization by a single surface ancestral species (Leys *et al*. 2003; Leys *et al*. submitted). There are a number of problems with obtaining similar estimates of the age of subterranean amphipod lineages or estimating how long calcrete populations of amphipods may have been isolated. First, related surface amphipod species are extinct throughout the study region, so there is a lack of any clear contrasts between surface and subterranean lineages to provide a maximum age of subterranean lineages. Second, the systematics of Australian crangonyctoid and hyalid amphipods is poorly known making it difficult to define the nearest related species. Third, unlike the water beetle fauna, there is only one potential case of a calcrete containing sister lineages of amphipod and the sister grouping received only moderate support, suggesting they are unlikely to have evolved from a common ancestor within a calcrete body. Fourth, our analyses do not contain all the potential calcrete populations of subterranean amphipods because of the problem of access to calcrete bodies, which rely on the availability of suitable wells or boreholes. Last, the ancient and stable landscape of the Yilgarn, emergent since the Proterozoic (Beard 1998), has provided sparse opportunity for geology to underpin molecular dates. In this respect, the Yilgarn is quite unlike geologically dynamic areas, such as the Tyrrhenian region, where there are well-dated geological events to complement molecular interpretations of stygofauna history (Ketmaier *et al*. 2003).

With the current data set, the most suitable nodes for obtaining a minimum date for the isolation of calcrete populations are from related lineages in geographically proximate calcrete bodies. We have identified a number of these nodes in Table 2. Using a rate of evolution of CO1 of 0.0125 substitutions per site per million years, proposed for subterranean isopods by Ketmaier *et al*. (2003), provides rough estimates of divergences in the range of 14.6 and 4.1 Ma (Table 2). While there are likely to be considerable errors in these estimates, they nevertheless suggest that populations have been isolated for a considerable period of time, and probably since the late Miocene or Pliocene.

The above dates for the isolation of amphipod populations are largely consistent with those obtained for the isolation of water beetle lineages (Leys *et al*. 2003), and with a model for the history of the calcretes proposed on the basis of geological data by Morgan (1993). Morgan proposed that calcretes were originally deposited by precipitation from the groundwater during an arid phase of the Oligocene around 37–30 Ma. Following a change to a wetter climate and reactivation of river flow during the Miocene (30–10 Ma), springs were extensively developed and caves/karst features formed within the calcrete. These developments would potentially have opened up the habitat for colonization by species living in surface water, or in the hyporheic zone, in either alluvial deposits or fractured rock aquifers. This wet phase was then followed by a period of aridity in central Australia, that started in the north around 14 Ma and, with varying intensity (becoming more arid 12, 9, 5 and 3 Ma: Stein & Robert 1986), extended southwards reaching the southern part of the Yilgarn by 5 Ma (Clarke 2001). As the aridity deepened, groundwater would have become the principle permanent waters remaining throughout the region. Lesser climatic fluctuations occurred within the Quaternary allowing lake systems to flood and dry repeatedly despite the prevailing arid conditions (Bowler *et al*. 2001). It might be expected that such flood events would lead to geneflow among calcrete populations, or even the potential for further colonization events by surface beetles and amphipods. However, our analyses show no evidence for geneflow or new colonization events throughout the Quaternary. Although the timing of the divergence of geographically proximate calcrete populations of amphipods coincides with the onset of aridity after the Miocene, we cannot rule out the possibility of an earlier colonization history, when calcretes first became available as a suitable habitat for stygofauna.

Species boundaries

There has been a recent suggestion by Lefébure *et al*. (2006b) that species of crustacean can be delimited on the basis of

CO1 patristic distances of the order of 0.16 substitutions/site. Although there may be problems in using a single mtDNA marker to delimit species boundaries (e.g. Mallet & Willmott 2003; Moritz & Cicero 2004), the large divergences of CO1 (> 18.8%, with one exception of 10.2%; see Table 2) we have detected between calcrete populations fall outside this suggested threshold, providing some evidence that each calcrete population may indeed represent a distinct species. Morphological analyses (J. Bradbury *et al*., in preparation) provide further support for this notion, although distinct differences associated with each calcrete population are not evident. Similar findings have recently been reported by Finston *et al*. 2007) who found large genetic differences between populations of subterranean amphipods from different tributaries of the Pilbara Region, Western Australia, but little/no clear morphological divergence between each of the genetic lineages, despite millions of years of isolation. In contrast, stygobitic water beetle species show considerable morphological variation, particularly among species within a single calcrete (Watts & Humphreys 1999, 2000, 2001, 2003, 2004, 2006).

Modes of evolution of subterranean lineages

There are at least two potential hypotheses that might explain the evolution of subterranean lineages of amphipods in the Yilgarn region. One hypothesis is that each calcrete was separately colonized by one or more aquatic surface species, with each population independently evolving stygobitic characteristics (loss of eyes and pigment, attenuated bodies and/or appendages, Holsinger 1994; Culver *et al*. 1995) by a process of convergent/parallel evolution. A second hypothesis is that evolution of stygobitic characteristics occurred just once each for hyalid and crangonyctoid amphipods and the current distribution of stygobitic populations resulted from dispersal by stygobitic ancestors, followed by vicariance events that isolated populations within calcretes.

Our previous analyses of the water beetle fauna provided strong support for the former hypothesis, involving independent colonization events by a number of different widespread surface ancestors (Leys *et al*. 2003; submitted). Recently, Lefébure *et al*. (2006a) argued that a dispersal/vicariance hypothesis explained the phylogeographic history of a widespread subterranean amphipod species *Niphargus virei* in France, largely on the basis of the phylogeographic structure showing fragmentation events and recent range expansions, and a lack of morphological variation among different populations. The hyalid and crangonyctoid lineages of the Yilgarn calcretes are also cryptic in their morphology, but this cryptic nature seems to be a general feature of these two groups in Australia (Bradbury & Williams 1999; Finston *et al*. 2007; J. Bradbury, unpublished data). We have suggested above that the current structure of the matrix between calcretes appears to form a major barrier to geneflow through the groundwater between calcretes, providing tentative support to the hypothesis that colonization originally occurred from surface waters. However, we cannot rule out the possibility that subterranean amphipods may have colonized calcretes after dispersal in groundwater interstitials of river beds during a wet phase in the Miocene (i.e. ancestors were hypogean). If this latter hypothesis is correct, then at least two ancestral crangonyctoid species were involved, to explain the current phylogenetic pattern in the group, that is two major mtDNA lineages (A and B) overlap in their range within the Gascoyne and Murchison palaeodrainages (Fig. 3). Further phylogenetic analyses, incorporating morphological and molecular data from related epigean and subterranean lineages, are required to help distinguish between these two hypotheses.

Conclusion

Phylogeographic analyses provide strong evidence that the Yilgarn calcretes are equivalent to a 'subterranean archipelago', with each calcrete island showing millions of years of isolation. A range of scenarios, involving either dispersal/vicariance and/or independent evolution of stygobitic characters may account for the composition of their current biodiversity. This novel subterranean system offers a unique opportunity to further explore current theories on the evolution of subterranean animals, such as regressive evolution (e.g. Leys *et al*. 2005) and modes of speciation.

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Steve Cooper is a Senior Research Scientist who specializes in the application of molecular techniques to investigate the systematics, evolution and ecology of Australian fauna. John Bradbury is a taxonomist specializing on Australian amphipod fauna. Kathy Saint is a senior technician with expertise in molecular genetic analyses. Remko Leys is a Research Associate who specializes in the application of molecular data and molecular-clock estimates to study systematics and evolution of invertebrates. Andy Austin is the Director for the Australian Centre for Evolutionary Biology & Biodiversity at Adelaide University, and is interested in the systematics and evolution of Australian arthropods. Bill Humphreys is a Senior Curator with an interest in the diversity, ecology and evolution of subterranean animals.
