

Subterranean archipelago: mitochondrial DNA phylogeography of stygobitic isopods (*Oniscidea: Haloniscus*) from the Yilgarn region of Western Australia

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Abstract. The arid Yilgarn region of Western Australia contains numerous isolated calcrete aquifers, within which a diverse subterranean fauna has been discovered. Genetic and morphological studies of subterranean dytiscid beetles and amphipods have suggested that individual calcretes are equivalent to closed island habitats, which have been isolated for millions of years. Here we test this ‘subterranean island’ hypothesis further by phylogeographic analyses of subterranean oniscidean isopods (*Haloniscus*), using mitochondrial DNA (mtDNA) sequence data derived from the cytochrome *c* oxidase subunit I gene. Phylogenetic and population genetic analyses provided evidence for significant phylogeographic structuring of isopod populations, with evidence for at least 24 divergent mtDNA lineages, each restricted in their distribution to a single calcrete aquifer. The high level of divergence among calcrete populations (generally >25%) and several mtDNA lineages within calcretes (>16%) suggests that each lineage is likely to represent a distinct species. These analyses, together with comparative phylogeographic data from dytiscid beetles and amphipods, provide strong support for the ‘subterranean island’ hypothesis, applying to both air-breathing and fully aquatic arthropod groups. The finding of several epigeal lineages that grouped with stygobitic *Haloniscus* populations, and the overall phylogeographic structure of populations, suggests that the majority of stygobitic species evolved within individual calcretes following independent colonisation by epigeal ancestors.

Additional keywords: cytochrome *c* oxidase subunit I (COI), Isopoda, stygofauna.

Introduction

Previous morphological and molecular genetic studies have identified a unique and diverse subterranean aquatic invertebrate fauna (known collectively as stygofauna) within calcrete (limestone) aquifers of the arid Yilgarn region of central Western Australia (Fig. 1). The fauna comprises ~100 water beetle species (Dytiscidae, Watts and Humphreys 1999, 2000; 2001, 2003; 2004, 2006) and a variety of crustacean species in groups such as Bathynellacea (Cho 2005; Cho *et al.* 2006a, 2006b), Isopoda (Taiti and Humphreys 2001), Amphipoda (J. Bradbury, unpubl. data), Copepoda (Karanovic 2004) and Ostracoda (Karanovic and Marmonier 2002).

Taxonomic and molecular genetic studies (Cooper *et al.* 2002; Leys *et al.* 2003) of the dytiscid fauna showed that species are restricted in their distributions to single calcrete aquifers and

that cladogenesis coincided with a major period of aridity of the Australian continent in the Pliocene (~5–10 million years ago, Bowler 1976; Stein and Robert 1986). These analyses led to the proposal that calcrete aquifers (henceforth referred to as ‘calcretes’) are equivalent to closed island habitats, which have had little or no gene flow between them for millions of years. This hypothesis is significant because it suggests that each of the ~200 major calcretes in the Yilgarn region contains unique, locally endemic, stygobitic species. In a region where groundwater and the calcrete itself is heavily utilised for mining operations, such a proposal has major conservation and environmental management implications, with drawdown of groundwater below the level of the calcrete (~10 m) likely to be a significant threat to unique stygobitic species.

Further support for the ‘subterranean island’ hypothesis comes from a recent phylogeographic study of the amphipod fauna (Cooper *et al.* 2007). Each calcrete population of amphipods was found to contain one or more divergent mitochondrial DNA (mtDNA) lineages (monophyletic groups of mtDNA haplotypes), suggesting long-term isolation of each population. These analyses extended the scope of the ‘subterranean island’ hypothesis to include, in addition to air-breathing dytiscid beetles, entirely water-respiring arthropods and further suggested that the fine structure of alluvial deposits between calcretes is a major barrier to gene flow. In contrast, several taxonomic studies have proposed the existence of widespread species with distributions in more than one calcrete body, e.g. Bathynellacea (Cho 2005; Cho *et al.* 2006a, 2006b) and copepods (Karanovic 2004). It is, therefore, important to further examine the generality of the ‘subterranean island’ hypothesis using a range of taxa with differing life histories, dispersal abilities and environmental (e.g. salinity and desiccation resistance) tolerances. Here we test this hypothesis using phylogeographic analyses of oniscidean isopods.

To date, four stygobitic species of oniscidean isopod, three from the genus *Haloniscus* Chilton and one tentatively

ascribed to *Andricophiloscia* Vandel, have been described from two calcretes in the Yilgarn region (Taiti and Humphreys 2001). Three of the species were collected from a single calcrete. However, additional isopods have now been obtained from a further 23 calcretes and ongoing morphological studies suggest that they belong to *Haloniscus* (S. Taiti and W. F. Humphreys, unpubl. data). To date, *Haloniscus* also includes a widespread epigeal aquatic species, *H. searlei* Chilton, found associated with salt lakes in Tasmania, Victoria, South Australia and Western Australia (Ellis and Williams 1970) and a subterranean species, *H. anophthalmus* Taiti, Ferrara & Iliffe, from anchialine waters in New Caledonia (Taiti *et al.* 1995). In addition to the single Yilgarn species, *Andricophiloscia* includes a further two species, *A. melanesiensis* Vandel and *A. stephensi* Nicholls and Barnes, with the latter epigeal species being a terrestrial form distributed in Western Australia.

Although ongoing morphological studies suggest that calcrete populations of *Haloniscus* and *Andricophiloscia* represent distinct species (S. Taiti and W. F. Humphreys unpubl. data), our approach here is to make no assumptions on species boundaries and to examine the phylogeographic structure of isopod popula-

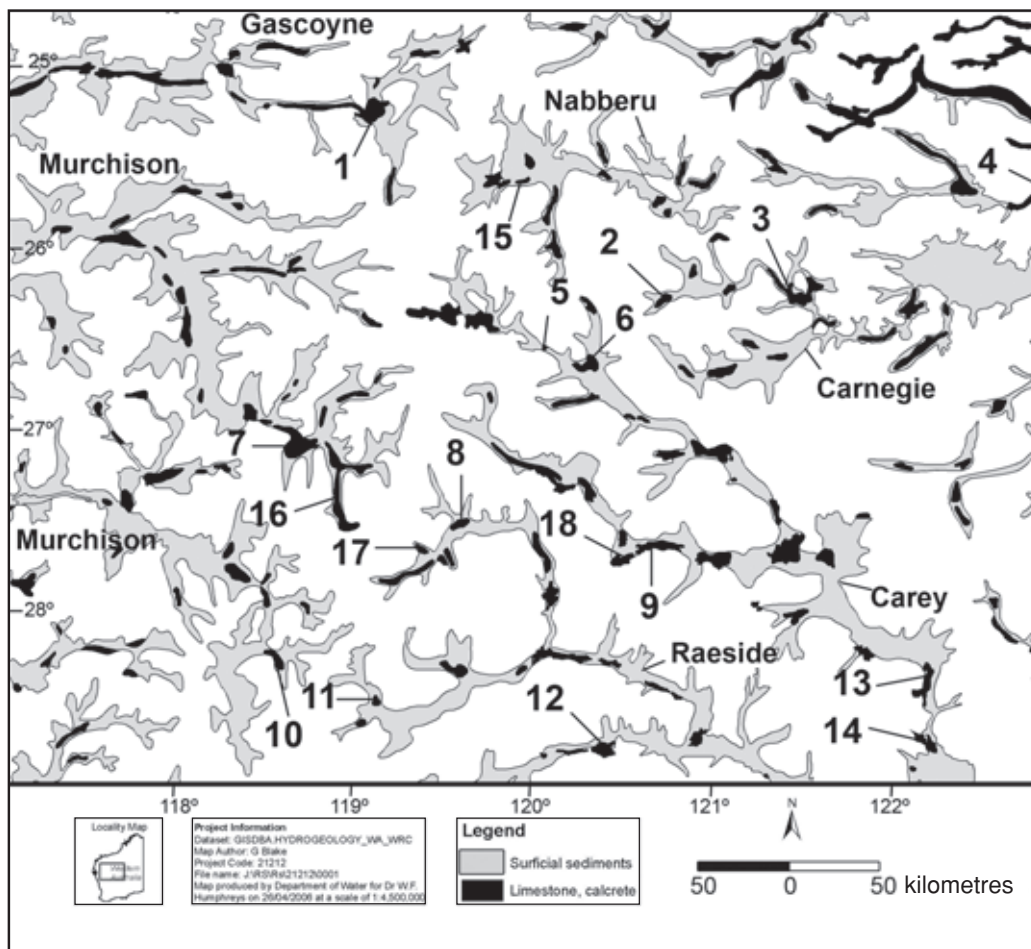


Fig. 1. Map of the northern Yilgarn Region of central Western Australia showing the location of calcrete (black) populations used in the isopod analyses. Further details of each population are given in Table 1. Grey shaded regions represent surficial sediments in the palaeodrainage systems and these are separated by exposures largely of Precambrian geology.

tions across their range in the Yilgarn region using a mtDNA marker, cytochrome *c* oxidase subunit I (COI). The prediction from the 'subterranean island' hypothesis is that calcretes should each contain populations showing long-term isolation, which would be supported by finding monophyletic groups of mtDNA haplotypes associated with a specific calcrete body (Avisé 1994).

A further aim of the study is to explore the factors that have led to the evolution of stygobitic species in the Yilgarn region. In addition to the isolation of calcrete bodies themselves, the location of calcretes along ancient river valleys, or palaeodrainages, may have influenced the phylogeographic structure of isopod populations. Different palaeodrainages are separated by divides largely comprising Precambrian rock and a similar regional divide separates drainages flowing west to the Indian Ocean and those flowing east towards central Australia and the Nullarbor Plain (Beard 1998; Fig. 1). One prediction is that isopods from adjacent calcretes of the same palaeodrainage channel are likely to be more closely related than isopods from different palaeodrainage channels or across the drainage divide, due to either historical or recent groundwater or surface water connections. Alternatively, surface ancestral species may have had considerable dispersal potential, leading to limited phylogeographic structuring of populations across the Yilgarn region before the colonisation of calcretes.

Materials and methods

Sampling methods

Isopods were selected from ethanol preserved samples collected between 1998 and 2006 from calcrete aquifers in 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 isopods 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. Isopod specimens from 18 calcretes were available for DNA sequence analyses. Many of these samples are also being used in ongoing morphological studies of the isopods (S. Taiti and W. F. Humphreys, unpubl. data). An additional nine specimens, representing a range of isopod families and genera, were also included in initial phylogenetic analyses as potential outgroups, including Tylidae: *Tylos neozelanicus* Chilton; Detonidae: *Deto marina* (Chilton), *Armadilloniscus ellipticus* (Harger); Philosciidae: *Laevophiloscia yalgooensis* Wahrberg, *Haloniscus anophthalmus* Taiti, Ferrara & Iliffe (New Caledonia); Porcellionidae: *Porcellionides pruinosus* (Brandt); and Flabellifera: *Pygolabis humphreysi* Wilson (Table 1).

mtDNA sequencing

DNA was extracted from dissected legs using a Puregene Extraction Kit (Gentra, Qiagen, www1.qiagen.com) according to the manufacturer's instructions. We PCR amplified a 708-bp region of the COI gene using the primers LCO1490 (5'-GGT-CAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994). Several additional samples that failed to PCR amplify for the 708-bp fragment were PCR amplified for short (~190 bp) fragments using the *Haloniscus*-specific primers M876 (5'-CARTTYCCAAAMCCNCCAATTA-3') and LCO1490, or M877 (5'-AATTTTATTACAACRTRATHAATATRCG-3') and HCO2198. PCR amplifications were carried out in 25-µL volumes with ~100 ng genomic DNA, 4 mM MgCl₂, 0.20 mM deoxyribonucleotide triphosphates (dNTPs), 1× PCR buffer (Applied Biosystems, www.appliedbiosystems.com), 6 pmol of each primer (Geneworks, www.geneworks.com.au) and 0.5 units of AmpliTaq Gold (Applied Biosystems). PCR amplification was performed under the following conditions: 94°C for 9 min, then 34 cycles of 94°C for 45 s; annealing 48°C for 45 s; 72°C for 60 s; with a final elongation step at 72°C for 6 min. PCR product was purified using Ultraclean PCR cleanup columns (MoBio Laboratories, www.mobio.com) and sequenced in both directions using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing was carried out on an ABI 3700 DNA analyser and raw sequences were edited and aligned using SeqEd ver. 1.0.3 (Applied Biosystems). Sequences have been submitted to GenBank (see Table 1 for accession numbers).

Phylogenetic analyses

Phylogenetic analyses of the COI sequence data were conducted using maximum parsimony (MP) in PAUP* ver. 4.0b10 (Swofford 2002) and a Bayesian inference (BI) approach using MRBAYES ver. 3.1.1 (Huelsenbeck and 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 branch swapping), with the exception of using random stepwise addition repeated 100 times. Character state optimisation for MP trees used the DELTRAN option. MP bootstrap analyses (Felsenstein 1985) were carried out using 500 bootstrap pseudoreplicates, employing a heuristic search option as above with random input of taxa and 'max trees' set to 100. Initial MP analysis of the entire dataset resulted in >100 000 equally parsimonious trees, most likely due to the presence of closely related haplotypes within calcrete populations. The MP analysis was, therefore, repeated with a reduced dataset of samples for each calcrete population, comprising two exemplars for each divergent mtDNA lineage, giving a total of 60 sequences in the input matrix. Bootstrap analyses of this reduced dataset were carried out as described above, except that 1000 pseudoreplicates were used.

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 and Crandall 1998), was found to be the most appropriate model to use in the BI analyses,

Table 1. Locations of calcrete populations and isopod samples, sample numbers (BES numbers held with the Western Australian Museum), GenBank accession numbers, patristic distances using a maximum likelihood model GTR+I+G (PML), and nucleotide diversity (π) indices within calcrete populations

Calcrete population ^A	<i>n</i>	BES numbers or codes	GenBank accession	PML ^B (%) ^C	π (%) ^C
Gascoyne drainage (west)					
1. Three Rivers Plutonic (S25.28313 E119.17573)	2	8624.1,2	–	0.6	–
Carnegie drainage (east)					
2. Jundee (S26.26876 E120.68094)	1	6601	EU364563	–	–
3. Lorna Glen (Site 1: S26.29826 E121.40341)	4	12880.1,2;12892.1, 2	EU364564–5	0–3.9	2.0
(Site 2: S26.25863 E121.40429)	3	12888, 12888.1,2	EU364566–8		
4. Jimmy's Well (S25.66058 E122.87469)	2	H.nsp.12, 12.2	EU364569	0	0
Nabberu drainage (east)					
15. Cunyu: Sweetwaters (S25.59375 E120.37241)	3	12860, 12860.1, 2	EU364570	0	0
Carey drainage (east)					
5. Millbillillie (Bubble Well)(site 1: S26.56072 E120.0409)	8	5637, 5637.1,2; 8101, 8101.1,2,3, 4;	EU364571–2	0–31.1	6.8
(site 2: S26.56362 E120.04265)	6	12910.1,2; 14282.0,1,2,3	EU364573–7		
6. Uramurdah Lake (site 1: S26.68763 E120.30268)	6	6455, 6456.1S; 12831.1, 2,3,4S;	EU364578–81	0–45.5	12.9
(site 2: S26.68762 E120.35283)	4	10527, 10527.2; 14289, 14289.1;	EU364582–4		
(site 3: S26.6876 E120.3078)	1	14299	EU364585		
9. Lake Miranda East (site 1: S27.66407 E120.61167)	1	10459	EU364586	0.2	0.2
(site 2: S27.6634 E120.6124)	1	14336	EU364587		
13. Laverton Downs(Northern site: S28.39652 E122.19766)	14	10290; 10291.1,2; 12011,16,20,21.1;	EU364588–95	0–36.5	11.3
(Southern site: S28.48423 E122.13336)	8	13157, 13167, 13173.1,2; 13180.1;			
		13186.2; LA16.1; 12005	EU364596–		
		12087; 12102.1,2; 13141; 13149.1,2;	WP64		
14. Mount Morgan (site 1: S28.73177 E122.1569)	2	10582.1,2	EU364601	0–6.5	3.7
(site 2: S28.73159 E122.15884)	2	11811, 11811.1	EU364602–3		
18. Lake Miranda West (S27.71085 E120.54332)	1	10532	EU364604		
Raeside drainage (east)					
8. Lake Mason (Salt Well)(site 1: S27.53999 E119.62427)	2	10410, 10411S	EU364605	0–23.0	7.2
(site 2: S27.586 E119.5218)	4	14360.0,1,2,3	EU364606–8		
11. Yuinmery South (S28.54862 E119.09113)	1	6655	EU364609	–	–
12. Perrinvale (S28.77504 E120.417)	2	10257, 10257.2	EU364610	0	0
17. Lake Mason South (S27.71371 E119.39969)	1	13232	EU364611	–	–
Murchison drainage (west)					
7. Yarrabubba Nowthanna (site 1: S27.06683 E118.67994)	1	13093	EU364612	1.0	1.0
(site 2: S27.06684 E118.67844)	1	13101	EU364613		
10. Windimurra (site 1: S28.2861 E118.5743)		8956, 8956.2, 13133, 13133.1,2	EU364614–5	0–0.5	0.1
16. Cogla Downs (S27.40129 E118.89793)	1	10445S	–	–	–
Epigeal taxa					
<i>Haloniscus searlei</i> (site 1: S32.00 E115.50)	1	6573	EU364616	0–5.9	2.9
(site 2: S32.228 E117.358)	1	9886.1	EU364617		
(site 3: S33.86667 E137.61667)	1	9887.1	EU364618		
(site 4: S33.40 E122.80)	1	9891.1	EU364619		
(site 5: S33.61667 E122.36667)	1	9892.1			
(site 6: S38.20 E143.10)	1	9912	EU364620		
(site 7: S38.16667 E124.40)	1	9913			
(site 8: S38.20 E142.86667)	1	9914.2	EU364621		
<i>Haloniscus</i> sp.: lake in Martinjini Nature Reserve (S30.302 E116.454)	1	9888.1	EU364622	–	–
<i>Haloniscus</i> sp.: Lake Goolry (S29.98333 E117.00)	1	9890	EU364623	–	–
Outgroup taxa					
<i>Tylos neozelanicus</i> (S36.95 E174.467)	2	11448, 11448.1	EU364624	–	–
<i>Deto marina</i> (S32.0167 E115.50)	1	9923	EU364625	–	–
<i>Haloniscus anophthalmus</i>	1	10201	EU364626	–	–
<i>Porcellionides pruinosus</i> (S31.9833 E115.5333)	1	9924, 9895	EU364627	–	–
<i>Pygobolis humphreysi</i> (S23.32944 E119.8433)	1	11441	EU364628	–	–
<i>Laevophiloscia yalgoensis</i> (S28.6013 E115.31531)	1	7416.1	EU364629	–	–
<i>Armadilloniscus ellipticus</i> (S31.95 E115.8667)	1	–	EU364630	–	–

^ANumbers represent distinct calcrete populations shown in Fig. 1. ^BPML was calculated using the GTR+I+G model with parameter settings as given in the methods. ^CIntra-calcrete diversity levels were calculated by pooling data from all sites in the calcrete.

based on the Akaike Information Criterion (AIC). 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 10 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 1.6% and the potential scale reduction factor (PSRF) was approximately one for all parameters, with the exception of the proportion of invariant sites and rate(A–T) parameters. This result suggested that convergence may not have been completed for the tree topology and some parameter estimates, which is likely to have resulted from some of the distantly related genera used in the analyses. A second analysis was therefore conducted using the oniscidean isopod species *T. neozelandicus*, *D. marina* and *H. anophthalmus* (New Caledonia) as outgroups, using an identical approach as described above, with the exception that five million generations were used for each of the two independent runs. In this BI analysis, the final standard deviation of split frequencies had reduced to 0.45% and the PSRF was 1.0 for all parameters, suggesting convergence had been reached. The likelihood values converged to relatively stationary values after ~5000 generations. A burnin of 1000 trees (equivalent to 100 000 generations) was chosen for each independent run of MRBAYES, with a >50% posterior probability consensus tree constructed from the remaining 98 002 trees (49 001 trees each run).

ML patristic distances (Lefébure *et al.* 2006b) between sequences were estimated using the GTR+I+G model of evolution and branch lengths and parameters were estimated for the MRBAYES consensus tree using PAUP*, with the optimality criterion set to maximum likelihood (ML). Estimates for these parameters were as follows: A–C = 0.39988, A–G = 9.64494, A–T = 1.93732, C–G = 0.71913, C–T = 10.97396, G–T = 1.0, I = 0.49929, G = 0.93092. The program ARLEQUIN ver. 3.01 (Excoffier *et al.* 2005) was used to estimate nucleotide diversity levels within calcrete populations and the epigean species *H. searlei*. An analysis of molecular variation (AMOVA) also was performed using ARLEQUIN to determine how genetic variation was partitioned, using an hierarchical structure: among palaeodrainages; among calcretes within palaeodrainages; and within calcretes (Excoffier *et al.* 1992). Sixteen separate calcrete populations of isopods from five palaeodrainages, excluding ‘Three Rivers Plutonic’ (Gascoyne palaeodrainage) and ‘Cogla Downs’ (Murchison), which each consisted of partial sequences only (Table 1), were defined *a priori* and included in the AMOVA. An analysis of this dataset using MODELTEST selected the transversion model (TVM) (Posada and Crandall 1998) with a proportion of invariant sites ($I = 0.5187$) and unequal rates among sites modelled with a gamma distribution ($G = 0.7429$) (Yang 1996) under the AIC. This model was used to derive a matrix with pairwise distances among haplotypes, which was imported into ARLEQUIN for the AMOVA. The significance of the estimates was tested using 1023 random permutations of the data.

Results

A 649-bp fragment of the COI gene was sequenced from 101 isopod samples, comprising nine outgroup samples, 10 epigean

Haloniscus samples and 82 subterranean isopod samples from 16 distinct calcretes (Table 1, Fig. 1). A further seven samples (including samples from two additional calcrete populations) failed to PCR amplify for the 649-bp COI fragment, but were successfully PCR amplified and sequenced for a 150-bp COI fragment (Table 1, Fig. 1). The 18 calcretes were distributed along six palaeodrainage channels with four (containing 14 calcretes) draining inland and two (containing four calcretes) draining to the Indian Ocean. All COI sequences showed an open reading frame and absence of insertions or deletions, suggesting they were derived from a functional COI gene. Furthermore, there was no evidence for multiple sequences being present in the dataset, suggestive of paralogous copies of COI (e.g. a nuclear pseudogene) being PCR amplified.

The COI sequence data showed high levels of nucleotide sequence divergence (ML patristic distance >10.7%) among calcrete populations, but generally low levels of sequence divergence (<6.9%) within calcretes, with several notable exceptions: Uramurdah Lake, Millbillillie (Bubble Well), Laverton and Lake Mason (Salt Well), where divergence levels were very high (>31%; Table 1). The COI partial sequences, which were excluded from further analyses, showed low levels of divergence from other samples from the same calcrete populations. However, COI partial sequences from the Three Rivers Plutonic (BES8624.1 and 2) and Cogla Downs (BES10445) calcretes showed high levels of divergence (>28.7%) from haplotypes from other calcrete populations, indicating that they represent divergent mtDNA lineages. Nucleotide diversity estimates within calcrete populations ranged between 0% and 12.9%, with high diversity indices found in the Uramurdah Lake, Millbillillie (Bubble Well), Laverton and Lake Mason calcretes (Table 1). Excluding these four populations, nucleotide diversity levels were generally low (<3.7%). Nucleotide diversity within the widespread epigean species *H. searlei* was 2.9%. AMOVA indicated that only 10.4% of the genetic variation was distributed among palaeodrainages, 63.7% of the total genetic variation was distributed among calcrete populations and 25.9% of the variation was distributed within calcretes, with permutation tests indicating that these values were significant ($P < 0.01$). After exclusion of two calcrete populations (‘Bubble Well’ and ‘Uramurdah Lake’) that contained multiple species based on morphological analyses (see below), a total of 11.8% of variation was distributed among palaeodrainages, 67.1% of the genetic variation was distributed among calcrete populations and 21.1% of the variation was distributed within calcrete populations.

Initial MP and BI analyses of the COI data, including nine samples of seven different potential outgroup isopod genera, supported the monophyly of a group containing the epigean species *H. searlei* and all the other calcrete populations to the exclusion of the outgroup genera (tree not shown). Given the divergent nature of many of these outgroups and the lack of convergence of the BI analyses (see methods), the phylogenetic analyses were repeated using the oniscidean isopods *T. neozelandicus*, *D. marina* and *H. anophthalmus* as outgroups. The ingroup contained the calcrete populations of isopods and the epigean lineages *H. searlei*, plus two other epigean samples (9890 and 9888.1) shown in the initial analysis to group with the calcrete populations. This second BI analysis showed strong convergence of tree topology and parameters after 10 million

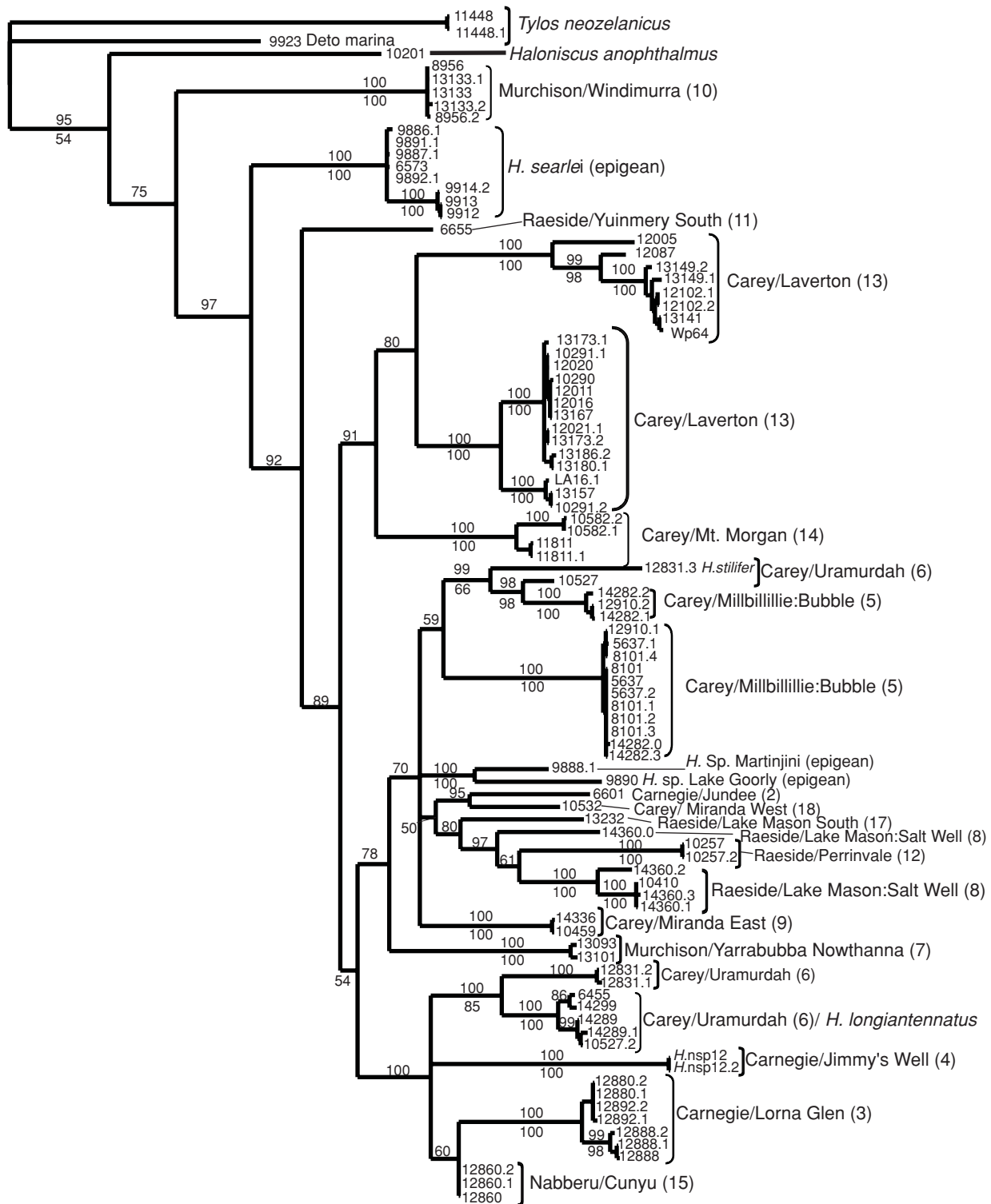


Fig. 2. Bayesian consensus tree (50% majority rule) generated from 98 002 trees sampled in two independent runs of MRBAYES (ver. 3.1.1). Sample codes for each specimen are given in Table 1, and palaeodrainage, followed by calcrete population name, is given for each group. Numbers in parentheses are calcrete population numbers shown in Fig. 1 and Table 1. Numbers adjacent to branches refer to Bayesian posterior probabilities (left or single numbers) and maximum parsimony bootstrap values obtained from 500 pseudoreplicates generated in PAUP* (right), with only numbers >50% shown.

generations and the Bayesian consensus tree, rooted using *T. neozelanicus* as the outgroup, is shown in Fig. 2. Both the MP and BI trees provided strong evidence for the existence of 24 divergent (ML patristic distances >10%) mtDNA clades, with haplotypes within each clade grouping with 100% bootstrap and 100% posterior probability support (Fig. 2). Each clade is restricted in its distribution to a single calcrete aquifer. MP analysis of a reduced dataset of samples for each calcrete population produced eight MP trees of length 1977 steps, each with a very similar topology to that presented in Fig. 2 (tree not shown). One difference in the topology was that *H. anophthalmus* from New Caledonia formed a sister group relationship with the calcrete population from Windimurra, supported by 59% of bootstrap pseudoreplicates.

Several calcretes (Uramurdah Lake, Millbillillie (Bubble Well), Lake Mason (Salt Well) and Laverton Downs) also were found to contain more than one divergent (ML patristic distances >10%) mtDNA lineage. The Uramurdah Lake calcrete, in particular, contained four divergent (>18%) mtDNA lineages. Two of these lineages correspond to the previously described species *H. longiantennatus* and *H. stilifer* (Tait and Humphreys 2001), as DNA sequences were derived from the same samples used in their taxonomic description for the former and from samples morphologically identical to the male paratype of *H. stilifer*. The Laverton calcrete contained at least four divergent (>10%) sister clades, two localised in the southern region of the calcrete and two localised in northern sites, ~13 km away. The Millbillillie (Bubble Well) calcrete contained two clades, one grouping with a population from Uramurdah to the exclusion of the second clade.

Discussion

The 'subterranean island' hypothesis

Phylogenetic analyses and AMOVA of the isopod fauna indicate that there is significant phylogeographic structuring of populations, with evidence for at least 24 divergent (>10%) mitochondrial lineages, each restricted in their distribution to a single calcrete aquifer. Calcrete populations generally consisted of related mtDNA haplotypes with low nucleotide diversities, with some exceptions where multiple divergent mtDNA lineages were found within calcretes. No calcrete population was found to share haplotypes or closely related haplotypes with any other calcrete population, which would be expected if there was ongoing gene flow among populations. This phylogeographic pattern is indicative of long-term isolation of calcrete populations (Avisé 1994). Similar findings of unique species (Watts and Humphreys 1999, 2000; 2001, 2003, 2004, 2006) or divergent mtDNA lineages (Cooper *et al.* 2002; Leys *et al.* 2003) restricted to a single calcrete were previously found for dytiscid diving beetles and, more recently, amphipods (Cooper *et al.* 2007). Similarly, Guzik *et al.* (2008) carried out a mtDNA phylogeography of the parabathynellids (Bathynellacea) and showed that divergent mtDNA lineages were each restricted to a single calcrete body. Taken overall, molecular analyses of the macro-invertebrate stygofauna provide strong support for the hypothesis that calcrete bodies are equivalent to closed 'subterranean islands'.

Previous estimates of divergence times for beetle and amphipod calcrete populations suggest that calcrete bodies have been

isolated for at least four million years (my) (Leys *et al.* 2003; Cooper *et al.* 2007), coinciding with a major period of aridity on the Australian continent (Bowler 1976; Stein and Robert 1986). Using a rate of evolution for COI of 0.0125 substitutions per site per million years, proposed for subterranean isopods by Ketmaier *et al.* (2003), a level of divergence of 10% for neighbouring calcrete populations Uramurdah Lake and Millbillillie (Bubble) equates roughly to 4 my. This divergence level represents a minimum between calcrete populations, with other nearby calcrete populations showing significantly higher levels of divergence (ML patristic distances >28% or ~11 my). Similarly, the divergences between epigeal and subterranean lineages (e.g. epigeal 9888.1 and Miranda east calcrete individuals, ML patristic distance 28.5%) were high. It is, therefore, possible that colonisation of the calcretes and isolation of populations occurred in a much earlier time period, perhaps during the Mid-Late Miocene. However, it is also possible, though unlikely given the slight variation seen in the widespread *H. searlei*, that divergence among lineages occurred before the colonisation of calcretes. Following warm-humid and wet conditions in the Early Miocene, regular flows in the palaeodrainage systems of western Australia are proposed to have stopped by the Mid Miocene (van der Graaff *et al.* 1977; Martin 2006). This irregular flow regime may have led to periodic isolation of populations in ground or surface water along the palaeodrainages and between different palaeodrainage systems. Further work is required to develop a more comprehensive picture of the phylogenetic relationships among calcrete populations of isopods and more rigorously estimate divergence times using relaxed molecular clock models of evolution.

The evolutionary origins of stygobitic isopods

There are at least two hypotheses that might explain the evolution of subterranean lineages of isopods in the Yilgarn region. One hypothesis is that each calcrete was separately colonised by one or more surface species, with each population independently evolving stygobitic characteristics (e.g. loss of eyes and pigment, attenuated bodies and/or appendages, Holsinger 1994; Culver *et al.* 1995) by a process of convergent/parallel evolution. This hypothesis was previously supported by analyses of the water beetle fauna in the Yilgarn region (Leys *et al.* 2003). A second hypothesis is that evolution of stygobitic characteristics occurred just once (or a few times at most) and the current distribution of stygobitic populations resulted from dispersal by stygobitic ancestors, followed by vicariance events that isolated populations within calcretes. Calcretes are thought to be dynamic entities, with carbonates being continuously deposited from or taken into solution, depending on the climatic conditions (Arakel 1986). Under this scenario, calcretes may have been previously fused and subsequently fragmented, or may have expanded leading to fusion of adjacent calcretes, thereby allowing dispersal. Support for this latter hypothesis of dispersal and vicariance comes from a phylogeographic study of a widespread subterranean amphipod species, *Niphargus virei* Chevreux, in a limestone karst system in France (Lefébure *et al.* 2006a). The two hypotheses are not necessarily mutually exclusive: the former hypothesis would be supported by the presence of epigeal lineages that are closely related to stygobitic lineages; and the latter hypothesis would lead to the prediction

that, owing to the limitations of dispersal through groundwater, phylogeographic structure might correspond to the distribution of calcretes along palaeodrainage channels, with nearby calcretes more likely to contain related populations. An absence of the latter pattern would lend support to the former hypothesis.

The finding of several epigeal lineages (*H. searlei* and two populations of unknown species from a lake in Martinjini Nature Reserve (9888.1) and Lake Goorly (9890)) that grouped with the stygobitic populations, lends some support to the former hypothesis. The *H. searlei* lineage was basal to a monophyletic group containing the majority of stygobitic isopod populations, with the exception of a population in the Windimurra calcrete. A second epigeal clade, containing the Martinjini and Lake Goorly lineages, grouped with several calcrete populations from the Carey, Carnegie and Raeside palaeodrainages. On the assumption that epigeal lineages were unlikely to have evolved from stygobitic ancestors, the phylogenetic analyses suggest there have been multiple (>3) independent colonisations of calcretes by surface ancestral species. In addition, it was apparent from the phylogenetic analyses that several calcrete populations from different palaeodrainage systems grouped closely together (e.g. Jundee and Miranda West of the Carnegie and Carey palaeodrainages respectively; Uramurdah, Jimmy's Well/Lorna Glen and Cunyu (Sweetwater) from the Carey, Carnegie and Nabberu palaeodrainages respectively, see Fig. 2), suggesting that their common ancestor was unlikely to be stygobitic and limited to dispersal through groundwater. However, it was also evident from the phylogenetic analyses that some nearby calcrete populations of the same palaeodrainage channel were sister lineages. For example, a monophyletic group of calcretes from the Raeside palaeodrainage was strongly supported and several neighbouring calcretes from Laverton and Mt Morgan of the Carey palaeodrainage and Millbillillie (Bubble) and Uramurdah of the Carey palaeodrainage also were found to group closely together (Fig. 2). Therefore, although the hypothesis of independent colonisation from surface ancestors seems most likely to explain the evolution of stygobitic isopod species, we cannot entirely rule out the hypothesis that some calcrete populations of isopods resulted from dispersal of stygobitic ancestral species through groundwater followed by vicariance events that isolated calcrete populations.

Species boundaries and sympatric species

With just one exception, calcrete populations showed patristic distances >25.3% and additional divergent lineages (>16%) existed within a further four calcretes (three within Laverton, four within Uramurdah Lake, two within Millbillillie (Bubble) and two within Lake Mason (Salt Well); see Table 1). Such levels of sequence divergence are suggestive of species-level differences among crustacean taxa (e.g. see Lefebvre *et al.* 2006b). Ongoing morphological studies also support the hypothesis that each of these lineages is likely to represent a distinct species (S. Taiti and W. F. Humphreys, unpubl. data). In two calcretes, Uramurdah Lake and Laverton, there also was evidence for sympatric sister lineages. A similar finding was found for the dytiscid diving beetles where 13 cases of sympatric sister species have been reported (Cooper *et al.* 2002; Leys *et al.* 2003; R. Leys, unpubl. data). In each case, sympatric beetle

species were found to be in distinct size classes, suggesting the possibility of niche partitioning and providing a potential case of sympatric speciation (R. Leys, unpubl. data). However, it is not yet clear whether there are any distinct phenotypic characteristics of sympatric sister lineages of isopods to help support the case for sympatric speciation. Further work also is required to assess whether these intra-calcrete sister lineages may have evolved within calcretes by micro-allopatric processes or by multiple colonisation of calcretes from the same ancestral source during different time periods.

Conclusion

Phylogeographic analyses of a variety of macro-invertebrate stygofauna provide strong evidence that the Yilgarn calcretes are equivalent to a 'subterranean archipelago', with each calcrete island showing millions of years of isolation and containing unique stygobitic species. The diversification of the isopod fauna bears some striking resemblances to the dytiscid beetles, with evidence for multiple independent colonisation events from surface ancestors and the existence of several sympatric sister lineages. These characteristics suggest the isopod fauna offers great potential for further analyses of modes of speciation and regressive evolution of subterranean animals.

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