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DNA BARCODING

DNA barcoding of stygofauna uncovers cryptic amphipod diversity in a calcrete aquifer in Western Australia's arid zone

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Abstract

The arid Yilgarn region of Western Australia contains numerous subterranean calcrete aquifers with unique assemblages of obligate groundwater invertebrates (stygofauna). We aimed to establish a DNA barcoding framework for the macro-invertebrates present in a single calcrete, as a basis for future assessment of biodiversity of the Yilgarn calcretes and for investigating food webs. Intense sampling of a bore field grid in the Sturt Meadows calcrete was undertaken to obtain representatives of the entire macro-invertebrate ecosystem. A 623-bp fragment of the mitochondrial cytochrome *c* oxidase 1 (*COI*) gene was used to provide DNA barcodes for stygobiont macro-invertebrates plus terrestrial organisms that are found in the calcrete. Phylogenetic analyses revealed the existence of 12 divergent monophyletic groups of haplotypes. Subterranean amphipods (Chiltoniidae) showed three groups of *COI* haplotypes with sequence divergences between them of >11%. Allozyme analyses found a large number of fixed allelic differences between these three amphipod groups, indicating that there are three morphologically cryptic species within the Sturt Meadows calcrete. Unlike the sister triplet of dytiscid beetles present, the amphipods are not sister clades and are more closely related to other Yilgarn and non-Yilgarn amphipods than to each other. Our results show that the aquifer contains at least 12 macro-invertebrate species and DNA barcoding provides a useful means for discriminating species in this system.

Keywords: allozymes, amphipods, *COI* mitochondrial DNA, cryptic species, DNA barcoding, stygofauna

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Introduction

Calcrete aquifers (calcretes) of the Yilgarn region of Western Australia provide habitat for a diverse fauna of obligate groundwater invertebrates, or stygofauna (Humphreys 2008). Over the last decade, since the discovery of this fauna, there has been a push to formally describe new stybogiont species, however, in some of the groups, such as the amphipods, progress has been impeded by their cryptic morphology. Previous biodiversity surveys have been conducted at a broad scale, sometimes sampling with only a single well or bore hole in a calcrete providing access to the groundwater, making it difficult to assess the total number of macro-invertebrate species present. Here, we use a molecular approach for estimating biodiversity in a single calcrete aquifer on the Sturt Meadows pastoral property, where an extensive bore field has enabled thorough sampling of the subterranean ecosystem.

Traditionally, the taxonomy of arthropods has been based on comparison of their morphology (Quicke 1993). However, it is now well established that morphologically cryptic species can and do occur in most organismal groups and biomes (Bickford *et al.* 2006; Pfenninger & Schwenk 2007). This phenomenon is likely to be even

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more common with stygofauna, where adaptation to the subterranean environment may lead to major modifications in morphology (Culver *et al.* 1995), while convergent evolution can mask species differences or lead to problems in the classification of species. Therefore, a combination of morphological examination along with molecular analyses is often required to identify and discover cryptic stygobitic species (Paquin & Hedin 2004; Bickford *et al.* 2006; Lefébure *et al.* 2006b; Trontelj *et al.* 2009).

DNA-based identification systems for species are especially useful where taxonomic expertise based on morphological traits is not readily available, with the 5′end of the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene proposed as a 'barcode' for all animal species (Hebert *et al.* 2003). *COI* barcodes have been successfully used to carry out biodiversity inventories of species of Lepidoptera from Costa Rica, with over 97% of the 521 species examined being distinguished by the barcode (Hajibabaei *et al.* 2006). However, focusing on a single gene risks overlooking new or rapidly diverging species, and analyses from several genomic regions in multiple individuals are sometimes required to distinguish between closely related species and to confidently define species boundaries (Mallet & Willmott 2003; Lefébure *et al.* 2006a). One approach, which is particularly useful for investigating species boundaries when there are cryptic species present in sympatry, is allozyme electrophoresis, which provides a rapid means of screening multiple nuclear markers (Richardson *et al.* 1986).

The Yilgarn calcretes are carbonate deposits, approximately 10-m thick, honeycombed with small holes and filled with groundwater, which were originally deposited upstream of salt lakes by evaporation of groundwater within palaeodrainage channels (Humphreys 2001). Each calcrete studied to date contains a combination of unique aquatic invertebrate species including predaceous dytiscid diving beetles (Coleoptera), large and small crustaceans (Syncarida, Isopoda, Amphipoda, Copepoda, Ostracoda), and worms (Oligochaeta) (Watts & Humphreys 2004). Phylogeographical studies of the diving beetles (Cooper *et al.* 2002; Leys *et al.* 2003), amphipods (Cooper *et al.* 2007), bathynellids (Guzik *et al.* 2008) and oniscidean isopods (Cooper *et al.* 2008) have found these taxa to be restricted in their distribution to single calcretes. This remarkable endemism points to the isolation of the individual calcretes in the groundwater system, with Cooper *et al.* (2002) describing them as 'islands under the desert'.

The calcretes contain the most diverse known assemblage of subterranean diving beetles in the world, with usually between one and four species per calcrete, each differing considerably in size (Balke *et al.* 2004; Watts & Humphreys 2004). Phylogenetic analyses using mitochondrial DNA (mtDNA) have identified 13 calcretes containing sympatric sister species of large and small diving beetles, and several plausible hypotheses have been proposed to explain their modes of speciation (Cooper *et al.* 2002; Leys *et al.* 2003). One hypothesis is that the different-sized diving beetles have arisen as a result of sympatric speciation driven by divergence into different ecological niches. Such divergences may have occurred by competition for resources if the various size diving beetle species within a calcrete are utilizing different trophic niches. To investigate this possibility, we have focused on predator/prey studies for a single calcrete at the Sturt Meadows pastoral property in Western Australia. This calcrete contains an extensive grid of bore holes allowing intensive sampling of the calcrete and a comprehensive assessment of the ecosystem present. Ecological surveys of the Sturt Meadows calcrete have identified a simple ecosystem containing seven stygobiont macro-invertebrate species, with a sister triplet of dytiscid beetle species as the likely top predators (Allford *et al.* 2008; Leys & Watts 2008). These beetles do not overlap in size and we hypothesize that each species feeds on different prey items.

In this study, universal arthropod primers (Folmer *et al.* 1994) were used to amplify *COI* mtDNA for all the taxa present and a phylogenetic approach was taken to identify taxa. Allozyme analyses were also used to investigate species boundaries in the Amphipoda. The resultant DNA barcodes will be ultimately used as a tool to provide species-specific markers for gut content analysis and as a basis for future biodiversity surveys of the Yilgarn calcretes.

Materials and methods

Taxonomic sampling

The Sturt Meadows calcrete in Western Australia (28°41′S 120°58′E) was accessed through a grid of bores drilled for mineral exploration (Fig. 1). The field contains 115 bore holes of between 5 and 11 m in depth in a 1.4×2.5 km (3.5 km^2) grid. Intensive sampling, by both hauling with a small weighted plankton net (250 μm) and pumping, was carried out over 16 months, leading to the collection of 6254 stygobitic macro-invertebrate specimens from 262 sampling events (Allford *et al.* 2008). Identification of macro-invertebrates was carried at least to order level, with family level identifications possible for copepods and amphipods and species-level for the beetles. Stygobiont invertebrates in the Sturt Meadows calcrete were identified by their taxonomic affinity and stygomorphic features, such as eyelessness and lack of colour. Terrestrial species that had fallen into the aquifer were also collected. Several troglobiont (terrestrial subterranean) specimens were excluded from the molecular analyses and kept for morphological taxonomic description due to their rarity. In total, 46 specimens belonging to eight groups (both stygobiont and terrestrial) were

Fig. 1 Map of Australia with the Sturt Meadows pastoral property in Western Australia marked. The expanded diagram shows the sample site at Sturt Meadows with the bore hole grid north and south of the road outlined in a black square. The shape of the calcrete is illustrated in black.

included in initial sequencing analyses. Subsequently, a further 87 amphipod specimens were sequenced for *COI* and a selection of these subjected to allozyme analysis (see below).

Vouchering specimens

All specimens were lodged with the Australian Biological Tissue Collection (ABTC) at the South Australian Museum and given an ABTC number. Specimen details including ABTC voucher number, identification, sampling location and specimen type are in the Supporting Information. Automontage digital images were taken of representative specimens and have been lodged with the South Australian Museum.

MtDNA extraction, amplification and sequencing

Individual specimens were preserved in 100% ethanol. DNA was isolated from one leg for each specimen >2 mm, or using the whole body for animals <2 mm long (i.e. copepods). DNA was extracted using the GENTRA method (Puregene) according to the manufacturer's protocol for fresh tissues. Polymerase chain reaction (PCR) amplification of a 623-bp fragment from the mitochondrial *COI* gene was carried out with the 'universal' primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al.* 1994) in 25 μL volumes containing 4 mM $MgCl_2$, 0.20 mm dNTPs, 1× PCR buffer (Applied Biosystems), 6 pmol of each primer and 0.5 U of AmpliTaq Gold (Applied Biosystems). PCR amplification was performed under the following conditions: 94 °C for 9 min; followed by 34 cycles of 94 °C for 45 s, annealing at 48 °C for 45 s, and 72 °C for 60 s; with a final elongation step at 72 °C for 6 min. PCR products were purified with a PCR Clean-up DNA purification kit (MoBio Laboratories Inc.) and sequencing was performed using the ABI prism Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems). Sequencing was carried out on an ABI 3700 DNA analyser and sequences were edited and manually aligned in SEQED version 1.0.3 (Applied Biosystems).

MtDNA analyses

The *COI* data were aligned by eye and translation of the DNA sequences to protein was carried out in MEGA v. 4 (Kumar *et al.* 2008) using the invertebrate mitochondrial genetic code, to check for the presence of nuclear paralogues. Sequences and traces were submitted to GenBank (GenBank Accession numbers FJ785739–FJ785818).

Neighbour-joining (NJ) trees were constructed for the initial sequencing analysis with the computer program PAUP* version 4.0b10 (Swofford 2002), using HKY85 distances (Hasegawa *et al.* 1985), aiming to cluster similar sequences into taxonomic groups rather than investigate their phylogenetic relationships (Hebert *et al.* 2003). A midpoint root was applied and the NJ tree was bootstrapped with 500 pseudoreplicates carried out using the same model of evolution used above. Sequences were checked for any close matches by an NCBI BLAST search of GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and taxa with close sequence similarity were included in the phylogenetic analyses.

A further 87 amphipods from Sturt Meadows were sequenced for *COI* and identical haplotypes were removed from the data set. Chiltoniid amphipod sequences (GenBank Accession numbers EF118196–EF188256; Cooper *et al.* 2007) originating from other Yilgarn calcretes at various distances from Sturt Meadows (Lake Mason, 178 km; Barwidgee, 171 km; Depot Springs North and South, 115 and 106 km; Mt Padbury, 432 km) were included in the expanded amphipod data

set. Epigean chiltoniid amphipods from The Fountain and Coward Springs of the Great Artesian Basin (GenBank Accession numbers EU886946 and EU886924; Murphy *et al.* 2008) 1600 km away in South Australia were also included in the analyses. Phylogenetic analyses with *Hyallella azteca* (GenBank Accession number DQ464727) as an outgroup, showed the sample from Coward Springs to be a sister lineage to amphipods from the Yilgarn and The Fountain, and the Coward Springs sequence was subsequently used as the outgroup in our analyses.

Maximum parsimony (MP) analyses for the amphipod haplotypes were conducted using PAUP*. MP bootstrap analyses (Felsenstein 1985) were carried out using 500 bootstrap pseudoreplicates, employing a heuristic search option with random input of taxa. The data set was then partitioned by codon, and ModelTest with AIC (Posada & Crandall 1998) was used to determine the best model of sequence evolution. Bayesian inference was carried out in MrBayes (Ronquist & Huelsenbeck 2003) for 1 000 000 generations, after a burn in of 100 000 generations. Parameter convergence for both runs was checked in Tracer v. 2.1 (Rambaut & Drummond 2005), by ensuring that effective sample sizes were >100 for all parameters.

Allozyme electrophoresis on Sturt Meadows amphipods

After removal of a leg for DNA sequencing, amphipods were snap frozen in liquid nitrogen and subsequently stored at −80 °C. Allozyme electrophoresis was performed on cellulose acetate gels (Cellogel©; MALTA) and stained for enzyme activity according to the principles and procedures of Richardson *et al.* (1986). To ensure that homogenates exhibited adequate enzyme activity after electrophoresis, only specimens >3 mm were selected (49 total). Twenty-six enzymes displayed banding patterns of sufficient activity and resolution to permit allozymic interpretation namely ACP, ACYC, ADH, ALD, ARGK, ENOL, EST, FDP, FUM, G6PD, GAPD, GOT, GPI, GPT, IDH, LDH, MDH, ME, MPI, NDPK, PEP-A, PGAM, PGM, PK, SORDH and TPI. Details of enzyme and locus abbreviations, enzyme commission numbers, electrophoretic conditions and stain recipes are presented in Richardson *et al.* (1986) or Wallman & Adams (2001). Allozymes were designated alphabetically and multiple loci, where present, were designated numerically, both in order of increasing electrophoretic mobility (e.g. Acp^a, Acp^b; *Got1*, *Got2*).

The genetic affinities of individuals from the allozyme study were explored using principal coordinates analysis (PCO), as implemented on a pairwise matrix of Rogers' (1972) genetic distance using PATN (pattern analysis package, DOS version; Belbin 1994). Allele frequencies and pairwise genetic distances (both percent fixed differences and unbiased Nei *D*s) for the three mtDNA clades were calculated as detailed in Murphy *et al.* (2008).

Results

Stygobiont invertebrate groups identified in the Sturt Meadows calcrete comprised three dytiscid beetle species, *Paroster macrosturtensis*, *Paroster mesosturtensis* and *Paroster microsturtensis*, chiltoniid amphipods between 2 and 5 mm in length, cyclopoid and harpactacoid copepods and several aquatic oligochaete worms (Fig. 2). Terrestrial invertebrates included mites (Acari), a centipede (Chilopoda), springtails (Collembola) and insect larvae. These were in the minority and most of the animals caught were stygofauna.

The primers of Folmer *et al.* (1994) successfully amplified the DNA of all the animals tested, with the exception of a few terrestrial specimens (unknown insect larvae and an oniscidean isopod). All sequences had an open reading frame with no evidence for stop codons or insertions/deletions, and hence it was assumed that the sequences are unlikely to be nuclear paralogues. Phylogenetic distance analyses revealed the existence of 12 distinct clusters of haplotypes (Fig. 3). The dytiscid beetle species form three distinct clades, each with 100% bootstrap support. Species-level identification of dytiscid larvae was made possible by including the *COI* sequence in the distance analysis, i.e. by barcoding (data not shown). Cyclopoid and harpactacoid copepods could be distinguished by their *COI* sequence and formed two well-supported clades. Collembola, oligochaete worms, the centipede and mites also clustered into their respective groups. Collembola, worms and the centipede had close BLAST matches on GenBank and these GenBank sequences were included in the phylogenetic analyses to assess further the robustness of our order-level taxonomy (Fig. 3). Pairwise genetic distances between Sturt Meadows fauna and their GenBank matches were 20% for collembola, 16–21% for worms and 18% for the centipede. Oligochaete worms showed considerable within-clade genetic heterogeneity, with four distinct lineages present among the six haplotypes examined and pairwise genetic distances ranging between 14% and 26%.

Sturt Meadows amphipods formed three distinct clades in the NJ analysis (Fig. 3). The expanded amphipod data set was partitioned by codon using models of sequence evolution for the first codon position (TrN+I+G), second codon position (K81uf+I) and third codon position (HKY+G). MP/Bayesian analysis including chiltoniid amphipods from other Yilgarn aquifers showed that the Sturt Meadows amphipods are not sister to each other (Fig. 4). Clade 1 is more closely related to stygobiont amphipods from Lake Mason (W.A.) and

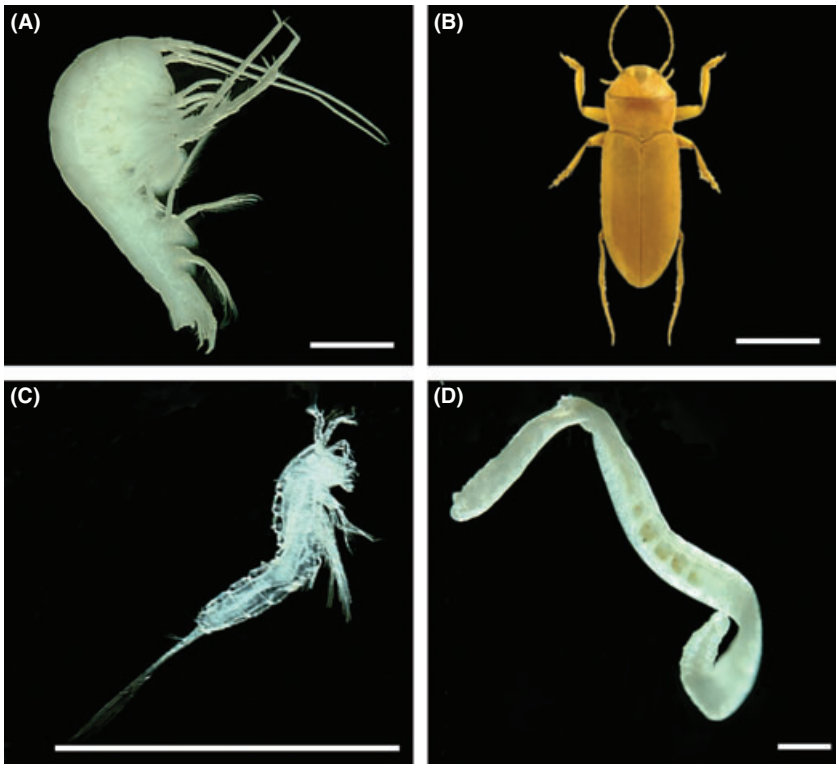

Fig. 2 Stygobiont macro-invertebrates present in the Sturt Meadows calcrete. (A) Amphipoda: Chiltonidae, (B) Coleoptera: Dytiscidae *Paroster macrosturtensis* (photograph by Chris Watts), (C) Copepoda: Harpactacoidea and (D) Oligochaeta, Scale bar = 1 mm.

epigean mound spring amphipods at The Fountain (South Australia) than to the other Sturt Meadows clades. Clade 2 forms a distinctive lineage without any close phylogenetic affinities, while clade 3 is more closely related to amphipods from the Yilgarn calcretes at Mount Padbury and Depot Springs than to clades 1 and 2. Each Sturt Meadows amphipod clade is well supported, with posterior probabilities of 1.00% and 100% bootstrap support. Pairwise distances (HKY85) between the clades range between 11% and 13%, with within-clade divergences below 3%.

Allozyme genotypes were able to be assigned at 31 putative allozyme loci for the 49 amphipods examined. PCO of these 49 individuals (Fig. 5) revealed three very distinctive genetic clusters, corresponding to the three clades identified independently by DNA barcoding. The allele frequencies for the three amphipod mitochondrial clades are presented in Table 1.

Based on the available data and notwithstanding the small sample size for clade 3, per cent fixed differences (% FD; allowing a 10% tolerance for shared alleles) ranged from 59% FD between clades 1 and 2 to 73% FD between clades 2 and 3, while Nei *D*-values ranged between 1.08 (clades 1 and 2) and 1.50 (clades 2 and 3). Of the 27 allozyme loci scored in all clades, seven (*Enol*, *Fdp*, *Gapd*, *Pgam*, *Pgm*, *Pk* and *Tpi*) were unequivocally diagnostic as to clade identity. Interestingly, although within-clade levels of genetic variability (as measured by direct count heterozygosity estimates, H_O) were similar for clades 1 ($H_O = 0.232 \pm 0.044$) and 3 ($H_O = 0.186 \pm 0.054$), clade 2 displayed much reduced levels of variability ($H_O = 0.013 \pm 0.009$).

Discussion

Biodiversity assessment of the Sturt Meadows calcrete

DNA barcoding has provided a useful approach for examining invertebrate diversity in the Sturt Meadows calcrete aquifer, with species-level diversity detected for a number of the groups that was not evident from other broad-scale studies of the Yilgarn calcretes. Three species of dytiscid beetle, at least three species of chiltoniid amphipods (separate at multiple allozyme loci) and single harpactacoid and cyclopoid copepod species were found. Four divergent lineages of oligochaete worms, a single mite, centipede and springtail (Collembola) species all clustered into distinct groups by phylogenetic analyses of a 623-bp *COI* fragment and each undoubtedly represents putative species. Species diversity appears to be richer than previously anticipated when examining the morphology of the macro-invertebrates present.

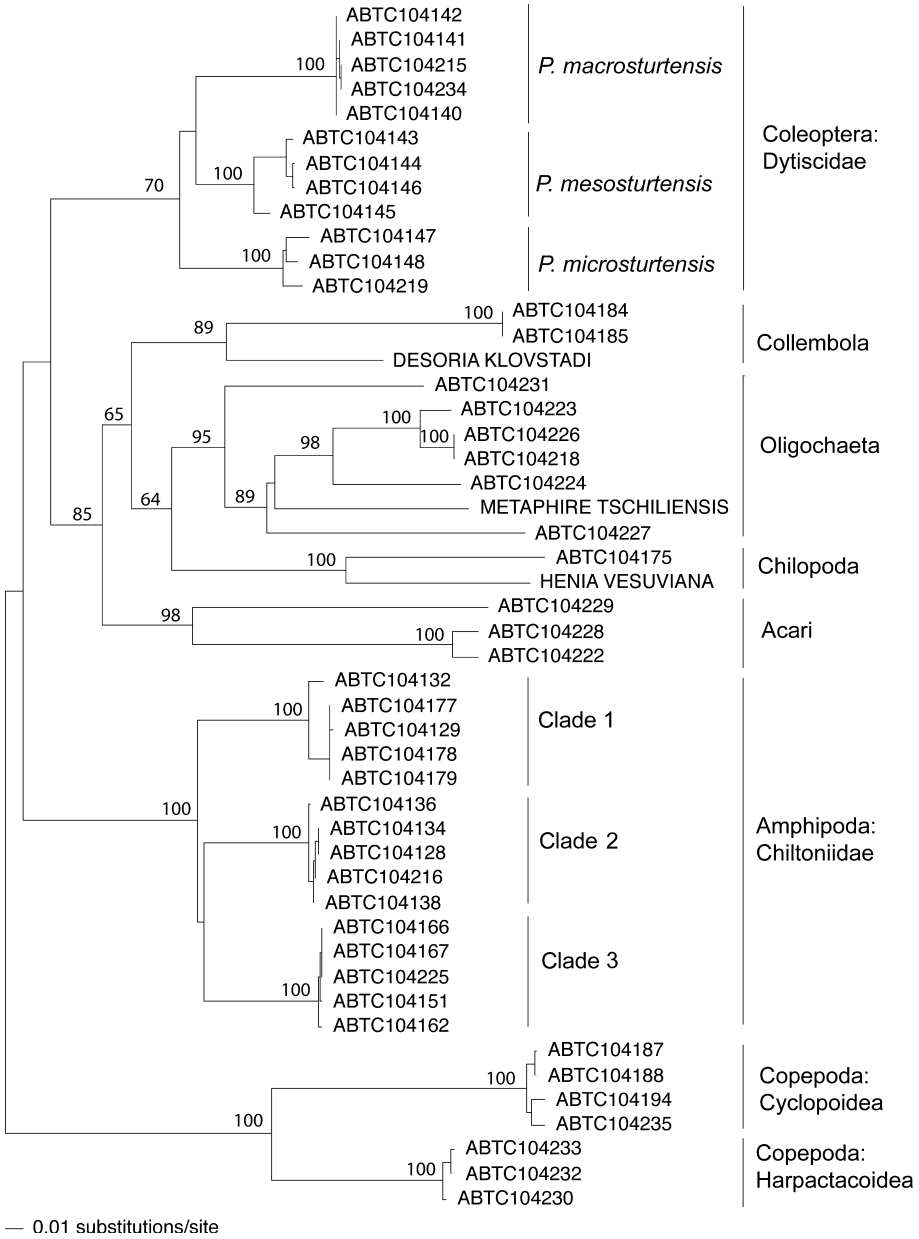

Fig. 3 Neighbour-joining tree for Sturt Meadows macro-invertebrates based on HKY85 distances. GenBank sequences were included from *Desoria klovstadi*, *Metaphire tschiliensis* and *Henia vesuviana* (GenBank Accession numbers DQ365786, DQ835676 and AY288754 respectively). Bootstrap values >50% are shown above the line.

Other than the beetles, all of the stygobiont invertebrate taxa present in the Sturt Meadows calcrete are likely to be new species. These potential beetle prey items can now be distinguished by a molecular barcode that enables us to develop molecular methods for studying natural predation events and test hypotheses on modes of speciation in the diving beetles.

Morphological analyses of the several troglobiontic specimens collected have revealed another macro-invertebrate group within the calcrete, i.e. the discovery of the first indigenous palpigrade from Australia (Barranco & Harvey 2008). Palpigrades are a well-known rainforest group and the recently described species, *Eukoenenia guzikae*, is highly likely to be a short range endemic, a pattern common within poorly dispursive members of Australian invertebrate fauna (Harvey 2002; Barranco & Harvey 2008). An additional species of troglobiontic isopod from the family Armadillidae has also recently been identified from the Sturt Meadows calcrete (S. Taiti, personal communication). Fifteen million years ago, the current

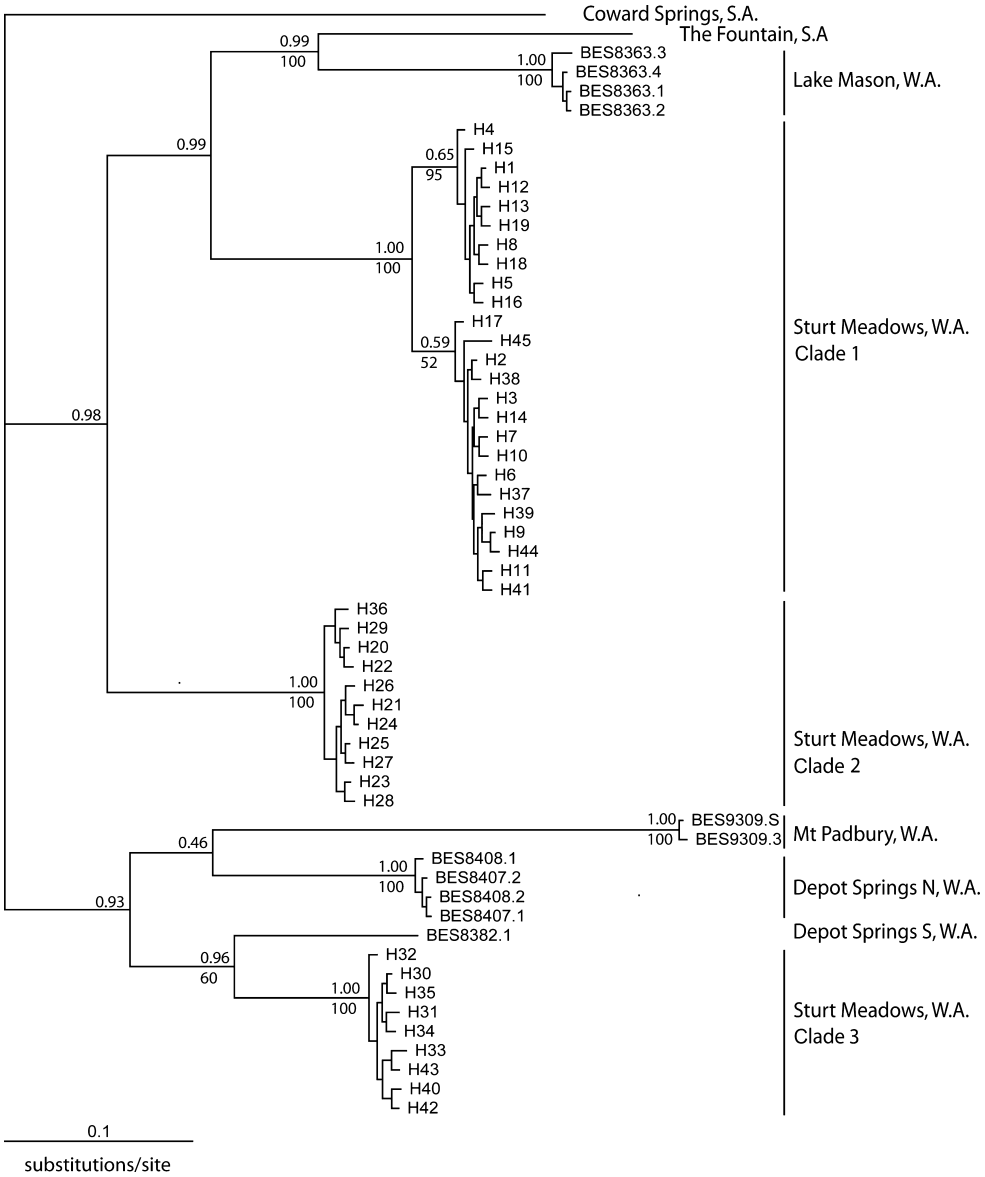

Fig. 4 Bayesian phylogenetic tree for Chiltoniid amphipod *COI* haplotypes (models and approach as given in the Materials and methods). Posterior probabilities are shown above the line and bootstrap values from the maximum parsimony analysis are shown below.

Australian arid zone had a warm and wet environment, with extensive rainforest, and the onset of aridity ~10 Ma resulted in the evolution of an arid-adapted biota (Byrne *et al*. 2008). It is hypothesized that invertebrates living above the water table in the calcretes represent a relictual rainforest fauna, driven underground during the drying out of the surface landscape, where they have then evolved troglomorphic features as an adaptation to the subterranean environment.

Cryptic diversity in the Sturt Meadows stygofauna

Amphipods, which were originally thought to comprise a single species from the family Chiltoniidae based on their morphology, form three divergent mitochondrial lineages and each can be separated at multiple allozyme loci indicative of their reproductive isolation. Each lineage has different phylogenetic affinities, being more closely related to epigean mound spring taxa from South Australia and other Yilgarn amphipods hundreds of kilometres away than they are to each other. Together, these molecular, phylogenetic and geographical data demonstrate that the Sturt Meadows calcrete contains three distinct evolutionary (and biological) species of chitoniid amphipod, and indicate the need for follow-up morphological taxonomic studies to formally describe them.

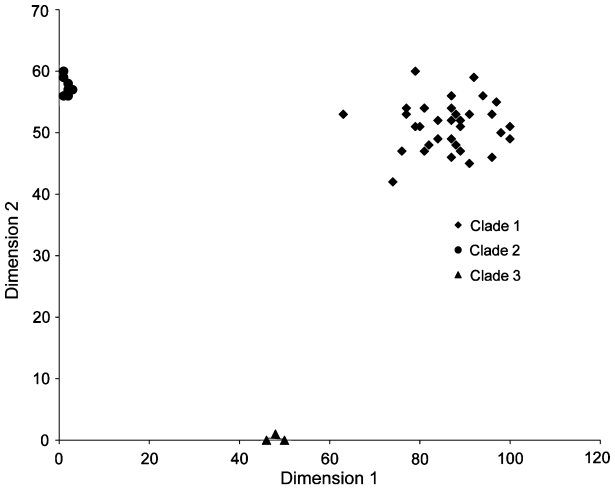

Fig. 5 Principle coordinates analysis for amphipod mitochondrial clades 1, 2 and 3. The relative PCO scores have been plotted for the first (*X*-axis) and second (*Y*-axis) dimensions, which individually explained 35% and 14%, respectively, of the total multivariate variation present. The number of points is less than the total number of individuals because some individuals share the same PCO scores in the first two dimensions.

Table 1 Allele frequencies at 31 putative allozyme loci for the three amphipod mitochondrial clades

Locus	Clade 1	Clade 2	Clade 3
Acp	c^{56},b^{40},a^{4} (36)	c (9)	d (4)
Acyc	e^{60},d^{21},c^{13},a^{6} (35)	e (9)	d^{87},b^{13} (4)
Ada	b^{45},d^{37},a^{18} (11)	c^{93},b^{7} (7)	—
Ald	a (4)	b (5)	b (2)
Argk	b (12)	b (7)	a (4)
Enol	c^{76},a^{24} (36)	d (9)	b (4)
Est	a (11)	a (7)	a (4)
Fdp	a (10)	c (6)	b (1)
Fum	a^{93},b^{5},c^{2} (21)	d (8)	b (3)
G6pd	e^{69},c^{29},a^{1},f^{1} (35)	c (9)	d^{83},b^{17} (3)
Gapd	b (12)	c (7)	a (4)
Got1	e^{83},f^{8},g^{7},c^{1},a^{1} (36)	g (9)	d^{50},e^{37},b^{13} (4)
Got2	d^{71},e^{25},c^{3},b^{1} (36)	d^{89},a^{11} (9)	d (4)
Gpi	f^{21},e^{18},d^{18},g^{11},h^{11},i^{8}, j^{8},b^{3},a^{1},k^{1} (36)	e (9)	a^{50},c^{38},f^{12} (4)
Gpt	b^{94},c^{6} (36)	a (9)	a (4)
Idh1	c^{86},b^{7},a^{4},e^{3} (28)	d (9)	b (3)
Idh2	f^{60},d^{27},e^{10},g^{3} (30)	h (9)	f^{50},b^{17},a^{17},c^{16} (3)
Ldh	a (4)	—	a (4)
Mdh1	a (4)	—	b^{50},c^{50} (3)
Mdh2	a (4)	—	b (2)
Me	a (7)	a (3)	b (4)
Mpi	d^{45},f^{26},c^{10},a^{6},e^{4},b^{4}, h^{3},g^{2} (34)	d (9)	c^{67},d^{33} (3)
Ndpk1	a^{95},b^{5} (11)	a (7)	a^{88},c^{12} (4)
Ndpk2	a (8)	b (3)	a (4)
PepA1	c^{97},b^{3} (31)	d (9)	a^{63},c^{25},d^{12} (4)
PepA2	c^{41},d^{32},b^{21},e^{4},a^{1},g^{1} (34)	e (9)	f (4)
Pgam	a^{50},c^{50} (4)	d (7)	b (3)
Pgm	b^{65},a^{26},c^{9} (31)	e (5)	d (2)
Pk	b^{83},a^{17} (9)	c (6)	d (3)
Sordh	c^{95},b^{5} (10)	c (6)	b^{83},a^{17} (3)
Tpi	c^{81},d^{19} (36)	e (9)	a^{88},b^{12} (4)
H_O ± SE	0.232 ± 0.044	0.013 ± 0.009	0.186 ± 0.054

H_O, observed heterozygosity estimates; —locus was not scorable in this taxon.

For polymorphic loci, the frequency of each allele is expressed as a percentage and shown as a superscript. Sample sizes for each locus are indicated in brackets; differences between different loci for the same taxon reflect the poor activity displayed by smaller individuals at some but not all loci.

The Sturt Meadows calcrete is the only subterranean aquifer identified thus far that contains more than one divergent lineage of chiltoniid amphipod (Cooper *et al.* 2007). This result is likely to reflect the extensive, fine-scale sampling that has been possible at Sturt Meadows rather than the lack of diversity in other aquifers. With respect to their distribution within the calcrete, each species has been found in sympatry in a number of bore holes. All three amphipod species cover the extent of the borehole grid, although species 2 and 3 are very patchy in their distribution. Interestingly, amphipod species 2 has much reduced levels of heterozygosity at the suite of allozyme loci examined, suggesting a demographical change (e.g. a recent bottleneck or expansion event) may have occurred in this species that is not evident in the other two species.

Finston *et al.* (2007) also found that subterranean amphipods from the Pilbara Region of northwest Western Australia contain highly divergent lineages in two morphologically cryptic genera. Sequence divergence levels between haplotypes were >22% in *Chydaekata* and >6% in *Pilbarus* (Finston *et al.* 2007). Studies on American desert spring amphipods have found provisional cryptic species, identified by a species screening threshold of 10 times the average intra-population *COI* divergence, with divergence levels >4% found between provisional species (Witt *et al.* 2006). As the three Sturt Meadows amphipods showed intra-population divergences of ~3%, this would represent a threshold value of 30% according to the criteria of Witt *et al.* (2006). With only 11–13% divergence between amphipod species found at Sturt Meadows, this result highlights the difficulty in imposing a *COI* threshold for species-level identification in amphipods using pairwise genetic distances alone. We propose therefore that the presence of divergent monophyletic lineages is best used as an indicator of the presence of cryptic species requiring further scientific investigation.

There was evidence for further cryptic species within the oligochaete worms, as they showed even greater levels of within-clade mitochondrial genetic divergence than the amphipods. Four haplotypes identified have more than 14% pairwise distance between them, strongly

suggesting that there are potentially four species of worms present in this aquifer. As only six individuals were sequenced, considerably more sampling is required to gain a reliable estimate of the number of species present.

Conservation of stygofauna

The barcoding approach offers considerable potential in this calcrete system for identifying distinct genetic lineages and assessing the level of species diversity in a diverse group of morphologically conservative macroinvertebrates. The Sturt Meadows calcrete is missing some of the other stygobiont taxa such as bathynellids, oniscidean isopods, candonid ostracods and hydrobiid gastropods found elsewhere in the calcrete aquifers of arid Australia. However, the *COI* fragment used here has been successfully amplified in both bathynellids and isopods (not yet attempted for ostracods and gastropods) and so could be employed more broadly for biodiversity assessment. Given the importance of being able to recognize short-range endemic species in regions where groundwater exploitation threatens habitat integrity, DNA barcoding is likely to prove essential for the rigorous assessment of desert aquatic invertebrate diversity (Finston & Johnson 2004; Witt *et al.* 2006; Finston *et al.* 2007; Humphreys 2008). As major project approvals in Western Australia require assessment of the subterranean fauna, there is an urgent need for rapid biodiversity assessment methods to determine the environmental impact of proposed mine developments, as well as assess the impact of mining operations over time.

Extreme environmental conditions within the underground ecosystem, such as low levels of dissolved oxygen and lack of light, can lead to convergence of morphology in subterranean animals. The convergence of morphological characters makes it difficult to carry out species-level surveys, especially when there is a scarcity of taxonomic knowledge for most of the groups involved. Only by building up a sequence database for macroinvertebrates present in this system will it be possible to identify the divergent lineages that are likely to signal the existence of cryptic species. The numerous isolated underground aquifers in this region clearly represent a biodiversity hotspot of macro-invertebrates, and it is probable that DNA barcoding will provide the only way in which the biodiversity present can be rapidly assessed.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 List of specimens used in this study with associated voucher information.

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