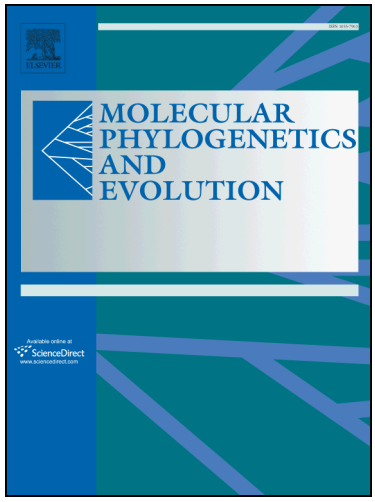

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Molecular systematics and biodiversity of oniscidean isopods in the groundwater calcretes of Central Western Australia

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Abstract

Groundwater calcrete aquifers of central Western Australia have been shown to contain a high diversity of stygobiont (subterranean aquatic) invertebrates, with each species confined to an individual calcrete and the entire system resembling a 'subterranean archipelago' containing hundreds of isolated calcretes. Here, we utilised alternative sampling techniques above the water table and uncovered a significant fauna of subterranean terrestrial oniscidean isopods from the calcretes. We explored the diversity and evolution of this fauna using molecular analyses based on one mitochondrial gene, Cytochrome C Oxidase Subunit I (*COI*), two Ribosomal RNA genes (*28S* and *18S*), and one protein coding nuclear gene, Lysyl-tRNA Synthetase (*LysRS*). The results from 12 calcretes showed the existence of 36 divergent DNA lineages belonging to four oniscidean families (Paraplatyarthridae, Armadillidae, Stenoniscidae and Philosciidae). Using a combination of phylogenetic and species delimitation methods, we hypothesized the occurrence of at least 27 putative new species of subterranean oniscideans, of which 24 taxa appeared to be restricted to an individual calcrete, lending further support to the "subterranean island hypothesis". Three paraplatyarthrid species were present on adjacent calcretes and these exceptions possessed more ommatidia and body pigments compared with the calcrete-restricted taxa, and are likely to represent troglophiles.

The occurrence of stenoniscid isopods in the calcretes of central Western Australia, a group previously only known from the marine littoral zone, suggests a link to the marine inundation of the Eucla basin during the Late Eocene. The current oniscidean subterranean fauna consists of groups known to be subtropical, littoral and benthic, reflecting different historical events that have shaped the evolution of the fauna in the calcretes.

Keywords:

Groundwater calcretes, molecular systematics, oniscidean isopods, species delimitation, subterranean fauna.

1. Introduction

Subterranean animals were once thought to only occur in humid and dark subsurface habitats of karst systems where limestone, gypsum and dolomite are the abundant minerals associated with caves and meso-caverns. Subsequently these faunas have been found also as significant inhabitants in non-karstic areas such as lava tubes and fractured basalts, in Hawaii and the Canary Islands (Howarth, 1983; Oromi and Martin, 1992). Subterranean fauna in Australia were previously known from classic Tertiary carbonate karsts, such as those at Cape Range and the Nullarbor, Western Australia, and the Undara lava tubes in Queensland. However, recent extensive exploration of subterranean groundwater-associated systems in the arid zone of Australia have revealed diverse hypogean invertebrate communities in non-karstic pisolite and fractured rock terrains in the Pilbara region, Western Australia, and the Ngalia Basin, Northern Territory (Taiti and Humphreys, 2001; Balke et al., 2004; Cho et al., 2006a; Watts and Humphreys, 2006) and in groundwater calcretes (hereafter 'calcretes') of the Yilgarn, Western Australia (Humphreys, 2006, 2008; Cooper et al., 2007, 2008; Guzik et al., 2008, 2009; Eberhard et al., 2008, 2009; Karanovic and Cooper, 2012). As a result, there has been a corresponding recent focus on research towards exploring and identifying this fauna and formally describing the new stygobiont (subterranean aquatic) species (Humphreys et al., 2009; Karanovic and Cooper, 2012; King et al., 2012).

Humphreys et al., (2009) documented a diverse faunal assemblage of subterranean invertebrates (8 classes, 13 orders and 34 families) occurring across Western Australian calcretes. Within the arid Yilgarn region of Western Australia (Fig. 1), numerous stygobiont species of diving beetles (Watts and Humphreys, 1999, 2006, 2009; Leys and Watts, 2008) and a range of crustacean taxa including Bathynellacea, Amphipoda, Isopoda, Copepoda and Ostracoda have been identified (Taiti and Humphreys, 2001, 2008; Karanovic and Marmonier, 2002; Karanovic, 2004; Cho, 2005; Cho et al., 2006a; Cho et al., 2006b; Guzik et al., 2008; Abrams et al., 2012; Karanovic and Cooper, 2012; King et al., 2012). Several molecular studies on components of this diverse fauna have shown that these calcretes are equivalent to 'subterranean islands' with each species, or divergent genetic lineage, restricted to a single calcrete (Cooper et al., 2002, 2007, 2008; Leys et al., 2003; Guzik et al., 2008, 2009, Karanovic and Cooper, 2012; King et al., 2012). Significantly, for environmental

managers, these calcretes are much smaller than the threshold for short range endemic taxa (10 000 km^2; Harvey, 2002) and within the range of IUCN Redlist criteria for listing species as Endangered (extent of occurrence < 5000 km^2) or Critically Endangered (extent of occurrence <100 km^2), if a threatening process is evident (IUCN Redlist). However, there are also exceptions to this pattern of strict endemicity, where adjacent calcretes contain morphologically identical species (e.g. *Limbodessus insolitus* Watts and Humphreys (Dytiscidae) in the adjacent calcretes Uramurdah Lake and Lake Violet; Watts and Humphreys, 2009), but where each calcrete population is typically associated with a divergent mtDNA clade, indicative of long-term isolation of populations.

Although the systematics and evolution of the stygofauna is reasonably well documented, little is known about the subterranean terrestrial fauna that is associated with the Yilgarn calcretes. Such terrestrial animals are commonly found in humid and dark subterranean habitats, such as air-filled caves, and smaller subsurface cavities/voids, which may also occur in the vadose zone of the calcretes. The latter is an unsaturated area between the surface and the top of the phreatic zone. In central Western Australian calcretes (North of 30°) the vadose zone may vary from 2-3 m in depth and is sometimes temporarily submerged as a result of occasional groundwater fluctuations during epiosodic wet periods (Humphreys, 2001; Watts and Humphreys, 2006). Our study was initiated as a result of the incidental collection of terrestrial invertebrates during stygofauna sampling. This fauna included various Crustacea, Insecta and Arachnida (Humphreys, 2008; Bradford et al., 2010), and the first endemic member of the Palpigradi in Australia (Barranco and Harvey, 2008). Following these discoveries, we undertook an intensive survey of the vadose zone of 12 calcretes using alternative sampling techniques designed to target the terrestrial invertebrate fauna.This survey uncovered a diverse assemblage of arachnids, including pseudoscorpions (Harrison et al., 2014), Collembola (Guzik et al., in prep), and an abundant oniscidean isopod fauna that is the focus of the current study.

Oniscidean isopods are the most diverse and successful group of crustaceans adapted to terrestrial life. Oniscideans occur in a wide range of terrestrial environments, ranging from wet tropical habitats to hot deserts, and from sea level to high elevations (Hornung, 2011). Some species are adapted to aquatic habitats and live in groundwater systems, caves and salt lakes (Hornung, 2011). However, until recently little was known about subterranean

oniscidean diversity in Australia. Taiti and Humphreys (2008) reported 28 new troglobiontic and stygobitic oniscidean species from Western Australia including *Styloniscus* (Styloniscidae) and *Adoniscus* (Olbrinidae) from the Pilbara; Stenoniscidae (unknown genus), and stygobitic *Haloniscus* (Philosciidae) from the Yilgarn region; *Troglarmadillo* (Armadillidae) from Cape Range, Pilbara, Nullarbor, and the Yilgarn region; *Hanoniscus* (family placement uncertain) from Cape Range and the Nullarbor; and *Laevophiloscia* (Philosciidae) from Nambung and Augusta cave areas. In addition, a new oniscidean family, Paraplatyarthridae, was recently discovered and described from the Yilgarn calcretes (Javidkar et al., 2015). Notably absent from Australian subterranean habitats is *Buddelundia* (Armadillidae), an arid adapted genus (Warburg, 1965).

The aim of the present study is to elucidate the diversity, phylogenetic relationships and distributional patterns of oniscidean isopod species associated with the calcretes of central Western Australia, using a multiple gene approach including both mitochondrial and nuclear genes, the latter including a new nuclear gene marker for isopods. In particular, we investigate whether the "subterranean island hypothesis" also applies to the subterranean terrestrial isopods found within the calcretes.

2. Material and Methods

2.1. Taxon sampling/Sorting

To collect the oniscidean fauna from calcretes, we used leaf litter traps made from 65 mm internal diameter PVC pipes, between 150-180 mm long and approximately 0.16-0.18 l in volume, and sealed at both ends. The pipes had numerous slots cut into them to allow invertebrates to freely enter the tubes (see Supplementary Figure B. 5). Traps were filled with microwave sterilised leaf litter, to ensure the absence of contaminating live invertebrates. They were then suspended, sometimes in pairs, on fine cord above the water table within unlined mineral exploration boreholes (Supplementary Figures B 1-4; see Table 2 for locality details) that had previously been fitted with a short, 110 mm diameter, PVC sleeve cemented in place to stabilize the bore opening and seal the base of the sleeve. A tight-fitting PVC cap was fitted to maintain humidity and prevent the intrusion of epigean

131 species. In total, 177 traps were deployed at 115 sites across 12 discrete calcretes along the
132 Carey, Raeside and Nabberu palaeodrainage systems (Fig. 1, Table 2). The litter traps were
133 left underground for 3-12 months to be colonised by invertebrates and sampling of the leaf
134 litter was carried out 2-3 times per year (between April and October).The Sturt Meadows
135 (SM) and Laverton Downs (LD) calcretes, each contained extensive arrays of mineral
136 exploration bores and were more intensively sampled with 45 traps (SM; 40 sites) and 30
137 traps (LD; 20 sites), respectively.

138 After recovery of the traps (Supplementary Figure B. 6), their contents were sealed in zip-
139 lock bags for transport to the Western Australian Museum where the living litter fauna was
140 extracted into 100% ethanol using two banks of 12 Tullgren funnels (BS00290; Burkard
141 Scientific, Uxbridge, United Kingdom). In addition, surface (epigean) isopods from five
142 Western Australian localities (Table 2) were collected by hand under/between crevices of
143 rotten/fallen tree branches and preserved in 100% ethanol. All specimens collected from
144 the calcretes were classified into two categories: 1) Group A: characterized by a completely
145 pale body (no visible chromatophores on the epithelium of the dorsal body), and lack of
146 ommatidia (no external eye structures recognisable), indicative of anophthalmy. Included in
147 this group, are also individuals with vestigial remnants of eye components (i.e. a single
148 ommatidium-like remnant of very reduced size, lacking external structure and pale. The
149 latter is most likely associated with the crystalline cone cells (Nilsson, 1978)). 2) Group B:
150 characterized by a partly pigmented body with a very diffuse pattern of chromatophores on
151 dorsal body to more concentrated. In this group, the external structure of eye ommatidia is
152 evident but the size of the ommatidia is reduced. Specimens classified in this group may
153 represent a case of microphthalmy.

154 Isopod samples were identified to family and genus level according to Dalens (1992),
155 Taiti et al., (1998), Taiti and Humphreys (2001), Schmidt (2002, 2003) and Poore (2002).

156

157 2.2. DNA extraction and sequencing

158 Three to six pereopods (except for male pereopod 7 which is important for
159 morphological diagnosis) were dissected from 100% ethanol-preserved animals and rinsed

in 10 mM Tris to remove the alcohol before the extraction process. Total genomic DNA was isolated using a Puregene Genomic DNA Purification Kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions (DNA purification from 5-10 mg fresh or frozen solid tissue), except that centrifugation times were increased to 20 min and 5 min for the DNA precipitation and wash steps respectively. In addition, for the DNA precipitation stage, after adding 100% Isopropanol, the solution was kept at -20°C overnight.

Four genes including the mitochondrial Cytochrome C Oxidase subunit 1 (*COI*), the nuclear Lysyl-tRNA Synthetase (*LysRS*), and two nuclear ribosomal genes: *LSU rRNA* (*28S*; D1-D3 region) and *SSU rRNA* (*18S*; core and variable regions C1, V1, C2, V2, C3) were PCR-amplified and sequenced (see Table 1 for primers). Primers for the *LysRS* gene were newly developed for this study using transcriptome data available from two species of *Paraplatyarthrus* and one *Porcellionides* (unpublished data; Javidkar et al., in prep.). *LysRS* encodes the enzyme Lysyl tRNA Synthetase which catalyses the covalent attachment of Lysine to the 3' end of the cognate tRNA (Lysyl-transfer RNA), which then incorporates Lysine into proteins during translation (Chan and Bingham, 1992; Freist and Gauss, 1995). A 791-643 bp region of *LysRS,* containing no introns (based on alignment of genomic sequences with the transcriptome data), was PCR-amplified and, being found to be phylogenetically informative, was sequenced for all taxa using Sanger sequencing methods.

PCR amplification of all genes involved an initial denaturation at 95°C for 10 min and 34 subsequent cycles of 94°C for 45 s, 48°C to 55°C (variable with respect to the target gene; see Table 1) for 45 s, 72°C for 1 min and a final extension of 72°C for 6 min, followed by a 2-min hold time at 25°C. For the samples which were not successfully amplified, or showed double bands in PCR amplification, different sets of primers were designed and used (Table 1). All PCRs were carried out on either Palm-Cycler thermal cyclers (Corbett, CG1-96) or Kyratec Supercycler thermal cyclers (SC300) using 25 µl reaction volumes consisting of nuclease-free water, 5 µl of 5 x Immolase PCR buffer (comprising 3.75 mM $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphae (dNTP) and 2.5 x BSA (0.25 mg/ml)), 1 µl of each primer (5 µM concentration for *COI* and *18S* primers, 7 µM for G2328 and 8 µM for G2329, 10 µM concentration for G2281, G2282, G2340 and G2341, 7 µM for 28srD1.2a and 5 µM for 28srd4.2b), 0.5 units of Immolase DNA polymerase, and 2-2.5 µl of ~1µg ml^{-1} DNA. Amplified PCR products were visualised on 1.5% agarose gels and purified using a PCR

multiscreen filter plate (Millipore). Purified PCR products were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified using a SEQ multiscreen filter plate (Millipore) and analysed on an ABI 3700 DNA capillary sequencer. Sequences were edited using Geneious Pro version 5.6.4 (http://www.geneious.com).

2.3. Phylogenetic analyses

Alignments were carried out using ClustalW (cost matrix: IUB, Gap open cost: 9, Gap extend cost: 3) allowing free end gaps. To conduct phylogenetic analyses, the data were partitioned into seven subsets including first, second and third codon positions of *COI*, *LysRS*, *28S* and conserved (C1, C2, C3) and variable (V1, V2) regions of the *18S* gene. Mrmodeltest version 2.3 (Posada and Crandall, 1998) was used to estimate the best nucleotide substitution model for each data subset using an Akaike Information Criterion (AIC) framework. A GTR+I+G (Rodríguez et al., 1990; Yang, 1996) was found to be the most appropriate nucleotide model for *COI* codon positions; HKY+I+G (Hasegawa et al., 1985; Yang, 1996) for *LysRS*; GTR+G (Rodríguez et al., 1990; Yang, 1996) for *28S;* SYM+I (Zharkikh, 1994) and K80+G (Kimura, 1980; Yang, 1996) for the core and variable regions of *18S,* respectively. Garli 2.0-win (Zwickl, 2006), which performs phylogenetic searches using the Maximum Likelihood (ML) criterion, was used to examine the best partitioning scheme for the dataset. Thirteen different partitions of *COI* first, second and third base codon positions, *LysRS*, *28S* and core and variable regions of *18S* (C1-C2-C3, V1-V2) were examined to calculate the lnL and AIC index for each partition (Table 3). To run individual partitioned models of ML for each scheme, the Garli configuration file was set for two independent search replicates and all parameters were unlinked. The subset specific rate multiplier was set to vary over data subsets and other settings of the Garli configuration file used default options and a fast ML stepwise-addition starting tree for the initial tree topology. The likelihood scores of the two independent runs were computed and the greater likelihood score was chosen for calculation of AIC scores. The AIC score of each partitioning scheme was calculated as AIC = 2 x (#parameters - lnL) and the lowest value was chosen as the best score.

220 According to Table 3, partition number 13 which treated each subset separately showed
221 the highest ML score (-14320.8661) and lowest AIC value (28747.7322), and, therefore, was
222 selected as the best partition scheme for phylogenetic analyses.
223 Bayesian Inference (BI) analyses of both single and combined datasets were performed
224 using the procedure of Markov Chain Monte Carlo (MCMC) convergence as implemented in
225 MrBayes version 3.2.0 (Huelsenbeck and Ronquist, 2005). All parameters were unlinked and
226 the rates were allowed to vary over the subsets. Two independent runs with four chains
227 were run simultaneously for 5 million generations, subsampling trees and parameters every
228 100 generations. The final standard deviation of split frequencies was less than 0.002
229 (except for single *Lysyl-tRNA* phylogeny which was 0.0038) and Potential Scale Reduction
230 Factor (PSRF) values for all parameters were 1.0, suggesting convergence had occurred. To
231 further assess convergence to the stationary distribution, the program Tracer version 1.5
232 (Rambaut and Drummond, 2003) was used. For each independent MrBayes run, a 25% burn-
233 in, equivalent to 12,500 samples, were discarded from the 50,001 samples sub-sampled
234 during the analysis (i.e. 37,501 samples were included). A 50% majority rule BI consensus
235 tree was constructed from the remaining trees and posterior probabilities were used to
236 assess the robustness of nodes. Five phylogenetic analyses were carried out, including 1)
237 that based on *COI* only (680 bp) to obtain a general picture of the subterranean oniscidean
238 diversity in the calcretes; 2) the nuclear gene, Lysyl-tRNA Synthetase (*LysRS*) only to
239 compare its topology and branching pattern with those of the *COI*; 3) variously combining
240 two genes *COI-Lysyl tRNA* (1434 bp), 4) three genes *COI-Lysyl tRNA-18S* (2114 bp), and 5) all
241 four genes *COI-Lysyl tRNA-28S-18S* (2781 bp) to reconstruct and compare oniscidean
242 relationships.

243 Each of the ML analyses, with the same gene partitioning scheme as used for BI
244 analyses, was carried out using Garli OSX version 2.0 (Zwickl, 2006). The ML analyses used
245 two search replicates; substitution models were unlinked and subset specific rates were
246 allowed to vary across partitions, and the number of bootstraps was set to 500 replicates.
247 Other parameters were set according to Garli configuration file defaults. As Garli does not
248 calculate consensus trees from bootstrap replicates, the Sumtree package under Dendropy
249 3.12.0 (Sukumaran and Holder, 2010), which is a Python (version 2.7.3) library for
250 phylogenetic computing, was used to generate 50% majority rule ML bootstrap consensus

trees. All trees were rooted using *Ligia* sp. (Isopoda; Diplochete), which successfully amplified for all markers. Figtree version 1.3.1 (Rambaut, 2009) was used to visualize phylogenetic trees. The inter lineage *COI* p-distances were calculated using Mega version 5.1 (Tamura et al., *2007*).

2.4. Species delimitation

We used single gene and multiple gene phylogenies and morphological evidence to obtain an estimate of the number of putative species using species delimitation methods. We applied the Poisson Tree Processes (PTP) and bPTP (a Bayesian implementation of PTP) models of species delimitation, which are based on the phylogenetic species concept (Eldredge and Cracraft, 1980; Davis and Nixon, 1992; Baum and Donoghue, 1995), to the *COI* and *LysRS* datasets using the PTP-master package (Zhang et al., 2013). BI consensus phylogenetic trees generated for the same genes using MrBayes version 3.2.0 were used as input. In addition, a species threshold of 12% *COI* p-distance was also utilised as a second criterion for species delimitation. This p-distance was based on a close relationship between two morphologically distinct *Paraplatyarthrus* species (lineages S4 and B17, Fig. 3; Javidkar, 2014), both occurring in calcretes of the same palaeodrainage. This species threshold is comparable to or higher than other thresholds proposed for crustaceans, including those from the Yilgarn region (e.g. Lefébure et al., 2006: 16% patristic; Guzik et al., 2011: 11% Kimura 2-Parameter (K2P); Abrams et al., 2012: 7.1% K2P).

3. Results

Approximately 1500 specimens identified as oniscidean isopods were collected from calcretes of the Yilgarn region between 2008 and 2012 (Supplementary Table A) of which 907 specimens were classified as Group A (troglomorphic) and 592 specimens as Group B (intermediate forms) (see Methods). Four oniscidean families were identified from the calcretes, namely, Armadillidae (*Troglarmadillo, Buddelundia* (surface species), and two unknown genera), Paraplatyarthridae (*Paraplatyarthrus*), Philosciidae (*Haloniscus*) and Stenoniscidae (unknown genus). Paraplatyarthrid isopods were the most frequently

collected family (n=1156) and Stenoniscidae the least collected (n=11 from the Laverton Downs calcrete; Windarra and Erlistoun sites). *Haloniscus* species are aquatic (Taiti and Humphreys, 2001) and their presence in the litter traps may have resulted from the traps occasionally being submerged in the groundwater due to water table fluctuations. We have included these aquatic taxa in the species delimitation and phylogenetic analyses as previous studies have only reported *COI* sequence data (Cooper et al., 2008).

In total, ~330 oniscidean *COI* sequences, most belonging to subterranean lineages, were generated from 12 calcretes and five surface localities (Table 2). We used these results to select samples for sequencing of *18S*, *28S* and *LysRS*, which resulted in the generation of 122 sequences of *18S*, 120 of *28S* and 100 of *LysRS*.

3.1. Single mitochondrial 'COI'and nuclear 'Lysyl-tRNA Synthetase' phylogenetic analyses

BI analysis of the *COI* data showed the presence of 36 divergent (with a minimum inter-lineage p-distance 1.8% between B13 and B14) mtDNA lineages associated with Group A (19) and Group B (14) samples (Table 2, Fig. 2; see Supplementary Figure A for the tree based on the whole *COI* sequence data).

The subterranean lineages of Armadillidae, Philosciidae and Stenoniscidae were each restricted in their distribution to a single calcrete (see Table 2). Three paraplatyarthrid Group B lineages showed the presence of identical or closely related haplotypes that were shared between two or more calcretes (B17 in Halfpenny, Nambi and Laverton Downs-Windarra; B10 in the Uramurdah and Bubble Well calcretes; B12 shared between Halfpenny and Nambi calcretes). All other paraplatyarthrid lineages were restricted to individual calcrete bodies. The *COI* BI phylogeny also showed a strongly supported lineage (Bayesian Posterior Probability (BPP) = 1) grouping a surface species collected from Mt Morgan (S4/MOR) with the subterranean lineages B18 (Barwidgee) and B17. The single nuclear gene BI phylogeny for *LysRS* (Fig. 3) showed a similar topology to that for *COI* for most lineages, but with a few exceptions; polytomies and some weakly supported nodes that were evident in *COI* paraplatyarthrid and armadillid lineages were resolved in the *LysRS* phylogeny.

However, relationships of Clade 4 (B13/15) and Clade 5 (S4/B17) with other clades were not resolved in the *LysRS* phylogeny.

The paraplatyarthrid inter-lineage p-distances ranged from 1.8% to 20.6% (average 16.3%) for *COI* and 0.2% to 8.1% for *LysRS* (Supplementary Tables B. 1, C. 1). The lowest *COI* p-distances corresponded to the B13-B14 (1.8%), B10-B11 (4.9%) and B9-B10 (4.9%) comparisons, while the highest paraplatyarthrid *COI* p-distance was for S1-B9 (20.6%). The lowest and highest p-distances for *LysRS* (average 5.3%) were for B9-B11/B10-B11 (0.2%) and B2-B12 (8.1%), respectively (*LysRS* p-distance for B13-B14 is not available). Among the armadillid lineages, the inter-lineage p-distances for *COI* varied from 17% (D1-D2, D4-D8) to a maximum of 26% between D3-D8 lineages (average 20%; Supplementary Table B. 2; N.B. *LysRS* was not amplified for all armadillid lineages and so the relevant p-distances are not available for comparsion). Philosciid lineages showed a minimum *COI* p-distance of 12% (C1-C2) and a maximum of 18% divergence (C1-C4 and C3-C4) (average 15%; Supplementary Table B. 3); the same lineages also presented a minimum *LysRS* p-distance of 1.1% (C1-C3) and a maximum of 3.7% (C2-C4) (Supplementary Table C. 2; average 2.1%). The p-distances for Stenoniscidae were 9.0% and 1.0% (A1-A2) for *COI* and *LysRS,* respectively.

3.2. Combined Phylogenetic Analyses

The BI and ML phylogenetic analyses of the data combined for *COI-LysRS* (Fig. 4), *COI-LysRS-18S* (Fig. 5) and all four genes *COI-LysRS-28S-18S* (Fig. 6) showed a consistent topology with high posterior probabilities and bootstrap support values for most nodes. As some genes did not amplify for some taxa it was not possible to generate a complete dataset comprising all four genes for the mtDNA lineages identified above, particularly those within the Armadillidae and Philosciidae. Therefore, the combined analyses did not include some lineages, although it was clearly amenable for reconstructing phylogenies that included all paraplatyarthrid lineages.

In the combined phylogenies, monophyly of all taxa within the Paraplatyarthridae (*Paraplatyarthrus*), Armadillidae (*Troglarmadillo*), Philosciidae (*Haloniscus*) and the Stenoniscidae lineages was strongly supported (PP = 1.00; BP = 100) (Figs. 4-6). The paraplatyarthrid lineages revealed five well supported and distinct lineages in both BI and ML analyses, referred to hereafter as Clade 1 to Clade 5 (PP = 1.00; BP ≥ 91). Clade 1

included taxa from Lake Miranda East/West (Group A), Cunyu (Group A), Sturt Meadows (Group B) and Lake Violet (Group B). Both the Lake Miranda East (B4) and West (B5) populations were sister to a group comprising Sturt Meadows (B1), Cunyu (B2) and Lake Violet (B3) calcrete lineages. The lineages B1 (Raeside), B2 (Nabberu) and B3 (Carey), belonging to different palaeodrainages, formed a highly supported group (PP = 1.00; BP = 100), and were more closely related to B4 and B5 from Lake Miranda East/West (Carey), which were sister to all other lineages, with high PP and BP support (Figs 4-6). The surface taxa from Jorgensen Park, Gooseberry Hills, Wooroloo and Moorapulling formed a second lineage (Clade 2) with high support (PP = 1.00; BP = 100), but their relationships with other clades were not resolved in the combined phylogenies. Clade 3 comprised Group B intermediate forms from Laverton Downs-Windarra, Nambi, Halfpenny Well, Uramurdah, Bubble Well, Lake Violet and a single species from the Laverton Downs calcrete (Quandong; Group A). Clade 4 comprised lineages B13-B15, all from the Laverton Downs calcrete (Quandong, Shady Well, Windarra sites; Group A), which formed a monophyletic group with Clades 1, 2 and 3 in all BI analyses (PP = 0.96, 0.91, 0.88 in the two, three and four genes combined analyses, respectively). Clade 5 included the surface species from Mt Morgan and lineage B17 (Group B) distributed in Nambi, Halfpenny and Laverton Downs-Windarra calcretes (PP = 1.00; BP = 98, 97). This clade was sister to all the remaining paraplatyarthrid clades 1-4 (PP = 1.00; BP = 100).

3.3. Species delimitation

The PTP model for species delimitation applied to the *COI* data resulted in an estimated 33 subterranean and five surface species. The model yielded 12 armadillid (11 subterranean and one surface), five philosciid (subterranean), two stenonisciid (subterranean), and 19 paraplatyarthrid (15 subterranean and four surface) species (Table 4). According to this model, the lineages comprising the paraplatyarthrid clade including B9, B10 and B11 (Group B intermediate forms) were estimated to be the same putative species. Similarly, the lineages of the paraplatyarthrid clade comprising B13 and B14 from Laverton Downs calcrete were identified as a single putative species. All armadillid, philosciid, stenoniscid and the rest of the paraplatyarthrid lineages were each estimated to be separate putative species. The bPTP model for the same gene led to an estimated 35 subterranean and five

surface species (Table 4) in which the subterranean paraplatyarthrid lineages, B9, B10 and B11, were each delimited as distinct species (Fig. 2).

The PTP model on the *LysRS* gene evaluated all armadillid, philosciid and stenoniscid lineages as distinct species (C5, D2, D5, D8, D9 and D10 were not amplified for *LysRS*) while the paraplatyarthrid lineages 'B6-B7-B12' (Group B), 'B9-B10-B11', 'B13-B15' (Group A), 'S1-S2-S3' (surface species) and 'B4-B5' (Group A) were considered the same species. The bPTP analysis on the same dataset generated the same results as the PTP model (Table 4, Fig. 3).

Based on the 12% p-distance divergence threshold, 26 subterranean oniscidean DNA lineages out of 41 (subterranean and surface) lineages were considered as putative species: nine paraplatyarthrid (11 including the surface lineages), 11 armadillid, five philosciid and one stenoniscid lineage (Table 4).

4. Discussion

4.1. Species boundaries and distributions - the "subterranean island hypothesis"

This is the first molecular study to explore the diversity and phylogenetic relationships of terrestrial isopods associated with calcretes in Australia. The phylogenetic analyses, based on a combination of four genes, including one mtDNA (*COI*) and three nuclear genes (*18S*, *28S* and *LysRS*) revealed significant genetic diversity within four oniscidean families, namely Armadillidae, Paraplatyarthridae, Philosciidae, and Stenoniscidae, all collected from subterranean sites. Paraplatyarthridae is regarded as a subtropical group (Javidkar et al., 2015), *Troglarmadill*o a genus within Armadillidae that is morphologically similar to specimens from the calcretes, is monotypic from caves in tropical North Queensland (*T. cavernae* Wahrberg 1922; Chillagoe Caves), while Stenoniscidae are usually found in littoral environments. These distributions for related taxa hint that the calcrete isopod fauna is relictual. In contrast to the current arid conditions on the surface, calcretes provide a warm, humid environment (Humphreys et al., 2009) that has possibly enabled the survival of taxa that previously inhabited rainforests, the latter being widespread on the Australian continent during the Mid-Miocene (see Byrne et al., 2008 and references therein). As such,

the isopod taxa within the calcretes would form part of a 'living zoological museum' representing ancestors of that climatic period for this region of Australia.

Except for three paraplatyarthrid lineages comprising Group B intermediate forms (B10 - Uramurdah and Bubble Well; B12– Halfpenny and Nambi, and B17 - Halfpenny, Nambi and Laverton Downs), all other subterranean lineages were restricted in their distribution to an individual calcrete body. This finding, and the associated high (10-26%) genetic divergences among lineages, is indicative of long-term isolation of populations in accordance with the "subterranean island hypothesis" (Cooper et al., 2002). This hypothesis is well supported by numerous taxonomic and phylogenetic analyses of the stygofauna, including dytiscid diving beetles, amphipods, the stygobitic isopod genus *Haloniscus*, and Parabathynellidae (Taiti and Humphreys, 2001; Cooper et al., 2002; Leys et al., 2003; Cooper et al., 2007, 2008; Guzik et al., 2008; Watts and Humphreys 1999, 2000, 2001, 2003, 2004, 2006; Abrams et al., 2012; King et al., 2012), as well as troglobiont pseudoscorpions (Harrison et al., 2014). Such high levels of genetic differentiation within oniscideans associated with isolation are not limited to the calcrete aquifers although they occur over a finer scale, < ca 360 km than that found in other studies. For example, high genetic divergence values were reported for intertidal *Ligia* over distances of about 2100 km, 585 km and 3770 km, for the rocky beaches of Gulf of California-Baja Peninsula (Hurtado et al., 2010), Hawaiian islands (Santamaria et al., 2013) and the Caribbean (Santamaria et al., 2014), respectively.

Prior to the current study, little was known about the subterranean terrestrial fauna associated with the calcretes, although Harrison et al. (2014) revealed the presence of seven pseudoscorpion mtDNA lineages each restricted to individual calcretes in the Yilgarn region. While the latter study was based on a relatively small sample size (n=32) of pseudoscorpions from the Yilgarn calcretes, the current study is based on collections of ~1500 specimens and sequence data from ~330 specimens, providing a more robust assessment of the distribution of species and their potential for movement through the landscape. However, access to sampling holes in several of the calcretes (e.g. Nambi and Halfpenny) was minimal and so we cannot rule out the possibility that several taxa are more widespread than our current data suggest. That said, the palaeovalley sediments on which the calcretes form comprise numerous clay sequences (Arakel et al., 1990; Humphreys, 2001) which appear to have inhibited subterranean movements of macro-invertebrates, such as water beetles and

amphipods (Cooper et al., 2002, 2007), between different calcretes. Given the phylogeographic pattern identified above, it appears that the alluvial matrix is also likely to be a major barrier for many oniscidean species.

The presence of the same *COI* haplotypes in some paraplatyarthrids that were classified as Group B intermediate forms (partial eyed and semi-pigmented) from calcretes over a distance of 75 km (Halfpenny-Nambi) to 125 km (Halfpenny-Laverton Downs) suggests there may have been relatively recent dispersal of these taxa. These shared localities are adjacent calcretes in the same palaeodrainage (Carey) system (Fig. 1), but there was no evidence for the same taxa being in calcretes from different palaeodrainage systems (e.g. Sturt Meadows in the Raeside palaeodrainage which was sampled intensively). Palaeodrainages provide a natural flow path for water, suggesting possible dispersal by episodic sheet floods, that often occur every few years (Wilford, 2000) and likely provide temporary moist environments (e.g. from decaying tree logs) over several months. Sampling of surface habitats revealed the presence of epigean Paraplatyarthridae in decaying vegetation, one of which (S4; not found in subterranean calcretes) grouped closely with lineages assigned to Group B intermediate forms (B17-18). However, the distribution of surface isopod species in the region is currently unknown and intensive sampling of epigean oniscideans would allow for a more comprehensive understanding of this fauna.

4.2. A new nuclear gene for isopod phylogenetics and species delimitation

LSU rRNA (*28S*) and SSU rRNA (*18S*) genes have been widely used for reconstruction of phylogenetic relationships among isopods (e.g. Wägele et al., 2003; Osborn, 2008). As the rRNA genes contain both variable and conserved regions, they have been useful for elucidating oniscidean relationships and have helped resolve some of the polytomies that occurred in the *COI*-based phylogeny. However, the rRNA genes provide little discrimination among closely related species and the variable regions can often be difficult to align among ingroup and outgroup taxa. In contrast, the Lysyl-tRNA Synthetase gene (*LysRS*), developed for the first time during this study from isopod transcriptome data, proved to be phylogenetically informative and highly discriminatory at the species level. The overall mean divergence of *LysRS* among paraplatyarthrid lineages was estimated at 0.052, which is

459 approximately three times less than that of *COI.* The BI *LysRS* phylogeny showed the same
460 topology as *COI* for the majority of clades, provided a better resolution of their
461 relationships, and higher posterior probability support levels for most nodes (Fig. 3). For
462 instance, Clade 1, consisting of the paraplatyarthrid B1 to B5, which was weakly supported
463 in the *COI* phylogeny (0.71), was strongly supported by the *LysRS* phylogeny (0.98). Although
464 aminoacyl-tRNA synthetases (aaRS's), including the Lysyl-tRNA Synthetase (*LysRS*) gene,
465 have been used for reconstruction of phylogenetic relationships in several studies (Brown
466 and Doolittle, 1995; Nagel and Doolittle, 1995; Brown et al., 1997), they need to be
467 considered more widely in terms of their phylogenetic utility.

468

469 ## 4.3. Species delimitation and assessment of oniscidean diversity

470 Intensive sampling of four main localities in the Laverton Downs calcrete, revealed the
471 presence of several divergent mtDNA lineages (Fig. 2, Clade 4: B13-16; Stenoniscidae A1-2)
472 that were interpreted by the PTP and bPTP analyses as representing multiple distinct
473 species. Previous molecular analyses of stygobitic diving beetles, amphipods and *Haloniscus*
474 isopods using *COI* sequence data from three of these sites (Quandong, Shady Well and Mt
475 Windarra) revealed similar phylogeographic patterns and divergent clades within species.
476 The single divergent clade of haplotypes associated with the Mt Windarra site but absent
477 from the northern sites (Shady Well and Quandong) probably resulted from an impediment
478 to gene flow caused by an intervening saltlake (playa), a pattern that has previously been
479 reported in stygobiontic dytiscid beetles, amphipods and aquatic isopods from the same
480 calcrete (Guzik et al., 2011). The occurrence of divergent lineages associated with the Mt
481 Windarra site for paraplatyarthids and stenoniscids suggest that similar evolutionary forces
482 may be operating to impede gene flow among oniscideans within the calcrete.

483 Such phylogeographic structure within a calcrete is problematic for species delimitation
484 models such as PTP, bPTP (Zhang et al., 2013) and GMYC (Pons et al., 2006, Fontaneto et al.,
485 2007), possibly leading to an over-estimate of the number of species present. The
486 delimitation models applied to analyse the nuclear gene *LysRS* also supported the presence
487 of 'clade 4', but indicated that it comprised a single species, a result corroborated by the
488 12% threshold method using the *COI* data. Although the stenoniscid lineages A1 and A2

from Laverton Downs Windarra and Erlistoun, showed 10% mitochondrial divergence and ~1% divergence for *LysRS*, data are currently insufficient and hence at this stage A1/A2 are considered to represent a single species.

With application of this combined approach, using the models of species delimitation on the *COI* and *LysRS* genes and a 12% threshold, the paraplatyarthrid lineages (B9 to B11) distributed in the Lake Uramurdah, Bubble Well, and Lake Violet calcretes were considered a single species. Divergence among the lineages was <4.6% and no significant morphological differences were detected among the taxa (Javidkar, 2014). These calcretes, although adjacent (1-35 km from each other) and within the same paleodrainage (Carey), each contain several distinctive stygofaunal species (e.g. parabathylellid crustaceans; Guzik et al., 2008) but, conversely, are also known to share dytiscid beetle species (*Limbodessus insolitus* Watts and Humphreys 2009 and *Limbodessus millbilliensis* Watts and Humphreys 2006; Watts and Humphreys, 2009 and references therein), suggesting there was connectivity between the calcretes in the past. Moreover, using the same approach, some other paraplatyarthrid lineages including 'B6, B7 and B12' (distributed in Laverton Downs Windarra, Halfpenny and Nambi), 'B4 and B5' (distributed in Lake Miranda East and West respectively) and the surface lineages 'S1, S2 and S3' from WA were evaluated as conspecific.

Finally, using the results of species delimitation methods on the *COI* and *LysRS* genes, phylogenetic structure and morphological evidence, one paraplatyarthrid lineage (B8: Laverton Downs, Quandong; no eyes with pale body, Group A) with at least 9.1% nucleotide divergence for *COI* from a group comprising three other paraplatyarthrid lineages (B6, B7 and B12; eyes of 3-5 ommatidia with semi-pigmented body, Group B), was considered a distinct species. With respect to this evidence, although the 12% mitochondrial threshold to delimit species largely led to similar results to the delimitation models based on the *LysRS* phylogeny, this threshold should be treated with caution as it failed to delimit some species which showed significant structuring for *LysRS* (i.e. B8). This result emphasises that multiple approaches for species delimitation should be used to best assess the number of putative species in the calcrete systems of Western Australia.

In general, based on a combination of methods, the results of single and multiple gene phylogenies, and species delimitation approaches (PTP, bPTP and use of a 12% threshold), a conservative assessment of the diversity in the surveyed calcretes is that there are at least 27 subterranean lineages (Table 4), each representing distinct species, within the 12 calcretes. Included in this estimate is an armadillid lineage that was amplified only for *LysRS* (D6 from Sturt Meadows). Of these 27 species, 22 are terrestrial species (subterranean terrestrial: including nine paraplatyarthrid, 12 armadillid and one stenoniscid species) and five are aquatic (stygofauna: *Haloniscus*). This estimate also includes 14 lineages characterised as Group A, and 11 lineages characterised as Group B (the grouping for the other two can not be confirmed; see Table 2). We henceforth refer to these lineages as species. The present study shows that the diversity of the oniscidean species is comparable with that of the stygofauna identified from the same 12 calcretes, where currently 23 dytiscid species, ~12 divergent (>10% p-distance) stygobitic isopod (*Haloniscus*) lineages and multiple amphipod species are known (see Cooper et al., 2008; Watts and Humphreys, 2009, and references therein; King et al., 2012), plus a suite of copepods (Karanovic, 2004; Karanovic and Cooper, 2012; Karanovic et al., 2015).

4.4. Evidence that the oniscidean fauna in the calcretes represent troglofauna.

A key question that requires consideration is whether the oniscidean fauna associated with the calcretes are soil dwellers (e.g., animals adapted to living in leaf litter and soil) or subterranean fauna, such as troglofauna, organisms regulary found in subterranean biotopes (e.g. calcretes or karst), which represent part or the whole of their natural habitat (Trajano, 2005). Under this latter definition, the oniscidean fauna occurring in the non-karst calcrete aquifers of central Western Australia should be classified as subterranean organisms. However, we further propose that several key features of the isopod fauna support the hypothesis that they are also troglofauna, with Group A individuals representing troglobites (subterranean animals whose source populations are strongly bound to hypogean habitats (Sket, 2008; Trajano, 2012). First, Group A individuals have very well defined troglomorphies, including an absence of, or highly reduced, eyes, a lack of pigment and relatively slender body form. There is evidence for these characteristics being apomorphic in some lineages (e.g., *Paraplatyarthrus subterraneus* Javidkar and King 2015,

549 Javidkar et al., 2015), suggesting they evolved following colonization of the hypogean
550 habitat from epigean ancestors. Although such characteristics can be found in soil dwelling
551 representatives of several invertebrate groups (e.g. millipedes; Polydesmoidea – Sket,
552 2008), we are not aware of any oniscidean species collected from soil and litter habitats that
553 have such troglomorphic characteristics, nor have any been identified- from field surveys or
554 in published or unpublished environmental reports. However, oniscideans with such
555 troglomorphies are known from cave habitats (e.g. *T. cavernae*; Chillagoe Caves, QLD).
556 Second, there is evidence for genetic isolation of all Group A species from species in
557 different calcretes, suggesting that each species is restricted to the hypogean habitat of a
558 calcrete. This phylogeographic pattern is consistent with the established endemicity of other
559 subterranean species, both stygobiont and troglobiont, in the same calcretes, including the
560 Laverton calcrete where substructuring is found either side of a salt lake (Taiti and
561 Humphreys, 2001; Leys et al 2003; Cho et al. 2006a; Cooper et al. 2007; Cooper et al. 2008;
562 Guzik et al. 2008; Watts and Humphreys 2009; Guzik et al. 2011; Karanovic and Cooper,
563 2012; Abrams et al 2012; King et al 2012; Harrison et al. 2014; Karanovic et al. 2015).
564 In contrast, Group B taxa, which show less extreme troglomorphic characteristics, are
565 likely to represent troglophiles (subterranean species able to live and reproduce
566 underground as well as in the epigean domain; i.e. source populations occur in both
567 hypogean and epigean habitats (Humphreys, 2000; Trajano, 2012). Although, to date, they
568 have only been collected from bore hole litter traps within calcretes, the sharing of mtDNA
569 haplotypes among specimens from adjacent calcretes in several taxa (e.g. B10 in URA and
570 BUB; B12 in HAW and NAM; B17 in LDW and HAW) suggests recent dispersal and, the lack of
571 connectivity of the calcrete matrix underground, suggests that animals most likely disperse
572 on the surface. Given that the surface landscape is usually extremely dry with no permanent
573 sources of water in creeks and lakes (annual rainfall < 200 mm and high potential
574 evaporation >3,000 mm per year: Mann and Horwitz, 1979), as mentioned above, surface
575 dispersal likely occurs following episodic sheet floods, along palaeodrainage systems
576 (Wilford, 2000). Confirmation of these hypotheses (i.e. Group A = troglobites; Group B =
577 troglophiles) requires further sampling of surface populations, particularly following rainfall
578 events, and subterranean sampling in the areas between the calcretes.
579

4.5. Stenoniscid species in arid central Western Australia

The possible new genus of Stenoniscidae recorded here (pers. comm., S. Taiti, 2012) is thought to be related to *Metastenoniscus* Taiti and Ferrara 1981 from South America (Venezuela) and Andaman Islands in the Indian Ocean, and Bali in Indonesia (Taiti and Humphreys, 2008). Stenoniscid isopods are a known littoral (coastal) group of oniscidean isopods (Schmidt, 2003), so the discovery of a stenoniscid isopod in the Laverton Downs calcrete was unexpected as the calcrete lies on the Yilgarn craton, more than 500 km from the nearest coastline, a landscape emergent since the Proterozoic (BMR Palaeogeographic Group 1990). Stenoniscids are not alone among the calcrete fauna for having marine affinities. Stygobiont species of *Halicyclops* (Cyclopoida: Cyclopidae) are found widely in calcretes of the Yilgarn region (Karanovic, 2004), and are also characteristic of marine littoral waters (coastal lagoons, estuaries, interstitial water of beaches and anchialine caves) around the World from about 60° N to 45° S (Rocha et al., 2000). In addition, a number of genera of Harpacticoida, that typically have similar marine affinities, are well represented by stygobiont species in the Yilgarn calcretes including *Schizopera* (Diosaccidae), *Hirtaleptomesochra, Novanitocrella, Parapseudoleptomesochra* and *Haifameira* (Ameiridae) (Karanovic, 2004; Karanovic and Cooper, 2012).

The occurrence of these littoral (Stenoniscidae and Copepoda) groups in the Laverton Downs calcrete may be linked to the marine inundation of the Eucla basin, comprising the Nullarbor Plain, which is located on the southern margins of the Yilgarn, Musgrave and Gawler Cratons across southern Australia, during the Late Eocene (Fig. 7). Geological evidence suggests that the palaeo-coastline of the Eucla Basin during the Cenozoic was most extended in the Late Eocene, with its northern most limits delineated by a set of palaeo-shorelines (Hou et al., 2008; Sandiford et al., 2009). Inset valleys incised into the base of the palaeovalleys and filled with shallow marine sediments dating from the early Mid Eocene are widespread throughout the eastern Yilgarn Craton (Broekert and Sandiford, 2005), indicating that marine transgressions likely developed some several hundred kilometres up the palaeovalleys that drained to the Eucla Basin (Alley et al., 1999).

The palaeo-shorelines extended further inland and their position expanded to the margin of the Neale Plateau in the northwest, including the lower Carey palaeodrainage,

and Barton barrier-Wilkinson estuary in the northeast (Clarke and Hou, 2000; Hou et al., 2008). The current distribution of stenoniscids at the Laverton Downs calcrete (Carey palaeodrainage) is close to the northern most marine inundation. When the sea retreated during the Oligocene/Miocene, it is likely that ancestral stenoniscids, which were stranded in the north-west, subsequently colonised the calcrete, perhaps as very early colonisers of the calcretes. Stenoniscid isopods may also have been able to survive on the shore-lines of playas (salt lakes) which are associated with the calcretes throughout the Yilgarn region. Humphreys et al. (2009) proposed that conditions equivalent to marine estuaries occur where calcretes and playas abut, potentially providing a suitable environment for the persistence of littoral taxa.

5. Conclusions

Groundwater calcretes in arid central Western Australia provide habitat for numerous, typically endemic, oniscidean isopods belonging to at least four families, with both markedly troglomorphic and intermediate forms present. At least 27 lineages were identified that most likely represent new species. This high level of diversity was found from the exploration of just 12 calcretes along three palaeodrainages. Given that there are more than 400 major calcrete deposits in the region, most of which are currently inaccessible for sampling, the number of undiscovered oniscidean taxa in the region is likely to be very large. With the exception of three paraplatyarthrid lineages with intermediate forms, found in more than one calcrete, all oniscidean lineages were endemic to individual calcrete bodies, supporting the “subterranean island hypothesis”. The oniscidean fauna in the Western Australian calcretes comprise subtropical (*Paraplatyarthrus*, *Troglarmadillo*), benthic (*Haloniscus*) and littoral (Stenoniscidae) species indicating that complex historical events were likely involved in shaping the composition of the fauna.

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Table 1. Primers and associated PCR annealing temperatures used for amplification of *COI*, *LysRS*, *28S* and *18S* for oniscidean Isopods. Primers indicated in bold refer to Forward.

Primer	Gene/ fragment amplified	Annealing Temperature (C)	Sequence (5′-3′)
LCO1490_t1[a]	*COI*;	48°	TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG
HCO2198_t1[a]	680 bp		CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA
M13F[b]	*COI*;	50°	TGTAAAACGACGGCCAGT
M13R[b]	680 bp		CAGGAAACAGCTATGAC
18s1.2F[c]	*18S*;	50°	TGCTTGTCTCAAAGATTAAGC
18sb5.0[c]	680 bp		TAACCGCAACAACTTTAAT
28srD1.2a[c]	*28S*;	50°	CCCSSGTAATTTAAGCATATTA
28srd4.2b[c]	867 bp		CCTTGGTCCGTGTTTCAAGACGG
G2328[e]	*LysRS*;	48°	GTGCCACYGCCAAACCT
G2329[e]	791 bp		CCATRCCCCAACCTSCTGT
G2340[e]	*LysRS*;	50°	GATCGTGTWTAYGAAGTYGGAAG
G2341[e]	643 bp		TCAAGAGCRGTACARWAGTTTTC
G2281[e]	*28S*;	55°	GSGATGCCGCGTWTGGGAGN
G2282[e]	630 bp		TTCACCGTCBVAGAGGCCGT

[a]Robin M. Floyd in BOLD, the barcode of life data system (http://www.boldsystems.org)
[b]Used for sequencing reactions, Messing (1983)
[c]Whiting (2001)
[e]This study

Table 2. Lineage codes, sampling locations and associated geographic coordinates, BES codes and GenBank accession numbers. Abbreviations for the calcretes, the associated paleodrainages and some localities from Western Australia are as follows: Calcrete areas: Laverton Downs-Windarra (LDW), Carey; Laverton Downs-Erlistoun (LDE), Carey; Laverton Downs-Quandong (LDQ), Carey; Laverton-Shady Well (LDS), Carey; Sturt Meadows (SM), Raeside; Cunyu (CUN), Nabberu; Lake Violet (LV), Carey; Lake Miranda East (LME), Carey; Lake Miranda West (LMW), Carey; Nambi (NAM), Carey; Uramurdah (URA), Carey; Bubble Well (BUB), Carey; Halfpenny Well (HAW), Carey; Barwidgee (BAR), Carey; Hinkler Well (HIN), Carey; Mt Morgans (MOR), Carey; Non-calcrete areas: Jorgensen Park, Kalamunda, WA (JOP); Gooseberry Hills, WA (GOO); Wooroloo, WA (WOO); Moorapulling Rd. (MOO), Marradong, WA. Numbers in parentheses are the code numbers on the map. Lineage codes indicated in bold, normal and italic fonts show Group A, Group B and surface species, respectively. A, (B and S), C and D lineage codes represent Stenoniscidae, Paraplatyarthridae, Philosciidae and Armadillidae, respectively.

Lineage codes	Family/Genus	Locality/ Coordination	BES numbers or JA Codes	GenBank accession number			
				COI	*LysRS*	*28S*	*18S*
	Stenoniscidae						
A1	new genus	LDW (6) S28.44388, E122.18681; S28.52602, E122.18787	16022; 16023	X	X	X	X
A2	new genus	LDE (5) S28.44388, E122.19568	16071	X	X	X	X
	Paraplatyarthridae						
B1	*Paraplatyarthrus*	SM (1) S28.70124, E120.90361; S28.7003, E120.9026	15551.8,9; 17225.1,2	X	X	X	X
B2	*Paraplatyarthrus*	CUN (14) S25.7806, E120.1075	15090.1,3	X	X	X	X
B3	*Paraplatyarthrus*	LV (11) S26.7091, E120.2357; S-26.709, E120.2346	15080;15097	X	X	X	X
B4	*Paraplatyarthrus*	LME (15) S27.66384, E120.61076; S27.6638, E120.6108	15543.3; 17215.2	X	X	X	X
B5	*Paraplatyarthrus*	LMW (16) S27.74667, E120.5266	15538.10	X	X	X	X
B6	*Paraplatyarthrus*	LDW (6) S28.4989, E122.1798	3U.1,3; 14632.3	X	X	X	X
B7	*Paraplatyarthrus*	NAM (7) S28.2351, E121.8306	17221.1	X	X	X	X
B8	*Paraplatyarthrus*	LDQ (3) S28.35515, E122.22551	16567.1	X	X	X	X
B9	*Paraplatyarthrus*	URA (12) S26.6876, E120.313; S26.6876, E120.3078	15088.1; 15087.1	X	X	X	X
B10	*Paraplatyarthrus*	URA (12); BUB (13) S26.6876, E120.313; S26.5607, E120.0409; S26.5607, E120.0409	15067.1; 15095.3; 15065.1	X	X	X	X
B11	*Paraplatyarthrus*	LV (11) S26.70923, E120.26404	16476.1,2	X	X	X	X
B12	*Paraplatyarthrus*	HAW (8); NAM (7) S27.6966, E121.3395; S28.2210, E121.8216	15071.2; 17222.1	X	X	X	X
B13	*P. subterraneus*	LDW (6) S28.50282, E122.17726	15525.15,25	X	X	X	X
B14	*P. subterraneus*	LDQ (3) S28.35515, E122.22551	16567	X	X	X	X

B15	*P. subterraneus*	LDW (6) S28.49937, E122.17838	15524.6	X	X	X	X

Table 2. Continued

Lineage codes	Fam/Gen	Locality/Coordination	BES numbers or JA Codes	GenBank accession number			
				COI	*LysRS*	*28S*	*18S*
B16	*P. subterraneus*	LDS (4) S28.4074, E122.1997	14605.1	X	X	X	X
B17	*Paraplatyarthrus*	HAW (8); NAM (7); LDW(6) S27.6966, E121.3395; S28.2223, E121.8201; S27.6966, E121.3395; S28.5052, E122.1804; S27.69661, E121.33953	15072.1; 15073; 15072; 17224.1,2; 16478.3	X	X	X	X
B18	*Paraplatyarthrus*	BAR (9) S27.1375, E120.9495	15062	X	X	X	X
	Philosciidae						
C1	*Haloniscus*	LME (15) S27.6792, E120.6019	15082	X	X	X	X
C2	*Haloniscus*	LV (11) S26.6876, E120.2977	15085	X	X	X	X
C3	*Haloniscus*	URA (12) S26.6876, E120.313; S26.6876, E120.3027	15088.2; 15089.3	X	X	X	X
C4	*Haloniscus*	LDW (6) S28.5002,E122.1785	15094.1,2	X	X	X	X
C5 Not seen	*Haloniscus*	LDS (4)	14621.1	X	X	X	X
	Armadillidae						
D1	*Troglarmadillo*	SM (1) S28.70118, E120.89849; S28.69663, E120.89953	15550.1; 16511.1	X	X	X	X
D2	*Troglarmadillo*	NAM (7) S28.22059, E121.81755	16469.1,2	X	X	X	X
D3	*Troglarmadillo*	LDS (4) S28.40376, E122.2037	15528.3	X	X	X	X
D4	*Troglarmadillo*	LV (11) S26.70903, E120.23463	16386.1	X	X	X	X
D5	*Troglarmadillo*	LDS (4)	14603.1	X	X	X	X
D6	*Troglarmadillo*	SM (1) S28.7003, E120.9026	17225.3	X	X	X	X
D7	*Troglarmadillo*	LMW (16) S27.74667, E120.5266	15537.7	X	X	X	X
D8	*Troglarmadillo*	LDW (6) S28.5047, E122.17794	15509	X	X	X	X
D9 Not seen	*Troglarmadillo*	HIN (10) S26.8644, E120.2874	15104.2	X	X	X	X
D10 Not seen	*Troglarmadillo*	BUB (13) S26.5607, E120.0409	15092.4	X	X	X	X
D11	unrecognised	LME (15) S27.6634, E120.6123	15103.3	X	X	X	X
D12	unrecognised	LME (15) S27.664, E120.6126	15096.2	X	X	X	X
S1	*Paraplatyarthrus*	JP; GOO	Ja126; Ja144	X	X	X	X
S2	*Paraplatyarthrus*	WOO	Ja148	X	X	X	X
S3	*Paraplatyarthrus*	MOO	Ja152; Ja155	X	X	X	X
S4	*Paraplatyarthrus*	MOR (2)	Ja100; Ja101	X	X	X	X
Buddelundia cf. labiata	Armadillidae	MOR (2)	Ja110	X	X	X	X

Table 3. Garli partitioning schemes, lnL, number of parameters and AIC values. The numbers in the partitioning scheme column denote: 1, 2 and 3 for the *COI* first, second and third codon positions, respectively; 4 for *LysRS*, 5 for *28S*, 6 and 7 for core and variable regions of *18S*, respectively.

partition scheme	lnL	Free parameters	AIC
Partition 1 (1,2,3,4,5,6,7)	-15497.45835	10	31014.9167
Partition 2 (1,2,3,4)(5,6,7)	-15159.26796	10+9+(2-1)=20	30358.53592
Partition 3 (1,2,3,4)(5)(6,7)	-15100.19882	10+9+3=22	30244.39764
Partition 4 (1,2,3,4)(5)(6)(7)	-15007.17497	10+9+6+2=27	30068.34994
Partition 5 (1,2,3) (4)(5,6,7)	-14905.8998	10+6+9=25	29861.7996
Partition 6 (1,2,3) (4)(5)(6,7)	-14847.37137	10+6+9+3=28	29750.74274
Partition 7 (1,2,3)(4)(5)(6)(7)	-14753.65397	10+6+9+6+2=33	29573.30794
Partition 8 (1,2)(3)(4)(5,6,7)	-14537.03664	10+10+6+9=35	29144.07328
Partition 9 (1,2)(3)(4)(5)(6,7)	-14478.38933	10+10+6+9+3=38	29032.77866
Partition 10 (1,2)(3)(4)(5)(6)(7)	-14387.31508	10+10+6+9+6+2=43	28860.63016
Partition 11 (1)(2)(3)(4)(5,6,7)	-14472.71086	10+10+10+6+9=45	29035.42172
Partition 12 (1)(2)(3)(4)(5)(6,7)	-14413.99034	10+10+10+6+9+3=48	28923.98068
Partition 13 (1)(2)(3)(4)(5)(6)(7)	-14320.8661	10+10+10+6+9+6+2=53	28747.7322

Table 4. The number of putative species based on the PTP, bPTP and 12% *COI* threshold for species delimitation of the subterranean/surface oniscidean species. N.B. the philosciid lineage C5, and the armadillid lineages D2, D5, D8, D9 D10, and *Buddelundia* cf. *labiata* were not amplified for *LysRS.*

		PTP		bPTP		12% threshold
		COI	*LyRS*	*COI*	*LysRS*	
Paraplatyarthridae	subterranean	15	9	17	9	9
	surface	4	2	4	2	2
Armadillidae	subterranean	11	6	11	6	11
	surface	1	-	1	-	1
Philosciidae	subterranean	5	4	5	4	5
	surface	-	-	-	-	-
Stenoniscidae	subterranean	2	2	2	2	1
	surface	-	-	-	-	-
Total subterranean		33	21	35	21	26
Total		38	23	40	23	29

Fig. 1. A map of the sampled groundwater calcretes and their positions in the palaeodrainages. Numbers refer to the calcretes as listed in Table 2. Black shaded areas indicate groundwater calcretes and grey shaded ones are palaeodrainage valleys.

Fig. 2. Majority rule consensus Bayesian Inference tree based on the mtDNA *COI* gene. The numbers next to the nodes are posterior probabilities. The clade labels comprise lineage specific and calcrete codes, respectively. Families identified include Stenoniscidae (A codes), Paraplatyarthridae (B codes, *Paraplatyarthrus*), Philosciidae (C codes, *Haloniscus*) and Armadillidae (D codes; D1-D10 for *Troglarmadillo*; D11 and D12 probably belong to distinct, currently undescribed genera). The blue, red and black bars show species delimitation using the PTP, bPTP and a 12% nucleotide sequence divergence threshold, respectively, for subterranean and surface species. The black stars denote lineages considered to be the same putative species based on the 12% threshold. The blue, black and red lineages represent Group A, Group B and surface species, respectively.

Fig. 3. Consensus *LysRS* Bayesian Inference tree. The numbers next to the nodes are posterior probabilities. The clade codes comprise voucher numbers and calcrete codes separated by an underscore, respectively. The labels next to the gray bars refer to lineage specific and calcrete codes. The blue and red bars show species delimitation using the PTP and bPTP, respectively, for subterranean and surface species. The black bars refer to the putative species based on the *COI* 12% threshold.

Fig. 4. Majority rule consensus BI tree for mtDNA *COI* and nuclear *LysRS* genes. The numbers next to the nodes are posterior probabilities. The clade labels comprise lineage specific and calcrete codes, respectively.

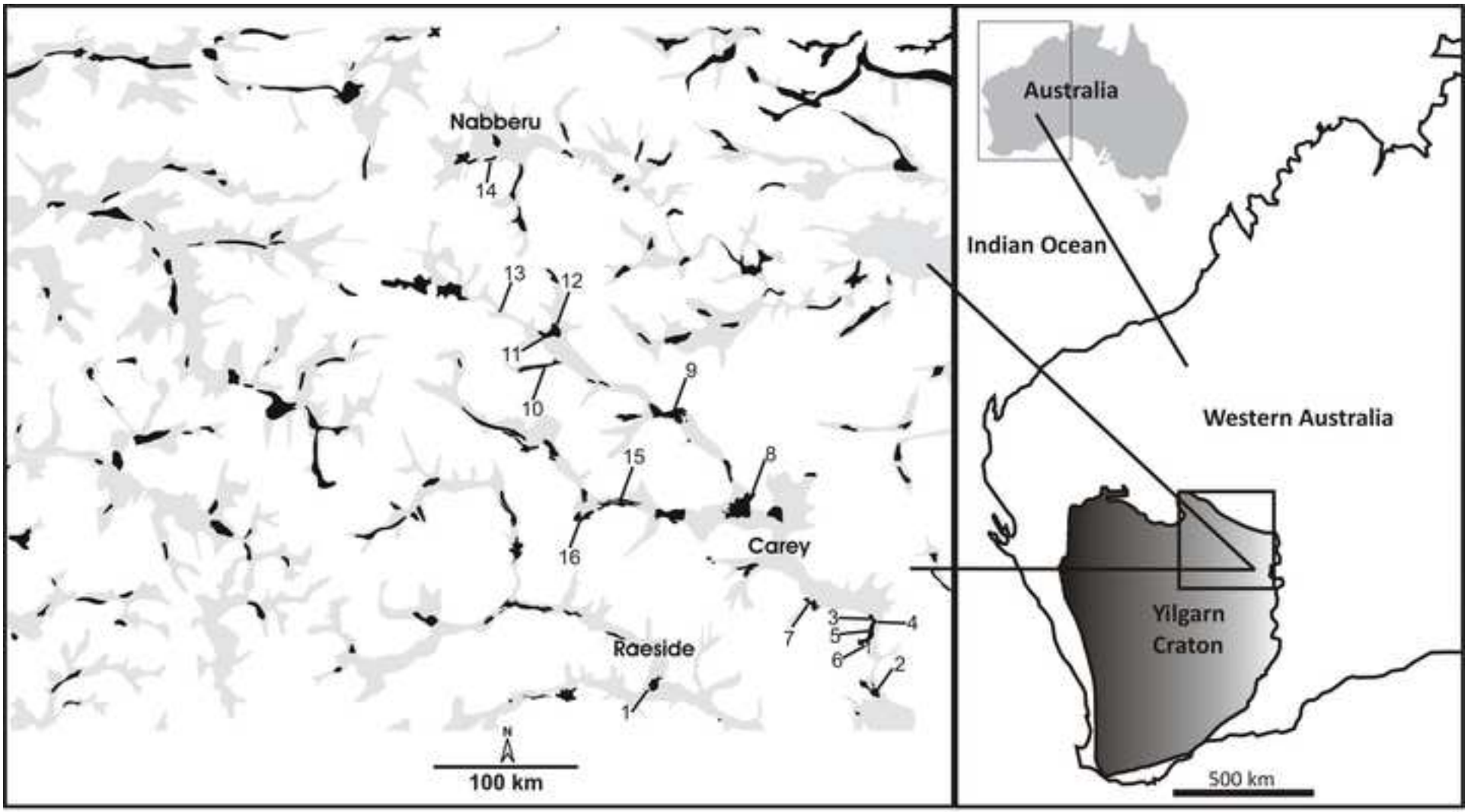
Fig. 5. Majority rule consensus BI tree based on three genes comprising *COI*, *LysRS* and *18S*. The numbers next to the nodes are posterior probabilities and ML bootstrap values, respectively. The clade labels include lineage specific and calcrete codes, respectively.

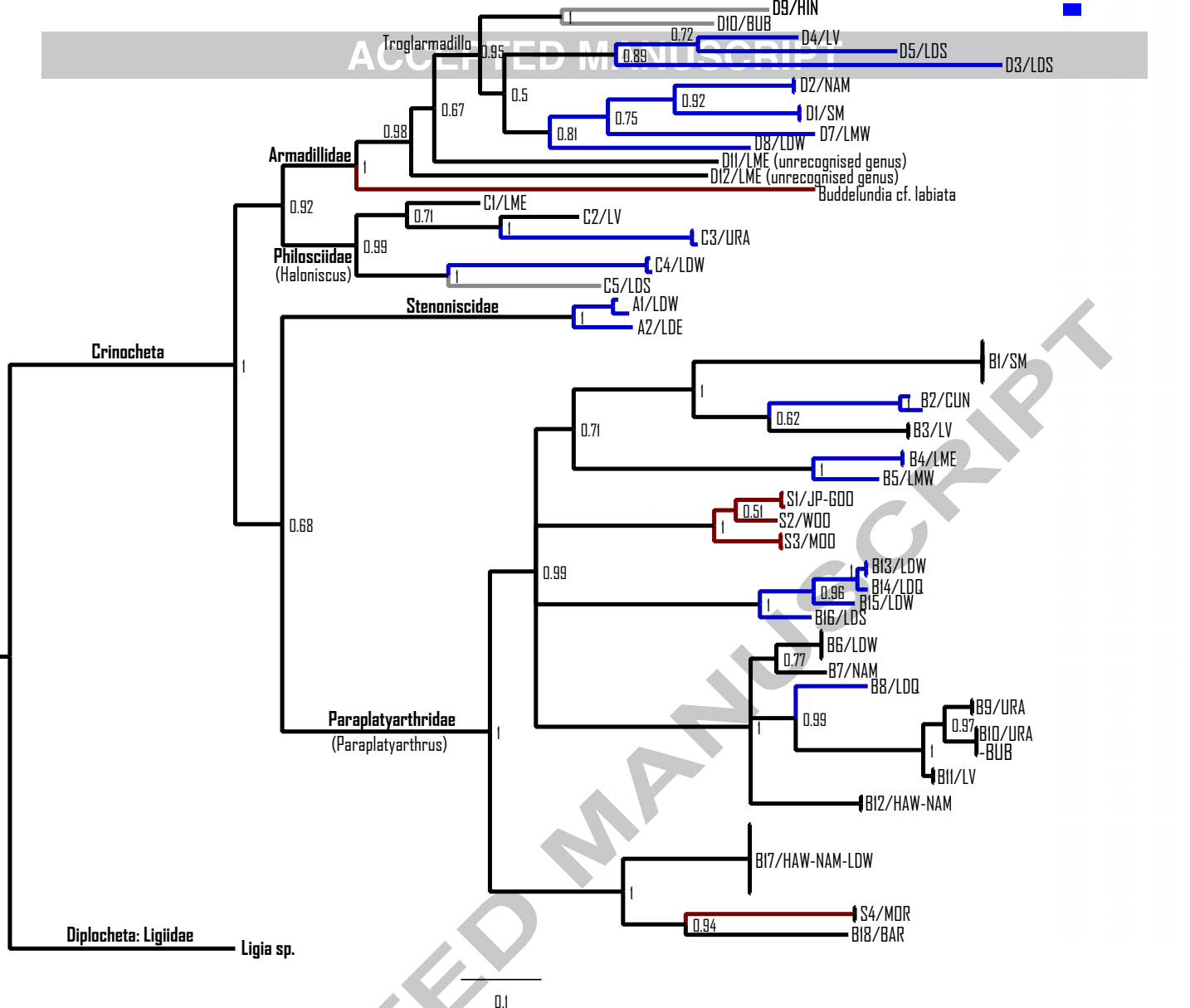
Fig. 6. Majority rule consensus BI tree based on four genes comprising *COI*, *LysRS*, *28S* and *18S*. The numbers next to the nodes are posterior probabilities and ML bootstrap values, respectively. The clade labels include lineage specific and calcrete codes, respectively.

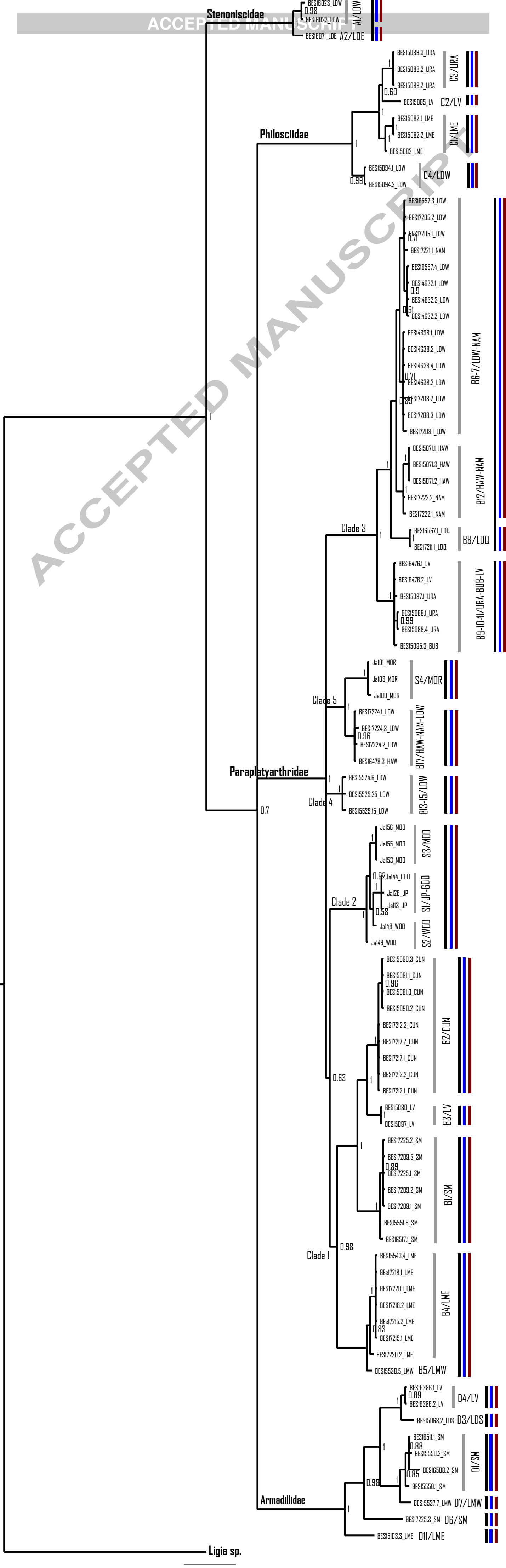
Fig. 7. The Eucla Basin and associated major palaeodrainages including Carey. Historical fluctuations in coastlines from the Cretaceous to present, which is inferred to have influenced the distribution of the littoral fauna in Australia, are indicated (composite map after Hou et al., 2003, 2008).

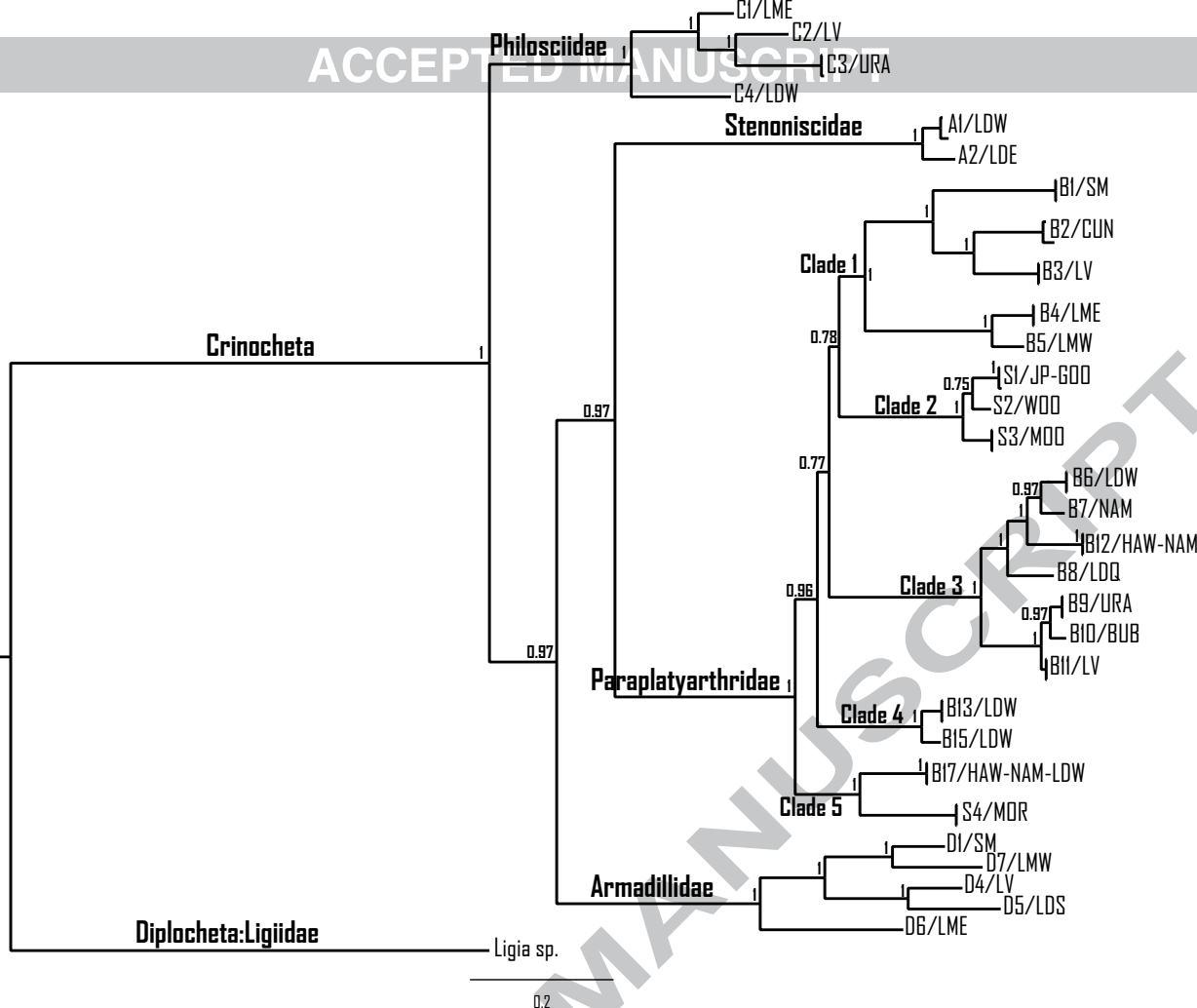
Supplementary figure A. Majority rule consensus Bayesian tree based on the whole mtDNA *COI* dataset. The numbers next to the nodes are posterior probabilities. The clade labels comprise haplotype vouchers and calcrete codes. The labels below the branches indicate lineage specific numbers. The red branches denote surface species.

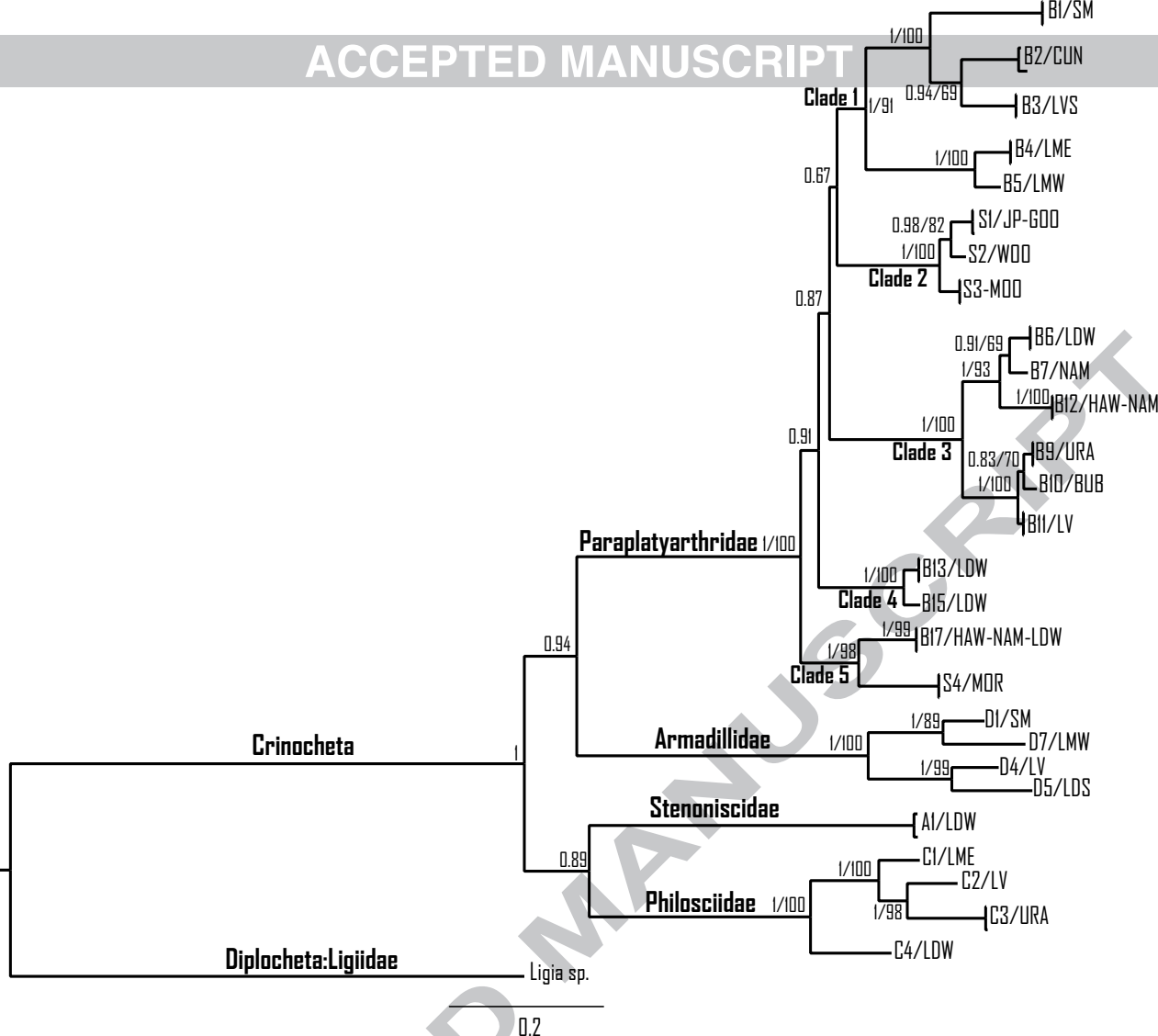
Supplementary figure B. Subterranean terrestrial sampling conducted in Western Australia. 1: Locating boreholes provided by mining companies using geographic coordinates 2: Borehole unearthed 3: PVC pipes stabilise the entrance of bores 4: Stabilised bore ready for subterranean samplings 5: Slotted litter trap to be set underground in a borehole 6: Recovered traps after 3 to 10 months.

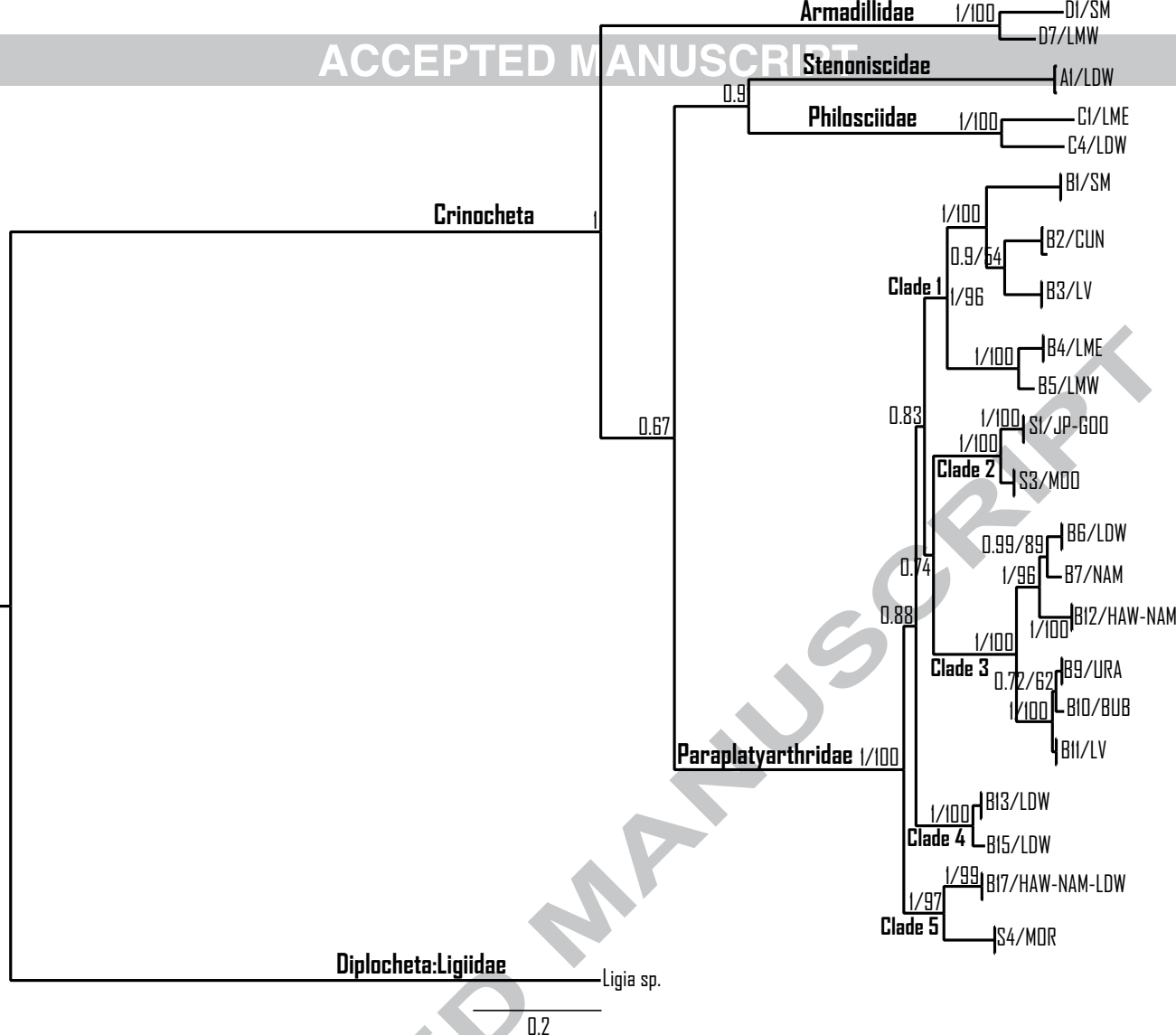


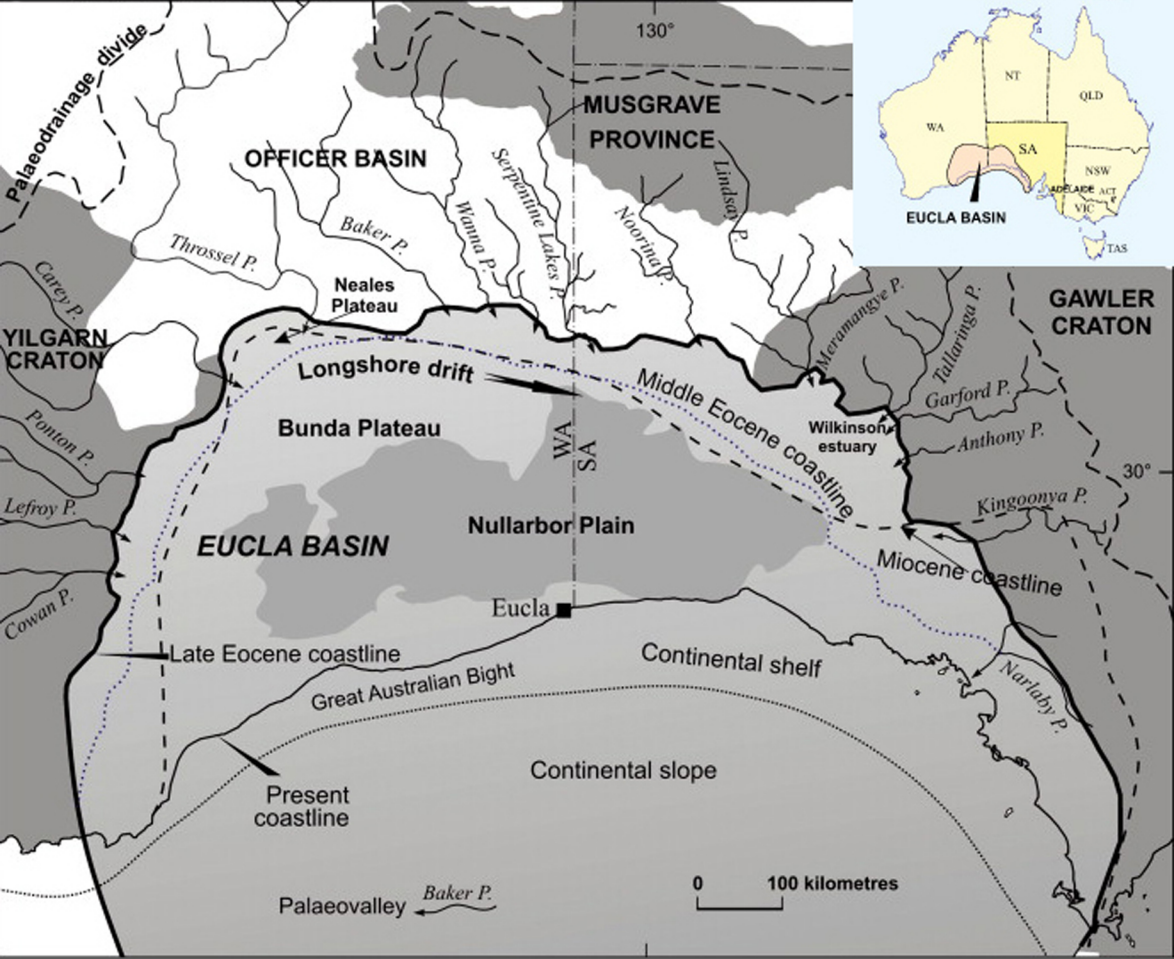











Palaeodrainage divide
130°
MUSGRAVE PROVINCE
OFFICER BASIN
Throssel P.
Baker P.
Wanna P.
Serpentine Lakes P.
Noorina P.
Lindsay P.
Carey P.
Neales Plateau
YILGARN CRATON
Longshore drift
Middle Eocene coastline
Bunda Plateau
WA
SA
Meramangye P.
Tallaringa P.
GAWLER CRATON
Garford P.
Wilkinson estuary
Anthony P.
30°
Ponton P.
Lefroy P.
Kingoonya P.
EUCLA BASIN
Nullarbor Plain
Miocene coastline
Eucla
Cowan P.
Late Eocene coastline
Continental shelf
Great Australian Bight
Narlaby P.
Continental slope
Present coastline
Palaeovalley
Baker P.
0
100 kilometres
NT
QLD
WA
SA
NSW
ADELAIDE
ACT
VIC
TAS
EUCLA BASIN

Highlights:

- A significant fauna of oniscidean isopods from the calcrete aquifers of central Western Australia were discovered and their phylogenetic relationships were investigated using multiple nuclear and mitochondrial genes.
- Using a combination of phylogenetic and species delimitation methods, and morphological evidence, at least 27 lineages of subterranean oniscideans were hypothesized to represent new species.
- Most species were found to be restricted in their distribution to individual calcrete aquifers in support of the “subterranean island hypothesis”, with exception of three paraplatyarthrid species found widespread across multiple calcrete aquifers.