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Scratching the surface of subterranean biodiversity: Molecular analysis reveals a diverse and previously unknown fauna of Parabathynellidae (Crustacea: Bathynellacea) from the Pilbara, Western Australia

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ABSTRACT

Like other crustacean families, the Parabathynellidae is a poorly studied subterranean and aquatic (stygobiontic) group in Australia, with many regions of available habitat having not yet been surveyed. Here we used a combined approach of molecular species delimitation methods, applied to mitochondrial and nuclear genetic data, to identify putative new species from material obtained from remote subterranean habitats in the Pilbara region of Western Australia. Based on collections from these new localities, we delineated a minimum of eight and up to 24 putative new species using a consensus from a range of molecular delineation methods and additional evidence. When we placed our new putative species into the broader phylogenetic framework of Australian Parabathynellidae, they grouped with two known genera and also within one new and distinct Pilbara-only clade. These new species significantly expand the known diversity of Parabathynellidae in that they represent a 22% increase to the 109 currently recognised species globally. Our investigations showed that sampling at new localities can yield extraordinary levels of new species diversity, with the majority of species showing likely restricted endemic geographical ranges. These findings represent only a small sample from a region comprising less than 2.5% of the Australian continent.

1. Introduction

The Pilbara is one of the oldest emergent surfaces on earth (Wiemer et al., 2018) and is recognised for its rich mineral deposits (iron ore in particular) (Summerfield, 2016). Its landscape is incised by major river valleys with rivers flowing from cyclonic and episodic rainfall during tropical summers (November-April), but drying during arid winters (May-October). However, permanent subterranean rivers flow as groundwater below the surface within alluvium (sand, silt, gravel deposited by flowing water), and these habitats have been found to comprise a biodiversity hotspot for subterranean aquatic organisms referred to as stygofauna (Wilson, 2003; Eberhard et al., 2005; Finston et al., 2007; Guzik et al., 2011a,b). Subterranean aquifers and their associated fauna are often closely linked to mineral formations (Mokany et al., 2019), and mining of iron ore in north-western Western Australia (WA), in particular, potentially poses significant threats to aquifer ecosystems through sealing of surface water and dewatering for extraction of minerals (Humphreys, 2008; Britt et al., 2017). Conservation concerns have increased the need for characterisation and description of subterranean species as part of the review process for environmental impact assessments, particularly by the mining industry (Humphreys, 2001; Eberhard et al., 2005; Hancock and Boulton, 2009). Intensive research and extensive environmental impact assessment surveys by environmental consultancies over the last decade have sampled a highly diverse endemic stygofauna in the Pilbara. These include stygofaunal groups such as copepod, ostracod and amphipod

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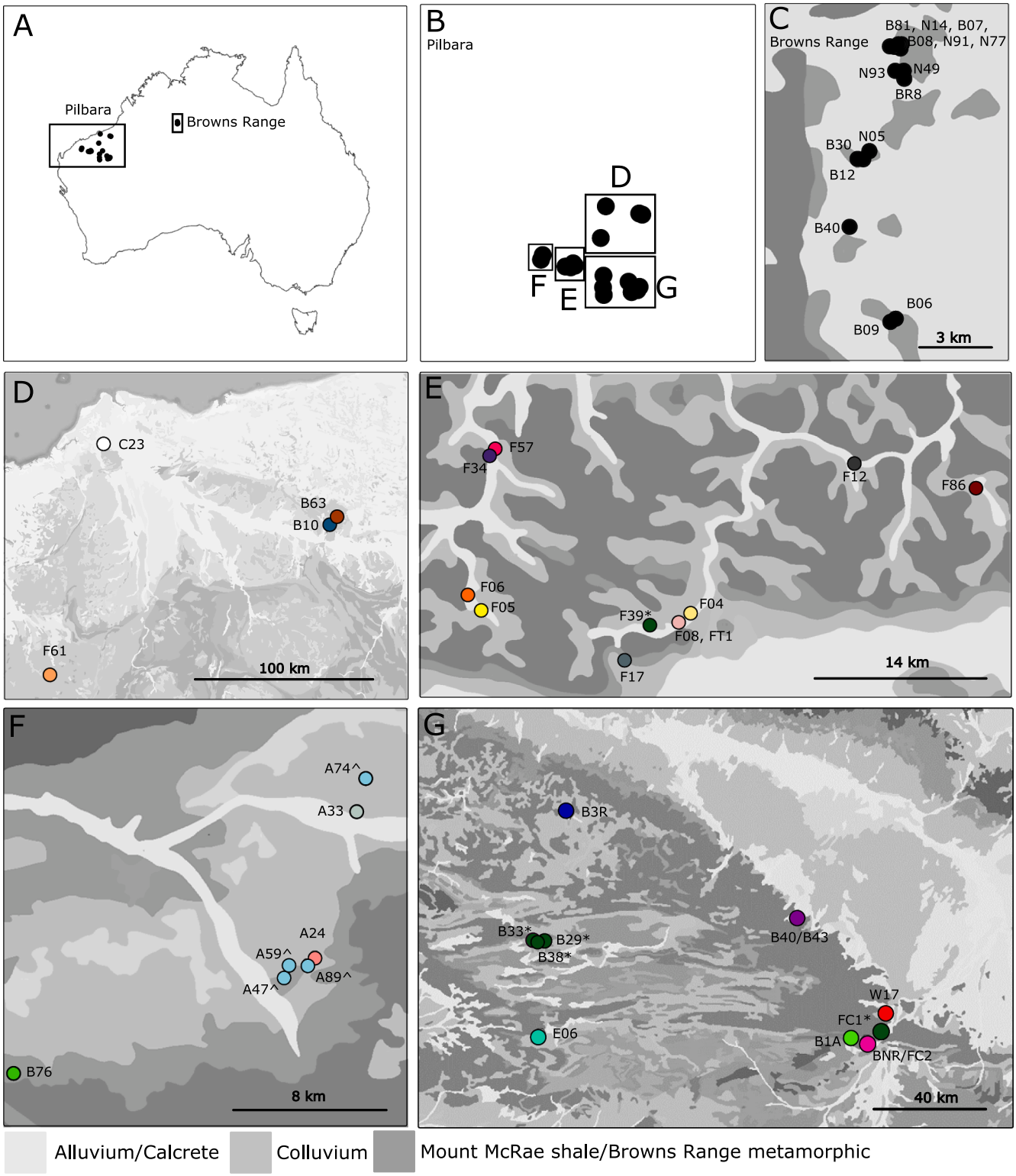

Fig. 1. New parabathynellid sampling locations in Australia. From left to right: A map of Australia with B Pilbara and C Browns Range sampling locations in boxes; inset map of the Pilbara with four key sampling regions within the Pilbara (E-G). Inset maps D-G show magnified views of each area and individual bores, labelled by code and coloured according to putative species present as per Fig. 2. ^ and * indicate bores sharing putative species in common. A generalised indication of underlying geological formations is provided in greyscale: light grey – Alluvium/Calcrete, middle grey – Colluvium, dark grey – Mount McRae shale/Browns range metamorphic. Map created using ArcMap v 10.3.1 (ESRI, 2017), data layers provided by Rio Tinto and from the AUSGIN Geoscience Portal 2017. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crustaceans (Eberhard et al., 2005; Reeves et al., 2007; Halse et al., 2014), as well as subterranean and terrestrial (troglofauna) groups such as pseudoscorpions (Edward and Harvey, 2008; Harrison et al., 2014) and schizomids (Harvey et al., 2008). Only a small proportion of this biodiversity has been formally assessed and described to date (Guzik et al., 2011a,b).

Parabathynellidae Noodt, 1965 are a relictual freshwater family of syncarid microcrustaceans that are known from around the world (Schminke, 1972, 1973; Cho et al., 2006b; Camacho et al., 2012). They are remarkably diverse in Australia (Humphreys, 2001; Finston and Johnson, 2004; Guzik et al., 2008; Asmyhr and Cooper, 2012) with a high proportion recently described from Western Australia (Cho, 2005; Guzik et al., 2008, 2011; Cho and Humphreys, 2010). Of the 45 known parabathynellid genera worldwide (Camacho et al., 2018), 11 have Australian representatives (48 species). These include four supposedly widely distributed genera: *Atopobathynella* Schminke, 1973, *Chilibathynella* Noodt, 1963, *Hexabathynella* Schminke, 1972, and *Notobathynella* Schminke, 1973. The remaining seven genera are endemic to Australia and include *Arkaroolabathynella* Abrams and King, 2013 (in Abrams et al., 2013), *Billibathynella* Cho, 2005, *Brevisomabathynella* Cho et al., 2006a, *Kimberleybathynella* Cho et al., 2005, *Lockyerenella* Little and Camacho, 2017, *Octobathynella* Camacho and Hancock, 2010a and *Onychobathynella* Camacho and Hancock, 2010a (Little and Camacho, 2017). To date, four species are known from the Pilbara, *Billibathynella cassidis* Hong and Cho, 2009, *Brevisomabathynella hahni* Cho and Humphreys, 2010, *Brevisomabathynella pilbaraensis* Cho and Humphreys, 2010 and *Atopobathynella schminkei* Cho et al., 2006b. Due to their high diversity, poor dispersal ability and role as bioindicators (Harvey et al., 2011; Korbel et al., 2013), parabathynellids are recognised as a significant conservation priority (Smith et al., 2016; Korbel et al., 2017).

Differences among parabathynellid species can be hard to detect using morphology alone (Abrams et al., 2012; Asmyhr and Cooper, 2012; Abrams et al., 2013). Convergent evolution of similar morphological traits means that species are often morphologically uniform and cryptic (Christiansen, 1961; Pipan and Culver, 2012; Camacho et al., 2014, 2018; Little and Camacho, 2017) due to life in lightless and confined habitats (Guzik et al., 2008; Bradford et al., 2010; Abrams et al., 2012; Asmyhr and Cooper, 2012). In addition, difficulties associated with sampling and a general sparsity of specimens (Abrams et al., 2013) means that the taxonomy for this group remains largely incomplete (Eberhard et al., 2005; Guzik et al., 2008). Molecular studies have been valuable for providing a first assessment of their species level diversity (Guzik et al., 2008; Humphreys, 2008; Little et al., 2016), particularly for cryptic taxa (Humphreys, 2001, 2008; Harvey, 2002; Finston and Johnson, 2004; Eberhard et al. 2005; Guzik et al., 2008; Abrams et al., 2012; Asmyhr and Cooper, 2012; Asmyhr et al., 2014b). Delineation of parabathynellid species from around Australia using molecular methods in the last 10 years has focused on several key groundwater systems, including alluvial systems in Queensland (QLD) (Cook et al., 2012; Little et al., 2016; Little and Camacho, 2017), South Australia (SA) (Abrams et al., 2012, 2013), New South Wales (NSW) (Asmyhr and Cooper, 2012), and calcrete aquifers of the Yilgarn region, WA (Guzik et al., 2008; Abrams et al., 2012, 2013). Each of these studies has demonstrated that species are endemic to a small part of each specific region. In some cases, genera are widespread across Australia, but, mostly, they are restricted regionally and no single species has been found in multiple regions. Follow-up taxonomic work that has described these new genera and species has reinforced observations of endemism and geographic isolation (Cho, 2005; Cho et al., 2005, 2006a, 2006b; Camacho and Hancock, 2010a, 2010b; Cho and Humphreys, 2010; Abrams et al., 2012; Little and Camacho, 2017).

In this study, our aim was to estimate the number of putative species for the north-western region of Western Australia, in particular the Pilbara, from where parabathynellids are known to occur, but are not well characterised (Hong and Cho, 2009). To do this, we aimed to delimit species using three genetic loci (*Cytochrome Oxidase* c *Subunit I* (*COI*), *12S* rRNA (*12S*) and nuclear *18S* rRNA (*18S*)). Based on previous research in other locations around Australia, we anticipated a high level of local endemicity in the Pilbara fauna. Secondly, substantial *COI* and *18S* sequence data for Parabathynellidae are available to assess relationships for the Australian fauna (Abrams et al., 2012; Cook et al., 2012; Asmyhr et al., 2014a, 2014b; Little et al., 2016) and, with this information, we aimed to place new putative species from the Pilbara within a current revised and expanded phylogenetic framework.

2. Materials and methods

2.1. Field collections and taxon sampling

Parabathynellids were collected from two separate regions in northern WA (the Pilbara and Browns Range; Fig. 1), and obtained from previous ecological surveys by three consultancies (Subterranean Ecology, Bennelongia Environmental Consultants and Stantec) between 2008 and 2017 (Fig. 1 and Supplementary Material Table S1). Specimens were collected from pre-existing bore holes using stygofaunal haul nets, and pumping water from aquifers. All specimens were preserved in 100% ethanol and stored in the Western Australian Museum's (WAM's) Crustacean (WAMC) collection. From WAM's long-term collection of subterranean fauna we were able to sequence 74 individuals from 32 boreholes broadly distributed across the Pilbara and more narrowly from 42 individuals at 15 bores from Browns Range (north-west WA). A key aim of this study was to place our Pilbara Parbathynellidae (Pilbara-only) into the broader Australian framework and for this reason we used all Australian parabathynellid sequences available to us, both unpublished data (KMA) and also published sequences of Australian Parabathynellidae from studies by: Abrams et al. (2012); Cook et al. (2012); Asmyhr et al. (2014a, 2014b); Little et al. (2016) (see Supplementary Material Table S1). The Browns Range individuals from the present study and also the additionally sequenced Pilbara individuals (Lineages B and C) (Abrams et al., 2013) were considered especially important because of their geographic proximity to our Pilbara individuals and their value as a geographically distinct north-east comparison to the Pilbara. Sequences from additional species of previously unpublished research by KMA from numerous locations around Australia were also included to further extend the range of geographic sampling. These included: *Chillibathynella* sp. 3, *C.* sp. 4, *Notobathynella* sp. 1, *Kimberleybathynella* sp.3, *K.* sp.4, *At.* sp.7, *At. wattsi* Cho et al., 2006b, *Arkaroolabathynella* sp. 1, *Ar.* sp. 2, *Billibathynella.* sp. 3, *Brevisomabathynella parooensis* Cho and Humphreys, 2010, *B. cunyuensis* Cho et al., 2006a, *At. hinzeae* (Cho et al., 2006b) (see Supplementary Material Table S1 for details).

Australian parabathynellids have been shown not to be monophyletic (Camacho et al., 2018) and a lack of bathynellacean fossils has made understanding their present distribution particularly challenging (Camacho and Valdecasas, 2008). At this time we have focussed on identification of potential new Australian species in the present study. Future research will aim to broaden the scope of the analyses to non-Australian taxa and to place the Australian parabathynellid fauna internationally, but such analyses will likely require additional genetic markers to be developed for parabathynellids (see Supplementary Material Table S1).

2.2. DNA extraction, amplification, and sequencing

DNA was extracted from body segments between segment 10 and the pleotelson (preserving significant characters for morphological studies) from approximately 190 individuals, using Qiagen DNeasy tissue and blood extraction kit under standard protocol. Use of degraded museum specimens can be a major limitation in DNA sequencing studies. Here we were able to successfully sequence 61% (116/190) of available specimens for one or more genes. The lower success rate for sequencing specimens was probably due to sub-optimal storage conditions during consultancy surveys. Fragments of the mtDNA gene *COI*, *12S* and *18S* genes were amplified using the following primers; *COI*: C1-J-1718F GGAGGATTTGGAAATTGATTAGTTCC and C1-J-2329 ACTGTAAATATATGATGAGCTCA (Simon et al., 1994). 12S: 12SCRF GAGAGTGACGGGCGATATGT and 12SCRR AAACCAGGATTAGATACC

CTATTAT (Wetzer, 2001). 18S[i] 18s1F TACCTGGTTGATCCTGCCAG TAG and 18S5R CTTGGCAAATGCTTTCGC (Giribet et al., 1996); (Whiting et al., 1997). 18S [ii] 18S3F GTTCGATTCCGGAGAGGGA and 18Sbi GAGTCTCGTTCGTTATCGGA (Giribet et al., 1996); (Whiting et al., 1997). 18S [iii] 18Sa2.0 ATGGTTGCAAAGCTGAAAC and 18S9R and GATCCTTCCGCAGGTTCACCTAC (Giribet et al.,' 1996; Whiting et al., 1997). PCR-amplifications were conducted in 25 μL volumes comprising 1 × PCR buffer (1 mM dNTPs and 3 mM $MgCl_2$), 1 unit of TAQ polymerase (MyTaq), approx. 1–5 ng of DNA template, 0.2 μM of each primer. PCR cycling conditions for *COI* consisted of a 5 min denaturing period at 95 °C, then 7 cycles of 95 °C for 30 s, 40 °C for 30 s, 72 °C for 60 s, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s with a final extension at 72 °C for 10 min. Conditions for *12S* were 95 °C for 5 min, then 40 cycles of 95 °C for 30 s, 47 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. *18S* was amplified as three fragments, under the following conditions: 95 °C for 5 min, then 35 cycles of 95 °C for 30 s, 49 °C for 30 s, and 72 °C for 45 s, with a final extension of 72 °C for 10 min. Products were visualised using the eGel Electrophoresis system (Life Technologies, Scoresby, Vic., Australia), on 2% agarose gels with ethidium bromide. Bidirectional sequencing was carried out at the Australian Genome Research Facility.

Any sequences new to this study were submitted to GenBank (GenBank numbers *COI* MK546312 - MK546373; *12S* rRNA MK573384 - MK573427; *18S* rRNA MK554585 - MK554634). For the expanded Australian parabathynellid phylogeny, representatives of all existing Australian lineages for *COI* and *18S* from published studies were downloaded from GenBank (see Supplementary Material Table S1 for details). No existing *12S* sequence data were available from GenBank, but *12S* sequences for Pilbara individuals were still retained in the final alignment for their informativeness and use in future work. GenBank sequences were aligned with the concatenated dataset, and trimmed to match the length of Pilbara samples for *COI* and *18S* rRNA.

2.3. Nucleotide analysis

Geneious v10.0.9 (Kearse et al., 2012) was used to edit and assemble *COI, 12S* and *18S* sequence data for Pilbara individuals. Forward and reverse sequence fragments were edited and trimmed, aligned, and a single consensus sequence was created. Consensus sequences of all sequenced individuals for each gene were aligned using MUSCLE (Edgar, 2004) and trimmed so that sequence lengths were the same amongst all individuals and the final *COI* and *18S* sequences were aligned with two outgroup taxa, Psammaspididae sp. (KX022576, KX022537) (Little et al., 2016) and Bathynellidae sp. (JQ446079, JN817410) (Abrams et al., 2012) using MUSCLE alignment (Edgar, 2004). There were no suitable outgroup sequences available for *12S*. The *COI* alignment was translated to amino acids using MEGA 7 (Kumar et al., 2016). FaBox (Villesen, 2007) was used to collapse the final sequence alignments into haplotypes using the 'DNA to haplotype collapser and converter'. The number of *COI* base substitutions per site between putative species and bores was estimated using the Kimura 2-parameter model (Kimura, 1980) with MEGA 7 (Kumar et al., 2016). Sequences for *COI*, *12S* and *18S* were combined into a single file using the 'concatenate alignment' feature in Geneious (Kearse et al., 2012).

2.4. Molecular phylogenetic analysis and species delineation of Pilbara-only data

The Pilbara-only alignment of *COI*, *12S* and *18S* was initially analysed independently of taxa from outside the region. This analysis comprised individual analyses of each gene region and then all three gene sets were concatenated. We examined the effect of converting *COI* to amino acids and removing the third codon position, but found no effect of these manipulations on tree topology, so nucleotide data including all codon positions were used for the final phylogenetic analyses. The final datasets (*COI*, *12S*, *18S* and concatenated alignment) were partitioned by codon position and gene, before using Partition Finder v2.1.1 (Lanfear et al., 2016a) to determine the models of evolution which best fit each partition. The best fitting models for *COI* 1st, 2nd and 3rd codon positions respectively were the General Time Reversible (GTR) model (Tavaré, 1986) + Gamma (G), Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) + Invariable sites (I) + G and HKY + G, and for *12S* and *18S* they were GTR + I + G and GTR + G respectively. The alignments were analysed using MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003) via the CIPRES Science Gateway v.3.3 (Miller et al., 2010). In MrBayes, four chains for the Markov chain Monte Carlo (MCMC) analysis (two runs) were run simultaneously for 50,000,000 generations, sampling every 100th tree, under the specified models of substitution for each partition. To ensure that the chains had been run for long enough to reliably estimate the posterior distributions of the parameters of interest (Lanfear et al., 2016b), MCMC convergence was assessed using Tracer 1.6 (Rambaut et al., 2015). Effective Sample Size (ESS) values were well above 200 for final analyses providing us with confidence that the impact of autocorrelation was not detrimental to the estimated parameters and resultant tree topologies. Convergence in analyses was assessed using Tracer 1.6 (Rambaut et al., 2015) to determine a burnin of 20%. A 50% majority rule Bayesian consensus tree generated from the remaining posterior distribution of trees. Posterior Probability (PP) support was used to assess confidence in resolved nodes. Resulting trees were edited for presentation using FigTree v. 1.4.3 (Rambaut, 2009).

Three methods of species delineation were used; two on each of the final *COI*, *12S* and *18S* genes for the Pilbara-only datasets, and an additional threshold approach for *COI*. Firstly, Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) was conducted on each gene alignment, with default settings except for relative gap width which was adjusted to one, and initial and recursive partitions calculated for Jukes-Cantor 69, Kimura K80, and simple distance models. Secondly, we employed the Poisson Tree Processes (PTP) (Zhang et al., 2013) analysis using the MrBayes gene tree as input and using both Maximum Likelihood (PTP) and Bayesian (bPTP) approaches with a burnin of 0.1 and 100,000 MCMC generations. Resulting trace plots were used to assess tree convergence. Species were also delineated using established *COI* Kimura-2-parameter model distance thresholds for parabathynellids in MEGA7 (Kumar et al., 2016) and using the Species Delimitation plugin in Geneious (Masters et al., 2011): 7.1% (Abrams et al., 2012), 11% (Guzik et al., 2011a,b) and generic crustacean threshold 17% (Costa et al., 2007) which also encompasses the threshold of 16% (Lefébure et al., 2006). Results of species delineation methods for each gene were compared (see Results), and an assessment of putative species was made based on a consensus of these observations.

To apply a unified species concept as per de Queiroz (2007) we incorporated the following lines of evidence: a) the consensus of molecular species delimitation results, b) monophyly of lineages based on tree topologies (Supplementary Material Figs. S1–S6) especially for resolved nodes of 0.90 PP for *COI* and *12S* (PP node support for *18S* was generally low so we down-weighted the results of this gene); c) geographic isolation (i.e. distance and geology) (as represented by colours in Fig. 1 and phylogenies in Fig. 2 and Supplementary Material Fig. S1-S6), were also used to corroborate species divisions (de Queiroz, 2007; Guzik et al., 2011a,b). A minimum of two for these criteria needed to be fulfilled for a lineage of individuals to recognised here as a putative species. For completeness, morphological examination and descriptions of the new species will be undertaken in future research.

2.5. Phylogenetic analysis of Australia-wide data

A second phylogenetic analysis was conducted on a combined data set of all available parabathynellid sequences from Australia. This Australia-wide data set comprised putative species representatives from

(caption on next page)

Fig. 2. Bayesian consensus tree of concatenated data (*COI, 12S, 18S*) for 73 individuals from the Pilbara, including final putative species divisions (Putative species). Outgroups included in the analyses not displayed. Coloured squares match geographic locations in Fig. 1. Black bars summarise delineation results. For *COI*, 1 = ABGD (initial) 2 = ABGD (recursive), 3 = bPTP, and for *12S* 1 = ABGD JC69 and Kimura K80 (initial and recursive), ABGD simple distance (initial), 2 = ABGD simple distance (recursive), 3 = bPTP. A more detailed summary of species delineation results is included in Supplementary Material Table S3. An X indicates an unavailable gene, * indicates sequences for which only *18S* was available, and grey bars are those that span across unavailable sequences (X's). Node labels are Bayesian posterior probabilities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

our Pilbara-only data set (Fig. 2), new sequences (including 42 sequences from Browns Range, 1000 km north-east of the Pilbara), and also existing (GenBank) sequences of individuals from previous studies (Supplementary Material Table S1). Analyses were conducted in exactly the same way as listed above for the Pilbara sequences.

2.6. Geographic locality data

The location data for specimens were converted to KML files and visualised using Google Earth v7.1 (Google Inc., 2017) and ArcMap v10.3.1 (ESRI, 2017). An indication of the underlying geology was added to maps using the open access lithostratigraphy layers in the Australian Geoscience Information Network Geoscience Portal (2017). Bores were coloured according to putative species sampled.

3. Results

3.1. Nucleotide analysis of Pilbara-only data

Specimens of parabathynellids were available for 32 boreholes throughout the Pilbara and sequenced for all three genes (*COI, 12S, 18S*) (Supplementary Material Table S1). A 609 bp fragment of the *COI* mtDNA gene was sequenced for 44 individuals (number of haplotypes (h) = 33). Of these *COI* sequences, 26 haplotypes were singletons and seven were represented by multiple individuals (for a list of all haplotypes see Supplementary Material Table S2). A 343 bp fragment of the *12S* rRNA gene was sequenced for 56 individuals (h = 26). Fifteen haplotypes were represented by multiple individuals and 11 were singletons (Supplementary Material Table S2). Thirty-eight individuals were sequenced for 1684 bp of the *18S* rRNA gene. Of these, 32 were distinct haplotypes, with 28 singletons and four found in multiple individuals (Supplementary Material Table S2). Concatenation of sequence data for the three genes resulted in an alignment of 74 sequences, 2,636 bp long. Sequence data from Pilbara individual WAMC57170 (putative species 24) were not included in the Pilbara-only analyses because of the high observed genetic distance compared to the other Pilbara parabathynellid sequences.

3.2. Pilbara genetic divergence and species delineation

A summary of molecular species delineation results and their geographic locations for putative species 1–23 are shown with the Bayesian consensus tree of concatenated data (*COI, 12S, 18S*) for all but putative species 24 of the Pilbara putative species (Fig. 2 and Supplementary Material Table S3). The following delineation methods were used for individual gene trees: *COI* (ABGD, bPTP, 7.1% threshold, 11% threshold and 17% threshold (Supplementary Material Table S3)); *12S* (ABGD and bPTP), and also an appraisal of monophyletic groups in the *18S* Bayesian tree topology (Supplementary Material Fig. S3). The putative species estimates varied between 10 and 21 for *COI* and 9–19 for *12S* (see Fig. 2 and Supplementary Table S3). Despite some differences in delineation results between genes, especially when data were missing, delineation methods showed the same major groupings (Fig. 2, Supplementary Material Table S3 and Supplementary Material Figs. S1–S6)). Final putative species numbers were arrived at using a final consensus of all methods as listed in the Materials and Methods (see Fig. 2 for final consensus summary).

For the 24 putative Pilbara species that were delineated based on the final consensus of all molecular species delineation methods (see results below; Fig. 2 and Supplementary Material Table S3), we observed *COI* genetic distances among putative species of 6.4–36% (Supplementary Material Table S4). It should be noted that Pilbara putative species 8, 9 and 10 were delineated by ABGD and bPTP methods for the *COI* gene, but did not meet the minimum 7.1% divergence threshold with 6.2–6.4% divergences between these three species. *COI* variation within putative species was usually less than 1%, and putative species 17 had the highest variation within a species at 2.8% (Supplementary Material Table S4). The genetic distances for *12S* among putative species had a range of 2–44%, and 0–1% within putative species (Supplementary Material Table S5). *18S* variation between putative species was unsurprisingly low at 0.1–6% (Supplementary Material Table S6).

3.3. Australia-wide phylogeny of Parabathynellidae

The phylogenetic analyses of all parabathynellids from Australia (see Fig. 3 for locality map) included sequence data from 54 taxa representing 10 known genera (Fig. 4; see also Supplementary Material Table S1). Our analyses showed the Pilbara individuals formed three separate clades, a Pilbara-only clade (putative species 1–19); four haplotypes (putative species 20–23) that grouped with, but were not necessarily the same species as, various *Atopobathynella* species (*Atopobathynella* sp. 5 (Pilbara)) (PP 85–100%), *Atopobathynella* sp. 7, *At. glenayleensis*, *At. hinzeae*, *At. wattsi* from the Yilgarn (PP 69%), and *Atopobathynella* sp. 3 (Pilbara) (PP 100%)); and finally one individual, WAMC57170 (putative species 24), which grouped with a *Hexabathynella* individual (SA) (PP 100%) (Fig. 4). Putative species 13 grouped very closely to Lineage B from Abrams et al. (2013), species 21 grouped with *At.* sp. 5 and species 23 with *At.* sp. 3, the latter two from Abrams et al. (2012), respectively. Putative species 2, 6 and 18 were not included in the Australia-wide phylogeny as only one gene was successfully sequenced for these species, and because the missing data adversely affected estimates of tree topology in the final phylogeny (results not shown).

The other newly-sequenced individuals from around Australia (*Chillibathynella* sp. 3, *C.* sp. 4, *Notobathynella* sp. 1, *Kimberleybathynella* sp.2, *K.* sp.3, *K.* sp.4, *A.* sp.7, *Arkaroolabathynella* sp. 1, *Ar.* sp. 2, *Billibathynella*. sp. 3, *Brevisomabathynella parooensis*, *Br. cunyuensis*, *At. wattsi*, *At. hinzeae* grouped as expected with representatives from respective genera (based on GenBank data). Further, of the 42 individuals from 14 Browns Range (north-west WA) bores, 30 sequences (h = 15) were obtained for *COI*; 42 individuals (h = 20) were obtained for *12S* rRNA; and 30 sequences (h = 9) for the *18S* rRNA gene. All haplotypes from Browns Range formed a single clade that was nested within *Atopobathynella* (PP 100%).

3.4. Geographic patterns

In total, 88% of putative species from the Pilbara were restricted to a point locality, i.e. a single borehole. The *Hexabathynella* specimen (putative species 24) was one of the most genetically distinct individuals (Supplementary Material Table S4), and also came from one of the most geographically isolated bores (Bore C23) (Fig. 1A). However, there were three key exceptions to this pattern of very short ranges. All *12S* haplotypes of putative species 3 (57109–11, 57113–16, 57181, 57184) were found in four bores (A47, A59, A74, A89) that had

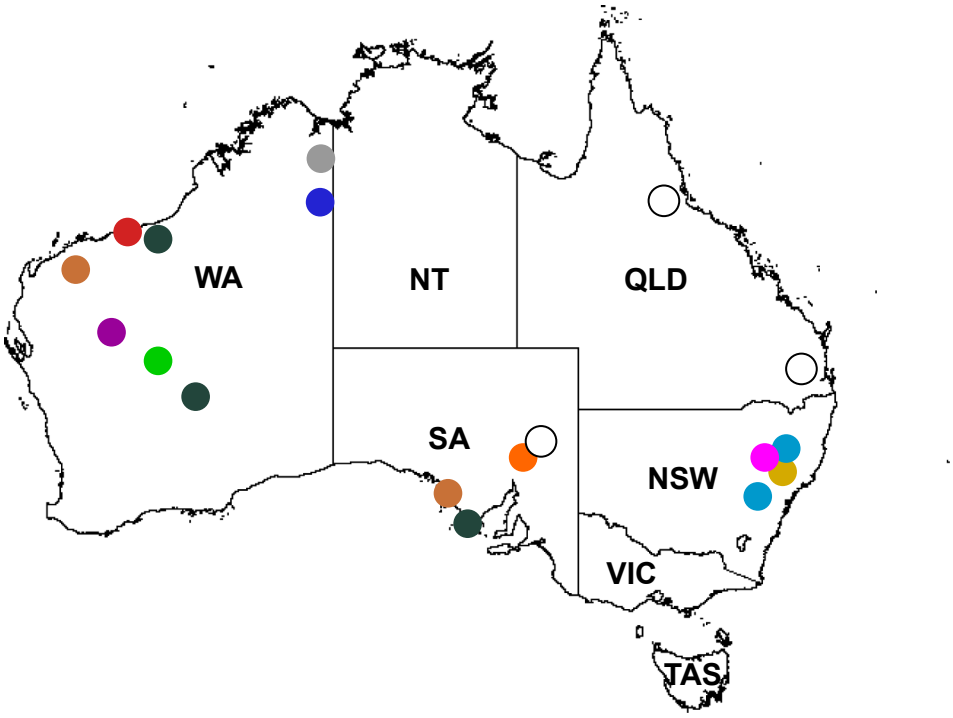

Fig. 3. Map of Australia shows locations of known and unknown Lineages A-C of Parabathynellidae and colours represent all groups examined in the present study: Red - Pilbara putative species; Green - *Brevisomabathynella*; Light Blue - *Chilibathynella*; Purple - *Billibathynella*; Pink - *Octobathynella*; Orange - *Arkaroolabathynella*; Light Green - *Lockyerenella*; Dark Blue - Browns Range haplotypes; Dark Green – *Atopobathynella* (*sensu lato* (*s.l.*)), i.e. in the broad sense); Grey - *Kimberleybathynella*; Yellow - *Notobathynella*; Brown - *Hexabathynella*, Black and open circles represent unnamed genera. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a linear range of ~12 km, with potential geological connectivity via an alluvium/colluvium network (Fig. 1C, light blue dots, Supplementary Material S1). Haplotypes of putative species 6 (57139–41, 57153–56) were found in two bores (FT1, F08) that were extremely close together, i.e. 2.5 km (Fig. 1B, pale pink dots, Supplementary Material S1). Finally, *COI* haplotype WAMC57195 and also *18S* haplotype WAMC54549 for putative species 13 were variously found in five bores (B29, B33, B38, F39, FC1) (Fig. 1D, dark green dots). Individuals shared closely related haplotypes across 3.5 km (B29, B33, B38) and even 250 km (FC1, F39) and, in the most extreme examples, shared the same haplotypes across 140 km. For instance, we observed a single haplotype shared among individuals from three key locations (bores B38, FC1 and F39). Bore B38 was 118 km from bore FC1 and 140 km from bore F39 in the opposite direction. Shared haplotypes across broad geographic locations for putative Species 13 were: the *18S* haplotype WAMC54549, which was found 118 km apart at bores FC1 (individual WAMC54549) and B38 (individual WAMC57196), and also the *COI* haplotype, WAMC57195 (represented by individuals WAMC57195 and WAMC57196 at bore B38), was sampled 140 km away at bore F39 (individual WAMC57202).

4. Discussion

The Pilbara has been identified as a region of high diversity for subterranean fauna (Eberhard et al., 2005; Guzik et al., 2008; Halse et al., 2014). Based on the results from this study, it is now clear that parabathynellids also comprise a significant component of this biodiversity, serving as bioindicators and potentially performing ecosystem services (Boulton et al., 2008; Hose and Stumpp, 2019). Using a consensus of multiple molecular species delineation approaches, monophyletic lineages and geographic isolation as criteria to delimit species (Fig. 2), we identified up to 24 putative new species at 32 point locations (bore holes) within the Pilbara bioregion (180,000 km^2) (Thackway and Cresswell, 1995). Based on the inclusion of our new putative species into an Australia-wide phylogeny of parabathynellids, it seems likely that these new Pilbara taxa are closely related to two known genera, *Atopobathynella* and *Hexabathynella*, and form one distinct clade (Pilbara-only clade Fig. 4). Our findings reveal a substantial increase in parabathynellid diversity for the Pilbara and Australia, which is likely to have only just scratched the surface of the full extent of their biodiversity even within this bioregion, given its broad geographic range and sparse sampling. These new species significantly expand the known diversity of Parabathynellidae in that they comprise a 22% increase to the 109 currently recognised species globally (the World Register of Marine Species database 2018).

4.1. New putative Pilbara parabathynellid species

Here we present a novel data set for new Australian parabathynellids. We have a minimum of eight delineated species based on results for when data from all three loci were available. These eight species (putative species 3, 8, 14, 16, 18, 20, 21, 24 (WAMC7170)) are the most conservative estimate of putative species for Pilbara parabathynellids based on the data presented here and met the criteria outlined under the unified species concept (de Queiroz, 2007). When all genes and all haplotypes were analysed, irrespective of missing data, a maximum of 24 new putative species from the Pilbara were identified using multiple species delineation approaches (Fig. 2 and Supplementary Material Table S3). These final putative species numbers were derived based on three criteria that provide evidence towards a unified species concept (de Queiroz, 2007): (a) the consensus of molecular species delimitation results, (b) monophyly of lineages based on tree topologies for resolved nodes and (c) geographic isolation (i.e. distance and geology) (as represented by colours in Fig. 1 and phylogenies in Fig. 2 and Supplementary Material Figs. S1–S6), were also used to corroborate species divisions. In molecular species delineation analyses, the ABGD and bPTP methods were largely consistent across the datasets, but at a minimum genetic divergence threshold of 7.1% (Abrams et al., 2012) for *COI*, three putative species; 8, 9 and 10 could not be separated (divergent in *COI* by 6.2–6.4%). Such divergences may reflect phylogeographic differentiation rather than fixed genetic differences between species, but with the current data this hypothesis is difficult to test. Some differences in delineation results were also observed between genes (putative species 10–21 for *COI* and 9–19 for *12S*) with the sources of these differences predominantly being missing data. For example, putative species 6 and 18 were sequenced for *12S*,

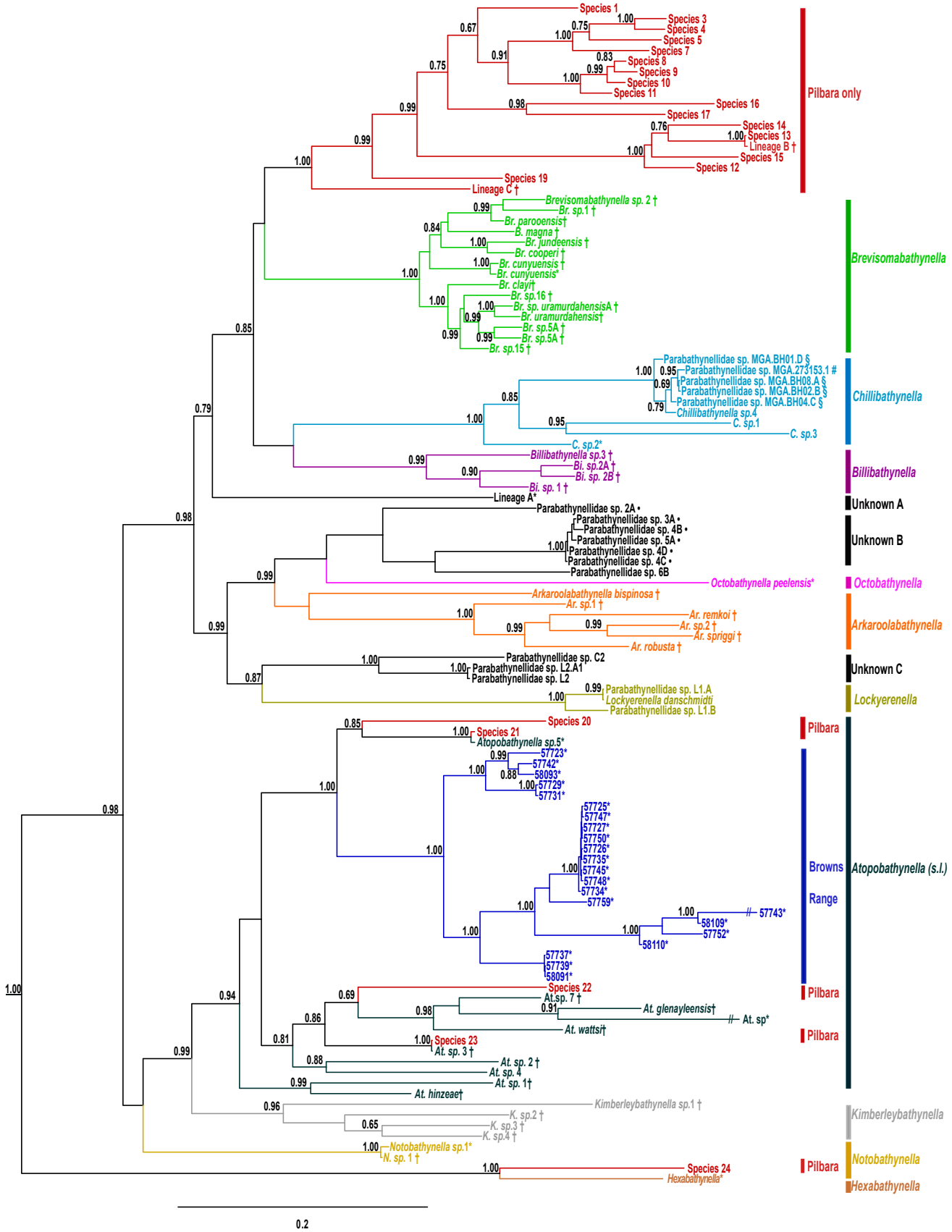

Fig. 4. Bayesian consensus tree of representative concatenated data (*COI, 12S, 18S*) for Australian Parabathynellidae from sequences of new Pilbara Putative species 1–19, 20–23 and 24 (as named in Supplementary Material S1 and Fig. 2) and also other published studies (tip symbols (after Supplementary Material S1): †Abrams et al. (2012); § Asmyhr et al., 2014a; # Asmyhr et al., 2014b; • Cook et al. (2012); ♦ Little et al. 2016)). Asterisks indicate sequences new to this study and not from the Pilbara. Outgroups included in the analyses are not displayed. Colours on branches and in text denote known and unknown Lineages A-C – see Fig. 3 for colour reference.

but not *COI*, and each showed high levels of *12S* divergence (> 11%) with other putative species, supporting their designation as distinct species.

For the remaining criteria, monophyly of lineages based on tree topologies for resolved nodes and geographic isolation we observed consistent results with the 24 delineated species. The majority of lineages that represented delineated species were reciprocally monophyletic with support greater than PP 90% for *COI* and 12S trees (Supplementary Material Figs. S1–S6). Geographic isolation was broadly observed for most of the putative species, with 88% of putative species from the Pilbara restricted to a point locality (single borehole), including the *Hexabathynella* specimen (putative species 24) which was genetically distinct and came from one of the most geographically isolated bores (Bore C23) (Fig. 1A). The only putative species that deviated from this pattern of geographic isolation, and showed connectivity of populations over substantial distances, were putative species 3, 6, 13, for which we present explanations below.

As with all molecular species delineation methods, we reiterate the caveat that not all criteria for a species agreed across all methods and genes, however, strong general support was consistent for the delineated species. Despite the minor differences in individual locus delineation results, major groupings for at least eight, but more realistically 22–24 putative species in all of these three gene trees, combined with their monophyly and geographic isolation, have formed a robust hypothesis and basis for examining species-level diversity of parabathynellids in the future using morphological analyses. For ease of interpretation, we will continue to use the estimate of 24 new species from the Pilbara in discussing our key findings with the possibility of as few as eight putative species.

Consistent with previous studies of this family (Guzik et al., 2008; Abrams et al., 2012; Cook et al., 2012; Little et al., 2016) and based on the current sampling, we observed that for the 24 new putative species 88% were found at individual locations indicating that species had narrow distributions (see Fig. 1 for map of species locations and Fig. 2 for phylogenetic relationships among haplotypes). We have observed that of the 24 putative species, seven showed *COI* haplotypes represented by multiple individuals and of these, three species shared haplotypes over areas greater than 3 km. More intensive and broader sampling would help to test these results further. We were also able to establish the endemism of these restricted haplotypes and species to the Pilbara by including them in our expanded Australian parabathynellid phylogeny (see details below). To explain the high number of species we see in the Pilbara, the hydrogeology of the region and the biology of this group is considered below.

The Pilbara has been identified as a centre for biodiversity in subterranean fauna. In their review of stygofauna for this region, Eberhard et al. (2005) identified the following criteria as key determinants of the high regional biodiversity in north-west WA: age of the landscape, existence of suitable subterranean aquatic habitat, and habitat fragmentation. As one of the most ancient and geologically stable regions in the world, the Pilbara has a diverse array of available subterranean habitats (e.g. alluvium/colluvium and calcrete profiles) that are known to yield exceptionally high levels of stygofaunal species richness (Eberhard et al., 2005). Alluvium/colluvium is well-known around the world as ideal habitat for stygofaunal species (Dole-Olivier et al. 1994). Parabathynellids are considered to be poor dispersers, heavily impacted by dispersal barriers (Asmyhr et al., 2014b) and subterranean habitat fragmentation and hydrological barriers, which serve to greatly reduce gene flow. These factors have undoubtedly contributed to high levels of speciation in these isolated habitats (Boulton et al., 2003; Eberhard et al., 2005; Finston et al., 2007; Guzik et al., 2011a,b; Halse et al., 2014). Our discovery of new species in almost every newly sampled location is, thus, well explained by the criteria for stygofaunal biodiversity as postulated by Eberhard et al. (2005), and we predict that future sampling of the Pilbara and north-western Australia will probably continue to increase the number of parabathynellid species for the region.

4.2. Evidence of contemporary connectivity and sampling limitations

River systems associated with the subterranean alluvium/colluvium habitat are known to episodically flood, potentially providing opportunities for dispersal by stygofauna down the catchment. Here we observed divergent putative species 3 and species 6 haplotypes in multiple closely positioned bores (Fig. 1B, pale pink dots, Fig. 2 and Supplementary Material S1)). We also observed shared haplotypes from putative species 13 across 140 km (putative species *COI* haplotype WAMC57195 and *18S* haplotype WAMC54549). These results could be explained as ancestral haplotype retention, however, the presence of a shared haplotype from *COI* across this distance is less straightforward. Stepwise dispersal mediated by cyclonic flooding events have been cited for dispersal of stygobiontic amphipods in the Pilbara, where gene flow within a tributary is thought to be facilitated by downstream dispersal during episodic flooding (Finston et al., 2007). Alternate explanations could include errors in the field or in the laboratory.

Endemism, and in particular, short-range endemism (*sensu* Harvey, 2002), has sometimes been identified as an artefact of insufficient sampling effort (Eberhard et al., 2009). Here we have conducted one of the most intensive studies yet of Australian parabathynellids, but sampling is still likely to have had a substantial impact on the observed frequency of species and haplotypes among locations. Typically, 1–5 individuals per bore were collected and sequenced, thus limiting sample size. However, it needs to be emphasised that sampling of stygofauna from the Pilbara can be exceptionally difficult with sample sizes usually being very low (Eberhard et al., 2009; Halse and Pearson, 2014). Travel and access to sites that are predominantly located on mining leases and sampling aquifers 60–80 m underground can be extremely difficult and prohibitively expensive. Successful collection of parabathynellids can also be problematic because individuals are caught very infrequently using haul net methodology. Haul nets are a targeted sampling approach (Allford et al., 2008), but collecting individuals of a particular type (i.e. parabathynellids) is largely random. To confirm species estimates into the future it will be critical to sample more exhaustively both within and between current and new bore locations with a view to sampling as many species as possible.

4.3. Molecular phylogeny of Australian Parabathynellidae

To provide context to our new putative species from the Pilbara, we incorporated their sequences into a phylogenetic analysis of available GenBank sequences from 10 known clades from around Australia (Fig. 4), sequences from additional individuals from Browns Range and elsewhere in Australia, and three unknown parabathynellid clades (Lineage A-C Fig. 4). This analysis (Fig. 4) confirms the uniqueness of the Pilbara fauna and builds significantly on the existing pool of known parabathynellid reference sequences. First, we observed a major clade that contained 16 species endemic to the Pilbara region (Pilbara-only), representing putative species 1–19, excluding species 2, 6 and 18 which were not included in the larger phylogeny. In this clade, multiple delineated species based on molecular data grouped together within a reciprocally monophyletic clade, with a distinct position in the phylogeny relative to all other parabathynellid genera, and with strong posterior probability support (PP 100%), as well as geographic isolation within the Pilbara bioregion. Putative species 2, 6 and 18 were also members of this Pilbara-only clade given their close relationships to putative species 1, 7 and 19 respectively (Fig. 2). Lineages B and C were also observed within the Pilbara-only clade. Sequenced prior to the current study (Abrams et al., 2013), these individuals were sampled from a location very close to all others that we have sequenced from the Pilbara (Supplementary Material Table S1). Second, three putative species were found to group closely with representatives of *Atopobathynella* (putative species 20–23). Third, we found a single putative

new species that closely grouped to *Hexabathynella* spp. (putative species 24), and finally we observed a second major clade that contained a number of likely new species from Browns Range, nested within *Atopobathynella*. All of the relevant nodes were well supported (PP 94–100%) and showed that, based on the current data, there are possibly two existing genera represented in the Pilbara (i.e. *Atopobathynella* and *Hexabathynella*) and a new Pilbara-only clade. Species from the Pilbara-only clade spanned the breadth of the sampled sites across all major groundwater drainages in the Pilbara. The remaining putative species 20–23 and the Browns Range clade formed relationships with known and undescribed species of the broader *Atopobathynella* lineage (*Atopobathynella sensu lato* (*s.l.*)) but were not reciprocally monophyletic. Putative species 20–21 grouped most closely with *Atopobathynella* sp. 5 and the Browns Range clade (PP 100%).

Our results of restricted phylogeography for parabathynellids are consistent with results for other syncarids in the Pilbara (Perina et al., 2018), elsewhere in Australia (e.g. Cho et al., 2005, 2006a; Camacho and Hancock, 2010a; Abrams et al., 2013; Little and Camacho, 2017) and on other continents (Schminke, 2011; Camacho et al., 2012). However, our Australia-wide phylogeny showed that *Atopobathynella* (previously known from only one location in the Pilbara (Abrams et al., 2012)) and *Hexabathynella* (previously unknown from the Pilbara) are possibly significantly expanded. *Atopobathynella* species formed a monophyletic group of representatives from the Yilgarn (central WA), SA, and now the Pilbara (Fig. 4). The widespread distribution of this genus is also supported by their characteristic morphology (Cho et al., 2006b) with the genus also being recorded from other locations in Australia (Victoria, Tasmania, Northern Territory), as well as New Zealand, India, and Chile (e.g. Cho et al., 2006b; Abrams et al., 2013; Bandari et al., 2017). For *Hexabathynella*, a strong sister relationship was observed between SA and Pilbara putative species 24, a finding that also potentially suggests long distance historical regional connections for this genus. *Hexabathynella* is described as spanning multiple continents, but the species within this genus are not widespread (Camacho et al., 2014, 2017a,b). Previous studies on Australian members of the genus have alluded to historically widespread distributions of stygobiontic ancestors with a subsequent constriction in range due to climate induced aridification of the landscape and reduction in available groundwater (Byrne et al., 2008; Abrams et al., 2012). Whilst we have not included any sequences of taxa from outside Australia, it is possible that *Atopobathynella* and *Hexabathynella* are monophyletic and widespread in Australia. However, numerous sub-lineages within *Atopobathynella*, in particular, were observed in our Australia-wide phylogeny, which raises the question of whether this genus represents a large, genetically diverse genus, or rather is better treated as numerous smaller genera. These findings of shared genera between regions are possibly not unexpected given the current morphological taxonomy, but they do appear counterintuitive given their low dispersal ability and short-range endemic distributions (see below), which is consistently found across Australia and elsewhere around the world (Schminke, 2011; Camacho et al., 2016). One possible explanation is that these groups historically had broad ancestral distributions and that the morphology of parabathynellid genera is highly conserved (Abrams et al., 2013). Together with the new Pilbara and Browns Range clades and related specimens, taxonomic treatment is now required.

4.4. Distribution patterns of Australian Parabathynellidae

The Australia-wide phylogeny showed that, aside from *Atopobathynella* and *Hexabathynella*, each sampled geographic region maintains its own unique parabathynellid taxa, especially at species, and often at generic level, consistent with previous studies (Guzik et al., 2008, 2011; Abrams et al., 2012, 2013). For instance, *Arkaroolabathynella* appears to be restricted to the Flinders Ranges and surrounds (SA), *Brevisomabathynella* to the Yilgarn and Pilbara (WA) (Cho and Humphreys 2010), *Billibathynella* to the Yilgarn and Pilbara (WA) (Hong and Cho, 2009), *Kimberleybathynella* to the Kimberley and Canning Basin (WA) (Cho et al., 2005), and *Lockyerenella* to Burdekin (QLD) (Little and Camacho, 2017). We incorporated representative haplotypes of all these genera in the current phylogeny, as well as new sequences from genera identified as *Octobathynella* (NSW) and *Chillibathynella* (NSW) and undescribed lineages sequenced from other locations in NSW, QLD, Lineage A (SA), Lineages B and C (Pilbara, WA) and 42 individuals from Browns Range. Significantly, our results have reinforced the premise of other stygobiont crustacean studies in that they display a high degree of regional endemism, as well as high species diversity with less than 9% overlap of species between regions (e.g. Humphreys, 2008; Watts and Humphreys, 2009; Karanovic and Cooper, 2011 and references within).

4.5. Conservation recommendations and conclusions

The Parabathynellidae have been studied from a number of locations around Australia (e.g. Cho et al., 2005; Guzik et al., 2008; Camacho and Hancock, 2010a; Abrams et al., 2012; Asmyhr and Cooper, 2012; Cook et al., 2012; Asmyhr et al., 2014b; Little et al., 2016; Little and Camacho, 2017), and provide an expanding framework that can be used for monitoring and conservation of species and the communities in which they reside. Typically, implications for conservation are dependent on the stygofaunal group and conservation efforts must be considered and be in-line with management priorities. Here, we record numerous new species, most likely many of them with extremely short geographical ranges, and an apparently general inability to disperse. Under these circumstances, even highly localised, small-scale environmental changes could result in the extinction of species. Protection and monitoring of subterranean habitats and their communities is the best way to conserve stygofaunal groups. Inclusion of groundwater dependent ecosystems in conservation planning is highly appropriate. Ongoing monitoring of subterranean systems for negative impacts during developments associated with mining, in particular, is critical for conservation management.

A substantial proportion of the previously recognised and sequenced parabathynellid lineages have already been reliably identified to genus level, and a good number of them have been formally described using morphological criteria. Description of new species is important because the most effective documentation of biodiversity for conservation management includes species names (Costello et al., 2015). However, as is usual for stygofauna, and indeed many invertebrate groups, their formal taxonomy is falling behind as the recognition of molecular Operational Taxonomic Units continues to increase. The relationships we highlight in this study indicate that parabathynellids may be an excellent candidate group for integrative 'turbo' taxonomy (Riedel et al., 2013) as a solution to the taxonomic impediment we see in Australia. Furthermore, the implementation of new high throughput environmental DNA metabarcoding for biodiversity assessment and monitoring is of intense interest for its augmentation of traditional monitoring and survey methods (Thomsen et al., 2012; Bohmann et al., 2014; Deiner et al., 2016; Goldberg et al., 2016). This is especially true for monitoring endangered, rare, and elusive taxa (Yu et al., 2012; Bohmann et al., 2014; Furlan et al., 2016) for stygofauna including Parabathynellidae. A robust Barcode Reference Library (BRL) is essential for effective detection of fauna using metabarcoding methods. Here we have made a significant first step in this direction for Parabathynellidae. Priorities to transform our consensus of species delimitation results into a parabathynellid BRL into the future include morphological verification and taxonomic diagnoses of delineated Pilbara species. Additional verification through more extensive stygofaunal sampling, barcoding of a much broader range of genes, and additional phylogenetic analyses will also be essential.

Declaration of Competing Interest

None.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.106643.

References

Abrams, K., Guzik, M., Cooper, S.J.B., Humphreys, W., King, R., Cho, J.L., Austin, A., 2012. What lies beneath: molecular phylogenetics and ancestral state reconstruction of the ancient subterranean Australian Parabathynellidae (Syncarida, Crustacea). Mol. Phylogenet. Evol. 64, 130–144.

Abrams, K.M., King, R.A., Guzik, M.T., Cooper, S.J.B., Austin, A.D., 2013. Molecular phylogenetic, morphological and biogeographic evidence for a new genus of parabathynellid crustaceans (Syncarida: Bathynellacea) from groundwater in an ancient southern Australian landscape. Invertebrate Syst. 27, 146–172.

Allford, A., Cooper, S.J.B., Humphreys, W.F., Austin, A.D., 2008. Diversity and distribution of groundwater fauna in a calcrete aquifer: does sampling method influence the story? Invertebrate Syst. 22, 127–138.

Asmyhr, M.G., Cooper, S.J.B., 2012. Difficulties barcoding in the dark: the case of crustacean stygofauna from eastern Australia. Invertebrate Syst. 26, 583–591.

Asmyhr, M.G., Hose, G., Graham, P., Stow, A.J., 2014a. Fine-scale genetics of subterranean syncarids. Freshw. Biol. 59, 1–11.

Asmyhr, M.G., Linke, S., Hose, G., Nipperess, D.A., 2014b. Systematic conservation planning for groundwater ecosystems using phylogenetic diversity. PLoS ONE 9 (12), E115132.

Australian Geoscience Information Network (AUSGIN), 2017. AUSGIN Geoscience Portal, powered by AuScope, Available from: http://www.geoscience.gov.au/.

Bandari, E., Shaik, S., Ranga Reddy, Y., 2017. A phylogenetic review of the genus Atopobathynella Schminke, 1973 (Crustacea, Malacostraca, Bathynellacea) with three new species from southeastern India. J. Nat. Hist. 51, 2143–2184.

Bohmann, K., Evans, A., Gilbert, M.T., Carvalho, G.R., Creer, S., Knapp, M., Yu, D.W., de Bruyn, M., 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends Ecol. Evol. 29, 358–367.

Boulton, A.J., Humphreys, W.F., Eberhard, S.M., 2003. Imperilled subsurface waters in Australia: Biodiversity threatening processes and conservation. Aquat. Ecosyst. Health Manage. 6, 41–54.

Bradford, T., Adams, M., Humphreys, W., Austin, A., Cooper, S., 2010. DNA barcoding of stygofauna uncovers cryptic amphipod diversity in a calcrete aquifer in Western Australia's arid zone. Mol. Ecol. Resour. 10, 41–50.

Boulton, A.J., Fenwick, G.D., Hancock, P.J., Harvey, M.S., 2008. Biodiversity, functional roles and ecosystem services of groundwater invertebrates. Invertebrate Syst. 22, 103–116.

Britt, A., Summerfield, D., Senior, A., Kay, P., Huston, D., Hitchman, A., Hughes, A., Champion, D., Simpson, R., Sexton, M., Schofield, A., 2017. Australia's Identified Mineral Resources 2017. Geoscience Australia, Canberra. http://dx.doi.org/10.11636/1327-1466.2017.

Byrne, M., Yeates, D.K., Joseph, L., Kearney, M., Bowler, J., Williams, M.A.J., Cooper, S.J.B., Donnellan, S.C., Keogh, J.S., Leys, R., Melville, J., 2008. Birth of a biome: insights into the assembly and maintenance of the Australian arid zone biota. Mol. Ecol. 17, 4398–4417.

Camacho, A.I., Hancock, P., 2010a. A new genus of Parabathynellidae (Crustacea: Bathynellacea) in New South Wales, Australia. J. Nat. Hist. 44, 1081–1094.

Camacho, A.I., Hancock, P., 2010b. First record of Syncarida from Queensland, Australia, with description of two new species of Notobathynella Schminke, 1973 (Crustacea, Bathynellacea, Parabathynellidae). J. Nat. Hist. 45, 113–135.

Camacho, A.I., Dorda, B.A., Rey, I., 2012. Undisclosed taxonomic diversity of Bathynellacea (Malacostraca: Syncarida) in the Iberian Peninsula revealed by molecular data. J. Crustac. Biol. 32, 816–826.

Camacho, A.I., Dorda, B.A., Rey, I., 2014. Iberian Peninsula and Balearic Island Bathynellacea (Crustacea, Syncarida) database. ZooKeys 386, 1–20.

Camacho, A.I., Brancelj, A., Dorda, B.A., Casado, A., Rey, I., 2016. New Parabathynellidae species in Africa: the first bathynellids from Chad and an assay of their phylogenetic position in the order Bathynellacea (Crustacea: Malacostraca) based on 18S sequences. J. Nat. Hist. 50, 2691–2726.

Camacho, A.I., Dorda, B.A., Chillón, B.S., Rey, I., 2017a. The collection of Bathynellacea specimens of MNCN (CSIC) Madrid: microscope slices and DNA extract. ZooKeys 678, 31–63.

Camacho, A.I., Mas-Peinado, P., Dorda, B.A., Casado, A., Brancelj, A., Knight, L.R., Hutchins, B., Bou, C., Perina, G., Rey, I., 2017b. Molecular tools unveil an underestimated diversity in a stygofauna family: a preliminary world phylogeny and an updated morphology of Bathynellidae (Crustacea: Bathynellacea). Zool. J. Linn. Soc. 183, 70–96.

Camacho, A.I., Mas-Peinado, P., Watiroyram, S., Brancelj, A., Bandari, E., Dorda, B.A., Casado, A., Rey, I., 2018. Molecular phylogeny of Parabathynellidae (Crustacea, Bathynellacea), and three new species from Thai caves. Contrib. Zool. 87, 227–260.

Camacho, A.I., Valdecasas, A.G., 2008. Global diversity of syncarids (Syncarida; Crustacea) in freshwater. Hydrobiologia 595, 257–266.

Cho, J.L., 2005. A primitive representative of the Parabathynellidae (Bathynellacea, Syncarida) from the Yilgarn Craton of Western Australia. J. Nat. Hist. 39, 3423–3433.

Cho, J.L., Park, J.G., Humphreys, W.F., 2005. A new genus and six new species of the Parabathynellidae (Bathynellacea, Syncarida) from the Kimberley region, Western Australia. J. Nat. Hist. 39, 2225–2255.

Cho, J.L., Park, J.G., Reddy, Y.R., 2006a. *Brevisomabathynella* gen. nov. with two new species from Western Australia (Bathynellacea, Syncarida): the first definitive evidence of predation in Parabathynellidae. Zootaxa 1247, 25–42.

Cho, J.L., Humphreys, W.F., Lee, D.S., 2006b. Phylogenetic relationships within the genus *Atopobathynella* Schminke (Bathynellacea: Parabathynellidae). Invertebrate Syst. 20, 9–41.

Cho, J.L., Humphreys, W., 2010. Ten new species of the genus *Brevisomabathynella* Cho, Park and Ranga Reddy, 2006 (Malacostraca, Bathynellacea, Parabathynellidae) from Western Australia. J. Nat. Hist. 44, 993–1079.

Christiansen, K.A., 1961. Convergence and parallelism in cave Entomobryinae. Evolution 15, 288–301.

Cook, B.D., Abrams, K.M., Marshall, J., Perna, C.N., Choy, S., Guzik, M.T., Cooper, S.J.B., 2012. Species diversity and genetic differentiation of stygofauna (Syncarida: Bathynellacea) across an alluvial aquifer in north-eastern Australia. Aust. J. Zool. 60, 152–158.

Costa, F.O., deWaard, J.R., Boutillier, J., Ratnasignham, S., Dooh, R.T., Hajibabaei, M., Herbert, P.D., 2007. Biological identifications through DNA barcodes: the case of the Crustacea. Can. J. Fish. Aquat. Sci. 64, 272–295.

Costello, M.J., Vanhoorne, B., Appeltans, W., 2015. Conservation of biodiversity through taxonomy, data publication, and collaborative infrastructures. Conserv. Biol. 29, 1094–1099.

Deiner, K., Fronhofer, E.A., Machler, E., Walser, J.C., Altermatt, F., 2016. Environmental DNA reveals that rivers are conveyer belts of biodiversity information. Nat. Commun. 7. https://doi.org/10.1038/ncomms12544.

de Queiroz, K., 2007. Species concepts and species delimitation. Syst. Biol. 56, 879–886.

Dole-Olivier, M.J., Marmonier, P., Creuze des Châtelliers, M., 1994. Interstitial fauna associated with the alluvial floodplains of the Rhone River (France). In: Gibert, J., Danielopol, D.L., Stanford, J.A. (Eds.), Groundwater Ecology. Academic Press, San Diego, pp. 314–345.

Eberhard, S., Halse, S., Humphreys, W., 2005. Stygofauna in the Pilbara region, north-west Western Australia: a review. J. Royal Soc. Western Australia 88, 167–176.

Eberhard, S.M., Halse, S.A., Williams, M.R., Scanlon, M.D., Cocking, J., Barron, H.J., 2009. Exploring the relationship between sampling efficiency and short-range endemism for groundwater fauna in the Pilbara region, Western Australia. Freshw. Biol. 54, 885–901.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Edward, K.L., Harvey, M.S., 2008. Short-range endemism in hypogean environments: the pseudoscorpion genera *Tyrannochthonius* and *Lagynochthonius* (Pseudoscorpiones: Chthoniidae) in the semiarid zone of Western Australia. Invertebrate Syst. 22, 259–293.

Environmental Systems Research Institute (ESRI), 2017. ArcMap version 10.3.1., Available from: http://desktop.arcgis.com/en/arcmap/.

Finston, T.L., Johnson, M.S., 2004. Geographic patterns of genetic diversity in subterranean amphipods of the Pilbara, Western Australia. Mar. Freshw. Res. 55, 619–628.

Finston, T.L., Johnson, M.S., Humphreys, W.F., Eberhard, S.M., Halse, S.A., 2007. Cryptic speciation in two widespread subterranean amphipod genera reflects historical drainage patterns in an ancient landscape. Mol. Ecol. 16, 355–365.

Furlan, E.M., Gleeson, D., Hardy, C.M., Duncan, R.P., 2016. A framework for estimating the sensitivity of eDNA surveys. Mol. Ecol. Resour. 16, 641–654.

Giribet, G., Carranza, S., Baguna, J., Riutort, M., Ribera, C., 1996. First molecular evidence for the existence of a Tardigrada + Arthropoda clade. Mol. Biol. Evol. 13, 76–84.
Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P., Gilbert, M., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol. Evol. 7, 1299–1307.
Google Inc., 2017. Google Earth version 7.1.8, Available from: https://www.google.com/earth/download/ge/agree.html.
Guzik, M.T., Abrams, K.M., Cooper, S.J.B., Humphreys, W.F., Cho, J.L., Austin, A.D., 2008. Phylogeography of the ancient Parabathynellidae (Crustacea: Bathynellacea) from the Yilgarn region of Western Australia. Invertebrate Syst. 22, 205–216.
Guzik, M.T., Cooper, S.J.B., Humphreys, W.F., Ong, S., Kawakami, T., Austin, A.D., 2011a. Evidence for population fragmentation within a subterranean aquatic habitat in the Western Australian desert. Heredity 107, 215–230.
Guzik, M.T., Austin, A.D., Cooper, S.J., Harvey, M.S., Humphreys, W.F., Bradford, T., Eberhard, S.M., King, R.A., Leys, R., Muirhead, K.A., 2011b. Is the Australian subterranean fauna uniquely diverse? Invertebrate Syst. 24, 407–418.
Halse, S., Pearson, G.B., 2014. Troglofauna in the vadose zone: comparison of scraping and trapping results and sampling adequacy. Subterranean Biol. 13, 17–34.
Halse, S., Scanlon, M., Cocking, J., Barron, H., Richardson, J., Eberhard, S., 2014. Pilbara stygofauna: deep groundwater of an arid landscape contains globally significant radiation of biodiversity. Rec. Western Australian Museum Suppl. 78, 443–483.
Hancock, P.J., Boulton, A.J., 2009. Sampling groundwater fauna: efficiency of rapid assessment methods tested in bores in eastern Australia. Freshw. Biol. 54, 902–917.
Harrison, S.E., Guzik, M.T., Harvey, M.S., Austin, A.D., 2014. Molecular phylogenetic analysis of Western Australian troglobitic chthoniid pseudoscorpions (Pseudoscorpiones: Chthoniidae) points to multiple independent subterranean clades. Invertebrate Syst. 28, 386–400.
Hasegawa, M., Kishino, K., Yano, T., 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.
Harvey, M.S., 2002. Short-range endemism among the Australian fauna: some examples from non-marine environments. Invertebrate Syst. 16, 555–570.
Harvey, M.S., Berry, O., Edward, K.L., Humphreys, G., 2008. Molecular and morphological systematics of hypogean schizomids (Schizomida: Hubbardiidae) in semiarid Australia. Invertebrate Syst. 22, 167–194.
Harvey, M.S., Rix, M.G., Framenau, V.W., Hamilton, Z.R., Johnson, M.S., Teale, R.J., Humphreys, G., Humphreys, W.F., 2011. Protecting the innocent: studying short-range endemic taxa enhances conservation outcomes. Invertebrate Syst. 25, 1–10.
Hong, S.J., Cho, J.L., 2009. Three new species of *Billibathynella* from Western Australia (Crustacea, Syncarida, Parabathynellidae). J. Nat. Hist. 43, 2365–2390.
Hose, G.C., Stumpp, C., 2019. Architects of the underworld: bioturbation by groundwater invertebrates influences aquifer hydraulic properties. Aquat. Sci. 81, 20.
Humphreys, W., 2001. Groundwater calcrete aquifers in the Australian arid zone: the context to an unfolding plethora of stygal biodiversity. Rec. Western Australian Museum 64, 63–83.
Humphreys, W.F., 2008. Rising from Down Under: developments in subterranean biodiversity in Australia from a groundwater fauna perspective. Invertebrate Syst. 22, 85–101.
Karanovic, T., Cooper, S.J.B., 2011. Third genus of parastenocaridid copepods from Australia supported by molecular evidence (Copepoda, Harpacticoida). Crustaceana Monographs: Studies on Freshwater Copepoda 16, 293–337.
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organisation and analysis of sequence data. Bioinformatics 28, 1647–1649.
Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
Korbel, K.L., Hancock, P.J., Serov, P., Lim, R.P., Hose, G.C., 2013. Groundwater Ecosystems Vary with Land Use across a Mixed Agricultural Landscape. J. Environ. Qual. 42 380-290.
Korbel, K., Chariton, A., Stephenson, S., Greenfield, P., Hose, G.C., 2017. Wells provide a distorted view of life in the aquifer: implications for sampling, monitoring and assessment of groundwater ecosystems. Sci. Rep. 7. https://doi.org/10.1038/srep40702.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T., Calcott, B., 2016a. PartitionFinder 2: new methods for selecting partitioned models of evolution formolecular and morphological phylogenetic analyses. Mol. Biol. Evol. 34, 772–773.
Lanfear, R., Hua, X., Warren, D.L., 2016b. Estimating the effective sample size of tree topologies from Bayesian phylogenetic analyses. Genome Biol. Evol. 8, 2319–2332.
Lefébure, T., Douady, C.J., Gouy, M., Gibert, J., 2006. Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. Mol. Phylogenet. Evol. 40, 435–447.
Little, J., Camacho, A.I., 2017. Morphological and molecular characterisation of a new genus and new species of Parabathynellidae (Crustacea: Syncarida) in Queensland, Australia. Invertebrate Syst. 31, 208–219.
Little, J., Schmidt, D.J., Cook, B.D., Page, T.J., Hughes, M.J., 2016. Diversity and phylogeny of south-east Queensland Bathynellacea. Aust. J. Zool. 64, 36–47.
Masters, B.C., Fan, V., Ross, H.A., 2011. Species delimitation – a geneious plugin for the exploration of species boundaries. Mol. Ecol. Resour. 11, 154–157.
Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES science gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), pp. 1–8.
Mokany, K., Harwood, T.D., Halse, S.A., Ferrier, S., 2019. Riddles in the dark: Assessing diversity patterns for cryptic subterranean fauna of the Pilbara. Divers. Distrib. 25, 240–254.
Noodt, W., 1963. Estudios sobre Crustaceos de aguas subterraneas, III. Crustacea. Syncarida de Chile Central. Investigaciones Zoológicas Chilenas 10, 151–167.
Noodt, W., 1965. Natürliches System und Biogeographie der Syncarida (Crustacea, Malacostraca). Gewässer und Abwässer 37 (38), 77–186.
Perina, G., Camacho, A.I., Huey, J., Horwitz, P., Koenders, A., 2018. Understanding subterranean variability: the first genus of Bathynellidae (Bathynellacea, Crustacea) from Western Australia described through a morphological and multigene approach. Invertebrate Syst. 32, 423–447.
Pipan, T., Culver, D.C., 2012. Convergence and divergence in the subterranean realm: a reassessment. Biol. J. Linn. Soc. 107, 1–14.
Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Mol. Ecol. 21, 1864–1877.
Rambaut, A., 2009. FigTree v1.4.3 https://github.com/rambaut/figtree/releases/tag/v1.4.3 (accessed 30 May 2019).
Rambaut, A., Suchard, M.A., Xie, D., Drummond, A.J., 2015. Tracer v1. 6. 2014 Computer program and documentation distributed by the author. https://github.com/beast-dev/tracer/releases/tag/v1.6 (accessed 30 May 2019).
Reeves, J.M., De Deckker, P., Halse, S.A., 2007. Groundwater ostracods from the arid Pilbara region of northwestern Australia: distribution and water chemistry. In: Ostracodology—Linking Bio-and Geosciences. Springer, Dordrecht, pp. 99–118.
Riedel, A., Sagata, K., Suhardjono, Y.R., Tänzler, R., Balke, M., 2013. Integrative taxonomy on the fast track-towards more sustainability in biodiversity research. Front. Zool. 10 (1), 15.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Schminke, H.K., 1972. *Hexabathynella halophila* gen. n., sp. n. und die Frage nach der marinen Abkunft der Bathynellacea (Crustacea: Malacostraca). Mar. Biol. 15, 282–287.
Schminke, H.K., 1973. Evolution, system und verbreitungsgeschichte der familie Parabathynellidae (Bathynellacea, Malacostraca). Mikrofauna des Meeresbodens 24, 219–408.
Schminke, H.K., 2011. Arthropoda: Crustacea: Malacostraca: Bathynellacea Parabathynellidae. In: Invertebrate Fauna of the world. Vol. 21. Seo-gu Incheon (Republic of Korea): National Institute of Biological Resources, pp. 1–244.
Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mictochondrial gene sequences and a compilation of polymerase chain reaction primers. Entomological Soc. Am. 87, 651–701.
Smith, R.J., Paterson, J.S., Launer, E., Tobe, S.S., Morello, E., Leijs, R., Marri, S., Mitchell, J.G., 2016. Stygofauna enhance prokaryotic transport in groundwater ecosystems. Sci. Rep. 6. https://doi.org/10.1038/srep32738.
Summerfield, D., 2016. Australian Resources Review: Iron. Geoscience Australia, Canberra, Australia.
Tavaré, S., 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Lectures on Mathematics in the Life Sciences, American Mathematical Society 17, 57–86.
Thackway, R., Cresswell, I.D. (Eds.), 1995. An Interim Biogeographic Regionalisation for Australia: A Framework for Establishing the National System of Reserves, Version 4.0. Australian Nature Conservation Agency, Canberra.
Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, T.P., Orlando, L., Eske, W., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. 21, 2565–2573.
Yu, D.W., Ji, Y., Emerson, B.C., Wang, X., Ye, C., Yang, C., Ding, Z., 2012. Biodiversity soup: metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. Methods Ecol. Evol. 3, 613–623.
Villesen, P., 2007. FaBox: an online toolbox for fasta sequences. Mol. Ecol. Notes 7, 965–968.
Watts, C.H.S., Humphreys, W.F., 2009. Fourteen new Dytiscidae (Coleoptera) of the genera *Limbodessus* Guignot, *Paroster* Sharp, and *Exocelina* Broun from underground waters in Australia. Trans. Royal Soc. South Australia 133, 62–107.
Wiemer, D., Schrank, C.E., Murphy, D.T., Wenham, L., Allen, C.M., 2018. Earth's oldest stable crust in the Pilbara Craton formed by cyclic gravitational overturns. Nat. Geosci. 11 (5), 357.
Wetzer, R., 2001. Hierarchical analysis of mtDNA variation and the use of mtDNA for isopod (Crustacea: Peracarida: Isopoda) systematics. Contrib. Zool. 70, 23–40.
Whiting, M.F., Carpenter, J.C., Wheeler, Q.D., Wheeler, W.C., 1997. The Strepsiptera problem: phylogeny of the holometabo-lous insect orders inferred from 18S and 28S ribosomal DNAsequences and morphology. Syst. Biol. 46, 1–68.
Wilson, G.D.F., 2003. A new genus of Tainisopidae fam. nov. (Crustacea: Isopoda) from the Pilbara Western Australia. Zootaxa 245, 1–20.
World Register of Marine Species 2018 Parabathynellidae Noodt, 1965. Accessed at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=390954 on 2018-12-18.
Zhang, J., Kapli, P., Pavlidis, P., Stamatakis, A., 2013. A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29, 2869–2876.