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What lies beneath: Molecular phylogenetics and ancestral state reconstruction of the ancient subterranean Australian Parabathynellidae (Syncarida, Crustacea)

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ABSTRACT

The crustacean family Parabathynellidae is an ancient and significant faunal component of subterranean ecosystems. Molecular data were generated in order to examine phylogenetic relationships amongst Australian genera and assess the species diversity of this group within Australia. We also used the resultant phylogenetic framework, in combination with an ancestral state reconstruction (ASR) analysis, to explore the evolution of two key morphological characters (number of segments of the first and second antennae), previously used to define genera, and assess the oligomerization principle (i.e. serial appendage reduction over time), which is commonly invoked in crustacean systematics. The ASR approach also allowed an assessment of whether there has been convergent evolution of appendage numbers during the evolution of Australian parabathynellids. Sequence data from the mtDNA *COI* and nDNA *18S* rRNA genes were obtained from 32 parabathynellid species (100% of described genera and ~25% of described species) from key groundwater regions across Australia. Phylogenetic analyses revealed that species of each known genus, defined by traditional morphological methods, were monophyletic, suggesting that the commonly used generic characters are robust for defining distinct evolutionary lineages. Additionally, ancestral state reconstruction analysis provided evidence for multiple cases of convergent evolution for the two morphological characters evaluated, suggesting that caution needs to be shown when using these characters for elucidating phylogenetic relationships, particularly when there are few morphological characters available for reconstructing relationships. The ancestral state analysis contradicted the conventional view of parabathynellid evolution, which assumes that more simplified taxa (i.e. those with fewer-segmented appendages and setae) are derived and more complex taxa are primitive.

1. Introduction

Although traditional morphological taxonomy has been used to infer species relationships for over 200 years, morphological approaches can be confounded by factors such as convergence and the presence of highly adaptive forms, resulting from strong and sometimes unusual selection pressures (Wiens et al., 2003; Daniels et al., 2006; Schönhofer and Martens, 2010). Modern approaches combining molecular data with morphological data have, in some cases, been able to overcome these confounding factors (e.g. Wahlberg et al., 2005; Edgecombe and Giribet, 2006; Pretti et al., 2009). Additionally, the use of molecular sequence data to reconstruct species relationships has made it possible to test the pattern of evolution for particular traits and identify ancestral character states and evolutionary history across taxa (Vanderpoorten and Goffinet, 2006; Schäffer et al., 2010), as well as revealing previously unrecognised levels of diversity (Wahlberg et al., 2005; Schäffer et al., 2010). Such analyses have highlighted that certain morphological traits may be ineffective for elucidating systematic relationships (e.g. counts of appendage segments, spines and setae in centropagid copepods (Adamowicz et al., 2007) and fairy shrimps (Weekers et al., 2002). There is, therefore, a need for additional studies that explore character state evolution in taxa where convergent evolution may be a confounding factor for systematics. Here we report on a study of parabathynellids of the crustacean superorder Syncarida.

Syncarida has fascinated and puzzled researchers since its discovery, because of its rarity and unique combination of characters, especially the complete lack of a carapace or carapace shield, which is unusual for malacostracan crustaceans (McLaughlin,

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1980). Of the two extant orders within the Syncarida (Anaspidacea, Bathynellacea), the Bathynellacea are an ancient lineage, which has maintained a 'primitive' morphology since the Carboniferous (Schminke, 1974). Nearly all bathynellaceans inhabit groundwater habitats in the interstitial spaces between sand grains (in caves, wells, springs and river beds) (Camacho and Valdecasas, 2008). Adaptation to interstitial habitats has constrained the size of bathynellaceans so that most are only 1–3 mm in length and they are often highly vermiform. Of the two families within the Bathynellacea, Bathynellidae and Parabathynellidae, the latter is better studied due to their ecological and morphological diversity (Schminke, 1974).

Parabathynellidae occurs on all continents except Antarctica, with 10 of 45 genera described from at least two continents (Camacho, 2006). However, most species have only been collected from one or a few localities contained within a limited area and nearly half of all parabathynellid genera are monotypic (Camacho and Valdecasas, 2008) (see Supplementary Table A). Further, extreme morphological simplification in parabathynellids has caused difficulties in defining genera (Camacho, 2005) and, consequently, assigning species because morphological convergence has likely obscured true phyletic ancestry and diversity. The characters typically used to define parabathynellid genera and species are the number of antennal and antennular segments, and the structure of the mouthparts and male thoracopod VIII (Schminke, 1973; Cho and Humphreys, 2010). Unique combinations of these characters define genera and species, but individually these characters do not seem to delineate species. This is exemplified by the widespread genus *Notobathynella* Schminke, 1973 which comprises species with a wide range of characters, overlapping those used to define other genera (Camacho and Hancock, 2011). Such a broad generic diagnosis makes it nearly impossible to systematically group genera and species in a meaningful way and elucidate phylogenetic relationships within the family. The number of segments of particular appendages has not only been used to define genera, but also to assess intergeneric relationships and determine primitive versus derived taxa. The oligomerization principle (i.e. serial appendage reduction over time), which is commonly invoked in crustacean systematics (Adamowicz et al., 2007), has also been used to infer which state is primitive or derived for a particular character, with many segments regarded as primitive and few segments considered to be derived. Since parabathynellids are highly convergent in morphology and relatively simplified compared with other malacostracans, it remains to be determined whether these and other morphological characters used to define genera and species are homoplastic, and consequently, not useful for inferring phylogenetic relationships.

Historically, the described Parabathynellidae were dominated by northern hemisphere taxa (Noodt, 1965); (Schminke and Noodt, 1988; Camacho et al., 2000). However, a recent increase in the discovery of groundwater fauna, particularly in Western Australia (Humphreys, 2008; Humphreys et al., 2009; Guzik et al., 2011a), has substantially boosted the study of parabathynellids from the southern hemisphere (Guzik et al., 2008; Hong and Cho, 2009; Camacho and Hancock, 2010; Cho and Humphreys, 2010). Of the eight genera (40 species) described from Australia, four are described from widely distributed genera (*Atopobathynella* Schminke, 1973, *Chilibathynella* Noodt, 1963, *Hexabathynella* Schminke, 1972, *Notobathynella*), while four are recently discovered and endemic to Australia (*Billibathynella* Cho, 2005, *Brevisomabathynella* Cho et al., 2006b, *Kimberleybathynella* Cho et al., 2005 and *Octobathynella* Camacho and Hancock, 2010). Interestingly, these genera are likely only to be the tip of a 'taxonomic iceberg' in terms of Australian parabathynellid diversity, as shown in recent studies by Guzik et al. (2008, 2011a). To date only one comprehensive molecular phylogenetic study has explored parabathynellid systematic relationships. Guzik et al. (2008) used molecular data from the *Cytochrome c Oxidase subunit 1* (*COI*) gene to investigate the diversity and phylogeography of parabathynellids within the arid Yilgarn region of Western Australia. This area has been shown to be a biodiversity hotspot for stygofauna (Humphreys, 2008; Humphreys et al., 2009). The Guzik et al. (2008) study uncovered seven putative new species with highly restricted distributions from the genera *Billibathynella* and *Brevisomabathynella*, and also drew attention to the difficulties of using morphology to elucidate the phylogenetic relationships among genera and species in this group.

The present study aims to investigate the diversity and evolution of Australian parabathynellids. In particular, we investigate the phylogenetic relationships amongst Australian parabathynellid genera using sequence data from the nuclear *18S* ribosomal RNA (rRNA) and mitochondrial *COI* genes. This phylogeny builds on the earlier work of Guzik et al. (2008), which solely examined Yilgarn species and genera, by increasing the distribution to Australia wide and including additional taxa and an additional marker. A further aim is to use the resultant phylogenetic framework, in combination with an ancestral state reconstruction analysis, to explore the evolution of two key morphological characters, previously used to define genera, and assess whether there had been convergent evolution of appendage numbers during the evolution of Australian parabathynellids. We also identify potentially new species using a combination of criteria such as degree of genetic divergence and distinctive morphological differences (see methods for more detail). Although this study is based solely on Australian taxa, our findings have broader implications for parabathynellid systematics at a global level.

2. Methods

2.1. Sampling

Parabathynellids were collected from various localities across Australia (Fig. 1), with a large proportion of the specimens being collected from calcrete aquifers (Fig. 2) in the Yilgarn region of Western Australia, but also other habitat types including the hyporheic zones and alluvial aquifers associated with the Hunter and Peel Rivers (New South Wales), and springs in the Flinders Ranges (South Australia). Sampling consisted of a combination of netting and pumping (following the same regimes of Cooper et al. (2007) and Hancock and Boulton (2008)). Locations of the sampled individuals are listed in Table 1. Where possible, multiple individuals per location were sequenced, to control for the possibility of sequencing errors and contamination. After ensuring that our sequence data were robust, identical sequences were excluded from the phylogenetic and character state analyses.

2.2. Criteria for assessing new species and genera

To assess species, both new and pre-existing, we used a combination of criteria including morphological characters based on previous descriptions, sequence divergence, a sister lineage relationship to two or more defined species (i.e. labelled position in phylogeny in Table 2) and geographical location (following the methods of Guzik et al., 2011a; Table 2). Genera were defined on morphological grounds as per generic diagnoses in the literature (Noodt, 1963; Schminke, 1972, 1973; Cho, 2005; Cho et al., 2005, 2006b; Camacho and Hancock, 2010; Table 3). Characters assessed included the number of segments in the antennule, antennae and thoracopodal exopods, number of spines on the furcal rami and uropodal sympod, the shape of the male thoracopod VIII, as well as the presence or absence of the epipod of thoracopod I and pleopods. Many specimens could be assigned to known genera based on key combinations of diagnostic characters, e.g.

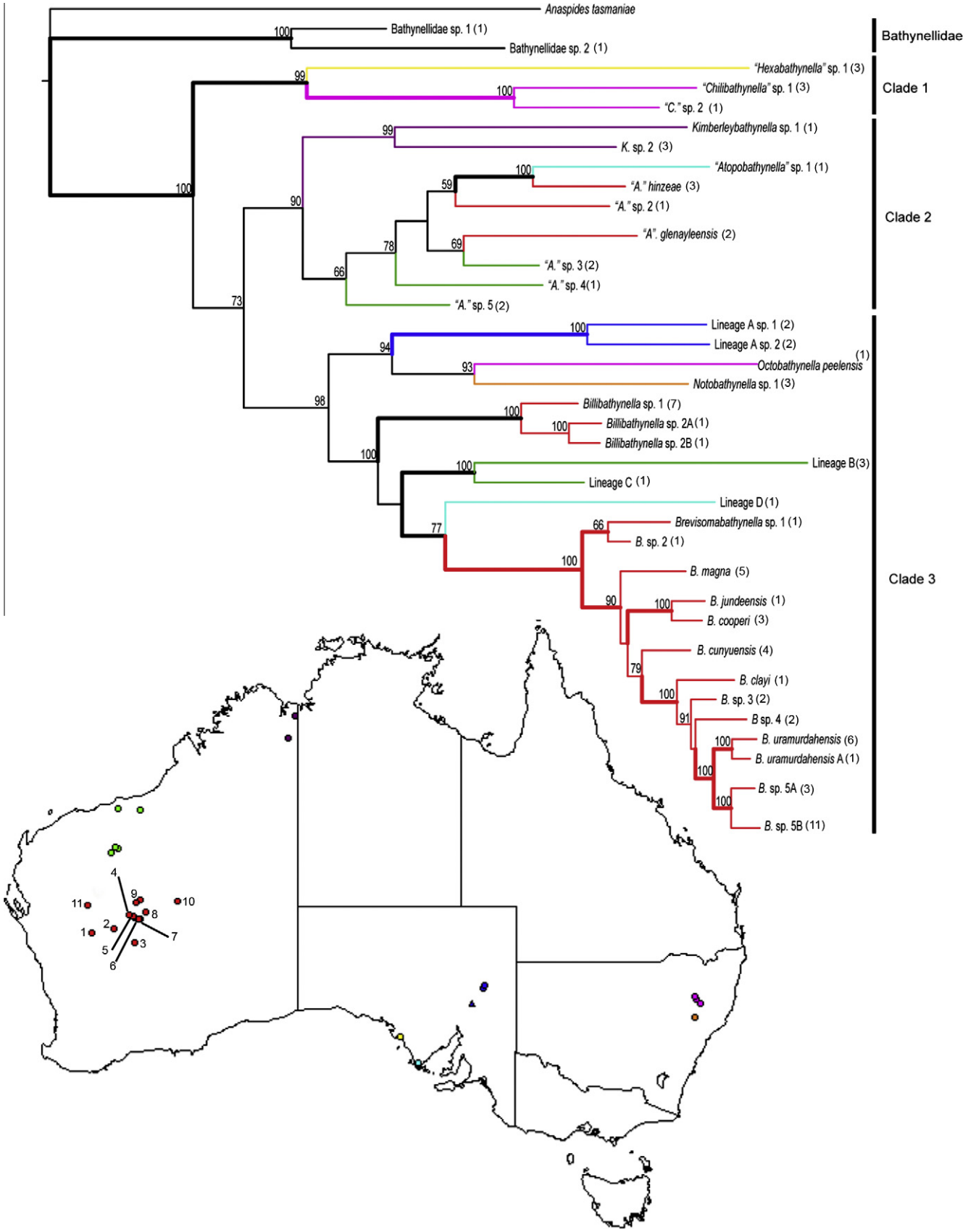

Fig. 1. Posterior probability (majority-rule) Bayesian consensus tree using *COI* and *18S* data with model partitioning, implemented in MRBAYES. Numbers on the nodes are Bayesian posterior probabilities and thicker lines represent nodes supported by Maximum parsimony and/or Maximum likelihood bootstrap values greater than 50%. Numbers in parentheses after taxon labels reflect the number of individuals sequenced to represent each taxon. The map of Australia shows the collection site of each species and the numbers correspond to calcrete numbers shown in Table 1 and Fig. 2. The species in the phylogeny are colour-coded to match the location from which they were collected. Parabathynellids are represented by coloured circles and bathynellids are represented by a blue triangle.

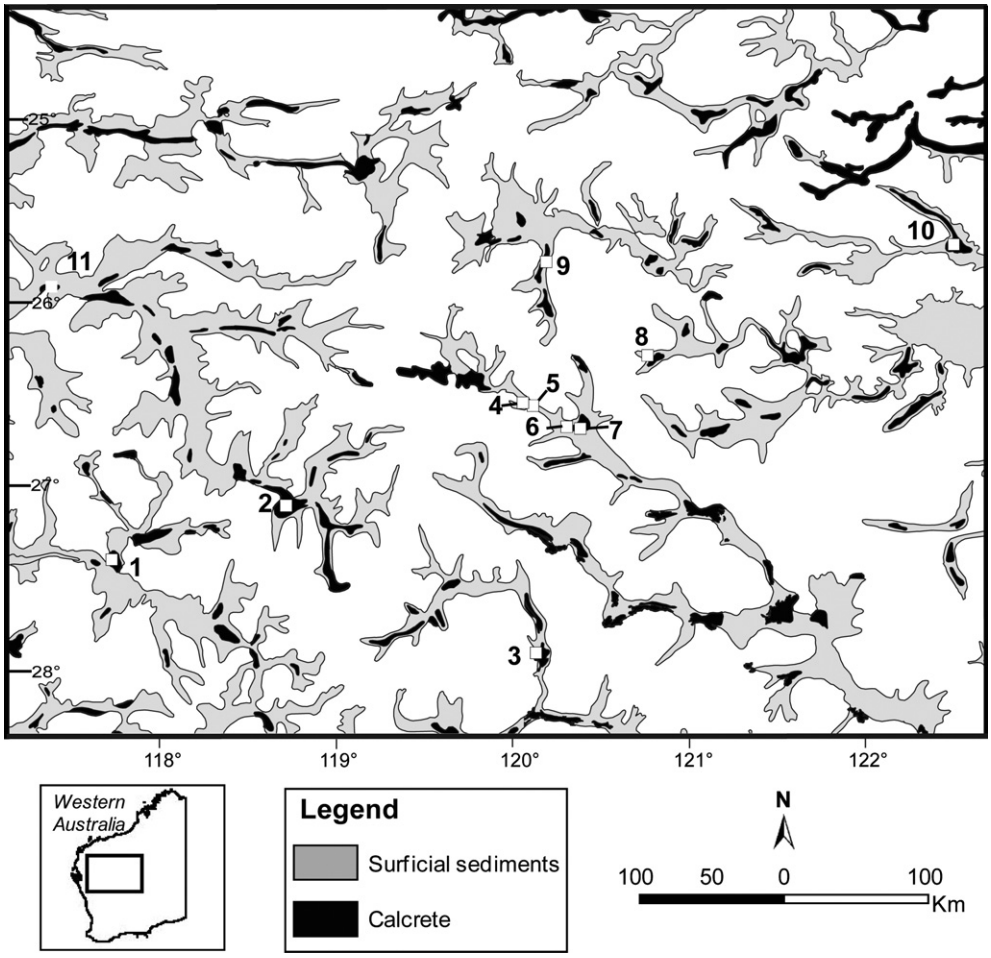

Fig. 2. Map of the northern Yilgarn Region of central Western Australia showing the location of calcretes (black) from which parabathynellids were collected. Grey shaded regions represent surficial sediments in the palaeodrainage systems and these are separated by exposures largely of Precambrian geology. Calcretes are numbered as follows: 1. Austin Downs; 2. Yarrabubba; 3. Depot Springs; 4. Yandil Magellan; 5. Bubble Well; 6. Lake Violet; 7. Uramurdah Lake; 8. Jundee; 9. Cunyu; 10. Carnegie Downs; 11. Moorarie.

Hexabathynella characteristically lacks thoracopod VII and has one to two-segmented thoracopodal exopods (Cho and Schminke, 2006) (see Table 3 for diagnostic characters of genera). Genera that are based on non-Australian type species, but contain putative Australian species (i.e. *Atopobathynella, Chilibathynella* and *Hexabathynella*) are denoted by inverted commas to express uncertainty of their congeneric status. Previous research (Guzik et al., 2008) has indicated that it is relatively common for parabathynellids to exhibit highly restricted distributions. Therefore, some doubt exists on whether the specimens in our study truly belong to cosmopolitan genera which were originally described from other continents, although our taxa generally match the diagnostic criteria for these genera. The described species included in this study were identified by an expert parabathynellid taxonomist (J.-L. Cho) who has described many of the Australian genera and species (Cho, 2005; Cho et al., 2005; Cho et al., 2006a,b; Cho and Humphreys, 2010). Additionally, we examined the sequence divergence within and between key clades to identify species. Since genetic divergence thresholds can vary amongst organisms (and differing opinions among researchers), we estimated the number of potential new species based on three different *COI* pairwise distance thresholds (using the Kimura-2-parameter model (Kimura, 1980): (1) $\geqslant$7.1% which is based on the *COI* divergence between two morphologically distinct, described parabathynellid species, *Brevisomabathynella cooperi* and *B. jundeensis* (Fig. 3); (2) $\geqslant$11% as suggested by Guzik et al. (2011a) and (3) $\geqslant$17% as suggested by Costa et al. (2007) based on broadly assessed divergences among decapod crustaceans. Additionally we calculated patristic distances, from the *COI* ML tree using the program Geneious Pro 5.4. (Drummond et al., 2011) and compared them with a threshold of (1) $\geqslant$0.075 substitutions per site (subst./site) which is based on the *COI* divergence between *Brevisomabathynella cooperi* and *B. jundeensis* and (2) $\geqslant$0.16 subst./site as suggested by Lefébure et al. (2006) based on broadly assessed divergences amongst various crustacean groups. Finally we took into account geographic location of potential species, i.e., if they were found in an isolated locality, or confined aquifer, with little possibility or evidence of migration to other locations, we considered this additional support for separate species status.

Eighty-five individuals representing two putative bathynellid species, nine described parabathynellid species, a further 23 putative parabathyenllid species (based on the criteria defined above; see Table 3) and all of the eight known Australian genera are represented in this study (i.e. 100% of described genera and ~25% of described species). Many crustacean taxa are difficult to amplify and sequence so most studies are limited to using the mitochondrial markers *16S* and *COI* and the nuclear markers *18S* and ITS (Giribet and Ribera, 2000; Regier and Shultz, 2001; Koenemann et al., 2010) The markers used here (*COI* and *18S*) were selected because they have proven useful for various levels of systematic studies in a range of organisms (Hebert et al., 2003) including crustaceans (Page et al., 2007; Wyngaard et al., 2010). *COI* is considered

Table 1
Locations of Bathynellacea samples and GenBank accession numbers.

Species	BES voucher number[a]	Collection site	Calcrete no.	Latitude	Longitude	GenBank accession numbers	
						COI	*18S*
"*Hexabathynella*" (Schminke, 1972) sp. 1	–	Port Kenny, SA	–	−33.1564	134.64456	JN817387	JQ446049
"*Chilibathynella*" (Noodt, 1963) sp. 1	–	Peel River, NSW	–	−31.08361	150.91167	JN817388	–
"*Chilibathynella*" sp. 2	–	Peel River, NSW	–	−31.3053	151.14	JN817389	–
"*Atopobathynella*" (Schminke, 1973) sp. 1	–	Uley, Port Lincoln, SA	–	−34.65712	135.60195	JN817390	JQ446050
"*Atopobathynella*" *hinzeae*	11166	Depot Springs, WA	3	−27.93010	120.05849	JN817391	JQ446051
"*Atopobathynella*" sp. 2	13493	Yarrabubba, WA	2	−27.2147	118.9186	EU350252	JQ446055
"*Atopobathynella*" *glenayleensis*	9961	Carnegie Downs, WA	10	−25.6685	122.3686	EU350256	JQ446052
"*Atopobathynella*" sp. 3	–	Yarrie Pit, Pilbara, WA	–	–	–	JN817392	JQ446053
"*Atopobathynella*" sp. 4	–	Marillana Creek, Pilbara, WA	–	–	–	JN817393	JQ446054
"*Atopobathynella*" sp. 5	–	Yarrie Station, Pilbara, WA	–	–	–	JN817394	JQ446057
Kimberleybathynella Cho, Park and Humphreys sp. 1	–	Kimberley region, WA	–	−16.692	128.4541	JN817395	–
Kimberleybathynella sp. 2	–	Kimberley region, WA	–	−15.4645	128.8928	JN817396	–
Lineage A sp. 1	–	Grindell's Hut, SA	–	−30.47716	139.21348	JN817397	JQ446056
Lineage A sp. 2	–	Bollabollana Spring, SA	–	−30.28742	139.28187	JN817398	–
Octobathynella peelensis (Camacho and Hancock, 2010)	–	Peel River, NSW	–	−30.9561	150.80167	JN817399	JQ446076
Notobathynella (Schminke, 1973) sp. 1	–	Hunter River, NSW	–	−32.0484	150.8194	JN817400	–
Billibathynella (Cho, 2005) sp. 1	14245	Austin Downs, WA	1	−25.874	117.4524	EU350247	JQ446060
Billibathynella sp. 2A, B	14775, 14777	Moorarie, WA	11	−27.41337	117.71122	JN817401,JN817402	JQ446059, JQ446058
Lineage B	–	Coondewanna Creek, Pilbara, WA	–	−23.0384	118.7503	JN817404	JQ446061
Lineage C	–	Marillana Creek, Pilbara, WA	–	−22.7073	118.9732	JN817405	JQ446062
Lineage D	–	Wanila, SA	–	−34.5907	135.602	JN817403	JQ446063
Brevisomabathynella clayi Cho et al., 2006b	14277	Uramurdah Lake, WA	7	−26.6876	120.3027	EU350240	JQ446066
Brevisomabathynella cooperi	14301B	Jundee, WA	8	−26.2827	120.6757	EU350254	JQ446065
Brevisomabathynella cunyuensis	13347	Cunyu, WA	9	−25.7642	120.1143	JN817408	JQ446075
Brevisomabathynella jundeensis	14301A	Jundee, WA	8	−26.2827	120.6757	EU350253	JQ446064
Brevisomabathynella magna	13331	Cunyu, WA	9	−25.5938	120.3724	EU350243	JQ446078
Brevisomabathynella uramurdahensis	6452	Uramurdah Lake, WA	7	−26.6878	120.3383	EU350236	JQ446072
B. uramurdahensis A	11147	Bubble Well, WA	5	−26.56073	120.04083	JN817407	JQ446073
Brevisomabathynella sp. 1	13479	Yandil, Magellan, WA	4	−26.545	119.9855	EU350241	JQ446071
Brevisomabathynella sp. 2	11138	Uramurdah Lake, WA	7	−26.6878	120.3274	JN817406	JQ446077
Brevisomabathynella sp. 3	13454	Lake Violet, WA	6	−26.6774	120.228	EU350232	JQ446070
Brevisomabathynella sp. 4	13457E	Lake Violet, WA	6	−26.6876	120.2977	EU350231	JQ446069
Brevisomabathynella sp. 5A	13385	Lake Violet, WA	6	−26.6828	120.221	EU350233	JQ446068
Brevisomabathynella sp. 5 B	13457C	Lake Violet, WA	6	−26.68758	120.29777	EU350230	JQ446074
Bathynellidae sp. 1	–	Lubra Water, SA	–	−31.33593	138.6013	JN817410	–
Bathynellidae sp. 2	–	Lubra Water, SA	–	−31.33593	138.6013	JN817409	JQ446079

[a] The BES voucher numbers link the specimens from Guzik et al. (2008) to the species in this study, because they have different names here since the taxa nomenclature has changed recently. Additionally, taxa without BES numbers have been supplied by institutions other than the WAM or consulting companies and have not been assigned museum collection numbers yet.

informative at species level (Lefébure et al., 2006), and *18S* has proven useful for examining higher-level crustacean relationships (Giribet and Ribera, 2000). These markers were also selected because there were numerous primers available to trial and modify and they amplified DNA most consistently across syncarid taxa. Primers for numerous other mitochondrial and nuclear genes (*16S*, NADH1, EF1-α, histone 3, wingless, *28S*, opsin, GAPDH, CAD, PEPCK, ANT, LTRS, ARGK) were trialed unsuccessfully which may be due in part, to the Bathynellacea being an extremely ancient lineage, making it difficult to find appropriate primers. Individuals are also very small, sometimes leading to problems in extracting sufficient DNA to PCR-amplify single copy nuclear genes using sub-optimal degenerate primers.

Anaspides tasmaniae (Anaspidacea: Syncarida, GenBank accession L81948) (Spears and Abele, 1997) was used as an outgroup, since the monophyly of the Parabathynellidae and Bathynellidae remains unconfirmed, and Anaspidacea is the sister lineage to the Bathynellacea and the only other extant order within the Syncarida.

2.3. Sequencing protocols

The molecular protocols used in this study were similar to those described in Guzik et al. (2008). Genomic DNA was extracted from specimens stored in 100% ethanol, using the Gentra Systems PUREGENE DNA Purification Kit. Where possible, one to three appendages were removed from a single side from each individual for DNA extractions. However, most specimens were small so whole individuals had to be used to provide sufficient material for the extractions. Every effort was made to retain voucher material for future morphological and molecular examination, with vouchers being lodged at the Western Australian Museum (WAM) or South Australian Museum (SAM). *COI* sequences were obtained for 87 individuals (2 Bathynellidae and 85 Parabathynellidae) and *18S* sequences were obtained from 41 individuals (1 Bathynellidae and 40 Parabathynellidae; Table 1). The *COI* sequences were translated into amino acid sequences to determine if any gaps or stop codons were present. Typically, a 592 base pair (bp) fragment of *COI* was amplified with the universal oligonucleotide primers

Table 2
Putative new species and the criteria used to delineate them.

Taxon (genetically distinct lineage)	Lowest K2P divergence (%)	*COI* thresholds						Geographic isolation			Position in phylogeny	Species-level morphological differences
		K2P thresholds				Patristic thresholds						
		Lowest patristic divergence	This paper threshold >7.1%	Guzik et al. (2011a,b) threshold >11%	Costa et al. (2007) threshold >17%	This paper threshold >0.075	Lefébure et al. (2006) threshold >0.16	Hydrogeological	Harvey 2002[a] threshold: <10,000 km^2	Eberhard et al. (2009)[b] threshold: 1000 km^2		
"*Hexabathynella*" sp.	27.1	0.491	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. sp. 1	18.4	0.3	✓	✓	✓	✓	✓	✓	✓	✓	✓	[c]
C. sp. 2	18.4	0.3	✓	✓	✓	✓	✓	✓	✓	✓	x	[c]
K. sp. 1	24.9	0.46	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
K. sp. 2	26.2	0.414	✓	✓	✓	✓	✓	✓	✓	✓	x	[c]
A. sp. 1	20.4	0.378	✓	✓	✓	✓	✓	✓	✓	✓	x	✓
A. sp. 2	15.8	0.167	✓	✓	x	✓	✓	✓	✓	✓	✓	✓
A. sp. 3	15.8	0.166	✓	✓	x	✓	✓	✓	✓	✓	x	[c]
A. sp. 4	20.7	0.179	✓	✓	✓	✓	✓	✓	✓	✓	✓	[c]
A. sp. 5	17.7	0.174	✓	✓	✓	✓	✓	✓	✓	✓	✓	[c]
Lineage A sp. 1	14.5	0.139	✓	✓	x	✓	x	✓	✓	✓	✓	✓
Lineage A sp. 2	14.5	0.139	✓	✓	x	✓	x	✓	✓	✓	x	✓
Notobathynella sp.	20.7	0.202	✓	✓	✓	✓	✓	✓	✓	✓	✓	[c]
Bi. sp. 1	9.4	0.1	✓	x	x	x	✓	✓	✓	✓	✓	✓
Bi. sp. 2A	9.4 (with *Bi.* sp. 1), 6.5 (with *Bi.* sp. 2B)	0.058	✓	x	x	x	x	✓	✓	✓	x	[c]
Bi. sp. 2B	6.5	0.058	x	x	x	x	x	Same calcrete as sp. 2A	✓	✓	x	[c]
Lineage B	25.3	0.219	✓	✓	✓	✓	✓	✓	✓	✓	✓	[c]
Lineage C	21.1	0.237	✓	✓	✓	✓	✓	✓	✓	✓	x	[c]
Lineage D	22.1	0.18	✓	✓	✓	✓	✓	✓	✓	✓	✓	[c]
Br. sp. 1	7.1	0.07	✓	x	x	x	x	✓	✓	✓	✓	✓
Br. sp. 2	7.1	0.07	✓	x	x	x	x	✓	✓	✓	x	[c]
Br. sp. 3	7	0.064	x	x	x	x	x	✓	✓	✓	x	[c]
Br. sp. 4	7.1	0.07	✓	x	x	x	x	✓	✓	✓	x	[c]
Br. sp. 5 A	6.2 (with *Br. uramurdahensis*), 5.7 (with sp. 5A)	0.054 (with *Br. uramurdahensis*), 0.053 (with sp. 5A)	x	x	x	x	x	✓	✓	✓	x	✓
Br. sp. 5 B	5.7	0.053	x	x	x	x	x	Same calcrete as 5A	✓	✓	x	[c]
Brevisomabathynella uramurdahensis A	4.8	0.046	x	x	x	x	x	Different calcrete but potentially connected	✓	✓	x	[c]

[a] Distance threshold for short-range endemic taxa suggested by Harvey (2002).
[b] Distance threshold for short-range endemic subterranean taxa suggested by Eberhard et al. (2009).
[c] Data deficient.

Table 3
Character variability in parabathynellid genera in Australia (modified from Camacho and Hancock (2010)). Abbreviations: A, absent; A1, antennule; A2, antenna; No., number; Mx1, maxillule; sgt, segment; Th I–VIII, thoracopod 1–8; min, minimum; max, maximum.

	Chilibathynella	*Hexabathynella*	*Atopobathynella*	*Kimberleybathynella*	*Notobathynella*	*Billibathynella*	*Brevisomabathynella*	*Octobathynella*	Lineage A
A1 No. sgt	7	6	6	6	6–7	7	7	8	7
A2 No. sgt	5–6	5	1	2	5–6	7	5	7	5
Labrum No. teeth	10–16	10–14	12–26	32–36	14–22	22–28	12–63	18–20	8–22
Mx 1 No. spines (distal)	5–6	4–6	5–6	5	6–7	7–10	5–7	7	6–9
Th I. Epipod	P/A	P/A	P/A	P/A	P/A	P	P	A	P
Exopod No. sgt. ThI	1	1	1	1	1–3	4–8	2–9	3	1–4
Th II	1	1–2	1	1	2–3	5–11	3–11	4	3–5
Th III–IV	1	1–2	1	1	3–4	5–12	3–12	4–5	3–6
Th. V–VII	1	1–2	1	1	2–4	4–13	2–11	3–5	3–6
Th VIII male shape	Rectangular	Rectangular	Semicircular	Hemispherical	Subglobular	Rectangular	Rectangular	Rectangular	Rectangular
Pleopod	P	P/A	P	P	A	P/A	P/A	A	A/P
Sympod spine type	Homonomous/ Inhomonomous	Homonomous	Homonomous/ Inhomonomous	Inhomonomous	Homonomous/ Inhomonomous	Homonomous	Homonomous	Inhomonomous	Inhomonomous
Sympod spine No.	8–11	2–8	5–17	6–20	6–13	13–28	6–20	10–12	7–17
Furcal rami spine No.	6–12	3	3–9	4–6	7–11	10–23	5–20	10–13	7–14
Length min.–max. (mm)	1.2–2.8	0.6–1.7	1.0–3.0	0.9–3.5	1.2–2.3	2.11–6.0	1.1–4.62	1.4–2.11	1.03–3.3
No. of species	3	22	11	6	9	4	12	1	4

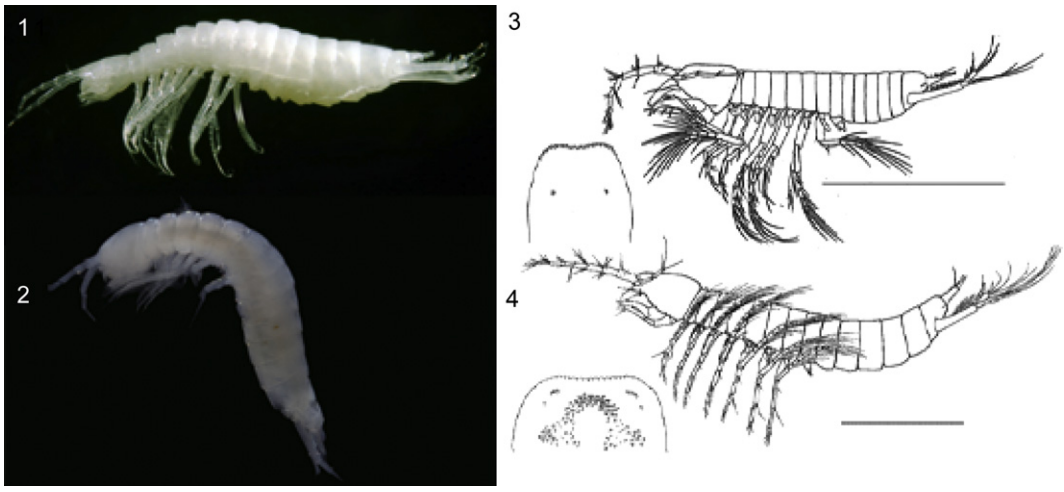

Fig. 3. *Brevisomabathynella* species display a variety of morphological forms, (1) *B. uramurdahensis* and (2) *B.* sp. 5 are sister species from closely located calcretes and are morphologically distinct yet are only 6.2% divergent for *CO1;* (3) *B. cooperi* and (4) *B. jundeensis* are sympatric sister species with distinctive morphological characters including mouthparts, e.g. the labrum shown to the left of the lateral habitus drawings.

C1-J-1718 (5′-GGAGGATTTGGAAATTGATTAGTTCC-3′) and C1-J-2329 (alias K525) (5′-ACTGTAAATATATGATGAGCTCA-3′) (Simon et al., 1994). A 500 bp fragment was amplified with the primers LCOI1490 (5′GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) for two individuals: BES 14277, BES 14301a. PCR amplifications for *COI* were carried out in 25 μl reactions containing PCR buffer, 0.1 units of AmpliTaq Gold® DNA Polymerase, (Applied Biosystems Inc.), 2–4 μl $MgCl_2$, 2.5 mM of each dNTP, 5.0 μM of each primer and ~1 ng of DNA. Thermal cycling occurred in an Eppendorf thermal cycler using the following conditions: enzyme activation at 94 °C for 9 min, followed by 35 cycles of 94 °C for 30 s, 47 °C for 30 s and 72 °C for 60 s with a final elongation step at 72 °C for 6 min. A 707 bp fragment of the *18S* region was amplified using

the primers 1.2F (5′-TGCTTGTCTCAAAGATTAAGC-3′) and b3.9 (5′-TGCTTTRAGCACTCTAA-3′) (Whiting, 2002) under thermal cycling conditions of 94 °C for 9 min for enzyme activation, then 94 °C for 2 min, followed by 40 cycles of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 60 s, then a final elongation step at 72 °C for 6 min. PCR products were purified using the Ultraclean PCR Clean-up Kit (MOBIO Laboratories Inc.) and sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). Amplified products were sequenced in both directions on an ABI PRISM 3700 (Applied Biosystems). Raw sequences were compared with their corresponding chromatograms to clarify ambiguous bases, using BioEdit version 7.0.1 (Hall, 1999) and Sequence Scanner version 1 (Applied Biosystems 2005). Sequences were aligned using Clustal W (Thompson et al., 1994) and checked by eye.

2.4. Sequence analysis

Nucleotide sequence composition statistics were estimated using MEGA 4.0 (Tamura et al., 2007). Phylogeny reconstruction of *COI* and *18S* sequence data involved Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) approaches, using separate and combined datasets, implemented in the programs MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), RaxML v. 7.2.3 (Stamatakis et al., 2008) and PAUP* 4.0b10 PC version (Swofford, 2002) respectively. Modeltest 3.7 (Posada and Buckley, 2004) was used to estimate the model which best fitted the nucleotide data, in combined and separate analyses, and the model selected by the Akaike Information Criterion was used in BI analyses (GTR + I + G: combined and *COI* datasets, and TVMef + G: *18S*). The dataset was partitioned by codon for *COI* and by gene using the above models in an unlinked analysis which allowed the rates to vary over the partitions. Bayesian analyses were run using four chains for 10 million generations in two independent runs, sampling every 100 generations. The program Tracer 1.5 (Rambaut and Drummond, 2003) was used to evaluate convergence to the stationary distribution. We observed effective sample size (ESS) values for all parameters to be well above 500, providing evidence that convergence had been reached. The likelihood values converged to relative stationarity after ~96,000 generations. A burnin of 15,000 was chosen and a strict BI consensus tree was constructed from the remaining 85,000 trees.

MP analysis was carried out using a heuristic unweighted parsimony search that involved tree-bisection-reconnection branch swapping and 10 multiple random addition sequence replicates. The DELTRAN method for character state optimisation was used to avoid erroneous branch length reconstructions caused by the ACCTRAN option (Mac version of PAUP* 4.0b10). Bootstrap analysis comprising 1000 replicates was undertaken for the heuristic search. ML analyses implemented in RAxML used 100 rapid bootstrap inferences and the likelihood of the best tree was optimised and evaluated under a gamma + P-Invariable model. Pairwise distances between sequences were estimated using the GTR + I + G model of evolution and branch lengths and parameters were estimated for the BI consensus tree using PAUP*, with the optimality criterion set to maximum likelihood.

2.5. Ancestral state reconstruction

Two morphological characters (number of segments in the (1) antennule and (2) antenna) were used in an ancestral state analysis because they are among the most commonly used characters in parabathynellid systematics, they have the potential to show a discrete, transitional series of evolution and the number of segments of these characters has often been used to suggest the ancestral or derived nature of the taxa being discussed. The number of segments in the antennules and antennae of each morphospecies were counted and coded as unordered, multistate characters. ML, MP and BI approaches were used to reconstruct ancestral states and compared with each other because each method varies in its assumptions and has advantages and disadvantages (Xiang and Thomas, 2008; Schäffer et al., 2010). Both MP and ML character optimisations were applied (using Mesquite version 2.74 Maddison and Maddison, 2011) to a set of 4000 trees generated by BI for 37 taxa (anaspid and bathynellid taxa were excluded in the Mesquite analyses) under the GTR + I + G model of DNA substitution. The Markov k-state 1 (Mk1) parameter model was used for ML reconstructions with equal probability for any particular character state change. We also used a BI approach to analyse ancestral states, using the 'Multistate' option in BayesTraits v1.0 (Pagel and Meade, 2006). This program has the advantage of testing numerous models by employing a reversible jump (RJ) Markov chain Monte Carlo (MCMC) which searches the posterior distribution of different models of evolution as well as the posterior distributions of the parameters of these models. Initially ML analyses were run to determine the 'optimal' rate parameters and likelihood for each tree, as suggested by the BayesTraits authors. Subsequently, ancestral states were reconstructed for four key nodes using MCMC methods using a RJ hyperprior with a gamma prior (exponential prior seeded from a uniform distribution on the interval 0–7 for antennule segment number and 0–19 for antenna segment number). We conducted numerous preliminary analyses to determine a *ratedev* which would produce an acceptance rate of proposed changes between 20% and 40%; the *ratedev* value was 0.055 for antennule segment number and 0.01 for antenna segment number. A burnin of 14 million generations for antennule segment number and 6 million generations for antenna segment number, and sampling every 500 generations were applied. Multiple runs were also conducted in order to determine the number of iterations required for parameters such as the likelihood and harmonic mean to reach convergence (140 million iterations for antennule segment number and 60 million for antenna segment number). The four reconstructed nodes were specified using the 'addMRCA' command. Alternative ancestral character states for nodes 1–4 were compared using the 'fossil' command to fix the nodes to each state and using BayesFactor (BF) tests to compare the harmonic means of the alternative states. Interpretation of BF followed Pagel et al. (2004), i.e. support for any particular state was considered positive when BF = 2{log [harmonic mean (best model)] – log [harmonic mean (alternative model)]} was >2, strong evidence for values >5, and very strong evidence for values >10. When BF values were close to the cut-off value of 2, analyses were repeated between one and five times to assess whether fluctuations in the harmonic means would affect the outcome.

3. Results

All *COI* sequences (~592 bp) were open reading frames with no evidence of gaps or stop codons, suggesting they were derived from functional *COI* genes. The *18S* sequence data aligned well, without gaps to an *Anaspides tasmaniae* reference sequence so a secondary structure model was not required to aid the alignment. The *COI* sequences comprised 56% variable sites and 49% parsimony informative sites. In comparison, the more conserved *18S* data comprised 23% variable sites and 13% parsimony informative sites.

3.1. Phylogenetic analysis

Individual gene trees for *COI* and *18S* were reconstructed and because no major phylogenetic incongruence in their topologies was observed, the datasets were combined for further phylogenetic analyses. Identical haplotypes were removed from the phylogenies

in order to visually simplify the shown trees. The ML tree had the same topology as the BI tree (Fig. 1), with the exception of a paraphyletic clade consisting of *Kimberleybathynella* intermixed with '*Atopobathynella*', however many of the bootstrap support values were low. Since the ML topology was consistent with the BI tree and the MP tree was less-well resolved (consisting of numerous polytomies although Bathynellidae, *Billibathynella, Brevisomabathynella*, '*Chilibathynella*' and Lineage A were supported as monophyletic clades), they are not shown here, but are available as supplementary data (Supplementary Figs. A and B). The impact of including six taxa for which *COI* sequences alone were available (i.e. *18S* data absent) was assessed by running analyses, either including or excluding these taxa. Their inclusion did not significantly weaken posterior probabilities, nor did it suggest any incompatible relationships in the BI analysis. The few differences in topology (described below) are most likely due to the lack of *18S* sequence data for three key genera ('*Chilibathynella*', *Kimberleybathynella* and *Notobathynella*). Based on these findings, and given the increased taxon representation, all taxa were included in the final BI and MP analyses. The missing *18S* sequence data are also likely to account for differences in topology between BI and ML analyses because RaxML cannot accommodate taxa coded as missing data. The results viewed in Tracer confirmed that all parameter estimates had converged and showed suitable ESS values (>500).

The BI tree (Fig. 1) generated from the 1299 bp combined dataset was used to assess whether the known genera are monophyletic. In total, 37 genetically divergent lineages were resolved, which include nine described species, 23 putative new parabathynellid species and two putative new bathynellid species. As discussed in the methods, we used at least two of the following criteria (genetic divergence, position in the phylogeny, morphological differences and geographical isolation) to identify putative species, and so from this point we refer to these genetically distinct lineages as 'species' (see Table 2). Based solely on divergence thresholds, we would recognise 23 putative new parabathynellid species when using the 7.1% *COI* threshold, 16 putative new species when using the 11% *COI* threshold and 12 putative new species when using the 17% *COI* threshold. Solely using a patristic threshold of 0.075 subst./site would result in the recognition of 16 new species, while applying the Lefebure et al. threshold of 0.16 subst./site, would result in the recognition of 15 new species. Additionally we observed two genetically distinct (16.1%) bathynellid lineages from the same spring which could be recognised as two new species based on the first and second *COI* thresholds.

Although the dataset only includes Australian taxa, BI analyses of the combined data provides evidence for the existence of two highly divergent monophyletic clades, corresponding to the Bathynellidae and Parabathynellidae (100% Bayesian posterior probability (BPP) and >74% ML bootstrap value). The Parabathynellidae shows a clear division into three major clades, each comprising multiple genera. Clade 1 consists of one new species of '*Hexabathynella*' and two new species of '*Chilibathynella*' (100% BPP, 95% MP bootstrap value). Clade 2 (90% BPP) contains seven species of '*Atopobathynella*' (66% BPP, 96% ML bootstrap value): '*A.*' *hinzeae, 'A'. glenayleensis*, and five new species, '*A.*' sp. 1–5, in addition to two new species (99% BPP): *Kimberleybathynella* sp. 1 and *K.* sp. 2.

Clade 3 (98% BPP, 96% ML bootstrap value) contains four described genera – the type species of the genus *Octobathynella peeleensis*, one new species of *Notobathynella*, two new species of *Billibathynella* (100% BPP, ML, MP) and 11 species of *Brevisomabathynella* (100% BPP, ML and MP bootstrap values), six of which are described (*B. clayi, B. cooperi, B. cunyuensis*, *B. jundeensis, B. magna, B. uramurdahensis*), and five of which are new. Clade 3 also contains four distinct lineages – Lineage A, containing two species (100% BPP), and Lineages B, C and D, each consisting of one species, which could not be readily assigned to existing genera.

3.2. Phylogenetic relationships

The supposed 'morphologically-primitive' genus *Billibathynella* (Cho, 2005), has a more apical position in the phylogeny with the most basal taxa being '*Hexabathynella*' + '*Chilibathynella*' (100% BPP, 74% ML bootstrap value) (Fig. 1). There is also a relatively well supported sister relationship between the '*Atopobathynella*' and *Kimberleybathynella* (90% BPP) lineages.

The phylogenetic position of Lineage D is uncertain; in MP analysis it groups with Lineage A + *Octobathynella* + *Notobathynella*, while in ML analyses it falls between *Billibathynella* and *Brevisomabathynella* (ML) or between Genus B + C and *Brevisomabathynella* in BI analysis, although the support is low (>77%) in all cases. There is also uncertainty in the phylogenetic position of Lineages B and C – the BI analysis weakly (46% BPP) supports it as sister to Lineage D + *Brevisomabathynella*, whereas the ML analysis places it as sister to *Billibathynella*, also with lower than 50% bootstrap support. Both analyses support the sister relationship between Lineages B and C (100% BPP, 89% ML), although in the *COI*-only dataset Lineage B had a strongly supported sister relationship to '*Hexabathynella*' (99% BPP).

Lineage A (comprising two distinct species) is a well-supported (95% BPP) clade in the BI tree, and is sister to a clade comprising *Notobathynella* and *Octobathynella*. BI and MP analyses, as well as shared morphological characters such as the male thoracopod VIII and mouthparts (Camacho and Hancock, 2010), suggest a close relationship between *Octobathynella* and *Notobathynella* (although not well-supported; only 50% BPP in the combined analysis, but 98% BPP in the *COI*-only dataset).

3.3. Genetic divergences

The average pairwise sequence divergence for *COI* among genera ranged between 18.4–28.2% (K2P) and 0.271–0.757 subst./site for patristic divergences. The average divergence amongst all parabathynellid species for *COI* was 24.2%/0.444 subst./site. The average *18S* sequence divergence among genera ranged between 3.1% and 8.8% and the average divergence amongst all parabathynellid species was 4%.

The average pairwise sequence divergence for *COI* among species within genera was highly variable, ranging from 9%/0.142 subst./site (*Billibathynella*, two species) to 32.5%/0.493 subst./site (*Kimberleybathynella*, two species). However, because the entire specimen of *Kimberleybathynella* sp. 2 was used for DNA extraction, it is not certain whether this taxon matches the morphological criteria for *Kimberleybathynella*. '*Atopobathynella*' displays the second highest but markedly lower average interspecific divergence of 20.6%/0.348 subst./site (seven species) and all taxa within this clade exhibit morphological characters consistent with the genus (Table 3). The average sequence divergence within genera for *18S* ranged between 0.1 (*Billibathynella*, two species) and 2.1% ('*Atopobathynella*', seven species). However, *18S* sequence data for more than one individual was only available for three genera (the latter two taxa and *Brevisomabathynella*).

The *COI* divergence among species within genera was also variable, and in some cases considerably low. For example, among 11 species of *Brevisomabathynella*, genetic divergences varied from 6.2% to 15.9% K2P and the patristic divergences ranged from 0.193 subst./site to 0.085 subst./site. *COI* divergences ranged from 7.1%/0.075 subst./site to 12.5%/0.131 subst./site among the six described *Brevisomabathynella* species (Table 4) but different body forms were observed between closely related species which were only 6.2%/0.054 subst./site divergent (*Brevisomabathynella uramurdahensis* and *Brevisomabathynella* sp. 5) (see Fig. 3), suggesting that our 7.1%/0.075 subst./site thresholds may be slightly high. The divergence among species in '*Atopobathynella*' was much greater,

ranging from 15.8%/0.255 subst./site to 24.6%/0.48 subst./site, with a divergence of 21.8%/0.394 subst./site observed between the described species '*A.*' *hinzeae* and '*A.*' *glenayleensis*. In comparison, the *18S* divergence was much lower *i.e.* ranging from 0.2–2.8% in *Brevisomabathynella* to 0.5–4.4% in '*Atopobathynella*'.

3.4. Ancestral state analysis

The results of the ancestral state analysis using ML and BI methods are summarised in Fig. 4. Results of the parsimony analysis are not shown as they are essentially identical to the ML results. The internal pie charts on the tree represent the relative likelihoods of alternative character states based on ML analysis and the external pie charts are based on Bayesian MCMC methods.

Overall, BI supports a trend of fewer antennule and antennal segments being the ancestral state and more being the derived state, although the BayesFactor tests were not always consistent or significant (see Table 5). In contrast to this, ML suggests that for the antennule, 7-segments is the ancestral state and that the other states evolved one (8-segments) to two (6-segments) times independently; and for the antenna, 5-segments is the ancestral state and the other states evolved one (1- and 2-segments) to three (8-segments) times independently.

4. Discussion

Here we present the first study to examine the diversity and phylogenetic relationships amongst genera and species of parabathynellids on a continent-wide scale. We also explored the evolution of two morphological characters, which are widely used for reconstructing parabathynellid phylogenetic relationships, to assess the oligomerization principle. Cladistic analysis of the relationships amongst multiple parabathynellid genera has only been undertaken once previously when Camacho et al. (2000) reconstructed the relationships amongst six related genera from the northern hemisphere. Instead, researchers have inferred relationships amongst genera based on phenetic similarities. A lack of comprehensive analysis is understandable given difficulties in accessing specimens, and their taxonomic intransigence, stemming from a combination of extreme morphological specialisation to confined interstitial spaces of subterranean groundwater and progenetic development (i.e. sexual maturation of an organism resulting in an adult descendent exhibiting the larval or juvenile morphology of its ancestor (Coineau, 2000, p. 194), which has led to a simplified body plan (Schminke, 1974, 1981). This tendency towards simplicity is clearly shown in the reduced number of ornaments on appendages, the reduced number of segments per appendage, and even the loss of whole appendages, particularly in *Hexabathynella* (Cho et al., 2006b). Consequently, morphological phylogenetic analysis of the group is strongly dependent on reductional characters, which often results in poor resolution of relationships among genera and species (Cho et al., 2006b).

4.1. Generic relationships amongst Australian parabathynellids

The Bayesian phylogeny revealed a clear division into three well-supported monophyletic clades. The first of the three major clades consisted of '*Hexabathynella*' and '*Chilibathynella*'. The basal positioning of '*Hexabathynella*' was unexpected because it is considered to be one of the most derived genera (Schminke, 1974), characterised by the absence of the 7th set of thoracopods and reduced thoracopods (1–2-segments) (Cho and Schminke, 2006). Schminke (1974) postulated that *Hexabathynella's* closest relative is *Notobathynella*, which he considered to be more primitive due to *Notobathynella* bearing more segments of the thoracopods and setae and spines of the mouthparts and uropod. Based on our analysis, '*Hexabathynella*' + '*Chilibathynella*' is sister to all other included taxa, and *Notobathynella* is in a more derived position in clade 3, which contains another putatively primitive genus, *Billibathynella* (Cho, 2005). These results suggest that some character states, previously assumed to be primitive, may be more recently derived, thus highlighting the value of including molecular data when evaluating parabathynellid systematics.

The second clade revealed a sister relationship of '*Atopobathynella*' and *Kimberleybathynella*, which is congruent with the morphological assessment that these genera are closely related, based on similarities in the form of the male thoracopod VIII and the one-segmented exopods on thoracopods I–VII (Cho et al., 2005). In fact, these genera are so morphologically similar that there has been some doubt as to whether *Kimberleybathynella* should be accorded separate genus status (Cho et al., 2005). Our study supports a hypothesis that these are two separate and divergent (*COI* divergence: 21.4%, 0.414 subst./site) monophyletic groups of species and so is consistent with an hypothesis of two distinct genera. Interestingly, *Atopobathynella* is widely distributed across Australia, with species found in South Australia, Western Australia, Northern Territory and Victoria (although we were unable to obtain specimens from the latter two regions for molecular sequencing). In contrast, *Kimberleybathynella* appears to be restricted to the Kimberley region of Western Australia (Cho et al., 2005). It has been suggested that *Atopobathynella* is closely related to *Chilibathynella* based on morphological characters such as one-segmented exopods of the thoracopods I–VII and furcal rami ornamented with numerous spines (Cho et al., 2006a, p. 33). However, our analysis supports a sister relationship between '*Chilibathynella*' and '*Hexabathynella*' rather than '*Atopobathynella.*'

With the exception of *Notobathynella* (which is also known from New Zealand and one species from Madagascar which is morphologically very distinctive (Drewes and Schminke, 2007)), clade 3 consists solely of Australian genera, namely: *Octobathynella*, *Billibathynella* and *Brevisomabathynella*. Clade 3 also contains four additional distinct lineages (A–D) which do not group closely with or within any of the known generic groups. Lineage A may represent a new genus based on: (1) a unique combination of morphological characters (see Table 3); (2) sequence divergence of approximately 21%/0.425 subst./site (the lowest *COI* divergence is 21%, between it and *Billibathynella*, the highest is 36%/0.615

Table 4
COI pairwise (K2P followed by patristic) genetic divergence between and within six *Brevisomabathynella* species.

COI divergence (K2P%/patristic subst./site)	*B. magna*	*B. jundeensis*	*B. cooperi*	*B. cunyuensis*	*B. clayi*	Divergence within species	No. specimens
B. magna	x					0.002–0.004	5
B. jundeensis	9.4/0.107	x				–	1
B. cooperi	9.1/0.082	7.1/0.075	x			0–0.002	3
B. cunyuensis	8.5/0.097	12.5/0.122	9.7/0.097	x		0.018	2
B. clayi	9.4/0.099	11.8/0.131	11.2/0.107	10/0.122	x	–	1
B. uramurdahensis	9.1/0.088	12.4/0.121	11.7/0.097	11.1/0.111	8.5/0.082	0–0.007	7

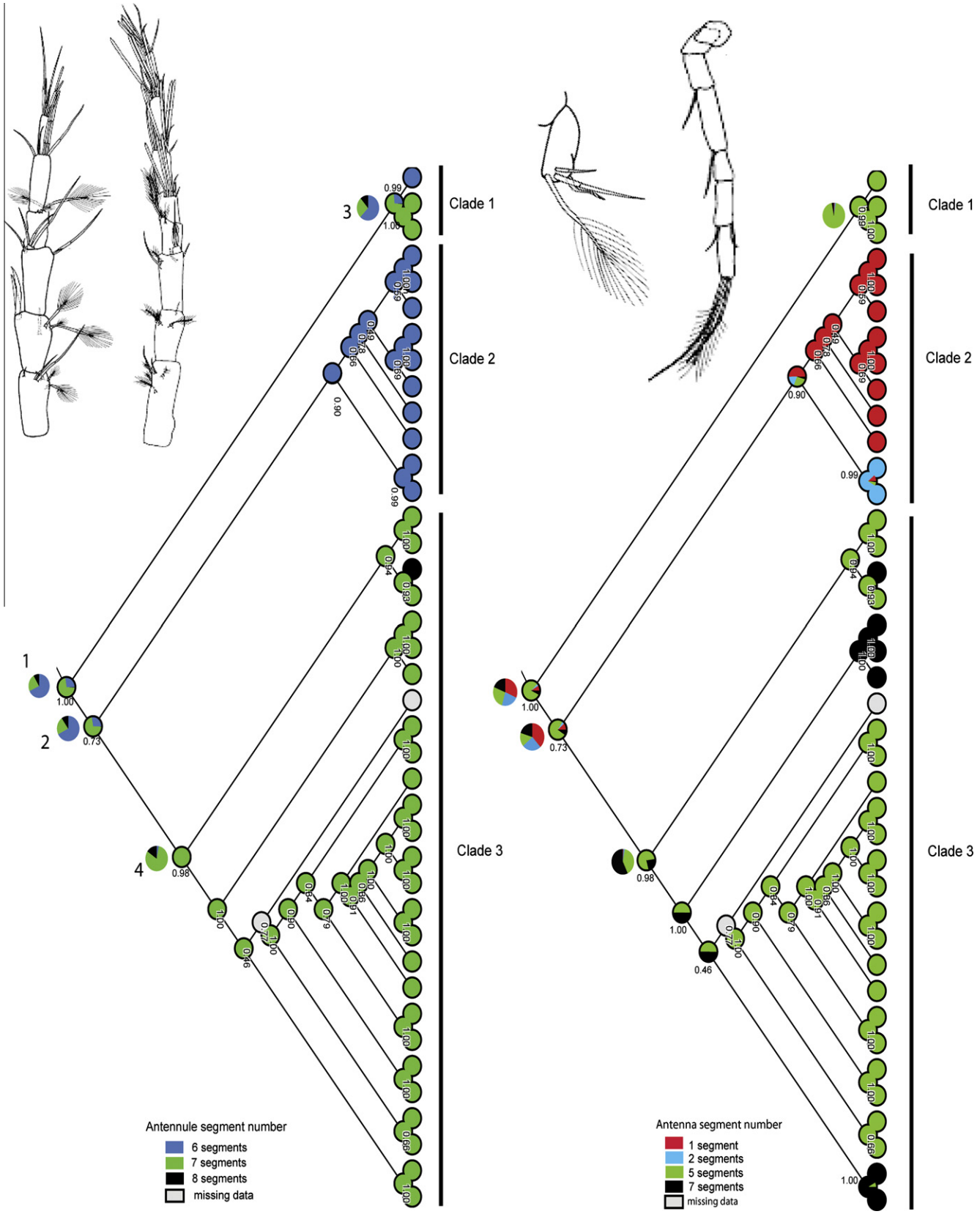

Fig. 4. Results of the ancestral state reconstruction analysis for antennule and antenna segment number based on Maximum likelihood and Bayesian approaches. The internal pie charts on the tree represent the relative likelihoods of alternative character states based on Maximum likelihood analysis and the external pie charts are based on Bayesian MCMC methods, using the programs Mesquite v2.7.4 (Maddison and Maddison, 2011) and Bayestraits v1.0 (Pagel and Meade, 2006) respectively. Diagrams of the antennule of *Atopobathynella wattsi* and *Octobathynella peelensis* and the antenna of *Atopobathynella glenayleensis* and *Octobathynella peelensis* are included to illustrate the minimum and maximum number of segments for each appendage, displayed in Australian parabathynellids.

Table 5
Results of the BayesFactor analysis. Antennule segment number: 6-, 7- or 8-segments. Antenna segment number: 1-, 2-, 5- or 7-segments. Abbreviations: $Hm^{6\text{-segments}}$ = harmonic mean when 6-segments is set with *fossil* command, BF = BayesFactor.

Test 1				Test 2			
Antennule segment number	Harmonic means for segment number		Larger Hm–smaller Hm	BF	Harmonic means	Larger Hm–smaller Hm	BF
Node 1	$Hm^{6\text{-segments}}$	−17.8729	7-segments–6 segments	7.4695[b6]	−18.7108	7-segments–6 segments	5.7936[b6]
	$Hm^{7\text{-segments}}$	−21.6076	8-segments –6 segments	16.0374[c6]	−21.6076	8-segments –6 segments	14.3615[c6]
	$Hm^{8\text{-segments}}$	−25.8916	8-segments –7-segments	8.5679[b7]	−25.8916	8-segments –7-segments	8.5679[b7]
Node 2	$Hm^{6\text{-segments}}$	−18.7052	7-segments–6 segments	5.3206[b6]	−17.4379	7-segments–6 segments	5.7703[b6]
	$Hm^{7\text{-segments}}$	−21.3655	8-segments –6 segments	10.9182[c6]	−20.3231	8-segments –6 segments	8.3352[b6]
	$Hm^{8\text{-segments}}$	−24.1643	8-segments –7-segments	5.5976[b7]	−21.6055	8-segments –7-segments	2.5649[a7]
Node 3	$Hm^{6\text{-segments}}$	−18.1262	7-segments–6 segments	0.6686	−18.1262	7-segments–6 segments	3.8257[a6]
	$Hm^{7\text{-segments}}$	−18.4605	8-segments –6 segments	6.7883[b6]	−20.0391	8-segments –6 segments	9.1798[b6]
	$Hm^{8\text{-segments}}$	−21.5204	8-segments –7-segments	6.1196[b7]	−22.7161	8-segments –7-segments	5.3541[b7]
Node 4	$Hm^{6\text{-segments}}$	−22.2591	6 segments–7-segments	7.4042[b7]	−22.2991	6 segments–7-segments	9.4871[b7]
	$Hm^{7\text{-segments}}$	−18.5570	6 segments–8-segments	2.3553[a8]	−17.5555	6 segments–8-segments	1.2438
	$Hm^{8\text{-segments}}$	−21.0814	8-segments–7-segments	5.0488[b7]	−21.6772	8-segments–7-segments	8.2433[b7]
Antenna segment number, Node 1	$Hm^{1\text{-segment}}$	−23.1589	2-segments–1-segment	0.6967	−23.1589	2-segments–1-segment	0.6967
	$Hm^{2\text{-segments}}$	−23.5072	5-segments–1-segment	0.0654	−23.5072	5-segments–1-segment	9.5503[b1]
	$Hm^{5\text{-segments}}$	−23.1916	7-segments–1-segment	5.7402[b1]	−27.9340	7-segments–1-segment	5.7402[b1]
	$Hm^{7\text{-segments}}$	−26.0290	2-segments–5-segments	0.6313	−26.0290	5-segments–2-segments	8.8536[b2]
			7-segments –2-segments	5.0435[b1]		7-segments–2-segments	5.0435[b2]
			7-segments–5-segments	5.6748[b2]		5-segments–7-segments	3.8101[a7]
Node 2	$Hm^{1\text{-segment}}$	−23.3800	2-segments–1-segment	18.4275[c1]	−23.9094	2-segments–1-segment	1.4210
	$Hm^{2\text{-segments}}$	−32.5938	5-segments–1-segment	2.1512[a1]	−24.6199	5-segments–1-segment	7.9072[b1]
	$Hm^{5\text{-segments}}$	−24.4556	7-segments–1-segment	0.0020	−27.8629	7-segments–1-segment	0.0812
	$Hm^{7\text{-segments}}$	−23.3810	2-segments–5-segments	2.1512[a5]	−23.9500	5-segments–2-segments	6.4861[b2]
			2-segments–7-segments	18.4255[c7]		2-segments–7-segments	1.3398
			5-segments–7-segments	2.1492[a7]		5-segments–7-segments	7.8260[b7]
Node 3	$Hm^{1\text{-segment}}$	−27.7246	1-segment–2-segments	3.7372[a2]	−27.7246	2-segments–1-segment	2.9840[a1]
	$Hm^{2\text{-segments}}$	−25.8560	1-segment–5-segments	3.7372[a5]	−29.2166	1-segment–5-segments	10.7551[c5]
	$Hm^{5\text{-segments}}$	−22.3470	7-segments–1-segment	5.1166[b1]	−22.3470	1-segment–7-segments	4.5387[a7]
	$Hm^{7\text{-segments}}$	−30.2829	2-segments–5-segments	7.0179[b5]	−25.4552	2-segments–5-segments	13.7391[c5]
			7-segments–2-segments	8.8538[b2]		2-segments–7-segments	7.5228[b7]
			7-segments–5-segments	15.8717[c5]		7-segments–5-segments	6.2164[b5]
Node 4	$Hm^{1\text{-segment}}$	−29.0656	1-segment–2-segments	3.1253[a2]	−26.9528	1-segment–2-segments	1.3898
	$Hm^{2\text{-segments}}$	−27.5029	1-segment–5-segments	10.1055[c5]	−26.2579	1-segment–5-segments	9.3269[b2]
	$Hm^{5\text{-segments}}$	−24.0128	1-segment–7-segments	9.0138[b7]	−22.2894	1-segment–7-segments	0.4166
	$Hm^{7\text{-segments}}$	−24.5586	2-segments–5-segments	6.9802[b5]	−26.7445	2-segments–5-segments	7.9371[b5]
			2-segments–7-segments	5.8885[b7]		7-segments–2-segments	0.9732
			7-segments–5-segments	1.0917		7-segments–5-segments	8.9103[b5]

[a] BF > 2 is positive evidence for either 6-, 7- or 8-segments as the ancestral condition using the criteria of Pagel et al. (2004).
[b] BF > 5 is strong evidence for either 6-, 7- or 8-segments as the ancestral condition using the criteria of Pagel et al. (2004).
[c] BF > 10 is very strong evidence for either 6-, 7- or 8-segments as the ancestral condition using the criteria of Pagel et al. (2004).

subst./site between it and *Kimberleybathynella*), which is consistent with that found between the other parabathynellid genera; (3) phylogenetic position, being a sister lineage to a clade comprising two distinct genera; and (4) their geographic isolation in South Australia, an area from which parabathynellids have not been described previously. We did not have enough specimens of Lineages B, C and D to conduct thorough morphological examinations, and therefore refrain from postulating what taxonomic rank they might warrant. However, we note that they do not group within any of the recognised genera and exhibit sequence divergences of 21/0.464 subst./site–38%/0.674 subst./site (Lineages B + C, *COI*) and 33%/0.507 (Lineage D) from taxa in other distinct genera. Additional sampling and further morphological investigation are required to determine whether these taxa should be given separate generic status.

Regarding relationships within clade 3, our study shows a sister lineage relationship between *Octobathynella* and *Notobathynella*, which is consistent with Camacho and Hancock's (2010) hypothesis based on these genera having a similar structure of the male thoracopod VIII and the maxillule bearing seven claws. Both taxa are from New South Wales, albeit from different river systems, the former is from the Peel River and the latter is from the Hunter River. These genera are sister to Lineage A, from the Flinders Ranges, South Australia. Interestingly, *Notobathynella* is morphologically similar to *Billibathynella* and therefore they are considered to be closely related (Hong and Cho, 2009), which is partially supported by our analysis as they are in the same clade. However, *Billibathynella* appears to be more closely related to Lineages B–D and *Brevisomabathynella*. Cho and Humphreys (2010) reported that *Brevisomabathynella* shares many of *Billibathynella's* generic characters, causing some uncertainty in the validity of having two separate genera. Our analysis is consistent with both hypotheses and further morphological analyses of Lineages B–D are required to determine whether there are enough distinctive morphological differences to maintain separate genera.

4.2. Parabathynellid species diversity

Here we report 23 new putative species (or 12 or 16 based on the more conservative higher *COI* thresholds), raising the total parabathynellid species in Australia from 35 to 58 (or 47 or 51 based on the higher *COI* thresholds), making it the most species rich continent to date (see (Camacho and Valdecasas, 2008) for a comparison of species numbers per continent). In comparison, the second richest continental region is Europe with 39 species (Camacho and Valdecasas, 2008), and this is probably the most well-sampled continent given its long history of subterranean biological research. Other likely hotspots for stygofauna and potentially parabathynellids, include largely unexplored regions such as Africa, South America and India (Guzik et al., 2011a). However, the number of parabathynellid species in Australia as it presently stands is likely to be a significant underestimate given that many

potential groundwater habitats in Australia have not yet been surveyed (Guzik et al., 2011a). Although very few individuals here represent known species, we were able to examine the relationships among nine known species from three genera. Interestingly, the two described *Atopobathynella* species included here, *A. hinzeae* and *A. glenayleensis*, from the Yilgarn Region, Western Australia, are more closely related to a species from South Australia and the Pilbara, Western Australia respectively than to each other. Our study also included the following six known species of *Brevisomabathynella* (Cho and Humphreys, 2010), *B. magna*, *B. jundeensis*, *B cooperi*, *B. clayi, B. cunyuensis* and *B. uramurdahensis. Brevisomabathynella* is a remarkable genus because it is unusually morphologically diverse, displaying a range of body types including 'squat', 'fat-bellied' and long, narrow forms (Cho and Humphreys, 2010). Cho and Humphreys (2010) hypothesised that the diversity of forms may be due to niche partitioning, with the co-occurrence of sister species, *B. jundeensis* and *B. cooperi*, providing evidence for this, because these species have markedly different body forms (the former being squat and the latter long and narrow). This sister lineage relationship is in accordance with previous research of Cooper et al. (2002) and Leys et al. (2003) which suggested that sympatric species pairs (and triplets) of stygobitic diving beetles inhabiting the Yilgarn calcrete system may have diversified through niche partitioning. Here, we have identified five new species of *Brevisomabathynella* and six additional species of *Brevisomabathynella* have been described based on morphological data (not yet sequenced), bringing the total species number to 17 (Cho and Humphreys, 2010). The richness of this genus is noteworthy given that nearly half of parabathynellid genera are currently monospecific (Camacho, 2006) (although it is noteworthy that many genera are described from a single sample collected in an entire country), while the two most species-rich genera contain 22 species (*Iberobathynella* and *Hexabathynella*). *Brevisomabathynella* is also noteworthy because its diversity of morphological forms is not accompanied by high genetic divergences. In fact, genetic divergences were surprisingly low, with divergences of 6.2%/0.054 subst./site seen between two morphologically distinct, undescribed species from separate calcretes. None of the genetic distances between known *Brevisomabathynella* species meet the Costa et al. (2007) threshold of 17% (K2P) or the Lefébure et al. (2006) of 0.16 subst./site and three species do not meet the Guzik et al. (2011a) 11% threshold. It appears that *Brevisomabathynella* may have undergone a relatively recent species radiation, which could have been caused by the formation and fragmentation of the Yilgarn calcretes.

Overall, our analyses revealed the first species of '*Hexabathynella*' from South Australia, two new species of '*Chilibathynella*' (*COI* sequence divergence of 18%/0.3 subst./site, *18S*: 6.6%), five new species of '*Atopobathynella*' ('*A.*' sp. 1–5, min. *COI* genetic divergence of 16%/0.272 subst./site) and two new species of *Kimberleybathynella* (*COI* divergence of 33%/0.493 subst./site). It is noteworthy that despite the high morphological similarity among species in the latter two genera, there is high interspecies genetic divergence (up to 25%/0.483 in '*Atopobathynella*' and 33%/0.493 subst./site in *Kimberleybathynella*), suggesting that relying solely on morphological data may underestimate species diversity for parabathynellids. Additionally, we have identified two new species of *Billibathynella* and five putative new species (Lineages A–D) which do not group within any of the recognised Australian genera.

High parabathynellid species diversity in Australia is not surprising given some arid areas have recently been recognised as stygofaunal 'hotspots' for other stygofauna (Humphreys, 2008; Eberhard et al., 2009; Guzik et al., 2011a). Parabathynellids have been collected from a range of habitats from beach sands and alluvial aquifers in New South Wales to springs in the Flinders Ranges, South Australia to calcrete aquifers in arid Western Australia (Hancock and Boulton, 2008; Camacho and Hancock, 2010; Cho and Humphreys, 2010). Thus far, the Yilgarn Region of Western Australia has yielded the highest number of new taxa, however this may be due to the extensive sampling conducted in the region, in addition to the unique nature of the calcrete aquifer system which is like a subterranean archipelago (Cooper et al., 2002, 2007, 2008; Leys et al., 2003; Guzik et al., 2008) allowing numerous opportunities for allopatric speciation through population fragmentation (e.g. (Guzik et al., 2011b). The locations of many of these habitats are extremely ancient. For example, the Flinders Ranges date to the Precambrian period and the Pilbara and Yilgarn cratons have been emergent above sea level since the Proterozoic, although the calcretes are geologically Tertiary (Knoll et al., 2004; Humphreys, 2008). Although there are no bathynellacean fossils, their pervasive presence in these ancient areas is consistent with their hypothesised ancient origin in the Upper Palaeozoic (Brooks, 1962; Schram, 1977). In recent years, each new area of Australia that has been explored for subterranean fauna has yielded new parabathynellid species; therefore we predict that further sampling will uncover a significant diversity of new species. Additionally, this diversity and the likelihood that they provide valuable ecological services such as biofiltration (Boulton et al., 2008) make them of high conservation significance.

4.3. Morphological convergence obscures true phyletic relationships

Although our ancestral state reconstruction analysis did not produce congruent results between methods, the Bayesian analysis produced some support for a trend of increasing segment number in derived taxa, contradicting the traditional view that fewer segments equate to a derived state. We consider the Bayesian approach to be somewhat more rigorous than ML and MP as it takes into account both mapping uncertainty (i.e. the error associated with reconstructing the evolution of a character on a given phylogenetic tree (Ronquist, 2004, p. 475) and phylogenetic uncertainty (i.e. the uncertainty in reconstructing character evolution owing to error in the phylogenetic estimate (Ronquist, 2004, p. 475). It also has the advantage of testing many models whereas ML analysis using Mesquite can only implement the Mk1 model, which may not be appropriate for all data sets (Ekman et al., 2008). The results of the present analysis do not provide clear evidence for evolution in one particular direction and in the terminal nodes of the phylogeny there is evidence for appendage number characters switching states relatively frequently, suggesting that caution should be applied when using these characters to assess phylogenetic relationships. Although these results are not definitive, they nonetheless do not support the traditional view that derived taxa are morphologically simple and primitive taxa are complex. Further, our phylogenetic analysis showed that the genus with highly reduced segmentation of the thoracopods and setation of the uropod and mouthparts (*Hexabathynella*) was one of the most basal taxa and the most highly segmented and setose genus (*Billibathynella*) was in a more derived position.

We observed that a six-segmented antennule is conserved in the closely related '*Atopobathynella*' and *Kimberleybathynella*. The ancestral state for clade 3 is seven-segments, which is seen in all taxa in this clade, except for *O. peelensis*, which has the unusual state of eight-segments, indicating that contrary to the suggestion of previous authors (Schminke, 1974; Cho, 2005), in some cases the addition of segments may represent the derived state. The ancestral state for antennal segment number was 1- or 2-segments, according to the BI analysis, with 1-segment being well-supported over 5- or 7-segments but not over 2-segments. This result was unexpected because the basal taxa in the phylogeny have 5-antennal segments and this is the most common state in parabathynellid species worldwide (A. Camacho, pers. comm.). However, this result may be due to

instability at the basal nodes of the phylogeny, possibly caused by a lack of taxa from outside Australia. Future studies may be able to resolve this problem with greater world-wide sampling. It is noteworthy that for the antenna, the highest number of segments (7 in *Octobathynella* and *Billibathynella*) is seen in the terminal parts of the phylogeny, while the least number of segments (1 in '*Atopobathynella*') is observed in lineages from a relatively basal part of the phylogeny.

Overall, we observed that the molecular data supported the distinction of currently described species and genera, suggesting that use of combinations of characters such as segment number and appendage ornamentation (i.e. number and position of spines and setae) are appropriate for alpha taxonomy. However, given the evidence for character state reversals and convergent evolution we suggest that caution needs to be applied when using the two characters examined here (i.e. antennule and antennae segment numbers) for phylogeny reconstruction. Indeed, Cho et al. (2006a) conducted a cladistic analysis of the relationships amongst species of *Atopobathynella*, and reported that state reversal occurred many times, causing a lack of resolution and support for their cladogram.

5. Conclusions

Molecular phylogenetic analyses of Australian parabathynellids have provided a framework for future research into parabathynellid systematics and revealed a high diversity of taxa in Australia. Our analyses further supported the monophyly of known genera defined by traditional morphological methods, suggesting that the commonly used generic characters are robust for recognising parabathynellid genera. However, caution needs to be shown when using morphological characters such as antenna and antennule segment numbers to elucidate phylogenetic relationships, due to evidence of their convergent evolution, as indicated by the results of the ancestral state reconstruction analysis. The current analysis contradicted the conventional view of parabathynellid evolution, which assumes that more simplified taxa (i.e. those with fewer-segmented appendages and setae) are derived and more complex taxa are primitive. To overcome difficulties in elucidating phylogenetic relationships and defining taxa, a combined molecular and morphological approach is recommended in future investigations into parabathynellid systematics.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.03.010.

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