Phylogenetics and biogeography of Australian subterranean Parabathynellidae

Brevisomabathynella leijsi (Parabathynellidae)

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DECLARATION

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Kym Abrams

This thesis is dedicated to my parents,

Berenise and Rashaad

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ABSTRACT

The putatively ancient subterranean crustacean family Parabathynellidae has been poorly studied, in part because of the problem of obtaining material from difficult to access subterranean habitats in which they live. Further, the systematics of the group has been complicated by their generally simplified morphology and isolated descriptions of new taxa in the absence of any phylogenetic framework. This thesis provides a comprehensive molecular systematics framework for Australian Parabathynellidae, which is used to explore phylogenetic relationships amongst parabathynellids, their diversity, some aspects of character evolution and their biogeographic history within Australia. In addition, taxonomic descriptions are provided for the first parabathynellid species from South Australia.

For the first data chapter molecular sequence data from the mitochondrial cytochrome c oxidase subunit 1 (*COI)* and *18S* rRNA genes were generated in order to examine phylogenetic relationships amongst Australian genera and assess the species diversity of this group within Australia. The resultant phylogenetic framework, in combination with an ancestral state reconstruction analysis, was used to explore the evolution of two key morphological characters 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 ancestral state reconstruction analysis was also used to determine whether there has been convergent evolution of appendage numbers during the evolution of Australian parabathynellids. 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. These analyses also revealed a remarkable diversity of parabathynellids. Additionally, ancestral state reconstruction analysis provided evidence for multiple cases of convergent evolution for the two morphological characters evaluated and 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.

The third chapter focuses on South Australia, where phylogenetic analyses revealed a previously unknown diversity of parabathynellids from South Australia, and a complex set of relationships with the New South Wales and Western Australian fauna. Additionally, the first parabathynellid genus from South Australia, *Arkaroolabathynella* gen. nov, is described and a key to and checklist of Australian parabathynellid genera is also provided.

The final data chapter used an expanded dataset to investigate the geographic distribution, history and evolutionary relationships of extant parabathynellids between subterranean bioregions in Australia. This study found evidence for significant regional biogeographic

structuring of parabathynellids at the genus and species levels, indicating a long and complex evolutionary history for these animals in Australia, likely shaped by fluctuating climates throughout the continent's development. The high incidence of regional endemism for parabathynellids is signifcant because it confirms the poor dispersal capability of these animals, which makes them particularly vulnerable to disturbance or destruction of their subterranean habitats.

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CHAPTER I

GENERAL INTRODUCTION

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Introduction to subterranean environments

Subterranean environments are considered to be 'extreme' habitats because they are characterized by complete darkness, periods of anoxia and highly variable food resources (Lefébure *et al.*, 2006b). These characteristics have led to convergence of morphological characters associated with adaptations to this environment in a diverse array of animal groups, ranging from arthropods to fish (Humphreys, 2001). Subterranean fauna can be separated into two groups according to habitat, namely stygofauna (aquatic subterranean fauna) and troglofauna (terrestrial subterranean fauna). Characteristic components of Australian stygofauna include Insecta (diving beetles), Oligochaeta and Mollusca. However, the dominant component of most stygofaunal communities typically comprises the Crustacea, particularly the amphipod, isopod, copepod and syncarid groups.

Morphological adaptations of subterranean fauna include a unique suite of regressive (pigment and eye loss) and progressive (appendage elongation, enhanced non-optic sensory organs) traits collectively termed troglomorphisms (Christiansen, 1962; Porter, 2007). Subterranean animals also exhibit ecological adaptations such as slow metabolisms, lowered fecundity, degeneration of circadian rhythms and increased life spans and egg volume, in comparison with surface species (Humphreys, 2000). These characteristics generally make species slow to recover from population decreases and, for stygofauna, vulnerable to perturbations of the groundwater system (Humphreys, 2000). Furthermore, due to these characteristics, subterranean fauna tend to be short-range endemics, i.e. they have a very small geographic range (Harvey, 2002). Collectively these factors make subterranean fauna a significant group of organisms for biodiversity conservation.

The unusual characteristics of subterranean fauna and the truncated ecosystems of subterranean environments also make these environments potential natural ecological and evolutionary laboratories (Poulson and White, 1969; Juan *et al.*, 2010). Some subterranean taxa are geographic or phyletic relicts, i.e. they are the remnants of populations that have become restricted to subterranean habitats at different times due to climatic change, vicariance or speciation (Humphreys, 2006). These fauna are of great scientific significance because they can be used to examine questions about the earth's past geological history and processes of evolution and speciation (Humphreys, 2006).

Recently, numerous genetic studies of stygobiont crustaceans have revealed that crustacean species diversity has been severely underestimated by morphological methods (Jarman and Elliott, 2000; Proudlove and Wood, 2003; Finston and Johnson, 2004; Lefébure *et al.*, 2006a; Guzik *et al.*, 2008; Trontelj *et al.*, 2009). This is likely due to the common occurrence of

cryptic (i.e. "two or more distinct species that are erroneously classified (and hidden) under one species name, because they are at least superficially morphologically indistinguishable" (Bickford *et al.,* 2007:149) speciation in crustaceans (Lefébure *et al.*, 2006a; Apte *et al.*, 2007; Finston *et al.*, 2007) as well as a lack of detailed taxonomic study and available specialist expertise. Moreover, stygobiont organisms exhibit troglomorphy which has been recognised as one of the most powerful examples of habitat-driven convergence of form (Porter and Crandall, 2003) resulting in a high incidence of cryptic species.

Biogeography of subterranean fauna

In the past, theories of the evolution of subterranean fauna have been controversial and many hypotheses are currently still a matter of debate. Some of the most vigorously debated issues include origins of subterranean species and the modes of speciation involved in the diversity of species (i.e. the climate relict vs. ecological/parapatric modes of speciation) and the role played by dispersal vs. vicariance to explain subterranean biogeographic barriers (see Porter, 2007 for a review). Theories to explain why and how epigean species invade and colonize subterranean habitats usually incorporate three phenomena: preadaptation of epigean ancestors, invasion and colonization by founder species, and speciation (Holsinger, 2000). Presently there are two commonly invoked hypotheses to explain the origins of subterranean species - the 'climatic relict' hypothesis and the 'adaptive shift' hypothesis (Howarth, 1973; Holsinger, 2000). The former hypothesis (Holsinger, 1988; Peck and Finston, 1993) was initially proposed for temperate ecosystems and postulates that subterranean organisms evolve from epigean organisms that invade subterranean habitats to escape unfavourable climatic conditions (e.g. glaciations or aridity) (Holsinger, 2000). Eventually, they become isolated in underground refugia when their epigean relatives become extinct. In this case, speciation is allopatric. In contrast, the 'adaptive shift' hypothesis, which was initially proposed for tropical subterranean fauna, postulates that subterranean organisms evolve from epigean ancestors that actively invade subterranean habitats in order to exploit new niches (Howarth, 1987; Desutter-Grandcolas and Grandcolas, 1996). In this case there is no period of physical isolation from surface relatives and speciation is sympatric or parapatric (Holsinger, 2000). The key factor that allows one model to be distinguished from the other model is the presence or absence of gene flow during divergence (Niemiller *et al.*, 2008; Juan *et al.*, 2010). Previous studies have provided support for both hypotheses (see Juan *et al.*, 2010 for a review). However, a lack of closely related extant epigean species may make it difficult to determine which of these hypotheses, if any, have played a role in the evolution of some taxa. Interestingly, *in situ* speciation through allopatric and possibly sympatric processes is also likely to have played an important role in the evolution of new species within cave systems (Juan *et al.*, 2010), especially after isolation within the cave system. The number of studies to

test the hypothesis of *in situ* speciation directly is limited (Guzik *et al.*, 2009 and Bradford *et al.*, in review), but has the possibility to explain high levels diversity found in some cave systems.

Since the late 1800s, there has been vigorous debate concerning the mechanisms responsible for the distribution of subterranean fauna, largely centered on the relative roles of different biogeographic models, particularly dispersal and vicariance (Porter, 2007). Most studies tend to side exclusively with either dispersal or vicariance (see Culver *et al.*, 2009), but recently it has been recognised that faunal distributions are more likely to be shaped by a combination of these events. Biogeography of subterranean populations is complicated further because current population distribution patterns have been shaped by dispersal and vicariant events which have occurred not only in the present species, but also in surface ancestral populations from which they arose. Since faunal distributions are most likely governed by complex internal (e.g. dispersal capability) and external (e.g. habitat connectivity, vicariant events) processes, it has become more important to understand the combination of factors causing current distribution patterns, rather than attributing them to one mechanism versus another (Porter, 2007). Detailed knowledge of faunal distributions and how they came about are important to managing organisms of conservation significance, such as stygofauna. For example, knowledge of an organism's historical and current dispersal abilities may allow its ability to survive changes to its habitat, caused by human activities or climatic events to be assessed. Further, levels of biodiversity can be estimated, especially where short range endemics are concerned.

Conservation importance and management of stygofauna

Approximately 97% of the world's available freshwater lies underground (Boulton *et al.*, 2003) and, with the increasing anthropocentric demand for water, groundwater systems are increasingly threatened. Stygofauna play an important role in maintaining groundwater quality through the provision of important ecosystem services such as water purification, bioremediation, nutrient cycling and water infiltration and transport (Boulton *et al.*, 2008). Amphipods, isopods and syncarids have been identified as some of the most important groups providing these services (Boulton *et al.*, 2008). A loss of stygofaunal biodiversity and its associated ecological services may, therefore, have grave impacts on groundwater systems.

Recently, a number of 'hotspots' of subterranean faunistic diversity have been identified in Australia, such as the Yilgarn, Pilbara and Kimberley regions of Western Australia (WA), and other potential hotspots include the Flinders Ranges of South Australia (SA) and alluvial aquifers of New South Wales (NSW). Unfortunately, these areas are often heavily exploited for mineral and aquatic resources (Hancock *et al.*, 2005; Boulton *et al.*, 2008). Some of the

major threats to groundwater systems and their associated stygofauna include quarrying and mining, agriculture, waste disposal and groundwater extraction (Boulton *et al.*, 2003). In Australia, the impact of land clearing and agriculture is particularly significant because it affects cave microclimates, nutrient and sediment inputs and hydrological regimes (Boulton *et al.*, 2003). Evidence of this can be seen in the loss of biodiversity in the cave fauna of karstic areas of NSW and Victoria, associated with removal of native vegetation cover (Hamilton-Smith and Eberhard, 2000). Human impacts on groundwater can also include changes to the hydrological cycle which can result in habitat loss, degradation or fragmentation, altered water quality and reduction or cessation of baseflow and spring discharge (Tomlinson and Boulton, 2008). Further, reduced groundwater discharge can severely threaten the ecology and biodiversity of many wetlands and rivers by limiting connectivity, affecting stream metabolism and failing to support dry season refugia (Boulton *et al*., 2008; Tomlinson and Boulton, 2008). A significant example of the consequences of over-extraction of groundwater is provided by the cessation of flow in one-third of the 3000 natural springs of the Great Artesian Basin (Worthington-Wilmer and Wilcox, 2007), resulting in the listing of mound springs communities under Commonwealth endangered species legislation.

Management of stygofauna is particularly difficult due to the general paucity of information on the hydrology and biodiversity of groundwater systems. This is a consequence of limited sampling of these habitats (often due to inaccessibility), few taxonomic specialists working on stygofauna, and a poor public understanding of the ecological value and variety of groundwater habitats (Boulton *et al.*, 2003). Australian stygofauna show a high degree of short-range endemism (mostly due to poor dispersal abilities) (Harvey, 2002; Harvey *et al.*, 2011), which makes them particularly vulnerable to threats from groundwater extraction and contamination. Detailed knowledge of the variability and distributions of subterranean species is required as a first step to protecting them from these threats.

The use of molecular data to inform conservation management

Presently it is recognized that we are in the midst of a biodiversity crisis, with species being lost to extinction before they have even been described. To combat this problem, molecular genetic methods can be employed to assist species identification, especially in the case of cryptic species which lack diagnostic morphological characters (Gaines *et al.*, 2005). Morphological convergence has not only caused species underestimation, but has also obscured true phyletic descent of groups thereby confounding systematic studies. Species underestimation due to morphological convergence is an important issue because a stable systematic framework is required to appropriately identify, manage and conserve stygofauna. Additionally, accurate species identifications and classifications are vital to conservation purposes since generally legal protection and management is based on government legislation

using conventional taxonomic distinctions (Gaines *et al.*, 2005; Harvey *et al.*, 2011). Advances in molecular genetics and the consequential increase in molecular studies of subterranean biota have allowed the testing of hypotheses on biogeography and modes of evolution within explicit phylogenetic frameworks. The consequence of this has been an exploration of species discovery, distributions and evolutionary history on an unprecedented scale.

Molecular techniques offer a large number of characters for phylogenetic studies, increasing the sensitivity and resolution of the analyses to investigate evolutionary hypotheses (Porter, 2007). Mitochondrial DNA (mtDNA) sequences are among the most commonly used genetic data in animal studies concerning population structure and species relationships (Gaines *et al.* 2005). This is mostly due to factors such as mtDNA's rapid rate of sequence divergence, relative ease of sequencing (Simon *et al.*, 2006), availability of universal primers and the ability to compare datasets across taxa (Gaines *et al.* 2005). However, in some cases mtDNA may not accurately reflect species relationships (Shaw, 2002). This is because each gene is used to generate a genealogy that only represents a single inference about an organism's evolutionary history, therefore the use of multiple unlinked loci is recommended to accurately reconstruct evolutionary history (Simon *et al.*, 2006). Other potential issues associated with mtDNA include the possible presence of pseudogenes which can mislead phylogenetic hypotheses; and saturation of nucleotide substitutions can affect our ability to estimate accurate species divergences and relationships (Brown *et al.*, 1979; Zhang and Hewitt, 2003). Nuclear DNA (nDNA) sequences can be used to provide independent estimates of phylogenetic relationships, which can either corroborate or conflict with phylogenies constructed with mtDNA data (Williams *et al.*, 2001; Gaines *et al.*, 2005). Nuclear genes have the added advantage of being useful in resolving higher taxonomic relationships (e.g. among genera and families) (Porter, 2007). In most cases (depending on time and funds) it is important to compare and combine nuclear and mitochondrial datasets (Shaw, 2002; Gaines *et al.*, 2005; Simon *et al*., 2006).

Today, DNA sequencing is increasingly being used to identify and evaluate species diversity (Bickford *et al.*, 2007). This is emphasized by the fact that numerous studies of widely diverse organisms such as frogs (Meegaskumbura *et al.*, 2002), tropical butterflies (Hebert *et al.*, 2004), fungi (Bidochka *et al.*, 2001) and subterranean amphipods (Lefébure *et al.*, 2006a) have identified cryptic species using DNA sequence data. One of the important difficulties of species' delineation using sequence data, is the amount of divergence required to accurately infer species' boundaries. Lefébure *et al.* (2006b) conducted a genetic study of various groups of crustaceans (using DNA sequences from Genbank), which led them to suggest that crustacean species can be delineated on the basis of cytochrome oxidase 1 (*COI)* patristic

distances of 0.16 or more substitutions/site. A similar study of numerous crustacean groups by Costa *et al.* (2007) suggested that crustacean species can be delimited by 16% Kimura-2-parameter (K2P) pairwise divergence. Studies by Cooper *et al.* (2007) and Finston *et al.* (2007), which found quite large genetic divergences (>18%) among populations of subterranean amphipods from the Yilgarn and Pilbara regions (Western Australia) respectively, where little or no clear morphological divergences were apparent, are congruent with the thresholds suggested above. However, different groups of organisms may have different divergence thresholds and this has led to controversy over the use of thresholds to infer species. While genetic divergence estimates can provide an invaluable tool for detecting and differentiating cryptic species, caution needs to be applied when using molecular thresholds. Although molecular methods are an important tool that can be used to aide and guide taxonomic distinction, an integrative taxonomy approach (Dayrat, 2005; Page *et al.*, 2005; Will *et al.*, 2005), maintaining a taxonomic framework based upon multiple types of biological information (e.g. molecular, morphological and ecological data, Will *et al.*, 2005; Porter, 2007), is recommended as use of only one data type may produce inconclusive results (Lee, 2004).

The impact of incorrect or incomplete species' identification is highlighted in a study by Finston *et al.* (2004), which conducted allozyme analyses on what was originally said to be 15 species of groundwater amphipods (based on morphology) of the genus *Chydaekata* Bradbury, 2000, which were each restricted to a single bore. The study concluded that there was not sufficient molecular evidence to support this number of species, but rather there were two distinct and widely distributed genetic groups present, and so mining was allowed to proceed in the area. The reverse situation can also occur where cryptic species cohabiting an area are not identified as separate species so their significance is not recognised and this could result in a loss of highly endemic species. For this reason it is important to employ both morphological and genetic data to identify and define species and/or evolutionarily significant units. Additionally, molecular data can also be used in phylogeographic studies, which investigate the geographical distribution of genealogical lineages, uniting phylogenetic relationships of populations with the geographical distributions of those phylogenetic groupings (Avise *et al.*, 1987; Avise, 2000). A phylogeographic perspective is an invaluable tool which can be used to make predictions about areas where novel taxa may occur, or which may be unusually diverse, thereby allowing new areas for biodiscovery to be targeted and increased conservation protection measures developed (Harvey *et al*., 2011).

High levels of subterranean faunal biodiversity, especially in Australia, have recently been highlighted (Guzik *et al*., 2011). Both new and well known subterranean environments are revealing unprecedented genetic and morphological diversity amongst the fauna. Whilst it is

known that these fauna have existed in these environments for many millions of years, biogeographic origins and patterns have yet to be compared at a continent-wide scale. The present study aimed to explore and compare species diversity, boundaries and biogeography of distinct biodiversity hotspots/regions in Australian subterranean fauna. One taxon group in particular, the Parabathynellidae, is used for this case study due to a number of characteristics that make it an excellent example for studying possible biogoegraphic events through vicariance and/or dispersal. In particular, parabathynellids are predicted to have limited dispersal abilities making it vulnerable to geographic isolation and also environmental perturbations (i.e. dewatering or contamination of groundwater dependent habitats). Further, it is predicted that parabathynellids have ancient origins within Australia (Cho, 2005) with this group inhabiting the subterranean realm prior to the break-up of Pangaea. This extensive history of groundwater-dependent life history can be used to examine ancient biogeographic origins. Interestingly though, it also means that the colonization of groundwater habitats is unlikely to be the same as for other subterranean taxa (i.e. dytiscid beetles, which colonized groundwater habitats in the last 8-3 MY Leys *et al.*, 2003).

Parabathynellidae – an example of stygofaunal biodiversity

The Parabathynellidae Noodt, 1965 is a useful group for investigating questions of species diversity and boundaries and biogeography. They belong to the syncarid order Bathynellacea Chappuis, 1915 which also contains the family Bathynellidae, Grobben 1904. Parabathynellidae contains 172 species within 47 genera (Camacho, 2006), and exhibits a worldwide distribution, except for the polar regions. Parabathynellids may be identified by an elongate, vermiform body with posteriorly-directed antennae, a labrum which is serrate or fringed with fine setae, lateral setae on the pleotelson and the first pleopods, if present, are one-segmented or represented by only two setae (Fig. 1.1) (Schram, 1986). They have been found in a variety of habitats such as wells, gravel banks of rivers, wet caves, springs, marine beaches and even a hot spring (55^{o} C) in Africa (Schminke and Noodt, 1988). Although very little is known about their biology and ecology, the remarkable variation in types of mouthparts, suggesting specialization on different types of food (Schminke, 1973), provides some insight into the feeding habits of parabathynellids. Generally, they are thought to be detritivores, feeding on bacteria, animal and vegetal remains, but two genera are known to be carnivorous (*Iberobathynella,* Schminke 1973 and *Brevisomabathynella* Cho *et al.*, 2006), feeding on copepods and ostracods respectively (Cho *et al.*, 2006a; Camacho and Valdecasas, 2008). The variability of parabathynellid mouthparts is significant because mouthpart characters are commonly used to define and distinguish genera and species (Schminke, 1973). The plasticity of these organs has likely contributed to the successful diversification of parabathynellids.

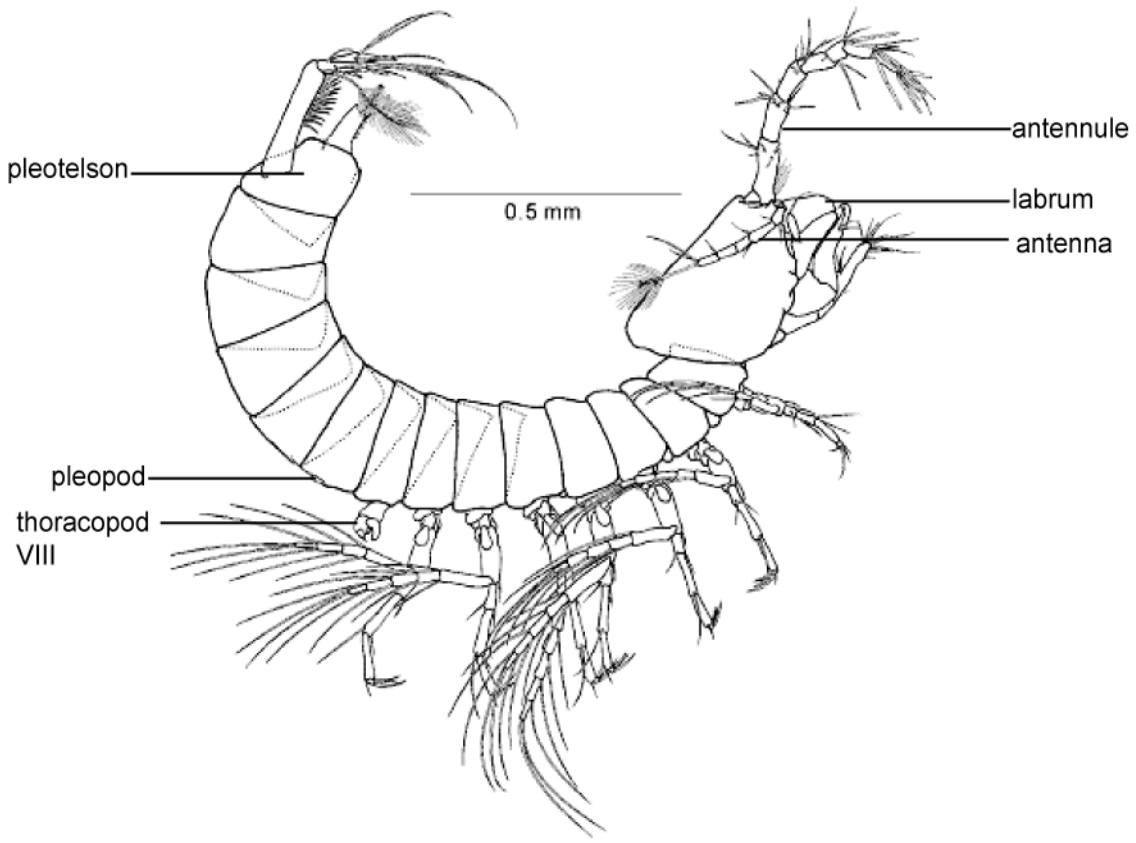

Fig. 1.1. Diagram of a parabathynellid, with important characters labeled. Modified from Hong and Cho (2009).

Less than a decade ago, the Australian parabathynellid fauna was poorly known and prior to 2005, only four genera had been described from Australia — *Notobathynella* Schminke, 1973, *Chilibathynella*, Noodt, 1963, *Atopobathynella* Schminke, 1973 and *Hexabathynella* Schminke, 1972. Recent exploration of Western Australia, however, partially due to extensive natural resource mining activities in the region, has led to the discovery of three new genera, *Kimberleybathynella* Cho *et al.*, 2005, *Billibathynella* Cho, 2005 and *Brevisomabathynella*. The discovery of a significant and diverse stygofauna in Western Australia led to further, more thorough explorations of the subterranean realm in other parts of Australia resulting in the discovery of *Octobathynella* Camacho and Hancock, 2010 and *Onychobathynella* Camacho and Hancock, 2012 from New South Wales (NSW) and the first parabathynellids described from Queensland (Camacho and Hancock, 2011) and the Northern Territory (Cho *et al.*, 2006b). There are currently nine genera containing 43 described species from Australia (see Checklist in Ch. III for complete listing).

One of the most speciose habitats for parabathynellids in Australia appears to be the groundwater calcrete aquifers (hereafter termed calcretes) of the arid western Yilgarn craton (Cho *et al.*, 2006b; Guzik *et al.*, 2008). In this region, the family is known from at least 49 discrete calcretes occurring in 13 palaeodrainage systems (Cho *et al.*, 2006b). The description of three new genera and 22 new species from the Yilgarn region, in the last five years,

emphasizes the immense diversity in this area. These parabathynellids have also been observed to have unusual characteristics because they are free-swimming and larger (generally 1-3 mm long) than those from other regions of the world (<1 mm). The largest (6.3 mm) and putatively the most morphologically primitive species, *Billibathynella humphreysi*, Cho, 2005 is found in this area.

Systematics and classification of parabathynellids

Although Schminke (1973) commented on the phylogenetic relationships among all known parabathynellid genera based on his intuitive ideas of how the group evolved; parabathynellid phylogenetic relationships have never been formally analysed and many new genera and species have been discovered since then. Therefore the phylogenetic relationships among Australian parabathynellid genera and the global fauna are unknown. This taxonomic uncertainty stems from a simplified body plan (Schminke, 1974) with few phylogenetically informative characters. Consequently, any morphological phylogenetic analysis of this group would be strongly dependent on characters displaying reduction of structures, which often results in poor resolution of relationships among genera and species (Cho *et al.*, 2006b). Additionally, the simplified and convergent nature of bathynellaceans makes them likely candidates for cryptic speciation. This is supported by prior studies of parabathynellids (Guzik *et al.*, 2008), and more recently for bathynellids, as a molecular analysis of *Vejdovskybathynella* Serban and Leclerc, 1984, uncovered the presence of two cryptic species (Camacho *et al*., 2011). In light of the high incidence of cryptic species in stygobiont crustaceans generally, it is reasonable to assume that there may be many cryptic parabathynellids. Given the taxonomic difficulties associated with this group, it is likely that molecular methods will better resolve relationships within the Parabathynellidae, as has been

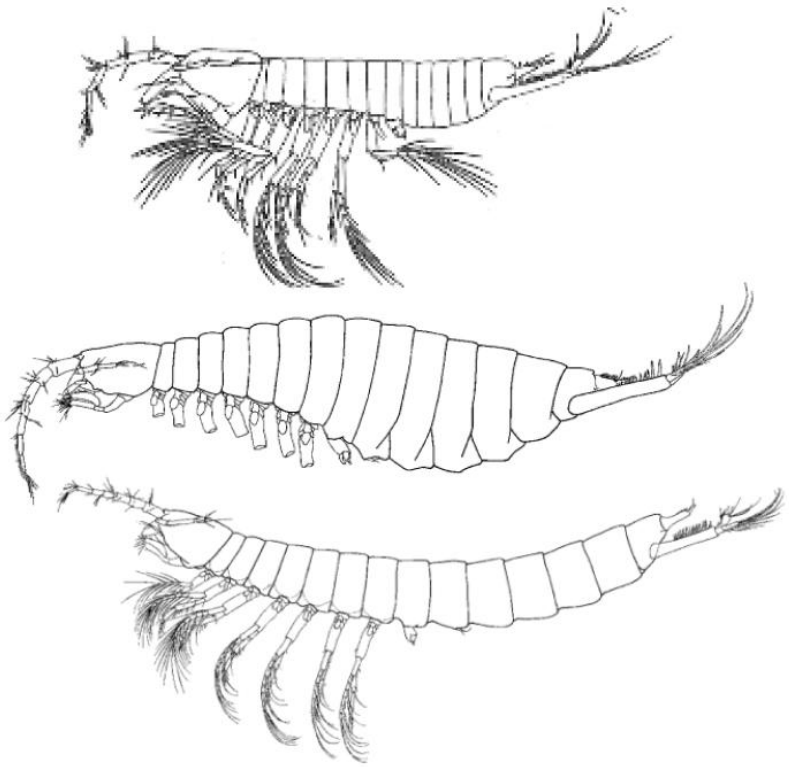

Fig. 1.2. Diversity of body forms observed in *Brevisomabathynella*. From top to bottom: *B. cooperi*, *B. uramurdahensis* and *B. magna*. Figure modified from Cho *et al.* (2006a) and Cho and Humphreys (2010).

recently achieved with stygobiont caridean and *Machrobrachium* shrimps (Murphy and Austin, 2005; Page *et al.*, 2007).

The phylogeny of a small number of Australian species have been investigated in three recent studies (Camacho, 2003; Cho *et al.*, 2006b; Cho and Schminke, 2006), but all were based on morphological characters only and each study focused on species within one genus. Recently, Guzik *et al*. (2008) investigated the relationships among parabathynellid species from calcrete aquifers in Western Australia using sequence data based on a region of the mitochondrial DNA gene *COI*. Their study included species from three genera — *Atopobathynella*, *Billibathynella* and *Brevisomabathynella.* They found that species grouped within their respective genera and formed monophyletic groups. They also uncovered a paraphyletic genus (Genus A in their study), which had *Brevisomabathynella* embedded within it. Further morphological analyses (Cho and Humphreys, 2010) confirmed that the species of Genus A were in fact members of *Brevisomabathynella*, despite their markedly different body forms (Fig. 1.2). This was remarkable because *Brevisomabathynella* was initially thought to only contain species with an unusually squat, 'pygmoid' body form (see Cho *et al.*, 2006a). Consequently, molecular data has revealed that the usually morphologically conservative parabathynellids may also contain exceptionally morphologically diverse genera.

The appearance of *Atopobathynella* in a basal position to the 'primitive' *Billibathynella* in Guzik *et al*.'*s* (2008) molecular phylogeny is a second significant and surprising finding because *Billibathynella* is thought to display highly primitive morphological characters (Cho, 2005) and be comparable to the putative stem species of Parabathynellidae, as hypothesized by Schminke (1973). This is significant because the conventional method for inferring the primitive versus derived nature of parabathynellids is to assume that multiple segments and setae in a species equate to that species being primitive, and reduced segments and setae equate to a species being more derived. A classic example of this is *Hexabathynella*, which is considered to be the most derived genus due to its small size, reduced number of thoracopodal segments and the absence of the seventh pair of thoracopods (Cho and Schminke, 2006). This method is commonly used in crustacean systematics and is discussed further in Chapter II. Additional sampling of other genera, particularly the putatively primitive *Notobathynella* and *Chillibathynella* will be included in this study to test intergeneric relationships and the conventional hypothesis that highly segmented genera are primitive. Additionally, a phylogeny which also includes sequence data from *18S*, a more slowly evolving nuclear gene, will be used to test and verify these relationships.

Parabathynellid biogeography

Of the 47 known parabathynellid genera, only 10 are described from two or more continents and are considered to have cosmopolitan distributions (see Appendix 1, Chapter II). 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). As parabathynellids are small, restricted to small areas of interstitial groundwater habitat and presumed to have poor dispersal abilities there has been much speculation about how genera could achieve global distributions (Schminke, 1974; Camacho *et al.*, 2006; Cho and Schminke, 2006). A lack of bathynellacean fossils and extant epigean ancestors has made understanding the present distribution of parabathynellids particularly challenging (Camacho and Valdecasas, 2008), although it is commonly believed that genera existed in widespread distributions before the breakup of Pangaea and that their present biogeography can be explained by vicariance and secondary 'local' dispersal (Schminke, 1981; Cho and Schminke, 2006). It has been largely assumed that parabathynellids are poor dispersers (Schminke, 1981; Schram, 2008) and therefore have highly restricted distributions, however, Camacho and Valdecasas, (2008) recently suggested that parabathynellid species may be more widely distributed than expected, with some species (e.g. *Iberobathynella imuniensis*) found to have broader (~20km) distributions after intensive sampling over many seasons in different habitats. Additionally, Cho *et al.* (2006b) recorded *Atopobathynella watts* Cho, 2006 from multiple calcretes (separated by 10's of kms), suggesting that parabathynellids may disperse between calcretes. However, it should be noted that neither the *I. imuniensis* nor *A. wattsi* distributions have been tested with molecular data. In the first broad scale attempt to analyse molecular mtDNA data in parabathynellids (Guzik *et al*., 2008), no evidence of dispersal of species between calcretes was found; instead numerous cases of highly restricted parabathynellid species and genera were reported. These findings are similar to recent molecular analyses involving other calcrete invertebrate fauna (amphipod crustaceans and beetles), which have been found to be restricted to individual calcretes (Leys *et al.*, 2003; Cooper *et al.*, 2007).

Despite the above possibly questionable examples of potentially widespread parabathynellid species and multiple genera with intercontinental distributions, the bulk of evidence, albeit mostly circumstantial, seems to support the hypothesis that parabathynellids are very poor dispersers. Further, given that they are highly prone to morphological convergence that has deeply confounded their systematics, it is possible that genera are not distributed on multiple continents. Rather, they may be morphologically convergent yet genetically and geographically distinct clades which are endemic to particular continents/regions. Given the

above information, further investigation is required to understand the phylogeny, phylogeography and dispersal abilities of parabathynellids.

Project aims

The overall aim of this thesis was to use molecular and morphological data to investigate the evolutionary history of Parabathynellidae using both molecular phylogenetics techniques and also morphological taxonomy, to explore and compare species diversity, boundaries and biogeography of distinct biodiversity hotspots/regions in Australia, with a view to informing the conservation management of this group and applying this knowledge to other subterranean fauna. Each of the results chapters have been written as stand-alone papers, with separate introduction, methods, results and discussion.

Specifically, this study aims to

1. Examine the phylogenetic relationships among Australian parabathynellid genera using mtDNA and nDNA sequence data to create a natural classification system for this family (Chapter II);
2. Assess the usefulness of some key morphological characters for elucidating evolutionary relationships using the molecular phylogeny combined with an ancestral reconstruction analysis approach (Chapter II);
3. Assess the diversity of this group within Australia and identify new taxa (Chapters II and III);
4. Describe four species from a new genus from South Australia and examine their phylogenetic relationships with other Australian taxa (Chapter III);
5. Investigate the biogeography of this group at a continental scale and the timing of their diversification (Chapter IV).

In the last section (Chapter V) a general discussion is presented on the broader implications of this study, especially where other subterranean fauna are concerned, its limitations and likely avenues for future research.

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CHAPTER II

WHAT LIES BENEATH: MOLECULAR PHYLOGENETICS AND ANCESTRAL STATE RECONSTRUCTION OF THE ANCIENT SUBTERRANEAN AUSTRALIAN PARABATHYNELLIDAE (SYNCARIDA, CRUSTACEA)

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Molecular Phylogenetics and Evolution (In review)

Statement of Authorship

This chapter is a submitted research article.

Kym Abrams (candidate)

Corresponding author: Prepared DNA extracts for PCR amplification, carried out DNA sequencing, analysed sequence data, wrote manuscript and produced all figures.

Signed Date 15/2/2012

Michelle T Guzik

Provided some DNA extracts and sequences for study, provided assistance with obtaining project funding, supervised the direction of the study, provided advice on analyses and critically reviewed manuscript.

I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 16/2/2012

Steven J. B. Cooper

Collected some of the samples for study, sought and won project funding, supervised the direction of the study, provided advice on analyses and critically reviewed manuscript.

I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 15/2/2012

William F. Humphreys

Collected some of the samples for study, provided assistance with obtaining project funding and critically reviewed manuscript.

I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 24/1/2012.

Rachael A. King

Provided taxonomic training to the first author and advice for the taxonomic component and critically reviewed manuscript.

I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 15/2/12

Joo-Lae Cho

Identified some of the taxa in the manuscript.

I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date Feb. 01. 2012

Andrew D. Austin

Provided some project funding and assistance with obtaining additional funding, supervised the direction of the study and critically reviewed manuscript.

I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 15/2/12

CHAPTER II

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. The resultant phylogenetic framework was used, 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.

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; 2005 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 unrecognized 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, character state evolution is explored for 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, 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 1-3mm 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 Appendix 2.1). 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) reported from Australia, four belong to widely distributed genera (see Appendix 2.1) (*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, the endemic Australian 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, the phylogenetic relationships amongst Australian parabathynellid genera are investigated 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. New species are also identified 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, the findings have broader implications for parabathynellid systematics at a global level.

Methods

Sampling

Parabathynellids were collected from various localities across Australia (Fig. 2.1), with a large proportion of the specimens being collected from calcrete aquifers (Fig. 2.2) 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 2.1. Where possible, multiple individuals per location were sequenced, to control for the possibility of sequencing errors and contamination. After ensuring that the sequence data were robust, identical sequences were excluded from the phylogenetic and character state analyses.

Criteria for assessing new species and genera

To assess species, both new and pre-existing, 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 2.2) and geographical location (following the methods of Guzik *et al.*, 2011a; Table 2.2) was used. Genera were defined on morphological grounds as per generic diagnoses in the literature (Noodt, 1963; Schminke, 1972, 1973; Cho, 2005; Cho *et al.*, 2005; Cho *et al.*, 2006b; Camacho and Hancock, 2010; Table 2.3). 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. *Hexabathynella* characteristically lacks thoracopod VII and has one to two-segmented thoracopodal exopods (Cho and Schminke, 2006) (see Table 2.3 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 this study truly belong to cosmopolitan genera which were originally described from other continents, although the 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; Cho *et al.*, 2006b; Cho and Humphreys, 2010). Additionally, the sequence divergence within and between key clades was examined to identify species. Since genetic divergence thresholds can vary amongst organisms (and differing opinions among researchers), the number of potential new species was estimated based on three different *COI* pairwise distance thresholds (using the Kimura-2-parameter model (Kimura, 1980): (1) ≥7.1 % which is based on the *COI* divergence between two morphologically distinct, described parabathynellid species, *Brevisomabathynella cooperi* and *B. jundeensis* (Fig. 2.3); (2) ≥11% as suggested by Guzik *et al.* (2011a) and (3) ≥17% as suggested by Costa *et al.* (2007) based on broadly assessed divergences among decapod crustaceans. Additionally, patristic distances were calculated, from the *COI* ML tree using the program Geneious Pro 5.4. (Drummond *et al.*, 2011) and compared with a threshold of (1) ≥0.075 substitutions per site (subst./site) which is based on the *COI* divergence between *Brevisomabathynella cooperi* and *B. jundeensis* and (2) ≥0.16 subst./site as suggested by Lefébure *et al.*, (2006) based on broadly assessed divergences amongst various crustacean groups. Finally geographic location of potential species was taken into account, i.e., if they were found in an isolated locality, or confined aquifer, with little possibility or evidence of migration to other locations, this was considered 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 2.3) and all of the eight known Australian genera are represented in this study (i.e. 100% of described genera and ~25% of described species). Crustacean taxa can be 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 informative at species level (Lefébure *et al.*, 2006), and *18S* has proven useful for examining higher-level crustacean relationships (Giribet and Ribera, 2000).

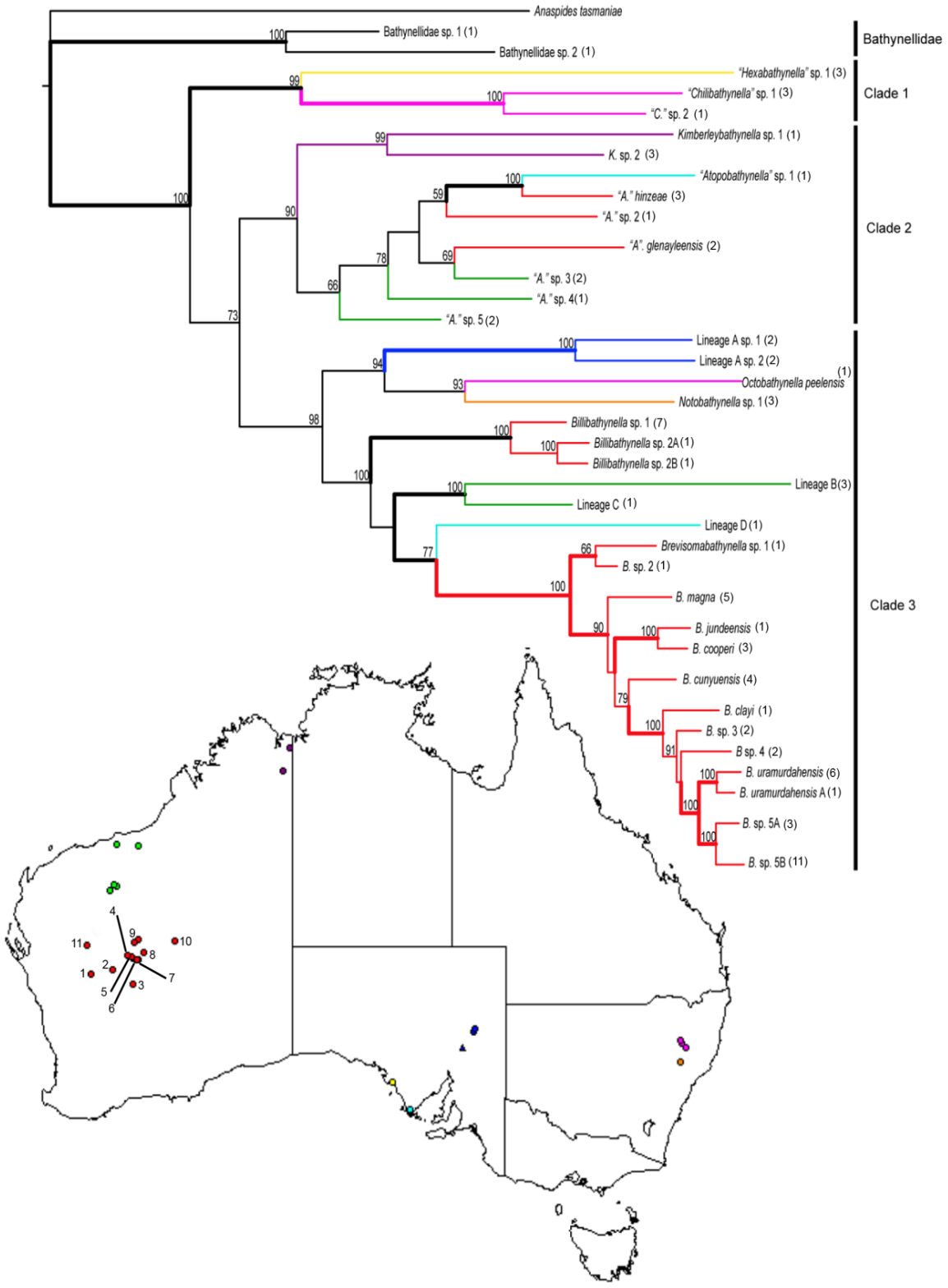

Fig. 2.1. Posterior probability (majority-rule) Bayesian consensus tree using *COI* and *18S* data with model partitioning, implemented in MRBAYES. Numbers on the nodes are Bayesian posterior probabilities and thicker lines represent nodes supported by Maximum parsimony and/ or Maximum likelihood bootstrap 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 2.1 and Fig. 2.2. 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.

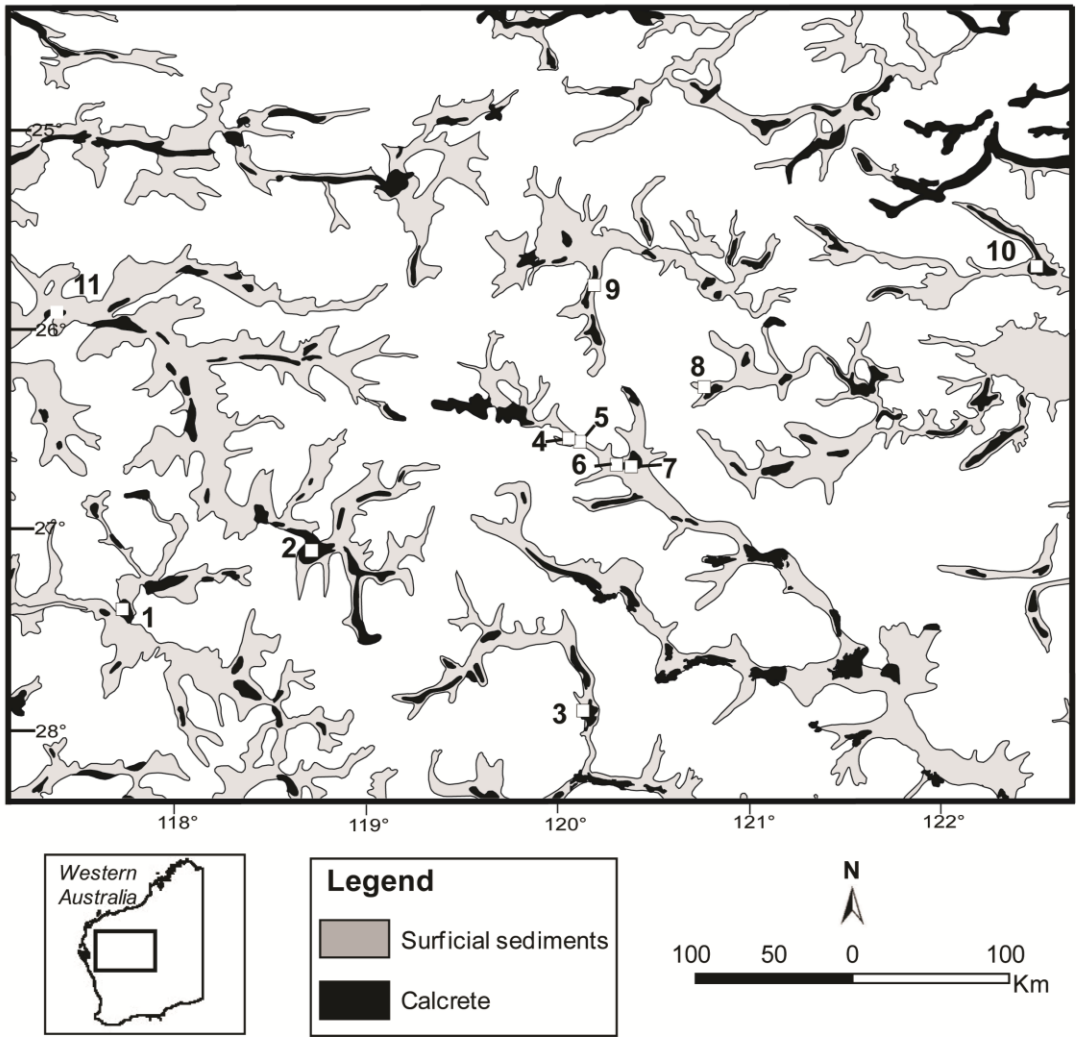

Fig. 2.2. Map of the northern Yilgarn Region of central Western Australia showing the location of calcretes (black) from which parabathynellids were collected. Grey shaded regions represent surficial sediments in the palaeodrainage systems and these are separated by exposures largely of Precambrian geology. 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.

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 Thompson, 1893 (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 considered the sister lineage to the Bathynellacea and the only other extant order within the Syncarida.

Table 2.1. Locations of Bathynellacea samples and GenBank accession numbers.

Species	BES voucher number*	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

Table 2.2. (continued)

Species	BES voucher number*	Collection Site	Calcrete no.	Latitude	Longitude	*GenBank accession numbers* *COI*	Species *18S*
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, Park and Reddy 2006	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

*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 yet been assigned museum collection numbers.

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 2.1). 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 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.), 2-4µl $MgCl_2$, 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^{o}C for 9 min, followed by 35 cycles of 94^{o}C for 30 s, 47^{o}C for 30 s and 72^{o}C for 60 s with a final elongation step at 72^{o}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^{o}C for 9 min for enzyme activation, then 94^{o}C for 2 min, followed by 40 cycles of 94^{o}C for 45 s, 52^{o}C for 45 s and 72^{o}C for 60 s, then a final elongation step at 72^{o}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.

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. Observed effective sample size (ESS) values for all parameters were 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.

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; Schaffer *et al.,* 2010). Both MP and ML character optimisations were applied (using Mesquite version 2.74 Maddison and Maddison, 2003) 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. A BI approach was also used 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 to 7 for antennule segment number and 0 to 19 for antenna segment number). Numerous preliminary analyses were conducted to determine a *ratedev* which would produce an acceptance rate of proposed changes between 20-40%; 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.

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.

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. 2.1), 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 Appendix 2.3 and 2.4. 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. 2.1) 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, at least two of the following criteria (genetic divergence, position in the phylogeny, morphological differences and geographical isolation) were used to identify putative species, and so from this point these

genetically distinct lineages are referred to as 'species' (see Table 2.2). Based solely on divergence thresholds, 23 putative new parabathynellid species would be recognised 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 Lefébure *et al*. threshold of 0.16 subst./site, would result in the recognition of 15 new species. Additionally two genetically distinct (16.1%) bathynellid lineages were observed from the same spring and they could be recognized 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): *"At." hinzeae, "At." glenayleensis,* and five new species, *"At."* 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 peelensis* Camacho and Hancock, 2010, 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 (*Br. clayi, Br. cooperi, Br. cunyuensis, Br. jundeensis, Br. magna, Br. 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.

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. 2.1). There is also a relatively well supported sister relationship between the *"Atopobathynella"* and *Kimberleybathynella* (90% BPP) lineages.

Table 2.2. Putative new species and the criteria used to delineate them.

			COI thresholds					Geographic isolation			Position in phylogeny	Species-level morphology differences
			K2P thresholds			Patristic thresholds						
Taxon (genetically distinct lineage)	Lowest K2P divergence (%)	Lowest patristic divergence	This paper threshold >7.1%	Guzik *et al*. 2011 threshold >11%	Costa *et al.* 2007 threshold >17%	This paper thresh-old >0.075	Lefébure *et al*. 2006 threshold >0.16	Hydrogeol-ogical	Harvey 2002* threshold: <10,000km^2	Eberhard *et al.* 2009$^+$ threshold: 1000km^2		
"Hexabathynella" sp.	27.1	0.491	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. sp. 1	18.4	0.3	✓	✓	✓	✓	✓	✓	✓	✓	✓	#
C. sp. 2	18.4	0.3	✓	✓	✓	✓	✓	✓	✓	✓	x	#
K. sp. 1	24.9	0.46	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
K. sp. 2	26.2	0.414	✓	✓	✓	✓	✓	✓	✓	✓	x	#
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	#
A. sp. 4	20.7	0.179	✓	✓	✓	✓	✓	✓	✓	✓	✓	#
A. sp. 5	17.7	0.174	✓	✓	✓	✓	✓	✓	✓	✓	✓	#
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	✓	✓	✓	✓	✓	✓	✓	✓	✓	#
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	#
Bi. sp. 2B	6.5	0.058	x	x	x	x	x	Same calcrete as sp. 2A	✓	✓	x	#
Lineage B	25.3	0.219	✓	✓	✓	✓	✓	✓	✓	✓	✓	#
Lineage C	21.1	0.237	✓	✓	✓	✓	✓	✓	✓	✓	x	#
Lineage D	22.1	0.18	✓	✓	✓	✓	✓	✓	✓	✓	✓	#
Br. sp. 1	7.1	0.07	✓	x	x	x	x	✓	✓	✓	✓	✓
Br. sp. 2	7.1	0.07	✓	x	x	x	x	✓	✓	✓	x	#
Br. sp. 3	7	0.064	x	x	x	x	x	✓	✓	✓	x	#

Table 2.2. (continued)

			COI thresholds					Geographic isolation			Position in phylogeny	Species-level morphology differences
			K2P thresholds			Patristic thresholds						
Taxon (genetically distinct lineage	Lowest K2P divergence (%)	Lowest patristic divergence	This paper threshold >7.1%	Guzik *et al*. 2011 threshold >11%	Costa *et al.* 2007 threshold >17%	This paper thresh-old >0.075	Lefébure *et al*. 2006 threshold >0.16	Hydrogeol-ogical	Harvey 2002* threshold: <10,000km^2	Eberhard *et al.* 2009$^+$ threshold: 1000km^2		
Br. sp. 4	7.1	0.07	✓	x	x	x	x	✓	✓	✓	x	#
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	#
Brevisomabathynella uramurdahensis A	4.8	0.046	x	x	x	x	x	Different calcrete but potentially connected	✓	✓	x	#

*Distance threshold for short-range endemic taxa suggested by Harvey 2002

$^+$ Distance threshold for short-range endemic subterranean taxa suggested by Eberhard *et al*. 2009

$^\#$ Data deficient

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).

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 (7 species) and all taxa within this clade exhibit morphological characters consistent with the genus (Table 2.3). The average sequence divergence within genera for *18S* ranged between 0.1 (*Billibathynella*, 2 species) and 2.1% (*"Atopobathynella"*, 7 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 2.4) but different body forms were observed between closely related species which were only 6.2% / 0.054 subst./site divergent (*Br. uramurdahensis* and *Br.* sp. 5) (see Fig. 2.3), suggesting that the 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 *"At." hinzeae* and *"At." glenayleensis*. In comparison, the *18S* divergence was much lower *i.e.* ranging from 0.2-2.8% in *Brevisomabathynella* and 0.5-4.4% in *"Atopobathynella."* K2P pairwise divergences between individuals are shown in Appendix 2.2.

Ancestral state analysis

The results of the ancestral state analysis using ML and BI methods are summarized in Fig. 2.4. 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 2.5). 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.

Table 2.3. 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

Table. 2.4 *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

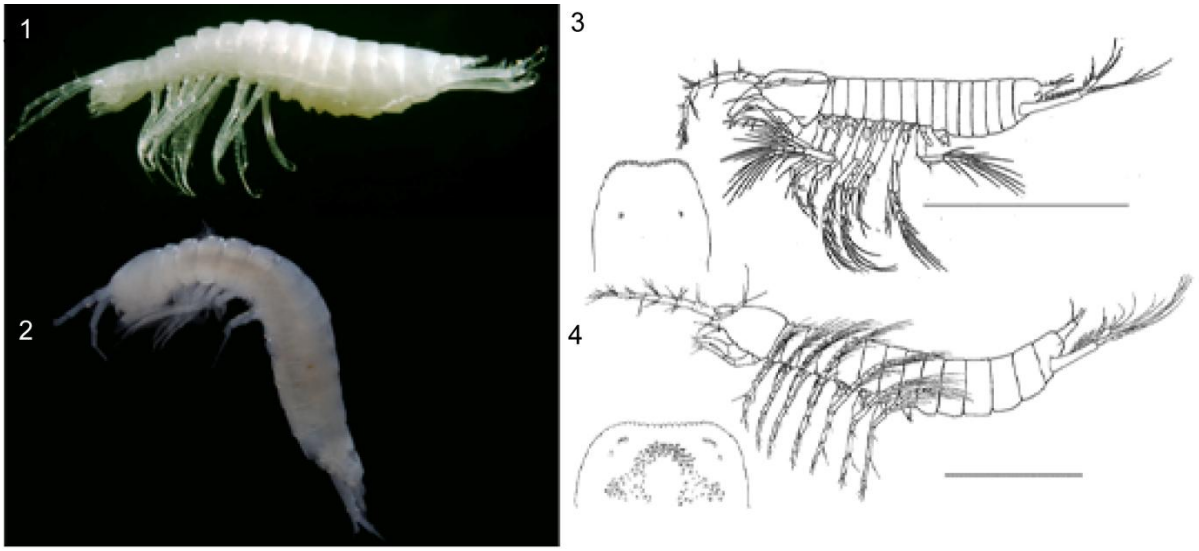

Fig. 2.3. *Brevisomabathynella* species display a variety of morphological forms, (1) *Br. uramurdahensis* and (2) *Br.* sp. 5 are sister species from closely located calcretes and are morphologically distinct yet are only 6.2 % divergent for *CO1;* (3) *Br. cooperi* and (4) *Br. jundeensis* are sympatric sister species with distinctive morphological characters including mouthparts, e.g. the labrum shown to the left of the lateral habitus drawings.

Discussion

This is the first study to examine the diversity and phylogenetic relationships amongst genera and species of parabathynellids on a continent-wide scale. Additionally, the evolution of two morphological characters, which are widely used for reconstructing parabathynellid phylogenetic relationships, are used 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

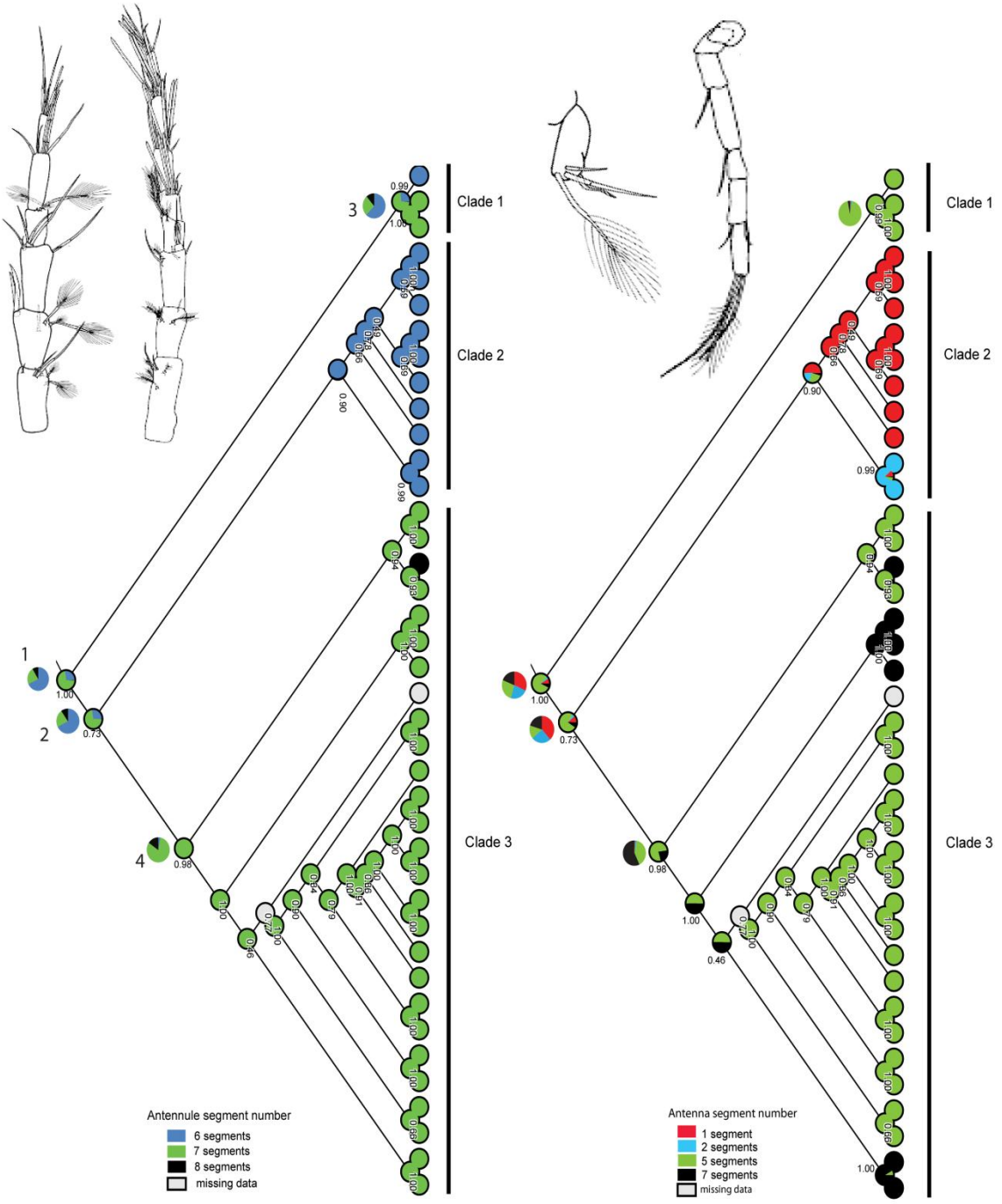

Fig. 2.4. Results of the ancestral state reconstruction analysis for antennule (left) and antenna (right) segment number based on Maximum likelihood and Bayesian approaches. The internal pie charts on the tree represent the relative likelihoods of alternative character states based on Maximum likelihood analysis and the external pie charts are based on Bayesian MCMC methods, using the programs Mesquite v2.7.4 (Maddison and Maddison 2003) 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 2.5. 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^{**6}	-18.7108	7-segments- 6 segments	5.7936^{**6}
	$Hm^{7\text{-segments}}$	-21.6076	8-segments - 6 segments	16.0374^{***6}	-21.6076	8-segments - 6 segments	14.3615^{***6}
	$Hm^{8\text{-segments}}$	-25.8916	8-segments - 7-segments	8.5679^{**7}	-25.8916	8-segments - 7-segments	8.5679^{**7}
Node 2	$Hm^{6\text{-segments}}$	-18.7052	7-segments- 6 segments	5.3206^{**6}	-17.4379	7-segments- 6 segments	5.7703^{**6}
	$Hm^{7\text{-segments}}$	-21.3655	8-segments - 6 segments	10.9182^{***6}	-20.3231	8-segments - 6 segments	8.3352^{**6}
	$Hm^{8\text{-segments}}$	-24.1643	8-segments - 7-segments	5.5976^{**7}	-21.6055	8-segments - 7-segments	2.5649^{*7}
Node 3	$Hm^{6\text{-segments}}$	-18.1262	7-segments- 6 segments	0.6686	-18.1262	7-segments- 6 segments	3.8257^{*6}
	$Hm^{7\text{-segments}}$	-18.4605	8-segments - 6 segments	6.7883^{**6}	-20.0391	8-segments - 6 segments	9.1798^{**6}
	$Hm^{8\text{-segments}}$	-21.5204	8-segments - 7-segments	6.1196^{**7}	-22.7161	8-segments - 7-segments	5.3541^{**7}
Node 4	$Hm^{6\text{-segments}}$	-22.2591	6 segments – 7-segments	7.4042^{**7}	-22.2991	6 segments – 7-segments	9.4871^{**7}
	$Hm^{7\text{-segments}}$	-18.5570	6 segments – 8-segments	2.3553^{*8}	-17.5555	6 segments – 8-segments	1.2438
	$Hm^{8\text{-segments}}$	-21.0814	8-segments – 7-segments	5.0488^{**7}	-21.6772	8-segments – 7-segments	8.2433^{**7}
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^{**1}
	$Hm^{5\text{-segments}}$	-23.1916	7-segments – 1-segment	5.7402^{**1}	-27.9340	7-segments – 1-segment	5.7402^{**1}
	$Hm^{7\text{-segments}}$	-26.0290	2-segments – 5-segments	0.6313	-26.0290	5-segments – 2-segments	8.8536^{**2}
			7-segments - 2-segments	5.0435^{**1}		7-segments – 2-segments	5.0435^{**2}
			7-segments – 5-segments	5.6748^{**2}		5-segments – 7-segments	3.8101^{*7}

Table 2.5. (continued)

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 2	$Hm^{1\text{-segment}}$	-23.3800	2-segments – 1-segment	18.4275*** [1]	-23.9094	2-segments – 1-segment	1.4210
	$Hm^{2\text{-segments}}$	-32.5938	5-segments – 1-segment	2.1512* [1]	-24.6199	5-segments – 1-segment	7.9072** [1]
	$Hm^{5\text{-segments}}$	-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* [5]	-23.9500	5-segments – 2-segments	6.4861** [2]
			2-segments – 7-segments	18.4255*** [7]		2-segments – 7-segments	1.3398
			5-segments – 7-segments	2.1492* [7]		5-segments – 7-segments	7.8260** [7]
Node 3	$Hm^{1\text{-segment}}$	-27.7246	1-segment – 2-segments	3.7372* [2]	-27.7246	2-segments – 1-segment	2.9840* [1]
	$Hm^{2\text{-segments}}$	-25.8560	1-segment – 5-segments	3.7372* [5]	-29.2166	1-segment – 5-segments	10.7551*** [5]
	$Hm^{5\text{-segments}}$	-22.3470	7-segments – 1-segment	5.1166** [1]	-22.3470	1-segment – 7-segments	4.5387* [7]
	$Hm^{7\text{-segments}}$	-30.2829	2-segments – 5-segments	7.0179** [5]	-25.4552	2-segments – 5-segments	13.7391*** [5]
			7-segments – 2-segments	8.8538** [2]		2-segments – 7-segments	7.5228** [7]
			7-segments – 5-segments	15.8717*** [5]		7-segments – 5-segments	6.2164** [5]
Node 4	$Hm^{1\text{-segment}}$	-29.0656	1-segment – 2-segments	3.1253* [2]	-26.9528	1-segment – 2-segments	1.3898
	$Hm^{2\text{-segments}}$	-27.5029	1-segment – 5-segments	10.1055*** [5]	-26.2579	1-segment – 5-segments	9.3269** [2]
	$Hm^{5\text{-segments}}$	-24.0128	1-segment – 7-segments	9.0138** [7]	-22.2894	1-segment – 7-segments	0.4166
	$Hm^{7\text{-segments}}$	-24.5586	2-segments – 5-segments	6.9802** [5]	-26.7445	2-segments – 5-segments	7.9371** [5]
			2-segments-7-segments	5.8885** [7]		7-segments-2-segments	0.9732
			7-segments-5-segments	1.0917		7-segments-5-segments	8.9103** [5]

*= BF > 2 is positive evidence for either 6-, 7- or 8-segments as the ancestral condition using the criteria of (Pagel *et al*. 2004).

**BF > 5 is strong evidence for either 6-, 7- or 8-segments as the ancestral condition using the criteria of (Pagel *et al*. 2004).

***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).

body plan (Schminke, 1974; Schminke, 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).

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 the 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 generic status (Cho *et al.*, 2005). This 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 it was not possible 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 pg:33). However, the 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 2.3); 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 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. There was insufficient material of Lineages B, C and D to conduct thorough morphological examinations, and therefore it is not possible to determine what taxonomic rank they might warrant. However, it is noteworthy that Lineages B-D do not group within any of the recognized 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, this 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 the phylogenetic 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. The analysis presented here 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.

Parabathynellid species diversity

Here 23 new putative species (or 12 or 16 based on the more conservative higher *COI* thresholds) are reported, 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, it was possible to examine the relationships among nine known species from three genera. Interestingly, the two described *"Atopobathynella"* species included here, *"At." hinzeae* and *"At." 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. This study also included the following six known species of *Brevisomabathynella* (Cho and Humphreys, 2010), *Br. magna*, *Br. jundeensis*, *Br. cooperi*, *Br. clayi*, *Br. cunyuensis* and *Br. 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) hypothesized that the diversity of forms may be due to niche partitioning, with the co-occurrence of sister species, *Br. jundeensis* and *Br. 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 within a calcrete body through niche partitioning. Here, five new species of *Brevisomabathynella* have been identified 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 comparatively 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, the 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" ("At."* 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, two new species of *Billibathynella* and five putative new species (Lineages A – D) which do not group within any of the recognized Australian genera have been identified.

High parabathynellid species diversity in Australia is not surprising given some arid areas have recently been recognized 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; Leys *et al.*, 2003; Cooper *et al.*, 2007; Cooper *et al.*, 2008; 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 hypothesized 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 it is likely 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.

Morphological convergence obscures true phyletic relationships

Although the 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. The Bayesian approach is 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, the 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.

This study 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 world-wide (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, 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, 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.

In summary, 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. The analyses presented here further supported the monophyly of known genera defined by traditional morphological methods, suggesting that the commonly used generic characters are robust for recognizing 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 2.1. A list of parabathynellid genera showing number of described species and their distribution

Genus	# Described species	Type species locality	Broader distribution
Acantobathynella Coineau, 1967	1	Ivory Coast only	-
Afrobathynella, Schminke 1976	1	South Africa	-
Batubathynella Schminke, 1973	1	Malaysia	-
Brasilibathynella Jakobi, 1958	1	Brazil	-
Californibathynella Camacho and Serban, 1998	1	USA	-
Ctenophallonella Coineau and Serban, 1978	1	South Africa	-
Guadalopebathynella Camacho and Serban, 1998	1	Spain	-
Haplophallonella Serban and Coineau, 1975	1	Ivory Coast	-
Heterodontobathynella Schminke, 1973	1	Uganda	-
Issykkulibathynella Serban, 1994	1	Russia	-
Lamtobathynella Serban and Coineau 1982	1	Ivory Coast	-
Montanabathynella Camacho, Stanford and Newell 2009	1	USA	-
Nilobathynella Dumont, 1984	1	Sudan	-
Nunubathynella Schminke, 1976	1	South Africa	-
Octobathynella Camacho and Hancock 2010	1	Australia	-
Odontobathynella Delamare and Serban, 1979	1	Brazil	-
Paraeobathynella Camacho 2005	1	Vietnam	-
Psalidobathynella Schminke, 1979	1	Venezuela	-
Sabahbathynella Schminke, 1988	1	Malaysia	-
Sinobathynella Camacho, Trontelj and Zagmajster, 2006	1	China	-
Sketinella Camacho 2005	1	Vietnam	-
Califobathynella Cho, 1997	2	USA	-
Hexaiberobathynella Camacho et Serban, 1998	2	Spain	-
Noodtibathynella Schminke, 1973	2	Brazil	South America
Parabathynella Chappuis, 1926	2	Serbia	Europe
Racovitzaibathynella Serban and Coineau, 1994	2	South Africa	-
Texanobathynella Delamare, Coineau et Serban, 1975	2	USA	-
Thermobathynella Capart, 1951	2	Zaire	South America

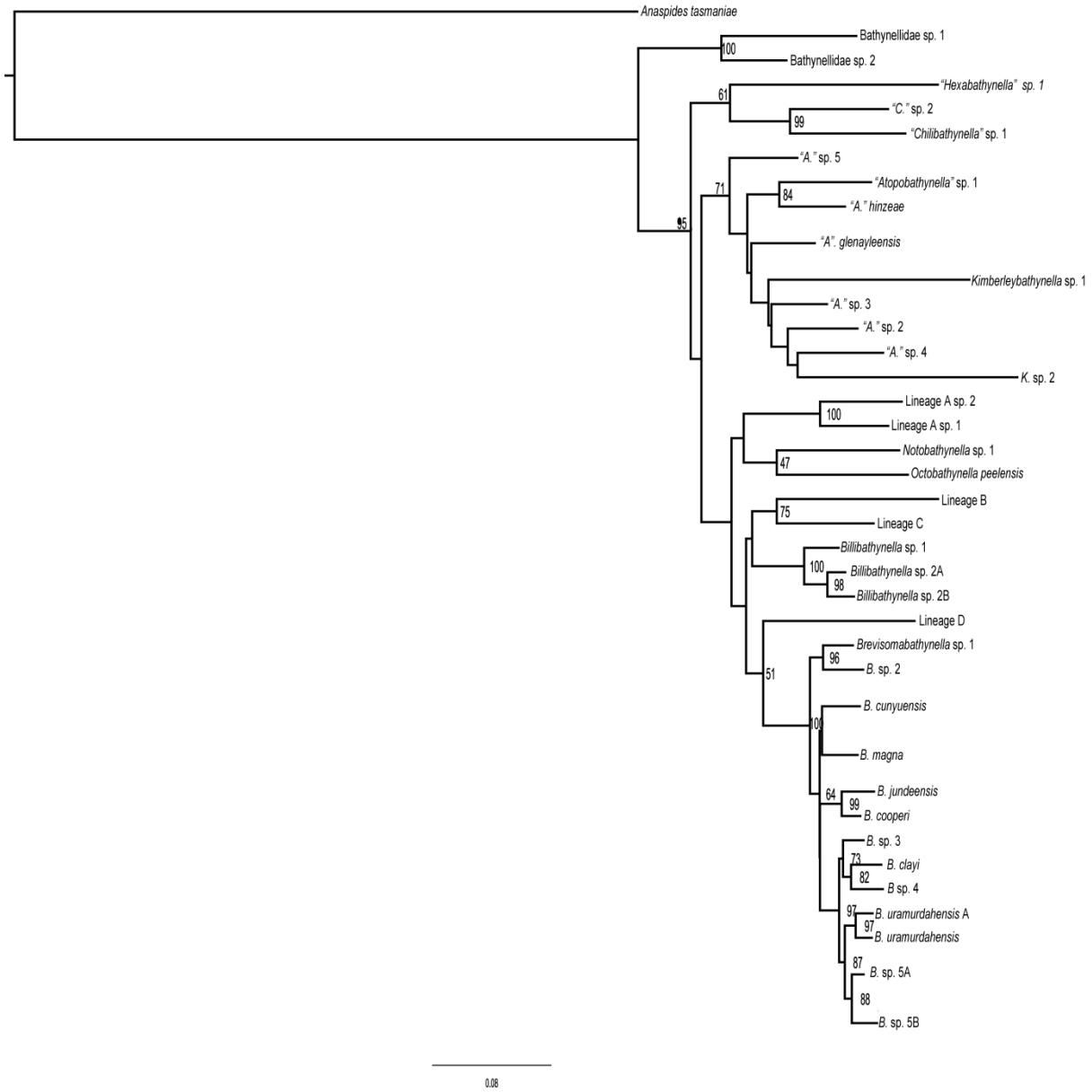
Appendix 2.1. (continued)

Genus	# Described species	Type species locality	Broader distribution
Chilibathynella Noodt, 1963	3	Chile	South America, Australia, India
Leptobathynella Noodt, 1963	3	Paraguay	South America
Nipponbathynella Schminke, 1973	3	Japan	-
Pacficabathynella Schminke and Noodt, 1988	3	USA	-
Paraiberobathynella Camacho and Serban, 1998	3	France	Europe, Africa
Billibathynella Cho, 2005	4	Australia	-
Eobathynella Birstein and Ljovuschkin, 1964	5	Russia	Eurasia, Japan
Parvulobathynella Schminke, 1973	5	Paraguay	South America, South Africa
Kimberleybathynella Cho, Park and Humphreys, 2005	6	Australia	-
Cteniobathynella Schminke, 1973	7	Zaire	Africa, South America
Allobathynella Morimoto and Miura, 1957	9	Japan	Japan, South Korea
Notobathynella Schminke, 1973	9	Australia	Madagascar, India, Australia, New Zealand
Habrobathynella Schminke, 1973	10	Madagascar	Madagascar, India
Atopobathynella Schminke, 1973	11	Chile	South America, Australia, India,
Brevisomabathynella Cho, Park and Reddy, 2006	12	Australia	-
Hexabathynella Schminke, 1972.	22	Madagascar	Madagascar, New Zealand, Australia, Africa, Europe, North America, South America
Iberobathynella Schminke, 1973	22	Spain	Europe

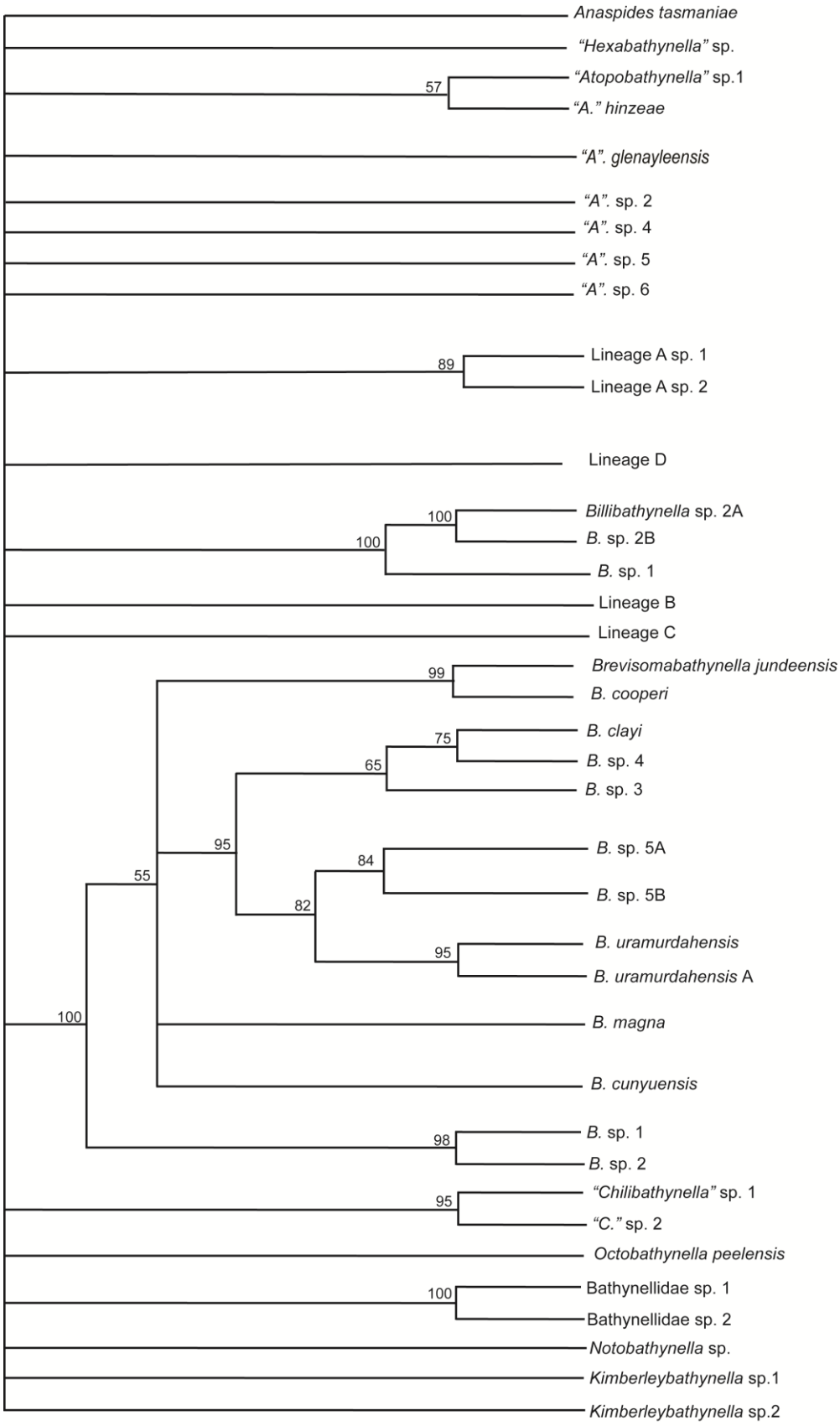
Appendix 2.2. *COI* pairwise (K2P) genetic divergences between parabathynellid individuals sequenced for this study.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1. *"Hexabathynella"* sp. 1																																		
2. *"Chilibathynella"* sp. 1	29																																	
3. *"Chilibathynella"* sp. 1	31	18																																
4. *Kimberleybathynella* sp. 1	31	36	36																															
5. *Kimberleybathynella* sp. 2	35	36	36	33																														
6. *"Atopobathynella" hinzeae*	29	31	25	29	26																													
7. *Atopobathynella glenayleensis*	31	31	29	28	30	22																												
8. *"Atopobathynella"* sp. 1	27	31	26	31	32	20	24																											
9. *"Atopobathynella"* sp. 2	32	28	25	25	26	18	20	24																										
10. *"Atopobathynella"* sp. 3	31	28	26	25	28	19	17	22	16																									
11. *"Atopobathynella"* sp. 4	32	33	31	27	27	22	25	24	21	21																								
12. *"Atopobathynella"* sp. 5	29	31	29	28	26	21	21	24	18	18	21																							
13. Lineage A sp. 1	29	33	29	33	29	26	31	25	23	27	29	25																						
14. Lineage A sp. 2	31	32	27	36	30	25	29	27	21	22	28	25	14																					
15. *Octobathynella peelensis*	35	31	34	33	36	27	31	32	29	27	30	30	33	28																				
16. *Notobathynella* sp. 1	30	32	29	30	28	21	28	25	26	27	24	23	26	29	24																			
17. *Billibathynella* sp. 1	28	26	25	30	28	22	26	26	25	24	26	19	21	23	27	23																		
18. *Billibathynella* sp. 2A	31	27	27	30	30	25	26	26	23	23	25	19	25	25	26	23	11																	
19. *Billibathynella* sp. 2B	31	27	28	31	30	25	27	27	24	24	26	21	23	25	25	25	9	6																
20. Lineage B	30	33	31	35	35	32	35	38	30	28	31	29	32	28	26	34	26	29	27															
21. Lineage C	31	32	29	31	34	26	31	28	25	28	30	25	27	26	29	25	22	24	22	30														
22. Lineage D	28	31	33	32	34	28	27	27	29	30	29	26	26	27	28	27	26	25	26	31	28													
23. *Brevisomabathynella clayi*	32	26	23	30	31	25	28	28	26	22	29	22	26	26	28	24	21	20	19	27	22	24												
24. *Brevisomabathynella cooperi*	29	29	24	31	32	25	27	28	25	25	31	22	26	24	26	24	18	20	18	27	23	26	11											
25. *Brevisomabathynella cunyuensis*	31	28	24	32	30	23	28	28	28	25	31	23	28	28	29	22	22	22	21	29	22	27	10	10										
26. *Brevisomabathynella jundeensis*	34	30	25	32	33	25	27	29	25	24	33	23	29	27	28	25	22	21	19	27	23	27	12	7	12									
27. *Brevisomabathynella magna*	31	28	21	29	29	23	26	26	26	21	27	21	25	25	26	23	18	19	18	26	23	29	9	9	8	9								
28. *Brevisomabathynella uramurdahensis*	32	26	21	30	33	24	26	27	24	24	29	21	26	26	23	22	21	19	18	28	21	24	9	12	11	12	9							
29. *B. uramurdahensis* A	31	27	23	31	34	25	27	30	27	26	31	22	26	27	25	22	22	20	20	29	24	27	9	13	11	14	10	5						
30. *Brevisomabathynella* sp. 1	30	27	23	31	32	25	27	26	26	23	30	20	24	22	25	23	18	18	18	26	22	24	11	9	10	10	8	10	11					
31. *Brevisomabathynella* sp. 2	33	31	25	32	31	29	29	30	26	26	32	23	25	25	28	24	20	21	20	25	21	27	14	11	11	12	11	14	15	7				
32. *Brevisomabathynella* sp. 3	31	27	23	30	33	24	26	27	25	25	29	22	26	26	27	23	21	19	18	28	21	22	8	10	9	12	10	7	8	9	13			
33. *Brevisomabathynella* sp. 4	29	26	26	29	34	26	27	28	26	23	30	23	27	27	27	24	22	21	20	27	23	24	7	12	11	14	11	9	9	11	16	8		
34. *Brevisomabathynella* sp. 5A	31	28	24	29	31	25	25	27	25	24	30	20	27	27	26	23	20	20	19	26	22	23	9	11	11	13	9	6	8	9	13	8	8	
35. *Brevisomabathynella* sp. 5 B	33	27	23	31	32	27	28	30	26	26	31	21	25	26	27	24	20	20	19	28	22	26	9	11	11	13	10	6	7	9	13	8	9	6

Appendix 2.3. Maximum likelihood consensus tree based on *COI* and *18S* data, implemented in RAxML.



Appendix 2.4. Maximum parsimony tree based on *COI* and *18S* data, implemented in Paup.



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CHAPTER III

MOLECULAR PHYLOGENETIC, MORPHOLOGICAL AND BIOGEOGRAPHIC EVIDENCE FOR A NEW GENUS OF PARABATHYNELLID CRUSTACEANS (SYNCARIDA: BATHYNELLACEA) FROM GROUNDWATER IN AN ANCIENT SOUTH AUSTRALIAN LANDSCAPE

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Statement of Authorship

This chapter is a submitted research article.

Kym Abrams (candidate)

Corresponding author: Prepared DNA extracts for PCR amplification, carried out DNA sequencing, analysed sequence data, produced taxonomic drawings and descriptions, wrote manuscript and produced all figures.

Signed Date 15/2/2012

Rachael A. King

Provided taxonomic training to the first author and advice for the taxonomic component, supervised direction of the study and critically reviewed manuscript. I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 15/2/12

Michelle T Guzik

Provided some DNA extracts and sequences for study, provided assistance with obtaining project funding, provided advice for molecular analyses and critically reviewed manuscript. I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 16/2/2012

Andrew D. Austin

Provided some project funding and assistance with obtaining additional funding, supervised the direction of the study and critically reviewed manuscript. I give consent for Kym Abrams (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 15/2/12

CHAPTER III

Preamble

This chapter is based on the phylogeny presented in Chapter II, but has a focus on the new genus described herein (Lineage A in Chapter II) and its phylogenetic relationships with other Australian taxa. Furthermore, two additional species of the new genus are included and the molecular phylogeny is used to demonstrate the monophyly of the genus. In addition, morphological data, species descriptions and a checklist and key to Australian genera are included in this chapter, setting it apart from Chapter II. The contents of this chapter have recently been submitted to *Invertebrate Systematics* for publication.

Abstract

The putatively ancient subterranean crustacean family Parabathynellidae has been poorly studied, in part because of the problem of obtaining material from difficult to access subterranean habitats in which they live. Further, the systematics of the group has been complicated by their generally simplified morphology and isolated descriptions of new taxa in the absence of any phylogenetic framework. Using material from comprehensive field surveys and mitochondrial Cytochrome c oxidase subunit 1 (*COI*) and nuclear *18S* sequence data, plus morphology, a new genus is recognized, *Arkaroolabathynella* Abrams & King gen. nov., from underground waters in the Flinders Ranges, South Australia. *Arkaroolabathynella* contains four genetically and morphologically distinct species, described as *A. bispinosa* Abrams & King sp. nov., *A. remkoi* Abrams & King sp. nov., *A. robusta* Abrams & King sp. nov. and *A. spriggi* Abrams & King sp. nov. Phylogenetic analysis also revealed a previously unknown diversity of parabathynellids from South Australia, and a complex set of relationships with the New South Wales and Western Australian fauna. Additionally, this study showed that deep molecular divergences in parabathynellids are not always reflected in morphological divergence. A key to Australian parabathynellid genera and a checklist to species are also provided.

Introduction

The Parabathynellidae Noodt, 1965, are a worldwide family of tiny, enigmatic syncarid crustaceans which are confined to subterranean aquatic environments. Considered rare and often difficult to collect, very little is known about their biology, ecology or evolutionary relationships. The family comprises 47 described genera and 172 species (Camacho and Valdecasas, 2008) of which 10 genera are described from two or more continents and are considered to have a cosmopolitan distribution. In contrast, nearly half of all parabathynellid genera are monotypic (Camacho, 2006; see Appendix 1, Ch. II). The high incidence of

monotypic genera is indicative of the difficulties associated with parabathynellid systematics. Some of these difficulties include few experts working on the group, lack of sufficient specimens for descriptions, inadequate early descriptions (Camacho, 2005), in addition to the extreme morphological simplification and convergence, which has likely obscured their true phyletic ancestry and diversity.

The Australian parabathynellids have been, until recently, poorly studied. Schminke (1972, 1973) described the first parabathynellids from Australia, erecting the genera *Hexabathynella* Schminke 1972 (New South Wales), *Atopobathynella* Schminke 1973 (Victoria and Tasmania) and *Notobathynella* Schminke1973 (New South Wales and Tasmania). Schminke (1973) also described the first Australian representative of *Chilibathynella* Noodt, 1963, *Chilibathynella australiensis* Schminke (Victoria), a genus which is also known from Chile and India. Some 33 years later, exploration of the diversity of Western Australian subterranean aquatic fauna (stygofauna), fueled in part by environmental surveys associated with extensive natural resource mining activities in the region, led to the discovery of three new genera, *Brevisomabathynella* Cho *et al.*, 2006b (currently 12 described species), *Billibathynella* Cho, 2005 (four described species) and *Kimberleybathynella* Cho *et al.*, 2005 (six described species). Since then, six new species of *Atopobathynella* have been described from Western Australia and the Northern Territory (Cho *et al.*, 2006a). Most recently, Camacho and Hancock (2010, 2011, 2012) described two new genera, *Octobathynella* and *Onychobathynella*, from New South Wales and two new species of *Chilibathynella* and *Notobathynella*, the latter being the first parabathynellids recorded from Queensland. There are currently nine genera and 43 described species from Australia (see Checklist, Appendix 1, this Chapter).

South Australia is the only major region of the continent from which bathynellaceans have not been recorded, although an unidentified syncarid has been collected from the Flinders Ranges, over a decade ago (Cooling and Boulton, 1993). Given this and the high diversity of bathynellaceans found in a similarly ancient landscape such as the Yilgarn Region of Western Australia (Guzik *et al.*, 2008; Cho and Humphreys, 2010), it was predicted that the Flinders Ranges could harbor a highly diverse stygofauna (Guzik *et al.*, 2011). These ranges and hills are the remnants of an ancient chain of mountains located in the geographical centre of South Australia (Brandle, 2001). This area has a long and complex geological history, with some of its bedrock dating to pre-Cambrian times (Corbett, 1969). The region is unique because it spans two of Australia's major climatic regions, namely the temperate Bassian and arid Eyrean regions (Brandle, 2001). A combination of complex climate, geomorphology and geology has produced a relatively small region with remarkably high habitat diversity which, in turn, is reflected in the high diversity of plant and animal communities present (Brandle

2001). For example, the Flinders Ranges National Park, Management Plan has identified at least 12 “land systems” ranging from various types of woodlands to shrublands to grasslands, and these habitats shelter 86 reptile species, 10 amphibian species, 283 bird species and 34 mammal species (much of the historic mammal fauna is recently extinct due to the introduction of feral grazers and predators) (Smith, 1996).

The Flinders Ranges region is world-renowned for its Ediacaran fossil invertebrate fauna (Sprigg, 1947; Canfield *et al.*, 2007; Xiao and Laflamme, 2009), yet it is generally poorly studied in terms of its extant invertebrate biodiversity. Boulton and Williams (1996) identified a rich aquatic invertebrate fauna in the Ranges, however most of the species remain undescribed and little research has been published since then. Even less is known about the stygofauna of the region, although recent surveys indicate that it is remarkably diverse (Guzik *et al.*, 2011; R. Leys, pers. comm.). To date only one subterranean beetle (Leys *et al.*, 2010) and one crustacean species (*Brachina invasa* Barnard and Williams, 1995) have been described from this region, highlighting the general paucity of information available. The troglofauna (terrestrial subterranean invertebrates) is slightly better known due to investigation of pseudoscorpions (Moulds *et al.*, 2007) and guano- associated arthropod communities (Moulds, 2005).

This study represents a significant step towards documenting the subterranean diversity of this region and also expands knowledge of the distribution and diversity of parabathynellids within Australia. Here, material from comprehensive field surveys and sequence data from the mitochondrial Cytochrome c oxidase subunit 1 (*COI*) and nuclear *18S* gene regions is used to determine whether four taxa from the Flinders Ranges are genetically distinct and constitute a monophyletic group. Subsequently, the phylogenetic relationships of the Flinders Ranges taxa with other genera found in Australia are examined and the group is described as a new genus with four new species, based on molecular results and comparison of their morphology. Additionally, molecular data is presented for the first parabathynellids collected from the Eyre Peninsula of South Australia. A key to genera in Australia to facilitate their identification, and a checklist of species found on the continent is also provided.

Methods

Specimen sampling and taxon selection

The material used in this study was obtained using a combination of netting and pumping methods (following the same regimes as Cooper *et al.*, 2007 and Hancock and Boulton, 2008). The new species described here, were collected from the Flinders Ranges, South Australia (SA) (Fig. 3.1). Other material included in a previous broader molecular phylogeny (Fig. 3.1) (see Ch. II) was collected from additional sites in South Australia, the Pilbara, Kimberley and Yilgarn regions of Western Australia (WA), and the Hunter and Peel Rivers of New South Wales (NSW) (see Table 3.1, Ch. II). Where possible, multiple individuals per location were sequenced, to control for the possibility of sequencing errors and contamination. After ensuring that the sequence data were robust, identical sequences were excluded from the analyses. The type material of the species described below is deposited in the collection of the South Australian Museum (SAM, SAM C6940 – C6943, C7018-7023).

Thirty-eight bathynellacean individuals (36 Parabathynellidae and 2 Bathynellidae) were included in the molecular phylogeny. The majority of taxa were sequenced for the previous study (Ch. II), but two additional taxa are included here of the putative new genus, from the Flinders Ranges. Individuals included in the analyses represent eight of the nine known genera in Australia, as well as all four species of the genus described here. *Anaspides tasmaniae* Thompson, 1893 was used as the outgroup, due to its sister relationship with the Bathynellacea.

Sequencing protocols

The molecular and phylogenetic protocols used in this study are the same as those described in detail in Ch. II. In brief, genomic DNA was extracted from specimens stored in 100% ethanol, using the Gentra Systems PUREGENE DNA Purification Kit and voucher material was lodged at the Western Australian Museum (WAM) or South Australian Museum (SAM). The *COI* sequences were translated into amino acid sequences to determine if any gaps or stop codons were present.

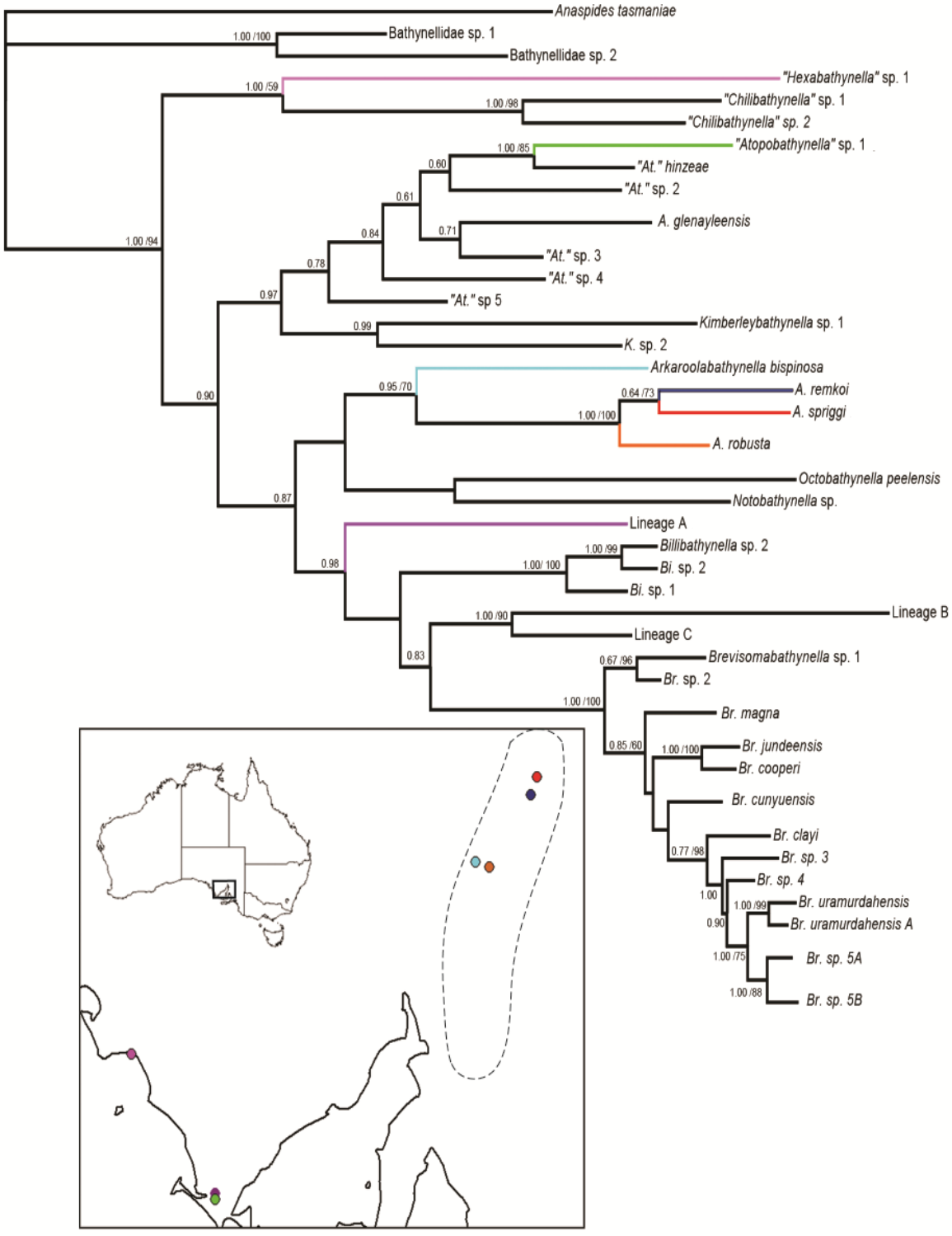

Fig. 3.1. Posterior probability (majority-rule) Bayesian consensus tree using *COI* and *18S* data with model partitioning, implemented in MRBAYES. Numbers on the nodes are Bayesian posterior probabilities followed by Maximum likelihood bootstrap values. The map of Australia shows the collection sites of the South Australian species, which are also colour-coded on the phylogeny as shown on the map. Area enclosed by dashed lines represents the Flinders Ranges.

PCR amplifications for *COI* and *18S* were carried out using the primers and thermal cycling conditions described in Ch. II. 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.

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 and maximum likelihood (ML) approaches, using separate and combined datasets, implemented in the programs MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and RaxML v. 7.2.3 (Stamatakis *et al.*, 2008) 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 Bayesian 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. Effective sample size (ESS) values for all parameters were 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 Bayesian consensus tree was constructed from the remaining 85,000 trees.

Maximum parsimony 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. Maximum likelihood 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 MrBayes consensus tree using PAUP*, with the optimality criterion set to maximum likelihood.

Criteria for assessing new species and genera

The criteria used to identify both new and previously described species and genera are outlined in detail in Ch. II. In brief, a combination of the following criteria were employed: 1) morphological differences based on characters identified in previous comprehensive descriptions of parabathynellid taxa (e.g. Cho *et al.*, 2005, 2006b); 2) levels of sequence divergence; 3) a sister lineage relationship to two or more defined species, and 4) geographical location (following the approach of Guzik *et al.* 2011). Many specimens could be assigned to known genera based on combinations of key diagnostic characters. Genera that have type species that are not from Australia but contain putative Australian species (*Atopobathynella*, *Chilibathynella* and *Hexabathynella*) are denoted by inverted commas to express uncertainty as to their congeneric status.

Explanation of setal formulae

Setal formulae are included in the systematic descriptions as the methodology of parabathynellid taxonomists who believed it to be an important set of characters to highlight in shorthand was followed. The setal formulae for the antenna, maxilla and thoracopods follow Schminke (1973), i.e. for the antenna, each number, or set of numbers, separated by "/" represents the number of setae (inner then outer) on each segment, with plumose setae shown in parentheses. For example, for the antenna of *A. spriggi* sp. nov. (0/0+0/1+0/1+1/4(1)), means the first and second segments do not have setae, the third segment has one inner seta, the fourth segment has one inner and one outer seta and the fifth segment has four setae, one of which is plumose. For the maxilla, each number separated by "-" represent the number of setae on each segment. For the thoracopodal exopod, each number or set of numbers, separated by "/" represents the number of segments of each exopod i.e. for the thoracopodal endopods (3+1/3+1/3+1/3(1)), means the first three segments have three inner and one outer setae and the fourth segment has three setae, two of which are robust in addition to one smaller seta, designated by parentheses.

Results

Molecular phylogeny

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 the *Anaspides tasmaniae* reference sequence so a secondary structure model was not required to aid the alignment. The *COI* sequences comprised 56% variable sites and 48% parsimony informative sites. In comparison, the more conserved *18S* data comprised 23% variable sites and 13% parsimony informative sites.

Not surprisingly, the relationships amongst genera are similar to those presented in Ch. II so they are discussed here only briefly, but with a focus on the phylogenetic placement of a putative new genus, and the evidence for its monophyly. With the additional two taxa, the only difference in topology between the Bayesian phylogeny in Ch. II and the one presented here is in the placement of Lineage A (Lineage D in Ch. II), which was sister to *Brevisomabathynella* in the previous phylogeny, but here is placed as the sister lineage to *Billibathynella* + Lineage B + Lineage C + *Brevisomabathynella*.

Maximum likelihood phylogenetic analysis (not shown) and Bayesian analysis provided largely congruent tree topologies consisting of three major clades within Parabathynellidae (differences discussed below), containing multiple monophyletic clades, many of which could be assigned to known genera. In the Bayesian and ML analyses, the first clade consists of a species of *"Hexabathynella"* from the Eyre Peninsula (J-L Cho pers. comm.), SA and two species of *"Chilibathynella"* (100% BPP, 95% MP bootstrap value) from New South Wales. In the Bayesian analysis, the second clade (97% BPP) contains seven species of "*Atopobathynella"* (78% BPP) from WA and SA and two species of *Kimberleybathynella* (99% BPP) from WA; while in the ML analysis, the second clade contains *Notobathynella* and is only weakly supported (26% ML bootstrap value) as sister to a paraphyletic clade of *Kimberleybathynella* and "*Atopobathynella"* species. In the Bayesian analysis, the third clade (87% BPP) comprises *Octobathynella* and *Notobathynella* from NSW, *Billibathynella* and *Brevisomabathynella* from WA and three distinct lineages, Lineages A – C from SA and WA each consisting of one species, which could not be readily assigned to existing genera. Additionally, this clade contains the four species of the new genus from the Flinders Ranges. In the ML analysis the third clade (46% ML bootstrap value) contains all of the aforementioned genera except *Notobathynella*.

In both analyses the four species of the new genus form a well-supported monophyletic clade (95% BPP, 70% ML bootstrap value). However, their sister group relationships differ slightly between analyses. In the Bayesian analysis (Fig. 3.1) it is weakly supported (51% BPP) as sister to *Octobathynella* + *Notobathynella*, whereas in the ML analysis the new genus is weakly supported (49% bootstrap value) as sister to *Octobathynella* only. The four species comprising the putative new genus were genetically distinct (based on Kimura-2-parameter distances), with the lowest *COI* divergences being ~13% between *A. spriggi* and *A. robusta*, and the highest being ~29%, between *A. spriggi* and *A. bispinosa.* The more conserved *18S* genetic divergences were much lower, ranging from 0.3 to 3.1%.

Morphological analysis

Examination of the Flinders Ranges specimens revealed a number of fixed morphological difference that supported the molecular data and the recognition of a new genus. The major defining characters of this genus include a seven-segmented antennule, five-segmented antenna, multi-segmented exopods of thoracopods I-VII, large, rectangular male thoracopod VIII without basipodal setae, uropod with numerous spines on the sympod, elongated furcal rami with two large terminal spines and numerous small spines on the inner margin and uneven sympodal spine row. All of the characters listed above, except the last one, are shared with *Brevisomabathynella* which, based on the phylogeny, is not closely related to the new genus (see Remarks below), thus highlighting the extremely morphologically convergent nature of this group. The four species described here can be distinguished morphologically, mainly based on differences in the number of setae and spines on particular appendages (see Table 3.1 and Remarks under each species).

Table 3.1. Morphological differences among species of *Arkaroolabathynella* gen. nov. Abbreviations: s= simple seta, pl = plumose seta, Th I = thoracopod I, A= absent, P= present.

Species	*A. spriggi*	*A. remkoi*	*A. robusta*	*A. bispinosa*
Body length (mm)	1.9 – 2.1	2.2 - 3.3	1.03	1.15 – 1.17
Antennule setation				
Segment 1	1 s, 4 pl	2 s, 2 pl	1 s, 1 pl	1 s 3 pl
Segment 2	2 s, 4 pl	3 s, 3 pl	3 s	3 s, 3 pl
Segment 3	3 s, apophysis with 3 s	3 s, apophysis with 3 s	apophysis with 3 s	3 s, apophysis with 3 s
Segment 4	1 s, apophysis with 2 pl	1 s, apophysis with 2 pl	1 s	2 s, apophysis with 2 pl
Segment 5	3 s, 2 aesthetascs	4 s, 2 aesthetascs	0/ 1 s	3 s, 2 aesthetascs
Segment 6	3 s, 3 aesthetascs	3 s, 2/3 aesthetascs	3 s, 3 aesthetascs	4 s, 2 aesthetascs
Segment 7	4 s, 3 aesthetascs	4 s, 3 aesthetascs	3 s, 1 aesthetasc	4 s, 3 aesthetascs
Setation of antenna	0/0+0/1+0/1+1/4 (1)	0/0+0/0+0/1+1/ 4 (1)	0/ 0+0/1+0/1+1/1	0+0/0+0/1+1/0+0/4 (1)
Labrum # teeth	17	22	8	10
Mandible				
No. teeth incisor process	4	4	4	4
No. teeth molar process	10	13	6	7
Maxillule				
No. spines proximal endite	4	4	4	4
No. spines distal endite	7	7	9	6
Setal formula of maxilla	2-3-11-6	4-4-10-5	2-2-5-7	4-3-9-6
Thoracopods I-VII				
No. segments exopod	3-4-5-5-5-5-4	4-5-6-6-6-6-4	1-3-3-3-3-3-3	1-3-3-3-3-3-3
Setal formula Th. I	3+1/3+1/3+1/3	1+1/3+1/0+1/-	2+1/1+1/1+1/3	2+1/3+1/2+1/4
Setal formula Th. II	1+1/3+1/1+1/4	1+1/3+1/0+1/3	0+1/1+1/0+1/3	1+1/2+1/0+1/4
Setal formula Th. III	1+1/4+1/0+1/4	1+1/3+1/0+1/4 or 3	0+1/0+0/0+0/3	1+1/2+1/0+1/3
Setal formula Th. IV	1+1/3+1/0+1/4	1+1/4+1/0+1/4	0+0/0+1/0+0/3	1+1/2+1/0+1/4
Setal formula Th. V	1+1/3+1/0+1/4	1+1/3+1/0+1/3	0+1/1+1/0+0/3	1+1/1+1/0+1/4
Setal formula Th. VI	1+1/3+1/0+1/4	1+1/4+1/0+1/4	0+0/0+0/0+0/3	1+1/1+1/0+1/3
Setal formula Th. VII	1+1/3+1/0+1/4	1+1/3+1/0+1/2	0+1/0+1/0+0/3	1+0/1+1/0+0/3
Pleopod	A	A	A	P
Uropod				
No. spines on sympod	17	17	7	10

Table 3.1. **(continued)**

Species	*A. spriggi*	*A. remkoi*	*A. robusta*	*A. bispinosa*
No. spines on endopod	1 large	1 large, 1 small	1 large	2 large, 2 small
Setae of endopod	4 (3 s, 1 pl)	4 (3 s, 1 pl)	4 (3 s, 1 pl)	4 (3 s, 1 pl)
No. setae on exopod	7	8	1	4
No. spines on furcal rami	14	13	7	10
No. setae on furcal rami	1 s, 2 pl	2 pl	2 pl	1 s, 2 pl

Key to parabathynellid genera found in Australia*

1. Antenna with 1 segment ..... ***Atopobathynella***

— Antenna with 2 segments ..... ***Kimberleybathynella***

— Antenna with 5 segments (Fig. 3.2A)..... 2

— Antenna with 6 segments ..... ***Notobathynella (N. remota only)***

— Antenna with 7 segments 7

2. Presence of 'claw-like' setae on antenna, antennule, thoracopods and uropod (Fig. 2B) ..... ***Onychobathynella***

— Presence of smooth setae on antenna, antennule, thoracopods and uropod 3

3. Male antennal organ present (Fig. 3.2C)..... 4

— Male antennal organ absent..... 5

4. Antennule with 6 segments; male thoracopod VIII basipodal seta present (Fig 3.2E); 6 pairs of thoracopods ..... ***Hexabathynella***

— Antennule with 7 segments (Fig. 3.2D); male thoracopod VIII basipodal seta absent; 7 pairs of thoracopods ..... ***Chilibathynella***

5. Male thoracopod VIII basipodal seta present (Fig. 3.2E) .... ***Notobathynella*** (most species)

— Male thoracopod VIII basipodal seta absent 6

6. Sympod even (Fig. 3.2F)..... ***Brevisomabathynella***[#]

— Sympod uneven (Fig. 3.2G) ..... ***Arkaroolabathynella* gen. nov.**

7. Antennule with 7 segments; thoracopod I epipod present (Fig. 3.2H); sympod even; male thoracopod VIII basipodal seta absent..... ***Billibathynella***

— Antennule with 8 segments; thoracopod I epipod absent; sympod uneven; male thoracopod VIII basipodal seta present ..... ***Octobathynella***

*****Note: This key is based on Australian taxa only. Additionally, the key is based on male characters, following convention of previous taxonomic studies. This is due to a lack of female characters appropriate for recognising genera.

Note: There is only one *Brevisomabathynella* species (*B. uramurdahensis*) which could be described as having an uneven sympod but it is morphologically and genetically distinct from *Arkaroolabathynella* gen. nov. and can be easily identified based on other morphological differences (see Cho and Humphreys 2010).

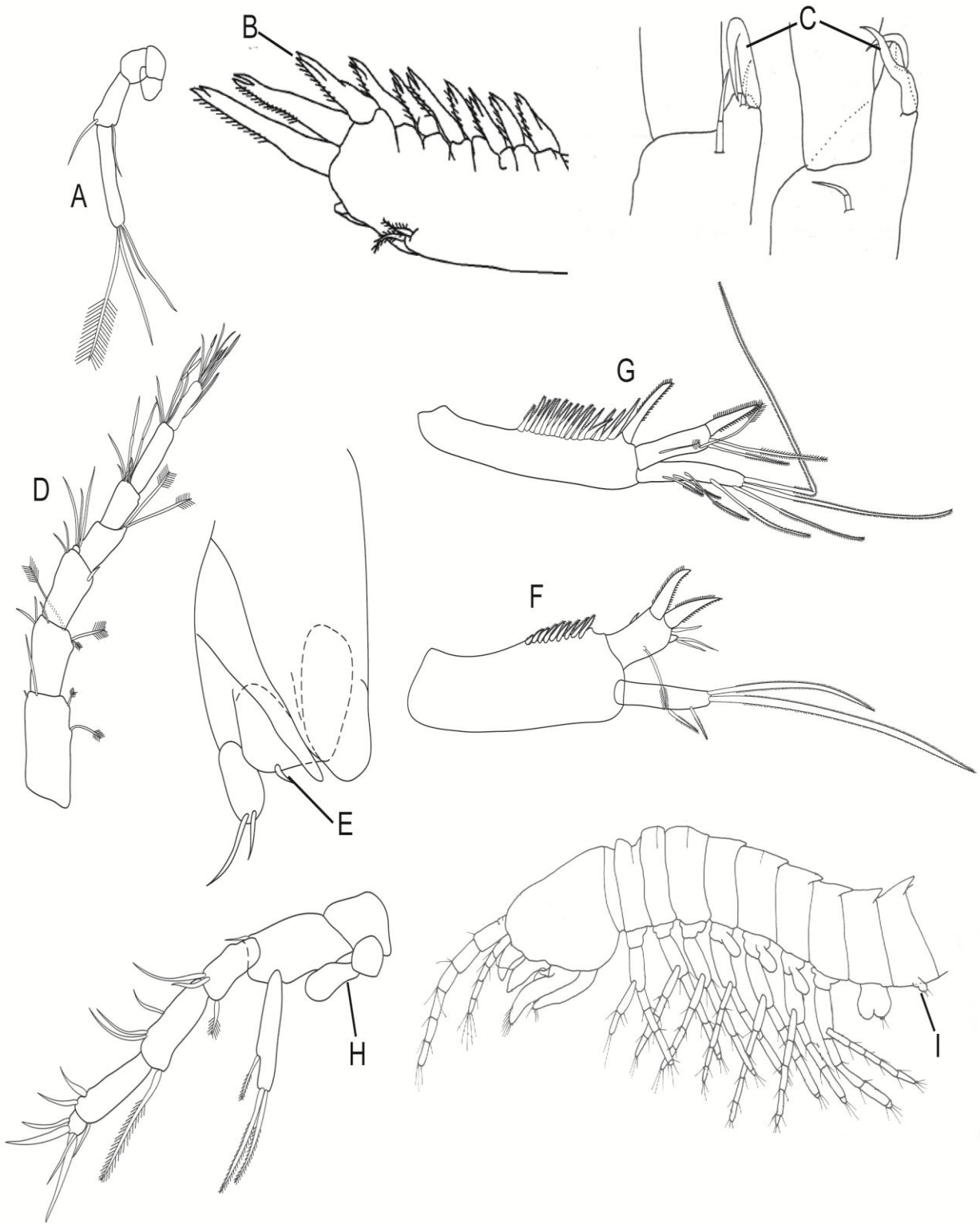

Fig 3.2. Morphological characters referred to in the keys: (A) antenna; (B) "claw-like" setae; (C) male antennal organ; (D) antennule; (E) basipodal seta on male Th. VIII; (F) even sympod; (G) uneven sympod; (H) epipod; (I) pleopod.

Taxonomy

Superorder **Syncarida** Packard, 1885

Order **Bathynellacea** Grobben, 1904

Family **Parabathynellidae** Noodt, 1965

Genus ***Arkaroolabathynella*** Abrams & King gen. nov.

Type species: *Arkaroolabathynella spriggi* Abrams & King sp. nov.

Included species: *A. bispinosa* Abrams & King sp. nov., *A. remkoi* Abrams & King sp. nov., *A. robusta* Abrams & King sp. nov., *A. spriggi* Abrams & King sp. nov.

Diagnosis

Antennule with seven segments. Antenna with five segments. Labrum with numerous (8-22) teeth on free margin. Mandibular palp with one segment. Maxilla with four segments. Thoracopods I-VII with exopod of 1-6 segments. Male thoracopod VIII almost rectangular, longer than wide; protopod protrudes at inner distal corner, epipod large, triangular, distal part covering penial region of protopod; basipod with or without setae, inner margin of basipod drawn out into projection. Uropod with numerous (7-17) spines on sympod; endopod with 1-2 terminal spines and 3-4 setae; exopod with numerous setae (1-8), without basiventral seta. Anal operculum slightly concave. Furcal rami elongated with two large terminal spines and numerous (7-14) spines on free margin.

Remarks

This genus shares many characters with *Brevisomabathynella*. These characters include, 1) the seven-segmented antennule, 2) five-segmented antenna, 3) the multi-segmented exopods of thoracopods I-VII, 4) the large, rectangular male thoracopod VIII without basipodal setae, 5) the uropod with numerous spines on the sympod, and 6) the elongated furcal rami with two large terminal spines and numerous small spines on the inner margin. Indeed, the only obvious difference between the two genera is the uneven spine row of the uropodal sympod of *Arkaroolabathynella* gen. nov.; *Brevisomabathynella* species have an even spine row. As both character states can be expressed within other genera (e.g. *Chilibathynella*, *Atopobathynella),* on its own, this would not be a good character for defining a new genus. However, there is additional data to support the recognition of this genus; first *Arkaroolabathynella* gen. nov is genetically distinct with an average K2P divergence of 20.3% for COI and 4.7%. for *18S.* Indeed, the molecular phylogeny suggests a closer relationship between *Arkaroolabathynella* gen. nov. and *Octobathynella* + *Notobathynella*, than between *Arkaroolabathynella* gen. nov. and *Brevisomabathynella*. Further support for the distinctiveness of *Arkaroolabathynella* gen.

nov. from *Brevisomabathynella* is provided by the large geographic separation of these genera, the former being from the Flinders Ranges, South Australia and the latter from Western Australia. *Arkaroolabathynella* gen. nov. can be distinguished from *Octobathynella* by the following characters: antennule with eight segments and the absence of an epipod on thoracopod I; and from *Notobathynella* by the absence of a basipodal seta on the male thoracopod VIII. Neither of these genera has been collected from the Flinders Ranges; *Notobathynella williamsi* Schminke 1973 has been described from Victoria (Schminke 1973), the closest record for the two genera to the Flinders ranges (100s km), but this region has no connection in its hydrogeology.

The four species of *Arkaroolabathynella* gen. nov. differ from each other in many aspects of their external morphology (see Table 3.2, and the key below). As the species range in size from 0.83 mm (*A. robusta*) to 3.3 mm (*A. remkoi*), it is plausible that many of the differences are meristic, as was found for *Brevisomabathynella* (Cho and Humphreys 2010).

Etymology. The name refers to the Arkaroola area of the Flinders Ranges from where the type species was collected.

Key to species of *Arkaroolabathynella* gen. nov.

1. Robust, simplified, short body form (Fig. 3.9G); single uropodal seta (Fig. 3.11C) .....***A. robusta*** sp. nov.
- Slender, elongate body form 2
2. Presence of pleopod (Fig. 3.2I); 2 large and 2 small spines on uropodal endopod (Fig. 3.5C) .....***A. bispinosa*** sp. nov.
- Absence of pleopod 3
3. Presence of a seta on third segment of antenna (Fig. 3.12C).....***A. spriggi*** sp. nov.
- Absence of setae on third segment of antenna (Fig. 3.6C); 1 tiny spine on uropodal endopod (Fig. 3.8C) .....***A. remkoi*** sp. nov.

Arkaroolabathynella bispinosa Abrams & King sp. nov.

(Figs. 3.3-3.5)

Material examined

Holotype (SAM C6940), male, 1.17 mm, Werta Spring, Gum Creek, Flinders Ranges, South Australia, 31° 9' 57.24"S, 138° 35' 13.596"E, Coll. R Leys, 4 October 2008.

Paratype (SAM C7018), female, 1.15 mm, same location as holotype, Coll. R. and P. Leys, 17 June 2009.

Description.

Male (SAM C6940), 1.17 mm. *Body* elongate (Fig. 3.3A), segments slightly widening towards posterior end. *Head* length slightly greater than width and about the length of the first three and a half thoracic segments combined.

Antennule (Fig. 3.3B) with seven segments; no sexual dimorphism; first segment bearing one simple and three plumose setae; second segment bearing three simple and three plumose setae, third segment bearing three simple setae and inner flagellum with three setae; fourth segment bearing two tiny simple setae and two plumose setae on outer distal apophysis; fifth segment bearing three simple setae and two aesthetascs; sixth segment bearing four simple setae and two aesthetascs; seventh segment bearing four simple setae and three aesthetascs. *Antenna* (Fig. 3.3C) with five segments; similar in length to the first segment of antennule; first, second and fourth segments small and of similar size, segments three and five of similar size. Setal formula: 0/0+0/1+1/0+0/4(1).

Labrum (Fig. 3.3D) with at least 10 teeth but is damaged so actual number unknown. *Mandible* (Fig. 3.3E) with incisor process of four teeth (slightly damaged so female mandible shown too, Fig 3.3I); molar process with seven spines; palp with one segment with one apical seta, palp slightly exceeds incisor process in length. *Maxillule* (Fig. 3.3F) proximal endite with four spines; distal endite with six spines; three simple setae on outer distal margin, most distal spine nearly twice as long as other inner spines. *Maxilla* (Fig. 3.3G) with four segments, setal formula: 4-3-9-6.

Thoracopods I–IV (Fig. 3.4A-D) increasing in size posteriorly. *Thoracopods IV–VII* (Figs. 3.4D-F, 3.5A) similar in size. *Thoracopods I–VII* each bearing one small epipod on protopod and one simple seta on basipod; the number of segments of exopod of thoracopods I–VII is: 1-3-3-3-3-3-3; all thoracopod exopods with two barbed setae on each segment, except Th I, IV and V which have three setae on the first segment; endopods with four segments, with all inner setae on segments simple, outer setae of first and second segments of Th I-VII plumose. Endopod setal formula: Th I: 2+1/3+1/2+1/4 (2), Th II: 1+1/2+1/0+1/4 (2),

Th III: 1+1/2+1/0+1/3 (2), Th IV: 1+1/2+1/0+1/4 (2), Th V: 1+1/1+1/0+1/4 (2), Th VI: 1+1/1+1/0+1/3 (1), Th VII: 1+0/1+1/0+0/3 (1). *Thoracopod VIII male* (Fig. 3.5B), approximately rectangular in frontal view, twice as long as wide; protopod massive, with prominent penial region with a distal opening; epipod large, triangular, its distal part nearly reaching the penial region of the protopod; basipod without setae, inner margin drawn out into projection; exopod one-third size of basipod, nearly oval-shaped, with one simple seta; endopod half as large as exopod, with two simple setae.

First pleopods present. *Uropod* (Fig. 3.5C) with sympod 3.3 times as long as wide, 3.6 times longer than endopod and 2.6 times longer than exopod; with nine barbed spines of similar size, increasing slightly towards the posterior end, and one most distal spine more robust and 2.3 times as long as the preceding spines; endopod shorter than exopod with two large spinous projections and two small spines, one plumose in the middle, one subterminal plumose seta and two terminal barbed setae on outer distal margin; exopod bearing four barbed setae. *Pleotelson* (Fig. 3.5D) with one seta near the base of the furcal rami on both sides; anal operculum slightly concave. *Furcal rami* (3.5E) 2.5 times as long as wide, with two large distal spines and seven smaller spines on inner margin, with two unequal plumose dorsal setae.

Female (SAM C7018), 1.15 mm. Identical to male except for following characters.

Antennule seventh segment with five simple setae and one aesthetasc.

Labrum (Fig. 3.5H) bearing 12 teeth. *Maxilla* setal formula: 2-4-10-3.

Thoracopods I-VII exopod segment number: 1-3-3-3-3-3-2. Setal formulae of endopods: Th I: 2+1/2+1/2+1/2 (0) (appears deformed), Th II: 1+0/1+1/0+1/3 (1), Th III: 1+0/2+1/0+1/4 (2), Th IV: 1+0/2+1/0+1/3 (1), Th V: 1+0/1+1/0+1/3 (1), Th VI: 1+0/1+1/0+1/3 (1), Th VII: 1+0/1+1/0+1/3 (1). *Thoracopod VIII* (Fig. 3.5F) tiny, elongate, separated, 1.5 times as long as wide.

Uropod sympod bearing nine spines. *Furcal rami* with nine spines on inner margin.

Variability

In one partial female paratype (lacking pleotelson which was used for DNA extraction) the following variation was observed: second segment of the antennules with two simple and three plumose setae, fifth segment with three simple setae and one aesthetasc on left side and two aesthetascs on the right side, seventh segment with four simple setae and two aesthetascs on one side and three simple setae and three aesthetascs on the other.

Remarks

This species is unique in having first pleopods and two large and two small spines on the uropodal endopod. It is most similar to *A. robusta* sp. nov. in terms of the number of segments of the thoracopodal exopods (identical in the male of both species) and similar number of teeth of the labrum (10 vs. 8) and spines of the mandibular molar process (7 vs. 6) and setae of the uropodal endopod (4 vs. 3).

Relationships

This is the most basal species within the genus (Fig. 3.1) and has the greatest interspecies sequence divergences. The K2P *COI* divergence with *A. spriggi* sp. nov. is 28.9%, with *A. remkoi* sp. nov. is 27.4% and with *A. robusta* sp. nov. is 27.3%. The K2P *18S* divergence with *A. remkoi* sp. nov. is 3.1% and with *A. robusta* sp. nov. is 3.1 %.

Etymology

The species name means “two spines” and refers to the two large spines on the uropodal endopod.

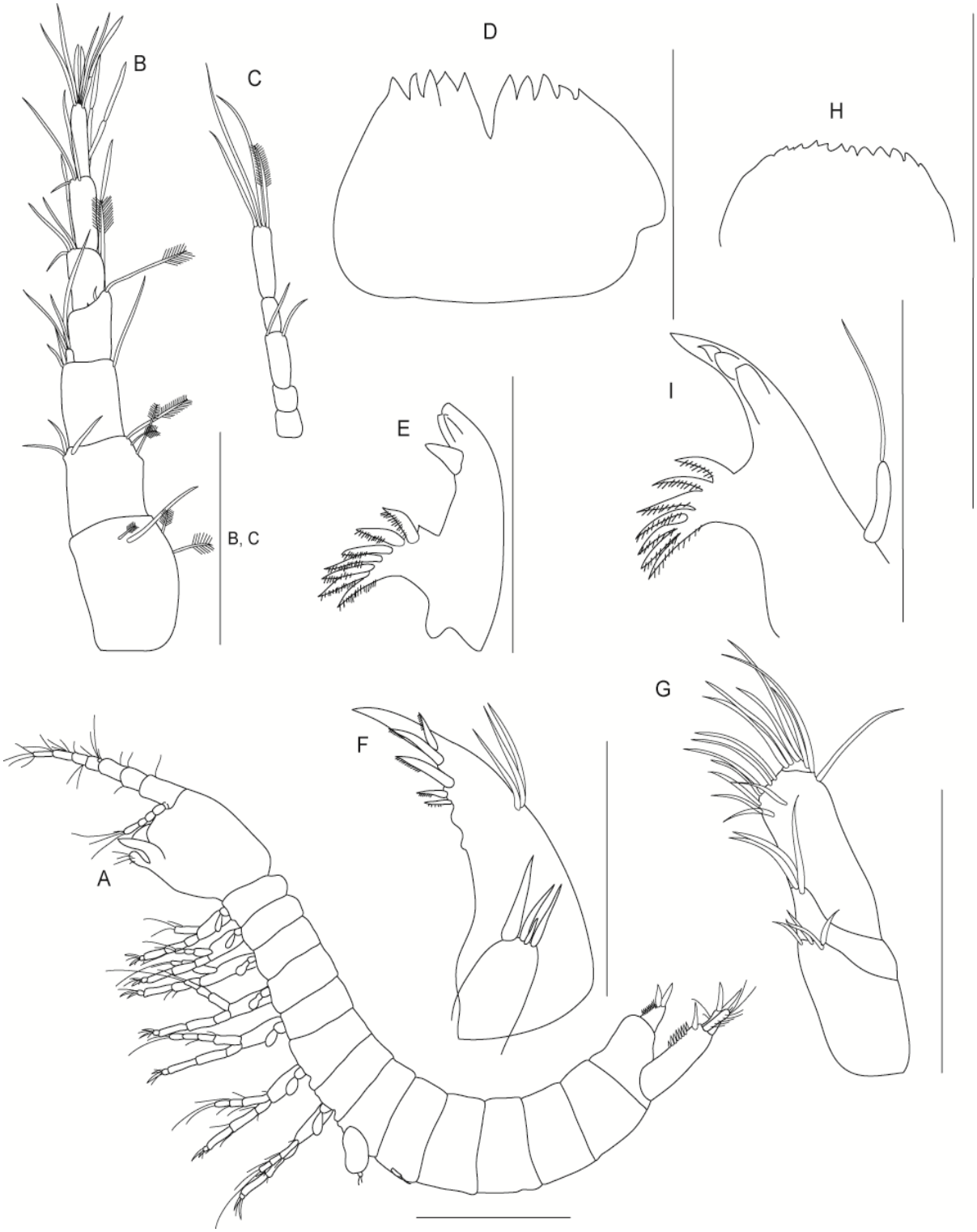

Fig. 3.3. *Arkaroolabathynella bispinosa* sp. nov. (male: holotype; female: allotype). (A) general habitus, male; (B) left antennule, male; (C) left antenna, male; (D) labrum, male; (E) left mandible, male; (F) left maxillule, male; (G) left maxilla, male; (H) labrum, female; (I) left mandible, female. Scale bars: A = 0.5mm; B - G, I = 0.1mm; H = 0.2mm.

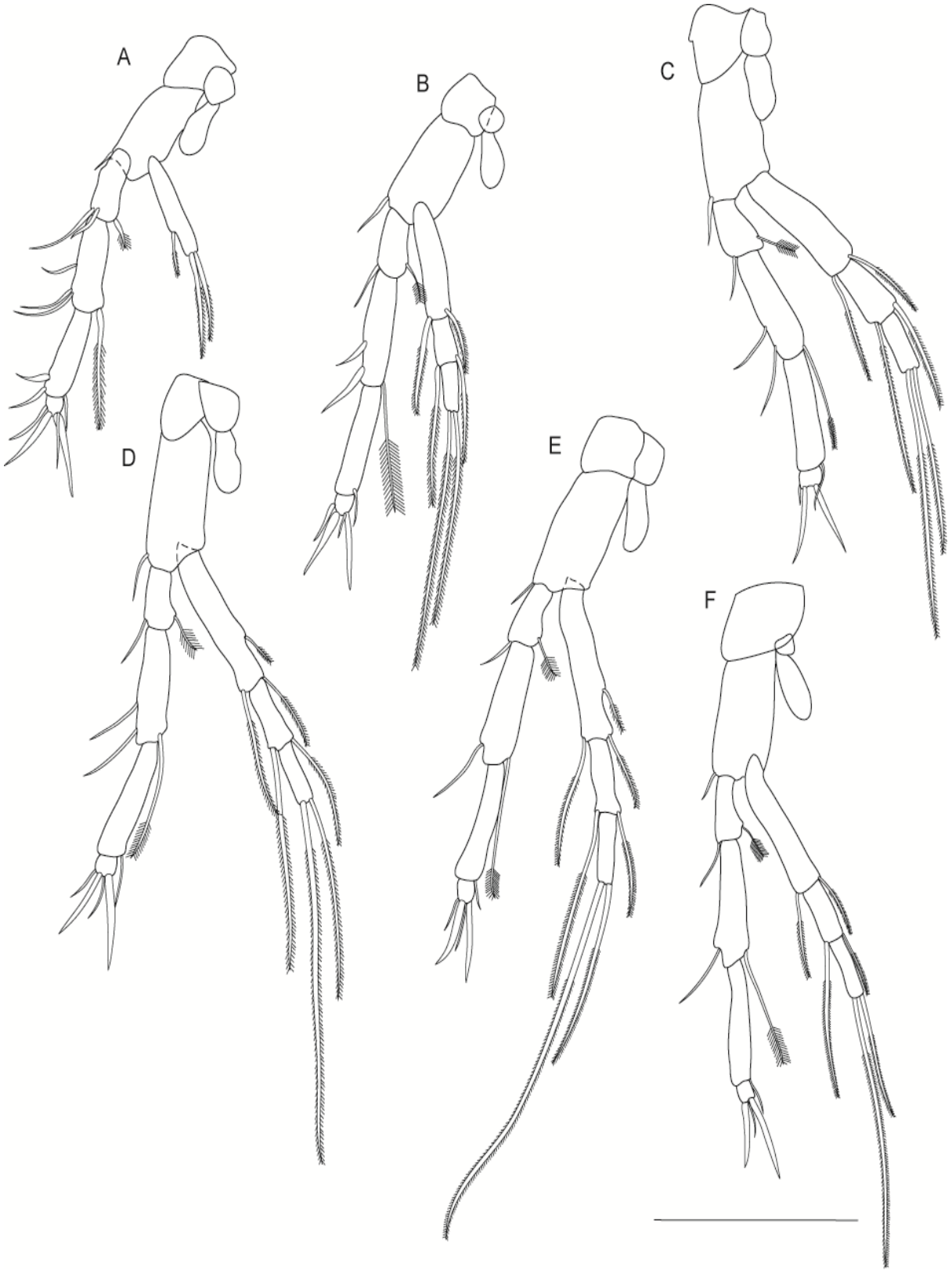

Fig. 3.4. *Arkaroolabathynella bispinosa* sp. nov. (male: holotype). A - F, thoracopods I - VI, male. Scale bars: 0.1mm.

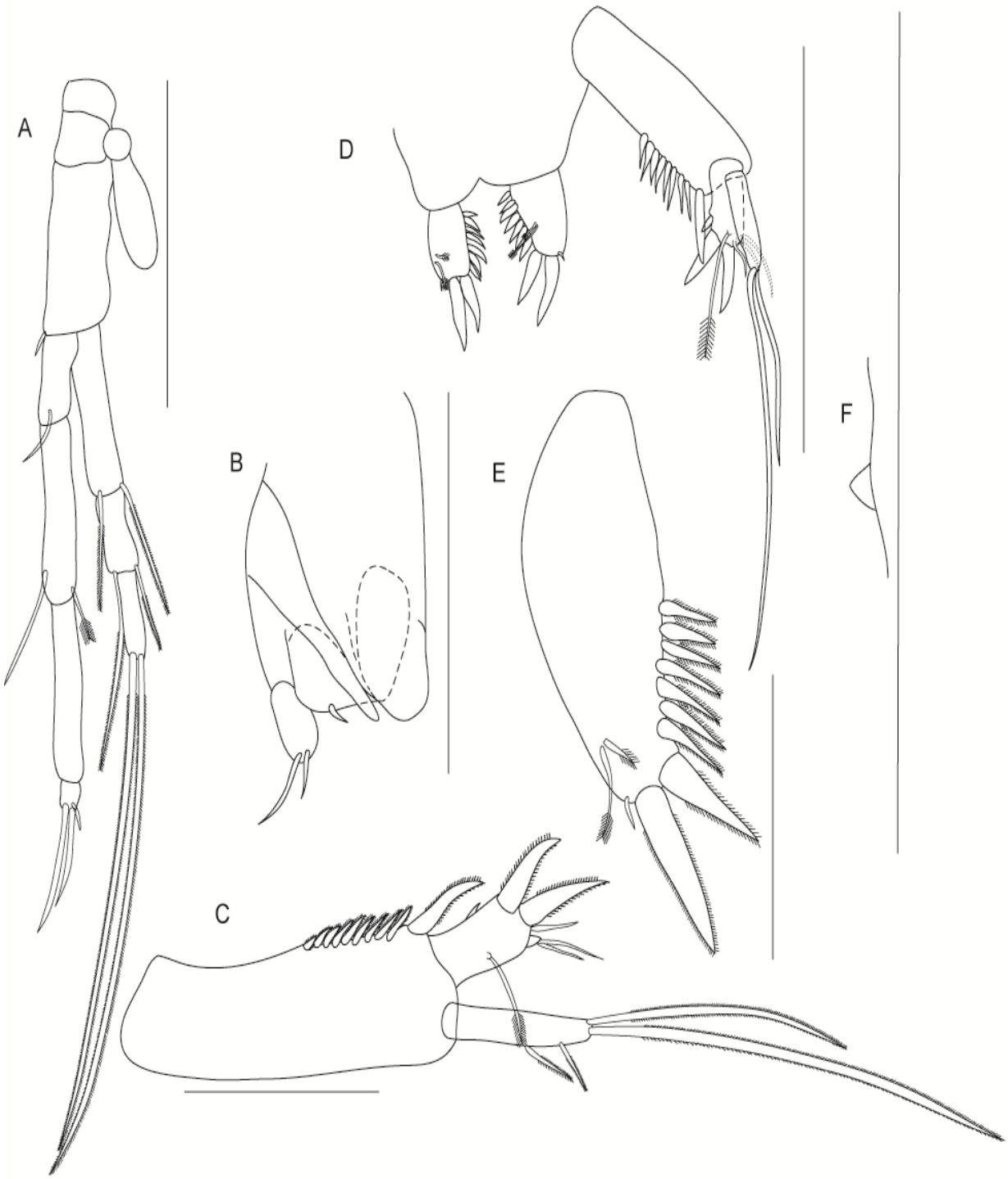

Fig. 3.5. *Arkaroolabathynella bispinosa* sp. nov. (male: holotype, female: allotype). (A) left thoracopod VII, male; (B) thoracopod VIII, male; (C) left sympod, male; (D) pleotelson, male; (E) left furcal ramus, male; (F) thoracopod VIII, female. Scale bars: A-C, E = 0.1mm; D, F= 0.2mm.

Arkaroolabathynella remkoi Abrams & King sp. nov.

(Figs. 3.6-3.8)

Material examined

Holotype (SAM C6492), male, 3.3 mm, Grindell's Hut, Flinders Ranges, South Australia, 30°28′ 37.92″S, 139°12′ 48.6″E, Coll. R Leys, 6 October 2008.

Paratypes (SAM C7019), female, 2.5 mm, and (SAM C7020), female, 2.21 mm. collected with holotype.

Description

Male (SAM C6942), 3.3 mm. *Body* elongate (Fig. 3.6A), segments slightly widening towards posterior end. *Head* length slightly greater than width and about the length of the first five thoracic segments combined.

Antennule (Fig. 3.6B) with seven segments; no sexual dimorphism; first segment bearing two simple and two plumose setae; second segment bearing three simple and three plumose setae, third segment bearing three simple setae and inner flagellum with three setae; fourth segment bearing one tiny simple seta on left A1 (but not on the right) and two plumose setae on outer distal apophysis; fifth segment bearing four simple setae and two aesthetascs; sixth segment bearing three simple setae and three aesthetascs (right A1 has 2 aesthetascs); seventh segment bearing four simple setae and three aesthetascs, setae positioned as seen in Fig. 3.6B. *Antenna* (Fig. 3.6C) with five segments; first three segments small and of similar size; last two segments of similar size but final segment is the longest. Setal formula: 0/0+0/0+0/1+1/4(1).

Labrum (Fig. 3.6D) with 22 teeth. *Mandible* (Fig. 3.6E) with incisor process of four teeth, molar process with thirteen spines; palp of one segment with one apical seta (female palp shown with mandible, Fig. 3.6F), nearly reaching incisor process. *Maxillule* (Fig. 3.6G) proximal endite with four spines; distal endite with seven spines; three simple setae on outer distal margin. Most distal spine slightly more than twice (2.3x) as long as other inner spines. *Maxilla* (Fig. 3.6H) four-segmented, setal formula 4-4-10-5.

Thoracopods I–IV (Fig. 3.7A-D) increasing in size posteriorly. *Thoracopods IV–VII* (Fig. 3.7D-F, 3.8A) similar in size. *Thoracopods I–VII* each bearing one small epipod on protopod and one simple seta on basipod; the number of segments of exopod of thoracopods I–VII is: 4-5-6-6-6-6-4; all thoracopod exopods with two barbed setae on each segment, except Th II, V and VII which have three setae on the first segment; endopod four-segmented, with all inner setae on segments simple and outer setae of first and second segments of Th I-VII are plumose. Endopod setal formula: Th I.: 1+1/3+1/0+1/- (broken), Th II:

1+1/3+1/0+1/3(0), Th III: 1+1/3+1/0+1/3(1) (Other thoracopod: 1+1/3+1/0+1/4 (2)), Th IV 1+1/4+1/0+1/4(2), Th V: 1+1/3+1/0+1/3(1), Th VI: 1+1/4+1/0+1/4(2), Th VII: 1+1/3+1/0+1/2(0) (Other thoracopod: 1+1/3+1/0+1/4(2)). *Thoracopod VIII male* (Fig. 3.8B). Approximately rectangular in frontal view, twice as long as wide; protopod massive, with prominent penial region with a distal opening; epipod large, triangular, its distal part shorter than the penial region of the protopod; basipod with two simple setae, inner margin of basipod drawn out into projection; exopod nearly half the size of basipod, nearly oval-shaped; endopod half as large as exopod, with two simple setae.

First pleopods. Absent. *Uropod* (Fig. 3.8C) with sympod 5.9 times as long as wide, 2.4 times longer than endopod and 1.7 times longer than exopod; with sixteen barbed spines of similar size, increasing slightly towards the posterior end, and one most distal spine more robust and nearly twice as long as the preceding spines; endopod shorter than exopod with one spinous projection and one small subterminal dorsal spine, one plumose seta near the base, and one subterminal plumose seta and two terminal barbed setae on outer distal margin; exopod bearing eight barbed setae. *Pleotelson* (Fig. 3.8D) with one seta near the base of the furcal rami on both sides; *anal operculum* slightly concave. *Furcal rami* (Fig. 3.8D) 2 times as long as wide, with two large distal spines and eleven smaller spines on inner margin, and with two unequal plumose dorsal setae.

Female (SAM C7019), 2.5 mm. Identical to male except for following characters.

Antennule fourth segment without simple setae, fifth segment with three simple setae and one aesthetasc, sixth segment with four simple setae and two aesthetascs.

Labrum bearing sixteen teeth. *Mandible* (Fig. 3.7F) molar process with eleven spines. *Maxillule* distal endite with nine spines. *Maxilla* setal formula: 3-4-10-6.

Thoracopods I-VII. Exopod segment number: 3-4-5-5-5-5-4. Seta formulae of endopods: Th I: 1+1/3+1/0+1/4 (2), Th II: 1+0/3+1/0+1/4 (2), Th III: 1+1/3+1/0+1/4 (2), Th IV: 1+1/3+1/0+1/4 (2), Th V:1+1/3+1/0+1/4 (2), Th VI:1+1/2+1/0+1/4 (2), Th VII:1+1/2+1/0+1/3 (1). *Thoracopod VIII female* (Fig. 3.8E) tiny, elongate, separated, 2.6x as long as wide.

Uropod sympod bearing fifteen spines. *Furcal rami* with thirteen spines on inner margin.

Variability

In one female paratype the following variation was observed: length 2.2 mm, number of exopodal segments of thoracopods I-VII: 3-4-5-5-5-4-3; the uropod sympod with 13 spines; furcal rami with 10 small spines.

Remarks

This species has the highest number of teeth on the labrum (22), spines on the molar process of the mandible (13), number of segments of the thoracopodal exopods and number of setae on the sympodal exopod (8). It is unique in having one tiny spine on the sympodal endopod, although *A. bispinosa* is similar in that it has two tiny spines on its endopod. *A. remkoi* is also the only species with setae on the basipod of the male thoracopod VIII.

Relationships

This species is sister to *A. spriggi* sp. nov. (Fig. 3.1) (K2P *COI* sequence divergence 14.6%). The K2P sequence divergence with *A. robusta* sp. nov. is 15.3% and with *A. bispinosa* sp. nov. is 27.4%. The *18S* K2P sequence divergence with the other two species is 3.1%.

Etymology

Named for Dr. Remko Leys who collected the specimens.

Fig. 3.6. *Arkaroolabathynella remkoi* sp. nov. (male: holotype; female: allotype). (A) general habitus, male; (B) left antennule, male; (C) left antenna, male; (D) labrum, male; (E) left mandible, male; (F) left mandible, female; (G) left maxillule, male; (H) left maxilla, male. Scale bars: A = 0.5mm; B-E, G, H = 0.1mm; F=0.2mm.

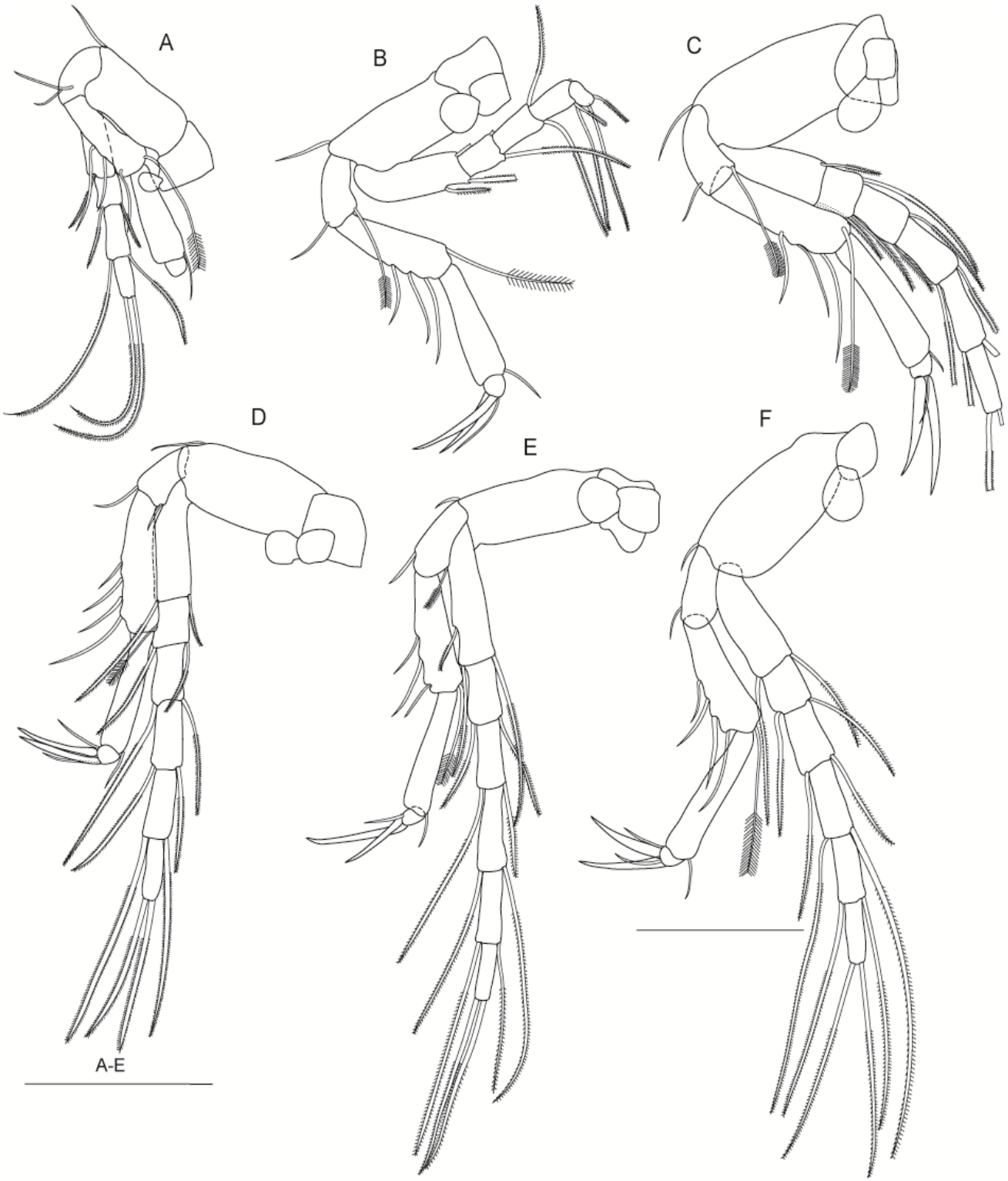

Fig. 3.7. *Arkaroolabathynella remkoi* sp. nov. (male, holotype): A - F, thoracopods I - VI. Scale bars: A - F = 0.1mm.

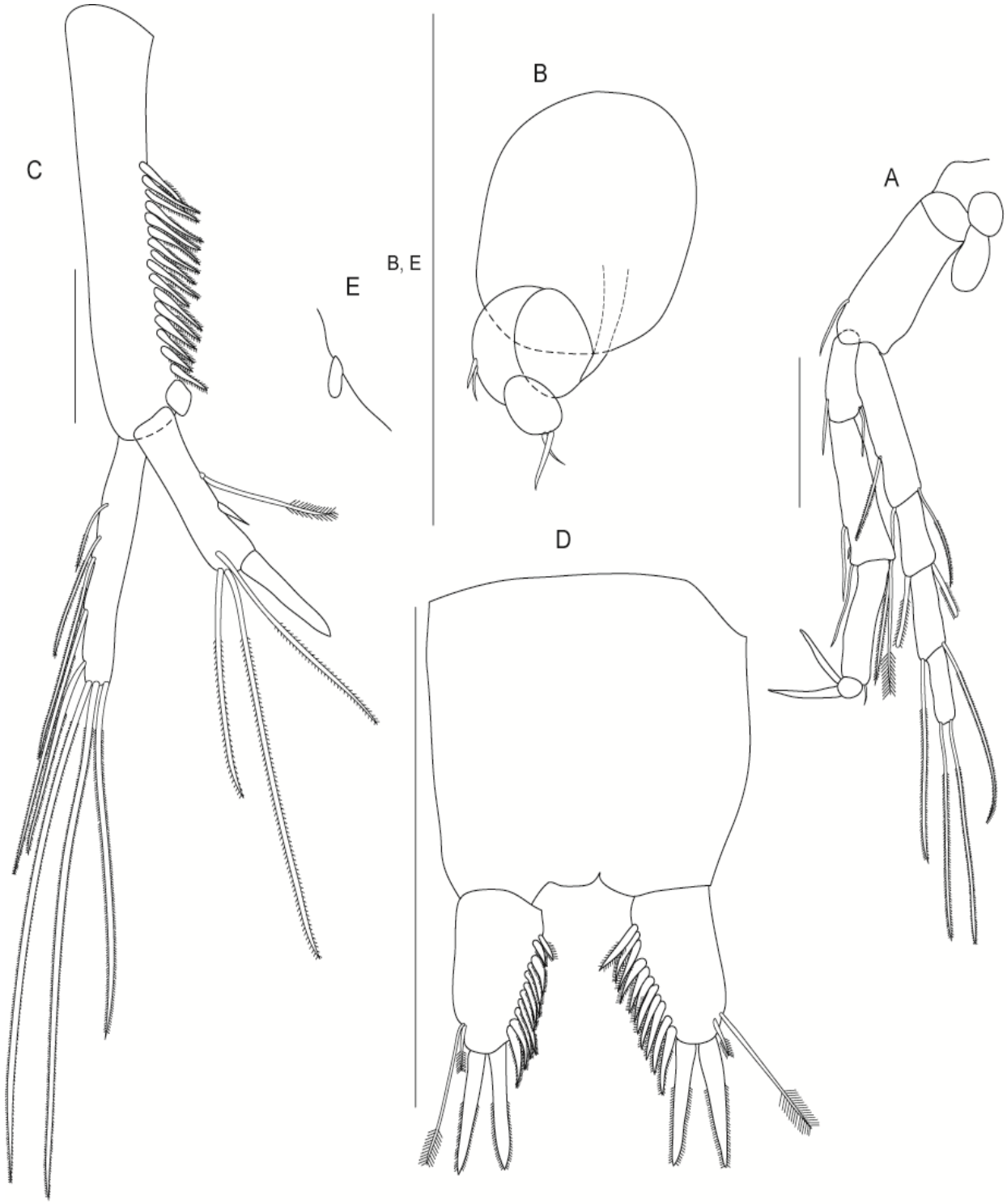

Fig. 3.8. *Arkaroolabathynella remkoi* sp. nov. (male: holotype; female: allotype). (A) left thoracopod VII, male; (B) thoracopod VIII, male; (C) sympod, male; (D) pleotelson, male; (E) thoracopod VIII, female. Scale bars: A, B, D, E = 0.1mm. C =0.2mm

Arkaroolabathynella robusta Abrams & King sp. nov.

(Figs. 3.9-3.11)

Material examined

Holotype (SAM C6941), male, 0.83 mm. Gum Creek, Yaltipena Spring, Flinders Ranges, South Australia, 31° 13′9.84″S, 138° 44′ 34.872″ E, Coll. R Leys, 6 October 2008.

Paratype, (SAM C7021) juvenile, 0.9 mm, collected with holotype.

Description

Male (SAM C6941). *Body* elongate (Fig. 3.9G) segments slightly widening towards posterior end. *Head* length slightly greater than width and about the length of the first three and a half thoracic segments combined. Specimen is partially damaged and deformed.

Antennule (Fig. 3.9A) damaged, with seven segments; first segment bearing one simple and one plumose seta; second segment bearing three simple setae, third segment bearing one simple seta and inner flagellum with two simple setae; fourth segment bearing one simple seta and has an outer distal apophysis with a broken seta; fifth segment without setae (right antenna has one seta); sixth segment bearing three simple setae and three aesthetascs; seventh segment bearing four simple setae and three aesthetascs, setae positioned as seen in Fig. 3.9A. *Antenna* (Fig. 3.9B) with five segments; first three segments small and of similar size; last two segments of similar size but final segment is the longest, setal formula: 0/0+0/1+0/1+1/1.

Labrum (Fig. 3.9C) with eight teeth. *Mandible* (Fig. 3.9D) with incisor process of four teeth; molar process with six spines; palp of one segment with one apical seta, slightly exceeding incisor process. *Maxillule* (Fig. 3.9E) proximal endite with four spines; distal endite with six spines; three simple setae on outer distal margin; most distal spine slightly longer than inner spines. *Maxilla* (Fig. 3.9F) four-segmented, setal formula: 2-2-5-7.

Thoracopods I–IV (Fig. 3.10A-D) increasing in size posteriorly. *Thoracopods IV–VI* (Fig. 3.10D-F) similar in size. *Th VII* (Fig. 3.11A) small, similar to size of Th II. *Thoracopods I–VII* each bearing one small epipod on protopod. Th I and IV with one simple seta on basipod. The number of segments of exopod of thoracopods I–VII is: 1-3-3-3-3-3-3. Th I exopod with two terminal barbed setae (one is broken). Thoracopod II – VII exopods without setae on first segment and with two barbed setae on final two segments, endopod four-segmented, with all inner setae on segments simple and outer setae of first and second segments of Th I-VII are plumose. Endopod setal formula: Th I: 2+1/1+1/1+1/3 (1), Th II: 0+1/1+1/0+1/3(1), Th III: 0+1/0+0/0+0/3 (1), Th IV: 0+0/0+1/0+0/3 (1), Th V: 0+1/1+1/0+0/3 (1), Th VI: 0+0/0+0/0+0/3 (1), Th VII: 0+1/0+1/0+0/3 (1). *Thoracopod VIII*

male (Fig. 3.11B) approximately rectangular in frontal view, twice as long as wide; protopod massive, with prominent penial region with a distal opening; epipod large, triangular, its distal part reaching the penial region of the protopod; basipod without setae, inner margin of basipod drawn out into projection; exopod one-third size of basipod, nearly oval-shaped; endopod half as large as exopod, with one simple seta.

First pleopods. Absent. *Uropod* (Fig. 3.11C) sympod 3.1 times as long as wide, 3.1 times longer than endopod and 2.7 times longer than exopod; with six barbed spines of similar size, increasing slightly towards the posterior end, and one most distal spine more robust and three times as long as the preceding spines; endopod slightly shorter than exopod with one spinous projection, one plumose seta, and one subterminal plumose seta and two terminal barbed setae on outer distal margin. Exopod bearing one barbed seta (damaged, possibly more setae in other specimens, see juvenile Fig. 3.11F). *Pleotelson* (Fig. 3.11D) without setae; *anal operculum* slightly concave. *Furcal rami* (Fig. 3.11E) 1.6 times as long as wide, with two large distal spines and five smaller spines on inner margin, and with two unequal plumose dorsal setae.

Female: unknown.

Variability

In one juvenile (Fig. 3.11F) the following differences were observed: sympod with five spines, sympodal endopod with three setae and furcal rami with eight spines.

Remarks

This species is remarkably short and robust compared with it congeners and has the lowest number of segments, spines and setae on all appendages. It is unique in having one very long seta on the uropodal exopod, although the specimen is fairly damaged and other specimens may have more than one seta. It has the least number of teeth (8) on the labrum compared with all other described Australian species.

Relationships

Although this species has similarly low numbers of segments and setae to *A. bispinosa* sp. nov., the molecular results suggest it is more closely related to *A. spriggi* sp. nov. and *A. remkoi* sp. nov. than *A.bispinosa* sp. nov. (Fig. 3.1). The K2P *COI* divergence with *A. spriggi* sp. nov. is 13.4%, with *A. remkoi* sp. nov. is 15.3% and with *A. bispinosa* sp. nov. is 27.3%. The K2P *18S* divergence with *A. remkoi* sp. nov. is 0.3% and with *A. bispinosa* sp. nov. is 3.1%.

Etymology

Named for its simplified, robust body in comparison with its congeners.

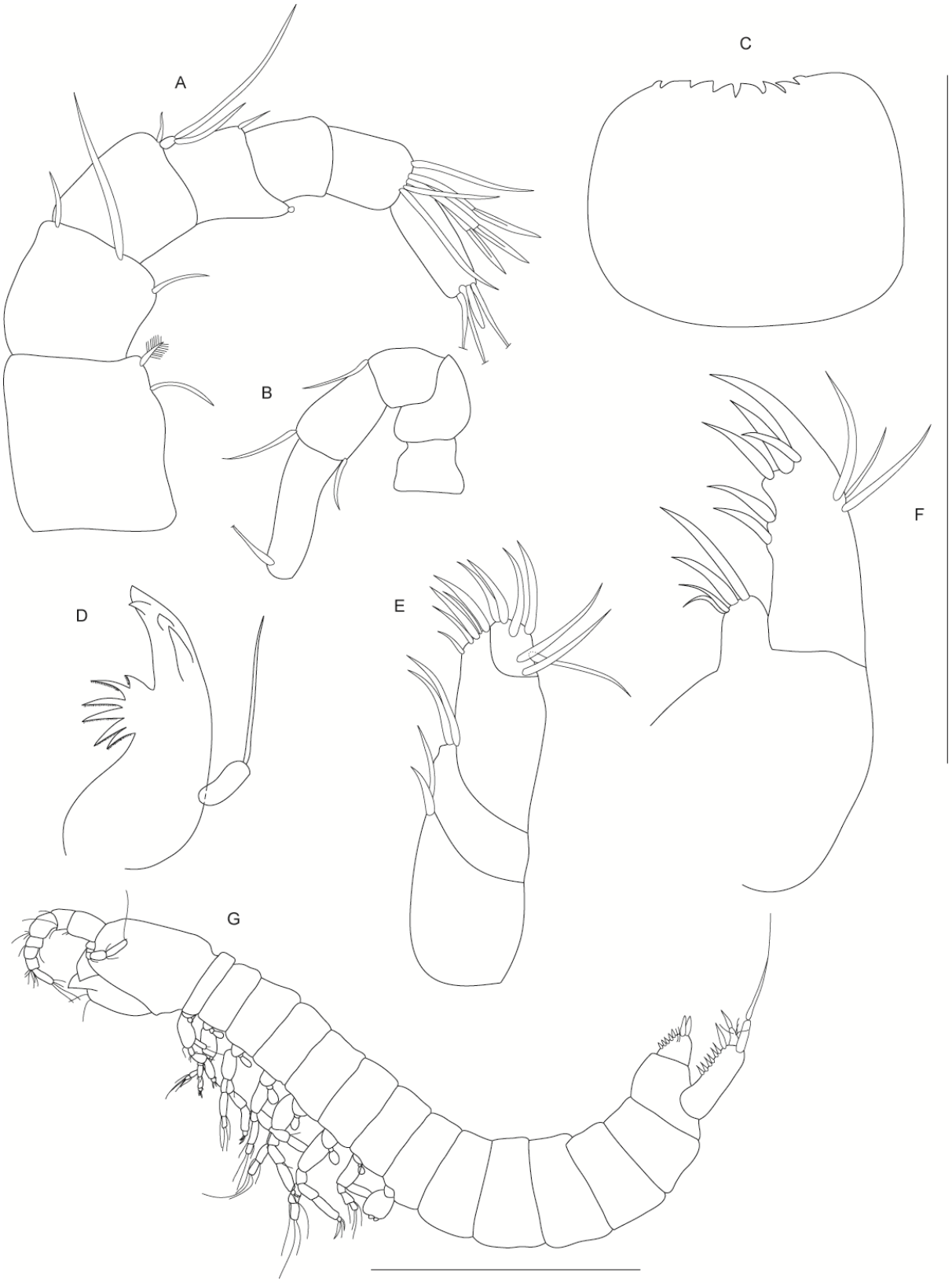

Fig. 3.9. *Arkaroolabathynella robusta* sp. nov. (male: holotype). (A) left antennule; (B) left antenna; (C) labrum; (D) left mandible; (E) left maxillule; (F) left maxilla; (G) general habitus. Scale bars: A-F=0.1mm; G=0.25mm

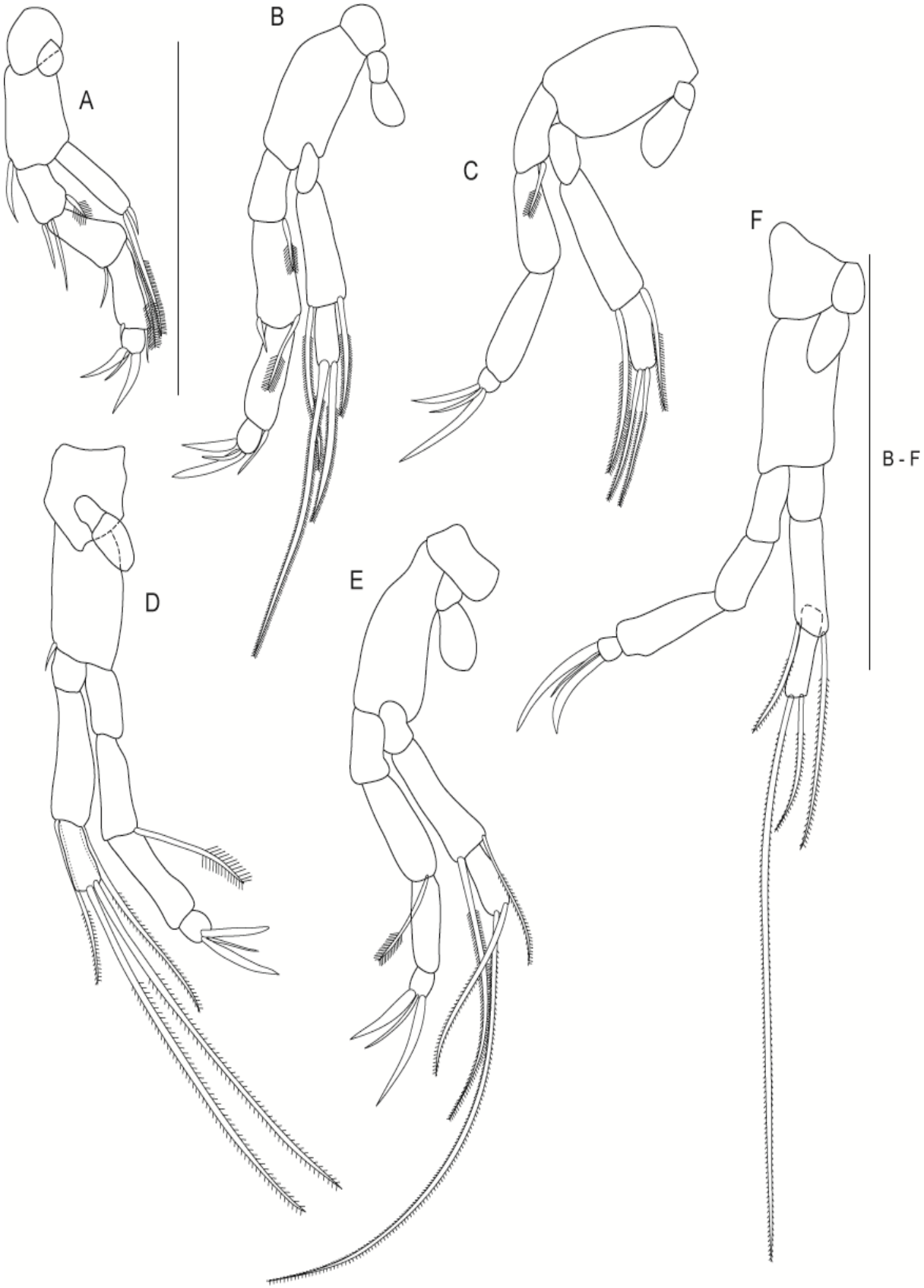

Fig. 3.10. *Arkaroolabathynella robusta* sp. nov (male: holotype). A - F, thoracopods I-VI, male. Scale bars: 0.1mm.

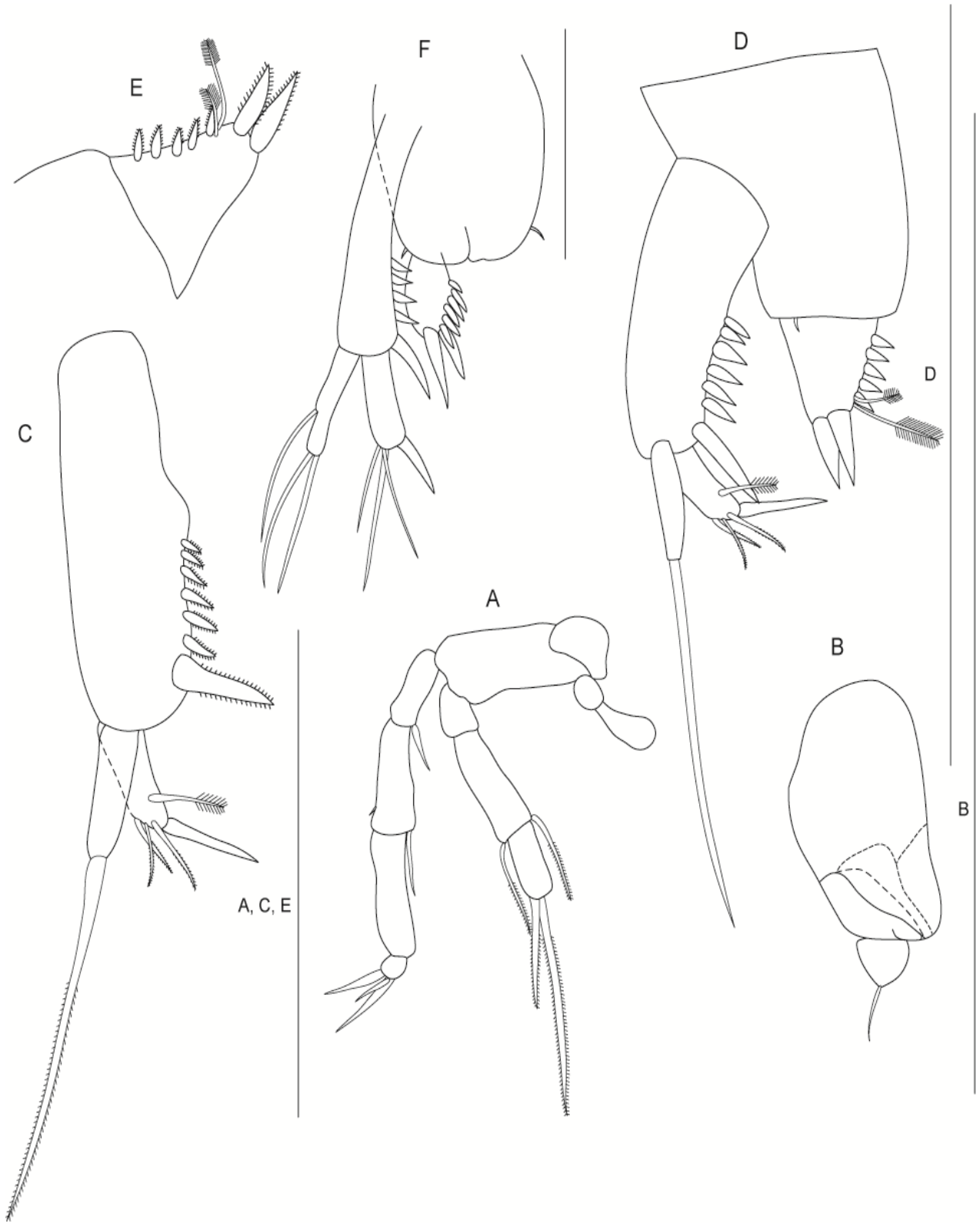

Fig. 3.11. *Arkaroolabathynella robusta* sp. nov. (male: holotype; juvenile: allotype). (A) Left thoracopod VII, male; (B) left thoracopod VIII, male; (C) sympod, male; (D) pleotelson, male; (E) furcal ramus, male; (F) pleotelson, juvenile. Scale bars: A - C = 0.1mm; D = 0.2mm.

Arkaroolabathynella spriggi Abrams & King sp. nov.

(Figs. 3.12-3.14)

Material examined

Holotype (SAM C6943). Male, 1.89 mm, Bollabollana Spring, Flinders Ranges, South Australia, 30°17′14.712″S, 139°16′ 54.732″E, Coll. R Leys and Dean Peani, 24 October 2002.

Paratypes (SAM C7022), female, 2.1 mm, and male (SAM C7023) collected with holotype.

Description

Male (SAM C6943), 1.89 mm. *Body* (Fig. 3.12A). Body elongate, segments slightly widening towards posterior end. *Head* length slightly greater than width and about the length of the first four thoracic segments combined.

Antennule (Fig. 3.12B). With seven segments; no sexual dimorphism. First segment bearing one simple and four plumose setae; second segment bearing two simple and four plumose setae, third segment bearing three simple setae and inner flagellum with three setae; fourth segment bearing one tiny simple seta and two plumose setae on outer distal apophysis; fifth segment bearing three simple setae and two aesthetascs; sixth segment bearing three simple setae and three aesthetascs; seventh segment bearing four simple setae and three aesthetascs, setae positioned as seen in Fig. 3.12B. *Antenna* (Fig. 3.12C) with five segments; similar in length to the first segment of antennule; first two segments small and of similar size, last three segments of similar size but final segment is the longest. Setal formula: 0/0+0/1+0/1+1/4(1).

Labrum (Fig. 3.12D) with 17 teeth. *Mandible* (Fig. 3.12E) with incisor process of four teeth, molar process with ten spines. Palp of one segment with one apical seta, slightly exceeding incisor process. *Maxillule* (Fig. 3.12F) proximal endite with four spines; distal endite with seven spines; three simple setae on outer distal margin, most distal spine nearly twice as long as other inner spines. *Maxilla* (Fig. 3.12G) four-segmented, setal formula: 2-3-11-6.

Thoracopods I–IV (Fig. 3.13A-D) increasing in size posteriorly. *Thoracopods IV–VII* (Fig. 3.13D-E, 3.14A–B) similar in size. Thoracopods *I–VII* each bearing one small epipod on protopod and one simple seta on basipod; the number of segments of exopod of thoracopods I–VII is: 3-4-5-5-5-5-4; all thoracopod exopods with two barbed setae on each segment, except Th II which has three setae on the first segment; endopod four-segmented, with all inner setae on segments simple and outer setae of first and second segments of Th I-VII are

plumose. Endopod setal formula: Th I: 3+1/3+1/3+1/3(1), Th II: 1+1/3+1/1+1/4(2), Th III: 1+1/4+1/0+1/4(2), Th IV: 1+1/3+1/0+1/4(2), Th V: 1+1/3+1/0+1/4(2), Th VI: 1+1/3+1/0+1/4(2), Th VII: 1+1/3+1/0+1/4(2). *Thoracopod VIII male* (Fig. 3.14C) approximately rectangular in frontal view, twice as long as wide; protopod massive, with prominent penial region with a distal opening; epipod large, triangular, its distal part reaching the penial region of the protopod; basipod without setae, inner margin of basipod drawn out into projection; exopod one-third size of basipod, nearly oval-shaped; endopod half as large as exopod, with two simple setae.

First pleopods. Absent. *Uropod* (Fig. 3.14D) sympod 6.5 times as long as wide, 2.9 times longer than endopod and 2.1 times longer than exopod; with sixteen barbed spines of similar size, increasing slightly towards the posterior end, and one most distal spine more robust and nearly twice as long as the preceding spines; endopod shorter than exopod with one spinous projection, one plumose seta near the base, and one subterminal plumose seta and two terminal barbed setae on outer distal margin; exopod bearing seven barbed setae. *Pleotelson* (Fig. 3.14E) with one seta near the base of the furcal rami on both sides; *anal operculum* slightly concave. *Furcal rami* (Fig. 3.14E) 2.7 times as long as wide, with two large distal spines and twelve smaller spines on inner margin, and with two unequal plumose dorsal setae.

Female (SAM C7022), 2.1 mm. Identical to male except for following characters.

Antennule. Seventh segment with two aesthetascs.

Labrum. Bearing 16 teeth. *Maxilla.* Setal formula: 2-4-11-6.

Thoracopods I-VII. Exopod segment number: 3-5-6-6-6-6-4. Setal formulae of endopods: Th I: 3+1/3+1/3+1/4(2), Th II:1+1/4+1/1+1/4(2), Th III: 1+1/4+1/1+1/4(2), Th IV:1+1/4+1/0+1/4(2), Th V: 1+1/3+1/0+1/4(2), Th VI: 1+1/3+1/0+1/4(2), Th VII: 1+1/2+1/0+1/4(2). *Thoracopod VIII female* (Fig. 3.14F) tiny, elongate, separated, 2.6x as long as wide.

Uropod sympod bearing eighteen spines. *Furcal rami* with fifteen spines on inner margin.

Variability

In one male paratype the following variation was observed: length 1.4 mm, number of exopodal segments of thoracopods I-VII: 3-5-6-6-6-5-4; the uropodal sympod with 17 spines; furcal rami with ten small spines.

Remarks

Arkaroolabathynella spriggi sp. nov. is most similar to *A. remkoi* sp. nov. (same number of spines on the distal endite of maxillule and sympod), however, it has numerous differences from its congeners such as the number of teeth on the labrum (17), setation of the antennae (0/0+0/1+0/1+1/4(1)), maxilla (2-3-11-6) and thoracopodal endopods. It also has the highest number of spines on the furcal rami (14 for the male, 15 for the female paratype).

Relationships

It is the sister species to *A. remkoi* sp. nov., however the lowest *COI* sequence divergence is with *A.robusta* sp. nov. (13.4%). The K2P *COI* divergence with *A.remkoi* sp. nov. is 14.6% and with *A. bispinosa* sp. nov. is 28.9%. There is no *18S* sequence data available for this species.

Etymology

Named for the Sprigg family who own the property from which the species was collected.

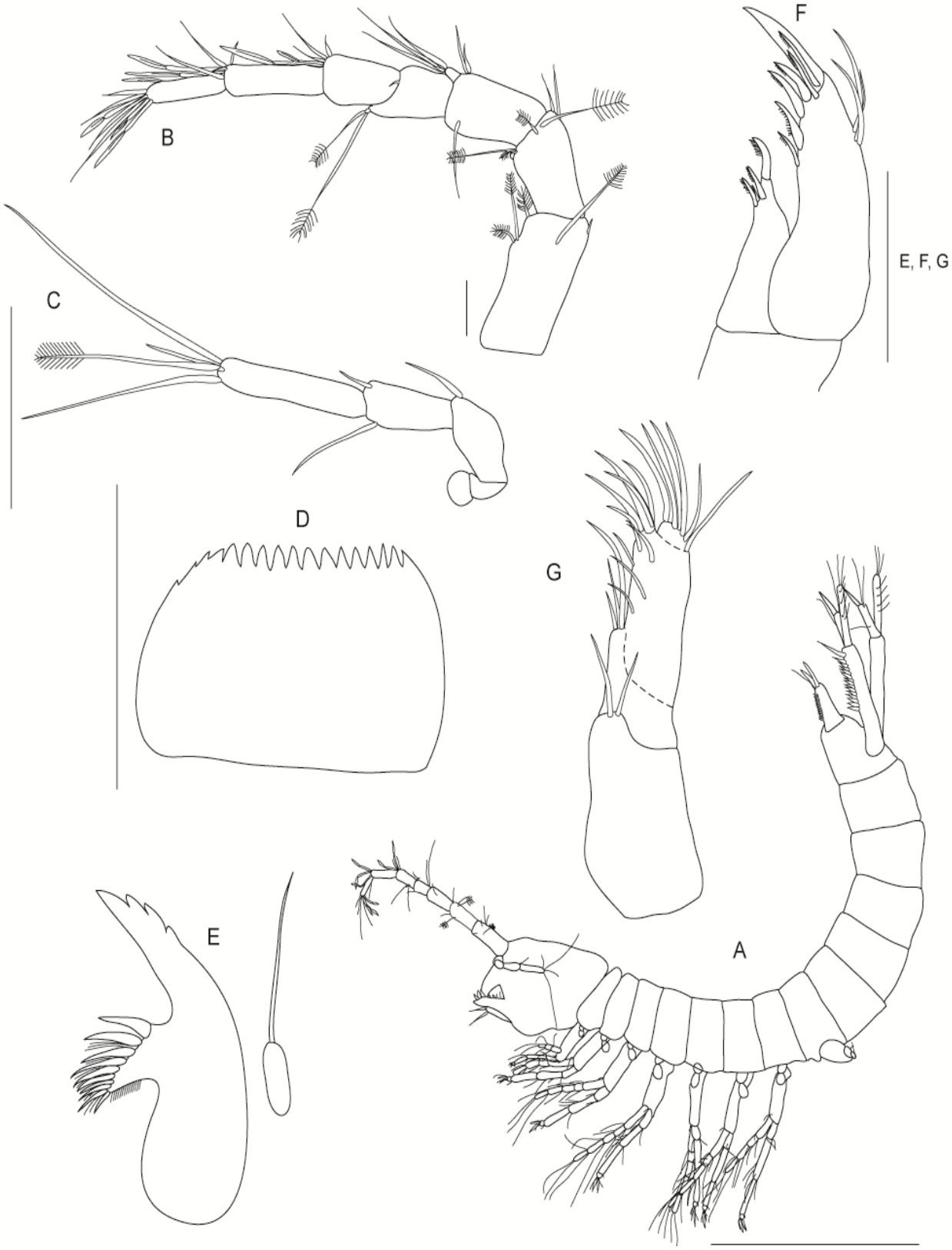

Fig. 3.12. *Arkaroolabathynella spriggi* sp. nov. (male: holotype). (A) general habitus; (B) left antennule; (C) left antenna; (D) labrum; (E) left mandible; (F) left maxillule; (G) left maxilla. Scale bars: A = 0.5mm; B - G = 0.1mm.

Fig. 3.13. *Arkaroolabathynella spriggi* sp. nov. (male, holotype): A - E, thoracopods I - V. Scale bars: A - E = 0.1mm.

Fig. 3.14. *Arkaroolabathynella spriggi* sp. nov. (male: holotype; female: allotype). (A) left thoracopod VI, male; (B) left thoracopod VII, male; (C) thoracopod VIII, male; (D) sympod, male; (E) pleotelson, male; (F) thoracopod VIII, female. Scale bars: A - D, F = 0.1mm; E =0.2mm.

Discussion

Recent surveys of stygofauna of South Australia have uncovered a wealth of unknown stygofaunal diversity, including parabathynellids, but most groups remain unstudied (R. Leys, pers. comm). The results indicate that there are at least four parabathynellid genera present in South Australia, including *Arkaroolabathynella* gen. nov. The other genera include a putative new species of *"Hexabathynella"* (J-L. Cho pers. comm.), a putative new species of *"Atopobathynella"* and an unknown lineage which could not be assigned to any described genera (Lineage A, Fig. 3.1).

Molecular and morphological analyses have revealed a monophyletic lineage comprising the first parabathynellid genus, *Arkaroolabathynella* gen. nov. to be described from the semi-arid Flinders Ranges. Although the molecular data are supported by morphological characters, there are no definitive apomorphic characters for this genus, instead there is a suite of synapomorphies, as has been found for other parabathynellid genera from Australia (see Ch. II; Cho 2005; Cho *et al.* 2005; Camacho and Hancock 2010; Cho and Humphreys, 2010). The lack of defining characters makes the recognition of new taxa particularly difficult and could lead to significant underestimation of parabathynellid diversity, if assessed using morphological criteria alone. Additionally, most of the defining suites of synapomorphies for parabathynellid genera overlap so extensively that it is extremely difficult, if not impossible, to assess intergeneric relationships based on morphology (see Table 3.2, Ch. II). In such situations, as demonstrated here, molecular data are essential for assessing phylogenetic relationships and species diversity.

One of the most important structures studied in parabathynellid systematics is the male thoracopod VIII. Although it is beyond the scope of the current study to comprehensively assess the usefulness of characters of thoracopod VIII for phylogenetic reconstructions, it is clear that taxa with similar-shaped thoracopod VIIIs grouped together in the molecular phylogeny. For example, all of the genera (possibly with the exception of lineages A-C which are morphologically very simplified) in clade 3 share a similarly-shaped, male thoracopod VIII, which is large and roughly rectangular in shape. Likewise, the other clades have distinctively-shaped male thoracopod VIIIs, with clade 2 containing genera ("*Atopobathynella", Kimberleybathynella*) with characteristically, unusually small, semi-circular male thoracopod VIIIs. Clade 1 contains genera that also have distinctive male thoracopod VIIIs but they are quite different to each other structurally (*"Hexabathynella"* has a characteristically elongated endopod (Cho and Schminke, 2006) while *"Chilibathynella"* has a characteristically "balloon-shaped" protopod (Reddy, 2006)). At present, no conclusions can be drawn regarding the usefulness of this character system as too few taxa have been

examined. However, further study including a broader sampling of taxa on a world-wide basis would be worthwhile.

Overall, the morphological analysis here suggests that the commonly used characters are useful for recognizing parabathynellid genera but are not as useful for elucidating phylogenetic relationships (see Ch. II), therefore a combined morphological and molecular approach is advocated here for all future studies of parabathynellid systematics.

Genetic diversity amongst species

To date, there is not enough genetic information available to comprehensively discuss genetic diversity within the Australian fauna, but for the few cases where sequence data are available for congeneric species, sequence divergence ranges between 4.4 – 13 % K2P (12 species of *Brevisomabathynella*) and 9.1% –19.7% (7 species of *"Atopobathynella")* for *COI*. In comparison, the divergence amongst *Arkaroolabathynella* gen. nov. species ranges between ~13 – 29%. Given that in Ch. II an average *COI* sequence divergence among genera from 16% to 27.9% was observed, *A. bispinosa* sp. nov. is highly divergent from its congeners However, because it s part of the same clade and shares numerous morphological characters with them, it is appropriate at this stage to place it in this genus.

Diversity of South Australian parabathynellids

The Flinders Ranges region has been noted for its antiquity and multi-layered geological history (Twidale and Bourne 1996), as indicated by the famous Ediacaran fossils dating from 600 million years ago (Sprigg, 1947). At present, the region contains a diverse range of plant and animal communities, believed to be a reflection of a frequently changing climate, and tectonic instability, which have potentially created numerous biogeographic barriers leading to speciation. Interestingly, *Arkaroolabathynella* gen. nov. is more closely related to taxa from New South Wales (Peel and Hunter Rivers) than to species from other regions of South Australia (i.e. *"Hexabathynella"* from Port Kenny, northern Eyre Peninsula, *"Atopobathynella"* sp. 1 from Uley and Lineage A from Wanila, southern end of the Eyre Peninsula). This study has revealed the presence of four deeply divergent species comprising the new genus which have only been collected from their type localities despite moderately thorough and repeated sampling throughout the Flinders Ranges area. The two geographically closest species (*A. spriggi* sp. nov. and *A. remkoi* sp. nov.) are resolved as sister species and, interestingly, are more closely related to the most geographically distant species, *A. robusta* sp. nov., than to *A. bispinosa* sp. nov., which is significantly more divergent from the other species. The presence of four distinct parabathynellid species in addition to multiple distinct lineages of bathynellids (K. Abrams unpubl. data; R. Leys pers. comm.) apparently restricted

to individual sites, in this relatively small region (~390 km, Brandle (2001)) hints at a complex and perhaps highly fragmented subterranean environment.

The other South Australian parabathynellids have affinities with the western side of the continent. These taxa are from a distinct biogeographical region known as the Eyre and York Block (Anon., 2005). Although the geographically closest taxa, (*"Atopobathynella"* sp. 1 and Lineage A), are phylogenetically very distant, both have closer links to Western Australia. For example, *"Atopobathynella"* sp. 1 is more closely related to congeneric Western Australian species and Lineage A is more closely related to Western Australian species from the unknown lineages B and C, and *Brevisomabathynella* (Fig. 3.1). This pattern of apparent wide disjunction between SA and WA taxa is congruent with the distribution of numerous Australian plants (see Conran and Lowrie (2007). Further, close phylogenetic relationships between amphipods from SA and WA have also been reported (Bradford *et al.*, 2010; King *et al.*, in press). The areas in which many parabathynellids are distributed (i.e. Flinders Ranges, Eyre Peninsula, Kimberley, Yilgarn and Pilbara) are all associated mainly with underlying Precambrian shield bedrock and sufficiently locally elevated to have avoided extensive marine incursions over the last 190 MYA (Conran and Lowrie, 2007). These areas are considered to be long-term refugia for many plant and animal species during past marine incursions and/or unfavourable climatic conditions (Tyler, 1985; Boardman, 1986; Gell and Bickford, 1996). The relationships of *"Atopobathynella"* sp. 1 and Lineage A with WA taxa potentially indicate that each of the clades to which they belong, descended from a widespread common ancestral population which became fragmented during past marine incursions and the disjunct distribution observed may be representative of vicariant remnant populations which survived and speciated in ancient, stable areas whilst their intervening habitat was inundated. Although the origins of parabathynellids and the timing of their colonization of Australia are unknown, their presence and diversity in ancient Australian landscapes such as the Flinders Ranges and Yilgarn region of Western Australia indicates that they have been in Australia for a significant period of time.

Restricted distributions

As discussed above, *Arkaroolabathynella* gen. nov. appears to be restricted to the Flinders Ranges area with individual species recorded only from single point localities. This restricted distribution is indicative of short-range endemism (Harvey, 2002), which is common to many other stygofauna (Humphreys, 2008; Guzik *et al.*, 2011 review), and is therefore of particular conservation significance. Other parabathynellid genera with restricted distributions include *Kimberleybathynella*, *Brevisomabathynella* and *Billibathynella*, which are only known from the Kimberley, Pilbara and Yilgarn regions of WA, respectively, and similarly comprise species mostly recorded from single aquifer systems (Cho, 2005; Cho *et al.*, 2005; Cho *et al.*,

2006b). Given the highly restricted distributions of Australian parabathynellids, it is likely that, in general, they are highly endemic and unlikely to be broadly distributed across multiple continents. Sequencing a wide sampling of species (including the types species) of cosmopolitan genera, such as *Hexabathynella*, *Atopobathynella*, and *Chilibathynella*, is required to test whether such genera are indeed monophyletic. However, given the common pattern of short range endemicity among parabathynellids it is likely that generic level taxa will be restricted to single continents, and that the current intercontinental congeners for such genera are likely not to be closely related.

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Appendix 3.1. Checklist of Australian parabathynellids

***Arkaroolabathynella* gen. nov.**

1. *A. bispinosa* sp. nov. Type locality: Werta Spring, Gum Creek, South Australia, Australia.Type depository: SAM, Australia.
2. *A. remkoi* sp. nov. Type locality: Grindell's Hut, Flinders Ranges, South Australia. Type depository: SAM, Australia.
3. *A. robusta sp*. nov. Type locality: Gum Creek, Yaltipena Spring, South Australia. Type depository: SAM, Australia.
4. *A. spriggi* sp. nov. Type locality: Bollabollana Spring, Flinders Ranges, South Australia. Type depository: SAM, Australia.

Atopobathynella Schminke, 1973. Syn.: *Parabathynella* Chappuis, 1926 partim.

5. *A. chelifera* Schminke, 1973. Type locality: Kiewa River, near Gundowing, Victoria, Australia. Type depository: personal collection of Schminke, Oldenburg.
6. *A. compagana* Schminke, 1973. Type locality: Dennick, Victoria, Australia. Type depository: personal collection of Schminke, Oldenburg.
7. *A. gascoyneensis* Cho, Humphries and Lee, 2006. Type locality: Gascoyne River terrace, Carnavon, Western Australia. Type depository: WAM, Australia.
8. *A. glenayleensis* Cho, Humphries and Lee, 2006. Type locality: Glenayle Station, Western Australia. Type depository: WAM, Australia.
9. *A. hinzeae* Cho, Humphries and Lee, 2006. Type locality: Depot Springs Station, Pilbara, Western Australia, Australia. Type depository: WAM, Australia.
10. *A. hospitalis* Schminke, 1973. Type locality: St Patricks River, near Targa, Tasmania. Type depository: personal collection of Schminke, Oldenburg.
11. *A. readi* Cho, Humphries and Lee, 2006. Type locality: Newhaven Station, Northern Territory. Type depository: WAM, Australia.
12. *A. schminkei* Cho, Humphries and Lee, 2006. Type locality: Fortescue River, Pilbara, Western Australia. Type depository: WAM, Australia.
13. *A. wattsi* Cho, Humphries and Lee, 2006. Type locality: Millbillillie Station, Western Australia. Type depository: WAM, Australia.

Brevisomabathynella Cho, Park and Reddy, 2006.

14. *B. changjini* Cho and Humphreys 2009. Type locality: Lorna Glen Station Bore Site 42, Carnegie Palaeodrainage,Western Australia. Type depository: WAM Australia.
15. *B. clayi* Cho and Humphreys 2009. Type locality: drainage between Lake Violet and Uramurdah calcretes, MEB site 266, Millbillillie Station, Carey Palaeodrainage, Western Australia. Type depository: WAM, Australia.
16. *B. cooperi* Cho, Park and Reddy, 2006. Type locality: Jundee Station (Carnegie drainage), JSP 1 South Hill Well BF, Jundee Mine, Gascoyne, Western Australia. Type depository: WAM, Australia.
17. *B. cunyuensis* Cho, Park and Reddy, 2006. Type locality: State Barrier Fence (Nabberu drainage), Cunyu Station, Western Australia. Type depository: WAM, Australia.
18. *B. eberhardi* Cho and Humphreys 2009. Type locality: Paroo Station, GSWA Bore #20(A), Western Australia. Type depository: WAM, Australia.
19. *B. hahni* Cho and Humphreys 2009. Type locality: Millstream Aquifer, piezometer 15B, Pibara, Western Australia. Type depository: WAM, Australia.
20. *B. jundeensis* Cho and Humphreys 2009. Type locality: South Hill Well BF, Jundee Mine, JSP 10, Jundee Station, Gascoyne, Western Australia. Type depository: WAM, Australia.

21. *B. leijsi* Cho and Humphreys 2009. Type locality: SBF calcrete, MEB site 36, Cunyu Station, Nabberu Palaeodrainage,Western Australia. Type depository: WAM, Australia.
22. *B. magna* Cho and Humphreys 2009. Type locality: Cunyu Station Site 272 Sweetwaters Well, Nabberu Palaeodrainage,Western Australia. Type depository: WAM, Australia.
23. *B. parooensis* Cho and Humphreys 2009. Type locality: Paroo Station GSWA 15 South, Carey Palaeodrainage, Western Australia. Type depository: WAM, Australia.
24. *B. pilbaraensis* Cho and Humphreys 2009. Type locality: Ethel Creek, Bore W230, Pilbara, Western Australia. Type depository: WAM, Australia.
25. *B. uramurdahensis* Cho and Humphreys 2009. Type locality: Uramurdah Lake, MEB site 264, Millbillillie Station, Carey Palaeodrainage, Western Australia. Type depository: WAM, Australia.

Billibathynella Cho, 2005.

26. *B. humphreysi* Cho 2005. Type locality: Gascoyne, Mt Padbury Station, Western Australia. Type depository: WAM, Australia.
27. *B. cassidis* Hong and Cho 2009. Type locality: Bore W6, Ethel Creek, Pilbara, Western Australia. Type depository: WAM, Australia.
28. *B. ilgarariensis* Hong and Cho 2009. Type locality: Ilgarari Creek-Yanneri Well, Bulloo Downs Station, Gascoyne, Western Australia. Type depository: WAM, Australia.
29. *B. wolframnoodti* Hong and Cho 2009. Type locality: Esso seismic uphole site 189, Neds Creek Station, Gascoyne, Western Australia. Type depository: WAM, Australia.

Chilibathynella Noodt, 1963.

30. *C. australiensis* Schminke, 1973. Type locality: Tambo River, Battle Point, Victoria. Type depository: personal collection of Schminke, Oldenburg.
31. *C. digitus* Camacho and Hancock 2012. Type locality: Department of Environment, Climate Change and Water (DECCW) groundwater monitoring bore number 30168, in the alluvial aquifer of the Peel River floodplain, Tamworth, NSW. Type depository: Museo Nacional de Ciencias Naturales, Madrid (MNCN).
32. *C. joshuai* Camacho and Hancock 2012. Type locality: Department of Environment, Climate Change and Water (DECCW) groundwater monitoring bore number 36442 in the alluvial aquifer beneath the Maquarie River floodplain, near Dubbo, NSW. Type depository: Museo Nacional de Ciencias Naturales, Madrid (MNCN).

Hexabathynella Schminke, 1972. Syn.: *Parabathynella* Chappuis, 1926 partim.

33. *H. decora* Schminke, 1973. Type locality: Hawkesbury River, near Windsor, NSW. Type depository: personal collection of Schminke, Oldenburg.
34. *H. halophila* Schminke, 1972. Type locality: Coledale beach, near Stanwell Park, NSW. Type depository: personal collection of Schminke, Oldenburg.

Kimberleybathynella Cho, Park and Humphreys, 2005.

35. *K. argylensis* Cho, Park and Humphreys, 2005. Type locality: Argyle Diamond Mine, Kimberley, Western Australia. Type depository: WAM, Australia.

36. *K. gigantea* Cho, Park and Humphreys, 2005. Type locality: Weber Plains, East Kimberley, Western Australia. Type depository: WAM, Australia.
37. *K. hexapoda* Cho, Park and Humphreys, 2005. Type locality: Weber Plains, East Kimberley, Western Australia. Type depository: WAM, Australia.
38. *K. kimberleyensis* Cho, Park and Humphreys, 2005. Type locality: Weber Plains, East Kimberley, Western Australia. Type depository: WAM, Australia.
39. *K. mandorana* Cho, Park and Humphreys, 2005. Type locality: Mandora, Western Australia. Type depository: WAM, Australia.
40. *K. pleochaeta* Cho, Park and Humphreys, 2005. Type locality: Argyle Diamond Mine, Kimberley, Western Australia. Type depository: WAM, Australia.

Notobathynella Schminke, 1973.

41. *N. remota* Schminke, 1973. Type locality: Gwydir River, near Yarrowyk, New South Wales. Type depository: personal collection of Schminke, Oldenburg.
42. *N. tasmaniana* Morimoto, 1978. Type locality: Exit Cave, Ida Bay Caves, near southeastern corner of Tasmania. Type depository: National Science Museum (Natural History), Tokyo.
43. *N. williamsi* Schminke, 1973. Type locality: Blackall Creek, Avon River, between Stratford and Llowalong, Victoria. Type depository: personal collection of Schminke, Oldenburg.
44. *N. octomura* Camacho and Hancock 2011. Type locality: Department of Natural Resources monitoring piezometer number 13700234A, near Bundaberg, Queensland. Type depository: Museo Nacional de Ciencias Naturales, Madrid (MNCN).
45. *N. pentatrichion* Camacho and Hancock 2011. Type locality: Department of Natural Resources monitoring piezometer number 13700234A, near Bundaberg, Queensland. Type depository: Museo Nacional de Ciencias Naturales, Madrid.

Octobathynella Camacho and Hancock 2010.

46. *O. peelensis* Camacho and Hancock 2010. Type locality: Tamworth, New South Wales. Type depository: Museo Nacional de Ciencias Naturales, Madrid (MNCN).

Onychobathynella Camacho and Hancock 2012

47. *O. bifurcata* Camacho and Hancock 2012. Type locality: hyporheic zone of the Hunter River at Aberdeen, NSW. Bore number 5P141. Type depository: Museo Nacional de Ciencias Naturales, Madrid (MNCN).

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CHAPTER IV

ANCIENT DIVERGENCES IN LONG EMERGENT LANDSCAPES: THE BIOGEOGRAPHICAL HISTORY OF AUSTRALIAN SUBTERRANEAN PARABATHYNELLIDAE

CHAPTER IV

Preamble

This chapter is based on an expanded dataset including that of Chapter II, but has a focus on the biogeography of parabathynellids throughout Australia, rather than the phylogenetic relationships amongst genera. This Chapter was set out as a separate manuscript to allow in depth explanations of the biogeographic relationships amongst taxa and determine significant regions for parabathynellid diversity. Additionally, molecular dating and ancestral area reconstruction analyses were included to explore the observed biogeographic patterns.

Abstract

Australian subterranean groundwater dependent ecosystems (GDEs) and their associated fauna may provide a valuable model system which can be used to understand evolutionary processes, speciation and biogeography. Molecular phylogenetic and ancestral area reconstruction approaches, using sequence data from the mtDNA *COI* and nDNA *18S* rRNA genes, were used to investigate the evolutionary relationships and biogeography of extant parabathynellids (Bathynellacea: Syncarida) from subterranean GDEs in Australia. Evidence for significant regional biogeographic structuring of parabathynellids at the genus and species levels was found, indicating a long and complex evolutionary history for these animals in Australia, likely shaped by fluctuating climates throughout the continent's history. This study proposes that parabathynellids represent an ideal indicator group that can be used to identify potentially significant biogeographic regions for other subterranean aquatic taxa.

Introduction

Approximately 70% percent of Australia is arid or semi-arid (Byrne *et al.*, 2008). However, the continent is known to contain a remarkable array of aquatic habitats, many of which are groundwater dependent ecosystems (GDEs) (Boulton *et al.*, 2003). Known Australian GDEs include cave and karst systems in eastern Australia (Thurgate *et al.*, 2001a., 2001b), freshwater springs in Queensland and South Australia (Worthington Wilmer and Wilcox, 2007; Murphy *et al.*, 2010) aquifers (Humphreys, 2006; Guzik *et al.*, 2011a), and a number of well recognised diversity hotspots reviewed by Humphreys (2008) and Guzik *et al.* (2011a). Additional Australian areas likely to contain high subterranean biodiversity, based on preliminary sampling of subterranean invertebrates, include the Flinders Ranges, South Australia (SA) (Moulds *et al.*, 2007; Guzik *et al.* 2011a; R. Leys pers.comm.; Ch. III) and alluvial aquifers associated with the Hunter and Peel Rivers of NSW (Tomlinson *et al.*, 2007; Hancock and Boulton, 2008). Subterranean GDEs are of particular interest for the complex fauna that they contain. Due to the unusual morphological and ecological adaptations

(detailed in Ch. I) exhibited by subterranean fauna and the truncated nature of subterranean ecosystems, these environments have the potential to serve as natural ecological and evolutionary laboratories (Poulson and White, 1969; Juan *et al.*, 2010). Further, subterranean taxa are potentially geographic or phyletic relicts, i.e. they may be the remnants of populations that have become restricted to subterranean habitats at different times due to climatic change, vicariance or speciation (Humphreys, 2006). These relictual fauna are of great scientific significance because they can be used to test hypotheses about the earth's past geological history and processes of evolution and speciation (Humphreys, 2006).

Biogeographic studies help to identify geographic barriers, species distributions and evolution across landscapes, and can provide information as to why particular areas have higher levels of endemism than others (Avise *et al.*, 1987; Avise, 2000; Riddle and Haffner, 2006; Beheregaray, 2008; Avise, 2009). Further, phylogeography (the study of the geographical distribution of genealogical lineages (Avise, 2000)) has widely been implemented to identify evolutionarily important areas and assess conservation priorities (Moritz and Faith, 1998; Harvey *et al.*, 2011). Biogeographic studies can also be used to understand the distribution and connections among GDEs. This understanding is required to provide a framework for ecological studies and to develop and manage these regions sustainably (Bowman *et al.*, 2010). At a regional scale, numerous phylogeographic studies of subterranean aquatic fauna (termed stygofauna) have helped to identify patterns of evolution among species to answer questions at a local scale (e.g. amongst calcretes and/or alluvial aquifers in a range of groups such as amphipod (Finston and Johnson, 2004; Cooper *et al.*, 2007) and isopod crustaceans (Cooper *et al.*, 2008; Finston *et al.*, 2009), and stygobiont beetles (Cooper *et al.*, 2002; Leys *et al.*, 2003; Guzik *et al.*, 2011b) . These regional studies have led to the development of novel hypotheses such as the subterranean island hypothesis (Cooper *et al.*, 2002, 2007), which postulates that in arid regions, aquifers are equivalent to closed island habitats which may have been isolated for millennia. This contrasts with the hypothesis developed for stygofauna from the Pilbara region in Western Australia, which postulates that the stygobiont fauna has become isolated in headwater tributaries as a result of reduced river flow and salinisation resulting from continental onset of aridity (Humphreys, 2001b; Finston *et al.*, 2007).

At a continental scale, within Australia, there is a significant lack of coverage in biogeographic studies for freshwater fauna (Murphy and Austin, 2005), let alone for subterranean aquatic (stygobiont) fauna in GDEs. The most comprehensive, freshwater, biogeographic study is that of Unmack (2001), which defined biogeographic regions of Australia based on data from freshwater fish. This model could be used to test the biogeographic diversity of subterranean GDEs, however, previous studies of surface

freshwater crustaceans (Austin *et al.*, 2003; Nguyen *et al.*, 2004) revealed incongruent results compared to those of Unmack's regions. Given that known stygobiont GDE hotspots are situated in different areas to the diverse epigean regions defined by Unmack (2001), and that epigean and subterranean taxa are likely to have different life histories and dispersal capabilities, it is likely that Unmack's study is not particularly applicable to stygobiont biogeography. Instead, regions of high diversity and endemicity for stygobiont parabathynellids in Australia are identified and the evolutionary links between these regions are investigated.

Parabathynellids are an ideal study taxon that can be used to help identify Australian stygobiont GDE regions because they are a significant component of freshwater stygofauna (subterranean and aquatic fauna), and are likely to have been present in Australia for a long time, given their high diversity and the deep divergences observed among genera (see Chapter II). Although parabathynellids have a world-wide distribution, excluding the polar regions, the majority of taxa have only been identified from single locations. Given the small size of parabathynellids (typically 1-3 mm) and their restriction to interstitial groundwater habitats, there has been much speculation about how they achieved a global distribution (Schminke, 1974; Camacho *et al.*, 2006; Cho and Schminke, 2006), in particular, because these taxa are considered to have weak dispersal abilities (Schminke, 1974; Camacho and Valdecasas, 2008). Many researchers support the hypothesis that parabathynellids reached a global distribution before the breakup of Pangaea and that their present biogeography can be explained by vicariance and secondary dispersal (Schminke, 1981b; Cho *et al.*, 2006a). A lack of bathynellacean fossils and extant epigean ancestors has made understanding their present distribution particularly challenging (Camacho and Valdecasas, 2008). Although compelling, the broad questions of parabathynellid origins *into* freshwater systems is beyond the scope of the current study. Rather, it investigates the more recent geographic distribution and evolutionary relationships of extant parabathynellids *between* subterranean GDEs in Australia.

Recent molecular studies of parabathynellid phylogenetics and phylogeography (Guzik *et al.*, 2008; see Ch. II) have provided evidence of multiple cases of endemic genera, restricted to particular regions of Australia (e.g. Yilgarn Region, Western Australia), which are geologically isolated . The high levels of endemism in parabathynellids, considered to be the product of poor dispersal capabilities (Schminke, 1981a; Schram, 2008) and the complex geological history of Australia, suggest that parabathynellids have the potential to serve as valuable indicators of subteranean biodiversity throughout Australia.

Here, a phylogenetic approach is used to examine levels of diversity within and between key groundwater regions of Australia and assess the history of connectivity between these regions for parabathynellids. These data are used to identify regions of high parabathynellid diversity and endemicity, and comment on whether past climatic events may have influenced the distinctiveness of the regional fauna.

Methods

Sampling

Eleven Bathynellidae and 48 Parabathynellidae specimens were obtained from various localities throughout Australia (Fig. 4.1). Samples were collected from four (i.e. Kimberley, Pilbara, Yilgarn regions of Western Australia and south-east of South Australia (only bathynellids were collected from the latter region) of the five biodiversity hotspot regions for subterranean fauna, as identified by Guzik *et al.* (2011a). In addition, samples from the Eyre Peninsula, Flinders Ranges and alluvial aquifers associated with the Hunter, Macquarie and Peel Rivers of New South Wales were included, as these regions have been suggested as further potential subterranean faunal hotspots by Guzik *et al.* (2011a).

Sampling consisted of a combination of netting and pumping (following the same regimes as Cooper *et al.*, (2007) and Hancock and Boulton, (2008)). The bathynellid samples represent undescribed species and the 48 parabathynellid samples represent eight known genera comprising 10 previously described species and one genus containing four new species, (treated in Chapter III). This dataset builds on that of Ch. II by including 10 new bathynellid and eleven new parabathynellid sequences. Locations of the majority of sampled individuals are listed in Ch. II and additional samples are listed in Table 4.1.

Table 4.1. List of additional taxa and their collection localities

Name	Latitudes	Longitudes	Region
Bathynellidae sp. 3	-30.32137	139.44052	Flinders Ranges, SA
Bathynellidae sp. 4	-30.35757	139.05407	Flinders Ranges, SA
Bathynellidae sp. 5	-31.42292	138.57402	Flinders Ranges, SA
Bathynellidae sp. 6	-27.0677	118.673	Yilgarn, WA
Bathynellidae sp. 7	-27.935	120.0839	Yilgarn, WA
Bathynellidae sp. 8	-35.2805	139.01086	Langhorn Creek, SA
Bathynellidae sp. 9	-34.0375	139.76578	Cadell, SA
Bathynellidae sp. 10	-37.54729	140.44706	Burra, SA
Bathynellidae sp. 11	-32.5289	151.0505	Hunter River, NSW
"Chilibathynella" sp. 3	-32.0492	150.9431	Hunter River, NSW
"Chilibathynella" sp. 4	-32.3148	148.6262	Macquarie River, NSW
Notobathynella sp. 1	-32.0064	150.5328	Hunter River, NSW
Kimberleybathynella sp. 2	-15.4645	128.8928	Kimberley, WA
Kimberleybathynella sp. 3	-15.8783	128.7321	Kimberley, WA
Kimberleybathynella sp.	-15.4645	128.8928	Kimberley, WA
Atopobathynella wattsi	-26.6845	120.2151	Yilgarn, WA
"Atopobathynella" sp. 6	-28.3970	122.2021	Yilgarn, WA
"*Atopobathynella"* sp. 7	-28.3911	122.2038	Yilgarn, WA
Arkaroolabathynella sp. 1	-30.4661	138.75962	Flinders Ranges, SA
Arkaroolabathynella sp. 2	-32.01318	139.82915	Crocker Well, SA
Billibathynella sp. 3	-25.1759	118.0614	Yilgarn, WA
Brevisomabathynella parooensis	-26.4004	119.7630	Yilgarn, WA

Fifty *COI* and 37 *18S* sequences were obtained from bathynellacean samples using the primers used previously and following the protocol detailed in Ch. II. 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 Geneious (Drummond *et al.*, 2011). Sequences were aligned using Clustal W (Thompson *et al.*, 1994) and checked by eye. It was not possible to obtain *18S* sequences for the genera *Notobathynella* and *Kimberleybathynella* and only one *18S* sequence was obtained from the Bathynellidae specimens. A sequence from *Anaspides tasmaniae* (Anaspidacea: Syncarida, Genbank accession L81948, Spears and Abele, 1997), a sister lineage to the Bathynellacea, was used as the outgroup.

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 and maximum likelihood (ML) approaches, using separate and combined datasets, implemented in the programs MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and RaxML v. 7.2.3 (Stamatakis *et al.*, 2008) respectively. Modeltest 3.7 (Posada and Buckley, 2004) was used to estimate the model which best fit the nucleotide data in combined and separate analyses, and the model selected by the Akaike Information Criterion was used in Bayesian 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 aforementioned models in an unlinked analysis which allowed the rates to vary over the partitions. Bayesian analyses were run using four chains for 9 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. Effective sample size (ESS) values for all parameters were observed 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 12,000 was chosen and a 50% majority-rule Bayesian consensus tree was constructed from the remaining 78,000 trees.

Maximum likelihood analyses used 100 rapid bootstrap inferences and the likelihood of the best tree was optimised and evaluated under a gamma model. Pairwise distances (Lefébure *et al.*, 2006) between sequences were estimated using the GTR+I+G model of evolution and branch lengths and parameters were estimated for the MrBayes consensus tree using PAUP*, with the optimality criterion set to maximum likelihood.

Molecular dating analyses

Evolutionary rate and divergence times were estimated using a Bayesian approach implemented in the program BEAST version 1.6.1 (Drummond and Rambaut, 2007). The time-scale of divergence of the parabathynellid phylogeny was investigated using a relaxed molecular clock and Uncorrelated Lognormal (UCLN) and exponential models. Due to a lack of fossil and geological data to calibrate the molecular clock, the invertebrate mtDNA clock rate of 0.0115 substitutions per site per million years (~2.3%) calculated by Brower (1994) was used. The combined dataset was analysed, and the GTR+I+G model of nucleotide substitution was implemented for *COI* and the HKY model was used for *18S* (this latter model was used as the model selected by MODELTEST was not available in BEAUTI), as suggested by MODELTEST 3.06. The Yule Prior was selected for the tree model and fixed for all partitions.

Two independent MCMC chains were run for 1 x 10^7 generations with sampling every 1000 generations with the default 10% burnin of the posterior samples. Tracer (version 1.5; Rambaut and Drummond 2009) was used to determine if the runs had reached convergence, based on effective samples sizes (ESS) above 300 and appropriate posterior probabilities, as suggested by the authors. To determine if the data fitted a molecular clock model, the coefficient of variation of rates and the rate of covariance were examined. Trees were annotated using Tree Annotator version 1.6.1 and viewed using Figtree version 1.2.2 (Rambaut, 2009).

Ancestral area reconstruction

Ancestral areas were inferred using a Bayesian approach to dispersal–vicariance analysis (Bayes-DIVA) (Ronquist, 1997; Nylander *et al.*, 2008) implemented in RASP 2.0 beta (formerly S-DIVA) (Yu *et al.*, 2010; Yu *et al.*, 2011). RASP uses the collection of trees from a Bayesian MCMC analysis and can handle optimization uncertainty in reconstructing biogeographic histories (Yu *et al.*, 2011). To account for phylogenetic uncertainty in the biogeographic analysis, 1000 trees from MrBayes' output of combined *COI* and *18S* data were used.

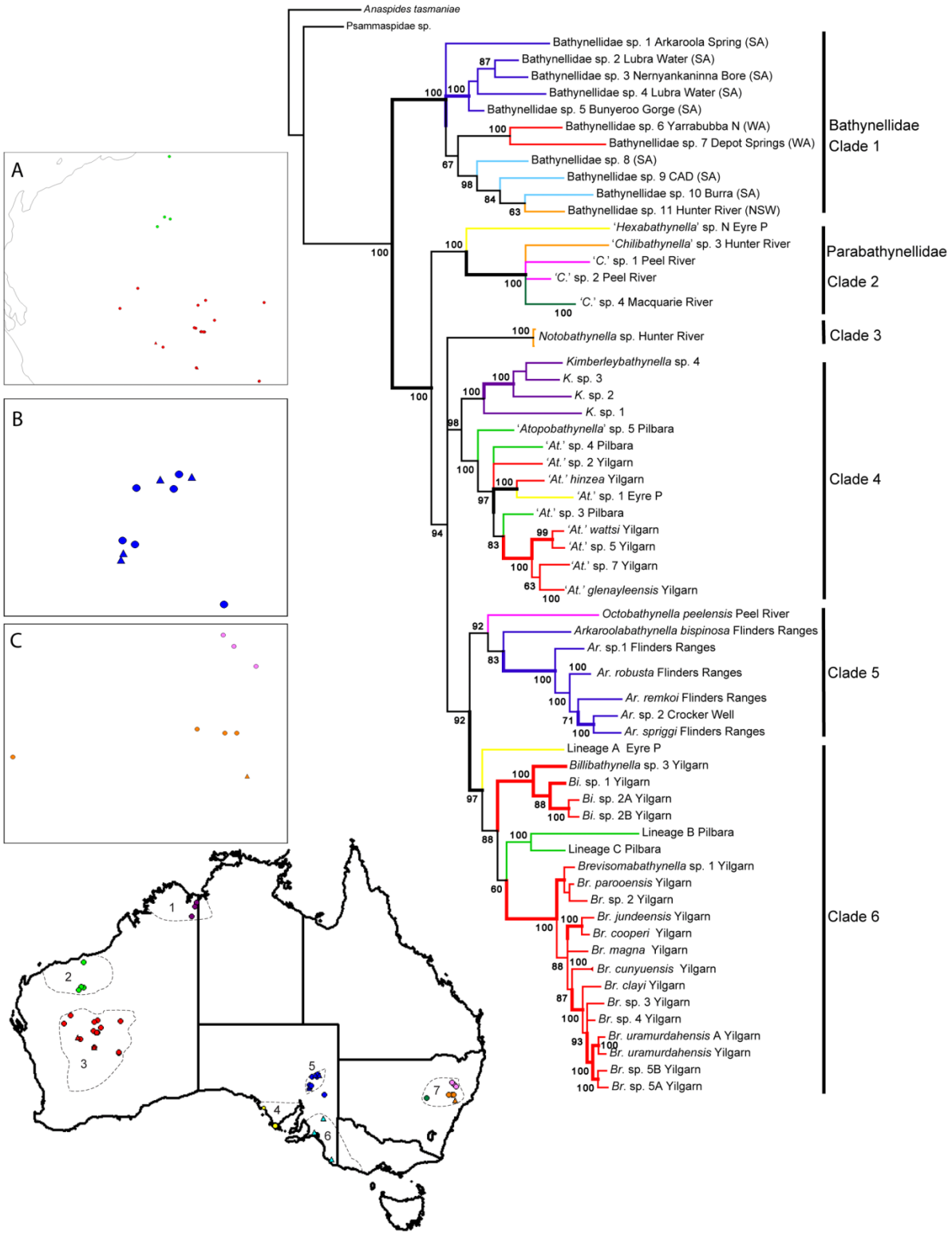

Fig. 4.1. Posterior probability (majority-rule) Bayesian consensus tree using *COI* and *18S* data with model partitioning, implemented in MR BAYES. Numbers on the nodes are Bayesian posterior probabilities and thicker lines represent nodes supported by Maximum likelihood bootstrap values greater than 50%. The map of Australia and inset maps show the collection site of each species. Inset map A corresponds to regions 2 and 3, B corresponds to region 5 and C corresponds to region 7. The numbers on the map represent regions discussed in the text, 1 – Kimberley, 2 – Pilbara, 3 – Yilgarn, 4 – Eyre Peninsula, 5 – Flinders Ranges, 6 – south-east, 7 – NSW alluvial aquifers. 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.

The number of cycles was set to 50,000 and the root distribution was set to wide (although the null option was also explored, data not shown). A variety of maximum areas (2, 7 and 8) was tested, but only the results using maxareas = 8 are shown as parabathynellids are found in all of these regions, although data for all regions was not obtained. All other options remained as default. Eight geographic areas are defined: A—Kimberley, B—Pilbara, C—Yilgarn, D—Eyre Peninsula, E—Flinders Ranges, F—south-east SA, G— New South Wales, H — Tasmania (see Fig. 4.3). These geographic boundaries are based on the current distribution of syncarid species.

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 (~706 bp) aligned without gaps to an *Anaspides tasmaniae* reference sequence so a secondary structure model was not required to aid the alignment. The *COI* sequences comprised 58% variable sites and 51% parsimony informative sites. In comparison, the more conserved *18S* data comprised 23% variable sites and 13% parsimony informative sites.

Phylogenetic analysis

Individual gene trees for *COI* and *18S* showed no major phylogenetic incongruence in their topologies, therefore, the datasets were combined for further phylogenetic analyses. BI and ML analyses produced well-resolved and well-supported trees (Fig. 4.1) and were almost identical in topology.

The phylogeny (Fig. 4.1) generated from the 1298 bp combined dataset was used to assess biogeographic relationships amongst taxa. The phylogenetic relationships and identification of species and monophyletic clades are discussed in Ch. II, so the relationships of the additional taxa only are included here are discussed. A total of 59 genetically distinct lineages were resolved, which includes 11 described species. Of the 11 additional specimens included, two from the Hunter and Macquarie Rivers grouped within *"Chilibathynella"* Noodt, 1963 (*"C."* sp. 3 and 4), two from the Kimberley Region grouped within *Kimberleybathynella* Cho *et al.*, 2005 (*K.* sp. 3 and 4), and one from the Flinders Ranges (*Ar*. sp. 1) and one (*Ar.* sp. 2) from Crocker Well, SA grouped within *Arkaroolabathynella* Abrams and King gen. nov. Of the additional specimens from Yilgarn calcretes, two unidentified specimens ("*At*." sp. 6 and 7) and one identified as *"Atopobathynella" wattsi* (Cho *et al.*, 2006a), from Yilgarn calcretes, grouped within *"Atopobathynella",* one specimen grouped within *Billibathynella* Cho, 2005 (*Bi.* sp. 3) and one unidentified and one specimen identified as *Brevisomabathynella parooensis* based on morphology (Cho and Humphreys, 2010), grouped within *Brevisomabathynella* Cho *et al*., 2006b.

Biogeography of bathynellaceans

The Bathynellacea is resolved into six major clades, each comprising multiple genera and lineages. Many of the genera are restricted to a particular region, corresponding to suggested subterranean hotspots. Clade 1 consists of a well-supported (100% BBP, 97 ML% bootstrap value) group of 11 lineages belonging to the family Bathynellidae, which can be further divided into three sub-clades. The first subclade consists of a single SA lineage, the second consists of four SA lineages and the third consists of two WA lineages, three SA lineages and one NSW lineage.

The remaining major clades (2-6) form a single monophyletic group comprising Parabathynellidae (100% BP/ 85% ML bootstrap value). Clade 2 consists of one species of *"Hexabathynella"* Schminke, 1972 from SA and four lineages of *"Chilibathynella"* from the Peel, Macquarie and Hunter Rivers, NSW (100% BPP, 99% ML bootstrap value). Clade 3 consists of a single lineage of *Notobathynella* Schminke, 1973 from the Hunter River, NSW. Clade 4 (98% BPP, 63% ML bootstrap value) contains nine lineages (including three described species) of *"Atopobathynella"* (100% BPP): one from Uley (southern Eyre Peninsula), SA and eight from WA (five from the Yilgarn and three from the Pilbara), in addition to four lineages of *Kimberleybathynella* from the Kimberley, WA (100%BPP, 67% ML bootstrap value). Clade 5 (although not robustly supported, 92% BPP) comprises five lineages (including four described species of *Arkaroolabathynella*) from the Flinders Ranges, SA, one lineage from a fractured rock aquifer, approximately 191km east of the Flinders Ranges and the monotypic species *Octobathynella peelensis* Camacho and Hancock, 2010 from the Peel River, NSW. Clade 6 (97% BPP) consists of Lineage A from Wanila, SA (northern Eyre Peninsula), three species of *Billibathynella* from the Yilgarn, WA, Lineages B and C from the Pilbara, WA and 14 species of *Brevisomabathynella* from the Yilgarn, WA. There was, however a difference in the placement of Lineage A in the BI vs ML trees; it is sister to *Billibathynella* + Lineage B + Lineage C + *Brevisomabathynella* (97% BPP) in the BI tree, but sister to *Brevisomabathynella* in the ML tree.

Regional biogeography: the calcretes of the Yilgarn Region, WA

In total, 24 species were observed from 13 calcretes. Yilgarn species were from clades 4 and 6, representing three described species of '*Atopobathynella'* ("*At." hinzea, "At." wattsi, "At." glenayleensis*), three unknown species of *Billibathynella* and eight previously described (*Br. clayi, Br. cooperi, Br. cunyuensis, Br. eberhardi, Br. jundeensis, Br. magna, Br. parooensis, Br. uramurdahensis*) and five putatively new species of *Brevisomabathynella.*

No species were shared among calcretes, suggesting that species were restricted to individual calcretes aquifers. There was little geographic structure in the phylogeny, as clades which

contained calcrete taxa (clades 4 and 6) included taxa from multiple calcretes spanning different palaeodrainages. For example, species from clade 4 were from five calcretes from four palaeodrainages: Yarabubba South (Murchison palaeodrainage), Depot Springs (Raeside palaeodrainage), Carnegie Downs (Burnside palaeodrainage), Hinkler Well and Laverton (both Carey palaeodrainage). All of these species belonged to *"Atopobathynella"* and can be separated into two sub-clades. According to the phylogeny, *"At."* sp. 6 and *"At."* sp. 2 are more closely related to each other and *"At."* sp. 1 from South Australia than to the other calcrete taxa. The species *"At." wattsi*, *"At."* sp. 7 and *"At."* sp. 8 from calcretes in the Carey palaeodrainage formed a well-supported (100% BPP, 89% bootstrap value) sub-clade with *"At." glenayleensis* from the Burnside palaeodrainage and the sister taxon to this clade being *"At."* sp. 3 from the Pilbara.

Additionally, the species from clade 6 were from eight calcretes spanning five palaeodrainages: Austin Downs (Murchison); Milgun and Moorarie (Gascoyne); Millbillillie, Lake Violet, Paroo and Uramurdah Lake (Carey); Cunyu (Nabberu); Jundee (Carnegie). The former two palaeodrainages drain to the Indian Ocean, while the latter three drain to the interior. The former three calcretes contained distinct species of *Billibathynella* and the latter calcretes contained distinct species of *Brevisomabathynella*. Although the support values for *Brevisomabathynella* interspecies' relationships were fairly low, it was observed that generally the species were not grouping by geographic location (i.e. nearby calcretes showing closer phylogenetic relationships). For example, the Cunyu species are not sister taxa nor are the Uramurdah species sister taxa, although there is one case of sister taxa, *Br. cooperi* and *Br. jundeensis* inhabiting the same calcrete. However, there is some evidence for species grouping by palaeodrainage channel; for example, two groups of species (*Br.* sp. 1, 2 and *Br. parooensis*; and *Br. clayi* to *Br*. sp. 5) from calcretes in the Carey palaeodrainage show close phylogenetic relationships, although the two groups are not most closely related to each other. "*Atopobathynella"* species were found in palaeodrainages on both sides of the drainage divide (the Western Shield, on which the Yilgarn is found, is divided north to south by a drainage divide that separates those catchments draining to the west and those draining inland to the east (Beard 1998).

The Pilbara and Kimberley regions, WA

Five distinct parabathynellid lineages from the Pilbara were observed, three of which are most likely new *"Atopobathynella"* species and two of which did not group with any recognized genera. Of the five basins in this region, sampling was limited to the De Grey (*"At."* sp. 5 and Lineage C) and Fortescue (*"At."* sp. 4 and Lineage B) basins. Coverage of the Kimberley region was also limited, but even this small sample has identified four deeply divergent (20.8-

31.7 % K2P *COI*) lineages, three of which are from sites North of Lake Argyle (*K*. sp. 2-4) and one from a site South of Lake Argyle (*K*. sp. 1).

The Flinders Ranges, Eyre Peninsula and south-east, SA

The Flinders Ranges contains five distinct parabathynellid species from *Arkaroolabathynella* (four of which are described) and five lineages of Bathynellidae. All species were from separate drainages except *Ar. spriggi* and Bathynellidae sp. 3, which shared the same drainage, but were collected from different sites. There is also one species of *Arkaroolabathynella* from Crocker Well, approximately 191 km south-east of the Flinders Ranges, which is the sister species to *Ar. spriggi*. The bathynellid lineages here form a monophyletic sub-group, to the exclusion of a second group consisting of bathynellids from the south-east region of SA (three distinct lineages), the Yilgarn region and NSW (Hunter River).

Alluvia associated with NSW rivers

Octobathynella peelensis and two new species of *"Chilibathynella"* were recorded from the Peel River. *Octobathynella* appears to be most closely related to *Arkaroolabathynella* from South Australia. One new species of *"Chilibathynella"*, a new species of *Notobathynella* and one unknown species of bathynellid from the Hunter River were also recorded. Additionally, a new species of *"Chilibathynella"* was collected from the Macquarie River. The *"Chilibathynella"* species are resolved, with high support, as sister to the *"Hexabathynella"* species from the northern Eyre Peninsula, SA. However, the position and closest relatives of *Notobathynella* are unresolved, while the Hunter River bathynellid is most closely related to SA taxa from south-east SA.

Molecular dating

The results of the molecular dating analysis are shown in Fig. 4.2. As the phylogeny has deeply divergent lineages and the *COI* data are likely to be affected by saturation, the estimated dates for the deeper nodes should be considered uncertain. However, dates from the more recent nodes (e.g. those within the *Brevisomabathynella* clade) are likely to be more accurate. Exponential and lognormal molecular clocks were used to explore the effect of model choice on the data, but only the results from the lognormal model are shown because the results were so similar.

Applying the invertebrate mtDNA clock rate gave divergence times of the root nodes for individual genera ranging from 19.9 – 56.52 MYA (95% HPD; lower: 14.85 MYA, upper: 71.08 MYA). Divergence times for the sympatric sister species pair *Br. cooperi* and *Br. jundeensis* (Jundee calcrete) was 6.79 MYA (95% highest posterior density (HPD); lower:

3.46 MYA; upper: 10.57 MYA). Estimated divergence dates for other nodes are shown in Fig 4.2.

Ancestral area reconstruction

The results of the ancestral state analysis using Bayesian inference methods are summarized in Fig. 4.3. Although a variety of maximum areas (2, 7 and 8) was tested, only the results for maxareas = 8 are shown as they are essentially identical in their reconstructions of the most likely ancestral area and its support. Additionally, the analysis was run with the root distribution set to 'null' and 'wide' (as suggested in the manual), but this caused no significant differences in the results.

Bayes-DIVA analysis reconstructed NSW (G) as the ancestral area for most of the nodes in the backbone of the tree, including node I, representing Parabathynellidae (95.6% marginal probability). NSW was also reconstructed as the most likely ancestral area for clades 2 (node II), 4 (node III) and 5 (node IV), although there was some lower support for other areas, particularly for clade 2 (Fig. 4.3). The analysis suggests the ancestral area for the only widespread genus, "*Atopobathynella"* (node V) is the Pilbara (98.15% marginal probability).

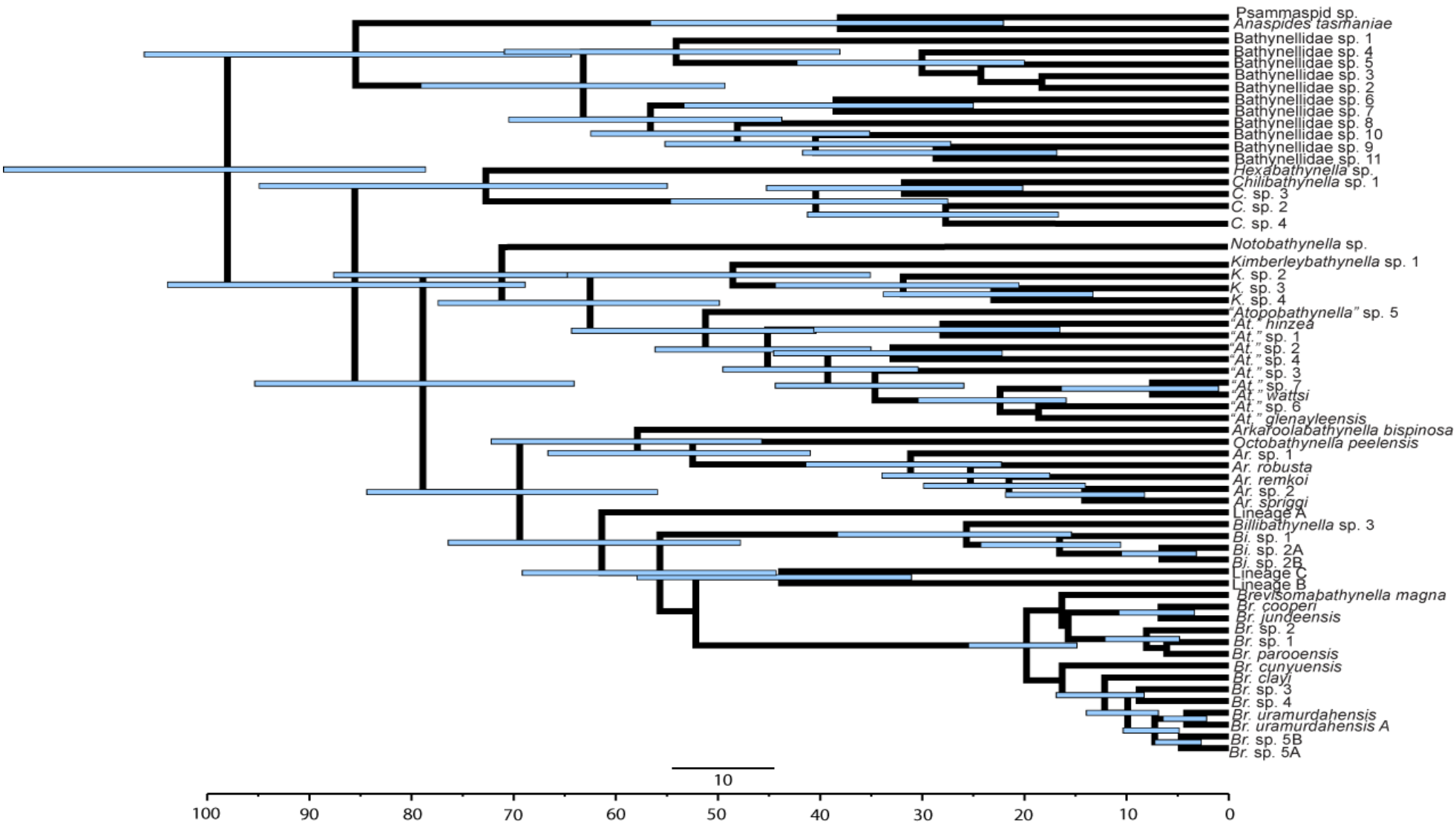

Fig. 4.2. Maximum credibility ultrametric tree for bathynellaceans inferred from a relaxed molecular clock using BEAST. Node bars are 95% confidence intervals. Scale bar is in millions of years ago (MYA), starting from the present (0 MYA) at the tips of the nodes.

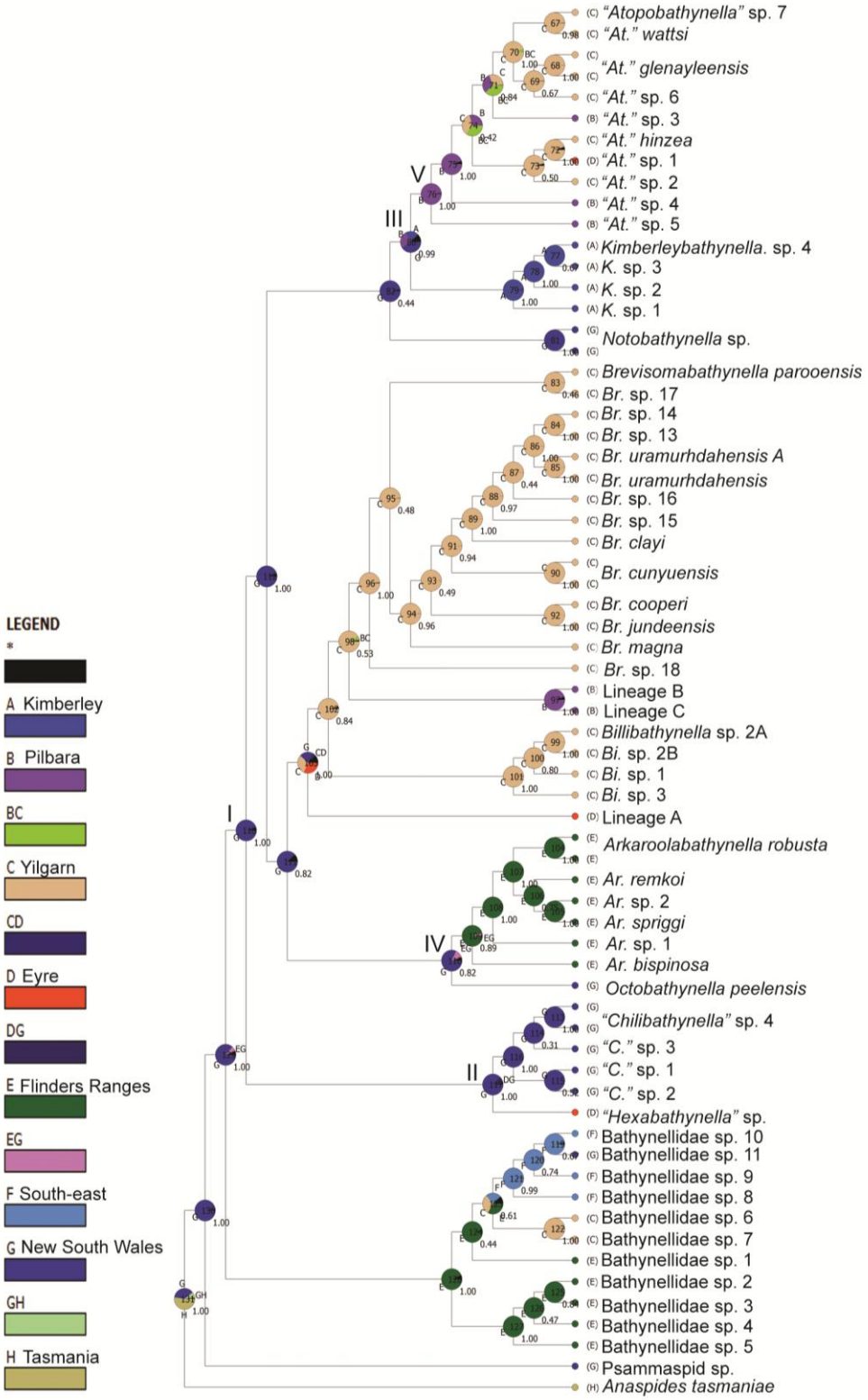

Fig. 4.3. Graphical results of ancestral distributions reconstructed at each node of the phylogeny of Bathynellacea using Bayesian MCMC analysis, implemented in RASP. Pie charts at each node show probabilities of alternative ancestral ranges. Numbers I – V represent nodes discussed in the text, letters beside nodes represent areas shown in the colour key and small numbers represent posterior probability support. Colour key displays possible ancestral ranges at different nodes; black with an asterisk represents all other possible ancestral ranges. A – Kimberley, B –Pilbara, C – Yilgarn, D – Eyre Peninsula, E – Flinders Ranges, F – south-east, G – New South Wales, H –Tasmania.

Discussion

Phylogenetic analyses of bathynellaceans indicate that there is significant biogeographic structuring at multiple levels. Overall, the data suggest that Australia's parabathynellids are highly diverse, with each region containing a unique and distinct parabathynellid fauna. Further, the data show numerous incidences of relationships between disjunct regions. Below, connections between regions inferred from the relationships among bathynellacean taxa and the diversity and distinctiveness of each region are discussed. As only three bathynellids were collected from the south-east SA region, there is not enough information to comment on whether it may be a significant area for bathynellaceans, therefore this region and group are not discussed further.

A brief palaeoclimatic history of Australia

Australia's geological and palaeoclimatic history has been reviewed extensively (see Quilty, 1994; Lear *et al.*, 2000; Zachos *et al.*, 2001; Hall, 2002) but a brief account is presented here as it is required to understand how changing climates may have shaped the biota of the present. Data from sediments and fossil plants and animals suggest that Australia has transitioned from widespread very wet, aseasonal climates in the Palaeogene to the present-day predominantly dry and seasonal climates (Byrne *et al.*, 2011). As Australia became much drier between the Eocene and the present, the arid zone expanded to cover ~70% of the continent, and mesic environments contracted to the eastern, south-east and south-west margins of the continent (Byrne *et al.*, 2011). Although the exact timing of climatic drying is poorly known, sedimentary and palaeontological evidence suggests that the periods between 37-30 MYA, 10-5 MYA and <2 MYA-present were particularly dry (Fig. 4.4). In contrast, the periods from 66-37 MYA and 30-10 were significantly wetter, with a multitude of ancient rivers and lakes (Fig. 4.4). These alternating wet and dry periods are likely to have strongly affected the present-day biota by causing high rates of extinction of mesic taxa, but driving speciation and expansion of arid-adapted taxa (Byrne *et al.*, 2008; Byrne *et al.*, 2011). Marine incursions of the southern part of the continent, particularly the Eucla basin/ Nullarbor, during wetter time periods, are another important factor which may have influenced speciation and species distributions (Fig. 4.4).

Fig. 4.4. Palaeogeographic maps representing summaries of the palaeogeographic features for a period of time based on sedimentological or structural changes (modified from BMR Palaeogeography Group). Dark blue areas represent marine environments, light blue shading represents shallow seas, yellow and orange shading represent lake and river deposits and dark brown shading represents areas which have been emergent for most of Australia's history.

Diversity and deep divergences in ancient landscapes

Phylogenetic analyses revealed that most parabathynellid genera are restricted to major bioregions and likely represent ancient divergences. For example, *Kimberleybathynella*, *Arkaroolabathynella* (possibly excluding *Ar*. sp. 2), *Billibathynella* and *Brevisomabathynella* are restricted to the Kimberley, Flinders Ranges and Yilgarn regions. Both of the latter genera also contain described species from the Pilbara region which were not represented in the phylogenetic analysis, reinforcing the results of regional and genus level endemism. All of these regions have been emergent since Pre-Cambrian times (Beard, 1998). Previous research focusing on the biota of the Western Shield and Kimberley has demonstrated the distinctiveness of the regional biota and led to hypotheses of vicariant speciation events in response to major climatic events and geographic barriers being the most likely explanation for the distinctive regional faunas (Cooper *et al.*, 2007; Bowman *et al.*, 2008; Humphreys 2008). Here, the regional distinctiveness and its potential causes is explored for parabathynellids.

Overall, the diversity and strong regional structuring suggest that parabathynellids have been common and diverse in ancient, long-emergent landscapes of Australia. This is congruent with observations of other Australian freshwater crustaceans such as phreatoicid isopods (Wilson and Johnson, 1999), which similarly display a high degree of endemism at genus and higher levels, and are largely distributed in areas which have not been subject to marine incursions during the continent's history. The Pilbara and Yilgarn regions are a good example of regional distinctiveness, irrespective of their geographical proximity (Humphreys, 2008), as they display a high diversity of copepod and isopod species, with only 4% and 8% overlap between regions, respectively (Humphreys, 2008). Various authors attribute the diversification of stygofauna in the Pilbara and Yilgarn regions to the onset of extreme aridity in the late Miocene to Pliocene (Bradbury and Williams, 1997; Humphreys, 2001a; Finston *et al.*, 2009). The diversification seen in *Brevisomabathynella* coincides with the late Miocene, suggesting that aridification may have played a role in this relatively rapid diversification event.

According to the data and analyses undertaken here, much of the diversification of parabathynellids appears to have happened before the major aridification events after the late Miocene. This is discussed briefly below, however, the need for caution in interpreting the molecular clock results is emphasized as *COI* is not ideal for dating deep historical events, due to its high rate of evolution and AT bias in invertebrates, leading to saturation. In future, data from more slowly-evolving genes is likely to be useful in reanalyzing the dates recovered here, to assess their accuracy. The current study suggests potential divergence events dating back to the mid-Tertiary and Cretaceous. If these dates are reasonably accurate it is plausible

that a once widespread fauna may have been subject to vicariant differentiation as has been proposed for freshwater phreatoicidean isopods (Wilson and Johnson 1999). Although land forms such as the Yilgarn/Pilbara Cratons and the Kimberley have remained emergent since Pre-Cambrian times; marine incursions in the Cretaceous and Tertiary (Wilson and Johnson, 1999; Martin, 2006) and alternating wet/dry periods may have removed intervening habitats and prevented gene flow between them. Support for this hypothesis is seen in the results of the widespread genus *"Atopobathynella"*, which has a distribution spanning the Pilbara, Yilgarn and Eyre Peninsula regions (and also containing described species from south-eastern and central Australia). Each region has distinct *"Atopobathynella"* lineages but the lineages within regions do not form monophyletic groups. According to the results, the genus may have originated-at about 51.9 MYA (95%HPD, lower: 39.84 MYA, upper: 64.52 MYA), with species diversification occurring between 45 and 7.5 MYA. There is evidence for a connection between the Yilgarn and Eyre Peninsula (sister species relationship between *"At." hinzea* and *"At."* sp. 1) at least 28.61 MYA (95% HPD; lower: 16.98 MYA, upper: 41.13 MYA) and between the Pilbara and Yilgarn regions (*"At."* sp. 3 is basal to a group of four Yilgarn species) at least 37.08 MYA (95% HPD; lower: 26.49 MYA, upper: 48.54 MYA). The mean and upper limit dates are consistent with wetter time periods (see Fig. 4.4) for which there is sedimentary evidence for potential river connections between the Yilgarn and Eyre Peninsula and Pilbara regions, which could have been interrupted by a marine incursion of the Nullarbor (Hill, 1994).

In contrast to a previous hypothesis that parabathynellids may have evolved in the ancient Yilgarn craton (Cho, 2005), ancestral state reconstruction suggests that the region of New South Wales containing the Hunter, Macquarie and Peel Rivers, was the ancestral area for parabathynellids. The high diversity of genera (*"Chilibathynella", "Hexabathynella", Notobathynella, Octobathynella* and *Onychobathynella*) in this region, and its surrounds, lends further support to this hypothesis. Additionally, NSW, although with high marginal probabilities, is identified as the ancestral area for almost all of the basal nodes of the phylogeny.

Regional connections

The following connections were observed between regions, based on repeated relationships between taxa: (a) a strong connection between SA + NSW, (b) SA + WA and (c) Yilgarn + Pilbara regions. Although there is considerable uncertainty in the dates at internal nodes, it is likely that the observed regional connections pre-date the aridity phase that commenced in the late Miocene. As highlighted above, pre-Miocene times are hypothesized to have been warmer and wetter climates than the present (Hill, 1994; Martin, 2006; Byrne *et al.*, 2011). It is possible that the fluctuating appearance and disappearance of large water bodies (e.g. lakes

and rivers) and changing sea levels over this time period played an important role in shaping the patterns seen here for parabathynellids.

The SA + NSW connection is complex, with each of the individual sub-regions of SA (Eyre Peninsula, Flinders Ranges and south-east region) connected to NSW, via sister taxa relationships at the genus level, i.e. "*Hexabathynella"* + *"Chilibathynella"*, *Arkaroolabathynella* + *Octobathynella* and the bathynellid lineages from south-east SA + one lineage from NSW. The divergence times estimated for these splits appear to pre-date the Miocene, perhaps dating back to wetter time periods when large river and lake systems formed in central and eastern Australia, potentially providing a connection between them (Fig. 4.4). The SA + WA connection, includes two incidences of Eyre Peninsula taxa related to WA taxa. Close phylogenetic relationships between SA and WA taxa are also observed for amphipods (Bradford *et al.*, 2010; King *et al.*, in press). Based on the phylogeny the SA+WA connection could date back to considerably wetter periods, approximately 28.61 MYA (95% HPD; lower: 16.98 MYA, upper: 41.13 MYA) (*"At." hinzea* and *"At."* sp. 1) and 61.4 MYA (Clade 6). Although marine incursions may have played a role in shaping this pattern, it is hypothesized that the connection observed here may be explained by ancient rivers (evidenced by known palaeodrainages) that flowed east towards central Australia and the Nullarbor (Beard 1998). The Yilgarn and Pilbara connection is difficult to explain given that, generally, there are inexplicable faunall differences (e.g. high diversity of dytiscid diving beetles in the Yilgarn and absence of beetles in the Pilbara) between these contiguous regions (Humphreys, 2008). The phylogeny indicates common ancestors for Pilbara and Yilgarn taxa estimated at 37-50.9 MYA and ancestral area analyses reconstructed the Pilbara as the ancestral region for the widespread *"Atopobathynella."*

Regional endemism

Current research (this Chapter and Ch. II) has shown that Australia's parabathynellid fauna is remarkably diverse. This study has also demonstrated that each region contains its own endemic fauna (e.g. *Kimberleybathynella* restricted to the Kimberley, *Brevisomabathynella* and *Billibathynella* restricted to the Pilbara and Yilgarn), even in relatively poorly sampled areas (i.e. Flinders), highlighting high levels of short-range endemic taxa in each of the major regions. Previous studies have shown that these regions contain other distinctive stygobiont fauna, for example, species of candonine ostracods (Karanovic, 2004), a variety of copepods, and a phreatoicidean isopod that is apparently sister to all other Australasian Phreatoicidae in the Kimberley (Wilson and Keable, 1999) in the Kimberley; an unparalleled diversity of dytiscid diving beetles in the Yilgarn (Watts and Humphreys, 2009 and references therein), and a remarkably high diversity of endemic candonine ostracods in the Pilbara (Karanovic and Marmonier, 2003; see Humphreys, 2008 for a summary). Although little is known about

the stygobiont fauna of the Flinders Ranges, Eyre Peninsula and NSW aquifers, given the distinctive nature of their parabathynellid fauna elsewhere, it is likely that similar processes to those that shaped the diversification and distinctiveness of the family, may have had a similar effect on other co-occurring stygobiont groups.

Numerous authors have found evidence for increased species diversification in central Australia corresponding to the spread of aridity during the late Miocene and Pliocene periods (e.g. beetles (Leys *et al.*, 2003), isopods (Finston *et al.*, 2009), amphipods (Cooper *et al.*, 2007)). The estimated dates show a similar species radiation in the arid Yilgarn but also show earlier episodes of diversification, for example the species radiation in the Flinders Ranges (mean 56.52 MYA; 95% HPD, lower: 42.75 MYA, upper: 71.08 MYA), coinciding with a warm-wet period following a cool-dry period (Fig. 4.4). These apparent 'bursts' of evolution may have been mediated by multiple ancestral parabathynellid species inhabiting the hyporheic zones associated with rivers which existed before the onset of dry periods in the late Eocene and Late Miocene. An example of a relatively recent burst of evolution/speciation is *Brevisomabathynella*, which shows divergence of species coinciding with the onset of aridification in the Late Miocene period (Guzik *et al.*, 2008). This finding is further supported here with inclusion in the analyses of additional species. Each species is endemic to an individual calcrete aquifer, despite some aquifers being less than one km apart (e.g. species from Lake Violet and Uramurdah calcretes that are ~800 m apart). This is congruent with the findings of Guzik *et al.* (2008), providing further support for the subterranean archipelago hypothesis and indicating that the Yilgarn is an important region for parabathynellid species diversity.

Overall, it is clear that parabathynellids are common and diverse in ancient, long-emergent landscapes of Australia and that they display strong regional endemism at the genus and species levels. This pattern is suggestive of a long and complex evolutionary history, largely shaped by vicariance due potentially to marine incursions and climatic fluctuations. The evidence here supports connections between disparate regions such as NSW with the Eyre Peninsula,and Flinders Ranges with the Western Shield.

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CHAPTER V

GENERAL DISCUSSION

CHAPTER V

Synthesis

This study is the first to use molecular genetic approaches to explore the relationships within the Parabathynellidae at a continental scale. It provides the first robust phylogeny for the family and will serve as a framework for all future research on the group. Although Guzik *et al.* (2008) provided the first genetic study to explore relationships amongst parabathynellid taxa, their study was restricted to three genera from the calcrete aquifers of the Yilgarn region, Western Australia. Here, I have greatly expanded on their study by including all known genera and a third of the known species from Australia, sampling from various types of aquifers across the continent, and using a nuclear gene to aid in resolution of the deeper nodes of the phylogeny.

With the use of a molecular phylogenetic approach I have shown that each known genus, defined by traditional morphological methods, is monophyletic, suggesting that the commonly used generic characters are robust for defining distinct evolutionary lineages. However, ancestral state reconstruction (ASR) analysis provided evidence for multiple cases of convergent evolution for the two commonly used morphological characters (number of segments of the first and second antennae), 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. I also used the ASR approach to assess the oligomerization principle (i.e. serial appendage reduction over time), which is commonly invoked in crustacean systematics. My results contradict 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. This result will likely influence the future taxonomy and phylogenetics of parabathynellids (discussed below).

My study has also contributed to scientific knowledge of parabathynellid systematics, by identifying 32 genetically distinct lineages, the majority of which are probably new species. As part of this work the first genus and four species of parabathynellids from South Australia have been described. Following on from Guzik *et al.*'s (2008) study, this project has revealed a high diversity of parabathynellids which indicates that Australia could be a hotspot for the family. This may be due to the continent's plethora of diverse subterranean aquatic systems ranging from alluvial to calcrete aquifers. Given that this study found a new genus in South Australia (SA), it is likely that as new areas are explored, many additional new taxa will be discovered.

In this study, the identification and description of new species has been guided by DNA sequence data and, given the current biodiversity extinction crisis, the use of molecular methods is vital to 'rapid' assessment of the diversity of Australia's parabathynellid fauna and to inform their conservation management. The molecular (*COI* sequences that can serve as DNA barcodes) and morphological data (in the form of a key to genera) that this study has generated are a significant contribution to conservation management for this group because they provide a framework that can be used by government agencies and private consulting companies to identify parabathynellids. These identification tools are valuable given that these animals are commonly misidentified and their diversity has been greatly underestimated in the past. For example, *COI* sequences can be obtained from parabathynellids collected from mining exploration surveys and compared with those generated by my study to determine whether they are known or are new species inhabiting the site in question.

Finally, my study has shown a high incidence of short-range endemicity for parabathynellids at the species level. Given this and their poor dispersal abilities, it is likely that parabathynellids are truly vicariant and this calls the so-called cosmopolitan genera into question. In addition, it confirms that parabathynellids are an interesting target group for further biogeographic studies.

Future directions

Generic level systematics

Showing that morphologically simplified parabathynellids are not necessarily derived indicates that a comprehensive review of global parabathynellid relationships is required as previous assessments of relationships, albethey mostly intuitive, have probably been misled by the oligomerization principle and unrecognised high levels of convergent characters. A global assessment of relationships was unfortunately beyond the scope of this study due to a lack of suitable material. In future any broad scale study will require significant collaboration among syncarid workers, and targeted collecting where few specimens have so far been forthcoming – in this respect Africa, Madagascar, India, New Zealand and South America will be particularly important. The results of my project indicate a strong pattern of restricted distributions for parabathynellid taxa, which is incongruent with the current cosmopolitan genera, e.g. *Hexabathynella*, *Atopobathynella*, and *Chilibathynella*. The inclusion of species of these taxa from each continent in a global molecular phylogeny would provide a test of whether these genera are monophyletic or not. However, given the observed common pattern of short-range endemicity among Australian parabathynellids it is plausible, if not highly likely, that generic level taxa are vicariant and restricted to single continents, and that the

current intercontinental genera are likely not to be closely related, given the level of morphological convergence revealed in this study.

It is well recognised that multiple genes should be employed to assess the phylogenetic relationships of organisms in order to establish more robust relationships (Cunningham, 1997). Although the two amplified genes (*COI* and *18S*) provided relatively consistent and well-resolved phylogenies, the support for the deeper nodes in the phylogeny was not particularly high and the position of *Notobathynella* remains unresolved. It is likely that as new taxa are added, additional sequence data will be required to resolve the phylogeny, particularly for deeper nodes. However, results of this study showed it can be very difficult to acquire or develop primers that can successfully amplify a range of markers for syncarids and other crustaceans. For example, in this study amplification of a number of genes besides *COI* and *18s* was attempted with little (*16S* and *28S*) or no success (*wingless, EF-1alpha, opsin,* NADH1, histone 3, GAPDH, CAD, PEPCK, ANT, LTRS, ARGK). This is probably due to the primers not being similar enough to the target DNA to enable the annealing process to occur. This could be due to parabathynellids being an ancient group and therefore having significant time to diverge from other crustacean lineages from which universal primers have been designed. Recent advances in sequencing technology (e.g. second and third generation sequencing; see Shendure and Ji (2008)) will likely play an important role in future systematics studies for parabathynellids. These techniques are advantageous in producing vast amounts of genetic data and freeing the user from relying on published sequences and primers, and having to design new primers. Another profitable avenue for future research, would be to include more characters that have been used to infer phylogenetic relationships in ASR analyses to assess levels of convergence for each character and determine their suitability for elucidating relationships. Evaluation of characters of the male thoracopod VIII would be particularly interesting, given that the results from Ch. III suggest a potential pattern of genera with uniquely-shaped thoracopod VIII's among the included species. However, evaluation of characters relating to this structure will be difficult, due to numerous older species' descriptions providing incomplete or no description of the male thoracopod VIII and for some species, the male is completely unknown.

Family level systematics

To date, bathynellacean systematics is still controversial. For example, there is ongoing debate concerning the number of families within the order. Recently, the order has been considered to contain two families, Bathynellidae and Parabathynellidae (Camacho and Valdecasas, 2008; Drewes and Schminke, 2011). However, Noodt (1965) established the family Leptobathynellidae to contain *Leptobathynella* Noodt (1963) and *Brasilibathynella*

Jakobi (1958). Schminke (1973) synonymised this family with Parabathynellidae, reasoning that some African species were intermediate in form between Leptobathynellidae and Parabathynellidae, although he did consider the above two genera plus *Parvulobathynella*, *Acanthobathynella* and the '*Cteniobathynella* subgroup' to be a distinct lineage within Parabathynellidae. However, some authors (Coineau and Serban, 1973; Delamare Debouttéville and Serban, 1974; Coineau and Serban, 1978) have consistently maintained the validity of Leptobathynellidae, and subsequently Serban (1980) resurrected the family based on the uniqueness of the mandibular morphology in comparison with the other two families. Reddy *et al.* (2011) argues that Cho (1997) relegated the Leptobathynellidae to subfamily level without providing any reason, although nobody challenged this decision until Reddy's recent morphological study. Although resolution of the relationships within Bathynellacea was beyond the scope of my project; inter- and intra-family relationships could potentially be solved in the future, through the use of molecular data from a broad representation of taxa across the superfamily.

Biogeography of Australian stygofauna

In Ch IV, I explored the geographic relationships amongst Australian parabathynellids and attempted to define bioregions of particular significance for the family. I found that parabathynellids are common and diverse in ancient, long-emergent landscapes of Australia and display strong regional endemism at the genus and species levels. Areas with diverse and distinctive parabathynellid faunas included the Yilgarn, Pilbara, Flinders Ranges and Hunter and Peel River aquifers. Apart from the Yilgarn and Pilbara regions, the stygofauna of the other regions is poorly known, while numerous regions, particularly in eastern Australia are yet to be surveyed. Our understanding of the evolution of the biota of these regions would also be better informed by future comparisons of the distribution and relationships of other crustaceans in these regions (particularly amphipods, isopods, copepods and ostracods). Unfortunately, at this stage, there are no molecular phylogenies available for any of these crustacean groups, sampled at a continent-wide scale, as was undertaken, at least in part, for this study. However, a regional study of parastenocarid copepods by Karanovic and Cooper (2011) has also found evidence for short-range endemism and diverse but distinctive copepod faunas for particular regions (namely the Yilgarn and Pilbara regions). In future, comparative biogeographic approaches could also be used to test hypotheses of whether particular geological and climatic events affected different taxa in similar ways (e.g. increased or decreased speciation).

Conservation and management of subterranean fauna

Over the past two decades, studies have uncovered a wealth of stygofauna throughout Australia. The better-studied western side of the continent currently contains the highest diversity of stygofauna (Guzik *et al*., 2011), but other areas such as alluvial aquifers in New South Wales (Hancock and Boulton, 2008) and springs in the Flinders Ranges (R. Leys, pers. comm.) are proving to contain substantial diversity, although studies of the latter areas are only at an early stage. The Yilgarn, Pilbara and Cape Range areas have been found to contain distinctive regional faunas and this led Humphreys (2008) and Guzik *et al*. (2011) to hypothesize that other regions of Australia may also contain distinctive stygofaunal communities. This study has added to the growing body of literature highlighting the high incidence of short-range endemicity for stygobiontic animals by providing evidence for very restricted distributions for parabathynellids, with no evidence of species dispersal between regions or even within regions (e.g. the Yilgarn taxa). Additionally, this study found that each region of Australia sampled for parabathynellids, contained a unique fauna. New species were discovered in each region, suggesting that there is a wealth of fauna yet to be discovered, particularly from little explored areas such as the Eyre Peninsula of SA.

In order to appropriately manage stygofauna, it is important to have a thorough understanding of their diversity and distribution, particularly because they tend to be restricted to subterranean habitats which are under increasing pressure from both pastoralism and the mining industry. Management of subterranean ecosystems and stygofauna is hindered by numerous obstacles including: a shortage of taxonomic expertise, lack of data from replicated surveys, incomplete coverage of habitat types, lack of standardised protocols for sampling and processing, lack of identification keys, a need for centralised repository of reference material, and a need for a central database adhering to nationally-agreed standards (Tomlinson and Boulton, 2008). In addition, there is a lack of policy specifically related to stygofauna protection for most Australian states and territories (Tomlinson and Boulton 2008). Western Australia currently has the best protection policy for stygofauna, as their Environment Protection Agency requires environmental approval for major resource projects which entail documentation of species diversity and distributions, in addition to ensuring that species are not threatened with extinction through the actions of the resource acquirer (Environment Protection Authority, 2003, 2007). There is an urgent need for the other Australian jurisdictions to provide similar protection for stygofauna in order for Australia to meet its obligation to conserve biodiversity through the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). Studies of groundwater ecosystems and their associated stygofauna are vital to groundwater management because they can be used to

identify groundwater habitat types and to aide conservation planning, design of monitoring programs and sustainable water use (Danielopol *et al.*, 2008; Tomlinson and Boulton, 2008).

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