# Molecular Systematics and Biogeographic History of Oniscidean Isopod Troglofauna in Groundwater Calcretes of Central Western Australia

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A Thesis Presented for the Degree of Doctor of Philosophy
March 2014

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This study was funded by an ARC linkage grant (#LP100200494) to Andrew D. Austin, Steven J. B. Cooper, William F. Humphreys, Mark S. Harvey and Mark I. Stevens; an ABRS travel grant (BUR212-28) awarded to Seyedmohammad Javidkar and the participating industry partners, Minara Resources, South Australian Museum and Western Australian Museum.

*This thesis is dedicated to:*

*My parents, Ebrahim and Zahra*

*And my wife, Nahid*

# ABSTRACT

Groundwater calcretes of central Western Australia have revealed an extraordinary diversity of short-range endemic invertebrate subterranean fauna. Although considerable attention has been given to the aquatic dwellers of the calcretes (stygofauna), the subterranean terrestrial fauna of the calcretes (troglofauna), particularly the oniscidean isopods, have been poorly studied. This thesis, including four data chapters, presents the results of multiple-gene and morphological analyses to establish a phylogenetic framework for elucidation of species diversity, systematics, and the biogeographic history of oniscidean isopod troglofauna in arid central Western Australia.

The first data chapter focuses on higher level systematic relationships of the isopod fauna. In order to examine the monophyly of the family Platyarthridae, representatives of the main oniscidean families and genera from Australia, South America, Africa and Europe were analysed using molecular and morphological approaches, including data from a Scanning Electron Microscopy study. The phylogenetic analyses of mitochondrial and nuclear genes (*COI*, *18S*, and *28S*) showed that Platyarthridae is polyphyletic, and also revealed a very distinct Australian lineage with a unique water conducting system on antenna 2. Based on both morphological and molecular data, a new southern hemisphere oniscidean family, Paraplatyarthridae, occurring from subtropical/temperate to arid regions of Australia and South America, is proposed and described.

The second data chapter focuses on the molecular systematics, species diversification and distributional patterns of the oniscidean troglofauna in calcrete aquifers of central Western Australia. The results, based on morphological and multiple-gene molecular approaches, reveal a significant diversity of oniscidean DNA lineages. The application of different species delineation methods, suggests the existence of 28 putative species belonging to four oniscidean families, which most likely represent distinct undescribed species. The phylogenetic analyses show (with some exceptions) that the majority of oniscidean DNA lineages were restricted in their distribution to individual calcrete bodies, lending support to the hypothesis that individual calcretes are equivalent to “Subterranean Islands”. In addition, the occurrence of subtropical, littoral and benthic oniscidean groups in the calcretes suggests complex historical events, including the marine inundation of the

Eucla basin during the late Eocene, have shaped the taxonomic representation of the current oniscidean troglofauna.

The third data chapter investigates the biogeographic history of the widespread genus *Paraplatyarthrus*, which showed noticeable morphological diversity, from troglophilic to troglobitic forms. The phylogenetic and molecular clock dating analyses provided evidence that evolutionary transitions from surface to subterranean habitats took place from the late Miocene, and further indicated that troglophile ancestral species independently colonised the calcrete aquifers. These findings support both the climatic relict and adaptive shift hypotheses to explain the evolution of the oniscidean isopod troglofanua with aridity being a significant driver of diversification underground.

The final data chapter comprises the morphological description of five new species of the genus *Paraplatyarthrus* (Paraplatyarthridae fam. nov.) and provides a key to their identification.

# ACKNOWLEDGEMENTS

Thanks be to God, the assisting hands never touched but always felt, the light never seen but always lit my way and the fellow whose sound never heard but his words always perceived.

This PhD thesis would never have been possible without the guidance of my strong supervisory panel: Prof. Steven Cooper, Prof. Andrew Austin, Prof. William (Bill) Humphreys and Dr Rachael King. My very special thanks to Steve that I found him always supportive, encouraging and very keen to help me throughout my PhD. Here, I want to thank him again, even though I know this gratitude is not enough, for accepting my request to do this wonderful opportunity of doing a PhD, training me, making me familiar with new techniques and methods in this area, always responding to my questions with patience and going through all the thesis drafts with professional and constructive comments, suggestions and corrections. My special thanks to Andy who accepted me as his PhD student and kindly supported me/my PhD project both academically and financially. Thanks to Andy, also, for reading the entire thesis and the associated articles in a time when he was loaded with lots of PhD/honours dissertations and for all his professional comments. Thank you to Bill, for his invaluable help in the field, sharing the incredible collection of subterranean fauna with me and his nice corrections/suggestions on my PhD drafts. In my numerous encounters, I found him so enthusiastic to discuss my results and further improve my thesis. I must also thank Rae, Bill's wife, for her excellent hospitality and support during my stay in Western Australia for field trips. Thank you to Rachael, who helped me on species descriptions and went through my PhD drafts with both professional taxonomic comments and precise corrections. My special thanks also to Kathy Saint who trained me in the molecular lab and was always so keen to help and guide me with patience. I sincerely thank Dr Terry Bertozzi who kindly assisted me with the bioinformatics section and helped me to analyse the NGS data.

I must thank Profs. Stefano Taiti and Helmut Schmalfuss for their invaluable comments and sending critical specimens for molecular and morphological experiments. Thank you to Ms Lynette Waterhouse from Adelaide Microscopy for training and helping me in SEM techniques. Thanks to Dr Simon Judd who kindly sent important specimens for further lab

experiments. I would like to thank Andy Austin's lab group: Dr Michelle Guzik and Dr Mark Stevens for their help and arrangements in DNA barcoding; Dr Gary Taylor, Dr John Jennings and Dr Simon Tierney, for support in Microscopy and the associated imaging studies and feedback on my talk presentations.

Thank you to my friends Mohammad Kurghi, Alejandro Velasco-Castrillon and Rebecca Kittle who provided me with support and made nice moments through my PhD life.

My deepest thanks, which certainly cannot be adequately expressed, to my lovely wife, Nahid, for her love, help, and support and stood by me in every single moment of my life with her. I owe my sincerest thanks to my mother, Zahra, and my father, Ebrahim, who always encouraged and supported me both in life and my studies; to my dear brother, Mohsen, and my lovely sister, Fatemeh, who helped me in every possible moment and were always supporting and encouraging me with their best wishes.

I would like to thank the funding bodies Australian Research Council and the Australian Biological Resources Study for providing grants for lab experiments/equipments, field and conference trips. Finally, I would like to thank the School of Earth and Environmental Sciences of the University of Adelaide, for part funding my PhD stipend and providing project support funds.

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# CHAPTER I:

## GENERAL INTRODUCTION

## ISOPODA CLASSIFICATION AND PHYLOGENY

The order Isopoda with around 10,000 described species worldwide, classified in 10 suborders, is considered the most diverse group of crustaceans of the superorder Peracarida Calman 1905, being found from the deepest oceans to inland ecosystems. This group is characterised by brooding their young in a marsupium formed from thin plates on the basal segments of their legs (Brusca & Wilson, 1991; Poore, 2002; Schotte *et al*. 2008). Isopods are distinctive among crustaceans owing to the lack of a carapace and the associated gills, and, instead, they use gill-like pleopods on the posterior body for respiration.

The suborder Oniscidea Latreille 1802, referred to under the common names of woodlice, slaters or pillbugs, are terrestrial isopods that generally live in humid places and feed on dead plant material (Poore, 2002). According to Schmalfuss (1998), more than 4,000 nominal species classified into 33 families (Gruner, 1993) have been reported. However, Schmalfuss (2004) reduced this number to 3637 valid species and listed the taxa alphabetically regardless of their systematic position. Despite most species being found in humid habitats, some species occur in semi-arid to arid regions where microclimatic conditions associated with humidity, such as under stones, fallen bark and leaf litter, together with sophisticated behavioural and morphological adaptations, allow them to persist (Marikovskij, 1969; Linsenmair & Linsenmair, 1971; Paoli *et al.* 2002; Schmidt, 2002). Contrary to terrestrial oniscidean species, some species such as those of *Haloniscus* Chilton 1920 have secondarily become aquatic in inland waters, for example, *H. searlei* Chilton 1920 which occurs in inland salt waters, and the subterranean *Haloniscus* in groundwater calcretes of Western Australia (Taiti & Humphreys, 2001; Cooper *et al.* 2008). Some families including Scyphacidae Dana 1852, Halophilosciidae Verhoeff 1908, Actaeciidae Vandel 1964, Olibrinidae Budde-Lund 1913, Tylidae Dana 1852 and most species of Ligiidae Leach 1814 are halophilic inhabitants of supralittoral zones.

The phylogenetic relationships among terrestrial isopods and the various marine groups are poorly known (Martin & Davis, 2001). Based on cladistic analyses of morphological characters it has been proposed that terrestrial isopods are monophyletic and originated from aquatic isopods only once (Schmalfuss, 1989; Wägele, 1989; Brusca & Wilson, 1991; Erhard, 1996; Tabacaru & Danielopol, 1996; Erhard, 1998). The monophyly of Oniscidea is supported by several synapomorphies, of which the occurrence of a water conducting

system (WCS) composed of scale rows on the ventral side of the coxal plates is the most prominent one (Schmidt, 2008). The Oniscidea consist of five major groups, each of which is considered monophyletic: Ligiidae, with about 80 species distributed in seashores or terrestrial habitats with high humidity; Tylidae, including about 20 species distributed in seashores, with just one inland terrestrial species; Mesoniscidae Verhoeff 1908 (*Mesoniscus*) with just two species; Synocheta Legrand 1946 with about 650 species that are limited to relatively moist inland environments; and Crinocheta Legrand 1946, which are the most diverse group of Oniscidea comprising approximately 2,750 species with complex lungs on the pleopod exopodites and adaptations to relatively drier terrestrial environments, in comparison with species of other groups (Schmidt, 2008).

Holdich *et al.* (1984) classified oniscidean isopods into two infraorders, Tylomorpha (including Tylidae) and Ligiamorpha, comprising three sections, Crinocheta, Synocheta and Diplocheta Vandel 1957 (Ligiidae and Mesoniscidae). The Crinocheta were divided into Oniscoidea for families that were thought to lack pseudotrachae (lungs) and Porcellionoidea for families with pseudotrachae on the pleopod exopodites. Schmalfuss (1989) did not use the infraorder level for higher classification of oniscideans, instead using only the four sections: Crinocheta, Synocheta, Microcheta (Mesoniscidae) and Diplocheta. However, Schmalfuss's new section (Microcheta) and the associated classification were not widely accepted as his synthesis was based on relatively few characters. Wägele (1989), in his cladistic phylogeny of isopods, showed Diplocheta (Ligiidae, Mesoniscidae and Tylidae) forming a sister relationship with the Synocheta-Crinocheta group, although in the latter group most nodes were unresolved or weakly supported. In this classification the occurrence of pseudotrachae on the dorsal pleopod exopodites was considered an important character for assessing relationships within Crinocheta, while the lack of pseudotrachae was regarded as a primitive character or a secondary loss.

Erhard (1998) conducted research on the phylogenetic-systematic relationships amongst representatives of Ligiidae, Tylidae, Mesoniscidae, Synocheta and Crinocheta (but did not mention which species were used to code characters) using comparative anatomical investigations including, among other characters, the morphology of the pleons, and proposed a modified classification. His cladistic phylogeny (Figure 1.1) showed a sister relationship between Ligiidae and Holoverticata (Tylidae, Mesoniscidae, Synocheta and

Crinocheta). Within Holoverticata, Tylidae formed a sister lineage to the monophyletic Orthogonopoda (Mesoniscidae, Synocheta, Crinocheta), within which Synocheta and Crinocheta formed a sister group classified as Euoniscoida. These relationships are partly in agreement with those revealed by molecular phylogenies based on the mitochondrial Large Sub-Unit (*LSU*) *rRNA* and nuclear Small Sub-Unit (*SSU*) *rRNA* genes (Michel-Salzat & Bouchon, 2000; Mattern & Schlegel, 2001). Mattern and Schelegel's work (the *SSU rRNA* conserved region phylogeny) supported the relationships among Crinocheta, Synocheta and Diplocheta based on previous morphological analyses, but the monophyly of Oniscidea was not confirmed by this analysis.

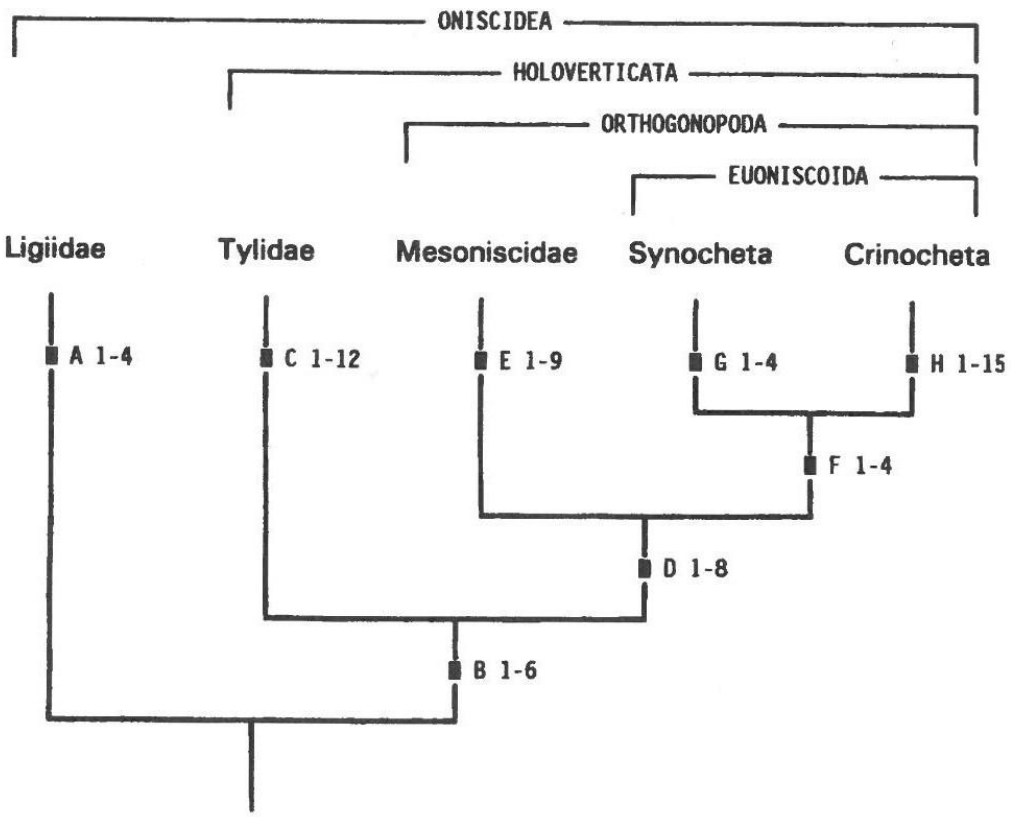


**Fig. 1.1.** Cladistic phylogeny and classification of Oniscidea by Erhard, 1998 (black squares refers to apomorphies of each group; see the reference cited for details).

Two hypotheses have been proposed to explain the phylogenetic relationships among Crinocheta, Synocheta and Mesoniscidae. Legrand (1946), Wägele (1989), Schmalfuss (1989) and Erhard (1995, 1997) proposed a sister relationship between Crinocheta and Synocheta,

while Tabacaru and Danielopol (1996) favoured a sister group relationship between Synocheta and Mesoniscidae.

The molecular phylogeny of 42 aquatic and terrestrial crustacean species based on the *LSU rRNA* gene did not show Oniscidea as monophyletic, but revealed *Ligia* Fabricius 1798, *Ligidium* Brandt 1833 and *Tylos* Latreille 1826 grouping closely with some aquatic isopod species (Michel-Salzat & Bouchon, 2000). The cladogram of the same authors also revealed Crinocheta and Synocheta to be monophyletic and sister to each other. Schmidt (2008) argued that as the Michel-Salzat and Bouchon's cladogram was reconstructed using only a neighbour joining algorithm, their analyses were insufficient to be considered more reliable than the original classification based on numerous/complex morphological apomorphies, which supported the monophyly of Oniscidea (Schmalfuss, 1974, 1989; Wägele, 1989; Brusca & Wilson, 1991; Erhard, 1995; Tabacaru & Danielopol, 1996; Erhard, 1998).

Crinocheta, which includes the majority (approximately 80%) of described species of Oniscidea, is considered a well supported monophyletic group with several synapomorphies, including the muscular structure of the pleons, the structure of the male copulatory apparatus, marsupium, mouthparts and stomach (Schmidt, 2008). The phylogenetic relationships among the families of Crinocheta are poorly studied and most have been examined on the basis of a single or few species. Several families, including Scyphacidae, Philosciidae Kinahan 1857, Platyarthridae Verhoeff 1949, Dubioniscidae Schultz 1995, Trachelipodidae Strouhal 1953 and Porcellionidae Brandt & Ratzeburg 1831, have been recognised as paraphyletic (Erhard, 1995; Schmidt, 2002, 2003). In Schmidt's (2002, 2003) study, which involved phylogenetic analyses using morphological data to elucidate the relationships between crinochetan families and to reconstruct the ground pattern of Crinocheta, most of the inclusive major clades were not named, as the morphological data to support their monophyly were insufficient (Figure 1.2). Mattern and Schlegel (2001), who constructed molecular phylogenies of 12 species of Oniscidea using the *SSU rRNA* gene (*18S*), revealed a group consisting of Oniscidae, Philosciidae, Platyarthridae, Armadillidiidae Brandt 1833 and Cylisticidae Verhoeff 1949, which was part of a comprehensive clade together with Trachelipodidae and Porcellionidae.

Although there have been several studies elucidating relationships between the higher oniscidean taxa, nothing is known about relationships within oniscidean families.

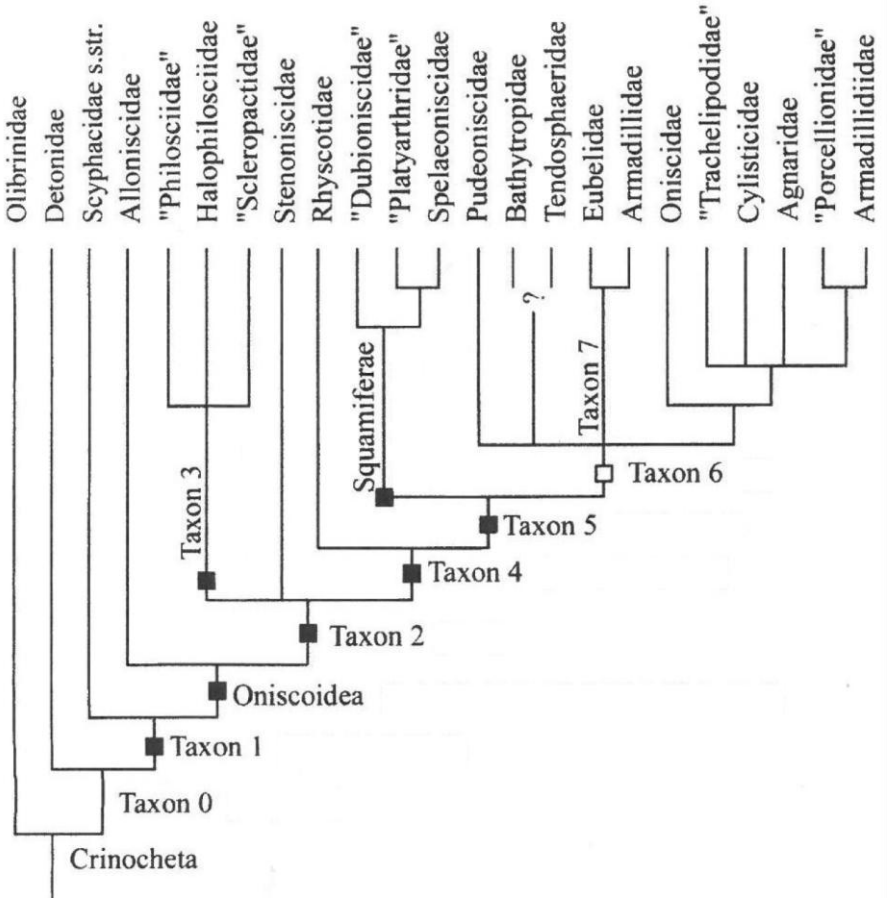


**Fig. 1.2.** Phylogenetic relationships within Crinocheta based on morphological data (After Schmidt, 2002).

## SUBTERRANEAN ECOSYSTEMS, CALCRETE AQUIFERS OF CENTRAL WESTERN AUSTRALIA AND THE ASSOCIATED DIVERSITY

Subterranean environments, which are subsurface spaces and cavities, include many kinds of caves (e.g. karst, lava tubes) and interstitial habitats such as littoral sea bottoms, freshwater lake bottoms, hyporheic zones and the associated aquifers with/without connection with surface waters. The modern definition of subterranean environments is even broader with inclusion of subterranean fissures, cracks and other microspace substrates, which may harbour significant subterranean fauna (Culver & Pipan, 2009).

In Australia, subterranean environments and the associated fauna are widely distributed. Some major regions embracing important subterranean habitats are as follows (Humphreys, 2008; EPA, 2012):

1) Western Australia with over 4,000 species, of which only 18% are described species (Guzik *et al*. 2010), comprises significant diversity of subterranean fauna reported from regions including the Nullarbor, a vast area of limestone plain and the largest arid karst area in the world, Cape Range and Barrow Island, the Pilbara, the Yilgarn region, a vast area extending from below the Pilbara to the arid and semi-arid areas of the Midwest, Murchison and Goldfields, Kimberley, and the Southwest (Figure 1.3).

2) Lake Lewis, Ngalia Basin, Northern Territory.

3) South Australia such as the Gambier Karst region (Grimes, 2006).

4) New South Wales and Jenolan Caves in the Great Dividing Range.

5) Far North Queensland and Undara Lava Tube.

6) Christmas Island (Indian Ocean, south of Java).

7) Tasmania, such as Ida Bay Karst in southern Tasmania which includes one of the richest obligate cave faunas in Tasmania and temperate zone Australia (Eberhard, 1990).

Groundwater calcretes occurring in Western Australia (north of 30° S) are non-pedogenic isolated calcretes which are approximately 10 m but up to 30 m thick (Anand *et al*. 1997; Humphreys *et al.* 2009). There are more than 200 major calcretes in Western Australia varying in size from one square kilometre in area to as large as 100 km long and 10 km wide; these large calcretes are situated towards the central zone of the calcretes distribution while the smaller ones are usually on the margin (Mann & Horwitz, 1979). The climate of the area is arid with less than 200 mm mean annual rainfall and evaporation of more than 3000 mm per year (Mann & Horwitz, 1979). The groundwater salinity in the calcretes of central Western Australia varies from saline to hyper-saline (1,000-18,300 mg/L, data after Watts & Humphreys, 2006), while in calcretes of the Pilbara region water remains fresh throughout the depth profile (Humphreys, 2006). The low and periodic average rainfall, high evaporation and the low movement of groundwater are thought to be the main

factors driving the formation of calcretes such as those that developed extensively during the Tertiary (Mann & Horwitz, 1979; Humphreys *et al.* 2009).

Some stages for the formation of groundwater calcretes have been described by Mann and Horwitz (1979), which are different from those of pedogenic calcretes. The stages are as follows:

1) Existence of a wide drainage system, shallow groundwater under arid climate with low rainfall.

2) Periodic recharge, transportation of calcium and carbonate ions into the water table, precipitation of calcium carbonate in the water table as a result of near-surface evaporation or evapotranspiration and concentration processes.

3) Development of carbonate precipitation in phreatic zone with upward pressure.

4) Carbonate precipitation continuing towards maturation; newly precipitated carbonates push the old ones above the water table during which various processes such as recrystalisation, hardening, silicification, and manganese staining occur accordingly, resulting in further development of the groundwater calcretes.

The separation of Gondwana from the early Cretaceous to Eocene, the migration of the Australian continent northward and the concomitant historical climate changes in the Paleogene and Neogene significantly contributed to shaping the river systems and groundwater calcretes of the Yilgarn Craton of Western Australia. These groundwater calcretes, based on geological and paleoclimatic evidence, are proposed to have been formed during 37-30 Mya (the Late Eocene to Early Oligocene) within the paleodrainages owing to global cooling and dry conditions (Morgan, 1993). As a result of a sea level rise during 30-10 Mya (Early Oligocene to Middle Miocene), a warmer and wetter period occurred in Australia; springs were significantly developed in this period in the paleodrainages and other inland areas; calcretes were being dissolved in groundwaters which may have resulted in formation of caves (Morgan, 1993; Langford *et al*. 1995) or other groundwater habitats. The time period between 10 Mya to the present is characterised by a temperature decline and sea level fall associated with the expansion of the Antarctic ice cap. This period, in general, represents the onset of Australian aridity during which surface rivers

stopped flowing, salt lakes developed, calcrete deposition started once again, and extensive pedogenic calcretes also developed showing the influence of increasing aridity in their development (Van der Graaff *et al*. 1977; Morgan, 1993).

Over the last two decades, groundwater calcretes of Western Australia have been shown to have an extraordinary invertebrate stygofauna (the aquatic dwellers of subterranean habitats) and troglofauna (the terrestrial inhabitants of subterranean environments), entirely new to science. These faunal discoveries represented a huge advance in the knowledge of subterranean biology in Australia. According to Humphreys *et al.* (2009), eight classes, 14 orders and 36 families of subterranean invertebrates have been identified from Western Australian aquifers. Also Included among the fauna are diverse higher taxa considered to be ancient freshwater lineages, namely bathynellaceans, tainisopidean and phreatoicidean isopods, amphipods of the family Crangonyctidae and candonine ostracods (Bradbury, 1999; Humphreys, 2001; Wilson, 2001; Karanovic & Marmonier, 2003; Wilson, 2003; Humphreys, 2008). Calcretes of the arid Yilgarn region contain more than 100 species of subterranean diving beetles (Watts & Humphreys, 1999; Leys *et al.* 2003; Watts & Humphreys, 2006; Leys & Watts, 2008; Watts & Humphreys, 2009) and a range of crustacean species such as Bathynellacea, Amphipoda, Isopoda, Copepoda and Ostracoda (Taiti & Humphreys, 2001; Karanovic & Marmonier, 2002; Karanovic, 2004; Cho, 2005; Cooper *et al.* 2008).

Generally, subterranean habitats contain locally endemic fauna owing to the hydrological isolation of the habitats and the low dispersal ability of the fauna (Trontelj *et al*. 2007). By definition, species are considered to be short-range endemic if their natural geographic range is less than 10,000 $km^2$ (Harvey, 2002). Short-range endemism in Western Australian calcretes, such as reported for subterranean diving beetles, isopods, amphipods, parabathynellids and copepods, is a common pattern with each species confined to an individual calcrete aquifer body (Taiti & Humphreys, 2001; Cooper *et al*. 2002; leys *et al*. 2003; Karanovic, 2004; Cooper *et al*. 2007, 2008; Guzik *et al*. 2008; Bradford *et al*. 2010).

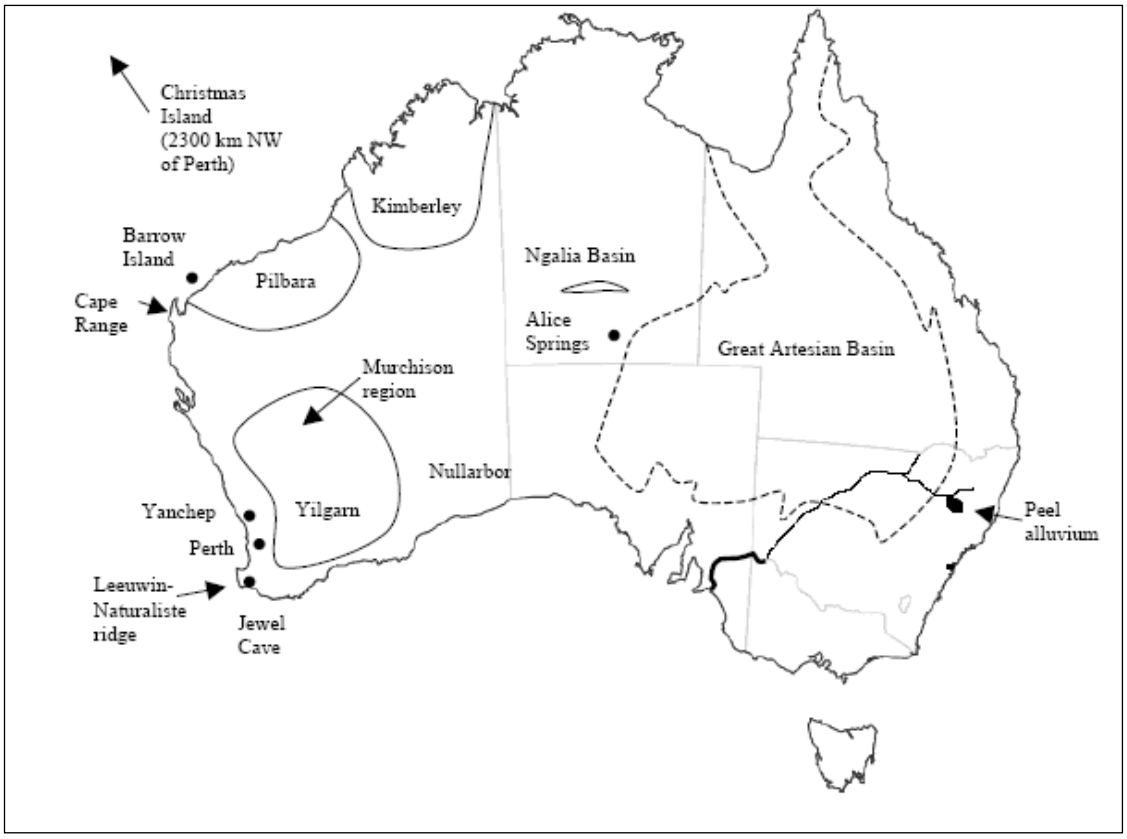


**Fig. 1.3.** Location of the subsurface groundwater dependent ecosystems in Australia (after Tomlinson & Boulton, 2008).

With respect to the confined distributions found in various stygofaunal groups, deduced using molecular and morphological analyses, the "Subterranean Island" hypothesis was proposed (Cooper *et al*. 2002; Leys *et al*. 2003). This hypothesis proposes that subterranean species in groundwater calcretes are confined into individual calcrete aquifers with no gene flow between them due to the between-calcrete geologic matrices of fine alluvial deposits with layers of clay acting as a barrier. This confinement is believed to be as a result of vicariance associated with aridification from the late Miocene (Byrne *et al*. 2008) which accordingly resulted in isolation of stygobitic invertebrates. Although this hypothesis has been well supported through studies of various invertebrate groups, the distribution of the invertebrate troglofauna is unknown. Moreover, although there has been considerable attention paid to identifying the stygofauna within the calcretes, little is known about the diversity of troglofauna.

## TROGLOFAUNA ASSOCIATED WITH THE WESTERN AUSTRALIAN CALCRETES

Troglofauna are communities of terrestrial animals found in humid and dark underground habitats such as geologic or geomorphic environments, air-filled caves and smaller subsurface cavities. Terrestrial subterranean fauna are classified into three categories; trogloxene, troglophile and troglobite. Trogloxenes are animals usually found in subterranean environments that must leave it during some periods to fulfil biological requirements such as to obtain food and for reproduction. Troglophiles are facultative inhabitants of subterranean environments which are able to live in both hypogean and epigean habitats. The third category, troglobites (troglobionts), is a term used to describe subterranean species, both terrestrial and aquatic taxa that are obligate subterranean inhabitants, not able to survive in epigean environments (Trajano, 2005; Culver & Pipan, 2009). Troglobites possess specific characteristics such as loss/reduction of eyes, loss of pigmentation, loss of wings in pterygote hexapods, elongation of appendages, slender body form, increased life span, low metabolism (in some species), decrease in the number of eggs, increase in egg volume, increase in extra optical sensory structures (Gibert & Deharveng, 2002; Culver & Pipan, 2009). Christiansen (1962) suggested the term, troglomorphy, to describe the traits under convergent evolution in subterranean inhabitants. Troglomorphies comprise both losses/reduction (referred to as regressive evolution) as well as the gaining of traits.

In general, different taxonomic groups such as gastropods, isopods, beetles, spiders, turbellarians, millipedes, amphipods, decapods, collembolans, diplurans and pseudoscorpions have been recognized to include troglobitic species (Barr & Holsinger, 1985). Previously, terrestrial subterranean faunas were considered to show low diversity in environments with arid or semi arid climates. However, further investigations in different parts of Australia have indicated that arid and semi-arid zones comprise a significant diversity of troglofauna (Edward & Harvey, 2008). Western Australia has surprisingly shown a spectrum of subterranean habitats associated with troglofauna, with recent environmental impact assessment surveys revealing diverse troglobitic assemblages in non-karstic terrains of the Pilbara region. Eberhard *et al*. (2008, 2009) reported a significant terrestrial subterranean fauna including arachnids (Schizomida, Araneae, Pseudoscorpionida, Palpigrada), myriapods (Diplopoda, Chilopoda, Symphyla, Pauropoda), hexapods (Diplura,

Thysanura, Coleoptera, Hemiptera, Blattodea) and crustaceans (Isopoda) in the iron-ore rocks of the Pilbara region. They asserted that the high level of troglobitic richness in their surveys was comparable to or more than that found in karst terrains and lava tubes. In addition, short-range to regionally widespread distribution patterns were reported for the collected species. For example, five new species of the genus *Tyrannochthonius* and three new species of *Lagynochthonius* (Pseudoscorpiones: Chthoniidae) were described from surveys of the Pilbara region, the Gasgoyne region and Barrow Island, all possessing typical troglomorphic traits such as loss of eyes and attenuated appendages (Edward & Harvey, 2008). Except for *T. aridus* Edward and Harvey 2008, which is epigean and relatively widespread, all the new troglobites appear to show short-range endemism since they have been collected only from a few localities and may be localised to limestone habitats or mesa formations. Barranco and Harvey (2008) reported for the first time a new species of *Eukoenenia* from the subterranean environments of the Yilgarn region. This new palpigrade, *E. guzikae* Barranco and Harvey 2008, was shown to be the first indigenous member of the order Palpigradi in Australia. Numerous taxonomic groups including Isopoda (Philosciidae, *Troglarmadill*o sp.), Symphyla, Diplura (Japygidae, Parajapygidae, Campodeidae) and Coleoptera (Curculionidae) have been identified from the Carina and other iron ore deposits of the Yilgarn region (Bennelongia, 2009), and all are likely to contain large numbers of new species.

Taiti and Humphreys (2008) reported 28 new species of troglobitic and stygobitic oniscidean isopods from Western Australia including the Pilbara, Cape Range, Nambung, Augusta, Yilgarn and Nullarbor areas. Of these, 13 species of *Haloniscus* (Philosciidae) were recognized from the Yilgarn region, each of which was restricted to a single calcrete body, thus supporting the “subterranean island” hypothesis. Cooper *et al*. (2009) conducted preliminary research on the groundwater calcretes of central Western Australia to explore their troglobitic diversity. A rich array of invertebrate groups of troglobites were recorded including pseudoscorpions, spiders, mites, palpigrades, beetles, silverfish, plant hoppers, cockroaches, millipedes, centipedes, springtails and slaters/sowbugs. Accordingly, the DNA barcoding analyses of the collected oniscideans, based on Cytochrome C Oxidase subunit I gene (*COI*) sequences, revealed 22 divergent lineages, each of which was confined to a single groundwater calcrete body. These initial findings were considered as a basis to develop the

present study with more sampling coverage of the calcrete aquifers, using more molecular and morphological data required to explore the diversity of the oniscidean troglofauna.

## COLONISATION AND EVOLUTION OF SUBTERRANEAN FAUNA

Two general hypotheses, referred to as the climatic relict hypothesis (CRH) and adaptive shift hypothesis (ASH) have been proposed to explain the evolution of troglobites/stygobites. According to the CRH, an ancestral epigean population, pre-adapted to subterranean life, invades underground habitats as a result of extreme and inhospitable surface conditions (for example, aridification or glaciation) while the remaining epigean populations migrate or become extinct. Following a long period of isolation and acquiring specific adaptations to subterranean environments such as caves or other geological refuges, the hypogean populations become distinct troglobitic species as a result of allopatric speciation (Peck & Finston, 1993; Desutter-Grandcolas & Grandcolas, 1996; Danielopol & Rouch, 2005). Adaptive shift is a phenomenon in which an ancestral species living on the surface, but pre-adapted to subterranean life, may actively exploit hypogean habitats as new resources once they become available. The subterranean population evolves in parapatry with the surface population, with selection driving its adaptation to the hypogean habitat. These adaptations further restrict gene flow at the boundary of the two populations ultimately leading to parapatric speciation (Howarth, 1980, 1987; Howarth & Houch, 2005).

Desutter-Grandcolas & Grandcolas (1996), by studying different combinations of the present-day distribution of epigean and troglobitic species, speciation modes (allopatric and parapatric) and past environmental events, developed eight scenarios for the evolution of troglobitic life. They hypothesised that each allopatric and parapatric present day distribution could potentially have happened under four different variables, including speciation modes (parapatric/allopatric speciation) and favourable/unfavourable past environmental conditions. The same authors argued that the CRH and ASH are not the only hypotheses to explain subterranean evolution, but merely two among eight possible scenarios.

Distribution of subterranean fauna throughout underground habitats is shaped by two biogeographic factors including dispersal and vicariance. Dispersal is the movement of

organisms from one area to another causing range expansion/change in natural distribution of species, while vicariance is the separation of populations by a geographic barrier which accordingly leads to the occurrence of closely related species in separated areas (Humphries & Parenti, 1999). Holsinger (1991), in a review of subterranean amphipods, suggested that their limited dispersal ability and local endemism make vicariance a better explanation for the evolution of amphipods, than dispersal. However, Holsinger suggested further biogeographic analyses of other subterranean crustacean groups, such as isopods, should be carried out to determine whether there is congruence in phylogenetic and distribution patterns among these groups to lend support to the vicariance hypothesis.

According to Cooper *et al*. (2007), two modes of evolution, namely dispersal/vicariance and independent colonization by surface ancestors, could be hypothesised for stygobitic amphipods of central Western Australia. On the basis of the first model, populations of stygobitic amphipods colonized calcrete aquifers following underground dispersal during a wet period of the Miocene and subsequently became isolated within calcretes by vicariance events (aridification). The second hypothesis predicts that each groundwater calcrete was colonized separately by independent epigean ancestors and the isolated populations finally evolved subterranean characteristics. Molecular data and features of the environment such as the current physical structure of the matrix between the groundwater calcretes, which is postulated as a barrier to gene flow, support the second hypothesis, that several aquatic surface ancestors colonized the calcretes and, accordingly, became isolated within the calcretes following aridification. In contrast, a dispersal/vicariance model of colonization has been proposed for the European amphipod *Niphargus virei* Chevreux 1896, in France, with phylogeographic analyses showing cryptic diversity, fragmentation events and recent dispersal via vicariant barriers (Lefébure *et al*. 2006). Culver *et al*. (2009) found evidence for the importance of both vicariance and dispersal (Figure 1.4) in determining spatial patterns of stygobitic karst fauna in Europe and the United States. The same authors stated the relative importance of each model varies from region to region.

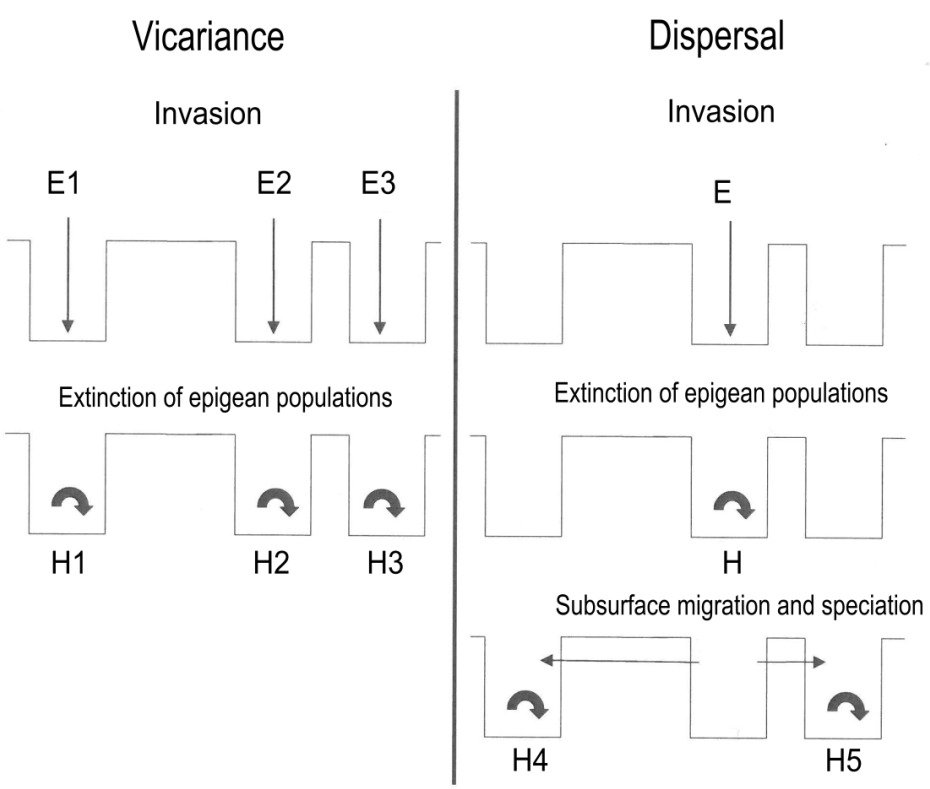


**Fig. 1.4.** Vicariance and dispersal models of speciation in caves. According to the vicariance model, three conspecific epigean populations (E1, E2 and E3) colonise the caves; following the extinction of epigean populations, the cave populations evolve into three distinct species. In the dispersal model, one epigean population (E) invades a cave and the epigean population becomes extinct; subsequently, the two other caves are colonised by subsurface dispersal and form separate species, under the assumption that dispersal events are rare (modified figure and description after Culver *et al*. 2009).

Although colonisation events and the associated vicariance and dispersal models for spatial patterns of stygobitic invertebrates in subterranean habitats have been relatively well documented in Western Australia and other parts of the world, the relative importance of the above models and the role of historical events in shaping the evolution of the invertebrate troglofauna in the groundwater calcretes of central Western Australia are unknown.

## AIMS OF PROJECT

The general aim of my project was to elucidate the systematics, diversity and evolution of isopod troglofauna occurring in groundwater calcretes of central Western Australia using both molecular and morphological approaches.

The specific aims were to:

1. Investigate the systematic relationships of the Western Australian oniscidean taxa attributed to Platyarthridae with platyarthrid type representatives and other oniscidean higher taxa using a multiple-gene phylogenetic approach and morphological analyses that include a Scanning Electron Microscopy study (Chapter II).
2. Examine the monophyly of the family Platyarthridae using representative taxa from Australia, South America, Africa and Europe (Chapter II).
3. Elucidate the diversification, phylogenetic relationships, species delineation and distributional patterns of oniscidean isopod troglofauna in groundwater calcretes of central Western Australia using a multiple-gene approach, with application of a Next Generation Sequencing approach for detection of new nuclear markers (Chapter III).
4. Elucidate the historical factors which have shaped the diversification, distribution and speciation modes of subterranean paraplatyarthrid isopods in the groundwater calcretes of central Western Australia using molecular phylogenetic analyses (Chapter IV).
5. Describe a selection of the new species of the new family Paraplatyarthridae (Chapter V).

In the last chapter (Chapter VI) a general discussion presents a synthesis of the research and broader implications of this study and discusses the limitations and requirements for future research.

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# CHAPTER II:

# MOLECULAR PHYLOGENY OF ISOPODS ATTRIBUTED TO THE PLATYARTHRIDAE (CRUSTACEA, ISOPODA, ONISCIDEA) REVEALS A NEW SOUTHERN HEMISPHERE ONISCIDEAN FAMILY

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This chapter will be submitted to either the Journal of Zoologica Scripta or PLOS ONE

## STATEMENT OF AUTHORSHIP

# Statement of Authorship

**Title of Paper:** Molecular phylogeny of isopods attributed to the Platyarthridae (Crustacea, Isopoda, Oniscidea) reveals a new Southern Hemisphere oniscidean family

**Publication Status:** Publication style

**Publication Details:** This chapter is going to be submitted to the Journal of Zoologica Scripta

## Author Contributions

By signing the statement of authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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**Contribution to the Paper:** Carried out both morphological and molecular lab experiments, analysed sequence data, produced morphological drawings and figures and wrote the manuscript.

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## ABSTRACT

The family Platyarthridae (Isopoda: Oniscidea)**,** with seven recognised genera, has a worldwide geographic distribution. Although the genera share some common characters, the monophyly of these genera is doubtful as the characters seem inadequate, owing to convergent evolution, to characterize a phylogenetically solid group. In order to test the monophyly of Platyarthridae, representatives of the main genera were analysed using molecular and morphological approaches including data from a Scanning Electron Microscopy (SEM) study. The in-group examined comprised *Trichorhina* spp. (Australia, South America), *Niambia* spp. (Australia, Africa), *Platyarthrus* spp. (Europe), and species of non-platyarthrid families including Armadillidae, Philosciidae, Armadillidiidae, Stenoniscidae, *Haloniscus* spp., Oniscidae, Detonidae, Styloniscidae, Trichoniscidae and Ligiidae. Combined and individual Bayesian phylogenetic analyses of the group using two nuclear (*SSU rRNA* (776 bp), *LSU rRNA* (891 bp)) and one mitochondrial gene (*Cytochrome C Oxidase subunit I* (677bp)) strongly showed Platyarthridae was not monophyletic, but the group comprises multiple divergent clades that are distinct from those represented by the non-platyarthrid families. The phylogenetic analyses recovered a strongly corroborated clade that is clearly distinguishable from platyarthrid species based on both morphological and molecular evidence. The clade is morphologically diagnosed on the basis of a combination of characters including setae ornamentation on the dorsal body, the structure of the second antenna, the fusion of postfrons and profrons in the cephalothorax, and tooth characteristics on the outer endite of the first maxilla. Based on these findings a new southern hemisphere oniscidean family, Paraplatyarthridae, is proposed which occurs in the subtropical and arid regions of Western Australia and South America.

**Keywords:** Oniscidean isopods, Phylogeny, Paraplatyarthridae, Systematics, Western Australia.

## INTRODUCTION

Oniscidean isopods (Crustacea, Malacostraca, Peracarida), are one of the largest isopod suborders with over 3,600 described species (*sensu* Schmalfuss, 2003) classified into 33 families (Gruner, 1993). Oniscidean species are unique among the crustaceans by being fully adapted to terrestrial life, with diagnostic features of the suborder including the presence of complex water conducting systems (Verhoeff, 1920; Hoese, 1981, 1982; Wägele, 1989). Even though the monophyly of terrestrial isopods is supported by morphological investigations (Schmalfuss, 1974, 1989), the relationships of oniscidean groups to one another and to marine relatives are still poorly understood (Martin & Davis, 2001). Crinochete oniscideans (Crinocheta Legrand 1946), comprising about 2,750 species in 28 families are the largest and most diverse group of oniscideans. They are considered to be a well-supported monophyletic group based on several synapomorphies associated with the muscular structure of pleons, and the structure of the male copulatory apparatus, marsupium, mouthparts and stomach (Schmidt, 2008).

Platyarthridae Verhoeff 1949 (Oniscidea Latreille 1802, Crinocheta), with over 120 nominal species, has been widely reported from tropical, subtropical and temperate regions, in substrates such as leaf litter, rotten wood and moist soil. The systematics of the family is problematic, however, seven genera are currently recognized: *Platyarthrus* Brandt 1833 (Palaearctic except for one species occurring in India), *Trichorhina* Budde-Lund 1908 (Central and South America, Africa, Europe, Burma to Australia), *Lanceochaetus* Schmalfuss and Ferrara 1978 (Africa), *Niambia* Budde-Lund 1904 (mainly in Africa), *Gerufa* Budde-Lund 1909 (Africa), *Echinochaetus* Ferrara and Schmalfuss 1983 (Africa), and *Cephaloniscus* Ferrara and Taiti 1989 (Malaysia).

The family is generally recognised by the two-jointed flagellum, the presence of modified scale-setae on dorsal segments, absence of pleopodal lungs, and an inability to roll up. However, the monophyletic status of the family is considered doubtful by some authors as these characters are thought to have evolved independently within other oniscidean isopod groups (Ferrara & Taiti, 1989; Mattern, 2003; Schmidt, 2003). Despite several cladistic revisions, the family continues to be poorly defined. Collinge (1943) suggested a distinct family, Trichorhinidae, for the genera *Trichorhina*, *Gedania* Budde-Lund 1912 (synonym of *Trichorhina*), *Bathytropa* Budde-Lund 1885 and *Calycuoniscus* Collinge 1915, but this was not

widely accepted. Vandel (1946) included the genera *Trichorhina*, *Calycuoniscus*, *Labyrinthasius* Verhoeff 1929 and *Platyarthrus* with two- or three-jointed second antennal flagellum in Squamiferidae Vandel 1946 (=Platyarthridae), while later authors considered those with a two-jointed second antenna (*Trichorhina*, *Platyarthrus*) to comprise a distinct family, Platyarthridae. Taiti *et al.* (1992) considered the family Bathytropidae Vandel 1952 as a possible synonym of Platyarthridae due to the lack of explicit morphological differences, and Schmidt (2008) found the Bathytropidae to be an artificial collection of taxa. Schmidt (2003) redefined the Squamiferae Vandel 1946 to comprise Platyarthridae, Dubioniscidae Schultz 1995 and Spelaeoniscidae Vandel 1948 based on several synapomorphies including shell-shaped tergal scale-setae with narrow bases, outer teeth group on the outer endite of the first maxilla broader than half of apical margin, and fusion of the distal two articles of the maxilliped palp. The same author proposed that Platyarthridae and Dubioniscidae were most probably paraphyletic, while Spelaeoniscidae constituted a monophyletic group with several autapomorphies.

Schmidt (2002) suggested a re-examination of *Trichorhina* species owing to incongruence in the position of noduli laterales, which are located close to the posterior margin of the tergites in *Trichorhina tomentosa* Budde-Lund 1893, whereas they are distant from the posterior margin in some other species of *Trichorhina*, the latter potentially being distantly related species. Schmidt (2002) also noted that as gland pores have been reported on the coxal plates of *Niambia* species, their classification within Platyarthridae also requires reassessment.

### *EVOLUTION OF THE WATER CONDUCTING SYSTEM AND LUNGS (PSEUDOTRACHEAE)*

The retention of water, or fluid to moisten pleopods, which are the gas-exchange organs, and preventing its loss through physiological and morphological means are the most important adaptations of oniscideans to terrestrial life. In platyarthrids, coxal plates and pleonal epimera (3-5) are enlarged, making individuals capable of sticking their bodies firmly to the substratum (typically referred to as a “clinger” type; Schmidt, 2002), except for some *Platyarthrus* species which are “creepers” (personal communications, S. Taiti). This modification is thought to effectively decrease water loss through evaporation by enclosure of air between the ventral side of the animal and the substrate. Another prominent morphological feature, well-developed in Oniscidea, is the water conducting system, which

is considered a synapomophy of all oniscidean isopods (Verhoeff, 1920; Hoese, 1981, 1982; Wägele, 1989). The water conducting system is composed of a series of interconnected channels of scale rows on the ventral side of the coxal plates, linking the bilateral maxilliped glands in the cephalothorax to an area of the first pair of pleopods, through which fluids (water and urine) are transmitted via capillary action (Carefoot, 1993). Hoese (1981, 1982) distinguished two types of water conducting systems within the Oniscidea; open and closed. In the open conducting system (*Ligia*-type), which occurs in Ligiidae Leach 1814, Tylidae Dana 1852, Synocheta Legrand 1946 and Mesoniscidae Verhoeff 1908 there are scale rows on pereopods 6 and 7 where water moves by capillary action to join the ventral scale row channels. In the closed system (*Porcellio*-type), which is present in most Crinocheta, water conduction involves the ventral channels as well as dorsal exoskeletal articulations that circulate fluid (urine) at each articulation from one ventral channel to the other. Despite numerous studies on the morphology and taxonomy of terrestrial isopods, the evolution of the water conducting system is poorly known.

In terrestrial isopods respiration takes place primarily within the pleopods which are five pairs of biramous appendages (Schmidt & Wägele, 2001). In putatively primitive forms of Oniscidea, respiration is performed through the pleopodal endopods which function similarly to gills (Verhoeff, 1917, 1920; Hoese, 1983; Kummel, 1984). In Oniscidea adapted to terrestrial life, specialized structures for respiration, called lungs or pseudotracheae, have evolved on the pleopod exopods. Major types of lungs reported in Oniscidea include: a) uncovered lungs, considered as the simplest type, which are composed of a wrinkled respiratory area on the dorsal surface of the exopods directly exposed to the air, b) partially uncovered lungs, where the rest of the pulmonary area develops into the walls of the exopods, and c) covered lungs (monospiracle and polyspiracle), which are internal tubuliform cuticular respiratory areas deeply wedged into the exopod walls and in connection with the exterior via spiracles (Paoli *et al.* 2002). In species of Ligiidae, Platyarthridae, Mesoniscidae, Stenoniscidae Verhoeff 1908 and Trichoniscidae Sars 1899 lungs are absent and it has been proposed that lungs in Platyarthridae were probably lost secondarily (Mattern & Schlegel, 2001; Schmidt & Wägele, 2001). The genus *Niambia* is the only representative of Platyarthridae containing a distinct respiratory area of uncovered lungs on all pleopodal exopods of most species (e.g. Schmalfuss & Ferrara, 1978; Ferrara & Taiti, 1981; Taiti & Ferrara, 1991, 2004). Recently, it has been suggested that lungs have

evolved multiple times independently within oniscidean isopods (Ferrara *et al*. 1994; Taiti *et al*. 1998) so their presence or absence is not regarded as a suitable character for reconstruction of phylogenetic relationships (Mattern & Schlegel, 2001; Schmidt & Wägele, 2001).

Detailed surveys in recent years have identified a diverse assemblage of terrestrial oniscidean isopods inhabiting the subterranean voids above the water table in groundwater calcrete (carbonate) aquifers of central Western Australia. These calcretes act as isolated 'subterranean islands' for a variety of invertebrate species, including dytiscid beetles, amphipods, isopods and bathynellids (Cooper *et al*. 2002; Leys *et al*. 2003; Cooper *et al*. 2007, 2008; Guzik *et al.* 2008, 2009; Humphreys *et al*. 2009; King *et al*. 2012). Numerous oniscideans recently collected from these calcretes were provisionally ascribed to the genus *Trichorhina* (Platyarthridae) but their identity remained uncertain. This study examines the systematic relationships of these Western Australian taxa using a multiple gene phylogenetic approach and morphology that include detailed Scanning Electron Microscopic examination of the water conducting system. The hypothesis that the Platyarthidae form a monophyletic group is tested using representative taxa from Australia and other continents. Subsequently, owing to the robust position of the Australian/South American taxa within the phylogeny and their significant divergence from Platyarthridae, a new family based on a new genus and species is described.

## MATERIAL AND METHODS

### *COLLECTING METHODS*

Oniscidean species used in this study were collected using several techniques. Subterranean species from groundwater calcretes of central Western Australia (Table 2.3) were collected using slotted PVC pipes filled with sterilized leaf litter, which were left in non-lined mineral exploration boreholes (1-3 m underground) for 6 to 12 months for colonisation by troglobitic fauna. After recovery of the traps, their contents were sealed in zip lock bags and transported to the Western Australian (WA) Museum for processing. Isopod samples were extracted from the leaf litter using Tullgren funnels and specimens were preserved in 100% ethanol. After transporting the preserved samples to the Adelaide laboratory, the specimens were kept in a -20°c freezer.

Surface terrestrial isopod species were collected by hand from different habitats including arid and temperate regions in Western Australia. The sampling was carried out by searching under stones, rotten wood material beside trees/shrubs and specifically in crevices of broken tree trunks where they were found frequently. *Ligia* Fabricius 1798 specimens were also caught by hand from a rocky shore beach in Rapid Bay, South Australia while *Deto marina* Chilton 1884 was sampled from a sandy beach in Hallet Cove, SA. All specimens were preserved in 100% ethanol and kept in a -20° freezer prior to DNA extraction. Collection data, vouchers and accession numbers of species used both for molecular and morphological work are listed in Table 2.3. Some oniscidean species used only for morphological studies (old material-DNA degraded) were kindly donated by colleagues (see acknowledgments).

### *DNA EXTRACTION AND SEQUENCING*

Three to six pereopods (except for male pereopod 7 which is important for morphological diagnosis) were dissected from 100% ethanol-preserved animals and rinsed in 10 mM Tris to remove alcohol before the extraction process. Total genomic DNA was isolated using a Puregene Genomic DNA Purification Kit (Gentra systems, www.gentra.com) according to the manufacturer's instructions (DNA purification from 5-10 mg fresh or frozen solid tissue) with the following minor modifications. For DNA precipitation samples were centrifuged at 12500 rpm for 20 min and 5 min (the step containing 70% ethanol), respectively.

To obtain partial sequences of mtDNA, Cytochrome C Oxidase subunit 1 (*COI*) gene ~677 bp were amplified using the universal primers LCO1490_t1 and HCO2198_t1 (designed by Robin M. Floyd at BOLD: The Barcode of Life Data system; see Table 2.1). PCR amplification of all *COI* sequences involved an initial denaturation at 95°C for 10 min and 34 subsequent cycles of 94°C for 45 sec, 48°C for 45 sec, 72°C for 1 min and a single final extension of 72°C for 10 min, followed by a 2-min hold time of 25°C. For the sequencing reactions M13F and M13R primers were used (Messing, 1983).

Small Subunit Ribsomal rRNA (*SSU rRNA gene=18S*) is amongst the most frequently used nuclear marker for the reconstruction of deep phylogenetic relationships among animals. While in general the length of the *SSU rRNA* gene in most metazoans varies between 1800-1900 bp, the longest metazoan *SSU rRNA* gene reported to date (3537 bp) belongs to the oniscidean isopod, *Cubaris murina* Brandt 1833 (Mattern & Schlegel, 2001). Crease and

Colbourne (1998) reported the unusual length of this gene in the crustacean *Daphnia pulex* is due to hypervariable regions or expansions in V2, V4, V7 and V9 with increases in V4 and V7 being the largest. Hypervariable regions have been found useful for reconstruction of phylogenetic relationships in the genus *Cicindela* (Coleoptera) and the marine isopod family Serolidae (Vogler *et al*. 1997; Held & Wägele, 1998). Mattern (2003), who conducted a molecular study based on the entire *SSU rRNA* gene to examine relationships within Oniscidea, discussed how the conserved regions are useful for elucidation of basal phylogenetic splits within Oniscidea and Crinocheta, whereas variable regions are better suited for analysis of branching patterns between closely related species. Approximately 776 bp including core and variable regions C1, V1, C2, V2 and C3 of the nuclear *SSU rRNA* gene (*18S*) were amplified using 18s1.2F and 18sb5.0 primers (Whiting, 2002; Table 2.1). The PCR amplification profile was set for a 10-min single initial denaturation at 95°C; 34 cycles of 45 sec at 94°C, 45 sec at 50°C, 1 min at 72°C and a single final elongation cycle of 6 min at 72°C followed by a hold time step of 25°C for 2 min.

The sequences (~891 bp) of the nuclear *LSU rRNA* gene (*28S*) surrounding D1 to D3 were amplified using universal primers 28srD1.2a and 28srd4.2b (Whiting 2002; Table 2.1). For those samples that failed to PCR, new primers G2281 and G2282 (Table 2.1) were designed for PCR and sequencing amplifications. Cycling conditions for the *28S* sequences consisted of one single cycle at 95°C for 10 min, 34 cycles of 94°C for 45 sec, 50°C (for universal primers) to 55°C (for new primers) for 45 sec, 72°C for 1 min and a single step 6-min final extension of 72°C followed by an incubation stage at 25°C for 2 min.

All PCRs were carried out on either a Palm-Cycler thermal cycler (Corbett, CG1-96) or Kyratec Supercycler thermal cycler (SC300) using 25 µl reaction volumes consisting of 15.4 µl of nuclease-free molecular water, 5µl of 5 x Immolase PCR buffer (comprising 3.75 mM $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphae (dNTP) and 2.5X BSA (0.25mg/ml)), 1 µl of each primer (5 µM concentration for *COI* and *18S* primers, 10 µM concentration for G2281 and G2282, 7 µM for 28srD1.2a and 5 µM for 28srd4.2b), 0.1µl of Immolase DNA polymerase (concentration of 5u/µl) and 2-2.5 µl of ~1µg/ml DNA. Amplified PCR products were then identified using 1.5% agarose gel electrophoresis in 1 x TBE. A Millipore Multiscreen Vacuum Manifold with a 96-well PCR and SEQ multiscreen filter plates were used to clean up the PCR and sequencing products. Purified PCR products were sequenced in both directions using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Applied

Biosystems) and analyzed on an ABI 3700 DNA capillary sequencer. Sequences were edited in Geneious Pro version 5.6.4.

**Table 2.1.** Primers used for amplification of *COI*, *18S* and *28S* in the oniscidean Isopods.

| Primer | Direction | Gene | Sequence (5'-3') |
|---|---|---|---|
| LCO1490_t1 | F | *COI* | TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG |
| HCO2198_t1 | R | *COI* | CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA |
| M13F | F | *COI* | TGTAAAACGACGGCCAGT |
| M13R | R | *COI* | CAGGAAACAGCTATGAC |
| 18s1.2F | F | *18S* | TGCTTGTCTCAAAGATTAAGC |
| 18sb5.0 | R | *18S* | TAACCGCAACAACTTTAAT |
| 28srD1.2a | F | *28S* | CCCSSGTAATTTAAGCATATTA |
| 28srd4.2b | R | *28S* | CCTTGGTCCGTGTTTCAAGACGG |
| G2281 | F | *28S* | GSGATGCCGCGTWTGGGAGN |
| G2282 | R | *28S* | TTCACCGTCBVAGAGGCCGT |

### *ALIGNMENT, TAXON SAMPLING AND PHYLOGENETIC ANALYSES*

As the *18S* gene consists of regions with different rates of sequence divergence including slow-evolving (core/conserved regions) and fast evolving (variable) regions, a four-step process was followed for alignment of the sequence data. The whole dataset was first aligned using ClustalW (gap opening cost of 9, gap extension cost of 3, an IUB cost matrix and free end gaps) within Geneious Pro 5.6.4, which led to the alignment of the conserved regions. In the next step the preliminary alignment was aligned to an annotated oniscidean *18S* alignment to identify the core and variable regions (Mattern & Schlegel, 2001). After identifying the conserved (for ease of use, C1, C2 and C3 nomenclature was used for conserved regions in the alignment) and variable (V1 and V2) regions an additional regional alignment was applied for the variable regions using the Muscle alignment plug-in for Geneious Pro 5.6.4 (according to the default settings). In the last step the alignment was checked by eye and refined manually where there were obviously incorrectly aligned positions such as displaced single nucleotides considering the presence of known monophyletic taxa in the alignment. No gene positions or nucleotide regions were removed from the alignment. *COI* and *28S* alignments were generated in ClustalW with the same opening and extending gap penalties as for *18S*. The Domain regions in the *28S* isopod alignment were identified manually using the annotated *28S* sequence for *Drosopohila melanogaster* (Tautz *et al.* 1988).

For the individual *18S* phylogenetic analysis, the in-group comprised 14 oniscidean genera belonging to 10 known families. To examine the monophyly of Platyarthridae, the examplar species included nine subterranean/surface species ascribed to Platyarthridae from Western Australia (one additional species, not included in the molecular study, from Jorgensen Park, Kalamunda, WA was just subjected to Scanning Electron Microscopy experiments), *Trichorhina tomentosa*, which is the type species of the genus and the main representative of Platyarthridae (N.B. the morphological description of the family is based on this species, Schmidt, 2003), one new species of *Trichorhina* from Brazil, *Platyarthrus hoffmannsegii* Brandt 1833, the type species of *Platyarthrus* from Italy, and two species of *Niambia* from Botswana (Africa) and Australia. Also a number of additional oniscidean sequences were included in the *18S* analysis to expand the family/genus representation and were either sequenced as part of this study or were downloaded from the NCBI GenBank database. These included representatives of Armadillidae Brandt & Ratzeburg 1831, Armadillidiidae Brandt 1833, Detonidae Budde-Lund 1906, Ligiidae, Oniscidae Latreille 1802, Philosciidae Kinahan 1857 (including *Philoscia muscorum* Scopoli 1763, *Haloniscus* spp., and an unknown genus/species from Australian, Taxon 11), Stenoniscidae, Styloniscidae Vandel 1954 and Trichoniscidae (see Table 2.3). Four crustacean species available on Genbank for *18S*, *Orchestia* sp., *Gammaracanthus lacustris* Sars 1867 (Amphipoda), *Heterocarpus* sp. (Decapoda) and *Brevisomabathynella magna* Cho & Humphreys 2010 (Parabathynellidae) were selected as out-groups.

For the phylogenetic analysis of the combined data (*COI, 18S* and *28S*), the number of in-group taxa was reduced to eight known oniscidean families (10 genera) as the three molecular markers were not available for all in-group taxa. No likely outgroups were available on Genbank for all three genes and so *Sphaeroma serratum* Fabricius 1787 (Isopoda, Sphaeromatidea) was selected and sequenced for *COI, 18S* and *28S* as part of this study. This species was also used as the out-group for the individual *28S* analysis. Genbank accession numbers are included in Table 2.3, and new sequence data provided as supplementary files. The same taxa used for the combined phylogeny were used for the individual *28S* phylogenetic analysis. The individual *28S* phylogeny is not shown here as the number of taxa was the same as in the combined analysis, but its topology is described for nodes relevant to the current study. Additional platyarthrid species, including *Platyarthrus aiasensis* Legrand 1954 (Italy), *P. costulatus* Verhoeff 1908 (Italy) and *Trichorhina*

*anophthalma* Arcangeli 1936 (Portugal), could not be used for molecular analysis as their DNA was too degraded, but they were subjected to morphological examinations.

In order to estimate the best nucleotide substitution model for the presumed data partitions, MrModeltest 2.3, which is a modified version of Modeltest 3.6 (Posada & Crandall, 1998), under an Akaike Information Criterion (Posada & Buckley, 2004) framework was used. Nucleotide models were selected for all data subsets; GTR+I+G (Rodrígue *et al.* 1990; Yang, 1996) for 1$^{st}$ (1) and 2$^{nd}$ (2) codon positions of *COI*, combined 1$^{st}$-2$^{nd}$ *COI* codon positions (1,2), full *COI* (1,2,3) and combined *COI-18S-28S* (1,2,3,4,5,6), HKY+G (Hasegawa *et al.* 1985; Yang, 1996) for *COI* 3$^{rd}$ position (3), SYM+I+G (Zharkikh 1994; Yang, 1996) for full *18S* (4,5), while SYM+G (Zharkikh, 1994; Yang, 1996) and GTR+G (Rodríguez *et al.* 1990; Yang, 1996) were chosen for the *18S* core components C1-C2-C3 (4) and *18S* expansion elements V1-V2 (5), respectively. A GTR+G (Rodríguez *et al.* 1990; Yang, 1996) model was found to be the most appropriate model for *28S* (6) and combined *18S-28S* (4, 5, 6). Garli 2.0-win (Zwickl, 2006), which performs phylogenetic searches using the Maximum Likelihood (ML) criterion was used to examine the best partitioning scheme for the dataset. Eleven different partitions of *COI* first, second and third base codon positions, core and variable regions of *18S* (C1-C2-C3, V1-V2) and *28S* were examined to calculate the lnL and AIC index for each partition (Table 2.2).

To run individual partitioned models of ML for each scheme the Garli configuration file was set for two independent search replicates and all parameters were unlinked (except for partition 1 in which data subsets were assumed to be a single dataset). The subset-specific rate multiplier was set to vary over data subsets, and other settings of the Garli configuration file were according to the default. The likelihood scores of the two independent runs were computed and the greater likelihood score was chosen for calculation of AIC scores. The AIC score of each partitioning scheme was calculated as AIC = 2x (#parameters - lnL) and the lowest value was chosen as the best score.

**Table 2.2.** Garli partitioning schemes, lnL, number of parameters and AIC values. The numbers in the partitioning scheme column denote: 1, 2 and 3 for the *COI* first, second and third codon positions respectively; 4 and 5 for the core and variable regions of the *18S* gene respectively; 6 for the *28S* gene.

| Partitioning schemes | lnL | Parameters (#free parameters + (subsets-1)) | AIC |
|---|---|---|---|
| P1: (1,2,3,4,5,6) | -19325.64397 | 10 | 38671.28794 |
| P2: (1,2,3)(4,5)(6) | -18753.05649 | 10+7+9+(3-1)=28 | 37562.11298 |
| P3: (1,2)(3)(4,5)(6) | -18380.06583 | 10+5+7+9+(4-1)=34 | 36828.13166 |
| P4: (1)(2)(3)(4,5)(6) | -18296.9014 | 10+10+5+7+9+(5-1)=45 | 36683.8028 |
| P5: (1,2,3)(4,5,6) | -18901.43427 | 10+9+(2-1)=20 | 37842.86854 |
| P6: (1,2)(3)(4,5,6) | -18527.9075 | 10+5+9+(3-1)=26 | 37107.815 |
| P7: (1)(2)(3)(4,5,6) | -18432.12293 | 10+10+5+9+(4-1)=37 | 36938.24586 |
| P8: (1,2,3)(4,5,6) | -18901.43314 | 10+9+(2-1)=20 | 37842.86628 |
| P9: (1,2,3)(4)(5)(6) | -18597.46653 | 10+6+9+9+(4-1)=37 | 37268.93306 |
| P10: (1,2)(3)(4)(5)(6) | -18224.22739 | 10+5+6+9+9+(5-1)=43 | 36534.45478 |
| P11: (1)(2)(3)(4)(5)(6) | -18128.45071 | 10+10+5+6+9+9+(6-1)=54 | 36364.90142 |

According to Table 2.2, partition number 11 (P11), which treats each subset separately with the highest maximum likelihood score (-18126.42588) and lowest AIC value (36362.85176), was selected as the best partition scheme for phylogenetic analyses.

For phylogenetic analyses of the individual *18S*, *28S* and combined *COI-18S-28S* a Bayesian Inference approach was employed using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001). Posterior probabilities were used to examine the robustness of the nodes. The P11 partitioning scheme, with separate unlinked models for each of the six data partitions, was used for the Bayesian Inference (BI) analysis of the combined data. All parameters were unlinked and the rates were allowed to vary over the subsets. Two independent runs with four different chains were run simultaneously for five million generations, subsampling trees every 100 generations. In this Bayesian analysis, the final standard deviation of split frequencies reached 0.08% and PSRF values for all parameters were 1.0, suggesting convergence had occurred. For each independent MrBayes run, a 25%-burn-in, which equated to 12,500 samples, was discarded from the 50,001 samples obtained during the analysis (37,501 samples were included). To further assess convergence to the stationary distribution, the software package Tracer version 1.5 (Rambaut & Drummond, 2003) was used. The effective sample size (ESS) for all parameters of the combined runs

(run1 and 2) were between 1,047 (TL) and 60,732 (alpha2). A 50% majority rule BI consensus tree was constructed from the remaining trees and visualized using the program Fig Tree version 1.3.1 (Rambaut, 2009). Individual Bayesian analyses were also performed on *18S* (partitioned by core and variable regions, each with separate nucleotide substitution models), using a similar analysis to that given above (10 million generations, 25% burnin, unlinked models and rates variable over subsets) and *28S* data (one million generations and a 25% burnin value). The single Bayesian analyses converged on the stationary distributions with standard deviations of split frequencies of 0.21% for *18S* and 0.86% for *28S*. SPSS Statistics version 19 was used for GC content comparisons.

### *SCANNING ELECTRON MICROSCOPY (SEM)*

In order to dry specimens prior to SEM, depending on whether they were fresh or preserved in 100% ethanol, two different chemical methods were used. As most of the samples were brittle and susceptible to being damaged during any handling, an Electric point drier was not used. Freshly collected samples were fixed for 24 h in EM fixative (4% parafromaldeyde/1.25% glutaraldehyde in PBS, plus 4% sucrose, Ph 7.2). Samples were washed in a buffer (PBS + 4% sucrose) for 5 min then post-fixed in 2% $O_sO_4$ (osmium tetroxide) for 1 h. SEM samples were then dehydrated using 70% ethanol (2 changes of 10 min each), 90% ethanol (2 changes of 10 min each) and 100% ethanol (3 changes of 15 min each). Samples were placed in a 1:1 solution of HMDS (hexamethyldisilazane) and 100% ethanol for 10 min and then transferred to 100% HMDS for 2 changes of 10 min each. After removing the HMDS, samples were air dried (all the chemical treatments were done under a fume hood). For specimens that had been preserved in 100% ethanol a different drying procedure was employed. SEM samples were placed in small petri dishes of 1:1 solution of HMDS and 100% ethanol for 15 min. After discarding, the samples were immersed in 100% HMDS for three changes of 15 min, 15 min and 20 min, respectively. SEM samples were then air dried.

All dried samples and associated body parts were mounted on metal stubs using paper sticks and coated with carbon/gold. Philips XL20 and Philips XL40 instruments in Adelaide Microscopy (The University of Adelaide) were used to examine specimens.

**Table 2.3.** Taxonomy, geographic location and accession number of species examined (stars denote Genebank accession numbers for *18S*).

| Family | Species | Locality | Voucher number | GenBank accession number |
|---|---|---|---|---|
| Armadillidae | *Troglarmadillo* sp. 1 | Sturt Meadows calcrete, E Murchison, W. Australia | BES15550.1 | |
| Armadillidae | *Troglarmadillo* sp. 2 | Lake Miranda West calcrete, E Murchison, W. Australia | BES15537.4 | |
| Armadillidae | *Troglarmadillo* sp. 3 | Laverton Downs, Shady Well, E Murchison, W. Australia | BES15068.2 | |
| Armadillidiidae | *Armadillidium vulgare* | Adelaide, S. Australia | JA253-2012 | |
| Detonidae | *Deto marina* | Hallet Cove, S. Australia | JA259-2012 | |
| Ligidae | *Ligia oceanica* | Galicia, Spain | | *AF255698 |
| Ligidae | *Ligia* sp. | Rapid Bay, S. Australia | JA257-2012 | |
| Oniscidae | *Oniscus asellus* | Bielefeld, Germany | | *AF255699.1 |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 1 | Mt Morgans, E Murchison, W. Australia | JA103-2011 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 2 | Marradong, W. Australia | JA152-2011 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 3 | Sturt Meadows calcrete, E Murchison, W. Australia | BES15551.9 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 4 | Lake Miranda east calcrete, E Murchison, W. Australia | BES15543.3 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 5 | Lake Miranda west calcrete, E Murchison, W. Australia | BES15538.10 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus subterraneus* (genus & sp. n.) Taxon 6 | Laverton Downs Windarra calcrete, E Murchison, W. Australia | BES15524.6 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 7 | Cunyu calcrete, E Murchison, W. Australia | BES15090.1 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 8 | Uramurdah Lake calcrete, E Murchison, W. Australia | BES15088.1 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 9 | Lake Violet, E Murchison, W. Australia | BES16476.3 | |
| Paraplatyarthridae (family n.) | *Paraplatyarthrus* (genus n.) Taxon 10 | Porto, Alegre Belein Novo, RS, Brazil | Ja244-2011 | |
| Philosciidae | *Philoscia muscorum* | Leipzig, Germany | | |
| "Philosciidae" | *Haloniscus* sp. 1 | Laverton Downs Windarra calcrete, E Murchison, W. Australia | BES15094.2 | |

| | | | | |
|---|---|---|---|---|
| "Philosciidae" | *Haloniscus* sp. 2 | Lake Violet calcrete, E Murchison, W. Australia | BES15085 | |
| "Philosciidae" | *Haloniscus* sp. 3 | Lake Miranda east calcrete, E Murchison, W. Australia | BES15082 | |
| "Philosciidae" | *Haloniscus* sp. 4 | Uramurdah calcrete, E Murchison, W. Australia | BES15088.2 | |
| "Philosciidae" | Genus indet./ Taxon 11 | Douglas Scrub, S. Australia | JA249-2012 | |
| Platyarthridae | *Niambia* sp. 1 | Bostwana, Africa | JA245-2012 | |
| Platyarthridae | *Niambia* sp. 2 | Adelaide, S. Australia | JA225-2012 | |
| Platyarthridae | *Platyarthrus aiasensis* | Capo S. Marco, Sinis Peninsula, Sardegna, Italy | | |
| Platyarthridae | *Platyarthrus costulatus* | Giara di Gesturi, Sardegna, Italy | | |
| Platyarthridae | *Platyarthrus hoffmannseggi* | Monte Moreilo, N of Florence, Italy | Ja204-2011 | |
| Platyarthridae | *Trichorhina anophthalma* | De Santo António, Algar, Portugal | - | |
| Platyarthridae | *Trichorhina tomentosa* | Varginha Clube, MG, Brazil | JA248-2012 | |
| Porcellionidae | *Porcellionides pruinosus* | Laverton Downs, Mt Windarra, E Murchison, W. Australia | | |
| Stenoniscidae | Genus indet. | Laverton Downs, Mt Windarra calcrete, E Murchison, W. Australia | BES16023 | |
| Styloniscidae | *Styloniscus* sp. | Douglas Scrub, S. Australia | JA250-2012 | |
| Trachelipodidae | *Orthometopon dalmatinum* | Ionian Island, Greece | - | |
| Trachelipodidae | *Trachelipus cavaticus* | Crete Island, Greece | - | |
| Trichoniscidae | *Haplophthalmus danicus* | Leipzig, Germany | | *AJ287066 |
| Trichoniscidae | *Trichoniscus pusillus* | Leipzig, Germany | | *AJ287067 |
| **Out-groups** | | | | |
| Gammaracanthidae | *Gammaracanthus lacustris* | Savonranta, Finland | | *JF966191.1 |
| Pandalidae | *Heterocarpus* sp. | Taiwan | | *JF346257 |
| Parabathynellidae | *Brevisomabatynella magna* | Cunyu, W. Australia | | *JQ446078.1 |
| Sphaeromatidae | *Sphaeroma serratum* | Hallet Cove, S. Australia | JA263-2012 | |
| Talitridae | *Orchestia* sp. | Hallet Cove, S. Australia | JA261-2012 | |

## RESULTS

### *INDIVIDUAL AND COMBINED PHYLOGENETIC ANALYSES*

Thirty-two (*COI, 18S*) to 31 (*28S*) oniscidean species representing 10 known isopod families and a likely new family were sequenced (see Table 2.3 for Genbank accession numbers; new sequence data given in supplementary files). The Individual *18S* phylogenetic tree (Figure 2.1) was generated from a Bayesian Inference analysis. The monophyly of platyarthrid species including *Niambia* spp., *P. hoffmannseggii* and *T. tomentosa* was not supported, with a group including *Niambia* spp., and *P. hoffmannseggii* being more closely related to *Armadillidium vulgare* Latreille 1804 (Armadillidiidae), *Oniscus asellus* Linnaeus 1758 (Oniscidae) and *Philoscia muscorum* Scopoli 1763 (Philosciidae). A second group, henceforth referred to as the new family Paraplatyarthridae (described below), comprising nine Australian taxa and one South American species, was highly divergent from all other platyarthrid species. The Australian taxa were sister to the South American species with high posterior probability support (0.96). Interestingly, the new family is sister to a clade comprising Stenoniscidae and *Haloniscus* Chilton 1920 with a high posterior probability support value (0.95). The *18S* Bayesian phylogeny also provided strong support for the monophyly of Synocheta Vandel 1952 including *Haplophthalmus danicus* Budde-Lund 1880, *Styloniscus* Dana 1853 (identification is based on an individual female specimen) and *Trichoniscus pusillus* Brandt 1833. The section Diplocheta Vandel 1957, here comprising *Ligia oceanic* Linnaeus 1767 and *Ligia* sp., grouped outside a clade comprising Synocheta/Crinocheta. The individual *28S* BI phylogeny showed no major conflicting results with the phylogeny based on *18S*. The *28S* tree further supported the divergence of the new family from all included platyarthrid species. A clade with a 0.95 posterior probability support included all platyarthrid species (excluding Paraplatyarthridae) and *A. vulgare*, in which *Niambia* spp. and *P. hoffmannseggii* formed a sister group supported by a 0.97 posterior probability, while *T. tomentosa* (Platyarthridae) and *A. vulgare* formed a distinct sister clade of low support (0.69). The *28S* phylogeny also showed a highly supported sister relationship (1.00) between *Troglarmadillo* spp., and *D. marina*.

The species of *Haloniscus*, which are ascribed to Philosciidae Kinahan 1857 by some authors, were not monophyletic with *P. muscorum*. *Haloniscus* formed a highly supported clade (1.00) which was sister to an unknown genus attributed to Philosciidae (based on

morphology) while *P. muscorum* linked closely with *O. asellus* (Oniscidae). The clade comprising *Haloniscus* spp. and the unknown philosciid genus was placed as a sister to the family Stenoniscidae with high support (1.00).

The 50% majority rule BI tree based on the combined *COI*, *18S* and *28S* data (Figure 2.2) showed similar deep-level relationships to that found in the individual gene trees. As expected, support levels for nodes in the single gene phylogenies were all noticeably increased by combining the three gene data and applying a 6-partitioned model-based scheme. A clade with high posterior probability support (0.98) comprised all included platyarthrid species (excluding Paraplatyarthridae) and *A. vulgare*, in which *Niambia* spp. was closely related to *P. hoffmannseggii* (1.00). *A. vulgare* and *T. tomentosa* were respectively sister lineages to the platyarthrid clade, each with high posterior probability support (1.00, 0.98). Again the new family Paraplatyarthridae is highly divergent from all platyarthrid species and has closest affinity (1.00) to a clade including Stenoniscidae and *Haloniscus* spp. (1.00). In addition, the combined BI phylogeny suggests high support (1.00) for a sister relationship between *Troglarmadillo* spp. and *D. marina*. Further, Crinocheta and Synocheta sections are sister to each other with high support (0.99) while Diplocheta is a sister to the Crinocheta-Synocheta clade (1.00).

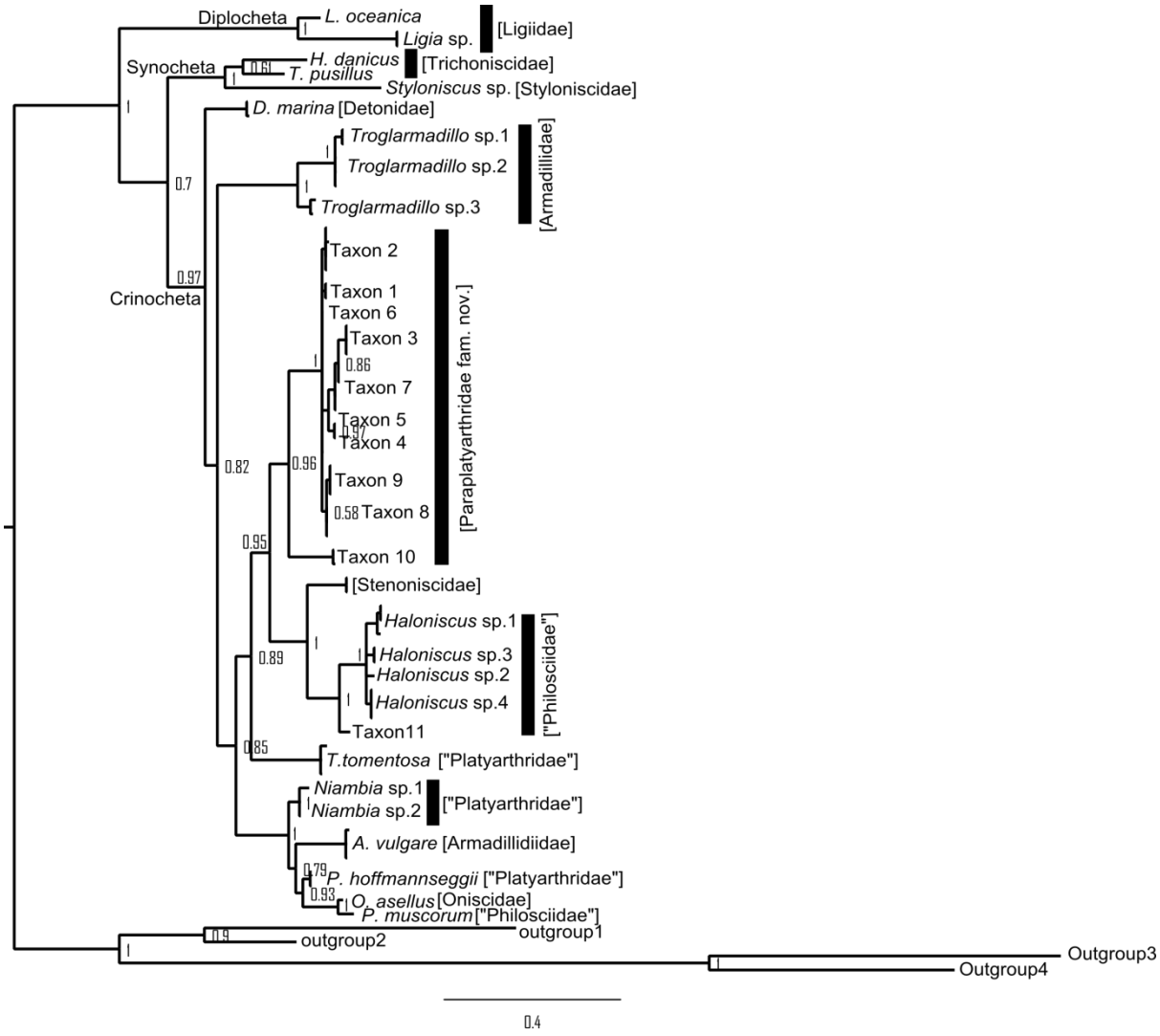



**Fig. 2.1.** The 50% majority rule posterior probability tree from the Bayesian Inference analysis of *18S* using 32 in-group species of 11 known/new oniscidean families, four crustacean out-groups and 776 bp positions of core (C1, C2, C3) and variable (V1, V2) regions. The families are shown in square brackets and those indicated in quotes are not monophyletic. Numbers adjacent to the nodes are Bayesian posterior probabilities. Outgroups 1, 2, 3, and 4 refer to *Heterocarpus* sp., *Brevisomabathynella magna*, *Gammaracanthus lacustris* and *Orchestia* sp. respectively.

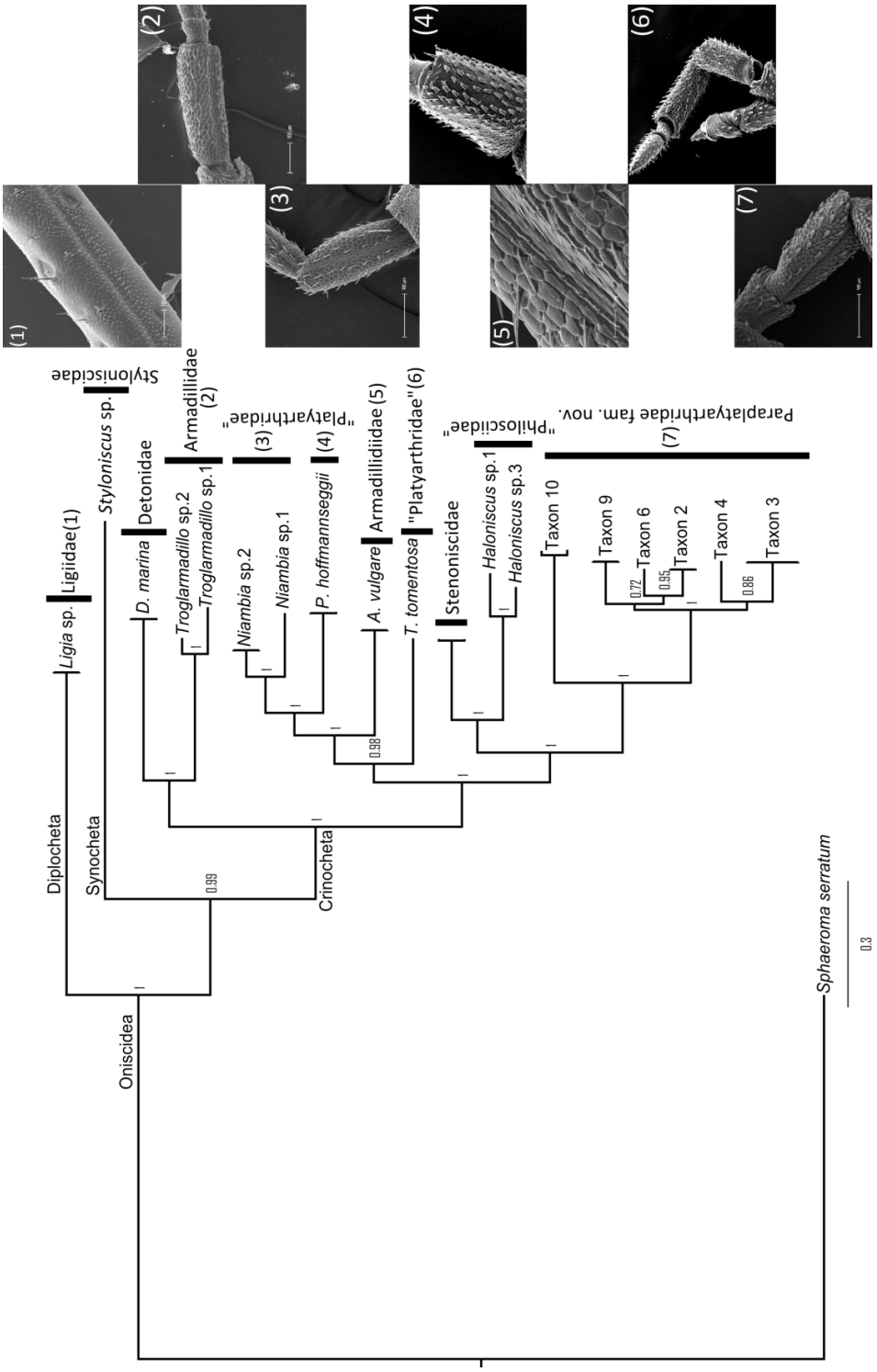



**Fig. 2.2.** A 50% majority rule posterior probability tree from a Bayesian Inference (BI) analysis comprising the mitochondrial gene (*COI*) and two nuclear ribosomal genes (*18S* and *28S*). Taxa include 20 in-group species belonging to 9 known/new oniscidean families and one species of Flabellifera as an out-group. The data comprised 2306 bp partitioned by three codon positions of *COI*, *18S* conserved and core regions and *28S* (6 partitions in total). The families are shown in square brackets and those indicated in quotes are not monophyletic. Numbers adjacent to the nodes are Bayesian posterior probabilities. The micrographs denote the second antenna structures.

### *V1/V2 LENGTH VARIATION, GC CONTENT AND MOLECULAR SIGNATURES*

The *18S* V1 region ranged from 13 bp in *D. marina*, *Styloniscus* sp. and *T. pusillus* to 52 bp in *L. oceanica*. There was no variation in the length of V1 among Australian species of the new family (30 bp) but not the South American taxon which was 38 bp (Table 2.4). On average, the V2 region is more than two times longer than V1. In oniscidean species the sequence length of the *18S* V2 region varied from 10 bp in *Styloniscus* sp. (the shortest region in expansion elements) to 76 bp in *D. marina*. In the new family the length of V2 ranged from 49 bp to 54 bp in Australian species while it was 59 bp in the South American species. The percentage GC content of the *18S* expansion elements was significantly more than that of the core regions ($t=4.94$, $P<0.05$). While the GC content was approximately constant across the *18S* core regions, it was variable in the expansion elements of the examined oniscidean species. The comparison of the two ribosomal genes showed that the total *28S* (D1-D3) is richer ($t=-13.59$, $P<0.05$) in GC than that of total *18S* (57.7% in *28S* compared with 51.2% in *18S*). In the *18S* alignment, considering that our dataset begins at the 52nd nucleotide from the start of the gene, there is a 19-21bp gap (Figure 2.3) in all sequences of Crinocheta and Synocheta in the C1 region relative to species of Diplocheta. Before and after the gap an immediate "TGT" sequence is probably a characteristic of Crinochete and Synochete isopods.

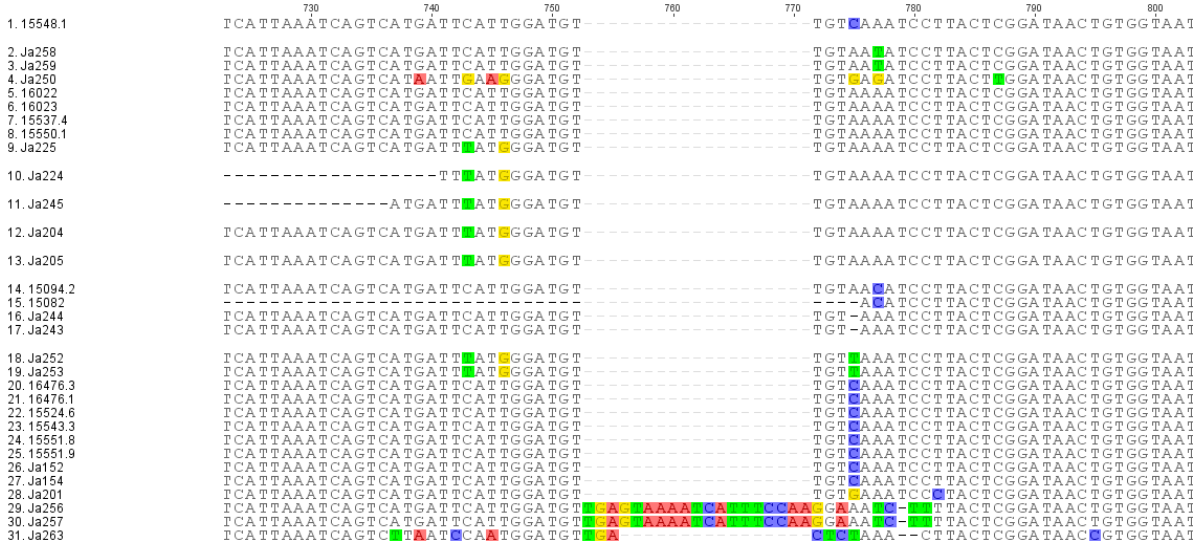



**Fig. 2.3.** Part of the alignment of *18S* core element (1) showing a ~21 bp gap in all examined Crinocheta and Synocheta species. In Diplocheta (*Ligia* sp., *L. oceanica*) this gap is covered by 19-21 nucleotides.

**Table 2.4.** Length of *18S* expansion elements and GC content of *18S* and *28S* in the species examined.

| Oniscidean species | ***18S* Expansion elements Length (bp)** | | **GC contents (%)** | | | |
|---|---|---|---|---|---|---|
| | V1 | V2 | *18S* Expansion regions | *18S* Conserved regions | *18S* total | *28S* total (D1-D3) |
| ***A. vulgare*** | 15 | 56 | 60 | 49.8 | 51 | 59.6 |
| ***D. marina*** | 13 | 76 | 46.1 | 50 | 49.5 | 54 |
| ***Haloniscus* sp. 1** | 19 | 59 | 47.4 | 49.7 | 49.4 | 51.9 |
| ***Haloniscus* sp. 2** | 19 | 60 | 45 | 51.9 | 51 | - |
| ***Haloniscus* sp. 3** | 19 | 62 | 44.3 | 50.5 | 49.6 | 54.2 |
| ***Haloniscus* sp. 4** | 19 | 62 | 45.7 | 51.5 | 50.6 | - |
| ***H. daniscus*** | 14 | 28 | 45.2 | 49.1 | 48.8 | - |
| ***Ligia* sp.** | 34 | 35 | 50.7 | 49.9 | 50 | 56 |
| ***L. oceanica*** | 52 | 36 | 60.2 | 48.8 | 50.3 | - |
| ***Niambia* sp. 1** | 17 | 65 | 64.6 | 50.5 | 52.5 | 57.5 |
| ***Niambia* sp. 2** | 17 | 66 | 61.4 | 50.2 | 52.1 | 58.3 |
| ***O. asellus*** | 15 | 64 | 62.8 | 50.6 | 52.2 | - |
| ***Philoscia muscorum*** | 14 | 65 | 63.3 | 50.2 | 51.8 | - |
| ***P. hoffmannseggii*** | 14 | 65 | 64.6 | 50.1 | 52 | 58.1 |
| **Stenoniscidae, unknown genus** | 21 | 71 | 54.3 | 50.9 | 51.5 | 59.2 |
| ***Styloniscus* sp.** | 13 | 10 | 60.9 | 49.8 | 50.3 | 58.4 |
| **Taxon 1 (fam. nov.)** | 30 | 49 | 65.8 | 50 | 52 | - |
| **Taxon 2 (fam. nov.)** | 30 | 49 | 65.8 | 50.3 | 52.2 | 58.1 |
| **Taxon 3 (fam. nov.)** | 30 | 49 | 67.1 | 50.5 | 52.6 | 57.3 |
| **Taxon 4 (fam. nov.)** | 30 | 53 | 66.3 | 50.2 | 52.3 | 58.7 |
| **Taxon 5 (fam. nov.)** | 30 | 51 | 65.4 | 50.2 | 52.1 | - |
| **Taxon 6 (fam. nov.)** | 30 | 49 | 65.8 | 50.2 | 52.1 | 57.8 |
| **Taxon 7 (fam. nov.)** | 30 | 49 | 67.1 | 50.4 | 52.6 | - |
| **Taxon 8 (fam. nov.)** | 30 | 54 | 66.7 | 51.8 | 54 | - |
| **Taxon 9 (fam. nov.)** | 30 | 54 | 66.7 | 50.4 | 52.7 | 57 |
| **Taxon 10 (fam. nov.)** | 38 | 59 | 50 | 51.4 | 51.2 | 60 |
| **Taxon 11** | 19 | 62 | 46.9 | 49.7 | 49.3 | - |
| ***Trichoniscus pusillus*** | 13 | 28 | 51.2 | 50.2 | 50.3 | - |
| ***T. tomentosa*** | 14 | 49 | 56.3 | 50.4 | 51 | 58.5 |
| ***Troglarmadillo* sp.1** | 23 | 38 | 55.7 | 50.1 | 50.7 | 59.7 |
| ***Troglarmadillo* sp.2** | 23 | 38 | 57.4 | 50.1 | 50.8 | 60.9 |
| **Average** | 22.87±9.1 | 52.8±14.96 | 57.5±8.2 | 50.3±0.65 | 51.2±1.24 | 57.7±2.2 |

### *STRUCTURE OF THE WATER CONDUCTING SYSTEM ON THE SECOND ANTENNAE*

Using SEM, it was discovered that a previously unreported structure, a furrow, exists on the antennal peduncle in Australian species of the new family, Paraplatyarthridae. This furrow contains elongated modified hair-like setae which possibly act as capillary setae (Figure 2.6A-B). No similar furrow was found in *T. tomentosa* (Figure 2.4A), *T. anophthalma* (Figure 2.4C), *P. hoffmannsegii* (Figure 2.4B), *P. aiasensis* (light microscopy) or *P. costulatus*

(light microscopy). In addition, in Australian paraplatyarthrid taxa the ventral side of the antennal peduncle possesses scale setae similar to those occurring on the dorsal body (Figure 2.6A). In addition, the distal article of the antennal flagellum possesses tightly-packed elongated setae, which are directed towards the apical cone (Figure 2.6C-D). In *Niambia* the capillary system on the second antennae comprises elongate tightly-packed setae, but no furrow is present (Figure 2.4E-F). No specific antennal capillary furrow occurs in *P. pruinosus* (Porcellionidae) (Figure 2.5E) except for longitudinal depressions along the second antenna, which does not seem to be homologous with the oniscidean capillary furrows. In *A. vulgare* (Armadillidiidae) the furrow is wider consisting of two rows of scale-like setae (not elongated), separated by stout pointed setae medially. In addition, the cuticular scales on the second antenna are sub-rectangular and do not overlap (Figure 2.4D). The capillary furrow in *Orthometopon dalmatinum* Verhoeff, 1901 (Trachelipodidae) has a distinct morphology in which capillary setae are elongated and arranged in three rows: on the peduncle, the setae on the medial row are straight whereas the two rows either side include elongate setae, which bend inwards over the medial row. The antennal furrow on the flagellum is not as deep as that of the peduncle and includes capillary setae which are set upwards (Figure 2.5A-B). *Trachelipus cavaticus* Schmalfuss, Paragamian & Sfenthourakis 2004 (Trachelipodidae) possesses a different type of capillary furrow in which the furrow is not demarcated by cuticular scales laterally, but possesses relatively thickened setae that are not tightly-packed (Figure 2.5C-D). In *Ligia*, a furrow occurs on the peduncle of the second antenna, but it lacks any setae (Figure 2.5F).

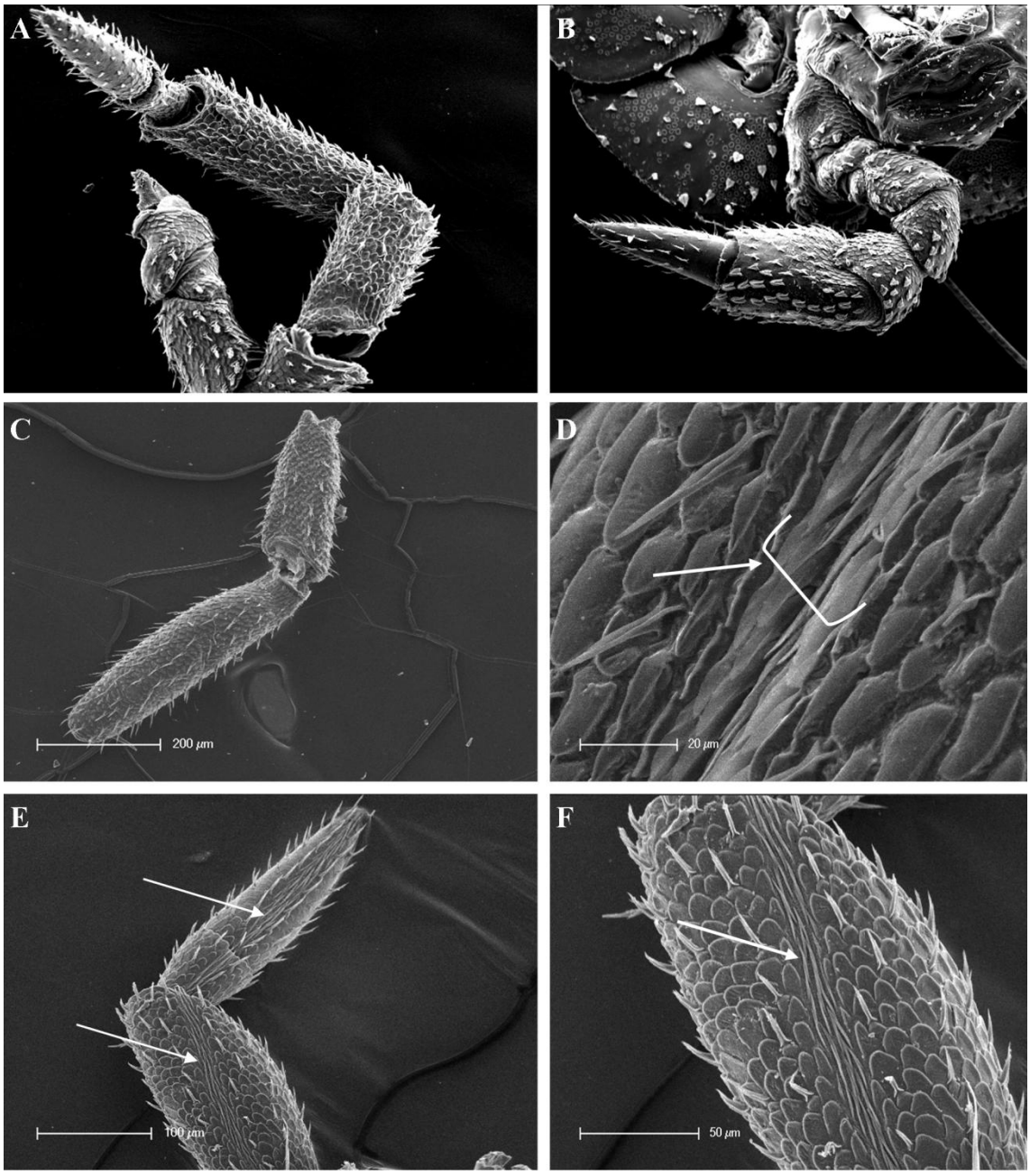



**Fig. 2.4.** Morphology of the second antenna water conducting system. Capillary system not present in A: *Trichorhina tomentosa*; B: *Platyarthrus hoffmannseggii*; C: *Trichorhina anophthalma* ("Platyarthridae"); D: *Armadillidium vulgare* (Armadillidiidae), capillary furrow; E: *Niambia* Sp. ("Platyarthridae"), capillary system with setae but lacking a furrow; F: *Niambia* sp., close up showing capillary setae.

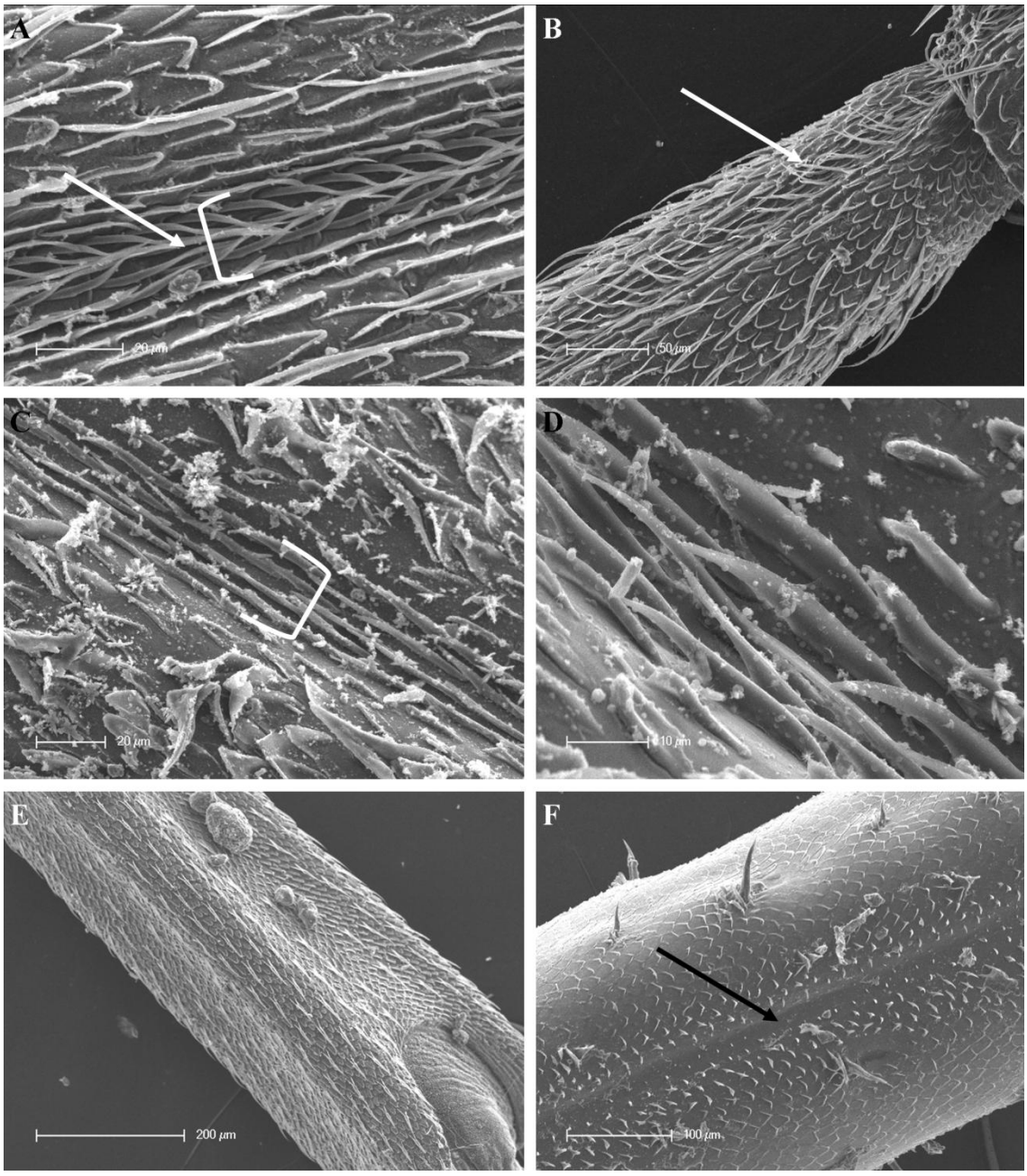



**Fig. 2.5.** Morphology of the second antenna the capillary system. A: *Orthometopon dalmatinum* ("Trachelipodidae"), capillary furrow on the peduncle; B: *Orthometopon dalmatinum,* capillary setae on the flagellum; C: *Trachelipus cavaticus* ("Trachelipodidae"), furrow on the peduncle and associated capillary setae; D: *Trachelipus cavaticus*, close up of the furrow and thickened setae; E: *Porcellionides pruinosus* (Porcellionidae)*,* no furrow on the peduncle; F: *Ligia* sp., furrow on the peduncle lacking capillary setae.

***SYSTEMATICS***

Order **Isopoda**

Suborder **Oniscidea** Latreille 1802

Section **Crinocheta** Legrand 1946

Family **Paraplatyarthridae** Javidkar & King **fam. nov**.

Type genus ***Paraplatyarthrus*** Javidkar & King **gen. nov.**

**Diagnosis.** Dorsal body entirely covered with fan-like scale setae from cephalothorax to pleotelson. Second antenna peduncle ventrally with fan-like scale setae and a furrow containing elongated hair-like capillary setae that form part of the water conducting system; flagellum 2-jointed. Head with postfrons and profrons fused. Maxilla 1 outer endite with 4 + 4/5 teeth, outer 4 teeth with 1 comparatively shorter stout tooth.

**Description.** Body length 2.5 - 5.5 mm (from the anterior part of cephalothorax to pleotelson tip). The included species are of the "clinger type" with large coxal plates and pleon epimera. Dorsal body is covered with fan-like scale setae from the cephalothorax to the pleotelson. The body pigmentation, depending on the life style, is variable from fully pigmented in surface species to completely pale in troglobitic ones.

Cephalic lateral lobes are present from small to comparatively enlarged forms. Supraantennal line present in the form of lines of cuticular scales compressed (Figure 2.7B). Frontal line is absent. The Postfrons completely fused with profrons (Figure 2.7E). Number of ommatidia variable from a maximum of 7 in surface species to eyeless in subterranean species. First antenna 3-jointed (Figure 2.7A). Second antenna flagellum 2-jointed, the apical cone short with longitudinal sutures and lateral setae at the basal part, the top of cone circular with very small setae in a circle (Figure 2.6D); second antenna ventral side (peduncle) with fan-like scale setae and a distinct furrow containing modified elongate hair-like setae which forms part of the so-called Water Conducting System (WCS, Figure 2.6A-B).

Mandibles with 1-2 plumose setae on the hairy lobe and 1 stout plumose seta between the lobe and the pars molaris; pars molaris on both mandibles with a tuft of plumose setae. Maxilla 1 outer endite with outer group of 4 simple large teeth, one smaller than the other but stout, inner group with a combination of 4 to 5 more slender cleft/simple teeth; inner endite with 2 stout plumose setae. Maxilla 2 either with a suture (line) delimiting the lobes or the suture absent or vestigial. Maxilliped endite with 1 large subapical seta; basal article

of the palp with two large setae; distal articles of the palp fused with simple and tuft of setae on inner side.

Noduli laterales present. Tergites 2-7 epimera with a fine suture originating from the posterior epimera and extending towards the anterior end.

Pereopods 1-7, with both simple and large serrate setae on inner side of propodus, carpus and merus; ischium, merus and carpus with few large serrate setae on outer apical part; dactylus with a long simple seta, the outer claw straight or sickle-shaped, the inner claw simple and situated at the base of the outer claw.

Pleopods with no dorsal respiratory fields. Male pleopod 1 endopodite straight, much longer than exopodite, dorsal spermatic furrow narrow. Genital papilla ventral sheath triangular with pointed or rounded tip; the ventral sheath surpassed by a long lobe with genital orifices most probably situated at the apical corners. Male pleopod 2 endopodite slender and longer than that of pleopod 1.

Pleopod 2-5 exopodites with both marginal simple and serrate setae on inner side.

Uropod exopodite dorsoventrally flattened, exceeding the pleotelson; endopodite laterally flattened.

**Etymology.** The family name is derived from the name of the type genus, *Paraplatyarthrus*.

**Distribution.** Subtropical and arid regions of Western Australia and South America

**Remarks.** Recognition of this new family is based on both the results of the molecular phylogeny (s 1, 2) and several diagnostic morphological characters evident from examination of 10 species comprising both surface and subterranean taxa. Paraplatyarthridae is distinguishable from all other oniscidean families by the combination of characters above. It is superficially most similar to Platyarthridae, however based on *T. tomentosa* as representative of the family (Schmidt, 2003), it lacks a capillary furrow on the second antenna and scale setae on the ventral side of the antenna (Figure 2.4A), compared with the unique antennal furrow which is developed in all Paraplatyarthridae. An antennal furrow is also lacking in all *Platyarthrus* species examined. The postfrons is clearly delimited from the profrons in *T. tomentosa* (Figure 2.7F), while in paraplatyarthrid species it is fused (Figure

2.7E). In all paraplatyarthrids there are also four outer teeth including one stout smaller tooth on the outer endite of the first maxilla (Figure 2.8A), whereas in Platyarthridae the short stout tooth is absent. *Trichorhina anophthalma* from Portugal (not included in the molecular phylogeny due to degraded DNA) also lacks a capillary furrow on the second antenna (Figure 2.4C), has prominent delimitation of the postfrons from the profrons, and 3 outer teeth present on the apical margin of the first maxilla outer endite. Therefore, based on these characters *T. anophthalma* clearly fits into the Platyarthridae as a valid member of *Trichorhina*. In addition, all paraplatyarthrid species have a supraantennal line, while it is absent in true members of *Trichorhina* (i.e. those congeneric with *T. tomentosa*). In paraplatyarthrid species the supraantennal line is weaker than that of *Niambia* species in which it is solid.

Based on our molecular results and morphology assessment we propose that the new family comprises two genera; *Paraplatyarthrus* gen. n., described below, which is restricted to Australia and is represented by multiple species (Taxon 1-9, Figures 2.1-2), and a separate genus from South America (Porto, Alegre Belein Novo, Brazil), represented by Taxon 10 which is sister to *Paraplatyarthrus*. However, we refrain from formal description of the South American genus until more material becomes available (N.B. it is currently only known from a few female specimens) and South American oniscideans, particularly species described under *Trichorhina*, can be examined in more details.

Genus ***Paraplatyarthrus*** Javidkar & King **gen. nov**.
Type species ***Paraplatyarthrus subterraneus*** Javidkar & King **sp. nov**.

**Diagnosis.** Fan-like scale setae covering the body smooth. Maxilliped endite with 2 small arrow-like setae on distal margin. Pereonal tergite 7 with 2 noduli laterales on each side (4 on the whole pereonite; Figure 2.7C), pereonal tergites 1-6 with 1 nodulus lateralis on each side (2 on the whole pereonite).

**Etymology.** The name of the genus is derived from the prefix ‘para’ meaning ‘near’ and Platyarthridae due to its general morphological similarity with *Trichorhina* and *Platyarthrus*.

**Distribution.** Known from terrestrial and subterranean habitats in arid and temperate regions of Western Australia

**Remarks.** *Paraplatyarthrus* species possess two prominent arrow-like setae on the outer apical margin of the maxilliped endite which are absent in all other oniscidean species examined including the South American paraplatyarthrid species (Taxon 10) (i.e. not to be recognizable under light microscopy). These specialized setae have not been reported previously in the oniscidean literature and their presence/absence is likely a good diagnostic trait to separate *Paraplatyarthrus* and the South American species. However, it will be essential to examine additional South American species to confirm this. Moreover, *Paraplatyarthrus* and the South American species (Taxon 10) can also be separated on the structure of the scale setae on the tergites (smooth in *Paraplatyarthrus*, ribbed in Taxon 10), and the number of noduli laterales on the pereonal tergite 7 (2 on each side in *Paraplatyarthrus*, 1 on each side in Taxon 10). Given these differences and the level of divergence between *Paraplatyarthrus* species and South American Taxon 10 in the molecular analysis (Figure 2.1), we propose that the latter species represents a distinct genus, as discussed above. Below we describe a single taxon (Taxon 6 in Figures 2.1-2) as the type species of the genus, but are preparing a comprehensive revision of the genus as a separate study, to be published elsewhere.

***Paraplatyarthrus subterraneus*** Javidkar & King **sp. nov.** (Figures 2.8-10)

**Holotype. Male, WAM C53623 (BES15525.19)**, Laverton Downs Windarra calcrete, Eastern Murchison region, Western Australia, AUSTRALIA; 28.50282$^{o}$S, 122.17726$^{o}$E. 13 July 2010, W. F. Humphreys & S. J. B. Cooper, WA Museum

**Paratypes.** 5 males including WAM C53624 (BES15525.24), WAM C53625 (BES15525.16), WAM C53626 (BES15525.12), WAM C53627 (BES15525.2), WAM C53628 (BES15525.3); 1 female WAM C53629 (BES15525.4); same locality/collectors' as holotype (all deposited in the Western Australian Museum).

**Description. Male (Holotype)**, body length 3.3 mm (range for paratypes 3.0-3.8 mm). No pigmentation, the whole body pale and eyeless typical of a true troglobitic form. A single scale seta present at the top of the supraantennal line in the middle (Figure 2.7B). Cephalon

lateral lobes present but not enlarged. Fan-like scale setae on dorsal body serrate at the top of sheath (Figure 2.6E-F); cuticular scales either crescent-shaped or curved and pointed at the top (Figure 2.6E-F). Antenna 1 3-jointed, medial article shortest, distal article longest, bearing 4 pairs of aesthetascs, each with a very fine longitudinal suture medially (Figure 2.7A). Antenna 2 flagellum 2-jointed, basal article about 1/3 the length of the distal article, dorsal and lateral sides with simple setae, ventral side with hair-like setae lying along each other in 2/3 rows towards the top.

Left mandible (not shown) pars molaris with a tuft of about 7 plumose setae; hairy lobe attached to lacinia mobilis bearing 2 plumose seate, the top is covered with a few small fine setae; 1 plumose seta occuring between the lobe and pars molaris. Right mandible pars molaris with a tuft of about 8 plumose setae; 1 plumose seta on hairy lobe, very small fine setae around the base of plumose setae; 1 single plumose seta between hairy lobe and pars molaris closer to lobe (Figure 2.8A); lacinia mobilis coronate. Maxilla 1 outer endite with an outer group of 4 teeth covering about 60% of the marginal area, one smaller than the others but stout, inner group of 4 bifurcate teeth and 1 simple tooth (Figure 2.8B); inner endite with 2 apical stout plumose setae, apical outer corner with 2 very fine setae close to each other (Figure 2.8C). Maxilla 2 apically bilobate; inner lobe smaller than outer lobe, with thick sensilla on distal margin; the lobes delimited by a fine suture; outer lobe with very fine small setae from apical margin towards subapical area (Figure 2.8D). Maxilliped endite with 1 large seta close to subapical inner corner; basal article of palp with 2 setae, one on inner side the longest, distal articles fused, with 1 large proximal seta, a medial tuft of 3 large slender setae and an apical tuft of a few long setae; the outer margin of palp with 1 medial fine seta and 2 distal fine setae (Figure 2.8E).

Epimeron 1 bluntly projected anteriorly; in dorsal view, posterolateral corner of pereonites 1-3 rounded; posterolateral corner of pereonites 4-7 posteriorly directed. Noduli laterales present on pereonal tergites (Figure 2.11).

Pereopod 1 carpus inner margin densely covered with serrate large setae, dense tuft of fine setae present medially near distal margin; propodus with both simple setae and a few large serrate setae; dactylus with a long narrow seta exceeding the claws, outer claw sickle-shaped; inner claw shorter, situated at basal posterior of outer claw (Figure 2.9A); carpus inner side sexually dimorphic in density of setae (male with inner side more plumose). Pereopod 7 not showing any significant sexual dimorphism; carpus and merus inner side

with serrate setae but less dense in comparison with those of pereopod 1; carpus, merus and ischium outer apical margin with large serrate setae; ischium inner side with few short simple setae; basis apical inner side with a single simple long seta (Figure 2.9B).

Pleon outline continuous with pereon. No dorsal respiratory field on the pleopods. Pleopod 1 endopodite straight, with very fine small setae on medial and apical parts (Figure 2.10A); exopodite shorter with no marginal setae (Figure 2.10B). Pleopod 2 endopodite long and slender (Figure 2.10C); exopodite large, with 4 marginal (inner side) long serrate setae (Figure 2.10D). Pleopod 3, 4, 5 exopodites with 3, 4 and 4 marginal serrate setae, respectively (Figure 2.10E, F, G). Pleotelson triangular and pointed. Uropodal exopodites well surpassing pleotelson; endopodites slightly exceeding pleotelson.

**Etymology.** The species name is derived from the Latin word ‘subterraneus’ (meaning subterranean) due to its troglobitic life.

**Distribution.** The new species is confined to an individual calcerete aquifer, Laverton Downs, Eastern Murchison region, WA, Australia.

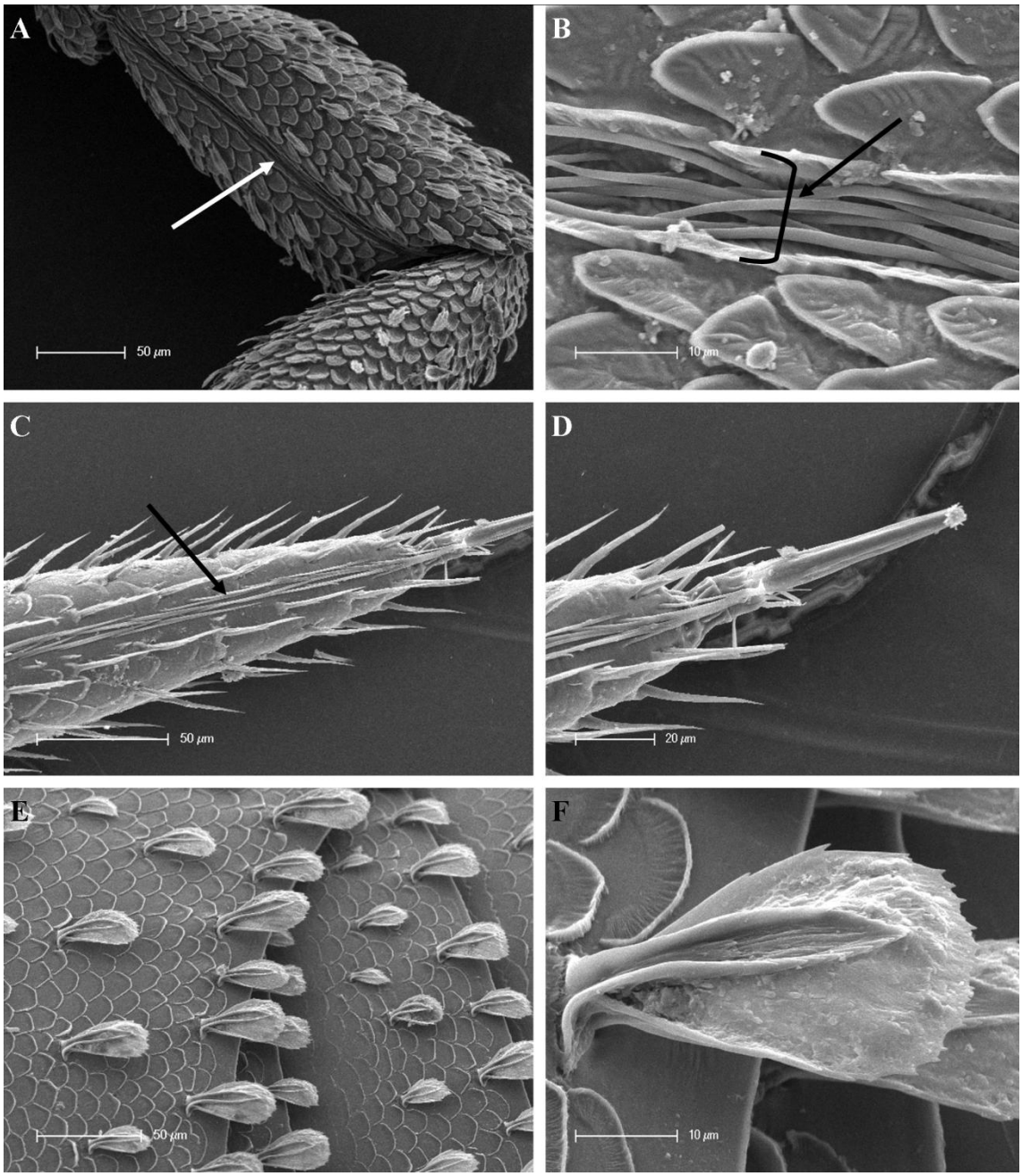



**Fig. 2.6.** *Paraplatyarthrus* gen. nov., morphology of the second antenna. A: Capillary furrow on the peduncle and fan-like scale setae scattered around the furrow (Jorgensen Park, Kalamunda, WA); B: Taxon 1, Close up of the capillary furrow and the associated elongated hair-like capillary setae; C: Taxon 1, Distal article of the flagellum showing the tightly-packed setae; D: Taxon 1, Apical cone of the flagellum; E: Taxon 1, Dorsal scale setae; F: Taxon 1, Close up of one scale seta and crescent cuticles.

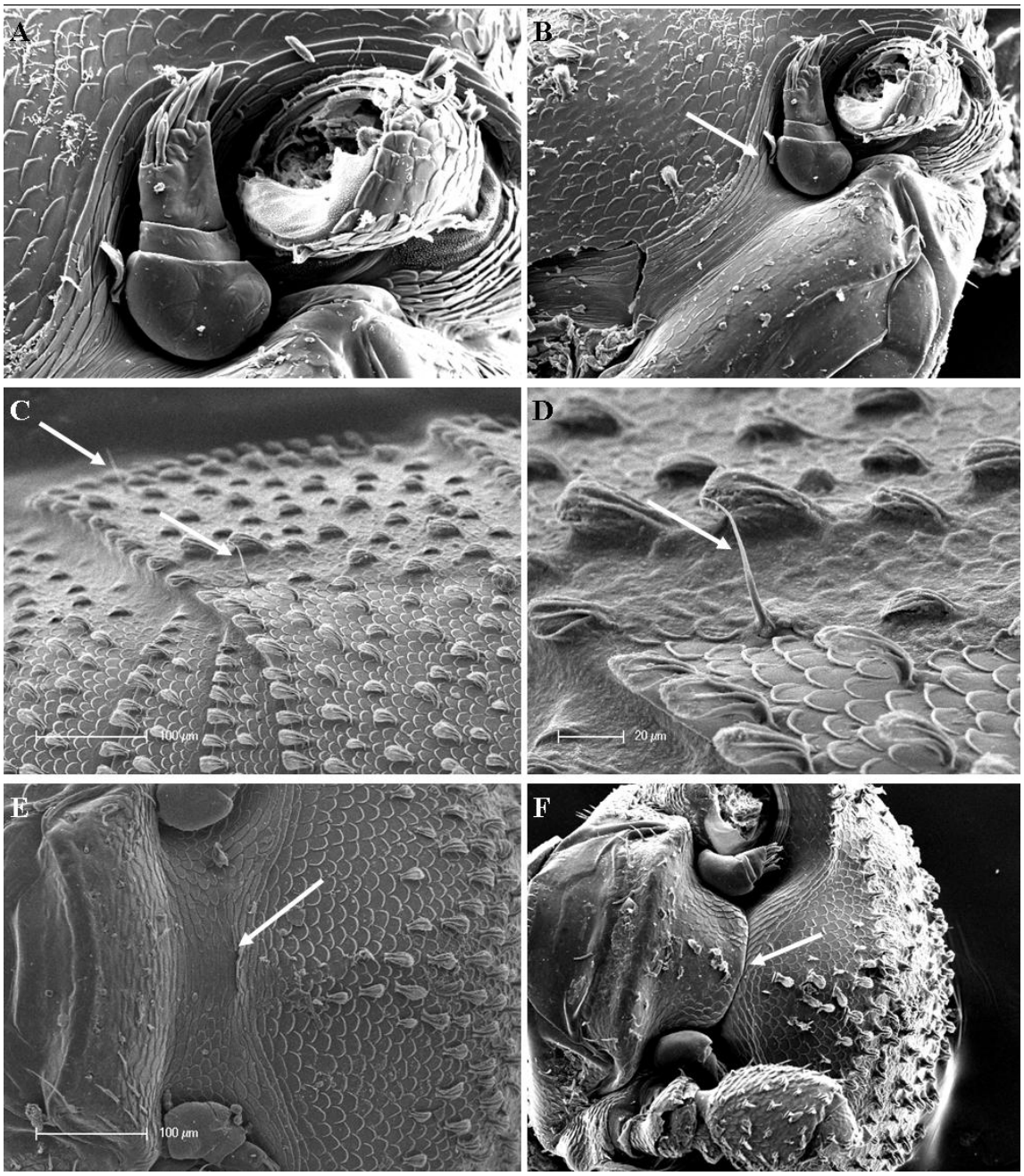



**Fig. 2.7.** *Paraplatyarthrus* gen. nov. (A-E): A: Taxon 6, Close up of first antenna; B: Taxon 6, Supraantennal line; C: Noduli laterales on tergite 7 (Jorgensen Park, Kalamunda, WA); D: Close up of nodulus lateralis on the same tergite (Jorgensen Park, Kalamunda, WA); E: Postfrons fused to the profrons (Jorgensen Park, Kalamunda, WA); F: *Trichorhina tomentosa* ("Platyarthridae"), the postfrons obviously delimited from the profrons.


**Fig. 2.8.** *Paraplatyarthrus subterraneus* sp. nov. (Holotype, ♂), mouth parts: A: Right Mandible; B: Maxilla 1 outer endite; C: Maxilla 1 inner endite; D: Maxilla 2; E: Maxilliped (note the arrow-like setae on the apical outer corner of the endite). Scale bars: 0.1 mm.

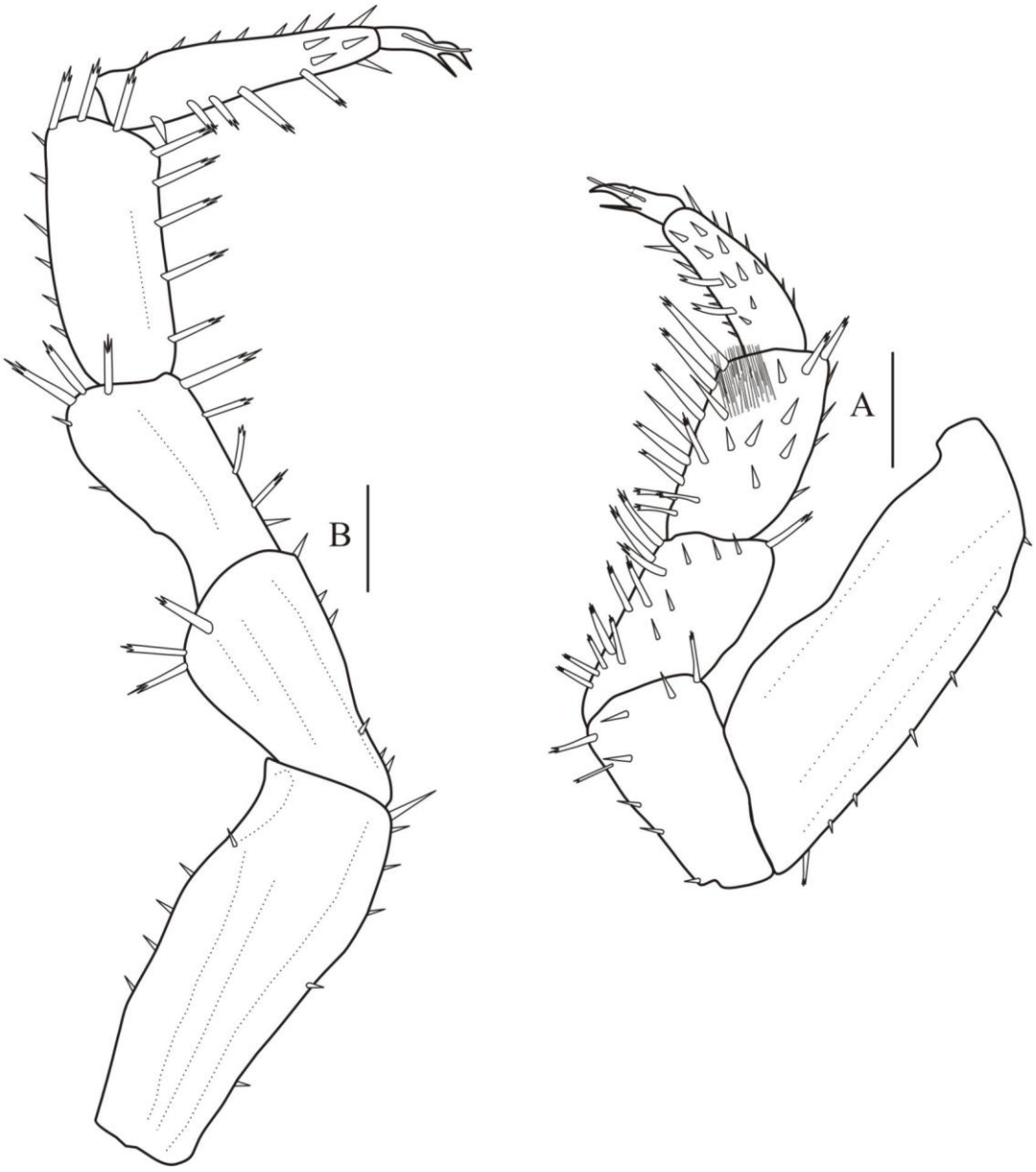



**Fig. 2.9.** *Paraplatyarthrus subterraneaus* sp. nov. (Holotype, ♂), A: Pereopod 1; B: Pereopod 7. Scale bars: 0.1 mm.

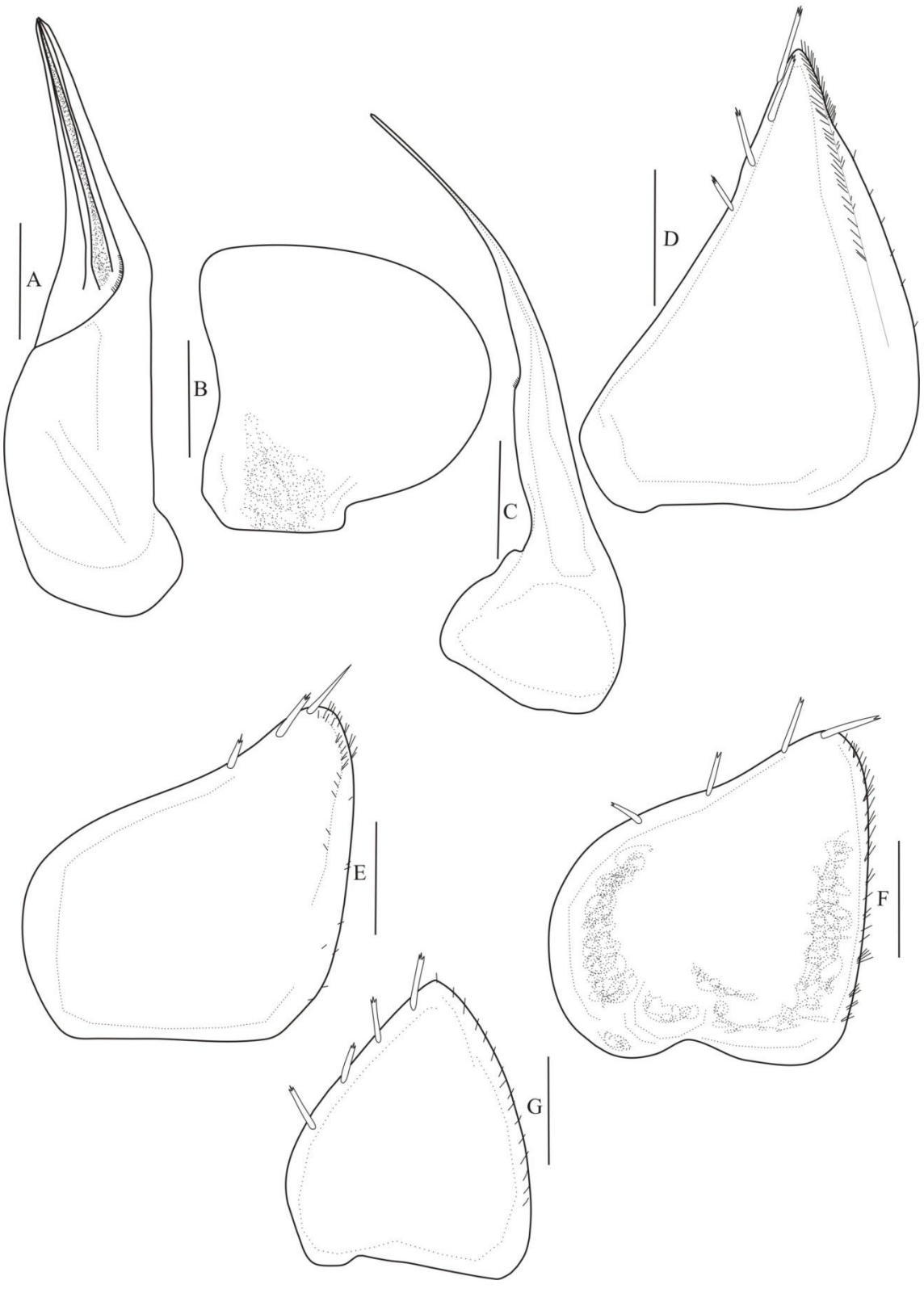
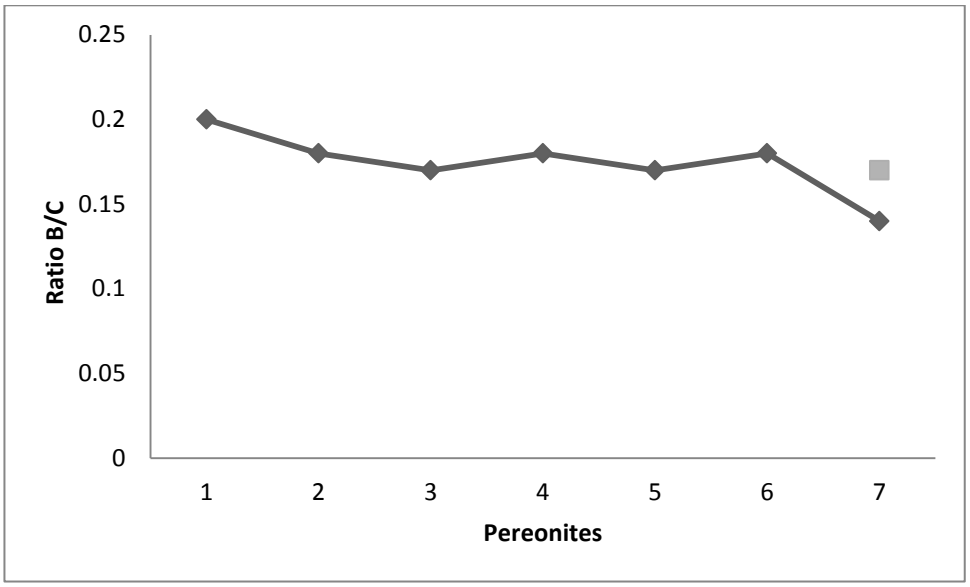



**Fig. 2.10.** *Paraplatyarthrus subterraneaus* sp. nov. (Holotype, ♂), A: pleopod 1 endopodite; B: Pleopod 1 exopodite; C: Pleopod 2 endopodite; D: pleopod 2 exopodite; E: pleopod 3 exopodite; F: pleopod 4 exopodite; G: pleopod 5 exopodite. Scale bars: 0.1 mm.



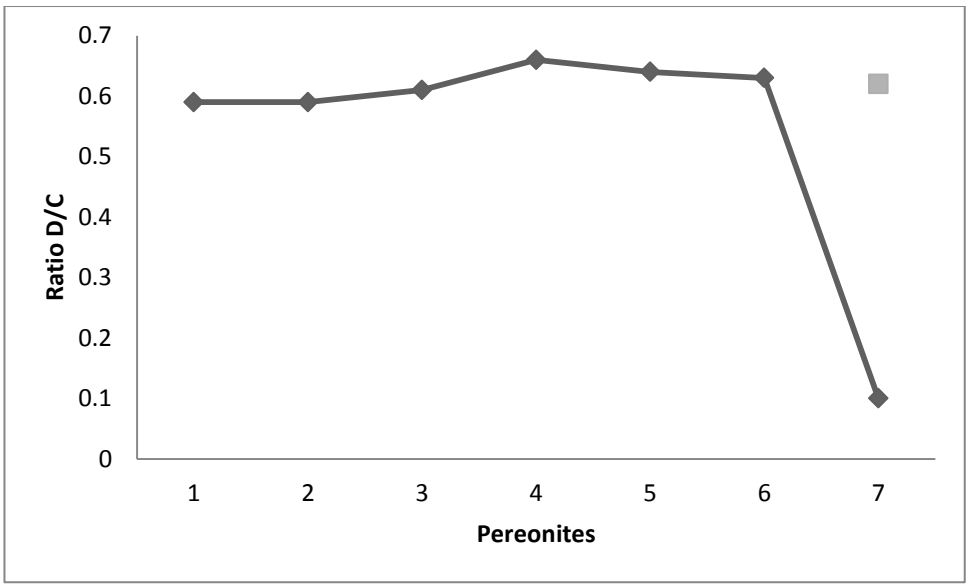




**Fig. 2.11.** *Paraplatyarthrus subterraneus* sp. nov. Paratype, ♀ (WAM C53629 (BES15525.4)). Relative position of the noduli laterales on pereonites 1 to 7 defined by the rations B/C and D/C. B = distance from the nodulus lateralis to the posterior margin of the pereonite, C = maximum length of the pereonal tergite (antero-posterior); D = distance from the nodulus lateralis to the lateral margin of the pereonite.

## DISCUSSION

### *MOLECULAR PHYLOGENY AND RELATIONSHIPS*

This study represents the first molecular phylogeny of platyarthrid isopods to test their monophyly with respect to 10 other related families. The topologies obtained, including both individual and combined gene analyses, strongly indicate the polyphyly of taxa currently considered to be members of Platyarthridae and show the presence of a divergent clade comprising Australian and South American taxa. This clade is herein described as a new family, Paraplatyarthridae. Detailed morphological examination using SEM of Australian and South American species confirmed its higher systematic status within Oniscidea. The Paraplatyarthridae can be diagnosed by a unique combination of morphological characters including the presence of fan-like scale setae on the dorsal body; a two-jointed second antenna flagellum with a ventral side comprising a furrow and fan-like scale setae; fusion of the postfrons and profrons; and the outer endite of the first maxilla with 4/4+5 teeth with the outer 4 teeth including one shorter stout tooth.

Both individual *18S* and combined phylogenies showed the Paraplatyarthridae to be sister to a group comprising the genus *Haloniscus* and the family Stenoniscidae. The *18S* and combined phylogenies revealed that relationships among additional species designated as platyarthrids are even more complex, with *T. tomentosa* not forming a monophyletic group with *Niambia* spp. and *Platyarthrus hoffmannseggii*. Molecular phylogenetic analysis of oniscidean taxa based on the whole *SSU rRNA* gene showed Trachelipodidae Strouhal 1953 and Platyarthridae (just two species studied, *T. tomentosa* and *P. schoblii*) as well as the genera *Porcellio* Latreille 1804 and *Porcellionides* Miers 1878 were not monophyletic (Mattern, 2003). The species *T. tomentosa* branched off at the basal part of the phylogeny while *P. schoblii* was sister to a clade including *Oniscus asellus* and *Philoscia muscorum*. Mattern and Schlegel (2001) presented a phylogeny based on the entire *18S* gene and found support for a group comprising Oniscidae Latreille 1802; Philosciidae, Platyarthridae, Cylisticidae Verhoeff 1949 and Armadillidiidae, which was part of a more inclusive clade containing Trachelipodidae and Porcellionidae Brandt & Ratzeburg 1831. Our *18S* phylogeny revealed the same result, in which *P. hoffmannseggii* was the sister lineage to a group comprising *O. asellus* (Oniscidae) and *P. muscorum* (Philosciidae). Overall, there was high posterior probability support for a group consisting of *P. hoffmannseggii*, *O. asellus*

(Oniscidae), *P. muscorum* (Philosciidae), *A. vulgare* (Armadillidiidae) and *Niambia* spp. As the combined phylogeny did not include the taxa *O. asellus* and *P. muscorum,* so *P. hoffmannseggii* here is sister to a clade consisting of *Niambia* species, with *A. vulgare* a sister lineage to the *P. hoffmannseggii*/*Niambia* clade, followed by *T. tomentosa* which is robustly placed as sister to the whole group. Although *P. hoffmannseggii* and *Niambia* species are sister lineages in the combined phylogeny, in the *18S* topology *P. hoffmannseggii* is a sister lineage to a clade comprising *O. asellus* and *P. muscorum*. The most likely reason for this difference is the absence of the latter group in the combined analysis. The phylogenetic topologies here and those of Mattern and Schlegel (2001) differ from the cladogram reconstructed on the basis of morphological data only (Schmidt, 2002; Leistikow, 2001) where the Platyarthridae was found to be a sister lineage to Spelaeoniscidae.

In the current study, the systematic position of *Niambia* is still uncertain and more oniscidean taxa are needed to further resolve its relationships. The occurrence of an undescribed species of *Niambia* in Australia (Adelaide) may be due to its introduction from Africa as it was found beside a river within an urban area. *Niambia capensis* Dollfus 1895 is the only reported widespread species which is native to South Africa but has been introduced to different parts of the world including the USA (California, in a sandy intertidal zone) and New Zealand (Maloney *et al.* 2007; report from New Zealand: Pers. Comm. S. Taiti).

Mattern (2003) found a sister relationship between *T. tomentosa* and *Cubaris murina* (Armadillidae) but the phylogenies here do not support any close relationships between *T. tomentosa* and Armadillidae (*Troglarmadillo* spp.), assuming that Armadillidae is monophyletic. Instead, the combined phylogenetic analysis suggested a sister relationship between Armadillidae (*Troglarmadillo* spp.) and *Deto marina*. The systematic position of *Troglamardillo* within Armadillidae is still ambiguous. Taiti *et al.* (1998) suggested that the validity of this genus, established by Arcangeli (1957) for *Armadillo cavernae,* is doubtful as the type specimens were damaged in the cephalon and pleon.

In the world catalog of terrestrial isopods, 56 species of *Trichorhina* have been recorded, mostly from the Neotropical region (Schmalfuss, 2003). Following identification of some new species of *Trichorhina* from Brazil the total number of identified species of *Trichorhina* increased to 64 (Wahrberg, 1922; Lewis 1998a; Araujo & Almerão, 2007; Souza *et al.* 2011). With respect to the convergent morphology in some characters, such as the existence of

scale setae on the dorsal body occurring among members of the new family Paraplatyarthridae and genus *Trichorhina*, it is likely *Trichorhina* is polyphyletic. While most species of the *Trichorhina* have tropical distributions, paraplatyarthrid species are subtropical and temperate in their distribution, mostly occurring in Western Australia from the west and south-west to arid regions in central Western Australia, within which most species have a subterranean lifestyle. In the current study, the South American species ascribed to *Trichorhina,* which was sister to all Australian paraplatyarthrid species, was also collected from a subtropical region in Brazil. The systematic position of the two Australian described species *Trichorhina australiensis* Wahrberg 1922 *(*Western Australia) and *Trichorhina tropicalis* Lewis 1998b (Queensland) and an undescribed species ascribed to *Trichorhina* from Lord Howe Island (Lewis, 1998b) is still ambiguous as samples of these species were unavailable. Moreover, the morphological descriptions of these taxa do not provide key diagnostic characters to determine whether they in fact belong to the Paraplatyarthridae. Additional morphological study of several Australian platyarthrid species from the Western Australian Museum collection, which have been designated as *Trichorhina* (DNA was too degraded for sequencing), showed that none of them belong to *Trichorhina*. A further comprehensive molecular and morphological study of all Australian species attributed to *Trichorhina* is required to determine whether they belong to the Paraplatyarthridae and, indeed, whether true Platyarthridae occur on the continent. With respect to the current study, the systematic position of other platyarthrid genera including *Echinochaetus*, *Lanceochaetus*, *Cephaloniscus* and *Gerufa* is still ambiguous. These genera have been placed within Platyarthridae based on general morphological characters which are shown here to be insufficient for defining a reliable monophyletic group due to convergence. Our phylogenetic results, supported by morphological data, indicate the existence of at least three higher lineages (four if *Niambia* is considered separate from *Platyarthrus*) within Platyarthridae.

The genus *Haloniscus*, which has a controversial taxonomic history, was first considered a member of the family Oniscidae by Williams (1970) and then included in the Philosciidae by Vandel (1973). The systematic position of *Haloniscus* is still unclear and is regarded as an unplaced taxon by Poore (2002). In our combined phylogeny, *Haloniscus* showed a strong sister relationship with Stenoniscidae, and was well separated in the *18S* tree from *P. muscorum* representing Philosciidae (Figure 2.1), which suggests an affinity between

*Haloniscus* and Philosciidae is unlikely. Taiti *et al.* (1995), based on a morphological description of a new aquatic species of *Haloniscus* from New Caledonia, discussed a possible relationship between *Alloniscus* Dana 1854 (Alloniscidae) and *Haloniscus* on the basis of the lack of noduli laterales, second antennal flagellum with three articles, and the same structure of mouthparts. The addition of members of Alloniscidae Schmidt 2003 in future phylogenetic studies would be valuable to resolve this issue. According to the *18S* phylogeny, subterranean *Haloniscus* spp. formed a highly supported sister relationship with a surface species from South Australia (Taxon 11). Since Taxon 11 was represented solely by female specimens its generic identity remains uncertain.

Holdich *et al.* (1984) recognized three sections within the Infraorder Ligiamorpha Vandel 1943 (suborder Oniscidea) including Crinocheta, Synocheta and Diplocheta, whereas Schmalfuss (1989) did not use an infraorder classification and divided the Oniscidea into four sections comprising Crinocheta, Synocheta, Diplocheta and Microcheta Schmalfuss 1989, but this was not followed by Martin and Davis (2001) in their updated classification of crustaceans. Salzat and Bouchon (2000) generated a phylogeny of 31 genera of aquatic and terrestrial crustaceans using partial sequences of the mitochondrial *16S* rRNA gene. The same authors showed that Synocheta and Crinocheta were sister lineages, with Ligiidae and Tylidae in basal positions in the tree. In this respect, our combined phylogeny is in agreement with that of Salzat and Bouchon's (2000).

### *MORPHOLOGICAL COMPARISONS*

*Niambia,* which is widely distributed in southern Africa, was previously distinguished from *Trichorhina* based on the presence of a frontal line which is absent in *Trichorhina*, but it has also been noted that this distinction is uncertain (Vandel, 1959; Schmalfuss & Ferrara, 1978). According to our SEM studies, *Niambia* can be separated from *Trichorhina* and Paraplatyarthridae by the structure of the water conducting system (WCS) on the second antenna which comprises a simple line of capillary setae, rather than a furrow (Figure 2.4E-F), whereas this structure is absent in *Trichorhina* and *Platyarthrus*. In Paraplatyarthridae the second antenna possesses a deep furrow containing elongated capillary setae which is observable even by light microscopy. In addition, *Trichorhina* species do not have any supraantennal line while this line is well-developed in *Niambia*. Furthermore, in *Niambia* the postfrons is fused with the profrons providing an additional character to help distinguish

*Niambia* and *Trichorhina*. In the *Niambia* species examined here, tracheal systems were missing in all pleopod exopodites which is in agreement with Vandel's (1959) study who stated they were absent in *Niambia*. SEM examination of the new family Paraplatyarthridae shows they also lack any tracheal system in the pleopod exopodites, which are also absent in *Trichorhina*.

Based on the results of the current study Platyarthridae, as previously defined, undoubtedly represents a complex of families. It is also evident that described species attributed to *Trichorhina* should be re-examined to verify their generic placement. As Paraplatyarthridae appear to have a subtropical distribution in Australia and South America, the species occurring in these biomes and currently ascribed to *Trichorhina* should be treated with caution. Two species of *Trichorhina* have been described from Australia, one occurring in Western Australia (*T. australiensis*) and the other in Queensland (*T. tropicalis*). Also, a number of undescribed species from Australia exist in collections from Lord Howe Island (Pacific) and the Northern Territory, which have been tentatively assigned to *Trichorhina* (Lewis, 1998b; Moulds & Bannink, 2012); all these species are in need of reassessment. To date, no *Trichorhina* species have been found associated with groundwater calcretes in central Western Australia (Javidkar unpublished observations). Rather, these calcretes contain numerous subterranean species of Paraplatyarthridae.

Examination of previously collected species deposited in the WA Museum, uncovered a species (WAM-C12903) from Rottnest Island (WA) which had been ascribed to *Trichorhina* (material too old for sequencing). Surprisingly, based on the diagnostic characters identified as part of this study, it appears this specimen belongs to neither Paraplatyarthridae nor *Trichorhina* but matches those of *Niambia*. Along with the species collected from Adelaide, this is the second species of *Niambia* occurring in Australia. However, the question remains whether these taxa occur naturally on the continent or have been introduced. More collections, targeting creeks and port lands, are required to identify the distribution of the genus in Australia which is probably much wider.

SEM studies on the structure of the second antenna furrow on the oniscidean families Trachelipodidae, Porcellionidae, Armadillidae, Armadillidiidae and Ligiidae revealed numerous fixed differences, suggesting it is a robust character for taxonomic and phylogenetic evaluations at the family level. In *Troglarmadillo* (Armadillidae), the furrow includes elongated setae which are similar to that of Paraplatyarthridae, but no fan-like scale

setae are present on the second antenna peduncle. Schmalfuss (1998) stated that when the second antennae are in contact with wet substrates, water (or fluid) can be either absorbed into the ventral water conducting system to regulate the water budget or emitted as excretion by capillary action. In the *Ligia* species examined there is just one simple furrow along the second antenna from the peduncle to the flagellum which does not include any capillary setae. It is still not clear whether this furrow is a primitive form homologous to capillary furrows in other oniscidean species or it is related to a different second antennal structure (Figure 2.5F). The SEM results also show the cuticle of the second antennae in *Ligia* (associated with the littoral zone) is relatively smooth and resembles more closely the condition found in aquatic isopod species. In other oniscidean species examined associated with terrestrial environments, the cuticle on the second antennae is well-developed, for example, in Paraplatyarthridae where they are significantly protruding and lie on each other. Schmalfuss (1978), who examined the structure of oniscidean cuticles using SEM, considered it to have anti-adhesive functions, preventing tiny wet particles from sticking to the body surface.

## CONCLUSION

Molecular phylogenetic analysis demonstrates that the family Platyarthridae represents a complex of distantly related oniscidean lineages and indicates that morphological convergence exists for characters traditionally used to diagnose the family. The species of Paraplatyarthridae fam. nov., previously accommodated within Platyarthridae, was found to be highly divergent and is erected here based on its robust postion in the molecular phylogeny and morphological evidence that confirms its unique position within Oniscidea. Re-examination is warranted of all species previously ascribed to *Trichorhina* based on new morphological characters identified here to distinguish them from the new family which is distributed in Australian and South American subtropical and arid regions. The inclusion of other platyarthrid genera comprising *Cephaloniscus*, *Echinochaetus*, *Lanceochaetus* and *Gerufa* into a more comprehensive molecular phylogeny is needed to elucidate their relationships with both the new family and other platyarthrid lineages.

## ACKNOWLEDGEMENTS

The authors sincerely thank Dr Stefano Taiti, for his invaluable comments on the manuscript and for providing critical samples for both the molecular and morphological study, Dr Helmut Schmalfuss, who kindly provided some oniscidean samples for SEM studies, Ms Lynette Waterhouse from Adelaide Microscopy, for training and helping with SEM techniques, Nahid Shokri Bousjein, who voluntarily assisted with field samplings, and Dr Andrew Hosie, for providing oniscidean samples from the Western Australian Museum. We are grateful to Ms Kathy Saint (SA Museum) who helped in the molecular laboratory at the University of Adelaide and the Austin labgroup for their help and comments. The research was supported by an ARC linkage grant (LP100200494) to the authors, a National Geographic grant to SJBC and WFH, and a PhD scholarship from the University of Adelaide to MJ.

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# CHAPTER III:

# MOLECULAR SYSTEMATICS AND DIVERSIFICATION PATTERNS OF ONISCIDEAN ISOPOD TROGLOFAUNA IN GROUNDWATER CALCRETES OF CENTRAL WESTERN AUSTRALIA

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## STATEMENT OF AUTHORSHIP

# Statement of Authorship

**Title of Paper:** Molecular Systematics and Diversification patterns of Oniscidean Isopod Troglofauna in Groundwater Calcretes of Central Western Australia

**Publication Status:** Publication style

**Publication Details:** This chapter is going to be submitted to the Journal of Molecular Phylogenetics and Evolution.

## Author Contributions

By signing the statement of authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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## ABSTRACT

Groundwater calcretes of central Western Australia have recently been shown to contain an extraordinary diversity of stygobiont invertebrates that display short-range endemism. In the present study, we have utilised alternative sampling techniques and uncovered a significant oniscidean troglobiont fauna from the calcretes. We explore the diversity, species delimitation and evolution of this fauna using molecular analyses based on one mitochondrial gene, Cytochrome C Oxidase Subunit I (*COI*), two Ribosomal RNA genes (*28S* and *18S*), and one protein coding gene, Lysyl-tRNA Synthetase (*LysRS*) developed for the first time here on isopod species using an Illumina next generation sequencing technique. The results from 12 calcrete aquifers show the existence of 37 divergent DNA lineages belonging to four oniscidean families (Paraplatyarthridae, Armadillidae, Stenoniscidae and Philosciidae). On the basis of a combination of species delineation methods (Poisson Tree Processes, General Mixed Yule Coalescent, 12% *COI* divergence species threshold), the results of single/multiple gene phylogenies and morphological evidence, 28 putative new species of oniscidean troglofauna were identified. The majority of these lineages were restricted to individual calcrete bodies, and the number of lineages within each calcrete was variable: from 10 (Laverton Downs) to one (Hinkler). In addition, suites of distinct morphological characters (eye ommatidia and pigmentation coverage) were noted that correlated with lineages. Our analyses lend support to the hypothesis that individual calcretes are equivalent to subterranean islands, with the exception of three paraplatyarthrid troglophile lineages that were found to be distributed in multiple calcretes. The occurrence of stenoniscid lineages in the calcretes of central Western Australia, a group previously only known from the littoral zone, suggests a previous connection with the marine inundation of the Eucla basin during the Late Eocene. The current oniscidean troglofauna consists of subtropical, littoral and benthic groups reflecting different historical events that have shaped the evolution of the fauna in the groundwater calcretes.

**Keywords:** central Western Australia, diversity, Oniscidean isopods, phylogeny, troglofauna.

## INTRODUCTION

Subterranean fauna were once thought to occur in humid and dark subsurface habitats of karst systems where limestone, gypsum and dolomite are the abundant minerals in caves and meso-caverns (Howarth, 1983). However, subsequently it has been found that they are also significant inhabitants of underground geological structures in non-karstic areas such as fractured basalts in Hawaii and the Canary Islands (Howarth, 1983; Oromi & Martin, 1992). Subterranean fauna in Australia were known from classic Tertiary carbonate karsts, such as Cape Range, Western Australia, and pseudokarstic lava flows in Queensland. However, recent discoveries in the arid zone of Western Australia have revealed the existence of diverse hypogean invertebrate communities in non-karstic fractured rock terrains in the Pilbara region and in groundwater calcretes of the Yilgarn region (Humphreys, 2006; Cooper *et al.* 2007, 2008; Guzik *et al.* 2008, 2009; Eberhard *et al.* 2009; Karanovic & Cooper, 2012). As a result, there has been a corresponding recent focus on research towards exploring and identifying this fauna and formally describing the new stygobiont (subterranean aquatic) species (Humphreys *et al.* 2009; Karanovic & Cooper, 2012; King *et al.* 2012).

Humphreys *et al.* (2009) documented a considerable number of subterranean higher taxa including 8 classes, 13 orders and 34 families of invertebrates occurring in Western Australian calcretes. Within the arid Yilgarn region of Western Australia (Figure 3.1), there exist numerous species of stygobitic diving beetles (Leys *et al.* 2003; Watts & Humphreys, 1999, 2006, 2009; Leys & Watts, 2008) and a range of crustacean species such as Bathynellacea, Amphipoda, Isopoda, Copepoda and Ostracoda (Taiti & Humphreys, 2001; Karanovic & Marmonier, 2002; Karanovic, 2004; Cho, 2005; Cho *et al.* 2006a; Cho *et al.* 2006b; Guzik *et al.* 2008; Taiti & Humphreys, 2008; Abrams *et al*. 2012; King *et al.* 2012). Karanovic & Cooper (2012) documented an unprecedented diversity of subterranean copepods (genus *Schizopera*) in a single calcrete aquifer of the Yilgarn region, using molecular and morphological evidence, which equalled 67% of the copepod taxa previously recorded in that region. Several studies on these diverse stygobitic faunas have shown the groundwater calcretes of central Western Australia are equivalent to subterranean closed islands with each species found to be restricted to single calcrete bodies (Cooper *et al.* 2002; Leys *et al.* 2003; Cooper *et al.* 2007, 2008; Guzik *et al.* 2008, 2009, Karanovic & Cooper,

2012; King *et al.* 2012), with the taxa showing short-range endemism under the criteria proposed by Harvey (2002).

Although the systematics and evolution of the stygofauna of the Yilgarn calcretes is reasonably well documented, little is known about the troglofauna, including troglobionts (obligate cavernicoles) and troglophiles (facultative cavernicoles), which are communities of terrestrial animals found in humid and dark subterranean habitats such as air-filled caves and smaller subsurface cavities/voids. Incidental collection of troglobionts during stygofauna sampling in the calcretes (Humphreys, 2008; Bradford *et al*. 2010), including various orders of Crustacea, Insecta and Arachnida, showed that a troglobiont community occurs in the air-filled parts of the calcretes and this included the first indigenous member of the order Palpigradi in Australia (Barranco & Harvey, 2008). Similar sampling results, although with a different fauna, was also found in fractured rock and pisolite deposits in the arid Pilbara region to the north (Eberhard *et al.* 2008; Bennelongia, 2009; Eberhard *et al.* 2009). Initial molecular data, using a fragment of *COI*, reported by Cooper *et al.* (2009, unpublished) on the groundwater calcretes of the Yilgarn region showed a remarkable diversity of DNA lineages of oniscidean isopod troglofauna.

Oniscidean isopods are the most diverse and successful group of isopods that are fully adapted to terrestrial life. Oniscidean species occur in a wide range of terrestrial environments, ranging from tropical wet habitats to dry deserts, at sea level to higher elevations (Hornung, 2011). Some species are adapted to specialised aquatic habitats and live in groundwater systems, caves and salt lakes (Hornung, 2011). Recent surveys of the groundwater calcretes of Western Australia have shown that oniscideans are an abundant component of the terrestrial subterranean fauna in the calcretes. However, little is currently known about subterranean oniscidean diversity and systematic relationships. Taiti and Humphreys (2008) reported 28 new troglobitic and stygobitic oniscidean isopod species from Western Australia including *Styloniscus* (Styloniscidae) and *Adoniscus* (Olbrinidae) from the Pilbara; Stenoniscidae (new genus), and stygobitic *Haloniscus* (Philosciidae) from the Yilgarn region; *Troglarmadillo* (Armadillidae) from Cape Range, Pilbara, Nullarbor, and the Yilgarn region; *Hanoniscus* (unplaced genus) from Cape Range and the Nullarbor; *Laevophiloscia* (Philosciidae) from Nambung and Augusta cave areas. A new oniscidean family, Paraplatyarthridae, was recently discovered (chapter 2), and described because of its

abundance in calcrete troglofauna samples and the notable morphological diversity in eye ommatidia and pattern of body pigmentation found in the group.

The aim of the present study is to elucidate oniscidean isopod species diversity, phylogenetic relationships and distributional patterns of the terrestrial oniscidean isopods in the groundwater calcretes of central Western Australia, using a multiple gene approach. We include both mitochondrial and nuclear genes, with application of paired end Illumina Next Generation Sequencing to identify new nuclear markers. In addition, the role of historical events, which have shaped the current structure of oniscidean fauna in the calcretes, is discussed.

## MATERIAL AND METHODS

### *FIELD SAMPLING/SORTING*

In order to collect troglofauna, borehole traps (henceforth referred to as trog-traps) made from 65mm internal diameter PVC pipes, measured variably between 150-180 mm long, approximately 0.16-0.18 litres in volume, were used. The pipes contained slits, so that invertebrates could enter the tubes, and lids to seal both ends (see Appendix 3.3E). Traps were filled with heat-sterilised (using a microwave) leaf litter, to ensure the absence of contaminating live invertebrates. The trog-traps were placed 50-100 cm above the water table within unlined mineral exploration boreholes (see Table 3.2 for locality details) using a cord attached to the traps (Appendix 3.3A-D). To maintain the humidity of the bores and promote the retention of moisture in the leaf litter, the bores were plugged with PVC pipe, and capped. Sampling was carried out 2-3 times per year (between April and October) and trog-traps were left underground for 6-10 months (in some cases 12 months) to be colonised by invertebrates. In total, 12 groundwater calcretes occurring in the Carey, Raeside and Nabberu palaeodrainages were targeted for troglofauna (Figure 3.1, Table 3.2).

After recovery of the traps, their contents were sealed in zip-lock bags for transport to the Western Australian Museum where the living fauna in the litter was extracted into 100% ethanol by means of Tullgren funnels (Appendix 3.3H). Surface isopod species from Western Australia were collected by hand under/between crevices of rotten/fallen tree branches and

preserved in 100% ethanol. Specimens caught during samplings were classified into three groups comprising a) troglobite, which included specimens characterized by their pale body and lack of or significantly reduced eyes b) troglophile including specimens with partly pigmented body and presence of eyes, and c) surface specimens with fully pigmented body and developed eyes.

Morphospecies were identified to family/genus level according to Dalens (1992), Taiti *et al*. (1998), Taiti and Humphreys (2001), Schmidt (2002, 2003) and Poore (2002).

### *DNA EXTRACTION AND SEQUENCING*

Three to six pereopods (except for male pereopod 7 which is important for morphological diagnosis) were dissected from 100% ethanol-preserved animals and rinsed in 10 mM Tris to remove the alcohol before the extraction process. Total genomic DNA was isolated using a Puregene Genomic DNA Purification Kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions (DNA purification from 5-10mg fresh or frozen solid tissue), except that centrifugation times were increased to 20 mins and 5 mins for the DNA precipitation and wash steps respectively.

Four genes including the mitochondrial Cytochrome C Oxidase subunit 1 (*COI*) gene, the nuclear Lysyl-tRNA Synthetase (*LysRS*), and two nuclear ribosomal genes: *LSU rRNA* (*28S*; D1-D3 region) and *SSU rRNA* (*18S*; core and variable regions C1, V1, C2, V2, C3) were subjected to PCR amplifications. PCR amplification of all genes involved an initial denaturation at 95°C for 10 mins and 34 subsequent cycles of 94°C for 45s, 48°C to 55°C (variable with respect to the target gene; see Table 3.1) for 45s, 72°C for 1 min and a final extension of 72°C for 6 mins. For the samples which were not successfully amplified, or showed double bands in PCR amplification, different sets of primers were designed and used (Table 3.1).

All PCRs were carried out on either Palm-Cycler thermal cyclers (Corbett, CG1-96) or Kyratec Supercycler thermal cyclers (SC300) using 25 µl reaction volumes consisting of 15.4 µl of nuclease-free molecular water, 5 µl of 5X Immolase PCR buffer (comprising 3.75 mM $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphae (dNTP) and 2.5X BSA (0.25 mg/ml)), 1 µl of each primer (5 µM concentration for *COI* and *18S* primers, 7 µM for G2328 and 8 µM for G2329, 10 µM concentration for G2281, G2282, G2340 and G2341, 7 µM for 28srD1.2a and 5 µM for 28srd4.2b) and 0.1 µl of Immolase DNA polymerase (5 u/µl). Amplified PCR

products were visualised on 1.5% agarose gels and purified using PCR multiscreen filter plates (Millipore). Purified PCR products were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified using a SEQ multiscreen filter plates (Millipore) and analysed on an ABI 3700 DNA capillary sequencer. Sequences were edited using Geneious Pro version 5.6.4 (http://www.geneious.com).

Table 3.1. Primers and the associated PCR annealing temperatures used for amplification of *COI*, *LysRS*, *28S* and *18S* in the oniscidean Isopods. Primers indicated in bold refer to forward primers.

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

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

## *IDENTIFYING A NEW CANDIDATE NUCLEAR GENE FOR PHYLOGENETICS USING NEXT GENERATION SEQUENCING (NGS) ANALYSES.*

### RNA extraction and preparation of cDNA library

Three different groups of specimens preserved in *RNAlater* (Qiagen), including 12 specimens of troglophiles (*Paraplatyarthrus* sp1., Laverton Downs Windarra, labelled as G1), seven specimens of troglobites (*Paraplatyarthrus* sp2., Laverton Downs Windarra, labelled as G2) and two large specimens of *Porcellionides pruinosus* (Mt Windarra, Laverton Downs, labelled as G3) were used for RNA extractions. The oniscidean taxa were selected as closely and distantly related species to identify conserved regions within gene markers. The cephalothorax of specimens of each group was dissected in sterile Petri dishes within *RNAlater* to avoid RNA degradation during handling. The head samples of each group were then pooled within sterile/RNAase-DNAase safe 2 ml vials and, briefly centrifuged and the remaining *RNAlater* removed. RNA was extracted using a QIAGEN RNeasy Plus Micro Kit without using a carrier. A fluorometer was used to estimate the amount of RNA extracted for each group. This revealed that 39.62 ng (2.83 ng/µl), 139.86 ng (9.99 ng/µl) and 338.8 ng (24.2 ng/µl) of RNA was extracted for G1, G2 and G3, respectively. The extracted RNA was then stored at -70°C prior to additional downstream applications.

### cDNA synthesis

To generate full length cDNA as libraries for Illumina next generation sequencing, the Clontech SMARTER PCR cDNA Synthesis Kit protocol was followed according to the manufacturer's manual (www.clontech.com), except for the ss cDNA (single stranded) synthesis step for full length cDNA synthesis, where the incubation time was extended to 90 mins.

In the Double Stranded cDNA (ds cDNA) synthesis step, 10 µl of ss cDNA was used as the starting material and the number of PCR cycles was initially set to 15 cycles. During the optimisation stage to find the most efficient number of PCR cycles for the ds cDNA synthesis, 20, 19 and 17 cycles were found to be best for G1, G2 and G3, respectively based on visualisation in 1.5% agarose gels.

An UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Inc.) was used to purify PCR-amplified cDNA products according to the manufacturer's manual, except that steps 11 to 13

were repeated. The cDNA was quantified using a fluorometer. In total, 1.739 µg (G1: 34 ng/µl), 2.15 µg (G2: 45.9 ng/µl) and 1.18 µg (G3: 43.8 ng/µl) of PCR-amplified cDNA were purified for downstream procedures.

**Analysis of Illumina transcriptome data**

The cDNA libraries including G1, G2 and G3 were sequenced using an Illumina HiSeq 2000 platform with 100bp paired-end reads. The number of reads obtained was 24,795,047 (4.96 Gb), 21,896,830 (4.38 Gb) and 23,941,206 (4.79 Gb) for G1, G2 and G3, respectively.

FastQC version 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of the illumina reads after each step in the quality control process. Tagcleaner version 0.12 (Schmieder *et al*. 2010) was used to trim the SMARTer II A Oligonucleotide (5'–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3') and 3′ SMART CDS Primer II A (5'–AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$N$_{-1}$N-3') used in the cDNA synthesis (N= A, C, G or T; N$_{-1}$= A, G or C). Trimmomatic version 0.22 (Lohse *et al*. 2012) was used to remove adapters, short and long poly A and T tails and other illumina-specific sequences from the reads (Module: ILLUMINACLIP). Sequences shorter than 30 bases after trimming were discarded resulting in paired and unpaired fastq files for each group.

*De novo* assembly of the transcriptome data was carried out using the Trinity software package (version r2012-06-08) on a DELL PowerEdge R910 server with 512GB RAM. The assembled sequence data were then subjected to BLAST analyses, with the G3 data blasted against data from the other two groups to identify contigs that were likely to be orthologous based on an e-value of $10^{-6}$ or less. A BLASTX analysis was also used to search the GenBank protein database to both determine the likely gene identity of assembled contigs and identify suitable gene models to help with the localisation of exon/intron boundaries. For all BLASTX analyses, the branchiopod crustacean *Daphnia pulex,* for which a comprehensive database of genomic and transcript data are available, was found to be the closest subject to most of the G3 contig data. For those markers that no genomic/transcript sequences were found to match crustacean sequences using BLASTX, *Drosophila melanogaster* was used as a reference to identify the potential position of exons in the transcriptome data and potentially locate the position of introns. MUSCLE alignment followed by MAFFT alignment (algorithm: E-INS-I; scoring matrix: 200Pam/k=2; Gap open penalty: 1.53) implemented in

Geneious Pro 5.6.4 was used to align the selected markers and the associated annotated references (*D. pulex* or *D. melanogaster*). All primer designs were carried out using Primer3 version 0.4.0 (Koressaar & Remm, 2007).

***PHYLOGENETIC ANALYSES***

Alignments were carried out using ClustalW (cost matrix: IUB, Gap open cost: 9, Gap extend cost: 3) allowing free end gaps. To conduct phylogenetic analyses, the data were partitioned into seven subsets including first, second and third codon positions of *COI*, *Lysyl tRNA Synthetase*, *28S* and conserved (C1, C2, C3) and variable (V1, V2) regions of the *18S* gene. Mrmodeltest version 2.3 (Posada & Crandall, 1998) was used to estimate the best nucleotide substitution model for each data subset using an Akaike Information Criterion (AIC) framework. A GTR+I+G (Rodríguez *et al.* 1990; Yang, 1996)) was found to be the most appropriate nucleotide model for *COI* codon positions; HKY+I+G (Hasegawa *et al.* 1985; Yang, 1996) for *Lysyl tRNA Synthetase*; GTR+G (Rodríguez *et al.* 1990; Yang, 1996) for *28S;* SYM+I (Zharkikh, 1994) and K80+G (Kimura, 1980; Yang, 1996) for the core and variable regions of *18S,* respectively. Garli 2.0-win (Zwickl, 2006), which performs phylogenetic searches using the maximum likelihood (ML) criterion, was used to examine the best partitioning scheme for the dataset. Thirteen different partitions of *COI* first, second and third base codon positions, *Lysyl tRNA Synthetase*, *28S* and core and variable regions of *18S* (C1-C2-C3, V1-V2) were examined to calculate the lnL and AIC index for each partition (Table 3.3). To run individual partitioned models of ML analyses for each scheme, the Garli configuration file was set for two independent search replicates and all parameters were unlinked. The subset specific rate multiplier was set to vary over data subsets and other settings of the Garli configuration file were according to the default. The likelihood scores of the two independent runs were computed and the greater likelihood score was chosen for calculation of AIC scores. The AIC score of each partitioning scheme was calculated as AIC = 2x (#parameters - lnL) and the lowest value was chosen as the best score.
According to Table 3.3, partition number 13 which treated each subset separately showed the highest ML score (-14320.8661) and lowest AIC value (28747.7322), and, therefore, it was selected as the best partition scheme for phylogenetic analyses.

Bayesian Inference (BI) analyses of both single and combined datasets were performed using the procedure of Markov Chain Monte Carlo (MCMC) convergence as implemented in

MrBayes version 3.2.0 (Huelsenbeck & Ronquist, 2005). All parameters were unlinked and the rates were allowed to vary over the subsets. Two independent runs with four chains were run simultaneously for 5 million generations, subsampling trees and parameters every 100 generations. The final standard deviation of split frequencies reached less than 0.002 (except for single *Lysyl-tRNA* phylogeny which was 0.004) and PSRF values for all parameters were 1.0 suggesting convergence had occurred. To further assess convergence to the stationary distribution, the program Tracer version 1.5 (Rambaut & Drummond, 2003) was used. For each independent MrBayes run, a 25% burn-in, equivalent to 12,500 samples, were discarded from the 50,001 samples sub-sampled during the analysis (i.e. 37,501 samples were included). A 50% majority rule BI consensus tree was constructed from the remaining trees and posterior probabilities were used to assess the robustness of nodes. Five phylogenetic analyses were carried out, including one based on *COI* only (680 bp) to obtain a general picture of the subterranean oniscidean diversity in the groundwater calcretes; a single phylogeny of the nuclear gene, Lysyl-tRNA Synthetase (*LysRS*) to compare its topology and branching pattern with those of the mitochondrial *COI*, and three analyses variously combining two genes *COI-Lysyl tRNA* (1434 bp), three genes *COI-Lysyl tRNA-18S* (2114 bp) or four genes *COI-Lysyl tRNA-28S-18S* (2781 bp) to reconstruct and compare oniscidean relationships.

Each of the combined dataset ML analyses, with the same gene partitioning scheme as used for BI analyses, was carried out using Garli OSX version 2.0 (Zwickl, 2006). The ML analyses were set to carry out two search replicates; substitution models were unlinked and subset specific rates were allowed to vary across partitions and the number of bootstraps set to 500 replicates. Other parameters were set according to Garli configuration file defaults. As Garli does not calculate consensus trees from bootstrap replicates, the Sumtree package (Sukumaran & Holder, 2010) under Dendropy 3.12.0 (Sukumaran & Holder, 2010), which is a Python (version 2.7.3) library for phylogenetic computing, was used to make 50% majority rule ML bootstrap consensus trees. All phylogenetic trees were rooted using *Ligia* sp. as the out-group, selected based on results from chapter 2. Figtree version 1.3.1 (Rambaut, 2009) was used to visualize phylogenetic trees. The inter lineage *COI* p-distances and the Transition to Transversion ratios (Tr/Tv) were calculated using Mega version 5.1 (Tamura *et al. 2007*).

### *SPECIES DELIMITATION*

For species delineation, both Poisson Tree Processes (PTP; Zhang *et al*. 2013) and General Mixed Yule Coalescent (GMYC; Pons *et al*. 2006; Fujisawa & Barraclough, 2013) models were used on the mitochondrial *COI* data using the PTP package (Zhang *et al*. 2013). To perform a *de novo* species delimitation using the stand-alone PTP model, a Maximum Likelihood tree using Garli 2.0 was used as input. An ultrametric tree (time calibrated) obtained by BEAST version 1.6.1 (Drummond *et al.* 2006; Drummond & Rambaut, 2007) was used for the GMYC model. In addition, a conservative species threshold (12% *COI* p-distance) based on a sister relationship between two described *Paraplatyarthrus* species (chapter 5), both occurring in the same palaeodrainage, with consideration of the morphological evidence, was used as the third criterion for species delimitation. To make a final decision on the number of putative species a combination of species delimitation methods, the results of single nuclear and multiple gene phylogenies and morphological evidence were used.

Table 3.2. Lineage codes, locations of isopod lineages and the associated geographic coordinates, BES codes and GenBank accession numbers (available after journal submission). Abbreviation for the calcretes and some localities from Western Australia as follows: Calcrete areas: Laverton Downs-Windarra (LDW); Laverton Downs-Erlistoun (LDE); Laverton Downs-Quandong (LDQ); Laverton Downs-Shady Well (LDS); Sturt Meadows (SM); Cunyu (CUN); Lake Violet (LV); Lake Miranda East (LME); Lake Miranda West (LMW); Nambi (NAM); Uramurdah (URA); Bubble Well (BUB); Halfpenny Well (HPW); Barwidgee (BAR); Hinkler Well (HIN); Mt Morgans (MOR); Non-calcrete areas: Jorgensen Park, Kalamunda, WA (JOP); Gooseberry Hills, WA (GOO); Wooroloo, WA (WOO); Moorapulling Rd. (MOO), Marradong, WA. Numbers in parentheses are the code numbers on the map. Lineage codes indicated with bold, normal and grey-shaded fonts show troglobites, troglophiles and surface species respectively.

| **Lineage codes** | **Family/Genus** | **Locality/Coordination** | **BES numbers or JA Codes** | **GenBank accession number** | | | |
|---|---|---|---|---|---|---|---|
| | | | | ***COI*** | ***LysRS*** | ***28S*** | ***18S*** |
| **A1** | Stenoniscidae/ unknown genus | LDW (6) S28.44388, E122.18681; S28.52602, E122.18787 | 16022; 16023 | X | X | X | X |
| **A2** | Stenoniscidae/ unknown genus | LDE (5) S28.44388, E122.19568 | 16071 | X | X | X | X |
| B1 | Paraplatyarthridae/ *Paraplatyarthrus* | SM (1) S28.70124, E120.90361; S28.7003, E120.9026 | 15551.8,9; 17225.1,2 | X | X | X | X |
| **B2** | Paraplatyarthridae/ *Paraplatyarthrus* | CUN (14) S25.7806, E120.1075 | 15090.1,3 | X | X | X | X |
| B3 | Paraplatyarthridae/ *Paraplatyarthrus* | LV (11) S26.7091, E120.2357; S26.709, E120.2346 | 15080;15097 | X | X | X | X |
| **B4** | Paraplatyarthridae/ *Paraplatyarthrus* | LME (15) S27.66384, E120.61076; S27.6638, E120.6108 | 15543.3; 17215.2 | X | X | X | X |
| **B5** | Paraplatyarthridae/ *Paraplatyarthrus* | LMW (16) S27.74667, E120.5266 | 15538.10 | X | X | X | X |
| B6 | Paraplatyarthridae/ *Paraplatyarthrus* | LDW (6) S28.4989, E122.1798 | 3U.1,3; 14632.3 | X | X | X | X |
| B7 | Paraplatyarthridae/ *Paraplatyarthrus* | NAM (7) S28.2351, E121.8306 | 17221.1 | X | X | X | X |
| **B8** | Paraplatyarthridae/ *Paraplatyarthrus* | LDQ (3) S28.35515, E122.22551 | 16567.1 | X | X | X | X |
| B9 | Paraplatyarthridae/ *Paraplatyarthrus* | URA (12) S26.6876, E120.313; S26.6876, E120.3078 | 15088.1; 15087.1 | X | X | X | X |
| B10 | Paraplatyarthridae/ *Paraplatyarthrus* | URA (12); BUB (13); MIL (13) S26.6876, E120.313; S26.5607, E120.0409; S26.5607, E120.0409 | 15067.1; 15095.3; 15065.1 | X | X | X | X |
| B11 | Paraplatyarthridae/ *Paraplatyarthrus* | LV (11) S26.70923, E120.26404 | 16476.1,2 | X | X | X | X |
| B12 | Paraplatyarthridae/ *Paraplatyarthrus* | HPW (8); NAM (7) S27.6966, E121.3395; S28.2210, E121.8216 | 15071.2; 17222.1 | X | X | X | X |
| **B13** | Paraplatyarthridae/ *Paraplatyarthrus* | LDW (6) S28.50282, E122.17726 | 15525.15,25 | X | X | X | X |
| **B14** | Paraplatyarthridae/ *Paraplatyarthrus* | LDQ (3) S28.35515, E122.22551 | 16567 | X | X | X | X |
| **B15** | Paraplatyarthridae/ *Paraplatyarthrus* | LDW (6) S28.49937, E122.17838 | 15524.6 | X | X | X | X |
| **B16** | Paraplatyarthridae/ *Paraplatyarthrus* | LDS (4) S28.4074, E122.1997 | 14605.1 | X | X | X | X |

Table 3.2. Continued

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

Table 3.3. Garli partitioning schemes, lnL, number of parameters and AIC values. The numbers in the partitioning scheme column denote: 1, 2 and 3 for the *COI* first, second and third codon positions respectively; 4 for *LysRS*, 5 for the *28S*, 6 and 7 for core and variable regions of the *18S* gene respectively.

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

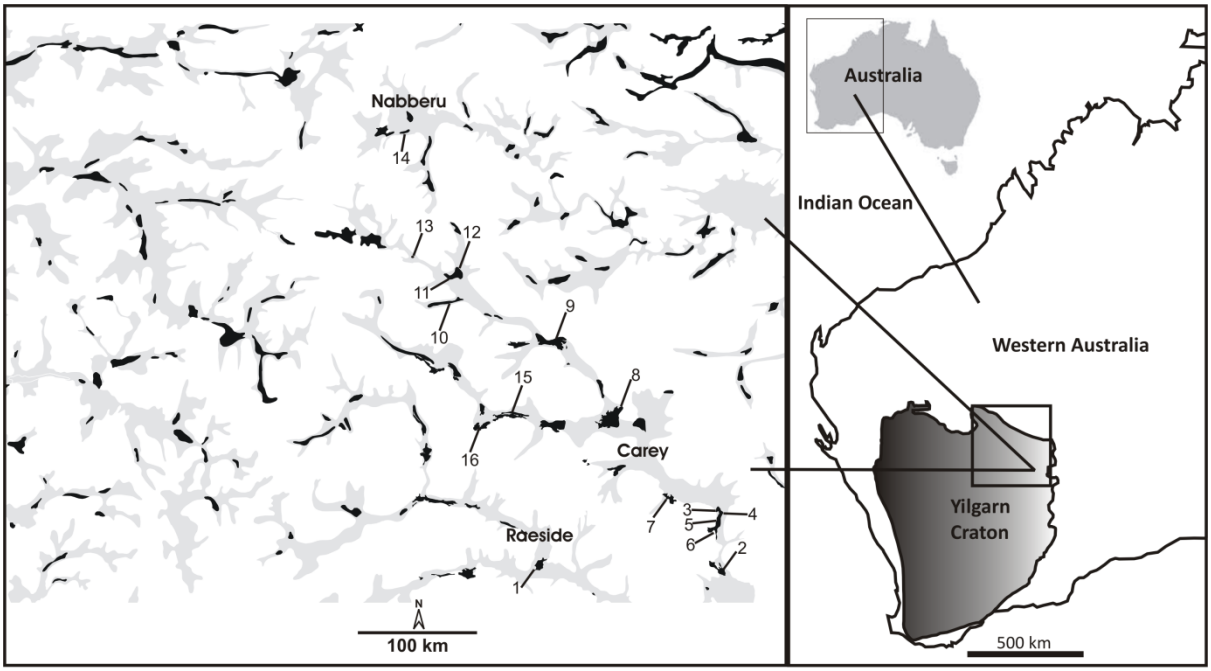


Fig. 3.1. A map of the sampled groundwater calcretes and their positions in the palaeodrainages. Numbers refer to the calcretes as listed in the Table 3.2. Black shaded areas indicate groundwater calcretes and grey shaded ones are palaeodrainage valleys.

## RESULTS

Approximately 1500 specimens identified as oniscidean isopods were collected from groundwater calcretes of the Yilgarn region between 2008 and 2012 (Appendix 3.1). In total, 907 specimens were classified as troglobite and 592 as troglophile (see Methods). Four oniscidean families including Paraplatyarthridae (genus *Paraplatyarthrus*), Armadillidae (genera *Troglarmadillo, Buddelundia* (surface species), and two unrecognised genera), Philosciidae (genus *Haloniscus*) and Stenoniscidae (unknown genus) were identified from the calcretes. Paraplatyarthrid isopods were found to be the most frequently collected oniscidean group (n=1156) by the trog-traps. In contrast, Stenoniscidae with just 11 specimens collected from the Laverton Downs calcrete (Windarra and Erlistoun sites) was the least abundant troglobitic group collected.

In order to develop new molecular markers for phylogenetic analyses using the Illumina next generation sequence data, 23 nuclear markers were chosen for primer design and PCR-amplification, of which one marker, identified as the Lysyl-tRNA Synthetase gene (*LysRS*), was found to successfully PCR-amplify across the majority of taxa examined. This gene encodes the enzyme Lysyl tRNA Synthetase which catalyses the covalent attachment of Lysine to the 3' end of the cognate tRNA (Lysyl-transfer RNA), which would then add Lysine into proteins during translation (Chan & Bingham, 1992; Freist & Gauss 1995). The exon 3 of *LysRS,* which is 949 bp in length, was found to be conserved and phylogenetically informative, and therefore was further sequenced for all taxa using Sanger sequencing methods. The NGS statistics for each oniscidean group are given in Table 3.4.

Table 3.4. Illumina next generation sequencing statistics for the three oniscidean isopod groups examined.

| Groups | Number of reads | Number of contigs | Number of reads > 1 kb | Min read length | Max read length |
|---|---|---|---|---|---|
| **G1** | 24,795,047 | 40,461 | 6,602 | 201 | 17,556 |
| **G2** | 21,896,830 | 46,114 | 9,089 | 201 | 17,251 |
| **G3** | 23,941,206 | 37,368 | 4,720 | 201 | 14,232 |

326 oniscidean *COI* sequences, most belonging to subterranean lineages, were generated from 12 groundwater calcretes and five surface localities (Table 3.2). As well, 122 sequences of *18S*, 120 of *28S* and 100 of *LysRS* were also produced.

### *SINGLE MITOCHONDRIAL "COI" AND NUCLEAR "LYSYL-tRNA SYNTHETASE" ANALYSES; GC CONTENT AND TRANSITION/TRANSVERSION ESTIMATION*

Bayesian phylogenetic analyses of the *COI* data (Figure 3.2; between one to five sequence representatives were selected per lineage; see Appendix 3.2 for the tree based on the whole *COI* sequence data) showed 36 subterranean mtDNA lineages (41 with surface species with the exception of *Buddelundia cf. labiata*) belonging to Stenoniscidae (A codes), Paraplatyarthridae (B codes, *Paraplatyarthrus*), Philosciidae (C codes, *Haloniscus*) and Armadillidae (D codes; D1-D10 for *Troglarmadillo*; D11 and D12 probably belong to distinct, currently undescribed genera).

The subterranean lineages of Armadillidae, Philosciidae and Stenoniscidae were each restricted in their distribution to a single calcrete aquifer. Three paraplatyarthrid clades showed the presence of identical or closely related haplotypes that were shared between two or more calcretes (B17 in Halfpenny, Nambi and Laverton Downs-Windarra; B10 in the Uramurdah and Bubble Well calcretes; B12 shared between Halfpenny and Nambi calcretes). All other paraplatyarthrid lineages were found to be restricted to individual calcrete bodies. The *COI* BI phylogeny also showed a strongly supported clade grouping the single surface species collected from Mt Morgan with B18 (Barwidgee) and B17 subterranean lineages. The single nuclear Bayesian phylogeny of the *LysRS* (Figure 3.3) showed the same topology as *COI* for the majority of the clades with only a few exceptions; polytomies that were evident in *COI* armadillid and paraplatyarthrid clades were resolved in the *LysRS* phylogeny. However, the phylogenetic relationships of the B13/15 clade and a well-supported group comprising S4/B17 with other clades were not resolved in the *LysRS* phylogeny.

The paraplatyarthrid inter-lineage *COI* p-distances ranged from 1.8% to 20.4% (Appendix 3.4). The lowest p-distances corresponded to the B13-B14 (1.8%), B10-B11 (4.9%) and B9-B10 (4.9%) comparisons. Among the armadillid lineages, the p-distance varied from 17% (D1-D2, D4-D8) to a maximum of 26%, occurring between D3-D8 lineages (Appendix 3.5). Philosciid lineages showed a minimum p-distance of 12% between C1-C2 and a maximum of

18% divergence in the C1-C4 and C3-C4 pair-wise comparisons (Appendix 3.6). A 9% p-distance was calculated between A1-A2 lineages of Stenoniscidae.

Oniscidean GC contents (Table 3.6) showed a significant difference between the four genes ($F= 8.233$, $P<0.05$). The *28S* GC content (oniscidean average 52.7%) was significantly higher than those of *COI* (35.4%), *LysRS* (38.7%) and *18S-V* (45.7%) while comparisons of *COI*, *Lysyl* and *18S-V* did not show a significant difference in GC content ($P>0.05$).

A total ML estimation of Transition/Transversion bias (R) in different genes across the higher lineages ranged from 1.1 in *18S* core regions to 4.88 in *COI*. The observed proportion of transitions to transversions at the first, second and third codon positions of *COI* across all lineages were 5.51, 2.10 and 6.23, respectively. A minimum Tr/Tv value of 0.74 was estimated for the *18S* variable regions (V1-V2) of paraplatyarthrid lineages, while the highest value (51.46) was estimated for the *18S* core regions (C1-C2-C3) of *Haloniscus* lineages (Table 3.6).

***SPECIES DELIMITATION***

The PTP model for species delimitation of oniscidean isopods collected from subterranean and surface habitats predicted 33 subterranean and five surface species ($\lambda_s =$ 5.59 as speciation rate per substitution; $\lambda_c =$ 88.04 as the rate of within species branching events per substitution (coalescent rate); Maximum log-likelihood score = 122.98; P-value = $1.11e^{-16}$ ). The model yielded 12 armadillid (11 subterranean and one surface species), five philosciid, two stenonisciid (all subterranean), and 19 paraplatyarthrid (15 subterranean and four surface) species (Table 3.5). The GMYC model resulted in 36 subterranean and five surface species (highest log-likelihood (LLH) = 40.30; P-value = 0).

A 12% *COI* p-distance divergence between two sister paraplatyarthrid species S4 and B17, which are considered as separate species (described in chapter 5) based on molecular and morphological evidence, was used as a threshold for species delineation of the crinochete oniscidean isopod troglofauna and the associated paraplatyarthrid surface species. In total, using the species delineation threshold defined, 26 subterranean oniscidean DNA lineages out of 41 (subterranean and surface) can be considered as putative species: nine paraplatyarthrid (11 including the surface lineages), 11 armadillid, five philosciid and one stenoniscid lineage (Table 3.5).

Based on the fourth criterion, the combination of methods utilising the results of single and multiple gene phylogenies together with morphological evidence, 26 subterranean lineages (the same recognition pattern as the 12% threshold) plus two more lineages, including one armadillid amplified only for *LysRS* (D6 from Sturt Meadows) and one more Paraplatyarthrid lineage that appeared to be a troglobitic form (B8 from Laverton Downs Quandong, Clade 3) with at least 9.1 % divergence from other paraplatyarthrid lineages, were considered as distinct putative species (28 subterranean species).

Table 3.5. The number of putative species based on the PTP, GMYC and 12% threshold for species delimitation of the subterranean/surface oniscidean species.

| | | PTP | GMYC | 12% threshold |
|---|---|---|---|---|
| **Paraplatyarthridae** | subterranean | 15 | 18 | 9 |
| | surface | 4 | 4 | 2 |
| **Armadillidae** | subterranean | 11 | 11 | 11 |
| | surface | 1 | 1 | 1 |
| **Philosciidae** | subterranean | 5 | 5 | 5 |
| | surface | - | - | - |
| **Stenoniscidae** | subterranean | 2 | 2 | 1 |
| | surface | - | - | - |
| **Total subterranean** | | 33 | 36 | 26 |
| **Total** | | 38 | 41 | 29 |

### *COMBINED PHYLOGENETIC ANALYSES*

The BI and ML phylogenetic analyses of the combined genes including *COI-LysRS* (Figure 3.4), *COI-LysRS-18S* (Figure 3.5) and *COI-LysRS-28S-18S* (Figure 3.6) showed a consistent topology with high posterior probabilities and bootstrap support values for most nodes. As some genes did not amplify for some taxa it was not possible to generate a complete dataset comprising all four genes for all of the mtDNA lineages identified above, particularly those within the Armadillidae and Philosciidae, so the combined analyses did not include some lineages. However, the combined dataset was amenable for reconstructing phylogenies that included all major paraplatyarthrid lineages.

In the combined phylogenies, monophyly of all calcrete lineages of Paraplatyarthridae (*Paraplatyarthrus*), Armadillidae (*Troglarmadillo*), Philosciidae (*Haloniscus*) and the

Stenoniscidae lineages was strongly supported (Figures 3.4-6). Combined phylogenies of paraplatyarthrid lineages revealed the occurrence of five well supported distinct clades in both BI and ML analyses. Clade 1 included both troglobitic (Lake Miranda East/West and Cunyu) and troglophilic (Sturt Meadows, Lake Violet) taxa. Both Lake Miranda East and West were sister to a group comprising Sturt Meadows, Cunyu and Lake Violet calcrete lineages. The lineages, B1 (Raeside), B2 (Nabberu) and B3 (Carey), belonging to different palaeodrainages, formed a highly supported group (PP = 1.00; BP = 100), and were more closely related to B4 and B5 from Lake Miranda East/West (Carey), which were sister to all other groups, with high PP and BP support (Figures 3.4-6). The surface taxa from Jorgensen Park, Gooseberry Hills, Wooroloo and Moorapulling formed a second clade (Clade 2) with high support (PP = 1.00; BP = 100), but their relationships with other lineages were not strongly supported in the combined phylogenies. Clade 3 comprised troglophilic species from Laverton Downs Windarra, Nambi, Halfpenny Well, Uramurdah, Bubble Well, Lake Violet and a single troglobitic species from the Laverton Downs calcrete (Quandong). The lineages B13-B16, all troglobitic forms from the Laverton Downs calcrete (Quandong, Shady well, Windarra), constituted a fourth clade (Clade 4) which is sister to Clades 1, 2 and 3 in all Bayesian analyses (high support in the single *COI* (PP = 0.96) and combined *COI-LysRS* (PP = 0.99) phylogenies). A fifth clade (Clade 5) included the surface species from Mt Morgan and the troglophilic lineage B17 distributed in Nambi, Halfpenny and Laverton Downs-Windarra calcretes (PP = 1.00; BP = 98, 97). This clade formed a sister lineage in the tree relative to a group comprising all the remaining paraplatyarthrid clades (PP = 1.00; BP = 100).

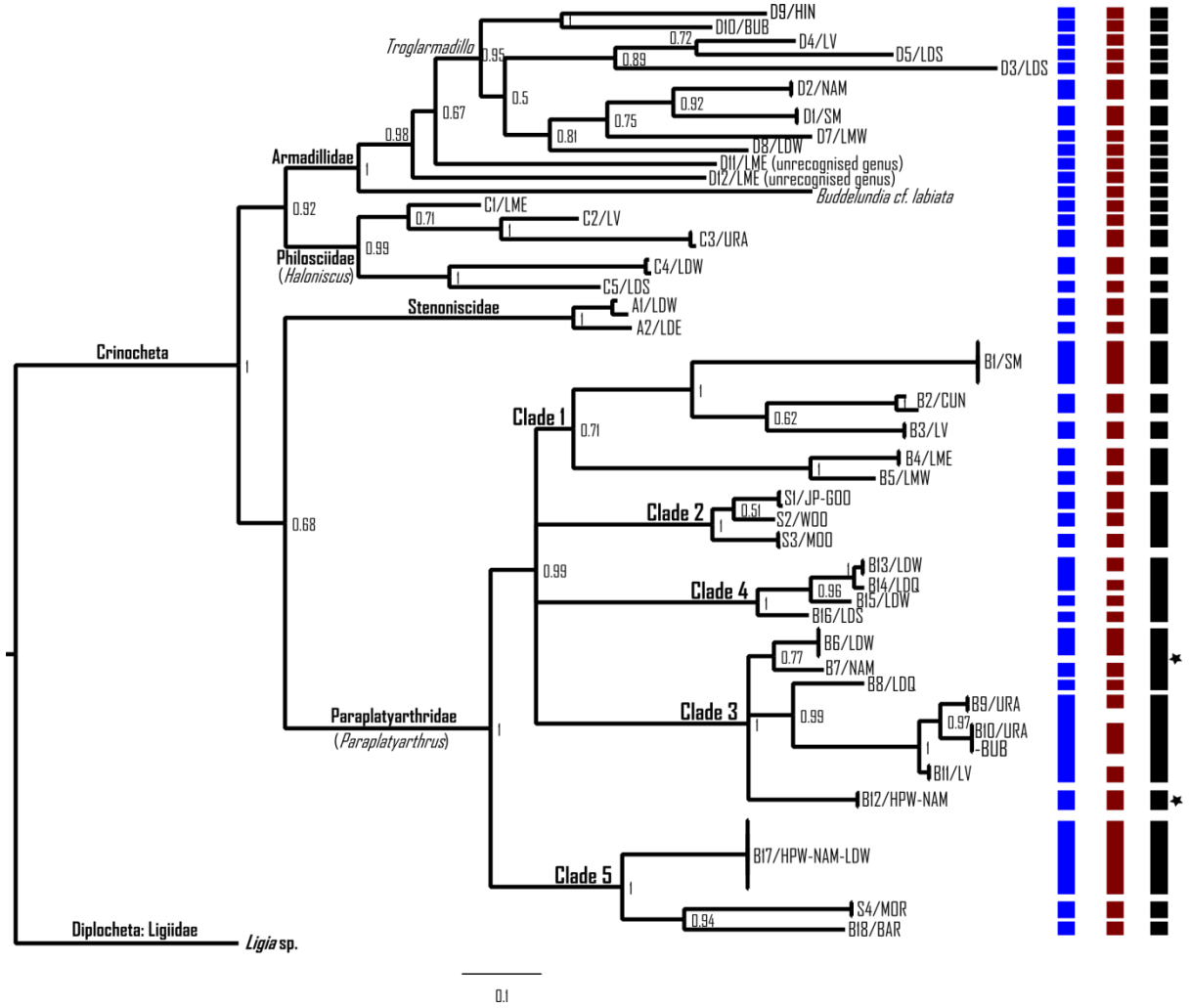


Fig. 3.2. Majority rule consensus Bayesian Inference tree based on the mtDNA *COI* gene. The numbers next to the nodes are posterior probabilities. The clade labels comprise lineage specific and calcrete codes, respectively. The blue, red and black bars show species delimitation using the PTP, GMYC and a 12% nucleotide sequence divergence threshold, respectively, for subterranean and surface species of oniscidean crinochete isopods in Western Australia. The black stars denote lineages considered as the same putative species based on the 12% threshold.

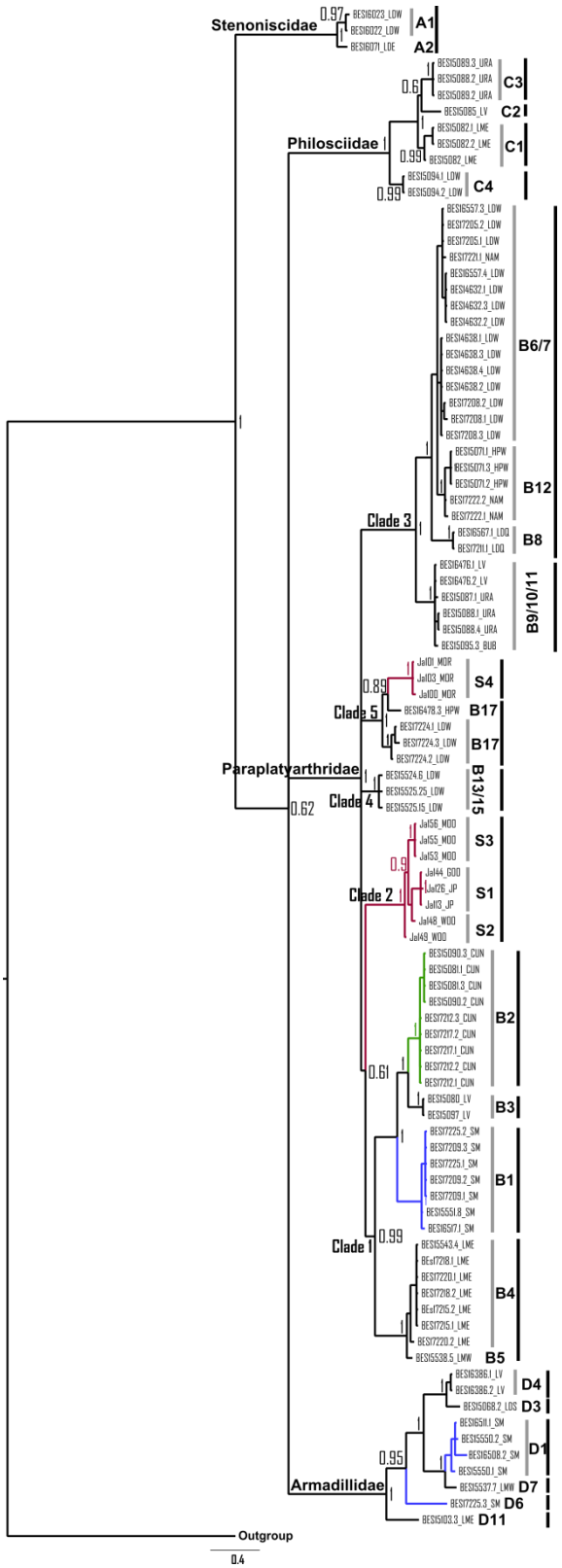


Fig. 3.3. Consensus *LysRS* Bayesian Inference tree. The numbers next to the nodes are posterior probabilities. The clade codes comprise voucher numbers and calcrete codes separated by an underscore, respectively. The colour coded branches referred to as Carey (black), Raeside (blue) and Nabberu (green) palaeodrainages; red branches denote surface species. The labels next to the grey bars refer to lineage specific codes. The black bars refer to the putative species based on the *COI* 12% threshold.

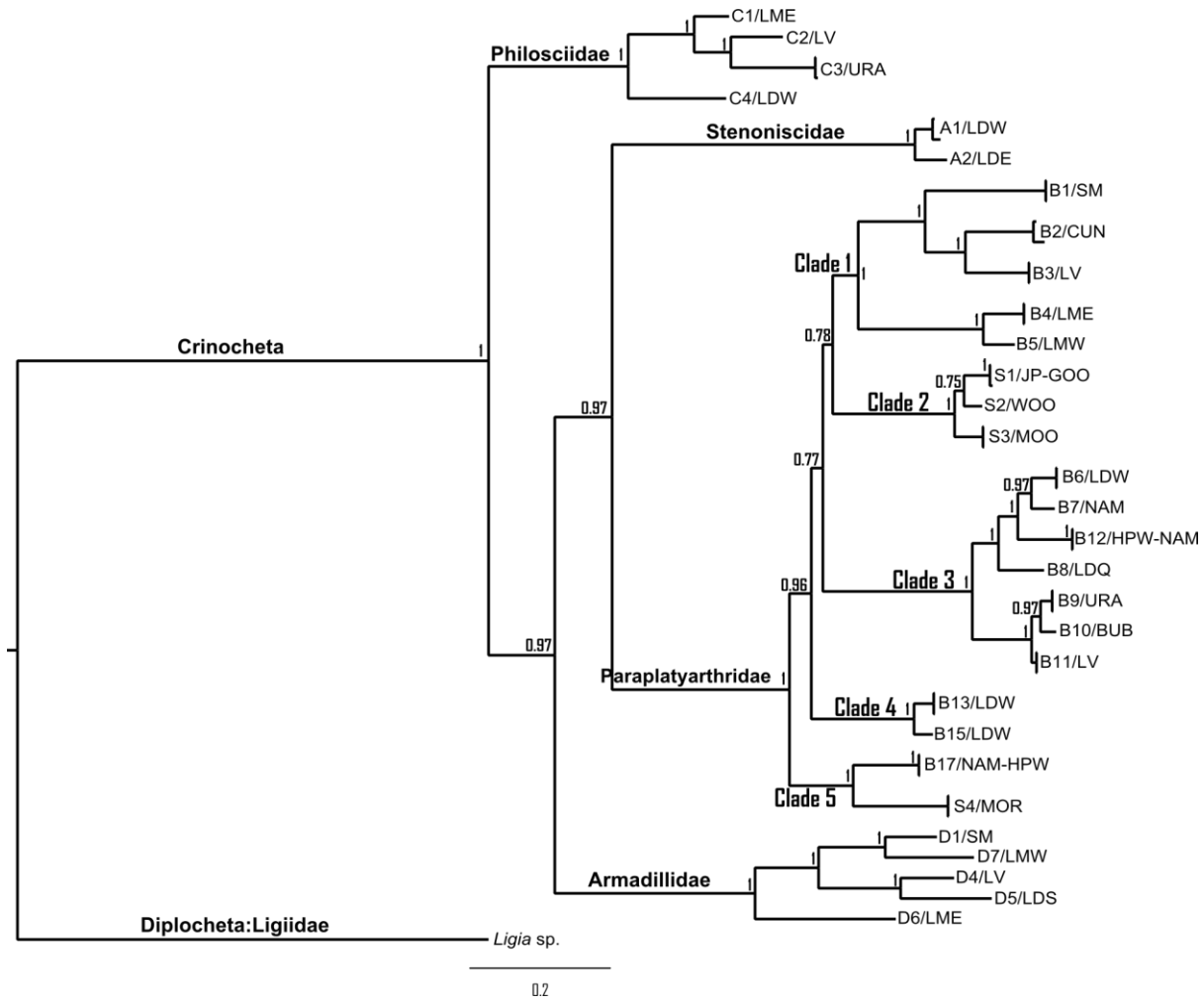


Fig. 3.4. Majority rule consensus Bayesian tree of mtDNA *COI* and nuclear *LysRS* genes. The numbers next to the nodes are posterior probabilities. The clade labels comprise lineage specific and calcrete codes, respectively.

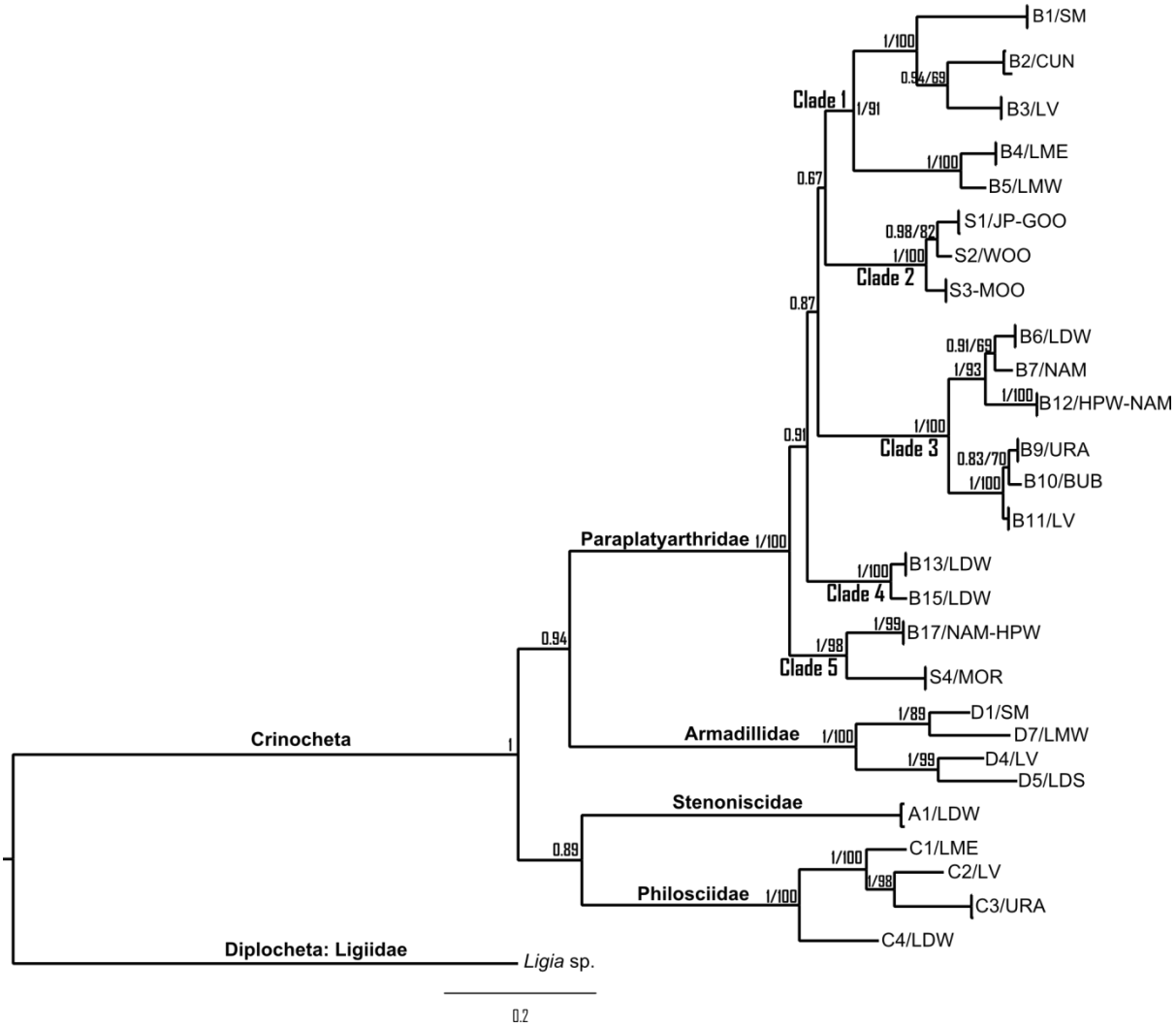


Fig. 3.5. Majority rule consensus Bayesian tree based on three genes comprising *COI*, *LysRS* and *18S*. The numbers next to the nodes are posterior probabilities and Maximum Likelihood bootstrap values, respectively. The clade labels include lineage specific and calcrete codes.

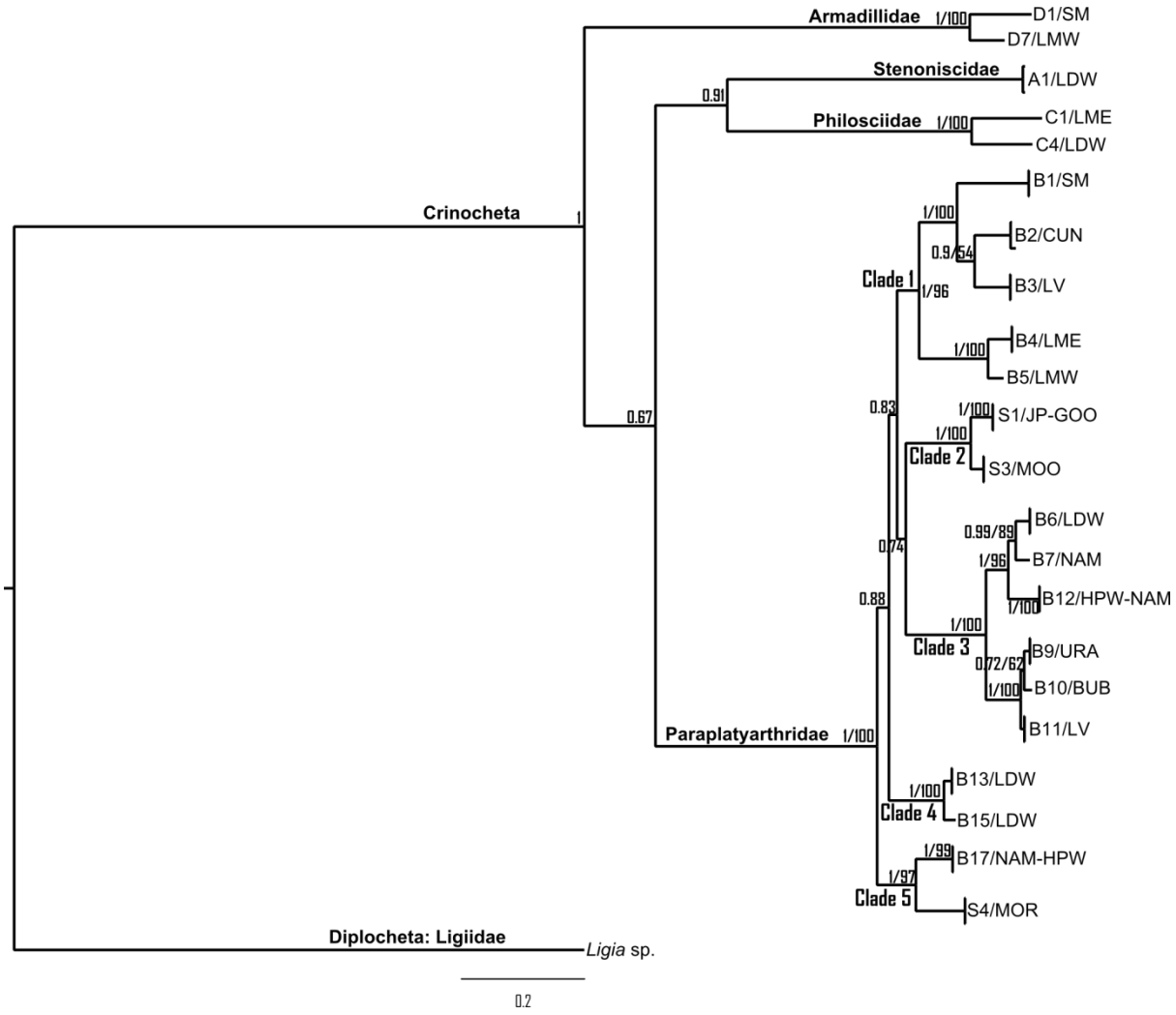


Fig. 3.6. Majority rule consensus Bayesian tree based on four genes comprising *COI*, *LysRS*, *28S* and *18S*. The numbers next to the nodes are posterior probabilities and Maximum Likelihood bootstrap values, respectively. The clade labels include lineage specific and calcrete codes.

Table 3.6. Minimum to Maximum *COI* and Lysyl-tRNA Synthetase (*LysRS*) p-distances given as a percentage, the ratio of Transitions to Transversions using Maximum Likelihood and GC content percentage in *COI*, *LysRS Synthetase*, *28S*, *18S* core regions (C1-C2-C3) and *18S* variable regions (V1-V2). The higher lineage codes are Stenoniscidae (A), Paraplatyarthridae (B), Philosciidae (C) and Armadillidae (D).

| **Higher lineages** | **P distance % (Min-Max)** | | **ML Tr/Tv (R)** | | | | | | | | **GC content (%)** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *COI* | *LysRS* | *COI* (1+2+3) | *COI* 1CODON | *COI* 2CODON | *COI* 3CODON | *Lysyl-tRNA* | *28s* | *18s-C* | *18s-V* | *COI* | *Lysyl-tRNA* | *28s* | *18s-C* | *18s-V* |
| B | 1.8-20.4 | 0.3-7.9 | 3.58 | 5.51 | 2.10 | 6.23 | 2.80 | 4.92 | 1.08 | 0.74 | 33.5 | 38.8 | 53.0 | 48.5 | 50.1 |
| C | 12-18 | 1.1-3.7 | 5.29 | | | | 4.00 | - | 51.46 | 1.42 | 38.2 | 38.4 | 47.2 | 49.2 | 34.5 |
| D | 17-26 | 1-4 | 14.94 | | | | 3.67 | - | 1.49 | 2.94 | 39.7 | 38.2 | 54.7 | 48.4 | 32.8 |
| A | 9 | - | 5.19 | | | | - | - | - | - | 40.6 | 36.9 | 51.6 | 48.7 | 46.8 |
| Average | - | - | 4.88 | | | | 2.44 | 2.42 | 1.10 | 1.29 | 35.4 | 38.7 | 52.7 | 48.6 | 45.7 |

## DISCUSSION

### *DIVERSITY, DIVERGENCES AND SPECIES DELINEATION*

The current research is the first molecular study to explore diversity, species delimitation and phylogenetic relationships of troglofauna associated with the calcrete aquifers in Western Australia. The phylogenetic analysis based on *COI* showed significant mtDNA lineage diversity within four subterranean terrestrial oniscidean families, namely Armadillidae, Philosciidae, Paraplatyarthridae and Stenoniscidae. The *COI* BI phylogeny of the subterranean isopod species showed a remarkable spectrum of oniscidean mtDNA lineage diversity across the calcretes. In total, 37 subterranean DNA lineages were identified from the groundwater calcretes, including 18 paraplatyarthrid (22 with surface lineages) belonging to genus *Paraplatyarthrus*, 12 armadillid (*Troglarmadillo* and two more belonging to undescribed genera), five philosciid (*Haloniscus*) and two stenoniscid (unknown genus) lineages. The paraplatyarthrid lineages, collected in all calcrete aquifers using trog-trap sampling, were found to be the most abundant subterranean isopod group. In contrast, Stenoniscidae with just 11 specimens collected was found to be the least abundant group represented in collections. However, a limitation is that the method of sampling may not reflect the actual diversity/frequency that was present for all the groups.

The present study showed the diversity of the oniscidean isopod troglofauna in the calcrete aquifers is comparable with that of invertebrate stygofauna discovered in this region. Such a concentrated oniscidean diversification has also been documented for the stygobitic isopod *Haloniscus*, with evidence for 24 divergent mtDNA lineages in calcrete aquifers of the Yilgarn region, each restricted in their distribution to an individual calcrete body (Cooper *et al.* 2008). A high level of divergence (generally more than 25%) among calcrete populations of the stygobitic *Haloniscus* has been previously documented in calcrete aquifers of the Yilgarn region (Cooper *et al.* 2008). The presence of *Haloniscus* both in groundwater and terrestrial parts of the calcretes may be a result of trog-traps drowned by water table fluctuations. However, there is still some possibility that *Haloniscus* can move between the aquatic and terrestrial parts of the calcretes suggesting a transitional life. Cooper *et al.* (2007), by conducting a molecular phylogenetic analysis of amphipod species in groundwater calcretes of central Western Australia documented a minimum 10.2% inter-lineage divergence among calcrete populations. Subsequently, it was reported that the

amphipod calcrete populations may indeed represent distinct species (Bradford *et al.* 2010), of which three have recently been described as new sympatric genera (King *et al*. 2012). The calculated genetic distances in oniscidean troglofauna showed high inter-lineage divergences which averaged 20% in *Troglarmadillo* (Armadillidae), 16.3% in *Paraplatyarthrus* (Paraplatyarthridae), 15% in *Haloniscus* and 9.9% in Stenoniscidae.

In order to delineate the oniscidean subterranean species, the PTP and GMYC models of species delimitation, based on the phylogenetic species concept (Eldredge & Cracraft, 1980; Cracraft, 1983; Nixon & Wheeler, 1990; Davis & Nixon, 1992; Baum & Donoghue, 1995) were used. The PTP model yielded 38 subterranean (33) and surface (5) oniscidean putative species, all representing undescribed taxa. According to this model, the lineages of a troglophile paraplatyarthrid clade including B9, B10 and B11 were considered to belong to the same putative species; similarly, the lineages of a paraplatyarthrid troglobite clade comprising B13 and B14 from Laverton Downs calcrete were identified as a single putative species. All armadillid, philosciid, stenoniscid and the rest of the paraplatyarthrid lineages were each considered as individual putative species based on the PTP model.

The GMYC model yielded 41 subterranean (36) and surface (5) putative species, which essentially considered all oniscidean lineages as putative species. Lohse (2009) argued that the GMYC model (Pons *et al*. 2006, Fontaneto *et al*. 2007) may overestimate the species number if incomplete sampling of demes is involved in the coalescent process. The same authors found this model could artificially yield clusters recognised as separate species when approximately less than 20% of all demes were sampled. The number of paraplatyarthrid putative species by the GMYC model may also be overestimated as a result of the lack of sampling across the calcretes. The PTP model proved to be more conservative compared to the GMYC model as it recognised some of the within calcrete structures/nearby calcretes as the same putative species. The latter finding is consistent with previous studies of stygofauna within calcretes such as Laverton Downs, where considerable intra-specific variation was reported (Guzik *et al*. 2011).

Crinochete oniscidean isopods, including both subterranean and some surface species, were also delineated based on a conservative threshold (12% p-distance equal to about 14% Patristic distance) calculated between two closely related paraplatyarthrid sister lineages

occurring in the same palaeodrainage (S4, surface species and B17, troglophile) considered as distinct species based on several autapomorphies (Chapter 5). By application of this threshold to delineate species, the number of paraplatyarthrid mtDNA lineages reduced to 11 distinct putative species. As a result, the occurrence of some sister lineages within the same calcretes such as troglobites from Laverton Downs (B13-B16), Lake Miranda (B4 and B5) or the widespread troglophiles plus one troglobite lineage and surface lineages including (B9-B11), (B6, B7, B8 and B12) and (S1-S3) were considered as the same putative species but showing significant intraspecific variation. For example, the lineages of the paraplatyarthrid troglophile clade, including B9 to B11 that were distributed in Uramurdah, Bubble Well, and Lake Violet calcretes, with 4.6% nucleotide sequence divergence, showed no significant morphological differences (unpublished data). These calcretes, although adjacent (10-35 km distance from each other) in the same paleodrainage (Carey), contain a quite distinctive stygofauna, particularly dytiscid beetles and parabathynellid crustaceans (Guzik *et al*. 2008; Cooper *et al*. 2008; see Watts & Humphreys, 2009 and references therein). This delineation is in agreement with morphological assessment and description of the new *Paraplatyarthrus* species (chapter 5) and the associated threshold, at least for paraplatyarthrid lineages, is conservative enough to avoid overestimation of species. Lefébure *et al.* (2006) suggested a 16% Patristic threshold (0.16 subst./site) for species delineation of crustacean groups. Guzik *et al.* (2011) considered 11% K2P threshold for species delineation of Australian subterranean invertebrates, while Abrams *et al.* (2012) used a lower threshold (7.1% K2P (0.075 Patristic distance)) for species delineation of parabathynellid crustaceans that also differed morphologically. However, with respect to the high level of sequence divergence, ranging from 17% to 26% (>16%, Lefébure's threshold), for the subterranean armadillid lineages and 12% to 18% for the philosciid lineages, each lineage most likely represents a distinct species. The two stenoniscid lineages (9% p-distance) from the same calcrete, Laverton Downs, were considered to belong to the same putative species as they did not meet the criteria for at least one of the thresholds. Comparatively, the 12% threshold for species delineation of the subterranean crinochete oniscidean species appeared as the most conservative, congruent with single/multiple-gene phylogenies and morphological evidence conducted on paraplatyarthrid species (chapter 5). For instance, according to the single nuclear *LysRS* phylogeny (Figure 3.3), some calcrete lineages (such as Laverton Downs and Lake Miranda East and Lake Miranda West) showed that haplotypes were shared or very

closely related among these lineages. Interestingly, the species number estimated by the PTP model was closer to that of the 12% threshold.

As application of a constant divergence threshold may also underestimate species richness, a combination of species delimitation methods considering the results of single/multiple gene phylogenies and morphological evidence was also used to estimate the number of putative species. On the basis of these criteria, one troglobitic paraplatyarthrid lineage (B8: Laverton Downs, Quandong; no eyes with pale body) with at least 9.1% nucleotide divergence from other paraplatyarthrid lineages, considered to be conspecific with the troglophile lineages (B6/B7 and B12; eyes of 3-5 ommatidia with semi-pigmented body) using the threshold, was recognised as a distinct species. In the *LysRS* phylogeny, B8 appeared as a sister group to a clade comprising B6-B7-B12 with no shared haplotypes. However, the estimation of species number using the combined criteria does not differ significantly from the 12% threshold, showing the utility of the threshold at least for paraplatyarthrid species delimitation. Taken overall, 28 subteranean oniscidean lineages are recognised as putative species including 10 paraplatyarthrid, 12 armadillid, five philosciid and one stenoniscid species.

### *DISTRIBUTION PATTERNS*

Except for three paraplatyarthrid troglophile lineages (B10 - Uramurdah and Bubble Well; B12 - Halfpenny and Nambi, and B17 - Halfpenny, Nambi and Laverton Downs-Windarra), all other oniscidean lineages were restricted in their distribution to an individual calcrete body. The presence of the same *COI* troglophile haplotypes over a distance of 35 km (Uramurdah-Bubble Well) to 125 Km (Halfpenny-Laverton Downs, Windarra) suggests there may have been recent dispersal of these paraplatyarthrid troglophiles.

The restriction of the troglobitic oniscidean lineages to individual calcrete bodies, and the associated high (12-26%) genetic divergences among lineages (as putative species), is indicative of long-term isolation of populations in accordance with the 'subterranean island' hypothesis (Cooper *et al.* 2002). This hypothesis has been well supported by numerous taxonomic and phylogenetic analyses on stygobitic fauna including dyticid diving beetles, amphipods, the stygobitic isopod genus *Haloniscus*, and Parabathynellidae (Watts & Humphreys 1999, 2000; Taiti & Humphreys, 2001; Watts & Humphreys, 2001; Cooper *et al.*

2002; Leys *et al.* 2003; Watts & Humphreys, 2003, 2004, 2006; Cooper *et al.* 2007, 2008; Guzik *et al.* 2008; Abrams *et al.* 2012; King *et al.* 2012).

Calcretes are reportedly separated by a matrix of fine alluvial deposits containing layers of clay with limited air-filled voids and this matrix most likely limits dispersal of macro-invertebrates such as water beetles and amphipods (Cooper *et al*. 2002, 2007). Given the phylogeographic pattern identified above, it appears that the alluvial matrix is also a major barrier for oniscidean troglofauna. The presence of some widespread oniscidean troglophile lineages found to occur in multiple calcretes (B10, B12 and B17) may be due to dispersal on the surface during wet periods and colonisation of nearby calcretes when conditions become arid. However, such movement between the calcretes can be considered as very recent events owing to the occurrence of similar haplotypes. The widespread troglophile lineages were found among nearby calcretes in the same palaeodrainage (Carey). Despite extensive collections conducted around the Sturt Meadows calcrete (Raeside palaeodrainage) no widespread troglophile lineages were collected.

Guzik *et al*. (2011) conducted research on three stygobitic diving beetles, one amphipod species and a lineage of isopods using *COI* sequence data from three main sampling sites across the Laverton Downs calcrete, including Quandong, Shady Well and Mt Windarra, to explore their phylogeographic patterns. They report a single divergent clade of haplotypes for each species at the southern Mt Windarra site, which was absent from the northern sites, and concluded that the occurrence of the salt lake adjacent to Mt Windarra most likely underlies the pattern of phylogeographic structure. The occurrence of such distinct divergent lineages was also observed for paraplatyarthrid isopods (B15) in a southern site of the Laverton Downs calcrete belonging to the same putative species. The common patterns of phylogeographic structure point to similar evolutionary forces operating but additional samples and genetic markers need to be examined to further verify these findings.

### *NUCLEAR MARKER CANDIDATES AND RELATIONSHIPS*

*LSU rRNA* (*28S*) and *SSU rRNA* (*18S*) genes have been widely used for reconstruction of phylogenetic relationships in isopod species. As the rRNA genes contain both variable and conserved regions they were found useful for elucidating oniscidean relationships and helped resolve the polytomies that occurred in the *COI*-based phylogeny owing to

saturation. The Lysyl-tRNA Synthetase gene (*LysRS*), selected using the Illumina Next Generation Sequencing technique, was amplified and developed here for the first time in isopod species. The Exon 3 of the gene was identified based on the annotated intron/exon structure of the crustacean *Daphnia pulex* which was then targeted for sequencing in the oniscidean taxa. This gene was found to be an informative nuclear marker for phylogenetic reconstruction, with a moderate evolutionary rate between the *28S* and *COI* genes. The overall mean divergence of *LysRS* among the paraplatyarthrid lineages was estimated at 0.052, which is approximately three times less than that of mtDNA *COI.* The BI *LysRS* phylogeny showed the same topology and branching pattern as mitochondrial *COI* for the majority of clades, a better resolution of the relationships, and stronger posterior probability support levels for most of the nodes (Figure 3.3). For instance, Clade 1, consisting of the paraplatyarthrid B1 to B5 lineages, which was weakly supported in the *COI* phylogeny (0.71), was strongly supported by the *LysRS* phylogeny (0.99). The usefulness of aminoacyl-tRNA synthetases (aaRS's) including the Lysyl-tRNA Synthetase (*LysRS*) gene for reconstruction of phylogenetic relationships such as the "tree of life" has been highlighted in several studies (Brown & Doolittle, 1995; Nagel & Doolittle, 1995; Brown *et al.* 1997). It is proposed that they are both ubiquitous and ancient and believed to have evolved very early in evolutionary time owing to their critical role in translation (Diaz-lazcoz *et al.* 1998).

The paraplatyarthrid lineages were the main focus of the current study and, according to all combined phylogenetic analyses, there was strong support for the existence of five major clades. The first clade included five divergent troglobitic/troglophile lineages from calcretes at Sturt Meadows (B1), Cunyu (B2), Lake Violet (B3), Lake Miranda East/West (B4 and B5), of which the Lake Miranda lineages were sister to a group comprising B1, B2 and B3. As this clade includes lineages belonging to different palaeodrainages (Sturt Meadows from Raeside, Cunyu from Nabberu and Lake Violet and Lake Miranda E/W from Carey) their grouping together suggests that the common ancestor was most likely a widespread surface species. Cooper *et al.* (2008) also documented closely related groups of stygobitic *Haloniscus* calcrete populations belonging to different palaeodrainages.

Clade 2 included surface lineages showing strong branching patterns. The relationship of this clade with the incorporated paraplatyarthrid lineages is ambiguous, as no phylogenies strongly supported its affinities with other clades. According to field observations (by MJ),

this clade appears to be widespread in temperate regions of Western Australia. Clade 3, which was classified here as containing both troglobite and troglophile individuals, comprised seven mtDNA lineages considered to belong to three distinct species and found at Laverton Downs Windarra (B6), Nambi (B7), Laverton Downs Quandong (B8), Halfpenny and Nambi calcretes (B12) and Uramurdah, Bubble Well, Lake Violet (B9, B10 and B11). The relationships between the three putative species and the associated lineages were not resolved in the *COI* BI phylogeny, but they were strongly supported in the single nuclear *LysRS* and combined phylogenies. Clade 4 comprised just one putative troglobitic species (12% threshold) found in the Laverton Downs calcrete and showed strong population structuring from Laverton Downs Windarra to the northern most site, Quandong (B13 to B16). Clade 5, which always appeared as sister to the other four clades in all phylogenetic analyses except for the *LysRS* phylogeny, consisted of three surface and troglophile lineages. Lineage B17, which showed a widespread distribution from Nambi, Halfpenny to Laverton Downs/Windarra calcretes, formed a strongly supported sister clade to the surface species from Mt Morgans (S4) in all phylogenetic analyses. In contrast to Clade 1, other subterranean paraplatyarthrid clades (3 to 5) were only found distributed along the same palaeodrainage (Carey).

### *THE ROLE OF HISTORICAL EVENTS IN THE FORMATION OF THE ONISCIDEAN TROGLOFAUNA IN WESTERN AUSTRALIA*

The occurrence of both subtropical (Paraplatyarthridae) and littoral (Stenoniscidae) faunas in the same aquifers, such as the lineages observed in the Laverton Downs calcrete, indicates that the groundwater calcretes of central Western Australia have experienced complex historical geological/climatic events. The occurrence of otherwise littoral stenoniscids in arid central Western Australia can be linked to the marine inundation of the Eucla basin, comprising the Nullarbor Plain, which is located on the margins of the Yilgarn, Musgrave and Gawler Cratons in southern Australia, during the Late Eocene (Figure 3.7). The unknown genus of Stenoniscidae (possibly a new genus) is related to *Metastenoniscus,* which is strictly littoral, and which has been recently reported from South America (Venezuela) and some Indian Ocean islands such as Bali (Taiti & Humphreys, 2008).

The geological evidence suggests that the palaeo-coastline of the Eucla Basin during the Cenozoic was most extended in the Late Eocene, with its northern most limits delineated by

a set of palaeo-shorelines (Hou *et al*. 2008; Sandiford *et al*. 2009). During the Late to Mid Eocene and Late Eocene, marine transgressions developed some several hundred kilometres up the palaeovalleys in the Eucla Basin (Alleys *et al*. 1999). The palaeo-shorelines extended further inland and their position expanded to the margin of the Neale Plateau in the northwest and Barton barrier-Wilkinson estuary in the northeast (Clarke & Hou, 2000; Hou *et al*. 2003, 2006). The current distribution of stenoniscid populations at the Laverton Downs calcrete (Carey palaeodrainage), suggests it was close to the northern most marine inundation. When the sea retreated during the Oligocene/Miocene, it is likely that the stenoniscid populations, which were stranded in the northwest, subsequently colonised the groundwater calcrete, perhaps as very early colonisers of the calcretes.

Groundwater calcretes distributed within palaeodrainages were proposed to have been formed during Late Eocene to Early Oligocene (37-30 Ma) owing to global cooling and dry conditions on the Australian continent (Morgan, 1993). It is likely that formation and further development of calcretes were enhanced by the subsequent dry periods during the Oligocene and following the Late Miocene. Several studies have shown that ancestral invertebrate surface species colonised the groundwater calcretes of central Western Australia as a result of a major aridification event that started from the Mid Miocene (10.4 Ma) onwards (Cooper *et al.* 2002; Leys *et al.* 2003; Cooper *et al.* 2007, 2008; Guzik *et al.* 2008). It is possible that the Miocene aridification has also been the major driving force for colonisation of subterranean habitats which may have provided a suitable substrate for the ancestral subtropical and benthic oniscidean surface species to survive.

The troglofauna in groundwater calcretes of central Western Australia now comprise subtropical elements which were most likely widespread in Australian subtropical rainforests (*Paraplatyarthrus*, *Troglarmadillo*), benthic (*Haloniscus*) and littoral (Stenoniscidae) faunas. The occurrence of these different faunas within the same habitat suggests the groundwater calcretes harbour relictual taxa belonging to different historical geological/climatic periods.

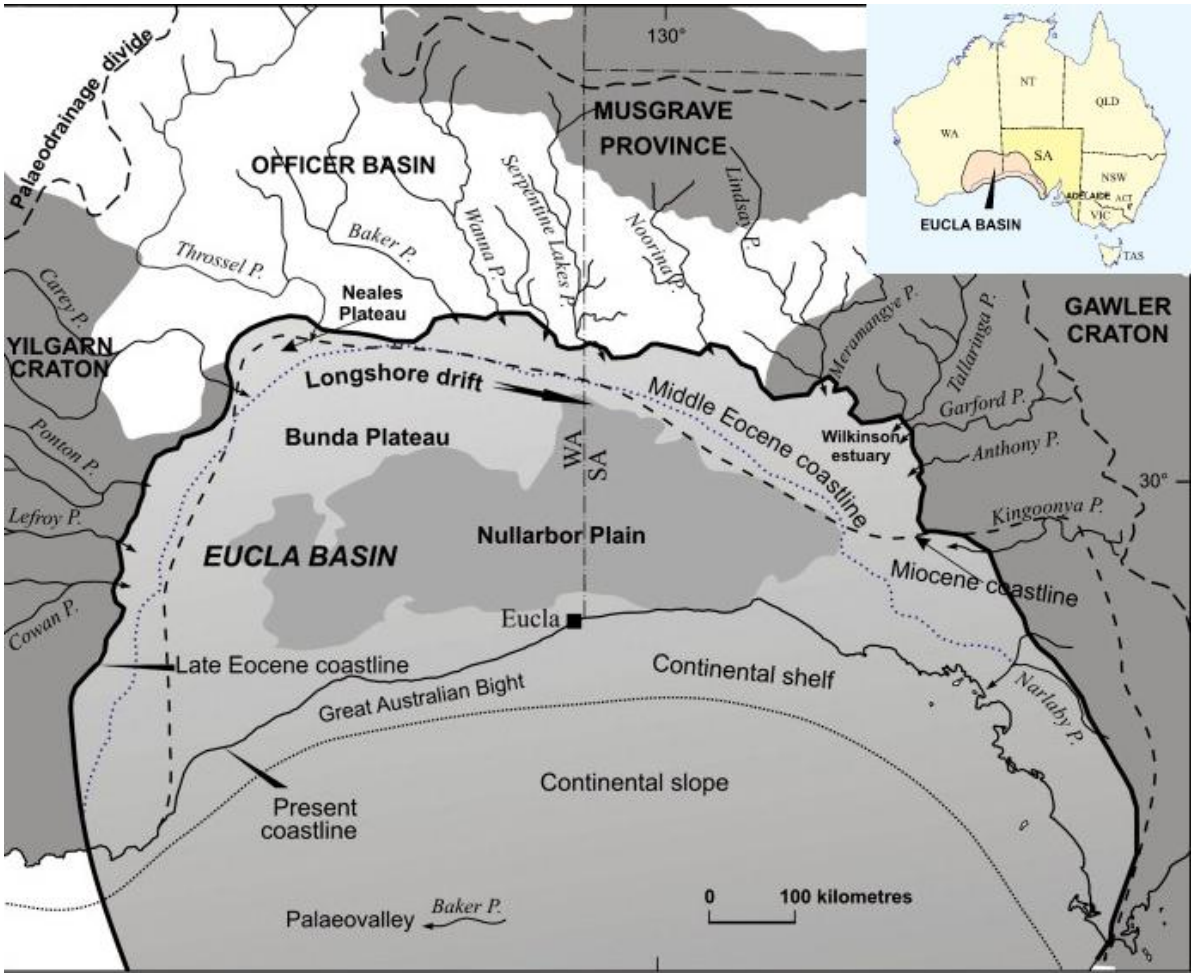


Fig. 3.7. The Eucla Basin and the associated major palaeodrainages including Carey. Historical fluctuations in coastlines from the Cretaceous to present, which is inferred to have influenced the distribution of the littoral fauna in Australia, are indicated (composite map after Hou *et al.* 2003, 2008).

## CONCLUSION

Groundwater calcretes in arid central Western Australia possess numerous significant and diverse oniscidean isopod lineages belonging to at least four families, with both troglobitic and troglophilic forms present. Twenty-eight lineages were identified that most likely represent new species. This considerably high level of diversity was found from the exploration of just 12 calcrete aquifers along three palaeodrainages. Given that there are approximately 200 major calcrete deposits in the region, the number of undiscovered oniscidian taxa in the region is likely to be very large. With the exception of just three troglophile paraplatyarthrid lineages found in more than one calcrete aquifer, most

oniscidean lineages were restricted to individual calcrete bodies, supporting the subterranean island hypothesis. The oniscidean fauna in calcrete aquifers of central Western Australia comprise subtropical (*Paraplatyarthrus*, *Troglarmadillo*), benthic (*Haloniscus*) and littoral (Stenoniscidae) species indicating that complex historical events were likely involved in shaping the make-up of the fauna. The occurrence of an otherwise littoral family in the remote arid region (Laverton Downs), most probably is associated with the marine inundation of the Eucla basin which extended into the inland and palaeovalleys during Late Eocene.

## ACKNOWLEDGEMENTS

Special thanks are due to Ms Kathy Saint for her help in the Molecular lab, Stefano Taiti for his advice on the taxonomy of isopods, and the WA museum technician, Julianne Waldock, who helped sort samples. We also thank Flora, Peter and Paul Axford (Sturt Meadows pastoral property) for their hospitality and Minara Resources (Murrin Murrin operations) environmental officers who facilitated use of a vehicle, freight transportation and accommodation during field trips in WA. We are also thankful to Andy Austin's lab group for their help and advice. The current research was supported by an ARC linkage grant (#LP100200494) to ADA, SJBC, WFH and MS and a National Geographic grant to SJBC and WFH.

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Appendix 3.1. Oniscidean troglofauna collected from the calcrete aquifers of central Western Australia. Data include BES codes, the number of oniscidean samples collected from each borehole, family, coordinates, calcretes and the associated palaeodrainganes from 2008-2012.

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 14602 | 14 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Jul-08 |
| 14604 | 2 | Paraplatyarthridae | Troglophile | -28.70028 | 120.89543 | Sturt Meadows | Raeside | Jul-08 |
| 14605 | 1 | Paraplatyarthridae | Troglobite | -28.40743 | 122.1997 | Laverton Downs-Shady Well | Carey | Jul-08 |
| 14606 | 6 | Paraplatyarthridae | Troglophile | -28.22232 | 121.82014 | Nambi | Carey | Jul-08 |
| 14611 | 1 | Paraplatyarthridae | Troglophile | -28.69934 | 120.89443 | Sturt Meadows | Raeside | Jul-08 |
| 14613 | 1 | Paraplatyarthridae | Troglobite | -27.66405 | 120.61015 | Lake Miranda East | Carey | Jul-08 |
| 14618 | 2 | Paraplatyarthridae | Troglobite | -28.38932 | 122.19853 | Laverton Downs-Shady Well | Carey | Jul-08 |
| 14619 | 3 | Paraplatyarthridae | Troglophile | -26.70905 | 120.23217 | Lake Violet | Carey | Jul-08 |
| 14620 | 6 | Paraplatyarthridae | Troglobite | -27.66405 | 120.61147 | Lake Miranda East | Carey | Jul-08 |
| 14622 | 6 | Paraplatyarthridae | Troglobite | -27.66401 | 120.61264 | Lake Miranda East | Carey | Jul-08 |
| 14623 | 1 | Paraplatyarthridae | Troglophile | -28.70124 | 120.90361 | Sturt Meadows | Raeside | Jul-08 |
| 14625 | 2 | Paraplatyarthridae | Troglophile | -28.7029 | 120.8923 | Sturt Meadows | Raeside | Jul-08 |
| 16614 | 4 | Paraplatyarthridae | Troglophile | -26.68757 | 120.30777 | Uramurdah | Carey | Jul-08 |
| 16618 | 3 | Paraplatyarthridae | Troglophile | -28.49933 | 122.17988 | Laverton Downs Windarra | Carey | Jul-08 |
| 16619 | 17 | Paraplatyarthridae | Troglobite | -25.78064 | 120.10745 | Cunyu | Nabberu | Jul-08 |
| 16621 | 50 | Paraplatyarthridae | Troglophile | -28.23914 | 121.83547 | Nambi | Carey | Jul-08 |
| 16627 | 45 | Paraplatyarthridae | Troglobite | -25.76419 | 120.11429 | Cunyu | Nabberu | Jul-08 |
| 16629 | 83 | Paraplatyarthridae | Troglobite | -28.50282 | 122.17726 | Laverton Downs Windarra | Carey | Jul-08 |
| 16630 | 40 | Paraplatyarthridae | Troglobite | -28.50282 | 122.17726 | Laverton Downs Windarra | Carey | Jul-08 |
| 16631 | 2 | Paraplatyarthridae | Troglophile | -26.68757 | 120.30777 | Uramurdah | Carey | Jul-08 |
| 16632 | 1 | Paraplatyarthridae | Troglobite | -28.49937 | 122.17838 | Laverton Downs Windarra | Carey | Jul-08 |
| 16633 | 1 | unknown | Troglobite | -26.68760 | 120.30271 | Uramurdah | Carey | Jul-08 |
| 16637 | 6 | Philosciidae | Troglobite | -28.49933 | 122.17988 | Laverton Downs Windarra | Carey | Jul-08 |
| 16638 | 12 | Armadillidae | Troglophile | -27.34220 | 120.12112 | Yeeleerie Altona | | Jul-08 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 16642 | 25 | Armadillidae | Troglophile | -27.66340 | 120.61231 | Lake Miranda East | Carey | Jul-08 |
| 16655 | 1 | Armadillidae | Troglobite | -26.68757 | 120.30777 | Uramurdah | Carey | Jul-08 |
| 16657 | 1 | destroyed | | -28.39835 | 122.19866 | Laverton Downs-Shady Well | Carey | Jul-08 |
| 16658 | 2 | Armadillidae | Troglobite | -28.40743 | 122.20380 | Laverton Downs-Shady Well | Carey | Jul-08 |
| 16659 | 4 | Paraplatyarthridae | Troglophile | -26.70907 | 120.23568 | Lake Violet | Carey | Jul-08 |
| 15062 | 1 | Paraplatyarthridae | Troglophile | -27.13748 | 120.94945 | Barwidgee | Carey | Mar-09 |
| 15064 | 1 | Paraplatyarthridae | Troglophile | -27.69661 | 121.33953 | Halfpenny Well | Carey | Mar-09 |
| 15065 | 3 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Mar-09 |
| 15067 | 2 | Paraplatyarthridae | Troglophile | -26.68762 | 120.313 | Uramurdah | Carey | Mar-09 |
| 15068 | 2 | Armadillidae | Troglobite | -28.40557 | 122.19962 | Laverton Downs-Shady Well | Carey | Mar-09 |
| 15071 | 2 | Paraplatyarthridae | Troglophile | -27.69661 | 121.33953 | Halfpenny Well | Carey | Mar-09 |
| 15072 | 2 | Paraplatyarthridae | Troglophile | -27.69661 | 121.33953 | Halfpenny Well | Carey | Mar-09 |
| 15073 | 2 | Paraplatyarthridae | Troglophile | -28.22232 | 121.82014 | Nambi | Carey | Mar-09 |
| 15075 | 5 | Paraplatyarthridae | Troglobite | -28.50282 | 122.17726 | Laverton Downs Windarra | Carey | Mar-09 |
| 15076 | 1 | Paraplatyarthridae | Troglophile | -28.23914 | 121.83547 | Nambi | Carey | Mar-09 |
| 15077 | 1 | Paraplatyarthridae | Troglophile | -28.23914 | 121.83547 | Nambi | Carey | Mar-09 |
| 15080 | 1 | Paraplatyarthridae | Troglophile | -26.70907 | 120.23568 | Lake Violet | Carey | Mar-09 |
| 15081 | 3 | Paraplatyarthridae | Troglobite | -25.78064 | 120.10745 | Cunyu | Nabberu | Mar-09 |
| 15082 | 3 | Philosciidae | Troglophile | -27.67919 | 120.60191 | Lake Miranda East | Carey | Mar-09 |
| 15084 | 1 | Paraplatyarthridae | Troglobite | -27.66405 | 120.61015 | Lake Miranda East | Carey | Mar-09 |
| 15085 | 1 | Philosciidae | Troglophile | -26.68761 | 120.29771 | Lake Violet | Carey | Mar-09 |
| 15087 | 2 | Paraplatyarthridae | Troglophile | -26.68757 | 120.30777 | Uramurdah | Carey | Mar-09 |
| 15088 | 2 | Paraplatyarthridae | Troglophile | -26.68762 | 120.313 | Uramurdah | Carey | Mar-09 |
| 15088 | 2 | Philosciidae | Troglobite | -26.68762 | 120.313 | Uramurdah | Carey | Mar-09 |
| 15089 | 3 | Philosciidae | Troglobite | -26.6876 | 120.30271 | Uramurdah | Carey | Mar-09 |
| 15090 | 3 | Paraplatyarthridae | Troglobite | -25.78064 | 120.10745 | Cunyu | Nabberu | Mar-09 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 15092 | 3 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Mar-09 |
| 15093 | 1 | Paraplatyarthridae | Troglobite | -28.38932 | 122.20076 | Laverton downs-Shady Well | Carey | Mar-09 |
| 15094 | 2 | Philosciidae | Troglobite | -28.5002 | 122.17849 | Laverton Downs Windarra | Carey | Mar-09 |
| 15095 | 2 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Mar-09 |
| 15096 | 2 | Armadillidae | Troglophile | -27.66401 | 120.61264 | Lake Miranda East | Carey | Mar-09 |
| 15097 | 1 | Paraplatyarthridae | Troglophile | -26.70903 | 120.23463 | Lake Violet | Carey | Mar-09 |
| 15102 | 2 | Armadillidae | Troglobite | -28.40565 | 122.2026 | Laverton downs-Shady Well | Carey | Mar-09 |
| 15065 | 1 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Mar-09 |
| 15079 | 1 | Paraplatyarthridae | Troglophile | -28.70124 | 120.90361 | Sturt Meadows | Raeside | Mar-09 |
| 15091 | 1 | Armadillidae | Troglobite | -28.40743 | 122.2038 | Laverton Downs-Shady Well | Carey | Mar-09 |
| 15095 | 1 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Mar-09 |
| 15096 | 1 | Armadillidae | Troglophile | -27.66401 | 120.61264 | Lake Miranda East | Carey | Mar-09 |
| 15103 | 1 | Armadillidae | Troglophile | -27.6634 | 120.61231 | Lake Miranda East | Carey | Mar-09 |
| 15104 | 2 | Armadillidae | not seen | -26.86436 | 120.28737 | Hinkler Well | Carey | Mar-09 |
| 16615 | 1 | Paraplatyarthridae | Troglobite | -28.40743 | 122.19970 | Laverton Downs-Shady Well | Carey | Apr-09 |
| 16616 | 51 | Paraplatyarthridae | Troglobite | -28.50282 | 122.17726 | Laverton Downs Windarra | Carey | Apr-09 |
| 16617 | 20 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Apr-09 |
| 16620 | 1 | Paraplatyarthridae | Troglobite | -28.38932 | 122.19853 | Laverton Downs-Shady Well | Carey | Apr-09 |
| 16622 | 5 | Paraplatyarthridae | Troglophile | -27.13758 | 120.94638 | Barwidgee | Carey | Apr-09 |
| 16623 | 5 | Paraplatyarthridae | Troglophile | -28.71371 | 120.89305 | Sturt Meadows | Raeside | Apr-09 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 16624 | 16 | Paraplatyarthridae | Troglobite | -28.49937 | 122.17838 | Laverton Downs Windarra | Carey | Apr-09 |
| 16625 | 10 | Paraplatyarthridae | Troglophile | -26.70903 | 120.23463 | Lake Violet | Carey | Apr-09 |
| 16628 | 2 | Paraplatyarthridae | Troglophile | -28.69948 | 120.90356 | Sturt Meadows | Raeside | Apr-09 |
| 16635 | 6 | Armadillidae | Troglobite | -28.40743 | 122.19970 | Laverton Downs-Shady Well | Carey | Apr-09 |
| 16636 | 1 | Armadillidae | Troglobite | -28.38932 | 122.19853 | Laverton Downs-Shady Well | Carey | Apr-09 |
| 16639 | 1 | Armadillidae | Troglobite | -26.86436 | 120.28737 | Hinkler Well | Carey | Apr-09 |
| 16640 | 1 | Armadillidae | Troglobite | -28.71374 | 120.88898 | Sturt Meadows | Raeside | Apr-09 |
| 16641 | 3 | Armadillidae | Troglophile | -26.86436 | 120.28737 | Hinkler Well | Carey | Apr-09 |
| 16643 | 1 | Armadillidae | Troglobite | -28.40566 | 122.20686 | Laverton Downs-Shady Well | Carey | Apr-09 |
| 16651 | 1 | Armadillidae | Troglobite | -28.39835 | 122.19866 | Laverton Downs-Shady Well | Carey | Apr-09 |
| 16652 | 1 | Armadillidae | Troglobite | -28.40376 | 122.20370 | Laverton Downs-Shady Well | Carey | Apr-09 |
| 16653 | 1 | Armadillidae | Troglobite | -26.56067 | 120.04092 | Bubble Well | Carey | Apr-09 |
| 16654 | 1 | Armadillidae | Troglobite | -28.70831 | 120.89117 | Sturt Meadows | Raeside | Apr-09 |
| 16656 | 1 | Armadillidae | Troglobite | -26.70903 | 120.23463 | Lake Violet | Carey | Apr-09 |
| 15509 | 1 | Armadillidae | Troglobite | -28.5047 | 122.17794 | Laverton Downs Windarra | Carey | Jul-10 |
| 15522 | 3 | Philosciidae | Troglobite | -28.5002 | 122.17849 | Laverton Downs Windarra | Carey | Jul-10 |
| 15523 | 9 | Philosciidae | Troglobite | -28.49933 | 122.17988 | Laverton Downs Windarra | Carey | Jul-10 |
| 15524 | 7 | Paraplatyarthridae | Troglobite | -28.49937 | 122.17838 | Laverton Downs Windarra | Carey | Jul-10 |
| 15525 | 27 | Paraplatyarthridae | Troglobite | -28.50282 | 122.17726 | Laverton Downs Windarra | Carey | Jul-10 |
| 15526 | 14 | Armadillidae | Troglobite | -28.39835 | 122.19866 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15527 | 1 | Armadillidae | Troglobite | -28.40195 | 122.19967 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15528 | 5 | Armadillidae | Troglobite | -28.40376 | 122.2037 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15529 | 1 | Armadillidae | Troglobite | -28.40377 | 122.1996 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15530 | 1 | Armadillidae | Troglobite | -28.38932 | 122.20076 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15531 | 7 | Armadillidae | Troglobite | -28.39291 | 122.19971 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15532 | 13 | Armadillidae | Troglobite | -28.38932 | 122.19853 | Laverton Downs-Shady Well | Carey | Jul-10 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 15533 | 1 | Armadillidae | Troglobite | -28.40566 | 122.20686 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15534 | 2 | Armadillidae | Troglobite | -28.40565 | 122.2026 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15535 | 4 | Armadillidae | Troglobite | -28.40557 | 122.19962 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15536 | 3 | Armadillidae | Troglobite | -28.40743 | 122.2038 | Laverton Downs-Shady Well | Carey | Jul-10 |
| 15537 | 18 | Armadillidae | Troglobite | -27.74667 | 120.5266 | Lake Miranda West | Carey | Jul-10 |
| 15538 | 16 | Paraplatyarthridae | Troglobite | -27.74667 | 120.5266 | Lake Miranda West | Carey | Jul-10 |
| 15539 | 1 | Paraplatyarthridae | Troglobite | -27.66401 | 120.61264 | Lake Miranda East | Carey | Jul-10 |
| 15540 | 1 | Armadillidae | Troglophile | -27.66401 | 120.61264 | Lake Miranda East | Carey | Jul-10 |
| 15541 | 4 | Armadillidae | Troglophile | -27.6634 | 120.61231 | Lake Miranda East | Carey | Jul-10 |
| 15542 | 2 | Paraplatyarthridae | Troglobite | -27.66406 | 120.61214 | Lake Miranda East | Carey | Jul-10 |
| 15543 | 10 | Paraplatyarthridae | Troglobite | -27.66384 | 120.61076 | Lake Miranda East | Carey | Jul-10 |
| 15544 | 1 | Armadillidae | Troglophile | -27.66406 | 120.61163 | Lake Miranda East | Carey | Jul-10 |
| 15545 | 11 | Paraplatyarthridae | Troglobite | -27.66405 | 120.61015 | Lake Miranda East | Carey | Jul-10 |
| 15546 | 10 | Armadillidae | Troglobite | -28.69669 | 120.89852 | Sturt Meadows | Raeside | Jul-10 |
| 15547 | 5 | Paraplatyarthridae | Troglophile | -28.69847 | 120.89652 | Sturt Meadows | Raeside | Jul-10 |
| 15548 | 2 | Paraplatyarthridae | Troglophile | -28.69934 | 120.89443 | Sturt Meadows | Raeside | Jul-10 |
| 15549 | 4 | Paraplatyarthridae | Troglophile | -28.69948 | 120.90356 | Sturt Meadows | Raeside | Jul-10 |
| 15550 | 2 | Armadillidae | Troglobite | 28.70118 | 120.89849 | Sturt Meadows | Raeside | Jul-10 |
| 15551 | 26 | Paraplatyarthridae | Troglophile | -28.70124 | 120.90361 | Sturt Meadows | Raeside | Jul-10 |
| 16525 | 1 | Armadillidae | Troglophile | -28.44156 | 122.18609 | Laverton Downs-Erlistoun | Carey | Aug-11 |
| 16530 | 4 | Philosciidae | Troglobite | | | unknown | | Aug-11 |
| 16551 | 3 | Paraplatyarthridae | Troglobite | -28.51738 | 122.18187 | Laverton Downs Windarra | Carey | Aug-11 |
| 16553 | 5 | Armadillidae | Troglobite | -28.39839 | 122.19863 | Laverton Downs-Shady Well | Carey | Aug-11 |
| 16555 | 7 | Paraplatyarthridae | Troglophile | -28.49933 | 122.17988 | Laverton Downs Windarra | Carey | Aug-11 |
| 16557 | 11 | Paraplatyarthridae | Troglophile | -28.50020 | 122.17849 | Laverton Downs Windarra | Carey | Aug-11 |
| 16562 | 1 | Armadillidae | Troglobite | -28.40566 | 122.20686 | Laverton Downs-Shady Well | Carey | Aug-11 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 16565 | 1 | Philosciidae | Troglobite | -28.34065 | 122.21134 | Laverton Downs-Quandong | Carey | Aug-11 |
| 16567 | 2 | Paraplatyarthridae | Troglobite | -28.35515 | 122.22551 | Laverton Downs -Quandong | Carey | Aug-11 |
| 16569 | 2 | Stenoniscidae | Troglobite | -28.35513 | 122.21736 | Laverton Downs -Quandong | Carey | Aug-11 |
| 16573 | 1 | Paraplatyarthridae | Troglobite | | | unknown | | Aug-11 |
| 16581 | 3 | Philosciidae | Troglobite | -28.43617 | 122.18816 | Laverton Downs-Erlistoun | Carey | Aug-11 |
| 16603 | 2 | Paraplatyarthridae | Troglophile | -28.49933 | 122.17988 | Laverton Downs Windarra | Carey | Aug-11 |
| 16605 | 1 | Armadillidae | Troglobite | -28.49933 | 122.17988 | Laverton Downs Windarra | Carey | Aug-11 |
| 16605 | 1 | Paraplatyarthridae | Troglobite | -28.49933 | 122.17988 | Laverton Downs Windarra | Carey | Aug-11 |
| 16609 | 1 | Armadillidae | Troglobite | -28.52734 | 122.18539 | Laverton Downs Windarra | Carey | Aug-11 |
| 16458 | | Armadillidae | Troglophile | -26.70905 | 120.23217 | Lake Violet | Carey | Oct-11 |
| 16459 | 1 | Armadillidae | Troglobite | -28.22232 | 121.82014 | Nambi | Carey | Oct-11 |
| 16461 | 1 | Armadillidae | Troglobite | -26.70903 | 120.23463 | Lake Violet | Carey | Oct-11 |
| 16463 | 50 | Paraplatyarthridae | Troglobite | -27.66405 | 120.61015 | Lake Miranda East | Carey | Oct-11 |
| 16466 | 1 | Philosciidae | Troglophile | -27.67919 | 120.60191 | Lake Miranda West | Carey | Oct-11 |
| 16469 | 5 | Armadillidae | Troglobite | -28.22059 | 121.81755 | Nambi | Carey | Oct-11 |
| 16472 | 8 | Paraplatyarthridae | Troglobite | -27.66384 | 120.61076 | Lake Miranda East | Carey | Oct-11 |
| 16475 | 5 | Paraplatyarthridae | Troglobite | -27.66401 | 120.61264 | Lake Miranda East | Carey | Oct-11 |
| 16476 | 4 | Paraplatyarthridae | Troglophile | -26.70923 | 120.26404 | Lake Violet | Carey | Oct-11 |
| 16477 | 54 | Paraplatyarthridae | Troglobite | -27.66340 | 120.61231 | Lake Miranda East | Carey | Oct-11 |
| 16477 | 11 | Armadillidae | Troglobite | -27.66340 | 120.61231 | Lake Miranda East | Carey | Oct-11 |
| 16478 | 5 | Paraplatyarthridae | Troglophile | -27.69661 | 121.33953 | Halfpenny Well | Carey | Oct-11 |
| 16480 | 4 | Paraplatyarthridae | Troglobite | -27.74669 | 120.52682 | Lake Miranda West | Carey | Oct-11 |
| 16481 | 15 | Paraplatyarthridae | Troglophile | -28.71371 | 120.89305 | Sturt Meadows | Raeside | Oct-11 |
| 16503 | 5 | Armadillidae | Troglophile | -28.70022 | 120.89334 | Sturt Meadows | Raeside | Oct-11 |
| 16505 | 1 | Armadillidae | Troglobite | -28.69847 | 120.89652 | Sturt Meadows | Raeside | Oct-11 |
| 16506 | 3 | Armadillidae | Troglobite | -28.69841 | 120.89449 | Sturt Meadows | Raeside | Oct-11 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 16508 | 3 | Armadillidae | Troglobite | -28.70290 | 120.89230 | Sturt Meadows | Raeside | Oct-11 |
| 16511 | 2 | Armadillidae | Troglobite | -28.69663 | 120.89953 | Sturt Meadows | Raeside | Oct-11 |
| 16515 | 3 | Armadillidae | Troglobite | -28.70120 | 120.89747 | Sturt Meadows | Raeside | Oct-11 |
| 16517 | 3 | Paraplatyarthridae | Troglophile | -28.69934 | 120.89443 | Sturt Meadows | Raeside | Oct-11 |
| 16522 | 1 | Armadillidae | Troglobite | -28.70034 | 120.90260 | Sturt Meadows | Raeside | Oct-11 |
| 16022 | 1 | Stenoniscidae | Troglobite | -28.52602 | 122.18681 | Laverton Downs | Carey | Oct-11 |
| 16023 | 1 | Stenoniscidae | Troglobite | -28.52602 | 122.18787 | Laverton Downs | Carey | Oct-11 |
| 16071 | 1 | Stenoniscidae | Troglobite | -28.44388 | 122.19568 | Laverton Downs-Erlistoun | Carey | Oct-11 |
| 15934 | 1 | Philosciidae | Troglobite | -28.35380 | 122.22078 | Laverton Downs-Erlistoun | Carey | Oct-11 |
| 15948 | 4 | Stenoniscidae | Troglobite | -28.44027 | 122.18851 | Laverton Downs-Erlistoun | Carey | Oct-11 |
| 15950 | 1 | Paraplatyarthridae | Troglophile | -28.23649 | 121.82920 | Nambi | Carey | Oct-11 |
| 16342 | 4 | Paraplatyarthridae | Troglophile | -26.56067 | 120.04092 | Bubble Well | Carey | Oct-11 |
| 16398 | 1 | Paraplatyarthridae | Troglobite | -27.74669 | 120.52682 | Lake Miranda West | Carey | Oct-11 |
| 16386 | 2 | Armadillidae | Troglobite | -26.70903 | 120.23463 | Lake Violet | Carey | Oct-11 |
| 14632 | 59 | Paraplatyarthridae | Troglophile | -28.4989 | 122.1798 | Laverton Downs- Windarra | Carey | May-12 |
| 14633 | 1 | Stenoniscidae | Troglobite | -28.4043 | 120.2082 | Laverton Downs-Shady Well | Carey | May-12 |
| 14634 | 4 | Paraplatyarthridae | Troglophile | -28.6963 | 120.9020 | Sturt Meadows | Raeside | May-12 |
| 14636 | 12 | Armadillidae | Troglobite | -28.3952 | 120.2001 | Laverton Downs-Shady Well | Carey | May-12 |
| 14637 | 1 | Stenoniscidae | Troglobite | -28.4980 | 122.1803 | Laverton Downs- Windarra | Carey | May-12 |
| 14638 | 100 | Paraplatyarthridae | Troglophile | -28.4980 | 122.1813 | Laverton Downs- Windarra | Carey | May-12 |
| 14639 | 4 | Armadillidae | Troglobite | -28.7160 | 120.8935 | Sturt Meadows | Raeside | May-12 |
| 14641 | 5 | Armadillidae | Troglobite | -28.4060 | 120.2052 | Laverton Downs-Shady Well | Carey | May-12 |
| 14642 | 7 | Armadillidae | Troglobite | -28.3321 | 122.2087 | Laverton Downs-Quandong | Carey | May-12 |
| 14643 | 1 | Armadillidae | Troglobite | -28.4006 | 120.2011 | Laverton Downs-Shady Well | Carey | May-12 |
| 14644 | 8 | Armadillidae | Troglobite | -28.6999 | 120.9009 | Sturt Meadows | Raeside | May-12 |
| 14645 | 3 | Philosciidae | Troglobite | -28.4366 | 122.1865 | Laverton Downs-Erlistoun | Carey | May-12 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 17203 | 21 | Paraplatyarthridae | Troglophile | -28.6993 | 120.8944 | Sturt Meadows | Raeside | May-12 |
| 17204 | 1 | Armadillidae | Troglobite | -28.3339 | 122.2108 | Laverton Downs-Quandong | Carey | May-12 |
| 17205 | 5 | Paraplatyarthridae | Troglophile | -28.5161 | 122.1833 | Laverton Downs- Windarra | Carey | May-12 |
| 17206 | 1 | Armadillidae | Troglobite | -28.7070 | 120.8926 | Sturt Meadows | Raeside | May-12 |
| 17207 | 1 | Paraplatyarthridae | Troglobite | -28.4061 | 120.2011 | Laverton Downs-Shady Well | Carey | May-12 |
| 17208 | 10 | Paraplatyarthridae | Troglophile | -28.4980 | 122.1798 | Laverton Downs- Windarra | Carey | May-12 |
| 17209 | 8 | Paraplatyarthridae | Troglophile | -28.7016 | 120.8989 | Sturt Meadows | Raeside | May-12 |
| 17210 | 1 | Paraplatyarthridae | Troglophile | -26.5607 | 120.0409 | Bubble Well | Carey | May-12 |
| 17211 | 3 | Armadillidae | Troglobite | -28.3538 | 122.2208 | Laverton Downs-Quandong | Carey | May-12 |
| 17212 | 4 | Paraplatyarthridae | Troglobite | -25.7642 | 120.1143 | Cunyu | Nabberu | May-12 |
| 17213 | 1 | Paraplatyarthridae | Troglophile | -28.6994 | 120.8954 | Sturt Meadows | Raeside | May-12 |
| 17214 | 1 | Paraplatyarthridae | Troglophile | -28.5170 | 122.1813 | Laverton Downs- Windarra | Carey | May-12 |
| 17214 | 1 | Armadillidae | Troglobite | -28.5170 | 122.1813 | Laverton Downs- Windarra | Carey | May-12 |
| 17215 | 54 | Paraplatyarthridae | Troglobite | -27.6638 | 120.6108 | Lake Miranda East | Carey | May-12 |
| 17216 | 5 | Armadillidae | Troglobite | -28.2192 | 121.8190 | Nambi | Carey | May-12 |
| 17217 | 7 | Paraplatyarthridae | Troglobite | -25.7726 | 120.1108 | Cunyu | Nabberu | May-12 |
| 17218 | 8 | Paraplatyarthridae | Troglobite | -27.6641 | 120.6115 | Lake Miranda East | Carey | May-12 |
| 17219 | 1 | Armadillidae | Troglobite | -28.3393 | 122.2128 | Laverton Downs-Quandong | Carey | May-12 |
| 17220 | 32 | Paraplatyarthridae | Troglobite | -27.6641 | 120.6102 | Lake Miranda East | Carey | May-12 |
| 17221 | 2 | Paraplatyarthridae | Troglophile | -28.2351 | 121.8306 | Nambi | Carey | May-12 |
| 17222 | 2 | Paraplatyarthridae | Troglophile | -28.2210 | 121.8216 | Nambi | Carey | May-12 |
| 17223 | 14 | Armadillidae | Troglophile | -27.6640 | 120.6126 | Lake Miranda East | Carey | May-12 |
| 17224 | 57 | Paraplatyarthridae | Troglophile | -28.5052 | 122.1804 | Laverton Downs- Windarra | Carey | May-12 |
| 17225 | 9 | Paraplatyarthridae | Troglophile | -28.7003 | 120.9026 | Sturt Meadows | Raeside | May-12 |
| 17225 | 1 | Armadillidae | Troglophile | -28.7003 | 120.9026 | Sturt Meadows | Raeside | May-12 |
| 17227 | 2 | Armadillidae | Troglobite | -28.7029 | 120.8923 | Sturt Meadows | Raeside | May-12 |

Appendix 3.1. Continued

| BES codes | no. | Family | Group | Latitiude | Longitude | Calcrete aquifer | Palaeodrainage | Date |
|---|---|---|---|---|---|---|---|---|
| 17228 | 1 | Paraplatyarthridae | Troglophile | -28.6971 | 120.8959 | Sturt Meadows | Raeside | May-12 |
| 17228 | 2 | Armadillidae | Troglophile | -28.6971 | 120.8959 | Sturt Meadows | Raeside | May-12 |
| 17229 | 30 | Paraplatyarthridae | Troglobite | -27.7467 | 120.5268 | Lake Miranda West | Carey | May-12 |
| 17230 | 14 | Armadillidae | Troglobite | -28.4006 | 120.2011 | Laverton Downs-Shady Well | Carey | May-12 |

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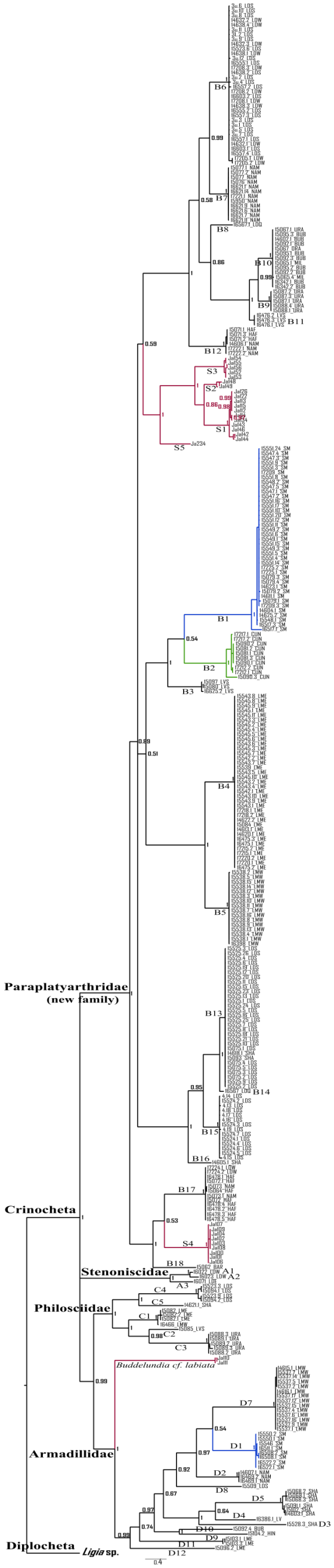
Appendix 3.2. Majority rule consensus Bayesian tree based on the *COI* gene. The numbers next to the nodes are posterior probabilities. The clade labels comprise haplotype vouchers and calcrete codes. The labels below the branches indicate lineage specific numbers.



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Appendix 3.3. Troglofauna sampling conducted in Western Australia. **A:** Locating boreholes provided by mining companies using geographic coordinates and red tags set on the ground **B:** Borehole unearthed **C:** PVC pipes stabilise the entrance of bores **D:** Stabilised bore ready for subterranean samplings **E:** Slotted borehole trap filled with leaf litter to be set underground **F:** Recovered traps after 6 to 10 months **G:** Collecting leaf litter from borehole traps **H:** Tullgren funnels to extract troglofauna from leaf letter.

Appendix 3.4. *COI* p-distance matrix of Paraplatyarthridae/*Paraplatyarthrus* mtDNA lineages indicated by B codes in the groundwater calcretes of central Western Australia. S codes refer to surface species.

| | B1 | B2 | B3 | B17 | S4 | B18 | S1 | S2 | S3 | B6 | B7 | B8 | B12 | B9 | B10 | B11 | B4 | B5 | B13 | B14 | B15 | B16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B1 | | 0.159 | 0.153 | 0.180 | 0.177 | 0.185 | 0.200 | 0.187 | 0.196 | 0.181 | 0.177 | 0.175 | 0.189 | 0.188 | 0.183 | 0.182 | 0.179 | 0.187 | 0.178 | 0.170 | 0.176 | 0.179 |
| B2 | | | 0.130 | 0.187 | 0.183 | 0.174 | 0.191 | 0.180 | 0.183 | 0.194 | 0.179 | 0.185 | 0.193 | 0.194 | 0.197 | 0.189 | 0.189 | 0.201 | 0.175 | 0.170 | 0.182 | 0.178 |
| B3 | | | | 0.161 | 0.158 | 0.152 | 0.170 | 0.161 | 0.171 | 0.174 | 0.159 | 0.167 | 0.160 | 0.172 | 0.172 | 0.172 | 0.177 | 0.187 | 0.163 | 0.152 | 0.154 | 0.166 |
| B17 | | | | | 0.123 | 0.128 | 0.192 | 0.176 | 0.188 | 0.158 | 0.161 | 0.150 | 0.163 | 0.179 | 0.190 | 0.177 | 0.175 | 0.190 | 0.159 | 0.155 | 0.161 | 0.161 |
| S4 | | | | | | 0.129 | 0.178 | 0.164 | 0.187 | 0.183 | 0.184 | 0.166 | 0.179 | 0.197 | 0.193 | 0.193 | 0.196 | 0.196 | 0.156 | 0.158 | 0.164 | 0.169 |
| B18 | | | | | | | 0.177 | 0.159 | 0.165 | 0.167 | 0.178 | 0.167 | 0.174 | 0.183 | 0.193 | 0.184 | 0.187 | 0.197 | 0.158 | 0.158 | 0.159 | 0.170 |
| S1 | | | | | | | | 0.067 | 0.096 | 0.179 | 0.189 | 0.182 | 0.185 | 0.206 | 0.201 | 0.189 | 0.188 | 0.189 | 0.175 | 0.176 | 0.174 | 0.187 |
| S2 | | | | | | | | | 0.082 | 0.180 | 0.175 | 0.162 | 0.170 | 0.184 | 0.172 | 0.172 | 0.181 | 0.178 | 0.151 | 0.149 | 0.156 | 0.161 |
| S3 | | | | | | | | | | 0.186 | 0.195 | 0.179 | 0.181 | 0.192 | 0.194 | 0.192 | 0.189 | 0.198 | 0.170 | 0.171 | 0.169 | 0.179 |
| B6 | | | | | | | | | | | 0.072 | 0.098 | 0.112 | 0.128 | 0.139 | 0.125 | 0.203 | 0.194 | 0.202 | 0.204 | 0.199 | 0.193 |
| B7 | | | | | | | | | | | | 0.091 | 0.104 | 0.119 | 0.122 | 0.112 | 0.195 | 0.185 | 0.188 | 0.187 | 0.181 | 0.191 |
| B8 | | | | | | | | | | | | | 0.104 | 0.108 | 0.115 | 0.097 | 0.175 | 0.175 | 0.172 | 0.168 | 0.162 | 0.170 |
| B12 | | | | | | | | | | | | | | 0.136 | 0.136 | 0.137 | 0.198 | 0.184 | 0.195 | 0.185 | 0.185 | 0.187 |
| B9 | | | | | | | | | | | | | | | 0.049 | 0.046 | 0.194 | 0.198 | 0.193 | 0.182 | 0.172 | 0.187 |
| B10 | | | | | | | | | | | | | | | | 0.049 | 0.177 | 0.177 | 0.198 | 0.183 | 0.178 | 0.182 |
| B11 | | | | | | | | | | | | | | | | | 0.187 | 0.183 | 0.189 | 0.181 | 0.180 | 0.184 |
| B4 | | | | | | | | | | | | | | | | | | 0.090 | 0.185 | 0.178 | 0.180 | 0.165 |
| B5 | | | | | | | | | | | | | | | | | | | 0.186 | 0.188 | 0.180 | 0.181 |
| B13 | | | | | | | | | | | | | | | | | | | | 0.018 | 0.061 | 0.086 |
| B14 | | | | | | | | | | | | | | | | | | | | | 0.059 | 0.090 |
| B15 | | | | | | | | | | | | | | | | | | | | | | 0.086 |
| B16 | | | | | | | | | | | | | | | | | | | | | | |

Appendix 3.5. *COI* p-distance matrix of Armadillidae/*Troglarmadillo* mtDNA lineages indicated by D codes in the groundwater calcretes of central Western Australia.

| | D1 | D7 | D3 | D4 | D2 | D8 | D6 | D9 | D5 | D10 |
|---|---|---|---|---|---|---|---|---|---|---|
| D1 | | 0.21 | 0.23 | 0.20 | 0.17 | 0.18 | 0.18 | 0.21 | 0.22 | 0.23 |
| D7 | | | 0.24 | 0.22 | 0.20 | 0.21 | 0.19 | 0.22 | 0.25 | 0.18 |
| D3 | | | | 0.23 | 0.23 | 0.26 | 0.20 | 0.22 | 0.24 | 0.25 |
| D4 | | | | | 0.20 | 0.17 | 0.23 | 0.20 | 0.19 | 0.21 |
| D2 | | | | | | 0.21 | 0.17 | 0.18 | 0.24 | 0.19 |
| D8 | | | | | | | 0.20 | 0.22 | 0.20 | 0.18 |
| D6 | | | | | | | | 0.20 | 0.20 | 0.22 |
| D9 | | | | | | | | | 0.21 | 0.19 |
| D5 | | | | | | | | | | 0.21 |
| D10 | | | | | | | | | | |

Appendix 3.6. *COI* p-distance matrix Philosciidae/*Haloniscus* mtDNA lineages indicated by C codes in the groundwater calcretes of central Western Australia.

| | C1 | C2 | C4 | C3 | C5 |
|---|---|---|---|---|---|
| C1 | | 0.12 | 0.18 | 0.15 | 0.16 |
| C2 | | | 0.17 | 0.13 | 0.16 |
| C4 | | | | 0.18 | 0.17 |
| C3 | | | | | 0.15 |
| C5 | | | | | |

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# CHAPTER IV:

# BIOGEOGRAPHIC HISTORY OF THE GENUS *PARAPLATYARTHRUS* (PARAPLATYARTHRIDAE: ONISCIDEA: ISOPODA) IN GROUNDWATER CALCRETES OF WESTERN AUSTRALIA

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This chapter will be submitted to the Journal of Biogeography

## STATEMENT OF AUTHORSHIP

# Statement of Authorship

**Title of Paper:** Biogeographic History of the Genus *Paraplatyarthus* (Paraplatyarthridae: Oniscidea: Isopoda) in Groundwater Calcretes of Western Australia

**Publication Status:** Publication style

**Publication Details:** This chapter is going to be submitted to the Journal of Biogeography

## Author Contributions

By signing the statement of authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

**1. Name of Principal Author (Candidate):** Mohammad Javidkar

**Contribution to the Paper:** Carried out molecular lab experiments, analysed sequence data, produced all figures and wrote the manuscript.

**Signature:** **Date:** 19/02/2014

**2. Name of Co-Author:** Steven J. B. Cooper

**Contribution to the Paper:** Provided project funding, assisted in field collections, supervised the direction of the study, gave advice on analyses and critically reviewed the manuscript.

**Signature:** **Date:** 7/3/2014

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| **Contribution to the Paper:** Collected a significant portion of the samples, provided assistance to obtain project funding, and critically reviewed the manuscript.<br><br>**Signature:** **Date:** 19/2/2014 |

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| **4. Name of Co-Author:** Rachael A. King |
| **Contribution to the Paper:** Provided advice on this chapter and critically reviewed the manuscript.<br><br>**Signature:** **Date:** 7/3/2014 |

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|---|
| **5. Name of Co-Author:** Simon Judd |
| **Contribution to the Paper:** Collecting surface species from Western Australia, critically reviewed the manuscript.<br><br>**Signature:** **Date:** 12/3/2014 |

| |
|---|
| **6. Name of Co-Author:** Andrew D. Austin |
| **Contribution to the Paper:** Provided funding for the project, supervised the direction of the study and critically reviewed the manuscript.<br><br>**Signature:** **Date:** 12/3/2014 |

## ABSTRACT

Groundwater calcrete aquifers in arid central Western Australia have been shown to contain a very diverse invertebrate groundwater fauna (stygofauna). Recent survey work has also uncovered a diverse oniscidean isopod troglofauna, including species of the genus *Paraplatyarthrus* (Isopoda). The aim of the present study was to investigate the mode of evolution of this genus and the timing of transitions from epigean to subterranean habitats. Phylogenetic relationships among the isopod troglofauna from 12 groundwater calcretes along three palaeodrainage systems were assessed using one mitochondrial gene *Cytochrome C Oxidase subunit 1 (COI)* and two nuclear markers, *Lysyl-tRNA Synthetase (LysRS)* and *SSU rDNA* (*18S*) genes. The phylogenies and relaxed molecular clock analyses of combined mitochondrial-nuclear genes showed evolutionary transitions took place approximately from 10.5 to 1.75 Mya coinciding with the aridification of Australia that started from the late Miocene and providing support for the climatic relict hypothesis for the evolution of troglofauna. However, the molecular evidence for dispersal of some troglophiles to the surface, probably during wet periods, and active colonisation of other calcrete aquifers, also suggested a role for the adaptive shift hypothesis during the evolution of the troglobitic isopods. The phylogenetic analyses, together with the morphological evidence, demonstrate that the troglobites most likely evolved from troglophile ancestors. In cases where groundwater calcretes contained multiple species, each calcrete was found to have been independently colonised by multiple troglophilic ancestral species.

**Keywords:** Adaptive shift hypothesis, climate relict hypothesis, colonisation, phylogeny, groundwater calcretes, troglofauna.

## INTRODUCTION

Two main hypotheses have been proposed to explain the diversity and evolution of subterranean animals: the 'climatic relict' and 'adaptive shift' hypotheses (Banarescu, 1975; Howarth, 1981; Sbordoni, 1982; Barr & Holsinger, 1985; Howarth, 1987; Rouch & Danielopol, 1987; Peck & Finston, 1993). Under the Climatic Relict Hypothesis (CRH), surface species, pre-adapted to subterranean life, passively colonise underground habitats and evolve into troglobites, following the extinction of surface populations, the latter resulting from unfavourable extrinsic factors such as Pleistocene glaciations. Consequently, the CRH involves a process of allopatric speciation. The CRH is proposed to explain the present distribution of cave fauna in Europe and North America, where Pleistocene glaciations/post-glaciations resulted in extinction of surface populations, and the aquatic subterranean fauna (stygofauna) of Western Australia, where surface populations were influenced by aridification (Holdhaus, 1933; Barr, 1968; Chapman, 1982; Peck, 1984; Leys *et al.* 2003; Cooper *et al.* 2007). However, the occurrence of troglobitic species in the caves of tropical regions with closely related epigean relatives living in nearby surface habitats, suggested that drastic climatic fluctuations were not a prerequisite to troglobitic speciation and led to the proposal of the Adaptive Shift hypothesis (ASH) (Howarth, 1973; Peck, 1975; Chapman, 1976; Howarth, 1981; Chapman, 1982; Howarth, 1987; Peck & Finston, 1993). Under the ASH, epigean populations actively colonised underground habitats to utilise new resources, and evolved into troglobites in parapatry with the surface population. Peck & Finston (1993) reported several troglobitic invertebrates living in parapatry with their putative surface ancestors in lava tubes of the Galapagos Islands. Howarth & Hoch (2012) also documented a noticeable list of Hawaiian lava tube fauna including species of Cixiidae, Philosciidae, Gryllidae, Lycosidae, Noctuidae, Reduviidae and Carcinophoridae where parapatric speciation had most likely occurred with closely related epigean species in nearby surface habitats.

Although it is widely accepted that the majority of subterranean species evolve from surface species (CRH or ASH models), it has been recently reported that there are cases of subterranean taxa that underwent diversification underground from stygobitic/troglobitic ancestors (Stepien *et al*. 2001; Finlay *et al*. 2006; Guzik *et al*. 2009; Guzik *et al*. 2011; Ribera

*et al*. 2010; Leijs *et al*. 2012). For example, the occurrence of sympatric sister pairs or triplets of diving beetle species in calcrete aquifers of central Western Australia represent a case where speciation may have occurred underground within the calcretes (Cooper *et al*. 2002; Leys *et al*. 2003; Leys & Watts 2008; Guzik *et al*. 2009). However, an alternative model of speciation involving repeated colonisation events from the same ancestral species at different time periods may also explain this pattern of diversification. Leijs *et al*. (2012), using molecular phylogenetic and statistical models, showed it was unlikely that this model explains the high number of sister dytiscid species, supporting the hypothesis of speciation underground.

Groundwater calcretes of the arid central Western Australia (WA) have recently been shown to contain a significant diversity of invertebrate stygofauna, such as diving beetles and a range of crustacean species (Watts, 1985; Watts & Humphreys, 2009 and references therein; Taiti & Humphreys, 2001; Karanovic & Marmonier, 2002; Karanovic, 2004; Karanovic & Cooper 2012). Phylogenetic and molecular clock analyses suggest that species have been restricted to individual calcrete bodies for millions of years as a result of aridification which commenced during the Late Miocene in Australia (Leys *et al.* 2003; Cooper *et al.* 2007, 2008). Despite considerable attention given to evolutionary studies of the aquatic subterranean fauna (stygofauna), the terrestrial dwellers of the calcrete aquifers are currently poorly understood. Javidkar *et al.* (Chapter 3) reported a diverse isopod troglofauna belonging to four oniscidean families including Armadillidae, Stenoniscidae, Philosciidae and a new family Paraplatyarthridae. Isopods of the genus *Paraplatyarthrus* (Paraplatyarthridae) were found to be one of the most frequent and diverse groups of troglobites/troglophiles in calcrete aquifer samples. The same authors, using a multi-gene phylogenetic approach and species delimitation methods, documented 28 subterranean lineages (considered as putative species) of which 10 belonged to the genus *Paraplatyarthrus*. The latter showed significant morphological diversity, ranging from lineages with no/significantly reduced eyes and pale body, here considered as troglobites, to those with eyes and semi-pigmented body, here defined as troglophiles. As some clades included troglophile, troglobites and surface species with highly supported sister relationships, they provide the potential to investigate the timing of the evolution of the troglobite/troglophile lineages. The present research utilises the same dataset (three genes

including *COI, Lysyl tRNA Synthetase* genes, *18S*) as for Chapter 3 with the exception that additional surface species from Western Australia were added to the sequence data.

The aim of this study is to use phylogenetic and molecular clock analyses to elucidate the historical factors which have shaped the diversification and distribution of subterranean paraplatyarthrid isopods in the groundwater calcretes of central Western Australia. In addition, we assess which model(s), CRH or ASH or speciation underground, can be applied to the evolution of the oniscidean troglofauna.

## MATERIAL AND METHODS

### *FIELD SAMPLING/SORTING*

In total, 18 subterranean (12) and surface (6) taxa belonging to the genus *Paraplatyarthrus* and distributed in Western Australia were targeted as the main group in this study (see Appendix 4.1 for troglomorphic characteristics of the subterranean lineages). Troglofauna were collected using borehole traps (trog-traps) made from 65 mm internal diameter PVC pipes measured variably between 150 mm-180 mm long, with lids to seal both ends, and an approximately 0.16-0.18 litre volume containing sterilized (with microwave) leaf litter. The trog-traps had slits on their surface, to allow invertebrates to enter and colonise the leaf litter. The trog-traps were set 50-100 cm above the water table within unlined mineral exploration boreholes (MEBs). To maintain the humidity of the bores, which is essential for troglofauna, the bores were plugged with PVC pipe and capped. Sampling was carried out two times a year (May and October) and the trog-traps were left underground for six months (in some cases one year) to be colonized by invertebrates. In total, 12 groundwater calcretes belonging to three palaeodrainages including Carey, Raeside and Nabberu were utilised for sampling the troglofauna (see both Figure 4.1 and Table 4.2). These calcretes represented the currently known localities available with MEBs that could be feasibly surveyed over a 10 day field trip.

After recovery of the traps, their contents were sealed in zip-lock bags for transport to the Western Australian Museum where the living fauna in the litter was extracted into 100% ethanol by means of Tullgren funnels. Surface species from Western Australia were collected

by hand under/between crevices of rotten/fallen trunks and preserved in 100% ethanol (Table 4.2).

### *DNA EXTRACTION AND SEQUENCING*

Three to six pereopods (except for male pereopod 7 which is important for morphological diagnosis) were dissected from 100% ethanol-preserved animals and rinsed in 10 mM Tris to remove the alcohol before the extraction process. Total genomic DNA was isolated using a Gentra Puregene Genomic DNA Purification Kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions (DNA purification from 5-10 mg fresh or frozen solid tissue) with the following minor modifications. For the stage of DNA precipitation samples were centrifuged at 12500 rpm for 20 mins and 5 mins (the step containing 70% ethanol) respectively.

To obtain partial sequences of mtDNA, a 681 bp fragment of the Cytochrome C Oxidase subunit1 gene (*COI*) was amplified using the universal primers LCO1490_t1 and HCO2198_t1 (designed by Robin M. Floyd in BOLD: The barcode of life data system; see Table 4.1). PCR amplification of all *COI* sequences involved an initial denaturation at 95°C for 10 mins and 34 subsequent cycles of 94°C for 45 seconds, 48°C for 45 s, 72°C for 1 min and a single final extension of 72°C for 10 mins followed by a 2-mins hold time of 25°C. For the sequencing reactions M13F and M13R primers were used (Messing 1983; Table 4.1).

A 751 bp region of the nuclear *Lysyl-tRNA Synthetase* (*LysRS*) protein coding gene was amplified using the designed primers G2328 and G2329 (Table 4.1). For the samples which were not successful or showed double bands in PCR amplifications, internal designed primers G2340 and G2341 (Table 4.1), which amplified a ~643 bp region of the gene, were used. PCR amplifications of all sequences involved an initial denaturation cycle of 95°C for 10 mins and 34 subsequent cycles of 94°C for 45 s, 48°C (for original primers)/50°C (for internal primers) for 45 s and 72°C for 1 min, followed by a final extension step of 72°C for 6 mins.

A 674 bp fragment, including core and variable regions C1, V1, C2, V2 and C3 of the nuclear *SSU rRNA* gene (*18S*) were amplified using 18S1.2F and 18Sb5.0 primers (Whiting 2001; see Table 4.1). The PCR amplification profile was set for a 10-min single initial denaturation at 95°C; 34 cycles of 45 s at 94°C, 45 s at 50°C, 1 min at 72°C and a single final elongation cycle of 6 mins at 72°C.

All PCR experiments were carried out on either a Palm-Cycler thermal cycler (Corbett, CG1-96) or a Kyratec Supercycler thermal cycler (SC300) using 25 µl reaction volumes consisting of 15.4 µl of nuclease-free molecular water, 5 µl of 5X Immolase PCR buffer (comprising 3.75 mM $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphae (dNTP) and 2.5X BSA (0.25 mg/ml)), 1 µl of each primer (5 µM concentration for *COI* and *18S* primers, 7 µM for G2328 and 8 µM for G2329, 10 µM concentration for G2340 and G2341), 0.1 µl of Immolase DNA polymerase (5 u/µl) and 2-2.5 µl of ~1 µg/ml DNA. A Millipore Multiscreen Vacuum Manifold with a 96-well PCR and SEQ multiscreen filter plates were used to clean up the PCR and sequencing products. Purified PCR products were sequenced in both directions using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and analysed on an ABI 3700 DNA capillary sequencer. Sequences were edited by Geneious Pro version 5.6.4.

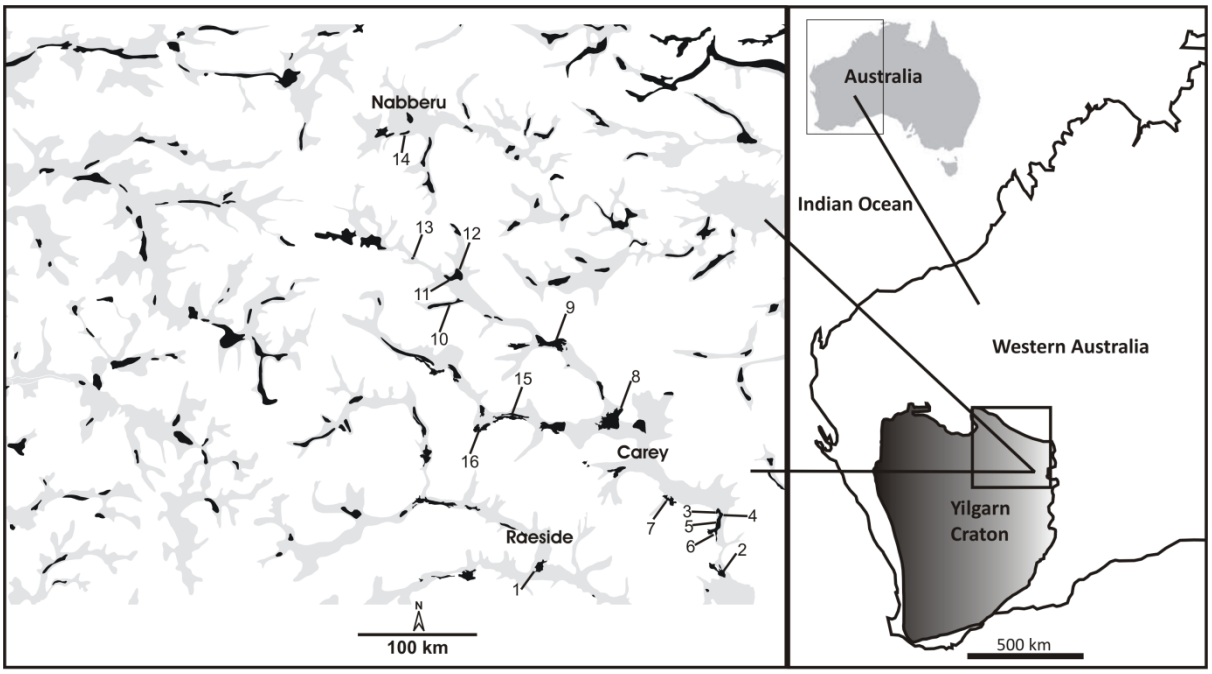


Fig. 4.1. A map of the sampled groundwater calcretes and their positions in the palaeodrainages. Numbers refer to the calcretes as listed in Table 4.2. Black shaded areas indicate groundwater calcretes and grey shaded ones are palaeodrainage valleys.

Table 4.1. Primers used for amplification of *COI*, *LysRS* and *18S* in the oniscidean isopods.

| Primer | Direction | Gene | Sequence (5'-3') |
|---|---|---|---|
| **LCO1490_t1** | F | *COI* | TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG |
| **HCO2198_t1** | R | *COI* | CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA |
| **M13F** | F | *COI* | TGTAAAACGACGGCCAGT |
| **M13R** | R | *COI* | CAGGAAACAGCTATGAC |
| **G2328** | F | *LysRS* | GTGCCACYGCCAAACCT |
| **G2329** | R | *LysRS* | CCATRCCCCAACCTSCTGT |
| **G2340** | F | *LysRS* | GATCGTGTWTAYGAAGTYGGAAG |
| **G2341** | R | *LysRS* | TCAAGAGCRGTACARWAGTTTTC |
| **18s1.2F** | F | *18S* | TGCTTGTCTCAAAGATTAAGC |
| **18sb5.0** | R | *18S* | TAACCGCAACAACTTTAAT |

Table 4.2. Lineage codes, locations of Isopod lineages and the associated geographic coordinates, WA Museum BES codes and GenBank accession numbers (available upon acceptance of the chapter for publication; see supplementary data). Abbreviation for the calcretes and some localities from Western Australia are as follows: Calcrete areas: Laverton Downs-Windarra (LDW); Laverton Downs-Erlistoun (LDE); Sturt Meadows (SM); Cunyu (CUN); Lake Violet (LV); Laverton Downs-Quandong (LDQ); Lake Miranda East (LME); Lake Miranda West (LMW); Nambi (NAM); Uramurdah (URA); Bubble Well (BUB); Lake Violet (LV); Halfpenny Well (HPW); Mt Morgans (MOR); Non-calcrete areas: Jorgensen Park, Kalamunda, WA (JOP); Gooseberry Hills, WA (GOO); Wooroloo, WA (WOO); Moorapulling rd (MOO), Marradong, WA; NNW of Mt Saddleback, WA (MOU); Forest block, NNW of Quindanning, WA (QUI). Numbers in parentheses are the code numbers on the map (Figure 4.1). Troglobite, troglophile and surface species have been indicated by bold, simple and grey-shaded respectively.

| **Taxa** | **Locality/Coordination** | **BES/Ja Codes** | **GenBank Accession Numbers** | | |
|---|---|---|---|---|---|
| | | | ***COI*** | ***LysRS*** | ***18S*** |
| Taxon1 | SM (1)<br>S28.70124, E120.90361;<br>S28.7003, E120.9026 | 15551.8, 9;<br>17225.1, 2 | X | X | X |
| **Taxon2** | CUN (14)<br>S25.7806, E120.1075 | 15090.1, 3 | X | X | X |
| Taxon3 | LV (11)<br>S26.7091, E120.2357;<br>S26.709, E120.2346 | 15080; 15097 | X | X | X |
| **Taxon4** | LME (15)<br>S27.66384, E120.61076;<br>S27.6638, E120.6108 | 15543.3;<br>17215.2 | X | X | X |
| **Taxon5** | LMW (16)<br>S27.74667, E120.5266 | 15538.10 | X | X | X |
| Taxon6 | LDW (6)<br>S28.4989, E122.1798 | 3U.1, 3;<br>14632.3 | X | X | X |
| Taxon7 | NAM (7)<br>S28.2351, E121.8306 | 17221.1 | X | X | X |

Table 4.2. Continued

| Lineage codes | Locality/Coordination | BES/Ja Codes | GenBank Accession Numbers | | |
|---|---|---|---|---|---|
| | | | *COI* | *LysRS* | *18S* |
| **Taxon8** | LDQ (3)<br>S28.35515, E122.22551 | 16567.1 | X | X | X |
| Taxon9 | URA, P1 (12)<br>S26.6876, E120.313;<br>S26.6876, E120.3078;<br>S26.6876, E120.313; | 15088.1;<br>15087.1;<br>15067.1 | X | X | X |
| | BUB, P2 (13)<br>S26.5607, E120.0409;<br>S26.5607, E120.0409 | 15095.3;<br>15065.1 | X | X | X |
| | LV, P3 (11)<br>S26.70923, E120.26404 | 16476.1, 2 | X | X | X |
| Taxon10 | HPW (8); NAM (7)<br>S27.6966, E121.3395;<br>S28.2210, E121.8216 | 15071.2;<br>17222.1 | X | X | X |
| **Taxon11** | LDW (6)<br>S28.50282, E122.17726;<br>S28.49937, E122.17838 | 15525.15, 25;<br>15524.6 | X | X | X |
| Taxon13 | HPW (8); NAM(7);<br>LDW(6)<br>S27.6966, E121.3395;<br>S28.2223, E121.8201;<br>S27.6966, E121.3395;<br>S28.5052, E122.1804;<br>S27.69661, E121.33953 | 15072.1;<br>15073;<br>15072;<br>17224.1,2;<br>16478.3 | X | X | X |
| Taxon15 | JP; GOO | Ja126; Ja144 | X | X | X |
| Taxon16 | WOO | Ja148 | X | X | X |
| Taxon17 | MOO | Ja152; Ja155 | X | X | X |
| Taxon18 | MOR(2) | Ja100; Ja101 | X | X | X |
| Taxon19 | MOU<br>S32°54′29.49″,<br>E116°25′9.04″ | Ja215; Ja216;<br>Ja217 | X | X | X |
| Taxon20 | QUI<br>S32°58′18.34″,<br>E116°30′52.76″ | Ja221; Ja222 | X | X | X |

### *PHYLOGENETIC ANALYSES*

As two more surface species collected from Western Australia were added to the sequence dataset, phylogenetic analyses were carried out to assess their affinity to other surface and subterranean species. The DNA sequences were aligned using ClustalW (cost matrix: IUB, Gap open cost: 9, Gap extend cost: 3) allowing free ends gaps. For phylogenetic analyses, the data were partitioned into six subsets including first, second and third codon

positions of *COI*, *LysRS* and conserved (C1, C2, C3) and variable (V1, V2) regions of *18S*. MrModeltest version 2.3 (Posada & Crandall, 1998) was used to determine the best nucleotide substitution model for each data subset using the Akaike Information Criterion (Posada & Buckley, 2004). A GTR+I+G (Rodríguez *et al.* 1990; Yang, 1996) model was found to be the most appropriate model for *COI* first and second codon positions; HKY+I+G (Hasegawa *et al.* 1985; Yang, 1996) for the *COI* third codon position and *LysRS*; SYM+I (Zharkikh, 1994) and HKY (Hasegawa *et al.* 1985) models for the core and variable regions of *18S* respectively. Phylogenetic analyses using Bayesian Inference (BI) were carried out using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2005), using unlinked models for each partition. Two independent analyses, with four chains per each analysis, were run simultaneously for 5 million generations, sub-sampling trees every 100 generations. The final standard deviation of split frequencies for the combined (three genes) BI phylogeny was 0.0016, and PSRF values for all parameters were 1.0, suggesting convergence had occurred. For each independent MrBayes analysis, a 25%-burn-in was used, equating to 12500 samples being discarded from the 50001 samples sub-sampled during the analysis (37501 samples were included). To assess convergence to a stationary distribution, the software package Tracer version 1.5 (Rambaut & Drummond, 2009) was used. A 50% majority rule BI consensus tree was constructed from the remaining trees and posterior probabilities were used to examine the robustness of the nodes. For the combined datasets, a Maximum Likelihood (ML) analysis with the same gene partitioning scheme similar to that of the BI was carried out using Garli OSX version 2.0 (Zwickl, 2006). ML analyses used two search replicates; save tree parameter every 100 times; substitution models unlinked and subset specific rates were allowed to vary across partitions. Support for clades was estimated using non-parametric bootstrapping with 500 pseudo-replicates. Other parameters were set according to the Garli configuration file defaults. As Garli doesn't calculate consensus trees from bootstrap replicates, the Sumtree package (Sukumaran & Holder, 2010) under Dendropy 3.12.0 (Sukumaran& Holder, 2010), which is a Python (version 2.7.3) library for phylogenetic computing, was used to make a 50% majority rule ML bootstrap consensus tree.

All phylogenetic trees were rooted using *Ligia* sp. and a paraplatyarthrid taxon from South America (considered as a distinct genus, see Javidkar *et al.* Chapter 2) as out-groups. Figtree

version 1.3.1 (Rambaut, 2009) was utilized to visualize phylogenetic trees. The specimens from Barwidgee (9) and Hinkler Well (10) calcretes (Figure 4.1) were not analysed in this study owing to unsuccessful PCR amplifications for *LysRS*.

### *ESTIMATION OF DIVERGENCE TIMES*

In order to infer the ages of the *Paraplatyarthrus* clades, analyses of the sequence data were conducted using BEAST version 1.6.1 (Drummond *et al.* 2006; Drummond & Rambaut, 2007). A well-supported sister relationship between the South American and Australian paraplatyarthrids was demonstrated using the combined mitochondrial and nuclear phylogeny of oniscidean isopods (Chapter 2). Andújar *et al*. (2012) conducted research assessing methodological decisions on rate and node age estimations of the genus *Carabus* (Coleoptera), and argued that when deep nodes are affected by saturation causing incorrect branch length estimations, errors in node ages will be extrapolated to recent parts of the tree leading to older time estimations for recent nodes rather than true time estimates. To avoid this problem, the South American paraplatyarthrid taxon was considered the most appropriate option as the out-group to reduce the problem with saturation. Under the assumption that this sister relationship resulted from Gondwanan vicariance, the time-point for the separation of Gondwanan continents, with respect to the Australia-Antarctica split (Lawver & Gahagan, 2003), as the minimum age of the root between the Australian and South American paraplatyarthrids, was considered an appropriate time to calibrate the isopod time tree. Moreover, the combination of both mitochondrial and nuclear markers in our dating phylogeny can effectively reduce the erroneous behaviour of individual genes and increase the accuracy of estimation of evolutionary rates and node ages.

To estimate divergence times for the internal nodes of a combined phylogeny based on two protein coding genes, including *COI* and the nuclear *LysRS* (the *18S* gene could not be amplified for some taxa so it was not used for the molecular timing analyses), two different approaches were used. On the basis of the first approach, the age of the root was set based on the separation of Antarctica from Australia (~50 MYA, Lawver & Gahagan, 2003), whereas the second approach employed the mtDNA *COI* clock rate of 0.0125 substitutions per site per million years for the subterranean aquatic stenasellid isopods (Ketmaier *et al*. 2003) to calibrate the time tree. In total, considering both the approaches, 12 different models from a combination of: uncorrelated lognormal and uncorrelated exponential relaxed clock models

to account for lineage-specific rate heterogeneity; Yule and Birth-Death processes for the tree priors; and normal/exponential prior distributions for the root height were used to assess the consistency of age estimates across the models. For all analyses, both genes were assumed to have separate substitution models, a single clock model and a single tree. A GTR+I+G model was selected for the *COI* gene allowing for partitioning into three codon positions and a HKY+G model was chosen for the *LysRS* gene. Markov chains were run for 50000000 generations, sampling every 1000 generations. Convergence diagnostics were assessed using Tracer Version 1.5 (Rambaut & Drummond, 2009).

The plots of lineages through time (LLT) were estimated using the software package Tracer version 1.5 (Rambaut & Drummond, 2009).

## RESULTS

### *SEQUENCES AND PHYLOGENETIC ANALYSES*

The sequence data of the three genes belonging to the genus *Paraplatyarthrus* generated in chapter 3 and those additional sequence data from some surface species were used for phylogenetic analyses. The haplotypes of individuals within each taxon that were very similar in sequence were pruned from the final dataset prior to the analyses (i.e., one to three sequences were selected as the representatives of each taxon).

The majority rule BI consensus tree showed a similar topology to the 50% ML consensus bootstrap tree. The ML analysis of the combined three genes after final optimization resulted in a tree with a likelihood score of -9894.21.

In general, phylogenetic analyses (Figure 4.2) resulted in five strongly supported major clades. Clade 1, with high support values (PP = 1.00, BS = 85%), included taxa (1-5) with both troglobite and troglophile forms. In this clade, taxa 2 and 3 formed a sister group to a lineage comprising taxon 1 with high support (PP = 1.00, BS = 99%); a sister group comprising taxa 4 and 5 (PP = 1.00, BS = 100%) was sister to the group comprising taxa 1, 2 and 3. Clade 2, comprising surface taxa 15, 17, 19, and 20, was supported with high node support values (PP = 1.00, BS = 100%). Within Clade 2, taxon 19 formed a sister lineage to a group comprising taxa 15, 17 and 20 (PP = 1.00, BS = 100%). Clade 3, comprising taxa 6, 7, 10 and 9, was

strongly supported (PP = 1.00, BS = 100%) by the BI and ML analyses. Within this clade, taxa 6, 7 and 10 grouped together with high support (PP = 1.00, BS = 97%), while taxon 9 formed a sister lineage (Taxon 8 did not amplify for *18S*). Clade 4 (troglobite taxon 11) and Clade 5 (including surface and troglophile taxa 13 and 18) weakly grouped, but the relationships between taxa within each clade were fully resolved. Taxa 13 and 18 formed a sister group with high support (PP = 1.00, BS = 98%).

### *GEOGRAPHIC DISTRIBUTION OF ONISCIDEAN TROGLOFAUNA*

Clade 1 including taxa 1 to 5 (taxa 4 and 5 are likely to be conspecific; see Chapter 3), and comprising both troglobite/troglophile forms represents species each restricted to an individual calcrete body. Clade 3, comprising both troglophiles (the conspecific taxa 6, 7, and 10; Taxon 9) and a single troglobite (taxon 8 occurring in Laverton Downs Quandong), distributed in the Carey palaeodrainage, showed some troglophile taxa had comparatively widespread distributions: taxon 9 occurred in the adjacent calcretes, Uramurdah, Bubble well and Lake Violet South with P2 which included shared haplotypes between Bubble Well and Uramurdah; the conspecific taxa 6, 7 and 10 occurred in Nambi, Halfpenny and Laverton Downs calcretes. Taxon 13, which grouped with one surface species from Mt Morgans, appeared widespread, being distributed in the Nambi, Halfpenny and Laverton Downs calcretes. The possibility that the occurrence of shared haplotypes in multiple calcretes resulted from a mix-up of samples during field collections or Tullgren funnel extractions was ruled out, as analyses of additional samples from each calcrete that had been independently collected confirmed the presence of the shared haplotypes within each calcrete. Taxon 11 (troglobite) comprising two divergent lineages was confined to the Laverton Downs calcrete. Molecular phylogenies showed the three sympatric species in the Laverton Downs calcrete, including taxa 11 (troglobite, Laverton Downs Windarra), 6 (troglophile, Laverton Downs Windarra) and 8 (troglobite, Laverton Downs Quandong) are highly divergent from each other and do not form a monophyletic group.

### *ESTIMATION OF DIVERGENCE TIMES AND COLONISATION EVENTS*

The maximum clade credibility trees using 12 different models recovered by BEAST (Figure 4.3; Table 4.3) generated consistent topologies which were also similar to those of the BI and ML phylogenies; the only exception was the position of Clade 5 which appeared basal to the whole clade of Australian paraplatyarthrids (except for the LYN model which

showed a weakly supported sister relationship between Clades 4 and 5). The ages of several key nodes with the highest probability density (95% HPD) support, including the sister troglobite (H), sister troglophiles (P, J; L, M), sister troglophile-troglobite (G, K) and surface-troglophile (O) are given in Table 4.3. Considering all the models, in Clade 3, the divergence time of the troglophile sister taxa 6 and 7 (node M) was estimated to be between 2.85 (EBDN) to 5.44 (LYE) Mya (model acronyms are given in Table 4.3); the divergence time between the same sister group and troglophile taxon 10 (L) was estimated to be between 5.25 (EBDN) to 9.19 (EYE) Mya. The divergence time between the troglobite taxon 8 and a troglophile group comprising taxa 6, 7 and 10 (node K) was estimated to be between 7.18 (LYN) to 13.33 (EYE) Mya. The age of the node for the conspecific troglophile taxon 9 group (J node) was estimated to be between 2.3 (LYN) to 8.01 (EYE) Mya, with the internal P node estimated to be between 1.75 (LYN)-4.34 (EYE) Mya. In Clade 1: the divergence time between the sister troglobite-troglophile group comprising taxa 2 and 3 (G), was estimated to be between 7.05 (EBDE) to 12.45 (LBDN) Mya; the age of the node for the sister conspecific troglobite taxa 4-5 was estimated to be approximately 4.31 (H) to 8.19 (EYE) Mya. In Clade 5, the divergence time between the troglophile taxon 13 and the surface species taxon 18 (O) was estimated to be between 10.74 (LYN) to 20.88 (LBDN) Mya.

With application of the Gondwanan separation (~50 Mya) time for the age of the root (South American taxon as the out-group) to calibrate the time tree, the *COI* rate of evolution was estimated to be 0.0115 substitutions per site per million years for the paraplatyarthrid isopods. Moreover, for the combined *COI-lysRS* dataset, using the Ketmaier's *COI* rate of evolution (0.0125) for the subterranean aquatic stenasellid isopods, the age of the root between the Australian and South American platyarthrid isopods was estimated to be 39.24 Mya (CEY), 49.72 Mya (CLY), 58.12 Mya (CEBD) and 67.33 Mya (CLBD) for each of the different models applied.

The number of lineages through time (LTT) plot using the Yule model of speciation showed that the lineage diversification over time was pretty constant and speciation events have been relatively continuous from 20 Mya to present (e.g. LYN model; Figure 4.4A). The application of a Birth-Death model of speciation resulted in a LTT plot showing a gradual exponential increase in the number of lineages over time approximately from 10 Mya (e.g. EBDN model Figure 4.4B).

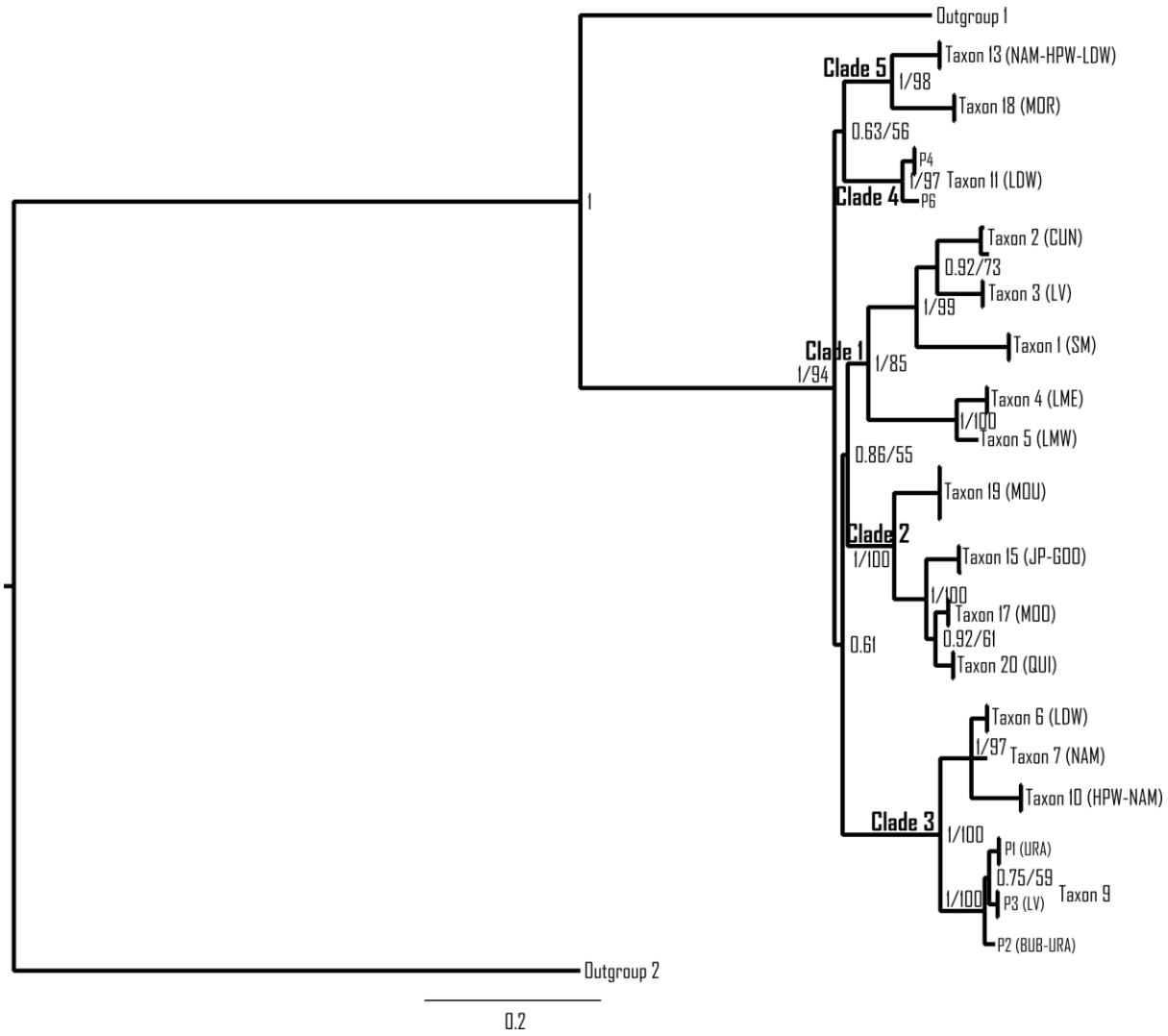


Fig. 4.2**.** Majority rule consensus BI tree on the basis of three genes comprising *COI*, *LysRS* and *18S*. The numbers next to the nodes are posterior probabilities and maximum likelihood bootstrap values respectively. The codes in parentheses refer to the groundwater calcretes or surface localities. Outgroups 1 and 2 refer to the South American paraplatyarthid and *Ligia* sp. respectively.

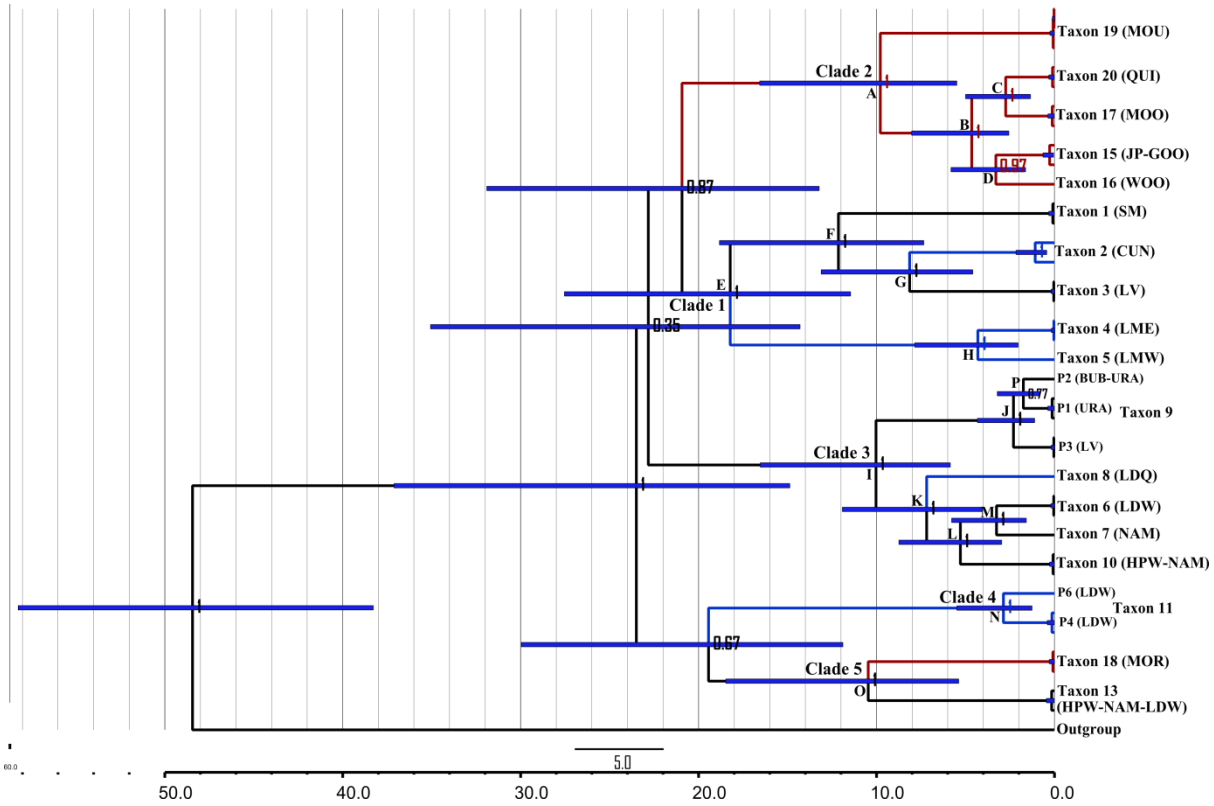


Fig. 4.3**.** A calibrated evolutionary time tree (*COI* and *lysRS* data, 1432 bp; LYN model), inferred from the BEAST analysis of the subterranean and surface species of the genus *Paraplatyarthrus* (Paraplatyarthridae) in Western Australia. The out-group refers to a paraplatyarthrid taxon from South America (Brazil). Numbers next to the nodes indicate posterior probabilities. The colour coded branches referred to as troglophile (black), troglobite (blue) and surface species (red). The node bars indicate 95% highest posterior density (HPD) intervals for nodes. The scale bar is age in millions of years.

Table 4.3. Age of nodes (millions of years) and the HPD with 95% confidence intervals for 12 different models used in BEAST molecular clock analyses of sequence data from *COI* and *LysRS* genes. Abbreviations for the models are as follows: Time calibration based on the Gondwanan separation: **LYN**: Uncorrelated Lognormal with Yule process and Normal prior for the root height; **LYE**: Uncorrelated Lognormal With Yule process and exponential prior for the root height; **LBDN**: Uncorrelated Lognormal with Birth-Death process and Normal prior for the root height; **LBDE**: Uncorrelated Lognormal with Birth-Death process and Exponential prior for the root height; **EYN**: Uncorrelated Exponential with Yule process and Normal prior for the root height; **EYE**: Uncorrelated Exponential with Yule process and Exponential prior for the root height; **EBDN**: Uncorrelated Exponential with Birth-Death process and Normal prior for the root height; **EBDE**: Uncorrelated Exponential with Birth-Death process and Exponential prior for the root height. Time calibration based on the *COI* rate: **CYL**: Uncorrelated Lognormal with Yule process; **CLBD**: Uncorrelated Lognormal with Birth-Death process; **CEY**: Uncorrelated Exponential with Yule process; **CEBD**: Uncorrelated Exponential with Birth-Death process. G, H, J, K, L, M, N, O and P represent the nodes shown in Figure 4.3.

| | G | H | J | K | L | M | N | O | P |
|---|---|---|---|---|---|---|---|---|---|
| **LYN** | 8.14 (4.59-13.11) | 4.31 (2.04-7.86) | 2.3 (1.11-4.33) | 7.18 (4.05-11.92) | 5.29 (2.97-8.75) | 3.26 (1.58-5.78) | 2.87 (1.26-5.49) | 10.47 (5.38-18.47) | 1.75 (0.81-3.22) |
| **LYE** | 12.25 (7.61-17.9) | 7.46 (4.09-12.2) | 4.62 (2.58-7.52) | 11.41 (7.38-16.57) | 8.68 (5.46-12.79) | 5.44 (2.91-8.66) | 5.46 (2.67-9.54) | 18.03 (9.78-29.66) | 3.26 (1.72-5.4) |
| **LBDN** | 12.45 (6.97-19.01) | 7.3 (3.48-12.02) | 4.98 (2.64-7.99) | 11.46 (6.81-17.0) | 8.56 (4.83-12.95) | 5.26 (2.58-8.55) | 5.62 (2.56-9.85) | 20.88 (11.05-33.26) | 3.25 (1.56-5.43) |
| **LBDE** | 11 (6.18-16.32) | 6.53 (3.43-10.68) | 3.94 (2.15-6.34) | 10.05 (6.2-14.75) | 7.63 (4.54-11.38) | 4.7 (2.43-7.52) | 4.68 (2.2-8.03) | 16.28 (8.46-27.51). | 2.76 (1.41-4.6) |
| **EYN** | 10.32 (4.33-18.65) | 7.71 (2.18-16.12) | 7.73 (2.35-15.46) | 12.77 (5.62-21.45) | 8.72 (3.5-15.75) | 4.91 (1.4-10.16) | 7.82 (1.48-19.97) | 16.39 (5.08-34.41) | 4.17 (1.05-9.56) |
| **EYE** | 10.68 (4.44-18.43) | 8.19 (2.3-17.04) | 8.01 (2.59-15.5) | 13.33 (5.88-21.87) | 9.16 (3.8-16.13) | 5.21 (1.49-10.55) | 8.29 (1.66-21.87) | 17.62 (5.45-35.99) | 4.34 (1.34-9.69) |
| **EBDN** | 6.46 (1.81-13.92) | 4.77 (0.93-12.02) | 4.36 (0.9-10.82) | 8.09 (2.38-16.81) | 5.25 (1.38-11.45) | 2.85 (0.64-6.9) | 4.28 (0.59-13.71) | 10.93 (2.12-29.75) | 2.24 (0.4-6.13) |
| **EBDE** | 7.05 (1.79-14.55) | 4.9 (0.89-12.07) | 4.58 (0.77-10.56) | 8.66 (2.59-17.02) | 5.61 (1.58-12.14) | 2.98 (0.64-7.35) | 4.54 (0.6-15.37) | 11.61 (1.58-30.97) | 2.38 (0.36-5.89) |
| **CLY** | 11.29 (6.85-16.52) | 5.81 (3.24-9.25) | 2.83 (1.65-4.22) | 9.77 (6.63-13.62) | 6.97 (4.46-9.97) | 4.28 (2.21-6.69) | 3.52 (1.79-5.87) | 17.11 (9.38-26.55) | 2.1 (1.12-3.31) |

Table 4.3. Continued

| | G | H | J | K | L | M | N | O | P |
|---|---|---|---|---|---|---|---|---|---|
| **CLBD** | 11.4 (6.6-16.95) | 5.74 (2.89-9.35) | 2.68 (1.5-4.1) | 9.84 (6.39-13.99) | 6.73 (4.08-9.91) | 3.94 (1.86-6.39) | 3.35 (1.59-5.72) | 17.74 (9.42-28.47) | 1.93 (0.97-3.09) |
| **CEY** | 8.07 (3.95-13.25) | 5.0 (1.77-10.08) | 3.44 (1.28-6.76) | 8.7 (4.78-14.06) | 5.0 (2.83-9.8) | 2.28 (1.18-6.29) | 3.85 (1.06-9.85) | 14.01 (5.27-26.91) | 2.09 (0.78-4.34) |
| **CEBD** | 8.34 (3.82-14.34) | 4.95 (1.78-10.62) | 2.99 (1.16-6.05) | 8.8 (4.53-14.94) | 5.48 (2.6-9.68) | 3.0 (1.12-5.99) | 3.24 (0.96-8.43) | 14.99 (4.8-32.42) | 1.8 (0.7-3.7) |

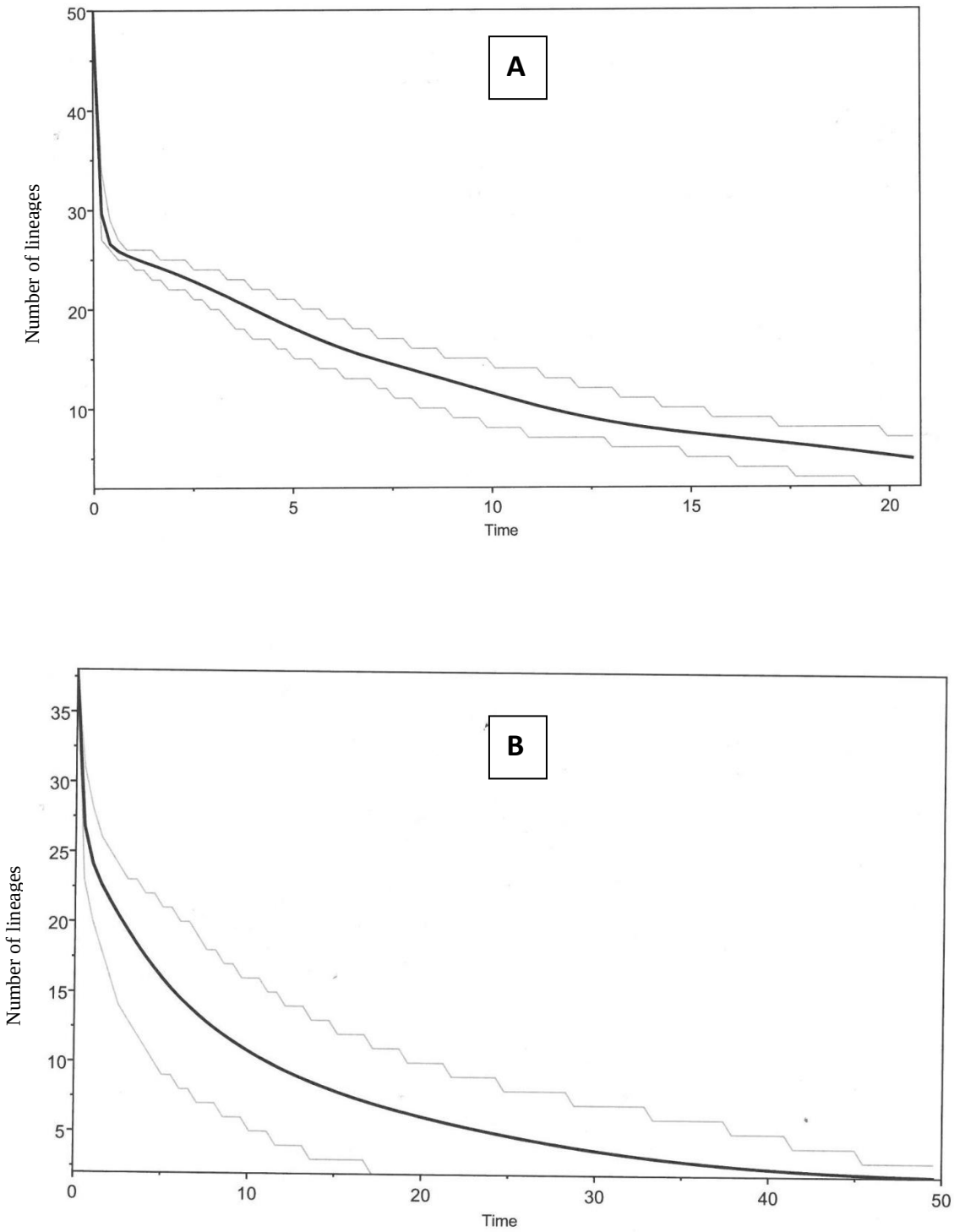


Fig. 4.4. Number of *Paraplatyarthrus* lineages through time (LTT plot); A: LYN model, B: EBDE model. The time axis represents million of years

## DISCUSSION

### *MOLECULAR DATING AND COLONISATION EVENTS*

The current study is the first attempt to elucidate the biogeographic history of troglofauna from groundwater calcretes of central Western Australia, providing a comparison with previous studies based on the stygofauna. The estimated divergence times inferred from the BEAST analyses give an approximate timeframe in which the oniscidean troglofauna might have colonised the subterranean habitats. These time-points can be estimated using the sister relationships between troglophiles-troglobites (G, K nodes), sister troglobite lineages in adjacent calcretes (H node), sister troglophile lineages (L, M, J, P nodes) and surface-troglophile (O node) groups. Among the 12 models used in this study, the LYN model produced comparatively narrow "95% highest probability density (HPD)" intervals, indicating stronger temporal signals (Pagán & Holmes, 2010), for all nodes. As a result, the LYN was selected as the most appropriate model for time interpretations. In general, the models EBDN and EBDE produced relatively similar results to the LYN model, while use of the EYE and EYN models led to broad confidence intervals (95% HPD) for most of the nodes and seemed to have overestimated the divergence times.

Independent estimates of divergence times, based on the *COI-LysRS* dataset calibrated by the *COI* rate of 0.0125 substitutions per site per million years for subterranean aquatic stenasellid isopods (Ketmaier *et al*. 2003), ranged from 39.42 (CEY) to 67.33 (CLBD) Mya for the divergence time of South American-Australian paraplatyarthrids. These time estimates, especially those based on the CLY model (49.72 Mya), are close to that of Cenozoic Gondwanan continental separations used for the calibration of the combined tree. On the basis of the Antarctic-Australia split (50 Mya) to calibrate the *COI* time tree, the *COI* rate of evolution for the paraplatyarthrid isopods was calculated at 0.0115 substitutions per site per million years. This estimated rate is the same as Brower's (1994) calculation for the rate of evolution of arthropod mtDNA and very similar to the Ketmaier *et al*. (2003) rate (see above).

According to the LYN model, in general, the timing of colonisation events ranged from 10.47 to 1.75 Mya. Divergence time estimates at the nodes G, K and H (troglophile-troglobite, troglobite-troglobite) suggested colonisation events occurred between 8.14 to

4.31 Mya which may reflect the time period during which troglobites evolved in isolation within the calcretes. This time estimation for the evolution of paraplartyarthrid troglobites is relatively similar to that estimated for stygobitic dytiscid diving beetles (9-4 Mya; Leys *et al*. 2003). The node N of the conspecific troglobite lineages in Laverton Downs most likely represents lineage diversification within the calcretes. However, the time point of this node (~2.87 my) possibly underestimates the minimum time point when the troglobite was present in the calcrete, as *LysRS* was not amplified for other more divergent conspecific lineages (based on mtDNA sequence data), which were not included in the molecular clock analyses (see Chapter 3).

In the reconstructed phylogenies, the sister relationships between the troglophile and troglobite species and the molecular evidence for the dispersal of troglophiles suggest that the troglobites most likely evolved from a troglophile ancestor rather than an epigean species. If so, troglophiles would have been able to move from calcrete aquifers to the surface and actively or passively colonised other calcretes. The lack of troglophile-troglobite sister relationships for several of the troglobites, taxon 11 (Laverton Downs) and the conspecific taxa 4 (Lake Miranda East) and 5 (Lake Miranda West), may also represent insufficient sampling effort in the region. The colonisation of subterranean habitats by pre-adapted populations, or troglophiles, has already been proposed as one step in the process of troglobite evolution (Holsinger, 2000; Trajano & Cobolli, 2012). This model predicts the colonisation of underground by troglophiles happens during most favourable environmental conditions when reproductive success and population size are at their peak. Interestingly, the molecular data lend support to the hypothesis that paraplatyarthrid troglophiles are able to actively disperse among distinct calcrete aquifers. Evidence for this is the occurrence of identical or near identical troglophile haplotypes shared between the Halfpenny and Nambi (taxon10) calcretes, among Halfpenny, Nambi and Laverton Downs (taxon 13) calcretes, and between Uramurdah and Bubble Well calcretes (taxon 9, P2), suggesting that dispersal most probably occurred during a recent wet period, potentially facilitated by flooding of the palaeodrainage valley. However, the possibility that some troglobites may have evolved directly from an epigean ancestral species that may have later become extinct in the region cannot be entirely ruled out.

The colonisation of subterranean environments and evolution of paraplatyarthrid troglofauna, in some aspects, is different from many of the stygobitic invertebrates (with some exceptions; e.g. bathynellids which naturally live in interstitial environments and are already adapted to living underground) of central Western Australia; first, there is no evidence for the existence of stygobitic intermediate forms (i.e. stygophiles) that are capable of dispersing via surface habitats during favourable conditions; second, it was proposed that stygofauna most likely evolved from epigean ancestors; third colonisation events by invertebrate stygofauna occurred in a relatively small time frame between 9 and 4 million years ago followed by long-term isolation of populations/species within the calcretes (Leys *et al*. 2003), whereas those of oniscidean troglofauna suggest a continuous and dynamic process of troglophilic dispersal and colonisation events, with the troglobite species showing long term isolation within the calcretes. Cooper *et al*. (2007), based on the amount of divergence between stygobitic amphipod lineages in proximate calcrete bodies, and using a rate calibration of *COI* based on isopods (Ketmaier *et al*. 2003), roughly estimated the time of divergence in the range of 14.6 and 4.1 Mya, suggesting isolation within individual calcretes since the late Miocene or Pliocene.

### *EVOLUTION OF PARAPLATYARTHRUS TROGLOFAUNA: CLIMATIC RELICT AND ADAPTIVE SHIFT HYPOTHESES*

The climatic relict (CRH) and adaptive shift hypotheses (ASH) (Peck & Finston, 1993; Howarth, 1981, 1987) represent two alternative scenarios to explain the evolution of troglobitic and troglophilic *Paraplatyarthrus* fauna. According to the ASH, once the groundwater calcretes were available as a suitable habitat, *Paraplatyarthrus* isopods would have colonised the calcretes and evolved into troglobites or troglophiles in parapatry with surface populations. Support for this hypothesis would be evident if there were close sister relationships between subterranean and surface species in the phylogenetic trees and the existence of related surface populations in the region. In contrast, the CRH assumes *Paraplatyarthrus* isopods passively colonised the groundwater calcretes and evolved into troglobites or troglophiles following the extinction of surface ancestral populations due to harsh and unfavourable surface conditions. If this was the case, the divergence time for the key nodes representing sister relationships between troglobite, troglophile and surface-

species should be in a time period after the late Miocene when the Australian continent experienced major aridification.

In the phylogenetic analyses no close sister relationships between surface and subterranean species were uncovered. The only exception was the sister group including taxon 13 (Nambi, Halfpenny, Laverton Downs-Windarra) and taxon 18 (Mt Morgans). Moreover, except for a few specimens from Mt Morgan no surface populations were found during the field sampling of the arid central Western Australia, in contrast to temperate areas in Western Australia where surface specimens were collected frequently. According to the molecular clock analyses, the approximate transition times for colonisation of groundwater calcretes by troglobites and troglophiles in the arid central Western Australia were estimated to occur from 10.5 Mya. These estimated transition times are concurrent with the cold/dry periods from the late Miocene (Markgraf *et al*. 1995) in Australia. The Australian aridification in the late Miocene is largely linked to major changes in the Pacific oceanic circulation owing to the progressive northward drift of the Indo-Australian plate and the growth of the Antarctic ice cap (Bowler, 1976; Stein & Robert, 1986; Martin & McMinn, 1994; Martin, 1998; Sniderman *et al*. 2007). Moreover, in general, the LTT plots, using the Birth-Death speciation model, show an increase in the number of lineages approximately from 10 Mya coincident with the Australian aridification and a noticeable diversification commenced from this period. However, the LTT plots, using the Yule model of speciation, did not show an exponential increase in the number of lineages, but indicated the speciation/lineage diversification events over evolutionary times were relatively continuous from the late Miocene to present. These differences may have resulted from the direct influence of each of the priors on the tree branching pattern. With respect to the evidence presented, the CRH model is a probable hypothesis to explain the evolution and diversification of the *Paraplatyarthrus* troglofauna. This model was also proposed to explain the evolution of Australian stygobitic dytiscid diving beetles in groundwater calcretes of central Western Australia with aridity from the late Miocene as the main driver for the transition to subterranean life (Leys *et al*. 2003). However, there is also evidence for speciation underground in this group (Leijs *et al*. 2012).

Although there is support for the CRH, there also is evidence to suggest the ASH model may have played a role during the evolution of the isopod fauna: 1) the troglophile taxa 9, 10

and 13 with similar haplotypes occurring in multiple distinct calcretes provided strong evidence that troglophile populations have shifted from hypogeal habitats (here calcrete aquifers), probably during a wet period, to the surface and actively dispersed to other nearby calcrete aquifers. 2) the early Pliocene in Australia (5.3-3.6 Mya) is characterised by wet and warm conditions, consistent with the contraction of the Antarctic ice cap which accordingly caused a sea level rise and basin flooding (Martin, 2006; Byrne *et al*. 2008). This relatively brief period, was again followed by the dry and cool late Pliocene. Moreover, ice age cycles during the Pleistocene also resulted in wetter conditions during inter-glacials. With respect to the occurrence of populations of the same species in multiple calcretes (i.e. intraspecific taxa 6, 7 and 10; taxon 9) it is possible that these wet periods may have facilitated dispersal on the surface by troglophiles and their active colonisation of other calcrete aquifers, and facilitating gene flow in parapatry with closely related troglobites. In addition, it is possible that further related surface species occurring above/near the calcretes have not been collected, as surveys to date have been limited. The surprising discovery of an extant surface species in Mt Morgans (Carey palaeodrainage), forming a sister relationship with the troglophile taxon 13, reinforces the idea that some patchily distributed surface species, closely related to troglophiles or troglobites, may still occur in the region. The ASH model has also been supported for the Hawaiian cave adapted isopods, genus *Littorophiloscia,* which most likely evolved from a marine littoral ancestor which colonised a terrestrial subterranean habitat, based on a close sister relationship between a surface-dwelling species and the cave species, and, also, the known parapatric distributions of species (Rivera *et al*. 2002).

Taken overall, both CRH and ASH models are supported to explain the evolution of the paraplatyarthrid troglofauna in calcrete aquifers of central Western Australia. The results show that a combination of Australian aridification from the late Miocene and the intermittent adaptive shifts associated with the troglophile dispersal ability during short humid intervals are likely to be the major drivers of the evolution of paraplatyarthrid troglofauna.

### *MULTIPLE OR SINGLE ANCESTORS?*

In calcretes which contained more than one species, the phylogenetic relationships between *Paraplatyarthrus* calcrete lineages, together with morphological evidence (Chapter

5), suggest that multiple troglophile ancestors independently colonised the calcrete aquifers. The cases include the occurrence of several distantly related species within the same calcrete, such as those within Nambi, Laverton Downs, Halfpenny and Lake Violet. For example, of three distinct taxa occurring in Nambi, one was more closely related to a surface species from Mt Morgans and a subterranean population from Barwidgee (see Chapter 3) than it was to the other two taxa. Colonisation of the groundwater calcretes by multiple epigean ancestors has been documented for some invertebrate stygobitic fauna such as dytiscid diving beetles, isopods of the genus *Haloniscus* and paramelitid/chiltoniid amphipods (Leys *et al.* 2003; Cooper *et al*. 2007; Cooper *et al*. 2008). Finston *et al*. (2009) also proposed that multiple distantly-related surface ancestors colonised the subterranean refugia as a result of Tertiary aridification events for the stygobitic isopod *Pygolabis* lineages of the Pilbara region in Western Australia. The same authors stated these lineages lack any closely related epigean ancestor in the region as a result of the lack of permanent surface water.

## CONCLUSION

The molecular clock analyses provide evidence that colonisation of the groundwater calcretes of central Western Australia occurred during a time period coinciding with aridification of Australia from the late Miocene. The apparent sister relationships found between the troglophilic and troglobitic species in the reconstructed phylogenies, together with molecular evidence for troglophiles ability to disperse across the landscape, reinforce the hypothesis that troglobites most likely evolved from ancestral troglophiles. The results based on the time estimations of colonisation events which coincide with aridification of Australia after the late Miocene and also strong evidence for troglophile dispersal, probably during short humid intervals, suggest both passive and active colonisations of the calcrete aquifers from the late Miocene to the present, supporting both the climatic relict and adaptive shift hypotheses to explain the evolution of *Paraplatyarthrus* troglofauna.

## ACKNOWLEDGEMENTS

We are grateful to the WA museum technician, Julianne Waldock for the initial specimen sorting and field equipment, MINARA resources environmental officers who facilitated the use of a field vehicle, and accommodation during field trips in WA. We are also thankful to Andy Austin's lab group for their help and advice. The current research is supported by an ARC linkage grant (#LP100200494) to the authors and a National Geographic grant to SJBC and WFH.

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Appendix 4.1. Number of eye ommatidia and body pigmentation patterns in paraplatyarthrid troglofauna from the groundwater calcretes of central Western Australia. See Table 4.2 for abbreviation of the calcretes and surface localities. Troglobite, troglophile and surface species have been indicated by bold, simple and grey-shaded respectively.

| Taxa | Locality | ommatidia/colour | Body pigmentation |
|---|---|---|---|
| Taxon 1 | SM | 3 reduced black ommatidia | Partly-pigmented |
| **Taxon 2** | CUN | 3 significantly reduced (vestigial) orange ommatidia | Pale |
| Taxon 3 | LV | 3 reduced black ommatidia | Pale |
| **Taxon 4** | LME | 1 significantly reduced black ommatidium | Pale |
| **Taxon 5** | LMW | 1 significantly reduced black ommatidium | Pale |
| Taxon 6 | LDW | 3 reduced black ommatidia | Partly-pigmented/Pale |
| Taxon 7 | NAM | 4-5 reduced black ommatidia | Partly-pigmented/Pale |
| **Taxon 8** | LDQ | No ommatidia | Pale |
| Taxon 9 | URA/BUB/ LV | 5 reduced black ommatidia | Partly-pigmented |
| Taxon 10 | HPW/NAM | 5 reduced black ommatidia | Partly-pigmented |
| **Taxon 11** | LDW | No ommatidia | Pale |
| Taxon 13 | HPW/NAM/LDW | 5 reduced black ommatidia | Partly-pigmented/Pale |
| Taxon 15 | JP; GOO | 6 developed black ommatidia | Fully pigmented body |
| Taxon 16 | WOO | 5 developed black ommatidia | Fully pigmented body |
| Taxon 17 | MOO | 7 developed black ommatidia | Fully pigmented body |
| Taxon 18 | MOR | 5 developed black ommatidia | Fully pigmented body |
| Taxon 19 | MOU | 5 developed black ommatidia | Fully pigmented body |
| Taxon 20 | QUI | 4-5? developed black ommatidia | Fully pigmented body |

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# CHAPTER V:

# FIVE NEW SPECIES OF *PARAPLATYARTHRUS* (ISOPODA, ONISCIDEA, PARAPLATYARTHRIDAE) FROM WETERN AUSTRALIA

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This chapter will be submitted to the Journal of ZooKeys

**STATEMENT OF AUTHORSHIP**

**Title of Paper:** Five New Species of *Paraplatyarthrus* (Isopoda, Oniscidea, Paraplatyarthridae) from Western Australia

**Publication Status:** Publication style

**Publication Details:** This chapter is going to be submitted to the Journal of Zookeys

# Author Contributions

By signing the statement of authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

**1. Name of Principal Author (Candidate):** Mohammad Javidkar

**Contribution to the Paper:** Carried out morphological lab experiments, produced all morphological drawings and figures, analysed the data and wrote the manuscript.

**Signature:** **Date:** 19/02/2014

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**Contribution to the Paper:** Supervised morphological analyses and descriptions of new species, contributed to text, revised manuscript drafts.

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## ABSTRACT

A new family, Paraplatyarthridae, has been recently identified from the groundwater calcretes of central Western Australia and subtropical regions of Brazil. Accordingly, five new subterranean and surface species of the nominal genus *Paraplatyarthrus* are described from Western Australia including *P. crebesconiscus* sp. nov., *P. cunyuensis* sp. nov., *P. nahidae* sp. nov., *P. occidentoniscus* sp. nov., and *P. pallidus* sp. nov. A key to the identification of species is provided.

**Keywords:** Oniscidea, *Paraplatyarthrus*, troglofauna, Western Australia.

## INTRODUCTION

A significant and diverse subterranean invertebrate fauna, including both stygofauna and troglofauna, exists in Western Australian groundwater calcrete aquifers (Bradbury, 1999; Taiti & Humphreys, 2001; Cooper *et al*. 2002; Karanovic & Marmonier, 2002, 2003; Leys *et al.* 2003; Karanovic, 2004; Cho, 2005; Cooper *et al.* 2007, 2008; Taiti & Humphreys, 2008; Guzik *et al.* 2008; Humphreys *et al.* 2009; Guzik *et al*. 2009; Karanovic & Cooper, 2012; King *et al.* 2012). Following the recognition of a highly diverse Western Australian oniscidean isopod troglofauna, primarily using mitochondrial DNA analyses, a multi-gene phylogenetic analysis, together with a comparative morphological study, led to the recognition of a new terrestrial oniscidean family, Paraplatyarthridae (Chapter 2). This new family is comprised of species distributed in subtropical/arid regions of Australia and South America (Chapter 2). To validate the new family, a single species has been described, *Paraplatyarthrus subterraneus*, from a distinct subterranean calcrete aquifer system in central Western Australia. However, the presence of additional congeneric species has been recognised in samples from across Western Australia, including both subterranean and surface species (Chapters 2, 3).

Using a multiple gene approach and species delineation methods, 10 putative and highly divergent paraplatyarthrid species have been identified from calcrete aquifers of central Western Australia, of which five species had adequate samples of males and females for morphological analyses. Here, morphological information, together with molecular data from Chapter 3, is used to describe these five new species of *Paraplatyarthrus* which include both subterranean and surface dwelling taxa.

## MATERIAL AND METHODS

The methods for collecting subterranean and surface specimens have been described in chapter 3. Most specimens were preserved in 100% ethanol (for DNA extraction) and subsequently became brittle, making dissections difficult. To avoid too much damage to the specimens during dissection they were mounted in a drop of water-based lubricant (equate)

in a Petri dish and then 100% ethanol was added to the Petri dish, causing the lubricant drop to stabilize the mounted samples during dissection.

Illustrations were carried out using an Olympus compound microscope BX53 equipped with a drawing arm. Digital images were taken using a Visionary Digital BK+ Imaging System, with either a 65 mm Varifocal lens (5D, 1.4X extension, 5X) or a long distance microscope lens K2 (7D, P1, 10X). Source images were stacked using Zerene Stacker version 1.04 and enhanced using Adobe Photoshop CS5. All type material is deposited with the Western Australian Museum (WAM).

Specimens were classified into three groups comprising: a) troglobites, which included subterranean specimens characterized by their pale body and lack of or significantly reduced (vestigial) eyes, b) troglophiles, including subterranean specimens with a partly pigmented body and reduced eyes, and c) surface species with a fully pigmented body and developed eyes.

## KEY TO SPECIES OF *PARAPLATYARTHRUS* FROM AUSTRALIA

1. Maxilla 2 with no delimiting line between lobes (see Figure 5.17E).....*P. pallidus*

—Maxilla 2 with delimiting line between lobes (see Figure 5.1F) ..... **2**

2. Eyes absent (no ommatidia present) (see Figure 5.22H) .....*P. subterraneus*

—Eyes present or significantly reduced (3-5 ommatidia present)..... **3**

3. Male genital papilla apically truncated (Figure 5.6C); eyes with 3 very reduced orange ommatidia (Figure 5.22C) ..... *P. cunyuensis*

—Male genital papilla apically rounded (Figure 5.19A); eyes with 3-5 black ommatidia..... **4**

4. Cephalic lateral lobes developed (Figure 5.9H); male pleopod 1 exopodite with developed posterior point (Figure 5.11B) .....*P. nahidae*

—Cephalic lateral lobes not developed (Figures 5.22C, E); male pleopod 1 exopodite with no or a weak posterior point (Figure 5.7B)..... **5**

5. Eyes with 3 ommatidia.....*P. occidentoniscus*

—Eyes with 5 ommatidia..... *P. crebesconiscus*

## SPECIES DESCRIPTIONS

***Paraplatyarthrus crebesconiscus*** Javidkar & King **sp. nov.**

**Holotype.** Male, WAM C 54789 (BES16478.3), Halfpenny Well calcrete, Millbillillie pastoral station, Eastern Murchison region, Western Australia, Australia; 27.69661°S, 121.33953°E. Oct 2011. W. F. Humphreys & S. J. B. Cooper.

**Paratypes.** 3 females including WAM C 54790 (BES16478.2), WAM C 54791 (BES16478.4), WAM C 54792 (BES16478.5); 1 male WAM C 54793 (BES16478.1). Same locality and collection data as holoptype.

**Description. Male (WAM C 54789),** Body length 2.7 mm, cephalon and posterior body weakly pigmented. Cephalic lateral lobes not enlarged. Eyes with 5 black ommatidia. Supraantennal line present. Antenna 1 3-jointed, medial article shortest, distal article longest (Figure 5.1A). Antenna 2 flagellum 2-jointed (Figure 5.1B), basal article shorter, about 1/3 the length of distal article. Left mandible pars molaris (Figure 5.1C) with 6 to 7 plumose setae; hairy lobe bearing 2 plumose setae; 1 long plumose seta occurring between lobe and pars molaris. Right mandible pars molaris with about 6 plumose setae; 1 plumose seta on hairy lobe and 1 single plumose seta between pars molaris and hairy lobe. Maxilla 1 outer endite (Figure 5.1D) with outer group of 4 teeth covering about 65%of marginal area, one smaller than the others, inner group of 3 cleft teeth, 1 simple and 1 stalk-like tooth; inner endite (Figure 5.1E) with 2 stout apical plumose setae closer to inner corner, 2 very fine setae on subapical outer marginal corner. Maxilla 2 (Figure 5.1F) apically bilobate; inner lobe comparatively smaller than outer one, with thick sensilla on distal margin; inner and outer lobes delimited by a fine suture; subapical region of lobes covered with very fine small setae. Maxillipedal endite with 1 large seta close to subapical inner corner, with 2 short apical arrow-like setae; basal article of palp with 2 setae, one on inner side longest, distal articles fused, with 1 large proximal seta, a medial tuft of 2 large and 1 smaller setae and an apical tuft of probably 2 long setae (Figure 5.1G).

Epimeron 1 rounded anteriorly; in dorsal view, posterolateral corner of pereonites 1-4 rounded; posterolateral corner of pereonites 5-7 posteriorly directed (Figure 5.21B). Pleonal epimeron 5 reaching (but not surpassing) uropodal sympodite. Noduli laterales present on

pereonal tergites (Figure 5.4A, B); tergite 7 with 2 noduli laterales; D/C ratios not constant in tergites 1-7.

Pereopod 1 (Figure 5.2A) carpus inner margin densely covered with long serrate setae, tuft of fine setae present medially near distal margin; propodus with both small simple and large serrate setae; dactylus with a long seta not exceeding the claws, outer claw relatively straight, with a small depression on medial part, inner claw shorter and situated at the basal posterior of the outer claw. Pereopod 7 (Figure 5.2B) carpus not showing any sexual dimorphism.

Pleon outline continuous with pereon. Pleopod 1 endopodite (Figure 5.3A) slender, with simple apex, medial margin with a group of fine setae, very fine setae also close to tip of endopodite; exopodite (Figure 5.3B) heart-shaped with posterior point not developed. Genital papilla (Figure 5.2C) ventral sheath apically pointed, surpassed by a long rounded lobe. Pleopod 2 endopodite (Figure 5.3C) with distal half slender, not reaching to posterior apex of pleopod 4 exopodite. Pleopod 2-5 exopodites (Figures 5.3D, E, F, G) with 4 to 5 cleft and simple long setae. Pleotelson triangular and pointed. Uropodal exopodites dorso-ventrally flattened, surpassing pleotelson, with a suture on outer margin; endopodites laterally flattened, slightly exceeding pleotelson; uropodal sympodite with an elongated circumflex-shaped incision (^).

**Diagnosis.** Eyes with 5 ommatidia; cephalic lateral lobes not developed; male pleopod 1 exopodite with weak posterior point.

**Variability**. Body length 2.7 mm - 3.5 mm. Some individuals have stronger pigmentation on dorsal body (i.e. semi-pigmented; Figure 5.22A).

**Distribution.** This species has been recorded from calcrete aquifers at Halfpenny Well (Millbillillie pastoral station), Nambi pastoral station and Laverton Downs pastoral station (Mt Windarra), Eastern Murchison region, WA, Australia.

**Remarks.** *P. crebesconiscus,* representing a troglophilic form, is a sister group to the surface species *P. nahidae* based on the molecular phylogenies (Chapter 3). Unlike *P. nahidae*, body pigmentation is semi-pigmented to pale, head with cephalic lobes not developed and male pleopod 1 exopodite with weak posterior point.

**Etymology.** The species name is composed of the Latin word ‘crebesco’ (meaning widespread) and ‘oniscus’ referring to its comparatively widespread distribution in the calcrete aquifers.

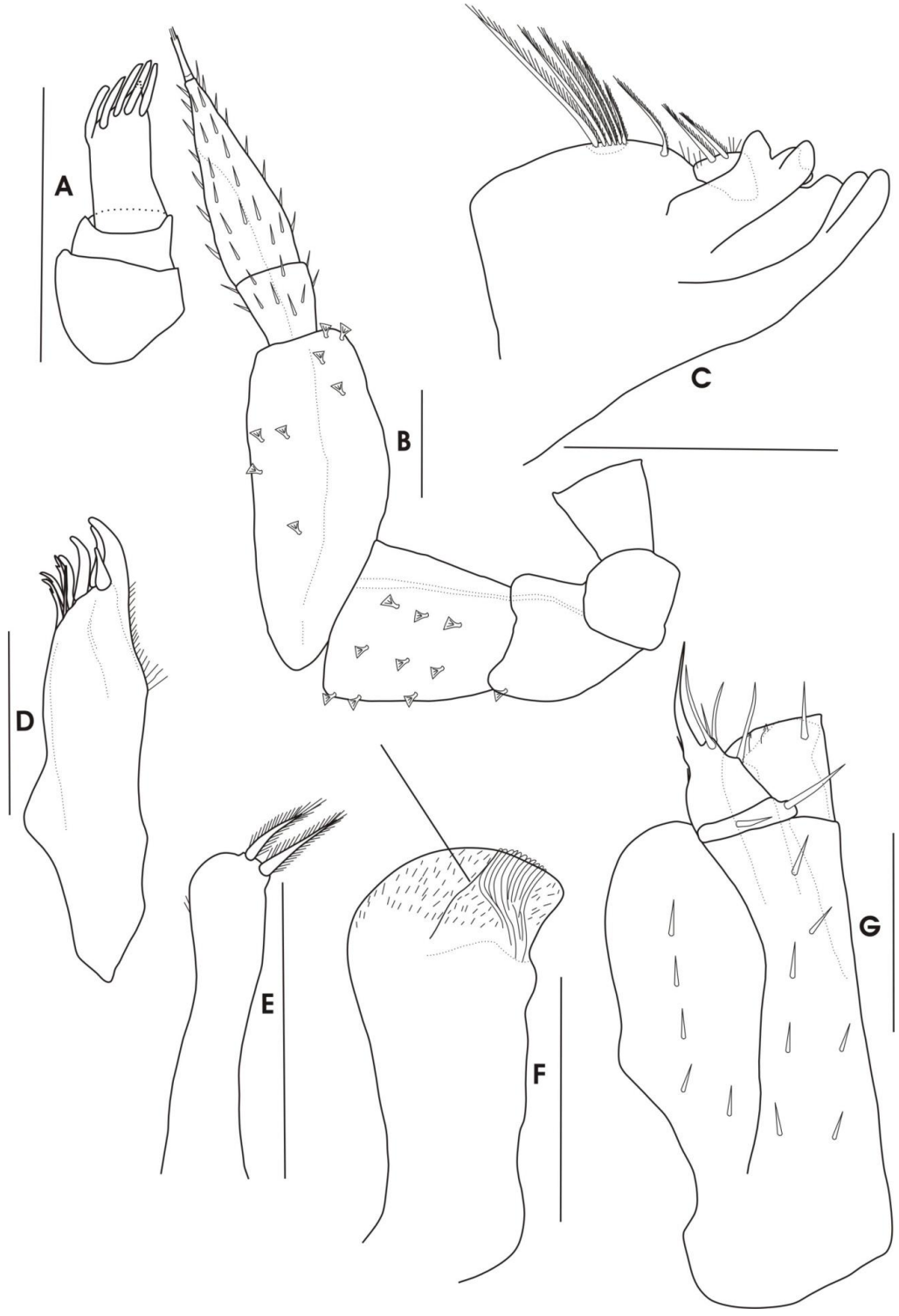



**Fig. 5.1.** *Paraplatyarthrus crebesconiscus* sp. nov. (Holotype, ♂), A: antenna 1; B: antenna 2; C: left mandible; D: maxilla 1 outer endite; E: maxilla 1 inner endite; F: maxilla 2, the arrow shows the suture delimiting the lobes; G: maxilliped. Scale bars: 0.1 mm.

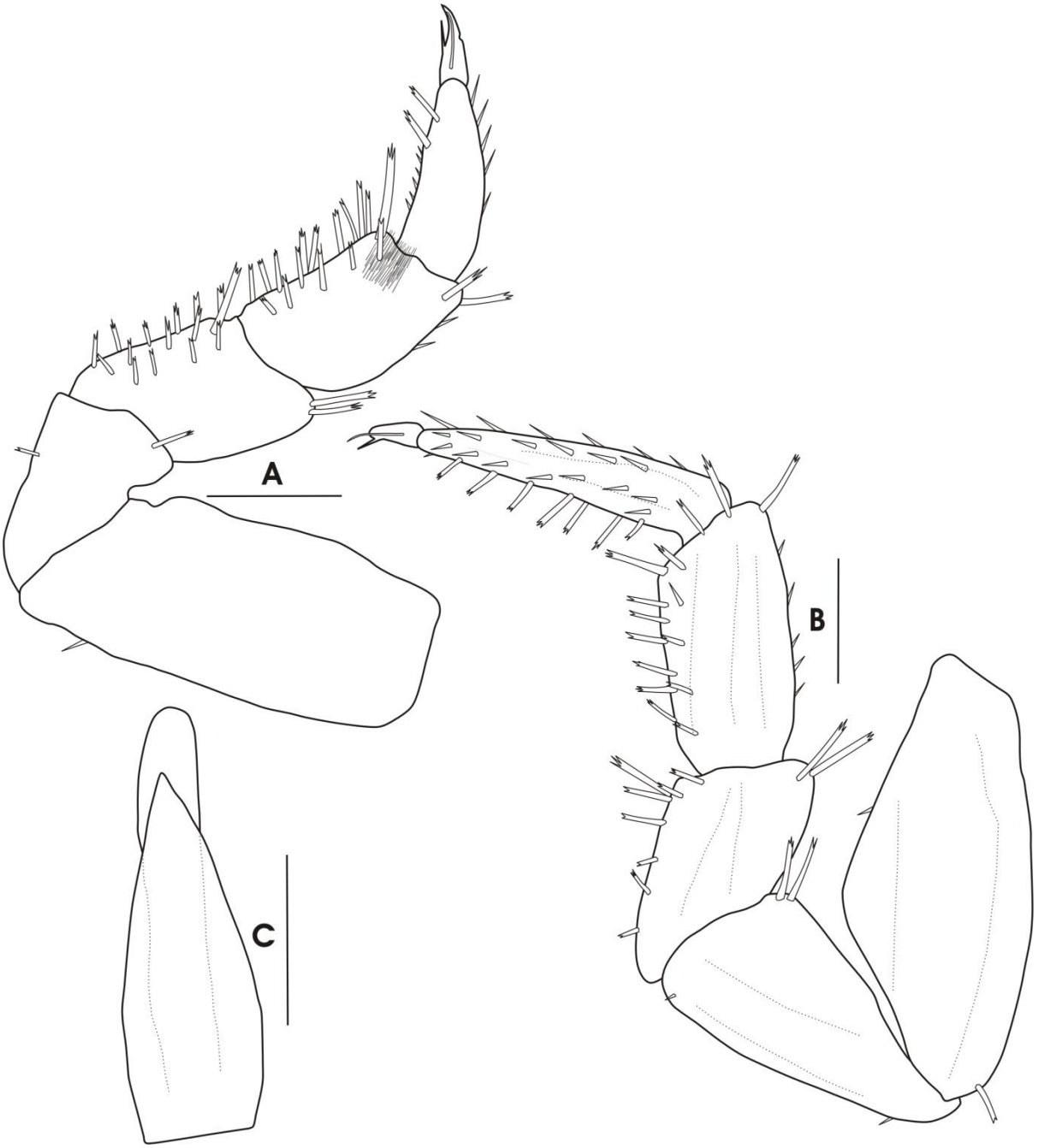



**Fig. 5.2.** *Paraplatyarthrus crebesconiscus* sp. nov. (Holotype, ♂), A: pereopod 1; B: pereopod 7; C: genital papilla. Scale bars: 0.1 mm.

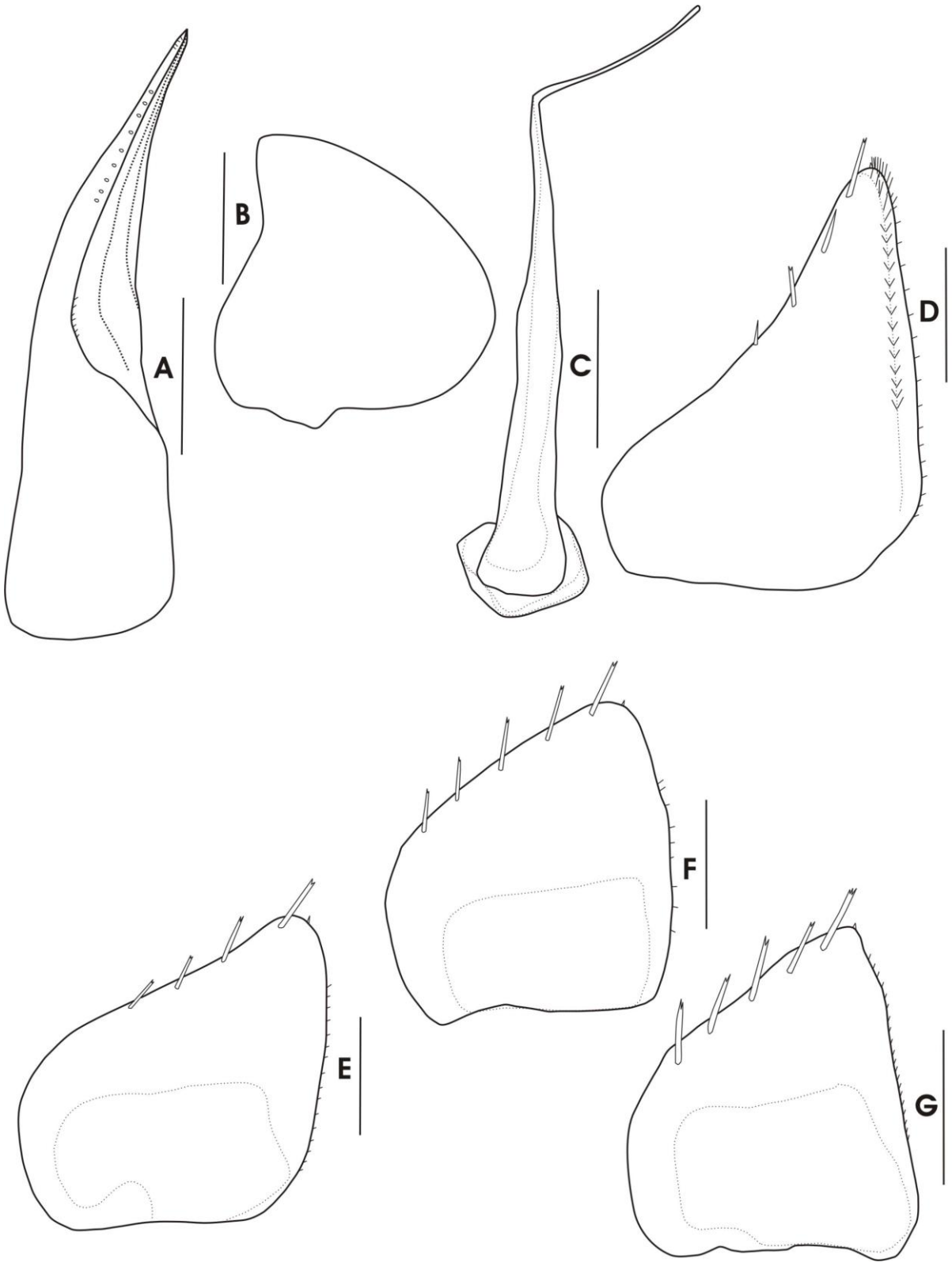



**Fig. 5.3.** *Paraplatyarthrus crebesconiscus* sp. nov. (Holotype, ♂), A: pleopod 1 endopodite; B: pleopod 1 exopodite; C: pleopod 2 endopodite; D: pleopod 2 exopodite; E: pleopod 3 exopodite; F: pleopod 4 exopodite; G: pleopod 5 exopodite. Scale bars: 0.1 mm.

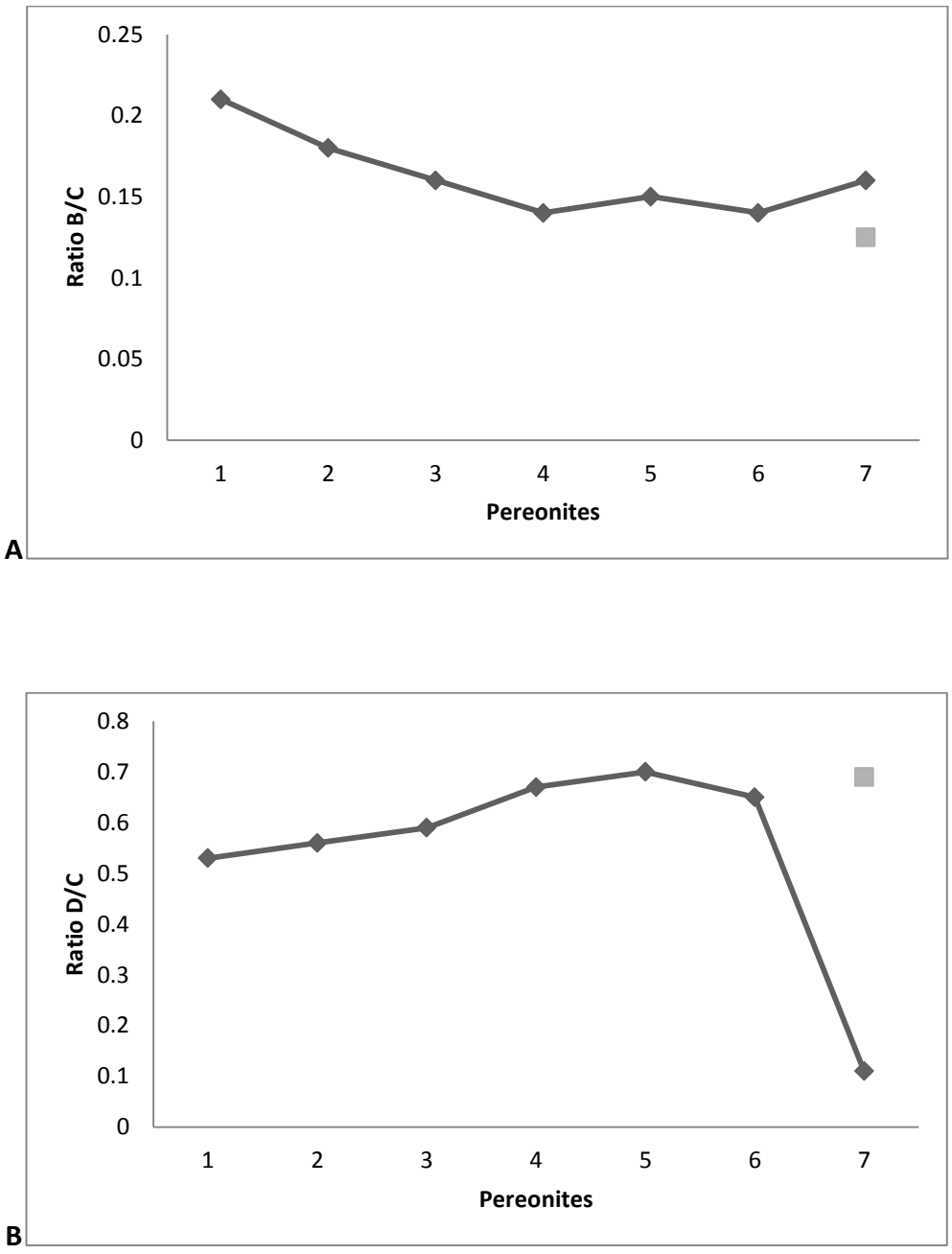



**Fig. 5.4.** *Paraplatyarthrus crebesconiscus* sp. nov. (Paratype, ♀), relative position of the noduli laterales (black and gray) on pereonites 1 to 7 defined by the ratios B/C (A) and D/C (B). B = distance from the nodulus lateralis to the posterior margin of the pereonite, C= maximum length of the pereonal tergite (antero-posterior); D = distance from the nodulus lateralis to the lateral margin of the pereonite.

***Paraplatyarthrus cunyuensis*** Javidkar & King **sp. nov.**

**Holotype.** Male, WAM C 54809 (BES17212.2), State Barrier Fence calcrete, Cunyu pastoral station, Eastern Murchison region, Western Australia, Australia; 25.7642°S, 120.1143°E. May 2012. W. F. Humphreys & S. J. B. Cooper.

**Paratypes.** 6 females, WAM C 54810 (BES17217), WAM C 54811 (BES17217.1), WAM C 54812 (BES17217.2) (25.7726°S, 120.1108°E; W. F. Humphreys & S. J. B. Cooper; May 2012); WAM C 54813 (BES17212.1) (Same locality and collection data as holoptype ); WAM C 54814 (BES15090.2), WAM C 54815 (BES15081.1) (25.78064°S, 120.10745°E; W. F. Humphreys & S. J. B. Cooper; March 2009); 1 male, WAM C 54816 (BES15081.2) (25.78064°S, 120.10745°E; W. F. Humphreys & S. J. B. Cooper; March 2009). All paratypes from State Barrier Fence calcrete, Cunyu pastoral station, Eastern Murchison region, Western Australia, Australia.

**Description. Male (WAM C 54809)**, Body completely pale (Figure 5.22B). Cephalon lateral lobes present but not developed. Eyes with 3 very reduced (vestigial) orange ommatidia (Figure 5.22C). Antenna 1 3-jointed (Figure 5.5A), medial article shortest, distal and basal articles the same size but longer than medial one. Antenna 2 flagellum 2-jointed, basal article short about 1/3 of distal article (Figure 5.5B). Left mandible (Figure 5.5C) pars molaris with about 5 plumose setae; hairy lobe bearing 2 plumose setae; 1 plumose seta between lobe and pars molaris. Right mandible pars molaris with a few long plumose setae; 1 relatively long plumose seta on hairy lobe, 1 smaller down lobe. Maxilla 1 outer endite (Figure 5.5D) with an outer group of 4 teeth covering about 65% of marginal area, inner group of 2 cleft, 2 truncated and 1 simple tooth; inner endite (Figure 5.5E) with 2 stout plumose setae. Maxilla 2 (Figure 5.5F) apically bilobate, inner lobe smaller than outer one, bearing thicker sensilla on distal part, a fine suture delimiting lobes, subapical region of lobes covered with very fine small setae. Maxilliped endite (Figure 5.5G) with 1 large seta close to subapical inner corner, 2 very short apical arrow-like setae; basal article of palp with 2 setae, one on inner side longest, distal articles fused, with 1 long proximal seta, 2 medial smaller setae and an apical tuft of a few long setae, 2 small setae on medial outer margin of palp.

Epimeron 1 rounded anteriorly; in dorsal view, posterolateral corner of pereonites 1-3 rounded; posterolateral corner of pereonites 4-7 posteriorly directed (Figure 5.21A). Pleonal epimeron 5 posterior corner not surpassing uropodal sympodite. Noduli laterales present on

pereonal tergites (Figure 5.8A, B); tergite 7 with 2 noduli laterales relatively at same distance to posterior margin; D/C ratio is relatively constant, except for the one next to the lateral margin in tergite 7.

Pereopod 1 carpus inner margin not dense, with a few long and short serrate setae, tuft of fine setae present (Figure 5.6A); propodus with both small simple and large serrate setae; dactylus with a long fine seta not surpassing claws, outer claw relatively straight, with a small depression on medial part, inner claw shorter, situated at basal posterior of outer claw. Pereopod 7 not showing any sexual dimorphism (Figure 5.6B). Pleon outline continuous with pereon. Pleopod 1 endopodite (Figure 5.7A) with simple apex, distal part with very fine small setae; exopodite heart-shaped, posterior point not developed (Figure 5.7B). Genital papilla ventral sheath apically pointed and surpassed by a long lobe which is apically truncated and subapically protruded (Figure 5.6C). Pleopod 2, 3 and 5 exopodites with 1 marginal simple seta (Figure 5.7C, D, F), pleopod 4 exopodite with 2 marginal setae (Figure 5.7E). Pleotelson triangular, with rounded tip. Uropodal exopodites dorso-ventrally flattened, well-developed and surpassing pleotelson, with a suture on outer margin; endopodites laterally flattened, slightly exceeding pleotelson, insertion (a bit upper) at almost same level as exopodite; uropodal sympodite with an extended circumflex-shaped incision (^), with rounded apex (not reaching proximal sympodite) on outer side (Figure 5.6D).

**Diagnosis.** Eyes with 3 very reduced (vestigial) orange ommatidia; male genital lobe apically truncated, subapically protruded; D/C ratio is relatively constant, except for the one next to the lateral margin in tergite 7.

**Variation.** Body length 3.0 mm - 4.5 mm; pleotelson is somewhat pointed in a few individuals.

**Distribution.** The new species is restricted into an individual calcrete aquifer, Cunyu State Barrier Fence calcrete, Eastern Murchison region, WA, Australia.

**Remarks.** *P. cunyuensis*, representing a troglobitic form, can be easily distinguishable from other species based on the pale body and the occurrence of 3 significantly reduced (vestigial) orange ommatidia which showed to be easy characters with no variation for quick

identifications. Although *P. occidentoniscus* with 3 ommatidia seems similar to *P. cunyuensis* the colour and the size of ommatidia, which are black and comparatively much bigger in *P. occidentoniscus*, are highly diagnostic.

**Etymology.** The species name refers to its confined distribution in the Cunyu calcrete aquifer.

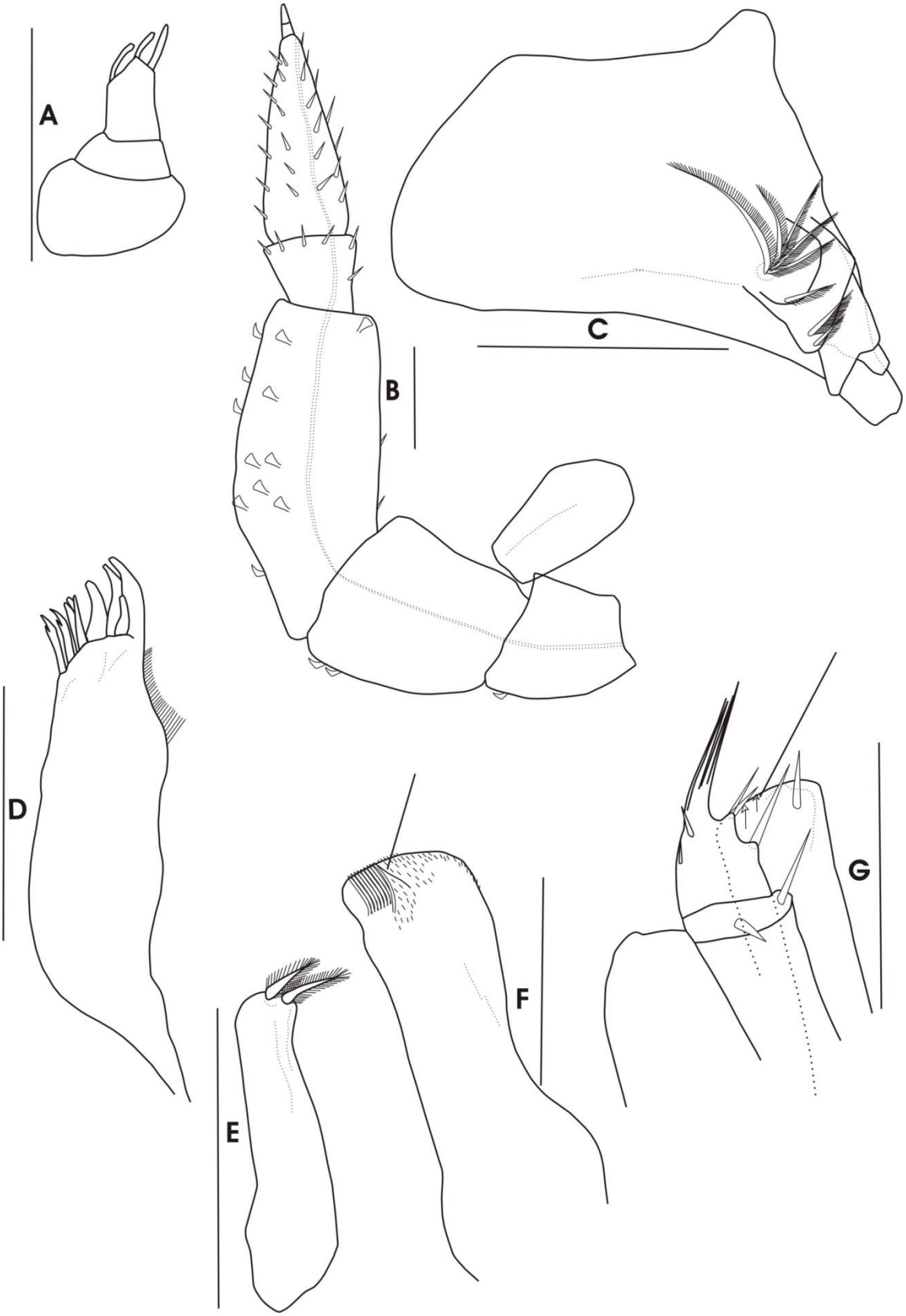



**Fig. 5.5.** *Paraplatyarthrus cunyuensis* sp. nov. (Holotype, ♂), A: antenna 1; B: antenna 2 (Paratype); C: left mandible; D: maxilla 1 outer endite; E: maxilla 1 inner endite; F: maxilla 2, the arrow shows the line delimiting the lobes; G: maxilliped. Scale bars: 0.1 mm.

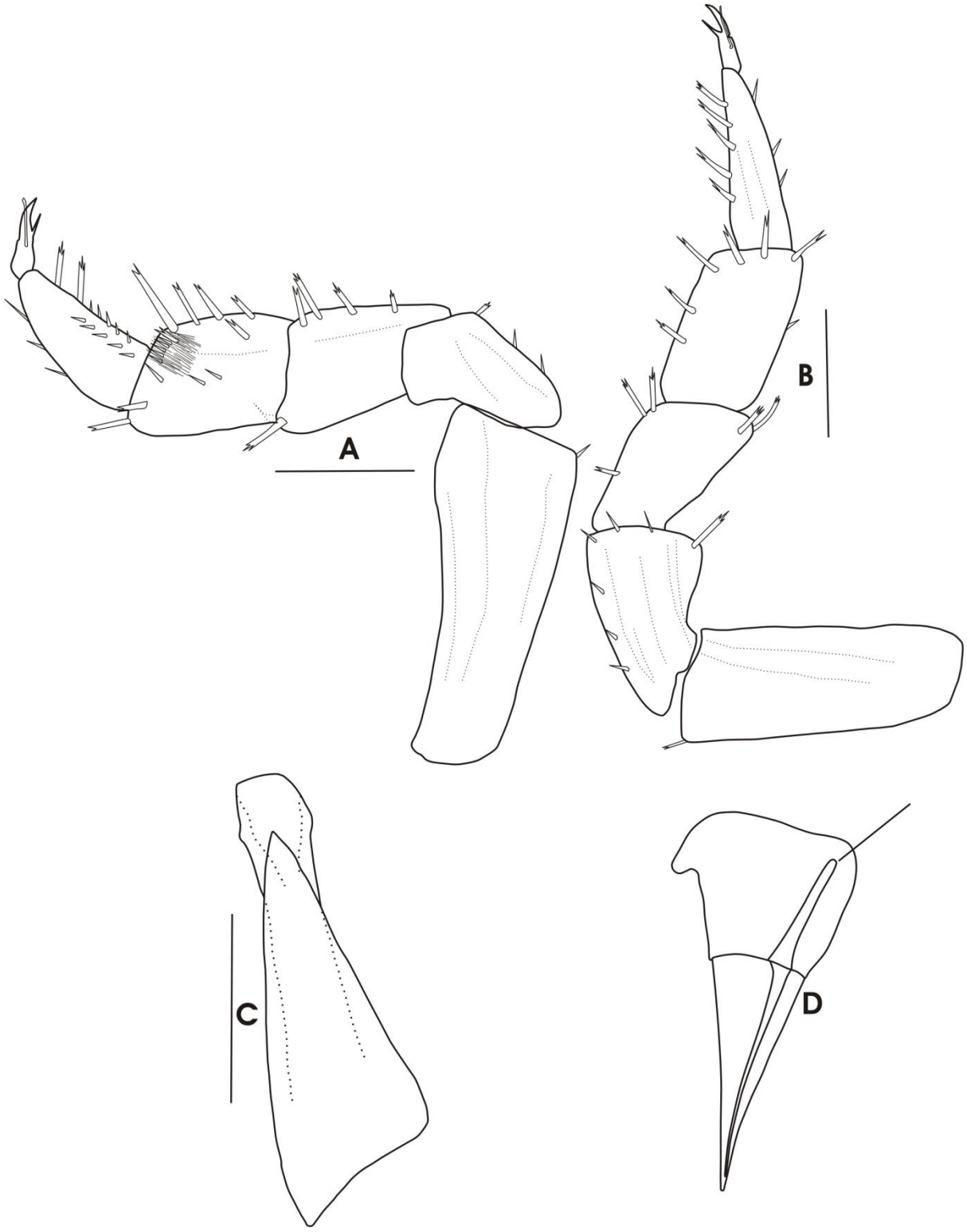



**Fig. 5.6.** *Paraplatyarthrus cunyuensis* sp. nov. (Holotype, ♂), A: pereopod 1; B: pereopod 7; C: genital papilla (Paratype); D: uropod (Paratype). Scale bars: 0.1 mm.

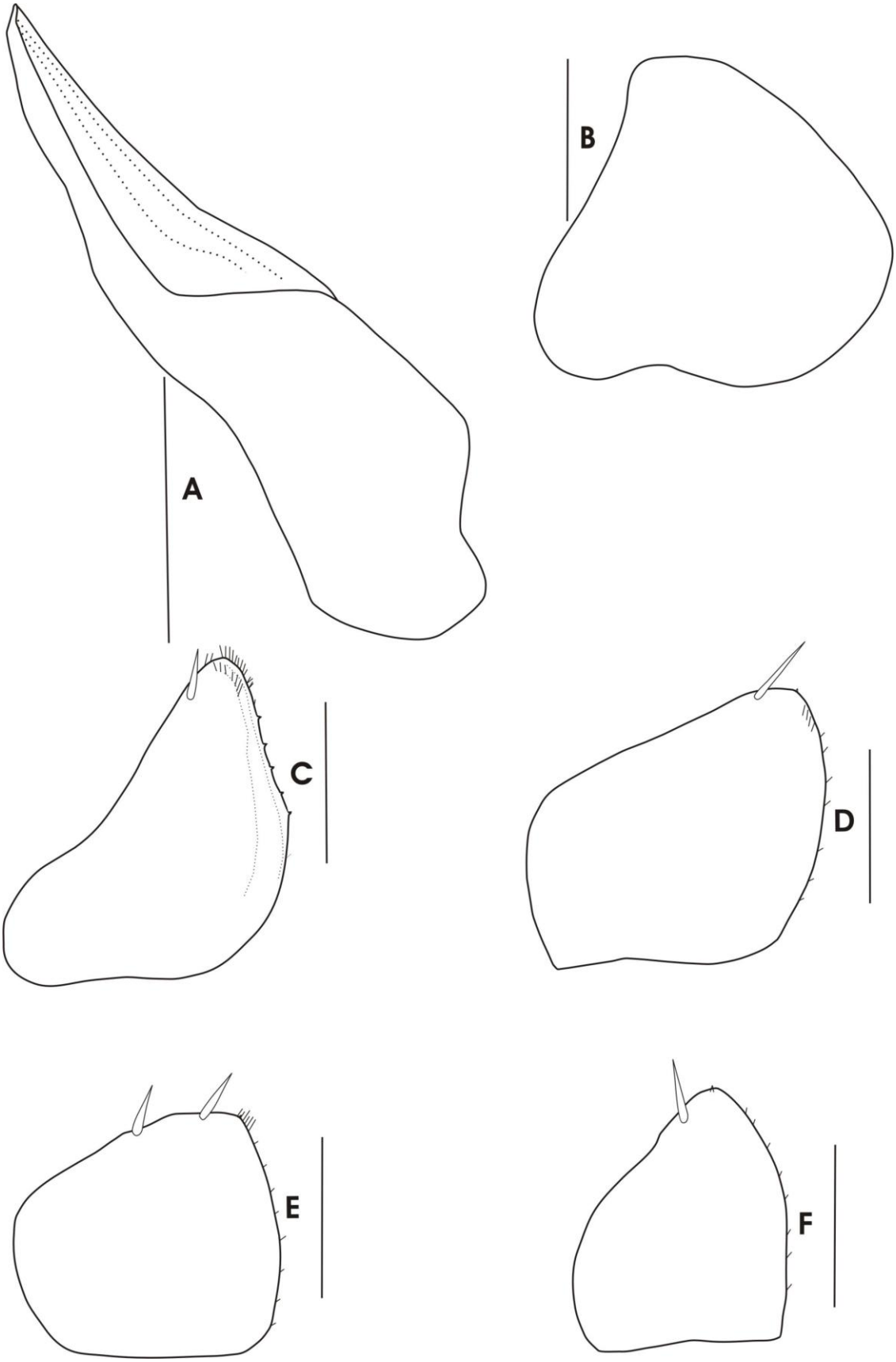
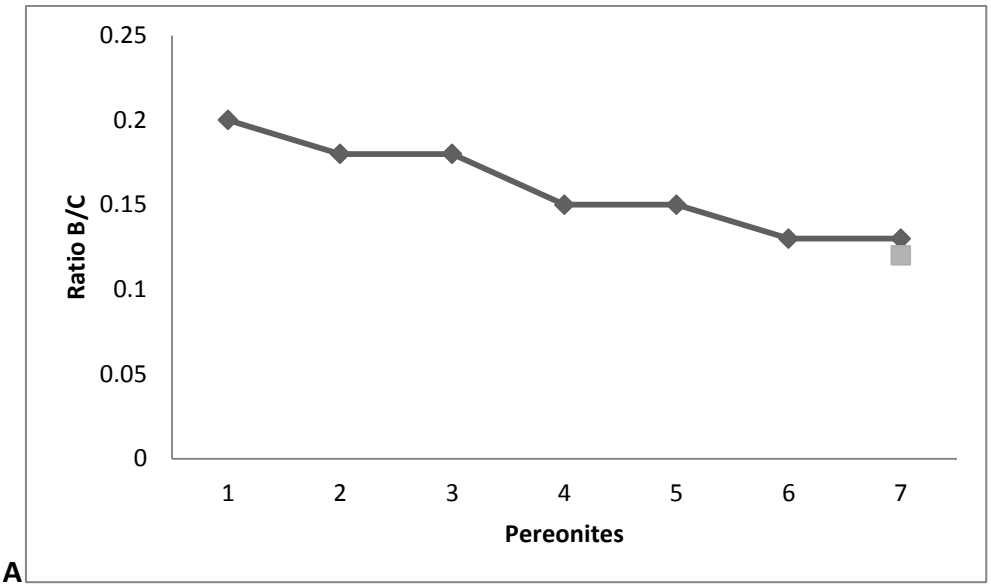
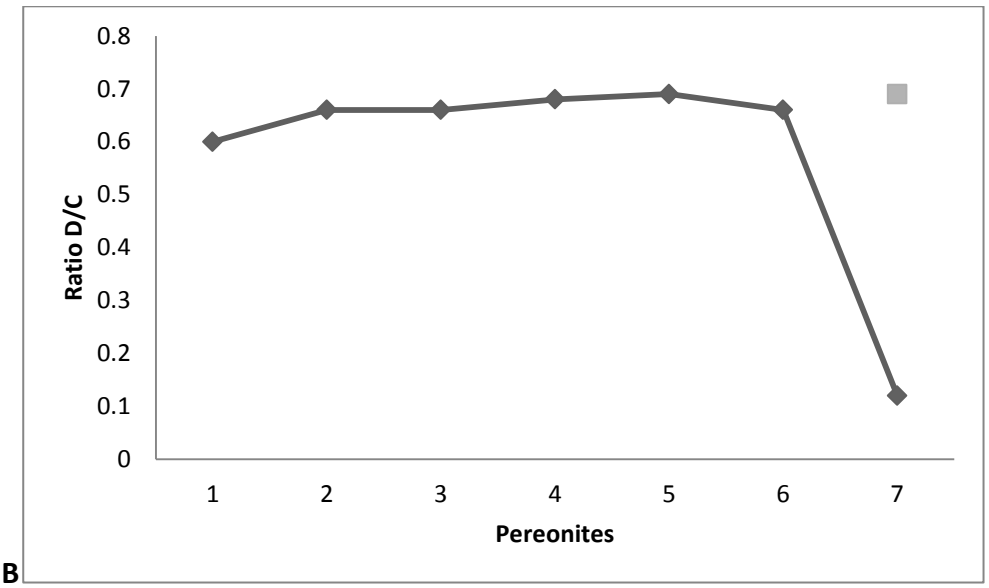



**Fig. 5.7.** *Paraplatyarthrus cunyuensis* sp. nov. (Holotype, ♂), A: pleopod 1 endopodite; B: pleopod 1 exopodite (Paratype); C: pleopod 2 exopodite; D: pleopod 3 exopodite; E: pleopod 4 exopodite; F: pleopod 5 exopodite. Scale bars: 0.1 mm.




A




B

**Fig. 5.8.** *Paraplatyarthrus cunyuensis* sp. nov. (Paratype, ♀), relative position of the noduli laterales (black and gray) on pereonites 1 to 7 defined by the ratios B/C (A) and D/C (B). B = distance from the nodulus lateralis to the posterior margin of the pereonite, C = maximum length of the pereonal tergite (antero-posterior); D = distance from the nodulus lateralis to the lateral margin of the pereonite.

***Paraplatyarthrus nahidae*** Javidkar & King **sp. nov.**

**Holotype.** Male, WAM C 54785 (JA100), Mt Morgans calcrete, Eastern Murchison region, Western Australia, Australia; 28.73272°S, 122.15430°E, 8 Aug 2011, Coll. M. Javidkar and W. F. Humphreys.

**Paratypes.** 2 males (WAM C 54786 (JA103), WAM C 54787 (JA105)); 1 female (WAM C 54788 (JA104)); 2 additional paratype specimens (WAM C 54817 (JA101), WAM C 54818 (JA102)) are kept on SEM stubs (WAM). Same locality and collection data as holoptype

**Description. Male (WAM C 54785 (JA100)),** body length 5.0 mm, fully pigmented from head to pleotelson (Figure 5.22D). Cephalon lateral lobes developed, with straight sides and apex (Figure 5.9H). 1 single *nodulus lateralis* occurring on the profrons. Eyes with 5 ommatidia. Supraantennal line present. Antenna 1 3-jointed, medial article shortest, distal article longest (Figure 5.9A). Antenna 2 flagellum 2-jointed (Figure 5.9B), basal article short, less than half length of distal article (about 0.35 of the distal). Left mandible (Figure 5.9C) pars molaris with a tuft of 6 plumose setae; hairy lobe bearing 2 plumose setae, the top covered with small fine setae, with a few fine setae down the lobe; 1 plumose seta between lobe and pars molaris. Right mandible pars molaris with several long to short plumose setae (about 8); 1 plumose seta on hairy lobe and 1 plumose seta between hairy lobe and pars molaris; several very fine setae between hairy lobe and pars molaris. Maxilla 1 outer endite (Figure 5.9D) with an outer group of 4 teeth covering about 67%of marginal area, one smaller than the others, inner group of 4 cleft teeth and 1 simple tooth; inner endite (Figure 5.9E) with 2 stout apical plumose setae closer to inner corner. Maxilla 2 (Figure 5.9F) apically bilobate; inner lobe relatively large, with thick sensilla on distal margin, inner and outer lobes delimited by a fine suture; the apical margin and subapical region of lobes covered with very fine small setae. Maxillipedal endite (Figure 5.9G) with 1 large seta close to the subapical inner corner, with 2 very short apical arrow-like setae; basal article of palp with 2 setae, one on inner side longest, distal articles fused, with 1 large proximal seta, a medial tuft of 2 large and 2 smaller setae and an apical tuft of few long setae; outer margin of palp with 1-2 fine setae.

Epimeron 1 bluntly projected anteriorly; in dorsal view, posterolateral corner of pereonites 1-3 rounded; posterolateral corner of pereonites 4-7 posteriorly directed (Figure

5.21A). Noduli laterales present on pereonal tergites (Figure 5.12A, B); B/C ratio on tergite 1 less than 0.2 (0.18); tergite 4 with noduli laterales most distant from the lateral margin (D/C ratio more than 0.8); tergite 5 with noduli laterales closest to posterior margin; tergite 7 with 2 noduli laterales; D/C ratios not constant in tergites 1-7.

Pereopod 1 (Figure 5.10A) carpus inner margin densely covered with long serrate setae, two types of long cleft setae and one simple and short recognisable, dense tuft of fine setae present medially near distal margin; propodus with both small simple and large serrate setae; dactylus with a long narrow seta not exceeding claws, outer claw relatively straight, inner claw shorter and situated at basal posterior of outer claw. Pereopod 7 (Figure 5.10B) carpus not showing any sexual dimorphism.

Pleon outline continuous with pereon (Figure 5.22D). Pleopod 1 endopodite (Figure 5.11A) moderately apically acute, with narrow spermatic furrow and a row of very small spine-like setae along medial margin; exopodite (Figure 5.11B) with a prominent posterior point and no marginal setae. Genital papilla (Figure 5.11A) ventral sheath apically rounded and surpassed by a long rounded-tip lobe. Pleopod 2 endopodite (Figure 5.11C) long, reaching to base of pleopod 5, with a small depression on medial endopodite, with tuft of very fine setae posteriorly. Pleopods 2-5 (Figures 5.11D, E, F, G) exopodites with 5-6 marginal long serrate setae. Pleotelson pointed (Figure 5.22D). Uropodal exopodites dorso-ventrally flattened and well developed, longer than the pleotelson, with a suture on outer margin; endopodites laterally flattened, slightly exceeding pleotelson; uropodal sympodite (Figure 5.10C) with an elongated suture.

**Diagnosis.** Body fully pigmented (surface species), the posterior point of male pleopod 1 exopodite developed, a single nodulus lateralis on the profrons of the cephalon, and the cephalic lateral lobes developed.

**Variability**. Body length 4.5 mm to 5.5 mm.

**Distribution.** Mt Morgans borefield, Eastern Murchison region, WA, Australia.

**Remarks**. *P. nahidae*, representing a surface species, is similar to *P. crebesconiscus* with 5 ommatidia but the size of each ommatidium is bigger and fully developed in *P. nahidae*. *P.*

*nahidae* is easily distinguished from other species here based on a fully pigmented body, male pleopod 1 exopodite posterior point developed and enlarged cephalic lateral lobes.

**Etymology.** This species is named for Nahid Shokri (wife of M. Javidkar), for her significant support during this research.

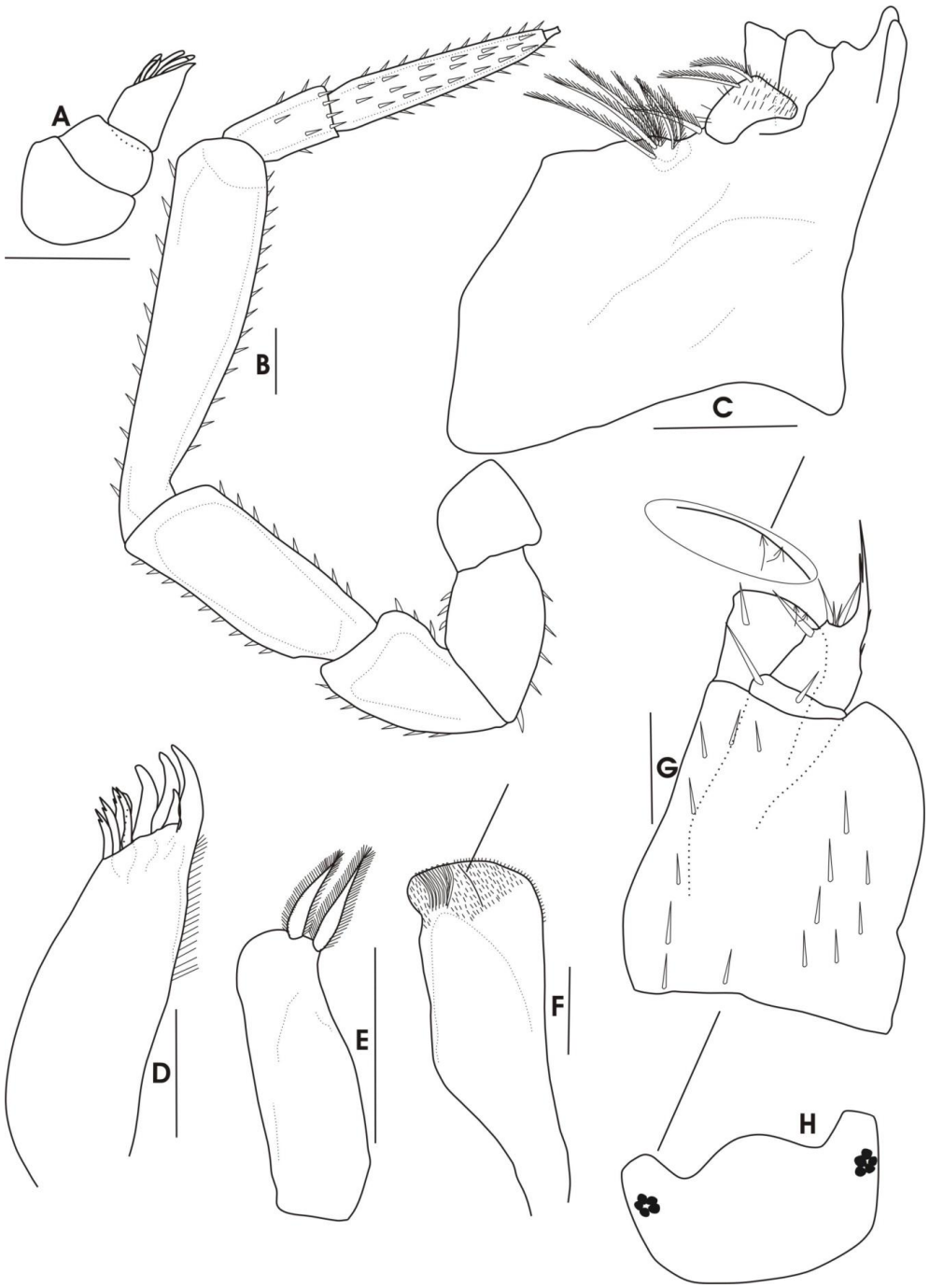



**Fig. 5.9.** *Paraplatyarthrus nahidae* sp. nov. (Holotype, ♂), A: antenna 1; B: antenna 2; C: left mandible; D: maxilla 1 outer endite; E: maxilla 1 inner endite; F: maxilla 2, the arrow shows the delimiting line between the lobes; G: maxilliped, the arrow shows a close-up of the arrow-like setae; H: cephalon, the arrow shows the developed cephalic lateral lobes. Scale bars: 0.1 mm.

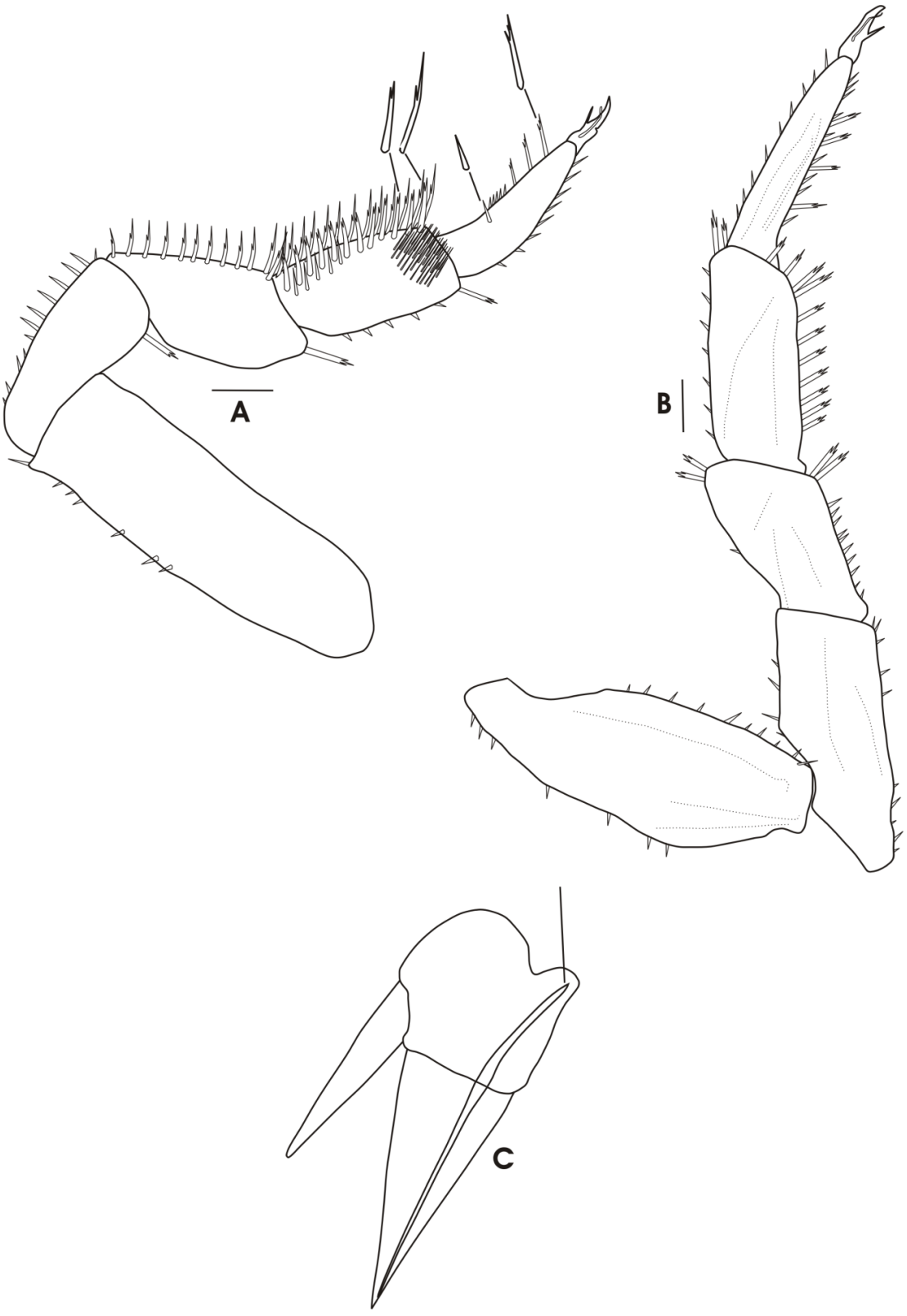



**Fig. 5.10.** *Paraplatyarthrus nahidae* sp. nov. (Holotype, ♂), A: pereopod 1, the arrows show different types of setae on the carpus and propodus; B: pereopod 7; C: uropod. Scale bars: 0.1 mm.

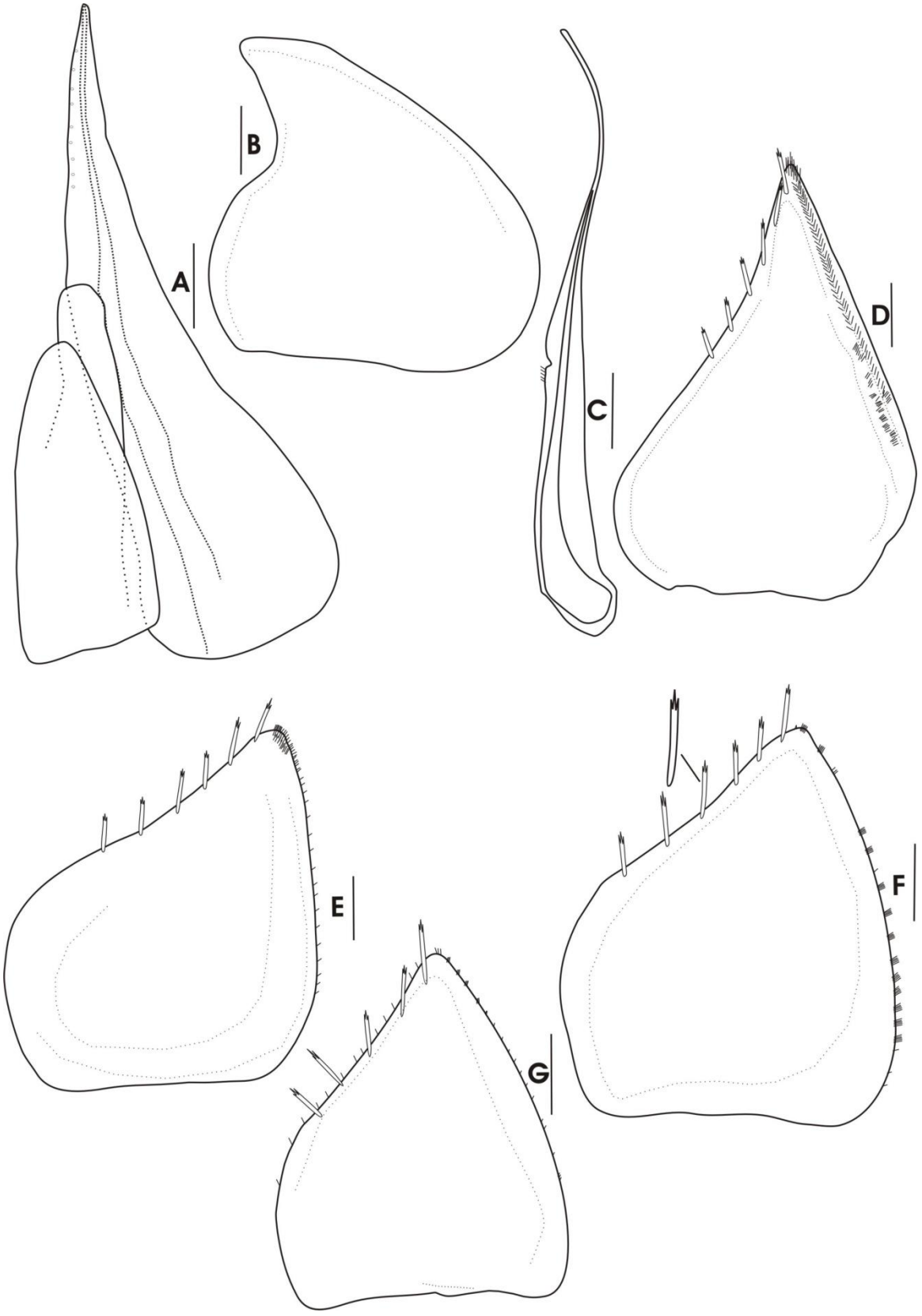
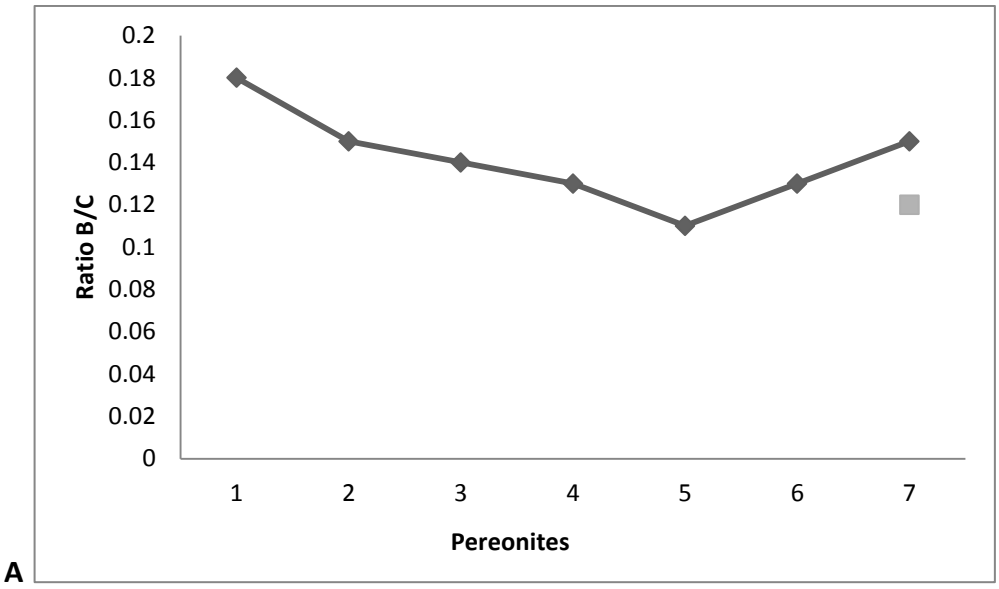
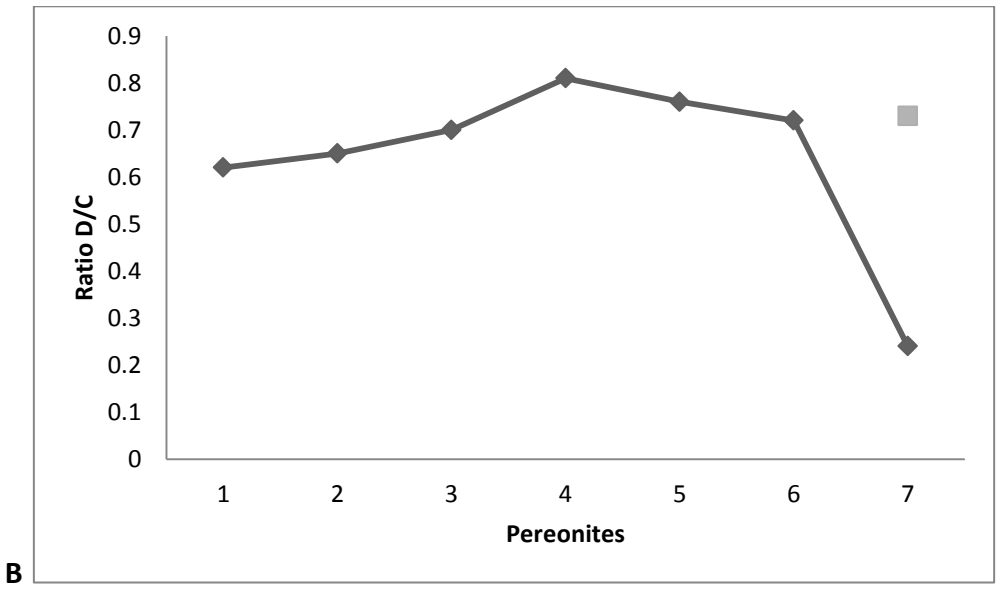



**Fig. 5.11.** *Paraplatyarthrus nahidae* sp. nov. (Holotype, ♂), A: pleopod 1 endopodite and genital papilla; B: pleopod 1 exopodite; C: pleopod 2 endopodite; D: pleopod 2 exopodite; E: pleopod 3 exopodite; F: pleopod 4 exopodite, the arrow shows the serrate setae; G: pleopod 5 exopodite. Scale bars: 0.1 mm.




A




B

**Fig. 5.12.** *Paraplatyarthrus nahidae* sp. nov. (Paratype, ♂), relative position of the noduli laterales (black and gray) on pereonites 1 to 7 defined by the ratios B/C (A) and D/C (B). B = distance from the nodulus lateralis to the posterior margin of the pereonite, C = maximum length of the pereonal tergite (antero-posterior); D = distance from the nodulus lateralis to the lateral margin of the pereonite.

***Paraplatyarthrus occidentoniscus*** Javidkar & King **sp. nov.**

**Holotype.** Male, WAM C 54794 (BES15551.10), Sturt Meadows pastoral station calcrete, Eastern Murchison region, Western Australia, Australia; 28.70124°S, 120.90361°E, Oct 2011, Coll. W. F. Humphreys & S. J. B. Cooper.

**Paratypes.** 4 females including WAM C 54795 (BES15551.12), WAM C 54796 (BES15551.23), WAM C 54797 (BES15551.4), WAM C 54798 (BES15551.22); 4 males, WAM C 54799 (BES15551.14), WAM C 54800 (BES15551.3), WAM C 54801 (BES15551.1), WAM C 54802 (BES15551.17). Same locality and collection data as holoptype.

**Description. Male (WAM C 54794)**, Body pale. Cephalic lateral lobes small and rounded. Eyes with 3 black ommatidia. Supraantennal line present. Antenna 1 3-jointed (Figure 5.13A), medial article shortest, distal and basal articles approximately the same size. Antennal 2 flagellum 2-jointed (Figure 5.13B), basal article shorter, about 1/3 length of distal one. Left mandible pars molaris with 5-6 plumose setae; 2 plumose setae on hairy lobe, 1 plumose seta between lobe and pars molaris. Right mandible pars molaris (Figure 5.13C) with about 6 plumose setae; hairy lobe bearing 2 plumose setae on top and down lobe, with a few single fine setae between the 2 plumose setae. Maxilla 1 outer endite (Figure 5.13D) with an outer group of 4 teeth covering about half of marginal area, one smaller than the others, also with inner group of 3 cleft teeth, 1 simple and 1 stalk-like tooth; inner endite (Figure 5.13E) with 2 stout apical plumose setae closer to inner corner (plumose setae very close to each other so they can appear as a single stout plumose seta), 1 single very fine seta on subapical outer marginal corner. Maxilla 2 (Figure 5.13F) apically bilobate; inner lobe slightly smaller than outer one, with thick sensilla on distal margin; inner and outer lobes delimited by a fine suture; subapical region of lobes covered with very fine small setae. Maxillipedal endite (Figure 5.13G) with 1 large seta close to subapical inner corner, with 2 very short apical arrow-like setae; basal article of palp with 2 setae, distal articles fused, with 1 large proximal seta, 2 medial large setae and an apical tuft of a few long setae.

Epimeron 1 rounded anteriorly; in dorsal view, posterolateral corner of pereonites 1-3 rounded; posterolateral corner of pereonites 4-7 posteriorly directed (Figure 5.21A). Pleonal epimeron 5 not reaching uropodal sympodite. Noduli laterales present on pereonal tergites

(Figure 5.16A, B); tergite 7 with 2 noduli laterales; tergites 6 and 7 (the one distant to lateral margin) with noduli laterales closest to posterior margin.

Pereopod 1 (Figure 5.14A) carpus inner margin with both small simple setae and long and short serrate setae but not dense, tuft of fine setae present; propodus with both small simple and large serrate setae; dactylus with a long narrow seta not exceeding the claws, outer claw sickle-shaped with a small depression on medial part, inner claw shorter, situated at basal posterior of outer claw. Pereopod 7 (Figure 5.14B) not showing any sexual dimorphism.

Pleon outline continuous with pereon. Pleopod 1 endopodite (Figure 5.15A) slender, with simple apex including very fine setae, very fine setae in medial part; exopodite (Figure 5.15B) heart-shaped, with no posterior point or setae on margins. Genital papilla (Figure 5.15A) ventral sheath apically pointed and surpassed by a long distally rounded lobe. Pleopod 2 endopodite (Figure 5.15C) very slender; exopodite with 2 long marginal setae and very fine setae on other side (Figure 5.15D). Pleopod 3-5 exopodites (Figures 5.15E, F, G) with 3 simple and serrate marginal long setae.

Pleotelson triangular and pointed. Uropodal exopodites dorso-ventrally flattened surpassing the pleotelson, with a suture on outer margin; endopodites laterally flattened, inserted at approximately same level (a bit upper) as exopodites; uropodal sympodite with an extended circumflex-shaped incision (^) on outer side.

**Diagnosis.** Cephalic lateral lobes not developed; male pleopod 1 exopodite with no posterior point; eyes with 3 black ommatidia.

**Variability.** Body length 2.0 - 4.5 mm. Body pigmentation variable from semi- to weakly-pigmented to pale (Figures 5.22E, F)

**Distribution.** This new species is confined to a single calcrete aquifer, Sturt Meadows pastoral station, Eastern Murchison region, WA, Australia.

**Remarks**. *P. occidentoniscus*, representing a troglophilic form, is sister to the group of *P. cunyuensis* and an undescribed taxon from the Lake Violet calcrete aquifer from Western Australia based on the molecular phylogenies (Chapter 3). This new species is diagnosed

from other species by a combination of characters including eyes with 3 black ommatidia, weak cephalic lateral lobes and male pleopod 1 exopodite with no or very weak posterior point.

**Etymology.** The species name is composed of the Latin 'occidente' meaning west, referring to its Western Australian distribution, plus oniscus.

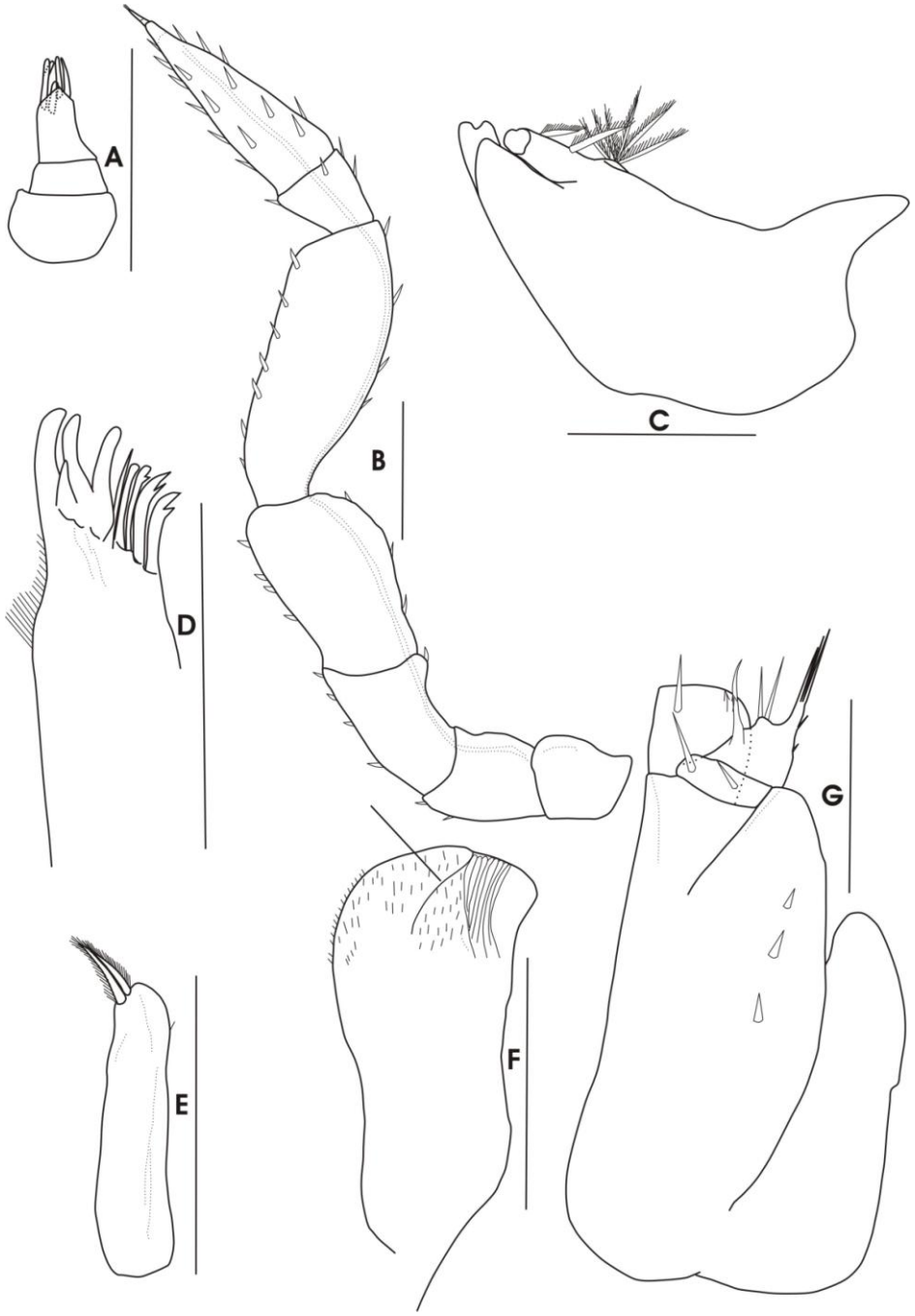



**Fig. 5.13.** *Paraplatyarthrus occidentoniscus* sp. nov. (Holotype, ♂), A: antenna 1; B: antenna 2; C: right mandible (Paratype); D: maxilla 1 outer endite; E: maxilla 1 inner endite; F: maxilla 2 (the arrow shows the line delimiting the lobes); G: maxilliped. Scale bars: 0.1 mm.

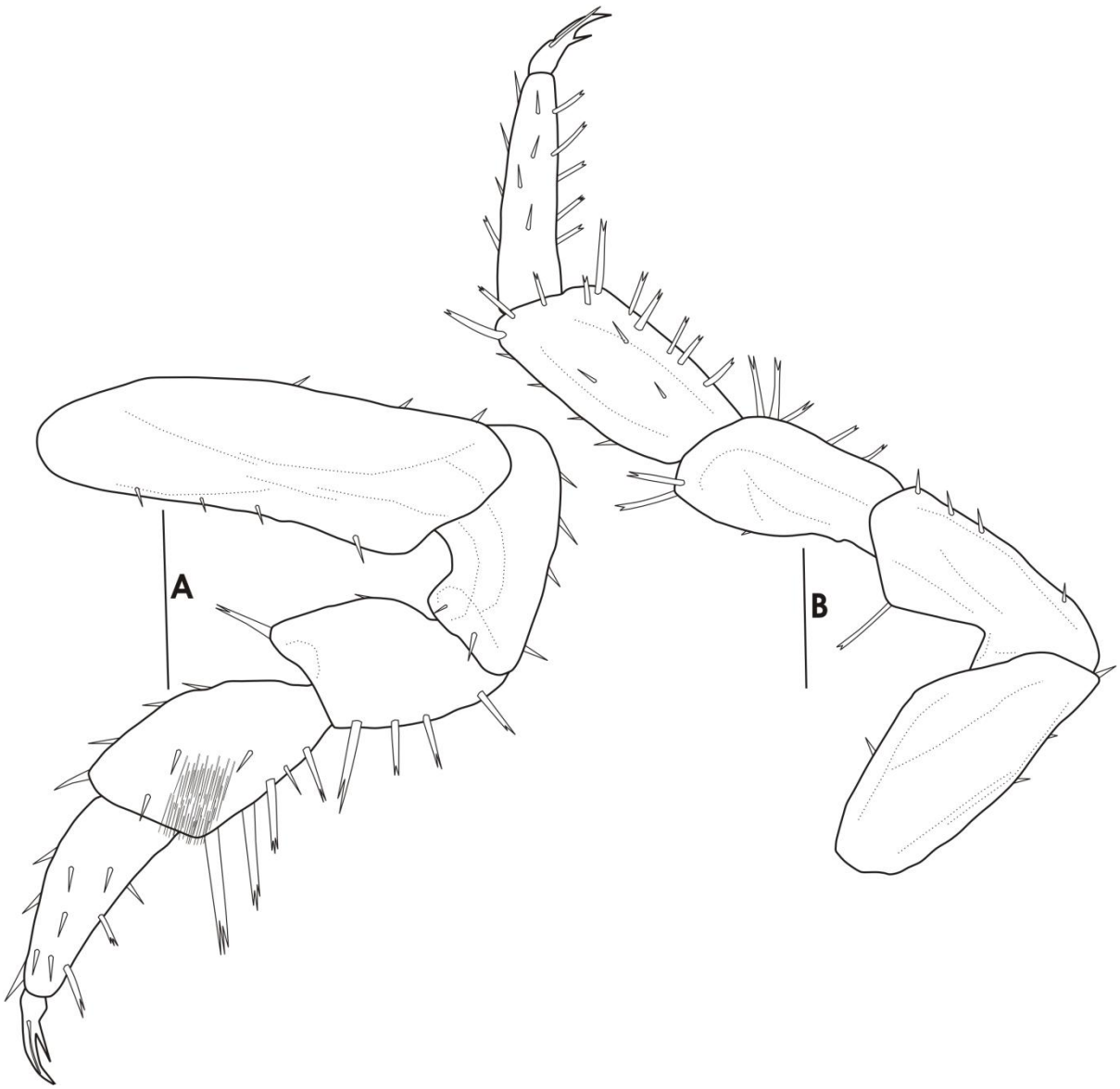



**Fig. 5.14.** *Paraplatyarthrus occidentoniscus* sp. nov. (Holotype, ♂), A: pereopod 1; B: pereopod 7. Scale bars: 0.1 mm.

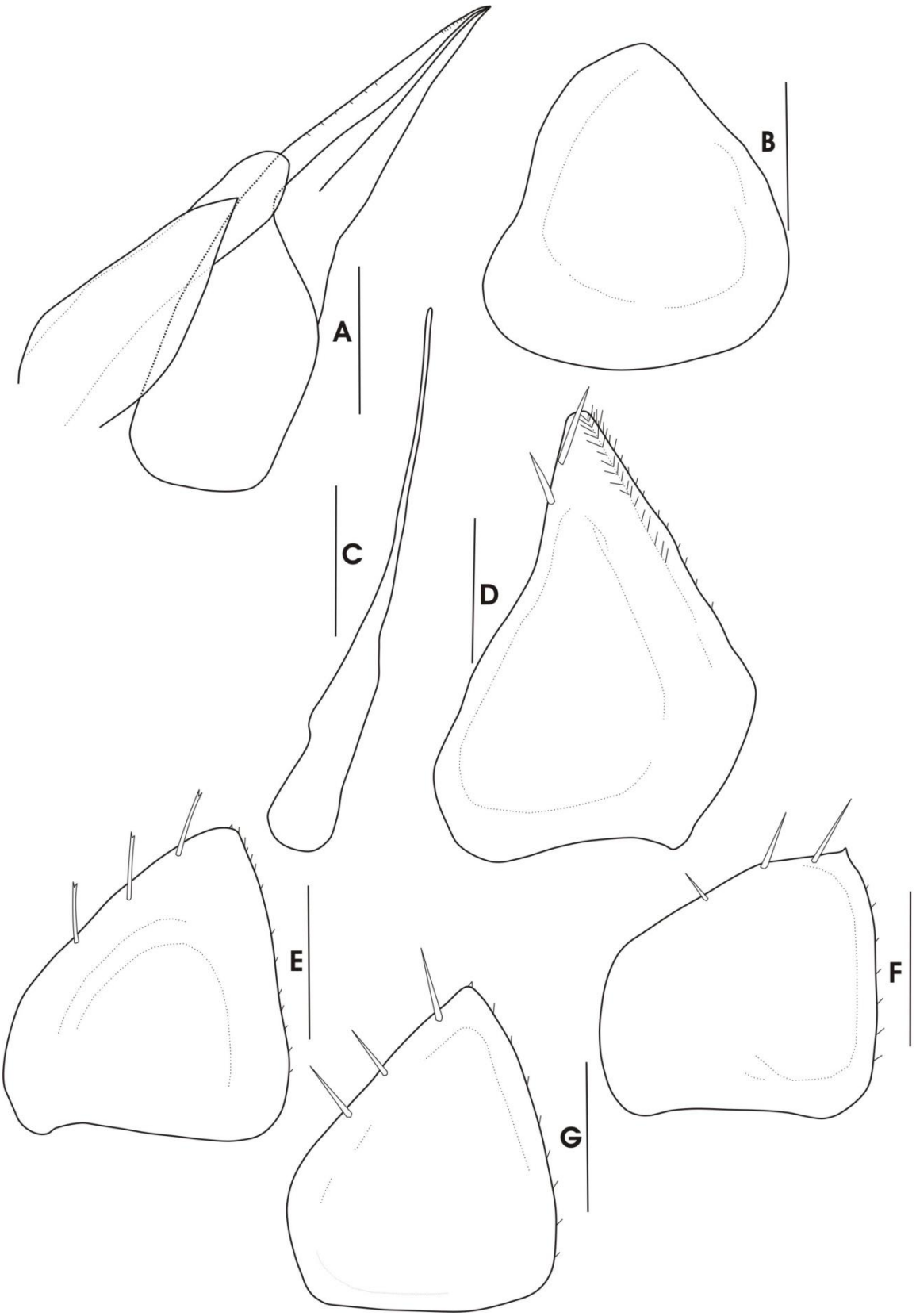
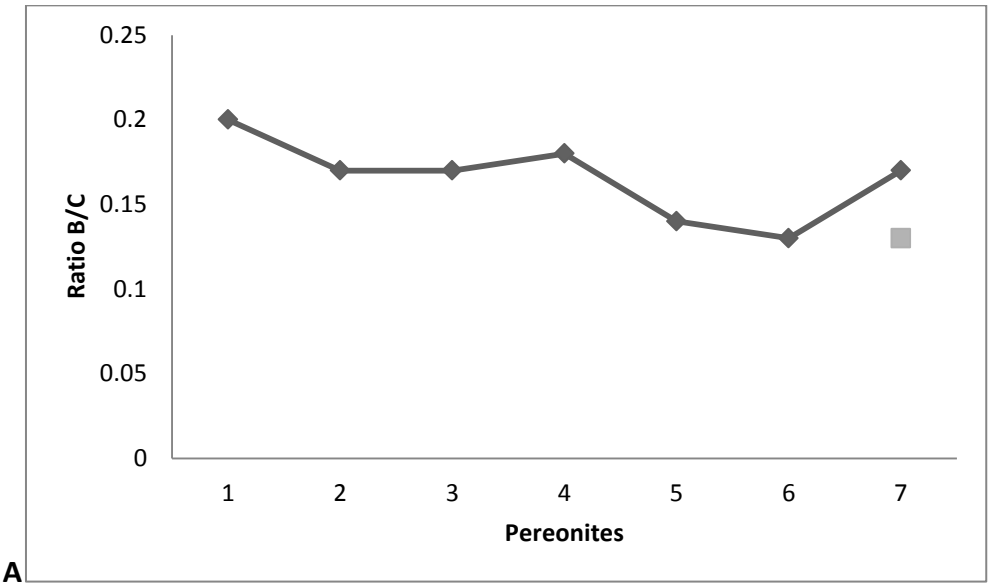
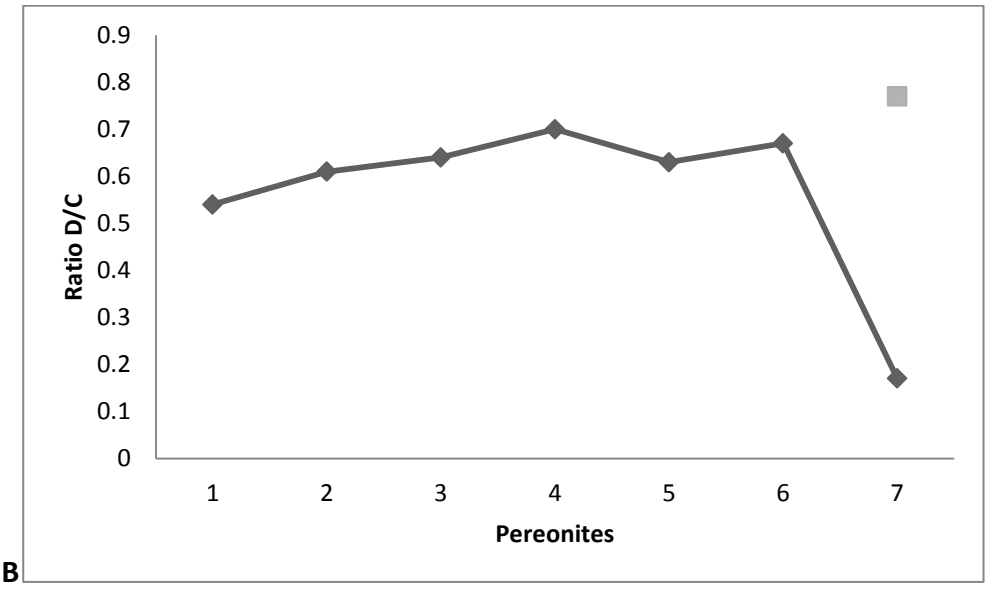



**Fig. 5.15.** *Paraplatyarthrus occidentoniscus* sp. nov. (♂), A: pleopod 1 endopodite and genital papilla (Paratype); B: pleopod 1 exopodite (Paratype); C: pleopod 2 endopodite (Paratype); D: pleopod 2 exopodite (Paratype); E: pleopod 3 exopodite (Paratype); F: pleopod 4 exopodite (Holotype); G: pleopod 5 exopodite (Paratype). Scale bars: 0.1 mm.




A




B

**Fig. 5.16.** *Paraplatyarthrus occidentoniscus* sp. nov. (Paratype, ♀), relative position of the noduli laterales (black and gray) on pereonites 1 to 7 defined by the ratios B/C (A) and D/C (B). B = distance from the nodulus lateralis to the posterior margin of the pereonite, C = maximum length of the pereonal tergite (antero-posterior); D = distance from the nodulus lateralis to the lateral margin of the pereonite.

***Paraplatyarthrus pallidus*** Javidkar & King **sp. nov.**

**Holotype.** Male, WAM C 54803 (BES15545.2), Lake Miranda East calcrete aquifer, Yakabindie pastoral station, Eastern Murchison region, Western Australia, Australia; 27.66405°S, 120.61015°E. Jul 2010. S. J. B. Cooper & W. F. Humphreys.

**Paratypes.** 2 females including WAM C 54804 (BES15545.8), WAM C 54805 (BES15545.3); 3 males, WAM C 54806 (BES15545.11), WAM C 54807 (BES15545.10), WAM C 54808 (BES15545.6). Same locality and collection data as holotype.

**Description. Male (WAM C 54803)**, Body length 2.7 mm, completely pale (Figure 5.22G). Cephalon lateral lobes with straight sides. Eyes significantly reduced, with only 1 black ommatidium (troglobite). Supraantennal line present. Antenna 1 3-jointed, medial article shortest, distal article longest, seemingly bearing 6 aesthetascs on top (Figure 5.17A). Antenna 2 flagellum 2-jointed, basal article short, about 1/3 of distal one. Left mandible (Figure 5.17B) pars molaris with about 8 plumose setae; hairy lobe bearing 2 plumose setae, with no small fine setae; 1 plumose seta between lobe and pars molaris. Right mandible pars molaris with probably more than 4 plumose setae; 1 plumose seta on hairy lobe, 1 plumose seta between hairy lobe and pars molaris. Maxilla 1 outer endite (Figure 5.17C) with an outer group of 4 teeth covering about 65% of marginal area, inner group of 2 cleft, 2 simple and 1 truncated tooth; inner endite (Figure 5.17D) with 2 stout apical plumose setae. Maxilla 2 (Figure 5.17E) not apically bilobate (delimiting line hardly recognisable); inner lobe with thick sensilla contracted into a small area on distal inner corner. Maxillipedal endite (Figure 5.17F) with 1 large seta close to subapical inner corner, 2 very short apical arrow-like setae; basal article of palp with 2 setae, distal articles fused, with 1 large proximal seta, a medial tuft of 2 or 3 simple setae and an apical tuft of few long setae.

Epimeron 1 bluntly projected anteriorly; in dorsal view, posterolateral corner of pereonites 1-4 rounded; posterolateral corner of pereonites 5-7 posteriorly directed (Figure 5.21B). Pleonite 5 posterior corner reaching uropod sympodite but not surpassing it. Noduli laterales present on pereonal tergites (Figure 5.20A, B); tergite 7 with 2 noduli laterales, at approximately same distance from posterior margin; tergites 2-6 with noduli laterales at approximately same distance from posterior margin; D/C ratio is relatively constant, except for the one next to the lateral margin in tergite 7.

Pereopod 1 carpus inner side with long simple and serrate setae, tuft of fine setae present medially near distal margin (Figure 5.18A); propodus with both small simple and large serrate setae; dactylus with a long seta exceeding the claws, outer claw relatively straight, inner claw shorter and situated at the basal posterior of the outer claw. Pereopod 7 carpus not showing any sexual dimorphism (Figure 5.18B). Pleon outline continuous with pereon. Pleopod 1 endopodite (Figure 5.19A) moderately acute, with narrow spermatic furrow and a few very fine setae close to distal part; exopodite (Figure 5.19B) with no marginal setae, posterior point not developed. Genital papilla ventral sheath apically pointed and surpassed by a long rounded-tip lobe (Figure 5.19A). Pleopod 2 endopodite (Figure 5.19C) long and very slender closer to distal end. Pleopod 2-5 exopodites with 3 medium to large simple and serrate setae (Figures 5.19D, E, F, G). Pleotelson pointed. Uropodal exopodites dorso-ventrally flattened and surpassing the pleotelson, with a suture on outer margin; endopodites laterally flattened, slightly exceeding pleotelson; uropodal sympodite with an extended incision and truncated apex linking proximal and distal sympodite (Figure 5.18C).

**Diagnosis.** Single significantly reduced black ommatidium and pale body; maxilla 2 with no delimiting line between the lobes, the inner lobe contracted into a small area in the distal inner apical corner; uropodal sympodite with incision on outer margin, apically truncated and merging with the proximal sympodite; D/C ratio is relatively constant, except for the one next to the lateral margin in tergite 7).

**Variability.** Mean body length 2.7 mm – 4.0 mm.

**Distribution.** The new species is restricted to a single calcrete aquifer, Lake Miranda East, Yakabindie pastoral station, Eastern Murchison region, WA, Australia.

**Remarks.** According to the molecular phylogenies conducted in Chapter 3, *P. pallidus* forms a basal lineage relative to a group including *P. cunyuensis*, *P. occidentoniscus* and an undescribed divergent taxon from the lake Violet calcrete aquifer (Western Australia). *P. pallidus*, representing a troglobitic form, is easily distinguishable from other species based on 1 single significantly reduced ommatidium, pale body, the lack of the delimiting line in Maxilla 2 and the state of incision on the uropod sympodite merging to the proximal sympodite.

**Etymology.** The new species name is taken from the Latin ‘pallidus’ alluding to its pale body.

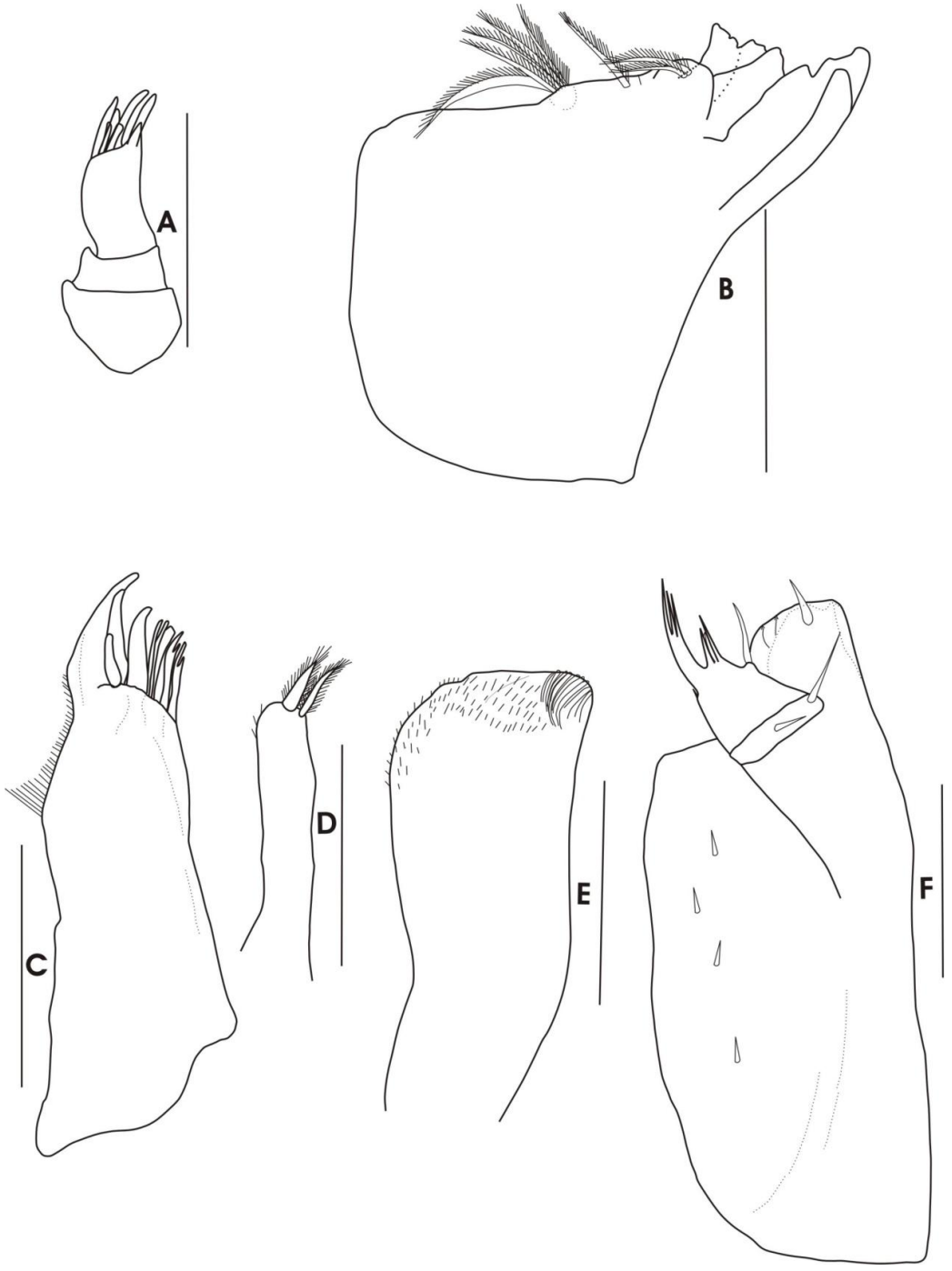



**Fig. 5.17.** *Paraplatyarthrus pallidus* sp. nov. (Holotype, ♂), A: antenna 1; B: left mandible; C: maxilla 1 outer endite; D: maxilla 1 inner endite; E: maxilla 2; F: maxilliped. Scale bars: 0.1 mm.

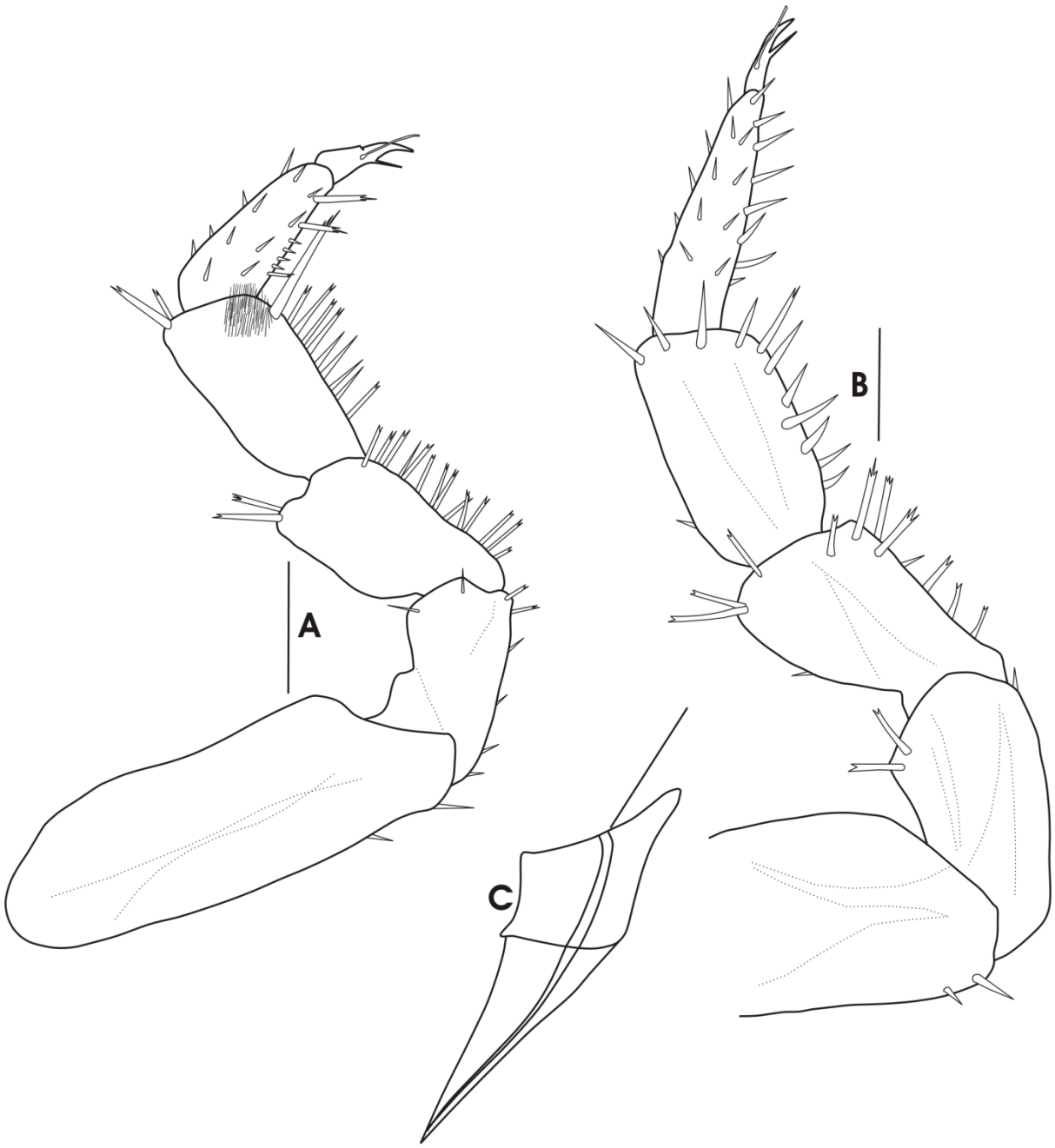



**Fig. 5.18.** *Paraplatyarthrus pallidus* sp. nov. (Holotype, ♂), A: pereopod 1; B: pereopod 7; C: uropod (Paratype). Scale bars: 0.1 mm.

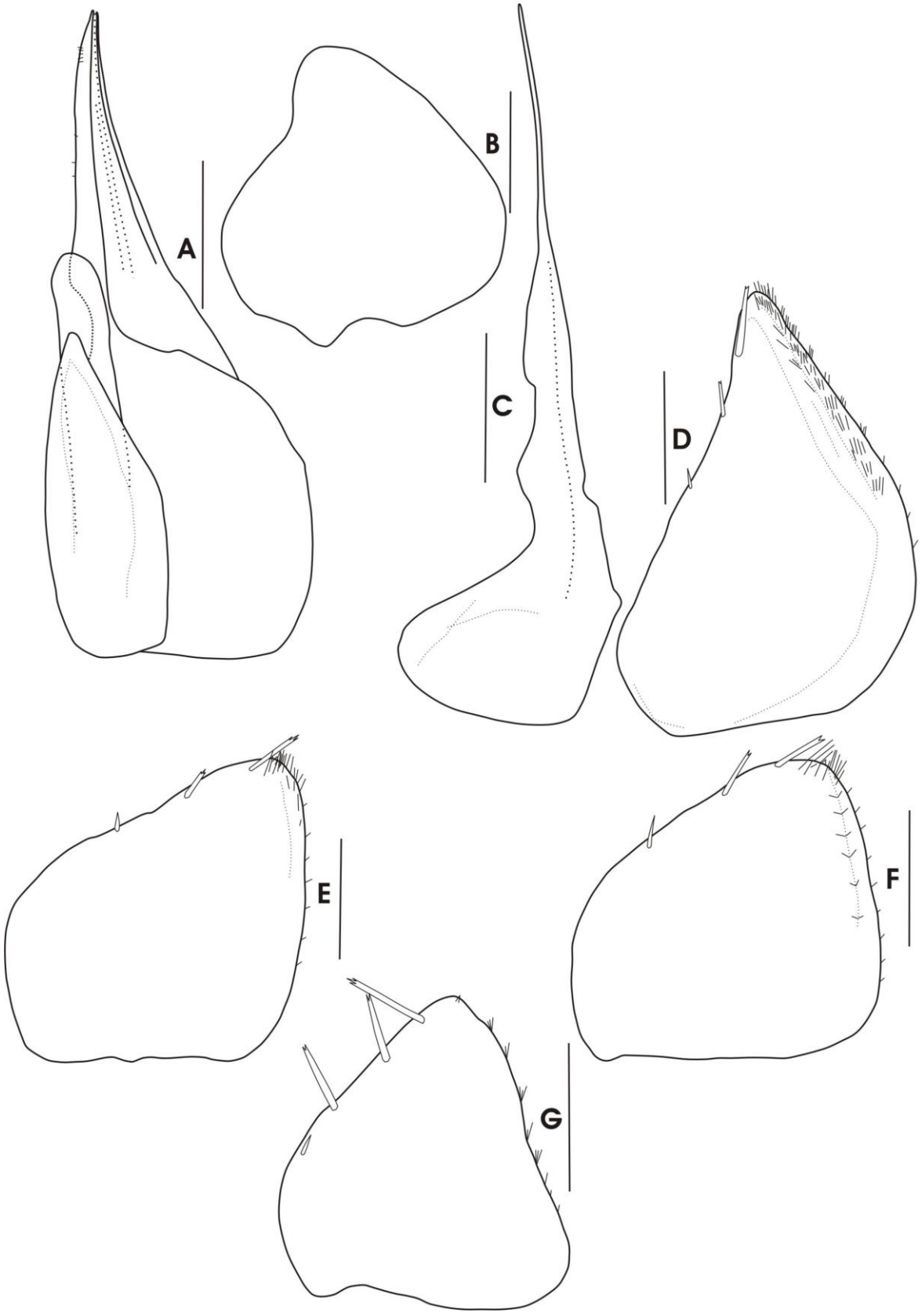
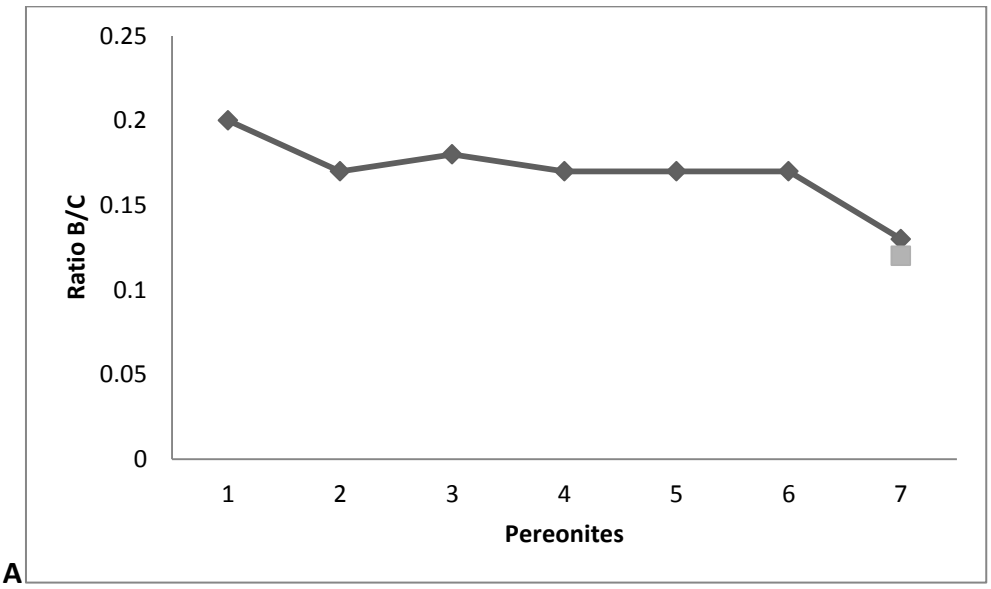



**Fig. 5.19.** *Paraplatyarthrus pallidus* sp. nov. (Holotype, ♂), A: pleopod 1 endopodite and genital papilla; B: pleopod 1 exopodite; C: pleopod 2 endopodite; D: pleopod 2 exopodite; E: pleopod 3 exopodite; F: pleopod 4 exopodite; G: pleopod 5 exopodite. Scale bars: 0.1 mm.

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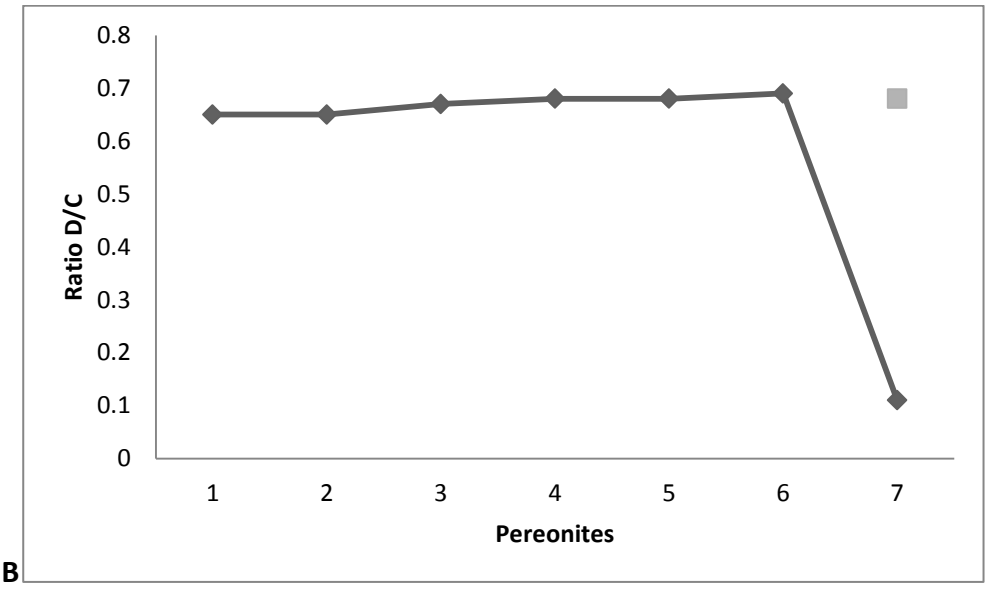


B

**Fig. 5.20.** *Paraplatyarthrus pallidus* sp. nov. (Paratype, ♀), relative position of the noduli laterales (black and gray) on pereonites 1 to 7 defined by the ratios B/C (A) and D/C (B). B = distance from the nodulus lateralis to the posterior margin of the pereonite, C = maximum length of the pereonal tergite (antero-posterior); D = distance from the nodulus lateralis to the lateral margin of the pereonite.

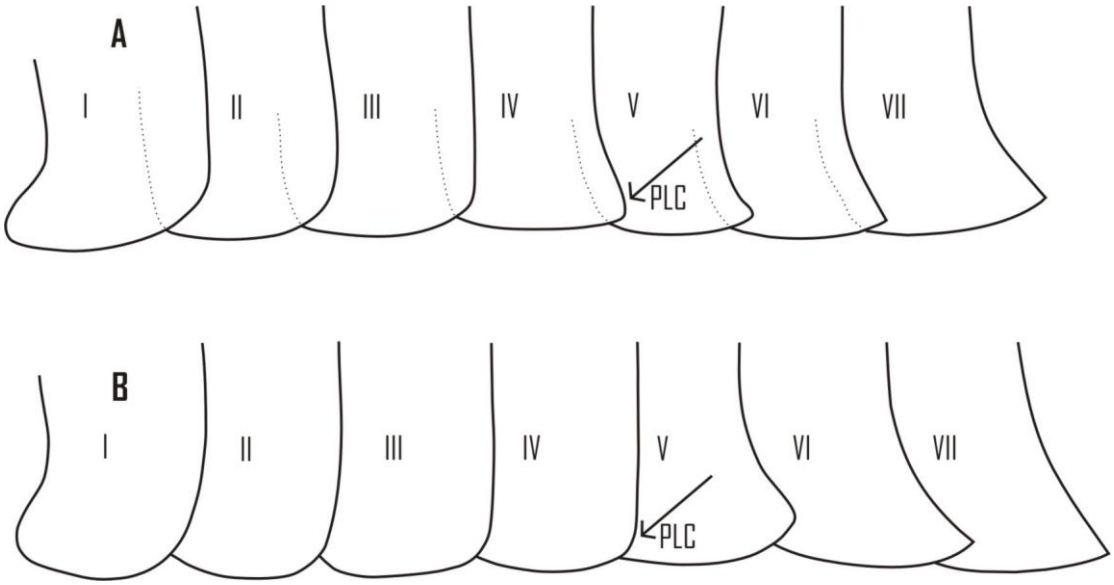


**Fig. 5.21**. The position of posterolateral corner (PLC) of epimera 1 to 7 in dorsal view; Type A: PLC in pereonal epimera 1-3 rounded, in 4-7 posteriorly directed; Type B: PLC in pereonal epimera 1-4 rounded, in 5-7 posteriorly directed.


**Fig. 5.22. A:** *P. crebesconiscus* sp. nov.; **B & C:** *P. cunyuensis* sp. nov., the arrow shows the cephalic lateral lobe; **D:** *P. nahidae* sp. nov.; **E & F:** *P. occidentoniscus* sp. nov., the arrow shows the cephalic lateral lobe; **G:** *Paraplatyarthrus pallidus* sp. nov.; **H:** *P. subterraneus* sp. nov.; scale bar of the image D: 0.5 mm, scale bar in other images: 1 mm. All images are paratype specimens.

## DISCUSSION

This study describes five new species of *Paraplatyarthrus* from central Western Australia, and is the first contribution to begin the process of formally documenting the diversity of Paraplatyarthridae. The species described here are considered as congeneric with *P. subterraneus* and distinct species based on the results of the multi-gene analyses (Chapter 2) and application of species delimitation methods (Chapter 3). Distributional patterns of the described subterranean species include both restricted and relatively widespread taxa: *P. pallidus* (Lake Miranda East; troglobite), *P. cunyuensis* (Cunyu; troglobite) and *P. occidentoniscus* (Sturt Meadows; troglophile) were only recorded from single calcrete aquifer bodies, and so are likely to have distributions that match the area of the calcretes, i.e. only a few square kilometres. The troglophile *P. crebesconiscus* was found to occur in multiple distinct calcretes including Nambi, Halfpenny and Laverton Downs, based on evidence from the molecular analyses (Chapter 3). To date, the surface species *P. nahidae*, has only been recorded from a single site at Mt Morgans in Western Australia, but this general area has not been very well collected and the species may well have a broader distribution than this.

The relative position of the noduli laterales to the lateral margins of the pereonites was found to be useful for distinguishing *P. pallidus, P. cunyuensis* and *P. subterraneus* from other paraplatyarthrid species. In these species, D/C ratios are relatively constant in tergites 1-7 (except for the one next to the lateral margin in tergite 7), whilst there is significant variation in the other paraplatyartrid species described here for this character. Although the descriptions are based on limited material (so the D/C ratio was not used in the key) the species boundaries are consistent with the phylogenetic analyses. The data presented in Chapter 3 shows *P. pallidus* and *P. cunyuensis* are in the same major clade (Clade1); however, the position of *P. subterraneus* in relation to other species was not robustly resolved.

The morphology of the posterior corners of tergites 1 to 4 in dorsal view, used in the species' descriptions, showed some variability within *P. pallidus* and *P. crebesconiscus* so this character was not used to diagnose these species. For example, in some individuals of both species the posterior corners of tergite 4 were posteriorly directed. It is possible that the

direction of the posterior corner can be influenced by muscle movements when the animal flexes upwards or inwards or alternatively, it may be an artefact caused by fixing the animals in absolute ethanol. This character has been used previously in determination of some Australian *Trichorhina* species (Lewis, 1998a, 1998b). However, in future, this character should be treated with some caution with a large number of individuals being required to assess its variability.

## ACKNOWLEDGEMENTS

We are grateful to Julianne Waldock (WA Museum) who helped with samples. We also thank Flora, Peter and Paul Axford (Sturt Meadows pastoral property) for their hospitality and Minara Resources (Murrin Murrin Operations) environmental officers who facilitated use of a vehicle, freight transportation and accommodation during field trips in WA. Thanks to the Austin lab group (The University of Adelaide) for their help and advice. This research was supported by an ARC linkage grant (#LP100200494) to ADA, SJBC and WFH, a National Geographic grant to SJBC and WFH, and an Adelaide University PhD Scholarship to MJ.

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# CHAPTER VI:

# GENERAL DISCUSSION

## SYNTHESIS

This study includes four results chapters each of which is presented as an independent manuscript, that will be submitted for publication, depicting different aspects of my PhD project on the systematics, species diversity, distributional patterns, and the biogeographic history of isopod troglofauna from groundwater calcretes of central Western Australia. In general, the molecular and morphological analyses provide, for the first time, a detailed systematic study of a major isopod group, Platyarthridae, which led to the identification of a new oniscidean family, Paraplatyarthridae. The analyses further revealed a significant diversity of oniscidean isopod troglofauna in the calcrete aquifers, the historical time periods which may represent the evolutionary transition of the fauna to subterranean realms, the models to account for their evolution and the morphological description of a number of associated new species. This research project helps provide a theoretical and practical framework to the 1) systematics of Oniscidea, 2) evolution of the arid-zone isopod troglofauna and 3) conservation management of isopod troglofauna in the calcrete aquifers.

Following initial discoveries on the invertebrate troglofauna in the groundwater aquifers of central Western Australia prior to the current study, a group of oniscidean isopod troglofauna were ascribed as members of the family Platyarthridae (genus *Trichorhina*), but remained enigmatically defined. Moreover, all Australian related surface species collected during environmental assessment projects were commonly attributed to Platyarthridae. This family was previously shown to be a paraphyletic taxon, together with Scyphacidae, Philosciidae, Dubioniscidae, Trachelipodidae and Porcellionidae, on the basis of a morphological-based phylogeny (Schmidt, 2002). Mattern (2003) also showed using phylogenetic analyses of the *18S* gene from Trachelipodidae and Platyarthridae (just two species included, *Trichorhina tomentose* and *Platyarthrus schoebli*) that the genera *Porcellio* and *Porcellionides* were not monophyletic. Despite the results of these phylogenetic analyses, no decisions were made to resolve the classification of Platyarthridae, probably owing to insufficient morphological evidence and the need for incorporation of more platyarthrid taxa and molecular markers. For the first time here, multiple gene phylogenies (*COI, 18S*, *28S*) and a detailed morphological assessment using Scanning Electron Microscopy (SEM), were used to test the monophyly of the family Platyarthridae. The phylogenetic

analyses with inclusion of several Australian taxa attributed to *Trichorhina* and species of *Trichorhina*, *Platyarthrus* and *Niambia* from South America, Africa and Europe, as the main representatives of Platyarthridae, showed that the family is polyphyletic, comprising multiple divergent clades which are distinct from non-platyarthrid families. The molecular phylogenies revealed the occurrence of a very distinct clade, previously believed to belong to the genus *Trichorhina*, from Australia and South America. As a result, with respect to the robust phylogenetic position of this clade within Oniscidea and the accompanying morphological data, a new family Paraplatyarthridae, occurring in the subtropical and arid regions of Australia and South America, was erected, which is entirely distinguishable from other platyarthrid species using the new characters revealed as part of this study.

My thesis also contributed to the molecular systematics and elucidation of oniscidean isopod species diversity in 12 groundwater calcretes of central Western Australia. To assess species diversity and phylogenetic relationships of the oniscidean troglofauna, four molecular markers including one mitochondrial (*COI*) and three nuclear genes were analysed. The latter included two ribosomal (*18S* and *28S*) and one protein coding gene, *LysRS,* developed for the first time in isopod species using the Illumina next generation sequencing technique. The mitochondrial lineages, delimited using the PTP, GMYC models, and a 12% nucleotide sequence threshold for species delimitation, showed the existence of 28 putative taxa belonging to four oniscidean families; Paraplatyarthridae, Philosciidae, Armadillidae and Stenoniscidae which most likely represent distinct species, new to science. This study also revealed that the majority of the oniscidean lineages were restricted to individual calcrete aquifers, supporting the “subterranean island” hypothesis. Most of the taxa, therefore, showed short-range endemism, with the exception of three paraplatyarthrid troglophile species found to occur in multiple calcretes. The occurrence of two littoral stenoniscid lineages in the Laverton Downs calcrete suggested a faunal connection with the marine inundation of the Eucla Basin during the Late Eocene, in accordance with the available geological data. Together with the presence of subtropical and benthic species my results indicate that a variety of distinct historical events have shaped the evolution of the oniscidean fauna in the groundwater calcretes. Interestingly, the stenoniscid populations were only recorded in the Laverton Downs calcrete and were not found further north and

west in central Western Australia, further supporting its connection with the Eucla Basin marine transgressions.

My study also examined the phylogenetic relationships and biogeographic history of the genus *Paraplatyarthrus* at a broader scale from groundwater calcretes in arid central Western Australia with taxa from some temperate terrestrial regions of Western Australia. Relaxed molecular clock analyses of the combined mitochondrial-nuclear gene sequence data (*COI-LysRS*) revealed that evolutionary transitions to subterranean habitats most likely took place after the late Miocene, following the onset of aridity on the Australian continent, thus supporting the climatic relict model of evolution of subterranean animals (Barr & Holsinger, 1985; Holsinger, 2000). However, the molecular data and field collections showing the presence of widespread troglophilic lineages, and historical evidence for the resurgence of the wet and warm environmental conditions during the early Pliocene and Pleistocene in Australia, also lend support to the feasibility of the adaptive shift model (Howarth, 1981, 1987; Peck & Finston, 1993; Holsinger, 2000). Overall, both models are likely to provide an explanation of the evolution and diversification of the paraplatyarthrid troglofauna in groundwater calcretes of central Western Australia. In addition, the associated phylogenetic analyses, together with morphological evidence, showed that the groundwater calcretes were likely to have been independently colonised by troglophile ancestors; i.e. isopods pre-adapted to living under ground, but capable of surviving on the surface when conditions were favourable (Trajano & Cobolli, 2012).

Finally, in my last data chapter, I described five new species of the genus *Paraplatyarthrus* using morphological data, and a key to facilitate their identification. These were also shown to be very divergent clades using molecular evidence (Chapter 3).

## FUTURE RESEARCH DIRECTIONS

As discussed, the multiple-gene phylogeny of the oniscidean higher taxa together with morphological evidence revealed that the family Platyarthridae was polyphyletic, containing highly divergent taxa which were previously classified within the same family owing to morphologies now interpreted as convergent. Moreover, the molecular phylogenies showed

that even the well-established platyarthrid taxa, including *T. tomentosa*, *P. hoffmannseggii* and *Niambia* species, did not form a monophyletic group relative to other oniscidean families that were included in the analyses. The systematic status and phylogenetic relationships of the other platyarthrid genera, including *Cephaloniscus*, *Echinochaetus*, *Lanceochaetus* and *Gerufa*, are also unclear. Their inclusion in a more comprehensive phylogeny was beyond the capacity of this project, due to the limitations of accessing exemplar specimens from Africa and Malaysia, but this will be necessary in the future to assess their affinity to the new family and *Trichorhina* (as the representative of Platyarthridae), *Niambia* and *Platyarthrus* taxa, in a more comprehensive way.

With respect to the newly defined diagnostic morphological characters, the paraplatyarthrid species are easily distinguishable from "Platyarthrid" taxa, and accordingly these can be used in museum collections for correct identification and species assignment. Up until now, two species of *Trichorhina* including *T. australiensis* from Western Australia, *T. tropicalis* from Queensland and a few undescribed species from Lord Howe Island and the Northern Territory, have been attributed to Platyarthridae; these need to be examined in future studies. In the current research, despite numerous collections conducted in the groundwater calcretes, no *Trichorhina* species has been found, but these calcretes comprise a significant diversity of paraplatyarthrid species. Additional morphological examinations of the Western Museum collection, which had been attributed to *Trichorhina*, showed that none of the specimens actually belonged to this genus. As a result, the occurrence of *Trichorhina* in Australia is uncertain and identification of platyarthrid taxa commonly ascribed to *Trichorhina* worldwide, mostly from South America and Africa, should be treated with caution, considering the relationships outlined here.

Despite both molecular and morphological evidence for the presence of a South American paraplatyarthrid taxon, I did not describe this species as all individuals were female. With respect to its high level of molecular divergence relative to the Australian taxa, and with supportive morphological evidence, this species may very well also represent a distinct genus.

The diversity (28 species) of oniscidean troglofauna, assessed using molecular phylogenetic analyses and morphological data in the current study, was only from 12

calcrete aquifers associated with three palaeodrainages, whereas there are approximately 200 major calcrete aquifers in central Western Australia. This implies that the number of oniscidean species, currently undescribed, in this region could be as many as 400 species. In future a more comprehensive sampling of calcrete aquifers needs to be carried out to estimate the level of oniscidean diversity across the whole region. Moreover, the collecting method used, involving trog-traps with litter, may have under-estimated the actual diversity in the calcrete aquifers. For instance, in several cases the traps had been drowned beneath the water table due to seasonal groundwater fluctuations so, I recommend using modified trog-traps to resolve the problem.

So far, Stenoniscidae in Australia has only been recorded from the Laverton Downs calcrete and its distribution pattern in other parts of Australia is poorly known. Although the occurrence of this littoral stenoniscid species at Laverton Downs may be linked to the marine inundation of the Eucla basin in concordance with geological evidence, further confirmation of this hypothesis is required using molecular clock analyses including additional species of stenoniscids from coastal Australia. According to this hypothesis, stenoniscid populations should still be present in coastal regions of the Eucla basin, so future samplings should aim to target this region. In addition, the generic classification of Stenoniscidae in Australia is still unknown. Two genera of Stenoniscidae including *Stenoniscus* from North America and Europe, and *Metastenoniscus* from South America (Venezuela) and some Indian Ocean Islands such as Bali have been identified. Taiti and Humphreys (2008) stated that the Australian stenoniscids (Laverton Downs) are more related to *Metastenoniscus*. However, to elucidate the affinity of the Australian populations in terms of their generic level status, a molecular phylogenetic approach is required, accompanied by a morphological examination of museum collections, to resolve the systematics of stenoniscid isopods from Australia.

Molecular divergence and phylogenetic analyses conducted to investigate biogeographic patterns and evolution of the paraplatyarthrid troglofauna supported both the CRH and ASH models. Although the adaptive shift model was supported by the data here, owing to the evidence for dispersal from the hypogean habitats (groundwater calcretes) to surface environments, presumably during favourable conditions (i.e. wet periods), active colonisation of hypogean environments directly by epigean ancestors and parapatric speciation, is still a potential alternative for the evolution of the fauna. The supporting

evidence is the occurrence of a single surface species from Mt Morgans and its sister relationship with the troglophile taxon 13 (Nambi, Halfpenny, Laverton Downs, Windarra), which accordingly reinforces the likelihood that we have not adequately surveyed the whole region for possible surviving related surface species. Therefore, a more comprehensive sampling is necessary to further determine whether the ASH model is a potential alternative hypothesis for the evolution of paraplatyarthrid troglobites. Moreover, with respect to the dispersal ability of some troglophiles to move between calcretes and possible hybridisations between troglophile-troglobite species, we recommend detailed molecular analyses should be carried out to test for the potential of hybridisation events and gene flow between troglophile and troglobite sister taxa.

### *CONSERVATION AND MANAGEMENT*

Studies over the last two decades in Australia have shown that invertebrate subterranean faunas, including both troglofauna and stygofauna, are significant components of underground environments such as non-karstic fractured rock terrains in the Pilbara and groundwater calcretes in arid central Western Australia (Watts & Humphreys, 1999, 2006, 2009; Taiti & Humphreys, 2001; Karanovic & Marmonier, 2002; Leys *et al.* 2003; Karanovic, 2004; Cho, 2005; Humphreys, 2006; Leys & Watts, 2008; Cooper *et al.* 2007, 2008; Guzik *et al.* 2008, 2009; Humphreys *et al.* 2009; Eberhard *et al.* 2009; Karanovic & Cooper, 2012; King *et al.* 2012). My study also showed a significant diversity of undescribed oniscidean troglofauna in terrestrial parts of the groundwater calcretes and the majority of them were found to be restricted to individual calcrete aquifers and could be classified as short-range endemics. These data not only inform the conservation management of the fauna uncovered, but also assist with the environmental review process for industrial developments and water utilisation associated with the calcrete aquifers in the region. As the isopod troglofauna are strictly dependent on soil humidity associated with the groundwater so, in conservation planning, environmental monitoring and groundwater assessments, their management should be simultaneously linked to the management of aquifers and the associated aquatic fauna. Moreover, for sustainable management of the fauna, it has been proposed that surface waters and the associated groundwater aquifers should be managed together using a whole-of-resource approach (DLWC, 2002).

The major threats from the increasing mining activities in Western Australia to subterranean fauna in calcrete aquifers, which may potentially lead to a reduction in biodiversity or even their extinction, include contamination and changes to the water table such as dewatering, water abstraction and water injection. For example, Bennelongia (2010) estimated that the injection of unused water by Iron Ore mining activities in the Pilbara below the water table at sites away from mine pits may result in the mounding of the water table and loss of up to 19% of the troglofauna habitat within the injection area. In calcrete aquifers of central Western Australia, where short-range endemicity of troglofauna is a common pattern, environmental assessments of water-related industrial activities/utilisation should seriously consider vulnerability of the fauna. In addition, as these calcretes are in fact shallow aquifer deposits, any mis-calculations of water usage regimes may cause serious damage to the extant biodiversity or even extinction of the whole local fauna.

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