Modes of Speciation in Subterranean Diving Beetles from a Single Calcrete Aquifer in Central Western Australia

Paroster macrosturtensis (Dytiscidae)

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This thesis is dedicated to my children

Nikita and Jack

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ABSTRACT

Calcrete aquifers from the Yilgarn region in central Western Australia's arid zone contain a highly diverse range of obligate groundwater invertebrate species (stygofauna), with many endemic to single calcretes. Phylogenetic studies on the diving beetles from the Yilgarn calcretes suggest a scenario of invasion of the subterranean environment by several surface dwelling ancestors during aridification of the region since the Miocene. This system is ideal for examining modes of speciation within a closed calcrete body and in particular the relative roles of allopatric, parapatric and sympatric speciation in the generation of diving beetle (Dytiscidae) species diversity. Previous phylogenetic analyses have identified 13 independent cases of sympatric sister species pairs of large and small diving beetles in separate calcretes, suggesting the potential for their speciation in sympatry as a result of ecological niche partitioning. A single calcrete at Sturt Meadows contains a sympatric sister triplet of large and small diving beetles (*Paroster macrosturtensis, P. mesosturtensis, P microsturtensis*), and can be accessed by an extensive grid of mineral exploration bores (3.5km^2, 115 bores), allowing intensive sampling for population genetic studies and biodiversity assessment. Comparative phylogeographic analyses by others on these *Paroster* beetle species found no evidence for long term allopatric processes operating within the calcrete, although any fragmentation event that could have led to the evolution of the three beetle species may not have persisted post-speciation, and thus would not been seen in their gene genealogies.

The aim of this study was to investigate the possibility that the three beetle species at Sturt Meadows may have evolved by sympatric speciation due to trophic niche partitioning. Two main approaches were used to achieve this aim. First, whether the different beetle species have different trophic niches was determined and, second, whether micro-allopatric processes, such as fragmentation events, may have led to the evolution of the three beetle species was investigated. To detect evidence for such fragmentation events, a comparative phylogeography of chiltoniid amphipods in the Sturt Meadows calcrete was undertaken.

A DNA barcoding framework was established for the macro-invertebrates in the Sturt Meadows calcrete, in order to obtain sequence information on potential prey groups for the diving beetles. A 623-bp fragment of the mitochondrial cytochrome *c* oxidase 1 (*COI*) gene was amplified from stygofauna plus terrestrial organisms that were found in the calcrete. Phylogenetic analyses revealed the existence of 12 divergent monophyletic groups of haplotypes, including three unrelated lineages of chiltoniid amphipod that are

morphologically cryptic. Allozyme analyses on the amphipods showed them to be three species that can be separated at multiple allozyme loci.

Spatial variation in genetic diversity was assessed for the chiltoniid amphipods, enabling a comparative phylogeography of the three species across the bore field. There was no common pattern in the gene trees of the amphipod species, so no evidence for three isolation events in the diversification of the diving beetles at this site. However, high haplotype diversity coupled with low nucleotide diversity, plus signatures of population expansion and isolation by distance in two out of three species, match previous findings for diving beetles at the same site and indicate the potential for micro-allopatric evolutionary processes to have operated within the calcrete. Isolation of populations in pockets of favourable habitat (refugia) within the calcrete followed by expansion events, are proposed as the most likely generator of population genetic diversity, and are thought to be related to water level changes in the aquifer.

Trophic niche partitioning in the sister triplet of large and small *Paroster* diving beetles and their larvae was investigated by molecular amplification of small fragments of the *COI* gene identified by the barcode. Amphipods (Chiltoniidae) and copepods (Harpacticoida, Cyclopoidea) were chosen for the analysis as they were the most abundant potential prey items in the calcrete. There was not complete trophic partitioning in the adult beetles, with all three species feeding on amphipods and copepods. As the trophic analysis was molecular, differences in size of the prey were not tested for. There was some evidence for preferential feeding on particular prey species by the adult beetle species, however, small sample sizes precluded making comparisons between their larvae. It is thought that in the impoverished environment of the aquifer, the adult beetles are scavengers and opportunistic feeders, as well as active predators. Stable isotope analysis confirmed that the three diving beetle species are feeding on similar food items, and indicated a separate source of carbon and nitrogen to the tree roots as the basis of the food web of the calcrete.

In summary, there was no evidence for complete trophic niche partitioning in the adult diving beetles of different sizes that could have led to their speciation in sympatry. Any further investigation of trophic differences needs to concentrate on preferential feeding in the adults, and the trophic niche of the beetle larvae which are active predators. The potential for micro-allopatry in the diversification of the different sized diving beetles at Sturt Meadows has been identified, through congruence in current population genetic patterns for the amphipods and the diving beetles. Stygofauna in the calcrete have high genetic diversity, which is thought to be the result of historical water level fluctuations leading to frequent population bottlenecks, followed by range expansion after aquifer recharge. Identification of

at least 12 macro-invertebrate species in a single calcrete increases the total number of stygobytic and troglobytic species discovered in the Yilgarn, and has enabled estimates to be made of possible numbers of subterranean species in the region still to be discovered.

The presence of large and small dytiscid beetles in multiple calcretes that are sympatric sister species still points to some common ecological niche differentiation within the calcrete environment. It is hypothesized that there could be depth partitioning in the different sized diving beetles related to their oxygen requirements. The population genetic and ecological data generated at Sturt Meadows provide a baseline for the Yilgarn calcretes. Extending such a study to examine spatial and ecological differentiation in sympatric sister species of subterranean diving beetles across multiple calcretes, would be a powerful approach in the investigation of modes of speciation.

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CHAPTER II

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CHAPTER III

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CHAPTER V

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GENERAL INTRODUCTION

Sympatric sister species of subterranean diving beetles from the Sturt Meadows calcrete, in non-overlapping size classes; From left to right, *P. microsturtensis* (1.8 mm), *P. mesosturtensis* (2 mm), *P. macrosturtensis* (4 mm). Picture by Chris Watts.

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CHAPTER I

In this chapter I will introduce the topic of speciation, with particular reference to evidence of the process of sympatric speciation. All other literature review topics, including DNA barcoding, phylogeography and predator-prey studies, are covered in the results chapters.

Subterranean diving beetles from the Yilgarn calcretes

Phylogenetic studies employing mitochondrial DNA on the diving beetles (Dytiscidae) from the Yilgarn calcrete aquifers in Western Australia, showed that the stygobiontic beetles evolved following an invasion of the subterranean environment by several surface dwelling ancestors during a period of aridification 5-10 million years ago (Cooper *et al.*, 2002; Leys *et al.*, 2003). As surface waters have dried out speciation is thought to have occurred due to the isolation of calcretes, with the majority of species evolving due to allopatric speciation. The result was a remarkable number of new species of diving beetles that contribute to a biodiversity hotspot of groundwater invertebrates living under the desert (Watts & Humphreys, 1999, 2000, 2001, 2003, 2004, 2006, 2009). Within individual calcretes a pattern has been observed of large and small sister species of diving beetles, which has been repeated at least 13 times (Cooper *et al.*, 2002; Leys *et al.*, 2003; Leys & Watts, 2008). Speciation into the different size classes appears to have occurred in the isolated habitat of the calcrete and the number of independent replicates makes comparative analyses for testing hypotheses on the modes of speciation possible.

Speciation

Mayr's (1963) biological species concept introduced the idea of species as reproductively isolated groups. „Good" species rarely interbreed, even when overlapping in their distribution, i.e. when they are sympatric (Maynard Smith, 1966). Coyne and Orr (2004) extended the biological definition of a species to include the possibility of some gene flow between closely related species. They describe the process of speciation as the evolution of reproductive isolating barriers.

Understanding how speciation occurs remains a fundamental problem in biology (Dieckmann & Doebeli, 1999) and, from this emerges a central question: How does the

evolutionary process result in discrete groups of organisms living together in nature, rather than an organic continuum (Coyne & Orr, 2004)? Proposed modes of speciation include allopatric, parapatric and sympatric divergence. It is thought that many species have arisen through allopatric speciation, where an ancestral species is divided by a geographical barrier (Mayr, 1963; Coyne & Orr, 2004). Natural selection or genetic drift, acting on the two or more populations, then produces reproductive isolating barriers over time. Parapatric speciation also infers separation by distance, with species divergence occurring under conditions of limited gene flow, often just at a boundary between two sub-populations that may have diverged initially in allopatry (Coyne & Orr, 2004).

More difficult to show is evidence for sympatric speciation, where new species are formed within a freely interbreeding population. The process of sympatric speciation involves the evolution of reproductive isolation without a barrier caused by geography, but due to the biology of the organism (Maynard Smith, 1966). Speciation is then driven by selection for ecological divergence (genetic, morphological or behavioural differences), and sexual selection for mates belonging to the same group. The difficulty with the concept of sympatric speciation is that if selection is causing a population to split into two, recombination will presumably breakdown the gene complexes that could produce reproductive isolation (Felsenstein, 1981). In order to reduce recombination events, preferential (assortative) mating with like individuals, or close linkage of genes involved in reproductive isolation with genes under selection is hypothesized (Maynard Smith, 1966; Tregenza & Butlin, 1999).

Within a population under natural selection, incipient species must differ genetically in their preference for niches and their ability to survive in these niches, with certain alleles allowing individuals to have a selective advantage (Maynard Smith, 1966). Under disruptive natural selection, intermediate phenotypes will be disadvantaged, leading to the formation of two distinct ecotypes (Darwin, 1859). For a stable polymorphism in a population occupying two niches to result in speciation, reproductive isolation must occur. Assortative mating, with individuals mating preferentially with those that choose the same niche, is a mechanism for reproductive isolation based on habitat (Maynard Smith, 1966). Organisms that mate on or near their preferred resource are thought to be more likely to undergo sympatric speciation (Maynard Smith, 1966; Coyne & Orr, 2004).

Support for ecologically driven sympatric speciation has come from theoretical models (Dieckmann & Doebeli, 1999; Higashi *et al.*, 1999; Kondrashov & Kondrashov, 1999; Gavrilets, 2004). Mathematical models of sympatric speciation do produce reproductive isolation in potentially interbreeding populations, with favourable conditions being strong

disruptive ecological selection, strong mate preference, low cost to being choosy, low recombination between mating and ecological loci, and high mutation rates (Gavrilets, 2004). Sympatric speciation is thought to be most likely to occur on colonisation of new, empty habitats (Tregenza & Butlin, 1999). The difficulty is in determining how often the conditions modelled by theorists occur in nature (Bolnick & Fitzpatrick, 2007; Gavrilets & Vose, 2007; Gavrilets *et al.*, 2007).

Empirical evidence of the process of sympatric speciation comes from cases of small monophyletic groups of sympatric sister species in isolated habitats (Bolnick & Fitzpatrick, 2007; Fitzpatrick *et al.*, 2009).). Populations can be considered sympatric when individuals can encounter one another with moderately high frequency, with ecologically separated populations being sympatric if there is contact along ecotones (Mallet *et al.*, 2009). Isolated habitats may be oceanic islands, as in the case of the *Howea* palms endemic to Lord Howe Island (Savolainen *et al.*, 2006), or crater lakes as for cichlid fish species in Nicaragua (Barluenga *et al.*, 2006a) and the Cameroon (Schliewen *et al.*, 1994; Schliewen & Klee, 2004). The host for a parasite also constitutes an isolated habitat, with host switching thought to be the cause of sympatric speciation in brood-parasitic indigobirds (Sorenson *et al.*, 2003), and sympatric sister species of phytophagous insects (Berlocher & Feder, 2002).

Coyne and Orr (2004) state that ruling out an allopatric phase in the biogeographic and evolutionary history is critical when proposing sympatric speciation. Savolainen *et al.* (2006) found when examining DNA - based phylogenies for the two sister species of *Howea* palms endemic to Lord Howe Island, that the species diverged approximately 1-2 mya, well after the island was formed. The distribution of the sister species overlap by 20% and, as they are wind-pollinated, it is considered to be unlikely that they have ever been separated by distance. Genetic structuring is low in both species of *Howea*, and spatial separation is thought to have had a limited role in their speciation ie. they are proposed as a case for sympatric speciation (Babik *et al.* 2009). The two species differ in flowering time and soil pH preference, traits which are proposed to have evolved due to disruptive natural selection acting on the ancestral *Howea* (Savolainen *et al.*, 2006; Gavrilets & Vose, 2007). Such speciation can be proposed as sympatric, as long as there has been no environmental effect on the onset of flowering time (Gavrilets & Vose, 2007), and no occasional migrants can reach the island from the mainland (Butlin *et al.*, 2008). Evidence of reproductive isolation between the two species comes from the finding that potential hybrids between the two species of palm were very rare (Savolainen *et al.*, 2006; Babik *et al.*, 2009).

Barluenga, *et al.* (2006a) also hypothesised disruptive natural selection as the driving force in the speciation of two species of Midas cichlids from the 5 km wide volcanic crater,

Lake Apoyo in Nicaragua. They found that the species have different habitat preferences with one limnetic and one benthic form, and subsequently occupy different trophic niches. Comparison of their mitochondrial DNA showed evolution of the limnetic form from the benthic form less than 10 000 years ago, a time frame that is supported by a mathematical model of the system (Gavrilets *et al.*, 2007). Evidence for reproductive isolation comes from laboratory experiments where the two species have been found to mate assortatively (Baylis, 1976), and it is thought that differences in courtship behaviour now cause their reproductive isolation. An alternate scenario of multiple colonization and hybridization was proposed by Schliewen *et al.* (2006), however, it was rejected as incompatible with the molecular evidence (Barluenga *et al.*, 2006b; Gavrilets *et al.*, 2007).

An alternative method for assessing the prevalence of sympatric speciation is by age-range correlation (Barraclough & Vogler, 2000). Fitzpatrick and Turelli (2006) used association of phylogenetic relationship and geographic range overlap to examine geographic modes of speciation in 14 different mammal clades. Allopatric species are expected to have no initial range overlap with some intersection of ranges occurring with time. Sympatric speciation is proposed if there is 100% initial range overlap, which would diminish with time since divergence. For most of the sister clades there was no consistent correlation between geographic range overlap and time since divergence, which could be due to species ranges having altered too rapidly during their evolutionary history (Fitzpatrick & Turelli, 2006). The authors concluded that even with the limitations of their age-range correlation technique, there was a lack of evidence for general allopatric divergence in these groups of mammals, and that a mixture of parapatric and sympatric modes of speciation are a possibility.

Although studies that propose sympatric speciation in nature all have their critics, it appears to be the best hypothesis for origin of the *Howea* palms and cichlid fish, whose habitats are isolated from multiple invasions and where mobility within the habitat is not restricted. The question now is how common is sympatric speciation in nature, and what is the role of natural selection and biology of the organism in divergence (Bolnick & Fitzpatrick, 2007)? As more research accumulates on geographic patterns, it is clear that categorising speciation as allopatric, parapatric or sympatric is a simplistic view of a continuum, with complete allopatry and sympatry at the extremes, and parapatric speciation the most likely scenario (Butlin *et al.*, 2008; Fitzpatrick *et al.*, 2009; Mallet *et al.*, 2009). However, such a geographic (spatial) framework is useful in explaining patterns in nature (Coyne & Orr, 2004; Mallet *et al.*, 2009). Fitzpatrick *et al.* (2009) propose that only by including ecological and genetic factors affecting divergence, will it be possible to understand the evolutionary processes behind these patterns and the prevalence of sympatric speciation.

Sympatric sister species from isolated habitats such as caves, where range shifts and secondary contacts are unlikely, are interesting cases in the investigation of the prevalence of sympatric speciation (Bolnick & Fitzpatrick, 2007). Coyne and Orr (2004) state that for the process of sympatric speciation to be proposed, species must be sympatric sister species, that is overlapping in their distribution and reproductively isolated, with no period of allopatry in their biogeographic and evolutionary history. The aim of the present study has been to investigate evidence for sympatric speciation, based on the criteria of Coyne and Orr (2004), in subterranean diving beetles from the Yilgarn calcretes of central Western Australia. The importance in speciation of the diving beetles of natural selection and biology of the organism has also been investigated.

The Sturt Meadows calcrete as a model system for studying modes of speciation

Details of the field site at Sturt Meadows, including structure of the calcrete, are presented in Chapter II. This calcrete aquifer contains a sister triplet of diving beetles in separate size classes (*Paroster. microsturtensis*, *P. mesosturtensis*, *P. macrosturtensis* - see title photo, page 1) that overlap in their distribution, raising the potential that sympatric speciation has occurred in this system. Two approaches have been undertaken in order to investigate modes of speciation in these diving beetles from the Sturt Meadows calcrete. First, by investigating the trophic niche occupied by the large and small beetles, the possibility of trophic partitioning as a driving force in beetle speciation was assessed. Second, by comparing phylogeographic structure between amphipod species that are sympatric with the diving beetles, the possibility that fragmentation of populations or micro-allopatry has driven the diversification of the beetle fauna was investigated, with the simplest hypothesis being that there were two isolation events in their evolutionary history.

Project AIMS addressed in separate results chapters

Each of the results chapters have been written as stand-alone papers, with separate introduction, methods, results and discussion. Chapter III is already published and the order that the data is presented follows that of the published paper (see Appendix IV). These three results chapters address the three aims of the study listed below.

Aim 1 Establish a DNA barcode of species present to enable identification of potential diving beetle prey items (Chapter III)

Aim 2 Compare the phylogeographic structure of three amphipod species, and determine whether there are congruent patterns between stygofauna indicative of historical geographic barriers to gene flow, and/or congruent population genetic patterns between stygofauna indicative of micro-allopatric processes (Chapter IV)

Aim 3 Investigate whether different sized beetle sister species occupy separate trophic niches within a calcrete, by molecular amplification of gut contents with species specific primers (Chapter V)

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GEOLOGY OF THE STURT MEADOWS CALCRETE DEPOSIT

Erosion zone, showing the soil profile and calcrete (whitish rock) at Sturt Meadows, Yilgarn Region, central Western Australia

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CHAPTER II

This study, involving a detailed biodiversity assessment and fine scale population genetic analyses of stygofauna, was possible due to the presence of a grid of mineral exploration bores at the Sturt Meadows pastoral property, which enabled access to the calcrete. This chapter presents what has been undertaken to map the calcrete deposit at Sturt Meadows, and what can be determined of the structure of the sub-surface environment. The only exposed area at this site is the erosion zone (see title photo, page 13), so knowledge of the structure of the calcrete environment in which the stygofauna live comes from lithology of the drill cores. This data was identified after the study was complete, and the aim in summarizing the drill core information was to explain the spatial biological patterns of the amphipod fauna presented in Chapter IV, to better infer the underlying processes of speciation. As no imaging of the pore sizes and connectivity within the calcrete was available, the system was treated as an area where actual distance between sampling points in the bore grid is unknown, and thus *a priori* predictions about population structure were not made.

Exploration of the Sturt Meadows calcrete in Western Australia by Anaconda Nickel Limited

In 2000-2001 Anaconda Nickel Limited tested a calcrete deposit located on Old Station Well exploration lease area E37/645 in Western Australia, latitude 28˚ 42´ S and longitude 120˚ 53´ E (Fig. 1A and B). This deposit lies within a broad ephemeral drainage approximately 7 km north of the playa Lake Raeside, straddling an east-west track known as the Ida Valley Road, and approximately 9 km west of the Sturt Meadows homestead (Fig. 1C).

Calcretes of Tertiary age or younger appear on the LEONORA SH 51-01 1:250 000 geological map sheet produced by AGSO – Geoscience Australia (Dept. of Industry, Science and Resources, Canberra) in 2001. The closest calcretes, with the rock code Czk, to the Sturt Meadow‘s deposit shown on the surface mapping are over 85 km away to the north-east and north-west, and there is no connecting surface water. On 29 Nov. 2000, Anaconda Nickel Limited produced a map of the calcrete resources in the region that was included as Figure 8 in their environmental impact statement for the Mt Margaret Nickel-Cobalt project. This shows extensive calcretes in the main watercourse that includes the Sturt Meadows area. The subsequent drill grid was located adjacent to, but not on some of these mapped occurrences. Further regional mapping at a scale of 1:4 500 000 was produced for Dr W. F. Humphreys by G. Blake of the Western Australian Department of Water on 26 April 2006 – project code 21212. Although none of the mapping has produced an accurate definition of the extent of

the Sturt Meadows calcrete, with the extent of the deposit determined from satellite images and not from ground mapping, approximate size and shape has been taken from the regional map (project code 21212) for use in this study (Chapters III and IV).

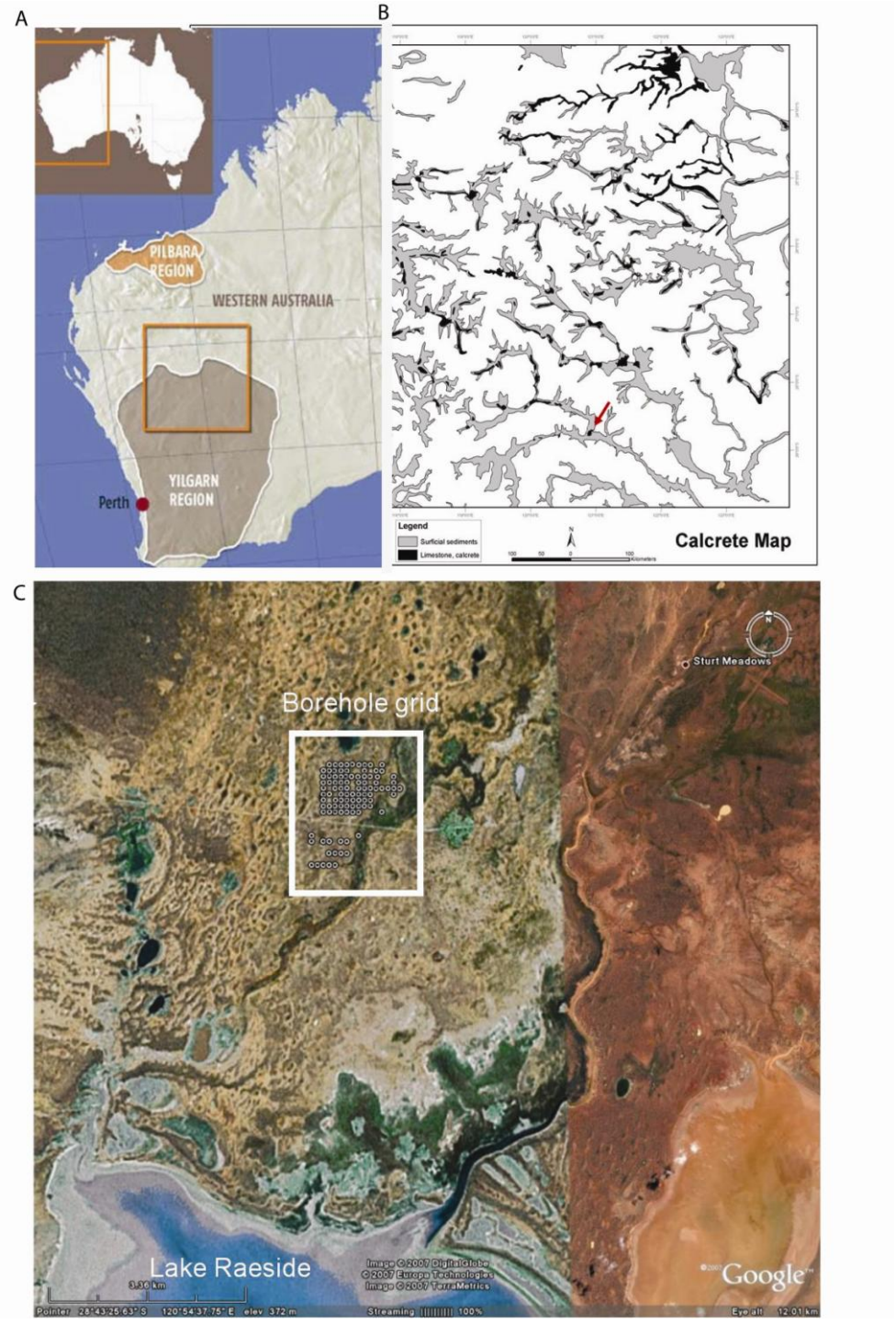


Fig. 1 Locality map showing the Sturt Meadows calcrete.
A. Location of major palaeodrainage and calcrete study area over the northern Yilgarn region of Western Australia. **B.** Interpreted detail of palaeodrainage and calcrete within study area. Red arrow marks the location of the Sturt Meadows calcrete. **C.** Satellite image showing location of mineral exploration bore field drilled into the Sturt Meadows calcrete by Anaconda Nickel Limited.

In May 2002 Anaconda produced Technical Report 1252 for the period 3 December 2000 to 2 December 2001. This contains geological details obtained from collecting and testing samples over 1 m depth intervals from 160 reverse circulation percussion drill holes SMRC 0001-0160. Of these, 129 were drilled to 11 m, 20 to 5 m and the remaining 11 from 8-15 m for a total of 1656 m. Fifty-one bores were drilled in a grid south of the Ida Valley Road and 109 in a grid to the north. Layout of the bore hole grid, with bores 100 m apart along rows, is shown in Figure 2. Approximately 2 kg sub-samples were collected via a two-tier riffle-splitter from each metre of drilling. The samples were dispatched to Ultratrace laboratories in Perth and analysed for a suite of elements by the inductively-coupled plasma optical emission spectroscopy (ICPOES) method. Elements analysed were calcium, magnesium, iron, aluminium, silica, copper, chromium, arsenic, manganese, zinc, nickel, cobalt, uranium and thorium. Results are shown on cross section plans TR9670xIDWCa001-004 (Appendix 1 of Technical Report 1252) for calcium, magnesium, silica, aluminium and iron.

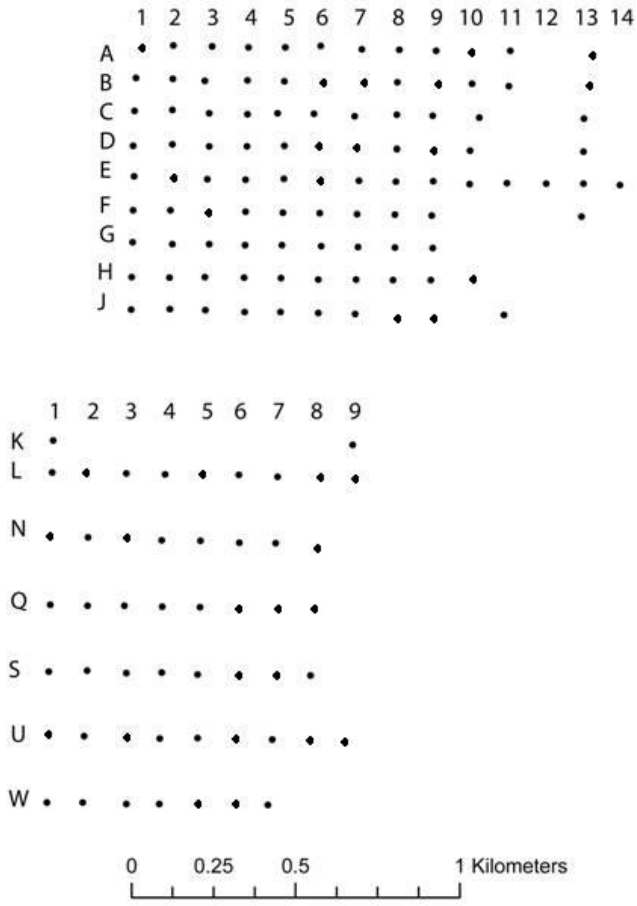


Fig 2. Layout of the bore holes at Sturt Meadows. Rows are identified by a letter, and holes in each row by a number.

Lithology derived from the drill core logs

The lithologies identified from the drill samples follow and expand on those shown in a Schematic Cross Section of a Calcrete Profile (Fig. 5, Mt Margaret Nickel-Cobalt Project PER). This shows topsoil over vadose calcrete (Czk) which extends from the top of the ground surface to the water table, overlying phreatic calcrete (Czks) that contains the groundwater, over a clay/sand aquifer. Drilling at the Sturt Meadows site shows topsoil and clay over part of the drill grid areas, as well as extensive areas where Czk is dominant. Topsoil, where recorded, is generally only 1-2 m thick. The surface clay is from 1 m with occasional holes showing it is up to 4 m thick. Holes with substantial clay content were generally drilled only to a depth of 5 m. Czk has a calcium content in the range 17-25%. Czks, with a calcium range of 25-35%, were identified as underlying Czk and generally overlying clay. Apart from the calcium content, the difference between vadose and phreatic calcrete is that vadose calcrete is wet but not saturated and phreatic calcrete is below the water table. The combined calcrete thickness often reaches and may well exceed the common drill depth of 11 m. The clays often contain 15-20% calcium and have, in places, been included in the tonnage calculations for the overall calcium resource. There are also a number of samples where Czk and Czks are mixed with clay. Czk has been identified to a depth of 13 m. Czk has also been identified as intermixing with Czks, generally with Czks becoming dominant with depth. Clays also occur intermixed with the calcretes throughout the sequence in some drill holes. Silica occurs in some holes within Czks. Granite is found at the bottom of drill hole D13.

Drill samples show that the holes recorded as dry along the eastern side of the grid contain significant quantities of clay, sometimes mixed with calcrete. This clay essentially runs parallel to the modern drainage feature that forms the eastern boundary of the grid. Due to the number of holes that only go to 5 m, it is not clear whether the clay forms a complete barrier at depth between the gridded area and any calcretes to the east of this drainage feature. North of the Ida Valley road, drill holes at the western extremity of the grid show considerable clay and intermixing of the calcretes. This may indicate that there is another watercourse feature along the western boundary that is now covered by sand dunes encroaching from the west.

A schematic cross section for grid row J (Fig. 3) has been compiled from the lithologies provided on resource section plan TR9670xIDWCa002 (Appendix 1 of Technical

Report 1252). It shows phreatic calcrete, underlying vadose calcrete, with clay pockets and a clay barrier to the east.

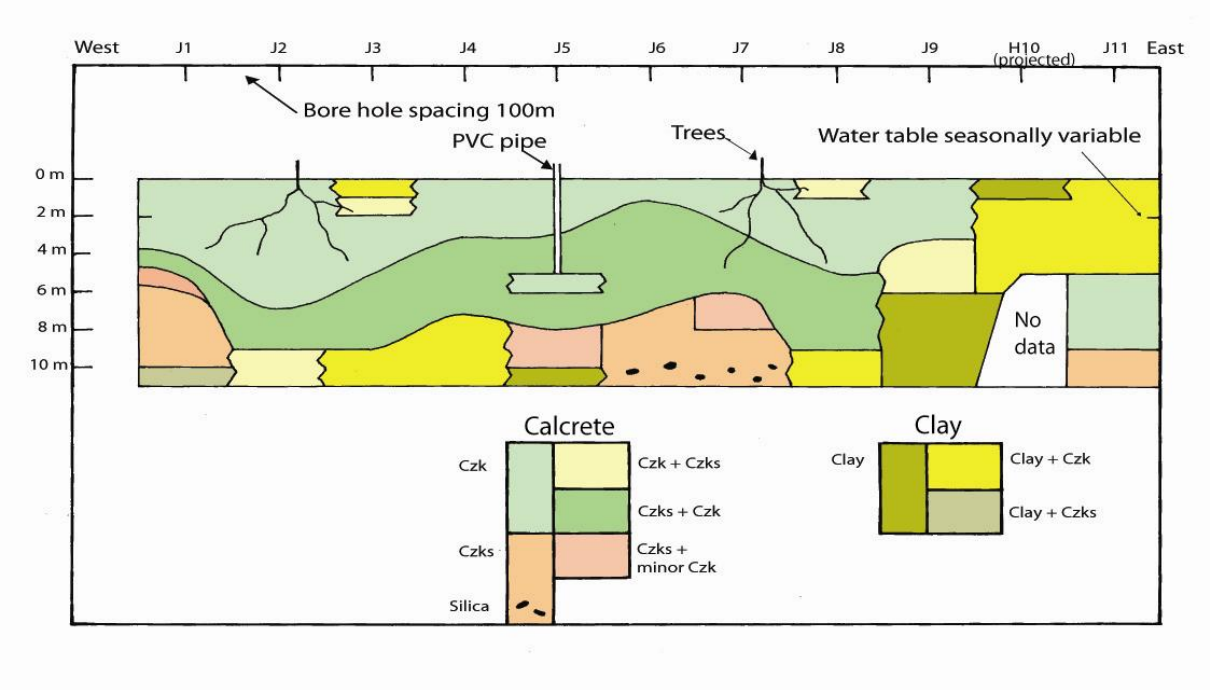


Fig. 3 Diagrammatic cross-section of the Sturt Meadows calcrete along bore hole gridline J. Czk, vadose calcrete; Czks phreatic calcrete.

A system of classification for the lithologies in each drill hole has been devised for this study (Table 1), in order to establish if variations in the underwater geological environment show any correlation with different groupings of species observed (see Chapter IV). It is unlikely that localized surface occurrences of soil and clay that do not extend below the water table at approximately 2 m will have any significant influence on the underwater environment. Hence, this material has been combined with the immediate underlying lithology. The summary of the lithologies in each drill hole is presented in Table 1.

Table 1. Lithology of the drill hole logs showing thickness in metres and the classification of the rock. * = drill hole terminated at 5 m depth; d = hole recorded as dry when drilled.

A = CALCRETE - combined vadose and phreatic calcretes with minor clay layers
B = CALCRETE - as above with substantial clay content less than 50%
C = CLAY - with substantial calcrete content less than 50%
D = CLAY - includes minor calcrete in some samples

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	8A	4B	6A	10A	11A	10A	10A	10A	10A	8A	5D		5B	
	3C d	7C	5B	1D		1B	1B	1B	1C	3B	*		6C	
										d				

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
B	9A 2B	8A 3B	9A 2B	8C 3B	10A 1C	10A 1B	10A 1C	10A 1C	4D 7C	8A 3B	5D *		6A 5C	
C	11C	7A 4B	11A	11A	10A 1C	10A 1B	10A 1B	10A 1B	9A 2C	5C *			5A 6C	
D	5A 6B	10A 1B	9A 2B	9A 2B	11A 2D	4C 1A	10A 1C	11A 2D	11A	11C			9C	
E	4A 7C	11A	10A 1D	10A 1C	10A 1B	10A 1B	10A 1B	10A 1B	9A 2C	5C *	5C *	3A 2B *	6A 5C	5A 6B
F	4A/7C	11A	10A 1C	10A 1B	10A 1C	10A 1B	8A 3C	9A 2B	9A 2C	5C *d			8A 3B	
G	11B	10A 1C	10A 1C	10A 1C	10A 1D	10A 1C	6C 3A 2C	11A	11A	5C *d				
H	9A 2D	5C*	11A 2B	10A 1C	10A 1D	10A 1D	11A	10A 1C	8A 1C	5C *d				
J	10A 1C	9A 2B	9A 2C	7A 4C	10A 1D	11A	11A	9A 2C d	6A 5D		6A 5C			
K	7A 4D								2A 4C 5D					
L	7A 4B	2A 4C 5D	7A 4D	8A 3C	11A 2D	11A 2D	6A 5B	5D *d	5C *d					
N	3A 3B 5C	6A 5B	7A 4B	9A 2D	7A 4D	7A 4D	7A 4B	5C *d						
Q	6A 5D	4A 5C 2D	5D *	5D *	8C 3D d	6A 5D	3A 3C 4D d	5D *d						
S	7A 4D	7A 2D	7A 1C	11A	7A 4D	7A 4C d	9A 2D	5D *d						
U	8A 3D	7A 4D	8A 3D	7A 4D	7A 4D	9A 2D d	6B 5D	5D *d	5C *d					
V		7A 4D												
W	7A 4D	7A 4D	7A 4D	7A 4D	6A 5D d	5D *d	10B 1D							

Mapping the summarized lithology data onto the bore grid showed that the calcrete deposit in the area covered by the grid lies mainly to the west of a roughly north-south trending clay zone (Zone C-D; Fig. 4), that approximately follows the present-day drainage feature visible on the satellite image (Fig. 1C). The calcrete is divisible into two major and three minor local zones, interspersed with clay (Fig. 4).

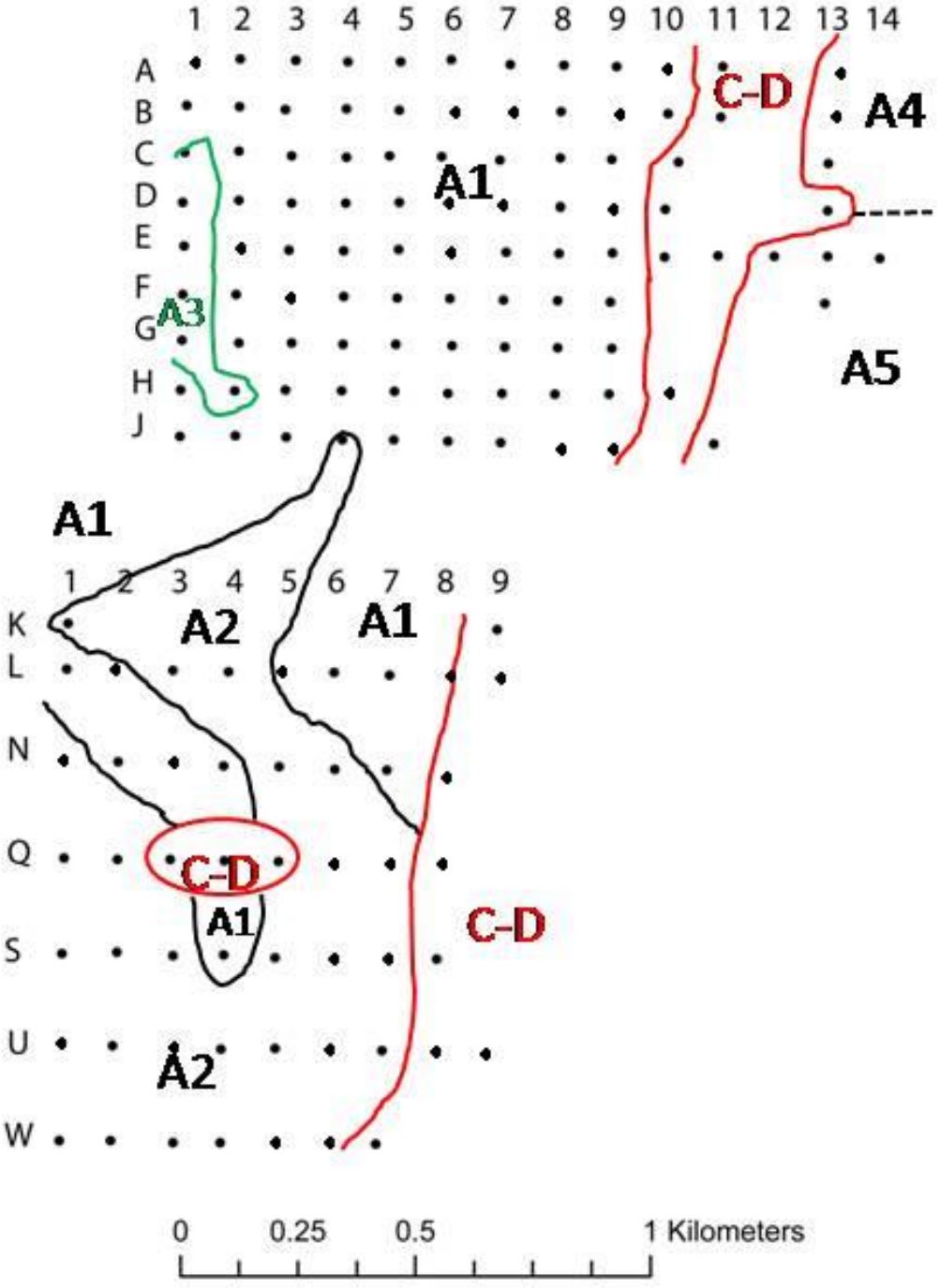


Fig. 4 Lithology zones in the Sturt Meadows bore hole grid.

Zone A1 Contains calcrete that is at least 11 m thick and may well continue below this depth– most drill holes stopped at this level. Some holes intersected some clayey material in the bottom 1 m. Drill holes A2, B4, B9, D6, G7, L2 and N1 contain clay pockets.

Zone A2 This zone is mainly south of the roadway that separates the grid. Calcretes are thinner than in Zone A1. They average 6–8 m in thickness and overlie Clay.

Zone A3 Along the north-western margin from grid C1 to G1 & H2, and contains generally < 5 m of calcrete overlying clay containing up to 25% silica. Drill logs indicate that this may be a sedimentary margin.

Zone A4 Lies in the north-eastern corner of the grid and to the east of the main clay divide. Contains calcrete generally <7 m thick, overlying clay.

Zone A5 Immediately south of Zone A4. Drill hole D14 at the junction of these two zones contains granite below 9 m depth. This zone has calcrete underlying clay that extends below the surface for 5–6 m. Insufficient data is available to indicate whether the calcrete is linked from east to west under the main clay divide. Holes B9 and D5 also have calcrete underlying 4 m of clay.

Zone C-D Clay with some calcrete. Many holes drilled into this zone are only 5 m deep. Localized pockets occur in holes A2, B4, B9, D6, G7, H2, L2, and N1. There is a clay zone in grid line Q extending from east of hole Q2 to east of Q5. This may provide a barrier to movement of groundwater in the southern part of the grid area.

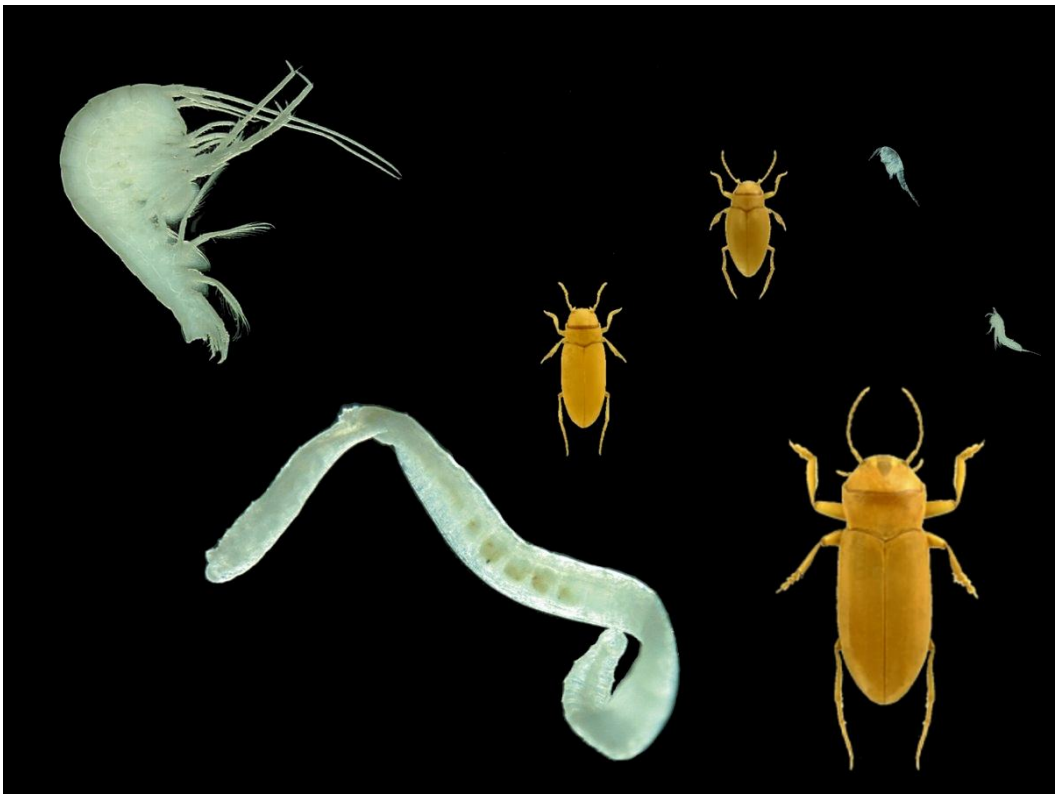
The major zones A1 and A2 differ in thickness. Zone A2 is shallower and would contain less phreatic calcrete.

Data Sources

Mt Margaret Nickel-Cobalt Project, Public Environment Report (PER) for Anaconda Nickel Limited, by URS, Western Australia (Ref: KMF/31059-018-4000-071/DK:502-F2272.3/DOC/PER; December 2000).

Sturt Meadows Project Technical Report 1252, Old Station Well E37/645 (May 2002), produced by Anaconda Nickel Limited (Western Australian Mineral Exploration Index, Item 11845).

DNA BARCODING OF STYGOFAUNA UNCOVERS CRYPTIC AMPHIPOD DIVERSITY IN A CALCRETE AQUIFER IN WESTERN AUSTRALIA'S ARID ZONE



Stygofauna from the Sturt Meadows calcrete

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Molecular Ecology notes (2009) in press

Aim 1 Establish a DNA barcode of species present to enable identification of potential diving beetle prey items

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Statement of Authorship

This chapter is a published research article (Appendix IV).

Bradford T, Adams M, Humphreys WF, Austin AD, Cooper SJB (2010) DNA barcoding of stygofauna uncovers cryptic amphipod diversity in a calcrete aquifer in Western Australia'a arid zone. *Molecular Ecology Resources* **10**, 41–50. doi: 10.1111/j.1755-0998.2009.02706.x

Bradford T (Candidate)

Corresponding author: Collected specimens and sorted through past collections for additional specimens, prepared extracts for PCR amplification and allozymes electrophoresis, carried out DNA sequencing, analysed sequence data, wrote manuscript and produced all figures.

Signed... Date.....23/3/10.....

Adams M

Carried out allozyme electrophoresis, conducted allozyme analyses and evaluated the manuscript.

I give consent for T. Bradford (candidate) to include this paper for examination towards the degree of Doctor of Philosophy.

Signed..... .Date.....24/3/10.....

Humphreys WF

Identified species to the lowest taxonomic level, sought and won funding, supervised the direction of study and evaluated manuscript.

I give consent for T. Bradford (candidate) to include this paper for examination towards the degree of Doctor of Philosophy.

Signed... Date.....24/3/2010

Austin AD: Supervised the direction of study and evaluated manuscript.

I give consent for T. Bradford (candidate) to include this paper for examination towards the degree of Doctor of Philosophy.

Signed..... Date.....24/3/2010

Cooper SJB: Sought and won funding, supervised the direction of study and evaluated manuscript.

I give consent for T. Bradford (candidate) to include this paper for examination towards the degree of Doctor of Philosophy.

Signed..... Date.....24/3/2010

CHAPTER III

Abstract

The arid Yilgarn region of Western Australia contains numerous subterranean calcrete aquifers with unique assemblages of obligate groundwater invertebrates (stygofauna). We aimed to establish a DNA barcoding framework for the macro-invertebrates present in a single calcrete, as a basis for future assessment of biodiversity of the Yilgarn calcretes and for investigating food webs. Intense sampling of a bore field grid in the Sturt Meadows calcrete was undertaken to obtain representatives of the entire macro-invertebrate ecosystem. A 623-bp fragment of the mitochondrial cytochrome *c* oxidase 1 (*COI*) gene was used to provide DNA barcodes for stygobiont macro-invertebrates plus terrestrial organisms that are found in the calcrete. Phylogenetic analyses revealed the existence of twelve divergent monophyletic groups of haplotypes. Subterranean amphipods (Chiltoniidae) showed three groups of *COI* haplotypes with sequence divergences between them of > 11%. Allozyme analyses found a large number of fixed allelic differences between these three amphipod groups, indicating that there are three morphologically cryptic species within the Sturt Meadows calcrete. Unlike the sister triplet of dytiscid beetles present, the amphipods are not sister clades and are more closely related to other Yilgarn and non-Yilgarn amphipods than to each other. Our results show that the aquifer contains at least 12 macro-invertebrate species and DNA barcoding provides a useful means for discriminating species in this system.

Introduction

Calcrete aquifers (calcretes) of the Yilgarn region of Western Australia provide habitat for a diverse fauna of obligate groundwater invertebrates, or stygofauna (Humphreys, 2008). Over the last decade since the discovery of this fauna there has been a push to formally describe new stygobiont species, however, in some of the groups, such as the amphipods, progress has been impeded by their cryptic morphology. Previous biodiversity surveys have been conducted at a broad scale, sometimes sampling with only a single well or bore hole in a calcrete providing access to the groundwater, making it difficult to assess the total number of macro-invertebrate species present. Here, we use a molecular approach for estimating biodiversity in a single calcrete aquifer on the Sturt Meadows pastoral property, where an extensive bore-field has enabled thorough sampling of the subterranean ecosystem.

Traditionally the taxonomy of arthropods has been based on comparison of their morphology (Quicke, 1993). However, it is now well established that morphologically cryptic species can and do occur in most organismal groups and biomes (Bickford *et al.* 2006; Pfenninger & Schwenk 2007). This phenomenon is likely to be even more common with stygofauna, where adaptation to the subterranean environment may lead to major modifications in morphology (Culver *et al*. 1995), while convergent evolution can mask species differences or lead to problems in the classification of species. Therefore, a combination of morphological examination along with molecular analyses is often required to identify and discover cryptic stygobitic species (Paquin & Hedin, 2004; Bickford *et al.*, 2006; Lefebure *et al.*, 2006b; Trontelj *et al.*, 2009).

DNA-based identification systems for species are especially useful where taxonomic expertise based on morphological traits is not readily available, with the 5‘end of the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene proposed as a ‗barcode‘ for all animal species (Hebert *et al.*, 2003). *COI* barcodes have been successfully used to carry out biodiversity inventories of species of Lepidoptera from Costa Rica, with over 97% of the 521 species examined being distinguished by the barcode (Hajibabaei *et al.*, 2006). However, focussing on a single gene risks overlooking new or rapidly diverging species, and analyses from several genomic regions in multiple individuals are sometimes required to distinguish between closely related species and to confidently define species boundaries (Mallet & Willmott, 2003; Lefebure *et al.*, 2006a). One approach that is particularly useful for investigating species boundaries when there are cryptic species present in sympatry is allozyme electrophoresis, which provides a rapid means of screening multiple nuclear markers (Richardson *et al*. 1986).

The Yilgarn calcretes are carbonate deposits, approximately 10 m thick, honeycombed with small holes and filled with groundwater, which were originally deposited upstream of salt lakes by evaporation of groundwater within palaeodrainage channels (Humphreys, 2001). Each calcrete studied to date contains a combination of unique aquatic invertebrate species, including predaceous dytiscid diving beetles (Coleoptera), large and small crustaceans (Syncarida, Isopoda, Amphipoda, Copepoda, Ostracoda) and worms (Oligochaeta) (Watts & Humphreys, 2004). Phylogeographic studies of the diving beetles (Cooper *et al.*, 2002; Leys *et al.*, 2003), amphipods (Cooper *et al.*, 2007), bathynellids (Guzik *et al*. 2008) and oniscidean isopods (Cooper *et al.*, 2008), have found these taxa to be restricted in their distribution to single calcretes. This remarkable endemism points to the isolation of the individual calcretes in the groundwater system, with Cooper *et al.* (2002) describing them as ―islands under the desert".

The calcretes contain the most diverse known assemblage of subterranean diving beetles in the world, with usually between 1-4 species per calcrete, each differing considerably in size within calcretes (Balke *et al.*, 2004; Watts & Humphreys, 2004). Phylogenetic analyses using mtDNA have identified 13 calcretes containing sympatric sister species of large and small diving beetles, and several plausible hypotheses have been proposed to explain their modes of speciation (Cooper *et al.*, 2002; Leys *et al.*, 2003). One hypothesis is that the different-sized diving beetles have arisen as a result of sympatric speciation driven by divergence into different ecological niches. Such divergences may have occurred by competition for resources if the various size diving beetle species within a calcrete are utilizing different trophic niches. In order to investigate this possibility we have focussed on predator/prey studies for a single calcrete at the Sturt Meadows pastoral property in Western Australia. This calcrete contains an extensive grid of bore holes allowing intensive sampling of the calcrete and a comprehensive assessment of the ecosystem present. Ecological surveys of the Sturt Meadows calcrete have identified a simple ecosystem containing seven stygobiont macro-invertebrate species, with a sister triplet of dytiscid beetle species as the likely top predators (Allford *et al.*, 2008; Leys & Watts, 2008). These beetles do not overlap in size and we hypothesize that each species feeds on different prey items.

In this study, universal arthropod primers (Folmer *et al.* 1994) were used to amplify *COI* mtDNA for all the taxa present and a phylogenetic approach was taken to identify taxa. Allozyme analyses were also used to investigate species boundaries in the Amphipoda. The resultant DNA barcodes will ultimately be used as a tool to provide species-specific markers for gut content analysis and as a basis for future biodiversity surveys of the Yilgarn calcretes.

Materials and Methods

Taxonomic Sampling

The Sturt Meadows calcrete in Western Australia (28˚41‘S 120˚ 58‘E) was accessed through a grid of bores drilled for mineral exploration (Fig. 1). The field contains 115 bore holes of between 5-11m in depth in a 1.4 km X 2.5 km (3.5 km^2) grid. Intensive sampling, by both hauling with a small weighted plankton net (250 μm) and pumping, was carried out over 16 months, leading to the collection of 6254 stygobitic macro-invertebrate specimens from 262 sampling events (Allford *et al.*, 2008). Identification of macro-invertebrates was carried at least to order level, with family level identifications possible for copepods and amphipods and

species-level for the beetles. Stygobiont invertebrates in the Sturt Meadows calcrete were identified by their taxonomic affinity and stygomorphic features, such as eyelessness and lack of colour. Terrestrial species that had fallen into the aquifer were also collected. Several troglobiont (terrestrial subterranean) specimens were excluded from the molecular analyses and kept for morphological taxonomic description due to their rarity. In total, 46 specimens belonging to eight groups (both stygobiont and terrestrial) were included in initial sequencing analyses. Subsequently, a further 87 amphipod specimens were sequenced for *COI* and a selection of these subjected to allozyme analysis (see below).

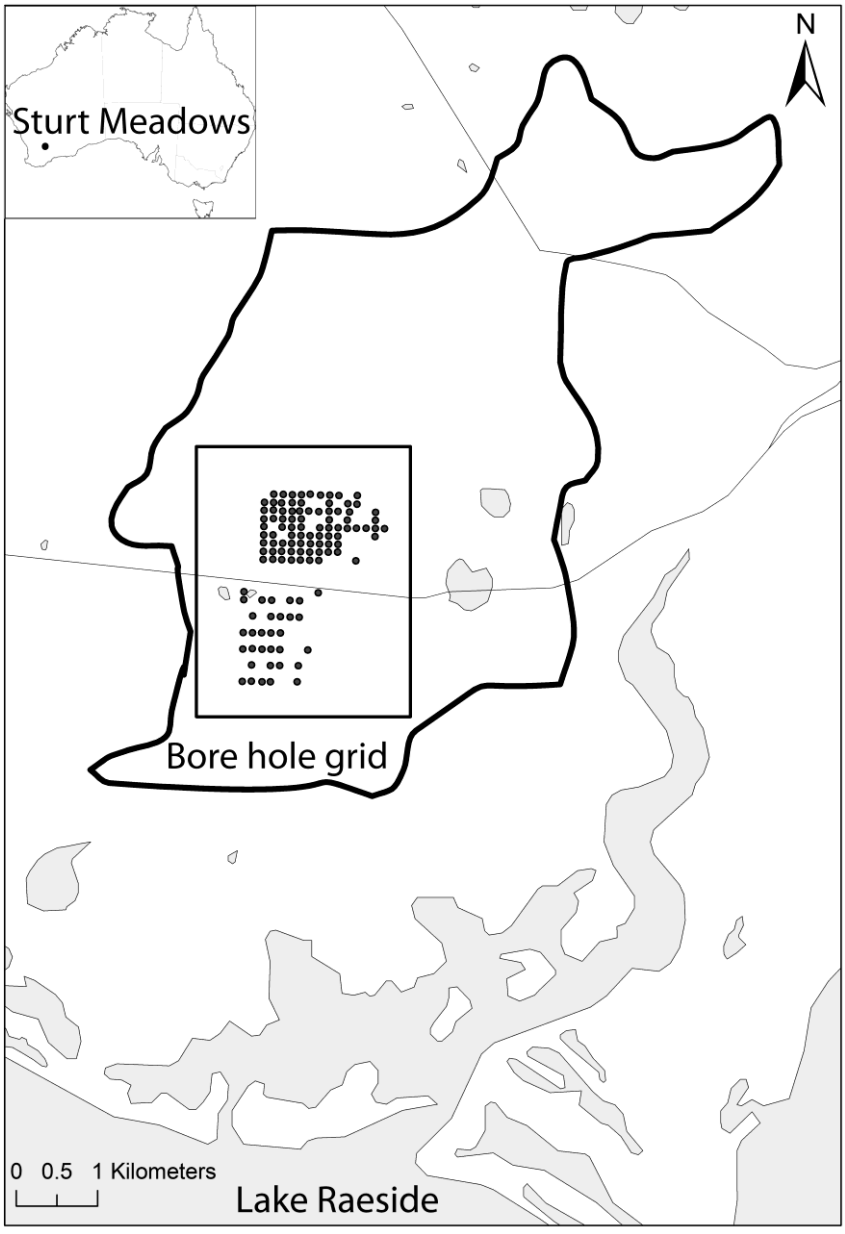

Fig. 1 Map of Australia with the Sturt Meadows pastoral property in Western Australia marked. The expanded diagram shows the sample site at Sturt Meadows with the bore hole grid north and south of the road outlined in a black square. The shape of the calcrete is illustrated in black.

Vouchering specimens

All specimens were lodged with the Australian Biological Tissue Collection (ABTC) at the South Australian Museum and given an ABTC number. Specimen details including ABTC voucher number, identification, sampling location and specimen type are in Appendix I. Automontage digital images taken of representative specimens and have been lodged with the South Australian Museum.

MtDNA extraction, amplification, and sequencing

Individual specimens were preserved in 100% ethanol. DNA was isolated from one leg for each specimen > 2 mm, or using the whole body for animals < 2 mm long (i.e. copepods). DNA was extracted using the GENTRA method (Puregene) according to the manufacturer's protocol for fresh tissues. PCR amplification of a 623-bp fragment from the mitochondrial *COI* gene was carried out with the ~~"u~~niversal" primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al.*, 1994) in 25 μL volumes containing, 4 mM $MgCl_2$, 0.20 mm dNTPs, 1× PCR buffer (Applied Biosystems), 6 pmol of each primer and 0.5 U of Ampli*Taq* Gold (Applied Biosystems). PCR amplification was performed under the following conditions: 94 °C 9 min, then 34 cycles of 94 °C 45 s; annealing 48 °C 45 s; 72 °C, 60 s; with a final elongation step at 72 °C for 6 min. PCR products were purified with a PCR Clean-up DNA purification kit (MoBio Laboratories Inc., Solana Beach, CA) and sequencing was undertaken using the ABI prism Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, CA). Sequencing was carried out on an ABI 3700 DNA analyser and sequences were edited and manually aligned in SeqEd version 1.0.3 (Applied Biosystems).

MtDNA analyses

The *COI* data were aligned by eye and translation of the DNA sequences to protein was carried out in MEGA v. 4 (Kumar *et al.* 2008) using the invertebrate mitochondrial genetic code, to check for the presence of nuclear paralogs. Sequences and traces were submitted to GenBank (GenBank accession numbers FJ785739 - FJ785818).

Neighbour Joining (NJ) trees were constructed for the initial sequencing analysis with the computer program PAUP*version 4.0b10 (Swofford, 2002), using HKY85 distances (Hasegawa *et al.* 1985), aiming to cluster similar sequences into taxonomic groups rather than investigate their phylogenetic relationships (Hebert *et al.*, 2003). A midpoint root was applied and the NJ tree was bootstrapped with 500 pseudoreplicates carried out using the same model of evolution used above. Sequences were checked for any close matches by an NCBI BLAST search of GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and taxa with close sequence similarity were included in the phylogenetic analyses.

A further 87 amphipods from Sturt Meadows were sequenced for *COI* and identical haplotypes were removed from the dataset. Chiltoniid amphipod sequences (GenBank accession numbers EF118196 - EF188256; Cooper *et al.* 2007) originating from other Yilgarn calcretes at various distances from Sturt Meadows (Lake Mason, 178 km; Barwidgee, 171 km; Depot Springs North and South, 115 and 106 km; Mt Padbury, 432 km) were included in the expanded amphipod dataset. Epigean chiltoniid amphipods from The Fountain and Coward Springs of the Great Artesian Basin (GenBank accession numbers EU886946 and EU886924; Murphy *et al.*, 2008) 1600 km away in South Australia were also included in the analyses. Phylogenetic analyses with *Hyallella azteca* (GenBank accession number DQ464727) as an outgroup, showed the sample from Coward Springs to be a sister lineage to amphipods from the Yilgarn and The Fountain, and the Coward Springs sequence was subsequently used as the outgroup in our analyses.

Maximum parsimony (MP) analyses for the amphipod haplotypes were conducted using PAUP*. MP bootstrap analyses (Felsenstein, 1985) were carried out using 500 bootstrap pseudoreplicates, employing a heuristic search option with random input of taxa. The dataset was then partitioned by codon, and Modeltest with AIC (Posada & Crandall, 1998) used to determine the best model of sequence evolution. Bayesian inference was carried out in MrBayes (Ronquist & Huelsenbeck, 2003) for 1 million generations, after a burn in of 100 000 generations. Parameter convergence for both runs was checked in Tracer

v. 2.1 (Rambaut & Drummond 2005), by ensuring that effective sample sizes were > 100 for all parameters.

Allozyme electrophoresis on Sturt Meadows amphipods

After removal of a leg for DNA sequencing, amphipods were snap frozen in liquid nitrogen and subsequently stored at -80˚C. Allozyme electrophoresis was undertaken on cellulose acetate gels (Cellogel©, MALTA, Milan) and stained for enzyme activity according to the principles and procedures of Richardson *et al.* (1986). To ensure that homogenates exhibited adequate enzyme activity after electrophoresis, only specimens > 3mm were selected (49 total). Twenty-six enzymes displayed banding patterns of sufficient activity and resolution to permit allozymic interpretation, namely: ACP, ACYC, ADH, ALD, ARGK, ENOL, EST, FDP, FUM, G6PD, GAPD, GOT, GPI, GPT, IDH, LDH, MDH, ME, MPI, NDPK, PEP-A, PGAM, PGM, PK, SORDH, and TPI. Details of enzyme and locus abbreviations, enzyme commission numbers, electrophoretic conditions, and stain recipes are presented in Richardson *et al.* (1986) or Wallman and Adams (2001). Allozymes were designated alphabetically and multiple loci, where present, were designated numerically, both in order of increasing electrophoretic mobility (e.g. *Acp*^{a}, *Acp*^{b}; *Got1*, *Got2*).

The genetic affinities of individuals from the allozyme study were explored using Principal Co-ordinates Analysis (PCO), as implemented on a pairwise matrix of Rogers' genetic distance (Rogers, 1972) using PATN (Pattern Analysis Package, DOS version; Belbin, 1994). Allele frequencies and pairwise genetic distances (both percent fixed differences and unbiased Nei Ds) for the three mtDNA clades were calculated as detailed in Murphy *et al.* (2008).

Results

Stygobiont invertebrate groups identified in the Sturt Meadows calcrete comprised three dytiscid beetle species, *Paroster macrosturtensis, P. mesosturtensis* and *P. microsturtensis*, chiltoniid amphipods between 2 and 5 mm in length, cyclopoid and harpacticoid copepods

and several aquatic oligochaete worms (Fig. 2). Terrestrial invertebrates included mites (Acari), a centipede (Chilopoda), springtails (Collembola) and insect larvae. These were in the minority and most of the animals caught were stygofauna.

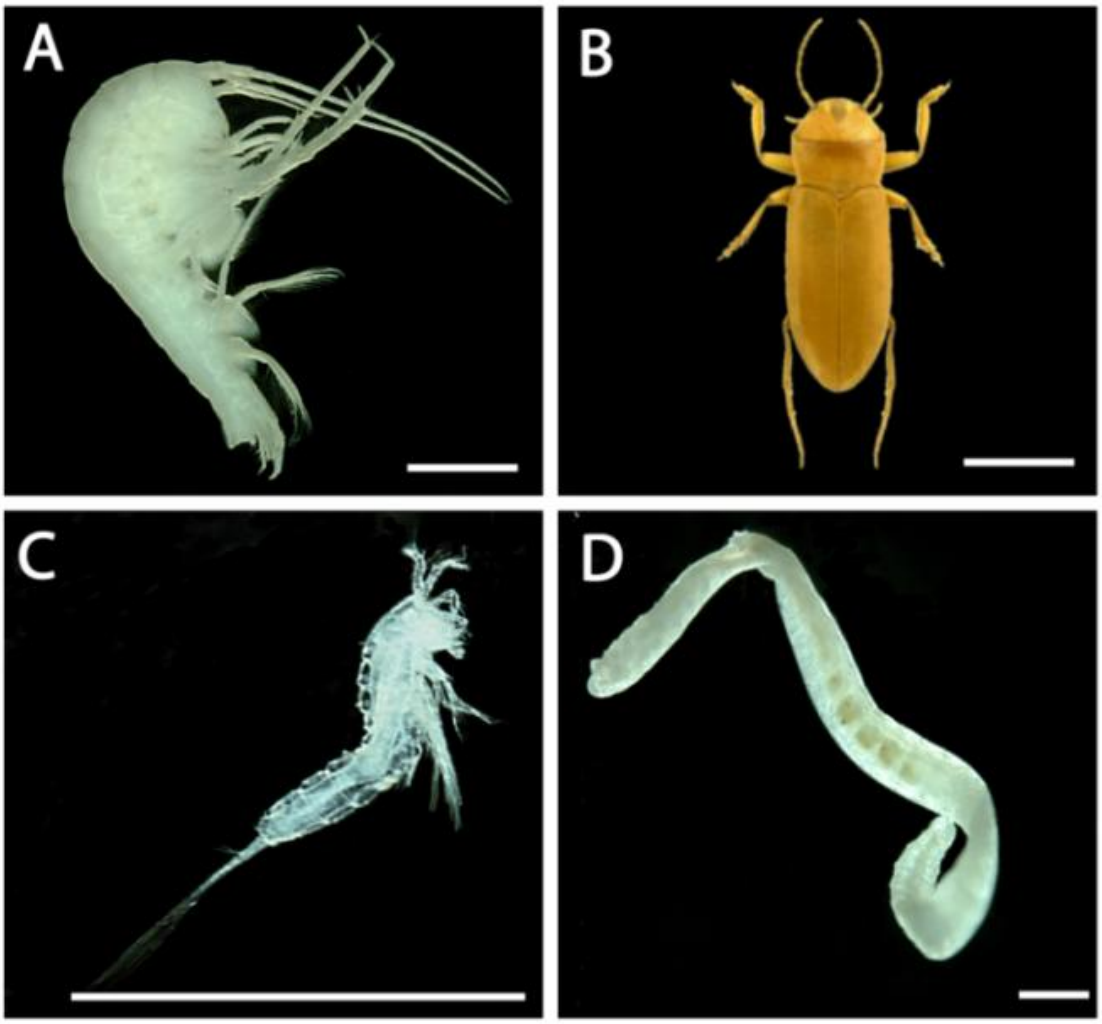

Fig. 2 Stygobiont macro-invertebrates present in the Sturt Meadows calcrete.
A. Amphipoda: Chiltonidae, **B.** Coleoptera: Dytiscidae *Paroster macrosturtensis* (photograph by Chris Watts), **C.** Copepoda: Harpacticoida and **D.** Oligochaeta, Scale bar = 1mm.

The primers of Folmer *et al.* (1994) successfully amplified the DNA of all the animals tested, with the exception of a few terrestrial specimens (unknown insect larvae and an oniscidean isopod). All sequences had an open reading frame with no evidence for stop codons or insertions/deletions, so it was assumed that the sequences are unlikely to be nuclear paralogs. Phylogenetic distance analyses revealed the existence of 12 distinct clusters of haplotypes (Fig. 3). The dytiscid beetle species form three distinct clades, each with 100% bootstrap support. Species level identification of dytiscid larvae was made possible by including the *COI* sequence in the distance analysis, i.e. by barcoding (data not shown).

Cyclopoid and harpacticoid copepods could be distinguished by their *COI* sequence and formed two well supported clades. Collembola, oligochaete worms, the centipede and mites also clustered into their respective groups. Collembola, worms and the centipede had close BLAST matches on GenBank and these GenBank sequences were included in the phylogenetic analyses to further assess the robustness of our order level taxonomy (Fig. 3). Pairwise genetic distances between Sturt Meadows fauna and their GenBank matches were 20% for collembola, 16-21% for worms and 18% for the centipede. Oligochaete worms showed considerable within-clade genetic heterogeneity, with four distinct lineages present among the six haplotypes examined and pairwise genetic distances ranging between 14-26%.

Sturt Meadows amphipods formed three distinct clades in the Neighbour Joining analysis (Fig. 3). The expanded amphipod dataset was partitioned by codon using models of sequence evolution for the first codon position (TrN+I+G), second codon position (K81uf+I) and third codon position (HKY+G). MP/Bayesian analysis including chiltoniid amphipods from other Yilgarn aquifers showed that the Sturt Meadows amphipods are not sister to each other (Fig. 4). Clade 1 is more closely related to stygobiont amphipods from Lake Mason (Western Australia) and epigean mound spring amphipods at The Fountain (South Australia) than to the other Sturt Meadows clades. Clade 2 forms a distinctive lineage without any close phylogenetic affinities, while clade 3 is more closely related to amphipods from the Yilgarn calcretes at Mount Padbury and Depot Springs than to clades 1 and 2. Each Sturt Meadows amphipod clade is well supported, with posterior probabilities of 1.00 and 100% bootstrap support. Pairwise distances (HKY85) between the clades range between 11-13%, with within-clade divergences below 3%.

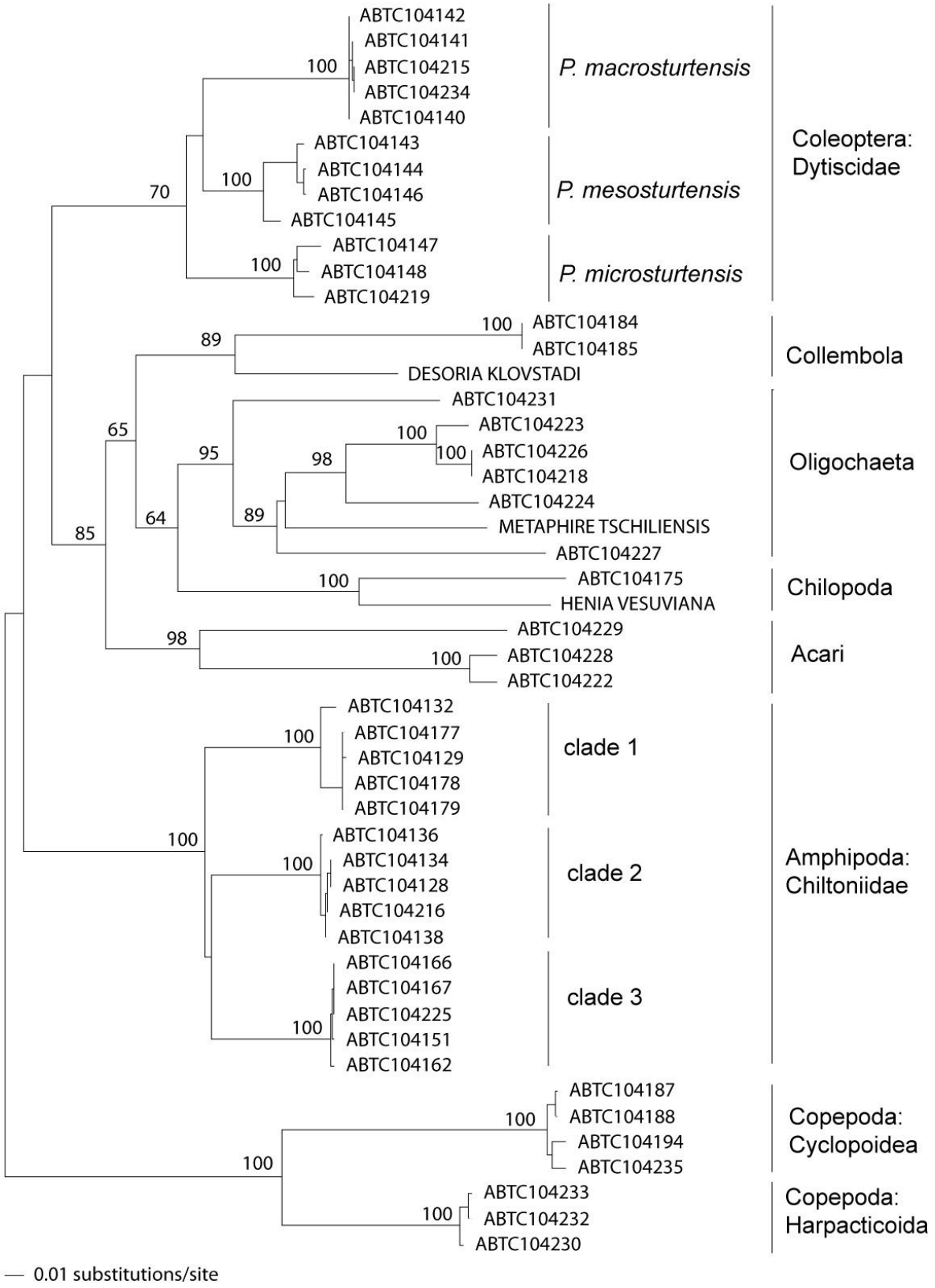

Fig. 3 Neighbour joining tree for Sturt Meadows macro-invertebrates based on HKY85 distances. GenBank sequences were included from *Desoria klovstadi*, *Metaphire tschiliensis* and *Henia vesuviana* (GenBank accession numbers DQ365786, DQ835676 and AY288754 respectively). Bootstrap values > 50% are shown above the line.

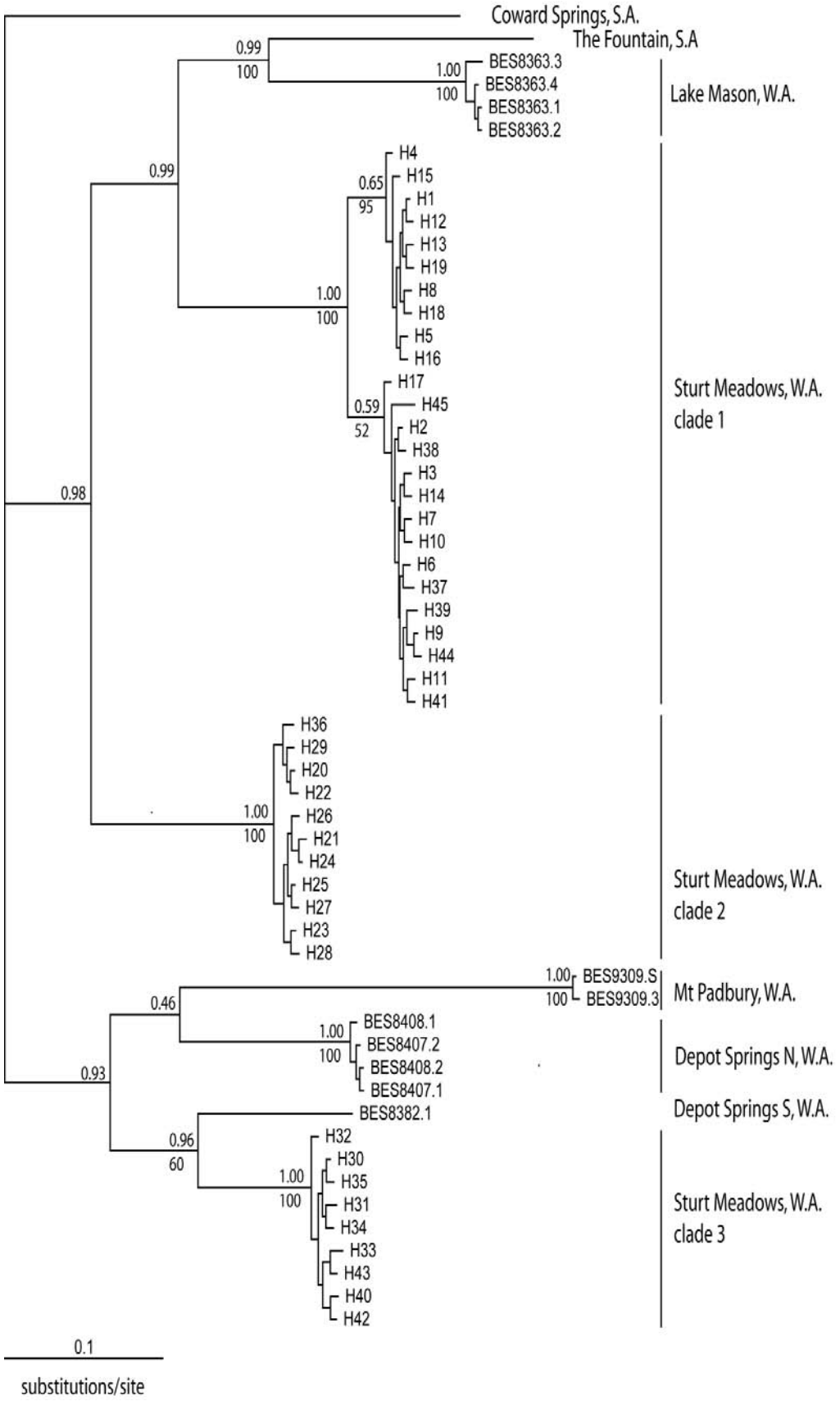

Fig. 4 Bayesian phylogenetic tree for Chiltoniid amphipod *COI* haplotypes (models and approach as given in the Methods). Posterior probabilities are shown above the line and bootstrap values from the Maximum Parsimony analysis are shown below.

Allozyme genotypes were able to be assigned at 31 putative allozyme loci for the 49 amphipods examined. PCO of these 49 individuals (Fig. 5) revealed three very distinctive genetic clusters, corresponding to the three clades identified independently by DNA barcoding. The allele frequencies for the three amphipod mitochondrial clades are presented in Table 1.

Based on the available data and notwithstanding the small sample size for clade 3, percent fixed differences (%FD; allowing a 10% tolerance for shared alleles) ranged from 59 %FD between clades 1 and 2 to 73 %FD between clades 2 and 3, while Nei D values ranged between 1.08 (clades 1 and 2) and 1.50 (clades 2 and 3). Of the 27 allozyme loci scored in all clades, seven (*Enol*, *Fdp*, *Gapd*, *Pgam*, *Pgm*, *Pk*, and *Tpi*) were unequivocally diagnostic as to clade identity. Interestingly, although within-clade levels of genetic variability (as measured by direct count heterozygosity estimates, H_O) were similar for clades 1 ($H_O = 0.232 \pm 0.044$) and 3 ($H_O = 0.186 \pm 0.054$), clade 2 displayed much reduced levels of variability ($H_O = 0.013 \pm 0.009$).

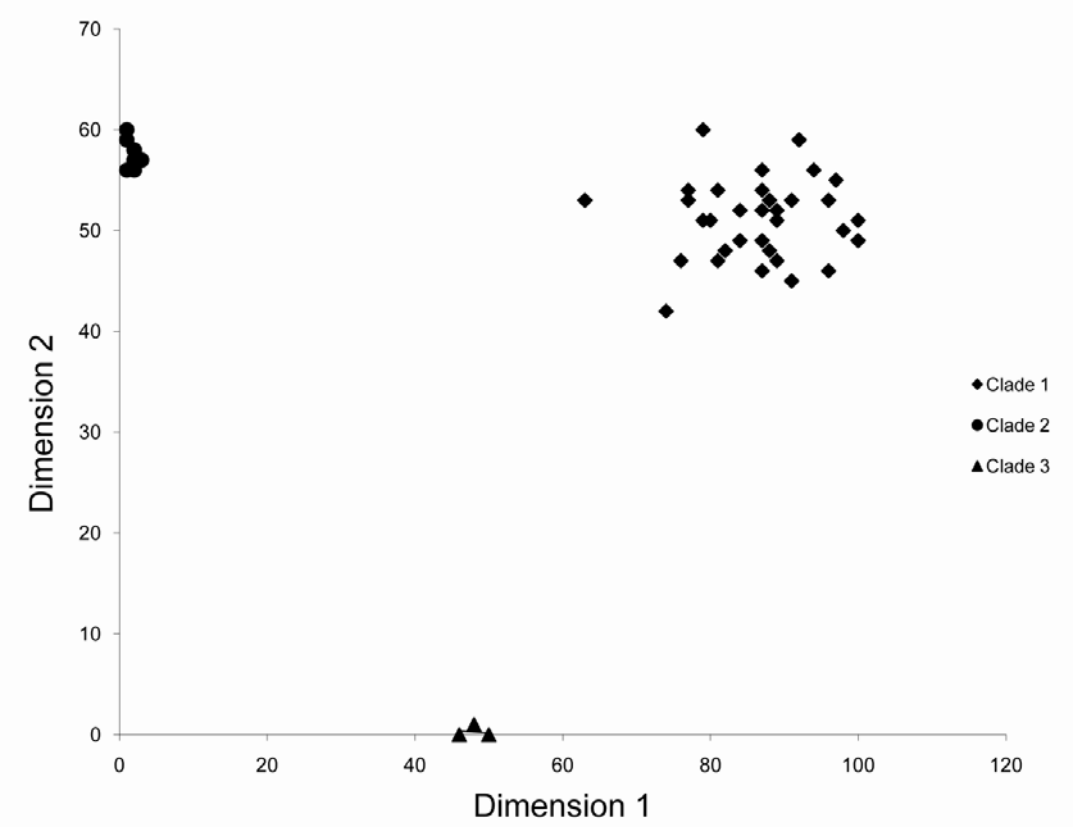

Fig. 5 Principle co-ordinates analysis for amphipod mitochondrial clades 1, 2 and 3. The relative PCO scores have been plotted for the first (X-axis) and second (Y-axis) dimensions, which individually explained 35% and 14% respectively of the total multivariate variation present. The number of points is less than the total number of individuals because some individuals share the same PCO scores in the first two dimensions.

Table 1 Allele frequencies at 31 putative allozyme loci for the three amphipod mitochondrial clades. For polymorphic loci, the frequency of each allele is expressed as a percentage and shown as a superscript. Sample sizes for each locus are indicated in brackets; differences between different loci for the same taxon reflect the poor activity displayed by smaller individuals at some but not all loci. A dash indicates the locus was not scorable in this taxon. (H_O = observed heterozygosity estimates).

Locus	**clade 1**	**clade 2**	**clade 3**
Acp	c^{56},b^{40},a^{4} (36)	c (9)	d (4)
Acyc	e^{60},d^{21},c^{13},a^{6} (35)	e (9)	d^{87},b^{13} (4)
Ada	b^{45},d^{37},a^{18} (11)	c^{93},b^{7} (7)	-
Ald	a (4)	b (5)	b (2)
Argk	b (12)	b (7)	a (4)
Enol	c^{76},a^{24} (36)	d (9)	b (4)
Est	a (11)	a (7)	a (4)
Fdp	a (10)	c (6)	b (1)
Fum	a^{93},b^{5},c^{2} (21)	d (8)	b (3)
G6pd	e^{69},c^{29},a^{1},f^{1} (35)	c (9)	d^{83},b^{17} (3)
Gapd	b (12)	c (7)	a (4)
Got1	e^{83},f^{8},g^{7},c^{1},a^{1} (36)	g (9)	d^{50},e^{37},b^{13} (4)
Got2	d^{71},e^{25},c^{3},b^{1} (36)	d^{89},a^{11} (9)	d (4)
Gpi	f^{21},e^{18},d^{18},g^{11},h^{11},i^{8},j^{8},b^{3},a^{1},k^{1} (36)	e (9)	a^{50},c^{38},f^{12} (4)
Gpt	b^{94},c^{6} (36)	a (9)	a (4)
Idh1	c^{86},b^{7},a^{4},e^{3} (28)	d (9)	b (3)
Idh2	f^{60},d^{27},e^{10},g^{3} (30)	h (9)	f^{50},b^{17},a^{17},c^{16} (3)
Ldh	a (4)	-	a (4)
Mdh1	a (4)	-	b^{50},c^{50} (3)

Locus	clade 1	clade 2	clade 3
Mdh2	a (4)	-	b (2)
Me	a (7)	a (3)	b (4)
Mpi	d^{45},f^{26},c^{10},a^{6},e^{4},b^{4},h^{3},g^{2} (34)	d (9)	c^{67},d^{33} (3)
Ndpk1	a^{95},b^{5} (11)	a (7)	a^{88},c^{12} (4)
Ndpk2	a (8)	b (3)	a (4)
PepA1	c^{97},b^{3} (31)	d (9)	a^{63},c^{25},d^{12} (4)
PepA2	c^{41},d^{32},b^{21},e^{4},a^{1},g^{1} (34)	e (9)	f (4)
Pgam	a^{50},c^{50} (4)	d (7)	b (3)
Pgm	b^{65},a^{26},c^{9} (31)	e (5)	d (2)
Pk	b^{83},a^{17} (9)	c (6)	d (3)
Sordh	c^{95},b^{5} (10)	c (6)	b^{83},a^{17} (3)
Tpi	c^{81},d^{19} (36)	e (9)	a^{88},b^{12} (4)
H_O ± S.E.	0.232 ± 0.044	0.013 ± 0.009	0.186 ± 0.054

Discussion

Biodiversity assessment of the Sturt Meadows calcrete

DNA barcoding has provided a useful approach for examining invertebrate diversity in the Sturt Meadows calcrete aquifer, with species-level diversity detected for a number of the groups that was not evident from other broad-scale studies of the Yilgarn calcretes. Three species of dytiscid beetle, at least three species of chiltoniid amphipods (separate at multiple allozyme loci) and single harpacticoid and cyclopoid copepod species were found. Four divergent lineages of oligochaete worms, a single mite, centipede and springtail (Collembola)

species all clustered into distinct groups by phylogenetic analyses of a 623-bp *COI* fragment and each undoubtedly represent putative species. Species diversity appears to be richer than previously anticipated when examining the morphology of the macro-invertebrates present. Other than the beetles, all of the stygobiont invertebrate taxa present in the Sturt Meadows calcrete are likely to be new species. These potential beetle prey items can now be distinguished by a molecular barcode that enables us to develop molecular methods for studying natural predation events and test hypotheses on modes of speciation in the diving beetles.

Morphological analyses of the several troglobiontic specimens collected have revealed another macro-invertebrate group within the calcrete, i.e. the discovery of the first indigenous palpigrade from Australia (Barranco & Harvey, 2008). Palpigrades are a well known rainforest group and the recently described species, *Eukoenenia guzikae,* is highly likely to be a short range endemic, a pattern common within poorly dispursive members of Australian invertebrate fauna (Harvey, 2002; Barranco & Harvey, 2008). An additional species of troglobiontic isopod from the family Armadillidae has also recently been identified from the Sturt Meadows calcrete (S. Taiti pers. comm.). Fifteen million years ago (mya) the current Australian arid zone had a warm and wet environment, with extensive rainforest, and the onset of aridity ~ 10 mya resulted in the evolution of an arid adapted biota (Byrne *et al.*, 2008). It is hypothesized that invertebrates living above the water table in the calcretes represent a relictual rainforest fauna, driven underground during the drying out of the surface landscape, where they have then evolved troglomorphic features as an adaptation to the subterranean environment.

Cryptic diversity in the Sturt Meadows stygofauna

Amphipods, that were originally thought to comprise a single species from the family Chiltoniidae based on their morphology, form three divergent mitochondrial lineages and each can be separated at multiple allozyme loci indicative of their reproductive isolation. Each lineage has different phylogenetic affinities, being more closely related to epigean mound spring taxa from South Australia and other Yilgarn amphipods hundreds of kilometres away than they are to each other. Together, these molecular, phylogenetic and geographic data demonstrate that the Sturt Meadows calcrete contains three distinct evolutionary (and

biological) species of chitoniid amphipod, and indicate the need for follow-up morphological taxonomic studies to describe them formally.

The Sturt Meadows calcrete is the only subterranean aquifer identified thus far that contains more than one divergent lineage of chiltoniid amphipod (Cooper *et al.*, 2007). This result is likely to reflect the extensive, fine-scale sampling that has been possible at Sturt Meadows rather than the lack of diversity in other aquifers. With respect to their distribution within the calcrete, each species has been found in sympatry in a number of bore holes. All three amphipod species cover the extent of the borehole grid, though species 2 and 3 are very patchy in their distribution. Interestingly, amphipod species 2 has much reduced levels of heterozygosity at the suite of allozyme loci examined, suggesting a demographic change (e.g. a recent bottleneck or expansion event) may have occurred in this species that is not evident in the other two species.

Finston *et al.* (2007) also found that subterranean amphipods from the Pilbara Region of north-west Western Australia contain highly divergent lineages in two morphologically cryptic genera. Sequence divergence levels for *COI* between haplotypes were > 22% in *Chydaekata* and > 6% in *Pilbarus* (Finston *et al.*, 2007). Studies in American desert springs amphipods have found provisional cryptic species, identified by a species screening threshold of 10 times the average intra-population *COI* divergence, with divergence levels > 4% found between provisional species (Witt *et al.*, 2006). As the three Sturt Meadows amphipods showed intra-clade divergences of ~ 3%, this would represent a threshold value of 30% according to the criteria of Witt *et al.* (2006). With only 11-13% divergence between amphipod species found at Sturt Meadows, this result highlights the difficulty in imposing a *COI* threshold for species level identification in amphipods using pairwise genetic distances alone. We propose therefore that the presence of divergent monophyletic lineages is best used as an indicator of the presence of cryptic species requiring further scientific investigation.

There was evidence for further cryptic species within the oligochaete worms, as they showed even greater levels of within-clade mitochondrial genetic divergence than the amphipods. Four haplotypes identified have more than 14% pairwise distance between them, strongly suggesting that there are potentially four species of worms present in this aquifer. As only six individuals were sequenced, considerably more sampling is required to gain a reliable estimate of the number of species present.

The barcoding approach offers considerable potential in this calcrete system for identifying distinct genetic lineages and assessing the level of species diversity in a diverse group of morphologically conservative macro-invertebrates. The Sturt Meadows calcrete is missing some of the other stygobiont taxa found elsewhere in the calcrete aquifers of arid Australia such as bathynellids, oniscidean isopods, candonid ostracods and hydrobiid gastropods. However, the *COI* fragment used here has been successfully amplified in both bathynellids and isopods (not yet attempted for ostracods and gastropods) and so could be employed more broadly for biodiversity assessment. Given the importance of being able to recognize short range endemic species in regions where groundwater exploitation threatens habitat integrity, DNA barcoding is likely to prove essential for the rigorous assessment of desert aquatic invertebrate diversity (Finston & Johnson, 2004; Witt *et al.*, 2006; Finston *et al.*, 2007; Humphreys, 2008). As major project approvals in Western Australia require assessment of the subterranean fauna, there is an urgent need for rapid biodiversity assessment methods to determine the environmental impact of proposed mine developments, as well as assess the impact of mining operations over time.

Extreme environmental conditions within the underground ecosystem such as low levels of dissolved oxygen and lack of light, can lead to convergence of morphology in subterranean animals. The convergence of morphological characters makes it difficult to carry out species-level surveys, especially when there is a scarcity of taxonomic knowledge for most of the groups involved. Only by building up a sequence database for macro-invertebrates present in this system will it be possible to identify the divergent lineages that are likely to signal the existence of cryptic species. The numerous isolated underground aquifers in this region clearly represent a biodiversity hotspot of macro-invertebrates, and it is probable that DNA barcoding will provide the only way in which the biodiversity present can be rapidly assessed.

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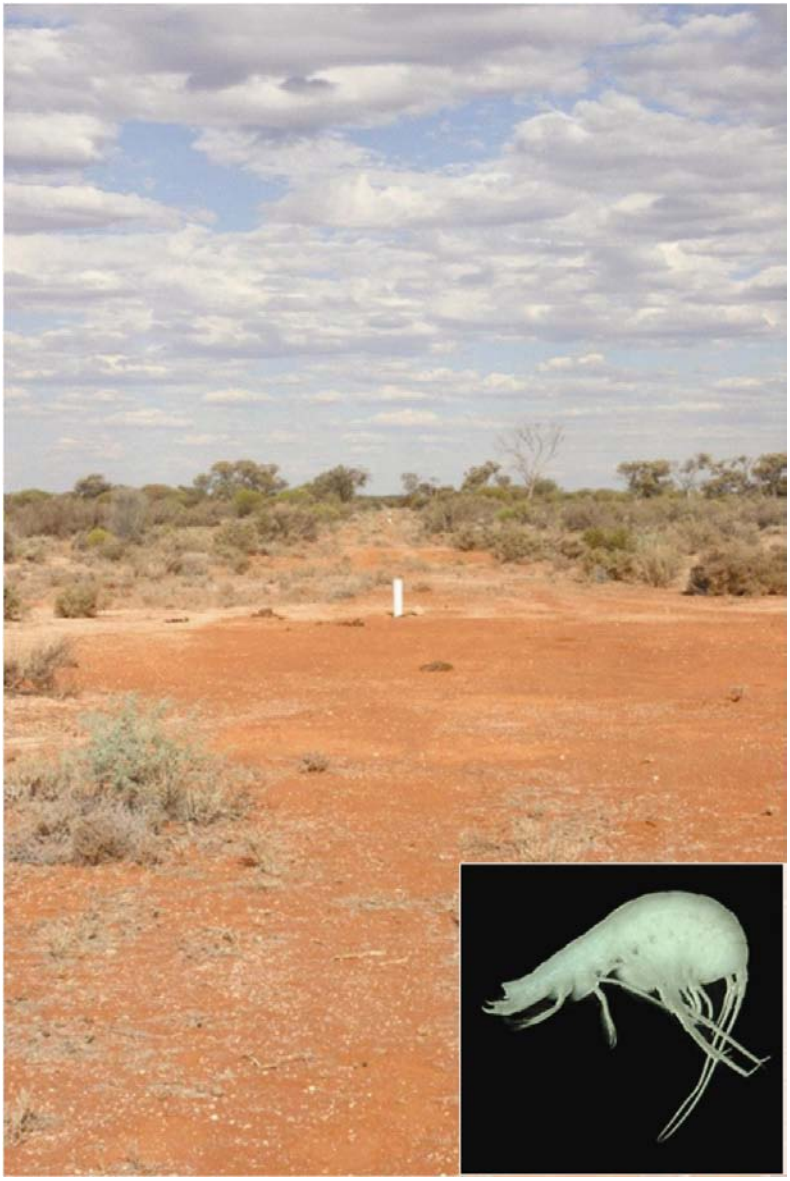
COMPARATIVE PHYLOGEOGRAPHY OF STYGOBIONT AMPHIPODS REVEALS COMMON HISTORICAL EVOLUTIONARY PROCESSES IN STYGOFAUNA FROM A SINGLE CALCRETE AQUIFER



Bore hole line at Sturt Meadow, with insert showing a chiltoniid amphipod

Aim 2 Compare the phylogeography of three amphipod species, looking for congruent phylogeographic structure between stygofauna that indicates a historical geographic barrier to gene flow, and congruent population genetic patterns between stygofauna indicative of micro-allopatric processes

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CHAPTER IV

Abstract

Calcrete aquifers from the Yilgarn region of central Western Australia contain a highly diverse range of obligate groundwater invertebrate species that are each endemic to single aquifers. We are interested in the modes of speciation that have generated this enormous diversity of species and, in particular, what diversifying forces may have operated within the closed system of a calcrete body. Fine-scale phylogeographic structuring was investigated in three sympatric species of amphipod (Chiltoniidae) in order to search for evidence of common population genetic structure in stygofauna endemic to a single calcrete aquifer, i.e. is there evidence for past geographic isolation of populations. Genetic diversity in amphipod mitochondrial DNA (cytochrome *c* oxidase subunit I gene) and allozymes was examined across a 3.5 km^2 grid of 115 bores drilled into the aquatic environment. Stygobitic amphipods were found to have high levels of haplotype diversity coupled with low nucleotide diversity. Mitochondrial phylogeographic structuring was found for one of the chiltoniid species, which was not seen in nuclear markers, consistent with an environment that is not homogeneous rather than a stable geographic barrier separating two demes. Signatures of population expansion and isolation by distance in two out of three species, match previous findings for diving beetles at the same site. Isolation of populations in pockets within the calcrete, followed by expansion events, are proposed as the most likely generator of population genetic diversity, with restricted gene flow across the calcrete common to both stygobitic amphipods and beetles. There was no evidence for common population fragmentation events during the evolution of amphipods in this portion of the calcrete.

Introduction

Understanding how speciation occurs remains a fundamental problem in biology (Coyne and Orr, 2004). We are interested in what processes lie behind the patterns of species diversity seen in stygofauna, and in particular subterranean diving beetles (Dytiscidae), from the multiple subterranean groundwater calcretes in central Western Australia. The observation that individual calcretes can contain endemic diving beetles in separate size classes that are sympatric sister species (Cooper *et al.*, 2002; Leys *et al.*, 2003), has led to investigation of the

potential for sympatric speciation within individual aquifers. Comparative phylogeographic analyses of stygofauna have been undertaken from a single calcrete aquifer in order to generate baseline data for population genetic structuring and investigate geographic modes of speciation within the calcrete.

Obligate subterranean groundwater species (stygofauna) generally form fragmented populations, caused by geographic barriers to gene flow in karst and hydrological factors of their aquatic environment (Zasek *et al.*, 2009). The result of such population fragmentation is that many stygobionts have very small distributions (Humphreys, 2008; Trontelj *et al.*, 2009). It is thought that cave species most likely evolved from independent colonisation events from the surface (Sbordoni *et al.*, 2000). Cases where species appear to have evolved within a cave locality are rare, with multiple sympatric sister species of dytiscid diving beetles from the calcretes of the Yilgarn region of central Western Australia appearing to be an exception (Cooper *et al.*, 2002; Leys *et al.*, 2003; Guzik *et al.*, 2009).

Population fragmentation leads to a distinct pattern in gene trees which is likely to be shared by organisms experiencing the same diversifying forces (Avise, 2000). Reconstructing population history of multiple taxa from the same community can enable the underlying processes, that have caused the current patterns of biodiversity, to be better understood (Bermingham & Moritz, 1998; Avise, 2000). The comparative phylogeography approach has been used as a means of identifying refugia and evidence of population expansion, with Carnaval *et al.* (2009) predicting high genetic diversity and strong population structure within populations in refugia relative to unstable areas which are recently colonized. Many phylogeographic studies of stygobiontic organisms have been undertaken at a regional scale, with the aim of understanding the evolutionary processes that have shaped broad distribution patterns (Cooper *et al.*, 2007; Lefébure *et al.*, 2007; Carlini *et al.*, 2009; Finston *et al.*, 2009). A few studies have focused on population genetic structuring at the level of individual caves, such as those for *Astyanax* fish in Mexico (Strecker *et al.*, 2003) and subterranean crayfish in south-eastern United States (Buhay & Crandall, 2005), and at the level of an individual calcrete aquifer for subterranean diving beetles in the Yilgarn region of central Western Australia (Guzik *et al.*, 2009).

The Yilgarn craton covers 720 000 km^2 and has been above sea level since the Palaeozoic, representing a long, stable non-marine period of geological time (Morgan, 1993; Humphreys, 2001). Calcrete deposits were thought to have been precipitated from groundwater 37-30 Mya along paleodrainage channels, with karst features forming and groundwater flow activated 30-10 Mya (Morgan, 1993). Over 200 major calcretes are found

in the Yilgarn and they can be between 2 and 200 km^2. The calcretes provide habitat for aquatic invertebrates in this arid region, as they are lenses of porous limestone, approximately 10 m thick, filled with groundwater. Between 14-5 Mya, onset of aridity caused drying up of permanent surface waters (Li & McGowran, 2000), and active colonization of the sub-surface calcretes by epigean aquatic species is proposed (Humphreys, 2001). Opportunities for dispersal between calcretes are limited, and molecular phylogenetic studies of Yilgarn stygofauna ,including crangonyctoid and chiltoniid amphipods (Cooper *et al.*, 2007), dytiscid diving beetles (Cooper *et al.*, 2002; Leys *et al.*, 2003), *Haloniscus* isopods (Cooper *et al.*, 2008) and parabathynellids (Guzik *et al.*, 2008), have shown monophyletic mitochondrial DNA (mtDNA) lineages restricted to individual calcretes. These data support the hypothesis of multiple colonisations by epigean ancestors, followed by isolation of macro-invertebrate populations within single calcretes. As a result we can study speciation of stygofauna, without the confounding influence of gene flow from related populations in different calcretes.

Subterranean diving beetles from the Yilgarn calcretes are the most extensively studied of the Yilgarn stygofauna in terms of their taxonomy and evolutionary biology, as they are incredibly diverse in this region. Ninety-five species have been described formally, with usually between one and three endemic species that are in non-overlapping size classes found in individual calcretes (Watts & Humphreys, 1999, 2000, 2001, 2003, 2004, 2006, 2009; Cooper *et al.*, 2002; Leys & Watts, 2008). Molecular clock estimates date the divergence of the diving beetle lineages to 10-5 Mya (Leys *et al.*, 2003; Guzik *et al.*, 2009). Evolution of these diving beetles into obligate subterranean species is thought to have resulted from their physical isolation in individual calcretes, with new species forming due to allopatric divergence (Cooper *et al.*, 2002; Leys *et al.*, 2003; Guzik *et al.*, 2009).

Phylogenetic analysis using mitochondrial DNA identified 13 cases of sympatric sister species of large and small diving beetles in single calcretes (Cooper *et al.*, 2002; Leys *et al.*, 2003; Leys & Watts, 2008). These diving beetle species could be the result of repeated colonisation events, with speciation in isolation. However, this process is thought to be unlikely, due to the multiple independent cases of sympatric sister species, and given the number of ancestral species predicted to have been available for colonisation (Leys *et al*. in revision). The presence of sympatric sister species that are different sizes, raises the potential for sympatric speciation to have occurred in the system with divergence as a result of ecological niche partitioning. Empirical studies that lend support to sympatric speciation as a result of ecological specialization are few, but come mostly from island systems (Savolainen

et al., 2006) or isolated lakes (Schliewen *et al.*, 1994; Barluenga *et al.*, 2006; Olafsdottir *et al.*, 2007). Coyne and Orr (2004) state that for sympatric speciation to be proposed, species need to be sympatric, sister species that are reproductively isolated, and there be no period of allopatry in their evolutionary and biogeographic history. There is the potential for fragmentation of populations or micro-allopatry to have caused speciation in diving beetles within the calcrete, if populations become isolated due to water level fluctuations, salinity gradients or by calcrete fission or fusion, with subsequent gene flow resulting in present day range overlap. Guzik *et al*. (2009) have tested this scenario in a sister triplet of large and small diving beetles (*Paroster macrosturtensis, P. mesosturtensis, P microsturtensis*) from a single calcrete at Sturt Meadows, via comparative phylogenetic analyses of their mtDNA. High haplotype diversity was found to be a result of isolation by distance within the calcrete, and micro-allopatry could not be ruled out as a potential diversifying force. There was no evidence for long term allopatric processes operating in the diving beetles, i.e. enough to lead to reciprocally monophyletic groups of mtDNA haplotypes in different parts of the calcrete. However, it is possible that any allopatric fragmentation process that may have led to the evolution of the three beetle species may not have occurred again after speciation, and comparison with other stygofauna from the same area is required to identify isolation events that could have led to their speciation.

The Sturt Meadows calcrete also contains three cryptic species of chiltoniid amphipod that belong to separate evolutionary lineages (Chapter III, Bradford *et al.*, 2010). The Chiltoniidae amphipod lineage is thought to be between 50-70 Mya old based on molecular clock analyses (R. Leys unpublished data), and ancestral species may have colonized the calcrete at an earlier or similar time point to the dytiscid beetles, while surface waters were still present. In this study a comparative phylogeography of chiltoniid amphipods at the same field site has been undertaken in order to answer the question: Could fragmentation of populations or micro-allopatry have led to diversification of the diving beetle fauna? The main assumption being that factors affecting dispersal would be very similar among species and higher taxa, and that since surface waters have dried up any speciation has occurred in isolation. Multiple genetic markers were analysed, including mitochondrial cytochrome *c* oxidase subunit I (*COI*) sequence and allozyme data, in order to account for the time any signal of fragmentation may take to develop in the genome. Identification of similar branching in the gene trees of amphipods present at the same site is thought to indicate past barriers to gene flow, and congruent population genetic patterns between stygobitic species indicative of micro-allopatric processes operating within the calcrete.

Materials and Methods

Samples

The Sturt Meadows calcrete (~ 43km^2) can be accessed by a 3.5 km^2 grid of mineral exploration bores (Fig. 1, Chapter III). Analyses of the drill samples show topsoil, clay and vadose calcrete, overlaying phreatic calcrete which is over a clay/sand aquifer (Fig. 3, Chapter II; Anaconda Nickel Limited Technical Report 1251, 2002). Bores were sampled for amphipods between 2003 and 2008 (Allford *et al.*, 2008; Chapter III, Bradford *et al.*, 2010). A total of 253 specimens were collected from 62 bores during this time period.

There are three cryptic species of stygobitic amphipod belonging to the family Chiltoniidae present in the Sturt Meadows calcrete aquifer (Chapter III, Bradford *et al.* 2010). Amphipods collected were sequenced for *COI* (see below) and typed to biological species 1, 2 or 3, based on clustering of groups of haplotypes in a phylogenetic analysis (Fig. 4 in Chapter III). In total, 149 specimens of chiltoniid species 1, 89 chiltoniid species 2, and 15 chiltoniid species 3 were collected, with larger specimens also analysed for allozymes. The amphipod species were found to be sympatric in their distribution, i.e. occupying a number of the same bores (Fig.1). Sample locations with *COI* haplotypes present are listed in Appendix II. Bore hole E12 yielded the most specimens, with 40 individuals collected over successive field trips.

DNA extraction, amplification and sequencing

One leg was removed from each amphipod and DNA extracted using the GENTRA method (Puregene) according to the manufacturer‘s protocol for fresh tissues. The amphipod body was then frozen in liquid nitrogen for allozyme electrophoresis or preserved in 100% ethanol as a voucher for taxonomic description. Partial mtDNA sequences (638bp) from the cytochrome *c* oxidase subunit 1 (*COI*) gene were PCR-amplified using the arthropod universal primers of Folmer *et al.* (1994). PCR amplification was carried out using primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) in 25 µl volumes containing, 4 mM $MgCl_2$,

0.20 mm dNTPs, 1× PCR buffer (Applied Biosystems), 6 pmol of each primer and 0.5 U of Ampli*Taq* Gold (Applied Biosystems). PCR was performed under the following conditions: 94 °C 9 min, then 34 cycles of 94 °C 45 s; annealing 48 °C 45 s; 72 °C, 60 s; with a final elongation step at 72 °C for 6 min. PCR products were purified with a PCR Clean-up DNA purification kit (MoBio Laboratories Inc., Solana Beach, CA) and sequencing was undertaken using the ABI prism Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, CA). Sequencing was carried out on an ABI 3700 DNA analyser and sequences were edited and manually aligned in SeqEd version 1.0.3 (Applied Biosystems). Sequences were aligned by eye and translation to amino acid level was used to verify the correctness of the alignment and the absence of nuclear paralogs.

Network analyses

Genealogical relationships among sequences within each species of amphipod were estimated by constructing statistical parsimony networks in TCS (Clement *et al.*, 2000) with a 95% parsimony limit according to the method of Templeton *et al.* (1992).

Estimation of population genetic structure

The calcrete aquifer at Sturt Meadows is completely enclosed making it difficult to have *a priori* assumptions about any physical barriers to gene flow to help define potential sub-populations. The number of individuals of a single species per bore was generally less than five, so intra-specific comparisons were not made between individual bores due to small sample sizes. Instead of comparing individual bores, spatial analysis of molecular variance, or SAMOVA (Dupanloup *et al.*, 2002), was used to statistically group bores. SAMOVA uses a simulated annealing procedure to find groups of sub-populations that are geographically adjacent and genetically similar, and maximises the genetic differentiation between groups, with the criterion that there is no isolation by distance within groups. The output can be used with the program ARLEQUIN version 3.11 (Excoffier *et al.*, 2005) to estimate the proportion of genetic variation partitioned within and between subpopulations using the fixation indices Φ_{CT} (the variance among groups relative to the total variance), Φ_{ST} (the variance among

subpopulations relative to the total variance) and Φ_{SC} (the variance among subpopulations within groups). The SAMOVA program was run at increasing values of k (number of distinct populations) until a maximum possible was reached (here, the total number of bores present). A plot the of Φ_{CT} values generated in the analysis for different values of k, was undertaken to identify the best model of population structure (bore groups within each species), based on the criteria of maximising Φ_{CT} and where the curve forms a plateau.

Historical demography

Demographic analyses were carried out for the three species of amphipod, in order to determine if similar population processes have shaped their history. The two bore groups within species 1, identified in the SAMOVA analysis, were also included in these analyses to explore the apparent phylogeographic structure present. Genetic diversity indices were calculated using the method of Nei (1987) in ARLEQUIN. Haplotype diversity (*h*), defined as probability that two randomly chosen haplotypes are different in the sample, with standard deviation for the sampling process; nucleotide diversity(π), which is the mean number of differences between all pairs of haplotypes in the sample, with standard deviations for both the sampling and the stochastic processes; and number of polymorphic sites (S) were calculated. Genetic diversity across the bore field was represented by Landscape Shape Plots generated in the program Alleles In Space (AIS; Miller, 2005). The X and Y coordinates on the landscape plot are the midpoints in each edge of the triangulation in a connectivity network between sampling locations, and genetic distance between observations (vertices of the triangles) is represented as surface heights (Miller, 2005). Population comparisons of genetic structure within species 1 were made by AMOVA in ARLEQUIN for the SAMOVA defined groups.

Summary statistics that utilize different sequence information were estimated in ARLEQUIN to look for evidence of population expansion or selection. Mismatch distributions of pair-wise distances between haplotypes were derived and compared to a simulated distribution with parameters estimated under the sudden expansion model. Population growth leaves a characteristic signature in the distribution of nucleotide site differences between pairs of individuals and a uni-modal Poisson distribution was considered to be a sign of population growth (Rogers & Harpending, 1992). Tests of selective neutrality undertaken were Tajima‘s D, which uses information of mutation frequency with an excess of

singletons indicative of population expansion or selection (Tajima, 1989) and Fu's *Fs* which uses information from the haplotype distribution (Fu, 1997). R_2, a test statistic which is based on the difference between number of singletons and average number of nucleotide differences (Ramos-Onsins & Rozas, 2002), was calculated using the program DnaSP version 4.50.3, with 10 000 coalescent simulations under a neutral infinite-sites model (Rozas *et al.*, 2003). Both Fu's Fs and R_2 have been found to be the most powerful statistical tests for detecting population growth under a range of different population growth scenarios, assuming no selection (Ramos-Onsins & Rozas, 2002).

Isolation by distance (IBD; Wright, 1843) was investigated using the Mantel test (Mantel 1967) in AIS, following the approach of Slatkin (1993). This test examines any significant association between genetic (uncorrected (p) distances between sequences) and geographical distance matrices of observations across the landscape.

Allozyme population study

Allozyme electrophoresis was performed on cellulose acetate gels (Cellogel©; MALTA) and stained for enzyme activity according to the principles and procedures of Richardson *et al.* (1986). To ensure that homogenates exhibited adequate enzyme activity after electrophoresis, only specimens > 3 mm were selected. The initial allozyme screen of amphipods from the Sturt Meadows calcrete found chiltoniid species 2 to be largely homozygous at multiple loci and only six specimens of species 3 were suitable for allozyme electrophoresis (Chapter III, Bradford et al., 2010), so the population study focussed on loci found to be polymorphic in chiltoniid species 1. An additional 41 amphipod specimens (> 3 mm) were collected for the allozyme population study to try to increase sample sizes to the level of those in the mtDNA dataset. When genotyped based on their allozymes (Chapter III, Bradford et al., 2010), 34 were found to be species 1, six were species 2 and one was species 3. Of the 26 enzymes that displayed banding patterns that enabled allozymic interpretation (Chapter III, Bradford et al., 2010), 11 were polymorphic at the population level for chiltoniid species 1 (ENOL, G6PD, GOT1, GOT2, GPI, IDH, MPI, PGM, PEP-A2, PEPB, and TPI). In total, 117 specimens of chiltoniid species 1 from 30 bores were subsequently stained for these allozymes. To enable an inter-specific comparison of heterozygosity estimates across the bore hole grid, 15 specimens of chiltoniid species 2 from seven bores, and seven specimens of species 3 from five bores, were also analysed at these loci. Details of enzyme and locus abbreviations,

enzyme commission numbers, electrophoretic conditions and stain recipes are presented in Richardson *et al.* (1986) or Wallman and Adams (2001). Allozymes were designated alphabetically and multiple loci, where present, were designated numerically, in order of increasing electrophoretic mobility. Allele frequencies were calculated as detailed in Murphy *et al.* (2008).

Estimation of population genetic structure from the chiltoniid species 1 allozyme dataset

Statistical evidence for departure from Hardy-Weinberg expectations or linkage disequilibrium at the four individual sites with good sample sizes (n > 10; B4, B11, F4, H2), and for the meta-population (all individuals with n > 2), was determined using the computer program GENEPOP version 3.4 (Raymond & Rousset, 1995). Population genetic structure between individual bores where n > 10 was investigated by estimating pair-wise F_{ST}'s in ARLEQUIN. Statistical significance was determined at the $P < 0.05$ level, with Bonferroni correction for multiple tests (Bonferroni, 1936). For the meta-population, genetic structure within and between individual bores, and mtDNA SAMOVA groups, was evaluated by AMOVA in ARLEQUIN.

Population genetic structure within chiltoniid species 1 was investigated without assuming *a priori* population groupings using two approaches. STRUCTURE version 2.2 (Pritchard *et al.*, 2000) was chosen as a spatially non-informative clustering method, and GENELAND (Guillot *et al.*, 2005a; Guillot *et al.*, 2005b) as a spatially explicit method that utilises the geographic location of individuals within the clustering algorithm. Both these methods do not incorporate any prior information on number of populations. This is important in the calcrete environment, where barriers to gene flow are difficult to identify and actual underground distance between sample locations is not known. Not using any pre-defined structure also enabled an independent search for populations for comparison with the mtDNA dataset results.

The model based method implemented in STRUCTURE uses a Bayesian approach to cluster individuals based on genotypes at multiple loci and finds groups of individuals (for different values of k, the predefined number of groups (K)) where loci are not in linkage disequilibrium. The log probabilities of the data given K[ln $P(X/K)$] were calculated for each value of K, as well as the second order rate of change of the likelihood function with respect

to *K* (Evanno *et al.* 2005). The true number of populations can be identified in an *ad hoc* manner, using the maximum value of the likelihood returned by STRUCTURE. Five independent simulations were run for each value of k (1–8), with 1 000 000 Markov Chain Monte Carlo (MCMC) iterations carried out after a burn-in of 500 000. The admixture model was used with correlated allele frequencies, a combination that was found by (Falush *et al.*, 2003) to be best for detecting subtle population structure. Mean maximum likelihood was plotted against population size to determine the number of populations in the data.

The program GENELAND (Guillot *et al.*, 2005a; Guillot *et al.*, 2005b) uses Bayesian inference of the parameters of the spatial model through the MCMC algorithm to identify clusters in individual multi-locus genetic data. The number of populations (k) was searched for in the clustering and geographic location was included in the analysis. The correlated allele model was used, as allele frequencies tend to be similar in different populations, and this model is recommended for detecting subtle population structure. The MCMC chain was run multiple times for 1 000 000 iterations in order to estimate k, with uncertainty on coordinates set to 50 m as a conservative estimate of home range size. Two populations were found in the clustering. Ten runs were then carried out with k fixed (k = 2) and the one with the highest log posterior density chosen to best represent the pattern in the data. The initial peak in log posterior density in the best run was used to give a burn in time of 200 000 generations. A contour map of the posterior mode of population membership was drawn to visualize population boundaries within the study site, and F statistics (Wright, 1931) calculated by GENELAND were used as a measure of genetic structuring. Principle coordinates analysis (PCO) was carried out using PATN (pattern analysis package, DOS version; Belbin, 1994) on a pair-wise matrix of Rogers (1972) genetic distance, to explore the genetic affinities of individuals from the allozyme study, based on the two GENELAND populations. The Mantel test for IBD was carried out in AIS, as above.

Results

Species distribution

Chiltoniid species 1, 2 and 3 have overlapping distribution patterns, with all three species sympatric in two (E5 and E12) out of the 62 bores where amphipods were collected and 19 bores contained two or more amphipod species (Fig. 1). Distribution was patchy for all three

species, with chiltoniid species 1 found in 45%, species 2 in 17% and species 3 in 10% of bores.

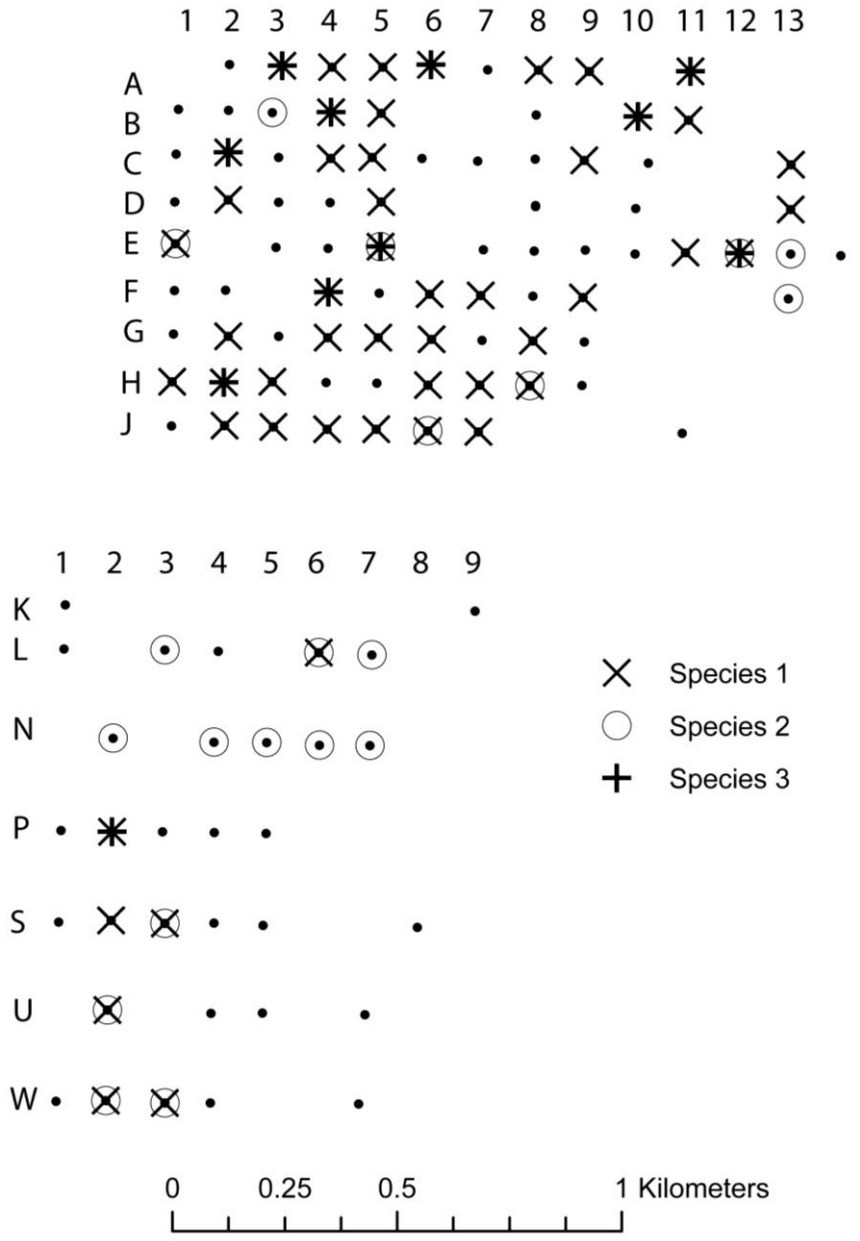

Fig. 1 Geographical distribution of chiltoniid species 1, 2 and 3 across the bore hole grid. Location in the grid is identified by a letter followed by a number.

Haplotype networks

Partial *COI* sequences of 638bp were obtained for the 253 samples in the mtDNA dataset and all could be translated into amino acids with no unexpected stop codons. Two hundred and ten sites were found to be parsimony informative. Sequences were AT rich (A = 0.26, C =

0.15, G = 0.20, T = 0.40), as has been observed in other invertebrate mtDNA (Guzik *et al.*, 2009).

Haplotype networks showed that there is some population genetic diversity in the amphipods at this fine spatial scale. Networks were similar for species 2 and 3 (Fig. 2B, C), with both having one common haplotype distributed across the sampled landscape which appears internal in the network and is thus hypothesized as being ancestral (Clement *et al.*, 2000). Phylogeographic structure was found for species 1, with two major haplotypes separated by nine mutational steps found roughly in the eastern and western parts of the bore hole grid (Fig. 2A). A radiation of singletons could be seen in the network tips for species 1, from both of the common haplotypes (Fig. 2A), and for species 3 (Fig. 2C), indicative of a recent expansion event, or a locus undergoing selection.

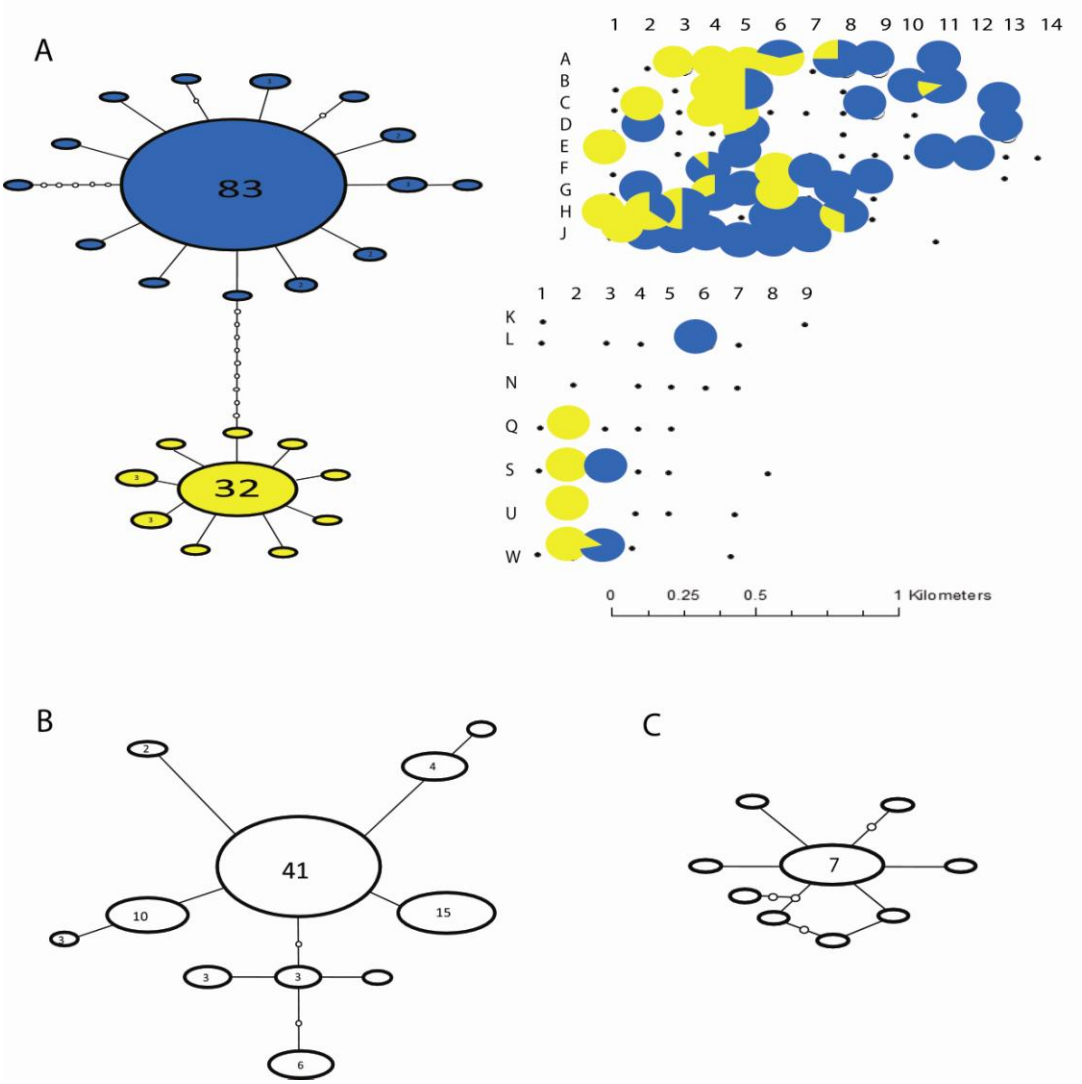

Fig. 2 Statistical parsimony networks of mtDNA diversity.
A. Chiltoniid species 1, **B.** species 2 and **C.** species 3. Small open circles indicate unsampled haplotypes and numbers in the elipses are the sample size where greater than one. Colour in the circles represents the major haplotype groupings for chiltoniid species 1.

SAMOVA analysis was undertaken to estimate the optimal number of populations from the genetic data of species 1 and 2, with species 3 excluded from the analysis due to small sample size. Species 2 showed no levelling off of Φ_{CT} with increasing number of populations, suggesting population structuring at the level of individual bores (Fig. 3A). For species 1, Φ_{CT} reduced after k = 2, suggesting that one or two populations is the optimal population structure for partitioning the genetic variation (Fig. 3A). SAMOVA group membership largely corresponded with that seen for haplotype group 1 or 2 across the bore field (Fig. 2A), with bores containing both major haplotypes assigned as being more like SAMOVA group 1 or 2 in this analysis (Fig. 3B).

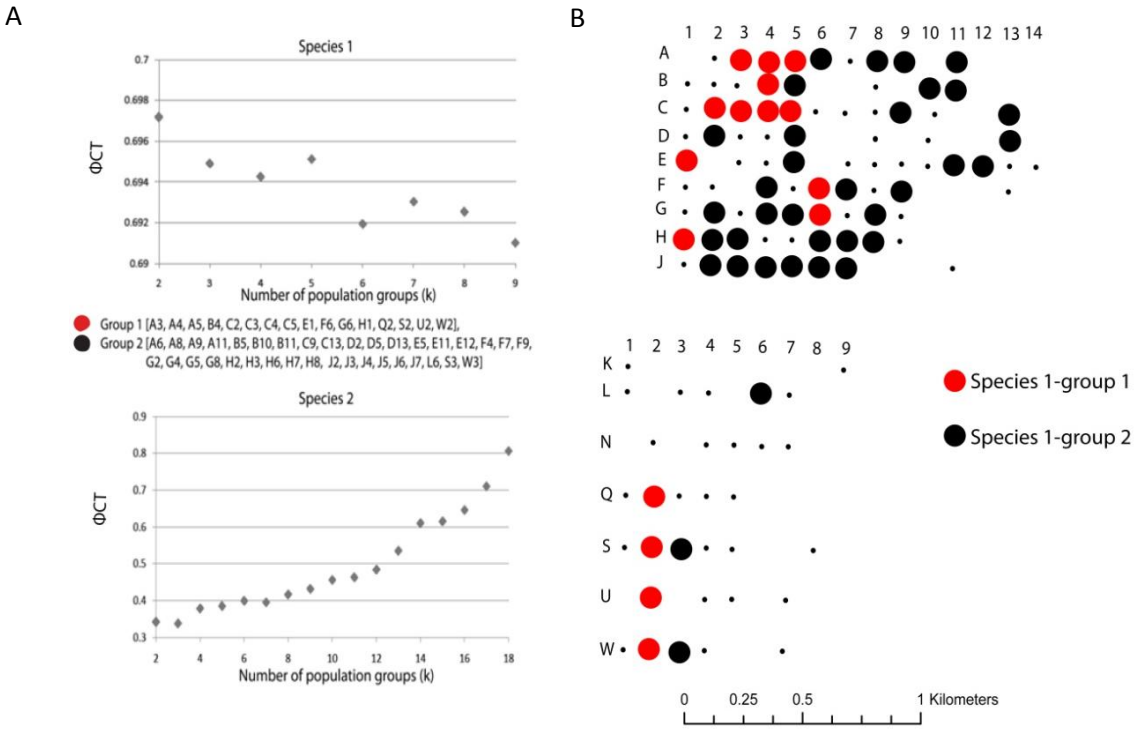

Fig. 3 Results of a SAMOVA analysis of chiltoniid species 1 and 2.
A. Plots of Φ_{CT} values for each value of k (the overall number of populations) for species 1 and 2. The assignment of bore hole sites to groups for k = 2 in species 1 is shown below the top graph. **B.** The distribution of the two groups (indicated by red and black circles) of species 1 across the bore hole grid.

Strong genetic structure was found within species 1, with a significant Φ_{ST} value of 0.71 (Table 1). Most of the variation was found among the SAMOVA bore groups, with the remainder within individual bores.

Table 1 AMOVA for the two SAMOVA bore groups of amphipod species 1.

Group	**Source of variation**	**Sum of squares**	**Variance components**	**% of variation**	**Fixation Indices**
Species 1	Among bore groups	158	4.014 Va	69.72	$\Phi_{CT} = 0.70$*
	Within bore groups	93.98	0.075 Vb	1.3	$\Phi_{SC} = 0.01$*
	Within bores	161.84	1.668 Vc	28.98	
	Total	413.81	5.757		$\Phi_{ST} = 0.71$*

*significant at the $P < 0.05$ level

Overall mtDNA diversity for each amphipod species, and within species 1 for the two SAMOVA groups, is summarized in Table 2. Species 1 was found in the largest numbers across most bores, with correspondingly the highest haplotype diversity and polymorphic sites of the three species. Levels of nucleotide diversity were low for all species examined, with species 1 at 0.009, slightly higher than species 2 and species 3 at 0.003. In contrast to the low levels of nucleotide diversity, haplotype diversity was high and ranged between 0.53-0.80. Thus haplotype diversity was at a similar order of magnitude for each amphipod species.

Table 2 Molecular diversity indices for the three species of amphipod plus species 1 SAMOVA groups. *n,* total number of individuals; S, number of polymorphic sites; *h,* haplotype diversity, π, nucleotide diversity as mean number of pair-wise differences; r, correlation of genetic and geographic distance using the Mantel test. P value in bold is statistical significance at the 0.05 probability level; NS, not significant.

Group	***n***	***n*/ bore**	**# bores**	**# hap**	**S**	***h***	**π**	**r**
Species 1	149	1-14	52	25	37	0.645 +/- 0.038	0.009 +/- 0.005	0.10 **P = 0.001**
Species 1 - group 1	23	1-3	16	8	7	0.715 +/- 0.093	0.001 +/- 0.001	-0.09 **NS**
Species 1 - group 2	126	1-14	36	17	31	0.532 +/- 0.050	0.006 +/- 0.003	0.02 **NS**
Species 2	89	1-35	20	11	12	0.744 +/- 0.040	0.003 +/- 0.002	0.10 **P = 0.006**
Species 3	15	1-2	11	9	10	0.800 +/- 0.108	0.003 +/- 0.002	-0.18 **NS**

The highest level of mtDNA diversity was found between bores in the north-western corner of the bore field and lowest genetic diversity was between bores in the south-eastern corner, a pattern that was shared for all three species (Fig. 4). Species 1, with the largest sample size, showed more pockets of low genetic diversity in the southern end of the bore field, which did not appear to be related to the number of individuals collected from these sites. Sample size was a limitation with species 3, however the bores to the south were more closely related (haplotype 30; Appendix II).

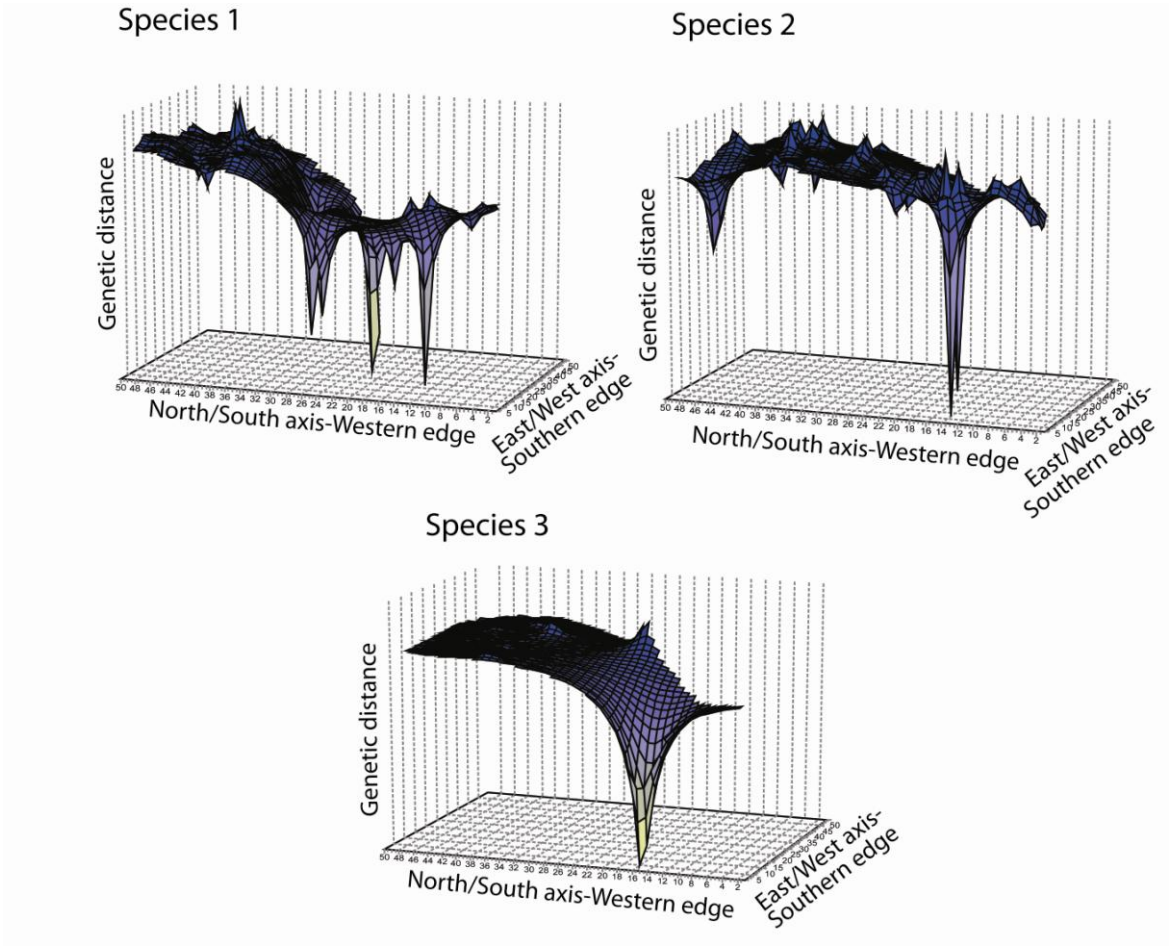

Fig. 4 Spatial patterns of mtDNA diversity across the sampled landscape for the three amphipod species. X and Y axes correspond to midpoints between sampling locations and height represents genetic distances between bores. Large genetic distances are indicated by the dark blue peaks and locations of high genetic similarity by troughs with paler shading.

Isolation by distance, with a significant positive correlation between genetic distance and geographic distance, was found for both species 1 and 2, but not for species 3 (Table 2). Within species 1, there was no evidence for isolation by distance within each of the two separate SAMOVA populations, indicating that gene flow may be restricted between but not within these groups.

Historical demography

Population genetic summary statistics, Tajima's D, Fu's *Fs* and R_2 are presented in Table 3. Negative values found for *Fs* and D for all three species indicate a departure from the Wright-Fisher neutral model (Wright, 1931). However, *Fs,* D and R_2 were significant only for chiltoniid species 3, i.e. it is a population undergoing expansion/selection. Within species 1, the SAMOVA group 1 was significant for all three summary statistics, whilst group 2 was not significant.

Table 3 Tests for demographic expansion for the three species of amphipod. D, Tajima's (1989) D; *Fs*, Fu's (1997) *Fs*; R_2, Ramos-Onsins, Rozas (2002) R_2. P value in bold is statistical significance at the 0.05 probability level; NS, not significant.

Group	**D**		***Fs***		**R_2**	
Species 1	-0.456	**NS**	-2.544	**NS**	0.0858	**NS**
Species 1 - group 1	-1.646	**P = 0.036**	-5.063	**P < 0.001**	0.133	**P = 0.007**
Species 1 - group 2	-0.933	**NS**	-1.191	**NS**	0.088	**NS**
Species 2	-0.852	**NS**	-2.638	**NS**	0.095	**NS**
Species 3	-1.732	**P = 0.032**	-5.313	**P < 0.001**	0.147	**P = 0.008**

The mismatch distribution for species 3 closely matched the Poisson distribution expected for a population undergoing expansion, using a demographic model of sudden expansion (Fig. 5). The hypothesis of recent demographic expansion using the mismatch distribution was rejected for species 2. For species 1, the hypothesis of expansion was not rejected, which is the opposite of what was found with the other summary statistics. When the mismatch distributions for the two populations within species 1 were considered, the hypothesis of expansion was rejected. This is a different result to what was seen with the other equivalent test statistics for species 1-group 1.

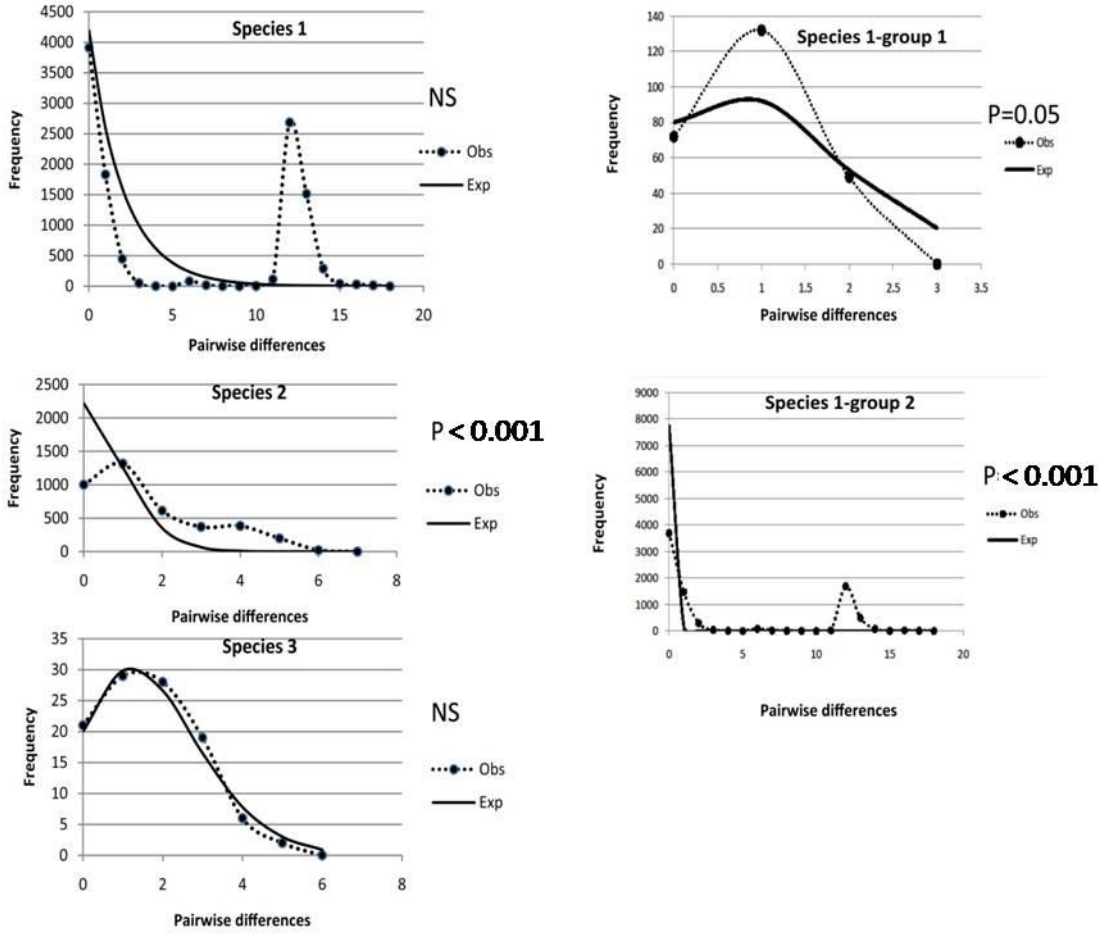

Fig. 5 Mismatch distributions of pair-wise differences between haplotypes for the three species of amphipod and the two populations of species 1. The frequency distribution of observed pair-wise differences is shown as a dotted line and the distribution estimated under the sudden expansion model is represented by a solid line. Null hypothesis is that the distribution fits a model of expansion. P value is statistical significance at the 0.05 probability level; NS, not significant.

Allozyme allele frequencies were scored at 11 loci and found to be polymorphic for chiltoniid species 1 in the initial overview screen. Of the 117 individuals screened for species 1, number of samples per bore varied between one and 14, with 10 out of the 30 bores having only one individual sampled. The allele frequencies at these 30 sites for species 1 are presented in Appendix IIIA. Average number of alleles per locus was six, with number of alleles ranging from three (ENOL, TPI, GPT) up to 13 (GPI). Heterozygosity estimates were high in each bore, and varied between 0.167 -0.583 across the sites. Results for the small allozyme dataset of chiltoniid species 2 (n = 15) and 3 (n = 7) are presented in Appendix IIIB. Species 2 was largely homozygous, with an average number of alleles per locus of 1.6 and heterozygosity levels of 0-0.09 for the different sites. Species 3 showed greater genetic variation at these loci, with an average of 2.6 alleles per locus. Heterozygosity levels for species 3 of 0.29-0.37 were more in the region of those seen for species 1.

When assessing genotype frequencies in species 1, there was no statistical evidence for departure from Hardy-Weinberg equilibrium (HWE) at $P < 0.05$ or linkage disequilibrium at the individual sites with good sample sizes ($n > 10$; B4, B11, F4, and H2). For the entire population of species 1, there was significant departure from HWE at $P < 0.05$.

Pair-wise differences at the level of individual bores with $n > 10$ in species 1, found B4 to be significantly different from H2, F4 and B11 (Table 4). F4 and B11 were not significantly different from each other in this analysis. H2 was significantly different from B11, but not from F4 when Bonferroni's adjustment was applied.

Table 4 Pair-wise F_{ST}'s between bores > 10 individuals for chiltoniid species 1

	B4	**B11**	**F4**	**H2**
B4	0			
B11	0.1433*	0		
F4	0.10322*	-.0144	0	
H2	0.14549*	0.0659*	0.0339	0

*significant at the $P < 0.05$ level, with Bonferroni correction

Analysis of the whole species 1 allozyme dataset using STRUCTURE was unsuccessful as determination of k was found to be ambiguous. Likelihood values fluctuated with increasing values of k, and an indication of the number of clusters could not be gained for our dataset with this method.

Analysis of the whole species 1 allozyme dataset using GENELAND showed two populations, with bores A3 (n = 1) and B4 (n = 12) genetically distinct from the other sites when all 11 loci were analysed using the correlated allele model (Fig. 6). Thus, B4 appears to be a genetically distinct population, both by spatial clustering analysis on the whole dataset and pairwise differences between individual bores with good sample sizes . Great genetic differentiation was found for species 1 allozyme loci, with a F_{ST} value of 0.18 (Wright, 1978). Principle coordinates analysis showed the two GENELAND populations as overlapping groups (Fig. 7). There was no evidence for IBD across the bore hole grid in the species 1 allozyme data, with the correlation between geographic and genetic distance in the Mantel Test not significant (r = 0.04).

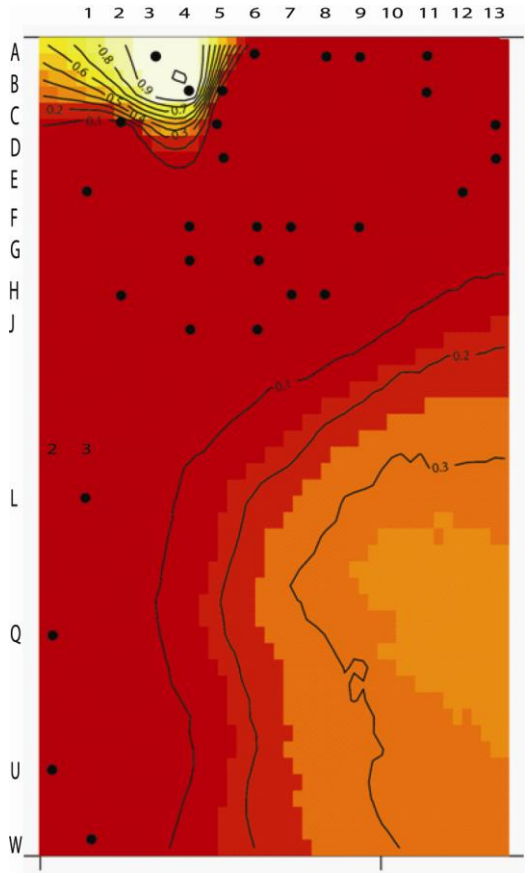

Fig. 6 Map of posterior probabilities of population membership (number of GENELAND populations = 2) for chiltoniid species 1. Lighter areas correspond to higher probability to belong to population 1, and bores where individuals sampled for allozymes came from are indicated with black dots.

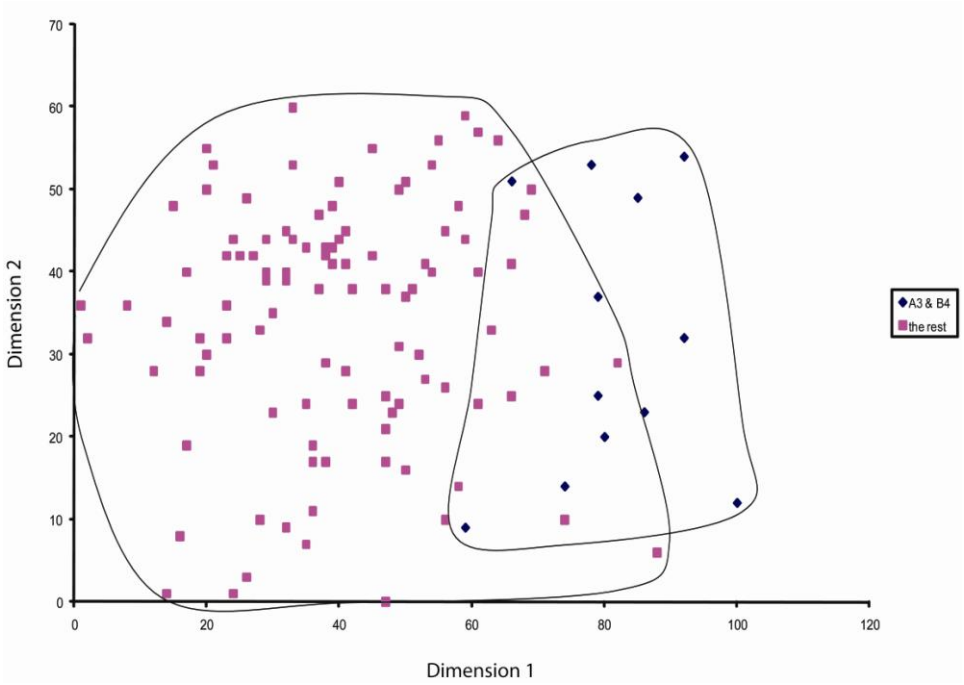

Fig. 7 Principle coordinates analysis for chiltoniid species 1 GENELAND populations. The relative PCO scores have been plotted for the first (X-axis) and second (Y-axis) dimensions.

SAMOVA mtDNA groupings did not explain the genetic variation in the species 1 allozyme dataset, with the AMOVA showing only 4.29% of the genetic variation being between SAMOVA bore groups (Table 5). The majority of the genetic variation was at the level of individuals. There was some sub-structuring in species 1, based on allozyme data, with a F_{IT} value of 0.12. There was quite high among group variation in the allozyme data for such a small spatial scale, with a F_{CT} value of 0.04. However none of the fixation indicies were significant at the $P<0.05$ level.

Table 5 AMOVA on allozyme data for the two mtDNA SAMOVA groups of chiltoniid species 1.

Group	Source of variation	Sum of squares	Variance components	% of variation	Fixation Indices
Species 1	Among bore groups	10.90	0.1018 Va	4.29	F_{CT} = 0.04
	Within bore groups	42.40	0.1427 Vb	6.01	F_{SC} = 0.06
	Within bores	173.89	0.0433 Vc	1.82	
	Within individuals	192	2.087	87.88	
	Total	419.19	2.3748		F_{IT} = 0.12

Discussion

Potential modes of speciation within the Yilgarn calcrete range from allopatric (a physically divided population) through parapatric (i.e. short periods of isolation and reconnection of populations) to completely sympatric (no extrinsic barrier to gene flow) (Butlin *et al.*, 2008). This study has focussed on evaluating the geographic context of these historical speciation events, by comparing current patterns of population genetic structure between stygofauna that form a closed community within a single calcrete. The three species of chiltoniid amphipod found in the Sturt Meadows calcrete are sympatric (all three can be found in the same bore), though they are patchily distributed across the grid of bores available for sampling. However, in contrast to the beetles, the amphipods are from completely separate evolutionary lineages (Chapter III, Bradford *et al.* 2010), so sympatric speciation is not being proposed in their evolution; rather they are likely to be the result of colonisation of the calcrete by three distinct epigean ancestors. They coexist with the triplet of large and small diving beetles, that are sympatric sister species, studied previously at this site (Guzik *et al.*, 2009). It is possible that during the course of speciation in the beetle fauna from the Sturt Meadows calcrete, there has been both spatial and ecological differentiation (Butlin *et al.*, 2008; Fitzpatrick *et al.*, 2009). Comparison can now be made of population genetic patterning between stygobitic crustaceans (Chiltoniidae) and beetles (Dytiscidae), with congruence in pattern proposed as evidence for common processes that may promote population divergence within the calcrete.

Phylogeographic patterns and demographic events

A spatial population genetic pattern was found only in chiltoniid species 1, based on mtDNA, with two divergent haplotype groupings distributed roughly in the eastern and western parts of the bore field. The two groups overlap in their distribution, and there is strong genetic structuring between them, largely as a result of variation between the bores grouped in the mtDNA SAMOVA analysis. When multiple allozyme loci were examined, there was departure from HWE indicating sub-structuring in the data or selection acting on the different loci. Both landscape genetic analyses across the bore field, and pair-wise differences for individual bores, identified two populations of species 1, which could not be separated by multivariate analysis. This genetically distinct population at the north-western end of the sample site, is a subset of a population identified in the mtDNA analysis. The two allozyme

populations showed subtle population genetic structure, which was remarkable considering the small spatial scale and size and patchiness of the sampling. There was no evidence of isolation by distance in the allozyme data. The smaller effective population size for mtDNA, and generally higher nucleotide substitution rate compared to genes within the nuclear genome, will result in a more rapid response to various population phenomena that influence genetic diversity (e.g. effective population size changes, drift or selection) than a neutral nuclear marker (Zink & Barrowclough, 2008). Different genetic sub-structuring found from allozyme data indicates that though there is either physical or reproductive isolation of populations, there is not an obvious common allopatric barrier across the calcrete.

A similar haplotype network to chiltoniid species 1, with two divergent haplotype groups, was found for the diving beetle *P. mesosturtensis* (Guzik *et al.*, 2009). However, there was no evidence for a spatial separation of haplotype groups, and the pattern was proposed to be the result of stochasticity in coalescence of genes (Guzik *et al.*, 2009). There was a lack of any population genetic structuring in chiltoniid species 2 and 3 (though sample sizes were very low for species 3 and the population analyses may have been compromised to some extent), as well as the other two diving beetle species, across the bore field (Guzik *et al.*, 2009). There is no common spatial pattern that would indicate a geographic barrier to gene flow, and as such no evidence of past allopatry in this portion of the calcrete. However, there are similar population genetic network patterns for both crustaceans and beetles, indicating that the environment is not homogeneous and spatial factors are important within the calcrete.

Signatures of population expansion and isolation by distance were found in two of the three chiltoniid species. The two SAMOVA populations of chiltoniid species 1 have different summary statistics, with group 1(western) undergoing expansion and/or selection and group 2 (eastern) not showing such a pattern. The hypothesis of expansion was rejected for both populations of species 1 based on the mismatch distribution, though when the groups were combined the hypothesis of expansion was not rejected. Lack of evidence for non-neutral processes in group 1 seen in the other tests coincides with findings by others that the mismatch distribution is a weaker test for detecting population growth (Ramos-Onsins & Rozas 2002). Chiltoniid species 3 appears to be a population undergoing expansion without IBD. In species 2, genetic variation across the calcrete appears to be the result of IBD, with no significant population expansion. Homozygosity at multiple allozyme loci suggests that the population may have been through a bottleneck. Two out of the three species of diving beetles also showed isolation by distance, with signatures of population expansion in all three species (Guzik *et al.*, 2009). Comparison of the historical demography of these multiple

stygobitic species suggests that the calcrete environment is dynamic, with changes in water levels and salinity perhaps being key drivers in the demographic changes in populations.

Chiltoniid species 3 could either be rare or at the edge of its range and more abundant in other areas. Signatures of population expansion with the absence of IBD, suggests that the bore field is at the edge of its range. The site has been extensively sampled over a three year period in all seasons, so this restricted distribution is unlikely to be the result of the sampling regime but rather reflects its abundance in the bore field. This may be an indication of different micro-habitat preferences between the three species of amphipod, however no morphological differences have been identified among taxa that might indicate occupation of separate ecological niches. It is perhaps more likely that patchy distribution coupled with extinction/colonisation dynamics in the calcrete allow persistence of all three species, despite having very similar habitat preferences. Different micro-habitat preferences have been found in subterranean amphipods from the Pilbara (north-western Western Australia), with *Nedsia* sp. associated with alluvial sediments, but *Pilbarus millsi* restricted to areas of higher calcrete composition in the same catchment (Finston & Johnson, 2004). However, lack of available bores across the entire Sturt Meadows calcrete makes it impossible to directly examine any areas abundant in species 3 to infer habitat preferences.

Genetic diversity and distribution across the bore field

Stygobiont amphipods at Sturt Meadows all had very low levels of nucleotide diversity, but high haplotype diversity, as also found by Guzik *et al.* (2009) for the dytiscid beetles in the same calcrete. This pattern can result from population bottlenecks followed by range expansion leading to an excess of new mutations, with singletons largely responsible for the high haplotype diversity (Avise, 1994). Range expansion followed by periods of isolation has also been identified as a mechanism for increased genetic variation in subterranean crayfish (Buhay & Crandall, 2005). These results support the theory of Barr (1968) that after the initial bottleneck during colonisation of the subterranean environment, range expansion and population growth can lead to high genetic diversity in cave fauna. There is evidence from other studies that cave populations can have higher genetic diversity (Buhay & Crandall, 2005; Verovnik *et al.*, 2005), or lower genetic diversity related to population bottlenecks (Strecker *et al.*, 2003; Carlini *et al.*, 2009), compared to surface populations of the same

species. However, no surface populations of the Sturt Meadows chiltoniid species exist for such a comparison.

There was concordant variation in the distribution of genetic diversity for the amphipods across the calcrete, with low genetic diversity in the south. Analyses of the drill cores shows the calcrete to be a lot thicker in the north of the site (>/= 11 m) compared to the southern sites in which the calcrete was 5-8 m thick (Fig. 4 in Chapter II; Anaconda Nickel Limited Technical Report 1251, 2002). It is possible that when the water table is low, populations may go extinct there and the lower genetic diversity is indicative of recent range expansion at a time of higher water table. The diving beetles also showed spatial variation in genetic diversity, again with troughs in the southern sites. However, diversity patterns were not concordant among sister species of diving beetle, potentially as a result of competition for resources and/or habitat between the two small species (Guzik *et al.*, 2009). The Sturt Meadows diving beetles have a very different life style to the amphipods. They are poor swimmers, crawling on the calcrete rock and surfacing for air, whilst the amphipods are free-swimming, with no requirement to come to the surface. Rather, they seem to be associated with the presence of tree roots on which they are likely to graze. Patchiness in distribution of the amphipods and beetles across the bore field may reflect scarce food resources in the subterranean environment and interconnectivity of the calcrete, with potentially some large open spaces. Recharge of the aquifer occurs after heavy rain (Allford *et al*. unpublished data), resulting in seasonal variation in level of the water table and presumably access to different areas of the calcrete. Pleistocene climate fluctuations (Byrne *et al.*, 2008) could also have influenced water levels in the aquifer and population genetic diversity. It is hypothesized that these water level changes led to frequent isolation in small areas resulting in population bottlenecks and, when followed by range expansion after recharge, could result in the high haplotype diversity seen in all three chiltoniid species and the very high haplotype diversity in the diving beetles.

Modes of speciation within a single calcrete

Under the hypothesis of pure allopatric speciation in the diversification of the diving beetles at Sturt Meadows, we would expect evidence for three isolation events in other co-distributed species. It cannot be known how long the amphipods have been present in the calcrete, however, unlike the beetles, their divergence pre-dates formation of the calcretes (R. Leys

unpublished Chiltoniid phylogeny) and the assumption is that they have always had some range overlap. There were no common fragmentation patterns evident from the mtDNA phylogeographies of the three amphipod species, suggesting that no long-term barrier to gene flow has been present in this portion of the calcrete.

Congruence between the amphipods and the beetles at Sturt Meadows comes from analysis of historical demography and molecular diversity indices. The common pattern is of high haplotype diversity coupled with low nucleotide diversity, with populations undergoing expansion events and/or showing isolation by distance. This congruence among stygofauna in population processes at the same site suggests that isolation is a diversifying force in the calcrete, i.e. micro-allopatry. The ability for gene flow in this portion of the calcrete is evident from the lack of spatial structure in the haplotype networks. However, gene flow and retention of ancestral diversity are hard to tell apart and the future use of microsatellite markers would be a way of differentiating between the two. The spatial pattern evident in one chiltoniid species 1 appears to be the result of isolation with partial gene flow (parapatry). Genetic sub-structuring is also seen in the allozyme data for species 1 indicating that This study has identified that there is some spatial differentiation in the calcrete, with genetic diversity varying across the landscape, and it is hypothesized that isolation in habitable pockets with local extinction events, followed by population expansion, would lead to a patchy population distribution with high haplotype diversity, i.e. multiple independent localized refugia. The identification by Guzik *et al.* (2009) that the Sturt Meadow‘s sister species of beetles are sympatric with range overlap at very fine spatial scale can be considered in itself as evidence for sympatric speciation to have operated in their divergence (Fitzpatrick *et al.*, 2009). However, there is also evidence for micro-allopatry in the diversification of diving beetles and amphipods at the Sturt Meadow‘s site, so the strict criteria of Coyne and Orr (2004) for sympatric speciation cannot be met. Evaluation of ecological differentiation in the diving beetles, which is implied by their different sizes, is required to try and understand the importance of natural selection in the evolution of the diving beetle fauna in the Yilgarn calcretes.

Conclusion

After colonisation of the Sturt Meadows calcrete by three separate lineages of chiltoniid amphipod (Chapter III, Bradford et al., 2010), diversification has continued in isolation,

enabling us to investigate what evolutionary processes have shaped the patterns of genetic diversity that we see today in the calcrete environment. Comparative phylogeography of the amphipods and diving beetles (Guzik *et al.*, 2009) from the Sturt Meadows bore field has provided baseline genetic data for the Yilgarn calcretes, as it is the only within-calcrete population study carried out on this unique system to date. Congruence in spatial genetic variation among the three unrelated lineages of amphipod, which have been isolated for approximately the same period of time, indicates that population processes are constrained by their subterranean habitat. Periods of population isolation followed by range expansion appears to be the main diversifying force in both the chiltoniid amphipods and dytiscid beetles (Guzik *et al.*, 2009), with restricted gene flow indicative of a combination of a structured subterranean environment and the size and mobility of the stygofauna. It is hypothesized that there is a continual process of population contraction and expansion within the calcrete, related to changes in water level, due to episodic rainfall patterns in the region (Leys *et al.*, 2003), with the potential of Pleistocene climate fluctuations (Byrne *et al.*, 2008) having long term effects on distribution of populations and groundwater levels. Patchiness in stygofauna distribution makes it unlikely that the same regions in the calcrete would support populations during dry periods or over a long period of time, and it is proposed that there would be multiple independent localised refugia within the calcrete. Future research on speciation in the sympatric sister species of large and small diving beetles plans to explore any ecological differentiation within the calcrete and spatial differentiation over broader distances.

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IDENTIFICATION OF NATURAL PREDATION EVENTS IN DIVING BEETLES (DYTISCIDAE) FROM A CALCRETE AQUIFER IN THE ARID YILGARN REGION OF CENTRAL WESTERN AUSTRALIA

Paroster macrosturtensis investigating an amphipod

Aim 3 Investigate whether different sized beetle sister species occupy separate trophic niches within a calcrete, by molecular amplification of gut contents with species specific primers

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CHAPTER V

Abstract

Calcrete aquifers from the Yilgarn region of central Western Australia contain a diverse range of obligate groundwater invertebrates (stygofauna), each species endemic to a single calcrete. Modes of speciation that have produced this diversity of species are of interest, in particular the relative roles of allopatric, parapatric and sympatric speciation in the generation of species diversity on the calcrete ‗islands'. Previous phylogenetic analyses, based on mitochondrial DNA sequence data, have identified in separate calcretes 13 independent cases of sympatric sister species pairs of large and small diving beetles (Dytiscidae), suggesting that they may have speciated in sympatry. The possibility of ecological differentiation within the calcrete has been investigated in this study, by examining trophic niche partitioning in a sister triplet of large and small diving beetles (Dytiscidae) from a single aquifer. Diving beetles were sampled from a 3.5 km^2 grid of 115 bore holes. Fragments of the *COI* gene, specific to amphipods (Chiltoniidae) and copepods (Harpacticoida, Cyclopoidea) that are possible prey items, were amplified from the intestines of beetle adults and larvae. There was not complete trophic partitioning in the adult beetles, with all three species feeding on amphipods and copepods. Small sample sizes precluded making similar comparisons between the larvae. It is hypothesized that in the impoverished environment of the aquifer, the adult beetles are scavengers and opportunistic feeders, as well as active predators.

Introduction

The Yilgarn calcrete aquifers in central Western Australia are a biodiversity hotspot for subterranean diving beetles (Dytiscidae), and provide a fascinating system for studying modes of speciation in discrete ‗island' environments where opportunities for dispersal are limited. There is a repeated pattern of calcretes containing two to three beetle species in separate size classes (Cooper *et al.*, 2002; Watts & Humphreys, 2009), suggesting that there is some form of ecological niche occupied by either large or small beetle species that is common to the calcrete environment. The presence of sympatric sister species of large and small beetles in multiple separate calcretes (Cooper *et al.*, 2002; Leys *et al.*, 2003; Leys & Watts, 2008) raises

the potential that a sympatric mode of speciation (divergence with gene flow) has operated within the calcrete, with divergence due to ecological differentiation (Fitzpatrick *et al.*, 2009). The aim of this study is to investigate the potential for sympatric speciation to have occurred in the Yilgarn dytiscid beetles by disruptive natural selection as a result of trophic niche partitioning.

The Dytiscidae are predaceous diving beetles with a worldwide distribution that commonly occur in slow moving or stagnant water, living in the gravel or mud at the margins of lakes and ponds, bodies of water in caves, temporary pools, slow-moving rivers or in lakes (Hawkeswood, 1987). Adults are smooth with a streamlined, oval body and flattened hind legs, which they move simultaneously when swimming (Lawrence & Britton, 1994). Dytiscids undergo complete metamorphosis, with distinct larval, pupal and adult stages, and eggs are usually deposited out of water (Lawrence & Britton, 1994). Larvae have large heads with grooved mandibles, elongate bodies and fringed swimming legs and grow through several moults resulting in three different instars. (Hawkeswood, 1987)

Dytiscids prey on all forms of aquatic life (Larson *et al.*, 2000). Adults bite off fragments of their prey and their mandibles are adapted for chewing (Hawkeswood, 1987). Deding (1988) carried out gut content analysis of large and small dytiscids and found that prey size was proportional to beetle body length, with some prey items being over-represented in the gut contents when compared to availability in the pond, indicating food preferences. Dytiscid larvae are very effective predators, and will attack animals much larger than themselves. They are external feeders that take liquid food through a narrow groove along the inner margins of their mandibles, sucking up the body fluids of their prey, or the remains that they have liquefied with digestive enzymes (Hawkeswood, 1987).

Diving beetles that are exclusively subterranean have been described from many areas around the world (U'eno, 1957; Castro & Delgado, 2001; Watts & Humphreys, 2009). Adaptation to life underground has resulted in features common to stygobitic dytiscids, including reduced eyes, lack of pigmentation, thin exoskeleton, absent or vestigial metathoracic flight wings, and longer and more abundant setae (Spangler, 1986). Little is known about the ecology of stygobitic diving beetles. Castro and Delgado (2001) observed the stygobitic dytiscid *Iberoporus cermenius* for three months in aquaria and found that they fed on both live and freshly dead animals, with group feeding occurring rapidly after one beetle had discovered its prey. U′eno (1957) also observed stygobitic dytiscids in aquaria feeding on live (copepods) and dead food (amphipods, isopods). To date there is no record of

stygobitic diving beetles breeding in aquaria, so understanding of their oviposition, larval food habits, and their pupation is based on that seen in their surface relatives.

Phylogenetic studies on the diving beetles from the Yilgarn calcretes imply a scenario of invasion of the subterranean environment by several surface dwelling ancestors during a period of aridification 10-5 mya (Cooper *et al.*, 2002; Leys *et al.*, 2003; Guzik *et al.*, 2009). It is hypothesized that as surface waters dried out, diversification has occurred in the numerous isolated calcretes in the region, which are lenses of porous limestone filled with groundwater (Humphreys, 2001a). The outcome is reflected in the remarkable number of new species of diving beetle (95), which are part of the subterranean biodiversity recently recorded from groundwaters under the desert of Australia (Watts & Humphreys, 1999, 2000, 2001, 2003, 2004, 2006, 2009). Within individual calcretes a pattern has been observed of large and small sympatric sister species of diving beetles, based on mitochondrial DNA analyses of beetles found from 13 separate calcretes (Cooper *et al.*, 2002; Leys *et al.*, 2003; Leys & Watts, 2008). Plausible hypotheses for how speciation has occurred range from micro-allopatric divergence due to historical barriers to gene flow within the calcretes, through to sympatric speciation due to niche partitioning with disruptive natural selection resulting from competition for resources. Indirect evidence for the potential of micro-allopatry in diving beetle speciation within the calcretes comes from congruent population genetic patterns found in three sympatric sister species of beetles (Guzik *et al.*, 2009) and three unrelated lineages of chiltoniid amphipods (Chapter IV), from a single calcrete aquifer at Sturt Meadows. The potential for ecological differentiation in the Yilgarn dytiscid beetles is currently unknown and requires evidence that large and small beetle species do occupy separate ecological niches within the calcrete.

It is hypothesized that the different-sized beetles, and/or their larvae, could occupy separate trophic niches within the calcrete. Investigation of trophic niches is possible using molecular markers to delineate predator-prey food webs (Symondson, 2002; Sheppard & Harwood, 2005). Polymerase chain reaction (PCR) amplification of gut contents using species-specific primers enables very small fragments of prey DNA to be detected, which is essential when examining the products of digestion (King *et al.*, 2008). This technique requires specific primer design with assay optimization to be confident of detecting the products of digestion without cross-amplification from the predator (King *et al.* 2008). Only known prey items can be analysed by this method, as specific primers have to be designed for each taxon. Detection time in the gut has been found to be inversely proportional to fragment length (Symondson, 2002), and the rate of digestion will influence detection time, with rates differing considerably between predator species from a few hours up to a week (Symondson,

2002; Sheppard & Harwood, 2005). Field collection studies followed by molecular detection methods have successfully identified natural predation events in generalist invertebrate predators in agricultural ecosystems (Agusti *et al.*, 2003; Harper *et al.*, 2005; Read *et al.*, 2006). Whether the species detected in the gut are the result of predation or scavenging cannot be resolved by PCR gut content analysis, because carrion prey can be detected as efficiently as fresh prey items (Juen & Traugott, 2005). Application of this method to the Yilgarn dytiscids would enable us to identify ingestion events (trophic niche) in the different sized species that are difficult to observe in their natural environment, and allow the inclusion of dytiscid larvae, which are liquid feeders, in the analysis.

The single calcrete at Sturt Meadows contains three sympatric sister species of diving beetles which do not overlap in size, namely *Paroster macrosturtensis*, *P. mesosturtensis* and *P. microsturtensis,* with body lengths of 3.6 - 4.1 mm, 1.9 - 2.3 mm, and 1.7 - 1.8 mm, respectively (Leys & Watts, 2008) (see title photo, page 1). The approach in this study is to identify the source of the degraded DNA that is the product of beetle digestion, by designing PCR primers that amplify short group-specific DNA fragments from common potential prey items in this relatively simple subterranean system (in terms of macro-invertebrate species diversity). Barcoding of invertebrates present in the Sturt Meadows calcrete has identified, in addition to the dytiscid beetles, three species of chiltoniid amphipod, four divergent lineages of oligochaete worms, single species of harpacticoid and cyclopoid copepods, and single species of mite, centipede and Collembola that are thought to be troglobiontic (Chapter III; Bradford et al., 2010). This sequence information enables us to identify regions of the mitochondrial *CO1* gene that can serve as group-specific markers. Dytiscid beetles, and in particular their larvae, are likely to be the top predators in the calcrete. Of the potential prey items available, the stygobiontic chiltoniid amphipods and harpacticoid and cyclopoid copepods were chosen for the dytiscid predation study as they are by far the most abundant, and have distinctive size ranges from 1-2 mm for the copepods and 2-5 mm for the amphipods.

Further information on the trophic ecology of the dytiscids has been sought from the stable isotope signatures of the different species and their potential food. Stable isotope ratios of nitrogen and carbon have been determined, to elucidate the food-web in the calcrete and any trophic niche partitioning between the beetle species (Ehleringer *et al.*, 1986; West *et al.*, 2006). By investigating the trophic niche occupied by large and small predaceous beetles, an insight into the food-web of this unusual system, where direct observation is difficult, can be gained, as well as an understanding of whether trophic partitioning could have been a driving

force in dytiscid speciation. By observing these dytiscid beetles in the laboratory, the aim is to add to the information available on the behaviour of subterranean dytiscids.

Methods

Sampling of dytiscid beetles

Adult and larval dytiscids in the Sturt Meadows calcrete aquifer were sampled by haul netting via abandoned mineral exploration bore holes, with 10 hauls of the net through the water column found to be the most efficient collecting method (Allford *et al.*, 2008). Beetles were sorted from other stygofauna present in the calcrete under a microscope in the field and identified to species level (Watts & Humphreys, 2009). At the time of sampling, dytiscid larvae had not been formally described, so they were sequenced for *COI* and typed to species level based on clustering in phylogenetic distance analyses (see below). Diving beetles collected for the feeding trials were transported to the laboratory in rainwater, as the calcrete water quickly became anoxic, even when filtered through a 2 µm membrane. Individual dytiscid adults and larvae were rinsed with rain water to prevent contamination of gut content assays by calcrete water, which would contain decaying macro-invertebrates. Specimens were then blotted dry and killed by snap freezing in liquid nitrogen, as rapid preservation is required to prevent any further decay of the DNA (King *et al.*, 2008).

DNA extraction

Diving beetles and larvae were thawed to room temperature, and whole body DNA extracts were prepared using the DNeasy Blood and Tissue Kit (QIAGEN), according to the manufacturer‘s instructions.

Amplification and sequencing of dytiscid larvae DNA

In order to enable species level identifications of the larvae, polymerase chain reaction (PCR) amplification of a 623-bp fragment from the mitochondrial *COI* gene was carried out with the

universal primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al.*, 1994) in 25 µl volumes containing 4 mM $MgCl_2$, 0.20 mm dNTPs, PCR buffer (Applied Biosystems), 6 pmol of each primer and 0.5 U of Ampli-Taq Gold (Applied Biosystems). PCR amplification was performed under the following conditions: 94 ˚C for 9 min; followed by 34 cycles of 94 ˚C for 45 s, annealing at 48 ˚C for 45 s, and 72 ˚C for 60 s; with a final elongation step at 72 ˚C for 6 min. PCR products were purified with a PCR Clean-up DNA purification kit (MoBio Laboratories Inc.) and sequencing was performed using the ABI prism Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems). Sequencing was carried out on an ABI 3700 DNA analyser and sequences were edited and manually aligned in SEQED version 1.0.3 (Applied Biosystems).

Phylogenetic analyses to barcode dytiscid larvae

Paroster macrosturtensis, P. mesosturtensis and *P. microsturtensis sequences* (GenBank accession numbers FJ785739- FJ785750; Bradford *et al.* 2010; see Appendix I) were included in the analysis. The *COI* data were aligned by eye and Neighbour-joining (NJ) trees were constructed with the computer program PAUP* version 4.0b10 (Swofford, 2002), using HKY85 distances. A midpoint root was applied and the NJ tree was bootstrapped with 500 pseudo-replicates carried out using HKY85 distances (Hasegawa *et al.*, 1985). Clustering of the larval sequences with *P. macrosturtensis, P. mesosturtensis* or *P. microsturtensis* monophyletic clades (including sequence data from known adult specimens of each species) was used to identify them to species level.

Primer design and calibration

Primers specific for prey groups, were designed to amplify small fragments (100-300bp) of DNA, to detect the products of digestion. The aim was to be able to screen for presence/absence of taxon-specific PCR products on an agarose gel. Mitochondrial *COI* data from the three diving beetle species, single harpacticoid and cyclopoid copepod species and the three chiltoniid amphipod species (GenBank accession numbers FJ785739- FJ785750,

FJ785766- FJ785772, FJ785785- FJ785789, FJ785801- FJ785805, FJ785806- FJ785808, FJ785815, FJ785816; Bradford *et al.* 2009; see Appendix I) were aligned in CLUSTAL X (Thompson *et al.*, 1997), in order to cover the level of intra-specific variation in *COI* data obtained previously (Chapter III, Bradford *et al.* (2009). Group-specific primers were designed using the computer software AMPLICON (Jarman, 2004).

In order to minimize cross-amplification of non-targets, primers were chosen with high melting temperatures. PCR conditions for amplifying *COI* (as above) were optimized for individual primer pairs, screening a range of temperatures for the highest possible annealing temperature (T_A) that still maximized amplicon yield. Increasing the PCR cycle number to 40 repeats was found to improve the sensitivity of the assay, without compromising specificity of the primers. All of the primers were tested for cross-reactivity against the other prey species being amplified, as well as the three dytiscid species. It is possible that the primers can cross-react with non-target organisms, such as other invertebrates or micro-organisms in the calcrete. To test this, amphipod amplicons from two individuals of the three dytiscid species were sequenced (as above) and aligned with amphipod sequences using SEQED version 1.0.3 (Applied Biosystems).

Feeding trials

Feeding trials were conducted in order to test the ability of the PCR assay to detect food items in whole beetle extracts, as well as give an indication of time post-feeding that a prey item can be detected. Feeding trials were limited to those animals that could be induced to feed in captivity, but the significant problems that were experienced in keeping stygofauna alive outside their specialized environment severely limited the trials that could be undertaken. A few diving beetles of each species have been kept in rainwater in the laboratory at 25˚C in the dark for several years, fed monthly on epigean chiltoniid amphipods. However, it was found that most of the diving beetles collected did not survive the first few weeks of captivity and there was a sufficient number for a feeding trial only of *P.mesosturtensis*, the most abundant of the dytiscids in the calcrete, and so collected in the largest numbers. No dytiscid larvae survived capture for longer than 24 h, as they are soft bodied and appear to be affected by the collecting process. Large numbers of prey items were required to encourage the beetles to feed, and these were provided using epigean amphipods from the Adelaide Hills in South Australia (Chiltoniidae; 4-8 mm long), as those from Surt Meadows were too valuable to be

used as food. Thus, ability of the PCR assay to detect gut contents and the detection period from whole body extracts, and to give an indication of detection periods and digestion times of dytiscids, is based on feeding trials with epigean prey species not found in the Sturt Meadows calcrete. Hence, caution is needed in interpreting the results in relation to the natural predation study, as the rate of digestion may differ between beetle species and life stages, and primer pairs may have different detection sensitivities.

Mitochondrial *COI* was amplified from four epigean amphipod specimens and specific primers were designed according to the protocol above. Field collected *P. mesosturtensis* were starved for at least 24 h in the dark at a constant temperature of 25˚C. Beetles were then fed live epigean amphipods that had been injured. Animals observed to feed were transferred to separate containers filled with rainwater. Beetles were harvested at 0, 6, 24, 48, 72, 96 h post-ingestion. Ten beetles were used per time-point, with any that died during the trial being removed from the experiment. DNA extracts were prepared as above, and all amplified successfully with *COI* universal primers before being used in the gut content assay. Epigean chiltoniid amphipod specific primers (see Table 1) were added to a PCR containing beetle DNA to test for any cross taxon amplification. Amphipod extract was included as a positive control, and *P. mesosturtensis* starved for at least 2 weeks, calcrete water and PCR water as further negative controls. PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. Each extract was scored for presence/absence of a band at 134 bp.

Natural predation study

Forty-eight *P. macrosturtensis* adults and 22 larvae, 45 *P. mesosturtensis* adults and 10 larvae and 53 *P. microsturtensis* adults and 4 larvae were collected for the field study. Specimens were washed in rainwater, dried, killed, stored separately and then DNA extracted as above. All extracts were amplified with *COI* universal primers (see Table 1 for all primer details), then positives were screened singly with group specific primers. Initially, extracts were amplified with *Paroster* species-specific primers, in order to look for any evidence of predation among species. Extracts were then amplified with amphipod and copepod specific primers. Prey target extract diluted 1/10 was included as a positive control, and specific beetle extract (from a leg) included in the negative controls. Presence of target DNA in the gut was identified by a clear band of the correct size on an agarose gel. Field trial results

were then collated and the null hypothesis that each beetle species has the same level of predation on each prey item was tested by a Chi-square test (Fisher & Yates, 1963).

Carbon and nitrogen isotope analysis

Stable isotope ratio analysis (SIRA) was conducted on 5 mg samples of vegetation from the calcrete (tree roots and leaves), and up to 1.5 mg samples of *P. macrosturtensis*, *P. mesosturtensis, P. microsturtensis* and chiltoniid amphipods, snap frozen in liquid nitrogen. Copepods had to be excluded as their small size was out of the detection range of the analyser. Total carbon and nitrogen and the fractionation of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ determinations were made on a modified Europa Roboprep CN Elemental Analyser (EA) attached to a Finnigan Mat Conflo III and Finnigan 252 (Environmental Isotopes Pty Ltd, N.S.W.). Samples were analysed relative to internal gas standards calibrated using international nitrogen isotope standards IAEA-N1 ($\delta^{15}N$ = 0.43 ‰ AIR) and IAEA-N2 ($\delta^{15}N$ = 20.41 ‰ AIR) (Bohlke & Coplen, 1995) and carbon isotope standards (NBS-22 (δ13C = -30.03 ‰ VPDB) and ANU SUCROSE (δ13C = -10.3 ‰ VPDB) (Coplen *et al.*, 2006). Carbon isotope data is expressed using the δ notation where:

$$\delta^{13}C = \frac{^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{reference} * 1000}{^{13}C/^{12}C_{sample}}$$

Values are reported in permil (‰) relative to the international standard VPDB which is defined by the International Atomic Energy Agency standard, IAEA-NBS19 ($\delta^{13}C$ = +1.95‰) (Coplen, 1995). Nitrogen isotope data is expressed in a similar fashion:

$$\delta^{15}N = \frac{^{15}N/^{14}N_{sample} - ^{15}N/^{14}N_{reference} * 1000}{^{15}N/^{14}N_{sample}}$$

and reported in permil relative to the AIR scale defined by IAEA standard IAEA-N-1 (+0.43 ‰) (Bohlke & Coplen, 1995). Errors were estimated using laboratory standards run regularly and standard deviations of better than ± 0.15 ‰ were achieved for C and N isotope values.

Results

New group-specific primers

Group-specific primers with their amplicon size and annealing temperature used are listed in Table 1. As the three species of chiltoniid amphipod are cryptic in their morphology, the initial aim was to design primers to identify them as a group. However, the lineages proved to be too divergent to be recognized by a single primer pair and two sets of primers were used. Amplicons for all analyses were all between 100 and 300 bp, except for *P. macrosturtensis* where the smallest specific fragment of *COI* that could be amplified was 438 bp.

Primers were tested for cross-amplification of DNA with other groups from the natural predation assay and all were found to be specific to the species or group for which they were designed (Fig. 1). To check if the PCR product amplified was the prey item only, an amphipod specific PCR product was directly sequenced from each of the three dytiscid species, and compared with the initial amphipod sequences used in primer design. All were found to match the amphipod sequence.

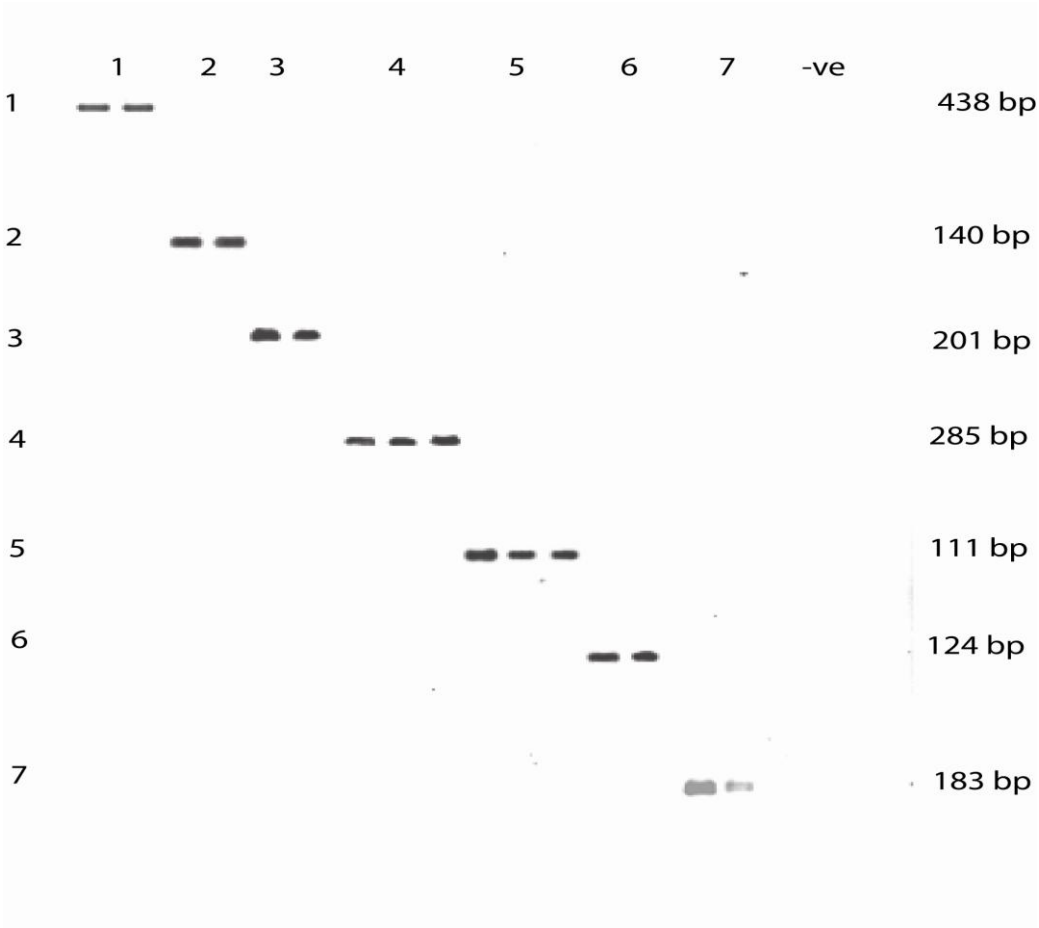

Fig. 1 Cross-amplification by group-specific primers with DNA from predators and prey analysed in the field study. Amplicons for each set of primers were run on 1.5% agarose gels and stained with ethidium bromide. Each row is a separate gel.
Primers specific for each group numbered below are to the left of the gel. DNA extracts for each group numbered below are above the gel.
1, *P. macrosturtensis*; **2**, *P. mesosturtensis*; **3**, *P. microsturtensis;* **4**,Cyclopoidea Sp.; **5**, Harpacticoida Sp.; **6**, Chiltoniidae Sp. 1; **7**, Chiltoniidae Spp. 2 & 3;-ve, PCR water.
Amplicon size is shown to the right of the gel.

Table 1 Details of group-specific primer sequences (5' - 3') plus expected product size and annealing temperature

Species	Name	Forward primer	Name	Reverse primer	Size	T_A
P. macrosturtensis	M791	GGGCAGAACTGGGAAATCCC	M792	CAACAATAGAAGGGCCGTG	438	60
P. mesosturtensis	M837	CCCATTATCAGCAGGAATCGCT	M838	GGCTGTAATTCCCACACGATCATACA	140	60
P. microsturtensis	M795	CAGTTTATCCACCATTATCTGCGGGG	M796	GCATAGTAATAGCCCCAGCC	201	62
Chiltoniidae *Sp. 1*	M849	GTGACAGCTCATGCTTTTGT	M850	CCCAACACCACTCTCTACTA	124	60
Chiltoniidae *Spp. 2 & 3*	M950	GACTTGTTCCTCTAATATTGG	M952	GCACCAGCTAAATGTAAAG	183	55
Cyclopoidea *Sp.*	M797	AATAGAACTTGGTCAACCAGGGGGG	M798	GAAAATAGCAAAATCAACTGCGGG	285	60
Harpacticoida *Sp.*	M845	ACAGCCTGGAGGAGGGATTATTAAT	M846	ACATATGTCTGGTGCTCCCAA	111	60
epigean amphipod *Sp*	M677	TTATTCGATCTGAGTTAAGTGCC	M678	ATTACCAAATCCTCCGATCATAAC	134	60

Feeding trials

Detection times of the amphipod prey were long, with 2/4 animals positive 96 h after the initial predation event by *P. mesosturtensis*. Number of positives varied at each time point; with 2/4 at 72 h, 4/8 at 48 h, 4/5 at 24 h, 5/5 at 6 h and only 6/8 animals detected as positive immediately after feeding. No amphipod DNA was detected in the calcrete water, PCR water or *P. mesosturtensis* specimens that had not been fed.

Field-caught beetle adults and larvae

Results for the natural predation study are shown in Table 2. There was a greater number of positive samples for *P. macrosturtensis*. Beetles of each of the dytiscid species feed on amphipods plus harpacticoid and cyclopoid copepods, despite the great difference in size. A chi-squared test rejects the hypothesis that the beetles all have the same level of predation on amphipods (Table 2). *Paroster microsturtensis* showed a two-fold greater number of positive signals for harpacticoid copepods compared to the other beetle species, though this difference was not significant. *Paroster macrosturtensis* larvae fed on the same prey items as the adults. *Paroster mesoturtensis* larvae were shown to feed on amphipods and harpacticoid copepods, and *P. microsturtensis* larvae were shown to feed on cyclopoid and harpacticoid copepods. Numbers of *P. microsturtensis* and *P. mesosturtensis* larvae were too low to enable inter-specific comparisons.

Table 2. Natural predation study, showing presence of prey-specific PCR product as a fraction of the total number of individuals, with percent positive in brackets. Prey items are shown on the top of the table and predators in the left column. Chi-square tests were carried out with the null hypothesis that each beetle species has the same level of predation on each prey item; ** = significant at the probability level of 0.01; *** = significant at the probability level of 0.001; NS = not significant.

	Chitoniidae *Sp. 1*	Chiltoniidae *Spp. 2 & 3*	Cyclopoidea *Sp.*	Harpacticoida *Sp.*
Adults	**	***	**NS**	**NS**
P. macrosturtensis	33/48 (69%)	20/48 (42%)	2/48 (4%)	3/48 (6%)
P. mesosturtensis	16/45 (36%)	3/45 (7%)	2/45 (4%)	4/45 (9%)
P. microsturtensis	15/53 (28%)	8/53 (15%)	2/53 (4%)	9/53 (17%)
Larvae	**NS**	**NS**	**NS**	**NS**
P. macrosturtensis	15/22	10/22	3/22	6/22
P. mesosturtensis	5/10	3/10	0/10	1/10
P. microsturtensis	0/4	0/4	1/4	1/4

Behavioural observations of the diving beetles in captivity

All three *Paroster* species come to the surface, although they were not observed hanging head-down from the surface film as seen in epigean species that store air under their elytra. They frequently had bubbles trapped around the abdomen and bubbles were also present on the calcrete substrate. Beetles preferred to crawl on the substrate (calcrete rock or a tissue), rather than to swim, but will swim to ascend to the surface and drop down (negatively buoyant) to descend. Only *P. macrosturtensis* (large beetle) was observed completely leaving and re-entering the water by crawling on the container surface, with the smaller species apparently unable to break through the water surface tension. Loss of ability to control their negative buoyancy during collection and sorting appeared to be the main cause of death in captive beetles.

Paroster macrosturtensis could catch and feed on the epigean amphipods, which were slightly larger than themselves, while the small beetle species required amphipods to be injured to slow them down, before they could feed. Group feeding occurred commonly, with presence of one beetle on a prey item attracting others in the same container. No scavenging behaviour was observed in the captive beetle species and they did not prey on each other. However, scavenging by beetles was observed during the collection process, when they were in close contact with dead animals in the 5 ml collection tube. Beetle larvae preyed on live amphipods, copepods and adult beetles in the collection tube.

Stable Isotope Analysis

Results from the stable isotope analysis are shown in Figure 2. Signatures of nitrogen and carbon for all samples analysed (plants, crustaceans and beetles) had some degree of range overlap, with no distinct levels of separation between groups for nitrogen (food source) or carbon (energy source). A plant sampled for leaves from the surface and roots from the bore had very high average values for $\delta^{15}N$ of 15 ‰ and 14 ‰, respectively. Amphipods were lighter by 1 ‰ in average nitrogen isotope ratio when compared to the tree roots. The three dytiscid beetle species had the same average value of $\delta^{15}N$ (15 ‰), and were shifted towards more positive in $\delta^{15}N$ by 2 ‰ when compared to the amphipods.

Plant leaves (-16 ‰) were enriched by 4 ‰ in carbon isotope ratio compared to roots (-20 ‰), which were not from the same plant. Complete overlap was found between amphipods and plants in the range of carbon isotope ratios (-15 to -21 ‰). Ratios varied by 1‰ among the beetle species, with *P. mesosturtensis*, *P. microsturtensis* and *P. macrosturtensis* at -20 ‰, -21 ‰ and -22 ‰, respectively. Carbon isotope ratios were more negative for the dytiscid beetles in comparison with the amphipods by 1-2 ‰.

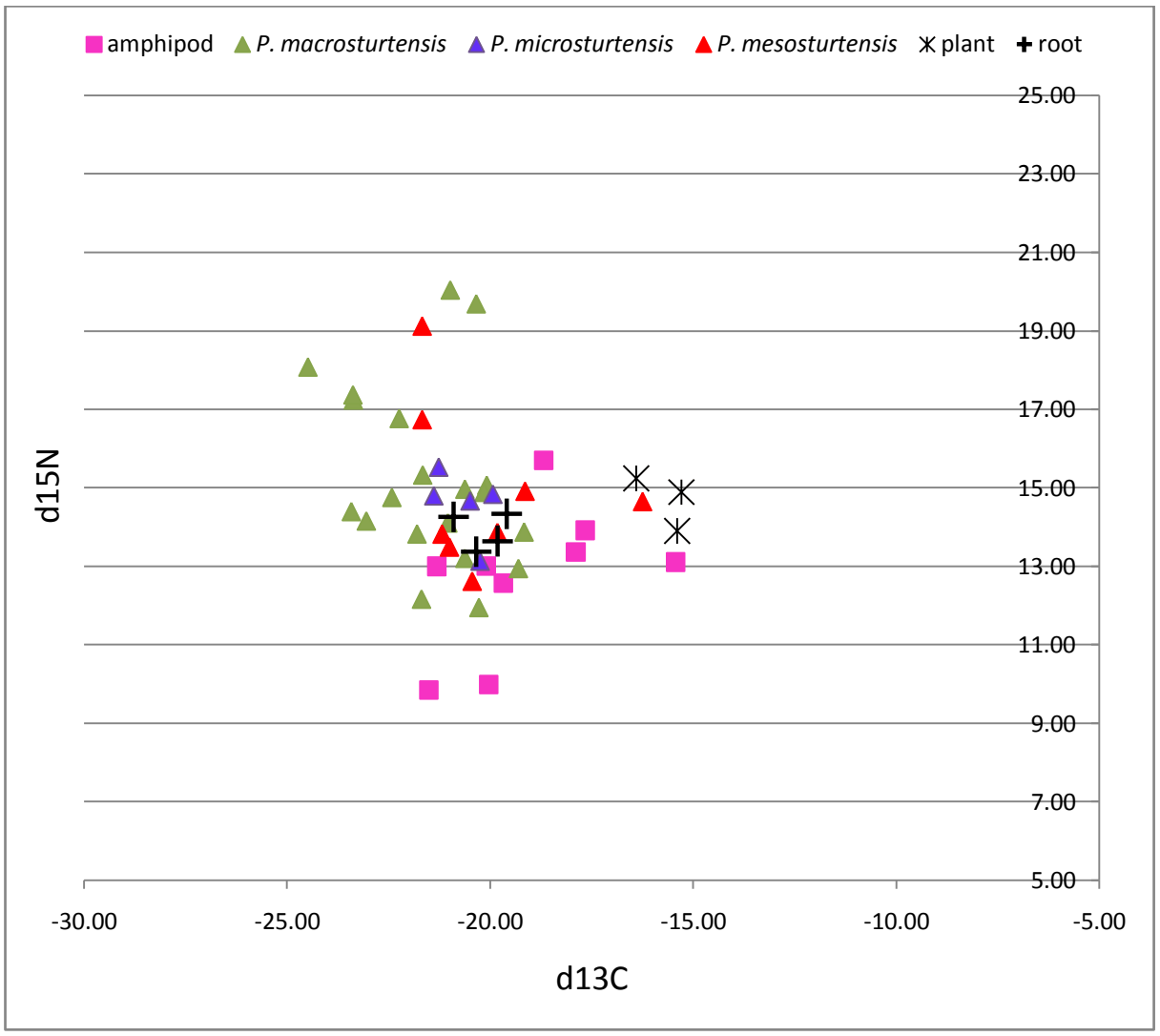

Fig. 2 Comparison of $\delta^{13}C$ ‰ and $\delta^{15}N$ ‰ for stygofauna and plants from the Sturt Meadows calcrete

Discussion

Molecular detection of predation events in the Sturt Meadow's dytiscids

Molecular methods have been successfully used in this study to identify harpacticoid and cyclopoid copepods and chiltoniid amphipods as the prey of dytiscid beetles in the enclosed calcrete aquifer at Sturt Meadows. Different beetle species were found to prey on both crustacean groups. There was evidence for niche partitioning, with trends towards preferential feeding by *P. macrosturtensis* on amphipods, and *P. microsturtensis* on harpactioids, though many more samples are required to confirm the latter result. These

patterns make sense in regards to the size of the predator. Such trends are of interest as there may be times when harpacticoids or amphipods are very abundant, giving beetles that can catch them an advantage, and complete trophic partitioning may not be necessary to promote ecological speciation. It is likely that any differences found may reflect the sensitivity of the assay, and that there will be a bias towards more positives in the larger beetles. This bias is indicated by the results of the feeding trials, which showed that there is the potential for false negatives in *P. mesosturtensis*, combined with the greater number of positive amplicons for *P. macrosturtensis*. Preferential feeding by different sized beetles on the different crustacean groups remains a possibility, but this was unable to be tested using molecular methods due to the difficulty of carrying out feeding trials with stygobitic species. Ideally, all three species of beetle, both adults and larvae, should be trialled, with all prey groups tested for in the natural predation study (King *et al.*, 2008).

The beetle larvae are of particular interest in any study on beetle feeding as they are highly predaceous and the active feeding stage of the beetle (Larson *et al.* 2001). Dytiscid larvae will rapidly attack all other invertebrates they come into contact with, and had to be separated from other stygofauna immediately on collection. The larvae develop through three instars before pupating, and spend the greater proportion of their time in the third instar (Larson *et al.*, 2000). *Paroster macrosturtensis* larvae examined were all larger than the adult beetles and both copepod and amphipod groups could be detected as prey items. *Paroster mesosturtenis* larvae were found to feed on amphipods and harpacticoid copepods, and *P. microsturtensis* larvae were shown to feed on both copepod groups. Only by increasing the number of samples can any species differences in trophic niche for the larvae of the different dytiscid species be determined. The aquifer has been extensively sampled over a two year period and there does not appear to be a particular breeding season for the dytiscids, presumably related to the observation that temperature in the calcrete remains very constant throughout the year, fluctuating only by a couple of degrees (A. Allford, unpublished data). Flushes of larvae have been found previously after aquifer recharge, when nutrients are presumed to be washed into the system and numbers of crustaceans present multiply (A. Allford, unpublished data). During the sampling period of this study, the major cyclonic events that bring rain episodically to the region missed Sturt Meadows and numbers of dytiscid larvae present remained at a low level.

Not all individual dytiscids tested in the natural predation assay showed evidence for feeding on copepods or amphipods. These animals are by far the most abundant food items in the calcrete, although there is the potential that the other macro-invertebrate groups (either

alive or dead) are being fed on by the dytiscids. Apart from the limitations of the molecular assay in detecting predation, it is likely that animals in the natural environment would frequently have empty guts due to difficulties locating food items. Indeed, styogfauna are patchily distributed across this site (Guzik *et al.*, 2009; Chaper IV), indicative of numerous small areas of suitable habitat. Digestion times appear to be long in the dytiscids tested, and an extended time course for the feeding trials is required to obtain an estimate of digestion times in these subterranean species. However, given the pattern of fewer positives with time it is also possible that digestion is destroying the DNA. Compared to surface relatives, subterranean species typically have reduced metabolism as an adaptation to the energy-poor sub-surface environment (Culver *et al*. 1995), a premise supported by the observation that subterranean dytiscids can live for up to eight months without food (U'eno, 1957).

Stable isotope analysis of the calcrete

Understanding of the food-web in an enclosed aquifer can be gained by stable isotope analysis, with consumers thought to be slightly enriched in $\delta^{13}C$ (1-2 ‰) and $\delta^{15}N$(2-4 ‰) relative to their food source (Ehleringer *et al.*, 1986; Humphreys, 1999). However, potential trajectories of trophic increments in stable isotope ratios could not be predicted in this system, due to overlap between the stable isotope signatures of the plants and animals analysed, indicating the presence of other sources of nitrogen and carbon as the basis of the calcrete food-web. Stable isotope analysis of the three dytiscid beetle species found them to be similar in their trophic level. There was a small separation in carbon isotope ratio of 1 ‰ between species, although probably not enough to indicate utilization of different food sources between them, taking into account the spread of the data and differences in sample sizes.

Plant leaves and roots from Sturt Meadows (along with those from other calcretes; data not shown) had very high nitrogen isotope ratios, making them unlikely to be nitrogen fixers (Ehleringer *et al.*, 1986). The vegetation analysed was not at a lower trophic level compared to the amphipods and beetles, indicating that it is not the source of carbon and nitrogen in this system. Organic carbon fixed by photosynthesis at the surface is thought to be the main energy source for subterranean animals. The range of carbon isotope ratios found for the beetles and amphipods indicates that they are potentially using carbon derived from C3 plants (-20- -35‰, Ehleringer *et al.*, 1986), although wider sampling of the surface vegetation, is required to test this hypothesis.

High levels of nitrogen isotope ratios for amphipods and beetles are similar to that seen for other subterranean animals, such as predatory fish ($\delta^{15}N$ = 12 to 14 ‰; Humphreys 1999; Humphreys, 2001b), amphipods ($\delta^{15}N$ = 13 ‰; Pohlman, 1997) and atyid shrimps at specific sites ($\delta^{15}N$ = 10 ‰; Humphreys, 2001b). The amphipods are associated with tree roots and were initially thought to be herbivorous, although the trophic level of stable isotope ratios indicates an omnivorous and perhaps a scavenging life style, which has been suggested as an adaptation to subterranean life by Humphreys (2001b) for atyid shrimps. Amphipods do not appear to be the main food source for the diving beetles as they have less negative carbon isotope ratios, implying that the beetles are heavily utilizing another food source, such as the copepods.

Values of carbon are largely conserved in food chains and provide information about the energy base. The copepods that are identified as prey items in this study could be feeding on a ^{13}C depleted source of organic carbon such as a bacterial film. It is possible that chemotrophs ie. organisms that derive energy from chemical reactions, are the basis of the food-web in the calcrete, as $\delta^{13}C$ values for chemoautotrophic bacteria can be very low (-35 *‰)* and could heavily bias the carbon isotope ratio data. Use of bacteria as a food source has been proposed as causing more negative carbon ratios in atyid shrimps from anchialine cave systems (Pohlman, 1997; Humphreys, 1999; Humphreys, 2001b). Microbial communities in the Yilgarn calcrete aquifers have not yet been characterized and it is not known if the stygofauna present could graze on bacterial films. It has been suggested by Humphreys *et al.* (2009) that the Yilgarn calcretes could support chemoautotrophic sulphur bacteria, with strong physico-chemical gradients, combined with the high sulphate and nitrate levels found in groundwater, resulting in a cascade of microbiological communities. Increasing the sensitivity of our analyses to include stable isotope determinations of the copepods and any biofilm within the groundwater would be of great interest in understanding the food-chain in this system.

Dytiscid behavioural observations

The dytiscids at Sturt Meadows appear to be scavengers, opportunistic feeders and probably active predators, as has been found for stygobiontic diving beetles from the northern hemisphere (U'eno, 1957; Castro & Delgado, 2001). Previous lack of evidence for subterranean dytiscids surfacing (Castro & Delgado, 2001) has led to the proposal that they obtain oxygen through their cuticle and lead a fully aquatic lifestyle (Franciscolo, 1983).

However, the Sturt Meadow's dytiscids were all observed surfacing, even although they did not hang at the surface, as seen in epigean dytiscids (Larson *et al.*, 2000). It is possible that they are capturing bubbles of air at the surface as well as from their calcrete substrate, and obtaining oxygen in this way, as from a physical gill, where a bubble at the tip of the abdomen enables the exchange of O_2 from the water and CO_2 from the sub-elytral space into the water along concentration gradients (Larson *et al.*, 2000). The need to surface will restrict the depths at which dytiscids at Sturt Meadows can live; the calcrete is more than 11m thick at the northern end of this site (Fig. 4, Chapter II; Anaconda Nickel Limited Technical Report 1251, 2002). Ecological differentiation within the calcrete is potentially a result of how the large and small dytiscids are obtaining oxygen, with the small *P. mesosturtensis* found throughout the water column and the large *P. macrosturtensis* restricted to the upper 500 mm (Allford *et al.*, 2008), as they may need to make more trips to the surface for oxygen. Data on depths at which *P. microsturtensis* is found is yet to be determined, though the trend towards feeding on harpacticoids suggests that they are at lower levels in the water column (W. Humphreys, pers. comm.). Any correlation of water chemistry, which alters with depth (Humphreys *et al.* 2009), with the distribution of the dytiscid species is a focus for further investigation, although, water chemistry was found to have a low predictive power of distributions in a study of dytiscid assemblages in a Swedish lake system (Nilsson & Soderberg, 1996). As only the large beetle species appeared to be able to break the surface tension at the air/water interface it is possible that the small beetles pupate underwater, as has been proposed by Ueno (1957).

Modes of speciation in the dytiscid beetles

There is no evidence for complete trophic niche partitioning within this calcrete for the dytiscid beetles, with the adult beetles thought to be scavengers and group feeders, as well as active predators. It is possible that there is ecological differentiation as a result of partial tropic partitioning, with specialities or preferences in food items, however the number of samples would have to be much greater to test this. Also, there is still the potential for trophic niche partitioning in the larvae, which are active predators. The repeated observation of multiple instances of large and small beetles in the same calcrete, and in particular those that are sympatric sister species, raises the potential that there will be ecological niche partitioning within the calcrete. It would be of interest to the study of speciation to extend sample

numbers, in particular for the larvae to enable their inter-specific comparison, and repeat the trophic study in the other calcretes which contain sympatric sister species of beetles associated with different sized crustacean groups. Also of interest in studying modes of speciation in the Yilgarn dytiscids, would be the depths occupied by the different sized beetles within the calcrete, particularly if species differences in amount of surfacing for oxygen required could result in spatial partitioning down the water column.

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GENERAL DISCUSSION

CHAPTER VI

Speciation in the sympatric sister species of diving beetles from the Yilgarn calcretes

Coyne and Orr (2004) state that for the process of sympatric speciation to be proposed, species must be sympatric sister species that are reproductively isolated, with no period of allopatry in their biogeographic and evolutionary history (before speciation). The occurrence of sympatric sister species of dytiscid beetles in the Yilgarn region of W. A. may have been the result of at least three main processes

1. repeated colonisation from the same ancestral species with divergence in allopatry;
2. intra-calcrete speciation in sympatry due to ecological niche partitioning, or ;
3. intra-calcrete speciation through micro-allopatric processes.

This study has examined the potential for intra-calcrete divergence for the beetles at the Sturt Meadows calcrete, by investigating the case for ecological niche partitioning and micro-allopatric processes operating.

Examination of trophic niche partitioning that may have led to the different size classes of beetle and ecological speciation in sympatry, found trends towards preferential feeding, though no complete separation in food items consumed. There was also no evidence for any common fragmentation events that could have led to allopatric speciation in the beetles, from the study of comparative phylogeography of the amphipods that are presumed to have either predated or colonised the aquifer at a similar time point to the beetles. However, other forms of micro-allopatric divergence were evident with common population genetic patterns found for amphipod and beetle species (Guzik *et al.*, 2009). Evidence for populations undergoing expansion in all three beetle species and two out of three amphipod species, plus isolation by distance in two out of the three species of beetle and amphipod, indicate that population isolation followed by expansion events generate high genetic diversity in calcrete fauna. As such, the diving beetles fail the fourth criterion of Coyne and Orr (2004), i.e. that there must be no evidence for allopatry in the evolutionary history for the process of sympatric speciation to be proposed. The calcrete matrix is variable in structure and historical variation in water levels would change which areas of the calcrete can be accessed by stygobiontic invertebrates. The ‗pure' allopatric model of speciation seems unlikely to operate in this system, unless there have been major historical calcrete fission and/or fusion events. It is quite likely that historical water level changes in the aquifer have led to both micro-allopatric and parapatric divergence in the beetles, making it impossible to meet the criteria of Coyne and Orr (2004) for the Sturt Meadows calcrete. This does not

mean that sympatric speciation was not the initial driver in the speciation process for either two or all three of the beetle species. Our understanding of the process of speciation is still very limited, with empirical studies to date suggesting the spectrum of geographic isolation from allopatry to sympatry is possible. As such the criteria of Coyne and Orr (2004), though useful in describing patterns in nature, are overly simplistic as they make the assumption that allopatric speciation is the null hypothesis. The pattern of large and small beetles is repeated across calcretes even when there is clear evidence of multiple colonising species of different ancestry (Cooper *et al.* 2002; Leys *et al.* 2003), implying that common patterns of ecological differentiation exist in all the calcretes. The presence of sympatric sister species of large and small beetles in multiple calcretes still suggests an adaptive radiation on colonisation of the sub-surface as the most likely driver in their speciation.

Genetic diversity in stygofauna from the Yilgarn calcretes

The two datasets for stygofauna from the Sturt Meadows calcrete showed high haplotype diversity coupled with low nucleotide diversity, with some haplotypes being maintained in populations that are quite divergent (Chapter IV; Guzik *et al.* 2009). These stygobiontic amphipods and beetles (Guzik *et al.*, 2009) have population genetic patterns indicative of bottlenecks followed by range expansion. Regions of lower genetic diversity in the amphipod species were found in a shallower area of the calcrete, potentially as a result of past extinction of populations at low aquifer levels and recent range expansion into this area at a time of higher water. The water levels in the aquifer are thought to be linked to surface rainfall, with cyclonic events that impact on the region likely to be a major factor in aquifer recharge. Thus, population retraction and expansion events could occur over a cycle of several years, potentially generating the high haplotype diversity seen in stygofauna from the calcretes. Pleistocene climate fluctuations would have impacted on the subterranean environment by altering the groundwater levels and access to different areas of the calcrete, with local population extinctions predicted at times of low water level. Ice age maxima were associated with increased aridity and low temperatures in the arid zone (Byrne *et al.* 2008), though the low temperatures may have also reduced evaporation, so it is difficult to predict their influence on water table levels.

It would have furthered the phylogeographic and population genetic study of amphipod species at Sturt Meadows to go beyond genetic diversity indices and test statistics,

to obtain estimates of effective population size, evidence for past population bottlenecks, and estimates of migration rates between the two populations of chiltoniid species 1. Allozyme data indicated the potential for a severe bottleneck in chiltoniid species 2, though the lack of variation in itself made these markers uninformative and the generation of microsatellite markers would enable testing for past bottlenecks in this species (Cornuet & Luikart, 1996). A single mitochondrial marker can be used to obtain estimates of fluctuating population sizes using the computer program BEAST (Drummond *et al.*, 2005) which is orientated towards rooted, time measured phylogenies. The problem with applying this method to the Sturt Meadows calcrete was the lack of any fossil data to calibrate the phylogeny and the inaccuracies involved in estimates of population size that result from applying a general invertebrate molecular clock (A. Drummond pers. comm.). Also, the data will only reflect the stochastic behaviour of a single marker, and these results cannot be relied on when trying to infer what actually happened to the population size. The program MIGRATE (Beerli & Felsenstein, 2001) also can be used to provide estimates of population size and past migration rates (M) between populations. There is a risk, however, when using a single mitochondrial gene of over-interpreting the data (Knowles & Maddison, 2002) and a sequence dataset with mitochondrial and unlinked non-recombining nuclear loci, or a microsatellite dataset, would enable independent replicates of gene history (Beerli, 2006), and give more confidence in the estimates of these particular population genetic parameters. A statistical phylogeographic approach could then be undertaken, where alternate hypotheses for historical evolutionary processes are represented as mathematical models and level of fit to the observed data is then evaluated (Johnson & Crandall, 2009). Of interest for chiltoniid species 1 would be testing whether there has been past separation of populations ($M = 0$) with recent limited gene flow ($0<M<0.5$; Butlin *et al.*, 2008).

Ecological niche partitioning in the diving beetles from the Sturt Meadows calcrete

Searching for ecological niches that are specific to each of the large and small species in separate calcretes appears to be a promising line of investigation for gaining an understanding of speciation in these beetles. This study identified the potential for differences in food preferences in the adult beetles. The large species, *P. macrosturtensis* appeared to prefer amphipods and the smallest species, *P. microsturtensis,* showed a trend towards preference for harpacticoid copepods. Food preferences are of interest as partial trophic partitioning with

a greater ability to access a certain food source would be a selective advantage in times when such animals are abundant. As the beetles appear to be opportunistic group feeders as well as scavengers, it is not surprising that no complete trophic partitioning was found, and large sample numbers would be required to pick up any differences in abilities of adults to feed on live animals.

Although dytiscids are thought of as predaceous, a possible shift towards a scavenging life style in the adults may be an adaptation to a nutrient poor subterranean environment. Sampling of the aquifer over three years has found that stygofauna are patchily distributed, presumably related to availability of potential habitat. It is thought that periods with plentiful crustaceans as available prey items may be linked to aquifer recharge, which appears to be driven by local rainfall (A. Allford, unpublished data). Rainfall records at the Sturt Meadows pastoral property indicate that major rainfall and, hence, recharge events are likely to be very sporadic (Axford family, pers. comm.) and could occur at intervals of several years. Even if metabolism is slow, it is not surprising that all sources of animal food would be accessed by the beetles, which can live up to three years and maybe longer, based on data from beetles kept in captivity. Stable isotope ratios of the beetles and plants from the area do not indicate any reliance on vegetation as a food source as has been indicated in studies on surface dytiscids (Deding, 1988).

All current evidence points to dytiscid larvae being solely predaceous, making identification of any trophic niche partitioning potentially more straightforward for this life history stage. A complicating factor, however, is that dytiscid larvae go through three instars. Although only second and third instars at Sturt Meadows have been collected, second instars are smaller (Alarie *et al.*, 2009) and may access different prey items. Examination of third instar larval morphology of the Sturt Meadow's dytiscids by Alarie *et al.* (2009) found *P. macrosturtensis* to be distinguished from *P. mesosturtensis* and *P. microsturtensis* by its larger size and disproportionately large head capsule, and that the three species differed in the amount of broadening of the head capsule with *P. microsturtensis* > *P. mesosturtensis* > *P. macrosturtensis*. Enlargement and broadening of the head capsule are thought to increase the ability to catch prey in a non-visual manner (Alarie *et al.*, 2009). Amphipods and copepods in the calcrete are highly mobile and species differences in larval morphology may be adaptive for catching different prey. Numbers of larvae appear to increase after recharge of the aquifer (A. Allford & S. Cooper pers. obs.) indicating that breeding/egg hatching is linked to the availability of potential prey items. For the hypothesis of trophic niche partitioning between the dytiscid larvae to be tested, molecular amplification of gut contents of greater numbers of

specimens are required, by timing a collection trip to Sturt Meadows after a heavy rainfall event.

Partitioning of the three *Paroster* species down the water column at Sturt Meadows, suggested by Allford *et al.* (2008), is a possibility that has not been investigated in this study. The trend towards preference for feeding on harpacticoid copepods by *P. microsturtensis* is a potential indicator that they can survive lower in the water column, as harpacticoids are thought to be bottom dwellers (W. Humphreys pers. comm.). Both cyclopoid and harpacticoid copepods are very fast movers and it remains to be tested whether the beetles can catch them alive. An interesting finding from keeping diving beetles in aquaria is that the beetles surface but do not seem to hang at the surface to take on air under the elytra. The reason for surfacing is unknown, however capturing bubbles from the surface or the substrate seems to be the most likely method of respiration, with the added possibility of transfer of oxygen through the cuticle. Kehl and Dettner (2009) suggest that there may be tracheated setae on the elytra of Australian subterranean dytiscids for cuticular respiration, though examination of the Sturt Meadow's species did not find any evidence for this (C. Watts pers. comm.). It would be very interesting to see how often the different sized beetles need to surface and if this limits the depths at which they can live, with smaller beetles potentially able to take more of their oxygen requirements through the elytra. Partitioning down the water column as a result of oxygen requirement differences, which may further influence abilities to prey on certain food groups, is a potential isolating mechanism related to their biology that could have resulted in speciation in sympatry, and is certainly worth investigating further.

Conservation of stygofauna in the Yilgarn

The stygofauna from the Yilgarn have largely been described over the last decade (Humphreys, 2008), with over 300 stygal species now known from the Yilgarn calcretes (EFN groundwater ecosystems workshop, Darwin 2009). An important outcome of this study has been the assessment of species richness at Sturt Meadows which identified at least 12 macro-invertebrate species in this calcrete, increasing the total number of stygobiontic and troglobiontic species discovered in the Yilgarn. Mitochondrial lineages do not necessarily equate to species but provide an estimate of potential numbers that require further investigation. This was found for the morphologically cryptic amphipods when an allozymes

dataset was assembled, confirming the presence of three biological species. There are approximately 210 calcretes in the Yilgarn system, and if the dataset at Sturt Meadows alone is used for extrapolation of potential species numbers, there could be 2000 species yet to be discovered in the region. The Sturt Meadow's calcrete is missing a number of the crustacean groups that are common to other calcretes in the Yilgarn, particularly *Haloniscus* isopods and bathynellaceans. Also, the troglofauna above the water table has only just recently been surveyed using litter traps for troglobionts, rather than the chance captures from the water with a haul net. Hence, though sampling at Sturt Meadows over a three year period has probably revealed most of the macro-invertebrate fauna, there may be many more troglobiont and possibly micro-invertebrates in this single calcrete. Also, as sample sizes for sequencing were quite small, it is possible that other cryptic species might exist but be unsampled. So the potential number of species simply gives an idea of what might be present and, considering the rate of species discovery so far, an indication of the enormous amount of work left to do in discovering and describing the stygofauna and troglofauna of the Yilgarn calcretes.

In order to conserve the stygofauna and troglofauna in the Yilgarn it is important to know what is actually there, as the calcrete system is under pressure from both pastoralism and the mining industry. The Environmental Protection Agency requires an environmental approval for major resource projects in Western Australia, and there is a requirement to document the diversity and distribution of species. If they are short-range endemics (Harvey, 2002), then there is the risk of extinction if there is over-exploitation of the groundwater. Study of amphipods from the Sturt Meadow's calcrete identified the risk of local population extinctions with lowering of the water table below 11 m.

Very little is known about the biology and ecology of the calcrete fauna, with this study being the first to investigate the food web of a calcrete. Interestingly, the signatures of isotopes of carbon indicated the potential that chemo-autotrophic bacteria may be the basis of the food chain. As such, it would be important to classify what bacteria are present in the calcrete, either by DNA barcoding or analysis of functional groups. If micro-organisms are the energy source for the system then their conservation is required to maintain ecosystem function, which is of particular concern if the aim is to preserve the calcrete fauna in the face of development of groundwater resources.

In conclusion

Cave faunas provide fascinating systems for investigating the process of speciation, with the Yilgarn calcrete system of particular interest because of the very limited opportunities for dispersal between calcretes, enabling the investigation of such processes in isolation. Speciation in the Yilgarn calcretes is largely thought to have been the result of divergence in allopatry when colonization of the sub-surface occurred as a result of climate change during the Miocene. The presence of large and small dytiscid beetles in multiple calcretes, including those that are sympatric sister species, points to some sort of adaptive shift post colonisation, with common ecological niche differentiation within the calcrete environment. It is hypothesized that there could be depth partitioning in the different-sized diving beetles related to their oxygen requirements, or food preferences in the adults and/or larvae. Within the calcrete, isolation has been identified as a significant diversifying force, with the potential for micro-allopatric speciation and parapatric speciation of stygofauna at this fine spatial scale, that possibly relate to calcrete thickness and groundwater levels. The population genetic and ecological data generated at Sturt Meadows provide a baseline for the Yilgarn calcretes. Extending such a study to examine spatial and ecological differentiation in sympatric sister species of subterranean diving beetles across multiple calcretes, would be a powerful approach in the investigation of modes of speciation and add to what is known about the poorly understood process of speciation.

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List of specimens with associated voucher information

Specimen voucher	GenBank acc. #	Identification	Haplotype	Locality	State	Country	Latitude Longitude	Specimen type
ABTC104142	FJ785739	*Paroster macrosturtensis*		Sturt Meadows	W.A.	Australia	28.7070609 S 120.8966821 E	DNA extract
ABTC104140	FJ785740	*P. macrosturtensis*		Sturt Meadows	W.A.	Australia	28.69714209 S 120.8958614 E	DNA extract
ABTC104141	FJ785741	*P. macrosturtensis*		Sturt Meadows	W.A.	Australia	28.69903573 S 120.9040314 E	DNA extract
ABTC104215	FJ785742	*P. macrosturtensis*		Sturt Meadows	W.A.	Australia	28.69904646 S 120.9050506 E	DNA extract
ABTC104234	FJ785743	*P. macrosturtensis*		Sturt Meadows	W.A.	Australia	28.70258161 S 120.8977979 E	DNA extract
ABTC104143	FJ785744	*P. mesosturtensis*		Sturt Meadows	W.A.	Australia	28.71056923 S 120.8904648 E	DNA extract
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Specimen voucher	GenBank acc. #	Identification	Haplotype	Locality	State	Country	Latitude Longitude	Specimen type
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ABTC104109	FJ785816	Chiltoniidae	Hap43	Sturt Meadows	W.A.	Australia	28.69637498 S 120.8958668 E	DNA extract
ABTC104111	FJ785817	Chiltoniidae	Hap44	Sturt Meadows	W.A.	Australia	28.69540402 S 120.9030068 E	DNA extract
ABTC104124	FJ785818	Chiltoniidae	Hap45	Sturt Meadows	W.A.	Australia	28.69621404 S 120.8934528 E	DNA extract
ABTC103996		Chiltoniidae	Hap1	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC103997		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC103998		Chiltoniidae	Hap1	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC103999		Chiltoniidae	Hap1	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC104000		Chiltoniidae	Hap1	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC104002		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC104010		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69981357 S 120.8958131 E	DNA extract
ABTC104011		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69981357 S 120.8958131 E	DNA extract
ABTC104012		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69981357 S 120.8958131 E	DNA extract
ABTC104013		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69981357 S 120.8958131 E	DNA extract
ABTC104014		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69981357 S 120.8958131 E	DNA extract
ABTC104016		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.70168039 S 120.8998418 E	DNA extract
ABTC104017		Chiltoniidae	Hap3	Sturt Meadows	W.A.	Australia	28.70258161 S 120.8977979 E	DNA extract
ABTC104018		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.70258161 S 120.8977979 E	DNA extract
ABTC104019		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.70255479 S 120.8957863 E	DNA extract
ABTC104023		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69526991 S 120.8978623 E	DNA extract
ABTC104024		Chiltoniidae	Hap5	Sturt Meadows	W.A.	Australia	28.69621404 S 120.8958668 E	DNA extract
ABTC104025		Chiltoniidae	Hap1	Sturt Meadows	W.A.	Australia	28.6962355 S 120.8968699 E	DNA extract
ABTC104026		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.6962355 S 120.8968699 E	DNA extract
ABTC104028		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69637498 S 120.9030068 E	DNA extract

Specimen voucher	GenBank acc. #	Identification	Haplotype	Locality	State	Country	Latitude Longitude	Specimen type
ABTC104029		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69637498 S 120.9030068 E	DNA extract
ABTC104031		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69726547 S 120.9050614 E	DNA extract
ABTC104032		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69726547 S 120.9050614 E	DNA extract
ABTC104033		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69726547 S 120.9050614 E	DNA extract
ABTC104039		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.70072016 S 120.8958024 E	DNA extract
ABTC103875		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.6953772 S 120.9000134 E	DNA extract
ABTC103877		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69991549 S 120.9009039 E	DNA extract
ABTC103878		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.71601948 S 120.8934528 E	DNA extract
ABTC103880		Chiltoniidae	Hap6	Sturt Meadows	W.A.	Australia	28.69637498 S 120.9030068 E	DNA extract
ABTC103881		Chiltoniidae	Hap1	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC103882		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.70247969 S 120.8937371 E	DNA extract
ABTC103916		Chiltoniidae	Hap2	Sturt Meadows	W.A.	Australia	28.69903573 S 120.9040314 E	DNA extract
ABTC104035		Chiltoniidae	Hap28	Sturt Meadows	W.A.	Australia	28.69903573 S 120.9040314 E	DNA extract
ABTC104036		Chiltoniidae	Hap25	Sturt Meadows	W.A.	Australia	28.69903573 S 120.9040314 E	DNA extract
ABTC104037		Chiltoniidae	Hap26	Sturt Meadows	W.A.	Australia	28.69903573 S 120.9040314 E	DNA extract
ABTC104040		Chiltoniidae	Hap25	Sturt Meadows	W.A.	Australia	28.7070609 S 120.8966821 E	DNA extract
ABTC104041		Chiltoniidae	Hap23	Sturt Meadows	W.A.	Australia	28.7070609 S 120.8966821 E	DNA extract
ABTC104042		Chiltoniidae	Hap23	Sturt Meadows	W.A.	Australia	28.71243068 S 120.8925462 E	DNA extract
ABTC103874		Chiltoniidae	Hap25	Sturt Meadows	W.A.	Australia	28.69903573 S 120.9040314 E	DNA extract
ABTC103883		Chiltoniidae	Hap25	Sturt Meadows	W.A.	Australia	28.71415802 S 120.8913982 E	DNA extract
ABTC103884		Chiltoniidae	Hap25	Sturt Meadows	W.A.	Australia	28.70881506 S 120.8945792 E	DNA extract
ABTC103885		Chiltoniidae	Hap23	Sturt Meadows	W.A.	Australia	28.70880433 S 120.8935225 E	DNA extract
ABTC103886		Chiltoniidae	Hap23	Sturt Meadows	W.A.	Australia	28.70696434 S 120.8925462 E	DNA extract
ABTC103887		Chiltoniidae	Hap23	Sturt Meadows	W.A.	Australia	28.71243068 S 120.8925462 E	DNA extract

ABTC103888	Chiltoniidae	Hap23	Sturt Meadows	W.A.	Australia	28.70886334 S 120.8956361 E	DNA extract
ABTC103889	Chiltoniidae	Hap23	Sturt Meadows	W.A.	Australia	28.70886334 S 120.8956361 E	DNA extract
ABTC103891	Chiltoniidae	Hap24	Sturt Meadows	W.A.	Australia	28.70881506 S 120.8946061 E	DNA extract
ABTC103892	Chiltoniidae	Hap25	Sturt Meadows	W.A.	Australia	28.70701262 S 120.89562 E	DNA extract
ABTC103912	Chiltoniidae	Hap26	Sturt Meadows	W.A.	Australia	28.69903573 S 120.9040314 E	DNA extract
ABTC103917	Chiltoniidae	Hap25	Sturt Meadows	W.A.	Australia	28.7159712 S 120.8913606 E	DNA extract
ABTC104001	Chiltoniidae	Hap30	Sturt Meadows	W.A.	Australia	28.70161601 S 120.8937263 E	DNA extract
ABTC104020	Chiltoniidae	Hap30	Sturt Meadows	W.A.	Australia	28.69529137 S 120.8948904 E	DNA extract
ABTC104021	Chiltoniidae	Hap30	Sturt Meadows	W.A.	Australia	28.69529137 S 120.8948904 E	DNA extract

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Haplotype and sample locality list for the amphipod phylogeography study in Chapter IV

List of the sampling localities (bore holes with corresponding waypoints), sample size per bore (*n*) and DNA haplotypes (Hap) for each species of amphipod. Haplotype sample sizes (in brackets where number of specimens with a particular haplotype is > 1) and GenBank Accession numbers for the sequences obtained in the study are included.

Bore	**latitude/ longitude**	**Species 1**			**Species 2**			**Species 3**		
		n	**Hap**	***COI***	***n***	**Hap**	***COI***	***n***	**Hap**	***COI***
A3	-28.69529137/ 120.8948904	1	4	FJ785787				2	30(2)	FJ785761
A4	-28.69531819/ 120.8958882	1	1	FJ785785						
A5	-28.69531819/ 120.8969021	1	1	FJ785785						
A6	-28.69526991/ 120.8978623	5	1(3)	FJ785785				1	32	FJ785764
			17	FJ785798						
			2	FJ785752						
A8	-28.6953772/ 120.9000134	4	1	FJ785785						
			2(3)	FJ785752						
A9	-28.69540402/ 120.9010273	2	2(2)	FJ785752						
A11	-28.69540402/ 120.9030497	4	2(4)	FJ785752				2	30	FJ785761
									42	FJ785815
B3	-28.69622477/ 120.8946919				2	25(2)	FJ785802			
B4	-28.69621404/ 120.8958668	2	5(2)	FJ785788				1	43	FJ785816

B5	-28.6962355/ 120.8968699	4	1(2)	FJ785785			
			2(2)	FJ785752			
B10	-28.69630524/ 120.9020036	1	2	FJ785752	1	40	FJ785813
B11	-28.69637498/ 120.9030068	1	2(9)	FJ785752			
		4	6(2)	FJ785789			
			41(2)	FJ785814			
			44	FJ785817			
C2	-28.69701871/ 120.8938068	3	1(2)	FJ785785	1	33	FJ785806
			15	FJ785796			
C3	-28.69712063/ 120.8948153	1					
C4	-28.69714209/ 120.8958614	1	5	FJ785788			
C5	- 28.69712063/ 120.8966875	1	16	FJ785797			
C9	-28.69717964/ 120.9009254	2	2(2)	FJ785752			
C13	-28.69726547/ 120.9050614	8	2(8)	FJ785752			
D2	-28.69796821/ 120.8938068	1	2	FJ785752			
D5	-28.69801113/ 120.8968699	10	1(3)	FJ785785			
			2(7)	FJ785752			
D13	-28.69816133/ 120.9050506	1	2	FJ785752			

Bore	latitude/ longitude	Species 1			Species 2			Species 3		
		n	**Hap**	***COI***	***n***	**Hap**	***COI***	***n***	**Hap**	***COI***
E1	-28.69884261/ 120.8927554	2	16	FJ785797	1	27	FJ785803			
			18	FJ785799						
E5	-28.69890162/ 120.8968538	1	2	FJ785752	1	26	FJ785758	1	35	FJ785808
E11	-28.699025/ 120.9029531	1	2	FJ785752						
E12	-28.69903573/ 120.9040314	4	2(2)	FJ785752	35	25(20)	FJ785802	1	34	FJ785807
			9(2)	FJ785755		26(12)	FJ785758			
						28(2)	FJ785804			
						29	FJ785805			
E13	-28.69904646/ 120.9050506				2	25(2)	FJ785802			
F4	-28.69981357/ 120.8958131	9	1	FJ785785				1	31	FJ785763
			2(8)	FJ785752						
F6	-28.69985112/ 120.8978355	1	1	FJ785785						
F7	-28.69987794/ 120.8988601	3	2	FJ785752						
			3	FJ785786						
			39	FJ785812						

F9	-28.69991549/ 120.9009039	3	2(2)	FJ785752						
			38	FJ785811						
F13	-28.69994768/ 120.9050077				5	25(3)	FJ785802			
						26(2)	FJ785758			
G2	-28.70069333/ 120.8938068	2	2(2)	FJ785752						
G4	-28.70072016/ 120.8958024	3	1	FJ785785						
			2	FJ785752						
			7	FJ785790						
G5	-28.70072016/ 120.8968109	1	2	FJ785752						
G6	-28.70075234/ 120.8978784	1	16	FJ785797						
G8	-28.70078989/ 120.8999008	2	2(2)	FJ785752						
H1	-28.70159992/ 120.892691	1	8	FJ785751						
H2	-28.70161601/ 120.8937263	11	1(7)	FJ785785				2	30(2)	FJ785761
			2(4)	FJ785752						
H3	- 28.70159992/ 120.8946919	2	9	FJ785755						
			19	FJ785800						
H6	-28.70166966/ 120.8978033	1	2	FJ785752						
H7	-28.70166966/ 120.8988386	2	2(2)	FJ785752						

Bore	latitude/ longitude	Species 1			Species 2			Species 3		
		n	Hap	*COI*	*n*	Hap	*COI*	*n*	Hap	*COI*
H8	-28.70168039/ 120.8998418	6	1(2)	FJ785785	1	28	FJ785804			
			2	FJ785752						
			37	FJ785810						
			38(2)	FJ785811						
J2	-28.70247969/ 120.8937371	1	2	FJ785752						
J3	-28.70250651/ 120.8947134	1	10	FJ785791						
J4	-28.70255479/ 120.8957863	4	2(4)	FJ785752						
J5	-28.70255479/ 120.896768	1	11	FJ785792						
J6	-28.70258161/ 120.8977979	2	2	FJ785752	2	21	FJ785757			
			3	FJ785786		29	FJ785805			
J7	-28.70260307/ 120.8988118	1	2	FJ785752						
L3	-28.70696434/ 120.8925462				2	23	FJ785760			
						26	FJ785758			
L7	-28.7070609/ 120.8966821				4	22	FJ785801			
						23(2)	FJ785760			
						25	FJ785802			

N2	-28.70872506/ 120.8915061				2	24(2)	FJ785759			
N4	-28.70880433/ 120.8935225				3	23(3)	FJ785760			
N5	-28.70881506/ 120.8945792				2	20	FJ785756			
						25	FJ785802			
N6	-28.70881506/ 120.8956361				2	23(2)	FJ785760			
N7	-28.70887407/ 120.8966392				2	20	FJ785756			
						24	FJ785759			
Q2	-28.71059605/ 120.8914894	2	1	FJ785785				2	30(2)	FJ785761
			13	FJ785794						
S2	-28.71236631/ 120.8914679	1	1	FJ785785						
S3	-28.71243068/ 120.8925462	1	14	FJ785795	2	23(2)	FJ785760			
U2	-28.71415802/ 120.8913982	3	1(3)	FJ785785	4	20(2)	FJ785756			
						25	FJ785802			
						27	FJ785803			
W2	-28.7159712/ 120.8913606	1	1	FJ785785	2	25(2)	FJ785802			
W3	-28.71601948/ 120.8934528	7	2(5)	FJ785752	14	25(7)	FJ785802			
			12	FJ785793		29(2)	FJ785805			
			45	FJ785818		36(5)	FJ785809			

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Allozyme allele frequencies for the amphipod phylogeography study in Chapter IV

Appendix III. Allozyme profiles at 11 variable loci. Where a locus is polymorphic, the frequency of the more common alleles are expressed as percentages and shown as superscripts, and the frequency of the rarest allele can be calculated by subtraction. The number in brackets refers to the number of individuals sampled at this site. H_O = observed heterozygosity count; S.E. = standard error.

A. Allozyme profiles for chiltoniid species 1 from 30 sites.

Site	*Enol*	*G6pd*	*Got1*	*Got2*	*Gpi*	Gpt	*Idh1*	*Mpi*	*Pgm*	*PepB*	*PepA2*	*Tpi*	H_O ± S.E.
A3(1)	c	d	d	e	h^{50},n^{50}	b	b^{50},c^{50}	e^{50},g^{50}	e	d	b^{50},c^{50}	e	0.333 ± 0.142
A6(4)	e	e	d^{75},e^{13},f^{12}	e^{87},c^{13}	$p^{37},j^{25},o^{13},f^{13},l^{12}$	b	c	f^{63},e^{25},h^{12}	e^{67},c^{17},g^{16}	d	c^{63},b^{25},d^{12}	d^{75},e^{25}	0.326 ± 0.101
A8(2)	e^{75},c^{25}	e^{75},a^{25}	d	e^{75},c^{25}	j^{75},h^{25}	b	c	f^{75},e^{25}	g^{50},e^{25},h^{25}	d	c^{75},d^{25}	d^{50},e^{50}	0.375 ± 0.090
A9(2)	e	e^{75},d^{25}	d	e^{75},g^{25}	j^{50},h^{25},n^{25}	c^{75},b^{25}	c	f^{75},g^{25}	e^{50},g^{25},h^{25}	d	d	d^{50},e^{50}	0.417 ± 0.120
A11(3)	e^{83},c^{17}	e^{67},d^{17},f^{16}	d^{83},f^{17}	e	$h^{33},j^{33},g^{17},n^{17}$	b	c	f^{50},g^{33},e^{17}	$g^{49},e^{17},c^{17},h^{17}$	d^{67},e^{17},f^{16}	c^{67},d^{33}	d	0.444 ± 0.118
B4(12)	e^{54},c^{42},b^{4}	e^{62},d^{38}	d^{83},a^{17}	e^{92},f^{4},g^{4}	$n^{46},h^{33},l^{13},j^{4},p^{4}$	b^{88},c^{12}	$c^{50},b^{42},e^{4},f^{4}$	$c^{58},g^{21},f^{17},h^{4}$	e^{54},g^{46}	d	d^{62},c^{38}	d^{71},e^{29}	0.368 ± 0.052
B5(2)	e^{75},c^{25}	e	d	e	h^{50},j^{25},o^{25}	b		a^{50},f^{50}	e	d	c^{75},d^{25}	d^{75},e^{25}	0.318 ± 0.122
B11(14)	e^{79},c^{21}	$e^{81},d^{11},c^{4},f^{4}$	d^{79},f^{14},e^{7}	e^{82},c^{14},g^{4}	$j^{29},i^{18},l^{17},h^{14},n^{11},o^{7},g^{4}$	b^{89},c^{11}	c^{89},b^{7},e^{4}	f^{75},g^{14},e^{11}	$g^{67},h^{18},e^{11},b^{4}$	$d^{75},c^{11},f^{11},g^{3}$	$c^{46},d^{29},b^{21},a^{4}$	d^{82},e^{18}	0.405 ± 0.042

C2(2)	e^{75},c^{25}	e	d^{75},f^{25}	e^{75},a^{25}	j^{75},n^{25}	b	c^{75},b^{25}	e^{50},f^{25},g^{25}	g^{50},h^{50}	d^{75},f^{25}	c	d	0.417 ± 0.104
C5(3)	e^{67},c^{33}	e	d	e	$j^{33},p^{33},l^{17},c^{17}$	b	c^{83},f^{17}	f^{67},g^{33}	e^{50},g^{33},c^{17}	d^{83},e^{17}	d^{67},c^{33}	d	0.278 ± 0.099
C13(3)	e^{83},c^{17}	e^{83},d^{17}	d^{67},e^{17},f^{16}	e^{50},g^{33},c^{17}	i^{67},l^{17},n^{16}	b	c^{67},b^{33}	g^{50},f^{33},c^{17}	e^{50},g^{50}	d	c^{50},d^{33},b^{17}	d	0.389 ± 0.090
D5(8)	c^{75},e^{25}	e^{87},d^{13}	d^{63},f^{37}	e^{81},g^{19}	$j^{38},n^{25},h^{18},g^{13},i^{6}$	b	c	f^{50},g^{36},e^{14}	g^{62},e^{38}	d^{50},f^{50}	$c^{38},b^{31},d^{25},a^{6}$	d^{88},e^{12}	0.436 ± 0.086
D13(1)	e	e	d^{50},f^{50}	e	l^{50},p^{50}	b	c	f^{50},g^{50}	e^{50},g^{50}	d	b^{50},d^{50}	d	0.417 ± 0.149
E1(1)	e	b^{50},e^{50}	d^{50},f^{50}	e	j^{50},p^{50}	b^{50},c^{50}	b^{50},c^{50}	f	g^{50},h^{50}	d	c^{50},d^{50}	d	0.583 ± 0.149
E12(1)	e		d^{50},e^{50}	e^{50},g^{50}	j^{50},l^{50}	b	c	e	c^{50},h^{50}	d^{50},f^{50}	d	d	0.455 ± 0.157
F4(11)	e^{77},c^{23}	e^{77},d^{18},a^{5}	d^{77},f^{18},b^{5}	e^{77},g^{23}	$j^{36},i^{14},l^{14},n^{14},h^{8},f^{5},e^{5},o^{4}$	b	c^{94},a^{6}	$f^{49},g^{32},i^{9},e^{5},a^{5}$	g^{65},e^{20},h^{15}	d	$c^{35},d^{35},b^{20},e^{5},h^{5}$	d^{82},e^{13},b^{5}	0.405 ± 0.077
F6(1)	c^{50},e^{50}		d	e	n	b	c	c^{50},g^{50}	c^{50},g^{50}	d	b^{50},c^{50}	d	0.364 ± 0.152
F7(3)	e^{83},c^{17}	e^{67},d^{33}	d	e	j^{83},g^{17}	b	c	f^{67},g^{33}	g^{83},c^{17}	d^{83},c^{17}	c^{67},b^{17},d^{16}	d^{83},e^{17}	0.250 ± 0.073
F9(2)	e	e	d^{75},f^{25}	e^{75},g^{25}	j^{50},n^{25},o^{25}	b	c^{75},e^{25}	f^{75},g^{25}	g	d	c^{75},b^{25}	d	0.292 ± 0.096

Site	*Enol*	*G6pd*	*Got1*	*Got2*	*Gpi*	Gpt	*Idh1*	*Mpi*	*Pgm*	*PepB*	*PepA2*	*Tpi*	H_O ± S.E.
G4(2)	e^{75},c^{25}	d^{50},e^{50}	e^{75},d^{25}	e^{50},b^{25},g^{25}	l^{75},n^{25}	b	c	f^{75},g^{25}	g	d^{75},f^{25}	c^{50},d^{50}	d	0.458 ± 0.114
G6(1)	e	e	d	e	h^{50},j^{50}	b	c	b	g	c^{50},d^{50}	d	d	0.167 ± 0.112
H2(14)	e	e^{57},d^{43}	d^{96},f^{4}	e^{64},g^{32},c^{4}	$j^{43},h^{25},l^{25},n^{4},o^{3}$	b^{89},c^{11}	c^{96},a^{4}	$f^{36},g^{28},c^{25},b^{7},a^{4}$	g^{82},e^{18}	d^{86},f^{14}	$c^{61},d^{24},b^{11},f^{4}$	d^{89},e^{11}	0.346 ± 0.070
H7(1)	c^{50},e^{50}	d	d	e	j	b	c	e^{50},f^{50}	g	d	c^{50},f^{50}	d	0.250 ± 0.131
H8(6)	e^{75},c^{25}	e^{90},d^{10}	d^{83},b^{17}	e^{92},c^{8}	$h^{25},j^{25},i^{17},n^{17},o^{16}$	b^{92},d^{8}	c	f^{83},g^{9},e^{8}	h^{50},e^{33},g^{17}	d	c^{50},d^{42},b^{8}	d^{92},e^{8}	0.336 ± 0.084
J4(4)	e^{75},c^{25}	d^{75},e^{25}	d^{88},f^{12}	e	$i^{38},j^{25},l^{25},o^{12}$	b	c^{50},a^{33},e^{17}	f^{62},g^{25},e^{13}	e^{50},g^{33},h^{17}	d^{67},c^{17},f^{16}	c^{50},b^{33},d^{17}	d^{63},e^{37}	0.451 ± 0.114
J6(2)	e	e	d	g	i^{50},n^{50}	b	b^{50},c^{50}	b^{50},c^{50}	g^{75},h^{25}	d	b^{50},d^{50}	d	0.375 ± 0.139
L3(1)	e	e	d^{50},f^{50}	e	j	b^{50},c^{50}	c	g	c^{50},g^{50}	f	c^{50},d^{50}	d	0.333 ± 0.142
Q2(1)	e	e	d	c^{50},e^{50}	k^{50},o^{50}	b	c	e^{50},h^{50}	e^{50},h^{50}	d	c	d^{50},e^{50}	0.417 ± 0.149
U2(2)	e	e^{75},d^{25}	d^{50},f^{50}	e	n^{50},l^{25},o^{25}	b	c	b^{50},e^{25},h^{25}	h^{50},g^{25},e^{25}	d^{75},f^{25}	c^{50},a^{25},d^{25}	d	0.458 ± 0.130
W3(7)	e^{93},c^{7}	e	d^{86},f^{14}	e^{79},g^{21}	$j^{43},o^{15},n^{14},g^{14},h^{7},l^{7}$	b	c^{92},b^{8}	$f^{43},g^{29},b^{21},c^{7}$	$g^{43},e^{36},i^{14},c^{7}$	d^{71},f^{22},c^{7}	$d^{36},b^{29},c^{21},f^{14}$	d^{64},e^{36}	0.371 ± 0.077

B. Allozyme profiles a for chiltoniid species 2 and 3.

Taxon	Site	*Enol*	*G6pd*	*Got1*	*Got2*	*Gpi*	*Gpt*	*Idh1*	*Mpi*	*Pgm*	*PepB*	*PepA2*	*Tpi*	$H_O \pm$ S.E.
Sp. 2	E12(3)	f	d	f	e	i	a	d	f	-	f	e	f	0.000 ± 0.000
	H8(1)	f	d	f	e	i	a	d	f	j	f	e	f	0.000 ± 0.000
	L7(2)	f	d	f	e	i	a	d	f	j	f	e	f	0.000 ± 0.000
	S3(1)	f	d	f	a^{50},e^{50}	i	a	d	f		f	e	f	0.091 ± 0.091
	U2(1)	f	d	f	e	i^{50},l^{50}	a	d	f	j	f	e	f	0.083 ± 0.083
	W2(1)	f	d	f	e	i	a	d	f	j	f	e	f	0.000 ± 0.000
	W3(6)	f	d	f^{92},g^{8}	e	i^{92},m^{8}	a	d	f	j^{84},h^{8},b^{8}	f	e	f	0.056 ± 0.031
Sp. 3	A3(2)	d	e	d^{50},c^{25},a^{25}	e	d^{50},g^{25},j^{25}	a	b	d^{75},f^{25}	d	b^{75},d^{25}	g	a^{75},c^{25}	0.292 ± 0.114
	B4(2)	d^{75},a^{25}	e^{75},b^{25}	$a^{25},b^{25},c^{25},d^{25}$	d^{50},e^{50}	d^{75},b^{25}	a	b	d	d^{75},f^{25}	b^{50},a^{25},d^{25}	g	a	0.333 ± 0.112
	C2(1)	d	b^{50},e^{50}	c	e	d^{50},g^{50}	a	b	d^{50},f^{50}	-	b^{50},f^{50}	g	a	0.364 ± 0.152
	E1(1)	d	e	a^{50},d^{50}	e	a^{50},d^{50}	a	b	-	a^{50},f^{50}	b	g	a^{50},c^{50}	0.364 ± 0.152
	H2(1)	d	-	c^{50},d^{50}	e	d^{50},g^{50}	a	-	-	-	b^{50},d^{50}	g	a	0.375 ± 0.183

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