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ORIGINAL ARTICLE

Evidence for population fragmentation within a subterranean aquatic habitat in the Western Australian desert

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The evolution of subterranean animals following multiple colonisation events from the surface has been well documented, but few studies have investigated the potential for species diversification within cavernicolous habitats. Isolated calcrete (carbonate) aquifers in central Western Australia have been shown to contain diverse assemblages of aquatic subterranean invertebrate species (stygofauna) and to offer a unique model system for exploring the mechanisms of speciation in subterranean ecosystems. In this paper, we investigated the hypothesis that microallopatric speciation processes (fragmentation and isolation by distance (IBD)) occur within calcretes using a comparative phylogeographic study of three stygobiontic diving beetle species, one amphipod species and a lineage of isopods. Specimens were sequenced for the mitochondrial cytochrome *c* oxidase 1 gene from three main sites: Quandong Well, Shady Well (SW) and Mt. Windarra (MW), spanning a 15 km region of the Laverton Downs Calcrete. Phylogenetic and haplotype network analyses revealed that each species possessed a single divergent clade of haplotypes that were present only at the southern MW site, despite the existence of other haplotypes at MW that were shared with SW. IBD between MW and SW was evident, but the common phylogeographic pattern most likely resulted from fragmentation, possibly by a salt lake adjacent to MW. These findings suggest that microallopatric speciation within calcretes may be a significant diversifying force, although the proportion of stygofauna species that may have resulted from *in situ* speciation in this system remains to be determined.
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Introduction

The shift from an epigean existence to cave life and subsequent isolation can typically lead to dramatic speciation events and morphological adaptation (Niemiller *et al*., 2008). This process of speciation by transition from surface to subterranean environments is suggested as the main mechanism for the evolution of subterranean species, although there is some debate about whether it requires extinction of the surface ancestral species (climate relict hypothesis; for example Peck and Finston (1993)) or can proceed in the presence of gene flow between the surface and subterranean populations (adaptive shift hypothesis; for example Desutter-Grandcolas and Grandcolas (1996)). However, given the extraordinary number of species observed in cave systems (Poulson and Culver, 1969; Humphreys, 2000, 2008; Buhay *et al*., 2007), this transition, from surface to subterranean environments, is unlikely to be the sole source of species diversification in cave animals and it is possible that some species evolved from troglomorphic ancestors fully adapted to life underground (Juan and Emerson, 2010; Juan *et al*., 2010; Ribera *et al*., 2010). The difficult nature of gaining access to cave habitats makes this latter hypothesis difficult to investigate; however, a number of key studies have examined these questions (Caccone, 1985; Buhay and Crandall, 2005; Guzik *et al*., 2009). They observe that, internally, cavernicolous habitats maintain complex genetic systems that, undisturbed, are largely stable over time and can maintain significant levels of genetic diversity (Culver, 1970a, b; Culver *et al*., 1973). This, despite the substantial climatic oscillations during the Pleistocene, suggests that cave systems may share similarities with other well-known closed ecosystems in which sympatric, parapatric or microallopatric (allopatry *in situ*) speciation has been documented (for example, sticklebacks in Nicaraguan crater lakes (Taylor and McPhail, 2000), palms on Lord Howe Island (Savolainen *et al*., 2006), spiders on Hawaiian Islands (Gillespie, 2004)). A cave system in the desert of outback Australia provides a further opportunity to investigate the possibility of *in situ* diversification of subterranean animals.

Calcrete (carbonate) aquifers in the Yilgarn region of Western Australia comprise some of the greatest diversity of short-range endemic (*sensu*; Harvey (2002)) cave

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species in the world. Each aquifer comprises similar stygobiontic organisms: crustaceans (for example, amphipods, copepods, isopods, bathynellids), oligochaetes (segmented worms) and water beetles (Dytiscidae; Humphreys, 2000), but each maintains a distinct set of species. The subterranean Dytiscidae (Watts and Humphreys, 2006) exemplify this diversity and are thought to represent the world's largest groundwater beetle fauna by a factor of 10 (Humphreys, 2006). The origin of this fauna is thought to lie in the mid-late Miocene when Central and Western Australia was a stable mesic habitat (Leys *et al*., 2003; Byrne *et al*., 2008). Subsequent aridification led to relictualisation of the fauna to habitats that retained water (for example, calcrete aquifers, mound springs and groundwater-fed lakes). Very likely, significant speciation probably occurred through vicariance at the time of colonisation and calcrete enclosure.

Evidence suggests that *de novo* speciation may also have occurred *in situ* well after these vicariance events (Cooper *et al*., 2002; Leys *et al*., 2003). A repeated pattern of two to three species of stygobiontic diving beetles is consistently observed within isolated aquifers, some of which are clear sympatric sister species (Leys *et al*., 2003; Watts and Humphreys, 2006; Guzik *et al*., 2009). To date, this pattern has been observed in 29 calcretes in which each species occupies non-overlapping size classes, ranging from large (~5 mm) to very small (~1 mm) beetles (Watts and Humphreys, 1999, 2000, 2001, 2003, 2004, 2006, 2009). Phylogenetic analyses using mitochondrial DNA (mtDNA) data have shown that 13 of these calcretes contain sympatric sister species (two with a sister triplet) and have further suggested that species were isolated within single calcretes ~3–10 million years ago (mya; Cooper *et al*., 2002; Leys *et al*., 2003; Leys and Watts, 2008). Microallopatric or sympatric speciation is a plausible scenario for the origin of these species, in addition to other geographic modes of speciation between these two extremes (Butlin *et al*., 2008). Alternatively, repeated colonisation from the same ancestral source may also lead to such patterns. Currently, it is difficult to test the scenario of sympatric speciation, but identifying whether microallopatric speciation processes, such as population fragmentation, have operated within calcrete aquifers is a distinct possibility.

Guzik *et al*. (2009) investigated the population genetic and phylogeographic structure of three sympatric sister species of dytiscid diving beetle from a single aquifer. Their results revealed evidence for isolation by distance (IBD) effects in two of the three beetle species over a very small spatial scale. However, the study area of 3.5 km^2 at Sturt Meadows Calcrete (SMC), although large in size relative to the inhabiting organisms, was not large enough to detect possible evidence of population fragmentation in the three species. In the present study, we gained access to a substantially larger sampling region (up to 15 km between samples compared with ~3.5 km) in a calcrete aquifer that lies ~135 km northeast of SMC and on a different palaeodrainage channel, Carey drainage (east), known as the Laverton Downs Calcrete (LDC). As in the previous study, we used a comparative phylogeographic approach using three dytiscid beetle species, *Limbodessus lapostae* Watts and Humphreys (1999), *L. windarraensis* Watts and Humphreys (1999) and *L. palmulaoides* Watts and Humphreys (2006), which vary in size from the smallest to the largest (1.3, 2.2 and 4.2 mm, respectively). The three species are congeneric and sympatric but are not sister species (Leys *et al*., 2003; Leys, unpublished). It is most likely that these three species evolved from three distinct ancestral species that were already reproductively isolated before their colonisation of and enclosure within the aquifer. An undescribed chiltoniid amphipod species and a lineage of *Haloniscus* isopods were also analysed to further examine whether similar geographic patterns of population subdivision applied to other taxa in the aquifer. The *Haloniscus* isopods were examined previously using mtDNA sequence analyses (Cooper *et al*., 2008), in which they were shown to form a reciprocally monophyletic clade, and a number of lineages were observed from LDC specimens with some evidence for phylogeographic structure. We included all of them in the current analyses and use the term 'isopods' to broadly identify them.

The specific aim of this study was to investigate broad-scale (distances up to 15 km in an aquifer ~90 km^2 in size) population genetic structuring within a single calcrete aquifer for three dytiscid beetles, an amphipod and a lineage of isopods. We have used a mitochondrial gene marker and a multispecies phylogeographic approach to determine whether there were concordant patterns of genetic structure among taxa. It was predicted that if fragmentation of the range of each species had occurred in the past, the signal of this event should (a) be present and shared in the genes of each species and (b) reflect robust monophyletic groups of haplotypes with long branches connecting each group (Avise, 1994). In the absence of or with reduced recombination, as in mitochondrial markers, this fragmentation signal may still be present within extant populations even if gene flow subsequently recommenced. In the event of evidence for fragmentation, we aimed to test a hypothesis of IBD or vicariance (that is, potential microallopatry) within the subterranean aquatic habitat.

Materials and methods

Sampling

The three species of Dytiscidae, the amphipod and isopods were collected (years 2005–2007) from three key locations in the LDC of the Yilgarn region (Figure 1 and Appendix 1). Two of these locations comprise borefields, similar to that of SMC (Guzik *et al*., 2009), that are ~9.5 km apart. These borefields are named Mt. Windarra (MW) and Shady Well (SW) consecutively from south to north. Each borefield contains a grid of up to 20 mineral exploration bores, but MW has substantially fewer bores than SW. At the time these bores were established, their primary purpose was not for biological study, which was also the case at the SMC (Guzik *et al*., 2009). Another site ~6.5 km further north of the SW borefield, Quandong Well (QW), an active pastoral well, provided additional access to the calcrete. Specimens were collected using plankton nets as per Allford *et al*. (2008). The use of this method captured many different stygobiontic invertebrates and yielded large numbers of *L. windarraensis* but fewer specimens of *L. lapostae* and *L. palmulaoides*. The amphipod was collected relatively frequently, whereas the isopods were rare and considered potentially benthic.

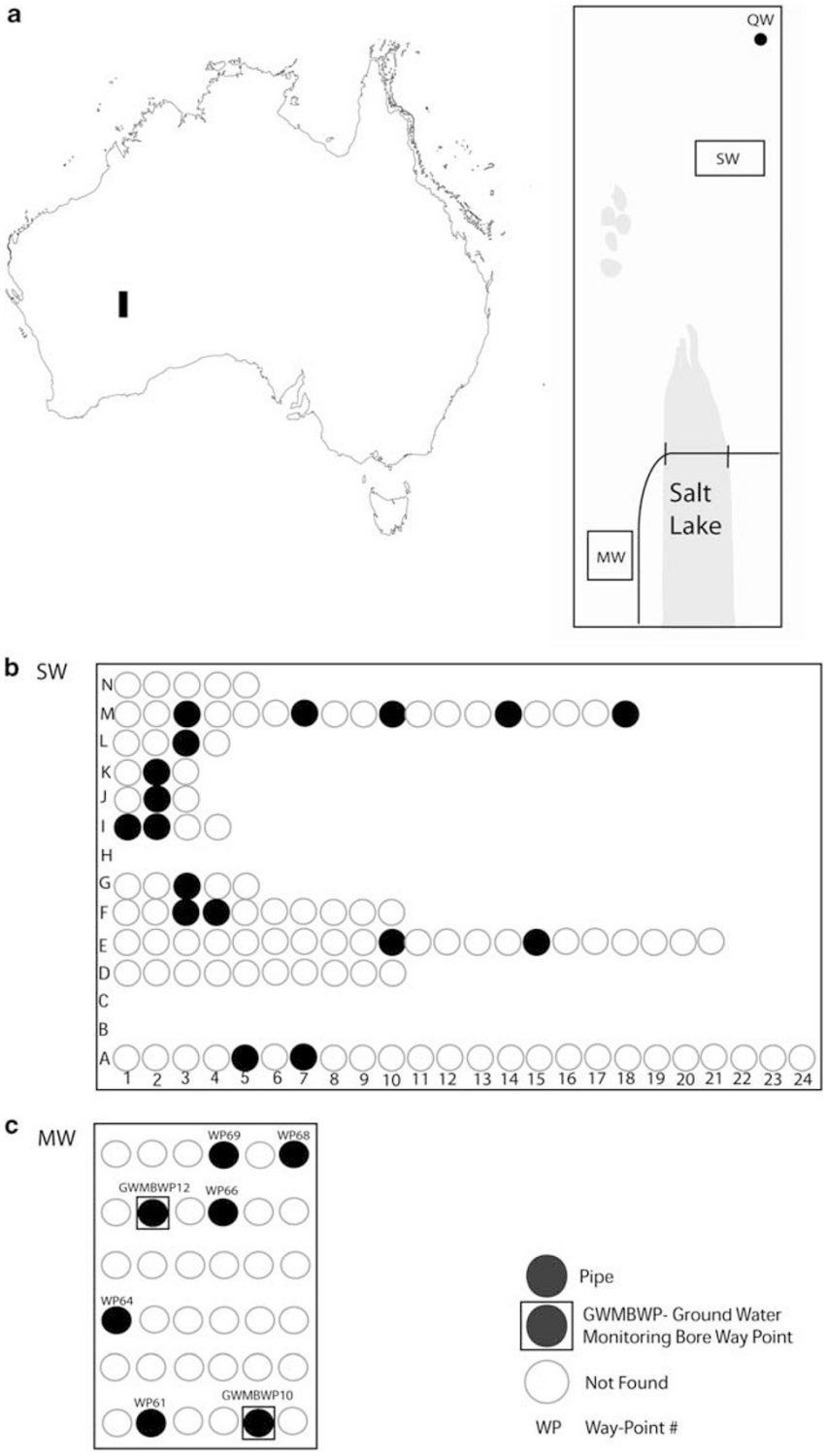

Figure 1 (**a**) Schematic map of Australia with location of LDC and a diagram of borefield locations at LDC, MW, SW and QW. Schematic diagram of borefields at (**b**) SW and (**c**) MW.

Specimens were preserved in the field using 100% ethanol and stored at −20 °C until DNA extraction. Voucher specimens of each of the three beetle species have been lodged in the South Australian Museum. Frozen DNA vouchers have also been lodged at the South Australian Museum's Evolutionary Biology Unit, located at the University of Adelaide.

Sequencing and alignment

PCR was used to amplify a 689-bp fragment of the mtDNA cytochrome *c* oxidase subunit 1 (*cox1*) gene. The primers used for beetles were C1-J-2183 (5′-CAACATTT ATTTTGATTTTTTGG-3′; Simon *et al*., 1994) and UEA10 (5′-TCCATTGCACTAATCTGCCATATTA-3′; Lunt *et al*., 1996); for the amphipods: LCO1490 (5′-GGTCAAC

AAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAA CTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*., 1994); and the same primers as used in Cooper *et al*. (2008) for the isopods. PCR was carried out on a PalmCycler v.2.1.7 (Corbett Research, Sydney, Australia) thermocycler using 25 μl reaction volumes consisting of 17.4 μl of nuclease-free H_2O, 2.5 μl of 2.5 × buffer, 0.5 mM of dNTP's, 1.0 μl of 5 μM of each primer and 0.1 μl of HotmasterTaq (Eppendorf AG, Hamburg, Germany). The cycling conditions for *cox1* consisted of one cycle at 94 °C for 2 min and 40–45 cycles (94 °C, 30 s; 48–54 °C, 30 s; and 72 °C, 60 s), followed by a final incubation step at 24 °C for 3 min. Amplified PCR products were then identified using gel electrophoresis. PCR products were purified using the AMPure (Agencourt Bioscience Corporation, Danvers, MA, USA) system according to the manufacturer's protocol. Purified products were sequenced using the ABI Prism Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in 10 μl reaction volumes, according to the manufacturer's protocol.

The sequence alignment program BioEdit v7.0.5.3 (Hall, 1999) was used to edit and align sequences. Unique haplotypes were identified using the program FaBox (Villesen, 2007). PAUP* 4.0b10 (Swofford, 2002) was used to compute corrected (HKY) pairwise genetic distances among haplotypes.

Phylogenetic analysis

To examine relationships within and among species and haplotypes, mtDNA *cox1* sequences were analysed using a phylogenetic approach in the beetles and isopods. Phylogenetic relationships of amphipod populations were not presented here because of the high similarity between haplotypes among sampled individuals. Instead, haplotype networks were a better representation of these data (see below). Two out-group beetle taxa were chosen, *Bidessodes limestonensis* (Watts and Humphreys, 2003) and *Bidessodes gutteridgei* (Watts and Humphreys, 2003), which are distant relatives from the same tribe of diving beetles as the *Limbodessus* sampled from LDC (Bidessini) (Leys *et al*., 2003), and their sequences were obtained from Genbank (Genbank accession: AY350880 and AY350894, respectively). For the LDC isopod out-groups, we chose their nearest relatives on the basis of the phylogeny of Cooper *et al*. (2008). In that study, the taxa (BES10582: EU364601 and BES 11811: EU64602) from the Mt. Morgan calcrete on the Carey palaeodrainage channel formed a monophyletic relationship with those from LDC. In addition to new sequence data for the isopods, a number of sequences were obtained from Genbank (BES12005, 12087, 12102, 13141, 13149.1: EU364595-600 and BES10291.1 (13167), 10291.2 (13157), 12021.1 (13173.2), BES13173.1, 13180.1, 13186.2: EU36 4589-4). Phylogenetic reconstruction using a Bayesian approach was implemented with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The model that best fit the higher level sequence data for both beetles and isopods was estimated with MODELTEST 3.7 (Posada and Crandall, 1998) for nucleotide data under an Akaike Information Criterion framework. Models were tested for all three codon positions: the HKY + I + G model was favoured for the first and second, and GTR + I + G for the third position. The nucleotide sequence data were partitioned by codon position and each partition was started independently with a different model (listed above). All parameters were unlinked and the rates were allowed to vary over the partitions. This approach circumvents part of the problem of saturation at the third codon position because each position is treated independently and not assumed to be evolving at the same rate. Four chains were run simultaneously for 5 000 000 generations in two independent runs, sampling trees every 100 generations. To evaluate convergence to the stationary distribution, the program Tracer 1.4 (Drummond and Rambaut, 2007) was used. The likelihood values converged to relative stationarity after about 75 000 generations. A burn-in of 1 250 000 was chosen and a 50% consensus tree was constructed from the remaining trees.

Population analyses

A hierarchical approach was taken to examine the partitioning of genetic variation within and between populations. The highest population level consisted of the three main sites from south to north that were separated by more than 6.5 km: MW, SW and QW, described here as 'borefield'. Although QW is a single bore, we also use the term 'borefield' as a general term for the three broad populations. The next hierarchical level was that of individual 'bore holes', which permitted a fine-scale examination of the genetic variance. ARLEQUIN v.3.1 (Excoffier *et al*., 2005) was used to carry out analysis of molecular variance, determine nucleotide compositions, as well as carry out population comparisons. ARLEQUIN was also used to test for population differentiation using 100 000 Markov chain steps and 10 000 dememorisation steps, at $P = 0.05$. TCS v.1.21 (Clement *et al*., 2000) was used to generate and arrange haplotype networks for each of the three beetles and the amphipod at a 95% connection limit. Networks were not presented for the isopods as this parsimony-based analysis does not adequately reflect the distinctive relationships among some of the haplotypes.

It must be noted here that, distinct from our *a priori* population groupings discussed above, our results also revealed genetically distinct clades among all taxa. Individuals bearing haplotypes for one of these clades were localised in their distribution to the MW borefield (MW-only clade) and the other haplotypes were found to be widespread in their distribution and were generally found in all borefields (widespread-LAV clade). This pattern was found particularly for the three beetle species and to a lesser extent for the isopods (see results section for details). The amphipods showed some evidence of genetic differentiation relative to geographic location, but haplotypes were too closely related to warrant discussion of an MW-only clade. Therefore, these were also included in some of the population analyses.

The demographic history of individual populations was assessed with ARLEQUIN v3.1 by computing Fu's (Fu, 1997) F_s (F_s), Tajima's (Tajima, 1989) D (D), parameters for the model of population expansion (time since expansion (τ), and relative population sizes before (θ_1) and after (θ_2) expansion) and for the continent-island model of demographic expansion ($\tau = 2T\mu$, $\theta = 2N\mu$ and $M = 2Nm$, where T = number of generations before spatial expansion, μ = mutation rate, N = size of deme (assumed constant) and m = fraction of individuals from

a deme exchanging with other demes). The generalised least-squares approach (Schneider and Excoffier, 1999) in ARLEQUIN was used to test the empirical mismatch distribution against a model of demographic expansion. We used DnaSP v4.20.2 (Rozas *et al*., 2003) to calculate R_2 (Ramos-Onsins and Rozas, 2002) for detecting population growth in the same genetic populations on the basis of the number of segregating sites: an assumption of no recombination and 10 000 replicates. The model of IBD was tested using a Mantel test following the approach of Slatkin (1993) using the Alleles In Space program (Miller, 2005) with 10 000 randomisations. The tests were conducted with pairwise comparisons between each of the three borefields rather than all three at once. The rationale for this was that an artificial IBD signature was observed when all three locations were tested because of the presence of genetically distinct haplotypes at the MW borefield only (MW-only clade see below). This IBD signature did not reflect the presence of widespread and shared haplotypes at all borefields (widespread-LAV clade haplotypes) because of the MW-only clade haplotypes being found only in the southern borefield. The pairwise Mantel test allowed us to test for IBD between borefields while minimising the impact of the MW-only clade.

Estimating coalescent time of mtDNA sequences

BEAST v1.4.7 (Drummond and Rambaut, 2007) was used to estimate the coalescence time (that is, time to most recent common ancestor of mtDNA sequences for each species). The subprogram BEAUti v1.4.7 (Drummond and Rambaut, 2007) was used to create input .xml files, and Tracer v1.4 (Drummond and Rambaut, 2007) was used to analyse the parameter distributions estimated from BEAST. An UPGMA starting tree was estimated under the HKY + I + G model in which (a) base frequencies were estimated, (b) codon positions were partitioned (positions 1 + 2, 3) and (c) the parameters, substitution model across codon positions and rate heterogeneity model were unlinked. The substitution rate was fixed at 0.0115 (standard arthropod mtDNA molecular clock of 2.3% divergence per million years (Brower, 1994)), and a relaxed clock (uncorrelated lognormal) was used. A number of different models built earlier were implemented separately on each of the LDC species using BEAST (for example, Yule speciation, and coalescent: expansion, exponential, constant and Bayesian Skyline). We found that each of the previous models made little difference to the final time estimate and that these estimates were very similar (within 0.2 mya); therefore, we present results using the Yule model only. Each analysis was run for 10 000 000 generations, with sampling every 100 generations, and the burn-in was 25% of the total sampled trees (that is, 25 000). Each analysis was run multiple times and all estimated dates were consistent with those presented here.

Results

Mitochondrial *cox1* sequences of 689-bp length were obtained from *L. lapostae* ($n = 55$, $h = 15$), *L. windarraensis* ($n = 84$, $h = 30$) and *L. palmulaoides* ($n = 27$, $h = 21$), of 618 bp for the amphipod species ($n = 80$, $h = 43$) and of 605 bp ($n = 42$, $h = 23$) for the isopods (see Appendix 1 for details of haplotypes and location data). All sequences were free of insertions and deletions, and comprised an open reading frame, as expected of a functional gene, suggesting that the sequences were unlikely to represent mitochondrial pseudogenes. Sequences were deposited at Genbank: amphipods: ALA1-43, HQ535666-708; beetles: *L. palmulaoides*: LLA1-21, HQ535709-29; *L. lapostae*: SLA1-15, HQ535730-44; *L. windarraensis*: MLA1-30, HQ535745-74; and isopods: ILA7-8 (HQ535781-82), ILA15-23 (HQ535789-97).

Phylogenetic analysis

Each species showed significant phylogenetic structuring, with multiple divergent monophyletic groups of haplotypes. The Bayesian tree of haplotypes for all three beetle species (Figure 2) showed that each species possessed a small clade of individuals found only from the southern MW location (herein coded MW-only clades). All remaining individuals were widespread and represented throughout the LAVerton region by what we refer to here as the widespread-LAV clade for which haplotypes were generally found at all three borefields (that is, MW, SW and QW). The isopods (Figure 3) showed a similar pattern, with the presence of one divergent clade almost entirely restricted to MW and a second major clade restricted to SW + QW, the latter containing two subclades, referred to here as ISOLAV1 (SW + QW haplotypes) and ISOLAV2 (SW haplotypes). The only discrepancies in the geographic patterns between isopods and all other taxa were that the MW clade contained a single divergent haplotype (BES 12005: ILA6) from the SW region and there were no shared haplotypes between the MW and SW + QW regions.

Genetic distances

Corrected genetic distances (HKY model of base substitution) among and within the three beetle species showed that, for most individuals of the same species, genetic distance was rarely more than 2% (data not shown). Tables 1 and 2 show the percentage genetic distance between clades from locations throughout LDC. Consistent with the phylogenetic trees, the MW-only clade individuals in each species were substantially more divergent than the rest (widespread-LAV clade individuals; Table 1). In *L. lapostae* and *L. palmulaoides*, divergences were 0–3% among all haplotypes of each species. In *L. windarraensis,* seven haplotypes from southern MW sites (WP12, WP64 and WP69) showed high divergence (1–4%) compared with all others. Further, several haplotypes from these bores were also shared with individuals from the SW bores (widespread-LAV clade). Genetic distances were the highest between 'known' species at 11–15% (Table 1), which is consistent with levels of inter-specific divergence for stygobiontic (Guzik *et al*., 2009) and epigean dytiscid congeners for *cox1* (Ribera *et al*., 2003). The amphipod showed low levels of divergence among individuals, ranging between 0 and 2% (for instance, 2% divergence between ALA24 and ALA46). The most divergent haplotypes were from MW but these differences were not as great as those within beetle species. In contrast to the other species, the two major clades of isopods were highly divergent from each other (19–21% among haplotypes from each clade) and genetic divergence among haplotypes was also high within each clade (0–12%; Table 2). Within the ISOLAV1

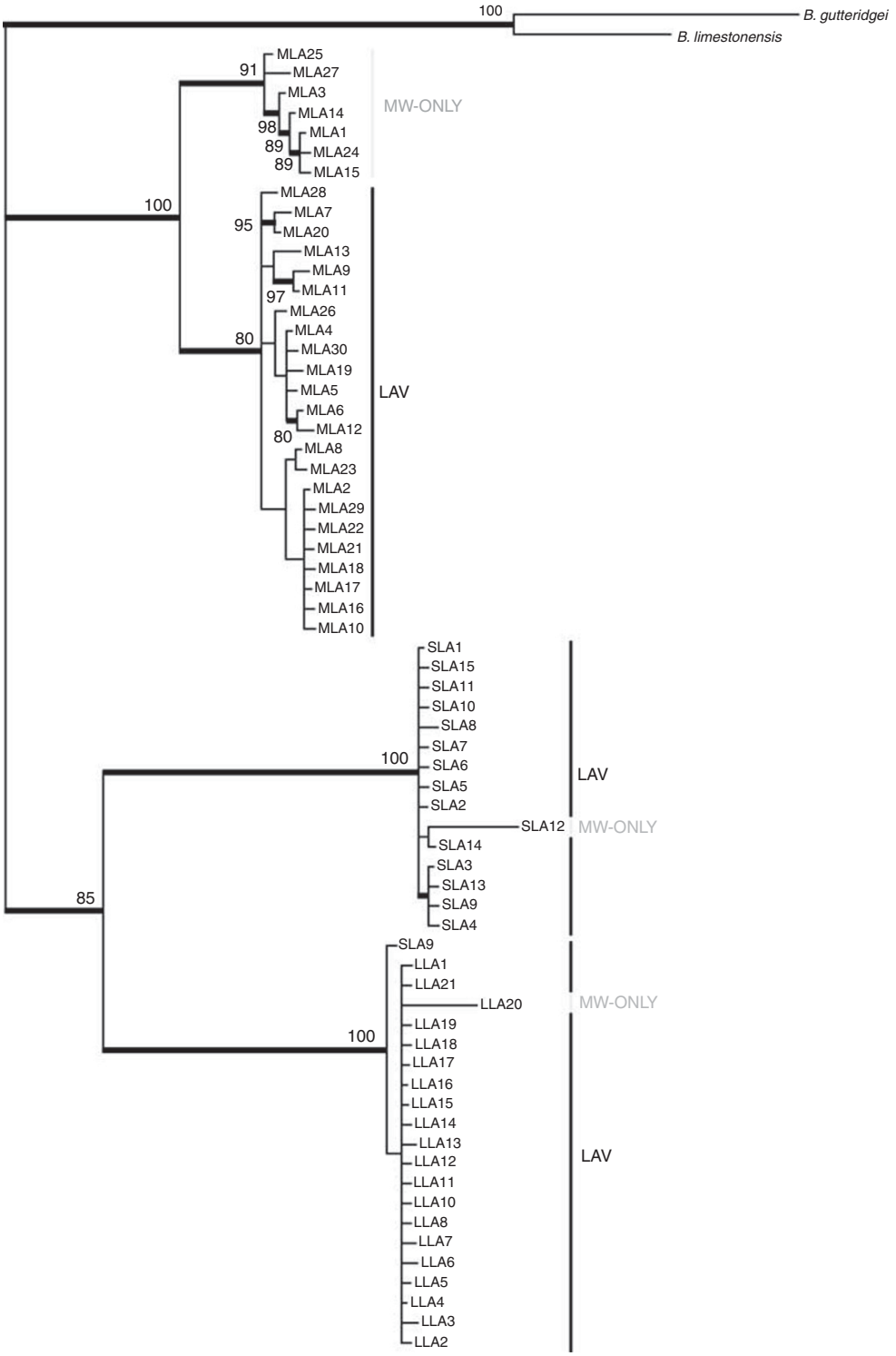

Figure 2 Consensus phylogeny estimated using a Bayesian approach that employed the HKY + I + G model of nucleotide evolution for haplotypes of *L. lapostae*, *L. windarraensis* and *L. palmulaoides*, with out-group taxa *Bidessodes gutteridgei and B. limestonensis*. Lists of specimens and haplotypes are given in the Appendix 1. Haplotype acronyms reflect small (S), medium (M) and large (L) size of the beetle species from LDC: MLA, *L. windarraensis*; SLA, *L. lapostae;* and LLA, *L. palmulaoides*. Both the MW-only clades and the widespread-LAV (LAV) clades are noted. Thickened black lines and numbers on branches indicate Bayesian posterior probabilities >80%.

and ISOLAV2 subclades, divergences were typically 0–3%. Two private haplotypes that grouped closely with the MW-only clade were found only once in our samples (BES 12005 (ILA5) and 12087 (ILA6)) and were very divergent (9–12%) from all others from MW.

Molecular diversity

Haplotype diversity (h) was generally high for all regions in all species (Table 3) with the lowest estimates observed at SW and QW for *L. windarraensis* and *L. lapostae* ($h = 0.66$–0.86). In contrast, nucleotide diversity

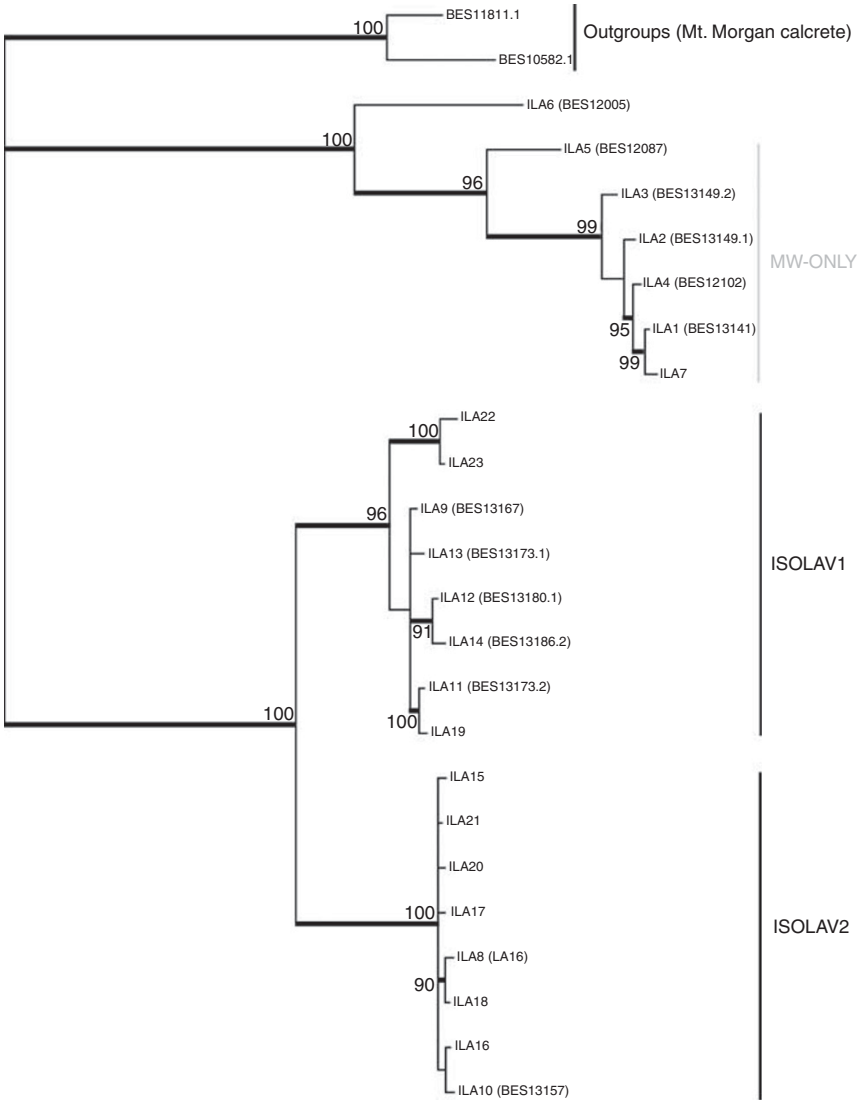

Figure 3 Consensus phylogeny estimated using a Bayesian approach that employed the GTR + I + G model of nucleotide evolution for haplotypes of the *Haloniscus* isopod. Numbers in brackets indicate sequences taken from Cooper *et al*. (2008) from LDC, with unknown outgroup taxa (BES 11811.1 and BES 10582.1) from Mt. Morgan calcrete. Both the MW-only clades and the two widespread-LAV clades (ISOLAV1 and ISOLAV2) are noted. Thickened black lines indicate Bayesian posterior probabilities $>80\%$.

(polymorphism) represented by the mean number of pairwise differences (π) was consistently low at SW and QW for each of the beetle species and in the amphipod at all locations, whereas the MW borefield showed very high nucleotide diversity for the beetles and isopods ($\pi > 8$ average differences) because of the presence of highly divergent haplotypes in the MW borefield. The isopods also showed very high nucleotide diversity ($\pi = 23.9$) at SW.

Haplotype networks

All species showed typical patterns of expansion (or selection) in haplotype networks in which a single dominant haplotype has experienced multiple single-point mutations, leading to many novel and rare haplotypes (Figure 4). In addition, as mentioned above, the beetle species all showed strikingly divergent haplotypes that were solely from MW populations and did not join the networks at 95% confidence in statistical parsimony analyses (Figure 4). The amphipod (Figure 4d) also showed some geographic divergence among individuals from this site; however, they differed less than that observed in the beetles. Amphipod haplotypes were mostly shared between the major populations of MW, SW and QW, excluding individuals from the MW-only clades. We have not shown isopod networks because of the lack of connectivity between numerous clades/haplotypes at the 95% confidence interval. We consider that the relationships between haplotypes were better represented by the phylogenetic analyses.

Population structure

In an analysis of molecular variance, we examined three borefields that provided access to LDC (MW, SW and QW), with borefields comprising numerous individual

Table 1 Percentage of genetic distance (HKY model of substitution) between major clades in each of three stygobiontic beetle species from LDC

	L. lapostae			*L. windarraensis*			*L. palmulaoides*		
	SLA12	*SLA01*	*SLA2*	*MLA1*	*MLA4*	*MLA5*	*LLA01*	*LLA20*	*LLA2*
L. lapostae									
SLA12	—								
SLA01	**<1**	—							
SLA2	**3**	**3**	—						
L. windarraensis									
MLA1	12	11	12	—					
MLA4	12	12	13	**4**	—				
MLA5	12	12	13	**4**	**<1**	—			
L. palmulaoides									
LLA01	14	14	15	14	13	12	—		
LLA20	15	15	15	14	13	13	**<1**	—	
LLA2	15	14	15	13	12	12	**3**	**3**	—

Abbreviations: *L*, *Limbodessus;* LDC, Laverton Downs Calcrete.
Haplotype codes represent species and the haplotype identification number, that is, *L. lapostae* (SLA), *L. windarraensis* (MLA), and from *L. palmulaoides* (LLA; see also Figure 2). Haplotypes belonging to the MW-only clade are noted in grey and haplotypes from the widespread-LAV clade are in black. Representative haplotypes were compared to demonstrate variation within (bold font) and between (normal font) species.

Table 2 Percentage of genetic distance (HKY model of substitution) between major clades of stygobiontic *Haloniscus* isopods from LDC

	MW-only			*ISOLAV1*			*ISOLAV2*	
	ILA7	*ILA5**	*ILA6**	*ILA14*	*ILA22*	*ILA23*	*ILA21*	*ILA10*
MW-only								
ILA7	—							
ILA5*	**7**							
ILA6*	**12**	**11**						
ISOLAV1								
ILA14	20	19	20					
ILA22	22	20	19	**3**				
ILA23	21	20	19	**3**	**<1**			
ISOLAV2								
ILA01	20	20	21	9	9	9		
ILA21	20	20	21	9	9	9	**<1**	

Abbreviations: LDC, Laverton Downs Calcrete; MW, Mt. Windarra.
Haplotype codes represent isopods (I) and the haplotype identification number (see also Figure 3). Haplotypes belonging to the MW-only clade are noted in grey and haplotypes from the widespread-LAV clade are in black. Representative haplotypes were compared to demonstrate variation within (bold font) and between (normal font) species. Outliers were noted with *.

bore holes at the former two locations and one at QW. Overall, substantial genetic differentiation was observed in the MW borefield (Table 4). In beetles, the majority of genetic variation was observed within individual bores and was significant in *L. windarraensis*, the amphipod and the isopods (60.53–80.27%, $P \leqslant 0.003$). We attribute this diversity within bores to the two major haplogroups existing within sites at MW. However, some of the genetic variation was also attributed between borefields for the beetles (24.34–41.07%), but only significantly so for the isopod and the amphipod (35.11–74.16%, $P \leqslant 0.01$). A confounding factor in beetle data was the presence of both types of highly divergent haplotypes at the southern MW borefield, but not so in the northern borefields (SW and QW). The clearest association between genetic data and spatial location was observed in isopods and the amphipod.

All Mantel test results (Table 5) for the isopods, by borefield, showed a moderate to strong correlation between genetic and geographic distance ($r = 0.28$–0.93, $P < 0.01$). A confounding factor was the presence of three clear genetic lineages (MW-only; ISOLAV1; and ISOLAV2) that were each restricted in their distribution to distinct geographic locations in the LDC. We deemed it inappropriate to continue with these tests because of the confounding influence of the deep genetic differences among individuals that, in some cases, may represent species level differences. The remaining taxa showed some evidence of IBD between sites. Between SW and MW ($\sim$9.5 km), *L. windarraensis* showed IBD that was significant ($r = 0.14$, $P < 0.01$) and the amphipod showed similar correlations between genetic and geographic distance ($r = 0.30$, $P < 0.01$). Between QW and MW, the amphipod showed significant IBD ($r = 0.23$, $P < 0.01$) and for other taxa, the r values were high but not significant over this $\sim$15 km distance. For *L. windarraensis,* these results were unsurprising, again because of the presence of highly divergent lineages, such as those at MW. Interestingly, the comparison between SW and QW revealed no evidence of IBD over a substantial distance ($\sim$6.5 km) in any of the species (excluding *Haloniscus* discussed above). It should also be noted that *L. palmulaoides* was not sampled at QW.

Historical demography

Some evidence for a departure from neutrality and population expansion in *cox1* was observed in the LDC system. Negative and significant estimates of F_s, D and significant R_2 estimates were observed in SW and QW populations of *L. lapostae* and *L. palmulaoides,* and the amphipod (Table 3). Selection is typically indicated by an excess of identical haplotypes, which is seen in only a few of these populations, although these tests are not able to distinguish between selection and demographic

Table 3 Molecular diversity indices and population demographic parameters for three beetle species, the amphipod and isopods from three borefields on LDC: MW, SW and QW

Statistics	n	h	S	*h*	π	F_s	*D*	Demographic expansion				R_2
								τ	θ_0	θ_1	*SSD*	
L. lapostae												
MW	2	2	17	1.0 ± 0.1	17.0	2.8	0	—	—	—	—	0.1
SW	45	12	13	0.7 ± 0.1	0.9	−9.1*	−2.1*	1.0	0	Inf.	0.004	0.1*
QW	8	5	4	0.9 ± 0.1	1.4	−2.0*	−0.5	1.5	0	Inf.	0.02	0.2
L. windarraensis												
MW	39	16	46	0.8 ± 0.1	14.2	2.2	1.1	26.1	0.002	24.0	0.06*	0.1
SW	40	14	20	0.8 ± 0.1	4.3	−1.9	−0.3	7.7	0.002	7.0	0.06	0.1
QW	5	3	11	0.7 ± 0.2	5.4	2.5	0.2	9.5	0.002	11.8	0.3	0.2
L. palmulaoides												
MW	4	3	17	0.8 ± 0.2	8.7	2.4	−0.7	17.1	0	3.4	0.2	0.3
SW	23	19	23	1.0 ± 0.03	2.6	−19.8*	−2.2*	2.6	0	Inf.	0.01	0.1*
Amphipod												
MW	35	23	27	1.0 ± 0.03	4.9	−12.6	−0.9	6.6	0	14.3	0.006	0.1
SW	25	18	15	1.0 ± 0.02	1.6	−21.3	−2.1*	1.7	0.01	Inf.	0.003	0.1*
QW	20	12	19	0.9 ± 0.1	2.5	−6.3	−2.0*	1.3	1.0	Inf.	0.003	0.1*
Isopods												
MW	13	7	85	0.9 ± 0.1	19.5	4.8	−1.3	8.1	0.004	12.1	0.04	0.2
SW	27	17	55	0.9 ± 0.04	23.9	1.3	2.6	45.6	0	61.87	0.07*	0.1
QW	2	2	3	1.00 ± 0.5	3.0	1.1	0	0	0	0	0	0.3

Abbreviations: *D*, Tajima's (1989) *D*; F_s, Fu's (1997) F_s; *h*, haplotype diversity; inf, infinite estimate; *L*, *Limbodessus;* LDC, Laverton Downs Calcrete; MW, Mt. Windarra; *n*, total number of individuals; QW, Quandong Well; R_2, Ramos-Onsins and Rozas's (2002) R_2; *S*, number of polymorphic sites; SSD, sum of squatter deviations between the observed and the expected mismatch as a test statistic; SW, Shady Well; π, nucleotide diversity as mean number of pairwise differences in the population; −, not estimated.
Model of demographic expansion parameters, where τ is an index of time since the expansion expressed in units of mutational time; θ_0 and θ_1 are pre- and post-expansion values for the mutation parameter (that is, $2N\mu$, where N is the effective female population size and μ is the mutation rate per gene per generation); *, significance tests where $P<0.05$.

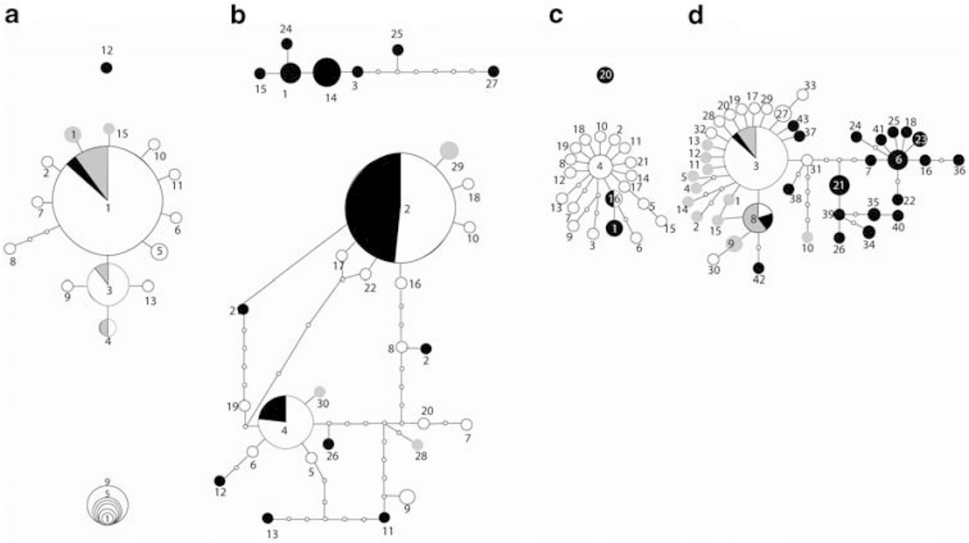

Figure 4 Haplotype network of (**a**) *L. lapostae*, (**b**) *L. windarraensis*, (**c**) *L. palmulaoides* and (**d**) the amphipod. Each haplotype is represented by a circle, with the size of the circle corresponding to the number of individuals sharing the haplotype (key bottom left corner) and the proportion of haplotypes shaded indicating the fraction of individuals from a given location possessing that haplotype, with black representing MW; white, SW; and grey, QW. Each line represents a single base difference between two haplotypes, and missing haplotypes are the smallest circles.

processes (Ballard and Whitlock, 2004). It has been demonstrated that R_2 and F_s are powerful tests of population expansion and R_2 is particularly useful when sample size is low, whereas F_s is useful when sample size is high (Ramos-Onsins and Rozas, 2002). Evidence of demographic expansion was also found using the mismatch analyses. There was no significant difference between the observed and expected mismatch

Table 4 AMOVA results estimated with ARLEQUIN (Excoffier *et al*., 2005) using three borefields on LDC (MW, SW and QW) and individual bores within each borefield to examine patterns of spatial genetic structure in three stygobiontic beetles *L. lapostae*, *L. windarraensis* and *L. palmulaoides,* the amphipod and isopods

Species	*Sum of squares*	*Variance*	*Variation (%)*	*Fixation index*
L. lapostae				
Among borefields	8.86	0.46 Va	41.07	Φ_{SC}: −0.03
Among bores within borefields	4.73	−0.02 Vb	−1.60	Φ_{ST}: 0.39
Within bores	29.50	0.67 Vc	60.53	Φ_{CT}: 0.41
L. windarraensis				
Among borefields	71.76	1.43 Va	24.34	Φ_{SC}: −0.06
Among bores within borefields	45.39	−0.27 Vb	−4.61	Φ_{ST}: 0.20[a]
Within bores	320.46	4.71 Vc	80.27	Φ_{CT}: 0.24[a]
L. palmulaoides				
Among borefields	6.93	0.80 Va	32.92	Φ_{SC}: 0.03
Among bores within borefields	10.87	−0.05 Vb	−1.92	Φ_{ST}: 0.31
Within bores	30.13	1.67 Vc	69.00	Φ_{CT}: 0.33
Amphipod				
Among borefields	55.90	0.93 Va	35.11	Φ_{SC}: 0.11[a]
Among bores within borefields	30.82	0.20 Vb	7.35	Φ_{ST}: 0.42[a]
Within bores	96.50	1.53 Vc	57.54	Φ_{CT}: 0.35[a]
Isopod				
Among borefields	768.29	34.94 Va	74.16	Φ_{SC}: 0.52[a]
Among bores within borefields	276.02	6.30 Vb	13.35	Φ_{ST}: 0.88[a]
Within bores	152.98	5.88 Vc	12.49	Φ_{CT}: 0.74[a]

Abbreviations: AMOVA; analysis of molecular variance; *L*, *Limbodessus;* LDC, Laverton Downs Calcrete; MW, Mt. Windarra; QW, Quandong Well; SW, Shady Well.
[a]Significance tests where $P<0.05$.

Table 5 Results of Mantel test for IBD where a pairwise comparison between three borefields on LDC: MW, SW and QW were conducted for three stygobiontic beetles *L. lapostae*, *L. windarraensis* and *L. palmulaoides,* an amphipod and the isopods

	MW	*SW*
SW		
L. windarraensis	0.14 ($P<0.001$)[a]	—
L. palmulaoides	0.40 ($P=0.04$)	—
L. lapostae	0.63 ($P=0.03$)	—
Amphipod	0.30 ($P<0.001$)[a]	—
Isopods	0.93 ($P<0.001$)[a]	—
QW		
L. windarraensis	0.04 ($P=0.7$)	0.08 ($P=0.13$)
L. palmulaoides	NA	NA
L. lapostae	0.51 ($P=0.11$)	0.08 ($P=0.20$)
Amphipod	0.23 ($P<0.001$)[a]	0.03 ($P=0.18$)
Isopods	0.88 ($P=0.009$)	0.28 ($P<0.001$)

Abbreviations: IBD, isolation by distance; *L*, *Limbodessus;* LDC, Laverton Downs Calcrete; MW, Mt. Windarra; NA, not available; QW, Quandong Well; SW, Shady Well.
[a]Indicates significant difference from the null hypothesis of no IBD with Bonferroni correction.

distributions under the demographic expansion model in all species, excluding *L. windarraensis* at MW and the isopod at SW (Table 3).

Estimates of the parameter τ (time since expansion) under the model of demographic expansion (Table 3) indicated most bore populations of *L. lapostae* and *L. palmulaoides,* and the amphipod showed relatively recent (low) demographic expansion events in all northern bore holes ($\tau<3$), whereas the QW population of *L. windarraensis* and the MW borefield of the amphipod showed medium ages of τ (6.6–9.5). Particularly interesting was the ancient demographic expansion found at populations MW in the two species that had high sample sizes at these sites (*L. windarraensis* and *L. palmulaoides*; 17.1–26.1). The estimates of τ, θ_0 and θ_1 under the demographic expansion model indicated that, in most cases, the relative population size is likely to have undergone substantial demographic expansion in all three beetle species at some point in time.

Coalescent timing

The BEAST analyses showed a total coalescent time of approximately 1–2 mya for *L. lapostae* and *L. palmulaoides* (Table 6), whereas an older total time to coalescence of 2.6 mya was observed for *L. windarraensis*. Further, dating of time to coalescence for the divergent clades revealed dates of 3–400 000 years for *L. windarraensis* (MW-only), *L. lapostae* (widespread-LAV) and *L. palmulaoides* (MW-only), whereas *L. windarraensis* (widespread-LAV) had a date of 800 000 years. Estimates of time since the last common ancestor for the LDC amphipods was a mean of ~7.5 mya, suggestive of a divergence time much earlier in history than for the *L. windarraensis* (MW-only), *L. lapostae* (widespread-LAV) and *L. palmulaoides* (MW-only) clades. In contrast, the BEAST analyses on the isopod lineages failed to reach convergence, with effective sample size values below 100, despite running chains of >10 000 000 generations. Given the very large margins of error and our low confidence in the estimates, we excluded these results from the study and considered the results of the isopods qualitatively.

Table 6 Estimates of time since most recent common ancestor (time per million years) for three stygobiontic beetle and the amphipod based on Yule (speciation) coalescent model using a Bayesian coalescent approach with BEAST (Drummond and Rambaut, 2007)

Species	*Clade*	*Mean*	*s.d. of mean*	*Median*	*95% HPD lower*	*95% HPD upper*	*ACT*	*ESS*
L. lapostae	Widespread-LAV	0.3	3.3E-03	0.2	0.1	0.4	1.6E+04	623.8
	Total	1.4	9.5E-03	1.3	0.7	2.2	5870.2	1702.0
L. windarraensis	MW-only	0.4	4.0E-03	0.4	0.2	0.7	6711.9	1488.6
	Widespread-LAV	0.8	6.2E-03	0.8	0.5	1.2	1.1E+04	918.5
	Total	2.6	1.7E-02	2.5	1.6	3.8	8398.1	1189.7
L. palmulaoides	Widespread-LAV	0.3	4.4E-03	0.3	0.1	0.4	2.6E+04	387.3
	Total	1.3	9.1E-03	1.2	0.7	2.0	6276.4	1591.9
Amphipod	Total	7.5	4.0E-02	7.4	4.7	10.7	6355.4	1572.0

Abbreviations: ACT, auto-correlation time; ESS, effective sample size; HPD, highest posterior density; *L, Limbodessus;* MW, Mt. Windarra. Each species was examined as a whole group (total) and within each of the two major intra-specific clades observed in this study, that is, the MW-only clade and the widespread-LAV clade.

Discussion

The inherently parapatric and allopatric nature of the epigean-subterranean nexus (Niemiller *et al*., 2008) is indisputably a key speciation mechanism in cave environments; however, *in situ* speciation (that is, speciation within cavernicolous habitats) is also predicted to have had an impact (Juan *et al*., 2010). Speciation from an obligate subterranean-adapted ancestor within the cave environment has rarely and only recently been explored (Caccone, 1985; Buhay and Crandall, 2005; Guzik *et al*., 2009). There is some evidence for intra-calcrete speciation by the presence of consistent size class variation among beetles species within calcretes of the Yilgarn region of Western Australia, some of which are sister species (Cooper *et al*., 2002; Leys *et al*., 2003). In this paper, we have investigated whether there is evidence for population fragmentation with a possibility for microallopatric population processes within a single calcrete for several cave-adapted species that may potentially result in *de novo* speciation following adaptation to obligate subterranean life.

Our previous study demonstrated that stygobiontic dytiscid diving beetles maintain thriving populations with high genetic (haplotype) diversity despite their likely isolation from the surface environment since the last ~3–10 million years (Guzik *et al*., 2009). The levels of intra-calcrete diversification were high within a very small sample region with evidence of IBD detected in two of the three species of beetles. However, evidence for population fragmentation within species was not observed. Here, we examined genetic diversity and population genetic structure of stygobionts on a much broader spatial scale (LDC sample region of ~15 km transects through a calcrete ~25 km long and ~90 km^2 in area). We used a comparative approach using three beetle species, a single chiltoniid amphipod species and *Haloniscus* isopods, the latter potentially represented by multiple species (see below). Consideration of several stygobiontic species from a larger sample region permitted the investigation of multispecies population fragmentation. Significantly, our findings generally revealed a shared evolutionary history among the species in this habitat.

The phylogeographic pattern

Comparable phylogeographic patterns among unrelated taxa within an ecological system are strong evidence of a shared spatiotemporal history (Bermingham and Moritz, 1998; Carstens *et al*., 2005; Sunnucks *et al*., 2006). At LDC, five unrelated stygobiontic taxa shared a divergent pattern of spatially associated genetic divergence. Each taxon maintained a genetically distinct clade of mtDNA haplotypes that showed up to 5% divergence within each taxon, and much higher in the isopod samples. In subsequent sections, we primarily discuss the results obtained from beetles and amphipods as isopods revealed much larger divergences (~21%) between lineages, suggesting a different temporal scale to their phylogeographic history. For all taxa, the individuals that yielded divergent haplotypes were sampled from the southernmost MW borefield (that is, the MW-only clade). In contrast, other individuals sampled from this location shared haplotypes with the northern SW (~11 km) and QW (~5 km from SW) borefields (that is, the widespread-LAV clade). The processes that have led to this pattern are possibly twofold.

On first inspection, a model of IBD in which individuals from nearby bores are more closely related to each other than to distant bores may explain our observations. IBD was cited as a key mechanism of diversification at the previously studied SMC aquifer (Guzik *et al*., 2009) and is expected to be used over the spatial scales examined in the LDC. Results of the Mantel tests for all taxa, excluding isopods, showed that there was evidence of IBD between the southern MW borefield and the two northern SW/QW borefields that could be because of the high divergence levels of MW-only haplotypes. However, there was no evidence of IBD between the two northern borefields SW/QW. The lack of IBD between the two northern sites indirectly suggests that distances of ~6.5 km are not necessarily major barriers to dispersal in the LDC for these taxa. Therefore, we suggest that the divergence between widespread-LAV and MW-only haplotypes is unlikely the product of a purely IBD process. The results of nucleotide diversity ($\pi = 8.7$–17.0) and the level of divergence (4%) among different haplotypes in the stygobiontic beetles at the southern MW borefield were substantially higher than those found in the SW/QW borefields (Table 3), as well as those previously seen in beetle species from the SMC aquifer in a similarly small geographic area (3.5 km^2; Guzik *et al*., 2009). High nucleotide and haplotype diversity, such as that observed here, even within bore holes, is indicative of a large stable population with a long evolutionary history, allowing retention of ancient

haplotype lineages and/or secondary contact between two differentiated populations (Grant and Bowen, 1998). Because we generally see two major clades of haplotypes in all the taxa rather than multiple divergent clades, we consider the latter scenario most probable.

Under a scenario of historical population fragmentation, individuals would have to be restricted to the southern region of the aquifer and physically separated over time from populations in the north. Given sufficient time for isolation, a clear signature of divergent haplotypes in reciprocal monophyly for MW versus SW/QW borefields would be expected. However, although there is evidence for divergent haplotypes, there is no support for reciprocal monophyly among the geographic locations, with several widespread-LAV haplotypes shared between the MW and SW/QW borefields. Either fragmentation has not persisted long enough to provide sufficient time to achieve reciprocal monophyly or, as suggested earlier, there has been long-term fragmentation of populations with recent secondary contact (Taberlet *et al*., 1998; Hewitt, 2000). This latter hypothesis of post-isolation colonisation, in which a population is fragmented by a geographic barrier (vicariance) and subsequently rejoined, has been demonstrated in many epigean species (Zamudio and Savage, 2003; Phillips *et al*., 2004; Hoskin *et al*., 2005; Pinceel *et al*., 2005). Such a scenario has also been documented from a broad range of cavernicolous habitats and is a plausible hypothesis for the taxa within the LDC aquifer system (Cobolli Sbordoni *et al*., 1990; Crouau-Roy and Bakalowicz, 1993; Buhay and Crandall, 2005; Hunter *et al*., 2008).

Short-term vicariance and allopatric divergence with secondary contact

On the basis of the current data, there appears to be a unidirectional gene flow from the north to the south, as indicated by the presence of widespread-LAV haplotypes found at the southern MW borefield and an absence of divergent MW-only haplotypes in northern sites. We mention this directionality with caution because, although our sampling was rigorous, especially for *L. windarraensis* and the amphipod in SW bores, there is always a possibility that these haplotypes are rare and remain unsampled from the northern sites. However, in the strict sense, the presence of mechanisms of unidirectional gene flow (for example, fast currents) is probably limited in this type of system, because of the generally flat topology of the desert landscape and the low flow of groundwater through the water table (Humphreys *et al*., 2009). Instead, we propose a scenario in which a barrier may have existed close to the MW borefield that, when removed, permitted gene flow between MW and the population on the other side of the barrier. This scenario, in which MW is located in a zone of introgression between two genetically divergent populations, may explain the presence of some shared haplotypes between the SW/QW and MW borefields and the absence of MW-only clade individuals at SW/QW borefields, which is outside the zone of introgression. We suggest that for the latter population there has been insufficient time for gene flow to spread divergent MW haplotypes to SW/QW. One possible barrier is a salt lake that lies immediately adjacent to MW (Figure 1).

Salt lakes are typically associated with calcrete formation and can be a source of strong saline stratification for proximate groundwater habitats (Humphreys *et al*., 2009), and marked salinity gradients have been recorded in a number of calcrete aquifers (Mann and Deutscher, 1978; Humphreys *et al*., 2009). Individuals at LDC may have been isolated on either side of a salinity cline or else separated by an intrusion of hypersaline water. An alternative hypothesis is that of aquifer drawdown (Culver and Sket, 2000; Culver *et al*., 2000; Leys *et al*., 2003), in which part of the water body at the MW borefield may have been partitioned from the rest of the calcrete, leading to isolation of populations in distinct refugia in different regions of the calcrete. The cause of such aquifer drawdown could potentially be external climate change events resulting from aridification of Western Australia during the Pleistocene and/or because of cycles of aridity resulting from ice ages (Byrne *et al*., 2008). At this stage, we cannot discriminate between these hypotheses but future ecological studies may help to resolve the question.

One additional explanation for the phylogeographic pattern observed here is that the clades may reflect divergences that occurred before, or during colonisation of, the subterranean habitat. In the current study, we made the assumption that, unlike the beetle triplet from the previously studied SMC aquifer (Guzik *et al*., 2009), the LDC species have evolved from different ancestral surface species that colonised the calcrete independently. The basis for this assumption is that the LDC species do not form a monophyletic clade of sister species in comprehensive phylogenetic analyses of *Limbodessus* species from Yilgarn calcretes (Leys *et al*., 2003; Leys, unpublished). Therefore, it is possible that the population differentiation that we observe here may reflect allopatric diversification through multiple colonisation events in different regions of the calcrete by the same ancestral species. These events are thought to occur commonly in cave habitats in which the nexus between subterranean and epigean habitats remains open for extended periods of time (Soulier-Perkins, 2004; Villacorta *et al*., 2008). However, the finding that multiple taxa shared a similar phylogeographic structure within a $\sim$15 km transect of the LDC is unlikely to be explained by random colonisation events in different regions of the calcrete. Further, we see an overlap in the intra-specific coalescent estimates of time to most recent common ancestor for haplotypes of the LDC and SMC aquifers. In particular, the oldest intra-specific coalescent times occurred in the last 2–3 mya (total *L. windarraensis* (LDC, Table 6) and in total *Paroster mesosturtensis* (SMC, Table 4, Guzik *et al*., 2009)), with the majority of intra-specific coalescent times $<$1.5 mya (for example, *L. lapostae* and *L. palmulaoides*; SMC: *Paroster macrosturtensis* and *Paroster microsturtensis*). Major differences in the scale of sampling in the current study and that in Guzik *et al*. (2009) are likely to have led to considerable variation in the intra-specific coalescent times of taxa from the two calcretes. In particular, the colonisation/speciation/demographic history of the taxa from the two aquifers, as well as the stochastic variation in the process of lineage sorting of haplotypes, has probably impacted TMRC estimates. However, despite this variation, the times are generally lower than the predicted timing for the enclosure and isolation of Yilgarn calcretes

(that is, 3–10 mya; Leys *et al*., 2003; Cooper *et al*., 2007, 2008). Therefore, we believe that the phylogeographic patterns within taxa from the LDC aquifer most likely resulted from intra-calcrete processes (for example, fragmentation events) after colonisation, rather than from processes that occurred before, or during colonisation of, the subterranean habitat.

Overall, our population demographic results are consistent with the observed structure of the haplotype networks and a model of constant population size was not supported. Given that a range of taxa showed signals of expansion for multiple analyses, it is unlikely that selective sweeps (Turelli and Hoffmann, 1991; Ballard and Kreitman, 1995; Ballard and Rand, 2005) and/or stochastic variation in the coalescent time of the mtDNA were responsible for the significance of these test statistics. Demographic estimates and parsimony network analyses suggested that the MW bores incurred older demographic expansions (*L. windarraensis* and *L. palmulaoides* MW-only: $\tau = 17.1–26.1$) than that estimated for other parts of the aquifer ($\tau = 1.0–9.5$). This difference could be due to a bias because of the presence of divergent haplotype groups at MW. The differences in divergence time between species could also be due to sampling bias, as *L. windarraensis* was more frequently captured than *L. lapostae* and *L. palmulaoides*. We consider it to be more likely that multiple isolation and expansion events have occurred at different times within the aquifer (that is, the temporal patterns of expansion are different for each species) as indicated by BEAST analyses and estimates of τ. These observations are consistent with that of Guzik *et al*. (2009) for the SMC aquifer.

Crustacean population genetic structure and cryptic species

The level of genetic divergence observed for the crustacean taxa was distinctly different from that of beetles. Among the isopods, divergences were deep and suggest species level differences (see below), whereas the amphipods revealed a more recent expansion. In the amphipod species, there was some evidence of genetic divergence (2% between widespread-LAV and the MW-only clade), but it was smaller than that of beetles (4% intra-specific divergence) and isopods (1–20% divergence). These results may represent differences in mobility, life history or behaviour among the taxa, which were discussed in our earlier study of groundwater beetles (Guzik *et al*., 2009). The current study also includes isopods and amphipods, which are entirely aquatic and have no larval stage and, hence, are likely to have very different dispersal rates, life history traits and environmental tolerances (such as tolerance to different salinity levels). It is thought that the *Haloniscus* isopods are benthic-dwelling organisms that feed on detritus and are likely to be tolerant of high salinity levels, given that the related surface dwelling species of *Haloniscus* live on salt lakes (Bayly and Ellis, 1969). The adults are some of the largest of all stygobiontic invertebrates within calcretes ($\sim$5–6 mm in body length and $\sim$1 mm in body width; Taiti and Humphreys, 2001) and they crawl on the calcrete surface rather than swimming through the groundwater. Therefore, they are likely to have more localised ranges and reduced rates of dispersal compared with the smaller beetle and amphipod species. The amphipod is thought to scavenge and disperse widely, because of its small size ($<$2–3 mm) and active juvenile and adult stages, which are capable of both swimming and crawling in the calcrete. They are known to inhabit the upper layers of the water column and are probably the most frequently sampled invertebrate in aquifers after copepods (Allford *et al*., 2008; Eberhard *et al*., 2009). Their tolerances to physicochemical differences in the groundwater may also vary widely (Berezina *et al*., 2001) and may potentially lead to distinct niche partitioning among zones within a salinity gradient (Fenchel and Kolding, 1979; Williams, 2003).

It is unlikely that the widespread-LAV and MW-only clades of the beetles and amphipods represent incipient species, and probably reflect historically divergent populations. Conversely, the isopods showed the presence of two highly divergent (19–21%; Table 2) lineages (MW-only and ISOLAV1 + 2) that are most likely separate species (based on proposed species thresholds within crustaceans; Lefébure *et al*., 2006). Within each putative species were additional divergent ($\sim$9%) lineages (that is, ISOLAV1 and ISOLAV2) or private haplotypes (ILA5 (BES 12005) and ILA6 (BES12087); Table 2). There is the possibility that ILA5 and ILA6 haplotypes may not be true representatives of the *cox1* mtDNA gene. In single locus studies, pseudogenes can provide spurious results and for the mtDNA *cox1* gene nuclear mitochondrial (numt) pseudogenes have been shown to inflate the number of identified species (Song *et al*., 2008). However, our data did not reveal any of the typical indicators of numts (that is, in-frame stop codons, high numbers of point mutations and routine multiple PCR product results such as multiple bands on gels and double peaks, background noise and so on); therefore, we consider it unlikely that pseudogenes have influenced our results.

The origins of these isopod lineages are likely to be more complex than our explanations for the beetles and amphipod; however, the near fixation of each 'species' to either the MW or SW end of the calcrete is still consistent with our hypothesis of a barrier to dispersal within the system. However, either the two 'species' diverged before their isolation within the calcrete, with the barrier leading to one predominating by chance in the MW region and the other predominating in the SW region, or, alternatively, fragmentation occurred much earlier in the history of the calcrete and led to a complete barrier to gene flow and speciation for the large-bodied isopods, which also may have colonised the LDC during a much earlier time period than the beetles and amphipods. Currently, we are unable to distinguish between these hypotheses, although it is interesting to note that the private haplotype ILA5 (BES 12087 from SW), which groups closely with the MW-only haplotypes, is likely to be a relictual haplotype from this early fragmentation process and has been retained by chance in the northern part of the calcrete. Additional relictual haplotypes and analyses incorporating nuclear gene markers are likely to shed further light on the history of these calcrete populations.

Conclusion

We have now examined the genetic diversity and population genetic structure of subterranean species

within two independent calcrete aquifers in the Yilgarn region. It is clear that individual aquifers vary in their faunal composition and the population genetic substructure of species. Interestingly though, a number of generalisations regarding the calcrete habitat can now be made on the basis of the combined results of these two studies. First, we see that large and genetically diverse populations of stygobiontic organisms are maintained, with persistence over millions of years, despite major changes in climate during the Pleistocene (Byrne *et al*., 2008). The presence of shared haplotypes throughout both calcretes suggests that connectivity through large regions of the calcrete aquifers exists with preferred pockets of habitat that can facilitate genetic differentiation. It is thought that the connectivity within aquifers is complex, as suggested by evidence for IBD over very short spatial scales at both SMC and LDC, with connectivity among regions likely to vary over time depending on the level of groundwater. The implications of our findings for speciation are that there is clear potential for population genetic fragmentation and in turn speciation to occur from a single troglomorphic ancestral species *in situ* of the aquifer. However, whether this mode of *in situ* speciation is responsible for a significant proportion of stygofauna species within the Yilgarn calcretes is yet to be determined.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A

Geographic coordinates of sampling sites for stygofauna from LDC and the number of individuals and their *cox1* haplotypes from each location

Boregrid/Borehole	*Latitude*	*Longitude*	*L. lapostae*		*L. windarraensis*		*L. palmulaoides*		*Amphipod*		*Isopod*	
			n	*Haplotypes (SLA)*	n	*Haplotypes (MLA)*	n	*Haplotypes (LLA)*	n	*Haplotypes (ALA)*	n	*Haplotypes* (ILA)
SW												
A5	−28.41151	122.20300			2	2, 8	1	6				
A7	−28.41150	122.20508							2	5, 6	1	21
A21	−28.4129	122.2179										
D3	−28.4073	122.1997										
E3	−28.4056	122.1996									3	16, 17, 20
E6	−28.4056	122.2027									4	10, 15, 18
E10	−28.40430	122.20817							5	3, 7, 8, 9	1	18
F3	−28.40243	122.20105	11	1, 3, 4, 5	12	2, 4, 7, 9, 10, 18, 19, 20	6	2, 4, 5, 16, 19	3	21, 22, 23	3	8, 10, 16
G3	−28.40062	122.20112	1	10	5	2, 9	1	15				
I1	−28.39701	122.19903	2	2, 3								
I2	−28.39703	122.20011	2	5, 9	1	4	1	9			7	9, 10, 11, 19
I4											1	9
J2	−28.39522	122.20012	15	1, 3, 11, 13	3	2, 4	1	21			5	9, 11, 13, 14
K2	−28.39340	122.20011	9	1, 3, 6, 8	3	2, 4, 6	4	7, 8, 17, 18				
M3	−28.38984	122.20114	3	1, 3	1	2					1	9
M7	−28.38985	122.2038			1	22					1	12
M10	−28.38986	122.20831	2	1, 7	12	2, 4, 5, 16, 17	7	4, 6, 10, 11, 12, 13, 14	2	3, 4		
N4	−28.38801	122.20218					2	3, 4				
WP60	−28.50332	122.18198			1	2						
MW												
WP61	−28.5034	122.179			1	21						
WP64	−28.5015	122.179			15	1, 2, 3, 14, 24, 25, 26, 27					1	1
WP66	−28.4989	122.180			2	2, 4					6	2, 3, 5, 6, 7
WP68	−28.4980	122.181									3	2, 3, 7
WP69	−28.498	122.18	2	1, 12	6	1, 2, 4, 17	4	1, 16, 20				
GWMB10												
GWMB12	−28.4989	122.1780			14	2, 3, 4, 11, 12, 13, 14, 15, 23			15	3, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20	3	1, 4
QW	−28.34509	122.2051	8	1, 3, 4, 14, 15	5	28, 29, 30					2	22, 23

Abbreviations: *L*, *Limbodessus*; LDC, Laverton Downs Calcrete; MW, Mt. Windarra; QW, Quandong Well; SW, Shady Well.
Letters in brackets indicate the prefix for haplotypes in each species. Some isopod haplotypes are from Cooper *et al*. (2008) and their corresponding sequence codes are: ILA1-6 (BES 13141, 13149.1-0.2, 12102, 12087 and 12005) and ILA8-14 (LA16, BES 13167, 13167, 13157, 13173.2, 13180.1, 13173.1 and 13186.2).