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Cryptic speciation in two widespread subterranean amphipod genera reflects historical drainage patterns in an ancient landscape

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Abstract

The landscape of the Pilbara region of Western Australia has been relatively unchanged for 100 million years. The ancient river systems of this region might be expected to be sources of isolation and divergence for aquatic species. Hence, the occurrence of widespread groundwater taxa in this landscape offers the opportunity to examine associations between genetic diversity and drainage patterns. *Pilbarus* and *Chydaekata* are two widespread genera of subterranean amphipods endemic to the Pilbara, each occupying multiple tributaries. We used molecular data to examine the roles of drainage patterns in structuring genetic diversity. Gene flow within a tributary may be facilitated by the occasional occurrence of these amphipods in springs, which results in their downstream dispersal during episodic flooding. However, tributary boundaries may form hydrological barriers to gene flow, resulting in localised isolation of populations and divergence. Samples of both genera, collected throughout three river basins, were examined for sequence divergence in the cytochrome *c* oxidase I mitochondrial gene. There was no evidence of contemporary gene flow among populations of either genus, and each tributary contained highly divergent lineages, which were not associated with similar morphological differentiation. This suggests cryptic speciation has occurred, and similar phylogenetic signals in both taxa imply similar evolutionary histories. Surface populations may have been driven into subterranean refugia by the cessation of flow in the rivers, associated with Tertiary climate change, while morphological evolution may have been constrained by stabilising selection. The lack of congruence between molecular diversity and morphology raises important practical issues for conservation.

Keywords: COI, conservation, haplotype, morphology, mtDNA

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Introduction

Drainage structure can be a powerful force in shaping the genetic structure of populations of freshwater taxa (Hughes *et al*. 2000; Waters *et al*. 2001; Cook *et al*. 2002, 2006; McGlashan & Hughes 2002; Hughes & Hillyer 2003). In particular, the hydrological barriers imposed by river catchment boundaries appear to restrict movement between drainages, even in areas of low topographic relief and major flooding events (Cook *et al*. 2002), and in animals with good dispersal abilities (Hughes & Hillyer 2003). Less well-understood are the barriers these hydrological boundaries impose on groundwater taxa. Groundwater aquifers are defined by both geological structure, which affects barriers and void space, and by hydrological processes; vertical shifts of the water table can connect or isolate discrete aquifers. Historical changes to surface and subterranean drainage patterns may add another dimension to the structure, while seasonal patterns of recharge and occasional major events of flooding provide the potential for mixing along surface or hyporheic pathways.

Molecular analyses of groundwater fauna have revealed highly subdivided populations, and many instances of cryptic speciation. In some cases, genetic differentiation among populations has been linked to hydrological patterns (Gooch & Hetrick 1979; Kane *et al*. 1992) and

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processes (Eberhard *et al*. 2005a) but other processes, such as ecological restrictions (Caccone 1985), geological barriers (Humphreys & Adams 1991), climatic and geological events (Ketmaier *et al*. 2003; Lefébure *et al*. 2006), and differing invasion scenarios (Fong & Culver 1994) also contribute to the genetic structuring of populations.

The potential is high for some of these processes to be operating in the Pilbara, a large (178 000 km^2) region of northwest Western Australia. The Pilbara is an ancient, stable landscape, which has been relatively unchanged for 100 million years (Frakes *et al*. 1981). This includes the ancient river systems, with flow-paths that have been extant since the Mesozoic (Beard 1998), and catchment structure that might be expected to be sources of isolation and divergence for aquatic species. Bradbury & Williams (1997a) and Humphreys (2001) have proposed that increasing aridity in the northwest of Australia during the Tertiary, as a result of continental drift and climatic fluctuations, would have driven freshwater species into subterranean refugia, the only remaining sources of freshwater. Restriction to confined aquifers would promote isolation and diversification. Indeed, the diverse groundwater fauna of the Pilbara contains a significant proportion of narrow endemics (Humphreys 1999, 2001; Eberhard *et al*. 2005b), of which some species are known from single catchments and their associated aquifers, and occasionally, single sampling sites (usually a bore or well) within an aquifer (Poore & Humphreys 1998, 2003; Bradbury 2000; Wilson 2003). Hence, the presence of widespread groundwater genera in the Pilbara provides an opportunity to examine patterns of population structure with respect to hydrology, and to test hypotheses regarding patterns and timing of groundwater invasions.

Two such groundwater amphipod genera, *Pilbarus* and *Chydaekata*, are widespread in the Pilbara. *Pilbarus* is monotypic (Bradbury & Williams 1997b), while *Chydaekata* comprises 15 species, 14 of which were described from an ~15–20 km section of the Fortescue River near Ethel Creek, in the upper Fortescue Valley (Bradbury 2000). The validity of these species is under question (Finston *et al*. 2004; this study), so until such time that a taxonomic re-evaluation of the genus is published, we will refer to what appears to be a single species of *Chydaekata* at Ethel Creek showing morphological variation among individuals as *C. acuminata* Bradbury, the type species of the genus. Specimens of both genera have subsequently been collected from springs and groundwater sites in multiple tributaries and river basins of the Pilbara, though their distributions are allopatric (Department of Conservation and Land Management, unpublished data). Regardless of the differing vertical distributions of collecting sites, all specimens of both genera possessed fully stygobitic characteristics.

This study examines the genetic structure of populations of *Pilbarus* and *Chydaekata*, to test for an association of genetic divergence with hydrological structure. Dispersal within tributaries may be facilitated by the occasional occurrence of these amphipods in surface waters and the extreme flooding associated with cyclonic rains when floodwaters may stretch hundreds of kilometres in regions with little topographic relief. In this case, there would be evidence of connectivity among sites in the form of shared haplotypes. If tributary boundaries constitute barriers to gene flow, we would expect molecular structure to be associated with hydrological structure, and indeed if the hypothesis of isolation during the Tertiary is correct (Bradbury & Williams 1997a; Humphreys 2001), we would expect the lineages to be ancient.

In addition to its rich subterranean fauna, the Pilbara is also an economically important region because it contains substantial deposits of iron ore (Twidale *et al*. 1985), and much mining occurs below the water table. Legislation to protect species requires that mining be interrupted or halted if narrowly endemic species have ranges restricted to areas of mining impact (detailed in Finston *et al*. 2004). Hence, to manage both fauna and resources, it is important to develop an understanding of the distribution of subterranean biodiversity in the Pilbara. Here, we examine sequence variation in the mitochondrial gene cytochrome *c* oxidase I (COI) in samples of *Pilbarus* and *Chydaekata* from 13 tributaries throughout three river basins. This hierarchical sampling scheme was designed to: (i) examine the diversity and distributions of haplotypes in the two genera; (ii) test the prediction that gene flow is restricted among tributaries; and (iii) provide information for the management of the fauna, by identifying the level at which hydrological structure needs to be maintained in order to maintain biodiversity.

Methods

Sampling

Approximately 450 bores and wells were sampled from five drainage basins in the Pilbara (see Eberhard *et al*. 2005c for sampling details). Of those, sites located in approximately 25 separate tributaries in three of the basins contained *Pilbarus* and *Chydaekata.* In this study, samples were used from 40 bores in 13 tributaries from the three drainage basins. Four tributaries were sampled in the Ashburton River basin, one in the Onslow Coast basin, and eight in the Fortescue River basin (Table 1; Fig. 1). We aimed to sequence DNA from at least 10 individuals per tributary, but due to the rarity of specimens at some sites, this was not always possible. Marillana and Weeli Wolli Creeks are adjoining tributaries, while all other sites represent separate tributaries. In all, 94 specimens identified as either *Pilbarus* or *Chydaekata* were included in the present study, with specimens possessing sternal gills assigned to *Chydaekata* (Bradbury 2000), and those lacking

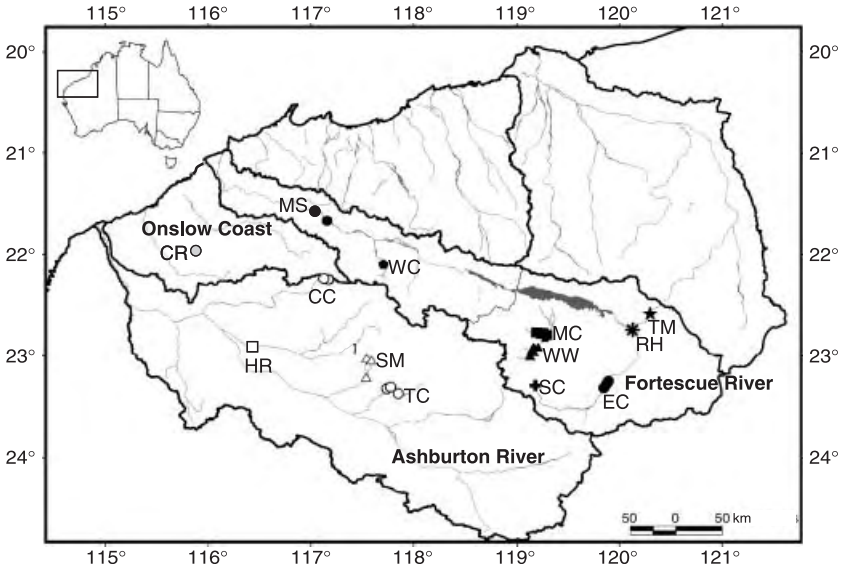

Fig. 1 Map of the Pilbara, Western Australia, showing river basin boundaries (thick lines) and major creeks (thin lines). Open symbols, Ashburton basin: circle, Caves Creek (CC); square, Hardey River (HR); triangle, Seven Mile Creek (SM); octagon, Turee Creek (TC); black filled symbols, Fortescue basin: circle, Millstream (MS); pentagon, Weelamurra Creek (WC); square, Marillana Creek (MC); triangle, Weeli Wolli Creek (WW); star, Tuccamunna (TM); asterisk, Roy Hill (RH); cross, Spearhole Creek (SC); octagon, Ethel Creek (EC); grey filled symbols, Onslow Coast basin: circle, Cane River.

Table 1 Primers used for amplification of samples of *Molina pleobranchos* and *Pilbarus*

Site	Forward primer	Reverse primer	Fragment length
All sites except those below	LCOI490 5′-GGTCAACAAATCATAAAGATATTGG-3′	HCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	658
Millstream (MS3)	5′-TCTGAATTAAGAGCCCCAGGT-3′	5′-GGTCACCTCCACCACTAGGA-3′	651
Weelamurra Ck. (WC1, WC2)	5′-TGGGCAAGAATGCTAGGAAC-3′	5′-CGCCACTAGGGTCAAAGAAT-3′	600
Millstream (MS1, MS2)	5′-TGGGCAAGAATGCTAGGAAC-3′	5′-CGCCACTAGGGTCAAAGAAT-3′	600
Onslow Coast (CR1)	LCOI490	5′-CACCACCTCCTCTAGGATCG-3′	633
Wyloo (HR1)	LCOI490	5′-TGCACCTGCTAGAACAGGAA-3′	614

sternal gills assigned to *Pilbarus* (Bradbury & Williams 1997b; Appendix). Other characters, which apparently differentiate the two genera, such as the shape of the gnathopods, the robustness of the body, and setation on the body and limbs (Bradbury 2000), showed overlap across samples and genera (this study; T. L. Finston, M. S. Johnson, S. M. Eberhard, J. Cocking, J. McRae, S. A. Halse, B. Knott, unpublished data), and hence were not used, allowing assignment to one of the two genera to be unambiguous, as the gills are the only diagnostic character to distinguish them. *Pilbarus millsi* was sequenced from its type locality at Millstream, and specimens of *Chydaekata* were sequenced from type localities in Ethel Creek. In addition, a haplotype of *Molina pleobranchos* Bradbury (2000) from near its type locality at Millstream was used as an outgroup.

DNA methods

Whole genomic DNA was extracted from frozen or alcohol-preserved specimens. Specimens stored in alcohol were rinsed twice in a 10-mM Tris solution to remove the alcohol before the extraction process. One or two legs were dissected from each individual, and the remainder of the body was returned to ethanol, and deposited in the collections of either the Department of Conservation and Land Management or the Western Australian Museum. Digestions on the legs were carried out at 60 °C in 50 μL of a proteinase K extraction buffer (Schwenk 1996) for between 16 h and 20 h. Following a 10 minute cycle at 96 °C, the entire extract was frozen and stored, ready for direct use in the polymerase chain reactions (PCRs). For most specimens, a 710-base-pair (bp) fragment of the 3′ end of the COI gene was amplified, using the primers LCOI490 and HCO2198 as described by Folmer *et al*. (1994; Table 1). Specimens from the Onslow Coast (CR), Hardey River (HR), Millstream (MS) and Weelamurra Creek (WC) could not be amplified using these primers, so internal primers were designed using the software package PRIMER 3 (Rozen & Skaletsky 2000; Table 1). The 25 μL PCRs used 0.2 mM dNTPs, 4.0 mM $MgCl_2$, 1 × buffer, 12.5 pmoles of each primer, 1 U *Taq*, and 2.5 μL template, either at full concentration or at 1/10 dilutions. Sequences were cleaned using the UltraClean PCR Clean-up DNA purification kit (MoBio Laboratories) prior to sequencing. The sequencing reaction was carried out using the BigDye V3 Ready Reaction Mix (ABI PRISM), and the products were sequenced using both

primers on an ABI 373 automated sequencer (Applied Biosystems). The first sequence analysed, SWL10a, was compared with sequences from GenBank, to verify that the sequence came from an amphipod, using BLAST (Altschul *et al*. 1997). Sequences were aligned with GENEDOC (Nicholas & Nicholas 1997), using default settings, and edited by eye.

Phylogenetic analyses

Phylogenetic relationships among haplotypes were analysed using MRBAYES version 3.0 (Ronquist & Huelsenbeck 2003; http://morphbank.ebc.uu.se/mrbayes/). Distance matrices were obtained using MEGA version 3.0 (Kumar *et al*. 2004). PAUP (Swofford 2001) and MODELTEST (Posada & Crandall 1998) were used to identify the model of sequence evolution that best fit the data for use in the phylogenetic and distance analyses. The general time-reversible model with a significant proportion of invariable sites and gamma distributed rate heterogeneity (GTR + I + G) was selected as the best fit. The proportion of invariable sites (I) was estimated to be 0.451, and the gamma shape parameter (α) was estimated at 0.5626. Maximum-likelihood analyses with 100 bootstrap replicates were performed in PAUP, using the appropriate model of sequence evolution, and utilising nearest-neighbour branch-swapping, with the starting trees obtained by neighbour-joining. Further, a Bayesian approach was used to calculate likelihood (Holder & Lewis 2003) and provide posterior probabilities of individual nodes. The GTR model with gamma distributed rates was used. The Markov chain Monte Carlo (MCMC) simulation was run in MRBAYES, and was started with a random tree. Four chains, each of 10^6 cycles, were run. An initial burn-in of 10 000 generations (= 100 trees) was required for the ln likelihood values to reach stability. A majority-rule consensus tree was constructed in PAUP, excluding these trees. A specimen of *Molina pleobranchos*, from the family Paramelitidae was used to root the tree.

Rate heterogeneity and divergence times

In order to justify the use of a molecular clock, tests for equal rates of nucleotide substitution between lineages were carried out using GRATE (Müller *et al*. 2004; http://www.botanik.uni-bonn.de/system/downloads/) and PAUP. This method was chosen over other methods of rate testing, as it implements the parameters defined by the model of evolution identified as the best fit to the data (here, GTR + I + G) and allows for grouping of haplotypes into predefined morphological or ecological groups (Müller *et al*. 2004). Rate heterogeneity was tested for all pairwise sequences of *Pilbarus* and *Chydaekata*, using *Molina* as the outgroup. We compared the results of the two methods to estimate the time to the most recent common ancestor (TMCRA) between pairs of tributaries by applying a standard molecular clock for COI, and using a coalescent-based approach. First, in the absence of a clear fossil or geological record, we used the commonly cited range of 1.4 to 2.6% sequence divergence per million years in COI for crustaceans in implementing the molecular clock (Knowlton *et al*. 1993; Knowlton & Weigt 1998). Corrected pairwise nucleotide sequence divergence (Tamura & Nei 1993) was used for the distance matrix, because the very large divergences obtained suggest saturation at the third codon, which would lead to an under-estimation of divergence times. This correction method, which accommodates gamma distributions, allows for unequal nucleotide frequencies and transition/transversion ratios, and variation in the substitution rate among sites, was calculated in the Distances module in MEGA. We used the smallest corrected distance between haplotypes in each comparison of tributary pairs to obtain TMRCA. Second, we used MDIV (Nielsen & Wakeley 2001), which uses Markov chain Monte Carlo (MCMC) methods to find likelihood functions and posterior distributions of a model of coalescence to estimate Φ (a standardized measure of N_e and mutation rate) and TMCRA between tributaries. Available models of nucleotide evolution were limited, so the HKY model of site substitutions was used, as this model allows for multiple mutations at each site, and differences in the nucleotide frequencies and transition/transversion ratios. 10^6 cycles were run for each comparison, with a burn-in of 10^5 cycles. TMRCA was converted to years by multiplying by Φ and generation time. A generation time of 8 years was used because stygobionts show reduced metabolic rates and growth (Gilbert *et al*. 1994), with time to sexual maturity at 8–10 years or more for some stygobitic amphipods and isopods (Dickson & Holsinger 1981; Rouch & Danielopol 1999). We chose the lower end of the range because these estimates come from temperate species, where groundwater temperatures are generally lower than those found in the Pilbara.

Haplotype distribution

For each genus, analyses of the distribution of haplotypes were carried out in ARLEQUIN version 2.0 (Schneider *et al*. 2000) to identify the hydrological levels that most contribute to genetic variation. To test the significance of variation within and among tributaries, a hierarchical analysis of molecular variance (AMOVA) was performed for each genus, using the AMOVA/MSN module. The levels in the hierarchy were 'among tributaries' and 'within tributaries' for both *Pilbarus* and *Chydaekata*. Only those tributaries where more than one bore was sampled were included in the analyses. The analyses of molecular variance used both haplotype frequency and divergence between sequences, and Φ_{ST}, roughly equivalent to Wright's F_{ST}, was calculated. Significance of the Φ statistics and variance was tested by 1000 permutations.

Results

Sequencing of the COI gene resulted in an alignment of 658 base pairs for most individuals, but shorter sequences were used for those individuals of *Pilbarus* in which internal primers were used. There were 34 haplotypes (see Appendix for GenBank Accession numbers). Where multiple bores were sampled in a tributary, the number of haplotypes detected varied among tributaries, ranging from a single haplotype of *Pilbarus* at Caves Creek to eight haplotypes of *Chydaekata* at Weeli Wolli Creek. These values were expressed as haplotype ratios (HR; number of haplotypes/number of individuals), and ranged from 0.1 at Caves Creek to 1.0 at Hardey River, Millstream (excluding *Molina pleobranchos*), and Cane River (see Appendix). Importantly, haplotypes were confined to single tributaries, although haplotypes were frequently shared among bores within a tributary. For example, in *Pilbarus*, all 10 individuals collected from four different bores in Caves Creek shared the same haplotype (Appendix). Likewise, haplotype WW1 from Weeli Wolli Creek was found in four bores, and occurred in both subterranean (1026 and JSE14) and surface (Spring2 and WWS) sites. Despite the use of specimens of three described species of *Chydaekata* from their type localities at Ethel Creek, there was no molecular evidence for the presence of multiple species at that site, which is consistent with previous morphological and allozyme analyses which cast doubt on the original taxonomy (Finston *et al*. 2004). Haplotype EC2 was found in all eight bores (WB23-1, WB23-4, WP126, WP126nre, W116, W152, W245 and W270) and in 17 of the 18 individuals sequenced. Haplotype EC1 was detected in a single individual, and differed from EC2 by 0.2% sequence divergence (T-N corrected).

Phylogenetic analysis

Both ML and Bayesian analysis generated phylogenetic trees with identical general topographies. The Bayesian tree (shown) showed two major clades, corresponding to *Pilbarus* and *Chydaekata* (Fig. 2). *Chydaekata* was composed of five lineages (A–E, Fig. 2), and *Pilbarus* was composed of four lineages (F–I; Fig. 2). Lineage C corresponded to haplotypes of *Chydaekata acuminata* from the type locality at Ethel Creek, and lineage I contained haplotypes of *P. millsi* from its type locality at Millstream. Here, lineages were defined as clusters of haplotypes characterised by short internodal distances, but long tip to node lengths between other such clusters, and generally arising from single tributaries. Posterior probabilities for all major nodes were 1.00 (Fig. 2). In the ML analysis, the *Pilbarus* clade was well supported by bootstrapping, with all internal clades bar one (Seven Mile Creek — SM) receiving support of 90% or greater (Fig. 2). The *Chydaekata* clade was less well-supported, with bootstrap values ranging from 61 to 100% (Fig. 2). With few exceptions, the phylogeny reflected the hydrological structure of tributaries and basins: in both genera, lineages tended to correspond to tributaries, and in *Pilbarus*, lineages fell into two clades, corresponding to the Ashburton (lineages F–H) and Fortescue (lineage I) basins. There were three deviations from a strictly hydrological pattern to the molecular diversity. First, the haplotype of *Pilbarus* from the Onslow Coast basin (CR1) occurred in lineage G, containing haplotypes from Seven Mile Creek (SM) in the Ashburton River basin, although it had a relatively long branch length (Fig. 2). Second and third were the occurrences of haplotypes from Marillana Creek (MC) and Weeli Wolli Creek (WW) in the single lineage E, and haplotypes from Caves Creek (CC) and Hardey River (HR) in the single lineage F.

Divergence rates and molecular clock analysis

There was no evidence for rate heterogeneity among lineages. Tests for differences in number of substitutions per site (d) among sequences assigned to either *Pilbarus* or *Chydaekata* showed no significant differences between pairwise comparisons ($d = 0.110 \pm 0.068$; not significant). Sequence divergence was lowest among haplotypes within single tributaries for both genera, ranging from 0 to 1.8%. Sequence divergence between haplotypes from different tributaries within a basin did not exceed 18.0% in *Pilbarus* (Table 2). In contrast, in *Chydaekata*, sequence divergence exceeded 20% between haplotypes in different tributaries for all comparisons, with the exception of Marillana Creek and Weeli Wolli Creek, which connect downstream of the two areas sampled. At these two sites, per cent sequence divergence between haplotypes ranged from 0.7 to 1.1%. Divergence between haplotypes of *Pilbarus* in different basins ranged from 12 to 23% between the Ashburton and Fortescue, 21.5 to 22.4% between the Onslow Coast and Fortescue, and 2 to 16% between the Onslow Coast and Ashburton. The lower value in the latter comparison occurred due to the high similarity of the Cane River haplotype in the Onslow Coast basin to haplotypes from Seven Mile Creek in the Ashburton River basin.

Both methods of calculating TMRCA gave similar estimates, with the estimate from MDIV generally falling within or below the range obtained using the molecular clock (Table 2). Using the lowest pairwise divergence between haplotypes of *Pilbarus* and *Chydaekata*, TMRCA for the two genera was 16.2 million years ago (Ma; MDIV) but ranging from 7.6 to 26.5 Ma (molecular clock).

Haplotype distribution

There were high levels of genetic differentiation at the tributary level (Table 3). The AMOVA showed that for both genera, the majority of the overall variance was partitioned

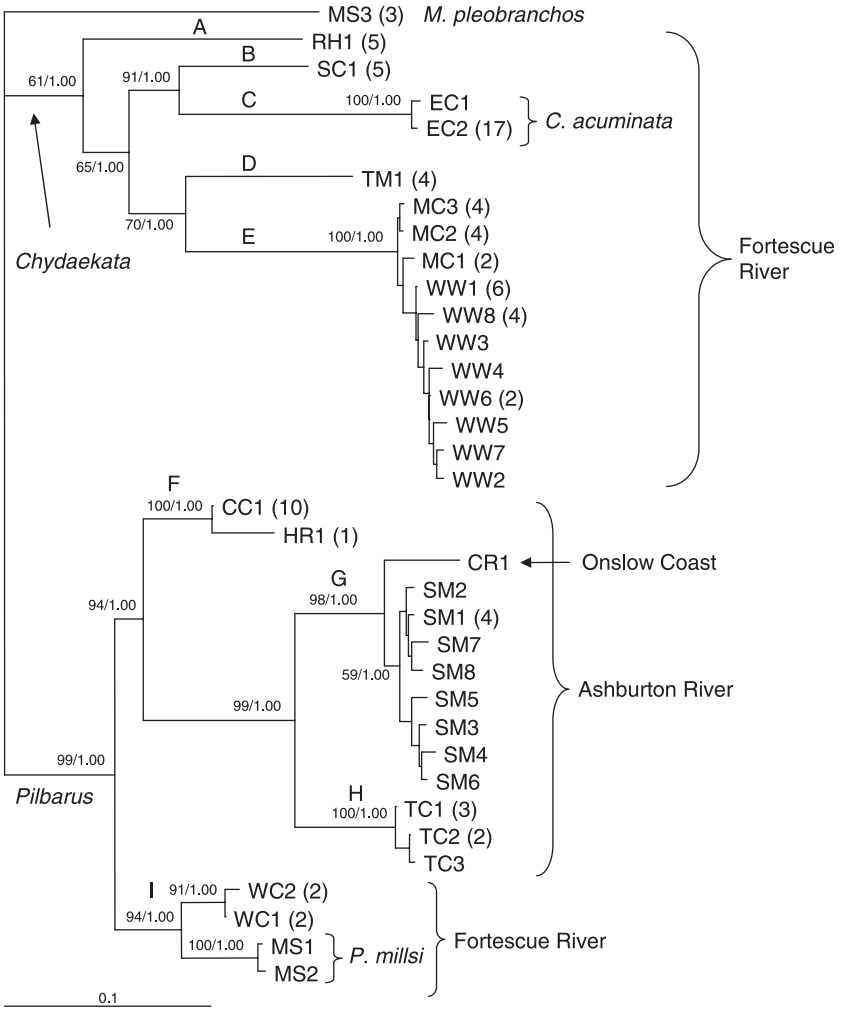

Fig. 2 Most probable tree based on maximum likelihood and Bayesian likelihood analyses for 94 individuals of *Pilbarus* and *Chydaekata*. *Molina pleobranchos* is used as an outgroup. Bootstrap values/posterior probabilities are shown on branches. Number of individuals represented by each haplotype is shown in parentheses for $n > 1$.

to variance among tributaries (98.7% and 96.7% for *Chydaekata* and *Pilbarus*, respectively), reinforcing the finding of a lack of gene flow across tributary boundaries.

Discussion

In summary, we found no evidence of contemporary gene flow among tributaries for either amphipod genus. Instead, each separate tributary contained haplotypes that were highly divergent from haplotypes in other tributaries, indicating that the lineages are ancient. Further, similar phylogeographic patterns were observed in both genera — the patterns of ancient coalescent processes mirrored the hydrological structure of surface tributaries and basins. The extent of haplotype divergence suggests cryptic speciation has occurred across tributaries, and similar phylogenetic signals in the two genera imply similar evolutionary histories.

There was a clear pattern of correspondence between mitochondrial lineages and surface hydrological structure. The two genera are composed of multiple monophyletic lineages, corresponding to tributaries, and separated by sequence divergences ranging from 22 to 32% in *Chydaekata*, and from 6 to 23% in *Pilbarus*. The only exception to this pattern was that haplotypes in the two contiguous tributaries Weeli Wolli Creek and Marillana Creek occupied the same lineage. While haplotypes were not shared between the two tributaries, their low sequence divergence (0.7 to 1.1%) indicates a much more recent connection than between other tributaries.

The analysis of molecular variance further emphasised the hierarchical genetic subdivision of populations within each genus: differences among haplotypes within single tributaries contributed little to overall variation; in contrast, differences among haplotypes from different tributaries constituted the vast majority of overall variation. Identical haplotypes at multiple sites within tributaries, such as Caves Creek, Weeli Wolli Creek, and Ethel Creek, however, suggest localised gene flow within tributary boundaries. This may be facilitated by the formation of calcretes near the water table, which when overlain with alluvial sediments, may act as corridors between calcrete patches. Indeed, the aquifer at Ethel Creek, where a common widespread haplotype occurred over approximately

Table 2 Pairwise measures of divergence between haplotypes of *Chydaekata* and *Pilbarus* between tributaries. Above diagonal: range of sequence divergence using the Tamura & Nei (1993) distance measure. Below diagonal: time to most recent common ancestor (TMRCA) in million years. First number: estimated from MDIV, values were converted to years by multiplying by θ and assuming 1 generation = 8 years; followed by estimated range using molecular clock for COI (in parentheses; see text for calibration details)

Chydaekata

	Ethel	Spearhole	Marilliana	Tuccamunna	Weeli Wolli	Roy Hill
Ethel	*	0.226–0.228	0.280–0.302	0.292–0.294	0.286–0.304	0.241–0.244
Spearhole	7.0 (8.7–16.1)	*	0.238–0.244	0.271	0.224–0.235	0.318
Marillana	8.9 (10.8–20.0)	7.2 (9.2–17.0)	*	0.251–0.257	0.005–0.011	0.294–0.301
Tuccamunna	7.6 (11.2–20.8)	4.4 (10.4–19.4)	7.0 (9.6–17.9)	*	0.246–0.256	0.236
Weeli Wolli	8.2 (11.0–20.4)	6.9 (8.6–16.0)	1.3 (0.19–0.36)	6.6 (9.5–17.6)	*	0.304–0.319
Roy	7.4 (9.3–17.4)	4.8 (12.2–22.7)	6.0 (11.3–21.5)	4.0 (9.1–16.9)	7.1 (11.7–22.8)	*

Pilbarus

	Caves	Seven Mile	Turee	Hardey	Weelamurra	Millstream	Cane
Caves	*	0.156–0.169	0.175–0.182	0.020	0.121–0.123	0.144–0.145	0.164
Seven Mile	5.5 (6.0–11.1)	*	0.088–0.106	0.155–0.174	0.182–0.195	0.191–0.204	0.022–0.036
Turee	6.8 (6.7–12.5)	3.8 (3.4–6.3)	*	0.180–0.190	0.224–0.231	0.240	0.119
Hardey	3.6 (0.77–1.4)	4.0 (6.0–11.1)	4.6 (6.9–12.8)	*	0.150–0.153	0.163	0.164
Weelamurra	5.6 (4.7–8.7)	4.6 (7.0–13.0)	5.0 (8.6–16.0)	4.0 (5.8–10.7)	*	0.059	0.215
Millstream	5.8 (5.5–10.3)	4.7 (7.3–13.6)	5.2 (8.8–16.4)	3.7 (6.3–11.6)	3.0 (2.3–4.2)	*	0.224
Cane	6.4 (6.3–11.7)	2.0 (0.85–1.6)	3.9 (4.4–8.1)	3.1 (6.3–11.7)	4.5 (8.3–15.4)	4.5 (8.6–16.0)	*

Table 3 Analysis of molecular variance: partitioning of total genetic variance (% Variance), and Φ statistics (Φ_{ST}) at two hierarchical levels across the Pilbara. Significance of the values of Φ_{ST} indicated with *

A. *Chydaekata*

Comparison	% variance	Φ_{ST}
Among tributaries	98.7	0.99*
Within tributaries	1.3	
		*$P < 0.001$

B. *Pilbarus*

Comparison	% variance	Φ_{ST}
Among tributaries	96.7	0.97*
Within tributaries	3.3	
		*$P < 0.001$

20 km, is considered to behave as a single hydrologic system, with hydraulic connectivity between the alluvium and the calcrete deposits (Barnett & Commander 1985). Likewise, haplotype WW1 occurred in both subterranean and surface sites in Weeli Wolli Creek, indicating not only lateral connections, but vertical connections within a tributary.

Phylogeographic patterns showing strong geographical clusters of haplotypes are indicative of a former population becoming fragmented into isolated populations at approximately the same time (Templeton 1998). Historical processes in the Pilbara provide a mechanism for the observed fragmentation patterns in the present study. Increasing aridity has been suggested as the impetus for driving the aquatic fauna of the Pilbara to seek refuge in subterranean habitats (Bradbury & Williams 1997a; Humphreys 2001). Early studies of palaeoclimates suggested that regular flow in the rivers had stopped before the mid-Miocene, as the region experienced episodic changes between humid and arid climates during the Tertiary (van de Graaff *et al*. 1977; Frakes *et al*. 1981), but a later study suggests that these events may have occurred more recently, with aridity apparently taking hold in the Pilbara in the Pliocene (MacPhail & Stone 2004). The allopatric distribution of the two genera (the apparent restriction of *Chydaekata* to the upper Fortescue, while *Pilbarus* occupies the lower end of the basin) is probably maintained by the peculiar hydrology of the basin: the upper Fortescue has drained internally since the Miocene, while the lower Fortescue is drained by the South Fortescue River towards the coast (Barnett & Commander 1985), the same divide that separates the only two species in Australia of the order Spelaeogriphacea (Poore & Humphreys 2003). Our MDIV estimates of the ages of lineages are consistent with some estimates of these major climatic events, with divergence between haplotypes of *Chydaekata* and *Pilbarus* (approximately 16 Ma) coinciding with the change in flow pattern in the Fortescue River in the Miocene, while divergence between haplotypes in different tributaries within both groups (approximately 2.0–8.9 Ma) coincide with Pliocene events. Despite likely errors in estimates of divergence

times due to rate heterogeneity, ancestral polymorphism and errors associated with both measures of divergence and calibration of clocks (Arbogast & Slowinski 1998), our estimates agree closely with the estimated time that diving beetles moved into calcrete aquifers in the Yilgarn region, to the south of the Pilbara, which apparently occurred as the deepening aridity spread from north to south (Leys *et al*. 2003). The lower MDIV estimates compared to those using the molecular clock may indicate that we underestimated generation times in these amphipods, or that the molecular clock is running faster than in other crustaceans upon which the calibrations were made.

Phylogenetic patterns may also give insight into the occurrence and timing of historical changes to the hydrology of the Pilbara. River capture may explain the odd affinity of the Onslow Coast haplotype from the Cane River (CR1) with haplotypes from Seven Mile Creek (SM) in the Ashburton River. Historical shifts in hydrological pathways have occurred between the Ashburton, Fortescue and Onslow basins, with some events occurring as recently as the Holocene (Barnett & Commander 1985). There is evidence from river gravels (van de Graaff *et al*. 1976) that during the progressive deformation of Cape Range in the Middle Miocene–Late Pliocene, the Ashburton River passed close to Northwest Cape (Wyrwoll *et al*. 1993). The uplift would have forced the mouth of the Ashburton northwards to its present position at Onslow, a process making it likely to capture the head streams of more northerly drainages, such as the Cane River. Our estimates of TMRCA for CR1 and haplotypes from Seven Mile Creek (0.85–2.5 Ma) suggest a Pliocene or Pleistocene connection between these two basins.

The monophyletic clades A–I, grouped by tributary, provide evidence that there has been no recent gene flow among tributaries, and that the amphipods in each lineage have evolved independently for millions of years. By current DNA standards for species-level divergence for surface water crustaceans (Rocha-Olivares *et al*. 2001; Wetzer 2001), these lineages would be considered different species. More importantly, other stygobitic populations in similar contexts, with similar levels of genetic divergence, have been shown morphologically to be different species (Cooper *et al*. 2002; Leys *et al*. 2003; Keable & Wilson 2006). Of these studies, the subterranean diving beetles (Cooper *et al*. 2002; Leys *et al*. 2003) are well differentiated morphologically; in contrast, the crustaceans (isopods; Keable & Wilson 2006) show only subtle morphological differences. In *Pilbarus* and *Chydaekata*, genetic diversity appears not to have been translated into similar levels of morphological diversification. Since their descriptions in 1997 and 2000, respectively, no new species in either genus have been described. While there are differences in characters such as the number of spines and setae on the limbs and body among individuals, there are no clear diagnostic characters to separate populations of each genus over their broad geographical ranges. Our findings highlight the need for a full taxonomic re-evaluation of both genera. Future taxonomic work on the amphipods of the Pilbara should focus on identifying differences among individuals in different tributaries, preferably utilising SEM and traits of genital morphology.

However, from a conservation perspective, when legislation is designed to protect *species*, not *lineages*, can we demonstrate that genetic diversity among morphologically cryptic allopatric populations equates to speciation or any meaningful, functional differences between lineages? Empirical evidence for reproductive isolation among such lineages is scarce. Allozyme analysis confirmed the sympatric occurrence of divergent lineages of the North American amphipod *Hyalella azteca*, which were thought to have arisen allopatrically in separate glacial refugia (Witt & Hebert 2000). Similarly, allopatrically derived lineages of the water flea *Daphnia obtusa* occasionally occur sympatrically (Penton *et al*. 2004); however, in that example, reproductive isolation could not be confirmed with allozymes. We were unable to test directly for reproductive isolation among lineages of *Pilbarus* and *Chydaekata* due to a lack of sympatric occurrences of haplotypes. While we have no direct evidence of reproductive isolation among these lineages based on morphology, allozymes showed a similar pattern of lineages associated with tributaries and no current gene flow among allopatric populations of *Pilbarus* and *Chydaekata* (Finston & Johnson 2004). Hence, these lineages do fit the criterion for 'evolutionarily significant units' (ESUs) for conservation, *sensu* Moritz (1994); that is, preserving these lineages ensures the preservation of the evolutionary processes contributing to genetic diversity. However, the biological significance of the differences between lineages remains to be demonstrated.

The deep genetic divergence in *Pilbarus* and *Chydaekata* at the tributary level, which is so far unlinked to any apparent pattern of morphological variation, highlights a disconnection between molecular and morphological evolution. Our findings demonstrate that reliance on the current morphological taxonomy underestimates underlying genetic diversity. Future research should be focused on identifying morphological, functional or ecological differences between the genetically different lineages. Nevertheless, this study has contributed to changing perspectives on patterns of diversity of freshwater crustaceans in general, and stygobitic amphipods of the Pilbara region in particular. The present study substantiates the importance of regional, rather than local, hydrological processes underlying the genetic diversity of these amphipods. It is not yet clear at what scale processes driving morphological diversity operate.

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Appendix

Sites from which samples were collected and hierarchical placement within tributaries and basins; S denotes samples collected from surface springs; H, hyporheic. Morphological identifications (M) are indicated by M (*Molina*) P (*Pilbarus*) and C (*Chydaekata*); T, sampled from type locality. Total, number of individuals sequenced possessing the haplotype; HR, haplotype ratio = number of haplotypes/number of individuals, calculated for each tributary. Voucher numbers correspond to Western Australia Museum collections except where noted: *, Department and Conservation and Land Management collections

Drainage basin	Tributary (HR)	Site	M	Haplotype	Total	Genbank accession number	Voucher number
Ashburton	Caves Creek (0.10)	HDM012	P	CC1	1	DQ256029	WAM C 38128
		HS1	P	CC1	1	DQ256028	PSS095*
		HW1	P	CC1	2	DQ256032, DQ256037	WAM C 38127
		SWL10	P	CC1	6	DQ256030–DQ256031, DQ256033–DQ256036	WAM C 38119-38124
	Hardey River (1.0)	Wyloo3	P	HR1	1	DQ490125	PSS362*
	Seven Mile Creek (0.73)	99RS3	P	SM1	4	DQ256038–DQ256039, DQ256040 DQ256041	PSS047* WAM C 38105
				SM2	1	DQ256042	WAM C 38106
				SM8	1	DQ256048	WAM C 38107
		PSPRSLK20	P	SM3	1	DQ256043	PSS177*
				SM4	1	DQ256044	
		PSPRSLK24	P	SM5	1	DQ256045	PSS178*
				SM6	1	DQ256046	
		PSPRSLK36	P	SM7	1	DQ256047	PSS045*
	Turee Creek (0.50)	COB12	P	TC1	3	DQ256022–DQ256024	WAM C 38173
				TC2	1	DQ256025	
		PF010-4	P	TC2	1	DQ256026	PSS052*
				TC3	1	DQ256027	
Fortescue	Ethel Creek (0.11)	WB23-1	C	EC1	1	DQ256006	WAM C 38225-38226
				EC2	1	DQ256000	
		WB23-4	C[T]	EC2	1	DQ679985	BES0331
		W116	C	EC2	1	DQ679986	WAM C 38214
		WP126	C[T]	EC2	4	DQ255995–DQ255996, DQ256001–DQ256002	WAM C 38190-38191 WAM C 38215-38216
		WP126nre	C	EC2	2	DQ255997, DQ256004	WAM C 38219
		W152	C[T]	EC2	3	DQ679981–DQ679982 DQ679983	WAM C 38199-38200 WAM C 38223
		W245	C	EC2	4	DQ255998–DQ255999, DQ256003, DQ267095	WAM C 38227-38230
		W270	C	EC2	1	DQ679984	WAM C 38231
	Marillana Creek (0.30)	Discharge	C	MC2	2	DQ255987–DQ255988	WAM C38165
		M2	C	MC1	2	DQ255980–DQ255981	WAM C 38159
		M5	C	MC3	2	DQ255982, DQ255985	WAM C 38155-38156
		M7	C	MC3	2	DQ255983–DQ255984	WAM C 38157-38158
		MNEW1	C	MC2	2	DQ255986, DQ255989	WAM C 38152-38153
	Millstream (1.0)	PSW011	P[T]	MS1	1	DQ490126	PSW011*
				MS2	1	DQ490127	
		COW1	M	MS3	3	DQ255960–DQ255962	PSS121*
	Roy Hill (0.20)	Aerodrome	C	RH1	5	DQ256007–DQ256011	BES11806, BES11809
	Spearhole Creek (0.20)	GMP077	C	SC1	5	DQ255990–DQ255994	
	Tuccamunna (0.25)	Roy Hill 1	C	TM1	4	DQ256012–DQ256015	PSS324*
	Weelamurra Creek (0.50)	PSB013S[H]	P	WC1	2	DQ256016–DQ256017	PSB013S*
				WC2	2	DQ256018–DQ256019	
	Weeli Wolli Creek (0.47)	1026	C	WW1	3	DQ255974–DQ255976	WAM C 38133, 38135, 38188
				WW2	1	DQ255978	WAM C 38143
		HD21	C	WW3	1	DQ255967	WAM C 38149
		JWB04	C	WW4	1	DQ255979	
		JSE6	C	WW8	1	DQ255970	WAM C 38171
		JSE14	C	WW1	1	DQ255977	WAM C 38205
		SC5	C	WW8	3	DQ255968–DQ255969, DQ255971	WAM C 38168-38170
		Spring2[S]	C	WW1	1	DQ255972	WAM C 38114
		WWS[S]	C	WW1	1	DQ255973	WAM C 38113
		WB2	C	WW5	1	DQ255963	WAM C 38147
		WB3	C	WW6	2	DQ255964–DQ255965	WAM C 38130-38131
				WW7	1	DQ255966	
Onslow Coast	Cane River (1.0)	Red004	P	CR1	1	DQ490128	PSS375*