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Islands under the desert: molecular systematics and evolutionary origins of stygobitic water beetles (Coleoptera : Dytiscidae) from central Western Austalia

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Abstract. Calcrete aquifers in the Yilgarn Craton of central Western Australia have recently been found to contain a rich invertebrate stygofauna, including the world's most diverse collection of stygobitic dytiscid water beetles. Our aim was to determine the evolutionary relationships between 11 species of stygobitic dytiscids and epigean species of the tribe Bidessini. Phylogenetic analyses of 1431 base pairs of mitochondrial DNA (mtDNA), including segments of *cytochrome oxidase 1*, *16S rRNA*, $tRNA_{leu}$ and *NADH dehydrogenase 1* genes, revealed that 10 of the 11 species form a natural group within the Bidessini and are most closely related to epigean taxa from the genera *Allodessus* Guignot, *Boongurrus* Larson, *Limbodessus* Guignot and *Liodessus* Guignot. The analyses support the morphological taxonomy of the stygobitic beetles at the species level, but generic level classification is not concordant with mtDNA lineages. Sympatric species of the large *Tjirtudessus* Watts & Humphreys and smaller *Nirridessus* Watts & Humphreys are more closely related to each other than either are to their congeners, suggesting a possible case of sympatric speciation. The analyses indicate that there have been multiple independent origins of stygobitic dytiscids and that origins correlate with the onset of aridity during the Miocene and also provide evidence that each calcrete aquifer may represent a 'subterranean island'.

Introduction

It has recently been discovered that calcrete (limestone) aquifers of the Yilgarn Craton in central Western Australia contain one of the world's most diverse collections of subterranean water beetles (Coleoptera : Dytiscidae; Watts and Humphreys 1999, 2000, 2001). Associated with the dytiscid water beetles is a rich invertebrate stygofauna (ground water fauna) comprising mainly Crustacea such as Bathynellacea, Amphipoda (Crangonyctoidea and Ceinidae), Ostracoda, Copepoda (Harpacticoidea and Cyclopoidea), and Isopoda : Oniscoidea (*Haloniscus* spp.), and Oligochaeta (Phreodrilidae), most of which are yet to be formally described. The calcretes consist of shallow isolated deposits of limestone along ancient river (palaeodrainage) systems, and are thought to have formed following the onset of continental aridity after the start of the Oligocene (Morgan 1993).

Of the stygobitic dytiscids collected to date, four new genera (*Tjirtudessus* Watts & Humphreys, *Nirridessus* Watts & Humphreys, *Kintingka* Watts & Humphreys and *Nirripirti* Watts & Humphreys) and seventeen new species have been formally described (Watts and Humphreys 1999, 2000, 2001). The taxa were collected from 11 discrete calcretes, of which five calcretes contained two species and one contained three species in sympatry. The beetles possess traits of animals adapted to underground life (Fig. 1). These include the complete loss of eyes and pigment, flattened body shape, long sensory bristles and reduction of wings. To date, each species appears to be unique to an individual calcrete aquifer. In Western Australia, there are about 210 major calcrete aquifers (approximately 50–1000 km^2 in area) associated with five major drainage systems, plus many smaller deposits, some of which have recently yielded new dytiscid species (C. H. S. Watts and W. F. Humphreys, unpublished data). Similar habitats occur throughout arid central Australia. Only 10% of these major calcretes from three drainages have so far been sampled, so the diversity in stygobitic dytiscids (and probably other stygofauna) is likely to be substantial.

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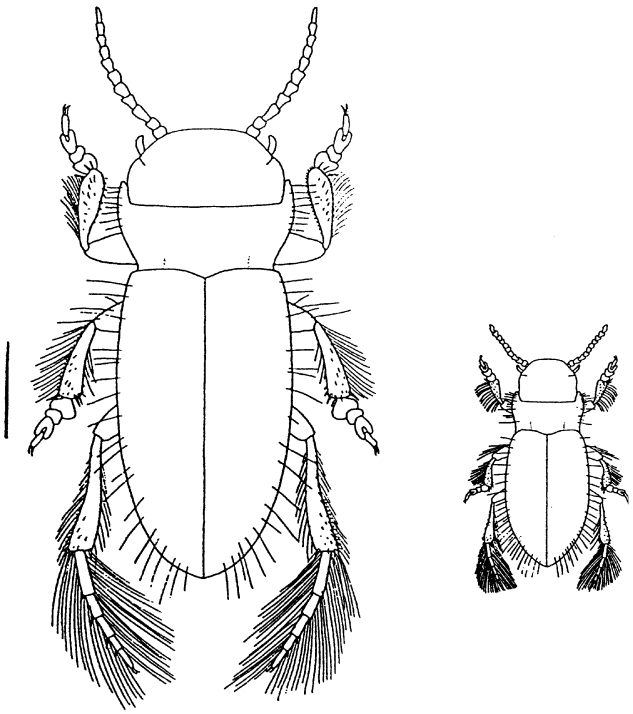

Fig. 1. Dorsal view of *Tjirtudessus magnificus* (left) and *Nirridessus cueensis* (right). Scale bar: 1 mm.

Watts and Humphreys (1999, 2000) proposed that *Tjirtudessus* and *Nirridessus* belong to the tribe Bidessini based on a number of morphological characters including the synapomorphy of two-segmented parameres (Biström 1988). The tribe Bidessini includes a variety of surface genera (e.g. *Allodessus* Guignot and *Liodessus* Guignot) with widespread distributions across Australia (Watts 1978). Relationships of these stygobitic genera within the Bidessini are uncertain, although Watts and Humphreys (1999) predicted that their sister-groups were likely to be found within the current Australian bidessines, such as *Liodessus*, *Limbodessus* Guignot or *Boongurrus* Larson. The genus *Nirripirti* comprises one species that has single-segmented parameres and other morphological characters that are characteristic of the tribe Hydroporini (Watts and Humphreys 2001). The systematic affinities of the genus *Kintingka* are unknown because only a single female specimen has been collected from this genus and a male is required to assess the number of segmented parameres.

The number of genera described by Watts and Humphreys (1999, 2000, 2001) reflects the extraordinary morphological diversity of the stygobitic dytiscids relative to epigean taxa that are, in contrast, conservative in their morphology (Biström 1988). Currently, there is no phylogenetic basis to the generic level classification based on morphology. It also is likely that adaptation to a subterranean environment has resulted in significant convergent evolution in morphological characters that will obscure phylogenetic relationships. For this reason we have assessed the systematics of the stygobitic dytiscids and their potential epigean relatives using molecular genetic data. This paper reports the first molecular phylogenetic analysis of the stygobitic dytiscids using mitochondrial DNA sequence data. We have focused this analysis on relationships among species of *Tjirtudessus* and *Nirridessus* and members of the tribe Bidessini, and have included data from *Nirripirti hinzeae* Watts & Humphreys to test whether it may also be a member of the tribe Bidessini. We were unable to include *Kintingka* in our analyses due to the lack of an available specimen. In addition, we investigate the number of origins of subterranean taxa and provide preliminary analyses of the age of divergence of stygobitic lineages.

Methods

Taxon sampling

Thirty-one species of dytiscids were included in our analyses (Table 1). We aimed to sequence two or three specimens per species to control against possible sequencing and contamination problems and errors in identification or labelling of samples during molecular procedures. However, additional specimens for sequencing were only available for 20 of the 31 species (Table 1). Eighteen epigean species are from the tribe Bidessini, and represent nine genera. Eleven stygobitic species were collected from seven different calcretes in central Western Australia (Fig. 2). These samples represent three of the four described genera, with *Kintingka* excluded due to lack of an available specimen. Of these, the following pairs of species were found in sympatry, each pair in different calcretes: *Nirridessus cueensis* and *Tjirtudessus magnificus*; *Nd. pulpa* and *T. eberhardi*; *Nd. masonensis* and *T. raesideensis*; and *Nd. fridaywellensis* and *Nirripirti hinzeae*. The latter species, *Np. hinzeae*, was included in our analyses to test whether it falls within the tribe Bidessini. *Necterosoma dispar* from the tribe Hydroporini and *Carabhydrus niger* from the tribe Carabhydrini are used as outgroups for the phylogenetic analyses. Recent molecular analyses by Ribera *et al.* (2002) have provided confirmation that these taxa represent appropriate outgroups to the tribe Bidessini and provided further evidence for the monophyly of the Bidessini, in support of previous morphological analyses (Biström 1988).

DNA extractions

Dytiscids were stored at either –80°C or in 100% ethanol. DNA was extracted from the entire body using DNAzol (Molecular Research Center, Cincinnati, Ohio) (Chomczynski *et al.* 1997) with slight modifications. Prior to centrifugation, the homogenate was incubated at 55°C for two hours with proteinase K (400 µg mL^{-1}; Sigma, St. Louis, MO) after which DNA was precipitated overnight at –20°C with 100% ethanol.

Polymerase chain reaction (PCR) amplification and sequencing

Two regions of the mitochondrial genome were amplified and sequenced using PCR methods. A 611-bp region of the 3′-end of the *cytochrome oxidase 1* (*CO1*) gene was amplified using primers M80 (forward, 5′-TACAGTTGGATTAGACGTTGATAC-3′) and M70 (reverse, 5′-TCCATTGCACTAATCTGCCATATTA-3′) (UAE 9 and 10; Lunt *et al.* 1996). Some taxa failed to amplify, but a newly developed forward primer M312 (forward, 5′-TACCGAGCTTATTT-TACTTCAG-3′) designed internal to, but with a 3-bp overlap with, M80 proved successful in such taxa. Primer combination M202 (forward, 5′-CAACATTTATTTTGATTTTTTGG-3′, alias Jerry; Simon *et al.* 1994) and M70 also usually gave satisfactory PCR amplifications.

Table 1. Twenty epigean species and eleven stygobitic species used in phylogenetic analysis
All species are from Australia with the exception of *Liodessus* sp. g and *Liodessus* sp. r, which are from Irian Jaya, Indonesia. All epigean species are from the tribe Bidessini with the exception of *Necterosoma dispar* and *Carabhydrus niger* (outgroup species). Stygofauna are shown in bold and sample locations are shown on the map in Fig. 2. Further information about specimens can be obtained from the Australian Biological Tissue Collection (ABTC) using the given voucher numbers. The number of specimens sequenced for each species is indicated by *n*

Species	ABTC	*n*	Location
Allodessus bistrigatus (Clark)	70192–4	4	Forreston, SA
Bidessodes bilita Watts	9601	2	Orbost, Vic
Bidessodes mjobergi (Zimmerman)	9411	1	Petford, Vic
Boongurrus rivulus Larson	9451	1	Herberton, Qld
Boongurrus, sp. nov.	75354–5	2	Wittenoon, WA
Carabhydrus niger Watts	75356–7	2	Detention River, Tas
Clypeodytes migrator (Sharp)	9460	2	Townsville, Qld
Gibbidessus chipi Watts	75358	1	Casterton, Vic
Hydroglyphus balkei Hendrich	9413	2	Petford, Qld
Hydroglyphus daemeli (Sharp)	9298	1	Townsville, Qld
Limbodessus compactus (Clark)	75359–61	3	Maryborough, Vic
Liodessus amabilis (Clark)	9230	1	Yunti, SA
Liodessus dispar (Sharp)	9576	2	Pinjarrah, WA
Liodessus inornatus (Sharp)	9543,9564	3	Pemberton, WA
Liodessus praelargus (Lea)	9705	2	Nangwarry, SA
Liodessus shuckhardi (Clark)	75362	1	Maryborough, Vic
Liodessus sp. r	75363–4	2	West Papua, Indonesia
Liodessus sp. g	75365–6	2	West Papua, Indonesia
Necterosoma dispar (Germar)	70201–2	2	Forreston, SA
Nirridessus challaensis Watts & Humphreys	75367	1	Challa Stn, WA
Nirridessus cueensis Watts & Humphreys	75368–9	2	Cue, WA
Nirridessus fridaywellensis Watts & Humphreys	75370	1	Depot Spring Stn, WA
Nirridessus hinkleri Watts & Humphreys	75371–3	3	Hinkler Well, WA
Nirridessus masonensis Watts & Humphreys	75374–5	2	Lake Mason Stn, WA
Nirridessus pulpa Watts & Humphreys	75376–7	2	Paroo, WA
Nirridessus windarraensis Watts & Humphreys	75378–9	2	Mount Windarra, WA
Nirripirti hinzeae Watts & Humphreys	75380	1	Depot Springs Stn, WA
Tjirtudessus eberhardi Watts & Humphreys	75381–2	2	Paroo, WA
Tjirtudessus magnificus Watts & Humphreys	75383–4	2	Cue, WA
Tjirtudessus raesidensis Watts & Humphreys	75385	1	Lake Mason Stn, WA
Uvarus pictipes (Lea)	75386	1	Byenup lagoon, WA

A 355-bp segment spanning 179 bp at the 3′-end of the large ribosomal subunit (*16S rRNA*) gene, the entire 76 bases of $tRNA_{leu}$ and the first 100 bases of *NADH dehydrogenase subunit 1* (*ND1*) was also amplified using the primers M223 (reverse, 5′-GGTCCCTTACGAATTTGAATATATCCT-3′, ND1A; Simon *et al.* 1994) and M313 (forward, 5′-CGACCTCGATGTTGAATTAA-3′; R. Buzila, personal communication). The primers M14 (forward, 5′-CGCCTGTTTATCAAAAACAT-3′, 16Sar; Simon *et al.* 1994) and M13 (reverse, 5′-CCGGTCTGAACTCAGATCACGT-3′, 16Sbr; Simon *et al.* 1994) were also used to amplify 511 bases of the 3′-end of the *16S rRNA* gene. In a number of cases the combination of primers M14 and M223 proved successful for amplifying a single 820-bp fragment of *16S-tRNA-ND1*.

Standard PCR amplifications included 1× reaction buffer (Perkin Elmer, Boston, MA), 0.2 mM of each dNTP, 5 pM of each primer, 1 unit of Amplitaq Gold (Perkin Elmer) and 2 mM of $MgCl_2$ in a 50 μL reaction volume. PCR amplifications were carried out on either an OMN-E 500 (Hybaid, Ashford, UK) thermocycler or a PC-960G Gradient thermal cycler (Corbett Research, Mortlake, NSW) for 1 cycle of 92°C for 9 min and 35 cycles (94°C, 45 s; 48°C, 45 s; and 72°C, 60 s), followed by a final incubation step at 72°C for 6 min. PCR products were purified using the UltraClean™ PCR Clean-up DNA purification kit (MoBio Laboratories Inc., Solana Beach, CA) according to the manufacturer's protocol.

Sequencing was performed using the ABI Prism™ Big Dye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, CA) in 20 μL or 10 μL reaction volumes according to the manufacturer's instructions. PCR primers were used as sequencing primers and each fragment was sequenced on both strands. Reaction products were purified by ethanol precipitation (as specified by ABI) and sequenced on ABI 373 (version 3.0) automated DNA sequencers. Sequence files were edited using SeqEd version 1.0.3 (Applied Biosystems) and a consensus of bi-directional sequencing was determined. Sequences were manually aligned using Se-Al (Rambaut 1995). Sequences have been submitted to GenBank (Accession numbers: AF484126–56, AF485931–61).

Phylogenetic analyses

Phylogenetic analyses of aligned sequence data were carried out using the program PAUP* version 4.0b8 (Swofford 2001), unless otherwise stated. The two data sets, *CO1* and *16S-tRNA-ND1*, were analysed

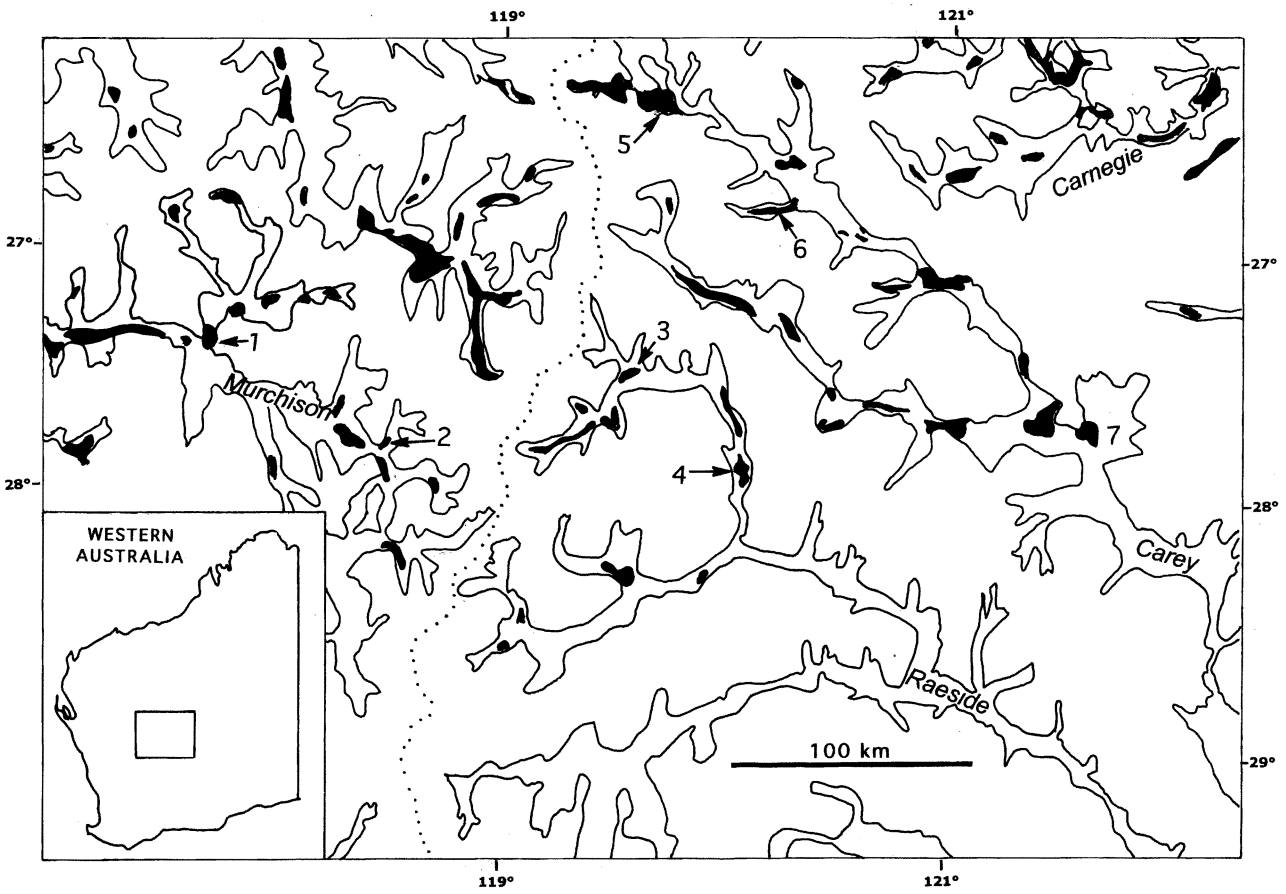

Fig. 2. Map showing the location of palaeodrainage channels (solid lines) containing the discrete surface expressions of groundwater calcretes (black) in which the stygobitic Dytiscidae mentioned in the text occur. Murchison palaeodrainage: 1, Cue; 2, Challa. Raeside palaeodrainage: 3, Lake Mason; 4, Depot Springs. Carey palaeodrainage: 5, Paroo; 6, Hinkler Well; 7, Mount Windarra. The dotted line denotes the surface drainage divide (Beard 1998), inland drainage to the east, Indian Ocean drainage to the west.

together. The four gene partitions are mitochondrial, and hence are considered to be of one locus. Chi-square analyses were used to test for homogeneity of base frequencies among taxa for each gene region, as implemented in PAUP*. Maximum parsimony (MP) analyses were performed using heuristic searches with 200 random addition sequences to search for the most parsimonious trees from different islands of trees. Bootstrapping (Felsenstein 1985) with 1000 pseudoreplicates and the heuristic search option was used to examine the robustness of nodes in the resulting tree. Maximum likelihood (ML) analyses were also carried out. To find the most appropriate model of molecular evolution for our data set, a series of nested likelihood ratio tests were performed on the combined data set using MODELTEST 3.06 (Posada and Crandall 1998) and PAUP*. However, rather than a Jukes-Cantor tree, ML scores from different evolutionary models were calculated using the tree from our above MP analysis. The significance of the likelihood ratios of different pairs of models was tested against a chi-squared distribution (Posada and Crandall 1998).

The size of the data set and the large number of parameters to be estimated made it computationally intensive to estimate parameters during ML tree search methods. Instead, initial model-parameters were calculated from the most parsimonious tree. These parameters were then used as starting values in a series of ML searches using the PUZZLE option in PAUP* (Strimmer and von Haeseler 1996). A number of iterative heuristic searches with previously estimated parameters, using the resulting tree for estimating new parameters, were performed until parameters, likelihood scores and tree topology did not change. These 'final' parameters were then used for a ML heuristic search. Maximum likelihood analyses were performed using heuristic searches with 10 random additions of sequences to search for the shortest likelihood tree from different islands of trees. Bootstrapping with 100 'fast'-replicates and the heuristic search option was used to examine the robustness of nodes in the resulting tree.

Estimation of divergence dates

To obtain estimates of divergence times of several nodes in the phylogeny, we used a distance-based method. Although there is large variation in the estimated divergence times using distance methods because of variation in molecular rates across branches of a topology (e.g. Sanderson 1997), the method is appropriate for obtaining conservative estimates of the nodes of interest. Because appropriate fossils of bidessine dytiscids are not available, we used *CO1* sequence data from the Canary Islands beetle genera *Pimelia* (Juan *et al.* 1995) and *Hegeter* (Juan *et al.* 1996), to calibrate molecular clocks using biogeographic distribution data and emergence times of the islands. The relationship of the maximum emergence times of the islands and the molecular distance of the island-specific taxa since divergence using first and second nucleotide positions of *CO1*, but not third positions, proved to be linear for divergence times of up to at least 20 million years

Table 2. Base frequencies per gene fragment and codon position, chi-square tests of homogeneity of base frequencies across taxa, number of parsimony informative sites and total number of sites

	A	C	G	T	χ^2	P	No. inf. sites	Total sites
CO1								
1st	0.25624	0.15439	0.22677	0.36259	10.86	1.0	41	203
2nd	0.18824	0.24902	0.13720	0.42554	2.40	1.0	12	204
3rd	0.44238	0.10679	0.03446	0.41637	106.19	0.1170	157	204
All	0.29550	0.17015	0.13283	0.40151	30.78	1.0	210	611
ND1								
1st	0.15095	0.03561	0.28954	0.52391	26.32	1.0	9	33
2nd	0.14344	0.06557	0.12193	0.66906	6.29	1.0	4	33
3rd	0.40371	0.00105	0.17068	0.42456	66.22	0.9718	26	32
All	0.23126	0.03432	0.19438	0.54004	26.04	1.0	39	98
tRNAleu								
All	0.31542	0.04724	0.16489	0.47244	11.66	1.0	21	78
16S								
All	0.34652	0.08938	0.16038	0.40372	20.58	1.0	172	644

ago (mya) (Juan *et al.* 1995, 1996). They are, therefore, suitable for our purpose because the calcretes are likely to be of a similar age. To be able to compare the distance data using the most appropriate ML model of sequence evolution for our data set (see above) with the data of the Canary Island beetles, we obtained sequences of the Canary Island beetles through GenBank (accession no's X97209–23 and Z71707–27) and calibrated molecular clocks for these two beetle genera using the same model of sequence evolution as for our dytiscids. Subsequently, the estimated clock rates were used to infer minimum and maximum divergence times using pairwise distances of the dytiscid nodes of interest.

Results

A total data set of 1431 bp of aligned sequences was established for 31 species. Duplicate samples of species showed nearly identical mtDNA sequences (<0.5% divergence) and were pruned from the data set. Within the final data set of 31 taxa, 30.8% (n = 442) of the sites are parsimony informative. Table 2 gives an overview of the base frequencies, results of chi-square tests of homogeneity of base frequencies across taxa, number of parsimony informative sites and the total number of sites per gene fragment and codon position. No significant heterogeneity of base frequencies across taxa was found. Third codon positions for the protein-encoding genes *CO1* and *ND1* showed apparent AT-bias, 85.9 and 82.8% respectively. AT-bias was also apparent for all nucleotides of the $tRNA_{leu}$ and *16S* sequences, respectively, 78.8 and 75.0%. Strong AT-bias in mitochondrial genes has been found in several insect orders (Simon *et al.* 1994; Jermiin and Crozier 1994). Coding regions of *CO1* and *ND1* were found to be open reading frames.

Phylogenetic analyses

Maximum parsimony analysis with all characters unweighted resulted in a single most parsimonious tree after 200 random sequence additions. The resulting tree (tree length = 2141 steps) is shown in Fig. 3. Excluding *Nirripirti hinzeae*, all the stygofauna are found in a strongly supported clade (bootstrap value 99%) together with species of the epigean genera *Allodessus*, *Boongurrus*, *Limbodessus* and *Liodessus*. The relationships among the stygofauna and epigean genera remain largely unresolved, except for the stygobitic species *Nirridessus cueensis* and *Tjirtudessus*

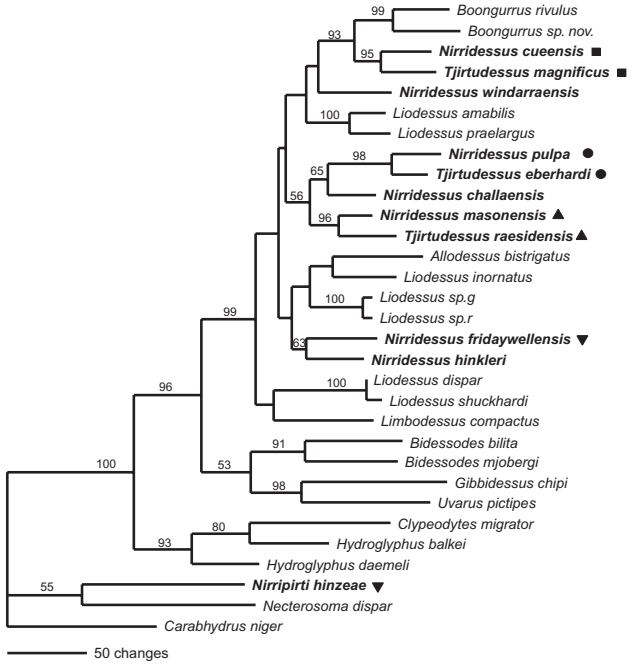

Fig. 3. Maximum parsimony tree of length 2141 steps resulting from analyses of 1431 bp of combined *CO1*, *16S rRNA-tRNA*$_{leu}$-*ND1* sequences using a heuristic search with 200 random additions in PAUP*. Consistency index = 0.402, rescaled consistency index = 0.443 and homoplasy index = 0.598. Numbers next to branches refer to percentages of 1000 bootstrap pseudoreplicates. Stygobitic dytiscids are shown in bold, and codes are given in Table 1.

Fig. 4. Phylogenetic tree (-ln likelihood = 11255.8) resulting from ML analysis of 1431 bp of combined *CO1*, *16S rRNA-tRNA$_{leu}$-ND1* sequences from dytiscids using a heuristic search with 10 random additions in PAUP*. A general time reversible substitution model was used with equal transition rates, nucleotide frequencies estimated empirically, gamma rate variation (α = 0.569303), proportion of invariant sites (i = 0.410879), and rate parameters (A–C 1.415, A–G 15.74, A–T 4.33, C–G 0.3823, C–T 15.74, G–T 1.00). Numbers above branches refer to percentages of 100 'fast' bootstrap pseudoreplicates. Stygobitic dytiscids are shown in bold, and codes are given in Table 1. Highlighted nodes (*a–e*) are those for which divergence times have been estimated (Table 3). Sympatric taxa are denoted by symbols: ■ Cue, ● Paroo, ▲ Lake Mason, ▼ Depot Spring Station.

magnificus, which show a close relationship with the two epigean species of *Boongurrus* (bootstrap support 93%). The three sympatric species-pairs (*Nd. cueensis* and *T. magnificus*; *Nd. pulpa* and *T. eberhardi*; and *Nd. masonensis* and *T. raesideensis*) appear to be sister-species, supported by bootstrap values of 95–98%. Two additional allopatric stygobitic species, *Nd. hinkleri and Nd. fridaywellensis*, form a clade, but the bootstrap support for this node is low (63%). Among Australian members of the tribe Bidessini there was good bootstrap support for two major lineages, one including members of the genera *Hydroglyphus* Motschulski and *Clypeodytes* Regimbart, and a second group comprising all the remaining Bidessini taxa.

Maximum likelihood analyses were performed using a restricted GTR + g + i model of sequence evolution (Rodríguez *et al.* 1990) with equal transition rates. The ML tree (-ln = 11255.8) is shown in Fig. 4. The tree topology is very similar to the MP tree, and only the low bootstrap (<50%) support nodes from the MP tree show a different branching order in the ML analysis (Fig. 4).

Molecular clock estimates

Likelihood ratio (LR) tests performed using MODELTEST showed that the ML value of a tree calculated without a molecular clock enforced was significantly larger ($P < 0.01$, d.f. = 29) for both the Rambaut (LR = 163.664) and the Rambaut multi-dimensional (LR = 163.662) molecular clock models, indicating rejection of a global molecular clock model for our mitochondrial data set. However, it is possible that local molecular clocks may apply in different lineages of our phylogeny so we proceeded to estimate the range of possible divergence times, but emphasise that these divergence times should be treated with some caution.

In order to calibrate molecular clock rates of *CO1*, we re-analysed sequence data available from GenBank for species of the tenebrionid beetle genera *Pimelia* and *Hegeter* (Juan *et al.* 1995, 1996) from the Canary Islands. First and second codon positions of *CO1* were used, and pairwise distances were calculated using the Tamura–Nei (Tamura and Nei 1993) model of sequence evolution with unequal base frequencies and three substitution types. Graphical analyses of maximum age of the beetle groups endemic to the islands, based on the geological age of the islands using the data in Juan *et al.* (1995, 1996) and the molecular distance between the beetle groups, revealed (almost) linear relationships (data not shown). From these relationships we calibrated *CO1* molecular clocks for the two beetle genera, 1% sequence divergence per 3.226 million years for *Pimelia* and 2.941 million years for *Hegeter* respectively. These clock rates were then used to calculate minimum and maximum divergence times using Tamura–Nei pairwise distances for selected nodes in the dytiscid phylogeny. For pairwise comparisons, we used the top 15 species in the ML phenogram of Fig. 4. The estimated divergence times of several comparisons are given in Table 3.

The well resolved sister-group relationships of the three sympatric stygofauna species-pairs (nodes *a–c*) show estimated divergence times that differ among each other more than two-fold, ranging from 3.8 million years to 9.0 million years. The only well-supported split between epigean and stygofauna species is represented by node *e* in the phylogeny (Fig. 4). Transition to the subterranean environment represented by the species *Nd. cueensis* and *T. magnificus* is estimated to have taken place 9.0–12.5 mya. Similar, but slightly older divergence times are found for comparisons among the non-sympatric stygofauna and among the remaining epigean and stygofauna species. Overall, the earliest transitions to the subterranean environment point to a time approximately 15.8 mya.

Discussion

Systematics

Molecular phylogenetic analyses based on mtDNA provide strong support for the morphological taxonomy of the

Table 3. Minimum and maximum divergence times estimated for pairwise comparisons of sympatric stygofauna, non-sympatric stygofauna and epigean *v.* stygofauna, using the Canary Islands tenebrionid beetle genera *Pimelia* and *Hegeter* for calibrating *CO1* molecular clock rates

The node designations are presented in Fig. 4

Comparison	Number	Node	Min.–max. divergence time (mya)
Sympatric stygofauna			
Nd. cueensis–T. magnificus	1	*a*	6.7–7.3
Nd. pulpa–T. eberhardi	1	*b*	3.8–4.2
Nd. masonensis–T. raesideensis	1	*c*	8.2–9.0
Non-sympatric stygofauna			
Nd. fridaywellensis–Nd. hinkleri	1	*d*	11.2–12.3
All others	36	–	6.0–15.8
Epigean versus stygofauna			
Boongurrus–Nd. cueensis, *T. magnificus*	4	*e*	9.0–12.5
All others	45	–	5.2–15.7

stygobitic dytiscids at the species level, with each described species being represented by a distinct mitochondrial lineage of considerable divergence (>3.8 mya, see further discussion below). The analyses also provide strong evidence that the genera *Nirridessus* and *Tjirtudessus* are members of the tribe Bidessini. This grouping was originally proposed by Watts and Humphreys (1999, 2000) based on a number of morphological characters, most notably the synapomorphy of two-segmented parameres (Biström 1988). Within the Bidessini, species of *Nirridessus* and *Tjirtudessus* are most closely related to species of *Liodessus*, *Limbodessus*, *Allodessus* and *Boongurrus*. Species of *Boongurrus*, in particular, were suggested by Watts and Humphreys (1999) to be likely relatives because they show signs of incipient existence underground, being often found in sand and gravel at the headwaters of streams. Larson and Storey (1994) suggested that *Boongurrus* is in the process of losing its cervical stria, thought to be a phylogenetically significant character in the Bidessini, which is absent in *Nirridessus* and *Tjirtudessus*, and possibly an adaptation to a subterranean existence. Other morphological characters that support the relationship of *Nirridessus* and *Tjirtudessus* with *Liodessus*, *Limbodessus* and *Boongurrus* include the presence of pronotal plicae, form of the parameres, simple central lobe of aedeagus, lack of or weak development of elytral setae, lack of sutural striae, weak punctation and lack of basal carinae on the epipleura (Watts and Humphreys 1999). The genus *Nirripirti* (Watts and Humphreys 2001) was found to group outside the Bidessini, but our data are insufficient to assess the systematic affinities of this genus. The presence of single-segmented parameres and additional morphological characters in both adults and larvae suggest *Nirripirti* belongs to the tribe Hydroporini (Watts and Humphreys 2001), but further molecular analyses, including a wider range of outgroups and genera of the tribe Hydroporini, are required to test this proposal.

Although morphological and molecular data are consistent with respect to species taxonomy and tribal relationships, the two data types are incompatible with respect to generic level classifications. The mtDNA phylogeny provides strong evidence that *Nirridessus* and *Tjirtudessus* are not distinct evolutionary lineages. In particular, the three species of *Tjirtudessus* are each more closely related to a sympatric species of *Nirridessus* (bootstrap support >93%) than other congeneric species. It is possible that the molecular phylogeny does not reflect the true species phylogeny because it is based on a single genetic marker (the mitochondrial genome) and/or sequences may represent nuclear copies of the mitochondrial genome (Zhang and Hewitt 1996). These possibilities, however, are unlikely to result in such clear and replicated patterns of relationship between the species in sympatry. In addition, the genes we have sequenced showed stationarity of base frequencies, with AT-bias typical of insect mtDNA and open reading frames in *CO1* and *ND1*, providing some evidence that they are mitochondrial in origin. Alternatively, our favoured hypothesis is that the morphological characters used to define the genera have resulted from convergent or parallel evolution. *Tjirtudessus* was originally distinguished from *Nirridessus* by its large size (>3 mm), cordate shape, subobsolete pronotal plicae and metacoxal lines, and bow-shaped protibiae (Watts and Humphreys 1999). With inclusion of further species from each genus, the morphological distinction between *Tjirtudessus* and *Nirridessus* has become weaker and size appears to be the only remaining character distinguishing them (C. H. S. Watts, unpublished data).

Origins of stygobitic dytiscids

The molecular phylogeny provides evidence for at least three independent origins of subterranean dytiscids from surface ancestors. One origin is apparent from the well-supported

monophyletic group comprising two species of the epigean *Boongurrus* and a sister-group containing the sympatric stygobitic *Nd. cueensis* and *T. magnificus*. A second clearly independent origin is of the species *Nirripirti hinzeae*, which falls outside the Bidessini. The seven remaining stygobitic species provide a third origin, although their relationships with epigean species are not clear and their monophyly is not supported in either the MP or ML trees. Therefore, it is likely that there are many additional independent origins of stygobitic taxa (see discussion below), but that there are insufficient epigean lineages, most likely due to extinction, to provide the contrasts.

We have attempted to place a date on the divergences between stygobitic and epigean lineages using a calibration based on the evolution of Canary Island tenebrionid beetles (Juan *et al.* 1995). Although these dates are likely to be subject to large error, they provide some idea of the magnitude of relative divergence times between taxa. Divergence times among sister-taxa within calcrete aquifers range between 3.8–9.0 mya, and those between epigean and stygobitic lineages range between 9.0 and 12.5 mya. These dates are consistent with the proposed timing of calcrete deposition following dry climatic conditions during the Oligocene and subsequent history of the region (Morgan 1993). Following a wetter climate and reactivation of river flow during the Middle Miocene (17–10 mya), Morgan (1993) suggested that springs were extensively developed and caves formed within the calcrete. These developments would potentially have provided suitable habitats for stygofauna to evolve and following an increase in aridity (10 mya to the present) allowed air to penetrate deep into calcretes. These springs have now been replaced by calcrete mounds, which are commonly found throughout the Yilgarn, and probably continue to provide a source of air into the calcretes. The dates we have estimated further suggest that there has been long-term isolation of species in each calcrete aquifer.

In the following discussion we consider the origin of extant stygobitic dytiscid species and whether they most likely originated by dispersal of stygobitic ancestors between calcretes or from epigean ancestors. Given that stygobitic dytiscids lack functional wings, their possible dispersal is likely to be restricted to locations within palaeodrainages and, most likely, to adjacent calcrete aquifers. The drainage divide, which separates palaeodrainages that drain into the Indian Ocean from those draining to the interior of the continent (Beard 1998), is likely to have been a major barrier to their dispersal. Similarly, the different palaeodrainage channels, which were incised into Archaean rocks by the Permian, are likely to be insurmountable barriers to stygobitic species. The calcretes are deposited from groundwater flow within the complex alluvial deposits in the palaeochannels immediately upstream of salt lakes (playas). Between the calcretes, the alluvial deposits are probably too fine – they contain layers of clays – to enable access from groundwater to the air-filled voids required for respiration by both adult and larval dytiscids (Spangler and Decu 1999). The absence of dytiscids outside calcrete aquifers, despite extensive sampling, supports this notion (Watts and Humphreys 1999, 2000). However, calcretes are dynamic entities, being continuously deposited from and taken into solution (Arakel 1986). Hence, in response to climatic change they may expand or contract in size, fragment and re-fuse, and sometimes possibly even fusing with adjacent calcretes. Despite this possibility, to date there is no evidence of conspecific taxa or closely related lineages in adjacent calcretes. Furthermore, the mtDNA data indicate that species from calcretes within the same drainage system are often more closely related to species in different drainages or across the drainage divide than species within the same drainage and adjacent calcretes, excluding species in sympatry. For example, *Nd. hinkleri* from the Carey palaeodrainage is more closely related to *Nd. fridaywellensis* from the Raeside palaeodrainage than it is to other Carey palaeodrainage species. Similarly, *Nd. challaensis* from the Austin palaeodrainage that flows to the Indian Ocean has its nearest relatives with species from the Carey palaeodrainage that flows to the interior. These findings suggest that the individual calcrete aquifers were colonised by widespread epigean ancestors and not by stygobitic ancestors. Hence, adaptation to life underground and speciation then proceeded independently within individual calcrete aquifers. The finding that each calcrete aquifer studied to date contains a unique assemblage of stygobitic dytiscids, and probably Crustacea, further supports this hypothesis, suggesting that the calcretes can be viewed as 'subterranean islands' comprising subterranean lakes in the desert.

The finding that three large/small pairs of sympatric stygobitic dytiscids (i.e. *Nirridessus v. Tjirtudessus*) are sister-species lead to questions about their evolutionary origins. In each case, the *Tjirtudessus* species are between 1.6 and 2.1 times longer than their sympatric *Nirridessus* sister-species (Fig. 1). One other pair of sympatric species (*Nd. fridaywellensis* and *Np. hinzeae*) are not sister-species, but the two taxa share the same pattern in size, with *Np. hinzeae* being 1.7 times longer than *Nd. fridaywellensis* (Watts and Humphreys 2001). The latter two taxa clearly arose after colonisation of the calcrete aquifer by two phylogenetically distinct ancestral species. A similar origin for the three remaining sympatric species-pairs would require that for each pair, the two ancestral taxa were sister-species prior to colonisation or that colonisation occurred by the same ancestral species on two separate occasions. Although such patterns of colonisation are possible, they would seem unlikely to be replicated multiple times in calcretes. We propose two alternative hypotheses for their origin. Firstly, the taxa may have originated by sympatric speciation driven by natural selection on

morphological characters involved in adaptation to alternative niche partitions within the calcrete aquifer. Recent theoretical studies suggest that phenotypic divergence and speciation can proceed by 'disruptive' natural selection and assortative mating without isolation of individuals (Dieckmann and Doebeli 1999; Tregenza and Butlin 1999). Potentially similar cases of parallel divergence in sympatry from a common ancestor are found among several fish taxa, including three-spined sticklebacks, where small, slender, 'limnetic' and larger, more robust, 'benthic' species exploit alternative niche partitions in lakes of recently glaciated areas (Schluter and McPhail 1992; see Schluter 2000 and references there in). Alternatively, the taxa may have evolved within the calcrete through some process of allopatric speciation following size partitioning of animals within the calcrete aquifer and periods of physical isolation of regions of the calcrete aquifer, perhaps by fluctuations in water depth or by fusion and fission of adjacent calcretes. Our data are currently insufficient to distinguish these hypotheses.

Conclusions

Our phylogenetic analyses of mtDNA provide evidence for multiple independent origins of stygobitic dytiscids in calcrete aquifers of the Yilgarn Craton. The finding that calcretes may represent 'subterranean islands', with independently evolved taxa, will make them fertile grounds for fascinating evolutionary research, including studies of speciation and regressive evolution. The large numbers of calcretes that have yet to be surveyed suggest that the diversity of stygobitic dytiscids that are likely to be found in the future will be considerable, and provide a wealth of material for this research.

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