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EVOLUTION OF SUBTERRANEAN DIVING BEETLES (COLEOPTERA: DYTISCIDAE: HYDROPORINI, BIDESSINI) IN THE ARID ZONE OF AUSTRALIA

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Abstract.—Calcrete aquifers in arid inland Australia have recently been found to contain the world's most diverse assemblage of subterranean diving beetles (Coleoptera: Dytiscidae). In this study we test whether the adaptive shift hypothesis (ASH) or the climatic relict hypothesis (CRH) is the most likely mode of evolution for the Australian subterranean diving beetles by using a phylogeny based on two sequenced fragments of mitochondrial genes (*CO1* and *16S-tRNA-ND1*) and linearized using a relaxed molecular clock method. Most individual calcrete aquifers contain an assemblage of diving beetle species of distantly related lineages and/or a single pair of sister species that significantly differ in size and morphology. Evolutionary transitions from surface to subterranean life took place in a relatively small time frame between nine and four million years ago. Most of the variation in divergence times of the sympatric sister species is explained by the variation in latitude of the localities, which correlates with the onset of aridity from the north to the south and with an aridity maximum in the Early Pliocene (five mya). We conclude that individual calcrete aquifers were colonized by several distantly related diving beetle lineages. Several lines of evidence from molecular clock analyses support the CRH, indicating that all evolutionary transitions took place during the Late Miocene and Early Pliocene as a result of aridification.

Key words.—Adaptive shift, aridification, climate relict, molecular clock, phylogeny.

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The world's largest biodiversity of subterranean diving beetles (Coleoptera: Dytiscidae) has recently been reported from groundwater calcrete (terrestrial limestone) aquifers (hereafter referred to as ''calcretes'') in the arid zone of inland Australia. Until now, 54 species have been described, with individual calcretes containing one to five unique species (Watts and Humphreys 1999, 2000, 2001, 2003). With the exception of two recently discovered but still undescribed species with greatly reduced eyes, all subterranean species lack eyes, have strongly reduced wings and pigment, and have altered body shapes as compared to their phylogenetically closest surface relatives (Fig. 1). These morphological characters are typically found in obligate subterranean insects (Culver et al. 1995) and indicate that the species of diving beetles are highly adapted to and obligate inhabitants of subterranean waters (stygobites). In Western Australia, there are at least 210 major calcretes (50–1000 km^2) and many smaller calcretes, with similar habitats throughout arid inland Australia. Only about 25% of these major aquifers have been sampled to date, so the diversity in stygobitic beetles is likely to be considerable.

Morphological and molecular phylogenetic analyses have shown that the beetles belong to two different subfamilies, the Hydroporinae and the Copelatinae, with the majority of species placed in two tribes (Bidessini and Hydroporini) within the Hydroporinae (Watts and Humphreys 1999, 2000, 2001, 2003; Cooper et al. 2002). Molecular phylogenetic analyses of evolutionary relationships among 11 subterranean species and 18 epigean (living in surface water) species of the Bidessini provided preliminary evidence that the majority of the subterranean species evolved independently from widespread epigean ancestors (Cooper et al. 2002). However, in three independent cases, pairs of species from individual calcrete aquifers originally placed in distinct genera (*Tjirtudessus* and *Nirridessus*) were found to be sister species, suggesting that speciation may have occurred in sympatry after colonization of the calcrete by a single ancestral species. Molecular clock analysis using pairwise distance data also suggested that the evolutionary transitions to subterranean life took place at some time during the Middle to Late Miocene (12–5 million years ago; mya; Cooper et al. 2002). Our aim here is to investigate the evolutionary processes and timing of events that have generated this extraordinary diversity of dytiscid species.

Modes of Evolution of Subterranean Fauna

The adaptation of organisms to a cave or groundwater environment has been the subject of many studies (Culver and Wilkens 2001; Holsinger 2000; Sbordoni et al. 2001; Rivera et al. 2002). Two general hypotheses are currently in use to test evolutionary transitions of epigean to stygobitic species. According to the climatic relict hypothesis (CRH: Barr 1968; Banarescu 1975; Sbordoni 1982; Barr and Holsinger 1985; Peck and Finston 1993), an epigean species preadapted to underground life (e.g., living in leaf litter or under stones in a stream) may survive and adapt to cave or groundwater life when the surface environment becomes unsuitable due to a climate change (e.g., glaciation or aridification). According to the adaptive shift hypothesis (ASH: Rouch and Danielopol 1987; Desutter-Grandcolas and Grandcolas 1996), an epigean species preadapted to subterranean life may actively enter cave or groundwater habitats once they become accessible. Restricted gene flow and divergent selection between the parent and the subterranean population will eventually lead to speciation driven by adaptive shifts in the subterranean population.

The above hypotheses can be tested by examining the phylogenetic contrasts of epigean and subterranean species, taking into account the geographic distribution of the contrasting

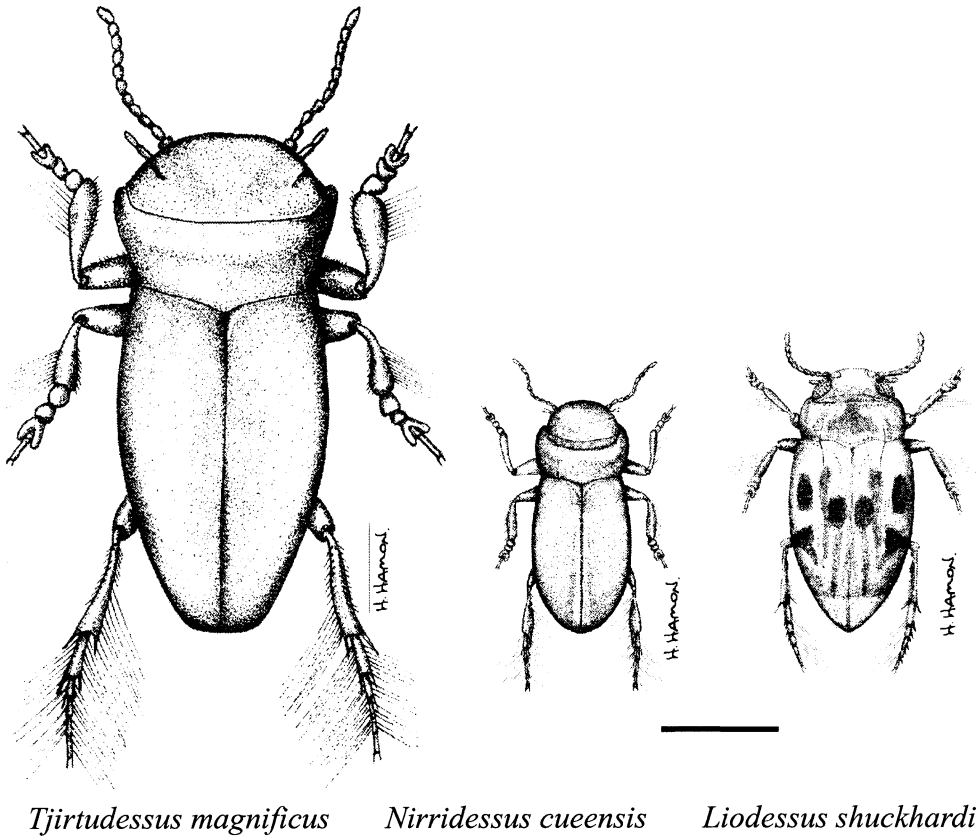

FIG. 1. Habitus of subterranean and epigean species. *Tjirtudessus magnificus* and *Nirridessus cueensis* are sympatric sister species, *Liodessus shuckhardi* is an epigean species. Scale bar = 1 mm.

species. If the ancestral species only survived in a geographical area outside the influence of climatic change, then the phylogenetic prediction of the CRH is that the epigean ancestor and the subterranean species are allopatric sister species, unless all epigean ancestor populations became extinct, leaving only a group of subterranean relict species. Whereas the phylogenetic prediction of the ASH shows the epigean ancestor and the subterranean adapted lineages as closely related parapatric sister species (for diagrams see Rivera et al. 2002). Although differences between the two hypotheses with respect to the predicted phylogenetic pattern and the geographical distribution of the epigean and subterranean species seem clear, it might be more difficult to distinguish the predicted patterns using real phylogenies when some of the ancestral epigean species have become extinct or have altered distributions. However, valuable additional information for testing both hypotheses can be gained by correlating divergence times of the evolutionary contrasts between epigean and subterranean lineages or correlating the branching pattern and timing among subterranean taxa with data of changing paleoclimate or geological data about the appearance of new habitat.

Calcrete Formation and Paleoclimates

All known Australian stygobitic waterbeetles are restricted to individual calcretes, so it is important to understand the paleoclimatic conditions and the geological history of the calcretes to interpret the evolutionary processes that have led to this great diversity of subterranean species. The calcretes form by precipitation of carbonates from shallow groundwater along the groundwater flow paths in paleodrainage channels. Calcretes form just upflow of the salt lakes that comprise such a prominent feature of arid Australia and that are scattered along the paleodrainages. The calcretes, which are generally about 10 m thick, occur in arid climates (annual rainfall $<$ 200 mm, annual evaporation $>$ 3000 mm; Mann and Horwitz 1979).

Although global climatic patterns are well established, the detailed paleoclimate of Australia in the Tertiary is poorly known, especially in the northwest. However, there are several lines of evidence from paleobotany especially palynology, deep oceanic clay deposits, geomorphology, and soils, that lead to a largely consistent interpretation of the general climatic trends (summarized in Martin 1998). The paleodrainage valleys in inland Australia contained active rivers at least until the Eocene, when rainforest covered Australia. Calcretes were formed within the paleochannels during the Late Eocene to Early Oligocene (37–30 mya) as a result of the dry conditions on the Australian continent due to global cooling.

During the Late Oligocene to Middle Miocene (30–10 mya) the Australian climate was warm temperate with periods of humidity and high rainfall regimes over much of southern Australia (Langford et al. 1995). Active rivers existed in the

paleovalleys of the Yilgarn craton of central Western Australia and other areas in inland Australia, as well as numerous freshwater lakes. Calcrete formations existed and were in a process of karstification (Morgan 1993) and may have become available as cave and or groundwater habitats during this period. Such an environment would provide a range of habitats suitable for diving beetles.

From the Middle to Late Miocene (10 mya onward) temperatures dropped and the climate became much drier (Markgraf et al. 1995). The onset of aridity started earlier in the northwest than in the southeast (Martin and McMinn 1994; Martin 1998). The permanent rivers ceased flowing and salt lakes developed (van der Graaff et al. 1977). Rainfall was periodical and unpredictable, but calcrete formations remained charged as part of the groundwater flow path and locally by recharge from episodic rainfall (Morgan 1993). Aridification of inland Australia started in the north around 14 mya and extended southward during the Late Miocene (10.4–5.3 mya) but with periods of greater aridity around 12, nine, five, and three mya (Stein and Robert 1986); the southern part of the Yilgarn has been arid only for the last five million years (Clarke 2001). The largely arid conditions prevailed until the present time.

Epigean diving beetles living in permanent rivers and streams would have survived unpredictable and intermittent drying of surface rivers due to the onset of aridity, either by migrating to places where surface water remained or by adapting to the hyporheic zone (below the surface flow) of the river. Species adapting to the hyporheic zone might eventually colonize and remain isolated in the calcretes when the hyporheic zone became unsuitable due to clogging of the sediment spaces (internal colmation; Brunke and Gonser 1997) with fine sands and clays. At present calcretes are the only permanent freshwater habitats for macro- and mesofauna through much of the arid zone of inland Australia.

Thus, the geological history of the calcretes and that of the paleoclimates provide us with a time frame that can be used to infer the timing of the evolutionary transitions that gave rise to the subterranean diving beetles. As has been explained above, evidence from geology and paleoclimates indicate that the calcretes became available as a subterranean niche long before the conditions above ground became unfavorable for epigean waterbeetles. Therefore, by incorporating historical data we might be able to distinguish both hypotheses. Under the ASH, it would be expected that colonization of the new subterranean habitats and associated speciation would have occurred from 37 mya until aridification made surface existence impossible. Alternatively, under the CRH, colonization and diversification of subterranean species would be limited to periods of maximum aridification (12–5 mya).

In this paper, we investigate the evolutionary history of the subterranean diving beetles using a phylogeny based on mitochondrial DNA (mtDNA) sequence data. The use of molecular data for inferring phylogenetic relationships opens the possibility to estimate divergence times of nodes when molecular rates in the tree can be calibrated. The use of molecular clocks has often been criticized because of accumulating data that rates of molecular evolution may not be constant (Wu and Li 1985; Muse and Weir 1992; Sorhannus and Van Bell 1999). However, recent methods that incorporate models of change of molecular rates in and among branches (Sanderson 1997, 2002a; Thorne et al. 1998; Huelsenbeck et al. 2000a; Kishino et al. 2001) make it possible to calculate ultrametric trees and estimate divergence times when the age of one or more nodes can be calibrated. In the absence of fossil data for our group, we developed a method for calibrating the nodes in the phylogenetic tree by using external calibrated clock rates. We extend the dataset from our previous analysis (Cooper et al. 2002) by adding new species belonging to the Bidessini from new localities across a wider geographical range and by adding species belonging to an additional tribe, the Hydroporini. The inclusion of species from two independent lineages that frequently are found in sympatry allows us to investigate whether the same evolutionary forces acted on each lineage.

Materials and Methods

Taxon Sampling

Sixty species of diving beetles (Dytiscidae) belonging to the tribes Bidessini and Hydroporini both classified in the subfamily Hydroporinae (Miller 2001) were included as ingroup taxa in our analyses. As outgroup taxa we used an Australian *Copelatus* species that belongs to the subfamily Copelatinae, the closest sister group comprising Australian taxa to the Hydroporinae (Miller 2001). We have chosen to use a single outgroup for both tribes, rather than analyzing both tribes separately using a different outgroup for each tribe, because tribal relationships within the Hydroporinae are not fully resolved (Miller 2001), which will make the choice of an appropriate outgroup difficult. Moreover, analyzing the species belonging to the two tribes in a single dataset with a single outgroup has the advantage that it will allow direct comparison of evolutionary trends in the two tribes.

The species used for the analyses in this paper are presented in the Appendix along with locality data (drainage, calcrete), voucher numbers of the Australian Biological Tissue Collection (ABTC), and GenBank accession numbers. Figure 2 shows a map of the sampled calcretes and their relative positions in the paleodrainages.

Of the Bidessini, we included several epigean representatives of all known Australian genera except *Terradessus* (which is a tiny terrestrial species from high-altitude rain forests in northern Queensland). We also included two still undescribed species of *Liodessus* from mountaintops in western Papua, Indonesia (kindly provided by M. Balke), as well as the majority of the subterranean taxa discovered during the last five years (Watts and Humphreys 1999, 2000, 2001, 2003). Subterranean taxa not included in this study were those that are known only from a small number of specimens (*Nirridessus hahni*, *N. morgani*, and *Kintingka kurutjutu*) or that failed to amplify in polymerase chain reactions for some of the genes (*N. lapostae*).

Of the Hydroporini, we included three *Paroster* species and *Necterosoma dispar* as representatives of epigean species. Preliminary phylogenetic analysis with the stygobitic *Nirripirti hinzeae* and a variety of Hydroporine genera showed that *Paroster* was the closest relative to *Nirripirti* (Ribera et

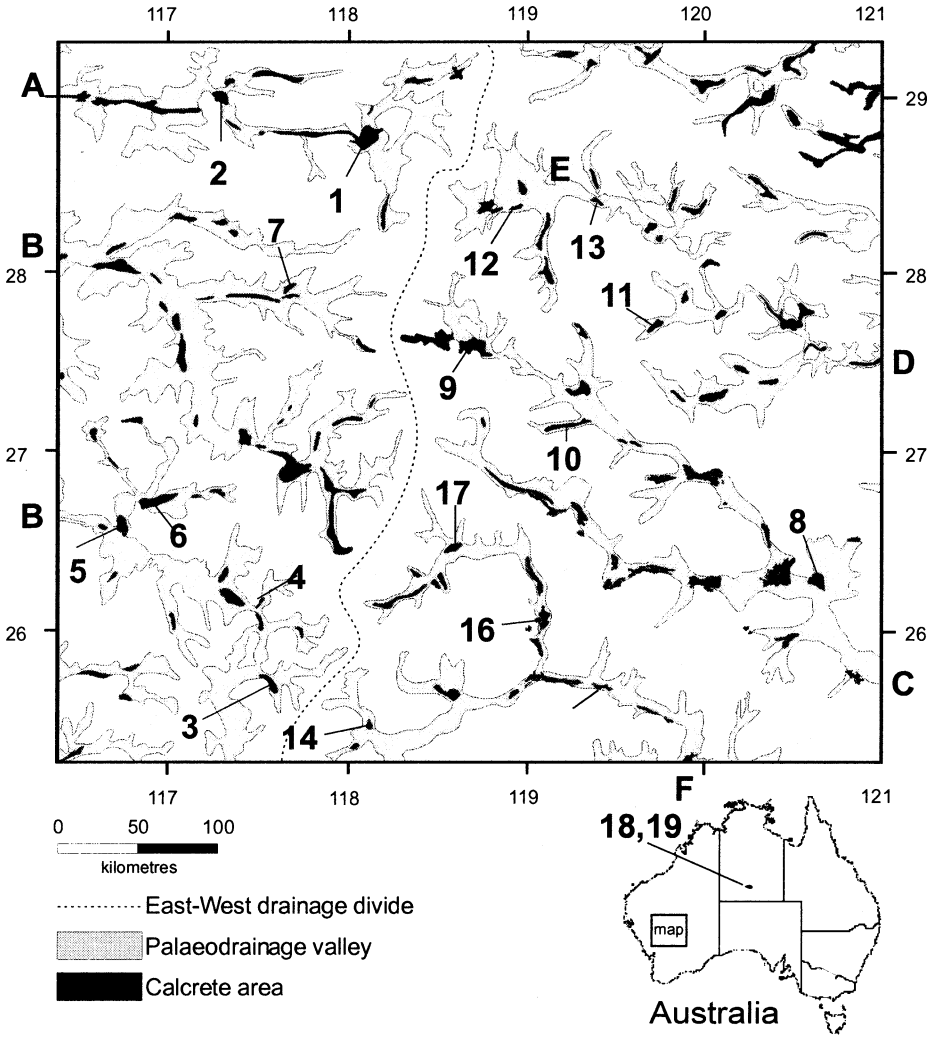

FIG. 2. A map of the sampled calcretes and their relative positions in the paleodrainages. Letters and numbers refer to the paleodrainages and calcretes, respectively, as listed in the Appendix.

al. 2002). We included all stygobitic hydroporine species known at the time.

All stygobitic species were collected by sampling wells and bores in calcretes in the Australian arid zone. The majority of the sample localities are from the Yilgarn craton in Western Australia and the remaining localities are from the Ngalia Basin, north of the Macdonnell Ranges, Northern Territory. Three species included in our analyses (*Boongurrus rivulus*, *B. occidentalis*, and *Liodessus gemellus*) were collected from the hyporheos of streams at localities peripheral to the arid zone. All of the surface species, except the widespread species *Allodessus bistrigatus* and *Limbodessus compactus*, were collected from localities around Australia with higher annual rainfall. For the distribution of the epigean species, we refer to Watts (1985).

Several species have not been formally described, and, therefore, only provisional names are given. However, the unique ABTC voucher numbers that are linked to those undescribed species will make association with the future descriptions possible.

DNA Methods

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing were performed as described in Cooper et al. (2002). Two regions of the mitochondrial genome were amplified and sequenced using PCR methods. An 822-bp region of the 3′ end of the cytochrome oxidase subunit 1 (*CO1*) gene was amplified using primers M202 (forward, 5′-CAA CAT TTA TTT TGA TTT TTT GG-3′, alias Jerry; Simon et al. 1994) and M70 (reverse, 5′-TCC ATT GCA CTA ATC TGC CAT ATT A-3′; UEA9 and 10, Lunt et al. 1996). An 820-bp fragment of the large-subunit RNA (16S)-tRNA^{leu}-NADH dehydrogenase subunit 1 (*16S-tRNA^{leu}-ND1*) was amplified using the primers M14 (forward, 5′-CGC CTG TTT ATC AAA AAC AT-3′, 16Sar; Simon et al. 1994) and M223 (reverse, 5′-GGT CCC TTA CGA ATT TGA ATA TAT CCT-3′, ND1A; Simon et al. 1994). In some cases it was necessary to use internal primers to amplify two overlapping fragments to obtain the whole 820-bp fragment of 16S-tRNA^{leu}-ND1 or overlapping fragments of the 822-bp fragment of the CO1 gene. Information about the internal

primers was published in Cooper et al. (2002). SeqEd version 1.0.3 (Applied Biosystems, Scoresby, Victoria, Australia) was used to edit chromatogram files, determine a consensus sequence from each strand, and manually align sequences across specimens. Secondary structure and conserved motifs of the RNA genes of insects (Buckley et al. 2000) and spiders (Masta 2000) were used to identify homologous positions in the sequences of 16S and $tRNA^{leu}$ and to assign nucleotide and indel sites to stems or loops.

Phylogenetic Analyses

Phylogenetic analyses of aligned sequence data were carried out using the program PAUP* version 4.0b8 (Swofford 2001) and BAMBE version 2.06b (Simon and Larget 2000). The two mitochondrial datasets, *CO1* and *16S-tRNA-ND1*, are considered to be of one locus and were thus analyzed together. 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 additions of 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.

The size of the dataset and the large number of model parameters that needed to be estimated for ML analyses and to obtain ML bootstrap support values for the nodes made it computationally too intensive to be analyzed with the methods used in PAUP*. Instead, we used Bayesian methods of phylogenetic inference, which significantly reduced the computational burden. The advantage of using Bayesian analysis over parsimony or likelihood tree reconstruction methods is that it gives reliable information about the correctness of the obtained tree topology, as well as information on the (posterior) probability of the individual nodes (comparable, but not the same as bootstrap values), given the data and the model of molecular evolution used (Huelsenbeck et al. 2000b; Rokas and McVean 2000). Moreover, it was estimated that using the Bayesian analysis would reduce calculation times by a factor of at least 20 compared to the time needed for ML bootstrapping.

Here, we used BAMBE version 2.06b (Simon and Larget 2000) for phylogenetic inference. The GTR model of molecular evolution (Rodríguez et al. 1990) was used with specific model parameters for each of the five data partitions in a combined analysis: first, second, and third codon positions of the coding genes *CO1* and *ND1* combined, and stems and loops for the RNA genes, 16S and $tRNA^{leu}$ combined. Analyses were run on a SUN (Gordon, New South Wales, Australia) computer following the suggested procedure of the Markov chain Monte Carlo (MCMC) convergence in the BAMBE (ver. 2.01) manual (Simon and Larget 2000). Initial runs were performed with a random starting tree, with global updating of parameters in the burn-in loop and local updating of parameters in the main loop to test whether the likelihoods of the MCMC cycles approached stability. Several short runs were performed to adjust the tuning factors until the parameter acceptance rates were within a 15–40% range. Several longer runs started with random seeds were conducted, each with 50,000 cycles, in which the final parameter values and tree topology of the previous run were used as priors to investigate whether these runs resulted in tree topologies with similar posterior probabilities and estimated parameter values. Finally, four runs, each of 10^6 cycles, started with random seeds, were performed. The saved tree topologies and parameter values of those runs were summarized together when they converged to similar final parameter values to obtain a final tree topology, the posterior probabilities, and the basic statistics of branch lengths and parameter values.

Estimation of Divergence Times

We used the phylogram resulting from the Bayesian analysis as the preferred tree topology for estimation of divergence times.

A semiparametric method (Sanderson 2002a) was used to linearize the tree topology. This method allows evolutionary rates to vary between branches within certain limits using a penalized likelihood function. This function includes a roughness penalty, which increases when rate differences in adjacent branches across a tree are large, and a smoothing parameter, which controls the trade-off between the smoothing of rate changes across adjacent branches and the goodness of fit in the model. Cross-validation procedures for finding the optimal smoothing parameters were performed by setting the age of the root of the tree to an arbitrary value of 100 million years. The program r8s version 1.05b (Sanderson 2002b) was used to carry out the abovementioned procedures. The program estimates divergence times and molecular rates when the age of at least one of the nodes is given. Analysis using r8s were performed using the penalized likelihood method and the quasi-newton algorithm, using 2000 iterations, repeated 10 times using different random combinations of initial divergence times.

Often fossil data are used for calibrating divergence times, but fossils belonging to the beetle groups in our dataset that might suit such a purpose are not available. Instead, in some cases geological data might be used to calibrate nodes in a tree. To avoid circularity, however, we cannot use the geological age estimates of the calcretes because a major objective of this paper is to test whether the age of divergences of subterranean lineages in the beetle phylogeny correspond with climatic history and the formation of calcretes.

In the absence of either a fossil or geological record, the molecular clock rates of taxonomic groups as close as possible to the target group might be used instead to obtain estimates of divergence times. A preliminary molecular clock for arthropod mtDNA of 2.3% pairwise sequence divergence per million years (Brower 1994; equivalent to a rate of 0.0115 substitutions per site per million years) is often used for questions concerning relatively recent divergences (Knowles 2000; Trewick and Wallis 2001).

Divergence times of the nodes in the diving beetle tree were calibrated using an iterative approach by adjusting the age of the root until an average rate of substitution was found (calculated using the program r8s) comparable to the preliminary mtDNA clock rate of Brower (1994). In this manner

TABLE 1. Parameter statistics from Bayesian analysis for different data partitions, standard deviation in parentheses. (A) Frequency of nucleotide bases, percentage of A-T bias, and the probability (*P*) of the chi-square test of nucleotide bias among taxa. (B) General time reversible substitution rates and relative substitution rates.

	1st positions	2nd positions	3rd positions	Stems	Loops
(A)					
pi-A	0.2122 (.0153)	0.1985 (.0188)	0.3735 (.0151)	0.2578 (.0152)	0.3144 (.0169)
pi-G	0.2965 (.0199)	0.1577 (.0175)	0.0500 (.0050)	0.2419 (.0168)	0.2417 (.0181)
pi-C	0.1969 (.0176)	0.2121 (.0200)	0.0981 (.0050)	0.0998 (.0128)	0.0620 (.0109)
pi-T	0.2943 (.0169)	0.4316 (.0236)	0.4785 (.0155)	0.4004 (.0197)	0.3819 (.0198)
% A-T bias†	50.66	63.01	85.19	65.82	69.64
χ^2†	52.26	11.07	357.54	33.94	37.11
P†	1.00	1.00	0.00	1.00	1.00
(B)					
rate AC	0.1076 (.0445)	0.1905 (.0950)	0.3582 (.0444)	0.1600 (.0632)	0.1539 (.0901)
rate AG	1.8589 (.1576)	3.1543 (.2539)	2.3368 (.1694)	3.1457 (.1728)	2.5082 (.1869)
rate AT	0.9430 (.0916)	0.9894 (.1301)	0.1789 (.0149)	1.2225 (.1027)	1.7017 (.1324)
rate CG	0.0225 (.0168)	0.3478 (.1234)	0.1751 (.0891)	0.1370 (.0664)	0.0885 (.0812)
rate CT	2.9060 (.1833)	1.1632 (.1697)	2.9160 (.1556)	1.0772 (.1359)	1.3374 (.2062)
rate GT	0.1621 (.0331)	0.1548 (.0584)	0.0351 (.0220)	0.2576 (.0494)	0.2102 (.0555)
Relative rate†	0.5052	0.1303	3.3973	0.4922	0.5830

† Calculated using PAUP*.

we avoided using pairwise distances, which can lead to serious problems when calculating divergence times both because of the nonindependence among the comparisons and because pairwise distances of taxa separated by the same node often vary considerably due to the stochastic nature of nucleotide substitution (Bromham and Penny 2003). Confidence intervals of divergence times were then calculated for nodes using a cut-off value of four, corresponding to 95% intervals (Cutler 2000) following the methods in r8s.

RESULTS

Sequencing of the *CO1* and *16S-tRNA^{leu}-ND1* mitochondrial gene fragments resulted in 1648 aligned nucleotide sites, of which two small segments in the 16S gene comprising 33 bp were excluded prior to analysis because of unalignable indels. Duplicate samples of species showed nearly identical mtDNA sequences (< 0.5% difference) and were pruned from the dataset prior to the analyses. Table 1a shows the average and standard deviation of the nucleotide frequencies, nucleotide bias, and overall chi-square values of tests for homogeneity of base frequencies across taxa for five different data partitions. Base composition bias among taxa was found to be significant only for third codon positions. A-T bias of third codon positions was most extreme in a hydroporine species, 92.5% in *Nirripirti hamoni*, but very high A-T bias was also found in several bidessine species: 91.6% in *Bidessodes limestonensis*, 90.3% in *Nirridessus bialveus*, 90.0% in *N. fridaywellensis*, and 89.8% in *N. cunyuensis*. The lowest values were found in the bidessine *N.* sp. 1 (78.3%) and the hydroporine *Paroster gibbi* (78.6%). No apparent phylogenetic structure in the distribution of the bias was found. Transition rates in the diving beetles were very high relative to transversion rates (Table 1b) and may provide a noisy phylogenetic signal, particular at third codon positions. The high A-T bias, found for third positions, may have been the result of directional mutations.

Maximum Parsimony Analysis

Given the heterogeneity of base frequencies and possible noise caused by high transition rates of third codon positions, we investigated whether a priori weighting of third position transitions of the coding genes affected the resolution of the resulting phylogenetic trees. Three different weights were applied to transitions of third codon positions: unweighted (= 1), weight = 0.1, and weight = 0. Applying different weights to transitions of the third codon positions only had a minor affect on tree topology, but had some effect on bootstrap support levels. Some taxa within the clade that forms the sister group of *Allodesus bistrigatus* (Fig. 3) had unstable positions. Unweighted transitions of third positions resulted in less overall bootstrap support and caused some branches to collapse under the 50% bootstrap consensus mark. However, more branches were resolved using third position transition weights of 0.1 or 0.0 and, although the same branches were supported, the values in general were slightly higher for weights 0.1. These a priori experiments show that parsimony analysis improves by down-weighting partitions that are suspected to be noisy. The bootstrap support values for the nodes in the MP analysis with third codon position weighted 0.1 are presented in Figure 3. Overall, the MP tree is well supported by bootstrap values: most of the branches in the Hydroporini clade, the basal nodes of the Bidessini clade, and all sympatric sister species in the Bidessini clade show bootstrap support levels greater than 50%, whereas the internal short branches in the Bidessini showed low bootstrap support (< 50%).

Maximum-Likelihood Analysis

ML analysis was performed using Bayesian methods in the program BAMBE (ver. 2.06a; Simon and Larget 2000). The GTR model of molecular evolution with site-specific substitution rates (five sites and six substitution classes) was used for each of the codon positions of the coding genes and for

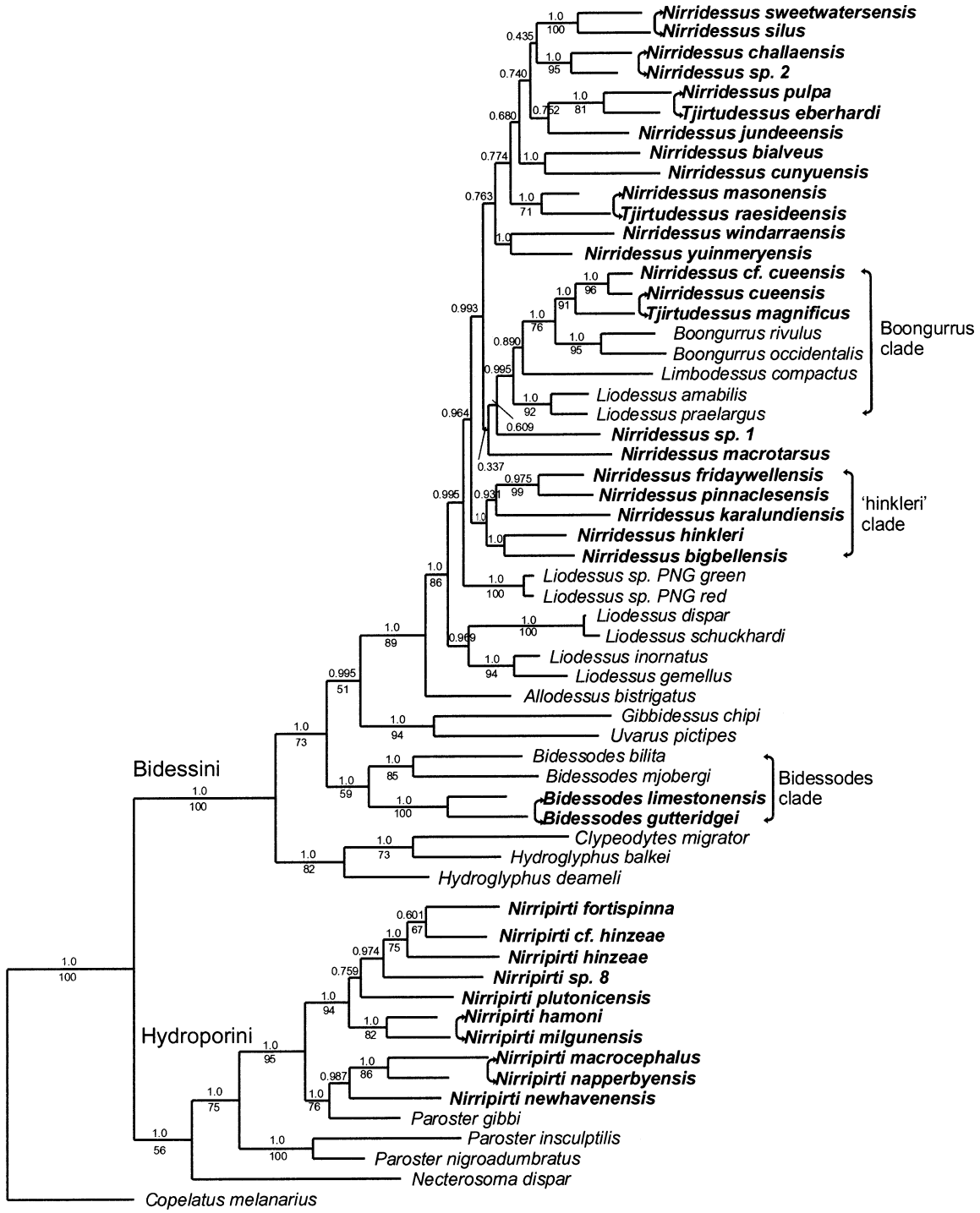

FIG. 3. Most probable tree topology based on Bayesian phylogenetic analysis. Posterior probabilities are shown above the branches, and parsimony bootstrap values (> 50%) are shown below the branches. Subterranean species are in bold, and sympatric sister species are shown with double-headed arrows.

stems and loops of the noncoding genes. Several initial runs were needed to find the most suitable parameter update values for our dataset. Four final runs, each started with a different random seed converged to similar likelihood values and tree topologies. The sampled trees of the independent runs were therefore summarized together, with the first 4000 trees (the burn-in phase of the MCMC) of each run excluded. The statistics of the GTR-model parameters for each of the partitions are presented in Table 1. The most probable tree topology had a posterior probability of 0.032. Several other tree topologies, with similar but slightly lower posterior probabilities (0.020–0.030), differed mainly with respect of the placement of the bidessine species *Nirridessus macrotarsus*, *N. jundeeensis*, and *N.* sp. 1. The posterior probabilities of the

nodes in the most probable phylogenetic tree inferred from Bayesian analysis are presented in Fig. 3. In general, this tree topology does not differ from the previous MP analyses. In almost all cases nodes that are supported by bootstrap values larger than 50% show posterior probabilities of 1.0 or very close to 1.0. Most of the uncertainty in the topology of the tree is found in the clade that represents the sister group of the *hinkleri* clade. Within this clade, the species belonging to the *Boongurrus* clade form a phylogenetically stable group. Furthermore, there are a number of species that are found in well-supported (posterior probability 1.0) sister pairs, several of them being sympatric sister species. Overall, there is little uncertainty in the tree topology and, therefore, this mitochondrial gene tree might be considered a reliable basis to test evolutionary hypotheses.

Geographical Distribution of Lineages with Subterranean Species

Apart from the eight pairs of sympatric sister species (42% of the localities) that occur in both tribes and across the whole tree, there is no apparent geographical structure in the tree: species from calcretes belonging to the same drainage system do not group together. The five species in the *hinkleri* clade, for instance, are found in five isolated calcretes belonging to three entirely separate paleodrainage systems including both sides of the divide between oceanic and interior drainage. A similar pattern is found for other clades in the tree. Several of the localities contain beetles belonging to different tribes (21% of the localities), the actual number of these localities being higher because not all species were available for molecular analysis. Additionally, several localities contain beetles belonging to different clades within the same tribe (21% of the localities). Such a pattern is consistent with several coexisting, widespread ancestral species that simultaneously colonized the calcretes of different drainage systems.

Calibration and Estimation of Divergence Times

The phylogenetic tree of Figure 3 was transformed into an ultrametric tree using semiparametric rate smoothing (Sanderson 2002a). The optimal smoothing parameters as determined by cross validation, appeared to be different for the two tribes in the tree: respectively 1.0 for the Bidessini and 10.0 for the Hydroporini. The phylogenies of the two tribes were therefore linearized separately.

To calibrate the divergence times in the linearized diving beetle tree, the invertebrate mtDNA clock rate of 0.0115 substitutions per site per million years calculated by Brower (1994) was used. Figure 4 shows the mean molecular rates for the two waterbeetles tribes inferred by substituting different divergence dates for the root node. Applying the invertebrate mtDNA clock rate gave divergence times of the root node for the Hydroporini and Bidessini trees of 21.5 and 28.2 mya, respectively.

Subsequently, these values were used as independent age estimates for the root nodes to calculate confidence intervals for a range of internal nodes in the phylogeny. The linearized trees are presented in Figure 5. Relevant nodes in the trees are numbered and divergence time estimates with confidence intervals for these nodes are presented in Table 2. The accuracy of the estimates (95% confidence intervals) ranged from 1.66 to 3.63 million years. Individual divergence time estimates of the eight pairs of sympatric sister species (nodes 1–8) varied within a range of 3.61–8.70 mya. However, regression analysis of the latitude of the localities with the estimated divergence times shows that 83% of the variation in divergence times is due to variation in latitude (mean square regression = 2.637, residual = 0.528; Fig. 6). Sympatric sister species from northern localities diverged earlier than those from southern localities. Interestingly, with the single exception of two closely related taxa *Nirridessus cueensis* and *N.* cf. *cueensis* from adjacent calcrete aquifers, all other splits that include subterranean species are of similar age or older than the sympatric sister species pairs. Divergence time estimates of the sympatric species pairs therefore represent a minimum age of the subterranean lineages. However, several species of epigean diving beetles, for example, belonging to the genera *Boongurrus* and *Liodessus*, have much more recent divergence dates than the youngest divergence dates of the subterranean lineages. Finally, five nodes (Table 2, nodes 9–13), chosen because they represent evolutionary contrasts between surface and subterranean lineages, show a relatively large range of divergence times (5.99–18.68 mya). These estimates represent maximum ages of the subterranean lineages (but, see Discussion).

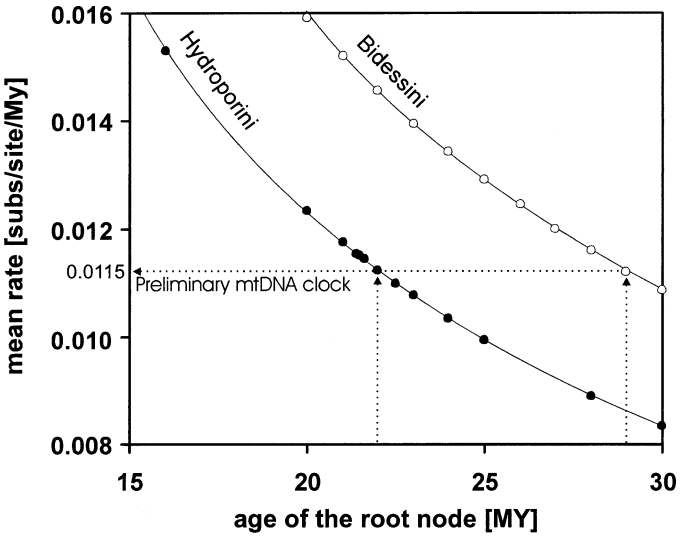

FIG. 4. Calibration of the diving beetle trees. Black dots, Hydroporini data; open circles, Bidessini data. The trees were calibrated by iteration of the age of the root node (million of years; see Fig. 4) until mean rates in the trees equaled the preliminary clock of Brower (1994). See Materials and Methods for details.

DISCUSSION

Evidence for Multiple Independent Origins of Subterranean Diving Beetles

The traditional way of investigating evolutionary trends is by mapping character states to the tips of the branches of a phylogenetic tree and then performing a reconstruction of the internal branches using MP (Maddison and Maddison 1998). The MP reconstruction of ancestral states, with epigean as the ancestral character state, shows us four gains (nodes 9–

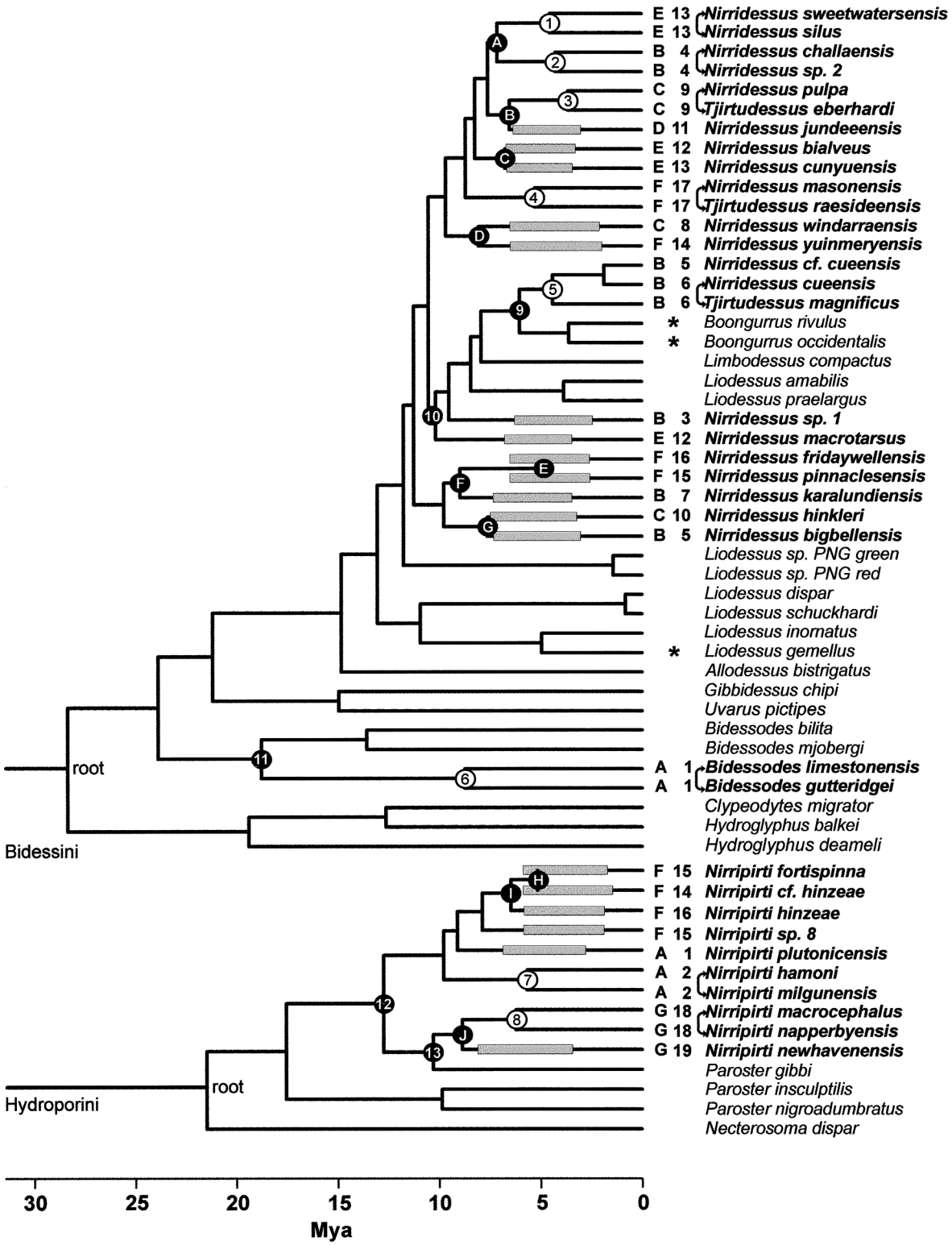

FIG. 5. Linearized and calibrated phylogenetic tree. Subterranean species are in bold, and sympatric sister species are shown with double-headed arrows. Letters at the tip of the branches show drainage systems, and numbers calcrete aquifers, as listed in the Appendix. The divergence times of the nodes with black dots show maximum estimates of transitions to the subterranean environment, the open circles show minimum transition times based on sympatric sister species. The letters and numbers in the nodes are listed in Table 2. Bars in the branches represent 95% confidence intervals of predicted transition times.

TABLE 2. Divergence time estimates in millions of years (95% confidence interval).

Taxa	Node	Calibrated with the mitochondrial DNA clock of Brower (1994)
Sympatric sister species		
Nirridessus sweetwatersensis–N. silus	1	4.52 (3.55–5.56)
Nirridessus challaensis–N. sp. 2	2	4.23 (3.33–5.31)
Nirridessus pulpa–Tjirtudessus eberhardi	3	3.61 (2.87–4.50)
Nirridessus masonensis–Tjirtudessus raesideensis	4	5.25 (4.11–6.60)
Nirridessus cueensis–Tjirtudessus magnificus	5	4.38 (3.50–5.43)
Bidessodes limestonensis–B. gutteridgei	6	8.70 (6.88–10.51)
Nirripirti hamoni–N. milgunensis	7	5.66 (4.66–6.75)
Nirripirti macrocephalus–N. napperbyensis	8	6.21 (5.25–7.27)
Evolutionary contrasts		
Boongurrus clade	9	5.99 (4.99–7.29)
Nirridessus s.l. clade	10	11.70 (10.22–13.36)
Bidessodes clade	11	18.68 (16.86–20.46)
Nirripirti clade 1	12	12.74 (11.64–13.87)
Nirripirti clade 2	13	10.28 (9.13–11.47)
Allopatric lineages		
Nirridessus sweetwatersensis–N. challaensis	A	7.08 (5.99–9.54)
Nirridessus pulpa–N. jundeeensis	B	6.48 (5.45–7.67)
Nirridessus bialveus–N. cunyuensis	C	6.70 (5.62–7.95)
Nirridessus windarraensis–N. yuinmeryensis	D	8.01 (6.67–9.77)
Nirridessus fridaywellensis–N. pinnaclesensis	E	4.82 (3.76–6.11)
Nirridessus fridaywellensis–N. karalundiensis	F	8.92 (7.54–10.46)
Nirridessus hinkleri–N. bigbellensis	G	7.57 (6.13–9.15)
Nirripirti fortispinna–N. cf. *hinzeae*	H	5.17 (4.37–6.06)
Nirripirti fortispinna–N. hinzeae	I	6.45 (5.61–7.38)
Nirripirti macrocephalus–N. newhavenensis	J	8.85 (7.79–10.02)

12) and two losses (nodes 13, 14). However, the use of parsimony in this case is dubious, because many of the subterranean lineages contain species that are confined to isolated calcretes that often belong to different paleodrainage systems. It is very unlikely that species once they are adapted to life in subterranean water (loss of eyes, wings, and pigment) will be able to disperse and colonize other isolated calcretes, especially those in different paleodrainages, sometimes over hundreds of kilometers apart and with no known groundwater connections. If dispersal were possible, it would be expected that related species would be found in adjacent calcretes within a single paleodrainage system. To date, there is only one case of related species (*Nirridessus cueensis* and *N.* cf. *cueensis*) in adjacent calcretes and these calcretes were possibly connected at some time in the past (see below). Excluding these taxa, we propose that for all other subterranean lineages with allopatric taxa each of these taxa evolved independently from epigean ancestors.

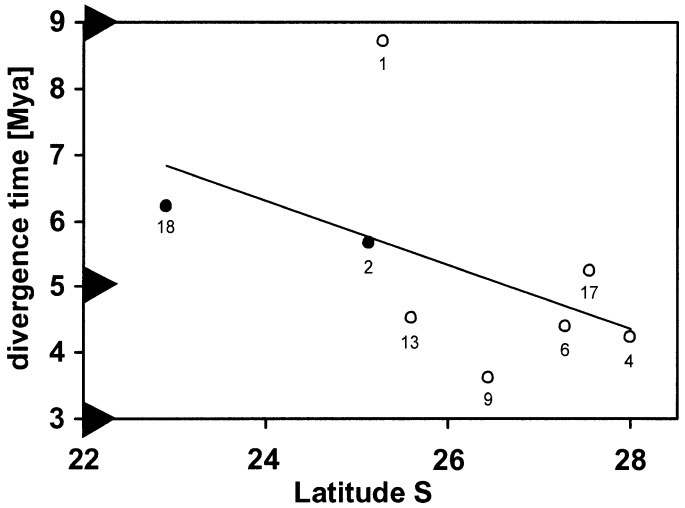

FIG. 6. Latitudinal variation in divergence times of eight sympatric sister pairs. The black triangles on the vertical axis show periods of maximum aridity, the open circles show species pairs belonging to the Bidessini, the black dots show species pairs belonging to the Hydroporini. The numbers refer to the localities listed in the Appendix.

There are probably eight additional independent adaptations to the subterranean environment in the ancestral lineages of the eight sympatric sister species pairs in our phylogeny (Fig. 5). We have previously argued that the sympatric sister species most likely evolved within a calcrete from a subterranean ancestor (Cooper et al. 2002). Character state reconstruction, taking into account that gene flow between calcretes virtually ceases following adaptation to the subterranean environment, reveals 26 independent gains and no losses, which is in sharp contrast with the maximum parsimony reconstruction. Overall, there is evidence for 18 independently evolved subterranean lineages within the Bidessini and another eight independently evolved lineages in the Hydroporini. As far as we are aware, this is the largest number of independently evolved subterranean diving beetle lineages recorded from a single region.

Molecular Dating and the Timing of Transitions

The estimated divergence times inferred from our analyses, allowing for variable rates of molecular evolution, give a

timeframe in which the diving beetles might have made the transitions to the subterranean environment. It should be noted, however, that the inferred timeframe could be subject to error due to several causes. First, we used the penalized likelihood approach (Sanderson 2002a) to calculate a linearized tree that attempts to minimize the rate differences between parent-child branches across the whole tree. It is possible that the subterranean taxa have a naturally higher rate of evolution because they all share the same life-history strategies associated with the subterranean environment. In comparison to related epigean species, obligate subterranean inhabitants typically have lower fecundity, smaller population sizes, and longer generation times (Martin and Palumbi 1993; Hüppop 2000) and are therefore likely to have smaller effective population sizes. If this indeed is the case, the timing of the nodes leading to the subterranean species might be slightly overestimated but it is unlikely to change the overall picture. Second, the absence of fossil data required us to use external data to calibrate the diving beetle molecular clock and these may not be representative of rates of evolution of diving beetles. However, the rate we used from the preliminary arthropod mtDNA clock of Brower (1994) is similar to the several other calibrated invertebrate mtDNA clocks (DeSalle et al. 1987; Russo et al. 1995; Fleischer et al. 1998; Farrell 2001).

Maximum time estimates of the transitions are suggested by the nodes that represent epigean-subterranean contrasts (Fig. 5, nodes 9–13). By further examination of the branching pattern of the subterranean species, however, it is possible to get more accurate information on the maximum transition times. It is reasonable to assume that transitions to subterranean life occurred somewhere along the branch from the contrasting node to either the tip of a subterranean species (no examples in our phylogeny) or to the next node connecting a pair of sympatric sister species (e.g., Fig. 5: branches connecting nodes 9–5 and nodes 11–6). However, in lineages consisting of subterranean allopatric species, given our arguments above (Discussion, first paragraph) that each of these species evolved independently from epigean ancestors, the transitions should have taken place after the last speciation event (i.e., nodes connecting subterranean allopatric sister taxa). Therefore, the divergence times of pairs of subterranean allopatric species (Fig. 5, nodes A–J) would give better estimates of maximum divergence times than estimates from the phylogenetic contrasts. These estimates range from 4.82 to 8.92 mya (Table 2).

We previously argued that the sympatric sister species most likely diverged from a common ancestor within each calcrete (Cooper et al. 2002), although the mode of speciation is currently unclear. Assuming that speciation occurred soon after isolation in the calcretes, the divergence of the sympatric species should provide minimum estimates for the transitions. These estimates vary within a relatively small range from 3.61 to 8.70 mya (Fig. 5, Table 2). Most of the variation is explained by latitude and coincides with the onset of an aridity maximum around five mya (Fig. 6) that progressed from the north to the south (Stein and Robert 1986).

The overall transition times can therefore be narrowed down to between 8.92 and 3.61 mya (Table 2, Fig. 5: node F and node 3, respectively). The actual transition times, however, may vary with the local circumstances. We have shown that the latitude of the localities explained most of variation in the age estimates of the nodes leading to the sympatric sister species and that these estimates are remarkably similar. The divergence times of the sympatric sister pairs would provide the best estimates for the transition times, assuming that the transitions in the sympatric sister species took place just prior to or during speciation.

We postulate that similar transition times for the other subterranean lineages might have occurred, because many of those lineages are in sympatry with one of the sympatric species pairs. To investigate whether this is a reasonable assumption, we predicted the transition times for all other subterranean species using the latitudes of their present localities and the regression data of the latitudes and divergence time estimates of the sympatric sister species. The predicted 95% confidence intervals for the transition times are shown using horizontal bars in Figure 5. Most predicted transition times are in the branches well past the previous node, showing no conflict with the previously discussed relative timing of the transitions in the branches of subterranean lineages of allopatric species. There are only two pairs of species, one from each tribe (node E: *N. fridaywellensis* and *N. pinnaclesensis*; node H: *N. fortispinna* and *N.* cf. *hinzeae*), for which the predicted transition time interval overlaps with the estimated divergence time of the two species, and both pairs are from adjacent calcretes belonging to the same paleodrainage. A possible explanation here is that populations of a single ancestral species in each of the tribes were isolated at different parts of the drainage systems just prior to a period of maximum aridity.

In summary, the use of the preliminary mtDNA clock of Brower (1994) to calibrate the diving beetle phylogeny provides us with estimates of the timing of the evolutionary transitions from epigean to subterranean life. These transition dates support the idea that subterranean diving beetles in the Australian arid zone evolved about five mya.

Evolution of Subterranean Diving Beetles

As mentioned in the introduction, the phylogenetic predictions of the adaptive shift hypothesis (ASH) and climatic relict hypothesis (CRH) differ with respect to the geographical distribution of epigean and subterranean sister lineages. Also, it was proposed that the divergence times of these sister lineages should fit either the time of emergence of new habitat (ASH) or the episodes of climatic change (CRH). Our phylogenetic tree does not show incidences of contrasts of epigean and subterranean parapatric sister species, which would have supported the ASH. The only epigean species that live in the arid zone are *Allodessus bistrigatus* and *Limbodessus compactus*, but neither species shows a close relationship with any of the subterranean species. Two nodes (nodes 9 and 11, Fig. 5) possibly represent allopatric contrasts, although in both cases further speciation had taken place in both the epigean as well as the subterranean lineages. Because epigean ancestral lineages might have become extinct or evolved into subterranean species or because the distributions of epigean species later changed with the environment and so obscured the testable patterns, the use of phylogenetic

patterns linked to species distributions in this case is inconclusive for testing ASH and CRH models based on their phylogenetic pattern alone. A similar conclusion was reached by Rivera et al. (2002).

However, we found that the estimated times of transitions to the subterranean environment, especially those of the sympatric sister species pairs, coincide with a period of maximum aridity in Australia about five mya (Stein and Robert 1986). These transition times are distinct from the time when calcretes would have become available as new habitat for stygofauna (from about 30 mya onward). Our analyses therefore support the CRH, suggesting that climate change, in this case aridification, was likely to have been the main driving force for the evolution of the subterranean diving beetles.

There are several points that together provide additional support for the CRH. First, the sympatric sister species belonging to the two tribes have similar divergence times, with most of the variation explained by the latitude of calcrete localities, which correlates with the progression of aridity from the north (Stein and Robert 1986). Second, with only one exception, there are no divergences in subterranean lineages that are younger than the divergence times of sympatric sister species, suggesting either that surface species were absent after that time or that conditions for transitions to subterranean life were not favorable. The one exception involves the species *Nirridessus cueensis* and *N.* cf. *cueensis*, which are found in adjacent calcretes and diverged from a common ancestor about two mya. It is most likely that they speciated as subterranean animals, probably due to physical isolation of the calcretes that once belonged to a single unit. Finally, diving beetles that occur in the hyporheos of rivers with unpredictable surface flow but not in the hyporheos of permanent rivers (e.g., *Boongurrus rivulus*, *B. occidentalis*, and *Liodessus gemellus*), point to the possible role of climate and how preadaptations to the subterranean environment might establish.

This timing is consistent with the view that a large component of both distributional and phyletic relicts in the Australian cave fauna may be attributed to the onset of aridity (Humphreys 1993a, 1993b, 1999, 2000). For example, using a variety of paleoclimatic, paleobotanic, geological, and allozyme data, but in the absence of phylogenetic evidence, Humphreys (1993b) suggested that in several taxa speciation events in Cape Range, northwestern Australia, occurred in the time interval three to seven mya associated with increasing aridity. Modes of speciation of subterranean animals have previously been investigated using molecular clock methods by applying allozyme data, genetic distance methods, and Nei's (1975) codon substitution rate for allozymes. Sbordoni et al. (1980) showed that the stygobitic isopods *Monolistra* diverged from a marine ancestor in the Late Miocene due to the Mediterranean salinity crisis. Culver and Wilkins (2001) discussed that several lineages of the amphipod *Gammarus minor* independently colonized different cave basins about one mya. Sbordoni et al. (1990) showed using newt and beetle groups from the Pyrenees, Sardinia, and Corsica, which include cave species, that estimates of divergence between the geographic lineages is in good agreement with the geological dating of the vicariance events resulting from tectonic movements of microplates. More recently, Caccone et al. (1997) and Caccone and Sbordoni (2001) used the newt and beetle studies to estimate evolutionary rates for some mtDNA genes and to investigate whether these rates are constant, but they did not attempt to estimate the age of the cave-adapted species.

In conclusion, our phylogenetic analyses using estimated divergence times based on molecular clock methods linked to species distributions provide strong evidence that aridification led to multiple independent origins of subterranean diving beetle species in isolated calcretes of the Yilgarn region of Western Australia. Together with a lack of evidence for the adaptive shift hypothesis, this strongly supports the climatic shift hypothesis and that climate change, in this case aridification, was the main driving force of the evolution of the subterranean waterbeetles.

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Literature Cited

Banarescu, P. 1975. Principles and problems of zoogeography. Nolit, Belgrade.

Barr, T. J. 1968. Cave ecology and the evolution of troglobites. Pp. 35–102 *in* T. H. Dobzhansky, M. K. Hecht, and W. C. Steere, eds. Evolutionary biology. Vol. 2. Plenum Press, New York.

Barr, T. C., and J. R. Holsinger. 1985. Speciation in cave faunas. Annu. Rev. Ecol. Syst. 16:313–337.

Bromham, L., and D. Penny. 2003. The modern molecular clock. Nat. Rev. Genet. 4:216–224.

Brower, A. V. Z. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc. Natl. Acad. Sci. USA 91:6491–6495.

Brunke, M., and T. Gonser. 1997. The ecological significance of exchange processes between rivers and groundwater. Freshw. Biol. 37:1–33.

Buckley, T. R., C. Simon, P. K. Flook, and B. Misof. 2000. Secondary structure and conserved motifs of the frequently sequenced domains IV and V of the insect mitochondrial large subunit rDNA gene. Ins. Mol. Biol. 9:565–580.

Caccone, A., and V. Sbordoni. 2001. Molecular biogeography of cave life: a study using mitochondrial DNA from Bathysciine beetles. Evolution 55:122–130.

Caccone, A., M. C. Milinkovitch, V. Sbordoni, and J. R. Powell. 1997. Mitochondrial DNA rates and biogeography in European newts (genus *Euproctus*). Syst. Biol. 46:126–144.

Clarke, J. D. A. 2001. Chapter 5 Ancient landforms of Kambalda and their significance to human activity. Pp. 49–57 *in* V. A. Gostin, ed. Gondwana to greenhouse: Australian environmental geoscience. Geological Society of Australia Special Publ. 21.

Cooper, S. J. B., S. Hinze, R. Leys, C. H. S. Watts, and W. F. Humphreys. 2002. Islands under the desert: molecular systematics and evolutionary origins of stygobitic water beetles (Co-

leoptera: Dytiscidae) from central Western Australia. Invertebr. Syst. 16:589–598.
Culver, D. C., and H. Wilkens. 2001. Critical review of the relevant theories of the evolution of subterranean animals. Pp. 381–398 *in* H. Wilkens, D. C. Culver, and W. F. Humphreys, eds. Ecosystems of the world. Vol. 30, Subterranean ecosystems. Elsevier, Amsterdam.
Culver, D. C., T. C. Kane, and D. W. Fong. 1995. Adaptation and natural selection in caves: the evolution of *Gammarus minus*. Harvard Univ. Press, Cambridge, MA.
Cutler, D. J. 2000. Estimating divergence times in the presence of an overdispersed molecular clock. Mol. Biol. Evol. 17: 1647–1660.
DeSalle, R., T. Freedman, E. M. Prager, and A. C. Wilson. 1987. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J. Mol. Evol. 26:157–164.
Desutter-Grandcolas, L., and P. Grandcolas. 1996. The evolution toward troglobitic life: a phylogenetic reappraisal of climatic relict and local habitat shift hypotheses. Mem. Biospeol. 23: 57–63.
Farrell, B. D. 2001. Evolutionary assembly of milkweed fauna: cytochrome oxidase I and the age of *Tetraopes* beetles. Mol. Phylogenet. Evol. 18:467–478.
Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.
Fleischer, R. C., C. E. McIntosh, and C. L. Tarr. 1998. Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. Mol. Ecol. 7:533–545.
Holsinger, J. R. 2000. Ecological derivation, colonization, and speciation. Pp. 399–415 *in* H. Wilkens, D. C. Culver, and W. F. Humphreys, eds. Ecosystems of the world. Vol. 30, Subterranean ecosystems. Elsevier, Amsterdam.
Huelsenbeck, J. P., B. Larget, and D. Swofford. 2000a. A compound Poisson process for relaxing the molecular clock. Genetics 154: 1879–1892.
Huelsenbeck, J. P., B. Rannala, and J. P. Masly. 2000b. Accommodating phylogenetic uncertainty in evolutionary studies. Science 288:2349–2350.
Humphreys, W. F. 1993a. Cave fauna in semi-arid tropical Western Australia: a diverse relict wet-forest litter fauna. Mem. Biospeol. 20:105–110.
———. 1993b. The significance of the subterranean fauna in biogeographical reconstruction: examples from Cape Range peninsula, Western Australia. Rec. West. Aust. Mus. (Suppl.) 45: 165–192.
———. 1999. Relict stygofaunas living in sea salt, karst and calcrete habitats in arid northwestern Australia contain many ancient lineages. Pp. 219–227 *in* W. Ponder and D. Lunney, eds. The other 99%: the conservation and biodiversity of invertebrates. Transactions of the Royal Zoology Society of New South Wales, Mosman 2088.
———. 2000. Relict faunas and their derivation. Pp. 417–432 *in* H. Wilkens, D. C. Culver, and W. F. Humphreys, eds. Ecosystems of the world. Vol. 30, Subterranean ecosystems. Elsevier, Amsterdam.
Hüppop, K. 2000. How do cave animals cope with the food scarcity in caves? Pp. 159–188 *in* H. Wilkens, D. C. Culver, and W. F. Humphreys, eds. Ecosystems of the world. Vol. 30, Subterranean ecosystems. Elsevier, Amsterdam.
Kishino, H., J. L. Thorne, and W. J. Bruno. 2001. Performance of a divergence time estimation method under a probabilistic model of rate evolution. Mol. Biol. Evol. 18:352–361.
Knowles, L. L. 2000. Test of Pleistocene speciation in montane grasshoppers (genus *Melanoplus*) from the sky islands of western North America. Evolution 54:1337–1348.
Langford, R. P., G. E. Wilford, E. M. Truswell, and A. R. Isern. 1995. Paleogeographic atlas of Australia. Vol. 10, Cainozoic. Australian Geological Survey Organisation, Canberra.
Lunt, D. H., D. X. Zhang, J. M. Szymura, and G. M. Hewitt. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol. Biol. 5: 153–165.
Maddison, W. P., and D. R. Maddison. 1998. MacClade, analysis of phylogeny and character evolution. Ver. 3.08. Sinauer, Sunderland, MA.
Mann, A. W., and R. C. Horwitz. 1979. Groundwater calcrete deposits in Australia: some observations from Western Australia. J. Geol. Soc. Aust. 26:293–303.
Markgraf, V., M. McGlone, and G. Hope. 1995. Neogene paleoenvironmental and paleoclimate changes in southern temperate ecosystems, a southern perspective. Trends Ecol. Evol. 10(4): 143–147.
Martin, A. P., and S. R. Palumbi. 1993. Body size, metabolic rate, generation time and the molecular clock. Proc. Natl. Acad. Sci. USA 90:4087–4091.
Martin, H. A. 1998. Tertiary climatic evolution and the development of aridity in Australia. Proc. Linn. Soc. NSW 119:115–136.
Martin, H. A., and A. McMinn. 1994. Late Caenozoic vegetation history of northwestern Australia from the palynology of a deep sea core (ODP Site 765). Aust. J. Bot. 42:95–102.
Masta, S. E. 2000. Mitochondrial sequence evolution in spiders: intraspecific variation in tRNAs lacking the TΨC arm. Mol. Biol. Evol. 17:1091–1100.
Miller, K. B. 2001. On the phylogeny of the Dytiscidae (Insecta: Coleoptera) with emphasis on the morphology of the female reproductive system. Entomol. Scand. 32:45–92.
Morgan, K. H. 1993. Development, sedimentation and economic potential of paleoriver systems of the Yilgarn Craton of Western Australia. Sediment. Geol. 85:637–656.
Muse, S. V., and B. S. Weir. 1992. Testing equality of evolutionary rates. Genetics 132:269–276.
Nei, M. 1975. Molecular population genetics and evolution. North-Holland, Amsterdam.
Peck, S. B., and T. L. Finston. 1993. Galapagos island troglobites: the questions of tropical troglobites, parapatric distributions with eyed-sister-species, and their origin by parapatric speciation. Mem. Biospeol. 20:19–37.
Ribera, I., J. E. Hogan, and A. P. Vogler. 2002. Phylogeny of *Hydraphagan* water beetles inferred from 18S rRNA sequences. Mol. Phylogenet. Evol. 23:43–62.
Rivera, M. A. J., F. G. Howarth, S. Taiti, and G. K. Roderick. 2002. Evolution in Hawaiian cave-adapted isopods (Oniscidea: Philosciidae): vicariant speciation or adaptive shifts? Mol. Phylogenet. Evol. 25:1–9.
Rodríguez, F., J. F. Oliver, A. Marín, and J. R. Medina. 1990. The general stochastic model of nucleotide substitutions. J. Theor. Biol. 142:485–501.
Rokas, A., and G. McVean. 2000. A Bayesian guide to tree felling. Trends Ecol. Evol. 15:396.
Rouch, R., and D. L. Danielopol. 1987. L'origine de la faune aquatique souterraine, entre le paradigme du refuge et le modèle de la colonisation active. Stygologia 3:345–372.
Russo, C., N. Takezaki, and M. Nei. 1995. Molecular phylogeny and divergence times of drosophilid species. Mol. Biol. Evol. 8:49–70.
Sanderson, M. J. 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol. Biol. Evol. 14:1218–1231.
———. 2002a. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol. Biol. Evol. 19:101–109.
———. 2002b. R8s, version 1.05 (beta) user's manual. Univ. of California, Davis, CA.
Sbordoni, V. 1982. Advances in speciation of cave animals. Pp. 219–240 *in* C. Barigozzi, ed. Mechanisms of speciation. A R Liss, New York.
Sbordoni, V., A. Caccone, E. De Matthaeis, and C. Sbordoni. 1980. Biochemical divergence between cavernicolous and marine Sphaeromidae and the Mediterranean salinity crisis. Experientia 36:48–49.
Sbordoni, V., A. Caccone, G. Allegrucci, and D. Cesaroni. 1990. Molecular island biogeography. *In* Biogeographical aspects of insularity. Atti Conv. Lincei 85:55–83.
Sbordoni, V., G. Allegrucci, and D. Cesaroni. 2001. Population genetic structure, speciation and evolutionary rates in cave-

dwelling organisms. Pp. 453–477 *in* H. Wilkens, D. Culver, and W. F. Humphreys, eds. Ecosystems of the world. Vol. 30, Subterranean ecosystems. Elsevier, Amsterdam.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651–701.

Simon, D. L., and B. Larget. 2000. Bayesian analysis in molecular biology and evolution. (BAMBE). Vers. 2.06 beta. Dept. of Mathematics and Computer Science, Duquesne University, Pittsburgh, PA.

Sorhannus, U., and C. Van Bell. 1999. Testing for equality of molecular evolutionary rates: a comparison between a relative rate test and a likelihood rate test. Mol. Biol. Evol. 16:849–855.

Stein, R., and C. Robert. 1986. Siliclastic sediments at sites 588, 590 and 591: Neogene and Paleogene evolution in the southwest Pacific and Australian climate. Pp. 1437–1445 *in* Initial reports of the Deep Sea Drilling Project 90. U.S. Government Printing Office, Washington, D.C.

Swofford, D. L. 2001. PAUP*: phylogenetic analysis using parsimony (* and other methods). Version 4.0b8. Sinauer, Sunderland, MA.

Thorne, J. F., H. Kishino, and I. S. Painter. 1998. Estimating the rate of evolution of the rate of molecular evolution. Mol. Biol. Evol. 15:1647–1657.

Trewick, S. A., and G. P. Wallis. 2001. Bridging the 'Beech-gap': New Zealand invertebrate phylogeography implicates Pleistocene glaciation and Pliocene isolation. Evolution 55:2170–2180.

van der Graaff, W. J. E., R. W. A. Crowe, J. A. Bunting, and M. J. Jackson. 1977. Relict Early Cainozoic drainages in arid Western Australia. Z. Geomorphol. Neue Folge 21:379–400.

Watts, C. H. S. 1985. A faunal assessment of the Australian Hydradephaga. Proc. Acad. Nat. Sci. Philadelphia 137:22–28.

Watts, C. H. S., and W. F. Humphreys. 1999. Three new genera and five new species of Dytisicdae (Coleoptera) from underground waters in Australia. Rec. S. Aust. Mus. 32:121–142.

———. 2000. Six new species of *Nirridessus* Watts and Humphreys and *Tjirtudessus* Watts and Humphreys (Dytiscidae: Coleoptera) from underground waters in Australia. Rec. S. Aust. Mus. 33: 127–144.

———. 2001. A new genus and six new species of Dytiscidae (Coleoptera) from underground waters in the Yilgarn paleodrainage system of Western Australia. Rec. S. Aust. Mus. 34: 99–114.

———. 2003. Twenty-five new Dytiscidae (Coleoptera) of the genera *Tjirtudessus* Watts & Humphreys, *Nirripirti* Watts & Humphreys and *Bidessodes* Regimbart, from underground waters in Australia. Rec. S. Aust. Mus. 36:135–187.

Wu, C. I., and W. H. Li. 1985. Evidence for higher rates of nucleotide substitution in rodents than in man. Proc. Natl. Acad. Sci. USA 82:1741–1745.

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Appendix

Epigean species and subterranean species used in the phylogenetic analysis. Information about specimens can be obtained from the Australian Biological Tissue Collection (ABTC) using the given voucher numbers. Acronyms for tribe names are B, Bidessini; C, Copelatini; H, Hydroporini. (A) *n* refers to the number of specimens sequenced. All epigean species are from Australia with the exception of *Liodessus* sp. PNG green and *Liodessus* sp. PNG red, which are from West Papua, Indonesia. (B) Subterranean species are listed with respect to locality. Paleodrainage valleys are in bold with the position relative to the drainage divide in parentheses. Calcrete aquifers with latitude and longitude information (in decimal degrees) of the sample localities are numbered. Sympatric sister species are indicated with vertical lines. GenBank accession numbers in bold refer to new sequences.

(A)

Species	Tribe	Location	ABTC	GenBank accession *CO1*	GenBank accession *16S-tRNA-ND*
Allodessus bistrigatus (Clark)	B	Forreston, SA	70192–70194	AF484126	AF485931
Bidessodes biliti Watts	B	Orbost, Vic	9601	AF484127	AF485932
Bidessodes mjoberg (Zimmerman)	B	Petford, Vic	9411	AF484128	AF485933
Boongurrus rivulus Larson	B	Herberton, Qld	9451	AF484129	AF485934
Boongurrus occidentalis Watts	B	Wittenoom, WA	75354, 75355	AF484150	AF485955
Clypeodytes migrator (Sharp)	B	Townsville, Qld	9460	AF484130	AF485935
Copelatus melanarius Sharp	C	Bendolba, NSW	9337	**AY350898**	**AY353845**
Gibbidessus chipi Watts	B	Casterton, Vic	75358	AF484132	AF485937
Hydroglyphus balkei Hendrick	B	Petford, Qld	9413	AF484133	AF485938
Hydroglyphus deameli (Sharp)	B	Townsville, Qld	9298	AF484134	AF485939
Limbodessus compactus (Clark)	B	Maryborough, Vic	75359–75361	AF484155	AF485960
Liodessus amablis (Clark)	B	Yunti, SA	9230	AF484136	AF485941
Liodessus dispar (Sharp)	B	Pinjarrah, WA	9576	AF484137	AF485942
Liodessus inornatus (Sharp)	B	Pemberton, WA	9543, 9564	AF484138	AF485943
Liodessus gemellus (Clark)	B	Moro Gorge, SA	78655	**AY350901**	**AY353848**
Liodessus praelargus (Lea)	B	Nangwarry, SA	9705	AF484139	AF485944
Liodessus shuckhardi (Clark)	B	Maryborough, Vic	75362	AF484156	AF485961
Liodessus sp. PNG green	B	Irian Jaya, Indonesia	75365, 75366	AF484140	AF485945
Liodessus sp. PNG red	B	Irian Jaya, Indonesia	75363, 75364	AF484141	AF485946
Necterosoma dispar (Germar)	H	Forreston, SA	70201, 70202	AF484144	AF485949
Paroster gibbi Watts	H	Casterton, ViC	78588, 78589	**AY350895**	**AY353842**
Paroster insculptilis (Clark)	H	Forreston, SA	78586, 78567	**AY350876**	**AY353823**
Paroster nigroadumbratus (Clark)	H	Forreston, SA	78584, 78585	**AY350877**	**AY353824**
Uvarus pictipes (Lea)	B	Byenup Lagoon, WA	75386	AF484154	AF485959

(B)

	Tribe	*n*	ABTC	GenBank accession *CO1*	GenBank accession *16S-tRNA-ND1*
A. Gascoyne drainage (west)					
1. Three Rivers (S25.28313 E119.17573)					
Nirripirti plutonicensis	H	16	78578, 78580, 78632–78647	**AY350892**	**AY353839**
Bidessodes limestonensis	B	1	78581	**AY350880**	**AY353827**
Bidessodes gutteridgei	B	2	78577, 78579	**AY350894**	**AY353841**
2. Milgun (S25.12276 E118.09555)					
Nirripirti hamoni	B	1	78583	**AY350878**	**AY353825**
Nirripirti milgunensis	B	1	78582	**AY350879**	**AY353826**
B. Murchison drainage (west)					
3. Windimurra (S28.28614 E118.57430)					
Nirridessus sp. 1	B	1	78555	**AY350889**	**AY353836**
4. Challa (S27.98842 E118.51756)					
Nirridessus challaensis	B	1	75367	AF484142	AF485947
Nirridessus sp. 2	B	3	78556–78558	**AY350903**	**AY353850**
5. Austin Downs (S27.41337 E117.71122)			78560, 78561, 78593, 78684,		
Nirridessus bigbellensis	B	5	78698	**AY350902**	**AY353849**
Nirridessus cf. *cueensis*	B	10	78559, 78562, 78614–78621	**AY350888**	**AY353835**
6. Nannine (Cue) (S27.26968 E117.98967)					
Nirridessus cueensis	B	6	75368, 75369, 78622–78625	AF484143	AF485948
Tjirtudessus magnificus	B	2	75383, 75384	AF484149	AF485954
7. Karalundi (S26.07950 E118.41220)					
Nirridessus karalundiensis	B	2	78549, 78550	**AY350891**	**AY353838**
C. Carey drainage (east)					
8. Windarra (S28.47824 E122.13719)					
Nirridessus windarraensis	B	2	75378, 75379	AF484148	AF485953

APPENDIX. Continued.

(B)

	Tribe	*n*	ABTC	GenBank accession *CO1*	GenBank accession *16S-tRNA-ND1*
9. Paroo (S26.43389 E119.77722)					
Tjirtudessus eberhardi	B	2	75381, 75382	AF484152	AF485957
Nirridessus pulpa	B	2	75376, 75377	AF484151	AF485956
10. Uramurdah Lake (S26.87524 E120.20150)					
Nirridessus hinkleri	H	3	75371–75373	AF484146	AF485951
D. Carnegie drainage (east)					
11. Jundee (S26.28265 E120.67652)					
Nirridessus jundeeensis	B	2	78563, 78564	**AY350887**	**AY353834**
E. Nabberu drainage (east)					
12. Cunyu, Mineral Bore (S25.78.73 E120.10749)					
Nirridessus bialveus	B	2	78573, 78574	**AY350904**	**AY353851**
Nirridessus macrotarsus	B	3	78575, 78576, 78605	**AY350881**	**AY353828**
13. Cunyu, Sweetwater Well (S25.59375 E120.37241)					
Nirridessus silus	B	2	78568, 78569	**AY350883**	**AY353830**
Nirridessus sweetwatersensis	B	2	78570, 78571	**AY350882**	**AY353829**
Nirridessus cunyuensis	B	1	78572	**AY350893**	**AY353840**
F. Raeside drainage (east)					
14. Yuinmery (S28.54862 E119.09113)					
Nirridessus yuinmeryensis	B	2	78552, 78553	**AY350890**	**AY353837**
Nirripirti cf. *hinzeae*	H	1	78551	**AY350885**	**AY353832**
15. Pinnacles (S28.25744 E120.12690)					
Nirridessus pinnaclesensis	B	2	78612, 78613	**AY350899**	**AY353846**
Nirripirti sp. 8	H	1	78554	**AY350884**	**AY353831**
Nirripirti fortisspinna	H	2	78610, 78611	**AY350900**	**AY353847**
16. Depot Springs (S28.06007 E120.06744)					
Nirridessus fridaywellensis	B	1	75370	AF484145	AF485950
Nirripirti hinzeae	H	1	75380	AF484135	AF485940
17. Lake Mason (S27.5400 E119.62428)					
Nirridessus masonensis	B	2	75374, 75375	AF484147	AF485952
Tjirtudessus raesidensis	B	1	75385	AF484153	AF485958
G. NT drainage					
18. Napperby NT (S22.90891 E132.72908)					
Nirripirti macrocephalus	H	1	78566	**AY350886**	**AY353833**
Nirripirti napperbyensis	H	1	78592	**AY350896**	**AY353843**
19. Newhaven NT (S22.71519 E131.16636)					
Nirripirti newhavenensis	H	2	78590, 78591	**AY350897**	**AY353844**