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FIRST MOLECULAR DATA ON THE WESTERN AUSTRALIAN *DIACYCLOPS* (COPEPODA, CYCLOPOIDA) CONFIRM MORPHO-SPECIES BUT QUESTION SIZE DIFFERENTIATION AND MONOPHYLY OF THE *ALTICOLA*-GROUP

BY

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ABSTRACT

Size differentiation has been considered an important phenomenon in evolution, and in situ speciation was hypothesized in the past for the parapatric subterranean Western Australian *Diacyclops* Kiefer, 1927 species from the *alticola*-group, based on morphological evidence. Aims of this study are to: derive their preliminary molecular phylogenies based on mitochondrial (12S) and nuclear (18S) genes; test if morpho-species are supported by molecular data; examine monophyly of the *alticola*-group; and test whether the size differences evolved in situ after colonization by a single ancestral species or resulted from different phylogeny. Analyses of the 12S sequences reveal at least six well defined clades, each corresponding to one morpho-species. The divergences are very high between all species, suggesting only a remote relationship, with those between sympatric species with significant size difference being in excess of 27%. Surprisingly, all analyses show very high bootstrap values for the clade formed by two cosmopolitan surface-water species, *Diacyclops bisetosus* (Rehberg, 1880) and *D. bicuspidatus* (Claus, 1857), despite numerous morphological differences. The 18S dataset also supports only a remote relationship between *Diacyclops scanloni* Karanovic, 2006 and two other Western Australian members of the *alticola*-group: *D. humphreysi* s. str. Pesce & De Laurentiis, 1996 and *D. sobeprolatus* Karanovic, 2006. Preliminary analyses suggest absence of in situ speciation and parallel evolution in the Western Australian *Diacyclops*, interspecific size differentiation being a result of different phylogeny. The *alticola*-group may be polyphyletic, and we recognize morphological characters that define two main lineages. A possibility of cryptic speciation in the cosmopolitan *D. bisetosus* is also suggested, and several sequences of *Diacyclops* available from GenBank are recognized either as contamination or misidentification.

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RÉSUMÉ

La différenciation par la taille a été considérée comme un phénomène important dans l'évolution, et l'hypothèse de la spéciation in situ a été proposée dans le passé pour les espèces souterraines parapatriques du genre *Diacyclops* Kiefer, 1927 appartenant au groupe *alticola*, à partir des données morphologiques. Les objectifs de cette étude sont : élaborer leur phylogénie moléculaire préliminaire à partir de gènes mitochondriaux (12S) et nucléaires (18S) ; tester si les espèces morphologiques sont soutenues par les données moléculaires ; examiner la monophylie du groupe alticola ; et enfin tester si les différences de taille ont évolué in situ après colonisation par une seule espèce ancestrale ou si elles résultent d'une phylogénie différente. Les analyses des séquences de 12S révèlent au moins six clades bien définis, correspondant chacun à une espèce morphologique. Les divergences sont très élevées entre toutes les espèces, suggérant seulement une relation très éloignée, avec celles parmi les espèces sympatriques ayant une différence de taille significative de plus de 27%. De façon surprenante, toutes les analyses montrent des valeurs de bootstrap très élevées pour le clade formé par deux espèces cosmopolites d'eaux de surface, *Diacyclops bisetosus* (Rehberg, 1880) et *D. bicuspidatus* (Claus, 1857), malgré de nombreuses différences morphologiques. De même, les données du 18S soutiennent seulement une relation éloignée entre *Diacyclops scanloni* Karanovic, 2006 et deux autres membres du groupe *alticola* d'Australie Occidentale : *D. humphreysi* s. str. Pesce & De Laurentiis, 1996 et *D. sobeprolatus* Karanovic, 2006. Des analyses préliminaires suggèrent l'absence de spéciation in situ et une évolution parallèle chez les *Diacyclops* d'Australie Occidentale, la différence de taille interspécifique étant le résultat d'une phylogénie différente. Le groupe *alticola* pourrait être polyphylétique, et nous reconnaissons les caractères morphologiques qui définissent deux lignées principales. Une possibilité de spéciation cryptique chez l'espèce cosmopolite *D. bisetosus* est aussi suggérée, et plusieurs séquences de *Diacyclops* disponibles sur GenBank sont reconnues soit comme contamination, soit comme fausse identification.

INTRODUCTION

Subterranean waters of Western Australia are becoming known as a significant hot-spot for faunal diversity on a global scale (Humphreys, 2008; Guzik et al., 2011), with numerous isolated calcrete aquifers that lie along palaeodrainage channels, and range in diameter from tens of kilometres to hundreds of meters (Humphreys, 2001, 2006). Highly porous and carbonate rich calcrete sediments represent an ideal habitat for various groups of stygofauna (aquatic subterranean fauna), including dytiscid beetles (Watts & Humphreys, 2006), amphipods (Finston et al., 2007), isopods (Wilson, 2008), bathynellids (Cho et al., 2006a, b), ostracods (Karanovic, 2007) and copepods (Karanovic, 2004, 2006). The majority of stygobitic species evolved within individual calcretes following independent colonization by epigean ancestors (Cooper et al., 2002, 2007, 2008; Leys et al., 2003; Guzik et al., 2008; Leys & Watts, 2008). The diversity of stygofauna is mostly dependent on the size of the calcrete, and typically includes one to three species from each major group, most of them endemic to that site (Karanovic, 2004, 2006, 2007; Finston et al., 2007; Leys & Watts, 2008). An example of a typical Western Australian calcrete is that at Sturt Meadows, where multiple studies from a very dense grid of bores revealed only two copepod species (Allford et al., 2008; Bradford et al., 2010). Some other recent studies (Karanovic & Cooper, 2011a, b, 2012) have

TABLE I
Body length of six *Diacyclops* Kiefer, 1927 taxa from the Pilbara region

Species	Female length (μm)				Male length (μm)			
	Minimum	Maximum	Average	*n*	Minimum	Maximum	Average	*n*
D. cockingi	409	802	597	30	375	662	514	17
D. einslei	477	604	527	8	446	452	448	3
D. h. humphreysi	360	488	432	18	321	404	372	6
D. h. unispinosus	326	492	418	18	324	377	352	5
D. scanloni	474	712	610	8	448	546	496	7
D. sobeprolatus	423	715	514	12	388	429	403	5

All data from Karanovic (2006); see text for authors of the specific names.

shown that larger calcretes may harbor a much more diverse copepod fauna, with up to four sympatric harpacticoid congeners and up to ten copepod species in a single bore. In these cases, a significant size differentiation among sympatric congeners was observed, which suggested this process to be potentially an important evolutionary force in subterranean habitats.

The only other well documented case of closely related sympatric congeners of copepods with a significant size differentiation was that of the genus *Diacyclops* Kiefer, 1927 in the Pilbara region of Western Australia (Karanovic, 2006), although this was never tested using molecular tools. Body length information was not a very good indicator of their size on its own (table I), because the copepod body has telescopic somites that can be extended or contracted depending on many factors during and after their collection and fixation (Huys & Boxshall, 1991). The difference in size, however, was so significant and devoid of intermediate stages, that one was led to hypothesize their separate specific statuses even during the preliminary identification and sorting under the dissecting microscope, often before even their generic status could be established with any certainty (fig. 1). Apart from their size, most other morphological characters are highly conservative. Six species were recorded so far from the Pilbara region, and one subspecies is endemic to Barrow Island, all of them belonging to the *alticola*-group: *Diacyclops cockingi* Karanovic, 2006; *Diacyclops einslei* De Laurentiis, Pesce & Humphreys, 1999; *Diacyclops humphreysi* s. str. Pesce & De Laurentiis, 1996; *Diacyclops humphreysi unispinosus* Karanovic, 2006; *Diacyclops scanloni* Karanovic, 2006; *Diacyclops sobeprolatus* Karanovic, 2006; and *Diacyclops reidae* De Laurentiis, Pesce & Humphreys, 1999 (see Pesce & De Laurentiis, 1996; De Laurentiis et al., 1999; Karanovic, 2006). Karanovic (2006), however, considered the validity of *D. reidae* problematic, possibly being described after an aberrant specimen of *D. einslei*. Only two other *Diacyclops* species are known from Australia, both very remotely related to the members of the *alticola*-group and to each other, and both known

Fig. 1. Two morphologically very similar and sympatric *Diacyclops* Kiefer, 1927 species from the Pilbara region with a significant size difference (both collected at the FMG tenement Solomons, from bore SM2872, 21 January 2010): A, *D. humphreysi humphreysi* Pesce & De Laurentiis, 1996; B, *D. scanloni* Karanovic, 2006, with somewhat squashed prosome. Scale bar 0.2 mm. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/15685403.

from surface waters in eastern Australia: the cosmopolitan *D. bisetosus* (Rehberg, 1880), and the Tasmanian-Victorian endemic *Diacyclops cryonastes* Morton, 1985 (see Morton, 1985; Dussart & Defaye, 2006). Additionally, the cosmopolitan *Diacyclops bicuspidatus* (Claus, 1857) has been recorded recently in New South Wales (Karanovic, unpublished data), but its presence in Australia (along with that of *D. bisetosus*) could be a result of anthropogenic translocation associated with early shipping activities (Karanovic, 2005; Karanovic & Krajicek, 2012).

It is beyond the scope of this paper to revise the taxonomy of the genus *Diacyclops*, which is the largest Cyclopidae Rafinesque, 1815 genus (Dussart & Defaye, 2006), and recognized to be polyphyletic or at least paraphyletic by many researchers (Monchenko & Von Vaupel Klein, 1999; Monchenko, 2000; Karanovic, 2005). The general agreement among taxonomists seems to be that the

genus would have to be split into several monophyletic lineages, many of which are recognized as species groups today (Reid & Strayer, 1994; Pesce, 1996), but revised together with the closely related genus *Acanthocyclops* Kiefer, 1927. The *alticola*-group was proposed by Karanovic (2006) for six subterranean *Diacyclops* species and one subspecies from Western Australia, in addition to the Indian *D. alticola* Kiefer, 1935 and the Madagascan *D. longifurcus* Shen & Sung, 1963. They all have a 12-segmented female antennula, three-segmented rami of all swimming legs, and the outer apical spine on the fourth leg endopod longer than the inner one.

Recent intensive sampling of two areas in the Pilbara, done as a part of impact assessment and monitoring projects for the mining industry, produced several specimens of four closely related species from the *alticola*-group, which gave us an opportunity to study them using molecular tools. They were found to live in sympatry (Karanovic, 2006), always exhibiting a significant difference in size (fig. 1). Body size determines many aspects of life history, such as energy balance, resource utilization, competition, dispersal or reproduction rates (Kubota & Sota, 1998; Sota et al., 2000; Leyequién, 2006). Differences among similar species whose distributions overlap geographically are normally accentuated in areas where the species live sympatrically, but are minimized or lost in those where their distributions do not overlap (Brown & Wilson, 1956), and character displacement has been considered an important phenomenon in speciation (Mayr, 1963; Nagel & Schluter, 1998; Berner et al., 2009). The process is driven by competition for limited resources (Bolnick & Fitzpatrick, 2007), and in subterranean interstitial environments size differentiation would enable different closely related species to explore and utilize voids of different size, thus avoiding competition (Gibert et al., 1994; Culver & Pipan, 2009). The process often results in parallel speciation (Rundle et al., 2000).

This is a phenomenon well known in Australian calcrete habitats for diving beetles, where the fauna of a single calcrete typically consists of three species of very different sizes, with 13 cases of sympatric sister species pairs being reported in different calcretes (Leys et al., 2003; Leys & Watts, 2008). Even sympatric speciation was considered at one stage as a possible explanation (Cooper et al., 2002, 2008; Leys et al., 2003; Bradford et al., 2010), however, evidence for considerable population structuring within calcretes makes it difficult to rule out parapatric or allopatric modes of speciation (Guzik et al., 2008; Juan et al., 2010). Although theoretical work suggests that speciation can occur despite initially high gene flow, empirical evidence for sympatric (Savolainen et al., 2006; Ryan et al., 2007) or parapatric (Foster et al., 2007; Quesada et al., 2007) speciation remains thin (Berner et al., 2009). In copepods, some recent studies (Karanovic & Cooper, 2012) on the genus *Schizopera* Sars, 1905 in a small subterranean area in the Yilgarn region documented closely related sympatric species with a significant body size difference. At least three different size classes, and with at

least two species in each size class, suggested a possibility of interspecific size differentiation as a main evolutionary mechanism, as well as parallel evolution of similar traits (size in this case). However, molecular phylogenies based on a 623-bp fragment from the mitochondrial COI gene revealed that both explosive radiation and multiple colonisations were responsible for this richness, but no evidence for parallel evolution was found, interspecific size differentiation probably being a result of different phylogeny.

Aims of this study were to: derive molecular phylogenies of Australian *Diacyclops* species based on mitochondrial and nuclear genes; test if morpho-species are supported by molecular data; examine monophyly of the *alticola*-group; and test if the size differentiation is a result of parallel evolution or different phylogeny. To test if the *Diacyclops* morpho-species are a result of in situ speciation (and parallel evolution) or different phylogeny (and thus colonisation history), we examined them for mitochondrial 12S rRNA and nuclear 18S rRNA haplotypes. For phylogeny to have a significant influence, populations of the same ecomorph must be more closely related to each other than to populations of different ecomorphs (Rundle et al., 2000). Investigating these phenomena in different copepod orders (Harpacticoida and Cyclopoida) and in different regions (Yilgarn and Pilbara), and comparing them with studies on diving beetles, may allow us to exclude any phylogenetic or historical environmental influence. This can hopefully lead to more comprehensive conclusions about size differentiation in subterranean habitats, as well as about the origin and evolution of stygofauna in different regions. The genus *Diacyclops*, for example, is completely absent from the Yilgarn region (Karanovic, 2004), while it is a dominant element in the fauna of the neighbouring Pilbara region (Karanovic, 2006).

MATERIAL AND METHODS

Most samples studied here were collected in the Fortescue Metals Group Ltd (FMG) Solomon tenement, Pilbara region of Western Australia, by a private environmental consulting company (Subterranean Ecology), and entrusted to the senior author for morphological identification (table II). Several samples were collected from the BHP Billiton (BHP) OB23 tenement, also in the Pilbara region, and also by Subterranean Ecology. They resulted from various impact assessment and monitoring projects. Specimens were collected from or near proposed or existing mine sites, but due to the sensitivity of such data no further information about mining operations or plans will be given here. Locality data and number of specimens analysed for this study are listed for every species, including precise coordinates (table II). These samples were collected with haul-nets (mesh size 50 or 150 μm) from groundwater bores. Bores are holes mainly made by mining companies or agricultural enterprises for the purpose of water monitoring and

TABLE II

List of material examined with specimen numbers for different sequences; see text for generic names and authors of the specific names

Species	Country	Locality	Coordinates	Date	Collector	12S	18S
D. bicuspidatus	Ukraine	Kiev, Khotov, pond	50.331°N 30.466°E	21 Apr 2010	V. Monchenko	Q15, Q16	–
D. bisetosus	Japan	Shiga, Maibara, rice paddy	35.369°N 136.346°E	07 Oct 2009	T. Karanovic	Q11, Q12, Q14	Q33
D. humphreysi	Australia	WA, FMG, Solomon, bore SM2308	22.123°S 117.747°E	25 Jan 2010	E. Volschek	Q05, Q06	Q29
		WA, FMG, Solomon, bore NILE	22.124°S 117.868°E	24 Jan 2010	E. Volschek	Q01, Q02	Q28, S04
D. scanloni	Australia	WA, FMG, Solomon, bore SM3633	22.122°S 117.872°E	20 Jan 2010	E. Volschek	Q09	Q31
		WA, FMG, Solomon, bore SM2872	22.124°S 117.871°E	21 Jan 2010	E. Volschek	Q07	S05
D. sobeprolatus	Australia	WA, BHP, OB23, bore W262	23.306°S 119.862°E	22 Nov 2009	P. Bell	Q10	Q32
		WA, BHP, OB23, bore W152	23.266°S 119.885°E	22 Nov 2009	P. Bell	Q03, Q04	–
M. albidus	Australia	WA, Perth, Lake Richmond	32.283°S 115.712°E	11 Dec 2009	T. Karanovic	Q17, R38	Q35
E. serrulatus	Germany	Hamburg, pond	53.602°N 9.938°E	09 Apr 2010	T. Karanovic	–	W06
	Poland	Wigry, pond	54.078°N 23.084°E	11 Oct 2010	D. Vondrák	–	X37
	Czech	Tupadly, pond	50.447°N 14.472°E	30 Apr 2010	D. Vondrák	–	W10

abstraction or mineral exploration, usually from 5 to 20 cm in diameter, and lined entirely, or in part, by PVC tubing (the casing). Haul-nets are simple plankton nets of a different size suitable for the bore; collars can range from 20 to 150 mm in diameter and are made of stainless steel. Weighed nets (using simple fishing leads) were lowered down into the bore with a bottle screwed on its distal part and then hauled through the water column, usually six times. Samples were preserved in the field in cold 100% ethanol, kept on ice or in a refrigerator, and sorted in a laboratory. Four species from the *alticola*-group were collected in these two locations: *Diacyclops cockingi*, *Diacyclops humphreysi* s. str., *Diacyclops scanloni* and *Diacyclops sobeprolatus*; the first one only represented with several decomposed specimens that were not suitable for PCR-amplification.

Two other *Diacyclops* species were included in our molecular analysis, both cosmopolitan and surface-water dwellers, and both previously reported from Australia: *Diacyclops bicuspidatus* and *D. bisetosus* (table II). *Macrocyclops albidus* (Jurine, 1820) was intended as an outgroup for our molecular analyses. Specimens of *Eucyclops serrulatus* (Fischer, 1851) were used as an additional outgroup in our 18S analyses. These samples were collected with plankton nets and preserved in 96% or 99.9% ethanol.

All specimens were examined morphologically in propylene glycol ($CH_3CH(OH)CH_2OH$) prior to DNA extraction using a dissecting microscope Leica M205C, and a compound microscope Leica MB2500, equipped with phase-interference kit and N-PLAN objectives (especially using the 63× dry objective). After examination they were returned in 100% ethanol. Morphological terminology follows Karanovic (2008), while biospeleological terminology follows Humphreys (2000).

DNA was extracted from individual whole specimens in 30 μl proteinase K solution, using the protocol of Schwenk et al. (1998). Fragments of two different genes (mitochondrial 12S rRNA (430 bp), and nuclear 18S rRNA (650 bp)) were amplified using a combination of primers given in table III. The 35 μl PCR reaction was done in a Bio-Rad iCycler Thermal Cycler and contained 7 μl of the DNA template, 1× PCR buffer, 0.2 mM deoxynucleotides, 2.5 mM $MgCl_2$, 0.4 μM primers and 0.6 U *Taq* polymerase. The PCR protocol consisted of 4 min initial denaturation at 95°C, followed by 40 cycles consisting of denaturation at 94°C for 45 s, annealing at 48°C (for 18S) or 60°C (for 12S) for 45 s and extension at 72°C for 1.5 min. A final extension at 72°C lasted for 6 min. PCR products were purified and sequenced on ABI automatic capillary sequencer (Macrogene, Seoul, South Korea) using primers marked in table III.

Obtained sequences were checked manually and aligned for each gene separately by the ClustalW algorithm (Thompson et al., 1994) in MEGA version 5 (Tamura et al., 2011). Most variable loop regions in 12S sequences could not be

TABLE III
List of primers

Gene	Primer	Sequence (5′ → 3′)	Reference
12S	L13337-12S*	YCTACTWTGYTACGACTTATCTC	Machida et al. (2004)
12S	H13845-12S	GTGCCAGCAGCTGCGGTTA	Machida et al. (2004)
18S	18s329	TAATGATCCTTCCGCAGGTT′	Spears (1992)
18S	18sI*	AACTCAAAGGAATTGACGG′	Spears (1992)

* Primer used for sequencing reaction.

reliably aligned, and were excluded from further analyses by processing the 12S alignment in Gblocks Server v. 0.91b (Castresana, 2000), using default settings but allowing gaps within blocks. We thus obtained a 403 bp long alignment (96% of the original 419 bp). The 18S dataset could be aligned unambiguously, resulting in a 596-bp-long alignment. Each dataset was analysed in MEGA version 5 (Tamura et al., 2011) with (1) maximum likelihood (ML) analysis using the General Time Reversable model with uniform rates (GTR) and the Close-Neighbour-Interchange (CNI) method, (2) maximum parsimony (MP) analyses using the CNI method on Random Trees and (3) neighbour joining (NJ) analysis using the Kimura 2-parameter (K2P) model, with gaps treated with partial deletion. One thousand bootstrap replicates were performed to obtain a relative measure of node support for the resulting trees. Average pairwise NJ distances for each dataset were also computed in MEGA version 5 using the K2P model. GenBank numbers for specimens listed in table II (in brackets) as follows: JN656684 (Q17), JN656666 (Q35), JX134402 (X37), JX134394 (W06), JX124393 (W10), JX236042 (Q16), JX236043 (Q15), JX236044 (Q14), JX236045 (Q12), JX236046 (Q11), JX236047 (Q10), JX236048 (Q09), JX236049 (Q07), JX236050 (Q06), JX236051 (Q05), JX236052 (Q04), JX236053 (Q03), JX236054 (Q02), JX236055 (Q01), JX236056 (Q33), JX236057 (Q32), JX236057 (Q28), JX236059 (Q29), JX236060 (S04), JX236061 (Q31), JX236062 (S05). BLAST analyses of GenBank were also done using MEGA.

RESULTS

DNA was extracted and 12S and 18S fragments were successfully PCR-amplified from 16 and 11 whole copepod specimens respectively (table II). BLAST analyses of GenBank, and also comparisons with our unpublished sequences of other cyclopoid genera, revealed that the sequences obtained are copepod in origin and not contaminants, and three of the GenBank 18S sequences (HQ008752.1, AY643529.1 and HQ008745.1), from *Diacyclops crassicaudis* (G. O. Sars, 1863), *Acanthocyclops vernalis* (Fischer, 1853) and *A. brevispinosus* (Herrick, 1884), re-

spectively, were included in our analyses (deposited by Grishanin et al., 2005; Wyngaard et al., 2011). A number of other 18S sequences of both identified and unidentified species ascribed to the genus *Diacyclops* are available from GenBank, as unpublished results from the Lake Baikal sequencing project (GU066263, 066268-066272, 066274, 066275, 066277-066281 and 066285-066289), but unsuccessful alignments with our 18S sequences exposed these as probably not copepod in origin. Impossible alignment also suggested that the 18S sequence published for *Diacyclops uruguayensis* (Kiefer, 1935) by Wyngaard et al. (2011) is either a contamination or a misidentification (GenBank accession number HQ008753.1). Our results represent the first 12S sequence data for the genus *Diacyclops*.

The ingroup taxa formed a monophyletic group in all analyses, and the topology of the resulting cladograms did not differ significantly depending on the phylogenetic method used. Relatively high retention and consistency indexes in the MP analyses (for 12S: No. of trees = 2; Ci = 0.777, Ri = 0.863; for 18S: No. of trees = 49; Ci = 0.852; Ri = 0.913) suggested that our data were relatively robust and informative for the analysis, despite short fragments of each gene.

Basic frame of phylogeny based on the 12S sequence dataset (fig. 2) revealed at least six well defined clades, most supported with high bootstrap values, and each corresponding to one previously recognized morpho-species. The average pairwise distances between *Macrocyclops albidus* and any of the *Diacyclops* species were in excess of 35% (table IV), and this result is not surprising, as *Macrocyclops* Claus, 1893 and *Diacyclops* belong to two different subfamilies of the family Cyclopidae Rafinesque, 1915 (see Boxshall & Halsey, 2004; Dussart & Defaye, 2006), and the former shows most morphological character states in their plesiomorphic form in the whole family (Karanovic & Tang, 2009). All morpho-species are also well defined, with the lowest 12S divergences (ranging from 22.6 to 23.5%) being those between the two cosmopolitan species (*Diacyclops bicuspidatus* and *Diacyclops bisetosus*). This is a surprising result considering their numerous morphological differences (they differ much more morphologically than any of the members of the *alticola*-group; see Dussart, 1969; Monchenko, 1974), but the clade was well supported in all our analyses (98% in ML, see fig. 2; 99% in MP and NJ). Surprisingly high divergences between the three Western Australian members of the *alticola*-group indicate that they are not as closely related as previously thought, and as suggested by their conservative morphology (Karanovic, 2006), and the monophyly of the *alticola*-group group was not supported in any of our analyses. Two sympatric species from the Solomon tenement, *Diacyclops humphreysi* and *Diacyclops scanloni* (fig. 1), are only remotely related, with the pairwise distances all being in excess of 27%, with an average value of 28%. Our analyses suggest a sister relationship between *Diacyclops sobeprolatus* and *D. humphreysi*, which are also morphologically most similar species (Karanovic, 2006), but the support for this clade is not very high in our ML analysis (49%,

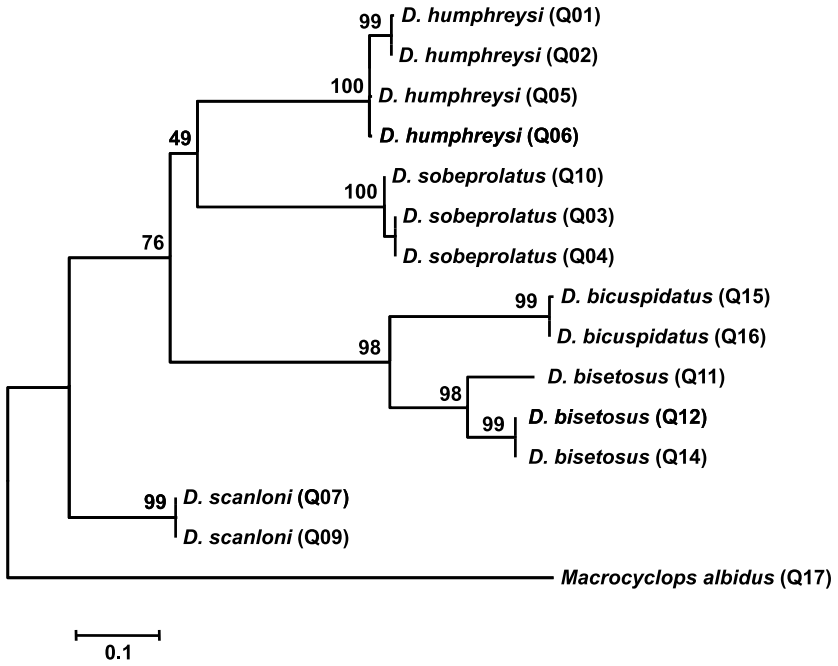

Fig. 2. Maximum likelihood (ML) tree based on 12S data from five *Diacyclops* Kiefer, 1927 species from eight different locations, constructed using MEGA v 5.0.3 and General Time Reversable model with uniform rates (GTR) and Close-Neighbour-Interchange (CNI) method. The outgorup is *Macrocyclops albidus* (Jurine, 1820), from Lake Richmond in Western Australia. The cladogram is drawn to scale, specimen codes in brackets correspond to those in table II, and the numbers above branches represent bootstrap values from 1000 pseudoreplicates.

see fig. 1) and it is only slightly better in our NJ analysis (78%). Despite great morphological similarity (they can be distinguished confidently only by the relative length of the dorsal caudal seta and body size when found together), the pairwise distances between these two species are surprisingly high, being between 27 and 30.4% (table IV), which suggests a long evolutionary history in this group of

TABLE IV

Average pairwise NJ distances (Kimura 2-parameter model) among 12S sequences between six morpho-species of cyclopoid copepods (lower diagonal) and within morpho-species (diagonal)

Species	1	2	3	4	5	6
1 *Diacyclops humphreysi*	0.029					
2 *Diacyclops sobeprolatus*	0.291	0.008				
3 *Diacyclops scanloni*	0.280	0.306	0.000			
4 *Diacyclops bisetosus*	0.343	0.337	0.332	0.071		
5 *Diacyclops bicuspidatus*	0.362	0.365	0.342	0.229	0.005	
6 *Macrocyclops albidus*	0.442	0.430	0.357	0.412	0.443	–

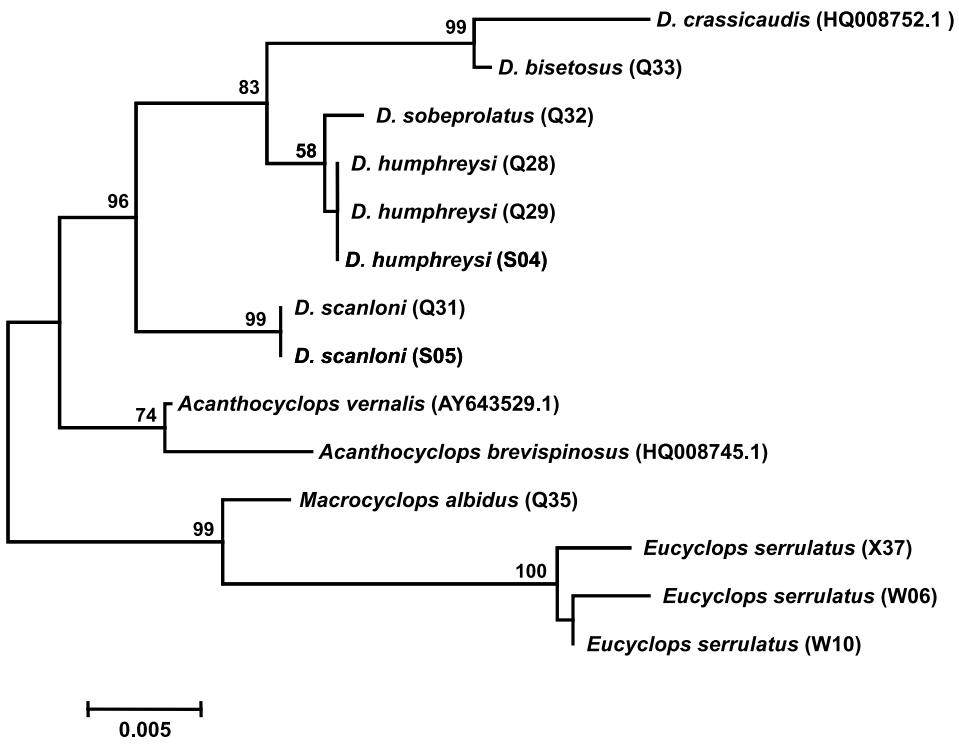

Fig. 3. Maximum likelihood (ML) tree based on 18S data from five *Diacyclops* Kiefer, 1927 and two *Acanthocyclops* Kiefer, 1927 species (from eight and two locations respectively), constructed using MEGA v 5.0.3 and General Time Reversable model with uniform rates (GTR) and Close-Neighbor-Interchange (CNI) method. The outgorups are *Macrocyclops albidus* (Jurine, 1820) from Lake Richmond in Western Australia, and *Eucyclops serrulatus* (Fischer, 1851) from Poland, Germany, and the Czech Republic. Sequences for *Acanthocyclops vernalis* (Fischer, 1853), *A. brevispinosus* (Herrick, 1884), and *Diacyclops crassicaudis* (G. O. Sars, 1863) are from GenBank (accession numbers in brackets). The cladogram is drawn to scale, specimen codes in brackets correspond to those in table II, and the numbers above branches represent bootstrap values from 1000 pseudoreplicates.

subterranean *Diacyclops* species in Western Australia. The clade that suggests a sister relationship of the *bicuspidatus/bisetosus* and *humphreysi/sobeprolatus* clades is moderately supported in the ML analysis (76%, fig. 2).

The highest divergences within morpho-taxa were those between three specimens of *D. bisetosus* (from 0 to 10.6%), which all came from the same rice paddy (table II), and this result indicates a possibility of cryptic speciation in this cosmopolitan species. Four specimens of *D. humphreysi* from two different bores show divergences between 0.2 and 4.4%, and three specimens of *D. sobeprolatus* from two bores differ from 0 to 1.2%. These are all indicative of intraspecific variability (Lefébure et al., 2006; Karanovic & Krajicek, 2012). Most specimens

TABLE V

Average pairwise NJ distances (Kimura 2-parameter model) among 18S sequences between nine morpho-species of cyclopoid copepods (lower diagonal) and within morpho-species (diagonal)

Species	1	2	3	4	5	6	7	8	9
1 *Diacyclops humphreysi*	0.000								
2 *Diacyclops sobeprolatus*	0.002	–							
3 *Diacyclops scanloni*	0.014	0.012	0.000						
4 *Diacyclops bisetosus*	0.012	0.014	0.026	–					
5 *Diacyclops crassicaudis*	0.021	0.022	0.035	0.008	–				
6 *Acanthocyclops vernalis*	0.017	0.019	0.014	0.022	0.031	–			
7 *Acanthocyclops brevispinosus*	0.024	0.026	0.020	0.029	0.038	0.007	–		
8 *Macrocyclops albidus*	0.029	0.031	0.026	0.035	0.044	0.022	0.022	–	
9 *Eucyclops serrulatus*	0.043	0.040	0.042	0.048	0.050	0.038	0.038	0.022	0.016

(excluding those of *D. bisetosus*) that came from the same locality showed zero divergence between their sequences.

Our 18S sequence dataset was somewhat limited (table II) but all morpho-species are well supported clades in this analysis as well, and the ingroup (*Diacyclops* + *Acanthocyclops*) is well defined (fig. 3). Members of the subfamily Eucyclopinae Kiefer, 1927 (*M. albidus* and *E. serrulatus*) form a well supported clade (99% support in ML), which is in contrast to some recent studies involving larger datasets (Wyngaard et al., 2011). Also, the genus *Diacyclops* is well supported, while the monophyly of the *alticola*-group is not. The average divergence rates between taxa (table V) are much smaller than those recoded for 12S, but this was expected, as 18S is a highly conservative gene (Pesole et al., 1999; Audzijonyte et al., 2005; Karanovic & Krajicek, 2012). Also not surprisingly, the 18S sequences show no intraspecific variability even between different sites (specimens of *D. humphreysi* and *D. scanloni* were collected at two different sites each), except in *Eucyclops serrulatus* which is probably a species-complex. The most interesting result of our 18S phylogenetic analyses is a very remote relationship of *D. scanloni* and two other Western Australian members of the *alticola*-group (fig. 3), with the average pairwise distances all in excess of 1.2%. Two widely distributed and surface water species, *D. bisetosus* and *D. crassicaudis*, form a well supported clade (99% in ML). All analyses also suggested a sister relationship between the surface-water *Diacyclops* clade (*bisetosus/crassicaudis*) and the *humphreysi/sobeprolatus* clade, which may indictate that the *alticola*-group is in fact polyphyletic. The 18S cladogram (fig. 3) also supports a sister relationship between *D. sobeprolatus* and *D. humphreysi*, just as the 12S sequence data (fig. 2) and morphological characters (Karanovic, 2006) do, but the support for this clade is again not high in any of our analyses (58% in ML).

DISCUSSION

The key findings of this study of the Australian *Diacyclops* are that morphospecies are well supported with molecular data despite their conservative morphology, the *alticola*-group is most probably polyphyletic, and our preliminary analyses suggest absence of in situ speciation and parallel evolution, with the interspecific size differentiation being a result of different phylogeny instead. A possibility of cryptic speciation in the cosmopolitan *Diacyclops bisetosus* is also suggested, and several 18S sequences of *Diacyclops* available from GenBank are recognized either as contamination or misidentification.

Among seven Australian taxa of the *alticola*-group two lineages were recognized on the basis of the presence/absence of inner seta on the first exopodal segments of all swimming legs (see key to species in Karanovic, 2006: 99), although this character was not considered as phylogenetically informative, and the monophyly of the Australian taxa was advocated. The first group, with the inner seta present, included *Diacyclops einslei*, *Diacyclops reidae* and *Diacyclops scanloni*; the second group included *Diacyclops cockingi*, *Diacyclops humphreysi* s. str., *Diacyclops humphreysi unispinosus* and *Diacyclops sobeprolatus*. Our phylogenetic analyses based on both 12S and 18S sequences suggest that the relationship between *D. scanloni* on one side and the *humphreysi/sobeprolatus* clade on the other is much more remote than what morphological data would suggest. The 18S cladogram (fig. 3) even suggests a sister relationship between the surface-water *bisetosus/crassicaudis* clade and the *humphreysi/sobeprolatus* clade, which would render the *alticola*-group polyphyletic. This sheds a new light on the phylogenetic importance of the inner setae on the first exopodal segments, and forced us to re-examine other morphological characters in the two groups (all published in Karanovic, 2006). The fifth leg looks very different in these two groups, with a much more slender distal segment and longer apical seta in *D. cockingi*, *D. humphreysi* s. str., *D. humphreysi unispinosus* and *D. sobeprolatus*, while the apical seta is much shorter and apical spine more robust in *D. einslei*, *D. reidae* and *D. scanloni* (see figs. 24E, 28F, 31A, 36D, G, 38C, 46E in Karanovic, 2006). The molecular and morphological analyses suggest that these two groups may represent two monophyletic lineages, which originated from different surface-water ancestors. They probably reduced the number of antennular segments through convergent evolution in subterranean habitats, where long antennulae may be a disadvantage for exploring smaller crevices in interstitial spaces. All seven Western Australian endemics have the outer apical spine on the fourth leg endopod longer than the inner one, which is a character they share with the surface-water cosmopolitan *D. bicuspidatus*, but not with *D. bisetosus*. This was the main reason we included both in our molecular analysis (besides both being recorded in Australia previously), as

we expected this character also to be reflected in our cladograms (i.e., we expected *D. bicuspidatus* to be a sister clade of the *alticola*-group, and *D. bisetosus* to be a sister clade of the former). That, however, was not the case, and the 12S cladogram suggests that the two surface-water species are more closely related to each other than to any of the Western Australian congeners (fig. 2). A possible polyphyly of the *alticola*-group in a well defined zoogeographical region shows that molecular characters will have to be considered in any future revision of the genus.

Relatively high divergence rates between three specimens of *D. bisetosus* (in excess of 10%; fig. 2; table IV) are generally indicative of distinct species by comparison with other crustaceans, even for much faster evolving genes like the COI (Lefébure et al., 2006), and are well within accepted values for distinct species in better studied non-related animal groups (Seddon et al., 1998). As all three specimens came from the same rice field (table II), this may suggest a possibility of two cryptic species in this complex. This is not surprising, as Monchenko (2000) found evidence for cryptic speciation in the *D. bicuspidatus* complex using cross-breeding studies, and Karanovic & Krajicek (2012) discovered cryptic speciation in the *Macrocyclops albidus* complex using a combined morphological/molecular approach. These are all cosmopolitan freshwater taxa, with a long and troubled taxonomic history (Dussart & Defaye, 2006), which may owe their very wide distribution to anthropogenic translocation associated with early shipping activities (Karanovic, 2005; Karanovic & Krajicek, 2012), or any subsequent human-mediated passive dispersal mechanism (fisheries, aquaculture, aquaristics, etc.).

Our analyses of both 12S and 18S sequences present preliminary evidence for absence of in situ speciation and parallel evolution in the Western Australian *Diacyclops*, interspecific size differentiation being probably a result of different phylogeny. This is most apparent in the case of two sympatric species in the FMG Solomon tenement, *D. humphreysi* s. str. and *D. scanloni*, which show a remarkable size differentiation (fig. 1). Both 12S and 18S data (figs. 2, 3) show that these two species are only remotely related. This inidicates that their size difference did not originate in response to a recent parapatry, driven by competition for limited resources. However, it should be said that the fact that two taxa come from different ancestors in the phylogeny does not rule out that selection in the aquifer could drive or maintain their size difference. Very high divergence values among the Western Australian *Diacyclops* species (especially for the 12S sequences) suggest that they may be an old component of the stygofauna in this arid Australian state, possibly originating from different surface-water species that lived here during a more humid climate in the Pliocene (Byrne et al., 2008). This is, however, just a speculation, as no molecular clock calibrations were used in our analyses.

The main conclusions of this study are similar to those of Karanovic & Cooper (2012), who examined a possibility of size differentiation in a different group

of copepods, with different genes, and in a different region. They studied an explosive radiation of the harpacticoid genus *Schizopera* in one of the larger calcretes in the Yilgarn region, combining haplotype frequency of the mtCOI gene and comparative morphology of microcharacters. There, up to four, and commonly three, species live sympatrically in the same bore, almost always with a significant difference in size. They described eight new species and subspecies from that small area, and suggested a possibility of another three cryptic species. However, they found no evidence for in situ speciation and parallel evolution with character displacement, the interspecific size difference being a result of different phylogeny in all cases. Reconstructed phylogenies revealed that both explosive radiation and multiple colonisations were responsible for this extraordinary richness, that sister species have parapatric distributions and niche partitioning in the area of overlap but no difference in size, and that *Schizopera* is a recent invasion in these habitats. In situ speciation from the same ancestral source is still to be found in cyclopoid or harpacticoid copepods, as opposed to more than 13 documented cases in dytiscid beetles (Cooper et al., 2002, 2008; Leys et al., 2003; Leys & Watts, 2008; Bradford et al., 2010), which may imply that different evolutionary forces are at work in different stygofauna groups. Karanovic & Cooper (2011b) provided evidence that even two different harpacticoid genera have a different colonisation history in the same palaeochannel in Western Australia, with the members of the genus *Kinnecaris* Jakobi, 1972 colonising the channel downstream and being represented just with allopatric species, and the genus *Schizopera* colonising upstream and with numerous sympatric and parapatric species. These two genera, however, belong to two different families, one of which has most of its diversity in marine environments (Karanovic & Cooper, 2012), while the other is freshwater in origin and probably started colonising subterranean waters in Australia just after the Permo-Carboniferous glaciations (Karanovic, 2004, 2006; Karanovic & Cooper, 2011a, b), which spread throughout much of what subsequently had become the Gondwana supercontinent and covered the entire Australian plate (Frakes, 1999; Playford, 2003). Both molecular and morphology based phylogenetic studies continue to provide new and amazing insights into the evolution of Darwin's wrecks of ancient life (Juan et al., 2010), and we hope they will stimulate other areas of research in subterranean environments, as well as their conservation and responsible management. This paper presents only preliminary results, based on a limited dataset, but they come from some of the remotest corners of our planet, where sampling is further complicated by restricted access due to numerous mining tenements.

Amplification success rates were different for the two chosen genes, those for the 12S being much higher (close to 90%) than those for the 18S (slightly below 50%). This is surprising, given that the 12S is a faster evolving gene.

Low amplification rates may be partly due to a relatively small size of copepod specimens and correspondingly low amount of DNA isolate, but more probably because we are yet to find an optimal procedure and combination of primers for this group and each gene (Karanovic & Cooper, 2011b). We did, however, test most primers available for copepods, and spent a lot of time on the optimization of the PCR protocol (finding the optimal annealing temperature on the temperature gradient). Recently, Karanovic & Krajicek (2012) were able to detect cryptic speciation in a global study of the *Macrocyclops albidus* complex, using 12S in combination with three other genes (16S, 18S, and cytB) and morphological microcharacters. Bláha et al. (2010) detected a possible cryptic species in the *Acanthocyclops vernalis* complex, also using 12S. This gives us confidence in the divergence values interpretation in the genus *Diacyclops*, as all three genera live in similar habitats and belong to the same family and their genes should evolve at similar rates. In the *M. albidus* complex, just as in most other animal groups, of the four genes cytB evolves fastest, followed by 12S, 16S and 18S. Possibilities of cryptic speciation in the cosmopolitan *D. bisetosus* (suggested by our 12S analyses; fig. 2, table IV) and *Eucyclops serrulatus* (suggested by our 18S analyses; fig. 3, table V) are thus worth investigating further with more markers and in combination with a study of morphological microcharacters.

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