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Phylogenetic placement and description of *Ngaliadessus humphreysi* gen. et sp. nov., Watts & Villastrigo (Coleoptera: Dytiscidae), a subterranean diving beetle from the Ngalia Basin in central Australia

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Abstract

The largest diversity in the world of subterranean diving beetles (Dytiscidae) has been discovered in underground waters of the Australian arid zone. The majority of species are from the Dytiscidae genera *Limbodessus* Guignot, 1939 (Bidessini) and *Paroster* Sharp, 1882 (Hydroporini) and are distributed within two major regions: calcrete islands of central Western Australia and the Ngalia Basin of the Northern Territory. Here, we use an integrative approach based on morphological and molecular analyses to describe *Ngaliadessus humphreysi* **gen. et sp. nov.** Watts & Villastrigo representing a new genus and species of stygobiotic Bidessini collected from a single well in the Ngalia Basin. Phylogenetic analyses using whole mitochondrial genome, Histone 3 and 18S rRNA data, representing a comprehensive coverage of Bidessini genera, support the distinction of the genus and species as a separate evolutionary lineage sister to the Australasian genus *Limbodessus* and the widely distributed genus *Allodessus* Guignot, 1953. Our study further confirms that the Ngalia Basin, containing 13 subterranean dytiscid species from four distinct genera, is one of the most speciose areas within the world's most diverse hotspot of subterranean diving beetles.

KEYWORDS

aquifer, Bidessini, calcrete, groundwater, mitochondrial genome, phylogenetic analyses, water beetle

Christopher H. S. Watts and Adrián Villastrigo contributed equally as first authors.

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INTRODUCTION

Australia is home to the most diverse assemblage of stygobiotic (obligate subterranean aquatic) diving beetles in the world (Austin et al. 2023; Balke et al. 2004). At present, there are more than 100 species described, the majority of which belong to the genera *Limbodessus* Guignot, 1939 and *Paroster* Sharp, 1882, with several species also belonging to *Neobidessodes* Hendrich & Balke 2009, and *Exocelina* Broun, 1886 (Balke et al. 2004; Watts & Humphreys 2009 and references therein; Eberhard et al. 2016; Nilsson & Hájek 2023). The stygobiotic Dytiscidae occur in a variety of habitats, including isolated calcrete (carbonate) aquifers of the Yilgarn region of Western Australia (Cooper et al. 2002; Humphreys et al. 2009; Watts & Humphreys 2009 and references therein), alluvial aquifers in eastern (Watts et al. 2007, 2008) and southern Australia (Leys et al. 2010), and calcareous areas of the Ngalia Basin of central Australia (Balke et al. 2004; Watts & Humphreys 2006).

The shallow aquifers of the Ngalia Basin are known to have a rich aquatic subterranean fauna, including Amphipoda, Isopoda, Syncarida, Copepoda, Gastropoda and Coleoptera (Balke et al. 2004; Cho et al. 2006; Ponder et al. 2022; Watts & Humphreys 2006; S. Taiti, pers. comm.). From Coleoptera alone, 11 stygobiotic diving beetle (Dytiscidae) species in three genera, *Exocelina* (subfamily Copelatinae), *Limbodessus* (subfamily Hydroporinae, tribe Bidessini) and *Paroster* (subfamily Hydroporinae, tribe Hydroporini), have been described (Watts & Humphreys 2009, and references therein). However, in September 2015, several adult and larval specimens were collected from Sullivans Well in the Ngalia Basin. Previously no Dytiscidae were known from the well, but three stygobiotic species of *Paroster* are known from the nearby Herbert Well (Watts & Humphreys 2003), possibly in the same aquifer. Initial morphological examination of the Sullivans Well beetles indicated they were neither *Paroster*, nor *Limbodessus*; one species of the latter is known from a more distant aquifer of the basin (Watts & Humphreys 2006). Here, we use an integrated approach based on morphology and DNA sequence analyses to investigate the generic assignment and species status of these specimens. Phylogenetic analyses based on whole mitochondrial genomes and nuclear data from representative Bidessini genera, and both adult and larval morphology provided evidence that our specimens belong to a previously unknown lineage in the tribe Bidessini, which we describe here as a new genus and species.

MATERIALS AND METHODS

Samples

Specimens (four adults and two larvae) were collected in September 2015 by SJBC, WFH and DNS from Sullivans Well in the Ngalia Basin, Northern Territory (22.73614°S, 132.46104°E; Figure 1), using small, weighted, plankton nets. Three of the adults were preserved in RNAlater (sample code BES18753) and one further adult and two larvae were preserved in 100% alcohol (sample code BES18752). Two adult specimens (including the holotype from BES18752) and two larvae were mounted on microscope slides in Euparal and examined using a NIKON 568 microscope equipped with both plain and dark field options, a Nikon DS-V11 camera, and an associated NIS Elements imaging and stacking system. Line drawings were made from montaged images and enhanced in Adobe Photoshop. A colour image of the adult was taken using the same microscope, again enhanced in Adobe Photoshop.

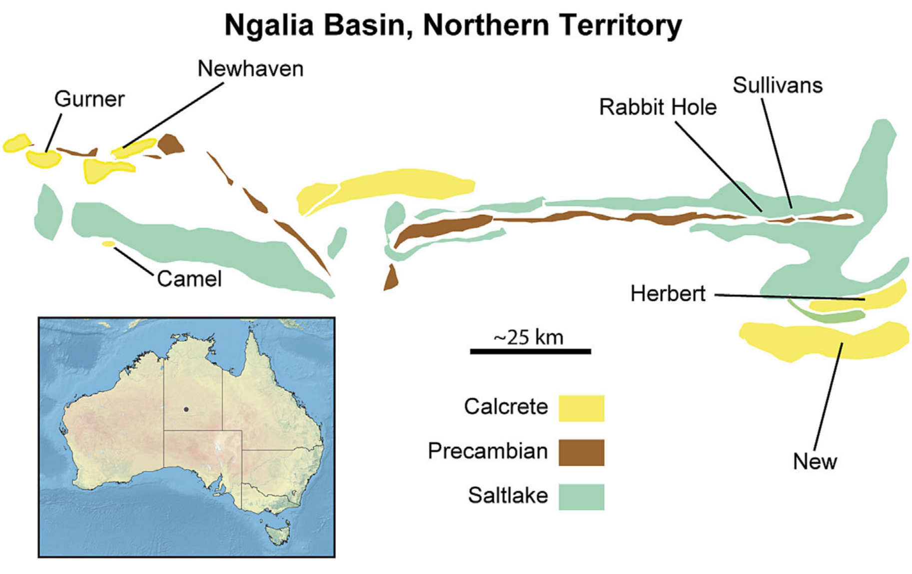

FIGURE 1 A geological representation of the Ngalia Basin with subterranean groundwaters and its main sampling sites for stygobiotic taxa, including Sullivans Well. The geology was determined from Australian Geological Survey 1: 250 000 maps SF52-12 Mt Doreen (1995) and Napperby SF53-09 (1982) and information compiled by Edgoose and Ahmad (2013). Inset shows the approximate location of the Ngalia Basin in central Australia.

Molecular data

We included specimen BES18753.1, one of the adult specimens (see Systematics section), in targeted exon capture analyses that were conducted using methods outlined in Langille et al. (2022). Briefly, these methods involved DNA extraction using the Gentra method (Gentra Systems, Inc.), followed by targeted exon capture using RNA baits designed to capture the exons of phototransduction genes (Langille et al. 2022). Exon capture libraries were sequenced on an Illumina MiSeq platform (Australian Genome Research Facility [AGRF] in Adelaide, Australia). The sequence data generated (sequence code 28095) were found to have mitochondrial DNA (mtDNA) sequence 'bycatch', which was used to assemble its complete mitochondrial genome and retrieve an internal fragment of the small ribosomal subunit (18S) and Histone 3 nuclear markers. The mitochondrial genome was assembled using the iterative DeBruijn graph assembler IDBA-UD (Peng et al. 2012) with the following parameters: mink = 60, maxk = 250 and step = 10. The output contigs were blasted against already published references sequences of other Hydroporinae beetles (Villastrigo et al. 2021) for cytochrome oxidase subunit I (*COI*) and 16S rRNA genes. The mitochondrial genome was annotated using the Mitos 2 Web Server (Donath et al. 2019) and refined with already published reference mitochondrial genomes. We used the same methodology to retrieve molecular data from a representative species of the related genus *Neobidessodes*, *N. mjobergi* (Zimmermann, 1922) and we incorporated low-coverage whole-genome sequencing data from *Allodessus bistrigatus* (Clark, 1862) following the procedure by Villastrigo et al. (2023). All newly generated data were uploaded into the GenBank nucleotide database (Table S1).

Additional sequencing of *COI* was performed to associate adults and larvae. DNA was extracted from the posterior part of the body of a single diving beetle larva from Sullivans Well using the Gentra Puregene DNA Purification method and the head was kept as a voucher. PCR-amplification of the 5′ end of *COI* was carried out using primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). PCRs were carried out in a 25 μl reaction volume consisting of nuclease-free water, 1× Immolase PCR buffer, 1.5 mM MgCl2, 0.8 mM dNTP mix, 0.05 mg/mL BSA, 0.24 μM primer, 0.5 units Immolase DNA polymerase (Bioline; NSW, Australia) and approximately 1 ng of extracted DNA. Cycling conditions were 95°C for 5 min, followed by 38 cycles of 95°C for 45 s/48°C for 45 s/72°C for 60 s, with a final extension at 72°C for 10 min. Product purification and Big Dye Terminator sequencing were conducted by the AGRF. The sequence trace file was edited in GENEIOUS version 10.2.4 (https://www.geneious.com). The *COI* sequence was then matched to that found from the exon capture libraries for the Ngalia Basin diving beetle. The *COI* sequence was submitted to Genbank (accession number OQ470206).

Taxon sampling and phylogenetic analysis

We used a comprehensive coverage of the Australasian Bidessini genera, together with other Bidessini genera, for phylogenetic analyses, compiling data from mitochondrial genomes, 18S rRNA and Histone 3 nuclear genes (Table S1). We followed a model selection approach in IQTree 2.1.3 (Kalyaanamoorthy et al. 2017; Minh et al. 2020). The best partition scheme and evolutionary models from IQTree were used in the Bayesian phylogenetic inference software BEAST v1.10.4 (Table 1; Suchard et al. 2018) using a Yule speciation process as a tree prior for 100 million generations. We ran two analyses under alternative clock hypothesis per partition: (1) strict clock and (2) uncorrelated lognormal relaxed. Both analyses were compared using Path sampling/Stepping-stone sampling scores (Baele & Lemey 2013; Baele et al. 2012). The MCMC trace files generated by BEAST were checked using Tracer v1.7.1 (Rambaut et al. 2018) and a 10% burnin fraction was discarded, prior to calculation of Bayesian posterior probabilities (BPPs).

RESULTS

Phylogenetic analyses

Phylogenetic analysis revealed a general topology for the tribe Bidessini with moderate to high support (BPPs > 0.92) in most of the branches (Figure 2). Uncertain relationships among genera were detected in early diverging branches, although the new taxon was detected in a strongly supported clade (BPP = 0.98) together with the genera *Allodessus*, *Gibbidessus* Watts, 1978, *Kakadudessus* Hendrich & Balke 2009, *Limbodessus*, *Neobidessodes* and *Uvarus* Guignot, 1939. Relationships within this clade were mostly strongly supported (BPP > 0.96), with strong support (BPP = 0.96) for a sister relationship of the new genus to a group comprising *Allodessus* and *Limbodessus* (BPP = 1).

TAXONOMY

Ngaliadessus gen. nov., Watts & Villastrigo

https://zoobank.org/urn:lsid:zoobank.org:act:5EF9D105-F888-4EBE-9317-78FD4F497A71

Type species: *Ngaliadessus humphreysi* sp. nov., Watts & Villastrigo by current designation (monotypy).

TABLE 1 Marginal likelihood estimate for model comparison of BEAST analyses using path sampling and stepping-stone sampling (higher values indicate a better model).

	Path sampling	Stepping-stone sampling
Strict clocks	−70 468.22	−70 468.04
Lognormal relaxed clocks	−70 121.46	−70 121.27

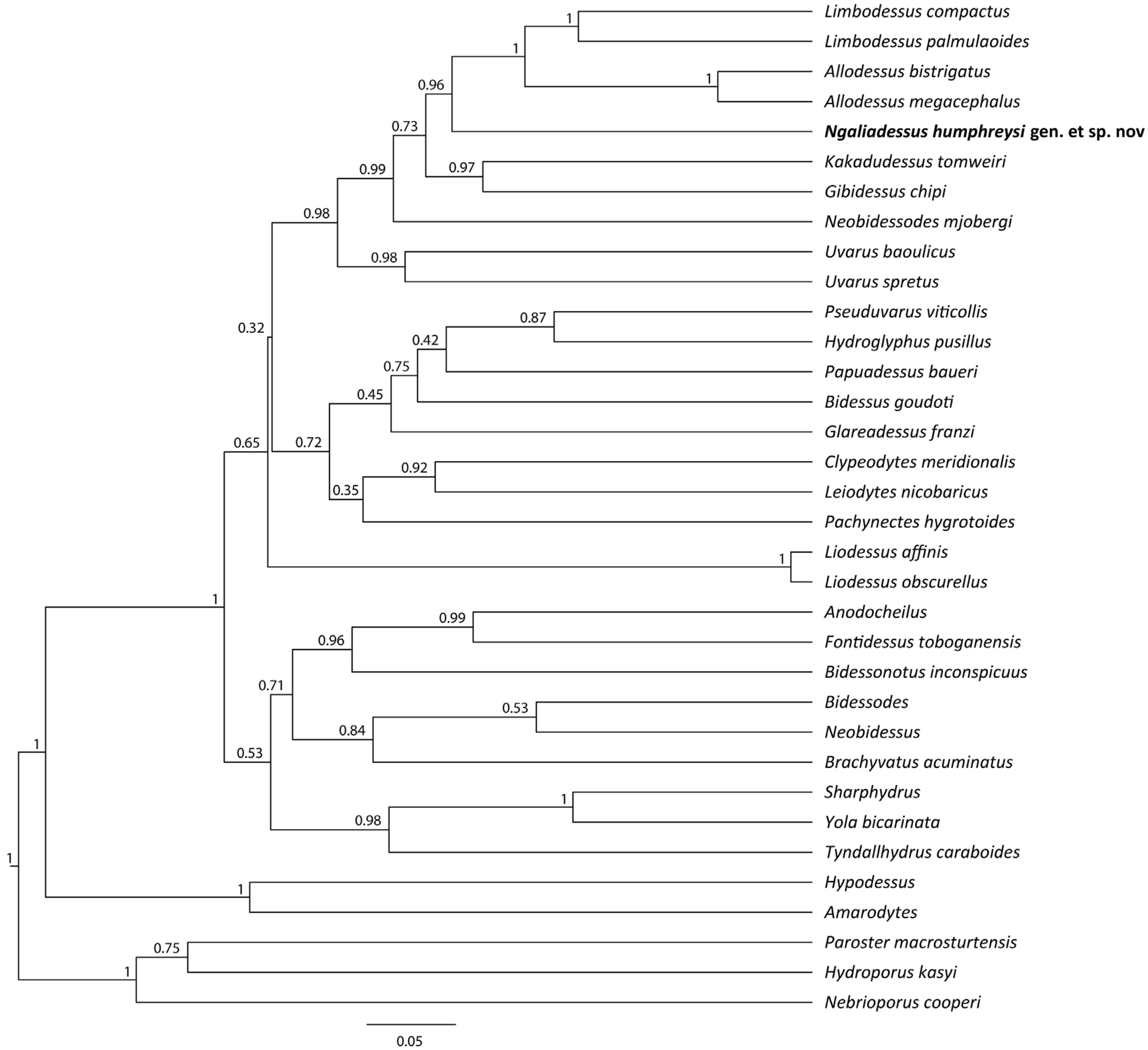

FIGURE 2 BEAST maximum clade credibility tree of Bidessini based on a combination of whole mitochondrial genome sequences, Histone 3 and 18S sequences (Table S1). The new genus/species is shown in bold, and numbers on branches represent Bayesian posterior probabilities.

Description

Small (<3 mm long), integument soft, head without eyes or eye remnants, clypeus not bordered, pronotum without basal stria, elytra without basal stria, without sutural stria, punctures sparce, setae sparce restricted to margins. Prosternal process narrow, reaching mesoventrite, suture between first and second ventrites complete, protarsus 5-segmented, mesofemur without basal spines on posterior margin, metatibia weakly curved. Spermatheca with well-developed triangular spine. Male not known.

Etymology

Named after the Ngalia Basin groundwater system, the type locality for the genus.

Ngaliadessus humphreysi sp. nov., Watts & Villastrigo

(Figures 3-4)

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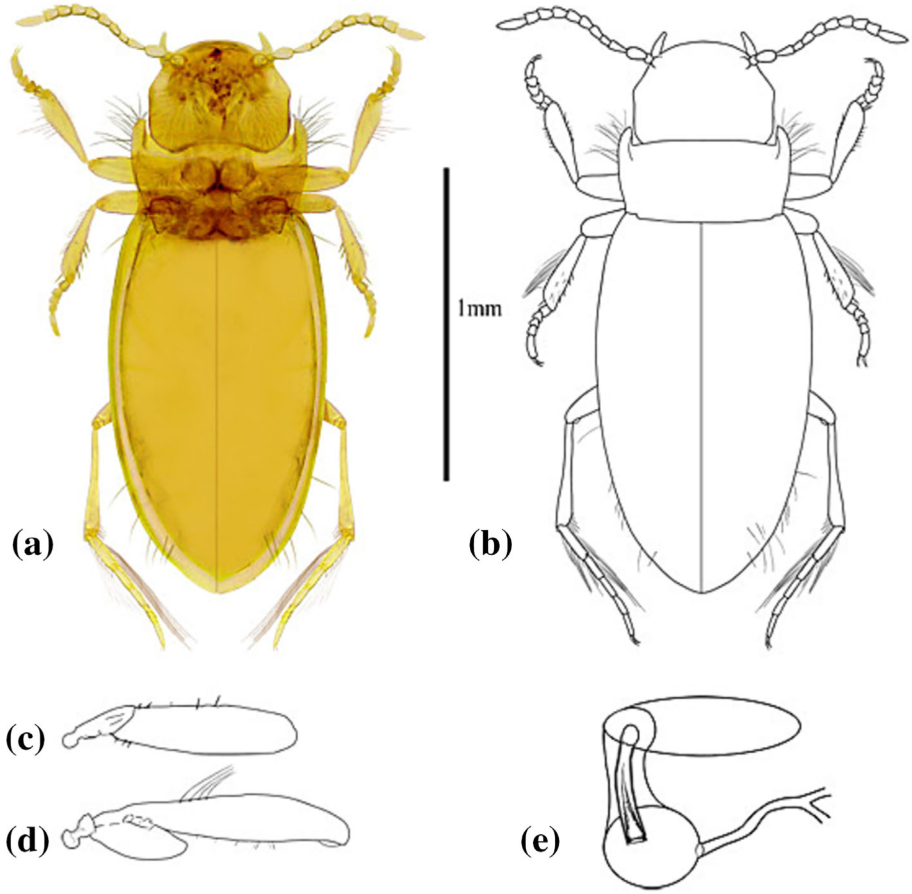

FIGURE 3 Adult *Ngaliadessus humphreysi* sp. nov., Watts & Villastrigo. (a) Dorsal view of adult; (b) line drawing; (c) line drawing of ventral surface of mesotrochanter and mesofemur; (d) line drawing of ventral surface of metatrochanter and metafemur; (e) portion of spermatheca with internal spine.

Type locality: Australia, Northern Territory, Sullivans Well – Napperby Station. (22.73614°S, 132.46104°E).

Material examined

Holotype

Female (Museum and Art Gallery of the Northern Territory, MAGNT I011303), Sullivans Well, Napperby Station 16/9/2015, collected by S. Cooper, W. Humphreys, and D. Stringer.

Paratype

1 female, partial, consisting of head capsule, abdomen and elytra, lacking appendages, mounted on same microscope slide as holotype MAGNT I011303. Two DNA samples (BES18753.1 and BES18753.2), stored at the South Australian Museum, represent female specimens that were morphologically identical to the holotype MAGNT I011303. Specimen BES18753.1 was used for the exon capture analyses to generate mitochondrial genome data (accession numbers are available in Table S1).

Description

The description below is based on female specimens only as no males were available.

Adult female

Size, colour and habitus
Habitus as in Figure 3. Length 2.7 mm; relatively flat, moderately constricted at junction of pronotum and elytra; elongate, elytra almost parallel-sided; uniformly testaceous; hindwings absent.

Head
Narrower than elytra, shiny, smooth, reticulation virtually absent, punctures sparse, very small; widening from apex

to about middle, then roundly angled to almost parallel sided; lacking setae, eye remnant absent other than very small crease. Antenna relatively short, relatively stout antennomeres 3–10 weakly widening towards apex on anterior edge, antennomeres broadening slightly towards apex, of approximately similar lengths except for antennomere 11 which is noticeably longer than antennomere 10. Each antennomere, except antennomer 1 with some very small setae on anterior side apically, antennomeres 5–10 slightly expanded on anterior apical corner. Maxillary palpus elongate, palpomere 4 as long as palpomeres 1–3 combined.

Pronotum

A little narrower than elytra; anterolateral angles projecting strongly forwards, projection about half length of rest of pronotum, posterior angles bluntly rounded, overlaying head somewhat; surface smooth, reticulation obsolete, punctures very weak, sparse, some weak but relatively long setae at sides; without stria.

Elytra

Not fused; sides slightly widening in middle, smooth, very weakly reticulate, very sparsely covered with small punctures; epipleuron weakly differentiated from rest of elytron, thin, weakly expanded close to front of elytra.

Ventral surface

Prosternal process very narrow between procoxae, then rapidly widening before smoothly narrowing to near apex where it sharply narrows to point, reaching mesothorax, sides subparallel, apex rounded. Metathorax relatively narrow, metaventral wings short. Metacoxal plates broad, pear-shaped, large, shiny, virtually nonreticulate. Sutural line between ventrites 1 and 2 complete, ventrites 3–5 mobile, sparsely covered with small punctures.

Legs

Protibia narrowly club-shaped, row of very short setae along inner edge, 5–6 setae along both inner and outer edges and a group of strong spines at apex, protarsus 5-segmented, tarsomeres weakly expanded, apices not strongly bifid, tarsomere 1 about 2× as long as broad, tarsomere 2 as wide as tarsomere 1, about half its length, tarsomere 3 slightly longer than tarsomere 1 deeply bifid, tarsomere 4 about length of tarsomere 3, narrower, moderately curved, claws curved relatively short, tarsomeres with some short setae on inner angles; profemur stout, elongate. Mesotrochanter small, oval; mesofemur relative thin, elongate boat-shaped with some weak spines near base and along basal half of anterior edge; mesotibia shorter than mesofemur, club-shaped with number of stout spines towards apex and along anterior edge; mesotarsi much weaker than protarsi, tarsomere 1 1.5× longer than wide, tarsomeres 2 and 3 similar, about half as long as tarsomere 4, smaller than tarsomere 3, tarsomere 5 elongate, weakly curved almost 3× length of tarsomere 1. Metatrochanter relatively large, elongate oval, apex bluntly pointed; metafemur relatively narrow, broadest in middle, lacking spines, with 4 long setae along dorsal edge; metatibia curved, widening towards apex, with field of long stiff setae close to apex on inner angle otherwise without spines or setae; metatarsus elongate, with swimming hairs, tarsomere 1 longest, tarsomeres 2 and 3 shorter, equal length, tarsomeres 4 and 5 similar length, slightly shorter than tarsomere 3, tarsomeres 1 and 2 in combination slightly shorter than others, claws very weak. Spermatheca with elongate, triangular spine (Figure 3).

Larvae

Two specimens, slide mounted, same data as holotype, one complete other only head capsule. By size and lack of egg busters we judge both to be second instar (SAMA 25-039823; SAMA 25-039824).

Colour and habitus

Body uniformly very pale, unpigmented. Head capsule, abdominal segment 8 and urogomphi as in Figure 4.

Head capsule

Longer than broad, with small lamellae clypeales; without stemmata (Figure 4a–c). Maximum width just posterior to where eyes would be, with slight neck constriction; nasal elongate, rounded apically, sinuate laterally; dorsal surface with some setae on margin near apex; margin of ventral surface from apex to near antennae with band of lamellae clypeales of varying sizes and some patches of small lamellae clypeales in middle near apex, two longer setae near base of antennae. Antenna elongate with four antennomeres, shorter than head; antennomere 1 short, relatively broad; antennomeres 2 and 3 approximately similar in length; antennomere 4 short, elongate, pointed. Mandible broad basically, distal half projecting inwards and upwards, apex sharp, mandibular channel present. Maxillary palpus elongate, relatively thin, composed of three palpomeres; palpi 2 and 3 subequal in length. Epidysal suture weak in specimens. Legs long, thin, tarsi moderately long thin, sparce setae on hind margins, without swimming hairs.

Abdomen

Abdominal segment eight longest, completely sclerotised, setae only on margins, siphon short, subconical with one set of ABC pores (Michat et al. 2012) (only one very small pore was detected). Most of second segment of both urogomphi missing from specimens; first segment moderately long, little less than twice length of abdominal segment 8, with three long setae on shaft and two long setae at end (Figure 4d).

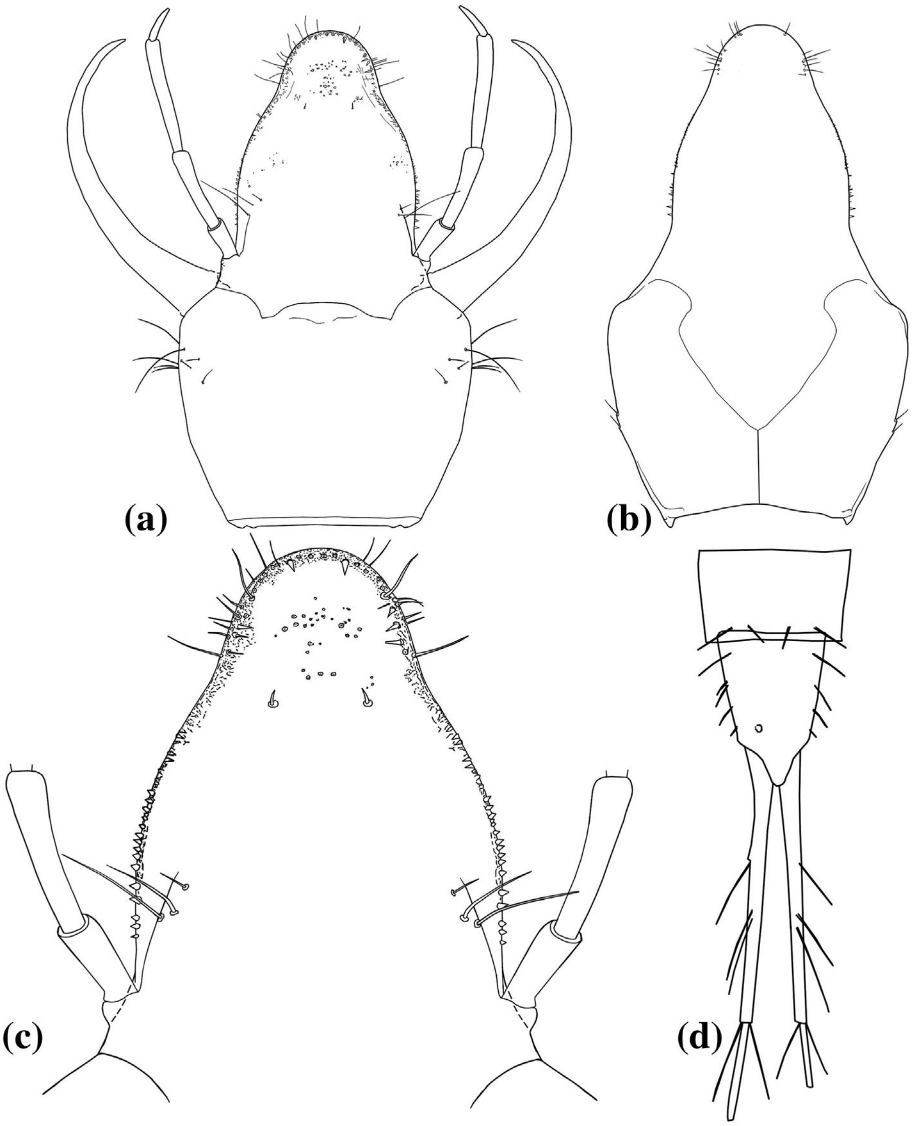

FIGURE 4 Line drawings of larva of *Ngaliadessus humphreysi* sp. nov.: (a) ventral view of head capsule; (b) dorsal surface of head capsule; (c) enlarged view of ventral surface of head capsule; (d) dorsal view of abdominal segment 8 and basal segment of urogomphi; small circle on segment 8 represents only visible pore.

Etymology

Named after William Humphreys in recognition of the outstanding research he conducted in the subterranean biology field for more than three decades, and his numerous field trips to discover, collect and describe, in partnership with Chris Watts and other colleagues, the majority of stygobiotic dytiscid species currently known in Australia. The specific name is a noun in the genitive case.

DISCUSSION

Generic placement

The species belongs in the Dytiscidae subfamily Hydroporinae based both on morphological and molecular data. Within this subfamily – and thus, excluding *Exocelina*, two genera in two tribes, Hydroporini and Bidessini were present in the Ngalia Basin and are known to have stygobiotic species: *Paroster* in the Hydroporini, and *Limbodessus* in the Bidessini. The genus *Neobidessodes* (tribe Bidessini) has stygobiotic species in WA, which we also consider below for the generic placement.

Adults of *N. humphreysi* have curved metatibia which are expanded towards the apex (Figure 3a,b) and have a spine in the spermatheca (Figure 3e), both characters defining the tribe Bidessini (Miller & Bergsten 2016). This would rule the species out of the tribe Hydroporini, and therefore of *Paroster*, the most species rich genus of stygobiotic Dytiscidae in the Ngalia Basin. Within the Bidessini the lack of spines on the basal posterior edge of the mesofemur suggests that it is not a *Limbodessus* as does the lack of pronotal striae although both these character states are weak or absent in some stygobiotic

Limbodessus, particularly small ones. Neither of the two, albeit poorly known, stygobiotic species of *Neobidessodes* from Western Australia appear morphologically close to the new species (Hendrich & Balke 2009; Watts & Humphreys 2003). The placement of these two species, particularly the small *Neobidessodes gutteridgei* (Watts & Humphreys 2003) in *Neobidessodes* depended strongly on sequence data linking them to the relatively abundant terrestrial species of *Neobidessodes* found in the northern parts of the Northern Territory (Hendrich & Balke 2009). Potentially, the species could be linked to one of the epigean Bidessini that occur in Australia: *Allodessus*, *Gibbidessus*, *Kakadudessus* and *Hydroglyphus* Motschulsky, 1853. It differs from all of these genera in a number of adult and larval characters, but most of these are related to morphological convergence due to its subterranean lifestyle (Culver & Pipan 2015). Importantly, phylogenetic analyses indicate that it is located in an isolated branch as a sister lineage to a group comprising *Allodessus* and *Limbodessus* (Figure 2), which suggests the taxon has an independent origin.

The larvae lack lateral projections on the nasal and have only one pair of pores on abdominal segment 8 (pores ABC sensu Michat et al. 2012) which rules out *Paroster* (and the tribe Hydroporini). The larvae resemble those of stygobiotic *Limbodessus* (Michat et al. 2012) and *Neobidessodes* (Michat et al. 2010) but it is "difficult to find characters that unambiguously separate (*Limbodessus)* from all other Bidessini larvae" (Michat et al. 2012). The larvae of the new species are distinctive, particularly in their elongate nasal with only a few setae on the dorsal surface and lamellae clypeales of varying sizes on the edge of the ventral surface as far back as the base of the antennae (Figure 4). The mandibles are also unusually elongate and, possibly as a consequence, thin.

In summary, the species is undoubtedly a member of the tribe Bidessini, but placement in a known genus based on the morphology of either the adult or the larva is not supported. The molecular phylogenetic analysis places the species as a sister, but distantly related, to a group comprising *Limbodessus* and *Allodessus*, and it appears to be distantly related to other Bidessini genera present in the area both epigean and terrestrial (Figure 2). We, therefore, place this species in a new genus, despite the absence of a male, on the arguments given above.

The finding of a new genus of dytiscid beetles in the Ngalia Basin brings the number of genera to four (*Exocelina*, *Ngaliadessus*, *Limbodessus* and *Paroster*) and number of species to 13 in this basin, making it a hotspot of stygobiotic dytiscid diversity. The other major centre of stygobiotic dytiscid diversity is the Yilgarn region of central Western Australia, which contains around 91 dytiscid species from four genera, distributed in 49 isolated groundwater calcretes, each containing between one and three species (Cooper et al. 2002; Watts & Humphreys 2009 and references therein; Eberhard et al. 2016). The Yilgarn species are mostly from the genera *Limbodessus* and *Paroster*, with one species from the genus *Exocelina* and two species from the genus *Neobidessodes*. Surface taxa are known for each of these genera but are unknown for *Ngaliadessus*. However, it is most likely that the ancestor of *N. humphreysi* was a surface (epigean) species, given the lack of related stygobiotic taxa in the Ngalia Basin and that surface beetle species represent the ultimate ancestral form for insect stygobionts (Cooper et al. 2023), with several notable exceptions (Faille et al. 2010; Langille et al. 2021; Leijs et al. 2012; Leys et al. 2003; Ribera et al. 2010). Due to the arid nature of the surface environment this ancestor is likely to be extinct in the region. Nonetheless, the possibility remains that epigean *Ngaliadessus* species are extant in rainforest or interstitial environments, as has been found for relatives of the other known stygobiotic taxa.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest with the publication of this manuscript.

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