Technical note

A test case for the use of DNA barcoding using *Berosus* (Order: Coleoptera)

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INTRODUCTION

Most identification of aquatic invertebrate specimens is currently achieved using morphological characters. However, often the diagnostic features required for species level identification only occur in a particular gender or life-history stage, and so achieving the lowest taxonomic resolution is not always possible. DNA barcoding, a technique used to describe identification of an organism by sequencing a section of DNA (~648 base pairs) for the Cytochrome Oxidase I mitochondrial gene (COI), provides a valuable tool to assist with these problem areas in species-level identification.

During a recent survey of wetlands in the Goldfields, aquatic beetles in the genus *Berosus* (Family Hydrophilidae) were collected from 11 of the 14 main survey locations and opportunistically hand-collected from four additional locations. However, at two-thirds of these sites (10 of the 15 locations) only larvae were collected and therefore no species level identifications were possible. The aim of undertaking molecular work on this group of aquatic beetles was to investigate whether DNA could readily be extracted from collected specimens, and if so, could DNA barcoding assist with species level identification of unknown *Berosus* larvae.

METHODS

Berosus larvae and adults (the latter morphologically identified to species level) collected from the Goldfields wetlands survey (Quinlan et al. 2016) were used for the test case, with several additional specimens of *Berosus* collected from previous Parks and Wildlife wetland surveys included to develop a more complete dataset of COI sequences. These additional specimens were adults also morphologically identified to species level. Specimens of another beetle (the gyrinid *Dineutus australis*) and coenagrionid damselflies were included to provide outgroups and a comparison for DNA extraction success. All individuals used in this molecular work are detailed in Table 1.

DNA was extracted using a standard 'Salting-out' technique (http://www.liv.ac.uk/~kempsj/IsolationofDNA.pdf), with the only modification being a proportionate scaling down in the volumes of materials used. DNA quality and quantity was assessed by electrophoresis on a 1% agarose gel and the results viewed on an imaging machine. Concentrations of DNA in ng μl^{-1} were recorded using a Qubit 2.0 Fluorometer.

Polymerase chain reaction (PCR) was used to amplify the mitochondrial (COI) gene using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). Final concentrations in a 25 µl PCR reaction mix were: 1 × PCR Buffer, 0.06 units Taq polymerase (Invitrogen), 3 mM $MgCl_2$, 0.02% Bovine Serum Albumin, 0.2 µM each dNTP, 0.4 µM each of the forward and reverse primers and sterile distilled water. Differing volumes of DNA template were used in the PCR reaction: 2 µl for samples that appeared to have high concentration DNA (10+ ng μl^{-1}) and 8.2 µl for low concentration samples. Samples were amplified under the following PCR conditions: 94 °C for 3 min, (94 °C 30 sec, 46 °C 30 sec, 46 °C 30 sec, 72 °C 30 sec) 35 times, 72 °C for 2 min, 20 °C for 1 min. The PCR product was checked by electrophoresis on a 1% agarose gel and the results viewed on an imaging machine. The PCR product (~23 µl) was then sent to the Australian Genome Research Facility (AGRF; Nedlands, WA) for Sanger sequencing (dual direction).

Sequences were trimmed and edited manually in the program *BioEdit* (version 7.2.5; Hall 1999) and then aligned using the ClustalW function in the same program. Sequences were then checked for compatibility against all taxonomic groups in the Genbank online

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DNA database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD =Web&PAGE_TYPE=BlastHome), using the BLAST function. Sequences appeared to match to at least family level and genus level for some individuals, confirming our identifications at a broader taxonomic level. Species level matches were made difficult due to an under-representation of Australian aquatic invertebrate species in the database.

Visualisation of a phylogenetic tree was conducted in MEGA (version 6.0; Tamura et al. 2013) using the Maximum Likelihood method, 2000 Bootstrap replications, General Time Reversible model, and default settings for the remainder. Information used in the analyses was derived from a ~648 base-pair fragment of the mitochondrial COI gene.

RESULTS AND DISCUSSION

DNA was successfully extracted from most specimens, and of those that were unsuccessful, most were specimens collected from old surveys where the material was not well preserved and from samples where formalin had been used in the past. Specimens clustered into six distinct phylogenetic groups (excluding outgroups), with a different adult *Berosus* species occurring in each (Fig. 1). This separation of each *Berosus* species is supported by high bootstrap values, with the exception being the node supporting *Berosus pulchellus*. The bootstrap value for *B. pulchellus* is weak, and the long branch lengths suggest that there may be additional genetic differentiation between the two specimens, requiring additional investigation. This species was not collected from the Goldfields survey, and the two specimens sequenced were from the Kimberley region and Katjarra (Carnarvon Range) in the Little Sandy Desert. Additional specimens from a range of locations would help to resolve the detail of this group.

From this phylogenetic tree we can, however, match all unidentified larvae collected during the survey to a particular *Berosus* species with confidence (Table 1). There is some variation within clades (e.g. *Berosus nutans*) but this is not considerable, with only very short branch lengths displayed. Outgroups (*Dineutus australis* and Coenagrionidae) were easily discernible from the *Berosus* clusters (Fig. 1). We acknowledge that these results are only based on one individual collected

Table 1

Individual specimens (including site code and survey information) used for molecular work. Identification (Lowest ID) of *Berosus* specimens before and after the completion of the molecular work is also displayed.

Site Code	Survey	Larvae/ Adult	Lowest ID before molecular work	Lowest ID after molecular work	Accession number (Parks and Wildlife database)
GOL01	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus macumbensis*	Ki8
GOL02	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus macumbensis*	Ki9
GOL04	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus nutans*	Ki11
GOL05	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus munitipennis*	Ki12
GOL06	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus macumbensis*	Ki13
GOL10	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus approximans*	Ki14
GOL12	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus nutans*	Ki15
GOL13	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus nutans*	Ki22
GOL14	Goldfields wetlands survey	Larvae	*Berosus* sp.	*Berosus dallasi*	Ki16
ADS31	Goldfields ad-hoc collection	Larvae	*Berosus* sp.	*Berosus nutans*	Ki17
ADS33	Goldfields ad-hoc collection	Larvae	*Berosus* sp.	*Berosus nutans*	Ki18
ADS33	Goldfields ad-hoc collection	Larvae	*Berosus* sp.	*Berosus nutans*	Ki19
ADS35	Goldfields ad-hoc collection	Larvae	*Berosus* sp.	*Berosus nutans*	Ki20
GOL03	Goldfields wetlands survey	Adult	*Berosus approximans*	—	Ki10
GOL06	Goldfields wetlands survey	Adult	*Berosus macumbensis*	—	Ki25
GOL14	Goldfields wetlands survey	Adult	*Berosus dallasi*	—	Ki24
ADS30	Goldfields ad-hoc collection	Adult	*Berosus munitipennis*	—	Ki21
CRS12	Katjarra survey	Adult	*Berosus pulchellus*	—	Ki31
RCM12	Resource condition monitoring	Adult	*Berosus pulchellus*	—	Ki26
RCM15	Resource condition monitoring	Adult	*Berosus nutans*	—	Ki28
Outgroups					
CRS09	Katjarra survey	Adult	*Dineutus australis*	—	Ki1
CRS09	Katjarra survey	Adult	*Dineutus australis*	—	Ki2
CRS04	Katjarra survey	Adult	*Dineutus australis*	—	Ki3
CRS04	Katjarra survey	Adult	*Dineutus australis*	—	Ki4
CRS3A	Katjarra survey	Adult	Coenagrionidae	—	Ki6
CRS3A	Katjarra survey	Adult	Coenagrionidae	—	Ki7

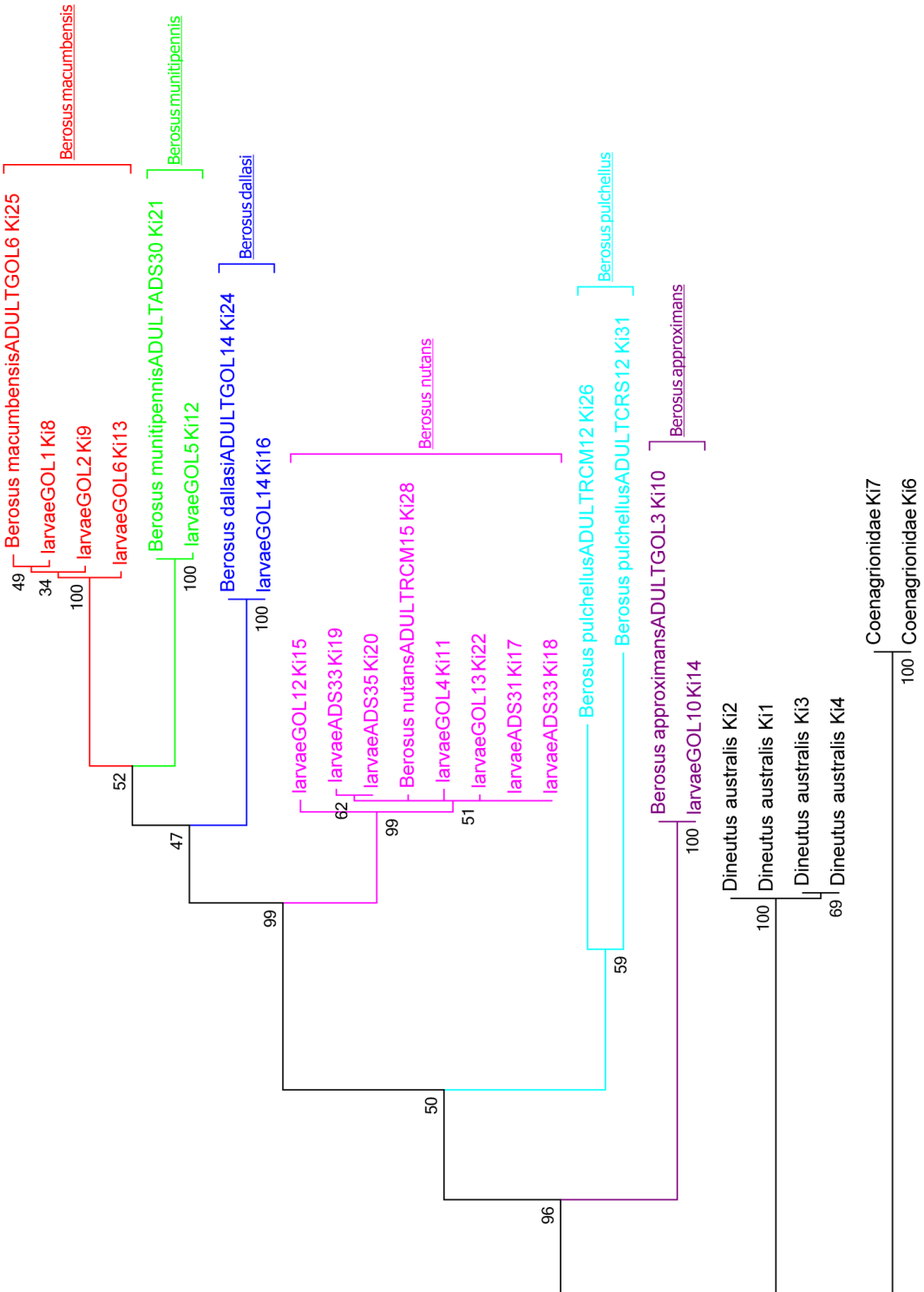

Figure 1. Maximum likelihood phylogenetic tree (MEGA v6.0) of unknown larvae and morphologically identified adults of the genus Berosus *from the Goldfields wetlands survey and additional Parks and Wildlife surveys. Specimen, site code and accession number is provided (refer to Table 1 for more detail). The tree is rooted on outgroups* (Dineutus australis *and Coenagrionidae). Values above nodes are bootstrap percentages, estimating the reliability of each node.*

at each site from the Goldfields survey, and ideally a dataset containing replicates from each site would have provided a more robust analysis. However, this test case demonstrates proof of concept, and it is expected that in time, with the addition of specimens (adult and larvae) from future surveys, this dataset will develop and be of a more robust nature.

This work has meant an increase from 33% to 100% species-level identification for *Berosus* specimens collected from 15 locations during the survey, using molecular methods over standard morphological methods. This in turn has enabled a more complete dataset to be used in community analyses. Taxonomic resolution to species level is important when investigating presence/absence at sites, community composition and, importantly, understanding the conservation value of wetlands. This work also contributes valuable information to understanding the distribution patterns of *Berosus* species within the Goldfields region. This approach can readily be extended to other invertebrate groups for which there are similar limits to morphological identification.

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