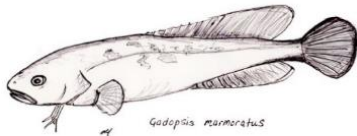


AQUASAVE - NatureGlenelgTrust



Ecology, Monitoring, Conservation



# Molecular taxonomy of Australia's endemic freshwater crayfish genus *Euastacus* (Parastacidae), with reference to priority 2019–20 bushfire-impacted species – 2022 update

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Grandjean**

*This report has been updated with the addition of  
targeted sequences for select species*

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We respectfully acknowledge the Traditional Owners of the lands on which this work took place, their Elders past, present and emerging, the First Nations of Australia, and their cultural, social, environmental, spiritual, and economic connection to their lands and water.

## Executive summary

*Euastacus* is the most threatened genus of endemic Australian freshwater crayfish, with 80% of its species considered threatened and listed under International Union for Conservation of Nature (IUCN) threat categories ([Furse and Coughran 2011](#); [Richman et al. 2015](#)). The 2019–20 Australian megafires in eastern Australia were predicted to have impacted 22 species of *Euastacus* (or 40% of described species) ([Legge et al. 2021a](#); [Legge et al. 2021b](#)).

The present study, forming part of a multi-faceted project initiated in response to the anticipated bushfire impact, is the most comprehensive molecular taxonomic analyses conducted on *Euastacus* to date. It utilised targeted field collection and took advantage of existing curated sample collections to assemble a comprehensive crayfish tissue collection which was used to generate 234 new *Euastacus* sequence datasets using genome skimming. Integration with all publicly available COI gene sequences (submitted to GenBank) achieved a combined dataset with representation of all known species of *Euastacus* as well as several putative new species. Overall, the analyses provided support for the distinctiveness of all described species of *Euastacus* (with a few exceptions) but revealed a very high and unanticipated level of cryptic diversity. All priority species (except the undescribed *E. sp. 2* as well as *E. gumar* and *E. pilosus*) could be unambiguously distinguished. Within the priority species, divergent lineages were identified associated with seven existing species, and most possibly representing nine new putative species. Two of these putative species (*E. sp. nov. 4*, *E. sp. nov. 5*) occur across bushfire-affected regions. All of the other 34 non-priority species of *Euastacus* were supported. Similar to the priority species, significant cryptic or unrecognised diversity was discovered. A total of 13 distinct cryptic lineages associated with 11 of these non-priority species was found, with an additional five sample groupings appear as additional distinct lineages that are not closely associated with any named species (from our study or GenBank sequences).

Taken together, these analyses provide a molecular taxonomic basis for the presence of up to 82 putative species within *Euastacus*, with as many as 27 being new species; this represents an approximate 50% increase in the number of species within the genus. It is acknowledged that additional lines of evidence (e.g., morphology, geography and ecology) and, potentially,

the analysis of additional sequences (i.e., nuclear markers), will be necessary to fully evaluate the status of these putative species to support formal description of new species.

Our study greatly improves understanding of the species-level diversity within *Euastacus* and represents the most comprehensive study of any of the speciose genera of freshwater crayfish worldwide. Nevertheless, some uncertainty as to the completeness of the taxonomic framework for the genus remains, due, in part, to incomplete geographic sampling and collecting, but also the unexpected extent of cryptic diversity. Ongoing research on the taxonomic and genetic variation within *Euastacus* is required, which is recommended to focus on:

- Comprehensive field surveys of all described and new putative species, where previously identified knowledge gaps exist;
- Further sequencing of additional tissue samples to resolve taxonomic uncertainties and species boundaries;
- Analysis of nuclear data to support findings based on mitochondrial data;
- Characterisation of full geographic range as well as morphological and ecological characteristics of new putative species;
- Formal description of well supported new putative species; and
- Ensuring ongoing resources are allocated to fulfil the necessary taxonomic revisions for *Euastacus* supported with molecular genetic data.

Broader conservation recommendations are:

- Formal listing assessment of greater number of species against EPBC Act criteria;
- Development of a formal Action Plan for the conservation and recovery of the genus *Euastacus*; and
- Direct and meaningful attention in management initiatives for, and conservation of, species of *Euastacus*.

This study provides a robust basis for the future conservation and management of species of *Euastacus*. The primary outcome of this study is a call to better understand, conserve and manage the substantially increased diversity of species in the genus *Euastacus*, a critical component of Australia's distinct freshwater fauna, and a denizen of fire-prone regions of eastern Australia.

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## 1. Background

Freshwater crayfish are a diverse taxonomic group, with more than 590 currently described species across four families worldwide (Parastacidae, Cambaridae, Cambaroididae and Astacidae) found in a wide range of aquatic habitats ([Ahyong 2014](#); [Crandall and De Grave 2017](#); [Richman et al. 2015](#)). In many parts of the world freshwater crayfish are under threat with at least one-third of all species considered at risk of extinction. The endemic Australian freshwater crayfish genus *Euastacus* is considered the most threatened genus of freshwater crayfish genus in the world, with 80% of its currently described species listed under International Union for Conservation of Nature (IUCN) threat categories ([Furse and Coughran 2011](#); [Richman et al. 2015](#)).

Freshwater crayfish are primarily threatened by over-exploitation and fragmentation of habitats and climate change ([Collen et al. 2014](#); [Hossain et al. 2018](#); [Richman et al. 2015](#)). Although not immediately obvious, bushfires followed by indirect impacts on aquatic habitats have emerged as a key threatening process for species of *Euastacus* ([Legge et al. 2021b](#)). Indeed, the 2019–20 Australian megafires that burned over 10 million hectares of the Australian continent ([Bowman et al. 2020](#); [Ward et al. 2020](#)) were predicted to have impacted 22 species of *Euastacus* (or 40% of the known species level diversity) ([Legge et al. 2021a](#); [Legge et al. 2021b](#)). Extinction risk in freshwater crayfish is significantly linked to range size, body size and habitat specialisation ([Bland 2017](#)). For species of *Euastacus*, their distinctive life history traits, including limited dispersal, slow growth, late maturity, slow population turnover (high longevity) and low egg production (fecundity), are anticipated to further heighten their vulnerability to environmental perturbations such as bushfires.

Urgent conservation actions are necessary for freshwater crayfish, especially for species of *Euastacus*. To this end, the ‘Saving the Spinys: urgent actions to conserve the *Euastacus* freshwater crayfish’ project, funded by the Australian Government’s Bushfire Wildlife and Habitat Recovery Program, addressed recovery actions for the 22 bushfire-impacted priority species of *Euastacus* (but with broader focus on the entire genus). This multi-faceted project incorporates field surveys and specimen collection, taxonomic identification, evaluation of the feasibility of conservation translocations and conservation assessments (against Australian *Environmental Protection and Biological Conservation Act 1999* (EPBC Act) criteria)

along with the collection of molecular genetic data to support taxonomic analyses and the application of environmental DNA (eDNA) methodology to assist range determination. The present report details the results of the molecular taxonomic component of the project designed to independently evaluate the taxonomic status of described species of *Euastacus* and the identify unrecognised diversity of possible taxonomic or conservational significance.

### 1.1 Current species status of *Euastacus*

*Euastacus* is a member of Australia's diverse and distinctive freshwater crayfish fauna. The genus currently includes 53 described species distributed from southern mainland Australia to northern Queensland (Figure 1-1 and 1-2, Table 1-1 and 1-2). An additional three putative new species have been recently identified (e.g., *Euastacus* sp. 1; *Euastacus* sp. 2 and *Euastacus* sp. 3) but require molecular and formal taxonomic evaluation. The Saving the Spynys project has a focus on the 22 priority species (Table 1-1) believed to be most impacted by the 2019–20 Australian megafires ([Legge et al. 2021a](#); [Legge et al. 2021b](#)).





Table 1-1. Summary of the 22 priority bushfire-related species of *Euastacus* (conservation status is **CR** = Critically Endangered; **EN** = Endangered; and **VU** = Vulnerable). EPBC = Australian Environment Protection and Biodiversity Conservation (EPBC) Act; IUCN = International Union for Conservation of Nature Red List of Threatened Species.

Species name		Species authority	Status	
Scientific	Common		Global (IUCN)	National (EPBC)
<i>Euastacus bidawalus</i>	East Gippsland Spiny Crayfish	Morgan, 1986	EN	-
<i>Euastacus clarkae</i>	Ellen Clark's Crayfish	Morgan, 1997	EN	-
<i>Euastacus claytoni</i>	Clayton's Spiny Crayfish	Riek, 1969	EN	-
<i>Euastacus crassus</i>	Alpine Crayfish	Riek, 1951	EN	-
<i>Euastacus dalagarbe</i>	Mud Gully Crayfish	Coughran, 2005	CR	-
<i>Euastacus diversus</i>	Orbost Spiny Crayfish	Riek, 1969	EN	-
<i>Euastacus gamilaroi</i>	Gamilaroi Spiny Crayfish	Morgan, 1997	CR	-
<i>Euastacus girurmulayn</i>	Smooth Crayfish	Coughran, 2005	CR	-
<i>Euastacus gumar</i>	Blood Crayfish	Morgan, 1997	EN	-
<i>Euastacus guwinus</i>	Tianjara Crayfish	Morgan, 1997	CR	-
<i>Euastacus jagabar</i>	Blue-Black Crayfish	Coughran, 2005	CR	-
<i>Euastacus jagara</i>	Jagara Hairy Crayfish	Morgan, 1989	CR	-
<i>Euastacus morgani</i>	Morgan's Crayfish	Coughran & McCormack, 2011	-	-
<i>Euastacus pilosus</i>	Hairy Cataract Crayfish	Coughran & Leckie, 2007	EN	-
<i>Euastacus polysetosus</i>	Many-bristled Crayfish	Riek, 1951	EN	-
<i>Euastacus riei</i>	Riek's Spiny Crayfish	Morgan, 1997	EN	-
<i>Euastacus simplex</i>	Simple Crayfish	Riek, 1956	VU	-
<i>Euastacus spinichelatus</i>	Small Crayfish	Morgan, 1997	EN	-
<i>Euastacus suttoni</i>	Sutton's Crayfish	Clark, 1941	VU	-
<i>Euastacus sp. 1</i>	Arte Spiny Crayfish	-	-	-
<i>Euastacus sp. 2</i>	Cann Spiny Crayfish	-	-	-
<i>Euastacus sp. 3</i>	West Snowy Spiny Crayfish	-	-	-

There are an additional 34 described species of *Euastacus* that were not specific priorities for this study (Table 1-2) but were included to quantify variation within and between species across the genus to ensure the objective establishment of species boundaries, and to enable the correct taxonomic identification of diversity within the priority species group.

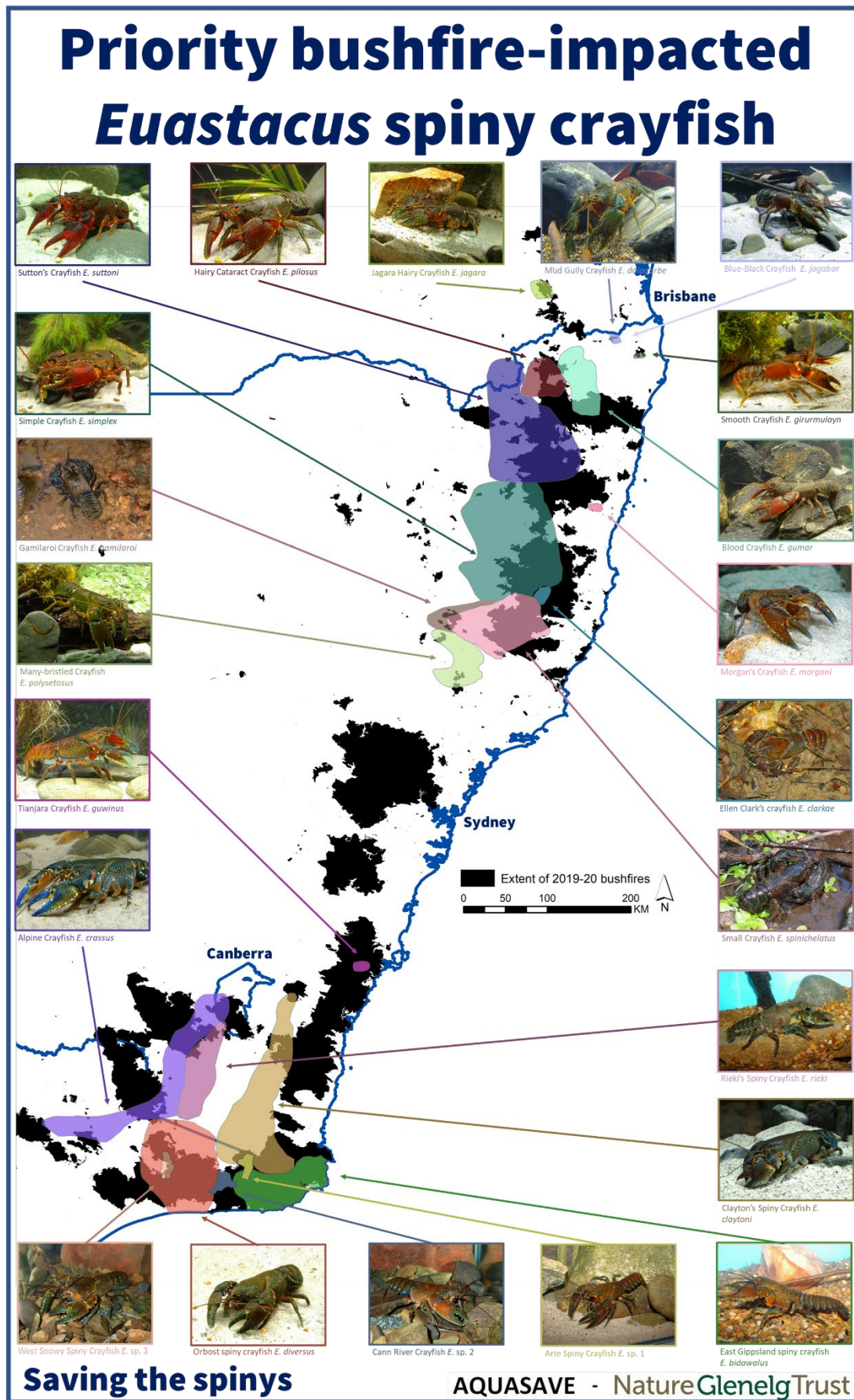


Figure 1-2. Summary of the 22 priority *Euastacus* species including known indicative range.

Table 1-2. Summary of the non-priority bushfire-related species of *Euastacus* (conservation status is **CR** = Critically Endangered; **EN** = Endangered; and **VU** = Vulnerable). EPBC = Australian Environment Protection and Biodiversity Conservation (EPBC) Act; IUCN = International Union for Conservation of Nature Red List of Threatened Species.

Species name		Species authority	Status	
Scientific	Common		Global (IUCN)	National (EPBC)
<i>Euastacus angustus</i>	The Narrow Dwarf Crayfish	Coughran & Dawkins, 2013	CR	-
<i>Euastacus armatus</i>	Murray Spiny Crayfish	(von Martens, 1866)	DD	-
<i>Euastacus australasiensis</i>	Australian Crayfish	(Milne Edwards, 1837)	LC	-
<i>Euastacus balanensis</i>	Balan Spiny Crayfish	Morgan, 1988	EN	-
<i>Euastacus bindal</i>	Mt Elliot Crayfish	Morgan, 1989	CR	CR
<i>Euastacus binzayedii</i>	Binzayeds Crayfish	Coughran & Furse, 2013	CR	-
<i>Euastacus bispinosus</i>	Glenelg Spiny Crayfish	Clark, 1936	VU	EN
<i>Euastacus brachythorax</i>	Short Thorax Crayfish	Riek, 1969	EN	-
<i>Euastacus dangadi</i>	Small Spiny Crayfish	Morgan, 1997	LC	-
<i>Euastacus dharawalus</i>	Fitzroy Falls Spiny Crayfish	Morgan, 1997	CR	CR
<i>Euastacus eungella</i>	Eungella Spiny Crayfish	Morgan, 1988	CR	-
<i>Euastacus fleckeri</i>	Flecker's Crayfish	Watson, 1935	EN	-
<i>Euastacus guruhgi</i>	Swollen Crayfish	Coughran, 2005	CR	-
<i>Euastacus hirsutus</i>	Southern Hairy Crayfish	(McCulloch, 1917)	EN	-
<i>Euastacus hystricosus</i>	Condondale Spiny Crayfish	Riek, 1951	EN	-
<i>Euastacus kershawi</i>	Gippsland Spiny Crayfish	Smith, 1912	LC	-
<i>Euastacus maccai</i>	Terrestrial Crayfish	McCormack & Coughran, 2008	EN	-
<i>Euastacus maidae</i>	Hinterland Crayfish	Riek, 1956	CR	-
<i>Euastacus mirangudjin</i>	Orange-bellied Crayfish	Coughran, 2002	CR	-
<i>Euastacus monteithorum</i>	Monteith's Crayfish	Morgan, 1989	CR	-
<i>Euastacus neodiversus</i>	South Gippsland Spiny Crayfish	Riek, 1969	EN	-
<i>Euastacus neohirsutus</i>	New Hairy Crayfish	Riek, 1956	LC	-
<i>Euastacus reductus</i>	Remote Crayfish	Riek, 1969	LC	-
<i>Euastacus robertsi</i>	Robert's crayfish	Monroe, 1977	CR	-
<i>Euastacus setosus</i>	Mount Glorious Spiny Crayfish	(Riek, 1956)	CR	-
<i>Euastacus spinifer</i>	Giant Spiny Crayfish	(Heller, 1865)	LC	-
<i>Euastacus sulcatus</i>	Mountain Crayfish	Riek, 1951	VU	-
<i>Euastacus urospinosus</i>	Maleny Crayfish	Riek, 1956	EN	-
<i>Euastacus valentulus</i>	Powerful Crayfish	Riek, 1951	LC	-
<i>Euastacus vesper</i>	Cudgegong Giant Spiny Crayfish	McCormack & Ahyong, 2017	CR	-
<i>Euastacus woiwuru</i>	Central Highlands Spiny Crayfish	Morgan, 1986	NT	-
<i>Euastacus yanga</i>	Variable Spiny Crayfish	Morgan, 1997	LC	-
<i>Euastacus yarraensis</i>	Southern Victorian Spiny Crayfish	(McCoy, 1888)	VU	-
<i>Euastacus yigara</i>	Cardwell Hairy Crayfish	Short & Davie, 1993	CR	-

## 1.2 Taxonomic history of *Euastacus*

*Euastacus* has a long taxonomic history (Figure 1-3), with the first species, *Euastacus australasiensis* (Australian Crayfish), formally described in 1837 ([Milne Edwards 1837](#)). In the next hundred years (to the 1940s), a further eight species were described, but since that time there has been an acceleration of taxonomic discovery in the genus. There have been distinct periods where species discoveries have been prominent, such as the 1950s (10 species: [Riek 1951](#); [Riek 1956](#)), the 1980s (seven species: [Morgan 1986](#); [Morgan 1988](#); [Morgan 1989](#)) and the 1990s (10 species: [Morgan 1997](#); [Short and Davie 1993](#)). In the twenty years between 2002 and 2021, a further 11 species were described ([Coughran 2005](#); [Furse et al. 2013](#); [McCormack and Coughran 2008](#)), with *Euastacus vesper* (Cudgegong Giant Spiny Crayfish) most recently described ([McCormack and Ahyong 2017](#)).

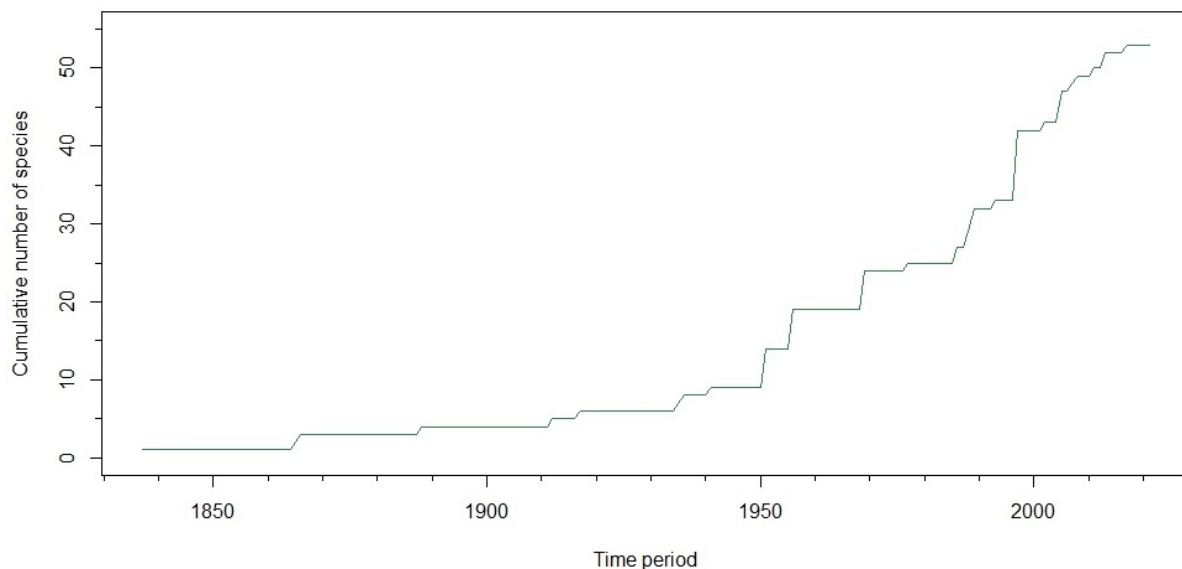


Figure 1-3. Summary of description of species of *Euastacus* over time.

Prior to 2005, species of *Euastacus* were established based solely on morphology (diagnostic anatomical characteristics, meristic traits and morphometrics). Molecular genetic insight into species boundaries in the genus were developed from the first comprehensive sequencing and phylogenetic study of the genus was by Shull et al. ([2005](#)). Their analyses, using mitochondrial (COI, 12S, 16S) and nuclear (28S) gene regions sequenced from 129 individuals from 40 (of the 43 known at the time) species, confirmed the validity of the majority of described species whilst also revealing cryptic diversity in several lineages. Subsequent species- or regionally-focused molecular genetic and phylogenetic analyses provided further

insight into species boundaries in the genus (e.g., [Baker et al. 2004](#); [Coughran et al. 2015](#); [Furse et al. 2013](#); [Hurry et al. 2015](#); [Ponniah and Hughes 2004](#); [Van Der Wal and Ah Yong 2019](#)). Nevertheless, the taxonomic status of several known or suspected species remained undescribed or unresolved, and further cryptic diversity was suspected.

Molecular genetic approaches are now routinely employed to support morphology-based taxonomic identification, new species delimitation and range determinations, as well as identifying biodiversity that may not be easily achieved using conventional approaches (e.g., identification of juvenile specimens and morphologically cryptic diversity). Molecular taxonomic and phylogenetic reviews have now been implemented for all freshwater crayfish groups ([Owen et al. 2015](#)) as well as specific genera, including *Cambarellus* ([Pedraza-Lara et al. 2012](#)), *Cambarus* (i.e., devil crayfish: [Glon et al. 2018](#)), *Cherax* ([Munasinghe et al. 2004](#)), *Engaeus* ([Gan et al. 2018](#); [Schultz et al. 2009](#)), *Fallicambarus* ([Ainscough et al. 2013](#)), and *Pacifastacus* ([Larson et al. 2016](#)). The sophistication of molecular genetic approaches to address phylogenetic and taxonomic questions has advanced rapidly in recent years. Historically, a range of molecular markers and PCR-based Sanger sequencing have been applied to a range of crayfish groups. Recently, low coverage next generation DNA sequencing (i.e., genome skimming) has proved effective for recovering whole animal mitochondrial genome sequences (i.e., mitogenomes) coupled with high copy number nuclear genes which provides cost effective data sets for robust phylogenetic and taxonomic analyses, including for freshwater crayfish ([Gan et al. 2018](#); [Tan et al. 2021](#)). The rapid and cost-effective nature of genome skimming presents an opportunity to apply this approach to large-scale investigation of intra and interspecific variations to better resolve fine scale molecular taxonomic and phylogenetic relationships, establish species boundaries and reveal cryptic diversity. These investigations provide a sound foundation for targeted morphological studies and formal species description and identification of conservationally significant geographic diversity.

### 1.3 Project objectives

We utilised a multi-gene molecular approach to estimate phylogenetic relationships within and among species of *Euastacus*. The specific objectives of the study were to:

- Obtain and process tissues (DNA extraction, library preparation) and sequence samples for all priority *Euastacus* (and non-priority species where available);
- Access previously collected sequences available from the NCBI database (GenBank);
- Generate a curated georeferenced database for all sequenced samples; and
- Undertake molecular genetic analyses to inform species identification and taxonomy.

This study represents the most comprehensive molecular taxonomic analyses of *Euastacus* so far and will underpin future taxonomic, conservation, management and research priorities.

## 2. Methods

### 2.1 Tissue sample collection and sequence datasets

The study incorporated an initial main sequencing analysis, which was supplemented by smaller targeted sequencing to clarify uncertainty raised during the initial analysis (Figure 2-1 and Table A1-1 and Table A2-1). For both, new tissue samples were obtained for sequencing and compiled sequences from GenBank to represent as many species of *Euastacus* as possible. Multiple samples were available for most species allowing the assessment of intraspecific and geographic variation to improve delineation of species boundaries and identify conservationally significant units. Field surveys focused on obtaining tissue samples from the 22 priority species of *Euastacus* across bushfire-impacted areas. These new tissue samples were also supplemented with samples from historical tissue collections (private and public) obtained over the past 10 years from priority and non-priority species from the same geographic regions, and held or accessed by the project team and collaborators ([S. Ah Yong, unpublished](#); [M. Lintermans, unpublished](#); [R. McCormack, unpublished](#); [T. Raadik, unpublished](#); [Whiterod et al. 2017](#)). Additional unpublished sequences for two *Euastacus* species sequenced at the Australian Genome Research Facility (AGRF) were included (F. Wedrowicz, Federation University, unpublished).

All tissue samples, typically obtained from leg muscle, were taken from crayfish collected by hand capture, dip netting, trapping and electrofishing, in accordance with all relevant collection permits and animal ethics approvals. Most specimens were returned alive to the point of capture, with a subset retained as vouchers for detailed morphological analysis. All tissues were sequenced at the Deakin Genomic Centre (Deakin University).

Additional sequences were sourced from the National Center for Biotechnology Information (NCBI) nucleotide database (GenBank) with an initial focus on the Cytochrome Oxidase I (COI) barcoding gene region (e.g., [Baker et al. 2004](#); [Coughran et al. 2015](#); [Crandall et al. 1999](#); [Furse et al. 2013](#); [Hurry et al. 2015](#); [Lawler and Crandall 1998](#); [Munasinghe et al. 2003](#); [Ponniah and Hughes 2004](#); [Shull et al. 2005](#); [Toon et al. 2010](#); [Van Der Wal and Ahyong 2019](#)) as well as complete mitogenome sequences for three species (*E. armatus*, *E. spinifer* and *E. yarraensis*: [Gan et al. 2016](#); [Gan et al. 2018](#)) (Table 2-1, Table A1-1 and Table A2-1). Additionally, sequences from the complete mitogenome of the Tasmanian Giant Freshwater Crayfish (*Astacopsis gouldi*) ([Gan et al. 2018](#)), representing the sister genus to *Euastacus*, was used as the outgroup for phylogentic-based analyses (Table A1).

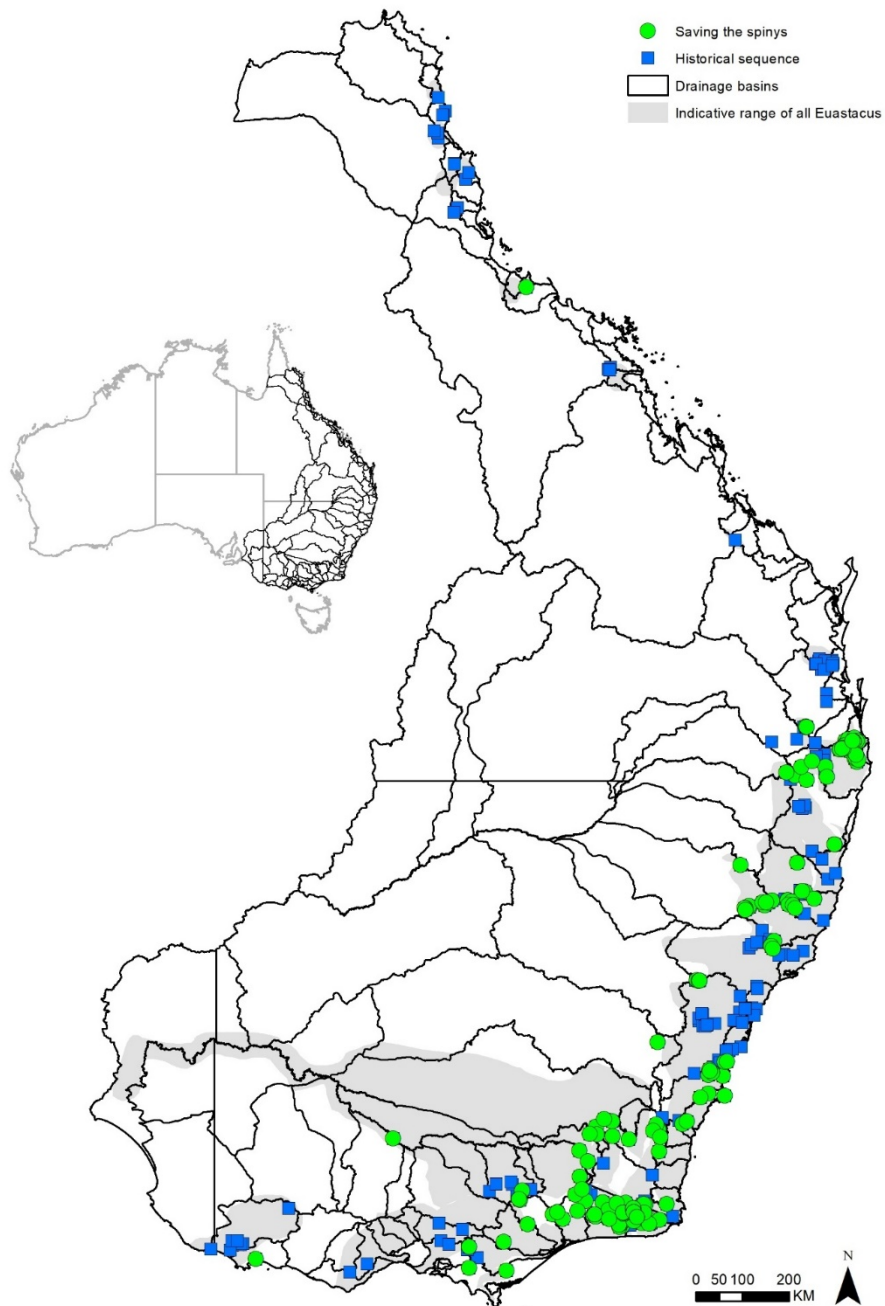


Figure 2-1. Location of samples (main and supplementary) sequenced for this project (●) including GenBank sequences (■) obtained from GenBank across eastern Australia. River basins (black outline). Indicative range (grey shade) for all species of *Euastacus* is also shown.



Table 2-1. Summary of studies from which GenBank sequences for *Euastacus* were sourced.

Study	Gene region	No. of sequences	Focus of study
Baker et al. (2004)	COI, 16S	21	Phylogenetic structuring and cryptic diversity in Australian freshwater macro
Coughran et al. (2015)	COI	80	Redescription of <i>E. diversus</i>
Furse et al. (2013)	COI	3	Description of two new species ( <i>E. angustus</i> , <i>E. binzayedii</i> )
Gan et al. (2016)	Whole mitogenome	1	First complete mitogenome of species of <i>Euastacus</i>
Gan et al. (2018)	Whole mitogenome	2	Molecular genetic evolution specifically in burrowing Australian freshwater crayfish
Hurry et al. (2015)	COI	15	Population structure and phylogeographic divergence in <i>E. urospinosus</i>
Miller et al. (2014)	COI	7	Population structure of <i>E. bispinosus</i>
Munasinghe et al. (2004)	COI, 12S, 16S	1	Systematics of <i>Cherax</i> genus
Ponniah and Hughes (2004)	COI, 16S	16	Evolution of QLD species of <i>Euastacus</i>
Shull et al. (2005)	COI, 12S, 16S & 28S	120	Phylogenetics of <i>Euastacus</i> genus
Toon et al. (2010)	COI, 16S & 18S, 28S	2	Evolutionary processes in Southern Hemisphere crayfishes
Van Der Wal and Ahyong (2019)	COI	57	Genomic analysis of <i>E. spinifer</i>

## 2.2 Genomic DNA extraction

From each tissue sample, approximately 50 mg of muscle was dissected from ethanol-preserved samples and cut into smaller pieces before an overnight digestion in 380µL lysis buffer (50mM Tris-HCl, 10mM EDTA, 20% SDS) and 20 µL Proteinase K (>600mAU/mL). Protein precipitation was performed by adding 100 µL saturated >5M KCl and incubated on ice. Following the addition of 1x volume of chloroform, the aqueous layer containing the DNA was transferred into a new tube. DNA was precipitated using 1x volume of isopropanol and washed using 1mL 80% ethanol before being eluted in 100 µL of elution buffer (10mM Tris-HCl, 1mM EDTA).

## 2.3 Sample processing, sequencing and bioinformatics

Genomic DNA for each sample was quantified with high-sensitivity assays (Invitrogen, USA) using a Qubit 4.0 Fluorometer (Invitrogen, USA) and the quality of the DNA assessed on 4200 TapeStation System (Agilent, USA). Between 10–500 ng of the genomic DNA was used for library preparation using Illumina DNA Prep kit (Illumina, San Diego, USA) according to the

manufacturer's instructions. Quantification and size estimation of the libraries was performed on both the Qubit 3.0 Fluorometer and 4200 TapeStation System (Agilent, USA). Next, the library was normalised to 2 nM and sequenced on a NovaSeq Sequencer (2 × 150 bp or 2 × 250 bp paired-end reads) (Illumina, San Diego, CA) at the Deakin Genomics Centre.

The bioinformatics workflow (see Figure 2-2) followed, with some modifications, the methods outlined by Grandjean et al. (2017) and Tan et al. (2021). Sequences generated from the partial genome sequencing of each sample were initially preprocessed to remove adapters and low-quality sequences. The resulting quality-filtered reads were then assembled using a combination of: (i) Novaplasty implemented on the Galaxy web server, (ii) Mitoz (Meng et al. 2019), and (iii) Geneious-based pipelines utilizing both mapping to a complete mitogenome of a closely related species or iterative mapping using seed sequences, usually COI or 16S rRNA sequences from GenBank. All mapped or recruited sequences were then reassembled using the Geneious Assembler (which identifies mitogenome circularity) and, where necessary, polished with the Geneious implemented Spades assembler.

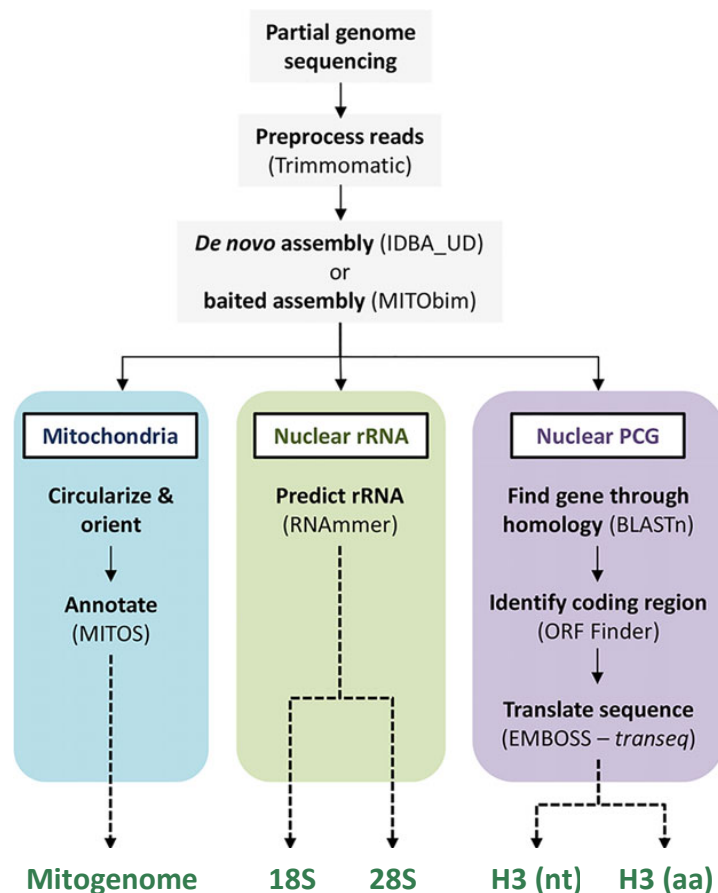


Figure 2-2. Genome skimming workflow used to recover the mitogenome and high copy number nuclear genes from partial genome scans (from Grandjean et al. 2017).

Preliminary analyses indicated that all three methods gave almost identical results when applied to the same data set, but with the Geneious pipelines having a slightly lower error rate (resolution of ambiguous bases). A similar approach was taken for recovering the nuclear 18S rRNA and 28S rRNA gene using Geneious pipelines to map sequences to a related species followed by polishing with the Spades assembler and determination of gene boundaries using BLAST searches and RNAmmer 1.2 Server ([www.cbs.dtu.dk/services/RNAmmer](http://www.cbs.dtu.dk/services/RNAmmer); Lagesen et al. 2007).

## 2.4 Taxonomic analyses

Distanced-based analyses were conducted by implementation of Geneious pipelines using the Neighbour Joining and UPGMA routines to construct trees and dendrograms based on various combinations of mitogenome sequences. As the level of genetic divergence was quite low, only nucleotide data were used, with the Jukes-Cantor method used as the genetic distance model (Jukes and Cantor 1969). Several datasets were analysed using distance approaches, comprising: (1) concatenation of the six longest mitochondrial genes (COI, ND4, ND5, CYTB, 16S and 12S genes); (2) COI; (3) 16S; (4) 12S; and (4) all COI gene fragments available from GenBank combined with the COI data from our study, and divided in three geographic groupings for ease of presentation.

## 3. Results

### 3.1 Sampling and datasets

Next generational sequence (genome skims) datasets were processed for 234 samples to extract mitochondrial genes from the initial main analysis (202 new and two unpublished sequences; coded: CA201 to CA202) and the supplementary targeted analysis (32 sequences; CA210 to CA241). Across both analyses, samples were obtained from 173 waterways over 35 river basins across eastern Australia, from the Glenelg River Basin (*E. bispinosus*) in southwestern Victoria to the Brisbane River Basin (*E. jagara*) in southern QLD. The study could not sequence samples from one priority species (*E. guwinus*) due to delayed field surveys. Sequences were obtained for seven species for which genetic data were not previously available (three priority species: *E. girurmulayn*, *E. jagabar*, and *E. simplex*; and four non-priority species: *E. guruhgi*, *E. hirsutus*, *E. maccai*, and *E. neodiversus*). Further, for all

described species, an attempt was made to obtain samples from multiple locations for sequencing (up to 26 locations for *E. bidawalus*).

The COI sequences were extracted from during this study were supplemented with 326 publicly available COI sequences, providing representation of all recognised species of *Euastacus* based on sample labelling, along with the three putative new species. The combined sequence dataset consisted of a total of 560 COI sequences (including the *A. gouldi* outgroup), allowing the investigation of taxonomic boundaries for the priority species within the context of sequences notionally representing all recognised species within the genus.

### 3.2 Genome skimming summary

Most tissue samples utilised in this study had sufficient quantity of intact DNA to be subjected to genome skimming resulting in typically the generation of from 2–4 gb of raw data (8,000,000–26,000,000 reads). A small number of samples with degraded DNA generated fewer reads (i.e., 300,000–500,000 reads) but successful mitogenome recovery was still possible in most cases. As a result, mitochondrial genes were successfully recovered from 200 samples sequenced at the Deakin Genomics Centre as well as the two unpublished sequence datasets provided by Federation University. Circularised mitogenomes were recovered from 188 of these samples ranging in length from 15,348–17,816 bp. For 14 samples the highly variable Control Region, which also often contains repetitive elements, could not be fully recovered, and therefore these mitogenomes could not be circularised. The typical 13 mitochondrial protein-coding genes and two ribosomal RNA genes (12S, 16S) were recovered from all samples ([Grandjean et al. 2017](#)). Complete or near-complete sequences were recovered for the two ribosomal nuclear genes 18S rRNA (1869–1885 bp) 28S rRNA (4144–5391 bp) from a subsample these datasets (n= 107 and n= 63, respectively), with this work ongoing.

### 3.3 Tree-based and taxonomic analyses

The initial analyses were based on the mitogenome dataset of 206 sequences, which included the 202 sequences generated from this study (200 sequenced and assembled during this study, two unpublished from Federation University sequenced by the AGRF and assembled for this study) along with published *E. armatus*, *E. yarraensis* and *E. spinifer* mitogenomes and

the published mitogenome of *A. gouldi*, with the latter used as the outgroup (Table A1-1). The supplementary targeted analyses included 32 sequences (Table A2-1). Overall, this study included the first sequences for five priority and five non-priority species of *Euastacus*, but did not have access to samples of *E. guwinus* (noting that GenBank sequences are available for this species).

The initial main concatenated six gene tree provided confirmation of 19 described species and two of the putative species that were a priority in this study, based on clustering patterns and levels of divergence, with each of 17 clusters with two or more samples receiving 100% bootstrap support (Figure 3-1, Figure A2-1 and Table 3-1). Overall. The species *E. clarkae* was represented by a single sample but showed substantial genetic divergence from all other samples or clades at a level typical for that seen among valid species. The putative undescribed species, *E. sp. nov. 2* was not supported whilst no sequences. Further, based on the genetic divergence alone, these data bring into question the taxonomic status of *E. pilosus*. While the four samples of this species form a tight monophyletic group, they show only limited divergence from the three samples of *E. gumar* that occurred that appears consistent with geographically divergent lineages rather than distinct taxa.

Seven of the priority species showed substantial levels of intraspecific variation that suggests the presence of a possible cryptic or sibling species associated with each of these species. For these pairs of species, the nominal species was assigned based on the proximity of samples to the type locality, whereas the divergent sister lineage was label with the 'cf' notation (i.e., 'compare with' or 'close to') to convey the species it is most similar to (e.g., *E. cf. bidawalus*, *E. cf. claytoni*). In addition, another unexpected finding was the presence of divergent samples that did not share clear affinities with any single species and that potentially also represent up to two new species: *E. sp. nov. 4* and *E. sp. nov. 5*. While these putative species are represented by small sample sizes (1 and 4, respectively), they displayed significant genetic divergence from all other samples.

Table 3-1. Status of the 22 priority bushfire-impacted *Euastacus* based on the molecular taxonomic analyses.

Scientific name	Status	Notes
<i>Euastacus bidawalus</i>	Valid species + 2 divergent lineages	One divergent sequence sympatric with two Genbank sequences of <i>E. bidawalus</i> sequences whereas the second divergent lineage from outlying location in southern NSW.
<i>Euastacus clarkae</i>	Valid species	
<i>Euastacus claytoni</i>	Valid species + 1 divergent lineage	Divergence of northern ( <i>E. cf. claytoni</i> ) and southern ( <i>E. claytoni</i> ) extent of range.
<i>Euastacus crassus</i>	Valid species + 1 divergent lineage	Divergence of western ( <i>E. cf. crassus</i> ) and eastern ( <i>E. crassus</i> ) extent of range
<i>Euastacus dalagarbe</i>	Valid species + 1 divergent lineage	Two sequences from single location (Running Creek tributary) extend <i>E. dalagarbe</i> range into QLD, which co-occur with the divergent lineage ( <i>E. cf. dalagarbe</i> ).
<i>Euastacus diversus</i>	Valid species	
<i>Euastacus gamilaroi</i>	Valid species	
<i>Euastacus girumalayn</i>	Valid species	
<i>Euastacus gumar</i>	Part of 2 taxa with uncertain status	Limited divergence from <i>E. pilosus</i> .
<i>Euastacus guwinus</i>	Valid species	Based on GenBank sequence only.
<i>Euastacus jagabar</i>	Valid species	
<i>Euastacus jagara</i>	Valid species	
<i>Euastacus morgani</i>	Valid species + 1 divergent lineage	
<i>Euastacus pilosus</i>	Part of 2 taxa with uncertain status	Limited divergence from <i>E. gumar</i> .
<i>Euastacus polysetosus</i>	Valid species	
<i>Euastacus rieki</i>	Valid species + 1 divergent lineage	The valid species ( <i>E. rieki</i> ) occurs in NSW whereas the <i>E. cf. rieki</i> is presently known from northern Victoria.
<i>Euastacus simplex</i>	Valid species	
<i>Euastacus spinichelatus</i>	Valid species + 2 divergent lineages	Divergent lineages ( <i>E. cf. spinichelatus</i> 1 and <i>E. cf. spinichelatus</i> 2) occur across a limited extent of the Hastings River Basin.
<i>Euastacus suttoni</i>	Valid species	
<i>Euastacus</i> sp. 1	Valid species	
<i>Euastacus</i> sp. 2	Not supported	
<i>Euastacus</i> sp. 3	Valid species	

More broadly, the concatenated six gene analysis included 76 samples representing 20 recognised non-priority *Euastacus* species, including five species (*E. angustus*, *E. binzayedii*, *E. hirsutus*, *E. maccai*, and *E. neodiversus*) sequenced for the first time via this study. These data support the distinctiveness of all of these 20 non-priority species, and like the priority species, revealed a high level of cryptic diversity (Table 3-2). Divergent lineages were identified for six species, potentially representing new species based on divergence levels equivalent to

established pairs of species (e.g., *E. cf. binzayedii*, *E. cf. hirsutus*, *E. cf. reductus*, *E. cf. woiwuru*, and *E. cf. yanga*). Individual gene-based analyses for COI, 16S and 12S were broadly consistent, recovering similar species delimitation for priority and non-priority species as the six-gene analysis, but with reduced level of bootstrap support for some lineages.

The second set of analyses combined the COI dataset (1535 bp sequences) from the main analyses of the present study (note supplementary sequences have not been included) with 326 COI gene sequences available from GenBank generated from PCR-based sequencing (mean = 640.5 bp; range: 393–669 bp). The complete COI dataset representing 528 individual sequences is presented separately for three geographically-based (southern, central and northern) tree groupings (Figure 3-2, 3-3 and 3-4). The combined COI analyses largely confirms species delimitation and cryptic diversity revealed in the first analyses, but further significant cryptic diversity is apparent throughout the genus, with the inclusion of these GenBank sequences. With the combined analyses, 20 of the 30 nominal and putative species identified in the initial analyses (using six-gene; COI; 16S; 12S datasets from our study) were also recovered as monophyletic groups, where there are matching sequences (which ranged between 1 to 25 sequences). Most, but not all, of the names assigned to GenBank sequences matched the nomenclature applied in this study with further details provided below. Two of the priority species, *E. polysetosus* and *E. guwinus*, not sampled in this study, were each represented by GenBank COI sequences which were genetically divergent at a level consistent with well-established species of *Euastacus*.

The combined COI analyses confirm the validity of all 34 non-priority species (Table 3-2 and Table 3-3). Within 10 of these species, highly divergent lineages were apparent, suggesting additional cryptic species, which has been previously noted for several of these species ([Hurry et al. 2015](#); [Shull et al. 2005](#); [Van Der Wal and Ahyong 2019](#)). The patterns of cryptic diversity revealed by the first analyses, is therefore evident across most of the extent of the genus in Australia. Several species from far northern QLD (e.g., *E. balanensis*, *E. robertsi*), as well as more broadly distributed species across Victoria (e.g., *E. kershawi* and *E. yarraensis*), are seen to be represented by at least two lineages. A further five undescribed species from New South Wales (NSW), which were anticipated by previous studies ([Baker et al. 2004](#); [Van Der Wal and Ahyong 2019](#)), are also supported by our results. This included groupings of sequences from: (1) the Clyde River-Jervis Basin (MK881011.1 and MK881012.1); (2) the Karuah and Hunter

basins (MK880959.1 to MK MK880962.1); (3) the Hunter Basin (MK880963.1, MK MK880964.1); (4) the Hawkesbury Basin (AY380463.1 to AY380465.1); and (5) the George River Basin (AY380466.1 to AY380468.1, AY380475.1 to AY380478.1). Overall, these analyses provide a molecular taxonomic basis for the recognition of 82 putative species within *Euastacus*, with as many as 27 being new undescribed species; this represents an approximate 50% increase in the number of species within the genus.

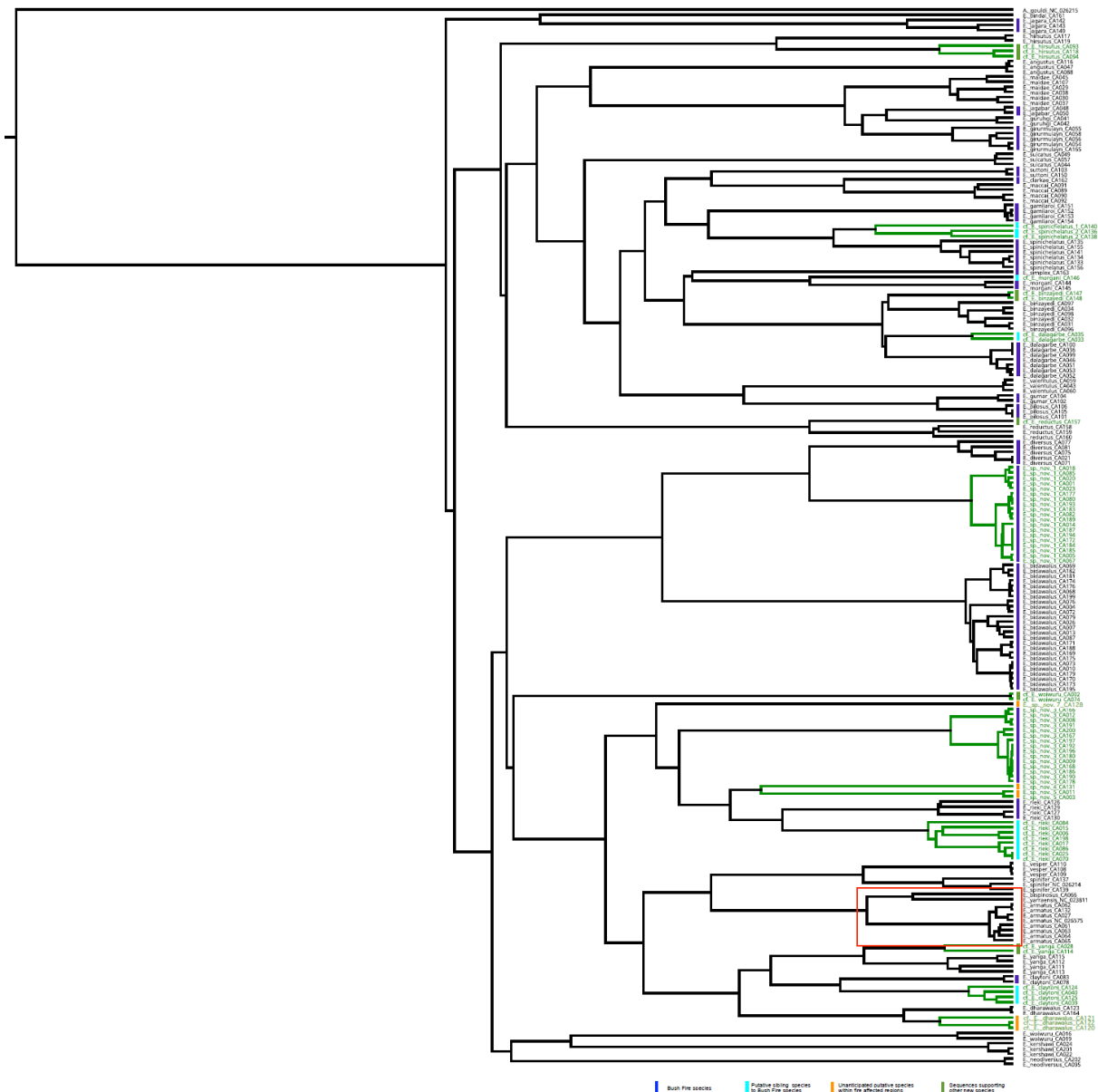


Figure 3-1. Concatenated six-gene (COI, ND4, ND5, CYTB, 16S and 12S) tree for *Euastacus* sequenced from this study (main sequences only). Colour coding is: priority bushfire-impacted species (I); putative new species divergent from bushfire-impacted species (I), unanticipated putative new species within fire-affected region (I); and new species supported only by GenBank COI sequences (green text). The red box highlights the mean divergence between well-established species (*E. armatus*, *E. bispinosus* and *E. yarraensis*) as a benchmark for the molecular taxonomic analyses.

A high resolution of the concatenated six-gene tree can be found [here](#).



Southern

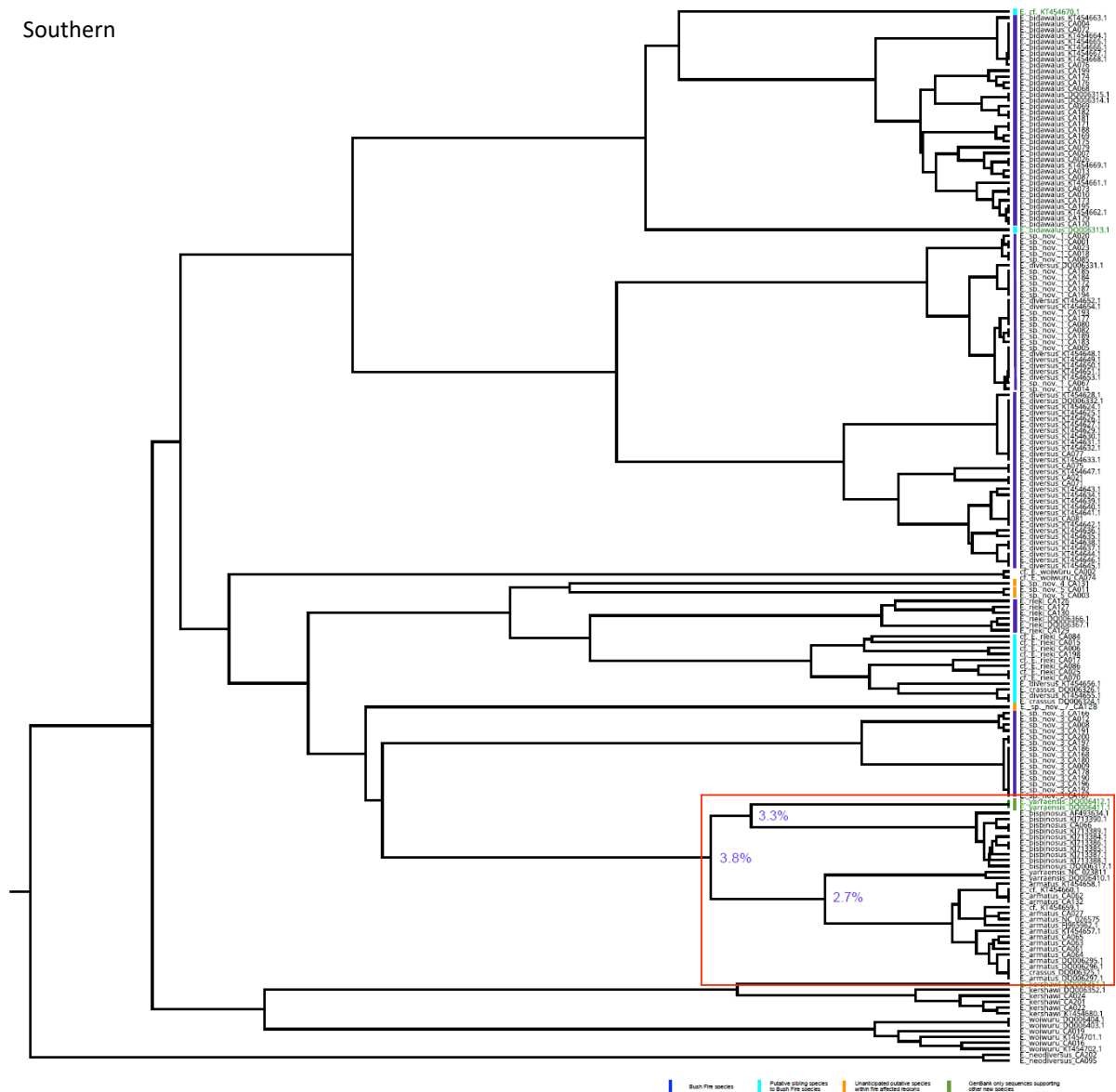


Figure 3-2. The combined COI gene tree for southern species of *Euastacus* relying on sequences from this study (main sequences only) as well as GenBank COI sequences publicly available (obtained from Genbank). Colour coding is: priority bushfire-impacted species (I); putative new species divergent from bushfire-impacted species (I), unanticipated putative new species within fire-affected region (I); and new species supported only by GenBank COI sequences (green text). The red box highlights the mean divergence between well-established species (*E. armatus*, *E. bispinosus* and *E. yarraensis*) as a benchmark for the molecular taxonomic analyses.

A high resolution of the southern combined COI gene tree can be found [here](#).

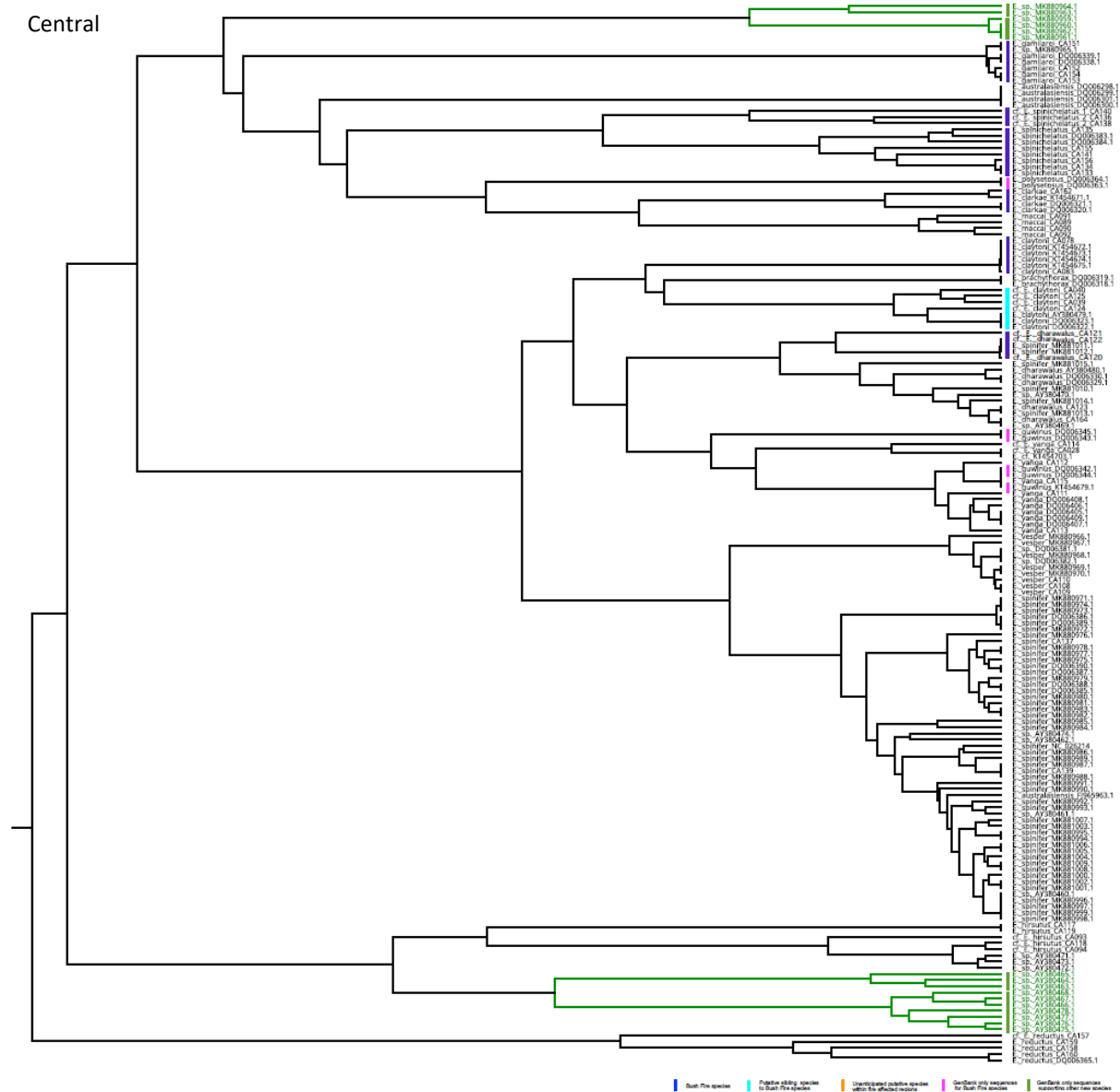


Figure 3-2. The combined COI gene tree for central species of *Euastacus* relying on sequences from this study (main sequences only) as well as GenBank COI sequences publicly available (obtained from GenBank). Colour coding is: priority bushfire-impacted species (I); putative new species divergent from bushfire-impacted species (I), unanticipated putative new species within fire-affected region (I); and new species supported only by GenBank COI sequences (green text).

A high resolution of the southern central COI gene tree can be found [here](#).

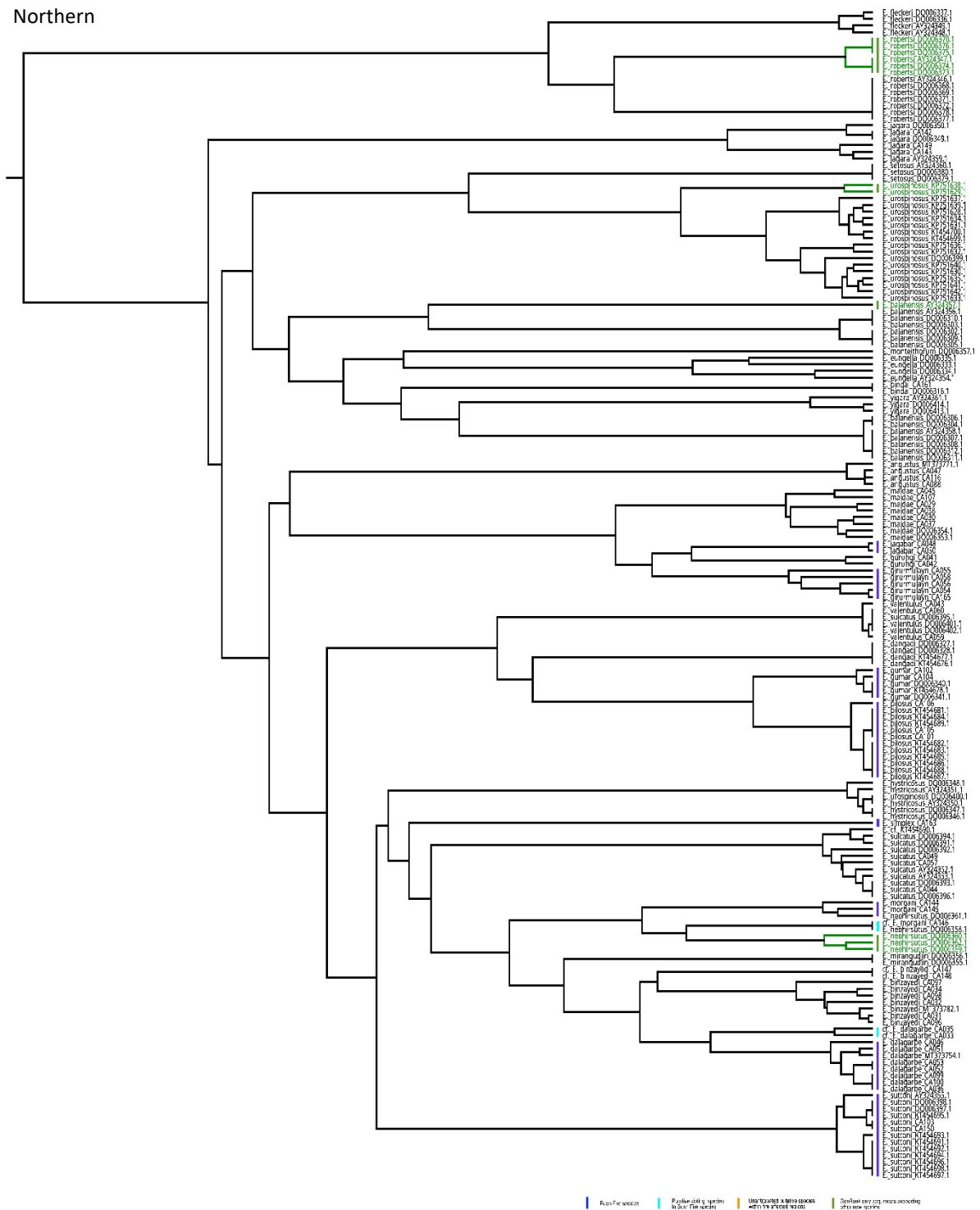


Figure 3-4. The combined COI gene tree for northern species of *Euastacus* relying on sequences from this study (main sequences only) as well as GenBank COI sequences publicly available (obtained from GenBank). Colour coding refers to (b) new species (green text) supported only by GenBank COI (only) sequence. Colour coding is: priority bushfire-impacted species (I); putative new species divergent from bushfire-impacted species (I), unanticipated putative new species within fire-affected region (I); and new species supported only by GenBank COI sequences (green text).

A high resolution of the southern central COI gene tree can be found [here](#).

Table 3-2. Status of the non-priority species of *Euastacus* on the basis of the molecular taxonomic analyses.

Scientific name	Status	Notes
<i>Euastacus angustus</i>	Valid species	
<i>Euastacus armatus</i>	Valid species	
<i>Euastacus australasiensis</i>	Valid species	
<i>Euastacus balanensis</i>	Valid species + 2 distinct lineages	Previously identified divergence ( <a href="#">Shull et al. 2005</a> ) of Barron River and northern Mulgrave-Russell Rivers basins ( <i>E. balanensis</i> ) with southern Mulgrave-Russell Rivers Basin ( <i>E. cf. balanensis</i> ) confirmed but with sympatric cryptic diversity evident ( <i>E. cf. balanensis</i> 2) in Mulgrave River.
<i>Euastacus bindal</i>	Valid species	
<i>Euastacus binzayedii</i>	Valid species + 1 distinct lineage	Two sequences (as <i>E. cf. binzayedii</i> ) from Springbrook region of South Coast Basin (QLD), diverged from <i>E. binzayedii</i> in areas of the Lamington National Park.
<i>Euastacus bispinosus</i>	Valid species	
<i>Euastacus brachythorax</i>	Valid species	
<i>Euastacus dangadi</i>	Valid species	
<i>Euastacus dharawalus</i>	Valid species + 1 distinct lineage	<i>Euastacus dharawalus</i> from Shoalhaven and Hawkesbury basins with divergent lineage ( <i>E. cf. dharawalus</i> ) from the Clyde River-Jervis Bay Basin.
<i>Euastacus eungella</i>	Valid species	
<i>Euastacus fleckeri</i>	Valid species	
<i>Euastacus guruhgi</i>	Valid species	
<i>Euastacus hirsutus</i>	Valid species + 1 distinct lineage	Wollongong Coast Basin (NSW) samples aligned to <i>E. hystricosus</i> whereas <i>E. cf. hystricosus</i> occurs to the south in the Shoalhaven River Basin
<i>Euastacus hystricosus</i>	Valid species	
<i>Euastacus kershawi</i>	Valid species + 1 distinct lineage	<i>Euastacus kershawi</i> in vicinity of type locality in Latrobe Basin whereas divergent lineage ( <i>E. cf. kershawi</i> ) broadly occurring across Bunyip, Thomson, Snowy and East Gippsland basins.
<i>Euastacus maccai</i>	Valid species	
<i>Euastacus maidae</i>	Valid species	

Table 3-3. Status of the non-priority species of *Euastacus* on the basis of the molecular taxonomic analyses.

Scientific name	Status	Notes
<i>Euastacus mirangudjin</i>	Valid species	
<i>Euastacus monteithorum</i>	Valid species	
<i>Euastacus neodiversus</i>	Valid species	
<i>Euastacus neohirsutus</i>	Valid species	
<i>Euastacus reductus</i>	Valid species + 2 distinct lineages	The valid taxon ( <i>E. reductus</i> ) from upper Karuah, Manning and Hunter River basins (NSW) was divergent from <i>E. cf. reductus</i> in the Hastings River Basin to the north as well as a <i>E. cf. reductus</i> 2 from lower (intermediately located between first two distinct lineages).
<i>Euastacus robertsi</i>	Valid species + 1 distinct lineage	North-south divergence in the most northerly distributed <i>Euastacus</i> in QLD: <i>E. robertsi</i> in Endeavour River Basin) and <i>E. cf. robertsi</i> in the Daintree River Basin. Previously inferred by Shull et al. (2005).
<i>Euastacus setosus</i>	Valid species	
<i>Euastacus spinifer</i>	Valid species	
<i>Euastacus sulcatus</i>	Valid species	
<i>Euastacus urospinosus</i>	Valid species + 1 distinct lineage	As revealed by Hurry et al. (2015), the Conondale National Park population was divergent from others.
<i>Euastacus valentulus</i>	Valid species	
<i>Euastacus vesper</i>	Valid species	
<i>Euastacus woiwuru</i>	Valid species + 1 distinct lineage	<i>Euastacus woiwuru</i> from Bunyip, Yarra and Goulburn River basins in Victoria divergent from sequences from upper Mitchell River Basin.
<i>Euastacus yanga</i>	Valid species + 1 distinct lineage	Divergence of northern ( <i>E. yanga</i> ) and southern ( <i>E. cf. yanga</i> ) extent of range.
<i>Euastacus yarraensis</i>	Valid species + 1 distinct lineage	Eastern ( <i>E. yarraensis</i> ) and western ( <i>E. cf. yarraensis</i> ) divergence evident.
<i>Euastacus yigara</i>	Valid species	

### 3.4 Priority species accounts

The following section provides detailed individual accounts of the results of the genetic analyses for the priority species (in alphabetical order) with divergence levels based on the COI gene sequence comparisons. The outcomes of the targeted sequencing are included

#### *Euastacus bidawalus*

Valid species

*Euastacus bidawalus* was described by Morgan, 1986 with the type locality of Chandlers Creek (East Gippsland Basin) in eastern Victoria ([Morgan 1986](#)). For the present study, a total of 25 samples were sequenced (Figure 3-5). The species was confirmed as distinct, with mean divergence of 7.8% (range: 7.0–8.4%), from the nearest



related clusters (i.e., *E. diversus* and *E. sp. nov. 1*). Several samples assigned in the field as other species (such as *E. sp. nov. 2*), clustered with *E. bidawalus* samples without showing significant genetic divergence and there was no evidence of the presence of more than one species. Nevertheless, intra-specific variation within *E. bidawalus* was evident (mean divergence: 1%; range: 0–1.6%), suggestive of geographically-based patterns of divergence, some of which may have conservation significance.

All but one of the 13 GenBank (COI) sequences from GenBank labelled as *E. bidawalus* clustered closely with sequences from our study, further supporting its validity. Interestingly, two highly divergent GenBank sequences were apparent. A single sample (GenBank accession number: KT454670.1) from the Nadgee River in NSW, a location not represented in our study, suggests the possibility of geographically-based speciation and requires further investigation. In addition, another GenBank sequence (DQ006313.1, labeled as ‘unidentified’ on GenBank) was highly divergent despite being from a crayfish that is potentially sympatric with two other crayfish for which GenBank sequences are available (DQ006314.1 and DQ006315.1 labelled as *E. bidawalus*), suggesting the possibility of further unrecognised speciation associated with this species. Targeted sequencing of a sample (CA218) from this location was assigned as *E.*



clustering with the sequence in our study. Nevertheless, the data suggest the possibility of some geographically-based variation between samples (DQ006320.1, DQ006321.1) in the vicinity of the type locality and others (CA162; KT454671.1), which deserves further investigation, especially given the low sample representation (i.e., four samples). This study extends the range of the species into the upper Macleay River Basin.

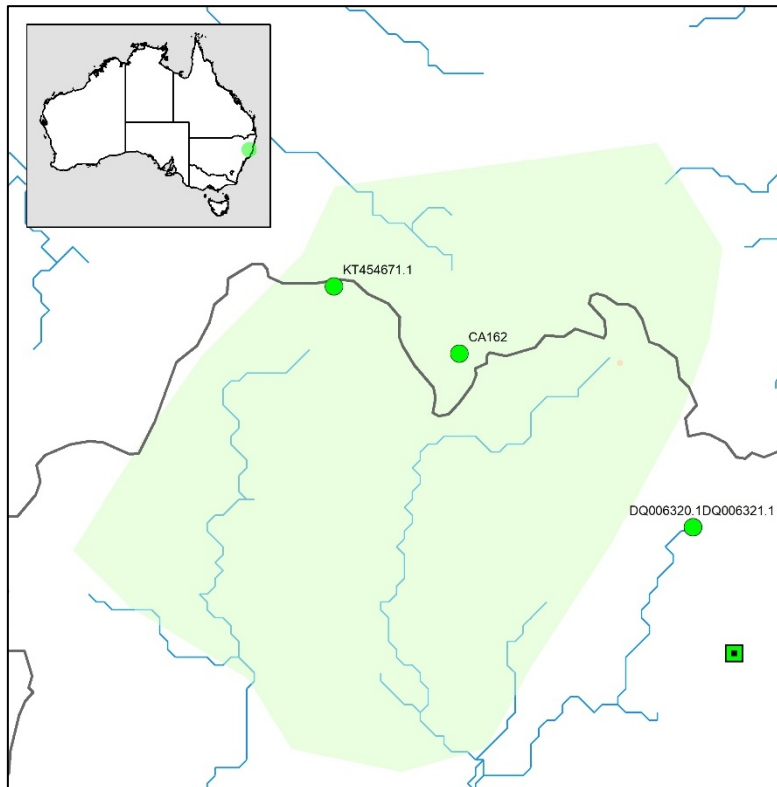


Figure 3-6. Location of *Euastacus clarkae* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in the Hastings River Basin (grey outline). The type locality of *E. clarkae* is shown (■) along with location of *E. clarkae* sequences (●).

### *Euastacus claytoni*

Valid species + 1 divergent lineage

This species is relatively broadly distributed (indicative Extent of Occurrence (EOO): 3000 km<sup>2</sup>) across southeastern NSW and eastern Victoria. *Euastacus claytoni* was described by Riek in 1969 with the type locality from the MacLaughlin River in the Snowy River Basin, NSW ([Riek 1969](#)).





Six samples identified as *E. claytoni* were sequenced for this study (Figure 3-7), which were all divergent from samples obtained from the closely related *E. yanga* (mean: 5.4%; range: 4.8–6.1%), supporting the validity of the species (Shull et al. 2005). Within *E. claytoni*, there was significant geographical variation with two samples (CA078; CA083) from the southern range of the species (labelled as *E. claytoni* due to proximity to type locality) being quite divergent from four northern range samples (CA039; CA040; CA124; CA125) labelled as *E. cf. claytoni* (mean: 4.4%; range: 4.2–4.6%).

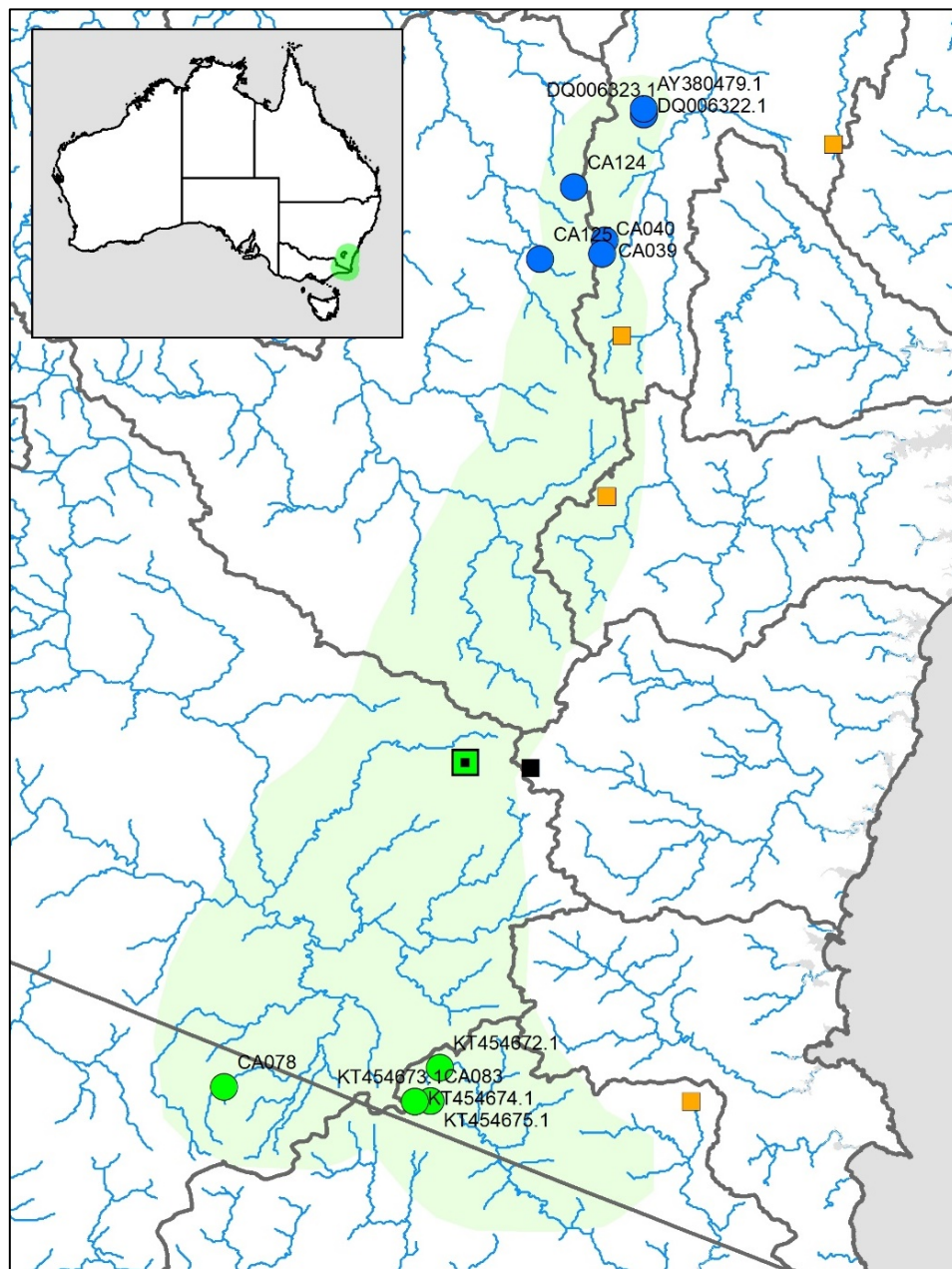


Figure 3-7. Location of *Euastacus claytoni* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in river basins (grey outlines). The type locality is shown (green square) along with distinct lineages are indicated as *E. claytoni* (green circle); and *E. cf. claytoni* (blue circle). For comparative purposes, geographically close species (*E. yanga* and *E. cf. yanga* (orange square) and *E. brachythorax* (black square) are also shown.

Seven GenBank (COI) sequences (*E. claytoni*: KT454672.1 to KT454675.1; *E. cf. claytoni*: DQ006322.1, DQ006323.1, and AY380479.1) confirm this pattern of divergence, consistent with the possibility that the northern (from Murrumbidgee River and Shoalhaven River basins) and southern (Snowy River and East Gippsland basins) populations of *E. claytoni* represent cryptic speciation. Further, sequences from GenBank indicate that the *E. claytoni* samples are divergent from the two *E. brachythorax* GenBank sequences (DQ006318.1, DQ006319.1), a species not sampled in our study. Further field surveys and sequencing of samples are required to resolve the range of *E. claytoni* and *E. cf. claytoni*, particularly between the north-south demarcation identified here.

### *Euastacus crassus*

Valid species + 1 divergent lineage

*Euastacus crassus* is a high-altitude species thought to occur across a relatively broad range across southern NSW, northern Victoria and the Australian Capital Territory (ACT). The species was described by Riek in 1951 from the upper Murrumbidgee River Basin in the ACT ([Riek 1951](#)). The species is difficult to identify in the field – the main sequencing analyses revealed that four samples anticipated to be *E. crassus* were quite divergent from each other and clustered with other samples identified as distinct taxa (*E. cf. claytoni*, *E. sp. nov. 1*, and two samples under *E. cf. rieki*). This necessitated the targeted sequencing of an additional four samples, which were able to identify *E. crassus* (CA237, CA241) as well as a divergent lineage of *E. cf. crassus* (CA211, CA213). This divergent lineage aligned with the *E. sp. nov. 7* from the main sequencing analyses, which has now been assigned as *E. cf. crassus*. Of the three GenBank sequences labelled as *E. crassus*, one appears to be a misidentification (as it matches closely with *E. armatus*). The other two samples are similar to each other and cluster with samples identified incorrectly as *E. diversus*, which in turn cluster more distantly with samples from this study labelled as *E. cf. rieki*.

These analyses appear to redefine and reduce the geographic extent of the *E. crassus* (i.e., it appears to only be distributed across the northern extent of what was thought to be its range) and highlight a divergent lineage. *Euastacus crassus* appears to occupy a narrower range, restricted to northern ACT, than previously anticipated with the distinct *E. cf. crassus* possibly occurring more broadly to the west of the ACT. Further sampling and sequencing is required to better understand the range of *E. crassus* and the divergent lineage.

### *Euastacus dalagarbe*

Valid species

The species was formally described by Coughran in 2005 from a restricted range (indicative EOO = 50 km<sup>2</sup>) across the Richmond River Basin in the Border Ranges National Park in northern NSW ([Coughran 2005](#)). The *E. dalagarbe* samples sequenced in this study confirm its validity as a distinct species given its level of divergence (mean: 3.8%; range: 3.2–4.2%) from its nearest relative, *E. binzayedii*. Of importance are two samples (CA099; CA100) that extend the known range of the species into the Logan-Albert Rivers Basin in Lamington National Park, QLD. Targeted sequencing of additional samples (CA231, CA233) identified further *E. dalagarbe* locations in the region.



Potential cryptic diversity was also revealed with two samples (CA033; CA035), labelled as *E. cf. dalagarbe*, that effectively co-occur with the new *E. dalagarbe* samples, differing by 2.8% (range: 2.5–3.0%), which represents significant differences given the sequences are from sympatric specimens. Similarly, a further two samples (CA147; CA148), are sufficiently divergent (mean: 3.7%; range: 3.8–3.6%) from *E. binzayedii* (labelled as *E. cf. binzayedii*) as well as being geographically separated (occurring to the east of all known *E. binzayedii* samples in the South Coast Basin) of the *E. binzayedii* cluster (n=6).

The intraspecific variation within *E. dalagarbe* (mean: 0.04%; range: 0.0–0.8%) and *E. binzayedii* was relatively low (mean: 0.9%; range: 0.1–1.4%), as expected for range restricted species. The single GenBank sequence for each species (*E. dalagarbe*: MT373754.1; *E. binzayedii*: MT373782.1) cluster consistently with the species as defined here. Whilst these findings corroborate the validity of *E. dalagarbe* (as well as that of *E. binzayedii*) and extend its range, the extent of cryptic diversity across a relatively small geographic area necessitates more detailed study to better define taxonomic boundaries and identify conservationally significant populations.

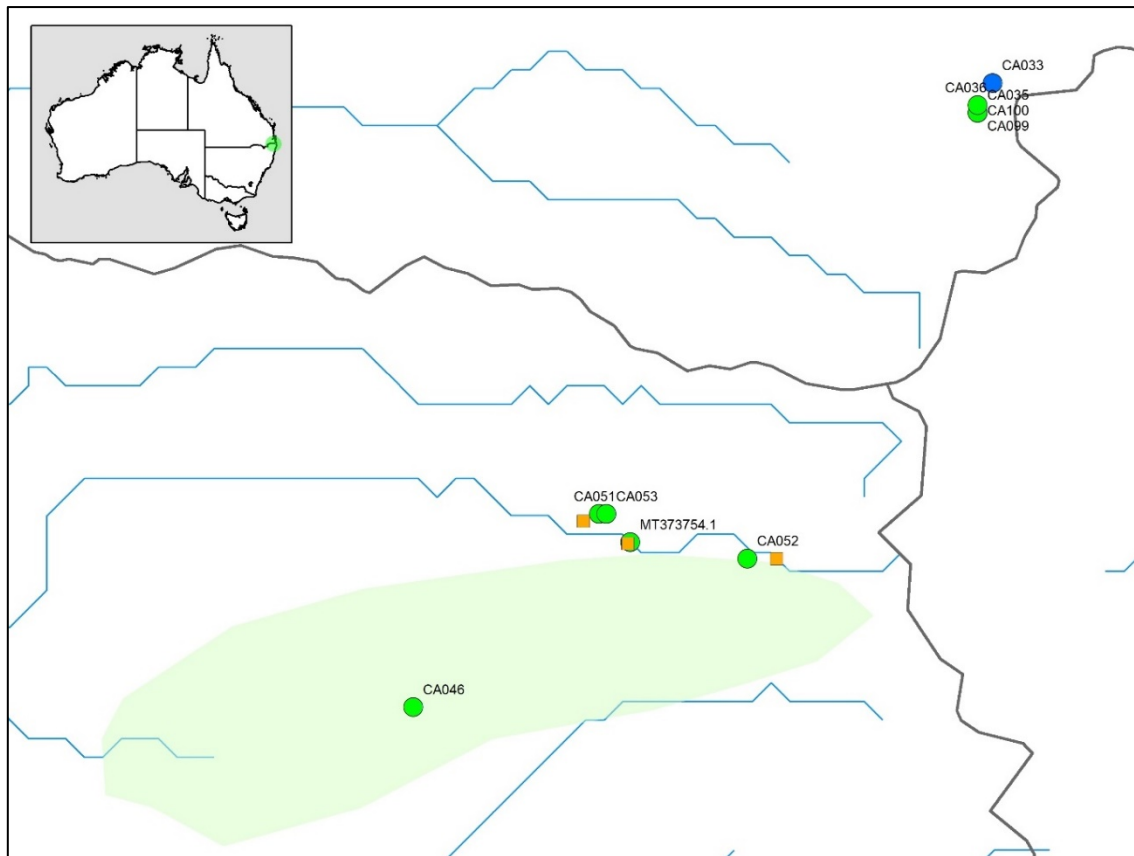


Figure 3-8. Location of *Euastacus dalagarbe* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in the Richmond River Basin (grey outline). The type locality is shown (L) along with the distinct lineages of *E. dalagarbe* (●); *E. cf. dalagarbe* 1 (●); and the geographically close species (*E. angustus*) (■).

### *Euastacus diversus*

Valid species

*Euastacus diversus* Riek, 1969 was described on the basis of a limited number of specimens obtained from a single location (Riek 1969). The phylogenetic analyses of Shull et al. (2005) recognised the species and Coughran et al. (2015) provided a redescription of the species based on new morphological and genetic information. The present study corroborated the species status of *E. diversus*, with our samples being divergent (mean: 4.5%; range: 4.2–4.8%) from the most closely related, but geographically disjunct, *E. sp. nov. 1*, an undescribed species supported as valid by this study (Figure 3-9).

The mean intraspecific divergence among the *E. diversus* samples was 1.5% (range: 0–2.1%) and within *E. sp. nov. 1* it was 0.6% (range: 0–1.2%). The addition of the GenBank COI sequences confirmed the distinction between these two groups and the GenBank confusion with respect to the identification of *E. diversus*. Twenty-five GenBank sequences, labelled as *E. diversus*, clustered with the five samples of *E. diversus* from this study, consistent with our

concept for this species. These samples also highlight geographically-based variation within our more narrowly defined *E. diversus*. A further eight GenBank sequences, labelled as *E. diversus*, were found to cluster with *E. sp. nov. 1*, and *E. diversus* has also been historically confused with the *E. rieki* complex and *E. crassus* with two of these sequences clustering with the *E. rieki* complex.

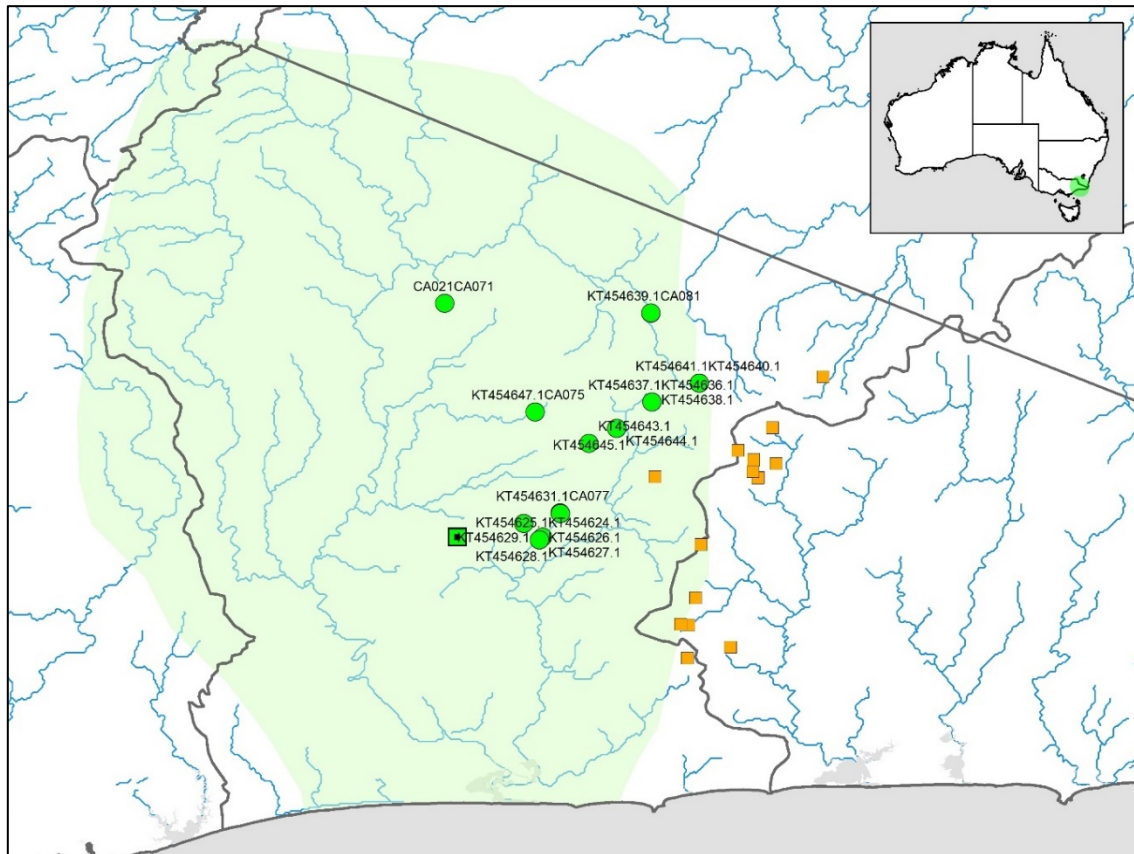


Figure 3-9. Location of *Euastacus diversus* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for sequences) in waterways across the indicative range (green shade) in river basins (grey outlines). The type locality is shown (□) along with *E. diversus* (●) and, for comparative purposes, the geographically close *E. sp. nov. 1* (■) is also shown.

*Euastacus gamilaroi*

Valid species

This species occurs across a very restricted range (indicative EOO = 10 km<sup>2</sup>), having been described by Morgan in 1997 from a few specimens from the in the upper Namoi River Basin ([Morgan 1997](#)). Consistent with Shull et al. ([2005](#)), this study supported *E. gamilaroi* as genetically distinct on the basis of four samples from separate populations (CA151–



CA154), with limited intra-specific variation (mean: 0.1%; range: 0–0.2%). The *E. gamilaroi* lineage was most closely related to the geographically proximate *E. spinichelatus* cluster (mean divergence: 8.6%; range: 7.8–8.9%), which occurs to the east of the *E. gamilaroi* samples (Figure 3-10). Two additional sequences (CA224, CA226), identified as *E. spinichelatus* in the field, aligned with *E. gamilaroi*, act to extend the eastern extent of the range of the species.

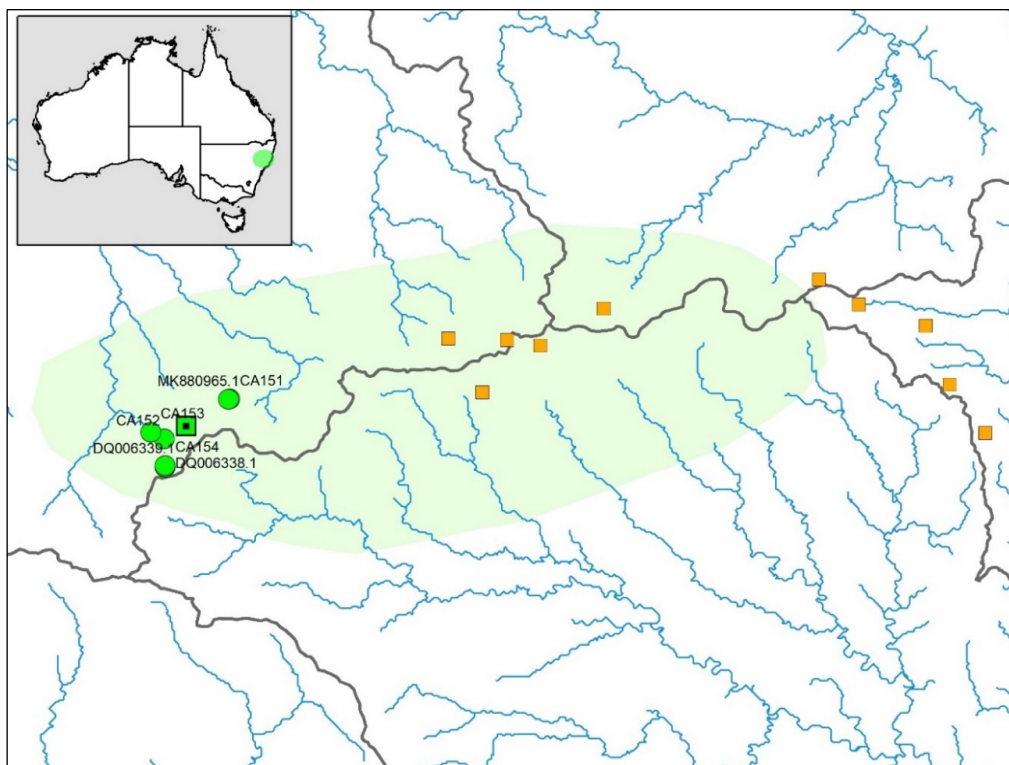


Figure 3-10. Location of *Euastacus gamilaroi* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in river basins (grey outline). The type locality is shown (■) along with *E. gamilaroi* (●). For comparative purposes, geographically close species (*E. spinichelatus*; *E. cf. spinichelatus* 1; and *E. cf. spinichelatus* 2 (■)) are also shown.

Three GenBank COI sequences (DQ006338.1; DQ006339.1; MK880965.1), one of which was submitted as an unknown species, clustered closely with the samples from our study confirming the identity of this species.

### *Euastacus girurmulayn*

Valid species



*Euastacus girurmulayn* was described by Coughran in 2005 from the Tuntable Creek (Richmond River Basin) in Nightcap National Park, northern NSW ([Coughran 2005](#)). The current study is the first to corroborate its validity (with no GenBank sequences available). Five *E. girurmulayn* sequences cluster tightly with limited divergence

(mean: 1.1%; range: 0.1–1.6%), distinct from a cluster containing *E. jagabar* and *E. guruhgi* (mean: 3.7%; range: 3.0–4.1%). The collection localities of samples from this study (Figure 3-11) increase the southern extent of the distribution, with two targeted sequence (CA232, CA234) furthering increasing the range to the east and northeast, for this range restricted species (indicative EOO = 10 km<sup>2</sup>). Future studies need to focus on more comprehensive surveying and sample collection to better delimit the range of this species in relation to the closely related *E. jagabar* and *E. guruhgi*.

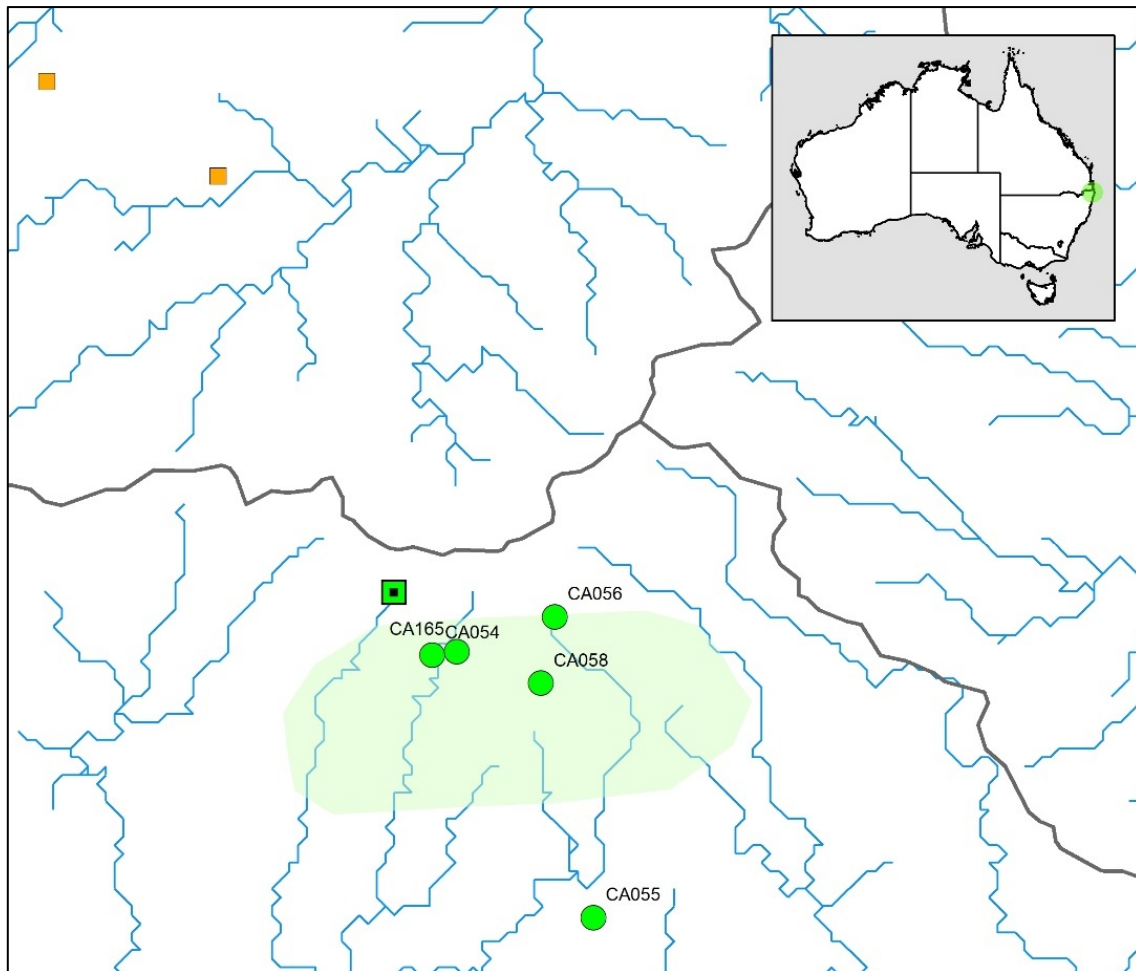


Figure 3-11. Location of *Euastacus girurmulayn* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in river basins (grey outline). The type locality is shown (■) along with *E. girurmulayn* (●). For comparative purposes, geographically close species (*E. guruhgi*) (■) is also shown.

### *Euastacus gumar* and *Euastacus pilosus*

Single valid species



*Euastacus gumar* (above left) was described by Morgan in 1997 from two sites in the upper Richmond River Basin ([Morgan 1997](#)). Coughran and Leckie ([2007](#)) identified *E. pilosus* (above



right) as morphologically distinct (but similar – see above) from *E. gumar*, and the two species are known to occur in separate areas of the upper Richmond River Basin (Figure 3-12). In this study, two samples of *E. gumar* (CA102; CA203) and three samples *E. pilosus* (CA101; CA105; and CA106) form a distinct cluster, which is further differentiated into two groupings that correspond to two genetic groups. The two samples subject to targeting sequencing (CA222, CA235) fall within the distinct cluster (with one within each of the genetic groups).

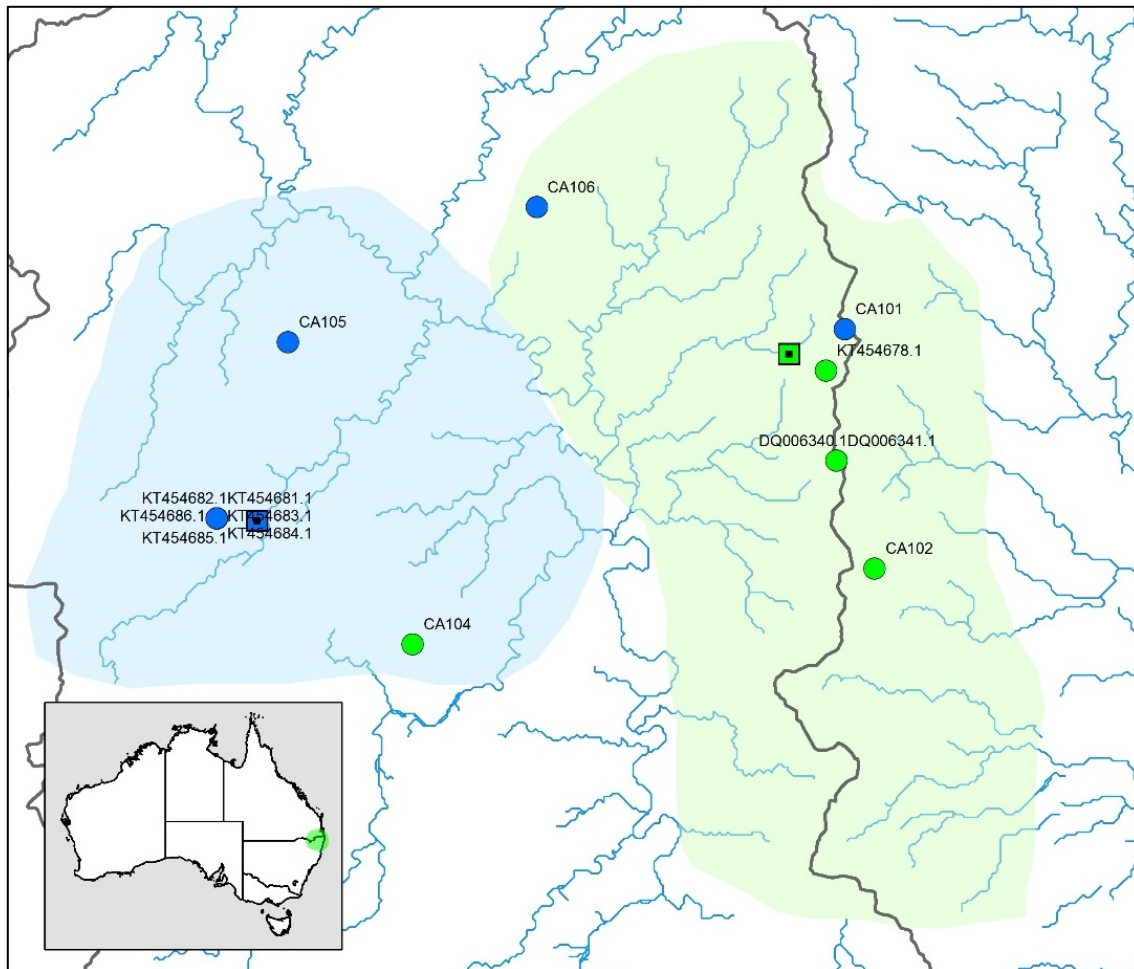


Figure 3-12. Location of *Euastacus gumar* and *Euastacus pilosus* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range of *E. gumar* (green shade) and *E. pilosus* (blue shade) in river basins (grey outline). The type locality for *E. gumar* (■) and *E. pilosus* (■) with sequence locations for each grouping (*E. gumar*: ●; *E. pilosus*: ●).

However, the level of genetic divergence between the *E. gumar* and *E. pilosus* groupings (mean: 2.0%; range: 1.9–2.0%), was the lowest found between pairs of described species in this study. Nevertheless, there was only limited variation within each group (mean intraspecific divergence: 0.2% for *E. gumar*; 0.1% for *E. pilosus*) and the 12 GenBank COI sequences including three for *E. gumar* (DQ006340.1, DQ006341.1, and KT454678.1) and nine

for *E. pilosus* (KT454681.1 to KT454689.1), clustered into the same two groups identified in this study. The geographic location of samples is largely allopatric except for samples near the type locality of *E. gumar*, but incongruent with the known range of each species. Whilst there is some uncertainty regarding the taxonomic status of *E. gumar* and *E. pilosus* – i.e., whether they are closely related sibling species, or two divergent lineages within a single species (*E. gumar*) – the established morphological differences indicate conservation-relevant divergence. Further work is needed to resolve the taxonomic relationship between the two species, with the strong possibility that *E. pilosus* is a variant of *E. gumar*.

### *Euastacus guwinus*

*Euastacus guwinus* was described by Morgan in 1997 from a very restricted range (indicative EOO = 10 km<sup>2</sup>) in the Tianjara Creek (Shoalhaven River Basin), southern NSW ([Morgan 1997](#)). Whilst no samples were sequenced as part of this study - one sample subject to targeted sequencing (CA217) was thought to be from this species but was identified as *E. yanga*.



Valid species

Five COI sequences labelled as *E. guwinus* are available on GenBank. Two of these COI sequences (DQ006343.1, DQ006345.1) from Tianjara Creek, are highly divergent and distinctly cluster, indicating it to be the true *E. guwinus* within the *E. yanga* species complex, whereas the other three COI sequences (DQ006342.1, DQ006344.1, KT454679.1) labelled as *E. guwinus* were found to cluster closely with *E. yanga* samples (from this study as well as GenBank COI sequences labelled as *E. yanga*). These three sequences maybe from misidentified samples of *E. yanga*, a species that co-occurs with *E. guwinus* at this locality. Further research on the definition of *E. guwinus* and its relationship with *E. yanga* is required by sequencing a greater range of samples and an examination of variation in diagnostic taxonomic characters for both species.

*Euastacus jagabar*

## Valid species

*Euastacus jagabar* was described by Coughran in 2005 from a tributary of Sheepstation Creek (Richmond River Basin) in the Border Ranges National Park in northern NSW ([Coughran 2005](#)). Two samples (CA048; CA050) of *E. jagabar* were sequenced which were found to be distinct from *E. guruhgi* (mean divergence: 3.1%;

range: 3.0–3.1%). With no sequences of this species available on GenBank, this study is the first to confirm its distinctiveness using molecular data. Despite being highly range restricted (indicative EOO = 10 km<sup>2</sup>), more comprehensive surveys are required to better define the distribution of this species.

*Euastacus jagara*

## Valid species

*Euastacus jagara* was described from six samples collected in 1973 from Flaggy Creek (Brisbane River Basin), southern QLD ([Morgan 1988](#)). The present study included three samples (CA124; CA143; CA149) from the southern border of the indicative range of the species (Figure 3-13), which were highly divergent from other species, with



mean divergence of 10.3% (range: 10.2–10.4%) from *E. bindal*, the most closely related species. Despite the samples originating from only a limited geographic range (reflecting the restricted range of the species), variation within *E. jagara* was significant (mean: 1.6%; range: 0.5–2.1%). The three GenBank COI sequences (DQ006349.1; DQ006350.1; AY324349.1) match closely with samples from our study confirming the identification of this species and reinforcing the strong geographic pattern of divergence relating to high-altitude areas (of Brisbane River and Condamine-Culgoa Rivers basins) compared to lower altitude areas of the

Condamine-Culgoa Rivers catchment. Even though *E. jagara* is a range restricted species, additional sampling is required to better establish geographic diversity within the species.

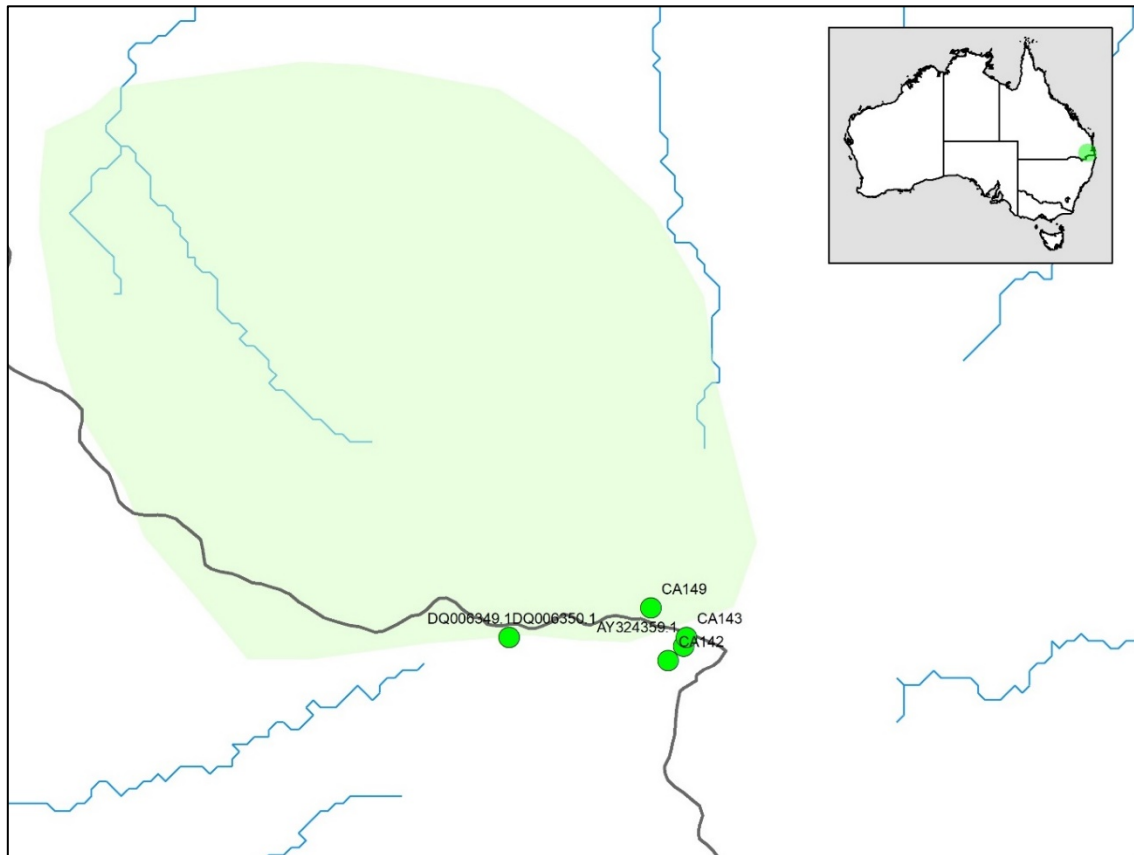


Figure 3-13. Location of *Euastacus jagara* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in river basins (grey outline). The type locality is shown (E) along with the location of *E. jagabar* (●) sequences.

### *Euastacus morgani*

Valid species



*Euastacus morgani* is one of the more recently described species ([Coughran and McCormack 2011](#)), with this study incorporating three samples collected from its restricted range (indicative EEO: <math><10\text{ km}^2</math>). These samples cluster together and are distinct from the next most similar species, *E. simplex* that is represented by a single

sample, with a mean genetic divergence of 8.3% (range: 7.8–8.6%). For *E. morgani*, two samples show little divergence (0.7%), but the third sample was surprisingly divergent given

the close proximity of all samples (mean divergence: 4.9%), indicating the possibility of the presence of a separate species. The inclusion of COI GenBank sequences indicates that there is a complicated and unresolved relationship with *E. neohirsutus*, which was not sampled in this study. One *E. neohirsutus* sequence matched closely with our narrowly defined *E. morgani*; one was identical to *E. cf. morgani* and another three form a distinct cluster at levels of divergence consistent with a separate species. This confirms there are two genetically divergent crayfish within the putative range of *E. morgani* that are likely to be sympatric. As a consequence, further studies of the relationships within and between *E. morgani* and *E. neohirsutus* and a review of morphological variation are required.

### *Euastacus polysetosus*

Valid species

*Euastacus polysetosus* has an indicative range of 750 km<sup>2</sup> in the upper Manning River Basin in NSW ([Riek 1951](#)). The initial sequencing did not account for *E. polysetosus*, but the targeted sequencing included a single sample of the species (CA215). This sample aligned with the two GenBank COI sequences, which collectively were sufficiently divergent from the genetically closest samples identified as *E. clarkae*, to confidently conclude it is a distinct taxon. *Euastacus polysetosus* also requires additional survey work and sample collection to better document the taxonomic relationships and geographic variability for this species.

### *Euastacus rieki*

Valid species + 1 divergent lineage



*Euastacus rieki* is known from a relatively broad range (indicative EOO = 5000 km<sup>2</sup>) across the alpine country of southern NSW and the ACT, with the type locality from Mount Kosciuszko in the Snowy River Basin ([Morgan 1997](#)). This study sequenced 12 samples that spanned the majority of the range of the species, except within the ACT.

These samples fell into two distinct and geographically separated clusters (mean divergence: 5.1 %; range: 4.7–5.5%), one consistent with the previous phylogenetic analyses of ([Shull et al. 2005](#)), whereas the other is a new cryptic lineage (which was anticipated by the project

team). The northern cluster was represented by samples (CA126–CA130) from the upper Murrumbidgee River Basin in southern NSW (referred to as *E. rieki* given proximity to type locality) with the southern cluster of samples (labeled as *E. cf. rieki*) are from the Snowy River, Tambo River and Upper Murray basins of eastern Victoria (Figure 3-14). Compared with the difference between these two groups, the intra-cluster differences are low: the mean divergence for *E. rieki* was 1.3% (range: 0.2–1.6%) and for *E. cf. rieki*, 1.8% (range: 0.0–2.7). Two GenBank COI sequences cluster closely with *E. rieki*, whereas another four COI sequences, labelled as *E. diversus* or *E. crassus*, aligned with the *E. cf. rieki* cluster. Targeted sequencing was able to identify *E. rieki* from the southern ACT (CA236, CA38–CA240) and Goobarragandra River in NSW (CA212) as well as extending the range of *E. cf. rieki* (CA216). Whilst the sample coverage provides a broad understanding of the range of *E. rieki* and *E. cf. rieki*, additional sequencing of samples obtained from comprehensive field surveys across the range of each species is required. That said, these findings coupled with known morphological differences will enable formal description of *E. cf. rieki* in the near future.

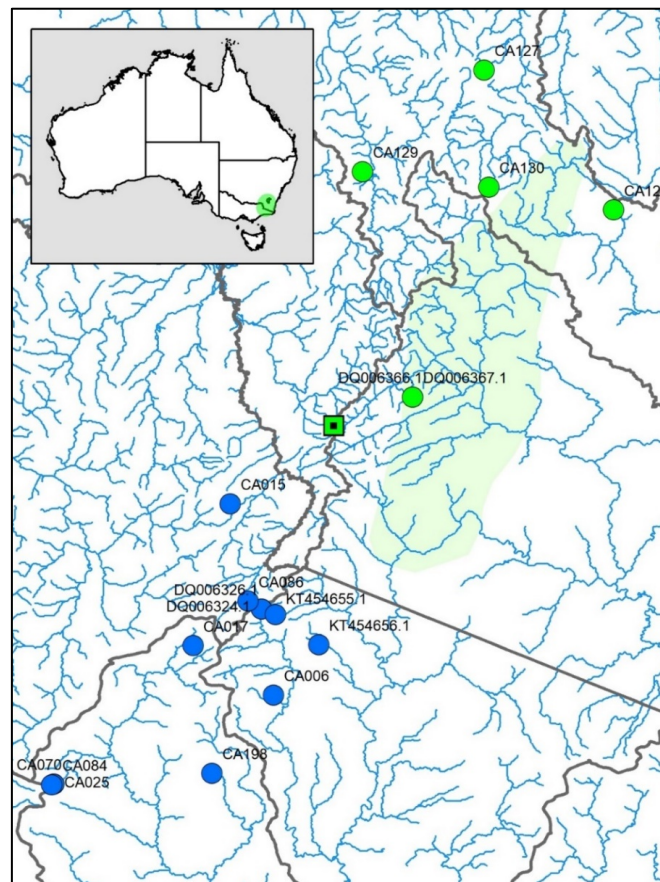


Figure 3-14. Location of *Euastacus rieki* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in river basins (grey outline). The type locality is shown (■) along with distinct lineages of *E. rieki* (●) and *E. cf. rieki* (●).

*Euastacus simplex*

Valid species



*Euastacus simplex* is broadly distributed (indicative EOO = 20,000 km<sup>2</sup>) across the Macleay River and Gwydir River basins, with the type locality in the former basin ([Riek 1951](#)). Two samples identified as *E. simplex* in the field were sequenced in this study, with one sample (CA162) from the Macleay River Basin clustering with GenBank *E.*

*clarkae* sequences (see *E. clarkae* account), whereas the other sample (CA163) was strongly divergent from other samples and is identified as *E. simplex*, which was supported by a targeted sequence (CA227). There are no GenBank sequences for this species, so with its field identification and location in the known range, the two samples provide the first genetic data for *E. simplex*. Unfortunately, the species has experienced declines in range and no longer occurs in the vicinity of the type locality (R. McCormack, unpublished data), but additional sequencing of samples across the current range of the species is warranted.

*Euastacus spinichelatus*

Valid species + 2 divergent lineages

Another species formally described by Morgan in 1997 (from 11 specimens collected in 1981), *E. spinichelatus* is known from high altitude regions of the Hasting River, Manning River and Macleay River basins ([Morgan 1997](#)). This study revealed a species complex with genetically diverse samples from across its known range (Figure 3-15), which are otherwise clearly divergent (mean divergence: 8.6%; range: 7.8–8.9%) from the related lineage, *E. gamilaroi*.



Within the *E. spinichelatus* species complex, six samples (CA133–CA135; CA140, CA141; CA155; CA156) labelled as *E. spinichelatus* sensu stricto are divergent (mean: 4.9%; range: 4.4–5.1%) from a second cluster of three samples referred to as *E. cf. spinichelatus*. Within

the *E. cf. spinichelatus* cluster another two divergent groups are apparent (mean: 3.0%; range: 3.0–3.1%): *E. cf. spinichelatus* 1 (CA140) and *E. cf. spinichelatus* 2 (CA136; CA138), with mean divergence of 0.4% revealed between the *E. cf. spinichelatus* 2 sequences. Two GenBank COI sequences labelled *E. spinichelatus*, from samples collected from close to the type locality for this species, cluster closely with one of our samples (CA135) of *E. spinichelatus* sensu stricto. There was a strong geographic correlation with respect to the three divergent lineages, with *E. spinichelatus* sensu stricto occurring across headwaters of Hasting River, Manning River, Macleay River and Namoi River basins, whereas *E. cf. spinichelatus* 1 and *E. cf. spinichelatus* 2 occur across a limited extent of the Hastings River Basin. The samples from the Namoi River Basin (CA155; CA156) act to extend the known range of this species complex. Priorities for further research include resolving the taxonomic status of *E. cf. spinichelatus* 1 and *E. cf. spinichelatus* 2 and surveying across the western extent of *E. spinichelatus* to resolve its range.

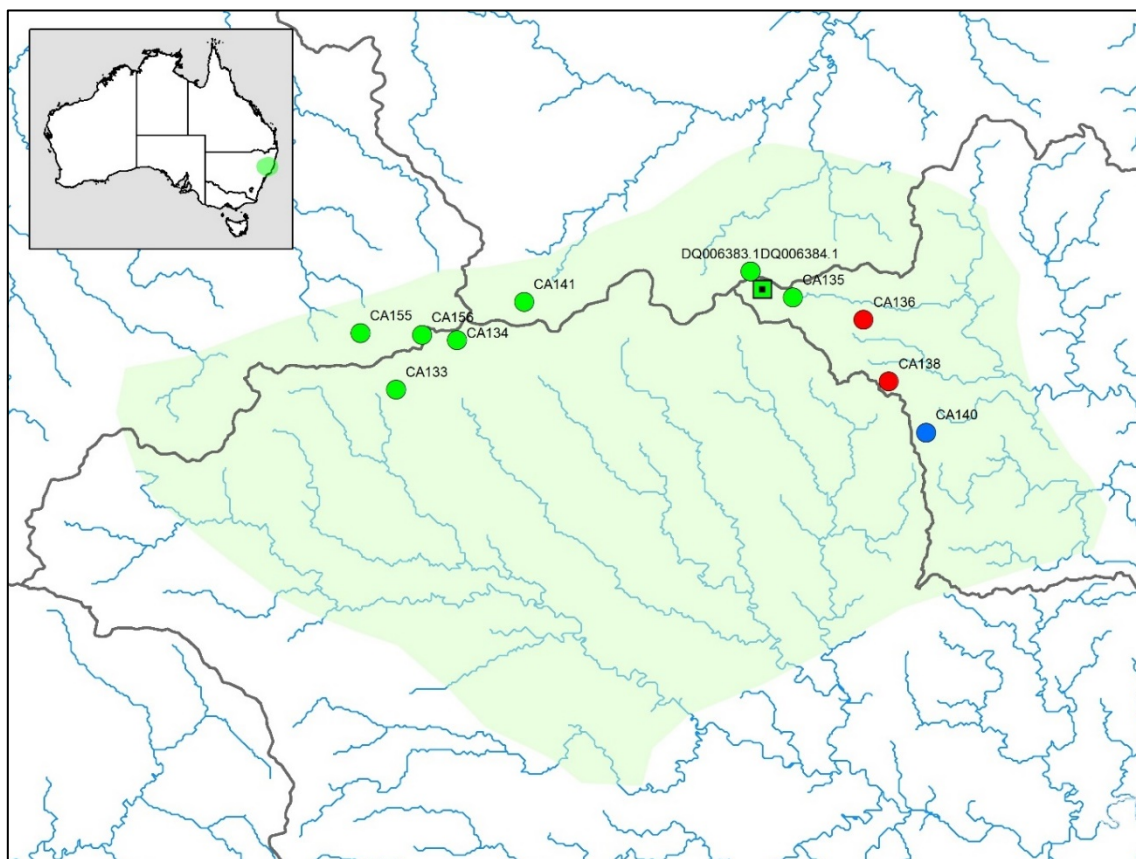


Figure 3-15. Location of *Euastacus spinichelatus* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in the river basins (grey outline). The type locality is shown (green square) along with distinct lineages of *E. spinichelatus* (green dot); *E. cf. spinichelatus* 1 (blue dot); and *E. cf. spinichelatus* 2 (red dot).



***Euastacus suttoni***

Valid species



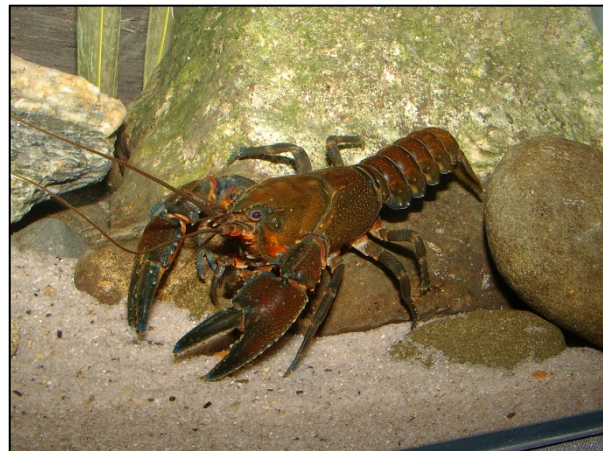
*Euastacus suttoni*, the most broadly distributed priority species (indicative EOO = 20,000 km<sup>2</sup>), was described from the Border Rivers Basin ([Clark 1941](#)). The two sequences (CA103; CA150) of *E. suttoni* in this study clustered together and were distinct from all other species. The mean divergence from the most genetically similar cluster (*E. clarkae* –

*E. maccai* cluster) was 7.5% (range: 7.2–7.8). The eleven GenBank COI sequences all cluster closely with the two samples sequenced for this study, which, taken together, cover most of the geographic range of the species.

***Euastacus* sp. nov. 1**

Valid species

*Euastacus* sp. nov. 1 is known from the Bemm River Catchment (East Gippsland Basin), with this putative species being confirmed genetically as a new distinct species related most closely to, and previously confused with, *E. diversus* (Figure 3-16). Nineteen samples were sequenced for this study, which were found to cluster



together and to be distinct from *E. diversus* (mean: 4.5%; range: 4.2–4.8%). Within *E. sp. nov. 1*, divergence was low (mean: 0.6%; range: 0–1.2%). There were eight GenBank COI sequences (labelled as *E. diversus*) were assigned to *E. sp. nov. 1*.

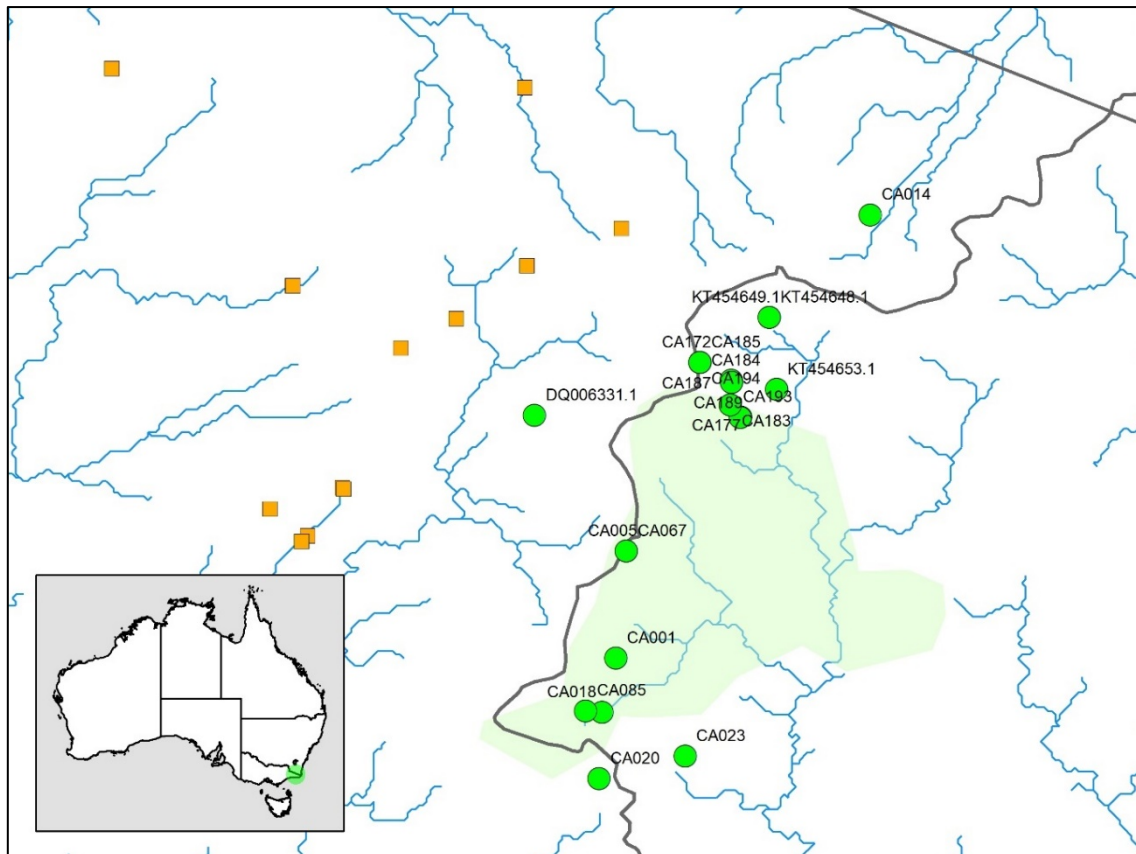


Figure 3-16. Location of *Euastacus sp. nov. 1* in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in river basins (grey outline). The distinct lineage *E. sp. nov. 1* (●) and, for comparative purposes, the geographically close *E. diversus* (■) are shown.

This study establishes that the species mostly occurs across the Bemm River Catchment of the East Gippsland Basin, but three sequences from the Snowy River Basin also aligned with *E. sp. nov. 1*. One was a GenBank COI sequence (DQ006331.1) from Ellery Creek (western extent of range); but individuals from this location are morphological consistent with *E. diversus*, so further sequencing of additional samples would be required to investigate the possibility of introgression. The formal description of this species is in preparation (e.g., McCormack and Fetzner in prep).

### *Euastacus sp. 2*

Not supported

This putative species was not supported as a candidate species with the 11 samples that were field-identified associated with other species (10 samples associated with the diverse *E. bidawalus* cluster and one sample (CA080) from the Bemm River was associated with *E. sp. nov. 1*).

*Euastacus* sp. nov. 3

Valid species



The present study sequenced 15 samples anticipated to be *E. sp. nov. 3* across its (currently known) range in the Snowy River Basin of eastern Victoria (Figure 3-17), which were significantly divergent (mean: 8.1%; range: 7.4–8.8%) from the most closely related cluster (comprising *E. sp. nov. 4*, *E. sp. nov. 5*, and the *E. rieki* species complex).

Despite geographically restricted surveys to date, intra-specific variation (mean divergence: 0.8%; range: 0.0–2.0%) was evident, with one group from a restricted range ( $n=11$ ) and the other from two locations to the south of the main group. Further survey work is required to characterise geographic variation throughout the anticipated geographic range of the species. As expected, there were no GenBank COI sequences found to match this species. The formal description of this species is underway.

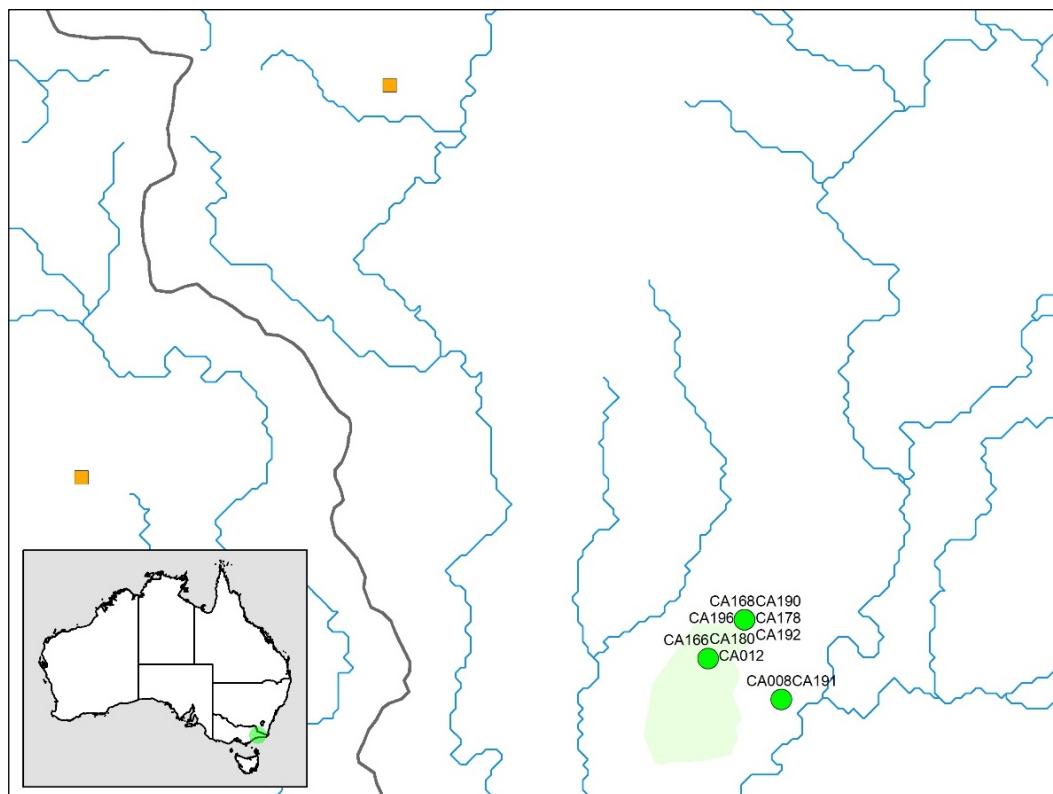


Figure 3-17. Location of *Euastacus* sp. nov. 3 in Australia (green dot in insert) and regionally (main map). The regional map shows sample sequences (with study code or GenBank accession number for GenBank sequences) in waterways across the indicative range (green shade) in river basins (grey outline). The distinct lineage *E. sp. nov. 3* (●) and, for comparative purposes, the most geographically close species (*E. cf. rieki*, ■) are shown.

### 3.5 *Unexpected putative species from bushfire-impacted regions*

In addition to the cryptic diversity revealed within described species (highlighted in Section 3.4), several unexpected putative species were discovered, each of which are potentially bushfire-impacted. This section provides a brief account of these taxa.

#### *Euastacus* sp. nov. 4

Three samples grouped distantly with the *E. rieki* complex cluster of samples and were not close to any GenBank COI sequences. There was significant divergence (mean: 5.5%; range: 5.5–5.5%) between the two clusters formed by the three samples, which is consistent with the geographic origins of the samples. One sample (CA131) from the Ruby Creek in the Upper Murray Basin (NSW) is hypothesised to represent a new species (e.g., *E. sp. nov. 4*) but additional survey work and sample collection will be required to clarify this.

#### *Euastacus* sp. nov. 5

The other two of three samples, mentioned above, from the Mitchell River Basin (CA011) and Tambo River (CA003) in eastern Victoria, are also highly divergent from all other samples. Sequencing of two targeted samples (CA219, CA225) provide support and act to slightly extend its known occurrence. This putative species (*E. sp. nov. 5*) is only known from two other locations in the Tambo River Basin and is a priority for further research to determine its distribution.

## 4. The way forward

### 4.1 *Summary*

The study provides the most comprehensive molecular taxonomic study conducted on *Euastacus* so far, and for any major group of freshwater crayfish worldwide. It builds on previous studies by incorporating targeted field surveys and existing curated sample collections to allow for the generation of 234 new sequences from *Euastacus* samples. We used the latest sequencing techniques to generate genome skim datasets that enabled the recovery of full mitogenome sequences, coupled with the extraction of nuclear genes to provide a multi-gene foundation to robustly resolve species boundaries, identify cryptic

species and establish geographic diversity. Further, integrating our results with all available COI gene region sequences from GenBank achieved a combined dataset of 560 sequences representing all but one of the known species of *Euastacus* as well as a significant number of new and undescribed species.

Overall, the analyses provided support for the unambiguous recognition of all currently recognised species of *Euastacus* (with two exceptions) and revealed a very high and unanticipated level of undocumented diversity. Importantly, this study provides the first molecular data for seven described species that support original morphological-based taxonomic assessments.

Of the priority species, samples thought to represent a putative new species, *E. sp. 2*, were genetically similar to other species groupings. Samples identified as *E. gumar* and *E. pilosus* were found to represent closely related lineages, with the degree of divergence between them the lowest for all validated species. Further, the geographic distribution of these samples did not match with their established geographic distributions. Thus, further study may indicate that *E. pilosus* is a junior synonym of *E. gumar*. Among the priority species, divergent lineages were found associated with five species (referred to as sibling species with the designation cf.), which gave rise to the possibility of six additional new putative species that should be added to the priority list. There were also two additional unexpected putative cryptic species (*E. sp. nov. 4*, *E. sp. nov. 5*), that were not closely associated with any existing species, but which occur within bushfire-affected regions.

All 34 non-priority species were supported, and like the priority species, also revealed a high level of unexpected cryptic diversity. There were 12 distinct lineages revealed across 11 non-priority species, whereas five sample groupings appear as distinct lineages that did not match any sequenced samples (from our study or from GenBank).

Taken together, these analyses provide a molecular taxonomic basis for the recognition of up to 82 species of *Euastacus*, with as many as 27 being putative new species, representing a more than 50% increase in the number of species for the genus. This is a remarkable finding for a major and conspicuous biotic component of Australia's waterways. It is acknowledged that additional studies on morphology, geographical distributions and ecology will be necessary to fully evaluate the new taxonomic hypotheses put forward in this study to determine the need for, and basis of, formal descriptions of new species.

## 4.2 Implications for conservation

The 2019–20 Australian megafires profoundly impacted animal and plant species, including species of *Euastacus* ([Legge et al. 2021a](#); [Legge et al. 2021b](#)). The present study forms part of a multi-faceted project initiated in response to the anticipated impacts on species of *Euastacus* believed to be threatened by the megafires. By broadening the scope of the molecular study to the whole genus, it was possible to provide a much more complete understanding of species boundaries and cryptic diversity. The finding that almost all formally described species are taxonomically valid based on genetic data ensures they remain relevant to conservation management. Indeed, more than 80% of the known species of the genus are threatened ([Richman et al. 2015](#)), so urgent conservation actions are necessary. It is anticipated that formal listing assessment of priority species by EPBC Act legislation will lead to increased conservation effort directed toward these species (underway). Many of the 27 putative new species identified in this study may warrant conservation listing, and we recommend a precautionary approach where meaningful conservation actions can be implemented before formal taxonomic recognition and description of these cryptic species. Further, most non-priority species are equally threatened and require formal assessment of conservation status. The primary implication of this study is a call to better understand, conserve and manage the substantially increased diversity of species within *Euastacus*.

The findings of this study have practical, relevant implications for the conservation of *Euastacus*. The analysis of genetic sequences from newly collected samples has, in many cases, acted to refine the known range of many species in the genus. For some described species, such as *E. dalagarbe*, the known range has increased, but for others, including *E. claytoni* (and *E. cf. claytoni*) and *E. rieki* (and *E. cf. rieki*), the discovery of two divergent lineages of taxonomic significance act to split the former known range into two smaller ranges. In many cases, new putative species were identified from areas where species of *Euastacus* had not previously been recorded, further emphasising the need to improve knowledge on the distribution of species of the genus. This may be in terms of mitigation of future disturbances, such as bushfires, drought and those imposed by climate change with the latter posing significant threat to *Euastacus* ([Bland 2017](#); [Hossain et al. 2018](#); [Richman et al. 2015](#)). Conservation actions, such as translocations ([see Zukowski et al. 2021](#)) and habitat protection and restoration, must draw on the findings of this study. Further, the fact that

protected areas (i.e., national parks) represent significant habitat for species of *Euastacus* (including many of the priority species as well as new putative species) highlights the great need to specifically consider *Euastacus* in the management of these areas.

It is anticipated that the current focus on conservation status (listing assessment against EPBC Act criteria) will facilitate the incorporation of priority species of *Euastacus* into management and lead to meaningful conservation actions and outcomes. It is recommended to extend the listing assessment project to consider all members of the genus and to other genera of vulnerable freshwater crayfish (e.g., *Engaeus*). Action plans have been developed for many Australian taxonomic groups, such as birds ([Garnett et al. 2011](#)), mammals ([Woinarski et al. 2019](#)), lizards and snakes ([Chapple et al. 2019](#)). A similar action plan for *Euastacus* would consolidate understanding and prioritise management and conservation actions. Further, an assessment of extinction risk, similar to what has been achieved for other taxonomic groups ([Geyle et al. 2021](#); [Geyle et al. 2020](#); [Lintermans et al. 2020](#)), would emphasise the urgency required to conserve species of *Euastacus*. Ultimately, this study, and other related projects, must lead to direct and meaningful action for the management and conservation for the conservation of the spiny crayfish species represented by *Euastacus*.

#### 4.3 Recommendations

This study greatly advances our understanding of the diversity of *Euastacus*. Nevertheless, some uncertainty and knowledge gaps remain regarding the full level of taxonomic and phylogenetic diversity within the genus, as the range of most species is yet to be fully documented. There is the real possibility that additional cryptic species remain to be discovered, especially from poorly sampled and unsampled geographic regions. Further, many of the putative cryptic species identified in this study are only represented by a small number of samples, so it is essential that additional targeted collections are conducted to properly determine the distribution of these potential new taxa and obtain voucher specimens for morphological and taxonomic study. Thus, continued research attention must now be focused on understanding the full diversity within *Euastacus*, combined with the rigorous establishment of species boundaries and formal taxonomic descriptions of new species where these are warranted. Aside from the conservation recommendations (above), a range of recommendations relating to this study are therefore evident. Namely:

- Comprehensive field surveys of all described and new putative species throughout their known distributions and including type localities;
- Further sequencing of additional samples to resolve taxonomic uncertainties;
- Delimitation of geographic range and characterisation of morphological variation;
- Habitat requirements and ecology of new putative species;
- Formal taxonomic description of new species; and
- Ensure ongoing resources are allocated to new taxonomic treatments of *Euastacus*.

The greater field survey effort, utilising traditional and environmental DNA (eDNA) methods, with additional molecular and morphological analyses will be critical to better resolve taxonomic uncertainties and the range of species identified in this study. Formal description of newly discovered species, and redescription of existing species will be necessary, which will require an understanding of morphological and ecological variation across the full geographic ranges of these species. This will necessitate an ongoing commitment to the allocation of resources to continue our progress in the taxonomic understanding of species of *Euastacus*, which is critical to the conservation and management of a critically important and vulnerable component of Australia's distinctive inland aquatic biodiversity.



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## Appendix 1

Table A1-1. Details for samples utilised in the molecular taxonomic analyses of *Euastacus*. Samples obtained through targeted surveys or historical collections (*S. Ahyong*, unpublished; *M. Lintermans*, unpublished; *R. McCormack*, unpublished; *T. Raadik*, unpublished; *F. Wedrowicz*, unpublished; *Miller et al. 2014*; *Whiterod et al. 2017*).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. sp. nov. 1</i>	CA001	East Gippsland	Arte River tributary	Pikes Hill Track, Errinundra	VIC	-37.53	148.78
<i>E. cf. woiwuru</i>	CA002	Mitchell River	Humffray River	West Humffray Road, Wonnagatta	VIC	-37.09	146.85
<i>E. sp. nov. 5</i>	CA003	Tambo River	Deptford Creek	Engineers Road, Stirling	VIC	-37.45	147.68
<i>E. bidawalus</i>	CA004	East Gippsland	Wingan River tributary	Drummer Road, Genoa	VIC	-37.48	149.38
<i>E. sp. nov. 1</i>	CA005	East Gippsland	Little Goolengook River	Greens Road, Errinundra National Park	VIC	-37.46	148.79
<i>E. cf. rieki</i>	CA006	Snowy River	Billy Plain Creek tributary	Falls Track, Wulgulmerang West	VIC	-37.11	148.12
<i>E. bidawalus</i>	CA007	East Gippsland	Thurra River tributary	Womb Hill Track, Noorinbee North	VIC	-37.46	149.25
<i>E. sp. nov. 3</i>	CA008	Snowy River	Running Creek	Jacksons Crossing Road, Buchan	VIC	-37.41	148.31
<i>E. sp. nov. 3</i>	CA009	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. bidawalus</i>	CA010	East Gippsland	Buldah Creek	North Buldah Track, Buldah	VIC	-37.23	149.15
<i>E. sp. nov. 5</i>	CA011	Mitchell River	Pheasant Creek tributary	Pheasant Creek Track, Wentworth	VIC	-37.37	147.49
<i>E. sp. nov. 3</i>	CA012	Snowy River	Basin Creek tributary	Unnamed track, W Tree	VIC	-37.39	148.27
<i>E. bidawalus</i>	CA013	East Gippsland	Genoa Creek	Genoa Creek Track, Genoa	VIC	-37.48	149.55
<i>E. sp. nov. 1</i>	CA014	Snowy River	Queensborough River tributary	Hensleigh Creek Road, Bendoc	VIC	-37.24	148.95
<i>E. cf. rieki</i>	CA015	Upper Murray River	Dinner Creek tributary	Dinner Creek Track, Tom Groggin	VIC	-36.64	148.01
<i>E. woiwuru</i>	CA016	Thomson River	Stoney Creek	Stoney No. 5 Track, Walhalla East	VIC	-37.90	146.54
<i>E. cf. rieki</i>	CA017	Tambo River	Straight Creek	Tailings Dam Road, Brumby	VIC	-36.99	147.92
<i>E. sp. nov. 1</i>	CA018	East Gippsland	Arte River	Glen Arte Road, Cabbage Tree Creek	VIC	-37.57	148.77
<i>E. woiwuru</i>	CA019	Bunyip River	Crystal Creek	Stoll Road, Jindivick	VIC	-37.99	145.88
<i>E. sp. nov. 1</i>	CA020	Snowy River	Cabbage Tree Creek tributary	Blairs Shortcut Track, Cabbage Tree Creek	VIC	-37.61	148.77
<i>E. diversus</i>	CA021	Snowy River	Minchin Creek	Bowens Track, Snowy River National Park	VIC	-37.14	148.45
<i>E. kershawi</i>	CA022	East Gippsland	Arte River	Arte Road, Cabbage Tree Creek	VIC	-37.55	148.80

Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. sp. nov. 1</i>	CA023	East Gippsland	McKenzie River tributary	Maidens Track, Club Terrace	VIC	-37.60	148.83
<i>E. kershawi</i>	CA024	Thomson River	Valencia Creek tributary	Blanket Wood Track, Wrathung	VIC	-37.56	147.00
<i>E. cf. rieki</i>	CA025	Tambo River	Sheepstation Creek tributary	Mount Delusion Track, Brookville	VIC	-37.33	147.58
<i>E. bidawalus</i>	CA026	East Gippsland	Log Bridge Creek	Stockyard Road, Noorinbee	VIC	-37.54	149.22
<i>E. armatus</i>	CA027	Ovens River	Buckland River	Buckland River Road, Buckland	VIC	-36.92	146.90
<i>E. cf. yanga</i>	CA028	Tuross River	Tuross River tributary	Peters Road, Badja State Forest	NSW	-36.15	149.54
<i>E. maidae</i>	CA029	Tweet River	Bilambil Creek	Tomwin Road, Tomwin	NSW	-28.24	153.38
<i>E. maidae</i>	CA030	South Coast	Tallebudgera Creek	Springbrook National Park	QLD	-28.23	153.29
<i>E. binzayedi</i>	CA031	Logan-Albert Rivers	Canungra Creek tributary	Lamington National Park Road, Lamington National Park	QLD	-28.25	153.15
<i>E. binzayedi</i>	CA032	South Coast	Coomera River tributary	Coomera Circuit, Lamington National Park	QLD	-28.22	153.19
<i>E. cf. dalagarbe</i>	CA033	Logan-Albert Rivers	Christmas Creek	Stinson Wreck, Lamington National Park	QLD	-28.32	153.12
<i>E. binzayedi</i>	CA034	South Coast	Rifle Bird Creek	Lower Bellbird Circuit, Lamington National Park	QLD	-28.20	153.19
<i>E. cf. dalagarbe</i>	CA035	Logan-Albert Rivers	Running Creel tributary	Stretcher track, Lamington National Park	QLD	-28.32	153.12
<i>E. dalagarbe</i>	CA036	Logan-Albert Rivers	Running Creel tributary	Stretcher track, Lamington National Park	QLD	-28.32	153.12
<i>E. maidae</i>	CA037	South Coast	Mudgeeraba Creek	Austinville Road, Austinville Forest Reserve	QLD	-28.18	153.30
<i>E. maidae</i>	CA038	South Coast	Currumbin Creek tributary	Cougal Cascade, Springbrook National Park	QLD	-28.24	153.35
<i>E. cf. claytoni</i>	CA039	Shoalhaven River	Bush Paddock Creek	Nicks Road, Tallaganda State Forest	NSW	-35.72	149.53
<i>E. cf. claytoni</i>	CA040	Shoalhaven River	Little Crow Valley Creek	South Forest Way, Tallaganda State Forest	NSW	-35.74	149.53
<i>E. cf. claytoni</i>	CA040	Shoalhaven River	Little Crow Valley Creek	South Forest Way, Tallaganda State Forest	NSW	-35.74	149.53
<i>E. guruhgi</i>	CA041	Tweet River	Byrrill Creek tributary	Mebbin Forest Road, Mebbin National Park	NSW	-28.41	153.20
<i>E. guruhgi</i>	CA042	Tweet River	Byrrill Creek tributary	Mount Warning Forest Hideaway, Byrrill Creek	NSW	-28.44	153.25
<i>E. valentulus</i>	CA043	Tweet River	Cabbage Tree Creek	Byrrill Creek Road, Byrrill Creek	NSW	-28.43	153.21
<i>E. sulcatus</i>	CA044	Tweet River	Quambatook Creek tributary	Boundary Track, Crystal Creek Rainforest Retreat	NSW	-28.25	153.30
<i>E. maidae</i>	CA045	Tweet River	Quambatook Creek	Boundary Track, Crystal Creek Rainforest Retreat	NSW	-28.25	153.30
<i>E. dalagarbe</i>	CA046	Richmond River	Sheep Station Creek	Tweed Range Scenic Drive, Border Ranges National Park	NSW	-28.40	153.04
<i>E. angustus</i>	CA047	Richmond River	Brindle Creek	Red Cedar Loop, Border Ranges National Park	NSW	-28.38	153.07

Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. jagabar</i>	CA048	Richmond River	Sheep Station Creek tributary	Booyong Walk, Border Ranges National Park	NSW	-28.40	153.02
<i>E. sulcatus</i>	CA049	Richmond River	Sheep Station Creek tributary	Rosewood Loop Track, Border Ranges National Park	NSW	-28.40	153.02
<i>E. jagabar</i>	CA050	Richmond River	Sheep Station Creek	Booyong Track bridge, Border Ranges National Park	NSW	-28.40	153.02
<i>E. dalagarbe</i>	CA051	Richmond River	Brindle Creek tributary	Brindle Creek Road, Border Ranges National Park	NSW	-28.38	153.07
<i>E. dalagarbe</i>	CA052	Richmond River	Brindle Creek tributary	Brindle Creek Walk, Border Ranges National Park	NSW	-28.38	153.09
<i>E. dalagarbe</i>	CA053	Richmond River	Brindle Creek tributary	Brindle Creek Road, Border Ranges National Park	NSW	-28.38	153.07
<i>E. girumalayn</i>	CA054	Richmond River	Terania Creek tributary	Terania Creek Road, Nightcap National Park	NSW	-28.57	153.31
<i>E. girumalayn</i>	CA055	Richmond River	Coopers Creek	North Rocks Road, Nightcap National Park	NSW	-28.65	153.35
<i>E. girumalayn</i>	CA056	Richmond River	Rocky Creek	Gibbergunyah Range Road, Nightcap National Park	NSW	-28.56	153.34
<i>E. sulcatus</i>	CA057	Richmond River	Rocky Creek	Gibbergunyah Range Road, Nightcap National Park	NSW	-28.56	153.34
<i>E. girumalayn</i>	CA058	Richmond River	Gibbergunyah Creek	Rummery Road, Nightcap National Park	NSW	-28.58	153.34
<i>E. valentulus</i>	CA059	Richmond River	Repentance Creek	Whian Whian Conservion Park	NSW	-28.60	153.38
<i>E. valentulus</i>	CA060	Tweet River	Commissioners Creek	Commissioners Creek Road, Commissioners Creek	NSW	-28.50	153.34
<i>E. armatus</i>	CA061	Murrumbidgee River	Talbingo Reservoir	Honeysuckle arm, Talbingo Reservoir	NSW	-35.68	148.32
<i>E. armatus</i>	CA062	Upper Murray River	Murray River	Towong	NSW	-36.14	148.00
<i>E. armatus</i>	CA063	Murray-Riverina	Murray River	River track, Gunbower National Park	NSW	-35.90	144.41
<i>E. armatus</i>	CA064	Lachlan River	Abercrombie River	Hell Hole, Ballyroe	NSW	-34.05	149.51
<i>E. armatus</i>	CA065	Macquarie-Bogan Rivers	Cudgegong River	Gays Place, Kelgoola	NSW	-32.85	150.27
<i>E. bispinosus</i>	CA066	Portland Coast	Fitzroy River	Fitzroy River Road, Tyrendarra	VIC	-38.22	141.76
<i>E. sp. nov. 1</i>	CA067	East Gippsland	Little Goolengook River	Greens Road, Errinundra National Park	VIC	-37.46	148.79
<i>E. bidawalus</i>	CA068	East Gippsland	Lockup Creek tributary	Whitegum Road, Noorinbee	VIC	-37.42	149.10
<i>E. bidawalus</i>	CA069	East Gippsland	Combienbar River tributary	Unnamed track, Combienbar	VIC	-37.47	148.99
<i>E. cf. rieki</i>	CA070	Tambo River	Sheepstation Creek	Mount Delusion Track, Brookville	VIC	-37.33	147.58
<i>E. diversus</i>	CA071	Snowy River	Minchin Creek	Bowens Track, Snowy River National Park	VIC	-37.14	148.45
<i>E. bidawalus</i>	CA072	East Gippsland	Karlo Creek	Princes Highway, Alfred National Park	VIC	-37.54	149.38
<i>E. bidawalus</i>	CA073	East Gippsland	Buldah Creek	North Buldah Track, Buldah	VIC	-37.23	149.15



Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. cf. woiwuru</i>	CA074	Mitchell River	Humffray River tributary	West Humffray Road, Wonnagatta	VIC	-37.08	146.84
<i>E. diversus</i>	CA075	Snowy River	Rodger River	Waratah Flat Road, Snowy River National Park	VIC	-37.28	148.57
<i>E. bidawalus</i>	CA076	East Gippsland	Coolwater Creek	tributary, Princes Hwy, Mt Drummer	VIC	-37.56	149.35
<i>E. diversus</i>	CA077	Snowy River	Martin Creek	Orbost-Bonang Road, Nurran	VIC	-37.42	148.60
<i>E. claytoni</i>	CA078	Snowy River	Reedy Creek	Lower Bendoc Road, Bendoc	VIC	-37.15	148.89
<i>E. bidawalus</i>	CA079	East Gippsland	Hopping Joe Creek tributary	Hopping Joe Forest Road, Bondi State Forest	NSW	-37.18	149.27
<i>E. sp. nov. 1</i>	CA080	East Gippsland	Kanuka Creek tributary	Errinundra Road, Goongerah	VIC	-37.35	148.86
<i>E. diversus</i>	CA081	Snowy River	Bonang River	Bonang Highway, Bonang	VIC	-37.15	148.72
<i>E. sp. nov. 1</i>	CA082	East Gippsland	Errinundra River tributary	Errinundra Road, Combienbar	VIC	-37.37	148.87
<i>E. claytoni</i>	CA083	East Gippsland	Taskers Creek	Bondi Forest Way , Bondi Forest	NSW	-37.17	149.24
<i>E. cf. rieki</i>	CA084	Tambo River	Sheepstation Creek tributary	Mount Delusion Track, Brookville	VIC	-37.33	147.58
<i>E. sp. nov. 1</i>	CA085	East Gippsland	Arte River	Larissa Lane, Cabbage Tree Creek	VIC	-37.57	148.76
<i>E. cf. rieki</i>	CA086	Upper Murray River	Painters Creek	Limestone-Black Mountain Road, Alpine National Park	VIC	-36.88	148.06
<i>E. bidawalus</i>	CA087	East Gippsland	Beehive creek tributary	Wb Line Road, East Gippsland	VIC	-37.33	149.25
<i>E. angustus</i>	CA088	Richmond River	Brindle Creek tributary	Brindle Creek Track, Border Ranges National Park	NSW	-28.38	153.09
<i>E. maccai</i>	CA089	Manning River	Macdonald River	Back Creek Road, Nowendec	NSW	-31.35	151.65
<i>E. maccai</i>	CA090	Namoi River	Macdonald River	Retreat Road, Retreat	NSW	-30.63	151.11
<i>E. maccai</i>	CA091	Hastings River	Forbes River tributary	Racecourse Trail, Forbes River	NSW	-31.14	152.35
<i>E. maccai</i>	CA092	Namoi River	South Head Creek	Snowball Road, Nundle State Forest	NSW	-31.45	151.28
<i>E. cf. hirsutus</i>	CA093	Shoalhaven River	Broughton Creek tributary	Foxground Road, Foxground	NSW	-34.70	150.77
<i>E. cf. hirsutus</i>	CA094	Shoalhaven River	Barrengarry Creek tributary	Belmore Falls Road, Robertson	NSW	-34.63	150.56
<i>E. neodiversus</i>	CA095	South Gippsland	Macks Creek tributary	Bulga Park Road, Tarra Bulga National Park	VIC	-38.46	146.60
<i>E. binzayedi</i>	CA096	Logan-Albert Rivers	Morans Creek	O Rielly's Forest Retreat, O'Rielly	QLD	-28.23	153.13
<i>E. binzayedi</i>	CA097	Logan-Albert Rivers	Lightning Creek	Lightening Falls Track, Lamington National Park	QLD	-28.26	153.16
<i>E. binzayedi</i>	CA098	South Coast	Nixon Creek tributary	Daves Creek Circuit, Binna Burra, Lamington National Park	QLD	-28.22	153.20
<i>E. dalagarbe</i>	CA099	Logan-Albert Rivers	Running Creel tributary	Stretcher track, Lamington National Park	QLD	-28.32	153.12

Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. dalagarbe</i>	CA100	Logan-Albert Rivers	Running Creel tributary	Stretcher track, Lamington National Park	QLD	-28.32	153.12
<i>E. pilosus</i>	CA101	Clarence River	Gorge Creek	Gorge Creek Road, Richmond Range National Park	NSW	-28.73	152.75
<i>E. gumar</i>	CA102	Richmond River	Cherry Tree Creek	Mallanganee National Park, Mallanganee NSW	NSW	-28.93	152.77
<i>E. suttoni</i>	CA103	Clarence River	Basket Swamp Creek	Basket Swamp National Park, out of Tenterfield	NSW	-28.91	152.15
<i>E. gumar</i>	CA104	Clarence River	Long Creek	Long Gully Road, Girard State Forest	NSW	-29.00	152.38
<i>E. pilosus</i>	CA105	Clarence River	Morgans Creek	Rivertree Firetrail, Boorook	NSW	-28.74	152.28
<i>E. pilosus</i>	CA106	Clarence River	Yabbra Creek tributary	Yabbra Plains Road, Yabbra National Park	NSW	-28.63	152.49
<i>E. maidae</i>	CA107	Tweet River	Quambatook Creek tributary	Boundary Track, Crystal Creek Rainforest Retreat	NSW	-28.25	153.30
<i>E. vesper</i>	CA108	Macquarie-Bogan Rivers	Cudgegong River	Cudgegong Road, Kelgoola	NSW	-32.87	150.30
<i>E. vesper</i>	CA109	Macquarie-Bogan Rivers	Cudgegong River	Kelgoola Picnic Area, Coricudgy Road, Kelgoola	NSW	-32.86	150.31
<i>E. vesper</i>	CA110	Macquarie-Bogan Rivers	Cudgegong River	Kelgoola Picnic Area, Coricudgy Road, Kelgoola	NSW	-32.86	150.31
<i>E. yanga</i>	CA111	Shoalhaven River	Trimbles Creek tributary	Moss Vale Road, Barrengarry	NSW	-34.68	150.49
<i>E. yanga</i>	CA112	Clyde River-Jervis Bay	Flat Rock Creek	Turpentine Road, Yerriyong	NSW	-35.04	150.49
<i>E. yanga</i>	CA113	Shoalhaven River	Jinden Creek	Jinden Ridge Road, Jinden	NSW	-35.88	149.56
<i>E. cf. yanga</i>	CA114	East Gippsland	Imlay Creek	Stink Trail, Mount Implay National Park	NSW	-37.17	149.68
<i>E. yanga</i>	CA115	Shoalhaven River	Tianjara Creek	Braidwood Road, Tianjara	NSW	-35.11	150.33
<i>E. angustus</i>	CA116	Richmond River	Brindle Creek	Brindle Creek Track, Border Ranges National Park	NSW	-28.38	153.07
<i>E. hirsutus</i>	CA117	Wollongong Coast	American Creek	Windy Gully Cemetery, Kembla Heights	NSW	-34.43	150.80
<i>E. cf. hirsutus</i>	CA118	Shoalhaven River	Barrengarry Creek	Belmore Falls, Wildes Meadow	NSW	-34.64	150.56
<i>E. hirsutus</i>	CA119	Wollongong Coast	Byarong Creek	Valley Drive, Figtree	NSW	-34.42	150.85
<i>E. cf. dharawalus</i>	CA120	Clyde River-Jervis Bay	Buckenbowra River	Corn Trail, Buckenbowra	NSW	-35.63	149.99
<i>E. cf. dharawalus</i>	CA121	Clyde River-Jervis Bay	Cat Creek	Beecroft Peninsula	NSW	-35.08	150.80
<i>E. cf. dharawalus</i>	CA122	Clyde River-Jervis Bay	Currowan Creek	Lyons Road, Currowan	NSW	-35.58	150.08
<i>E. dharawalus</i>	CA123	Hawkesbury River	Wingecarribee River tributary	Illawarra Highway, Moss Vale	NSW	-34.58	150.51
<i>E. cf. claytoni</i>	CA124	Murrumbidgee River	Molonglo River	Wild Cattle Flat Road, Captains Flat	NSW	-35.63	149.48
<i>E. cf. claytoni</i>	CA125	Murrumbidgee River	Careys Creek	Jerangle Road, Anembo	NSW	-35.75	149.43

Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. rieki</i>	CA126	Murrumbidgee River	Alum Creek	Bobeyan Road, Shannons Flat	NSW	-35.92	148.95
<i>E. rieki</i>	CA127	Murrumbidgee River	Peppercorn Creek tributary	Peppercorn Lean To, McLeods firetrail	NSW	-35.58	148.64
<i>E. cf. crassus</i>	CA128	Murrumbidgee River	Bulls Flat Creek	Goobarragandra Powerline Road, Goobarragandra	NSW	-35.53	148.48
<i>E. rieki</i>	CA129	Murrumbidgee River	New Maragle Creek	Boundary Road, Nurenmerenmong	NSW	-35.83	148.34
<i>E. rieki</i>	CA130	Murrumbidgee River	Nungar Creek	Schofields Trail, Kosciuszko National Park	NSW	-35.87	148.65
<i>E. sp. nov. 4</i>	CA131	Murray-Riverina	Ruby Creek	Elliot Way, Nurenmerenmong	NSW	-35.82	148.20
<i>E. armatus</i>	CA132	Murray-Riverina	Bogong Creek	Geehi Walls Trail, Geehi	NSW	-36.35	148.17
<i>E. spinichelatus</i>	CA133	Manning River	Mukki Creek	Wild Cattle Creek Road, Nowendoc State Forest	NSW	-31.41	151.57
<i>E. spinichelatus</i>	CA134	Manning River	Back Creek	Ralphs Fire Trail, Riamukka State Forest	NSW	-31.36	151.64
<i>E. spinichelatus</i>	CA135	Namoi River	Fenwicks Creek	Fenwicks Road, Doyles River State Forest	NSW	-31.31	152.02
<i>E. cf. spinichelatus 1</i>	CA136	Hastings River	Tobins River tributary	Seaview Road, Cottan-Bimbang National Park	NSW	-31.33	152.10
<i>E. spinifer</i>	CA137	Manning River	Cells River	Myrtle Scrub Road , Cottan-Bimbang National Park	NSW	-31.39	152.03
<i>E. cf. spinichelatus 1</i>	CA138	Hastings River	Stockyard Creek	Oxley Highway, Cottan-Bimbang National Park	NSW	-31.40	152.12
<i>E. spinifer</i>	CA139	Hastings River	Stockyard Creek	Oxley Highway, Cottan-Bimbang National Park	NSW	-31.40	152.12
<i>E. cf. spinichelatus 2</i>	CA140	Hastings River	Doyles River	Causeway Road, Cottan-Bimbang National Park	NSW	-31.46	152.17
<i>E. spinichelatus</i>	CA141	Macleay River	Tia River	Brackendale Road, Riamukka State Forest	NSW	-31.31	151.72
<i>E. jagara</i>	CA142	Brisbane River	Flaggy Creek	The Winder, Main Range National Park	QLD	-27.92	152.34
<i>E. jagara</i>	CA143	Condamine-Culgoa Rivers	Dalrymple Creek (north branch)	Lookout Road, Main Range National Park	QLD	-27.97	152.38
<i>E. morgani</i>	CA144	Clarence River	Burrell Creek	Bindarri National Park, Ulong	NSW	-30.23	152.92
<i>E. cf. morgani</i>	CA145	Clarence River	Burrell Creek	Bindarri National Park, Ulong	NSW	-30.23	152.92
<i>E. cf. morgani</i>	CA146	Clarence River	Little Nymboida River	Bindarri National Park, Ulong	NSW	-30.23	152.92
<i>E. cf. binzayedi</i>	CA147	South Coast	Boy-Ull Creek tributary	Repeater Station Road, Springbrook	NSW	-28.23	153.27
<i>E. cf. binzayedi</i>	CA148	South Coast	Boy-Ull Creek tributary	Repeater Station Road, Springbrook	NSW	-28.23	153.27

Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. jagara</i>	CA149	Brisbane River	Blackfellow Creek	Winder Trail, Main Range National Park	QLD	-27.96	152.37
<i>E. suttoni</i>	CA150	South Coast	Palling Yard Creek	Mount Norman Road, Girraween National Park	QLD	-28.84	152.00
<i>E. gamilaroi</i>	CA151	Namoi River	South Head Creek	Boundary Road, Nundle	NSW	-31.42	151.28
<i>E. gamilaroi</i>	CA152	Namoi River	Nuggetty Creek	Verden Road, Hanging Rock State Forest	NSW	-31.47	151.20
<i>E. gamilaroi</i>	CA153	Namoi River	Burrows Creek	Unnamed track, Hanging Rock State Forest	NSW	-31.46	151.18
<i>E. gamilaroi</i>	CA154	Namoi River	Burnt Hut Creek	upstream Sheba Dam, Hanging Rock State Forest	NSW	-31.50	151.20
<i>E. spinichelatus</i>	CA155	Namoi River	Macdonald River	Thunderbolts Way, Walcha	NSW	-31.35	151.53
<i>E. spinichelatus</i>	CA156	Namoi River	Cobrabold River	Hell Hole Road, Riamukka State Forest	NSW	-31.35	151.60
<i>E. cf. reductus</i>	CA157	Hastings River	Cobrabald Creek	Cobrabald Road, Bellangry State Forest	NSW	-31.28	152.53
<i>E. reductus</i>	CA158	Manning River	Cedar Creek tributary	Karuah River Road, Avon State Forest	NSW	-32.09	151.76
<i>E. reductus</i>	CA159	Karuah River	Hotel Creek tributary	Wang Road, Chichester State Forest	NSW	-32.19	151.70
<i>E. reductus</i>	CA160	Hunter River	Jerusalem Creek	Jerusalem Track, Barrington Tops National Park	NSW	-32.24	151.73
<i>E. bindal</i>	CA161	Haughton River	Alligator Creek	Unnamed track, Mount Elliot	QLD	-19.48	146.98
<i>E. clarkae</i>	CA162	Macleay River	Kunderang Creek tributary	Racecourse Trail, Werrikimbe National Park	NSW	-31.14	152.30
<i>E. simplex</i>	CA163	Macleay River	Styx River tributary	Boundary Trail, Styx River State Forest	NSW	-30.59	152.20
<i>E. dharawalus</i>	CA164	Shoalhaven River	Wildes Meadow Creek	Wildes Meadow Road, Wildes Meadow	NSW	-34.60	150.52
<i>E. girumalayn</i>	CA165	Richmond River	Bat Cave Creek	Protestor Falls, Nightcap National Park	NSW	-28.57	153.31
<i>E. sp. nov. 3</i>	CA166	Snowy River	Basin Creek tributary	Unnamed track, W Tree	VIC	-37.39	148.27
<i>E. sp. nov. 3</i>	CA167	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. sp. nov. 3</i>	CA168	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. bidawalus</i>	CA169	East Gippsland	Combienbar River	Buldah Trail Road, Combienbar	VIC	-37.33	149.05
<i>E. bidawalus</i>	CA170	East Gippsland	Ino Creek	Quadra Link Road, Bendoc	VIC	-37.27	149.04
<i>E. bidawalus</i>	CA171	East Gippsland	Combienbar River	Buldah Trail Road, Combienbar	VIC	-37.33	149.05
<i>E. sp. nov. 1</i>	CA172	East Gippsland	Errinundra River West Branch	Errinundra Road, Goongerah	VIC	-37.34	148.84
<i>E. bidawalus</i>	CA173	East Gippsland	Ino Creek	Quadra Link Road, Bendoc	VIC	-37.27	149.04
<i>E. bidawalus</i>	CA174	East Gippsland	Lockup Creek	Whitegum Road, Noorinbee	VIC	-37.42	149.11

Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. bidawalus</i>	CA175	East Gippsland	Combienbar River	Buldah Trail Road, Combienbar	VIC	-37.33	149.05
<i>E. bidawalus</i>	CA176	East Gippsland	Lockup Creek	Whitegum Road, Noorinbee	VIC	-37.42	149.11
<i>E. sp. nov. 1</i>	CA177	East Gippsland	Saint John Creek	Errinundra Road, Goongerah	VIC	-37.36	148.86
<i>E. sp. nov. 3</i>	CA178	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. bidawalus</i>	CA179	East Gippsland	Lock Creek	Quadra Link Road, Bendoc	VIC	-37.25	149.04
<i>E. sp. nov. 3</i>	CA180	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. bidawalus</i>	CA181	East Gippsland	Lockup Creek	Whitegum Road, Noorinbee	VIC	-37.42	149.11
<i>E. bidawalus</i>	CA182	East Gippsland	Tonghi Swamp Creek	Parsons Track, Club Terrace	VIC	-37.50	149.05
<i>E. sp. nov. 1</i>	CA183	East Gippsland	Saint John Creek	Errinundra Road, Goongerah	VIC	-37.36	148.86
<i>E. sp. nov. 1</i>	CA184	East Gippsland	Errinundra River West Branch	Errinundra Road, Goongerah	VIC	-37.34	148.84
<i>E. sp. nov. 1</i>	CA185	East Gippsland	Errinundra River West Branch	Errinundra Road, Goongerah	VIC	-37.34	148.84
<i>E. sp. nov. 3</i>	CA186	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. sp. nov. 1</i>	CA187	East Gippsland	Errinundra River West Branch	Errinundra Road, Goongerah	VIC	-37.34	148.84
<i>E. bidawalus</i>	CA188	East Gippsland	Combienbar River	Buldah Trail Road, Combienbar	VIC	-37.33	149.05
<i>E. sp. nov. 1</i>	CA189	East Gippsland	Kanuka Creek	Errinundra Road, Goongerah	VIC	-37.35	148.86
<i>E. sp. nov. 3</i>	CA190	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. sp. nov. 3</i>	CA191	Snowy River	Running Creek	Jacksons Crossing Road, Buchan	VIC	-37.41	148.31
<i>E. sp. nov. 3</i>	CA192	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. sp. nov. 1</i>	CA193	East Gippsland	Kanuka Creek	Errinundra Road, Goongerah	VIC	-37.35	148.86
<i>E. sp. nov. 1</i>	CA194	East Gippsland	Errinundra River West Branch	Errinundra Road, Goongerah	VIC	-37.34	148.84
<i>E. bidawalus</i>	CA195	East Gippsland	Lock Creek	Quadra Link Road, Bendoc	VIC	-37.25	149.04
<i>E. sp. nov. 3</i>	CA196	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. sp. nov. 3</i>	CA197	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29
<i>E. cf. rieki</i>	CA198	Tambo River	Wilkinson Creek	Wilkinson Log Road	VIC	-37.30	147.97
<i>E. bidawalus</i>	CA199	East Gippsland	Lockup Creek	Whitegum Road, Noorinbee	VIC	-37.42	149.11
<i>E. sp. nov. 3</i>	CA200	Snowy River	Camp Creek	Tulloch Ard Road, W Tree	VIC	-37.37	148.29

Table A1-1 (cont'd).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. kershawi</i>	CA201	Snowy River	Martin Creek	Bonang Road, Norran	VIC	-37.45	148.58
<i>E. neodiversus</i>	CA202	South Gippsland	Ruby Creek	Kardella-Fairbank Road, Arawata	VIC	-38.40	145.88
* <i>E. spinifer</i>	NC_026214.1	Hastings River	Ellenborough River	Elands	NSW	-31.566	152.342
* <i>E. armatus</i>	NC_026575.1	Ovens River	Ovens River	Great Alpine Road, Harrietville	VIC	-36.887	147.065
* <i>E. yarraensis</i>	NC_023811.1	Barwon River	Retreat Creek	Cape Otway Road, Birregurra	VIC	-38.328	143.907
* <i>Astacopsis gouldi</i>	NC_026215.1	Smithton-Burnie Coast	Big River	Wynard	TAS	-40.98	145.72

\*Sample from Gan et al. (2018).

\*Samples from Gan et al. (2016).

## Appendix 2

Table A2-1. Details for additional 32 samples utilised in the molecular taxonomic analyses of *Euastacus*. Samples obtained through targeted surveys or historical collections (C. Austin, unpublished; R. McCormack, unpublished; T. Raadik, unpublished).

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. cf. yarraensis</i>	CA210	Otway Coast	Aire River	Great Ocean Road, Genaire	VIC	-38.75	143.50
<i>E. cf. crassus</i>	CA211	Murrumbidgee River	Machine Creek	Argalong Road, Argalong	NSW	-35.31	148.41
<i>E. rieki</i>	CA212	Murrumbidgee River	Yarrangobilly River tributary	Snowy Mountain Highway, Yarrangobilly	NSW	-35.71	148.53
<i>E. cf. crassus</i>	CA213	Murrumbidgee River	Micalong Creek tributary	Brindabella Road, Mount Cromwell	NSW	-35.31	148.64
<i>E. reductus</i> 2	CA214	Karuah River	Trapyard Creek	Trapyard Road, Wallamba Nature Reserve, Wang Wauk	NSW	-32.14	152.29
<i>E. polysetosus</i>	CA215	Manning River	Unnamed drain	Gloucester Tops, Barrington Tops National Park	NSW	-32.06	151.60
<i>E. cf. rieki</i>	CA216	Upper Murray River	Glen Wills Creek tributary	Omeo Highway, Glen Valley	VIC	-36.85	147.51
<i>E. yanga</i>	CA217	Clyde River-Jervis Bay	Wandandian Creek tributary	Jerrawangala National Park	NSW	-35.12	150.36
<i>E. bidawalus</i>	CA218	East Gippsland	Dingo Creek	Euchre Valley Nature Drive, Lind National Park	VIC	-37.58	148.97
<i>E. sp. nov. 5</i>	CA219	Tambo River	Deptford Creek	Engineers Road, Stirling	VIC	-37.46	147.69
<i>E. binzayedii</i>	CA220	Logan-Albert Rivers	Noringalala Creek	Green Mountains Section, Lamington National Park	QLD	-28.25	153.18
<i>E. cf. reductus</i>	CA221	Hastings River	Starrs Creek	Starrs Creek Road, Coorabakh National Park	NSW	-31.70	152.51
<i>E. pilosus</i>	CA222	Clarence River	Pretty Gully	Pretty Gully downstream crossing Paddys Flat Road	NSW	-28.76	152.42
<i>E. cf. reductus</i>	CA223	Hastings River	Frenchs Creek	Frenchs Creek Road, Birriwal-Bulga National Park	NSW	-31.56	152.20
<i>E. gamilaroi</i>	CA224	Manning River	Tomalla Creek	xing Cowsby Rd, Nundle State Forest	NSW	-31.45	151.41
<i>E. sp. nov. 5</i>	CA225	Tambo River	Witch Creek tributary	Boomerang Spur Track, Brookville	VIC	-37.37	147.56
<i>E. gamilaroi</i>	CA226	Manning River	Washpool Gully tributary	Hanging Rock State Forest	NSW	-31.51	151.23
<i>E. simplex</i>	CA227	Macleay River	Bullock Creek	B78 Grafton Rd, Ebor	NSW	-30.47	152.30

Species	Sample code	River basin	Waterway	Location	State	Latitude	Longitude
<i>E. sp. nov. 11</i>	CA228	Logan-Albert Rivers	Long Creek	Mount Philips Road, Border Ranges National Park	NSW	-28.31	152.87
<i>E. sp. nov. 11</i>	CA229	Logan-Albert Rivers	Camp Creek	Mount Philips Road, Running Creek	QLD	-28.31	152.85
<i>E. sp. nov. 11</i>	CA230	Logan-Albert Rivers	Camp Creek	Mount Philips Road, Running Creek	QLD	-28.31	152.85
<i>E. dalagarbe</i>	CA231	Richmond River	Gradys Creek	Lost World Wilderness, Border Ranges National Park	NSW	-28.37	153.11
<i>E. girurmulayn</i>	CA232	Richmond River	Coopers Creek tributary	Goonengerry National Park	NSW	-28.58	153.41
<i>E. dalagarbe</i>	CA233	Logan-Albert Rivers	Running Creek tributary	Lamington National Park	QLD	-28.35	153.11
<i>E. girurmulayn</i>	CA234	Tweed River	Chowan Creek	Mount Jerusalem National Park	NSW	-28.47	153.38
<i>E. gumar</i>	CA235	Clarence River	Peacock Creek	Peacock Creek Camping Area, Richmond Range National Park	NSW	-28.66	152.72
<i>E. rieki</i>	CA236	Murrumbidgee River	Cotter River	Cotter source, Namadgi National Park	ACT	-35.76	148.86
<i>E. crassus</i>	CA237	Murrumbidgee River	Bushrangers Creek	Warks Road, Namadgi National Park	ACT	-35.40	148.80
<i>E. rieki</i>	CA238	Murrumbidgee River	Snowy Flat Creek	Mount Franklin Road, Namadgi National Park	ACT	-35.56	148.79
<i>E. rieki</i>	CA239	Murrumbidgee River	Nass Creek	Old Boboyan Road, Namadgi National Park	ACT	-35.86	148.99
<i>E. rieki</i>	CA240	Murrumbidgee River	Orroral River	Homestead Crossing, Namadgi National Park	ACT	-35.65	148.97
<i>E. crassus</i>	CA241	Murrumbidgee River	Gibraltar Creek	Above Gibraltar Falls, Paddys River	ACT	-35.49	148.93



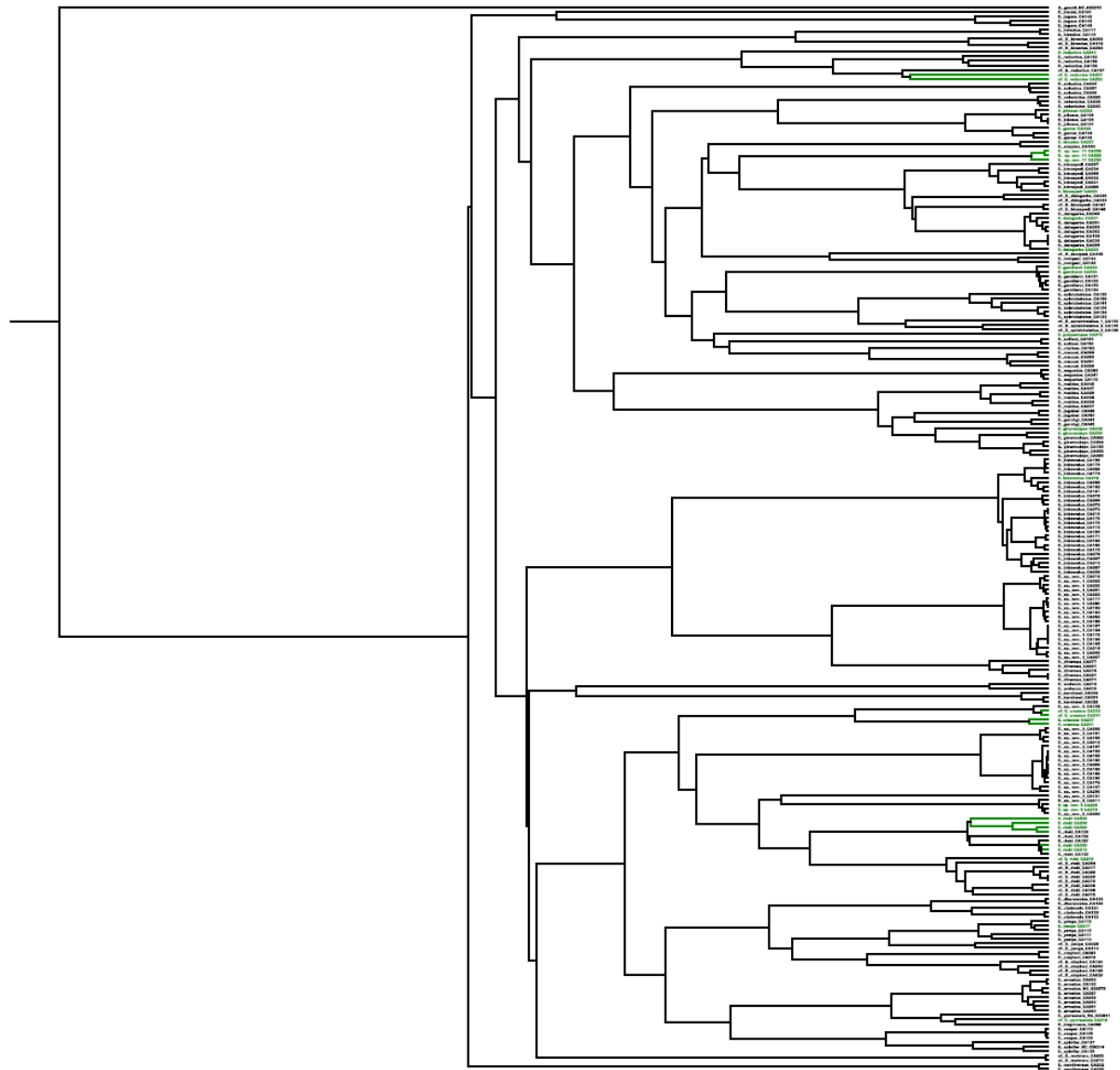


Figure A2-1. The final concatenated six-gene (COI, ND4, ND5, CYTB, 16S and 12S) tree for *Euastacus* sequenced from this study (both initial main and supplementary analyses, which are indicated in green text).

A high resolution of the concatenated six-gene tree can be found [here](#).