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Phylogeny and biogeography of the freshwater crayfish *Euastacus* (Decapoda: Parastacidae) based on nuclear and mitochondrial DNA

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Abstract

Euastacus crayfish are endemic to freshwater ecosystems of the eastern coast of Australia. While recent evolutionary studies have focused on a few of these species, here we provide a comprehensive phylogenetic estimate of relationships among the species within the genus. We sequenced three mitochondrial gene regions (COI, 16S, and 12S) and one nuclear region (28S) from 40 species of the genus *Euastacus*, as well as one undescribed species. Using these data, we estimated the phylogenetic relationships within the genus using maximum-likelihood, parsimony, and Bayesian Markov Chain Monte Carlo analyses. Using Bayes factors to test different model hypotheses, we found that the best phylogeny supports monophyletic groupings of all but two recognized species and suggests a widespread ancestor that diverged by vicariance. We also show that *Euastacus* and *Astacopsis* are most likely monophyletic sister genera. We use the resulting phylogeny as a framework to test biogeographic hypotheses relating to the diversification of the genus.

Keywords: Crayfish; Phylogeny; Biogeography; Evolution; Australia

1. Introduction

1.1. Crayfish phylogenetics

Freshwater crayfish are thought to have a monophyletic origin sometime between 185 and 225 million years ago (Crandall et al., 2000b; Scholtz and Richter, 1995). The Southern Hemisphere crayfish family Parastacidae forms a monophyletic sister group to the Northern Hemisphere crayfish (Crandall et al., 2000b). Phylogenetic analyses have successfully estimated relationships in the family Parastacidae at the generic level (e.g., Crandall et al., 1999, 2000a,b; Lawler and Crandall, 1998). Maximum-likelihood, minimum evolution, and parsimony analyses have all shown strong support for monophyletic groupings of most of the recognized genera in the family (Crandall et al., 1999, 2000b). However, phylogenetic analyses have not been performed as extensively below the genus level, such as for species of the genus *Euastacus* Clark (Decapoda: Parastacidae).

One of the outstanding issues within the Parastacidae is the grouping of the spiny crayfish, *Euastacus* and *Astacopsis* Huxley (Decapoda: Parastacidae), as separate genera. *Euastacus* inhabits only mainland Australia, while *Astacopsis* is endemic to Tasmania. Morgan (1997) classified *Euastacus* as the sister genus to *Astacopsis* and suggested that a host of morphological characters (podobranchial and telson structure, abdominal

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spination, genital papilla shape, abdominal width, and a longitudinal rostral carina) presented enough differences to justify the classification of *Euastacus* and *Astacopsis* as separate genera. However, Austin (1996) found that the allozyme electrophoretic variation was much greater between species of *Cherax* than the variation between *Euastacus* and *Astacopsis*, suggesting that they are not genetically unique enough to warrant separate genera. Lawler and Crandall (1998) suggested that *Astacopsis* was not monophyletic, but in a later study concluded that *Euastacus* and *Astacopsis* were probably distinct sister taxa (from minimum evolution and parsimony analyses), or that *Astacopsis* was derived from *Euastacus* (from maximum-likelihood analysis) (Crandall et al., 1999). Both studies used only the 16S rRNA mitochondrial gene region.

Results of phylogenetic analyses within and among crayfish genera often have depended on the method of analysis. Attempts to uncover these relationships and better understand the evolutionary histories in this family are commonly confounded by conflicting relationships depending on the optimality criterion used, and confidence in some of the conclusions drawn from the resulting phylogenies is low because of a lack of support at many of the major nodes (Crandall et al., 1999, 2000a,b; Ponniah and Hughes, 2004). With all phylogenetic methods, choice of the model of evolution is important for accurate estimation of evolutionary relationships (Hillis et al., 1994; Huelsenbeck, 1995). Bayesian methods allow for partitioned modeling of molecular evolution across different gene regions and therefore should, in theory, provide more accurate estimates of evolutionary relationships by using a more biologically realistic mixed model of evolution (Ronquist and Huelsenbeck, 2003). Thus, our study will implement this new method and compare the results of more complex modeling to traditional methods.

1.2. Classifying Euastacus

The amount of morphological variation between different populations of single species has made it difficult to rigorously delimit species within the genus *Euastacus* (see Morgan, 1997; Riek, 1969). Morphological variation between populations of the same species is common when the species inhabits a large range (e.g., *E. spinifer*, *E. australasiensis*, *E. yanga*, and *E. woiwuru*) or even in species with a narrow range (*E. neohirsutus*) (Morgan, 1997). However, on occasion there is little morphological variation between populations across a large range, as is the case with *Euastacus armatus* (Morgan, 1997). In this case, measurable genetic variation still exists (Versteegen and Lawler, 1997), suggesting that molecular techniques will yield greater resolution for delimiting species and recovering their relationships. Morgan (1997) suggests that many of these populations may be semi-isolated, which could possibly increase speciation rates. If this is true, phylogenetic data will aid in better understanding the evolutionary processes occurring throughout the genus.

1.3. Biogeography of Euastacus

Euastacus crayfish are endemic to the eastern and southeastern coast of Australia (Fig. 1). There are now 43 named species distributed throughout Queensland, New South Wales, and Victoria (Table 1) (Coughran, 2002; Morgan, 1986, 1988, 1989, 1997; Short and Davie, 1993). Most *Euastacus* species live in cool streams surrounded by forests, often in areas where human growth, activity, and environmental modification threaten, limit, or decrease their population sizes (Horwitz, 1995; Merrick, 1997). *Euastacus* are a useful group for broad biogeographical studies because the genus is generally distributed along a north–south axis. In northern Queensland, *Euastacus* species are cold-adapted specialists restricted to mountain refuges that rise from the Wet Tropics lowlands (Nix, 1991; Ponniah and Hughes, 2004). Extensive biogeographical studies have been done of both vertebrates and invertebrates in this northern area (Bell et al., 2004; Hugall et al., 2002; O'Connor and Moritz, 2003; Schneider et al., 1998), many producing similar phylogeographic patterns, most notably a break across the Black Mountain Corridor that separates *E. robertsi* and *E. fleckeri* from the rest of the genus. Ponniah and Hughes (2004) used the linear distribution of *Euastacus* to test whether the Queensland species diverged by a simultaneous vicariance event or by south to north dispersal. They concluded that there was simultaneous vicariance of at least two ancestral Queensland lineages. An increase in temperature and decline in moisture probably caused the ancestral lineages to retreat higher onto the mountains, stopping gene flow between populations and leading to divergence into modern Queensland species. In New South Wales and Victoria, the general pattern of distribution is that lowland *Euastacus* species are physically larger and also have larger distributions, while the highland species have smaller bodies and generally smaller distributions (Morgan, 1997). This biogeographical study will incorporate these southern species along with the Queensland species already mentioned, to examine the geographical history of the entire genus.

The aims of this study are to estimate a detailed phylogeny of all *Euastacus* species using nucleotide sequence data from the 16S rRNA, 12S rRNA, and cytochrome *c* oxidase subunit I (COI) mitochondrial gene regions, and from the 28S rRNA gene region of the nuclear genome. To estimate this phylogeny as robustly as possible, we will compare three methods of phylogenetic analysis [parsimony (MP), maximum likelihood (ML), and Bayesian], and the effects of choosing mixed models over

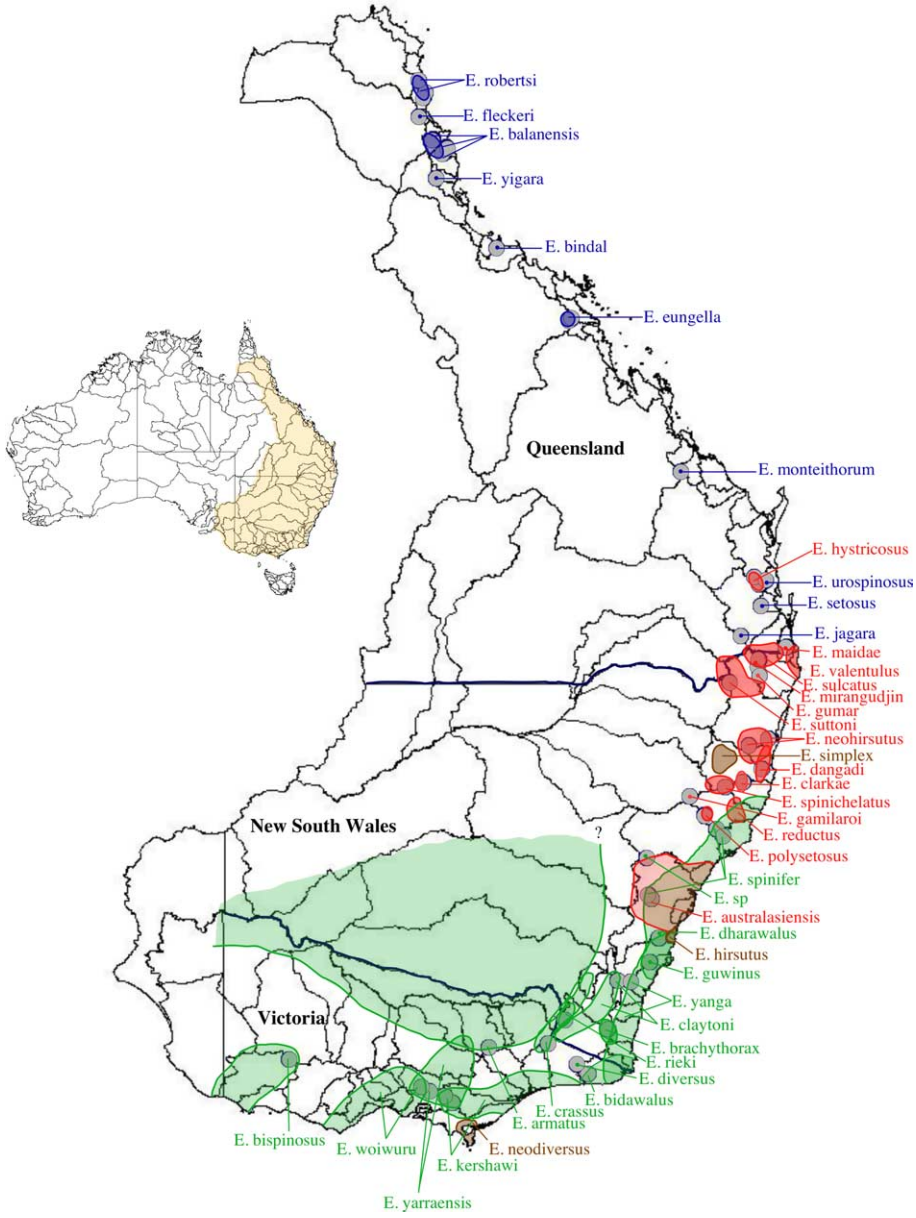

Fig. 1. Collection sites and distributions of 43 *Euastacus* species. Colors correspond to those of Figs. 2 and 3.

a single model when using multiple genes in a dataset. With the resulting phylogeny, we will test hypotheses of biogeographical distribution within the genus as well as the relationship between *Euastacus* and *Astacopsis*.

2. Materials and methods

2.1. Sampling and DNA extraction

Samples were collected by hand between 1992 and 2004 (Table 2). Gill or ovary tissue was stored in 100% ethanol (some older samples were stored in 70% ethanol for several years before extraction) and stored at −80°C after extraction. One sample from the Museum Victoria was included in this analysis (*E. diversus*) and had been stored in formalin. Sampling localities and geographic distributions are shown in Fig. 1.

Paranephrops and *Cherax* species were collected and used as outgroups. *Astacopsis* is clearly the most closely related to *Euastacus*, but it is uncertain whether the two groups are monophyletic so it would be premature to root the tree with *Astacopsis*. Using the phylogeny from Crandall et al. (1999), it appears that *Paranephrops* may be closely related to *Euastacus* but there is no support for their position within the clade. Some *Cherax* species are broadly sympatric with *Euastacus* yet occupy a distinct microhabitat and are clearly a monophyletic group distinct from *Euastacus* (Crandall et al., 1999). Therefore, *Cherax* as well as *Paranephrops* were collected

Table 1
Forty-three *Euastacus* species

Species	State	IUCN Red List Status (IUCN, 2001)
E. armatus (von Martens, 1866)	NSW/VIC	Vulnerable (A1ade)
E. australasiensis (Milne Edwards, 1837)	NSW	
E. balanensis Morgan, 1988	QLD	
E. bidawalus Morgan, 1986	NSW/VIC	
E. bindal Morgan, 1989	QLD	Endangered (B1+2c)
E. bispinosus Clark, 1936	VVIC	Vulnerable (A1ade)
E. brachythorax Riek, 1969	NSW	
E. clarkae Morgan, 1997	NSW	
E. claytoni Riek, 1969	NSW	
E. crassus Riek, 1969	NSW	Endangered (B1+2c)
E. dangadi Morgan, 1997	NSW	
E. dharawalus Morgan, 1997	NSW	
E. diversus Riek, 1969	VIC	Endangered (B1+2c)
E. eungella Morgan, 1988	QLD	Vulnerable (B1+2c)
E. fleckeri Watson, 1935	QLD	Vulnerable (B1+2c)
E. gamilaroi Morgan, 1997	NSW	
E. gumar Morgan, 1997	NSW	
E. guwinus Morgan, 1997	NSW	
E. hirsutus (McCulloch, 1917)	NSW	
E. hystricosus Riek, 1951	QLD	Vulnerable (B1+2c)
E. jagara Morgan, 1988	NSW	Endangered (B1+2c)
E. kershawi (Smith, 1912)	VIC	
E. maidae (Riek, 1956)	QLD	Endangered (B1+2c)
E. mirangudjin Coughran, 2002	NSW	
E. monteithorum Morgan, 1989	QLD	Endangered (B1+2c)
E. neodiversus Riek, 1969	VIC	Vulnerable (B1+2c)
E. neohirsutus Riek, 1956	NSW	
E. polysetosus Riek, 1951	NSW	
E. reductus Riek, 1969	NSW	
E. rieki Morgan, 1997	NSW	
E. robertsi Monroe, 1977	QLD	Endangered (B1+2c)
E. setosus (Riek, 1956)	QLD	Vulnerable (B1+2c)
E. simplex Riek, 1956	NSW	
E. spinichelatus Morgan, 1997	NSW	
E. spinifer (Heller, 1865)	NSW	
E. sulcatus Riek, 1951	QLD/NSW	
E. suttoni Clark, 1941	QLD/NSW	
E. urospinosus (Riek, 1956)	QLD	Endangered (B1+2c)
E. valentulus Riek, 1951	QLD/NSW	
E. woiwuru Morgan, 1986	VIC	
E. yanga Morgan, 1997	NSW	
E. yarraensis (McCoy, 1888)	VIC	
E. yigara Short and Davie, 1993	QLD	Endangered (B1+2c)

NSW, New South Wales; QLD, Queensland; VIC, Victoria. A1ade, estimated population reduction of at least 50% over the last ten years from direct observation, levels of exploitation, and the effects of competitors, pathogens, or pollutants. B1+2c, extent of occurrence estimated to be less than 5000 km^2 (endangered) or 20,000 km^2 (vulnerable), estimates indicating severely fragmented populations or known to exist at no more than five locations (endangered) or 10 locations (vulnerable), and continuing decline in area, extent and/or quality of habitat.

along with *Euastacus* samples and used as outgroups, while *Astacopsis* was analyzed as part of the ingroup.

DNA was extracted using a cell-lysis protocol as described in Crandall et al. (1999). Approximately, 5–15 mg of tissue was placed in 800 µl of cell-lysis solution (10 mM Tris base, 100 mM EDTA, 2% SDS, pH 8.0), to which 9 µl of proteinase K (10 mg/ml) was added. The samples were incubated overnight at 55 °C and mixed continually on a rotator. A volume of 180 µl of 5 M NaCl was added to the mixture and vortexed, then centrifuged to pellet out the salt. The supernatant was transferred to a clean cryo-tube and 420 µl of ice-cold isopropanol was added and mixed slowly. The mixture was centrifuged at 13,000 rpm for 10 min. The supernatant was removed and the DNA pellet was washed with 500 µl of 70% ethanol, then mixed on a cell rotator for approximately 1 h. The supernatant was removed and the DNA pellet was dried for 15 min at 55 °C in a dry vacuum, then resuspended in 30–100 µl TLE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Tissue that had been stored for more than a few months or kept in 70% ethanol was extracted using the DNeasy Tissue Kit (Qiagen, Inc.). One sample had been fixed in formalin (*E. diversus* KC 2773) and was extracted using a modified extended digestion technique (Bucklin and Allen, 2001; Diaz-Cano and Brady, 1997). Extractions were checked on a 1.5% agarose gel and diluted with purified water to obtain an approximate DNA concentration of 10 µg/ml, estimated according to the brightness of the bands in the gel.

2.2. Amplification, sequencing, and alignment

PCR products were obtained for each specimen using 50 µl reactions (see Table 3 for primers, annealing temperature, and PCR product size) with the following combination of reagents: 0.5× PCR buffer, 1.25 mM each dNTP, 2.5 mM magnesium chloride, 1 µM each primer, 0.6 U of *Taq* DNA polymerase, and 15 ng of sample DNA. PCR was performed on a Peltier Thermal Cycler machine with a standard three-step denaturation, annealing, and extension protocol with temperatures given in Table 3. Internal primers were used to sequence a larger 28S region; however, only the first 586 bp of the region were conserved enough to align and include in the dataset. To amplify the formalin-fixed sample, a consensus sequence was made from previous *Euastacus* data collected, and new internal primers were designed to amplify each gene in small fragments approximately 200 bp long (Table 3).

PCR products were purified using a Montage PCR_{96} plate (Millipore). Sequencing reactions were done using the ABI Big-dye Ready-Reaction Kit with a 1/8 reaction, and sequences were generated on an Applied Biosystems 3730 XL Automated Sequencer.

Nucleotide sequences were checked and cleaned in Sequencher 4.2 (Gene Codes Corporation). Each gene region was aligned using Clustal X (Thompson et al., 1997) and adjusted by hand in MacClade 4.05 OS X (Sinauer Associates, Inc.) to fix obvious errors in alignment. The different gene regions were then concatenated for a single data file for subsequent analyses. The resulting

Table 2
Sample collection information

Species	Sample	Site	State	Latitude	Longitude	Date	Queensland Museum Reg. No.
Astacopsis gouldi	KC2308	Mersey River	TAS		05/01		
A. tricornis	KC0614	Huon River	TAS		02/07/93		
A. tricornis	KC0615	Twin Creeks	TAS		02/07/93		
Cherax cuspidatus	KC2697	Culmaron Ck	NSW	28.85S	152.74E	02/05/02	QMW 26579
C. parvus	KC2665	O'Leary Ck	QLD	17.95S	145.65E	05/31/02	QMW 26639
C. quadricarinatus	KC2692	U. of QLD	QLD	27.53S	152.92E	10/06/02	
E. armatus	KC2653	Buffalo R	VIC	36.99S	146.80E	03/10/02	QMW 26582
E. armatus	KC2723	Buffalo R	VIC	36.99S	146.80E	03/10/02	QMW 26582
E. armatus	KC2724	Buffalo R	VIC	36.99S	146.80E	03/10/02	QMW 26582
E. australasiensis	KC2834	Govetts Leap Brook	NSW	33.64S	150.31E	01/07/04	QMW 27483
E. australasiensis	KC2836	Govetts Leap Brook	NSW	33.64S	150.31E	01/02/04	QMW 27496
E. australasiensis	KC2637	Govetts Leap Brook	NSW	33.64S	150.31E	02/12/02	QMW 26586
E. australasiensis	KC2707	Govetts Leap Brook	NSW	33.64S	150.31E	02/12/02	QMW 26586
E. balanensis	KC2782	Kauri Ck	QLD	17.10S	145.59E	22/11/94	
E. balanensis	KC2783	Kauri Ck	QLD	17.10S	145.59E	22/11/94	
E. balanensis	KC2784	Russel R	QLD	17.40S	145.81E	19/11/94	
E. balanensis	KC2785	Russel R	QLD	17.40S	145.81E	19/11/94	
E. balanensis	KC2786	Mulgrave R	QLD	17.27S	145.87E	22/11/96	
E. balanensis	KC2787	Mulgrave R	QLD	17.27S	145.87E	22/11/96	
E. balanensis	KC2667	Kauri Ck	QLD	17.10S	145.59E	06/03/02	QMW 26587
E. balanensis	KC2735	Kauri Ck	QLD	17.10S	145.59E	06/03/02	QMW 26587
E. balanensis (n. sp?)	KC2666	Summit Ck	QLD	17.40S	145.82E	06/02/02	QMW 26594
E. balanensis (n. sp?)	KC2734	Summit Ck	QLD	17.40S	145.82E	06/02/02	QMW 26594
E. balanensis (n. sp?)	KC2625	Summit Ck	QLD	17.40S	145.82E	11/27/95	QMW 26595
E. bidawalus	KC2650	Dingo Ck	VIC	37.58S	148.97E	03/20/02	QMW 26588
E. bidawalus	KC2721	Dingo Ck	VIC	37.58S	148.97E	03/20/02	QMW 26588
E. bidawalus	KC2840	Dingo Ck	VIC	37.58S	148.97E	01/05/04	QMW 27482
E. bindal	KC2690	North Ck	QLD	19.48S	146.97E	07/21/02	QMW 26590
E. bispinosus	KC0631	Burrong Falls	VIC	37.25S	142.40E	02/11/93	
E. brachythorax	KC2647	Rutherford Ck	NSW	36.61S	149.41E	03/18/02	QMW 26593
E. brachythorax	KC2718	Rutherford Ck	NSW	36.61S	149.41E	03/18/02	QMW 26593
E. clarkae	KC2630	Cockerawombeeba Ck	NSW	31.19S	152.37E	02/07/02	QMW 26597
E. clarkae	KC2700	Cockerawombeeba Ck	NSW	31.19S	152.37E	02/07/02	QMW 26597
E. claytoni	KC2640	Lowden Ck	NSW	35.51S	149.60E	02/16/02	QMW 26600
E. claytoni	KC2711	Lowden Ck	NSW	35.51S	149.60E	02/16/02	QMW 26600
E. crassus	KC2649	Buchan R	VIC	36.90S	148.09E	03/19/02	QMW 26601
E. crassus	KC2720	Buchan R	VIC	36.90S	148.09E	03/19/02	QMW 26601
E. crassus (n. sp?)	KC2654	Buffalo R	VIC	36.99S	146.80E	03/10/02	QMW 26596
E. dangadi	KC2628	Eungai Ck	NSW	30.90S	152.79E	02/06/02	QMW 26605
E. dangadi	KC2699	Eungai Ck	NSW	30.90S	152.79E	02/06/02	QMW 26605
E. dharawalus	KC2638	Wildes Meadow Ck	NSW	34.61S	150.52E	02/13/02	QMW 26607
E. dharawalus	KC2708	Wildes Meadow Ck	NSW	34.61S	150.52E	02/13/02	QMW 26607
E. diversus	KC2773	Ellery Ck	VIC	37.37S	148.73E	06/25/94	
E. diversus	KC2841	Martins Ck	VIC	38.45S	143.58E	01/05/04	
E. eungella	KC2732	Cattle Ck	QLD	21.03S	148.60E	05/29/02	QMW 26608
E. eungella	KC2663	Cattle Ck	QLD	21.03S	148.60E	05/29/02	QMW 26608
E. eungella	KC2671	Cattle Ck	QLD	21.06S	148.56E	02/01/02	QMW 26608
E. fleckeri	KC2668	Leichhardt Ck	QLD	16.60S	145.28E	06/04/02	QMW 26611
E. fleckeri	KC2736	Leichhardt Ck	QLD	16.60S	145.28E	06/04/02	QMW 26611
E. gamilaroi	KC2632	Burrows Ck	NSW	31.50S	151.20E	02/08/02	QMW 26621
E. gamilaroi	KC2702	Burrows Ck	NSW	31.50S	151.20E	02/08/02	QMW 26621
E. gumar	KC2644	Culmaron Ck	NSW	28.84S	152.74E	03/04/02	QMW 26622
E. gumar	KC2715	Culmaron Ck	NSW	28.84S	152.74E	03/04/02	QMW 26622
E. guwinus (cf?)	KC2842	Tianjarra Ck	NSW	35.11S	150.33E	01/06/04	QMW 27485
E. guwinus (cf?)	KC2642	Tianjarra Ck	NSW	35.11S	150.33E	02/18/02	QMW 26623
E. guwinus (cf?)	KC2713	Tianjarra Ck	NSW	35.11S	150.33E	02/18/02	QMW 26623
E. guwinus (cf?)	KC2709	Tianjarra Ck	NSW	35.11S	150.33E	02/13/02	QMW 26625
E. hystricosus	KC2672	Stony Ck	QLD	26.86S	152.73E	03/26/92	
E. hystricosus	KC2673	Stony Ck	QLD	26.86S	152.73E	03/26/92	
E. hystricosus	KC2691	Booloumbah Ck	QLD	26.69S	152.62E	09/29/02	QMW 26628
E. jagara	KC2763	Shady Ck	QLD	27.97S	152.32E	27/09/00	
E. jagara	KC2764	Shady Ck	QLD	27.97S	152.32E	27/09/00	

(*continued on next page*)

Table 2 (*continued*)

Species	Sample	Site	State	Latitude	Longitude	Date	Queensland Museum Reg. No.
E. kershawi	KC2656	Moe R	VIC	38.20S	146.03E	03/21/02	QMW 26629
E. kershawi	KC2657	Labertouche Ck	VIC	38.05S	145.84E	03/21/02	QMW 26630
E. maidae	KC2658	Tallebudgera Ck	QLD	28.23S	153.31E	04/22/02	QMW 26632
E. maidae	KC2729	Tallebudgera Ck	QLD	28.23S	153.31E	04/22/02	QMW 26632
E. mirangudjin	KC2646	Ironpot Ck	NSW	28.50S	152.73E	03/04/02	QMW 26633
E. mirangudjin	KC2717	Ironpot Ck	NSW	28.50S	152.73E	03/04/02	QMW 26633
E. monteithorum	KC2765	Kroombit Ck	QLD	24.36S	151.00E	02/12/98	
E. neohirsutus	KC2837	Middle Ck	NSW	30.36S	152.49E	01/10/04	QMW 27494
E. neohirsutus	KC2740	Greenes Falls	QLD	30.23S	152.92E	10/01/02	QMW 26636
E. neohirsutus	KC2627	Little Nymboida R	NSW	30.23S	152.92E	02/05/02	QMW 26638
E. neohirsutus	KC2629	Middle Ck	NSW	30.36S	152.49E	02/06/02	QMW 26650
E. neohirsutus	KC2698	Middle Ck	NSW	30.36S	152.49E	02/06/02	QMW 26650
E. polysetosus	KC2633	Dilgry R	NSW	31.89S	151.52E	02/09/02	QMW 26640
E. polysetosus	KC2703	Dilgry R	NSW	31.89S	151.52E	02/09/02	QMW 26640
E. reductus	KC2846	Problem Ck	NSW	32.23S	151.76E	01/09/04	QMW 27488
E. rieki	KC2648	Wragges Ck	NSW	36.38S	148.46E	03/18/02	QMW 26644
E. rieki	KC2719	Wragges Ck	NSW	36.38S	148.46E	03/18/02	QMW 26644
E. robertsi	KC2776	Roaring Meg R	QLD	16.08S	145.42E	17/11/96	
E. robertsi	KC2777	Roaring Meg R	QLD	16.08S	145.42E	17/11/96	
E. robertsi	KC2778	Hilda Ck	QLD	16.16S	145.37E	07/11/94	
E. robertsi	KC2779	Hilda Ck	QLD	16.16S	145.37E	07/11/94	
E. robertsi	KC2780	Annan Ck	QLD	15.82S	145.28E	04/11/94	
E. robertsi	KC2781	Annan Ck	QLD	15.82S	145.28E	04/11/94	
E. robertsi	KC2670	Horans Ck	QLD	15.82S	145.28E	06/05/02	QMW 26646
E. robertsi	KC2738	Parrot Ck	QLD	15.82S	145.28E	06/05/02	QMW 26646
E. robertsi	KC2669	Parrot Ck	QLD	15.82S	145.28E	06/05/02	QMW 26647
E. robertsi	KC2737	Parrot Ck	QLD	15.82S	145.28E	06/05/02	QMW 26647
E. robertsi (n. sp.?)	KC2674	Hilda Ck	QLD	16.16S	145.37E	11/07/94	
E. setosus	KC2693	Greenes Falls	QLD	27.32S	152.76E	10/01/02	QMW 26649
E. setosus	KC2739	Greenes Falls	QLD	27.32S	152.76E	10/01/02	QMW 26649
E. sp.	KC2705	Cudgegong R	NSW	32.85S	150.24E	02/11/02	QMW 26581
E. sp.	KC2635	Cudgegong R	NSW	32.85S	150.24E	02/11/02	QMW 26581
E. spinichelatus	KC2631	Joyces Ck	NSW	31.28S	151.97E	02/08/02	QMW 26652
E. spinichelatus	KC2701	Joyces Ck	NSW	31.28S	151.97E	02/08/02	QMW 26652
E. spinifer	KC2636	Jamieson Ck	NSW	33.73S	150.38E	02/12/02	QMW 26585
E. spinifer	KC2706	Jamieson Ck	NSW	33.73S	150.38E	02/10/02	QMW 26585
E. spinifer	KC2634	Problem Ck	NSW	32.23S	151.76E	02/10/02	QMW 26642
E. spinifer	KC2704	Problem Ck	NSW	32.23S	151.76E	02/10/02	QMW 26642
E. spinifer	KC2643	Mammy Johnsons Ck	NSW	32.35S	151.94E	11/21/96	QMW 26654
E. spinifer	KC2714	Mammy Johnsons Ck	NSW	32.35S	151.94E	11/21/96	QMW 26654
E. sulcatus	KC2645	Bundoozle Flora Reserve	NSW	28.61S	152.70E	03/04/02	QMW 26655
E. sulcatus	KC2716	Bundoozle Flora Reserve	NSW	28.61S	152.70E	03/04/02	QMW 26655
E. sulcatus	KC2660	Tallebudgera Ck	QLD	28.23S	153.31E	04/22/02	QMW 26657
E. sulcatus	KC2731	Tallebudgera Ck	QLD	28.23S	153.31E	04/22/02	QMW 26657
E. sulcatus	KC2659	Tallebudgera Ck	QLD	28.23S	153.31E	04/22/02	QMW 26658
E. sulcatus	KC2730	Tallebudgera Ck	QLD	28.23S	153.31E	04/22/02	QMW 26658
E. suttoni	KC2626	Washpool Ck	NSW	28.97S	152.07E	02/04/02	QMW 26663
E. suttoni	KC2696	Washpool Ck	NSW	28.97S	152.07E	12/19/01	QMW 26664
E. urospinosus	KC2767	Blackall Range	QLD	26.77S	152.86E	10/01/95	
E. urospinosus	KC2838	Skene Ck	QLD	26.68S	152.87E	01/12/04	QMW 27489
E. valentulus	KC2661	Tallebudgera Ck	QLD	28.23S	153.31E	04/22/02	QMW 26667
E. valentulus	KC2662	Cougal Ck	QLD	28.21S	153.34E	04/22/02	QMW 26668
E. woiwuru	KC2652	Dobsons Ck	VIC	37.87S	145.33E	03/22/02	QMW 26669
E. woiwuru	KC2722	Dobsons Ck	VIC	37.87S	145.33E	03/22/02	QMW 26669
E. yanga	KC2835	Burrawang Ck	NSW	34.62S	150.54E	01/06/04	
E. yanga	KC2639	Burrawang Ck	NSW	34.62S	150.54E	02/13/02	QMW 26626
E. yanga	KC2710	Burrawang Ck	NSW	34.62S	150.54E	02/13/02	QMW 26626
E. yanga	KC2641	Monga NP	NSW	35.56S	149.92E	02/16/02	QMW 26671
E. yanga	KC2712	Monga NP	NSW	35.56S	149.92E	02/16/02	QMW 26671
E. yarraensis	KC2831	Love Ck	VIC	38.48S	143.58E	01/01/04	
E. yarraensis	KC2832	Love Ck	VIC	38.48S	143.58E	01/01/04	
E. yarraensis	KC2651	Cockatoo	VIC	37.94S	145.49E	03/21/02	QMW 26674
E. yigara	KC2664	O'Leary Ck	QLD	17.95S	145.65E	03/31/02	QMW 26675

Table 2 (*continued*)

Species	Sample	Site	State	Latitude	Longitude	Date	Queensland Museum Reg. No.
E. yigara	KC2733	O'Leary Ck	QLD	17.95S	145.65E	03/31/02	QMW 26675
Paranephrops planifrons	KC2741	Stockton, S. Island	NZ			11/26/02	
P. zealandicus	KC2742	Waipahi R, S. Island	NZ			11/02	
P. zealandicus	KC2743	Waipahi R, S. Island	NZ			11/02	

Table 3
Primers and their conditions (25 μl PCRs)

Gene region	Primer sequence 5′–3′	Fragment size (bp)	Anneal temperature (°C)	Reference
12S:		1f/1r 343	50	
12s1f	CTT KAA ATT YAA ARA ATT TGG CGG	1f/2r 175	45	This study (MPL)
12s2r	TTC TAA RRT ATA AGC TGC ACC			This study (EAS)
12s2f	GTA TAC CGT CAT TAT YAG ATA AC	2f/1r 206	44	This study (EAS)
12s1r	AGC GAC GGG CGA TAT GTA C			This study (MPL)
16S:		L/1472 503	50	
16sL	CGC CTG TTT AAC AAA AAC AT	L/L1r 216	50	Crandall and Fitzpatrick, 1996
16sL1r	ACT TTA TAG GGT CTT ATC GTC C			This study (EAS)
16sL2f	GAA TTT AAC TTT TGA GTG ARA AGG C	L2f/L2r 228	50	This study (EAS)
16sL2r	TAA TTC AAC ATC GAG GTC GCA AAC			This study (EAS)
16sL3f	AAT TAC TTT AGG GAT AAC AGC G	L3f/1472 159	50	This study (EAS)
1472	AGA TAG AAA CCA ACC TGG			Crandall and Fitzpatrick, 1996
COI:		caf/cabr 702	48	
COIcaf	CTA CAA ATC ATA AAG ATA TTG	caf/3r 281	45	This study
COI3r	ACT ATS CCY CTT GTT AGG AG			This study (EAS)
COI2f	TGG RGG ATT CGG AAA YTG ACT TG	2f/2r 278	45	This study (EAS)
COI2r	TAG CGG TKG TTA TRA AGT TTA CTG C			This study (EAS)
COI3f	AAC TAT CGC MCA YGC RGG AGC	3f/cabr 330	45	This study (EAS)
COIcabr	CTT CAG GGT GAC CAA AAA ATC			This study
28s:		4.8a/7b1 900–1300	48–51	
rD4.8a	ACCTATTCTCAAACTTTAAATGG	4.8a/rev1 226	45	Whiting et al., 1997
28srev1	TGTTACACACTCCTTAGCGG			This study
rD5a	GGYGTTGGTTGCTTAAGACAG	5a/rev2 228–273	45	Whiting et al., 1997
28srev2	ACGCCGGATCCCTTCAGCGC			This study
28sfor3	GCCCTTAAAATGGTATGGCGC	for3/rev3 284–329	48	This study
28srev3	TTGCCTTGGGCTTAGGAGCG			This study
rD6.2b	AATAKKAACCRGATTCCCTTTCGC			Whiting et al., 1997
rD7b1	GACTTCCCTTACCTACAT			Whiting et al., 1997

alignment is available at the MPE website and the corresponding author's website (http://inbio.byu.edu/Faculty/kac/crandall_lab/pubs.html) and resulting sequences were individually deposited into GenBank.

2.3. Testing models of evolution

Each gene region as well as the combined dataset was run through Modeltest 3.06 PPC (Posada and Crandall, 1998) to find the best model of evolution for the data. The model with the best maximum-likelihood score using the Akaike Information Criterion (AIC) was chosen (Akaike, 1973). Theoretically, AIC reduces the number of unnecessary parameters that contribute little to describing the data by penalizing more complex models (Burnham and Anderson, 2002; Nylander et al., 2004). The combined model was used in the ML and single model Bayesian approaches; individual gene region models were used in the mixed model Bayesian analyses.

2.4. Evaluating congruence of datasets

The incongruence length difference (ILD) test (Farris et al., 1994) is a poor test for the combinability of data partitions into a single dataset (Hipp et al., 2004; Yoder et al., 2001). Therefore, we follow Wiens' (1998) suggestion to estimate a Bayesian phylogeny for each region and determine if there were any strongly supported conflicting clades between gene regions. Here, we consider that bootstrap support (BS) higher than 70% and a posterior probability (pP) $\geqslant 0.95$ are considered strong support for a clade (Wilcox et al., 2002; Wong et al., 2004).

2.5. Phylogenetic inference

MrBayes v3.0b4 (Ronquist and Huelsenbeck, 2003) was used to obtain a Bayesian phylogeny for the combined dataset of all four genes. We ran different types of Bayesian Markov Chain Monte Carlo (MCMC)

analyses to examine the effect of model choice and starting tree on the resulting parameters and likelihood scores. For each analysis we ran four Markov chains simultaneously, starting each chain from a random tree for three million generations, sampling from the chain every 5000th tree; this produced 601 total trees for each run. The prior for each of these analyses was of equal probability for each tree topology, since no other prior information was available.

The first analysis was run with all four genes constrained under a single model. This analysis was performed twice and is referred to as the single model analysis. The mixed model version of MrBayes allows different likelihood model parameters to be set for each partition of the data, so we also performed a mixed model analysis (four runs each), with each run started from a random tree, and refer to this as our mixed model analysis.

The mixed model analysis was performed under two conditions, the first with all parameters linked except branch lengths (unlinked branch lengths are considered proportional), and the second with substitution rates, character state frequencies, gamma shape parameter, and proportion of invariable sites unlinked across the four partitioned regions. These analyses are referred to as the linked and unlinked mixed model analyses. Each of these analyses started with a random tree, but we ran the latter analysis a second time using the maximum-likelihood tree as the initial tree (see below) in an attempt to improve the MCMC search.

Convergence and mixing were checked for each model analysis before combining independent runs as indicated by Huelsenbeck et al. (2001, 2002) and Nylander et al. (2004). To monitor convergence, we checked the plateau phase of all parameter plots and discarded the generations from the burn-in phase. We then compared the 95% credibility interval of each parameter for significant differences as indicated by non-overlapping intervals. Finally, we examined the resulting 50% majority rule consensus tree topology from each run by graphing a bivariate plot of clade probabilities from two analyses to calculate their correlation coefficient. We then combined the trees generated from the independent analyses into one 50% majority rule consensus tree. To compare model analyses, we calculated Bayes factors ($2\log_e(B_{10})$), which is the ratio of the harmonic means of the likelihoods of two models (M_1 vs. M_0) (Nylander et al., 2004).

Phylogenies were also estimated using the maximum-likelihood approach as implemented in PAUP* v4.0b10 (Swofford, 2002). Heuristic searches were performed with 10 random sequence additions and tree bisection–reconnection (TBR) branch swapping (Allen and Steel, 2001). Nodal support was assessed using the non-parametric bootstrap procedure (Felsenstein, 1985) with 100 bootstrap replicates, TBR branch swapping, and 10 random addition replicates. Each replicate took approximately 2 days to run on a Mac OSX G5, so bootstrap values were estimated on a reduced dataset with only one to two members of each species included.

A parsimony tree was estimated using heuristic searches in PAUP* v4.0b10 (Swofford, 2002) under the same conditions as above, with gaps considered missing data. Bootstrap values were estimated using the same method as above but with 1000 bootstrap replicates.

2.6. Alternative hypothesis testing

Alternative biogeographical hypotheses were tested in an ML framework using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) in PAUP* (using a RELL distribution and 1000 replicates) and in a Bayesian framework using posterior probabilities.

3. Results

3.1. Collection and sequencing

This study is based on 40 *Euastacus* species collected from the wild. Only *E. simplex*, *E. hirsutus*, and *E. neodiversus* are missing because they were not found in their area of occurrence, although *E. neodiversus* has recently been collected by other researchers (Andrew Murray, personal communication). More extensive fieldwork must be done to determine if there are still wild populations of the other two species in existence. The total dataset for this study includes 129 specimens (Table 2), with each taxon having all four gene regions sequenced; the combined dataset contained 2109 characters. All new data (512 sequences) have been deposited in GenBank under Accession Numbers DQ006289–DQ006800. In addition, four previously published *Euastacus* sequences were also used in our data analyses (AY324335, AY324337, AY324340, and AY324341).

3.2. Congruence and models of evolution

The Bayesian trees based on the four distinct gene regions recovered similar topologies; there were two conflicting clades among all four trees ($pP \geqslant 0.95$), regarding the monophyly of *E. balanensis* (possibly paraphyletic with *E. yigara*) and the monophyly of *E. robertsi* (possibly polyphyletic with *E. fleckeri*), but since these are small topological differences compared to the overall topology of the tree, we combined the four datasets into one. The individual gene trees had only 7–14 nodes supported by a $pP \geqslant 0.95$, while those recovered from the combined data had 20–27 nodes supported by a $pP \geqslant 0.95$; this shows promise for the use of multiple gene regions, including nuclear loci, in the estimation of crayfish relationships.

Our combined dataset resulted in a best likelihood score for the transversional model with invariable sites and rate heterogeneity (TVM + I + Γ), a subset of the General Time Reversible model (Rodríguez et al., 1990). The estimated nucleotide frequencies were as follows: πA = 0.3128, πC = 0.1897, πG = 0.1819, and πT = 0.3155. The substitution model incorporated the following rate matrix: [A–C] = 1.7312, [A–G] = 7.9981, [A–T] = 1.0407, [C–G] = 0.6953, [C–T] = 7.9981, and [G–T] = 1.0000. The shape parameter of the discrete gamma distribution was estimated to be 0.6467 with the proportion of invariable sites equal to 0.5803. The models of evolution for the individual gene regions were 12S: GTR + I + Γ, 16S: K81uf + I + Γ, CO1: TrN + I + Γ, and 28S: GTR + I + Γ. These were used to determine the number of substitution types and the inclusion of a gamma rate distribution and/or proportion of invariable sites in the Bayesian analyses. For a more specific description of the models see Posada and Crandall (2001).

3.3. Phylogeny estimation

All parameters and likelihoods of the 14 Bayesian runs converged, with non-significant differences based on the 95% credible interval. The correlation between pP from independent runs was very high ($r > 0.997$). Therefore, trees resulting from independent runs (excluding the burn-in phase) were combined.

A Bayes factor comparison of the models used in the Bayesian analyses showed that the unlinked mixed model approach showed the best fit to the data (Table 4). The Bayes factor is not a statistical test with an exact cutoff number that leads to rejection of a hypothesis, but rather compares the relative merits of competing models. Kass and Raftery (1995) suggested that a value of $2\log_e(B_{10})$ greater than 10 shows very strong evidence against M_0. The Bayes factors supporting the unlinked mixed model analysis over the single model and linked mixed model analyses were at least 12 times that number, so we feel confident in rejecting those two models. The Bayes factor comparing the two unlinked mixed model analyses (user tree = random vs. user tree = ML) was smaller ($2\log_e(B_{10}) = 13.34$); however, it is still greater than the suggested value. Interestingly, our random starting tree option gave a better likelihood score than when starting with a maximum-likelihood tree. This is presumably a convergence problem when starting with a suboptimal tree from which the parameter values were obtained resulting in a much longer convergence time. We re-ran these analyses with eight starting chains instead of four and for 10 million generations instead of 3, but obtained the same results. So, if this is a convergence problem, it may take substantially more effort to converge on an optimal likelihood with an ML starting tree rather than a random starting tree. Nylander et al. (2004) showed that the most important factor in model choice was allowing rate heterogeneity within a partition, but that allowing heterogeneity across partitions is also important, as it is shown in this study. We therefore chose the consensus tree of all four unlinked multiple model analyses starting with a random user tree as the best description of our data to obtain our resulting phylogeny (Fig. 2).

The unlinked mixed model Bayesian phylogeny shows several well-supported groups within the genus. The first is what we call the southern group (see Fig. 2), which consists of 16 species supported by a pP of 1.00. Several well-defined subgroups within this clade are also recovered with high pP values. A central group of 18 species is recovered (pP = 0.64), but the placement of *E. australasiensis*, *E. maidae*, and *E. reductus* differs among the trees estimated from each analysis type. Excluding these three species, the remaining central species are recovered with a pP = 1.00. The northern group of 10 species is not recovered as monophyletic, but we will discuss it as a single unit because of their geographic proximity. *E. fleckeri* and *E. robertsi* are sister species (pP = 1.00) and are recovered as basal to the entire genus with pP = 1.00 supporting monophyly of the rest of the genus. These northern species extend from just above the Queensland border to Mt. Finnigan in Northern Queensland. The linked mixed models analysis recovers *E. fleckeri* and *E. robertsi* as the most basal, but the relationships of the remaining northern, central, and southern groups are different. The single model analysis recovers an inverted topology within the genus, with the central group as basal and *E. fleckeri* and *E. robertsi* as the most derived. The unlinked mixed model analysis with a defined starting tree had a similar topology to the analysis starting from a random tree, except the northern group (excluding *E. fleckeri* and *E. robertsi*) was recovered as monophyletic. All three models recover *Astacopsis* as basal to all of *Euastacus*.

Table 4
Bayes factor comparing various models

Model comparison (M_1/M_0)	Model likelihood (harmonic mean)		Bayes factor		Evidence against M_0
	$\log_e f(X\|M_1)$	$\log_e f(X\|M_0)$	$\log_e B_{10}$	$2\log_e(B_{10})$	
Mixed linked models (MML)/Single model (SM)	−18872.24	−18939.15	66.91	133.82	Very strong
Mixed unlinked models (MMU) (usertree = random)/MML	−18512.36	−18872.24	359.88	719.76	Very strong

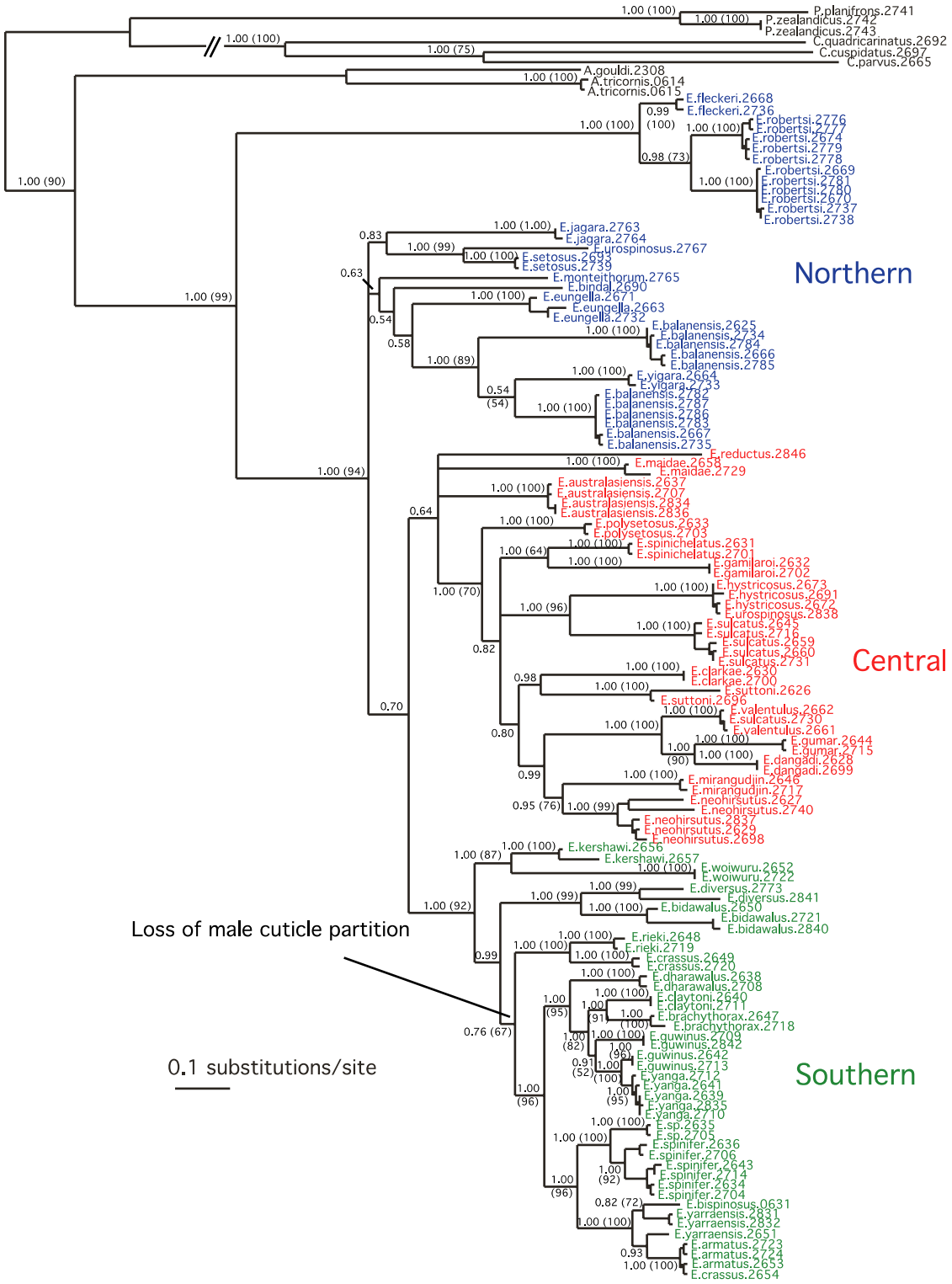

Fig. 2. Bayesian consensus tree of 2120 trees of similar likelihood scores after burnin calculated using unlinked mixed models. Clade posterior probabilities are shown on nodes with bootstrap values from a parsimony analysis of the same dataset (1000 replicates) shown in parentheses.

We incorporated our combined dataset model of evolution to estimate a phylogeny using maximum likelihood as an optimality criterion (Fig. 3). The southern and central groups are still monophyletic and distinct from the rest of the genus (BS = 94 and 80), except that *E. australasiensis*, *E. reductus*, and *E. maidae* are not part of the central group. *Astacopsis* is basal to *Euastacus*, and *E. fleckeri*, and *E. robertsi* are the most ancient *Euastacus* lineage. However, the northern group is not the next most basal group after the *fleckerilrobertsi*

Fig. 3. Maximum-likelihood phylogeny estimated using the model of evolution TVM + I + G with bootstrap values estimated from a reduced dataset analysis (100 replicates).

group. The ML tree is nearly identical to the linked mixed models Bayesian tree. There are two important considerations when comparing the ML and Bayesian analyses: first, the Bayes factor rejected a topology nearly identical to the ML tree (the linked mixed models analysis) when compared to the unlinked mixed models analysis; second, the computational time of running four Bayesian runs was less than the time needed for one ML

heuristic analysis of a dataset of this size. In both of these, the analysis using Bayesian unlinked mixed models is superior to the ML analysis.

Our dataset consisted of 639 parsimony-informative characters out of 2109 bp. Our MP analysis recovered 613 equally parsimonious trees. All nodes deeper than the species level were present in a strict consensus tree. All nodes supported with BS > 70 above the species level were identical to those in the Bayesian tree (Fig. 2). The MP tree recovers the southern and central groups (although the central group is supported with only a moderate bootstrap value of 70). The *fleckeri/robertsi* group is recovered with strong support (BS = 100), and also with strong support (BS = 94) for its position as the sister clade of all other *Euastacus*. The next most basal group is the northern group, which again is recovered as paraphyletic.

3.4. Biogeographical hypothesis testing

The tests described above were used to test hypotheses of relationships between specific groups. This would allow us to clarify the position of these groups to better infer the historical processes that occurred in *Euastacus* and *Astacopsis*. One hundred percent of the Bayesian trees in the post-burn-in distribution from the unlinked mixed model analysis show *Astacopsis* as the sister group to the genus *Euastacus*. The hypothesis of *Astacopsis* being derived from within *Euastacus* was found to be significantly worse than *Astacopsis* as sister to *Euastacus* using the SH test ($P = 0.007$). All of the Bayesian trees also show *E. fleckeri* and *E. robertsi* basal to the rest of the genus as opposed to derived from within the genus ($P < 0.04$ for SH test testing the two alternative hypotheses). Only 70% of the trees show the central and southern groups as a clade with all of the northern species basal. Excluding *E. fleckeri* and *E. robertsi*, an insignificant number of trees show the southern group as basal to the genus (pP < 0.001) but 29% of the trees show a basal central group. Excluding the *fleckeri/robertsi* clade again, the SH test score of a basal southern group is significantly worse ($P < 0.03$), but the scores of the central or the northern group being basal are not significantly different. We cannot definitively say then whether the northern or central group is basal, but we can reject the southern clade as being the center of origin of the genus.

Although the sampling was not extensive for every species, it appears that most species are monophyletic, with the possible exception of *E. balanensis* and *E. guwinus*. *Euastacus balanensis* is paraphyletic on the parsimony and Bayesian trees, with *E. yigara* included within the clade. Monophyly of *E. balanensis* is not rejected by the SH test ($P > 0.3$) and has a pP = 0.2. In all trees, however, it appears that *E. balanensis* has as much, if not more, genetic differentiation between the two main populations tested (Mt. Bartle Frere vs. Lamb Range) as is found between other sister species groups (e.g., *E. brachythorax/E. claytoni, E. crassus/E. rieki, E. hystricosus/E. sulcatus*). Two specimens of *E. guwinus* appear in all trees to be basal to a monophyletic clade of *E. guwinus* and *E. yanga*. *Euastacus guwinus* is sympatric at its single locality with *E. yanga*, and although morphologically it is most similar to *E. dharawalus* (Morgan, 1997), there is very little genetic differentiation between *E. guwinus* and *E. yanga* as seen by the short branch lengths connecting them. Three other samples [*E. urospinosus* (KC2838), *E. sulcatus* (KC2730), and *E. crassus* (KC2654)] do not group with their own species but group quite well with other species (pP = 1.00 in separate gene trees and all combined phylogenies); there could be several explanations for this, including possible misidentifications (Crandall and Fitzpatrick, 1996). However, these samples were re-extracted and resequenced to guard against contamination problems and identical sequences were obtained from the new extractions.

4. Discussion

4.1. Taxonomic classifications

This discussion will be restricted to the unlinked mixed model Bayesian phylogeny except where there are alternative relationships supported by other optimality criteria. In these cases, we will refer explicitly to the optimality criterion which estimated the relationship in question. Monophyly of each species was established in all but a few cases. These few cases warrant further examination through additional collecting and molecular work to accurately delimit species boundaries.

Nearly all previous attempts to estimate relationships between species of *Euastacus* have been based on morphological characters alone. Riek (1969) divided the genus into four groups according to the number of spines on the carpus and estimated relationships between the groups. His groupings are not consistent with the central and southern groups; carpal spination is probably a relatively divergent characteristic. Morgan (1997) believed the presence of a male cuticle partition was a more conservative character and divided the genus into two groups: those with and those without the partition. He suggested that the presence of a cuticle partition was plesiomorphic, but a loss of the character occurred early, before the genus had radiated throughout eastern Australia. It appears that his grouping based on the loss of the male cuticle partition is accurate, and that the partition was lost only once. All of the species without a partition, with the possible exception of *E. rieki* and *E. crassus*, form a monophyletic group with a posterior probability of 1.00 and bootstrap values of 95 (MP) and 92 (ML) (*E. dharawalus* to *E. spinifer*).

The two excluded species can also be included as basal to the rest of that monophyletic group, but with a pP of only 0.76 (MP bootstrap = 67, ML bootstrap = 66). Therefore, we can be fairly confident in saying the male cuticle partition was lost only once. Morgan then divided the groups into species complexes based on morphological characters and geographical proximity. Within the northern and central groups, many of Morgan's (1997) groupings based on morphological similarity and geographical proximity are concordant with our findings. This is less the case in the southern clade, in which species are much more widespread and morphological variation is greater.

The Bayesian, ML, and MP trees strongly show that *Astacopsis* and *Euastacus* are sister genera and are different enough that each warrants generic status. Monophyly of *Astacopsis*, however, must be cautiously interpreted because only two of the three *Astacopsis* species were included in the study. The most intriguing part of this conclusion is that *Astacopsis* is restricted to the island of Tasmania, while the most basal *Euastacus* lineage appears in northeast Australia.

4.2. Biogeographical history of Euastacus

There are two hypotheses concerning the formation of the current species distributions in this genus: vicariance or dispersal. Testing the different biogeographical hypotheses supports the placement of *E. fleckeri* and *E. robertsi* basal to the rest of the genus. The deep split between the *fleckeri*/*robertsi* group and the rest of the genus is consistent with the phylogenies of several other vertebrate and invertebrate species separated by the Black Mountain Corridor (Hugall et al., 2002), and the strong morphological divergence between these two species and the remainder of the genus, suggestive of an ancient vicariant split, has been discussed by Morgan (1988, 1997). This suggests that for some faunal groups, like the freshwater crayfish, the Black Mountain Corridor is a much older barrier to the gene flow than the Pleistocene as suggested in some faunal groups. There is no significant support for the placement of the remaining three major lineages in relation to each other, except that the southern group is certainly not basal. The Bayes factor supports the acceptance of the unlinked mixed model topology (which is quite similar to the MP topology) over the other hypotheses. These trees recover a topology consistent with a north to south dispersal of the genus, while the ML tree lacks nodal support for the deep nodes.

The relatively short internal branch lengths and longer tips suggest that there was a rapid historical radiation within the four lineages, especially inside the central and southern groups, followed by isolated divergence. If this is the case, it may be difficult to fully resolve the *Euastacus* phylogeny even with increasing amounts of data (see Poe and Chubb, 2004). However, rapid diversification leading to an unresolved phylogeny could be indicative of vicariance (Hoelzer and Melnick, 1994). Ponniah and Hughes (2004) concluded that for the Queensland taxa there was probably a vicariant event of at least two ancestral lineages that gave rise to current Queensland taxonomic diversity. Similarly, our phylogeny suggests that there were four ancestral lineages in Australia that may have diversified into the current 44 taxa by simultaneous vicariance. Future studies may want to investigate further these hypotheses regarding the evolution of the four ancestral lineages and the evolution of the current taxa, especially with respect to other freshwater organisms that may or may not show similar patterns of divergence.

5. Conclusions

This is the first attempt to reconstruct a robust phylogeny of the entire *Euastacus* genus. When comparing methods of phylogenetic reconstruction, partitioning and modeling the data by individual gene regions produced superior results to analyses with a single model applied to the entire dataset and these results were very similar to those using parsimony, although with reduced nodal support in the parsimony analysis. With this more complex modeling that better reflects the underlying biology of the genes used to estimate evolutionary histories, we better resolve the relationship of *Euastacus* and *Astacopsis* as monophyletic sister genera. The final *Euastacus* phylogeny supports the monophyletic groupings of a central and a southern clade along with a more ancient northern clade. Our resulting tree now sets the stage for future investigations dealing with phylogeography, taxonomy, conservation, and coevolution within this most interesting group of spiny crayfish.

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