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ORIGINAL ARTICLE

Gondwanan radiation of the Southern Hemisphere crayfishes (Decapoda: Parastacidae): evidence from fossils and molecules

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ABSTRACT

Aim The sequential break-up of Gondwana is thought to be a dominant process in the establishment of shared biota across landmasses of the Southern Hemisphere. Yet similar distributions are shared by taxa whose radiations clearly post-date the Gondwanan break-up. Thus, determining the contribution of vicariance versus dispersal to seemingly Gondwanan biota is complex. The southern freshwater crayfishes (family Parastacidae) are distributed on Australia and New Guinea, South America, Madagascar and New Zealand and are unlikely to have dispersed via oceans, owing to strict freshwater limitations. We test the hypotheses that the break-up of Gondwana has led to (1) a predominately east–west (((Australia, New Zealand: 80 Ma) Madagascar: 160–121 Ma) South America: 165–140 Ma), or (2) a southern (((Australia, South America: 52–35 Ma) New Zealand: 80 Ma) Madagascar: 160–121 Ma) pattern for parastacid crayfish. Further, we examine the evidence for a complete drowning of New Zealand and subsequent colonization by freshwater crayfish.

Location Southern Hemisphere.

Methods The evolutionary relationships among the 15 genera of Parastacidae were reconstructed using mitochondrial [16S, cytochrome *c* oxidase subunit I (COI)] and nuclear (18S, 28S) sequence data and maximum likelihood and Bayesian methods of phylogenetic reconstruction. A Bayesian (MULTIDIVTIME) molecular dating method using six fossil calibrations and phylogenetic inference was used to estimate divergence time among crayfish clades on Gondwanan landmasses.

Results The South American crayfish are monophyletic and a sister group to all other southern crayfish. Australian crayfish are not monophyletic, with two Tasmanian genera, *Spinastacoides* and *Ombrastacoides*, forming a clade with New Zealand and Malagasy crayfish (both monophyletic). Divergence of crayfish among southern landmasses is estimated to have occurred around the Late Jurassic to Early Cretaceous (109–178 Ma).

Main conclusions The estimated phylogenetic relationships and time of divergence among the Southern Hemisphere crayfishes were consistent with an east–west pattern of Gondwanan divergence. The divergence between Australia and New Zealand (109–160 Ma) pre-dated the rifting at around 80 Ma, suggesting that these lineages were established prior to the break-up. Owing to the age of the New Zealand crayfish, we reject the hypothesis that there was a complete drowning of New Zealand crayfish habitat.

Keywords

Continental drift, fossil calibration, Gondwana, historical biogeography, MULTIDIVTIME, **Parastacidae, phylogenetics, vicariance.**

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INTRODUCTION

Vicariance, through the break-up of the ancient landmass of Gondwana, has traditionally been considered a dominant process in the evolution of Southern Hemisphere biota. Support stems from the distribution of related animal taxa, such as freshwater crayfish, gondwanatherian mammals and abelisaurid dinosaurs on at least several of the Gondwanan landmasses of Africa, India, South America, Australia and New Guinea, Zealandia (New Zealand and New Caledonia) and Madagascar (Krause *et al.*, 1997; Sampson *et al.*, 1998; Crandall *et al.*, 2000a; Sanmartín & Ronquist, 2004). The idea that vicariance was a dominant process hinged on the assumption that Gondwanan taxa were poor dispersers and that long oceanic distances were therefore a barrier to dispersal. Recent advances in molecular analysis and phylogenetics have challenged the dominant role of vicariance and the ancient origin of taxa as empirical data have emerged supporting more recent divergences than expected among some taxa based on plate tectonic theory and Gondwanan drift (Briggs, 2003; McGlone, 2005; de Queiroz, 2005). While the close proximity of Madagascar to the African coast (400 km) has potentially allowed colonization, even in taxa thought to be poor transoceanic disperses (Yoder *et al.*, 2003; Vences *et al.*, 2004; Yoder & Nowak, 2006), evidence is mounting that transoceanic dispersal is also possible over much greater distances, e.g. Africa to Australia (Baum *et al.*, 1998), and potentially using intermediary islands (Cooper *et al.*, 2001; Chapple *et al.*, 2009). This does not mean that vicariance has not been important in the formation of Gondwanan biota (e.g. Noonan & Chippindale, 2006; Azuma *et al.*, 2008; Vargas-Ramírez *et al.*, 2008) but reflects a recent focus on the contribution of dispersal to Southern Hemisphere biogeography (McGlone, 2005). The relative contribution of vicariance and dispersal may be specific to the taxon of interest. In plants, non-random and directional dispersal events have been important, whereas animals tend to show a history where vicariance plays a larger role (Sanmartín & Ronquist, 2004). Furthermore, vicariance and dispersal are non-exclusive hypotheses and together are likely to have contributed to the distribution of species. The southern beech genus *Nothofagus* (Knapp *et al.*, 2005) and the araucarian genus *Agathis* (Knapp *et al.*, 2007) are two taxa in which both processes have played a role in their evolution and distribution. While dispersal and vicariance have been important in establishing current distributions, habitat and climate have played a significant role by restricting or facilitating dispersal, whether it be transoceanic or overland, such that distributions often reflect climate zones and wind directions (McLoughlin, 2001; Sanmartín & Ronquist, 2004). Therefore, biogeography requires an understanding of the biological processes and inclusion of fossils as well as the interpretation of phylogeny (Cook & Crisp, 2005).

The sequence of fragmentation of Gondwana after it began around 165 Ma is generally agreed upon (see Storey, 1995; Sanmartín & Ronquist, 2004; Upchurch, 2008). Herein, we present a brief summary of the main events of consequence to our study. The break-up of Gondwana began around 165 Ma, when India and Madagascar began to rift from Antarctica and Australia, following which, Madagascar (and India) broke away (*c.* 160 Ma) from Africa (Fig. 1a). This initial rifting divided Gondwana into east (Antarctica and Australasia) and west (Africa and South America) continents by around 140 Ma. The persistence of a land bridge, connecting east and west Gondwana, may have facilitated dispersal between southern South America, Antarctica and Australia until 31 Ma (Lawver & Gahagan, 2003). By 121 Ma, Madagascar had attained its current position off the African coast and later separated from India around 88–84 Ma (Fig. 1b) (Rabinowitz *et al.*, 1983). A land bridge may have connected Madagascar to the rest of Gondwana with the exception of Africa at the Kerguelen Plateau (Krause *et al.*, 1997) until late in the Cretaceous, around 80 Ma. Africa and South America were separating by 135 Ma, with the opening of the South Atlantic Ocean, and Africa became isolated from the rest of Gondwana around 110–95 Ma. Zealandia, a largely submerged Gondwanan continent, broke away from Australia and Antarctica *c.* 80 Ma (Fig. 1c) (Molnar *et al.*, 1975). New Zealand and New Caledonia were fully or at least partially submerged during the Cretaceous to Oligocene (LeMasurier & Landis, 1996; Landis *et al.*, 2008).

A number of hypotheses may explain the biotic patterns seen in the Southern Hemisphere today; these can be tested using a phylogenetic time divergence framework. A dispersal hypothesis predicts a younger age for taxa than would be expected based on the sequence of the Gondwanan break-up. Under a vicariance hypothesis, divergence times among Gondwanan biota are expected to match or pre-date the sequence of the break-up. Vicariance through a sequential break-up of Gondwana has led to a southern Gondwanan pattern (SGP), seen in many animal distributions (Sanmartín & Ronquist, 2004). The SGP assumes that taxa made use of the land bridge connecting South America and Australia via Antarctica until 31 Ma. However, patterns resulting from vicariance are likely to be more complex, depending on the ancestral distribution and dispersal ability of particular species, which may have facilitated or limited the use of historical land bridges to disperse to other landmasses. For example, assuming taxa did not disperse via the land bridge between South America and Australia, we would expect an east–west pattern of divergence [i.e. ((Africa, South America) (Australia, New Zealand))] and an estimated divergence time between South American and Australian taxa of around 165 Ma rather than 31 Ma. Based on the sequential break-up of Gondwana, Madagascar (and India) split from the rest of Gondwana around 165–160 Ma. However, the age of the Malagasy biota may be much younger, around 80 Ma, for taxa that made use of a land bridge at the Kerguelen Plateau. The biogeography of New Zealand is also complex. It is contended that a vicariance origin of New Zealand biota is unlikely because most or all potential New Zealand refuges sank below sea level after the sub-continent Zealandia drifted from Australia and Antarctica around 80 Ma until around 26 Ma when it re-emerged (Landis

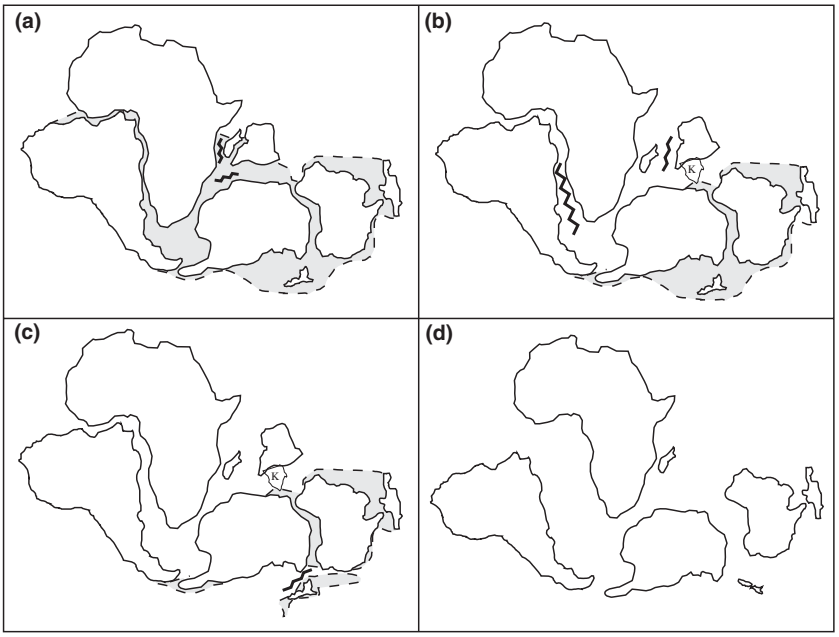

Figure 1 Gondwanan break-up through time based on Storey (1995), Sanmartín & Ronquist (2004) and Upchurch (2008). The shaded area indicates hypothesized land above sea level connecting present-day continents and islands. The zigzag line shows the origin of rifting leading to the separation of continents and islands. (a) Around 165 Ma, Gondwana begins to break up as India separates from Antarctica and Australia followed by Madagascar rifting from Africa around 160 Ma. (b) Madagascar and India continue to drift until Madagascar obtains its current position around 121 Ma. K indicates the position of the Kerguelen Plateau forming around the Late Cretaceous. Africa separates from South America between 135 and 110 Ma. (c) Around 80 Ma, Zealandia is rifting from Antarctica and Australia and, as it does so, it stretches and thins and large areas are submerged below sea level. (d) By 31 Ma, the Drake Passage connecting South America to Antarctica is open, and by 35 Ma Australia is fully isolated from Antarctica.

et al., 2008). A New Zealand drowning and dispersal hypothesis would predict an age of New Zealand biota of < 26 Ma. Alternatively, a pattern of Gondwanan vicariance for New Zealand biota would be supported if the age of its flora and fauna concurred with the drifting of New Zealand (80 Ma).

Freshwater crayfishes of the family Parastacidae have a broad Southern Hemisphere distribution. They are found on several (South America, Australia and New Guinea, New Zealand and Madagascar) of the large Gondwanan landmasses (Fig. 2), making them an ideal candidate group by which to test hypotheses about the break-up of Gondwana and its contribution to current distributions. They are a morphologically and ecologically diverse (Riek, 1972; Richardson & Swain, 1980; Horwitz, 1994) and species-rich group (175 species) (Crandall & Buhay, 2007), thus providing a large sample size and consequently a robust framework to test hypotheses of dispersal and vicariance. There have been some recent revisions of Australian taxa (Horwitz & Adams, 2000; Hansen & Richardson, 2006) and new species descriptions of crayfish from South America (Rudolph & Crandall, 2005, 2007), Madagascar (Boyko *et al.*, 2005) and New Guinea (Lukhaup & Pekney, 2006; Lukhaup & Herbert, 2008). Several species of crayfish from Madagascar (Jones *et al.*, 2007, 2009) and Australia (Horwitz, 1995; Merrick, 1995; Horwitz & Adams, 2000) are under threat from the introduction of invasive crayfish, clearing of riverine habitat and human water-use practices (e.g. dams, irrigation). Understanding evolutionary relationships can provide key insights for the management and protection of these species (e.g. Whiting *et al.*, 2000; Pérez-Losada *et al.*, 2009).

The infra-order Astacidea (Decapoda) is composed of three freshwater crayfish families, Parastacidae, Astacidae and Cambaridae, and a family of marine clawed lobsters, Nephropidae. Parastacidae is a monophyletic sister group to its Northern Hemisphere counterparts Astacidae and Cambaridae (Crandall *et al.*, 2000a). A sister relationship between nephropids and all freshwater crayfish is supported by molecular data (Crandall *et al.*, 2000a) and divergence estimates (Porter *et al.*, 2005), suggesting a single origin of freshwater crayfish on the supercontinent Pangaea. South-eastern Australia has the highest species richness of parastacids, thus representing the centre of biodiversity for the southern crayfish (Crandall & Buhay, 2007). This biodiversity, along with high levels of morphological and ecological diversity, has prompted a large amount of scientific interest in Australasian (Australia, New Guinea and New Zealand) crayfish, which in turn has resulted in hypotheses concerning the evolution of parastacid crayfish. Based on 16S molecular data, an early phylogenetic study sampling all (10) Australasian genera recognized at that time suggested they are not monophyletic but are arranged into three clades with New Zealand crayfish nested within an Australian crayfish clade (Crandall *et al.*, 1999). The first clade

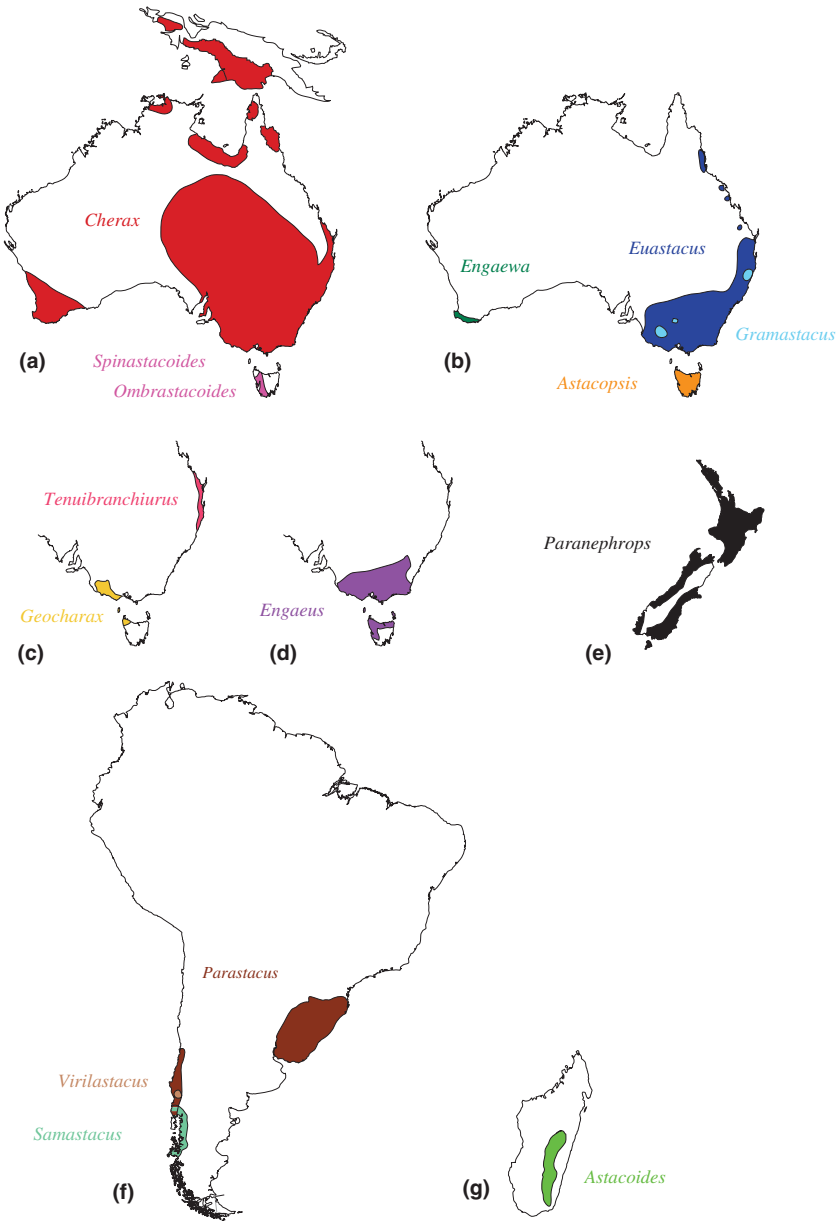

Figure 2 Distribution map of: Australian freshwater crayfish: (a) *Cherax*, *Spinastacoides*, *Ombrastacoides*, (b) *Euastacus*, *Gramastacus*, *Astacopsis*, *Engaewa*, (c) *Tenuibranchiurus*, *Geocharax*, (d) *Engaeus*; New Zealand freshwater crayfish: (e) *Paranephrops*; South American freshwater crayfish: (f) *Parastacus*, *Virilastacus*, *Samastacus*; and Malagasy freshwater crayfish: (g) *Astacoides*. Distributions are based on Horwitz (1994), Crandall *et al.* (1999, 2000b), Horwitz & Adams (2000), Shull *et al.* (2005), Hansen & Richardson (2006) and Dawkins *et al.* (in press).

included *Engaeus*, *Tenuibranchiurus*, *Geocharax*, *Gramastacus*, *Cherax* (all from Australia, Fig. 2) and was sister to *Euastacus*, *Astacopsis* (both from Australia with *Astacopsis* endemic to Tasmania), *Paranephrops* (New Zealand) and *Parastacoides* (now *Ombrastacoides* and *Spinastacoides*, Hansen & Richardson, 2006) (Tasmanian endemics). *Engaewa* (Western Australia endemic) made up the third clade, sister to all other species. The affinity between *Astacopsis* and *Euastacus* and between *Engaeus* and *Tenuibranchiurus* agreed with earlier morphological hypotheses related to ecological habitat (strong versus moderate burrowers) (Riek, 1972). Other relationships, such as the placement of *Engaewa* and *Geocharax*, differed from morphological hypotheses, suggesting inconsistency between the morphological and molecular characters (Crandall *et al.*, 1999). Crandall *et al.* (2000b) conducted a subsequent study on the position of South American crayfish (Chile, Brazil), showing that the three genera form a monophyletic group but nested within the Australasian crayfish. *Virilastacus* and *Samastacus* (Chilean endemics) formed a monophyletic sister clade, which was in turn a sister clade to *Parastacus* (Chile and Brazil). The three South American genera were closely affiliated with the New Zealand *Paranephrops* and the Australian *Parastacoides*. A phylogeographic study of New Zealand freshwater crayfish, *Paranephrops*, showed a close relationship between the two endemic species and estimated their divergence from Australian crayfish to have occurred around 50 Ma (Apte *et al.*, 2007). Few studies have attempted to reconstruct relationships among Malagasy crayfish or determine their placement within Parastacidae. A molecular study reconstructing genetic relationships using 16S data shows their placement as unresolved (Sinclair *et al.*, 2004).

To date, published phylogenies of Southern Hemisphere crayfish have been at least partially unresolved. Increasing taxon sampling and/or increasing character sampling are two possible ways to improve the power and accuracy of phylogenetic analysis (Cummings & Meyer, 2005). Recent studies have focused on branches of the parastacid tree to resolve lower-level relationships with increased taxon sampling (Shull *et al.*, 2005; Schultz *et al.*, 2007) and multiple molecular markers (Munasinghe *et al.*, 2003; Shull *et al.*, 2005). By omitting taxa/genera that are possibly nested within the true species tree, there is the potential for constructing incorrect evolutionary relationships. Conversely, sampling a large number of species for a single gene shows a high level of paraphyly within some genera (Sinclair *et al.*, 2004) that may be real or due to lack of a phylogenetic signal. An example is the previously unresolved relationship between *Euastacus* and *Astacopsis*. Early molecular work based on a single gene suggested that these genera were paraphyletic, and either that *Euastacus* was derived from *Astacopsis* (Lawler & Crandall, 1998) or that it was the ancestral form (Crandall *et al.*, 1999), depending on the taxa sampled. Using multiple gene markers, Shull *et al.* (2005) were able to support a reciprocally monophyletic sister relationship between these genera. The next obvious step in resolving the Parastacidae tree is the addition of independent molecular markers across all genera.

The aims of this study are to estimate an accurate phylogenetic hypothesis of the relationships among the genera of the parastacid freshwater crayfish and then use this phylogeny to estimate divergence times within Parastacidae to test biogeographic hypotheses relating to the break-up of Gondwana. We assess the contribution of vicariance and the sequence of break-up via the rifting of Gondwanan landmasses to current parastacid distributions. In particular, we investigate phylogenetic relationships within Parastacidae: (1) to test whether branching pattern and timing of divergence coincides with continental drift; (2) if it does, to test alternative hypotheses of an east–west Gondwanan pattern: (((Australia, New Zealand: 80 Ma) Madagascar: 160–121 Ma) South America: 165–140 Ma) or a southern Gondwanan pattern: (((Australia, South America: 31 Ma) New Zealand: 80 Ma) Madagascar: 160–121 Ma); and (3) to test the hypothesis of a drowning and re-emergence of New Zealand.

MATERIALS AND METHODS

Taxon sampling

Our sampling focused on genus-level representation of Parastacidae for a molecular phylogenetic analysis (Table 1). To represent the genera proportionally, we sampled a greater number of those taxa that have the highest morphological and species diversity, such as the Australian genera, *Cherax*, *Engaeus* and *Euastacus*. We also sampled crayfish from all Gondwanan landmasses except for New Guinea. The genus *Cherax* is distributed broadly across Australia with a few recently described species from New Guinea (Fig. 2a); however, as this study is at the genus level we have not included crayfish species from New Guinea as the centre of diversity for this genus is clearly in Australia. In total, we selected 54 ingroup taxa from the 15 genera of Parastacidae and seven outgroups, including representatives of the two Northern Hemisphere crayfish families, Cambaridae (*Cambarellus shufeldtii*, *Orconectes virilis*, *Procambarus clarkii*) and Astacidae (*Astacus astacus*, *Pacifastacus leniusculus*), the clawed lobster Nephropidae (*Homarus americanus*) and a thalassinidean from the family Callianassidae (*Sergio mericeae*).

Molecular and sequence analysis

DNA was extracted from crayfish tissue using a Qiagen DNeasy® tissue extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. Polymerase chain reaction (PCR) and cycle sequencing reactions were performed for two mitochondrial genes, 16S rRNA (Crandall & Fitzpatrick, 1996) and cytochrome *c* oxidase subunit I (COI; Folmer *et al.*, 1994), and two nuclear genes, 18S rRNA (Whiting *et al.*, 1997; Whiting, 2002) and 28S rRNA (Whiting *et al.*, 1997; Whiting, 2002), for each sample using published primer sets. The PCR conditions were as follows: initial denaturation at 95 °C for 3 min followed by 25 cycles of 95 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 7 min. Annealing temperatures were calculated from primer base pair composition. The amplified product was cleaned with Millipore Multiscreen® PCR (Millipore, Billerica, MA, USA) plates. Sequencing reactions were performed in both directions using a Big-Dye Ready-Reaction kit (Applied Biosystems, Foster City, CA, USA) and cleaned with Sephadex® G-50 columns (GE Healthcare, Piscataway, NJ, USA). Sequences were generated on an Applied Biosystems 3730xl automated sequencer. Nucleotide sequences were edited in SEQUENCHER 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA).

Phylogenetics

Each dataset was aligned in MAFFT v.6 (Katoh *et al.*, 2002; Katoh & Toh, 2008) using the L-INS-i method. Poorly aligned blocks of sequence data and large indels were removed with GBLOCKS (Castresana, 2000) retaining half-gap positions. Although in some cases the use of GBLOCKS reduces the power of the analysis and can lead to lower bootstrap support of the phylogeny, studies suggest that the resulting tree better represents the true phylogeny by removing noise and ambiguity from the dataset (Talavera & Castresana, 2007). Models of evolution were selected for each gene using MODELTEST (Posada & Crandall, 1998) with the Akaike information criterion (AIC). We used maximum likelihood (ML) and a Bayesian framework with Markov chain Monte Carlo (BMCMC) inference to estimate phylogenies using the four-gene aligned dataset. The ML analysis was run in RAxML (Stamatakis, 2006) at the CIPRES portal (Stamatakis *et al.*,

Table 1 Taxonomy, location and GenBank accession data for Parastacidae and outgroups included in this study. Accession numbers in bold were sequenced in this study.

Species	Country	16S	18S	28S	COI
Parastacoidea Huxley, 1879					
Astacopsis gouldi Clark, 1936	Australia	DQ006547	DQ079737	DQ079772	DQ006289
Astacopsis tricornis Clark, 1936	Australia	DQ006548	**EU921123**	**EU921135**	DQ006290
Cherax albidus Clark, 1936	Australia	AF135971	**FJ965974**	**FJ966009**	**FJ965956**
Cherax cairnsensis Riek, 1969	Australia	**EU921120**	**EU921124**	**EU921132**	**EU921142**
Cherax cuspidatus Riek, 1969	Australia	DQ006550	EU920960	EU920996	DQ006292
Cherax dispar Riek, 1951	Australia	AF135974	**FJ965975**	**FJ966010**	**FJ965957**
Cherax glaber Riek, 1967	Australia	AF135978	DQ079745	DQ079783	**FJ965958**
Cherax parvus Short & Davie, 1993	Australia	DQ006551	**FJ965976**	**FJ966011**	DQ006293
Cherax quadricarinatus (von Martens, 1868)	Australia	DQ006552	**EU921125**	**EU921139**	DQ006294
Cherax quinquecarinatus (Gray, 1845)	Australia	AF135976	**FJ965977**	**FJ966012**	**FJ965959**
Tenuibranchiurus glypticus Riek, 1951	Australia	AF135998	**FJ965978**	NA	**FJ965973**
Engaeus cunicularius (Erichson, 1846)	Australia	AF135980	**FJ965979**	**FJ966013**	NA
Engaeus fossor (Erichson, 1846)	Australia	**EU921121**	**EU921126**	**EU921134**	**EU921144**
Engaeus laevis (Clark, 1941)	Australia	**FJ965951**	**FJ965980**	**FJ966014**	NA
Engaeus lyelli (Clark, 1936)	Australia	**FJ965950**	**FJ965981**	AY211979	NA
Engaeus sericatus Clark, 1936	Australia	AF135981	**FJ965982**	**FJ966015**	**FJ965960**
Engaewa reducta Riek, 1967	Australia	**FJ965955**	**FJ965983**	**FJ966016**	**FJ965971**
Engaewa similis Riek, 1967	Australia	AF135982	**FJ965984**	**FJ966017**	NA
Euastacus armatus (von Marten, 1866)	Australia	AF044242	**FJ965985**	**FJ966018**	**FJ965962**
Euastacus australasiensis (H. Milne-Edwards, 1837)	Australia	AF044243	**FJ965986**	**FJ966019**	**FJ965963**
Euastacus balanensis Morgan, 1988	Australia	DQ006560	**FJ965987**	**FJ966020**	DQ006302
Euastacus bidawalus Morgan, 1986	Australia	DQ006573	**FJ965988**	**FJ966021**	DQ006315
Euastacus eungella Morgan, 1988	Australia	DQ006593	EU920964	EU921001 EU921002	DQ006335
Euastacus fleckeri Watson, 1935	Australia	DQ006595	**FJ965989**	**FJ966022**	DQ006337
Euastacus maidae (Riek, 1956)	Australia	DQ006610	**FJ965990**	**FJ966023**	DQ006354
Euastacus neohirsutus Riek, 1956	Australia	DQ006617	**FJ965991** **FJ965992**	**FJ966024**	DQ006362
Euastacus rieki Morgan, 1997	Australia	DQ006622	**FJ965993**	**FJ966025**	DQ006367
Euastacus robertsi Monroe, 1977	Australia	DQ006633	EU920962	EU920988	DQ006378
Euastacus setosus (Riek, 1956)	Australia	DQ006635	**FJ965994**	**FJ966026**	DQ006380
Euastacus spinichelatus Morgan, 1997	Australia	DQ006638	EU920963	EU920989	DQ006383
Euastacus spinifer (Heller, 1865)	Australia	DQ006645	**FJ965995**	**FJ966027**	DQ006390
Euastacus sulcatus Riek, 1951	Australia	DQ006651	**EU921127**	**EU921133**	DQ006396
Euastacus suttoni Clark, 1941	Australia	DQ006653	**FJ965996**	**FJ966028**	DQ006398
Euastacus valentulus Riek, 1951	Australia	DQ006656	**FJ965997** **FJ965998**	**FJ966029**	DQ006402
Euastacus woiwuru Morgan, 1986	Australia	DQ006657	**FJ966001**	**FJ966030**	DQ006403
Euastacus yanga Morgan, 1997	Australia	DQ006663	**FJ965999**	**FJ966031**	DQ006409
Euastacus yigara Short & Davie, 1993	Australia	DQ006668	**FJ966000**	**FJ966032**	DQ006414
Geocharax gracilis Clark, 1936	Australia	AF235992	AF235968	**EU921140**	**EU921145**
Gramastacus insolitus Riek, 1972	Australia	AF135993	**FJ966002**	**FJ966033**	**FJ965961**
Spinastacoides insignis (Clark, 1939)	Australia	AF135996	**FJ966003**	**FJ966034**	**FJ965966**
Ombrastacoides huonensis Hansen & Richardson, 2006	Australia	AF135997	**EU920956**	**EU920995**	**EU921143**
Paranephrops planifrons White, 1842	New Zealand	AF135995	**EU921128**	**EU921141**	DQ006415
Paranephrops zealandicus (White, 1847)	New Zealand	DQ006670	**FJ966004**	**FJ966037**	DQ006416
Astacoides madagascariensis (H. Milne-Edwards & Audouin, 1839)	Madagascar	**FJ965952**	**FJ966005**	**FJ966035**	**FJ965964**
Astacoides crosnieri Hobbs, 1987	Madagascar	**EU921122**	**EU921129**	**EU921136**	**EU921147**
Astacoides betsileoensis Petit, 1923	Madagascar	EU920912	EU920955	EU920992	EU921146
Astacoides caldwelli (Bate, 1865)	Madagascar	**FJ965953**	**FJ966006**	**FJ966036**	**FJ965965**
Parastacus brasiliensis (von Martens, 1869)	Brazil	AF175244	**EU921130**	**EU921138**	EF599158
Parastacus defossus Faxon, 1898	Brazil	AF175243	EU920953	EU920991	**FJ965968**
Parastacus pilimanus (von Martens, 1869)	Brazil	AF175246	**FJ966007**	**FJ966038** **FJ966039**	**FJ965967**
Parastacus pugnax (Poeppig, 1835)	Chile	AF175237	AF235969	**FJ966040**	EF599157

Table 1 Continued

Species	Country	16S	18S	28S	COI
Parastacus varicosus Faxon, 1898	Brazil	EU920933	EU920954	EU920990	**FJ965969**
Samastacus spinifrons (Phillipi, 1882)	Chile	AF175241	**EU921131**	**EU921137**	EF599159
Samastacus sp.	Chile	**FJ965954**	**FJ966008**	**FJ966041**	**FJ965970**
Virilastacus araucanius (Faxon, 1914)	Chile	AF175236	AF235970	**FJ966042**	EF599156
Astacoidea Latreille, 1802					
Astacus astacus (Linnaeus, 1758)	Europe	AF235983	AF235959	DQ079773	AF517104
Pacifastacus leniusculus (Dana, 1852)	USA	AF235985	AF235961	DQ079806	**EU921148**
Cambarellus schufeldtii (Faxon, 1884)	USA	AF235986	AF235962	DQ079778	**EU921149**
Orconectes virilis (Hagen, 1870)	USA	AF235989	AF235965	DQ079804	AF474365
Procambarus clarkii (Girard, 1852)	USA	AF235990	**EU920952**	**EU920970**	AY701195
Nephropoidea Dana, 1852					
Homarus americanus H. Milne-Edwards, 1837	USA	HAU11238	AF235971	DQ079788	DQ889104
Callianassoidea Dana, 1852					
Sergio mericeae Manning & Felder, 1995		DQ079733	DQ079768	DQ079811	**FJ965972**

COI, cytochrome oxidase *c* subunit I; NA, data not available.

2008) using partitioned models so that parameters were estimated for each gene independently. Node support was estimated with 1000 bootstrap pseudoreplicates. The BMCMC analysis was run in MrBayes (Yang, 1997; Ronquist & Huelsenbeck, 2003) on the partitioned dataset with 1×10^7 generations sampled every 1000 generations. The analysis was run four times starting from random trees. Model parameters were unlinked, treated as unknown variables with uniform default priors and estimated as part of the analysis. Convergence and mixing were assessed for all the parameters in Tracer v.1.4 (Drummond & Rambaut, 2007). All sample points prior to reaching stationarity were discarded as burn-in. The posterior probabilities for individual clades obtained from separate analyses were compared for congruence and then combined and summarized on a 50% majority-rule consensus tree (Huelsenbeck & Imennov, 2002; Huelsenbeck *et al.*, 2002).

Fossil calibration points

Fossil calibration points used in conjunction with molecular data increase the accuracy in estimating divergence times by adding informative constraints to estimated parameters. The recent find of body and trace parastacid crayfish fossils, *Palaeoechinastacus australianus*, from the Otway and Strzelecki Group (south-eastern Australia), has dated southern crayfish to the Early Cretaceous (106–116 Ma) (Martin *et al.*, 2008). Here, we use the mean age of fossils (Table 2) from representative crayfish taxa *Astacus* (Van Straelen, 1928; Imaizumi, 1938) and *Procambarus* (Feldmann *et al.*, 1981) and two species of parastacid crayfish, *Palaeoechinastacus australianus* (Martin *et al.*, 2008) and *Paranephrops fordycei* (Feldmann & Pole, 1994). We also included a fossil calibration for the split between Astacidea and Thalassinidea at the root node (Amati *et al.*, 2004). All fossils were set as lower calibration limits following Porter *et al.* (2005). As the time-divergent software requires one maximum node limit set, we used the break-up of Pangaea set as 185 Ma between Astacoidea and Parastacoidea (Crandall *et al.*, 2000a).

Estimating divergence times

A Bayesian approach following Thorne & Kishino (2002) and implemented in the program multidivtime (http://statgen.ncsu.edu/thorne/multidivtime.html) was used to estimate divergence times. This analysis allows estimation of relative divergence times using multiple genes without enforcing a molecular clock, instead allowing rate variation through time. Fossil data were used to place bounds on nodes to estimate the rate and timing of divergences among taxa. The ML tree was used as the best-supported phylogeny in the subsequent analysis to estimate divergence times. Parameters of the evolutionary model were estimated under the F84 + G model (Felsenstein, 1984) in paml (Yang, 1997, 2007). This model is less parameterized than the best-fit models selected by Modeltest. However, previous studies (Yang & Yoder, 2003, and references therein) have shown that it is actually the parameter of rate variation among sites that has the greatest effect on divergence time estimation. Branch lengths and the corresponding covariance matrix were estimated in estbranches (multidivtime package). After an initial burn-in of 10^5, we sampled 5×10^5 every 100 iterations. The distance between root and tip (rttm) was given as 250 Ma. We used a prior for the substitution rate (rtrate) of 0.12 per 100 Ma, calculated from the median amount of evolution between the ingroup root and the ingroup tips. All other parameters were selected following suggestions in the manual. Each set of start parameters was run using the dataset three times. To test the influence of priors on posterior values, we reran the program three times using a range of prior values. We compared the effect of increasing the mean of the prior distribution (rttm) to 110% (275 Ma)

Table 2 Fossil calibrations and mean age used in divergence time estimations. The node denotes the placement of the fossil on the crayfish chronogram. All fossils belong to the infra-order Astacidea.

Family	Species	Reference	Geological period (Ma)	Node
Chimaerastacidae	*Chimaerastacus*, *Pacifluvialis*	Amati *et al.* (2004)	Mid Triassic (Late Ladinian) (227–234)	C1
Parastacidae	*Palaeoechinastacus australianus*	Martin *et al.* (2008)	Early Cretaceous (106–116)	C3
Parastacidae	*Paranephrops fordycei*	Feldmann & Pole (1994)	Early Middle Miocene (Otaian–Lillburnian) (21.7–12.7)	C4
Astacidae	*Astacus licenti*	Van Straelen (1928)	Late Jurassic (144–159)	C5
Astacidae	*Astacus spinirostris*	Imaizumi (1938)	Late Jurassic (144–159)	C5
Cambaridae	*Procambarus primaevus*	Feldmann *et al.* (1981)	Late Early Eocene (52.6–53.4)	C6

Calibration C2 is 185 Ma, based on the splitting of Pangaea used as an upper limit.

and 120% (300 Ma). We also compared the influence of the standard deviation of the prior distribution (rttmsd) by increasing the value from 10% to equalling the prior distribution.

Testing alternative topologies

Alternative hypotheses concerning the sequence of Gondwanan break-up were constructed in MacClade v.4 (Maddison & Maddison, 1989) by rearranging clades restricted to each continent and island. The hypotheses tested were southern Gondwanan vicariance [i.e. (((Australia, South America) New Zealand) Madagascar)] and east–west Gondwanan vicariance [i.e. (((Australia, New Zealand) Madagascar) South America)]. These topologies were then compared with our best hypothesis of relationships using the topological test of Shimodaira & Hasegawa (1999) (SH test) as implemented in paup* v.4b10 (Swofford, 2002) and Bayesian inference. One thousand replicates were performed for the SH test, resampling the partial likelihoods for each site (RELL model). Bayesian topological tests were conducted as described in Huelsenbeck *et al.* (2002).

RESULTS

Phylogenetic relationships

New 16S, COI, 18S and 28S DNA sequences were submitted to GenBank under the accession numbers shown in Table 1. The full alignment of four genes was 5365 base pairs (bp) long. After alignment and removal of difficult to align regions, the dataset was reduced to 4094 bp. The GTR+I+G model was selected with Modeltest as the best-fit model for all four genes. GTR+G was chosen over the fully parameterized model GTR+I+G, as the former model allows for low rates at sites such that it approximates the latter and avoids the problems associated with simultaneously estimating gamma and the proportion of invariable sites (Yang, 2006).

The ML and BMCMC phylogenetic analyses provided almost identical reconstructions and strong node support for parastacid species placement within genera. The only disagreement between trees was the placement of several species of *Cherax* (*Cherax dispar*, *Cherax cairnsensis*, *Cherax parvus*) which had low nodal support in both analyses. As our focus was to estimate divergence times among genera, and we found no conflict between analyses at this level, we proceeded with further analysis using the ML tree (Fig. 3). With respect to the relationships within Astacidea, monophyly of the Northern (Astacoidea) and Southern (Parastacoidea) Hemisphere crayfish was recovered with strong nodal support. All genera where multiple species were included in the analysis (except *Engaeus*) were monophyletic with high nodal support. *Engaeus* was paraphyletic with *Engaeus lyelli* basal to a *Gramastacus*/*Tenuibranchiurus*/*Geocharax* grouping. A more intensive study on this group has shown that *Engaeus lyelli* is indeed peculiar with respect to the rest of this genus (Schultz *et al.*, 2009). South American crayfish (*Virilastacus*, *Samastacus*, *Parastacus*) were supported as monophyletic and were the most basal clade with respect to all other southern crayfish. Relationships among crayfishes from Australia were not monophyletic. The majority of Australian crayfish formed one clade that was sister to a clade that included *Paranephrops* (New Zealand), *Astacoides* (Madagascar) and a second group of Australian crayfish, *Spinastacoides* and *Ombrastacoides*, which are endemic to Tasmania. Although the relationships among *Paranephrops*, *Astacoides*, *Spinastacoides* and *Ombrastacoides* have low nodal support, the placement of these four genera as sister to all other Australian crayfish was supported. The main Australian clade was therefore made up of the other eight Australian genera, which comprises three divergent groups: *Euastacus*/*Astacopsis*, *Engaewa*/*Engaeus*/*Gramastacus*/*Tenuibranchiurus*/*Geocharax* and *Cherax*.

Estimating divergence times

Multiple independent runs of multidivtime using the same and different starting parameters (e.g. rtrate and samples) resulted in very similar divergence estimates and 95% confidence intervals. Results varied by up to 1% when the rttm was increased to 275 and 300 Ma (Table 3). Decreasing the standard deviation of the priors (rttmsd, rtratesd) to 10% of the value of rttm and rtrate resulted in a difference of up to 5% in some estimates; however, it had little effect on the magnitude of the confidence intervals.

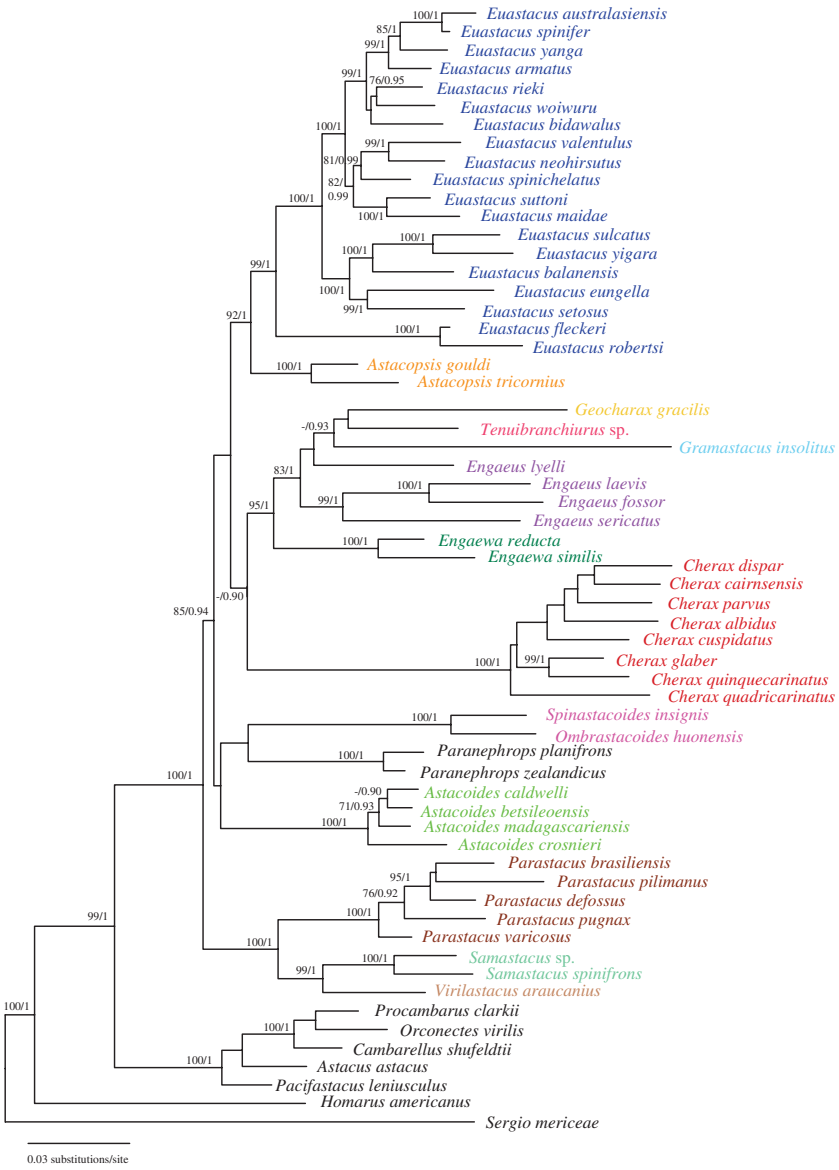

Figure 3 Maximum likelihood (ML) and Bayesian mixed-model trees of DNA sequence data for Parastacidae. Outgroups include *Homarus americanus* and *Sergio mericeae* and representatives of Astacidae and Cambaridae. Branch lengths are shown proportional to the amount of change along the branches in the ML tree. Bootstrap proportions > 70% for the ML analysis and posterior probabilities > 0.90 for the Bayesian analysis are shown on individual nodes.

Based on fossil calibrated, multi-locus divergence time, the origin of Parastacidae was estimated to be around 177–185 Ma (Fig. 4). Radiation of the parastacids commenced shortly after, establishing the South American lineages (158, 136–178 Ma) and subsequently two clades, representing most of the Australian lineages and the Malagasy (*Astacoides*)/New Zealand (*Paranephrops*)/Australian (*Spinastacoides*, *Ombrastacoides)* lineages (152, 129–173 Ma). Radiation of South American lineages started *c.* 116 Ma (89–144 Ma) and the three genera were established around 85 Ma (57–117 Ma). Although an early origin for *Astacoides* (147, 122–169 Ma), *Paranephrops* and *Ombrastacoides*/*Spinastacoides* (136, 109–160 Ma) was calculated, diversification among extant lineages occurred more recently, in the last 50 Myr. Divergence between the two species of New Zealand crayfish was estimated to be relatively recent, around 22 Ma (7–42 Ma). Among the Australian taxa (excluding *Ombrastacoides*/*Spinastacoides*) early diversification established three groups of crayfish: *Engaewa*/*Engaeus*/*Gramastacus*/*Tenuibranchiurus*/*Geocharax* and *Cherax* (134 Ma) and *Euastacus*/*Astacopsis* (116 Ma).

Testing alternative topologies

The southern Gondwanan vicariance hypothesis (((Australia, South America) New Zealand) Madagascar) was significantly different from the parastacid consensus tree in the Bayesian topology analysis [posterior probability (PP) < 0.001], although the more conservative SH test was not significant ($P = 0.091$). Neither topology test rejected an east–west Gondwanan hypothesis (((Australia, New Zealand) Madagascar) South America) (Bayesian, PP = 0.084; SH test, $P = 0.335$). This suggests the east–west Gondwanan pattern explains the data better than the southern Gondwanan pattern.

Table 3 Divergence time estimates and confidence intervals (95%) of freshwater crayfish taxa selected for comparison to show variation produced by using different priors in MULTIDIVTIME (MDT). MDT1 uses the standard priors with a root-to-tip mean (rttm) of 250 Ma and equal standard deviation. The standard deviation of rttm and rtrate was decreased to 10% of the prior estimates for MDT2. The rttm was increased to 275 and 300 for MDT3 and MDT4, respectively. Each estimate is taken at the crown node representing the radiation of that taxon.

Taxon (node)	MDT1	MDT2	MDT3	MDT4
Astacidea (A)	241 (231–268)	234 (231–242)	242 (231–268)	242 (231–270)
Freshwater crayfish (B)	183 (177–185)	183 (179–185)	183 (177–185)	183 (177–185)
Astacoidea (C)	154 (152–161)	155 (152–162)	155 (152–162)	155 (152–162)
Parastacoidea (D)	158 (136–178)	154 (138–168)	158 (135–178)	157 (135–178)
South American (E)	116 (89–142)	114 (87–139)	116 (89–143)	116 (88–143)
Australia I (F)	124 (98–149)	120 (97–142)	124 (97–150)	123 (97–149)
Australia II (G)	116 (89–144)	116 (89–141)	117 (89–145)	116 (88–144)
Cherax (H)	76 (54–103)	63 (44–85)	76 (54–103)	76 (53–103)
Astacoides (I)	49 (29–75)	47 (26–73)	50 (29–75)	49 (28–75)
Paranephrops (J)	22 (7–42)	22 (4–46)	22 (7–44)	22 (7–42)

DISCUSSION

Our study had two main goals at the outset. First, we hoped to provide a robust phylogenetic estimate of the evolutionary relationships among genera of freshwater crayfish from the family Parastacidae. We used complete sampling of all genera coupled with multiple genes to estimate these relationships. Second, we tested alternative vicariance hypotheses and estimated divergence dates of the parastacid crayfish using a combination of molecular phylogenetic dating with constrained nodal divergence times based on the fossil record. Here we discuss our results concerning these two goals.

Parastacid systematics

Several hypotheses have been put forward in the literature detailing the relationships among parastacids based on morphology, molecular markers or a combination of the two (Crandall *et al.*, 1999, 2000b; Schultz *et al.*, 2009). Until now, these studies have not made use of multiple markers nor have they included representatives of all genera. Thus, herein we present the most up-to-date phylogeny of the Parastacidae. Our resulting phylogeny suggests that crayfish are not monophyletic with respect to the four southern landmasses. Rather, two genera of Australian crayfish restricted to the island of Tasmania, *Ombrastacoides* and *Spinastacoides*, are nested with genera endemic to New Zealand and Madagascar. Paraphyly of Australian crayfish suggests that factors other than continental drift were also important in the early diversification of parastacids. These include isolation among drainage basins on Gondwanan landmasses and Antarctica potentially facilitating dispersal among continents prior to drift. The sister relationship between New Zealand crayfish and Tasmanian crayfish had been previously suggested (Crandall *et al.*, 1999). The position of Malagasy crayfish, however, was unknown. Our results suggest that the Malagasy genus *Astacoides* is sister to the New Zealand/Tasmanian clade of *Paranephrops* (New Zealand), *Ombrastacoides* and *Spinastacoides*, albeit with low support. All other Australian genera are supported as one phylogenetic group. The three South American genera, *Parastacus*, *Virilastacus* and *Samastacus*, also form one phylogenetic group. The affinity among South American crayfish supports an earlier hypothesis proposed by Starobogatov (1995) and supported by Crandall *et al.* (2000b) that *Parastacus*, *Virilastacus* and *Samastacus* form a group to the exclusion of Australian parastacids.

The relationships among Australian species generally showed strong node support, while the phylogenetic position of some Australian genera remains difficult to resolve. Our study agreed with Shull *et al.* (2005); *Euastacus* and *Astacopsis* are monophyletic sister taxa rather than paraphyletic (Lawler & Crandall, 1998; Crandall *et al.*, 1999). A sister relationship was suggested but not strongly supported between *Euastacus*/*Astacopsis* and the *Engaewa*/*Engaeus*/*Gramastacus*/*Tenuibranchiurus*/*Geocharax* and *Cherax* clade. The relationship between *Engaeus*/*Gramastacus*/*Tenuibranchiurus*/*Geocharax* and *Cherax* was moderately supported (64%/0.89). Although this topology was inconsistent with earlier single-locus (Crandall *et al.*, 1999) and morphological (Riek, 1972) studies, it was congruent with recent single-locus and multi-locus studies (Schultz *et al.*, 2007, 2009). Our findings augment earlier studies through a broader sampling and the inclusion of outgroups, which strongly supports independence of South American crayfish early in the evolution of parastacids, subsequently followed by the radiation and divergence of Australian, New Zealand and Malagasy crayfishes.

Biogeography, divergence times and vicariance hypotheses

Fossil-calibrated molecular estimates suggest that Parastacidae originated around 183 Ma (177–185 Ma), and subsequent divergence established four lineages of crayfish by the Late Jurassic. Radiation within the southern crayfish lineage followed, establishing almost all recognized extant genera by the end of the Early Cretaceous. This estimated early origin

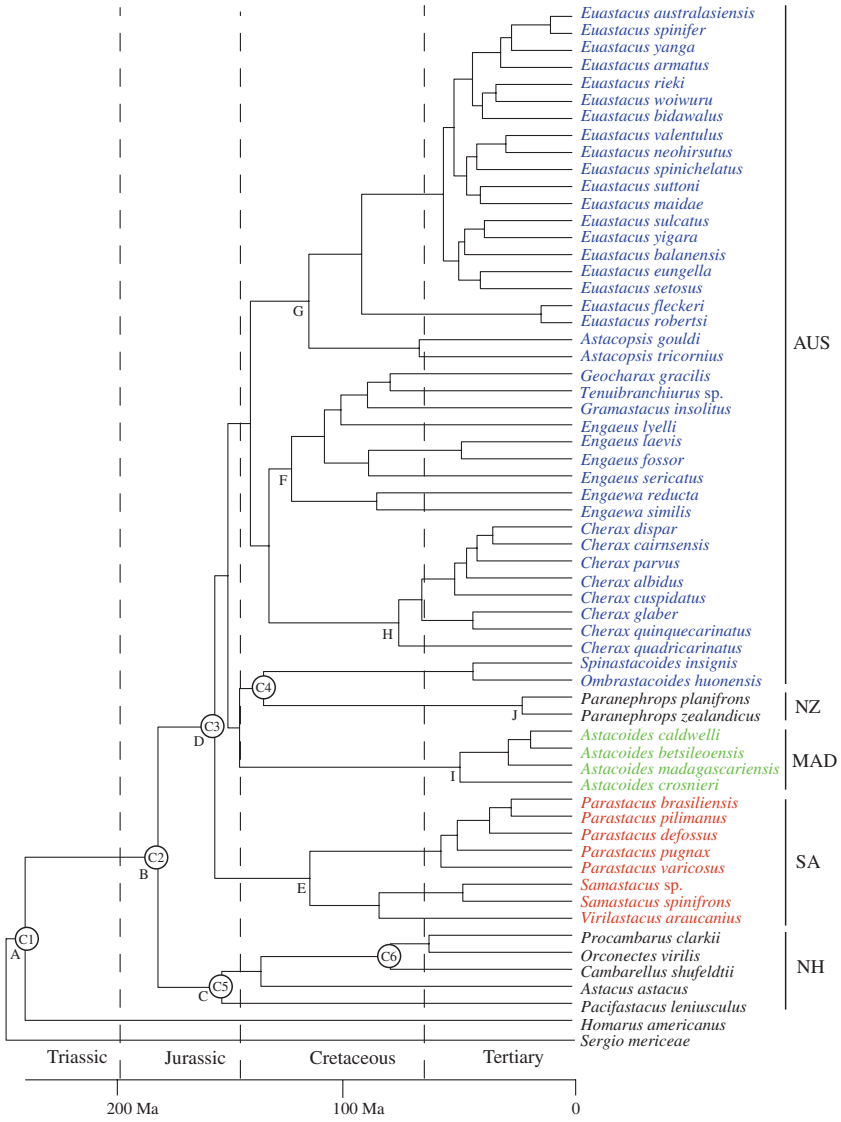

Figure 4 Divergence time chronogram of Astacidea and selected outgroups (*Homarus americanus*, *Sergio mericeae*) estimated using the maximum likelihood (ML) topology. The geographic distribution of crayfish species, AUS (Australia), NZ (New Zealand), MAD (Madagascar), SA (South America) and NH (Northern Hemisphere), is indicated to the right of the tree. Fossil calibration estimates are shown on the nodes (C1–C6) and refer to dates given in Table 2. Major geological periods are separated by the dashed line. The crown nodes for estimated time of radiation used in Table 3 are labelled A–J.

and radiation of parastacid crayfish is consistent with previous studies (Crandall *et al.*, 2000a; Rode & Babcock, 2003; Porter *et al.*, 2005; Bedatou *et al.*, 2008) and supports the idea that ancestral taxa were widespread across Gondwanan landmasses prior to the break-up.

To test between the Gondwanan vicariance hypotheses, the divergence dates among crayfish from different landmasses must concur with or pre-date the estimated age of continental rifting. The divergence of South American crayfish from other parastacids occurred around 158 Ma. This is consistent with the idea of the early splitting (165–140 Ma) of Gondwana into eastern (Madagascar, India, Australia, Antarctica and New Zealand) and western (South America, Africa) landmasses. The east–west divergence of parastacids was also supported by topology tests. Palaeogeographic reconstructions suggest the southern portion of South America remained in contact with Antarctica until the Drake Passage opened around 31 Ma (Lawver *et al.*, 1992; Lawver & Gahagan, 2003). The warm temperate climate of the Late Cretaceous (80–90 Ma) may have facilitated dispersal of temperate biota from the Andes along a continuous mountain chain through Antarctica to Australia (Woodburne & Zinsmeister, 1982; Woodburne & Case, 1996). A southern Gondwanan supercontinent also explains why there is no evidence of abelisaurid dinosaurs being present in Africa (Sampson *et al.*, 1998). Even so, our estimates of divergence time and the Bayesian topology test suggest that the land bridge between South America and Antarctica was an unlikely dispersal passage for crayfish. It is also unlikely that this passage explains the absence of crayfish on Africa since parastacid diversification began around 178–135 Ma, suggesting that crayfish were already present on South America and eastern Gondwana prior to the break-up of Gondwana. The three genera of South American crayfish, *Parastacus*, *Samastacus* and *Virilastacus*, were established by around 85 Ma. They are distributed on either side of the South American continent. Trace fossils from central Patagonia identified as Astacidea from the Late Jurassic to the Late Cretaceous suggest that during this period, crayfish were

spread throughout southern South America (Bedatou *et al.*, 2008). *Parastacus* has a disjunct distribution on either side of South America (Chile, Argentina and Brazil) that is characteristic of South American biota fragmented by the formation of the Andes (e.g. Pérez-Losada *et al.*, 2004; Ruzzante *et al.*, 2006). The single species we included of Chilean representatives of this genus, *Parastacus pugnax*, was nested within *Parastacus*. It is possible that the current disjunct distribution reflects vicariance of a once widespread species of crayfish as a result of the formation of the Andes. However, without complete sampling of South American taxa, we cannot rule out other potential explanations.

Radiation of eastern Gondwanan crayfish (Australia, Madagascar and New Zealand) began around 152 Ma. The initial divergence within eastern Gondwana established a group that contains all extant Australian species (excepting *Ombrastacoides* and *Spinastacoides*) and a group leading to the Malagasy (around 146 Ma), New Zealand and the other Australian lineages. The timing of the origin of *Astacoides* is consistent with a separation of Madagascar from Australia/Antarctica/New Zealand between 160 and 121 Ma, providing further support for the Gondwanan vicariance hypothesis among some crayfish lineages. Furthermore, there is no evidence to suggest that the rise of the Kerguelen Plateau around 120–80 Ma (Krause *et al.*, 1997) was used by crayfish for dispersal between Madagascar/India and Antarctica/Australia/New Zealand after the initial break-up of the continents.

The divergence between New Zealand *Paranephrops* and the Tasmanian crayfish *Ombrastacoides* and *Spinastacoides* (around 136 Ma, 109–160 Ma) occurred earlier than predicted by the rifting of New Zealand and Australia around 80 Ma (Weaver *et al.*, 1994). The early divergence time suggests that speciation among these crayfish pre-dates the separating Gondwanan landmasses. Potentially, the sister taxa of *Paranephrops* were distributed elsewhere, such as Antarctica, and have since gone extinct. Interestingly, we have no evidence of diversification within *Paranephrops* until around 22 Ma, with the origin of the two extant taxa. This is consistent with the divergence of these species corresponding to a proposed vicariant event associated with the reduction of proto-New Zealand to several widely spaced small islands, estimated to have occurred around 27 Ma (McDowall, 2005). There is also fossil evidence of a third *Paranephrops* species (*Paranephrops fordycei*) known from this time (12.7–21.7 Ma; Feldmann & Pole, 1994). The recent time of diversification within New Zealand crayfish may be a result of a fragmented landscape as predicted by the partial drowning and re-emergence of New Zealand, rather than complete drowning and recolonization. The estimated time of divergence of New Zealand and Australian crayfish pre-dating the estimated time of re-emergence of New Zealand (around 26 Ma) is consistent with the idea that at least some land remained above sea level as refugia. It is interesting that New Caledonia, which is also suggested to have submerged at least partially (see Ladiges & Cantrill, 2007, for a review), does not have any freshwater crayfish. The history and origin of crayfish on New Zealand remains a complex question, and a more detailed study of relationships between New Zealand and Tasmanian crayfish, including multiple genes and broader sampling of each genus, would yield a better insight into the evolutionary history of these genera.

The radiation of Australian crayfish throughout the Cretaceous and into the recent has established a highly diverse group. South-eastern Australia is one of the most biodiverse regions for crayfish, second only to the southern Appalachian Mountains of the south-eastern United States (Crandall & Buhay, 2007). Crayfish have diversified and filled many niches across Australia. In some cases, a vicariant origin explains the distribution of divergent species such as crayfish distributed on isolated mountaintops (Ponniah & Hughes, 2004). In other cases, the history is less clear, with closely related genera/species sharing habitat within streams. Two species-rich groups of interest are *Cherax* and *Euastacus*. Based on molecular data, they appear to have diverged from other parastacids in the Early Cretaceous, and yet major diversification within these groups did not occur until much later (76 Ma, 54–103 Ma and 57 Ma, 37–81 Ma). This more recent but broad radiation may suggest diversification into new niches or signal that climatically induced habitat change occurred at the Cretaceous–Tertiary boundary. These divergence dates are considerably older than those estimated by Schultz *et al.* (2009) based on a Bayesian analysis of 16S data. Their analysis assumed a constant and known substitution rate from crabs without incorporating fossil data and resulted in divergence time estimates of 20–50 Ma for the *Engaewa* clade and 15–40 Ma for the *Cherax* clade. Our analysis did not incorporate any fossil calibrations within this group, and estimation of divergence time for nodes further from fossil calibrations may increase error. It is interesting that by using a more robust four-gene dataset and including fossil data, we estimate relatively older origins and diversification of genera. Although beyond the scope of this study, it would be interesting to compare these radiations with other genera of Australian crayfish and Australian biota in general.

CONCLUSIONS

Herein we present the most up-to-date phylogeny for parastacid crayfish inclusive of all genera and representative of their Gondwanan distribution. Robust empirical evolutionary studies rely on a combination of geological hypotheses, molecular, morphological and biological data, as well as the fossil record, to uncover the history of the taxa of interest. Parastacid crayfish have a Gondwanan distribution, show deep divergences based on combined fossil and molecular data consistent with estimates of continental drift, and have limited oceanic dispersal abilities, making them ideal candidates for testing Gondwanan vicariance hypotheses. An interesting outcome of this study is that crayfish do not have the typical southern Gondwanan biogeographical pattern based on palaeogeographic evidence (((southern South America, Australia) New Zealand) Madagascar) seen in many animals (Sanmartín &

Ronquist, 2004). The most basal split in the parastacid crayfish separates South American crayfish from all others, even though geological data suggest that a land bridge allowed dispersal of organisms between eastern and western Gondwana until 31 Ma. Although the sequential break-up of Gondwana may underlie the overall biogeographical pattern seen in parastacids, an important factor is also likely to be the historical isolation of river systems and the sheer magnitude of distances across the Gondwanan supercontinent. All extant Southern Hemisphere crayfishes are distributed on landmasses that, prior to the break-up of Gondwana, were separated from each other by Antarctica. This separation by a continent that eventually lost all its endemic crayfish is likely to affect the phylogenetic tree we are able to build from the extant taxa available and needs to be taken into account when considering the evolution of this group.

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BIOSKETCH

Alicia Toon has an interest in phylogenetics, phylogeography, speciation and adaptive trait evolution. Our working group is part of the US National Science Foundation Decapod Assembling the Tree of Life program (http://decapoda.nhm.org/) and we are interested in uncovering decapod relationships to test ecological, morphological, evolutionary and biogeographical hypotheses.

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