The Timing of the Diversification of the Freshwater Crayfishes

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ABSTRACT

Freshwater crayfish (Astacidea) serve as model organisms for many diverse disciplines, from neurology to toxicology, and have been the focus of many physiological, ecological, and molecular-based studies. Although much of the recent work has focused on the evolutionary history, phylogeography, and conservation biology of freshwater crayfishes, estimations of their divergence times and radiations have never been made. Recently, divergence time estimations for decapods provided the first proposed molecular-timing hypothesis involving freshwater crayfish. In this study we focus specifically on estimating divergence among Astacidea. We employ a Bayesian method implemented in multidivtime for timing estimation, calibrated with multiple fossils including a Parastacoidea fossil newly discovered in Australia. With our narrow taxonomic focus, we increase the accuracy and provide divergence estimations more specific to freshwater crayfish. Our molecular time estimation supports a late Permian to early Triassic divergence from Nephropoidea with radiation and dispersal before the breakup of Pangaea, as well as subsequent speciation and radiation prior to or directly associated with Gondwana and Laurasia disassembly. The breakup of Gondwana and Laurasia resulted in the separation of Parastacoidea and Astacoidea during the Jurassic. The hypothesized divergence and radiation of these two superfamilies are also supported by our molecular time estimations. For the three families of crayfish, we estimate the Astacidae radiation at ~153 million years ago (MYA), the Cambaridae radiation at ~90 MYA, and diversification of Parastacidae at ~161 MYA.

1 INTRODUCTION

Freshwater crayfish have a worldwide distribution, occurring on all continents except Antarctica and Africa excluding Madagascar. They are placed in the infraorder Astacidea, which includes three superfamilies: 1) Astacoidea—Northern Hemisphere crayfish, 2) Parastacoidea—Southern Hemisphere crayfish, and 3) Nephropoidea—the clawed lobsters. The crayfish form a monophyletic group (Crandall et al. 2000b) and have ~640 described species (Crandall et al. 2008) with Nephropoidea, the clawed lobsters, hypothesized as their sister group (Crandall et al. 2000a). Parastacoidea contains one family, Parastacidae, with 15 genera (*Astacoides*, *Astacopsis*, *Cherax*, *Engaeus*, *Engaewa*, *Euastacus*, *Geocharax*, *Gramastacus*, *Ombrastacoides*, *Paranephrops*, *Parastacus*, *Samastacus*, *Spinastacoides*, *Tennuibranchiurus*, and *Virilastacus*) and 176 species. Astacoidea contains two families, Astacidae and Cambaridae. Astacidae has three genera (*Pacifastacus*, *Astacus*, *Austropotamobius*) (Hobbs 1974) to six genera (Starobogatov 1995), depending on whose taxonomy one prefers, and 16–39 species. Cambaridae has 2 subfamilies (Cambarellinae and Cambarinae) containing 11 genera (*Barbicambarus*, *Bouchardina*, *Cambarellus*, *Cambarus*, *Distocambarus*, *Fallicambarus*, *Faxonella*, *Hobbseus*, *Orconectes*, *Procambarus*, *Troglocambarus*), plus a distinct genus *Cambaroides* that appears to be more distantly related to these two subfamilies; Cambaridae has a total of 445 species (see Crandall & Buhay 2008 for a recent summary).

Freshwater crayfish relationships at higher taxonomic levels are well understood. The two superfamilies are monophyletic sister clades, and Parastacidae and Astacidae are monophyletic (Crandall et al. 2000b; Rode & Babcock 2003). Cambaridae is paraphyletic, as one of its genera, *Cambaroides*, is in a basal lineage to Astacidae and the rest of the Cambaridae genera (Braband et al. 2006; Crandall et al. 2000b). Most of the taxonomic relationships within Cambaridae are currently best explained by Hobbs' (1989) taxonomic revision. The following taxonomic groups within *Cambarinae* have been evaluated since Hobbs' (1989) revision: the genus *Orconectes* (Taylor and Knouft 2006); subgenus *Crockerinus* within *Orconectes* (Taylor and Hardman 2002); the subgenus *Scapulicambarus* within *Procambarus* (Busack 1989); and the subgenus *Aviticambarus* within *Cambarus* (Buhay et al. 2007). Within Astacidae, the taxonomy within *Astacus* and *Pacifastacus* is based on Hobbs' (1989) morphological taxonomic revision. The taxonomy within *Austropotamobius* was recently examined by Grandjean et al. (2000), Zaccara et al. (2004), and Fratini et al. (2004), all of whom reported multiple cryptic subspecies. However, Starobogatov (1995) provided a comprehensive overview of the Astacidae that resulted in 6 genera and 36 species, but his proposed taxonomy has not yet taken hold in the literature. The Astacidae in general is in need of a detailed examination to unify the diversity of ideas concerning its taxonomy.

The first comprehensive phylogenetic hypothesis of the Parastacoidea was morphologically based on male genitalia, cephalothorax, chelae, and body shape (Riek 1969). Studies that followed addressed the relations within this family using morphological, protein, and molecular data (Austin 1995; Crandall et al. 1995; Patak & Baldwin 1984; Patak et al. 1989; Riek 1972). These studies included limited sampling of genera and had conflicting results. The study by Crandall et al. (2000a) established well-supported relations within this family by analyzing 13 of the then 14 genera using mitochondrial DNA. Out of the now 15 genera in Parastacoidea, eight have been recently evaluated taxonomically and/or phylogenetically: *Engaewa* (by Horwitz and Adams 2000), *Cherax* (by Austin 1996), *Euastacus* (by Schull et al. 2005), two new genera *Spinastacoides* and *Ombrastacoides* (by Hansen and Richardson 2006), and *Engaeus*, *Geocharax*, and *Gramastacus* (by Schultz et al. 2007).

Through these recent studies, the problems of determining relationships among the freshwater crayfish become very apparent. Studies have not been fully comprehensive and have been limited in taxonomic sampling, due in part to the large number of freshwater crayfish taxa and their global distribution. The genetic and protein studies have shown high morphological and habitat variation within species and have demonstrated that convergent evolution is common (Braband et al. 2006; Crandall & Fitzpatrick 1996; Taylor & Hardman 2002). Additionally, these studies have revealed multiple cases of paraphyly, discovery of cryptic species, and even some unsupported described species (e.g., Austin 1996; Grandjean et al. 2000; Hansen & Richardson 2006; Schull et al. 2005; Schultz et al. 2007; Crandall et al. 2008). As a result, Sinclair et al. (2004) proposed the completion of a worldwide phylogeny based on multiple mitochondrial and nuclear genes. Because of the group's extensive convergent evolutionary history, only through molecular analysis and full taxonomic coverage will it be possible to infer the relationships within this group. While this goal is yet to be achieved, here we report on a phylogenetic status of the major genera of freshwater crayfish and the associated divergence times to put such a phylogeny into a temporal perspective.

Recently, Porter et al. (2005) published a phylogeny and associated divergence time estimates for the decapods as a whole. This study was the first molecular-based time hypothesis that included the freshwater crayfish. The goal of that study was to estimate decapod divergences; hence, only two of their fossil calibrations came from within the infraorder Astacidea. Multiple studies have shown that the most important factor affecting molecular divergence time estimation is the number and distribution of the calibration points throughout the tree (Lee 1999; Porter et al. 2005; Thorne & Kishino 2002; Yang & Yoder 2003; Yoder & Yang 2000). In this study we focus specifically on estimating divergence among Astacidea. By including multiple fossil calibrations and a specific taxonomic focus we increase the accuracy and can provide divergence estimations more specific to freshwater crayfish events. The use of molecular-based divergence time estimates has improved the

understanding of the timing of evolutionary processes and events. A molecular time estimate for crayfish is particularly interesting because the current hypotheses of the divergence times correlates with estimates of the timing of the breakup of Pangaea and disassembly of Gondwana and Laurasia (Ahyong & O'Meally 2004; Crandall et al. 2000b; Porter et al. 2005; Rode & Babcock 2003). We test the hypotheses that freshwater crayfish diverged from *Nephropoidea* (clawed lobsters) during the Permian or Triassic, and that Parastacoidea (Southern Hemisphere) and Astacoidea (Northern Hemisphere) divergence occurred during the Jurassic (Ahyong & O'Meally 2004; Crandall et al. 2000b; Porter et al. 2005; Rode & Babcock 2003), using a comprehensive phylogeny at the genus level for the major lineages of freshwater crayfish.

2 METHODS

2.1 *Taxon sampling, DNA extraction, PCR, and sequencing*

Crayfish species were chosen to represent major crayfish lineages in order to date the divergence times of these major groups (Table 1). Multiple sequences were obtained from GenBank, and the remaining sequences were generated by Toon et al. (in prep.), as indicated by an asterisk in Table 1. Although specifics can be obtained from Toon et al. (this volume), crayfish collection, preservation, DNA extraction, and amplification were completed following protocols and methods described in Crandall & Buhay (2004) and Crandall & Fitzpatrick (1996) for 16S rDNA (~500 bp; Crandall & Fitzpatrick, 1996), 12S rDNA (~400; Mokady et al. 1999) and COI (~700 bp; Folmer et al. 1994), and two nuclear genes: 18S (~2,000 bp; Whiting et al. 1997) and 28S (~3,000 bp; Whiting et al. 1997).

2.2 *Phylogenetic analyses*

Astacoidea and Parastacoidea were aligned separately using MAFFT (Katoh et al. 2002; Katoh et al. 2005) implementing the G-INS-I alignment algorithm and then combined using the MAFFT profile alignment option with default parameters for each gene. *Homarus americanus* and *Sergio mericeae* were then aligned to the ingroup using MAFFT profile alignment for each gene. This multiple sequence alignment program has been shown to provide quick and accurate results by Notredame et al. (2000) and Katoh et al. (2005). The iterative algorithms used by MAFFT allow for repeatability of alignment. GBlocks 0.91b (Castresana 2000) was used to objectively trim sections of the alignment with questionable homology using the default parameter with the exception of the allowed gap positions parameter. The latter was set to allow gaps that are present in at least half of the sequences (Talavera & Castresana 2007). Models of evolution for each alignment were estimated in ModelTest (Posada & Crandall 1998) using the AIC criteria (Akaike 1973) to compare and choose best-fit models for the different gene partitions.

Phylogenies were estimated using maximum likelihood (ML) and Bayesian optimality criteria, with RAxML (Stamatakis 2006) and MrBayes (Ronquist & Huelsenbeck 2003), respectively (see Palero & Crandall, this volume, for a general description of these approaches). RAxML is a unique ML program in that it allows the use of multiple models, therefore giving better ML estimates. We partitioned the data set by gene and applied the model GTR+I+G to each gene allowing independent parameters to be estimated during analysis. We selected the tree with the best ML score after multiple independent runs with random starting positions and assessed confidence in nodal support through 1000 bootstrap pseudoreplications. Bayesian analysis was performed in MrBayes, in which four independent runs starting from random trees were run using the default flat priors for 5×10^6 generations sampling every 100 generations. We also ran two independent MrBayes runs with the same settings using the best RAxML tree as a start tree. The negative log likelihood posterior distribution was checked for convergence and length needed for burn-in using the program Tracer

Table 1. Taxa and GenBank accession numbers associated with each sample. Asterisks (*) indicate sequences from Toon et al. (submitted).

Taxon	Gene 12S	16S	18S	28S	CO1
Astacidea Latreille 1802					
Astacoidea Latreille 1802					
Astacus astacus (Linnaeus1758)	EU920881*	AF235983	AF235959	DQ079773	AF517104
Cambarellus shufeldtii (Faxon1884)	EU921117*	AF235986	AF235962	DQ079778	EU921149*
Cambaroides japonicus (de Haan 1841)	EU921118*	AF235987	DQ079742	DQ079779	no seq
Cambarus maculates (Hobbs & Pflieger 1988)	EU921119*	AF235988	AF235964	DQ079780	no seq
Orconectes virilis (Hagen 1870)	EU920900*	AF235989	AF235965	DQ079804	AF474365
Pacifastacus leniusculus (Dana 1852)	EU921116*	AF235985	AF235961	DQ079806	EU921148*
Procambarus clarkii (Girard 1852)	EU920901*	AF235990	EU920952*	EU920970*	AY701195
Parastacoidea (Huxley 1879)					
Astacoides betsileoensis (Petit 1923)	EU920882*	EU920912*	EU920955*	EU920992*	EU921146*
Astacoides crosnieri (Hobbs 1987)	EU921112*	EU921122*	EU921129*	EU921136*	EU921147*
Astacopsis tricornis (Clark 1936)	DQ006419	DQ006548	EU921123*	EU921135*	DQ006290
Cherax cairnsensis (Riek 1969)	EU921113*	EU921120*	EU921124*	EU921132*	EU921113*
Cherax quadricarinatus (von Martens 1868)	DQ006423	DQ006552	EU921125*	EU921139*	DQ006294
Engaeus fossor (Erichson 1846)	EU921114*	EU921121*	EU921126*	EU921134*	EU921144*
Euastacus sulcatus (Riek1951)	DQ006525	DQ006651	EU921127*	EU921133*	DQ006396
Geocharax gracilis (Clark 1936)	EU921115*	AF235992	AF235968	EU921140*	EU921145*
Paranephrops planifrons (White 1842)	DQ006544	AF135995	EU921128*	EU921141*	DQ006415
Ombrastacoides huonensis (Hansen & Richardson 2006)	EU920905*	AF135997	EU920956*	EU920995*	EU921143*
Parastacus brasiliensis (von Martens 1869)	EF599134	AF175244	EU921130*	EU921138*	EF599158
Samastacus spinifrons (Phillipi 1882)	EF599136	AF175241	EU921131	EU921137	EF599159
Nephropoidea (Dana, 1852)					
Homarus americanus (H. Milne-Edwards 1837)	DQ298427	HAU11238	AF235971	DQ079788	DQ889104
Outgroup					
Thalassinidea					
Callianassoidea (Dana 1852)					
Sergio mericeae (Manning & Felder 1995)	EU920909*	DQ079733	DQ079768	DQ079811	no seq

v1.4 (Rambaut & Drummond 2007) across all Bayesian runs. Converging MrBayes runs were combined after independent analysis and deletion of burn-in. Nodal confidence for the Bayesian trees was assessed using posterior probabilities compiled from the set of trees post-burn-in. We compared the support indices from our RAxML and MrBayes hypothesis and chose the phylogeny with the highest number of well-supported nodes considering bootstrap values ≥ 70 and Bayesian posterior probabilities ≥ 95 as high support for use in our molecular clock estimation.

2.3 *Fossil calibrations*

The fossil record is being continually updated, and relationships based on it are constantly being reanalyzed. The recent discovery of a new Australian fossil *Palaeoechinastacus australianus* (Martin et al. 2008) doubles the previously recorded geological time range of the family *Parastacidae* (Hasiotis 2002; Rode & Babcock 2003; Sokol 1987, 1988). Because fossil calibrations are a major source of error in molecular timing estimation, it is imperative to use multiple calibrations to get the best possible estimation, thus reducing the inherent amount of error associated with the fossil record (Table 2). Along with fossil calibrations, many studies have incorporated time estimations of vicariate events associated with the split in major land masses such as Pangaea, Laurasia, and Gondwana (Bocxlare et al. 2006; Porter et al. 2005). Our choice of Bayesian molecular time

Table 2. Fossil calibrations used for divergence time estimations, with the node referring to placement of the fossil on the crayfish chronogram.

Taxonomy	Species	Reference	Geologic (MYA)	Node
Infraorder Astacidea **Family** Chimaerastacidae	*Chimaerastacus pacifluvialis*	Amati et al. 2004	Mid Triassic (Upper Ladinian) 227–234	C1
Family Parastacidae	*Palaeoechinastacus australianus*	Martin et al. 2008	Early Cretaceous 106	C3
	Paranephrops fordycei	Feldmann & Pole 1994	early middle Miocene (Otaian-Lillburnian) 21.7–12.7	C4
Family Astacidae	*Astacus licenti*	Van Straelen 1928	Late Jurassic 144–159	C5
	Astacus spinirostris	Imaizumi 1938	Late Jurassic 144–159	C5
Family Cambaridae	*Procambarus primaevus*	Feldmann et al. 1981	Late early Eocene 52.6–53.4	C6

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

estimation requires that we have an estimation of at least one upper time limit (i.e., maximum age). Following Porter et al. (2005), we used the split of Pangaea at 185 MYA as an upper limit calibration for the divergence of the superfamilies Astacoidea and Parastacoidea (Crandall et al. 2000b). All other calibrations are estimated as the mean date of the fossil and set as the lower limit calibration indicating the absolute minimum age of the calibrated group (Porter et al. 2005). Additionally, we incorporated fossil calibrations for the origin of the family Astacidae and the split between Astacidea and Thalassinidea as the root node for our phylogenetic and molecular time estimation (Amati et al. 2004; Imaizumi 1938; Van Straelen 1928). Finally, we included three additional fossil calibrations: one to calibrate the genus *Procambarus* in Cambaridae and two to represent the family Parastacidae (Feldmann 2003; Feldmann et al. 1998; Martin et al. 2008). We agree with Porter et al. (2005) and others that trace fossil burrows are difficult to associate with crayfish with any amount of certainty (Babcock et al. 1998; Hasiotis 2002). Therefore, we chose to include only fossil records from descriptions of preserved animals. Our choice not to use trace fossils and to set each fossil calibration as the lower limit makes our estimate more conservative, while still allowing us to account for the fossil species existing for an undetermined amount of time before the actual fossilization event.

2.4 *Divergence time estimation*

Freshwater crayfish divergence times were estimated using the multi-locus Bayesian method of Thorne and Kishino (2002) as implemented in the Multidivtime package (http://statgen.ncsu.edu/thorne/multidivtime.html). This approach was built on the continual improvements of molecular clock theory and applications (Kishino et al. 2001; Thorne et al. 1998). This method allows the use of multiple genes while not requiring a full taxa set for all genes included, does not assume a molecular clock in branch estimation, and allows for multiple calibrations. The use of multiple genetic loci and multiple fossil calibrations improves divergence times and rate estimations (Pérez-Losada et al. 2004; Porter et al. 2005; Thorne & Kishino 2002; Yang 2004; Yang & Yoder 2003; Yoder & Yang 2000). Multidivtime estimates times and rates by minimizing the discrepancies in

branch lengths and by minimizing rate changes over branches. This Bayesian method employs the rate evolution model of Thorne et al. (1998) and Kishino et al. (2001), which averages rates using a Markov chain Monte Carlo (MCMC) process.

We used three different parameter settings for Multidivtime. First, rttm and rttmsd (distribution of time separating the ingroup root from the present and the standard deviation, respectively) were set to 2.5 (250 MYA), and rtrate and rtratesd (prior evolutionary rate and standard deviation, respectively) were set to 0.0136 substitutions per million years. Second, rttm and rttmsd were set at 2.38 (238 MYA), and rtrate and rtratesd were set to 0.015 substitutions per million years, to see the effect of placing it closer to the age of the fossil calibration. Third, the rttm and rttmsd were set at 3.5 (350 MYA), and the rtrate and rtratesd were set to 0.0102 substitutions per million years to explore the effects of perturbations to the rttm setting. For each parameter setting, we applied two different burn-in period settings, 10^4 and 10^6 steps, combined with 5×10^5 samples collected at every 100th cycle. The default settings were used for the rest of the required parameters. A total of 12 runs were completed with three independent random starts for each parameter and burn-in period setting. The three runs for each burn-in and parameter setting were checked, and the set with the most consistent estimations was chosen for our time estimation.

3 RESULTS

3.1 *Phylogenetics*

All models selected by ModelTest were nst=6 with gamma and invariable sites (16S, 18S, and CO1=TVM+I+G ; 28S=TrN+I+G; and 12S=GTR+I+G). There are a limited number of models in RAxML and MrBayes; therefore, the GTR+G+I model was chosen for each partition, allowing the respective programs to estimate the parameters during phylogenetic estimation. The RAxML best tree likelihood score was -24658.608503. Our RAxML tree compared to our Bayesian tree resulted in fewer nodes with high bootstrap support ($\geq$ 70) and Bayesian posterior probabilities ($\geq$ 95). Therefore, the Bayesian tree was used for the molecular divergent time estimation (Fig. 1). The relationships within Astacidea are concordant with recent studies placing the genus *Cambariodes* basal to both Astacidae and Cambaridae. Although *Astacus* and *Pacifastacus* fall out independently, they both fall between the paraphyletic Cambaridae. Parastacids reflect the same relationships as in Crandall et al. (2000b), the most extensive study of the entire family to date.

3.2 *Divergence time estimations*

Changing the rttm parameter, defined as the distribution of time separating the ingroup root from the present, to 2.386 and 3.5 hardly affected the results, with the largest difference in estimations being 3×10^5 years (Table 3). Pérez-Losada et al. (2004) and Porter et al. (2005) found similar results using even larger perturbations and also reported a minimal effect on the overall time estimation. The burn-in period setting of 10^6 steps produced three nearly identical independent time estimations. From these three estimates, we chose the estimation with the smallest 95% posterior intervals for the chronogram (Fig. 1 & Table 3).

Our divergence time estimates between the crayfish lineages (Astacoidea and Parastacoidea) and Nephropoidea is ~239 MYA (node 38). The divergence time estimates for the Northern Hemisphere families resulted in Astacidae divergence ~153 MYA (node 25) being significantly older than Cambaridae divergence at ~90 MYA (node 22). Parastacidae (the Southern Hemisphere crayfish) divergence time is estimated at ~161 MYA (node 36) with the genera having much older divergence times than Northern Hemisphere crayfish.

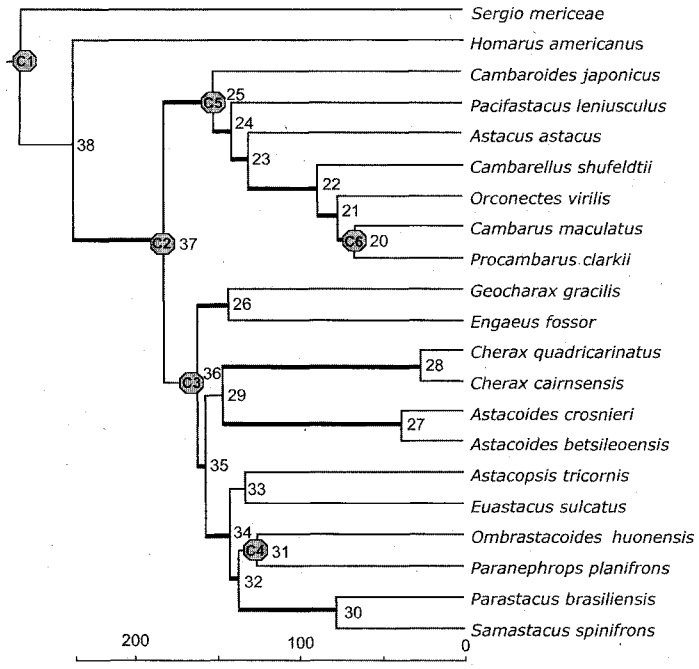

Figure 1. Crayfish divergence time chronogram estimated with a Bayesian tree topology. Bolded branches indicate posterior probability of 1. Nodes labeled C1–C6 indicate locations of fossil calibration (Table 2). Node number refers to the estimated time and 95% posterior interval (Table 3).

Table 3. Node time estimations referring to crayfish chronogram (Fig. 1). Time is represented in MYA with 95% interval, standard deviation, and well-supported ML bootstrap and Bayesian posterior probability.

Node	Time MYA	95% Posterior Interval Lower	Upper	Standard Deviation	ML Bootstraps	Bayesian Posterior Probability
20	67.342	53.461	96.797	11.820	97	1
21	77.593	56.790	109.350	13.966	100	1
22	90.413	63.279	125.150	16.161	100	1
23	132.263	100.796	150.774	13.184	82	1
24	143.006	117.570	154.648	9.769	-	1
25	153.367	151.552	157.798	1.698	100	1
26	144.531	128.907	157.830	7.363	99	1
27	37.916	6.370	73.685	17.888	100	1
28	25.915	12.882	45.609	8.481	100	1
29	147.774	130.894	161.587	7.834	-	-
30	78.473	40.3	109.408	17.520	100	1
31	127.486	102.616	149.049	11.846	-	-
32	138.331	115.897	156.189	10.326	87	1
33	135.304	111.904	153.525	10.688	87	-
34	144.026	123.144	160.854	9.653	80	1
35	158.120	143.756	169.560	6.56	-	1
36	161.875	150.093	171.880	5.542	100	-
37	183.459	179.650	184.957	1.446	100	1
38	239.345	230.789	258.697	7.587		

4 DISCUSSION

4.1 *Phylogeny and divergence time estimations*

The phylogenetic results were consistent with the most recent molecular studies for freshwater crayfishes (Crandall et al. 2000b; Porter et al. 2005; Rode & Babcock 2003). The tree is generally well supported with the monophyly of the freshwater crayfish being recovered in 100% of the Bayesian posterior distributions. Most lineages within the Parastacidea are similarly supported, with a few of the deeper nodes having low support values. Our divergence time estimations support the divergent time hypotheses of Crandall et al. (2000b), Rode and Babcock (2003), Ahyong & O'Meally (2004), and Porter et al. (2005). In the most current divergence hypothesis, Porter et al. (2005) estimated the divergence between the crayfish lineages Astacoidea and Parastacoidea from Nephropoidea at ~278 MYA. Our estimation of ~239 MYA (node 38) differs probably because of the calibration of the node prior to this estimation in each study. Although both studies used *Chimaerastacus pacifluvialis* (C1) as a lower limit, we additionally used it as a guideline to estimate the time from the root to the tip, setting it at 250 MYA. Our estimation falls between their two estimations when they calibrated the previous node as a lower limit and when it was calibrated as an upper and lower bound. We estimate the Astacidae radiation at ~153 MYA (node 25), fitting within the range of the fossils used for calibration. We include *Cambaroides japonicus* in this estimation due to consistent placement of this genus within the Astacidae (Braband et al. 2006). Therefore, our estimate is significantly older than the Astacidae radiation estimate of Porter et al. (2005). Although their actual estimation is not reported, a visual inspection of the chronogram of Porter et al. (2005) reveals a similar estimation when including *Cambaroides japonicus*. The Cambaridae radiation was estimated at ~90 MYA (node 22), which coincides with Porter et al. (2005). These divergence estimates support the idea that Astacoidea diversified and was widespread before the split of Laurasia during the late Cretaceous (Owen 1976) ~65 MYA.

The diversification of Parastacidae was calibrated with a new fossil dated to 106 MYA (Martin et al. 2008), which resulted in our estimated divergence time of ~161 MYA. This divergence time suggests that older Parastacidae fossils are likely to be found in Australia. The first stages of Gondwana separation are estimated to have begun ~150 MYA with the separation of South America and Africa from Antarctica-India-Madagascar-Australia-New Zealand (Wit et al. 1999). Veevers (2006) estimates a later separation of Africa-India from Australia-Antarctica-South America at ~132 MYA. Regardless of the specific Gondwana breakup theory ascribed, the divergence time estimates between South America and Australia-New Zealand crayfish (node 32) and the Madagascar and Australian crayfish (node 29) can be explained by vicariance associated with the disassembly of Gondwana. The split between *Ombrastacoides* (Australia) and *Paranephrops* (New Zealand) (node 32) ~127 MYA is also consistent in that vicariance may have happened before or in sync with this separation, which is commonly estimated at ~90 MYA, but rifting may have begun as early as ~110-115 (Stevens 1980, 1985).

4.2 *Interpreting results*

Molecular time estimations are prone to multiple errors, partially due to complete reliance on fossil calibration, in which there is an inherent amount of error, including incorrect assignment of fossils, error in chronological and date assignment, and introduced topological errors in the phylogenetic estimation (Graur & Martin 2004). With the amount of possible error, it is encouraging to get results that are consistent with the current fossil record and/or that are supported by theories of distribution and divergence. Although most time estimations were discussed as point estimation (the expected estimate of posterior distribution), readers should be aware of, and consider, the 95% posterior interval for all estimations. The Bayesian method employed is one of the few methods that allows the user to set minimum age fossil calibrations, but in doing so it results in a larger variance, increasing

the size of the posterior age interval. By setting fossil calibration intervals instead of minimum age estimates, you can effectively reduce the amount of variance resulting in a reduced size of the posterior age distribution. In the future, molecular clock estimates may consider using *Astacus licenti* and *Astacus spinirostris* fossil calibrations (C5) for Astacidae as an interval calibration instead of minimum age for two reasons. First, it is supported by two independent fossils. Second, our point estimation fits within the fossil estimated time interval. Including more upper limit calibrations or employing calibration intervals reduces the size of posterior interval estimates.

5 CONCLUSIONS

Our molecular clock estimation supports a late Permian to early Triassic divergence of freshwater crayfishes from Nephropoidea with radiation and dispersal before the breakup of Pangaea. Subsequent speciation and radiation prior to, or directly associated with, Gondwanan and Laurasian breakup resulted in the separation of the superfamilies Parastacoidea and Astacoidea during the Jurassic, thus supporting current divergent time estimations (Ahyong & O'Meally 2004; Crandall et al. 2000b; Porter et al. 2005; Rode & Babcock 2003). The hypothesized divergences and radiation of the two superfamilies attributed to the breakup of Laurasia and Gondwana are supported by our molecular time estimations. We do not expect this to be the last molecular divergence estimation for freshwater crayfishes, and we expect future estimates to improve in accuracy with the discovery of new fossils and new molecular dating techniques.

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