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Patterns of molecular diversity in wild stocks of the redclaw crayfish (*Cherax quadricarinatus*) from northern Australia and Papua New Guinea: impacts of Plio-Pleistocene landscape evolution

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SUMMARY

1. Analysis of mitochondrial and nuclear DNA (microsatellites) in 379 individuals, collected from 15 localities in northern Australia and Papua New Guinea (PNG), demonstrated that wild redclaw crayfish (*Cherax quadricarinatus*) populations consist of two highly divergent Australian lineages and two PNG lineages.
2. The disjunction between the two Australian lineages occurs over a distance of approximately 200 km in the south-western corner of the Gulf of Carpenteria. These data conflict with an earlier study that detected no significant differentiation in 23 variable allozyme loci in redclaw sampled from northern Australia, but concur broadly with the previous recognition of two morphologically distinct species (*C. quadricarinatus* and *C. bicarinatus*) across northern Australia, and a third species in PNG (*C. albertsii*).
3. The inferred timing and patterns of divergence evident in the molecular data presented here closely align with a similar pattern reported in a co-distributed freshwater decapod crustacean, and broadly reflect patterns in some vertebrate taxa with similar distributions across northern Australia and PNG.
4. These congruent patterns most probably reflect periodic Plio-Pleistocene land and freshwater connections between Australia and New Guinea.

Keywords: Australia, evolution, Lake Carpentaria, New Guinea, phylogeography

Introduction

Parastacid crayfishes represent an important ecological and evolutionary component of Southern Hemisphere fresh waters (Riek, 1969; Crandall *et al.*, 1999). Australia has the highest diversity of southern freshwater crayfishes (Parasticidae) (Riek, 1969; Crandall *et al.*, 1999), and while freshwater crayfish form an important component of an otherwise depauperate freshwater fauna in Australian rivers, little is known about the extent of intraspecific variation in the major species. *Cherax quadricarinatus* Von Martens 1868, or 'redclaw', occurs naturally in the rivers of northern Australia – from the Kimberley region of north-western Western Australia across the north to the Gulf of Carpentaria and Cape York. The species also occurs in some rivers in the southern half of Papua New Guinea (PNG) – a distribution pattern which suggests that intraspecific variation in these wild stocks may be high. In support, Gu, Mather & Capra (1995) demonstrated significant genetically based variation among inbred lines developed from distinct wild populations sourced from three geographically

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discrete Gulf of Carpentaria river stocks. Along similar lines, variation in morphology, behaviour, habitat preference and some quantitative traits, including maximum body size, growth rate and salinity tolerance, have been reported among wild *C. quadricarinatus* stocks in Australia (Austin, 1996).

The high degree of morphological variation evident in many *Cherax* species has led to problems with their systematics that, until recently, has been based largely on comparisons of external morphological characters (Riek, 1969; Austin, 1996). A study that examined both morphometric and genetic data (allozyme variation) in *Cherax destructor* Clark 1936 revealed two distinct morphotypes that corresponded with significant levels of genetic divergence, supporting subspecies status for *C. albidus* (Campbell, Geddes & Adams, 1994). Similarly, two genetically distinct allopatric forms of *Cherax tenuimanus* Smith 1912 have recently been described (Austin, 1996; Nguyen *et al.*, 2002). Recognition of discrete forms within what was previously considered to be a single species of *C. destructor*, and potentially also *C. tenuimanus*, has important implications for their conservation and management. The taxonomy of *C. quadricarinatus*, the focus of the current study, has also been in doubt for some time.

Cherax quadricarinatus as it is currently known, was originally described as three taxa (Fig. 1) based on external morphology (Nobili, 1901; Riek, 1969). Eastern populations in Queensland were classified as *C. quadricarinatus* von Martens 1868, western populations in the Northern Territory were classified as *C. bicarinatus* Gray 1845, and PNG populations as *C. albertsii* Nobili, 1901; based on small differences in the male genitalia, cephalothorax, number of rostral spines, chelae and body shape (Nobili, 1901; Riek, 1969). *Cherax bicarinatus* differs from *C. quadricarinatus* in the shape of the areola and length of the rostral carinae in addition to a slight difference in the rostral formula (Riek, 1969), while *C. albertsii* was described as possessing narrower claws relative to the other two 'species' (Nobili, 1901). The natural distributions of the three taxa were considered to be disjunct at the time, and this was used to support their recognition as discrete species. Riek's (1969) classification remained unchallenged until a later study by Austin (1996) of the three taxa. Patterns of variation at 23 variable allozyme loci used in that study could not distinguish among them, and they were as a consequence considered to belong to a single morphologically variable species (Austin, 1996). Austin (1996) concluded that any morphological differentiation observed was likely to reflect morphological plasticity in response to different environmental regimes, rather than evidence for discrete species. Morphological plasticity is common in decapod crustaceans, as evidenced in a study of *Macrobrachium australiense* Holthuis 1950, which showed that rostral morphology (a character used widely in decapod taxonomy) was influenced strongly by the environment (Dimmock, Williamson & Mather, 2005).

One important discovery since Riek's (1969) systematic review of *Cherax* is that the natural distributions of western and eastern Australian redclaw stocks he recognized as *C. bicarinatus* and *C. quadricarinatus*, respectively, were not accurate, and redclaw apparently occur almost continuously across the major rivers that flow into the Gulf of Carpentaria and the Timor Sea east of Darwin. Since only low levels of allozyme differentiation were evident among Riek's (1969) allopatric 'species', Austin (1996) argued on distributional and genetic evidence that the two Australian forms and the PNG form should be reclassified as a single species under the name *C. quadricarinatus*.

Macaranas *et al.* (1995) in contrast had identified a single fixed allozyme difference, at a Carbonic anhydrase locus (CA), an enzyme not screened by Austin (1996), between western and eastern redclaw populations across northern Australia. Detection of a fixed allozyme difference contrasts with Austin's (1996) suggestion that *C. quadricarinatus* are genetically homogeneous across their natural range in northern Australia. CA catalyses the hydration of CO_2 to provide H^+ and HCO_3^- for use in sodium and chloride uptake (Henry, 1988). This is an important metabolic process during ion exchange in freshwater crayfish, especially during the intermoult (Wheatly & Gannon, 1995). Macaranas *et al.* (1995) hypothesized that the CA fixed difference could be related to regional differences in local water chemistry experienced by western (alkaline water) and eastern (acidic water) populations across northern Australia respectively. However, whether this difference reflects historical isolation and independent evolution, or results from extreme selection on pH regulation associated with regional differences in environmental water chemistry, is unknown.

External phenotype can be affected by the environment, and so taxonomic delineations based solely on

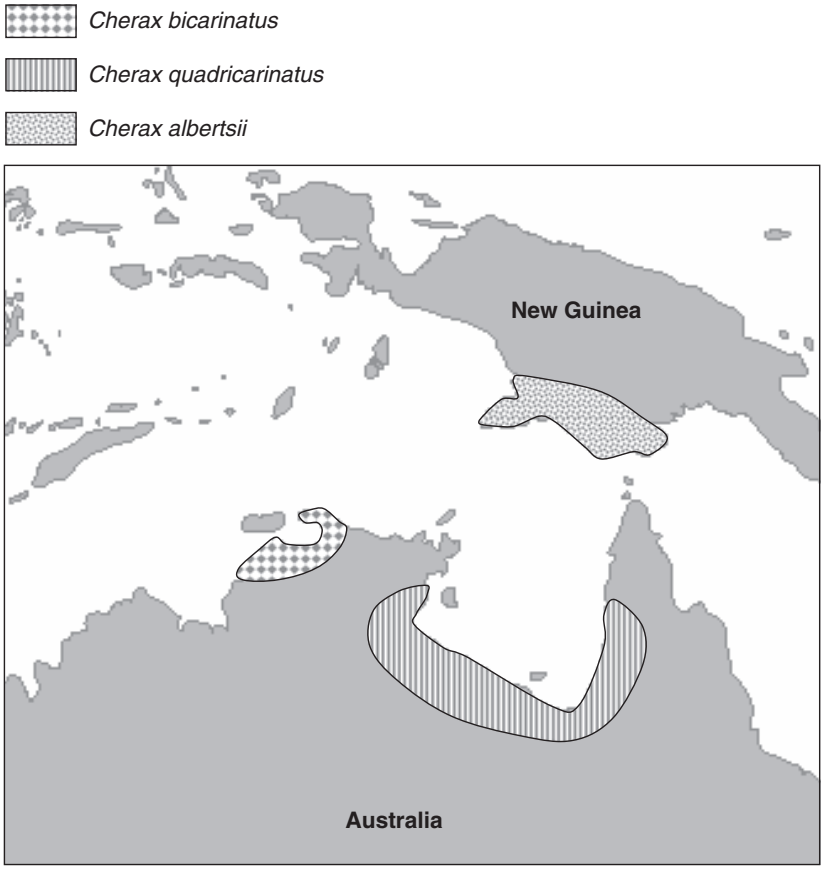

Fig. 1 Riek's (1969) classification based on morphological differences of *Cherax* in northern Australia and southern New Guinea.

morphometrics need to be interpreted with caution, as does the use of allozyme data in decapod crustaceans (Hedgecock, Tracey & Nelson, 1982). In contrast, a vast body of literature now exists (e.g. Avise, 1994; Bouchon, Souty-Grosset & Raimond, 1994; Horovitz & Meyer, 1995) that has demonstrated the ability of molecular markers, including mitochondrial DNA (mtDNA) and nuclear DNA (nDNA; e.g. microsatellites), to delineate lineage and/or species boundaries effectively for sympatric taxa that display little or limited morphological variation (Moritz & Joseph, 1993; Daniels *et al.*, 2003; Gouws, Stewart & Daniels, 2004).

The extensive natural distribution of redclaw across northern Australia and in southern PNG, where environmental conditions vary widely, suggests that genetic variation in wild populations could be high, although how this variation may be distributed geographically, is currently unknown. Recent studies of geographical variation in mtDNA and nDNA in a co-distributed freshwater decapod crustacean (*Macrobrachium rosenbergii* De Man 1879) suggests that significant divergence can evolve in wild stocks that are structured regionally across northern Australia and New Guinea (De Bruyn, Wilson & Mather, 2004a,b; De Bruyn & Mather, 2007). In addition, earlier studies of diadromous and brackishwater species show similar phylogeographic patterns across this region (*Lates calcarifer* Bloch 1790 – Chenoweth *et al.*, 1998; *Scylla serrata* Forskål 1775 – Gopurenko & Hughes, 2002). Thus, information on the patterns of genetic diversity in wild redclaw stocks could be central to future efforts aimed at conserving this species, which is currently listed as 'Vulnerable' on the IUCN Red List (IUCN, 2007; http://www.iucnredlist.org). The objective of the current study, therefore, was to characterize both mtDNA and nDNA (microsatellite) variation and to investigate the population structure and systematic status of wild *C. quadricarinatus* populations.

Methods

Sampling

We sequenced a 523 base pair (bp) fragment of the mitochondrial Cytochrome oxidase I gene (COI), and a

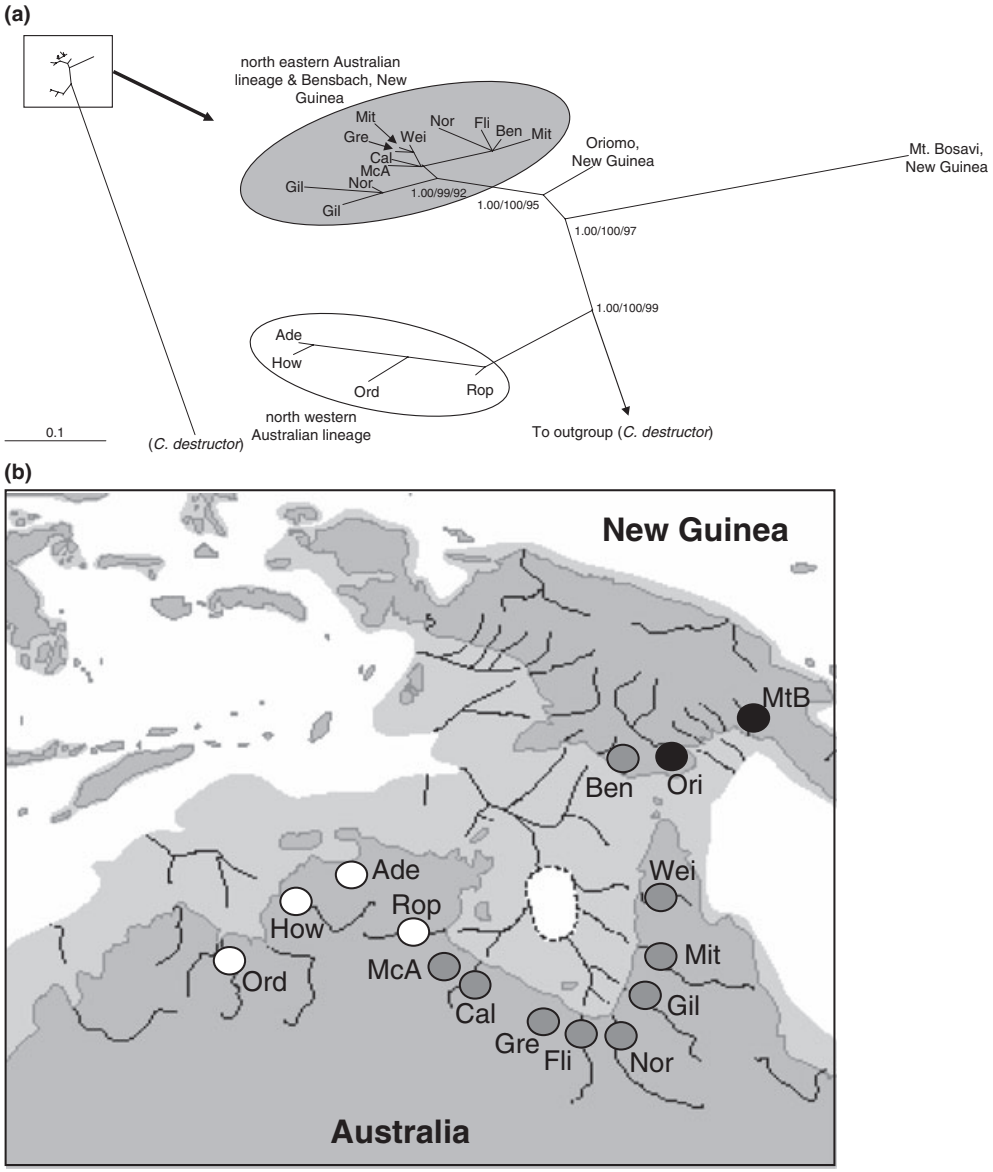

Fig. 2 (a) Bayesian consensus tree for 18 unique concatenated mitochondrial (COI & 16S) haplotypes obtained from sampling 30 *Cherax quadricarinatus* from 15 locations across northern Australia and southern New Guinea. Values at tree nodes indicate (in order): Bayesian posterior probabilities, neighbour-joining bootstraps, maximum-likelihood bootstraps. (b) Map of sampling locations showing the distribution of lineages. Light grey shading on map indicates −120 m sea level contour, and Lake Carpentaria and major freshwater catchments at this time are shown (Voris, 2000).

482 bp fragment of the mitochondrial 16S rRNA gene, in individuals sampled widely from 15 locations across northern Australia and southern PNG (Fig. 2; see Table 1 for sample sizes). In addition, we genotyped 379 individuals at three microsatellite loci developed for *C. quadricarinatus* (Baker *et al.*, 2000), sampled from 12 locations across northern Australia. As only small sample sizes were available from PNG, this precluded microsatellite analyses of these samples.

DNA extraction

Initial COI sequence data obtained using whole genomic DNA suggested that pseudogenes may have been present. This phenomenon has been reported in a wide range of organisms, including a closely related species, *C. destructor* (duplication of 16S rRNA gene region) (Nguyen *et al.*, 2002). To overcome the potential for amplifying pseudogenes, a specific

Table 1 Sample sizes (mtDNA and nDNA) and molecular diversity indices for *Cherax quadricarinatus* based on three microsatellite loci

	COI	16S	Microsatellites			
Locality	*n*	*n*	*n*	NA	H_E	H_O
Kununurra Dam, Ord drainage, WA (Ord)	2	2	22	16	0.59	0.59
Howard R., Howard drainage, NT (How)	2	2	30	34	0.87	0.58
Adelaide R., Adelaide drainage, NT (Ade)	2	2	24	26	0.78	0.71
Roper R., Roper drainage, NT (Rop)	2	2	11	18	0.78	0.48
McArthur R., McArthur drainage, NT (McA)	2	2	25	23	0.64	0.55
Calvert R., Calvert drainage, NT (Cal)	2	2	23	22	0.67	0.35
Gregory R., Gregory drainage, Qld (Gre)	2	2	33	20	0.68	0.43
Flinders R., Flinders drainage, Qld (Fli)	2	2	32	11	0.65	0.61
Norman R., Norman drainage, Qld (Nor)	2	2	32	35	0.81	0.53
Gilbert R, Gilbert drainage, Qld (Gil)	2	2	34	32	0.73	0.61
Mitchell R., Mitchell drainage, Qld (Mit)	2	2	34	40	0.84	0.57
Weipa R., Mission drainage, Qld (Wei)	2	2	30	20	0.68	0.70
Mt Bosavi, Kikori drainage, PNG (MtB)	2	2	–	–	–	–
Oriomo R, Oriomo drainage, PNG (Ori)	2	2	–	–	–	–
Bensbach R, Bensbach drainage, PNG (Ben)	2	2	–	–	–	–

n, sample size; NA, number of alleles at all loci, mean expected (H_E) and observed (H_O) heterozygosity respectively.

mtDNA extraction protocol was employed to isolate mtDNA and to remove nDNA contamination before amplification. mtDNA was extracted using a modified protocol developed by Tamura & Aotsuka (1988) as follows: approximately 100 mg of *C. quadricarinatus* muscle tissue or tail clip was digested in 1 mL homogenizing buffer (0.25 M sucrose, 10 mM EDTA and 30 mM Tris–HCl pH 7.5) and 10 μL of 20 mg/mL proteinase K for 1–2 h. This solution was then centrifuged at 10 000 *g* for 1 min to pellet nuclei and cellular debris. The supernatant was recovered and centrifuged at 12 000 *g* for 10 min to pellet the mitochondria. The pellet was resuspended in 100 μL 10 mM Tris–EDTA pH 8.0 and 10 mM EDTA. Resuspended mitochondria were combined with 200 μL of freshly prepared 0.18M NaOH containing 1% sodium dodecyl sulphate and samples were incubated on ice for 5 min. One hundred and fifty microlitre of ice-cold solution of 3 M potassium and 5 M acetate was then added, and the sample incubated on ice for a further 5 min. This mixture was centrifuged at 12 000 *g* for 5 min and the supernatant was recovered. Standard phenol : chloroform/isoamyl procedures were then employed, followed by ethanol precipitation, to recover mtDNA (Sambrook, Fritsch & Maniatis, 1989). Whole genomic DNA was extracted using standard phenol : chloroform extraction methods, and collected by ethanol precipitation, for subsequent microsatellite analyses.

Genotyping

Universal crustacean 16S rRNA primers (Crandall & Fitzpatrick, 1996) were employed in addition to COI primers (Palumbi *et al*., 1991) successfully to amplify *C. quadricarinatus* mtDNA. The degenerate primer (COf-L) was replaced with a specific primer (COcq-L: 5′-CGGCATAGTCTCACACATCG-3′) designed here for *C. quadricarinatus* to ensure specificity during PCR, and to provide unambiguous sequence results. Thermal cycling was performed on a PTC-100 thermocycler (MJ Research Inc., Waltham, MA, U.S.A.) under the following conditions: 3 min denaturation at 94° C, followed by 30 cycles of 30 s at 94° C, 30 s at 55° C, 30 s at 72° C and a final 10 min extension at 72° C, before cooling to 4° C for 10 min. PCR products were visualized on a 1% agarose gel, before purifying according to the QIAquick PCR purification preps (QIAGEN Inc., Doncaster, Australia) protocol. DNA sequencing was conducted at the Australian Genome Research Facility at the University of Queensland, Brisbane, Australia. Both strands of the PCR product were sequenced. The genomic library used to develop seven-specific microsatellite markers for *C. quadricarinatus* was initiated from an individual sampled from the Flinders River in north-eastern Australia. Procedures for the construction and screening of the genomic library for microsatellites, and PCR conditions, are outlined in Baker *et al.* (2000). Western

C. quadricarinatus populations would not amplify at all seven loci. After extensive experimental manipulation that involved changing PCR conditions and redesigning primers, western *C. quadricarinatus* populations would amplify at only three of the seven loci. Microsatellite analyses presented here are therefore restricted to data from three loci (CQ003; CQ004; CQ006) that amplified successfully in both eastern and western individuals. Screening of microsatellite variation was conducted using hex-labelled primers on a Gelscan 2000 (Corbett Research, Mortlake, Australia) system, and gels analysed using ONEDSCAN software (Scanalytics, Rockville, MD, U.S.A.).

Phylogenetic inference

Sequences were edited in CHROMAS 2.13 (Technelysium Pty Ltd, Eden Prairie, MN, U.S.A.) and aligned with ClustalW (Thompson, Higgins & Gibson, 1994) online using the Australian National Genomic Information Service (ANGIS). To determine whether the mitochondrial regions employed in the present study were evolving according to neutral expectations, we employed Fu & Li's (1993) *D* and *F* neutrality tests in DNASP version 3.99 (Rozas *et al.*, 2003). We investigated whether sequences had reached substitution saturation by plotting separately the number of transitions or transversions between pairs of haplotypes versus the Kimura two-parameter genetic distances that corrects for multiple hits. We first used the two mtDNA data sets independently, and then jointly (concatenated), to construct a Bayesian 50% majority consensus tree in MRBAYES version 3.1 (Ronquist & Huelsenbeck, 2003). The posterior probabilities of phylogenetic trees were estimated by 2×10^7 generation Metropolis-coupled Markov chain Monte-Carlo (MCMCMC) algorithms (GTR+Γ+I model; default priors; four chains, chain temperature = 0.2), with parameters estimated from the data set. Convergence of the MCMCMC was indicated when the average standard deviation of split frequencies fell below 0.00001, and the Potential Scale Reduction Factor (PSRF) was approximately one. For comparison, pairwise nucleotide differences among mtDNA haplotypes were calculated in MEGA version 3 (Kumar, Tamura & Nei, 2004) using an appropriate substitution model as determined in MODELTEST (Posada & Crandall, 1998). A gamma value for each gene fragment determined using PAUP* version 4 (Swofford, 2004) was used to account for variable substitution rates at different nucleotide sites. The resulting distance matrix was used to construct trees with Neighbour-Joining (NJ) algorithms. A quartet-puzzling algorithm was employed in TREE-PUZZLE (Schmidt *et al.*, 2002) to construct maximum-likelihood (ML) trees. Statistical support for nodes for the ML and NJ trees was tested using 1000 bootstrap replicates (Felsenstein, 1985). We first constructed the 16S phylogenetic trees using sequences for the *C. quadricarinatus* sister species *C. rhynchotus* and *C. sp* New Guinea-DM-2003 (Munasinghe, Burridge & Austin, 2004), downloaded from GenBank (accession numbers: AY191774 & AY191775, respectively) as outgroups. The topologies of the tree did not differ using these outgroups or the closely related *Cherax* species *C. destructor* as the outgroup. We therefore used *C. destructor* as the outgroup, as tissue samples were available for us to extract and sequence both COI and 16S for this species, to add to our concantenated data set. Sequences or tissue samples for other *Cherax* species (e.g. *C. barretti* Clark 1941, *C. wasselli* Riek, 1969) with geographically proximate distributions to *C. quadricarinatus* were unavailable for use as outgroups. Thus, to eliminate the possibility that our genetically divergent choice of outgroup was affecting tree topology, we verified the intraspecific topology against an unrooted median joining network implemented in NETWORK (Bandelt, Forster & Rohl, 1999).

Population genetic analyses

For microsatellite data, allele frequencies, expected (H_E) and observed (H_O) heterozygosities, and tests for linkage disequilibrium (LD) and Hardy–Weinberg equilibrium (HWE) (Table 1) were performed in GENEPOP (Raymond & Rousset, 1995) and GENALEX version 1.5 (Peakall & Smouse, 2001). Unbiased estimates of Fisher's exact test employing the Markov chain method (10 000 iterations) were used to calculate values of significance for all tests performed in GENEPOP. F_{ST} estimates were calculated (Weir & Cockerham, 1984) and tested for significance using 1000 random permutations in GENALEX. Hierarchical population genetic structure was examined using AMOVA (Excoffier, Smouse & Quattro, 1992) and tested for significance using 1000 random permutations in GENALEX. A multilocus model-based Bayesian clustering method, implemented in the program STRUCTURE (Pritchard, Stephens & Donnelly, 2000),

was also used to estimate population structure. The method places individuals into K clusters based on posterior probabilities, where K is chosen *a priori*, but can be varied across independent runs. Three independent runs (10^6 iterations; 50 000 burn-in) of each K were calculated, with K ranging from 1 to 14. We used Mantel tests in IBD (Bohonak, 2002) to determine whether a correlation existed between pairwise F_{ST} versus geographical distance. Because *C. quadricarinatus* populations are effectively distributed in two dimensions rather than along linear habitat features, data were log-transformed as advocated by Rousset (1997).

We were interested in assessing if gene flow occurs between western and eastern Australian lineages (see *Results*) of *C. quadricarinatus*, and the approximate time of onset of lineage divergence. Pairwise comparisons of individuals sampled from the Roper versus the McArthur Rivers, using both the mtDNA 16S & COI (under a HKY model) data, and the microsatellite data (under an SMM), were considered together within an isolation with migration model using the program IM (Hey & Nielsen, 2004). This work was carried out by using the resources of the Computational Biology Service Unit from Cornell University. In order to estimate time of splitting and bidirectional gene flow rates, we used the mtDNA COI molecular clock rate of 1.4×10^{-8} derived independently by Knowlton & Weigt (1998) and Morrison, Rió & Duffy (2004) for decapod shrimps, and scaled rates for other loci on the COI rate. Following a discarded burn-in period of 10^5 steps, individual simulations were run at least three times (with a different random seed) for 60 million updates or more to ensure similar distributions were being obtained. To ensure adequate mixing of the Markov chain, we used a heating scheme of between 5 and 10 chains. We ran the program until the smallest ESS estimates were >300, and update rates were >20%. This pairwise comparison was chosen as these locations are geographically proximate (<200 km distant), and are found either side of the division between the eastern and western Australian lineages (see *Results*). Note that the two populations compared do not represent their immediate closest relatives based on mtDNA relationships, but this lineage-specific approach has nonetheless proven useful in studies of other taxa (e.g. Won & Hey, 2005; Hoelzel *et al.*, 2007). We also attempted to estimate migration rates and time of splitting for populations within lineages, although convergence was not reached. We did not attempt to estimate these parameters for the New Guinean lineages, as sample sizes were small, and molecular analyses were limited only to mtDNA data.

Results

Phylogenetic relationships based on mtDNA

Neutrality tests indicated that both the 16S rRNA (D: −1.180, $P > 0.10$; F: −1.157, $P > 0.10$) and COI (D: −0.666, $P > 0.10$; F: −0.713, $P > 0.10$) data sets were evolving according to neutral expectations. There was no evidence for saturation in either data set (16S rRNA $R^2 = 0.984$; COI $r^2 = 0.994$, data plots not shown). 16S rRNA sequence data for 30 *C. quadricarinatus* individuals sampled from 15 discrete drainage basins across northern Australia and southern New Guinea revealed 10 unique haplotypes. No 16S variation was detected within any sample site, and several haplotypes were distributed across drainage basins. For example, individuals from the Adelaide and Howard Rivers exhibited the same haplotype, while individuals from three of the western Gulf of Carpentaria rivers (McArthur, Calvert, Gregory), and three of the eastern Gulf rivers (Gilbert, Norman, Jardine), respectively, also shared a single common haplotype.

Cytochrome oxidase I mtDNA sequence data were more variable, with 18 unique haplotypes revealed among the same 30 individuals. As before, variation within rivers was limited or non-existent. As phylogenetic relationships derived from both the 16S rRNA and COI mtDNA data sets were essentially the same, the two data sets were concatenated and the results presented here, based on 1005 bp of combined mtDNA sequence. All methods of phylogenetic reconstruction consistently resolved four discrete well-supported *C. quadricarinatus* lineages (Fig. 2). Each of the three PNG sites represented a genetically distinct lineage. One of the PNG lineages (Bensbach River samples) grouped with all Australian samples from the McArthur River eastward, while the fourth purely Australian lineage encompassed all samples from the Roper River westward (see Fig 2). All sequences generated from this study have been deposited with GenBank (accession numbers: EU244878–EU244912).

Population genetic analyses

No significant LD was identified for microsatellite locus-pair population comparisons. Probability tests detected seven significant deviations from HWE out of 42 comparisons (across populations: How, Rop, Cal, Gre, Nor, Gil, Mit). Five of seven departures from HWE were evident at the CQ004 locus because of heterozygote deficiencies. This could result from the presence of null alleles or the Wahlund effect. When this locus was discarded from the analyses it did not significantly alter our results, so statistical analyses presented here included locus CQ004. Molecular diversity indices are presented in Table 1. AMOVA supported the existence of significant microsatellite variation both within and among populations (60% of variance within populations, 40% among populations; $P < 0.001$; 10 000 iterations). STRUCTURE results based on the microsatellite data set from the Australian samples supported the presence of significant divergence between the eastern and western Australian lineages inferred above from the mtDNA data. The east–west split was observed in all runs of K set from 1 to 10, with individuals sampled from either side of the mtDNA break falling into broadly overlapping clusters either side of this break, but with no clusters crossing this divide. From $K = 11$–14, this pattern eroded as individuals from the same predefined population were split into separate clusters. Log probability values for K ranged from −10 247.8 ($K = 1$) to −5934.7 ($K = 12$), with 12 being the most probable number of populations inferred using STRUCTURE. At higher values of K, most individuals sampled from a single site clustered into their discrete *a priori* population of origin, although some individuals had partial membership in multiple clusters. This was most obvious in the McArthur River population, and to a lesser extent in the Roper River population, possibly reflecting either past admixture of these neighbouring groups, or continuous gradation of allelic frequencies across regions. Similarly, pairwise F_{ST} matrices based on microsatellite loci (65 cases; range 0.076–0.576) showed significant population structure among all sites (Table 2). The lowest F_{ST} estimate was observed for the geographically proximate Mitchell versus Norman pairwise comparison, while the highest F_{ST} value was obtained for the geographically distant Ord (western) versus Flinders (eastern) comparison. All pairwise comparisons after Bonferroni corrections were significant ($P < 0.01$; 1000 permutations). Regressions obtained using permutated Mantel tests (microsatellites: $r = 0.311$, $P = 0.330$) were not significantly different from zero. Mantel tests indicated that isolation-by-distance has played little or no role in structuring genetic variation. This suggests that a stepping-stone model, which may be expected in some freshwater taxa because of genetic exchange among adjacent drainages, is not an adequate representation of population genetic structure in *C. quadricarinatus*. An island model may more accurately represent population structure in redclaw. Isolation-with-Migration results, derived from the combined mtDNA and microsatellite data sets, indicate that the eastern and western Australian lineages have been

Table 2 Population differentiation of Australian *Cherax quadricarinatus* measured by F_{ST}

	Ord	How	Ade	Rop	McA	Cal	Gre	Fli	Nor	Gil	Mit	Wei
Ord	–											
How	0.391	–										
Ade	0.473	0.298	–									
Rop	0.500	0.363	0.428	–								
McA	0.378	0.237	0.284	0.317	–							
Cal	0.385	0.241	0.297	0.328	0.116	–						
Gre	0.358	0.214	0.269	0.315	0.150	0.148	–					
Fli	0.576	0.404	0.480	0.513	0.310	0.352	0.305	–				
Nor	0.350	0.204	0.256	0.300	0.113	0.142	0.106	0.228	–			
Gil	0.403	0.252	0.307	0.348	0.139	0.199	0.158	0.326	0.091	–		
Mit	0.291	0.182	0.244	0.276	0.104	0.129	0.098	0.275	0.076	0.094	–	
Wei	0.376	0.266	0.332	0.377	0.169	0.189	0.191	0.395	0.178	0.210	0.095	–

Comparisons based on three microsatellite loci (below diagonal). All pairwise comparisons were significant ($P < 0.01$) after 1000 permutations.

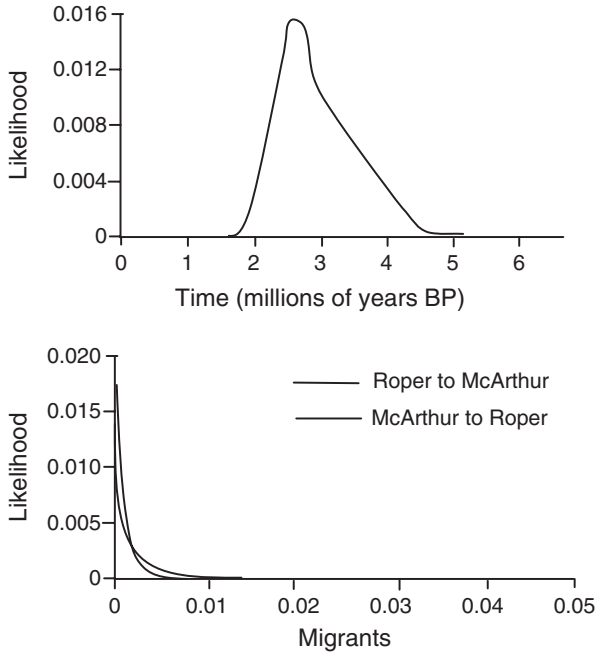

Fig. 3 Posterior probability distributions and 95% confidence intervals for time of splitting and migration for the Roper (north-western Australia lineage) versus McArthur (north-eastern Australian lineage) pairwise comparison, estimated in IM. Illustrated results are based on $\mu = 1.4 \times 10^{-8}$.

separated for approximately two million years, with little or no subsequent gene flow (*m* estimates highly skewed to zero) (Fig. 3). This timing is based on a 'phylogenetic' mutation rate calibrated for marine shrimp (Knowlton & Weigt, 1998; Morrison *et al*., 2004), and should thus be interpreted with caution. Nonetheless, it is probable that these two lineages have been separated for a considerable evolutionary timeframe.

Discussion

Phylogenetic relationships among wild Australian redclaw samples, based on mtDNA, accord broadly with Riek's (1969) description of two morphologically distinct species that he designated *C. quadricarinatus* and *C. bicarinatus*, sampled from eastern and western regions across northern Australia respectively (Figs 1 & 2). This pattern also accords with Macaranas *et al*.'s (1995) finding of a fixed allozyme difference between eastern and western forms. One exception to these patterns was our finding that samples from the Roper River grouped together with 'north-western' samples, despite the Roper River's clear palaeo-connection to the Carpentaria Basin (Fig. 2). Possible explanations for this pattern include a geologically recent river capture event, an intermittent freshwater connection, or possibly anthropogenic movement of individuals from the north-west into the Roper Drainage Basin. Further evidence for significant divergence in Australian redclaw is suggested by experimental laboratory-based mating and mate choice trials, conducted between individuals sampled from the north-eastern (Weipa River) and north-western (Howard River) *C. quadricarinatus* wild stocks, which resulted in no successful matings, although successful matings were achieved routinely between individuals sampled from within either the north-eastern or north-western geographic distributions (K. Ogden, unpubl. data). While this experiment was undertaken under artificial conditions, it provides indirect support that eastern and western lineages of *C. quadricarinatus* are highly divergent.

The deep divergence between eastern and western forms of redclaw was also evident in the microsatellite data set (STRUCTURE), while the failure of four microsatellite loci (developed from an eastern individual) to amplify in north-western samples indicated that the lineages have probably been evolving independently for a considerable time. Indeed, a rough estimation of the onset of splitting between the two lineages suggests they probably began to diverge around the Pliocene–Pleistocene boundary, some two million years ago (Fig. 3). Moreover, all evidence suggests that, since then, gene flow between the two forms has been negligible or absent.

Results here contrast with those of Austin (1996), who reported no significant difference among Australian (and New Guinean) samples of redclaw, and he used these data to reject Riek's (1969) conclusions. Consequently, since Austin's (1996) work, *C. quadricarinatus*, *C. bicarinatus* and *C. albertsii* have been treated as a single taxon. The existence of two distinct lineages of redclaw in Australia raises the question as to whether or not the lineages represent two discrete biological species. Unless the two lineages can be found in sympatry, it is difficult to assess whether the lineages are reproductively isolated and hence will not recognize each other as potential mates under natural conditions. A contact or hybrid zone could potentially exist somewhere within the *c*. 200 km zone in the south-western corner of the Gulf of Carpenteria between the Roper R. (north-western form) and

McArthur R. (north-eastern form) and, although samples were sought in this area, unfortunately none were found.

Levels of genetic divergence detected among the two lineages are large, and similar levels of divergence have been documented between other *Cherax* lineages that have been recognized as subspecies, e.g. *C. destructor destructor* and *C. destructor albidus* exhibit 4.7% sequence divergnce at 16S RNA. Thus, the 3.9% sequence divergence reported here between the north-eastern and north-western Australian redclaw lineages suggests that current taxonomic designations may require revision. The two lineages may warrant sub-specific or higher taxonomic status, but at the very least should be managed as evolutionarily significant units (Moritz, 1994). If phylogenetic distance alone was the sole criterion for erecting species or sub-species status, however, the two discrete PNG lineages (Mt Bosavi, Oriomo) would also warrant such recognition, as they are >5% divergent from all other lineages, respectively, for the 16S rRNA marker.

Interestingly, a close genetic relationship was observed between north-eastern Australian samples and a single PNG lineage (Bensbach) (Fig. 2). This relationship indicates a fairly recent freshwater connection between north-eastern Australia and New Guinea. Indeed, the vast Lake Carpentaria (Torgersen *et al*., 1983, 1985) that existed during the Pleistocene on the Arafura Shelf landbridge between Australia and New Guinea, connected the two landmasses as recently as 10 000 years ago (Torgersen *et al*., 1983, 1985; Chivas *et al*., 2001). Recent geological evidence suggests that, just prior to the incursion of sea water around 10 000 years ago, the lake was fresh and almost full, approaching a surface area of 600×300 km and a depth of up to 15 m (Chivas *et al*., 2001; Reeves *et al*., 2007). It is probable that the Bensbach River drained into Lake Carpentaria at this time (Voris, 2000), as would most north-eastern Australian rivers sampled here (Fig. 2). This would have created potential for admixture of redclaw from these sites. As redclaw crayfish are an obligate freshwater species, a more recent (marine) dispersal pathway between these locations can be rejected with some confidence.

Two alternative scenarios can potentially explain the presence of two discrete redclaw lineages in Australia. First, following evolution of ancestral redclaw in northern Australia, probably during the Miocene (Munasinghe *et al*., 2004), geomorphological events and/or eustatic change may have resulted in geographical barriers that restricted gene flow between north-eastern and north-western populations, sometime around the Pliocene–Pleistocene boundary. These redclaw populations may subsequently have evolved independently, and diverged under the influence of genetic drift and/or selection, probable colonization/founder events, and range expansions to their current distributions. In support, Macaranas *et al.* (1995) proposed that differentiation via selection related to water chemistry may have influenced the genetic structure of redclaw across northern Australia. Basalt soils in eastern northern Australia produce acidic waters, and ancient coral reefs that are now exposed limestone escarpments produce alkaline waters in western northern Australia. If this scenario is correct, the phylogeny also suggests that the presence of redclaw in PNG probably results from at least two independent colonizations, one (or more) during the Miocene, followed by a New Guinean radiation and subsequent lineage diversification (e.g. Oriomo, Mt Bosavi), and one much more recent (Bensbach), probably via Lake Carpentaria as outlined above. A second scenario is that redclaw evolved first in New Guinea, and two independent colonizations of Australia occurred at times of lowered sea levels when northern Australia was connected to New Guinea, followed by back-migration from north eastern Australia into the Bensbach site much more recently. Further work will be required to investigate these hypotheses.

Patterns of population genetic structure reported here for *C. quadricarinatus* concur broadly with that of another decapod crustacean (the Giant freshwater prawn, *Macrobrachium rosenbergii*), that is co-distributed across northern Australia and New Guinea (De Bruyn, Wilson & Mather, 2004b). While *M. rosenbergii* is a predominantly freshwater species, it is more tolerant of saline conditions. Independent evolutionary lineages of *M. rosenbergii* were identified in New Guinea, as well as 'eastern' and 'western' lineages across northern Australia, although the boundary between the two lineages fell to the west of the divide reported here for Australian redclaw lineages. Results from the *M. rosenbergii* study also indicated that Lake Carpentaria played a significant role in facilitating past gene flow in this species. Close genetic relationships between northern Australia and New Guinea

have also been observed in other terrestrial (green python – *Morelia viridis* Schlegel 1872, Rawlings & Donnellan, 2003; Elapidae snakes, Wüster *et al*., 2005) and freshwater (Melanotaeniidae fishes, McGuigan *et al*., 2000) taxa. In particular, the phylogenetic analyses of three genera of elapid snakes is noteworthy, as this study identified a similar scenario of repeated (land) crossings between Australia and New Guinea between the late Miocene and Pleistocene, followed by a much more recent connection (late Pleistocene) between north-eastern Australian and New Guinean populations of taipans (*Oxyuranus scutellatus* Peters 1867) . Taken together, this suggests that the patterns of genetic divergence in fresh water, and to a lesser extent in some terrestrial fauna, have been influenced in similar ways by historical geomorphological events that resulted in periodical fresh water and terrestrial connections between New Guinea and the Australian mainland during, and prior to, the Pleistocene.

Within Australia, microsatellite clustering algorithms indicate that individuals sampled from each of the 12 sampled sites in modern times constitute independent populations, that together with the highly significant pairwise F_{ST} estimates suggests that gene flow among sites is limited. Speculation that redclaw can disperse across adjacent drainage basins at times of flood, either via floodplains or flood plumes, therefore seems highly unlikely in the populations examined here (except possibly the Roper River, see above). Although metapopulation processes such as extinction and colonization events can result in elevated estimates of F_{ST} (Whitlock & McCauley, 1990), the extensive and consistently high levels of differentiation observed here for redclaw argue for substantial genetic isolation of populations present in discrete drainage basins. A number of wild redclaw populations are currently under threat from impoundments, over-fishing and pollution from agricultural runoff (Yen & Butcher, 1997). With limited (if any) gene flow occurring among major drainage basins, independent evolution of modern redclaw populations in catchments has important management repercussions for conserving genetic diversity *in situ*. Since genetic diversity is partitioned among catchments, if populations within a catchment go extinct, it is unlikely that recolonization will occur, at least over short evolutionary timeframes.

In conclusion, results presented here, and from our previous studies on freshwater crustacea (De Bruyn *et al*., 2004a,b; De Bruyn & Mather, 2007) from the northern Australian/New Guinean interface, highlight the biogeographical importance of drainage history. The well-defined palaeodrainage (Voris, 2000) makes this region a model system for understanding the determinants of population genetic structuring in freshwater systems.

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