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Molecular signatures of Pleistocene sea-level changes that affected connectivity among freshwater shrimp in Indo-Australian waters

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Abstract

A major paradigm in evolutionary biology asserts that global climate change during the Pleistocene often led to rapid and extensive diversification in numerous taxa. Recent phylogenetic data suggest that past climatic oscillations may have promoted long-distance marine dispersal in some freshwater crustacea from the Indo-Australian Archipelago (IAA). Whether this pattern is common, and whether similar processes are acting on diversification below the species level is unknown. We used nuclear and mitochondrial molecular variation in a freshwater-dependent decapod crustacean (*Macrobrachium rosenbergii*), sampled widely from the IAA, to assess the impact of Pleistocene sea-level changes on lineage diversification in this species. Fitting of an isolation with migration model enabled us to reject ongoing migration among lineages, and results indicate that isolation among both mainland–mainland and mainland–island lineages arose during the mid-Pleistocene. Our data suggest a scenario of widespread marine dispersal during Pleistocene glacial maxima (in support of the 'Pleistocene marine dispersal hypothesis') when sea levels were low, and geographical distances between fresh watersheds were greatly reduced, followed by increased isolation as sea levels subsequently rose.

Keywords: biogeography, climate change, dispersal, evolution, phylogeography, Last Glacial Maximum

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Introduction

The impact that Pleistocene [~2 million years (Myr) to 10 000 BP] climate change had on driving evolutionary diversification has been debated for more than a century (Darwin 1859; Wallace 1862; Hofreiter *et al*. 2003). Accumulating evidence from Europe and North America indicates that glacial events during the Pleistocene epoch resulted in major shifts in species distributions (Avise 2000; Hewitt 2000; Davis & Shaw 2001), and may have been the principal factor that contributed to the decline and eventual extinction of some species (Guthrie 2003; Shapiro *et al*. 2004). As climate oscillated through the Pleistocene, fossil (Coope 1994; Bennett 1997) and other evidence (reviewed in Hewitt 2000) show that the geographical distributions of some species responded by expanding and contracting their ranges during times of glacial cycling. Furthermore, these responses may have been swift, and may have occurred repeatedly (Hewitt 2000). Understanding the causal mechanisms that generate (or extinguish) biodiversity is critical for safeguarding our natural resources, and is of particular urgency in regions of high species richness.

The Indo-Australian Archipelago (IAA) houses one of the highest levels of species richness and endemism in the world (Myers *et al*. 2000). Four of the world's 25 biodiversity 'hotspots' overlap within this region (Mittermeier *et al*. 1999). Many theories have been proposed to explain such high levels of diversification in the IAA, although no consensus has been reached. Major hypotheses include: (i) *centre of accumulation hypotheses* such as the plate tectonic evolution hypothesis (Wallace 1869; Archbold *et al*. 1982; Audley-Charles 1983), whereby Southeast Asia is a region

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of admixture between predominantly Oriental and Australasian or earlier Gondwanan biotas brought into contact through plate tectonic coalescence; or (ii) *vicariance hypotheses* [e.g. eustasy (Heaney 1986; Schmitt *et al*. 1995) and refugium hypotheses (Brandon-Jones 1998; Hewitt 2000; Gathorne-Hardy *et al*. 2002)], in which cyclical Pleistocene sea-level fluctuations resulted in numerous speciation events as populations were isolated repeatedly, sometimes into discrete refugia. It is apparent from the literature that most studies on the evolution of the biota conducted in this region, however, focus on organisms that are able to disperse widely through relatively continuous habitat for a significant proportion of their recent history, often leading to inconclusive results (Heaney 1986; Schmitt *et al*. 1995; Briggs 2000; Hewitt 2000; Brown & Guttman 2002). In contrast, freshwater taxa are ideal model organisms for the current field of research, as they reflect well the underlying biogeographical history of a given region (e.g. Bermingham & Avise 1986) due to limited dispersal abilities — their requirement for freshwater should restrict them.

Few studies have been carried out on freshwater taxa from the IAA, although an increasing body of molecular work is accumulating on freshwater crustacea (de Bruyn *et al*. 2004a; Murphy & Austin 2005; Page *et al*. 2007). Phylogenetic studies on freshwater shrimp (e.g. *Caridina,* Page *et al*. 2007; *Macrobrachium*, de Bruyn *et al*. 2004a; Murphy & Austin 2005) from the IAA provide strong support for the centre of accumulation hypothesis, which predicts an exchange of components of the Oriental and Australian freshwater fauna during the Miocene, as the Sunda (Oriental) and Sahul (Australian) continental shelves collided (Hall 2002). Intriguingly, these studies also suggest a more recent (probably of Pleistocene origin) intercontinental exchange of freshwater taxa, hereafter referred to as the 'Pleistocene marine dispersal hypothesis', which, in effect, is an extension of the 'vicariance hypotheses' outlined above; in terrestrial taxa, Pleistocene lowering of sea levels associated with glacial cycling resulted in a sundering of formerly contiguous populations in the IAA, whereas in freshwater taxa, the exact same process resulted in a significant reduction in geographical distances between fresh watersheds (Fig. 1), presumably facilitating dispersal of a subset of the freshwater biota exhibiting some degree of saltwater tolerance. Nonetheless, vicariant biogeographers (see Cowie & Holland 2006; for review) continue to argue for the primacy of ancient vicariance over dispersal in the construction of continental biotas, viewing such (dispersal) data as stochastic noise. As Page *et al*. (2007) pointed out, however, transoceanic dispersal 'appears to have been responsible for formation of much of the Australian continental freshwater biota'.

While phylogenetic data provide valuable insight into the processes likely acting on interspecific differentiation, whether these processes are commonplace and are acting below the species level is unclear (Page *et al*. 2007). For example, our earlier work (de Bruyn *et al*. 2004a) found a sharp genetic break across Huxley's Line between eastern and western forms of the giant freshwater prawn (*Macrobrachium rosenbergii* De Man, 1879) dating back to the Miocene. Later work at the population level within these two forms (de Bruyn *et al*. 2004b, 2005), however, found evidence for widespread dispersal (gene flow) during the Pleistocene. This was most probably facilitated by the expansion of fresh watersheds during times of glacial maxima; for example, sites on the Sahul and Sunda shelves, respectively, which are today separated by ocean, were connected by vast freshwater systems during the Pleistocene (e.g. Lake Carpentaria, Sahul Shelf; Siam and Malacca Straits River systems, Sunda Shelf) (Voris 2000). While these results provide strong evidence that the expansion of freshwater systems during the Pleistocene promoted gene flow in some freshwater aquatic taxa, they do not corroborate the Pleistocene marine dispersal hypothesis, which posits long-distance oceanic dispersal of freshwater taxa during the Pleistocene. The occurrence of *M. rosenbergii* on true oceanic islands (discussed further below) in the IAA does, however, lend some credence to this hypothesis, as it suggests that oceanic dispersal has played, or continues to play, a role in the evolutionary history of this species.

The giant freshwater prawn, *M. rosenbergii*, is an ideal candidate species to investigate the influence of Pleistocene sea-level changes on intraspecific diversification, for reasons mentioned above, and because it is widely distributed (Fig. 1) and locally abundant throughout the IAA. *M. rosenbergii* is most often associated with coastal river systems, as it inhabits freshwater as an adult, but requires brackish water for larval development. Females migrate from freshwater into estuarine areas to spawn, where free-swimming larvae hatch from eggs attached to the females' abdomen. Larval duration is approximately 3–6 weeks, following which juveniles migrate upstream to freshwater habitat. The ability of larvae to tolerate marine conditions is unknown. Laboratory studies, however, indicate that adults do not survive in marine conditions for more than a week, although a small percentage of postlarvae may survive for up to 20 days (Sandifer *et al*. 1975). Even given their apparent limited euryhalinity, *M. rosenbergii* are found on some true oceanic islands (e.g. Christmas Island, Palau, Sulawesi, the Philippine Archipelago), which suggests that at least limited marine dispersal occurred in the past. Two discrete forms of *M. rosenbergii* have been recognized, based on morphological (Lindenfelser 1984), allozyme (Lindenfelser 1984), and mitochondrial DNA (mtDNA) (de Bruyn *et al*. 2004a) variation, with the boundary between these forms coinciding with Huxley's extension of Wallace's Line (Fig. 1) (de Bruyn *et al*. 2004a). Time of divergence between the two forms most likely dates back to the Miocene, some

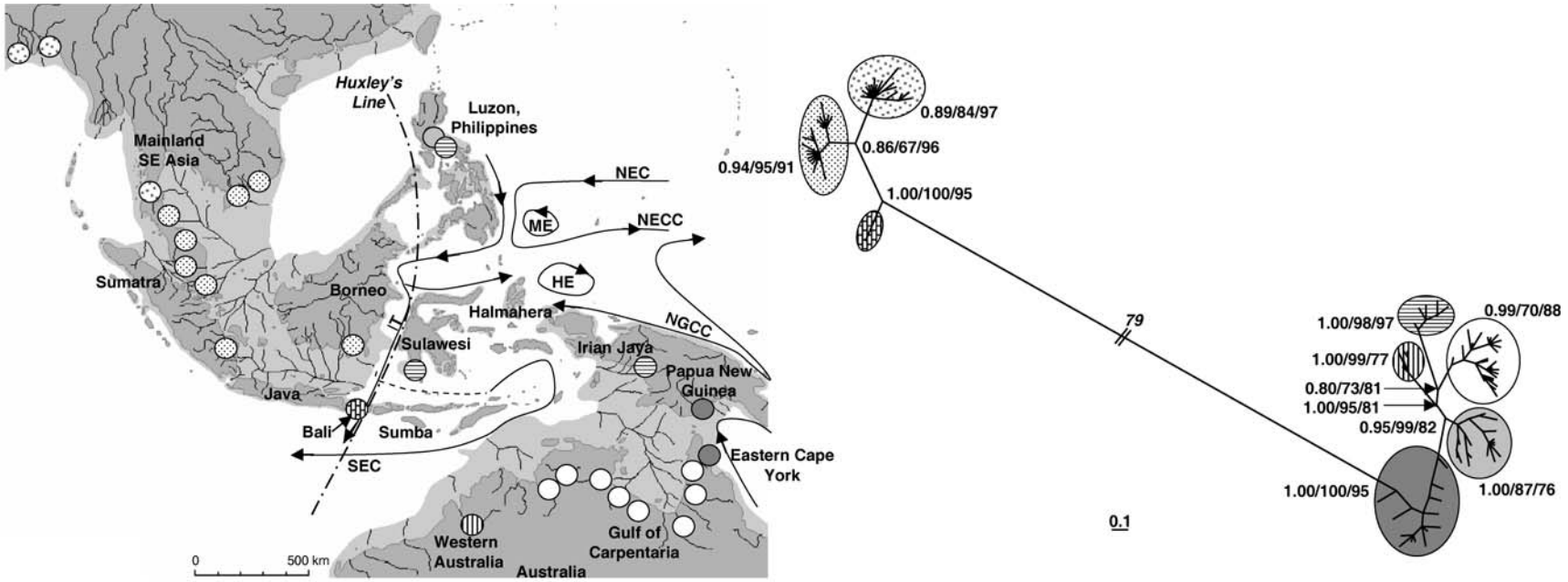

Fig. 1 Map of sampling locations showing the distribution of lineages, and Bayesian consensus tree for 93 unique mitochondrial COI haplotypes obtained from sampling 861 *Macrobrachium rosenbergii* from 26 locations east and west of Huxley's Line. Major surface currents of interest are shown. Dashed line indicates seasonally reversing currents. North Equatorial Current (NEC); North Equatorial Counter Current (NECC); New Guinea Coastal Current (NGCC); Indonesian Throughflow (IT; location of Makassar Strait); Mindanao Eddy (ME); Halmahera Eddy (HE). Light grey shading on map indicates –120 m sea-level contour, and major fresh watersheds at this time are shown (Voris 2000). Values at tree nodes indicate (in order): Bayesian posterior probabilities, neighbour-joining bootstraps, maximum-likelihood bootstraps.

Table 1 Molecular diversity indices for *Macrobrachium rosenbergii* (eastern form) based on mtDNA COI region sequences and five microsatellite loci. *n*, sample size; NH, number of haplotypes; values of haplotype diversity (*h*), nucleotide diversity (π), θ_S, are presented with standard errors in parentheses. NA, number of alleles at all loci, mean expected (H_E) and observed (H_O) heterozygosities, respectively

	mtDNA					Microsatellites			
Locality	*n*	NH	*h*	π	θ_S	*n*	NA	H_E	H_O
Lennard R, W/A, Australia (WA)	18	5	0.66(0.102)	0.0029(0.0020)	1.74(0.90)	20	73	0.76	0.86
Keep R, N/T, Australia (KE)	31	4	0.50(0.088)	0.0010(0.0009)	0.75(0.47)	39	99	0.85	0.90
Katherine R, N/T, Australia (KA)	33	3	0.27(0.094)	0.0006(0.0006)	0.74(0.46)	41	99	0.81	0.85
Roper R, N/T, Australia (RO)	22	1	—	—	—	45	74	0.50	0.53
Limmen Bight R, N/T, Australia (LB)	27	4	0.21(0.103)	0.0004(0.0005)	0.78(0.49)	29	82	0.89	0.95
McArthur R, N/T, Australia (MC)	49	4	0.32(0.078)	0.0030(0.0019)	1.79(0.78)	55	106	0.89	0.94
Norman R, Qld, Australia (NO)	44	6	0.48(0.086)	0.0030(0.0019)	2.07(0.88)	53	118	0.89	0.95
Archer R, Qld, Australia (AR)	25	3	0.50(0.098)	0.0024(0.0017)	1.32(0.70)	25	88	0.89	0.95
Wenlock R, Qld, Australia (WE)	40	5	0.61(0.052)	0.0036(0.0022)	2.11(0.90)	39	97	0.86	0.91
Olive R, Qld, Australia (ECY)	16	3	0.65(0.081)	0.0086(0.0049)	2.96(1.35)	16	52	0.82	0.93
Fly R, Papua New Guinea (PNG)	39	9	0.48(0.097)	0.0035(0.0022)	4.49(1.63)	48	107	0.77	0.96
Ajkwa R, Irian Jaya (IJ)	42	4	0.46(0.069)	0.0021(0.0015)	0.93(0.52)	49	106	0.67	0.70
Maros R, Sulawesi (SU)	35	1	—	—	—	35	33	0.54	0.57
Plandez/Pulilan R, Luzon, Philippines (PH)	36	14	0.89(0.032)	0.0106(0.0057)	5.55(1.97)	47	101	0.77	0.82

5–12 million years ago (Ma) (de Bruyn *et al*. 2004a). Taxonomists have recently suggested that these two forms should be elevated to specific taxonomic status: the western form *Macrobrachium schenkeli*, and the eastern form *Macrobrachium rosenbergii* (Wowor 2004).

Understanding the timing of last contact among geographically widespread *M. rosenbergii* populations in the IAA, including those found on true oceanic islands, is thus critical. Do the data support a Pleistocene, Miocene or even earlier connection between populations? Or is there evidence for ongoing dispersal that would challenge either of the two main hypotheses considered here? To investigate these questions, we present new combined with previously reported mitochondrial data (de Bruyn *et al*. 2004b, 2005) from across the entire natural distribution of *M. rosenbergii*, that is, from both the eastern and western forms (Fig. 1), in order to identify relationships among lineages and potential colonization routes, and assay nuclear variation in populations of the eastern form of *M. rosenbergii*, including populations recently sampled from the true oceanic islands of Sulawesi (Indonesia) and Luzon (Philippines).

Materials and methods

Sampling

To investigate the evolutionary impact that Pleistocene glacial events had on the history of *Macrobrachium rosenbergii*, we genotyped 541 individuals at six Mendelian-inherited microsatellite loci (Chand *et al*. 2005), sampled from 14 locations east of Huxley's Line [*sensu* the eastern form of *M. rosenbergii* (de Bruyn *et al*. 2004a)] (Fig. 1). We also sequenced a 602 base pair (bp) fragment of the mitochondrial cytochrome oxidase I gene (COI) in individuals sampled from Bali and Sulawesi (Indonesia), Luzon (Philippines) and Eastern Cape York (Australia) (see Table 1 for sample sizes), and combined these data with COI sequences previously reported in de Bruyn *et al*. (2004b, 2005). This represents a total sample of 861 individuals in our COI data set (n = 457 for the eastern form).

Genotyping

Methods for genomic DNA extractions, polymerase chain reaction (PCR) amplification and sequencing of a 602-bp fragment of the mtDNA COI region, and PCR amplification and screening of six microsatellite loci are available elsewhere (de Bruyn *et al*. 2004b; Chand *et al*. 2005). The microsatellite flanking sequences and primers are available on GenBank under Accession nos AY791965–AY791970, and COI sequences: AY554293–AY554327, AY614545–AY614587, DQ060194–DQ060208, representing 93 unique haplotypes.

Phylogenetic inference

We determined that the TrN model of substitution (Tamura & Nei 1993) plus invariable sites (I) and a gamma distribution (Γ) of rate heterogeneity across variable sites provided the best fit to our COI data set with the program MODELTEST 3.06 (Posada & Crandall 1998). The estimated parameters under this model were Γ = 5.8053, I = 0.8099 and Ti/Tv = 4.60. The TrN model and its estimated parameters were used for subsequent analyses where appropriate. Using the complete mtDNA data set, we constructed a

Table 2 Population differentiation measured by F_{ST}. Comparisons based on mtDNA COI sequences (above diagonal) and five microsatellite loci (below diagonal). All pairwise comparisons were significant after 1000 permutations, except where indicated by NS (not significant)

	KA	KE	RO	MC	WE	NO	AR	LB	WA	IJ	PNG	ECY	SU	PH
KA	—	0.161	0.935	0.755	0.554	0.558	0.757	0.945	0.943	0.940	0.928	0.868	0.988	0.720
KE	0.004 NS	—	0.895	0.740	0.538	0.572	0.725	0.920	0.928	0.932	0.921	0.854	0.980	0.709
RO	0.048	0.039	—	0.765	0.647	0.489	0.820	0.980	0.950	0.945	0.929	0.860	1.000	0.714
MC	0.034	0.028	0.011 NS	—	0.448	0.525	0.544	0.095	0.888	0.891	0.892	0.830	0.926	0.704
WE	0.025	0.033	0.029	0.032	—	0.303	0.373	0.634	0.861	0.877	0.869	0.780	0.916	0.651
NO	0.027	0.039	0.035	0.030	0.009 NS	—	0.492	0.723	0.879	0.891	0.890	0.816	0.928	0.679
AR	0.020	0.022	0.019	0.024	0.001 NS	0.009 NS	—	0.771	0.888	0.896	0.899	0.812	0.953	0.618
LB	0.051	0.046	0.030	0.039	0.047	0.038	0.043	—	0.948	0.939	0.926	0.867	0.993	0.717
WA	0.037	0.043	0.058	0.049	0.049	0.056	0.045	0.059	—	0.918	0.908	0.825	0.966	0.697
IJ	0.049	0.049	0.049	0.046	0.025	0.030	0.019	0.054	0.060	—	0.912	0.849	0.236	0.604
PNG	0.033	0.038	0.045	0.038	0.021	0.025	0.021	0.058	0.047	0.022	—	0.386	0.942	0.742
ECY	0.107	0.110	0.111	0.107	0.085	0.080	0.088	0.118	0.098	0.075	0.070	—	0.893	0.562
SU	0.098	0.098	0.117	0.111	0.092	0.100	0.094	0.099	0.136	0.056	0.067	0.185	—	0.648
PH	0.039	0.043	0.060	0.049	0.033	0.037	0.038	0.065	0.071	0.037	0.025	0.107	0.082	—

Bayesian consensus tree in MRBAYES version 3.1 (Ronquist & Huelsenbeck 2003). The posterior probabilities of phylogenetic trees were estimated by 2×10^6 generation Metropolis-coupled Markov chain Monte-Carlo algorithms (four chains, chain temperature = 0.2), with parameters estimated from the data set. For comparison, we also constructed bootstrapped (1000 pseudo-replicates) maximum-likelihood and neighbour-joining trees in TREE-PUZZLE (Schmidt *et al*. 2002) and MEGA version 3.1 (Kumar *et al*. 2004), respectively. A 95% parsimony network was constructed from the 'eastern' mtDNA sequences using TCS version 1.13 (Clement *et al*. 2000), and the topology was then verified against a median-joining network implemented in NETWORK (Bandelt *et al*. 1999). Cavalli-Sforza & Edwards (1967) chord distance (D_{CE}) was used to construct a neighbour-joining phylogenetic tree in PHYLIP version 3.5c (Felsenstein 1993) from raw 'eastern' microsatellite allelic frequencies. Support for tree nodes was assessed by bootstrapping over loci (1000 iterations).

Population genetic analyses

For microsatellite loci, 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). 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. Population divergence (Table 2) was estimated by computing Φ_{ST} for the COI data set in ARLEQUIN (Schneider *et al*. 2000), and F_{ST} for the microsatellite data set in FSTAT (Goudet 1995). A hierarchical analysis of molecular variance (AMOVA) was performed in ARLEQUIN, as were molecular diversity calculations for mtDNA (Table 1). We tested our COI data set for lineage expansion events using Tajima's D, Fu's F_s, and mismatch distributions calculated in ARLEQUIN. Tajima's D and Fu's F_s were developed originally as tests for selection, but in its absence, negative values are considered evidence of an expanding 'population'. Populations that have been stable historically are predicted to display a multimodal mismatch distribution, while those that have expanded recently are predicted to display a unimodal distribution. The validity of the model was tested using the parametric bootstrap approach implemented in ARLEQUIN under both the sudden expansion and the spatial expansion models, where P = (number of SSDsim = SSDobs)/B. We used Mantel tests in IBD (Bohonak 2002) to determine whether a correlation existed between pairwise $\Phi/(1-\Phi)$ and F_{ST}, respectively, vs. geographical distance. Data were analysed both untransformed, or with only geographical distance log-transformed (Rousset 1997).

Timing of demographic events

We estimated the chronology of pairwise lineage, and lineage-specific population divergence times, respectively, in the eastern form of *M. rosenbergii*, utilizing data from the mtDNA COI (under an HKY model) and five of the six (see Results) microsatellite loci (under an SMM) analysed together, considered within an isolation with migration model using the program IM (Hey & Nielsen 2004). The model was applied under a Bayesian framework that provides estimates for the posterior probability density of the model parameters, given the data. 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 *et al*. (2004) for Caridean shrimp (the infra-order to which

Macrobrachium belongs), and scaled rates for other loci on the mtDNA rate. We used uniform (i.e. uninformative) prior distributions. In effect, this makes the posterior distributions proportional to likelihood distributions, with the peaks of the likelihood values equivalent to maximum-likelihood estimates (Nielsen & Wakeley 2001). Following a 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 30 and 50 chains. We ran the program until the smallest ESS estimates were greater than 300, and update rates were greater than 20%. For credibility intervals, we assessed the 90% highest posterior density (HPD) interval; that is, the boundaries of the shortest span that incorporates 90% of the posterior density of a parameter. Pairwise comparisons were chosen to address key questions regarding time of splitting and gene flow within and among *M. rosenbergii* lineages.

Results

Phylogenetic relationships

The phylogeny supports previous work showing significant differentiation among eastern and western forms of *Macrobrachium rosenbergii* (Fig. 1) (Lindenfelser 1984; de Bruyn *et al*. 2004a; Wowor 2004). Within the 'eastern' form, the 58 unique COI haplotypes define five well-supported lineages (Figs 1 and 2). These lineages comprised samples from: Western Australia (WA); the Lake Carpentaria region (today's Gulf of Carpentaria), Australia [LC (de Bruyn *et al*. 2004b)]; Papua New Guinea and Eastern Cape York, Australia (PNG/ECY); Luzon Island, Philippines (PH); and a final clade comprising individuals from Irian Jaya, Sulawesi and Luzon Island, Philippines (IJ/SU/PH) (Figs 1 and 2). Further support for this geographical pattern of genetic differentiation in the eastern form of *M. rosenbergii* is provided by a phenogram based on microsatellite variation (Fig. 2). Relationships among populations based on nuclear data were largely concordant with that estimated from the COI data, although bootstrap support was low in some cases. The close relationship between PNG and ECY was supported, as was that between IJ, SU and PH. Notable differences in the nuclear phylogeny were: (i) the intermediate placement of the KA and KE populations relative to the WA and Gulf of Carpentaria populations; (ii) the Gulf of Carpentaria populations were further subdivided into two groups comprising populations from the western (RO, LB and MC), and those from the eastern Gulf (NO, WE and AR), suggesting a more recent connection among these sites, which is consistent with predictions based on geography (Figs 1 and 2).

Population genetic analyses

No significant LD was identified for microsatellite locus-pair population comparisons. Probability tests detected 12 significant deviations from HWE out of 84 comparisons. Seven of 12 departures from HWE were evident at the *Mr*-95 locus due to heterozygote deficiencies. This could result from the presence of null alleles or the Wahlund effect. To eliminate the possibility that null alleles might bias our results, we removed locus *Mr*-95 from the analyses. Analysis of molecular variance (AMOVA) supported the existence of significant structure among lineages indicated by the phylogenetic data (COI: $\Phi_{ST} = 0.900$, $\Phi_{CT} = 0.769$, $\Phi_{SC} = 0.568$, $P < 0.001$; 10 000 iterations). Moreover, pairwise F_{ST} matrices based on both mtDNA (91 cases; range 0.161–1.000) and microsatellite loci (86 cases; range 0.019–0.185) showed significant population structure among most sites (Table 2). Only five pairwise comparisons (after Bonferroni corrections; among populations from the LC clade) based on microsatellite data were nonsignificant (range 0.004–0.011). *M. rosenbergii* in these locations are believed to have been connected by a freshwater lake (Lake Carpentaria) that existed ~80 000–8500 BP in what is today the marine Gulf of Carpentaria (de Bruyn *et al*. 2004b). Regressions obtained using permutated Mantel tests (COI: untransformed $r = 0.053$, $P = 0.400$, transformed $r = 0.220$, $P = 0.065$; microsatellites: untransformed $r = 0.192$, $P = 0.165$, transformed $r = 0.249$, $P = 0.135$) were not significantly different from zero. Mantel tests indicated that isolation by distance has played little or no role in structuring genetic variation. These data suggest a stepping-stone model, which may be anticipated in some freshwater taxa due to genetic exchange among adjacent drainages, is not an adequate representation of population genetic structure in *M. rosenbergii*.

Recombination and selection

Macrobrachium rosenbergii mtDNA displayed no signs of recombination [four-gamete test (Hudson & Kaplan 1985): $R = 0.001$, $P = 0.159$] or selection [McDonald–Kreitman test (McDonald & Kreitman 1991): no differences in the ratios of nonsynonomous to synonymous changes within and between 'species', in this case the eastern and western forms of *M. rosenbergii*; Fisher's exact test, $P = 1.000$], and has been evolving at a relatively constant rate (log-likelihood ratio test: $\chi^2 = 67.53$, d.f. = 56, $P > 0.10$). These features allowed us to determine times of divergence within and among lineages under an 'isolation with migration' model (Nielsen & Wakeley 2001). This approach is particularly well suited to unraveling a range of demographic parameters within recently diverged populations or species, as it can discriminate between ongoing migration and recent divergence (Palsbøll *et al*. 2004),

Fig. 2 (a) 95% parsimony network for 58 unique mitochondrial COI haplotypes obtained from sampling 457 *Macrobrachium rosenbergii* from 14 locations in the Indo-Australian Archipelago east of Huxley's Line. Closed circles indicate inferred missing haplotypes. Dashed lines indicate alternative inferred connections among haplotypes; (b) Neighbour-joining phenogram depicting genetic relationships based on Cavalli-Sforza and Edwards' chord distances (D_{CE}) among 14 *M. rosenbergii* populations sampled from the IAA east of Huxley's Line. The percentage of bootstrap replicates (n = 1000) over five microsatellite loci is indicated by the values on the nodes; (c) Map showing sampling locations east of Huxley's Line. Light grey shading on map indicates –120 m sea-level contour, and Pleistocene drainage basins are shown (Voris 2000). Populations are as follows, PNG/ECY clade: Fly R, Papua New Guinea (PNG), Olive R, Eastern Cape York (ECY); PH clade: Plandez/Pulilan R, Luzon, Philippines (PH); WA clade: Lennard R, Western Australia (WA); IJ/SU/PH clade: Maros R, Sulawesi (SU), Ajkwa R, Irian Jaya (IJ), Plandez/Pulilan R, Luzon, Philippines (PH); LC clade: Keep R (KE), Katherine R (KA), Roper R (RO), Limmen Bight R (LB), McArthur R (MC), Norman R (NO), Wenlock R (WE) and Archer R (AR).

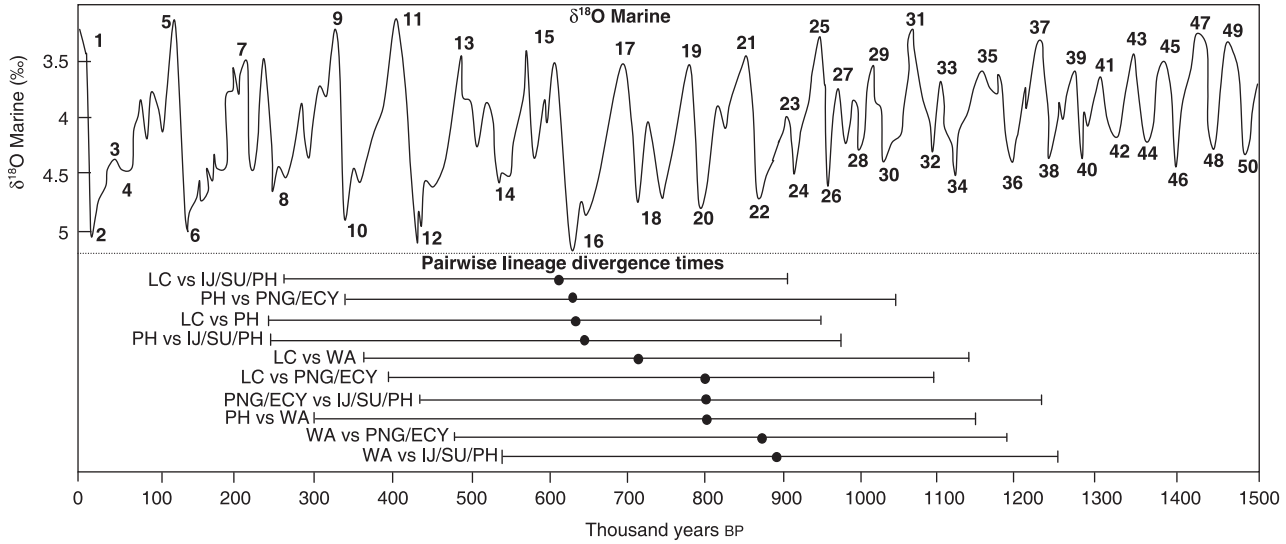

Fig. 3 Estimated timing of the beginning of divergence for *Macrobrachium rosenbergii* pairwise lineage comparisons and their relation to Pleistocene glacial maxima inferred from 57 globally distributed $\delta^{18}O$ records (after Lisiecki & Raymo 2005). Illustrated results are based on $\mu = 1.4 \times 10^{-8}$. Closed circles indicate the peak of the posterior density distribution for the time of splitting parameter *t* calculated in IM with 90% highest (and lowest) posterior densities illustrated by error bars. All times fall within periods of glacial maxima (MIS 22, 20, 18 and 16). Lineage-specific population pairwise divergence times are not shown, but do fall within MIS 4–2 (20 cases: range 31 000–17 000 BP). Pairwise lineage divergence times as follows (thousand years BP): LC vs. IJ/SU/PH (610); PH vs. PNG/ECY (622); LC vs. PH (635); PH vs. IJ/SU/PH (641); LC vs. WA (712); LC vs. PNG/ECY (798); PNG/ECY vs. IJ/SU/PH (798); PH vs. WA (803); WA vs. PNG/ECY (867); WA vs. IJ/SU/PH (880).

and does not assume an equilibrium model, i.e. sampled populations may have separated recently (in evolutionary terms).

Demographic events in M. rosenbergii

To gain a more accurate estimate of the age of the eastern form of *M. rosenbergii*, we estimated the divergence time (using COI data only, in IM) between the eastern ($n = 457$) and the western [$n = 404$ (de Bruyn *et al*. 2005)] forms of *M. rosenbergii*, sampled from across their entire distributions. Timing of the beginning of divergence between the two forms is consistent with an ancient separation (beginning approximately 5.6 Ma) with little or no subsequent gene flow (*m* estimate highly skewed to zero). All 'eastern' pairwise lineage parameter estimates of the isolation with migration model, fit using the IM program, are shown in Figs 3 and 4 and in the Supplementary material. A subset of key lineage-specific pairwise population comparisons is also presented. There is a strong signal of restricted gene flow in our data, with most estimates of *m* highly skewed to zero, even among lineage-specific population comparisons. All estimates of ancestral and extant effective population sizes were large, ranging from ~1–1.2 million individuals for the east/west comparison, to 400 000–122 000 for lineages, which is to be expected due to the local abundance of these macro-invertebrates. Indicators of lineage expansion (Table 3) suggest that expansion has occurred in all lineages except for WA.

Discussion

We propose that the deep east/west split observed in our mitochondrial data results from ancestral *Macrobrachium rosenbergii* dispersing across what is today known as the Makassar Strait (Fig. 1). This may have been facilitated by the meeting of the Sunda and Sahul Shelves during the Miocene (Hall 2002). Subsequent isolation of the two forms possibly arose via vicariance (divergence beginning ~5.6 Ma; Fig. 4) due to extremely high sea levels resulting from climatic change. Benthic $\delta^{18}O$ data suggest that global sea levels over the past 5.5 Myr achieved their maximum height approximately 5.1 Ma (Marine Isotope Stage T7) (Lisiecki & Raymo 2005). The direction of this dispersal is unclear from our data; however, because *M. rosenbergii* appears closely allied to the Indian subcontinental species *M. gangeticum* and *M. malcolmsonii* (Johnson 1973; Short 2004), we can speculate that the eastern form is probably derived from the more ancestral western type. Unfortunately, molecular data (or tissue samples) for these other *Macrobrachium* taxa were unavailable for phylogenetic analyses to test this hypothesis. Phylogenetic relationships among lineages are consistent with morphological variation (Short 2004), with northwestern populations from the western form at one end of the spectrum, and Western Australian (WA) individuals from the eastern form at the other (Fig. 1). Interestingly, Western Australia is the only eastern lineage that did not display evidence for expansion

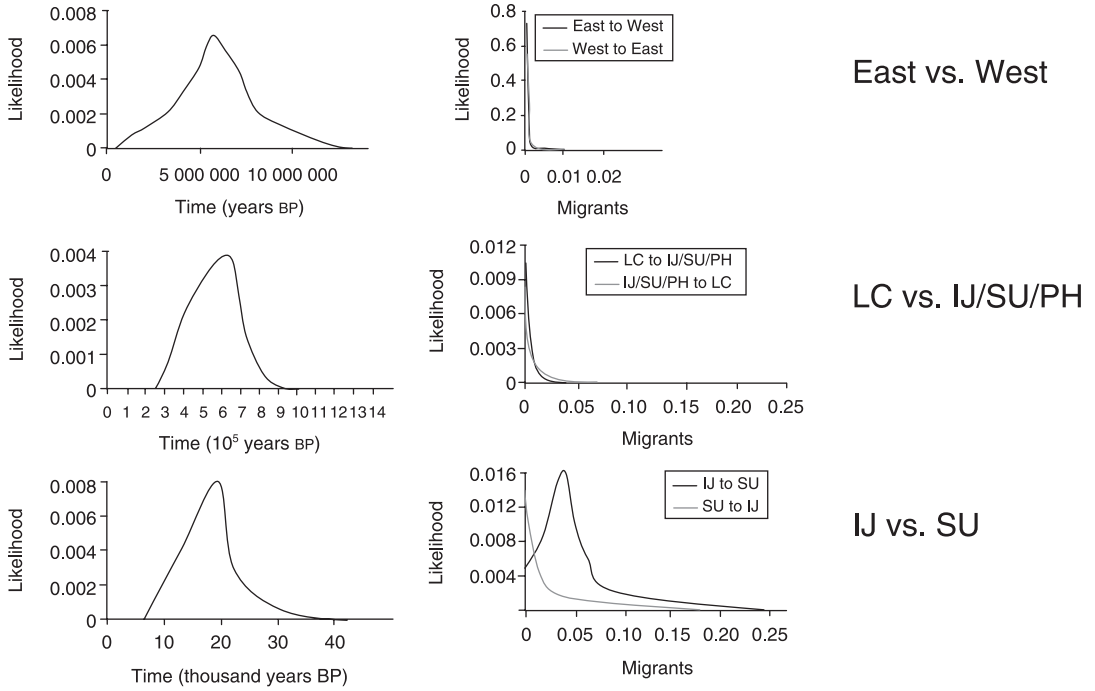

Fig. 4 Examples of the marginal likelihood surfaces for time of splitting and migration for pairwise 'species', lineage, and population comparisons, estimated in IM, indicating bounds of 90% highest posterior densities (HPD). Illustrated results are based on $\mu = 1.4 \times 10^{-8}$.

Table 3 Indicators of demographic/spatial expansion events in *Macrobrachium rosenbergii* (eastern form) based on mtDNA COI sequences. Tajima's *D* and Fu's F_s values and mismatch distribution *P* values are shown. Expansion events are indicated in all lineages except for WA

Lineage	Tajima's *D*	Fu's *Fs*	Mismatch sudden expansion *P*	Mismatch spatial expansion *P*
PNG/ECY	–0.65	–0.37	0.55	0.75
PH	–0.36	–3.23	0.35	0.65
PH/IJ/SU	0.26	1.00	0.35	0.40
LC	–0.44	–4.82	0.06	0.45
WA	0.00	0.16	0.00	0.04

(Table 3), a pattern that may indicate that this lineage is at equilibrium.

While individuals from Bali are considered most similar to the eastern form based on morphology, the nearest eastern relative to the western type remained unresolved in this study (Short 2004). Short (2004) suggested that the Philippine, New Guinea or northeast Australian types were likely candidates. Our data suggest that individuals from Bali and PNG/ECY are more closely related than other eastern vs. western lineage comparisons (Fig. 1). How this pattern arose is unclear, as one might expect a closer relationship between Bali and geographically proximate locations such as Sulawesi or Western Australia. If one had to speculate, one possible mechanism is historical dispersal facilitated by past ocean currents. During the Miocene, if the South Equatorial Current (SEC; Fig. 1) flowed through what is today the Gulf of Carpentaria, passing through the narrow gap between the Cape York Peninsula and southern New Guinea, instead of deflecting to the west as it currently does, this may have provided a vehicle for dispersal (of larvae?) from Bali to PNG/ECY. Colonization of the IAA east of Huxley's Line may then have proceeded from PNG/ECY. Phylogenetic tree topology supports this hypothesis. Support for the PNG/ECY lineage being older than other eastern lineages is also suggested by a pattern of high mitochondrial and nuclear diversity (Table 1), and large genetic distances (Figs 1 and 2) among haplotypes (both within and among the Fly and Olive River populations, i.e. the PNG/ECY lineage). A similarly high level of molecular diversity is evident for samples from Luzon (Table 1), although this is probably partly due to the admixture of two divergent lineages (see Results).

The chronology of colonization from PNG/ECY to the rest of the IAA east of Huxley's Line is not obvious, although the mitochondrial data (tree, network and Φ_{ST}) do suggest that the PH lineage is most closely related to PNG/ECY, and could possibly have been colonized next. The microsatellite data, however, are more ambiguous, suggesting that IJ or PH may be candidates. No matter the

direction of colonization, all evidence suggests a fairly rapid radiation among lineages. There may of course be other unsampled populations east of Huxley's Line that display greater affinity to the Bali population, and the Lesser Sunda Islands east of Bali are likely candidates. *M. rosenbergii* have been sampled in the past from Sumba in this region, and also from Halmahera in the Maluku Islands farther north, but there is no record of the species occurring elsewhere in the region (D. Wowor, personal communication). It is unlikely that sufficient freshwater habitat exists in this region to support viable natural populations of *M. rosenbergii* (D. Wowor, personal communication); however, this requires further investigation. Interestingly, the Eastern Cape York (ECY) region has long been regarded as the most likely colonization route for many, if not most, freshwater fishes and invertebrates (including *Macrobrachium* species; Short 2004; Murphy & Austin 2005) into Australia from the north, which is consistent with the close association shown here between the PNG and ECY populations. The region has the highest diversity of Australian freshwater fish species, many of which are closely allied to taxa from southern New Guinea (Unmack 2001).

The question then arises as to what mechanism has since maintained the deep split between the eastern and western forms subsequently? The Indonesian Throughflow may have limited dispersal of *M. rosenbergii* across Huxley's Line, after the initial dispersal across this barrier hypothesized above. The Indonesian Throughflow carries a huge volume of fast-flowing water (~12 Sverdrup, Sv = 106 $m^3 s^{-1}$; Meyers *et al*. 1995) between the islands of Borneo and Bali to the west, and Sulawesi and Lombok to the east (Fig. 1). The Makassar Strait, through which the Indonesian Throughflow travels, acts as the western boundary for the Australian and Asian biotic transition zone (Wallacea). Indeed, of all vertebrate groups, the distributions of primary freshwater fish fauna most clearly demarcate this boundary (Moss & Wilson 1998).

Relationships among western lineages of *M. rosenbergii* have been described elsewhere (de Bruyn *et al*. 2005), as were the effects of Lake Carpentaria on gene flow in the eastern form (de Bruyn *et al*. 2004b). Within the eastern form of *M. rosenbergii*, a fairly recent connection (in evolutionary terms) among lineages is suggested by the shallow parsimony network and microsatellite tree (Fig. 2), a scenario supported by pairwise comparisons of lineage divergence times (Figs 3 and 4 and Supplementary material). The timing of these events (nine cases; range 886 000–610 000 BP) may be coincident with a disruption of dispersal after periods of low global sea level during glacial maxima (Marine Isotope Stages 22, 20, 18 and 16; Fig. 3). Pleistocene sea levels in the IAA are believed to have dropped periodically by as much as 120 m (Voris 2000), although some authors have suggested that the fall in sea levels may have been greater and up to 150 m (Chappell & Shackleton 1986). Such a dramatic decrease in sea level would have exposed vast areas of the Sahul Shelf, revealing extensive fresh watersheds (Voris 2000), and would have reduced oceanic distances between landmasses considerably (Fig. 1).

In contrast to the land bridge that united Australia and New Guinea for much of the Pleistocene (Fig. 1), the true oceanic islands of Sulawesi (Indonesia) and Luzon (Philippines) (Fig. 1) are believed to have never been physically linked to other major landmasses in this region (Hall 2002). The tectonic history of the Philippine Archipelago, in particular, is extremely complex. Reconstructions by Hall (1998) suggest that the main landmass of the Philippines originated as a series of island arcs far out in the Pacific Ocean more than 50 Ma. As the Australian continent moved northward towards the Asian continent, plate tectonic movement formed undersea volcanoes, which gradually emerged from the sea and underwent considerable tectonic movement and rotation. The Philippine Archipelago may have only taken on its current shape over the last 5–10 Myr, but the geological history of the region is still poorly understood. Similarly, Sulawesi has a complex history, and is believed to be a composite landmass of different geological origins. Geologists suggest that: (i) the SE arm of the island and possibly parts of the northern arm may have been emergent approximately 20 Ma; (ii) central Sulawesi was emergent during at least part of the Miocene; (iii) the microcontinental blocks of Banggai-Sula and Buton-Tukang Besi, which rifted from the Australian-New Guinea continent during the late Mesozoic, were accreted onto eastern Sulawesi during the Miocene or Pliocene; and (iv) Sulawesi finally took on its present shape between the Pliocene and the present (Hall 1998; Moss & Wilson 1998). For Wallacea as a whole, Hall (2001) postulated that most of the smaller islands only emerged within the last 5 Myr, and therefore the biota can only have populated much of Wallacea during this period. Taken together, lineage-specific data suggest that during mid-Pleistocene glacial cycles (Marine Isotope Stages 22, 20, 18 & 16; Fig. 3), dispersal between both mainland-mainland and mainland-island sites, was extensive. Since this time, gene flow has been restricted (IM analyses: all pairwise lineage *m*-values highly skewed to zero; Fig. 4 and Supplementary material), and lineage sorting has lead to geographically restricted reciprocally monophyletic lineages. This is particularly evident in our mtDNA data set (Fig. 2) — as expected from theoretical expectations of a fourfold reduction in effective population size for mtDNA (Birky *et al*. 1989).

The IJ/SU/PH lineage and the PH lineage, taken together, however, provide an exception to a mid-Pleistocene restriction of dispersal. The occurrence of these two divergent (9 bp) mitochondrial lineages sampled from the same location (Plandez/Pulilan River, Luzon Island, Philippines) suggests a secondary dispersal (introgression) event into

the PH site. All four haplotypes in the IJ/SU/PH clade were sampled from the IJ site. All SU samples (n = 35) were fixed for one of these haplotypes, while nine individuals sampled from PH were fixed for another (Fig. 2), indicating founder events at these sites, i.e. a fairly recent dispersal of IJ individuals into the SU and PH sites. Indeed, estimating times of divergence (Fig. 4; Supplementary material) for these populations (three cases; range 27 000–20 000 BP) provides support for a second round of overseas dispersal during a glacial maximum, namely leading into the Last Glacial Maximum. Estimates of posterior density distributions of the migration parameter, m, support the directionality of dispersal outlined above, and also suggest that ongoing dispersal (gene flow) is limited or nonexistent.

To examine further the effects of glacial maxima on *M. rosenbergii* dispersal, we estimated pairwise population divergence times within all other lineages (PNG/ECY & LC) where multiple populations were present. Estimates of population divergence times (Fig. 4 and Supplementary material) provide further support for dispersal during periods of extremely low sea levels leading into the Last Glacial Maximum (PNG vs. ECY, 22 000 BP; KE vs. KA, 24 000 BP). Indeed, the fact that estimates of the migration parameter between these pairwise population comparisons were also highly skewed toward zero support a pattern of restricted dispersal since this time. Similarly, estimates of pairwise times of divergence (15 cases; range = 31 000–17 000 BP), and m values again highly skewed to zero (data not shown) among all other populations that comprise the LC clade, support the contention that they were connected recently, probably via Lake Carpentaria, during Marine Isotope Stages 4–2 (~90 000–10 000 BP) leading into the Last Glacial Maximum, but are now more isolated from one another (de Bruyn *et al*. 2004b). The probability that all times of divergence coincided with Pleistocene glacial maxima by chance alone is extremely unlikely (sign tests: all pairwise lineage divergence times, nine cases, P = 0.00391; all pairwise population divergence times, 20 cases, P = 0.00000191).

We acknowledge that a caveat on our interpretation of the timeframe involved, regarding putative mechanisms generating the observed patterns in our data, depends to a large extent on the molecular clock calibrated independently by Knowlton & Weigt (1998) and Morrison *et al*. (2004). Recent work on birds and mammals suggests that the intraspecific rate of evolution may be significantly faster than the 'phylogenetic' rate, presumably resulting from deleterious mutations not yet purged from the genome (reviewed in Ho *et al*. 2005; Penny 2005). If this were the case in our data, time of splitting between lineages, and populations within lineages, would be considerably younger. Nonetheless, timing would still be consistent with a Pleistocene origin of lineage diversification in the eastern form of *M. rosenbergii*, albeit younger than that proposed here.

Results presented here provide strong support for the 'Pleistocene marine dispersal hypothesis'. During the Pleistocene epoch, Marine Isotope Stage 16 (~630 000 BP) and the Last Glacial Maximum (~30 000–18 000 BP) were two periods when Antarctic ice sheets were at their maxima, and thus when global sea levels are believed to have been at their lowest (Fig. 1) (Epica Community Members 2004). If the molecular clock we use here is accurate, these two episodes of extreme reductions (Lisiecki & Raymo 2005) in global mean sea level appear to have played the major roles in facilitating both widespread overseas, and mainland–mainland, dispersal in eastern *M. rosenbergii* (Fig. 3), before lineages and populations within lineages, respectively, began to diverge. Recurring chronologically nested (lineage and lineage-specific population) dispersal events, resulting in genetic exchange during periods of extreme climatic change, may explain why the eastern form of *M. rosenbergii* has persisted for so long (~5 Myr), yet shows such low levels of genetic differentiation [i.e. has not speciated (Lindenfelser 1984; de Bruyn *et al*. 2004a; Wowor 2004; this study)] for a freshwater-dependent species over such large natural geographical distributional scales. A phylogenetic study of members of the speciose, globally distributed freshwater shrimp genus *Macrobrachium* hints at a similar scenario, albeit at the species level, of an evolutionary history of the genus shaped largely by historically widespread dispersal (Murphy & Austin 2005). Page *et al*. (2007) reported similar findings for the freshwater shrimp genus *Caridina*. That a large proportion of the speciation events among *Macrobrachium* taxa date back to the climatically unstable Miocene era (and to the Pleistocene) suggests that climate, and possibly dramatic fluctuations in sea level that occurred during this time (Hallam 1984), may have driven interspecific differentiation in this genus in a similar way to the generation of intraspecific diversification reported here.

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Mark de Bruyn completed his PhD under the supervision of Peter Mather at QUT in 2006. This is the final paper from that dissertation, which investigated the evolutionary history and biogeography of *M. rosenbergii*. M.dB.'s current research at the University of Durham utilises ancient DNA techniques to address questions regarding evolution, mutation rate, and the impact of Holocene climate change on distribution patterns. P.M. directs research in the Molecular Ecology lab at QUT, and has broad interests in the evolution and ecology of aquatic (and other) taxa.

Supplementary material

Marginal likelihood surfaces for time of splitting and migration for pairwise lineage and population comparisons, estimated in IM, indicating bounds of 90% highest posterior densities (HPD). All other pairwise population comparisons ($n = 16$) are not shown, but time of splitting does fall within MIS 4–2 (range 31 000–17 000 BP), with m values highly skewed to zero. Note that the scale of the x- and y-axes change throughout the series. Illustrated results are based on $\mu = 1.4 \times 10^{-8}$.

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