

Short communication

Huxley's line demarcates extensive genetic divergence between eastern and western forms of the giant freshwater prawn, *Macrobrachium rosenbergii*

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Abstract

Phylogenetic analysis of representatives from 18 wild populations of the giant freshwater prawn, *Macrobrachium rosenbergii*, utilising a fragment of the 16S rRNA mitochondrial gene, identified two major reciprocally monophyletic clades either side of a well-known biogeographic barrier, Huxley's line. The level of divergence between the two clades (maximum 6.2%) far exceeds divergence levels within either clade (maximum 0.9%), and does not concord with geographical distance among sites. 'Eastern' and 'western' *M. rosenbergii* clades have probably been separated since Miocene times. Within-clade diversity appears to have been shaped by dispersal events influenced by eustatic change.

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1. Introduction

Prawns of the genus *Macrobrachium* Bate, 1868 (Crustacea: Palaemonidae) are a highly diverse group of decapod crustaceans found in circumtropical marine-, estuarine-, and fresh-waters. Much debate has surrounded the systematic relationships of many species within this group (e.g., Holthuis, 1950, 1995; Johnson, 1973; Pereira, 1997), which has until recently been based exclusively on comparisons of external morphological characteristics. Molecular genetic approaches to resolving systematic questions in *Macrobrachium* have only been applied recently, when Murphy and Austin (2002) recognised that species and genus level designations did not correspond to traditional morphology-based classification schemes.

Macrobrachium rosenbergii, the giant freshwater prawn, is found in coastal river systems from Pakistan in the west to Vietnam in the east, across SE Asia, and

south to Papua New Guinea and northern Australia. Gravid females migrate from freshwater to estuarine areas, satisfying larval requirements for brackish water for survival and early development, where free-swimming larvae hatch and metamorphose into post-larvae, before migrating to freshwater after 3–6 weeks (New and Singholka, 1985). Several studies have reared *M. rosenbergii* larvae to post-larvae stage in artificial seawater (Sandifer and Smith, 1979; Smith et al., 1976), and considering this in light of a relatively prolonged larval duration, suggests that marine dispersal may play a previously unrecognised role in the life-history of this species.

Two forms of *M. rosenbergii* ('eastern' and 'western') have been described independently (De Man, 1879; Johnson, 1973), although the species is currently considered to be monophyletic. Lindenfelser (1984) analysed morphometric and allozyme data, and concluded that the boundary for eastern and western *M. rosenbergii* forms correspond approximately with Wallace's line (although Philippine samples were assigned to the eastern form, thus Huxley's line would seem a more appropriate boundary than Wallace's line; see Fig. 1).

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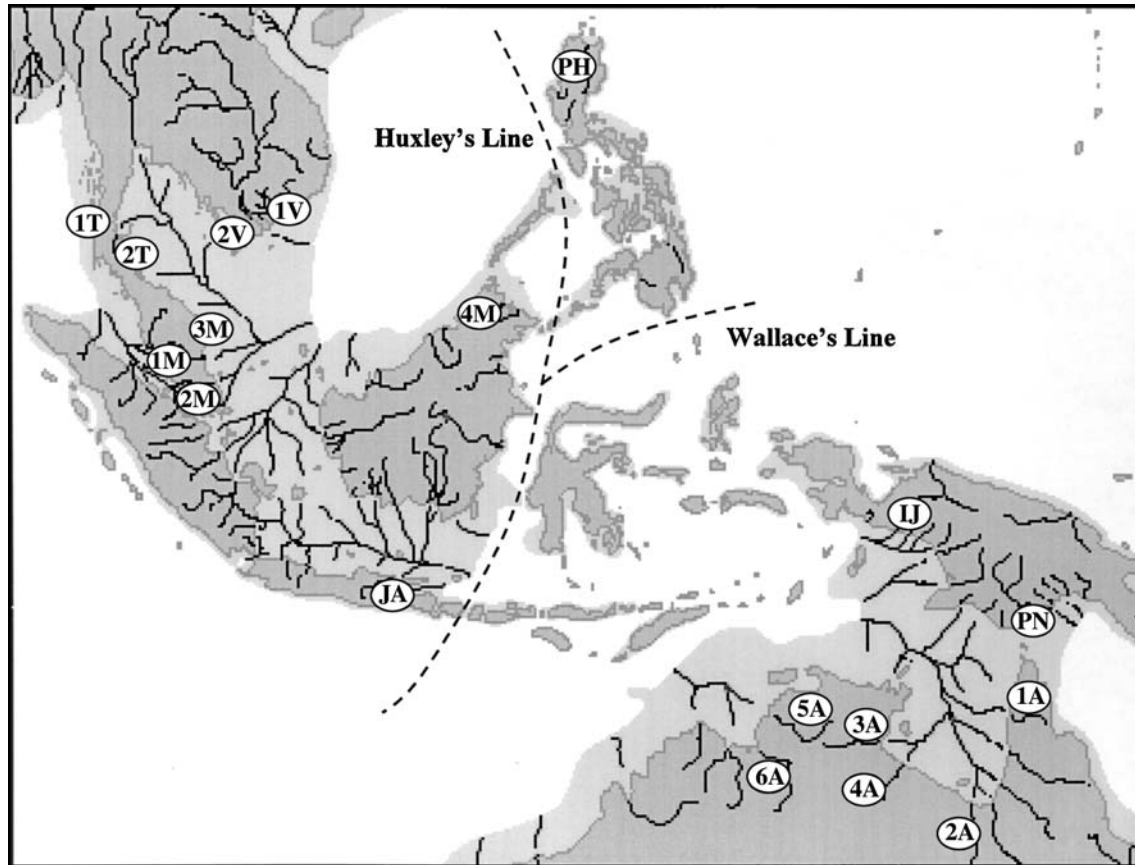


Fig. 1. Locations sampled for *Macrobrachium rosenbergii*. Light grey shading indicates -120 m sea-level contour. Pleistocene drainage basins indicated on map (Voris, 2000). Haplotype labels correspond to Appendix A. (Map adapted with kind permission Harold K. Voris and the Field Museum of Natural History, Chicago, USA; Voris, 2000.)

Malecha (1977, 1987) and co-workers (Hedgecock et al., 1979) recognised three 'geographical races'; an eastern, a western, and an Australian 'race,' based on allozyme and morphological data. Wowor and Ng (2001) regard the eastern and western forms of *M. rosenbergii* as two distinct species, based on adult morphological characters. Thus, *M. rosenbergii* as currently recognised taxonomically may be polytypic both regionally and perhaps even within biogeographic regions. Hence, the goal of the present study was to examine the evolutionary relationships among wild *M. rosenbergii* stocks at a regional scale, using 16S ribosomal RNA mitochondrial DNA (mtDNA) sequences, and relate the findings to the biogeographical history of the region.

2. Materials and methods

2.1. Specimens, DNA extraction, amplification, and sequencing

Prawns used in this study were collected from localities indicated in Appendix A and Fig. 1. *Macrobrach-*

ium australiense and *Macrobrachium lar* were used as outgroup taxa. Tissue samples were incubated overnight at 55°C in $500\ \mu\text{l}$ extraction buffer ($100\ \text{mM}$ NaCl, $50\ \text{mM}$ Tris, $10\ \text{mM}$ EDTA, and 0.5% SDS) containing $20\ \mu\text{l}$ of $10\ \mu\text{g}/\mu\text{l}$ Proteinase K (Sigma). Total genomic DNA was extracted using standard phenol:chloroform extraction methods. A 472-bp region of the mitochondrial 16S ribosomal gene was amplified using primers 16SAR and 16SBR (Palumbi et al., 1991). DNA sequencing was conducted at the Australian Genome Research Facility, Brisbane, Australia; using an ABI 377 automated DNA sequencer. Both strands of the PCR product were sequenced. Because mtDNA sequences were invariant among five individuals from each of four sampling sites (Mekong and Dongnai, Vietnam; Wenlock, Australia; Plandez/Pulilan, Philippines; de Bruyn et al., unpublished data), a single sequence from each sampling site was considered to be representative for phylogenetic analyses.

2.2. Phylogenetic analyses

Consensus sequences were aligned using ClustalX (Thompson et al., 1997). A total of $472\ \text{bp}$ were aligned

for analysis (see Appendix A for GenBank accession numbers). Saturation of nucleotide substitutions in the dataset was tested. A bootstrapped (1000 pseudoreplicates) maximum parsimony (MP) and neighbour-joining (NJ) phylogeny was constructed using MEGA version 2.1 (Kumar et al., 2001), based on Kimura 2-parameter distances (Kimura, 1980). A quartet-puzzling maximum-likelihood tree using the Hasegawa–Kishino–Yano (HKY) sequence evolution model (Hasegawa et al., 1985) was constructed in TREE-PUZZLE (Strimmer and von Haesler, 1996), using 1000 iterations of the puzzling process. Finally, a log-likelihood ratio test was carried out in TREE-PUZZLE that compared trees generated under the assumption of a molecular clock, to trees unconstrained by any such assumption (Felsenstein, 1988).

3. Results

A total of 472 bp of the 16S mitochondrial gene were amplified successfully for 18 *M. rosenbergii* individuals and two outgroup species. Of these, 90 variable sites were detected, of which 59 were phylogenetically informative. All sequences were found to be AT-rich (62.9%). Nucleotide substitutions (excluding outgroups) favoured transitions over transversions, yielding a transition/transversion ratio of 3.3. No evidence of saturation was evident. Kimura 2-parameter sequence divergences ranged from 5.1 to 6.2% between haplotypes from eastern and western *M. rosenbergii* samples, 0.0–0.6% among western samples, and 0.0–0.9% among eastern samples. A single deletion was observed in the dataset, for the *M. australiense* outgroup sequence. The log-likelihood ratio test rejected the assumption of clock-like behaviour. Two major reciprocally monophyletic *M. rosenbergii* clades (Fig. 2) were identified; corresponding geographically with the east/west disjunction reported previously (Lindenfelser, 1984). Bootstrap support for these clades was high in all cases. Relationships within the two clades were resolved to varying degrees.

4. Discussion

Variation in 16S rRNA sequences for *M. rosenbergii* support Lindenfelser's (1984) recognition that wild stocks comprise two major clades, restricted to either side of Huxley's line (Fig. 1). The level of sequence divergence observed between the two clades exceeds interspecific 16S rRNA divergence levels reported for diverse crustacean taxa, including penaeid prawns (Tong et al., 2000) and freshwater crayfish (Grandjean et al., 2002). The significant phylogenetic break between east-

ern and western haplotypes observed indicates the coalescence for these two clades was probably of mid to late Miocene origin, and approximates 5.3–11.7 million years before present (BP), based on 16S rRNA molecular clocks calibrated for porcelain crabs (0.53%/MY; Stillman and Reeb, 2001) and fiddler crabs (0.96%/MY; Sturmbauer et al., 1996; these values represent upper- and lower-bound extremes for crustacean 16S rRNA molecular clocks identified in a literature search). This estimate should be approached with caution, however, due to the rejection of clock-like behaviour of the dataset.

Wallace's line has long been recognised as a major biogeographical barrier. Huxley (1868) modified Wallace's line by extending it into the Philippines, based on zoological data, linking the island of Palawan to the western (Oriental) group, and the rest of the Philippine Archipelago to the eastern (Australasian) group. Data presented here clearly links a region of the Philippines (Luzon) to the eastern group. Tree topology indicates that the Australian OTUs (Operational Taxonomic Units) are basal to the remaining eastern OTUs examined. The unexpectedly low degree of divergence (1–2 bp) between the Philippine OTU and the rest of the eastern OTUs suggests recent gene flow has occurred. This has presumably been facilitated by larval marine dispersal, as the Philippine and Australian/New Guinea landmasses have been geographically distant since at least Miocene times (Hall, 1996). Tree topology indicates that gene flow has occurred from a southerly (Australian) to northerly (Philippines) direction, consistent with major ocean current movements in the region (South Equatorial Current; Gordon and Fine, 1996), although this remains to be rigorously tested with a more comprehensive dataset. Similar genetic signatures of Australian–Philippine dispersal events have been observed in a number of marine species (reviewed by Benzie, 1998).

The Mekong OTU appears ancestral to all other western OTUs. Sabah (Borneo) and Java cluster together, while all other western OTUs (Mainland Malaysia, Thailand, and Vietnam) apart from SW Thailand share identical 16S rRNA haplotypes. Reconstructions of Pleistocene drainage basins on the Sunda Shelf (Voris, 2000) suggest that the ancient Mekong drainage system has long been isolated from all other Pleistocene drainages identified. The Sabah drainage remained isolated throughout the Pleistocene, while the East Sunda River system, which encompassed the locality of the Javan OTU, drained eastward to exit near Bali, possibly restricting westward dispersal of *M. rosenbergii*. The SW Thai OTU would also have remained isolated during this time, while all other western OTUs would have been incorporated into either the Siam or Malacca Straits River Systems (Voris, 2000) that may have coalesced at some stage in the past. Ongoing gene-flow

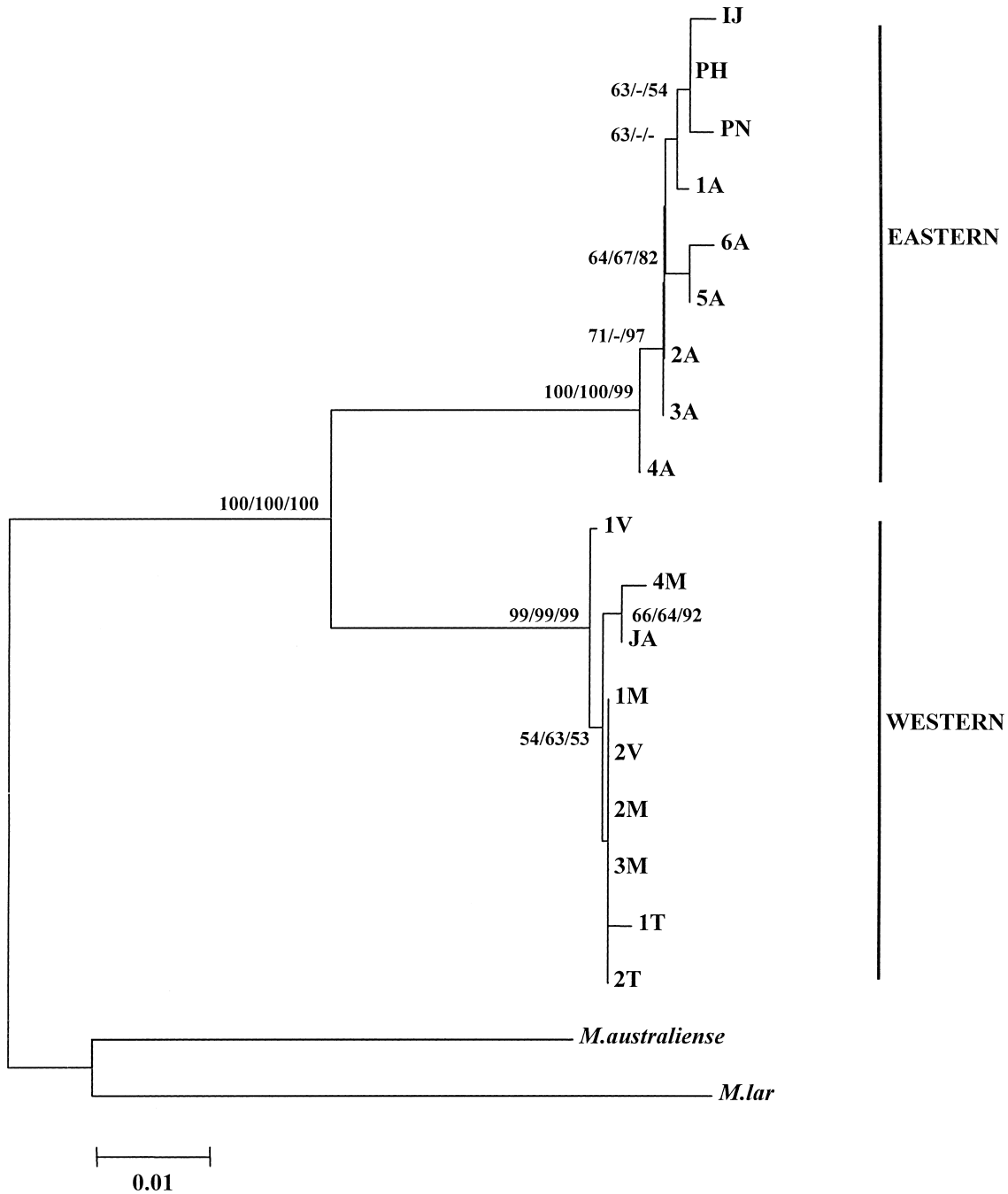


Fig. 2. Neighbour-joining distance tree of the relationships between *Macrobrachium rosenbergii* 16S rRNA haplotypes. Haplotype labels correspond to Appendix A. Bootstrap values (percentages) are shown for nodes with support >50%. Values correspond to neighbour-joining firstly, maximum-parsimony secondly, and maximum-likelihood thirdly. The trees produced by all three methods of analyses were not significantly different in topology.

amongst these localities, however, cannot be ruled out at present. The possibility that some form of selective sweep has produced the patterns observed in this study would appear unlikely, given the concordance of mt DNA (this study), allozymes (Hedgecock et al., 1979; Lindenfelser, 1984; Malecha, 1977, 1987), and morphological characters (De Man, 1879; Johnson, 1973; Lindenfelser, 1984; Malecha, 1977, 1987; Wowor and Ng, 2001).

5. Conclusion

Significant mtDNA divergence between eastern and western *M. rosenbergii* clades supports previous conclusions (De Man, 1879; Johnson, 1973; Lindenfelser, 1984; Malecha, 1977, 1987; Wowor and Ng, 2001) that *M. rosenbergii* may actually represent two distinct phylogenetic ‘species.’ Regardless of whether specific status is accorded to the eastern and western forms,

the divergence levels presented here are highly relevant for conservation of wild stocks. A number of intriguing questions regarding the evolutionary history of *M. rosenbergii* have been raised by this study. If marine larval dispersal has occurred between New Guinea/Australia and the Philippine Archipelago, why does that not appear to be the case between sites separated by lesser geographic distances (e.g., between Sabah and the Philippines) either side of Huxley's line? Can ancient vicariant events explain the divergence between eastern and western clades? Could the ancestral (Australian and Vietnamese) haplotypes represent lineages that persisted in Pleistocene refugia (sensu Hewitt, 1996) during periods of glacial maxima? Future directions for our research on *M. rosenbergii* will address these questions utilising mitochondrial COI markers in conjunction with nuclear markers.

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Appendix A

Samples used in this study for mitochondrial DNA extraction

Collection site location	Site abbr.	Eastern or western type	GenBank Accession Nos.
Bahand R, NW Peninsula Malaysia	1M	Western	AY203912
Semenyih R, SW Peninsula Malaysia	2M	Western	AY203915
Setiu R, NE Peninsula Malaysia	3M	Western	AY203904
Sandakan R, Sabah, Malaysia	4M	Western	AY203905
Mekong R, Vietnam	1V	Western	AY203914
Dongnai R, Sth Vietnam	2V	Western	AY203907
Kraburi R, SW Thailand	1T	Western	AY203908
Tapi R, SE Thailand	2T	Western	AY203911
Bengawan R, Java, Indonesia	JA	Western	AY203913
Plandez/Pulilan R, Luzon, Philippines	PH	Eastern	AY203910
Fly R, Papua New Guinea	PN	Eastern	AY203906
Ajkwa R, Irian Jaya, Indonesia	IJ	Eastern	AY203909
Wenlock R, Qld, Australia	1A	Eastern	AY203918
Leichardt R, Qld, Australia	2A	Eastern	AY203919
Roper R, NT, Australia	3A	Eastern	AY203920
McArthur R, NT, Australia	4A	Eastern	AY203921
Katherine R, NT, Australia	5A	Eastern	AY203917
Ord R, WA, Australia	6A	Eastern	AY203916
<i>Macrobrachium australiense</i>			AY203922
<i>Macrobrachium lar</i>			AY203923

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