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An allozyme study of the blue swimmer crab, *Portunus pelagicus* (Crustacea : Portunidae), in Australia: stock delineation in southern Australia and evidence for a cryptic species in northern waters

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Abstract. Allozyme analysis was used to examine the species-level systematics and stock structure of the Australian blue swimmer crab *Portunus pelagicus*. Fifty-seven crabs from eight sites were screened in an overview study for allozyme variation at 35 loci. This overview study revealed the presence of two species, differing at a Nei *D* of 0.14 (2% fixed differences), in the Darwin region of northern Australia. One of these species corresponds to the common *P. pelagicus* found throughout Australia, whereas the other is most likely either an undescribed 'cryptic' species, or the east-Asian species *P. trituberculatus*. In total, 609 *P. pelagicus* from 11 sites covering three regions in South Australia and two regions in the Northern Territory were then genotyped at seven polymorphic loci and these data assessed, using goodness-of-fit and *F*-statistics, for the existence of subpopulations. Four discrete subpopulations could be discerned, namely West Coast, Spencer Gulf, and Gulf St Vincent in South Australia, and Darwin–Gove in the Northern Territory. No evidence of population substructuring among sites within each subpopulation was evident from the allozyme data. The results support the current recognition of the three South Australian regions as separate stocks, and suggest that a taxonomic revision of Indo-Pacific *Portunus* is warranted.

Introduction

The blue swimmer crab, *Portunus pelagicus* (Linnaeus), is a large, edible species inhabiting coastal areas throughout the Indo-West Pacific from east Africa to Japan and northern New Zealand (Kailola *et al.* 1993). Within Australia, *P. pelagicus* is found in a continuous distribution extending northwards from south-west Western Australia around to southern New South Wales (Fig. 1). In addition to this essentially tropical–subtropical distribution, *P. pelagicus* is also located in three geographically isolated regions within temperate South Australia: 'West Coast', Spencer Gulf, and Gulf St Vincent (Fig. 1). *P. pelagicus* can survive in the three South Australian regions only because summer water temperatures are high enough to allow growth and reproduction to occur (Smith 1982). The blue swimmer crab and other 'tropical–subtropical' species that also have geographically isolated populations within South Australia (e.g. the western king prawn, *Penaeus latisulcatus* (I. Potter *et al.* 1991), and the mangrove, *Avicennia marina* (Duke 1991)) may have been separated from more northern populations during the past 10 000 years when seas rose to their present levels (Williams *et al.* 1993) and the climate cooled across southern Australia (Harrison 1993).

The life cycle of *P. pelagicus* consists of a larval phase and a crab phase (Yatsuzuka 1962). The larval phase is planktonic and lasts for at least three weeks (Meagher 1971), with the total lifespan lasting up to three years (Kailola *et al.* 1993). On the basis of the planktonic nature and extended duration of the larval phase and the high degree of mobility during the crab phase in *P. pelagicus* (Edgar 1990; M. Potter *et al.* 1991), the potential for genetic exchange between geographically separated populations appears to be high. Nonetheless, striking differences in colour and pattern morphology plus minor differences in structural morphology have been noted in *P. pelagicus* across its Australian range (e.g. Stephenson 1968; Meagher 1971). Such differences have led to the suggestion of population substructuring or even the existence of subspecies (Stephenson 1972; M. A. Potter, personal communication). Local fishermen have also speculated about the presence of separate inshore and offshore stocks of *P. pelagicus* in Spencer Gulf, South Australia. As a consequence of their geographic and historical isolation, it is even possible that the South Australian population may represent a separate, 'cryptic' species.

P. pelagicus is subject to commercial and recreational harvesting Australia-wide, with substantial commercial fisheries existing in South Australia, Western Australia, Queensland, and New South Wales (Kumar 1997). Although there is already one reported case of stock collapse in *P. pelagicus* from Streaky Bay in the West Coast region of South Australia (Grove-Jones 1987), commercial and recreational fisheries across Australia are set to expand (Kumar

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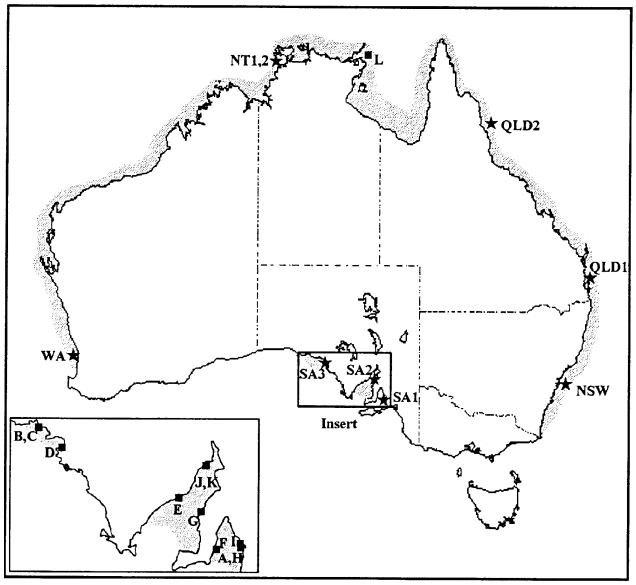

Fig. 1. Distribution of *Portunus pelagicus* in Australia (adapted from Kailola *et al.* 1993) and the locations of collecting sites for the sample sets used in the overview (★) and population studies (■) on *P. pelagicus*. WA, Perth; NT1, NT2, Darwin; L, Gove; QLD2, Cairns; QLD1, Brisbane; NSW, Sydney; SA1, A, H, St Kilda; SA2, Port Hughes; SA3, D, Streaky Bay; B, C, Denial Bay; E, Cowell; J, K, Whyalla; G, Port Broughton; F, Ardrossan; I, Port Gawler. Sample sets SA1, A, H, I, and F represent the Gulf St Vincent region; sample sets SA2, E, G, J, and K represent the Spencer Gulf region; and sample sets SA3, B, C, and D represent the West Coast region.

1997). Proper management of these fisheries requires knowledge of the degree of independence of crabs from different regions, in particular the extent to which discrete stocks (whether they be species, subspecies or subpopulations) can be identified and mapped geographically. The need for a thorough examination of systematics and population structure is emphasized by recent advances in the aquaculture of *P. pelagicus* in South Australia (Anon. 1997). Future success in any broadly based aquaculture industry across Australia may depend on the availability of such fundamental information about the biology of blue swimmer crabs.

Molecular genetic markers are now recognized as forming an integral part of any investigation into the systematics and population structure of animal groups (Avise 1994; Hillis *et al*. 1996). Allozyme electrophoresis has long been used by systematists and population geneticists for assessing genetic variability, population structure and the existence of 'cryptic' species in problematic groups of organisms (Richardson *et al.* 1986). In particular, it has been used widely on marine crustacea (Tracey *et al.* 1975; Burton *et al.* 1979; Mulley and Latter 1981; Stevens 1991; Pannacciulli *et al.* 1997), including crabs (Bert 1986; Kordos and Burton 1993; McMillen-Jackson *et al.* 1994). The present study describes a large-scale allozyme study of the systematics and population structure of *P. pelagicus* throughout Australia, with an emphasis on the three disjunctive regions within South Australia.

Materials and methods

General protocol

This project was performed in three stages: a pilot study, an overview study, and a population study. The pilot study assessed the effect of freeze–thawing on tissues and determined the tissue type most suitable for general use. The effect of freeze–thawing was investigated because significant losses of allozyme activity or resolution can sometimes result for marine invertebrates when homogenates have undergone more than one freeze–thaw event (Adams, unpublished). The overview study examined as many loci as practicable from 57 blue swimmer crabs that were collected across the geographic range of *P. pelagicus* within Australia. Its aims were to assess whether there was any genetic evidence for the existence of 'cryptic' species and to determine whether sufficient polymorphic loci existed to enable a full assessment of population substructuring. Richardson *et al*. (1986) suggested that a detailed allozyme study of population structure requires a minimum of six polymorphic loci, with the average frequency of the common allele at each locus not exceeding 90%. Given that sufficient polymorphic loci were found in the overview study, it was then possible to carry out the population study to assess the population structure of *P. pelagicus*. This involved the characterization of larger sample sets for those loci found to be polymorphic and for which individual genotypes were readily distinguishable on zymograms.

Collection of samples

Blue swimmer crabs were captured with dab nets, hoop nets, and commercial crab pots and trawl nets. For the pilot study, a few animals were transported whole to the laboratory where tissues were removed, prepared, and electrophoresed with and without prior freezing. Samples of leg muscle, abdominal muscle, gill, and hepatopancreas were also obtained from a single individual and compared for most of the enzymes run in the overview study. Subsequent to the pilot study, all South Australian animals were sampled directly in the field by removal of one of the walking legs and allocation of fresh leg muscle samples to two separate Eppendorf tubes for immediate snap freezing in liquid nitrogen. Animals collected outside South Australia were either frozen whole at the point of capture and then air-freighted on dry ice to Adelaide, or air-freighted live to Adelaide. All tissues and whole animals were kept at –80°C until required for analysis.

For the overview study, nine sample sets (WA, NT1, NT2, QLD1, QLD2, NSW, SA1, SA2 and SA3) were collected from eight regional centres around Australia including the three geographically isolated regions within South Australia (Fig. 1). In total, 12 sample sets (A–L) from nine sites were collected from South Australia and the Northern Territory for the population study (Fig. 1). Sampling targets were between five and ten crabs per sample set for the overview study and 50 per sample set for the population study; in most cases these targets were achieved. As part of the population study, replicate sample sets were also taken at one site in each of the three South Australian regions. Sample sets A and H were used to assess temporal heterogeneity within a site. They were both taken from St Kilda, the former in November 1993, and the latter in April 1994. All other sample sets for the population study were collected in March or April of 1994. Sample sets J and K assessed small-scale spatial heterogeneity within the Whyalla site; set J was collected from 'inshore' waters whereas set K was collected from 'offshore' waters approximately 5 km from set J. Sample sets B and C were taken on consecutive days from the Denial Bay site. Together these replicate sample sets provided a robust measure of within-site variability, ensuring that any between-site heterogeneity was not simply an artifact of the sampling protocol.

Sample preparation

Tissues were homogenized by sonication in two volumes of a buffered homogenizing solution (0.02 M Tris–HCl, pH 7.4, containing 0.2% 2-mercaptoethanol and 0.2 mg mL^{-1} NADP). Homogenates were then centrifuged at 10 000*g* for 10 min, and the supernatant either used directly for electrophoresis or stored at –20°C as separate 5–10 μL aliquots inside glass capillary tubes.

Allozyme electrophoresis

Allozyme electrophoresis was undertaken on cellulose acetate gels ('Cellogel') according to the principles and methods detailed in Richardson *et al*. (1986). The following 30 enzymes were surveyed successfully in the overview study: aminoacyclase (ACYC, EC 3.5.1.14), adenosine deaminase (ADA, EC 3.5.4.4), alcohol dehydrogenase (ADH, EC 1.1.1.1), fructose-bisphosphate aldolase (ALD, EC 4.1.2.13), aldehyde dehydrogenase (ALDH, EC 1.2.1.5), arginine kinase (ARGK, EC 2.7.3.3), enolase (ENOL, EC 4.2.1.11), fructose-bisphosphatase (FDP, EC 3.1.3.11), fumarate hydratase (FUM, EC 4.2.1.2), glyceraldehyde-3-phosphate dehydrogenase (GAPD, EC 1.2.1.12), lactoylglutathione lyase (GLO, EC 4.4.1.5), aspartate aminotransferase (GOT, EC 2.6.1.1), glycerol-3-phosphate dehydrogenase (GPD, EC 1.1.1.8), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), alanine aminotransferase (GPT, EC 2.6.1.2), isocitrate dehydrogenase (IDH, EC 1.1.1.42), cytosol aminopeptidase (LAP, EC 3.4.11.1), L-lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), nucleoside-diphosphate kinase (NDPK, EC 2.7.4.6), dipeptidase (PEPA, EC 3.4.13.), tripeptide aminopeptidase (PEPB, EC 3.4.11.), proline dipeptidase (PEPD, EC 3.4.13.), phosphoglycerate mutase (PGAM, EC 5.4.2.1), phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), phosphoglycerate kinase (PGK, EC 2.7.2.3), phosphoglucomutase (PGM, EC 5.4.2.2), pyruvate kinase (PK, EC 2.7.1.40), and triose-phosphate isomerase (TPI, EC 5.3.1.1). Nomenclature for enzymes, loci, and alleles follows Adams *et al*. (1987).

Data analyses

Genetic distances in the overview study were calculated as corrected Nei Distances (Nei 1978). Genetic relationships among regions and taxa were also displayed visually as a dendrogram, constructed by the Unweighted Pair Group Method of Analysis (UPGMA, Sneath and Sokal 1973).

The assessment of stock structure for the population study was undertaken in two ways. Firstly, χ^2 analyses were used at each of the selected polymorphic loci to *(1)* test the presumed independence of genetic markers by conducting between-locus contingency analyses to search for any association between the genotypes displayed at different loci, *(2)* test individual data sets for departure from the genotypic expectations under Hardy-Weinberg equilibrium, and *(3)* compare different data sets for heterogeneity of allele frequencies. These analyses were repeated at several steps in a hierarchy from sample set through to region and beyond, as recommended by Richardson *et al*. (1986). The hierarchical structure used herein involved 13 sample sets (A–L plus NT1+NT2 from the overview study) taken from 10 sites among five geographic regions (Gulf St Vincent, Spencer Gulf, West Coast, Darwin, and Gove) in two states (South Australia, and Northern Territory; Fig. 1). In all cases, alleles were pooled into two classes to ensure that small expected values would not be encountered, and Yate's correction for continuity was used when testing 2 × 2 contingency tables (Zar 1984).

Tests for deviation from Hardy-Weinberg were undertaken by use of the computer program BIOSYS-1 (Swofford and Selander 1981), and locus-by-locus contingency analyses and tests for between-sample set heterogeneity of allele frequencies were calculated by two BASIC programs written by one of us (MA). For each step in the hierarchical testing procedures of *(1)–(3)* above, significance values from the χ^2 analyses were adjusted to allow for multiple tests of the same hypothesis by using the sequential Bonferroni technique (Rice 1989) with an initial α-level of 0.05.

The second category of stock structure analysis involved the use of *F*-statistics according to the rationale of Weir and Cockerham (1984) and the computer program of Weir (1990). For subpopulations in Hardy-Weinberg equilibrium and which have identical allele frequencies at all loci, both F_{IS} (called *f* by Weir 1990*)* and F_{ST} (called θ by Weir 1990) are equal to zero. Here, the 95% confidence limits for both F_{ST} and F_{IS} are empirically calculated by jackknifing and bootstrapping the data. Any value of F_{ST} for which the lower 95% confidence limit is greater than zero is then indicative of the existence of significant genetic divergence among the subpopulations under examination. With respect to F_{IS}, any value which is significantly greater than zero indicates that inbreeding is occurring within some subpopulations, whereas a significant negative value suggests the avoidance of mating of relatives (Weir 1990). As with the χ^2 analyses, *F*-statistics were applied to several of the hierarchical levels between sample set and metapopulation.

Results

Pilot study

Allozyme activity and resolution of tissue homogenates were not affected by more than one freeze–thaw event. The most suitable tissue for use in this project proved to be leg muscle, because it displayed adequate levels of allozyme activity and resolution at all 30 enzymes, and was the most economical to sample in the field. It was therefore used as the sole tissue type in the overview and population studies. Although hepatopancreas displayed a few 'tissue-specific' loci which were either weak or absent in leg muscle, and abdominal muscle displayed adequate levels of allozyme activity and resolution at all 30 enzymes, it was felt that the need to kill so many crabs and the extra field work involved in collecting these tissues could not be justified. This was particularly the case given that the pilot study revealed a sufficient number of polymorphic enzymes in muscle alone. In contrast to the other tissues, gill homogenate displayed either poor activity or no activity for most of the enzymes.

Overview study

In total, 35 presumptive loci were scorable from the 30 enzymes examined in muscle. Of these, the following 21 loci were invariant in all 57 animals sampled : *Ada*, *Adh*, *Ald*, *Fdp*, *Fum*, *Gapd*, *Got-1*, *Gpd-1*, *Gpd-2*, *Gpi*, *Gpt*, *Idh*, *Ldh*, *Mdh*, *Ndpk*, *PepB-1*, *PepC*, *PepD-1*, *6Pgd*, *Pgk*, and *Pk*. Of the remaining 14 loci, with one exception, all sample sets displayed genotype frequencies which were consistent with the presence of a single panmictic population at that site. The one significant exception was the Darwin sample NT1 where the allozyme data were clearly heterogeneous at several loci. The most striking indication of this heterogeneity was at the locus *Aldh-1*, where a single individual was homozygous for *Aldh-1*^{c}, the only allele displayed by the other 47 animals sampled from around Australia (Table 1). Of the remaining nine animals sampled, eight were homozygous for the *Aldh-1*^{a} allele, whereas one individual was heterozygous for these two alleles. The loci *Enol* and *Glo*, either monomorphic or virtually monomorphic elsewhere, also showed the same pattern in the NT1 sample. In both cases the same individuals constituted the 1:1:8 split into the three groups, as was evident at *Aldh-1* (Table 1).

In order to further explore the heterogeneity evident in NT1, an additional 42 animals (sample set NT2) were sampled from the Darwin area. These animals were screened for the loci found to be commonly polymorphic in blue swimmer crabs, and also for the three loci which were heterogeneous in the overview study. The combined data from the NT2 and NT1 crabs indicate that there are two distinct

Table 1. Allele frequencies (%) at 14 polymorphic loci from the overview study on *Portunus pelagicus*

Size of each sample set is shown in parenthesis. The combined data for the Darwin site after screening 42 additional crabs from sample set NT2 is shown in boldface. Dashes indicate those loci not used to characterize the additional crabs from NT2. See Fig. 1 for details of sample set codes. Pp, *Portunus pelagicus*; Hyb, hybrid; Psp, *Portunus* sp.

Locus	Allele	Overview study										NT1+NT2		
		SA1 (7)	SA2 (7)	SA3 (7)	WA (6)	NSW (4)	QLD1 (11)	QLD2 (5)	(1)	NT1 (1)	(8)	**Pp (42)**	**Hyb (1)**	**Psp (9)**
Acyc	f					12								
	e				17		10	10				**16**		
	d	71	93	64	50	88	85	80	100	100	100	**65**	**100**	**100**
	c	29	7	29	25							**8**		
	b			7	8		5	10				**11**		
Aldh-1	d											**1**		
	c	100	100	100	100	100	100	100	100	50		**98**	**50**	
	b											**1**		
	a									50	100		**50**	**100**
Aldh-2	g											**2**		
	f											**1**		
	e											**3**		
	d	21	29	71	42	37	45	40		50	100	**53**	**50**	**100**
	b	79	71	29	58	63	55	60	100	50		**41**	**50**	
Argk	b	71	93	93	50	37	14	40	100	100	100	**18**	**100**	**100**
	a	29	7	7	50	63	86	60				**82**		
Enol	b					12				50	87		**50**	**89**
	a	100	100	100	100	88	100	100	100	50	13	**100**	**50**	**11**
Glo	b									50	94	**7**	**50**	**89**
	a	100	100	100	100	100	100	100	100	50	6	**93**	**50**	**11**
Got-2	b					17								
	a	100	100	100	100	83	100	100	100	100	100	–	–	–
Lap	b			7										
	a	100	100	93	100	100	100	100	100	100	100	–	–	–
Mpi	b			7										
	a	100	100	93	100	100	100	100	100	100	100	–	–	–
PepA	c				50	75	91	70	100	100	100	**92**	**100**	**100**
	b	100	100	100	50	25	9	30				**7**		
	a											**1**		
PepB-2	d											**1**		
	c	7					10				12	**10**		**11**
	b	43	64	79	58	50	45	70		100	88	**67**	**100**	**89**
	a	50	36	21	42	50	45	30	100			**22**		
PepD-2	c	57	36	29	17		4					**3**		
	b	43	64	71	83	75	64	90	100	100	100	**76**	**100**	**100**
	a					25	32	10				**21**		
Pgm	c	57	64	86	50		27	30		50	100	**17**	**50**	**100**
	b	43	36	14	50	87	68	70	100	50		**81**	**50**	
	a					13	5					**2**		
Tpi	b	93	100	100	100	100	100	100	100	100	100	–	–	–
	a	7												

genetic groups present within the Darwin region (Table 1). The two groups show a fixed allelic difference at the *Aldh-1* locus, major differences in allele frequency at *Argk*, *Enol*, *Glo*, and *Pgm*, and smaller but still notable differences in allele frequency at *Acyc* and *Aldh-2* (Table 1). The single individual from the NT1 sample which was labelled as a 'hybrid' is heterozygous at the appropriate alleles for five of the seven key loci, and has a genotype consistent with it being a hybrid at the remaining two loci (Table 1).

Levels of genetic divergence between the two genetic groups found at the Darwin site and the other sample sets from around Australia indicate that there are two taxa represented by the samples (Fig. 2), one taxon being distributed throughout Australia and the other being restricted to the Darwin site. For convenience, we hereinafter refer to the widely-distributed taxon as *Portunus pelagicus* and the northern taxon as *Portunus* sp. The two taxa are genetically similar, with an average level of genetic divergence of 0.14 Nei *D* (but only 2% 'fixed' or fully-diagnostic differences), compared with a maximum level of within-species genetic divergence of 0.04 Nei *D* in *P. pelagicus*. The ratio of the two taxa at the Darwin site varied from 1:8 *P. pelagicus*:*P.* sp. (plus one hybrid) in the NT1 sample to 41:1 in the NT2 sample. The genetic distances also suggest that South Australian *P. pelagicus* are genetically distinct from those found elsewhere in Australia (Fig. 2), an inference that is explored in detail below.

Population study

As well as identifying the presence of two taxa within Australian blue swimmer crabs, the overview study also found seven loci (*Acyc*, *Aldh-2*, *Argk*, *PepA*, *PepB-2*, *PepD-2*, and *Pgm*) in *P. pelagicus* for which the average frequency of the most common allele was less than 90%. Six of these loci were also polymorphic within the South Australian regions, whereas *PepA* showed substantial apparent divergence between the South Australian regions and the other Australian regions. All seven loci were used in the population study of stock structure. An examination of the estimates of allele frequencies from the eight regions in the overview study indicated that all seven loci showed quite substantial differences in allele frequency (i.e. >30%) for at least one between-region comparison. The initial target of 50 crabs per sample set was deemed sufficient to adequately characterize sample sets for the purposes of statistical analysis (see Table 5.2, Richardson *et al*. 1986).

Allele frequencies at the seven polymorphic loci for the 11 South Australian sample sets (A–K), the Gove sample set (L, only 18 crabs available), and the Darwin 'sample set' (NT1+NT2, data taken from the overview study) are presented in Table 2. Table 2 summarizes the genotypic data of 549 crabs from South Australia and 60 crabs from the Northern Territory, a total of 4263 genotypes. Because of space considerations, the actual genotypic data are not presented here, but are available from the junior author on request.

Between-locus contingency analyses

Locus-by-locus contingency analyses were performed on all 13 sample sets, on the three pools of replicate sample sets (A+H, J+K, and B+C), and on the pooled data from within

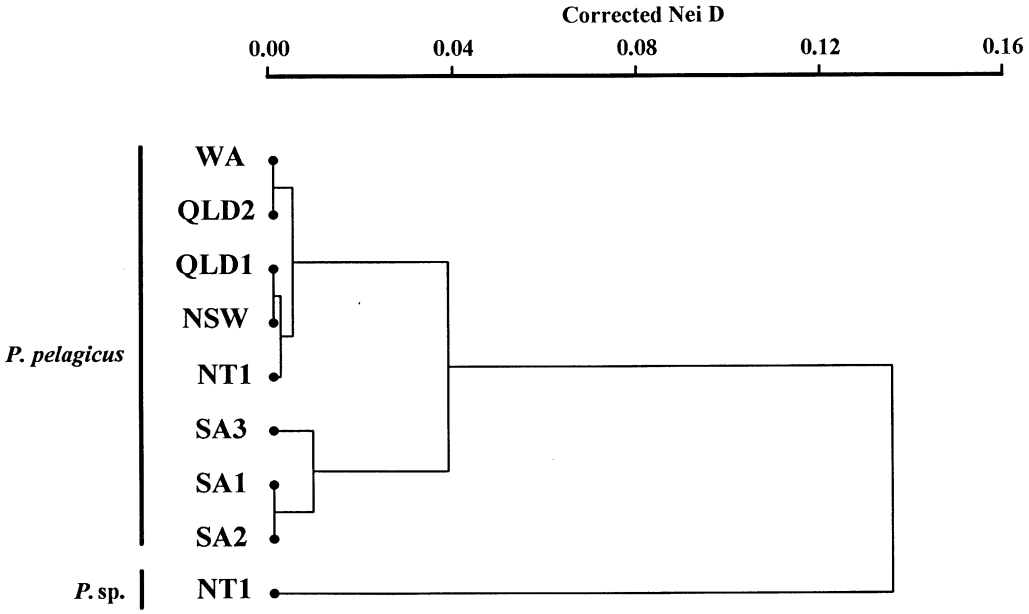

Fig. 2. Dendrogram depicting levels of genetic divergence among the sample sets surveyed in the overview study on *Portunus pelagicus*. See Fig. 1 for details of sample set codes and the text for further information.

Table 2. Allele frequencies (%) from the population study on *Portunus pelagicus*

Included are the 11 South Australian sample sets A–K, the Darwin sample set NT1+NT2, and the Gove sample set L. Maximum sizes of each sample set are shown in parentheses. See Fig.1 for details of sample set codes

Locus	Allele	Gulf St Vincent				Spencer Gulf				West Coast			Northern Territory	
		A (50)	H (49)	I (50)	F (50)	G (50)	J (50)	K (50)	E (50)	D (50)	B (50)	C (50)	NT1+NT2 (42)	L (18)
Acyc	e	1					1						16	17
	d	79	84	74	82	71	75	81	73	58	58	64	65	69
	c	15	15	16	10	13	14	10	19	37	42	33	8	8
	b	3		9	8	13	9	7	8	3		2	11	6
	a	2	1	1		3	1	2		2		1		
Aldh-2	g												2	
	f												1	
	e							1					3	
	d	33	33	33	39	35	41	33	39	56	63	48	53	44
	c		1	1										
	b	67	66	66	61	65	59	66	61	44	37	52	41	53
	a													3
Argk	b	63	72	69	72	90	89	94	89	92	97	99	18	25
	a	37	28	31	28	10	11	6	11	8	3	1	82	75
PepA	d							1						
	c	4	13		3	3	3	5					92	89
	b	96	87	100	97	97	97	94	100	100	100	100	7	11
	a												1	
PepB-2	d												1	
	c	8	6	6	6	7	5	3	1	1			10	5
	b	65	56	52	55	58	48	55	66	70	70	73	67	81
	a	27	38	42	39	35	47	42	33	29	30	27	22	14
PepD-2	c	45	38	38	44	35	26	28	36	2	5	1	3	2
	b	55	62	62	56	65	74	72	64	98	95	99	76	67
	a												21	31
Pgm	d			1					1			1		
	c	69	66	65	68	72	66	68	71	92	84	88	17	11
	b	31	34	34	32	28	34	32	28	8	16	11	81	83
	a												2	6

each of the three regions of South Australia. Significance values from the 1787 individual tests are not displayed because of space considerations; however, at all three hierarchical levels of testing there were no significant associations between genotypes displayed at different loci. Thus there was no evidence for linkage, non-Mendelian factors, or departure from the null hypothesis of panmixia at any of the three hierarchical levels. The seven genetic markers used in this study are therefore able to provide independent tests of population structure in *P. pelagicus*.

Departure from Hardy–Weinberg expectations

There were no significant departures from Hardy–Weinberg expectations in any of the tests at the levels of sample set, replicate sample set, or site (Table 3). Thus, there is no evidence that the sample sets from within any of the regions were drawn from anything other than a single panmictic population.

Comparisons of allele frequency

Having established that there was no apparent population substructuring within sample sets, a comparison of allele frequencies between sample sets was performed, again in a hierarchical manner. Tests at the replicate sample set level showed no significant heterogeneity (Table 4). Hence, it was appropriate to pool the replicate sample sets into three larger units to achieve greater statistical power in the analysis. Comparisons of allele frequencies between these new data sets at the site level also revealed no significant heterogeneity (Table 4). Sample sets from within each of the three regions were therefore pooled in order to conduct regional comparisons for South Australia.

Table 3. χ^2 values for genotypic expectations under Hardy-Weinberg equilibrium at various levels of sampling for the population study on *Portunus pelagicus*

See Fig. 1 for details of sample set codes. All χ^2 values are non-significant at $P = 0.05$ by sequential Bonferroni; –, no test possible

Hierarchical level	Geographic focus	Data sets tested	No. animals	Locus *Acyc*	*Aldh-2*	*Argk*	*PepA*	*PepB-2*	*PepD-2*	*Pgm*
Sample set	St Kilda	A	50	0.46	2.45	0.49	0.09	0.29	1.47	1.42
	St Kilda	H	49	0.53	0.08	0.04	1.15	3.97	1.46	0.08
	Port Gawler	I	50	1.03	0.59	0.62	–	1.97	0.02	0.49
	Ardrossan	F	50	0.13	2.03	2.13	0.05	1.15	0.15	0.33
	Port Broughton	G	50	3.69	0.01	0.62	0.05	0.01	0.49	0.42
	Whyalla	J	50	2.57	2.30	0.33	0.05	0.09	3.71	0.24
	Whyalla	K	50	0.03	1.96	0.20	0.20	1.47	0.42	0.01
	Cowell	E	50	0.95	2.03	0.76	–	0.59	2.39	6.79
	Streaky Bay	D	50	2.68	0.15	1.71	–	1.02	0.02	0.38
	Denial Bay	B	50	1.12	0.26	0.05	–	2.83	0.14	3.28
	Denial Bay	C	50	0.10	2.04	0.01	–	0.07	0.01	0.93
	Darwin	NT1+NT2	42	7.00	3.01	1.51	0.36	0.01	0.36	2.05
	Gove	L	18	sample size too small						
Replicate sample set	St Kilda	A+H	99	1.02	0.82	0.58	0.87	0.99	2.72	0.38
	Whyalla	J+K	100	1.15	4.44	0.13	0.22	1.02	0.75	1.62
	Streaky Bay	B+C	100	0.26	1.29	0.42	–	1.09	0.10	1.15
Site	Gulf St Vincent	A+F+H+I	199	0.11	0.29	2.75	0.56	0.43	1.96	0.25
	Spencer Gulf	E+G+J+K	200	0.89	4.81	0.44	0.19	0.01	1.30	2.57
	West Coast	B+C+D	150	1.85	1.33	2.61	–	2.06	0.11	0.42

Table 4. χ^2 values and results from significance tests for heterogeneity of allele frequencies at all hierarchical levels of sampling for the population study on *Portunus pelagicus*

See Fig. 1 for details of sample set codes. GSV, Gulf St Vincent; SG, Spencer Gulf; WC, West Coast; *$P < 0.05$ using sequential Bonferroni; –, no test possible

Hierarchical level	Geographic focus	Data sets compared	Locus *Acyc*	*Aldh-2*	*Argk*	*PepA*	*PepB-2*	*PepD-2*	*Pgm*
Replicate sample set	St Kilda	A *v.* H	0.02	0.03	1.61	4.31	1.28	0.79	0.06
	Whyalla	J *v.* K	0.43	0.77	1.03	0.47	0.72	0.03	0.02
	Denial Bay	B *v.* C	1.37	3.97	0.27	–	0.10	1.59	0.37
Site	GSV	A+H *v.* I	0.00	0.00	0.00	8.49	1.90	0.13	0.18
		A+H *v.* F	1.12	0.86	0.30	3.00	0.79	0.15	0.01
		I *v.* F	1.11	0.35	0.01	1.52	0.08	0.52	0.09
	SG	J+K *v.* E	2.09	0.04	0.11	3.99	5.50	2.15	0.33
		J+K *v.* G	0.00	0.05	0.00	0.27	1.05	1.67	0.56
		E *v.* G	0.93	0.19	0.00	1.49	1.04	0.00	0.00
	WC	B+C *v.* D	0.00	0.00	4.55	–	0.00	0.02	1.77
Region	South Australia	GSV *v.* SG	0.01	0.29	52.35*	1.60	0.00	8.16*	0.25
		GSV *v.* WC	48.10*	29.98*	77.79*	13.99*	13.73*	130.81*	40.02*
		SG *v.* WC	48.61*	24.22*	8.17*	7.74*	13.82*	85.67*	34.07*
Region	Northern Territory	L *v.* NT1+NT2	0.11	1.21	0.71	0.10	1.21	0.59	0.32
State	Australia	GSV *v.* L+NT1+NT2	4.84	13.40*	102.23*	381.95*	5.20	8.70*	103.53*
		SG *v.* L+NT1+NT2	4.76	10.28*	251.51*	413.51*	5.24	0.75	112.54*
		WC *v.* L+NT1+NT2	44.64*	0.30	271.35*	380.26*	0.24	54.30*	210.96*

All three regional comparisons for South Australia showed significant differences in allele frequency at two or more loci (Table 4). The geographically adjacent Gulf St Vincent and Spencer Gulf regions showed significant differences at two of the seven loci, whereas, strikingly, the West Coast region was genetically distinct at all seven loci when

compared to the other two regions (Table 4). Thus, in summary, there is strong evidence that each of the three regions within South Australia can be defined as a discrete subpopulation or stock. In contrast, the one regional comparison for the Northern Territory revealed no significant differences in allele frequency (Table 4) and therefore no evidence of population substructuring between the Gove and Darwin regions. Although the smaller sample sizes involved in this comparison increase the chance of a Type II error (i.e. falsely accepting the null hypothesis of no structuring), inspection of the allele frequencies displayed at the two sites reveals that they show little qualitative divergence at any locus (Table 2).

Comparisons between each of the three South Australian regions and a combined Northern Territory data set (L+NT1+NT2) revealed significant differences in allele frequency at either four or five of the seven loci (Table 4). This demonstrates that the combined sample sets from the Northern Territory constitute a fourth subpopulation within Australian *P. pelagicus*. The allele frequencies that characterize the four subpopulations at the seven loci demonstrate that the Northern Territory subpopulation is the most divergent genetically, with a high frequency of the alleles *Argk*^{a}, *Pep-A*^{c}, and *Pgm*^{b}, none of which are common elsewhere, and unique alleles at moderate frequency for two other loci (*Acyc*^{e} and *PepD-2*^{a}; Table 5).

Although the sample sizes for the two Queensland sites sampled in the overview study are too small for detailed statistical analysis, allele frequencies reveal that the composite of QLD1 and QLD2 has clear genetic affinities with the Northern Territory subpopulation (Table 5). Most alleles occur at similar frequencies in both samples, with the maximum difference in allele frequency being 22% for *PepB-2*^{a}. In particular, the key alleles that identify the Northern Territory subpopulation from the South Australian subpopulations are all present at similar frequencies, both in the composite Queensland sample (Table 5) and in the original QLD1 and QLD2 subsets (Table 1). This suggests that the Northern Territory subpopulation may have quite a wide geographic distribution.

Within South Australia, the West Coast subpopulation is more divergent from the others, with a distinctive pattern of allele frequency at five loci (*Acyc*, *Aldh-2*, *PepB-2*¸ *PepD-2*, and *Pgm*; Table 5). The Gulf St Vincent and Spencer Gulf subpopulations are mainly distinguishable from one another by a large difference in allele frequency at *Argk* (21%) and a smaller difference at *PepD-2* (10%; Table 5). Their allelic profiles at all other loci are very similar, sharing all but one of the rare alleles (P < 10%) found in South Australia (Table 5).

Table 5. Summary of allele frequencies (%) for each of the four subpopulations in *Portunus pelagicus*

GSV, Gulf St Vincent; SG, Spencer Gulf; WC, West Coast; NT, Northern Territory. Allele frequencies for Queensland (QLD) are also shown, compiled by pooling data from sample sets QLD1 and QLD2 as shown in Table 1. Maximum sample sizes are shown in parentheses

Locus	Allele	Subpopulations				
		GSV (199)	SG (200)	WC (150)	NT (60)	QLD (16)
Acyc	e				16	10
	d	80	75	60	67	83
	c	14	14	37	8	
	b	5	9	2	9	7
	a	1	2	1		
Aldh-2	g				2	
	f				1	
	e				2	
	d	34	37	56	49	44
	c	1				
	b	65	63	44	45	56
	a				1	
Argk	b	69	90	96	20	22
	a	31	10	4	80	78
PepA	c	5	3		91	84
	b	95	97	100	8	16
	a				1	
PepB-2	d				1	
	c	7	4		8	6
	b	57	57	71	72	53
	a	36	39	29	19	41
PepD-2	c	41	31	3	3	3
	b	59	69	97	73	72
	a				24	25
Pgm	c	67	69	88	15	28
	b	33	31	12	82	69
	a				3	3

F-statistics

None of the F_{ST} values at the site level was significantly different from zero (Table 6), indicating no evidence of genetic divergence within any of the three South Australian regions. Between-region comparisons for South Australia revealed one case of significant genetic divergence when all 11 sample sets were combined (Table 6). This F_{ST} value becomes non-significant when the West Coast sample sets are removed from the analysis (Table 6), indicating that the existence of this subpopulation is fully supported by *F*-statistics. In contrast, the F_{ST} value for the eight sample sets from Gulf St Vincent and Spencer Gulf is not significantly greater than zero, thus offering no unequivocal support for the recognition of these gulfs as separate subpopulations. This outcome contrasts with that obtained by the earlier analysis addressing homogeneity of allele frequencies. Nevertheless, the value of F_{ST} involved, 0.011, is the largest non-significant value for F_{ST} in Table 6, and its associated 95% confidence interval only just includes zero. As such, the *F*-statistics support the notion of two subpopulations near the point of displaying significant genetic divergence from one another. As with the earlier analyses, no population substructuring was demonstrable by F_{ST} analysis between the two Northern

Table 6. Summary of *F*-statistics for three hierarchical levels of sampling from the population study on *Portunus pelagicus*

95% confidence intervals are shown in parentheses immediately after the F_{ST} and F_{IS} values. See Fig. 1 for details of sample set codes. GSV, Gulf St Vincent; SG, Spencer Gulf; WC, West Coast; *n*, total number of sample sets; **P* < 0.05

Hierarchical level	Geographic focus	Data sets tested		F_{ST} Mean 95%CI	F_{IS} Mean 95%CI
Site	GSV	A,H,I,F		–0.001 (–0.005 to 0.007)	0.012 (–0.041 to 0.069)
	SG	J,K,E,G		0.000 (–0.005 to 0.005)	0.061 (0.014 to 0.107)*
	WC	B,C,D		0.003 (–0.004 to 0.013)	–0.080 (–0.106 to 0.006)
Region	South Australia	GSV,SG	(*n*=8)	0.011 (–0.002 to 0.035)	0.037 (0.008 to 0.069)*
		GSV,SG,WC	(*n*=11)	0.073 (0.037 to 0.125)*	0.015 (–0.004 to 0.035)
	Northern Territory	NT1+NT2,L	(*n*=2)	–0.005 (–0.012 to 0.002)	0.061 (0.004 to 0.104)*
State	Australia	GSV,NT1+NT2,L	(*n*=6)	0.179 (0.052 to 0.360)*	0.025 (–0.029 to 0.065)
		WC,NT1+NT2,L	(*n*=5)	0.344 (0.097 to 0.626)*	–0.028 (–0.053 to 0.034)

Territory regions (Table 6). The two between-state comparisons undertaken, using either the Gulf St Vincent region or the West Coast region to represent South Australia, both displayed large, significantly-positive values for F_{ST} (Table 6), offering strong support for recognizing the genetic distinctiveness of a northern subpopulation.

There were three instances where F_{IS} was significantly greater than zero (Table 6). Such an outcome is usually interpreted as an indication of inbreeding (i.e. the tendency for related animals to mate with one another) within one or more of the sample sets. The three cases concerned were the four sample sets within Spencer Gulf, the eight sample sets for Spencer Gulf plus Gulf St Vincent (which therefore include the aforementioned sample sets), and the two sample sets from the Northern Territory. A more detailed examination of the data reveals the reasons for these apparent departures from panmixia within some sample sets. Deleting sample set E in the first two cases is sufficient to restore F_{IS} to a non-significant value (results not shown). This occurs because sample set E displays a marked deficiency of heterozygotes at *Pgm*, ($\chi^2 = 6.79$; Table 3), and to a lesser extent at the loci *Aldh-2* and *PepD-2*. Similarly, a noticeable heterozygote deficiency also occurs at *Acyc* in the Darwin sample set ($\chi^2 = 7.00$; Table 3), and removal of this locus from the analysis causes F_{IS} to drop to a non-significant value (results also not shown). Thus, although there is some evidence of non-random mating, this result stems mainly from four cases of heterozygous deficiency at individual loci in two sample sets, none of which are significant under the sequential Bonferroni correction.

Discussion

Systematics

Although genetically distinct, the specimens of *P. pelagicus* and *P.* sp. detected in the overview study could not be separated by use of the morphological key of Stephenson (1972). Nevertheless, a *post hoc* comparison indicated that the two groups displayed subtle differences in gross morphology, most notably as follows: *(1) P.* sp. were generally smaller in overall size than *P. pelagicus*; *(2)* the ninth epibranchial spines were longer (relative to the carapace) and more curved in *P.* sp. than *P. pelagicus*; and *(3)* there were differences in dorsal pigmentation between the two species.

There are three possibilities regarding the systematic identity of the *P.* sp. specimens. They may correspond to an undescribed cryptic species within *P. pelagicus*. Alternatively, they may be representatives of the already-described species *P. trituberculatus* (Miers). The morphological description of the latter species is almost identical to that of *P. pelagicus* (see Stephenson 1972), to the point where its specific status has been questioned (Stephenson and Campbell 1959). Such morphological ambiguity between two valid species could account for the results of this study. *P. trituberculatus* is believed to be restricted to south-east and east Asia (Stephenson 1972; Lim and Hirayama 1991) and this would also help explain why specimens of *P.* sp. were collected only from the region of Australia closest to south-east Asia. The *P.* sp. (or *trituberculatus*) specimens could represent rare and possibly transient immigrants from the north, and the dramatic change in the ratio of *P. pelagicus* to *P.* sp. from the NT1 sample to the NT2 sample would be due to the strong element of chance associated with the micro-distribution of migratory *P.* sp. crabs.

A third hypothesis to explain the presence of two taxa in the Darwin region is that there are two forms of *P. pelagicus*, one restricted to Australia and another occupying peripheral parts of northern Australia and other regions of the Indo-West Pacific. Such a situation is believed to exist for another portunid, *Scylla serrata*, with two morphologically distinct forms present within northern Australia (Kailola *et al.* 1993). However, this situation can occur only if the two forms migrate to and from allopatric breeding grounds, since the existence of separate forms (i.e. *P. pelagicus* and *P.* sp. in the

present study) without genetic interchange in true sympatry represents the working definition of distinct biological species. The detection in the present study of a single *P. pelagicus* × *P.* sp. hybrid crab from the Darwin site suggests that the two taxa do not have fully allopatric breeding grounds and therefore argues against such an explanation for blue swimmer crabs. The occasional appearance of F_1 hybrids is however consistent with the existence of two separate species.

Turning to the specimens ascribable to *P. pelagicus*, the allozyme data provide no evidence that the marked colour and pattern variations noted for blue swimmer crabs from different regions are due to the presence of cryptic species or subspecies. It is probable that much of the variation in pigmentation for *P. pelagicus* is largely due to phenotypic plasticity, as such characteristics can change markedly over time in laboratory-held specimens (Meagher 1971; Bryars, unpublished). Nevertheless, given the subtle morphological differences which distinguish *P.* sp. from *P. pelagicus* in the Darwin region, one cannot reject the possibility that other taxa may still be present among Australian *P. pelagicus* (or indeed that *P.* sp. is more widely distributed across the north of Australia). 'Unusual' blue swimmer crabs displaying the features of *P.* sp. described earlier have also been reported from north Queensland waters (M. A. Potter, personal communication). Any future allozyme analysis which compares 'unusual' specimens with typical blue swimmer crabs is likely to provide a determination of whether or not such specimens are conspecific with *P. pelagicus*. Such molecular characterization would be best carried out as the starting point for a much-needed reassessment of the morphological criteria used to determine species boundaries among Indo-Pacific *Portunus*.

The systematic uncertainty outlined above must cast doubt over some of the results of earlier studies on *P. pelagicus*. For example Weng (1992), in a study of *P. pelagicus* from the southern part of the Gulf of Carpentaria and Moreton Bay in Queensland, found marked differences in the population structure and dynamics of the two regions; in particular, Gulf crabs were found to be far smaller than Bay crabs. Significantly, Weng (1992) was unable to fully explain these differences. However, based upon what has been observed in the present study it is quite possible that the two distinct taxa identified here were unknowingly sampled by Weng (1992). If this was the case, then Weng's (1992) data would not be able to give an objective picture of population structure in the two regions. Ambiguity of this sort reiterates the importance of systematics and of the need to unequivocally identify whether the populations under study are conspecific. It is also possible that Weng (1992) had sampled from two different stocks of *P. pelagicus* (see below).

Population structure

As the three South Australian subpopulations of *P. pelagicus* are geographically isolated and genetically distinct from those of all other regions of Australia, the present fisheries strategy of managing these stocks separately should be maintained. Implications for the aquaculture of *P. pelagicus* in South Australia are also apparent from the findings of the present study. Outside of South Australia, there is no evidence that *P. pelagicus* constitute anything other than a single stock. Nevertheless, the lack of detailed sampling throughout the range, particularly in the east and west, must render this a tentative conclusion at this stage. Additional animals need to be run at these seven loci and/or other molecular genetic markers before this working hypothesis can be accepted.

Although the extent of population substructuring in *P. pelagicus* outside of South Australia is unclear, the allozyme data do suggest that the northern subpopulation (characterized by the Darwin and Gove samples) may be quite widespread. Such a situation might be expected for *P. pelagicus* based upon its continuous distribution in northern Australia (Fig. 1) and the dispersal potential of both larvae and crabs (see earlier). It might also be anticipated that larval and crab dispersal could facilitate substantial gene flow along the west and east coasts of Australia. A low level of genetic heterogeneity has already been found for species with planktotrophic larvae along the Western Australian coastline (Johnson and Joll 1993) where the Leeuwin Current is known to have a significant impact on the larval dispersal of several species including the western rock lobster, *Panulirus cygnus* (Pearce and Phillips 1994). Similarly, on the east coast of Australia, the East Australian Current may be particularly important for the alongshore larval dispersal of species such as the eastern Australian king prawn, *Penaeus plebejus* (Ruello 1975).

The genetic data presented here provide strong evidence that gene flow is restricted between the West Coast, Spencer Gulf, and Gulf St Vincent subpopulations in South Australia. Crabs are not found between these three regions, and this implies that 'inter-regional' larval dispersal is restricted; a conclusion supported by other work (Bryars, unpublished). Therefore the persistence and population dynamics of each subpopulation must be dependent on its own larval supply. In terms of fisheries management this means that overfished stocks in one subpopulation will not be replenished by larval immigrants from another subpopulation. This is clearly shown in the West Coast subpopulation where overfishing occurred in the Streaky Bay area during the 1980s (Grove-Jones 1987), yet despite the existence of large stocks in Spencer Gulf and Gulf St Vincent, there has obviously been little or no successful larval immigration from these regions into the Streaky Bay area where crab numbers remain relatively low.

The West Coast subpopulation was found to be more genetically divergent from the Spencer Gulf–Gulf St Vincent subpopulations than the Spencer Gulf and Gulf St Vincent subpopulations were from each other. Such an outcome could arise for any of three reasons. Firstly, there may be occasional episodes of gene flow between Spencer Gulf and Gulf St Vincent, enough to keep these two stocks from diverging to any great extent. A second possibility is that the

impact of random genetic drift and/or selection pressures has been different for each subpopulation and unrelated to their time since isolation. Alternatively, the levels of genetic divergence might simply reflect the historical events that have influenced the isolation times of the three subpopulations and therefore the time available for genetic divergence. Under this explanation, the West Coast subpopulation would have been the first to become isolated from those to the east. The time of separation of the three subpopulations is difficult to determine but probably occurred less than 10 000 years ago when the sea was ~50 m below its present level (Williams *et al.* 1993). During the past 10 000 years the sea rose to its present level (~6000 years BP, Williams *et al.* 1993) and the climate cooled in southern Australia (Harrison 1993). Because of the bathymetry of the present-day South Australian coastline, such a rise in sea level is likely to have resulted in the West Coast stock being isolated before the Spencer Gulf and Gulf St Vincent stocks became isolated from one another (Bryars, unpublished).

South Australian *Portunus pelagicus* are genetically more similar to one another than to those elsewhere in their Australian range (Fig. 2, Table 5). If allozyme divergence does reflect time since isolation, then it also follows that the South Australian subpopulations were isolated from those found in the eastern and western parts of Australia before their isolation from one another. No firm evidence is available to test this contention, although it is the authors' belief that this also occurred around 10 000 years BP. Clearly the concepts of simultaneous isolation of all subpopulations and of allozyme divergence being proportional to time cannot both be in operation. It remains to be seen which of the two is inappropriate.

Results of this study do not support the idea of separate inshore and offshore stocks within the Spencer Gulf region, and there was also no evidence of substructuring within the West Coast and Gulf St Vincent regions. However, despite genetic homogeneity within each of the three recognizable South Australian stocks, the results do not necessarily indicate substantial gene flow (and therefore substantial larval and/or crab movements) within each stock as only a few individual migrations per generation may be required to maintain genetic homogeneity (Kimura and Maruyama 1971). Therefore in terms of fisheries management the genetic data do not imply that overfishing in one area within a stock will be quickly replenished by immigrants from another area within the same stock. This is particularly important for the Spencer Gulf stock where substructuring may still occur although it was not detected in this study. Richardson (1982), in a population genetic study of the western king prawn, *Penaeus latisulcatus*, found genetic homogeneity between specimens from Gulf St Vincent and nearby Investigator Strait. Based on other evidence, however, the author still suggested that substructuring might occur between these two regions. In order to further investigate the fine-scale substructuring of the stocks of West Coast, Spencer Gulf, and Gulf St Vincent *Portunus pelagicus*, other techniques such as crab tagging, mitochondrial DNA, and DNA microsatellite analysis may be required. The suggestion of inbreeding from the F_{IS} values for a few sites, although based on only a limited number of loci, strengthens the case for such additional investigations.

In summary, this allozyme study has demonstrated the existence of two taxa within Australian blue swimmer crabs and provided evidence for the existence of a minimum of four stocks within the widespread species *P. pelagicus*. These unequivocal outcomes highlight the importance of conducting a molecular systematic study on every 'species' under close human scrutiny, whether for harvesting, management, aquaculture, or conservation purposes. All of these situations involve the input of significant human resources, and yet there is no guarantee that the systematic framework (both at the species and the population and stock level) currently underpinning the group in question is the correct one, where such a framework does not incorporate both molecular and morphological information.

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