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ORIGINAL ARTICLE

Sympatric seagrass shrimp show similar structure and selection along the seashore: a comparison of two cryptic *Phycomenes zostericola* lineages

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Abstract

Small crustaceans are diverse and abundant in seagrass habitats and provide many ecological services. Further information is needed about their long- and short-term response to habitat fragmentation and loss. Shrimp specimens were sampled from coastal seagrasses: latitudes 9°–27° S (Torres Strait to Moreton Bay, Queensland, Australia) and longitudes 141°–153° E (Weipa to Moreton Bay, Queensland, Australia). The seagrass shrimp *Phycomenes zostericola* (Caridea: Decapoda) underwent phylogeographic analysis using mitochondrial and nuclear sequence data. Lineage relationships were investigated and two deeply divergent sympatric lineages of *P. zostericola* were discovered in northern samples. The two lineages display vastly different demographic histories in spite of similar overall phylogeographic patterns. The two lineages appear to be in secondary contact with each other, detailing independent histories of sea level change, long distance colonisation and habitat fragmentation. Their regional structure is evidence that the potential for dispersal is only realised under specific environmental conditions. Nuclear data, used to determine reproductive status between lineages, instead displayed a non-random latitudinal geographic distribution suggesting directional selection, possibly in response to ocean temperatures. The differences between the two deeply divergent lineages highlight the importance of understanding variability among cryptic taxa and their response to habitat change.

Introduction

Caridean shrimp are a diverse and abundant component of seagrass fauna (De Grave 1999). Seagrass shrimp enhance the nursery function of seagrass habitats and make a valuable indirect contribution to commercial fisheries, as they are an abundant and palatable food source for juvenile fisheries species (Heck *et al.* 2003). Although phylogeographic studies on caridean shrimp are not uncommon, most in Australia focus on freshwater species (Hughes *et al.* 1995; Hurwood & Hughes 2001). Broad-scale connectivity studies on small marine shrimp were undertaken in South Africa (Teske *et al.* 2007). However, little other phylogeographic research has been done on shrimp in seagrass systems. Seagrass habitats are becoming increasingly fragmented and lost due to anthropogenic influences (Orth *et al.* 2006; Connolly 2009). As this vital habitat becomes lost, it becomes ever more urgent that we understand how seagrass fauna responds to change in general and specifically for individual species. These questions are further complicated by the presence of cryptic species and the similar or divergent responses that they may have to environmental change.

Cryptic species are not rare in the sea; they are common among crustaceans and can be found at almost any depth and at all latitudes (Knowlton 1993) in the presence or

absence of obvious biogeographical boundaries (Hellberg 1998; Knowlton & Weigt 1998; Barber *et al.* 2000; Mathews *et al.* 2002). To manage marine biodiversity effectively it is vital that we identify cryptic diversity. It is also important to understand the environmental and biological drivers of new diversity. Divergence between species may be accelerated by selection, which can operate over very short temporal scales (see, Hilbish 1985) and small spatial scales ranging from kilometres for the copepod *Tigriopus californicus* (Burton 1997) to meters for the intertidal gastropod *Littorina saxatilis* (Wilding *et al.* 2001).

Species that are exposed to the same long-term extrinsic barriers to gene flow should display similar intraspecific phylogenetic structuring in a geographically concordant manner (Avise *et al.* 1987; Avise 2000). This is true, for example, in Torres Strait, where a shallow stretch of sea extends between Australia and Papua New Guinea. Fluctuating sea levels here sundered populations east and west by the repeated formation of a land bridge on the Sahul shelf (Galloway & Kemp 1981). Evidence of divergence can be found in multiple marine species either side of the Torres Strait (Lavery *et al.* 1996; Benzie *et al.* 2002; Crandall *et al.* 2008).

Phycomenes zostericola is an abundant caridean shrimp species with a broad coastal distribution (northern Western Australia, Northern Territory, Queensland and New South Wales) and is closely associated with their seagrass habitat (Bruce 2008; Haig *et al.* 2010). They thus provide an ideal study organism to explore broad-scale patterns of connectivity among seagrass-associated fauna. Initial analyses of *P. zostericola* populations revealed two deeply divergent lineages. Of the two lineages, lineage one (hereafter referred to as L1) has been shown to display a complex evolutionary history shaped by fluctuating sea levels and available refugia along a variable continental shelf (Haig *et al.* 2010). Here we examine the phylogeographic patterns of lineage two (L2) and the evolutionary relationship between the two lineages (L1 and L2).

This paper reports the discovery and comparison of two mitochondrial lineages of *P. zostericola.* We assess both lineages for morphological differences and use nuclear markers to test the hypothesis that the two lineages are reproductively isolated. Using coalescent analysis and comparative phylogeography we determined variations in demographic response to sea level change and coastal habitat among cryptic populations of seagrass-associated caridean shrimp.

Material and Methods

Sampling strategy

Sample sites were chosen to encompass the range of the species *Phycomenes zostericola* in Queensland and were designed to test genetic breaks along the Queensland coastline (Table 1 and see Haig *et al.* 2010). Specimens of *P. zostericola* were obtained from 19 of the 26 seagrass habitats sampled along the Queensland coastline. The smallest distance between adjacent sites where L2 was caught was 25 km (Ugar Island to Erub Island) and the greatest was 1080 km (Torres Strait to Townsville). All sites were <100 m from shore and were sampled at times when water was no more than 2 m deep.

Morphological examination

A caridean taxonomic key (Wadley 1978) and the taxonomic description of *Phycomenes zostericola* (Bruce 2008) were used to search for diagnostic morphological characters between lineages (most notably for *Phycomenes* species is the presence of a triangular median process on the forth thoracic sternite). All morphological traits listed in Bruce's (2008) description of *P. zostericola* were compared between several individuals from each lineage from across the sampled range. To confirm our findings we also provided several individuals from each lineage across the sampled range to Dr A. J. Bruce, a caridean taxonomic specialist from the Queensland Museum, for identification.

DNA extraction

The distal abdominal segments of shrimp were sectioned to avoid the potential contamination by food contents partially digested in the foregut. Total genomic DNA was extracted using a modification of the cetyl trimethyl ammonium bromide (CTAB)/phenol-chloroform DNA extraction protocol (Doyle & Doyle 1987) and stored in 50 μl of water at <4 °C.

Polymerase chain reaction amplification and sequencing

An ~800-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, a ~300-bp fragment of the nuclear protein-encoding histone H3, and ~600-bp fragment of the nuclear protein-coding gene myosin heavy chain (MyHC) was amplified by polymerase chain reaction (PCR). The COI gene was chosen due to the high level of intraspecific diversity found in other crustaceans (Cook *et al.* 2008), and histone (H3) is a highly conserved nuclear gene which has been used to investigate interspecies diversity (Porter *et al.* 2005). Like the burrowing shrimp *Alpheus lottini* (Williams *et al.* 2002), *Phycomenes zostericola* may also carry a single copy of MyHC showing intraspecific variation. PCRs were run in an Eppendorf Mastercycler Gradient or an Applied Biosystems Geneamp PCR System 2700 and 9700 (Applied Biosystems, Mulgrave, Victoria, Australia). PCR

conditions for the amplification of COI can be found in Haig *et al.* (2010). PCRs contained approximately 40 ng of template DNA, 0.5 each of 1 M primer MyHC1124 forward (5′ 5′AAG CTC GAG TCT GAC ATC A3′) and MyHC1806 reverse (5′GCA CTT CCT CAG GTT CTT CT3′; Williams *et al.* 2002), 0.5 mM dNTP (Astral Scientific, Caringbah, NSW, Australia), 2 mM $MgCl_2$, 1 μl of 10× polymerase reaction buffer and 0.02 units of *Taq* polymerase (Fisher Biotech, Subiaco, WA, Australia) adjusted to a final volume of 10 μl. PCR for MyHC followed the thermo-cycling profile of: 5 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C followed by an extension step of 5 min at 72 °C and a final hold at 4 °C. PCR success was visualised under UV light by running the product through a 1% agarose gel containing ethidium bromide. All PCR products were run alongside a 1-kb DNA size ladder (Invitrogen, Victoria, Australia). PCR product was purified with the exonuclease I-shrimp alkaline phosphatase (exo-sap) method, using 7 μl PCR product, 1 μl shrimp alkaline phosphatase (Promega, Fitchburg, WI, USA), and 0.25 μl exonuclease I (Fermentas, Burlington, ON, Canada) and a two-step thermal cycling profile of: 35 min at 35 °C and 20 min at 80 °C. Sequencing reactions contained 1 μl purified product, 1 μl forward primer, 0.5 μl BIGDYE V1.1 (Applied Biosystems), and 2 μl BigDye 5× sequencing buffer (Applied Biosystems) and 5.5 μl of distilled water at the following thermal cycling profile: 1 min at 96 °C; 30 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C; and a hold at 4 °C. Sequencing was conducted on a 3130 × l Capillary Electrophoresis Genetic Analyser (Applied Biosystems). Sequences were aligned and edited using SEQUENCHER version 4.1.2 (Gene Codes Corporation, Ann Arbor, MI, USA). The COI and MyHC datasets were aligned at default settings. No gaps occurred in any alignments. As crustaceans may contain pseudogenes of mitochondrial fragments (Song *et al.* 2008), all mitochondrial sequences were transformed into amino acids and scanned for internal stop codons.

Other nuclear loci trialled

The amplification of multiple anonymous loci and also the nuclear gene 28S rRNA (Mitsuhashi *et al.* 2007) was trialled on several individuals from different locations and both lineages. These failed to yield any variable sites within or between the two divergent lineages (data not shown). Elongation factor-1α (Williams *et al.* 2001) and the internal transcribed spacer gene (ITS II region, between the 5.8S and 28S genes; Ji *et al.* 2003) resulted in multiple bands and messy product; further, due to ITS existing in multiple copies within individuals, both genes were deemed unsuitable for this study.

Mitochondrial COI likelihood methods

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.* 2007). The net mean genetic distance between the 159 samples (*Phycomenes zostericola* L1 n = 127; *P. zostericola* L2 n = 32) used 1000 bootstrap replicates of a maximum composite likelihood model. Outputs provided a divergence estimate and standard error. The divergence estimate was calculated with a 1.4% Myr^{-1} evolutionary rate for the crustacean mitochondrial gene COI (Knowlton & Weigt 1998). A maximum parsimony phylogeny was then inferred from 1000 replicates (model = HKY + I + G) and is taken to represent the evolutionary relationship between the two lineages. Maximum likelihood trees were obtained using RAXML (Stamatakis *et al.* 2005; Stamatakis 2006) via the CIPRES web portal version 1.15 (Miller *et al.* 2009) with bootstrap replicates of 1000 (Stamatakis *et al.* 2008). Trees were viewed and edited in the program DENDROSCOPE V2.3 (Huson *et al.* 2007).

Population analyses

No internal stop codons were found, permitting all fragments to be used in data analyses. For the COI dataset, estimates of haplotype and nucleotide diversity were calculated using DNASP 4.0 (Rozas *et al.* 2003). Pair-wise comparisons of F_{ST} and Φ_{ST} values were calculated in ARLEQUIN 3.1 using 1000 bootstrap repetitions (Excoffier *et al.* 2005) and a modified false discovery rate (FDR) correction (Narum 2006) where alpha = 0.05 and probability values <0.0114 were considered significant. A Mantel test (1000 permutations) was used to test for isolation-by-distance. The input matrix compared distance values obtained from Google earth© (straight line distances conforming to the shape of the coastline in kilometres) against Slatkin's linearized Φ_{ST} values. Geographical structure was analysed using a hierarchical analysis of molecular variance (Excoffier *et al.* 1992). The significance of the following fixation indices (Φ_{ST}, Φ_{SC} and Φ_{CT}) were tested using permutation procedures outlined in Excoffier *et al.* (1992).

Populations were grouped to test for the significant partitioning of among group genetic variation (Φ_{CT}) based on the hypothesis that *Phycomenes zostericola* L1 and L2 are morphologically identical and are probably similar in biology and habitat choice and so were expected to display similar phylogeographic patterns. *Phycomenes zostericola* L1 show divergence between Torres Strait Islands and Queensland populations (Haig *et al.* 2010); therefore, L2 populations were expected to display a similar structure. The geographic structure was also investigated using a simulated annealing approach in

SAMOVA (spatial analysis of molecular variance; Dupanloup *et al.* 2002), which was used to partition populations that are geographically similar and maximally differentiated (thus providing the highest Φ_{CT} values). SAMOVA was run for 10,000 iterations for K = 2–5 using 100 initial conditions.

Haplotype network and nested clade phylogeographic analysis (NCPA)

Haplotype networks were constructed using statistical parsimony (95% probability cut-off) in TCS software, version 1.21 (Clement *et al.* 2000). Several ambiguous loops were resolved using some predictions from coalescent theory before clades were nested (Templeton & Sing 1993; Posada & Crandall 2001). Clades were nested by hand using rules from Templeton *et al.* (1987, 1995) and Templeton & Sing (1993) . Input files for the geographical locations and nested haplotype design were run by GEODIS 2.5 (Posada *et al.* 2000). The output was assessed using the latest version of the GEODIS inference key (15 December 2008) to infer biological explanations for clades found to display significant structure. Although nested clade phylogeographic analysis has been the subject of recent criticism (Petit 2008, and references therein) it remains a valuable phylogeographic tool (Templeton 2008), particularly when inferences are corroborated by independent analyses.

Population demographic expansion

Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) tests were used to determine whether sequence variation was consistent with predictions under the neutral model. Tajima's D compares two estimates of diversity that should be the same under neutrality. Fu's F_S is more sensitive to demographic expansion; it is based on the haplotype frequency distribution conditional on the genetic diversity of the sample. Negative values in both analyses result from an excess of substitutions relative to expectations for a constant-sized population and may be interpreted as evidence for recent population expansion. *Phycomenes zostericola* L2 haplotypes were nested into four clade levels. To test for clade demographic expansion, the two clades from level three were grouped for neutrality tests and mismatch analysis. Parameters of the mismatch distributions, with 95% confidence intervals, were calculated using a generalised least-square approach (Schneider & Excoffier 1999; Excoffier 2004) with the addition of Harpending's raggedness index (Harpending 1994). Time as scaled by mutation (tau) and time (ka) was calculated using a 1.4% Myr^{-1} mutation rate (Knowlton & Weigt 1998).

Coalescent analysis

The hypothesis that the two lineages and the populations within them may have each experienced isolation and vicariance due to the last glacial maximum (LGM) was tested using coalescent analysis on the mitochondrial dataset. Population divergence times (t), effective population size (N_e) and the time to most recent common ancestor (TMRCA) were calculated using the IM program (Hey & Nielsen 2004) for both *Phycomenes zostericola* lineages and also between Queensland and Torres Strait populations belonging to L2. As there was an absence of genetic structure between populations within both Queensland and Torres Strait for L2, populations were grouped into region. The t value was estimated between each pair of adjacent sample locations, as well as the N_e value for each. Preliminary analyses using wide intervals were used to determine prior distributions. Metropolis coupling was used to swap between 10 and 20 chains with heating of all chains set at g1 = 0.8, g2 = 0.9 to ensure the effective sampling of parameter space. All runs were executed for 3 million updates. Posterior distributions (including 90% credibility intervals) provided estimates of population divergence time (t), contemporary and ancestral theta (θ_{1or2} and θ_A). To convert divergence time into years, a sequence divergence rate of 1.4% $Myr^{-1}$for COI was used, which is the calibrated evolutionary rate for COI in caridean shrimp (Knowlton & Weigt 1998; Morrison *et al.* 2004) and with a generation time of 1 year, with generation time taken to mean the mid-point between becoming a reproductive individual and dying (A. J. Bruce, Queensland Museum, personal communication). Runs were repeated for each pair-wise comparison of adjacent populations to ensure parameter estimates were consistent.

Results

Morphology

Individuals from the two lineages were found to be morphologically indistinguishable from the *Phycomenes zostericola*-type material described in Bruce (2008).

Sequencing results

Sequencing usually resulted in good quality sequences that were easy to align. A total of 279 individual specimens of *Phycomenes zostericola* L1 from 19 of the 27 sampled locations and 58 individual specimens of *P. zostericola* L2 from 10 of the sites where L1 co-occurred underwent molecular examination (see Table 1). All individuals from both lineages were analysed for a 590-bp segment of the mitochondrial gene COI. Sequencing of

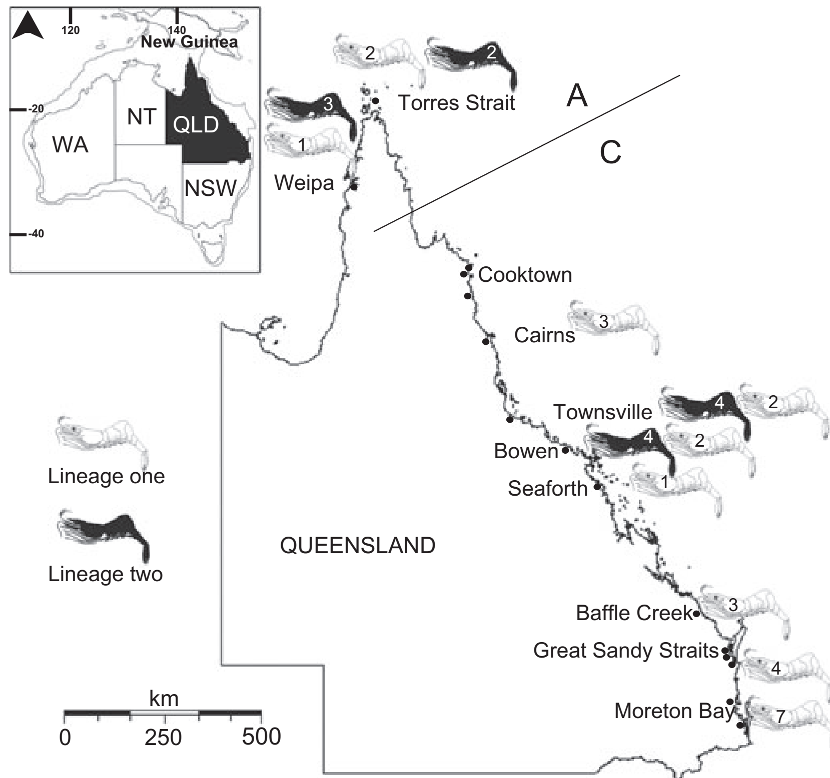

Fig. 1. A map of Queensland, Australia, displaying regions where each mitochondrial lineage of *Phycomenes zostericola* was sampled for the nuclear gene myosin heavy chain (MyHC). Numbers on shrimp indicate total individuals sampled at that location. A line indicates the geographical separation of the two MyHC alleles. A single nucleotide polymorphism in the MyHC gene occurs as an adenine (A) in northern individuals or as a cytosine (C) in southern individuals. Inset map of Australia shows northern orientation (arrow) and the states where *P. zostericola* have been caught (Bruce 2008): WA, Western Australia; NT, Northern Territory; QLD, Queensland; NSW, New South Wales.

the nuclear gene MyHC resulted in a 610-bp segment and contained a single nucleotide polymorphism (SNP) at position 233, either an adenine or a cysteine. Both lineages L1 and L2 displayed both polymorphisms. For both lineages an adenine was found in all 'northern' individuals (Torres Strait islands and Weipa) and a cysteine was found in all 'southern' individuals (populations along the east coast of Queensland and south of ~15° S) (Fig. 1). Aligning *P. zostericola* MyHC sequences with other palaemonid specimens, and also *Drosophila melanogaster* and *Homarus gammarus* MyHC sequences available from GenBank did not yield any useful information on codon positioning and hence we could not determine the non/synonymous nature of the SNP. The H3 primers yielded 309 bp of clean sequence for eight L2 and seven L1 individuals; all sequences were identical. The following sequences were submitted to GenBank (L1 COI accession numbers GU576176–GU576454; L2 COI; L1 and L2 MyHC and H3 accession numbers JN82236–JN82263).

Deep divergence of the mitochondrial gene COI

Divergence between the two *Phycomenes zostericola* lineages was estimated at 5.6% (SE ± 1%) using a maximum composite likelihood model. The grouping of two lineages is supported by 100% bootstrap support and within each lineage a north–south grouping was supported by 96% (L1) and 71% (L2) bootstrap values. Using a 1.4% Myr^{-1} divergence rate (Knowlton & Weigt 1998) the two *P. zostericola* lineages were calculated to have diverged during the mid Pliocene; 3.92 Ma (SE ± 0.7 Myr). The maximum parsimony tree found the same pattern and (using 10 individuals from each lineage) provides a visual representation of the result found in the above analyses (Fig. 2).

Population analyses

A full report of the genetic structure of L1 populations can be found in Haig *et al.* (2010). A high level of diversity and regional structuring was observed for L2 populations. Of the 58 L2 sequences used in population analyses, 32 haplotypes, with 40 segregating sites and 16 parsimony informative sites, were found in the 590-bp-long COI segment. Site haplotype diversity ranged from 0 to 1 (Table 2). No correlative relationships were found between nucleotide diversity and geographic location to support a range expansion hypothesis. In 45 pair-wise population comparisons 23 F_{ST} values were significant (Table 2); 15 pair-wise F_{ST} comparisons remained significant under false discovery rate (FDR) correction (Narum 2006). Significant structure was observed between Torres Strait islands and Queensland populations (Table 2). The reduction in significant pair-wise comparisons after the

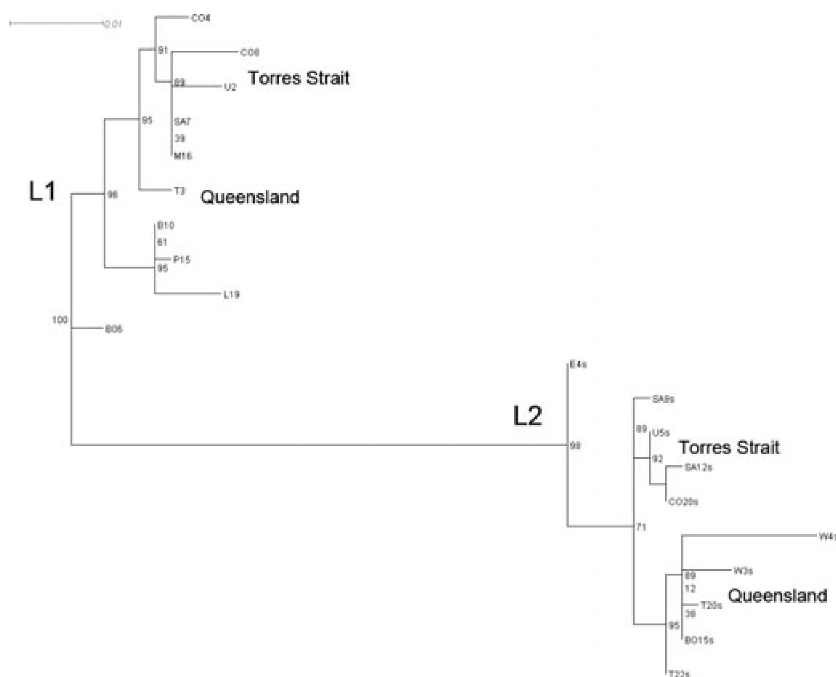

Fig. 2. The evolutionary history of two *Phycomenes zostericola* lineages inferred from a maximum likelihood tree using RAXML (Stamatakis *et al.* 2005; Stamatakis 2006) via the CIPRES web portal version 1.15 (Miller *et al.* 2009) with bootstrap replicates of 1000 (Stamatakis *et al.* 2008). Trees were viewed and edited in the program DENDROSCOPE V2.3 (Huson *et al.* 2007). Scale bar indicates percentage of nucleotide divergence. Numbers on tree indicate bootstrap values. Torres Strait: CO, Coconut Island; E, Erub Island; M, Moa Island; SA, Saibai Island; U, Ugar Island. Queensland (from north to south): W, Weipa; T, Townsville; P, Pumicestone passage; L, Loders Creek. Small s after site code indicates L2.

Table 1. Regions in Queensland where *Phycomenes zostericola* were caught and number of individuals belonging to each mitochondrial COI lineage.

Sampled location	Latitude	Longitude	Lineage L1	Lineage L2
Weipa	12°40′32.44″ S	141°52′44.73″ E	13	11
Torres Strait islands	10°35′10.04″ S	142°13′18.03″ E	69	34
	09°22′18.46″ S	142°37′02.14″ E		
	09°36′38.88″ S	143°46′25.10″ E		
Cairns/Cooktown	15°27′18.88″ S	145°13′43.27″ E	22	0
	16°54′00.77″ S	142°45′27.20″ E		
Townsville	19°11′18.25″ S	146°40′56.97″ E	14	9
Bowen	20°01′05.09″ S	148°15′00.21″ E	15	4
Dingo Beach/Seaforth	20°05′17.29″ S	148°25′51.79″ E	35	0
	20°52′04.90″ S	148°56′59.13″ E		
South East Queensland	24°31′24.05″ S	152°02′57.48″ E	111	0
	27°57′13.11″ S	153°24′38.92″ E		
Total			279	58

Latitude and longitude indicate either a single or a range of sampling locations.

FDR correction did not change any overall patterns of genetic differentiation. An isolation-by-distance effect was detected using a Mantel test ($r = 0.68$; $P \leq 0.002$). A significant proportion of the genetic variance observed among populations was significant regardless of hierarchy (Table 3). Grouping Weipa samples with Torres Strait samples did not yield a significant Φ_{CT} value. Genetic structure between Torres Strait Islands and Queensland was evident in all SAMOVA partitions (Table 3). The largest Φ_{CT} value observed using SAMOVA was for $K = 2$. Little change in Φ_{ST} was observed between $K = 2$ and $K = 5$ (Table 3).

Nested clade phylogeographic analysis

The nesting of the haplotype network identified 32 haplotypes, nested into 19 one-step clades, 7 two-step clades, 3 three-step clades, and 1 four-step clade (Fig. 3). Only the total cladogram (nesting level 4) had a significant chi-squared value (61.80, $P \leq 0.001$). Using the chain of inference it was not possible to resolve between fragmentation and isolation by distance due to inadequate sampling (data not shown). Although intermediate sites were sampled, no L2 individuals were found between Torres Straits and Townsville. The large physical distances

Table 2. Pair-wise F_{ST} (lower matrix) and Φ_{ST} (upper matrix) estimates calculated in Arlequin (Excoffier *et al.* 2005) for *Phycomenes zostericola* L2 from Queensland and Torres Strait coastal seagrass habitats.

					Torres Strait Islands							Queensland		
Location	n	nh	h	π	CO	SA	M	TL	Y	R	U	W	T	BO
CO – Coconut Island	7	5	0.857	0.007		−0.046	0.091	0.001	0.029	−0.038	0.125	**0.380**	**0.467**	**0.368**
SA – Saibai Island	8	7	0.964	0.005	−0.005		0.209	0.166	0.150	−0.011	**0.201**	**0.462**	**0.579**	**0.497**
M – Moa Island	4	1	0.000	0.000	0.186	0.230		0.385	0.000	0.095	−0.081	**0.633**	**0.795**	**0.795**
TI – Thursday Island	2	2	1.000	0.003	0.104	0.026	0.724		0.250	−0.005	0.347	**0.577**	**0.743**	0.669
Y – Yam Island	3	1	0.000	0.000	0.127	0.172	0.000	0.647		0.004	−0.154	**0.606**	**0.776**	**0.759**
E – Erub Island	4	3	0.833	0.005	−0.129	−0.037	0.167	0.111	0.077		0.134	**0.523**	**0.662**	**0.568**
U – Ugar Island	6	2	0.333	0.001	0.112	0.155	−0.081	0.510	−0.154	0.043		**0.649**	**0.799**	**0.804**
W – Weipa	11	10	0.982	0.007	**0.067**	0.027	**0.360***	0.013	**0.317***	**0.078**	**0.292***		0.007	−0.067
T – Townsville	9	8	0.972	0.003	**0.071**	0.032	**0.384***	0.020	**0.337***	0.085	**0.311***	−0.029		0.091
BO – Bowen	4	3	0.833	0.005	0.153	0.091	**0.583**	**0.111**	**0.520**	0.167	**0.458**	−0.012	−0.027	

n, number; nh, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; bold values are ≤0.05; F_{ST} values with an asterisk are ≤0.0114 under an FDR correction (Narum 2006).

Table 3. Analysis of molecular variance (AMOVA, above) and spatial analysis of molecular variance (SAMOVA, below) of *Phycomenes zostericola* L2; results calculated in ARLEQUIN (Excoffier *et al.* 2005) and SAMOVA (Dupanloup *et al.* 2002) for mtDNA cytochrome *c* oxidase subunit I (COI) using haplotype frequency differences between grouped populations. Probability values shown in parentheses.

					Percent variation		
	Hypothesised genetic break analysed using AMOVA	Φ_{CT}	Φ_{SC}	Φ_{ST}	Among group	Among population within group	Within population
	Torres Strait/Queensland	0.637 (<0.05)	$0.021^{n.s.}$	0.637 (<0.001)	63	1	36
K	SAMOVA Population Groups						
2	1 Weipa, Townsville, Bowen 2 Saibai, Coconut, Moa, Yam, Erub, Ugar, and Thursday islands	0.599 (0.008)	$0.030^{n.s.}$	0.611 (0.000)	60	1	39
3	1 Weipa, Townsville, Bowen 2 Thursday island 3 Saibai, Coconut, Moa, Yam, Erub, and Ugar islands	0.581 (0.002)	$0.03^{n.s.}$	0.594 (0.000)	58	1	40
4	1 Townsville, Weipa 2 Bowen 3 Thursday Is. 4 Saibai, Coconut, Moa, Yam, Erub and Ugar islands	0.559 (0.002)	$0.032^{n.s.}$	0.573 (0.000)	56	1	43
5	1 Townsville 2 Weipa, Bowen 3 Erub Is. 4 Thursday, Moa, Yam, and Ugar islands 5 Saibai and Coconut islands	0.554 (0.001)	$-0.111^{n.s.}$	0.504 (0.000)	55	−5	50

Torres Strait: Coconut Island, Erub Island, Moa Island, Saibai Island, Ugar Island; Queensland: Weipa, Townsville and Bowen; n.s., non-significant.

between Torres Strait islands and Townsville was a likely contributor to the lack of resolution observed.

Population demographic expansion

Neutrality and mismatch analyses were not tested on clade level 3-2 due to its small sample size (n = 6). Tests of neutrality for Tajima's D were significantly negative for nested clade 3-1 (−1.80, $P \le 0.011$) and clade 3-3 (−2.06, $P \le 0.006$). Fu's F_S neutrality tests were significantly negative for nested clade 3-1 (−6.78, $P \le 0.000$) and clade 3-3 (−10.14, $P \le 0.000$). The parameters of the mismatch distribution for nested clade levels 3-1 and 3-3 could not reject a sudden population expansion model (Table 4).

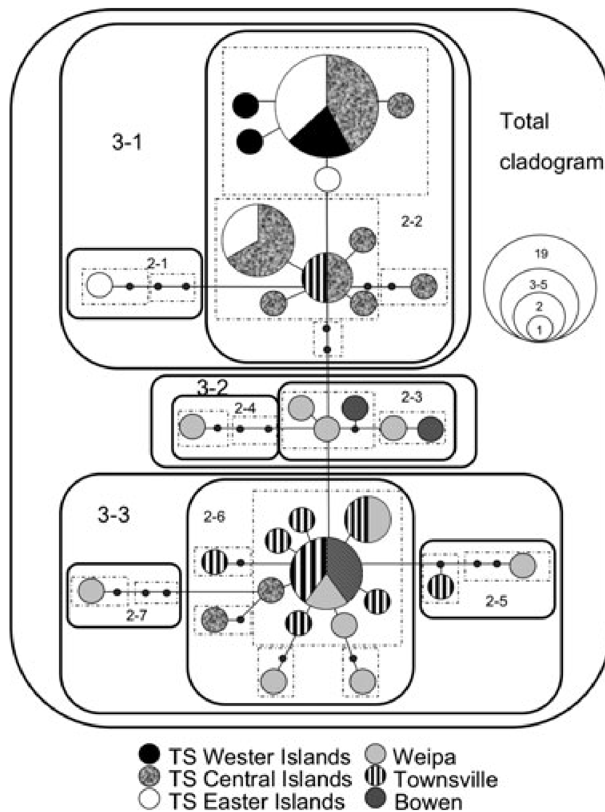

Fig. 3. Nested parsimony network for 32 *Phycomenes zostericola* L2 haplotypes, computed by TCS using 590 bp of the cytochrome oxidase subunit I (COI) gene with 95% confidence interval. The circle size indicates number of individuals sharing a haplotype; shade or pattern indicates sample location. Connecting bars indicate one base pair mutation between haplotypes and black circles indicate additional base pair mutations. Nesting levels are boxed into clade levels one (broken lines) to four (total cladogram). TS (Torres Strait) Wester Islands: Moa and Thursday islands; TS Central Islands: Saibai, Coconut and Yam islands; TS Easter Islands: Ugar and Erub islands.

Population expansions estimated using tau values date well within the Pleistocene epoch: c. 16.3 ka for nested clade level 3-1 and c. 204.5 ka for nested clade level 3-3 (Fig. 4).

Coalescent analysis

Coalescence for Torres Strait island and Queensland mainland populations was estimated to have occurred during the Pleistocene and the TMRCA existed ~647 ka (Fig. 4). Population divergence times and effective population size estimates for some locations have very broad posterior densities, which are probably due, in part, to sample size and the use of a single genetic locus. Regardless of this, Torres Strait islands and the Queensland mainland populations were estimated to have exchanged as little as one migrant every 6 years. Even though effective population estimates had broad posterior distributions, the large numbers (in the millions) were likely true and similar to *Phycomenes zostericola* L1 (Haig *et al.* 2010).

Discussion

The two *Phycomenes zostericola* lineages were morphologically indistinguishable. In the description of *P. zostericola* (Bruce 2008), some size-related morphological variation was observed between two ovigerous female specimens caught in Moreton Bay, southeast Queensland. Bruce (2008) concluded that either substantial variation occurs within the species or, less likely, a second taxon was involved. The two distinct genetic lineages were observed to be morphologically identical; Dr Bruce of the Queensland museum also confirmed this identification. In this study, all 111 specimens of *P. zostericola* sampled from southeast Queensland belonged to the L1, lending support to Bruce's (2008) suggestion of high within-species morphological variation. In sites where the two lineages occurred sympatrically, L1 was always more abundant, which may either suggest low numbers of L2 or a sampling bias towards the capture of L1. In two alpheid shrimp species, sympatric sister species occupy the same habitat type, although patches were dominated by either one or the other sister taxon (Mathews *et al.* 2002). Further research is required to determine whether microhabitat and resource partitioning occurs between the two lineages, and if this has led to reproductive isolation.

A deep mitochondrial divergence for morphologically identical lineages

The deep divergence between the two *Phycomenes zostericola* lineages (5.6% ± 1) is estimated to have occurred

Table 4. Results of neutrality tests, Tajima's D and Fu's F_S and parameters of the mismatch distribution for three-step clades from a nested clade phylogeographic analysis (NCPA) of *Phycomenes zostericola* L2 populations from Queensland.

Population	Tajima's D	Fu's F_S	Tau	t (years)	Hri
Clade 3-1	−1.80 (0.011)	−6.78 (0.000)	0.135 (0–0.506)	16 300 (0–61 300)	0.046 (1.000)
Clade 3-3	−2.06 (0.006)	−10.14 (0.000)	1.689 (0.508–4.639)	204 500 (61 500–561 600)	0.041 (0.602)

Mismatch parameters are: time as scaled by mutation (tau) and time since expansion (t) calculated using the 1.4% Myr^{-1} mutation rate. Harpending's raggedness index (Hri) was also applied to mismatch data. Probability values (P) or upper and lower bounds of the 95% confidence intervals are in parentheses.

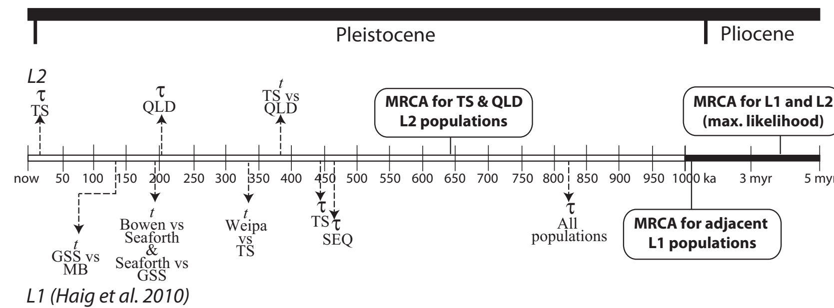

Fig. 4. Time line in thousands to millions of years, showing Pleistocene and Pliocene epochs adjacent to the summarised results of maximum likelihood analysis, neutrality tests and coalescent analysis for *Phycomenes zostericola* L1 (below) and L2 (above). GSS, Great Sandy Strait populations; MB, Moreton Bay populations; SEQ, Southeast Queensland populations; τ, time of clade demographic expansion; t, time of population coalescence, MRCA, time of most recent common ancestor between either adjacent populations within a lineage or mitochondrial lineages; TS, Torres Strait populations; QLD, Queensland populations.

during the Pliocene, approximately 3.2–4.6 Ma. The depth of the mitochondrial divergence between the two *P. zostericola* lineages suggests speciation (Knowlton & Weigt 1998). However, results from nuclear loci failed to reveal whether the two divergent lineages were reproductively isolated. Evidence of mitochondrial divergences in other invertebrate species suggests that the divergence observed in *P. zostericola* is well within the range of that observed for sibling species. For example, reproductive isolation was observed in the fresh-water shrimp *Paratya australiensis* (Cook *et al.* 2006) and marine snapping shrimp *Alpheus armillatus* (Mathews *et al.* 2002) with <5% mitochondrial sequence divergence. Further, mitochondrial divergences of between 2 and 12% are congruent with species delineation for sand prawns (Teske *et al.* 2009), insects (Monaghan *et al.* 2005; Pons *et al.* 2006), gastropods (Duda *et al.* 2008) and echinoderms (Boissin *et al.* 2008). The divergence between the two *P. zostericola* lineages is recent on an evolutionary timescale and does not yet display variation among the highly conserved and slow evolving nuclear genes (MyHC and H3) trialled during this study.

Secondary contact for two divergence lineages

Phycomenes zostericola occur in seagrass meadows (predominantly *Zostera muelleri* – formerly known as *Zostera capricorni*) from northern Western Australia to New South Wales (Bruce 2008). The two *P. zostericola* lineages were sympatric in the north (from Weipa to Bowen) but along the Queensland coast only L1 was caught in seagrasses south of Bowen. Any number of scenarios may have led to the current geographical distribution of the two lineages (Hellberg 1998; Barber *et al.* 2000; Mathews *et al.* 2002; Mathews 2007). Whilst it is possible that the most recent common ancestor of the two lineages was an Australian resident, there is greater evidence supporting the hypothesis that Queensland is the location for post divergence secondary contact between the two *P. zostericola* lineages. The data suggest that L1 arrived much earlier than L2; L1 populations coalesce around 1 Ma, whereas L2 populations appear to have coalesced more recently (Fig. 4). Further, demographic changes evident from neutrality testing show that L2 has a much more recent history along the Queensland coast compared with L1. Such data for each *P. zostericola* lineage supports the hypothesis that L1 arrived to northeastern Australia first and populations expanded prior to the subsequent arrival and expansion of L2 populations. Under this scenario, L2 populations were not capable or are yet to experience environmental conditions suitable for a southern range expansion. Further investigation is required to determine whether L1 has a greater capacity for dispersal and colonisation or has evolutionary adaptations for cooler southern waters. Sympatric sister pairs of the snapping shrimp *Alpheus angulatus* and *Alpheus armillatus* diverged subsequent to the closure of the Isthmus of Panama and are currently in secondary contact (Mathews *et al.* 2002). Although sibling *Alpheus* spp. occupy similar microhabitats they exhibit patterns of habitat partitioning, suggesting that sympatry and niche competition in these two recently diverged species has selected for high levels of behavioural incompatibility (Mathews *et al.* 2002). *Phycomenes zostericola* lineages are similar in that they also occupy the same microhabitat; whether they use spatial or biological resource partitioning or exhibit competitive behaviours is not known and would make a very interesting study.

Demographic differences and similar phylogeographic structure

The two *Phycomenes zostericola* lineages display vastly different demographic histories despite having a similar

intraspecific genetic structure. L2 had slightly less variation (h = 0.89) and a more recent history (τ = 0.14 and 1.69) than that of L1 (h = 0.96, τ = 3.8 and 6.8; Haig *et al.* 2010). Both lineages shared a large population size, very little gene exchange between regions, and high within and among population diversity. The regional structuring between Torres Strait islands and Queensland populations was also similar, observed in the nested clade phylogeographic analysis and supported by analyses of molecular variance (AMOVA and SAMOVA). The pair-wise F_{ST} values for L2 between Torres Strait and Queensland sites (F_{ST} 0.1–0.6) were slightly higher than those observed in L1 (F_{ST} 0.1–0.4; Haig *et al.* 2010), although the same pattern of divergence between the two regions was evident. An isolation-by-distance effect was confirmed using a Mantel test. However, as no L2 individuals were caught between Torres Strait and Townsville it is difficult to determine whether the isolation is a result of divergent onshore currents at 15° S or the effects of vast and rapid sea level fluctuations across a shallow, narrow stretch of continental shelf throughout the Pleistocene (see Haig *et al.* 2010). Sea-level fluctuations separated marine populations east and west of Torres Strait and exposed most of the north Queensland continental shelf (Poore 1994). Such sea-level fluctuations during the Pleistocene were thought responsible for the morphological divergence among marine sponges (Hooper & Ekins 2004) and the genetic divergence within species of portunid crab (*Scylla serrata*, Gopurenko & Hughes 2002), penaeid prawn (*Penaeus monodon*, Benzie *et al.* 1992), asteroid (*Linckia laevigata*, Williams & Benzie 1997), and estuarine fish (*Lates calcarifer*, Chenoweth *et al.* 1998). Both *P. zostericola* lineages show significant divergent structure between Queensland and Torres Strait populations, indicating a source population for Torres Strait outside Queensland waters, possibly in either the Arafura or Coral Seas. Further sampling from both east (in the Coral Sea) and west (Papua New Guinea, Arafura Sea and northern Australia) is necessary to determine not only the potential source of *P. zostericola* populations but also the location of the divergence between the two lineages.

Populations shaped by changing sea levels

Both *P. zostericola* lineages experienced population expansions prior to the last glacial maximum (Haig *et al.* 2010). Data for L2 describes two population expansions, both evident in the network as star-like phylogenies (Slatkin & Hudson 1991). Neutrality tests date the expansions of Torres Strait island populations (clade 3-1) at ~16 ka and Queensland populations (clade 3-3) at ~204 ka. Population expansions coincide with high sea level periods (sea levels estimated from environmental peaks in oxygen isotopes; Lisiecki & Raymo 2005). The demographic expansion of Torres Strait L2 populations (~16 ka) was probably facilitated by a rising sea level and a corresponding increase in available seagrass habitat after the last low sea-level stand, which left Torres Strait completely exposed ~18 ka (Larcombe *et al.* 1995). The apparent expansion and persistence of Queensland populations (>204 ka) throughout periods of lowered sea level strongly suggest that seagrass refugia were present along the central Queensland coastlines.

Latitudinal patterns in MyHC gene

The MyHC gene has proven useful in other phylogeographic studies on caridean shrimp (Williams *et al.* 2002). In this study the MyHC showed only a single nucleotide polymorphism in both lineages, which was not enough variation to either support or contradict the mitochondrial data. In both lineages the northern populations were fixed for adenine and the southern populations for cytosine. There are two plausible explanations for non-random geographic distribution of MyHC alleles. First, the nuclear mutation displays a geographical selection gradient from north to south in both mitochondrial lineages, in which case the mutation either predates the mitochondrial divergence or has occurred recently and independently in both lineages. The second explanation is that the pattern occurred by chance. The MyHC gene is a protein-coding gene responsible for the development and function of muscle fibres (Mogami *et al.* 1986), so a mutation may alter an individual's physical aptitude for movement, for example, to escape from predators. The MyHC gene is a useful indicator of temperature adjustment by populations of fish (Vornanen 1994; Liang *et al.* 2007) and gammarid amphipods (Rock *et al.* 2009), both of which display different MyHC isoforms under different environmental temperature conditions. The early results for *P. zostericola* populations suggest an influence of latitudinal temperature gradients, presenting an exciting opportunity for ocean temperature-based research.

Conclusion

Little is understood about broad-scale historical connectivity or biological diversity among populations of seagrass fauna. The demographic response to sea-level change and habitat fragmentation differs between the two lineages of *Phycomenes*. The results from the nuclear gene may indicate a latitudinal cline for this species but further research is needed on this. Included in climate change predictions is a dubious future for seagrass habitats and communities. There is an increasing and immediate need for a better understanding of seagrass species connectivity

on both ecological and evolutionary scales. This study provides a valuable starting point to a better understanding of the history, connectivity and structure of two permanent and abundant seagrass inhabitants.

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