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Gene flow, colonisation and demographic history of the flat oyster *Ostrea angasi*

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Abstract. The Australian flat oyster *Ostrea angasi* is currently being assessed for its potential as a species for culture in New South Wales. It is considered important to determine the population genetic structure of wild stocks among estuaries before translocation of juveniles (spat) for growout in order to avoid possible deleterious effects of hybridisation of genetically divergent stocks (i.e. outbreeding depression). Five estuaries were sampled in southern New South Wales as well as another four from across the natural range of the species in Australia. Sequence analysis of a 594 base pair fragment of the mitochondrial *cytochrome oxidase I* gene was used to determine the degree of population structuring inferred from pairwise Φ_{ST} estimates and spatial analysis of molecular variance analysis. The analyses revealed that there is no significant genetic differentiation among the sampled New South Wales estuaries ($P > 0.05$) and all eastern samples represent a geographically homogeneous population. This essentially removes any potential constraints on broodstock sourcing and spat translocation within this region. Although levels of differentiation among all sites varied, little divergence was evident across the entire range of the sample. Furthermore, the study revealed extremely low levels of divergence between *O. angasi* and its northern hemisphere congener, *O. edulis*, raising the possibility that *O. angasi* may have only recently colonised Australian estuaries.

Extra keywords: aquaculture, *cytochrome oxidase I*, *Ostrea edulis*, outbreeding depression, population structure.

Introduction

Oysters have been cultured on the east coast of Australia for more than 130 years. The industry has relied primarily on the Sydney rock oyster (*Saccostrea glomerata*) with the only significant exception being the exotic Pacific oyster (*Crassostrea gigas*). Legal farming of *C. gigas* in New South Wales has been confined to Port Stephens where, and only since 1991, local growers were given special dispensation to do so. Elsewhere in New South Wales, Queensland and Victoria, the Pacific oyster is considered a noxious species. The total annual production of farmed *S. glomerata* in New South Wales has markedly declined from a peak level of ~160 million oysters in the mid 1970s to ~100 million in 1990, a level at which it has since stabilised. Major reasons for the 40% decline in production between the mid 1970s and 1990 and lack of growth since have been an escalation of costs of production (especially labour) above farm-gate prices and periodic outbreaks of two major diseases, QX disease and winter mortality, both of which can cause episodic losses of up to 80% (Nell 2001). One way to address the constraints to a re-expansion of the industry is to diversify production into alternative high-value oyster species that may be less vulnerable to disease.

The flat oyster *Ostrea angasi* (Sowerby, 1871) appears to be a good candidate for diversification for several reasons. During pilot commercial farming since 1998 (Heasman and Lyall 2000), the species seems to be less vulnerable to major diseases, displays rapid growth (potentially twice as fast as *S. glomerata*) and has a high market value (up to 50% higher farm-gate market value than *S. glomerata* in 2002–2003) (Heasman *et al*. 2004). Furthermore, *O. angasi* is considered an Australian endemic.

Unlike rock oysters that release their gametes into the water column where fertilisation takes place, *O. angasi* brood larvae in their mantles. *Ostrea angasi* larvae are released when they average 170–180 μm and attain competence to settle and metamorphose at a mean size of 320–340 μm (Hickman and O'Meley 1988). Developmental times for larvae vary with temperature. Required periods reported for hatchery-reared larvae are from 12 to 15 days at 21°C (Hickman and O'Meley 1988) and from 9 to 14 days at 25°C (M. P. Heasman, unpublished data). A shorter period of 7 days

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was reported for *O. edulis* larvae reared at 20–22°C (Utting and Spencer 1991).

Dispersal among populations of sessile marine organisms relies largely on the passive dispersal capability of pelagic larvae on ocean currents (Brown 1991). Therefore, an abbreviated planktonic phase should result in a reduced passive dispersal capacity for *O. angasi* compared with broadcast spawners such as *C. gigas*, whose larvae may remain in the water column for two to three times longer. It should be noted, however, that long-distance dispersal can be achieved by sessile marine organisms via the process of rafting (an individual attaching to a free-floating substrate). Foighil *et al*. (1999) invoked rafting to explain the trans-Pacific distribution of the flat oyster *O. chilensis*. Nevertheless, a growing number of genetic studies are revealing that many marine taxa with a planktonic larval phase do not fully realise their dispersal potential. That is, the extent of potential dispersal estimated from the duration of the larval phase and the velocity of ocean currents is not supported by the distribution of alleles (e.g. Barber *et al*. 2002; Gilg and Hilbish 2003; Taylor and Hellberg 2003 and references therein). Whether larval duration and the nature of ocean currents adjacent to the study region (the East Australian Current that flows in a southerly direction) have the potential to homogenise allele frequencies among estuaries along the south-east Australian coastline is unknown for *O. angasi*. A phylogeographic study on the scleractinian corals using nuclear *ITS1–2* ribosomal DNA (rDNA) suggests highly restricted dispersal among south-east Australian populations (Rodriguez-Lanetty and Hoegh-Guldberg 2002), indicating that passive planktonic dispersal may somehow be limited in this region. Similarly, an allozyme study of a muricid snail shows significant structure across the range of the *O. angasi* sample sites in southern New South Wales (Hoskin 2000). Although this species displays direct larval development (i.e. non-planktonic), extensive adult rafting has been invoked to explain its widespread distribution and hence a reliance on oceanic currents for dispersal. Interestingly, the greatest level of population structuring occurs on the southern New South Wales coast, once again providing evidence that factors can limit ocean current dispersal in this region.

With restricted dispersal/gene flow, populations are likely to differentiate genetically as a consequence of the rise of new mutations, the effects of random genetic drift and natural selection owing to adaptations to local environments. Therefore, owing to specific life-history traits (i.e. larval brooding, sessile adult phase), *O. angasi* may be expected to display some degree of genetic structuring, even over relatively small geographic distances. Studies using the mitochondrial DNA (mtDNA) *12S* gene (Diaz-Almela *et al*. 2004) and microsatellite markers (Launey *et al*. 2002) found significant genetic differentiation in the northern hemisphere congeneric *O. edulis* among Atlantic and Mediterranean populations over similar spatial scales to that examined in the present study.

Furthermore, dispersal over the entire range of *O. angasi* across southern Australia in a single generation is highly improbable. Therefore, a signature of isolation by distance among geographically separated stocks may be anticipated, where geographic distance is positively correlated with genetic distance (Slatkin 1993). A slight isolation by distance effect was detected across the range of *O. edulis* (Diaz-Almela *et al*. 2004).

The presence of stock structure in aquaculture species has implications for culture production. Marine aquaculture generally relies on sourcing of broodstock from one or a few locations, followed by the widespread translocation of juveniles (spat) to growout facilities in suitable habitats (usually within the natural range of the species). It is therefore desirable to determine the stock structure of the species in the wild before translocations. This is in order to avoid potentially deleterious consequences of escapes from culture (generally considered to be inevitable (Cross 2000)), and the possibility of culture stocks interbreeding with local wild populations (Youngson *et al*. 2001), hence mixing divergent stocks. When genetically divergent stocks interbreed, a decline in fitness in the population may result (i.e. outbreeding depression) owing to either a deviation of the hybrid phenotype from local optima (Allendorf and Waples 1996) or through the disruption of locally co-adapted gene complexes (Templeton 1986). Therefore, if significant genetic structure is present in *O. angasi* populations, this attribute would need to be considered when spat were produced in hatcheries for wild growout so as to avoid the potential for contaminating wild gene pools with non-indigenous genes.

The specific aim of the present study, therefore, was to determine the genetic structure of *O. angasi* populations in southern New South Wales in order to make more informed management decisions about how broodstock should be sourced from the wild for culture and subsequent translocation of spat for growout. If structure is detected among estuaries, then separate broodstocks will be needed from each discrete wild population and the resulting spat should only be stocked in their natal locations. This would significantly raise the costs and effort required in producing spat for culture. Conversely, if no structure is found, these constraints on hatchery production are largely removed.

Rapidly evolving mtDNA has been used extensively to determine if localised populations are genetically differentiated from each other, and the approach has been applied successfully in the aquaculture industry (Liu and Cordes 2004). A smaller effective population size owing to maternal inheritance and a haploid nature dictates that populations will diverge faster for mtDNA genes than for nuclear genes. Therefore, if structure exists, a mtDNA marker, being more sensitive to genetic drift, will be likely to detect it. An added advantage of mtDNA sequence analysis is that it can be informative with respect to the history of a population/species so that questions may be addressed relating to how long extant

populations have been separated and if there have been historical fluctuations in population demography (e.g. population declines/expansions).

Materials and methods

Sampling

Samples were collected from five estuaries in southern New South Wales (Fig. 1). These sites were Bateman's Bay, Narooma, Bermagui, Merimbula and Pambula. To determine the population structure, between 14 and 18 samples from each of these estuaries were used in the study (specific sample sizes are given in Table 1). Additionally, to place the level of genetic differentiation among New South Wales estuaries into context, oysters were also collected from four additional sites representing the wider natural range of *Ostrea angasi* in Australia. These sites were Port Phillip Bay in Victoria, Bicheno in Tasmania, Streaky Bay in South Australia and Albany in Western Australia (Fig. 1).

DNA extraction, polymerase chain reaction and sequencing

Total genomic DNA was extracted using a protocol modified from Doyle and Doyle (1987). The specific protocols used are outlined in Hurwood and Hughes (1998). A fragment of the mitochondrial *cytochrome oxidase I* (*COI*) gene was amplified using the following polymerase chain reaction protocol. Primers LCO-1490 and HCO-2198 (Folmer *et al*. 1994) were used to amplify ~670 base pairs (bp). Reactions contained 30 nmol each of dATP, dGTP, dCTP and dTTP (Roche, www.roche-applied-science.com/, verifed October 2005), 2 units of *Taq* DNA polymerase (Roche), 2.5 mmol $MgCl_2$, 5 μL of 10× polymerase reaction buffer (Roche), 1.0 μmol of each primer, 0.2 μg of template DNA, 1.0 μL of dimethyl sulfoxide (DMSO) and adjusted to a final volume of 50 μL with distilled and deionised water (ddH_2O). DNA was initially denatured at 95°C for 5 min, then 15 cycles of 95°C denaturing for 30 s, 40°C annealing for 30 s and 72°C extension for 1 min. The annealing temperature was then raised to 55°C for another 20 cycles with denaturing and extension temperatures as before. All samples were sequenced using the light strand primer (LCO-1490) only, whereas six samples were sequenced for both strands to verify correct amplification of the gene fragment. DNA sequencing was undertaken by the Australian Genome Research Facility, Brisbane, Australia, using an ABI 377 automated sequencer (Amersham Biosciences, Little Chalfont, UK).

Statistical analysis

Relationships among mtDNA haplotypes were displayed using a minimum spanning cladogram estimated in the TCS programme (Clement *et al*. 2000), which provides the 95% parsimoniously plausible branch connections between haplotypes. Two methods were employed to determine the level of genetic structuring among samples. First, the proportion of genetic variation among pairs of estuaries (Φ_{ST}, an analogue of F_{ST}) was estimated using ARLEQUIN Version 2.000 (Schneider *et al*. 2000). This method incorporates genetic variation arising from both haplotypic frequency differences among sampling sites and levels of sequence divergence among haplotypes. Divergence among haplotypes was determined using the K2P (Kimura 1980) distance method. The Exact Test of Raymond and Rousset (1995) was used to establish whether Φ_{ST} values were significantly larger than zero. As multiple tests increase the risk of Type I error, a Bonferroni correction is sometimes applied to pairwise Φ_{ST} analysis to adjust the α-level. However, the Bonferroni procedure inflates the probability of committing Type II errors and it has been argued that it lacks biological justification (Moran 2003). Therefore, any genetic structure that is present may be incorrectly discarded after correction for multiple comparisons. Instead, the Bernoulli equation was implemented to assess the probability of the observed frequency of significant Φ_{ST} values occurring by chance (Moran 2003).

The second method of determining geographical structure of the samples involved the spatial analysis of molecular variance (SAMOVA) method of Dupanloup *et al*. (2002), which groups populations that are geographically homogeneous. This analysis uses a simulated annealing approach to maximise the proportion of the total genetic variation among groups of population (F_{CT}).

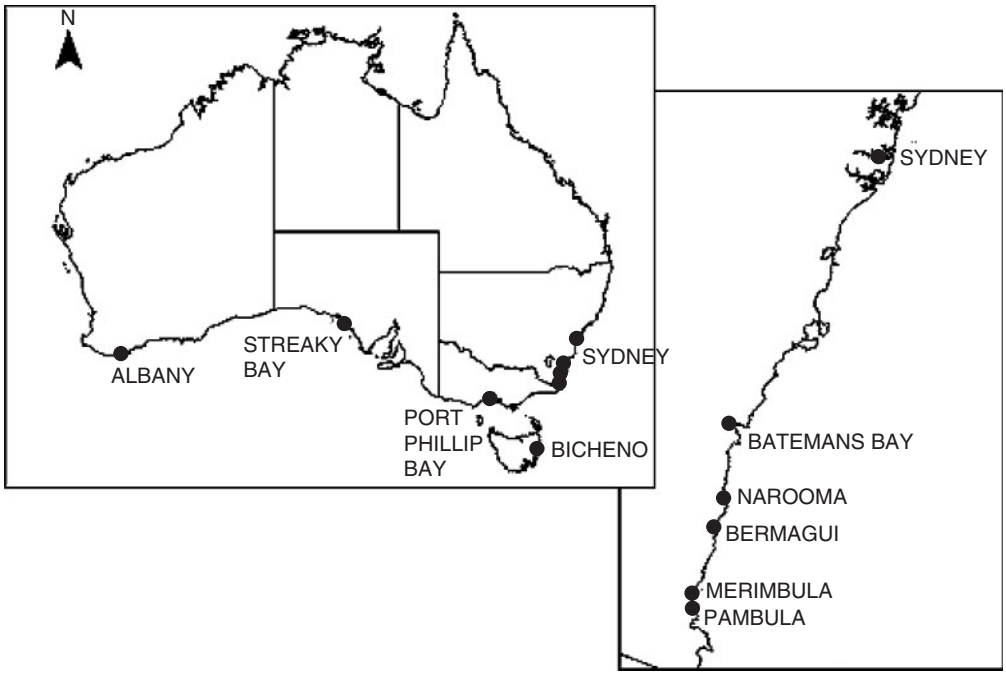

Fig. 1. Map of Australia showing sampling sites for *Ostrea angasi*. Inset shows sampling sites in southern New South Wales.

Table 1. Haplotype frequency and sample size per sampling site for *Ostrea angasi*

	BB $n=18$	Na $n=14$	Be $n=16$	Me $n=15$	Pa $n=18$	PPB $n=5$	Bic $n=6$	SB $n=5$	Al $n=4$
Oa1	5	4	3	5	2	2	2	2	
Oa2			6	3	3				
Oa3	8	5	4	4	8		1		1
Oa4					1				
Oa5	1				1				
Oa6					1				
Oa7					1				
Oa8	2								
Oa9	1								
Oa10		2			1				
Oa11		1							
Oa12	1								
Oa13								1	
Oa14								1	
Oa15									1
Oa16								1	
Oa17									1
Oa18									1
Oa19						1			
Oa20						1			
Oa21						1			
Oa22							1		
Oa23							1		
Oa24				1			1		
Oa25				1					
Oa26				1					
Oa27		1							
Oa28		1							
Oa29			1						
Oa30			1						
Oa31			1						

BB, Bateman's Bay; Na, Narooma; Be, Bermagui; Me, Merimbula; Pa, Pambula; PPB, Port Phillip Bay; Bic, Bicheno; SB, Streaky Bay; Al, Albany.

To determine whether a signature of isolation by distance was evident across the range of *O. angasi*, a Mantel's test (Mantel 1967) was performed in ARLEQUIN. This test was used to estimate the significance of the correlation between pairwise genetic distance among sampling sites using Slatkin's linearised F_{ST} (Slatkin 1995) and $\log_{10}$ straight line geographic distance among sites. A pattern of isolation by distance would be supported by a positive correlation between genetic differentiation and geographic distance. The test incorporated 1000 permutations.

Demographic history was investigated by determining the distribution of the number of pairwise differences among individuals. Commonly referred to as a mismatch distribution (Harpending *et al*. 1993; Rogers 1995), this method can differentiate between populations that have remained stable over time and those that have expanded rapidly in the relatively recent past. The mismatch distribution was generated using DnaSP Version 4.00 (Rozas *et al*. 2003) with 99% confidence intervals calculated using ARLEQUIN. The expansion model is based on three parameters: θ_0 (expected mean pairwise differences before an expansion), θ_1 (expected mean pairwise differences after an expansion), and τ (the date of the expansion measured in units of mutational time ($\tau = 2ut$; t is the time in generations, and u is the mutation rate per sequence and per generation)) (Rogers and Harpending 1992). The relationship between τ and generation time allows an estimate of the timing of any inferred demographic change in the population. Various mutation rates for invertebrates have been calibrated for the *COI* fragment used in the present study. There is no specific molecular clock for *Ostrea* species; consequently, the calibration of marine bivalve molluscs reported by Marko (2002) (ranging from 0.67 to 1.2% per million years) was used to convert units of mutational time into the number of years since any inferred demographic fluctuation occurred. Conformity to the population expansion model was tested further using 1000 coalescent simulations in order to test the significance of both the R_2 statistic of Ramos-Onsins and Rozas (2002) and Fu's (1997) F_S statistic in DnaSP. R_2 is based on the difference between the number of singleton mutations and the average number of nucleotide differences whereas F_S is based on Ewens' (1972) sampling distribution of θ predicting high numbers of singleton mutations as a result of an expansion. R_2 is expected to be significantly small and F_S significantly negative if a population expansion has occurred.

Results

A total of 101 individuals from nine sampling sites were sequenced. Of the 670 bp of *COI* amplified, 594 bp were used for further analysis in the present study. Sequencing identified 30 variable sites resulting in 31 unique haplotypes (sequences submitted to GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html, verified October 2005) with accession

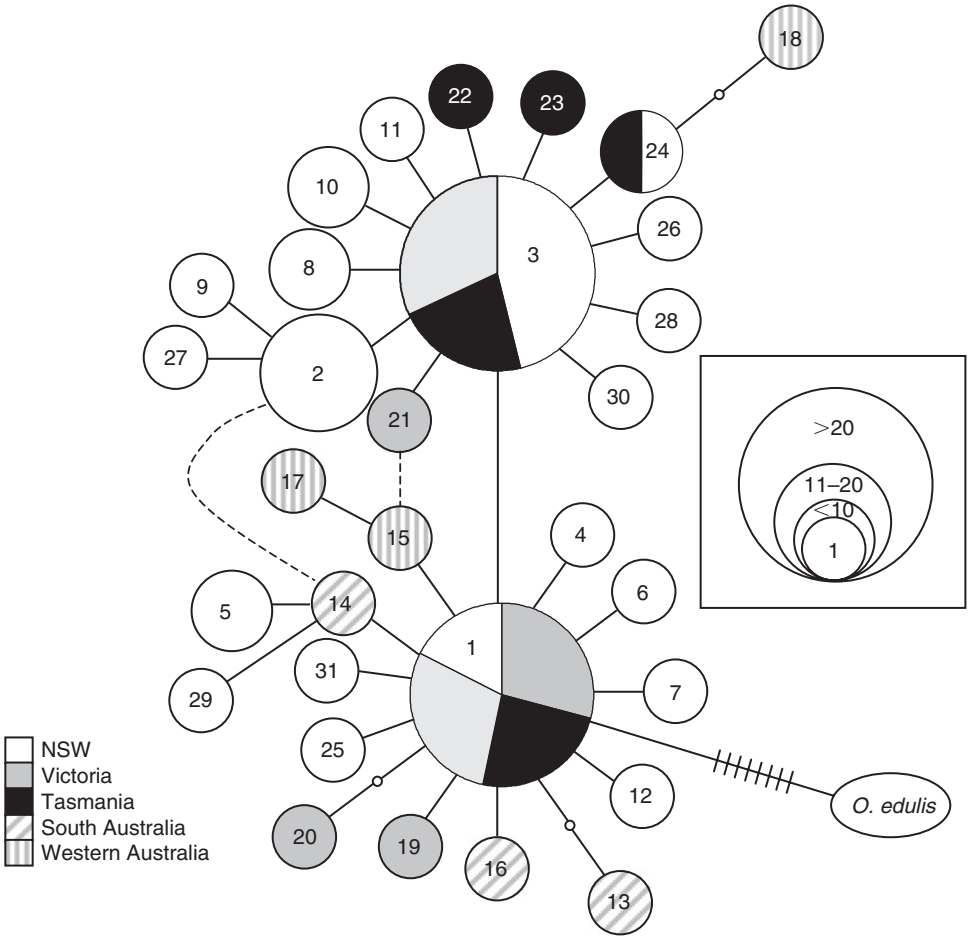

Fig. 2. Reconstructed haplotype network for *Ostrea angasi* haplotypes (circles) and for *O. edulis* (ellipse). Solid lines represent single base pair differences among haplotypes and dashed lines indicate alternative connections. Size of the circles represents the frequency of each haplotype in the sample and pie segments indicate the relative frequency per sampling site. The smallest circles represent hypothesised haplotypes that were not detected in the sample. The line with crosshatching between Oa1 and *O. edulis* (GenBank accession number AF120651) represents eight bp differences.

numbers DQ078638–DQ078668). All point mutations were transitions with 27 at the third codon position whereas the remaining four were first codon position mutations. Of these four, only one mutation altered the amino acid sequence. The number of bp differences among individual haplotypes is displayed in a minimum spanning network (Fig. 2). Six haplotypes were found in more than a single sampling site (Oa1, Oa2, Oa3, Oa5, Oa10 and Oa24) whereas the rest were site specific (Table 1). Of these six, haplotypes Oa2 and Oa10 were detected in New South Wales sites only. Both haplotypes Oa1 and Oa3 were widespread across much of the range of the species.

The global Φ_{ST} revealed that a significant proportion of the genetic variation detected in the present study occurred among sampling sites ($\Phi_{ST} = 0.1483$; $P = 0.004$). Levels of genetic differentiation among sampling locations (pairwise Φ_{ST}) indicated that no structure was evident among the five estuaries on the southern coast of New South Wales (Table 2). Pairwise comparisons among more distant sites showed varying levels of differentiation that is not apparently correlated with geographic distance. For example, the Port Phillip Bay sample was significantly differentiated from almost all New South Wales sites, yet was not differentiated from Albany in Western Australia. Overall, 15 out of 36 pairwise comparisons showed a significant Φ_{ST}. The probability of this outcome occurring by chance owing to multiple comparisons is remote ($P = 5.9 \times 10^{-11}$ according to the Bernoulli equation (Moran 2003)). This result confirms that the population structure observed is real, at least among some sites.

Given the above pattern of differentiation, it is not surprising that the Mantel's test revealed no correlation between genetic and geographic distance ($r = -0.058$; $P = 0.641$). However, the SAMOVA analysis identified three population groupings ($F_{CT} = 0.19482$; $P < 0.001$). The first group contained all New South Wales sites together with the Tasmanian and Victorian populations. The South Australian

Table 2. Pairwise Φ_{ST} estimates (lower diagonal) and levels of significance (upper diagonal) among all sites for *Ostrea angasi*

	BB	Na	Be	Me	Pa	PPB	Bic	SB	Al
BB	–	ns	ns	ns	ns	*	ns	*	*
Na	−0.0084	–	ns	ns	ns	*	ns	**	*
Be	0.0500	0.0664	–	ns	ns	*	ns	**	*
Me	−0.0277	−0.0070	0.0035	–	ns	ns	ns	*	ns
Pa	−0.0211	−0.0188	−0.0053	−0.0372	–	*	ns	*	*
PPB	0.1362	0.1528	0.2178	0.1174	0.1572	–	ns	ns	ns
Bic	−0.0201	−0.0258	0.0828	−0.0280	−0.0070	0.0409	–	*	ns
SB	0.2119	0.2705	0.2531	0.1921	0.2176	0.0003	0.2052	–	**
Al	0.1636	0.1614	0.2196	0.1436	0.1660	−0.0530	0.0293	0.1406	–

BB, Bateman's Bay; Na, Narooma; Be, Bermagui; Me, Merimbula; Pa, Pambula; PPB, Port Phillip Bay; Bic, Bicheno; SB, Streaky Bay; Al, Albany; ns, not significant; *$P < 0.05$; **$P < 0.01$.

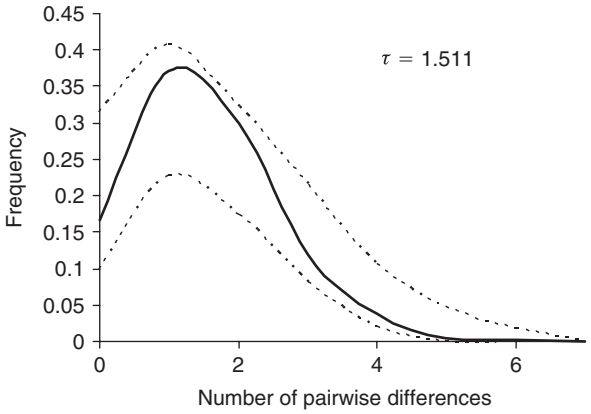

Fig. 3. Mismatch distribution of the number of pairwise differences among all samples for *Ostrea angasi*. Solid line represents the observed frequency and dashed lines are the 99% confidence interval under the population expansion model.

and Western Australian populations each represented a group on their own. This result supports the idea that geographic distance may play a role in the genetic structuring of the species across its natural range.

All methods used to test for historical demographic fluctuations revealed strong support for a rapid population expansion with a smooth curve in the mismatch distribution (Fig. 3). Coalescent simulations showed that the probability of getting a smaller R_2 (0.0240) or a more negative F_S (−33.893) by chance was extremely small ($P < 0.001$ for both estimates). The application of molecular clock estimates with a value for $\tau = 1.511$ suggests that an expansion occurred Australia-wide ~320 to 570 thousand years ago. Furthermore, a very significant negative value for F_S indicates high interdeme migration at the time of expansion and therefore supports a geographical range expansion (Ray *et al*. 2003).

Discussion

There are no reliable methods that will determine the likely consequences of hybridisation between populations/taxa with a high degree of certainty owing to the complex nature of gene interactions. The simplest indicators, however, relating to the potential beneficial or detrimental effects of outcrossing appear to be either genetic or geographic distance (Edmands and Timmerman 2003). The levels of genetic differentiation revealed in the current study provide evidence that the geographically proximate sampling sites in New South Wales represent a single panmictic population. From a management perspective, therefore, the present study has provided no evidence to suggest that broodstock origin and translocation of *Ostrea angasi* spat for growout would create potential problems associated with outbreeding, at least in New South Wales. It should be stressed, however, that these results reflect population structure determined by a single locus. A multilocus analysis (including nuclear genes) would be required to verify the conclusions arising from the present study.

Given the life history traits of *O. angasi* and the apparent limit to dispersal seen in other planktonic species in the region, it is surprising that such low levels of genetic differentiation were detected across the range of the species in southern Australia. Although levels of population differentiation were non-significant in New South Wales, as well as with certain other pairwise comparisons from more geographically distant estuaries suggesting a moderate degree of gene flow among all sites (Table 2), it should be noted that the majority of shared haplotypes were internal in the minimum spanning network (Table 1; Fig. 2). Under coalescent principles, internal haplotypes in a cladogram are likely to represent ancestral types (Crandall and Templeton 1996) whereas tip haplotypes are considered to be more recently derived. If the observed pattern of low genetic differentiation was owing to moderate levels of contemporary gene flow, then it may be expected that some tip (recently derived) haplotypes would be detected in more than a single site. The only occurrence of this was haplotype Oa10 found in Narooma and Pambula (Table 1), suggesting that gene flow does occur on a relatively small geographic scale among New South Wales estuaries. However, the low degree of genetic differentiation

between Albany in the west and some eastern samples, combined with what is known about *O. angasi* life history traits, is problematic. A more likely scenario is that this pattern may be explained by a historical dispersal event or range expansion as suggested by the demographic analysis. However, low statistical power to detect significant differentiation owing to insufficient sampling cannot be discounted.

The signature of population expansion, possibly associated with a range expansion, indicates that gene flow may have been widespread in the relatively recent evolutionary past of *O. angasi*. The estimate of an expansion in the late Quaternary coincides with significant fluctuations in sea level and climatic conditions in southern Australia (Twidale 1980). Although speculative, these factors may have provided habitat more suitable for *O. angasi* and provided oceanic conditions more favourable for the facilitation of widespread gene flow.

Another consideration in attempting to explain the unexpected lack of genetic differentiation across vast geographic distances for an essentially sessile organism is the possibility of human-mediated dispersal. The potential for inadvertent dispersal of marine species (especially species with a planktonic larval phase) via ballast water in ocean-going vessels is immense (Carlton and Geller 1993). Indeed, *O. angasi*'s northern hemisphere congener, *O. edulis*, has recently been detected in Western Australia (Morton *et al*. 2003). If this temperate species has been able to survive a trans-equatorial translocation and establish a population in Australia, then it is conceivable that there could be extensive gene flow among Australian ports. If it is assumed that structure existed before recent translocation (at least between the east and west coasts of Australia), then it may be expected that an admixture of divergent lineages would be evident in the data, which was not the case. However, several processes such as historical founder events or bottlenecks and natural selection may slow the accumulation of deep mtDNA lineages (Grant and Bowen 1998).

Further to the detection of *O. edulis* in Australia, it is interesting to note the level of genetic differentiation between this species and *O. angasi*. Sequence data from a sample of *O. edulis* from Europe (GenBank accession number AF120651; Giribet and Wheeler 2002) showed only eight bp differences from *O. angasi* haplotype Oa1. The degree of differentiation among species is of the same magnitude as seen among the entire Australian *O. angasi* sample (i.e. ~1% divergence). Additionally, a study on genetic diversity of *O. edulis* across its natural range in Europe (Diaz-Almela *et al*. 2004) using the more slowly evolving mitochondrial *12S* gene showed up to 3% divergence among sites. These data suggest that some samples of European *O. edulis* are more closely related to *O. angasi* than they are to other conspecifics. A simple supposition is that these two taxa could be, in fact, the same species. The same hypothesis was proposed by Kenchington *et al*. (2002) who compared nuclear ribosomal gene fragments. Unresolved taxonomy could present serious implications for the development of *O. angasi* as a culture species in Australia. These taxonomic inconsistencies are currently under investigation.

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