

Lack of genetic introgression between wild and selectively bred Sydney rock oysters *Saccostrea glomerata*

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ABSTRACT: Sydney rock oysters *Saccostrea glomerata* are among the most important estuarine species on the eastern coast of Australia and also the basis of a major aquaculture industry. The industry now largely relies on Sydney rock oysters that have been selectively bred for fast growth and disease resistance. Selectively bred *S. glomerata* are currently farmed in estuaries that also sustain wild populations of Sydney rock oysters, providing the opportunity for interbreeding. This has led to concern that gene flow from farmed, selectively bred oysters could alter the genetic variability of wild oyster populations. Here, we use next-generation genotype-by-sequencing to test for genetic introgression between wild and farmed, selectively bred (B2 line) Sydney rock oysters from 2 sites in the Georges River, NSW. Strong genetic partitioning was identified between the wild and selectively bred populations. There was no evidence of gene flow in the form of introgression, even though selectively bred B2 oysters have been farmed in the Georges River since the early 1990s. Contrary to our expectations, we also found significantly higher levels of genetic diversity and heterozygosity in the selectively bred population relative to the wild population. The relatively low level of genetic diversity that we detected in wild oysters may be particularly relevant to the adaptive capacity of this species in the wild.

KEY WORDS: Gene flow · Aquaculture · Selective breeding · Population genetics

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INTRODUCTION

Oysters are environmentally and economically important organisms (Schroback et al. 2014). They form the structural and trophic foundations of coastal environments worldwide, sustaining significant levels of biodiversity and productivity (Newell 2004, Coen et al. 2007, Grabowski & Peterson 2007, Beck et al. 2011). Oyster reefs in coastal estuaries were historically extensive. However, more than 85% of oyster reefs have now been lost globally (Beck et al. 2011). These declines are due to increased coastal development, the degradation of water quality and excessive harvesting of oysters (Grabowski & Peterson 2007). Past human exploitation of natural oyster beds has selectively harvested the largest and pre-

sumably fastest-growing individuals (Gaffney 2006). As a result, it is possible that any remnant oyster populations have also been depleted of genes for fast growth and resilience (Allendorf et al. 2008). Some evidence suggests that wild oyster populations have decreased resilience to environmental stressors (Lenihan et al. 1999, Butt et al. 2006) and are more susceptible to disease relative to historical levels (Lenihan et al. 1999, Beck et al. 2011). If true, this depletion of natural resilience in wild oyster populations may have reduced their capacity to deal with the fast pace of environmental change resulting from global climate change and increasing human development in coastal areas. By corollary, the decline of natural oyster populations and the associated loss of essential ecosystem services from coastal estuaries could have

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increasingly severe environmental and ecological impacts.

In southeastern Australia, Sydney rock oysters *Saccostrea glomerata* Gould, 1980 are the most common native oyster species (Nell 2001). Their natural range is extensive (see Fig. 1B), covering the length of the Australian east coast from Victoria to Queensland, and around the northern coast as far as Western Australia (Nell 2001). However, Sydney rock oyster populations are now classified as functionally extinct in the wild, meaning they have declined in abundance to the point that they can no longer provide ecosystem services such as reducing turbidity, improving water quality and cycling nutrients (Coen et al. 2007, Beck et al. 2011, Diggles 2013). Despite this decline of wild populations, large numbers of Sydney rock oysters are now actively farmed in the states of New South Wales (NSW) and southern Queensland (see Fig. 1B). Sydney rock oyster farming is the largest aquaculture industry in NSW, with commercial farming dating back to 1870 (Banks et al. 2006, O'Connor & Dove 2009). Sydney rock oysters currently comprise over half of the edible oysters produced in Australia each year, with the annual harvest exceeding 96 million oysters worth AUD\$100 million (Banks et al. 2006, O'Connor & Dove 2009, Schrobback et al. 2014). Oyster spat (juveniles) for farming were traditionally caught within each growing area, and were often translocated between estuaries. However, the development of hatchery technology now provides a more centralised source of spat for the Sydney rock oyster aquaculture industry. Hatchery production has also enhanced selective breeding programmes that have been in operation since 1990 (Nell 2001, Nell & Hand 2003). These selective breeding programmes initially aimed to increase the growth rates of Sydney rock oysters via mass selection (Nell & Hand 2003). In 1997, the programme was expanded to include selection for disease resistance, due to major outbreaks of 2 diseases (winter mortality and QX disease) that heavily impacted commercial production of Sydney rock oysters (Nell & Hand 2003, Simonian et al. 2009). The hatchery-reared 'B2' line is now the main commercial product from these breeding programmes. It has been mass selected for resilience against QX disease and winter mortality over 6 generations.

The genetic relationships between farmed Sydney rock oysters and their wild conspecifics are likely to have been substantially altered by industry practices of hatchery production and selective breeding. Increased reliance on hatchery-produced spat means that new recruits for oyster crops in particular grow-

ing areas are no longer sourced locally. It is also likely that differences in genetic constitution between farmed and wild oysters are continuing to increase because most hatchery-produced spat are now produced by selective breeding programmes that have substantially altered many traits. Selectively bred Sydney rock oysters grow faster than wild oysters (Nell & Perkins 2005), have developed dual resistance to the 2 major infectious diseases (Nell & Perkins 2006) and are more resilient to a range of environmental stressors (Parker et al. 2011, 2012). These selectively bred oysters are currently farmed in growing areas that also contain wild oyster populations. As oysters reproduce by non-selective broadcast spawning (Parker et al. 2009), the coexistence of wild and selectively bred oysters provides the opportunity for interbreeding. This is of concern, as the resulting gene flow has the potential to alter the genetic variability and structure of wild populations.

The potential impacts of such gene flow remain unknown. Selective breeding programmes generally have small founder population sizes and, therefore, have increased potential for inbreeding depression due to high levels of relatedness between individuals. This can decrease levels of genetic variation and fitness for traits other than those under active selection (English et al. 2001, Green et al. 2009). If gene flow is occurring between wild and farmed oysters, reduced genetic variation within the farmed, selectively bred populations could be transferred into wild populations, with potentially widespread consequences at the ecosystem, community and population levels (Hughes et al. 2008). Alternatively, gene flow could transfer beneficial alleles for fast growth, disease resistance and environmental resilience from the farmed, selectively bred populations into local wild populations (Parker et al. 2012). Introgression of these beneficial genes could help to protect wild populations against QX disease, winter mortality, ocean acidification and potentially other factors associated with environmental change.

Introgression has been previously observed from domesticated animals into closely related wild populations, with evidence for both beneficial and detrimental outcomes. In the marine environment, introgression between wild and escaped Atlantic salmon *Salmo salar* from aquaculture farms has been widely studied. Decreased growth at some temperatures (Harvey et al. 2016) and decreased fitness due to altered maturation patterns (Yates et al. 2015) was detected among hybrids of wild and farmed salmon. In contrast, increased genetic diversity was evident

in salmon from the Gulf of Finland that had interbred with escaped aquaculture stock (Ozerov et al. 2016). Aside from farmed fish species, the majority of studies examining the effects of cultured or genetically altered organisms on wild populations have focused on terrestrial ecosystems, mainly involving crop species (Stewart et al. 2003, Warwick et al. 2009). Among the terrestrial animals studied, the transfer of a major histocompatibility complex allele from domestic goats to the Alpine ibex *Capra ibex* in Switzerland has been shown to increase immunity in the wild ibex population (Grossen et al. 2014). In contrast, hybridisation between domestic dogs and the grey wolf *Canis lupis* in Italy (Randi et al. 2014), and between domestic cats and the European wildcat *Felis silvestris silvestris* in Scotland (Beaumont et al. 2001) and Hungary (Pierpaoli et al. 2003), has reportedly reduced the natural genetic variation in the corresponding wild populations.

Little research has assessed the possible deleterious impacts of hatchery-produced or selectively bred oysters on the genetic structures of local wild oyster populations. Similarly, few studies have assessed the potentially beneficial use of selectively bred oysters in ecological restoration projects, even though hatchery-produced spat are already being used to boost population sizes in areas where wild oysters have declined (Kingsley-Smith et al. 2009). Some oyster restoration projects in the United States already use hatchery-produced, selectively bred Eastern oysters *Crassostrea virginica*, although their impact on the wild population is largely unknown (Carlsson et al. 2008).

To rectify the lack of relevant population genetic data for *S. glomerata*, the current study uses next-generation genotype-by-sequencing to investigate the genetic structures of wild Sydney rock oyster populations and their relationships to the sympatric, selectively bred B2 line. The data are used to test the hypothesis that gene flow resulting in genetic introgression has occurred between the farmed and wild populations of Sydney rock oysters in a single coastal estuary where selectively bred oysters have been farmed since 1990 (Nell et al. 2000).

MATERIALS AND METHODS

Collection of oyster samples

Sydney rock oysters *Saccostrea glomerata* were collected from 2 sites in the Georges River, New South Wales; Woollooware Bay ($34^{\circ}02'14.2''$ S $151^{\circ}08'51.5''$ E) and Quibray Bay ($34^{\circ}01'29.7''$ S $151^{\circ}10'50.3''$ E; Fig. 1A). Both sites experience QX disease outbreaks each year. Woollooware Bay is more heavily affected by QX disease than Quibray Bay, and Quibray Bay also experiences outbreaks of winter mortality (Nell 2006). Wild spat caught within the estuary have been farmed in the Georges River since the 1880s. Selectively bred oysters, including the B2 line and its progenitors, have been grown at both sites since 1990 (Nell et al. 2000). Approximately 85% of the oysters currently farmed at the 2 sites are selectively bred B2 oysters, with the remainder wild caught within the Georges River estuary (M. Dove, NSW Department of Primary Industries [DPI], pers. comm.).

Three groups of oysters (comprising 12 oysters per group) were collected within each of the 2 bays. The

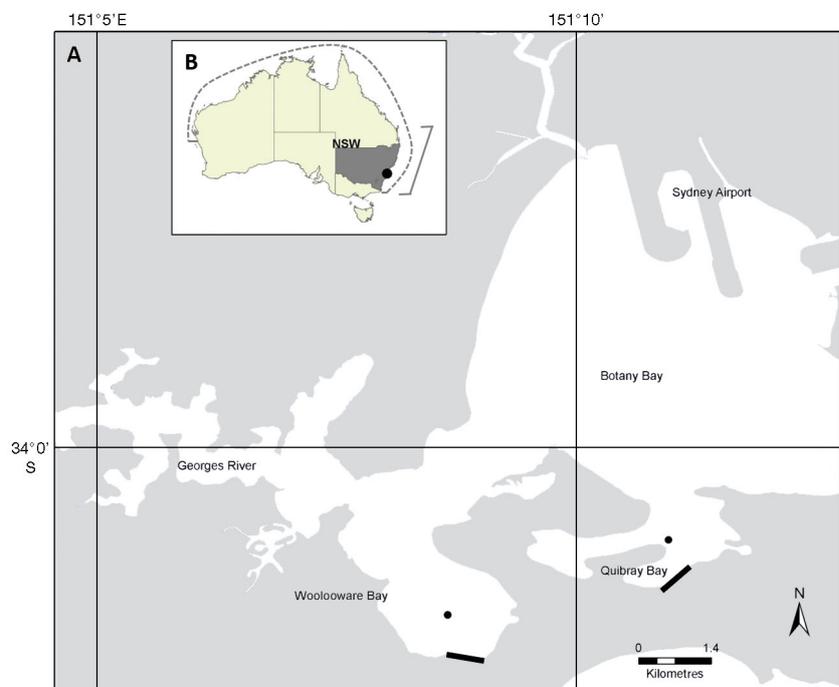


Fig. 1. Map of the 2 sampling sites in the Georges River estuary, New South Wales: Quibray Bay and Woollooware Bay. (A) Black circles indicate farming leases where B2 and overcatch oysters were collected and black bars indicate the corresponding areas of shoreline where wild-caught oysters were collected. (B) Dotted line indicates the natural distribution of Sydney rock oysters in Australia and solid line indicates the major farming area (image courtesy of New South Wales Department of Primary Industries)

3 groups from each bay were; (1) wild oysters collected from a 10–20 m stretch of shoreline approximately 1 km from oyster farming leases (designated wild-caught, WC); (2) oysters collected from sticks or pylons supporting oyster farming racks (designated overcatch, OC); (3) selectively bred, 6th generation B2 oysters from within the farming racks (designated B2). The progenitors of the B2 line were established from just 140 parents, taken from the Georges River and 3 other rivers in northern NSW that had been affected by severe annual outbreaks of QX disease (Nell 2006). The B2 oysters collected from the Georges River were kindly supplied by NSW DPI.

In addition to oysters from the Georges River, 2 reference groups were also provided by NSW DPI. These were hatchery-reared 6th generation B2 (designated hatchery B2, HB2, $n = 11$) and 'control' oysters (designated hatchery controls, HC, $n = 10$) grown in Port Stephens, New South Wales ($32^{\circ}44'12.5''$ S $152^{\circ}3'18.9''$ E). The Port Stephens estuary is approximately 170 km north of the Georges River estuary and has never experienced outbreaks of QX disease or winter mortality (Nell 2006). The hatchery B2 oysters were of the same stock as those collected in the Georges River (M. Dove, NSW DPI, pers. comm.). The hatchery controls were 6th generation progeny of the same wild oysters used as the founder population of the B2 selective breeding line. Unlike the B2 line, HC oysters have never been subjected to selective breeding.

Tissue samples from all oysters ($n = 93$) were collected in February 2015. After shucking, 5×15 mm portions of gill tissue were excised, immediately placed in 95% ethanol and stored at 4°C . The tissues were further dissected into 4×4 mm portions that were stored in 70% ethanol for transport and subsequent molecular analyses.

Next-generation nucleotide sequencing, bioinformatics and genotyping

Tissue subsections from all 93 oysters were processed for sequencing and genotyping at Diversity Arrays Technology (DArT). DNA extractions, sample preparation and sequencing followed the DArT-Seq™ protocol to identify single nucleotide polymorphisms (SNPs). The DArTSeq™ protocol is a genotyping-by-sequencing approach using DArT markers (Jaccoud et al. 2001, Luikart et al. 2003) and Illumina sequencing platforms (Sansaloni et al. 2011). It facilitates the identification and genotyping

of thousands of SNPs that are evenly distributed throughout the genome of the target species (Petroli et al. 2012). Detailed descriptions of DArT marker technologies for SNP discovery and genotyping are provided in Jaccoud et al. (2001) and Sansaloni et al. (2011).

In brief, DNA was extracted using GenCatch™ Blood and Tissue Genomic Mini Prep Kits (Epoch Biolabs) in accordance with the manufacturer's instructions. Purified DNA was held in a $1\times$ solution of MultiCore™ restriction enzyme buffer (Promega). Aliquots of all DNA samples were electrophoresed on 0.8% agarose gels pre-stained with GelRed™ (Biotium; Huang et al. 2010) to confirm that they contained high-molecular-weight DNA. Each DNA sample (100 ng) was then digested using 2 restriction enzymes (*PstI* and *SphI*) and ligated to adapters specific to these enzymes. The *PstI* adapter contained an Illumina flow cell attachment sequence, a sequencing primer and a barcode sequence unique for each individual sample.

Samples were then purified with PCR clean up kits (Qiagen) and amplified by PCR with primers specific to both the adapter and barcode sequences. The PCR conditions included 1 min initial denaturation at 94°C , then 30 cycles of denaturation (20 s, 94°C), annealing (30 s, 58°C) and extension (45 s, 72°C), with a final extension of 7 min at 72°C . Following PCR, all samples were pooled together in equal molar quantities. The pooled samples were then diluted and denatured with NaOH prior to hybridisation to the flow cell. An Illumina HiSeq-2500 single read platform was used to sequence the library. This process used 77 cycles and produced reads of equal length (65 bp). To ensure reproducibility, technical replicates were created by carrying approximately 30–40% of the samples through a second run of the library preparation protocol and through subsequent downstream analyses (Donnellan et al. 2015).

Illumina HiSeq2500 software was used to convert the resulting raw sequence data to .fastq files. Reads from individual oysters were de-multiplexed using the individual-specific barcode sequence ligated to the samples. All reads were then subjected to quality control. This involved checking for contaminants using GenBank viral and bacterial sequences and an in-house DArT database (Diversity Arrays Technology). Any reads with Phred (Ewing et al. 1998) quality scores <25 were also removed. The remaining reads were assessed using the DArT proprietary pipeline DArTSoft14™ (Diversity Arrays Technology) to identify and call SNPs.

This pipeline is comparable to the STACKS pipeline (Catchen et al. 2013). However DArTSoft14™ calls the sequence clusters for all the pooled samples first, followed by the calling of the sequence clusters for each individual. Sequence clusters found to be monomorphic were removed. SNPs were then identified and filtered to further ensure quality. Any SNPs that did not occur in both homozygous and heterozygous forms were removed and the balance of read counts evaluated for each allele. SNPs with a read depth <5 and reproducibility <95% were removed. Any locus with a very high read depth was also removed so that only SNPs with an average ratio of read depth between alleles of 0.70 were retained.

The final SNP dataset provided by DArT was further filtered for missing data at both the individual and locus level. As a result, only individuals and loci with ≥95% available data were retained. The data were screened for allele coverage, with any SNPs displaying a read depth <10 removed from the dataset (Lemay & Russello 2015). SNPs were also filtered for minor allele frequencies <5%, as low-frequency SNPs are known to create biases when analysing genetic signatures of selection (Roesti et al. 2012). Lastly, in cases where multiple polymorphisms were found within the same sequence length, only one SNP was retained and all other duplicates were removed to avoid bias due to physical linkage (Lemay & Russello 2015). There is no reason to suspect systematic bias as a result of these filtering steps, which are commonplace in population genetics analyses. This final filtered dataset is publicly available on Dryad Digital Depository (<http://dx.doi.org/10.5061/dryad.32q80>).

After filtering, the dataset was converted into the genotype coding system appropriate for use in the Microsoft Excel add-in package GenAlEx version 6.5 (Peakall & Smouse 2006, 2012). GenAlEx was used for preliminary data exploration and for export of the data into the Arlequin project (.arp) format. All other required file types were created from .arp files using the file conversion program PGDSpider version 2.0.8.3 (Lischer & Excoffier 2012).

Detecting loci potentially under selection

F_{ST} outlier tests were used to detect loci that were potentially under selection. Although the exact methods for these tests vary between programs, the common process involves identifying loci that have F_{ST} values outside of the range expected to occur

through genetic drift. Low F_{ST} values indicate balancing or negative selection, while high F_{ST} values indicate disruptive or positive selection (Lewontin & Krakauer 1973). In the current study, we undertook F_{ST} outlier tests in 3 independent programs.

Potential F_{ST} outliers were first detected using FDIST tests in Lositan (Antao et al. 2008) implementing the methods of Beaumont & Nichols (1996). The options for 'force mean F_{ST} ' and 'neutral mean F_{ST} ' were selected, as recommended by Antao et al. (2008). The false discovery rate was set at 0.05, number of simulations set at 1 000 000 and CIs set at 99.5%. All other settings were left at default (mutation model = infinite alleles). Lositan is simulation-based, so 2 independent sets of simulations were run to generate a consensus to identify markers putatively under selection.

In addition to Lositan, potential F_{ST} outliers were detected using the Bayesian simulation method of Beaumont & Balding (2004) in BayeScan version 2.1 (Foll & Gaggiotti 2008). All the default parameters were used (sample size = 5000; thinning interval = 10; pilot runs = 20; pilot run length = 5000; additional burn in = 50 000; prior odds for neutral model = 10). 'SNP genotypes matrix data' was also selected. Outputs were analysed with all default parameters and a false discovery rate of 0.05 (Foll & Gaggiotti 2008, Foll 2012), using the 'plot_bayescan' function in R (R Development Core Team 2011).

Potential F_{ST} outliers were also detected by the coalescent method of hierarchically structured populations in Arlequin version 3.5.2.2 (Excoffier & Lischer 2010), using a variation of the method of Beaumont & Nichols (1996). Default parameters were used (20 000 coalescent simulations, 10 groups and 100 demes). Outliers were identified using a threshold p-value of <0.05 and the type of selection (positive vs. negative) was determined based on F_{ST} values. Loci with negative F_{ST} values were deemed to be under negative selection, while loci with large, positive F_{ST} values were deemed to be under positive selection.

A consensus list of outliers under putative selection was constructed using results from all 3 F_{ST} outlier tests described above. Outliers that were identified as under selection across all 3 F_{ST} outlier tests were removed from the original data to produce a dataset comprising only loci incorporating putatively selectively neutral SNPs. This edited dataset containing neutral SNPs was used for all subsequent analysis, unless stated otherwise. Creating a more conservative list of neutral loci by removing all loci identified as under selection across all 3 outlier tests made no difference to the results.

Population structure and introgression

Summary statistics were calculated for each sample group, as well as totals for the pooled wild and B2 groups. Observed and expected heterozygosities were calculated in GENEPOP version 4.6 (Rousset 2008). Deviations from Hardy-Weinberg equilibrium (HWE) were also calculated per population and per locus, using the exact tests method of Guo & Thompson (1992) and 1000 permutations. F_{IS} values and 95% CIs were calculated using the 'divBasic' function in the diveRsity package of R (Keenan et al. 2013), using default parameters and 1000 bootstraps. Pairwise F_{ST} values were calculated for each pair of sample groups using the diveRsity function 'diffCalc' (Keenan et al. 2013), which implements the methods of Weir & Cockerham (1984). Default parameters and 500 bootstraps were used to calculate 95% CIs for each of the values. POPTREE2 was used to generate a neighbour-joining tree based on pairwise F_{ST} values with 15 000 bootstraps (Takezaki et al. 2010). In addition, the loci were screened for fixed or private alleles in the different sample groups. Where necessary, the significance of differences in fixed or private allele frequencies between oyster populations was determined by chi-squared analysis. Genetic diversity across sample groups was then assessed by principal component analysis (PCA) in the adegenet package of R (Jombart 2008) using the multivariate method described by Jombart et al. (2010).

A discriminant analysis of principal components (DAPC) was also undertaken in adegenet to determine the likely number of genetically distinct populations present within the dataset. The function 'find.clusters' was used to determine K , the number of genetically distinct groups present within a dataset of all oysters without any prior grouping information (Jombart 2013). This value of K was then used to run the function 'dapc'. A new dataset was then created based on the population groupings identified in the DAPC. Membership probabilities to these groups were plotted for each individual using the function 'compplot'. Summary statistics and frequencies of fixed and private alleles were subsequently re-calculated across the re-grouped dataset. In addition, individual observed heterozygosities were calculated for both the neutral and positive-selection datasets. The resulting distribution of heterozygosity frequencies were compared across the newly identified genetic groups using a 2-sample Kolmogorov-Smirnov test.

RESULTS

SNP databases

DArT genotyped 93 individuals to produce a dataset of 15250 SNPs (average read depth 41.8). This level of SNP identification is within the range expected, as reported for similar studies using DArT (Lind et al. 2017). Filtration steps addressing missing data, duplicate sequences, read depth and minor allele frequencies narrowed this dataset to 1200 SNPs (average read depth 67.1), equating to approximately 0.01% of the genome (genome size 788 Mb; D. Powell, University of the Sunshine Coast, pers. comm.).

Two independent simulations of this dataset of 1200 SNPs using Lositan identified 49 loci under balancing selection and 29 loci under positive selection. In contrast, BayeScan detected 18 loci under positive selection and no loci under balancing selection, whilst Arlequin identified 122 loci under balancing selection and 122 loci under positive selection. Only 11 loci were identified as under positive selection by all 3 forms of outlier analysis. Hence, 2 final datasets were assembled. One contained the 11 loci under positive selection (mean $F_{ST} = 0.3529$) and the second comprised the remaining 1189 selectively neutral loci (mean $F_{ST} = 0.0467$).

Population structure and introgression

Summary statistics for the 8 sample groups are shown in Table 1. Expected heterozygosities (H_e) for the different sample groups ranged from 0.2706 to 0.2835, while observed heterozygosities (H_o) were between 0.1968 and 0.2643. F_{IS} values ranged from 0.0133 to 0.2363. F_{IS} values for the pools of all B2 oysters and all wild oysters were 0.1276 (95% CI: 0.0770–0.1723) and 0.2225 (95% CI: 0.1828–0.2566), respectively. The pools of B2 sample groups and wild sample groups both deviated significantly from HWE (<0.001); however, no single locus deviated from HWE across all of the sample groups. Pairwise F_{ST} values for each of the sample groups ranged from 0.0033 to 0.0972 (Table 2). All combinations of wild vs. selectively bred sample groups had higher pairwise F_{ST} values (ranging from 0.0654 to 0.0972), with 95% CIs that did not include zero. All pairs of wild sample groups had lower pairwise F_{ST} values (ranging from 0.0033 to 0.0116), with all 95% CIs including zero. All pairs of selectively bred sample groups had lower pairwise F_{ST} values (ranging from 0.0150 to 0.0224), with all 95% CIs including zero. The neighbour-joining tree in Fig. 2 shows these differences.

Table 1. Summary statistics for each of the 8 sample groups of oysters based on 1189 neutral single nucleotide polymorphisms, including mean observed (H_o) and expected (H_e) heterozygosities, F_{IS} values and p-values testing the null hypothesis that there was no deviation from Hardy-Weinberg equilibrium (HWE)

Sample group	Group code	n	H_o	H_e	F_{IS}	HWE
Hatchery B2	HB2	11	0.2329	0.2810	0.1296	0.3680
Hatchery control	HC	10	0.2065	0.2792	0.2148	0.0005
Quibray Bay B2	QB2	9	0.2643	0.2835	0.0133	1
Quibray Bay overcatch	QOC	12	0.2067	0.2805	0.2263	<0.0001
Quibray Bay wild-caught	QWC	12	0.1968	0.2706	0.2363	<0.0001
Woolooware Bay B2	WB2	12	0.2380	0.2724	0.0915	0.5615
Woolooware Bay overcatch	WOC	12	0.2454	0.2807	0.0864	0.9975
Woolooware Bay wild-caught	WWC	12	0.2231	0.2770	0.1568	0.0171
Total B2	B2	32	0.2437	0.2823	0.1276	<0.0001
Total wild (overcatch, wild-caught and hatchery controls)	Wild	58	0.2161	0.2790	0.2225	<0.0001

Table 2. Pairwise F_{ST} values for all wild and selectively bred oyster sample groups; see Table 1 for a description of sample group codes. Bold text indicates selectively bred sample groups, while plain text indicates wild sample groups. * indicates values significantly different to zero based on 95% CIs

	HB2	QB2	WB2	HC	QOC	WOC	QWC	WWC
HB2								
QB2	0.0214							
WB2	0.0224	0.0150						
HC	0.0654*	0.0682*	0.0913*					
QOC	0.0679*	0.0699*	0.0962*	0.0037				
WOC	0.0655*	0.0673*	0.0937*	0.0098	0.0080			
QWC	0.0683*	0.0689*	0.0972*	0.0039	0.0033	0.0116		
WWC	0.0717*	0.0688*	0.0957*	0.0070	0.0034	0.0088	0.0037	

The DAPC identified 2 distinct clusters of oysters ($K = 2$, Bayesian information criterion = 496.5) based on allele frequencies in the selectively neutral dataset (Fig. 3A). The 2 clusters comprised 61 and 32 oysters. These numbers corresponded to the sample groups containing all wild and hatchery control oysters ($n = 58$) and the sample groups containing the B2 oysters ($n = 35$), with the exception of 3 Quibray Bay B2 (QB2) individuals that fell into the wild and hatchery control cluster (Fig. 3B). Membership probabilities confirmed confidence in the assignment of individuals into each cluster, and clearly identified wild genotypes for the 3 QB2 individuals (Fig. 4). Therefore, these 3 individuals were deemed to be outliers likely derived from inadvertent mixing of populations during farming (see Discussion), and so were removed from subsequent analyses. PCA of the selectively neutral dataset visualised the 2 clusters of oysters identified in the DAPC (Fig. 5). There was overlap between the sample groups within each cluster, yet no overlap was evident between the 2 clusters.

There were 61 instances of fixed alleles across the 8 separate sample groups. These fixed alleles fell in 26 loci (25 neutral, 1 under positive selection). Some alleles were fixed in more than one sample group. No private alleles were found within any of the single sample groups. However, comparison of the 2 DAPC clusters (wild and hatchery controls and B2) identified 112 private alleles. Of these, 77 were found

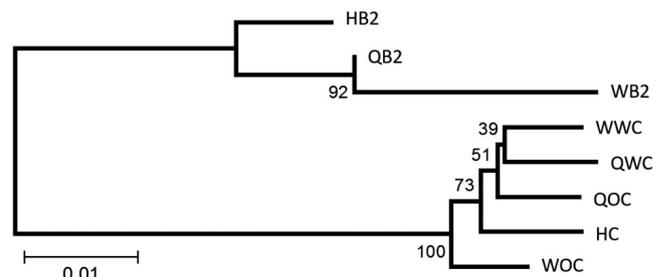


Fig. 2. Neighbour-joining tree based on pairwise F_{ST} values for the 8 sample groups, with the bootstrap values (%) for 15 000 replicates on each node. See Table 1 for a description of sample group codes

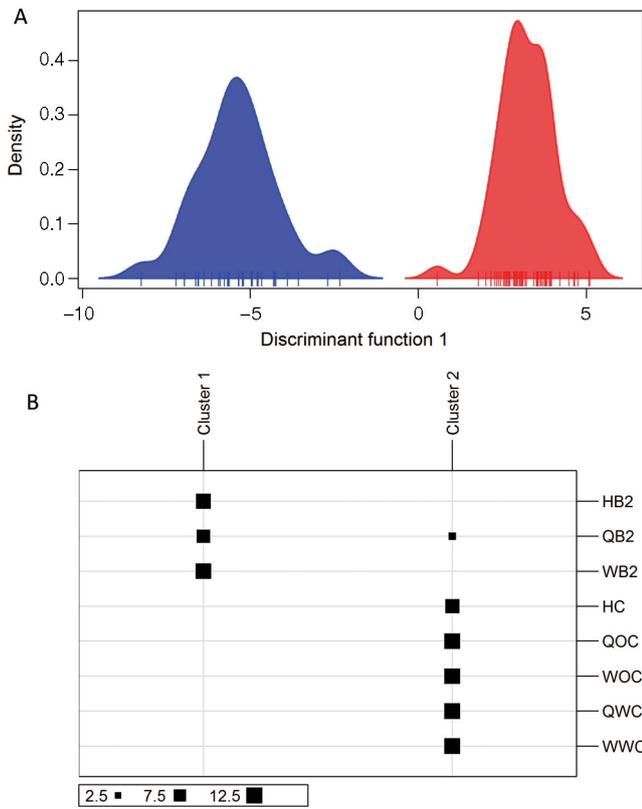


Fig. 3. Genetic clusters determined by discriminant analysis of principal components based on single nucleotide polymorphism data. (A) Density of the values of the first discriminant function across the 2 clusters, where cluster 1 (blue) comprises the B2 individuals and cluster 2 (red) comprises the wild and control oysters. (B) Allocation of individuals from each sample group into each of the clusters. See Table 1 for a description of sample group codes

exclusively in the wild and hatchery control cluster (all neutral loci) and 35 were found exclusively in the B2 group (30 neutral loci, 5 loci under positive selection). This represented a significant difference in the frequencies of private alleles between the 2 clusters ($\chi^2 = 8.0046$; $df = 1$; $p = 0.0047$). Three loci (all selectively neutral) were fixed across all B2 individuals, whilst none were fixed across all wild and hatchery control individuals.

Significant differences in the frequencies of individual observed heterozygosities between the B2 and wild and hatchery control clusters were evident for both the neutral loci and the loci under positive selection (2-sample Kolmogorov-Smirnov tests; both $p < 0.001$). Mean individual observed heterozygosity across the neutral loci was higher in the B2 group (mean \pm SD: 0.2429 ± 0.0511) than in the wild and hatchery control cluster (0.2153 ± 0.0446 ; Fig. 6A). Loci under positive selection also had higher mean

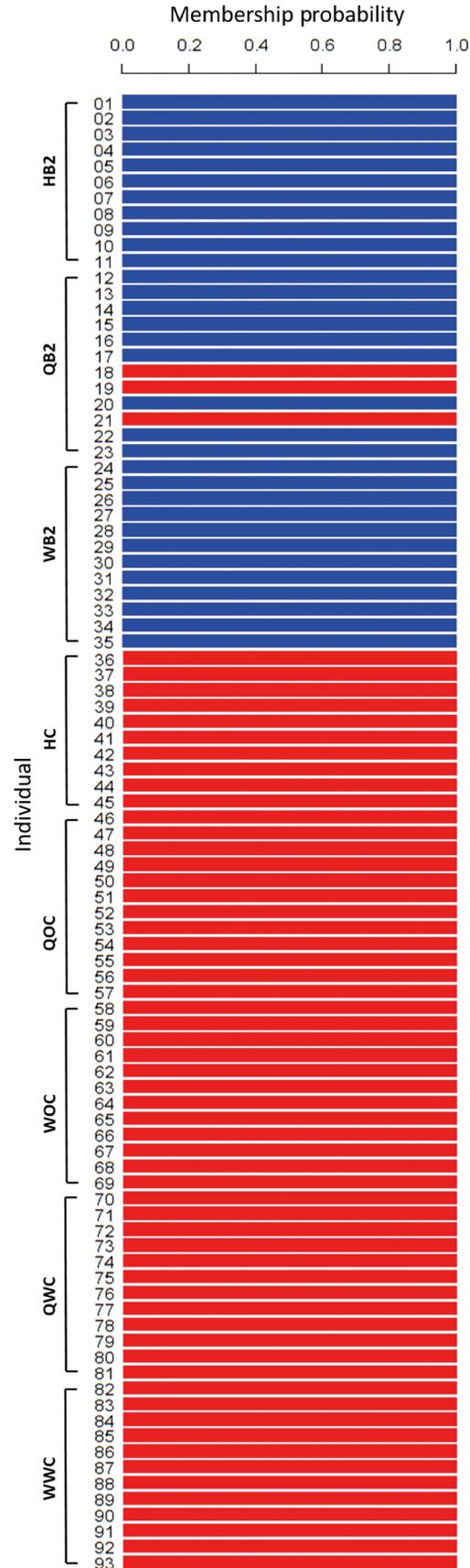


Fig. 4. Membership probabilities for each individual (1 to 93, grouped by sample group) to the 2 clusters determined by discriminant analysis of principal components based on single nucleotide polymorphism data. Cluster 1 (blue) comprises the B2 individuals and cluster 2 (red) comprises the wild and control oysters, with the exception of 3 QB2 individuals (see 'Discussion' for an explanation). See Table 1 for a description of sample group codes

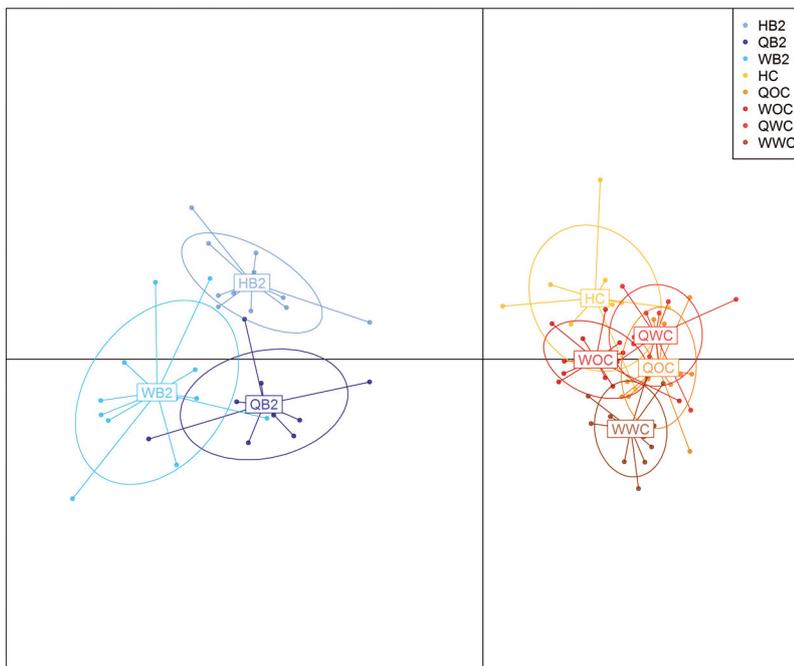


Fig. 5. Principal component analysis of variation between individual oysters ($n = 90$) based on 1189 selectively neutral single nucleotide polymorphisms. Each point represents an individual oyster. Ovals represent 95% CIs for each sample group. See Table 1 for a description of sample group codes

individual observed heterozygosity in the B2 cluster (0.3485 ± 0.1946) relative to the wild and hatchery control cluster (0.0547 ± 0.0717 ; Fig. 6B).

DISCUSSION

This study has shown that the selectively bred B2 line of Sydney rock oysters is genetically distinct from wild Sydney rock oysters, and there is no evidence of introgression occurring from farmed B2

oysters into the wild population growing in the Georges River, NSW. DAPC (Fig. 3), membership probabilities (Fig. 4) and PCA (Fig. 5) all identified a clear distinction between the wild and farmed B2 sample groups. The large number of private alleles (112; 9.3%) identified between the B2 oysters and the wild groups is also indicative of limited gene flow between the 2 clusters. This is further supported by the pairwise F_{ST} values and neighbour-joining tree (Fig. 2), which reflect differentiation between the wild and B2 sample groups. All wild-wild comparisons and all B2-B2 comparisons have lower pairwise F_{ST} values and CIs including zero, while all wild-B2 sample group pairs have higher pairwise F_{ST} values and CIs that do not include zero. All of these data suggest that genetic introgression between the farmed B2 oysters and the wild oyster population has not occurred in the Georges River.

The only discrepancies in the distinction between the B2 and wild population were 3 oysters collected from the B2 farming racks at Quibray Bay (sample group QB2). These oysters appeared to have wild, rather than B2 genotypes. There are several explanations for the occurrence of these oysters. The most pragmatic explanation is that these 3 oysters were wild-caught oysters that were mistakenly placed in the B2 farming racks. Spat of both B2 and wild-caught oysters are produced in the Port Stephens hatchery and subsequently farmed in the Georges

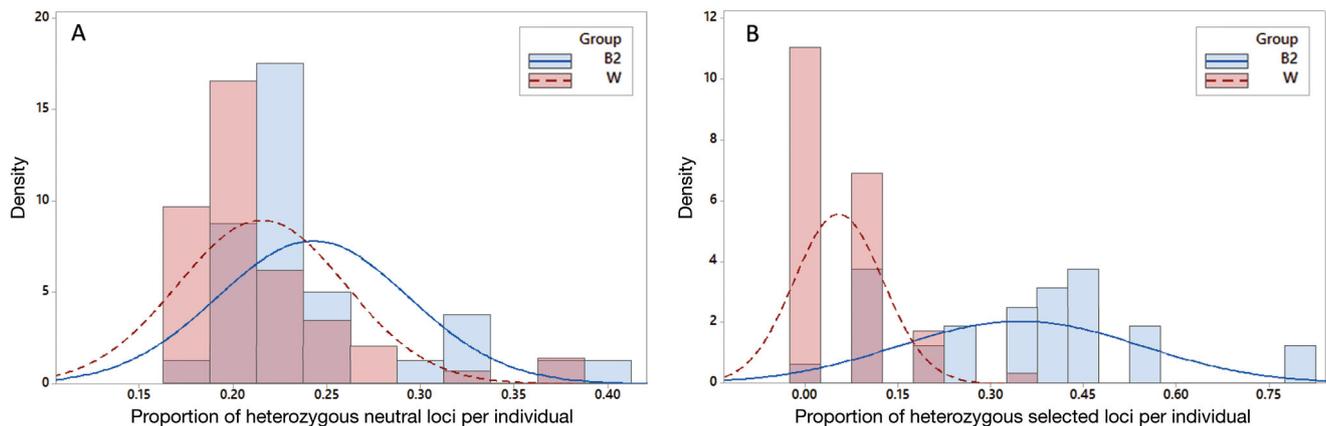


Fig. 6. Individual observed heterozygosities for selectively bred (B2, blue) and wild and hatchery control (W, red) oysters. Data are presented for (A) neutral loci and (B) loci identified as under positive selection

River, where racks containing each type of oyster are often processed side by side. These farming practices provide the opportunity for accidental mixing of the 2 types of oysters. It is also feasible that cross-contamination or hybridisation could occur in the hatchery (W. O'Connor, NSW DPI, pers. comm.). However, none of these explanations are consistent with gene flow from the farmed B2 oysters into the wild population because the gene flow implied by the presence of these oysters is in the opposite direction (wild into B2).

There are a number of factors that may account for the lack of observed introgression between the populations investigated in this study. Wild and selectively bred Sydney rock oyster gametes may have a limited capacity for cross fertilisation, or hybrid spat may have low survival rates compared to wild spat. The selectively bred line has been spawned under hatchery conditions for several generations and so may have developed fertilisation or larval characteristics that are not suited to recruitment in the wild. Alternatively, the lack of interbreeding between wild and selectively bred Sydney rock oysters might be the result of farming practices. Farmed oysters are generally sold once they reach a defined size class in order to return the most profit. For Sydney rock oysters, harvesting usually occurs when they reach sexual maturity. Gravid oysters are said to be 'fat' (and so more saleable) due to their engorged reproductive organs. Therefore, it is likely that the majority of farmed oysters are sold before they have had the opportunity to spawn within the estuary. A further reason for the lack of introgression may be the relative size of oyster populations in the Georges River. Anecdotal evidence (W. O'Connor, NSW DPI, pers. comm.) suggests that the number of wild oysters growing in the Georges River may be orders of magnitude greater than the farmed B2 population. In this case, the number of gametes from wild oysters may simply overwhelm those from the farmed population. Selectively bred oysters have only been farmed in the Georges River since the 1990s, so it is possible that longer periods of interaction with wild oysters may be necessary for introgression to become apparent.

It is also possible that introgression has occurred in the Georges River, but was not detected in this study. Larval dispersal patterns of Sydney rock oysters are poorly understood (Banks et al. 2006). It is not known how long larvae spend in the water column or how far they travel before settling and metamorphosing into sessile juveniles. The circulation patterns of the Georges River estuary are also not well documented,

so the direction that larvae travel and exchange patterns between bays remain unknown. Although our study accounted for potential larval dispersal by choosing 2 bays that are geographically separated by approximately 10 km of coastline, it is possible that larvae produced in these bays may have settled further afield. More extensive studies of Sydney rock oysters within other bays in this estuary will help to clarify whether the effects of introgression can be detected elsewhere, particularly if hybrid larvae are travelling further from the farming areas than expected.

It is also important to note that the current study focused on the detection of sustained levels of introgression, where gene flow between populations leads to hybridisation and further backcrossing (Anderson & Hubricht 1938). Our experimental design was based on relatively low sample sizes from each population ($n = 9-12$ for each sample group at each sampling site). Such small sample sizes are relatively common in studies of marine species involving comparable SNP technologies, e.g. restriction site associated DNA sequencing (Keller et al. 2013: $n = 10$, Reitzel et al. 2013: $n = 6-9$, Lah et al. 2016: $n = 3-10$). Studies using DArT sequencing also report similar sample sizes (Lambert et al. 2016). Our small sample sizes may not have detected low levels of gene flow between the farmed and wild oyster populations that does not result in introgression. Such gene flow has been observed in other oyster species. For instance, Carlsson et al. (2008) found an extremely low level of hybridisation (0.6%) between wild and selectively bred eastern oysters *Crassostrea virginica* in Chesapeake Bay, USA. This was despite the release of approximately 18.5 million selectively bred spat within this region for population restoration projects. Carlsson et al. (2008) also reported no significant increase in the frequency of haplotypes known to be common in the selectively bred oysters but rare in the wild oysters. Similar or even lower levels of hybridisation could be occurring between the farmed B2 oysters and the wild population in the Georges River. However, if such low levels of hybridisation occur, the sheer number of wild oysters in the Georges River combined with the relatively small number of samples collected in this study would have made it unlikely for us to detect these occasional hybrids.

Rather than identifying substantial gene flow between the selectively bred B2 oysters and wild populations, our data reflected a surprisingly high level of genetic differentiation between the populations (Fig. 2). This genetic differentiation was high when

only selectively neutral loci were considered, and also when only considering loci under selection. The effects of mass selection for disease resistance and fast growth are reflected by the identification of numerous outlier loci with higher F_{ST} values than expected under neutrality in the B2 population. These selective forces, combined with unknown effects of hatchery rearing, may explain some of the differentiation between B2 and wild oysters. Until further genomic data become available for the Sydney rock oyster, we cannot be confident of the location or function of potentially adaptive SNPs, and simply regard their presence as a signature of divergent selection.

Our data also revealed substantial differences in the levels of genetic variation within the B2 and the wild Sydney rock oyster populations. The variability of neutral markers is lower in wild oysters than in B2 oysters (Table 1). This pattern was also evident in the individual heterozygosities observed for B2 and wild oysters across both the selectively neutral dataset and the loci under positive selection (Fig. 6). In both cases, the B2 oysters were found to have significantly higher levels of observed individual heterozygosities. Higher heterozygosity in selectively bred oysters was contrary to expectations. The B2 oysters were expected to have lower heterozygosity than the wild oysters due to both the selection for fast growth and disease resistance, and increased inbreeding resulting from the small size of the founding parental population for the B2 line. While the progenitors of the B2 line were established from just 140 parents, they were sourced from 4 geographically separated rivers in NSW. This may explain the higher level of genetic variation within the B2 line relative to the wild oysters collected from a single geographic location. The greater genetic diversity of B2 oysters relative to the wild population also contrasts with data from other oyster species. Studies of *Pinctada maxima* (Lind et al. 2009), *Crassostrea gigas* (Hedgcock & Sly 1990) and *C. virginica* (Carlsson et al. 2006) have consistently found lower genetic diversity in hatchery-reared and/or selectively bred oysters than in wild populations after as little as one generation.

The limited genetic diversity of wild Sydney rock oysters relative to the B2 line suggests that the genetic variation of wild Sydney rock oyster populations may differ substantially from those of other broadcast spawning bivalves. We suggest that it may reflect ongoing reductions in effective population size, potentially owing to disease outbreaks, previous overharvesting, and limited gene flow and larval dispersal in the Georges River. The impacts of disease may also explain the significantly lower than

expected heterozygosities of the hatchery control oyster group as the progenitors were from locations that experience QX disease. There is also evidence for comparable bottlenecks in other oyster species. For instance, Hedgcock & Sly (1990) identified a heterozygote excess in the first generations of 2 groups of hatchery-produced Pacific oysters *C. gigas*, whilst there was a heterozygote deficit in the wild founder population. B2 oysters have been selected for resistance to QX disease and winter mortality and so suffer far lower levels of mortality (Nell & Perkins 2006). Hence, the B2 oysters may not have been subjected to the bottleneck evident in their wild conspecifics.

CONCLUSIONS

This study has shown that there is no sustained gene flow in the form of genetic introgression occurring from the farmed, selectively bred Sydney rock oysters into the wild populations, in the only estuary where selectively bred Sydney rock oysters have been consistently farmed for a substantial period of time. Other significant findings of this study are that there is substantial genetic differentiation between selectively bred and wild Sydney rock oysters, and that wild Sydney rock oyster populations have significantly lower genetic variation relative to the farmed, selectively bred oysters. The lower genetic variation in the wild oysters is contrary to common assumptions about selective breeding programmes, and has significant implications for both the design of such programmes and the use of selectively bred populations for the restoration of wild oyster reefs.

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