Safeguarding our Sydney Rock Oyster Industry Against QX Disease

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Abbreviations

AGRF – Australian Genomic Research Facility

DPIRD – Department of Primary Industries and Regional Development

EBV – Estimated breeding value

EMAI - Elizabeth Macarthur Agricultural Institute

FCS – Foreign contamination screen

IPC – Internal process control

ITS – Internal transcribed spacer

LKB - Lime Kiln Bay

MLST – Multi-locus sequence typing

ONT – Oxford Nanopore technology

PSFI – Port Stephens Fisheries Institute

qPCR – Quantitative polymerase chain reaction

QX – Queensland unknown

rRNA – Ribosomal ribonucleic acid

RRRO – Richmond River rock oyster

SNV – Single nucleotide variation

SRO - Sydney rock oyster

YC – Year class

Executive Summary

What the report is about

This report outlines a NSW Department of Primary Industries and Regional Development (NSW DPIRD) investigation into two critical and interlinked challenges facing the Sydney rock oyster (SRO) industry: the ongoing threat posed by QX disease and the need to optimise selective breeding strategies to improve disease resistance, while informing future biosecurity policy. Specifically, the study examines whether genetic differences in the QX disease agent (*Marteilia sydneyi*) explain why disease outbreaks occur in some estuaries but not others and evaluates the performance of selectively bred oysters incorporating Richmond River Rock Oyster (RRRO) genetics across multiple years, life stages, and disease exposure events. Together, these findings aim to support evidence-based biosecurity decision-making and strengthen long-term industry resilience.

Background

The dissemination of QX disease has continued progressively across SRO-producing estuaries in NSW and Queensland over the past five decades, with recent outbreaks in Port Stephens highlighting its continued spread. In severe outbreaks, QX disease can cause mortalities of up to 90%, representing a major economic threat to one of Australia's most important aquaculture industries and the largest in NSW.

Although the parasite responsible for QX disease (*M. sydneyi*) has reportedly been detected in many estuaries, disease expression is inconsistent, and the reasons for this remain unclear. This uncertainty complicates biosecurity policy, particularly restrictions on oyster movements between estuaries classified as high- medium- or low-risk. One hypothesis is that *M. sydneyi* may comprise multiple strains or cryptic species with differing pathogenicity, similar to patterns observed in related *Marteilia* species overseas.

At the same time, selective breeding for QX resistance remains the primary management tool available to industry. The NSW DPIRD SRO breeding program has achieved steady gains in QX survival, and oysters originating from the Richmond River, where severe QX is epizootics have resulted in strong natural selection. However, the extent, consistency, and risks associated with incorporating RRRO genetics into the broader breeding program require further evaluation.

Aims/objectives

The specific aims of the study were to:

- Develop and apply a genetic typing framework for *M. sydneyi* to determine whether genetic differences exist among *M. sydneyi* populations from estuaries with differing QX disease risk.
- Confirm the presence or absence of *M. sydneyi* in estuaries currently classified as low risk for QX disease.
- Evaluate the QX disease resistance of selectively bred SRO families, including those incorporating RRRO genetics, across multiple sites, life stages, and exposure events.
- Assess the contribution of RRRO genetics to overall breeding program performance using survival, growth, and meat condition metrics.

Methodology

This project consisted of two main components: genetic characterisation of *M. sydneyi* across estuaries and evaluation of QX resistance in selectively bred oyster families including those with RRRO genetics.

For the pathogen component, archival QX-positive samples from high- and medium-risk estuaries were genetically analysed using newly available genome data for *M. sydneyi*. A genetic typing approach was developed, targeting the ribosomal RNA array to assess genetic diversity within and between estuaries. Extensive field sampling was also undertaken in estuaries classified as low risk for QX disease across three years, with oysters screened using a sensitive qPCR assay to detect *M. sydneyi*.

For the breeding component, oyster families from the 2022- and 2023-year classes, both with and without RRRO parentage, were assessed for QX survival as spat and adults. Trials were conducted across multiple QX-exposure sites and seasons, including single and repeated disease exposures. In addition to survival, growth and meat condition were measured, and all data were integrated into the SRO selective breeding database to generate Estimated Breeding Values (EBVs) and overall family rankings using the program's established selection index.

Results/key findings

No *M. sydneyi* was detected in more than 1,800 oysters sampled from low-risk estuaries over three seasons, despite targeted sampling during peak infection periods. This finding contrasts with earlier reports of parasite detection in these estuaries and suggests that *M. sydneyi* may be absent, rare, or previously misidentified. Consequently, direct genetic comparisons between parasites from high- and low-risk estuaries were not possible; however, these findings support the existing movement restrictions of oysters from high or medium risk estuaries to low-risk estuaries

Genetic analysis of *M. sydneyi* from high- and medium-risk estuaries revealed very limited between-estuary diversity. Core ribosomal genes were identical across all samples, and variability within the more variable ITS regions occurred primarily within individual samples rather than consistently between locations. These patterns are inconsistent with the presence of distinct cryptic species or geographically structured pathogenic strains and suggest that observed genetic variation may reflect within-host diversity rather than biologically meaningful strain differences. Overall, there was little evidence to support the hypothesis that differences in QX disease expression among estuaries are driven by fundamentally different *M. sydneyi* strains.

Selective breeding trials demonstrated continued improvement in QX resistance across year classes, confirming the effectiveness of the SRO breeding program. Families incorporating RRRO genetics were frequently among the top-performing groups for survival in high QX risk estuaries at both spat and adult stages, often outperforming non-RRRO families on average. However, performance among RRRO families was highly variable, with some ranking among the lowest-performing families for QX survival and overall selection index.

These results indicate that RRRO genetics can significantly enhance survival in high QX risk estuaries when combined with high-performing SRO breeding lines but also carry risks if incorporated without careful selection. The findings reinforce the importance of structured crossing, rigorous field testing, and cautious integration of RRRO material before broader commercial deployment.

Implications for relevant stakeholders

This study highlights that current evidence does not strongly support strain-based biosecurity differentiation among estuaries, with the exception of low-risk estuaries, where the presence of *M. sydneyi* was not confirmed and is considered questionable. This work supports the continued use of selective breeding, particularly the measured incorporation of RRRO genetics, as a cornerstone of QX disease management.

Recommendations

A summary of recommendations from this project are as follows:

- **Strengthen surveillance in low-risk estuaries:** Expand spatial and temporal monitoring across additional seasons and estuaries, using improved molecular tools (including pan-*Marteilia* assays) to confirm parasite presence, species diversity, and associated biosecurity risks.
- **Investigate drivers of QX disease expression:** Assess environmental factors and potential intermediate hosts that may influence transmission and explain why disease occurs in some estuaries but not others, to support accurate risk classification.
- **Refine and extend the selective breeding program:** Continue multi-season and multi-site QX exposure trials, including repeated exposure scenarios, to validate and sustain gains in disease resistance.
- **Accelerate genetic progress while maintaining balance:** Increase use of genomic markers for QX resistance and monitor correlated traits such as growth, meat condition, and robustness to ensure long-term improvement without loss of genetic diversity.

Keywords

Sydney rock oyster (SRO, *Saccostrea glomerata*, *Marteilia sydneyi*, QX disease, genetic typing, selective breeding

Introduction

Outbreaks of QX disease in Port Stephens in 2022 and 2023 mark the continued spread of this disease into Sydney rock oyster (SRO)-producing estuaries in NSW and QLD over the last 50 years. In severe years, QX can cause up to 90% mortalities in affected stock, therefore this disease poses a major threat to an industry that is of substantial economic, historic and cultural value.

Despite the apparent presence of the causative agent (*M. sydneyi*) in nearly all estuaries undertaking SRO production, disease only occurs in some, and biosecurity protocols further complicate SRO farming as stocks from high-risk estuaries cannot be moved into estuaries that have a lower QX disease risk rating.

Reasons behind the expression of QX disease in some estuaries but not others currently remain unknown. In France, *Marteilia refringens*, was originally thought to be a widespread cause of marteiliosis in the European flat oyster (*Ostrea edulis*) as well as mussels belonging to the genus *Mytilus*. *M. refringens* O (oyster) type and M (mussel) type, which were originally described due their differential pathogenicity in the respective hosts, have more recently been found to constitute separate (cryptic) species, with M type being renamed as *Marteilia pararefringens*. One hypothesis that required testing is that a similar situation may exist in Australia with *M. sydneyi* constituting more than one species and with the more pathogenic strains being responsible for QX disease outbreaks. Historically these questions could not be meaningfully answered due a lack of genetic information about *M. sydneyi*; however, NSW DPIRD has recently undertaken a genome sequencing project on *M. sydneyi* to facilitate strain comparison. Therefore, one aim of this project is to characterise *Marteilia* strains from estuaries where disease occurs and compare with those from estuaries where disease does not occur, to better inform biosecurity policies. If the *M. sydneyi* strains are identical across estuaries, then this may enable biosecurity policy to be modified to allow oyster translocations across so called “high” and “low” risk estuaries. However, if strains do differ across high and low risk estuaries, then any biosecurity policy will be aimed at protecting estuaries not currently experiencing QX outbreaks from the introduction of high pathogenicity strains.

QX disease remains as the primary threat for SRO production. Due to significant knowledge gaps in how this disease is transmitted, the use of selectively bred QX resistant oysters is the main management tool used to enable cultivation to continue in estuaries where the disease is enzootic. QX survival is a quantitative and a responsive trait where applied breeding offers a good solution to increase QX survival with significant economic benefits for industry. QX survival breeding involves challenging SRO families with QX disease in the field and breeding from families with highest survival. This method works well to increase resistance, however, best results for improving QX survival are achieved through a combination of breeding and management practices to minimise impacts. It is recommended to use oysters selected for QX survival as a risk management strategy to reduce stock losses before a QX disease outbreak occurs in an estuary. When oysters selected for QX survival are used in estuaries affected by QX, it is important to deploy spat when *M. sydneyi* infections have ceased and harvest these oysters prior to a second disease exposure.

This relies on specific timing of commercial hatchery production and fast oyster growth which is a trait under selection in combination with QX survival. Field exposures that run over two seasons of QX disease are now used to increase survival following consecutive outbreaks. Other diseases or factors that compromise SRO health prior to or during *M. sydneyi* infections also reduce the effectiveness of breeding. Increasing genetic gains for QX survival has been the primary objective of the breeding program since its inception (Nell et al. 2000). A genomics project is currently underway which aims to identify genetic markers for QX disease resistance to increase genetic progress for this trait. Batches of Richmond River Rock oyster (RRRO) produced by NSW DPI have shown high levels of QX disease

survival. Prior studies on RRROs suggest that genetically they are classified as SROs but they appear to have developed significant resistance, presumably due to years of exposure to QX in the Richmond River estuary where the disease in enzootic and multiple severe epizootics have resulted in strong natural selection favouring QX resistant oysters.

Preliminary experimental evidence suggests that RRROs display enhanced survival when exposed to QX disease, justifying their inclusion in the selective breeding program. Therefore, the second aim of this project is to assess QX survival of current RRRO families across multiple years of QX exposure and compare these results to other QX-resistant families in the breeding program. This information will be used in this project to formulate a breeding plan to create additional families using batches of RRROs that have been assessed for QX survival.

Objectives

1. To use previously generated genomic data from *M. sydneyi* to develop a multilocus sequence typing (MLST) scheme for *Marteilia* strains from SROs

2. To screen samples collected from low-risk estuaries for *M. sydneyi* using qPCR

3. To compare *Marteilia* MLST profiles of qPCR positive samples from low-risk estuaries with those from *M. sydneyi* MLST profiles from high-risk estuaries.

4. To create 10 additional families (in addition to the families produced for the 2023-year class breeding run) that have a parent sourced from the Richmond River

5. To assess the QX survival of the 10 additional 2023-year class families as spat, adults and over two seasons at multiple sites

6. To generate QX spat survival estimated breeding values (EBVs) for families with RRRO parents produced for the YC2022 and 2023-year classes

7. To collect performance data (QX survival and growth) that allows comparison of RRROs and commercial families from the Sydney Rock Oyster breeding program over two consecutive seasons of QX disease.

Methods

Genetic typing of *M. sydneyi*

Curation of QX-positive archival material from high and medium risk estuaries

Frozen archival material stored at EMAI, representing QX-affected oysters collected from high and medium risk estuaries was used in this study. Material used for initial comparison of strains across estuaries is shown in Table 1.

Table 1. Archival material used for genetic typing of *M. sydneyi* across high and medium risk estuaries.

Reference numbers	Year of collection	Estuary	Risk rating
M16-04749	2016	Hawkesbury River, NSW	High
M16-06827	2016	Georges River, NSW	High
M16-06983	2016	Brunswick River, NSW	Medium
M20-05667	2020	Macleay River, NSW	High (but with intermittent outbreaks)
M21-11844, M20-06326, M19-03170	2019, 2020, 2021	Richmond River, NSW	High
M22-17260, M22-17262, M22-17475	2022	Stradbroke Island, QLD	N/A*
M23-06441	2023	Port Stephens, NSW	High

*Estuaries in Queensland are not QX risk rated.

Sampling of low-risk estuaries

Sampling of oysters from estuaries at low risk of QX disease was guided by prior surveillance studies conducted across a number of NSW estuaries and in Wallis Lake. Estuaries with the highest reported incidence of *M. sydneyi* based on previous molecular testing were targeted for screening (Table 2).

Table 2. Summary of historical findings of *M. sydneyi* in low-risk estuaries in NSW based on PCR testing

Year	Estuary	No. sampled	Reported Prevalence	Study
2002	Wallis Lake	100	7%	FRDC 2001-214-DLD
	Tuross Lake	100	19%	FRDC 2001-214-DLD
	Wagonga Inlet	100	25%	FRDC 2001-214-DLD
2003	Wallis Lake	150	0%	FRDC 2001-214-DLD
	Tuross Lake	150	0%	FRDC 2001-214-DLD
	Wagonga Inlet	150	0%	FRDC 2001-214-DLD
2005	Wallis Lake	150	21%	NSW DPI surveillance

In this study, random sampling of SROs was undertaken in Wallis Lake and Wagonga inlet in 2023 and again in Wallis Lake, Tuross Lake and Wagonga Inlet in 2024 and 2025. Sampling of 150 oysters at a given site was chosen to give 95% confidence of detecting *M. sydneyi* infection at a prevalence of 2%, while sampling of 250 oysters gave >99% confidence of detecting infection at 2% prevalence (Cannon and Roe, 1982). All samples were collected during the months where *M. sydneyi* is expected to be active

(March-May). A mix of wild and cultivated oysters were collected to account for any natural resistance to *M. sydneyi* in wild oysters, and any potential effects related to the translocation or origin of cultivated oysters. Because rainfall appears to be a significant trigger for movement of *M. sydneyi* into SROs, rainfall data for each estuary derived from the Bureau of Meteorology website was collated for the 2 weeks prior to each sampling (https://www.bom.gov.au/climate/data/).

Sample processing and DNA extraction

Archived (frozen) samples were thawed and a 1.5 mm cube of digestive gland was aseptically dissected and placed in a sterile microfuge tube. For fresh samples, collected during this study from estuaries at low risk of QX disease, oysters were scrubbed, aseptically shucked, dissected in cross-section using a sterile scalpel blade, and then 2 × 1.5 mm cubes of digestive gland were either used to make cytological impression smears by dabbing the tissue onto a glass slide or removed into a sterile microfuge tube for DNA extraction.

All archived and fresh samples were extracted using the MagMAX™ Core DNA extraction kit (ThermoFisher Scientific) using overnight proteinase K digestion followed by extraction according to workflow D on a Kingfisher™ Flex or Kingfisher™ Duo instrument. An in-house internal process control (IPC) was added to the lysis buffer prior to extraction to monitor for downstream PCR inhibition.

Molecular testing of oysters from low-risk estuaries for *M. sydneyi*

All SROs collected from estuaries at low risk of QX disease were screened for *M. sydneyi* using a qPCR method developed previously (Jenkins et al., 2023). Briefly, 2 µL DNA extracted from the digestive gland of each oyster was tested in a 20 µL reaction containing 1 × Environmental Mastermix (ThermoFisher Scientfiic), 0.5 µM of QX-F and QX-R primers and 0.2 µM QX-Probe. Reactions also contained primers (0.3 µM IPC-F and 0.3 µM IPC-R) and probe (0.2 µM IPC-Probe) for amplification of the IPC. Thermal cycling consisted of 1 cycle of denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 60 s. All assays were run on a QuantStudio S5 instrument (ThermoFisher Scientific). Test acceptance criteria were a standard curve with an R^2 value of > 0.98 and a reaction efficiency between 85-110%. PCR reactions were re-run if the IPC fell outside of a pre-defined cycle threshold (Ct) range of 26-32.

Genome filtering, annotation and alignment

Filtering of assembled contigs

In a previous study, *M. sydneyi* sporonts were purified from infected oysters obtained from the Richmond River in 2020. The sporonts were sequenced with a combination of Illumina short read sequencing and Oxford Nanopore long read technology (ONT). While sporonts had been purified using density gradient centrifugation, DNA from *Saccostrea glomerata* and from other contaminating microeukaryotes and bacteria were represented amongst the assembled contigs. Prior to annotation of the genome, the assembled contigs were filtered for contaminating DNA through the NCBI Foreign Contamination Screen (FCS) suite using the FCS-GX module Version 0.5.0 (https://github.com/ncbi/fcs/wiki/FCS-GX). Contigs were filtered through Blobtoolkit (https://github.com/blobtoolkit/blobtoolkit) to further filter non-target DNA and CoverM (https://github.com/wwood/CoverM) to calculate and remove contigs with low coverage. Contigs removed from the assembly were < 1000 bp in length, recommended for exclusion by FCS-GX and, due to the very low GC content of the *M. sydneyi* genome, had a GC content of > 30%.

M. sydneyi *genome annotation*

Annotation of the filtered assembly was performed with the Funannotate v1.8.15 pipeline. Prior to training, the assembly was prepared with the funannotate clean, sort and mask commands with defaults. The Ab initio gene predictors Augustus v3.5.0 (https://github.com/Gaius-Augustus/Augustus),

SNAP v2013_11_29, GlimmerHMM v3.0.4 and Genemark-ES v4.72 were trained with ONT unassembled mRNA longreads and the cleaned sorted and masked ONT assembly with the --max_intronlen flag set to 20000. Gene prediction was performed with "funannotate predict" using mRNA read alignment, and trained ab initio predictors, with the --organism flag set to "other". Eggnog-mapper was used to provide functional data through ortholog clustering externally at http://eggnogdb.embl.de/#/app/emapper with protein fasta input from funannotate. InterProScan v5.55-88 was called using the funannotate iprscan command, and Ribosomal RNA subunits were predicted with Barrnap v0.9. For the funannotate annotate command the --busco_db flag was set to "protists".

Mapping of *Marteilia* sp. sequences to *M. sydneyi* scaffold 1

To facilitate the design of primers for amplification and sequencing of the *Marteilia* rRNA array from archival samples, existing sequence data for other *Marteilia* species, including data for individual gene regions and larger gene assemblies, were mapped to scaffold 1 of the *M. sydneyi* genome (containing the rRNA array) using Geneious R7. *Marteilia* species including in the alignment were *M. refringens* (MH304627, MH304628, MH304629, MH304635, MH304636, MH304637, MH356753, MH304854, MH304855, MH304856, MH304861, MH304862, MH342044, MH342613) *M. pararefringens* (MH304630, MH304631, MH304632, MH340638, MH304639, MH304640, MH304851, MH304852 MH304857, MH304858, MH304859, MH304860), *M. cochillia* (KF278722), *M. cocosarum* (ON320534), *M. octospora* (OK042968, OK042969) and *M. tapetis* (AB823743).

Primer design

Primers were designed to amplify across a ~7000 bp region of the *M. sydneyi* genome to allow for comparison of sequences across samples from high- and medium-risk estuaries. While presumed to be *M. sydneyi*, the identity of the *Marteilia* species associated with low-risk estuaries has not been confirmed, therefore primers were also designed to amplify other members of the *Marteilia* genus in addition to *M. sydneyi,* to account for potential sequence variations.

All primers had a melting temperature of 60°C ± 2°C as determined by Geneious and were compared to the GenBank nucleic acid database (using MegaBLAST) to check for specificity. Primers were also checked for hairpin formation and self-dimerisation using OligoCalc (BioTools). All primers used for PCR are listed in Table 3 and their position on the rRNA array is shown in Figure 1A.

PCR conditions

All PCR reactions were performed in a reaction volume of 20 µL and contained 2 × MyTaq HS mix (Meridian Bioscience, Cincinnati, USA) 0.2 µM each primer, and 2 µL of template DNA. Thermal cycling for each PCR consisted of a denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30s and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min.

PCR products were subjected to automated electrophoresis using D5000 ScreenTapes on a TapeStation 4150 system (Agilent Technologies, Santa Clara, USA) according to the manufacturer's instructions.

Amplicon sequencing

Amplicons were sequenced using a combination of Sanger and ONT sequencing. For Sanger sequencing, amplicons were purified using the Qiaquick PCR purification kit (Qiagen, Alameda, USA) according to the manufacturer's instructions and sequenced at the Australian Genome Research Facility (AGRF; Westmead, Australia).

Table 3. Sequences of primers designed to amplify across regions of the rRNA array of *Marteilia* spp.

Primer name	Primer sequence 5' – 3'
Msyd 18S FUp	GCTTCCGACATGACGAGAGG
18S F	TGGTCGATCCCGCCACC
Msp 18S F1	ACGGATATCTCTGGTAAGCTAGAGC
Msp 18S F2	CGAATAAGTCCCTGTCCTTTGTACA
Msp 18S R1	GTTGATAGGTGGCAGTCTCGTG
Msp 18S R2	CTTGACGCTGACGATRCATTGG
Msp 5.8S F	TGGACCGCTCGGATCGTG
Msp 5.8S R	CAGATTGCTGCGTCCTTCTAC
Msp 28S F1	GTAGCGCCGCRAACTTAAAC
Msp 28S R1	GTTGGTTTCTCTTGCTGCCG
Msp 28S F2	TCACCGATCGAATCGTGCG
Msp 28S R2	CATGATAAGCACACGCGACG
Msp 28S F3	GTGAAAGACCATTGACGCAGT
Msp 28S R3	CACCAGGTTAGCGAGCGAG
Msp 28S R4	ACTTGGCACGGCTTCGAAC

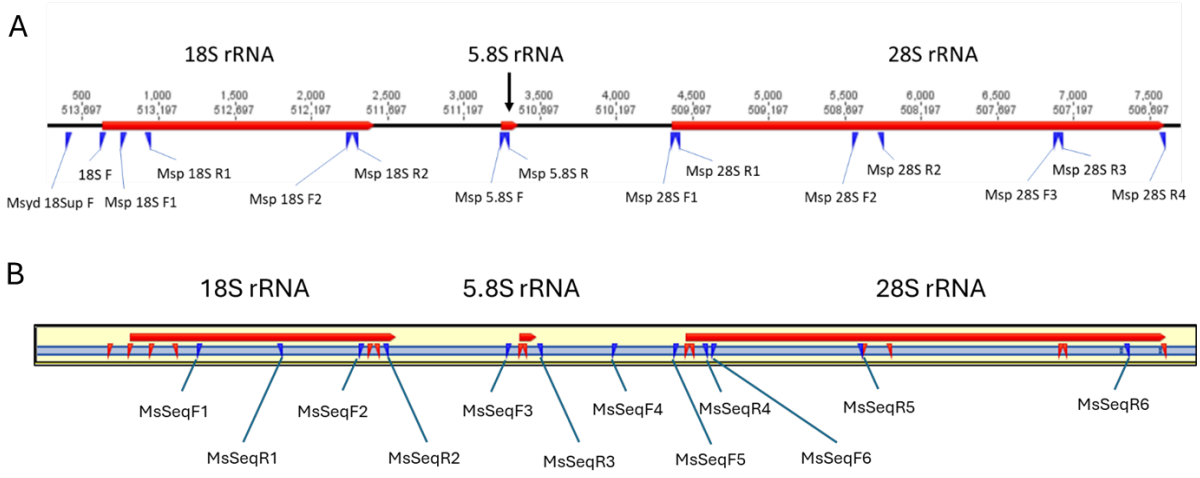

Figure 1. Locations of PCR (A) and sequencing primers (B) on the rRNA array.

Sanger sequencing was conducted using the primers listed in Table 3 as well as the additional internal sequencing primers listed in Table 4. The locations of the sequencing primers on the M. sydneyi rRNA array are shown in Figure 1B.

Table 4. Sequencing primers used in this study

Primer name	Primer Sequence (5'-3')
MsSeqF1	CGAGTGAGAACGTATTGTCGG
MsSeqR1	CGGTCGGCATAGTTTGGG
MsSeqF2	GGATTGCAACTGTTCCTCGTG
MsSeqR2	CCACCATCGACGTTCAGAAG
MsSeqF3	TCCGTCTCATGCAATCGTCG
MsSeqR3	GTATCCGGCTCTCGACCG
MsSeqF4	GACGAAGAGCAGTCGATCG
MsSeqR4	GTCGTCTGACGTCACAACTCC
MsSeqF5	GTGTAGTTCTGTTTGTTGTGGC
MsSeqR5	CGTAGATGATGCCGACTTCCG
MsSeqF6	TCGCCATAGAGAGCGAACG
MsSeqR6	CCAGGATAGGAAGAGCCGACA

ONT sequencing was used to resolve multiple sequence types within the one sample and was performed on additional samples from the same estuaries as outlined in Table 5. Amplicon sequencing libraries were generated directly from diluted PCR reactions using the Native Barcoding Kit 96 V14 (Oxford Nanopore Technologies, Oxford UK). Amplicon sequencing was performed on a v10.4.1 MinION or Flongle flowcell. Raw sequencing reads were quality filtered using fastp v0.24.1 (Reference: https://doi.org/10.1093/bioinformatics/bty560) and chopper v0.10.0 (Ref: https://doi.org/10.1093/bioinformatics/btad311). Consensus sequences were generated from filtered reads using Amplicon_sorter v2025-05-28 (Ref: https://doi.org/10.1002/ece3.8603).

Sequence assembly and alignment

Sequences of individual amplicons from each sample were aligned into contiguous sequences using the de novo assembly option and each contig was then aligned against the *M. sydneyi* rRNA array derived from the genome sequence using Geneious R7.1.

Table 5. Samples subjected to Oxford nanopore (ONT) sequencing

Estuary	Samples sequenced
Hawkesbury River, NSW	M16-04749-6, M16-04749-13
Georges River, NSW	M16-06827-85, M16-06827-88
Brunswick River, NSW	M16-06983-41, M16-06983-43, M16-06983-44
Macleay River, NSW	M20-05667-13, M20-05667-16, M20-05667-18, M20-05667-19, M20-05667-20, M20-05667-24
Richmond River, NSW	M20-06326-17, M20-06326-20, M20-06326-24, M20-06326-25, M20-06326-27, M20-06326-24, M21-11844-24, M21-11844-25, M21-11844-33
Stradbroke Island, QLD	M22-17260-16, M22-17260-24, M22-17262-10, M22-17475-7
Port Stephens, NSW	M21-12915-14, M21-12915-21, M23-06441-24

Selective breeding program

Previous incorporation of Richmond River Rock Oysters into the SRO breeding program

In 2018 NSW DPIRD produced the first breeding line incorporating RRRO genetics by mass spawning Rock Oysters collected from the Richmond River and assessed the QX resistance of the line in the Hawkesbury River in the 2018-2019 QX season. Survival of this line was 89.8%, however, QX disease was not detected during the exposure trial, and the survival of the line did not differ significantly from the controls. In 2020, this RRRO line was utilised in the SRO breeding program to produce four pair mated families for additional assessment for QX disease resistance. Assessment of these families for one adult exposure to QX disease in 2022, gave between 51 to 95% survival depending on the family. In 2022, the original 2018 RRRO breeding line and the higher ranking QX resistant RRRO families produced in 2020, were crossed with high-ranking families in the DPIRD SRO breeding program, to produce 17 SRO families incorporating RRRO genetics. These families formed the basis for this project and underwent assessment for QX disease resistance in this project (see further details below).

Incorporation of Richmond River Rock Oysters into the Sydney Rock Oyster breeding program in this study – 2023-year class

An objective of this project was to create a minimum of 10 families, in addition to those created above, that have a parent sourced from RRROs. Do to this, in 2023, 12 families were created by crossing the high-ranking families in the SRO breeding program, with RRRO parents. Specifically, five families were produced utilising the higher-ranking QX resistant RRRO families produced in 2020 and a further seven families were produced utilising new RRRO genetics.

Assessing QX disease resistance and performance in the 2022 and 2023-year class RRROs families

Assessments for QX disease resistance in the 2022 and 2023-year class families were conducted following a single QX disease exposure of spat and a single QX disease exposure of adults. Assessments were done at three QX exposure sites, Karuah River (Port Stephens, NSW), Tilligerry Creek (Port Stephens, NSW) and Lime Kiln Bar (LKB, Georges River, NSW) (Figure 2).

In addition to QX disease resistance, the 2022 and 2023-year class families were also assessed for critical performance traits of growth and meat condition. A field trial was set up for each year class in Cromarty Bay (Port Stephens, NSW) according to the deployment and sampling schedule in Table 6. For each trial, three replicates of 50 oysters per family were stocked in 8mm mesh trays and a subset of 12 oysters were sampled at two timepoints during the trial to assess their growth (total weight) and meat condition.

For the 2023 families, a fourth adult QX assessment site in Coba Bay (Hawkesbury, NSW) was set up with a sub-set of families and run by industry. Across each exposure site, three replicates of 50 oysters per family were stocked in trays or Zapco baskets and their survival was monitored according to the deployment and sampling schedule in Table 6. Briefly, spat were deployed when they were approximately 2-3 months old and their final QX survival assessment was measured when they were approximately 9 months old. Adults were deployed when they were approximately 10-11 months old and their final QX survival assessment was measured when they were approximately 20-21 months old.

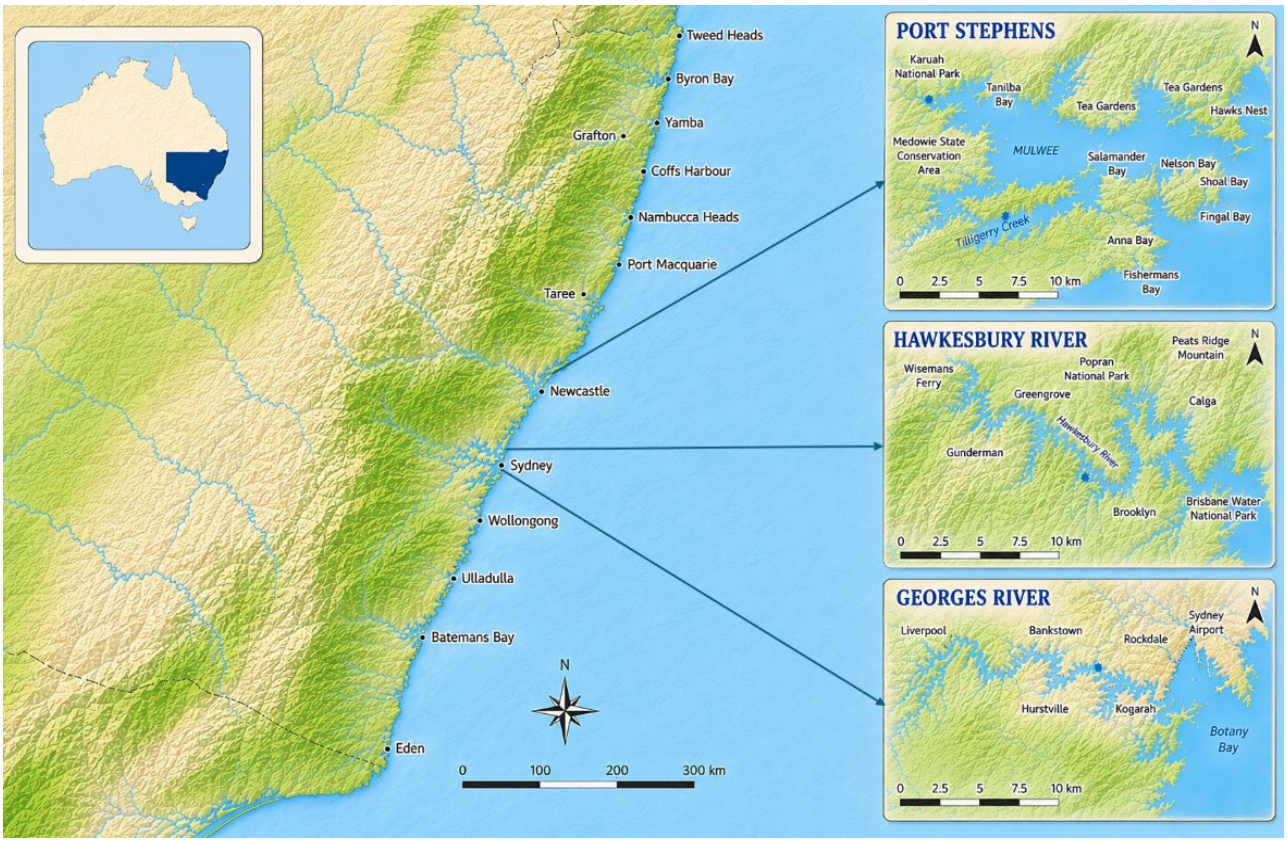

Figure 2. Location of QX and growth and condition field trials in NSW.

Survival and performance data from each trial was uploaded into the CSIRO SRO Selective Breeding Database to calculate the Estimated Breeding Value (EBV) for QX survival, growth/ total weight and meat condition for each family. EBVs of families with RRRO genetics were compared with those without RRRO genetics produced in the same year class.

Table 6. Deployment and measurement schedule of QX disease resistance and growth and condition assessment trials for the 2022- and 2023- year class SRO Breeding Program families

Field trial	Deployment	Measurement(s)	Notes
YC2022 QX Spat	December 2022	July 2023	Low prevalence of QX disease recorded in Tilligerry in 2023 season (based on Port Stephens QX Surveillance data).
YC2022 QX Adult	August – September 2023	July 2024	QX disease not recorded in Tilligerry in 2024 season and low prevalence of QX disease in Karuah. Oysters from LKB sent for QX disease assessment via qPCR on the 22/03/2024. Infection confirmed in 80%of individuals.
YC2023 QX Spat	January 2024	July 2024	QX disease not recorded in Tilligerry in 2024 season and low prevalence of QX disease in Karuah (based on Port Stephens QX Surveillance data).
YC2023 QX Adult	August – September 2024	June - July 2025	Low prevalence of QX disease recorded in Tilligerry in 2025 season (based on Port Stephens QX Surveillance data).
YC2022 Growth and Condition	August 2023	December 2024 May 2024	
YC2023 Growth and Condition	September 2024	January 2025 May 2025	

NB: YC = Year Class; Only final QX survival measurement dates included in table

Results and Discussion

Genetic typing of *M. sydneyi*

Sampling of low-risk estuaries

A total of 1,809 oysters were sampled across three estuaries at low risk of QX disease ie: estuaries where the parasite had been detected by molecular means in prior studies (Adlard and Wesche 2005). Estuaries where the prevalence of the parasite was previously determined to be relatively high (Table 2) were specifically chosen. For Wallis Lake, sampling of oysters was undertaken across 5 locations where prior NSW DPIRD surveillance had indicated that the prevalence of *M. sydneyi* was highest (Figure 3), namely around Cockatoo Island, the Eastern side of Regatta Island, Wallamba Broadwater, the Western end of the Coolongolook River and between Wallis Island and Godwin Island (Figure 4A).

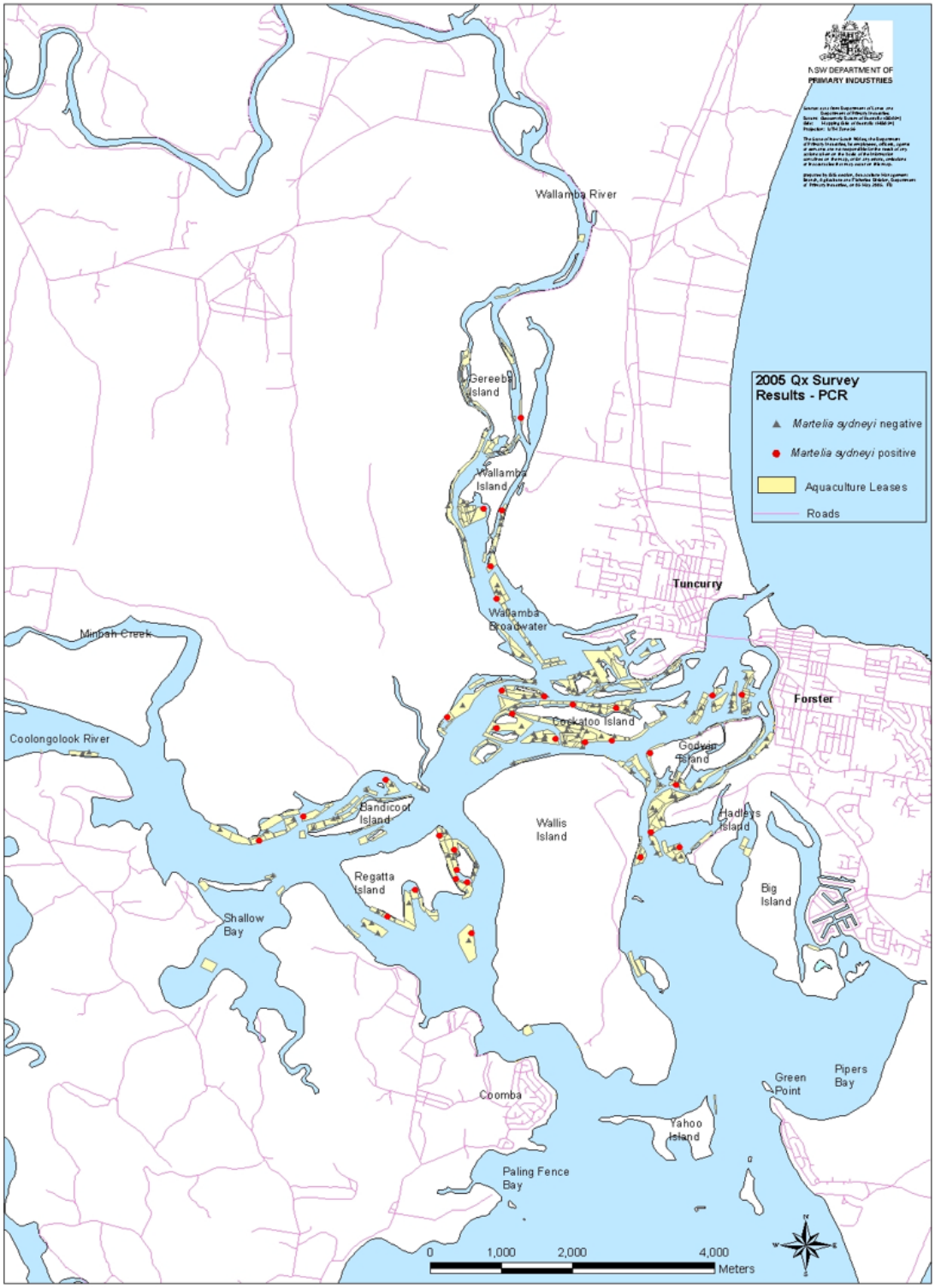

Figure 3. Sampling locations and results of the 2005 PCR survey of *M. sydneyi* in Sydney rock oysters collected from Wallis Lake (NSW DPI, 2005). Grey triangles indicate *M. sydneyi* negative oysters; red circles indicate *M. sydneyi* positive oysters.

For Tuross Lake and Wagonga Inlet where information on locations of prior positive detections was not available, 50 random collection points were generated across known cultivation areas of each estuary

shown in Figures 4B and 4C. A mix of wild and cultivated SROs were collected from each estuary as detailed in Table 7. Cumulative rainfall totals in the week and fortnight prior to each sampling period are also shown in Table 7. Significant rainfall occurred in the fortnight prior to each sampling, with 20 to > 100 mm of rain recorded locally, suggesting that seasonal conditions were conducive to transmission of *M. sydneyi* to SROs.

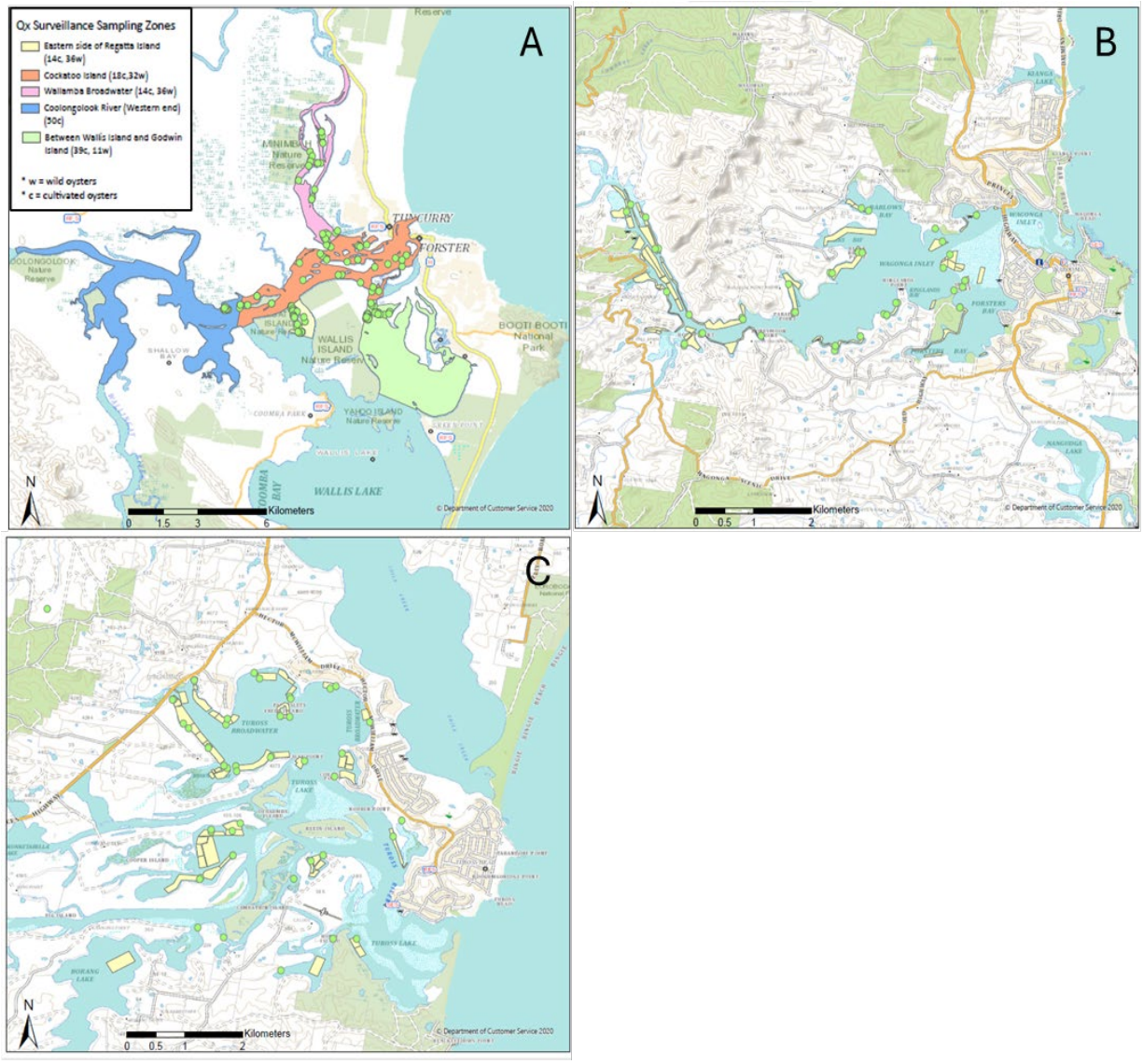

Figure 4. Maps of the low risk estuaries sampled in this study including the northern end of Wallis Lake including Wallamba Broadwater, around Wallis Island and the Coolongoolook River (A), Wagonga Inlet from Forsters Bay through to Frenchmans Gully (B) and Tuross Lake including Tuross Broadwater, Bowns Bay and Horse Island (C) with individual sampling locations indicated by green circles.

PCR screening of SROs collected from low-risk estuaries

All 1,809 SROs collected from 3 estuaries at low risk of QX disease and across three sampling years were screened for *M. sydneyi* using a qPCR method developed in project FRDC project 2021-129. All tests met the pre-defined test quality criteria and were therefore deemed valid. All 1,809 oysters tested negative for *M. sydneyi*. As the *M. sydneyi* qPCR is known to be significantly more sensitive method for detection of the parasite than cytology (Jenkins et al. 2023), the smears prepared from these oysters were not further examined.

Development of a genetic typing scheme for *M. sydneyi* using available genome data

Mapping of Marteilia *sequences to the* M. sydneyi *rRNA array*

The annotation of the *M. sydneyi* genome was complicated due to the divergent nature of this paramyxid parasite and the lack of homology between predicted open reading frames and known genes with described functions. Thus, the annotated genome contains many hypothetical genes. Studies on other paramyxids have involved the ribosomal RNA array and this region has been used recently to delineate the closely related species *M. refringens* and *M. pararefringens*. The availability of data for the rRNA array for a range of other *Marteilia* spp. also facilitated sequence comparisons. The sequence region was also one area of the *M. sydneyi* genome which was readily identifiable in the annotation. The rRNA array contains a mix of conserved rRNA genes and variable internal transcribed spacer regions suitable for delineation of both closely related and less closely related species. For these reasons, the rRNA array was selected for the purposes of genetic typing.

Table 7. Samples collected and cumulative rainfall recorded in the week and fortnight prior to sample collection from estuaries at low risk of QX disease

Estuary	Date sampled	No. wild oysters	No. cultivated oysters	Total oysters sampled (n)	Cumulative rainfall in the week prior to sampling(mm)	Cumulative rainfall in the fortnight prior to sampling (mm)
Wallis Lake	May 2023	75	75	150	27.4 mm	64.6 mm
	March 2024	115	135	250	23.2 mm	51.4 mm
	March 2025	160	95	255	8 mm	72 mm
Wagonga Inlet	May 2023	75	75	150	2 mm	21.7 mm
	March 2024	125	125	250	92.2 mm	95.2 mm
	April 2025	128	124	252	0 mm	105.4 mm
Tuross Lake	March 2024	140	110	250	78.4 mm	87.2 mm
	April 2025	128	124	252	115.6 mm	117.4 mm
Total		**946**	**863**	**1,809**		

Primers for genetic typing of Marteilia strains

Primers were designed to target all *Marteilia* spp. to account for potential sequence differences, particularly given that the *Marteilia* sp. previously identified in SROs from estuaries at low risk of QX disease had not been sequenced. While the rRNA genes from reference *Marteilia* strains shared significant sequence identity with *M. sydneyi*, the ITS regions mapped poorly due to extensive sequence variability in these regions (Figure 5).

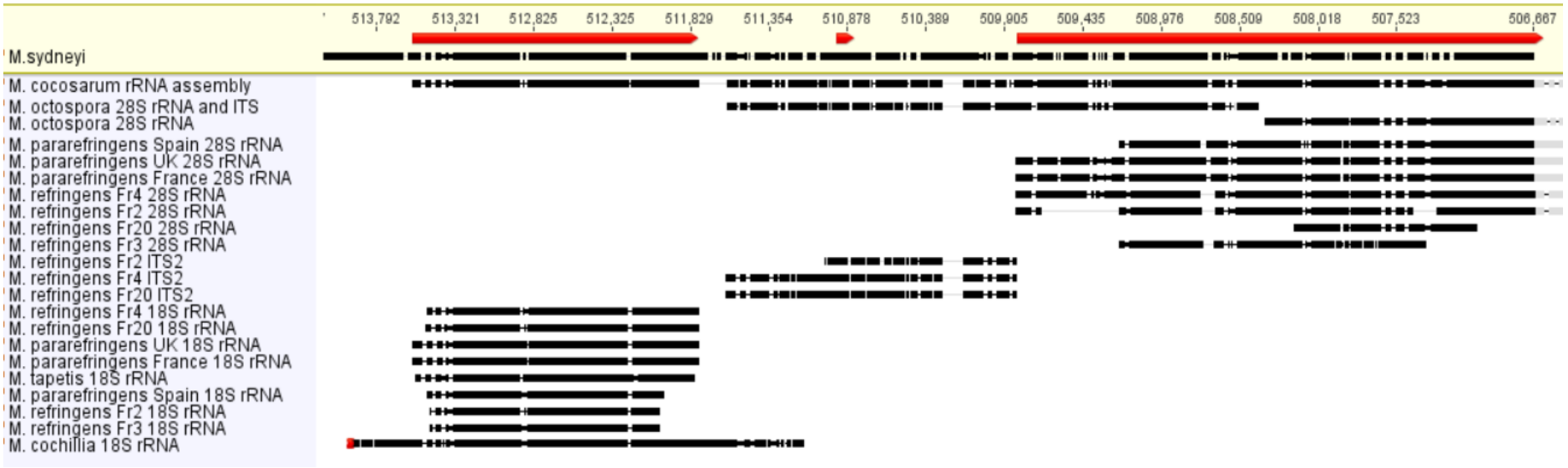

Figure 5. Sequences from reference *Marteilia* sequences mapped to the rRNA array of *M. sydneyi*.

A series of PCR assays were designed using the rRNA alignment of *Marteilia* sequences to amplify across the length of the rRNA array; however, because of the sequence variability across *Marteilia* spp. within the ITS regions, primer target sequences were all within the rRNA genes but allowing amplification across the ITS regions in addition to the rRNA genes (Figure 1).

PCR amplification of the rRNA array

The amplicon pairs used for sequence typing and the length of each amplicon is given in Table 8. All archival samples tested amplified successfully in each of the PCR assays.

Table 8. Genomic regions covered and predicted amplicon length for each primer pair

Primer pair	Genomic region covered	Predicted amplicon length
Msyd 18S FUp/Msp 18S R1	Upstream of 18S rRNA and 5' end of 18S rRNA	547 bp
18S F/ Msp 18S R1	5' end of 18S rRNA	314 bp
Msp 18S F1/Msp 18S R2	18S rRNA	1542bp
Msp 18S F2/Msp 5.8S R	3' end of 18S rRNA and ITS1	1059 bp
Msp 5.8 F/Msp 28S R1	5.8S rRNA and ITS2	1163 bp
Msp 28S F1/Msp 28S R2	5' end of 28S rRNA	1382 bp
Msp 28S F2/Msp 28S R3	28S rRNA	1357 bp
Msp 28S F3/Msp 28S R4	3' end of 28S rRNA	713 bp

Sequence comparisons

Comparison of *M. sydneyi* strains from high and medium risk estuaries to those from low-risk estuaries was not possible due to the lack of detection of *M. sydneyi* in SROs from low-risk estuaries. However, sequencing of the rRNA array of *M. sydneyi* was undertaken from archived material to determine the genetic diversity of *M. sydneyi* within high-risk estuaries and between high and medium-risk estuaries.

Comparison of the sequences to the reference *M. sydneyi* rRNA array (ie: obtained from genome sequencing of purified sporonts from SROs obtained from the Richmond River in 2020) suggests only limited sequence diversity amongst *M. sydneyi* strains in high and medium QX risk estuaries. All 18S rRNA, 5.8S rRNA and 28S rRNA sequences were identical across sampling locations and where examined, within a sampling location (i.e. where there were multiple samples examined from the same location). However, ribosomal rRNA genes can be inadequate for identification of cryptic species; indeed, the more variable ITS 1 and ITS 2 regions have been used to discriminate *Marteilia* refringens "O" and "M" types and designate these as different species, *M. refringens* and *M. pararefringens* (Kerr et al. 2018). A total of 32 candidate signatures that differentiate *M. refringens* and *M. pararefringens* including 11 in the ITS 1 region, 12 in the ITS 2 region and 1 in the 28S rRNA gene, with the remaining sequence variations in the IGS region after the 3' end of the 28S rRNA gene (not examined here) were identified in that study. Despite this, only 5 sites within the ITS 1 region were found to be invariant within each species when all available sequence data was included in the analysis (Kerr et al., 2018). While some sequence variation was observed in both the ITS 1 and ITS 2 regions of the *M. sydneyi* samples examined in this study (Table 9), these single nucleotide variations (SNVs) largely represented within-sample variations, with Sanger sequencing revealing "double peaks" at the variant sites (Figure 6A). Two of the 3 samples sequenced from Queensland (Stradbroke Island) contained a number of SNVs relative to the reference sequence, but without any evidence from Sanger sequencing of within-sample variation on the chromatograms (Figure 6B).

Table 9. Single nucleotide variations observed in the rRNA array of *M. sydneyi*

Sample	Estuary of origin	Alignment position	Gene region	Ambiguity
M16-04749-6	Hawkesbury River	1,912	ITS 1	A or T
		2,580	ITS 1	A or G
		3,309	ITS 2	A or C
M16-6827-85	Georges River	1,912	ITS 1	A or T
		3,309	ITS 2	A or C
M16-06983-41	Brunswick River	3,309	ITS 2	A or C
M20-05667-13	Macleay River	1,912	ITS 1	A or T
		3,309	ITS 2	A or C
		2,540	ITS 1	A or G
M16-06326-17	Richmond River	1,912	ITS 1	A or T
		3,309	ITS 2	A or C
M21-11844-24	Richmond River	1,912	ITS 1	A or T
		2,540	ITS 1	A or G
		3,309	ITS 2	A or C
M22-17260-24	Stradbroke Is	3,309	ITS 2	A or C
		3,430-3,454	ITS 2	Deletion
		3,679	ITS 2	G or T
M22-17262-10	Stradbroke Is	3,309	ITS 2	A → C
		3,324	ITS 2	C → A
		3,344	ITS 2	A → C
M22-17475-7	Stradbroke Is	3,309	ITS 2	A → C
		3,344	ITS 2	A → C
		3,430-3,454	ITS 2	Deletion
		3,740	ITS 2	C or T
M23-06441-24	Port Stephens	1,219	ITS 1	A or T
		2,540	ITS 1	A or G
		2,580	ITS 1	A or G
		3,309	ITS 2	A or C
		3,344	ITS 2	A or C
		3,430-3,454	ITS 2	Deletion

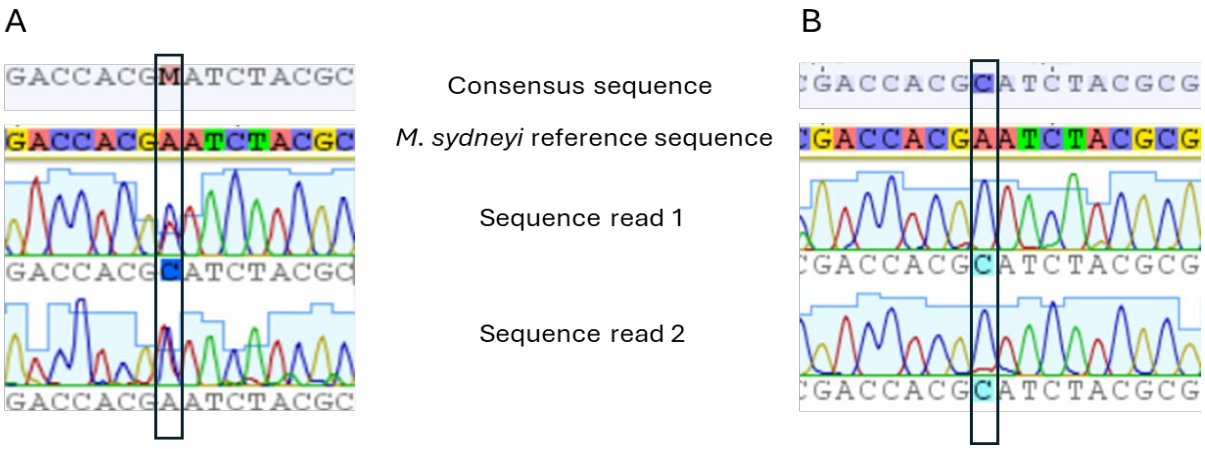

Figure 6. (A) An example of within-sample sequence variation within the ITS 2 region of the *M. sydneyi* rRNA. (B) An example of a SNV relative to the reference *M. sydneyi* sequence with no evidence of within-sample variation.

While these SNVs varied relative to the reference sequence, variations at these sites were not unique, with samples from other locations also containing these variants. In addition to the SNVs identified, Sanger sequencing revealed a 25 bp deletion in the ITS2 region (at positions 3430-3454) in 2 out of the 3 samples sequenced from Queensland and the sample from Port Stephens (Table 9), raising the question of whether these represented distinct strains. Close inspection of the sequence traces from the other samples revealed that some samples had low quality sequence between positions 3430 and 3454, with double peaks evident.

To determine whether sequence variants both with and without the ITS2 deletion existed in the remaining samples as well as additional samples from the same estuaries, the region of interest was amplified using primers Msp 5.8S F and Msp 28S R1 and then subjected to ONT sequencing. The presence/absence of the deletion as well as the proportion of reads containing the ITS2 indels was determined and is shown in Table 10. All individual samples contained both ITS2 sequence variants (with and without the deletion), including those in which initial Sanger sequencing had implied either an insertion or a deletion as indicated in Table 9. The insertion/deletion was present in close to equal proportions of ONT reads for most of the samples. Samples from Stradbroke Island contained the biggest variation in the proportion of within-sample sequence variants with between 12% and 75% of samples containing the insertion respectively. There was otherwise no discernible pattern in the proportion of sequence variants across estuaries.

It should be noted that the indel in the *M. sydneyi* ITS2 region is located between a set of direct 7-bp repeats (Figure 7) and the deletion of this region in some sequences may have arisen via slip-strand mispairing during DNA replication. As such, the likelihood of a mutational event at this site may be higher.

The presence of SNVs and the ITS2 indel within (rather than between) individual samples could have a number of different explanations, including the presence of heterozygous variants, the presence of multiple copies of the ITS2/rRNA array in the *M. sydneyi* genome arising from gene duplication events, or allelic variation of *M. sydneyi* within individual samples (infection with polyclonal parasites). While the presence of heterozygous variants could explain the proportion of within-sample reads being close to 50%, this would require the lifecycle stage of *M. sydneyi* within the oyster to be diploid; however, preliminary K-mer analysis of the draft *M. sydneyi* genome suggests that purified sporonts of *M. sydneyi* are haploid (D. Bogema, personal communication). Furthermore, parasitic protozoa are frequently haploid or are haploid for the majority of their lifecycle. Lifecycle stages responsible for dispersal, such as the sporont phase of *M. sydneyi*, are typically in the haploid state (Nuismer and Otto, 2004) making this explanation less likely. Similarly, preliminary assemblies of the *M. sydneyi* genome did not identify multiple copies of the ITS2 region or rRNA

Table 10. Percentage of ONT reads within each sample containing the ITS2 insertion.

Estuary	Sample ID	Total number of reads	% reads with ITS2 insertion
Hawkesbury River	M16-04749-6	27,004	63%
	M16-04749-13	24,304	53%
Georges River	M16-06827-85	30,784	63%
	M16-06827-88	26,382	53%
Brunswick River	M16-06983-41	22,010	51%
	M16-06983-43	27,273	59%
	M16-06983-44	18,208	62%
Macleay River	M20-05667-13	3,837	57%
	M20-05667-16	95	36%
	M20-05667-18	4,799	54%
	M20-05667-19	4,874	45%
	M20-05667-20	13,064	48%
	M20-05667-24	4,428	42%
Richmond River	M20-06326-17	27,145	66%
	M20-06326-20	38,199	65%
	M16-06326-24	7,276	70%
	M16-06326-25	6,282	53%
	M16-06326-27	7,464	68%
	M21-11844-24	26,666	67%
	M21-11844-25	27,224	61%
	M21-11844-33	31,473	61%
Stradbroke Island	M22-17260-16	7,682	49%
	M22-17260-24	5,992	37%
	M22-17262-10	4,506	75%
	M22-17475-7	6,730	12%
Port Stephens	M23-06441-24	6,764	44%
	M21-12915-14	8,145	55%
	M21-12915-21	6,401	57%
	M21-12915-24	7,928	34%

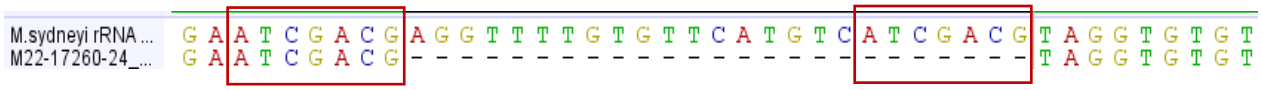

Figure 7. Alignment showing the 25bp indel in the ITS2 region of *M. sydneyi*. The 7-bp direct repeats are boxed in red.

array in the genome with microheterogeneity between copies, although this requires confirmation with an improved genome assembly. Allelic variation due to infection with multiple genetically distinct strains may explain the presence of within-sample SNVs and ITS2 variants. Polyclonal infections are known to be more common in situations where transmission rates are high; however, sequence variants were present within samples from all estuaries where *M. sydneyi* was detected, including the Brunswick River (a medium risk estuary).

Unfortunately, no *M. sydneyi* was detected in the low QX risk estuaries examined, despite extensive sampling over 3 seasons; therefore, comparison of *M. sydneyi* sequences from these estuaries was not possible. The lack of *M. sydneyi* detection in these estuaries despite reports of relatively high PCR prevalence in prior investigations (up to 25% of samples tested, Table 2) is noteworthy. There could be a number of explanations for the lack of molecular detections in this study, including seasonal factors (low *M. sydneyi* prevalence during the seasons and estuaries examined) or the use of an updated *M. sydneyi* PCR in this study. While SRO infection with *M. sydneyi* does vary with seasonal factors, this study was designed to detect *M. sydneyi* during peak periods of activity (March-May) and tested samples over 3 seasons. During this period QX disease was known to be active in other (high-risk) estuaries in NSW, indicating that general conditions were conducive to infection. Previous testing of samples from low-risk estuaries relied on a conventional PCR and electrophoretic detection of amplicons; however, no sequence confirmation of *M. sydneyi* presence was undertaken and/or recorded. As such, it is mpossible to confirm that *M. sydneyi* was being specifically detected in these prior studies. It therefore remains possible, that low risk estuaries do not harbour *M. sydneyi*. Indeed, during the course of this study, an unrelated investigation identified a novel *Marteilia* species related to *Marteilia tapetis* in mussels from the south coast of NSW (Ngo, 2025). Similarly, a parasite morphologically consistent with *Marteilia* sp. was also detected in wild caught flat oysters (*Ostrea angasi*) sampled from Bermagui and Narooma at prevalences of around 1% (Heasman et al., 2004). The specific identity of the latter parasite has not been determined but given that other *Marteilia* species clearly exist in NSW estuaries, it is therefore possible that prior molecular detections of *Marteilia* in SROs from low-risk estuaries were of another species. An alternative hypothesis is that *M. sydneyi* was not detectable in SROs collected during this study but may have been present in the alternate host(s). This hypothesis seems less likely given that seasonal conditions conducive to *M. sydneyi* transmission to SROs were present across all 3 years, including sampling following significant rainfall events and during the peak period for QX disease (March-May). Furthermore, in the case of Wallis Lake, SROs were sampled from specific locations within the estuary that had historical molecular detections of *Marteilia* (Figure 3 and 4A).

Perhaps the simplest explanation for the earlier molecular detections of *Marteilia* in the low-risk estuaries re-examined here is that the positive oysters had been translocated from areas were QX disease was enzootic. Indeed, the prior survey work was undertaken before the establishment of formal risk rankings for NSW estuaries; therefore, translocation of infected stock was likely. Furthermore, it is not stated in these previous reports whether the positive detections were in cultivated or wild oysters, which is an important omission as detections in wild oysters would indicate local endemicity of the parasite, while detections in cultivated oysters would favour translocation as an explanation. The lack of positive detections in the current study suggests that even if *M. sydneyi* were introduced to low-risk estuaries previously, the parasite has failed to establish locally, which is not unexpected for parasites with complex lifecycles. Findings from the prior survey work suggested that the observed parasite existed only in immature “aberrant” forms, with no evidence of sporulation, which is consistent with the idea that if *M. sydneyi* was present, it was unable to complete its lifecycle under the conditions encountered in low-risk estuaries.

While this study suggests that there may be some variability in *M. sydneyi* strains, we found little evidence for the presence of cryptic species in SROs from medium and high-risk estuaries, as has been established for *M.*

refringens and *M. pararefringens* in Europe. Firstly, while co-infections can occur, *M. refringens* and *M. pararefringens* display different host preferences (*M. refringens* for oysters and *M. pararefringens* for mussels), and pathogenicities in their respective hosts, and there is clear geographical separation of the two species with only *M. pararefringens* occurring in northern Europe (Kerr et al., 2018). Consistent sequence differences in the ITS1 and ITS2 regions of *M. refringens* and *M. pararefringens* have also been described. In contrast, there were no consistent differences discernible in the *M. sydneyi* sequence variants observed in this study, as all samples contained within-sample variations. In addition, no geographical differences were observed in the distribution of *M. sydneyi* sequence variants detected here, and prior literature has not identified these parasites in any other bivalve species, so there is no evidence for variant strains infecting different hosts.

Selective breeding program

Performance of 2022-year class families

Survival following a single QX exposure of spat during the 2023 QX season

QX surveillance data for the 2023 season confirmed the presence of QX disease in Karuah River and low prevalence in Tilligerry Creek. No surveillance or pathology examination was done to confirm the presence of QX disease in LKB, however, annual outbreaks of QX disease at this site have historically been observed (Reid and Bone. 2020), suggesting a high risk of exposure during the trial period.

Survival of the 2022-year class spat during the 2023 QX season was high across all sites, with a mean estimated breeding value (EBV) for spat survival following a single confirmed (Karuah and Tilligerry Creek) and presumed (LKB) QX exposure of 82 ± 0.01%. Despite, this high overall survival, survival of spat differed among the families (Figure 8), with the mean percentage survival of families ranging from 50 – 99% in Tilligerry Creek, 60 – 97% in Karuah River, and 55 – 99% in LKB (Fig. 2). As QX disease prevalence was low or unconfirmed at some sites, survival patterns should be interpreted cautiously and may not solely reflect responses to QX disease exposure.

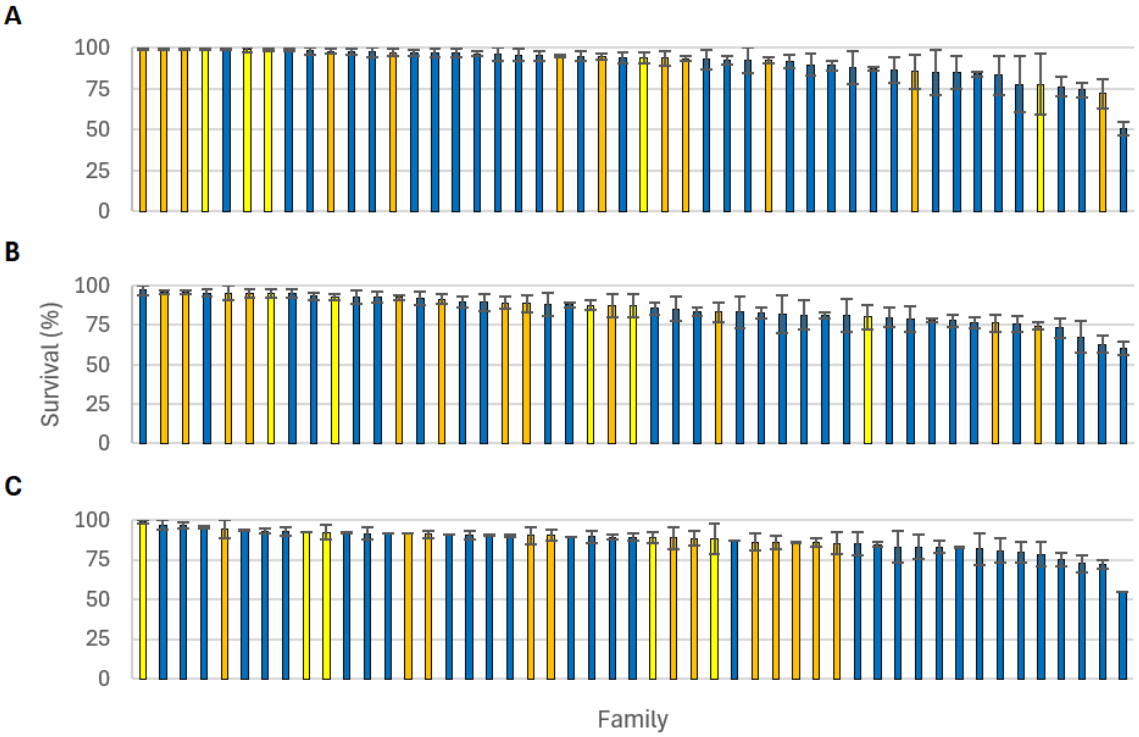

Figure 8. Mean percentage survival of the 2022-year class families following a single QX disease exposure of spat during the 2023 season at A) Tilligerry Creek, Port Stephens; B) Karuah River, Port Stephens; and C) Lime Kiln Bay, Georges River. Blue bars represent non-RRRO families, yellow bars represent YC2018 RRRO families and orange bars represent YC2020 RRRO families.

This result was like those found in previous year classes, with different levels of QX disease resistance measured among families in all past QX exposure trials. Many of the RRRO families had high spat survival following both confirmed and presumed exposure to QX disease, with 4 – 7 of the top 10 surviving families having a RRRO parent, depending on the exposure site. Given that the RRROs were crossed with

the highest-ranking QX-resistant families in the SRO breeding program, the high survival observed among some RRRO-derived families may reflect, in part, the contribution of the SRO breeding program parent. However, not all RRRO families ranked highly for QX survival, with several families falling within the bottom third of rankings (Figure 8). This highlights the potentially high variability of genetics in the RRROs and the importance of structured crossing/ incorporation of RRROs with SROs of known QX performance to better manage these risks. Overall, the mean EBV for spat survival of the RRRO families across the sites was 87.09% ± 0.01, compared to 79.33 ± 1.95%, indicating that the mean spat survival of the RRRO families was higher than the non-RRRO families.

Survival following a single QX exposure of adults during the 2024 QX season

QX surveillance data for the 2024 season indicated a low prevalence of QX disease in the Karuah River and no detection in Tilligerry Creek. In contrast, qPCR examination confirmed QX infection in oysters from LKB, with infection detected in 80% of individuals tested.

Survival of the 2022-year class adults during the 2024 QX season was lower than that observed for spat, with a mean EBV for adult survival following a single confirmed (LKB) or presumed (other sites) QX exposure of 69 ± 0.02%. Lower survival in adults relative to spat has been consistently observed in previous year classes, with gains in adult survival tracking at the same trajectory but below those of spat.

Comparison among sites showed higher survival at the Port Stephens locations (Tilligerry Creek and Karuah River) relative to LKB (Figure 9). Given that QX disease was not detected in Tilligerry Creek and was present at low prevalence in the Karuah River, survival differences at these sites may be attributed to differences in QX exposure intensity.

As observed in spat, survival among families varied (Figure 9), ranging from 53–98% in Tilligerry Creek, 51–99% in the Karuah River, and 6–85% in LKB. Between five and seven of the top 10 families at each site had an RRRO parent. However, variability was again evident, with some RRRO families ranking in the bottom third.

The mean EBV for adult survival among RRRO families was 78.24% ± 0.03, compared to 63.99 ± 2.91% for non-RRRO families. While RRRO families generally showed higher survival, it is important to note that non-RRRO families have been selectively bred to balance improvements in QX resistance with the maintenance of genetic diversity. As such, relatively lower survival in these families is expected.

Performance of the 2023-year class families

Survival following a single QX exposure of spat during the 2024 QX season

No useful data was obtained for the 2023-year class QX spat survival. As mentioned above, in the Port Stephens sites, this was due to no detection of QX disease in Tilligerry Creek in the 2024 season and only low prevalence of QX disease detected in Karuah River. In LKB, high mortality was observed in the spat due to factors others than QX disease i.e. unseasonal biofouling with barnacles and mussels, combined with flat worm infestation and heavy siltation.

Survival following a single QX exposure of adults during the 2025 QX season

QX surveillance data for the 2023 season confirmed the presence of QX disease in Karuah River and low prevalence in Tilligerry Creek. No surveillance or pathology examination was done to confirm the presence of QX disease in LKB and Coba Bay.

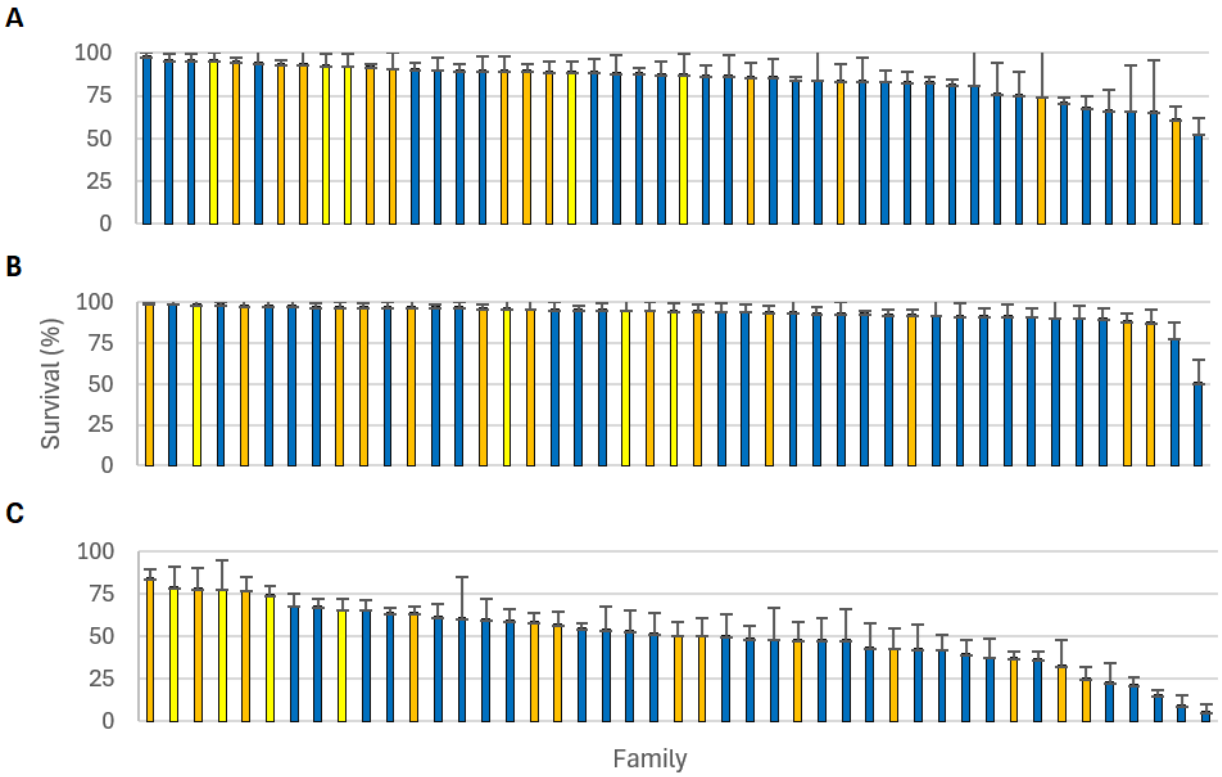

Figure 9. Mean percentage survival of the 2022-year class families across three sites following a single QX disease exposure of adults over the 2024 QX season at A) Tilligerry Creek, Port Stephens; B) Karuah River, Port Stephens; C) Lime Kiln Bay, Georges River. Blue bars represent non-RRRO families, yellow bars represent YC2018 RRRO families and orange bars represent YC2020 RRRO families.

Survival of the 2023-year class adults during the 2025 QX season was higher than both the 2022-year class spat and adults, with a mean EBV of 88 ± 0.01% for adult survival following a single confirmed (Karuah River) or presumed (other sites) exposure to QX. This likely reflects the cumulative genetic gains achieved through successive years of selection in the SRO breeding program, as well as potential additional gains associated with the incorporation of RRRO genetics into the program. As with the 2022-year class spat and adults, survival of adults from the 2023-year class varied considerably among the families at the Karuah River (43 – 96%), LKB (10 – 76%) and Coba Bay (11 – 81%) sites (Figure 10). Little variation in survival was observed among families at Tilligerry Creek (87 – 99%), likely reflecting the low prevalence of QX disease at this site in the 2025 season (based on QX surveillance data from Tilligerry Creek). Seven to eight of the top 10 survival families in Karuah River, LKB and Coba Bay, had a RRRO parent, indicating the potential for high QX resistance of these families. The lowest ranking family for survival in both LKB and Coba Bay, however, was a RRRO family, once again highlighting the variability in responses of the RRRO genetics and need to use caution when incorporating them into a breeding program (Figure 10). Overall, the mean EBV for adult survival of the RRRO families across the sites was 96.92 ± 4.69%, compared to 75.03 ± 0.03% in the non-RRRO families.

Growth and meat condition performance of the families

Growth and meat condition of the YC2022 and YC2023 families were assessed at Cromarty Bay, Port Stephens. QX surveillance did not detect the presence of QX disease at this site during the assessment periods.

The mean EBV for growth across the families was 23.81 ± 1.09% and 22.65 ± 1.24% for the YC2022 and YC2023 families, respectively, indicating that these families are growing, on average, 23.81% and 22.65% faster than wild SROs (Figures 11 and 12). The fastest-growing family had a growth EBV of 38.71% and was a non-RRRO family from the 2022-year class.

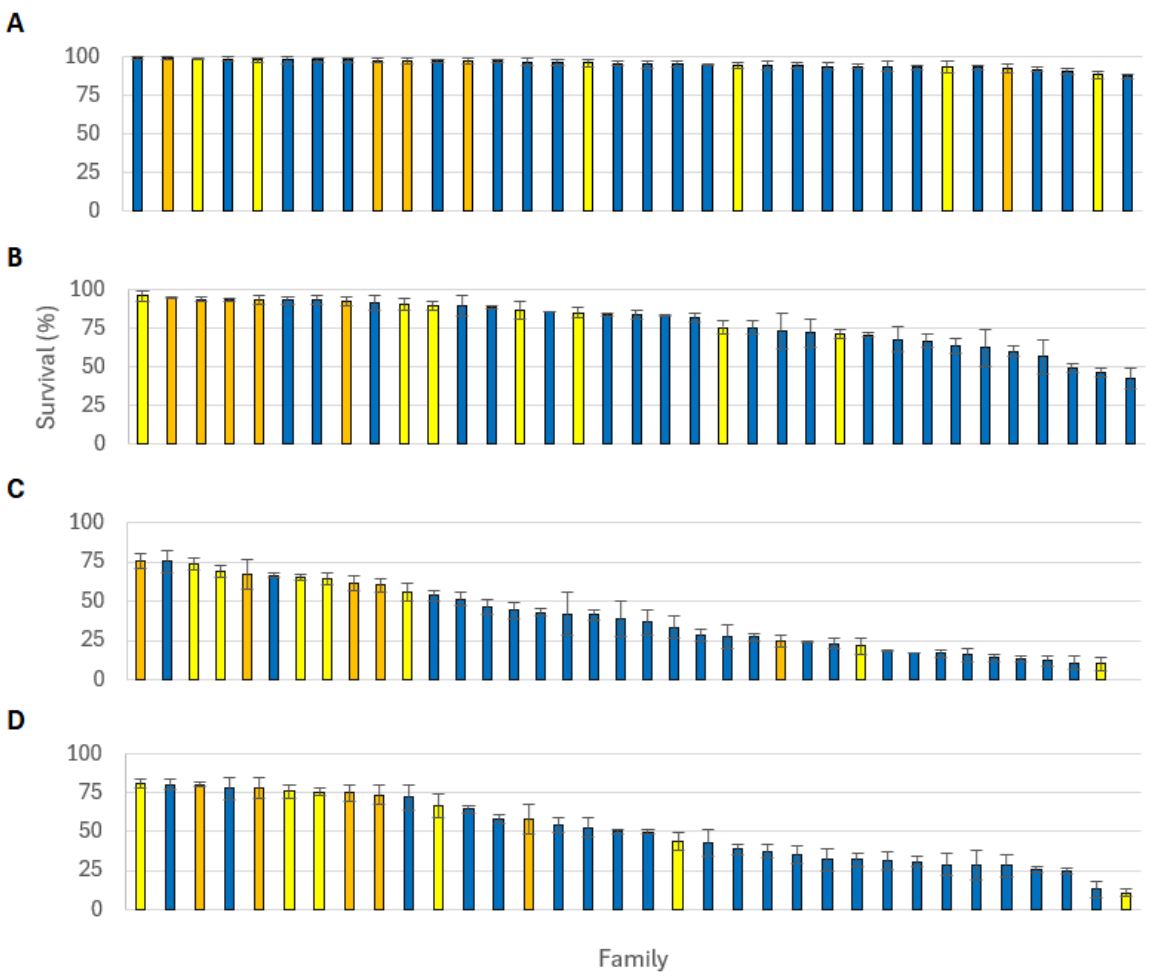

Figure 10. Mean percentage survival of the 2023-year class families across four sites following a single QX disease exposure of adults over the 2025 season at A) Tilligerry Creek, Port Stephens; B) Karuah River, Port Stephens; C) Lime Kiln Bay, Georges River; and D) Coba Bay, Hawkesbury River. Blue bars represent non-RRRO families, yellow bars represent new RRRO genetic families and orange bars represent YC2020 RRRO families.

Growth performance among families with RRRO genetics was variable across both year classes. While some of the top 10 ranked families for growth had a RRRO parent or grandparent (YC2022: 4/10; YC2023: 3/10), between 59% and 67% of families with RRRO genetics ranked in the bottom half of families for growth. In the 2023-year class, 7 of the 10 lowest-ranked families for growth had RRRO genetics (Figures 11 and 12). This suggests that some RRROs may exhibit reduced growth and as observed for QX resistance, highlights the need for caution when incorporating these lines into a breeding program. Reduced growth may represent a trade-off in some RRROs.

The mean EBV for meat condition was 0.49 ± 0.64% and 2.06 ± 0.72% for the YC2022 and YC2023 families, respectively. These values indicate that, on average, meat condition in these families is similar to that of wild oysters (meat condition EBV = 0%). However, substantial variability was observed among families, with EBVs ranging from -8.01% to 14.96% (Figures 11 and 12).

Many families with RRRO genetics exhibited high meat condition, with 6 of the top 10 ranked families having an RRRO parent or grandparent in both year classes (Figures 11 and 12). Nevertheless, variability in response was evident, with 6 families in the YC2022 year class and 3 families in the YC2023 year class displaying lower meat condition than wild oysters (i.e. EBV < 0%).

Family Rankings based on EBVs and SRO breeding program selection index

Top-performing families in the SRO breeding program are identified using a selection index that ranks families based on their EBVs for spat survival in high QX risk estuaries, growth (total weight), and meat condition, weighted at a ratio of 2:1:1, respectively. The top-ranking families are used to create the next generation and are more likely to be chosen for use in commercial hatchery runs than lower ranking families.

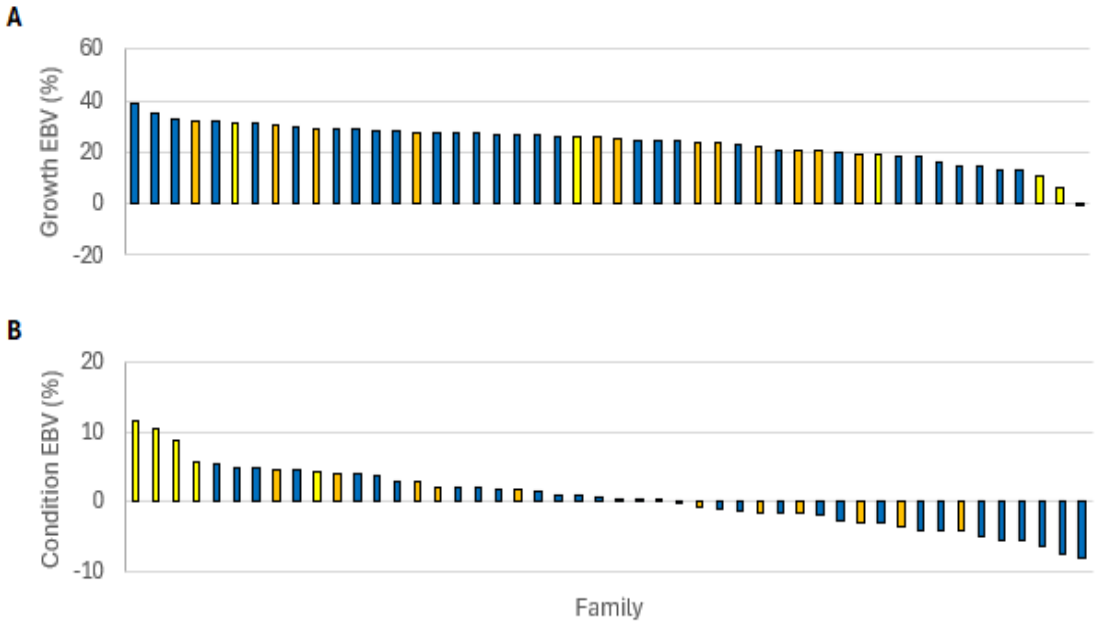

Figure 11. Estimated breeding values (EBVs) of the 2022-year class families for A) growth (weight selected) and B) meat condition. Families held at Cromarty Bay, Port Stephens. Blue bars represent non-RRRO families, yellow bars represent YC2018 RRRO families and orange bars represent YC2020 RRRO families.

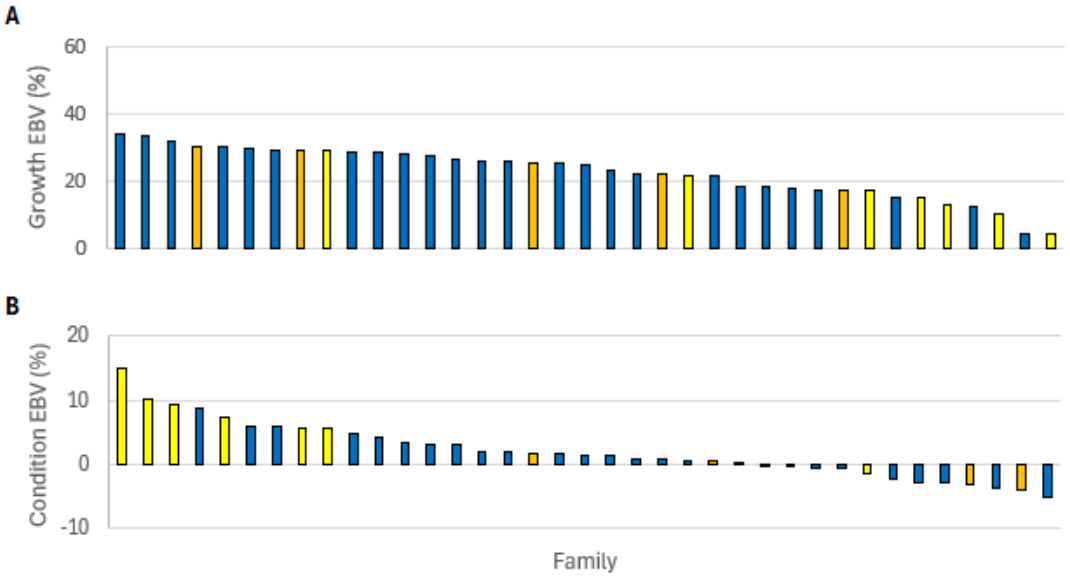

Figure 12. Estimated breeding values (EBVs) of the 2023-year class families for A) growth (weight selected) and B) meat condition. Families held at Cromarty Bay, Port Stephens. Blue bars represent non-RRRO families, yellow bars represent new RRRO genetic families and orange bars represent YC2020 RRRO families.

Ranking of the 2022 and 2023-year class families using the selection index indicated that RRRO families were frequently represented among the top-performing families within each year class (Tables 11 and 12). Specifically, seven of the top 10 families in the 2022-year class and five of the top 10 families in the

Table 11. Rankings of the 2022-year class families based on the Sydney Rock Oyster breeding program selection index.

Family Rank	Selection Index	Family Rank	Selection Index
1	95.15	25	73.31
2	94.51	26	73.23
3	94.00	27	72.78
4	91.33	28	72.68
5	88.70	29	71.73
6	88.58	30	71.72
7	85.19	31	71.43
8	83.89	32	71.02
9	82.44	33	70.99
10	81.84	34	70.93
11	81.55	35	70.63
12	79.78	36	70.16
13	78.63	37	70.14
14	78.39	38	69.17
15	77.72	39	69.02
16	77.56	40	68.99
17	77.340l	41	67.88
18	76.68	42	67.41
19	76.51	43	67.33
20	76.24	44	64.74
21	74.76	45	63.03
22	74.39	46	61.83
23	74.34	47	60.44
24	73.81	48	24.38

RRRO YC2018

RRRO YC2020

2023-year class contained RRRO genetics. However, consistent with patterns observed for survival, considerable variability was evident. Several RRRO families ranked among the bottom 10 families, including one RRRO family that ranked last in the 2023-year class (Tables 11 and 12). This variability highlights the heterogeneity of genetic performance within RRRO lines and reinforces the importance of structured crossing and the strategic incorporation of RRRO genetics with SRO lines of known QX performance to maximise gains while managing risk within the breeding program.

Table 12. Rankings of the 2023-year class families based on the Sydney Rock Oyster breeding program selection index.

Family rank	Selection Index
1	92.22
2	90.91
3	90.54
4	89.90
5	89.80
6	89.22
7	88.38
8	87.98
9	87.12
10	86.97
11	85.53
12	84.35
13	83.75
14	81.14
15	81.04
16	80.26
17	79.75
18	78.75
19	78.21
20	78.18

Family rank	Selection Index
21	77.87
22	77.75
23	77.74
24	76.75
25	76.39
26	76.02
27	75.85
28	75.57
29	75.38
30	75.19
31	73.39
32	73.18
33	72.62
34	69.80
35	68.59
36	67.56
37	65.73

RRRO new genetics

RRRO YC2020

Conclusion

This study represents the most comprehensive genetic characterisation of *Marteilia sydneyi* undertaken to date and provides important new insights into the distribution and diversity of this parasite in NSW and Queensland estuaries. By leveraging newly generated genome data, a genetic typing framework targeting the ribosomal RNA array was developed and applied to archival material from high- and medium-risk estuaries, alongside extensive molecular screening of oysters from estuaries classified as low risk for QX disease.

No *M. sydneyi* was detected in oysters sampled from low-risk estuaries across three consecutive sampling years, despite large sample sizes, targeted spatial sampling, and testing during periods of expected peak parasite activity. This finding contrasts with earlier reports of molecular detection in these estuaries and raises the possibility that *M. sydneyi* may be absent (failed to establish), occurs at extremely low prevalence, or was misidentified in prior studies due to limited assay specificity/lack of sequence confirmation. Due to the lack of molecular detection of *M. sydneyi* in the low-risk estuaries examined and the superior sensitivity of molecular testing, cytological evaluation was not pursued; however microscopic evaluation or preferably, the implementation of generic assays for *Marteilia* spp. may be warranted in future to capture the full diversity of species present in NSW waters. The results highlight the need for caution when interpreting historical detections and suggest that current estuary risk classifications may warrant re-evaluation as additional data become available.

Genetic analysis of *M. sydneyi* from high- and medium-risk estuaries revealed very limited sequence diversity across locations. All ribosomal RNA genes examined were identical among samples, and sequence variation within the internal transcribed spacer regions was observed primarily within individual samples rather than consistently between estuaries. Detailed analysis using both Sanger and ONT sequencing demonstrated that these variants, including a recurring ITS2 insertion/deletion, co-occurred within individual infections and did not show clear geographic structuring. This pattern is inconsistent with the presence of distinct cryptic species or stable, estuary-specific pathogenic strains analogous to those described for *Marteilia refringens* and *Marteilia pararefringens* overseas.

The within-sample sequence heterogeneity observed in this study is more consistent with allelic variation, polyclonal infections, or genomic features such as replication slippage in repetitive regions, rather than biologically discrete strain types. While the precise mechanisms underlying this variability remain unresolved, there is currently little evidence to support the hypothesis that differences in QX disease expression among estuaries are driven by fundamentally different *M. sydneyi* genotypes.

Together, these findings provide an important scientific foundation for future surveillance, biosecurity risk assessment, and targeted research into the non-genetic determinants of QX disease outbreaks.

This study demonstrates continued improvement in survival in high QX risk estuaries within the SRO breeding program across multiple year classes, life stages, seasons and field sites, while also highlighting the benefits and challenges associated with incorporating RRRO genetics. High overall survival of the 2022 and 2023-year class families following QX exposure (confirmed and presumed) confirms strong baseline resistance within the program, and sequential gains were observed across the year classes as seen previously within the SRO breeding program.

Across both spat and adult stages, 2022 and 2023-year class families containing RRRO genetics crossed with the highest-performing families in the SRO breeding program, were well represented among the highest-performing families based on their survival and "selection index" (combination of QX survival, growth and meat condition in a ratio of 2:1:1). These results indicate that RRRO genetics can contribute meaningfully to enhance survival in high QX risk estuaries when incorporated into the SRO breeding program. However, growth performance among RRRO families was variable, with a substantial proportion ranking in the lower half of families and suggesting that reduced growth may be a trade-off in some lines. In contrast, meat condition was generally comparable to or slightly above that of wild oysters on average, with a strong representation of RRRO families among the top performers, indicating promising potential for this trait, although some variability in performance was also evident across both year classes.

The observed variability in performance among RRRO families, including some families ranking among the lowest-performing families based on their survival, growth and selection index, highlights the potentially risks of using RRRO genetics and reinforces the need for cautious, structured integration with families of known performance.

Overall, these findings support the continued but measured use of RRRO genetics within the SRO breeding program, and thorough monitoring of performance before RRRO genetics are made available for commercial use.

Implications

The outcomes of this project have direct and indirect impacts on a range of end users, including government agencies responsible for biosecurity and fisheries management, the SRO industry, commercial hatcheries and consumers. By improving understanding of QX disease epidemiology and strengthening selective breeding strategies, the project delivers benefits that support industry resilience, inform policy decisions, and contribute to the long-term sustainability of oyster production in Australia.

The genetic typing work provides the most robust assessment to date of *Marteilia sydneyi* diversity in Australian estuaries. The absence of detectable *M. sydneyi* in low-risk estuaries across multiple years and the lack of

strong genetic differentiation among parasites from high- and medium-risk estuaries reduce uncertainty around the role of pathogen strain differences in QX disease expression. This evidence supports more informed, risk-based biosecurity decision-making and may contribute to future refinement of estuary risk classifications and oyster movement policies.

Improved confidence in the genetic uniformity of *M. sydneyi* reduces the likelihood that disease outbreaks are driven by the introduction of highly pathogenic strains, shifting the policy focus toward environmental drivers including vectors for transmission, local hydrological conditions, host susceptibility, and adaptive management strategies.

For SRO farmers in susceptible areas, QX disease represents the single greatest production risk, with severe outbreaks capable of causing up to 90% stock mortality. The demonstrated continued improvement in survival in high QX risk estuaries through selective breeding, including the effective incorporation of Richmond River Rock Oyster (RRRO) genetics, provides a tangible pathway to reduce stock losses and stabilise production.

Even modest improvements in survival can deliver substantial economic benefits. For example, a 10–20% increase in survival during a QX outbreak can translate to significant reductions in lost revenue, improved farm viability, and greater confidence to invest in infrastructure and stock. The identification of high-performing families using Estimated Breeding Values further enables industry to make informed choices about spat sourcing and risk management.

The genetic typing results also reduce the perceived risk associated with oyster movements between medium and high-risk estuaries, potentially enabling greater flexibility in future stock management if supported by complementary surveillance and policy review.

Extension and Adoption

PROGRESS AGAINST COMMUNICATION & EXTENSION PLAN:

Updates from the genetic typing component of this study have been:

- Uploaded onto the DPIRD website: https://www.dpi.nsw.gov.au/dpi/biosecurity/aquatic-biosecurity/aquaculture/oysters/nsw-dpird-qx-surveillance-and-research
- Presented at the FRDC Australasian Scientific Conference on Aquatic Animal Health and Biosecurity in July 2025
- Disseminated to interested stakeholders through periodic Shellfish Committee and the Aquaculture Research Advisory Committee meetings
- Presented to the Port Stephens QX Working Group in June 2024.
- Presented at the NSW Oyster Conference in Merimbula in May 2026.

The results for the incorporation of RRRO genetics into the NSW DPIRD SRO breeding program have been:

- Presented to the incoming NSW Farmers Oyster Committee on the 3rd October 2024.
- Presented at the NSW Oyster Conference in Port Macquarie in September.
- Presented at the Oysters Australia Unknown Oyster Mortality Workshop, 20th August 2025.
- Published in the SRO Breeding Program Update in the Oceanwatch newsletter.
- Presented at the NSW Oyster Conference in Merimbula in May 2026.

Adoption

Of the 29 RRRO families produced in year class 2022 and 2023 in this project, 22 have been identified as commercial families for hatcheries and made available for hatcheries to breed from to supply spat to industry.

In addition to communications outlined in the communication and extension plan, a report of the current progress for the incorporation of RRRO genetics into to the DPIRD SRO breeding program to the Port Stephens Oyster Farmers has been completed.

Recommendations and further development

The consistent failure to detect *M. sydneyi* in low-risk estuaries across three seasons warrants targeted follow-up. Future work should:

- Undertake expanded spatial and temporal surveillance, including additional seasons and estuaries at low risk of disease and
- Develop and apply new molecular assays (eg: pan-*Marteilia* assays) to determine the diversity of *Marteilia* species in bivalve production areas and the associated biosecurity risk
- Investigate whether environmental drivers for disease expression differ across estuaries, including the use of eDNA techniques to investigate parasite activity and the presence of potential intermediate hosts for transmission

This work is critical for validating estuary risk classifications and underpinning evidence-based biosecurity policy.

The breeding program is clearly delivering gains, but further refinement is recommended:

- Continue multi-season and multi-site QX exposure trials, including repeated exposure scenarios.
- Expand use of genomic tools and markers for QX resistance to accelerate genetic gain and reduce reliance on field challenges.
- Monitor correlated traits (growth, meat condition, robustness) to ensure balanced genetic progress.

This will sustain long-term gains while maintaining genetic diversity.

Project coverage

This project received media coverage on Landline in 2025 and is to appear in an upcoming issue of FRDC News.

Appendices

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