Investigation to address key policy gaps associated with the development of clam farming in South Australia

Genetic and health issues aligned to translocation and stock identification

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Contents

Contents iii
Acknowledgments v
Abbreviations v
1. Executive Summary 1
2. Introduction 6
3. Objectives 8
4. Method 9
4.1. Genetic studies 9
4.1.1. Sample collection and DNA extraction 9
4.1.2. Microsatellite development and data analyses 12
4.1.3. Species identification with mtDNA Cox I *barcode* 13
4.2. Risk mitigation strategies in relation to the development of clam aquaculture in SA 14
4.2.1. Techniques for practical differentiation between hatchery and wild mud cockle stocks 14
4.2.2. Risk mitigation strategies in relation to diseases and genetic contamination 14
5. Results and Discussion 15
5.1. Microsatellite markers in "*K. rhytiphora*" 15
5.2. Identification of *Katelysia* species in SA with *Cox I* barcoding 17
5.3. Risk mitigations in relation to the development of clam aquaculture in SA 21
6. Conclusion 30
7. Implications 31
Implications for aquaculture development 31
Implications for fishery management 31
8. Recommendations 32
Further development 32
9. Extension and Adoption 34
10. Appendices 35
10.1. List of researchers and project staff 35
10.2. References 35
10.3. Presentation at the workshop of National Aquatic Animal Health South Australia working group – 3 July 2012 40

Tables

Table 1. Descriptions of the shell characteristics used to distinguish between the *Katelysia species** 10
Table 2. "K. *rhytiphora*" sampling locations and the number of individuals used in genetic analyses 11
Table 3. Characteristics of microsatellites in *K. rhytiphora* species complex 17
Table 4. Population pairwise F_{ST} values (below diagonal) and effective number of migrants (Nm; above diagonal) among four populations along the SA coast 18
Table 5. Estimates of the uncorrected p-distance between sequences* 19
Table 6. Potential methods for differentiating between hatchery produced and wild clam stocks 25
Table 7. Practical ways to mitigate potential risks in mud cockle aquaculture development in SA 28

Figures

Figure 1. Annual global production of key bivalve aquaculture (FAO 2014). 6
Figure 2. Map of *K. rhytiphora* sampling localities in SA: Coffin Bay (▲), Streaky Bay (●) and Section Bank (■) 9
Figure 3. Map of sampling localities in Coffin Bay: Point Longnose (■) and Little Douglas (o) 10
Figure 4. Frequency distribution of 4 di-nucleotide repeat motifs and trinucleotide repeat motifs. 15
Figure 5. The *Cox I* phylogenetic tree revealed by the maximum likelihood method. PL: Point Longnose (Coffin Bay); LD: Little Douglas (Coffin Bay); ST: Streaky Bay; SE: Section Bank. The Cox I sequences of *Katelysia rhytiphora* (DQ184822.1), *Katelysia* sp 1 (DQ184824.1), *Katelysia* sp 2 DQ184825.1) and *Katelysia hiantina* (GQ855257.1) were sourced from GenBank (http://www.ncbi.nlm.nih.gov/genbank). 21
Figure 6. Difference in the morphology of *K. rhytiphora* shells during the hatchery phase ("hatchery shell") and the field phase in Coffin Bay ("grow-out shell"). 24

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Abbreviations

APVMA: Australian Pesticides and Veterinary Medicines Authority

Cox I: mitochondrial cytochrome oxidase I

DEWNR: Department of Environment, Water and Natural Resources (SA)

DNA: Deoxyribonucleic acid

DNP: dinucleotide repeat

DPTI: Department of Planning, Transport and Infrastructure (SA)

EPA: Environment Protection Authority (SA)

FAO: Food and Agriculture Organization of the United Nations

HNP: hexanucleotide repeat

PCR: polymerase chain reaction

PIRSA: Primary Industries and Regions South Australia

PNP: pentanucleotide repeat

SA: South Australia

SARDI: South Australian Research and Development Institute

SSR: simple sequence repeat

TNP: trinucleotide repeat

TTNP: tetranucleotide repeat

1. Executive Summary

What the report is about

This project aimed to fill some key knowledge gaps to assist in the development of a clam[1] / cockle aquaculture industry in South Australia (SA), specifically:

1. the population genetic structure of *Katelysia rhytiphora* in SA,
2. genetic contaminations associated with translocation of genetically dissimilar *K. rhytiphora* stock between locations,
3. biosecurity risks (disease status of mud cockles in SA is unknown), and
4. markers for practical differentiation between hatchery and wild cockle stocks.

Determining the genetic structure of *Katelysia rhytiphora* populations was the primary aim of the project and initially addressed with a microsatellite DNA technique. This species was selected as the FRDC project 2009/208 "Developing clam aquaculture in Australia: a feasibility study on culturing *Donax deltoides* and *Katelysia rhytiphora* on intertidal and subtidal leases in South Australia" indicated that it had the highest potential for aquaculture development in SA. The study was conducted in collaboration with Dr Klaus Oldach, SARDI Crop Improvement Program and Associate Professor Hongxia Wang, Institute of Oceanology, Chinese Academy of Sciences. The results indicate that the genetic structure of this species was more complex than initially anticipated and morphologically identical species (cryptic species) might exist. The *Cox I* barcoding technique was then employed as this emerging method is well suited to answering the taxonomic questions difficult to resolve on the basis of morphological characters.

A desktop study was conducted to investigate the published methods that could potentially be used to differentiate hatchery and wild cockle stocks. The methods can be grouped into three categories: a) chemical markers, b) DNA markers and c) physical markers.

Strategies that could be applied to mitigate or future research that is needed to address the risks identified for mud cockle aquaculture development in SA have also been discussed according to the information available to the project.

[1] In this report the terms "clam" and "cockle" are interchangeable.

Background

The need for the development of an Australian clam aquaculture industry is a direct outcome of the inability of the wild clam industry to meet the demand of a large international market prepared to pay a premium price for quality Australian clams. While commercial clam aquaculture has been successfully developed on a large scale overseas, none exists in Australia at this time. Over the last few years, a project on the feasibility of cockle aquaculture in SA (FRDC project 2009/208) has been completed and another looking at cockle stock enhancement has been initiated (FRDC project 2014/028 "Mud cockle (*Katelysia* spp.) stock enhancement/restoration: practical implementation and policy evaluation"). The current project builds on the outcomes from project 2009/208 (Gluis and Li 2014) to address the key gaps in the development of appropriate policy for cockle aquaculture in SA, which is targeted at ensuring the long-term viability of the wild harvest fishery and successful development of a sustainable aquaculture industry.

Objectives

The project objectives are:

1. To characterise the genetic population structure of *Katelysia rhytiphora* in South Australia.
2. To identify and evaluate method(s) for differentiation between farmed and wild clams.
3. To identify potential biosecurity issues relating to commercial clam aquaculture.
4. To identify practical ways to mitigate unaddressed risks associated with clam aquaculture development in SA.

Methodology

In this study samples of *K. rhytiphora* were collected from four localities in SA; Section Bank, Streaky Bay, Point Longnose (Coffin Bay) and Little Douglas (Coffin Bay).

The genomic DNA was extracted from *K. rhytiphora* using the AxyPrep Multisource Genomic DNA Miniprep Kit (AXYGEN). The development of microsatellite markers was achieved by the shotgun sequencing technique and expressed sequence tag (EST) mining for SSR loci. The SSRs screen was performed using the MISA software (http://pgrc.ipk-gatersleben.de/misa) after the removal of nonsense sequences with the Lucy and Seqclean program. Fifty primer pairs were then designed to amplify microsatellite regions using BatchPrimer3 (You et al. 2008).

Fourteen microsatellites were characterised and used to evaluate genetic variation and population genetic studies. Microsatellite data have been established for 28 individuals at Section Bank and 48 individuals at each of the remaining 3 localities.

DNA barcoding using the *Cox I* gene has recently become a popular tool to identify species in a variety of taxa, especially in morphologically identical species. In this study the *Cox I* gene was amplified using a standard invertebrate primer published by Folmer et al. (1994).

Results/key findings

In total 4,997 microsatellites were isolated from 254,769 contigs and singletons generated by the 454 Sequencer in the "*K. rhytiphora"* samples. Among them 14 microsatellite markers were developed and used for population genetic analyses. However, these analyses became invalid when it was recognised that multiple species had been included within the samples.

The results from the *Cox I* analyses revealed at least 3 species in the 18 individuals randomly selected from the "*K. rhytiphora*" samples that could be amplified using the standard primer pair. One species has a similar *Cox I* sequence to *K. rhytiphora* in the GenBank, the second similar to *Katelysia* sp., while the third does not match any of the species in this genus in GenBank, including *K. scalarina* and *K. hiantina*. Therefore, the number of *Katelysia* species in SA is more than the commonly agreed three species (*K. scalarina, K. peronii and K. rhytiphora*) that are identified morphologically.

Three types of methods that could potentially be applied to differentiate between farmed and wild clam stocks have been assessed using the published information available to the project, including: a) chemical markers (oxytetracyline, calcein, Alizarin red S, strontium chloride and non-toxic spray paint), b) a molecular marker (microsatellite), and c) a physical marker (hatchery spat shell colour). Both chemical and physical markers have successfully been applied to differentiate between the experimental and wild stocks in the studies conducted in the field. However, the assessment of these methods has typically only occurred over short periods (from a few days to a few months), with the longest being 15 months in oysters, which is still shorter than the at least 24 month period anticipated for the mud cockle aquaculture in SA.

Disease and pest information specific to Australian cockles is not currently available. In the absence of specific knowledge for mud cockle species, it is recommended that any Primary Industries and Regions South Australia (PIRSA) policy developed as part of this project that

addresses any risks associated with disease and pests be guided by existing protocols for mollusc species (e.g. PIRSA Disease Response Plan: Abalone Viral Ganglioneuritis 2013).

Implications for relevant stakeholders

The preliminary *Cox I* barcoding assessment has revealed multiple species in the "*K. rhytiphora*" samples used in this study. This has implications to cockle aquaculture development in Australia as different cryptic species might perform differently in the key traits important to aquaculture development such as growth rate, optimal temperature, sensitivities to different diseases, etc. For example, due to the existence of two cryptic species in kuruma shrimp, different optimal environmental conditions have been reported in this species complex (Tsoi et al. 2014), creating uncertainty in aquaculture practices.

The cornerstone of fisheries management relies on a sound taxonomic base and an understanding of how animals are grouped into management units (Hyde et al. 2014). The strong evidence of multiple genetically distinct species in the *K. rhytiphora* species complex warrants the reconsideration of the existing commercial mud cockle fishery management strategies in SA. The key challenge is the ability to distinguish these species practically.

Recommendations

Understanding the number of mud cockle species and population structure of individual species in SA is critical to both cockle fishery management and aquaculture development as they are strongly influenced by species biology. The results from this preliminary study have indicated that the *Cox I* barcoding technique is suitable for delimiting the *Katelysia* species in SA. However, the development of a new primer pair will be a priority as about 70% of Section Bank and Little Douglas individuals could not be amplified by the common *Cox I* primer pair, suggesting they might be a new species. In addition, at least a second barcoding gene is required to validate the *Cox I* results.

At the same time, the mud cockle species identified genetically should be described morphologically with the support of a bivalve taxonomist. When the relationship between the species in the genus *Katelysia* is established, the genetic structure of *K. rhytiphora* along the SA coast can then be revealed using molecular genetic techniques such as microsatellite markers.

The specifications of this project included the identification of a suitable marking technique to differentiate between farmed and wild cockle stock. From an assessment of techniques, it is

recommended that further investigation should focus on the two considered to have the most promise:

- oxytetracyline shell marking, as this creates a conspicuous ring on cockle shells, involves the use of the only chemical that has been approved for minor use by the Australian Pesticides and Veterinary Medicines Authority, and the burying behaviour of cockles may prevent the marking from fading due to the effect of sun light.
- the “hatchery shell” technique, where the hatchery environment readily results in distinguishing cockle shell morphological characteristics; this technique has been recommended by others for stock enhancement.

As these methods will not be entirely effective, a decision needs to be made first on what is an acceptable level of accuracy (e.g. 90%).

Keywords

Mud cockle, *Katelysia* spp, population genetics, microsatellites, mitochondria *Cox I*, barcoding, cryptic species, translocation, shell marking.

2. Introduction

Clam aquaculture is well developed in many countries overseas including Canada, China, Chile, France, Italy, Mexico, England, Spain, USA and Vietnam. Since 2008, the annual aquaculture production of clams has exceeded oysters and become the largest bivalve aquaculture sector in the world, with 5 million tonnes produced in 2012 (FAO 2014). However, clam culture on a commercial scale does not currently occur in Australia.

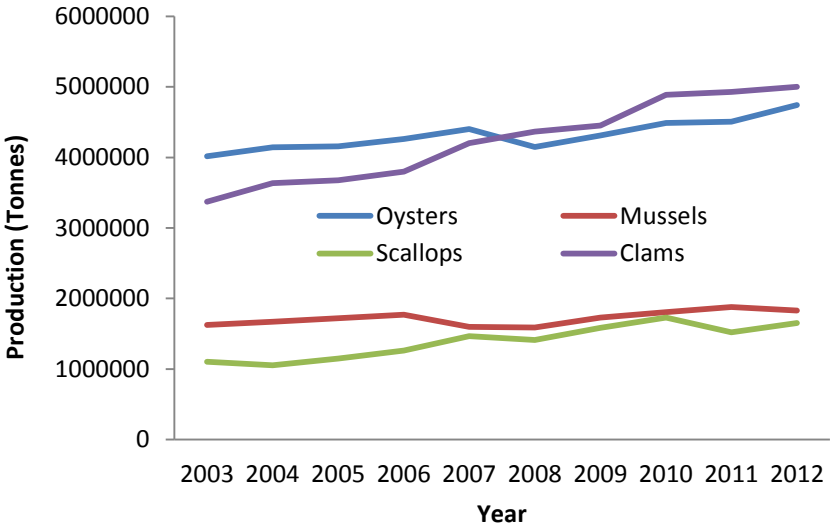

Figure 1. Annual global production of key bivalve aquaculture (FAO 2014).

Three bivalve species are currently farmed in South Australia (SA). These are the Pacific oyster (*Crassostrea gigas*), native flat oyster (*Ostrea angasi*) and blue mussel (*Mytilus galloprovincialis*). Of these, the Pacific oyster has the greatest production, with 5,710 tonnes produced in SA during 2012/13, worth an estimated $35 million (EconSearch 2014).

The need for the development of an Australian clam aquaculture industry is a direct outcome of the inability of the wild clam industry to meet the demand of a large international market prepared to pay a premium price for quality Australian clams. In SA, three species in the genus *Katelysia* are commercially harvested. They are *K. scalarina*, *K. rhytiphora* and *K. peronii*. *K. rhytiphora* occurs in shallow estuarine and marine embayments around the Australian temperate coastline from Western Australia to New South Wales (Roberts 1984). The commercial aquaculture possibilities for this species have been demonstrated in the recent study to evaluate clam culture on intertidal and subtidal leases in SA, with funding support from FRDC, SA Clam Aquaculture and SARDI (Gluis and Li 2014). The research has

established the technique for hatchery spat supply and initial field trials have shown potential in growth and survival performance at early grow-out stages.

To enable commercial-scale aquaculture production of mud cockle in SA, PIRSA Fisheries and Aquaculture is evaluating policies related to clam aquaculture development in SA to ensure the long-term viability of the wild harvest fishery and successful development of a sustainable aquaculture industry. The critical knowledge gaps identified for this assessment are: 1) population genetic structure of clam species in South Australia; 2) potential genetic and biosecurity risks of moving farming stocks between localities; and 3) effective and efficient methods to distinguish hatchery produced stock from wild stocks. Understanding these aspects will allow the development of more specific management strategies to control the potential adverse genetic and biosecurity impacts of farmed clams on the local wild population, thus protecting its genetic integrity if genetically divergent localised populations exist along the SA coastline. Practical identification of hatchery stock will ensure compliance with wild fishery, aquaculture and recreational harvesting policies.

The goal of this study was to develop background information to facilitate ecologically responsible and sustainable development of mud cockle aquaculture in SA.

3. Objectives

1. To characterise the genetic population structure of clams, *Donax deltoides* or *Katelysia rhytiphora* (to be determined) in South Australia.
2. To identify and evaluate method(s) for differentiation between farmed and wild clams.
3. To identify potential biosecurity issues relating to commercial clam aquaculture.
4. To identify practical ways to mitigate unaddressed risks associated with clam aquaculture development in SA.

4. Method

4.1. Genetic studies

4.1.1. Sample collection and DNA extraction

Samples of *K. rhytiphora* were collected in SA in the summer of 2011/12 and autumn of 2013 at four locations (Figures 2 and 3). These four locations were within the three existing mud cockle management zones; one in the Port River Zone (Section Bank), two in the Coffin Bay Zone (Point Longnose and Little Douglas) and one in the West Coast Zone (Streaky Bay).

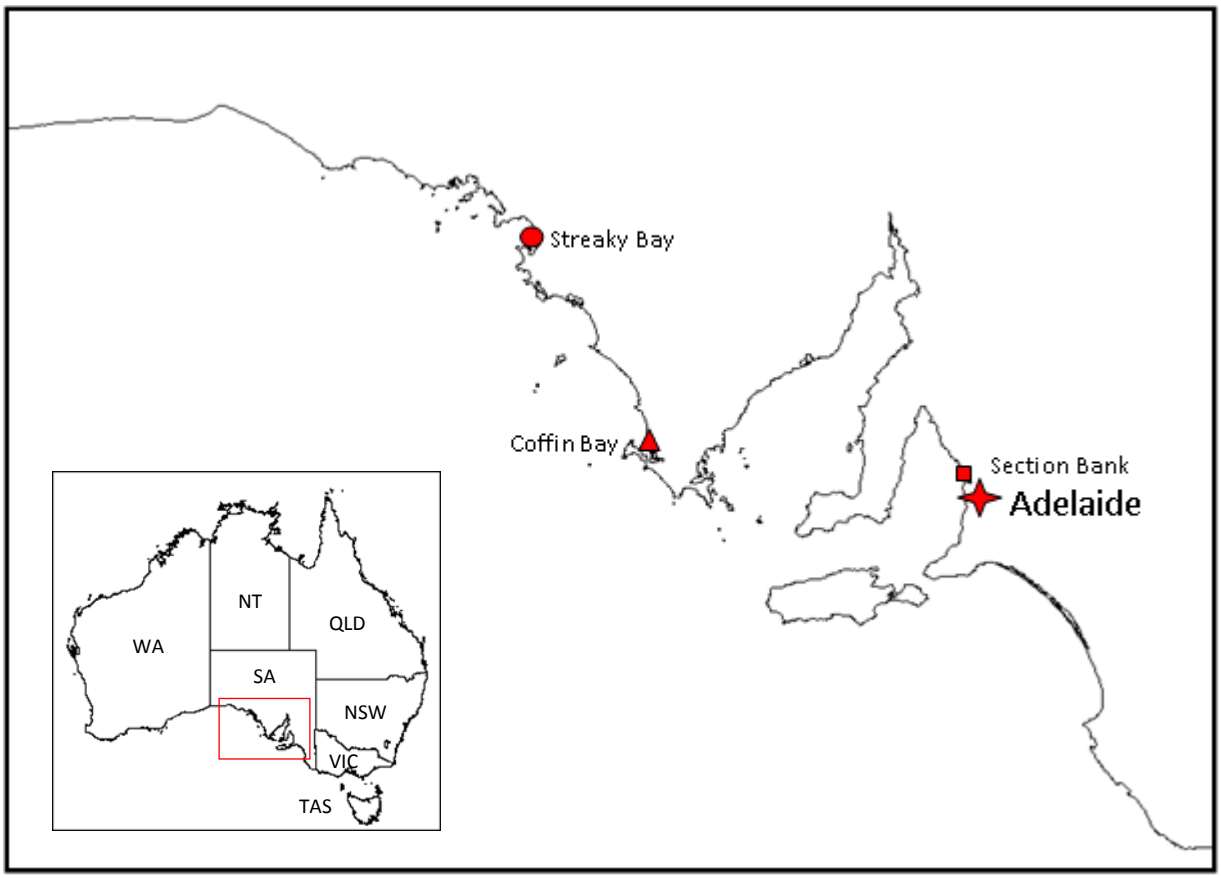

Figure 2. Map of *K. rhytiphora* sampling localities in SA: Coffin Bay (▲), Streaky Bay (●) and Section Bank (■).

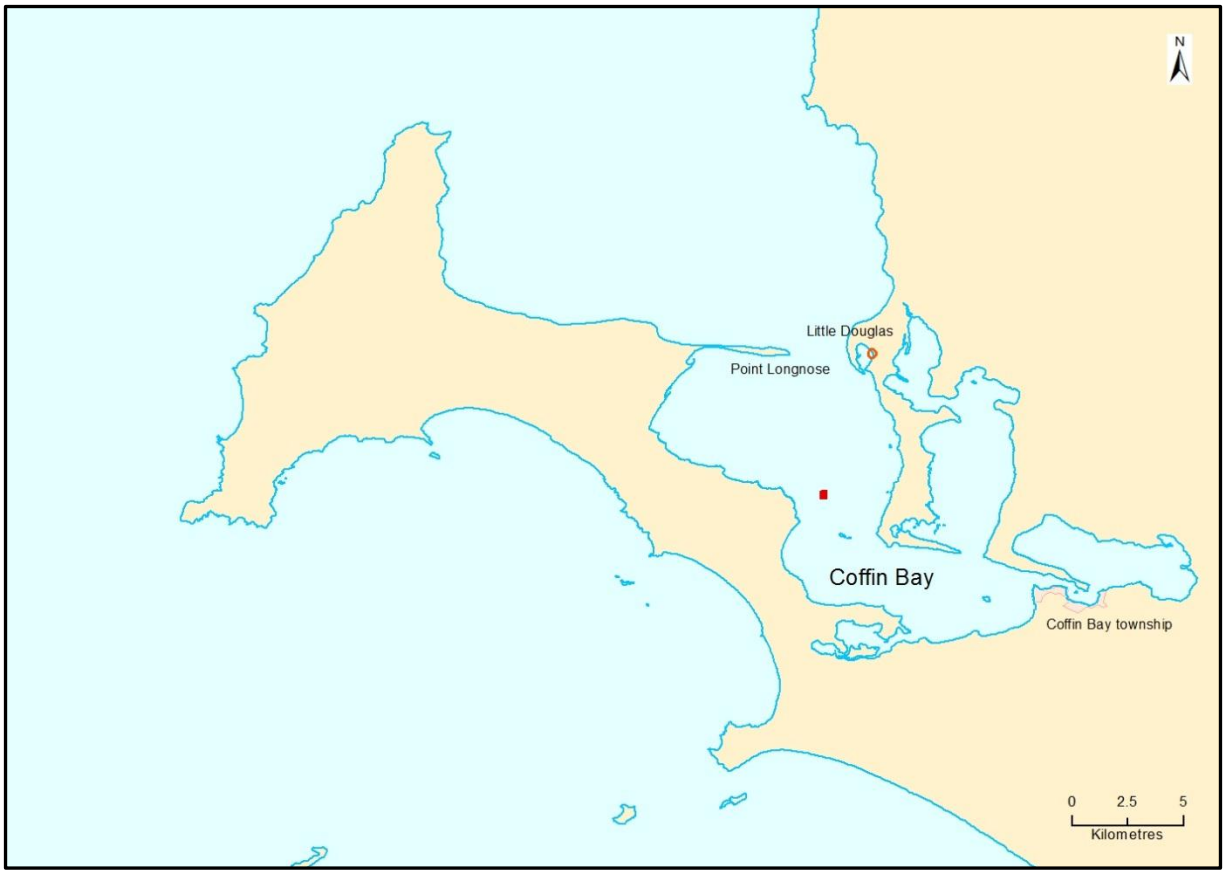

Figure 3. Map of sampling localities in Coffin Bay: Point Longnose (■) and Little Douglas (o).

The "*K. rhytiphora*" specimens used in this study were identified morphologically according to the taxonomic keys developed by Edward (1999) to distinguish SA mud cockle species (Table 1). These keys were similar to those published by Nielsen (1963), Roberts (1984) and Shepherd and Thomas (1989) for the identification of Australian mud cockle species, except that fine radial ridges were described by Edward (1999) in *K. rhytiphora* only whereas by other authors in both *K. peronii* and *K. rhytiphora*.

Table 1. Descriptions of the shell characteristics used to distinguish between the *Katelysia species**

Species	Description of shells
K. peronii	Flat concentric ridges; Exterior is cream and fine, brown to dark grey zigzag pattern crossing concentric ridges are sometimes present; Interior is yellow with purple markings covering an area ranging from muscle scar areas

	only to almost all of the shell;
	Posterior is the most rounded of all species.
K. rhytiphora	Rounded concentric ridges which give a "corrugated" look;
	Exterior is cream and blue to dark brown zigzag patterns crossing concentric ridges are often present;
	Fine radial ridges are always visible;
	Interior is yellow with purple markings covering an area ranging from muscle scar areas only to almost all of the shell;
	Shell is the most elongated of all species.
K. scalarina	Sharply raised concentric ridges, especially at the posterior edge;
	Exterior is cream to orange, but is usually mostly covered by blue, purple &/or brown zigzag patterns;
	Interior is white sometimes with a purple streak at posterior edge.

* From Edwards (1999).

The sample localities and number of individuals per locality used in the genetic structure analyses, which was based on the autumn 2013 samples, are provided in Table 2 as the quality of 2011/12 sample extractions was poor. The DNA kit used to purify the 2011/12 samples was most likely not suitable for the species investigated in this study.

Table 2. "*K. rhytiphora*" sampling locations and number of individuals used in genetic analyses

Sampling Locality	N
Coffin Bay – Point Longnose	48
Coffin Bay – Little Douglas	48
Streaky Bay	48
Section Bank	28

N = number of individuals

In the 2013 "*K. rhytiphora*" samples, approximately 0.3 cm^3 of muscle tissue was excised from each animal and total genomic DNA was extracted using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instruction. The DNA concentration was then analysed by NanoDrop 2000 (Thermo Scientific, DE, USA).

4.1.2. Microsatellite development and data analyses

Shotgun sequencing of *K. rhytiphora* species complex and mining of microsatellite loci

A total of 304,009 reads, with an average read length of 344 bp were generated from the genome DNA of Coffin Bay "*K. rhytiphora*" individuals through 454 sequencing. The accumulative length of the reads was 104.53 million bp. After the preprocessing with the Lucy and Seqclean program to remove nonsense sequences, 291,834 (96%) reads were left and then submitted for assembly. The MISA software (http://pgrc.ipk-gatersleben.de/misa) was used to screen for simple sequence repeats (SSRs). In the search for an SSR standard, we defined SSRs as dinucleotide repeat (DNP) ≥ 12 bases; trinucleotide repeat (TNP) ≥ 15 bases; tetranucleotide repeat (TTNP) ≥ 20 bases; pentanucleotide repeat (PNP) ≥20 bases; and hexanucleotide repeat (HNP) (and more) ≥ 24 bases (Cardle et al. 2000). Reverse-complement repeat motifs and translated or shifted motifs were grouped together (e.g. AC representing AC, CA, TG and GT) due to the double-stranded nature of DNA and the fact that the start site of a SSR could be considered arbitrary (Jurka and Pethiyagoda 1995). Primer pairs were designed to amplify microsatellite regions using BatchPrimer3 (Frank et al. 2008).

Microsatellite protocols

Fourteen microsatellites were isolated, characterised and used to evaluate genetic variation among samples. PCR was performed in 10 μL with the following conditions: 2.5 mM of $MgCl_2$, 0.25 mM of each dNTP, 1 μM of primers, 0.5 U of Taq polymerase, 1 μL of 10x buffer (Qiagen, Germany), 2 μL of Q solution (Qiagen), 30 ng of DNA template and sterile water to achieve the final volume.

The cycling PCR parameters were: 3 min of denaturation at 95 °C, followed by 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at the temperature optimized for each primer pair, 1 min of elongation at 72 °C, and a final elongation step of 5 min at 72 °C. The optimized annealing temperatures of different primer pairs are also listed in Table 3. PCR products along with DNA marker (pBR322 DNA/Msp Ⅰ markers) were electrophoresed on 8% polyacrylamide gel at 200v for 3 hours, and visualized by RedSafe DNA staining (iNtRon Biotechnology, USA). The images data was photographed and analyzed by the Quantity One Software (Bio-Rad, USA).

4.1.3. Species identification with mtDNA Cox I *barcode*

MtDNA *Cox I* barcoding

The results from microsatellite DNA analyses indicated that the genetic structures of the samples collected in this study might be more complex than initially anticipated. For example, the genetic difference between the two Coffin Bay populations (Point Longnose and Little Douglas) was greater than that between Point Longnose and Streaky Bay populations (Table 4) although the geographic distance between Point Longnose and Little Douglas is only about 4 km apart, whereas between Point Longnose and Streaky Bay is about 270 km apart (Figures 2 and 3). There was also missing data in three microsatellite markers in ~35% of the Little Douglas and Section Bank individuals. These data suggest that more complex genetic structures would exist in the *K. rhytiphora* samples originally identified by the *Katelysia* species identification keys developed by Edwards (1999). This poses the question of whether cryptic mud cockle species exist along the SA coast.

A pilot study to delimit the number of species in our samples with DNA barcoding was then initiated as this technique has recently emerged as a rapid method for species discovery, especially for those taxonomic problems difficult to resolve on the basis of morphological characters (Borisenko et al. 2008). For animal taxa, one of the most commonly used barcode regions is a short segment of approximately 600 base pairs of the mitochondrial cytochrome oxidase subunit I (*Cox I*).

In this study the *Cox I* was amplified with the common primer pair (Folmer et al. 1994):

LCO: GGTCAACAAATCATAAAGATATTGG
HCO: TAAACTTCAGGGTGACCAAAAAATCA

Amplification reactions were performed in a volume of 25 µL containing 1 µL of template DNA, 0.2 U Taq DNA polymerase (Promega), 2.5 µL 10×PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 µM each dNTP final concentration, and 5 pmoles of each primer.

PCR amplifications were conducted on a thermocycler TP650 (Takara Bio, Japan). The reaction profiles included an initial denaturation at 95 °C for 5 min, followed by 34 cycles, each consisting of 20 s denaturation at 94 °C, 30 s annealing at 50 °C, 30 s extension at 72 °C, and then a final 10 min extension at 72 °C. The PCR products were checked by electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized under UV light to confirm amplification and fragment sizes. Products were purified and sequenced by an ABI 3730xl DNA Analyzer (USA).

Genetic divergence

Alignments were edited by Mega 4.0 (Tamura et al. 2007). The computer-generated alignment was further adjusted manually for corrections and finally trimmed to obtain sequences of equal lengths. Mega 4.0 was used to calculate average uncorrected pairwise distances (*p*-distances) between different *Cox* I sequences.

4.2. Risk mitigation strategies in relation to the development of clam aquaculture in SA

4.2.1. Techniques for practical differentiation between hatchery and wild mud cockle stocks

A desktop study was conducted to identify techniques that have been used to differentiate between hatchery and wild bivalve stocks or experimental and wild bivalve stocks, including a) chemical markers, b) molecular markers, and c) physical markers. This study also considered their practical application, such as the toxicity of the chemicals assessed and the duration that a marker is conspicuous on animals in the field.

4.2.2. Risk mitigation strategies in relation to diseases and genetic contamination

This was a desktop study to identify the potential disease and genetic contamination risks due to aquaculture practices and discusses the potential strategies to mitigate the issues identified.

The biosecurity issues relating to the development of clam aquaculture in SA were also discussed at the Network for Aquatic Animal Health in South Australia meeting on 5 July 2012.

5. Results and Discussion

5.1. Microsatellite markers in "*K. rhytiphora*"

Using the Troll software, 4,997 microsatellites were isolated from a total of 254,769 contigs and singletons from the genome of "*K. rhytiphora*". These microsatellites could be classified into 1,987 dinucleotide repeats (DNPs), 1,159 trinucleotide repeats (TNPs), 1,023 tetranucleotide repeats (TTNPs), 128 pentanucleotide repeats (PNPs) and 700 other motif types of microsatellites including hexanucleotide repeats or repeats with more than 6 bases and compound repeat types. There were major differences in the relative abundances of specific repeat motifs; dinucleotide microsatellites were the dominant repeat type (39.8%), followed by trinucleotide (23.2%) and tetranuleotide (20.47%) microsatellites.

The most common motif type of DNPs was TA/AT (57.6%; Figure 4 Left), followed by AC/TG (30.7%) and TC/AG (11.4%). The GC/CG motif was rare in the data set. TNP motifs were dominated by AAC/TTG repeat, which was found in 384 loci (33.1%; Figure 4 Right). The least frequent TNP motif was GC-rich (CAG/TGG/TGC), and the accumulative total value was no more than 10% of TNPs (data not shown).

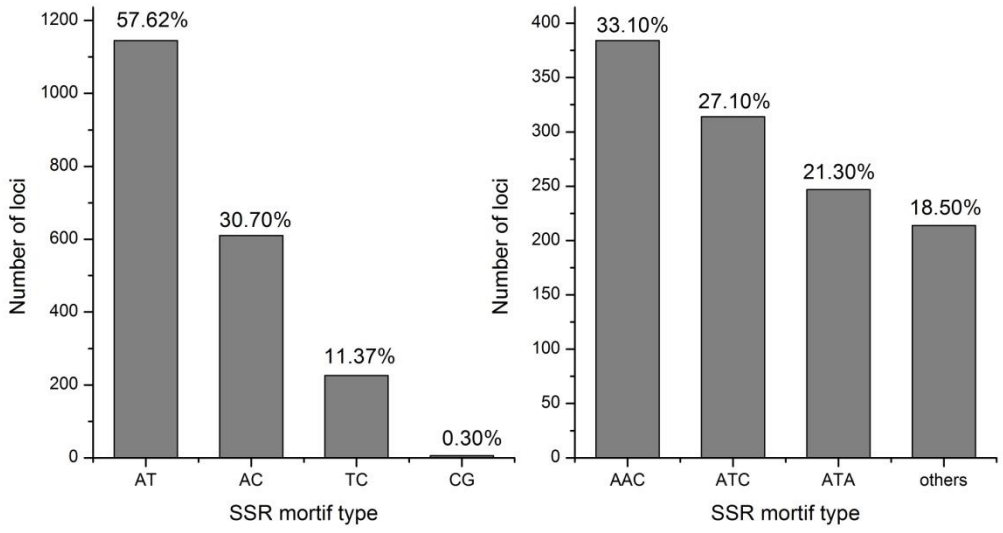

Figure 4. Frequency distribution of 4 di-nucleotide repeat motifs and trinucleotide repeat motifs.

According to the above information, 50 primer pairs were designed. Among them 17 pairs yielded clear bands in PCR reactions in most samples. Three pairs were subsequently dropped due to inconsistent amplification, resulting in a panel of 14. In the scoring gel data

points, single bands were regarded as homozygous for the given microsatellite locus, while different double bands were regarded as heterozygous.

In total 172 samples from four populations were genotyped using 14 microsatellite markers (Table 3). A total of 391 microsatellite alleles were detected, with an average of 26 alleles per marker. Among these microsatellite alleles, 80 displayed a frequency of more than 5% in the total sample and hence were classified as "common" alleles, while another 175 displayed frequencies between 1% and 5% and were classified as "less common" alleles. The 136 remaining alleles were classified as "rare" alleles with frequencies less than 1%.

Table 3. Characteristics of microsatellites in *K. rhytiphora* species complex

Locus	Primer sequence (5'-3')	Motif	Allele fragment	T_m (°C)
P3_AZGJU	**F:** CTTCTTTGAACATGTCCTCTG **R:** CAAAGGAGGATAATTGCTAAA	(TCATA)13	172	55
P4_CAWAZ	**F:** TTTTAGATTTGACACAGGATACA **R:** ATCTTTGACTTCAAGCGATG	(GAAT)21	150	58
P5_ANXFH	**F:** CGCCTAACCTCACTTGTATC **R:** TGACATGTATCATTTTACCAGAA	(TAGG)15	155	55
P6_BBUAE	**F:** CATTCCAGTTTTCTTTACACC **R:** AAACGTTATCCTACAGTCAACC	(AATC)10	243	58
P9_BKPA6	**F**: TAAAGAATAGAATGCGACCTG **R:** CAGGAAGTACGGTAACCTTG	(AATC)19	166	56
P10_BMGA6	**F:** CAACTTGACCTACTGAGTTCG **R:** AAAGTCCCGAAGCTTACTTAC	(AATC)16	138	58
P12_A6Z901	**F:** TGTAACTATAACAACCCATGTCA **R**: CACATTGATGTATTTAACACAGA	(TGAG)15	133	56
P13_BH77K	**F:** CTTGCAACTTAACACAAGACA **R:** TTATAGCGAAAATGACTACCG	(ATC)21	150	58
P28_CEACL	**F:** CAGAATAGAGGAAAACGCATA **R:** AAAAAGTGCTTTAGAAGTTTGG	(TAT)13	49	56
P29_AYZ5W	**F:** CTGCGCGAGTAATAGTAACAT **R:** ATTACCAGCACCATCATTAAC	(ATG)14	146	55
P31_BOTYM	**F:** GTATTGTTATTCATCGCCATC **R:** GCAATAATCCAAAACAGTGAT	(CAT)12	114	56
P32_BF3IJ	**F:** AAAAGCCACGTTCCAACAAC **R:** CGTGTGTGACTGTCCGTACC	(AC)16	133	64
P20_c01347	**F:** AGATTGTCTAAGCGGTTCTTT **R:** ATTACCGTTGATGTTGATGAT	(ATC)7	180	56
P34_c00950	**F:** TGAGTCAAGCAACTCTGAAAT **R:** TGCAGTAGTTGAAGGTATTTT	(TTCT)6	173	53

5.2. Identification of *Katelysia* species in SA with *Cox I* barcoding

The preliminary results suggest the genetic structure of mud cockles analysed in this study might be more complex than initially anticipated and morphologically identical species (cryptic species) might exist. For example, although the specimens collected at Point Longnose and Little Douglas were only about 4 km apart (Figure 3) and the distance between Point Longnose and Streaky Bay is about 280 km apart (Figure 2), the F_{ST} and effective number of migrant (Nm) values indicate that the Point Longnose population is more closely related to the Streaky Bay *K. rhytiphora* stock (F_{ST}=0.024; Nm=10.1305; Table 4) than the Little Douglas stock (F_{ST}=0.163; Nm=1.287). According to the classes for F_{ST} by Hartl and Clark (1997), there were little genetic differences between Point Longnose and Streaky Bay populations (F_{ST}<0.05), whereas the differences between two Coffin Bay populations (Point Longnose and Little Douglas) were great (F_{ST}=0.15-0.25). These genetic structures could also be explained by the effective number of migrants as high values indicate high connectivity between populations. In addition, there are missing data in a few microsatellite

markers (primarily P3_AZGJU, P4_CAWAZ and P34_c00950) in more than 35% of the Little Douglas and Section Bank individuals, suggesting the difference might exceed the level of variation within species.

Table 4. Population pairwise F_{ST} values (below diagonal) and effective number of migrants (Nm; above diagonal) among four populations along the SA coast

	Point Longnose	Streaky Bay	Little Douglas	Section Bank
Point Longnose		10.1305	1.287	1.887
Streaky Bay	0.02408		1.536	1.527
Little Douglas	0.16258**	0.13998**		4.591
Section Bank	0.15273**	0.14064**	0.05163**	

***P* < 0.001

To determine if cryptic species exist in the *K. rhytiphora* individuals used in this study a pilot investigation was initiated using *Cox I* DNA barcoding technique. This method offers considerable promise for solving some of the problems associated with traditional biological identifications (Johnson et al. 2008). The *Cox I* has proved to be a useful barcoding marker for invertebrate animals because “universal” invertebrate primers are available (Folmer et al. 1994, Hebert et al. 2003, Neigel et al. 2007).

The *Cox I* gene products were obtained from 39 Point Longnose (81%), 36 Streaky Bay (75%), 16 Little Douglas (33%) and 8 Section Bank (29%) individuals. Then products from 3 Point Longnose, 4 Streaky Bay, 9 Little Douglas and 2 Section Bank individuals (18 in total) were randomly selected and sequenced.

The uncorrected p-distance between any two sequences ranged from 0 to 2.093 (Table 5). The phylogenetic tree was generated by the maximum likelihood method using the *Cox I* sequences from both this study and those available in NCBI in the genus *Katelysia* (http://www.ncbi.nlm.nih.gov/genbank). Five groups with high bootstrap values (Figure 5) are revealed; 1) *K. scalarina*, 2) the *K. rhytiphora* clade, 3) the *Katelysia* sp (a) clade, 4) the *Katelysia* sp (b) clade and 5) *K. hiantina*.

Table 5. Estimates of the uncorrected p-distance between sequences*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. PL5		0.005	0.004	0.421	0.438	0.402	0.007	0.002	0.004	0.006	0.002	0.106	0.103	0.102	0.008	0.000	0.004	0.002	0.010	0.160	0.098	0.455	1.798
2. ST3	0.010		0.005	0.436	0.454	0.416	0.011	0.004	0.005	0.007	0.004	0.109	0.105	0.104	0.011	0.005	0.007	0.006	0.013	0.169	0.100	0.289	2.551
3. LD3	0.006	0.010		0.425	0.422	0.405	0.009	0.002	0.004	0.005	0.002	0.106	0.103	0.101	0.009	0.004	0.006	0.005	0.012	0.165	0.098	0.095	1.716
4. SE13	1.007	1.036	1.015		0.012	0.004	0.393	0.416	0.421	0.415	0.416	0.412	0.415	0.400	0.407	0.421	0.442	0.421	0.438	0.470	0.400	0.392	1.350
5. LD27	1.052	1.082	1.061	0.033		0.010	0.399	0.434	0.439	0.432	0.434	0.378	0.381	0.371	0.413	0.438	0.459	0.438	0.455	0.521	0.368	0.359	1.979
6. LD13	0.967	0.995	0.975	0.006	0.026		0.375	0.397	0.402	0.396	0.397	1.526	0.417	0.401	0.388	0.402	0.421	0.402	0.418	0.488	0.402	0.393	1.524
7. *Katelysia rhytiphora*	0.019	0.029	0.026	0.987	0.955	0.897		0.008	0.009	0.009	0.008	0.106	0.103	0.097	0.004	0.007	0.009	0.008	0.009	0.172	0.098	0.095	4.188
8. SE16	0.003	0.006	0.003	0.995	1.040	0.955	0.023		0.003	0.005	0.000	0.104	0.101	0.100	0.009	0.002	0.005	0.004	0.011	0.163	0.096	0.288	1.785
9. LD28	0.006	0.010	0.006	1.007	1.052	0.967	0.026	0.003		0.006	0.003	0.106	0.103	0.103	0.009	0.004	0.006	0.005	0.012	0.165	0.098	0.288	2.534
10. LD56	0.013	0.016	0.013	0.987	1.031	0.947	0.026	0.010	0.013		0.005	0.108	0.105	0.103	0.009	0.006	0.007	0.007	0.012	0.170	0.100	0.097	1.707
11. LD10	0.003	0.006	0.003	0.995	1.040	0.955	0.023	0.000	0.003	0.010		0.104	0.101	0.100	0.009	0.002	0.005	0.004	0.011	0.163	0.096	0.288	1.785
12. LD12	0.325	0.333	0.326	0.955	0.877	0.955	0.324	0.320	0.325	0.332	0.320		0.008	0.009	0.110	0.106	0.111	0.104	0.119	0.195	0.007	0.006	2.061
13. LD14	0.323	0.331	0.324	0.958	0.881	0.958	0.322	0.318	0.323	0.330	0.318	0.016		0.008	0.107	0.103	0.107	0.101	0.112	0.188	0.006	0.005	1.572
14. LD15	0.322	0.330	0.323	0.928	0.866	0.928	0.310	0.316	0.322	0.328	0.316	0.023	0.019		0.101	0.102	0.106	0.100	0.110	0.186	0.008	0.007	1.389
15. PL38	0.019	0.029	0.026	0.967	0.987	0.928	0.006	0.023	0.026	0.026	0.023	0.336	0.333	0.322		0.008	0.009	0.009	0.008	0.171	0.102	0.456	1.637
16. PL42	0.000	0.010	0.006	1.007	1.052	0.967	0.019	0.003	0.006	0.013	0.003	0.325	0.323	0.322	0.019		0.004	0.002	0.010	0.160	0.098	0.455	1.798
17. ST42	0.006	0.016	0.013	1.048	1.094	1.007	0.026	0.010	0.013	0.019	0.010	0.339	0.336	0.335	0.026	0.006		0.004	0.010	0.167	0.102	0.100	2.533
18. ST46	0.003	0.013	0.010	1.007	1.052	0.967	0.023	0.006	0.010	0.016	0.006	0.320	0.318	0.316	0.023	0.003	0.010		0.011	0.158	0.096	0.455	1.798
19. ST44	0.029	0.040	0.036	1.028	1.049	0.987	0.023	0.033	0.036	0.036	0.033	0.358	0.344	0.343	0.023	0.029	0.029	0.033		0.188	0.111	0.108	1.743
20. *Katelysia scalarina*	0.444	0.468	0.459	1.051	1.095	1.051	0.477	0.450	0.457	0.472	0.450	0.054	0.521	0.518	0.475	0.444	0.462	0.438	0.512		0.189	0.193	1.533
21. *Katelysia* sp. 2	0.311	0.319	0.312	0.928	0.853	0.928	0.310	0.306	0.311	0.318	0.306	0.016	0.013	0.019	0.322	0.311	0.324	0.306	0.343	0.523		0.005	1.540
22. *Katelysia* sp. 1	0.306	0.314	0.307	0.917	0.842	0.917	0.305	0.301	0.306	0.312	0.301	0.013	0.010	0.016	0.316	0.306	0.319	0.301	0.337	0.530	0.010		1.422
23. *Katelysia hiantina*	2.066	2.107	2.052	1.233	1.249	1.210	2.025	2.080	2.093	2.065	2.080	1.792	1.812	1.839	2.010	2.066	2.122	2.066	2.091	1.682	1.772	1.785	

* The p-distance values are shown below the diagonal.
The standard error estimates are shown above the diagonal.
The *Cox I* sequences of *Katelysia rhytiphora* (DQ184822.1) *Katelysia sp* 1 (DQ184824.1), *Katelysia sp* 2 (DQ184825.1) and *Katelysia hiantina* (GQ855257.1) were sourced from GenBank (http://www.ncbi.nlm.nih.gov/genbank).

The 18 individuals sequenced in this study belong to three clades; all 3 Point Longnose (PL) and 4 Streaky Bay (ST) individuals belong to the *K. rhytiphora* clade, the 9 Little Douglas (LD) specimen belong to the *K. rhytiphora*, *Katelysia* sp (a), and *Katelysia* sp (b) clades, respectively, and the 2 Section Bank (SE) specimen belong to the *K. rhytiphora* and *Katelysia* sp (b) clades, respectively. The genetic distances between the cockles in different clades showed 16% and 40% divergences, respectively. These levels of genetic differences between cryptic clades are comparable to species-level differences in the published studies in finfish (Lavoue et al. 2003, Thomas et al. 2014) but are higher than in other molluscan species (Won et al. 2003, Johnson et al. 2008) and the suggested divergence level for cryptic species complexes (Ward 2009).

It should also be noted that about 70% of LD and SE individuals could not be amplified by the common *Cox I* primer pair, suggesting more mud cockle species might exist. The universal Cox I primer pair has been used in our previous studies in other cockle species (Lu et al 2011) and successfully applied in the PL and ST samples in this study. Therefore, the individuals used in this study comprise at least three different species. Given this result, the shells of the 18 individuals used in the barcoding analysis were re-examined but were not able to be distinguished morphologically by identifying new shell characters.

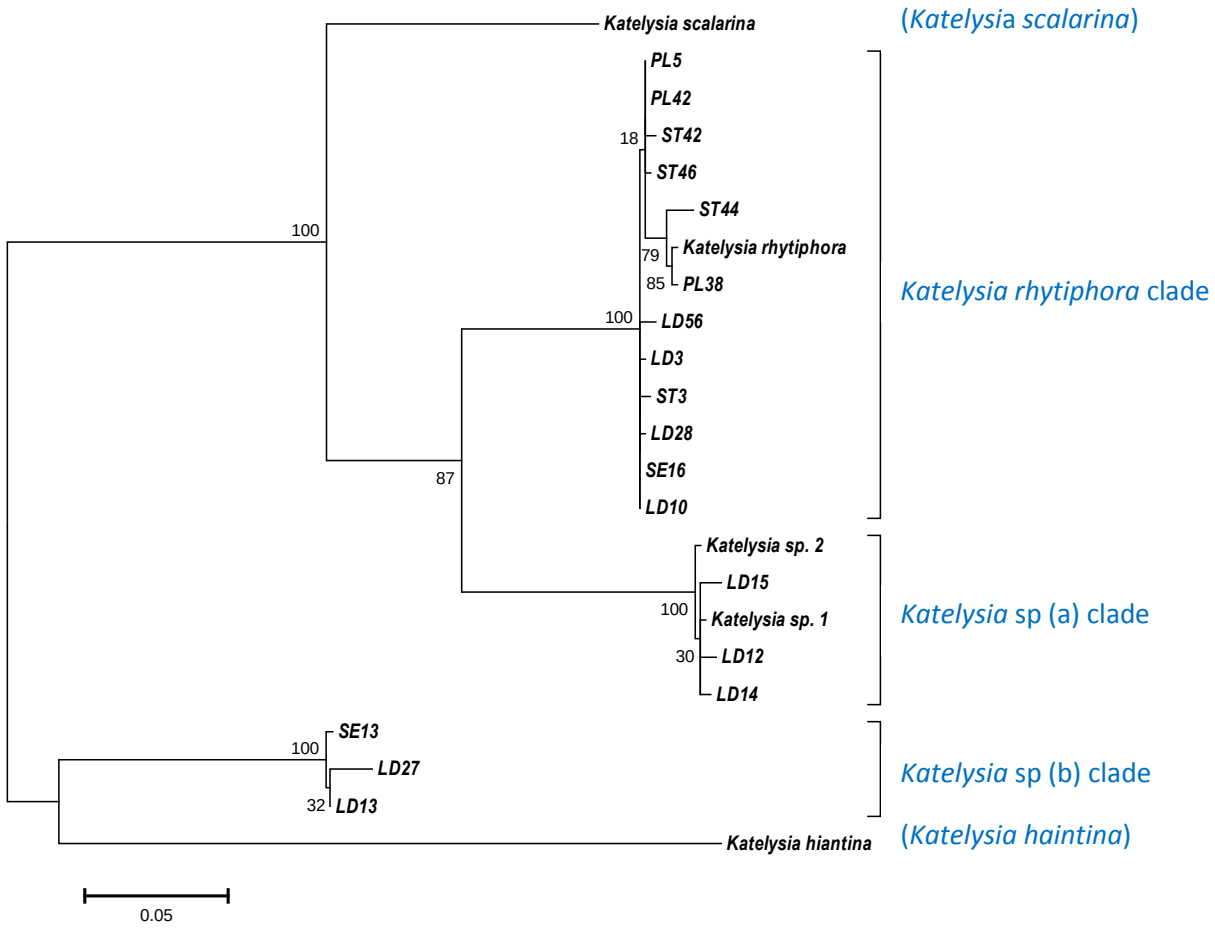

Figure 5. The *Cox I* phylogenetic tree revealed by the maximum likelihood method. PL: Point Longnose (Coffin Bay); LD: Little Douglas (Coffin Bay); ST: Streaky Bay; SE: Section Bank. The Cox I sequences of *Katelysia rhytiphora* (DQ184822.1), *Katelysia* sp 1 (DQ184824.1), *Katelysia* sp 2 DQ184825.1) and *Katelysia hiantina* (GQ855257.1) were sourced from GenBank (http://www.ncbi.nlm.nih.gov/genbank).

5.3. Risk mitigations in relation to the development of clam aquaculture in SA

The mud cockle *K. rhytiphora* is an important fisheries resource in SA. Currently, a study is underway to develop a strategy for stock enhancement/restoration at Section Bank. Commercial interests in cockle farming in SA are increasing as previous research has shown promising results in growth and survival rates in the field (Gluis and Li 2014). The key knowledge gaps identified for the commercialisation of the findings in these projects are 1) effective and efficient methods to distinguish hatchery produced stock from wild stocks, 2) potential genetic risks of farming or reseeding hatchery produced stocks, and 3) biosecurity risks of moving stocks between localities.

As outlined by Rothlisberg and Preston (1992) and Lucas et al. (2008) the ideal marking methods for reseeding programs should: 1) be able to mark small individuals, 2) be detectable in all subsequent life history stages (especially the adult), 3) be unique to the local population, 4) be suitable for identification of individuals or cohorts, 5) be inexpensive to apply and detect, 6) be harmless to the tagged animals and subsequent consumer, and 7) be acceptable to the public. For aquaculture programs, the marking methods also should: 8) persist for the farming period (at least two and half years in *K. rhytiphora* according to our estimation in FRDC 2009/208 clam aquaculture feasibility study; Gluis and Li 2014), and 9) have the level of identification accuracy required for compliance. It is very challenging to achieve the latter as it could not be found in the existing policy documents and has not been considered in the published studies. In addition, the published marking methods have only been assessed for a short period (Table 6) and most markers are slowly diminished by exposure to light or through abrasion.

Published studies have identified at least six chemicals that have been used to stain bivalve shells to assist field studies on bivalve ecology, physiology and reseeding strategies (Thrush et al. 1997, Cummings and Thrush 2004, Thebault et al. 2006, Riascos et al. 2007, Herrmann et al. 2009, Lucas et al. 2008, Lartaud et al. 2010, Mahe et al. 2010, Tada et al. 2010, Nedoncelle et al. 2013). In each study, the chemicals were evaluated for less than one year with the exception of manganese chloride that was assessed for 15 months in Pacific oysters. In addition, the suitability of some chemicals was species specific. For example, alizarin red S has been used successfully to mark the clams *Mercenaria mercenaria*, *Mya arenia* and *Mulinina lateralis* (Hidu and Hanks 1968). However, this chemical was found by Lucas et al (2008) as not ideal for marking the saucer scallop *Amusium balloti* as only 80% of individuals were marked and the mortality was about 40%. Strontium chloride was successful at staining the clam *Mesodesma donacium* but resulted in high mortality and slow growth rate in survivors (Riascos et al. 2007). The practicality of manganese chloride as a stain requires the use of a cathodoluminescence microscope, which will severely limit the application of this technique as this equipment is uncommon and cannot be readily accessed in most places.

Concerns on the toxicity of calcein and alizarin red on human have been raised, making them less ideal for commercial use than oxytetracyline as the latter antibiotic is approved for minor use (for specific species) by the Australian Pesticides and Veterinary Medicines Authority (Lucas et al. 2008). It should be noted that this potential toxic risk would be minimal in bivalves as the chance of accidental consumption of bivalve shells is extremely low. Although the fluorescence of oxytetracyline stain faded off after two years in the scallop shells trialed by Lucas et al. (2008; Wang, personal communication), this chemical might be suitable for *K.*

rhytiphora as this species is infaunal and the shells are mainly buried in the substrate. This type of behavior protects them from the light thus preventing the shell fluorescence fading.

In this study, as multiple species have been included in the microsatellite genetic analyses, the potential of a microsatellite genetic marker for determining the parental assignment of hatchery stock has not been assessed. However, research in other species has demonstrated that the number of microsatellites that will be required for parental assignment will depend on three key factors: 1) desired level of parental assignment success, 2) polymorphic levels of the markers available to the populations/stocks of interest and their linkages, and 3) anticipated number of broodstock contributing to the stock under investigation. For example, with 18 microsatellite loci developed in the sea cucumber *Holothuria scabra* the simulated assignment success remained close to 100% when the number of broodstock increased to 100 (Gardner et al. 2012). In the blue mussel *Mytilus galloprovincialis* a set of 19 microsatellite markers achieved 95% unambiguous assignment (Pino-Querido et al. in press). A study performed in the eastern oyster *Crassostrea virginica* achieved 100% assignment with 9 microsatellites (Wang 2006), whereas a similar number of microsatellites (10) produced only 63% assignment in a study in the mussel *M. galloprovincialis* (Nguyen et al. 2011). A range of parentage assignment successes has been reported in bivalve species using microsatellite markers (Lallias et al. 2010, Li et al. 2010, Wang et al. 2010, Nie et al. 2012, Morvezen et al. 2013). Therefore, research will be required to determine if this technique is suitable in *K. rhytiphora*.

It was noted in our previous project on the feasibility of aquaculturing *K. rhytiphora* that hatchery produced spat can be differentiated from natural seed by examining the “hatchery shell” which displays a polished appearance in comparison with the “grow-out shell” (Figure 6). However, for the shells decorated with grey bands this difference was less unobvious (Figure 6). As this observation was from a four month field experiment, further assessment will be required to determine if this character can persist until the cockles grow to market size. The polished “hatchery shell” has also been noted by Brooks et al. (2001) in the study on the littleneck clam (*Protothaca staminea*) stock enhancement program in Alaska and recommended as a potential mark to evaluate the success of the program.

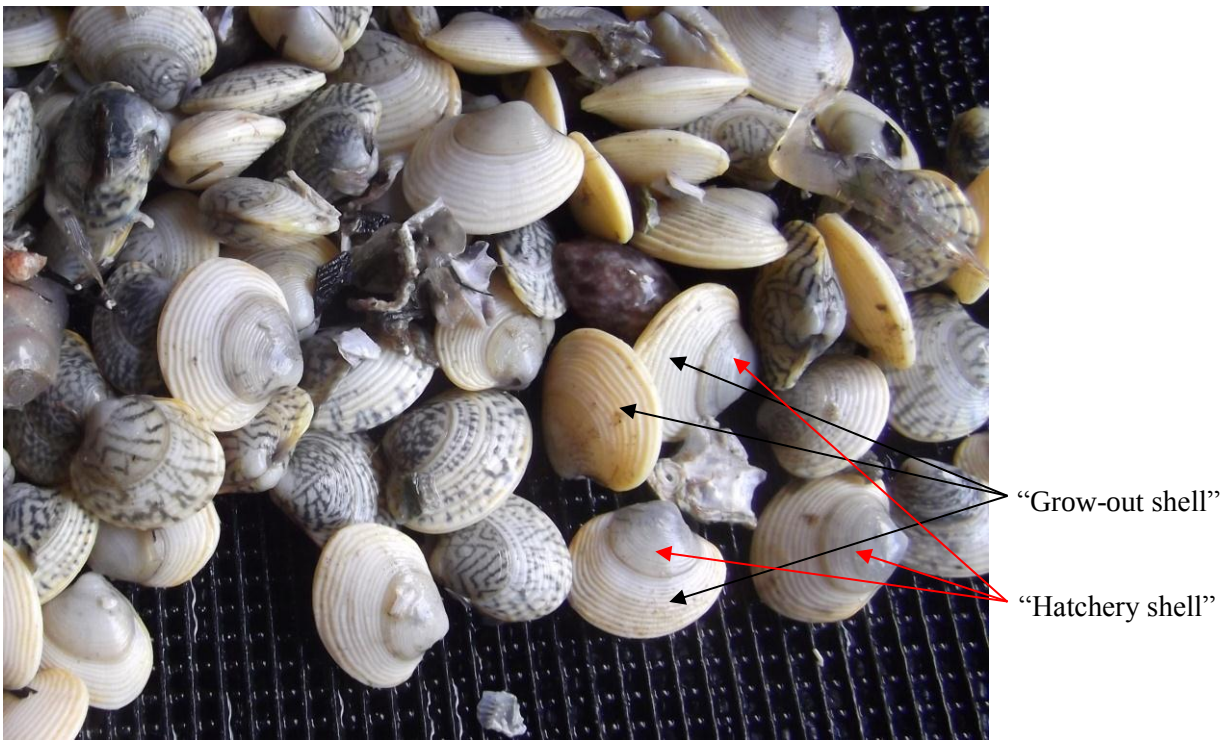

Figure 6. Difference in the morphology of *K. rhytiphora* shells during the hatchery phase ("hatchery shell") and the field phase in Coffin Bay ("grow-out shell").

Table 6. Potential methods for differentiating between hatchery produced and wild clam stocks

Techniques		Suitability for small individuals	Maximum period tested	Cohort identification	Requirement for applying	Requirement for detecting	Toxicity*		Tested on clam species	References
Types	Name						Bivalve	Human		
Chemical marker	Oxytetracyline (OTC)	Yes	10 months	Yes	Chemical concentration and staining duration optimisation	Fluorescent microscope	No impacts on subsequent performances have been reported if the animals have been treated with the optimal concentration and staining duration.	A broad spectrum antibiotics; used to treat bacteria in farmed fish; level 2 for toxicity and chronic categories in ChemGold.	No	Pirker and Schiel 1993, Day et al. 1995, Lucas et al. 2008
	Calcein	Yes	4 months	Yes	As above	As above	As above.	Not dangerous; level 1 for all hazard categories in Chemwatch.	Yes	Thebault et al. 2006, Riascos et al. 2007, Herrmann et al. 2009, Lucas et al. 2008, Mahe et al. 2010, Tada et al. 2010, Nedoncelle et al. 2013
	Alizarin red S	Yes	3 months	Yes	As above	As above	Higher staining mortality than calcein and OTC in scallops.	Suspected carcinogen; level 2 hazard ratings for toxicity, body contact and chronic categories in Chemwatch.	Yes	Hidu and Hanks 1968, Riascos et al. 2007, Lucas et al 2008, Herrmann et al. 2009
	Strontium chloride	Yes	Abalone and clam,	Yes	As above	As above	Mortality and slow growth	No data.	Yes, but at a high	Riascos et al. 2007, Tada et al. 2010

			20 days				rate.		concentration	
	Manganese chloride	Yes	Oysters 15 months; clams 12 days	Yes	As above	Cathodo-luminescence microscope	No impacts on subsequent performances have been reported.	Non-dangerous goods; level 2 for toxicity and chronic categories in Chemwatch.	Yes	Lartaud et al. 2010, Mahe et al. 2010
	Non-toxic spray paint	Yes	Cockles 28 days	Yes	No	No	No impacts on subsequent performances have been reported.	Refer to product information	Yes	Thrush et al. 1997, Cummings and Thrush 2004
Molecular marker	Microsatellites	Yes	All life history stages.	Yes	Markers	Facility for molecular genetic studies; expert advice.	N/A	N/A	Yes	Present study, Glover 2010, Yue et al. 2012, Zhang et al. 2013, Larrain et al. 2014
Physical marker	Shell color difference between spat and subsequent growth	Yes	4 months; > 2 years	Yes	Need to determine microalgal species and their combinations; percentage retain spat color in the subsequent growth in the field	No	N/A	N/A	Yes	Brooks 2001, Gluis & Li 2014

* ChemGold hazard categories: Flammability; Toxicity; Body Contact; Reactivity; Chronic.
* ChemGold hazard ratings: 0 = Minimum; 1 = Low; 2 = Moderate; 3 = High; 4 = Extreme.

Four key potential risks related to mud cockle aquaculture development in SA have been identified: 1) illegal collection of wild stock for farming or sale, 2) genetic contamination to local wild mud cockle population due to farming hatchery produced stock, 3) escape of farmed stock or invasion of wild stock into farming systems/regions, and 4) biosecurity risks.

Discussions on potential measures to address these risks are provided in Table 7. The key challenges to implement these measures are:

- The existence of cryptic species

  As cryptic species have been revealed in the "*K. rhytiphora*" samples at some locations, the spat produced using the broodstock collected from these regions would likely consist of multiple species and their hybrids if different species can be fertilized. If this is the case, the number of broodstock in each species would be lower than anticipated, thus resulting in lower than anticipated genetic diversity in each species. Additional genetic contamination would be produced due to hybridization between them.
- Differentiation between farmed and wild stocks

  It is anticipated that none of the methods listed in Table 6 would generate 100% accuracy in differentiating between hatchery produced and wild mud cockle stock due to fading of chemical markers over time, abrasion/fouling of "hatchery shell" or the limitation of genetic markers themselves such as mutations. It is also expected that a high number of microsatellite markers will be required as the fecundity of mud cockles is much lower than many other bivalves such as Pacific oysters. Therefore, to produce a similar number of spat for commercial production the number of broodstock mud cockles required would be higher than other species assuming a similar survival rate of all species.
- Biosecurity

  There is no report on health status of wild mud cockles in SA. However, heavy mortalities have occurred in some regions in SA in the past. For example, up to 90% of mud cockles were killed in Streaky Bay in December 2013. It is not clear if disease was a causative factor contributing to these events. Therefore, biosecurity measures should be in place if stock need to be translocated between regions, and if they don't exist at present, could be developed using the guidelines in related PIRSA protocols/policies, such as "Policy for the Release of Aquatic Resources" (http://pir.sa.gov.au/__data/assets/pdf_file/0019/246412/Policy_for_the_Release_of_Aquatic_Resources_-_April_2015.pdf).

Table 7. Practical ways to mitigate potential risks in mud cockle aquaculture development in SA

Risks identified	Potential mitigation strategies
Illegal collection of wild stock	To address this, a method that can differentiate between farmed and wild stocks is desirable. All potential methods and characters critical for stock differentiations are provided in Table 6. At this stage, none of them can be recommended as these methods have either not been evaluated long enough or have not been assessed in *Katelysis rhytiphora*. The risk of illegal collection of wild stock can be minimised if cockles are farmed in the areas where no or limited wild stock exists (assuming the area is suitable for cockle farming).
Genetic contamination	As preliminary mitochondrial DNA analyses conducted in the present study suggest that cryptic species might exist in the "*Katelysia rhytiphora*" samples collected in SA, strategies used to manage potential genetic contamination in farming "pure" species (e.g. ensuring genetic similarity between farmed and wild stocks) will not be applicable, unless the cryptic species are reproductively isolated from each other or "pure" species can be collected and confirmed from the surrounding regions.
Escape of farmed stock or invasion of wild stock	Escape of farmed stock 1. Stock escape This could happen if the farming system fails, although this is anticipated to be rare. The potential impact from escapees can be managed by: a) ensuring the genetics of farmed stock are similar to the local population, b) maintaining high genetic variation in the stock farmed, c) ensuring right mesh size for the cockles farmed, and d) the integrity of the structures holding the farm stock. 2. Reproduction of farmed stock Currently, no practical method can be applied to avoid the reproduction of farmed stock, unless the farmed stocks are harvested at a size prior to maturity or sterile farm stock is used. The former is unlikely as the size required by the market will probably be mature. In addition, as mud cockles do not spawn simultaneously and maintain spawning for more than six months in SA (Gorman et al. 2010), the business cannot afford not selling products for such a long period. The technique to produce sterile stock such as triploids has not been developed for mud cockle species. However, the potential impacts from the reproduction of farmed stock are minimal if the measures suggested to manage the potential impacts from escapees are implemented. Invasion of wild stock The potential impacts from invasion of wild stock depend on the methods that will be used for cockle farming. If farm stocks are reared in the substrate, the invasion of wild stock is not avoidable and the amount will depend on the density of surrounding stock and the recruitment rate (including those progenies bred from farmed stock). If suspended farming systems are used the invasion of wild stock would be minimal as newly metamorphosed spat are too small to be held in grow-out cages.
Biosecurity	As no infectious diseases specific to mud cockles have been reported in SA, the biosecurity measures adopted by the existing bivalve aquaculture industry in SA should also be applied to the mud cockle farming. In addition, the health status of stock should be assessed by a credited agency prior to hatchery produced spat being translocated to farm sites.

	A health survey of wild cockles in SA would be helpful for better understanding the potential biosecurity issues associated with cockle aquaculture development and specific mitigation strategy required.

6. Conclusion

In this study three cryptic species have been revealed by the *Cox I* barcoding technique in the *K. rhytiphora* samples collected in SA. This finding suggests that the mud cockle species in SA are much more taxonomically complex than the three species identified morphologically (*K. scalarina*, *K. peronii* and *K. rhytiphora*) and upon which the local fishery and its management is based. This result is preliminary as only 18 individuals have been analysed and validation with other barcoding markers is required.

A panel of 14 microsatellite markers has been developed and 11 of them have successfully been applied to all 172 *K. rhytiphora* samples initially used for genetic structure analysis, suggesting these markers would be suitable for the cryptic species identified. The remaining 3 (P3_AZGJU, P4_CAWAZ and P34_c00950) did not generate any data in some Section Bank and Little Douglas individuals, indicating they might be species specific.

All chemical and physical methods published for differentiating between experimental and wild bivalve stocks have been assessed only for a short period. The specifications of this project included the identification of a suitable marking technique to differentiate between farmed and wild cockle stock. From an assessment of techniques in previous and present use, it is recommended that further investigation should focus on the two considered to have the most promise:

- oxytetracyline shell marking, as this creates a conspicuous ring on cockle shells, involves the use of the only chemical that has been approved for minor use by the Australian Pesticides and Veterinary Medicines Authority, and the burying behaviour of cockles may prevent the marking from fading due to the effect of sun light.
- the “hatchery shell” technique, where the hatchery environment readily results in distinguishing cockle shell morphological characteristics; this technique has been recommended by others for stock enhancement.

As these methods will not be entirely effective, a decision needs to be made first on what is an acceptable level of accuracy (e.g. 90%).

7. Implications

In this study, cryptic species have been revealed by the *Cox I* barcoding in the "*K. rhytiphora*" samples. This finding has implications for both aquaculture development and wild fishery management.

Implications for aquaculture development

At the moment, the development of mud cockle aquaculture in SA is at its early stages and as such potential growers/investors are interested in establishing the performance of species (e.g. optimal temperature for growth and survival, tolerance to air exposure and diseases, etc). Different cryptic species may perform differently and if the crosses between cryptic species are viable it could be challenging to keep the desired species performance characteristics "pure". For example, the kuruma shrimp aquaculture in some Mediterranean countries involves two cryptic species *Penaeus japonicas* and *P. pulchricaudatus* (Turkmen 2007, Tsoi et al. 2014). As these two species have different environmental requirements and reproductive behaviors, it is essential to elucidate the species structure in these countries so that the species-specific farming techniques can be further improved.

Implications for fishery management

In SA the mud cockle fishery is based on three species: *K. scalarina*, *K. peronii* and *K. rhytiphora*. The total allowable commercial catch (TACC) is determined as a fraction of the harvestable biomass estimate for all *Katelysia* species in each management zone, up to a maximum harvest fraction of 7.5% (Dent et al. 2012, 2014). The results from preliminary *Cox I* barcoding analysis in this study suggest the *K. rhytiphora* cryptic species might exist along the SA coast. If one of the species is at a low abundance, matures at the same size but grows faster than others, this cryptic species would be more vulnerable to harvest pressures than others.

While this may warrant reconsideration of the fishery management strategy, a key limitation in implementing changes is the ability to distinguish among *Katelysia* species practically.

8. Recommendations

The following recommendations have resulted from the project findings with the aims to reveal the genetic structure of *K. rhytiphora* in SA and develop practical method to differentiate between hatchery produced and wild clam stocks:

A) Number of *Katelysia* species in SA

As mentioned previously, understanding the number of mud cockle species in SA will be critical to cockle fishery management and aquaculture development as both are based on species biology. The results from this preliminary study have indicated that the *Cox I* barcoding technique is suitable for delimiting *Katelysia* species in SA. However, the development of a new primer pair specific to mud cockle will be needed as about 70% of Section Bank and Little Douglas individuals could not be amplified by the common *Cox I* primer pair. This result also suggests at least another species might exist. In addition, a second barcoding gene is required to validate the *Cox I* results.

At the same time as further molecular studies are undertaken, the genetically identified mud cockle species also need to be described morphologically (if possible) to allow for practical differentiation for both aquaculture and fishery management.

B) Population genetic structure of *K. rhytiphora* in SA

When the relationship between the species in the genus *Katelysia* is established, the genetic structure of *K. rhytiphora* along the SA coast can then be revealed using molecular genetic techniques such as microsatellites, SNPs, etc.

C) Differentiation between hatchery produced and wild clam stocks

As none of the published methods that could potentially identify farmed from wild mud cockle stocks can provide 100% accuracy, an acceptable level of accuracy should be determined prior to further research of their suitability.

Further development

- Develop a *Cox I* primer pair specific to mud cockles in Australia to delimit the species in the genus *Katelysia* in SA and validate these results with other barcoding genes, such as the mitochondrial (16S and Cox III) and nuclear (ITS2) genes used by Li et al. (2013) to investigate the cryptic structures in the marine clam *Lasaea australis*.
- Investigate the taxonomy in the *Katelysia* genus using the samples that have been identified by barcoding genes.
- Investigate the population structure of *K. rhytophora* in SA with molecular techniques such as microsatellites, SNPs, etc.

- Investigate the suitability of microsatellite parentage assignment for differentiating between hatchery produced and wild *K. rhytiphora* stock.
- Long-term investigation (at least 2.5 years) of oxytetracyline and “hatchery shell” for differentiating between hatchery produced and wild *K. rhytiphora* stocks.
- Investigate if the cryptic *K. rhytiphora* species identified can be crossed to produce viable progenies.

9. Extension and Adoption

The finding of *K. rhytiphora* cryptic species in SA and its implication for aquaculture development and fishery management have been communicated to staff at PIRSA Fisheries and Aquaculture, potential cockle investors and cockle aquaculture and fishery researchers. These findings need to be considered in the development and implementation of fishery and aquaculture management strategies relating to *Katelysia* species. The extension and adoption also include a few ongoing project activities:

- Publication of this report at public domain.
- Preparation of a peer-reviewed paper on the microsatellites developed in this study.
- Presentations of project findings at relevant conferences/workshops.

10. Appendices

10.1. List of researchers and project staff

Professor Xiaoxu Li: SARDI Aquatic Sciences

Dr Kate Rodda: PIRSA Fisheries & Aquaculture

Dr Klaus Oldach: SARDI Sustainable Systems

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10.3. Presentation at the workshop of National Aquatic Animal Health South Australia working group – 3 July 2012

Project Title: Investigations to address key policy gaps associated with the development of clam farming in South Australia: genetic and health issues aligned to translocation and stock identification (FRDC Project 2010-233)

Principal Investigator: Xiaoxu Li (SARDI)

The need for the development of an Australian clam aquaculture industry is a direct outcome of the inability of the wild clam industry to meet the demand of a large international market prepared to pay a premium price for quality Australian clams. While clam aquaculture has been successfully developed on a large scale overseas, none exists in Australia at this time. Currently, a feasibility study to evaluate clams culture on intertidal and subtidal leases in SA is underway with the funding support from FRDC, SA Clam Aquaculture and SARDI (FRDC project 2009/208). This project builds on the outcomes from project 2009/208. A third project is being developed that investigates the issue of stock enhancement.

In this project, PIRSA Fisheries and Aquaculture is evaluating policies related to clam aquaculture development in SA to ensure the long term viability of both the wild harvest fishery and the successful development of a sustainable aquaculture industry.

The critical knowledge gaps identified for this assessment are:
1. population genetic structure of clam species in South Australia;
2. potential genetic and biosecurity risks of moving farming stocks between localities, and
3. effective and efficient methods to distinguish hatchery produced stock from wild stocks.

Understanding the population structure of this clam species and biosecurity risks of moving stocks will allow for the development of far more specific management strategies to control the potential adverse genetic and biosecurity impacts of farmed clams on the local wild population, thus protecting its genetic integrity if genetically divergent localised populations exist along the SA coastline. Practical identification of hatchery stocks will ensure compliance with wild fishery, aquaculture and recreational harvesting policies.

Gaps in our knowledge:

- Genetic structure of population in SA
- Genetic contaminations associated with translocation of cockle species between locations representing genetically dissimilar stock.
- Disease and susceptibility to different diseases in different environments (disease status for cockles is unknown)
- Interactions with wild populations of cockles and other species (e.g. competition for space (reseeding only) or food)
- Physical markers for practical differentiation between hatchery and wild cockle stock

Points for discussion (for a PIRSA Policy)

- Each population has its own disease status and the disease status is unknown for cockles, therefore:
    - use abalone as precautionary approach until more is known (e.g. PIRSA Disease Response Plan: Abalone Viral Ganglioneuritis 2013)
    - introduce controls
    - implement health checks
    - ensure that the broodstock is taken from the area where spat is to be released/farmed
- Risk of spreading disease from one location to another – what diseases are cockles susceptible to or able to transmit/harbor/act as host for?
- Considers issues relating to stock enhancement vs aquaculture activities.
    - Look at the stock enhancement policy being developed in Fisheries.
- Translocation – conditions under which it can occur (e.g. source of broodstock, disease risks, genetic contamination, risk of escape and competition with other species)
- Genetics – are populations in different areas distinct? (this is the purpose of project 2010/233)
- Source of spat for aquaculture – broodstock to be taken from near vicinity (depending on genetic diversity results)
- Structures – directly on seafloor – Environment Protection Authority (EPA), and Department of Environment, Water and Natural Resources (DEWNR) considerations, what habitat would be ok for this?
- What approvals would be required from other agencies – Department of Planning, Transport and Infrastructure (DPTI), EPA, and DEWNR?
- What licences/permits/authority is required to deal with access rights?
- What are the possible interactions (and impacts of) with other wildlife/spp. should cockles escape from the site or hatchery-reared cockles spawn?
- Methods to distinguish hatchery produced stock from wild stocks