

**Development of tools for the sustainable management of genetics
in polyploid Pacific oysters (*Crassostrea gigas*)**

Penny Alison Miller

Bachelor of Environmental Science (Marine Biology)

Bachelor of Science in Marine Biology (Honours)

Submitted in fulfilment of the requirements for the Doctor of Philosophy

School of Biological Sciences

University of Tasmania

July 2014

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Signed

A handwritten signature in black ink that reads "Penny Miller". The signature is written in a cursive style with a large initial 'P' and a long, sweeping flourish at the end.

Penny Miller

Date: 15/10/2014

Authority of Access

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

The publishers of the papers comprising Chapters 2 to 4 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Signed

A handwritten signature in black ink that reads "Penny Miller". The signature is written in a cursive style with a large initial 'P' and a long, sweeping flourish at the end.

Penny Miller

Date: 15/10/2014

Statement of co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Penny Miller, School of Biological Sciences

Anthony Koutoulis, University of Tasmania

Rene Vaillancourt, University of Tasmania

Nick Elliott, CSIRO

Peter Kube, CSIRO

John Henshall, CSIRO

Author details and their roles:

Paper 1, A genetic diversity study of cultured, naturalized and native Pacific oysters (*Crassostrea gigas*) determined from multiplexed microsatellite markers:

Located in chapter two.

Penny Miller was the primary author and with Anthony Koutoulis, Rene Vaillancourt, Nick Elliott and Peter Kube contributed to the idea, its formalisation and development, as well as assisted with refinement and presentation.

Paper 2, Genetic diversity and pedigree assignment in tetraploid Pacific oysters (*Crassostrea gigas*):

Located in chapter three.

Penny Miller was the primary author and with Anthony Koutoulis, Rene Vaillancourt, Nick Elliott and Peter Kube contributed to the idea, its formalisation and development, as well as assisted with refinement and presentation.

Paper 3, Assignment of parentage in triploid species using microsatellite markers with null alleles, an example from Pacific oysters (*Crassostrea gigas*):

Located in chapter four.

Penny Miller was the primary author and with Anthony Koutoulis, Rene Vaillancourt and Nick Elliott contributed to the idea, its formalisation and development. John Henshall was the primary designer of the software analysis. Anthony Koutoulis, Rene Vaillancourt, Nick Elliott and John Henshall assisted with refinement and presentation

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: _____

Anthony Koutoulis

Supervisor

School Of Biological Sciences

University of Tasmania

Rene Vaillancourt

Head of School

School of Biological Sciences

University of Tasmania

Date: _____

Acknowledgements

I would like to acknowledge the University of Tasmania, Australian Seafood CRC, CSIRO Food Futures Flagship and Shellfish Culture Ltd. for the funding of this research.

Foremost, I would like to thank my tireless team of supervisors. Anthony Koutoulis for his thorough understanding of university operations, Nick Elliott for his comprehensive knowledge of the aquaculture industry and Rene Vaillancourt for his attention to detail and molecular know how. I would like to thank you for sharing your knowledge, inspiration and experience and for responding to the countless emails and answering all my never ending questions.

A big thank you to Scott Parkinson, Michel Bermudes, Andy Day and all of the other staff at Shellfish Culture Ltd. for their support and constant supply of oyster samples. I am truly grateful for the hands on industry experience you gave me.

This research would not have been possible without the technical advice of a number of experts. I would like to thank Peter Kube for his valuable insights into project design and statistical analysis, Stan Allen for sharing his extensive knowledge of polyploid oysters with me and John Henshall for his wonderful software design. A big thank you to Tariq Ezaz who never once gave up hope on getting the chromosome spreads to work. Your enthusiasm and optimism were truly inspiring.

In terms of lab work, I would like to thank Sascha Wise for her consistent help and advice. I would also like to thank Bhumika Azad and Matt Young for going above and beyond to try to get the FISH analysis to work. Thank you to Aina Price, Jane Harbard and Rebecca Jones for sharing their lab and microscope skills with me.

Thank you to the following who helped in sample acquisition: Mike and Graeme Cameron and staff (Cameron of Tasmania), Matthew Cunningham and Ben Finn (Australian Seafood Industries), Mike Dove (Industry and Investment, NSW), Sylvie Lapègue (IFREMER, France), Jung-Ha Kang (NFRDI, Korea), Sharon Appleyard (CSIRO, Tasmania), Mathew Cook (CSIRO, Brisbane), Robert Green (Department of Primary Industries, Tasmania) and Michael Ezzy (volunteer, Tasmania).

Acknowledgments to the editors and reviewers of the Journal of Shellfish Research and the Journal of Aquaculture for their valuable input into chapters two and three and Ari Verbyla and David Lovell for their reviews of the statistical methods of chapter four.

Finally, I would like to thank my family. Words cannot express how grateful I am for your love and support. Without you this dissertation would still be a pipedream.

Thesis Abstract

The commercial production of triploid Pacific oysters (*Crassostrea gigas*) has grown rapidly in recent years. There is now a push to move away from commonly used mass spawning techniques towards single pair cross selective breeding programs in an effort to improve growth and disease resistance within the triploid product. Before this can be achieved, there is a need to understand some of the fundamental genetics behind polyploid production and to develop molecular tools and techniques that can be used in establishing breeding programs. This dissertation developed and utilised suites of microsatellite markers to determine the baseline diversity of native, naturalised and cultured diploid oysters. It was found that the high diversity within naturalised oysters may provide a genetic reservoir for future breeding programs. The same microsatellite markers were used to determine diversity and pedigree assignment within a mass spawned tetraploid population across two successive generations. The first generation showed a high diversity, which significantly decreased in the second generation produced via mass spawning. This was most likely due to a low number of effective broodstock and skewed parental contributions and highlights the benefits of using single pair crosses over mass spawning to control inbreeding. A method for assigning pedigrees in triploids, produced by crossing diploids with tetraploids, was developed. This method will allow the pedigree of strongly performing triploids to be traced back so that the same or closely related broodstock can be used in single pair cross selection programs to produce future generations. To determine the long term stability of tetraploid oysters, the aneuploid frequency was analysed using flow cytometry across three generations. No difference was observed which suggests that either aneuploidy is occurring at a lower rate than previously predicted or that aneuploid oysters are being removed from the system

through hatchery grading or early mortality. Flow cytometry is not sensitive enough to detect small scale chromosome loss. Hence, fluorescent in situ hybridisation (FISH) using microsatellite markers was trialled. This was unsuccessful due to the inconsistency of the markers. The molecular tools, techniques and results described within this dissertation will aid in the development of single pair cross selective breeding programs for the improvement of triploid oysters.

Contents

| | |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Chapter 1: Introduction | 1 |
| 1.1 What is polyploidisation? | 1 |
| 1.2 Polyploidisation in molluscs | 5 |
| 1.3 The history of Pacific oysters in Australia | 9 |
| 1.4 Triploid Pacific oyster production | 10 |
| 1.5 Potential issues associated with polyploids | 12 |
| 1.5.1 Breeding | 12 |
| 1.5.2 Inbreeding | 14 |
| 1.5.3 Aneuploidy | 16 |
| 1.6 Dissertation overview | 17 |
| 1.7 Conclusion | 19 |
| | |
| Chapter 2: Genetic diversity of cultured, naturalized and native Pacific oysters <i>Crassostrea gigas</i> determined from multiplexed microsatellite markers | 20 |
| 2.1 Introduction | 20 |
| 2.2 Materials and methods | 23 |
| 2.2.1 Sample collection and DNA extraction | 23 |
| 2.2.2 Microsatellite analysis and PCR conditions | 24 |
| 2.2.3 Data analysis | 27 |
| 2.2.3.1 Marker performance and measures of genetic diversity | 27 |
| 2.2.3.2 Bayesian analysis | 28 |
| 2.3 Results | 29 |
| 2.3.1 Marker performance | 29 |

| | |
|----------------------------------------------------------------------------------------------------------------------|-----------|
| 2.3.2 Measures of genetic diversity | 31 |
| 2.3.3 Bayesian analysis | 34 |
| 2.4 Discussion | 34 |
| | |
| Chapter 3: Genetic diversity and pedigree assignment in tetraploid Pacific oysters (<i>Crassostrea gigas</i>) | 38 |
| 3.1 Introduction | 38 |
| 3.1.1 Selection in polyploid oysters | 39 |
| 3.1.2 The importance of diversity within tetraploid oysters | 39 |
| 3.2 Materials and methods | 42 |
| 3.2.1 Sample collection | 42 |
| 3.2.2 DNA extraction and PCR | 43 |
| 3.2.3 Statistical methods | 44 |
| 3.2.3.1 Software | 44 |
| 3.2.3.2 Parental assignment | 46 |
| 3.3 Results | 46 |
| 3.3.1 Tetraploid diversity | 46 |
| 3.3.2 Pedigree analysis | 47 |
| 3.4 Discussion | 52 |
| 3.4.1 Tetraploid diversity | 52 |
| 3.4.2 Tetraploid assignment | 54 |
| 3.4.3 Unassigned tetraploids | 55 |
| 3.4.4 Conclusion | 57 |

| | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Chapter 4: Assignment of parentage in triploid species using microsatellite markers with null alleles, an example from Pacific oysters (<i>Crassostrea gigas</i>) | 58 |
| 4.1 Introduction | 58 |
| 4.2 Materials and methods | 61 |
| 4.2.1 Sample collection, DNA extraction and PCR | 61 |
| 4.2.2 Statistical method | 62 |
| 4.2.3 Simulated dataset | 66 |
| 4.2.4 Declaring parentage thresholds | 67 |
| 4.2.5 Statistical analysis of triploid oysters | 68 |
| 4.3 Results | 68 |
| 4.3.1 Software performance | 68 |
| 4.3.2 Triploid parental assignment | 69 |
| 4.4 Discussion | 73 |
| | |
| Chapter 5: Chromosome stability in successive generations of tetraploid oysters using flow cytometry | 78 |
| 5.1 Introduction | 78 |
| 5.2 Materials and methods | 80 |
| 5.2.1 Sample Collection | 80 |
| 5.2.2 Standard | 81 |
| 5.2.3 Flow cytometry | 81 |
| 5.2.4 Statistical methods | 82 |
| 5.3 Results | 82 |
| 5.4 Discussion | 84 |

| | |
|------------------------------------------------------------------------------------------------------------|------------|
| Chapter 6: Counting and identifying polyploid Pacific oyster (<i>Crassostrea gigas</i>) chromosomes | 87 |
| 6.1 Introduction | 87 |
| 6.2 Materials, methods and results | 90 |
| 6.2.1 Sampling | 90 |
| 6.2.2 Metaphase spreads | 90 |
| 6.2.3 FISH using microsatellite motifs | 92 |
| 6.2.4 FISH using PNA probe | 93 |
| 6.3 Discussion | 94 |
| | |
| Chapter 7: Conclusion | 97 |
| | |
| References | 104 |
| Appendix | 123 |
