Capturing and maintaining genetic diversity for the establishment of a long-term breeding program for barramundi (*Lates calcarifer*) aquaculture

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Contents

List of Tables

List of Figures

List of Appendices

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Shannon Loughnan

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Statement of Authorship

Chapter 1

S.L.

Chapter 2

Data collection : S.L., J.D., J.F.

Laboratory methods : S.L., J.D.

Statistical analysis : S.L.

Manuscript writing : S.L., N.R.

Chapter 3

Data collection : S.L.

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Chapter 4

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Laboratory methods : S.L., C.S.K.

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Chapter 5

Statistical analysis : S.L, N.R.

Manuscript writing : S.L, N.R.

Chapter 6

S.L.

Thesis summary

Mass spawning hatchery practices using small broodstock populations, in addition to the cannibalistic nature of some fish species, contribute to a reduction of genetic diversity from parent to offspring and throughout the juvenile grow-out stages. This is of concern when establishing a selective breeding program for such species because the genetic diversity that is captured in the start-up and initial generations of the program is the basic ingredient for future genetic improvement. The aim of this thesis was to examine methods for capturing and conserving genetic diversity in mass spawning barramundi (*Lates calcarifer*), when constructing a base population for a long-term selective breeding program for the species.

Involving 21 males and 12 females, the transfer of genetic diversity from broodstock to offspring in a large commercial mass spawn was investigated in chapter 2. Previous studies had indicated that substantial amounts of genetic diversity were lost using mass spawning techniques, which are normal practice for the commercial barramundi industry. A high participation rate of parents was detected among the large spawning group used in this study (n = 31). Broodstock contributions were skewed and the contribution by individual dams and sires was as high as 48% and 16% respectively at one day post hatch (dph). Barramundi progeny were monitored throughout the juvenile stages to investigate the conservation of genetic diversity, during the periods of larval metamorphosis and size grading (to inhibit cannibalism).

A reduction in allelic richness (A_r) was identified from broodstock to offspring at 1 dph, (A_r was 3.94 among broodstock and 3.52 among offspring sampled). However, no further loss of A_r or genetic diversity was detected in the offspring from

1 to 90 dph, which included the period of metamorphosis, multiple size grading events and losses through size culling, mortalities and the sale of juveniles. The effective population size (N_e) in the broodstock group ranged from 10.1 – 16.7, well below the broodstock census size of 33, whereas the rate of inbreeding was less than 5%. The results from the mass spawn provided reproductive and demographic parameters that could be used to inform the design of a base population for a barramundi selective breeding program.

In chapter 3, 407 mature captive broodstock under current use in eight commercial barramundi hatcheries were pedigree tested using 17 microsatellite markers, to determine their suitability for inclusion into a base population. Levels of genetic diversity within each hatchery and the degree of relatedness between individuals were estimated and compared. Genetic diversity was moderate within each broodstock group (A_r ranged from 2.67 – 3.42) and heterozygosity ranged from 0.453 – 0.537. Relatedness estimates within hatcheries were generally low and ranged from -0.003 to 0.273. Structure analysis revealed that captive Australian broodstock were broadly divided into two genetic stocks and suggested that hatchery individuals were either sourced from the two stocks or represented an admixture between them. From the results, an assessment was made of the genetic suitability of existing domesticated broodstock as contributors to the base population.

Chapter 4 sampled 1205 barramundi individuals from 48 wild sites covering a broad distribution range. Levels of wild genetic diversity were estimated and compared to captive groups from chapter 3. The wild collections were found to cover two broad ranging genetic stocks, an eastern and western stock and a central stock of genetic admixture (F_{ST} = 0.076). The majority of captive individuals were assigned to the eastern stock (59%), followed by the western stock (23%) and central

region of admixture (13%). Levels of genetic diversity, as determined by allelic richness (A_r), were slightly lower in the captive groups (average A_r = 3.15) when compared to the wild populations (average A_r = 3.40). Some genetic variation was unrepresented in the captive groups and it was concluded that the inclusion of wild individuals would enhance overall levels of genetic diversity in a base population for selective breeding.

Finally, a computer simulation model was developed in chapter 5 and used to compare different options for sourcing genetic variation for inclusion into the base population. It was assumed that the primary goal when establishing the base population would be to maximise genetic diversity. Candidates for inclusion into the synthetic base populations were selected according to levels of genetic diversity and relatedness. A range of options were tested, which included the use of candidates from both wild and captive populations. There was a significant reduction in the level of A_r between broodstock and offspring ($P < 0.05$) for many of the options. The best options for retaining genetic diversity were from the base populations constructed from an even representation of wild samples from genetic stocks (WSA_r, broodstock and offspring A_r was 5.21 and 4.75 respectively) and to select captive broodstock according to the lowest mean kinship levels (Cmk_r, broodstock and offspring A_r was 5.05 and 4.69 respectively). Five alternate base population sizes (N_c) were tested to estimate the effective population size (N_e) based on the variance of parental contribution and unequal sex ratio. N_e was 76, 85, 98, 105 and 115 from an N_c of 150, 180, 200, 230 and 250 respectively, and the rate of inbreeding (ΔF) ranged from 0.4 – 0.7%. Under the model presented in this study, an N_c of more than 213 broodstock individuals is required to achieve $N_e > 100$ and $\Delta F < 0.5\%$. The results suggested that a mixture of both wild and captive barramundi should be

included in the base population at the commencement of a selective breeding program for barramundi.

This thesis investigated the effects of hatchery practices, such as mass spawning and size grading on the conservation of genetic diversity. In addition, options for selecting candidates to compose a founding population were explored, and recommendations made to promote the longevity and impact of a selective breeding program for barramundi. The Australian industry has on hand a large number of mature captive broodstock that would be suitable for inclusion into a base population for barramundi selective breeding. However, it would be beneficial to include a selection of wild individuals from regions of high genetic diversity to strengthen the fitness of a base population at the commencement of a selective breeding program.

Summary of chapters

This thesis is presented as a series of manuscripts. Chapter 2 has been published, chapter 3 is under review and chapters 4 and 5 are manuscripts in preparation for publication.

Chapter 2 publication:

Broodstock contribution after mass spawning and size grading in barramundi (*Lates calcarifer*, Bloch).

Loughnan, S.R., Domingos, J.A., Smith-Keune, C., Forrester, J.P., Jerry, D.R., Beheregaray, L.B., Robinson, N.A. **Aquaculture** 2013, 404–405, 139–149.

Barramundi is naturally a mass spawning species, which can be induced to spawn in captivity under conditions that attempt to replicate the natural environment. Due to the high fecundity of females and the inclusion of numerous adults into a spawning group, the production of large quantities of larvae can be high. Relatively few breeders have the potential to supply a large proportion of the grow-out industry. However, the main complications identified by previous studies involving captive mass spawning barramundi, were the low participation rates for particular broodstock and highly skewed levels of parental contribution across all broodstock. With a limited number of contributors, inbreeding rates can be high and genetic diversity can be lost within offspring cohorts, which can complicate the selection of unrelated broodstock candidates for the next generation of breeders. Typically, small broodstock groups of 1 – 2 females and 3 – 5 males are constructed, not only due to high fecundity but space requirements and the costs of maintaining numerous adult barramundi can be high. In this study, a large mass spawn (12 females and 21 males)

not previously applied on this scale was carried out to investigate the level of parental contribution from a large mass spawning group, and the number of parent pair relationships that could be detected within the offspring. The offspring were sampled at regular intervals during grow-out, which provided the opportunity to investigate the conservation of genetic diversity throughout the period of size grading and culling for the avoidance of cannibalism. Previous studies have reported on a loss of genetic diversity by size grading, however, no study has yet monitored the maintenance of genetic diversity throughout the entire cannibalistic stage of juveniles. The major findings from this chapter include a high participation rate of both male and female broodstock and the subsequent production of a large number of parent pair combinations or families. Despite a high rate of participation, contribution levels were unequal and there was a high variance in family sizes. In addition, there was a slight loss of genetic diversity from broodstock to offspring but throughout the period of size grading and culling, no further loss of genetic diversity was detected. The results suggest that a mass spawning group of at least 30 barramundi individuals is required to achieve a high participation rate of breeders and to limit the loss of genetic variation transferred to the offspring.

Chapter 3 in review:

Genetic diversity and relatedness estimates for captive barramundi (*Lates calcarifer*) broodstock populations, informs efforts to form a base population for selective breeding.

Loughnan, S.R., Smith-Keune, C., Jerry, D.R., Beheregaray, L.B., Robinson, N.A.

Journal **Aquaculture.**

The Australian barramundi industry has on hand a large number of mature broodstock that are currently supplying the grow-out market, however, before selective breeding programs can begin, it is important to assess the levels of genetic diversity and relatedness of current captive broodstock populations. This has not yet been assessed for Australian captive stocks, nor has the application of such information been applied to establishing a base population for selective breeding. Due to the implications of mass spawning investigated in chapter 2, it is also unclear how this has impacted on genetic diversity and relatedness levels across the captive industry. To address these issues, microsatellite DNA markers were utilised to genotype barramundi broodstock from eight major Australian commercial hatcheries. Population structure analysis indicated that captive Australian broodstock were broadly divided into two genetic population groups, genetic diversity levels were moderate and a level of relatedness was detected in each broodstock group. The estimates of genetic diversity and relatedness derived from this study suggest that the Australian barramundi industry has on hand suitable broodstock candidates for the development of a base population for selective breeding from current captive stocks. Although, sourcing additional broodstock from wild regions of high genetic diversity could enhance the fitness of current captive stocks further. The results are discussed

with regard to broodstock management and the development of a base population for selective breeding using existing Australian broodstock.

Chapter 4 to be submitted:
Assignment of captive barramundi (*Lates calcarifer*) broodstock to wild Australian stocks guides captive base population recruitment for selective breeding.
Loughnan, S.R., Smith-Keune, C., Jerry, D.R., Beheregaray, L.B., Robinson, N.A.
Journal **Aquaculture.**

The quality of captive barramundi founder stocks can be enhanced and fitness maintained by including wild individuals from genetically diverse stocks at the commencement of a selective breeding program. Identifying which wild stocks to target can be aided with assignment tests, which can clarify the wild genetic origins of captive individuals and determine the degree of wild genetic diversity not currently represented in captive stocks. In chapter 3, levels of relatedness and genetic diversity were estimated for eight captive broodstock groups under current production, and in this chapter the individuals within each of these groups were assigned to their wild ancestral origins. Levels of genetic diversity and population structure were determined for wild barramundi samples from 48 sites with 16 polymorphic microsatellite loci. Two wild genetic stocks and a region of genetic admixture were detected and levels of genetic diversity were slightly higher in the wild sample collections than the captive groups. Upon developing a base population for the selective breeding of barramundi, wild locations demonstrating high levels of genetic diversity identified in this study should be accessed to gather broodstock candidates. Ideally, an even number of broodstock should be sourced from each of

the three wild genetic stocks, to lower the level of relatedness between individuals and to gather a broad range of genetic diversity for the founding population.

Chapter 5 to be submitted:

Comparison of the use of different source stocks for establishing base populations for selective breeding of barramundi (*Lates calcarifer*).

Loughnan, S.R., Smith-Keune, C., Jerry, D.R., Beheregaray, L.B., Robinson, N.A.

Journal **Aquaculture Research.**

To determine the most appropriate broodstock candidates to use when establishing a base population for barramundi selective breeding, a computer simulation model to predict the maintenance of genetic diversity at 16 microsatellite loci was developed. There are various methods for selecting broodstock candidates for inclusion into a base population, such as selecting according to kinship levels between individuals (mk_r) or choosing individuals from wild regions demonstrating high levels of genetic diversity. Both of these methods were tested in the simulation model. Synthetic base populations were developed from the observed genotypes of captive broodstock from eight hatcheries (accessed from chapter 3) and the genotypes from 48 wild sites were utilised from chapter 4. In addition, chapter 2 provided parental contribution probabilities, which were used to select male and female parents at the commencement of the simulation, to mimic the skewness of parental contribution that can occur in barramundi mass spawning. Overall, this chapter incorporated the findings of the previous studies and utilised the results to recommend the best method for selecting a base population. Under each option there was a loss of genetic diversity from each broodstock group to offspring, although the highest level of genetic diversity was maintained when selecting broodstock

according to low mean kinship values (mk_r). The results suggest that a base population of at least 213 individuals split into five spawning tanks of an equal sex ratio, will provide a N_e of 100 and ΔF of 0.2%. In addition, wild broodstock should be sourced from regions of high genetic diversity and combined with current captive broodstock that have been selected according to the lowest mk_r values. This will help to maintain founder genetic diversity and heterozygosity levels in subsequent generations.

1 General Introduction

1.1 Genetic improvement programs

Following multiple generations in captivity, domestication of animals occurs, reducing stress, which in turn can increase disease resistance and reduce mortality (Gjedrem et al., 1991). Selection for the genetic improvement of domesticated animals has been practiced for thousands of years. For fish, it is believed that domestication and selection began with carp-like species in China and Japan some 3000 – 4000 years ago and resulted in the many varieties of common carp, goldfish and koi of different forms and colours that are common today (Bardach et al., 1972).

The principal objective of a genetic improvement program is to achieve the highest genetic response possible, dependant on the amount of genetic variability available in the population (Davis and Hetzel, 2000). Attaining this, an increase in productivity, quality and most importantly profitability can all be expected. The design of a breeding program should include a formal definition of the breeding objective, which identifies the biological traits of a species that may be commercially important and estimates their relative economic values (Gjedrem et al., 2005). In addition, an estimation of the genetic parameters that describe populations and their differences, the evaluation of additive and non-additive genetic merit of individuals or families, and mating plans, should all be detailed (Davis and Hetzel, 2000). Overall, traits need to be chosen to move in the direction of the breeding objective, they need to be heritable and inexpensive to measure. Structured animal breeding programs for the selection of traits have been established for terrestrial livestock (Gjedrem, 2005). Molecular markers target genes or regions of DNA that exhibit differences among individuals (Ward et al., 2000) designed to detect differences in

DNA sequences (Davis & Hetzel 2000). They can assist in stock identification of families and individuals, as well as in the control of inbreeding and genetic improvement by selection for preferred gene combinations (Davis and Hetzel 2000). Molecular markers are capable of identifying marked genes known as quantitative trait loci (e.g. disease resistance and fast growth rate) from pedigree lines into commercial broodstock, while minimising the introduction of unwanted effects, such as inbreeding (Ward et al., 2000). Mitochondrial DNA (mtDNA) markers have traditionally been applied to population level studies and the inference of molecular relationships among closely related species (Meyer, 1993). Due to the maternal inheritance of mtDNA, it is more effective in comparing the genetic variability in wild populations and cultured stocks than a single nuclear (biparently inherited) locus (Cross, 2000), however, mtDNA represents only a single locus, which is unlikely to be informative for every question.

Protein electrophoresis or allozyme markers have typically been used in monitoring translocation and stocking regimes (Cross, 2000) and are not suitable for use in genetic improvement programs due to the need for fresh or frozen tissue from a variety of organs (i.e. liver, heart and muscle) and thus causing the death of the animals of interest. Allozymes do not detect large amounts of genetic variability (Cross, 2000) and due to the limited number of loci that can be surveyed (e.g. ~40), allozymes are not an effective marker when applied to aquaculture based programs. A molecular marker more suited is nuclear-encoded loci such as microsatellites.

Microsatellites are a form of repetitive sequence DNA, which are highly variable among individuals and exhibit large numbers of alleles (Cross, 2000). They are common throughout the genome, particularly in fish and a wide range of sample sources are suitable for the amplification of microsatellites via PCR, such as the non-

destructive sampling of scales and fin clips from fish (Schlotterer and Pemberton, 1994). Microsatellites have been detected in all eukaryotic genomes studied, although they are limited in plant and avian genomes (Zane et al., 2002). Advantageous to many fish species is the presence of larger numbers of alleles and high heterozygosity, more than those observed in mammals (O'Connell and Wright, 1997). Microsatellites are also considered to be selectively neutral (Cross, 2000), which is important for the inference of reproductive isolation based on allele frequency differences. Genome mapping and the detection of quantitative trait loci (QTL) require a large number of loci, and microsatellites have this advantage over other molecular markers (O'Connell and Wright, 1997). The major hindrance of utilising microsatellites is the complexity of initial development, often requiring significant genomic library screening experience, although recent next generation sequencing (NGS) techniques have made the development of microsatellites more cost effective.

The application of molecular markers and concept behind genetic improvement programs is similar across all species (Gjedrem, 2005), however, the design of each species-specific program can vary considerably. This is especially the case for aquaculture species which have diverse biological features and require special hatchery practices and design. Many fish species under aquaculture production are undomesticated but provide a high fecundity rate, practice external fertilisation and generally display higher levels of variation at genetic markers than terrestrial farmed animals (Chistiakov et al., 2006). These features enable much higher rates of genetic improvement to be achieved for many aquaculture species than for terrestrial livestock, although high fecundity rates can increase the risk of inbreeding and loss of genetic diversity.

Inbreeding is the mating of animals with recent common ancestry and the rate of inbreeding (ΔF) is measured as the probability that two genes at any locus are identical by descent. Once a selective breeding program is initiated the breeding population should be closed to further entry of new stock from other sources, which could dilute the genetic improvement made. In a closed population, some degree of inbreeding is inevitable after a few generations but problems can be avoided if inbreeding is limited by maintaining a large breeding population and if the founding base population is mostly unrelated. Generally accepted maximum levels of inbreeding in captive aquaculture populations have ranged from 0.5 – 1% per generation (Bentsen and Olesen, 2002; Fjalestad, 2005; Sonesson et al., 2005). For GIFT tilapia (Genetic Improvement of Farmed Tilapia, *Oreochromis niloticus*) ΔF has been estimated at 2% (Ponzoni et al., 2010), in rainbow trout (*Oncorhynchus mykiss*) 0.7% (Kause et al., 2005) and in Coho salmon (*Oncorhynchus kisutch*) ΔF ranged from 1.1 – 2.5% (Gallardo et al., 2004). To assist in reducing ΔF whilst maintaining genetic gain in future generations of livestock production, models have been developed to predict ΔF in populations under selection. Not only for random mating populations but also more complex breeding programs, which include non random mating and overlapping generations (Wray and Goddard, 1994; Woolliams and Bijma, 2000). Controlling ΔF can be more efficiently managed by including an optimised selection technique such as optimal genetic contributions, which restrict the ΔF applied (Hinrichs et al., 2006; Meuwissen et al, 2002). As the extent of genetic improvement with selective breeding is dependent on levels of genetic variation in the breeding population, the genetic diversity of the founding base population is also an important consideration when beginning selective breeding.

Captive stocks typically only represent a proportion of the genetic diversity available in wild populations. When establishing a base population for selective breeding, it is advantageous to include wild individuals to boost representative levels of genetic diversity. Relatedness levels amongst broodstock can also be lowered, which is an important factor when choosing individuals to construct broodstock groups for mass spawning. It is more difficult to control inbreeding and maintain genetic diversity in mass spawning species, as compared with paired mating. It is common that some broodstock in a mass or group spawning scenario will not always contribute to the spawn and if the broodstock group is small, contribution levels are more likely to be unequal and heavily skewed (e.g. Blonk et al., 2009; Chavanne et al., 2012; Frost et al., 2006; Hara and Sekino, 2003; Wang et al., 2008). This is accentuated if only one small broodstock group is utilised, rather than establishing multiple breeding groups. Because of the high fecundity of many aquaculture species, small base populations are generally maintained (e.g. Pacific oyster, *Crassostrea gigas* Boudry et al., 2002; mangrove red snapper, *Lutjanus argentimaculatus* Emata, 2003), and levels of inbreeding and loss of genetic diversity is therefore likely to be high. A large unrelated base population divided into multiple broodstock groups of equal sex ratio could be used in these instances, to help to conserve genetic diversity in future generations but more detailed knowledge about the spawning of these species would be needed to decide on the best strategy to use.

The initial development of a large and genetically diverse founder population has achieved positive genetic gains for some breeding programs (Gjedrem, 2010). For example, the base population for the Norwegian Atlantic salmon (*Salmo salar*) selective breeding program was originally developed from 40 wild river strains (Gjedrem et al., 1991) and the overall genetic gain for growth has been estimated at

115% when compared to wild stocks after five generations (Thodesen et al., 1999). A combination of four wild geographic strains from Africa and four established farmed strains from the Philippines were combined and successfully included into the first GIFT tilapia (*O. niloticus*) program in Asia (Eknath et al., 1993). The accumulated genetic gain in relation to the base population has been estimated at 85% after five generations of selection for growth (Eknath and Hulata, 2009). The capacity for genetic gain is limited by the extent of genetic diversity in small base populations and any loss of genetic diversity in first generation hatchery stocks is lost to all subsequent generations within a closed breeding program (Gjedrem, 2010).

Genetic diversity is described as the level of DNA variation within and among individuals, such as heterozygosity and the number of alleles present. High genetic variation generally refers to genetically fit stock containing a diverse genetic makeup, exhibiting a high survival rate and superior quality that can show a favourable increase in economic traits, such as rapid growth, preferred flesh quality and greater disease resistance. On the other hand, a decline in the rate of genetic diversity promotes genetic drift and can be detrimental to the overall performance of the cultured population and the life of a breeding program. Loss of genetic variation can occur within small populations, such as aquaculture stocks using a limited number of broodstock. This loss is due to sampling or genetic drift, and typically as a result of selection. Genetic variation is known to be the most important input for the development of captive stocks, and its loss is a constraint for any genetic improvement program (Freitas et al., 2007). To ensure the long-term sustainability of any selective breeding program it is important to capture and conserve the available genetic diversity.

Loss of genetic variation has been reported in many aquaculture populations, including abalone (*Haliotus rubra* and *H. midae*) (Evans et al., 2004a), white shrimp (*Litopenaeus vannamei*) (Freitas et al., 2007) and barramundi (*Lates calcarifer*) (Frost et al., 2006; Yue et al., 2002: Yue et al., 2009; Zhu et al., 2006a). Barramundi is a hardy euryhaline species, ideally suited to aquaculture due to its fast growth and tolerance for a wide range of environmental conditions. However, because barramundi is highly fecund and mass spawned with small broodstock population sizes, unequal parental contribution and subsequent high variance in family sizes can occur (Frost et al., 2006; Wang et al., 2008). It is particularly vulnerable to inbreeding and loss of genetic diversity in captive culture and there have been no detailed studies in the literature regarding alternative methods of design for establishing base populations, or about the size of a mass spawning base population needed to conserve genetic diversity and control inbreeding for selective breeding.

1.2 Barramundi (*Lates calcarifer*)

Barramundi or Asian seabass is distributed throughout the Indo-West Pacific region from northern Australia, throughout Southeast Asia to Taiwan (Grey, 1986). There are seven other species within the *Lates* genus (Froese and Pauly, 2010), many of which are either commercially fished or cultured. Some of the better known species are Nile perch (*L. niloticus*), which is commercially fished extensively in Africa and the Japanese *Lates* or akame (*L. japonicas*), which is commercially cultured in Japan. Productive wild fisheries exist for barramundi and captive production is increasing in regions of Southeast Asia and Australia, where barramundi is a highly valued recreational and food fish. Barramundi aquaculture in Australia is a developing industry, with recent growth in the number of license

holders and development approvals (ABARES 2011). Recent production figures have been recorded at 3190 tonnes for the period 2009 – 2010, valued at AU$27.5 million. In Southeast Asia, barramundi has been under intensive aquaculture production since the 1970's and were first successfully propagated in Thailand (Yue et al., 2009) for supplying fish to market and the restocking of native habitats. Since then, culture in the Philippines, Taiwan, Singapore and Malaysia have followed (Harvey et al., 1985). Hatchery production in Australia commenced in 1984 and the first commercial hatchery in north Queensland was initiated in 1986 (Tucker et al., 2002).

Throughout the native distribution range of barramundi, spawning is stimulated by the commencement of the rainy season, taking place in highly saline environments of lower estuaries and river mouths (Moore and Reynolds, 1982). Barramundi is a catadromous species and has both salt and freshwater requirements. Spawning, egg and early larval development all require salt water, whereas juveniles prefer estuarine and freshwater conditions where they grow and mature as males (Moore, 1982; Tucker et al., 2002). Following sexually maturity at 4 – 5 years, males begin to participate in spawning events, where they will spawn at least once before sex inversion at approximately seven years of age, and females may not commence egg production until they are eight years old (Davis, 1982; 1984). In captivity, males can change to females at approximately 3 – 4 years of age, although the time of sexual inversion can vary (Macbeth et al., 2002).

Barramundi naturally practice sex inversion or protandry, where all offspring are born male and later change to female. Females have shown evidence of being both complete and multiple spawners, where the complete ovary ripens and all eggs are shed at the one time. In larger females, the eggs develop sequentially and the fish

may spawn more than once in a season, shedding only 10% of their eggs at a time (Davis, 1984). As the length and weight of a female increases so does the fecundity, which has been estimated at 46 million eggs for a female of total length 1240 mm (Davis, 1984). Protandry can be problematic for a captive breeding program, as broodstock from alternate year classes need to be maintained to achieve the desired numbers of mature males and females at the same period of time (Robinson et al., 2010). This is particularly difficult when practising selective breeding, where generally the next generation of broodstock are selected from the same cohort. In some species, sex inversion can be induced via hormonal therapy to obtain both sexes at the desired stage of a breeding program (Peatpisut and Bart, 2010; Yeh et al., 2003). However, this is yet to be trialled with barramundi and although further investigations are also required for strip spawning and the cryopreservation of sperm, these methods may be more suitable for achieving the desired parent pair crosses (Leung, 1987; Macbeth and Palmer, 2011; Palmer et al., 1993).

Prior to the development of artificial reproduction techniques, barramundi were spawned in correspondence with the lunar cycle. In Southeast Asia, wild adults were captured and hormone induced in the field, eggs and milt stripped and collected, then transported to a grow-out facility where larvae were raised under semi intensive conditions (Tucker et al., 2002). More recently, barramundi production has become more intensive, not only in Asia but Australia, where most farmed barramundi are spawned in hatcheries and larvae are transferred to grow-out facilities. Rather than relying on barramundi broodstock to spawn naturally in the captive environment, the majority of hatcheries use hormones to induce the spawning process, such as human chorionic gonadotropin (HCG), gonadotropin releasing hormone analog (GnRHa), luteinising hormone-releasing hormone analogue (LHRHa), carp pituitary and

barramundi pituitary (Tucker et al., 2002). Together with a rise in water temperature, females are injected in the morning to spawn that night or the following day in salt water, whereas males are not normally injected and rely on the activity of the females to stimulate sperm release. Barramundi are generally mass spawned in a group of 1 – 2 females to 3 – 5 males (author's personal observations; Macbeth et al., 2002) and collected eggs are hatched artificially to improve survival rates.

Cannibalism occurs in a number of fish species under captive culture (e.g. giant grouper, *Epinephelus lanceolatus* Hseu et al., 2004 and Asian catfish, *Pangasianodon hypophthalmus* Baras et al., 2010) and is a major complication when rearing barramundi juveniles together. Constant size grading is required during the juvenile stages to remove any size variation throughout the cohort, a process that is both labour intensive and stressful on stock, however, the loss due to cannibalism can reach as high as 50% per day without size grading (Rutledge, 1991). Cannibalism in barramundi and other species has been attributed to an inadequate food source, low feeding frequency, high population density, minimal refuges, water clarity and light intensity (Curnow et al., 2006; Hecht and Pienaar, 1993; Parazo et al., 1991; Qin et al., 2004). Following size grading for the prevention of cannibalism, culling or the removal of size grades sometimes occurs to ensure a standard growth rate across the entire cohort (Macbeth et al., 2002), although both these hatchery practices can cause a loss of genetic diversity.

There are several important factors that need further exploration in order to capture and conserve high levels of founder genetic diversity for selective breeding of mass spawning fish species such as barramundi. Current hatchery methods utilising small population sizes have been shown to be unsustainable, resulting in a reduction of genetic diversity following each generation (Frost et al., 2006; Wang et

al., 2008). There is a need to know whether the use of larger mass spawning group sizes can result in a more even and reliable contribution by all individuals to the group spawn. We also need to know whether size grading to reduce cannibalism and the culling of extreme size grades, can significantly affect the loss of genetic diversity. In addition, it is important to know how existing genetic diversity among captive and wild populations can be best utilised and mixed to maximise the genetic diversity within the founding population for selective breeding. This thesis tests a large proportion of mature broodstock under current commercial production, along with representatives sampled from wild barramundi populations around Australia to explore how a base population of high genetic diversity can be formed and maintained for future generations of selective breeding.

1.3 Thesis scope and objectives

This thesis utilises genetic markers in the form of microsatellites to assess levels of genetic diversity, inbreeding and relatedness for the benefit of developing a base population for selective breeding of barramundi. Firstly, a large captive barramundi mass spawn at a scale not previously conducted was trialed in chapter 2 with the following objectives;

- To determine the effect of large scale mass spawning of barramundi on the maintenance of genetic diversity with selective breeding.
    - To investigate parental participation and levels of contribution from mass spawning broodstock to offspring.
    - To measure the effect of cannibalism and subsequent size grading on the maintenance of genetic diversity in offspring.

In planning for the development of a large and genetically diverse base population for barramundi selective breeding, chapter 3 examined levels of genetic diversity and relatedness within current captive broodstock, from multiple commercial hatcheries in Australia. The objectives of this chapter were;

- To evaluate levels of genetic diversity and relatedness in existing captive stocks.
- To determine whether existing captive stocks can provide suitable candidates for the development of a large and genetically diverse base population for selective breeding.

The wild origins of captive broodstock were then determined in chapter 4, utilising reference genetic data from a broad range of wild Australian locations. Levels of genetic diversity were also estimated in wild stocks and compared to levels currently represented in captive populations. The main objective of this chapter was;

- To assign current captive individuals to wild localities and identify genetic diversity not currently represented in captive stocks.

A computer simulation model was developed in chapter 5, which incorporated the results from the previous chapters to determine the most appropriate methods for conserving genetic diversity and controlling ΔF when designing a base population for barramundi selective breeding. The objectives of the final chapter were;

- To explore different options for selecting candidates to capture genetic diversity for inclusion into a base population.
    - Construct synthetic base populations according to levels of genetic diversity and relatedness, and apply these to a computer simulation model to predict the transfer and maintenance of genetic diversity in offspring.

2 Broodstock contribution after mass spawning and size grading in barramundi (*Lates calcarifer*, Bloch)

Publication; **Aquaculture 2013, 404 – 405, 139 – 149 (Appendix 2C)**

2.1 Abstract

Appropriately designed selective breeding programs are expected to limit the loss of genetic diversity and control levels of inbreeding, and to base selection decisions on data collected from many offspring of many families. Achieving a relatively even contribution by broodstock to subsequent generations is necessary and for many aquaculture species this is possible to control through paired mating. Barramundi (*Lates calcarifer*) provides an exception, because in captivity it is a species that mass spawn in small groups and whose offspring are repeatedly size graded in an effort to avoid cannibalism. Following mass spawning a large broodstock group of 33 barramundi, levels of parental contribution and multiple measures of genetic diversity were estimated over the course of repeated size grading events. Parentage was inferred using 17 microsatellite DNA loci. Twelve dams and 21 sires were artificially spawned over two nights and sampled at 1, 18 and 90 days post hatch (dph). Broodstock contributions were skewed and the contribution by individual dams and sires was as high as 48% and 16% respectively at 1 dph. Despite the unequal contribution and high variance in family sizes, 31 broodstock were detected as contributing to the spawning events and as a result up to 103 full-sibling families were detected (18 dph, n = 472). A reduction in allelic richness (A_r) was identified from broodstock to offspring at 1 dph, (A_r was 3.94 among broodstock and 3.52 among offspring sampled). However, no further loss of A_r or genetic diversity was detected in the offspring from 1 to 90 dph, which included the period of metamorphosis, multiple size grading events and losses through size culling, mortalities and the sale of juveniles. The effective census population size ratio (N_e/N_c) ranged from 0.31 – 0.51 at times of sampling, (N_e was calculated between 10.1 and 16.7, well below the broodstock census size of 33) and the rate of

inbreeding was less than 5%. This research provides valuable baseline data that can be used to make recommendations for the maintenance of genetic diversity and control of inbreeding for a barramundi selective breeding program. It also provides an example of what considerations need to be made for the genetic management of mass spawning and/or cannibalistic species.

2.2 Introduction

Understanding how genetic diversity is represented and maintained throughout the hatchery and production cycle is critical for the successful development of selective breeding programs in aquaculture. This is particularly evident for natural mass spawning species, where single pair mating cannot be conducted. Mass or group spawning (each female reproducing with many males and each male reproducing with many females randomly in a single tank) is a common method of breeding for a number of aquaculture species (e.g. common sole, *Solea solea* Blonk et al., 2009; gilthead seabream, *Sparus aurata* Chavanne et al., 2012; barramundi, *Lates calcarifer* Frost et al., 2006; Japanese flounder, *Paralichthys olivaceus* Hara and Sekino, 2003). Although this reproductive strategy can produce a large quantity of offspring and thus increase production, it can also promote heavily skewed levels of broodstock contribution and a high variance in family sizes, which can lead to a reduction in the effective population size (N_e) and an increase in the rate of inbreeding (ΔF) (Brown et al., 2005). Under captive culture, mass spawning is typically utilised for those species that naturally spawn in large congregations, although generally under this situation a limited number of sexually mature adults are utilised.

Low broodstock population sizes are typically employed for mass spawning species bred in captivity, because it is costly to maintain numerous adult fish. In addition, many species exhibit high fecundity, so that a small number of broodstock have the potential to fulfil seasonal production requirements (e.g. Pacific oyster, *Crassostrea gigas* Boudry et al., 2002; mangrove red snapper, *Lutjanus argentimaculatus* Emata, 2003). This may be appropriate for the commercial production of harvest fish where levels of genetic diversity are generally ignored, however, within the initial stages of a selective breeding program it is important to select a high number of founder broodstock from diverse ancestries, to maximise genetic diversity and actively avoid mating's between animals with recent common ancestry (Gjedrem, 2005). This important step not only assists in the maintenance of genetic diversity in future generations but it also reduces the extent of inbreeding.

Barramundi, or Asian seabass (*Lates calcarifer*), is a highly fecund, mass spawning catadromous species from the family Latidae, cultured mainly throughout Southeast Asia and Australia, with worldwide production increasing. As a mass spawning species, methods under captive culture involve the aggregation of conditioned, sexually mature broodstock, typically at the ratio of 1 – 2 females to 3 – 5 males (author's personal observations; Macbeth et al., 2002). Hormone induced spawning via luteinising hormone-releasing hormone analogue (LHRHa) injections and environmental manipulation, are generally necessary for final gonad maturation and to promote the release of gametes for artificial spawning (Tucker et al., 2002). Following hatching, heavy mortalities can occur among larvae during metamorphosis (Frost et al., 2006) and fingerling development phases, when intraspecific predation (cannibalism) can ensue (Parazo et al., 1991). Size grading of juvenile barramundi is used to reduce the incidence of cannibalism and produce a more uniform cohort for

stocking purposes, however, grading has the ability to alter the relative contributions of broodstock to the next generation of offspring and may consequently have a negative effect on the maintenance of genetic diversity (Frost et al., 2006).

Cannibalism is not only prevalent in Latidae but has also been reported within 36 other teleost families (Smith and Reay, 1991), many involved in aquaculture production, including Serranidae (giant grouper, *Epinephelus lanceolatus* Hseu et al., 2004) and Pangasiidae (Asian catfish, *Pangasianodon hypophthalmus* Baras et al., 2010). Cannibalism typically commences in barramundi fry after they have completed metamorphosis at approximately 15 – 20 days post hatch (dph) (Tookwinas, 1989) and continues until offspring reach an approximate total length of 100 mm (Qin et al., 2004). During grading, juveniles are divided into independent size grades, dependant on body size and some categories may be culled to achieve a uniform size across the cohort (Macbeth et al., 2002). It is possible that the disposal of size grades (culling) may contribute to the loss of genetic diversity (Frost et al., 2006), as discarded groups or even individuals may contain unique genetic variants or distinctiveness, which are excluded from the cohort and the contribution by some broodstock may be affected. Grading has also been employed to reduce social interactions and to improve the growth rate of silver perch, *Bidyanus bidyanus* (Barki et al., 2000) and captive sole, *Solea solea* (Blonk et al., 2010), and has been shown to result in the selection of animals of a particular gender when sexual dimorphism in body size occurs (e.g. Mediteranean sea bass, *Dicentrarchus labrax* Saillant et al., 2003). Molecular markers, such as microsatellite DNA, enable the reconstruction of family pedigrees to investigate the impact of size grading on broodstock contribution. They can also disclose levels of genetic variation in offspring of mass spawning species such as barramundi (Yue et al., 2002).

Microsatellites can be used to empirically reconstruct pedigrees, allowing unrelated animals to be chosen and mass spawned for breeding, so that the rate of inbreeding and loss of allelic diversity is limited with the production of each successive generation. In captive mass spawned barramundi, where no more than two dams were utilised for multiple spawns, microsatellites determined broodstock contributions as highly skewed (Frost et al., 2006). At 2 dph, Frost et al. (2006) detected the contribution of one sire as high as 77%, when three sires participated out of seven present in the tank and all dams and sires were injected with LHRHa. In an additional spawn under the same study, only three sires from a total of six were injected with LHRHa, with the contribution of one sire reaching over 60% at 2 dph. When 10 dams and 10 sires were all induced hormonally, Wang et al. (2008) recorded captive bred broodstock contributions as high as 98%, when five out of 20 broodstock contributed to the spawning. In an alternate spawning event using wild sourced broodstock that were again hormonally induced (n = 20), Wang et al. (2008) discovered that broodstock participation was high, with the involvement of 19 out of 20 parents, resulting in no single individual contributing greater than 36%. The level of participation and resulting contribution likely depends on broodstock weight and maturity (Brown et al., 2005) and mate competition, particularly due to the dominant behaviour of sires (Fessehaye et al., 2006; Weir et al., 2004) and the competiveness of sperm (Campton, 2004; Wedekind et al., 2007). The number of broodstock used and the quantity injected with LHRHa for artificial spawning, plus the timing of spawning are also likely to play an important role, with fertilisation more likely to occur between females and males spawning at approximately the same period of time.

Selective breeding programs for mass spawning barramundi have been initiated by Yue et al. (2009) in Asia and proposed by Robinson et al. (2010) in Australia, although the natural mass spawning nature of barramundi creates some obstacles. The main complications identified by previous studies involving captive mass spawning barramundi (Frost et al., 2006; Wang et al., 2008), were the low participation rates for particular broodstock and highly skewed levels of contribution across all broodstock. Understanding broodstock contribution and the transfer of genetic diversity of captive mass spawning barramundi under artificial spawning (as opposed to natural spawning), is not only of value to the development of a successful selective breeding program for the species but also for the restocking of wild fisheries and the maintenance of local genetic variation. In this study, a large mass spawn (12 dams and 21 sires) not previously applied on this scale, was carried out to examine these issues and to determine whether spawning's on this scale in multiple tanks could be applied to benefit a selective breeding program.

2.3 Materials and methods

2.3.1 Mass spawning of broodstock

The broodstock group consisted of captive bred stock, originally developed from wild individuals collected locally from the central Queensland region of Australia. The best performing broodstock were selected based on previous mass spawning events. Ideally an equal number of females and males were added to the spawning group, although no further females were available. As a result, additional males were added to the spawning group with the aim of developing a high number of families. Selected broodfish were sedated in a saltwater bath containing 40 ppm AQUI-S (Aquatic Diagnostic Services International) and a small segment of caudal

fin (*ca*. 1 cm^2) was removed for later DNA extraction and subsequent genotyping for pedigree determination. Fin clips were immediately stored for preservation in either 80% ethanol or DMSO-salt solution (20% DMSO, 0.25 M disodium-EDTA and NaCl to saturation at pH 8) (Seutin et al., 1991). Passive integrated transponder (PIT) tags implanted in each individual were scanned to provide a unique identification system. While sedated, all broodstock were cannulated to confirm sex with a 2.16 mm outside diameter catheter tube. Broodstock were then recovered from anaesthesia and placed back into their holding tank.

Twelve females (two of uncertain sex) and 21 males were conditioned for spawning, together in a 50,000 L fibreglass tank. The fish were fed a formulated diet (INVE Aquaculture) ad libitum, maintained at a constant water temperature of 28.5^{o}C and subjected to a 14 hour day length for 12 weeks. To determine their readiness for spawning, female broodstock were again sedated and cannulated as described above, and oocytes were collected using a catheter and inspected under a microscope. Oocytes of a diameter of 400 µm or more were considered appropriate for successful spawning. Whilst sedated, 10 females were injected with LHRHa (Syndel International Ltd), at a dosage rate of 50 $\mu g.kg^{-1}$ to assist in the release of eggs. A further two females, dams 06 and 10, were in the spawning tank but were not injected (sex uncertain at the time). Males were not induced to spawn using LHRHa, as the willingness of the females to release eggs due to hormone induction generally encourages the males to discharge sperm. Following recovery from sedation, all 10 females were released back into their spawning tank to circulate with the males and left to spawn over multiple nights. Following spawning each night, the water surface of the tank was directed into an external egg collection reservoir, where the eggs were caught in a 400 µm nylon mesh bag. The total egg count from

each spawning night was determined by counting a fixed volume under the microscope in a Sedgewick-Rafter slide. The fertilisation rate (%) of the spawn was determined, by observing the level of cell division and embryo development from multiple sub-samples under the microscope. All eggs from the first and second day of spawning were then transferred to two circular fibreglass tanks (1200 L) for incubation and hatching, and although the broodstock group continued to spawn on the third and subsequent nights no further eggs were collected.

2.3.2 Size grading and sampling

A random sample of whole larvae was collected at 1 dph for both the first (spawn A, n = 182) and second day of spawning (spawn B, n = 274), prior to the remaining larvae being transferred to two separate external grow out facilities at 3 dph for rearing. Sample sizes were restricted by the cost of genotyping, although based on previous studies (Frost et al., 2006; Yue et al., 2009) the sample sizes were deemed adequate. The 1 dph sample from spawn B was a key reference point used for many subsequent comparisons and the maximum number of samples was collected. Phenotypic parameters such as length and weight were collected from the 1 dph cohort and the results published in a study on the heritability of barramundi (Domingos et al., 2013, Domingos et al., 2014). The first grading event occurred at 18 dph, where the cohort was split into three size classes determined by the spacing of the grading device; small (<1.5 mm), medium (1.5 – 1.7 mm) and large (>1.7 mm) (see Appendix 2A). At these grading specifications, the larval rearing facility had discovered that cannibalism was effectively reduced in barramundi. Immediately following grading at 18 dph, random samples of whole larvae were collected from each size class for parentage analysis; small (n = 208), medium (n = 158) and large

(n = 106). A similar fraction of animals from each of the size classes were sampled. During each subsequent grading event following 18 dph, the offspring were sorted within their current size classes using increasingly wider spaced graders on each subsequent occasion. In some cases larger individuals from the small and medium size grades would be promoted to the medium and large size grades respectively (Appendix 2A). Size grading occurred on six occasions between 18 and 42 dph, followed by another six grading events between 42 and 90 dph, although samples were only DNA tested following size grading at 18 and 90 dph. At three grading events (18, 28 and 90 dph), the total estimated cohort size in the number of juveniles was provided and a representative percentage per size grade could be calculated. A final sample collection of 92 juveniles from each size grade was conducted after the last grading at 90 dph, where the cohort was divided according to average weight (4, 8 and 16 g), but similarly labelled as small, medium and large. At 90 dph, juveniles were large enough to take fin clips. Throughout the rearing stage, fish were removed from the population in three ways; by the sale of juveniles, size culling and general losses. During the monitoring period, 91% of the cohort from spawn B was either sold as live fingerlings, or removed as the result of size culling and general mortalities.

2.3.3 DNA extraction

DNA was extracted from broodstock fin clips using a CTAB (cetyl trimethylammonium bromide) protocol described by Adamkewicz and Harasewych (1996), with the following modifications; polyvinylpyrrolidone (PVP) and β-mercaptoethanol were excluded from the buffer mix, as they are both generally applied to mucous laden and tannin stained samples for the removal of polyphenols

present in some plants (Porebski et al., 1997). Tissue was incubated overnight at 55°C with 10 µL of Proteinase K (20 mg.mL^{-1}). Chloroform-isoamyl alcohol (24:1) was added and mixed with the digested samples, centrifuged and the upper aqueous phase transferred to tubes of cold isopropanol (600 µL) and stored in the freezer for at least 1 hour. After centrifuging (16,000 *g* for 30 min), the pelleted DNA was washed with 70% cold ethanol, air dried and resuspended in 50 µL of 1x TE. All isolated DNA from CTAB extractions were quantified with a spectrophotometer (Nanodrop Technologies ND-1000) and visualised on a 0.8% agarose gel.

Whole larval samples collected at 1 and 18 dph, and small segments of fin clips (*ca*. 2 mm^2) taken at 90 dph, were individually transferred into 96 well plates and DNA extracted in plate format by a modified Tween®-20 procedure, specifically developed for small tissue samples and larval DNA extraction (Taris et al., 2005). 100 µL of Tween®-20 lysate buffer (670 mM Tris-HCl pH 8.0, 166 mM Ammonium sulphate, 0.2% v/v Tween-20®, 0.2% v/v IGEPAL® CA-630 NP-40) and 5 µL of 20 mg.mL^{-1} Proteinase K were added to each sample and digested for a minimum of 4 hours at 55°C. The samples were then incubated at 95°C for 20 minutes to denature the Proteinase K, 100 µL of 1x TE buffer was added and the samples stored at -20°C overnight prior to PCR.

2.3.4 Batch sampling to discriminate non-contributors from low frequency contributors

Extra batches of eggs and whole larvae from each night of spawning at 1 dph were pooled directly prior to DNA extraction. Testing of these pooled egg/larvae samples was used to supplement the testing of individual larvae, as a cost effective approach to assist in the detection of particular broodstock that contributed at a low

frequency (undetected due to sampling error), or not at all to the batches. One batch of unhatched eggs and one of 1 dph larvae, each containing approximately 200 eggs or larvae per tube were collected from both spawn A and B (4 tubes in total). DNA extractions were performed on each tube as a single extraction (using the CTAB protocol described in section 2.3.3), combining all 200 samples per batch, with a final elution of 150 µL of 1x TE buffer. To assist in differentiating between alleles and stutter bands in the electropherograms and differential amplification in the pooled samples, the correction method developed by Kirov et al. (2000) was followed. For a minimum of four individuals that were not added to the pools, the peak heights of stutter patterns were measured using MegaBACE® Fragment Profiler® software, resulting in an average peak height for each stutter band (calculated in Excel, Microsoft Office). Under the correction method, all allele peak heights were reduced (excluding the longest and known as the first allele), some to levels that would dismiss them from being scored as a legitimate allele in the pool. To correct for differential amplification, the relative peak heights of alleles of heterozygous individuals were recorded (comparing all possible heterozygous allele combinations). The average height difference between adjacent alleles was used to calculate a relative weighting factor (W_i) for each allele (i) such that $W_i = H_a / H_i$ where H_a was the height of the longest allele and H_i was the height of the i^{th} allele. Beginning with the second shortest allele, the corrected allele height H'_i was then calculated as $H'_i = H_i W_i$.

2.3.5 PCR amplification

Two multiplex groups of 17 markers were selected from published *L. calcarifer* microsatellite loci. Multiplex one included markers *LcaM03* (Yue et al.,

2001), *LcaM16*, *LcaM40* (Yue et al., 2002), *Lca57* (Zhu et al., 2006a), *Lca154*, *Lca178* (Zhu et al., 2006b), *Lca287* and *Lca371* (Wang et al., 2007). Multiplex two included *LcaM08*, *LcaM20*, *LcaM21* (Yue et al., 2002), *Lca58*, *Lca64*, *Lca69*, *Lca70*, *Lca74* and *Lca98* (Zhu et al., 2006a). One primer from each pair was labelled with a fluorescent dye (HEX, TET or FAM) at the 5' end. PCR amplification occurred in a 10 µL multiplex reaction with approximately 40 ng genomic DNA, 10x primer mix (containing between 0.10 to 0.25 µM of each forward and reverse primer for multiplex one and 0.06 to 0.20 µM for multiplex two) and 2x Type-it® PCR Master Mix (Qiagen). Samples were denatured for multiplex one at 95°C for 5 min, followed by 10 cycles of 95°C for 30 s, 57°C for 90 s and 72°C for 30 s, then 20 cycles of 95°C for 30 s, 55°C for 90 s and 72°C for 30 s, followed by a final extension at 60°C for 45 min on a C1000 Thermal Cycler (Bio-Rad). Multiplex two followed the same amplification steps as above, although the final extension consisted of 60°C for 30 min. Following amplification, PCR products were diluted with 12 µL of water and desalted through Sephadex® 258 G-50 fine filtration 259 spin columns (GE Healthcare). Desalted PCR products were visualised on a 1.5% agarose gel prior to genotyping on a MegaBACE® 1000 DNA Analysis System (GE Healthcare). MegaBACE® software Fragment Profiler® was used for fragment analysis, where alleles were allocated with an identifying label.

2.3.6 Statistical analysis

Following the scoring of genotypes, MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004) was used to check for scoring errors, which can be caused by allele stutter and the presence of null alleles. Parentage analysis was performed using CERVUS 3.0.3 (Kalinowski et al., 2007), to determine broodstock contribution

to offspring and the total number of half (*HS*) and full-sibling (*FS*) families. Multiple unrelated families incorporated into a selective breeding program that demonstrate high levels of within family genetic diversity, can assist in the maintainance of genetic variation and help to control inbreeding in the long-term. Under the parentage program, broodstock allele frequencies were utilised for the simulation of parent pairs of known sex and the following parameters were utilised; the typing of 100% of loci, the allowance of a 1% error rate for scoring genotypes, the minimum number of typed loci was eight and 10,000 offspring were simulated. A strict confidence level (CI) of 95% was utilised to determine the most appropriate parent pair assigned to offspring. CERVUS was also utilised to calculate observed (H_o) and expected (H_e) heterozygosities, the number of alleles per locus (k), including the number of private alleles (k_a), where only one broodstock individual possessed that allele, which was considered rare in the population and provided a measure of genetic distinctiveness. The inbreeding coefficient (F_{is}), which measures the degree of random mating within populations, was estimated by the method of Weir and Cockerman (1984) using FSTAT 2.9.3.2 (Goudet, 2002). Significant departures from zero for F_{is} values were also calculated in FSTAT at the 0.05 level, for evidence of heterozygote deficiency or excess. Any deviation of observed from expected proportions under Hardy-Weinberg equilibrium (HWE) was calculated using GENEPOP 4.1 (Rousset, 2008). *P*-values were estimated using a Markov chain (MC) algorithm, beginning with a dememorisation step of 10,000, followed by 20 batches of 5000 iterations per batch. The level of significance was determined following sequential Bonferroni correction (Rice, 1988). Allelic richness (A_r) within each locus was estimated with FSTAT 2.9.3.2 (Goudet, 2002), which is a measure of the number of alleles independent of sample size and incorporates a rarefaction

approach (Hurlbert, 1971). The genetically effective population size (N_e) was estimated in a way that accounted for unequal sex ratio and variance in family sizes. The effect of variation in family size on the effective numbers of dams N_{ed} and sires N_{es} was calculated according to Frankham et al. (2002) as

$$N_{ed} = (N_d K_d - 1)/[K_d - 1 + (V_d/K_d)] \ and \ N_{es} = (N_s K_s - 1)/[K_s - 1 + (V_s/K_s)]$$

where N_d and N_s was the number of dams and sires respectively, K_d and K_s were the mean number of offspring per dam and sire, and V_d and V_s was the variance in contribution for dams and sires. To account for an uneven sex ratio, N_e was estimated as

$$N_e = 4N_{ed}N_{es}/(N_{ed} + N_{es})$$

The rate of inbreeding (ΔF) was computed according to Falconer (1989) as

$$\Delta F = 1/2(N_e)$$

Any significant differences in broodstock contribution levels between spawns A and B (at 1 dph), between sampling at 1, 18 and 90 dph of spawn B and between the size grades, were determined by Pearson's 2-sided chi-square-test, using the exact test option with a threshold for significance of 0.05, in IBM SPSS 20.0 following data transformation. The Mann-Whitney test was also calculated in SPSS, to detect for any significant differences between broodstock and offspring at three measures of genetic diversity; H_e, A_r and F_{is}. Relatedness and relationship inferences (kinship analysis) were estimated between broodstock pairs using ML-RELATE (Kalinowski et al., 2006), to determine the level of genealogical similarities within

the group via a maximum likelihood approach that corrects for the presence of null alleles.

2.4 Results

2.4.1 Broodstock contribution

Parentage assignment rates were 94% (95% confidence interval) for spawn A and ranged from 98% to 99% for spawn B. Broodstock contribution levels were skewed for both dams and sires over the two nights of spawning (Fig. 2.1 and 2.2) and an equal contribution (uniformity) from all 33 broodstock would have resulted in each dam and sire contributing to the production of 8.3% and 4.8% of offspring respectively. Dam 04 was the highest contributing dam to spawns A and B at 1 dph, assigned as the most likely parent of 48% and 30% of 1 dph larvae respectively (Fig. 2.1a). The highest contributing sires at 1 dph, were sire 03 (15%) to spawn A and sire 04 (16%) to spawn B (Fig. 2.2a). There was no significant difference in the level of broodstock contribution between spawns A and B at 1 dph (dams $P = 0.222$; sires $P = 0.242$). Similarly, there was no significant difference between the sampling events at 1 and 90 dph from spawn B for sires ($P = 0.117$), although there was a significant difference between the contributions of dams between 1 and 90 dph ($P < 0.05$), and also 18 and 90 dph ($P < 0.05$). Of the two dams that were not injected with LHRHa (dams 06 and 10, which were found to be dams from parentage analysis), only dam 06 was observed in the offspring from spawns A and B, although only a minor contribution was detected (< 3%) across all sampling events from this individual (Fig. 2.1). Dams 10 and 11 were not detected at any stage in the offspring and were considered as not participating in the spawning event over two nights. Besides dams 10 and 11, only sire 18 was undetected by 90 dph (Fig. 2.2b).

Small, medium and large size grades from spawn B

By monitoring the offspring population from spawn B throughout multiple size grading events up to 90 dph, it was possible to test for any impact of size grading on the contribution of broodstock to each of the size grades. Broodstock contribution levels to size grades were skewed and significant differences in the level of contribution were detected between some of the size grades for both dams and sires (Fig. 2.3 and 2.4). At 18 dph, broodstock contribution levels were significantly different between the small and medium size grades (dams $P < 0.01$; sires $P < 0.05$), and also between the medium and large groups for dams ($P < 0.01$). At 90 dph, a significant difference was detected between the small and large size grades (dams and sires $P < 0.01$), and also between the medium and large groups (dams $P < 0.01$; sires $P < 0.05$). The highest contributing dam at 18 and 90 dph was dam 08 (Fig. 2.1b), which was also a major contributor to the size grades, ranging from 20% to 44% (Fig. 2.3). Sires 03 and 13 were the greatest contributors at 18 and 90 dph respectively (Fig. 2.2b), and were also the major contributors to each of the size grades, ranging from 10% to 20% (Fig. 2.4). In general, broodstock found to have a higher participation rate in the spawning events, provided relatively even contribution levels across the alternate size grades, whereas broodstock with lower participation rates had more uneven contributions across the size grades. Following grading at 18 dph, the small size grade represented 78% of the remaining population, whilst the medium and large size grades represented 19% and 3% of the population respectively (see Appendix 2A). After grading at 28 dph (broodstock contribution not determined), the small, medium and large size grades were allocated 41%, 53% and 6% of the remaining population respectively, and by 90 dph, the small, medium and large size grades were distributed 24%, 62% and 14% respectively.

2.4.2 The production of half and full-sibling families

From a total of 10 dams (two dams were undetected) and 21 sires, the maximum number of full-sibling (*FS*) families detected was 103 at 18 dph from spawn B (n = 472, Table 2.1). The total number of *FS* families detected was dependant on sample size, as there was a considerable increase in the number of *FS* families at 18 dph when compared to 1 dph followed by a decrease at 90 dph, which was due to the quantity of samples collected (at 1 dph 78 families n = 274, at 18 dph 103 families n = 472, at 90 dph 77 families n = 276). As a result, the number of *FS* families detected per 100 offspring samples (FSn_{100}) was calculated at 1, 18 and 90 dph, as 28, 22 and 28, respectively. All 21 sires were detected as parents to the paternal half-sibs at 1 and 18 dph for spawn B, whilst a maximum of 10 dams were identified as parents of the maternal half-sibs (among offspring tested at 90 dph).

2.4.3 Genetic diversity

A total of 73 alleles (k) were recorded from the broodstock across 17 polymorphic microsatellite markers, ranging from two to eight alleles per locus and at an average of 4.3 alleles per locus (Table 2.2). Thirteen private alleles (K_a, an allele detected in only one broodstock individual) were detected and K_a contributed to 18% of the total number of alleles identified in the broodstock. The broodstock population conformed to Hardy-Weinberg equilibrium (HWE) over all loci, although there was a significant departure from zero for F_{is} values at two loci; *Lca*154 and *Lca*287 ($P < 0.05$), following sequential Bonferroni correction (Rice, 1988). Overall average relatedness was relatively low across the broodstock group (r = 0.08, maximum likelihood approach) at 95% confidence intervals, relatedness ranged from 0 – 0.35 for unrelated individuals, 0.09 – 0.38 for half-sibs, 0.30 – 0.82 for full-sibs

and 0.44 – 0.62 for parent offspring relationships. A high percentage of the parent pair combinations were estimated as having an unrelated relationship (83%), followed by half-sib (11%), full-sib (4%) and parent offspring (2%). Deviations from HWE and the presence of null alleles were detected in the offspring groups; at loci *Lca*287 ($P < 0.001$) for all sampling events, *Lca*371 (spawn A at 1 dph $P < 0.01$; spawn B at 1 dph $P < 0.05$) and *Lca*178 (spawn B at 1 dph $P < 0.05$).

Broodstock and 1 dph offspring from both spawns A and B

A loss in the number of alleles was detected when comparing 1 dph offspring to broodstock over the two nights of spawning. Eight alleles were undetected in the progeny from spawn A (Table 2.3), seven of those being private alleles detected in the broodstock, whilst six alleles were similarly undetected in the offspring from spawn B, which were all private alleles in the broodstock. A 15% and 11% reduction in allelic richness (A_r) from parent to offspring was detected at 1 dph, from spawn A and B respectively, however, there was no significant difference in the level of A_r between broodstock and offspring at 1 dph (spawn A, $P = 0.193$ and spawn B, $P = 0.339$). Over both spawning nights, expected heterozygosity (H_e) was lower in the offspring at 1 dph when compared to the broodstock population but there was no significant difference between the broodstock and offspring for H_e or F_{is} (Mann-Whitney tests). The number of broodstock that effectively contributed (N_e) to the spawn as detected at 1 dph was 10.1 for spawn A and 13.5 for spawn B, from a broodstock census size (N_c) of 33. From the estimates of N_e, the rate of inbreeding (ΔF) was calculated at 5% and 3.7% for spawn A and B respectively at 1 dph, and the N_e / N_c ratio ranged from 0.31 to 0.46.

Spawn B offspring 1 dph, 18 dph and 90 dph

Due to sampling error, the frequency of alleles derived from spawn B fluctuated from 1 to 90 dph, although there was no apparent loss of alleles by the final sample collection (Table 2.3). By 90 dph, the number of alleles including those deemed private in the broodstock actually increased when compared to 1 dph and no loss of genetic diversity was recorded when comparing offspring across 1, 18 and 90 dph, as measured by the non significant associations of H_e, A_r and F_{is} (Mann-Whitney tests). Average F_{is} was significantly different from zero in the offspring at both 18 and 90 dph ($P < 0.05$), except in the medium size grade at 18 dph ($P = 0.29$). Deviations from HWE were detected at locus *Lca*287 ($P < 0.001$), for each size grade sampled at 18 dph (excluding the large size grade) and 90dph.

Fate of rare alleles among the offspring

In total, five out of 13 alleles that were detected as private in the broodstock (allele 113 at locus *Lca*098; alleles 202 and 207 at locus *Lca*178; alleles 204 and 221 at locus *Lca*287) were not observed at any stage in the offspring and could be considered lost to the cohort (Appendix 2B). These five alleles were also not detected in the offspring population at 1 dph in the pooled egg and larvae samples. One of the private alleles belonged to sire 20, which was a very low contributor (< 2%) across both spawn A and B (Fig. 2.2). The remaining four private alleles belonged to dams 10 and 11 but neither dam contributed to the spawning events (Fig. 2.1). On the other hand, a high contributor such as dam 04 contributed as much as 30% to spawn B but only one private allele was observed for this individual (117 at *Lca*64), which had an allele frequency ranging from 0.030 – 0.132 among the offspring (Appendix 2B). In total, eight private alleles were detected in broodstock

that were low contributors to offspring at 1 dph (< 1.2%) and allele frequencies in the offspring for these eight alleles were no higher than 0.029.

2.5 Discussion

Broodstock contributions were skewed, although there was a high participation rate of broodstock in the spawning events, which resulted in a high number of full-sibling families. Individual broodstock contribution reached 48% and some significant differences in contribution levels between the size grades were detected. Unequal parental contribution and in some cases unequal sample size and sampling error, may have attributed to these results. Significant differences between parental contributions to the different size grades might be indicative of genetic or parental effects on early growth rate, as has been detected in other fish species such as European sea bass (Saillant et al., 2001). Contributions of up to 77% (Frost et al., 2006) and 98% (Wang et al., 2008) have been reported for individual barramundi broodstock under other mass spawning runs. Heavily skewed broodstock contribution levels have also been reported for other mass spawning aquaculture species (e.g. common sole, *Solea solea* Blonk et al., 2009; gilthead seabream, *Sparus aurata* Chavanne et al., 2012; Japanese flounder, *Paralichthys olivaceus* Sekino et al., 2003). For final gonad maturation and to promote the release of gametes for artificial spawning, the application of LHRHa was not beneficial for all dams. Dam 06 was not injected with LHRHa but in some cases its contribution level was greater than other dams within the broodstock group that had been injected, and despite dam 11 being injected with LHRHa it was not detected as contributing to either spawn A or B. No sires were injected with LHRHa, however, this did not impact on the participation rate of sires, as all were detected as contributing to the spawning events.

Unequal parental contributions did cause a reduction in the number of alleles from broodstock to offspring at 1 dph, although no further associated loss of genetic variation was detected from 1 to 90 dph due to putative larval mortalities throughout the period of metamorphosis, or from the effects of size grading, culling or the removal of juveniles for sales. Average A_r ranged from 3.33 – 3.55 in the offspring, whereas A_r was estimated at 3.94 in the broodstock group. Subsequent sampling at 90 dph (spawn B) showed a slightly higher average A_r when compared to 1 dph offspring, although the result was not significant (P = 0.876).

The effective number of broodstock contributing to the next generation (N_e) ranged from 10.1 – 16.7 for the two spawning events (N_c = 33), so that ΔF ranged from 3 – 5%. The range of inbreeding values far exceeded the generally recommended average of 0.5% for a population under a captive breeding program (Sonesson et al., 2005). If mass spawning were to be used for selective breeding of barramundi, careful consideration would need to be given to the relatedness of possible mate pairs in each spawning tank. For instance, using a cost-factor on inbreeding (see Brisbane and Gibson, 1995; Wray and Goddard, 1994) and including additional broodstock groups of diverse ancestry, would assist in limiting the level of inbreeding. Additional synchronous mass spawns would also need to be performed to boost family numbers. In other mass spawning species, variance in reproductive success among dams can differ greatly from that among sires (Gold et al., 2008; Gold et al., 2010), although little difference was detected in this study and therefore this factor would have little influence on the overall effective population size in this case.

The differences in broodstock contribution achieved in this barramundi mass spawn compared to previous experiments by other authors (Frost et al., 2006; Wang

et al., 2008), could be attributed to either differences in the nutritional conditioning and reproductive readiness of animals prior to spawning, the tank facilities used, the number of broodstock injected with LHRHa and the dosage, or the size of the spawning group. Complex behavioural cues may also lead to the stimulation of animals in the tank and could affect the success of the spawn. Another possibility is that the large number of broodstock used for the mass spawn in our study (compared to the smaller broodstock group sizes traditionally used within the industry), may have resulted in a greater and more even stimulation of the broodstock present. This could have resulted in more animals contributing to the spawning events and spawning occurring over a shorter time frame during each night, than was the case for other studies. Ultimately, to gain greater control over the production of family sizes and equalise broodstock contribution to the next generation of offspring, techniques for the collection of milt together with cryopreservation and the strip spawning of eggs should be investigated.

Reports of strip spawning are limited for barramundi, although the techniques have been developed (Leung, 1987; Palmer et al., 1993) and utilised successfully under some situations e.g. milt collected from spermiating wild stock (Palmer et al., 1993). Cryopreservation of sperm along with strip spawning of both males and females would be beneficial, as it would allow for tighter control over inbreeding and could eliminate the need for DNA testing. It may also overcome the main problem caused by protandry in barramundi, enabling the selection of broodstock candidates from the same generation to be mated. All barramundi are born as males, later changing to females at approximately 3 – 4 years of age in captivity, although the time of sexual inversion appears to be highly variable (Macbeth et al., 2002). Selective breeding programs for barramundi utilising strip spawning and

cryopreservation have been modelled and the use of these techniques would result in higher long-term benefit-cost ratios, compared to using mass spawning (Macbeth and Palmer, 2011; Robinson et al., 2010).

By pooling eggs and larvae, and DNA extracting as a batch, we were able to detect less frequent contributions to the spawns that may have otherwise been missed due to sampling error. Broodstock private alleles that were missing in the individual genotypes also went undetected in the pools, indicating that not all broodstock alleles were transferred to the offspring. Overall, the raw electropherogram patterns from the pooled genotypes helped to distinguish low contributors from non-contributors, although under the correction method for stutter many alleles were eliminated from the pools. Relative allele frequencies were not estimated from the pooled genotypes and subsequent correction for differential allele amplification proved difficult, because particular eggs or larvae may contribute more DNA to the pool than other individuals. There might be some cost benefits if pooled genotypes alone could be used to study the relative level of broodstock contribution and levels of genetic diversity (Skalski et al., 2006).

The ideal situation for a genetic improvement program is to have all broodstock contributing as evenly as possible, so that fewer offspring need to be reared, measured and genotyped. The pattern of broodstock contribution has been shown to have a large impact on the cost of the selective breeding program proposed for barramundi (Robinson et al., 2010). Stochastic simulation of breeding programs using mass selection, have indicated that more than 50 pairs of breeders and 30 – 50 progeny per parent pair need to be tested if inbreeding is to be limited to approximately 1% per generation, and to achieve a reasonable response to selection (Bentsen and Olesen, 2002). If parental contribution is reasonably even from a large

broodstock group, a random selection of offspring from each year's cohort would yield animals from many different and relatively evenly represented families for testing. Of course, some families will be poorly represented and therefore it would be necessary to use a higher number of broodstock to obtain adequate numbers of breeding pairs with sufficient numbers of progeny. However, with mass spawning a factorial mating pattern is achieved (each female reproducing with many males and each male reproducing with many females), so that both maternal and paternal half-sibs are produced. This is advantageous to a selective breeding program, as it allows minimisation of possible confounding between additive genetic, maternal and paternal effects (Gjerde, 2005). For a given number of spawning tanks under a balanced factorial mating design, less broodstock can be tested than for nested mating or single pair mating designs. For the mass spawning of barramundi in this study, the main limitation was not the number of spawning tanks required but the total costs of DNA testing and this is influenced by the evenness of broodstock contribution to the spawn. For instance, if 10 separate mass spawning's were carried out, each under identical conditions to the trial spawn in this study and if we aimed to continue DNA testing until we found 30 progeny from 50 separate pairs of breeders (as recommended by Bentsen and Olesen, 2002), then from our data we would have needed to DNA test approximately 1500 offspring per mass spawn. There are various strategies that could be adopted to reduce this number, such as performing more DNA tests from the tanks where the broodstock contribution is found to be more even, however DNA testing will still be a significant cost to the breeding program under a mass spawning situation.

2.6 Conclusion

In summary, a large number of half and full-sibling families could be produced for selective breeding from a mass spawn involving 33 barramundi broodstock, of which 31 were detected as contributing to the offspring. In addition, by combining offspring batches from multiple broodstock groups, the number of families detected could be increased. Due to unequal contribution and high variance in family sizes, there was an initial loss of allelic richness from parent to offspring at 1 dph but there was no further reduction of genetic variation due to size grading, or through the removal of offspring by either size culling, the sale of juveniles or general mortalities. Broodstock contribution was also variable across the two nights of spawning, resulting in some differences in the combination of parent pair crosses between spawn A and B. Therefore, we recommend monitoring parental contribution over multiple spawning nights, synchronising spawning in multiple tanks, and using more than 30 broodfish per spawning group, in order to maximise the transfer of genetic variation to the next generation of broodstock candidates.

Table 2.1 Number of full-sibling families (*FS*), the number of *FS* families detected per 100 offspring samples (FSn_{100}), maternal half-sibling (*Mhs*) and paternal half-sibling (*Phs*) families detected across the first (spawn A) and second night (spawn B) of spawning

		FS	FSn_{100}	*Mhs*	*Phs*
Spawn A					
1dph		59	32	7	19
Spawn B					
1dph		78	28	9	21
18dph	Total	103	22	9	21
	Small	74	36	8	19
	Medium	64	41	6	20
	Large	47	44	6	20
90dph	Total	77	28	10	20
	Small	47	51	9	17
	Medium	47	51	9	18
	Large	42	46	8	17

Table 2.2 Genetic diversity estimates for 33 broodstock; sample size (*N*), number of alleles (*k*)[a], number of private alleles (k_a)[a], allelic richness (A_r), observed (H_o) and expected (H_e) heterozygosities, and the inbreeding coefficient (F_{is})

Locus	*N*	*k*	k_a	A_r	H_o	H_e	F_{is}
LcaM03	33	2	-	2.00	0.273	0.282	0.034
LcaM08	33	3	1	2.55	0.152	0.144	-0.053
LcaM16	33	6	3	4.70	0.364	0.348	-0.046
LcaM20	33	4	1	3.57	0.455	0.403	-0.129
LcaM21	33	5	-	4.81	0.758	0.682	-0.113
LcaM40	33	3	-	3.00	0.515	0.664	0.227
Lca57	33	4	-	3.93	0.636	0.611	-0.042
Lca58	33	7	1	6.49	0.727	0.761	0.045
Lca64	33	8	1	7.57	0.909	0.859	-0.059
Lca69	33	3	-	2.82	0.394	0.418	0.058
Lca70	33	4	-	3.75	0.576	0.569	-0.012
Lca74	33	3	-	2.99	0.364	0.319	-0.143
Lca98	33	4	1	3.82	0.333	0.428	0.225
Lca154	33	4	-	3.82	0.697	0.545	-0.285*
Lca178	33	4	2	3.40	0.485	0.49	0.011
Lca287	33	7	3	5.73	0.545	0.697	0.220*
Lca371	33	2	-	2.00	0.576	0.441	-0.313
Total		73	13	3.94	0.515	0.509	-0.022

[a]Totals at *k* and k_a are counts, whilst the remaining totals are averages.

*Average F_{is} values significantly different from zero at the 0.05 level, following sequential Bonferroni correction for simultaneous tests (Rice, 1988) from 17 classes.

Table 2.3 Measures of genetic diversity; Sample size (N_c), number of alleles (k), number of private alleles (k_a), average observed (H_o) and expected (H_e) heterozygosities, allelic richness (A_r), average inbreeding coefficient (F_{is}), effective population size (N_e), rate of inbreeding (ΔF) and N_e/N_c ratio. Spawns A and B represent the first and second night of spawning respectively

		N_c	k	k_a	H_o	H_e	A_r	F_{is}	N_e	ΔF	N_e/N_c
Broodstock		33	73	13	0.515	0.509	3.94	-0.022	-	-	-
Spawn A											
1dph		182	65	6	0.475	0.488	3.33	0.028	10.1	0.050	0.31
Spawn B											
1dph		274	67	7	0.500	0.493	3.52	-0.013	13.5	0.037	0.46
18dph	Total	472	68	8	0.518	0.501	3.48	-0.041*	14.8	0.034	0.45
	Small	208	67	7	0.514	0.494	3.49	-0.048*	16.7	0.030	0.51
	Medium	158	67	7	0.502	0.498	3.45	-0.007	13.4	0.037	0.41
	Large	106	66	6	0.552	0.512	3.48	-0.087*	11.6	0.043	0.35
90dph	Total	276	68	8	0.531	0.498	3.54	-0.071*	14.8	0.034	0.45
	Small	92	66	7	0.518	0.497	3.53	-0.049*	14.6	0.034	0.44
	Medium	92	67	7	0.531	0.495	3.55	-0.088*	15.3	0.033	0.46
	Large	92	67	7	0.546	0.499	3.55	-0.080*	12.7	0.039	0.38

*Average F_{is} values significantly different from zero at the 0.05 level, following sequential Bonferroni correction for simultaneous tests (Rice, 1988) from 17 classes.

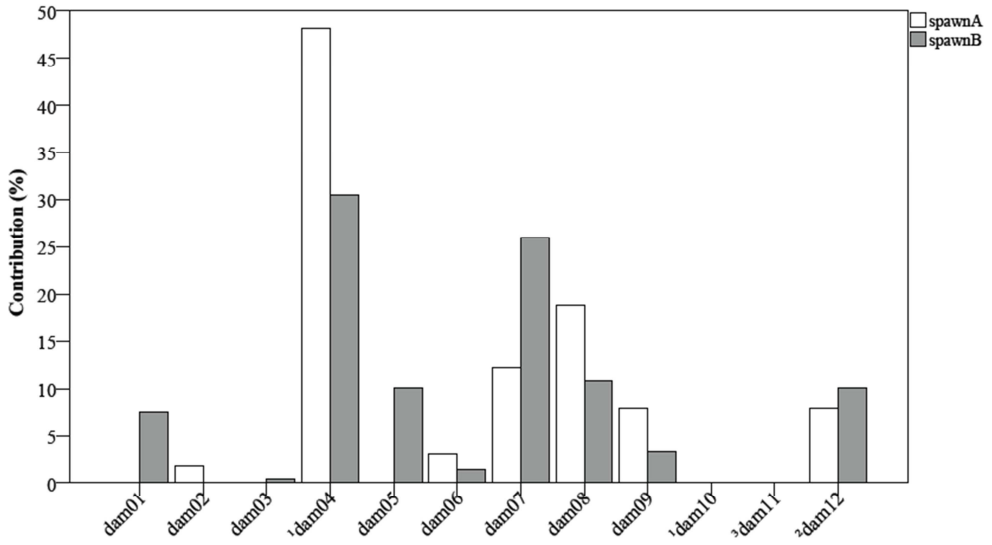


Fig. 2.1a

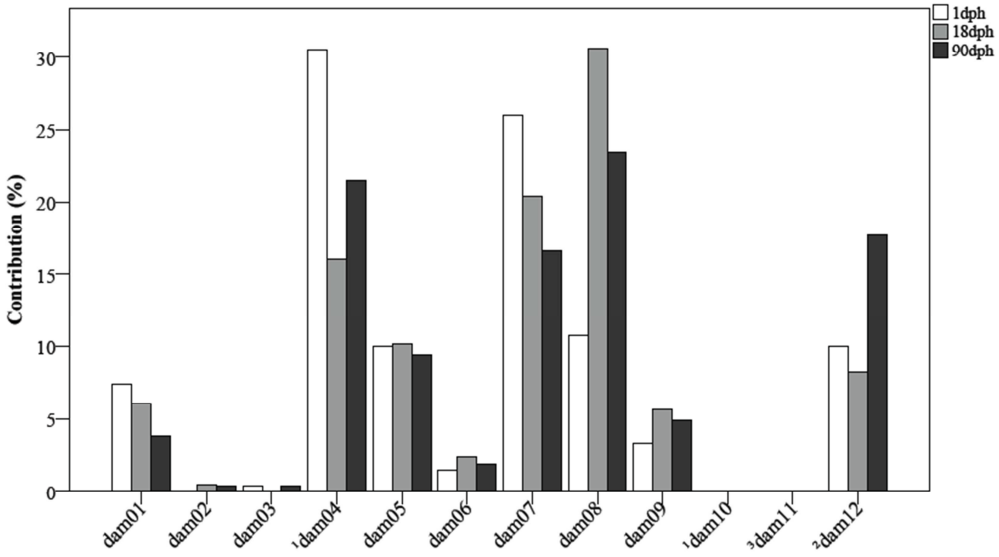


Fig. 2.1b

Figure 2.1 Dam contribution to offspring from spawn A and B at 1 dph (a), and from spawn B over three sampling events; 1, 18 and 90 dph (b). Numbers in superscript indicate the number of private alleles detected for the specified dam.

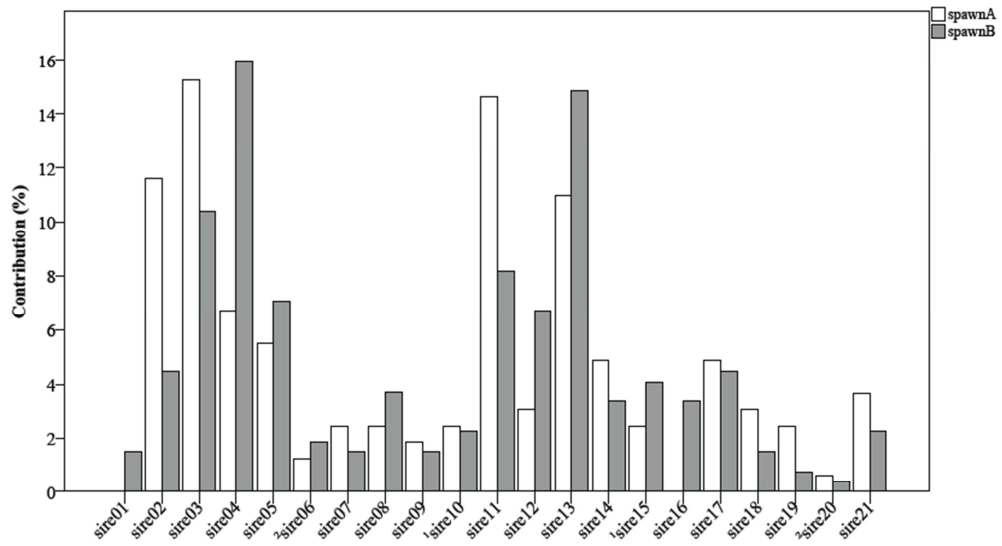


Fig. 2.2a

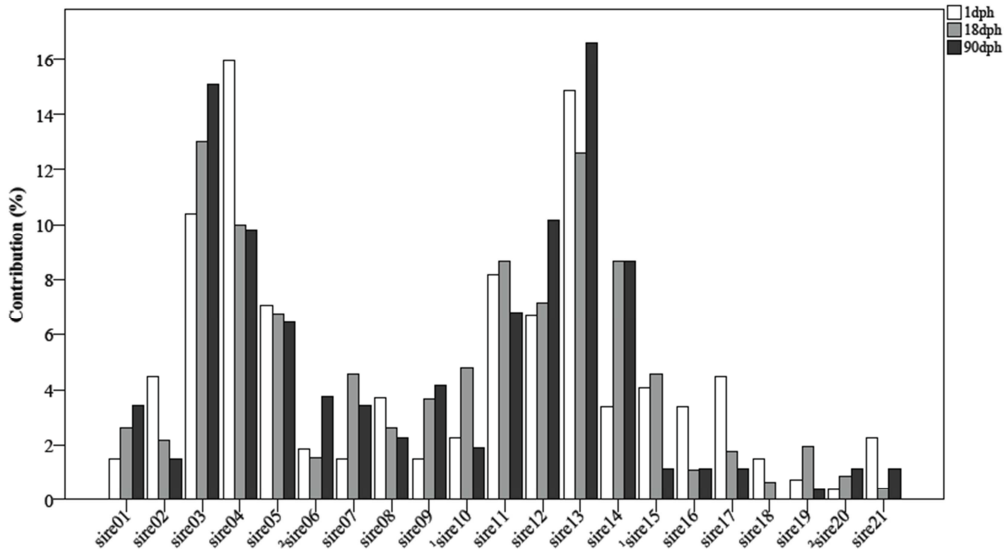


Fig. 2.2b

Figure 2.2 Sire contribution to offspring from spawn A and B at 1 dph (a), and from spawn B over three sampling events; 1, 18 and 90 dph (b). Numbers in superscript indicate the number of private alleles detected for the specified sire.

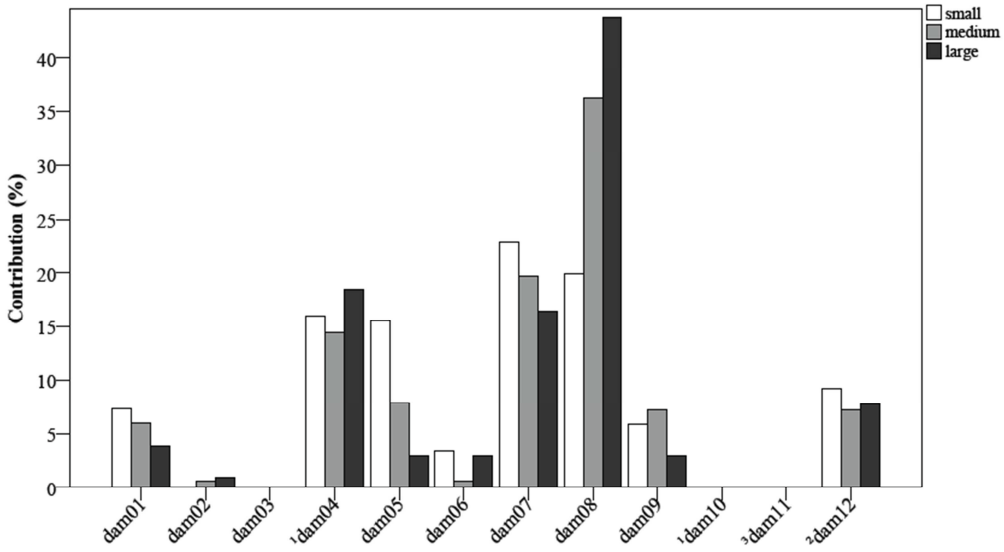


Fig. 2.3a

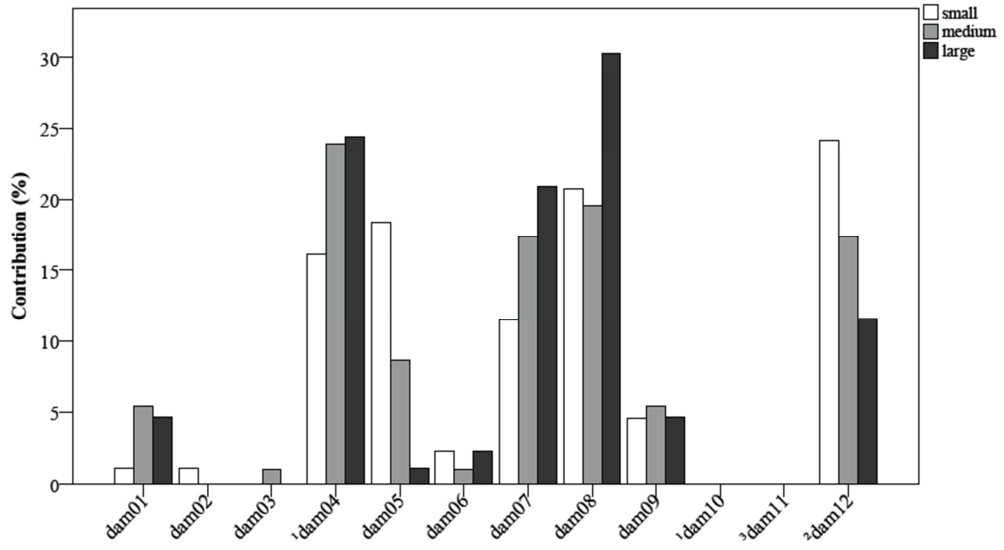


Fig. 2.3b

Figure 2.3 Dam contribution from spawn B at 18 dph (a) and 90 dph (b) for each size grade; small, medium and large. Numbers in superscript indicate the number of private alleles detected for the specified dam.

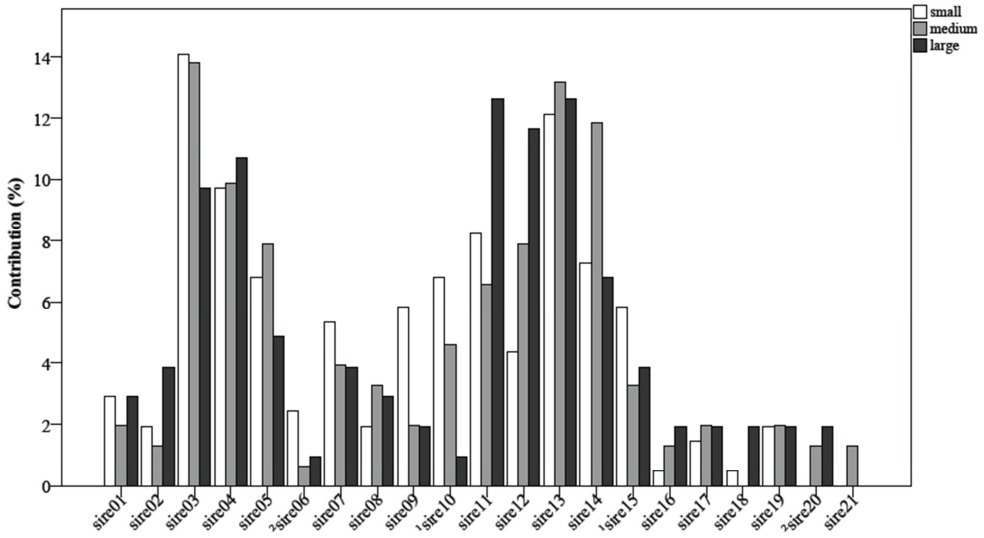


Fig. 2.4a

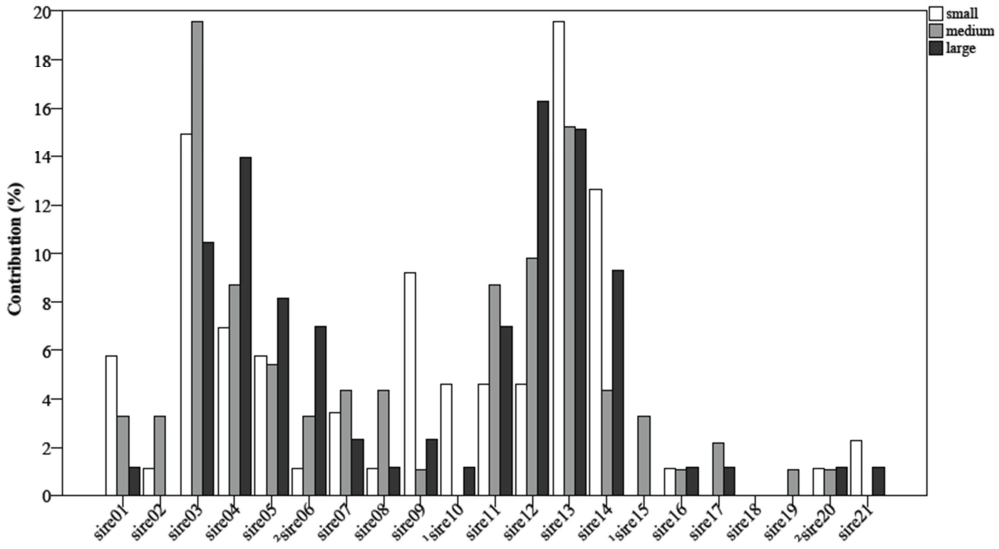


Fig. 2.4b

Figure 2.4 Sire contribution from spawn B at 18 dph (a) and 90 dph (b) for each size grade; small, medium and large. Numbers in superscript indicate the number of private alleles detected for the specified sire.

3 Genetic diversity and relatedness estimates for captive barramundi (*Lates calcarifer*) broodstock populations, informs efforts to form a base population for selective breeding

In review, journal **Aquaculture**

3.1 Abstract

Aquaculture of barramundi or Asian seabass (*Lates calcarifer*) is growing in both Australia and Southeast Asia and there is substantial interest to improve production efficiency through selective breeding. The establishment of a large and genetically diverse base population is a prerequisite for a sustainable and long-term productive breeding program. Accordingly, before selective breeding programs can begin for Australian barramundi it is important to assess the genetic diversity of current captive broodstock populations. To address this question, 407 captive barramundi broodstock from eight separate Australian hatcheries were genotyped using 17 polymorphic microsatellite DNA markers. A Bayesian structure analysis indicated that captive Australian broodstock are broadly divided into two genetic stocks. Multivariate analysis (discriminant analysis of principal components, DAPC) between individuals and pairwise F_{ST} between the hatcheries also supported the distinction for two stocks and suggested that hatchery individuals were either sourced from the two stocks or represented an admixture between them. Genetic diversity was low within each broodstock group (allelic richness ranged from 2.67 to 3.42 and heterozygosity ranged from 0.453 to 0.537). Relatedness estimates within hatcheries were generally low and ranged from -0.003 to 0.273. We recommend selecting captive individuals according to high levels of allelic richness and low levels of relatedness for the base selective breeding population, however, we also recommend the inclusion of genetically diverse wild individuals.

3.2 Introduction

The long-term success of closed selective breeding programs is contingent on the extent of genetic variation captured in the base population. A mating design that limits the rate of inbreeding (ΔF) and the loss of genetic diversity over subsequent generations can help to achieve this (Gjerde, 2005). Broad genetic diversity is desired as it leads to increased genetic diversity per generation, enables greater ability of the selected stock to adapt to new or changing conditions and/or greater ability to select for new traits of importance. The successful production of some aquaculture species can be partly attributed to the wide range of genetic variation captured in the base population at the commencement of the selective breeding program (e.g. Nile Tilapia *Oreochromis niloticus*, Eknath et al., 2007; Atlantic salmon *Salmo salar*, Gjedrem et al., 1991). Typically, a loss of genetic diversity occurs in all closed populations through genetic drift and this loss is increased with each generation of breeding if the genetically effective population size (N_e) is low (Frankham et al., 2002) and if the breeding of close relatives is not avoided. Inbreeding is known to lead to depression of fitness in fish (Wang et al., 2002) due to exposure of deleterious recessive genes and it can also reduce the potential for achieving genetic gain. Breeding programs without an adequate base population and/or with poorly managed "selective breeding" (Li et al., 2004; Schwartz and Beheregaray, 2008), could therefore result in a reduction in fitness and require regular supplementation with new animals to limit inbreeding depression of fitness and control the loss of genetic variation to acceptable levels.

Inbreeding depression has been well documented for small base populations (see Wang et al., 2002 for a review) including rainbow trout (*Oncorhynchus mykiss*), where a moderate impact on inbreeding depression for body weight at harvest ranged

from -1.6 to -4.5% per 10% unit increase in the rate of inbreeding (ΔF) (Pante et al., 2001). Limiting inbreeding becomes more difficult with successive generations of selective breeding, although it is generally accepted that a N_e greater than 100 resulting in ΔF less than 0.5% is sufficient each generation to avoid serious problems in captive populations (Fjalestad, 2005; Sonesson et al., 2005). Minimising the coancestry between individuals in the base population and during early generations after the breeding population is closed to new recruits gives the breeder the ability to make higher genetic gain per rate of inbreeding in subsequent generations. Therefore, it is important to select unrelated or distantly related stock containing high genetic diversity to found the base population for selective breeding.

For most aquaculture species, genetically diverse and structured wild stocks exist that can be accessed as a source of foundation animals for a selective breeding program. However, little research has been performed to determine the best means of capturing broad genetic variability and distinctiveness. When simulating the development of a base population, Holtsmark et al. (2006, 2008a, 2008b) demonstrated that the genetic variance and genetic gain could be increased by sampling fish from at least four genetically distinct subpopulations. Simulations have also demonstrated the benefits of utilising genetic markers for selecting candidates according to their contribution to total genetic diversity for the establishment of a base population and for the maintenance of diversity in subsequent generations (Hayes et al., 2006).

By utilising molecular DNA markers such as microsatellites the genetic diversity and relatedness of broodstock candidates can be estimated prior to selection. The relatedness or kinship between individuals x and y (r_{xy}) is a measure of the fraction of alleles that are identical by descent (IBD) and a pair of individuals

are deemed related if they share one or more alleles inherited from a common ancestor. By selecting parents based on r_{xy}, it is possible to limit the rate of inbreeding (Doyle et al., 2001; Rodzen et al., 2004; Sekino et al., 2004), maintain genetic variation within captive populations (Ballou and Lacy, 1995; Eding and Meuwissen, 2001) and identify broodstock groups of similar ancestries that have produced offspring with reduced production efficiency (Porta et al., 2006). Pairs of individuals with a lower value of r_{xy} compared to all other broodstock within a breeding group should be given breeding priority. Allelic richness (i.e. the average number of alleles per locus that takes into account differences in sample size) and rare or private alleles are other informative measures of genetic diversity within a population (Kalinowski, 2004; Loukovitis et al., 2012). Together with relatedness, the genetic diversity of source populations should be considered when selecting candidates for a breeding program.

Asian seabass (*Lates calcarifer*), also known as Barramundi in Australia has potential as a candidate species for genetic improvement, as its production in aquaculture is growing (ABARES, 2011), it demonstrates high fecundity (Palmer et al., 1993) and moderate heritability for economically valuable traits such as growth rate (Wang et al., 2008; Domingos et al., 2013). Barramundi readily spawn in captive culture and naturally breed in groups (mass spawn) providing the opportunity for creating numerous parent pair families. The mass spawning nature of this species means that there is little control over the contribution of individual broodstock to a particular spawning event (Frost et al., 2006; Wang et al., 2008; Loughnan et al., 2013) and N_e is therefore typically much less than the census size (N_c). As a result, a substantial number of unrelated broodstock are required to control ΔF and to provide a $N_e > 100$. Genetic markers that could be used to trace the pedigree of offspring

produced by mass spawning have already been developed for barramundi (Yue et al., 2001; 2002; Sim and Othman, 2005; Zhu et al., 2006a; 2006b; Wang et al., 2007; Zhu et al., 2008).

Reviews of genetic diversity and structure on both wild and captive barramundi populations have been conducted in the Asia-Pacific region and the results applied to the development of a selective breeding program (Zhu et al., 2006a; Yue et al., 2002; 2009). The genetic diversity within Australian captive stocks is yet to be assessed and the application of such information to establishing base populations for selective breeding has not been investigated. To address these issues, 17 microsatellite DNA markers were utilised to genotype barramundi broodstock from eight major Australian commercial hatcheries. Genetic diversity and relatedness estimates were investigated within each broodstock group and the results are discussed with regard to broodstock management and the development of a base population for selective breeding using existing Australian broodstock.

3.3 Materials and methods

3.3.1 Sampling, DNA extraction and genotyping

Barramundi broodstock samples (N_c = 407) were collected from eight commercial Australian hatcheries; one each in Western Australia (WA, N_c = 48) and the Northern Territory (NT, N_c = 71), and six in Queensland (QLD1, N_c = 58; QLD2, N_c = 14; QLD3, N_c = 111; QLD4, N_c = 80; QLD5, N_c = 9; QLD6, N_c = 16). Within each hatchery, all broodstock made accessible were sampled regardless of whether they were under current use, were listed as backup broodstock, or had not yet reached sexual maturity. At the time of sampling there were 136 females, 180 males and 91 fish of unknown sex. Relatively few of the broodstock under current use

were reportedly wild caught individuals (51 and 7 individuals from the NT and QLD4 groups respectively). The remaining broodstock were listed as captive bred fish following one or more generations of breeding, some acquired from or exchanged between hatcheries. The sample size (N_c = 407) was a high representation of the barramundi broodstock present in the Australian industry at the time of this study.

All broodstock were sedated in a saltwater bath containing 40 ppm AQUI-S (Aquatic Diagnostic Services International) and a small segment of caudal fin (*ca.* 1 cm^2) was removed and preserved in either 80% ethanol or DMSO-salt solution (20% DMSO, 0.25 M disodium-EDTA and NaCl to saturation at pH 8) (Seutin et al., 1991). Passive integrated transponder (PIT) tags implanted in each individual were scanned to provide unique identification. Whilst sedated, broodstock were cannulated with a 2.16 mm outside diameter (OD) catheter tube and the sex confirmed via observation of eggs or sperm under a microscope. Broodstock were then recovered from anaesthesia and placed back into their holding tanks as per standard industry practice.

Methods of DNA extraction were described in Loughnan et al. (2013) following the CTAB (cetyl trimethylammonium bromide) protocol described by Adamkewicz and Harasewych (1996). As for Loughnan et al. (2013), the same 17 microsatellite markers were amplified in two multiplex reactions using the polymerase chain reaction (PCR) procedures described therein. Multiplex one included markers *LcaM03* (Yue et al., 2001), *LcaM16*, *LcaM40* (Yue et al., 2002), *Lca57* (Zhu et al., 2006a), *Lca154*, *Lca178* (Zhu et al., 2006b), *Lca287* and *Lca371* (Wang et al., 2007). Multiplex two included *LcaM08*, *LcaM20, LcaM21* (Yue et al., 2002), *Lca58, Lca64*, *Lca69, Lca70, Lca74* and *Lca98* (Zhu et al., 2006a).

Genotyping was performed on a MegaBACE® 1000 DNA Analysis System (GE Healthcare) and MegaBACE® software Fragment Profiler® was used for fragment analysis.

3.3.2 Population analysis

To test for the presence of null alleles, large allele dropout and scoring errors MICRO-CHECKER 2.2.3 was utilised (van Oosterhout et al., 2004), applying 95% confidence intervals for Monte Carlo simulations. Null alleles were not accounted for when scoring genotypes. Following this, the average numbers of alleles (*A*), plus the expected (H_e) and observed (H_o) heterozygosities were estimated in GENALEX 6.5 (Peakall and Smouse, 2012). Allelic richness (A_r) and private allelic richness (PA_r) were estimated in HP-RARE 1.1 (Kalinowski, 2005), incorporating a rarefaction approach for a minimum of 14 genes per sample (7 diploid individuals). The inbreeding coefficient (F_{is}) and associated significance tests were calculated in FSTAT 2.9.3.2 (Goudet, 2002) using the Weir and Cockerham (1984) method followed by Bonferroni correction for multiple comparisons (Rice, 1988). Tests for Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) were calculated in GENEPOP 4.1 (Rousset, 2008) and significance also determined with sequential Bonferroni correction. Exact *P*-values under the Markov chain method were implemented with a dememorization step of 10,000 followed by 20 batches (100 batches for LD) of 5000 iterations per batch. Kruskal-Wallis tests were performed in IBM SPSS 20.0 for assessing whether broodstock groups differed statistically for three measures of genetic diversity; A_r, PA_r and H_e.

BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996; Piry et al., 1999) was utilised to check for signatures of recently reduced N_e within each broodstock group. Genetic bottlenecks have been detected in other captive aquaculture populations, such as largemouth bass (*Micropterus salmoides*, Bai et al., 2008) and gilthead sea bream (*Sparus aurata*, Loukovitis et al., 2012). The stepwise mutation model (SMM) and the two-phase model (TPM) were selected in BOTTLENECK and run for 1000 iterations as recommended for microsatellite applications (Luikart and Cornuet, 1998). The variance for TPM was set at 30 and the proportion of SMM in TPM was 70%. The mode-shift option was also applied to observe the distribution of allele frequencies (Luikart et al., 1998). A mode-shift is often found in populations that have experienced a recent bottleneck. Due to the relatively small number of markers available for bottleneck analysis (< 20) the more appropriate Wilcoxon's test was applied to the data (Piry et al., 1999).

Population structure was assessed across the 407 Australian captive broodstock to determine the number of genetic stocks represented across the industry and to aid in selecting candidates for a base population in a selective breeding program. A range of methods were utilised in the analysis of population structure. Firstly, the Bayesian method of individual clustering applied in STRUCTURE 2.3.3 was used (Pritchard et al., 2000) and accessed at the Bioportal computing resource (https:www.bioportal.uio.no/; Kumar et al., 2009). The most probable individuals were assigned to *k* groups with and without the use of sample location as a prior reference ('locprior'), a protocol designed to assess weak population structure. Admixture and correlated allele frequencies were applied for both models (Falush et al., 2003). Twenty replicate runs at each *k* (1 – 8) were performed (Gilbert et al., 2012). A burn in length of 100,000 iterations and one million MCMC repetitions

were applied for each run. The *q*-value threshold for assignment was > 0.90 to a single cluster and < 0.90 for the detection of admixture. STRUCTURE HARVESTER (Earl and vanHoldt, 2012) was used to assess the most likely number of genetic groups (*k*) represented in the dataset (Evanno et al., 2005). The admixture proportions of each individual over the 20 replicates were averaged for the best *k* using CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007) and barplots were designed in DISTRUCT 1.1 (Rosenberg, 2004).

The second method for the detection of population structure between captive broodstock used discriminant analysis of principal components (DAPC). DAPC assigns individual genotypes to predefined groups using a multivariate method (Jombart et al., 2010). R 3.0.1 programming language (R core team, 2013) utilised the R package adegenet (Jombart, 2008) for the calculation of DAPC. A neighbour-joining tree was constructed in MEGA 5.0 (Tamura et al., 2011) from Nei's (1978) standard genetic distance (calculated in SPAGeDi; Hardy et al., 2002). Finally, pairwise F_{ST} (Weir and Cockerham, 1984) and associated *P* values were estimated in GENALEX 6.5 and incorporated 999 permutations (Peakall and Smouse, 2012).

3.3.3 Relatedness estimates

The software COANCESTRY 1.0.1.2 (Wang, 2011) was utilised to estimate relatedness (r_{xy}) between each dyad (i.e. broodstock pairs) within each of the eight broodstock groups. The program incorporates seven relatedness and three inbreeding estimators, to enable selection of the most appropriate estimator for the data set. The best performing relatedness estimator depends on the dataset of each study and more specifically on the number of microsatellite markers and the levels of variation detected (Van de Casteele et al., 2001; Wang et al., 2011). The COANCESTRY

software incorporates Monte Carlo simulations, which were run with known allele frequencies calculated from the observed genotypes from all eight hatcheries. True relationship classifications, which provide specific genealogical relations were set at r_{xy} = 0.5 for parent-offspring (PO) and full sibs (FS), r_{xy} = 0.25 for half sibs (HS) and r_{xy} = 0 for unrelated (U), simulating 1000 dyads for each relationship type and with 1000 bootstraps to calculate 95% confidence intervals. Following the simulation, the best estimator that yielded a strong correlation between the true and estimated values was selected. This was the Queller and Goodnight (1989) estimator (r_{QG}), with a correlation coefficient of R = 0.79, $P < 0.05$ (Fig. 1). Ranging from -1 to 1, the Queller and Goodnight (1989) relatedness estimator has probably been the most widely chosen estimator for studies of kinship in both captive and wild populations (see Blouin, 2003 for a review) and it was applied to the empirical genotype dataset in this study to calculate r_{xy} estimates between all possible dyads. One-way ANOVA incorporating Tukey's post hoc tests were performed in IBM SPSS 20.0 to test for differences in r_{QG} between the broodstock groups.

3.4 Results

3.4.1 Genetic diversity and HWE

The average number of alleles per locus (A), A_r and PA_r were highest in the NT hatchery; with 5.6, 3.42 and 0.51, respectively (Table 1). This was the hatchery with the greatest number of reportedly wild caught broodstock. The lowest value of A_r was recorded for QLD6 (2.67) and PA_r was the lowest for QLD5 (0.03). Kruskal-Wallis tests revealed no significant differences in levels of A_r (P = 0.84) or H_e (P = 0.967) between the broodstock groups, however, there was a significant difference between the groups for PA_r ($P < 0.001$). An indication of inbreeding was detected

for the WA and QLD2 broodstock groups (F_{is}, $P < 0.05$) and F_{is} was significantly different from zero for both. There were significant deviations from HWE estimates at five loci ($P < 0.05$); *Lca*070 in the NT broodstock group, *LcaM*040 in the WA group, *Lca*058 for QLD3 and WA, *Lca*074 for QLD1 and at locus *Lca*287 deviations from HWE were detected in most groups except QLD2, 5 and 6. MICRO-CHECKER detected null alleles at six loci; *LcaM16* for NT, *LcaM*040 and *Lca*058 for WA, *Lca*069 for QLD3, *Lca*178 for QLD4 and *Lca*287 for NT, QLD1, QLD3, QLD4 and WA. Exact tests for the non-random association of alleles at different loci (linkage disequilibrium) revealed 46% of loci pairs presented significant *P* values ($P < 0.001$), following sequential Bonferroni correction (Rice, 1988). No bottleneck signatures were detected for the SMM or TPM mutation models within each broodstock group and the allele frequency distribution tests remained in a normal L-shaped distribution (Luikart et al., 1998). Due to deviations from HWE and the presence of null alleles in the majority of broodstock groups, *Lca287* was excluded from further analysis reducing the marker set to 16 loci.

3.4.2 Population structure of captive broodstock groups

The neighbour-joining tree of Nei's standard genetic distance (Figure 2) shows that broodstock from WA and NT was more closely related than the QLD broodstock groups. Similarly, QLD1 and QLD4 displayed closer similarity with each other as did QLD3 and QLD6. The most genetically distinct QLD group was QLD6 although this was on the same branch as QLD2 and QLD3. DAPC analysis revealed two major populations (Fig. 3); one including the individuals sampled from the QLD groups, whilst NT broodstock was located in the second group with WA individuals. The F_{ST} value across all eight hatcheries was 0.071 ($P < 0.001$) and a

significant difference ($P < 0.05$) was detected between most hatcheries except for some of the QLD groups (Table 2). A significant difference was detected between WA and NT broodstock groups ($F_{ST} = 0.061$), although the strongest levels of separation were identified between these two groups and the six hatcheries from QLD (F_{ST} ranged from 0.078 – 0.169).

There was little difference in the STRUCTURE output when incorporating either the 'no locprior' or 'locprior' models and as a result the output from 'no locprior' is presented. The most obvious Δk was two as determined by the method of Evanno et al. (2005) (Fig. 4). The barplot demonstrating these two genetic clusters (Figure 5) shows that the majority of WA and NT broodstock were allocated to stock one, whilst the bulk of broodstock from the QLD hatcheries represented stock two. Upon observing the average population threshold *q*-values of the broodstock groups, QLD1, 2, 3, 4 and 6 were allocated to stock two ($q > 0.90$), whilst QLD5 was predominantly assigned to stock one (Table 3). However, 18 individuals were allocated to stock one from the QLD3 broodstock group and a level of admixture was detected by *q* values < 0.90 (stock one 0.17, stock two 0.83). Similarly, the WA broodstock group also had *q* values < 0.90 (stock one 0.74, stock two 0.23) but was mostly assigned to stock one. No individuals were admixed within the WA broodstock although 12 were assigned to stock two. The NT group was allocated to stock one only. Admixture was detected between 1 – 4 individuals within five broodstock groups, however, admixture only accounted for 2% of the total individuals.

3.4.3 Broodstock relatedness

Relatedness was estimated separately within each broodstock group and the average within group relatedness was 0.118 (Fig. 6). The lowest level of relatedness was recorded for QLD2 (r_{QG} = -0.003 ± 0.020 SE) and the highest for QLD6 (r_{QG} = 0.273 ± 0.022 SE). These two broodstock groups contained small sample sizes (14 and 16 respectively) and showed relatedness values with larger standard errors than broodstock groups with higher numbers of individuals. The r_{QG} of QLD6 was significantly higher ($P < 0.05$) than every other group's estimate of r_{QG} (Table 4).

3.5 Discussion

Overall, barramundi broodstock representing the captive breeding population of the Australian industry contained genetic diversity levels of A_r = 3.15 and PA_r = 0.16, and a relatedness level of r_{QG} = 0.118. There was evidence of at least two genetic population groups (F_{ST} = 0.071). In a study investigating both captive and wild barramundi populations from the Asia-Pacific region (Yue et al., 2009), A_r ranged from 3.57 – 4.80 for three captive Australian populations, 6.65 and 7.89 for captive populations from Taiwan and Singapore respectively, and A_r ranged from 7.60 – 8.50 for four wild populations from Southeast Asia. When compared to this study, genetic diversity levels were higher in the barramundi populations from the Asia-Pacific region (Yue et al., 2009), although any direct comparison of genetic diversity between studies is difficult, due to differences in population size, demographic history and the incorporation of alternate genetic markers.

The results from this study provide a foundation for selecting a number of individuals for inclusion into a selective breeding program. Preference should be given to broodstock candidates whose addition would boost genetic diversity in the

base population and which share low levels of relatedness with other broodstock chosen for the base population. The results indicate that all eight hatcheries maintain some broodfish that do not share recent common ancestry to any other broodfish. This suggests that it should be possible to select a set of broodstock for the base population sharing little to no recent common ancestry. An approach for selecting mass spawning broodstock according to levels of relatedness has been developed and used by Doyle et al. (2001) and Sekino et al. (2004). The approach aims to minimise kinship (limit inbreeding) and maximise the conservation of rare alleles (maintain genetic variation) when establishing the base population and also for each subsequent generation of breeding. It is based on the relatedness coefficient r_{xy} and known as the mean kinship breeding strategy (mk_r), which was initially proposed by Ballou and Lacy (1995) but incorporated pedigree data rather than relatedness values.

For some of the broodstock groups, relatedness estimates may have been biased due to the small number of samples taken and differences in population size between the hatcheries. The exchange of broodstock between hatcheries and subsequent low levels of representative genetic diversity may have also contributed to the results. QLD2 recorded the lowest estimate of r_{QG} and higher levels of A_r and PA_r when compared with many of the other broodstock groups, although the small sample size of captive bred individuals whose exact genetic origins were unclear (N_c = 14) may have contributed to the result. In contrast, QLD6 individuals shared the most recent common ancestry as indicated by r_{QG} and recorded low levels of A_r and PA_r. The sample size was small (N_c = 16) and hatchery records showed that all broodstock sampled from QLD6 were captive bred. From hatchery records, the majority of individuals within each broodstock group were either sourced from the same wild locality or were descendants from the same captive group.

The population structure results suggested that the current captive individuals had been derived from two distinct genetic source stocks, which is supported by the findings of wild barramundi population genetic studies (Keenan, 1994; 2000; Chenoweth et al., 1998a; 1998b; Doupe et al., 1999). Similarly, the multivariate analysis from the DAPC method also displayed two main populations, supporting the existence of at least two genetically distinct stocks. This finding can also help to guide the choice of distantly related mates for group spawning and help to maximise levels of genetic variation. The two stocks must have evolved in isolation for some time for this genetic structure to exist and therefore it is possible that these stocks contain unique adaptive diversity, or diverged in such a way that their performance in aquaculture as pure bred or cross bred stock differs. The performance of cross bred stock needs to be evaluated to determine if cross breeding leads to a deterioration of fitness. In the absence of this information we would recommend avoiding cross breeding. The significant deviation of F_{is} from zero (deficiency of heterozygotes) could be caused by a Wahlund effect where hatchery populations are composed of animals sourced directly from two or more discrete wild stocks (Hartl and Clarke, 1997). When selecting broodstock candidates for a base population, it would be beneficial to mate unrelated individuals within each of the two stocks identified in this study, to help increase average heterozygosity and control the level of inbreeding within the founding group.

Two per cent of all broodstock were identified as admixed stock (Table 3). These could be animals that have been directly sourced from sites in the wild where admixture naturally occurs or might be the result of crosses between the two wild stocks that have occurred in captivity. Broodstock allocated to the first genetic stock (such as the individuals from NT) demonstrated the highest levels of A_r and PA_r,

(3.42 and 0.51 respectively in NT). All but two broodstock groups contained at least one individual from stock one and personal communications with hatchery managers revealed that these individuals were either originally sourced from a common wild region, or in some cases there was an exchange of captive bred broodstock between hatcheries. This may have resulted in the distribution of individuals from stock one, although in order to enhance current genetic diversity levels within the broodstock groups, sourcing individuals that represent both of the stocks detected in this study is recommended.

3.6 Conclusion

The eight barramundi hatcheries sampled for this study supply a large proportion of the broodstock under production in Australia, and as a result the reported levels of genetic diversity and relatedness were a thorough representation of those existing in the industry at the time of sampling. This study has focused on captive barramundi stocks and discusses ways in which these individuals could be utilised to establish a genetically diverse and unrelated base population for selective breeding. Captive spawners can be more productive than their wild counterparts and display less stress due to adaptation to the captive environment (Gjedrem, 2000). Many of the captive broodfish tested did not share any recent common ancestry with any other captive broodfish, therefore it would be possible to utilise existing captive broodfish in a way that totally avoids inbreeding in the initial generations of the breeding program. However, to further maximise levels of genetic diversity and reduce long-term inbreeding rates, it is recommended that a mixture of both captive bred and wild broodstock should be included in the base population. The introduction of wild individuals would increase N_e and the genetic diversity of the

base population. This would allow inbreeding to be limited to lower levels and provide a broader basis for future genetic improvement. Further work to investigate genetic diversity and structure among the widespread natural populations across northern Australia and to simulate different scenarios for establishing the base population, is needed so that efficient strategies for capturing new genetic diversity for barramundi selective breeding can be devised.

Population structure analysis detected that at least two wild genetic source stocks are represented within the captive populations. The Australian industry has access to natural populations of barramundi spanning coastal and river systems from Western Australia to central Queensland, and additional broodstock could be sourced from the most genetically diverse of these wild populations to boost the genetic diversity of the founding base population. Therefore we recommend that the industry composes the base population using captive individuals in a way that high A_r and low mk_r is achieved and using wild individuals such that variation in the wild genetic stocks is well represented.

Table 3.1 Measures of genetic diversity for eight captive barramundi broodstock groups based on 16 microsatellite DNA markers: Western Australia (WA), Northern Territory (NT) and Queensland (QLD). Population size (N_c), average number of alleles (A), mean observed (H_o) and expected (H_e) heterozygosities, mean allelic richness (A_r) and private allelic richness (PA_r), and the average inbreeding coefficient (F_{is})

Hatchery	N_c	A	H_o	H_e	A_r	PA_r	F_{is}
WA	48	4.2	0.469	0.497	3.18	0.19	0.069*
NT	71	5.6	0.503	0.509	3.42	0.51	0.020
QLD1	58	4.3	0.514	0.491	3.11	0.08	-0.038
QLD2	14	3.9	0.513	0.537	3.35	0.23	0.082*
QLD3	111	5.5	0.506	0.506	3.23	0.10	0.005
QLD4	80	4.4	0.513	0.518	3.19	0.06	0.016
QLD5	9	3.2	0.532	0.482	3.04	0.03	-0.042
QLD6	16	3.1	0.475	0.453	2.67	0.06	-0.014
Average‡	407	4.3	0.503	0.499	3.15	0.16	0.012

‡ N_c is the total count, whilst the remaining values are averages.

* significant at the 0.05 level following Bonferroni correction (Rice, 1988) from 16 classes.

Table 3.2 Pairwise F_{ST} values for eight captive barramundi groups. Significant values ($P < 0.05$) are depicted in bold between groups following Bonferroni correction from 16 classes

	WA	NT	QLD1	QLD2	QLD3	QLD4	QLD5
NT	**0.061**						
QLD1	**0.116**	**0.111**					
QLD2	**0.081**	**0.082**	**0.036**				
QLD3	**0.088**	**0.090**	**0.032**	0.019			
QLD4	**0.101**	**0.095**	**0.011**	0.006	**0.035**		
QLD5	**0.085**	**0.078**	**0.060**	0.006	**0.067**	0.020	
QLD6	**0.151**	**0.169**	**0.106**	**0.059**	**0.035**	**0.095**	**0.146**

P values were obtained using 999 permutations of the data.

Table 3.3 Assignment values from eight barramundi broodstock groups to stock one or two ($q > 0.90$), according to average admixture proportions in STRUCTURE. N_c is the population size of each broodstock group and the column of admixture represents individuals that recorded q values < 0.90. The percentages of individuals assigned to the three clusters are listed on the bottom row

Group	N_c	Stock one	Stock two	Admixture
WA	48	36	12	-
NT	71	67	-	4
QLD1	58	-	56	2
QLD2	14	1	13	-
QLD3	111	18	92	1
QLD4	80	3	75	2
QLD5	9	8	-	1
QLD6	16	-	16	-
Total	407	133	264	10
		33%	65%	2%

Table 3.4 Matrix of average relatedness estimates (r_{QG}) across eight captive barramundi broodstock groups, using the Queller and Goodnight (1989) estimator. Significant differences ($P < 0.05$) in r_{QG} between groups are depicted in bold

	WA	NT	QLD1	QLD2	QLD3	QLD4	QLD5	QLD6
WA	0.121							
NT	0.051	0.143						
QLD1	-0.062	-0.078	0.133					
QLD2	**-0.076**	**-0.092**	**0.012**	-0.003				
QLD3	-0.037	**-0.063**	**0.053**	**0.010**	0.089			
QLD4	**-0.076**	**-0.085**	**0.081**	0.026	0.010	0.064		
QLD5	-0.031	-0.030	0.019	**0.044**	-0.038	0.046	0.122	
QLD6	**-0.108**	**-0.166**	**0.007**	**0.015**	**0.117**	**-0.028**	**-0.127**	0.273

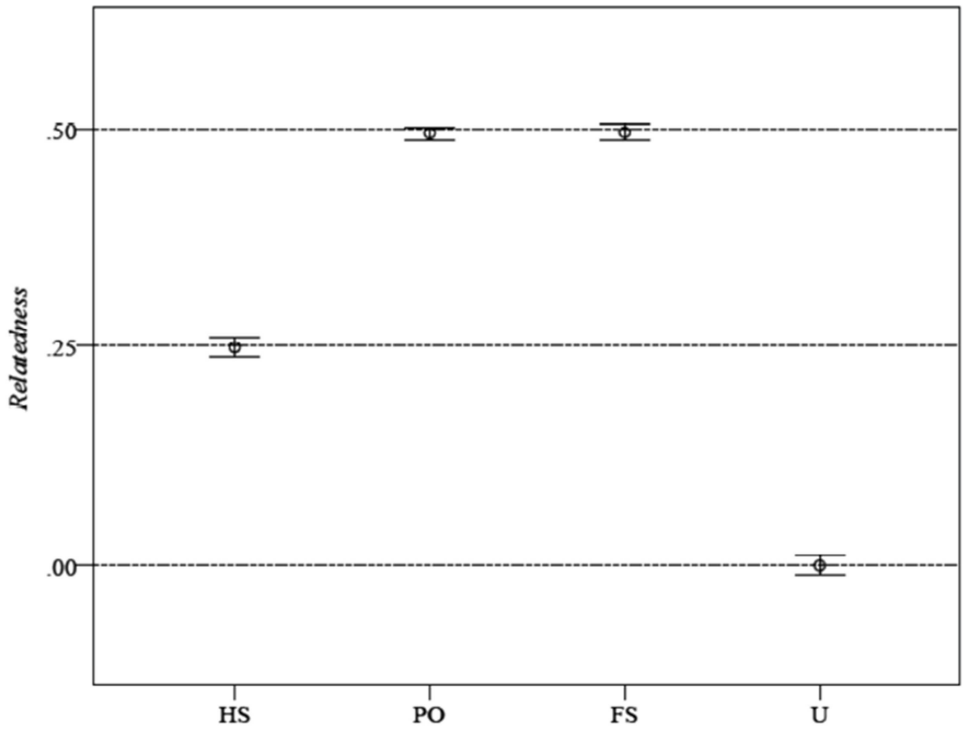


Figure 3.1 Relatedness values based on the Queller and Goodnight (1989) estimator. Mean values with error bars (SE) are displayed for the following relationship categories with true values represented by the dashed lines; HS, half-sibling (0.25); PO, parent-offspring (0.5); FS, full-sibling (0.5); U, unrelated (0). 1000 dyads were simulated for each category and the correlation coefficient was $R = 0.79$.

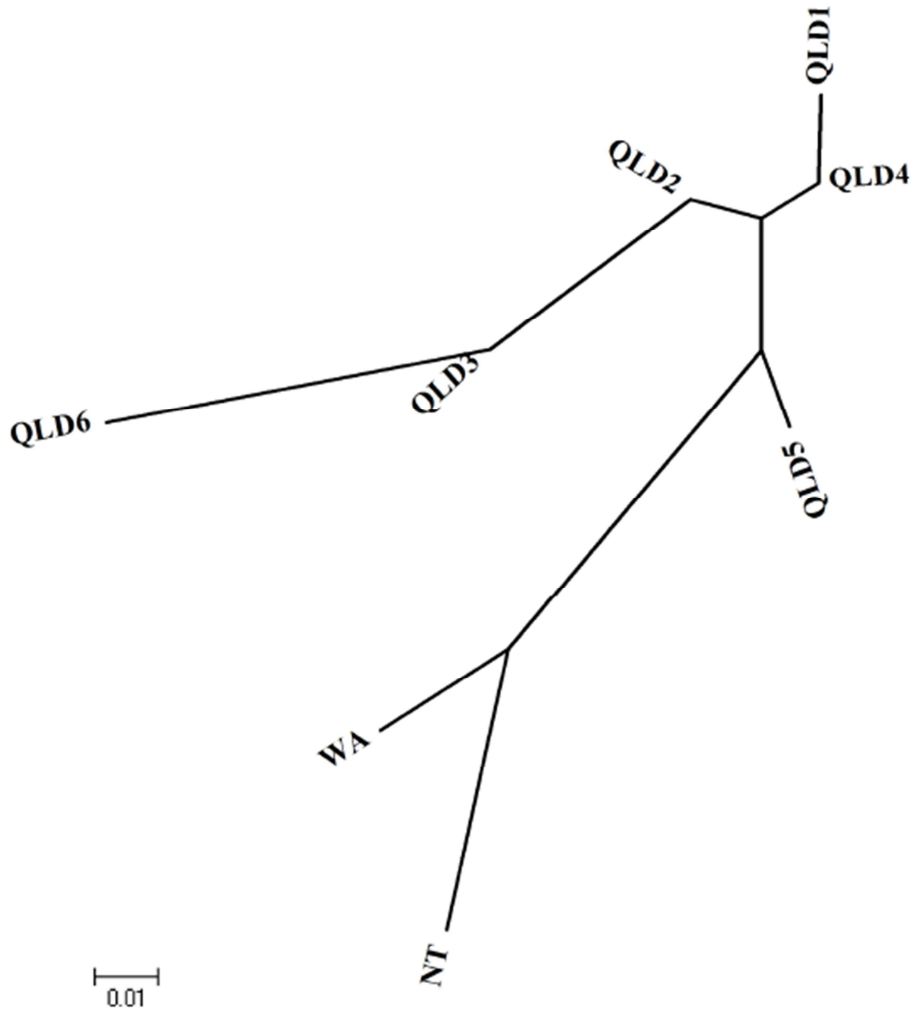


Figure 3.2 Unrooted Neighbour-joining tree of Nei's genetic distance (1978) drawn to scale for eight captive barramundi broodstock groups.

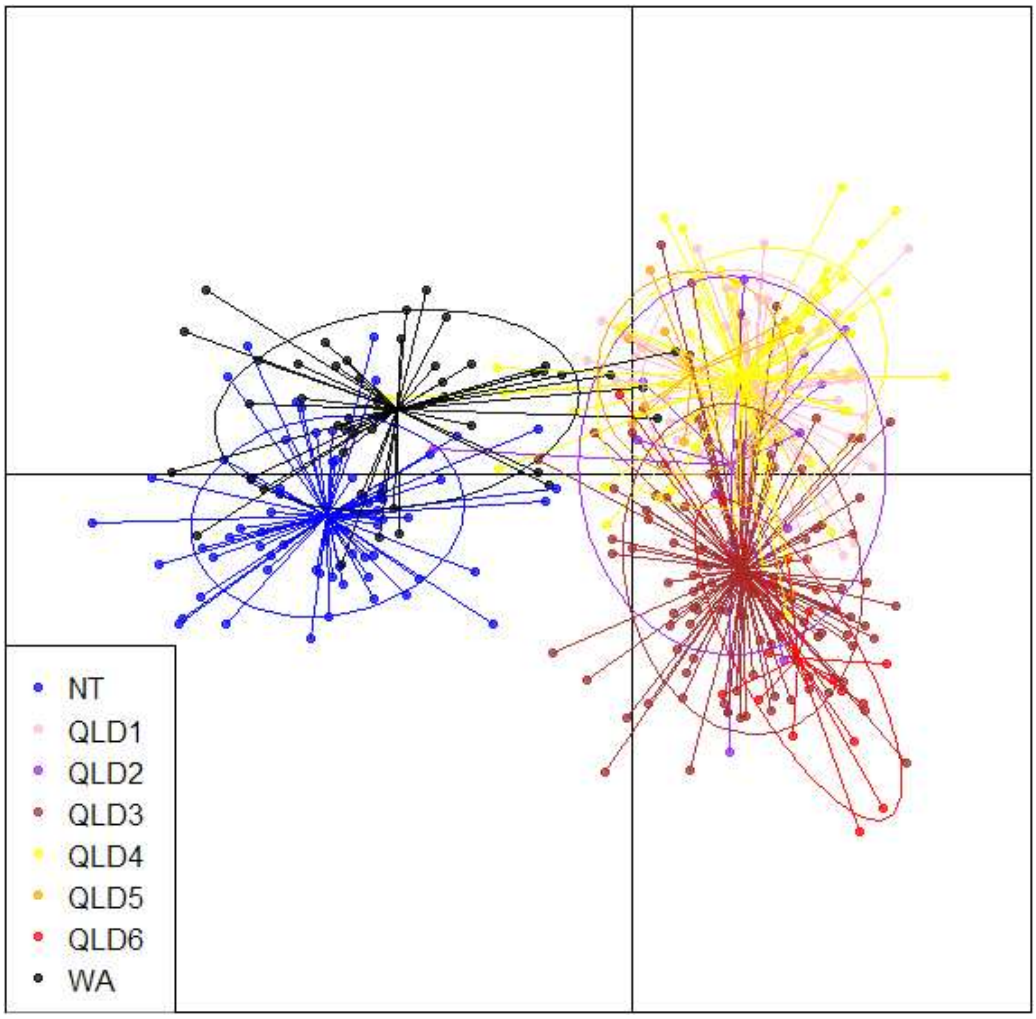


Figure 3.3 Scatterplots of the discriminant analysis of principal components (DAPC) for 407 individuals from eight *L. calcarifer* broodstock groups. Dots represent individual genotypes and colours represent broodstock populations. The first two principal components are represented by X and Y axes respectively.

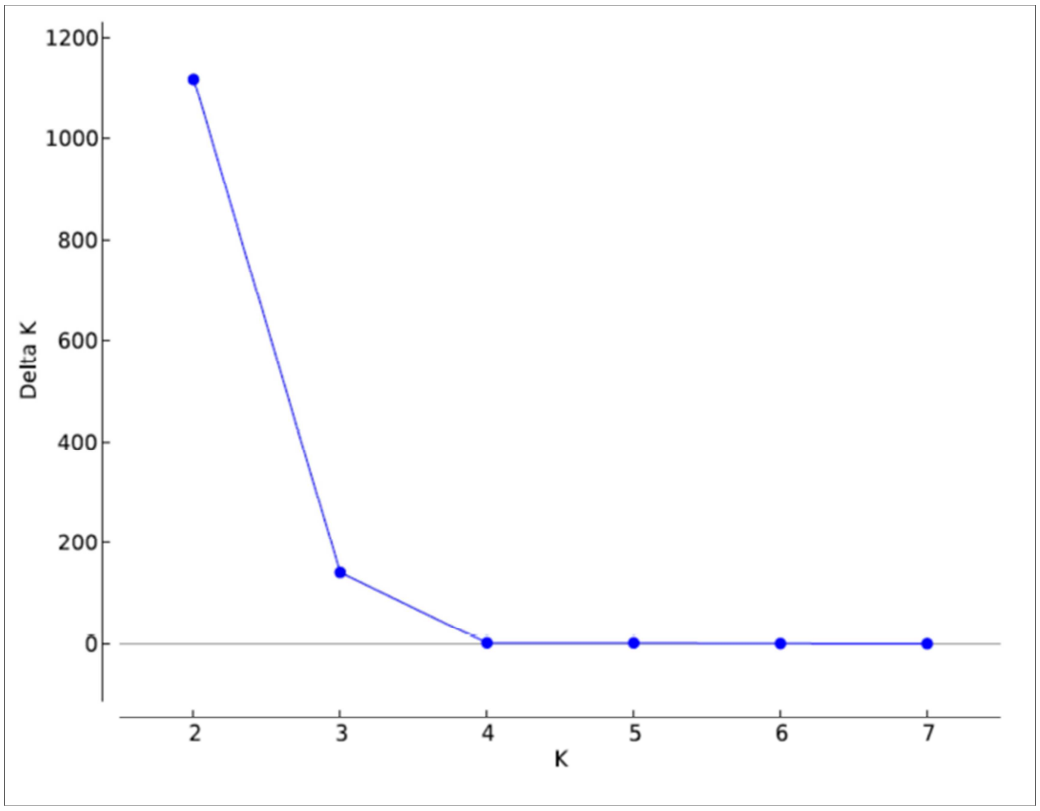


Figure 3.4 Delta *k* (Δ*k*) determined by the Evanno et al. (2005) method showing the most probable number of *k* groups (*k* = 2) from eight captive barramundi broodstock populations (N_c = 407) following 20 replications in STRUCTURE.

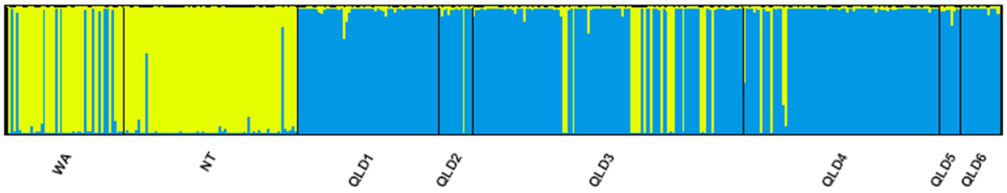


Figure 3.5 STRUCTURE barplot for eight captive barramundi hatchery groups (N_c = 407). Inferred number of populations (k) was equal to two; one represented in yellow and the other in blue. Broodstock groups are separated by a black line and each bar represents one individual.

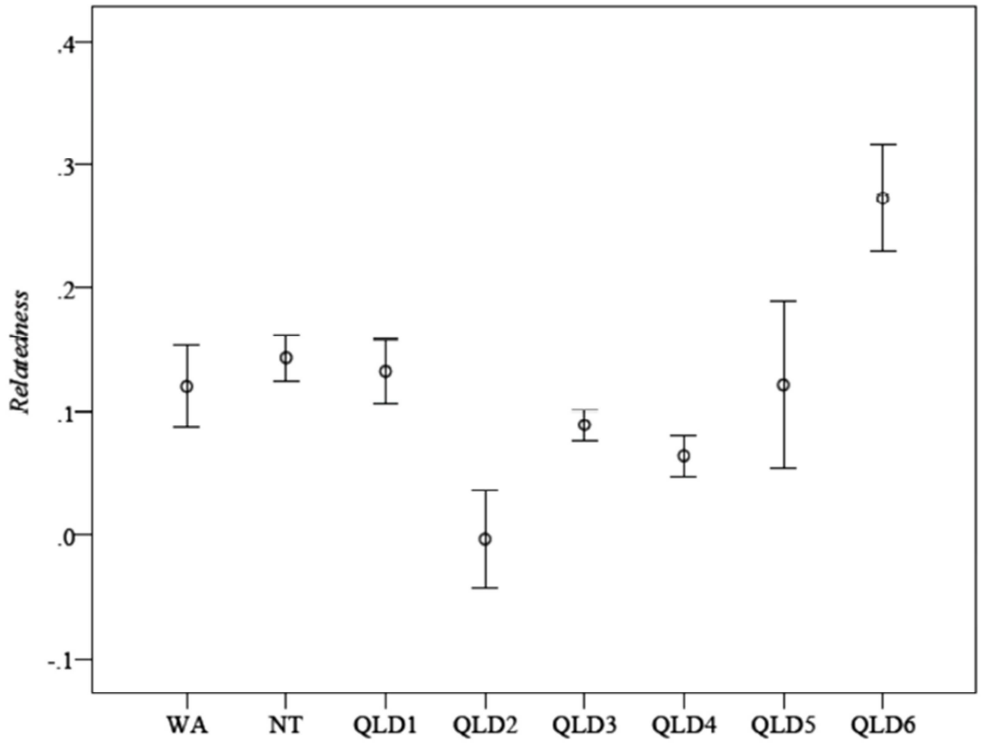


Figure 3.6 Relatedness estimates for eight captive barramundi broodstock groups as determined by the Queller and Goodnight (1989) estimator. Plots represent mean values of relatedness with error bars (SE).

4 Assignment of captive barramundi (*Lates calcarifer*) broodstock to wild Australian stocks guides captive base population recruitment for selective breeding

To be submitted, journal **Aquaculture**

4.1 Abstract

Understanding levels of genetic diversity and relatedness within current captive stocks of barramundi broodstock is important when designing a base population for selective breeding. However, the quality of founder stocks can be enhanced and maintained by including wild stocks of genetically diverse individuals. Assignment tests based on multilocus genotypes can potentially elucidate the wild genetic origins of captive individuals and determine if wild stocks are currently represented in captive stocks. To investigate the wild sources of existing captive barramundi broodstock, assignment tests incorporating 16 polymorphic microsatellite markers were used. Captive individuals from eight commercial barramundi hatcheries (n = 407) were compared with a large wild sample (n = 1205) obtained from 48 locations ranging from Broome in Western Australia (WA), and through the Northern Territory (NT), to the Mary River in Queensland (QLD). Two genetic stocks with broad distributions were detected in the wild: one eastern and one western Australian stock and a central genetically admixed region (across all samples F_{ST} = 0.076). The majority of captive individuals were assigned to the eastern stock (59%), followed by the western (23%) and central admixed region (13%), and 5% could not be assigned to any wild stocks. Levels of genetic diversity were slightly lower in captive groups (average allelic richness A_r = 3.15) compared to wild populations (average A_r = 3.40). For the western stock, the highest levels of diversity were detected at Swift Bay in WA (A_r = 3.64), whereas diversity was higher in the Archer River in QLD (A_r = 3.82) for the central admixed region and in the Burdekin River in QLD for the eastern stock (A_r = 3.46). Upon developing a base population for the selective breeding of barramundi, wild locations demonstrating high levels of genetic diversity identified in this study should be accessed to gather broodstock candidates. Ideally,

an even number of broodstock should be sourced from each of the two wild genetic stocks and central region of admixture, to lower the level of relatedness between individuals and to gather a broad range of genetic diversity for the founding captive population.

4.2 Introduction

The aim of selective breeding programs is to improve key production traits, including the enhancement of growth rates and the lowering of food conversion ratios (FCRs). Breeding programs can help to increase the efficiency and profitability of aquaculture production. Incorporating high levels of genetic diversity in a captive population can substantially increase the potential of aquaculture populations to respond to selection, however, without careful management genetic drift can rapidly reduce productivity through a decrease in genetic diversity within small captive populations (Frost et al., 2006; Norris et al., 1999). Within aquaculture stocks, genetic drift can be particularly strong as a small number of broodstock are typically used for spawning due to the high levels of fecundity frequently observed (e.g. Pacific oyster *Crassostrea gigas*, Boudry et al., 2002; mangrove red snapper *Lutjanus argentimaculatus*, Emata, 2003). In some cases, a loss of genetic variation in aquaculture populations has hampered the breeding program of interest. In one instance, up to 62% of common microsatellite alleles were lost with the production of first generation progeny from wild abalone broodstock (*Haliotus rubra* and *H. midae*) and the extent of loss of genetic variability in this founding population may have hampered the prospects for reseeding efforts (Evans et al., 2004a). Captive stocks of Brazilian white shrimp (*Litopenaeus vannamei*) also experienced a decline in genetic diversity due to population bottlenecks, and as a result, the incidence of

inbreeding and genetic drift increased, which may have limited the possibilities for genetic improvement in the affected population (Freitas et al., 2007). When alleles are not captured from wild individuals upon initiation of a breeding program, or when they are lost in first generation hatchery stocks, they may limit the breeding capacity for that stock (Gjedrem, 2010).

Analyses of population genetic datasets can be used to identify natural stock structure, which potentially enables the genetic assignment of captive fish to their wild genetic origins. This assists in identifying locations where populations contain high levels of genetic diversity and distinctiveness that may not be represented among the broodstock under current production (Benzie, 2000; Yue et al., 2009). If levels of genetic diversity are low and inbreeding rates high, captive populations should be enhanced with increased levels of diversity from suitable wild localities. However, in designing the base population at the commencement of a breeding program, genetic diversity levels should be maximised to avoid the need to introduce new unselected stock into a closed population. Determining the genetic origins of captive stocks is also valuable for fisheries management to ensure the reintroduction of individuals with a matching or similar genetic background compared to native stocks (Schwartz and Beheregaray, 2008; Shaddick et al., 2011). Assignment tests have been used for the management and conservation of fish stocks (Hansen et al., 2000; Hauser et al., 2006), identifying the farm of origin for recaptured escapees from natural populations (Glover et al., 2008; 2009; Zhang et al., 2013), detecting the geographic origin of commercial broodstock (De Innocentiis et al., 2005) and for food traceability (Yue et al., 2012). Assignment tests could provide a means for partitioning available genetic diversity into distinct types, such that founding animals

for the base population can be chosen in a way that maximises the total genetic diversity and the long-term sustainability of the breeding program.

Selecting broodstock candidates from a variety of geographic regions can increase the chance of including genetically diverse individuals into the founding population. For instance, for the Atlantic salmon (*Salmo salar*) selective breeding program in Norway samples were collected from 40 river systems (Gjedrem et al., 1991). Following five generations of selection for rapid growth the accumulated genetic gain was 115% compared to wild stocks of Atlantic salmon (Thodesen et al., 1999). The base population for the development of the Nile tilapia (*Oreochromis niloticus*) GIFT program (Genetic Improvement of Farmed Tilapias) comprised of four wild African strains and four captive bred stocks from Asia (Eknath et al., 1993). The best performing strain combinations were chosen to develop the base population and since then the distribution of GIFT derived strains has enhanced tilapia production worldwide (Eknath and Hulata, 2009). Outbreeding depression (OD) can occur in hybrid offspring produced from distinct strains or source stocks, subsequently causing a reduction in the fitness of the offspring. OD has been reported in mixed-source reintroductions into the natural environment of slimy sculpin (*Cottus cognatus*) (Huff et al., 2011) and in the release of hybrid pink salmon (*Oncorhynchus gorbuscha*) produced from odd and even year broodstock (Gharrett et al., 1999). OD can also potentially occur with the introduction of domesticated fish into wild populations (Tymchuk et al., 2007) including the introgression of captive escapees with wild stocks (McGinnity et al., 2003). Few selective breeding populations have been established using detailed knowledge about fish stock structure and genetic diversity. A number of studies concerning barramundi (*Lates calcarifer*) have considered how this information would be best utilised in choosing

founding stock when initiating selective breeding (Norfatimah et al., 2009; Yue et al., 2009; Zhu et al., 2006a).

Barramundi, or Asian seabass, has been under aquaculture production in Southeast Asia since the 1970s, commencing in Australia during the 1980s with production at 3190 tons in the years 2009 – 10 (ABARE, 2011). Wild barramundi are broad ranging and accessible across the tropical north of Australia and a number of large scale barramundi farms holding broodstock already exist. A selective breeding program for Asian seabass has been initiated in Southeast Asia (Yue et al., 2009) incorporating the selection of founder stocks from natural populations including Thailand, Malaysia, Singapore and Indonesia.

4.2.1 Population genetic structure of wild and captive barramundi stocks

In the Asia-Pacific region, Yue et al. (2009) examined the population structure of barramundi by sampling both wild and captive populations from Southeast Asia and captive populations from Australia. Significant differences amongst all populations were detected with clear differentiation between cultured Australian stocks and those from Southeast Asia. As a result, individuals from regions of high genetic structure were selected from wild populations in Southeast Asia and utilised in a selective breeding program in Singapore. Also sampling Southeast Asian populations, Zhu et al. (2006a) compared wild stocks to local captive broodstock groups and identified significant genetic differentiation between populations. The captive groups had recent Southeast Asian and Thailand origins resulting in a moderate level of genetic diversity, although the captive broodstock only contained a portion of the genetic diversity maintained in the wild populations. Norfatimah et al. (2009) investigated population structure of both wild and cultured stocks from

Peninsular Malaysia, which could be partitioned into three lineages within the region. Evidence was found of stock mixing between wild and cultured groups, which raised concerns about the translocation of aquaculture stock in the region.

Two main genetic stocks and a central region of admixture for wild Australian barramundi have been detected; an eastern stock from the central coast of QLD to Cape York, a western stock from Broome in WA to Darwin in the NT and a central region of admixture from Darwin to the QLD Gulf (Chenoweth et al., 1998a; 1998b; Doupe et al., 1999; Keenan 1994; 2000; Marshall, 2005). A historic land bridge between northern Australian and Papua New Guinea once existed, causing a barrier between east and west stocks across the Torres Strait (Chenoweth et al., 1998b). This division re-opened approximately 7000 years ago and is thought to have allowed mixing of the two metapopulations, leading to the admixed central region detected today. Although studies into wild Australian barramundi populations have been active in the past, to date there has been no attempt to utilise information for the development of a selective breeding program (Jerry and Smith-Keune, 2014). A major problem has been the diversity of past molecular methods used for each of the separate studies, such as allozymes (Keenan, 1994; Salini and Shaklee, 1987; 1988; Shaklee et al., 1993; Shaklee and Salini, 1983; 1985), mtDNA (Chenoweth et al., 1998a; 1998b; Doupe et al., 1999; Marshall, 2005) and microsatellites (Marshall, 2005), which makes it difficult to make comparisons between studies. In addition, the entire natural range of barramundi has not been fully covered in the past due to logistical problems.

In this study, genetic structure and diversity present in captive and wild Australian barramundi from across the entire range was characterised using genotypic data from 16 polymorphic microsatellite loci. Assignment tests were

employed to investigate the source of existing captive broodstock, which assisted in identifying wild regions of high genetic diversity that are not currently represented among captive stocks. These results were used to determine how to best establish a captive base population of barramundi in Australia, so that existing wild genetic diversity is well represented for initiating a selective breeding program.

4.3 Methods

4.3.1 Sampling, DNA extraction and genotyping

The population structure and genetic diversity of wild barramundi populations was investigated by genotyping fish from 48 locations ranging from Broome in Western Australia (WA) through the Northern Territory (NT) to the Mary River in south-east Queensland (QLD) (Fig. 4.1). A total of 1205 samples were collected by either governmental bodies or recreational fisherman (Table 4.1) and genotypes from microsatellite loci were obtained as part of a broader collaborative project with James Cook University in Townsville Australia (Jerry et al., 2013). At five locations (Daly R, Archer R, Bowling Green Bay, Burdekin R and Fitzroy R) replicate samples were collected < 10 km apart; one sample set was collected between 1988 and 1993 by C. Keenan (Keenan, 1994) and the more recent contemporary set was collected between 2006 and 2012. In total, 48 wild collections were incorporated in the analysis of population genetic diversity and structure. By accessing the historical and contemporary data sets any temporal shift in gene frequencies could be investigated, although this comparison was not conducted as part of this study. Similarly, developing a detailed study on the population genetics of natural barramundi stocks was not the primary aim of this study but rather the aim was a general analysis of natural and captive population structure, in order to allocate

captive broodstock samples to wild population sources as accurately as possible given the genetic data obtained.

Finclip samples were collected from barramundi broodstock held at eight commercial hatcheries in Australia (n = 407); one each in the Northern Territory (NT, n = 71) and Western Australia (WA, n = 48), and six in Queensland (QLD1, n = 58; QLD2, n = 14; QLD3, n = 111; QLD4, n = 80; QLD5, n = 9; QLD6, n = 16). The captive populations were sampled in a previous study (chapter 3) and the methods for collecting fin clips, DNA extraction, genotyping and scoring procedures are described in Loughnan et al. (2013). Similarly, all genotyping conducted for the 1205 wild and 407 captive samples were performed with the 17 microsatellite markers described in Loughnan et al. (2013). DNA extraction and methods leading up to genotyping were identical for both wild and captive samples, although all wild barramundi fragment analysis was performed using an ABI 3730 incorporating LIZ 550 as the size standard and fragment analysis was performed with GENEMAPPER® 4.1 software (Applied Biosystems). Captive samples were genotyped on a MegaBACE® 1000 DNA Analysis System (GE Healthcare) utilising ROX 500 size standard and MegaBACE® software Fragment Profiler® was used for fragment analysis. In order to calibrate the size of alleles in base pairs between the two DNA analysis systems, 19 captive broodstock individuals were incorporated as controls on the PCRs of wild samples being analysed with the ABI 3730 and allele labels were adjusted to enable direct comparison between the two datasets.

4.3.2 The genetic origin of captive stocks

Across the eight hatcheries, accurate records detailing the wild population origins for 24% of broodstock were available, whereas the remaining broodstock

were listed as captive bred. A comparison of these available records to the output from the assignment tests was used to assess the accuracy of assignment. In many cases broodstock individuals under current production were developed from captive bred ancestors and in these instances the origins of wild caught ancestors were not always recorded, nor had they been genotyped with molecular markers to determine their pedigrees. In addition, individuals were often exchanged between hatcheries for the purpose of introducing genetic diversity, however, this also had the potential for increasing genetic admixture within broodstock populations. Assigning captive or wild individuals accurately to genetic stocks or clusters becomes more difficult when levels of admixture are detected (Zhang et al., 2013).

Sampled broodstock making up the NT captive group (n = 71) contained 51 wild caught individuals from the Darwin Harbour region of NT principally from Bathurst Island to Shoal Bay (see Table 4.1), whilst the remaining 20 individuals were offspring from captive bred parents that were also originally sourced from these same locations. Of the 48 broodstock individuals included in the WA captive group, 36 were selected from captive grow-out populations. These were either acquired as offspring from QLD1 or the NT group, as first generation progeny of wild parents or pure wild stock. The final 12 captive bred broodstock from the WA group were collected from another interstate hatchery not sampled in this study but believed to have a genetic lineage tracing back to QLD parents (G. Partridge, personal communication). Capture records were limited for broodstock group QLD1 (n = 58) although it was assumed that all captive bred individuals originated from the central QLD region of the Johnston to Burdekin Rivers. The wild origins of captive bred QLD2 broodstock (n = 14) were mostly unknown, although records indicated that the QLD Gulf region was one area of origin. No wild locations were known for

broodstock group QLD3 (n = 111), although at least 15 individuals were captive bred from hatchery QLD1. Seven broodstock from QLD4 (n = 80) were wild caught from within the regions of the Johnston to Burdekin Rivers, whilst the remaining individuals were from unknown origins. Both broodstock groups QLD5 (n = 9) and QLD6 (n = 16) were captive bred individuals originally acquired from hatchery QLD3.

4.3.3 Data analysis

MICRO-CHECKER 2.2.3 was utilised to test for the presence of null alleles and scoring errors (van Oosterhout et al., 2004). The average number of alleles (A), expected (H_e) and observed (H_o) heterozygosities were estimated in GENALEX (Peakall and Smouse, 2012) for both the captive groups and the wild sample collections. Allelic richness (A_r) and private allelic richness (PA_r) were calculated in HP-RARE 1.1 (Kalinowski, 2005), incorporating a rarefaction approach for a minimum of 14 genes per sample. *Ar and* PA_r was calculated across 56 sample collections, combining the 48 wild sites and the 8 captive groups to determine a standardised measure. A_r and PA_r are measures of genetic diversity and rarefaction methods account for differences in sample size and number. Rarefaction limits sample sizes to a number less than or equal to the smallest sample size across populations (Hurlbert, 1971; Szpiech et al., 2008), resulting in a standardised level of A_r and PA_r. Private or unique alleles are those that are considered rare in a population, generally exhibiting low allele frequencies and are not found in other individuals or populations. A_r and PA_r were used as the principle measurements of genetic diversity between the wild and captive sample collections, while H_e and F_{is} were used as an indication of the extent of inbreeding within subpopulations. The

inbreeding coefficient (F_{is}) was calculated in FSTAT 2.9.3.2 (Goudet, 2002) and significance in heterozygote excess or deficiency ($P < 0.05$) was calculated using the method of Weir and Cockerham (1984) following Bonferroni correction for multiple comparisons (Rice, 1988). Tests for HWE were calculated in GENEPOP 4.1 (Rousset, 2008) and significance determined after sequential Bonferroni correction. Exact *P*-values under the Markov chain method were determined with a dememorization step of 10,000, followed by 20 batches of 5000 iterations per batch. To determine any significant differences between populations for H_e, A_r and PA_r, Mann-Whitney U-tests were performed in IBM SPSS 20.0.

4.3.4 Population structure and assignment tests

GENALEX 6.5 (Peakall and Smouse, 2012) was used to perform a principal component analysis (PCA) using Nei's genetic distance, combining the 48 wild sample sites and the eight captive broodstock groups. Pairwise F_{ST} values were also estimated in GENALEX for both the wild and captive populations, incorporating 999 permutations. The Bayesian method of individual clustering applied in STRUCTURE 2.3.3 (Pritchard et al., 2000) was used to test for genetic structure within the wild samples and broodstock groups, using the Bioportal computing resource (https:www.bioportal.uio.no/; Kumar et al., 2009). STRUCTURE analysis assigns the most probable individuals to *k* groups according to threshold *q*-values and enables the visualisation of the grouping of individuals into genetic clusters. The eight captive groups were treated as separate locations and added to the 48 wild collections in the STRUCTURE analysis, culminating to a total of 56 sample collections. Enabling sample location as a prior reference ('locprior') is designed to detect weak population structure and this was compared to the 'no locprior' model.

Admixture and correlated allele frequencies were considered in both models. Ten replicate runs at each *k* (1 – 48) were performed (Gilbert et al., 2012). A burn in length of 100,000 iterations and one million MCMC repetitions were performed for each run. STRUCTURE HARVESTER (Earl and vanHoldt, 2012) was used to detect the number of genetic groups (Δk) that best represented the data (Evanno et al., 2005). CLUMMP was used to average the admixture proportions for the best *k* of each individual over the 10 replicates (Jakobsson and Rosenberg, 2007) and barplots were designed in DISTRUCT 1.1 (Rosenberg, 2004).

Following the detection of genetic clusters in STRUCTURE, assignment tests were conducted using GENECLASS 2.0 (Piry et al., 2004). GENECLASS assigns or excludes individuals or groups to a reference population based on probability based exclusion. If an individual is rejected from all possible reference stocks it is an indication that the source is not represented in the dataset. Two broad wild genetic stocks and a central region of admixture were identified in the STRUCTURE analysis (see Results section 4.4.3) and these were used as reference populations for the assignment of the captive individuals in GENECLASS. The Bayesian method of Rannala and Mountain (1997) was utilised for computation and the simulation algorithm was of Paetkau et al. (2004), simulating 10,000 individuals for the detection of type I errors ($P < 0.01$). The default frequency level of 0.05 was used to assign or exclude any of the three stocks as the origin of an individual. An individual was assigned to a reference stock based on its highest probability. To test the accuracy of genetic assignment, self-assignment tests were undertaken on the three reference populations, using the direct assignment leave one out option.

4.4 RESULTS

4.4.1 Measures of genetic diversity and HWE within wild sample collections

Measures of genetic diversity are displayed in Table 4.1 for the 48 wild barramundi sample collections. The highest average number of alleles (*A*) and allelic richness (A_r) was detected for the Archer River, with values of 5.9 and 3.82 respectively. The highest private allelic richness (PA_r) was detected for the Alligator River (0.10). Average A_r and PA_r across all wild collections was 3.4 and 0.03 respectively. Cleveland Bay recorded the highest level of H_e (0.567), whereas average H_e for the wild collections was 0.52. F_{is} was significantly different from zero for Admiralty Gulf, Swift Bay and Darwin Harbour and overall F_{is} was slightly negative across all collections (-0.0002). At locus *Lca*287, many of the wild sample collections were not in HWE and null alleles were also detected for this marker. As a result, this locus was discarded from any further analysis of wild and captive populations.

4.4.2 Measures of genetic diversity and HWE within captive broodstock groups

For the eight broodstock groups, average A_r and PA_r were 3.15 and 0.03 respectively (Table 4.2). The highest levels of A_r and PA_r were recorded for the NT (A_r = 3.42) and QLD2 (PA_r = 0.14) broodstock groups. Deviations from HWE ($P < 0.05$) were detected for marker *Lca*040 and *Lca*058 for the WA hatchery, *Lca*070 in the NT hatchery and *Lca*074 for QLD1. Null alleles were detected at five loci; *Lca*16 (NT), *LcaM*040 and *Lca*058 (WA), *Lca*069 (QLD3) and *Lca*178 (QLD4), which may have driven the deviations from HWE. The loci demonstrating null alleles were not removed from the analysis because all wild populations (which were represented by larger samples), excluding *Lca*287, were in HWE and showed no null

alleles. Captive individuals that were closely related and demonstrated a deficiency of heterozygotes may have biased the results for detecting null alleles. Overall average H_e was 0.499 and at its highest in the QLD2 group. Average F_{IS} was positive (0.012) and significantly different from zero for both WA and the QLD2 hatcheries ($P < 0.05$). The average polymorphic information content (PIC) of the loci across the broodstock groups was 0.455. As determined by Mann-Whitney U-tests, there was no significant difference in levels of H_e, A_r or PA_r between the broodstock groups and the wild sample collections ($P = 0.82$ for all comparisons).

4.4.3 Population structure

Principal component analysis (PCA) revealed that 57% of the variation was explained within PC axis one, which shows two distinct clusters (Fig. 4.2). The first cluster includes wild collections from QLD, from Princess Charlotte Bay to the Mary River and all six broodstock groups from QLD (QLD1 – 6). All wild sample collections from WA and NT were included in the second cluster on PCA axis one, plus QLD sites from the Albert to the Escape Rivers. Only the broodstock groups from WA and NT were located in the second cluster on PC axis one. PC axis two explained 14% of the variation and could be divided into two smaller clusters, which were within the second cluster detected on PC axis one. The top cluster only included wild samples from NT and QLD, whereas the bottom cluster consisted of WA and NT wild samples. No broodstock groups were located in the top cluster although the bottom cluster included the WA and NT broodstock groups. Global estimates of pairwise F_{ST} was 0.076 ($P < 0.01$) across the 48 wild sample collections and 0.071 ($P < 0.001$) across the eight captive groups.

The most appropriate level of Δk as per Evanno et al. (2012) was two (Fig. 4.3, assessed across all wild and captive sample collections). Population structure was more defined for the 'locprior' model, particularly in the central admixed region and as a result this model is represented in the barplots for $k = 2$ (Fig. 4.4). The output from STRUCTURE revealed two distinct stocks; a western stock (Broome WA – Alligator R. NT, Fig. 4.4a), an eastern stock (Princess Charlotte Bay – Mary R. QLD, Fig. 4.4c) and a central region of admixture (Liverpool R. NT – Escape R., Fig. 4.4b). Upon visualisation of the barplot for the captive broodstock groups (Fig. 4.4d) the NT hatchery was allocated to the western stock, QLD1, 5 and 6 were allocated to the eastern stock and WA, QLD2, 3 and 4 contained a mixture of individuals from both the eastern and western stocks (or individuals that contained a level of admixture between the two stocks). The eastern stock conformed to the results from the PCA analysis, which was defined as cluster one on PCA axis one. The second cluster on PCA axis one included both the western stock and the central region of admixture, however, the top cluster on PCA axis two was defined as the central admixed region in STRUCTURE and the bottom cluster the western stock.

4.4.4 Measures of genetic diversity and HWE within three wild genetic stocks

As a result of the wild population genetic analysis, measures of genetic diversity and inbreeding were added to Table 4.1 for the two identified stocks (eastern and western) and central region of admixture. The highest levels of average A_r and PA_r were detected within the central admixed region with 3.56 and 0.04 respectively although average H_e was highest in the eastern stock at 0.53. Within the western stock, the highest levels of genetic diversity were recorded from samples at Swift Bay ($A_r = 3.64$) and the Alligator River ($PA_r = 0.10$). For the central admixed

region the Archer (A_r = 3.82) and Albert Rivers (PA_r = 0.07) recorded the highest levels. Within the eastern stock, the highest levels of genetic diversity were estimated for the Burdekin River (A_r = 3.46) and Hinchinbrook (PA_r = 0.04). There was no significant difference in levels of H_e, A_r or PA_r between the three wild stocks (P = 0.33). Overall levels of genetic diversity were similar across the three wild genetic stocks and the captive broodstock groups (Table 4.1 and 4.2). Average A_r (3.25 – 3.56) was slightly higher in each of the three wild stocks (broodstock groups, A_r = 3.15), however, average PA_r was only higher in the central admixed stock (0.04), when compared to the captive groups (0.03).

4.4.5 Direct assignment of broodstock individuals to wild populations

When self-assigning the wild samples (n = 1205) to the 48 sample collection sites using GENECLASS the success was low (19%, results not shown). In contrast, 90% of the wild samples were correctly self-assigned to the three broader stocks defined as the eastern and western stocks, and central region of admixture (Appendix 4A). Following self-assignment, all broodstock individuals were assigned to the three reference populations and the results are presented in Table 4.3. Only QLD6 broodstock were assigned to just one stock, which was the eastern. NT was assigned to both the western stock (82%) and central region (14%) and QLD1, 4 and 5 were assigned to the eastern stock (56 – 93%) and central region (7 – 44%). WA and QLD3 were assigned to the eastern (21 – 73%) and western (6 – 62%) stocks, and central region (14 – 15%). Overall, 59% of broodstock were assigned to the eastern stock, 23% to the western stock and 13% to the central region. Based on exclusion probabilities, 5% of captive individuals could not be assigned to any of the three stocks and were subsequently rejected from the analysis.

4.5 Discussion

In order to identify potentially uncaptured genetic diversity for a founding population for selective breeding in Australian barramundi, levels of genetic diversity have been estimated for captive broodstock groups under current production and wild samples covering a large distribution range for the species. Wild population structure analysis revealed two genetic stocks and a central region of admixture, and captive broodstock were found to be assigned to all three clusters. The results highlighted the levels of wild genetic diversity that had previously been captured in the broodstock and identified the level of wild genetic diversity that is still available to benefit captive breeding stocks of barramundi. Overall levels of genetic diversity were only slightly lower in the captive groups (average A_r = 3.15, PA_r = 0.03) than for each of the three wild stocks (average A_r = 3.40, PA_r = 0.03). A selective breeding program would benefit from sourcing further individuals from each of the two wild genetic stocks and central region of admixture, targeting localities that offer the highest levels of genetic diversity. The highest levels of genetic diversity were recorded in the central admixed region (A_r = 3.56, PA_r = 0.04), however, the majority of captive individuals were assigned to the eastern stock (59%). Levels of genetic diversity were lower in wild Australian populations than previously recorded for wild Southeast Asian populations (A_r ranged from 7.60 – 8.50) and this may be due to the latter region being at the centre of the natural range of barramundi (Yue et al., 2009), which probably represent the older populations of the lineage.

For this study, biosecurity issues were disregarded in order to identify the most suitable candidates for a founding population across the natural range of barramundi in Australia. However, previous population structure results have reported that

barramundi should not be transported between genetic stocks, to avoid any mixing of genetic strains from the chance of aquaculture escapees (Shaklee, 1993; Salini and Shaklee, 1988). State fisheries departments have also outlined specific regulations about the movement of barramundi between genetic stocks for aquaculture and restocking purposes (Grace et al., 2008). With the detection of two wild genetic stocks and a central region of admixture, it is suggested that hatcheries only access wild stock from their specific regions. However, there is natural mixing of the populations occurring in the central region which contains a mixture of alleles from both flanking eastern and western stocks, and it could be argued that Australian barramundi was historically one large panmictic population (Keenan, 1994), and that there would therefore be little value in maintaining the genetic differences between eastern and western regions.

Self-assignment results for the three wild stocks were high (90%), although GENECLASS still had difficulty in assigning captive individuals to just one stock at the exclusion level of 0.05. Broodstock individuals were assigned to a reference stock based on the highest probability, although in some cases assigned individuals to one stock were not fully excluded from the other two stocks. As a result, there was a chance that the individual could have originated from any of the three clusters, even though the reference population with the highest probability was chosen as the origin of the individual. The broad spatial scale of the two genetic stocks and central region of admixture covers a large natural range of barramundi and few individuals were excluded from all three reference populations (5%). The overall F_{ST} value between the wild sample collections was low (F_{ST} = 0.08) and this may have limited the power/sensitivity of the assignment tests, as the accuracy has been found to be greater when F_{ST} is > 0.1 (Cornuet et al., 1999). There are also other factors that can

affect the power of assignment tests, such as the number of population's sampled (Hansen et al., 2001), although in this study the number of sample sites was 48 and the three reference populations provided large sample sizes and covered a wide natural range of Australian barramundi. However, there are some areas that require further sampling to gather the full distribution range, such as the Pilbara region of WA and the QLD Gulf region. The number of loci and the level of polymorphism at loci can also affect the accuracy of assignment tests (Hansen et al., 2001). The impact of genetic drift, domestication and disruption of genotypes selected for adaptation can also impact on the probability of assigning captive broodstock to their wild origins correctly, due to changes in the allele frequencies of the captive individuals. In many cases broodstock were recorded as descendants of captive ancestors going back numerous generations.

High concordance was found between the hatchery records of known broodstock source localities and the output from the assignment tests based on the highest probability, indicating assignment was robust even given the broad spatial scales of the three reference populations. Hatchery records indicated that all of the NT captive broodstock were either wild individuals originally sourced from the Bathurst Island to Shoal Bay region (western stock) or were captive bred descendants from this area. The results of the assignment tests confirmed this, although some NT individuals were also assigned to the central admixed region (14%). In addition, the assignment tests also showed that WA broodstock had a mixed origin with lineages from all three genetic clusters, as per hatchery records. The majority of QLD broodstock individuals had high assignment rates to the eastern stock, although all the captive groups besides QLD6 contained some broodstock individuals that were assigned to the central admixed region.

From available records and the results from this study there was some evidence of the mixing of stock between hatcheries. Stock mixing could be beneficial for selective breeding as it would result in higher overall genetic diversity and could result in heterosis, however, a form of outbreeding depression (OD) is also possible if the fitness of such progeny in the culture environment is less than the fitness of pure stock (Gharrett et al., 1999; Huff et al., 2011; McGinnity et al., 2003; Tymchuk et al., 2007). Some degree of OD in the admixed central stock may explain why the genetic differences between the eastern and western stocks have persisted. Alternatively, there may not have been sufficient time since the opening of the land bridge for gene flow to homogenise these differences. This and previous genetic studies suggest relatively low levels of genetic structure and high levels of gene flow between Australian barramundi populations (Chenoweth et al., 1998a; 1998b; Doupe et al., 1999; Jerry and Smith-Keune, 2014; Keenan, 1994; Shaklee and Salini, 1983). However, there is some evidence of isolation and local adaptation to physiological thermal tolerances in barramundi from the eastern and western stocks (Edmunds et al., 2010; 2012; Newton et al., 2010). Heterosis or hybrid vigour is often observed when crossing stock from different strains of the same species (Goyard et al., 2008; Wachirachaikarn et al., 2009). When the selective breeding program is initiated, controlled common garden experiments would be carried out to assess the relative performance of different stock crosses, and emphasis on the different stocks for the production of subsequent generations would be weighted accordingly.

Overall, the 48 wild barramundi collections exhibited relatively even levels of genetic diversity, a typical finding for marine fish, which usually show relatively higher levels of dispersal when compared to freshwater fish (Ward et al., 1994). Natural and man-made barriers can restrict gene flow in freshwater fish and lead to

the creation of isolated subpopulations that can show a higher degree of population structure. Barramundi migrate between freshwater and marine habitats typically spawning at the mouths of estuaries. It is not known if mature barramundi show a preference to return to their natal breeding grounds to spawn but tagged fish have been shown to move between river systems throughout the marine environment (Davis 1986; Moore and Reynolds, 1982; Russell and Garrett, 1983). No significant barriers to migration are known to currently exist between the eastern and western stocks detected in this study. The observed pattern of stock structure and admixture is likely to have been caused by the isolated evolution of distinct eastern and western stocks when the Torres Strait land bridge existed for a period of about 110,000 years (Keenan, 1994; 2000). Approximately 7000 years ago the area flooded and reopened to migration, which seems to have occurred in a predominantly east to west direction causing a region of admixture in the Torres Strait. Support for the effect of this historic land bridge on local marine species in the region has also been shown in reef fishes (Mirams et al., 2011), sea turtles (Dethmers et al., 2006), prawns (Brooker et al., 2000) and sharks (Duncan et al., 2006). The slightly higher levels of genetic diversity detected in the central admixed region would be expected if the total species distribution consisted of two large stocks with gene flow between them. Evidence of the existence of natural gene flow between eastern and western barramundi stocks is also supported by previous studies using both allozyme loci (Keenan, 1994; Shaklee and Salini, 1983) and mitochondrial DNA markers (Chenoweth et al., 1998a; 1998b; Doupe et al., 1999) and the mixing of the two stocks may still be in progress.

Specific wild stocks and locations have been identified in this study that should be targeted in order to maximise genetic variation when initiating selective breeding.

The highest levels of genetic diversity detected in the western stock were found at Swift Bay in WA and the Alligator River in NT. For the central admixed region, genetic diversity was highest at the Archer and Albert Rivers in QLD and for the eastern stock the Burdekin River and Hinchinbrook region in QLD, recorded the highest levels of genetic diversity. One strategy is to select an equal number of males and females from each of the eastern and western stocks and/or from the central stock of admixture, at these sampling locations that show the highest levels of genetic diversity. Preferably, we would want to construct a large and broad ranging founding population at the commencement of a selective breeding program so that the broad genetic variation represented can yield a strong selection differential, although it is possible that some common or inferior genes would also be collected. However, it is not possible to evaluate the genetic merit of individuals, or even populations, when establishing new selective breeding programs without performing scientifically rigorous comparisons of performance in the same environment (which normally occurs as the selective breeding program gets underway). Also, genes that may be considered of little value to the current industry may become of high value later, as changes in the environment or industry occur (e.g. genes for resistance to specific diseases). Therefore it is important to capture as much broad genetic variation as possible when starting a selective breeding program so that genetic progress can be achieved.

It can be difficult to access the entire natural range of a species and there may be some areas that remain unrepresented when determining population structure, although the aim is to gather as close to a representative sample of the natural population as possible. Initially, excess broodstock should be collected because not all individuals will develop into successful breeders and the effective breeding size

of the group will always be less than the census size. The expense and space required to maintain numerous broodstock is also an important factor, although in the long term the benefits of increased production and lower inbreeding rates may outweigh the initial cost of establishing the base population. A selective breeding program making use of the captive broodstock existing in these eight hatcheries, would capture greater allelic diversity by accessing additional broodstock from an even representation of individuals from the wild stocks identified in this study.

4.6 Conclusion

The results from this study support the hypothesis for an east-west population split caused by a historic biogeographic barrier known as the Torres Strait land bridge in northern QLD (Chenoweth et al., 1998a; 1998b; Doupe et al., 1999; Jerry and Smith-Keune, 2014; Keenan, 1994; Shaklee and Salini, 1983). Secondary contact following the flooding of the Torres Strait to present day sea levels (~ 7000 years ago; Keenan, 1994) has caused an east to west direction of gene flow and a central region of admixture spreading from the QLD Gulf into NT. This area may provide a valuable resource of broodstock for developing a productive base population for a captive breeding program in barramundi, due to higher levels of genetic diversity contributed from both flanking eastern and western stocks. Levels of genetic diversity were similar for both the wild stocks and the broodstock, and this may be due to a sampling or founder effect with the repeated sampling of numerous different wild subpopulations as a source. Deviations from HWE and the appearance of null alleles in the captive populations could be due to a Wahlund effect (Hartl and Clark, 1997), where individuals in these particular captive populations were sourced from the eastern and western stocks and central region of admixture. The results

presented here provide valuable information regarding the origin of current barramundi broodstock under production, including the relative levels of natural genetic diversity available within these broodstock and throughout the species range. This information will be used to develop a plan for increasing the fitness and potential of captive stocks when establishing barramundi selective breeding programs in Australia and will serve as an example for the creation of genetic improvement programs for other species.

Table 4.1 Measures of genetic diversity and HWE for 48 wild barramundi sample sites from 16 microsatellite loci. Representing Western Australia (WA), Northern Territory (NT) and Queensland (QLD). Sample size (*N*), average number of alleles (*A*), mean observed (H_o) and expected (H_e) heterozygosities, mean allelic richness (A_r) and private allelic richness (PA_r) and the average inbreeding coefficient (F_{is}). Average measures are also provided for the two identified genetic stocks; eastern and western stocks, and the central region of admixture

Stock location	State	Code	*N*	*A*	H_o	H_e	A_r	PA_r	F_{is}
Broome	WA	BME	13	3.2	0.462	0.447	2.85	0.0549	0.007
St George Basin	WA	STG	30	4.5	0.536	0.528	3.38	0.0932	0.001
Admirality Gulf	WA	ADM	37	4.4	0.457	0.485	3.09	0.0073	0.072*
Swift Bay	WA	SWI	17	4.6	0.515	0.552	3.64	0.0082	0.098*
Drysdale River	WA	DRY	26	4.7	0.546	0.532	3.47	0.0033	-0.005
Salmon Bay	WA	SMB	25	4.9	0.550	0.526	3.46	0.0472	-0.025
King George River	WA	KGS	24	4.6	0.563	0.531	3.44	0.0077	-0.038
Berkeley River	WA	BER	24	4.8	0.537	0.536	3.57	0.0100	0.018
Helby River	WA	HEL	24	4.4	0.523	0.508	3.40	0.0003	-0.009
Bulla Nulla Creek	WA	NNC	21	4.3	0.478	0.480	3.18	0.0411	0.032
Ord River	WA	ORD	63	5.4	0.515	0.522	3.42	0.0024	0.022
Bonaparte Gulf	WA	KEE	26	4.9	0.529	0.517	3.48	0.0136	-0.003
Moyle River[k]	NT	MOYK	18	4.3	0.516	0.499	3.39	0.0057	-0.004
Daly River (2008)	NT	DLY	24	4.8	0.520	0.532	3.54	0.0034	0.045
Daly River[k] (1990)	NT	DLYK	22	4.8	0.549	0.524	3.51	0.0078	-0.023
Bathurst Island[k]	NT	BTIK	24	4.4	0.490	0.497	3.35	0.0625	0.035
Darwin Harbour[k]	NT	DHBK	23	4.8	0.470	0.490	3.41	0.0395	0.062*
Shoal Bay[k]	NT	SHOK	24	4.5	0.513	0.487	3.36	0.0268	-0.033
Mary River	NT	MRR	24	4.6	0.503	0.489	3.40	0.0077	-0.007
Alligator River	NT	ALG	13	4.3	0.538	0.496	3.53	0.0974	-0.039
Western stock[‡]			**502**	**4.6**	**0.516**	**0.509**	**3.39**	**0.0270**	**0.010**

(continued on next page)

Liverpool River	NT	LVP	32	4.1	0.498	0.515	3.15	0.0429	0.049
Arnhem Bay[k]	NT	ANBK	22	4.6	0.523	0.506	3.42	0.0523	-0.010
Roper River	NT	ROP	24	4.6	0.523	0.487	3.46	0.0677	-0.055
McArthur river	NT	MAC	24	4.8	0.513	0.514	3.54	0.0498	0.024
Albert River (2011)	QLD	ALB	24	5.3	0.511	0.528	3.70	0.0713	0.053
Leichhardt River[k] (1990/91)	QLD	LICK	24	4.9	0.520	0.502	3.55	0.0183	-0.016
Gilbert River	QLD	GIL	24	5.5	0.516	0.529	3.68	0.0549	0.047
Mitchell River[k]	QLD	MITK	24	5.0	0.541	0.527	3.67	0.0081	-0.004
Holroyd River[k]	QLD	HOLK	21	5.1	0.536	0.531	3.68	0.0488	0.016
Archer River (2011)	QLD	ARC	33	5.8	0.528	0.525	3.66	0.0213	0.009
Archer River[k] (1993)	QLD	ARCK	24	5.9	0.555	0.547	3.82	0.0385	0.007
Jardine River	QLD	JAR	16	4.4	0.539	0.509	3.52	0.0479	-0.028
Jackey Jackey Creek	QLD	JCK	30	4.7	0.557	0.542	3.42	0.0387	-0.010
Escape River	QLD	ESC	24	4.8	0.549	0.548	3.56	0.0322	0.019
Central region[‡]			**346**	**5.0**	**0.529**	**0.522**	**3.56**	**0.0423**	**0.007**
Princess Charlotte Bay	QLD	PCB	24	4.1	0.549	0.543	3.20	0.0431	0.009
Bizant River	QLD	PCB2	15	3.9	0.533	0.539	3.30	0.0017	0.045
Johnstone River	QLD	JOR	48	5.1	0.570	0.533	3.36	0.0153	-0.059
Hinchinbrook	QLD	HC	50	5.6	0.565	0.555	3.43	0.0436	-0.008
Cleveland Bay	QLD	CLE	23	4.6	0.584	0.567	3.44	0.0096	-0.008
Bowling Green Bay (2008)	QLD	BOW	24	4.5	0.555	0.535	3.24	0.0143	-0.016
Bowling Green Bay[k] (1988)	QLD	BOWK	24	4.4	0.534	0.540	3.31	0.0149	0.033
Burdekin River (2008)	QLD	BUR	24	4.6	0.581	0.549	3.46	0.0090	-0.036
Burdekin River[k] (1989/90)	QLD	BURK	24	4.6	0.583	0.552	3.37	0.0340	-0.034
Broad Sound[k]	QLD	BRDK	12	3.6	0.558	0.517	3.27	0.0033	-0.034
Fitzroy River (2008)	QLD	FTZ	23	3.3	0.509	0.461	2.68	0.0173	-0.080
Fitzroy River[k] (1988/90)	QLD	FTZK	24	4.4	0.570	0.541	3.25	0.0335	-0.032
Port Alma	QLD	PAF	24	3.7	0.480	0.453	2.85	0.0385	-0.036
Mary River[k]	QLD	MARK	18	4.4	0.552	0.539	3.39	0.0226	0.004
Eastern stock[‡]			**357**	**4.3**	**0.552**	**0.530**	**3.25**	**0.0215**	**-0.018**
All sample collections[‡]			1205	4.6	0.532	0.520	3.40	0.0303	-0.0002

[‡]*N* is the total count, whilst the remaining values are averages.

[*]Average F_{is} values significantly different from zero at the 0.05 level following Bonferroni correction for multiple comparisons (Rice, 1988) from 16 classes.

[k]Denotes temporal samples included from Keenan (1994), collected between 1988 and 1993 unless stated in parenthesis.

Table 4.2 Measures of genetic diversity and HWE for eight captive barramundi broodstock groups from 16 microsatellite loci; from the Northern Territory (NT), Queensland (QLD) and Western Australia (WA). Sample size (*N*), average number of alleles (*A*), mean observed (H_o) and expected (H_e) heterozygosities, mean allelic richness (A_r), private allelic richness (PA_r) and the average inbreeding coefficient (F_{is})

Hatchery	*N*	*A*	H_o	H_e	A_r	PA_r	F_{is}
WA	48	4.2	0.469	0.497	3.18	0.0019	0.069*
NT	71	5.6	0.503	0.509	3.42	0.0494	0.020
QLD1	58	4.3	0.514	0.491	3.11	0.0004	-0.038
QLD2	14	3.9	0.513	0.537	3.35	0.1356	0.082*
QLD3	111	5.5	0.506	0.506	3.23	0.0134	0.005
QLD4	80	4.4	0.513	0.518	3.19	0.0103	0.016
QLD5	9	3.2	0.532	0.482	3.04	0.0094	-0.042
QLD6	16	3.1	0.475	0.453	2.67	0.0318	-0.014
Multiple loci[‡]	407	4.3	0.503	0.499	3.15	0.0315	0.012

[‡]*N* is the total count, whilst the remaining values are averages.

[*]Average F_{is} values significantly different from zero at the 0.05 level following Bonferroni correction for multiple comparisons (Rice, 1988) from 16 classes.

Table 4.3 Assignment of eight captive barramundi broodstock groups in GENECLASS to two genetic stocks (western and eastern) and a central region of admixture. A count of assigned individuals is presented, followed by the % assigned in parenthesis. The number of individuals rejected from a stock was determined by exclusion probabilities ($P < 0.05$)

		Western stock	Central region	Eastern stock	Rejected from all
WA	Assigned	30 (62%)	7 (15%)	10 (21%)	
n = 48	Rejected	4	2	19	1 (2%)
NT	Assigned	58 (82%)	10 (14%)	-	
n = 71	Rejected	3	19	58	3 (4%)
QLD1	Assigned	-	4 (7%)	54 (93%)	
n = 58	Rejected	30	12	1	-
QLD2	Assigned	-	1 (7%)	10 (72%)	
n = 14	Rejected	10	5	3	3 (21%)
QLD3	Assigned	7 (6%)	15 (14%)	81 (73%)	
n = 111	Rejected	60	36	25	8 (7%)
QLD4	Assigned	-	11 (14%)	66 (82%)	
n = 80	Rejected	45	22	3	3 (4%)
QLD5	Assigned	-	4 (44%)	5 (56%)	
n = 9	Rejected	5	-	-	-
QLD6	Assigned	-	-	14 (88%)	
n = 16	Rejected	14	8	2	2 (12%)
	Total assigned	95 (23%)	52 (13%)	240 (59%)	
				Total rejected	20 (5%)

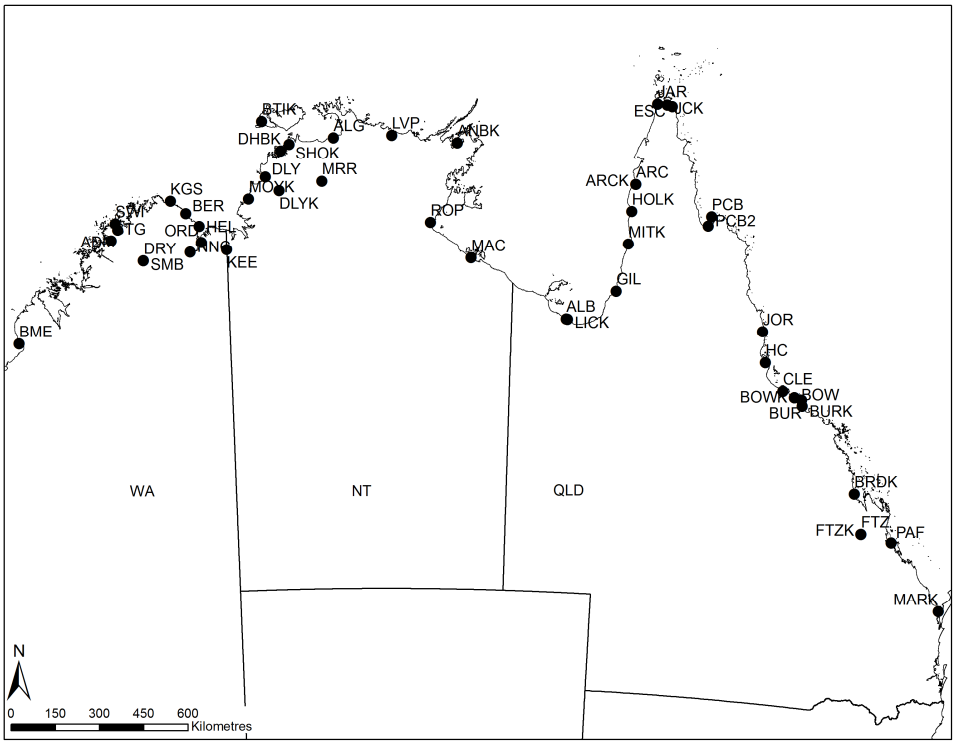


Figure 4.1 Map of 48 barramundi sample sites in Australia, where 1205 barramundi were collected for the study. See Table 4.1 for a description of the labels representing each collection site.

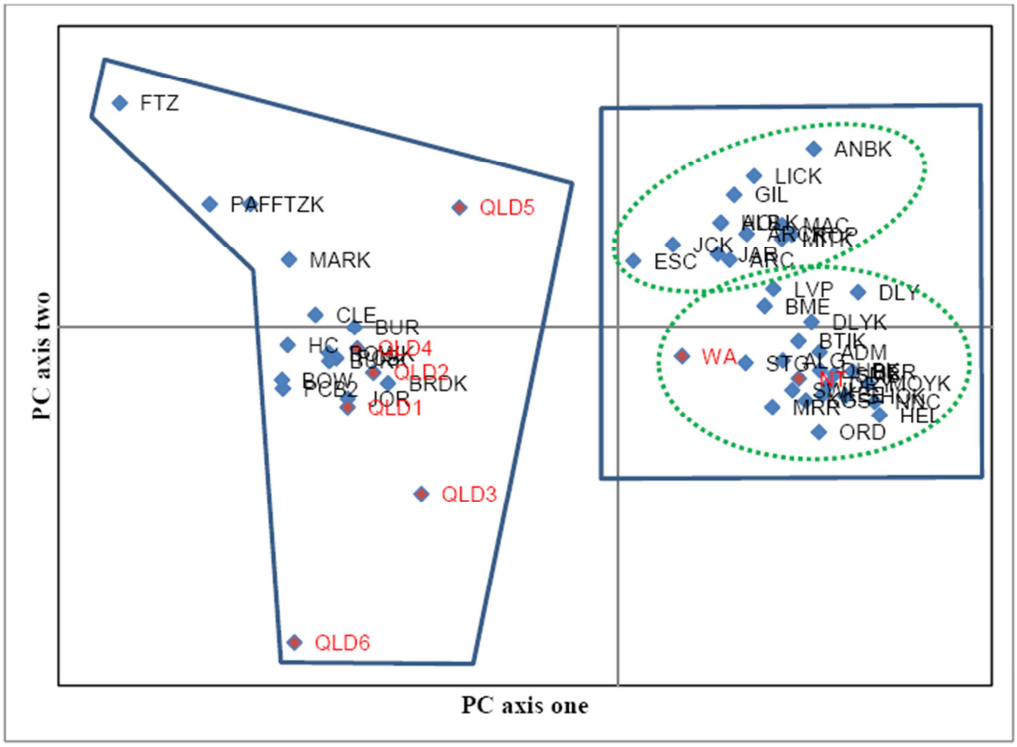


Figure 4.2 Plot of the first two principal coordinates of microsatellite variation using Nei's genetic distance. Each symbol corresponds to one of 56 barramundi subpopulations, including 48 wild sample collections (blue symbols) and eight captive broodstock groups (red symbols). See Tables 4.1 and 4.2 for a description of the labels representing each collection site. The variance explained on PC axis one was 58% and sample sites within the solid blue lines represent clusters one and two. PC axis two explained 14% of the variation and sample sites within the dashed green circles represent top and bottom clusters.

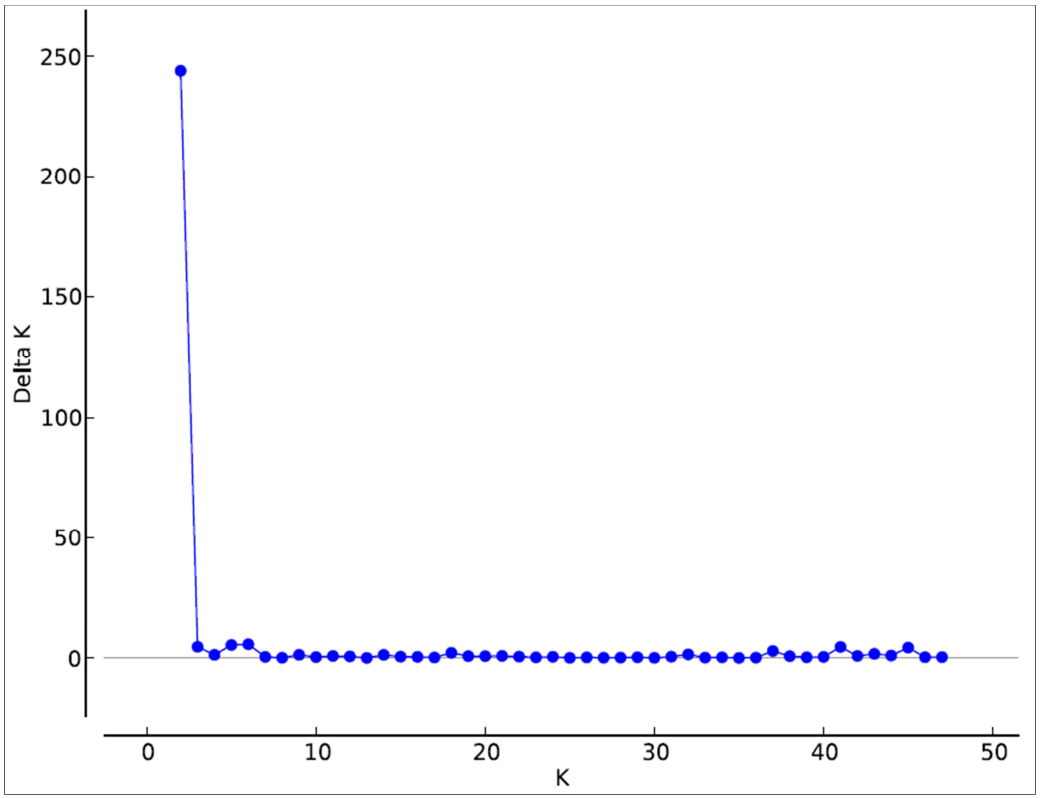
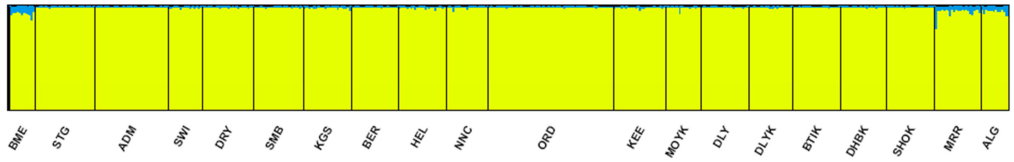
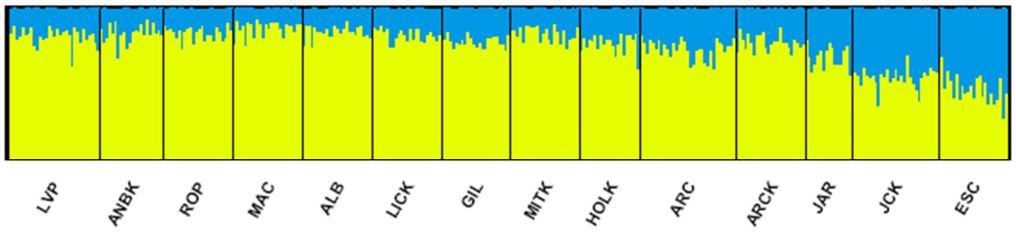


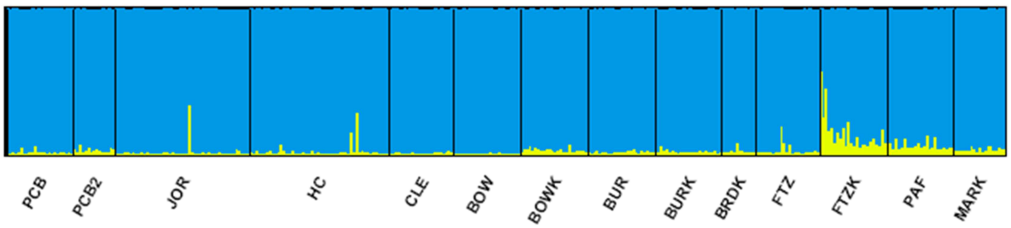
Figure 4.3 Delta *k* (Δ*k*) determined by the Evanno et al. (2005) method showing the most probable number of *k* groups (*k* = 2) from 56 subpopulations, including 48 wild sample collections (*n* = 1205) and eight captive broodstock groups (*n* = 407).



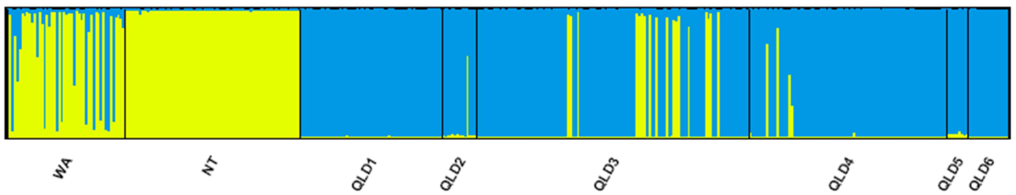
a.



b.



c.



d.

Figure 4.4 STRUCTURE barplots for 48 (*n* = 1205) wild sample collections (a – c) and eight (*n* = 407) broodstock groups (d). The inferred number of populations (*k*) was two. The wild samples are divided into a western stock (a), a central region of admixture (b) and an eastern stock (c). Sample sites and broodstock groups are separated by a black line and each bar represents one individual. See Tables 4.1 and 4.2 for a description of the labels representing each sample site.

5 Comparison of the use of different source stocks for establishing base populations for selective breeding of barramundi (*Lates calcarifer*)

To be submitted, journal **Aquaculture Research**

5.1 Abstract

A computer simulation model was developed to determine the most appropriate broodstock candidates to use when establishing a base population for barramundi selective breeding. The model predicts the allelic richness (A_r) expected at 16 microsatellite loci for five different options after initial mating of the founder broodstock. The input for the simulation was an actual dataset of genotypes from individuals sampled from two broad ranging wild genetic stocks and a region of genetic admixture, ranging from Western Australia, across the Northern Territory to Queensland. In addition, genotypes from eight captive barramundi populations existing in Australia were also included. The mean kinship between captive individuals (mk_r) was calculated using data from chapter 3 and A_r within wild sites was estimated with data from chapter 4. Individuals and populations were ranked according to mk_r and A_r respectively, for inclusion into a synthetic base population. Options tested for the source of founders were i) captive broodstock with the lowest mk_r (Cmk_r), ii) equal representation of two wild genetic stocks and a region of admixture selecting sites with the highest A_r (WSA_r), iii) wild sites with the highest A_r across the entire distribution range (WA_r), iv) one captive broodstock group combined with the highest A_r wild sites ($C1WA_r$), and, v) one captive broodstock group without additional wild sourced individuals (*C1*). Each option used a base population size of 150 individuals with an equal sex ratio. Parents were randomly distributed into five tanks (30 individuals per tank, each containing 15 males and 15 females) and each individual's contribution to the spawn was simulated based on parameters collected from a previous study of a mass spawning group. From the simulated gametes produced (containing alleles for the 16 loci) 100 offspring were generated per tank and each breeding program option was replicated 100 times. For

options WSA_r, WA_r, $C1WA_r$ and $C1$ there was a significant reduction in the level of A_r between broodstock and offspring ($P < 0.05$). However, levels of A_r were the highest for option WSA_r ($A_r = 4.75$). There was no significant difference in the level of A_r transferred from broodstock to offspring under option Cmk_r ($P = 0.09$). Five alternate base population sizes (N_c) were tested to estimate the effective population size (N_e). Average N_e was 76, 85, 98, 105 and 115 for an N_c of 150, 180, 200, 230 and 250 respectively, and the rate of inbreeding (ΔF) ranged from 0.4 – 0.7%. Under the model presented in this study, an N_c of more than 213 broodstock individuals is required to achieve $N_e > 100$ and $\Delta F < 0.5\%$. Overall, current captive broodstock maintained in the Australian industry have low mk_r and would be suitable for inclusion into a base population. However, the results indicate that the inclusion of wild individuals would significantly enhance levels of genetic diversity in a base population for the development of a selective breeding program.

5.2 Introduction

Small aquaculture broodstock populations typically represent a fraction of the genetic diversity available in wild stocks. The costs to maintain broodstock, space requirements and the fecundity of the species all affect the size of the base or founding population maintained. However, the long-term benefits gained by starting with a larger base breeding population (in terms of limiting inbreeding depression of fitness and maintaining high levels of genetic diversity) could outweigh the additional start-up costs. Breeding individuals should be chosen to capture as much of the wild representative genetic diversity as possible. This is important for ensuring the longevity of a closed selective breeding program. The extent of the genetic variation that is initially captured and maintained by the selective breeding

program ultimately limits the genetic response that is possible for traits under selection (Hayes et al., 2006).

A low effective number of breeding individuals, or genetically effective population size (N_e), can cause a loss of genetic diversity over time. N_e is positively correlated with the number of breeding individuals, or census size (N_c) but is not equal to N_c. This is due to unequal numbers of male and female broodstock and non-random variation in parental contribution to the production of offspring, which is due to differences in the fertility of parents, opportunities for reproduction and the survival of offspring. Hatcheries utilising a limited number of broodstock due to high levels of fecundity in mature females are at risk of losing genetic diversity (Boudry et al., 2002; Emata et al., 2003). The rate of inbreeding (ΔF) can be approximated as $1 / (2N_e)$. Typically in a mass spawning situation N_e is low. For example, in three gilthead seabream (*Sparus aurata*) broodstock groups that naturally mass spawn, N_e was between 14.0 – 18.3, the N_e / N_c ratio ranged from 0.29 – 0.33 and ΔF was therefore estimated between 3 – 4% (Brown et al., 2005). An average number of 53 broodstock of unequal sex ratio in each group were utilised, although the number of contributing parents was much less and ranged from 9 – 25.

The size of founding populations for selective breeding programs should be at a level that captures rare alleles and maintains available genetic diversity for the species, and enables inbreeding to be limited to acceptable levels. N_e and ΔF of more than 100 and less than 0.5% respectively, has been considered as an acceptable target for fish selective breeding programs (Fjalestad, 2005; Sonesson et al., 2005). In order to reach these targets, captive stocks may require enhancement with unrelated and genetically diverse individuals, possibly from other captive populations and/or wild genetic source stocks.

Whilst acquiring new broodstock recruits from genetically diverse wild stocks has advantages, maintaining current captive broodstock should not be discounted. This is because adaptation to the captive environment can lower the stress levels of broodstock and help to acclimatise the fish to spawning condition (Gjedrem, 2005). A combination of four wild geographic strains from Africa and four established farmed strains from the Philippines were successfully included into the base population of the first GIFT (Genetic Improvement of Farmed Tilapia, *Oreochromis niloticus*) program in Asia (Eknath et al., 1993). Farmed stocks of the species had become depleted and the injection of high levels of genetic diversity to create an enriched founder population was necessary. From this, 25 pure and crossbred groups that displayed the greatest additive genetic performance for growth were selected to form the founder population. As a result of the GIFT tilapia program, the accumulated genetic gain in relation to the base population has been estimated at 85% over five generations of selection for fast growth (Eknath and Hulata, 2009).

Regarding barramundi (*Lates calcarifer*), there are many groups of captive broodstock in hatcheries throughout Southeast Asia and Australia and the natural distribution range of the species is known and accessible. A large number of mature broodstock are present in Australian hatcheries and many of these individuals share no common ancestry with other captive individuals, and could be selected to provide levels of genetic diversity comparable to that existing within wild stocks (see chapters 3 and 4). Barramundi is a highly fecund mass spawning species and because of this, small broodstock groups have the ability to supply all the larval requirements for the entire industry. However, with small population sizes and a high chance of some individuals failing to participate in a spawning event, this can

result in a low level of N_e and high ΔF (Frost et al., 2006; Loughnan et al., 2013; Wang et al., 2008).

The aim of this study was to use a computer simulation model to compare options for the establishment of a base population at the commencement of a selective breeding program for barramundi. The simulation model was developed and utilised to construct a synthetic base population under several alternative broodstock choice scenarios (considering levels of relatedness and genetic diversity). Captive and wild barramundi recorded by two previous studies (chapters 3 and 4) were used as sources for genotyped animals. One generation of offspring was bred for each option, each option was replicated 100 times and levels of genetic diversity were estimated in the cohorts in order to predict the best method for constructing a base population that will conserve genetic diversity, limit inbreeding and maintain a high N_e for selective breeding.

5.3 Methods

5.3.1 Genetic data from captive and wild founders

Two datasets consisting of genotypes from 407 captive and 1205 wild barramundi at 16 microsatellite loci was used for the study (chapters 3 and 4). No stock performance information was available for the 407 captive broodstock, although it was determined that by cannulation inspections and from existing hatchery records there were 136 females and 180 males (chapter 3). Ninety-one individuals with unknown gender were randomly assigned a sex, male or female, with a probability of 0.5, making the final number of males and females 228 and 179 respectively. All samples from the targeted wild collections were also randomly

allocated a sex. The breeding simulation described below was designed to utilise these actual individual genotypes as input.

5.3.2 Structure of the captive breeding program

A simulation modelling the initial mating of founder broodstock for a captive selective breeding program was designed using the R 3.0.1 programming language (R core team, 2013). The program simulated the fate of allelic variation at 16 microsatellite loci in a breeding program with separate but synchronous mass spawning of five groups of fish, each group consisting of 30 mature broodstock. This was the same tank and mating structure modelled for other purposes and trialled in earlier papers (Loughnan et al., 2013; Robinson et al., 2010). An equal sex ratio of 15 males and 15 females was applied to each spawning tank in the model (see Appendix 5A for the full script).

5.3.3 Ranking of candidates for inclusion into the synthetic base population

To determine which 75 male and 75 female parents should be included in the simulation under the five scenarios described below, all captive broodstock were ranked on the basis of mean kinship (mk_r) and samples from wild locations were ranked according to allelic richness (A_r) at 16 loci. Preference was given to individuals with higher A_r and lower mk_r, as effective measures of genetic diversity.

To estimate mk_r, the relatedness estimator (r) of Queller and Goodnight (1989) was utilised in COANCESTRY 1.0.1.2 (Wang, 2011) to calculate r between every parent-pair combination across the eight hatcheries (n = 407). mk_r was then calculated from the estimates of r according to the modified methods of Doyle et al. (2001) and Sekino et al. (2004). The original *mk* strategy proposed by Ballou and Lacy (1995) assumed single pair mating and pedigree records, whereas the modified

methods incorporated *r* estimates rather than pedigree data and was applied to group or mass spawning species, a similar breeding strategy to barramundi. Relatedness was calculated once between each individual and every other individual in the candidate population and mk_r was calculated as

$$mkr_i = \sum_{j=1}^{N} f_{ij}/N$$

where f_{ij} is the kinship between *i* and *j* and *N* is the number of individuals in the population.

Selecting broodstock candidates from wild locations where the highest levels of A_r had been detected was performed in order to capture the highest genetic diversity. In a previous study (chapter 4), A_r was calculated using FSTAT 2.9.3.2 (Goudet, 2002) between the wild localities as

$$A_r = \sum_{i=0}^{n_1} \left[1 - \frac{\binom{2N-N_i}{2n}}{\binom{2N}{2n}}\right]$$

where N_i is the number of alleles of type i among the *2N* genes.

5.3.4 Options tested for source of founders

Captive broodstock with the lowest level of mk_r (Cmk_r)

Under this option, 75 male and 75 female captive broodstock (from a total of 407 individuals) with the lowest average mk_r were selected. Determining mk_r is an effective method for minimising kinship (limit inbreeding) and maximising the conservation of rare alleles (maintain genetic variation) between captive populations. Parents were randomly distributed into five tanks (30 different individuals per tank, each containing an equal sex ratio of 15 males and 15 females). The contribution of each parent to each spawning event was varied as described in detail below (see section 5.3.5). The average mk_r of the starting 30 broodstock within tanks one to five

was -0.069, -0.066, -0.066, -0.076 and -0.056 respectively, while average A_r was 5.52, 5.47, 5.48, 5.58 and 4.85 respectively.

Equal representation of each wild genetic stock, selecting sites with highest A_r (WSA_r)

Under this option, the top five sites with the highest A_r from each of the two wild genetic stocks and a central region of admixture (western, central and eastern, totalling 15 sites) were used as a source of animals (chapter 4). Population structure was previously detected between these regions and a representation of individuals from each would be expected to enhance the productivity of a captive broodstock population. Ten individuals from each of the five sites were randomly selected (Appendix 5B.1) within each stock and region of admixture. A_r ranged from 3.36 – 3.82 across the range of sample sites selected. Parents were randomly distributed into five tanks (30 individuals per tank), each containing an equal sex ratio of 15 males and 15 females. Average A_r was 5.64, 6.14, 5.71, 5.71 and 5.83 respectively for broodstock within the five spawning tanks.

Wild sites with highest A_r (WA_r)

Under this option, sample sites with the highest A_r across the entire distribution range irrespective of which genetic stock they belonged to were used as a source of animals (Appendix 5B.2). This option was tested to determine whether the highest levels of wild A_r could be maintained in subsequent generations. All of these sites belonged to the central region of admixture. A_r ranged from 3.66 – 3.82 and the Archer River recorded the highest level. To begin, all samples from the Archer River were allocated to the base population. Next, all samples from the second highest ranked site for A_r were then added (Albert River). This continued from the

highest to the lowest ranked sites for A_r, until the quota of 150 individuals (75 males and 75 females) was reached. Parents were randomly distributed into five tanks (30 individuals per tank), each containing an equal sex ratio of 15 males and 15 females. Average A_r was 5.70, 5.64, 5.79, 5.54 and 5.40 respectively for broodstock within the five spawning tanks.

One captive broodstock group (QLD1) combined with highest A_r wild sites ($C1WA_r$)

Under this option, all 58 broodstock individuals from captive group QLD1 (chapter 3) were selected and an additional 92 wild individuals added to give a total broodstock census size of 150 (75 male and 75 female). This option was developed to simulate the inclusion of wild individuals into a captive population, which is typically practiced in the industry, and its effect on the level of genetic diversity. Wild sites were selected according to the highest levels of A_r across the wild sample distribution range and these included the Archer, Albert, Gilbert and Holroyd Rivers from the central region of admixture (selected from Appendix 5B.2). Parents were randomly distributed into five tanks (30 individuals per tank), each containing an equal sex ratio of 15 males and 15 females. Average A_r was 5.09, 5.77, 5.52, 5.60 and 5.46 respectively for broodstock within the five spawning tanks.

Captive broodstock from QLD1 without additional wild sourced individuals (C1)

Under this option, the QLD1 broodstock group (n = 58) was used as one mass spawning broodstock group containing 26 females and 32 males, without the inclusion of wild individuals. This was for comparison to the results from the previous option ($C1WA_r$), for which QLD1 broodstock were combined with wild individuals. Average A_r for the broodstock group was 4.11.

5.3.5 Simulation of spawning events

First, each parent was assigned a level of contribution to each spawning event. It was assumed that the frequency with which each parent contributes gametes would be similar to the actual contribution frequencies determined from a trial spawn using 12 females and 21 males over two nights of spawning (Loughnan et al., 2013), which found that females 1 – 12 contributed at frequencies of 0.372, 0.208, 0.139, 0.092, 0.062, 0.051, 0.046, 0.021, 0.007, 0.002, 0.000 and 0.000 and that males 1 – 21 contributed at frequencies 0.134, 0.125, 0.122, 0.106, 0.072, 0.065, 0.053, 0.046, 0.039, 0.035, 0.032, 0.028, 0.023, 0.021, 0.021, 0.018, 0.016, 0.016, 0.014, 0.009 and 0.005. These frequency values were considered as measurements of individual spawning ability (*isa*). For the simulation, the *isa*'s for males *m* and females *f* in each spawning group were generated by randomly sampling from a pool of frequency values consisting of 100 of each of the actual contribution frequency values detected for male and female broodstock by Loughnan et al. (2013), without replacement. A corrected value of *isa* for each individual (*i*) was calculated for each set of *n* males or *n* females in each spawning group in the following manner;

$$isa_i = {uncorrected\ isa_i}\Big/{\sum_{i=1}^{n} uncorrected\ isa}$$

All loci were assumed to be independent and alleles were assumed to segregate randomly with meiosis according to the rules of Mendelian inheritance. A mixture of 1000 male and 1000 female gametes (Fig. 5.1) with alleles at 16 loci was created for each spawn by randomly selecting one allele for each locus from the parent of each gamete where the number of gametes (n_g) contributed by each parent *i* was

$n_{gi} = isa_i * 1000$

One hundred offspring were generated for each spawning group by randomly selecting male and female gametes without replacement from the mix for fertilisation. Each breeding program option was replicated 100 times and an average calculated.

5.3.6 Comparison of offspring allelic diversity between breeding programs

Following the output of offspring genotypes for each replicate, the inbreeding coefficient (F_{is}), number of alleles per locus (A) and allelic richness (A_r) were all calculated by the simulation model, with the inclusion of the gstudio (Dyer, 2012) package in R. A rarefaction option was chosen to calculate A_r, which standardises the sample sizes between testing populations and 999 permutations were applied. At the end of each simulation run, F_{is}, A and A_r were determined for the parents and averaged across the 100 replicates for the offspring from each spawning tank. Any significant differences between broodstock and offspring for levels of A_r were determined by two-sample Mann-Whitney U-tests in R.

5.3.7 Effective population size (N_e)

In order to determine the number of contributing parents required to reach an N_e and ΔF of more than 100 and less than 0.5% respectively, N_e and ΔF were estimated for each offspring cohort produced from ten replicates for five base population sizes; 150, 180, 200, 230 and 250. The base populations were developed by selecting an even number of wild samples from the two wild genetic stocks and central region of admixture (Appendix 5B.1 and 5B.3). One hundred offspring from each spawning tank were combined (n = 500) and the variance of parental contribution was determined using CERVUS (Kalinowski et al., 2007). This procedure was conducted for each of the 10 replicates and averaged across replicates.

A linear model with standard error (SE) was applied to estimations of N_e in R. The calculation of N_e accounted for variance in parental contribution and unequal sex ratios among contributing parents using the following equations;

$$N_{ed} = (N_d K_d - 1)/[K_d - 1 + (V_d/K_d)] \ and \ N_{es} = (N_s K_s - 1)/[K_s - 1 + (V_s/K_s)]$$

$$N_e = 4N_{ed}N_{es}/(N_{ed} + N_{es})$$

where N_{ed} and N_{es} were the effective number of dams and sires respectively, K_d and K_s were the mean number of offspring per dam and sire respectively, and V_d and V_s were the variance in contribution for dams and sires (Frankham et al., 2002). The rate of inbreeding (ΔF) was estimated from N_e according to Falconer (1996) as

$$\Delta F = 1/2(N_e)$$

In addition, the N_e / N_c ratio was estimated.

5.4 Results

5.4 .1 Genetic diversity

The results from the simulation runs are shown in Table 5.1 and in every case there was a loss of A and A_r from broodstock to offspring, and only for the Cmk_r option was the result non-significant ($P = 0.09$). The loss of A_r from broodstock to offspring was inversely proportional to the level of A_r in the parents and was related to how A_r was distributed among the parents. Broodstock and offspring under the WSA_r option showed the highest A_r (mean ± standard error of 5.21 ± 0.08 and 4.75 ± 0.07, respectively). Options WSA_r and WA_r showed the largest reductions in A_r from broodstock to offspring (-0.46 and -0.47 respectively). Besides *C1*, the Cmk_r option maintained the greatest level of A_r from broodstock (5.05 ± 0.11) to offspring (4.69 ±

0.10), where the base population was selected according to the lowest levels of kinship between captive individuals. Option Cmk_r using captive broodstock with the lowest mk_r, like option WSA_r, captured relatively high genetic diversity in broodstock and offspring (A_r of 5.05 and 4.69 respectively) but also resulted in the highest standard errors in A_r of all the options (SE of 0.11 and 0.10 respectively). Using a smaller number of unselected captive broodstock in the base population (*C1*) resulted in the lowest levels of A_r with 4.11 in the broodstock and 3.9 ± 0.01 for the offspring ($P < 0.01$). By enhancing the *C1* base population with the top ranking wild samples according to A_r, A_r levels increased to 4.56 ± 0.08 in the offspring. There was a significant difference in the level of A_r between offspring from *C1* and offspring from $C1WA_r$ ($P < 0.05$) when compared to the parents. An excess of heterozygotes was detected in each of the offspring populations according to F_{is}, although none were significantly different from zero.

5.4 .2 Effective population size (N_e)

The results for the estimations of N_e are presented in Table 5.2 for five base populations of alternate census sizes (N_c), 150, 180, 200, 230 and 250. For each option there were a number of broodstock that failed to contribute to the simulated spawn. For example, across the 10 replicates the average number of broodstock that did not contribute was 24 for N_c = 150, 35 for N_c = 180, 39 for N_c = 200, 52 for N_c = 230 and 57 for N_c = 250, and of those broodstock that did contribute some individuals dominated over others (e.g. the contribution of males ranged from 0.2 – 4.6% and females ranged from 0.2 – 7.0% for N_c = 150). The N_e / N ratio ranged from 0.45 – 0.51 and the level of inbreeding 0.4 – 0.7%. N_e was highest for a base population size of N_c = 250 resulting in N_e = 115 (ΔF = 0.4%) and when using a N_c

of 150 the estimated N_e was 76, which was below the recommended level of 100. According to the linear model represented in Figure 5.2, an N_c of 213 individuals would be required to achieve a N_e of 100.

5.5 Discussion

In this study, a computer simulation model was developed and applied to investigate options for establishing a base population for selective breeding, with the aim of capturing as much wild representative genetic diversity as possible and at a scale that will allow inbreeding to be limited to generally acceptable levels. The model assumed that parental contributions would vary in a similar manner to that observed by a previous study after a mass spawning trial over two nights (chapter 2; Loughnan et al., 2013). It is possible for barramundi to continually spawn for greater than two nights within the same mass spawning event (Tucker et al., 2002) and another stage of the simulation would need to be incorporated to demonstrate the respective parental contribution ratios for subsequent spawning nights. Each spawning tank was replicated 100 times and each replication selected a new contribution level of parents, which could be argued that multiple spawning nights are being represented, however, it would be more accurate to include actual contribution ratios for additional spawning nights in the simulation because it has been demonstrated that parental participation differs on alternate nights (chapter 2; Frost et al., 2006).

The simulation model demonstrated that by providing at least five spawning tanks, each with an equal sex ratio (15:15) for a total base population size of 150 individuals, it is only possible to maintain a N_e of approximately 77 and ΔF estimated at 0.7%. Ideally, to conserve genetic diversity and control inbreeding for

the longevity of a selective breeding program, it is generally considered that a N_e > 100 and ΔF < 0.5% should be maintained in the base population each generation (Fjalestad, 2005; Sonesson et al., 2005). To maintain genetic diversity and control inbreeding for future evolutionary change, a N_e of between 500 and 5000 has been estimated (Franklin and Frankham, 1998). Assuming the distribution of parental contributions used by the simulation model, a base population of 213 individuals would be required to maintain a N_e of 100. Increasing the size of the base population could be achieved by including wild individuals into a mixed base population with current captive broodstock, or by using only pure wild founders. Nonetheless, it could also be achieved from just using existing broodstock based on the mk_r values detected in this study. It may be difficult for a single hatchery to maintain a broodstock population size of 213. An alternative is to divide up the founding population between multiple hatcheries, which would help to include the entire industry in the program and also reduce the risk of stock loss when restricted to just one site. If a breeding program is going to be restricted to one site and the appropriate logistics are not available to hold the desired amount of adults, then the best recommendation is to develop as large as possible founding population from a broad ranging genetic base of unrelated individuals.

There was no significant reduction in A_r from broodstock to offspring and A_r was maintained at a high level among offspring when captive broodstock with the lowest level of relatedness (Cmk_r) were selected as founders for the selective breeding program. When broodstock were selected evenly from two wild genetic stocks and a central region of admixture (WSA_r), this resulted in the highest level of A_r among offspring in the base population. In order to make best use of existing domesticated stock, capture and conserve high A_r and limit the kinship between

founding individuals. The results from this study suggest that a combination of captive stock selected according to the lowest levels of mk_r and wild broodstock collected from regions of high A_r across the two genetic stocks and central region of admixture could be combined to form a genetically diverse base population of unrelated individuals. However, due to the alternate methods in developing the synthetic base populations, a direct comparison between the options cannot be compared.

In order to select the best candidates across the natural range of barramundi, no biosecurity restrictions were placed on transporting fish between genetic stocks for the simulation model. If translocation restrictions were to be enforced, it would be simple to adjust the model and only select candidates from the genetic stock of interest. The Australian barramundi industry has on hand a large number of mature broodstock from a diverse range of ancestral lineages that demonstrate low levels of relatedness (chapter 3). If the development of a selective breeding program was to have access to all current barramundi broodstock under production, as inferred in this study, then a N_e >100 and a low level of inbreeding could be achieved without including any wild individuals. However, a selective breeding plan would also have to contend with the protandrous life history of barramundi. Under selective breeding it would be difficult to mate current generation males with current generation females because all barramundi are born male, later changing to female (Macbeth et al., 2002). Suggestions have been made to overcome the implication of protandry in a selective breeding program, such as mating current generation males with previous generation females (Macbeth and Palmer, 2011; Robinson et al., 2010) or utilising a manual technique of strip spawning and the cryopreservation of male milt (Leung, 1987; Palmer et al., 1993). These techniques would give greater control over the

level of relatedness between parent-pairs within the base population and the level of inbreeding each generation. Other implications to the development of a selective breeding program are translocation and biosecurity issues and the high costs involved, due to the broad distribution range of both wild and captive barramundi across two genetic stocks and a region of genetic admixture.

In chapter 4, the range of genetic diversity levels were significantly different ($P < 0.05$) between the collection of wild samples ($A_r = 3.40$) and eight captive broodstock groups ($A_r = 3.15$). The wild samples were found to cover two spatially broad genetic stocks and a central region of admixture ($F_{ST} = 0.076$) that did not demonstrate high levels of structure within each stock, and the degree of gene flow throughout the sampled natural distribution range was high. The captive groups maintained a diverse range of individuals with origins from across the wild genetic stocks, so overall A_r was not considerably low within the hatcheries (ranging from 2.67 – 3.42). When captive broodstock with low mk_r (Cmk_r) were used as founding stock, A_r was maintained at higher levels ($A_r = 4.69$) than when a smaller number of founding stock were selected from one captive broodstock group only (*C1*, $A_r = 3.90$). However, the results of the simulations showed that captive broodstock groups could benefit from the inclusion of unrelated and genetically diverse wild individuals under some circumstances. A_r in the offspring was significantly less ($P < 0.01$) when only using broodstock from one hatchery to make the base population (*C1*), compared to option $C1WA_r$, which incorporated the same captive group but with the inclusion of additional wild individuals sampled from regions of high genetic diversity. By combining wild individuals with captive stock, there was an increase in levels of A_r and this could be further increased if the captive individuals were selected according to the lowest levels of mk_r.

Whilst the mk_r method for choosing broodstock candidates for the establishment of a base population proved efficient in this study, there are other methods of selection that could also be tested. Ballou and Lacy (1995) identified and tested a variety of techniques for measuring the genetic importance of individuals, including the *mk* method based on pedigrees. Other procedures included the founder importance coefficient (FIC), which standardises the genetic contribution of individuals, genome uniqueness (GU), which aims to conserve rare or unique alleles within a population and the method for the maximum avoidance of inbreeding (MAI), which maximises the effective population size. At the conclusion of testing, the *mk* method recorded the highest level of gene and allelic diversity in every replicate. The lowest inbreeding rates were estimated for the FIC strategy but FIC performed poorly in conserving gene and allelic diversity. The mk_r method of selection, utilising levels of *r* rather than pedigree data has been shown to capture a high proportion of allelic diversity in other captive finfish stocks (e.g. Doyle et al., 2001; Ortega-Villaizán et al., 2011; Sekino et al., 2004). The mk_r breeding strategy is similar to the optimal genetic contributions (OGC) method, which aims to maximise the genetic gain transferred onto the next generation, whilst restricting inbreeding (Hinrichs et al., 2006) but incorporates estimated breeding values (EBV) rather than mean kinship estimates.

In this study, both the mk_r and A_r methods for selecting the base populations maintained levels of A_r in the first generation of offspring. Over successive generations of selective breeding the continued maintenance of genetic diversity would depend on the number of breeding animals, spawning plan (number of tanks, stripping and cryopreservation) and factors affecting variability in reproduction and maturation. Ideally, the development of a base population at the commencement of a

selective breeding program would include a mixture of both captive broodstock and wild individuals, and the continuing breeding program would work towards conserving the level of genetic diversity set in the founding population into future generations. To assist in achieving this, it is necessary to improve the reliability of mating success and gain control over which fish breed with other fish. As it is desirable to close the breeding program after its establishment to the inclusion of additional stock, it is very important to ensure that a high level of genetic variability is captured when the selective breeding program is initiated. From our simulation of the base population for barramundi selective breeding, we recommend that a combination of captive stock selected according to the lowest levels of mk_r and wild broodstock collected from regions of high A_r across the two genetic stocks and region of admixture, would be the best combination to achieve a genetically diverse base population of unrelated individuals for initiating a selective breeding program for this species.

5.6 Conclusion

This study focused on a base population size of 150 individuals for the simulation model, consisting of 75 males and 75 females across five spawning tanks. A key assumption for all options was that parental contribution to the mass spawns would vary in a similar way to that observed in chapter 2 using a large mass spawning group (Loughnan et al., 2013). With a census size (N_c) of 150, the effective population size (N_e) was estimated at 76 and ΔF at 0.7%, which were outside the desired values of N_e >100 and rate of inbreeding (ΔF) < 0.5%. By increasing the N_c to 250 founding individuals of equal sex ratio, N_e was estimated at 115 and ΔF was 0.4%, which exceeded the preferred limits. For conserving genetic

diversity, selecting candidates according to mean kinship relatedness (mk_r) in captive stocks (Cmk_r) and choosing wild individuals from each of the genetic stocks (WSA_r) according to levels of allelic richness (A_r) were the best performing options. Current captive broodstock tested in this study demonstrated low mk_r values that were at appropriate levels to be used as founders. However, base populations using current captive broodstock groups would benefit by sourcing new individuals from wild regions of high genetic diversity, as this would lower mk_r values within the breeding group and result in higher A_r. The results from this study concerned the development of a base population for barramundi selective breeding and suggested additions to the model include the simulation of multiple generations and the inclusion of stock performance information. Domingos et al. (2013; 2014) utilised the offspring cohort sampled in chapter 2 and estimated heritability, genetic and phenotypic correlation between traits at harvest. Heritability estimates were as high 0.40 for growth related traits, demonstrating the amount of genetic gain that could be achieved when selecting animal's dependant on these traits. Incorporating heritability estimates into the model are possible and could help to simulate the impact of selecting for traits in future generations. The simulation model could be a valuable tool to apply to other mass spawning species under aquaculture production.

Table 5.1 Average measures of genetic diversity across 100 replicates for barramundi broodstock base populations and first generation offspring following mass spawning in five tanks. The broodstock size per spawning tank and the quantity of offspring generated per replicate (*N*), the average number of alleles per locus (*A*), mean allelic richness (A_r), mean expected (H_e) and observed (H_o) heterozygosities, and the average inbreeding coefficient (F_{is}). Values in parenthesis for the offspring are standard errors (SE) across the five spawning tanks for each option. Broodstock selected according to the lowest levels of captive mean kinship (Cmk_r), the highest levels of A_r from two wild genetic stocks and a central region of admixture (WSA_r), wild sites containing the highest levels of A_r irrespective of the genetic stock (WA_r), captive broodstock combined with wild individuals from sites of high A_r ($C1WA_r$), captive broodstock (*C1*)

(continued on next page)

Table 5.1 *(continued)*

Option	*N*	*A*	A_r	H_e	H_o	F_{is}
Cmk_r						
Broodstock	30	5.50 (0.14)	5.05 (0.11)	0.602 (0.004)	0.575 (0.011)	0.050 (0.012)
Offspring	100	5.23 (0.13)	4.69 (0.10)	0.587 (0.004)	0.611 (0.005)	-0.039 (0.003)
WSA_r						
Broodstock	30	5.85 (0.09)	5.21 (0.08)	0.566 (0.005)	0.537 (0.013)	0.049 (0.023)
Offspring	100	5.42 (0.08)	**4.75 (0.07)	0.553 (0.005)	0.578 (0.004)	-0.045 (0.003)
WA_r						
Broodstock	30	5.65 (0.07)	5.03 (0.05)	0.532 (0.003)	0.531 (0.005)	0.003 (0.007)
Offspring	100	5.21 (0.06)	**4.56 (0.04)	0.520 (0.003)	0.541 (0.003)	-0.040 (0.002)
$C1WA_r$						
Broodstock	30	5.53 (0.11)	4.98 (0.10)	0.547 (0.006)	0.521 (0.008)	0.043 (0.015)
Offspring	100	5.14 (0.10)	*4.56 (0.08)	0.534 (0.006)	0.561 (0.006)	-0.049 (0.003)
C1						
Broodstock	58	4.31	4.11	0.491	0.514	-0.047
Offspring	100	4.07 (0.01)	**3.90 (0.01)	0.485 (0.001)	0.499 (0.002)	-0.030 (0.001)

Mann-Whitney U-tests between broodstock and offspring for levels of A_r
`$^{*}$´0.05, `**´0.01

Table 5.2 Estimation of parental contribution and the effective population size (N_e) in barramundi broodstock, tested at five base population sizes selected from two wild genetic stocks and a central region of admixture (option WSA_r). The number of broodstock in the base population (N_c), the number of contributing sires (N_s) and dams (N_d) and the rate of inbreeding (ΔF). For five spawning tanks replicates 1 – 10 were combined for each base population and values in parenthesis are standard errors across the replicates

N_c	N_s	N_d	N_e	N_e / N	ΔF
150	71 (0.5)	55 (1.0)	75.6 (1.8)	0.51 (0.01)	0.007 (0.0001)
180	83 (0.9)	62 (1.1)	85.1(1.9)	0.47 (0.01)	0.006 (0.0002)
200	90 (0.7)	71 (1.1)	98.1 (2.3)	0.49 (0.01)	0.005 (0.0001)
230	101 (1.2)	77 (1.1)	104.6 (2.6)	0.45 (0.01)	0.005 (0.0002)
250	109 (0.8)	84 (1.1)	115.2 (2.9)	0.46 (0.01)	0.004 (0.0002)

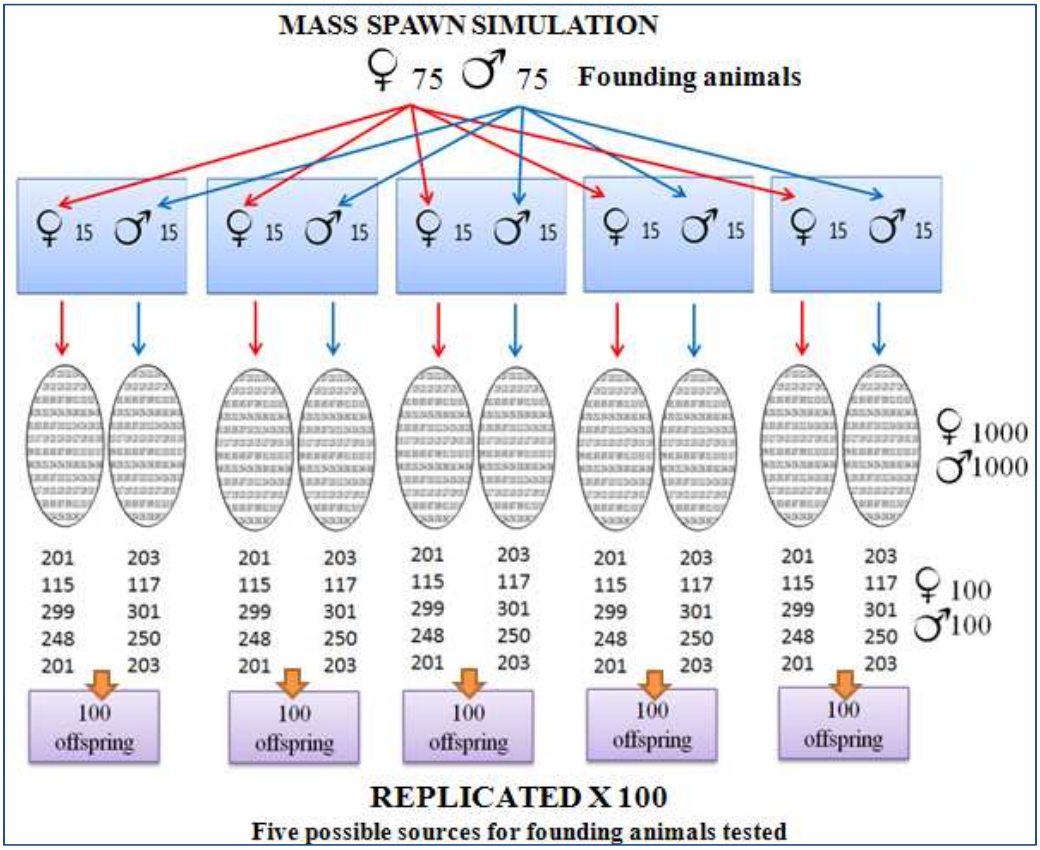


Figure 5.1. Schematic of the simulated mass spawn utilised in the model, demonstrating the selection of 150 broodstock divided into five spawning tanks of equal sex ratio, followed by the random pooling and selection of alleles to produce 100 offspring from each spawning tank.

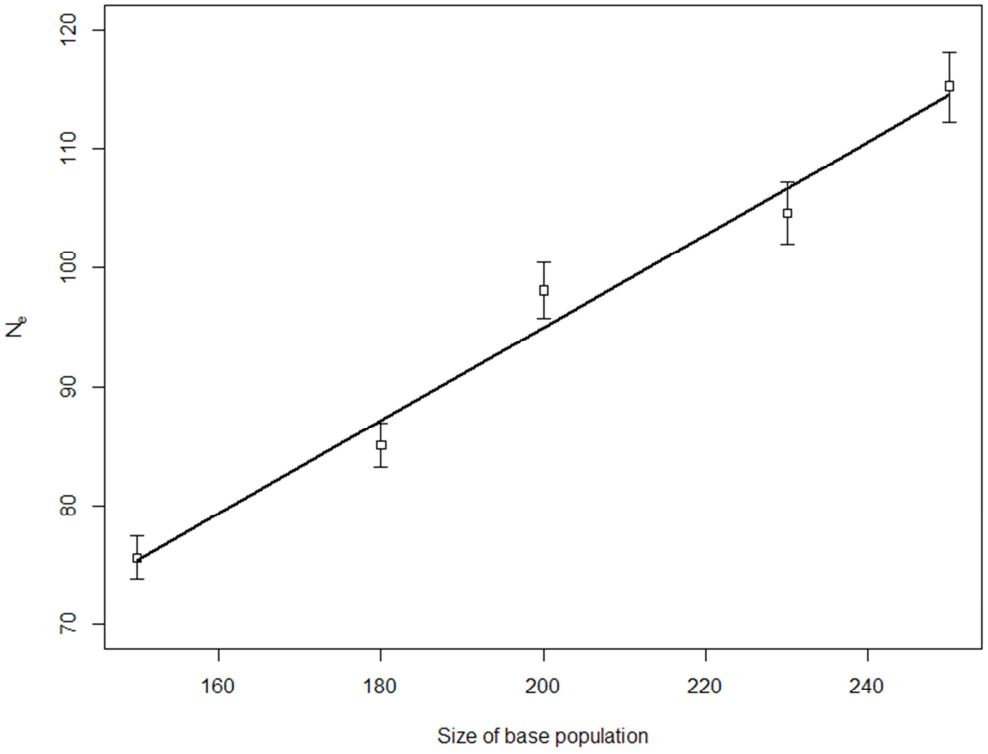


Figure 5.2 Plot of mean effective population size (N_e) with standard error (SE) across 10 replicates for five barramundi base population sizes; 150, 180, 200, 230 and 250. The base populations were selected from two wild genetic stocks and a central region of admixture (option WSA_r). The line of best fit was calculated according to a linear model resulting in y = 16.6, x = 0.392 and R^2 = 0.97.

6 General discussion

Barramundi is an ideal candidate for a selective breeding program in Australia. It is fast growing, highly fecund and well adapted to intensive aquaculture. There is high demand for fillet and live product, plus opportunities for increasing value-added products in the market. Most importantly, the Australian industry has direct access to a wide distribution range of wild stocks that can genetically enhance an already large number of mature captive broodstock under production. Current captive candidates could be selected to form a productive base population, as moderate levels of genetic diversity and relatedness were identified in chapter 3 across all hatcheries and evidence of at least two genetically differentiated stocks were detected. These two genetic stocks and a region of genetic admixture were also identified across the wild localities. However, uncaptured genetic diversity was recognised from assignment tests that could be used to supplement the genetic diversity detected among current captive stocks to benefit a selective breeding program. The Southeast Asian market for barramundi has already taken significant steps in sourcing and constructing a base population for the commencement of a selective breeding program (Yue et al., 2009). The Australian industry needs to follow suit and develop a centralised breeding program that will benefit all industry members.

This thesis has investigated the main steps in creating a productive base population from molecular genetic information, for the longevity of a selective breeding program for barramundi. Traditionally, only phenotypic information such as weight, total length and the fecundity of animals were utilised to select the best candidates for breeding, however, without pedigree data any control over inbreeding is limited. To date, molecular information has not been utilised for the development

of a base population (Goddard and Hayes, 2007), although the advantages are having greater control over inbreeding and maintaining a high genetic fitness. Ideally, a combination of both genetic and phenotypic information should be used to develop a base population, such as selecting current captive individuals according to prior spawning performance, of low relatedness and high genetic diversity. However, in order to make valid comparisons of breeding values, the animals to be compared need to be at a similar stage of development and to have experienced the same environmental conditions (e.g. derived from the same spawned batch of parents). Data like this normally becomes available after the breeding program has commenced.

It can be difficult to control inbreeding due to the high reproductive potential of many aquaculture species andcurrent mass spawning techniques are unsustainable for the conservation of genetic diversity and for the control of inbreeding. By utilising at least 33 broodstock individuals per mass spawning group, the results of chapter 2 demonstrated that a large number of half and full-sibling families can be developed. A high parental participation rate was achieved from the mass spawn although contribution levels were skewed and the variance of contribution large. A slight loss of genetic diversity was detected from broodstock to offspring, however, no further loss was recorded throughout the juvenile grow-out period, which included size grading and culling of juveniles. The high participation rate of parents was attributed to the changed dynamics of the larger spawning group utilised.

As determined by simulation, the best methods for developing a genetically diverse base population for barramundi selective breeding, was by choosing captive candidates according to the lowest mean kinship values (Cmk_r) and selecting individuals from wild regions of high genetic diversity, evenly selected across wild

genetic stocks (WSA_r). However, a direct comparison between the options could not be compared due to the different ways in developing the base populations, althoughthe Cmk_r option was determined as the best strategy tested from those developed due to the non significant loss of A_r from broodstock to offspring. In order to achieve a $N_e > 100$ and $\Delta F < 0.5\%$, a base population size of at least 213 individuals needs to be incorporated. The Australian industry has on hand suitable captive candidates to achieve the desired base population size and together with the inclusion of high quality wild individuals, founder genetic diversity can be maintained for future generations. Many commercial barramundi hatcheries are already proactive about the regular inclusion of new stock, either sourced from the wild or traded from other hatcheries. Often, pedigree records are not maintained and the selection of new individuals has been ad hoc. Southeast Asian wild barramundi stocks have demonstrated higher levels of genetic diversity than Australian stocks (Yue et al., 2009) and disregarding translocation issues the inclusion of Southeast Asian individuals would enhance the genetic fitness of a selective breeding program in Australia. However, the uncertain taxonomy of the Southeast Asian variant, particularly from Myanmar (Ward et al., 2008), may also restrict its inclusion into an Australian breeding program. Results from this thesis have helped to reconstruct pedigrees from molecular data and provided hatcheries with a direct genetic assessment of their stock on hand.

Under a selective breeding program, it is preferred to have a single breeding nucleus to manage broodstock and production, which then distribute offspring to multiplication hatcheries for grow-out. A single breeding nucleus is less costly to run, mate combinations can be arranged, pedigrees can be accurately tracked and inbreeding controlled. However, genotype by environment (G x E) interactions may

exist and if only using a single breeding nucleus, the best performing families in one production environment may not be the best performing families in another production environment (Domingos et al., 2013; Newton et al., 2010). Therefore, a single breeding program may not be able to meet the needs of the entire industry. With a centralised breeding nucleus, all industry will have access to the genetic gains achieved, although the dissemination of improved fish from a nucleus can be difficult for industry to accept due to the initial financial investment required. However, long-term production and financial gains can greatly exceed any initial trepidation.

6.1 Implications for barramundi selective breeding

1. A major implication of the results from this thesis for a barramundi selective breeding program is the space required to maintain sufficient numbers of mature broodstock in the base population. With the levels of parental contribution and skewness detected for the trial spawn undertaken in chapter 2, a large number of broodstock would be required to reach acceptable levels of N_e and ΔF (at least 213 individuals). Only a small number of Australian hatcheries under current production could maintain this quantity of broodstock, although distributing the founding population across multiple hatcheries could help to maintain a high N_e and increase industry involvement. It will be important to find a suitable site or sites with capacity to hold and spawn this large number of broodfish. The high fecundity of many aquaculture species, including barramundi, encourages some hatcheries to maintain small broodstock populations. If lower numbers (low N_e) were used in a closed breeding program, high ΔF and inbreeding depression of fitness may occur after successive generations, and loss of genetic variability may restrict the ability to make genetic gain.

2. The amount of fishing effort and pressure placed on natural stocks can be reduced if restocking programs, with a sound genetic basis are developed for barramundi. By improving understanding of the dynamics of group spawning and size grading for the prevention of cannibalism, restocking programs could benefit from the knowledge gained from this thesis and determine how to best manage spawning and size grading to limit inbreeding and loss of genetic diversity. In addition to boosting the conservation of genetic diversity in the wild through re-stocking programs based on the genetic knowledge from this thesis, any increase in captive barramundi production derived from genetic improvement can help to relieve commercial fishing pressures on wild stocks.

3. Most hatcheries sampled reported that wild broodstock were sourced from local regions, although the results from this thesis have detected a mix of genetic stocks in some hatcheries. Within wild barramundi populations, two genetic stocks (eastern and western stocks) and a region of genetic admixture were discovered, spanning central Queensland to Western Australia (Keenan, 1994; Salini and Shaklee, 1987; 1988; Shaklee et al., 1993; Shaklee and Salini, 1983; 1985). None of the hatcheries sampled actively spawn their fish for natural restocking purposes, however, all hatcheries and grow-out facilities should take precautions to prevent any accidental release of non-local captive stock into the natural environment. These precautions are needed to ensure eastern and western stocks are not mixed, as such releases could affect the natural genetic structure of wild populations. However, this thesis and Keenan (1994) have shown that gene flow within stocks is high and that there is a natural region of genetic admixture between stocks, both of which suggest that populations could quickly reach a new genetic equilibrium and that the fitness of

mixed fish stock in the natural environment is high. The central region of admixture is a combination of eastern and western stocks and any accidental release of captive fish into this region may not be detrimental to stock structure, although caution should be taken until further studies into introgression can determine the degree of impact if any. The effect of aquaculture escapees on natural stocks becomes more significant following many generations of selection in captivity, due to the change in allele frequencies, which can occur at a faster rate than in natural populations.

4. The majority of captive broodstock were assigned to the wild eastern stock (59%), followed by the western stock (23%) and central region of admixture (13%). In order to supply the maximum amount of genetic diversity available, the selective breeding program requires an equal representation from the two stocks, and the region of admixture could also be sampled because it contains genetic variation from both flanking eastern and western stocks. However, to assess the performance of pure stocks for traits of interest (i.e. rapid growth) in different environments, a diallel cross should be established and the results may favour the inclusion of a higher proportion of individuals from a specific stock. Steps need to be taken to coordinate the collection of broodstock candidates from the two wild stocks and region of admixture for inclusion into the base population for selective breeding. Within each of the two genetic stocks and region of admixture, some localities were found to contain more genetic diversity than others and emphasis should be placed on obtaining animals from these particular areas.

5. Within some hatcheries, estimated average relatedness levels were high and genetic diversity low (according to A_r). If these hatcheries continue with current practices, ΔF will increase and inbreeding depression of fitness could reduce growth

rates and lead to reduced resistance or tolerance to diseases and other stresses. Unrelated individuals from an alternate hatchery or wild stock should be injected into these broodstock groups, to increase levels of representative genetic diversity. Similarly, when constructing a base population for selective breeding, only broodstock of diverse ancestries should be included.

6. The amount of genetic testing and subsequent costs required for the construction of a diverse base population is an implication for the development of a selective breeding program. If using current captive broodstock tested in chapter 3, DNA tests to determine pedigrees have already been completed, however, it would be more than likely that additional stock would also require testing. In addition, each new generation of broodfish would also have to be DNA tested. With mass spawning and because of the variance in broodstock contribution that has been detected, a large number of offspring (1500 per mass spawn) would need to be DNA tested, in order to find sufficient representatives of each full sibling family to limit ΔF to 0.5% per generation and achieve a reasonable response to selection. Maintaining offspring in groups consisting of known family lines (if strip spawning could be used to control mating combinations) with tag identification would greatly reduce the amount of genetic testing needed and could result in greater cost efficiencies. Next generation sequencing (NGS) and SNP genotyping can produce large volumes of data, currently the methods are more expensive than traditional techniques, although it involves reduced labour and the output is greater. Applications of SNP genotyping include marker assisted selection and genetic mapping of QTLs, in many cases SNPs offer advantages over other genotyping methods in cost and efficiency. When the genome of a species is known, genomic

selection has the potential to explain all the genetic variance over the genome, although a large amount of markers and respective cost are required (Meuwissen et al., 2001). NGS can identify large quantities of SNPs and the latest technologies have reduced the cost of genotyping (Goddard and Hayes, 2007). The larger the number of markers available the less genotyping required.

7. Genetic diversity is the basis for selection and genetic improvement. By capturing high levels of genetic diversity in the founding population at the commencement of a selective breeding program and maintaining those levels, there should be greater scope for applying marker assisted selection (MAS) and making genetic improvement for traits of current and future interest. Traits such as rapid growth, flesh quality and disease resistance from infections such as betanodavirus (Hick et al., 2011) and *Streptococcus iniae* (Bromage and Owens, 2009), are important issues for the barramundi industry. Due to the high cost of feed, improving food conversion efficiency is also a trait of major priority.

8. The results from this thesis can be utilised for the management of other species under aquaculture production that have similar biology, spawning and hatchery requirements to barramundi. Mass spawning species that display high fecundity, such as Japanese flounder, *Paralichthys olivaceus* (Hara and Sekino, 2003) and gilthead seabream, *Sparus aurata* (Chavanne et al., 2012), have also shown to have highly skewed parental contributions to the next generation of offspring. Chapters 2 and 5 investigated methods into maintaining a high N_e, whilst dealing with unequal parental contribution. Other species that require size grading for the avoidance of cannibalism, such as giant grouper, *Epinephelus lanceolatus* (Hseu et al., 2004) and Asian catfish, *Pangasianodon hypophthalmus* (Baras et al.,

2010), where genetic diversity can be partitioned or lost, would also benefit from the knowledge generated in this thesis. Such as utilising a large number of broodstock to restrict the loss of genetic diversity to subsequent generations, from the techniques of size grading and culling.

6.2 Further studies

1. Future studies should involve refining methods for the genetic contribution of parents to offspring, such as experimenting with strip spawning and cryopreservation techniques, which would help to equalise parental contribution levels and direct the contribution of desired individuals. The flow on effects of this would be greater control over inbreeding, reductions in the number of broodstock that need to be maintained, a reduced need for DNA testing and the ability to mate same generation males and females to help overcome the implications caused by protandry in barramundi. Such developments would greatly reduce the costs and/or increase the rate of genetic gain (benefits) achievable from the breeding program. However, raising families separately until juvenile fish are large enough for identification tagging prior to pooling, can also be costly and requires a large amount of holding tanks. Previous studies have already modelled the development of a selective breeding program for the species that assume these issues can be overcome (Macbeth and Palmer, 2011; Robinson et al., 2010), however, further development of reproductive technologies for barramundi is required. Following hormone injections, barramundi can spawn for up to three consecutive nights (Tucker et al., 2002) and by increasing the sampling effort across all nights, the skewness of parental contributions could also be improved.

1. Protandry is a major issue for a selective breeding program because without the strip spawning and cryopreservation techniques described above, current generation stock cannot be mated together and a carefully planned breeding program incorporating overlapping generations would be required. It is also a major issue because the generation time (turn-over from one improved generation to the next) is limited by the age of female sexual maturity (around four years under most conditions). The time of sex-change and period of sexual maturation can be inconsistent, especially in captivity and methods into controlling these factors require further investigation. The process of sex-change is complex, species specific and controlled by gonadal steroids (Frisch, 2004; Guiguen et al., 1995). Manipulating the shift of gonadal steroids to induce female maturation at two years of age would greatly increase the rate of genetic improvement possible for barramundi.

2. Understanding the heritability (h^2, the total phenotypic variation that is genetic in origin) of traits is important in the development of a selective breeding program (Gjedrem, 2005). Wang et al. (2008) estimated the h^2 of growth traits in barramundi, which ranged from 0.22 – 0.25 for body weight. In addition, Domingos et al. (2013) investigated the h^2 of harvest growth traits and G x E interactions in barramundi. Average h^2 estimates for body weight ranged from 0.22 – 0.40 for fish reared in cages, intensive tanks and semi intensive ponds. No G x E interactions were detected, however, further studies into h^2 and G x E should be conducted into barramundi selected from the two genetic stocks and region of admixture identified in chapter 4.

6.3 Conclusion

Before breeding can commence, captive broodstock from populations identified in chapter 3 demonstrating high levels of allelic diversity and low levels of relatedness as compared with other captive individuals need to be gathered into a single breeding nucleus. This requires industry support from all hatcheries, funding and initial agreement on a site which is capable or needs expansion in order to hold the breeding nucleus. To enhance the fitness of captive broodstock groups, additional individuals should also be collected from wild regions of high genetic diversity identified in chapter 4.

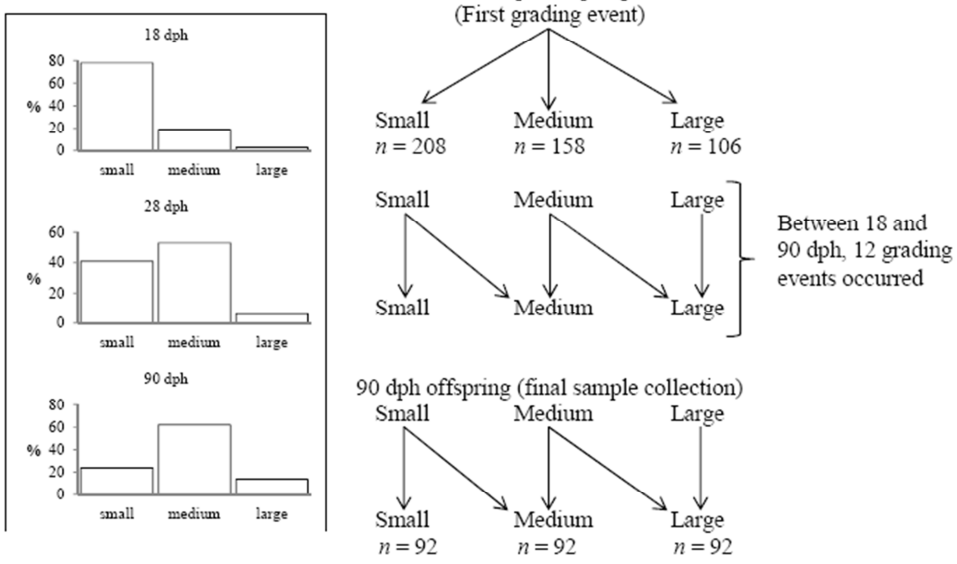
Many of the captive individuals tested did not share any recent common ancestry with any other captive broodfish, therefore it would be possible to utilise existing captive individuals in a way that totally avoids inbreeding in the initial generations of the breeding program. Although, in constructing the base population with the aim of further maximising levels of genetic diversity and reducing long-term inbreeding rates, it is recommended that a mixture of both captive bred and wild broodstock should be included. The introduction of wild individuals would increase N_e and the genetic diversity of the base population. This would allow inbreeding to be limited to lower levels and provide a broader basis for future genetic improvement. An eastern stock, western stock and a central region of admixture were identified from the barramundi wild population study, and this latter region may provide a valuable resource of broodstock for developing a productive base population. Levels of genetic diversity were similar for both the wild stocks and the captive broodstock and this may be due to sampling effects, with the repeated sampling of many different wild subpopulations as a source.

When mass spawning, it would be beneficial to monitor parental contribution over multiple spawning nights, synchronising spawning in multiple tanks, and use more than 30 broodfish per spawning group, in order to maximise the transfer of genetic variation to the next generation of broodstock candidates and reducing the skewness of parental contributions. N_e >100 and ΔF < 0.5% would be achieved by using 250 founding individuals of equal sex ratio as broodstock with each generation of breeding. Selecting candidates according to mean kinship (mk_r) in captive stocks (Cmk_r) and choosing wild individuals from each of the genetic stocks (WSA_r) according to levels of A_r would maximise the capture of genetic diversity in the founding population. Few current captive broodstock are highly related to each other and therefore there is broad scope for utilising the existing captive broodstock population as founders. Although, base populations using current captive broodstock groups would benefit by sourcing new individuals from wild regions of high genetic diversity, as this would lower mk_r values within the breeding group and result in higher A_r.

The results presented in this thesis provide valuable information regarding the origin of current barramundi broodstock under production, including the relative levels of natural genetic diversity available within these broodstock and throughout the species range. In addition, valuable information on the reproductive demographics is also presented and the maintenance of genetic diversity following mass spawning is discussed. This information will be used to develop a plan for increasing the fitness and potential of captive stocks, when establishing barramundi selective breeding programs in Australia and will serve as an example for the creation of genetic improvement programs for other species.

Appendix

Appendix 2A. Grading events and sample collections for spawn B, from the time of spawning to 90 dph. Bar charts represent the proportion of the cohort within the three size grades, on three occasions.



Appendix 2B. Allele frequencies for 17 microsatellite loci for broodstock and offspring divided into multiplex one (a) and two (b). Spawns A and B represent the first and second night of spawning respectively. The identification of sires or dams next to some allele labels indicates the detection of a private allele. Sample sizes are in parentheses, S, M and L represent the small, medium and large size grades respectively, - represents an allele not observed.

(continued on next page)

(a)		Broodstock	Spawn A	Spawn B								
Locus	Allele label	(33)	1dph (182)	1dph (274)	18dph (472)	S (208)	M (158)	L (106)	90dph (276)	S (92)	M (92)	L (92)
LcaM03	209	0.833	0.751	0.811	0.797	0.798	0.825	0.755	0.793	0.783	0.799	0.797
	212	0.167	0.249	0.189	0.203	0.202	0.175	0.245	0.207	0.217	0.201	0.203
LcaM16	(sire15) 201	0.015	0.006	0.009	0.012	0.015	0.006	0.015	0.002	-	0.005	-
	(dam12) 223	0.015	0.037	0.026	0.014	0.017	0.006	0.020	0.033	0.060	0.033	0.005
	224	0.803	0.825	0.807	0.824	0.834	0.815	0.817	0.788	0.772	0.786	0.808
	(sire06) 225	0.015	0.006	0.007	0.008	0.010	0.006	0.005	0.011	0.011	0.011	0.011
	226	0.091	0.101	0.095	0.080	0.071	0.102	0.064	0.100	0.109	0.110	0.082
	230	0.061	0.025	0.057	0.063	0.054	0.064	0.079	0.066	0.049	0.055	0.093
LcaM40	207	0.364	0.333	0.378	0.316	0.337	0.312	0.278	0.380	0.428	0.320	0.390
	208	0.242	0.241	0.220	0.241	0.259	0.237	0.212	0.221	0.200	0.291	0.171
	210	0.394	0.425	0.402	0.443	0.404	0.451	0.510	0.399	0.372	0.390	0.439
Lca57	202	0.242	0.385	0.336	0.265	0.287	0.252	0.242	0.291	0.317	0.261	0.295
	204	0.046	0.013	0.004	0.010	0.010	0.014	0.005	0.017	0.017	0.022	0.011
	205	0.561	0.363	0.500	0.505	0.518	0.469	0.530	0.467	0.494	0.484	0.420
	207	0.152	0.239	0.160	0.220	0.185	0.265	0.222	0.226	0.172	0.234	0.273
Lca154	201	0.136	0.017	0.085	0.072	0.086	0.074	0.040	0.086	0.103	0.099	0.055
	202	0.636	0.794	0.737	0.752	0.767	0.731	0.755	0.774	0.810	0.747	0.764
	204	0.197	0.160	0.105	0.112	0.088	0.125	0.140	0.095	0.071	0.099	0.115
	205	0.030	0.029	0.074	0.064	0.059	0.071	0.065	0.046	0.016	0.055	0.066
Lca178	(dam11) 202	0.030	-	-	-	-	-	-	-	-	-	-
	203	0.303	0.176	0.221	0.291	0.302	0.266	0.310	0.243	0.266	0.217	0.244
	204	0.652	0.824	0.779	0.709	0.698	0.734	0.690	0.757	0.734	0.783	0.756
	(dam10) 207	0.015	-	-	-	-	-	-	-	-	-	-
Lca287	(sire20) 201	0.015	0.015	0.029	0.005	-	0.003	0.015	0.044	0.043	0.033	0.055
	203	0.106	0.195	0.184	0.170	0.204	0.151	0.133	0.180	0.207	0.201	0.132
	(sire20) 204	0.015	-	-	-	-	-	-	-	-	-	-
	215	0.258	0.263	0.210	0.251	0.237	0.255	0.270	0.213	0.141	0.245	0.253
	216	0.470	0.509	0.511	0.508	0.464	0.537	0.551	0.500	0.505	0.478	0.516
	220	0.121	0.018	0.066	0.067	0.095	0.054	0.031	0.064	0.103	0.043	0.044
	(dam11) 221	0.015	-	-	-	-	-	-	-	-	-	-
Lca371	204	0.682	0.540	0.579	0.586	0.600	0.594	0.549	0.694	0.717	0.669	0.695
	205	0.318	0.460	0.421	0.414	0.400	0.406	0.451	0.306	0.283	0.331	0.305

(continued on next page)

(b)		Broodstock	Spawn A		Spawn B							
Locus	Allele label	(33)	1dph (182)	1dph (274)	18dph (472)	S (208)	M (158)	L (106)	90dph (276)	S (92)	M (92)	L (92)
LcaM08	(sire06) 111	0.015	-	0.006	0.004	0.010	-	-	0.009	0.006	0.005	0.017
	116	0.924	0.800	0.848	0.837	0.851	0.863	0.772	0.819	0.839	0.821	0.798
	118	0.061	0.200	0.146	0.159	0.139	0.137	0.228	0.172	0.156	0.174	0.185
LcaM20	102	0.758	0.912	0.828	0.855	0.851	0.857	0.862	0.892	0.898	0.913	0.865
	103	0.076	0.027	0.035	0.025	0.022	0.029	0.024	0.011	0.011	0.011	0.012
	(sire10) 105	0.015	0.005	0.002	0.014	0.022	0.010	0.005	0.009	0.011	-	0.018
	106	0.152	0.055	0.135	0.105	0.104	0.104	0.110	0.087	0.080	0.076	0.106
LcaM21	111	0.242	0.142	0.256	0.259	0.263	0.311	0.175	0.256	0.238	0.264	0.265
	113	0.485	0.579	0.472	0.452	0.438	0.423	0.521	0.443	0.388	0.478	0.459
	114	0.167	0.132	0.153	0.187	0.209	0.150	0.201	0.201	0.275	0.159	0.177
	116	0.030	0.132	0.074	0.044	0.045	0.042	0.046	0.055	0.063	0.038	0.065
	117	0.076	0.013	0.045	0.058	0.045	0.073	0.057	0.045	0.038	0.060	0.035
Lca58	(dam12) 105	0.015	-	-	0.007	0.012	0.004	-	0.030	0.023	0.036	0.034
	107	0.394	0.474	0.443	0.340	0.328	0.373	0.310	0.382	0.371	0.357	0.466
	109	0.061	0.105	0.037	0.062	0.076	0.052	0.051	0.045	0.045	0.043	0.052
	116	0.212	0.158	0.220	0.150	0.140	0.171	0.139	0.161	0.129	0.207	0.121
	118	0.197	0.053	0.098	0.156	0.206	0.111	0.120	0.158	0.212	0.129	0.103
	119	0.061	-	0.069	0.130	0.099	0.143	0.177	0.073	0.061	0.064	0.121
	130	0.061	0.211	0.134	0.155	0.140	0.147	0.203	0.152	0.159	0.164	0.103
Lca64	112	0.152	0.200	0.137	0.159	0.171	0.145	0.155	0.123	0.131	0.112	0.127
	113	0.106	0.082	0.112	0.093	0.101	0.095	0.073	0.093	0.101	0.090	0.089
	114	0.091	0.021	0.047	0.067	0.059	0.079	0.063	0.063	0.060	0.056	0.076
	(dam04) 117	0.015	0.132	0.078	0.039	0.040	0.030	0.053	0.058	0.065	0.067	0.038
	119	0.121	0.204	0.155	0.167	0.149	0.171	0.199	0.232	0.226	0.225	0.247
	120	0.121	0.114	0.137	0.131	0.124	0.115	0.170	0.123	0.071	0.129	0.171
	122	0.152	0.068	0.112	0.111	0.141	0.092	0.078	0.093	0.125	0.079	0.076
	126	0.242	0.179	0.222	0.233	0.215	0.273	0.209	0.214	0.220	0.242	0.177
Lca69	103	0.030	0.047	0.046	0.100	0.077	0.105	0.141	0.094	0.093	0.082	0.108
	104	0.727	0.676	0.705	0.653	0.718	0.611	0.587	0.640	0.692	0.679	0.545
	105	0.242	0.277	0.249	0.247	0.205	0.284	0.272	0.266	0.214	0.239	0.347
Lca70	103	0.030	0.031	0.013	0.004	0.003	0.003	0.010	0.004	-	0.005	0.006
	105	0.394	0.472	0.439	0.417	0.389	0.441	0.438	0.479	0.500	0.451	0.489
	106	0.530	0.491	0.524	0.571	0.606	0.546	0.538	0.511	0.494	0.538	0.500
	107	0.046	0.006	0.024	0.008	0.002	0.010	0.014	0.006	0.006	0.005	0.006
Lca74	105	0.091	0.091	0.125	0.103	0.118	0.087	0.100	0.129	0.137	0.130	0.118
	106	0.818	0.761	0.787	0.838	0.845	0.846	0.814	0.818	0.808	0.799	0.848
	120	0.091	0.148	0.088	0.058	0.037	0.067	0.086	0.053	0.055	0.071	0.034
Lca98	109	0.742	0.665	0.748	0.654	0.691	0.648	0.591	0.668	0.614	0.712	0.676
	111	0.121	0.291	0.190	0.258	0.198	0.273	0.351	0.261	0.284	0.234	0.267
	112	0.106	0.044	0.062	0.088	0.111	0.079	0.058	0.071	0.102	0.054	0.057
	(dam11) 113	0.030	-	-	-	-	-	-	-	-	-	-

Appendix 2C. Chapter 2 publication; **Aquaculture** 2013. 404 – 405, 139 – 149.

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Broodstock contribution after mass spawning and size grading in barramundi (*Lates calcarifer*, Bloch)

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ABSTRACT

Appropriately designed selective breeding programs are needed to limit the loss of genetic diversity and control levels of inbreeding, and to base selection decisions on data collected from many offspring of many families. Achieving a relatively even contribution by broodstock to subsequent generations is necessary and for many aquaculture species this is possible to control through mate pairing. Barramundi (*Lates calcarifer*) provides an exception, because it is a species that mass spawn in small groups and whose offspring are repeatedly size graded in an effort to avoid cannibalism. Following mass spawning a large broodstock group of 33 barramundi, levels of parental contribution and multiple measures of genetic diversity were estimated over the course of repeated size grading events. Parentage was inferred using 17 microsatellite DNA loci. Twelve dams and twenty-one sires were artificially spawned over two nights and sampled at 1, 18 and 90 days post hatch (dph). Broodstock contributions were skewed and the contribution by individual dams and sires was as high as 48 and 16% respectively at 1 dph. Despite the unequal contribution and high variance in family sizes, 31 broodstock were detected as contributing to the spawning events and as a result up to 103 full-sibling families were detected (18 dph, $n = 472$). A reduction in allelic richness (A_r) was identified from broodstock to offspring at 1 dph, (A_r was 3.94 among broodstock and 3.52 among offspring sampled). However, no further loss of A_r or genetic diversity was detected in the offspring from 1 to 90 dph, which included the period of metamorphosis, multiple size grading events and losses through size culling, mortalities and the sale of juveniles. The effective census population size ratio (N_e/N_c) ranged from 0.31 to 0.51 at times of sampling, (N_e was calculated between 10.1 and 16.7, well below the broodstock census size of 33) and the rate of inbreeding was less than 5%. This research provides valuable baseline data that can be used to make recommendations for the maintenance of genetic diversity and control of inbreeding for a barramundi selective breeding program. It also provides an example of what considerations need to be made for the genetic management of mass spawning and/or cannibalistic species.

1. Introduction

Understanding how genetic diversity is represented and maintained throughout the hatchery and production cycle is critical for the successful development of selective breeding programs in aquaculture. This is particularly evident for natural mass spawning species, where single pair mating cannot be conducted. Mass or group spawning (each female reproducing with many males and each male reproducing with many females randomly in a single tank) is a common method of breeding for a number of aquaculture species (e.g. Japanese flounder, *Paralichthys olivaceus* Hara and Sekino, 2003; barramundi, *Lates calcarifer* Frost et al., 2006; common sole, *Solea solea* Blonk et al., 2009; gilthead seabream, *Sparus aurata* Chavanne et al., 2012). Although this reproductive strategy can produce a large quantity of offspring and thus increase production, it can also promote heavily skewed levels of broodstock contribution and a high variance in family sizes, which can lead to a reduction in the effective population size (N_e) and an increase in the rate of inbreeding (ΔF) (Brown et al., 2005). Under captive culture, mass spawning is typically utilised for those species that naturally spawn in large congregations, although generally under this situation a limited number of sexually mature adults are utilised.

Low broodstock population sizes are typically employed for mass spawning species bred in captivity, because it is costly to maintain numerous adult fish. In addition, many species exhibit high fecundity, so that a small number of broodstock have the potential to fulfil seasonal

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production requirements (e.g. Pacific oyster, *Crassostrea gigas* Boudry et al., 2002; mangrove red snapper, *Lutjanus argentimaculatus* Emata, 2003). However, within the initial stages of a selective breeding program, it is important to select a high number of founder broodstock from diverse ancestries, to maximise genetic diversity and actively avoid mating's between animals with recent common ancestry (Gjedrem, 2005). This important step not only assists in the maintenance of genetic diversity in future generations but it also reduces the extent of inbreeding.

Barramundi, or Asian seabass (*L. calcarifer*), is a highly fecund, mass spawning catadromous species from the family Latidae, cultured mainly throughout Southeast Asia and Australia, with worldwide production increasing. As a mass spawning species, methods under captive culture involve the aggregation of conditioned, sexually mature broodstock, typically at the ratio of 1 to 2 females to 3 to 5 males (author's personal observations; Macbeth et al., 2002). Hormone induced spawning via luteinising hormone-releasing hormone analogue (LHRHa) injections and environmental manipulation, are generally necessary for final gonad maturation and to promote the release of gametes for artificial spawning (Tucker et al., 2002). Following hatching, heavy mortalities can occur among larvae during metamorphosis (Frost et al., 2006) and fingerling development phases, when intraspecific predation (cannibalism) can ensue (Parazo et al., 1991). Size grading of juvenile barramundi is used to reduce the incidence of cannibalism and produce a more uniform cohort for stocking purposes. However, grading has the ability to alter the relative contribution of broodstock to the next generation of offspring and may consequently have a negative effect on the maintenance of genetic diversity (Frost et al., 2006).

Cannibalism is not only prevalent in Latidae but has also been reported within 36 other teleost families (Smith and Reay, 1991), many involved in aquaculture production, including Serranidae (giant grouper, *Epinephelus lanceolatus* Hseu et al., 2004) and Pangasiidae (Asian catfish, *Pangasianodon hypophthalmus* Baras et al., 2010). Cannibalism typically commences in barramundi fry after they have completed metamorphosis at approximately 15–20 days post hatch (dph) (Tookwinas, 1989) and continues until offspring reach an approximate total length of 100 mm (Qin et al., 2004). During grading, juveniles are divided into independent size grades, dependant on body size and some categories may be culled to achieve a uniform size across the cohort (Macbeth et al., 2002). It is possible that the disposal of size grades (culling) may contribute to the loss of genetic diversity (Frost et al., 2006), as discarded groups or even individuals may contain unique genetic variants or distinctiveness, which are excluded from the cohort and the contribution by some broodstock may be affected. Grading has also been employed to reduce social interactions and to improve the growth rate of silver perch, *Bidyanus bidyanus* (Barki et al., 2000) and captive sole, *S. solea* (Blonk et al., 2010), and has been shown to result in the selection of animals of a particular gender when sexual dimorphism in body size occurs (e.g. Mediteranean sea bass, *Dicentrarchus labrax* Saillant et al., 2003). Molecular markers, such as microsatellite DNA, enable the reconstruction of family pedigrees to investigate the impact of size grading on broodstock contribution. They can also disclose levels of genetic variation in offspring of mass spawning species such as barramundi (Yue et al., 2002).

Microsatellites can be used to empirically reconstruct pedigrees, allowing unrelated animals to be chosen and mass spawned for breeding, so that the rate of inbreeding and loss of allelic diversity is limited with the production of each successive generation. In captive mass spawned barramundi, where no more than two dams were utilised for multiple spawns, microsatellites determined broodstock contributions as highly skewed (Frost et al., 2006). At 2 dph, Frost et al. (2006) detected the contribution of one sire as high as 77%, when three sires participated out of seven present in the tank and all dams and sires were injected with LHRHa. In an additional spawn under the same study, only three sires from a total of six were injected with LHRHa, with the contribution of one sire reaching over 60% at 2 dph. When 10 dams and 10 sires were all induced hormonally, Wang et al. (2008) recorded captive bred broodstock contributions as high as 98%, when five out of 20 broodstock contributed to the spawning. In an alternate spawning event using wild sourced broodstock that were again hormonally induced ($n = 20$), Wang et al. (2008) discovered that broodstock participation was high, with the involvement of 19 out of 20 parents, resulting in no single individual contributing greater than 36%. The level of participation and resulting contribution likely depends on broodstock weight and maturity (Brown et al., 2005) and mate competition, particularly due to the dominant behaviour of sires (Fessehaye et al., 2006; Weir et al., 2004) and the competiveness of sperm (Campton, 2004; Wedekind et al., 2007). The number of broodstock used and the quantity injected with LHRHa for artificial spawning, plus the timing of spawning are also likely to play an important role, with fertilisation more likely to occur between females and males spawning at approximately the same period of time.

Selective breeding programs for barramundi have been initiated by Yue et al. (2009) in Asia and proposed by Robinson et al. (2010) in Australia, although the natural mass spawning nature of barramundi creates some obstacles. The main complications identified by previous studies involving captive mass spawning barramundi (Frost et al., 2006; Wang et al., 2008), were the low participation rates for particular broodstock and highly skewed levels of contribution across all broodstock. Understanding broodstock contribution and the transfer of genetic diversity of captive mass spawning barramundi under artificial spawning (as opposed to natural spawning), is not only of value to the development of a successful selective breeding program for the species but also for the restocking of wild fisheries and the maintenance of local genetic variation. In this study, a large mass spawn (12 dams and 21 sires) not previously applied on this scale, was carried out to examine these issues and to determine whether spawning's on this scale in multiple tanks could be applied to benefit a selective breeding program.

2. Materials and methods

2.1. Mass spawning of broodstock

The broodstock group consisted of captive bred stock, originally developed from wild individuals collected locally from the central Queensland region of Australia. Selected broodfish were sedated in a saltwater bath containing 40 ppm AQUI-S (Aquatic Diagnostic Services International) and a small segment of caudal fin (*ca.* 1 cm^2) was removed for later DNA extraction and subsequent genotyping for pedigree determination. Fin clips were immediately stored for preservation in either 80% ethanol or DMSO-salt solution (20% DMSO, 0.25 M disodium-EDTA and NaCl to saturation at pH 8) (Seutin et al., 1991). Passive integrated transponder (PIT) tags implanted in each individual were scanned to provide a unique identification system. While sedated, all broodstock were cannulated to confirm sex with a 2.16 mm outside diameter (OD) catheter tube. Broodstock were then recovered from anaesthesia and placed back into their holding tank.

Twelve females (two of uncertain sex) and 21 males were conditioned for spawning, together in a 50,000 L fibreglass tank. The fish were fed a formulated diet (INVE Aquaculture) ad libitum, maintained at a constant water temperature of 28.5 °C and subjected to a 14 h day length for 12 weeks. To determine their readiness for spawning, female broodstock were again sedated and cannulated as described above, and oocytes were collected using a catheter and inspected under a microscope. Oocytes of a diameter of 400 μm or more were considered appropriate for successful spawning. Whilst sedated, 10 females were injected with LHRHa (Syndel International Ltd), at a dosage rate of 50 μg kg^{-1} to assist in the release of eggs. A further two females, dams 06 and 10, were in the spawning tank but were not injected (sex uncertain at the time). Males were not

induced to spawn using LHRHa, as the willingness of the females to release eggs due to hormone induction generally encourages the males to discharge sperm. Following recovery from sedation, all 10 females were released back into their spawning tank to circulate with the males and left to spawn over multiple nights. Following spawning each night, the water surface of the tank was directed into an external egg collection reservoir, where the eggs were caught in a 400 μm nylon mesh bag. The total egg count from each spawning night was determined by counting a fixed volume under the microscope in a Sedgewick-Rafter slide. The fertilisation rate (%) of the spawn was determined, by observing the level of cell division and embryo development from multiple sub-samples under the microscope. All eggs from the first and second day of spawning were then transferred to two circular fibreglass tanks (1200 L) for incubation and hatching, and although the broodstock group continued to spawn on the third and subsequent nights, no further eggs were collected.

2.2. Size grading and sampling

A random sample of whole larvae was collected at 1 dph for both the first (spawn A, $n = 182$) and second day of spawning (spawn B, $n = 274$), prior to the remaining larvae being transferred to two separate external grow out facilities at 3 dph for rearing. The 1 dph sample from spawn B was a key reference point used for many subsequent comparisons. The first grading event occurred at 18 dph, where the cohort was split into three size classes determined by the spacing of the grading device; small (<1.5 mm), medium (1.5–1.7 mm) and large (>1.7 mm) (see Appendix A). At these grading specifications, the larval rearing facility had discovered that cannibalism was effectively reduced in barramundi. Immediately following grading at 18 dph, random samples of whole larvae were collected from each size class for parentage analysis; small ($n = 208$), medium ($n = 158$) and large ($n = 106$). A similar fraction of animals from each of the size classes were sampled. During each subsequent grading event following 18 dph, the offspring were sorted within their current size classes using increasingly wider spaced graders on each subsequent occasion. In some cases larger individuals from the small and medium size grades would be promoted to the medium and large size grades respectively (Appendix A). Size grading occurred on six occasions between 18 and 42 dph, followed by another six grading events between 42 and 90 dph, although samples were only DNA tested following size grading at 18 and 90 dph. At three grading events (18, 28 and 90 dph), the total estimated cohort size in the number of juveniles was provided and a representative percentage per size grade could be calculated. A final sample collection of 92 juveniles from each size grade was conducted after the last grading at 90 dph, where the cohort was divided according to average weight (4, 8 and 16 g), but similarly labelled as small, medium and large. At 90 dph, juveniles were large enough to take fin clips. Throughout the rearing stage, fish were removed from the population in three ways; by the sale of juveniles, size culling and general losses. During the monitoring period, 91% of the cohort from spawn B was either sold as live fingerlings, or removed as the result of size culling and general mortalities.

2.3. DNA extraction

DNA was extracted from broodstock fin clips using a CTAB (cetyl trimethylammonium bromide) protocol described by Adamkewicz and Harasewych (1996), with the following modifications; polyvinylpyrrolidone (PVP) and β-mercaptoethanol were excluded from the buffer mix, as they are both generally applied to mucous laden and tannin stained samples for the removal of polyphenols present in some plants (Porebski et al., 1997). Tissue was incubated overnight at 55 °C with 10 μL of Proteinase K (20 mg mL^{-1}). Chloroform-isoamyl alcohol (24:1) was added and mixed with the digested samples, centrifuged and the upper aqueous phase transferred to tubes of cold isopropanol (600 μL) and stored in the freezer for at least 1 h. After centrifuging (16,000 *g* for 30 min), the pelleted DNA was washed with 70% cold ethanol, air dried and resuspended in 50 μL of 1× TE. All isolated DNA from CTAB extractions were quantified with a spectrophotometer (Nanodrop Technologies ND-1000) and visualised on a 0.8% agarose gel.

Whole larval samples collected at 1 and 18 dph, and small segments of fin clips (*ca.* 2 mm^2) taken at 90 dph, were all individually transferred into 96 well plates and DNA extracted in plate format by a modified Tween®-20 procedure, specifically developed for small tissue samples and larval DNA extraction (Taris et al., 2005). 100 μL of Tween®-20 lysate buffer (670 mM Tris–HCl pH 8.0, 166 mM Ammonium sulphate, 0.2% v/v Tween-20®, 0.2% v/v IGEPAL® CA-630 NP-40) and 5 μL of 20 mg mL^{-1} Proteinase K were added to each sample and digested for a minimum of 4 h at 55 °C. The samples were then incubated at 95 °C for 20 min to denature the Proteinase K, 100 μL of 1× TE buffer was then added and the samples stored at −20 °C overnight prior to PCR.

2.4. Batch sampling to discriminate non-contributors from low frequency contributors

Extra batches of eggs and whole larvae from each night of spawning at 1 dph were pooled directly prior to DNA extraction. Testing of these pooled egg/larvae samples was used to supplement the testing of individual larvae, as a cost effective approach to assist in the detection of particular broodstock that contributed at a low frequency (undetected due to sampling error), or not at all to the batches. One batch of unhatched eggs and one of 1 dph larvae, each containing approximately 200 eggs or larvae per tube were collected from both spawns A and B (4 tubes in total). DNA extractions were performed on each tube as a single extraction (using the CTAB protocol described in Section 2.3), combining all 200 samples per batch, with a final elution of 150 μL of 1× TE buffer. To assist in differentiating between alleles and stutter bands in the electropherograms and differential amplification in the pooled samples, the correction method developed by Kirov et al. (2000) was followed. For a minimum of four individuals that were not added to the pools, the peak heights of stutter patterns were measured using MegaBACE® Fragment Profiler® software, resulting in an average peak height for each stutter band (calculated in Excel, Microsoft Office). Under the correction method, all allele peak heights were reduced (excluding the longest and known as the first allele), some to levels that would dismiss them from being scored as a legitimate allele in the pool. To correct for differential amplification, the relative peak heights of alleles of heterozygous individuals were recorded (comparing all possible heterozygous allele combinations). The average height difference between adjacent alleles was used to calculate a relative weighting factor (W_i) for each allele (i) such that $W_i = H_a/H_i$ where H_a was the height of the longest allele and H_i was the height of the ith allele. Beginning with the second shortest allele, the corrected allele height H'_i was then calculated as $H'_i = H_i W_i$.

2.5. PCR amplification

Two multiplex groups of 17 markers were selected from published *L. calcarifer* microsatellite loci. Multiplex one included markers *LcaM03* (Yue et al., 2001), *LcaM16*, *LcaM40* (Yue et al., 2002), *Lca57* (Zhu et al., 2006a), *Lca154*, *Lca178* (Zhu et al., 2006b), *Lca287* and *Lca371* (Wang et al., 2007). Multiplex two included *LcaM08*, *LcaM20*, *LcaM21* (Yue et al., 2002), *Lca58*, *Lca64*, *Lca69*, *Lca70*, *Lca74* and *Lca98* (Zhu et al., 2006a). One primer from each pair was labelled with a fluorescent dye (HEX, TET or FAM) at the 5′ end. PCR amplification occurred in a 10 μL multiplex reaction with approximately 40 ng genomic DNA, 10x primer mix (containing between 0.10 and 0.25 μM of each forward and reverse primer for multiplex one and

0.06 to 0.20 μM for multiplex two) and 2× Type-it® PCR Master Mix (Qiagen). Samples were denatured for multiplex one at 95 °C for 5 min, followed by 10 cycles of 95 °C for 30 s, 57 °C for 90 s and 72 °C for 30 s, then 20 cycles of 95 °C for 30 s, 55 °C for 90 s and 72 °C for 30 s, followed by a final extension at 60 °C for 45 min on a C1000 Thermal Cycler (Bio-Rad). Multiplex two followed the same amplification steps as above, although the final extension consisted of 60 °C for 30 min. Following amplification, PCR products were diluted with 12 μL of water and desalted through Sephadex® 258G-50 fine filtration 259 spin columns (GE Healthcare). Desalted PCR products were visualised on a 1.5% agarose gel prior to genotyping on a MegaBACE® 1000 DNA Analysis System (GE Healthcare). MegaBACE® software Fragment Profiler® was used for fragment analysis, where alleles were allocated with an identifying label.

2.6. Statistical analysis

Following the scoring of genotypes, MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004) was used to check for scoring errors, which can be caused by allele stutter and the presence of null alleles. Parentage analysis was performed using CERVUS 3.0.3 (Kalinowski et al., 2007), to determine broodstock contribution to offspring and the total number of half (HS) and full-sibling (FS) families. Under the parentage program, broodstock allele frequencies were utilised for the simulation of parent pairs of known sex and the following parameters were utilised; the typing of 100% of loci, the allowance of a 1% error rate for scoring genotypes, the minimum number of typed loci was eight and 10,000 offspring were simulated. A strict confidence level (CI) of 95% was utilised to determine the most appropriate parent pair assigned to offspring. CERVUS was also utilised to calculate observed (H_o) and expected (H_e) heterozygosity, the number of alleles per locus (k), including the number of private alleles (k_a), where only one broodstock individual possessed that allele, which was considered rare in the population. The inbreeding coefficient (F_{is}), which measures the degree of random mating within populations, was estimated by the method of Weir and Cockerman (1984) using FSTAT 2.9.3.2 (Goudet, 2002). Significant departures from zero for F_{is} values were also calculated in FSTAT at the 0.05 level, for evidence of heterozygote deficiency or excess. Any deviation of observed from expected proportions under Hardy–Weinberg equilibrium (HWE) was calculated using GENEPOP 4.1(Rousset, 2008). P-values were estimated using a Markov chain (MC) algorithm, beginning with a dememorisation step of 10,000, followed by 20 batches of 5000 iterations per batch. The level of significance was determined following sequential Bonferroni correction (Rice, 1988). Allelic richness (A_r) within each locus was estimated with FSTAT 2.9.3.2 (Goudet, 2002), which is a measure of the number of alleles independent of sample size and incorporates a rarefaction approach (Hurlbert, 1971). The genetically effective population size (N_e) was estimated in a way that accounted for unequal sex ratio and variance in family sizes. The effect of variation in family size on the effective numbers of dams N_{ed} and sires N_{es} was calculated according to Frankham et al. (2002) as

$$N_{ed} = (N_d K_d - 1)/[K_d - 1 + (V_d/K_d)] \text{ and } N_{es} = (N_s K_s - 1)/[K_s - 1 + (V_s/K_s)] \quad (1)$$

where N_d and N_s was the number of dams and sires respectively, K_d and K_s were the mean number of offspring per dam and sire, and V_d and V_s was the variance in contribution for dams and sires. To account for an uneven sex ratio, N_e was estimated as

$$N_e = 4N_{ed}N_{es}/(N_{ed} + N_{es}) \quad (2)$$

The rate of inbreeding (ΔF) was computed according to Falconer (1989) as

$$\Delta F = 1/2(N_e) \quad (3)$$

Any significant differences in broodstock contribution levels between spawns A and B (at 1 dph), between sampling at 1, 18 and 90 dph of spawn B and between the size grades, were determined by Pearson's 2-sided chi-square-test, using the exact test option with a threshold for significance of 0.05, in IBM SPSS 20.0 following data transformation. We also calculated the Mann–Whitney test in SPSS, to detect for any significant differences between broodstock and offspring, for H_e, A_r and F_{is}. Relatedness and relationship inferences were estimated between broodstock pairs using ML-RELATE (Kalinowski et al., 2006), to determine the level of genealogical similarities within the group via a maximum likelihood approach that corrects for the presence of null alleles.

3. Results

3.1. Broodstock contribution

Parentage assignment rates were 94% (95% confidence interval) for spawn A and ranged from 98 to 99% for spawn B. Broodstock contribution levels were skewed for both dams and sires over the two nights of spawning (Figs. 1 and 2) and an equal contribution (uniformity) from all 33 broodstock would have resulted in each dam and sire contributing to the production of 8.3 and 4.8% of offspring respectively. Dam 04 was the highest contributing dam to spawns A and B at 1 dph, assigned as the most likely parent of 48 and 30% of

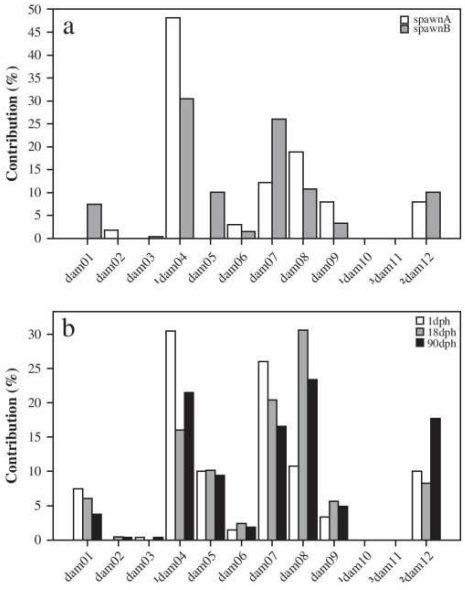

Fig. 1. Dam contribution to offspring from spawns A and B at 1 dph (a), and from spawn B over three sampling events; 1, 18 and 90 dph (b). Numbers in superscript indicate the number of private alleles detected for the specified dam.

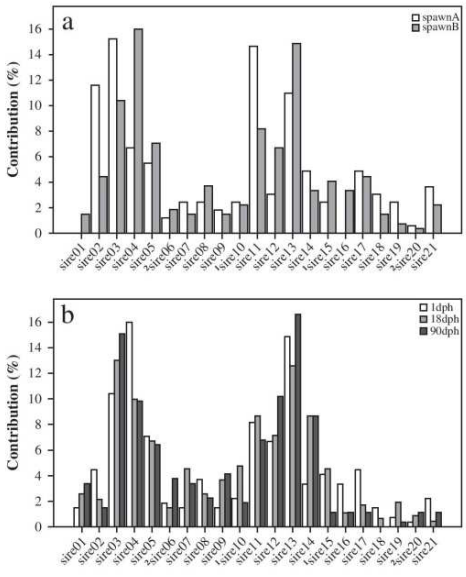

Fig. 2. Sire contribution to offspring from spawns A and B at 1 dph (a), and from spawn B over three sampling events; 1, 18 and 90 dph (b). Numbers in superscript indicate the number of private alleles detected for the specified sire.

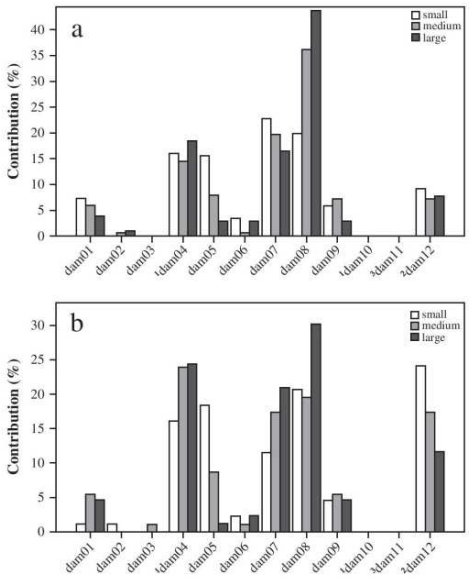

Fig. 3. Dam contribution from spawn B at 18 dph (a) and 90 dph (b) for each size grade; small, medium and large. Numbers in superscript indicate the number of private alleles detected for the specified dam.

1 dph larvae respectively (Fig. 1a). The highest contributing sires at 1 dph, were sire 03 (15%) to spawn A and sire 04 (16%) to spawn B (Fig. 2a). There was no significant difference in the level of broodstock contribution between spawns A and B at 1 dph (dams $P = 0.222$; sires $P = 0.242$). Similarly, there was no significant difference between the sampling events at 1 and 90 dph from spawn B for sires ($P = 0.117$), although there was a significant difference between the contributions of dams between 1 and 90 dph ($P < 0.05$), and also 18 and 90 dph ($P < 0.05$). Of the two dams that were not injected with LHRHa (dams 06 and 10, which were found to be dams from parentage analysis), only dam 06 was observed in the offspring from spawns A and B, although only a minor contribution was detected (<3%) across all sampling events from this individual (Fig. 1). Dams 10 and 11 were not detected at any stage in the offspring and were considered as not participating in the spawning event over two nights. Besides dams 10 and 11, only sire 18 was undetected by 90 dph (Fig. 2b).

3.1.1. Small, medium and large size grades from spawn B

By monitoring the offspring population from spawn B throughout multiple size grading events up to 90 dph, we were able to test for any impact of size grading on the contribution of broodstock to each of the size grades. Broodstock contribution levels to the size grades were skewed and significant differences in the level of contribution were detected between some of the size grades for both dams and sires (Figs. 3 and 4). At 18 dph, broodstock contribution levels were significantly different between the small and medium size grades (dams $P < 0.01$; sires $P < 0.05$), and also between the medium and large groups for dams ($P < 0.01$). At 90 dph, a significant difference was detected between the small and large size grades (dams and sires $P < 0.01$), and also between the medium and large groups

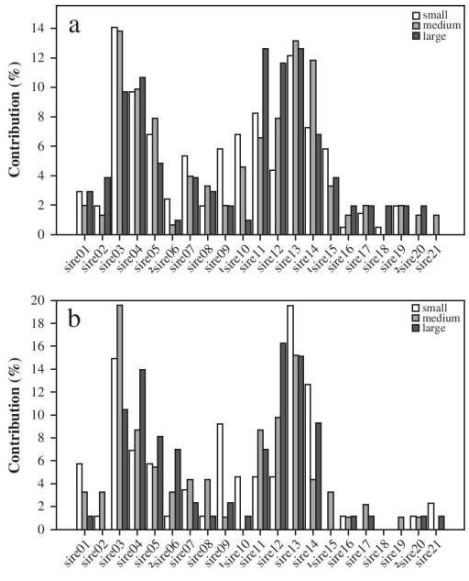

Fig. 4. Sire contribution from spawn B at 18 dph (a) and 90 dph (b) for each size grade; small, medium and large. Numbers in superscript indicate the number of private alleles detected for the specified sire.

(dams $P < 0.01$; sires $P < 0.05$). The highest contributing dam at 18 and 90 dph was dam 08 (Fig. 1b), which was also a major contributor to the size grades, ranging from 20 to 44% (Fig. 3). Sires 03 and 13 were the greatest contributors at 18 and 90 dph respectively (Fig. 2b), and were also the major contributors to each of the size grades, ranging from 10 to 20% (Fig. 4). In general, broodstock found to have a higher participation rate in the spawning events, provided relatively even contribution levels across the alternate size grades, whereas broodstock with lower participation rates had more uneven contributions across the size grades. Following grading at 18 dph, the small size grade represented 78% of the remaining population, whilst the medium and large size grades represented 19 and 3% of the population respectively (see Appendix A). At 28 dph (broodstock contribution not determined), the small, medium and large size grades were allocated 41, 53 and 6% of the remaining population respectively, and by 90 dph, the small, medium and large size grades were distributed 24, 62 and 14% respectively.

3.1.2. The production of half and full-sibling families

From a total of 10 dams (two dams were undetected) and 21 sires, the maximum number of full-sibling (*FS*) families detected was 103 at 18 dph from spawn B ($n = 472$, Table 1). The total number of *FS* families detected was dependant on sample size, as there was a considerable increase in the number of *FS* families at 18 dph when compared to 1 dph followed by a decrease at 90 dph, which was due to the quantity of samples collected (at 1 dph 78 families $n = 274$, at 18 dph 103 families $n = 472$, at 90 dph 77 families $n = 276$). As a result, the number of *FS* families detected per 100 offspring samples (FSn_{100}) was calculated at 1, 18 and 90 dph, as 28, 22 and 28, respectively. All 21 sires were detected as parents to the paternal half-sibs at 1 and 18 dph for spawn B, whilst a maximum of 10 dams were identified as parents of the maternal half-sibs (among offspring tested at 90 dph).

3.2. Genetic diversity

A total of 73 alleles (k) were recorded from the broodstock across 17 polymorphic microsatellite markers, ranging from two to eight alleles per locus and at an average of 4.3 alleles per locus (Table 2). Thirteen private alleles (K_a, an allele detected in only one broodstock individual) were detected and K_a contributed to 18% of the total number of alleles identified in the broodstock. The broodstock population conformed to Hardy–Weinberg equilibrium (HWE) over all loci, although there was a significant departure from zero for F_{is} values at two loci; *Lca*154 and *Lca*287 ($P < 0.05$), following sequential Bonferroni correction (Rice, 1988). Overall average relatedness was relatively low across the broodstock group ($r = 0.08$, maximum likelihood approach) at 95% confidence intervals, relatedness ranged from 0 to 0.35 for unrelated individuals, 0.09–0.38 for half-sibs, 0.30–0.82 for full-sibs and 0.44–0.62 for parent–offspring relationships. A high percentage of the parent pair combinations were estimated as having an unrelated relationship (83%), followed by half-sib (11%), full-sib (4%) and parent offspring (2%). Deviations from HWE and the presence of null alleles were detected in the offspring groups; at loci *Lca*287 ($P < 0.001$) for all sampling events, *Lca*371 (spawn A at 1 dph $P < 0.01$; spawn B at 1 dph $P < 0.05$) and *Lca*178 (spawn B at 1 dph $P < 0.05$).

Table 1
The number of full-sibling families (*FS*), the number of *FS* families detected per 100 offspring samples (FSn_{100}), maternal half-sibling (*Mhs*) and paternal half-sibling (*Phs*) families detected across the first (spawn A) and second night (spawn B) of spawning.

		FS	FSn_{100}	*Mhs*	*Phs*
Spawn A					
1 dph		59	32	7	19
Spawn B					
1 dph		78	28	9	21
18 dph	Total	103	22	9	21
	Small	74	36	8	19
	Medium	64	41	6	20
	Large	47	44	6	20
90 dph	Total	77	28	10	20
	Small	47	51	9	17
	Medium	47	51	9	18
	Large	42	46	8	17

Table 2
Genetic diversity estimates for 33 broodstock; sample size (N), number of alleles (k)[a], number of private alleles (k_a)[a], allelic richness (A_r), observed (H_o) and expected (H_e) heterozygosity, and the inbreeding coefficient (F_{is}).

Locus	N	k	k_a	A_r	H_o	H_e	F_{is}
LcaM03	33	2	–	2.00	0.273	0.282	0.034
LcaM08	33	3	1	2.55	0.152	0.144	−0.053
LcaM16	33	6	3	4.70	0.364	0.348	−0.046
LcaM20	33	4	1	3.57	0.455	0.403	−0.129
LcaM21	33	5	–	4.81	0.758	0.682	−0.113
LcaM40	33	3	–	3.00	0.515	0.664	0.227
Lca57	33	4	–	3.93	0.636	0.611	−0.042
Lca58	33	7	1	6.49	0.727	0.761	0.045
Lca64	33	8	1	7.57	0.909	0.859	−0.059
Lca69	33	3	–	2.82	0.394	0.418	0.058
Lca70	33	4	–	3.75	0.576	0.569	−0.012
Lca74	33	3	–	2.99	0.364	0.319	−0.143
Lca98	33	4	1	3.82	0.333	0.428	0.225
Lca154	33	4	–	3.82	0.697	0.545	−0.285*
Lca178	33	4	2	3.40	0.485	0.49	0.011
Lca287	33	7	3	5.73	0.545	0.697	0.220*
Lca371	33	2	–	2.00	0.576	0.441	−0.313
Total		73	13	3.94	0.515	0.509	−0.022

[a] Totals at k and k_a are counts, whilst the remaining totals are averages.
* Average F_{is} values significantly different from zero at the 0.05 level, following sequential Bonferroni correction for simultaneous tests (Rice, 1988).

3.2.1. Broodstock and 1 dph offspring from both spawns A and B

A loss in the number of alleles was detected when comparing 1 dph offspring to broodstock over the two nights of spawning. Eight alleles were undetected in the progeny from spawn A (Table 3), seven of those being private alleles detected in the broodstock, whilst six alleles were similarly undetected in the offspring from spawn B, which were all private alleles in the broodstock. A 15 and 11% reduction in allelic richness (A_r) from parent to offspring was detected at 1 dph, from spawns A and B respectively, however, there was no significant difference in the level of A_r between broodstock and offspring at 1 dph (spawn A $P = 0.193$ and spawn B $P = 0.339$). Over both spawning nights, expected heterozygosity (H_e) was lower in the offspring at 1 dph when compared to the broodstock population but there was no significant difference between the broodstock and offspring for H_e or F_{is} (Mann–Whitney tests). The number of broodstock that effectively contributed (N_e) to the spawn as detected at 1 dph, was 10.1 for spawn A and 13.5 for spawn B, from a broodstock census size (N_c) of 33. From these estimates of N_e, the rate of inbreeding (ΔF) was calculated at 5% and 3.7% for spawn A and B respectively at 1 dph, and the N_e /N_c ratio ranged from 0.31 to 0.46.

3.2.2. Spawn B offspring 1 dph, 18 dph and 90 dph

Due to sampling error, the frequency of alleles derived from spawn B fluctuated from 1 to 90 dph, although there was no apparent loss of

Table 3
Measures of genetic diversity; Sample size (N_c), number of alleles (k), number of private alleles (k_a), average observed (H_o) and expected (H_e) heterozygosity, allelic richness (A_r), average inbreeding coefficient (F_{is}), effective population size (N_e), rate of inbreeding (ΔF) and N_e/N_c ratio. Spawns A and B represent the first and second night of spawning respectively.

		N_c	k	k_a	H_o	H_e	A_r	F_{is}	N_e	ΔF	N_e/N_c
Broodstock		33	73	13	0.515	0.509	3.94	−0.022	–	–	–
Spawn A											
1 dph		182	65	6	0.475	0.488	3.33	0.028	10.1	0.050	0.31
Spawn B											
1 dph		274	67	7	0.500	0.493	3.52	−0.013	13.5	0.037	0.46
18 dph	Total	472	68	8	0.518	0.501	3.48	−0.041*	14.8	0.034	0.45
	Small	208	67	7	0.514	0.494	3.49	−0.048*	16.7	0.030	0.51
	Medium	158	67	7	0.502	0.498	3.45	−0.007	13.4	0.037	0.41
	Large	106	66	6	0.552	0.512	3.48	−0.087*	11.6	0.043	0.35
90 dph	Total	276	68	8	0.531	0.498	3.54	−0.071*	14.8	0.034	0.45
	Small	92	66	7	0.518	0.497	3.53	−0.049*	14.6	0.034	0.44
	Medium	92	67	7	0.531	0.495	3.55	−0.088*	15.3	0.033	0.46
	Large	92	67	7	0.546	0.499	3.55	−0.080*	12.7	0.039	0.38

* Average F_{is} values significantly different from zero at the 0.05 level, following sequential Bonferroni correction for simultaneous tests (Rice, 1988).

alleles by the final sample collection (Table 3). By 90 dph, the number of alleles including those deemed private in the broodstock actually increased when compared to 1 dph and no loss of genetic diversity was recorded when comparing offspring across 1, 18 and 90 dph, as measured by the non significant association of A_r (Mann–Whitney tests). Average F_{is} was significantly different from zero in the offspring at both 18 and 90 dph ($P < 0.05$), except in the medium size grade at 18 dph ($P = 0.29$). Deviations from HWE were detected at locus *Lca*287 ($P < 0.001$), for each size grade sampled at 18 dph (excluding the large size grade) and 90 dph.

3.2.3. Fate of rare alleles among the offspring

In total, five out of 13 alleles that were detected as private in the broodstock (allele 113 at locus *Lca*098; alleles 202 and 207 at locus *Lca*178; alleles 204 and 221 at locus *Lca*287) were not observed at any stage in the offspring and could be considered lost to the cohort (Appendix B). These five alleles were also not detected in the offspring population at 1 dph in the pooled egg and larvae samples. One of the private alleles belonged to sire 20, which was a very low contributor (<2%) across both spawns A and B (Fig. 2). The remaining four private alleles belonged to dams 10 and 11 but neither dam contributed to the spawning events (Fig. 1). On the other hand, a high contributor such as dam 04 contributed as much as 30% to spawn B but only one private allele was observed for this individual (117 at *Lca*64), which had an allele frequency ranging from 0.030–0.132 among the offspring (Appendix B). In total, eight private alleles were detected in broodstock that were low contributors to offspring at 1 dph (<1.2%) and allele frequencies in the offspring for these eight alleles were no higher than 0.029.

4. Discussion

Broodstock contributions were skewed, although there was a high participation rate of broodstock in the spawning events, which resulted in a high number of full-sibling families. Individual broodstock contribution reached 48% and some significant differences in contribution levels between the size grades were detected. Unequal parental contribution and in some cases unequal sample size and sampling error, may have attributed to these results. Significant differences between parental contributions to the different size grades might be indicative of genetic or parental effects on early growth rate, as has been detected in other fish species such as European sea bass (Saillant et al., 2001). Contributions of up to 77% (Frost et al., 2006) and 98% (Wang et al., 2008) have been reported for individual barramundi broodstock under other mass spawning runs. Heavily skewed broodstock contribution levels have also been reported for other mass spawning aquaculture species (e.g.; Japanese flounder, *P. olivaceus* Sekino et al., 2003; common sole, *S. solea* Blonk et al., 2009; gilthead seabream, *S. aurata* Chavanne et al., 2012;). For final gonad maturation and to promote the release of gametes for artificial spawning, the application of LHRHa was not beneficial for all dams. Dam 06 was not injected with LHRHa but in some cases its contribution level was greater than other dams within the broodstock group that had been injected, and despite dam 11 being injected with LHRHa it was not detected as contributing to either spawn A or B. No sires were injected with LHRHa, however, this did not impact on the participation rate of sires, as all were detected as contributing to the spawning events.

Unequal parental contributions did cause a reduction in the number of alleles from broodstock to offspring at 1 dph, although no further associated loss of genetic variation was detected from 1 to 90 dph due to putative larval mortalities throughout the period of metamorphosis, or from the effects of size grading, culling or the removal of juveniles for sales. Average A_r ranged from 3.33 to 3.55 in the offspring, whereas A_r was estimated at 3.94 in the broodstock group. Subsequent sampling at 90 dph (spawn B) showed a slightly higher average A_r when compared to 1 dph offspring, although the result was not significant ($P = 0.876$).

The effective number of broodstock contributing to the next generation (N_e) ranged from 10.1 to 16.7 for the two spawning events ($N_c = 33$), so that ΔF ranged from 3 to 5%. The range of inbreeding values far exceeded the generally recommended average of 0.5% for a population under a captive breeding program (Sonesson et al., 2005). If mass spawning were to be used for selective breeding of barramundi, careful consideration would need to be given to the relatedness of possible mate pairs in each spawning tank. For instance, using a cost-factor on inbreeding (see Brisbane and Gibson, 1995; Wray and Goddard, 1994) and including additional broodstock groups of diverse ancestry, would assist in limiting the level of inbreeding. Additional synchronous mass spawns would also need to be performed to boost family numbers. In other mass spawning species, variance in reproductive success among dams can differ greatly from that among sires (Gold et al., 2008, 2010), although little difference was detected in this study and therefore this factor would have little influence on the overall effective population size in this case.

The differences in broodstock contribution achieved in this barramundi mass spawn compared to previous experiments by other authors (Frost et al., 2006; Wang et al., 2008), could be attributed to either differences in the nutritional conditioning and reproductive readiness of animals prior to spawning, the tank facilities used, the number of broodstock injected with LHRHa and the dosage, or the size of the spawning group. Complex behavioural cues may also lead to the stimulation of animals in the tank and could affect the success of the spawn. Another possibility is that the large number of broodstock used for the mass spawn in our study (compared to the smaller broodstock group sizes traditionally used within the industry), may have resulted in a greater and more even stimulation of the broodstock present. This could have resulted in more animals contributing to the spawning events and spawning occurring over a shorter time frame during each night, than was the case for other studies. Ultimately, to gain greater control over the production of family sizes and equalise broodstock contribution to the next generation of offspring, techniques for the collection of milt together with cryopreservation and the strip spawning of eggs should be investigated.

Reports of strip spawning are limited for barramundi, although the techniques have been developed (Leung, 1987; Palmer et al., 1993) and utilised successfully under some situations e.g. milt collected from spermiating wild stock (Palmer et al., 1993). Cryopreservation of sperm along with strip spawning of both males and females would be beneficial, as it would allow for tighter control over inbreeding and could eliminate the need for DNA testing. It may also overcome the main problem caused by protandry in barramundi, enabling the selection of broodstock candidates from the same generation to be mated. All barramundi are born as males, later changing to females at approximately 3–4 years of age in captivity, although the time of sexual inversion appears to be highly variable (Macbeth et al., 2002). Selective breeding programs for barramundi utilising strip spawning and cryopreservation have been modelled and the use of these techniques would result in higher long-term benefit–cost ratios, compared to using mass spawning (Macbeth and Palmer, 2011; Robinson et al., 2010).

By pooling eggs and larvae, and DNA extracting as a batch, we were able to detect less frequent contributions to the spawns that may have otherwise been missed due to sampling error. Broodstock private alleles that were missing in the individual genotypes also went undetected in the pools, indicating that not all broodstock alleles were transferred to the offspring. Overall, the raw electropherogram patterns from the pooled genotypes helped to distinguish low contributors from non-contributors, although under the correction method for stutter many alleles were eliminated from the pools. Relative allele frequencies were not estimated from the pooled genotypes and subsequent correction for differential allele amplification proved difficult, because particular eggs or larvae may contribute more DNA to the pool than other individuals. There might be some cost benefits if pooled genotypes alone could be used to study the relative level of broodstock contribution and levels of genetic diversity (Skalski et al., 2006).

The ideal situation for a genetic improvement program is to have all broodstock contributing as evenly as possible, so that fewer offspring need to be reared, measured and genotyped. The pattern of broodstock contribution has been shown to have a large impact on the cost of the selective breeding program proposed for barramundi (Robinson et al., 2010). Stochastic simulation of breeding programs using mass selection, have indicated that more than 50 pairs of breeders and 30–50 progeny per parent pair need to be tested if inbreeding is to be limited to approximately 1% per generation, and to achieve a reasonable response to selection (Bentsen and Olesen, 2002). If parental contribution is reasonably even from a large broodstock group, a random selection of offspring from each year's cohort would yield animals from many different and relatively evenly represented families for testing. Of course, some families will be poorly represented and therefore it would be necessary to use a higher number of broodstock to obtain adequate numbers of breeding pairs with sufficient numbers of progeny. However, with mass spawning a factorial mating pattern is achieved (each female reproducing with many males and each male reproducing with many females), so that both maternal and paternal half-sibs are produced. This is advantageous to a selective breeding program, as it allows minimisation of possible confounding between additive genetic, maternal and paternal effects (Gjerde, 2005). For a given number of spawning tanks under a balanced factorial mating design, less broodstock can be tested than for nested mating or single pair mating designs. For the mass spawning of barramundi in this study, the main limitation was not the number of spawning tanks required but the total costs of DNA testing and this is influenced by the evenness of broodstock contribution to the spawn. For instance, if 10 separate mass spawning's were carried out, each under identical conditions to the trial spawn in this study and if we aimed to continue DNA testing until we found 30 progeny from 50 separate pairs of breeders (as recommended by Bentsen and Olesen, 2002), then from our data we would have needed to DNA test approximately 1500 offspring per mass spawn. There are various strategies that could be adopted to reduce this number, such as performing more DNA tests from the tanks where the broodstock contribution is found to be more even, however DNA testing will still be a significant cost to the breeding program under a mass spawning situation.

5. Conclusion

In summary, a large number of half and full-sibling families could be produced for selective breeding from a mass spawn involving 33 barramundi broodstock, of which 31 were detected as contributing to the offspring. In addition, by combining offspring batches from multiple broodstock groups, the number of families detected could be increased. Due to unequal contribution and high variance in family sizes, there was an initial loss of allelic richness from parent to offspring at 1 dph but there was no further reduction of genetic variation due to size grading, or through the removal of offspring by either size culling, the sale of juveniles or general mortalities. Broodstock contribution was also variable across the two nights of spawning, resulting in some differences in the combination of parent pair crosses between spawns A and B. Therefore, we recommend monitoring parental contribution over multiple spawning nights, synchronising spawning in multiple tanks, and using more than 30 broodfish per spawning group, in order to maximise the transfer of genetic variation to the next generation of broodstock candidates.

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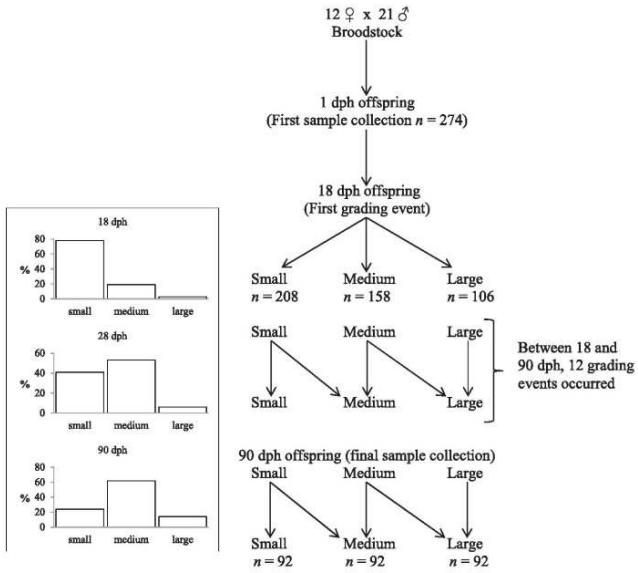
Appendix A

Grading events and sample collections for spawn B, from the time of spawning to 90 dph. Bar charts represent the proportion of the cohort within the three size grades, on three occasions.



Appendix B

Allele frequencies of 17 microsatellite loci for broodstock and offspring divided into multiplex one (a) and two (b). Spawns A and B represent the first and second night of spawning respectively. The identification of sires or dams next to some allele labels indicates the detection of a private allele. Sample sizes are in parentheses, S, M and L represent the small, medium and large size grades respectively, – represents an allele not observed.

(a)		Broodstock	Spawn A	Spawn B								
Locus	Allele label	(33)	1 dph (182)	1 dph (274)	18 dph (472)	S (208)	M (158)	L (106)	90 dph (276)	S (92)	M (92)	L (92)
LcaM03	209	0.833	0.751	0.811	0.797	0.798	0.825	0.755	0.793	0.783	0.799	0.797
	212	0.167	0.249	0.189	0.203	0.202	0.175	0.245	0.207	0.217	0.201	0.203
LcaM16	(sire15) 201	0.015	0.006	0.009	0.012	0.015	0.006	0.015	0.002	–	0.005	–
	(dam12) 223	0.015	0.037	0.026	0.014	0.017	0.006	0.020	0.033	0.060	0.033	0.005
	224	0.803	0.825	0.807	0.824	0.834	0.815	0.817	0.788	0.772	0.786	0.808
	(sire06) 225	0.015	0.006	0.007	0.008	0.010	0.006	0.005	0.011	0.011	0.011	0.011
	226	0.091	0.101	0.095	0.080	0.071	0.102	0.064	0.100	0.109	0.110	0.082
	230	0.061	0.025	0.057	0.063	0.054	0.064	0.079	0.066	0.049	0.055	0.093
LcaM40	207	0.364	0.333	0.378	0.316	0.337	0.312	0.278	0.380	0.428	0.320	0.390
	208	0.242	0.241	0.220	0.241	0.259	0.237	0.212	0.221	0.200	0.291	0.171
	210	0.394	0.425	0.402	0.443	0.404	0.451	0.510	0.399	0.372	0.390	0.439
Lca57	202	0.242	0.385	0.336	0.265	0.287	0.252	0.242	0.291	0.317	0.261	0.295
	204	0.046	0.013	0.004	0.010	0.010	0.014	0.005	0.017	0.017	0.022	0.011
	205	0.561	0.363	0.500	0.505	0.518	0.469	0.530	0.467	0.494	0.484	0.420
	207	0.152	0.239	0.160	0.220	0.185	0.265	0.222	0.226	0.172	0.234	0.273
Lca154	201	0.136	0.017	0.085	0.072	0.086	0.074	0.040	0.086	0.103	0.099	0.055
	202	0.636	0.794	0.737	0.752	0.767	0.731	0.755	0.774	0.810	0.747	0.764
	204	0.197	0.160	0.105	0.112	0.088	0.125	0.140	0.095	0.071	0.099	0.115
	205	0.030	0.029	0.074	0.064	0.059	0.071	0.065	0.046	0.016	0.055	0.066
Lca178	(dam11) 202	0.030	–	–	–	–	–	–	–	–	–	–
	203	0.303	0.176	0.221	0.291	0.302	0.266	0.310	0.243	0.266	0.217	0.244

(continued on next page)

Appendix B (*continued*)

(a)		Broodstock	Spawn A	Spawn B								
Locus	Allele label	(33)	1 dph (182)	1 dph (274)	18 dph (472)	S (208)	M (158)	L (106)	90 dph (276)	S (92)	M (92)	L (92)
	204	0.652	0.824	0.779	0.709	0.698	0.734	0.690	0.757	0.734	0.783	0.756
	(dam10) 207	0.015	–	–	–	–	–	–	–	–	–	–
Lca287	(sire20) 201	0.015	0.015	0.029	0.005	–	0.003	0.015	0.044	0.043	0.033	0.055
	203	0.106	0.195	0.184	0.170	0.204	0.151	0.133	0.180	0.207	0.201	0.132
	(sire20) 204	0.015	–	–	–	–	–	–	–	–	–	–
	215	0.258	0.263	0.210	0.251	0.237	0.255	0.270	0.213	0.141	0.245	0.253
	216	0.470	0.509	0.511	0.508	0.464	0.537	0.551	0.500	0.505	0.478	0.516
	220	0.121	0.018	0.066	0.067	0.095	0.054	0.031	0.064	0.103	0.043	0.044
	(dam11) 221	0.015	–	–	–	–	–	–	–	–	–	–
Lca371	204	0.682	0.540	0.579	0.586	0.600	0.594	0.549	0.694	0.717	0.669	0.695
	205	0.318	0.460	0.421	0.414	0.400	0.406	0.451	0.306	0.283	0.331	0.305

(b)		Broodstock	Spawn A	Spawn B								
Locus	Allele label	(33)	1 dph (182)	1 dph (274)	18 dph (472)	S (208)	M (158)	L (106)	90 dph (276)	S (92)	M (92)	L (92)
LcaM08	(sire06) 111	0.015	–	0.006	0.004	0.010	–	–	0.009	0.006	0.005	0.017
	116	0.924	0.800	0.848	0.837	0.851	0.863	0.772	0.819	0.839	0.821	0.798
	118	0.061	0.200	0.146	0.159	0.139	0.137	0.228	0.172	0.156	0.174	0.185
LcaM20	102	0.758	0.912	0.828	0.855	0.851	0.857	0.862	0.892	0.898	0.913	0.865
	103	0.076	0.027	0.035	0.025	0.022	0.029	0.024	0.011	0.011	0.011	0.012
	(sire10) 105	0.015	0.005	0.002	0.014	0.022	0.010	0.005	0.009	0.011	–	0.018
	106	0.152	0.055	0.135	0.105	0.104	0.104	0.110	0.087	0.080	0.076	0.106
LcaM21	111	0.242	0.142	0.256	0.259	0.263	0.311	0.175	0.256	0.238	0.264	0.265
	113	0.485	0.579	0.472	0.452	0.438	0.423	0.521	0.443	0.388	0.478	0.459
	114	0.167	0.132	0.153	0.187	0.209	0.150	0.201	0.201	0.275	0.159	0.177
	116	0.030	0.132	0.074	0.044	0.045	0.042	0.046	0.055	0.063	0.038	0.065
	117	0.076	0.013	0.045	0.058	0.045	0.073	0.057	0.045	0.038	0.060	0.035
Lca58	(dam12) 105	0.015	–	–	0.007	0.012	0.004	–	0.030	0.023	0.036	0.034
	107	0.394	0.474	0.443	0.340	0.328	0.373	0.310	0.382	0.371	0.357	0.466
	109	0.061	0.105	0.037	0.062	0.076	0.052	0.051	0.045	0.045	0.043	0.052
	116	0.212	0.158	0.220	0.150	0.140	0.171	0.139	0.161	0.129	0.207	0.121
	118	0.197	0.053	0.098	0.156	0.206	0.111	0.120	0.158	0.212	0.129	0.103
	119	0.061	–	0.069	0.130	0.099	0.143	0.177	0.073	0.061	0.064	0.121
	130	0.061	0.211	0.134	0.155	0.140	0.147	0.203	0.152	0.159	0.164	0.103
Lca64	112	0.152	0.200	0.137	0.159	0.171	0.145	0.155	0.123	0.131	0.112	0.127
	113	0.106	0.082	0.112	0.093	0.101	0.095	0.073	0.093	0.101	0.090	0.089
	114	0.091	0.021	0.047	0.067	0.059	0.079	0.063	0.063	0.060	0.056	0.076
	(dam04) 117	0.015	0.132	0.078	0.039	0.040	0.030	0.053	0.058	0.065	0.067	0.038
	119	0.121	0.204	0.155	0.167	0.149	0.171	0.199	0.232	0.226	0.225	0.247
	120	0.121	0.114	0.137	0.131	0.124	0.115	0.170	0.123	0.071	0.129	0.171
	122	0.152	0.068	0.112	0.111	0.141	0.092	0.078	0.093	0.125	0.079	0.076
	126	0.242	0.179	0.222	0.233	0.215	0.273	0.209	0.214	0.220	0.242	0.177
Lca69	103	0.030	0.047	0.046	0.100	0.077	0.105	0.141	0.094	0.093	0.082	0.108
	104	0.727	0.676	0.705	0.653	0.718	0.611	0.587	0.640	0.692	0.679	0.545
	105	0.242	0.277	0.249	0.247	0.205	0.284	0.272	0.266	0.214	0.239	0.347
Lca70	103	0.030	0.031	0.013	0.004	0.003	0.003	0.010	0.004	–	0.005	0.006
	105	0.394	0.472	0.439	0.417	0.389	0.441	0.438	0.479	0.500	0.451	0.489
	106	0.530	0.491	0.524	0.571	0.606	0.546	0.538	0.511	0.494	0.538	0.500
	107	0.046	0.006	0.024	0.008	0.002	0.010	0.014	0.006	0.006	0.005	0.006
Lca74	105	0.091	0.091	0.125	0.103	0.118	0.087	0.100	0.129	0.137	0.130	0.118
	106	0.818	0.761	0.787	0.838	0.845	0.846	0.814	0.818	0.808	0.799	0.848
	120	0.091	0.148	0.088	0.058	0.037	0.067	0.086	0.053	0.055	0.071	0.034
Lca98	109	0.742	0.665	0.748	0.654	0.691	0.648	0.591	0.668	0.614	0.712	0.676
	111	0.121	0.291	0.190	0.258	0.198	0.273	0.351	0.261	0.284	0.234	0.267
	112	0.106	0.044	0.062	0.088	0.111	0.079	0.058	0.071	0.102	0.054	0.057
	(dam11) 113	0.030	–	–	–	–	–	–	–	–	–	–

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Appendix 4A. Self-assignment of 1205 wild barramundi samples to two genetic stocks (western and eastern) and a central region of admixture in GENECLASS. Using the direct assignment Bayesian approach and the leave one out procedure, the overall number of individuals correctly assigned was 90%. The assignment of an individual was determined by the highest probability calculated. Emboldened numbers represent the number of individuals correctly assigned to their representative population and the last row displays the percentage of individuals correctly assigned. The remaining numbers represent misclassified individuals. Those rejected could not be assigned to any stock at the 0.05 level.

	Western	Central	Eastern	Rejected	Total
Western	**420**	41	0	4	465
Central	32	**334**	5	12	383
Eastern	2	24	**320**	11	357
Correctly assigned (%)	90%	87%	90%	$n = 27$	$n = 1205$

Appendix 5A. Simulation model of the initial mating of founder broodstock for a captive selective breeding program for barramundi, designed using R 3.0.1 programming language (R core team, 2013).

```
#set parameters#
library(gstudio)          #required package (Dyer, 2012)
setwd("E:\\")             #set the drive to read input files and write tables
nummale=15                #number of male spawners
numfem=15                 #number of female spawners
numpar=nummale+numfem   #for rarefaction
locnum=16                 # number of loci
off=100                   # number of offspring to generate
reps=100                  #number of spawn repeats
n=1                       #number of alleles to select
dams=read.table(file="filename.txt",header=F)       #female genotype input file
sires=read.table(file="filename.txt",header=F)      #male genotype input file
#start#
stats=matrix(0,reps,7)
#contribution from 12 females#
for (k in 1:reps) {
ProbDist=rep(0.372, times=100)
ProbDist=c(ProbDist,rep(0.208, times=100))
ProbDist=c(ProbDist,rep(0.139, times=100))
ProbDist=c(ProbDist,rep(0.092, times=100))
ProbDist=c(ProbDist,rep(0.062, times=100))
ProbDist=c(ProbDist,rep(0.051, times=100))
ProbDist=c(ProbDist,rep(0.046, times=100))
ProbDist=c(ProbDist,rep(0.021, times=100))
ProbDist=c(ProbDist,rep(0.007, times=100))
ProbDist=c(ProbDist,rep(0.002, times=100))
ProbDist=c(ProbDist,rep(0.000, times=100))
ProbDist=c(ProbDist,rep(0.000, times=100))
pbf=sample(ProbDist,numfem,replace=FALSE)      #contribution probability for
"numfem"
Total=sum (pbf)
pbf= pbf/Total            #sum of probability values equals 1
pbf1000=pbf*1000     #sum approximately 1000
pbf1000=round(pbf1000)      #round decimals to whole numbers
totalpbf1000=sum(pbf1000)  #total for building matrix
#create female gamete soup#
femgamsoup=matrix(0,totalpbf1000,16)      #develop matrix to store gamete soup
countgams=0                               #set the count of gametes to zero
initially
for (a in 1:numfem) {                     #runs loop for each female "a"
numgam=pbf1000[a]                  #number of gametes to generate for each
female "a"
```

```
if(numgam > 0){
for (i in 1:numgam) {                    #creates "numgam" lines of one female and 16
alleles
j=i+ countgams   #puts gametes in rows, accounting for number of gametes already
entered
z=sample(dams[a,2:3],n, replace=T)  #needed to get this as a single integer (not part
of a matrix). So pass to z first,
femgamsoup[j,1]=z[1,1]                   #then take z(1,1) and place in femgamsoup
matrix
z=sample(dams[a,4:5],n, replace=T)
femgamsoup[j,2]=z[1,1]
z=sample(dams[a,6:7],n, replace=T)
femgamsoup[j,3]=z[1,]
z=sample(dams[a,8:9],n, replace=T)
femgamsoup[j,4]=z[1,1]
z=sample(dams[a,10:11],n, replace=T)
femgamsoup[j,5]=z[1,1]
z=sample(dams[a,12:13],n, replace=T)
femgamsoup[j,6]=z[1,1]
z=sample(dams[a,14:15],n, replace=T)
femgamsoup[j,7]=z[1,1]
z=sample(dams[a,16:17],n, replace=T)
femgamsoup[j,8]=z[1,1]
z=sample(dams[a,18:19],n, replace=T)
femgamsoup[j,9]=z[1,1]
z=sample(dams[a,20:21],n, replace=T)
femgamsoup[j,10]=z[1,1]
z=sample(dams[a,22:23],n, replace=T)
femgamsoup[j,11]=z[1,1]
z=sample(dams[a,24:25],n, replace=T)
femgamsoup[j,12]=z[1,1]
z=sample(dams[a,26:27],n, replace=T)
femgamsoup[j,13]=z[1,1]
z=sample(dams[a,28:29],n, replace=T)
femgamsoup[j,14]=z[1,1]
z=sample(dams[a,30:31],n, replace=T)
femgamsoup[j,15]=z[1,1]
z=sample(dams[a,32:33],n, replace=T)
femgamsoup[j,16]=z[1,1]
}
countgams=countgams+numgam
}}
#contribution from 21 males#
ProbDist=rep(0.134, times=100)
ProbDist=c(ProbDist,rep(0.125, times=100))
ProbDist=c(ProbDist,rep(0.122, times=100))
ProbDist=c(ProbDist,rep(0.106, times=100))
ProbDist=c(ProbDist,rep(0.072, times=100))
ProbDist=c(ProbDist,rep(0.065, times=100))
```

```
ProbDist=c(ProbDist,rep(0.053, times=100))
ProbDist=c(ProbDist,rep(0.046, times=100))
ProbDist=c(ProbDist,rep(0.039, times=100))
ProbDist=c(ProbDist,rep(0.035, times=100))
ProbDist=c(ProbDist,rep(0.032, times=100))
ProbDist=c(ProbDist,rep(0.028, times=100))
ProbDist=c(ProbDist,rep(0.023, times=100))
ProbDist=c(ProbDist,rep(0.021, times=100))
ProbDist=c(ProbDist,rep(0.021, times=100))
ProbDist=c(ProbDist,rep(0.018, times=100))
ProbDist=c(ProbDist,rep(0.016, times=100))
ProbDist=c(ProbDist,rep(0.016, times=100))
ProbDist=c(ProbDist,rep(0.014, times=100))
ProbDist=c(ProbDist,rep(0.009, times=100))
ProbDist=c(ProbDist,rep(0.005, times=100))
pbm=sample(ProbDist,nummale,replace=FALSE)    #contribution probability for
"nummale"
Total=sum (pbm)
pbm= pbm/Total                #sum of probability values equals 1
pbm1000=pbm*1000              #sum approximately 1000
pbm1000=round(pbm1000)    #round decimals to whole numbers
totalpbm1000=sum(pbm1000)          #total for building matrix
#create male gamete soup#
malegamsoup=matrix(0,totalpbm1000,16)    #develop matrix to store gamete soup
countgams=0                   #set the count of gametes to zero initially
for (a in 1:nummale) {          #runs loop for each male "a"
numgam=pbm1000[a]          #number of gametes to generate for each male "a"
if(numgam > 0){
for (i in 1:numgam) {          # creates "numgam" lines of one male and 16 alleles
j=i+ countgams       #puts gametes in rows, accounting for number of gametes
already entered
z=sample(sires[a,2:3],n, replace=T)   #needed to get this as a single integer (not part
of a matrix). So pass to z first,
malegamsoup[j,1]=z[1,1]                #then take z(1,1) and place in malegamsoup
matrix
z=sample(sires[a,4:5],n, replace=T)
malegamsoup[j,2]=z[1,1]
z=sample(sires[a,6:7],n, replace=T)
malegamsoup[j,3]=z[1,]
z=sample(sires[a,8:9],n, replace=T)
malegamsoup[j,4]=z[1,1]
z=sample(sires[a,10:11],n, replace=T)
malegamsoup[j,5]=z[1,1]
z=sample(sires[a,12:13],n, replace=T)
malegamsoup[j,6]=z[1,1]
z=sample(sires[a,14:15],n, replace=T)
malegamsoup[j,7]=z[1,1]
z=sample(sires[a,16:17],n, replace=T)
malegamsoup[j,8]=z[1,1]
```

```
z=sample(sires[a,18:19],n, replace=T)
malegamsoup[j,9]=z[1,1]
z=sample(sires[a,20:21],n, replace=T)
malegamsoup[j,10]=z[1,1]
z=sample(sires[a,22:23],n, replace=T)
malegamsoup[j,11]=z[1,1]
z=sample(sires[a,24:25],n, replace=T)
malegamsoup[j,12]=z[1,1]
z=sample(sires[a,26:27],n, replace=T)
malegamsoup[j,13]=z[1,1]
z=sample(sires[a,28:29],n, replace=T)
malegamsoup[j,14]=z[1,1]
z=sample(sires[a,30:31],n, replace=T)
malegamsoup[j,15]=z[1,1]
z=sample(sires[a,32:33],n, replace=T)
malegamsoup[j,16]=z[1,1]
}
countgams=countgams+numgam
}}
#generate offspring#
#count rows in femgamsoup and malegamsoup#
dimfgs=dim(femgamsoup)
dimfgs1=dimfgs[1]
dimmgs=dim(malegamsoup)
dimmgs1=dimmgs[1]
#make vector for row numbers for each gamete soup#
vectfgs=c(1:dimfgs1)
vectmgs=c(1:dimmgs1)
offspring=matrix(0,off,33)     #offspring genotypes in rows, first column offspring
number
for (i in 1:off) {             #creates "off" lines of offspring genotypes
offspring[i,1]=i               #assigns offspring number
#pick gamete soup rows to sample#
progfem=sample(vectfgs,1, replace=FALSE)
progmale=sample(vectmgs,1, replace=FALSE)
#place female gametes in correct offspring column#
offspring[i,2]=femgamsoup[progfem,1]
offspring[i,4]=femgamsoup[progfem,2]
offspring[i,6]=femgamsoup[progfem,3]
offspring[i,8]=femgamsoup[progfem,4]
offspring[i,10]=femgamsoup[progfem,5]
offspring[i,12]=femgamsoup[progfem,6]
offspring[i,14]=femgamsoup[progfem,7]
offspring[i,16]=femgamsoup[progfem,8]
offspring[i,18]=femgamsoup[progfem,9]
offspring[i,20]=femgamsoup[progfem,10]
offspring[i,22]=femgamsoup[progfem,11]
offspring[i,24]=femgamsoup[progfem,12]
offspring[i,26]=femgamsoup[progfem,13]
```

```
offspring[i,28]=femgamsoup[progfem,14]
offspring[i,30]=femgamsoup[progfem,15]
offspring[i,32]=femgamsoup[progfem,16]
#place male gametes in correct offspring column#
offspring[i,3]=malegamsoup[progmale,1]
offspring[i,5]=malegamsoup[progmale,2]
offspring[i,7]=malegamsoup[progmale,3]
offspring[i,9]=malegamsoup[progmale,4]
offspring[i,11]=malegamsoup[progmale,5]
offspring[i,13]=malegamsoup[progmale,6]
offspring[i,15]=malegamsoup[progmale,7]
offspring[i,17]=malegamsoup[progmale,8]
offspring[i,19]=malegamsoup[progmale,9]
offspring[i,21]=malegamsoup[progmale,10]
offspring[i,23]=malegamsoup[progmale,11]
offspring[i,25]=malegamsoup[progmale,12]
offspring[i,27]=malegamsoup[progmale,13]
offspring[i,29]=malegamsoup[progmale,14]
offspring[i,31]=malegamsoup[progmale,15]
offspring[i,33]=malegamsoup[progmale,16]
}
#bring together male and female alleles and prepare in format for testing#
#using 'locus' in the loop puts the data in the correct format for testing#
loc=matrix(0,off,17)
colnames(loc)=c("ID","loc1", "loc2", "loc3", "loc4", "loc5", "loc6", "loc7",
"loc8", "loc9", "loc10", "loc11", "loc12", "loc13", "loc14", "loc15",
"loc16")
for (i in 1:off) {
loc[i,1]=i
loc[i,2]=locus(c(offspring[i,2],offspring[i,3]))
loc[i,3]=locus(c(offspring[i,4],offspring[i,5]))
loc[i,4]=locus(c(offspring[i,6],offspring[i,7]))
loc[i,5]=locus(c(offspring[i,8],offspring[i,9]))
loc[i,6]=locus(c(offspring[i,10],offspring[i,11]))
loc[i,7]=locus(c(offspring[i,12],offspring[i,13]))
loc[i,8]=locus(c(offspring[i,14],offspring[i,15]))
loc[i,9]=locus(c(offspring[i,16],offspring[i,17]))
loc[i,10]=locus(c(offspring[i,18],offspring[i,19]))
loc[i,11]=locus(c(offspring[i,20],offspring[i,21]))
loc[i,12]=locus(c(offspring[i,22],offspring[i,23]))
loc[i,13]=locus(c(offspring[i,24],offspring[i,25]))
loc[i,14]=locus(c(offspring[i,26],offspring[i,27]))
loc[i,15]=locus(c(offspring[i,28],offspring[i,29]))
loc[i,16]=locus(c(offspring[i,30],offspring[i,31]))
loc[i,17]=locus(c(offspring[i,32],offspring[i,33]))
}
#set each locus as "separated"#
loc1=locus(loc[,2],type="separated")
loc2=locus(loc[,3],type="separated")
```

```
loc3=locus(loc[,4],type="separated")
loc4=locus(loc[,5],type="separated")
loc5=locus(loc[,6],type="separated")
loc6=locus(loc[,7],type="separated")
loc7=locus(loc[,8],type="separated")
loc8=locus(loc[,9],type="separated")
loc9=locus(loc[,10],type="separated")
loc10=locus(loc[,11],type="separated")
loc11=locus(loc[,12],type="separated")
loc12=locus(loc[,13],type="separated")
loc13=locus(loc[,14],type="separated")
loc14=locus(loc[,15],type="separated")
loc15=locus(loc[,16],type="separated")
loc16=locus(loc[,17],type="separated")
#data.frame format for testing#
popn=data.frame(loc1,loc2,loc3,loc4,loc5,loc6,loc7,loc8,loc9,loc10,
loc11,loc12,loc13,loc14,loc15,loc16)
#calculate statistics for offspring#
inb=Fis(popn)                          #calculates F_is for each locus
inb=inb[!inb$Fis == "NaN",]            #removes any non numeric NaN
inb=mean(inb[,2])                      #calculates average F_is across all loci
het=He(popn)                           #calculates H_e for each locus
het=mean(het[,2])                      #calculates average H_e across all loci
hom=Ho(popn)                           #calculates H_o for each locus
hom=mean(hom[,2])                      #calculates average H_o across all loci
alls=allelic_diversity(popn, mode="A")       #number of alleles for each locus
alls=matrix(alls)
allmean=mean(alls)                     #calculates average number of alleles across all
loci
allsum=sum(alls)                       #total number of alleles
pms=999                        #number of permutations to run rarefaction for A_r
r1=rarefaction(loc1, mode = "A", size = numpar, nperm = pms)
r1=mean(r1)
r2=rarefaction(loc2, mode = "A", size = numpar, nperm = pms)
r2=mean(r2)
r3=rarefaction(loc3, mode = "A", size = numpar, nperm = pms)
r3=mean(r3)
r4=rarefaction(loc4, mode = "A", size = numpar, nperm = pms)
r4=mean(r4)
r5=rarefaction(loc5, mode = "A", size = numpar, nperm = pms)
r5=mean(r5)
r6=rarefaction(loc6, mode = "A", size = numpar, nperm = pms)
r6=mean(r6)
r7=rarefaction(loc7, mode = "A", size = numpar, nperm = pms)
r7=mean(r7)
r8=rarefaction(loc8, mode = "A", size = numpar, nperm = pms)
r8=mean(r8)
r9=rarefaction(loc9, mode = "A", size = numpar, nperm = pms)
r9=mean(r9)
```

```
r10=rarefaction(loc10, mode = "A", size = numpar, nperm = pms)
r10=mean(r10)
r11=rarefaction(loc11, mode = "A", size = numpar, nperm = pms)
r11=mean(r11)
r12=rarefaction(loc12, mode = "A", size = numpar, nperm = pms)
r12=mean(r12)
r13=rarefaction(loc13, mode = "A", size = numpar, nperm = pms)
r13=mean(r13)
r14=rarefaction(loc14, mode = "A", size = numpar, nperm = pms)
r14=mean(r14)
r15=rarefaction(loc15, mode = "A", size = numpar, nperm = pms)
r15=mean(r15)
r16=rarefaction(loc16, mode = "A", size = numpar, nperm = pms)
r16=mean(r16)
rare=data.frame(r1,r2,r3,r4,r5,r6,r7,r8,r9,r10,r11,r12,r13,r14,r15,r16)
rare=rowMeans(rare)
#collate statistics#
stats[k,1]=off          #number of offspring generated
stats[k,2]=allsum       #total number of alleles
stats[k,3]=allmean      #average number of alleles per locus
stats[k,4]=het          #average He
stats[k,5]=hom          #average Ho
stats[k,6]=inb          #average Fis
stats[k,7]=rare         #average Ar
genos=write.table(loc, file = "filename.csv", quote=FALSE, append=TRUE,
row.names=FALSE,col.names=FALSE)  #write genotypes to table if needed
}
stats_reps=write.table(stats, file = "filename.csv", append=FALSE,
quote=FALSE)                    #write each rep to table
off=mean(stats[,1])
allsum=mean(stats[,2])
allmean=mean(stats[,3])
het=mean(stats[,4])
hom=mean(stats[,5])
inb=mean(stats[,6])
rare=mean(stats[,7])
stats_avg=data.frame(off,allsum,allmean,het,hom,inb,rare)
stats_avg               #write statistics to screen
#calculate statistics for parents#
bstock=rbind(dams,sires)
parents=matrix(0,numpar,16)
for (p in 1:numpar) {
parents[p,1]=locus(c(bstock[p,2],bstock[p,3]))
parents[p,2]=locus(c(bstock[p,4],bstock[p,5]))
parents[p,3]=locus(c(bstock[p,6],bstock[p,7]))
parents[p,4]=locus(c(bstock[p,8],bstock[p,9]))
parents[p,5]=locus(c(bstock[p,10],bstock[p,11]))
parents[p,6]=locus(c(bstock[p,12],bstock[p,13]))
parents[p,7]=locus(c(bstock[p,14],bstock[p,15]))
```

```
parents[p,8]=locus(c(bstock[p,16],bstock[p,17]))
parents[p,9]=locus(c(bstock[p,18],bstock[p,19]))
parents[p,10]=locus(c(bstock[p,20],bstock[p,21]))
parents[p,11]=locus(c(bstock[p,22],bstock[p,23]))
parents[p,12]=locus(c(bstock[p,24],bstock[p,25]))
parents[p,13]=locus(c(bstock[p,26],bstock[p,27]))
parents[p,14]=locus(c(bstock[p,28],bstock[p,29]))
parents[p,15]=locus(c(bstock[p,30],bstock[p,31]))
parents[p,16]=locus(c(bstock[p,32],bstock[p,33]))
}
L1=locus(parents[,1],type="separated")
L2=locus(parents[,2],type="separated")
L3=locus(parents[,3],type="separated")
L4=locus(parents[,4],type="separated")
L5=locus(parents[,5],type="separated")
L6=locus(parents[,6],type="separated")
L7=locus(parents[,7],type="separated")
L8=locus(parents[,8],type="separated")
L9=locus(parents[,9],type="separated")
L10=locus(parents[,10],type="separated")
L11=locus(parents[,11],type="separated")
L12=locus(parents[,12],type="separated")
L13=locus(parents[,13],type="separated")
L14=locus(parents[,14],type="separated")
L15=locus(parents[,15],type="separated")
L16=locus(parents[,16],type="separated")
parents=data.frame(L1,L2,L3,L4,L5,L6,L7,L8,L9,L10,
L11,L12,L13,L14,L15,L16)          #set data.frame for testing
pinb=Fis(parents)                 #calculates F_is for each locus
pinb=pinb[!pinb$Fis == "NaN",]    #removes any non numeric NaN
pinb=mean(pinb[,2])               #average F_is across all loci
phet=He(parents)                  #calculates H_e for each locus
phet=mean(phet[,2])               #average H_e across all loci
phom=Ho(parents)                  #calculates H_o for each locus
phom=mean(phom[,2])               #average H_o across all loci
palls=allelic_diversity(parents, mode="A")  #number of alleles for each locus
palls=matrix(palls)
pallmean=mean(palls)        #calculates average number of alleles across all loci
pallsum=sum(palls)                #total number of alleles
pr1=rarefaction(L1, mode = "A", size = numpar, nperm = pms)
pr1=mean(pr1)
pr2=rarefaction(L2, mode = "A", size = numpar, nperm = pms)
pr2=mean(pr2)
pr3=rarefaction(L3, mode = "A", size = numpar, nperm = pms)
pr3=mean(pr3)
pr4=rarefaction(L4, mode = "A", size = numpar, nperm = pms)
pr4=mean(pr4)
pr5=rarefaction(L5, mode = "A", size = numpar, nperm = pms)
pr5=mean(pr5)
```

```
pr6=rarefaction(L6, mode = "A", size = numpar, nperm = pms)
pr6=mean(pr6)
pr7=rarefaction(L7, mode = "A", size = numpar, nperm = pms)
pr7=mean(pr7)
pr8=rarefaction(L8, mode = "A", size = numpar, nperm = pms)
pr8=mean(pr8)
pr9=rarefaction(L9, mode = "A", size = numpar, nperm = pms)
pr9=mean(pr9)
pr10=rarefaction(L10, mode = "A", size = numpar, nperm = pms)
pr10=mean(pr10)
pr11=rarefaction(L11, mode = "A", size = numpar, nperm = pms)
pr11=mean(pr11)
pr12=rarefaction(L12, mode = "A", size = numpar, nperm = pms)
pr12=mean(pr12)
pr13=rarefaction(L13, mode = "A", size = numpar, nperm = pms)
pr13=mean(pr13)
pr14=rarefaction(L14, mode = "A", size = numpar, nperm = pms)
pr14=mean(pr14)
pr15=rarefaction(L15, mode = "A", size = numpar, nperm = pms)
pr15=mean(pr15)
pr16=rarefaction(L16, mode = "A", size = numpar, nperm = pms)
pr16=mean(pr16)
prare=data.frame(pr1,pr2,pr3,pr4,pr5,pr6,pr7,pr8,pr9,pr10,
pr11,pr12,pr13,pr14,pr15,pr16)
prare=rowMeans(prare)
stats_parents=data.frame(numpar,pallsum,pallmean,phet,phom,
pinb,prare)
stats_parents
write.table(stats_parents, file = "filename.csv",
append=FALSE,quote=FALSE)
#finish#
```

Appendix 5B (1 – 3) Measures of genetic diversity and inbreeding from 16 microsatellite loci, shown for wild barramundi sample sites selected to represent base populations. Number of samples selected from each site (*N*), average number of alleles (*A*), mean allelic richness (A_r) and private allelic richness (PA_r), mean expected (H_e) and observed (H_o) heterozygosities, plus the average inbreeding coefficient (F_{is}). F_{is} was only significantly different from zero for Swift Bay ($P < 0.05$) and a summary of all measures is provided for the samples selected within the two genetic stocks and a region of admixture. Three states are represented; Western Australia (WA), the Northern Territory (NT) and Queensland (QLD). The tables were modified from chapter 4.

‡*N* is the total count, whilst the remaining values are averages.

*Average F_{is} values significantly different from zero at the 0.05 level, following Bonferroni correction for multiple comparisons (Rice, 1988).

kDenotes temporal samples included from Keenan (1994), collected between 1988 and 1993 unless stated in parenthesis.

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Appendix 5B.1

Wild barramundi samples selected to form the base population for option WSA_r (n = 150), which were divided into five spawning tanks (n = 30) according to sample sites with the highest levels of A_r from each of the two genetic stocks (eastern and western) and central region of admixture.

Stock location	State	Code	*N*	*A*	A_r	PA_r	H_e	H_o	F_{is}
Swift Bay	WA	SWI	10	4.6	3.64	0.008	0.552	0.515	0.098*
Berkeley River	WA	BER	10	4.8	3.57	0.010	0.536	0.537	0.018
Daly River (2008)	NT	DLY	10	4.8	3.54	0.003	0.532	0.520	0.045
Alligator River	NT	ALG	10	4.3	3.53	0.097	0.496	0.538	-0.039
Bonaparte Gulf	WA	KEE	10	4.9	3.48	0.014	0.517	0.529	-0.003
Western stock‡			50	4.7	3.55	0.027	0.527	0.528	0.005
Archer River[k] (1993)	QLD	ARCK	10	5.9	3.82	0.039	0.547	0.555	0.007
Albert River (2011)	QLD	ALB	10	5.3	3.70	0.071	0.528	0.511	0.053
Gilbert River	QLD	GIL	10	5.5	3.68	0.055	0.529	0.516	0.047
Holroyd River[k]	QLD	HOLK	10	5.1	3.68	0.049	0.531	0.536	0.016
Mitchell River[k]	QLD	MITK	10	5.0	3.67	0.008	0.527	0.541	-0.004
Central‡			50	5.4	3.71	0.044	0.532	0.532	0.024
Burdekin River (2008)	QLD	BUR	10	4.6	3.46	0.009	0.549	0.581	-0.036
Cleveland Bay	QLD	CLE	10	4.6	3.44	0.010	0.567	0.584	-0.008
Hinchinbrook	QLD	HC	10	5.6	3.43	0.044	0.555	0.565	-0.008
Mary River[k]	QLD	MARK	10	4.4	3.39	0.023	0.539	0.552	0.004
Johnstone River	QLD	JOR	10	5.1	3.36	0.015	0.533	0.570	-0.059
Eastern stock‡			50	4.9	3.42	0.020	0.549	0.570	-0.021

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Appendix 5B.2

Wild barramundi samples included in the base population for option WA_r, which were selected according to the highest levels of A_r across the entire sample range, irrespective of the genetic stock boundaries previously detected.

Stock location	State	Code	*N*	*A*	A_r	PA_r	H_e	H_o	F_{is}
Archer River[k] (1993)	QLD	ARCK	24	5.9	3.82	0.039	0.547	0.555	0.007
Archer River (2011)	QLD	ARC	33	5.8	3.66	0.021	0.525	0.528	0.009
Albert River (2011)	QLD	ALB	24	5.3	3.70	0.071	0.528	0.511	0.053
Gilbert River	QLD	GIL	24	5.5	3.68	0.055	0.529	0.516	0.047
Holroyd River[k]	QLD	HOLK	21	5.1	3.68	0.049	0.531	0.536	0.016
Mitchell River[k]	QLD	MITK	24	5.0	3.67	0.008	0.527	0.541	-0.004
Total[‡]			150	5.4	3.70	0.040	0.531	0.531	0.021

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Appendix 5B.3

Wild barramundi samples selected to form base populations of 180, 200, 230 and 250 individuals (for the N_e study), which were randomly divided into five spawning tanks of equal sex ratio. The sample sites were selected according to the highest levels of A_r from two genetic stocks (eastern and western) and central region of admixture.

Stock location	State	Code	*N*	*A*	A_r	PA_r	H_e	H_o	F_{is}
Swift Bay	WA	SWI	17	4.6	3.64	0.008	0.552	0.515	0.098*
Berkeley River	WA	BER	24	4.8	3.57	0.010	0.536	0.537	0.018
Daly River (2008)	NT	DLY	24	4.8	3.54	0.003	0.532	0.520	0.045
Daly River[k] (1990)	NT	DLYK	18	4.8	3.51	0.0078	0.549	0.524	-0.023
Western stock[‡]			83	4.8	3.57	0.007	0.542	0.524	0.013
Archer River[k] (1993)	QLD	ARCK	24	5.9	3.82	0.039	0.547	0.555	0.007
Albert River (2011)	QLD	ALB	24	5.3	3.70	0.071	0.528	0.511	0.053
Gilbert River	QLD	GIL	24	5.5	3.68	0.055	0.529	0.516	0.047
Holroyd River[k]	QLD	HOLK	11	5.1	3.68	0.049	0.531	0.536	0.016
Central[‡]			83	5.5	3.72	0.053	0.534	0.530	0.031
Cleveland Bay	QLD	CLE	47	4.6	3.44	0.010	0.567	0.584	-0.008
Hinchinbrook	QLD	HC	37	5.6	3.43	0.044	0.555	0.565	-0.008
Eastern stock[‡]			84	5.1	3.44	0.027	0.561	0.575	-0.008

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