Development of a Barramundi Selective Breeding Entity II

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Non-Technical Summary

2009/730 Development of a barramundi selective breeding entity II

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PROJECT OBJECTIVES:

- To develop a funded business entity that will run the Barramundi breeding program (BBP)
- To characterise broodstock available to the BBP and identify foundation stock
- To run a pilot scale trial of synchronized spawning to check/demonstrate feasibility of its use with the BBP
- To seek notional approval from Government agencies for the translocation of animals needed to establish the BBP and to supply farms

The first project objective was not achieved, but variation requests were approved which effectively added a final objective which was:

- To evaluate relevant data that should be collected by hatcheries and farms and to develop and establish a database that can be used to implement barramundi selective breeding

OUTCOMES ACHIEVED

Here we summarise the outcomes achieved by the project against each of the project objectives.

1 To develop a funded business entity that will run the BBP

Company structure defined, company (Barratek) registered and genetic business plan and lobbying document developed. The Barratek Board has met three times formally and a large number of times informally to progress the business model, including assessing options for funding. The model as proposed has been supported by industry and in principle by potential funders. Funding has been sought from a number of sources but at this stage no funding has been committed, but activity is ongoing beyond the completion of this CRC project. Globally tight economic times coinciding with reduced government expenditure has meant that funding via government sources at this stage appears unlikely. We are unsure when expenditure focussed on projects such as Barratek will increase. A range of other options are still being investigated.

2 To characterise broodstock available to the BBP and identify foundation stock

Samples have been collected from all farms and hatcheries who have indicated an interest in contributing broodstock. The PhD student (Shannon Loughnan) has re-extracted DNA from the wild population collection at James Cook University for the background genetic study of wild populations, and has completed the genotyping and analysis of these samples. All samples from potential broodstock in farms and hatcheries have been genotyped and analysed. Kinship relationships within and between hatchery populations have been determined. Wild genetic diversity has been found to be well represented in the existing captive broodstock population, and

the best sources of supplementary wild fish for initiating the selective breeding program have been determined (based on genetic diversity and stock structure).

3 To run a pilot scale trial of synchronized spawning to check/demonstrate feasibility of its use with the BBP

The spawning trial was completed (batches of fish were followed through the grading process until they reached 80mm). In the spawning tank were 11 females, 20 males and 2 animals of unknown sex (not canulated and not treated with hormones). Results from the parentage analysis show that on the first night 11 out of 12 females and 19 out of 21 males contributed to the spawning. On the second night 8 out of 12 females contributed and 21 out of 21 males contributed to the spawn. Overall, only one female (treated with hormones) did not contribute at all over the two consecutive nights, while all males contributed. This is a very positive result, and if the same result was replicated across 7-8 tanks when the breeding program starts, sufficient numbers of families would be produced contemporaneously by the breeding program for avoiding inbreeding depression of fitness and generating genetic improvement. Some small males were present that were only conditioned for around 2 weeks, and these males were found to contribute to the spawn (although the contribution was low, 1-2% for these newer fish, but enough for the breeding program). Of the two animals of unknown sex, both contributed to the spawn, one as a female (not treated with hormones) and one as a male.

4 To seek notional approval from Government agencies for the translocation of animals needed to establish the BBP and to supply farms

Notional approval has been received for the translocation of Barramundi across state/territory boundaries as a product of future selective breeding program activities from the Aquaculture Council, which consists of state and Territory representatives from around Australia.

5 To evaluate relevant data that should be collected by hatcheries and farms and to develop and establish a database that can be used to implement barramundi selective breeding

Traits and other factors that need recording were determined in consultation with farms and hatcheries. A database was devised and implemented and is now being used by the main barramundi hatcheries. Fact sheets advising farmers on the collection of data and use of the database have been produced and circulated among relevant hatcheries and farms.

In summary, we have made significant progress towards the establishment of the selective breeding program for barramundi by:

1. Registering a company for selective breeding of barramundi (Barratek) and producing a genetic business plan and lobbying document.
2. Identifying and characterising potential broodstock which could be used to establish the selective breeding program.
3. Demonstrating that sufficient broodstock will contribute to the spawning performed at GFB to allow for strong genetic improvement in key traits while limiting inbreeding and loss of genetic variability. Limiting loss of genetic variability and inbreeding is necessary so that the adaptability, robustness and fitness of the fish to diverse and changing environmental conditions can be maintained.
4. Obtaining notional approval from Government agencies for the translocation of animals needed to establish the BBP and supply farms.
5. Mapping existing genetic variation and making recommendations about where

to source stock to establish the base population for selective breeding
6. Devising and installing a database system and data collection processes that can be used to implement selective breeding

LIST OF OUTPUTS PRODUCED

- Business plan for Barratek
- Funding proposal/prospectus that aligns with the Barratek Business Plan
- Moreton Rye's report on commercialisation options
- PhD thesis
    - Loughnan S. (2013) Capturing and maintaining genetic diversity for the establishment of a long-term breeding program for barramundi (*Lates* calcarifer) aquaculture. PhD thesis, Flinders University.
- Article published in the journal Aquaculture on the trial spawning:
    - Loughnan S.R., Domingos J.A., Forrestor J., Smith-Keune C., Jerry D.R., Beheregary L.B., Robinson N.A. (2013). Broodstock contribution after mass spawning and size grading to prevent cannibalism in barramundi (*Lates calcarifer*, Bloch). *Aquaculture* **404**, 139-149.
- Contribution to article published with lead authorship by James Cook University:
    - Domingos J.A., Smith-Keune C., Robinson N., Loughnan S., Harrison P. and Jerry D.R. (2013) Heritability of harvest growth traits and genotype-environment interactions in barramundi *Lates calcarifer* (Bloch). *Aquaculture* **402**, 66-75.
- Manuscripts in preparation entitled:
    - Assignment of captive barramundi broodstock to wild Australian stock sources guides captive base population recruitment for selective breeding
    - Genetic diversity and relatedness estimates for captive barramundi (*Lates calcarifer*) broodstock populations informs efforts to form a base population for selective breeding
    - Comparison of the use of different source stock for establishing base populations for selective breeding of barramundi (*Lates calcarifer*).
- Database for implementing barramundi selective breeding
- Spreadsheets to assist the collection of data by farms
- Final report for project

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(Carolyn Smith-Keune and Dean Jerry). We are also grateful to Dr Ken Chapman at Good Fortune Bay Fisheries Pty Ltd (www.gfbfisheries.com) and Dr Paul Harrison at Mainstream Aquaculture Pty Ltd (www.mainstreamaquaculture.com) for assisting with the trial on mass spawning and providing access to the samples. Thanks also to all of the farms and hatcheries that contributed to the study including Challenger Tafe (Fremantle, WA), Coral Coast Mariculture (Bundaberg, QLD), Darwin Aquaculture Centre (Darwin, NT), Humpty Doo Barramundi (Humpty Doo, NT), King Reef Australia (Innisfail, QLD), Good Fortune Bay Fisheries (Bowen, QLD), JCU Aquaculture (Townsville, QLD), Jungle Creek Aquaculture (Innisfail, QLD), Mainstream Aquaculture (Werribee, VIC), Marine Produce Australia, (WA), Paradise Aquafarm Fish Hatchery (Gordonvale, QLD), PEJO Enterprises (Innisfail, QLD), Robarra Aquaculture Farm (Robe, SA) and WBA Hatcheries (West Beach, SA).

1. Introduction and Background

Australian aquaculture was worth an estimated $911.4 million in 2007/08. Barramundi aquaculture was worth $38.7 million (from 3,868 tonnes of fish produced) in 2007/08, placing barramundi as the third highest value finfish aquaculture in Australia. The Australian Barramundi Farmers Association (ABFA) estimates that current production is between 5 - 6,000 tonnes with a farm gate value of over $50 million per annum.

Barramundi was first farmed in Australia in the late 1980s and is now considered an established but still developing industry. Around 60% of Australian barramundi production is from Queensland, primarily North Queensland, where production occurs in freshwater, brackish and saltwater ponds, and raceways. The remaining 40% of production is mostly derived from the Northern Territory (utilising similar production systems to North Queensland), Northern Western Australia (using sea cages) and South Australia, Victoria, New South Wales and other states (using recirculating systems) whom produce mostly live fish for the domestic restaurant trade. Three to five million barramundi fingerlings per annum are also produced in Australia of which up to one million are stocked to fisheries (estimates derived from industry consultations and O'Sullivan and Savage (2009)). Breeding techniques for barramundi are well established and relatively routine. Larvae culture is also relatively routine.

The industry, however, is constrained in its future growth potential by the relatively high cost of production in Australia. Major factors causing the high cost of production relative to other countries are high labour costs and costs associated with environmental approvals and regulations. The current strong Australian dollar is compounding the issue, making imported barramundi ultra-competitive. Barramundi is farmed throughout South East Asia and exported to Australia. A selective breeding program for barramundi has been established in Singapore, and although it does not yet supply farms with improved stock, this program could potentially result in competitive advantages for overseas producers in the future.

The Queensland Department of Employment, Economic Development and Innovation (DEEDI) modelled the economics of barramundi production in 2008. Their model farm achieved a modest internal rate of return in the order of 13%, and noted that profit level is very sensitive to variations in yields and production times. Research co-funded by the ABFA has confirmed that genetic improvement achieved by selective breeding is the most effective means by which to achieve yield and production time improvements across the industry (Robinson et al 2010). However, to make genetic improvement, and for the industry to benefit from selective breeding, there is a need to apply sound genetic and economic principles to the management of the BBP. The intention of this project is to position the industry to begin a genetically and economically sustainable genetic improvement program for Barramundi that will provide large economic benefits in the future.

Commercialisation of selective breeding

Although the benefits from farming genetically improved stocks can be large (Gjedrem, Rye and Robinson 2012), selective breeding is an ongoing activity that requires considerable input of time and resources. Public funding sources have been used in the past to establish selective breeding programs for emerging industries. One example is that of the first modern selective breeding program for fish which was begun for Atlantic salmon in Norway (Gjedrem 2012). However, public funding, if

available at all, tends to only last for a short period of time, so all selective breeding programs need to plan to become self-sustainable operations soon after establishment. One of the difficulties with the commercialisation of selective breeding programs is that a few years of investment is required before the benefits (eg. faster growing fish) can be distributed and demonstrated to the industry and before the selective breeding entity can return a profit. Also, most breeding programs require a large up-front investment in infrastructure (tanks etc.). Like all businesses, it is crucial to have a good business plan and a strategy for attracting investment.

Broodstock availability and suitability for establishing the selective breeding program

Once a selective breeding program is established, the facility containing the breeding population (known as the breeding nucleus) is then normally closed to any further input of new animals. This is to prevent the dilution of the benefits from genetic improvement (by mating with unimproved stock, the BBP would take a backward step) and helps prevent the introduction of disease to the breeding population.

In order to make genetic improvement it is necessary to have genetic variation in the traits that are of interest to the BBP. These may be traits such as growth rate, which are currently of value to the industry, and also traits such as resistance to new diseases which might be of value in the future. It is also important when establishing the BBP that sufficient numbers of animals are used so that future inbreeding and loss of genetic diversity can be minimised. Therefore it is critical that when the BBP is established, that there is a good representation of natural genetic diversity among the founding animals and that there are sufficient numbers of founding animals used.

Inbreeding is the mating of individuals that share a common ancestor. One measure of inbreeding is “relatedness” which is the fraction of genetic material shared that is identical by descent (inherited from a common ancestor). Relatedness can be estimated by performing DNA tests. The risk of inbreeding is high in fish and shellfish if steps are not taken to avoid mating close relatives. This is because fish and shellfish have high fecundity, and many full siblings can be created from a single mating event. Inbreeding has been shown to have a number of negative effects on many fish populations:

1. Loss of genetic variance (animals become more similar which lowers the ability to make genetic improvement for any trait)
2. Inbreeding depression of fitness (increased homozygosity uncovers undesirable recessive genes that can lead to reduced survival, reproduction and growth rates). Fjalestad (2005) showed that out of 28 publications about inbreeding in fish and shellfish, 24 demonstrated inbreeding depression of between 0.9-9.3% per 10% of inbreeding.

It is normal practice to limit inbreeding to less than 0.5% per generation. This would be achieved in a randomly mating “ideal” population consisting of more than 100 broodstock.

Proof-of-concept trial spawning

Understanding how genetic diversity can be captured and maintained throughout the hatchery and production cycle is critical for the successful development of selective breeding programs in aquaculture. This is particularly important for natural mass spawning species, where single pair matings 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. gilthead seabream, *Sparus aurata* Chavanne et al., 2012; barramundi *Lates calcarifer* Frost et al., 2006), and although the technique 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 genetic drift and inbreeding depression (Brown et al., 2005; Frost et al., 2006). 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, because it is costly to maintain numerous adult fish and many species exhibit high fecundity so that a small number of broodstock have the potential to fulfil seasonal production requirements (e.g. gilthead seabream, *S. aurata* Brown et al., 2005; mangrove red snapper, *Lutjanus argentimaculatus* Emata, 2003; and Pacific oyster, *Crassostrea gigas* Boudry et al., 2002). 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 matings between animals with recent common ancestry. This important step can not only help for maintaining genetic diversity in future generations but it can also reduce the extent of inbreeding.

Barramundi, or Asian seabass (*L. calcarifer*), is a highly fecund, mass spawning catadromous species from the family Latidae, cultured throughout Southeast Asia, India, the Middle East and Australia. 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 (Macbeth et al., 2002). 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 (Palmer et al., 1993). 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 in 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 18 to 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 homogenous cohort for size (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 which are excluded from the cohort, and as the contribution by some broodstock may be affected. Grading has also been employed to reduce social interactions and to improve the growth rate of captive sole, *Solea solea* (Blonk et al., 2010) and silver perch, *Bidyanus bidyanus* (Barki et al., 2000), and has been shown to result in the selection of animals of a particular gender when sexual dimorphism in body size occurs (e.g. Mediterranean sea bass, *Dicentrarchus labrax* L., Saillant et al. 2003).

Molecular markers such as microsatellites, enable the reconstruction of family pedigrees to investigate the impact of size grading on broodstock contribution and levels of genetic variation in offspring for mass spawning species such as barramundi.

Microsatellites are highly polymorphic, co-dominant DNA markers that are abundant throughout eukaryotic genomes, including teleosts (Liu and Cordes, 2004). 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. Lind et al. (2010) incorporated microsatellites to monitor offspring survival and track relative changes in parental contributions for silver-lipped pearl oysters (*Pinctada maxima*), discovering variations in relative contribution over time and a high variance in family survival rates. In captive mass spawned barramundi, where no more than two females were utilized for multiple spawns, microsatellites determined broodstock contributions as highly skewed (Frost et al., 2006). At 1 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 were injected with LHRHa. In another instance 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 1 dph. When 10 females and 10 males were all injected with LHRHa, Wang et al. (2008) recorded individual broodstock contributions as high as 98%, when 5 out of 20 broodstock contributed to the spawning. In a follow up spawning event using the same broodstock, 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 is likely to depend on a number of factors, including broodstock weight and maturity (Brown et al., 2005) and mate competition, particularly due to the dominant behaviour of sires (Weir et al., 2004; Fessehaye et al., 2006) and the competiveness of sperm (Campton et al., 2004; Wedekind et al., 2007). The number of broodstock used and the quantity injected with LHRHa for artificial spawning, plus the timing of spawning is 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 programmes 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 and protandrous nature of barramundi creates some obstacles. The main complications identified by previous studies involving mass spawning barramundi (Frost et al., 2006, Wang et al., 2008), has been 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 programme 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 spawnings on this scale in multiple tanks could be applied to benefit a selective breeding programme.

Comparison of the use of different source stocks for establishing base populations for selective breeding

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 part of the 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.

Consultation

This project relates to the previously completed Seafood CRC project 2008/758 (Development of a genetic management and improvement strategy for Australian cultured barramundi) and 2009/738 (Development of a business plan for Barramundi selective breeding entity). The first of these projects identified key researchable constraints and practical options for beginning selective breeding. The project also developed a bioeconomic simulation model for barramundi selective breeding and used this to predict that substantial genetic and economic improvement was likely to result from a basic selective breeding program. The findings were that the BBP be established using a single facility and that we begin to develop the knowledge and skills necessary to establish and run the breeding program.

Following on from project 2008/758 the ABFA expressed a desire to investigate the business case for establishing a barramundi breeding centre, including a full investigation of issues and options involved in establishing the breeding centre, and to develop a timetable and facilitate the necessary steps to get such a business going.

Following further meetings and discussions with the industry, a document was produced and presented to ABFA in December 2009 by Graham Mair, Nick Robinson and Len Stephens on "Establishment of a barramundi selective breeding entity".

In September 2010 an ABFA Genetics Working Group agreed to move forward with this project for the development of a Barramundi selective breeding entity based on a managed centralized breeding program (with ABFA representation on the management) utilizing Good Fortune Bay's (GFB's) facilities at Bowen along the lines proposed in the business case presented out of CRC project 2009/738 (and subsequent breeding plan and costings) with a planned integration of a node at Darwin Aquaculture Centre (DAC).

In this project it was proposed to develop a not-for-profit entity that can be used to run the BBP, provide a proof-of-concept demonstration of how the breeding program could be run at a scale to achieve strong genetic improvement while limiting inbreeding and loss of genetic diversity, investigate the availability and suitability of potential founding broodstock for the BBP and clarify whether current translocation

regulations could be a barrier to the establishment and distribution of benefits from the program.

1.1 Need

The project will significantly fast track an important stakeholder processes initiated by the former Seafood CRC scoping study. This study identified a way forward for a nationally unified centralised breeding initiative that delivers selectively bred stock to the majority of the barramundi farming industry in Australia. The industry stakeholders were keen to maintain the momentum resulting from the former CRC study and seek funds to further develop and ratify the business model proposed in the study.

They were also keen to trial mass spawning as a possible option for generating the families needed by the program.

With a formalised and fully costed business proposal, and proof-of-concept demonstration, the stakeholders can pursue investment opportunities, including stakeholder investment. Without this project, these key steps towards a viable breeding facility would be slowed and thus adoption and benefits of the initiative would be significantly delayed.

1.2 Objectives

- To develop a funded business entity that will run the Barramundi breeding program (BBP)
- To characterise broodstock available to the BBP and identify foundation stock
- To run a pilot scale trial of synchronized spawning to check/demonstrate feasibility of its use with the BBP
- To seek notional approval from Government agencies for the translocation of animals needed to establish the BBP and to supply farms
- To evaluate relevant data that should be collected by hatcheries and farms and to develop and establish a database that can be used to implement barramundi selective breeding

2. Methods

Establishment of the new selective breeding entity was conditional on the successful achievement of the following tasks.

Task 1, June 2012 **Develop a funded business entity that will run the breeding program**

Task 1.1 June 2011 **Define BBP company structure, form initial management group, prepare prospectus including financial statements and describe the likely economic impact on the barramundi industry.**

The cost case and business model were developed by AEC Group. The cost case was based on the material gathered by Nick Robinson and Justin Forrester from the Good Fortune Bay hatchery. Estimates of the economic impact from the project on the overall industry were made and have been published as part of the previous project work (2009/738). The business case/prospectus will give the industry, investors and government agencies the confidence to invest in the new entity. The economic benefit case will be used to persuade government to invest through various funding arrangements.

In parallel with this project, Dr Morten Rye of the Akvaforsk Genetics Centre (a company based in Norway that has provided specialist genetic services and taken ownership in selective breeding programs around the world) was engaged by the Seafood CRC to write a review to assess the technical structure and the scope for commercialization of Australian breeding programs for Pacific oysters, Sydney rock oyster, barramundi and prawns.

Task 1.2, June 2011 **Negotiate and draft a lease agreement with the owner of a potential venue for housing the selective breeding program. (Len Stephens, ABFA)**

Barratek and the ABFA have agreed that the best placed venue to house the BBP is that of Good Fortune Bay (GFB). This approach was supported in the Morten Rye review.

At this stage there has been an agreement in principle to use the facilities at GFB. A final decision on venue and formal lease arrangements will be determined between Barratek and GFB when funding becomes available. Potential investors will also be consulted.

Task 1.3, June 2011 Attract funding (CRC and ABFA)

A strategy was implemented to attract funding from stakeholders, government (Commonwealth and Queensland) and other sources. The strategy was developed in two parts, 1) potential funding sources, requirements and tasks, and, 2) lobbying needed to increase the chance of open funding or to leverage special funds. The strategy was to be put in place in Nov-early December 2010 and lobbying was to begin December 2010. Funding applications were to be prepared and submitted by June 2011.

As a part of seeking funding a professionally prepared funding proposal/prospectus was developed that aligned with the Barratek Business Plan. This prospectus was a key component of all funding approaches.

The ABFA strategy was to approach and lobby local, state and Commonwealth government through Agencies, Local Members and Ministers seeking funding support. Lobbying was through the ABFA Executive and lobbyists aligned with companies involved with the ABFA.

Approaches were made to relevant Queensland Agencies, members and Ministers from the current and past Queensland government, as well as Federal Members and Ministers.

Although there was strong in-principle support for the concept and the business model proposed, the timing of the funding requests aligned with a time that has seen government funding reduced significantly at a state and commonwealth level. At this stage there has been no state or commonwealth government commitment to fund Barratek.

The concept of Barratek being an industry legacy project was informally floated to the CRC but it was felt that without an ongoing source of funding, or funding leverage, it did not meet the legacy projects parameters.

Currently the option of a Commercialisation Australia grant is being investigated.

Task 1.4, June 2012 Establish company (if appropriate or required – e.g. if funding commitments are secured) and recruit initial staff to initiate the BBP (CRC ABFA)

- A company name “Barratek” was registered.
- Representatives for the board of the company were appointed (ABFA Executive and CEO of the CRC – Marty Phillips, Mick Lisle, Bob Richards, Ken Chapman and Len Stephens) Marty Phillips is the interim Chair.
- A legal charter for the company was established.
- Roles for Queensland DEEDI and the Darwin Aquaculture Centre in the development of the Barramundi selective breeding entity were actively considered.

Task 2, May 2011 Characterise broodstock available to the BBP and identify foundation stock (Nick Robinson, Graham Dalton & Shannon Loughnan PhD student)

The CRC PhD student Shannon Loughnan has been involved in conducting this investigation. A background population genetic analysis for wild barramundi populations was conducted by James Cook University in parallel with this project. The analysis of the wild populations and comparison with captive broodstock will be conducted as part of CRC PhD project 2010/725. This will provide important baseline genetic information to use for identifying suitable foundation stock representing the significant pools of natural genetic diversity.

ABFA called on all barramundi hatcheries around Australia to provide a list of fish that could be contributed (loaned) as potential founding broodstock for the BBP. A program was arranged for the collection of DNA samples and PIT tagging of the broodstock.

A total of 419 broodstock samples were collected from eight hatcheries in Queensland, Northern Territory and Western Australia. Seventeen microsatellites markers were used to genotype broodfish and offspring using two multiplex PCR suites of eight (*Lca003*, *Lca016*, *Lca040*, *Lca057*, *Lca154*, *Lca178*, *Lca287*, *Lca371*) and nine (*Lca008*, *Lca020*, *Lca021*, *Lca058*, *Lca064*, *Lca069*, *Lca070*, *Lca074, Lca098*) microsatellite markers (methods described in detail below). All fish sampled were PIT tagged, and information was recorded about each fish on location including current sex and known source and history of the fish (hatchery, wild location etc).

The genotyping of 1205 wild barramundi samples, ranging from Western Australia to central Queensland was carried out in conjunction with our collaborators at James Cook University (Carolyn Smith-Keune and Dean Jerry). The collected samples were sourced from 48 localities by JCU, and following principal component analysis and a Bayesian clustering to look for population structure, the samples were grouped into 2 differentiated stocks and one region of admixture; Structure analysis of the wild population's revealed three distinct clusters; a western stock (WA01 – NT18, a central group of admixed stock (NT19 – QLD34) and an eastern stock (QLD35 – QLD48). Some admixture was also demonstrated within the wild populations of Broome (WA01) and the Fitzroy River (QLD46). NT and WA broodstock groups were allocated to the western stock, although the latter group displayed some admixture. A manuscript which fully describes these results is under preparation with leading authorship by our collaborators at James Cook University (Carolyn Smith-Keune and Dean Jerry). The 8 captive broodstock groups under aquaculture production were assigned to the most likely location of the wild stocks. Using assignment tests we determined the genetic origin of captive stocks and highlighted regions of wild stocks that are currently not represented within broodstock groups under production.

Task 3, May 2011 Trial spawning run to demonstrate use of mass spawning (Justin Forrester, Nick Robinson & Shannon Loughnan)

A trial mass spawning evaluation was carried out to test whether the breeding program will be able to generate sufficient numbers of families contemporaneously for avoiding inbreeding depression of fitness and generating genetic improvement.

Of key importance was to determine;

1. the level of contribution by different broodstock to the mass spawn and
2. whether the grading process significantly affects the contribution by different broodstock to the final breeding population.

Mass spawning of broodstock

The broodstock group consisted of both captive bred and wild sourced barramundi collected locally from coastal central Queensland, Australia.

Selected broodfish were sedated in a saltwater bath containing 40 ppm benzocaine (Aquatic Diagnostic Services International) and a small segment of caudal fin (~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 when collecting the sample from each individual. 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 and 21 males were conditioned for spawning, together in a 50,000L fibreglass tank. The fish were fed a formulated diet (INVE Aquaculture) ad libitum, maintained at a constant water temperature of 28.5^oC 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 dams were injected (excluding dam 06 and 10) with LHRHa (Syndel International Ltd) at 50 $\mu g.kg^{-1}$ to assist in the release of eggs. 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 dams 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 pool was directed into an external egg collection tank, 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. The fertilisation rate (%) of the spawn was also 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.

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. Spawn B was continually monitored throughout the grading period until 90 dph. 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). Immediately after grading, random samples of whole larvae were collected from each size class for parentage analysis; small (n = 208), medium (n = 158) and large (n = 106). During each subsequent

grading event following 18 dph, the offspring were sorted within their current size classes and recombined into small, medium and large groups. 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 (4g, 8g and 16g), but similarly labelled as small, medium and large. At 90 dph, juveniles were large enough to take fin clips. In between the 18 dph and 90 dph grading points, six additional grading events were performed; at 36 dph, 49 dph, 56 dph, 61 dph, 68 dph and 76 dph. Between 60% and 99% of the small size grade was culled at 56 dph, 61dph and 76 dph, as part of standard practice to achieve uniform growth within the cohort.

Batch DNA extractions to discriminate non-contributors from low frequency contributors

Batches of eggs and whole larvae from each day of spawning were pooled 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 for detecting whether particular broodstock contributed at 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, combining all 200 samples per batch. To assist in differentiating between alleles and stutter bands in the electropherograms of the pooled samples, the correction method proposed by Kirov et al. (2000) was applied. The peak heights of stutter patterns were measured for a minimum of four individuals per allele, resulting in an average peak height for each stutter band. Under the correction method, all allele peak heights are 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.

DNA extraction

DNA was extracted from broodstock fin clips and the pooled egg/larvae batches using a modified CTAB (cetyl trimethylammonium bromide) protocol (Adamkewicz and Harasewych, 1996). Polyvinyl pyrolidone (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 ^{o}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 x g for 30 min), the pelleted DNA was washed with 70% cold ethanol, air dried and resuspended in 50 µl of 1x TE for broodstock samples and 150 µl of 1x TE for pooled egg/larvae samples. 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 dph and 18 dph, and small segments of fin clips (~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 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 Proteinase K was added to each sample and digested for a minimum of 4 h at 55 ^{o}C. The samples were then incubated at 95 ^{o}C for 20 minutes to denature the Proteinase

K, 100 µl of 1x TE buffer was then added and the samples stored at -20 ^{o}C overnight prior to PCR.

PCR amplification

Two multiplex groups of 17 markers were selected from published microsatellite sequences for *Lates calcarifer*. 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). The second multiplex suite was developed and first used by Zhu et al. (2006a), to monitor the genetic diversity of captive stocks and the genetic structure of wild Asian seabass populations. 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 (at a volume of 1 µl for the CTAB DNA extracted samples and 0.5 µl for the Tween extracted larvae), 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 ^{o}C for 5 min, followed by 10 cycles of 95 ^{o}C for 30 s, 57 ^{o}C for 90 s and 72 ^{o}C for 30 s, then 20 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 90 s and 72 ^{o}C for 30 s, followed by a final extension at 60 ^{o}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 ^{o}C for 30 min. Following amplification, PCR products were diluted with 12 µl of water and desalted through a Sephadex 258 G-50 fine filtration 259 spin column (GE Healthcare). Desalted PCR products were visualised on a 1.5% agarose gel prior to genotyping on a MegaBACE 1000 DNA Analysis System (Amersham Biosciences). MegaBACE Fragment Profiler software (Amersham Biosciences) was used for fragment analysis, where alleles were allocated with an identifying label.

Statistical analysis

Following the scoring of genotypes, MICRO-CHECKER V2.2.3 (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 V3.0.3 (Kalinowski et al., 2007), to determine broodstock contribution to offspring and the total number of half and full-sibling families. Broodstock allele frequencies were utilised for the simulation of parent pairs of known sex and the following simulation parameters were utilized; 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 expected (H_e) and observed (H_o) heterozygosities, the number of alleles per locus, including the number of private alleles (where only one broodstock individual possessed that allele and it was considered rare in the population), which provides a measure of genetic distinctiveness within an individual or a population. The fixation index (F_{is}) was estimated by the method of Weir and Cockerman (1984) using GENEPOP V4.1 (Rousset, 2008). An indicator of the level of inbreeding, F_{is} describes the loss of heterozygosity relative to Hardy-Weinberg Equilibrium (HWE) expectations within subpopulations (Zhu et al., 2006a). Any deviation of observed from expected proportions under HWE was also calculated using GENEPOP V4.1. *P*-values were estimated using a Markov chain (MC) algorithm, beginning with a dememorization 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). By calculating the allelic richness (R_s), differences in sample size can be taken into consideration when comparing genetic diversity between batches (Loukovitas et al., 2011) and R_s within each locus was estimated with FSTAT V2.9.3.2 (Goudet, 2001), incorporating rarefaction to standardise R_s for interpopulation comparisons (El Mousadik and Petit, 1996) as;

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

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

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

$$N_{ed} = (N_d K_d - 1)/[K_d - 1 + (V_d/K_d)] \ and \ N_{es} = (N_s K_s - 1)/[K_s - 1 + (V_s/K_s)] \quad (2)$$

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

$$N_e = 4N_{ed}N_{es}/(N_{ed} + N_{es}) \quad (3)$$

N_e would equal *N* if the same number of male and female broodstock contributed equally to the next generation (Brown et al., 2005). The rate of inbreeding (ΔF) was computed according to Falconer (1996) as

$$\Delta F = 1/2(N_e) \quad (4)$$

Any significant difference in contribution levels between spawn A and B, between sampling at 1, 18 and 90 dph of spawn B and between size grades, were determined by the nonparametric Friedman two-way ANOVA, which was calculated with IBM SPSS V20 following data transformation. Relatedness was estimated within the broodstock group using COANCESTRY V1.0.0.1 (Wang, 2011), which calculates seven relatedness estimators and uses simulation analysis to determine the most appropriate estimator to apply. Observed allele frequencies were used to simulate 1000 individual multilocus genotypes, with 100 dyads from each true value relationship type; unrelated, parent-offspring, full and half sibling. 1000 bootstrapping samples were applied to estimate 95% confidence intervals for the relatedness coefficients. The relatedness estimator generating the highest correlation with the simulated values was applied to compare the relatedness amongst the broodstock.

Task 4, May 2011 **Seek approval from Government agencies for the translocation of animals needed to establish the program and to supply farms on the mainland (Graham Dalton and ABFA assisted by Graham Mair and Nick Robinson on the genetics side).**

The biosecurity arrangements, potential venues and predicted industry benefits were presented to Government Agencies. Approval was sought for translocation of animals into quarantine to found the selective breeding population and for regular translocation of certified disease free larvae from the selective breeding nucleus to farms in each state. It will be important to receive all approvals before the entity begins operation. A clear translocation policy for the selective breeding entity will be developed, accounting for current and future constraints to translocation, and conforming to State and Commonwealth Government legislations.

Achievement of the above tasks should give investors' confidence in the project and should provide strong incentives to invest.

Task 5, Oct 2013 **Establish barramundi database, enter data, train industry (Nick Robinson).**

The purpose of creating a database is to enable hatcheries to record essential information for barramundi selective breeding programs. This includes pedigree data, relationship estimate data for base stock, trait data, fish tracking data etc. The PI had already produced a genetic management database for yellowtail kingfish as part of project 2011/754. The yellowtail kingfish database was modified for the purposes of ABFA. The PI spent one week total with Good Fortune Bay and Darwin Aquaculture Centre, who both expressed interest in using the database, to train staff in use of the database while making changes to adapt the database to the barramundi situation. The PI designed new modules to query microsatellite relationship data in order to produced ranked lists of stock in terms of estimated relatedness. The PI devised a way to incorporate Shannon Loughnan's microsatellite relationship data into the database and also load existing site data from GFB and DAC into the database versions for each of those locations. Finally, the PI trained users at GFB and DAC about how to use the database (record and enter data, extract information etc.) and assist and make adjustments to the database as necessary.

The shell of the database, along with the relatedness data for existing broodstock, was made freely available to those industry participants who wished to use it. Individual industry participants will manage their own copy of the database and own the data they enter.

Task 6, June 2014 **Evaluation of key parameters for measurement of growth performance across the industry.**

A more thorough evaluation of relevant data that should be collected by hatcheries and farms, and methods to use for collecting this information across the industry was undertaken. Information needs to be obtained in a way that it can be easily collated and entered into a central database in the future if desired. Such information is needed in order to be able to assess performance across the industry and/or across the breeding population for genetic evaluation. Traits and data collection formats used by other industries were considered and adapted for barramundi when suitable. Factors that were considered included:

- Growth rates (gram over time)

- % Fillet recovery
- Water temperature
- Salinity
- % Survival
- Feed kg and type

The data collection format needs to provide adequate information, but not be excessively burdensome for industry to complete. An excel spreadsheet was developed that could be completed electronically (preferably) or printed for written entries. The spreadsheet also details the timing of measurements, and where and what to measure. The spreadsheet data was made into a format that can be easily/automatically uploaded into a future central database.

Additional tasks- Comparison of the use of different source stocks for establishing base populations for selective breeding

This additional work was conducted by Shannon Loughnan as part of his PhD thesis funded by the Australian Seafood CRC. The task and results are provided in a summarised form in this report (full details will be submitted as a manuscript to a peer reviewed international journal).

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) and A_r within wild sites was calculated using data from other chapters of Shannon's thesis. 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.

3. Results

Establishment of Barratek

Based on research projects undertaken by the CRC, the ABFA formally resolved to establish an independent company to manage the genetic improvement program. The constitution of the new company for Barramundi selective breeding, named "Barratek", was prepared by a law firm in conjunction with the ABFA and the CRC. The company is intended to be a not for profit company, limited by guarantee. The Seafood CRC owns the IP resulting from the barramundi genetics projects that have been funded through the CRC. This IP will be licensed to Barratek. Directors of Barratek have been nominated and appointed by ABFA.

There is some risk associated with this company, as with all new start-ups. The key risk will be to ensure that sufficient funds are available at all times. This risk can be covered in part by ensuring that Barratek has adequate insurance.

A full business model for the company was developed (Attached Appendix 1, ABFA Hatchery Business Model.xlsx). The model allows entry of core assumptions on revenue (value per larvae), capital purchases and operating items needed and calculates cash flows.

The AEC Group developed a business plan for Barratek which outlines the market for genetically improved barramundi, the business structure of the company (location, legal structure, board and operations overview), financial evaluation of the company (project funding, establishment capital costs, operational costs, revenue forecasts and financial feasibility) and sensitivity factors. The business plan is attached as Appendix 2.

The business plan and the funding proposal/prospectus outline the following need and business case for the Barratek business which would run the selective breeding program.

The Barratek entity and genetic improvement plan has broad support from the Australian barramundi industry, having resulted from recommendations from ABFA funded scientific research. Participating hatcheries in producing fingerlings from Barratek larvae are anticipated to be:

- Challenger Tafe (Fremantle, WA);
- Coral Coast Mariculture (Bundaberg, QLD);
- Darwin Aquaculture Centre (Darwin, NT);
- King Reef Australia (Innisfail, QLD);
- GFB Fisheries (Bowen, QLD);
- JCU Aquaculture (Townsville, QLD);
- Jungle Creek Aquaculture (Innisfail, QLD);
- Mainstream Aquaculture (Werribee, VIC);
- Paradise Aquafarm Fish Hatchery (Gordonvale, QLD);
- PEJO Enterprises (Innisfail, QLD);
- Robarra Aquaculture Farm (Robe, SA); and
- WBA Hatcheries (West Beach, SA).

These hatcheries constitute at least 80% of Australia's barramundi fingerling production and supply an even larger proportion of Australian commercial barramundi aquaculture producers.

Cumulative profits would begin after year 4 (Table 1).

Table 1. Barratek Cashflow Summary (Nominal $ Millions)

	Yr1	Yr2	Yr3	Yr4	Yr5	Yr6	Yr7	Yr8	Yr9	Yr10
Broodstock										
Total Cash Inflow	$0.080	$0.082	$0.105	$0.135	$0.172	$0.331	$0.357	$0.384	$0.413	$0.445
Total Cash outflow	-$0.650	-$0.625	-$0.641	-$0.673	-$0.690	-$0.707	-$0.725	-$0.743	-$0.762	-$0.781
Net Profit/Loss	**-$0.570**	**-$0.543**	**-$0.536**	**-$0.538**	**-$0.517**	**-$0.376**	**-$0.368**	**-$0.359**	**-$0.348**	**-$0.336**
20 mm fingerling										
Total Cash Inflow	$0.480	$0.480	$0.600	$0.750	$0.938	$1.211	$1.271	$1.335	$1.402	$1.519
Total Cash outflow	-$0.720	-$0.728	-$0.773	-$0.854	-$0.923	-$1.141	-$1.200	-$1.263	-$1.331	-$1.404
Net Profit/Loss	**-$0.240**	**-$0.248**	**-$0.173**	**-$0.104**	**$0.015**	**$0.069**	**$0.072**	**$0.072**	**$0.071**	**$0.115**
90 mm fingerling										
Total Cash Inflow	$0.900	$0.900	$1.125	$1.406	$1.758	$2.263	$2.376	$2.495	$2.620	$2.804
Total Cash outflow	-$1.107	-$1.257	-$1.304	-$1.426	-$1.492	-$2.145	-$2.242	-$2.347	-$2.461	-$2.586
Net Profit/Loss	**-$0.207**	**-$0.357**	**-$0.179**	**-$0.020**	**$0.266**	**$0.119**	**$0.135**	**$0.148**	**$0.159**	**$0.218**
Additional funding										
Grant	$1.000	$1.000	$1.000	$1.000	$1.000					
Summary										
Total Profit/Loss	-$0.017	-$0.148	$0.112	$0.337	$0.763	-$0.188	-$0.162	-$0.139	-$0.119	-$0.002
Cumulative	-$0.017	-$0.165	-$0.053	$0.285	$1.048	$0.860	$0.698	$0.560	$0.441	$0.439

Economic impact

Barratek has been modelled to yield a high short-term benefit to cost ratio for the barramundi industry (17:1, with a nominal economic effect on operating income of $10.2 million after 10 years of selective breeding, Robinson et al. 2010).

This level of improvement has been demonstrated as highly feasible both by the barramundi genetic work conducted by the Seafood CRC and by comparison to results achieved in other species. A classic example is the improvements made to broiler (meat) chickens through the 1960s, 70s and 80s. As of the 1990s a modern commercial broiler chicken grew three times faster and used half the feed to a given size as a 1960s commercial broiler chicken.

The result of being able to produce six times as many chickens for much the same capital and feed bill has transformed chicken meat from a luxury item to mass market protein source.

Analysis of the result for broiler chickens reveal that 85.3% of the growth improvement and 62.5% of the feed conversion improvement is due to genetic improvement (with the remainder due to improvements in diets). Similar results have been achieved for other major agriculture animals and for major aquaculture species such as salmon.

Australian barramundi farmers and ultimately Australian consumers will be the major beneficiaries from the development of Barratek. Additional income received by

farmers due to faster growing fish will primarily result in additional expenditure in the Australian economy through:

- Increased expenditure on wages to tend, harvest, process and transport fish;
- Increased investment in additional barramundi farming infrastructure;
- Additional seafood retailing and food services activity;
- Increased expenditure on aquaculture and marketing research.

Reduced production costs will also put significant downwards pressure on the prices paid for Australian barramundi by consumers.

Cost and timeframe

Leasing arrangements, in-kind support by ABFA members and the ability to leverage the expertise and systems of an existing facility mean that up-front capital requirements for establishing Barratek are minimal. None-the-less Barratek experiences a total funding shortfall over the establishment years of $4.5 million, before any contingency reserve.

As a not-for-profit company Barratek is unable to access finance or equity. ABFA has already invested significant funds in the foundation scientific research and business planning to date, and ABFA members have continued to invest significant time and other resources into developing the entity.

Barratek was planning to undertake genetic characterisation work and broodstock preparations in 2012 for its first breeding season in 2013. However, this has been delayed because the start-up investment funds needed to carry out this work are not yet available.

Barratek funding

Barratek has been established as a not for profit company, limited by guarantee, for the purposes of improving barramundi genetics, breeding the improved stock and supplying the Australian barramundi industry with larvae and fingerlings.

Grant funding of $1 million per annum for the first five years is required for Barratek to be established and reach a point of financial sustainability. This funding was sought from the Queensland Government in support of ensuring the long term viability of a major Queensland aquaculture industry. Unfortunately at this stage funding has not been forthcoming. Commonwealth funding has also been sought, but was also not successful.

In parallel with this project (2009/730), Dr Morten Rye from AFGC Norway undertook an independent review to assess the technical structure and the scope for commercialisation of Australian breeding programs for the Seafood CRC (attached as Appendix 3). The main findings outlined in the review were that:

- The Barratek initiative is solidly underpinned with extensive documentation of the sector's need for genetically improved seed and how a cost effective and bio secure program should be designed and implemented. The underlying documentation adequately discusses all relevant issues regarding the technical structure of a breeding program, the benefits and disadvantages of alternative models, and the risk factors involved.

- The proposed strategy of operating the program with one central breeding nucleus is strongly supported, and the GFB facility seems to be ideally equipped for this role.

- The operational budgets and cash flow analysis presented in the Barratek Business Plan are realistic and well documented, and there appears to be no recommendable alternatives to the presented centralized program structure for implementation of a sector-wide, effective and sustainable long term program to the sector.

- With the current low combined production volume of Barramundi in Australia, it is not realistic to assume that the sector itself can absorb the full costs of establishing a sector-wide breeding program and therefore needs substantial contributions from public funds (R&D grants or other). A breeding program may however have potential for significant revenues from international sales of genetically improved materials to other Barramundi producing countries in the region and this option should be actively explored, especially if it can provide access to start up funds.

The industry now appears to have reached a broad consensus on the way forward with a breeding program, based on the work done under the CRC, but have yet to commit to a funding structure for the program. In the present situation rather than relying on what appears to be an increasingly unlikely option of governmental support, it is strongly recommended that ABFA focuses its efforts on resourcing the start-up of the breeding program activity as outlined in the current plans. In parallel it should develop alternative mechanisms to support the program over the longer term, which may include industry support, international sales and/or international investments.

Ongoing lobbying is needed to increase the chance of open funding or to leverage special funds.

A lobbying document/prospectus was created by AEC Group called “Barratek Funding Proposal” (attached as Appendix 4) and has been the foundation for all contacts made seeking financial support for the concept

A strategy was developed to attract funding from stakeholders, government (Commonwealth and Queensland) and other sources. This has focussed on approaching local, state and Commonwealth government through Agencies, Local Members and Ministers seeking funding support. Lobbying was through the ABFA Executive and lobbyists aligned with companies involved with the ABFA.

i. Queensland State Government

The former Queensland Labor government were lobbied extensively to provide funding support for Barratek. Indications were that they were supportive in principle, however before this could be resolved there was a state election which saw a change in government.

The new Coalition government have been approached a number of times to resource Barratek but they have put in place a stringent fiscal program to reduce state debt. This has reduced the opportunity for funding for the project in the near future but there are ongoing discussions to attempt identify funding options. Again there is in principle support for the concept but no government resources currently available.

Due to the above this output has been only partially achieved. Expenses in the amount of $21,153 have been incurred in producing the business plan and prospectus.

ii. Commonwealth Government

The Federal government have been lobbied to identify possible funding options for Barratek. Although there appears to be in principle support, no funding options have been identified.

iii. Commercialisation Australia (http://www.commercialisationaustralia.gov.au)

Barratek might be entitled to apply for funding for:

- Skills and knowledge -$50,000 to access expert advice and services for e.g. capital raising or developing linkages with other businesses.
- Experienced executives- $350,000 over 2 years to engage a CEO
- Proof of Concept- $50,000 - $250,000 to assist establishing the commercial viability of the product
- Early stage commercialisation- $50,000 - $2 million to assist bringing the new product or service to market.

Eligibility:

- All applicants and body corporates must have combined earnings of <$50 million / year for last 5 years to be eligible for early stage commercial grants, <$10M per year for last 5 years to be eligible for other grants.
- Barratek must be a non tax-exempt company
- Comply with the equal opportunity for women in the workplace act
- Able to fund its share of the project costs
- Have ownership or beneficial use of IP for project (e. g. fish).

Assessment criteria:

- Show unable to fund ourselves and no other funding assistance available
- Significant market opportunities for outcome of project
- Compelling value proposition for new product or service
- Sound execution plan
- Appropriate management capability
- Significant national benefits resulting from project.

This option can be explored further when Barratek is able to fund its share of the project costs.

Characterisation of available broodstock and identification of foundation stock

Average relatedness for the broodstock within hatcheries is low in some cases (e.g. 0.05) and quite high in other cases (0.17) (Figure 1). This suggests that the broodstock represented from these hatcheries are in some cases sourced directly from diverse wild populations and that in other instances the broodstock are related to some extent (either families sourced from the same area, or close relatives produced in captivity). This is valuable information that will be used to guide the choice of animals for breeding.

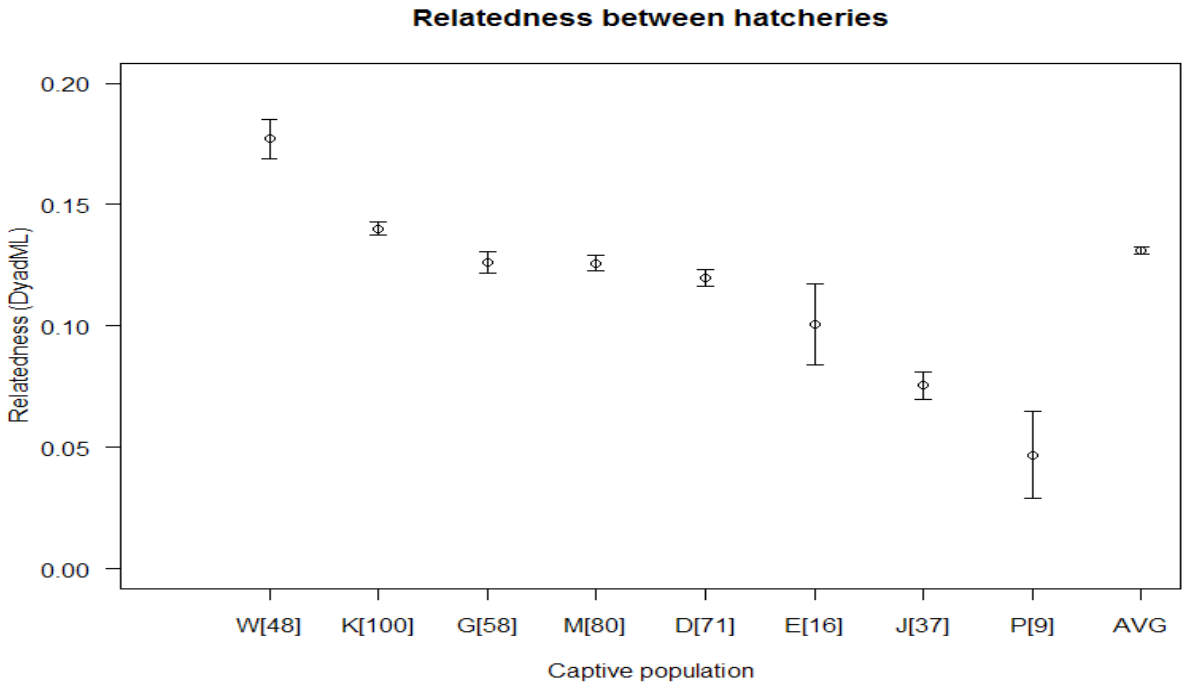


Figure 1. Relatedness measured among broodstock within sampled hatcheries (coded). AVG shows the average relatedness within hatcheries.

When the relatedness between broodstock sampled from different hatcheries is compared (Nei's genetic distance using an UPGMA unweighted pair group method to produce a neighbour joining tree), Queensland based broodstock form a diverse grouping (probably reflecting the diverse localities from which they are sourced) whereas WA and NT based broodstock form another distinct group (reflecting that the animals sampled from the WA hatchery were derived from NT stock) (Figure 2).

Overall it is apparent that the barramundi industry has an adequate base population at hand to develop a selective breeding program, although some new sources of broodstock to supplement the existing hatchery diversity may be suggested after wild population comparisons are made.

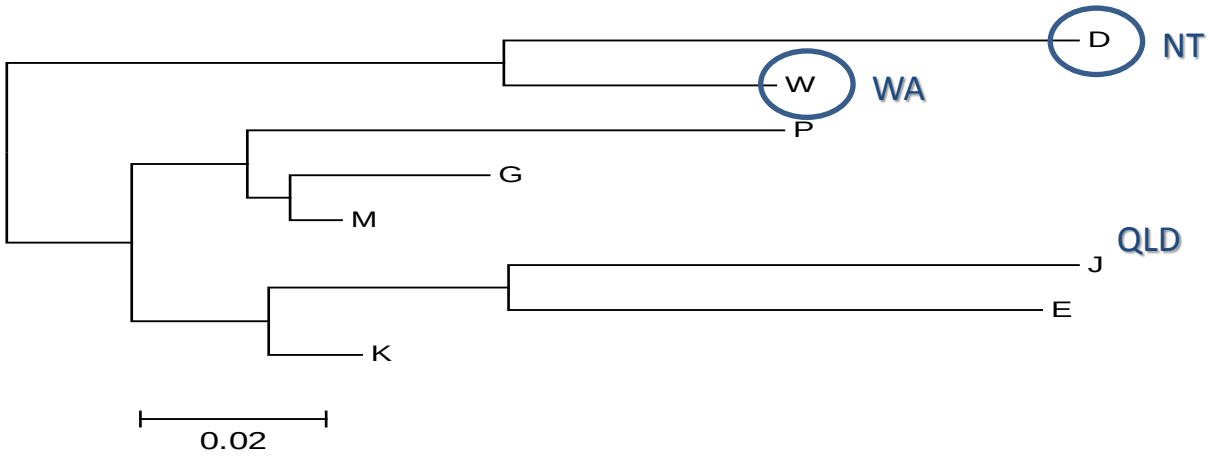


Figure 2. Neighbour joining tree showing the genetic relatedness between hatcheries

Genetic diversity was at its highest for QLD11 and QLD12, with a level of allelic richness (A_r) greater than 4 (Table 2). QLD18 recorded the lowest level of A_r and

subsequently the average number of alleles, although this may be attributed to the sample size of the population but also provides an interesting aspect of temporal sampling. Populations QLD18 and QLD19 were both sampled from the Fitzroy River tested temporally over time, with a difference between sampling events of nearly 20 years. A_r has been reduced over the years in QLD18. Overall, average allelic richness was relatively even across WA, NT and QLD with 3.5, 3.8 and 3.6 respectively. Although pairwise F_{st} estimates detected significant differences between the 21 wild populations, measures of genetic diversity were relatively even across all stocks and may reflect high rates of natural dispersal. Two-sided P-value tests between WA, NT and QLD groups revealed no significant differences in A_r between populations.

Measures of genetic diversity for the eight hatchery groups (Table 3) were lower than those calculated in the wild stocks. Allelic richness was no greater than 3.4 across all hatcheries. Knowledge of the origin of broodstock individuals can be beneficial to assignment tests, as we can compare the results back to the records maintained at the hatchery to determine the accuracy of the software. The original source of NT broodstock was wild caught stock from the Darwin Harbour region (NT06) with a small number of individuals from first generation aquaculture stock. WA broodstock were all selected from captive grow out populations, many sourced from another hatchery not represented here. Similarly, all QLD hatchery stocks are from captive grow out, where many hatcheries have exchanged individuals. Only QLD4 has a small number of wild individuals sourced locally from the Burdekin River region (QLD16).

The majority of captive QLD broodstock groups were assigned to QLD localities, and although the NT group was mainly assigned to NT stocks from Daly River to Shoal Bay, there was also a reasonable representation in WA04 (Table 4, Figure 3). The captive WA group was not only assigned to the three WA localities but there was also a high representation in QLD and the Daly River in the NT, evidence of a high rate of stock mixing with other hatcheries or directly acquiring broodstock from a broad range of wild localities. Where records had been maintained for the origin of broodstock, the majority were assigned correctly to their wild localities or the source localities of their parents. Most of the broodstock across the eight hatcheries are represented at two locations, QLD16 and QLD17. Eleven wild locations either had no captive stock assigned or the assignment rate was less than 10%.

Table 2.
Measures of genetic diversity for 21 wild barramundi catchments from 16 microsatellite loci, representing Western Australia (WA), Northern Territory (NT) and Queensland (QLD). Sample size (*N*), average number of alleles (*k*) and mean allelic richness (A_r).

Stock		*N*	*k*	A_r
Broome	WA01	13	3.2	3.04
St George Basin	WA02	30	4.5	3.69
Admirality Gulf	WA03	37	4.4	3.37
Swift Bay - Moyle R	WA04	268	6.6	3.87
Daly R	NT05	46	5.4	3.90
Bathurst Is - Shoal Bay	NT06	71	5.4	3.76
Mary R - Alligator R	NT07	37	5.3	3.85
Liverpool River	NT08	32	4.1	3.39
Arnhem Bay	NT09	22	4.6	3.80
Roper River	NT10	24	4.6	3.85
McArthur R - Leichardt R	QLD11	72	6.6	4.13
Gilbert R -Archer R	QLD12	126	7.2	4.14
Jardine River	QLD13	16	4.4	3.93
Jacky Jacky - Escape R	QLD14	54	5.6	3.84
PCB - Bizant R	QLD15	39	4.4	3.50
Johnston R - Burdekin R	QLD16	217	6.4	3.77
Broad Sound	QLD17	12	3.6	3.52
Fitzroy River - 2008	QLD18	23	3.3	2.85
Fitzroy River – 1988/90	QLD19	24	4.4	3.57
Port Alma	QLD20	24	3.7	3.11
Mary River	QLD21	18	4.4	3.76
	Total[‡]	1205	4.9	3.65

[‡]*N* is the total count, whilst the remaining total measures of genetic diversity are averages.
[*]Average F_{is} values were not significantly different from zero for both heterozygote deficiency and excess, following Bonferroni correction for multiple comparisons.

Table 3.
Measures of genetic diversity for 8 captive broodstock groups from the Northern Territory (NT), Queensland (QLD) and Western Australia (WA). Sample size (*N*), average number of alleles (*k*) and mean allelic richness (A_r).

Hatchery	*N*	*k*	A_r
NT	71	5.6	3.42
QLD1	58	4.3	3.11
QLD2	14	3.9	3.35
QLD3	111	5.5	3.23
QLD4	80	4.4	3.19
QLD5	9	3.2	3.04
QLD6	16	3.1	2.67
WA	48	4.2	3.18
Total[‡]	407	4.3	3.15

[‡]*N* is the total count, whilst the remaining total measures of genetic diversity are averages.

Table 4.
Assignment of 8 captive broodstock groups (first row) to 21 wild barramundi stocks (first column). The total proportion assigned is displayed in the final column.

	NT	QLD1	QLD2	QLD3	QLD4	QLD5	QLD6	WA	TOTAL
WA01								0.21	0.21
WA02	0.03			0.02				0.15	0.19
WA03									0.00
WA04	0.20						0.06	0.13	0.38
NT05	0.37	0.02	0.07	0.12				0.17	0.74
NT06	0.21			0.04				0.04	0.29
NT07	0.04			0.01	0.03			0.02	0.10
NT08									0.00
NT09				0.01	0.01				0.02
NT10				0.01					0.01
QLD11	0.08	0.02	0.14	0.05	0.04		0.06	0.02	0.41
QLD12	0.06		0.07	0.01	0.04	0.11		0.04	0.33
QLD13	0.01				0.01				0.03
QLD14		0.03		0.01	0.03				0.07
QLD15				0.03			0.06		0.09
QLD16		0.69	0.43	0.54	0.68	0.44	0.75	0.21	3.74
QLD17		0.21	0.29	0.14	0.18	0.44	0.06	0.02	1.33
QLD18									0.00
QLD19									0.00
QLD20									0.00
QLD21		0.03		0.04					0.07

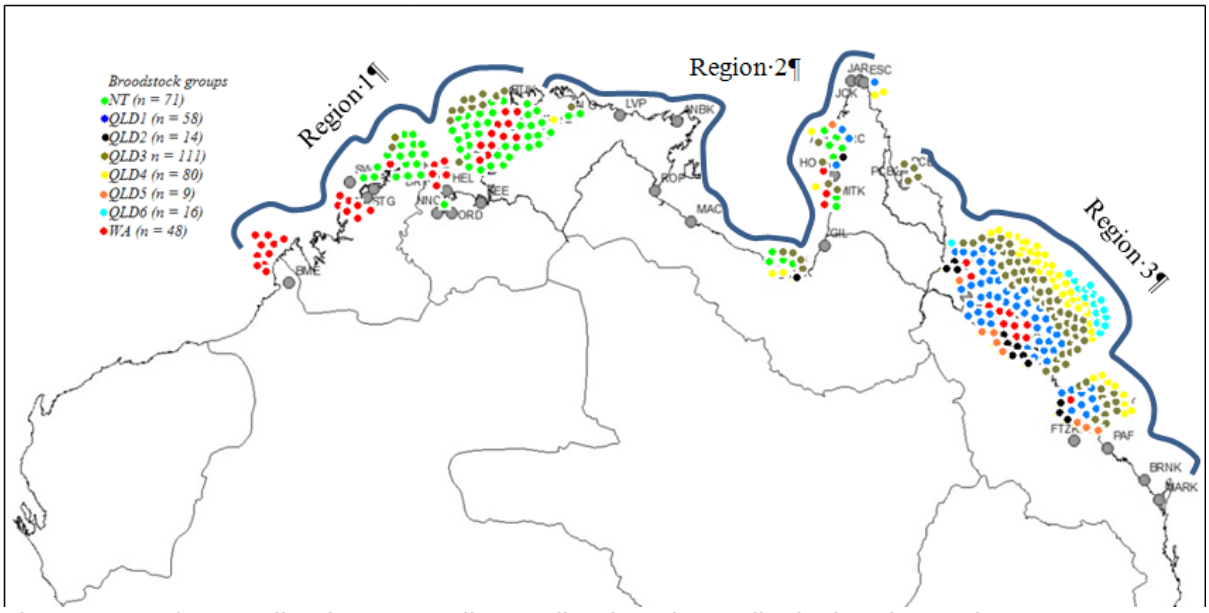


Fig. 3. Map of Australian barramundi sampling locations, displaying the assignment of 407 individuals from eight captive broodstock groups, as determined by GENECLASS. Each dot represents one individual from the corresponding broodstock group and sample sizes are provided in parenthesis. Region 1, Western stock consisting of catchments from Broome (WA) to Shoal Bay (NT); region 2, central region of admixture consisting of catchments from Mary River (NT) to Escape River (QLD); region 3, Eastern stock consisting of catchments from Princess Charlotte Bay (QLD) to Mary River (QLD). Wild population genetic samples obtained and analysis done as part of collaboration with James Cook University (Carolyn Smith-Keune and Dean Jerry, joint manuscript in preparation).

Broodstock contribution with synchronised spawning

Parentage assignment rates were 94% (95% CI) 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. 4 and 5) 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 spawn A and B at 1 dph, assigned as the most likely parent of 48% and 30% of 1 dph larvae respectively (Fig. 4a). The highest contributing sires at 1 dph were sire 03 (15%) to spawn A and sire 04 (16%) to spawn B (Fig. 5a). There was no significant difference in the level of broodstock contribution between spawn A and B at 1 dph (dams P = 1; sires P = 0.513) or between the sampling events of 1, 18 and 90 dph from spawn B (dams P = 0.741; sires P = 0.867). Of the two dams that were not injected with LHRHa (dams 06 and 10), only dam 06 was observed in the offspring from spawn A and B, although only a minor contribution was detected (< 3%) across all sampling events from this individual (Fig. 4). 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 90dph (Fig. 5b).

Small, medium and large size grades from spawn B

By monitoring the offspring cohort 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. An even representation of broodstock to each of the size grades is ideal, however when grading, the contribution of specific broodstock may be directed towards one or more size grades and the routine culling of fish from the small grade could have an effect on the overall contribution of broodstock and thus the number of families detected (Frost et al., 2006). Broodstock contribution levels to the size grades were skewed (Fig. 6 and 7), although there was no significant difference in the level of broodstock contribution between the small, medium and large size grades at either 18 dph (dams P=0.459, sires P=0.917) or 90 dph (dams P=0.832, sires P=0.950). The highest contributing dam at 18 and 90 dph was dam 08 (Fig. 4b), which was also a major contributor to the size grades, ranging from 20% to 44% (Fig. 6). Sire 03 and sire 13 were the greatest contributors at 18 and 90 dph respectively (Fig. 5b), and were also the major contributors to each of the size grades, ranging from 10% to 20% (Fig. 7). 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. The latter discrepancies may be due to reduced offspring numbers, caused by a reduction in sampling size and higher sampling error for broodstock with fewer offspring.

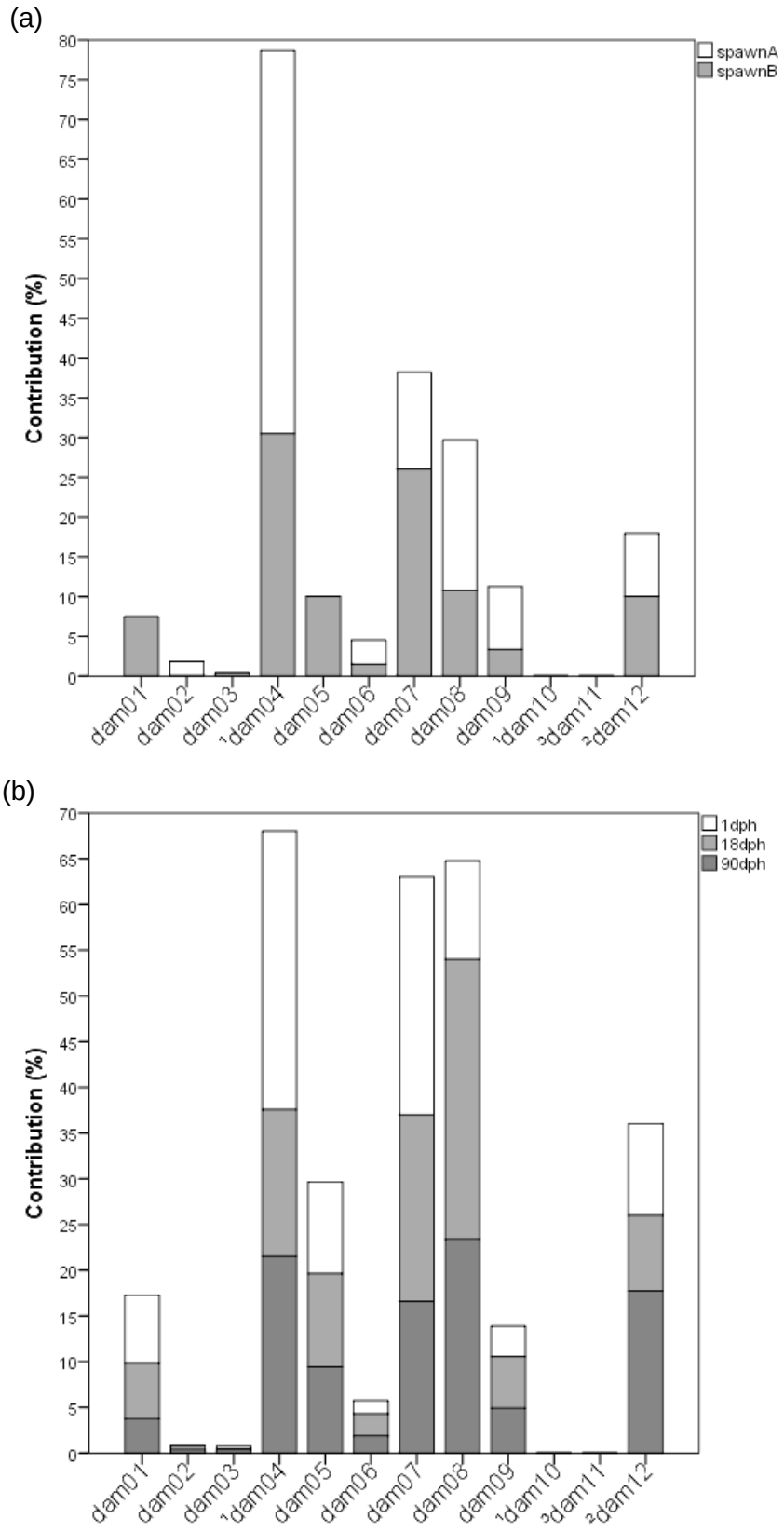
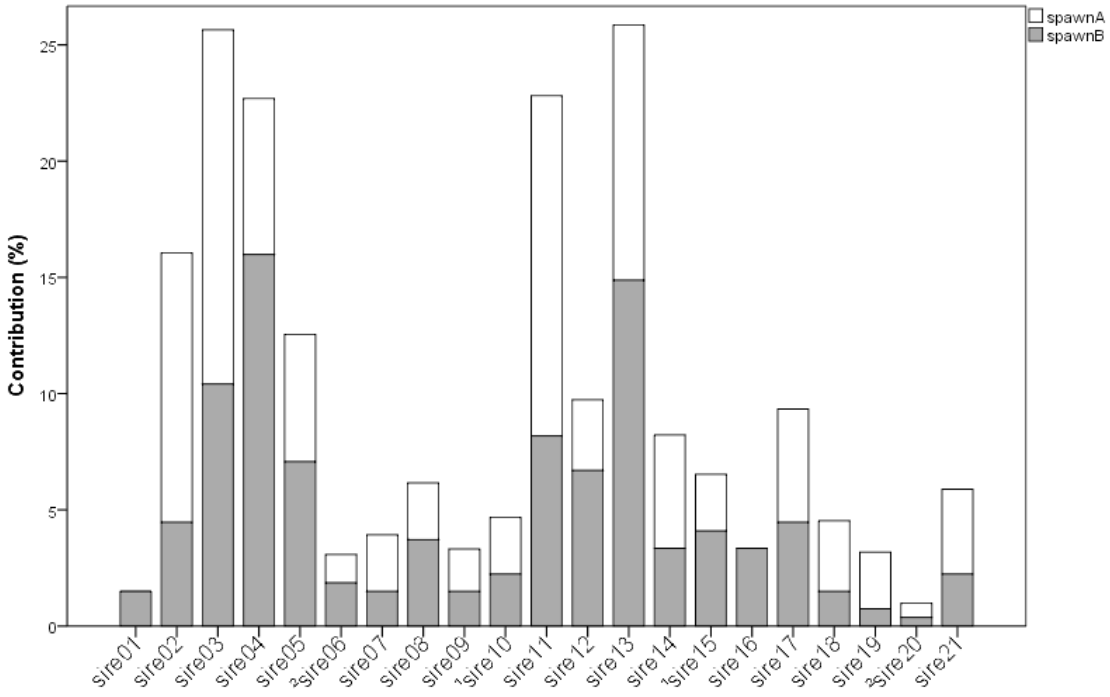


Fig. 4. Dam contribution to offspring from spawn A and B at 1dph (a), and from spawn B over three sampling events; 1, 18 and 90dph (b). Numbers in superscript indicate the number of private alleles detected for the specified dam.

(a)



(b)

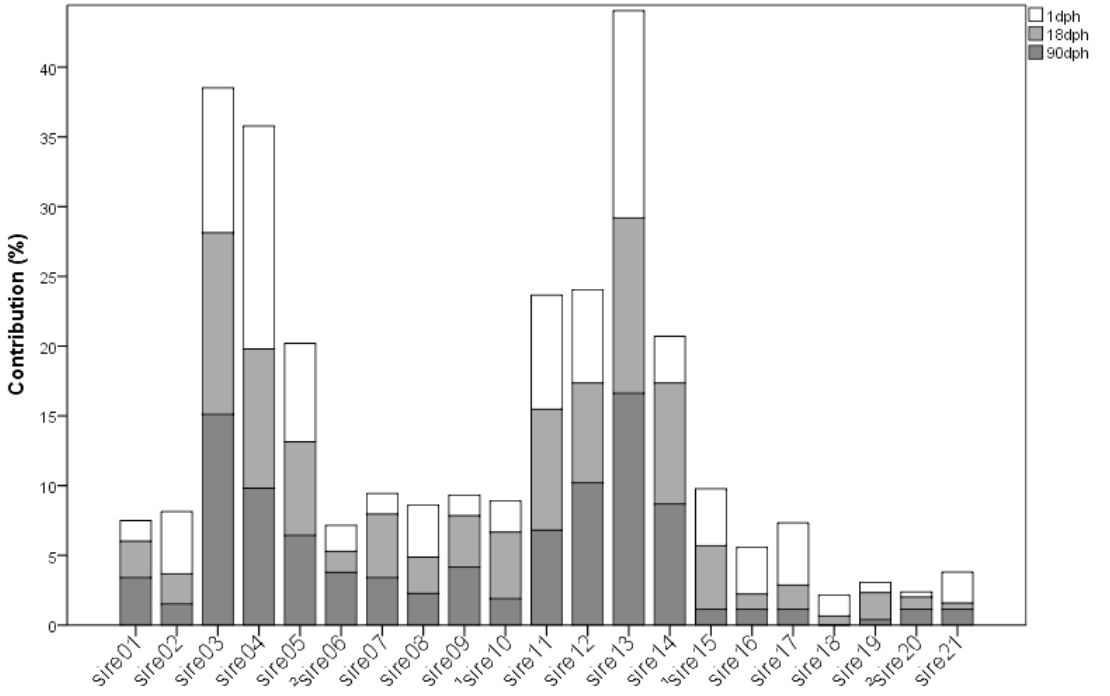
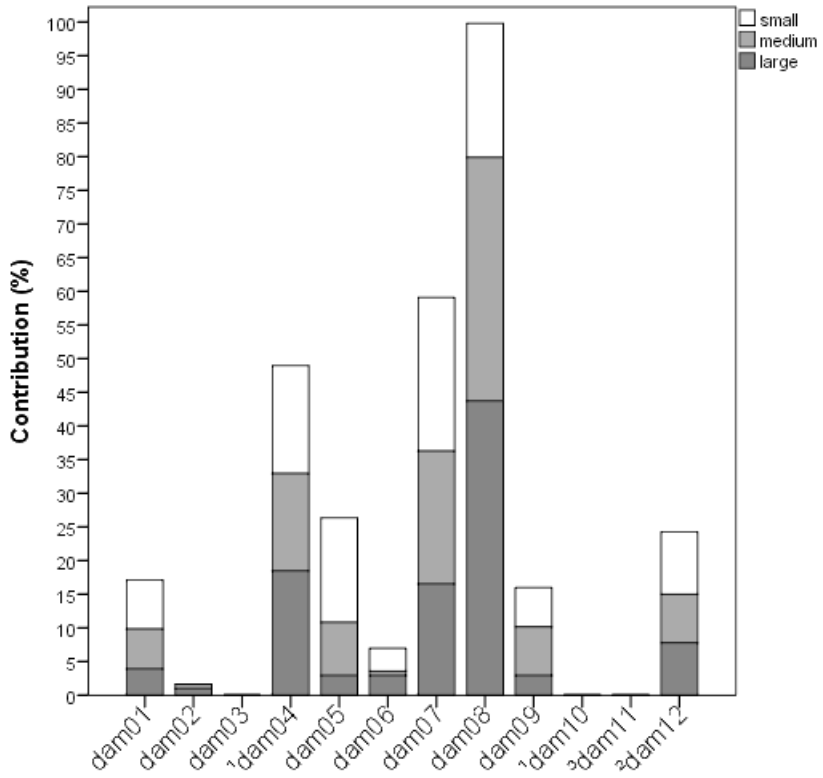


Fig. 5. Sire contribution to offspring from spawn A and B at 1dph (a), and from spawn B over three sampling events; 1, 18 and 90dph (b). Numbers in superscript indicate the number of private alleles detected for the specified sire.

(a)



(b)

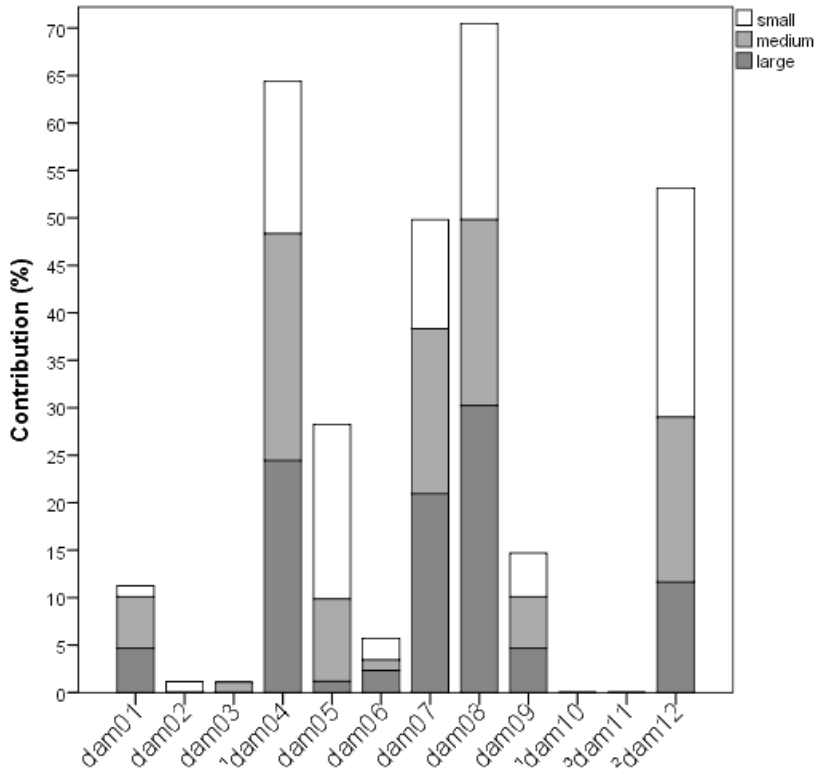
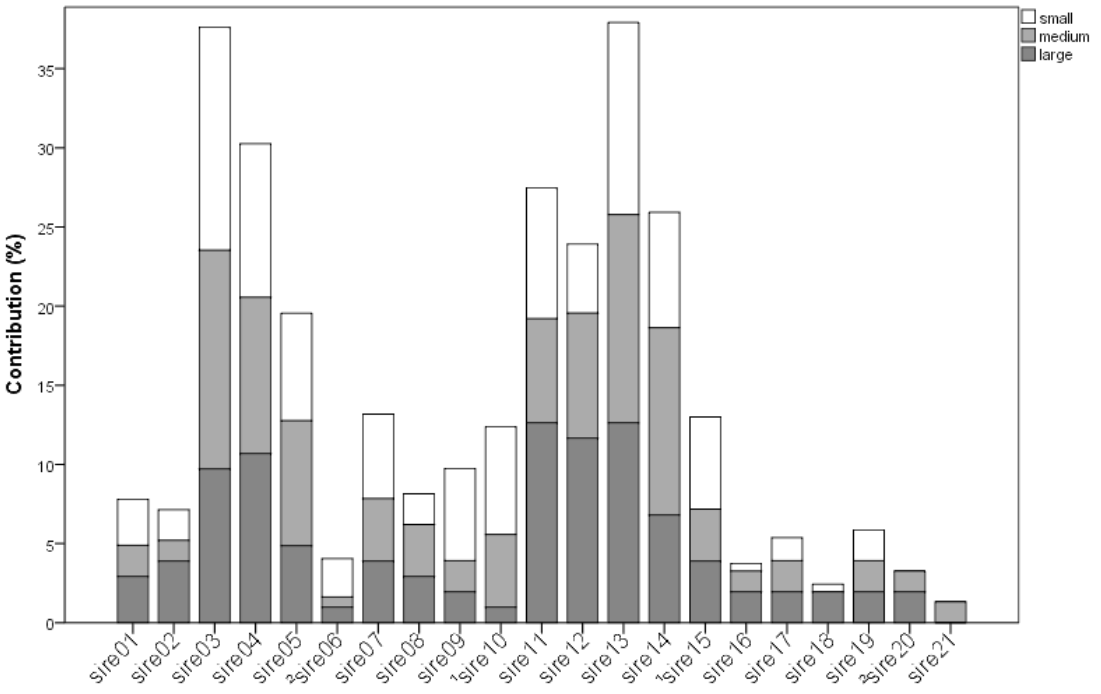


Fig. 6. Dam contribution from spawn B at 18dph (a) and 90dph (b) for each size grade (small, medium and large). Numbers in superscript indicate the number of private alleles detected for the specified dam.

(a)



(b)

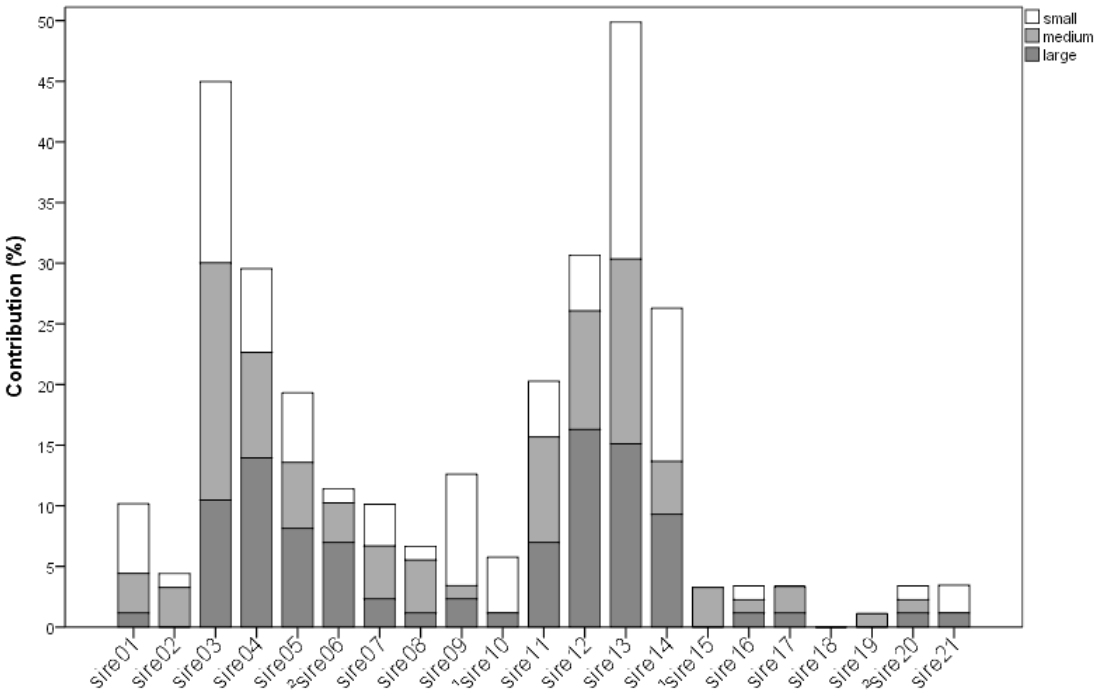


Fig. 7. Sire contribution from spawn B at 18dph (a) and 90dph (b) for each size grade (small, medium and large). Numbers in superscript indicate the number of private alleles detected for the specified sire.

The production of half and full-sibling families

From a total of 10 dams and 21 sires, the maximum number of full-sibling families detected was 103 at 18 dph from spawn B (Table 5, *n* = 472). The total number of full-sibling families detected was dependant on sample size, as there was a considerable increase 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 families detected per 200 samples (n_{200}) was calculated at 1, 18 and 90 dph as 57, 43 and 55 respectively. All 21 sires were detected as parents to the paternal half-sibs at 1 and 18 dph in spawn B, whilst a maximum of 10 dams were identified as parents of the maternal half-sibs (among offspring tested at 90 dph).

Genetic diversity

A total of 73 alleles were recorded in the broodstock across 17 polymorphic microsatellite markers, ranging from 2 to 8 alleles per locus and at an average of 4.3 alleles per locus (Table 6). Thirteen private alleles (an allele detected in only one broodstock individual) were detected and private alleles 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 F_{is} values were positive at seven individual loci, suggesting a slightly higher than expected number of homozygotes. Overall relatedness was low within the broodstock group (0.08), as calculated from the dyadic maximum likelihood (ML) estimator. The dyadic ML estimator returned the highest correlation with the true relatedness values (*r* = 0.83), when compared to six other relatedness estimators. 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 of genetic variation at microsatellite loci 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 7), 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 the allelic richness from parent to offspring was detected at 1 dph, from spawn A and B respectively. Over both spawning nights, observed heterozygosity was lower in the offspring at 1 dph, when compared to the broodstock population and average F_{is} was positive among the progeny sampled from spawn A. 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.

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 7). By 90 dph, the number of alleles including those deemed private in the broodstock actually increased when compared to 1 dph. The number of alleles across the size grades at both 18 and 90 dph ranged from 66 to 67. No loss of allelic

richness (R_s) was detected when comparing offspring across 1, 18 and 90 dph (R_s was higher at 90 dph possibly due to sampling error). H_o levels were higher than H_e in every case ($P < 0.001$) and deviations from HWE were detected at locus *Lca*287 ($P < 0.001$) for each size grade sampled at 18dph (excluding the large size grade) and 90dph.

Fate of rare alleles among the offspring

In total, five 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 that generation (Table 5). Following correction for allele stutter bands, pooled egg and larvae samples also failed to detect these five alleles in the population at 1 dph. One of the private alleles belonged to sire 20, whom was a very low contributor (< 2%) across both spawn A and B (Fig. 5). The remaining four private alleles belonged to dams 10 and 11 but neither dam contributed to the spawning events (Fig. 4). On the other hand, dam 04 contributed as much as 48% to spawn A but only one private allele was observed for this individual (117 at *Lca*064), which had an allele frequency ranging from 0.030 to 0.132 among the offspring (Table 8). In total, eight private alleles were detected in broodstock that were low contributors at 1 dph (< 1.2%), and allele frequencies in the offspring for these eight alleles were no higher than 0.029.

Table 5. The number of full-sibling (FS), maternal half-sibling (Mhs) and paternal half-sibling (Phs) families detected across the first (spawn A) and second night (spawn B) of spawning. The ratio of family number to sample size is presented in parentheses

		FS	*Mhs*	*Phs*
Spawn A				
1dph		59 (0.32)	7	19
Spawn B				
1dph		78 (0.28)	9	21
18dph	Total	103 (0.22)	9	21
18dph	Small	74 (0.16)	8	19
18dph	Medium	64 (0.14)	6	20
18dph	Large	47 (0.10)	6	20
90dph	Total	77 (0.28)	10	20
90dph	Small	47 (0.17)	9	17
90dph	Medium	47 (0.17)	9	18
90dph	Large	42 (0.09)	8	17

Table 6. Genetic diversity estimates for 33 broodstock; sample size (N), number of alleles (k)[a], number of private alleles (k_a)[a], allelic richness (R_s), expected (H_e) and observed (H_o) heterozygosity, fixation rate of inbreeding (F_{is}). P-value calculated at 17 microsatellite marker loci[b]

Locus	*N*	*k*	k_a	R_s	H_e	H_o	F_{is}
Lca003	33	2	-	2.00	0.282	0.273	0.034
Lca008	33	3	1	2.55	0.144	0.152	-0.053
Lca016	33	6	3	4.70	0.348	0.364	-0.046
Lca020	33	4	1	3.57	0.403	0.455	-0.129
Lca021	33	5	-	4.81	0.682	0.758	-0.113
Lca040	33	3	-	3.00	0.664	0.515	0.227
Lca057	33	4	-	3.93	0.611	0.636	-0.042
Lca058	33	7	1	6.49	0.761	0.727	0.045
Lca064	33	8	1	7.57	0.859	0.909	-0.059
Lca069	33	3	-	2.82	0.418	0.394	0.058
Lca070	33	4	-	3.75	0.569	0.576	-0.012
Lca074	33	3	-	2.99	0.319	0.364	-0.143
Lca098	33	4	1	3.82	0.428	0.333	0.225
Lca154	33	4	-	3.82	0.545	0.697	-0.285
Lca178	33	4	2	3.40	0.49	0.485	0.011
Lca287	33	7	3	5.73	0.697	0.545	0.220
Lca371	33	2	-	2.00	0.441	0.576	-0.313
Total		73	13	3.94	0.509	0.515	-0.022

[a]Totals at *k* and k_a are counts, whilst the remaining totals are averages.
[b]non significant departure from Hardy-Weinberg equilibrium at all loci following sequential Bonferroni correction (Rice, 1988).

Table 7. Measures of genetic diversity; Sample size (N_c), number of alleles (k), number of private alleles (k_a), expected (H_e) and observed (H_o) heterozygosity, allelic richness (R_s), fixation rate of inbreeding (F_{is}), effective population size (N_e), inbreeding coefficient (ΔF) and N_e/N_c ratio. Spawn A and B represent the first and second night of spawning respectively

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

Table 8. Allele frequencies of 17 microsatellite loci for broodstock and offspring, divided into multiplex one (a) and two (b). Spawn 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)	1dph (182)	1dph (274)	18dph (472)	S (208)	M (158)	L (106)	90dph (276)	S (92)	M (92)	L (92)
Lca003	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
Lca016	(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
Lca040	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
Lca057	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

(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)
Lca008	(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
Lca020	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
Lca021	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
Lca058	(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
Lca064	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
Lca069	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
Lca070	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
Lca074	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
Lca098	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	-	-	-	-	-	-	-	-	-	-

Comparison of different source stocks for establishing base populations for selective breeding

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.

Approval from government agencies for the translocation of animals needed to establish the program and supply farms on the mainland

Kerrod Beattie, Aquaculture Committee Member (Queensland), Department of Employment Economic Development and Innovation, wrote to ABFA 23 May 2011 after seeking resolution from State Aquaculture Committee (AC) members for the translocation of barramundi across state borders as a product of future selective breeding strategies for the barramundi industry.

The consensus from the state representative AC members was that, "....if these progeny were to be placed in closed-recirculating pond and/or tank production systems they would not pose a significant threat and so was supported (noting that some jurisdictions already allow this practice)." An exception raised by AC members was that, "some jurisdictions, however, require that stocks used in cage culture systems must be endemic to the area, therefore the selected lines proposed in this project might not be allowed to be placed in these systems."

The AC members also recommended that additional consultation with the Australian Government should occur if the activities fall outside of State or Territory jurisdiction and that disease protocols for translocation vary in each jurisdiction, and that these should requirements should be discussed with the relevant agencies prior to any translocation.

It was also suggested that the ABFA contact the office of the Gene Technology Regulator of the Department of Agriculture Fisheries and Forestry to hear their views on the proposal.

Database for selective breeding

An access database was created to allow the participants in the project to quickly and simply record data required for genetic management and to generate useful reports (Figure 8). The database is available on request from the Australian Seafood CRC for ABFA members (Appendix 5a). The following information can be entered into the database at the following stages in the process:

- Broodstock collection: Pit tag, sec, wild location, GPS, date collected, collector and weight at collection

- Quarantine: Tank ID, date of entry, current tank, feed type, feed weight, broodstock tank conditions, treatment type, treatment dose and treatment duration. For each fish within each tank, information about the PIT tag, transfers from and t tanks, fish weight and other observations can be recorded.
- Spawning events: Date of spawn, time, tank, total number of eggs, number of viable eggs, plate assessment data and egg incubation data.
- Grow out: Date recorded, transfers between tanks, number of fish, total weight, feed, feed intake, number of mortalities, temperature, flow rate and other comments can be entered.
- Tagging: PIT tag, date, spawn batch (F1 source or date collected if sourced from other localities)
- Mortalities: PIT tag, date found, cause, weight, other observations
- Sample for DNA testing: Date sampled, tube number, PIT, Facility, Wild location and comments
- Allocation of parentage from genotypes: PIT tag, dam PIT tag, sire PIT tag

All the above information exists in a series of tables in the background of the database. Data entry is done by pressing appropriate buttons and using drop down lists where appropriate. The drop down lists enforce adherence to use of set terms and descriptions so that the data can be simply and accurately categorised for reporting purposes. Drop down list categories include:

- Maturation terms
- Facility/tank names
- Disease status
- Wild source locations
- Cause of death terms
- Feed type
- Hormone type
- Treatment type

All data is linked and can be queried, and a number of set queries and reports have been created:

- Fish can be tracked and found
- Checks can be made on the biomass existing in tanks
- Transfers between tanks can be checked
- PIT tags by dates and current tank can be checked

The database was tested and improved in April 2013. More reports were created for different purposes. Data on existing broodfish was entered into the database. The database was rolled out to the Darwin Aquaculture Centre (DAC) and Good Fortune Bay (GFB) hatcheries. The database has been made freely available under agreement that the creator takes no responsibility for tailoring it to suit specific hatchery needs, or for any errors etc. that may exist, or may be introduced, with changes to the database.
Background tables in the database could be transferred to another database system in the future if that is required (eg. Microsoft SQL server). Nick Robinson has implemented a similar database transfer for another project. After transfer, the original Access database, containing queries, forms and reports, acts as a user-friendly “front end” and links to tables containing data on the Microsoft SQL server.

The server is secure, accessed seamlessly over the internet by the front-end, and contains all the raw data. In this way it is possible to reliably coordinate multiple users of the same database and to securely maintain and backup the data on a central server. It is also possible to restrict access to the raw data and raw database structure while giving users the freedom to create their own queries and reports based on the data.
When the database was rolled out to DAC and GFB a number of improvements were made and incorporated into the original database which was designed for yellowtail kingfish (in response to suggestions and requirements of the new users):

- A simple guide to use of the database was produced and distributed (Appendix 5b).
- The entry of data regarding broodstock within tanks (date of entry, PIT, tank, weight, stage of maturation, oocyte diameter, external markings, hormone type, hormone dose and other observations) was split from the entry of information regarding tank treatments and conditions.
- A much more comprehensive set of data entry fields was added regarding tank conditions and treatments. This includes selection of multiple feed types and quantities fed, treatment types, doses and durations, lighting and photoperiod type and duration, data reporter name, days old, salinity, dissolved oxygen, pH, Temperature, NH3, NO2, Algal density, algal/rotifer/artemia density etc, weight fed by feeders vs hand fed, clinical observations, mortalities and appetite etc.
- A summary report of animals sampled for DNA testing and tube numbers that can be sent to the laboratory with the samples was produced.
- A new facility was created so that DNA data on relatedness between animals can be entered, and four new reports can be made which order pair combinations of animals according to their relatedness (every animal with every other, all males with all females, all males with all other males and all females with all other females). This is useful for picking unrelated combinations of animals for group spawning.
- A table summarising the total food usage per batch can be easily generated. This is useful when comparing the performance of different batches under different diets.
- A batch delivery report which can be generated to be sent with larvae or fingerlings to customers (detailing and recording date delivered, batch number, company supplied, number delivered, average weight, weight range, average length, length range, date spawned, parentage, antibiotic use, deformities, feed type and pathology references) was created.
- A new report giving a general overview of PIT, generation, sex, weight at collection and current weight was also been created.
- Additional forms for entering customer details, staff details, and generation details were also created.

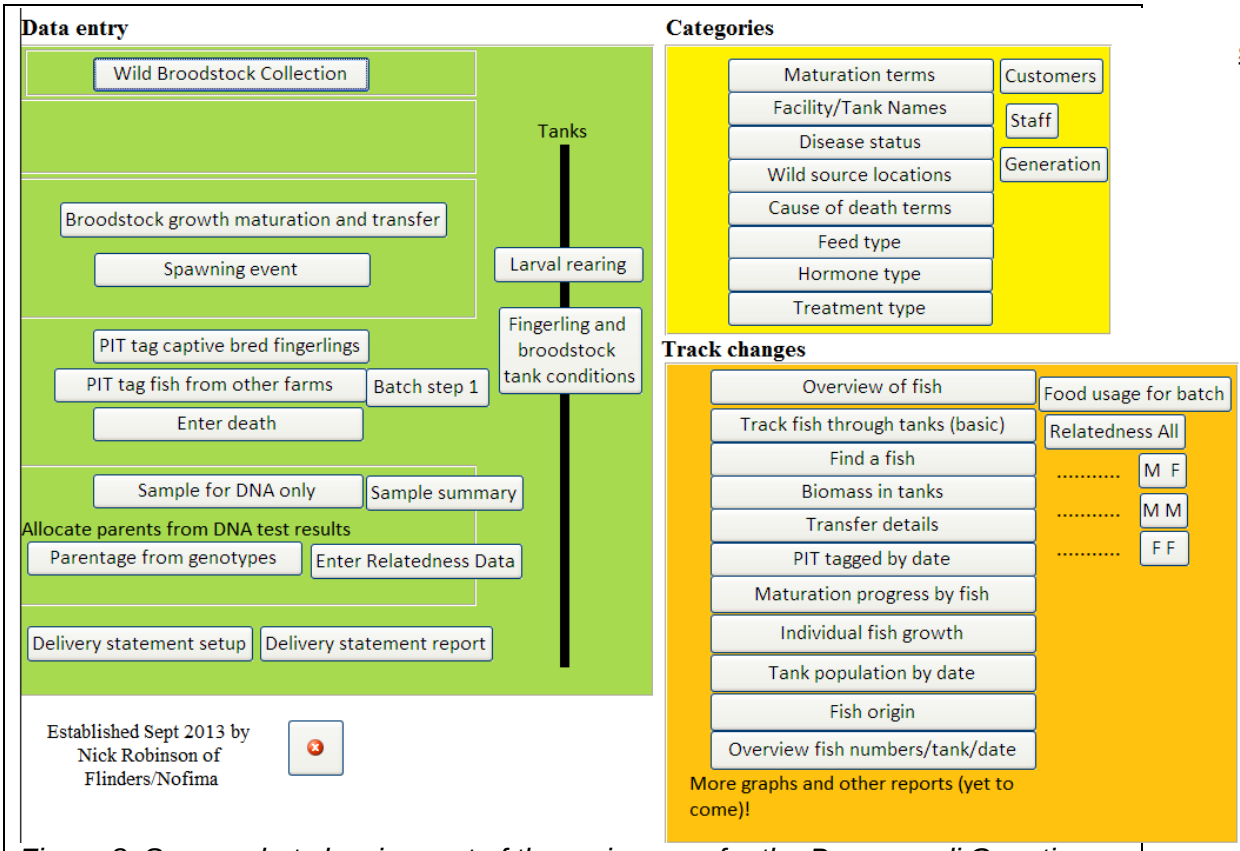


Figure 8. Screen shot showing part of the main menu for the Barramundi Genetic Management Database

Data collection – Tips on the collection of data for farm management

The spreadsheets are available on request from the Australian Seafood CRC (Appendix 6a)

This section is from a "tip sheet" was produced and distributed to barramundi farmers (Appendix 6b).

Tips on the collection of data for farm management

Nick Robinson,
Nofima and Flinders University

Data on the performance of animals and on the condition and treatment of ponds, tanks or cages of fish, can be used to help growers:

- Optimise feeding practices
- Improve growth rate
- Improve product quality
- Decrease mortalities
- Decrease risks of disease
- Manage genetics

All of the above leading to reduced costs and improved productivity and profits

The purpose of the attached simple data collection spreadsheets are to motivate barramundi farmers to collect data in a consistent and reliable fashion so that the data can be used to provide useful feedback on how to best optimise the performance of their stock. Collection of either hand-written or typed spreadsheets such as these, in a standard format, will allow the data to be easily uploaded into larger databases in the future, and could provide a means for farmers to make valid comparisons across farms if they wish to do so in the future.
The spreadsheets were prepared in consultation with a number of barramundi farmers. Farms may choose to use some or all of the sheets or fields. The sheets are designed to avoid repeat entry of information and to cover four different general data collection events:

1. Terms for farmer use - Setting standard terms to use for data collection
2. New batch - Starting with a new batch of animals
3. Fingerling and broodstock tank conditions - Regular collection of data on pond, cage or tank treatments or conditions.
4. Harvest - Collection of performance data at harvest or during grow out from a sample of fish in a batch or unit (pond, cage or tank).

There are some general principles that need to be followed when collecting such data.

A. Data needs to be consistent (so that individuals can be grouped according to environment, treatment or genetics)
    a. Repeated use of the same descriptive terms. Even small variations/errors can lead to problems. For instance, sometimes the first letter of a term might be capitalised (eg. “Male”), sometimes not (eg. “male”), making it difficult to pull all male animals into a list. Examples of such terms include:
        i. Set tank, pond or cage names (eg. “Tank 1”, “Tank 2” etc.)
        ii. Set terms describing the cause of death
        iii. Set terms describing the fillet colour
        iv. Etc
    b. Repeated use of the same measurement units should be used (eg. all weights should be specified in either grams or kilograms, but not both).
B. Data should be reliably cross-referenced
    a. PIT tags or other tags allow the performance of individuals to be tracked over time
    b. Spawn batch numbers or cohort IDs allow the farmer to trace back a batch of fish and relate the performance of the group to particular spawning events, environmental changes or treatments.
    c. Date/time of collection allows the farmer to track when a measurement was taken in relation to days since the batch was spawned
    d. By recording the tank, pond or cage the farmer can group animals grown in a common environment
C. Unnecessary repetition of data entry should be avoided. For instance, enter the date of collection once, not for every measurement made on fish on that date. But ensure that the date is captured and linked to the measurements made on that day.

D. If possible, automate data collection. PIT tag readers and balances can be linked to computers so that readings go directly into spreadsheets or databases. This reduces human error.
E. Check data for errors.
    a. Set thresholds which can be used to alert those handling and entering data to glaring mistakes. For instance, are there fish that are 10X heavier or 10X lighter than the average measured in the same batch? Could this be due to entry mistakes? Excel and Access can be used so that they warn you when glaring mistakes are made as the data is entered.
        i. Excel allows you to “validate input values”. Eg. Input values in a column could be limited to between 0.01 to 0.99. You can choose whether the data should be entered as whole numbers, decimals, lists, dates, times, text of specific lengths or whether any value can be accepted. Specific maximum and minimum values can be set. Excel will beep and give a message if the values are outside what is specified.
        ii. “Speak on enter” makes excel talk to you (ie. It says what you have entered as you enter it). This feature can be used to double check your typing.
    b. Where possible, enter the data directly into a computer based spreadsheet or table so that mistakes can be double-checked and corrected on-the-spot.
    c. Create a “list” in excel or access and only allow values that occur in that list so that consistent terms are used.
    d. Use the “auto complete” feature. Ie excel looks at previous entries and tries to complete the data entry for you. Hit enter if it has picked the correct entry to save time and avoid errors for long entries.
    e. Curate and proof test your data. Before uploading the data into your main database, run some checks on the data.
F. Consider having one person responsible for data entry in your organisation (somebody with good typing skills, experience, training and who has proven to be reliable at data entry). By having a single data handler you can avoid confusion (eg. due to multiple entry of the same data) and can improve the accuracy and usefulness of your data. But make sure there are others that are trained to take over the data entry in case.
G. Back up your data regularly and store it in a second location (or on “the cloud”).

The attached spreadsheets give a simple example that farmers may find useful to follow. All farms are welcome to add or modify fields in the sheets according to their own requirements. If you are unsure about how to use the features mentioned above in Microsoft Excel or Access just search on the internet for some tips. Please send your feedback for improvements to nick.robinson@nofima.no.

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4. Discussion

Commercialisation

Commercialisation of Barratek will only be possible if start-up funding is sourced or contributed by industry participants.

The business modelling estimates that within 5 years the BBP will be self-funding and generating at a profit. When genetic improvement can be demonstrated (evaluation of first and subsequent generations of selected stock) it is anticipated that industry take up of Barratek stock will increase.

As the profitability of barramundi farming in Australia further improves, due to the productivity increases made from the use of Barratek progeny, it is anticipated that interest and increased involvement in barramundi farming would follow as profitability increases.

Characterisation of available broodstock and identification of foundation stock

All eight hatcheries are involved in fingerling production for sale and transfer to grow out facilities both locally and overseas, and most of the eight also maintain a proportion to be grown at their own facility. Most of the fingerlings are grown as food fish for the restaurant trade, however, it is uncertain whether some of these juveniles are included in wild restocking practices. If this is the case the results presented here provide valuable information into the origin of broodstock, which can be crosschecked to the restocking locations to provide the correct genetic lineage prior to restocking. Captive bred broodstock that have been developed following multiple generations of captive culture should not be utilised as the founders of a wild restocking program, as adaptive genes can be influenced by the captive environment.

When broodstock origins were recorded by the hatcheries, assignment tests were able to allocate the majority of these individuals to their correct wild site. The results also detected that mixing of captive stocks between hatcheries occurs, particularly within the QLD groups and the WA hatchery which were found to hold stock from all three regions. Allelic richnesss did not significantly differ between any of the major wild genetic barramundi stocks. If genetic ‘hot spots’ of diversity exist amongst the wild localities, and if this diversity was not already represented in hatcheries, we would have recommended that broodstock be sourced from these regions for the selective breeding program.

Once the industry decides which existing broodstock fish can be used to found the breeding program, the information collected in this project will be used to determine whether and which wild populations need to be sampled to give adequate genetic diversity. This is ongoing work that will form part of Shannon Loughnan’s PhD thesis to be completed by the end of 2013. However, we can say at this stage that the current population of captive broodstock contains a large amount of the allelic richness found in wild populations around Australia, and would therefore be highly suited for establishing the selective breeding program.

In forming the foundation broodstock population we need to source a genetically diverse group of animals while at the same time giving more weight to populations that have proven to perform well under aquaculture conditions. In order to limit

inbreeding and loss of genetic diversity to acceptable levels we would recommend that more than 50 males and 50 females are bred each generation. Therefore we would recommend in broad terms using existing captive broodstock, which are genetically diverse, chosen as strong performers and sourced from multiple populations around the country, and supplementing numbers with mature fish caught from wild Queensland populations down the east coast, such as Qld 11 from McArthur and Leichardt River systems and Qld12 from Gilbert and Archer River systems where allelic richness is greatest.

Pilot scale trial of synchronized spawning

Although contributions were skewed, 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%, however there was no significant difference in the level of broodstock contribution between 1 and 90 dph from spawn B, or between the size grades at 18 or 90 dph, in spite of the majority of the small size grade being culled on three occasions. Contributions of up to 77% (Frost et al., 2006) and 98% (Wang et al., 2008) have been reported for individual barramundi broodstock under other spawning runs, and heavily skewed broodstock contributions are also common for other mass spawning aquaculture species (e.g. flounder, *Paralichthys olivaceus* Sekino et al., 2003; gilthead seabream, *Sparus aurata* Chavanne et al., 2012; common sole, *Solea solea* Blonk et al., 2009). For final gonad maturation and to promote the release of gametes for artificial spawning, the application of LHRHa proved beneficial to all dams, as demonstrated by the lack of or low levels of contribution detected by the two dams that were not injected in this study. 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 genetic diversity from broodstock to offspring at 1 dph as detected by R_s, 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 grading and culling. Average R_s ranged from 3.33 to 3.55 in the offspring, whereas R_s was estimated at 3.94 in the broodstock group. Higher levels of average R_s have been reported for other captive barramundi populations (Yue et al., 2009), ranging from 3.57 to 4.80 in three Australian farms and as high as 7.89 in a captive Singapore population and 6.65 in Taiwanese farmed stock. Subsequent sampling at 90 dph (spawn B) showed a higher average R_s when compared to 1 dph offspring and this is probably due to the larger available sample size and sampling error. This highlights that the comparison of R_s across different populations should be treated with some caution, especially when few markers or individuals are compared.

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 form of optimum contribution selection adapted to this situation (see Meuwissen and Sonesson, 2004), 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.

The differences in broodstock contribution achieved in this 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 could 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 may 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). Protandry in barramundi also presents a challenge for selective breeding and the cryopreservation of sperm would enable broodstock candidates from the same generation to be selected and mated. Selective breeding programmes for barramundi utilizing strip spawning and cryopreservation have been modelled and the use of these techniques would result in higher long-term benefit-cost ratios than the use of mass spawning (Macbeth et al., 2011; Robinson et al., 2010). Despite the benefits of strip spawning and cryopreservation, the feasibility of the technique remains uncertain for barramundi and further development is required.

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. Some 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. Relative allele frequencies were not estimated from the pooled genotypes because particular eggs or larvae may contribute more DNA to the pool than other individuals. For the calculation of relative allele frequencies from pools, some corrections would need to be made to account for stuttering and other PCR anomalies affecting electropherogram peak height. There might be some cost benefits if pooled genotypes alone could be used to study the relative level of contribution, although more research comparing predicted allele frequencies from pools to actual allele frequencies in the population would be needed. Selective DNA pooling has been used effectively to detect QTL for growth rate in aquaculture species (Baranski et al*.,* 2008) and DNA pooling is a popular method for large scale association studies for the detection of common diseases (Sham et al., 2002). Our study has shown that the absence or presence of private alleles in the pools, together with the allele frequencies calculated from genotyped individuals, assisted in reporting broodstock contribution and provided a cross check with parentage analysis software output. Depending on the probability of assignment for particular genotypes, calls by the parentage analysis software can be erroneous. Manual checking and additional markers may be needed when the probability of assignment is low to give certainty over parentage. Such checks would add additional expense to the selective

breeding programme and could hamper selection decisions when mass spawning is used.

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 programme 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 to 50 progeny per broodstock pair need to be tested if inbreeding is to be limited to less than 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 will 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 programme, as it allows minimization of possible confounding between additive genetic, maternal and paternal effects (Gjerde, 2005). For a given number of spawning tanks under a normal 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 is 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.

Establishing a base population of barramundi for selective breeding

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 earlier in this report using a large mass spawning group (Loughnan et al., 2013). With an N_c of 150, N_e was estimated at 76 and ΔF at 0.7%, which were outside the desired values of N_e >100 and 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 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 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. The simulation model could be a valuable tool to apply to other mass spawning species under aquaculture production.

5. Benefits and Adoption

As yet there is no selective breeding program established for Barramundi in Australia that can benefit from and adopt the results and recommendations of this study. However there at least two hatcheries that are armed with all the tools (databases, background knowledge etc) needed to run a selective breeding program We recommend that interested farmers or hatchery managers take the initiative to join together and invest to begin selective breeding using the recommendations in this report under appropriate genetic advice.

6. Further Development

The ABFA strongly endorses the Barratek model and believes it is key to improving productivity and profitability in the Australian barramundi farming industry.

The ABFA and the Board of Barratek are continuing to seek funding to start up the operation.

Specific actions in relation to the future of Barratek lie with the Board to determine the best course of action for Barratek.

A letter seeking expressions of interest for investment in barramundi selective breeding will be distributed with this report.

7. Planned Outcomes

Public Benefit Outcomes

The outcome of this project will be a commercial national selective breeding entity producing genetically superior barramundi larvae and juveniles for aquaculture from 2013 onwards. The selective breeding entity has been predicted to yield a high short-term benefit to cost ratio for the barramundi industry (17:1, with a nominal economic effect on operating income of $10.2 million after 10 years of selective breeding).

Barramundi farmers will be the major investors in this new entity and will be the major beneficiaries.

Private Benefit Outcomes

The benefits accrued from the project that may lead to positive private benefits outcomes include;

- The mapping of the genetic make-up of the broodstock used in the Australian barramundi farming industry - regardless of the short term future of Barratek industry members can utilise that information to improve their productivity
- Information from the successful mass spawning trial will allow the Australian barramundi farming industry to improve the transfer of genetic variation to the next generation of broodstock candidates.
- Information about sources of genetic variation in the wild stocks can be used by barramundi farmers and hatcheries to maintain a diverse and healthy farmed population.

- The Business model in place for Barratek provides opportunity for a whole of industry genetics program to be put in place when appropriate funding is sourced or industry investment is made.
- Data collection processes and tools (database) in place for implementing a selective breeding program

Linkages with CRC Milestone Outcomes

In laying the foundations for the development of a selective breeding program for Barramundi which will result in continuous improvement of production efficiency by the industry (kg production / unit pond, tank or cage) we address the CRC outcomes of “Substantially increasing the production and profitability of selected wild-harvest and aquaculture species” and “Removal or reduction of key production constraints in existing aquaculture systems”.

Specifically the project addresses:

Milestone 1.3.1 New genetic tools developed for genetic management and improvement of at least two aquaculture species, and

Milestone 1.3.2 Genetic parameters estimated for key commercial traits; genetic improvement programs designed and implemented for at least two aquaculture species

8. Conclusion

In summary, this project has set the foundations for creating continuous improvement of the efficiency of production by the Barramundi industry in Australia through the creation of an entity for the selective breeding and distribution of genetically improved fish. A company Barratek has been fully scoped and established to run the selective breeding program. A business plan and lobbying document have been created that detail the company and its operation, and present an attractive case for investment. The Australian Aquaculture Council has said that the translocation activities proposed by Barratek meet current state and territory guidelines. However, Barratech has so far failed to start the selective breeding process. Alternative sources of funds need to be investigated (including self-funding by interested industry participants).

A database of potential broodfish for establishing the selective breeding program and their genetic relationships has been established and analysed, and this has revealed where common ancestors exist and will also reveal any significant gaps in representation where additional fish from targeted wild populations may be needed.

The proof-of-concept trial spawning has shown that the genetic plan is a practical way forward. A large number of half and full-sib families could be produced for selective breeding from a mass spawn involving 33 broodstock, and 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 genetic variation from parent to offspring at 1 dph but there was no further reduction of genetic variation due to unequal broodstock contribution, size grading or the culling of the small size class. Grading methods, where under-sized animals were routinely discarded, did not affect the relative contribution made by different parents to the final batch of offspring. 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.

Genetic diversity within existing captive broodstock has been investigated and compared to wild populations around the Australian coast. Areas containing high genetic diversity, and existing stock structure boundaries, have been used as a guide for recommending where animals would be best sourced from to begin the selective breeding program. As the availability of captive broodstock is in constant change, some further evaluation may be required when the base population for selective breeding is finally formed.

A simulation model was developed and used to determine the most appropriate broodstock candidates to use when establishing a base population for barramundi selective breeding. Overall the current captive crop of broodstock would be suitable to use for inclusion into the base population, however the inclusion of wild individuals would enhance levels of genetic diversity substantially.

A database system and proposal for data collection has been devised and extended to industry to assist with the implementation of selective breeding. The industry is more aware about the importance of data collection and procedures that should be used.

Finally, an independent expert has reviewed the project and has provided support to the business model and genetic plan.

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10. Appendices, databases and spreadsheets (available from the Australian Seafood CRC on request to ABFA members)

1. Hatchery Business Model.xlsx
2. Business plan (ABFA Genetic Program Business Plan. Final.pdf)
3. Review to assess the technical structure and the scope for commercialisation of Australian breeding programs for the Seafood CRC by Dr Morten Rye (Morten Rye Review of Australian Breeding Programs and their Commercialisation_Final_Nov_2012_2.docx)
4. Lobbying document entitled “Barratek Funding Proposal” (Barratek Lobbying Document.pdf)
5. Database and guide
    - Barramundi selective breeding database (Barramundi_Database_30_sept_2013_DAC_3.accdb)
    - Easy Guide to the Barramundi Genetic Management Database.docx
6. Data collection spreadsheets and tipsheet
    - Barramundi Data Collection Spreadsheets-FARM.xlsx
    - Collection of barramundi data.docx

Photo: Barramundi Fingerlings, Nick Robinson, 2009