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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Mass spawning of broodstock

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

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

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

2.2. Size grading and sampling

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

2.3. DNA extraction

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

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

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

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

2.5. PCR amplification

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

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

2.6. Statistical analysis

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

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

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

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

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

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

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

3. Results

3.1. Broodstock contribution

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

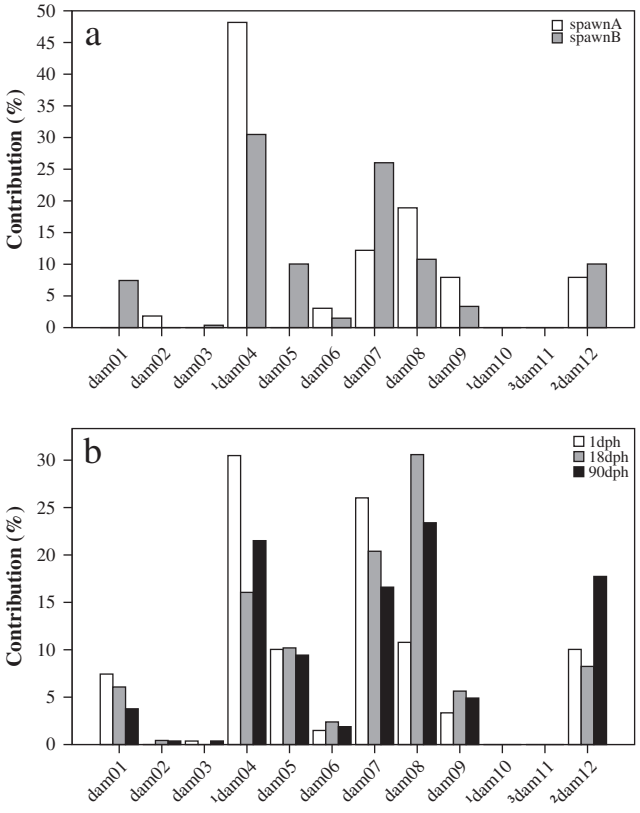

Fig. 1. Dam contribution to offspring from spawns A and B at 1 dph (a), and from spawn B over three sampling events; 1, 18 and 90 dph (b). Numbers in superscript indicate the number of private alleles detected for the specified dam.

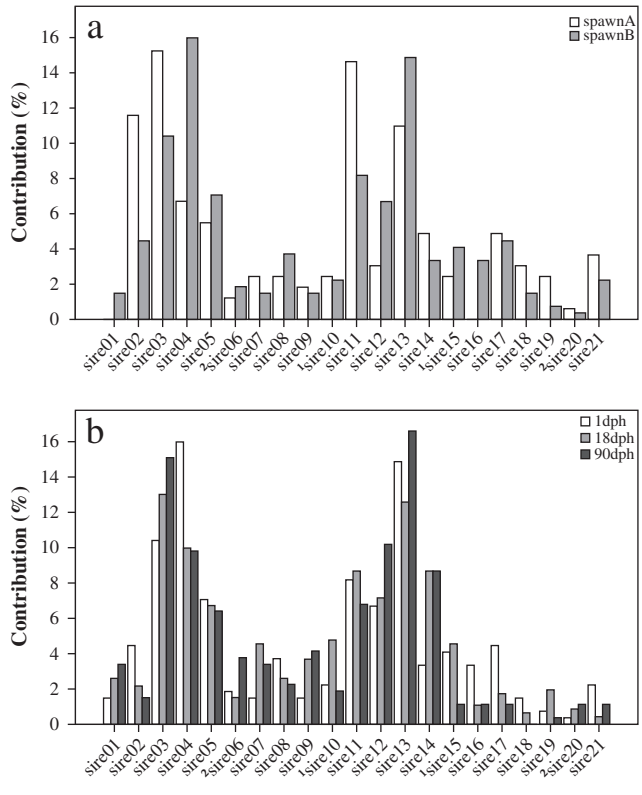

Fig. 2. Sire contribution to offspring from spawns A and B at 1 dph (a), and from spawn B over three sampling events; 1, 18 and 90 dph (b). Numbers in superscript indicate the number of private alleles detected for the specified sire.

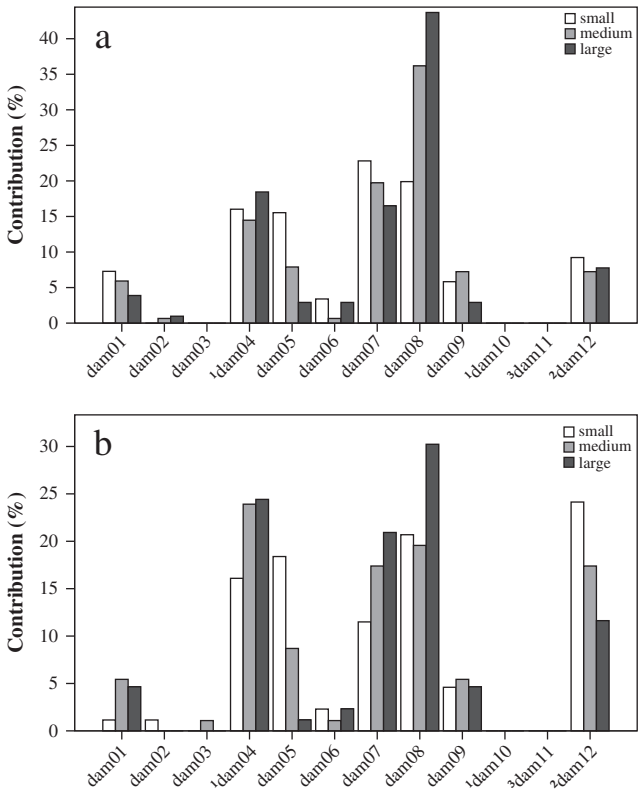

Fig. 3. Dam contribution from spawn B at 18 dph (a) and 90 dph (b) for each size grade; small, medium and large. Numbers in superscript indicate the number of private alleles detected for the specified dam.

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

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

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

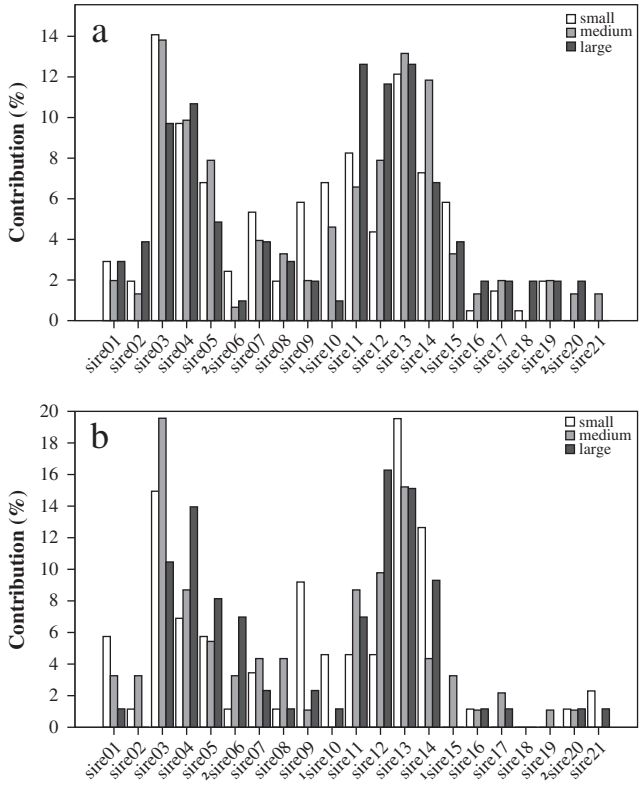

Fig. 4. Sire contribution from spawn B at 18 dph (a) and 90 dph (b) for each size grade; small, medium and large. Numbers in superscript indicate the number of private alleles detected for the specified sire.

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

3.1.2. The production of half and full-sibling families

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

3.2. Genetic diversity

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.2.3. Fate of rare alleles among the offspring

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

4. Discussion

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

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

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

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

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

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

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

5. Conclusion

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

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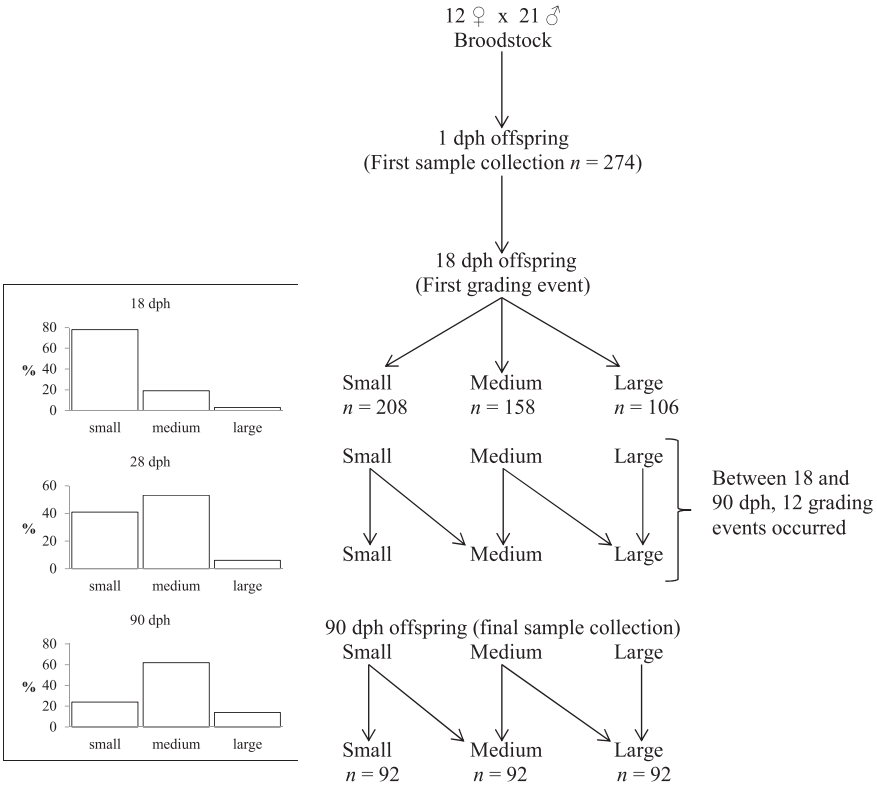
Appendix A

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



Appendix B

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

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

(continued on next page)

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Appendix B (*continued*)

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

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

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