

Unleashing the potential of all-female cobia farming

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Summary

Cobia is a pelagic fish with tropical and subtropical distribution across the globe and ideal biological attributes amenable to aquaculture: growth rates of five kg per year, excellent fillets for sashimi, relative ease of juvenile production and acceptance of formulated diets. Cobia has been farmed in Asia, the Americas and Australia. However, farmed stocks remain unimproved and the genetic basis for economically important traits are still unknown as there is very limited genomic information and resources available for the species. Given cobia is a mass-spawner, whereby single pair mating or artificial fertilization techniques are either impractical or not available for full control of the reproductive output, the development of a rapid, reliable and cost-effective genotyping strategy is the first critical genetic tool for the domestication of the species without inbreeding. DNA parentage analysis would enable our understanding of broodstock contribution to mass-spawning, and the estimation of relatedness, genetic parameters and breeding values of selected candidates.

In this study, previously reported microsatellite markers were scanned for polymorphisms and nine highly informative markers were incorporated into an optimized multiplex PCR suite (*PET_Rca1B-H09*, *PET_Rca1-A11*, *PET_Rca1B-F06*, *NED_Rca1-C04*, *NED_Rca1B-C06*, *VIC_Rca1B-E08A*, *VIC_Rca1-H08*, *FAM_Rca1-H01*, *FAM_Rca1B-D10*), which successfully amplified 97.5% of loci typed. A total of 77 alleles were identified within broodstock, averaging 8.55 (6~11) alleles per locus, 0.753 (0.69~0.82) of polymorphic information content and 0.837 (0.59~1) of observed heterozygosity. DNA parentage analysis of offspring produced in February 2018 yielded 95% success in assignment rate (74 out of 78 tested offspring). Seven families (out of two females and four males) were identified, with one of the females mothering 80% of the offspring. Paternal contributions to the spawning were less skewed, with three males contributing to ~30% of the offspring. This reliable and cost-effective genotyping strategy enabled rapid and unambiguous DNA parentage analysis for the first time in the species, and highlighted the efficacy of broodstock management at the Bribie Island Research Centre to reliably produce larvae from multiple families. The adoption of this standard marker panel in studies of wild and farmed populations will facilitate our understanding of the species diversity and contribute to the genetic improvement of cobia worldwide.

Keywords: Cobia, genetic improvement, multiplex PCR

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Background

Cobia (Rachycentron canadum) is an important commercial fish species with a widespread tropical and subtropical distribution across the globe which is being propelled into the scientific limelight for the biological attributes amenable to aquaculture - excellent quality fillets, high survival rates and most importantly due to its rapid growth rate of ~ 5 Kg in the first year. Cobia has been farmed in Asia, the American continent and more recently production has expanded to Queensland (QLD) in Australia (Lee *et al.*, 2015). However, the genetic basis for those valued characteristics are still unknown as there is very limited genomic information available for this species. There is an urgent need to develop genomic resources for this species to inform appropriate mating allocation strategies, inbreeding control, selective breeding programs and even chromosomal manipulations to exploit cobia's sexually dimorphic growth, whereby females grow 30% faster than males. Of particular importance to Queensland, the availability of all-female cobia juveniles would be of great benefit for prawn farmer's recently affected white-spot virus who want to explore alternative profitable crops. Cobia growth in SE Queensland is slower due to lower water temperatures, and thus shorter growing seasons than in the north of the state (Dutney *et al.*, 2010). By stocking all-females, farmers would not only obtain higher yields per hectare, but also harvest heavier fish which fetch a higher price per kg.

The development of a rapid, reliable and cost-effective genotyping strategy for cobia can be considered as the first critical genetic tool for the domestication of the species, in particular because cobia is a mass-spawner, whereby single pair mating or artificial fertilization techniques are not available for a full control of the reproductive output of the species. Such genotyping strategy, also known as DNA fingerprinting, would allow DNA parentage analysis techniques to contribute to our understanding of brood stock contribution to mass-spawning events and infer the relationships between captive individuals is a first step to avoid inbreeding and its negative consequences. DNA parentage analysis would also allow for whole genome sequencing of close related individuals, such as mother and son to facilitate the screening and identification of sex-specific loci.

In this study, a multiplex PCR suite of nine polymorphic microsatellite markers was developed and optimized to genotype F1 and F2 generations of broodstock held at the Bribie Island Research Centre, and offspring produced in a mass-spawning event during February 2018. In addition, DNA parentage analysis was carried out to identify the relative contribution of each brood stock, and the family crosses generated in the February 2018 spawning event.

The study originally undertook to compile a draft genome of cobia as the basis for identifying the sex determining system in this species, and use this information to develop a strategy for all female cobia production. DAF was unable to reach satisfactory agreement with BGI Australia, the agency initially earmarked to undertake the sequencing and genome construction, and no other agency could provide the services within the funding available. In addition, the recipient of the funding, Dr Jose Domingos, resigned from the Department in February 2018. A strategic decision was therefore made to undertake only the preliminary aspect of the study, that of developing an accurate, effective suite of microsatellite markers for parental determination in cobia.

Project Objectives

The initial study proposed to:

- Produce a first draft genome of cobia and identify sex-specific loci
- Elucidate the sex-determining system (e.g. XY or ZW) of cobia
- Develop a roadmap to attain all-female populations for grow-out via indirect feminisation

In light of the issues outlined above the objective was amended to:

- The development of a reliable and cost-effective genotyping strategy for DNA parentage analysis of cobia via single multiplex PCR of polymorphic microsatellite markers.

Methodology

Sample collection and DNA extraction

Cobia brood stock were anaesthetised with 20 ppt of AQUI-S® for weight checks and assessment of their reproductive conditions through cannulation biopsies, and a small piece of fin tissue (1 cm²) collected and preserved into individually labelled microfuge tubes containing 80% of ethanol for subsequent DNA extraction, PCR and genotyping. The spawning group comprised of two females (~ 27.5 Kg) with uniform eggs larger than 750 µm in diameter and four males (~ 22.1 Kg). Broodstock were induced to spawn through the injection of 0.05 µg LHRHa/Kg (2 females) and 0.025 µg LHRHa/Kg (3 males), as shown in Table 1. In addition, DNA was also extracted from fin tissues of 78 16 day-post-hatch larvae from the February 2018 spawn, two wild caught individuals from the Moreton Bay area and five samples donated from a Taiwanese farm, (supplied to DAF by staff from Rocky Point Aquaculture) to better inform allelic diversity and usefulness of each marker. DNA was extracted using Isolate II Genomic DNA Kit (Bioline), according to manufacturer's protocol and eluted in 30 µL of Elution Buffer G (Bioline). DNA quality was checked on a 1% TBE agarose gel containing GelGreen® (Biotium Inc.), where 2 µl of eluted DNA was loaded and electrophoresed for 30 min at 90 V and 400 mA.

Table 1 Details of cobia broodstock group selected and hormonally induced to spawn at the Bribie Island Research Centre (DAF-QLD) on 7 February 2018.

PIT Tag	Fin Clip	Sex	Origin	Weight (Kg)	Egg size	LHRHa (0.2 mg/ml)	Dose (µg/Kg)
91EB	C236	F	F2-13	25.0	~ 850 µm	1.25	0.050
4F6C	C245	F	F2-13	30.0	~ 750 µm	1.50	0.050
9CD6	C241	M	F2-14	23.4	-	0.60	0.026
5388	C237	M	F2-14	24.1	-	0.60	0.025
792C	C242	M	F2-14	18.0	-	0.45	0.025
A1FE	C240	M	F2-14	23.0	-	-	-

Development of a 9-marker multiplex suite and PCR amplification

A number of microsatellite markers have been developed (Pruett *et al.*, 2005, Renshaw *et al.*, 2005). Marker choice was based on a previous report (Lee *et al.*, 2015), where seven microsatellite markers (*Rca1B-H09*, *Rca1-A11*, *Rca1B-F06*, *Rca1-C04*, *Rca1B-E02*, *Rca1-B12*, *Rca1-E11*) had been previously investigated with limited success for progeny testing in Australia, whereby the last three markers were discarded due to high homozygosity and low number of alleles. In addition, another five markers (*Rca1B-E08A*, *Rca1-H08*, *Rca1B-C06*, *Rca1-H01*, *Rca1B-D10*) were added to the panel given their previously reported polymorphism (Renshaw *et al.*, 2005, Pruett *et al.*, 2006) and allelic size ranges allowing for a nine marker multiplex suite through the use of four 5' (5 prime, the end of DNA strand where fluorochrome resides) fluorochrome (FAM, VIC, NED, PET) labelling of the forward primer.

Forward (F) and reverse (R) primers published by Pruett *et al.* (2005) were first resuspended with 1X TE buffer to 100 µM and then combined into a 10X primer mix, according to the following volumes:

Rca1B-E08A – 4.2µl (F) VIC_CATATCAAGTCAATATCACAGACC, 4.2 µl (R) CCACGGAATAGCAGACTTTCTC;
Rca1-H08 – 8.0µl (F) VIC_GAGACCTACATGGCAGAAGGT, 8.0 µl (R) GACCACTCCTTTGAGGTCTCT;
Rca1B-C06 – 13.0 µl (F) NED_CCAGCATATCTCCTCTCAAGAA, 13.0 µl (R) GGCTTGAACCTTAACACAGCTCCT;
Rca1B-H09 -13.0 µl (F) PET_CATGTTATTCTCCAACATCATGG, 13.0 µl (R) GTGTATCCGCATACTTTTCAG;
Rca1-A11 – 10.0 µl (F) NED_CTACAGTGGTGTCCCTGTTAG, 10.0 µl (R) CAGTACATAGAGAAACAGGAGG;
Rca1B-F06 – 20.0 µl (F) PET_CAAGCAAATGCGTGGCCGA, 20.0 µl (R) CGTTAGCAACCACACGAGCTTG,
Rca1-C04 – 15.0 µl (F) NED_GACATCAAGTGGCACTTTG, 15.0 µl (R) CACTAACTTGTTCTCCTCTG;
Rca1-H01 – 15.0 µl (F) FAM_GTCCCAAGGGAATAGCGAAG, 15.0 µl (R) CCTCCAGACCAGACAGCAGA;
Rca1B-D10 -13.0 µl (F) FAM_GCAACTGCCTCCACCAATCA, 13.0 µl (R) CATGTGCATCGAAAGACAGAGA,

and 277.6 µl of Ultrapure water (Invitrogen), so adjusted to produce reasonable fluorescence intensities for each marker and to facilitate scoring of alleles.

PCR reactions consisted of 1.25 µl of 10X primer mix, 6.25 µl of 2X Type-it® PCR Buffer (Qiagen), 4.0 µl of water and 1 µl of DNA in a 12.5 µl reaction. Microsatellite amplification was performed on a Biorad Tetrad 2 Thermal Cycler (Biorad) using the following touch-down cycling conditions: 95 °C for five minutes 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, then a final step of 60 °C for 30 minutes. The success of PCR amplification was checked by visualisation on a 2.0% TBE agarose gel containing GelGreen® (Biotium Inc), where 2 µl of PCR products was loaded and electrophoresed for 40 minutes at 80 V and 400 mA. The remaining PCR products were diluted with 10 µl of water and desalted through a Sephadex® G-50 Fine (GE Healthcare) filtration spin column (3 min at 2000rpm). 1-2 µl of cleaned PCR products were transferred to a 96-well plate, dried at 60 °C and sent for genotyping.

Genotyping and parentage analysis

Capillary electrophoresis used for sizing of fluolabelled PCR fragments was performed at the Australian Genomics Research Facility and resultant fragment analysis was carried out using Geneious' Microsatellite plugin (Biomatters). Parentage assignment of genotyped offspring was performed using Cervus 3.0 (Kalinowski *et al.*, 2007). Samples with less than five markers genotyped (due to PCR or genotyping errors) were not tested for parentage analysis. Parentage assignment was considered successful when offspring were assigned with ≥95% confidence to a parental pair.

Results

The optimized genotyping protocol was able to successfully amplify all nine microsatellite markers in a single PCR, with 97.5% of loci typed. A total of 77 alleles were identified, averaging of 8.55 alleles per locus, 0.753 of polymorphic information content and 0.837 of observed heterozygosity (Table 2). Such high level of genetic diversity and polymorphism are critical for unambiguous parentage assignment.

Table 2. Summary statistics of allele frequency analysis for the cobia bloodstock and wild samples using 9-marker panel: number of alleles (*k*), polymorphic information content (PIC), observed heterozygosity (HObs) and alleles binned to their proximate size in bp.

Marker name	<i>k</i>	PIC	HObs	Alleles
Rca1B-E08A	6	0.669	1.000	185, 197, 200, 206, 212, 218
Rca1-H08	8	0.756	1.000	235, 243, 249, 271, 279, 281, 285, 287
Rca1B-C06	9	0.820	0.960	315, 327, 335, 343, 347, 351, 359, 379, 383
Rca1B-H09	9	0.731	0.778	166, 178, 182, 190, 194, 198, 202, 206, 210
Rca1-A11	7	0.752	1.000	159, 173, 175, 177, 183, 189, 191
Rca1B-F06	8	0.822	0.840	238, 242, 246, 274, 282, 322, 334, 342
Rca1-C04	9	0.753	0.667	232, 234, 238, 242, 246, 256, 260, 262, 268
Rca1-H01	10	0.755	0.692	272, 290, 294, 296, 300, 306, 310, 312, 316, 318
Rca1B-D10	11	0.723	0.593	136, 148, 156, 168, 172, 176, 180, 188, 192, 208, 220
Total / Average	77	0.573	0.837	-

Simulations of parental assignment in Cervus (Kalinowski *et al.*, 2007) for a situation where the genotypes of potential parents are known predicted that 100% of offspring could be correctly assigned to the parental pair with $\geq 95\%$ of confidence. In this study, two females (F2-2013 cohort) and four males (F2-2014 cohort) were allocated to spawn in the BIRC Finfish Building Tank 1 on 7 February 2018 and their larval offspring sampled 16 days post hatch. Here, 74 of the 78 larval offspring tested were assigned to their parental pair, reflecting a 95% success in assignment rate. Seven families (out of eight possible pairings) were identified (Fig. 1a), with one of the females mothering 80% of the offspring (Fig. 1b). Paternal contributions to the spawning were more even than females, with three males contributing to $\sim 30\%$ of the offspring (Fig. 1c).

Conclusions/Significance/Recommendations

- A reliable and cost-effective genotyping strategy for cobia *Rachycentron canadum* was developed in this study, where nine highly polymorphic microsatellite markers could be simultaneously amplified in a single multiplex PCR. As such, this methodology saves time, labour and reagents in the molecular laboratory, and genotyping costs.
- This technique has enabled reliable DNA parentage analysis for the first time in the species, which revealed that most brood stock selected and hormonally induced had participated in spawning and produced viable offspring. This also highlights the efficacy in the methodology employed by the cobia research team at BIRC for brood stock conditioning, reproductive assessment and induction procedures to reliably produce eggs and larvae. The high proportion of broodstock contributing to the spawning indicates that current broodstock husbandry

protocols, including the criteria used in the assessment of the suitability of broodstock for spawning, have led to the selection of broodstock which both produce good quality gametes and are reproductively competent.

- Authors encourage colleagues around the world to make use of this cobia marker panel and the methodology provided here, so that results from different wild and farmed populations can be easily compared, contributing to our understanding of the genetic diversity of this fabulous species.

Where to next

This marker panel can be used to genotype brood stock so that breeding of closely related individuals are avoided to minimize negative consequences of inbreeding depression. The use of pedigree mating enables a two-fold increase in the effective breeding efficiency (Tave, 1990), essential for managing breeding populations of large fish like cobia. This would allow breeders to apply high selective pressures while avoiding inbreeding, thus allowing for rapid genetic improvements. In addition, it can be used to determine the levels of genetic diversity in wild and farmed cobia populations and detect, and correct for, bottlenecks of farmed populations caused by domestication.

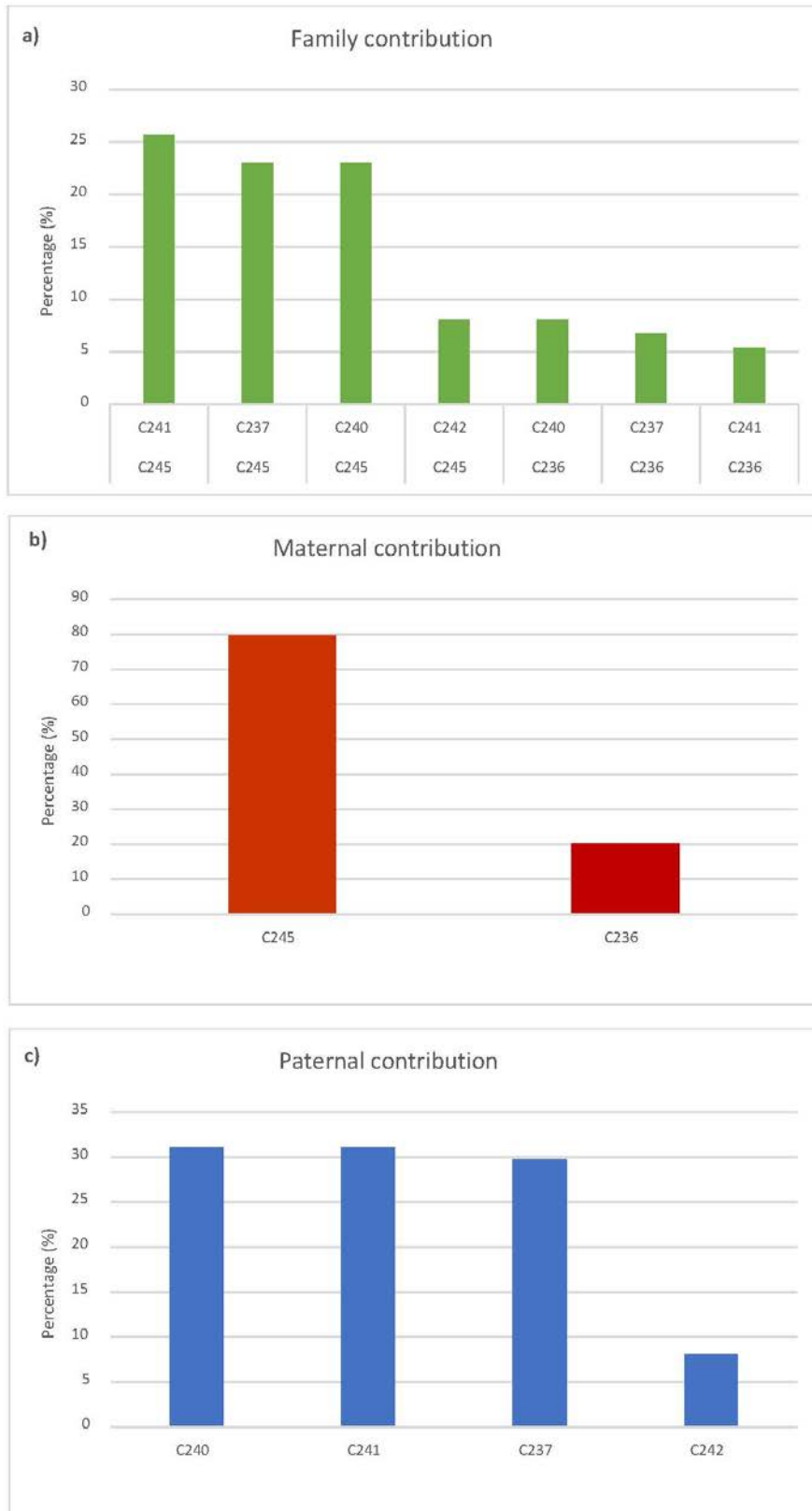


Figure 1. Family (a), maternal (b) and paternal (c) contribution of cobia to the February 2018 spawning in the Bribie Island Research Centre, identified through a nine microsatellite marker suite, optimized in this study to amplify from a single PCR assay.

Budget Summary

Goods/Services	Supplier	Expenditure \$ GST Inclusive
DNA Extraction kit and reagents	Bio line and Life Technologies Australia	\$900
PCR Reagents	Bio line Australia and Sigma Aldrich	\$766
Primers (18x)	Sigma Aldrich and Life technologies Australia	\$2300
Genotyping service Cost	Australian Genome Research Facility	\$610
TOTAL		\$4576
Original budget		\$8500

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