Aquaculture 459 (2016) 19–25

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

# A study into parental assignment of the communal spawning protogynous hermaphrodite, giant grouper (*Epinephelus lanceolatus*)

David Bright [a], Adam Reynolds [b], Nguyen H. Nguyen [a], Richard Knuckey [b], Wayne Knibb [a], Abigail Elizur [a,*]

[a] Genecology Research Centre, Faculty of Science, Health, Education and Engineering, The University of the Sunshine Coast, Queensland, Australia
[b] Finfish Enterprise, Cairns, Queensland, Australia

ARTICLE INFO

*Article history:*
Received 1 December 2015
Received in revised form 10 February 2016
Accepted 7 March 2016
Available online 8 March 2016

*Keywords:*
Grouper
Captive spawning
Parentage assignment
Microsatellites

ABSTRACT

Parental contributions of giant grouper to communal spawns in captivity are important for establishing genetic management of the species. In this study, we have followed the spawning dynamics of three males and three females over six to eight consecutive days, over three time periods. Polymorphic microsatellite markers were validated and utilised to successfully determine parentage in 574 offspring from 20 nights of spawns. Variation of both maternal and paternal contributions between nights in batches of spawns was significant ($P < 0.001$). Most paternal assignments were attributed to one dominant male who initiated each spawning batch, however, all males and females successfully mated over the spawning period. There was a significant ($P < 0.01$) trend towards a polygamous reproductive mode for giant grouper: in two of the three batches of spawns, where on some nights, eggs from all females were fertilised by multiple males. Genetic variation was assessed between parents and offspring. There was a loss of alleles on each spawning night, however, if offspring from a series of consecutive nights were combined, most or all of the genetic variation would be maintained in the $F_1$ generation. This research validates the use of molecular tools for genetic monitoring of giant grouper and improves the understanding of spawning dynamics of protogynous hermaphroditic communal spawners over time in an aquaculture setting.

*Statement of relevance:* The finding of this study shows that the dominant giant grouper male initiates spawning and is the primary contributor however other males contribute to spawns.

This contributes to our understanding of grouper spawning behaviour, and offers important information to grouper growers with respect to the need to collect eggs over consecutive days to ensure that the full genetic diversity is captured.



## 1. Introduction

The giant grouper, *Epinephelus lanceolatus*, of the family Serranidae has been IUCN listed as vulnerable due to overfishing, including destructive cyanide fishing (Halim, 2001; Mak et al., 2005; Sadovy, 1997). It is one of 159 species across 15 genera that make up the sub family Epinephelinae (Heemstra and Randall, 1993). It is one of the largest reef fish in the world and grows to 2.3 m, weighs up to 400 kg and lives for up to 40 years (Heemstra and Randall, 1993; Zeng et al., 2008). Giant grouper has a broad distribution, from East Africa to Hawaii, but has a low population density within this region (Lau and Li, 2000). To date, there have been no broad scale surveys to accurately assess the size of the population (Yang et al., 2011). Over the last two decades, along with other grouper species, the giant grouper has been a target for Southeast Asian and Indian Ocean fishermen, especially for the live reef food fish (LRFF) trade in Hong Kong and mainland China (Johannes and Kile, 2001; Mak et al., 2005; Muldoon et al., 2005; Shakeel and Ahmed, 1997; Tew et al., 2011).

Giant grouper, like many species in the Serranidae family, is a fecund, aggregative spawning protogynous hermaphrodite; however, little is known about reproductive sex ratio (number of males to females) at spawning aggregation sites in the wild. A sex ratio of one large male to up to five females has been reported for other aggregative spawners in the family (Rhodes and Sadovy, 2002). This can impact on the genetics of cultured giant grouper in two ways (Liu et al., 2005). Firstly, it is not possible to determine with confidence who the mother and father of offspring from a communal spawn are without some type of DNA tagging (Wang et al., 2010). The outcome of lack of parentage assignment in an aquaculture setting is that superior offspring selected for future broodstock may be related, leading to inbreeding. Secondly, only a small number of males may contribute to spawning, thereby reducing the genetic diversity of the offspring, which if released, may have a negative effect on genetic diversity of wild stocks (Allendorf and Phelps, 1980; Hara and Sekino, 2003; Wang et al., 2010). A recent study of giant grouper aquaculture in Taiwan highlighted the shortage of male broodstock relative to females and claimed that the majority of giant

* Corresponding author at: 90 Sippy Downs Drive, Sippy Downs, Queensland 4556, Australia.

http://dx.doi.org/10.1016/j.aquaculture.2016.03.013
0044-8486/

grouper in the marketplace are derived from inbred broodstock (Kuo et al., 2014).

Pedigree development and parentage assignment can be achieved using polymorphic DNA microsatellites to establish genotypes of offspring and where available, that of the broodstock (Wang et al., 2010). Giant grouper is a relatively new aquaculture species and, as such, there has been limited research into the genetic diversity of cultured or wild populations using molecular tools such as DNA microsatellites (Yang et al., 2011). Microsatellites are one of the most widely used molecular techniques for assessing the genetic variability and pedigree tracing of wild and cultured marine fish species (Antoro et al., 2006; Chistiakov et al., 2006; Liu and Cordes, 2004; Perez-Enriquez et al., 1999; Rhodes et al., 2003; Schunter et al., 2011; Wilson and Ferguson, 2002). Microsatellite markers, comparing allele number, heterozygosities and Wright's F-statistics including $F_{ST}$ values, have been used to estimate the genetic diversity within and between populations (including many grouper species) (Antoro et al., 2006; Hara and Sekino, 2003; King et al., 2001; Liu et al., 2005; Perez-Enriquez et al., 1999; Rhodes et al., 2003; Rivera et al., 2010; Schunter et al., 2011; Wang et al., 2010, 2011). Some giant grouper microsatellites (Rodrigues et al., 2011; Yang et al., 2011; Zeng et al., 2008) have been identified but have only been utilised in a few population studies or genetic breeding programs (Kuo et al., 2014). Kuo et al. (2014) tested the suitability of microsatellites from other grouper studies and highlighted six loci that would allow for high parentage assignment accuracy.

Giant grouper has spawned in captivity when there are at least two males and multiple females in a tank (Knuckey and Reynolds, unpublished). They spawn over a batch of six to eight nights at a certain time in the lunar cycle. To understand giant grouper captive spawning dynamics we set out to determine parental contribution in giant grouper communal spawns and how these vary over a batch of nights in a spawning period.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

Giant grouper broodstock were maintained at the Finfish Enterprises facility in Cairns, Queensland, Australia. Samples from 34 wild-caught broodstock and 576 eggs from 20 spawns from one spawning tank were collected. The spawning tank consisted of three males and three females which spawned over a four month period. The spawning events were on the 22–29/07/2012, 14–19/09/2012 and 14–21/10/2012 on a lunar cycle (six, six and eight nights per batch). Up to 30 samples were collected and analysed for each spawning night. Fin clip and egg samples were stored in collection tubes containing 70% ethanol. Total genomic DNA was extracted from fin clips using a modified salt (NaCl) extraction method (Lopera-Barrero et al., 2008). Samples of approximately 20 mg of giant grouper fin clips in Eppendorf micro tubes were mixed with 550 μl of lysis buffer (50 mM Tris–HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl) plus 1% SDS, digested by adding 8 μl of 0.2 mg/ml proteinase K, then incubated at 50 °C for 2 h during which time tubes were inverted and vortexed at 30 min intervals. After digestion, 300 μl of 5 M NaCl was added then samples were chilled on ice for 10 min before being centrifuged for 10 min at 13,000 rpm. The supernatant was transferred into new micro tubes and centrifuged for 15 min at 13,000 rpm. The supernatant was transferred to a new micro tube, containing 700 μl of ice-cold absolute ethanol and inverted 50 times to precipitate the DNA, then stored at 4 °C overnight. The next day, DNA samples were pelletised in a centrifuge at 13,000 rpm, washed with 700 μl of 70% ethanol and re-suspended in molecular grade water. Egg genomic DNA was extracted using a commercial DNA extraction kit due to very low expected yield from the fertilised eggs (DNeasy 96 Blood & Tissue Kit, QIAGEN).

### 2.2. Microsatellite markers and genotyping

Forty-eight DNA microsatellite markers were considered from three sources (Rodrigues et al., 2011; Yang et al., 2011; Zeng et al., 2008), of which a shortlist of 30 M-13 labelled microsatellite primer pairs was tested (Schuelke, 2000). Primers were initially tested using touchdown PCR in an attempt to amplify candidate loci from eight genomic DNA samples of giant grouper.

Amplification was conducted using an Eppendorf Thermal Cycler with each sample containing 15 μl:9 μl of molecular grade water, 1.5 μl of 10× PCR Buffer, 0.3 μl of 0.2 mM dNTPs, 1.125 μl of 1.875 mM $MgCl_2$, 0.3 μl of Taq polymerase, 1 μl of 50 ng genomic DNA template, 0.3 μl of 10 μM of both the forward primer and the fluorescent dye, and 0.6 μl of 10 μM reverse primer. The touchdown PCR conditions were 94 °C for 3 min followed by 20 cycles of 30 s at 94 °C, 30 s at 62 °C (decreasing by 0.5 °C per cycle) and 45 s at 72 °C, followed by 15 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C and a final extension of 10 min at 72 °C. PCR products were visualised with ethidium bromide on a 1.5% agarose gel to confirm that satisfactory PCR amplification has occurred and to determine amplification amounts so dilution rates for fragment analysis can be established. Fragment analysis of PCR products was carried out using the AB3500 genotyper with GeneScan LIZ 600 as a size standard (Applied Biosystems). Allele scoring was conducted using the computer program GeneMarker v. 1.95 (SoftGenetics LLC, State College, PA).

### 2.3. Parentage assignment

Seven primer pairs were selected for parentage assignment. Thirty-four broodstock and 576 offspring DNA were amplified, genotyped and scored following the above protocols. Parentage assignment was conducted using Cervus v. 3.0.3 (Kalinowski et al., 2007). Allele frequency analysis (including combined non-exclusion probabilities for first-, second, and parent-pairs), simulation of parentage analysis, and parentage analysis (parent pair- sexes known) were conducted with parentage assignment based on relaxed and strict LOD scores of 80% and 95% confidence. Number of alleles (N), homozygosity (Ho), heterozygosity (He), conformity to Hardy–Weinberg equilibrium (HWE) and polymorphic information content (PIC) were also calculated. Genotyping errors including the presence of null alleles, stuttering and large-allele dropout were assessed using Micro-checker v. 2.2.3 (Van Oosterhout et al., 2004).

### 2.4. Statistical analysis

Statistical analysis was performed on 576 fertilised egg samples collected over three successive spawning batches (20 nights in total). We conducted the following analyses:

#### 2.4.1. Parental contribution in spawning batches and nights

To examine variation in the contribution of parental breeders when they were assigned over a batch of nights or over the entire spawning period (three spawning batches over 20 nights), likelihood chi-square test in SAS (SAS Inc., 2009) and also reanalysed in SPSS version 22 (Nie et al., 1975). Further analysis investigated the contribution of sires and dams to total phenotypic variance of the observations (i.e. the proportion of offspring contributed by each pair of parents). In this analysis, the general linear mixed model included the fixed effects of spawning batch ($B$) or nights within a batch. The random terms in the mixed model were sires ($s$) and dam nested within sires ($d$). In mathematical notations, the model was written as:

$$y_{ijkl} = \mu + B_i + s_j + d_k(s) + e_{ijkl} \quad (1)$$

where $y_{ijkl}$ is the observed number of offspring, the μ is overall mean (constant), and $s$ and $d$ were as described above.