Backcross breeding between TGGG hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) and giant grouper (*E. lanceolatus*)

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Abstract

This paper reports on the first successful backcross breeding between TGGG hybrid grouper and GG in captivity. To establish a seed production technique for this new backcross grouper, eggs and larval development were observed. Three successful spawning was observed in November 2018, September 2019 and November 2019. The eggs were collected using stripping methods and the sperms were collected using sperms collector. About 1.2 to 1.3 litres of eggs were collected in each spawning. Eggs and larval development of backcross TGGG hybrid and GG were almost similar to its parental species and other *Epinephelus* species. Fertilized eggs were measured 0.860 to 0.915 mm in diameter, with oil globule 0.190 to 0.214 mm in diameter. Fertilization and hatching rate were in between 80 to 90 %. Newly hatched larvae range from 1.64 to 1.99 mm and commenced feeding at 60 d AH. Larval dorsal and pelvic spines started to develop from 9 d AH and fins were formed on 21 d AH. By the age of 30 d AH, the fish entered juvenile stage. At 60 d AH, the fry's body shape and colour resembled those of the adult. After more than 300 d AH, the fish reached 1 kg in weight. The backcross between female TGGG and male GG is expected to perform even better growth and survival rate than TGGG because the backcross carries more genetic material from the GG, which would be ideal for mass production in the aquaculture industry.

Keywords: Backcross breeding, *Epinephelus lanceolatus*, *Epinephelus fuscoguttatus*

*Bayu Aquaculture Sdn. Bhd. (BASB) is located at Kg. Serusop, Tuaran, Malaysia. BASB is the anchor company for aquaculture development in the Sabah Agriculture Blueprint 2020. BASB promotes production of quality fish for consumption without the use of chemical in the process.

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Introduction

Grouper aquaculture is carried out in tropical and subtropical areas throughout the world, but most production is from Asia (Rimmer and Glamuzina, 2017). In the Southeast Asian region, the most popular cultured groupers included the tiger grouper (*Epinephelus fuscoguttatus*), orange-spotted grouper (*E. coioides*) and giant grouper (*E. lanceolatus*) (Luan *et al*., 2016). The major grow-out sites for grouper in Malaysia are in Sabah (particularly Tuaran and Sandakan) and Sarawak, where wild seeds are available (Pomeroy, 2002). Groupers are high quality marine fish with increasing economic values. It was estimated almost 155 000 tonnes of groupers were produced in 2015 with a total value of USD 630 million (FAO, 2017). In order to meet the growing market demand, development of quality fish breeds can contribute to the increase of fish production.

In aquaculture, hybridization has long been practiced in various species of fishes to increase growth rate, improve flesh quality, increase disease resistance and environmental tolerance, and to improve other quality traits to make the fish more profitable (Rahman *et al*., 2013). Hybridization has been practice in groupers since 2006, with the first production of TGGG, a crossbreed between tiger grouper (TG; *E.fuscoguttatus*) and giant grouper (GG; *E. lanceolatus*) (Ch'ng and Senoo, 2008). The TGGG hybrid grouper is known as the most successful hybrid combination: has a wide range of environmental tolerance (Othman *et al*., 2015; De *et al*., 2016; Shapawi *et al*., 2018), better growth performance (Ch'ng and Senoo, 2008), and feeding performance (Othman *et al*., 2015).

Generally, hybrids species are consider sterile species because normally inter-specific hybrids cannot produce viable gametes (Bartley *et al*., 2001; Luin *et al*., 2013). Even so, there are cases of hybrids that show sexual maturation and fertility (Na-Nakorn *et al*., 2004; Wang *et al*., 2006), which indicates that producing second generation (F2) of hybrids and backcross breeding in fish is possible. In fact, F2 generation (Argue *et al*., 2003; Tymchuk and Devlin, 2005) and backcross experiment (Sui *et al*., 2011; Xu *et al*., 2011) in fish has been conducted in some studies. According to Shapawi *et al*. (2018), in comparison to the hybrid species, the following generation is characterized by greater hybrid vigor or positive heterosis characteristics.

In 2013, Luin *et al*. reported that the TGGG hybrid grouper could reach sexual maturation as evident from development of their gonads, which opened up the possibility to perform backcross breeding in grouper. In 2016, Luan *et al*. has successfully produce the eggs of backcross between OGGG hybrid grouper (orange spotted grouper, OG, *E.coioides* x giant grouper, GG, *E.lanceolatus*) and giant grouper (GG, *E.lanceolatus*) in captivity. However, fertilization and hatching rate were recorded the lowest compared to other hybrid groupers (Luan *et al*., 2016). In

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2018, Ching *et al*. reported the first natural spawning and fertilized eggs of F2 hybrid TGGG in captivity. Still, low survival rate remains the bottleneck that prevented the establishment of production.

The above studies have motivated the Bayu Aquaculture Sdn. Bhd. to conduct the present study, in an attempt to produce backcross bears close resemblance to GG, which is fast growing, has good organoleptic properties, and would likely exhibit higher resistance to common disease. To the best of our knowledge, this study was the first report on the successful backcross breeding between TGGG and GG in captivity. The backcross between female TGGG and male GG (Fig. 1) is expected to perform even better growth and survival rate than TGGG because the backcross carries more genetic material from the GG, which would be ideal for mass production in the aquaculture industry. This will then help to boost the fisheries sector in the country and also satisfy the increasing demand for grouper.

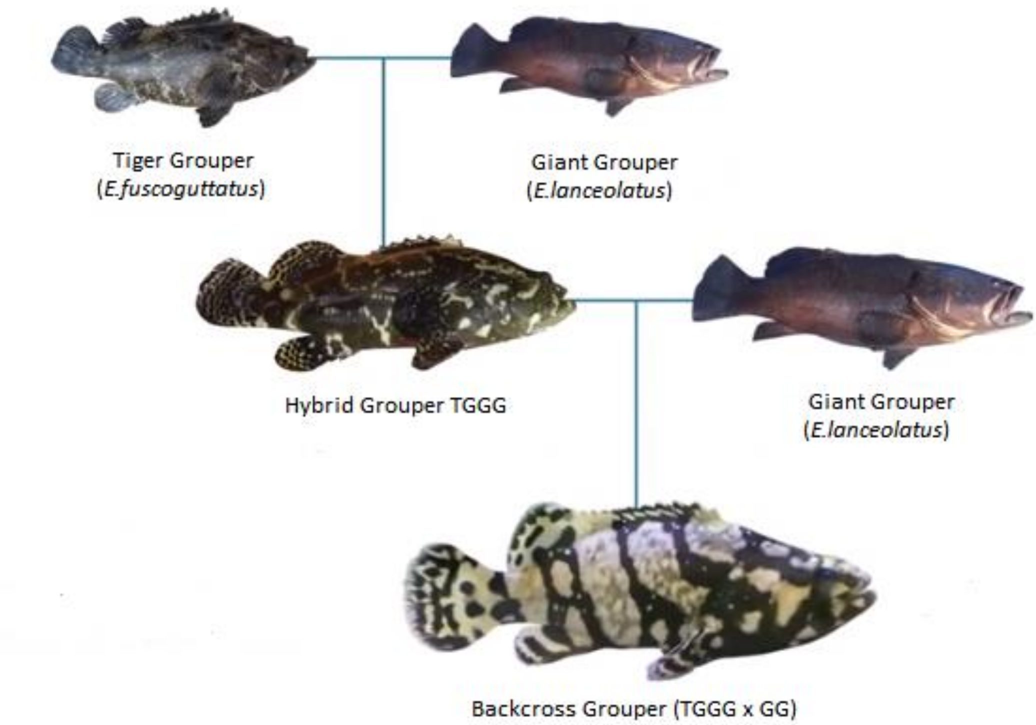

Figure 1: Concept of backcross breeding between female TGGG and male GG.

Materials and methods

Breeding and eggs incubation

The broodstock used in this study were reared in net cages located in Gaya Island, Sabah. The size of the net cages was 5 x 5 meter for GG and 4.5 x 4.5 meter for TGGG, with the depth of 3.5 meter. The broodstock were fed twice a week with enriched prey fish (*Sardinella* sp.) supplemented with fish oil, squid oil and vitamin premixes. The total length of the broods was in between 60 to 90 cm and weight was ranged from 40 to 60 kg for GG and 9 to 14 kg for TGGG.

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In the present study, five male of GG and three female of TGGG were involved in the spawning. After the maturity of the broods were checked and identified, the eggs were collected using stripping methods and the sperms were collected using sperms collector. The eggs and sperms were then mixed and washed. Fertilized eggs were then transferred to incubate at the fish farm of Bayu Aquaculture Sdn. Bhd. in Tuaran, Sabah. The size of eggs and development was observed under microscope and photographed using a digital camera. The fertilized eggs were incubated in a 15 x 6 x 1.3 meter concrete pond with 24 hour aeration. The water temperature, salinity, DO and pH during eggs incubation were 28 to 32℃, 30 ppt, 5.0 to 7.0 mg/l, and 7.8 to 8.0 respectively.

Larval and juvenile rearing

Hatched larvae were reared in the same pond where eggs incubated, and later transferred to round tank filled with 4 tonne seawater when the dorsal and pelvic spines began to retract. The rearing water temperature, salinity, DO and pH were 28 to 32℃, 30 ppt, 5.0 to 7.0 mg/l, and 7.8 to 8.0, respectively. Cleaning of tank's bottom was carried out daily to remove excess feed and dead larvae. Total length (TL) of larvae was measured from 0 d AH and larval development was observed under microscope and photographs were taken with a digital camera.

Different feeds were given at 3 times daily as shown in Figure 2. Larvae rely on egg yolk sac on 1 to 2 day after hatched (d AH). However, *Chlorella sp.*, were added to the rearing pond as water conditioner during that period. Rotifer *Brachionus sp*. (150 um) were given to larvae from 3 to 14 d AH. Commercial brine shrimp *Artemia salina* nauplii were introduced from 10 to 40 d AH, and formulated powder feed, Otohime (Marubeni Nisshin Food, Japan) was fed from 20 to 55 d AH. Copepod harvested from the sea was added into rearing tank from 24 to 60 d AH. Minced fish was added when the fish reached juvenile stage and onwards.

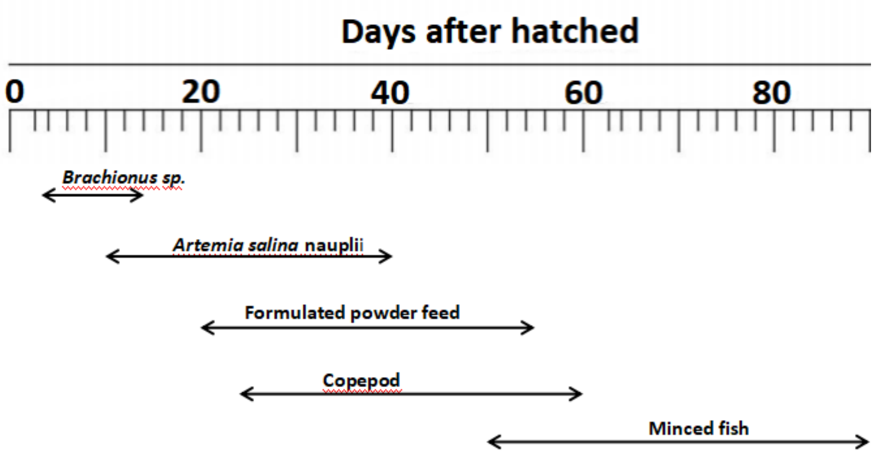

Figure 2: Feeding sequences of TGGG x GG backcross grouper

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Results

Spawning and eggs development

First successful spawning was observed in November 2018, followed by second and third batch in September 2019 and November 2019, respectively. About 1.2 to 1.3 litres of eggs were collected from the female TGGG in each spawning. Fertilization and hatching rate were in between 80 to 90%. Fertilized eggs were spherical, transparent, measured 0.860 to 0.915 mm in diameter, with oil globule 0.190 to 0.214 mm in diameter.

The eggs development is shown in Figure 3. At 10 hours after fertilized (h AF), the head and myomere were formed, with appearance of Kupper's vesicle. At 12 h AF, the tail has separated from the yolk sac. At 15 h AF, lens vesicle becomes visible and movement of embryo was observed. Hatching began at 18 h AF. Newly hatched larvae were measured 1.64 to 1.99 mm in total length (TL). The newly hatched larvae would stay on water surface, positioned upside down due to the yolk sac and oil globule and not swimming, but floating and drifting around the water column.

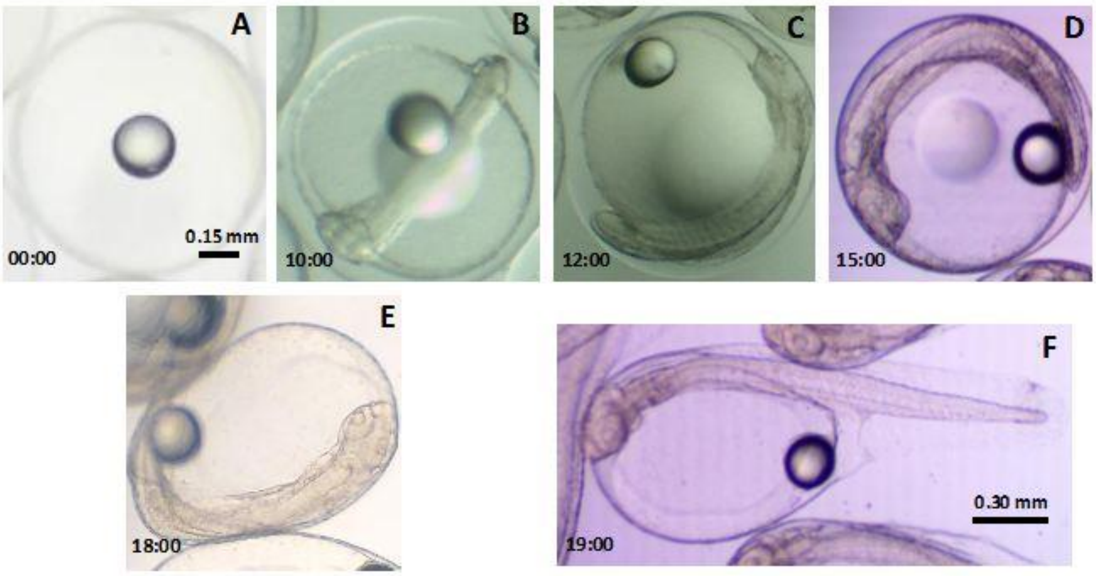

Figure 3: Egg development of TGGG x GG backcross grouper. A. Fertilized eggs; B. Head and myomere formed, Kupffer's vesicle appeared; C. Tail separated from yolk, optic vesicle appeared; D. Lens vesicle visible, embryo moving; E. Hatching commenced; F. Newly hatched larvae; number at the left corner show hours after fertilization; all the scales are same as shown in A at the right corner, except F.

Larval development and juvenile growth

The morphological changes in larvae and juvenile stage are illustrated in Figures 4 and 5, respectively. The correspondence between the morphological features and behavioral changes is summarized in Table 1. Total length (TL) changes of larvae are shown in Figure 6.

The TL of newly hatched larvae range from 1.64 to 1.99 mm. At 21 hour after hatched (h AH), the larvae eyes were formed but not pigmented. The anus part formed but not opened. At 24 h

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AH, the yolk sac reduced in size and the larvae started to perform vertical swimming. At 38 h AH, eyes pigmentation darken, anus part opened and digestive tract were formed. At 60 h AH, the yolk sac and oil globule were completely absorbed. The larvae mouth part opened, lower jaw and digestive tract started moving, and indicating that feeding process has begun. At 3 days after hatched (d AH), black pigmentation appeared at area above intestine. The larvae were actively swimming and fed on rotifer. At 5 d AH, melanophores on the area above intestine expanded and new spot of melanophores also appeared on the area between anus part and caudal fin.

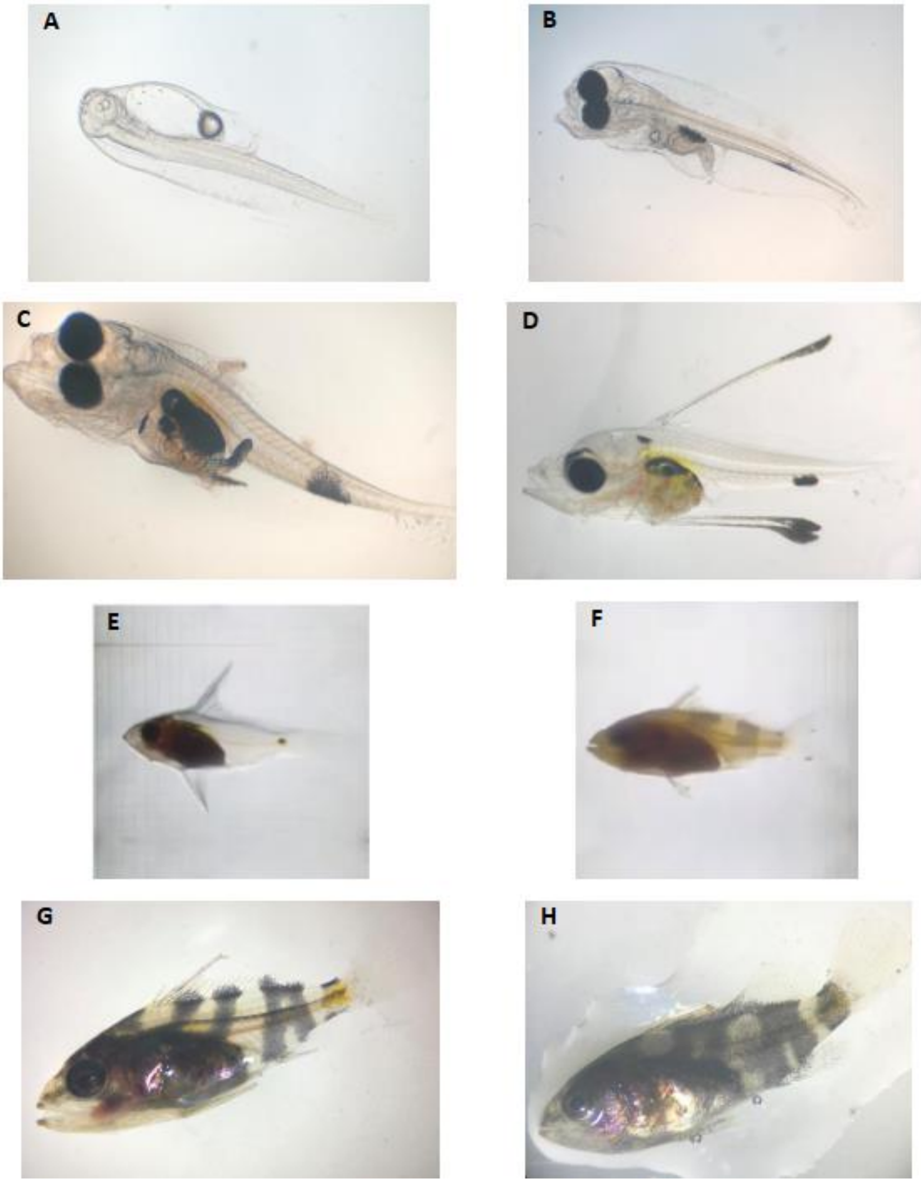

Figure 4: Morphological changes of TGGG x GG backcross grouper in larval stage. A. 1 d AH, 2.57mm TL; B. 3 d AH, 2.71mm TL; C. 9 d AH, 4.06mm TL, D. 14 d AH, 7.01mm TL, E. 21 d AH, 18.5mm TL, F. 30 d AH, 23.0mm TL, G. 35 d AH, 28.0mm TL; H. 40 d AH, 30mm TL.

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Figure 5: Morphological changes of TGGG x GG backcross grouper in juvenile stage. A. 60 d AH, 5.7cm TL; B. 80 d AH, 7.0cm TL; C. 120 d AH, 13.5cm TL; D. 170 d AH, above 20.0cm TL; E. More than 200 d AH, above 25.0cm TL; F. More than 400 d AH, 30.0cm TL; G. More than 300 d AH, reached 1kg in weight.

Dorsal pelvic spine started to develop from 9 d AH and fully elongated at 14 d AH. The spines were covered with melanophores. At that moment, the larvae were actively swimming and *Artemia salina* nauplii were being introduced into their diet. The larvae started to congregate at the center bottom of the tank. Dorsal and pelvic spines slowly retracted and fins were formed on 21 d AH. The larvae responded well to the formulated powder feed given.

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Table 1: Correspondence between morphological and behavioral changes backcrosses grouper TGGG x GG larvae.

Morphological changes	Day after hatched (d AH)	Behavioral changes
Hatching commenced.	0	Floating and drifting around the water column. Pelagic behaviour.
Eyes formed but not pigmented. Anus formed but not opened.	1	Relying on yolk sac for nutrient.
Eyes pigmented. Mouth opened and lower jaw moving. Movement in digestive tracts. Yolk sac and oil globule completely absorbed.	3	Swimming and started to feed on rotifer.
Dorsal and pelvic spines started to develop. Abdominal cavity deeply pigmented.	9	Actively swimming and feed on *Artemia salina* nauplii.
Dorsal and pelvic spines elongated, covered with melanophore. Pigmentation on head part. Coloured abdominal cavity.	14	Actively fed on *Artemia salina* nauplii. Congregated around central part of tank.
Dorsal and pelvic spines retracted. Dorsal and pelvic fins started to develop.	21	Responding well to formulate powder feed.
Body turned non-transparent. Development of brown colouration and dark patterns. Lateral line clearly seen. Dorsal, pelvic, anal and caudal fins well develop. Entering juvenile stage.	30	Actively preyed on mixed live feed of *Artemia salina* nauplii and copepod, with formulated powder feed.
Silvered abdomen. Juvenile stage.	40	Benthic habitat.

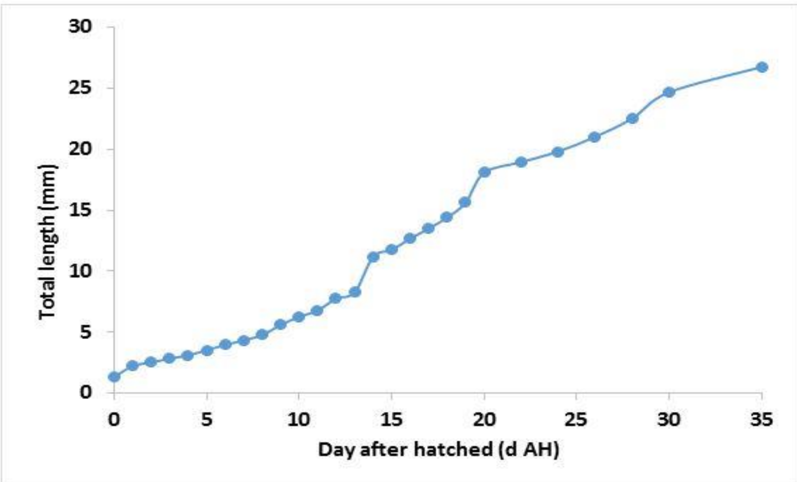

Figure 6: Growth of backcross grouper TGGG x GG larvae from 0 to 35 d AH.

By the age of 30 d AH, the larvae body was no longer transparent. Brown colouration and black stripes started to develop and lateral lines can be clearly seen. All the fins (dorsal, pelvic, anal and caudal) were well developing. The fish started to enter juvenile stage. At that stage, they were actively preying on combination of live feed consisting *Artemia salina* and copepod, and formulated powder feed. At 40 d AH, the fry reached 35 mm in TL, shifted to

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benthic habitat and looked more aggressive. After 60 d AH, the fry's body shape and colour resembled those of the adult and actively fed on minced fish. At 150 d AH, the juvenile reached 20 mm in TL, body colour and dark patterns turned darker. After more than 300 d AH, the fish reached 1 kg in weight.

Discussion

Eggs development

The fertilization and hatching rates in this study were in the range of 80% to 90%. The rates were similar with the F2 hybrid TGGG study by Ching *et al*. (2018), which reported the fertilization and hatching rates at 80.7% and 91.5%, and slightly higher than the maternal species, recorded at 86.8% and 87.2%, respectively by Ch'ng and Senoo (2008). Hatching time of the eggs in this study was 18 h AF, similar with the maternal species (Ch'ng and Senoo, 2008) but shorter than the paternal species, reported at 30 h AF by Garcia-Ortega *et al*. (2013). It is also shorter than the 19 h AF in TG (Boonlipatanon *et al*., 2002) and slightly longer than the 17:50 h AF in F2 hybrid TGGG (Ching *et al*., 2018). Temperature during eggs incubation in this study was 28 to 32 °C, more or less the same with Ch'ng and Senoo (2008), Boonlipatanon *et al*. (2002), and Ching *et al*. (2018) but higher than in Garcia-Ortega *et al*. (2013), which recorded at 28 to 29°C, 29 to 32°C, 29.5 to 30.5°C, and 27.6°C, respectively.

Fertilization and hatching rates are important criteria used to assess the eggs quality and often related to the nutritional requirements of broodstock's maturation and fecundity (Kjørsvik *et al*., 1990; Unuma *et al*., 2004; Ching *et al*., 2018). Broodstock in this study were reared in net cages, where the net mesh allows the water to pass freely, thus maintaining good water quality. The broodstock were also well-fed with nutritionally balanced diet with supplement. Physiologically, when fish are given with the required nutrient, they synthesize that nutrient for maintenance and growth and support various developmental processes throughout their life span and resulted in good quality of broodstock and offsprings. Differences in hatching time at similar water temperature are most likely a characteristic of the species, but most showed a close relationship with water temperature (Park *et al*., 2014).

Diameter of eggs (0.86 to 0.915 mm) in this study was considered similar to the paternal species (Garcia-Orgeta *et al*., 2013) and the TG (Lim, 1993), both recorded at 0.89 mm. Nevertheless, it was relatively larger than the maternal species, reported at 0.84 mm by Ch'ng and Senoo (2008), and other hybrid species (Koh *et al*., 2008; Koh *et al*., 2010; Addin and Senoo, 2011). It was also larger than the backcross between OGGG and GG, reported at 0.755 mm in diameter by Luan *et al*. (2016). It is known that egg and larval sizes are correlated and larger larvae tend to survive longer without food than those hatched from smaller eggs (Kjørsvik *et al*., 1990). Bigger sized eggs are assumed to have higher survival and growth potential in subsequent stages (Colin *et al*., 1996; Goncalves *et al*., 2011; Luan *et al*., 2016).

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Larval and juvenile development

Total length (TL) of newly hatched larvae in this study ranged from 1.64 to 1.99 mm, considered relatively similar when compared to the maternal species, reported at 1.99 mm by Ch'ng and Senoo (2008). It was also similar to the TG, reported at 1.80 to 1.90 mm by Lim (1993). Growth of backcross TGGG x GG was observed to be slower in the first few days after hatched compared to the parental species (Ch'ng and Senoo, 2008; Garcia-Orgeta *et al*., 2013), but caught up at later stage. TL of the backcross TGGG x GG was 2.69 mm in 2 d AH and 2.76 mm in 3 d AH, which was smaller than the TGGG, recorded at 2.81 mm in 2 d AH (Ch'ng an Senoo, 2008) and the GG, recorded at 2.9 mm in 3 d AH (Garcia-Ortega *et al*., 2013). However, in 20 d AH, TL of the backcross TGGG x GG reached 17.1 mm, while the size of TGGG was only 9.30 mm (Ch'ng and Senoo, 2008). In addition, TL of the backcross TGGG x GG reached 37.5 mm in 45 d AH, longer than the 35.4 mm recorded in the GG by Garcia- Ortega *et al*. (2013).

Backcrossed hybrids occasionally outperform the F1 hybrid and the parentals (Argue and Dunham, 1999). Rapid growth through hybrid vigor is observed in the F1 hybrid TGGG. Thus, it is expected that the offspring from backcross TGGG x GG may also inherit desirable growth traits that promote even faster growth than the parental species. Hybridization merits tend to be more affected by male parental species (Wang *et al*., 2006). This was also suggested in Koh *et al*. (2010), where TL of the hybrid with GG as paternal parent has faster growth than with TG as paternal parent. The offspring of backcross TGGG x GG is assumed to carry more genetic materials from the male GG, which could be the explanation of the faster growth of larvae in this study. However, since this is the first successful backcross breeding of grouper, further details studies is require to determine the growth-related traits.

Morphological and behavioral development of backcross TGGG x GG larvae to juveniles is almost similar to its parental species and other *Epinephelus* species (Heemstra and Randal, 1993; Tucker, 1999; Glamuzina *et al*., 2001; Ch'ng and Senoo, 2008; Gracia-Ortega *et al*., 2013). While the appearance of pigmentation represents the basic morphological characteristic of the grouper in its early larval stage, there were no distinct characteristic to differentiate it from other species of grouper larvae. In this study, pigmentation on larvae can be clearly observed at 3 d AH and the brown colouration with dark pattern which was similar to the parental species was developed at 30 d AH. The development of the dorsal and pelvic fin spines, another basic morphological characteristic of grouper larvae, were observed as early as 9 d AH, with melanophores on the tips. According to Kawabe and Kohno (2009), the spines play a role in maintaining position in the water column and act as anti-predator mechanism. When the length of dorsal and pelvic fin spines shortens and external melanistic pigmentation becomes more obvious, the juveniles enter the process of settling (Cunha *et al*., 2013).

Larval period in present study was about 30 d AH, which was similar to the martenal species (Ch'ng and Senoo, 2008). During the transition of fish larvae to juvenile stage, morphological, physiological and ecological changes occured (Powell *et al*., 1992; Chunha *et al*., 2013; Jayadi *et al*., 2017), such as scales covering the entire body (Kawabe and Kohna, 2009), shifting habitat from pelagic to benthic (Tucker, 1999), and cannibalism (Jayadi *et al*., 2017). In this study, behaviour of habitat preferences and development of spines were similar to the parental species and those of the other grouper. At 61 d AH, the juveniles backcross TGGG x GG reached 5 cm in TL and showing the adult-like features. At that stage, the juveniles exhibited greater tolerance to various environmental stress and can be used as important indicator for transfer and grading survivor (Kawabe and Kohno, 2009).

Major morphological deformities and large-scale mortality were not observed throughout the larvae and juvenile rearing period of backcross TGGG x GG. However, similar to other grouper species, cannibalism is the main factor responsible for the mortality in this study. Cannibalism can be minimized by feeding the fish well, weaning them as soon as possible and grading the fish regularly (Tucker, 2003). In the study by Ruangpanit and Yashiro (1995), the grading of fish is carried out once a week to reduce cannibalism, a necessary process which contributed to a 75% survival rate.

This study concluded the first record of backcross breeding in grouper between TGGG and GG with remarkable growth attained as fish has grown up to 1 kg in weight and reached nearly 30 cm in total length within a year. This is an important milestone achieved, especially for grouper aquaculture. Even so, survival rate remain as the main concern. Others include the optimum rearing condition and disease resistance which requires further investigation to promote better growth performance.

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