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Final report

Small research and development activity

project

Controlling giant grouper maturation, spawning and juvenile production in Vietnam, the Philippines and Australia

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Contents

1 Acknowledgments

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2 Executive summary

This Small Research Activity (SRA) was conducted with the long term view of developing a sustainable giant grouper (*Epinephelus lanceolatus*) aquaculture in the Southeast Asian region. Giant grouper is a fast growing species that has great aquaculture potential, however in both Vietnam and the Philippines this is hindered at present by a lack of knowledge regarding their reproductive and larval requirements which is essential for closing its life cycle. A reliable breeding program that prevents inbreeding is also currently not possible because there is no information regarding the genetics of giant groupers that are being collected from the wild and those that are already held in captivity. Research has not been initiated due to the high level of investment in holding and maintaining giant grouper broodstock. Therefore, in consultation with partners in the Philippines and Vietnam, the development of a comprehensive research project proposal led by the Australian partners was agreed upon. In the short term, this SRA was conceptualised towards establishing the logistics and infrastructure that are required for a large, 5 year project to develop sustainable giant grouper aquaculture.

The specific objectives of this SRA were:

- To learn from the experience in Taiwan through a study tour of giant grouper operations
- Analyse the genetics of the giant grouper broodstock in Vietnam to avoid inbreeding
- To participate in the 2012 breeding season of giant grouper in Vietnam
- To collect giant grouper broodstock in the Philippines

The study tour of giant grouper operations in Taiwan, by the leading Australian scientists (Prof Abigail Elizur and Dr Richard Knuckey), together with collaborators from SEAFDEC/AQD (Dr Felix Ayson and Dr Fiona Pedroso) and RIA1 (Dr Tran Dinh Luan and Mr Hoang Nhat Son) highlighted both the successes in establishing a vibrant giant grouper aquaculture and export market and the issues confronting their industry. Taiwan dominates the giant grouper culture in the SE Asian region, however biosecurity and technical inconsistency between and within operations are major challenges that have resulted in disease outbreaks and big losses to the industry. While farmers in Taiwan have successfully closed the life cycle of giant grouper in captivity, there is still a lack of thorough knowledge regarding the biology of the species. Inbreeding is also a significant issue as the genetic diversity of their existing broodstock has not been established, no genetic management is undertaken and mating of related individuals are most likely taking place.

The hormone delivery system produced at the University of the Sunshine Coast (USC) successfully induced spawning of the captive giant grouper broodstock in Vietnam. This technology avoids the necessity to strip eggs and sperm from the broodstock, an approach which was unsuccessfully attempted for this species previously. Coached by the Northern Fisheries Centre (NFC) partners, optimal water temperature and salinity for larval rearing have been obtained from preliminary larval rearing experiments. Survival of larvae has been achieved until Day 16 in indoor tanks and until Day 27 in outdoor tanks until fingerlings stage. There still are some fish at growout stage, however survival was compromised by VNN infections.

Molecular tools and DNA markers have been developed at USC. These markers were utilised to establish the parentage and genetic relatedness of giant grouper broodstock at

NFC and at RIA1 in Vietnam. Using these markers, a few individuals from the samples coming from Vietnam and Taiwan originally identified as *E. lanceolatus* have been recognised as hybrids or another grouper species, confirming the essential role of genetic management.

Through this SRA, the collaboration between the partners has been affirmed. The outputs achieved provide groundwork for the Phase 2 five year project that has been approved and is currently in the contract development stage. In particular, the genetic documentation of the broodstock at NFC and in Vietnam through the DNA markers developed during this SRA will help establish a genetics-based breeding program, which is one of the aims of the 5-year project. Through the success of the hormonal treatment in inducing spawning combined with the success of the preliminary larval rearing trials at RIA1, protocols towards closing the giant grouper life cycle are be available. In addition, this will enable the exploration of germ-cell transplantation and surrogate technologies as alternative approaches to giant grouper seed production. The optimised methods for identifying DNA markers, as well as the currently identified DNA markers, are ready for transfer to partners in Vietnam and the Philippines. Procurement of small (5-10 kg) giant grouper broodstock in the Philippines, currently in progress, will enable the characterisation of the reproductive biology of the species, including age at maturation and provide essential material for sex inversion trials.

The overarching impact of this work will be the development of a viable and sustainable giant grouper aquaculture industry in Vietnam and the Philippines.

Overall, achievements from this SRA have set the stage for the implementation of the proposed phase 2 project which provides a five year plan to establish the giant grouper aquaculture in Vietnam and the Philippines, together with securing knowledge and understanding of the biology of this species, to assist with the consistent reproduction, larval rearing and genetic management.

3 Background

Groupers are a significant component of the Live Reef Food-Fish trade in the SE Asian region. To meet market demand, grouper aquaculture is now well established, however only for one species, the Tiger grouper (*Epinephelus fuscogutattus*). The fast growing giant grouper (*E. lanceolatus*) has great aquaculture potential. Production of giant grouper juveniles has been achieved in some regional facilities but with very limited success. This limitation is partially due to a lack of knowledge on their maturation, spawning behaviour and larval development.

NFC has been very successful in closing the life cycle of the giant grouper. They now obtain ongoing spawning, have developed the protocols for successful larval rearing, and supplied farms with fingerlings for growout. In a scoping study that was carried out by Knuckey and Elizur in November 2012 at SEAFDEC/AQD in the Philippines and at RIA1 in Vietnam, it was confirmed that development of giant grouper aquaculture is of high priority in order to secure the markets for this species. Both overseas institutions are in a position to begin giant grouper aquaculture development, and have expressed their commitment to do so. Their extensive research experience on other grouper species arising from two previous ACIAR projects, FIS/97/73 Improved hatchery and grow-out technology for grouper aquaculture in the Asia-Pacific region and related FIS/2002/077, have led to a significant improvement in larval survival of tiger and mouse grouper (*Cromileptes altivelis*) and also a better understanding of their culture requirements. In the Philippines, this means that they can test their existing techniques of broodstock handling and larval rearing on giant grouper. However, they currently lack a sufficient captive giant grouper broodstock population, which will take time to secure and condition. In Vietnam, they already have a large number of giant grouper broodstock, but have been unable to achieve spawning while maintaining them under conditions successfully used for other grouper species. In addition, the genetic background and diversity of their giant grouper broodstock has not been assessed. Therefore, this SRA addressed these specific issues to enable the development and implementation of a much larger research project aimed towards establishing a sustainable giant grouper aquaculture in the region.

Benefits projected among the partner countries include an increase in research capability achieved from the cooperative sharing of knowledge and capacity that will result from combining participants' skills and resources. In Australia, sectors of the aquaculture industry, currently investing in grouper aquaculture, are limited by low numbers of available fingerlings will directly gain from the project outcomes. This will initially be within Queensland but will also extend to Western Australia and the Northern Territory. In the Philippines and Vietnam, benefits will be an increase in financial independence of small fish farmers and associated communities by improving the profitability of grouper culture. The availability of a reliable supply of high-value, fast growing giant grouper will also alleviate environmental concerns such as continued reliance on wild-caught fish or destructive fishing practices.

4 Objectives

The overall project aim was to achieve a more complete understanding of current giant grouper aquaculture and to investigate approaches to obtain consistent and controlled maturation and spawning of giant grouper that will enable successful larval rearing of captive grouper in tanks and cage systems. The specific objectives of the SRA were:

- To learn from the experience in Taiwan through a study tour of giant grouper operations
- Analyse the genetics of the giant grouper broodstock in Vietnam to avoid inbreeding
- To participate in the 2012 breeding season of giant grouper in Vietnam
- To collect giant grouper broodstock in the Philippines

The specific objectives of the SRA were aimed towards establishing the logistics and infrastructure that are required for the large, 5 year project that is proposed to start in mid-2013.

5 Methodology

5.1 Study tour of giant grouper operations in Taiwan

Two representatives from each country went together on a study tour of giant grouper operations in Taiwan from 24 - 30 June 2012. The group was composed of Prof Abigail Elizur and Dr Richard Knuckey from Australia; Dr Felix Ayson and Dr Fiona Pedroso from Seafdec/AQD in the Philippines; and Dr Tran Dinh Luan and Mr Hoang Nhat Son from RIA1 in Vietnam. The tour was organised with the assistance of Trade and Investment Queensland (TIQ) Office in Taipei. The group was accompanied by Mrs Clare Huang, TIQ Business Development Manager, on the first 4 days of the tour, and on the 5th day, by Mr Quentin Bai, TIQ Commissioner. The tour itinerary is attached as appendix 1.

5.2 Genetic analysis of giant grouper broodstock populations

5.2.1 Sample collection

All giant grouper broodstock in were tagged and tissue samples (fin clips) for DNA analysis were collected or supplied from four *E. lanceolatus* populations, as follows:

(1) From the Northern Fisheries Centre in Cairns, Australia: 34 samples from wild-caught broodstock and from two cohorts of their offspring from their breeding program, the latter captive bred cohorts comprising 15 samples in 2010 and 20 samples in 2011.

(2) From an aquaculture facility in Vung Tau, South Vietnam: two wild caught broodstock and 10 samples from fish that were imported as fingerlings from a Taiwanese *E. lanceolatus* aquaculture facility.

(3) From the RIA 1 research facility on Cat Ba Island in Vietnam: nine broodstock samples were collected, then a further 31 broodstock samples were further provided. All the RIA1 broodstock was tagged and genotyped as part of this project.

(4) From Taiwan: Ten samples were collected from a variety of locations, including an aquaculture facility, a University and a restaurant. This was conducted during the study tour described in Section 5.1.

All samples were stored in 70% ethanol until processing for DNA analysis.

5.2.2 Pedigree development and parentage assignment

Total genomic DNA was extracted from all samples described in Section 5.2.1 using a modified salt extraction method [5]. For pedigree development and parentage assignment, candidate DNA microsatellite markers from three published peer-reviewed work (1, 2, 3) were first evaluated. This was conducted by fragment analysis of the DNA samples on an AB3500 Genotyper, followed by computer analysis using the program GeneMarker v. 1.95 (SoftGenetics LLC, State College, PA). From the initial 48 DNA microsatellite markers that were tested, 8 were identified for further use. These 8 DNA markers were utilised to establish the pedigree and parentage of 69 giant grouper DNA samples consisting of 34 wild broodstock and two cohorts of 15 and 20 captive-bred offspring from two spawning events in 2010 and 2011, respectively, at NFC. Parentage was assigned using the Cervus v. 3.0.3 software [6].

5.2.3 Determining genetic diversity of giant grouper populations

Genetic diversity of the giant grouper populations was established by examining two datasets:

(1) DNA microsatellite data and (2) *Cox1* mitochondrial DNA data. The DNA microsatellite data obtained from the work described in Section 5.2.2 were subjected to a variety of statistical and genetic calculations using GENEPOP 4.1.3 software [7]. For the second dataset, a 655 bp DNA fragment of the *Cox1* gene located on the mitochondrial DNA was amplified and sequenced on 95 samples from the four populations (Australia, n=64 that includes hatchery reared offspring; Vung Tau, n=12; Cat Ba Island, n=9; and Taiwan, n=10) according to a DNA barcoding method [4]. In order to determine and compare phylogenetic relationships, a neighbour-joining tree and a maximum parsimony of taxa tree were constructed from the trimmed final fragment size of 490 bp with MEGA ver. 5.10 software [74] and Clustal W [8].

The work described in Sections 5.2.2 and 5.2.3 was conducted at the Genecology research laboratories of the University of the Sunshine Coast by David Bright (USC Honours student (2012) and PhD student (2013-)). Protocols for the sample collection had animal ethics approval.

5.3 Trials during 2012 breeding season in Vietnam

5.3.1 Induced spawning trials

Four induced spawning trials were conducted, 2 in tanks and 2 in cages. The spawning conditions in cages at Viet Hai –Cat Ba were: water temperature 28-29^{o}C; pH 8.0; salinity 30-32 ppt. The readiness of broodstock for induction was verified by sampling sperm and eggs through a gonadal biopsy, and observing them through the microscope (Figure 1C).

A. Monitoring broodstock cages B. Broodstock sampling C. Conducting gonadal biopsy

Figure 1. A. Regular cage monitoring, with Richard Knuckey and Adam Reynolds; B. Fish inspection with Richard Knuckey and Adam Reynolds; C. Conducting gonadal biopsy to verify maturational stage.

Fish were implanted with slow-release formulations of gonadotropin-releasing hormone analogue (GnRHa) at a dose of 50 ug/kg. The implants were prepared at University of the Sunshine Coast. GnRHa implantation was supervised by Richard and Adam. Details of the broodstock are shown in the Tables 1-4 below:

Table 1. Giant grouper induced on 26/09/2012 (Tank No 1)

No	Fish Tag	Gender	Weight (kg)
1	020415610967	Female	58
2	020415615582	Female	67
3	020415610272	Female	58
4	020415610906	Male	50
5	020415608381	Male	67

Table 2. Giant grouper induced on 27/09/2012 (Tank no. 2)

No	Fish Tag	Gender	Weight (kg)	Remarks
1	020415609848	Male	51	
2	020415609066	Female	43 (1)	died on 04/10
3	020415613437	Female	25	
4	020415611031	Female	42	
5	020415609652	Female	35 (2)	died on 02/10
6	020415610560	Male	44	

Table 3. Giant grouper induced on 25/09/2012 (**Cages B1 and B2)**

No	Fish Tag	Gender	Weight (kg)	Remarks
1	020415613477	Female	47	
2	020415614454	Female	42 (1)	died on 28/09/12
3	020415615724	Male	65	
4	020415610329	Male	57	
5	020415609447	Female	53	
6	020415614709	Female	50	
7	020415614300	Male	-	

Table 4. Giant grouper induced on 28/09/2012 (Cages A5 and A6)

No	Fish Tag	Gender	Weight (kg)
1	020415613499	Male	40
2	020415612711	Male	-
3	020415608340	Male	52
4	020415610357	Male	45
5	020415608911	Female	30
6	020415614171	Female	36

5.3.2 Larval rearing experiments

Experiment 1: Effect of water temperature on the development of giant grouper embryos and hatching rate

Five experimental rearing temperatures were tested: 23°C; 26°C; 29°C; 32°C and 35°C. Fertilised eggs from the same batch were distributed in 1 litre glass flasks, which were placed in 50 litre containers. Parameters monitored include duration of each developmental stage, hatching rate and larval quality.

Experiment 2: Effect of salinity on embryonic development and hatching rate

The water salinities evaluated were: 20‰; 24‰; 28‰; 32‰ and 36‰. Fertilised eggs used for the experiment were collected from the same batch and distributed in 1 litre glass flasks placed into 50 litre containers as in Experiment 1. Water was heated and maintained at 29-30°C and supplied with aeration. Parameters monitored include duration of each developmental stage, hatch rate and larval quality.

Experiment 3: Larval rearing trial in 8 m^3 indoor concrete tanks and 750 m^3 outdoor tanks

(a) Indoor 8 m^3 concrete tanks

The rearing techniques for gold spot grouper were applied. The environmental conditions were: salinity of 28-30‰; water temperature 22-27°C; dissolved oxygen 4.5-5.5 mg/L; water pH 8-8.2; lighting from 6:00 am to 10:00 pm. Twelve tanks were used for rearing with a stocking density of 10-15 larvae/litre. Feeding started from Day 3 with small strain rotifers (70-90 µm; *Proales similis*) at a density of 5-7 rotifers/ml until Day 5. From Day 6 to Day 10, *Brachionus plicatilis* (120-180 µm size rotifers) were used at 8-10 rotifers/ml, and then feed density was increased to 15 rotifers/ml from Day 11 onwards. Copepods were added from Day 12 at density of 2-3 copepods/ml, and increased to 3-5 copepods/ml from Day 15.

Chlorella sp. and *Nanochloropsis oculata* microalgae were supplied to the rearing tanks from Day 2 at a density of 3-5 x 10^5 cells/ml. Algal density was determined every morning. From Day 7 to Day 12, 10% of the water volume was exchanged daily. From Day 13 to Day 15, daily water exchange rate was increased to 20%.

(b) Outdoor 750 m^3 tanks covered with a polyethylene roof

Three tanks were used for rearing larvae at a stocking density of 2-4 larvae/litre. The rearing techniques for gold spot grouper were also applied. The environmental conditions were: salinity of 28-30‰; water temperature 24-27°C; dissolved oxygen 4.5-5.5 mg/L; water pH 8-8.2; ambient photoperiod. Feeding commenced on Day 3. Larvae were fed with rotifers (*Brachionus plicatilis*; 120-180 µm) at density of 8-10 rotifers/ml. Rotifer density was gradually increased up to 10-15 rotifers/ml until day 27. From Day 11, copepods were added as feeds at a density of 3-5 copepods/ml and increased to 5-7 copepods when larvae reached day 27. From Day 15, Vinh Chau Artemia (super small Artemia produced in Vietnam) was also added as feeds at 1 artemia /ml and maintained this density up to Day 27. Artemia was enriched with 20 g DHA Selco /kg Artemia. From Day 15, water exchange rate was 20%. Density of *Chlorella* sp. was maintained at 3-5 x 10^5 cells/ml.

6 Key results and outcomes

6.1 Study tour of giant grouper operations in Taiwan

The group visited 3 giant grouper farms mainly in the south of Taiwan. The group had discussions with key scientists involved in grouper aquaculture research in 2 universities (Graduate Institute of Aquaculture, National Kaohsiung Marine University; Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University) and 2 research institutes (Tungkang Biotechnology Research Center; Tainan Mariculture Research Center). In addition, a fish market and an aquaculture supplier were also visited. Several highly relevant data were obtained.

1. Taiwan has an established grouper industry that is largely based on 3 species of groupers, namely, the orange-spotted grouper (*Epinephelus coioides*), tiger grouper (*E. fuscoguttatus*) and giant grouper (*E. lanceolatus*).

2. Six other species of groupers have been bred in captivity in Taiwan. These are the coral grouper (*Plectropomus leopardus*), potato grouper (*E. tukula*), malabar grouper (*E. malabaricus*), speckled blue grouper (*E. cyanopodus)*, and yellow fin grouper (*E. bruneus*).

3. Production of grouper hybrids has also been achieved. Notable of which are crosses between giant grouper, and either orange-spotted grouper or tiger grouper. Purebred giant grouper are more difficult to produce than either tiger or gold-spotted grouper. Hybrids offer the possibility to produce more, faster growing grouper fry that display some of the fast growth rate of the giant grouper which is the fastest growing grouper species in the world. At the National Taiwan Ocean University, we saw adult hybrids of tiger and giant grouper and juvenile hybrids of orange-spotted grouper and giant grouper (Figure 2B & 2C). Both of these hybrids had a distinct pigmentation that was different from each other and either parent. Giant and tiger grouper hybrids have a faster growth rate than purebred tiger grouper and are reported to have an increased resistance to viral pathogens, in particular to noda virus. Staff from the University reported that so far the growth rate of giant grouper/gold-spotted grouper hybrids had been disappointing.

Figure 2. A) Study tour group with delegation from the National Taiwan Ocean University. Hybrid giant grouper at the university B) Giant grouper X tiger grouper C) Giant grouper X gold-spotted grouper.

4. Although some farms operate a complete lifecycle grouper production, the overall industry relies on the ability to break the production into sectors. Farms that operate a complete lifecycle production also sell eggs (NT$ 75,000/Kg giant grouper eggs) or larvae to third parties who will produce fingerlings for their own use and or sell on to others who operate a nursery or production facility. This provides additional return to operations that incur the expense of maintaining broodstock while reducing the overall risk to each participant in the production cycle. Grouper seed production or hatchery production, growing grouper fry from eggs until approximately two months old (roughly 2-3 cm in length), is done either in outdoor ponds or indoor hatchery facilities. The fingerling production, growing 3 cm fry until 8 cm fingerling, is done in indoor nursery facilities. An 8 cm grouper fingerling costs approximately NT 100/pc. The grow-out production is done mainly in outdoor ponds.

Figure 3. Long Diann Marine Biotechnology founder and Chairman, Mr Tai Kun Tsai.

Long Diann Marine Biotechnology is the single largest and most influential member in the grouper industry in Taiwan. The company is located in Fang-liao, Pingtung County in southern Taiwan. The Chairman and founder, Mr Dai Kun Tsai (Figure 3) is known as the “king of grouper” and his company has a dominant position on the culture of both giant grouper and potato grouper (*E. tukula*) where they currently have an exclusive ability to supply.

The company sells eggs to other industry members as well as having its own grow-out facilities in Taiwan and the Philippines.

5. Copepod pond production contributes much to the success of grouper larval rearing. It reduces cost of feed inputs to hatchery production. Copepods are harvested from open ponds and although this abundant supply has been very beneficial to efficient larval rearing, it remains a weakness in biosecurity.

6. Taiwan’s grouper market is mainly in Hong Kong and mainland China. The quantity of grouper imported is negatively impacted upon in time of significant financial uncertainty as has been shown recently (Table 5).

Table 5. Export of all groupers from Taiwan to China

Importation period	Quantity of grouper (Tonnes/day)
1985 – 1990	20
1991 – 1995	25
1996 – 2000	27
2001 – 2005	35
2005 – 2007	45
2008 – 2010	35
2011 - 2012	25

7. The desired harvest size for tiger grouper is 1.2 kg and giant grouper is 12-15 kg. The former is reached in 18 months while the latter is 3.5 years. The market price for grouper is calculated per 600 g and each species has a range of "standard" sizes that are marketed and the price varies for both species and market size class (Table 6).

Table 6. Market sizes and prices for a range of grouper species grown in Taiwan

Species	Market size	NT $/600g	AUS$/Kg (30NT$/AUS$)	Species	Market size	NT $/600g	AUS$/Kg (30 NT$/AUS$)
Green grouper	600 g	170	9.40	Giant grouper	600 g	800	44.40
	1 Kg	180	10.00		1.5 Kg	1200	66.70
	1.5 Kg	400	22.20		4.8 – 9 Kg	270	15.00
Tiger grouper	600 g	320	17.80		9 – 30 Kg	270	15.00
	2 Kg	600	33.30	Leopard coral grouper	600 g	1800	100.00
Potato grouper	600 g	460	25.60		2 Kg	3000	166.70
Blue speckled grouper	600 g	1000	55.60	Longtooth grouper	600 g	1200	66.70

When giant grouper is marketed in the larger size range (9 – 30 Kg), it is sold as portions with prices varying depending what part of the fish it represent (Figure 4)

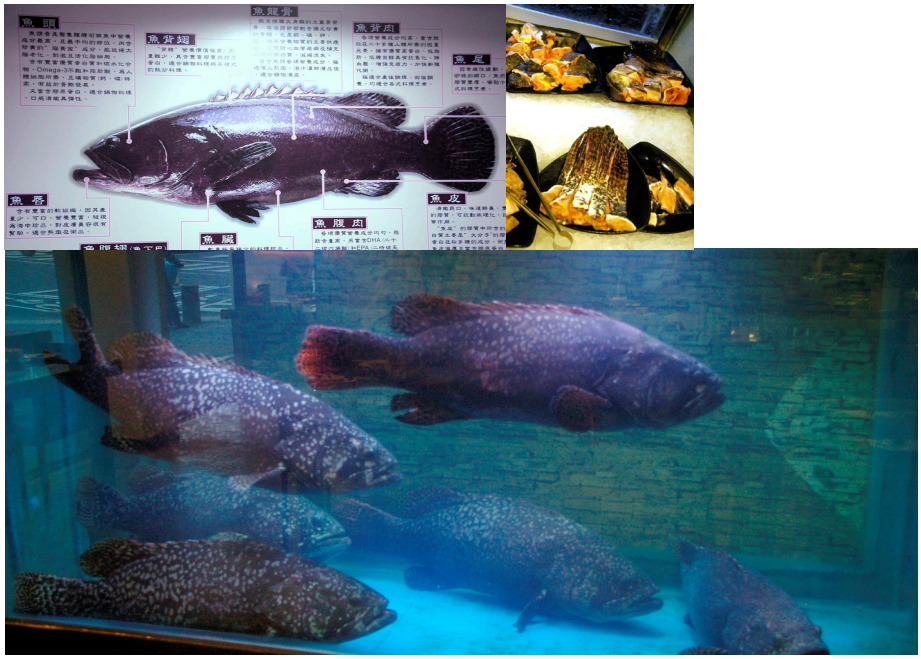

Figure 4. A restaurant in Kaohsiung that specializes in larger (15 Kg) giant grouper.

8. Commercial value of grouper is not only for food but also for cosmetics and anti-aging products. Specifically, extracts from giant grouper eggs are made into cosmetics and are already in the market. However, this is currently a small niche market.

9. There is active R & D for grouper in Taiwan. Research topics of interests include hybridization to produce faster growing and disease-resistant grouper fry, identifying molecular markers for specific traits, use of probiotics (herbal materials) for improved health management, strategies to control NNV occurrence in grouper farms.

10. The significant investment in the aquaculture of grouper in Taiwan is driven by the high market price of the fish. Taiwan is the dominant producer of giant grouper and now also potato grouper. However, the level of sophistication in hatcheries and grow-out facilities is highly variable even within a particular facility. This was evident in a private facility where the quality and cost of the infrastructure was very high (Figure 5) but biosecurity was low with larval diets sourced directly from adjacent, outdoor ponds. There are also few if any controls on people or equipment relocation within facilities. A general, low level of biosecurity is most likely the main reason behind the widespread problem of viral diseases that cause major losses in grouper. The main viral diseases are noda virus and iridovirus.

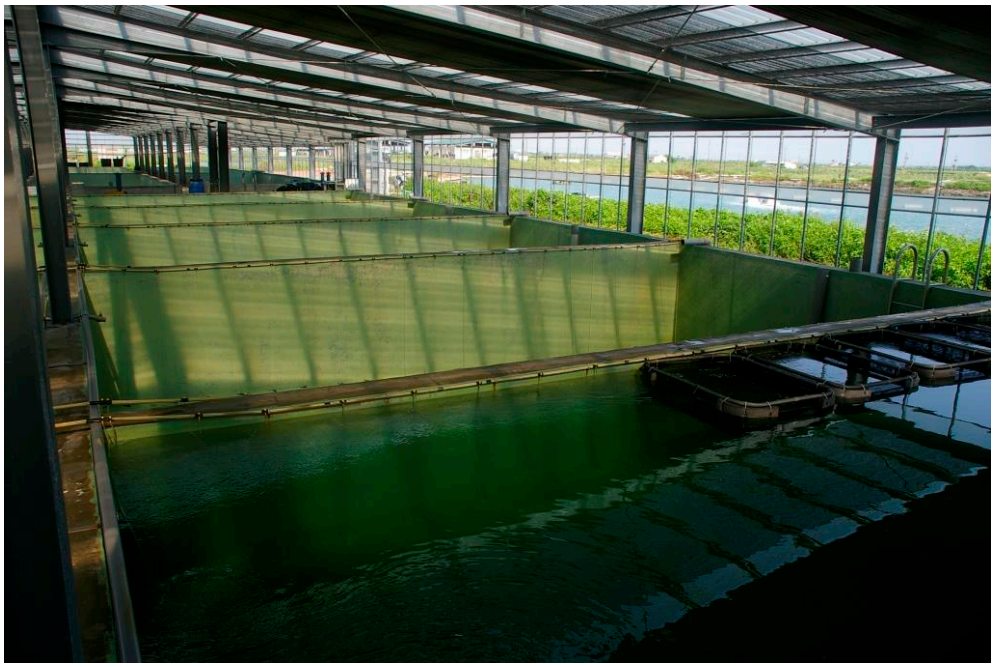

Figure 5. A new hatchery and nursery facility of high quality concrete construction and covered to avoid rain and birds but with generally low biosecurity protocols.

6.2 Genetic analysis of giant grouper broodstock populations

A modified salt extraction method for extracting DNA [70] was determined to be highly efficient. The cost to extract DNA was cheaper per sample and the concentration and purity of the extracted DNA was considerably higher and cleaner compared to previous extractions using commercial DNA blood and tissue extraction kits.

6.2.1 Pedigree and parentage

Eight DNA microsatellite markers (ELMS007, ELMS009, ELMS019, An2, An4, An8, An25, An31) were shown to reliably establish pedigree and parentage of giant grouper. These DNA markers were used to analyse 35 samples obtained from captive-bred offspring

consisting of two cohorts of 15 and 20 animals from two spawning events in 2010 and 2011, respectively, at NFC. Thirty three of the 35 offspring were assigned parent pairs at a strict confidence of >95%. The remaining two individuals were excluded from analysis due to failure to amplify at a minimum of five microsatellites. Parentage analysis indicated that the 2010 captive-bred cohort of 15 individuals were offspring of one male (NFC21) and two females (NFC20, NFC 25) (Figure 6). Analysis of the 2011 captive cohort of 20 individuals showed that they were offspring of only one male (NFC02) and only one female (NFC30) (Figure 7).

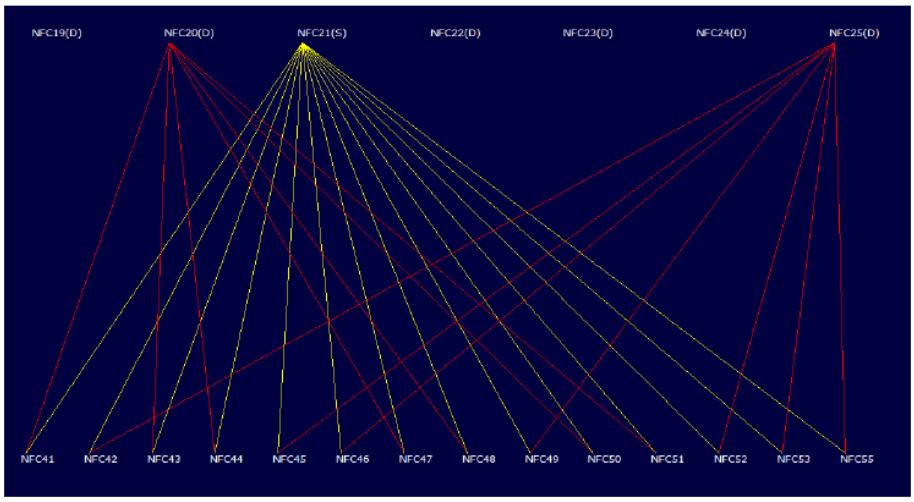

Figure 6. Pedigree view of the breeding interaction of *E. lanceolatus* 2010 cohort. (S) indicate sires or male parent, (D) indicate dams or female parent.

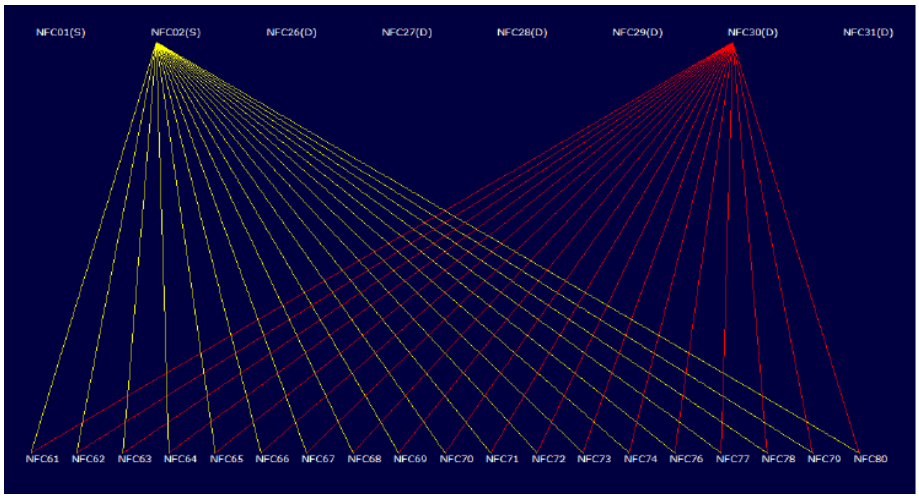

Figure 7. Pedigree view of the breeding interaction of *E. lanceolatus* 2011 cohort. (S) indicate sires, (D) indicate dams.

6.2.2 Genetic diversity

Four *E. lanceolatus* populations were originally considered based on sample site (Australia, Vung Tau, Cat Ba Island, and Taiwan). Analysis using the identified 8 DNA microsatellite markers showed great variation between the populations. However, analysis of the *cox1* mitochondrial DNA data showed that eight samples from Vung Tau and Taiwan were not in fact *E. lanceolatus*, but a species closely related to *E. fuscogattatus*. The discovery of a second grouper species (possibly *Epinephelus polyphekadion,* but insufficient data yet to confirm) during the *cox1* analysis in the Vung Tau, Vietnam and the Taiwanese populations have artificially inflated the apparent genetic variation between the

populations using the 8 DNA microsatellite markers. Because most of the giant groupers from Vietnam originated from Taiwan, samples from both these two locations were pooled and compared against the samples from Australia. The Australian population contained one allele that was most common, whereas the Vietnam-Taiwan population tended to have a more even distribution of alleles (Figures 8-15).

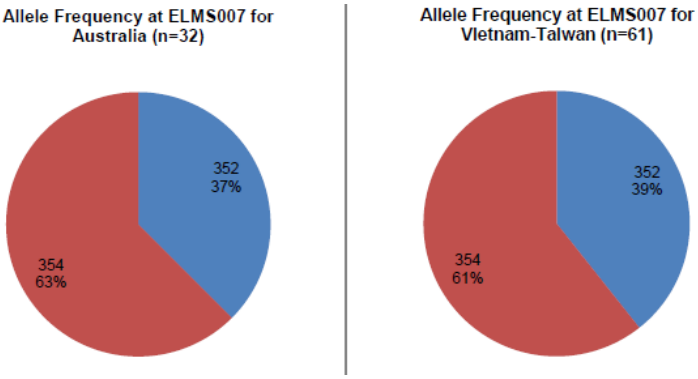

Figure 8. Allele frequencies of ELMS007, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

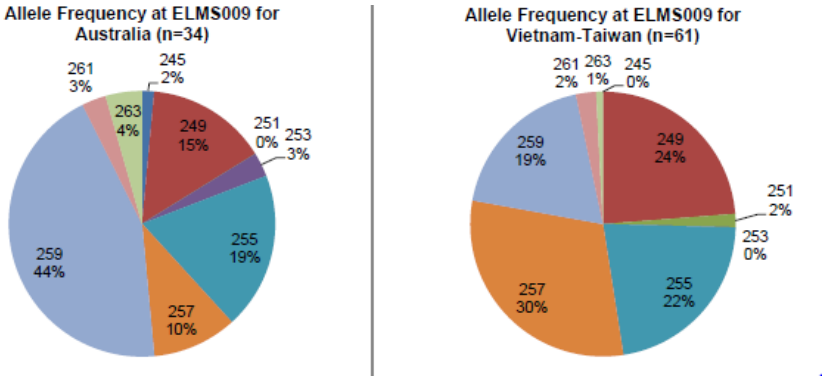

Figure 9. Allele frequencies of ELMS009, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

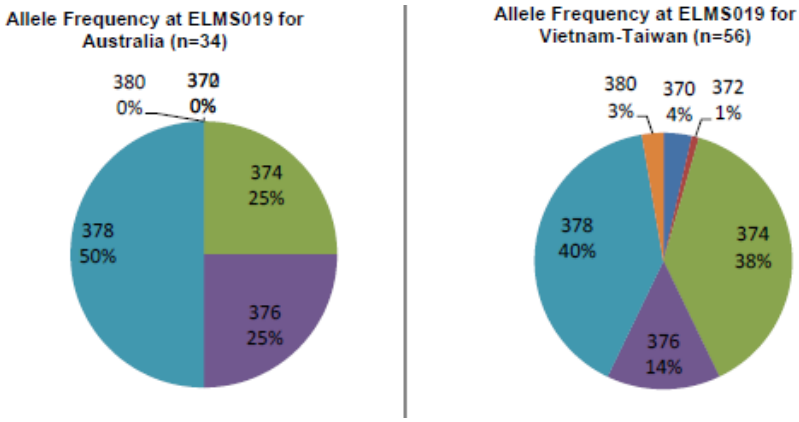

Figure 10. Allele frequencies of ELMS019, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

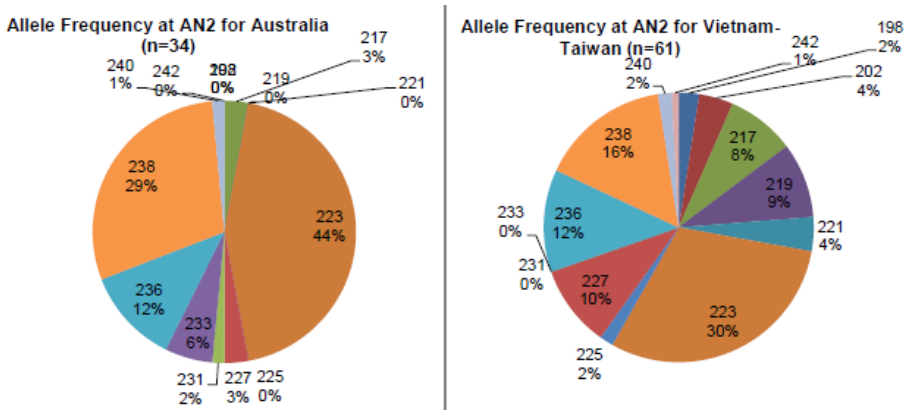

Figure 11. Allele frequencies of An2, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

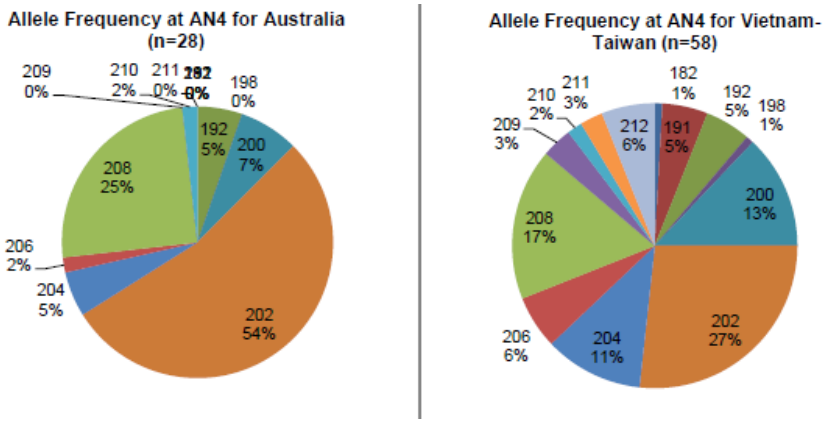

Figure 12. Allele frequencies of An4, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

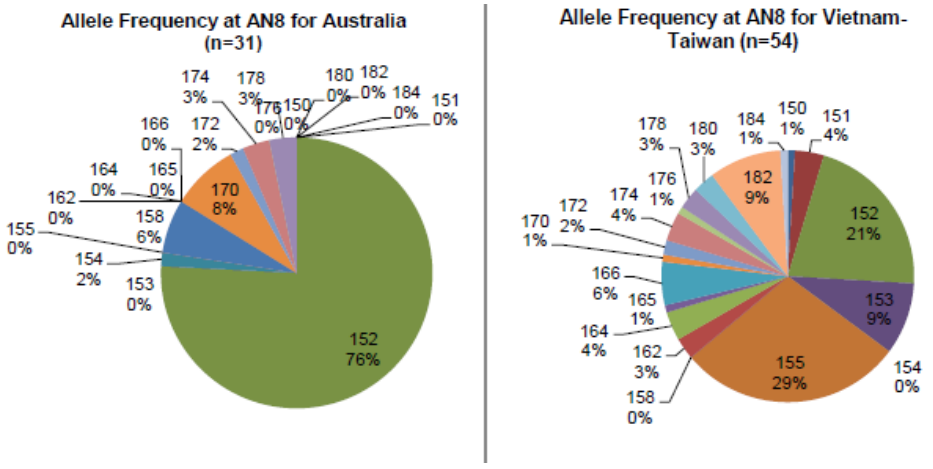

Figure 13. Allele frequencies of AN8, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

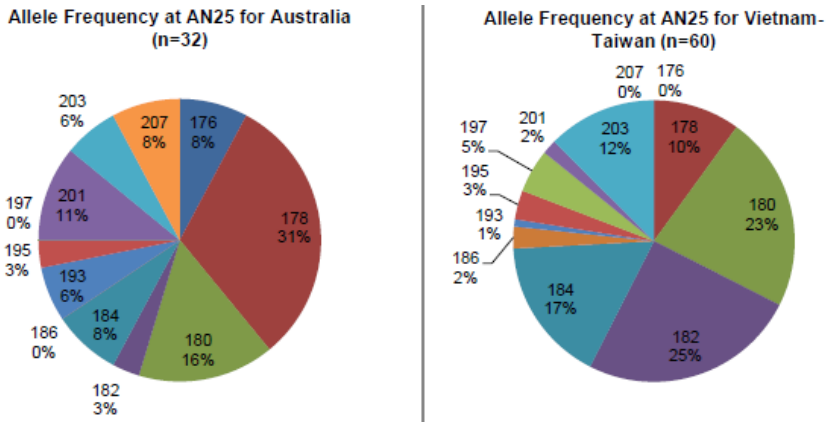

Figure 14. Allele frequencies of AN25, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

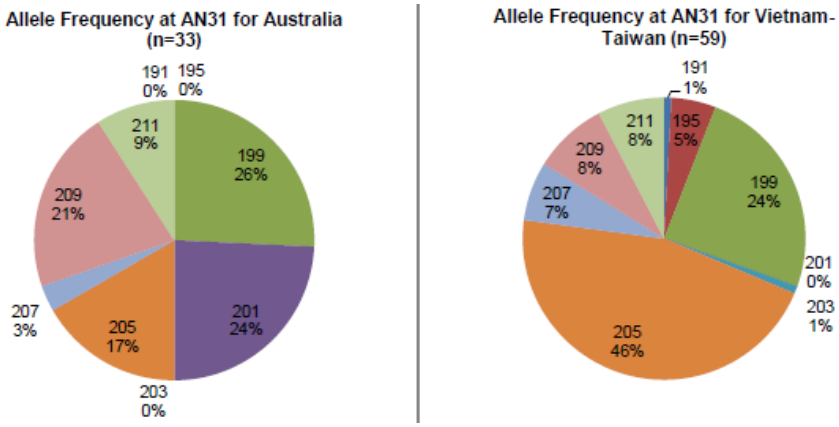

Figure 15. Allele frequencies of AN31, for Australia and Vietnam-Taiwan populations. N= number of genotyped individuals at each locus.

Phylogenetic relationship of the four *E. lanceolatus* populations was further examined using *cox1* mitochondrial DNA sequences. Results of the analysis revealed that of the 86 sequences analysed, 78 were indeed *E. lanceolatus* while 8 were found to be more closely related to *Epinephelus fuscogutattus* (Brown-marbled Grouper) (Figure 16).

Figure 16. Phylogenetic tree showing the 8 samples (in red box) not grouping with the confirmed giant grouper samples (in green box). The eight samples were found to be more closely related to *Epinephelus fuscogutattus* (Brown-marbled Grouper; also shown in the red box).

6.3 Breeding trials in Vietnam

6.3.1 Induced spawning trials

A total of 7 female and 4 male giant grouper broodstock were induced to spawn in tanks while 6 female and 7 male broodstock were induced to spawn in cages. Two female fish in tanks and one female broodstock in a cage died a few days after GnRHa implantation. All of the remaining females (n=10) spawned 6-7 days after the hormonal treatment for a consecutive 3-4 days. In total, 26.86 million eggs, 12.24 million fertilised eggs (45% hatching rate), and 8.47 million larvae (32% hatching rate) were produced. Detailed spawning data are shown in Tables 7- 10.

Table 7. Spawning data from Tank No 1.

Day/month	Eggs (Million)	Fertilization rate (%)	Fertilized eggs (Million)	Hatching rate (%)	Hatched larvae (Million)
3/10/2012	2.80	30	0.85	25	0.70
4/10/2012	2.30	48	1.10	41	0.95
5/10/2012	1.40	50	0.70	46	0.65
6/10/2012	0.52	58	0.30	37	0.19
Total	**7.02**	**42**	**2.95**	**35**	**2.49**

Table 8. Spawning data from Tank No 2.

Day/month	Eggs (Million)	Fertilization rate (%)	Fertilized eggs (Million)	Hatching rate (%)	Hatched larvae (Million)
3/10/2012	1.70	32	0.54	28	0.48
4/10/2012	1.94	72	1.40	67	1.30
5/10/2012	1.25	64	0.80	56	0.70
6/10/2012	0.90	28	0.25	17	0.15
Total	**5.79**	**52**	**2.99**	**45**	**2.63**

Table 9. Spawning data from Cage B1 and B2.

Day/month	Eggs (Million)	Fertilization rate (%)	Fertilized eggs (Million)	Hatching rate (%)	Hatched larvae (Million)
3/10/2012	1.20	0	0.00	0	0.00
4/10/2012	3.00	53	1.60	30	0.91
5/10/2012	3.00	50	1.50	30	0.89
6/10/2012	0.85	45	0.38	0	
Total	**8.05**	43	**3.48**	**22**	**1.80**

Table 10. Spawning data from Cage A5 and A6.

Day/month	Eggs (Million)	Fertilization rate (%)	Fertilized eggs (Million)	Hatching rate (%)	Hatched larvae (Million)
4/10/2012	3.40	32	1.10	18	0.60
5/10/2012	1.60	75	1.20	44	0.70
6/10/2012	1.00	52	0.52	25	0.25
Total	**6.00**	47	**2.82**	**26**	**1.55**

Broodstock management

Fish are reared in timber cages (3m x 6.5m x 3m; Figure 17B) at a stocking density of 8 – 12 fish/cage depending on the body weight of the fish. The cage nets are changed monthly for better water circulation and cleanliness. Giant grouper broodstock at Cat Ba rarely die due to disease. Regular cleaning of the cages and providing sufficient high quality food are being applied as preventive measures. Broodstock are fed with fresh trash fish at a daily intake of 1.5-2% body weight. Feeding is done once a day, but in winter, only once every two days. Feeding is modified based on the fish feeding behaviour. For 2.5 months before the spawning season, broodstock are fed twice a week with crabs and squid in the amount equal to 2% body weight as a nutritional supplement. From 2001 to present, almost all giant groupers have been reared according to this regime: normal feeding regime (non-spawning season) or intensive feeding (several months before spawning reason). Broodstock body weight usually increases by 3-6 kg/year. Broodstock implanted with GnRHa are fed with live crabs and squid with further supplementation of some vitamins. Fish are fed to satiation.

Spawning tanks (90 m^3) are provided with clean sea water that passed through a sand filter. In order to stimulate spawning, we designed a system that provides water flow going around the inside of the tank. Water exchange is about 30% a day. Fish in tanks usually stop feeding for 2 days after being transferred from cages. Fish usually spawned at midnight during the full moon.

Figure 17. Broodstock and algal culture facilities at RIA1, Vietnam.

6.3.2 Larval rearing trials

Experiment 1: Effect of water temperature on the development of giant grouper embryos and hatching rate

Embryo development was most rapid at 35°C (18h 50min) and the slowest rate at 26°C (20h 12min). The embryo developed normally and hatched at all temperature treatments, except at 23°C where the fertilized eggs proceeded to 16 cell embryo but did not develop further and then died out.

Hatch and deformity rate varied among the temperature treatments. At 29-32°C, the hatch rate was between 60.3 and 63.3%, significantly higher than all other treatments ($p<0.05$). However, there is no significant difference in hatch rate between 29°C and 32°C treatments ($p>0.05$). The lowest deformity was found at 29°C (2.5%). Occurrence of deformities was considerably higher at 32°C (10.3%). At 35°C, the lowest hatch rate and the highest deformity rate were observed (96.5%). Details of this experiment are shown in Table 11 below.

Table 11. Embryo development, hatching rate, deformity of eggs incubated at different water temperatures

Embryo stages	Temperature (°C)				
	23	26	29	32	35
2 -4 cells	29 min	24 min	20 min	18 min	18 min
16 cells	38 min	34 min	30 min	25 min	22 min
Morula	Development stopped.	3h20	3h00	2h46	2h10
Blastula	-	3h52	3h30	3h22	3h18
Gastrula	-	4h25	4h00	3h52	3h44
Neurula	-	15h58	15h30	15h05	14h50
Hatching time	-	20h12	19h25	19h05	18h50
Hatch (%)	0	29.1 ± 3.2^{a}	63.3 ± 2.4^{b}	60.3 ± 2.3^{b}	32.1 ± 4.5^{c}
Deformity (%)	0	4.1 ± 2.1^{a}	2.5 ± 0.5^{b}	10.3 ± 1.2^{c}	96.5 ± 3.4^{d}

Note: Different letters in the same row means significant difference (P<0.05)

Experiment 2: Effect of salinity on embryonic development and hatching rate

Embryos grew most rapidly at salinity of 36‰ (18h 30min) and slowest at 24‰ (19h 00min), however at salinity of 20‰, embryos stopped developing after reaching blastula stage. At the salinity range from 32 to 36‰, highest hatch rate (59.2 – 63.0%) and lowest deformity (1.7-1.9%) were observed. There is no significant difference in hatch rate and deformity rate between treatments at 32 and 36‰ (p>0.05). However, at salinity of 24‰, lowest hatch rate and highest deformity were observed (16.3% and 2.8%, respectively). Details are shown in Table 12.

Table 12. Time length, hatch rate, deformity rate at different salinity

Development stages	Salinity (‰)				
	20	24	28	32	36
2 -4 cells	22 min	24 min	20 min	18 min	18 min
16 cells	38 min	35 min	28 min	25 min	22 min
Morula	3h15	3h20	3h00	2h41	2h48
Blastula	Development stopped.	3h42	3h28	3h18	3h18
Gastrula	-	4h12	4h05	3h47	3h44
Neurula	-	15h32	15h22	15h02	14h50
Hatching time	-	19h00	18h51	18h33	18h30
Hatch (%)	0	16.3 ± 2.5^{a}	36.5 ± 2.3^{b}	63.0 ± 2.4^{c}	59.2 ± 2.5^{c}
Deformity (%)	0	2.8 ± 0.5^{a}	1.8 ± 0.7^{b}	1.7 ± 0.4^{b}	1.9 ± 0.6^{b}

Notes: Different letters in the same row means significant difference (p<0.05)

Experiment 3: Larval rearing trial in 8 m^3 indoor concrete tanks and 750 m^3 outdoor tanks

(a) Indoor 8 m^3 concrete tanks

Larvae usually used up their yolk at Day 3 after hatching. From Day 11, some spines appeared on the larvae. From Day 12, heavy larval mortalities were observed. There were about 350,000 to 400,000 larvae that were still healthy but they all died on Day 16. All samples taken from dead larvae were positive with VNN.

(b) Outdoor 750 m^3 tanks covered with a polyethylene roof

Day 3 from hatching, yolk was used up and larvae started feeding. When larvae reached Day 25, about 300 -320 larvae were counted. However, up to Day 28, most larvae died out. All samples of the dead larvae were positive with VNN. Larval developmental stages are illustrated in Figure 18.

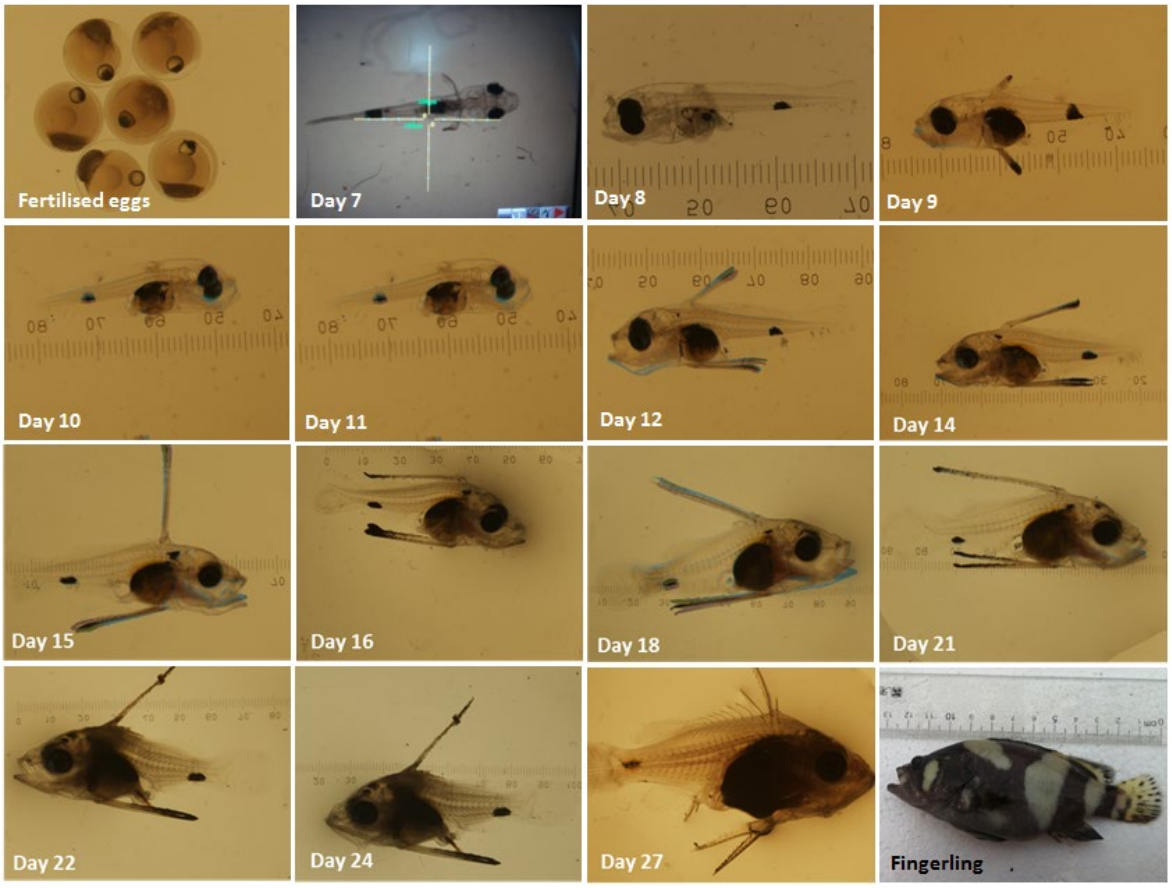

Figure 18. Development of giant grouper larvae until the fingerling stage. Fertilised eggs were obtained from broodstock which were induced to spawn with the GnRHa implants.

6.4 Grouper broodstock collection in the Philippines

The main activity of the SRA in the Philippines has been the acquisition of adult giant groupers from the wild to be used for the series of experiments that will be done for the next stage of the project.

For this, three new broodstock cages that measure 10 x 10 x 5 m (L x W x H) were constructed in Igang Marine Station (IMS) of SEAFDEC/AQD for holding the new giant groupers that are currently being acquired (Figure 19). Two of the cages are divided into 4 units each of 5 x 5 x 5 m. These cages will be used to hold only one size of breeders, especially the smaller ones, in one cage unit. The bigger size fish will be stocked in the larger cage. Considering the number of fish that will be collected, 3 more cages of the same size are being constructed to make the total number of cages to 6.

The fishermen in Palawan (South western Philippines) and Samar (Eastern Philippines) are currently collecting giant groupers for the project. During the early part of the Project, they were instructed to collect giant groupers of sizes ranging from 10 kg and up. Initial attempts to collect giant groupers of sizes between 15-30 kg proved to be very difficult because of the huge size. Although few fishes were collected by the fishermen, they did not survive. As a result, smaller size giant groupers with body weights ranging from 3-10 kg were decided to be collected instead. The fishermen were requested to collect about 60-80 pieces of giant groupers of the size range between 3-10 kg. Specifically, the instruction was to collect 10 pieces each of 3 kg, 4 kg, 5 kg, 6 kg, 7 kg, 8 kg, 9 kg and 10 kg size. These are ideal sizes for the experiments to determine the earliest size or age for sexual maturation and for the induction of sex reversal. At present, SEAFDEC/AQD has two giant groupers stocked in a cage in IMS and weigh about 50-100 kg. All giant groupers will be tagged and fin clipped for genetic analysis.

Aside from the newly-constructed cages to hold the giant grouper stocks, concrete broodstock tanks in SEAFDEC/AQD's Tigbauan Main Station are also available for actual breeding and spawning experiments.

The SEAFDEC/AQD Team also undertook initial discussions with a private company, Palawan Aquaculture Corporation, for collaboration in the giant grouper project. The company has existing giant grouper breeders: 8 pcs of 140-150 kg body weight (BW), 4 pcs of 80-100 kg BW, and 3 pcs of 15-20 kg BW stocked in cages (Figure 20). These can be used as alternative breeders for spawning purposes.

Figure 19. The newly-constructed cages for holding giant grouper broodstock collected from the wild.

Figure 20. Cages holding giant groupers in Palawan Aquaculture Corporation, in Palawan, southern Philippines.

7 Conclusions and future directions

The trip to Taiwan was an excellent opportunity for team building and understanding of the relative strengths and weaknesses of the three collaborating groups. The visit to the farms provided an understanding of the practices in Taiwan and the realisation of issues which highlighted the need to conduct a thorough understanding of giant grouper biology in order to establish a sustainable aquaculture for the species. Taiwan is currently the dominant producer of giant grouper in the SE Asian region. However, their culture techniques are highly variable even within a particular facility. Biosecurity is a major concern which is behind the widespread problem of viral diseases that cause major losses in grouper culture. Inbreeding is most likely a major issue as well, as there is no system for genetic management. Nevertheless, Taiwan has an active R & D for giant grouper. Areas of interests include hybridization to produce faster growing and disease-resistant grouper fry, identifying molecular markers for specific traits, use of probiotics (herbal materials) for improved health management, and strategies to control VNN occurrence in grouper farms. Yet, there is a big knowledge gap concerning giant grouper reproductive biology that remains to be investigated.

In Vietnam, a visit by Richard and Adam enabled the overseeing and supervision of GnRHa implantation, which successfully induced spawning in all of the treated broodstock. This is the first time giant grouper spawning was achieved in Vietnam. Optimal water temperature and salinity ranges for embryonic and larval development have been determined following the preliminary experiments on their rearing. Although massive mortalities have occurred, some giant grouper fingerlings have been produced, and many lessons learnt along the way in terms of handling, feeding and husbandry regimes. The existing giant grouper broodstock have been tagged and genotyped, which will facilitate genetic management. Infrastructure renovation (broodstock and spawning tanks) has been carried out and RIA1 are in position to start the full project. A full list of renovations undertaken is in the financial acquittal document.

In the Philippines, there has been a delay in sourcing fish however this is now being managed and giant grouper broodstock ranging in size from 3 to 10 kg are currently being sourced from the wild. Three cages have been constructed to hold the broodstock and three additional cages will be further constructed.

Relevant molecular and genetic tools were developed at USC. As a first step, a much cheaper DNA extraction method has been optimised. This is important in the context of developing affordable tools that are applicable in the Philippines and Vietnam, where cost of sample preparation is highly significant. A set of giant grouper DNA markers has been identified that enabled high confidence parentage assignment. For instance, the genetic structure of the giant grouper population at NFC has been established. In conjunction with DNA microsatellite markers, *Cox1* mitochondrial DNA sequence analysis was performed which enabled the proper identification of the grouper species from 4 locations, namely Australia, Vung Tau and Cat Ba Island in Vietnam, and Taiwan. A noteworthy finding was the identification of a species other than giant grouper (closely related to *Epinephelus fuscogutattus* or brown-marbled grouper) among the broodstock in Vung Tau, Vietnam, and samples collected from Taiwan. The use of DNA microsatellite makers in conjunction with *cox1* mitochondrial DNA sequencing can assist in (a) the development of a pedigree for use in selective breeding programs; (b) management of inbreeding in captive populations; and (c) perhaps help to optimize the mating/sex composition and numbers in captive breeding tanks. The use of these markers will facilitate further research into the population dynamics of this species in the wild and culture industry. Additional DNA markers are currently being developed at USC. During the phase 2 project, the application of DNA markers will be further expanded to identify associations between single

nucleotide polymorphisms (SNPs), genes and traits of fast growth and possibly disease resistance.

8 References

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9 Appendixes

9.1 Taiwan study tour itinerary

Queensland Government

Website: www.export.qld.gov.au
www.queensland.org.tw

Delegation
Prof. Abigail Elizur, Professor of USC **Dr. Richard Knuckey, Principal Scientist of NFC** **Dr. Felix Ayson, Director of SEAFDEC, Philippines** **Dr. Fiona Pedroso, Research Scientist of SEAFDEC, Philippine** **Dr. Tran Dinh Luan, Deputy Director of RIA1** **Mr. Hoang Nhat Son, Vice Director of National Broodsotck Center for marine species** *Accompanied by Claire Huang,* *Business Development Manager* *(Mobile: +886-933-080-242)*

Contact Officer:	Claire Huang, Business Development Manager Trade & investment Queensland Office Taiwan Suite 3202, 32nd Fl., International Trade Building, 333 Keelung Road, Section 1, Taipei 110, Taiwan 澳大利亞昆士蘭州貿易暨投資辦事處 □ □ 台北市基隆路1段333號32樓3202室
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□ □ □ □ □ □ □ □ □ □ 33□
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www.han-hsien.com.tw

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  台北市復興南路一段317號
  Tel: +886-2-5579-3888
  www.parktaipei.com

ITINERARY

Sunday, 24 June 2012

Time	Activity	Note
-	**Arrival in Kaohsiung & Check in at Urban Hotel Kaohsiung**	**It takes 25 minutes and cost about NT$300 by taxi from Kaohsiung international airport to Urban Hotel.**

Monday, 25 June 2012 (Casual wear is highly recommended for today's program)

Time	Activity	Note
10:00am	**Meet up at lobby of Urban Hotel Kaohsiung** ***Claire will go to Kaohsiung from Taipei in the morning.***	Accompany by Claire Huang +886-933-080-242
11:00am – 12:00pm	**Visiting Yong An grouper center in Kaohsiung** □ □ □ □ □ □ □ □ □ Tel: +886 7 6912031 □ □ □ □ □ □ □ □ □ □ □ 11□	Accompany by Claire Huang +886-933-080-242
12:05pm – 13:30pm	**Lunch break (Optional: □ □ □ □ □ □ □)** Tel: 07 691 0991 □ □ □ □ □ □ □ □ □ □ □ 6□ 10□	Accompany by Claire Huang +886-933-080-242
14:00pm – 15:30pm	**Visiting Ou A Liao fish market** **蚵仔寮港觀光魚市** Tel: +886 7 619 4100 □ □ □ □ □ □ □ □ □ □ □ 306□	Accompany by Claire Huang +886-933-080-242
15:30pm – 16:20pm	**Back to Urban Hotel Kaohsiung**	-

Tuesday, 26 June 2012

Time	Activity	Note

08:00am	**Meet up at lobby at Urban Hotel Kaohsiung**	-
09:00am – 11:00am	**Visiting Tungkang Biotechnology Research Center (grouper breeding, vaccine research and virus prevent research) in Pingtung** ***Contact: Dr. Tzy Ying Chen*** ***Tel:+886 8 8324121 ext. 211*** Address: □ □ □ □ □ □ □ □ □ □ □ □ 67□	Accompany by Claire Huang +886-933-080-242
11:10am – 12:25pm	**Lunch break in Pingtung** □ □ □ □ □ □ □ □ Tel: 08 833 8586 □ □ □ □ □ □ □ □ □ □ 76□	Accompany by Claire Huang +886-933-080-242
13:00pm – 14:00pm	**Visiting grouper farm (Long Diann Marine Bio Technology Co., Ltd) in Pingtung** ***Contact: Ms. Penny Cheng*** Tel: +886 8 8782971 □ □ □ □ □ □ □ □ □ □ □ □ 853□	Accompany by Claire Huang +886-933-080-242
14:00pm – 15:30pm	**Visiting another grouper farm which is nearby Long Diann grouper farm** ***Contact: Mr. Chen*** □ □ □ □ □ □ □ □ □ □ Tel: +886 927 282 679	Accompany by Claire Huang +886-933-080-242
15:30pm– 16:30pm	**Back to Urban Hotel Kaohsiung**	-

Wednesday, 27 June 2012

Time	Activity	Note
08:25am	**Meet up at lobby of Urban Hotel Kaohsiung**	-
09:00am – 10:30am	**Visiting Department & Graduate Institute of Aquaculture, National Kaohsiung Marine University** ***Professor Tom Hsiao, specialize in grouper*** Contact: Kay Chen, Assistant of International Office / 0937 679 689 Tel: +886 7 3617141 ext. 2346 Address: No. 142, Haijhuan Rd., Zanzih District, Kaohsiung City □ □ □ □ □ □ □ □ □ 142□	Accompany by Claire Huang +886-933-080-242
12:00pm – 12:50pm	**Lunch break at Salt Mountain in Cigu, Tainan** Tel: +886 6 780 0511 □ □ □ □ □ □ □ □ □ 66□	Accompany by Claire Huang +886-933-080-242

13:00pm – 15:00pm	**Visiting Mariculture Research Center (grouper fry breeding and production) in Tainan** □ □ □ □ □ □ □ □ □ ***Contact: Director Yeh□ □ □ □ □*** Tel: +886 6 788 0461 ext. 201 Address: □ □ □ □ □ □ □ □ □ □ 4□	Accompany by Claire Huang +886-933-080-242
15:00pm – 15:50pm	**Arrival at Tainan High Speed Rail Train station**	-
16:30pm – 18:06pm	**Back to Taipei by High Speed Rail Train**	-

Thursday, 28 June 2012

Time	Activity	Note
10:10am	**Meet up at lobby of Park Hotel Taipei**	Accompany by Claire Huang +886-933-080-242
10:30am – 11:30am	**Visiting MAGQU Co., Ltd (Grouper virus inspection equipment)** **http://www.magqu.com/about/index.php** ***Contact: Ms. Michelle Lu*** Tel: +886 2 8667 1897 Address: 4F No. 14 Lane 130 MinCyuan Rd., Xindian District New Taipei City □ □ □ □ □ □ □ □ □ 130□ 14□ 4□	Accompany by Claire Huang +886-933-080-242
11:30am	**Back to Park Hotel Taipei**	-

Friday, 29 June 2012

Time	Activity	Note
09:10am	**Meet up at lobby of Park Hotel Taipei**	Accompany by Quentin Bai, Commissioner of TIQ Claire Huang +886-933-080-242
10:00am – 12:00pm	• **Visiting National Taiwan Ocean University** **Department of Aquaculture, College of Life Sciences** • **Meeting with Ching-Fong Chang** **Distinguished Professor, Department of Aquaculture** ***Contact: Mario Huang□ □ □ □ □*** Tel: +886 2462 2192 ext.5203 / 0921 762 040 Address: 2 Pei-Ning Road,Keelung ,Taiwan	Accompany by Quentin Bai, Commissioner of TIQ Claire Huang +886-933-080-242

	基隆市中正區北寧路2號	
12:00pm – 12:50pm	**Back to Park Hotel Taipei**	-
12:50pm – 13:30pm	**Lunch & Recap meeting with Trade and Investment Queensland – Taiwan office**	Accompany by Quentin Bai, Commissioner of TIQ Claire Huang +886-933-080-242

Saturday, 30 June 2012

Time	Activity	Note
-	**Fly back to Brisbane / Philippine / Vietnam**	

END OF PROGRAM