GENOMIC LIBRARY CONSTRUCTION AND INHERITANCE PATTERNS ANALYSIS OF F1 GROUPER HYBRID: *Epinephelus coioides* X *Epinephelus lanceolatus*

LIM MING LEE

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Lim Ming Lee
PB20108056

CERTIFICATION

NAME : **LIM MING LEE**

MATRICE NO. : **PB20108056**

TITLE : **GENOMIC LIBRARY CONSTRUCTION AND INHERITANCE PATTERNS ANALYSIS OF F1 GROUPER HYBRID: *Epinephelus coioides* X *Epinephelus lanceolatus***

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VIVA DATE : **27th August 2012**

DECLARED BY

1. **SUPERVISOR**
   Dr. Kenneth F. Rodrigues

   Signature

2. **Examiner 1**
   Dr. Shafiquzzaman Siddiquee

3. **Examiner 2**
   Dr. Christopher Voo Lok Yung

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ABSTRACT

Genomic library construction and inheritance patterns analysis of F1 grouper hybrid: *Epinephelus coioides* X *Epinephelus lanceolatus*

The F1 hybrid of *Epinephelus coioides* and *Epinephelus lanceolatus* is a fast growing and well tolerance to crowing stress. An understanding of the underlying genomic inheritance patterns facilitates our understanding of the association between phenotypes and genotypes in F1 hybrid. A panel of thirty molecular markers was developed based on the partial small insert genomic library that constructed previously using shotgun approach. Testing against parental genotype and closely related grouper species revealed two markers which were unique to the original F1 hybrid; none of the primers amplified in the groupers *Epinephelus fuscoguttatus* and *Epinephelus corallicola;* seventeen markers were shared between *Epinephelus coioides* and *Epinephelus lanceolatus,* five were unique to *Epinephelus coioides* and seven were unique to *Epinephelus lanceolatus* including two heterozygous markers. These markers can be utilized to determine inheritance pattern of this F1 grouper hybrids and also genetic similarity and differentiation among all the tested groupers.

ABSTRAK

The F1 hibrid Epinephelus coioides dan Epinephelus lanceolatus adalah sejenis ikan yang cepat berkembang dan boleh tahan tekannan kesesakan.. Pemahaman corak warisan yang mendasari genomik akan memudahkan pemahaman kita bersekutu antara fenotip dan genotip dalam hibrid F1. Tiga puluh penanda molekul telah dibangunkan berdasarkan perpustakaan genomik perpustakaan yang dibina sebelum ini menggunakan pendekatan senapang patah. Ujian terhadap genotip ibu bapa dan spesies kerapu yang berkait rapat mendedahkan mendedahkan dua penanda molekul yang unik untuk hibrid F1 asal; tiada penanda molekul untuk ikan kerapu Epinephelus fuscoguttatus dan Epinephelus corallicola, 17 penanda molekul dikongsi antara Epinephelus coioides dan Epinephelus lanceolatus, lima penanda molekul adalah unik untuk coioides Epinephelus dan tujuh penanda molekul adalah unik untuk Epinephelus lanceolatus termasuk dua penanda heterozigot. Penanda ini boleh digunakan untuk menentukan corak pewarisan F1 kacukan kerapu ini dan juga persamaan atau perbezaan genetik di kalangan semua kerapu yang diuji.

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LIST OF ABBREVIATION / SYMBOLS

A	Adenine
AFLP	Amplified fragment length polymorphisms
blast	Basic local alignment search tool
blastn	Nucleotide-nucleotide blast
blastx	Nucleotide 6-frame translation protein
bp	Base pair
C	Cytosine
cm^2	centimeter square
cSNP	Coding region single nucleotide polymorphism
CoxI	Cytochrome oxidase subunit I gene
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
ddNTPs	Dideoxynucleotide triphosphates
E Value	Expect value
E. coioides	*Epinephelus coioides*
E. coli	*Escherichia coli*
E. corallicola	*Epinephelus corallicola*
E. fuscoguttatus	*Epinephelus fuscoguttatus*
E. lanceolatus	*Epinephelus lanceolatus*
EDTA	Ethylenediamminetetraacetate ion
EPA	Eicosapentaenoic acid
eSNP	Expressed single nucleotide polymorphisms

EST	Expressed sequence tags
F1	Filial 1
FASTA	FAST-All
G	Guanine
g	Gravity
GC	Guanine-cytosine
Indels	Insertion/deletion polymorphisms
ISSR	Inter-simple sequence repeat
IUCN	International Union for Conservation of Nature
Kb	Kilo base pair
MAS	Marker-assisted selection
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nano grams
nm	nano meter
nmol	nano moles
PCR	Polymerase chain reaction
PIC	Polymorphic information content
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RE	Restriction enzymes
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid

RT-PCR	Reverse transcription polymerase chain reaction
SCARs	Sequence characterized polymorphic regions
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphisms
T	Thymidine
Taq	*Thermus aquaticus*
TBE	Tris boric acid ethylenediamminetetraacetate
Tm	Melting temperature
TNTC	Too numerous to count
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
U/µl	Units per micro liter
UV	Ultraviolet
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background

Groupers are teleosts in the subfamily Epinephelinae of the family Serranidae that comprises about 159 species of marine fishes in 15 genera (Heemstra and Randall, 1993). Groupers are multicellular heterotrophs with a notochord that stiffened their bodies and allowed for better support. Groupers are bottom-associated fishes feed on a variety of fishes, larger crustaceans, and cephalopods and can be found in every ocean on the planet including tropical and subtropical (Heemstra and Randall, 1993). Majority of the groupers are monandric protogynous hermaphrodites but some also show to be gonochoristic (Heemstra and Randall, 1993; Erisman *et al.*, 2009; DeMartini *et al.*, 2011). Grouper play a vital role in keeping the balance of the coral reef (Cowen *et al.*, 2006). Groupers also well known of its economic value. However, overfishing has drastically damages the groupers numbers and result in several species of groupers are threatened with extinction of sexually mature individuals.

The high demand of groupers has prompted the need for cultivating grouper. In fact, groupers represent one of the important species for Asian aquaculture nowadays mainly in Hong Kong, China, Singapore and Taiwan. However, grouper culture in the Asia-Pacific region encounters several constraints to sustainable principally by the availability and quality of seeds from hatcheries. This phenomenon is mainly due to the lack of proper brood stock management practices coupled with poor and unreliable survival of larvae (Marte, 2003). Therefore, hybridization has been frequently applied by aquaculturists in the hope to boost up the quality of fish production by combining both desirable traits from each parent. Orange-spotted grouper (*Epinephelus coioides*) is famous of its

hardiness and tolerance to crowding and has become a prime candidate for commercial aquaculture (Ahmad *et al.*, 2000). Giant grouper (*Epinephelus lanceolatus*) on the other hand is also popular among farmers for its rapid growth. Reports showed that giant grouper able to grow up to 3 kg in their first year (Sadovy *et al.*, 2003). Therefore, hybridization of these two species is believed to produce a fast growing and hardy offspring.

The development of molecular markers has had a revolutionary impact on animal genetics including marine fishes. Molecular markers allow rapid progress in aquaculture investigations of genetic variability, parentage assignments, species identification, and linkage maps construction. Popular genetic markers used in aquaculture include allozymes, mitochondrial DNA, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), minisatellites, microsatellites, single nucleotide polymorphisms (SNP), and expressed sequence tags (EST) markers (Liu and Cordes, 2004). Many studies have been done on grouper species. For instance, the microsatellite method has been used to study genetic variation of grouper species (Antoro *et al.*, 2006; Ramirez, 2006; Wang *et al.*, 2007; Zeng *et al.*, 2008; Rodrigues *et al.*, 2009) and reported polymorphic loci in those populations. Recently, Chiu and other researcher also used multi-molecular marker that included random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and the cytochrome oxidase subunit I gene (*CoxI*) of mitochondrial DNA to analyze genetic variation in giant grouper or *Epinephelus lanceolatus* (Chiu *et al.*, 2012). In retrospect, molecular markers have become more popular and frequently used to study marine fishes including a vast variety of grouper species.

With regards of the numerous studies that done on different grouper species, limited studies have been conducted purely on grouper hybrids especially for those that done on molecular level or genetic investigation. Hence, a molecular study of F1 hybrid grouper is deeply needed. In this study, one of the F1 hybrid grouper of *Epinephelus coioides* and *Epinephelus lanceolatus* developed at the Borneo Marine Research Institute is the target species for this study (Chu *et al.*, 2010). A partial small insert genomic library provides genome database of this F1

hybrid grouper is needed in order to develop molecular markers. Testing of the developed molecular markers on other F1 grouper hybrid of *Epinephelus coioides* and *Epinephelus lanceolatus,* parental genotype and closely related grouper species discloses the number of loci inherited from each of the parental genotype and/or the uniqueness or polymorphic the developed molecular markers. Future studies using a larger number of loci that typically more than 400 genomic loci result in the development of a linkage map for groupers. Then, study into functional level of the obtained genomic loci simplify the process of choosing a grouper hybrid with desirable traits and realized the development of marker-assisted selection (MAS).

1.2 Objectives

The objectives of this study were:

a. To construct a partial small insert genomic library of the F1 hybrid of *Epinephelus coioides* and *Epinephelus lanceolatus* by using shotgun approach.
b. To design locus specific molecular markers for the F1 hybrid of *Epinephelus coioides* and *Epinephelus lanceolatus.*
c. To conduct cross amplification test and analyze inheritance patterns in the F1 hybrid of *Epinephelus coioides* and *Epinephelus lanceolatus.*

CHAPTER 2

LITERATURE REVIEW

2.1 Groupers

The word "grouper" come from a Portuguese name, *garoupa* that could trace back to an indigenous South American language. However, not all serranids are called groupers, others like sea basses, rockcod and hinds also included in this big family (Heemstra and Randall, 1993). The common name grouper is usually used by *Epinephelus* and *Mycteroperca* and some small genera likes *Anyperidon*, *Cromileptes*, *Dermatolepis*, *Gracila*, *Saloptia, Gonioplectrus*, *Niphon*, *Paranthias* and *Triso.* Besides, hamlets in the genus of *Alphestes*, the hinds in the genus of *Cephalopholis* and the *lyretails* in the genus of *Variola* are also named as grouper. Some other genus such as *Plectropomus* is commonly called as coralgroupers. All the mentioned genera are classified in the subfamily Epiphelinae (Heemstra and Randall, 1993). In short, the word "groupers" is usually taken as meaning the subfamily Epinephelinae.

Groupers are multicellular heterotrophs with a dominant notochord or backbone that stiffened and supported their bodies. This makes groupers to swim quicker and rigorously which seen to be a significant advantage over those lacking this trait. Over the time, these ancestral notochord groupers had evolved into a more easily regulated, lighter and flexible jaws, tail and fins (Craig and Daniel, 2001). This internal muscular-tendonous system surely could neutral buoyancy using swim bladder and improves the mouth structure for better feeding (Craig and Daniel, 2001). Groupers are not built for long-distance fast swimming thus they normally could weights up to 100 kg and over a meter in lengths.

As the major predators of the coral-reef ecosystem, most groupers feed on a variety of fishes, larger crustaceans, and cephalopods (Heemstra and Randall, 1993). Groupers do not have many teeth on the edges of their jaws, only some heavy crushing tooth inside the pharynx. They swallow prey from a distance by using a powerful sucking system of their mouth and gills that could easily suck in a large volume of water together with their prey in less than a second. They normally won't bit their prey into pieces before swallow. Groupers are a well known as ambush predators because they like to lie in coral or rocks waiting for their prey and attack or suck in their prey suddenly (Heemstra and Randall, 1993). They seldom chase to feed their prey in open water. Some other groupers like *Paranthias* species or *Epinephelus undulosus* are adapted for plankton feeding. They have numerous long gill rakers to easily feed on plankton. Besides hiding in coral or rocks, grouper also like to swim around or cruising the reef and shallows finding their meals. A study found out that the feeding habit of grouper is largely influence by their age (Craig *et al.*, 1999). Mature groupers like to feed on a variety of larger prey such as parrotfish, wrasses, damselfishes, snappers, octopus, crab, lobster, and crustaceans. On the other hand, the juvenile groupers like to feed on plankton, crustaceans, microalgae and other small microorganisms (Craig *et al.*, 1999). The mouth of grouper is not only useful and powerful for feeding but also good in forming shelter to protect themselves of being caught by other larger predator. This is done by digging their mouth into sand and jetting under big rock using their gills. The powerful gill muscles could lock themselves inside the shelter under big rocks or cave so that it is nearly impossible to pull them out.

Groupers are bottom-associated fishes that can be found in every ocean on the planet including tropical and subtropical. The specific habitat of groupers varies from species to species and again largely depends on the age of the organism (Craig *et al.*, 1999). Juvenile groupers like to live near to the shore or hide in seagrass beds or tidal pools. This is because this place is normally free from other larger predator and suitable to protect them until maturity. Hence, mature groupers generally travel to other place which is easier for them to look for their meals like coral reefs, estuaries or on rocky reefs of tropical and subtropical waters

(Craig *et al.*, 1999). Groupers could also be found in depths of 100 to 200 m and occasionally up to 500 m.

2.2 Breeding of Groupers

Groupers are solitary fishes, they group together only when it is time to aggregate or reproduce (Heemstra and Randall, 1993). During spawning season, groupers aggregate in large numbers up to 100,000 individuals at localized spawning sites. Sometimes, they might need to migrate from several kilometers in order to reach their destined spawning place. This large aggregation of grouper increases the probabilities of successful mating and chance of surviving. The time for grouper spawning is between May and August (Heemstra and Randall, 1993). Males are known to be able to spawn many times during the breeding period; but females can only spawn once a year (Heemstra and Randall, 1993). Studies showed that most groupers are monandric protogynous hermaphrodites meaning that they mature only as females but have the ability to change sex after sexual maturity (Heemstra and Randall, 1993; Erisman *et al.*, 2009; DeMartini *et al.*, 2011). Groupers grow approximately one kilogram per year and they turn into female when they grow up to three kilograms. Interestingly, after spawning as a female for one or more years as a female grouper may change sex and become a male (Heemstra and Randall, 1993). During sexual transition, the oocytes degenerate, the spermatogonia proliferate, and the ovary is transformed into a functional testis (Heemstra and Randall, 1993). The largest males often control the harems that contain three to fifteen females (Sadovy and Collin, 1995; Erisman *et al.*, 2009). Groupers often spawn in pair in which larger males will competitively eliminate smaller males from other female (Allsop and West, 2003; Munoz and Warner, 2003; Kuwamuru, 2004; Erisman *et al.*, 2009). Hence, if a small female grouper change sex, it definitely encounter problem to outcompete other larger male (Allsop and West, 2003; Munoz and Warner, 2003; Kuwamuru, 2004). Therefore, the sexual transition often performed by larger female groupers to increase their fitness and become more competitive in term of size. This is particularly important when the amount of large sexually mature male groupers decline drastically (Munoz and Warner, 2003). Unfortunately, once a female have transformed to a male, there is no turning back as they are unable to change back into female

again. This protogynous mode of reproduction is sometimes very complicated in certain species especially for those involve an exogenous or behavioural inducement of sexual transformation (Heemstra and Randall, 1993). This phenomenon is clearly seen in the situation where sexual transition happen over a broad range of size or age even for those female that have completed their transition (Heemstra and Randall, 1993).

On the other hand, some groupers are gonochoristic which mean individual specie is unisexual instead of bisexual (Erisman *et al.*, 2009). For example, grouper species like Nassau grouper or *Epinephelus striatus* (Sadovy and Colin, 1995) and leopard grouper, *Mycteroperca rosacea* (Erisman *et al.*, 2008) have been reported as a functional gonochores. They passed through bisexual phase when they are young and immature, and that no sign of sexual transition during adult stage. This reproductive strategy involves five times evolution in groupers independently (Erisman *et al.*, 2009). This evolution of gonochorism could be due to their reproduction behaviour like aggregating for spawning and hiding in their habitat. This may increase the survivor rate of the smaller male and thus increase the likelihood of a smaller male to compete with large male for reproduction (Munoz and Warner, 2003; Erisman *et al.*, 2008; Erisman *et al.*, 2009). The sperm production and larger teste size could also contribute for successfully compete for reproduction of smaller males (Sadovy and Colin, 1995; Munoz and Warner, 2003; Molloy *et al.*, 2007). In fact, gonochoristic groupers normally possess larger testes that comprise up to 10% of their body mass when compare with protogynous groupers in which the testes only comprise 1% of their body mass. This surely is a good indication that smaller grouper have evolved repeatedly and increased fitness in order to adapt better in the competing environment (Sadovy and Colin, 1995).

Finally, the shape of fertilized eggs of groupers is pelagic, spherical, and transparent with size of 0.70 to 1.20 mm in diameter. It will grow up to 1.85 mm when it hatched into a larva (Heemstra and Randall, 1993). The first feeding commences three days after hatching and by the time they normally will grow up to 2.6 mm in size. The shape of larvae is look like a kit and commonly known as a

"kite-shaped" body. An elongated second dorsal-fin spine and pelvic-fin spine are observed with a well-developed head spination and dense pigmentation on the dorsolateral surface of the body cavity (Heemstra and Randall, 1993).

2.3 Importance of Groupers

Groupers play an important role in the coral reef ecosystem. As a predator of the coral reef ecosystem, grouper serve to maintain the population certain species in the coral reef ecosystem to prevent the occurrence of overpopulation on the reef (Cowen *et al.*, 2006). In fact, there is a distinct relationship between groupers, shark, parrotfish, algae and coral reefs that must be place together to ensure the survival of the coral reef ecosystem (Cowen *et al.*, 2006). Another major importance of groupers as far as humans concerned is their economic value. According to the Food and Agriculture Organization of the United Nation, aqua farmers around the world bred approximately 78,425 tons of grouper in 2008 (Chiu *et al.,* 2012). Groupers comprise multi-million US dollars live reef fish market of the sea food trade centered and increasingly valuable as a commercial fishes in the fresh fish markets in tropical and sub tropical area. The wholesale premium market price can be up to US dollar of 100/kg in the Chinese live-fish markets like Hong Kong and South China (Asensio *et al.*, 2009). The increasing market demand of grouper surely will promote grouper fishing ground to develop rapidly.

However, the increasing demand of grouper has led to overfishing of grouper in many countries especially Asian countries. Overfishing especially during the groupers spawn season has drastically damages the numbers of sexually mature groupers. This will eventually led to a dramatic toll on the traits of the next generation and even kill entire populations. According to the data retrieved from IUCN or International Union for Conservation of Nature, twenty species of grouper are threatened with extinction (IUCN, 2007). Some grouper species of concern like orange spotted grouper or *Epinephelus coioides* assessed as being Near Threatened because the overall decline wild population especially juveniles fish have been taken extensively from South East Asia and also the losses of the key habitat (i.e. mangroves) for young *Epinephelus coioides* in some of the largest countries in Southeast Asia (Cornish and Harmelin, 2004). Another grouper

species of concern, giant grouper or *Epinephelus lanceolatus* is listed as Vulnerable on the Red List since the mid-1990s (Shuk and Ng, 2006). Giant groupers are well known for their delicious taste, high medicinal value and considered to confer good luck particularly to some Chinese. This in turn result in overfishing and the populations drop of giant grouper dramatically (Shuk and Ng, 2006). Other grouper species like *Epinephelus fuscoguttatus* have been assessed as being Near Threatened as little monitored over fishing especially during spawning season (Cornish, 2004), while there is insufficient information to make an informed assessment of *Epinephelus corallicola* (Rhodes *et al.*, 2008)

By the way, a depleted number of sharks have been related to the increase number in grouper populations (Cowen *et al.*, 2006). Besides, the increase number of groupers can result in reducing number parrotfish that responsible for maintaining the algae population in coral reefs, too. These in turn cause the overgrowing of algae in the coral reefs and result in death of the coral reef thus extinction of the entire coral ecosystem (Cowen *et al.*, 2006). On the other hand, a study conducted by American Museum of Natural History biologists revealed that increase number of groupers by banning fishing actually had positive effects on parrotfish on Caribbean reefs (Cowen *et al.*, 2006). With the decrease in fishing for about 20 years, parrotfish can to grow to a substantially large size than the average grouper could not swallow. This led to more and larger parrotfish and thus decreases the algal growth on the coral reefs that help in preserving the coral ecosystem.

In short, conservation efforts should emphasis on preserving the whole ecosystems and not only focuses on individual species genera in order to have a better output since all the populations in the ecosystems are close related and depend on each others. This can be done by preventing extinction of any species inside the ecosystems and that the intricate relationships between species are kept in a balance condition.

2.4 Aquaculture of Groupers

Aquaculture refers to the art, and business of cultivating marine or freshwater food fish. Nowadays, groupers represent an important species for Asian aquaculture mainly due to high profitability of its culture and the continuously increasing market demand for grouper meat especially in Asian countries. Hence, the culture technologies of groupers for several species are developed rapidly especially in Taiwan, China and Southeast Asia. Groupers culture was first introduced to Hong Kong and Taiwan in the early 1970s, and now occurred throughout Southeast Asia (Seng, 1998). Only selected species of groupers are being culture based on their commercial value and marker demand, thus around 20 species of them groupers are cultured in either small-scale or larger scale (Kongkeo and Phillips, 2002). For instances, the orange-spotted grouper (*E. coioides*), greasy grouper (*E. tauvina*), malabar grouper (*E. malabaricus)*, tiger grouper (*E. fuscoguttatus)*, giant grouper (*E. lanceolatus*), humpback grouper (*Cromileptes altivelis*), white grouper (*E. aeneus*), yellow-banded grouper (*E. awoara*), honeycomb grouper (*E. merra*), red grouper (*E. morio*), red spotted grouper, (*E. akaara*), dusky grouper (*E. marginatus*), leopard grouper (*Mycteroperca rosacea*), nassau grouper (*E. striatus*), and potato grouper (*E. tukula*) are commonly cultured globally. Increased global production of groupers was recorded with 60,774, 99,378, 163,093, and 198,690 metric ton for the year of 1990, 2000, 2005, and 2007, respectively (Hariskrishnan *et al.*, 2010).

Intensive farming is widely used for the aquaculture industry of groupers. This intensive culture of groupers often faces the problem of diseases which is largely caused by its crowding environment. This is particularly devastating when the incidences of infectious and non-infectious diseases happen that accelerated by crowding stress and eventually money loss (Heemstra and Randall, 1993). The common infectious disease are caused by virus, bacteria and parasites, while there are also several undiagnosed diseases of unknown origin that heavily impact the groupers aquaculture (Hariskrishnan *et al.*, 2010). One of the example is vibriosis caused by bacteria is a common problem for intensive farming of groupers that result in lethal red boil disease (Liao *et al.*, 1996). Other viral disease caused by nodaviruses and iridoviruses can result in viral nervous necrosis and sleepy