UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF CANDIDATE SINGLE NUCLEOTIDE POLYMORPHIC MARKERS IN BROWN-MARBLED GROUPER Epinephelus fuscoguttatus (FORSSKAL, 1775)

NUR DIYANA BINTI MOHAMAD TAHIR

IB 2021 1

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By

NUR DIYANA BINTI MOHAMAD TAHIR

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2021

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Dato' Mohamed Shariff bin Mohamed Din, PhD
Faculty : Institute of Bioscience

Brown -marbled grouper *Epinephelus fuscoguttatus* is a premium marine food fish with high demand in Asia. However, its aquaculture development is limited by broodstock availability with heavy reliance on wildstocks. The growth and stress polymorphic markers were explored using high-throughput sequencing and the genetic diversity of *E. fuscoguttatus* for potential use in selective breeding to facilitate the development of aquaculture. Thus, the first objective of this study was to investigate the salinity tolerance of *E. fuscoguttatus* and its effects on the serum cortisol levels, survival and growth in an aquaria setting. This was a preliminary study towards the identification of SNP markers later on. In this study, grouper juveniles (92.43 ± SEM 0.51 mm) were maintained in 31 ppt seawater for one-week acclimatization and transferred into five tanks with different seawater dilutions (5, 10, 15, 20, 25 ppt). The results revealed that serum cortisol of fish in high change of salinity (from 31 ppt to 15, 10 and 5 ppt) was significantly higher than the control group immediately after exposure. In the higher salinity change (from 31 to 5 ppt), the survival percentage was 50%, while no mortality observed in the lower change of salinity. Whereas after 2 weeks, a significant difference in the decrease of mean weight of fish in the higher change of salinities (from 31 to 15 ppt, 10 ppt, and 5 ppt) was observed compared to the control group indicating the effects of chronic stress on the growth performance of fish. In the aquaria experiment, some fish were more stress-tolerant than others could be used as potential as candidate for selective breeding. The second objective of this study was to measure the growth performance of *E. fuscoguttatus* in sea cage culture to detect the slow and fast growers for DNA sequencing. The fish were grown for 10 months in a floating cage where the morphometrics of 100 fish (215.10 ± SEM 21.64 mm) were measured and 20 fish were intramuscularly microchipped. At the end of 10 months, 10 fish recovered with microchip were categorized based on their size as fast- and slow-growers and processed for DNA extraction. The next objective was to assess the potential of a

novel high-throughput sequencing method on the muscle DNA samples. High-throughput sequencing using the double digestion restriction associated DNA sequencing (ddRADSeq) was done utilising two restrictive enzymes: *PstI* and *MspI*. The results showed 146,244 sequences and with 16172 SNP of 25 or less per sequence. Out of the 16172 SNP, 64% were identified with one SNP, while 5776 (35%) were identified with more than two SNPs. An overall F_{ST} for the two populations of fast- and slow-growers was extremely low (0.0705) with 21.2% - 26.1% polymorphic loci and high observed homozygosity as calculated by STACKS indicating inbreeding. The last objective of this study was to analyse the genetic diversity of *E. fuscoguttatus* and isolate and identify SNP markers using bioinformatic tools as candidate markers. Analysis of the 382 filtered SNP sequences using BLAST, GO and UniProt databases revealed 36 sequences which matched the *Epinephelus* spp. and 18 GO biological processes of 57 genes in the fast-and slow-growers related to growth and stress. Sequencing using the novel ddRAD Seq revealed four genes: *acss2, abhd15, lrp2* and *pdp1* as potential candidate markers that could assist in the selection of broodstock fish with superior traits. This is the first ddRADSeq performed in this species. The outcome of this study can contribute to understanding suitable bioinformatics pipelines, further adding to the limited genetic information available for establishing and identifying suitable markers for the future of assisted selective breeding of this species.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

IDENTIFIKASI CALON PENANDA POLIMORFISME NUKLEUTIDA TUNGGAL PADA KERAPU HARIMAU *Epinephelus fuscoguttatus* (FORSSKAL, 1775)

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Kerapu harimau (*Epinephelus fuscoguttatus*) adalah makanan laut premium yang mendapat permintaan yang tinggi di rantau Asia. Namun, perkembangannya di dalam sektor akuakultur terbantut kerana kebolehdapatan induk yang terhad dan pengantungan berat kepada ikan liar. Untuk membangunkan penternakan ikan kerapu harimau, penanda polimorfik pertumbuhan dan tekanan diteliti menggunakan penjujukan daya tinggi dan kepelbagaian genetik spesies ini disiasat sebagai potensi kegunaan di dalam pembiakan selektif. Objektif pertama kajian ini ialah menyiasat toleransi salinity dan kesan terhadap tahap kortisol, kelangsungan hidup dan pertumbuhan. Objektif pertama ini bertujuan sebagai kajian permulaan kearah SNP identifikasi kemudian. Dalam kajian ini, kerapu juvenil (92.43 ± SEM 0.51 mm) yang dikekalkan di dalam 31 ppt air laut telah dipindahkan ke lima tangki dengan pencairan air laut yang berlainan. Keputusan menunjukkan bahawa serum cortisol ikan dalam perubahan kemasinan yang tinggi (15, 10 dan 5 ppt) jauh lebih tinggi daripada kumpulan kawalan sebaik sahaja pendedahan. Dalam perubahan kemasinan yang lebih tinggi (5 ppt), peratusan kemandirian adalah 50% manakala tiada kematian dicerapkan dalam perubahan kemasinan yang lebih rendah. Selepas 2 minggu, perbezaan ketara diperhatikan pada berat ikan dalam perubahan kemasinan yang lebih tinggi (15 ppt, 10 ppt, dan 5 ppt) berbanding kumpulan kawalan dan ini menunjukkan kesan stres kronik kepada prestasi pertumbuhan ikan. Eksperimen di dalam akuaria ini menunjukkan bahawa sesetengah ikan kerapu mempunyai kerintangah stres lebih daripada ikan kerapu yang lain oleh itu berpotensi sebagai induk untuk digunakan di dalan pembiakan terpilih. Objektif kedua di dalan kajian ini adalah mengkaji prestasi pertumbuhan *E. fuscoguttatus* untuk pengesanan ikan pertumbuhan cepat dan lambat. Tempoh pertumbuhan 10 bulan dalam sangkar terapung dijalankan di mana morfometrik 100 ekor ikan (215.10 ± SEM 21.64 mm) diukur dengan 20 ekor ikan telah ditanda dengan menyuntik microcip secara intramuskular. Daripada jumlah ini, 10 sampel dari ikan

yang disuntik microcip (dikategorikan kepada pertumbuhan cepat dan lambat) kemudian dipilih untuk pengekstrakan DNA. Objektif seterusnya ialah mentaksir potensi penjujukan pencapaian tinggi novel terhadap sampel DNA otot *E. fuscoguttatus*. Penjujukan pencapaian tinggi menggunakan double digestion restriction associated DNA sequencing (ddRADSeq) telah diterokai untuk digunakan di dalam spesies ini menggunakan dua enzim restriktif: PstI dan MspI. Terdapat 146,244 urutan dengan polimorfisme nukleotida tunggal (SNP) dengan kebanyakan loci mengandungi satu SNP dan 5776 (35%) telah dikenal pasti dengan lebih daripada 2 SNP. FST secara keseluruhan untuk kedua-dua populasi pertumbuhan adalah sangat rendah (0.0705) dan 21.2% - 26.1% loci polimorfik seperti yang dikira oleh STACKS. Homozygositi yang lebih tinggi menunjukkan tahap silang dalam yang tinggi. Objektif terakhir pula ialah untuk menganalisa kepelbagaian genetic *E. fuscoguttatus* dan mengasingkan dan mengenal pasti calon marker SNP menggunakan alat bioinformatik. Analisis 279 urutan SNP ditapis menggunakan pangkalan data BLAST, GO dan UniProt mendedahkan 36 urutan yang sepadan dengan *Epinephelus* spp. dan GO proses biologi 28 gen yang berkaitan dengan pertumbuhan dan tekanan dalam ikan cepat pertumbuhan dan ikan lambat pertumbuhan. Penjujukan menggunakan ddRADSeq novel mendedahkan empat kandidat marker berpotensi iaitu *acss2*, *abhd15*, *lrp2* and *pdp1* yang boleh membantu dalam pemilihan individu yang mempunyai trait yang berkepentingan. Walaupun kajian ini adalah kajian yang pertama kali spesis ini menggunakan ddRADSeq, hasil kajian ini dapat menyumbang kepada pemahaman saluran paip bioinformat yang sesuai, dan menambah lagi maklumat genetik yang terhad untuk menubuhkan dan mengenal pasti penanda yang sesuai untuk membangunkan pembiakan selektif spesies ini masa depan.

ACKNOWLEDGEMENTS

Alhamdulillah. In the name of Allah, the omnipotent, the most merciful. I am deeply gratified for His gift and strength to complete my PhD programme.

My husband, Dr Wan Nor Fitri, who has given me the strength, support and advice when I needed them the most. To my daughter, Wan Nur Khadeeja, my constant companion throughout the writing of this thesis.

I would like to dedicate this achievement to my amazing parents; Rosnani Ibrahim and my late father, Mohamad Tahir bin Zainal Abidin for the love, support, and belief in me. Their prayer has pulled me through some difficult moments. My siblings; Zhafri and Fauzi for their support. My family in-laws – Juriah Md Hasan, Wan Jaafar Wan Mamat.

My sincere appreciation for the academic support that all my co-supervisors had provided; Prof. Dato' Dr Mohamed Shariff bin Mohamed Din, Dr Muhammad Fadhil Bin Syukri Ismail and Prof. Dr Fatimah Md Yusoff for their expertise in each of their field. They have cultivated the true passion for curiosity and to pursue a challenge with explanations and answers.

I thank Prof. Dr Jesse Faez Firdaus Abdullah, Dr Fuad Matori, Dr Sanjoy Banerjee and Dr Annas Salleh for the comments and critiques of my scientific studies.

Many thanks to Dr Pearl Kwan and Dr Akmal whose assistance and knowledge have helped me tremendously. Thank you to Dr Sabri, Danish, Fazley, Dr Azura, Dr Aslizah, Dr Rashidah, and Ms Kumari for their support and encouragement. Special thanks to Mr. Tahir for his kind assistance and technical expertise.

Thank you to the staff of Faculty of Veterinary Medicine especially the Aquatic Animal Health Unit and staff at the Institute of Bioscience particularly the Laboratory of Marine Biotechnology (MARSLAB) for their kind assistance.

I acknowledge Universiti Putra Malaysia for the Tenaga Akademik Muda Scheme and the Ministry of Higher Education Malaysia for the Project on the Higher Institution Centre of Excellence (HICoE) awarded to the Institute of Bioscience, Universiti Putra Malaysia, and the Ministry of Science, Technology and Innovation Malaysia under TechnoFund for the funding of part of this project.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 06 May 2021

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LIST OF ABBREVIATIONS

BD	Body depth
BLAST	Basic local alignment search tool
BW	Body width
BWT	Body weight
C	Celcius
Cm	Centimeter
DAH	Days after hatching
ddRAD	Double-digest restriction-associated DNA sequencing
DNA	Deoxyribonucleic acid
DoF	Department of Fisheries
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FCR	Feed conversion rate
F_{IS}	Inbreeding coefficient
g	Gram
GH	Growth hormone
GHR	Growth hormone receptor
GR	Glucocorticoids

HPA	Hypothalamic-pituitary-adrenal
HPI	Hypothalamic-pituitary-interrenal
HRP	Horseradish peroxidase
HSPs	Heat-shock proteins
HTS	High-throughput sequencing
HWE	Hardy–Weinberg Equilibrium
IGFBPs	Insulin-like growth factors binding proteins
IGFs	Insulin-like growth factors
IUCN	International Union for Conservation of Nature
KASPar	KBiosciences Competitive Allele Specific PCR SNP genotyping system
Kg	Kilogram
km	Kilometer
LF	Fork length
LTVs	Live fish transport vessels
M	Meter
MAF	Minor allele frequencies
MAS	Marker-assisted selection
MR	Mineralocorticoids
MRFs	Myogenic regulatory factors

MSTN	Myostatin
MT	Metric tonnes
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
Ppt	Part per thousand
RAD	Restriction-associated DNA
RNA	Ribonucleic acid
SD	Standard deviation
SEM	Standard error of mean
SGD	Sleepy grouper disease
SGR	Specific growth rate
SL	Standard length
SNP	Single nucleotide polymorphism
t	Tonne
TCU	Upper critical temperature
TL	Total length
TP	Pejus temperature
UPM	Universiti Putra Malaysia
VNN	Viral nervous necrosis

WGS	Whole genome sequencing
π	Estimate of nucleotide diversity

CHAPTER 1

INTRODUCTION

1.1 Background

The brown-marbled grouper is popular in the Southeast Asia region (Shapawi et al., 2014) and in recent years, has become a promising candidate for aquaculture due to its good market price (Rimmer and Glamuzina, 2017). Although there is a high market demand for this species (Yamamoto, 2006; Afero et al., 2010), the productions remain low (FAOSTAT, 2015). In Malaysia, the main issues in grouper aquaculture are low quality broodstock and seeds, high cost but a low yield of production and exposure to the environment leading to the occurrence of diseases (Hamzah et al., 2019). The industry is mainly focused on capture-based aquaculture (Ottolenghi et al., 2004) where fishers collect the seeds while the adult groupers aggregate to spawn, thus resulting in high mortality of groupers and other fishes (Rhodes et al., 2018). These unsustainable practices make them vulnerable to overexploitation (Rhodes et al., 2018).

Intensive farming exposes groupers to a variety of stress, such as changes in water conditions, and plagued by a host of diseases that lead to economic loss (Harikrishnan et al., 2011). The salinity of the aquatic environment affects metabolism, oxygen consumption, ammonia excretion, growth, and the survival of fish (Tsui et al., 2012). The practice of culturing groupers in floating cages exposes the fish to variability in salinity due to tropical weather conditions of heavy rainfalls (Huang et al., 2014) and poor water quality (Pomeroy et al., 2002). Low survival during culture is attributed to stress and diseases because of poor quality seeds from broodstock (Lim, 1993), making brown-marbled grouper one of the most difficult fish to culture.

Without strategic genetic improvement programmes, it is difficult to maintain viable and productive hatchery stock (Lind et al., 2012). Inbreeding can occur, causing deleterious alleles that will reduce trait qualities and survival rates (Kuo et al., 2014). Consequently, growth depression will occur (Pierre et al., 2008; Wang et al., 2015). This will compromise the broodstocks' fitness traits such as growth rate, fry survival (Pante et al., 2001) and disease resistance (Nurdalila et al., 2015). Thus, genotyping in the early stages of aquaculture can slow down the impact of inbreeding and improve broodstock management (Matusse et al., 2016). Genomic selection using genetic marker information will potentially facilitate selection for superior genotypes and improve broodstock quality (Crossa et al., 2017).

In aquaculture, high-throughput technology has only been applied to a few economically important fish species due to the significant effort required in terms of financial resources and human capacity (Lind, Ponzoni, Nguyen, & Khaw, 2012). Most DNA marker identification in Epinephilidae, including single nucleotide polymorphism (SNP) focuses on growth-related genes (Sonesson, 2003). Interests in the development of the hybrid *Epinephelus fuscoguttatus x Epinephelus lanceolatus* grouper add to some extent the genetic information of the brown-marbled grouper but remains limited with large gaps to fill (Yu et al., 2016). Currently, in the NCBI database, there are 698 genes listed for the *Epinephelus* sp., 37 genes in hybrid *E. fuscoguttatus x E. lanceolatus*, and only 13 genes annotated for brown-marbled grouper. At the time of this study, complete whole genome sequence (WGS) is currently not available for *E. fuscoguttatus*. Therefore, more genetic information is needed to develop and improve its broodstock with relation to genetic studies and possible linkage to phenotypes and genotypes of interest (Danzmann et al., 2016; Rasal et al., 2017).

Although WGS remains expensive for most organisms, genetic markers discovery would be very informative even without a complete genome sequence (Willing et al., 2011). Double-digest restriction-associated DNA sequencing (ddRAD), for the development of SNP markers allows for comparison of variant sites between individuals with reduced portion of genome sampled (Peterson et al., 2012). Advantages of ddRAD include the ability to multiplex many samples with relatively easy library preparations (Robledo et al., 2018). In aquaculture species, ddRAD has been used in tilapia (*Oreochromis niloticus*) (Palaiokostas et al., 2015), seabass (*Lates calcarifer*) (Wang et al., 2015) and even the orange-spotted grouper (*Epinephelus coiodes*) (Yu et al., 2016) but none has been applied in the brown-marbled grouper. This technique would be novel for the *E. fuscoguttatus*. Downstream biological inferences made for selective breeding will be affected by bioinformatics analysis and tools involved in ddRAD sequencing (Shafer et al., 2017). Marker-assisted selection (MAS) coupled with the classic domestication strategies to select individuals based on phenotypical characteristics and genotypic information (Lind et al., 2012) would preserve genetic variation and reduce consanguineous-derived negative effects (Matusse et al., 2016). The development of genomic resources of the brown-marbled grouper by identification of genes belonging to traits of key importance for profitability in broodstock (Hayes et al., 2007) will enable selective breeding programs utilising SNP markers and support better management applications.

1.2 Problem statement

1. In aquaculture, during rainy seasons, the salinity can rapidly decrease in grouper ponds (Cheng et al., 2013), causing stressful conditions

affecting growth rates (Sampaio & Bianchini, 2002) thus increasing the susceptibility of fish to diseases.

2. Inbreeding (Matusse et al., 2016) causes growth depression due to environmental stress sensitivity and trait depression (Pierre et al., 2008; Wang et al., 2014). The traditional phenotypical selection of broodstock currently practised for estimation of breeding values is not reliable.
3. Whole-genome sequencing and genetic improvement are limited for brown-marbled grouper due to financial resources and human capacity (Lind, Ponzoni, Nguyen, & Khaw, 2012). None of the 13 genes listed in NCBI for brown-marbled grouper relates to growth nor stress SNPs thus, limited genetic information and markers are currently available.

1.3 Justification

1. Brown-marbled grouper has a good market price and high demand in the market (Shapawi et al., 2014). However, the low survival during culture is attributed to stress and diseases because of poor quality seeds from broodstocks (Lim, 1993). Grouper cultured in floating cages are exposed to variability in salinity as a result of tropical weather condition of heavy rainfalls (Huang et al., 2014) with storm seasons (Cheng, Chen, & Chen, 2013) and poor water quality (Pomeroy et al., 2002). Therefore, there is a need to produce quality seeds that are stress-tolerant and therefore have better growth performance.
2. SNPs have a significant role in genetic studies and possible linkage to phenotypes and genotypes of interest (Danzmann et al., 2016; Rasal et al., 2017).
3. Double digestion restriction-associated DNA (ddRAD) sequencing methods for SNP development (Peterson et al., 2012) have not been described in the brown-marbled grouper.
4. Since there is limited information on SNP and ddRAD method for brown marble grouper, sets of bioinformatics tools can be explored and determined suitable for the analysis of data.
5. Ultimately, this study will be able to add knowledge on the genetic information of brown marble grouper. With the information at hand, better breeding programmes utilising marker-assisted selection (MAS) could generate high quality fingerlings.

1.4 Hypothesis

The hypothesis is SNP markers controlling stress tolerance and growth can be identified in brown-marbled grouper, as the fish show different stress tolerance level, survival, and growth rates, although reared in similar conditions.

Null hypothesis: No SNP markers identified in the brown-marbled grouper DNA are associated with stress and growth.

Alternate hypothesis: SNP markers identified in the brown-marbled grouper DNA are associated with stress and growth.

1.5 Objectives

The main objective of this study is to have a better understanding of the cortisol levels, survival and growth rate of brown-marbled groupers in different salinities in laboratory setting, mimicking the salinity fluctuations in cage culture condition. In addition, selecting fast and slow growth fish from cage culture for a novel sequencing method – ddRAD to identify SNP markers associated with growth and stress to enable selection of appropriate broodstock. Thus, the specific objectives are;

1. To investigate the salinity tolerance of *Epinephelus fuscoguttatus* by conducting a salinity stress challenge and analyse the serum cortisol levels, survival and growth.
2. Measurement of growth performance of *Epinephelus fuscoguttatus* in sea cage culture for the detection of slow and fast growers
3. To assess the potential of a novel high-throughput sequencing method on the *Epinephelus fuscoguttatus* DNA samples - the double digestion restriction-site associated DNA (ddRAD) using two enzymes *MspI* and *PstI*.
4. To analyse the genetic diversity of *Epinephelus fuscoguttatus* and isolate and identify SNP markers using bioinformatic tools as candidate markers for marker-assisted selective (MAS) breeding.

different species and suitable genes to be used in selection programs which are growth hormone (GH), insulin-like growth factors (IGFs) and myostatin (MSTN). At the time of this writing, other genes, including muscle fibre differentiation (SMYD1, RTN1, HSP90A), myoblast proliferation and cell cycle (DRG1, CEBPD), protein degradation pathways (MuRF1, MAFbx, CTSL1), muscle structural proteins (TnC, TnT2, actin2) and mitochondrial genes encoding for (NADH dehydrogenase subunit 1, cytochrome b, ATPase 6 (Bower & Johnston, 2010, (Salem et al., 2012) have been included in the list of potential genes for MAS breeding. Other genes identified in the current study were the toll-like receptors (TLRs) and osteonectin (sparc) from Table 4.11 and interleukin enhancer binding factor 2 (ILF2). The family of Toll-like receptors (TLRs) detect infections and recognise conserved pathogen structures are to induce immune effector molecules (Palti, 2011). They are present in various tissues such as the spleen, liver, and head kidney, as demonstrated in zebrafish, flounder and catfish (Rebl et al., 2010). Osteonectin (sparc) identified in the fast growers is expressed in nearly every organ and tissue during fish development, including skeletal tissues (Estêvão et al., 2005; Renn et al., 2006) and liaises the activities of various growth factors (Brekken & Sage, 2000). Those actions are associated with the calcification of fish scales, otoliths, teeth, and bones (Weigele et al., 2015). Interleukin enhancer binding factor 2 (ILF2) found in the slow growers also mediates cellular growth by inhibiting the stabilization of mRNA (Cheng et al., 2016).

The sequences of microsatellites found were mainly researched on grouper hybrid molecular characterisation (*E. lanceolatus X E. fuscoguttatus)* (Ching et al., 2016). The interest in hybridization was motivated by the need to meet grouper demand, and thus the genetic information was explored to further optimised the hybrid traits and potential (Shapawi et al., 2018). Isolation and characterization of microsatellite from *E. fuscoguttatus* genome preliminary studies were done by Mohd Azinuddin et al., (2011) to obtain genetic information on the species. In addition, the discovery of microsatellites using 454 pyrosequencing provided the tools to enhance population genetics studies and gene mapping and obtain reliable genetic variability estimates for groupers (Kubota et al., 2014). Identification of genes growth hormone-releasing hormone (GHRH) and the receptor, GHRHR related to growth patterns were investigated for potential use in broodstock management in the orange-spotted grouper (*E. coioides*) (Guo, Xia, Yang, Li, You, et al., 2015). A mixed marker of microsatellite and SNP approach may be the better option for mapping studies.

For both fast and slow-growth groups, the biological processes of cellular processes were up-regulated. For the fast growers, growth related processes will be the main focus as this is the main trait of interest in aquaculture. The present study identified genes including *lamtor2* involved in cellular growth regulation, *acss2* lipid biosynthetic processes, *hka2* glycogen metabolic process, *skil* response to growth factor, *grhl2* multicellular organism growth, and *nup98* regulation of glycolytic

process. The gene *acss2* may be a potential gene as studies showed that mice without ACSS2 exhibited a significant reduction in body weight (Huang et al., 2018). The previous study further concluded that ACSS2 protein promotes the selective regulation of genes involved in lipid metabolism via the systemic storage or metabolism of fat. Earlier studies on *hka2* were not extensive and only limited to studies on mice and rabbits (Zies, 2003). However, in mice, the gene is associated with potassium ion extra- and intracellularly for normal cell functions (Walter et al., 2016).

Immune genes identified, including the *tlr2,* which responses to inflammation, *adora2b* in cellular defence response, *rohu_023715* in activation of the innate immune response, and *nefl* in response to corticosterone. These immune genes were not identified in the slow growers. This could be possible due to allele dropout or error during PCR amplification, and thus the present study cannot deduce any comparison nor conclusion between the two groups.

In the slow-growth samples, *helq* is involved in double-strand break repair via homologous recombination, *abhd15* and *lrp3* in cellular lipid metabolic process, *loc106772934* in transcription by RNA polymerase III, *shc2* in intracellular signal transduction, *pdp1* in regulation of acetyl-CoA biosynthetic process from pyruvate, *lrp6* in cellular response to cholesterol and negative regulation of protein, *pol* in DNA integration and *plekhm3* in myoblast differentiation. The gene *abhd15* is promising as is required for the anti-lipolytic action of insulin in adipose tissue (Xia et al., 2018). According to the researchers, fatty acid mobilisation continues to occur in mice without ABHD15 despite treatments with insulin and glucose. The gene controlling low-density lipoprotein receptor-related protein 3 (LRP3) regulates the osteogenic and adipogenic differentiation (Elsafadi et al., 2017) and thus could be another potential candidate gene. The previous study linked the gene to inhibit the differentiation of adipocytes. A different study using *Drosophila* flies with PDP1 showed that although they appear to have normal muscle and gut function but they are severely growth delayed showing PDP1 is another potential candidate gene for growth selection (Reddy et al., 2000).

Thus, from the present study, four genes; *acss2, abhd15, lrp2* and *pdp1,* are proposed as candidate genes for further investigation as markers for growth. Although these genes have not been previously studied in fish, studies in other animals found various new genes involved in growth processes. This is not surprising as considering hundreds of genes and QTL regions are involved in growth and these markers can have positive or negative impacts on other genetic polymorphisms (Ulloa et al., 2015). Hui Yu et. al. (2016) investigated growth related traits in orange-spotted grouper and utilized 3029 SNPs to construct a genetic linkage map using a regression mapping algorithm. Furthermore, they identified 17 genes (*fez2, alg3, ece2, arvcf,*

sla27a4, sgk223, camk2, prrc2b, mchr1, sardh, pappa, syk, tert, wdrcp91, ftz-f1, mate1 and *notch1*) including three (*tert, ftz-f1* and *notch1*) that have been reported to be involved in fish growth. , The analysis performed by Ulloa et al. (2015) revealed 5 SNP associated with growth in key metabolic pathway genes, asparaginyl-tRNA synthetase (*Nars*), leiomodin2b (*Lmod2b*), CUB and zona pellucida-like domains 1 (*Cuzd1*), actin alpha skeletal muscle (*Acta1b*), and a novel protein with a PLAC8 family domain (*Plac8l1*). Two genes *nars* and *lmod2b*, had SNP with significant differences in the distribution of the minor alleles between phenotypes and these genes have not been linked to fish growth in earlier studies. From the findings, the authors concluded that these SNP could determine zebrafish individuals that are more efficient in terms of growth when fed different diet. Similarly, in our study, the findings based on the SNP sequences - four genes; *acss2, abhd15, lrp2* and *pdp1* as candidate genes that could assist selecting stock or individuals for economically important traits such as growth.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

Juvenile brown-marbled groupers was tolerant and able to survive in lower change of salinities ranging from 25 to 20 ppt without a significant increase in the serum cortisol level, compared to the control maintained at 31 ppt. Higher salinity changes of 15, 10 and 5 ppt showed an increase in the serum cortisol levels, indicating stress and an increase in the mortality rate. The growth of fish in the higher change of salinities showed no effect in short duration but after two weeks negative growth performance due tochronic stress. The implication of stress tolerance is an important factor in fish selective breeding. Fish that survived the salinity stress challenge can be selected as ideal candidates for breeding as osmoregulation and growth are governed by the same hormones and genes such as prolactin and growth hormone.

In the present study, the slow and fast growers were identified with the aid of microchip as identification for the purpose of monitored growth performance. The findings suggest that regardless of similar growth conditions as demonstrated in this study, there remains a size disparity among brown-marbled grouper in the grow-out stage. Climate data also suggest that the rainfall and temperature changes occurred throughout the study period could affect the salinity of the water affecting the fish. Out of the 20 fish microchipped, only 10 were selected for DNA sequencing to further understand the difference between the slow and the fast growers and the genes governing their growth.

The 279 SNPs of identical homozygous in two phenotypes of slow and fast growth were successfully mined using the ddRAD sequencing method utilising two enzyme cutters *PstI* and *MspI*. The number of SNP markers mined from the present study showed the abundance of markers can be obtained from a small sample size. Hence, the ddRAD sequencing technique was reliable for discovery of non-model species. However, the result showed that only identical homozygous pair within the two groups were recovered, which could be the result of closely related samples and showed high inbreeding levels. The inbreeding was supported by the extremely low F_{ST} value of 0.070526 and percent of polymorphic loci, which was 21.2% - 26.1%. Although it was confirmed that multiple breeding pairs of broodstock were used, the fish tested showed low differentiation. This further implies the serious issue of inbreeding situation in the local grouper aquaculture industry and the need to develop marker-assisted breeding to alleviate the situation. The low survival of groupers estimated to be around 40% may be associated with inbreeding and requires further investigation.

The study identified 382 SNP sequences and further analysis using databases such as BLAST, GO and UniProt were done and revealed potential candidate genes. Growth hormone receptor (GHR) gene, toll-like receptor 2 (TLR2) mRNA, *sparc* mRNA, insulin-like growth factor I (IGF1) gene and myogenic regulatory factor 4 (mrf4) gene were identified in the fast growth samples. And the slow growth samples of SNP sequences matched insulin-like growth factor binding protein 2 (IGFBP-2) gene, interleukin enhancer binding factor 2 gene, and growth hormone receptor 2 (GHR2) gene. The current study further proposed four genes: *acss2*, *abhd15*, *lrp2* and *pdp1*, for further investigation as candidate markers for marker-assisted selection. Although these genes have not been previously studied in fish, previous studies in other animals found various new genes involved in growth processes. The candidate markers are essential in establishing and identifying the markers for the future use in assisted selective breeding. The genetic information obtained in this study can be useful for future reference of the genetic studies on *E. fuscoguttatus*, thus contributing to establishing a more sustainable aquaculture industry whilst conserving the wildstock population.

Limitations of the study were:

- Information on broodstock was entirely dependent on the information provided by the farmer.
- The measurements in the growth monitoring project were recorded only at the beginning and at the end. No other measurement was taken in between the period to limit stress to the fish and possible death and monetary loss to the farmer.
- The availability of data from a bigger population, including the parent genetic information, could strengthen the association to the phenotypes in this species.
- Output data was huge and overwhelming, making selection of meaningful data time-consuming and requires basic bioinformatic knowledge. Applicable data mining requires additional knowledge and the use of supercomputer.

For further studies, the following are recommended:

- Include broodstock parents and the progenies and sampling of multiple populations from different geographic locations for sequencing to obtain the genetic information for QTL mapping, genetic stock identification and better association of the phenotype of interest to the genotype.

- Highly associated genes from the studies can be further validated and an assay developed especially for *E. fuscoguttatus* to assists in marker-assisted selective breeding.
- Characterisation of the SNPs to obtain more detailed information such as the intro/exon location of the polymorphisms
- For ease of experimental studies, internal and external tagging of animals should be done to facilitate close monitoring. External tagging for long-term studies is convenient for ease of visualisation but could also be lost during culturing period. Hence, external tagging with microchip is recommended.
- Latest sequencing method such as skim sequencing, may provide an alternative as the method comes with a library kit (instead of custom prepared such as in this study). This approach can reduce the error during library preparation and increase the quality of DNA to be sequenced for reliable results. Therefore, any association made between the genotype and phenotype can be further strengthened.

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BIODATA OF STUDENT

Nur Diyana was born in 1990 in Ipoh. After high school, she enrolled into Universiti Putra Malaysia in the Agricultural Science Foundation Programme. She graduated in 2010 and was accepted into the Degree of Veterinary Medicine in Faculty of Veterinary Medicine of the same university. After graduating as a Doctor of Veterinary Medicine in 2015, she was accepted into the Master of Science Programme in Institute of Bioscience, Universiti Putra Malaysia. During her studies, she was selected to go for a two-months laboratory training at the University of Glasgow, Scotland under the Global Health Fund in 2017. Following her return from Scotland, she entered into PhD programme. She is a mother to a daughter and a pet parent to two cats.

During her PhD candidature, she published peer-reviewed papers, participated in associations, courses and conferences as listed below:

Association

1. Malaysian Association of Veterinary Pathology (MAVP)
2. Malaysian Fisheries Society (MFS)

Courses

1. UK-Malaysia Vaccinology Workshop (2016), Institute of Bioscience, Universiti Putra Malaysia.
2. Agriculture IGNITE Workshop: Double-digestion restriction associated DNA (2016), MARDI.
3. Basic Transcriptome Genome Informatics Workshop (2016), Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia.
4. MYOHUN Antibiotic Resistance Workshop (2017), Faculty of Veterinary Medicine, Universiti Putra Malaysia.
5. Research training & affiliate member (2017), Institute of Biodiversity Animal Health & Comparative Medicine, University of Glasgow, Scotland.
6. Next-Generation Sequencing Data Analysis (2018), Malaysian Genome Institute, Kajang.

Conferences

1. Asian-Pacific Aquaculture (2018), Putra World Trade Centre, Kuala Lumpur
2. Seminar and Dialogue on R&D for sustainable Aquaculture and Fisheries (2019), Palm Garden Hotel, Putrajaya
3. 29th Veterinary Association Malaysia (VAM) Congress (2017), Holiday Inn Glenmarie.

LIST OF PUBLICATIONS

Journal Articles

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Book Chapter

1. Nur Diyana, M. T. (2019). Basic clinical examination of fish. In Annas Salleh (Ed.), *Basic histology and histopathology of fish* (pp. 1-6). Serdang, Selangor: UPM Press.

Conference Proceedings

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