การตรวจสอบพหุสัณฐานนิวคลีโอไทด์เดี่ยวของจีนที่เกี่ยวข้องกับการเติบโตของปลากะพงขาว
Lates calcarifer

นางสาวพรทิพย์ สวัสดิ์พานิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN GROWTH-RELATED

GENES OF ASIAN SEABASS *Lates calcarifer*

Miss Pornthip Sawatpanich

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

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Thesis Title	IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN GROWTH-RELATED GENES OF ASIAN SEABASS *Lates calcarifer*
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พรทิพย์ สวัสดิ์พานิช : การตรวจสอบพหุสัณฐานนิวคลีโอไทด์เดี่ยวของจีนที่เกี่ยวข้องกับการเติบโตของปลา กะพงขาว *Lates calcarifer*. (IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN GROWTH-RELATED GENES OF ASIAN SEABASS *Lates calcarifer*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ. ดร. เปี่ยมศักดิ์ เมนะเศวต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. บวรลักษณ์ คำน้ำทอง, ดร. ศิราวุธ กลิ่น บุหงา, , หน้า.

เครื่องหมายโมเลกุลที่สามารถนำมาใช้คัดเลือกลูกพันธุ์และพ่อแม่พันธุ์ปลาที่มีอัตราการเติบโตเร็วมี ประโยชน์ต่ออุตสาหกรรมการเพาะเลี้ยง จึงศึกษาโปรทีโอมิกส์ของโปรตีนทั้งหมดในตับของปลากะพงขาวจากบ่อเลี้ยง อายุ 4 เดือน ประกอบด้วยปลาที่มีขนาดใหญ่ (น้ำหนักตัวเฉลี่ย 28.53 ± 12.97 กรัม และความยาวตัวเฉลี่ย 13.30 ± 1.82 เซนติเมตร, *N* = 9) และปลาที่มีขนาดเล็ก (น้ำหนักตัวทั้งหมดเฉลี่ย 2.62 ± 0.74 กรัม และความยาวตัวทั้งหมด เฉลี่ย 6.00 ± 0.55 เซนติเมตร, *N* = 9) พบโปรตีนทั้งหมด 1578 โปรตีน จัดเป็นโปรตีนที่เหมือนกับโปรตีนที่ไม่ทราบชื่อ หรือไม่ทราบหน้าที่จำนวน 833 โปรตีน (52.8%) และโปรตีนที่ทราบหน้าที่จำนวน 745 โปรตีน (47.2%) โดยพบ 28 โปรตีนที่มีการแสดงออกแตกต่างกันอย่างมีนัยสำคัญทางสถิติระหว่างปลาขนาดใหญ่และปลาขนาดเล็ก เช่น calmodulin cAMP-dependent protein kise catalytic subunit beta, dual specificity phosphatase 6, fidgetin-like protein 1 และ clathrin coat assembly protein AP180-like เป็นต้น นอกจากนี้ พบโปรตีนที่ เกี่ยวกับเติบโตหลายโปรตีนด้วยกัน เช่น transforming growth factor beta-2 precursor, transforming growth factor beta-3 precursor, transforming growth factor-beta-induced protein ig-h3 precursor และ activin type IIB receptor (ActRIIB)

วิเคราะห์พหุสัณฐานนิวคลีโอไทด์เดี่ยว (สนิป) ในจีน *insulin-like growth factor II (IGF-II), myostatin (MSTN)* และ *ActRIIB* ในปลากะพงขาวอายุ 4 เดือน (*N* = 99) ที่เลี้ยงในบ่อซีเมนต์ ด้วยวิธี SSCP พบว่า ลักษณะพหุ สัณฐานตำแหน่ง intron ที่ 4 ของ *IGF-II* และตำแหน่ง loci 1 ของจีน *MSTN* ไม่มีความสัมพันธ์กับลักษณะฟีโนไทป์ ที่เกี่ยวข้องกับการเติบโต (น้ำหนักตัว, ความยาวตัว, น้ำหนักตับ และค่าดัชนีตับ) ในทางตรงกันข้าม ตำแหน่ง exon ที่ 4 ของจีน *IGF-II* พบว่า ปลาขนาดใหญ่ที่มีรูปแบบ SSCP แบบ B มีน้ำหนักตัวเฉลี่ย (30.32 ± 15.00 กรัม) มากกว่าปลาที่มี รูปแบบ SSCP แบบ C (19.66±5.10 กรัม) (*P* < 0.05) สำหรับตำแหน่ง loci 2 ของจีน *MSTN* พบว่าปลาที่มีรูปแบบ SSCP แบบ A จะมีน้ำหนักตัวเฉลี่ย ความยาวตัวเฉลี่ยและน้ำหนักตับเฉลี่ย (13.13±11.82 กรัม, 9.32±3.34 เซนติเมตร, 0.19±0.19 กรัม ตามลำดับ) มากกว่า ปลาที่มีรูปแบบ SSCP แบบ B (5.26±7.29 กรัม, 6.65±2.15 เซนติเมตร, 0.07±0.06 กรัม ตามลำดับ) (*P* < 0.05) สำหรับจีน *ActRIIB* พบว่าปลาที่มีรูปแบบ SSCP แบบ D มีน้ำหนักตัวเฉลี่ย ความยาวตัวเฉลี่ยและน้ำหนักตับเฉลี่ย (17.15±13.79 กรัม, 10.24±3.59 เซนติเมตร, 0.24±0.22 กรัม ตามลำดับ) มากกว่าแบบ A (2.64 กรัม, 6.10 เซนติเมตร, 0.08 กรัม ตามลำดับ) และ B (2.95±0.66 กรัม, 6.26±0.64 เซนติเมตร, 0.04±0.04 กรัม ตามลำดับ) (*P* < 0.05) และเมื่อพิจารณาเฉพาะในกลุ่มตัวอย่างปลาขนาดใหญ่พบว่า ปลาที่มีรูปแบบ SSCP แบบ D (น้ำหนักตัวเฉลี่ย 26.38±9.48 กรัม) แสดงลักษณะฟีโนไทป์ที่เกี่ยวข้องกับการเติบโตมากกว่าแบบ C (น้ำหนักตัวเฉลี่ย 17.77±5.02 กรัม) (*P* < 0.05) แต่ไม่พบความสัมพันธ์ระหว่างรูปแบบ SSCP และลักษณะฟีโนไทป์ที่ เกี่ยวข้องกับการเติบโตในกลุ่มตัวอย่างปลาขนาดเล็กในทุกจีนที่ทำการวิเคราะห์

นำตัวแทนปลาที่มีรูปแบบ SSCP แบบต่างๆ ของจีน *IGF-II* (exon ที่ 4) และ *ActRIIB* มาวิเคราะห์ลำดับนิ วคลีโอไทด์ พบสนิป $A/A_{72}C/C_{165}$, $G/G_{72}C/T_{165}$, $A/G_{72}C/C_{165}$ และสนิป $A/G_{144}C/T_{326}$, $A/G_{144}C/C_{326}$, $G/G_{144}C/T_{326}$, $G/G_{144}C/C_{326}$ ที่สอดคล้องกับรูปแบบ SSCP แบบ A-C และ A-D ของทั้งสองจีนตามลำดับ ผลการศึกษา แสดงให้เห็นว่า SSCP สามารถใช้วิเคราะห์สนิปในจีนที่สนใจของปลากะพงขาวได้อย่างมีประสิทธิภาพ

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PORNTHIP SAWATPANICH: IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN GROWTH-RELATED GENES OF ASIAN SEABASS *Lates calcarifer*. ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D., CO-ADVISOR: BAVORNLAK KHAMNAMTONG, Ph.D., SIRAWUT KLINBUNGA, Ph.D., pp.

Molecular markers that allow selection of juveniles and broodstock with a high breeding value for growth traits are useful for the aquacultural industry. In this study, cellular proteomics of hepatic tissues of large-sized (average body weight = 28.53 ± 12.97 g and average total length = 13.30 ± 1.82 cm, *N* = 9) and small-sized (average body weight = 2.62 ± 0.74 g and average total length = 6.00 ± 0.55 cm, *N* = 9) 4-month-old juveniles of the Asian seabass (*Lates calcarifer*) was examined. In total, 1578 protein homologues were identified and 833 proteins significantly matched either unnamed or unknown proteins (52.8%). The remaining 745 protein (47.2%) matched proteins with known functions. Twenty-eight proteins (e.g. calmodulin cAMP-dependent protein kise catalytic subunit beta, dual specificity phosphatase 6, fidgetin-like protein 1 and clathrin coat assembly protein AP180-like) showed significantly differential expression between large-sized and small-sized samples. In addition, several growth-related proteins, for example, transforming growth factor beta-2 precursor (TGF-β2 precursor), transforming growth factor beta-3 precursor (TGF-β3 precursor), transforming growth factor beta-2-induced protein ig-h3 precursor (TGF-β2-induced protein ig-h3 precursor) and activin type IIB receptor (ActRIIB) were also identified.

Single nucleotide polymorphism (SNP) in *insulin-like growth factor II* (*IGF-II*), *myostatin* (*MSTN*) and *ActRIIB* was further analyzed in 4-months-old juveniles cultured in concrete tanks (*N* = 99). Polymorphism of the intron 4 gene segment of *IGF-II* and *MSTN* loci 1 did not associated with growth-related parameters. In contrast, large-sized 4-month-old *L. calcarifer* carrying SSCP pattern B of the exon 1 gene segment of *IGF-II* possessed a greater average body weight (30.32 ± 15.00 g) than those carrying SSCP pattern C (19.66±5.10 g) (*P* < 0.05). For *MSTN* loci 2, results for overall samples revealed that 4-month-old fish carrying SSCP pattern A possessed a greater average body weight, total length and hepatic weight (13.13±11.82 g, 9.32±3.34 cm, 0.19±0.19 g, respectively) than those of fish carrying SSCP pattern B (5.26±7.29 g, 6.65±2.15 cm, 0.07±0.06 g, respectively) (*P* < 0.05). For *ActRIIB*, juvenile *L. calcarifer* exhibiting SSCP pattern D had a greater average body weight, total length and hepatic weight (17.15±13.79 g, 10.24±3.59 cm, 0.24±0.22 g, respectively) than those carrying SSCP genotypes A (2.64 g, 6.10 cm, 0.08 g, respectively) and B (2.95±0.66 g, 6.26±0.64 cm, 0.04±0.04 g, respectively) (*P* < 0.05). Considering only large-sized juveniles, those exhibiting SSCP pattern D (average body weight 26.38±9.48 g) had a greater growth-related parameters than those exhibiting SSCP pattern C (average body weight 17.77±5.02 g) (*P* < 0.05). Relationships between SSCP patterns of all examined genes and growth-related parameters were not statistically significant in small-sized fish (*P* > 0.05).

The PCR products of representative individuals exhibiting different SSCP patterns of *IGF-II* (exon 4) and *ActRIIB* were sequenced. Results indicated three (A/A$_{72}$C/C$_{165}$, G/G$_{72}$C/T$_{165}$ and A/G$_{72}$C/C$_{165}$) and four (A/G$_{144}$C/T$_{326}$, A/G$_{144}$C/C$_{326}$, G/G$_{144}$C/T$_{326}$ and G/G$_{144}$C/C$_{326}$) diplotypes, corresponding to SSCP patterns A-C and A-D of respective genes, were observed. Results demonstrated the potential of SSCP for identification of SNP in interested genes of *L. calcarifer*.

Field of Study: Biotechnology

Academic Year: 2013

Student's Signature ....

Advisor's Signature ....

Co-Advisor's Signature ....

Co-Advisor's Signature ....

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree celcius
dATP	deoxyadenocine triphosphate
dCTP	deoxycytocine triphosphate
dGTP	deoxyguanine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
M	molar
MgCl2	magnesium chloride
mg	milligram
ml	milliliter
mM	millimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris (hydroxyl methyl) aminomethane
μg	microgram
μM	micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

1.1 Background information

The Asian seabass (*Lates calcarifer*) is an economically important fish species in the tropical and subtropical regions in the Asia-Pacific. The global aquaculture production of *L. calcarifer* has increased from 1,403 tons in 1981 to 3,939 tons in 1988. Subsequently, its aquaculture production was increased to nearly 49,172 tons in 2009 and peaked at 75,405 tons in 2012 (FAO 2013; Figure 1.1).

In Thailand, spawning of Asian seabass had been successfully achieved by hormonal manipulation since 1973 which has made the Asian seabass fry readily available. Cage culture of the fish has been done mainly in the southern part of Thailand. However, culture has expanded to other parts of the country. The production is sold in the country or exported to neighboring countries such as Malaysia, Singapore, Hong Kong and Australia. The specific processed in aquaculture and various sizes of marketable fish will also influence the expansion of the industry and its foreign market.
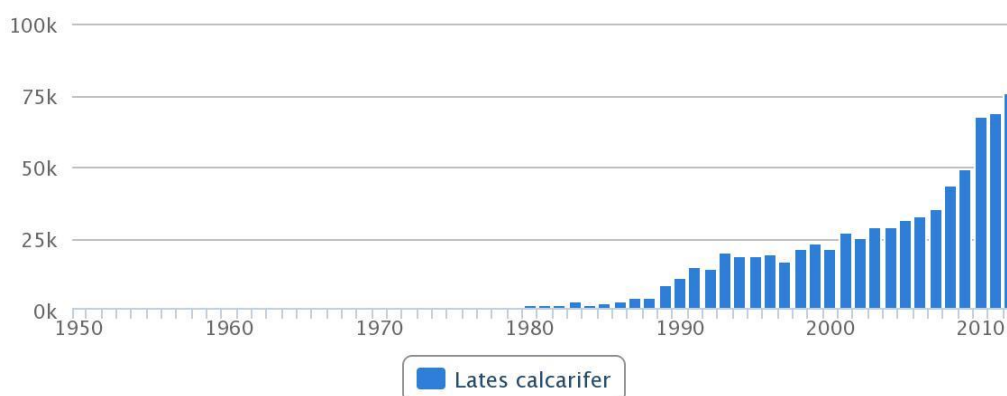
Figure 1.1 A diagram of the global aquaculture production of *L. calcarifer* during 1980-2012 (FAO 2013).

The fish has been successfully spawned by hormonal manipulation since 1973 in Thailand. The Asian seabass is more expensive than most other local fish species. The production of Asian seabass was in high demand.

Biotechnology and genetics have great potential to increase production from seabass farming and to help make its farming sustainable. Genetic improvement techniques for delivering genetic gain include formal definition of the breeding objectives, estimation of genetic parameters that describe populations and their differences, evaluation of additive and non-additive genetic merit of individuals or families and defining the structure of a breeding program in terms of breeding plans.

The culture period of this species to the market size was approximately 6-8 months. Presently, the production efficiency is decreased due to the lack of high quality broodstock and it presently takes a longer period of 10-12 months to get the market-sized fish. Selection for increased juvenile growth rates would be feasible in this species. Accordingly, genetic markers that allow selection of juveniles and broodstock with a high breeding value for growth rates would be useful for increasing the production efficiency of this species.

1.2 Objectives of this thesis

1. Determination of proteomic profiles in juvenile *L. calcarifer* having different growth rates by GeLC-MS/MS

2. Identification of single nucleotide polymorphisms (SNPs) in growth-related genes of *L. calcarifer* by single strand conformational polymorphism (SSCP) and DNA sequencing.

3. Determination of relationships between SSCP patterns of growth-related genes and growth parameters (e.g. body weight, total length, liver weight and hepatosomatic index (HSI) of juvenile *L. calcarifer*.

1.3 Biology of the Asian seabass (*L. calcarifer*)

1.3.1 Taxonomy of *L. calcarifer*

Asian seabass are taxonomically recognized as member the largest phylum in the animal kingdom, the Chordata. Taxonomic definition of the seabass is as folloes;

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Latidae

Genus: *Lates*

Species: *Lates calcarifer* (Bloch)

The scientific name of this species is *Lates calcarifer* (Mathew, 2009) and the common name is Asian seabass or Barramundi perch.

1.3.2 Morphology and distinctive characters of *L. calcarifer*

The morphological characters of *L. calcarifer* are well described. Its body is elongated, compressed, with deep caudal peduncle. Body large, elongate and stout, with pronounced concave dorsal profile in head and a prominent snout; concave dorsal profile becoming convex in front of dorsal fin. Mouth is large, slightly oblique, upper jaw reaching to behind eye; teeth villiform, no canine teeth present. Lower edge of pre-operculum is with strong spine; operculum with a small spine and with a serrated flap above original of lateral line. Dorsal fin with 7 to 9 spines and 10 to 11 soft rays; a very deep notch almost dividing spiny from soft part of fin; pectoral fin short and rounded; several short, strong serrations above its base; dorsal and anal

fins both have scaly sheath. Anal fin round, with three spines and 7–8 soft rays; caudal fin rounded. Scale large ctenoid (rough to touch) (Figures 1.2 and 1.3).

Two phases of colors are observed, either olive brown above with silver sides and belly in marine environment or golden brown in freshwater environment. In adult, it is usually blue-green or greyish above and silver below. Fins are blackish or dusky brown. Juveniles have mottled pattern of brown with three white stripes on head and nape, and white blotches irregularly placed on back. Eyes are bright pink, glowing at night.

Sexes of Asian seabass were difficult to identify. In during the spawning season can be identified. There were some dimorphic characters that were indicative of sex (Figure 1.4). Such as were the male has a more slender body than the female, weight of the female was heavier than males of the same size, during the spawning season, abdomen of the female was relatively more bulging than the males.
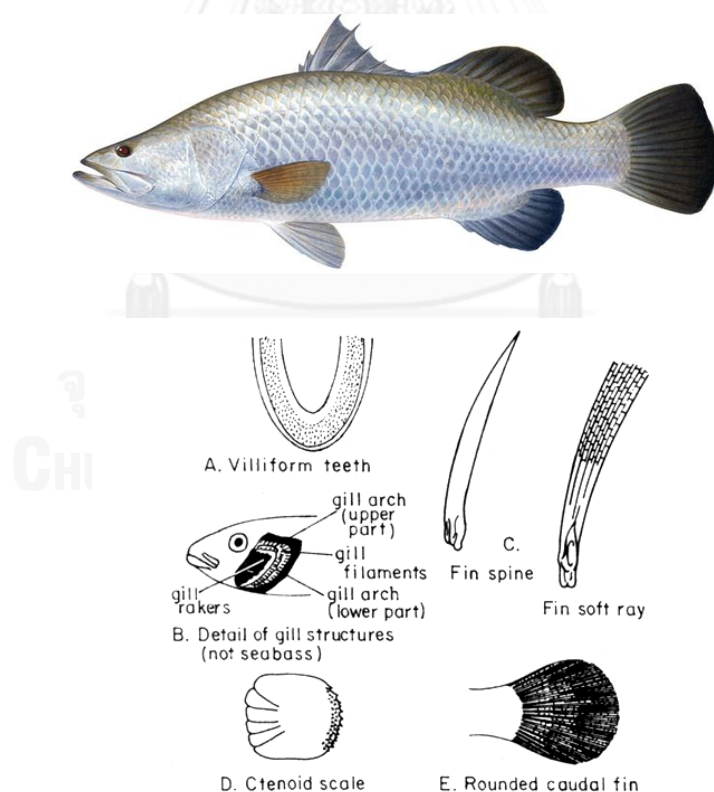
Figure 1.2 External morphology of the Asian seabass, *L. calcarifer* (http://www.fao.org/.../Q8694E02.gif)
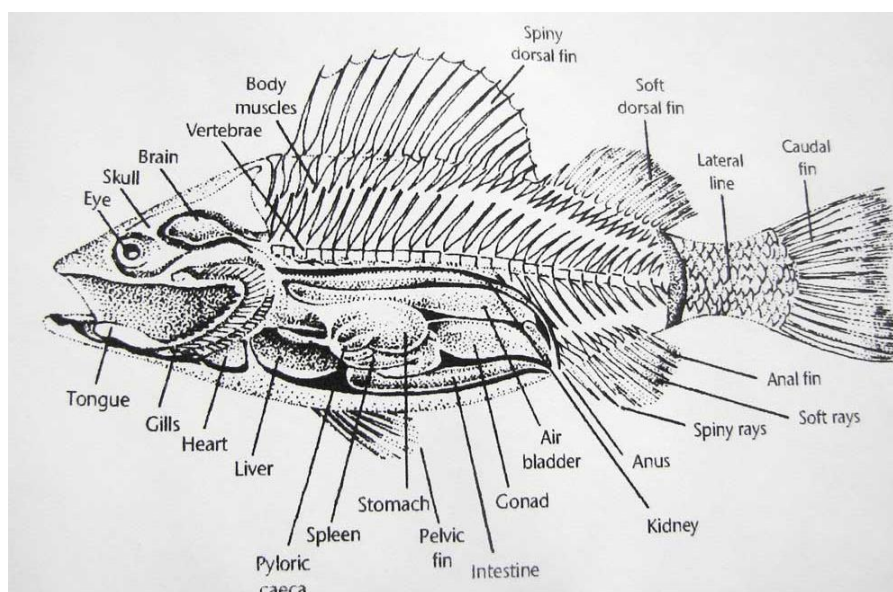
Figure 1.3 Internal morphology of fish (http://www.artscape.us/aquaculture/fish/fish_anatomy.gif)

Figure 1.4 Photograph of adult male and female of *L. calcarifer* (Mathew, 2009)

1.3.3 Life cycle of *L. calcarifer*

Asian seabass spends most of its growing period (2–3 years) in freshwater bodies such as rivers and lakes which are connected to the sea (Figure 1.5). It has a rapid growth rate, often attaining a size of 3-5 kg within 2–3 years. Adult fish (3-4 years) migrate towards the mouth of the river from inland waters into the sea where the salinity ranges between 30-32 ppt for gonadal maturation and subsequent spawning. The newly-hatched larvae (15–20 days old or 0.4–0.7cm) are distributed along the coastline of brackish water estuaries. After one year, juveniles move into coastal waters and then migrate upstream where adults reside for three to four years (Mathew, 2009).
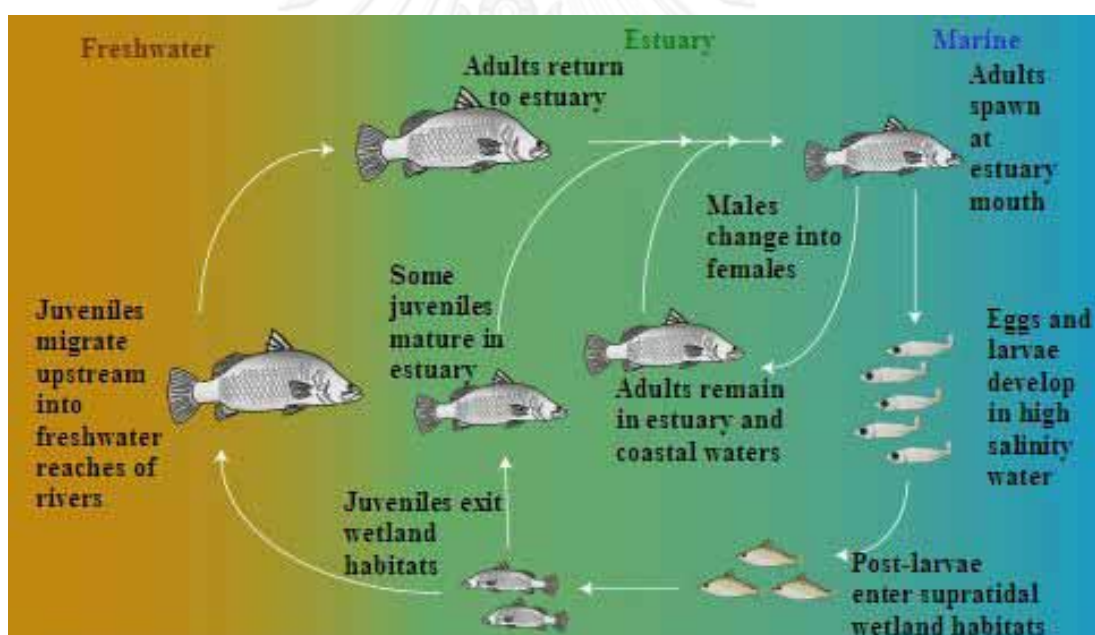
Figure 1.5 The life cycle of *L. calcarifer*. (http://eatingjellyfish.com/wp-content/.../Barramundi_life_cycle.jpg)

1.3.4 Distribution of *L. calcarifer*

Asian seabass is widely distributed in tropical and sub-tropical areas of the Western and Central Pacific and Indian Ocean, between longitude 50°E - 160°W latitude 24°N – 25°S (Fig. 1.6). It occurs throughout the northern part of Asia, southward to Queensland (Australia), westward to East Africa. It is found in coastal waters, estuaries and lagoons and usually occurs at depths of 10 to 40 m.
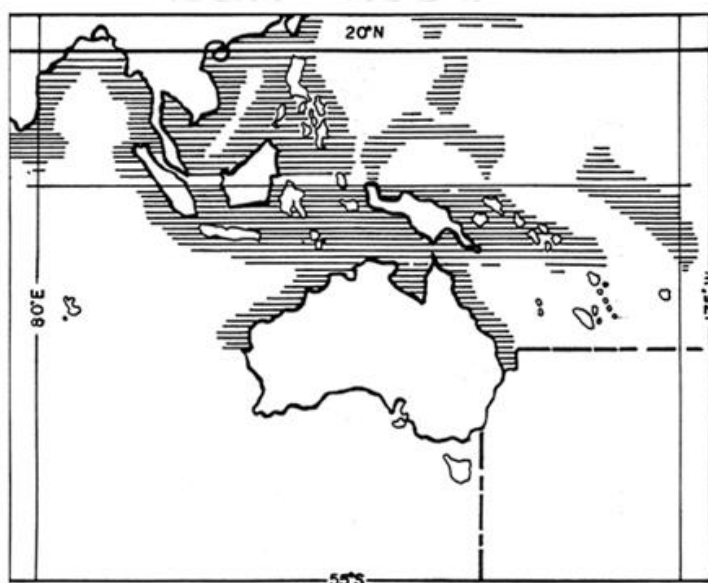
Figure 1.6 The geographic distribution of *L. calcarifer*. (FAO, 1974; http://www.fao.org/docrep/.../AC230E01.gif)

1.4 Molecular markers for genetic improvement of economically important species

Molecular genetic markers at the DNA level are the key players in animal genetics and can be applied for genetic improvement of various economically important species (Teneva, 2009). Molecular genetic markers are classified into two categories: type I which are those associated with genes of known function and type II markers which are associated with anonymous genomic segments (Liu and Cordes, 2004) (Table 1.1). Various types of molecular genetic markers are classified. These included hybridization-based DNA markers (e.g. Restriction Fragment Length Polymorphisms, RFLPs and oligonucleotide fingerprinting), PCR-based DNA markers (e.g. Random amplified Length Polymorphic DNAs, RAPDs, Simple Sequence Repeats or microsatellites, SSRs and Amplified Length Polymorphism, AFLPs) and DNA chip and sequencing-based DNA markers (e.g. single nucleotide polymorphisms, SNPs).

Table 1.1 Types of DNA markers, their characteristics, and potential applications (Lui and Cordes, 2004).

Marker type	Acronym	Requires prior molecular information?	Mode of inheritance	Type	Locus under investigation	Likely allele numbers	Polymorphism or power	Major applications
Allozyme	-	Yes	Mendelian, codominant	Type I	Single	2-6	Low	Linkage mapping, population studies
Mitocondrial DNA	mtDNA	No*	Maternal inheritance	-	-	Multiple haplotypes	-	Maternal lineage
Restriction fragment length polymorphism	RFLP	Yes	Mendelian, codominant	Type I or II	Single	2	Low	Linkage mapping
Random amplified polymorphic DNA	RAPD, AP-PCR	No	Mendelian, Dominant	Type II	Multiple	2	Intermediate	Fingerprinting for population studies, hybrid identification
Amplified fragment length polymorphism	AFLP	No	Mendelian, Dominant	Type II	Multiple	2	High	Linkage mapping, population studies
Microsatellites	SSR	Yes	Mendelian, codominant	Mostly type II	Single	Multiple	High	Linkage mapping, population studies, paternity analysis
Expressed sequence tags	EST	Yes	Mendelian, codominant	Type I	Single	2	Low	Linkage mapping, physical mapping, comparative mapping
Single nucleotide polymorphism	SNP	Yes	Mendelian, codominant	Type I or II	Single	2, but up to 4	High	Linkage mapping, Population studies?
Insertions/ deletions	Indels	Yes	Mendelian, codominant	Type I or II	Single	2	Low	Linkage mapping

* Conserved PCR primers can be adopted from sequence information from a related species.

1.4.1 Single Nucleotide Polymorphisms (SNPs)

SNPs are the most basic unit of genetic variation and represent the commonest class of DNA-based markers (Varshney et al., 2007). SNPs are one base changes including substitution, deletion or insertion occurring in the same genomic position of the DNA segments of difference individuals distributed with the frequency of more than 1% in the examined population (Thomas et al., 2009) (Figure 1.7).

SNPs have several advantages over other markers and are recognized as an important type of markers at present. There are four major reasons for the increasing interest in the use of SNPs as markers for genetic analysis (Beuzen et al., 2000). Firstly, SNPs are prevalent and provide more potential markers near or in any locus of interest than other types of polymorphism. In the human genome, there appears to be an SNP approximately every 1000 bases. Secondly, some SNPs are located in coding regions and directly affect protein function. These SNPs may be directly responsible for some of the variations among individuals in economic traits. Thirdly, SNPs are co-dominantly inherited, making them more suited as long-term selection markers. Finally, SNPs are most suitable for high throughput genetic analysis. Therefore, SNP is a marker of choice for association analysis of commercially important traits in various species.
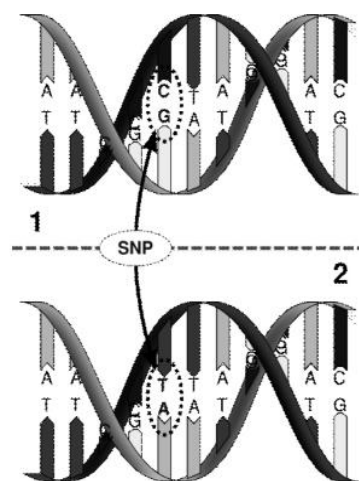
Figure 1.7 General illustration of single nucleotide polymorphism (SNP) (Thomas et al., 2009)

1.4.1.1 Classification of SNPs

SNPs can be classified are two types. Type I or coding SNPs are those located in exons, results in non-replacement (synonymous) and replacement (non-synonymous) of amino acid in the polypeptide chain. Type II or non-coding SNPs are those located in 5' or 3' non-transcribed regions, 5' or 3' untranslated regions, introns and intergenic spacers.

1.4.1.2 SNP discovery

SNP can be detected in the DNA sequences by several methods. DNA sequencing is the directed method for SNP identification (Figure 1.8). Nevertheless, several indirect methods can be used by single strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), allele-specific amplification (ASA) (Table 1.2).
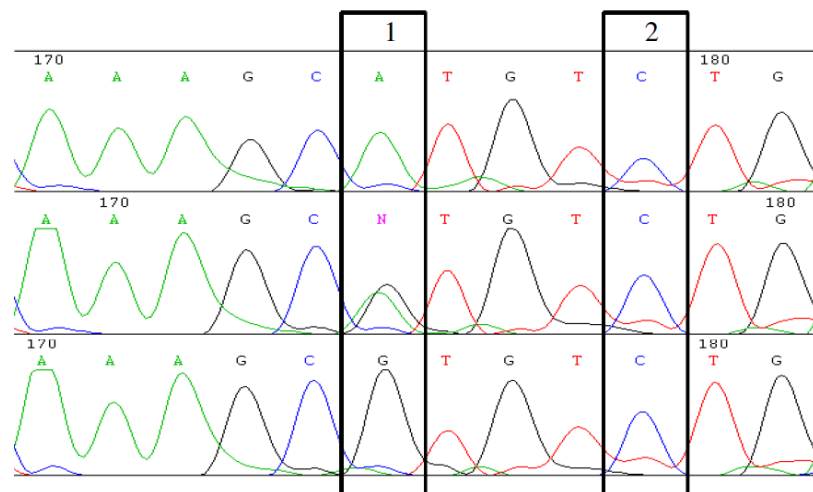
Figure 1.8 SNP discovery by alignment of sequence traces obtained from direct sequencing of genomic PCR products. Box 1: top sequence homozygote AA, middle sequence heterozygote AG and bottom sequence homozygote GG. Box 2: top and bottom sequences as heterozygote CT and the middle one as homozygote CC (Vignal et al., 2002).

Table 1.2 Comparison of selected mutation screening methods (Shastry, 2002).

Method	Fragment length (bp)	Advantage	Disadvantage	Efficiency
Single strand conformational polymorphism	~300	No expensive equipment	Small fragment, temperature variation	80
Heteroduplex analysis	300-600	No expensive equipment	Condition to be determined	80
Denaturing gradient gel electrophoresis	100-1000	Simple, long and short fragments	Gradient gel required, mutation in GC region may not be detected	100 with GC clamp
Enzymatic mismatch detection	300-1000	Long and short fragments	Identifies all kinds of mutations	100
Base excision sequence scanning	50-1000	Accurate	Expensive instruments	100
RNAase cleavage	1.6 kb	Longer fragment and rapid analysis	Requires special kit	100
Chemical cleavage	1-2 kb	Large fragment	Multi-steps, labor intensive and hazardous chemicals	100
DNA sequencing	500	Rapid and easy, no additional sequencing	Labor intensives	100

1.5 Molecular techniques used in this thesis

1.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is an analytical method used to separate components of a protein mixture based on their size. The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign. The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge and size. To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size. For this different protein molecules with different shapes and sizes, needs to be denatured (done with the aid of SDS) so that the proteins lost their secondary, tertiary or quaternary structure .The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) are separated by a molecular sieving effect based on size. After the visualization by a staining (protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker) (Figure 1.9).
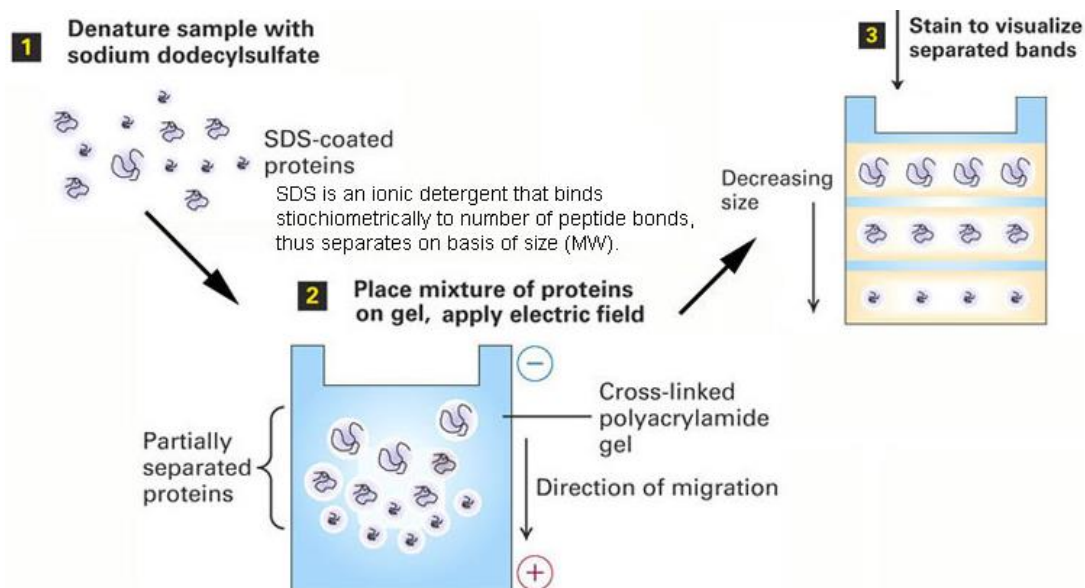
Figure 1.9 The SDS-PAGE process (http://www.chem.fsu.edu/chemlab/.../ background_clip_image010.jpg)

1.5.2 Mass spectrometry

Mass spectrometry is a highly sensitive technique of instrumental analysis of molecules. Currently, a wide range of mass spectrometry types that are specialized for the analysis of element, small gaseous molecules, or biomolecules and biopolymer, exists. Protein identification by this analysis used proteomically digested protein to give higher accuracy of identification than the intact proteins. Proteolysis is achieved using common enzymes such as trypsin prior to MS analysis. This enzyme hydrolyzes peptide bonds on the C terminal side of lysine (Lys) and arginine (Arg) residues, except when they are immediately followed by proline (Pro). Other enzymes such as pepsin, proteinase K and even chemical digestion using reagent such as cyanogenbromide (CNBr) can also be used for the protein digestion However, the use of CNBr yields large peptide fragment that may not useful for peptide sequencing by MS.

Mass spectrometers are made up of three functional units: an ion source, a mass analyzer, and a detector. For mass spectrometric analyzes, free gaseous ions are generated from the sample in the ion source and then focused into and ion beam in vacuum. The mass analyzer separates ions in this beam according to their mass/change (m/z)-ratio; these ions are then registered by detector. Individual measurements are plotted in a mass spectrum with m/z (x-axis) and intensity (y-axis).

Two techniques of mass spectrometry have established in biomolecular analysis; matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI).

Electrospray ionization involves spraying the analyte solution from a microcapillary that carries a high (negative or positive) potential in reference to the mass spectrometer. When the electrostatic force of the applied current exceeds the surface tension of the analyte solution, a Taylor cone forms at the tip of the microcapillary. Highly charged droplets from and solvent evaporation disintegrates them further to a fine spray. This analyte spray is they sucked into the evacuated mass. Analyzer through a microorifice. In the interface area, the droplets are dried and ion formation occurs. The working schematic of an ESI ion source is show in (Figure 1.10).

1.5.3 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The technique was developed by Kary Mullis in 1984 (Joshi and Deshpande, 2011). PCR is based on enzymatic replication of DNA, without using a living organism. This technique involves creating oligonucleotide primers that are complementary to sequence of the gene of interest (DNA template). The primers were designed to flank the interested gene sequence, usually 18-30 nucleotides in length. Million copies of the target DNA sequence can be synthesized from the low amount of starting the DNA template within a few hours.
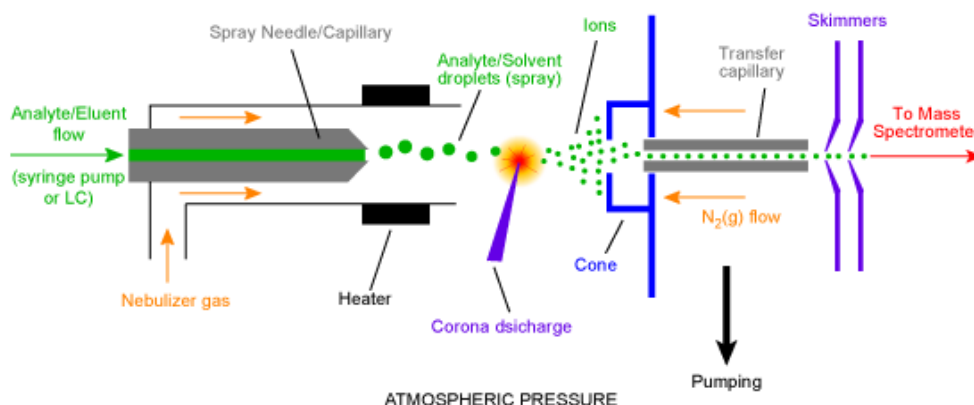
Figure 1.10 Schematic view of an electrospray ion source. Analyte solution is sprayed at atmospheric pressure, droplets enter the evacuated analyzer area through a microoorifice and anion beam is formed (http://www.bris.ac.uk/nerclsmsf/images/apci.gif)

The PCR components were composed of DNA template, a pair of primer for the target sequence, dNTPs (dATP, dCTP, dTTP and dGTP), PCR buffer and heat-stable DNA polymerase (usually *Taq* polymerase). There are three major steps involved in the PCR technique: denaturation, annealing, and extension (Figure 1.11). In step one; the DNA is denatured at high temperatures (from 90 - 97 degrees Celsius). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA. The cycle was repeated until the desired amount of DNA is obtained, usually 30-40 cycles, resulting in an exponential increase of the target DNA. The amplification product was determined by agarose gel electrophoresis.
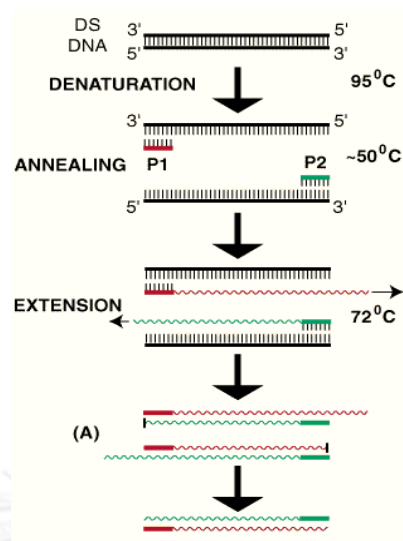
Figure 1.11 General illustration of polymerase chain reaction (PCR) for amplification of the target DNA (http://www.flmnh.ufl.edu/cowries/amplify.html)

1.5.4 Single-Stranded Conformational Polymorphism (SSCP)

SSCP was originally described by Orita et al. (1989). The SSCP analysis detects sequence variations (single-point mutations and other small scale changes) through electrophoretic mobility differences. These variations can potentially cause conformational changes in the DNA molecules. Under non-denaturing conditions and often reduced temperature, single-stranded DNA molecules can assume unique conformations that vary depending on their nucleotide sequences. These conformational changes can result in detectable differences in mobility as illustrated in Figure 1.12.

The major advantage of SSCP analysis is that a large number of individuals may be simultaneously genotyped. Heteroduplexes (product from misannealing of single stranded DNA from difference alleles) can occasionally be resolved from homoduplexes (products from annealing of single stranded cDNA of the same alleles) give additional information on the presence of variants. Therefore, SSCP is regarded as one of the potential techniques that can be used to detect low polymorphism in various species prior to conformational of the results by nucleotide sequencing.
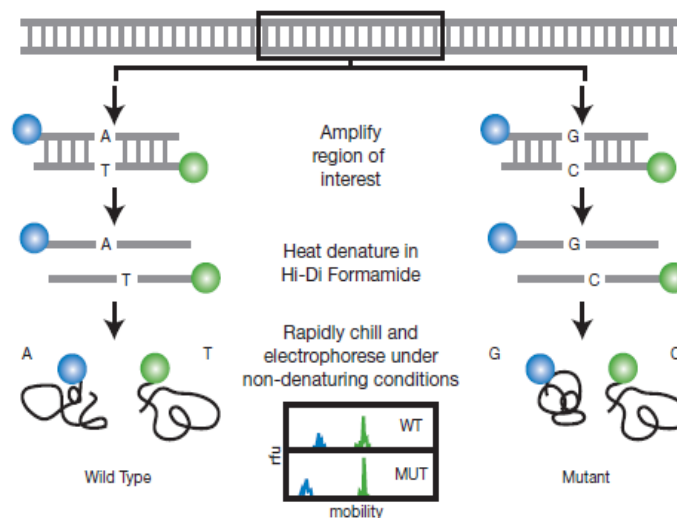
Figure 1.12 Schematic diagram of the SSCP technique

1.5.5 DNA sequencing

DNA sequencing techniques are key tools in many fields. DNA sequencing is the process for determine the order of the nucleotides bases; adenine (A), guanine (G), cytosine (C) and thymine (T) in a molecule of DNA. There are two main methods of DNA sequencing: the "chemical cleavage" procedure described by Maxam and Gilbert (1977) and the "enzymatic chain termination" procedure described by Sanger (1977). The disadvantage of the former method is that the procedure requires the use of several hazardous chemicals. Therefore, the latter is commonly used at present.

DNA polymerases copy single-stranded DNA templates, by adding nucleotides to a growing chain (extension product). Chain elongation occurs at the 3´ end of a primer, an oligonucleotide that anneals to the template. The deoxynucleotide added to the extension product is selected by base-pair matching to the template. The extension product grows by the formation of a phosphodiester bridge between the 3´-hydroxyl group at the growing end of the primer and the 5´-phosphate group of the incoming deoxynucleotide. DNA polymerases can also incorporate analogues of nucleotide bases. The dideoxy method of DNA sequencing developed by Sanger et

al. (1977) takes advantage of this ability by using 2′,3′-dideoxynucleotides as substrates When a dideoxynucleotide is incorporated at the 3′ end of the growing chain, chain elongation is terminated selectively at A, C, G, or T because the chain lacks a 3′-hydroxyl group (Figure 1.13).
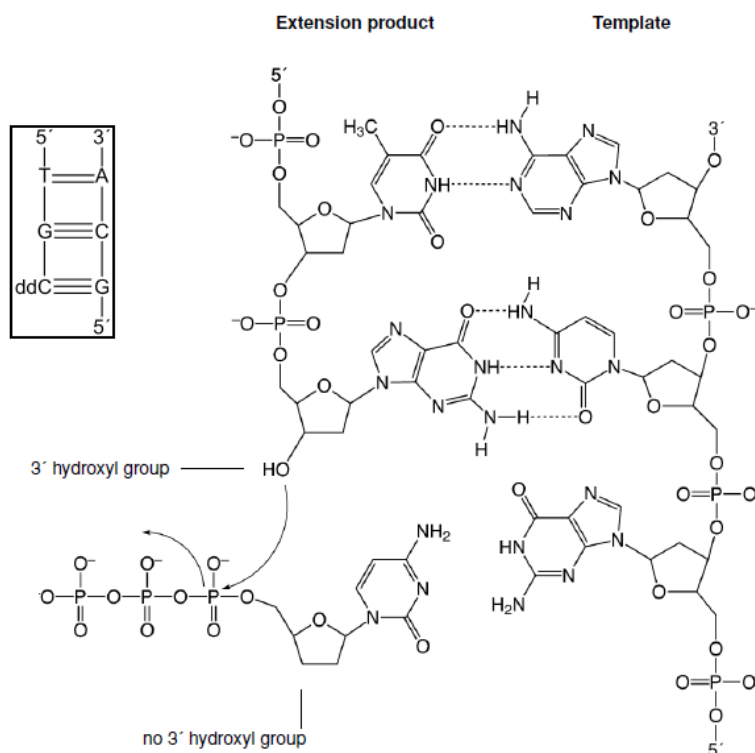
Figure 1.13 DNA strand synthesis by formation of phosphodiester bonds. The chain is terminated by the use of dideoxycytidine triphosphate (ddC) in place of deoxycytidine triphosphate (dCTP). The inset shows a schematic representation of the process.

For automated fluorescent sequencing, fluorescent dye labels are incorporated into DNA extension products using 5′-dye labeled primers (dye primers) or 3′-dye labeled dideoxynucleotide triphosphates (dye terminators). The most appropriate labeling method to use depends on your sequencing objectives, the performance characteristics of each method, and on personal preference. Automated DNA sequencers detect fluorescence from four different dyes that are used to

identify the A, C, G, and T extension reactions. Each dye emits light at a different wavelength when excited by an argon ion laser. All four colors and therefore all four bases can be detected and distinguished in a single gel lane or capillary injection (Figure 1.14).
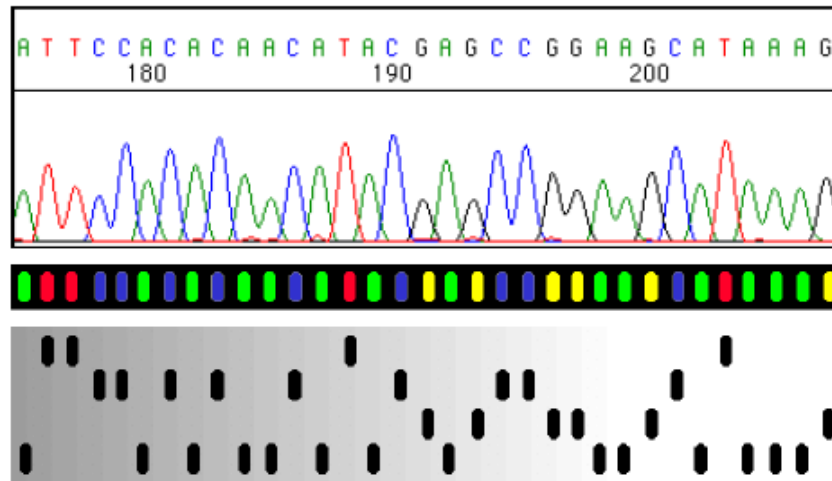
Figure 1.14 Four-color/one-lane fluorescent sequencing vs. one-color/four-lane method such as radioactive sequencing

1.6 Genes functionally involved with growth in fish

Growth performance was traits of major interest in fish production. Molecular information can improve the accuracy of breeding value estimation by the application of genetic markers linked to phenotypic traits. The candidate genes are commonly targeted based on prior knowledge of their role in regulation of specific metabolic pathways influencing a particular quantitative trait. In candidate gene studies, putative genes are first surveyed for polymorphisms and the statistical association between specific alleles and phenotypic expression of the trait of interest examined. If significant associations are found, this is taken as evidence that the gene is directly involved in the genetic control of the trait (De-Santis and Jerry, 2007).

In fish, growth regulation involves many components while the principal regulator of growth is the GH-IGF-I system (Benedet, 2008) (Figure 1.15). Other hormones such as thyroid hormones, insulin sex steroid and glucocorticoids also play in regulating growth and metabolism.

The growth hormone (*GH*) affects many functions, including somatic growth, energy metabolism, reproduction, feeding, osmoregulation and immune functions (Chang and Wong, 2009). The IGF axis is also commonly referred to as the Growth Hormone/IGF-I Axis. Insulin-like growth factor 1 (IGF-1) is mainly secreted by liver
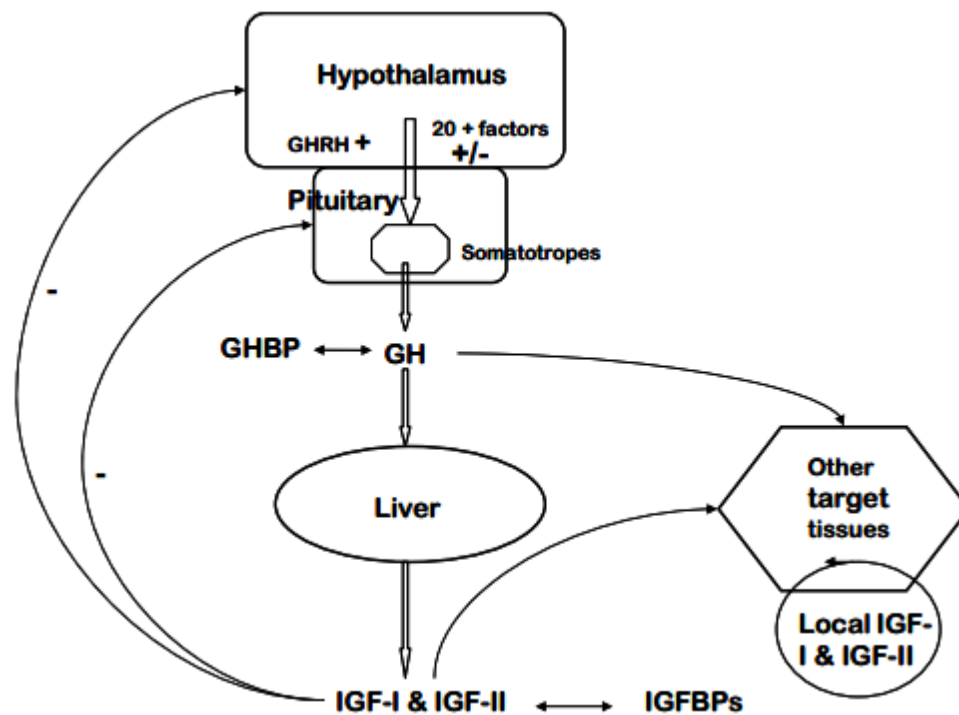
Figure 1.15 The model for GH-IGF-I-system. GH (growth hormone), GH-BP (GH binding protein), IGF-I (insulin-like growth factor I), IGF-II (insulin-like growth factor II), IGF-BP (IGF-binding protein), GHRH (growth hormone releasing hormone) **(Benedet, 2008).**

as a result of stimulation by growth hormone (GH). *IGF-I* is important for the regulation of normal physiology and insulin-like growth factor 2 (*IGF-2*) is thought to be a primary growth factor required for early development while IGF-1 expression is required for achieving maximal growth.

Gross and Nilsson (1999) examined polymorphism in *GH1* in the Atlantic salmon (*Salmo salar*) and its association with the weight of one-year-old progeny of the hatchery strain (graded into three-sized groups) Digestion of the *GH1* fragment with *Taq* I detected two novel polymorphism. Significant difference of genotype frequencies of *GH1* among the size group was detected ($P < 0.05$).

KANG et al. (2002) studied variation within the *GH* gene and its association with growth trait in the olive flounder (*Parlichtys olivaceus*). Polymorphism of various lengths was detected by Southern blot analysis and PCR-RFLP with *Sau*3AI. Association between variation in the GH gene and weight of three various sizes (large: BW > 15.4 g, *N* = 20; medium: BW = 8.2-12.2 g, *N* = 20 and small: BW < 5 g, *N* = 20) were analyzed. A total of 15 different genotypes were observed from the random association of six haplotypes. Significant heterogeneity of the GH gene with haplotype and genotype frequencies was detected among the different-sized groups.

Myostatin (MSTN) is a negative regulator of skeletal muscle growth and development. It is a member of the *transforming growth factor-beta (TGF-β)* superfamily. Wang et al. (2010) examined association of growth parameters with SNP in the *myostatin* gene (*MSTN*) of the mollusc (*Chlamys farreri*) using PCR-SSCP and DNA sequencing. Two mutations were found including A/G at position 327 in exon 2, which caused an amino acid change from Thr to Ala (Thr305Ala) and C/T at position 289 in exon3, which caused an amino acid change from Cys to Arg (Cys422Arg). Individuals possessing genotype GG of primer M5 had significantly higher body mass, soft-tissue mass, adductor muscle mass, shell length, shell height, absolute growth rate of shell height and body mass than those carrying genotypes AG and AA ($P < 0.05$).

Li et al. (2012) examined polymorphism of the *MSTN1* gene in spotted halibut (*Verasper variegatus*) and association between its promoter polymorphism and individual growth performance by directly sequencing. Five SNP existed in the promoter region, among which three (G-653T, T-355C and G-253A) were genotyped and tested for their association with growth traits (body length, body depth and total mass). Fish exhibiting genotype CC at the locus T-355C had significantly higher growth parameters than genotypes TC and TT ($P < 0.05$) in female spotted halibut. The results suggest that *MSTN* could be selected as a candidate gene for molecular breeding of *V. variegatus* stains with enhanced individual growth performance.

Correlation between genotypic and phenotypic variations in aquatic species is still not understood. In this thesis, relationships between SNP of growth-related genes and phenotypes (body weight, total length, hepatic weight and hapatosomatic index) of *L. calcarifer* were examined. Candidate markers for genetic improvement and selection of *L. calcarifer* is illustrated.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals

Specimens for proteomics based on one dimensional gel electrophoresis (SDS-PAGE) and nanoLC-MS/MS were 4-month-old *L. calcarifer* which divided two groups (*N* =9 for each group). For first group was large-sized (average body weight = 28.53 ± 12.97 g and average total length = 13.30 ± 1.82 cm) and second group was small-sized (average body weight = 2.62 ± 0.74 g and average total length = 6.00 ± 0.55 cm).

For SSCP and SNP analyses, cultured Asian seabass *Lates calcarifer* were collected from Chanthaburi Coastal Fisheries Research and Development Center (4-month-old juveniles cultured in concreted tanks, *N* = 99), Thailand. The body weight and total length of each fish were recorded and specimens were divided to 2 groups according to their body weight and total length (Table 2.1). For juvenile fish, only liver and muscle were collected and kept at -80°C and at -20°C until used.

Table 2.1 Experimental animals collected from the Coastal Fisheries Research and Development Center at Chanthaburi, Thailand

Sample	*N*	Average body weight (BW, mean ± SD)	Average total length (TL, mean ± SD)
Chanthaburi (4 month-old)	99		
- Large size	50	22.42 ± 8.82	12.20 ± 1.54
- Small size	49	2.69 ± 0.66	6.00 ± 0.55

2.2. Total protein extraction

Approximately 50 mg of the frozen liver of *L. calcarifer* was homogenized in the presence of liquid nitrogenand suspended in 10% trichloroacetic acid (TCA) in acetone including 0.1% dithiothreitol (DTT) and stored at -20℃ for 1 hour. After centrifugation at 10,000 g for 30 minutes at 4℃, the supernatant was removed and the protein pellet was washed three times with the acetone solution. The protein pellet was recovered by centrifugation at 12,000 g for 15 minutes at 4℃. The pellet was air-dried and dissolved in the 0.5% sodium dodecyl sulfate (SDS). The amount of extracted protein was measured (Lowry et al., 1951).

2.3 Determination of protein concentration by a Lowry-Peterson method

The protein pellet was resuspended in the 0.5% SDS and protein concentration was determined. Briefly, the protein solution was diluted with 0.5% SDS to the final volume of 10 μl and 200 μl of reagent A was added, vortexed and kept in the dark at room temperature for 30 minutes. Subsequently, 50 ul of reagent B was added and the reaction mixture was kept in the dark at room temperature for 30 minutes. The absorbance at 750 nm (OD_{750}) of each sample was measured by a spectrophotometer. The protein concentration of each sample was calculated using the standard curve, plotted between OD_{750} on the Y-axis and BSA protein concentration (μg/μl) on the X-axis.

2.4 One-dimensional gel electrophoresis

2.4.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Thirty micrograms of total proteins was mixed with 2X loading dye and boiled protein mixture at approximately 95℃ for 5 minutes and on ice for 5 minute. The mixture was loaded onto a 12.5% polyacrylamide gel (40% acrylamide in Tris-HCl pH 8.8, 10% SDS). To estimated size of polypeptides, low molecular weight protein standard marker (BioRad) was used. Electrophoresis was performed in SDS electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 0.1% SDS). The

protein separation was electrophoresed at 50 V for 30 minutes followed by 70 V for 3-4 hours.

2.4.2 Silver staining

At the end of each run, the gel protein was fixed in the fixing solution (50% methanol, 12% acetic acid and 50 ul of 37% formaldehyde to 100 ml fixing solution) for 30 minutes. The gel was removed in the washing solutions (35% ethanol) twice for 5 minute each and sensitizing in 0.02% sodiumthiosulfate for 2 minutes. After washing in distilled water twice for 5 minutes each, the gel was stained with staining solutions (0.2% silver nitrate) for 20 minutes and washed in distilled water for 30 seconds. The gel was shaken in the developing solutions (60% sodium carbonate, 0.04% sodium thiosulfate and 37% formaldehyde) until regarded protein bands were visualized and stopped quickly in the stopping solution (14.6% sodium EDTA) for 20 minutes. The gel was washed in distilled water 3 times for 5 minutes each and the gel was kept in 0.1% acetic acid at room temperature.

2.5 Mass spectrometry analysis

2.5.1 In-gel digestion

The protein bands were excised from silver-stained SDS-PAGE gels. The protein gels were subjected to in-gel digestion. The gel plugs were placed in a 96-well plate and subjected to in-gel trypsin digestion. The gel plugs were washed with sterile water and shaken at room temperature for 5 minutes. The gel plugs were dehydrated with 200 μl of 100% acetonitrile (ACN) twice and shaken at room temperature for 5 minutes each. The alkylation step using 50 μl of 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 hour and alkylated using 50 ul of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate at room temperature (dark) for 1 hour. After alkylation, the gel plugs were dehydrated twice time with 100% ACN for 5 minutes each. To perform in-gel digestion of proteins, 20 μl of trypsin solution (10 ng trypsin in 50% ACN and 10 mM ammonium bicarbonate) was added to the gel slices and incubated at room temperature for 20 minutes and 30% ACN (30 μl) was added to the gels and incubated at 37℃ for 3 hours or

overnight. To extract peptide digestion products, 30 ul of 50% ACN in 0.1% formic acid (FA) was added into the gels and shaken at room temperature for 10 minutes for three times. The pooled extracted peptides were dried by incubated at 40°C for 3-4 hours or overnight and kept at -80°C for mass spectrometric analysis.

2.5.2 nanoLC-MS/MS

The nano-electrospray liquid chromatography ionization tandem mass spectrometry (nonoESI-LC-MS/MS) was performed as followed. The extracted peptides submitted to an integrated the HCTultra ETD II system™ operated under HyStar™ (BrukerDaltonics). This system controlled by the Chromeleon Chromatography Management system and comprised a two-pump Micromass/Loading Iontrap system with an autosampler. Injected samples were first trapped and desalted on an AccLaimPepMap C18 u Precolumn Cartridge (5 um, 300-um inside diameter by 5 mm) for 3 minutes with 0.1% formic acid (FA) delivered by a loading pump at 20 ul/minutes, after which the peptides were eluted from the pre-column and separated on a nano column, AccLaimPepMap 100 C18 (15 cm x 3 um) connected inline to the mass spectrometer, at 300 nl/minutes using a 30 minutes fast gradient of 4 to 96% solvent B (80% CAN in 0.1% FA).

2.5.3 Database searches

After data acquisition, MS/MS ions from nanoLC-MS/MS were identified using MASCOT (http://www.matrixscience.com) searched against data of the National Central for Biotechnology Information (NCBI, nr). For MS/MS ion search, the peptide charge was 1+, 2+ and 3+, MS/MS ion mass tolerance was ±1.2 Da, fragment mass tolerance ±0.6 Da, and allowance for 1 miss cleavage. Variable modification was methionine oxidation and cysteine carbamidomethylation. Proteins with the highest score or higher significant score were selected. The significant hit proteins were selected according to Mascot probability analysis and regarded as positive identification after additional conformation with molecular weight (MW)/isoelectric point (p*I*) values.

2.6 Nucleic acid extraction and measurement

2.6.1 Genomic DNA extraction

Genomic DNA was extracted from frozen muscle of individual *L. calcarifer*by a phenol-chloroform-proteinase K method (Klinbunga et al., 2001). The muscle tissue (approximately 50 mg) was transferred to a 1.5 mlmicrocentrifuge tube containing 500 µl of the extraction TEN-buffer (100 mM Tris-HCl, 100 mM EDTA 250 mM NaCl; pH 8.0) and chopped into pieces using sterile scissors. Afterwards, 10% SDS and 10 mg/ml RNase A was added to the final concentration of 1.0% (w/v) and 100 µg/ml, respectively. The mixture was incubated at 37℃ for 1 hour. Subsequently, a 10 mg/ml of proteinase K solution was added to the final concentration of 200 µg/ml and incubated at 55℃ for 3-4 hours until tissue was dissolved. After that, an equal volume of buffer-equilibrated phenol was added and gently mixed for 5-15 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes at room temperature. The supernatant was transferred to a sterile microcentrifuge tube. This extraction process was then repeated once with phenol and phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform: isoamylalcohol (24:1). The final supernatant was transferred to a sterile microcentrifuge tube and an equal volume of TE buffer (10 mM Tri-HCl; pH 8.0 and 0.1 mM EDTA) was added. Subsequently, one-tenth volume of 3 M sodium acetate, pH 5.2 was added. The DNA was precipitated with two volumes of cold absolute ethanol and incubated at -80℃ for 30 minutes. The DNA was precipitated by centrifugation at 12,000 rpm for 10 minutes at 4℃. The DNA pellet was washed twice with 1 ml of 70% cold-ethanol for 15 minutes each and centrifuge at 12,000 rpm for 10 minutes at 4℃. The supernatant was removed and the DNA pellet was air-dried at room temperature for 15-20 minutes. The DNA pellet was dissolved in TE buffer (30-50 µl). The DNA solution was incubated at 37℃ for 1-2 hours and kept at 4℃ for immediately used or at -20℃ for a long storage.

2.6.2 Measurement of nucleic acids using spectrophotometry and agarose gel electrophoresis

2.6.2.1 Estimation of DNA and RNA concentrations by spectrophotometry

The concentration of extracted nucleic acid (DNA and RNA) was estimated by spectrophotometric measurement of UV absorption at wavelength 260 nm and 280 nm (OD_{260}/ OD_{280}). An OD_{260} of 1.0 is equivalent to a concentration of 50 μg/ml double stranded DNA, 40 μg/ml single stranded RNA and 33 μg/ml oligonucleotides (Sambrook and Russell, 2001). Therefore, the nucleic acid concentrations can be estimated in ug/ml by using the following equation;

Nucleic acid concentration (μg/ml) = OD_{260} x dilution factor x nucleic acid factor

;where nucleic acid factor = 50 ug/ml for DNA, 40 ug/ml for RNA and 33 ug/ml for oligonucleotides)

The purity of total nucleic acid can be made from OD_{260}/OD_{280} ratios. The OD_{260}/OD_{280} ratio of pure DNA is between of 1.8 to 2.0. The ratio lower than 1.8 indicated the contamination of protein or phenol whereas the ratio greater than 2.0 indicated the contamination of RNA in the DNA solution.

2.6.2.2 Estimation of quality and amount of extracted DNA using agarose gel electrophoresis

The quantification of extracted DNA can be roughly evaluated by agarose gel electrophoresis. The DNA sample was run through 0.8-1.0% agarose gel in 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.0 mM EDTA; pH 8.3) at 100 Volts. A rough estimated of DNA content can be obtained by comparing band intensity of extracted DNA with undigested λDNA as standards. After electrophoresis, the gel was stained with 0.5 ug/ml ethidiumbromide and visualized under the UV-transilluminator. The intensity of the fluorescence band comparing with undigested λDNA indicated the roughly amount of DNA.

2.7 Isolation of growth-related gene homologue using degenerate primers

2.7.1 Degenerate primer design

Candidate growth-related gene homologues identified from proteomic analysis were selected. Protein sequences from different species of these proteins were retrieved from GenBank and multiple aligned using Clustal W (Thompson et al., 1994). Degenerate primers for amplification of each gene were designed from the conserved region of each gene (Table 2.2) using Primer Premier 5.0. Subsequently, the amplification reaction was performed in a 25 μl reaction mixture (Table 2.3). The PCR product was eluted, cloned and sequencing by MACROGEN (Korea).

Table 2.2 Genes name, primer sequences, primer length and expected size of degenerate primer designed of growth-related proteins of *L. calcarifer* from proteomics results.

Gene	Sequence	Length (bp)	Size (bp)
PDZ-domain containing protein1	F: 5'-GGCGAGGAKGGTCACTCGATCMG-3'	23	309
	R: 5'-CAGRTARCASAGTTTRGSTTT-3'	21	
Proteasome subunit beta type 2	F: 5'-TGYGTBGGDGARGCHGGWGA-3'	20	264
	R: 5'-CCRTGDGCRGCRAASGGRGC-3'	20	
Cell division cycle 2-like protein kinase 5	F: 5'-AAYGAYAAAGGYGCBTTYTA-3'	20	285
	R: 5'-GTGATSACYTTRTTVGTRTABGG-3'	23	
DEAD (Asp-Glu-Ala-Asp) box polypeptide 56	F: 5'-GTNCCMACCAARGARCTGGG-3'	20	435
	R: 5'-TGCTGYAGCTGKSWGCTGTC-3'	20	
Acyl-CoA thioesterase 11	F: 5'-TACCTGAGYTACAAYAATGT -3'	20	378
	R: 5'-ACWGAACGCAGRGCGATCARRTA -3'	23	
14-3-3A2 protein	F: 5'-GCMTAYAAGAAYGTGGTRGG-3'	20	423
	R: 5'-GTGATSACYTTRTTVGTRTABGG-3'	23	

Table 2.3 PCR conditions used for amplification of cDNA and genomic DNA *L. calcarifer* using degenerate primers of various genes

Gene	dNTP (mM)	MgCl$_2$ (mM)	Primer (uM)	PCR conditions
PDZ-domain containing protein1	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 45 sec; 42℃, 1 min and 72℃, 1.30 min for 5 cycles and 94℃, 45 sec; 50℃, 1 min and 72℃, 1.30 min for 30 cycles and 72℃, 7 min for 1 cycle
Proteasome subunit beta type 2	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 30 sec; 50℃, 45sec and 72℃, 1.30 min for 35 cycles and 72℃, 7 min for 1 cycle
Cell division cycle 2-like protein kinase 5 (Cdk13)	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 30 sec; 50℃, 45sec and 72℃, 1.30 min for 35 cycles and 72℃, 7 min for 1 cycle
DEAD-box polypeptide 56	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 30 sec; 50℃, 45sec and 72℃, 1.30 min for 35 cycles and 72℃, 7 min for 1 cycle
Acyl-CoA thioesterase 11	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 30 sec; 50℃, 45sec and 72℃, 1.30 min for 35 cycles and 72℃, 7 min for 1 cycle
14-3-3A2 protein	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 45 sec; 42℃, 1 min and 72℃, 1.30 min for 5 cycles and 94℃, 45 sec; 50℃, 1 min and 72℃, 1.30 min for 30 cycles and 72℃, 7 min for 1 cycle

2.8 Identification of SNP in the amplified gene segment of *L. calcarifer* using single-stranded conformational polymorphism (SSCP) analysis

2.8.1 Primer design

PCR primers were designed from growth-related genes of *L. calcarifer* found in the GenBank (http://ncbi.nlm.nlh.gov) (Table 2.4) using Primer Premier 5.0. The amplification genomic DNA as expected from sequence of growth-related genes.

2.8.2 Polymerase Chain Reaction (PCR)

PCR amplification was performed in a 25 μl reaction mixture. The amplification profiles were carried out following condition described in Table 2.5. The amplification products are separated by using 1.2-2.0% agarose gel electrophoresis. The gel was stained with 0.5 μg/ml ethidium bromide and visualized under the UV-transilluminator.

Table 2.4 Genes, primer sequences, primer length and melting temperatures of primer designed of growth-related genes of *L. calcarifer* from GenBank.

Gene	Sequence	Length (bp)	Tm (°C)
Insulin-like growth factor I	F: 5'-ATCTCCTGTAGCCACACCCTCT-3'	22	68
	R: 5'-AGCCATAGCCTGGTTTACTGAA-3'	22	68
Insulin-like growth factor II (exon)	F: 5'-CGTTCTTGTTCAGTCTGCGTCC-3'	22	68
	R: 5'-AGTTTCCCAGTTGGTGAGGCTA-3'	22	66
Insulin-like growth factor II (intron)	F: 5'-CGTTCTTGTTCAGTCTGCGTCC-3'	22	68
	R: 5'-AGTTTCCCAGTTGGTGAGGCTA-3'	22	66
Follistatin	F: 5'-CGCACCAGACTGCTCCAACATC-3'	22	70
	R: 5'-CGTCACCTCAGGGCAAATCCGA-3'	22	70
Activintype IIB receptor	F: 5'-GACCACCTGAAGGGTAACAC-3'	20	62
	R: 5'-GAAGGAGTCTCGCTGGAAGT-3'	20	62
Myostatin	F: 5'-GCTTTGGGTCCAGTAGTTTTGA-3'	22	66
	R: 5'-TTATTGTCTCTGTGATGGCGTG-3'	22	68

Table 2.5 Genes name, the expected size, PCR conditions and PCR profiles used for amplification of genomic DNA of *L. calcarifer*

Gene	Size (bp)	dNTP (mM)	MgCl$_2$ (mM)	Primer (uM)	PCR Conditions
Insulin-like growth factor I	163	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 45 sec; 50℃, 1 min and 72℃, 1 min for 35 cycles and 72℃, 7 min for 1 cycle
Insulin-like growth factor II (exon)	225	0.2	2.5	0.2	94℃, 3 min for 1 cycle followed by 94℃, 45 sec; 61-55℃, 45sec and 72℃, 45 sec for 12 cycles (decreased for 2℃ every 3 cycles) and 94℃, 45 sec; 53℃, 45 sec and 72℃, 45 sec for 25 cycles and 72℃, 7 min for 1 cycle
Insulin-like growth factor II (intron)	434	0.1	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 1 min; 53℃, 45 sec and 72℃, 1 min for 35 cycles and 72℃, 7 min for 1 cycle
Follistatin	247	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 45 sec; 60℃, 1 min and 72℃, 1 min for 35 cycles and 72℃, 7 min for 1 cycle
Activintype IIB receptor	321	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 45 sec; 53℃, 45 sec and 72℃, 1 min for 35 cycles and 72℃, 7 min for 1 cycle
Myostatin	319	0.2	2.0	0.2	94℃, 3 min for 1 cycle followed by 94℃, 45 sec; 58℃, 45 sec and 72℃, 1 min for 35 cycles and 72℃, 7 min for 1 cycle

2.8.3 Agarose gel electrophoresis

The electrophoresis unit was prepared by cleaning the tray, running platform and combs. A 1x TBE buffer was added to a volumetric flask containing desired amount of powder agarose. The agarose was heated in microwave until the powder agarose completely dissolved. The melting gel was left at room temperature to approximately 50-55°C. The warm agarose solution was poured into the tray slowly and the comb was inserted. The agarose gel was allowed to set for 30-45 minutes and 1x TBE buffer was poured on the top of the gel and the comb was removed. The 1x TBE buffer was added in the gel chamber and the gel was placed in the gel chamber. The PCR product was mixed with the loading dye (0.25% bromophenol blue and 25% ficoll) and loaded into the well of agarose gel. A 100 bp DNA ladder was loaded alongside with the sample as a standard DNA marker. The sample was running at 100 volts until the tracking dye migrated about three-quartered of the gel. After electrophoresis, the gel was stained with 0.5 ug/ml ethidium bromide for 5 minutes and destained to remove unbond ethidium bromide by tap water for 15-20 minutes. The migrated DNA was visualized under a UV-transilluminator.

2.8.4 SSCP analysis

2.8.4.1 Preparation of glass plates

The two glass plates were washed thoroughly using water with detergent and rinsed under running tap water until no remain of detergent and air-dried. The glass plates were wiped three times by 95% ethanol with tissue paper. The long glass plate was coated with 1 ml of freshly prepared Bind silane (4 ul of Bind silane, Amersaham Biosciences, 980 ul of 95% ethanol and 10 µl of 5% gracial acetic acid) and left for approximately 10-15 minutes. After coated, the long glass was cleaned once with 95% ethanol. The short glass plate was coated with Repel silane (2% dimethyldichlorosilanein octamethylcyclotetrasitoxone) and left for approximately 10-15 minutes. After coated, the long glass was cleaned once with 95% ethanol. After the coated glass plates were assembled with a pair of 0.4 mm spacers.

2.8.4.2 Preparation of non-denaturing polyacrylamide gel electrophoresis

Different concentrations of low crosslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared from a 40% stock solution. The 40% stock solution was diluted by 10x TBE and sterile water to required percent gel concentration. A 40 ml of acrylamide gel solution was mixed and degassed under vacuum for 15 minutes. After degassed, 300 µl of 10% APS (prepared freshly) and 30 µl of tetramethylethylenediamine (TEMED). was added to the acrylamide gel solution. The mixture was mixed immediately and quickly poured to the casting gel cassette. The analytical comb was inserted into the prepared gel and allowed to polymerize for at least 4 hours or overnight.

2.8.4.3 Preparation of samples

Six microliters of each amplification product was mixed with four volumes (24 µl) of the SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH). The samples mixture was denatured at 95°C for 5 minutes and immediately cooled on ice for 5 minutes. The denatured product was electrophoretically analyzed in non-denaturing polyacrylamide gels at 200-250 volts for 15-16 hours at 4°C.

2.8.4.4 Silver staining

After electrophoresis, the gel plates were carefully separated apart. The long glass plates with the gel were transferred to a plastic tray containing 2 liters of the fix/stop solution (10% glacial acetic acid) and agitated well for 30 minutes with shaking. The gel was washed with deionized water three times for 10 minutes each and shaking. The gel was transferred to 1.5 liters of staining solution (0.1% silver nitrate) and agitated well at room temperature for 30 minutes. The gel washed in 1.5 liters of deionized water with shacking for 10 seconds and transferred to the developing solution. The developing solution was prepared by added 2.25 ml of formaldehyde and 300 ul of 10 mg/ml sodium thiosulfate to 3 liters of cold sodium carbonate solution (a half of the cold developing solution). The gel was shaken until the first bands are visible (usually 1.5-2 minutes) and then transferred to another tray

containing 1.5 liters of cold developing solution and shaked until band from every lanes were observed (usually 2-3 minutes). The fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes each. The gel was washed in deionized water for 15 minutes and air-dried at room temperature for 2-3 hours.

2.8.4.5 Data analysis for SSCP

Relationships between SSCP genotypes of each gene and growth parameters (body weight, total length, hepatic weight and hepatosomatic index) of 4-month-old seabass were statistically analyzed using one way analysis of variance (ANOVA) following by Duncan's new multiple range tests. When only two SSCP patterns were found in the examined sample, an independent t-test was applied. The significant differences were considered if P-value was less than 0.05 ($P < 0.05$).

2.9 Identification of polymorphic site in the growth-related genes of *L. calcarifer* from sequencing of cloned PCR products

2.9.1 PCR and electrophoresis

The amplification product of each SSCP pattern (N = 5 - 10 for each pattern for the total identified patterns) was amplified by PCR. The electrophoresis of the PCR product was fractionated through agarose gels in duplicate. One was run side-by-side with a 100 bp DNA markers and other was loaded into the distal well of the gel. After electrophoresis, the lanes representing the DNA marker and its proximal DNA sample were cut and stained with 0.5 ug/ml ethidium bromide for 5 minutes. The position of cut band was used to align for the position of non-stained target DNA band. The DNA fragment was excised from the gel with a sterile razor blade and put in a pre-weighed microcentrifuge tube.

2.9.2 Elution of DNA from agarose gel

DNA in pieces of excised gel (200-300 mg) was purified using an illustra^{TM} GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The capture buffer type 3 (500 µl) was added to the gel slices. The mixture was incubated at 60℃ for 15-30 minutes and the tube was inversed every 3 minutes until the gel pieces were completely dissolved. The gel solution was transferred into the assembled GFX MicroSpin column and incubated at room temperature for 1 minute before centrifuged at 13,000 rpm for 2 minutes. The GFX MicroSpin column was washed by an addition of 500 µl of wash buffer type 1 and centrifuged at 13,000 rpm for 1 minute. After discarding the flow-through, the column was dried by centrifugation at 13,000 rpm for 2 minutes. A dried column was placed in a new microcentrifuge tube and 15 µl of elution buffer type 4 was added to the center of column. The column was incubated at room temperature for 2 minutes before centrifugation at 13,000 rpm for 2 minutes. The eluted sample was stored at -20℃ until required.

2.9.3 Ligation of eluted DNA to pGEM$^{®}$-T Easy Vector

The gel-eluted PCR product was ligated to pGEM$^{®}$-T Easy Vector in a total volume of 10 µl containing 3.5 µl of gel-eluted DNA, 5 µl of 2x Rapid Ligation Buffer (60 mMTris-HCl, pH 7.8, 20 mM MgCl$_2$, 20 mM DDT, 2 mM ATP and 10% PEG 8000), 25 ng of the pGEM$^{®}$-T Easy Vector and 3 Weiss units of T4 DNA ligase. The ligation mixture was gently mixed by pipetting and incubated at 4℃ overnight.

2.9.4 Transformation of the ligation product into *E. coli* JM 109

2.9.4.1 Preparation of competent cells

For the preparation of bacterial cell starter, a single colony of *E. coli* JM 109 was inoculated in 5 ml of LB broth (1% bactotryptone, 0.5% bacto yeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37℃ for 16 hours. The starting culture was inoculated into 100 ml of LB broth and continued culture at 37℃ with vigorous shaking to the OD$_{600}$ of 0.4-0.6. The cultured cells were transferred to 50 ml tube and then chilled on ice for 30 minutes and centrifuged at 3,000 g for 15 minutes at 4℃.

After discarding cell medium, the cell pellets were resuspended in 30 ml of ice-cold MgCl$_2$-CaCl$_2$ solution (80 mM MgCl$_2$and 20 mM CaCl$_2$), chilled on ice for 45 minutes and centrifuge at 3,000 rpm for 15 minutes at 4℃. The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M CaCl$_2$ and divided into 100 ul aliquots. These competent cells were either used immediately or stored at -80℃ until needed.

2.9.4.2 Transformation of the ligation product to *E. coli* host cells

The competent cells were thawed on ice for 5 minutes. The ligation mixture (2-3 µl) was added and gently mixed by pipetting. The cell mixture was incubated on ice for 30 minutes. After incubated, the cells were heat-shock at 42℃ for exactly 45 seconds and immediately cooled on ice for 5 minutes. The cell mixture was transferred to the tube containing 1 ml of SOC medium (2% bactrotryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37℃ for 90 minutes. Afterward, the cultured cell was centrifuged at 8,000 rpm at room temperature for 1 minute. The cell pellet was resuspended in 100 µl of SOC medium and spread on a selective LB agar plate (containing 50 ug/ml of amplicillin, 25 µg/ml of IPTG and 20 µg/ml of X-gal). The spread agar plate was further incubated at 37℃ overnight (Sambrook and Russell, 2001). The recombinant cloned containing inserted DNA is white while those without inserted DNA are blue.

2.9.5 Detection of recombinant cloned by colony PCR

Colony PCR was performed in a total volume of 25 µl containing 1x buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 0.1 mM dNTP, 2.0 mM MgCl$_2$, 0.1 mM of pUC1 (5′-TCCGGCTCGTATGTTGTGTGG A-3′) and pUC2 (5′-GTGGTGCAAGGCGATTAAGTTGG-3′) primers and 0.5 units of *Taq*DNA polymerase. A recombinant colony was picked up by the sterile toothpick and mixed well in the amplification reaction. The PCR profile was pre-denaturing at 94℃ for 3 minutes, followed by 35 cycles of 94℃ for 30 seconds, 50℃ for 60 seconds and 72℃ for 90 seconds and the final extension was carried out at 72℃ for 7 minutes. The amplified

PCR product was analyzed by agarose gel electrophoresis and visualized under a UV-illuminator after ethidium bromide staining.

2.9.6 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was inoculated into 3 ml of LB broth containing 50 µg/ml of amplicillin and incubated at 37℃ with shaking at 250 rpm overnight. The recombinant plasmid was extracted using an illustra™plasmidPrep Mini Spin Kit (GE Healthcare). The culture cells were transferred into a 1.5 ml microcentrifuge tube and centrifuged at 12,000 g for 1 minute. The supernatant was discarded and the cell pellet was resuspended with 175 µl of the lysis buffer type 7 and mixed by vortexing. The mixture was then lysed with 175 µl of the lysis buffer type 8 and mixed by inverting the tube approximately 10 times. Additional, 350 µl of the lysis buffer type 9 was added and gently mixed. The cell mixture was centrifuged twice at 12,000 g for 15 minutes each. The supernatant was transferred into the illustra™ plasmid mini column and centrifuged at 12,000 g for 1 minute. The supernatant was discarded and the column was washed by 400 ul of wash buffer type 1 and centrifuge at 12,000 g for 1 minute. The column was dried by centrifugation at 12,000 g for 2 minutes. The column was placed in a sterile microcentrifuge tube and 30 µl of the elution buffer type 4 was added to elute the extracted plasmid DNA. The column was incubated at room temperature for 2 minutes and centrifuge at 12,000 g for a minute.

The insert size of recombinant plasmid was examined by digestion of the recombinant plasmid with *Eco* RI. The digestion was carried out in a total volume of 15 µl containing 1x buffer with BSA, 1 µl of recombinant plasmid and 3 units of *Eco* RI (Promega). The reaction was incubated at 37℃ for 3-4 hours or overnight and analyzed by agarose gel electrophoresis.

2.9.7 DNA sequencing

Nucleotide sequences of recombinant plasmids were examined by automated DNA sequencer using M13 forward and/or M13 reverse primers. Nucleotide sequencing was commercially serviced by MACROGEN (Korea). The nucleotide sequences were searched against the non-redundant nucleotide database in GenBank (http://www.ncbi.nlm.nih.gov/blast) using Blast*X*. The nucleotide sequences were multiple-alignment using clustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2).

2.10 Identification of polymorphic site in growth-related genes of *L. calcarifer* by PCR-direct sequencing

2.10.1 PCR products amplification

The amplification product of each SSCP pattern (N = 5-10 for each pattern for the total identified patterns) was amplified by PCR. The amplification product was performed in a 50 ul reaction mixture. The amplification product size was determined by electrophoresed through 1.5% agarose gel.

2.10.2 Purification of the PCR product

The amplification product was purified using an illustra™ GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The amplification product was transferred a sterile microcentrifuge tube and 500 µl of capture buffer type 3 was added. The solution was transferred into the assembled GFX MicroSpin column and incubated at room temperature for 1 minute and centrifuged at 8,000 g for 1 minute. The GFX MicroSpin column was washed by added 500 µl of wash buffer type 1 and centrifuge at 14,000 g for 1 minute. After discarding the flow-through, the column was dried by centrifugation at 14,000 g for 2 minutes. A dried column was placed in a new microcentrifuge tube. The addition of 20 µl of the elution buffer type 4 to the center of the column. The column was incubated at room temperature for 2 minutes before centrifugation at 14,000 g for 2 minutes. The eluted sample was stored at -20℃ until required. Nucleotide sequence of the purified PCR product was examined

by an automated DNA sequencer using primers of the corresponding growth-related genes by MACROGEN (Korea).

2.11 Characterization of *L. calcarifer* of *Cell division cycle 2-like protein kinase 5* (*Cdk13*) using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR)

2.11.1 Preparation of the 5' and 3' RACE template

Total RNA was extracted from liver of *L. calcarifer* using TRI Reagent. Messenger (m) RNA was purified using a QuickPrep micro mRNA Purification Kit. The RACE-Ready cDNA template was synthesized using a BD SMART^{TM} RACE cDNA Amplification Kit (BD Bioscience Clontech). The RACE-Ready cDNA template was prepared by combining 1.5 µg of hepatic mRNA with 1 µl of 5´CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5´RACE-PCR or 1 µl of 3´CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 3´RACE-PCR (Table 2.6). The components were mixed and briefly centrifuged. The reaction was incubated at 70°C for 2 minutes and snap-cooled on ice for 2 minutes. The reaction tube was briefly centrifuged. After that, 2 µl of 5x First-Strand buffer, 1 µl of 20 mM DDT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript reverse transcriptase were added. The reaction mixture was mixed by gently pipetting and briefly centrifuged. The tube was incubated at 42°C for 1.5 hours in a thermocycler. The first-strand reaction product was diluted with 125 µl of Tris-TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA) and heated at 72°C for 7 minutes. The RACE template was stored at -20°C until required.

Table 2.6 Primer sequence for the first-strand cDNA synthesis and RACE-PCR

Primer	Sequence
BD SMART™A Oligonucleotide	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
3'RACE-CDS Primer A	5'-AAGCAGTGGTATCAACGCAGAGTAC(T)$_{30}$ V N-3' (N=A, C, G or T; V=A, G or C)
5'RACE-CDS Primer A	5'-(T)$_{25}$ V N-3' (N=A, C, G or T; V=A, G or C)
10x Universal Primer A Mix (UPM)	Long (0.4 uM): 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACG CAGAGT-3' Short (2.0uM): 5'-CTAATACGACTCACTATAGGGC-3'
Nested Universal Primer A (NUP)	NUP (10 uM) 5'-AAGCAGTGGTATCAACGCAGAGT-3'

2.11.2 RACE-PCR of *Cdk13*

2.11.2.1 Primer design for RACE-PCR

A Gene-specific primer of *Cdk13* was designed from the partial cDNA sequence of *Cdk13* using Primer Premier 5. The antisense primer and nested primer for 5´ and/or 3' RACE-PCR were designed (Table 2.7).

Table 2.7 Gene specific primers (GSPs) and nested GSP used for isolation of the full-length cDNA of *Cdk13* of *L. calcarifer*

Gene specific primers	Sequence	Tm (°C)
5´RACE-*Cdk13*	5'-TGGCAGTAATCAAGACCCTCCAGCAGC-3'	84
3´NESTED-*Cdk13*	5'-GCTGCTGGAGGGTCTTGATTACTGCC-3'	82
3´RACE-*Cdk13*	5'-TGGACCACGACCTGATGGGTTTACTC-3'	80

2.11.2.2 RACE-PCR

The master mix for 5′ and/or 3′RACE-PCR (and semi-nested PCR) and the control reaction was prepared (Table 2.8). The PCR amplification was performed in a 25 µl reaction mixture. The reaction mixture including 2.5 µl of 10x Advantage® II PCR buffer, 1 µl of 10 mM dNTP mix, 0.5 µl of 50x Advantage® II polymerase mix and 13.5 µl of sterile deionized water was combined. The reaction was carried out for as described in Table 2.9.

Table 2.8 Compositions for amplification of 5' and 3' RACE-PCR of *Cdk13* of *L. calcarifer*

Component	5' RACE sample	3' RACE sample	GSP1 only (control)
5' or 3' RACE-ReadycDNA template	1.5 µl	1.5 µl	1.5 µl
10x UPM primer	5.0 µl	5.0 µl	-
10 uM GSP primer	1.0 µl	1.0 µl	1.0 µl
sterile deionized water	-	-	5.0 µl
Master mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

Table 2.9 The amplification condition for RACE-PCR of *L. calcarifer Cdk13*

Gene	Amplification conditions
5' and/or 3' RACE-PCR	94℃, 30 sec; 72℃, 3 min for 5 cycles followed by 94℃, 30 sec; 70℃, 30sec; 72℃, 3 min for 5 cycles and 94℃, 30 sec; 68℃, 30sec; 72℃, 3 min for 20 cycles and 72℃, 7 min for 1 cycle
5' and/or 3' (Semi-nested) RACE-PCR	94℃, 30 sec; 65℃, 30 sec and 72℃, 3 min for 25 cycles and 72℃, 7 min for 1 cycle

CHAPTER III

RESULTS

3.1 Cellular proteomics of hepatic proteins of large- and small-sized *L. calcarifer* analyzed by GeLC-MS/MS

Two groups of 4-month-old *L. calcarifer* (N = 9 for each group) including a larger size (average body weight = 28.53 ± 12.97 g and average total length = 13.30 ± 1.82 cm) and a smaller size (average body weight = 2.62 ± 0.74 g and average total length = 6.00 ± 0.55 cm) were used for cellular proteomics for isolation of candidate proteins related with growth of this species.

Thirty micrograms of total proteins from liver of each fish were size-fractionated. The electrophoresed proteins were eluted out from the gel slices and further analyzed further analyzed by nanoESI-LC-MS/MS. The raw data of protein from mass spectrometry were analyzed using DeCyder MS Differential Analysis software. The analyzed MS/MS data from DeCyder MS were submitted to database search using the MASCOT.

In total, 1578 protein homologues were identified. Examples of identified protein homologues were 14-3-3 Protein gamma-2 (Acc. No.: gi|308321470, Score: 8.09), activin type IIB receptor (Acc. No.: gi|291277947, Score: 2.00), transforming growth factor-beta (Acc. No.: gi|35902884, Score: 16.93), ubiquitin carboxyl-termil hydrolase BAP1 (Acc. No.: gi|255522786, Score: 12.94), dual specificity phosphatase 6 (Acc. No.: gi|38322768, Score: 4.83), fibroblast growth factor 8a (Acc. No.: gi|114306820, Score: 2.98), estradiol 17-beta-dehydrogese 1 (Acc. No.: gi|45387597, Score: 10.02), growth hormone 1 (Acc. No.: gi|188532133, Score: 4.45), growth hormone-regulated TBC protein (Acc. No.: gi|62955593, Score: 3.34), armadillo repeat-containing protein 10 (Acc. No.: gi|326680250, Score: 1.78), Squint (Acc. No.: gi|317419481, Score: 11.65) and dysferlin (Acc. No.: gi|380692350, Score: 5.49)

Six proteins were found only in large-sized fry while fifteen proteins were observed only in small-sized fry. Examples of protein homologues in the former group were cAMP-dependent protein kinase catalytic subunit beta (Acc. No.: gi|223649260, Score: 10.25), glutathione S-transferase kappa (Acc. No.: gi|239509199, Score: 4.30), clathrin coat assembly protein AP180-like (Acc. No.: gi|326675056, Score: 4.43) and guanylate cyclase 2G-like (Acc. No.: gi|326672477, Score: 1.97) while examples of those in the latter group were clathrin fidgetin-like protein 1 (Acc. No.: gi|192455670, Score: 11.67), connector enhancer of kise suppressor of ras 2 (Acc. No.: gi|189536778, Score: 1.50), pleckstrin homology-like domain family B member 3-like (Acc. No.: gi|292622857, Score: 4.00), spectrin beta chain, brain 4-like (Acc. No.: gi|189530996, Score: 3.85), retinitis pigmentosa GTPase regulator protein 2 ORF15 (Acc. No.: gi|282722852, Score: 1.78), unnamed protein product (Acc. No.: gi|47216520, Score: 9.00) and unnamed protein product (Acc. No.: gi|47223329, Score: 7.67) (Table 3.1).

Identified proteins found in liver of 4-month-old *L. calcarifer* were functionally categorized according to the biological process and molecular functions of their homologues using the Gene Ontology Categorizer (http://www.uniprot.org/). The most abundant protein found in this study were those classified as unnamed protein (484 proteins accounting for 30.7% of identified proteins) followed by those in unknown function (349 proteins accounting for 22.1% of identified proteins) (Figure 3.1).

Among proteins with known function, 124 proteins accounting for 7.9% of identified proteins in this study were categorized in regulation. They were, for example (growth hormone-regulated TBC protein 1, E3 ubiquitin-protein ligase CBL), 113 proteins accounting for 7.2% of identified proteins in this study were classified in binding. Examples of proteins in this functional category were ATP-dependent RNA helicase DHX8 and Insulin-like growth factor 1a receptor. Proteins in other functional categories found in at least 1.0% of the characterized hepatic proteins were transport proteins (44 proteins accounting for 2.8%; e.g. Na+/K+ ATPase beta subunit isoform 1, rabenosyn-5) and those in catabolic process (40 proteins

Table 3.1 Hepatic proteins showing significant differential expression between large-sized and small-sized L. calcarifer (4-month-old) collected from Chanthaburi Coastal Fisheries Research and Development Center, Thailand

Protein name	Score	Accession no.	Biological process	Molecular process	P-value	Intensity		Intensity ratio	
						Large-sized	Small-sized	Large-sized	Small-sized
39S ribosomal protein L23, mitochondrial [*Danio rerio*]	1.49	gi|50344758	Translation		0.0194	20.8493	16.7555	1	0.8036
Calmodulin [*Salmo salar*]	7.80	gi|209735446		Calcium ion binding	0.0429	19.6305	15.1501	1	0.7718
cAMP-dependent protein kise catalytic subunit beta [*Salmo salar*]	10.25	gi|223649260		ATP binding	0.0126	21.2308	0	1	0
Cytochrome c oxidase subunit 7C, mitochondrial precursor [*Salmo salar*]	2.97	gi|209730518			0.0304	17.9264	12.9659	1	0.7233
Dual specificity phosphatase 6 [*Tetraodon nigroviridis*]	4.82	gi|38322768	regulation of fibroblast growth factor receptor sigling pathway		0.0459	11.3474	16.1902	1	1.4268
E3 ubiquitin-protein ligase LNX [*Danio rerio*]	8.11	gi|115430089	Ubiquitin-dependent protein catabolic process		0.0049	20.1827	17.1532	1	0.8499
Fidgetin-like protein 1 [Danio rerio]	11.67	gi|192455670	ATP metabolic process		0.0119	0	21.3235	0	21.3235
Gamma-enolase [Salmo salar]	8.97	gi|213513750	Glycolysis		0.0012	16.6297	12.9216	1	0.7770
Globin X [Carassius auratus]	3.92	gi|56368398	Response to hypoxia		0.0329	14.5356	16.5826	1	1.14083
Glutathione S-transferase kappa [Hypophthalmichthys nobilis]	4.30	gi|239509199		Protein disulfide oxidoreductase activity	0.0130	22.3838	0	1	0
Glycine n-acyltransferase [Ictalurus furcatus]	5.42	gi|308321650		Glycine N-acyltransferase activity	0.0240	21.3179	18.4474	1	0.8654
Platelet receptor Gi24 precursor [Danio rerio]	4.92	gi|117606169			0.0110	18.1730	19.3752	1	1.0662

Table 3.1 Hepatic proteins showing significant differential expression between large-sized and small-sized *L. calcarifer* (4-months-old) collected from Chanthaburi Coastal Fisheries Research and Development Center, Thailand

Protein name	Score	Accession no.	Biological process	Molecular process	P-value	Intensity		Intensity ratio	
						Large-sized	Small-sized	Large-sized	Small-sized
PREDICTED: clathrin coat assembly protein AP180-like [Danio rerio]	4.43	gi|326675056	positive regulation of necrotic cell death		0.0013	21.9143	0	1	0
PREDICTED: complement C4-like [Danio rerio]	7.56	gi|125839308			0.0258	13.4275	17.7445	1	1.3215
PREDICTED: connector enhancer of kise suppressor of ras 2 [Danio rerio]	1.50	gi|189536778	regulation of signal transduction		0.0358	0	20.0379	0	20.0379
PREDICTED: guanylate cyclase 2G-like [Danio rerio]	1.97	gi|326672477			0.0314	18.9958	0	1	0
PREDICTED: pleckstrin homology-like domain family B member 3-like [Danio rerio]	4.00	gi|292622857			1.55E-05	0	14.5209	0	14.5209
PREDICTED: spectrin beta chain, brain 4-like [Danio rerio]	3.85	gi|189530996			0.0026	0	17.1939	0	17.1939
PREDICTED: thrombospondin-2 [Danio rerio]	16.49	gi|326672475	cell adhesion		0.0072	19.5482	15.9373	1	0.8153
Retinitis pigmentosa GTPase regulator protein 2 ORF15 [Danio rerio]	1.78	gi|282722852			0.0413	0	18.0884	0	18.0885
Short transient receptor potential channel 1 [Dicentrarchus labrax]	9.13	gi|317419981		calcium channel activity	0.0422	20.3218	17.6357	1	0.8678
Unmed protein product [Tetraodon nigroviridis]	9.89	gi|47179986			0.0313	21.8557	19.8092	1	0.9064
Unmed protein product [Tetraodon nigroviridis]	4.67	gi|47207691			0.0136	19.5328	17.4600	1	0.8939
Unmed protein product [Tetraodon nigroviridis]	9.00	gi|47216520			0.0006	0	21.2537	0	21.2537
Unmed protein product [Tetraodon nigroviridis]	7.67	gi|47223329			0.0344	0	20.8371	0	20.8371

Table 3.1 Hepatic proteins showing significant differential expression between large-sized and small-sized *L. calcarifer* (4-months-old) collected from Chanthaburi Coastal Fisheries Research and Development Center, Thailand (cont.)

Protein name	Score	Accession no.	Biological process	Molecular process	P-value	Intensity		Intensity ratio	
						Large-sized	Small-sized	Large-sized	Small-sized
Unmed protein product [Tetraodon nigroviridis]	3.19	gi|47223782			0.0259	19.5422	15.6148	1	0.7990
Unmed protein product [Tetraodon nigroviridis]	9.45	gi|47224651			0.0053	22.9589	21.3012	1	0.9278
Unmed protein product [Tetraodon nigroviridis]	6.21	gi|47226270			0.0144	22.0906	19.4059	1	0.8785

accounting for 2.5%; e.g. elongation factor EF1 alpha, catalase), transcription (25 proteins accounting for 1.6%; e.g. DNA-directed RNA polymerase I subunit RPA49, estrogen receptor alpha), metabolic process (21 proteins accounting for 1.3%; e.g. Methylmalonyl-CoA mutase, mitochondrial, Alanine--glyoxylate aminotransferase 2-like 1), development (18 proteins accounting for 1.1%; e.g. estradiol 17-beta-dehydrogenase 1, cortactin-binding protein 2), signaling pathway (16 proteins accounting for 1.0%; e.g. somatostatin receptor subtype 2, estrogen receptor beta), biosynthetic process (15 proteins accounting for 1.0%; e.g. mitochondrial ATP synthase subunit f, exostosin-1c),

Proteins in the remaining categories were found in relatively low frequencies including those in proteolysis (14 proteins accounting for 0.9%; e.g. bleomycin hydrolase, mitochondrial intermediate peptidase), DNA recombination (13 proteins accounting for 0.8%; e.g. ATP-dependent DNA helicase Q1, recombination activating gene 2), RNA processing (13 proteins accounting for 0.8%; e.g. pre-mRNA processing factor 8, cold shock domain-containing protein C2), oxidation-reduction process (11 proteins accounting for 0.7%; e.g. estradiol 17 beta-dehydrogenase 8, c-factor), transferase activity (10 proteins accounting for 0.6%; e.g. microsomal glutathione S-transferase-like, glutathione S-transferase theta), cell cycle (9 proteins accounting for 0.6%; e.g. cell division cycle 5-like protein, serine/threonine-protein kinase 10), translation (8 proteins accounting for 0.5%; e.g. 39S ribosomal protein L23, mitochondrial, 40S ribosomal protein S19), cell adhesion (8 proteins accounting for 0.5%; e.g. collagen alpha-1 (VII) chain, thrombospondin-2), cell communication (7 proteins accounting for 0.4%; e.g. PX domain-containing protein C6orf145, sorting nexin-7), cell differentiation (7 proteins accounting for 0.4%; e.g. gametogenetin-binding protein 2, Homeobox protein HMX3-B), immune response (7 proteins accounting for 0.8%; e.g. C-C chemokine receptor type 6, interleukin-8), recombination (6 proteins accounting for 0.4%; e.g. recombination activating protein 1, RAG1), glycolysis (5 proteins accounting for 0.3%; e.g. Fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase), DNA integration (4 proteins accounting for 0.3%; e.g. retrotransposable element Tf2 155 kDa protein type 1-like and Transposable element Tcb1 transposase), apoptotic process (4 proteins

accounting for 0.3%; e.g. protein yippee-like 3, BNIP2 motif-containing molecule at the C-terminal region 1), structural molecule activity (4 proteins accounting for 0.3%; e.g. Claudin-10, occludin), protein folding (4 proteins accounting for 0.3%; e.g. peptidyl-prolyl cis-trans isomerase FKBP10, ubiquitously-expressed transcript), respectively. In addition, 168 proteins (10.6%) collectively recognized in other functions were also identified.
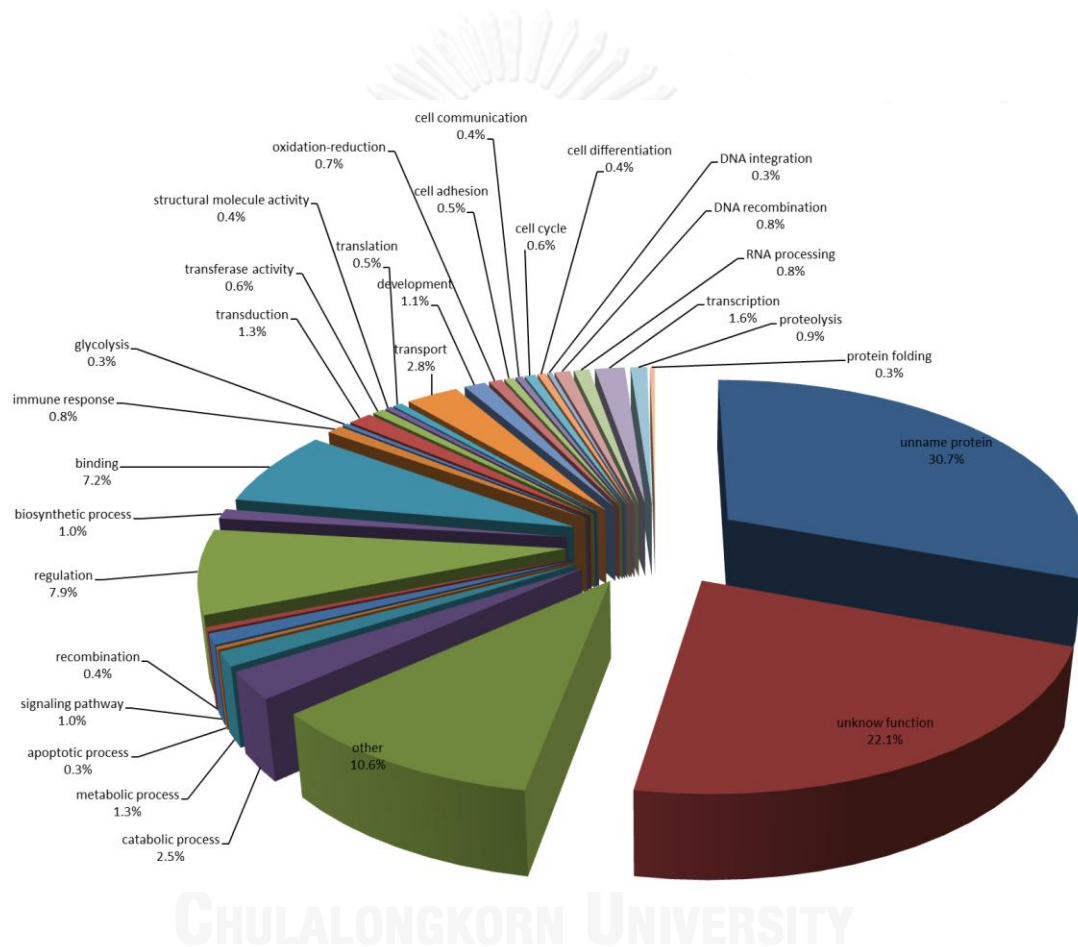
Figure 3.1 Functional classification of total numbers of hepatic proteins (1,578 proteins) identified from liver of 4-month-old *L. calcarifer* from Chanthaburi Coastal Fisheries Research and Development Center, Thailand.

Twenty-eight proteins showed significantly differential expression between large-sized and small-sized samples. Notably, stage-specific proteins were regarded as

differentially expressed protein in the present study. Examples of identified protein homologues were 39S ribosomal protein L23, mitochondrial (Acc. No.: gi|50344758, Score: 1.49), Calmodulin (Acc. No.: gi|209735446, Score: 7.80) cAMP-dependent protein kise catalytic subunit beta (Acc. No.: gi|223649260, Score: 10.25), Dual specificity phosphatase 6 (Acc. No.: gi|38322768, Score: 4.82), Fidgetin-like protein 1 (Acc. No.: gi|192455670, Score: 11.67), Glycine n-acyltransferase (Acc. No.: gi|308321650, Score: 5.42), Gamma-enolase (Acc. No.: gi|213513750, Score: 8.97) and clathrin coat assembly protein AP180-like (Acc. No.: gi|326675056, Score: 4.43).

The functional categories of these twenty-eight proteins were assigned. Unnamed protein (7 proteins accounting for 25.0%) was the most abundant functional group protein followed by those in the binding process, 3 (10.1%), unknown function, (2 proteins accounting for 7.1%), regulations, 1 (3.6%), catabolic process (1 protein accounting for 3.6%) cell adhesion (1 protein accounting for 3.6%), metabolic process (1 protein accounting for 3.6%), cell translation (1 protein accounting for 3.6%) and cell transferase (1 protein accounting for 3.6%). In addition, 4 proteins (14.3%) were collectively classified in other functions (Figure. 3.2).
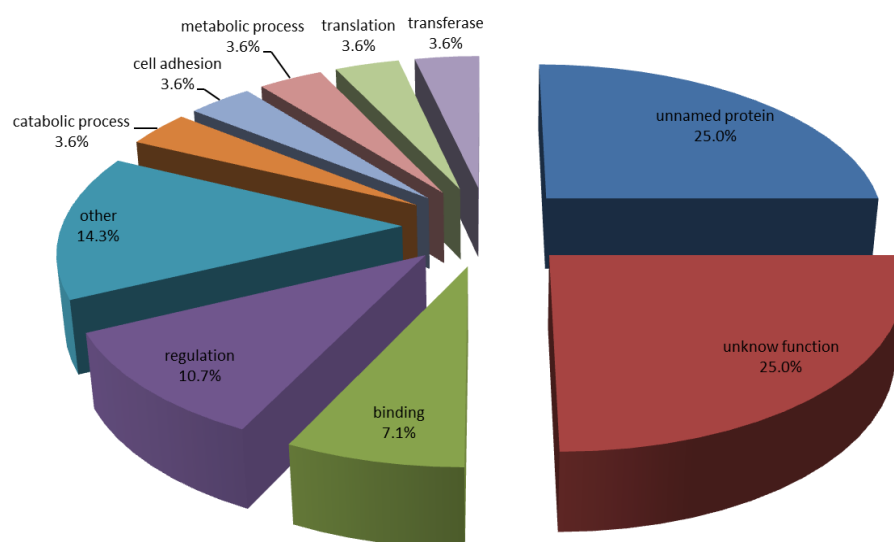
Figure 3.2 Functional classification of differentially expressed proteins (28 proteins) identified from liver of 4-month-old *L. calcarifer* from Chanthaburi Coastal Fisheries Research and Development Center, Thailand.

A large number of growth-related proteins were identified. Examples of growth-related proteins that more abundantly expressed (but not statistically significant) in hepatic tissue of large-sized juveniles than small-sized juveniles were transforming growth factor beta-2 precursor (TGF-ß2 precursor), transforming growth factor beta-3 precursor (TGF- ß3 precursor), and transforming growth factor-beta (TGF-ß)-induced protein ig-h3 precursor, proto-oncogene tyrosine-protein kise ROS, ras-related and estrogen-regulated growth inhibitor-like protein, BMP and activin membrane-bound inhibitor homolog, dysferlin, fibroblast growth factor receptor 1 isoform, growth hormone 1, myosin, light polypeptide 3, ataxin-7-like, epidermal growth factor receptor kise substrate 8-like protein 2-like, primary ciliary dyskinesia protein, tyrosine-protein kise Tec-like, WD repeat-containing protein 16-like isoform 2 and Ubiquitin-conjugating enzyme E2 C.

In contrast, proteins that more abundantly expressed (but not statistically significant) in the opposite direction were activin type IIB receptor (ActRIIB), type-1 angiotensin II receptor, ubiquitin carboxyl-termil hydrolase BAP1, dual specificity phosphatase 6, dual specificity protein kise Ttk, fibroblast growth factor 8a, fibroblast growth factor receptor 3 precursor, epidermal growth factor receptor precursor, growth hormone-regulated TBC protein 1, Insulin-like growth factor 1a receptor, monoacylglycerol lipase ABHD12, PX domain-containing protein C6orf145, stanniocalcin 1, armadillo repeat-containing protein 10 and PR domain zinc finger protein 16 isoform 2.

3.2 Amplification of growth-related genes using degenerate primers

From proteomic analysis, several growth-related proteins were found. Protein sequences of their homologues across different species were retrieved and multiple-aligned. A primer pair was designed from the conserved domain of a particular protein and the amplification product was further characterized.

3.2.1 *PDZ-domain containing protein 1*

Sequence alignment revealed large sequence divergence between *PDZ-domain containing protein 1* from different fish species (Figure 3.3). However, relatively high similarity was found at the PDZ domain sequence. Degenerate primers for amplification of the *PDZ-domain containing protein 1* gene segment were designed. PCR was carried out against the cDNA and genomic DNA template.

An intense band of approximately 220 bp was obtained from the cDNA template whereas several bands were observed from the genomic DNA template (Figure 3.4). Two amplified bands of approximately 220 and 400 bp were cloned and sequenced.

Blast analysis indicated that a 220 bp band (Figure 3.5A) did not significantly match sequence previously deposited in the databases while a 400 bp band (Figure 3.5B) showed the best hit with *unconventional myosin-VIIa*-like of *Oreochromis niloticus* (*E*-value = 3e-39; Figure 3.5C).

```
O.latipes      ---------MAAYKPRVISLTKKPRQTFGFFLRMELGEEGHLIRSLEMGGPAELAGIKDG 51
O.niloticus    ---------MAGYKPRVITLSKKPKQTFGFYLRVENGEEGHLIRCLEMGGPAELAGMKDG 51
T.rubripes     ---------MSGFRPKVISLNKKPGQTFGFYLRSEHGEEGHLIRCLEMGGPAELAGLKDG 51
D.rerio        ---------MAGPKPRIISLTKRESQGYGFYLRVEHGEEGHLIRALEMGGAAELAGLKDG 51
D.rerio-PDZ    DTEETTAGKMAGPKPRIISLTKRESQGYGFYLRVEHGEEGHLIRALEMGGAAELAGLKDG 60
Salmo salar    ---------MAGYKPRVIVLSKKPGHSFGFFLSVEKGEDGHLIRNLEMGGPAELAGLKDG 51
                        *:. :*::* *.*:   :  : :**:*   *  **:***** *****.*****:***

O.latipes      DRIIRVNGTFVDGLSHTEVVELVKNSGTSVTFHIMDESTYKEAKAKGVNLSEQHSTV-GS 110
O.niloticus    DRILRVNGTFVDERSHSEVVDLVKNSGTSVTFHILDESSYKQAKAQGVNLSSP-----GS 106
T.rubripes     DRLLRVNGTFVDEMPHGEVVDMVTSSGTSVTFYVLDEDSYKQAKAQGVDLANPQ----KT 107
D.rerio        DRILRVNNTFVDNLEHTQVVADLVRNSGMSVTLHVLGEEAYKTAKANNVNLADLQSLPGQS 111
D.rerio-PDZ    DRILRVNNTFVDNLEHTQVVADLVRNSGMSVTLHVLGEEAYKTAKANNVNLADLQSLPGQS 120
Salmo salar    DRILRVNGTYMDEMDHSQVVDLVKESGASVTFHVLDVASYKQAKTEGVDFSDPH-----P 106
                      **::***.*::*   * :*.::* .** ***::::.  :** **::.*:::.          .

O.latipes      KVIANGVAKGDPKPKLCYLVKSASGFEFSLFTSNDEEGLFITEVSPGGVAHKAGVRAKDR 170
O.niloticus    KPVANGVEKHRPKPKLCYLVKSSSSFGFSIRSVEGEHGLFMTEVTPGGTAERAGVRLNDR 166
T.rubripes     PAENNKVGAAVPKAKLCYLVNSISGFGFSLSSTEGNPGMFIKLVVPGSVAHNAGLSNNDR 167
D.rerio        QPTMNGVSTPAAKAKLCYLQKSSSGFGFSLKSTKGAHGIFMVDVVSGGVADQAGVKLGDR 171
D.rerio-PDZ    QPTMNGVSTPAAKAKLCYLQKSSSGFGFSLKSTKGAHGIFMVDVVSGGVADQAGVKLGDR 180
Salmo salar    KPTMNGVAGEAPKPKLCYLTKSSGGYGFSLRSVKGEVGMFMAEVNPGGVAERAGVKANDR 166
                    * *    .*.***** :*  ..: **:  : :.  *:*:  *  .*..*..**:     **

O.latipes      LLEVNGENVEDCTHEQVVNKIKQAGNTLMFLLLDEDADKVCRSQYAKMGSRLATVKFLPH 230
O.niloticus    LVEINGENVENITHDGAVDKIRRAGSHIMFLLVDKETDKFYQKTHAKIGSWLATTKYLPR 226
T.rubripes     LVELNGENIEGLSHSQVVEKIKKAGSSLMFLVVDAKTDEYYKNKSKTIGVWLASIKHLPH 227
D.rerio        LVEINSENVEHLVHDQIVQKVKAASDRLMLLLVDEEADRYFKSKNIQPGVSHATVKHLPH 231
D.rerio-PDZ    LVEINSENVEHLVHDQIVQKVKAASDRLMLLLVDEEADRYFKSKNIQPGVSHATVKHLPH 240
Salmo salar    LIEVNGENMENATHDQIVEKVKASGSRIMFLLVDEDTDKFYKNKHIRLGAWLATVKFLPL 226
                    *:*:*.**:*   *.   *:*:: :... :*:*::* .:*.  .:        *   *: *.**

O.latipes      KPRLVNLTKGPDGFGFVLREEPKQAGHFIKDIDRDSPAGRAGLKEMDRLVAVDGKEVDNC 290
O.niloticus    EPRIINITKGSDGYGFTLREEPSQAGHFIRDIERGSSAEKAHLKEKDRLVAVDGRDVESC 286
T.rubripes     KPRVADLSKGPTGFGFSLIYEQNKGGHFIKDIEINSPAQSSGLKEMDRLVAVNSKEVDSW 287
D.rerio        KPRIADMIKRADGYGFMLKEDPKRKGHCIGEIDKGSPAERAGMKDMDRLAAVNGEDIENC 291
D.rerio-PDZ    KPRIADMIKRADGYGFMLKEDPKRKGHCIGEIDKGSPAERAGMKDMDRLAAVNGEDIENC 300
Salmo salar    KPRIVDLFKGSDGYGYFLKAEPNKTGHFIKDIDRGSPADRAGLKEMDRLVAVEGEEVDSC 286
                    :**: :: *  . *:*:*  *   :  .: ** *:*:  .*.*    : :*: ***.**:..::.

O.latipes      SHEQVVEMFRQGGGECCLLVVDQETDQMYKLGKVSPMLFWEEMKDSNLPPSYTEVMNTAT 350
O.niloticus    SHEEVVDRIKQSGNKCCLLVVDKDTDQVYKLGKVSPLFMEEMNDSNSPPSYTEAVNLPI 346
T.rubripes     THDQVVDLIRQSGQSCCFLVMDKFTDKMYKLGNMPPLLFLDTLNDSSLPPSYSEALYLPT 347
D.rerio        KHEQVVEKICQ-GNKCCLLVLDAETDKIYKLGGVSPLLYWEEMRGSLLGYPDNEAVAIPA 350
D.rerio-PDZ    KHEQVVEKICQ-GNKCCLLVLDAETDKIYKLGGVSPLLYWEEMRGSLPGYPDNEAVAIPA 359
Salmo salar    SHEQVVDRIRQCGNKCCLLVVDEDTDTLYKMGGVSPLFYLEEMGFLLPASPP------PS 340
                    .*::**: : * * .*:*:**:*  ** :**:* *..*::: : :          .        .

O.latipes      PAQ---PDEKEE------------------------DLKPKLCRMEKSSDGYGFHLNG 382
O.niloticus    PTRSSTPAPERKE-----------------------ELRPKLCKLEKTSDGFGFHLNS 381
T.rubripes     HAQPSTPEPERRE-----------------------ELKPKLCRMQKSSGSFGFHLNG 382
D.rerio        VAAAVPAAAVAATPAPAATPAPAATPTPVPAAAEPADVDHKPKLCRLERTSAGFGFHLNG 410
D.rerio-PDZ    VAAAVPAAVVAATPAPAATPAPAATPTPVPAAAEPADVDHKPKLCRLERTSAGFGFHLNG 419
Salmo salar    YPECVPALAPATT-----------------------ALLKLCKMEKTSAGYGFHLNS 374
                    .    .                                 ***:::::* .:*****.
```

```
O.latipes     IQGVFGQFIKEVVKGGAADRAGLEDDDMVVEVNGVNVEQSGHQEVVEMIRRSGNSLEMLV 442
O.niloticus   IQGVCGHYLQNVVKGGVADKVGMENDDIVVEVNGVNVEQSHHDDVVKIIQGSGNYLEMLV 441
T.rubripes    IEGKDGHFLSEVVKDRAADVAGIKDGDILVEINGINVENRSHDEVVEMIHLSGNSLEMLV 442
D.rerio       IQGVPGQHIQEVVKGGAADRAGLVDDDVVVEVDGVNVEMSTHEQVVNLIRNSGDTLVLLV 470
D.rerio-PDZ   IQGVPGQHIQEVVKGGAADRAGLVDDDVVVEVDGVNVEMSTHEEVVNLIRNSGDTLVLLV 479
Salmo salar   IQGVCGLYINEVVKGGAADRVGMKDDDIVVEVDGVNVEQSSHEQVVELIRNSGSSLVLLV 434
              *:*   *  .:.:***. .** .*: :.*::**::*:***   *::**::*: **. * :**

O.latipes     ARKSVYDQLKAKGVSITRMLLGEEPKSQEPKSQE--------------HSPETSKKE-IE 487
O.niloticus   AKRSVYDKLKAQGVPITRQLLGETSYAQVHSAK---------------TPEASKRERLE 485
T.rubripes    ATKSVYNQLKANGVNITSQLLGECPEVQVQSTET-------------NRDERQLQDNSN 488
D.rerio       ADRKAYEHLKAKGIPITPQLLNKEPAADVPAP--------------AYTQVDGRKDTEK 515
D.rerio-PDZ   ADRKAYEHLKAKGIPITPQLLNKEPAADVPAP--------------AYTQVDGRKDTEK 524
Salmo salar   AGKQAYNHFKAKGVAITPQLLTPAPTARSPSPPPSPAQRHTPAQIHTPATLEEVREEEE 494
              * :..*:::**:*: ** **   .      .                   .: :

O.latipes     -----RPESPAEPAKQRTPSVSSSSSSAES------IDERL 516
O.niloticus   EEEEARPTTPTEPERQRNSSVSSSSSSAES------IDERM 519
T.rubripes    ----SRPETPTEAARERT---SSSSSEDS------LDEKL 515
D.rerio       D--IEKPATPPAPTRSRSSSSSSSSSSSSS-ASDDSEDERL 552
D.rerio-PDZ   D--IEKPATPPAPTRSRSSSSSSSSSSSSSSSASDDSEDERL 562
Salmo salar   E--EAKPDIPLPQTRERTPSVSSAASSCL-----SVDERL 527
                  :*   *   :.*.  **::*         **::
```

Figure 3.3 Multiple alignments of amino acid sequences of *PDZ-domain containing protein 1* from various species. Positions of degenerated primers are highlighted and the PDZ domain found in the deduced protein is underlined.

Figure 3.4 A 1.5% ethidium bromine-stained agarose gel showing the amplification product of degenerate primers for *PDZ-domain containing protein 1* against cDNA and genomic DNA of *L. calcarifer*. Lane M = a 100 bp DNA ladder. Lane 1 = the amplification products against the cDNA template. Lanes 2 and 3 = the amplification products against the genomic DNA template. Arrowheads indicated the amplification products that were cloned and sequenced.

A.

GGCGAGGATGGTCACTCGATCCGTGACAGAAATGACTCTCAGTGCTACTTATGCCTGAATGTCAGCATGTTTGGG
GGAATGTATGTGCCTCTTTATAATTGTGGGTGACTTGATGAGTGAATTTCTGTATGCTTGTGTAACAAGTGTGGA
CATAACTGAAGGGTATATGCATGGATGTGGGAGTGTGTGCGTGCGGATCGAGTGACCCTCCTCGCCA

B.

GGCGAGGATGGTCACTCGATCCGATCTTCGAAGGAGCACTGAAGGCCGAGCCGCTCAAAGACGAGATCTTCTGTC
AGATTCTCAAACAGCTCACAGATAATCACATCAAGTAAGTGTGAAACACACACACACACTCATTGTCAATAACTC
TGTCTGTGCCTTGGCTTTTTGCACAGAGTAAAGGCTGATACACAAACGATATAATTCTGTAAGATTTGAATAAAG
TATTTAACATGTGTGTGTTTGTGTGTAGGTACAGCGAGGAGAAGGGCTGGGAGCTGCTGTGGCTCTGCACTGGTT
TGTTTCCTCCCAGTAACATCCTGCTGCCTCACGTCCAGAAGTTCCTCCAGGCCAAGAAACACTATCCGCTGGCTC
CGGACTGCATGCAGCGCCTACAGAAAGCCTTACGGTAACACACACACACACTCTTAATGTTATATTCTTTCTTAC
ATACACTCTCTTATACATCCATCTCTCATCTCTTTTATTTGTTGCAGAAACGGATCGAGTGACCATCCTCGCCA

C.

PREDICTED: unconventional myosin-VIIa-like [Oreochromis niloticus]
Sequence ID: ref|XP_003453765.1|Length: 2179 Number of Matches: 1

Score = 153 bits(387), Expect = 3e-39
Identities = 82/131(63%), Positives = 82/131(62%), Gaps = 48/131(36%)
Frame = +3

Query 18   DPIFEGALKAEPLKDEIFCQILKQLTDNHIK*V*NTHTHSLSITLSVPWLFAQSKG*YTN 197
             D IFEGALKAEPLKDEIFCQILKQLTDNHIK
Sbjct 1760 DQIFEGALKAEPLKDEIFCQILKQLTDNHIK-------------------------- 1790

Query 198   DIIL*DLNKVFNMCVFVCRYSEEKGWELLWLCTGLFPPSNILLPHVQKFLQAKKHYPLAP 377
                              YSEEKGWELLWLCTGLFPPSNILLPHVQKFLQAKKHYPLAP
Sbjct 1791 ------------------YSEEKGWELLWLCTGLFPPSNILLPHVQKFLQAKKHYPLAP 1831

Query 378   DCMQRLQKALR 410
             DCMQRLQKALR
Sbjct 1832 DCMQRLQKALR 1842

Figure 3.5 Nucleotide sequences of the amplified products of approximately 220 bp (A) and 400 bp (B) generated from degenerate primers for *PDZ-domain containing protein 1*. Primer positions are underlined. (C) Blast analysis of the amplified 400 bp product against previously deposited sequences in GenBank.

3.2.2 *Proteasome subunit beta type 2*

Protein sequence alignments revealed high sequence similarity between *Proteasome subunit beta type 2* from different fish species (Figure 3.6). Degenerate primers for amplification of the *Proteasome subunit beta type 2* gene segments were designed. PCR was carried out against the cDNA template.

Several amplification bands were obtained. Of these, two intense band of approximately 280 and 300 bp was clearly observed (Figure 3.7). A 280 bp fragment was excised from the gel and cloned (Figure 3.8A). Blast analysis indicated that it showed the closest similarity with *protein DJ-1-like isoform 1* of *Oreochromis niloticus* (*E*-value = 3e-59; Figure 3.8B).

```
D.rerio       MEYLIGIQGPDFVLVAADNVAASSIIQMKHDYDKMFKLSEKILLLCVGEAGDTVQFAEYI 60
I.punctatus   MEYLIGIQGQDFVLVAADNVAASSIIKMKHDYDKMFKLSEKILLLCVGEAGDTVQFAEYI 60
O.mordax      MEYLIGIQGTDFVLVAADNVAASSIIQMKHDYDKMFKLSEKILLLCVGEAGDTVQFAEYI 60
O.niloticus   MEYLIGIQGQDFVLVAADNVAASSIIQMKHDYDKMFKLSEKILLLCVGEAGDTVQFAEYI 60
Salmo salar   MEYLIGIQGQDFVLVAADNIAANSIIQMKQDQDKMFKLSDKILLLCVGEAGDTVQFAEYI 60
O.mykiss      MEYLIGIQGQDFVLVAADNVAAHSIVKMKQDQDKMFKLSDKILLLCVGEAGDTVQFAEYI 60
              ********* **********:** **::**:* *******:*******************

D.rerio       QKNVQLYKMRNGYELSPAAAANFTRKNLADYLRSRTPYHVNLLLAGYDETDGPGLYYMDY 120
I.punctatus   QKNVQLYKMRNGYELSPAAAANFTRKNLADYLRSRTPYHVNLLLAGYDEADGPALYYMDY 120
O.mordax      QKNVQLYKMRNGYELSPSAAANFTRKNLADYLRSRTPYHVNLLLAGYDESDGPGLFYMDH 120
O.niloticus   QKNVQLYKMRNGYELSPAAAANFTRKNLADYLRSRTPYHVNLLLAGYDDTDGPGLYYMDH 120
Salmo salar   QKNIQLYKMRNGYELSPKAAANFTRKNLADYLRSRTPYHVNLLLAGFDETDGPGLYYMDH 120
O.mykiss      QKNIQLYKMRNGYELSPTAAANFTRKNLADYLRSRTPYHVNLLLAGFDETDGPGLYYMDH 120
              ***:*********** *************************:*::***.*:***:

D.rerio       LSALAKAPFAAHGYGAFLTLSILDRYYRPDLTREEAVDLLKKCLEELNKRFILNLPSFTV 180
I.punctatus   MSALAKAPFAAHGYGAFLTLSIMDRYYRPDLTREEALDLLKKCIEELNKRFILNLPSFTV 180
O.mordax      LSALAKAPFAAHGYGAFLTLSILDRYYRPELTRDEAVDLLKKCVEELNKRFILNLPSFSV 180
O.niloticus   LSSLAKAPFAAHGYGAYLTLSILDRYYRPDLSRDEAVDLLKKCVEELKKRFILNLPSFTV 180
Salmo salar   LSALAKAPFAAHGYGAYLTLSILDRYYRPDLTRDEAVGLLKKCVEELNKRFILNLPSFSV 180
O.mykiss      LSALAKAPFAAHGHGAYLTLSILDRYYRPDLTRDEAMDLLKKCIEELNHRFILNLPSFSV 180
              :*:**********:**:*****:******:*:*:**:.*****:***::*****:*****:*

D.rerio       RLIDKDGIHDMEKLP-VGRK-------- 199
I.punctatus   RLIDKDGIHDMEKLL-VGAK-------- 199
O.mordax      RLIDKEGIHDLEKIT-VGHK-------- 199
O.niloticus   RLIDKEGIHDLEKLT-LGAK-------- 199
Salmo salar   RLIDTEGIHDLEKLMPVGAKFVARAPSS 208
O.mykiss      RLIDKDGTHDLEKLIPVGAK-------- 200
              ****.:* **:**:  :* *
```

Figure 3.6 Multiple alignments of amino acid sequences of *Proteasome subunit beta type 2* from various species. Positions of degenerated primers are highlighted and the Proteasome domain found in the deduced protein is underlined.
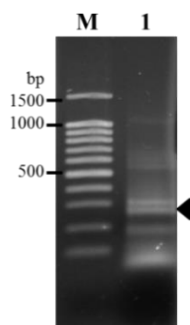
Figure 3.7 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of degenerate primers of *Proteasome subunit beta type 2* gene fragment against genomic DNA of *L. calcarifer*. Lane M = a 100 bp DNA ladder. Lane 1 = the amplification product against the genomic DNA template. An arrowhead indicated an amplified product that was cloned and sequenced.

A.

CCATGTGCGGCAAACGGAGCCCTTATGATGTGGTGCTTCTGCCAGGAGGAATGCCAGGGGCTCAGAATCTGGCCG
AGTCTCCTGCTGTGAAGGAGGTGCTGAAGGATCAAGATGGCAGAAAAGGCCTGATTGCAGCCATCTGTGCAGGTC
CCACCGCTCTTCTGGCGCATGGCATCGGCTTCGGCAGCACAGTCACTACACATCCTGCCATGAAGGAGAAGATGA
TGGCTGGAGACCACTATAAATATTCAGAAGCTCGAGTACAGAAGGATGGACATTACATCACCAGCCTCACCGACA
CAA

B.

```
PREDICTED: protein DJ-1-like isoform 1 [Oreochromis niloticus]
Sequence ID: ref|XP_003454576.1|Length: 189 Number of Matches: 1

Score = 191 bits(484), Expect = 3e-59
Identities = 91/93(98%), Positives = 92/93(98%), Gaps = 0/93(0%)
Frame = +3

Query  12   KRSPYDVVLLPGGMPGAQNLAESPAVKEVLKDQDGRKGLIAAICAGPTALLAHGIGFGST   191
            K+ PYDVVLLPGGMPGAQNLAESPAVKEVLKDQDGRKGLIAAICAGPTALLAHGIGFGST
Sbjct  63   KQGPYDVVLLPGGMPGAQNLAESPAVKEVLKDQDGRKGLIAAICAGPTALLAHGIGFGST   122

Query  192  VTTHPAMKEKMMAGDHYKYSEARVQKDGHYITS   290
            VTTHPAMKEKMMAGDHYKYSEARVQKDGHYITS
Sbjct  123  VTTHPAMKEKMMAGDHYKYSEARVQKDGHYITS   155
```

Figure 3.8 (A) Nucleotide sequence of the amplified product of approximately 300 bp generated from degenerate primers for *Proteasome subunit beta type 2*. The primer position is underlined. (B) Blast analysis of the amplified 300 bp product against previously deposited sequences in GenBank.

3.2.3 *14-3-3 A2*

Protein sequence alignments revealed high sequence similarity between *14-3-3 A2* from different fish species (Figure 3.9). Degenerate primers for amplification of this gene segment were designed. PCR was carried out against both the cDNA and genomic templates. A single discrete band of approximately 550 bp was generated from the cDNA template whereas several amplification bands were obtained from the genomic DNA template (Figure 3.10). A 280 bp fragment was excised from the gel and cloned (Figure 3.11A). Blast analysis indicated that it showed the closest similarity with *ribosomal protein S8* of *Solea senegalensis* (*E*-value = 6e-107; Figure 3.11B).

```
O.niloticus   MVDREQLVQKARLAEQAERYDDMAAAMKSVTELNEALSNEERNLLSVAYKNVVGARRSSW 60
I.furcatus    MVDREQLVQKARLAEQAERYDDMAAAMKSVTELNEALSNEERNLLSVAYKNVVGARRSSW 60
I.punctatus   MVDREQLVQKARLAEQAERYDDMAAAMKSVTELNEALSNEERNLLSVAYKNVVGARRSSW 60
Salmo salar   MVDREQLVQKARLAEQAERYDDMAAAMKSVTELNEALSNEERNLLSVAYKNVVGARRSSW 60
O.mykiss      MADREQLIQRARMAEQAERYDDMASAMKQVTELSEPLSNDDRNLLSVAYKNVVGARRSSW 60
D.rerio       MADREQLIQRARLAEQAERYDDMASAMKLVTELNEPLSNEDRNLLSVAYKNVVGARRSSW 60
              *.*****:*:**:************:*** ****.*.***::*****************

O.niloticus   RVISSIEQKTSADGNEKKIEMVRAYREKIEKELEAVCQDVLNLLDNFLIKNCSDTQHESK 120
I.furcatus    RVISSIEQKTSADGNEKKIEMVRAYREKIEKELETVCQDVLSLLDNYLIKNCSDAQHESK 120
I.punctatus   RVVSSIEQKTSADGNEKKIEMVRAYREKIEKELEAVCQDVLNLLDNFLIKNCSEAQHESK 120
Salmo salar   RVISSIEQKTSADGNEKKIEMVRAYREKIEKELETVCQDVLNLLDNFLIKNCNETQHESK 120
O.mykiss      RVTSSIEQRAMADGNDKKLELVKAYRETIEKELETVCQDVLNLLDQFLIKSCGEDQLESK 120
D.rerio       RVISSIEQKTAADGNEKKLELVRVYRETVEKELESVCQDVLTLLDQYLIKNCDETQVESK 120
              ** *****:: ****:**:*:*:.***.:*****:******.***::***.*.: * ***

O.niloticus   VFYLKMKGDYYRYLAEVATGEKRATVVESSEKAYNEAHEISKEHMQPTHPIRLGLALNYS 180
I.furcatus    VFYLKMKGDYYRYLAEVATGEKRSTVVESSEKAYNEAHEISKEHMQPTHPIRLGLALNYS 180
I.punctatus   VFYLKMKGDYYRYLAEVATGEKRAAVVESSEKAYSEAHEISKEHMQPTHPIRLGLALNYS 180
Salmo salar   VFYLKMKGDYYRYLAEVATGEKRAGVVESSEKSYSEAHEISKEHMQPTHPIRLGLALNYS 180
O.mykiss      VFYLKMKGDYYRYLAEVATAEKKKTSAVESSEGAYKEAYEISKS-MAATHPIRLGLALNFS 179
D.rerio       VFYLKMKGDYYRYLAEVATGEKRASAVESSEGAYKEAFDISKG-MPATHPIRLGLALNFS 179
              ******************.**:: .***** :*.**.:*** * .***********:*
```

```
O.niloticus    VFYYEIQNAPEQACHLAKTAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQDD 240
I.furcatus     VFYYEIQNAPEQACHLAKTAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQDD 240
I.punctatus    VFYYEIQNAPEQACHLAKTAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQDD 240
Salmo salar    VFYYEIQNAPEQACHLAKTAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQDD 240
O.mykiss       VFYYEIQNAPEEACKLAKEAFDEAIGHLDNLNEDSYKDSTLIMQLLRDNLTLWTSDQQDS 239
D.rerio        VFYYEIQNAPEQACQLAKEAFDDAIGHLDNLNEDSYKDSTLIMQLLRDNLTLWTSDQQDS 239
               ***********:**:*** ***:**..**.***************************.

O.niloticus    EGGEGNN 247
I.furcatus     EGGEGNN 247
I.punctatus    EGGEGNN 247
Salmo salar    EGGETNN 247
O.mykiss       EGGEAQP 246
D.rerio        EGGDANN 246
               ***: :
```

Figure 3.9 Multiple alignments of amino acid sequences of *14-3-3 A2* from various species. Positions of degenerated primers are highlighted and the 14-3-3 domain found in the deduced protein is underlined.
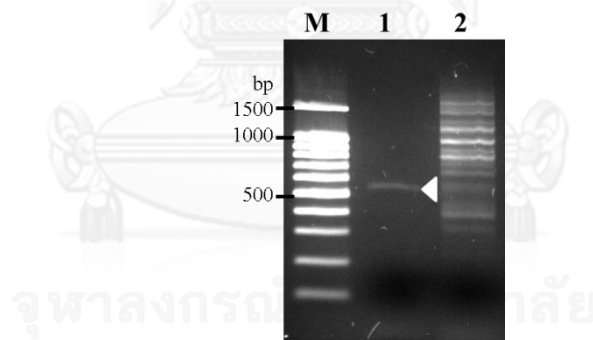
Figure 3.10 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *14-3-3A2* protein gene fragment against genomic DNA of *L. calcarifer*. Lane M = a 100 bp DNA ladder. Lane 1 = the amplification products against the cDNA template. Lane 2 = the amplification products against the genomic DNA template. An intense band of 550 bp (indicated by arrowhead) was cloned and sequenced.

A.

```
TGCATATAAGAATGTGGTAGGAACAAGAAGTACCGTGCTCTGAGGTTGGATGTTGGAAACTTCTCATGGGGCTCT
GAGTGCTGCACACGGAAGACCAGGATCATCGATGTGGTCTACAATGCCTCCAACAACGAGCTGGTCAGAACCAAG
ACCCTGGTGAAGAACTGCATCGTCCTCATCGACAGCCTTCCCTTCAGGCAGTGGTATGAGGCTCACTACGCCACT
CCTCTGGGACGCAAGAAGGGAGCCAAGCTGACTCCCGAGGAGGAAGAGGTCCTGAACAAGAAGCGGTCAAAGAGG
ACCCAGAAGAAATACGATGAGCGTAAGAAGACAGCCAAGATCAGTCCCCTCCTGGAGGAGCAGTTCCAGCAGGGA
AAACTGCTCGCTTGCATCGCCTCCAGACCCGGCCAGTGCGGTAGGGCAGACGGTTACGTCCTGGAAGGCAAAGAG
CTTGAGTTCTACCTGAGGAAGATCAAGGCCAAGAAAGGCAAATAGATGTACAGCTGTTTGATACGTCAATAAATT
CCTCCCACCACATTCTTGTAGGC
```

B.

```
ribosomal protein S8 [Solea senegalensis]
Sequence ID: dbj|BAF45896.1|Length: 208Number of Matches: 1

Score = 316 bits(810), Expect = 6e-107
Identities = 154/159(97%), Positives = 157/159(98%), Gaps = 0/159(0%)
Frame = +1

Query  16   GRNKKYRALRLDVGNFSWGSECCTRKTRIIDVVYNASNNELVRTKTLVKNCIVLIDSLPF  195
            G NKKYRALRLDVGNFSWGSECCTRKTRIIDVVYNASNNELVRTKTLVKNCIVL+DSLP+
Sbjct  50   GGNKKYRALRLDVGNFSWGSECCTRKTRIIDVVYNASNNELVRTKTLVKNCIVLVDSLPY  109

Query  196  RQWYEAHYATPLGRKKGAKLTPEEEEVLNKKRSKRTQKKYDERKKTAKISPLLEEQFQQG  375
            RQWYEAHYATPLGRKKGAKLTPEEEEVLNKKRSKRTQKKYDERKK AKISPLLEEQFQQG
Sbjct  110  RQWYEAHYATPLGRKKGAKLTPEEEEVLNKKRSKRTQKKYDERKKMAKISPLLEEQFQQG  169

Query  376  KLLACIASRPGQCGRADGYVLEGKELEFYLRKIKAKKGK  492
            KLLACIAS+PGQCGRADGYVLEGKELEFYLRKIKAKKGK
Sbjct  170  KLLACIASKPGQCGRADGYVLEGKELEFYLRKIKAKKGK  208
```

Figure 3.11 (A) Nucleotide sequence of the amplified product of approximately 550 bp generated from degenerate primers for *14-3-3A2 protein*. The primer position underlined. (B) Blast analysis of the amplified 550 bp product against previously deposited sequences in GenBank.

3.2.4 *DEAD (Asp-Glu-Ala-Asp) box polypeptide 56*

Protein sequence alignments revealed high sequence similarity between DEAD box polypeptide 56 from different fish species (Figure 3.12). Degenerate primers for amplification of the *DEAD box polypeptide 56* gene segments were designed. PCR was carried out against the cDNA template. An intense band of approximately 400 bp was clearly observed (Figure 3.13). This fragment was excised from the gel, cloned and sequenced (Figure 3.14A). However, blast analysis indicated that it showed the closest similarity with *warm temperature acclimation-related 65* kDa protein of *Acanthopagrus schlegelii* (*E*-value = 9e-47; Figure 3.15B).

```
T.rubripes    MASERLQFHEMGIDDRLLKAVADLGWSQPTLIQEKAIPLALEGKDLLARARTGSGKTAAY 60
O.niloticus   MAHERLQFHEMGLDDRLLKAVADLGWSKPTLIQEKVIPLALDGKDLLARARTGSGKTASY 60
O.latipes     MACERLQFHEMGLDDRLLKAVADLGWSQPTLIQEKAIPLALEGKDLLARARTGSGKTAAY 60
D.rerio       MEEDSVRFHEMGLDDRLLKALADLGWSQPTLIQEKAIPLVLEGKDLLARARTGSGKTAAY 60
              *   : ::****:*******:*****:*******.***.*:****************.*

T.rubripes    AVPVIQRILASKQSVREQDVKALILVPTKELGQQVQTMIRQLTAFCSRDVRVADVSGKAD 120
O.niloticus   GIPVIQRILASKQNVREQDVRALIVVPTKELGHQVQIMMRQLTAYCSRDIRVADISSKVD 120
O.latipes     AVPIIQHILTSKQSVREQAVRVLVLVPTKELGQQVQTMMRQLTSFCSRDVRVADISSKAD 120
D.rerio       AVPLIQRVLTSKQTVREQAVRAVVLVPTKELGQQVQTMIRQLTVYCSRDVRVADISGKAD 120
              .:*:**::*:*** ****  *:.:::*******:*** *:**** :****:****:*.*.*

T.rubripes    VSTQRPILMEKPDIVVGTPSRILAHLNAQNLLLHSSLEMLVVDEADLLFSFGFEADLKNL 180
O.niloticus   LSTQRPILMEKPDVVVGTPSRVLAHLNAQNLVLRSSLEMLVVDEADLIFSFGFESDLKNL 180
O.latipes     VSSQRPILMEKPDVVVGTPARVLAHINAHNLDLQASLEVLVVDEADLIFSFGFEADLKSL 180
D.rerio       LSAQKPILMEKPDIVVSTPSRIQAHINAQNLQLHSSLEMLVIDEADLLFSFGFEADLKNL 180
              :*:*:*********:**.**:*: **:**:** *::***:**:*****:******:***.*

T.rubripes    LCHLPKIYQSFLMSATLSEDVQSLKELLLHNPIILKLQGSQLPDSSQLQQYSINCEEEDK 240
O.niloticus   LCHLPNIYQSFLMSATFNEDVQTLKELLLHNPVILKLQGSQLPDSSQLQQYSIKCEEEDK 240
O.latipes     LCHLPKIYQSFLMSATFTEDVQALKELLLHNPVVLKLQGSQLPDSSQLQQYTIKCEEEDK 240
D.rerio       LCHLPKIYQAFLMSATLNDDVQALKELVLHNPVTLKLQGSQLPDSSQLQQFSIKCEEEDK 240
              *****:***:******:.:***:****:****: ****************:*:*:******

T.rubripes    FLLIYTLLKLRLVQGKTLLFVGAVDRSYRLKLFLEQFGIPTCVLNSELPVGSRCHIISQF 300
O.niloticus   FLLVYTLLKLCLVKGKTLLFVGTVDRCYRLKLFLEQFGIPACVLNSELPVQSRCHIIAQF 300
O.latipes     FLLVYTLLKLQLVRGKTLLFVGAVDRSYRLKLFLEQFSIPACVLNSELPVQSRCHIITQF 300
D.rerio       FLLIYTMLKLGLIQGKTLLFVSDVDRSYRLKLFLEQFSIPACVLNSELPVHSRCHIISQF 300
              ***:**:*** *::*******.  ***.*********.**:********* ******:**

T.rubripes    NQGFYDYIIAADEESLAVPAEGAASAEAAAGKGKKKKNSEKAGKSKDKEFGVSRGVDFQN 360
O.niloticus   NQGFYDYIIATDEQSLT---ETAAEPQTTAGK-EKKKTQKKGGKAKDKEYGVSRGVDFQN 356
O.latipes     NQGFYDIIIATDEQSLS------PPAGSSQTKGKEKKNADKGGKAKDKEFGVSRGVDFQN 354
D.rerio       NQGFYNYIIASDEQGLES---PAGSSQKTQEKGNKKKGTGK--KGKDKEYGVSRGIDFHN 355
              *****: ***:**:.*        .  :  * ::**   *  *.****:*****:**:.:*
```

```
T.rubripes    VSNVINFDFPTTVESYIHRVGRTARADNPGTALSFISHKEVALLSDVEEALTGDHSGSVL 420
O.niloticus   VANVINFDFPKTVESYIHRVGRTARADNPGTALSFVSHTELGLLSEVEEALTGDETNSPL 416
O.latipes     VANVINFDFPTTVESYIHRVGRTARADNQGTALSFISHTELPLLVEVEEALSTDNAESVL 414
D.rerio       VSNVINFDFPTSVESYIHRVGRTARADNPGTALSFISHAELSMLSEVENALTGDSNNCVL 415
              *:********.:*************** ******:** *: :* :**:**: *   . *

T.rubripes    KPYKFKMEEIEGFRYRCRDAMRSVTKQAVREARLKEIKQELLNSEKLKTYFEDNPRDLQL 480
O.niloticus   KPYEFKMEQIEGFRYRCRDAMRSVTKQAVKEARLKEIKQELLNSEKLKTYFEDNPRDLQL 476
O.latipes     KPYQFRMEEIEGFRYRCRDAMRAVTKQAVREARLKEIKQELLNSEKLKTYFDDNPRDLQL 474
D.rerio       KPYEFRMEEIEGFRYRCRDGMRSVTKQAVKEARLKEIKQELLNSEKLKTYFEDNPRDLQL 475
              ***:*:**:**********.**:******:***********************:*******

T.rubripes    LRHDKDLHPAVVKPHLRNLPDYLIPDTLRNVINPLSCKNRRKRKEKPKLGVVKTSFKN-T 539
O.niloticus   LRHDKDLHPAVVKPHLKNVPEYLIPEALKGVVHPLTSRRRRRKEIQRPGGVIKSSFKKNI 536
O.latipes     LRHDKDLHPAVVKPHMKNIPDYLIPQTLRGVVNPLSGRRKRWRVKPPAEGVARSSFKKND 534
D.rerio       LRHDKDLHPAIIKPHMKNVPEYLIPTALKSLVNPLNQRRKRKKVKSS--GVMLSSFKKNY 533
              **********::***::*:*:*:**** :*:.::::**. ..:* :      **  :***:

T.rubripes    RGKDPLKSFRYTRGKNRKGKAAQS 563
O.niloticus   QGKNPLKSFQYTGGKNRKGKASQS 560
O.latipes     PSRNPLKSFRYTRGRNKAAKAGKS 558
D.rerio       RGRNPLKSFRYAKKRRGEKKAGKP 557
                .::*****:*: :.   **.:.
```

Figure 3.12 Multiple alignments of amino acid sequences of *DEAD (Asp-Glu-Ala-Asp) box polypeptide 56* from various species. Positions of degenerated primers are highlighted and the DEXDc domain found in the deduced protein is underlined.
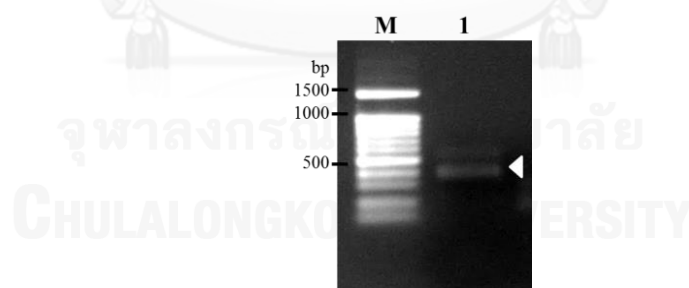
Figure 3.13 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *DEAD (Asp-Glu-Ala-Asp) box polypeptide 56* gene fragment against genomic DNA of *L. calcarifer*. Lane M = a 100 bp DNA ladder. Lane 1 = the amplification products against the cDNA template. An arrowhead indicated the band that was cloned and sequenced.

A.

<u>GTGCCCACCAAAGAACTGGGC</u>ATCGAGGGTCCCATTGAAGCTGCATTTGTCTGCGAGGATCATCACATTGCTCAC
ATCATCAAAGAGGGGAAGATTTATGATGTGGAGTTGAAGGCCAGCCCTCGCGTCGCAGCCAATGAGCGTCCAATT
TCCCTGCTGAAGAAGGTCGATGCTGCCATGTGCGATGCTACAGGAATTAAAGTGATCACAGGCAACCACTTGTGC
CACTTTGAGAGTACCATGGTGTTTGTTACTGCCAAGTCCTTGCCTGAGCAGAAAAGGGTATCCCTGGAGCTGTTC
GGCTGCGATCACTAAGAGGCTTGCTTGACAGGACTGACTAGATTAACAACACAGTAAAACGTGGAGAAACGACAG
CAGACAGCTACAGCAA

B.

```
warm temperature acclimation-related 65 kDa protein [Acanthopagrus
schlegelii]
Sequence ID: gb|ABL74446.1|Length: 425 Number of Matches: 1

Score = 166 bits(421), Expect = 9e-47
Identities = 80/101(79%), Positives = 84/101(83%), Gaps = 0/101(0%)
Frame = +1

Query  10   KELGIEGPIEAAFVCEDHHIAHIIKEGKIYDVELKASPRVAANERPISLLKKVDAAMCDA  189
            +ELGIEGPI+AAFVCEDHHIAHIIK  KIYDVELKASPRVA NER ISL  VDAAMCD
Sbjct  325  EELGIEGPIDAAFVCEDHHIAHIIKGNKIYDVELKASPRVAGNERTISLFDNVDAAMCDG  384

Query  190  TGIKVITGNHLCHFESTMVFVTAKSLPEQKRVSLELFGCDH  312
            TG+KVI GNH  HF ST FV +SLPEQ RVSLELFGCDH
Sbjct  385  TGVKVIKGNHFYHFISTHGFVAGRSLPEQHRVSLELFGCDH  425
```

Figure 3.14 (A) Nucleotide sequence of the amplified products of approximately 400 bp generated from degenerate primers for *14- DEAD box polypeptide 56*. The primer position is underlined. (B) Blast analysis of the amplified 400 bp product against previously deposited sequences in GenBank.

3.2.5 Acyl-CoA thioesterase 11

Protein sequence alignments revealed low sequence similarity outside the functional domain but high similarity at the domain region of *Acyl-CoA thioesterase 11* from different fish species (Figure 3.15). Degenerate primers for amplification of the *Acyl-CoA thioesterase 11* gene segments were designed. PCR was carried out against the cDNA and genomic DNA template.

Smear amplification products were observed from the genomic DNA template. Two intense band of approximately 250 and 200 bp were clearly observed (Figure 3.16). A 250 bp fragment was excised from the gel and cloned (Figure 3.17). Blast analysis indicated that it did not significantly match sequence previously deposited in the databases.

```
T.rubripes      MTPEDKS-DMVQYPVPIQGSEEGYRNPTELKMSQIVLPCHANHRGELSVGQLLKWMDSTA 59
T.nigroviridis  MTPEDNP-DTVQNPVPILRREEGDRNPTEVKMSQIVLPCHANHRGELSVGQLLKWMDSTA 59
O.latipes       MTSGQKDGDAMTDAQLIHADGEAYRNPSEVQMSQIVMPCHANHCGELSVGQLLKWMDSTA 60
O.niloticus     MTSENKDSDTMPDPLLIQESGEVYRNPTEVQMSQIVLPCHANHCGELSVGQLLKWMDSTA 60
                **. ::   * :  .  *      *  ***:*::*****:****** ***************

T.rubripes      CLSAERHAGCSCITASVDDIHFELTIGVGKVVNIIAKVNRAFTSSMEVGILVNCEDLYTD 119
T.nigroviridis  CLSAERHAGCSCITASVDDIHFEHTIGVGKVVNIIAKVNRAFTSSMEV----------- 107
O.latipes       CLSAERQAGCSCITASVDDIHFELTIGVGKVVNIKAKVNRAFTSSMEVGISVTCEDLYTG 120
O.niloticus     CLSAERHAGCSCITASVDDIHFEHTIGVGKVVNIIAKVNRAFTSSMEVGILVTCEDLYTD 120
                ******:*************** ********** *************

T.rubripes      RQWKVCQAFATFVARRTEAGKVQLKQVIPRTQLEQMEYSLAAERRRMRLLHAEIITDLLS 179
T.nigroviridis  --------------------QLKQVIPHTQLEQMEYSLAAERRRMRLLHAEIITDLLS 145
O.latipes       RQWKVCHAFATFVARRTETGKVQLKQVIPRTHMEQIEYSLAAERRRMRLIHAEIMTDLLS 180
O.niloticus     RQWKVCHAFATFVARRTEAGKVQLKQVIPRTQMEQMEYSLAAERRRMRLIHAEIITDLLS 180
                        *******:*::**:*************:****:***** :****:*****

T.rubripes      SSTAQPGECQEYQDAVPAEQTRVESVELVLPPHTNHQVSTFGGQIMAWMENVATISA--- 236
T.nigroviridis  SSTAQPGECQEYQDAVPAEQTRVESVELVLPPHTNHQVSTFGGQIMAWMENVATISARCV 205
O.latipes       SSTAQLGECQEYEGAVPAERTRVESVELVLPPHANHQVSTFGGQIMAWMENVATIAA--- 237
O.niloticus     SSTAQLGECQEYQDAVPAERTRVESVELVLPPHANHQVSTFGGQIMAWMENVATIAA--- 237
                ***** ******:.*****:*************:****************** **:*

T.rubripes      ----------------------------------------------------------
T.nigroviridis  SPSSRSWRFRSRWESSSSMSSQLHMPQTPSPQRRLNSRWPNDAAKPPLSEVRLSSYPLFK 265
O.latipes       ----------------------------------------------------------
O.niloticus     ----------------------------------------------------------
```

```
T.rubripes     ------------------------------------------------------------
T.nigroviridis GELNQLNLLFLKGQGHPTRPQRKQRTGVKRKNRHIRDSYRICGMLLLFLSRLWLHILPCR 325
O.latipes      ------------------------------------------------------------
O.niloticus    ------------------------------------------------------------


T.rubripes     ------------------------------------- SRLCNAHPTLRSIDMFHFRGPS 258
T.nigroviridis CEADVSLSCSLPRVHVRACHGVLTPKLKHCSLSLCFGCSRLCNAHPTLRSIDMFHFRGPS 385
Oryzias        ------------------------------------- SRLCKAHPTLRTIDMFHFRGPS 259
O.niloticus    ------------------------------------- SRLCNAHPTLRSIDMFHFRGPS 259
                                                     ****:******:**********
T.rubripes     HIGDRLVLKSIVNNSFKHSMEVGVCAEAYQGREPLRHINSAFMTFEVLDSNWKPRTLPRI 318
T.nigroviridis HIGDRLVLKSIVNNSFKHSMEVGVCAEAYQGREPLRHINSAFMTFEVLDSNWKPRTLPRI 445
O.latipes      HIGDRLVLKAIVNNSFKHSMEVGVCAEAYQGREPLRHINSAFMTFEVLDNERKPCTLPRI 319
O.niloticus    HIGDRLVLKAIVNNAFKHSMEVGVCAEAYQGGEPLRHINSAFMTFEVLDSDRKPCTLPRI 319
               *********:****:***************** *****************.: ** *****
T.rubripes     RPEPVDGKRRYQEAIARKKIRLDRKYIISCKQTEVPVSVPWDPSNQMYLSYNNVSALKLM 378
T.nigroviridis RPEPVDGKRRYQEAIARKKIRLDRKYIISCKQTEVPVS VPWDPSNQMYLSYNNVSALKLM 505
O.latipes      RPEPVDGKRRYVEAIARKKIRLDRKYIISCKQPQVSLSVPWDPSNQMYLSYNNVSALKLM 379
O.niloticus    RPEPVDGKRRFQEAIARKKIRLDRKYIISCKQTEVPLSVPWDPSNQMYLSYNNVSALKLM 379
               **********: ********************.:*.:*************** *********
T.rubripes     DARTNWVLTSEKNNVRLYTLEENHMLRFKVEMHVGVAAEQTFQLLSDLRRRKEWDQHYKE 438
T.nigroviridis DARTNWVLTSEKNNVRLYTLEENHMLRFKVETHVSIAAEQTFQLLSDLRRRKEWDHHYKE 565
O.latipes      DTRNNWVLTSEKNKVRLYTLEENHMLCFKVEMYVSVPAEQSFHLLSDLTRRKEWDRHYEQ 439
O.niloticus    DNRNNWVLASEKNKVRLYTLEENHMLCFKVEMNVRVSAEQTFHLLSDLRRRKEWDRHYEE 439
               * *.****:****:************* ****   * :.***:*:***** ******:**::
T.rubripes     CEAIMQVNEEDTLYRVTTPSVSKVGKDNDFILLASRRKPCDSRDPYLIALRSVSLPTHPP 498
T.nigroviridis CEAIMQVNEEDTLYRVTTPSVSKAGKDNDFILLASRRKPCDARDPYLIALRSVTLPTHPP 625
O.latipes      CEVIIQADEEDTLYRVVTPSVSKGGKGKDFILLASRRKPCDSRDPYLIALRSVTLPTHPP 499
O.niloticus    CEVINQADEDDTIYRVATPSVTKGGKGKDFILLASRRKPCDSGDPYLIALRSVTLPTHPP 499
               **.* *.:*:**:***.****:* **.:*************: **********:******
T.rubripes     TDAYTRGEVLCAGFTIREESGSLTKITYYNQATPGVLPYIATDIVGLSSSFYSSFSACSH 558
T.nigroviridis SDGYTRGEVLCAGFTIREESSSLTKITYYNQATPGVLPYISTDIVGLSSTFYSAFSACSL 685
O.latipes      TEDYTRGEVQCAGFTIREEESNSVTKLTYYNQATPGVLPYISTDIAGLSSGFYCTFSACSR 559
O.niloticus    TEEYTRGEVLCAGFTIWEESSSVTKITYYNQATPGVLPYISTDIAGLSSSFYSAFSACSQ 559
               ::.******.****** ***.*:**::****************:***.**** **.:*****
T.rubripes     FLEANKDVLTATFL----------- 572
T.nigroviridis FLEANKDVLTASFL----------- 699
O.latipes      FLETSEDTAVVDSAASKEEKSQQKE 584
O.niloticus    FLLANKDSLAALPPSAL-------- 576
               ** ::.:*  ..
```

Figure 3.15 Multiple alignments of amino acid sequences of *Acylt-CoA thioesterase 11* from various species. Positions of degenerated primers are highlighted and the START domain found in the deduced protein is underlined.
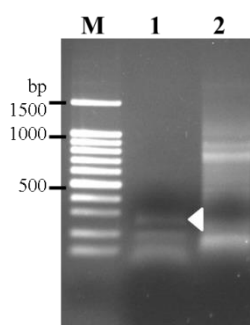
Figure 3.16 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *Acyl-CoA thioesterase 11* gene fragment against genomic DNA of *L. calcarifer*. Lane M = a 100 bp DNA ladder. Lane 1 = the amplification product against the cDNA template. Lane 2 = the amplification products against the genomic DNA template. An arrowhead indicates the positive amplification product that was cloned and sequenced.

ACAGAACGCAGGGCGATCAAGTATATAATTGTGGGTGACTTGATGAGTGAAAGTTTATAATCTTGAATCACATAC
ATTTCTGTATGCTTTTAGCGTGTAACAAGTGTGGACATAACTGCTGGCTTTCGTTGAAATGAACTACAACAGGTC
GTGGT

Figure 3.17 Nucleotide sequence of the amplified products of approximately 250 bp generated from degenerate primers for *Acyl-CoA thioesterase 11*. The primer position is underlined.

3.2.6 *Cell division cycle 2-like protein kinase 5 (cyclin-dependent kinase 13)*

Protein sequence alignments revealed low sequence similarity outside the functional domain but high similarity between sequences was found at the domain region of from different fish species (Figure 3.18). Degenerate primers for amplification of *Cell division cycle 2-like protein kinase 5* (hereafter called *cyclin-depaendent kinase 13, Cdk13*) gene segment were designed. PCR was carried out against the cDNA and genomic DNA template. No amplification product was observed from the cDNA template but a discrete band of 600 bp along with other faint bands was obtained when amplified against the genomic DNA template (Figure 3.19). A 600 bp fragment was excised from the gel and cloned. Nucleotide sequence obtained was 527 bp (Figure 3.20A). Blast analysis indicated that it showed the closest similarity with *cyclin-dependent kinase 13* of *Astyanax mexicanus* (*E*-value = 3e-39; Figure 3.20B).

```
T.rubripes       MPNSDVL-REGRG-RSPSAQRNANRQDRRSKPGSG-AAPRDRQREKRRSSVSRRRKRRRA 57
T.nigroviridis   ----------------------------------------------------------
D.labrax         MPNSEVL-REGRG-RSPSAQRNANRQDRRSKPGSG-AAQRDRHREKRRSSVSRRKKRRQA 57
O.niloticus      MPNSEVL-REGRG-RSPSAQRNASRQDRRSKPGSG-AAQRDRHREKRRSSASRRKKRRQP 57
O.latipes        MPNSEVL-RDGRGGRSPSELQNANRQGRRSKAASGTTAPRDRRREKGRSSASRRKKRRQQ 59
D.rerio          MPNPEIVGREGRG-RSPPPQR-SQREARRSKSRRE--RHRDG-KGRSASSSSRRKRHKRG 55


T.rubripes       KERSVWPSGAAGDHDNNRKSTFENEAELRTLVEYDDVSSQSERFSGSPSPRLDPHLDRLS 117
T.nigroviridis   ----------------------------------------------------------
D.labrax         RDKDLWPAGTTGDPDNDRKSTFEKEAELRTLVEYDDVSSQSERFSGSPSPKLDPHADRLS 117
O.niloticus      KERGLWHTGTIEDPDNDRRSAFEKDVELRTLVEYDDVSSQSERFSGSPSPKLDPHAESLT 117
O.latipes        KDRHLWHAVTTDGPD--KRSDFDKELELRTLVEYDDVSSQSERFSGSPSPKIDPHADNLP 117
D.rerio          REKQLQ------QPQPWQQQDALYDGDAGTLVEYDDVSSQSERFSGSPS----PRTDALS 105


T.rubripes       VDQLSDVDLGEYNGPSSPERSHPRGREEACINKKEPARGHSERSKPGKELGKKKERPSRE 177
T.nigroviridis   -----------------ERSHPRDRGEACINKKEPTRGHSERSKQGKELGKKKERPSRE 42
Dicentrarchus    VDRLSDVDYDD-NGPASPDRSHPRDREAVCIGKEEQTRGPSERSKQEKELARKKERQSRE 176
O.niloticus      ADRLSDVDFVDYNGPASPDRSHPREREAVSLSKDEQTRGPSERSRQEKELSKKKDRHSRE 177
O.latipes        ADPLSSVNFEY-NGPASLARGQLRDREAVYLSK--EFRGPSERSKQDKDLSKKKDHQSRE 174
D.rerio          VDRLSEAELQDCGYEAPAGREHNSSSR-------RRDGPAERIRSEK--DAKRNRSGRE 155
                                  * :                  * :** :  *    *::: .**
```

```
T.rubripes       RDSSRPRSRSSLSAPKNHRETSRSNDGNGKRPSAQ-SSQPAEKRDSKRHRSKTRSEKEPP 236
T.nigroviridis   TDPSKSKSRSSLSASKNHRAATRSSDGNGKRTSSQ-SSQPAEKRESKRHRSKTRLDKEPP 101
D.labrax         RDSSKVRSGGSLSGSKNHRDTTRSNDRNGKRTSTSQPSQSADKRDSKRHRSKTRSDKEPP 236
O.niloticus      RDSSKARSGGSLSGSKNHRDTTRSSDRNGKRTSTSQSSQSADKRDSKTHRSKTRSDKEPP 237
O.latipes        RDSLKVRTGGSLSGSK---TATRSGDKNGKRTSSS--SQSADKKNAKRHRSKTRTDKETP 229
D.rerio          QDAAFKSRSG-----------VRNHDSNGRKPSSA---SQADKKESRRHKSKPKPEKDAP 201
                  *.         .              *. * **::.*:    . *:*:::: *:**.: :*:.*

T.rubripes       SAYRDAPQSYRDDREDLRAYRRS-PGFKG----ESPYGTSYSYSYQSPAGYNQLASRRSP 291
T.nigroviridis   SAYRDAPQSYRDDREDLRAYRRS-PGFKA----ESPYGTSYSYTYQSPAGYSQLISRRSP 156
D.labrax         SAYR-------DDREDLRPYRRS-PGFKA----ESPYGTSYSYSYQFPGGYNQLISRRSP 284
O.niloticus      SAYRDAPQSYRDDREDLRAYRRS-PGFKS----ESPYGTSYSYNYQSPGGYNQLISRRSP 292
O.latipes        SAYRDAPQSYRNDREDPRAYRKS-PGFKT----DSPYGTSYSLNYQSPGNYAQLASRRSP 284
D.rerio          SAYREPPQAYRDDREELRAYRRSSPSFKATADNASPYGTSY-YGYQSPPANYQLIPWRSP 260
                  ****        :***: *.**:* *.**     *******   ** *    ** . ***

T.rubripes       TYGSKRLSPSTTYYSRDLDVYGAYGAPKSPGSYSSNKRKRSPTSPANWRRSPSYGRHSPY 351
T.nigroviridis   TYGSKRLSPSSAYYSRDLDVYGAYAAPKSPGSYSSNKRKRSPGSPANWRRSPSYGRHSPY 216
D.labrax         TYGSKRLSPSSTYYNRDGDSYGAYGMPKSPSSYSSNKRKRSPASPANWRRSPSFGRHSPY 344
O.niloticus      TYGSKRLSPSSTYYYSRDVDMYGSYGAPKSPSSYSSNKRKRSPVSPVNWRRSPSYGRHSPY 352
O.latipes        TYGSKRHSPSATYFNRD-DVYGGYGMPKSPTSYSSNKRKRSPASPANWRRSPSYGRHSPF 343
D.rerio          ----KKQSPTSTYY-RDPEMYAAYNALN-----SSGKRKRSPASPY-WRRSPSYGRHSPY 309
                  *: **::*:*: ** : *..*    :       **.****** **   ******:*****:

T.rubripes       EQGDFGSSPYGNRRRSRSPYRKSLSPSPDVRRTVRSRSRSPYSSSRHSRSRSRHRHSRSR 411
T.nigroviridis   EQADFGSSPYGNRRRSRSPYRKSLSPSPDVRRSARSRSRSPYTSSRHSRSRSRHRHSRSR 276
D.labrax         EQGEFGSSPYGNRRRSRSPYRKSLSPSPDVRRSARSRSRSPYSSSRHSRSRSRHRHSRSR 404
O.niloticus      EQGEFGSSPYGNRRRSRSPYRKSLSPSPDVRRSARSRSRSPYSTSRHSRSRSRHRHSRSR 412
O.latipes        EQGEFVSSPYGKRRKSRSPYRKSLSPSPDVRRPARSRSRSPFPSSRHSRSHSRHRHSRSR 403
D.rerio          EQGEFAGSPYGQRRRSRS-------PTPDTRRPVKSGSRSPYPSARHSRSRSRHRVSRSR 362
                  **.:* .****:**:***       *:**.**..:* ****:.::*****:**** ****

T.rubripes       SRPSSLSPSTLTFKSSLAAELSKQKKAKAAEAAAKAKTSSNTSTPTKGSSS---APQPSP 468
T.nigroviridis   SRPSSLSPSTLTFKSSLAAELSKQKRAKAAEAAAKAKTPSNASTPTKGSSS---APQPSP 333
D.labrax         SRPSSLSPSTLTFKTSLAAELSKQKKVKAAEAAAKAKNSSNTSTPTKGSSS---APQPSP 461
O.niloticus      SRPSSLSPSTLTFKTSLAAELSKQKKAKAAEAAAKAKNSSNTSTPTKGSSS---AHQPSP 469
O.latipes        SRPSSLSPSTLTFKSSLAAELSKQKKVKAAEAAAKAKNSSNTSTPTKMPSS---AHQPSP 460
D.rerio          SRPSSLSPSSLTLKSSLAAELSKQKKAKAAEAARAKNSSSNASTPTKGSSSSSNAPQPSP 422
                  *********:**:*:***********:.******   :..**:***** .**    * ****

T.rubripes       KNNSLR-KGRPPSPP-PERGPKTPTSSQPQSPSDRTSKKTSDPQSSKDREGKVKEDAVN- 525
T.nigroviridis   KVNHLARKGRPPSPP-PEKGPKTPTASEPQSPAEKTARKSTEHGS-KDRDGKGKEEPVT- 390
D.labrax         KSNHSSRKGRPPSPPQPEKGPRTPTSSQPQSPSDKS-KRTTDHQTSRDRDGKGKDDSVHR 520
O.niloticus      KSNHNSRKGRPPSPPQPEKGPRTPTSTQPQSPSERSSKKTTEHQTSRDRDVKGKDDSIHR 529
O.latipes        KSNHTAKKGRPPSPPAPEKGPRTPTSNQPQSPADKL-KKTAEHQSSS----KGKEDPLHR 515
D.rerio          VTNHAAKKTRPPSPPPPEKGPRTPVCSQPQSPVERPAKRSADVPLPG-RDAKLKEEKKK- 480
                   *     * ****** **:**:**...:****  ::  :::::          * *::

T.rubripes       --KKPSAAGQTKDKERPAGPQNSVMS-LPLPPTLLEHVDKGDSLKDTSLSGKK-KPERKP 581
T.nigroviridis   --KKTSAAGQNKDKERAAGPQSSAAP-LPLPPAAPEHTEKPESLKDGSLSGKK-KVEKKP 446
D.labrax         DKRKAPVSGTSKEKERATGPLISTLSSLPLPQTVLEHMDKGESLKDSSLSGKK-KSERKA 579
O.niloticus      DKRKAPASGQGKEKERTTGQAMSTLPLLPLPQTVIEHTDKGESLKDSSLSGKK-KSEKKA 588
O.latipes        DKKKAPASGQIKDKDRAAGPVISTLPQLPLPQIVAENADKGDNLKDGSLPGKK-KLEKKT 574
D.rerio          --------QAVKEKEKTVAPVVATHTPAANNMEHLEANDSVSSLKESSAGKKSSKPERKV 532
                          *:*::... :. . .     * :. ..**: *   *. * *:*
```

```
T.rubripes      RQLLSDLPLPPDFSAST-SSPHSPLEDK-RTQAARRRPKICGPRYGEFKETEIDWGKRCV 639
T.nigroviridis  RQLLADLPLPPDFSSST-SSPHSPQEDK-KSQAARKRPRICGPRYGEFKETEIDWGKRCV 504
D.labrax        RQLLSDLPLPPELTGGT-SSPHSPPEDK-KSQTLRRRPKICGPRYGEIKETEIDWGKRCV 637
O.niloticus     RQLLTDLPLPPELLGST-ASTQSPSDDK-KSQTIRRRPKICGPRYGEIKEIAIDWGKRCV 646
O.latipes       RQLLTDLPLPPELPCPS-VSPSSPPDKLEKSQTFRRRPKICGPRFGEIKEIEIDWGKRCV 633
D.rerio         RALLSDLPLPPELPGLTPASPLSPPDEK-KTPAPRRRPKICGPRFGEIKETEIDWGKRCV 591
                * **:******::   : *. ** :. :: : *:**:*****:**:**  *******

T.rubripes      DKFEIIGITGEGTYGQVYKAKDKDTGEMVALKKVRLDNEKEGFPITAIREIKILRQLNHK 699
T.nigroviridis  DKFEILGITGEGTYGQVYKAKDKDTGEMVALKKVRLDNEKEGFPITAIREIKILRQLNHK 564
D.labrax        DKFEIIGITGEGTYGQVYKAKDKDTAEMVALKKVRLDNEKEGFPITAIREIKILRQLNHK 697
O.niloticus     DKFEIIGITGEGTYGQVYKAKDKDTAEMVALKKVRLDNEKEGFPITAIREIKILRQLNHK 706
O.latipes       DKFEIIGITGEGTYGQVYKAKDKDTGEMVALKKVRLDNEKEGFPITAIREIKILRQLNHK 693
D.rerio         DKFEIIGITGEGTYGQVYKAKDKDTAELVALKKVRLDNEKEGFPITAIREIKILRQLNHK 651
                *****:*******************.*:******************************

T.rubripes      SIINMKEIVTDKEDALDFKNDKGAFYLVFEYMDHDLMGLLESGLVHFNENHIRSFMRQLL 759
T.nigroviridis  SIINMKEIVTDKEDALDFKNDKGAFYLVFEYMDHDLMGLLESGLVHFNENHIRSFMRQLL 624
D.labrax        SIINMKEIVTDKEDALDFKNDKGAFYLVFEYMDHDLMGLLESGLVHFNESHIKSFMRQLL 757
O.niloticus     SIINMKEIVTDKEDALDFKNDKGAFYLVFEYMDHDLMGLLESGLVHFNESHIKSFMRQLL 766
O.latipes       SIINMKEIVTDKEDALDFRNDKGAFYLVFEYMDHDLMGLLESGLVHFNESHIKSFMRQLL 753
D.rerio         SIINMKEIVTDKEDALDFKNDKGAFYLVFEYMDHDLMGLLESGLVHFNESHIKSFMRQLL 711
                ******************:*******************************.*.**:*******

T.rubripes      EGLDYCHKKNFLHRDIKCSNILLNNRGQIKLADFGLARLYNSEESRPYTNKVITLWYRPP 819
T.nigroviridis  EGLDYCHKKNFLHRDIKCSNILLNNRGQIKLADFGLARLYNSEESRPYTNKVITLWYRPP 684
D.labrax        EGLDYCHKKNFLHRDIKCSNILLNNKGQIKLADFGLARLYNSEESRPYTNKVITLWYRPP 817
O.niloticus     EGLDYCHKKNFLHRDIKCSNILLNNRGQIKLADFGLARLYNSEESRPYTNKVITLWYRPP 826
O.latipes       EGLDYCHKKNFLHRDIKCSNILLNNRGQIKLADFGLARLYNSEESRPYTNKVITLWYRPP 813
D.rerio         EGLDYCHKKNFLHRDIKCSNILLNNKGQIKLADFGLARLYNSEESRPYTNKVITLWYRPP 771
                *************************:*******************************

T.rubripes      ELLLGEERYTPAIDVWSCGCILGELFTKRPIFQANQELAQLELISRICGSPCPAVWPDVI 879
T.nigroviridis  ELLLGEERYTPAIDVWSCGCILGELFTKRPIFQANQELAQLELISRICGSPCPAVWPDVI 744
D.labrax        ELLLGEERYTPAIDVWSCGCILGELFTKKPIFQANQELAQLELISRICGSPCPAVWPDVI 877
O.niloticus     ELLLGEERYTPAIDVWSCGCILGELFTKKPIFQANQELAQLELISRICGSPCPAVWPDVI 886
O.latipes       ELLLGEERYTPAIDVWSCGCILGELFTKKPIFQANQELAQLELISRICGSPCPAVWPDVI 873
D.rerio         ELLLGEERYTPAIDVWSCGCILGELFTKKPIFQANQELAQLELISRICGSPCPAVWPDVI 831
                **************************:*******************************

T.rubripes      KLPFFHTMKPKKQYRRRLREEFAFIPPSALDLFDHMLNLDPSKRCAAEQALNSEFLRDVN 939
T.nigroviridis  KLPFFHTMKPKKQYRRRLREEFAFIPPSALDLFDHMLNLDPSRRCAAEQALHSEFLRDVN 804
D.labrax        KLPFFNTMKPKKQYRRRLREEFAFIPPSALDLFDHMLNLDPSKRCTAEQALGSEFLKDVD 937
O.niloticus     KLPFFNTMKPKKQYRRRLREEFAFIPPAALDLFDHMLNLDPSKRCTAEQALGSEFLKDVD 946
O.latipes       KLPFFNTMKPKKQYRRRLREEFAFIPPAALDLFDHMLNLDPGRRCTAEQALSSEFLKDVD 933
D.rerio         KLPYFNTMKPKKQYRRRLREEFAFIPLMALDLFDHMLALDPSKRCTAEQALNSDFLRDVD 891
                ***:*:******************** ********* *** .:**:***** *:*:**:

T.rubripes      PDKMPPPDLPLWQDCHELWSKKRRRQKQIPEELAAPKAPRKELGLDDSRSNTPQGFPPAT 999
T.nigroviridis  PDKMPPPDLPLWQDCHELWSKKRRRQKQVPEELAAPKAPRKELGPDDSRSNTPQGFPPSA 864
D.labrax        PDKMPPPDLPLWQDCHELWSKKRRRQKQIPEELAAPKAPRKELGLDDSRSNTPQGFPAPG 997
O.niloticus     PDKMPPPDLPLWQDCHELWSKKRRRQKQMPEELAAPKAPRKELGLDDSRSNTPQGLSAPG 1006
O.latipes       PDKMPPPDLPLWQDCHELWSKKRRRQKQMPEELVAPKAPRKELGLDDSRSNTPQGFAAPG 993
D.rerio         PAKMPPPDLPLWQDCHELWSKKRRRQKQMPEELTAPKAPRKELGLDDSRSNTPQGFGTAA 951
                * *************************:****.********** **********: ..
```

```
T.rubripes      GAKAPN--AAAPALLDPKASSSQLTQEQLAVLLNFLG-QPSSAVSTAQYVQSISAKVTQE 1056
T.nigroviridis  GAKAPN--AVASALLDPKASNSQLTQEQLAVLLNFLG-QPSAAVSASQYVQSASAKASQE 921
D.labrax        GIKAQN--AAASALLDAKGPNSQLTQEQLAVLLNFLG-QPKSAVNTAQFVQSMSTKVNQE 1054
O.niloticus     AIKAQN--AAASALLDPKGANSQLTQEQLAVLLNFLG-QPKSAVSTAQLVQSMSSKVNQE 1063
O.latipes       AMKTQN--AAASVLLESKGPNSQFTQEQLAVLLNLLG-QSKTAPS-------------- 1035
D.rerio         GGHKPQGSANAAGLLDPKSANSQLTQDQLAVLLNLLQSKSSAAGGSAQFMQTVSSKMNPE 1011
                . :  :   * *. **:.*...**:**:*******:*  :..:* .

T.rubripes      PPPQLK-NPPAADPADPLPPPPPP----PPQSTPSKPPQPAAPSGVPRTPPMPKPPSPPA 1111
T.nigroviridis  TTQQLAKNLPAADPAEPLPPPPPPGGRRPRQATPNKPPQPAAPLGVPRTPPMPKPPSPPA 981
D.labrax        TLQQLSKALAPSDPAEPPPQPTPT----------KPPQP------------------- 1083
O.niloticus     TLQQLSKALAPTDPAEPPLQPATA----------KPPPPTAPAGVPRTPPMPKPPSPPM 1112
O.latipes       --QCSPSSLRPSADQENPK--------------------------------------- 1052
D.rerio         TLQQLSKALPCGLPESERPPEPPALP-----KTSKALPAAASGPGAPRTPPMPKPPSPP- 1065
                                          .

T.rubripes      SAPPGIPEGEAAVATQTAMTMLLAQLLQAQQGQRSDRPDGVDAGDGANAAGAGGG-QPPP 1170
T.nigroviridis  CAPPGLAEGEAAVATQTAVTMLLAQLLQAQQG-------------------------- 1013
D.labrax        ----GIPEGEAAAATQTAMTMLLAQLLQAQQGQRQESSDGGEGAESNNPAGGAPGGQLLP 1139
O.niloticus     SAPSNIPEGEAAAATQTAMTMLLAQLLQAQHTQRQEPVDGGEGVESTNSAGGAAP--LLP 1170
O.latipes       ---------TSIANVRVNPGGWMAQLAACKTG------------------------S 1076
D.rerio         -GPQQADGESSASATQTAVTMLLAQLLNVQQG------APGDGTGFDGADGGVPNTAPAP 1118
                :   .:.      :***   :

T.rubripes      PEAKQPPEPSPISPGNRIGSAELLYSALSRLSEVGTILPPDKRPPEPPEPPPHADLDYRQ 1230
T.nigroviridis  -----------------------------------------------------------
D.labrax        PEVKQPPEPSPVSPGSPSSVYLFRETAELSEVGGVSILPPDQRPPEPPEPPPHADLDYRQ 1199
O.niloticus     PEVKQPPEPSPVSP--------------VSDVSGVSILPPDQRPPEPPEPPPHADLDYRQ 1216
O.latipes       WDTRVPNELHPV----------------------------------------------- 1088
D.rerio         PEIRQPPEPSPVSPDAEGGG-----VPSSGTLDSLSILPPDQRPPEPPEPPPCADLDYRH 1173

T.rubripes      PPPEPKTGHAPPMPSAGGGDGGRPPEPDYPPLPS--AEGYGGDYNHPPPPPFTPAGFSDG 1288
T.nigroviridis  ----------------GGDGGRPPEPDYPPLP---SEGYGGDYIYPPPPPFTPAGFSDS 1053
D.labrax        PPPEPKTGHAPPIASAGGGDGGRPPEPDYPPLPTAEGPGYGGDYSHPPPPPFTPAGFGEG 1259
O.niloticus     PPPEPKTGHAPPIASAGAGDGGRPPEPDYPPLPTAEGSGYGGEYSHPPPPPFTPAGFGES 1276
O.latipes       -----------------------------------------------------------
D.rerio         AP-----------ESRPAEPPRPPEPDYPPDGS----GYGGDFGRPPPP-FPHAGFSDS 1216

T.rubripes      YMGHMMGGGLPPHALNEVFSGPGHTAAAAAATSAAGVLAPPQPDLFPQVAGASGPS-MVF 1347
T.nigroviridis  YLGHMMGGGLPPHALREVFSGPGQTTAGAGA-----ILAPPQPDLFPQGGGVSGPSRMVF 1108
D.labrax        YMGHMLGGGLPPHALREVFSGPGQAAGSSAAAG---VLPPAHPDPFPPGAGATGPSSMVF 1316
O.niloticus     YMGHMLGGGLPPHTLREVFSGPGQAASSSTAPG---ALAPAHPDPFPPSAGASGPSSMVF 1333
O.latipes       -----------------------------------------------------------
D.rerio         YLGRMMGAGLPPHAP--------------------------LGPEAFARGDHGMVF 1246

T.rubripes      SGDKDHRFKYNHS-LPVEGQPNPSAIHMYNHGLPQKEGGPPP--IPAPGQPWGSPSQVGA 1404
T.nigroviridis  SGDKDHRFKYNHS-LPVEARPSPGAIHMYNHALPQKDGGPPP--IPAPGPPWGSPSQVGA 1165
D.labrax        TGDKDHRFKYNHSPLPVEGQPNPNAIHLYNHAMARKDGVPPPPPIPAPGQPWGSPSQVGA 1376
O.niloticus     TGDKDHRFEYNHSPLPVEGQSNPSAIHLYNHAMARKDGVPPPPPIPAPGQPWGSPSQVGA 1393
O.latipes       -----------------------------------------------------------
D.rerio         SGDQDHRFEYNHG------------------------PPP---PPPGQPWTSPSQS-- 1275
```

```
T.rubripes        PPLPLGFVPHVNSAATIRGRGLPF 1428
T.nigroviridis    PPLPLGFVPHVNSAATIRGRGLPF 1189
D.labrax          PPLPLGFVPHVNSTATIRGRGLPF 1400
O.niloticus       PPLPLGFVPHVNSTATIRGRGLPF 1417
O.latipes         -----------------------
D.rerio           ----------LGYAAALRGRGLPF 1289
```

Figure 3.18 Multiple alignments of amino acid sequences of *Cyclin-dependent kinase 13 (Cdk13)* from various species. Positions of degenerated primers are highlighted and the S_TKc domain found in the deduced Cdk13 protein is underlined.
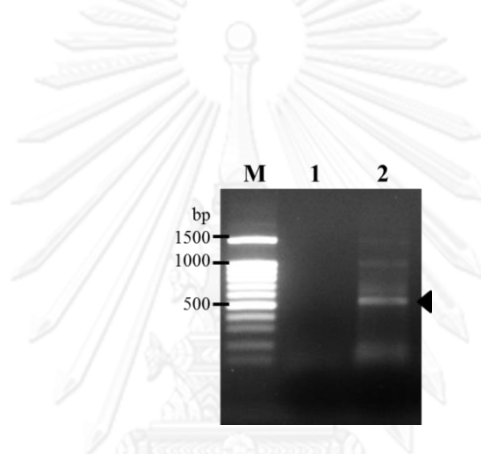
Figure 3.19 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *Cdk13* gene fragment against genomic DNA of *L. calcarifer*. Lane M = 100 bp DNA ladder. Lane 1 = the amplification products against cDNA template. Lane 2 = the amplification products against genomic DNA template. An arrowhead indicates the amplification product that was cloned and sequenced.

A.

<u>AATGATAAAGGTGCGTTTT</u>ATCTTGTATTCGAGTACATGGACCACGACCTGATGGGTTTACTCGAGTC
TGGCTTAGTTCATTTCAACGAAAGCCACATCAAGTCTTTCATGCGACAGCTGCTGGAGGGTCTTGATT
ACTGCCACAAGAAAAACTTTCTACACAGAGACATAAAGCGCTCTAATATTCTGCTCAATAACAAGTGA
GTCCTTGGGATCTCCACACTCAACCAGGACAAACACCTTTGTTGTATTTTATTACACTGTCAAACATG
TTTTCCAGCAAGATTTAATCAATGTTGTTTTTTCCCCTCCTAATCAGAGGTCAAATAAAGCTTGCAGA
CTTTGGTCTTGCTCGGCTGTATAACTCTGAAGAGAGGTGAGCCTCTTATATTCTTGCAAAATTTATCC
AACCTGCTAAGCTTTCTTGCTGTATCATCTTCCATATTTTATTTCCACCTATGTATGTGATTCAAGAT
TATAAACTGTATTCTCCTTTCCAGTCGACCGTATACCAATAAAGTGATCAC

B.

```
PREDICTED: cyclin-dependent kinase 13 [Astyanax mexicanus]
Sequence ID: ref|XP_007238721.1|Length: 1426 Number of Matches: 2


Range 1: 716 to 782
Score = 146 bits(369), Expect = 3e-39
Identities = 66/67(99%), Positives = 66/67(98%), Gaps = 0/67(0%)
Frame = +1


Query  1    NDKGAFYLVFEYMDHDLMGLLESGLVHFNESHIKSFMRQLLEGLDYCHKKNFLHRDIKRS  180
            NDKGAFYLVFEYMDHDLMGLLESGLVHFNESHIKSFMRQLLEGLDYCHKKNFLHRDIK S
Sbjct  716  NDKGAFYLVFEYMDHDLMGLLESGLVHFNESHIKSFMRQLLEGLDYCHKKNFLHRDIKCS  775

Query  181  NILLNNK  201
            NILLNNK
Sbjct  776  NILLNNK  782


Range 2: 782 to 800
Score = 40.8 bits(94), Expect = 3e-39
Identities = 18/19(95%), Positives = 19/19(100%), Gaps = 0/19(0%)
Frame = +3


Query  318  RGQIKLADFGLARLYNSEE  374
            +GQIKLADFGLARLYNSEE
Sbjct  782  KGQIKLADFGLARLYNSEE  800
```

Figure 3.20 (A) Nucleotide sequence of the amplified products using degenerate primers for *Cell division cycle 2-like protein kinase 5* (*cyclin-dependent kinase 13*). The primer position is underlined. (B) Blast analysis of the amplified product against previously deposited sequences in GenBank.

3.3 Isolation and characterization of the full-length cDNA of *L. calcarifer* of *Cdk13*

The primary 5′ and 3′ RACE-PCR generate smear amplification products (Figures 3.21A and 3.22A). Semi-nested PCR was carried out by amplification of the diluted primary RACE-PCR product with the same gene-specific primer and nested adapter primer. After electrophoresis, smear amplification products and several bands were obtained from semi-nested 5′- and 3′ RACE-PCR. A 2100 bp fragment from semi-nested 5' RACE-PCR (Figure 3.21B) and a 2000 bp fragment from nested 3' RACE-PCR (Figure 3.22B) were cloned and sequenced.
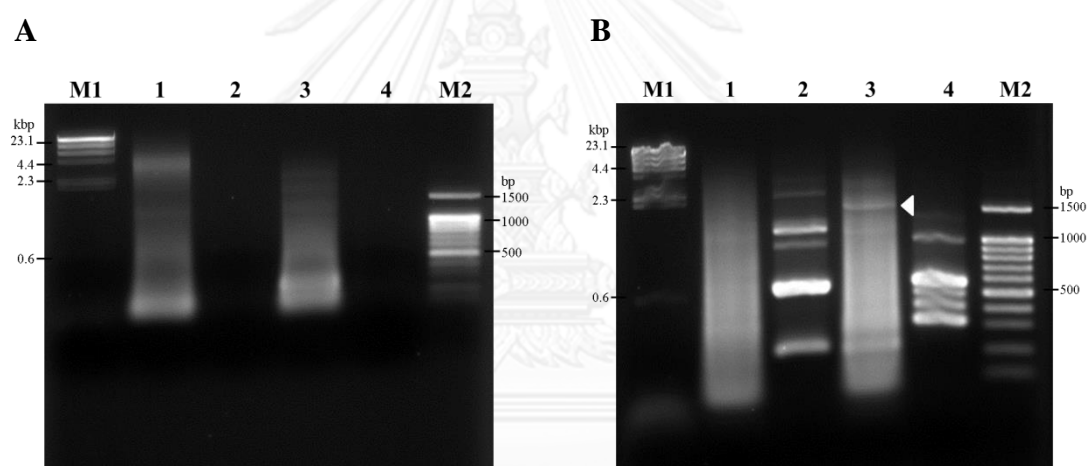
Figure 3.21 1.5% ethidium bromine-stained agarose gels showing the amplification product of 5' RACE-PCR of *Cdk13* of *L. calcarifer*. A primary 5' RACE-PCR against the hemocyte (lane 1, A) and the hepatic template (lane 3, A). The primer controls against respective templates were also included (lanes 2 and 4, A). A semi-nested 5' RACE-PCR was further carried out against the hemocyte (lane 1, B) and the hepatic template (lane 3, B). The primer controls against respective templates were also included (lanes 2 and 4, B). Lanes M1 = λDNA-*Hind* III. Lanes M2 = a 100 bp DNA ladder. An arrowhead indicated the amplified band that was cloned and sequenced.
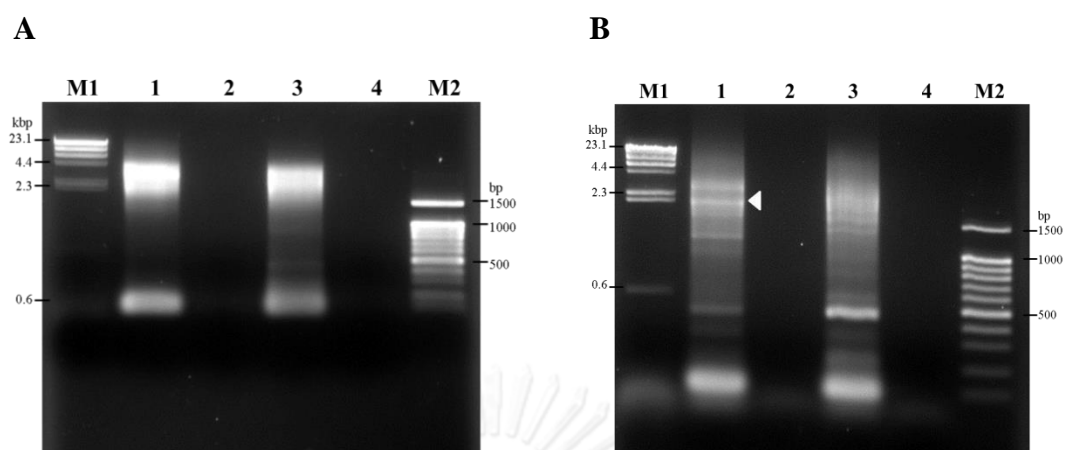
Figure 3.22 1.5% ethidium bromine-stained agarose gels showing the amplification product of 3' RACE-PCR of *Cdk13* of *L. calcarifer*. A primary 3' RACE-PCR against the hemocyte (lane 1, A) and the hepatic template (lane 3, A). The primer controls against respective templates were also included (lanes 2 and 4, A). A nested 3' RACE-PCR was further carried out against the hemocyte (lane 1, B) and the hepatic template (lane 3, B). The primer controls against respective templates were also included (lanes 2 and 4, B). Lanes M1 = λDNA-*Hind* III. Lanes M2 = a 100 bp DNA ladder. An arrowhead indicated the amplified band that was cloned and sequenced. An arrowhead indicates the amplification product that was cloned and sequenced.

Blast analysis indicated that their nucleotide sequences did not matched *Cdk13* but both 2100 and 2000 bp bands showed the closest similarity with *complement component c3* of *Epinephelus coioides* (*E*-value = 0.0; Figures 3.23 and 3.24).

A.

AAGCAGTGGTATCAACGCAGAGTTTGAGGTCAAAGAATATGTGTTGCCCAGTTTTGAGGTGAAGCTGACACCTAT
GAGTCCCTTCTTCTACGTGGACAGTCAAGACCTCACTGTCAACATCAAAGCTACGTATCTGTTTGGTGAAGAGGT
TGATGGGACAGCATATGTGGTATTTGGAGTTGTACATGAGCGTCAAAAGAGGAGCTTTCCAAGCTCACTTCAGAG
AGTGCCGATTGTGACAGGTGAAGGACAGGTCACACTGAAGAGAGAGCACATCGCACAGACCTTCCAACAAATCAA
CCAGCTGGTCGGGGAGCTCCATATTTGTAGCTGTCAGTGTGTTAACGGAGAGCGGTAGTGAGATGGTGGAGGCAGA
GTTGAGGGGTATCCAGATTGTCACATCACCCTATACCATCCACTTCAAGAAAACGCCCAAATATTTCAAACCAGG
GATGTCCTTCGATGTTTCGATTGAAGTTGTGAATCCAGATGAATCACCGGCACAAGGTATTCCAGTGGTGGTAGA
TCCAGGCATGGTGCGGGGTTTCACCGCAGCTAATGGCATGGCGAAGCTTACCATCAATACAGTGGCAGGAGAGCA
AAGGTTGACAATCACTGCAAAGACCAGTGATCCTCAAATTTCAGCTAACAGACAAGCATCAGCCACCATGGTAGC
TCTCCCATACCAAACTAAGAGTGACAACTACATCCACATAGGAGTGGATACAGCAGAGCTGCAATTAGGAGACAA
CCTGAAAATCAACCTCAACCTCAAGAGGCAGTCAAATGACAACACTGATATCACATACCTGATCCTGAGCAGGGG
CCAACTGGTCAAACATGGCCGATACAAGACAAGAGGCCAAGTACTGATATCTCTAATAATTCCCATCACCAAAGA
AATGCTGCCATCGTTCCGCATCATCGCCTACTACCATACAACTGGCAATGAAGTGGTATCAGACTCTGTTTGGGT
GGATGTCAAGGACTCTTGCATGGGCTCGCTGACGTTGGAATCATCGAGACCTGCTCCGTCTTATGAGCCTTCGCA
GGATGTTTGGTCTGAAGGTCACTGGAGATCCAGGGGCCACAGTGGGACTGGGTGCAGTTGACAAAGGCGTCTACG
TCCTGAATAACAAGCACCGCTCCCCCCAAAAAAAGTGTGGGACATTTTGGAGAAAATTGACACAGGTGGACCCCA
GTGGAAGGAAGAAAGAAATGGGGTGGTTCTCTCCACGCCGCGCCGGTGTTTTAATCCCCCACCTGTCTTTGGGAG
CCCCCCCGGCAAA

B.

```
complement component c3 [Epinephelus coioides]
Sequence ID: gb|ADU33222.1|Length: 1657Number of Matches: 1

Score = 590 bits(1521), Expect = 0.0
Identities = 304/385(79%), Positives = 336/385(87%), Gaps = 0/385(0%)
Frame = +1

Query  1     KQWYQRRVVEEYVLPSFEVKLTPMSPFFYVDSQDLTVSIKATYLFGEEVDGTAYVVFGVV    180
             + ++    V EYVLPSFEVKLTP SPFFYVDSQ+L V+IKATYLFGEEV+GTAYVVFGV+
Sbjct  223   QSYFAEFEVREYVLPSFEVKLTPDSPFFYVDSQELRVNIKATYLFGEEVEGTAYVVFGVM    282

Query  181   HERQKRSFPSSLQRVPIVTGEGQVTLKGEHITQTFQQINQLVGSSIFVAVSVLTESGSEM    360
              + QK+SFPSSLQRVP+  G G VTLK EHITQTF  I +LVG SIFVAVSVLTESGSEM
Sbjct  283   QDGQKKSFPSSLQRVPVGRGSGAVTLKREHITQTFPNILELVGKSIFVAVSVLTESGSEM    342

Query  361   VEAELRGIQIVTSPYTVHFKKTPKYFKPGMSFDVSIEVVNPDESPAQGIPVVVDPGMVRG    540
             VEAELR IQIVTSPYT+HF KTPKYFKPGMSFDV++EVVNPDE+PAQG+ VVVDPG V+G
Sbjct  343   VEAELRSIQIVTSPYTIHFTKTPKYFKPGMSFDVAVEVVNPDETPAQGVAVVVDPGNVQG    402

Query  541   FTAANGMAKLTINTVAGEQRLTITAKTSDPQISANRQASATMVALPYQTKSDNYIHIGVD    720
             FTAANGMA+LTINTVAG  RLTI A+T+DP+ISA RQA A+M A+PY TKS+NYIHIGVD
Sbjct  403   FTAANGMARLTINTVAGNARLTINARTNDPRISAERQARASMTAVPYATKSNNYIHIGVD    462


Query  721   TAELQLGDNLKINLNLKRQSNDNTDITYLILSRGQLVKHGRYKTRGQVLISLIIPITEEM    900
             TAELQLGDNLKINLNL RQ N N+D TYLILSRGQLVK GRYKTRGQVLISLI+PIT+EM
Sbjct  463   TAELQLGDNLKINLNLNRQENLNSDTTYLILSRGQLVKKGRYKTRGQVLISLIVPITKEM    522

Query  901   LPSFRIIAYYHTTGNEvvsdsvwvdvkdsCMGSLTLESSRPAPSYEPRRKFGLKVTGDPG    1080
             LPSFRI+AYYHT+GNEVVSDSVWVDV DSCMGSL LES R APSYEPRR FGLKVTGDPG
Sbjct  523   LPSFRIVAYYHTSGNEVVSDSVWVDVTDSCMGSLKLESLRAAPSYEPRRMFGLKVTGDPG    582

Query  1081  ATLGLVAVDKSV*LPEKQAPPPPKK    1155
             +T+GLVAVDK V +  KQ    KK
Sbjct  583   STVGLVAVDKGVFVLNKQHRLTQKK    607
```

Figure 3.23 (A) Nucleotide sequence of the amplification products generated from 5′RACE-PCR of *Cdk13*. The position of nested UPM primer is underlined. (B) Blast analysis of the amplified product against previously deposited sequences in GenBank.

A.

<u>AAGCAGTGGTATCAACGCAGAGT</u>TGTCGAAGAATATGTGTTGCCCAGTTTTGAGGTGAAGCTGACACCTATGAGT
CCCTTCTTCTACGTGGACAGTCAAGACCTCACTGTCAGCATCAAAGCTACGTATCTGTTTGGTGAAGAGGTTGAT
GGGACAGCATATGTGGTATTTGGAGTTGTACATGAGCGTCAAAAGAGGAGCTTTCCAAGCTCACTTCAGAGAGTG
CCGATTGTGACAGGTGAAGGACAGGTCACACTGAAGGGAGAGCACATCACACAGACCTTCCAACAAATCAACCAG
CTGGTCGGGAGCTCCATATTTGTAGCTGTCAGTGTGTTAACGGAGAGCGGTAGTGAGATGGTGGAGGCAGAGTTG
AGAGGTATCCAGATTGTCACATCACCCTATACCGTCCACTTCAAGAAAACGCCCAAATATTTCAAACCAGGGATG
TCCTTCGATGTTTCGATTGAAGTTGTGAATCCAGATGAATCACCGGCACAAGGTATTCCAGTGGTGGTAGATCCA
GGCATGGTGCGGGGTTTCACCGCAGCTAATGGCATGGCAAAGCTTACCATCAATACAGTGGCAGGAGAGCAAAGG
TTGACAATCACTGCAAAGACCAGTGATCCTCAAATTTCAGCTAACAGACAAGCATCAGCCACCATGGTAGCTCTC
CCATACCAAACTAAGAGTGACAACTACATCCACATAGGAGTGGATACAGCAGAGCTGCAATTAGGAGACAACCTG
AAAATCAACCTCAACCTCAAGAGGCAGTCAAATGACAACACTGATATCACATACCTGATCCTGAGCAGGGGCCAA
CTGGTCAAACATGGCCGATACAAGACAAGAGGCCAAGTACTGATATCTCTAATAATTCCCATCACCGAAGAAATG
CTGCCATCGTTCCGCATCATCGCCTACTACCATACAACTGGCAATGAAGTGGTATCAGACTCTGTTTGGGTGGAT
GTCAAGGACTCTTGCATGGGCTCGCTGACGTTGGAATCATCGAGACCTGCTCCGTCTTATGAGCCTCGCAGGAAG
TTTGGTCTGAAAGTCACTGGAGATCCAGGGGCCACATTGGGACTGGTGGCAGTTGACAAAAGCGTCTAACTCCCT
GAAAAACAAGCCCCGCCTCCCCCCAAAAAAAGGGTGGGGCATTTTTGGAGAAATTTAAACAAGCTGCACCCCACG
TGGAGGGAAGAAAGGATTTGGTGGGTTCTTCTATCCCGGGCGGGGGGGTTTAGTCCCCCCTCGTCTTTGGGATCCT
TCCCCCAAAAATAGAATTGTCACCCGCCCCCCGGGGAAAACAAGCCCCCCCTTACAAAAAGACCAACCACTTTTG
GGGGGCTTAAACAAAGATAAACACGACCCCTTGGGGGGGGGATAAGAGAAGACCCCTTTTCACTTTTGGAAAAAC
AGGGGTATCCCAAAGGCCCTTTGGGAATCTTCCTTTCCGGGAAAGACCGGCGGGAAAAAGGCCATTTCCCGCCAA
AACTACAAAATTTTTCCCCATAAACAATGGACCCGCCGTGCTGCCGCTTTTTTTTTTTTTTTTTTTTATCTCGG
TCATCAGTCTCACGGTATTCGGGTTTTTTGTTTTTTTTTTTTACACTTTCATAAAAACAAATAATAAAAGGACA
TTTAAGACAAAATAGATTTTTTTATTTTTTTATCTGAAAGTGCCCCCCCCCCACCCAAAAGCCCCCAAAAGGGGAG
AGGGGGGAAAAAGTTTTTTGGATCATTTTTTTTCCGGTAAAACCTTCTTTTTTTTTTTTTCGGGCTTTTCCCCCTGC
GGCCCCTGGGTGGTCAGGGGCCGTAACTTTGTGGCCCAATCCTTACGGGGTTAAAAGGGCCCGGAAATTGTGGTT
TGTAATGGGTATAAGGTAAAATTTAAAAAAAAAAAATGAAAAAGTA

B.

```
complement component c3 [Epinephelus coioides]
gb|ADU33222.1|Length: 1657 Number of Matches: 2

Score = 572 bits(1475), Expect = 0.0
Identities = 300/392(77%), Positives = 328/392(83%), Gaps = 0/392(0%)
Frame = +2

Query  17    AEFEVKEYVLPSFEVKLTPMSPFFYVDSQDLTVNIKATYLFGEEVDGTAYVVFGVVHERQ  196
             AEFEV+EYVLPSFEVKLTP SPFFYVDSQ+L VNIKATYLFGEEV+GTAYVVFGV+ + Q
Sbjct  227   AEFEVREYVLPSFEVKLTPDSPFFYVDSQELRVNIKATYLFGEEVEGTAYVVFGVMQDGQ  286

Query  197   KRSFPSSLQRVPIVTGEGQVTLKREHIAQTFQQINQLVGSSIFVAVSVLTESGSEMVEAE  376
             K+SFPSSLQRVP+  G G VTLKREHI QTF   I +LVG SIFVAVSVLTESGSEMVEAE
Sbjct  287   KKSFPSSLQRVPVGRGSGAVTLKREHITQTFPNILELVGKSIFVAVSVLTESGSEMVEAE  346

Query  377   LRGIQIVTSPYTIHFKKTPKYFKPGMSFDVSIEVVNPDESPAQGIPVVVDPGMVRGFTAA  556
             LR IQIVTSPYTIHF KTPKYFKPGMSFDV++EVVNPDE+PAQG+ VVVDPG V+GFTAA
Sbjct  347   LRSIQIVTSPYTIHFTKTPKYFKPGMSFDVAVEVVNPDETPAQGVAVVVDPGNVQGFTAA  406

Query  557   NGMAKLTINTVAGEQRLTITAKTSDPQISANRQASATMVALPYQTKSDNYIHIGVDTAEL  736
             NGMA+LTINTVAG  RLTI A+T+DP+ISA RQA A+M A+PY TKS+NYIHIGVDTAEL
Sbjct  407   NGMARLTINTVAGNARLTINARTNDPRISAERQARASMTAVPYATKSNNYIHIGVDTAEL  466

Query  737   QLGDNLKINLNLKRQSNDNTDITYLILSRGQLVKHGRYKTRGQVLISLIIPITKEMLPSF  916
             QLGDNLKINLNL RQ N N+D TYLILSRGQLVK GRYKTRGQVLISLI+PITKEMLPSF
Sbjct  467   QLGDNLKINLNLNRQENLNSDTTYLILSRGQLVKKGRYKTRGQVLISLIVPITKEMLPSF  526

Query  917   RIIAYYHTTGNEvvsdsvwvdvkdsCMGSLTLESSRPAPSYEPSQDVWSEGHWRSRGHSG  1096
             RI+AYYHT+GNEVVSDSVWVDV DSCMGSL LES R APSYEP +    +         G
Sbjct  527   RIVAYYHTSGNEVVSDSVWVDVTDSCMGSLKLESLRAAPSYEPRRMFGLKVTGDPGSTVG  586

Query  1097  TGCS*QRRLRPE*QAPLPPKKVWDILEKIDTG  1192
                     +      Q  L  KKVWDI+EK DTG
Sbjct  587   LVAVDKGVFVLNKQHRLTQKKVWDIVEKYDTG  618
```

Figure 3.24 (A) Nucleotide sequence of the amplification products generated from 3´RACE-PCR of *Cdk13*. The position of nested UPM primer is underlined. (B) Blast analysis of the amplified product against previously deposited sequences in GenBank.

3.4 Identification of SNP in growth-related genes by SSCP analysis

3.4.1 Genomic DNA extraction

Genomic DNA was extracted from frozen muscle of individual *L. calcarifer* by a phenol-chloroform-proteinase K method (Klinbunga et al., 2001). The quality of extracted genomic DNA was electrophoretically determined using a 0.8% agarose gel. High molecular weight DNA at the similar size as that of undigested λ-DNA (approximately 50 kb) along with degrade DNA was observed (Figure 3.25). The ratio of OD_{260}/OD_{280} of extracted genomic DNA was approximately 1.6-1.8 suggesting that the quality of extracted genomic DNA was acceptable for further applications.
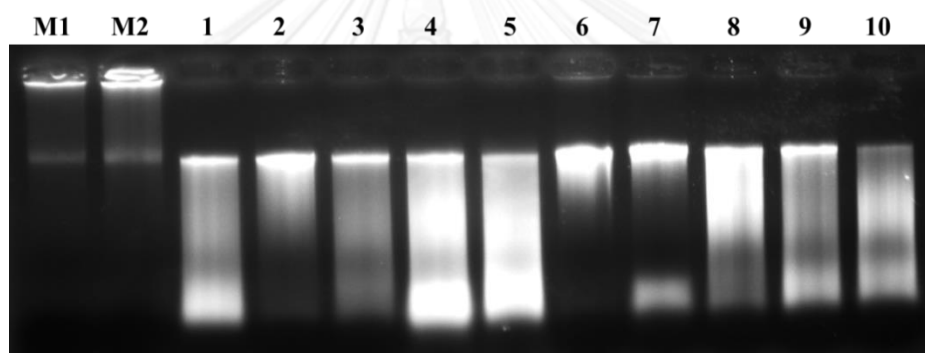
Figure 3.25 A 0.8% ethidium bromine-stained agarose gel showing the quality of genomic DNA extracted from muscle of *L. calcarifer*. Lanes M1 and M2 = 50 and 100 ng of undigested λ-DNA. Lanes 1-11 = genomic DNA extracted from muscle of ten individuals of *L. calcarifer* juveniles.

3.4.2 Amplification of growth-related gene segments by PCR

Four primer pairs were designed from previously deposited sequences in GenBank including *Insulin-like growth factor I* (*IGF-I*), *Insulin-like growth factor II* (*IGF-II*, 2 sets of primers) and *Myostatin* (*MSTN*). In addition, a pair of primers was designed from a gene sequence in GenBank where its protein homologue (*Activin type IIB receptor, ActRIIB*) was found from proteomic analysis of proteins in livers of 4-month-old *L. calcarifer*.

3.4.2.1 *Insulin-like growth factor I (IGF-I)*

Two amplification product of *IGF-I* gene segment against genomic DNA of 4-month-old *L. calcarifer* was obtained even though primers were designed from that of *L. calcarifer* (Acc. No: EU136176). One was the expected product size of 163 bp and the other was a larger product size of approximately 400 bp in size (Figure 3.26 and Table 3.2). Therefore, polymorphisms of these fragments were not further examined by SSCP analysis.
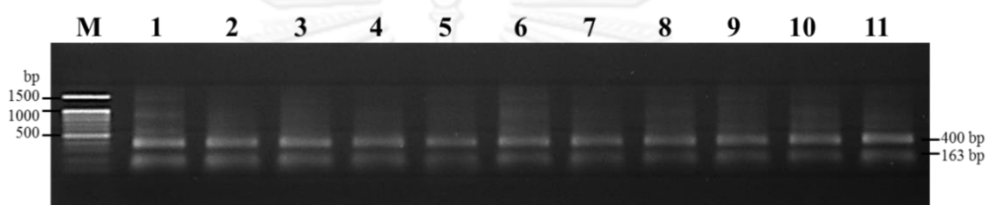
Figure 3.26 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *IGF-I* gene fragment against genomic DNA of individual *L. calcarifer* (4-month-old). Lane M = 100 bp DNA ladder. Lanes 1-11 =.the amplification products against genomic DNA of different individuals of *L. calcarifer*.

3.4.2.2 *Insulin-like growth factor II (IGF-II;* exon 4 portion)

A discrete 225 bp fragment was obtained from amplification of *IGF-II* against genomic DNA of 4-month-old *L. calcarifer* (Figure 3.27 and Table 3.2). The amplified *IGF-II* exon 4 gene segment was cloned and sequenced. Size of the expected and the amplified products from genomic DNA were identical suggesting that the amplified gene segment did not contain a large indel (insertion or deletion).

3.4.2.3 *Insulin-like growth factor II (IGF-II;* intron 1 portion)

A 343 bp fragment was obtained from amplification of *IGF-II* against genomic DNA of 4-month-old *L. calcarifer* (Figure 3.28 and Table 3.2). Nucleotide sequences of that in GenBank and the amplified products from genomic DNA were identical (Figure 3.29).

Figure 3.27 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *IGF-II* (exon 4 portion) gene fragment against genomic DNA of individual *L. calcarifer* (4-month-old). Lane M = 100 bp DNA ladder. Lanes 1-12 =.the amplification products against genomic DNA of different individuals of *L. calcarifer*.
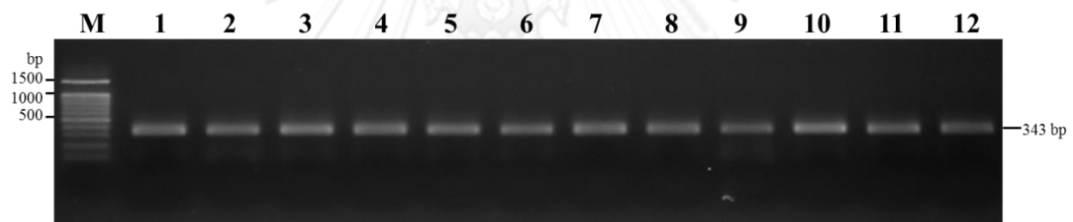
Figure 3.28 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *IGF-II* (intron 1 portion) gene fragment against genomic DNA of individual *L. calcarifer* (4-month-old). Lane M = 100 bp DNA ladder. Lanes 1-12 =.the amplification products against genomic DNA of different individuals of *L. calcarifer*.

A.

<u>GAAGTCCCAAGGAAGCAGCA</u>TGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGGAAGGCGGCCCAACGG
CTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGGAGGCAGGCGGAGAAGATCAAAGCACAGGAG
CAGGTTATCTTCCACAGGCCCCTGATCAGCCTTCCCAGCAAACTGCCTCCCGTCT<u>TGCTCACCACGGACAACTAT</u>

B.

```
IGF2-Query    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF2-Subject  GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
              ************************************************************
IGF2-Query    AAGGCGGCCCAACGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
IGF2-Subject  AAGGCGGCCCAACGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
              ************************************************************
IGF2-Query    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF2-Subject  AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
              ************************************************************
IGF2-Query    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF2-Subject  CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
              *********************************************
```

Figure 3.29 (A) Nucleotide sequence of the amplified *IGF-II* (intron 1 portion) gene segment. Positions of primers are underlined. (B) Pairwise alignment between nucleotide sequence from GenBank and that of the amplified *IGF-II* gene segment.

3.4.2.4 *Myostatin (MSTN)*

A 319 bp fragment (exon 1) was obtained from amplification of *MSTN* against genomic DNA of 4-month-old *L. calcarifer* (Figure 3.30 and Table 3.2). Size of the amplification products from genomic DNA and that of the expected product from genomic data were not different suggesting a lack of obvious indel in this gene region.
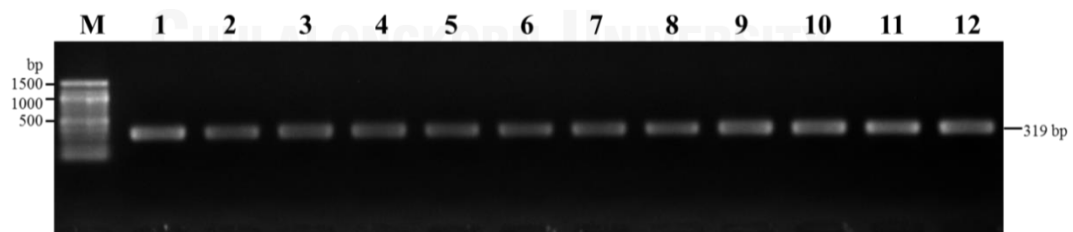
Figure 3.30 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *MSTN* gene fragment against genomic DNA of individual *L. calcarifer* (4-month-old). Lane M = 100 bp DNA ladder. Lanes 1-12 =.the amplification products against genomic DNA of different individuals of *L. calcarifer*.

3.4.2.5 *Follistatin (FST)*

The amplification product of *FST* against genomic DNA of 4-month-old *L. calcarifer* (Figure 3.31 and Table 3.2) was approximately 400 bp in length which is larger than that expected size (247 bp) from its cDNA sequence. The intron of approximately 150 bp is existent in the amplified region.
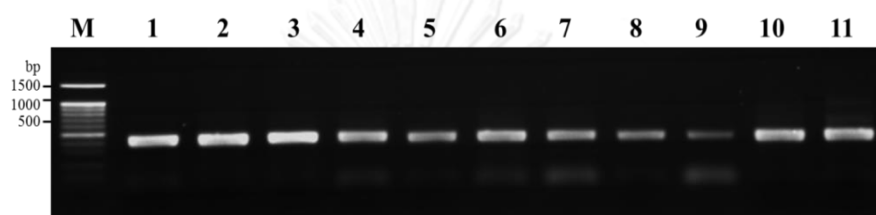
Figure 3.31 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *FST* gene fragment against genomic DNA of individual *L. calcarifer* (4-month-old). Lane M = 100 bp DNA ladder. Lanes 1-11 =.the amplification products against genomic DNA of different individuals of *L. calcarifer*.

3.4.2.6 *Activin type IIB receptor (ActRIIB)*

The amplification product size of *ActRIIB* against genomic DNA of 4-month-old *L. calcarifer* was 398 bp (Figure 3.32 and Table 3.2). The amplified fragment was cloned and sequence was cloned and sequenced and it contained an intron of 193 bp (Figure 3.33).
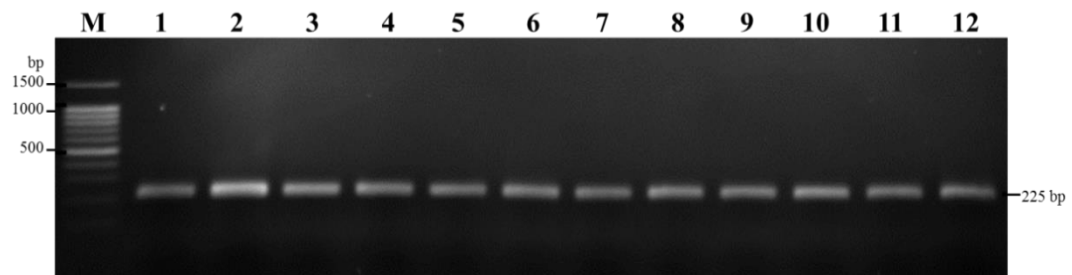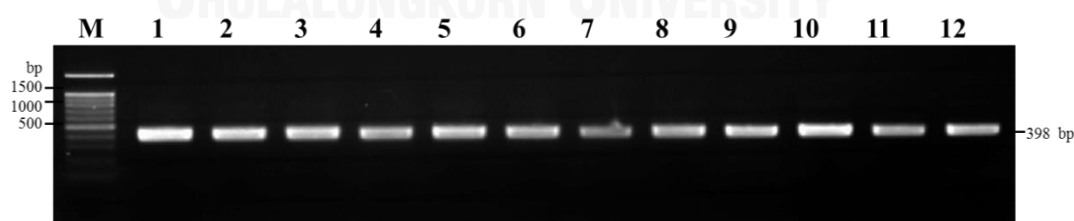

Figure 3.32 A 1.5% ethidium bromine-stained agarose gel showing the amplification products of *ActRIIB* gene fragment against genomic DNA of individual *L. calcarifer* (4-month-old). Lane M = 100 bp DNA ladder. Lanes 1-12 =.the amplification products against genomic DNA of different individuals of *L. calcarifer*.

A.

<u>GACCACCTGAAGGGTAACAC</u>TGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATGTCCCGCGGCTTGGCC
TACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAACCCACTATTGCACACAGGTATGCGAATCTG
TGCTTAATACACTGAAAACCAGACTTCATTTGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTG
TGCTCGATTTAGTAATTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA
CTGTAATGACTTCTAAAATGATTTTTCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCGAGATGATTTAACTGC
AGT<u>TATTGGAGACTTTGGGCTCG</u>

B.

```
ActRIIB-Query    GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG 60
ActRIIB-Subject  GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG 60
                 ************************************************************

ActRIIB-Query    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA 120
ActRIIB-Subject  TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA 120
                 ************************************************************

ActRIIB-Query    CCCACTATTGCACACAG------------------------------------------- 137
ActRIIB-Subject  CCCACTATTGCACACAG*GTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT* 180
                 *****************

ActRIIB-Query    ------------------------------------------------------------
ActRIIB-Subject  *TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA* 240

ActRIIB-Query    ------------------------------------------------------------
ActRIIB-Subject  *TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA* 300

ActRIIB-Query    ----------------------------GGACTTCAAGAGTAAGAATGTGATGCTTCG 167
ActRIIB-Subject  *CTGTAATGACTTCTAAAATGATTTTTCCAGG*GACTTCAAGAGTAAGAATGTGATGCTTCG 360
                                             ****************************

ActRIIB-Query    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG 205
ActRIIB-Subject  AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG 398
                 **************************************
```

Figure 3.33 (A) Nucleotide sequences of the amplified *ActRIIB* gene segments. Positions of primers were underlined. (B) Pairwise alignment between nucleotide sequence of *ActRIIB* previously deposited in GenBank and that of the amplified *ActRIIB* product. An intron sequence is italicized.

Table 3.2 Types of growth-related genes, expected sized and observed sized of *L. calcarifer* from GenBank and proteomics results.

Gene	Expected size (bp)	Observed size (bp)
Insulin-like growth factor I (*IGF-I*)*	163	163 and 400
Insulin-like growth factor II (*IGF-II*) (exon)*	225	225
Insulin-like growth factor II (*IGF-II*) (intron)*	343	343
Myostatin (*MSTN*)*	319	319
Follistatin (*FST*)*	247	400
Activin type IIB receptor mRNA (*ActRIIB*)**	205	398

* = primers were designed from the previously deposited sequences of *L. calcarifer* in GenBank;
** = primers were designed from a sequence in GenBank that encodes the protein homologue found in proteomic analysis of 4-month-old *L. calcarifer*

3.4.3 SSCP analysis

SSCP (Weder et al., 2001) are favored for identifying species origins of fish products due to their convenient and cost effective. Polymorphism of growth-related genes (*IGF-II*, *MSTN* and *ActRIIB*) was further analyzed. Subsequently, relationship between SSCP pattern and growth parameters (body weight, total length, hepatic weight and hepatosomatic index (HSI) were statically examined.

3.4.3.1 *IGF-II* (exon 4 portion)

Polymorphism of the amplified exon region (225 bp) of 99 individuals of 4-month-old *L. calcarifer* (*N* = 50 and 49 for large- and small-sized fish, respectively). Three polymorphic patterns (A, B and C) were observed (Figure 3.34). Disregarding sizes of specimens, the SSCP pattern C was found in 59 individuals while pattern A was found in 25 individuals and pattern B was found in 15 individuals.
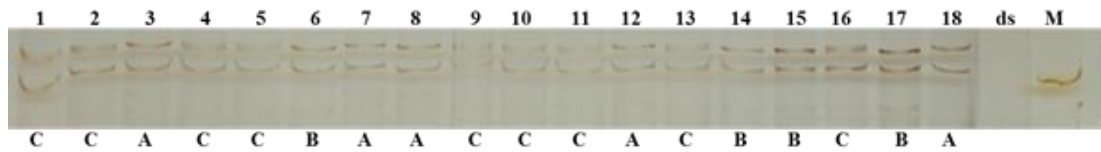
Figure 3.34 SSCP patterns of the *IGF-II* (an exon portion) gene segment against genomic DNA of different *L. calcarifer* individuals. Three polymorphic patterns were found. The denatured product was analyzed by a 15% non-denaturing polyacrylamide gel (37.5:1) at 250 V for 18 hr. Lane M = 100 bp DNA marker, Lane ds = non-denatured PCR product (double strand control) and Lanes 1-18 = denatured PCR product of *IGF-II* (an exon portion).

Considering distribution frequencies of SSCP patterns between large- and small-sized fish, the frequency of each pattern in different groups of samples were comparable fish. Relationships between SSCP patterns of the amplified exon region of *IGF-II* were tested against growth-related parameters. Based on the fact that, specimens was size-selected and cultured separately before samples were collected, statistical analysis was carried out separately between groups of samples.

In large-sized 4-month-old *L. calcarifer*, fish carrying SSCP pattern B possessed a greater average body weight (but not average total length, average hepatic weight and average hepatosomatic index) than those carrying SSCP pattern C ($P < 0.05$). Nevertheless, the result between those carrying SSCP patterns A and B was not different due to large standard deviation between these samples ($P > 0.05$). Relationships between SSCP patterns and growth-related parameters were not statistically significant in small-sized fish ($P > 0.05$) (Table 3.3).

Table 3.3 Relationships between SSCP genotypes of *IGF-II* (an exon 4 portion) and growth parameters of 4-month-old *L. calcarifer* (*N* = 99)

SSCP pattern	*N*	Average body weight ± SD (g)	Average total length ± SD (cm)	Average HP weight ± SD (g)	Average HSI ± SD (%)
Large-sized					
A	10	23.87 ± 6.78[ab]	12.33±1.23[a]	0.41±0.18[a]	1.74±0.58[a]
B	9	30.32 ± 15.00[a]	13.52±2.32[b]	0.38±0.19[a]	1.31±0.45[a]
C	31	19.66±5.10[b]	11.84±1.14[a]	0.29±0.14[a]	1.46±0.58[a]
Small-sized					
A	15	2.96±0.72[a]	6.17±0.56[a]	0.02±0.01[a]	0.80±0.51[a]
B	6	2.59±0.80[a]	6.00±0.78[a]	0.03±0.04[a]	0.88±0.86[a]
C	28	2.57±0.57[a]	5.93±0.49[a]	0.04±0.04[a]	1.56±1.66[a]

The same superscripts indicate that the body weight of fish possessing different genotypes were not significantly different ($P < 0.05$).

3.4.3.2 *IGF-II* (intron 1 portion)

The amplification product of the intron region of the *L. calcarifer* of *IGF-II* gene segment was 343 bp in size. Large- and small-sized 4-month-old *L. calcarifer* was genotyped. Two SSCP patterns were found (Figure 3.35) and the pattern A was distributed in a greater number of individuals than those of the pattern B in both large- (*N* = 50) and small-sized (*N* = 49) fish.

Relationships between SSCP genotype of this gene region and growth related parameters were examined. Results were not significant in both groups of samples and the average body weight, total length, hepatic weight and hepatosomatic index of fish exhibiting different SSCP genotypes within the same group were roughly comparable ($P > 0.05$) (Table 3.4).
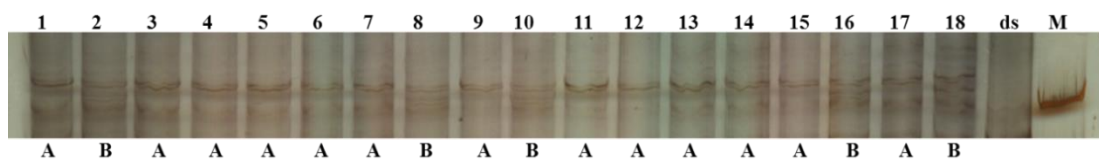
Figure 3.35 SSCP patterns of the *IGF-II* (intron portion) gene segment against genomic DNA of different *L. calcarifer* individuals. Two polymorphic patterns were found. The denatured product was analyzed by a 12.5% non-denaturing polyacrylamide gel (37.5:1) at 200 V for 16 hr. Lane M = 100 bp DNA marker, Lane ds = non-denatured PCR product (double strand control) and Lane 1-18 = denatured PCR product of *IGF-II* (intron portion).

Table 3.4 Relationships between SSCP genotypes of *IGF-II* (intron 1 portion) and growth parameters of 4-month-old *L. calcarifer* (*N* = 99)

SSCP pattern	*N*	Average body weight ± SD (g)	Average total length ± SD (cm)	Average HP weight ± SD (g)	Average HSI ± SD (%)
Large-sized					
A	36	22.29±7.27[a]	12.24±1.35[a]	0.33±0.16[a]	1.49±0.58[a]
B	14	22.76±12.29[a]	12.24±2.00[a]	0.32±0.17[a]	1.49±0.56[a]
Small-sized					
A	39	2.73±0.71[a]	6.04±0.59[a]	0.03±0.03[a]	1.09±1.03[a]
B	10	2.54±0.35[a]	5.90±0.33[a]	0.04±0.05[a]	1.84±2.20[a]

The same superscripts indicate that the body weight of fish possessing different genotypes were not significantly different ($P < 0.05$).

3.4.3.3 *MSTN*

The amplification product of 319 bp in size was obtained from amplification of *MSTN* gene segment against genomic DNA of *L. calcarifer.* SSCP analysis of 4-month-old *L. calcarifer* was carried out (N = 50 for large-sized samples and N = 49 for small-sized sample). Patterns of two different loci were observed suggesting that *MSTN* is multi-gene family. Three polymorphic SSCP patterns were identified from the first loci and two polymorphic SSCP patterns were found from the second loci (Figure 3.36).

Pattern distribution frequencies for loci 1 indicated that pattern A was the most common pattern followed by patterns B and C, respectively. Relationships between SSCP genotype of *MSTN* and growth-related parameters were examined. In this thesis, statistical analysis was also carried out in overall samples in case that at least one SSCP pattern was possessed by only one individual. In this study, pattern C of *MSTN* was found in only one individual. Results showed a trend that the average body weight, total length, hepatic weight and hepatosomatic index of fish exhibiting SSCP pattern A was sequentially greater than that of B and C, respectively. However, results were not statistically significant owing to large standard deviation between fish exhibiting different SSCP genotypes (P > 0.05).
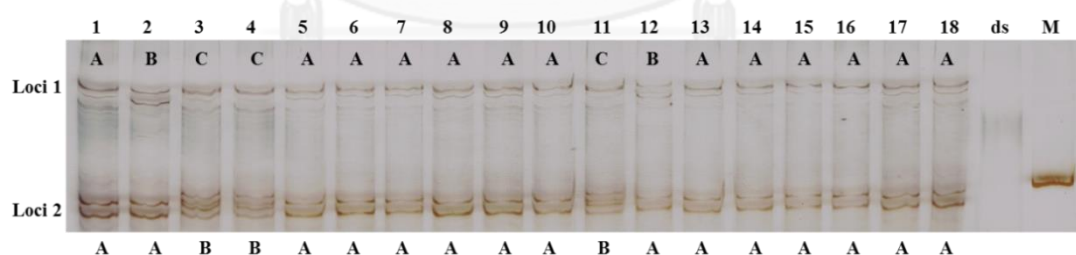
Figure 3.36 SSCP patterns of the *MSTN* gene segment against genomic DNA of different *L. calcarifer* individuals. SSCP analysis indicated 2 loci. Three and two polymorphic patterns were observed in loci 1 and 2, respectively. The denatured product was analyzed by a 15% non-denaturing polyacrylamide gel (75:1) at 200 V for 16 hr. Lane M = 100 bp DNA marker, Lane ds = non-denatured PCR product (double strand control) and Lanes 1-18 = denatured PCR product of *MSTN* gene.

Similar results were observed in the large-sized fish. Relationships between SSCP patterns and growth-related parameters were not statistically significant in small-sized fish ($P > 0.05$) (Table 3.5).

Table 3.5 Relationships between SSCP genotypes of *MSTN* (loci 1) and growth parameters of 4-month-old *L. calcarifer* (N = 99)

| SSCP pattern	N	Average body weight ± SD (g)	Average total length ± SD (cm)	Average HP weight ± SD (g)	Average HSI ± SD (%)
Total specimens					
A	83	13.57±12.11[a]	9.42±3.39[a]	0.20±0.20[a]	1.28±0.79[a]
B	10	9.47±8.62[a]	8.53±2.97[a]	0.14±0.14[a]	1.72±1.96[a]
C	6	5.26±7.29[a]	6.65±2.15[a]	0.07±0.06[a]	2.04±1.72[a]
Large-sized					
A	45	22.77±9.17[a]	12.30±1.58[a]	0.34±0.16[a]	1.51±0.58[a]
B	4	19.01±4.45[a]	11.84±1.14[a]	0.27±0.13[a]	1.41±0.43[a]
C	1	20.13	11.00	0.17	0.84
Small-sized					
A	38	2.68±0.65[a]	6.00±0.55[a]	0.05±0.06[a]	1.00±0.92[a]
B	6	3.11±0.68[a]	6.32±0.62[a]	0.04±0.03[a]	1.92±2.60[a]
C	5	2.28±0.34[a]	5.78±0.33[a]	0.03±0.03[a]	2.28±1.81[a]

The same superscripts indicate that the body weight of fish possessing different genotypes were not significantly different ($P < 0.05$).

For *MSTN* loci 2, only 2 SSCP patterns were found and distributed in 93 and 6 individuals, respectively. In large-sized sample pattern B was observed in one individual from 50 individuals examined. In small-sized samples, 44 individuals carried pattern A while 5 individuals exhibited pattern B.

Statistical analysis for overall samples revealed that 4-month-old fish carrying SSCP pattern A possessed a greater average body weight, total length and hepatic weight than those of fish carrying SSCP pattern B ($P < 0.05$) (Table 3.6).

Table 3.6 Relationships between SSCP genotypes of *MSTN* (loci 2) and growth parameters of 4-month-old *L. calcarifer* ($N = 99$)

| SSCP pattern	N	Average body weight ± SD (g)	Average total length ± SD (cm)	Average HP weight ± SD (g)	Average HSI ± SD (%)
Total specimens					
A	93	13.13±11.82[a]	9.32±3.34[a]	0.19±0.19[a]	1.32±0.98[a]
B	6	5.26±7.29[b]	6.65±2.15[b]	0.07±0.06[b]	2.04±1.72[a]
Large-sized					
A	49	22.47±8.90[a]	12.26±1.54[a]	0.33±0.16[a]	1.50±0.08[a]
B	1	20.13	11.0	0.17	0.84
Small-sized					
A	44	2.74±0.67[a]	6.04±0.56[a]	0.03±0.03[a]	1.14±0.35[a]
B	5	2.28±0.34[a]	5.78±0.33[a]	0.05±0.03[a]	3.00±0.01[a]

The same superscripts indicate that the body weight of fish possessing different genotypes were not significantly different ($P < 0.05$).

Statistical analysis could not be carried out in large-sized fish as only one individual possessed pattern B. Relationships between SSCP patterns and growth-related parameters were not statistically significant in small-sized fish ($P > 0.05$) (Table 3.6).

3.4.3.4 *ActRIIB*

The amplification products of 398 bp in size were obtained from amplification of the *ActRIIB* gene segment against genomic DNA of *L. calcarifer*. SSCP analysis of 4-month-old *L. calcarifer* ($N = 50$ for large-sized samples and $N = 49$ for small-sized samples). Four polymorphic patterns (A, B, C and D) were observed (Figure 3.37).

Pattern distribution frequencies across overall samples indicated that the two common patterns were C and D (N = 45 for each pattern) followed by patterns B (N = 8) and A (N = 1). Interestingly, patterns C and D were found in 23 and 27 examined large-sized individuals and patterns A and B were not distributed in small-sized samples while all four patterns (A, B, C and D) were found in small-sized samples (Table 3.7).
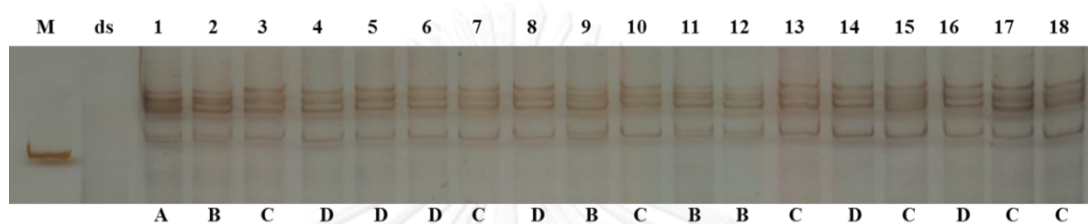
Figure 3.37 SSCP patterns of the *ActRIIB* gene segment against genomic DNA of different *L. calcarifer* individuals. Two polymorphic patterns were found. The denatured product was analyzed by a 12.5% non-denaturing polyacrylamide gel (37.5:1) at 200 V for 16 hr. Lane M = 100 bp DNA marker, Lane ds = non-denatured PCR product (double strand control) and Lanes 1-18 = denatured PCR product of *ActRIIB* gene.

Relationship between polymorphic patterns of *ActRIIB* and growth parameters of *L. calcarifer* across overall samples were examined and results indicated that 4-month-old *L. calcarifer* exhibiting SSCP pattern D had a greater average body weight, total length and hepatic weight than those carrying SSCP genotypes A and B which (P < 0.05) (Table 3.7). In addition, results between those exhibited SSCP patterns C and D due to large standard deviation between fish exhibiting these SSCP patterns (P > 0.05).

Considering only large-sized fish, those exhibiting SSCP pattern D had a greater average body weight, total length and hepatic weight than those exhibiting SSCP pattern C (P < 0.05). In contrast, growth-related parameters of those carrying SSCP pattern B, C and D were not statistically different in small-sized samples (P > 0.05) (Table 3.7).

Table 3.7 Relationships between SSCP genotypes of *ActRIIB* and growth parameters of 4-month-old *L. calcarifer* (*N* = 99)

SSCP pattern	*N*	Average body weight ± SD (g)	Average total length ± SD (cm)	Average HP weight ± SD (g)	Average HSI ± SD (%)
Total specimens					
A	1	2.64	6.10	0.08	3.03
B	8	2.95±0.66[a]	6.26±0.64[a]	0.04±0.04[a]	1.37±1.50[a]
C	45	10.11±8.31[ab]	8.66±2.93[b]	0.15±0.15[ab]	1.37±0.88[a]
D	45	17.15±13.79[b]	10.24±3.59[b]	0.24±0.22[b]	1.33±1.09[a]
Large-sized					
C	23	17.77±5.02[a]	11.35±1.19[a]	0.27±0.13[a]	1.51±0.62[a]
D	27	26.38±9.48[b]	13.00±1.41[b]	0.39±0.17[b]	1.48±0.53[a]
Small-sized					
A	1	2.64	6.10	0.08	3.03
B	8	2.95±0.66[a]	6.26±0.64[a]	0.04±0.04[a]	1.37±1.50[a]
C	22	2.64±0.77[a]	5.94±0.59[a]	0.03±0.03[a]	1.20±1.05[a]
D	18	2.69±0.67[a]	6.01±0.55[a]	0.02±0.03[a]	1.14±1.64[a]

The same superscripts indicate that the body weight of fish possessing different genotypes were not significantly different ($P < 0.05$).

3.5 Identification and characterization of SNP in growth-related genes (*IGF-II* (exon position) and *ActRIIB*) by DNA sequencing

To confirm polymorphic SNP in the amplified position previously examined by SSCP analysis, the PCR product of representative individuals exhibiting different SSCP genotypes of *IGF-II* and *ActRIIB* was clone and sequence. Nucleotide sequences of a particular gene were multiple-aligned for identification of SNP in each gene segment.

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3.5.1 *IGF-II*

The PCR products of representative individuals exhibiting SSCP patterns A (*N* = 11), B (*N* = 10) and C (*N* = 12) of *IGF-II* (exon position) were sequenced. Multiple sequence alignments were performed and shown in Figure 3.38.

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IGF-II-A-015    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-042    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-055    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-069    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-073    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-067    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-056    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-022    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-014    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-012    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-A-009    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-B-005    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-B-092    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
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IGF-II-B-078    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-B-064    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
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IGF-II-B-087    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
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IGF-II-C-077    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-C-060    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
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IGF-II-C-033    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-C-089    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-C-006    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-C-100    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-C-025    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
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IGF-II-C-029    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-C-051    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
IGF-II-C-008    GAAGTCCCAAGGAAGCAGCATGTGACCGTGAAGTATTCCAAATACGAGGTGTGGCAGAGG 60
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IGF-II-A-055    AAGGCGGCCCAACGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
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IGF-II-B-092    AAGGCGGCCCAGCGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
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IGF-II-B-036    AAGGCGGCCCAGCGGCTCCGGAGGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
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IGF-II-C-006    AAGGCGGCCCAGCGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
IGF-II-C-100    AAGGCGGCCCAGCGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
IGF-II-C-025    AAGGCGGCCCAACGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
IGF-II-C-053    AAGGCGGCCCAACGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
IGF-II-C-029    AAGGCGGCCCAACGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
IGF-II-C-051    AAGGCGGCCCAGCGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
IGF-II-C-008    AAGGCGGCCCAGCGGCTCCGGAGGGGTGTCCCCGCCATCCTGAGGGCCAAAAAGTTTCGG 120
                *********** .***********************************************

IGF-II-A-015    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-042    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-055    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-069    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-073    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-067    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-056    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-022    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-A-014    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
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IGF-II-B-078    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCATAGGCCCCTGATCAGC 180
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IGF-II-B-036    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
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IGF-II-C-077    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGG-CCCTGATCAGC 180
IGF-II-C-060    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-019    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-033    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-089    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-006    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-100    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-025    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-053    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-029    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-051    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
IGF-II-C-008    AGGCAGGCGGAGAAGATCAAAGCACAGGAGCAGGTTATCTTCCACAGGCCCCTGATCAGC 180
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IGF-II-A-015    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-042    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-055    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-069    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-073    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-067    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-056    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-022    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-014    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-012    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-A-009    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-005    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-092    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-004    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-078    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-064    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-036    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-063    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-071    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-087    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-B-093    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-077    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-060    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-019    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-033    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-089    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-006    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-100    GCTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-025    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-053    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-029    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-051    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
IGF-II-C-008    CTTCCCAGCAAACTGCCTCCCGTCTTGCTCACCACGGACAACTAT 225
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Figure 3.38 Multiple sequence alignments of *IGF-II* (exon region) gene segments amplified from genomic DNA of 4-month-old *L. calcarifer* individuals exhibiting different SSCP genotypes (A, B and C). Positions of SNP were highlighted.

Two SNP positions (72 and 165) were found from multiple alignments of nucleotide sequence of different SSCP genotype of *IGF-II* in the 4-month-old *L. calcarifer* (Table 3.8). Three SNP genotypes (A/A, G/G and A/G corresponding to SSCP pattern A, B and C, respectively) were observed at the position 72 of the amplified gene segment while two SNP positions (C/C and C/T corresponding to SSCP patterns A+C and B, respectively) were found at position 165 of the amplified exon region of *IGF-II*.

Considering polymorphism of these SNP simultaneously, three diplotypes (corresponding to SSCP genotype A, B and C) were observed. Genotypes of individuals carrying these diplotypes were $A/A_{72}C/C_{165}$, $G/G_{72}C/T_{165}$ and $A/G_{72}C/C_{165}$.

These diplotypes could differentiate those carrying SSCP genotype A, B and C unambiguously. Accordingly, results inferred for overall specimens of 4-month-old *L. calcarifer* were equivalent as those from SSCP analysis (Table 3.8).

Table 3.8 SNPs of *IGF-II* gene segment found in different diplotypes of 4-month-old *L. calcarifer* (*N* = 33)

Diplotype	SSCP pattern	*N*	SNP position	
			72	165
I	A	11	A/A	C/C
II	B	10	G/G	C/T
III	C	12	A/G	C/C

3.5.2 *ActRIIB*

The PCR products of individuals representing each SSCP pattern of *ActRIIB* (*N* = 1, 6, 5 and 5 for SSCP pattern A, B, C and D, respectively) were sequenced. Nucelotide sequences were multiple-aligned and shown in Figure 3.39.

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ActRIIB-A-001   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-B-039   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-B-038   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-B-029   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-B-011   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-B-002   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-B-009   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-C-061   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-C-085   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-C-053   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-C-057   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-C-086   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-D-064   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-D-063   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-D-056   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-D-090   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
ActRIIB-D-059   GACCACCTGAAGGGTAACACTGTGACCTGGACTGAGCTGTGTCACATAGCAGAGACCATG  60
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ActRIIB-A-001    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-B-039    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-B-038    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-B-029    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-B-011    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-B-002    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-B-009    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-C-061    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-C-085    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-C-053    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-C-057    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-C-086    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-D-064    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-D-063    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-D-056    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-D-090    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
ActRIIB-D-059    TCCCGCGGCTTGGCCTACCTCCATGAGGACATTCCCAGCTACAAGGGAGAGGGGCCGAAA  120
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ActRIIB-A-001    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-B-039    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-B-038    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-B-029    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-B-011    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-B-002    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-B-009    CCCACTATTGCACACAGGTATGCAAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-C-061    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-C-085    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-C-053    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-C-057    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-C-086    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-D-064    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-D-063    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-D-056    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-D-090    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
ActRIIB-D-059    CCCACTATTGCACACAGGTATGCGAATCTGTGCTTAATACACTGAAAACCAGACTTCATT  180
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ActRIIB-A-001   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-B-039   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-B-038   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-B-029   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-B-011   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-B-002   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-B-009   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-C-061   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-C-085   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-C-053   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-C-057   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-C-086   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-D-064   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-D-063   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-D-056   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-D-090   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
ActRIIB-D-059   TGATTTTAGATCTCATCTTAAATTAATGGTGCACTTTCCTGGTTGTGCTCGATTTAGTAA 240
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ActRIIB-A-001   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-B-039   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-B-038   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-B-029   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-B-011   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-B-002   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-B-009   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-C-061   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-C-085   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-C-053   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-C-057   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-C-086   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-D-064   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-D-063   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-D-056   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-D-090   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
ActRIIB-D-059   TTTTTAACCTGGAAAATAATGGACTAATTTATGTGACTCAACGTCTTTATAATTACGGCA 300
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ActRIIB-A-001    CTGTAATGACTTCTAAAATGATTTTTCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-B-039    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-B-038    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-B-029    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-B-011    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-B-002    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-B-009    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-C-061    CTGTAATGACTTCTAAAATGATTTTTCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-C-085    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-C-053    CTGTAATGACTTCTAAAATGATTTTTCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-C-057    CTGTAATGACTTCTAAAATGATTTTTCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-C-086    CTGTAATGACTTCTAAAATGATTTTTCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-D-064    CTGTAATGACTTCTAAAATGATTTTTCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-D-063    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-D-056    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-D-090    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
ActRIIB-D-059    CTGTAATGACTTCTAAAATGATTTTCCCAGGGACTTCAAGAGTAAGAATGTGATGCTTCG  360
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ActRIIB-A-001    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-B-039    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-B-038    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-B-029    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-B-011    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-B-002    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-B-009    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-C-061    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-C-085    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-C-053    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-C-057    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-C-086    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-D-064    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-D-063    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-D-056    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-D-090    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
ActRIIB-D-059    AGATGATTTAACTGCAGTTATTGGAGACTTTGGGCTCG  398
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Figure 3.39 Multiple alignment sequence of *ActRIIB* gene segments amplified from genomic DNA respective individuals of exhibiting different SSCP genotypes (A, B and C) 4-month-old *L. calcarifer.* The positions of SNP were highlighted and exon was underlined.

Two SNP positions (144 and 326) were found from multiple alignments of nucleotide sequence of four different SSCP genotype of *ActRIIB* in 4-month-old *L. calcarifer*. These SNPs were located in the intron region (Table 3.9).

Two SNP genotypes (A/G and G/G corresponding to SSCP pattern A+B and C+D, respectively) were observed at the position 144 of the amplified *ActRIIB* gene segment and two SNP positions (C/T and C/C corresponding to SSCP patterns A+C and B+D, respectively) were found at position 326 of the amplified intron region of *IGF-II*.

Considering polymorphism of these SNP simultaneously, four diplotypes (corresponding to SSCP genotype A, B, C and D) were observed. Genotypes of individuals carrying these diplotypes were $A/G_{144}C/T_{326}$, $A/G_{144}C/C_{326}$, $G/G_{144}C/T_{326}$ and $G/G_{144}C/C_{326}$. These diplotypes could differentiate those carrying SSCP genotype A, B, C and D unambiguously. Accordingly, results inferred for overall specimens of 4-month-old *L. calcarifer* were as the same as those from SSCP analysis.

Table 3.9 SNPs of *ActRIIB* gene segment found in different diplotypes of 4-month-old *L. calcarifer* (N = 17)

Diplotype	SSCP pattern	N	SNP position	
			144	326
I	A	1	A/G	C/T
II	B	6	A/G	C/C
III	C	5	G/G	C/T
IV	D	5	G/G	C/C

CHAPTER IV
DISCUSSION

Cellular proteomics for isolation of potential proteins functionally related with growth of *L. calcarifer*

Efficient growing phenotypes of aquatic species have a major influence on the profitability of food production. In addition, the use of selected populations of cultured species having fast growth rate has the potential to reduce aquaculture effluents leading to more environmentally sustainable production. Accordingly, successful selection for optimal growth rate or body weight is a key objective in aquaculture breeding programs.

Conventional selection is typically used to select for growth traits, however, it requires several generations to optimize genetic improvement. In addition, insight into the genetic bases of growth can be used to make better selection decisions. Molecular genetic markers have been applied to identify potential gene and protein markers associated with traits that genetic variation explaining phenotypic differences in growth.

Proteomics has been increasingly used in fish biology research. Proteomics has been applied primarily to investigate the physiology, development biology and the impact of contaminants in fish model species.

Wang et al. (2011) examined protein profiles in medaka (*Oryzias melastigma*) liver and brain experimentally exposed to acute inorganic mercury by two-dimensional gel electrophoresis (2-DE) and mass spectrometry. Results of protein expression identified were involved in oxidative stress, cytoskeletonal assembly, signal transduction, protein modification, metabolism and other related functions (e.g. immune response, ionoregulation and transporting).

Zhang et al. (2012) examined effects of perfluorononanoic acid (PFNA) exposure (0, 0.1, 0.5, and 1.0 mg/l for 180 days) in male zebrafish using 2DE and MALDI-TOF-MS/MS. A total of 57 proteins were successfully identified and functionally categorized including metabolism (e.g. amino acid metabolism and TCA cycle), structure and motility, stress and defense, signal transduction, and cell communication.

In addition, 2-DE and MALDI-TOF-TOF MS was used to examined estrogenicity of 17β-estradiol to screen hepatic responses in adult male zebrafish. Eight proteins were found to be up-regulated more than 2-fold, whereas five protein spots were down-regulated more than 2-fold after 1 nM E2 treatment for 14 days, which had caused histological effects in zebrafish livers. Differentially expressed proteins accounted for a variety of cellular biological processes, such as response to oxidative stress, cell surface receptor-linked signal transduction, oxidation–reduction and cellular calcium ion homeostasis (Jin et al., 2010).

The use of 2-DE for proteomic analysis is tedious and time consuming. In addition, it is difficult (or not possible) to identify proteins with very low and high molecular weight or those exhibiting very low or high p*I* simultaneously. In this thesis, one-dimensional gel electrophoresis (SDS-PAGE) was also used and the electrophoresed proteins were further characterized by nanoESI-LC-MS/MS. In this case, the protein staining method does not interfere the ability to compare whether the examined proteins were differentially expressed or not as the intensity of each protein in different specimens was evaluated from nanoESI-LC-MS/MS spectrum results.

Molecular mechanisms that regulate fish muscle degeneration/regeneration have received an interest at present. Muscle deterioration arises as a physiological response to elevated energetic demands of fish during sexual maturation and spawning. Salem et al. (2010) characterized proteomic profile in degenerating muscle of rainbow trout in relation to the female reproductive cycle using a LC/MS-based label-free protein quantification method and 146 significantly changed proteins in atrophying muscles was identified. Muscle atrophy was associated with decreased

abundance in proteins of anaerobic respiration, protein biosynthesis, monooxygenases, follistatins, and myogenin, as well as growth hormone, interleukin-1 and estrogen receptors. In contrast, proteins of MAPK/ERK kinase, glutamine synthetase, transcription factors, Stat3, JunB, Id2, and NFkappaB inhibitor, were greater in atrophying muscle.

In this thesis, cellular proteomics of hepatic tissues of large-sized and small-sized juveniles was carried out based on GeLC-MS/MS (sized fractionation of proteins by SDS-PAGE and characterization of electrophoresed proteins by nanoESI-LC-MS/MS). Among 1578 proteins identified, the most abundant proteins were unnamed proteins (484 proteins, 30.7%) followed by those with unknown function (349 proteins, 22.1%).

Functional categories were classified based on their gene ontology identifiers. Among proteins with known function, those classified into regulation (124 proteins, 7.9%) was the most abundant group followed by binding (113 proteins, 7.2%), transport proteins (44 proteins, 2.8%), catabolic process (40 proteins, 2.5%), metabolic process (21 proteins, 1.3%), development (18 proteins, 1.1%), signaling pathway (16 proteins, 1.0%) and biosynthetic process (15 proteins, 1.0%). Proteins in the remaining categories were found in relatively lower frequencies.

Although protein specifically found in large- and small-sized juvenile fish were identified, stage-specific proteins identified in this study were regarded as differentially expressed proteins and they should play the prominent role in different growth stage in *L. calcarifer*.

Examples of protein homologuess found only in the large-sized juveniles were cAMP-dependent protein kinase catalytic subunit beta, glutathione S-transferase kappa, clathrin coat assembly protein AP180-like and guanylate cyclase 2G-like. Cyclic AMP is a well-known second messenger, whose regulatory targets include kinase and GTP exchange factor activities. In somatic cells, cAMP functions both as a positive and a negative regulator of the mitotic cell cycle progression (depending on cell types) (Conti et al., 2002). The cAMP-protein kinase A (PKA) signaling pathway is

important for the regulation of cAMP levels which are necessary for the progression of cell division (Matten et al., 1994). Clathrin coat assembly protein AP180 plays an important role in clathrin-mediated endocytosis (Ford et al., 2001). Glutathione S-transferase is an antioxidant enzyme functionally involved in detoxification of reactive oxygen species (ROS).

Examples of proteins specifically found in small-sized *L.calcarifer* juveniles were connector enhancer of kise suppressor of ras 2, pleckstrin homology-like domain family B member 3-like, spectrin beta chain, brain 4-like, retinitis pigmentosa GTPase regulator protein 2 ORF15.

Several proteins were more differentially expressed towards the large-sized juvenile fish. These included 39S ribosomal protein L23, mitochondrial, calmodulin, cytochrome C oxidase subunit 7C, mitochondrial precursor, E3 ubiquitin-protein ligase LNX, gamma-enolase, glycine N-acyltransferase, thrombospondin-2, guanylate cyclase 2G-like ($P < 0.05$) while dual specificity phosphatase 6, globin X and complement C4-like were more abundantly expressed in hepatic tissue of small-sized than large-sized fish ($P < 0.05$). Functional involvement of these protein in growth of *L. calcarifer* should be further confirmed.

Many growth-related proteins were identified. Although their expression profiles in large- and small-sized juveniles of *L. calcarifer* seemed to be different. Results were not statistically significant owing to large standard deviation between groups of samples. Based on the fact that specimens used in the present study were not domesticated stocks but they were offspring of wild *L.calcarifer*. It is also important to consider non-significant growth-related proteins identified as the candidate markers for growth traits in *L. calcarifer*.

It has been reported that skeletal muscle growth is negatively regulated by several transforming growth factor ß (TGF- ß) molecules including activin A and myostatin, which signal through the activin type I and type II serine/threonine kinase receptors ((Link and Nishi, 1997); (Lee et al., 2005); (Lee et al., 2012)). In this study, transforming growth factor beta-2 precursor, transforming growth factor beta-3

precursor and transforming growth factor-beta-induced protein ig-h3 precursor were identified and they were more abundantly expressed in large-sized fish than small-sized juveniles.

Moreover, activin type IIB receptor (ActRIIB) was also identified. ActRIIB is the cell surface receptor for multiple TGF-β superfamily ligands. Myostatin signaling through ActRIIB is crucial for the regulation of muscle growth in teleost fish (Phelps et al., 2013). As a negative regulator of muscle growth and differentiation, the level of ActRIIB in large-sized juveniles was lower than that in small-sized juveniles of 4-month-old *L. calcarifer*.

The primary goal of genetic selection is the growth improvement of aquacultural species. Although the genes that affect a polygenetic trait such as growth can typically be identified through the genetic linkage maps, a number of potential candidate genes can be selected based on a known relationship between physiological or biochemical processes and a particular trait (KANG et al., 2002). The candidate genes encoding growth-related proteins are promising for the future development as gene-assisted selection (GAS) markers.

Identification of polymorphic growth-related genes in *L. calcarifer* and relationships between their SSCP genotypes and phenotypic parameters

Molecular markers linked with growth traits can be applied to select fry and/or broodstock which high growth performance in fish industry. Information on correlation between genotypic and phenotypic variation in the Asian seabass is limited.

Growth traits are physiological functions under the control of several genes and regarded as a phenotype under the control of quantitative trait loci. Genotyping animals for all the genes encoding a polygenic trait seems impractical and so it is more realistic to focus on only a few genes having effects that account for a significant part of the genetic variation in growth traits ((Li et al., 2011); (Dekkers, 2004); (Zhang et al., 2009)).

Previously, (He et al. (2012)) examined polymorphism in the *prolactin* gene (*PRL*) in *L. calcarifer* and its association with growth trait. SNP were found in intron 3 were significantly associated with growth traits ($P < 0.05$).

Gross and Nilsson (1999) examined polymorphism in the *growth hormone 1* (*GH1*) in the Atlantic salmon (*Salmo salar*) and its association with the weight of one-year-old progeny of the hatchery strain (graded into three-sized group) Digestion of the *GH1* fragment with *Taq I* detected two novel polymorphism. Significantly difference of genotype frequencies of *GH1* among the size group was detected ($P < 0.05$).

Xu et al. (2006) characterized two *parvalbumin* genes and their association with growth traits in Asian seabass (*Lates calcarifer*). Expression of *PVALB2* was detected only in muscle, brain, intestine and was up to 10-fold lower than *PVALB1* expression. A $(CT)_{17}$ microsatellite was identified in the 3'UTR of *PVALB1* and three SNP were identified in the third intron of *PVALB2*. The microsatellite in *PVALB1* was significantly associated with body weight and body length at 90 days post-hatch ($P < 0.01$) whereas SNPs in *PVALB2* were not associated with these important traits ($P > 0.05$).

From proteomic analysis, PDZ-domain containing protein 1, proteasome subunit beta type 2, 14-3-3 A2, DEAD box polypeptide 56, Acyl-CoA thioesterase 11 and cell division cycle 2-like protein kinase 5 (cyclin-dependent kinase 13) were selected for further analysis. Degenerate primers for amplification of genes encoding these proteins were designed. Nevertheless, blast analysis revealed that nucleotide sequences obtained did not match the target genes except that of Cdk13. Accordingly, SNP in sequences of genes functionally related with growth traits were studied instead. IGF-1 regulates MSTN signaling by inhibition of transcription factors responsible for the induction of the *MSTN* gene. In contrast, MSTN and ActRIIB are negative regulators for myoblast proliferation and differentiation. The nucleotide sequences of *IGF-I, IGF-II, MSTN* and *ActRIIB* of *L. calcarifer* available in GenBank were retrieved and primers for amplification of these gene segment were designed.

SNP by SSCP (SBS) analysis were applied and two bands were observed from the amplified *IGF-I* gene segment. This gene was not further analyzed. Specimens in this study was size-selected and cultured in separate concrete tanks. Therefore, distribution of large- and small-sized juvenile *L. calcarifer* was not normal distributed. As a result, association between SSCP genotypes of each gene and growth parameters in different groups of samples were statistically tested separately. If, however, at least one SSCP pattern in each sex was observed, statistical analysis also tested covering overall samples.

The large-sized juvenile seabass carrying different SSCP patterns of *IGF-II* (exon region), *MSTN* (loci 2) and *ActRIIB* exhibited different growth-related parameters. Results preliminary indicated that polymorphism of this genes was linked with growth-related parameters of non-selected *L. calcarifer*. Interestingly, pattern distribution frequencies of *ActRIIB* across overall samples indicated that patterns C and D were not found in large-sized juveniles while all four patterns (A, B, C and D) were found in small-sized samples.

It is not possible to obtain domesticated (or genetically improved) *L. calcarifer* families at present. Accordingly, juvenile L. calcarifer was collected from a fishery station. Therefore, association between SNP in *IGF-II* (exon region), *MSTN* (loci 2) and *ActRIIB* genes and age-specific growth rates of *L. calcarifer* should be confirmed using a growth-selected stock.

On the basis of SSCP analysis, it was indicated that polymorphism of *IGF-II* (exon region), *MSTN* (loci 2) and *ActRIIB* was significantly related with growth-related parameters of *L. calcarifer*. Considering pattern distribution frequencies of examined gene segment, *ActRIIB* seemed to be more potential marker than *IGF-II* (exon region), *MSTN* (loci 2).

DNA sequencing confirms that SSCP analysis is the potential method for SNP identification

Nucleotide sequences in representative individuals of 4-month-old *L. calcarifer* representing each SSCP pattern of *IGF-II* (exon region) and *ActRIIB* was further examined. Two SNP positions (72 and 165) were found in the former gene segment. At the position 72, SNP genotypes A/A, G/G and A/G corresponding to SSCP pattern A, B and C, were observed while SNP genotypes C/C and C/T corresponding to SSCP patterns A+C and B, were found at position 165. Considering polymorphism of these SNP simultaneously, three diplotypes; $A/A_{72}C/C_{165}$, $G/G_{72}C/T_{165}$ and $A/G_{72}C/C_{165}$ were found and these diplotypes corresponded to SSCP genotype A, B and C. Accordingly, differentiation of fish having different phenotypes in 4-month-old *L. calcarifer* can be assessed using SSCP analysis and SNP at the position 72 (single SNP genotype) or at both positions 72 and 165 (composite SNP genotypes, also called diplotypes).

Similarly, two SNPs (positions 144 and 326) were found in the *ActRIIB* gene segment of the 4-month-old *L. calcarifer*. At the position 144, SNP genotypes A/G and G/G corresponding to SSCP pattern A+B and C+D were observed while SNP genotypes C/T and C/C corresponding to SSCP patterns A+C and B+D were found at the position 326. Based on the fact that SSCP patterns A and B were not distributed in the large-sized juveniles, SNP at the position 144 could be used for initial elimination of small-sized fish from the remaining fish. Considering polymorphism of these SNPs simultaneously, four diplotypes; $A/G_{144}C/T_{326}$, $A/G_{144}C/C_{326}$, $G/G_{144}C/T_{326}$ and $G/G_{144}C/C_{326}$ corresponded to SSCP genotype A, B, C and D, were found. Therefore, differentiation of fish having different phenotypes in 4-month-old *L. calcarifer* can be assessed using SSCP analysis and/or composite SNP genotypes (diplotypes) at both positions 144 and 326.

Although SNP analysis by DNA sequencing is the most direct and reliable technique, results in this thesis demonstrated that SSCP analysis of *IGF-II* (exon region), *MSTN* (loci 2) and *ActRIIB* gene polymorphism was comparably potential and sufficient to examine relationships between their SSCP patterns and growth-related

parameters in *L. calcarifer*. Nevertheless, SSCP analysis of additional growth-related proteins identified by proteomics distribution and genetic should be further studied for identification of more growth-liked markers in this economically important species.

CHAPTER V

CONCLUSION

1. Cellular proteomics of hepatic tissues of large-sized and small-sized 4-month-old juveniles of *L. calcarifer* was examined.

2. A total of 1578 protein homologues were identified and 745 protein (47.2%) of these matched proteins with known functions in the databases.

3. Twenty-eight proteins (e.g. calmodulin, cAMP-dependent protein kise catalytic subunit beta and dual specificity phosphatase 6) showed significantly differential expression between large-sized and small-sized samples.

4. Several growth-related proteins (e.g. TGF-β2 precursor, TGF- β3 precursor, TGF- β2-induced protein ig-h3 precursor and *ActRIIB* were also identified.

5. SNP in *IGF-II*, *MSTN* and *ActRIIB* was further analyzed in 4-month-old juveniles cultured in concrete tanks (N = 99). Large-sized 4-month-old *L. calcarifer* carrying SSCP pattern B of the exon 1 gene segment of *IGF-II* possessed a greater average body weight than those carrying SSCP pattern C (P < 0.05).

6. Similarly, juvenile *L. calcarifer* carrying SSCP pattern A of *MSTN* loci 2, possessed a greater average body weight, total length and hepatic weight than those of fish carrying SSCP pattern B (P < 0.05).

7. Juvenile *L. calcarifer* exhibiting SSCP pattern D *ActRIIB*, had a greater average body weight, total length and hepatic weight than those carrying SSCP genotypes A and B (P < 0.05). Considering only large-sized fish, those exhibiting SSCP pattern D had a greater growth-related parameters than those exhibiting SSCP pattern C (P < 0.05).

8. Results from DNA sequencing indicated three ($A/A_{72}C/C_{165}$, $G/G_{72}C/T_{165}$ and $A/G_{72}C/C_{165}$) and four ($A/G_{144}C/T_{326}$, $A/G_{144}C/C_{326}$, $G/G_{144}C/T_{326}$ and $G/G_{144}C/C_{326}$) diplotypes corresponding to SSCP patterns A-C and A-D of *IGF-II* (exon 4) and *ActRIIB* genes, were observed This illustrated the potential of SSCP for identification of SNP in interested genes of *L. calcarifer*.

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