การสืบค้นเครื่องหมายโมเลกุลที่เกี่ยวข้องกับการเติบโตของหอยเป๋าฮื้อเขตร้อน *Haliotis asinina*

นางสาวธิดารัตน์ คล่องตรวจโรค

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IDENTIFICATION OF GROWTH-RELATED MOLECULAR MARKERS IN THE TROPICAL ABALONE *Haliotis asinina*

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จากการสืบค้นเครื่องหมายทางพันธุกรรมที่เกี่ยวข้องกับการเติบโตของหอยเป๋าฮื้อเขตร้อน *Haliotis asinina* ในระดับดีเอ็นเอ ด้วยเทคนิค AFLP โดยเบื้องต้นใช้ไพร์เมอร์จำนวน 64 คู่ไพร์เมอร์ และใช้ดีเอ็นเอต้นแบบที่มาจากการรวมดีเอ็นเอด้วยวิธี Bulked segregant analysis (BSA) ของหอยเป๋าฮื้อชุดที่ 1 ซึ่งแบ่งออกเป็น 2 กลุ่มคือ หอยเป๋าฮื้อที่เติบโตเร็ว (FG1, N =10) และหอยเป๋าฮื้อที่เติบโตช้า (SG1, N = 10) พบเครื่องหมายที่คาดว่าน่าจะจำเพาะต่อหอยเป๋าฮื้อที่มีการเติบโตเร็ว และช้าจำนวน 40 และ 43 เครื่องหมายตามลำดับ จากไพร์เมอร์ 44 คู่ แล้วตรวจสอบเครื่องหมายดังกล่าวด้วยการเพิ่มตัวอย่างชุดที่ 2 (FG2, N = 10; SG2, N = 10) พบว่าเครื่องหมายที่เกี่ยวข้องกับการเติบโตนั้นมีจำนวนลดลง โดยแสดงการจำเพาะต่อหอยเป๋าฮื้อที่มีการเติบโตเร็วและช้าเป็นจำนวน 2 และ 7 เครื่องหมายตามลำดับ จากไพร์เมอร์ 7 คู่ และจากการเพิ่มชุดตัวอย่างชุดที่ 3 (FG3, N = 10; SG3, N = 10) เปรียบเทียบร่วมกับตัวอย่างชุดที่ 1 และชุดที่ 2 ไม่พบว่ามีเครื่องหมายใดที่แสดงความจำเพาะต่อหอยเป๋าฮื้อที่มีการเติบโตเร็วและช้า จึงได้เพิ่มจำนวนคู่ไพร์เมอร์อีก 112 คู่ โดยใช้ดีเอ็นเอตัวอย่างชุดที่ 1, 2 และ 3 เป็นดีเอ็นเอต้นแบบ พบเครื่องหมายที่จำเพาะกับหอยเป๋าฮื้อที่เติบโตเร็ว 1 เครื่องหมาย (FGE3M12) ที่มีขนาด 225 คู่เบส จากไพร์เมอร์ *Eco* RI_{+3}3 และ *Mse* I_{+3}12 เมื่อนำเครื่องหมายดังกล่าวมาหาลำดับนิวคลีโอไทด์ และนำไปเปรียบเทียบกับข้อมูลในฐานข้อมูล ไม่พบว่ามีความคล้ายกับยีนใดๆ ในฐานข้อมูลที่เคยมีรายงาน จากนั้นออกแบบไพร์เมอร์จากลำดับนิวคลีโอไทด์ของ FGE3M12 เพื่อเปลี่ยนให้เป็นเครื่องหมาย SCAR นำมาตรวจสอบกับหอยเป๋าฮื้อแต่ละตัวที่เติบโตเร็ว (N = 9) และช้า (N = 9) ด้วยปฏิกิริยาลูกโซ่โพลีเมอเรส พบแถบดีเอ็นเอตามที่คาดหมายทั้งในหอยเป๋าฮื้อโตเร็วและช้า จึงนำมาตรวจสอบ single nucleotide polymorphism (SNP) ด้วยวิธี SSCP พบความแตกต่างในหอยเป๋าฮื้อแต่ละตัวทั้งที่เติบโตเร็วและช้า แต่ไม่พบ SNP ที่ใช้บ่งบอกความแตกต่างระหว่างหอยเป๋าฮื้อที่เติบโตเร็วและช้า

สำหรับการสืบค้นเครื่องหมายทางพันธุกรรมที่มีการแสดงออกแตกต่างกันระหว่างหอยเป๋าฮื้อที่มีการเติบโตเร็วและช้าในระดับ cDNA ด้วยเทคนิค RAP-PCR โดยใช้ไพร์เมอร์ทั้งหมด 43 คู่ไพร์เมอร์ พบเครื่องหมายที่มีการแสดงออกในหอยเป๋าฮื้อที่เติบโตช้าจำนวน 2 เครื่องหมายคือ SGRAP1 และ SGRAP2 โดยมีขนาด 210 และ 260 คู่เบส ตามลำดับ ซึ่งทั้งสองเครื่องหมายนี้ถูกสร้างโดยใช้ไพร์เมอร์ UBC101 และ UBC191 นำเครื่องหมาย SGRAP1 และ SGRAP2 มาหาลำดับนิวคลีโอไทด์และนำไปเปรียบเทียบกับข้อมูลในฐานข้อมูล พบว่ามีความคล้ายกับยีน ADP/ATP carrier protein (E-value=10^{-17}) และ Inosine triphosphatase (E-value=10^{-7}) ตามลำดับ เมื่อออกแบบไพร์เมอร์จากเครื่องหมายทั้งสองเพื่อตรวจสอบการแสดงออกที่แตกต่างกันระหว่างหอยเป๋าฮื้อที่เติบโตเร็ว (N = 5) และช้า (N = 5) โดยใช้วิธี RT-PCR พบว่าไพร์เมอร์ที่ออกแบบจากเครื่องหมายทั้งสองนั้น สามารถบ่งบอกการแสดงออกที่แตกต่างกันระหว่างหอยเป๋าฮื้อที่เติบโตเร็วและช้าได้ โดยไพร์เมอร์ที่ได้จาก SGRAP1 ให้ผลของการแสดงออกในหอยเป๋าฮื้อที่เติบโตช้ามากกว่าหอยเป๋าฮื้อที่เติบโตเร็วอย่างมีนัยสำคัญ ($p < 0.05$) และเช่นเดียวกัน ไพร์เมอร์ที่ได้จาก SGRAP2 ให้ผลการของการแสดงออกในหอยเป๋าฮื้อที่เติบโตช้ามากกว่าหอยเป๋าฮื้อที่เติบโตเร็วอย่างชัดเจน ในการค้นหายีน insulin-related peptide ในหอยเป๋าฮื้อด้วยปฏิกิริยาลูกโซ่โพลีเมอเรส โดยใช้ไพร์เมอร์ที่ออกแบบมาจากบริเวณที่ conserved ของลำดับกรดอะมิโนของ insulin-related peptide จากหอย *Aplysia californica* และ *Lymnaea stagnalis* ในฐานข้อมูล พบว่าลำดับนิวคลีโอไทด์ที่ได้ไม่เหมือนกับยีน insulin-related peptide ในฐานข้อมูล

ภาควิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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KEY WORD: tropical abalone/ *Haliotis asinina*/ GROWTH-RELATED MARKER
THIDARAT KLONGTRUAJROKE: IDENTIFICATION OF GROWTH-RELATED MOLECULAR MARKERS IN THE TROPICAL ABALONE *Haliotis asinina.* THESIS ADVISOR: ASSOC. PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D., 138 p. ISBN 974-53-1251-7

Growth-related DNA markers of abalone *Haliotis asinina* were identified by AFLP analysis. A total of 64 primer combinations was primarily screened with bulked genomic DNA of the first sample set, including fast growing (FG1, N = 10) and slow growing (SG1, N = 10) *H. asinina*. Forty candidate fast growing and 43 candidate slow growing related markers from 44 informative primers were further tested with both the first and the second sample set (FG2, N =10; SG2, N = 10). Only 2 candidate fast-growing and 7 candidate slow-growing related markers were found. To confirm the candidate markers, the third sample set (FG3, N = 10; SG3, N = 10) was tested but no growth-related markers was found. Then, 112 primer combinations were further used. As a result, one candidate of fast-growing related AFLP marker (225 bp) was found from primers *Eco* RI_{+3} 3 and *Mse* $I_{+3}12$ (FGE3M12). However, the sequence of FGE3M13 did not show significant matching to the sequences previously deposited in the GenBank (E-value cut off of 10^{-4}). SCAR marker was developed from candidate growth-related markers. Primers were designed for PCR amplification in genomic DNA of fast growing (N = 9) and slow growing (N = 9) *H. asinina.* The primers generated the expected amplicon in both fast growing and slow growing *H. asinina*. Therefore, the single nucleotide polymorphism (SNP) was further determined by SSCP analysis. The result showed that the SCAR marker was polymorphic, but not growth linked.

RAP-PCR analysis was carried out to isolate various types of expression markers in fast growing and slow growing *H. asinina*. Two slow-growing related expression markers were identified, SGRAP1 and SGRAP2 (210 bp and 260 bp respectively), through screening with 43 primer combinations. The two markers were generated from primers UBC101 and UBC191. SGRAP1 fragment showed a significant match to ADP/ATP carrier protein (E-value=10^{-17}), while the SGRAP2 fragment was homologue to inosine triphosphatase (E-value=10^{-7}). RT-PCR analysis was carried out using primers designed based on sequence of SGRAP1 and SGRAP2 markers. The analysis showed that the level of expression of SGRAP1 in slow-growing *H. asinina* was statistically significant than that of the fast-growing ones ($p<0.05$) and the level of expression of SGRAP2 in slow-growing *H. asinina* was obviously higher than that of the fast-growing ones. For the identification of insulin-related peptides homologue in *H. asinina,* using degenerated primers designed from insulin like peptides of the gastropod mollusks, *Lymnaea stagnalis* and *Aplysia californica,* the homologue in *H. asinina* was not identified.

Department Biotechnology Student's signature .....
Filed of study Biotechnology Advisor's signature .....
Academic year 2004 Co-advisor's signature .....

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Now, I would like to devote the final place here to my beloved family for being such a great support and the forever source of love and care to me. You are everything of mine. Thank you of being you.

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LIST OF ABBREVIATIONS

°C	degree celcius
μl	microlitre
μM	micromolar
bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid (disodium salt)
EtBt	ethidium bromide
IPTG	isopropyl-thiogalactoside
kb	kilobase pair
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mmol	millimole
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TE	tris EDTA
Tris	tris (hydroxy methyl) aminomethane

CHAPTER I

INTRODUCTION

1.1 General Introduction

Abalone as a member of univalve gastropod mollusks, has been economically important for many thousand years. Despite its costly price, abalone is increasingly popular worldwide due to its rich taste and high nutrients. Also, some groups of people such as the Chinese strongly believe that abalone is the best nourishment for a healthy life as it increases immunity in human beings.

The reason why the taste of abalone is considerably different from other seafood is its biological composition. Apart from its high protein at 18% and its very low cholesterol (Table 1.1), it specially consists of two kinds of amino acid, glutamic acid and glycine. Moreover, it has 5´nucleotide (AMP). All of the three elements help bring about the taste called *umami*, which is similarly tasteful as monosodium glutamate. Besides, the shell has very high stoma protein as mentioned above. And since the protein has some elements of collagen, the meat of the shell is magically soft and it is the softness that always calls most tasters back again to taste this expensive shell, abalone.

Additionally, abalone is not important only in the kitchen, but it also has some minor roles in ornament factory and decoration industry. Indeed, the shell of abalone can be also fashioned as accessories and other adornment things due to its unique

beauty. This can somehow support abalone trading in the world and helps strengthen abalone's popularity in a way.

Table 1.1 Nutrition information of abalone meat (100 g)

83 calories	35 mg magnesium
18 g protein	95 mg tin
0.1 g fat	0.01 mg vitamin B1
2.7 g carbohydrates	0.27 mg vitamin B2
59 mg cholesterol	0.07 mg vitamin B6
182 mg sodium	4.93 mg vitamin B12
229 mg potassium	0.62 mg vitamin E
16 mg calcium	0.1 mg nicotine
0.5 mg iron	8 mg folacin
0.9 mg zinc	

Nowadays, the outstanding producers of abalone in the world are Japan, China, Australia, the United States of America, New Zealand and South Africa. The biggest consumers are Hong Kong, China, and Japan. They consume 200-300 million USD per year, as showing in details in Table 1.2. Abalone is a highly valued species and will command as much as $100 per kilo (in the shell) or $35 per can.

Table 1.2 The countries importing abalone, ranked by the price.

Country	Type	Tonnes	value ($000's)	$Per kilo
China/Hong Kong	Meat canned	1,344	$110,130	$82.81
Japan	Meat canned	542	$40,705	$75.11
Japan	Meat frozen	341	$22,300	$65.39
China/Hong Kong	Meat frozen	687	$44,657	$65.00
USA	Meat canned	335	$20,100	$60.00
Japan	Fresh or chilled	419	$25,124	$59.96
USA	Meat frozen	184	$9,570	$52.02
Taiwan	Meat canned	649	$29,933	$46.13
China/Hong Kong	Fresh or chilled	790	$33,211	$42.06

Available at:
http://www.pir.sa.gov.au/pages/aquaculture/species_profiles/market_asses.pdf

To produce abalone, basically we have two ways: fisheries and cultures. However, since 1975 the number of fisheries has been greatly decreasing because of the downgrade of abalone natural habitats and a lack of a good management on the resources. At present, many countries are interested in developing abalone commercial aquacultures to its most effectiveness. So far, the quantity and quality of the cultures has been developed. The big producers are China and Taiwan (Chen, 1989). And from the most recent information, the total world production of abalone is estimated to be approximately 13,000 metric tons in 1999 from both fishery and aquaculture sectors. Abalone products are usually in fresh (with shell), frozen, canned and dried form.

Though 13,000 metric tons seem to be a great amount of production, nowadays abalone is still not produced enough for the vast market demand. Indeed, it

has been predicted that a potential demand and a supply in 2004 both of which are clearly not compatible, as shown in Fig. 1.1.

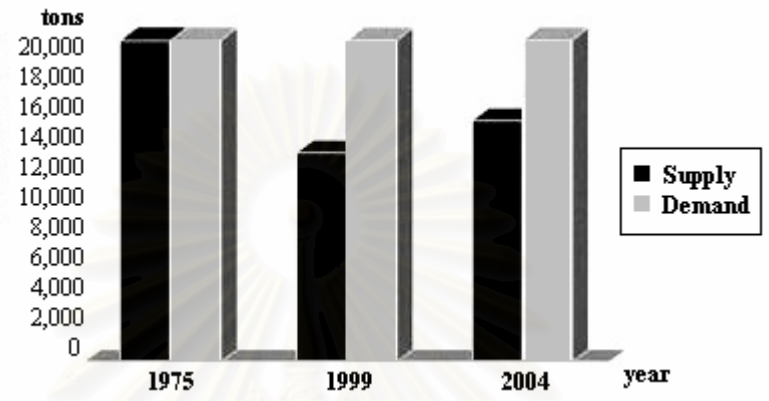

Figure1.1 Demand and supply of abalone (tons) (http://www.fishtech.com).

So far, approximately 75-100 species of abalone have been discovered both in tropical and temperate zones, but only around 20 species of all are noteworthy in terms of economic value (Table 1.3).

Table 1.3 Commercially important abalone species (Jarayabhand and Paphavasit, 1996).

Scientific name	Common name	Shell length (mm)
H. rufescens	Red	>275
H. fulgens	Green, southern green or blue	125-200
H. corrugata	Pink or corrugated	150-175
H. sorenseni	White or sorensen	125-200
H. assimilis	Threaded	<100
H. cracherodii	Black	75-125
H. walallensis	Flat or northern green	75-125
H. kamtschatkana	Pinto	100
H. discus hannai	Ezo awabi	180-200
H. discus	Kuro awabi, oni or onigai	200
H. diversicolor	supertexta* Tokobushi	50
H.gigantea	Madaka	250
H. sieboldii	Megae	170
*H. asinina**	Mimigai, donkey's ear	70-100
H. rubra	Black lip	120-140
H. laevigata	Green lip	130-140
H. roei	Roe's	70-80
H. iris	Paua or black	170
H. australis	Silver or queen paua	125
H. virginea	Virgin	70
H. tuberculata	Ormer	120
H. midae	Perlemon	90

* Tropical species

In Thailand, it is reported that there are three different species of tropical abalone: *Haliotis asinina*, *H. ovina* and *H. varia* (Nateewathana and Hylleberg, 1986). Among the three species, *H. asinina* is the most promising species for the culture industry due to its potential in culture, its biggest size, environmental tolerance and beneficial percentage ratio of meat weight to total weight of 85% (Singhagraiwan and Doi, 1993) (Fig. 1.2). Additionally, *H. asinina* has the fastest growth rate among the three abalone. It can reach the needed size to be sold in the market (60 mm) rather fast, only a year.

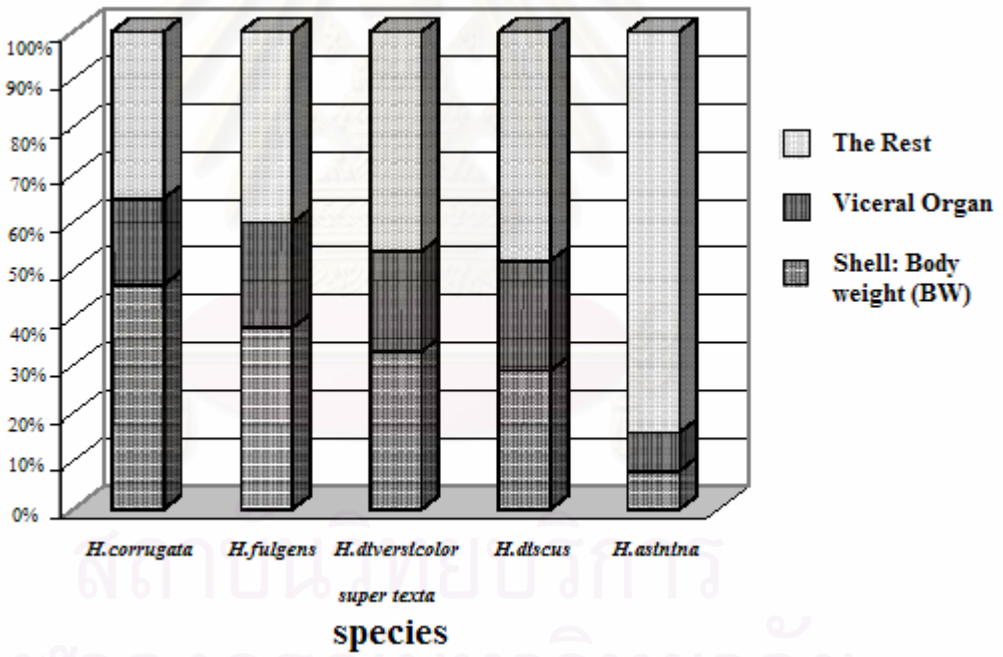

Figure 1.2 Eatable portion of *H. asinina* (http://phuketabalone.com).

From 1987 to 2003 Department of Fisheries has been doing research about *H. asinina* abalone culture in Thailand. As a result, the culture system has been respectively developed. Nowadays the shell can be sorted more effectively and be successfully cultured though without its wild broodstock, that can be a great asset to abalone commercial culture in Thailand to develop more and more in the future.

In terms of commercial benefits, Thai *H. asinina* is an attractive target to invest because the species is found very appropriate to commercially culture. It can be produced in two sizes, cocktail size (20-25g), sold at $25-38 per kilo for alive ones, and steak size (100g), sold at $60- 100 per kg for alive ones.

For its growth rate, the shell needs 8-12 months to be at cocktail size and another 1-1.5 year to be a steak size, the more expensive one. This is a big advantage over the Taiwanese species *H. diversicolor supertexta,* which needs 4-5 years to be at steak size. Also, within one year, the abalone can be mature in terms of sexuality, which is considered a great benefit. In addition, this species spawns throughout the year. What is more, today the spawning cycle is precisely predictable, resulting in more easiness in planning a commercial breeding program.

However, most of abalone food in Thailand is imported from foreign countries in terms of frozen and canned shells. Only some of it is from Thailand itself and the shells are not in a good quality and the quantity of them is not very certain. Nowadays, abalone commercial culture in Thailand is still limited because the shell needs more than a year to be cultured for a market size. Also, it needs more information and research about the biology, ecology, nutrition (both algal and artificial diets for the shell), grow-out technique, biotechnology (production of triploid abalone) and genetic (selection and hybridization) for the brighter future of tropical

abalone in Thailand. It can be clearly seen that it is very necessary to do more research for development, by using techniques, to increase not only the quantity but also the quality of Thai abalone, from both fisheries and cultures, and to decrease budget and risk in culture, all of which will help decrease the number of imports and increase the number of exports of the shells and consequently support Thai economy for the whole picture in the future.

1.2 Biology and ecology

1.2.1 Taxonomy of *Haliotis asinina*

The taxonomic definition of the tropical abalone, *H. asinina* is as follows (Hahn, 1989)

Kingdom: Animalia

Phylum: Mollusca

Class: Gastropoda

Subclass: Prosobranchia

Order: Archeogastropoda

Suborder: Zygobranchia

Superfamily: Pleurotomariacea

Family: Haliotidae

Genus: *Haliotis*

Species: *asinina*

Scientific name: *Haliotis asinina* (Linnaeus, 1758)

Common name: Mimigai, donkey's ear

1.2.2 Morphology and anatomy

Haliotis asinina is a member of the univalve gastropod mollusks. The name of the genus, Haliotis, stands for “sea ear” which clearly suggests the shape of the shell - if one sees the shell at its appearance, one will see it in the shape of spiral which can bring one’s mind to think of a typical snail with a little flatter shape.

Apparently, the color of the shell from the outside is greenish brown. Also, the area is rather tough, whereas, the inside part appears more smooth and pearly. In addition, the shell of the gastropod covers almost all the body of the shell and appears to have a row of holes around the left-hand side of the shell. In details, the holes are respectively bigger and bigger to the shell’s head so the biggest hole turns up to be the anterior holes around the head, while those holes at the back are often blocked. Anyway, these pores are functioned for respiration, waste removal and gamete releasing

Down to the foot, the foot of an abalone is in fact only a large muscle. Abalone uses this muscle in crawling on the seabed, as other typical snails. However, the shell does not use it moving on the sand. If it does, it might accidentally turn over itself and will finally victimize its life as a prey since the foot of abalone is not very suitable in using on such a place. That is why we can basically find abalone on hard rock and coral rather than sandy beach and so forth. What is more about the foot, there are some parts of the foot that are not totally covered by the shell. Indeed, the special revealed area amazingly looks like a pair of lips and is, as a result, often referred to as such similarity.

Besides the foot, next to the upper lip, there appears a series of tentacles, which should, presumably, be used to detect predators and food either through the sense of touch or taste. Next to the foot is the head. The head is typically snail-like, appearing with another series of tentacles as at the lips. These head tentacles are bigger, similarly to the eyestalks of land snails.

As for its mouth, the mouth is at the head below the lips. The mouth is a circular orifice amidst a piece of flesh called the oral disc. It has no teeth, but does have something tongue-like covering with special teeth called radula thier. The radula is for rasping food.

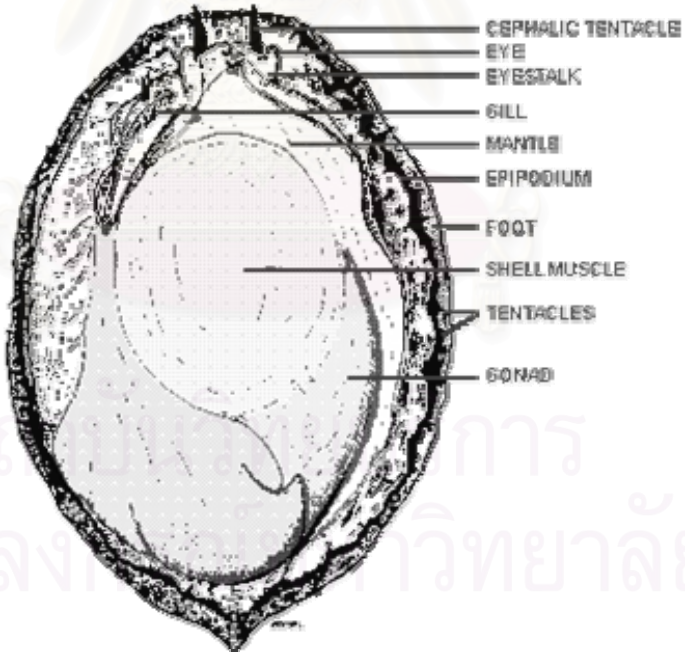

Figure 1.3 Anatomy of an abalone with shell removed (COX, 1962).

The shell's other internal organs are well arranged around the foot as well as underneath the shell (Fig.1.3).

Firstly, the gill chamber is located beside the mouth and under the respiratory holes. In respiration, water is first to be drawn in under the edge of the shell, and, after that, it will flow over the gills and at last out through the pores. As for waste and reproductive products, they are to be carried out along with this flow of water as well.

Coming to the most conspicuous organ, the shell's gonad is shaped as crescent. Its color can range from blue, green, to brown in female and always be creamy in male. However, both sex in immature age have their gonad in gray. The organ is located around the side of the shell but opposite the pores and extends itself to the rear of the abalone.

For its brain, as abalone has no obvious brain shape, it is considered a primitive animal. However, it does have a heart on its left side and have blood flows through the arteries, sinuses, and veins, assisted in the flowing by the surrounding tissues as well as muscles.

1.2.3 Reproduction

Abalone is not unisex. It does have separate sexes – male and female (Fig. 1.4). In reproducing, the shell releases sperms as well as eggs lay on the sea, letting high gamete densities to achieve fertilization. A reproductive strategy densely requires aggregated adults for success. When the gametes fuse, the fertilized egg will divide repeatedly and continuously and at last form a larva. The larvae will then go via a series of changes into a body form. The series of changes is called in terms of trochophore and veliger stages. The larvae are freely swimming for about a few days.

In this stage, they feed on plankton until their shells start to form a better shape. And when the shell forms, the juvenile abalones sink to the bottom clinging to rock and crevice with their single strong foot while feed on rock-encrusting coralline algae as well as on diatom and bacterial films. Once the shells are settled, they eat, grow and develop themselves to be adult abalones. And when they come to this stage, they will feed essentially on loose pieces of marine algae drifting with the surge or current. After the abalones start to develop and then become sexually mature. Finally, the cycle repeats itself. Most abalone species become grown-ups during the first and third year of their growing. And then, each abalone can last its life longer than 10 years.

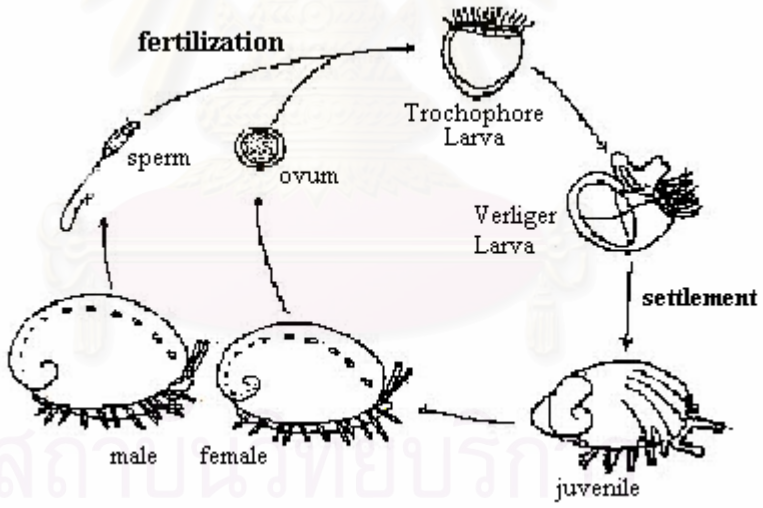

Figure 1.4 Life cycle of abalone (http://www.abalone.net).

1.2.4 Habitat and distribution

Their bodies' nature constrains them to live in certain appropriate habitats. Also, they have to behave properly in a certain way in those habitats. Geiger (2000) informed that *H. asinina* can be found in more than 100 places all over South East Asia, Japan and Australia (Fig. 1.5). As for Thailand, *H. asinina* can basically be found along the coasts of the upper Gulf of Thailand as well as the Andaman Sea (Nateewathana and Bussarawit, 1988).

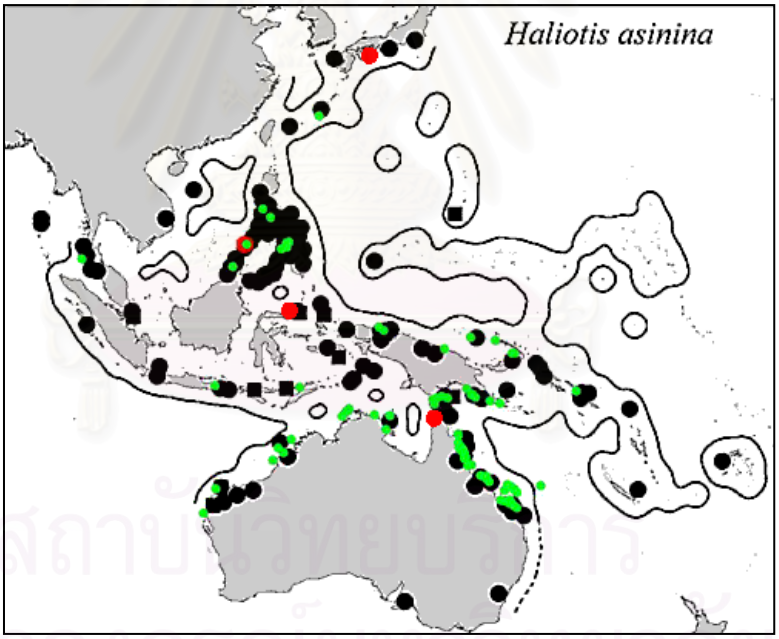

Figure 1.5 World wide distributions of abalone, *Haliotis asinina* (Geiger, 2000).

1.3 Molecular genetic techniques used in this thesis

Every organism is deem to mutate, as an ordinary way of them having normal cellular operations or interactions with the environment, which usually leads to genetic variation called polymorphism. In conjunction with selection and genetic drift, there arises genetic variation within and among individuals, species, and higher order taxonomic groups. For this variation to be useful to geneticists, it must be not only heritable but also discernable to the researcher, whether as a recognizable phenotypic variation or as a genetic mutation distinguishable by using molecular techniques.

To distinguish it, molecular genetic markers should be an effective tool since it can be adapted in a wide variety of disciplines ranging from forensics to evolutionary biology as well as applications in medicine, agriculture, and aquaculture. In the past, allozyme and mtDNA markers have been well known in genetic research. And nowadays, several marker types are also highly popular in genetics study. More recent marker types is also used for identification of genetic markers at the genomic DNA and the cDNA level. In the level of DNA, it includes amplified fragment length polymorphism (AFLP), single-strand conformation polymorphisms (SSCP) and DNA sequencing. As for in the level of cDNA, there are reverse transcription-PCR (RT-PCR) and RNA arbitrarily primed PCR (RAP-PCR), for instance.

1.3.1 Polymerase Chain Reaction (PCR)

Since PCR has been developed by Mullis et al. (1986), it has become a profound impact on molecular biology as well as has great potential as an important tool in detecting genetic polymorphism.

PCR is employed in the *in vitro* amplification of DNA at the logarithmic scale. Also, various components of the PCR reaction, such as Taq DNA polymerase, assay buffer, deoxynucleoside triphosphates, stabilizing agents, and primers greatly help make it possible for the DNA template to be sufficiently amplified *in vitro* to attain detectable quantities. Besides, PCR can generate a large number of copies of a specific genetic region from a small number of molecules. All of its benefits make clear why it is widely used in molecular biological studies.

In details, the region of the molecules to be amplified is called the target (possibly up to 10,000 base pairs long). In order to perform PCR, the nucleotide bases for two short flanking regions on either side of the target (usually 20–25 base pairs long) must be known. They are used to design primers for PCR.

A PCR cycle consists of three steps. The first step is called denaturation. In the step, the double-stranded DNA molecules are separated into single-stranded ones which are used as templates for the next two steps, the second step is called annealing. Primers anneal to the templates. During the third step which is called polymerase extension, DNA polymerase extends the annealed primers using the single-stranded molecules as templates. The process is repeated for many cycles (Fig. 1.6).

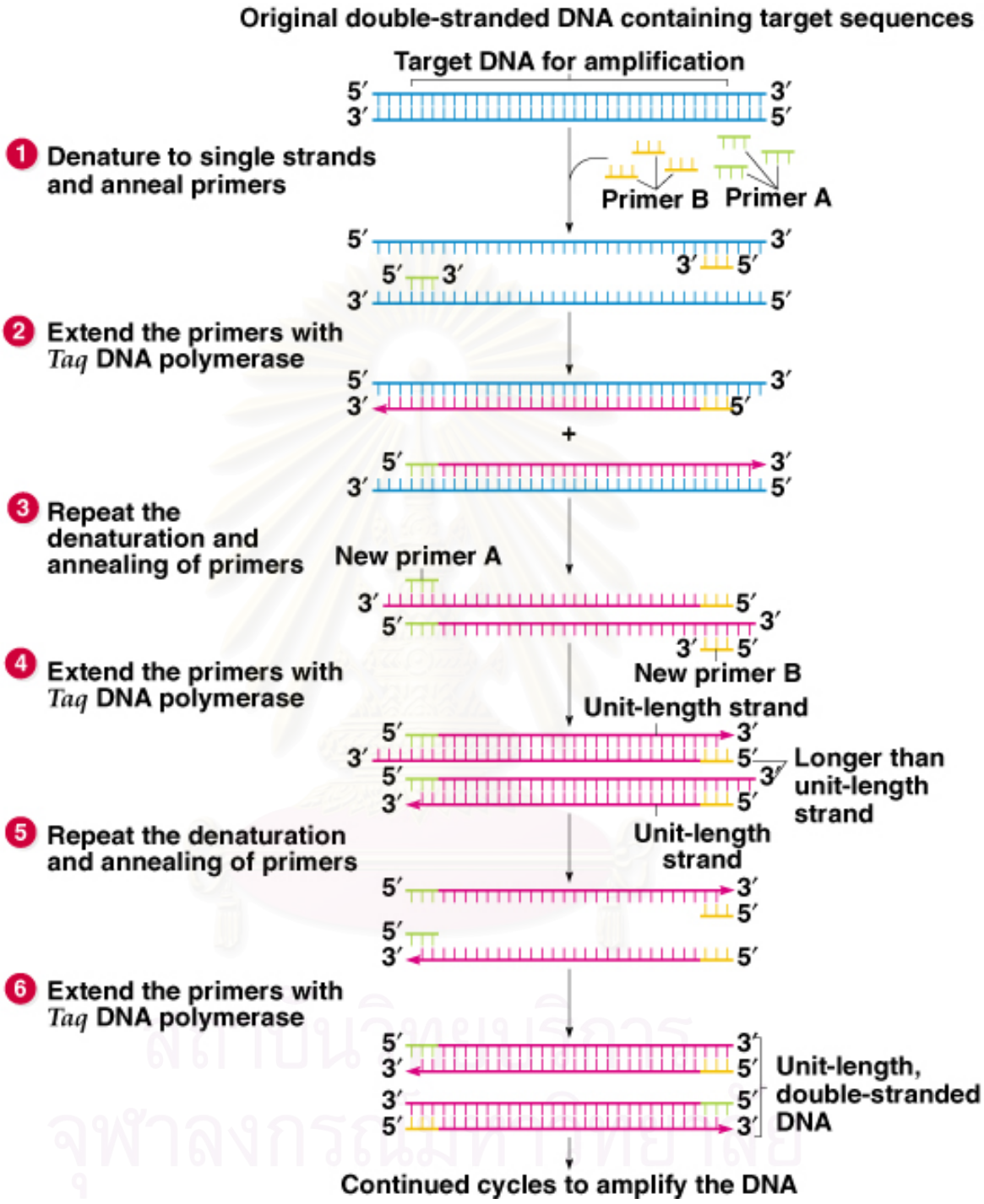

Figure 1.6 General illustration of the polymerase chain reaction for amplifying DNA.

1.3.2 Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a technique used in genome fingerprinting. The technique is based on the particular amplification of a subset of DNA fragments which are generated by restriction enzyme digestion. In details, DNA is digested with restriction endonucleases, and double-stranded DNA adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective nucleotides extending into the restriction fragments are added to the 3´ ends of the PCR primers, only in case a subset of the restriction fragments is recognized. In other words, only restriction fragments where the nucleotides flanking the restriction site match the selective nucleotides will be amplified. After that, the subset of amplified fragments will be analyzed by using denaturing polyacrylamide gel electrophoresis to generate the fingerprint (Fig. 1.7).

AFLP markers are not only highly polymorphic but also very reproducible; thus, they are considered so efficient a technique in analyzing DNA that the tool has revolutionized fingerprinting and diversity studies (Vos et al., 1995). Also, AFLP analysis can be used in detecting genetic variation throughout the genome. In doing that, it uses a pair of specific restriction enzymes and their corresponding adapters. The polymorphism is detected by using a number of selective bases following the restriction site. After the use, primers with one or no selective base then play the important role in a round of the first PCR reaction called preamplification. This reaction is diluted for use in a second round of PCR where primer pairs with 2 or 3 selective bases are used. In order to visualize the patterns, one of the PCR primers

contains either a radioactive or a fluorescent label, or, alternatively, the gels may be stained with ethidium bromide and the patterns may be examined under UV. However, we can also use the gel to see the patterns by using silver staining either.

Indeed, as AFLP combines the strengths of RFLP and RAPD, it requires a small amount of DNA as its first advantage. Also, it does not require any known sequence information or probes. Furthermore, it overcomes the problem of low reproducibility of RAPD. In addition, AFLP is able to produce the large number of polymorphic bands in a single analysis which significantly makes the price in experiments more economical as well as it helps make genetic analysis of close-related population possible.

The DNA polymorphisms identified by using AFLP are commonly inherited in Mendelian fashion and widely used in plant analysis as well as animal genetic mapping. Also it can be applied in medical diagnostics, phylogenetics studies and microbial typing. For instance, it was effectively employed in mapping QTLs for submergence tolerance in rice (Nandi et al., 1997), genotyping of *Campylobacter* strains isolated from poultry and humans (Duim et al., 1999), identification of characteristics genetic markers if they are able to distinguish the differences in chicken growing (Fumier et al., 2003), and, finally and recently, genome mapping in the Pacific oyster *Crassostrea gigas* Thunberg (Li and Guo, 2004)

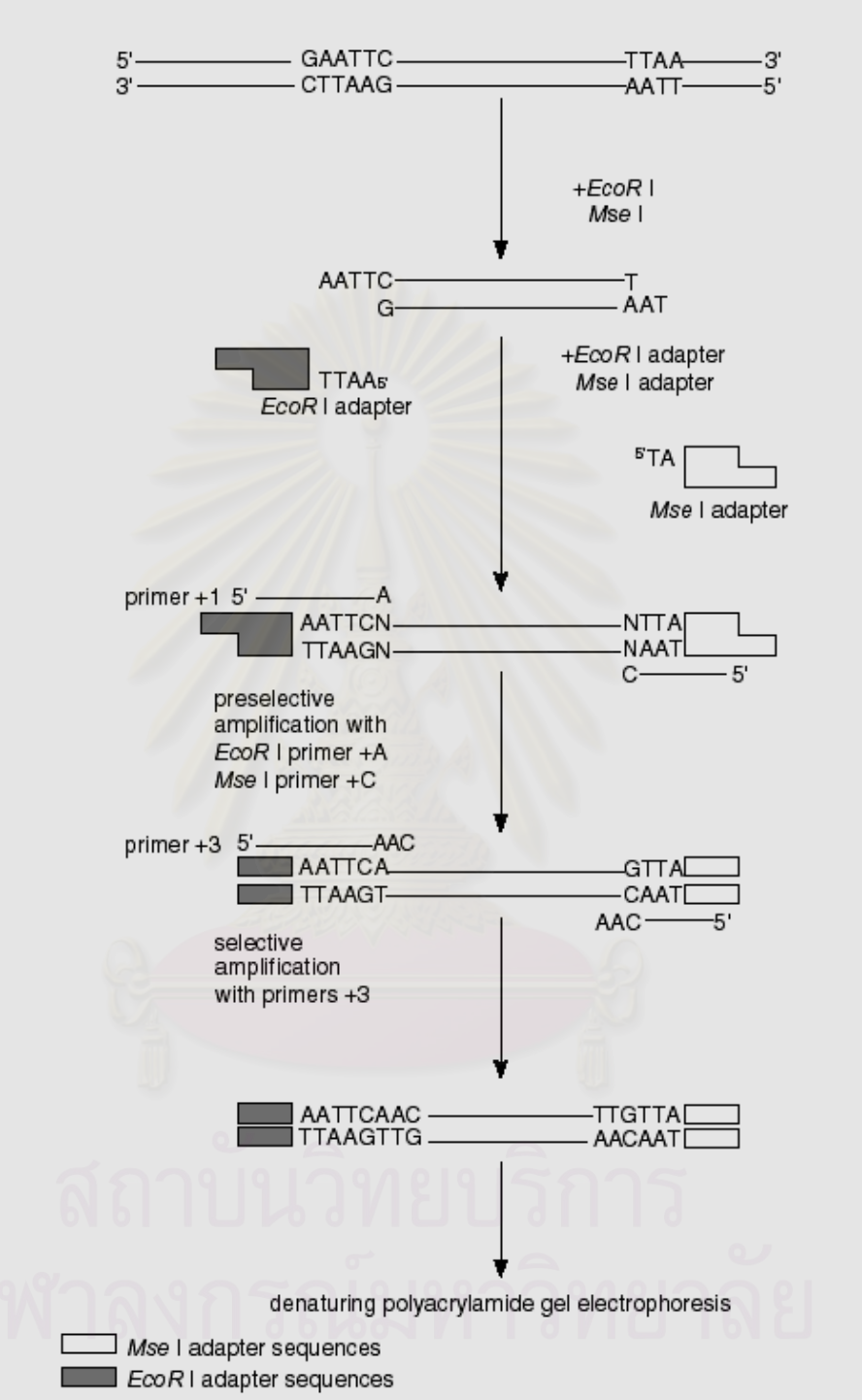

Figure 1.7 Flow chart of steps required for an AFLP analysis (AFLP® analysis System I, GIBCO/BRL).

1.3.3 Single-strand conformation polymorphisms (SSCP) analysis

In detecting mutation and analyzing variation, SSCP is the easiest and most-employed technique. After using the technique PCR in amplification of the specific place of interest, the aftermath DNA will be worked more. It will be denatured to create single-stranded molecules and loaded on non-denaturing gel (Orita *et al.,* 1989) (Fig. 1.8). In details, these mutations, after using the technique, will appear as new bands by using autoradiograms (radioactive detection), or silver staining of bands or the use of fluorescent PCR primers, which are subsequently detected by an automated DNA sequencer (non-radioactive detection).

Single-stranded DNA folds differently, depending on each single base. Also, some scientists believe that mutation-induced changes of tertiary structure of the DNA bring about differences in terms of mobility for each DNA strand. As for the non-denaturing gel, the composition and the running conditions of its can be varied: alteration of the temperature or the degree of cross-linking or the adding of glycerol or sucrose.

The technique SSCP is very suitable and effective as it is considered a sensitive, inexpensive, convenient, and rapid method for detecting sequence variation (Sekiya, 1993; Slabaugh et al., 1997; Sunnucks et al., 2000). To talk about more uses of SSCP, this technique has also been employed to characterize expressed products between two high-related member genes (Hedley et al., 1994), analyze the difference among members of a gene family (Hagiwara et al., 1998; Slabaugh et al., 1997), and produce SSCP markers for gene mapping (Slabaugh et al., 1997). Recently, the usefulness of this approach has been extended through the use of end-labeled primers and PCR amplification, by using a small amount of genomic DNA or RNA (Peterson

et al., 1995; Slabaugh et al., 1997). Moreover, it can use in analyzing the polymorphism of the goat growth hormone gene in 5' regulatory sequence (Li et al., 2004). Furthermore, recently SSCP screening can use in revealing a common DeltaC polymorphism, 254 bp 5' to the transcriptional start site (Smith et al., 2005).

However, this technique has some limitation in using. Temperature and degree of cross-linking affect SSCP. Also, the size of the PCR product may affect the sensitivity. Basically, the smaller the product the higher the sensitivity; therefore, the size should be limited in the optimum being 200-300 bp. (Dean and Milligan, 1998).

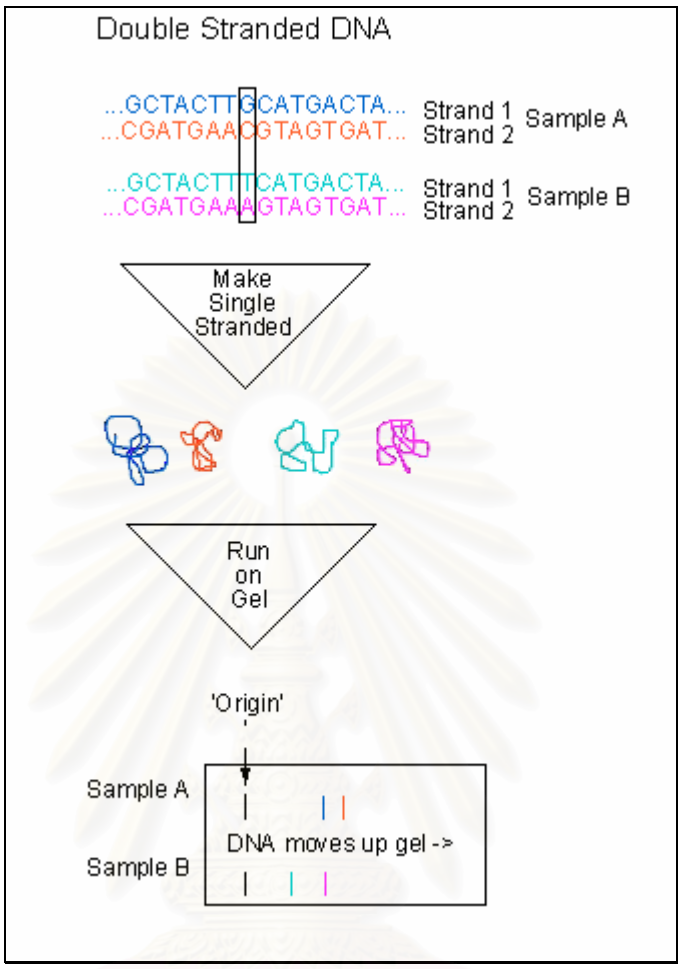

Figure 1.8 Schematic diagram-illustrating principles of SSCP analysis.

(http://www.amonline.net.au/evolutionary_biology/tour/sscp.htm)

1.3.4 DNA Sequencing

Polymorphisms at the DNA level can be studied by several methods. However, the most direct approach is determination of nucleotide sequence of a defined region. DNA sequencing provides not only a highly reproducible but also informative analysis of data (Weising et al., 1995).

To achieve sequencing of DNA, either chemical or enzymatic means can be employed. For the former one, the original technique for sequencing, Maxam and Gilbert sequencing, relying on the nucleotide-specific chemical cleavage of DNA, nowadays is not commonly used any more. On the other hand, the latter one, the enzymatic technique, Sanger sequencing, involves the use of dideoxynucleotides (2', 3'-dideoxy) that terminate DNA synthesis and is, therefore, also called dideoxy chain termination sequencing.

The Sanger DNA sequencing protocol utilizes dideoxynucleotides (ddNTPs) to terminate chain elongation during the *in vitro* synthesis of DNA from a cloned template. Synthesis is using a specific oligonucleotide primer initially. During the synthesis reaction a radioactive nucleotide, usually dATP, is incorporated into the elongating strands. Four separate reactions are carried out simultaneously, each of which contains all 4 dNTPs and a single ddNTP by the higher the concentration of ddNTP the more frequently chain elongation will terminate. As a result, one can regulate the extent of sequence information obtainable by varying the dNTP/ddNTP ratio. After following the extension reactions, the products are resolved by electrophoresis in a denaturing (urea) polyacrylamide gel. The results are obtained when the gel is dried and exposed to x-ray film. Bands near the bottom of the gel

represent short reaction products, i.e. closest to the 3'-end of the primer and those near the top the longest products.

Another possibility, automated sequencing machines can use ddNTPs that are each tagged with a different color fluorescent dye. Instead of performing four different reactions, DNA synthesis occurs in one tube. Then, the sequencing machine uses a light sensor to read the gel, identifying the bases by their different colors.

Additionally about the method, DNA sequencing is significantly appropriate for population genetic studies of various species. Not only that the DNA sequence of each individual is directly compared but also that the results of the level of genetic diversity between individuals within geographic samples, between geographic samples and species are particularly reliable. Besides, DNA sequencing can de used in coupling with RAPD and AFLP markers to convert dominant marker to sequence characterized amplified region (SCAR) marker that are co-dominantly segregated as well.

1.3.5 Reverse Transcription-PCR (RT-PCR)

RT-PCR is a rapid and quantitative method in analyzing the level of expression of genes. This technique utilizes the ability of reverse transcriptase (RT) to convert RNA into single-stranded cDNA and couple it with the PCR-mediated amplification of specific types of cDNAs presented in the RT reaction. The cDNAs produced during the RT reaction represent a window into the pattern of genes that are being expressed at the time the RNA was extracted. Total cellular RNA can be extracted from tissues or cells by any of several techniques and then used as a template for RT. In most cases, the RNA is primed using random primers. A small

aliquot of the RT reaction is then added to a PCR reaction containing primers specific to the sequences that one would like to amplify. Then, the products of the RT-PCR can be visualized as mentioned above.

1.3.6 RNA arbitrarily primed PCR (RAP-PCR)

RNA arbitrarily primed PCR (RAP-PCR) is one of the efficient techniques used in screening differential gene expression. Indeed, it is common to use the technique in identification of distinguishable phenotypes. Also, the tool can be used in detecting tissue and cell types, responses to hormones, growth factors, stress, and heterologous expression of certain genes.

RAP-PCR employs either single arbitrary primers (Welsh, 1992) or random hexanucleotide mixtures (Abu et al., 1996) in the first strand cDNA synthesis reaction. As for the second strand cDNA synthesis, we will use subsequent PCR in creating it by using one or two arbitrary primers. Due to low stringency temperatures, the arbitrary primer is able to bind at random sites within the template that show limited, but not complete complementarily

RAP-PCR provides many advantages. Firstly, it is simple and can be used to compare the fluctuations in gene expression between multiple samples simultaneously using small amounts of RNA. Moreover, since RAP-PCR employs short arbitrary primers, no prior sequence information about the organisms is needed. In addition, RAP-PCR can yield information on the overall patterns of gene expression between different cell types or between different physiological conditions of the same cell type. Comparison of the RAP-PCR fingerprints from these different experimental groups permits one to draw inferences regarding the overall cellular states of gene

expression and the interrelation between gene transcripts belonging to the same or different regulatory pathways (Perucho et al., 1995). The technique can be applied in many ways. For example, it investigated sex-specific gene expression in the nodule worm, *Oesophagostomum dentatum* (Strongylida) (Boag et al., 2000); identified differentially expressed genes during the development of the basidiomycete Lentinula edodes. Even more, recently it was employed to detect modification of mRNA expression in the freshwater bivalve *Unio tumidus* (Rodius, 2004).

1.4 Genetic study in abalone

Huang et al. (1997) sequenced growth-promoting genes of the blacklip abalone, *Haliotis rubra*. Two variable number of tandem repeats (VNTRs) were identified in abalone cDNA libraries. One contained a 33 bp repeat unit located in the 3′ untranslated region of a putative growth hormone (GH) gene. The other contained an 18 bp unit located in the 3′ untranslated region of a putative molluscan insulin-related peptides (MIP) gene. The designated primers were used in a primary population study of 100 blacklip abalone. All in all, it was found that the first tandem repeats included 7-20 repeats and the most frequent alleles are 16 and 17 repeats, whereas, another tandem repeats were found 4, 5, 6 and 7, and the most frequent ones are 5 and 6 repeats. It was concluded that these DNA minisatellites were useful in abalone study.

Muchmore et al. (1998) performed direct sequencing of genomic DNA for characterization of a satellite DNA in five species of eastern Pacific abalone. Phylogenetic trees of the consensus satellite sequences had the same topology as trees constructed for two abalone sperm acrosomal proteins. In 12 randomly picked clones

of the Red abalone (*H. rufescens*) *Sal* I satellite, 16 positions varied. In the Red abalone, the 290 bp *Sal* I satellite represents approximately 0.5% of total DNA. The species-specific consensus sequence of this satellite provides a molecular marker that could be used for identification of hybrid parentage, taxonomy, population identification, and forensic studies.

Huang et al. (2000) analyzed genetic structure of the populations of the blacklip abalone *Haliotis rubra* along Victoria, Australia by employing three types of molecular markers. The DNA markers contained 84 randomly amplified polymorphic DNA (RAPD) bands. They were amplified by using six random primers, which are two minisatellites (GHR and MIPR) and three microsatellites (RUBGT1, RUBCA1 and RUBGACA1). All of the three types of DNA markers showed significant subdivision in the *H. rubra* populations along the coastline. The genotypes of microsatellites indicated excessive homozygotes across all the populations at all three microsatellite loci.

Selvamani et al. (2001) genotyped individual *Haliotis asinina* Larvae by analyzing a set of polymorphic microsatellite loci. The analysis revealed that a single male fathered most of the veligers. And after observing, it suggested that highly controlled breeding practices may be required to ensure that the genetic diversity of an abalone population produced for aquaculture is maintained at the level of diversity of the original broodstock.

Li et al. (2003) developed microsatellite markers for a variety of abalones and recorded locus-specific homozygote excesses at population level for microsatellite loci and studied the mode of inheritance of 7 microsatellite loci in 4 families with a reciprocal cross of 2 females × 2 males to ascertain whether null alleles exist at

microsatellite loci in the Pacific abalone. All loci segregated codominantly, but only 3 loci *(Hdh1321, Hdh78,* and *Hdd108C)* confirmed to Mendelian segregation and can be used for parental analysis and population genetic studies. Two loci *(Hdh1761* and *Hdh1457)* confirmed Mendelian expectations in all families, while the remaining 2 loci *(Hdd114B* and *Hdd229)* showed deviation from Mendelian segregation in at least one family even though null alleles were considered. These results need further study to test the inheritance pattern for microsatellite markers in abalones before using them for population genetic or parentage analysis.

Klinbunga et al. (2003) analyzed genetic diversity of abalone in Thailand, which are *Haliotis asinina, H. ovina,* and *H. varia*, by polymerase chain reaction (PCR) of 18S and 16S rDNAs, with randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP). Species-specific RAPD markers were found in each abalone species. A dendogram obtained by the unweighted pair-group method revealed reproductively isolated gene pools of these abalone and indicated that *H. asinina* and *H. ovina* are genetically closer than *H. varia*. It was indicated that there was clear genetic differentiation between *H. ovina* originating from the Andaman Sea (west) and the Gulf of Thailand (east, $P < 0.0001$), whereas partial differentiation was observed between the Philippines and the remaining *H. asinina* samples ($P < 0.0021$). Species-specific PCR based on 16S rDNA polymorphism was successfully developed in *H. asinina* and *H. varia* but not in *H. ovina.*

Klinbunga et al., (2004) identified the species- and population-specific markers of abalone; *Haliotis asinina, H. ovina* and *H. varia* in Thai waters by a randomly amplified polymorphic DNA (RAPD) analysis. Fifteen species-specific and

six population-specific RAPD markers were identified. In addition, a 1650 bp band (UBC195) that was restricted to *H. ovina* from the Gulf of Thailand (east) was found. Twenty pairs of primers were designed and specificity-tested. Seven primer pairs (CUHA1, 2, 4, 11, 12, 13, and 14) were specifically amplified by *H. asinina* DNA, whereas a single pair of primers showed specificity with *H. ovina* (CUHO3) and *H. varia* (CUHV1), respectively. Four primer pairs, including CUHA2, CUHA12, CUHO3, and CUHV1 were further examined against 216 individuals of abalone. Results indicated the species-specific nature of all of them, except CUHO3. And CUHA2 can successfully identify the species-origin of the frozen, ethanol-preserved, dried and boiled *H. asinina.*

As described above, there have been several studies about genetic of abalone. However, the characteristics of growth in Thai abalone are still not studied yet. Therefore, this thesis aims to identify growth-related molecular markers in the tropical abalone *Haliotis asinina* and develop the markers to determine the differential growth of abalone *Haliotis asinina* in the hatchery.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- Automatic micropipette P20, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
- Electrophoresis apparatus
  - : Submerged agarose gel electrophoresis system, GelMateTM (Toyobo Co. Ltd., Japan)
  - : Vertical sequencing gel electrophoresis apparatus (Hoefer, USA)
  - : Power supply: Power PAC 3000 (Bio-RAD Laboratories, USA)
- -80^{o}C Freezer (REVCO, USA)
- -20^{o}C Freezer (Whirlpool)
- Incubator BM-600 (Memmert GambH, Germany)
- Light box 2859 SHANDON (Shandon/ scientific Co. Ltd., England)
- Microcentrifuge tube 0.2, 0.5, 1.5 ml (AxyGen, Inc., USA)
- Orbital shaker S03 (STUART SCIENTIFIC, UK)

- PCR Thermal cycler

    : Omnigene-E (Hybaid Limited, England)

    : Mastercycler (Eppendorf, Germany)

    : GeneAmp PCR system 9700 (Perkin Elmer, USA)

- Pipette tips 10, 20, 100, 1000 μl (AxyGen, Inc., USA)

- Refrigerated microcentrifuge kubota1300 (Kubota, Japan)

- Spectrophotometer DU 650 (Beckman, USA)

- Ultraviolet Transilluminator 2011 MACROVUE (LKB BROMMA)

2.1.2 Chemical agents

- Absolute ethanol (Merk, Germany)

- Acrylamide (Merk, Germany)

- Ammonium persulfate (Promega, USA)

- Agarose gel (Sekem, USA)

- Acetic acid glacial (Merk, Germany)

- Bacto-agar (Difco, USA)

- Bacto-tryptone (Difco, USA)

- Bacto-yeast extract (Difco, USA)

- Boric acid (Merk, Germany)

- Bromophenol blue (Merck, Germany)

- Chloroform (Merk, Germany)

- 100 mM dATP, dCTP, dGTP and dTTP (Promega, USA)

- Diethyl pyrocarbonate (DEPC, Sigma; USA)

- Ethidium bromide (Sigma, USA)

- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- Formamide (Gibco BRL, technologies, Co., USA)
- Glucose (Merck, Germany)
- Isopropanol (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- IPTG (New England Biolabs, Inc., USA)
- Magnesium chloride (BDH, England)
- Magnesium sulfate (Merck, Germany)
- N, N'-methylene-bis-acrylamide (Amersham, England)
- Phenol crystals (Carlo Erba, Italy)
- Potassium choride (Carlo Erba, Italy)
- Sodium acetate (Merck, Germany)
- Sodium chloride (BDH, England)
- Sodium citrate (Merck, Germany)
- Sodium dodecyl sulfate (Sigma, USA)
- Sodium hydroxide (Merck, Germany)
- Silver nitrate (Carlo Erba, Italy)
- Sodium carbonate (Merck, Germany)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Trizol reagent (Gibco BRL)
- Urea (APS Ajax Finechem, Australia)
- Xylene cyanol (Sigma, USA)

2.1.3 Enzymes

- *Alu* I (Promega, USA)

- DyNAzyme™ II DNA polymerase (Finnzymes, Finland)

- *Eco*RI (Promega, USA)

- Proteinase K (Gibco BRL, USA)

- RNase A (Sigma, USA)

- *Rsa* I (Promega, USA)

2.1.4 Reagent kits

- AFLP analysis system I kit (Gibco BRL, USA)

- ImProm-II™ reverse transcription system (Promega, USA)

- QIAquick™ gel extraction kit (Qiagen, Germany)

- QIAprep™ spin miniprep kit (Qiagen, Germany)

2.1.5 Cloning vector

- pGEM®-Teasy vector (Promega ,USA)

2.2 Samples

Juvenile abalone *H. asinina* were collected alive from Angsila Marine Biological Research Station, the Sichang Marine Science Research and Training Station, Chulalongkorn University, in Chonburi and were transported to Chulalongkorn University, Bangkok. The weights of individual abalone were recorded one by one. The samples were separated into two groups along with the weights – fast-growing ones and slow-growing ones at the same age. In the process of analyzing by using AFLP, the samples used are described in detail in Table 2.1. As for the analyzing by employing RAP-PCR, 11-month abalone *H. asinina* were sampled. Slow-growing and fast-growing ones were separated along with weight distribution. The average weight equaled 8.54 g. The standard deviation equaled 3.76. The slow-growing one was in the period of average weight minus 1 S.D. (3.8-4.7g), whereas the fast-growing group was in the period of average weight plus 1 S.D. (13.1-17.2 g).

Table 2.1 Abalone samples used in AFLP analysis

Fast-growing (FG)			**Slow-growing (SG)**		
FG1 (3 months old)	Sample	Weight (g)	**SG1** (3 months old)	Sample	Weight (g)
	1	2.40		1	0.22
	2	2.52		2	0.22
	3	2.63		3	0.29
	4	2.76		4	0.29
	5	2.91		5	0.36
	6	3.07		6	0.48
	7	3.08		7	0.50
	8	3.88		8	0.52
	9	4.04		9	0.58
	10	4.97		10	0.70
FG2 (3 months old)	Sample	Weight (g)	**SG2** (3 months old)	Sample	Weight (g)
	1	2.51		1	0.24
	2	2.55		2	0.26
	3	2.70		3	0.33
	4	3.12		4	0.38
	5	3.20		5	0.42
	6	3.27		6	0.43
	7	3.28		7	0.44
	8	3.37		8	0.49
	9	3.76		9	0.51
	10	4.04		10	0.64
FG3 (12 months old)	Sample	Weight (g)	**SG3** (12 months old)	Sample	Weight (g)
	1	13.46		1	1.02
	2	13.57		2	1.03
	3	13.83		3	1.08
	4	14.36		4	1.24
	5	14.75		5	1.35
	6	15.10		6	1.55
	7	15.17		7	1.58
	8	15.26		8	1.59
	9	17.21		9	1.63
	10	17.51		10	1.64

2.3 Nucleic acid extraction

2.3.1 DNA extraction

Firstly, genomic DNA was extracted from the foot tissue of each abalone by using a phenol-chloroform proteinase K method (Klinbunga et al., 1999). A piece of foot tissue was dissected out and placed in a prechilled microcentrifuge tube containing 500 μl of the TEN buffer (100 mM Tris-HCL, 100 mM EDTA, 250 mM NaCl, pH 8.0) and briefly homogenized with a micropestle.

The homogenate was added with 20% (w/v) SDS, briefly mixed, and incubated at 65 ^{o}C for an hour. After incubation, the solution was treated with 15 μl 0f Proteinase K solution (20 mg/ml). It was gently mixed and incubated at the same temperature for another 3 hours. RNase A (10 mg/ml) 10 μl was added and incubated at 37 ^{o}C for 1 hour. An equal volume of buffer-equilibrated phenol was added and gently mixed for 15 minutes. The solution was minutes. After that, the precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature and washed twice with 1 ml of 70% cold ethanol and allowed to air-dry. The DNA was resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA was incubated overnight at 37 ^{o}C for more complete solubilization and kept at 4 ^{o}C until further use.

2.3.2 RNA extraction

Total RNA was extracted from haemocytes. To collect the haemocytes, the incision in the foot was determined and haemolymph was collected from each abalone by using a 1.0 ml syringe pre-load with 200 μl of anti-coagulant (10% sodium acetate, W/V). Haemolymph was immediately centrifuged at 800xg for 10 minutes at 4 ^{o}C to

separate haemocytes from the plasma. The haemocytes pellet was resuspended in 500 µl of TRI REAGENT® and after that briefly homogenized. The homogenate was kept at room temperature for 5 minutes to achieve complete dissociation of nucleoprotein complexes. Then, 200 µl of chloroform was added and vigorously shaken for 15 seconds. The mixture was stored at room temperature for 2-5 minutes and centrifuged 12,000xg for 15 minutes at 4 ^{o}C. The colorless upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by the addition of 500 µl of isopropanol. The mixture was left at room temperature for 5-10 minutes and centrifuged at 12,000xg for 10 minutes at 4 ^{o}C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol. The RNA pellet was stored under 75% ethanol until used. When required, the samples were centrifuged at 12,000xg for 15 minutes at 4 ^{o}C. The supernatant was removed. The RNA pellet was briefly air-dried for 5-10 minutes. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water.

2.4 Measuring nucleic acid concentration using spectrophotometry

The concentration of extracted DNA or RNA sample was measured the optical density at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to concentration of 50 µg/ml double stranded DNA, 40 µg/ml single strand RNA and 33 µg/ml single strand DNA (Sambrook et al., 2001). Therefore, the concentration of DNA/RNA samples was estimated in µg/ml by employing the following formula.

$$[DNA] = OD_{260} \times \text{dilution factor} \times 50$$

$$[RNA] = OD_{260} \times \text{dilution factor} \times 40$$

The purity of DNA and RNA samples were evaluated from a ratio of OD_{260} /OD_{280}. The ratios of the purified DNA and RNA were approximately 1.8 and 2.0 respectively (Sambrook et al., 2001).

2.5 Synthesis of the first strand cDNA

Using an ImProm-II™ reverse transcription system kit (Promega), the first strand cDNA was synthesized from 1.5 μg of total RNA extracted from haemocytes of abalone as described by the manufacturer. Total RNA, 0.5 μg of oligo (dT_{15}) and approximate DEPC-treated H_2O in a final volume of 5 μl were combined. The reaction was incubated at 70 ^oC for 5 minutes and after that it was immediately placed on ice for at least 5 minutes. Then 5x reaction buffer, $MgCl_2$, dNTP mixed, RNasin were added to the final concentration of 1x, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 μl of ImProm-II™ reverse transcriptase was added and tenderly mixed by pipetting. The reaction mixture was incubated at 25 ^oC for 5 minutes and at 42 ^oC for 90 minutes. The reaction was terminated by incubation at 70 ^oC for 15 minutes in order to terminate the reverse transcriptase activity. Concentration and rough quality of first stranded cDNA was spectrophotometrically inspected (OD_{260}/OD_{280}) and electrophoretically analyzed (1.0% agarose gel) respectively. The first stranded cDNA was diluted to 200 ng/μl and kept at -20 ^oC until required.

For RNA arbitrarily primed (RAP) PCR analysis, the first stranded cDNA synthesis was carried out as described above except that 1 μM of each RAPD primer was used instead of 0.5 μM oligo (dT_{15}).

2.6 Bulked segregant analysis (BSA)

Bulk segregant analysis (BSA) (Michelmore et al., 1991) was employed in rapid markers' identification which was linked to any specific genes or genomic region. This technique is used in screening for differences, either in any characteristics or gender, in different pooled samples. Each pool or bulk contains individual selected having identical genotypes for a particular genomic region. Bulked segregant analysis was used to establish bulked DNA sample for AFLP analysis. The bulked was formed by pooling DNA from 10 individuals of abalone sample group (Table 2.1). The pooled DNA (250 ng) of the first sample set (FG1, N=10; SG1, N=10) was prepared for initial screening of candidate growth-related AFLP markers. Candidate AFLP markers from the first sample set were screened with the second sample set (FG2, N=10; SG2, N=10). Candidate markers from the second sample set were screened with the third sample set (FG3, N=10; SG3, N=10) of abalone *H. asinina*. For BSA of RAP-PCR analysis, total RNA, which was extracted from haemocyte of fast-growing (N=5) and slow-growing (N=3) abalone, was pooled as equivalent 1.5 μg and used as the template for the first strand cDNA synthesis and further RAP-PCR analysis as detail in 2.14.

2.7 Amplified Fragment Length Polymorphism (AFLP) analysis

2.7.1 Restriction enzyme digestion and adaptor ligation

Each bulked DNA (250 ng) was simultaneously digested with 2.5 units of *Eco* RI and *Mse* I in a 25 μl reaction mixture which contained 10 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate at 37 ^{o}C for approximately 4 hours. The reaction was terminated by incubation at 70 ^{o}C for 15 minutes. The *Eco* RI and *Mse* I adaptors (Table

2.2) were ligated to restricted genomic DNA by adding 24 ml of the adaptor ligation solution, which were the combination of *Eco* RI and *Mse* I adaptors, 0.4 mM ATP, 10 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate and 50 mM K-acetate, and 1 U of T4 DNA ligase. The reaction was incubated at 16 ^{o}C for approximately 16 hours.

2.7.2 Preamplification

An aliquot of the ligation product was ten-fold diluted with TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The preamplification reaction was carried out in a 50 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 30 ng of E_{+A} (5'-GAC TGC GTA CCA ATT CA-3') and M_{+C} (5'-GAT GAG TCC TGA GTA AC-3') primers, 1.5 units of DyNAzyme^{TM} II DNA polymerase (Finnzymes) and 5 μl of the diluted ligation product (1: 10) (or 0.5-1.0 μl of undiluted product). The process of PCR, by a Perkin Elmer 9700 thermocycler, consisted of denaturation at 94 ^{o}C for 30 seconds, annealing at 56 ^{o}C for 1 minute and extension at 72 ^{o}C for a minute. The preamplification product was then diluted for 12.5 fold.

2.7.3 Selective amplification

Selective amplification was done in a 20 (or 25) μl reaction volume, which contains 10 mM Tris-HCl, pH 8.8, 50 mM KCl 0.1% Triton X-100, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 30 ng of combinations of E_{+3} and M_{+3} primers, 1.5 units of DyNAzyme^{TM} II DNA polymerase (Finnzymes) and 5 μl of the diluted preamplification product (1: 50). The amplification reaction was then carried out by denaturation at 94 ^{o}C for 30 seconds, annealing at 65 ^{o}C for 45 seconds and extension

at 72 ^{o}C for 90 seconds for 2 cycles. And then, it was followed by 12 cycles of a touch down phase with lowering of the annealing temperature for 0.7 ^{o}C in every cycle. The amplification -- 94 ^{o}C for 30 seconds, 56 ^{o}C (or 53 ^{o}C) for 45 seconds and 72 ^{o}C for 90 seconds -- was performed for another 23-25 cycles. The final extension was carried out at 72 ^{o}C for 5 minutes.

2.8 Agarose gel electrophoresis

An appropriate amount of agarose (or metaphore) was weighed and mixed with an appropriate volume of 1xTBE (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3). The gel slurry was boiled in a microwave oven to let it conduct complete solubilization. Then, it was poured into the gel mold. A comb was inserted. The gel was left to solidify. When required, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1xTBE buffer covering the gel for approximately 0.5 cm. PCR products (10-25 µl) were mixed with the one-fifth volume of the 10x loading dye (0.25% bromophenol blue and 25% Ficoll in water) and loaded into the well. A 100 bp DNA ladder was employed as the standard marker. Electrophoresis was carried out in 1xTBE buffer at 100 volts until the bromophenol blue dye migrating to approximately one half of the gel. The electrophoresis gel was then stained with ethidium bromide solution (0.5 µg/ml) for 5-15 minutes and destained in running tap water removing unbound ethidium bromide from the gel. DNA fragments were visualized under a UV trans illuminator and photographed.

Table 2.2 AFLP primers and their sequences used for identification of growth-related markers in *H. asinina*.

Primer	Sequences
Adaptor sequences	
*Eco*RI adaptor	5′ -CTC GTA GAC TGC GTA CC-′3
	5′ -AAT TGG TAC GCA GTC TAC-′3
*Mse*I adator	5′ -GAC GAT GAG TCC TGA G-′3
	5′-TAC TCA GGA CTC AT-′3
Preamplification primers	
E_{+A}	5 ′- GAC TGC GTA CCA ATT CA-′3
M_{+C}	5 ′- GAT GAG TCC TGA GTA AC-′3
Selective amplification primers	
E_{+3}-1	E_{+A}-AC
E_{+3}-2	E_{+A}-AG
E_{+3}-3	E_{+A}-CA
E_{+3}-4	E_{+A}-CT
E_{+3}-5	E_{+A}-CC
E_{+3}-6	E_{+A}-CG
E_{+3}-7	E_{+A}-GC
E_{+3}-8	E_{+A}-GG
E_{+3}-9	E_{+A}-GT
E_{+3}-10	E_{+A}-GA

Table 2.2 (continued)

Primer	Sequences
E_{+3}-11	E_{+A}-TG
E_{+3}-12	E_{+A}-TC
E_{+3}-13	E_{+A}-TA
E_{+3}-14	E_{+A}-TT
E_{+3}-15	E_{+A}-AA
E_{+3}-16	E_{+A}-AT
M_{+3}-1	M_{+C}-AA
M_{+3}-2	M_{+C} -AC
M_{+3}-3	M_{+C} -AG
M_{+3}-4	M_{+C} -AT
M_{+3}-5	M_{+C} -TA
M_{+3}-6	M_{+C} -TC
M_{+3}-7	M_{+C} -TG
M_{+3}-8	M_{+C} -TT
M_{+3}-9	M_{+C} -GA
M_{+3}-10	M_{+C} -GT
M_{+3}-11	M_{+C} -GC
M_{+3}-12	M_{+C} -GG
M_{+3}-13	M_{+C} -CA
M_{+3}-14	M_{+C} -CT
M_{+3}-15	M_{+C} -CG
M_{+3}-16	M_{+C} -CC

2.9 Denaturing Polyacrylamide Gel Electrophoresis

2.9.1 Preparation of Glass Plate

The long glass plate was thoroughly cleaned with 2 ml of 95% commercial grade ethanol in one direction. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 µl of Bind silane; Pharmacia, USA, 995 µl of ethanol and 10 µl of 5% glacial acetic acid) and left for approximately 10-15 minutes. Excess binding solution was removed and the long glass plate was further cleansed with 95% ethanol for 3 times. For the short glass plate, it was also treated as the long one as described above with the significant exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled to each other with a pair of spacer in between. The bottom and both sides of assembled glass plates were sealed with the plastic tape.

2.9.2 Preparation of denaturing polyacrylamide gel electrophoresis

The products of AFLP as well as RAP-PCR were analyzed on the denaturing polyacrylamide gel. The denaturing polyacrylamid gel was prepared by combining 40 ml of the degassed acrylamide solution (19:1 acrylamide: bisacrylamide with 7 M urea in TBE buffer) with 240 µl of freshly prepared 10% ammonium persulphate and 24 µl of TEMED. The acrylamide solution was gently swirled and degassed for 20 minutes. The assembled plates used a 50 ml syringe. The filled plate sandwich was left in the horizontal position. The flat edge of the shark-tooth comb was then inserted. The gel was left at room temperature for 1 hour. After that, the polymerized gel was covered by water-soaked tissue paper and left at room temperature for 4 hours

(or overnight) for complete polymerization. When all was done, the spring clips and the sealing tape were carefully removed. The shark-tooth comb was rinsed with water.

2.9.3 Electrophoresis

The gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was carefully clamped with the integral gel clamps along with the sides of the sequencing apparatus. The upper and lower buffer chambers were filled with approximately 300 ml of 1xTBE. The shark-toothed comb was reinserted into the gel until the teeth just touched the surface of the gel. The gel was pre-runned at 30-40 W for 30 minutes.

The amplification products (6 μl) were mixed with 3 μl of loading dye (98% formamide, 200 mM EDTA, pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol) and then heated at 95 ^{o}C for 5 minutes before cooled down on ice for 3 minutes. The sample was carefully loaded into the well. Electrophoresis was carried out at 35-40 W for approximately 2.5 hours.

2.10 Silver staining

The gel plates were carefully separated by a plastic wedge. The long glass plate with the gel was placed in a plastic tray, containing 1.5 litres of the fix/stop solution, and then was well agitated for 40 minutes (25-30 minutes for SSCP). The gel was soaked in the deionized water and was shaken well for 2 minutes. The process was done three times. The gel was lifted out of the tray. The gel was transferred to 0.1% silver nitrate (1.5 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked, in 1.5 litres of deionized water, shaken with agitation and immediately placed in the tray containing 1.5 litres of the chilled developing solution. This step is very important that it should take no longer than 5-10 seconds to soak the gel in the water and transfer it to chilled developing solution. The gel was well agitated until the first bands could be seen (usually 1.5 – 2 minutes). The gel was then transferred to another tray containing 1.5 litres of chilled developer and shaken until bands from every lane were observed (usually 2-3 minutes). One litre of the fix/stop solution was directly added to the developing solution and shaken continuously for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was left at room temperature (SSCP gels) or at 80 ^{o}C for 2-3 hours (AFLP gels).

2.11 Cloning of AFLP fragments

2.11.1 Elution of DNA from polyacrylamide gels

Candidate growth-related AFLP fragments were excised from the gel using a sterile razor blade and washed 3 times for 30 minutes each at room temperature with 200 μl of sterile deionized water. Twenty microlitres of water was then added and incubated overnight at 37 ^{o}C. Reamplification of the target fragment was carried out by using the same PCR recipes, as those of selective amplification, with the exception that 100 ng of each primer and 5 μl of the eluted AFLP product were used. The amplification condition was composed of 5 cycles of 94 ^{o}C for 30 seconds, 42 ^{o}C for 1 minute and 72 ^{o}C for 1 minute, followed by additional 35 cycles at higher stringent annealing temperature at 50 ^{o}C. The final extension was performed at 72 ^{o}C for 7 minutes. The reamplified product was electrophoretically analyzed through a 1.5-1.8% agarose gel at 100 volt for approximately 40 minutes.

2.11.2 Elution of DNA from agarose gels

The required DNA fragment was fractionated through agarose gels in duplication. One was run side-by side with 100 bp DNA marker and the other loaded into the distal well of the gel. After electrophoresis, both lanes, the DNA standard one and its proximal DNA sample one, were cut and stained with ethedium bromide for 5 minutes. Position of the DNA markers and the EtBr-stained fragment were used to align the position of the non-stained target DNA fragment.

The cut DNA fragment (unstained) was transferred into a microcentrifuge tube and weighed. DNA was eluted out from the agarose gels using a QIAquick gel

extraction kit (QIAGEN), according to the protocol recommended by the manufacturer. The eluted sample was stored at -20 ^{o}C until required.

2.11.3 Ligation of PCR product to vector

The ligation reaction was set up in the total volume of 10 μl. The reaction volume contained 3 μl of the gel-eluted PCR product, 25 ng of pGEM®-T easy vector, 5 μl of 2x rapid ligation buffer (60 mM Tris-HCl pH 7.8, 2.0 mM DTT, 2mM ATP and 10% PEG 80000) and 3 Weiss unit of T4 DNA ligase. The ligation mixture was carefully mixed by pipetting and then was incubated at 4 ^{o}C overnight.

2.11.4 Preparation of host cell

A colony of *E. coli* stain XL1 blue was cultured as the starter in 15 ml of LB broth (1% bactrotryptone, 0.5% bacto yeast extract and 0.5% NaCl) medium containing 12.5 μg/ml tetracycline. Then, it was shaken at 37 ^{o}C overnight. Two and a half milliliters of the starter were added in 250 ml of LB broth medium and the culture was left for the cell to complete its growth at 37 ^{o}C with vigorous shake for 3-4 hours (OD600 ~ 0.5-0.8). The cells were then chilled on ice for 15-30 minutes, and centrifuged in a cold rotor (Beckman J2-21, USA) at 4,000xg for 15 minutes. After the supernatant was removed, pellet was washed by resuspending in a total of 250 ml of cold sterile water and then it was centrifuged again. The pellet was further washed with 125 ml of cold water and followed by 5 ml of ice cold sterile 10% glycerol. Finally, the cells were resuspended to a final volume of 500-750 μl, aliquoted (40 μl) into 1.5 ml microcentrifuge tubes and stored at -80 ^{o}C until required.

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

The portion of the ligation mixture was transformed into host cell *E. coli* strain XL1 blue by electroporation procedure (Dower et al., 1988). The apparatus was set as the following: 25 μF of Gene Pulser apparatus, 200Ω of the Pulse controller unit and 2.5 kV of the Gene pulser apparatus. Forty microliters of the cell suspension were gently set on ice. Then, 1 μl of ligation mixture was added and mixed well. Then, it was left on ice for 1 minute. After that, the mixture of cells and DNA was applied into a cold 0.2 cm cuvette. After one pulse was applied, the cells were immediately resuspended with 1 ml of SOC medium (2% bactotryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37 ^oC for 1 hr. At the end of the incubation period, the cultured cell suspension was centrifuged at 12,000 rpm for 20 seconds at room temperature.

The pellet was gently resuspended in 100 μl of SOC and spread on the LB agar plates, which contained 50 μg/ml ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-gal. After that it was incubated at 37 ^oC overnight. As a result, the recombinant clones, containing inserted DNA, are white, whereas those without inserted DNA are blue.

2.11.6 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 each of dATP, dTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 0.2 μM of SP6 (5'-ATT TAG GTG ACA CTA TAG AA-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') primers in 1 unit of DyNAzyme™ II DNA Polymerase. A recombinant colony was scraped by the

micropipette tip and mixed well in the amplification reaction. The PCR profiles were predenatured at 94 ^{o}C for 3 minutes and followed by 30 cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 60 seconds and 72 ^{o}C for 90 seconds. The final extension was carried out at 72 ^{o}C for 7 minutes. The result of PCR products was electrophoretically analyzed through agarose gel.

2.11.7 Plasmid extraction

A recombinant plasmid was incubated into 3 ml of LB broth containing 50 μg/ml of amplicillin. Then the incubation at 37 ^{o}C with constant shaking at 250 rpm overnight was further determined. The cell culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 g for 1 minute. The cell pellet was collected by using a QIAprep spin miniprep kit (QIAGEN). The cell pellets were resuspended in 250 μl of the P1 buffer. A 250 μl of the P2 buffer was added to the tube and gently inverted 4-6 times. Then, 350 μl of the N3 buffer was added. The tube was thoroughly and gently inverted 4-6 times and centrifuged at 12,000 rpm for 10 minutes. The supernatant was carefully collected and applied to the QIAprep column and centrifuged at 12,000 rpm for 1 minute. The flow-through solution was discarded. The QIAprep spin column was washed by adding 500 μl of the PB buffer and centrifuged at 12,000 rpm for 1 minute. The flow-though solution was discarded. The PE buffer (750 μl) was added and centrifuged as mentioned. After the flow-through solution was discarded, the QIAquick column was recentrifuged to remove the trace amount of the washing solution. The column was then placed into a sterile 1.5 ml microcentrifuge tube. Plasmid DNA was eluted out by adding 40-50 μl of the EB buffer (10 mM Tris-HCl, pH 8.5) to the center of the QIAprep column and then was

left for 1 minute before being centrifuged at 12,000 rpm for 1 minute. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.12 DNA sequencing and primer design

The recombinant plasmid was sequenced with a Thermo sequenase fluoresent labelled primer cycle sequencing kit (Amersham Biosciences, Sweden), using the M13 reverse or M13 forward primers on an automated DNA sequencer (MegaBace 1000 Ameraham BioSciences). The PCR primers were designed by using computer program OLIGO 4.0 (Table 2.3).

2.13 Polymerase chain reaction

The PCR reactions were performed in a 25 μl reaction volume which contained 10 mM Tris-HCl, (pH 8.8), 50 mM KCl and 0.1% Triton X-100, 2.0 mM $MgCl_2$, 100 μM dNTPs, 1 U of DyNAzyme^{TM} DNA polymerase, 0.4 μM of each primer and 50 ng of genomic DNA. The amplification condition was carried out as described in Table 2.4.

Table 2.3 Primers designed from a candidate growth-related AFLP marker of *H. asinina*.

Primer	Sequence	Tm (^{o}C)	Expected size (bp)
FGE3M12-F	5′-ACT GAC TTC ACA TAT TGT ACC′-3	58	176
FGE3M12-R	5′-GAA GAC TAA CCT AAG TGA ACG′-3	60	

Table 2.4 PCR profiles and composition for specificity test of a SCAR marker derived from the candidate growth-related AFLP marker of *H. asinina.*

Primer	$MgCl_2$ (mM)	Primer (μM)	PCR condition
FGE 3M12	2	0.4	94 ^{o}C 1 min; 1cycle
			followed by
			94 ^{o}C for 30 s 54 ^{o}C for 30 s 72 ^{o}C for 1 min } 30 cycles
			72 ^{o}C for 2 min; 1 cycle

2.14 RNA arbitrarily primed (RAP)-PCR

The first strand cDNA was synthesized from 1.5 μg of pooled total RNA which was extracted from haemocyte of fast-growing (N=5) and slow-growing (N=3) abalone. Three different primers (1μM each of UBC 101, UBC 119 and OPA 01) were used for the first strand cDNA synthesis.

One microgram of the first stranded cDNA was used as a template for PCR amplification in 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25 ^{o}C, 50 mM KCl and 0.1 Triton X-100, 2 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP, 1 unit of DyNAzyme™ II DNA Polymerase, 1 μM, each of the first arbitrary primers (that used for the first strand cDNA synthesis) and the second primers (the first primer or one of those in Table 2.5).

PCR was performed by predenaturation at 94 ^{o}C for 3 minute and then was followed by 40 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 36 ^{o}C for 60 seconds, and extension at 72 ^{o}C for 90 seconds. The final extension was carried out for 72 ^{o}C. Five microlitres of the amplification product were electrophoretically analyzed in order to answer whether the amplification was successful. Six microlitres of the PCR product were mixed with one-half volume of formamide loading buffer and denatured at 94 ^{o}C for 10 minutes prior to electrophoresis through a 4.5% denaturing polyacrylamide gel. Size-fractionated bands were visualized by silver staining. The gel was dried overnight and photographed under fluorescence light using Camera Pentax K1000.

2.15 Cloning and characterization of candidate growth differential expression markers from RAP-PCR analysis

Fragments showing presence/absence and differential expression were considered. Interesting bands were eluted out from the gel and reamplification steps were carried out as described previously with an exception that 0.2 μM of each primer and 0.2 μM of dNTPs were used. The amplification conditions were composed of 1 cycle of 94 ^{o}C for 3 minutes, following by additional 25-35 cycles at 94 ^{o}C for 30 seconds, 36 ^{o}C for 1 minute and 72 ^{o}C for 90 seconds. The final step was performed at 72 ^{o}C for 7 minutes. The reamplified product was electrophoretically analyzed through 1.5% agarose gel at 100 volts for approximately 40 minutes.

The DNA fragment was excised from the agarose gel, cloned and sequenced. Primer pairs were designed, commercially synthesized and used for RT-PCR amplification of fast growing and slow growing *H. asinina*.

Table 2.5 Sequence of arbitrary primers used for screening differential-growth expression markers in *H. asinina* employing RAP-PCR analysis.

Primer no.	Primer name	Sequence
1	UBC 101	GCGCCTGGAG
2	UBC 119	ATTGGGCGAT
3	UBC 122	GTAGACGAGC
4	UBC 128	GCATATTCCG
5	UBC 135	AAGCTGCGAG
6	UBC 138	GCTTCCCCTT
7	UBC 158	TAGCCGTGGC
8	UBC 169	ACGACGTAGG
9	UBC 174	AACGGGCAGC
10	UBC 191	CGATGGCTTT
11	UBC 217	ACAGGTAGAC
12	UBC 273	AATGTCGCCA
13	UBC 428	GGCTGCGGTA
14	UBC 457	CGACGCCCTG
15	UBC 459	GCGTCGAGGG
16	OPA 01	CAGGCCCTTC
17	OPA 07	GAAACGGGTG
18	OPA 09	GGG TAACGCC
19	OPA 17	GACCGCTTGT
20	OPB 10	CTGCTGGGAC

2.16 Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The first strand cDNA was synthesized total RNA extracted from haemocytes of fast-growing and slow-growing abalone as described in 2.3.2 and 2.5.

2.16.1 Primers

Primers were designed from candidates of growth-differential expression markers in *H. asinina* with RAP-PCR analysis. β-actin gene was used as an internal control. Sequences of design primers were shown in Table 2.6

2.16.2 Determination of PCR condition

Amplification reactions were performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25 ^{o}C, 50 mM KCl and 0.1% Triton X-100, 1.5-2.5 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP, 1 unit of DyNAzyme™ II DNA Polymerase, 0.2-0.4 μM of the each interested primers.

2.16.2.1 $MgCl_2$ concentration

The optimal $MgCl_2$ concentration of each primer pair (between 1-3 mM $MgCl_2$) was examined using the standard PCR condition. The $MgCl_2$ concentration giving the highest specificity was chosen.

2.16.2.2 Cycle number

The PCR amplifications were carried out at different cycles using the optimal concentration of $MgCl_2$ and analyzed by gel electrophoresis. The number of cycles that still provided the PCR product in the exponential range and did not reach a plateau level of amplification were chosen. The most appropriate condition that results from the determination of PCR condition mentioned earlier were chosen for the process of PCR amplification of growth-differential expression marker in *H. asinina*, as described in detail in Table 2.7.

2.16.3 Gel electrophoresis and quantitative analysis

The ratio between the interesting gene and the internal control products was determined by electrophoresis on 1.5-2.0% agarose gels. Six microlitres of PCR products were combined with ¼ volume of the gel-loading dye before loaded to the agarose gel. A DNA ladder (100 bp marker) was used as a standard DNA marker. After electrophoresis at 100 volts, the gel was stained with 2.5 μg/ml EtBr for 5 minutes and destained in distilled water for 15 minutes. The intensity analysis was performed with Gel Documentation System (GeneCam FLEX1, SynGene) and further quantified by the employment of the Gene tools analysis software.

Table 2.6 Sequence, melting temperature of primers and size of the expected amplification product of the candidate growth-differential expression markers in *H. asinina* from RAP-PCR analysis (slow-growing related expression markers) and internal control (β-actin).

Primer	Sequence	Tm (^{o}C)	Expected size (bp)
SGRAP1-F	5′-GAT CGT ATC AAT AGG GTA GG-3′	58	167
SGRAP2-R	5′-TGG ACT GTA CAG AGG TTT CG-3′	60	
SGRAP2-F	5′-CCC TGA TAC TCT GGC AAG-3′	56	179
SGRAP3-R	5′-CTT GCC AGA GTA TCA GGG-3′	56	
β-actin (internal control)			
β-actin-F	5′-GAA CTT GCT GTG TGA ATT TTG TTT GTT G-3′	55	220
β-actin-R	5′-CGA CGT AGC TGT CCT TCT GAC CCA-3′		

Table 2.7 PCR profiles and condition for testing of primers from the candidate growth-differential expression markers in *H. asinina* from RAP-PCR analysis (slow-growing related expression markers) and internal control (β-actin).

Primers	cDNA template (ng)	$MgCl_2$ (mM)	Primer (μM)	PCR condition
SGRAP1	800	2.5	0.4	94 °C 1 min; 1cycle followed by 94 °C for 30 s 55 °C for 30 s 72 °C for 30 s } 24 cycles 72 °C for 1 min; 1 cycle
SGRAP2	400	1.5	0.2	94 °C 1 min; 1cycle followed by 94 °C for 1 min 50 °C for 1 min 72 °C for 1 min; } 28 cycles 72 °C for 7 min; 1 cycle
β-actin	800	1.0	0.2	94 °C 1 min; 1cycle followed by 94 °C for 30 s 55 °C for 30 s 72 °C for 30 s; } 22 cycles 72 °C for 1 min; 1 cycle

2.17 SSCP analysis

2.17.1 Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel was used for size-fractionation of both single and double-strand DNA. The glass plates (PROTEIN II xi Cell) were cleaned and prepared as described earlier. Different concentration of low crosslink non-denaturing polyacrylamide gels (37.5: 1 of acrylamide and bis-acrylamide) was prepared by dilution of a 40% stock solution to required gel concentration. The acrylamide gel solution (30-40 ml) was mixed with glycerol (5% or 10% concentration), as well as 240 μl of 10% APS and 24 μl of TEMED. The analytical comb was inserted into the prepared gel and polymerized for 4 hours or overnight. In the SSCP analysis, 6 μl of the amplified products were mixed with 24 μl of the SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in a boiling bath for 5 minutes and immediately cooled on ice for 3 minutes. The denatured PCR products were electrophoretically analyzed on native polyacrylamide gels (a different gel concentration of 37.5:1 crosslink without glycerol) at 250-300 volts for 16-24 hours at 4 ^{o}C. The electrophoresed bands were visualized by silver staining, as described earlier, with the exception that the gel was rinsed 3 times for 3 minutes each after the fix/stop step.

2.18 Identification of insulin-related peptides homologue in *H. asinina*

2.18.1 Degenerated primer designation and polymerase chain reaction

In order to design degenerated primers, amino acid sequence of cDNA encoding molluscan insulin-related peptide (MIP; GenBank accession number: X06983), molluscan insulin-related peptideII (MIP II; P25289), and molluscan

insulin-related peptide III (MIP III; S69155) from *Lymnaea stagnalis,* and insulin precursor from *Aplysia californica* (AAF80383) were aligned using Clustal X program. Two generated primers were designed from relatively conserved region of insulin-related peptide genes for PCR amplification (expected size approximately at 180 bp; Table 2.8).

The first strand cDNA synthesized from abalone haemocyte was used as a template for PCR amplification. The PCR reactions were performed in a 25 μl reaction volume which contained 10 mM Tris-HCl, (pH 8.8), 50 mM KCl and 0.1% Triton X-100, 1 mM $MgCl_2$, 100 μM dNTPs, 1 U of DyNAzyme^{TM} DNA polymerase, 0.2 μM of each primer and 400 ng of cDNA template. The amplification condition was carried out as described in Table 2.9.

Table 2.8 The sequences of the two degenerated primers from alignment of the amino acid sequences of insulin-like peptide genes from *Lymnaea stagnalis* and *Alypsia californica* by Clustal X program.

Primer	Sequence
IGF-F	5′-ACA TCG CCG TGG SGT STG YGG′-3
IGF-R	5′-TGT CCC TTG TGT CCT GTC ARR TA′-3

Abbreviations: S = G/C Y = C/T R = A/G

Table 2.9 PCR profiles and conditions for testing degenerated primers.

Primer	**$MgCl_2$ (mM)**	**Primer (μM)**	**PCR condition**
IGF	1	0.2	94 °C 1 min; 1cycle followed by 94 °C for 1 min 50 °C for 1 min 72 °C for 1 min } 5 cycles 94 °C for 1 min 55 °C for 1 min 72 °C for 1 min } 35 cycles 72 °C for 7 min; 1 cycle

2.18.2 Cloning and characterization of insulin-like peptide homologue of *H. asinina*

The amplified product was electrophoretically analyzed through 1.5% agarose gel. The interesting PCR product was excised out and purified from the agarose gel using a QIAquick^{TM} gel extraction kit (QIAGEN). Eluted DNA was cloned and detected recombinant clone by colony PCR as previously described. The colony PCR products were electrophroresed through 1.2% agarose gel and visualized after ethidium bromide staining. Colony PCR products containing an insert were separately digested with *Alu* I and *Rsa* I in a 15 µl reaction volume containing 10x buffer (6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl and 1 mM DDT, pH 7.5 for *Alu* I and 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DDT, pH 7.9 for *Rsa* I), 0.1 mg/ml BSA, 2 units of each enzyme and 4 µl of the colony PCR products. The reaction was electrophoresed through 1.5% agarose gel and visualized after ethidium bromide staining.

Recombinant plasmid was extracted using a QIAprep^{TM} spin miniprep kit (QIAGEN) and sequenced. Primer pairs were designed (Table 2.10) and used for RT-PCR amplification of fast-growing and slow-growing *H. asinina*. In the process of RT-PCR, the appropriate condition of the optimal $MgCl_2$ concentration and cycle number of PCR condition were properly determined as described earlier. The most appropriate condition from the determination of PCR condition was chosen for the process of PCR amplification (Table 2.11). Then, semi-quantitative analysis by electrophoresis was determined, using β-actin gene as an internal control.

Table 2.10 Sequence, melting temperature of primers and the expected size of amplification product of IGFH primers.

Primer	**Sequence**	**Tm (^{o}C)**	**Expected size (bp)**
IGFH-F	5′-AGG AAG GTA AAA AGT GTC ACG C-3′	59	154
IGFH-R	5′-AAT GTT TGT TTG ACG GAC GGA C-3′	59	

Table 2.11 PCR profiles and condition for testing primers

Primers	**cDNA template (ng)**	**$MgCl_2$ (mM)**	**Primer (μM)**	**PCR condition**
IGFH	400	1.0	0.1	94 ^{o}C 1 min; 1 cycle followed by 94 ^{o}C for 1 min 59 ^{o}C for 1 min } 28 cycles 72 ^{o}C for 1 min 72 ^{o}C for 7 min; 1 cycle

2.19 Identification of 3´-end of insulin-related peptides homologue in *H. asinina*

To identify 3´-end of insulin-related peptides homologue in *H. asinina*, IGFH-F primer, a specific primer, designed in the conserved region and oligo (dT_{20}), were used to amplify the cDNA synthesized from abalone haemocyte. The PCR reactions were performed as above. The amplified product was electrophoretically analyzed through 1.5% agarose gel. The interesting PCR product was excised out and purified from the agarose gel using a QIAquick™ gel extraction kit (QIAGEN). Eluted DNA was cloned and detected recombinant clone by colony PCR as previously described. Recombinant plasmid containing an insert was extracted and sequenced as described in 2.11.7 and 2.12, respectively.

CHAPTER III

RESULTS

3.1 Genomic DNA samples

Genomic DNA was extracted from the foot tissue of *H. asinina* by using the proteinase K/phenol-chloroform extraction method described in 2.3.1. From agarose gel electrophoresis, high molecular weight genomic DNA was about 23.1 kb, which indicates good quality of extracted DNA (Fig 3.1). The quantity of genomic DNA was spectrophotometrically estimated. The OD_{260}/OD_{280} ratio was approximately 1.8 indicating its high purity.

3.2 Bulked segregant analysis (BSA) combined with Amplified fragment length polymorphism (AFLP) analysis for screening of candidate growth-related markers in *H. asinina*

To identify molecular makers linked with growth of *H. asinina*, the strategy of the bulked segregant analysis combined with the AFLP methods were employed since the techniques enabled us to rapidly identify. This BSA method was able to reduce the sample size while allow the rapid screening of polymorphic markers. DNA sample from each of *H. asinina*, segregated into both fast-growing and slow-growing (as shown in Table 2.1), were pooled (bulked segregant). Pooled genomic DNA of fast-growing (N=10) and slow-growing (N=10) *H. asinina* were subjected to AFLP analysis, as from now on the fast-growing and the slow-growing ones will be referred as FG and SG respectively. Each bulk DNA was digested with *Eco* RI

and *Mse* I and ligated to the corresponding adaptors as described in 2.7.1. The ligation products were preamplified using E_{+A} and M_{+C} primers. Smear preamplification products with the molecular weight greater than 300 bp indicate successful digestion and ligation of *H. asinina* genomic DNA (Fig 3.2). The preamplification products were then subjected to selective amplification with combinations of selective primers (three selective nucleotides, Table 3.1).

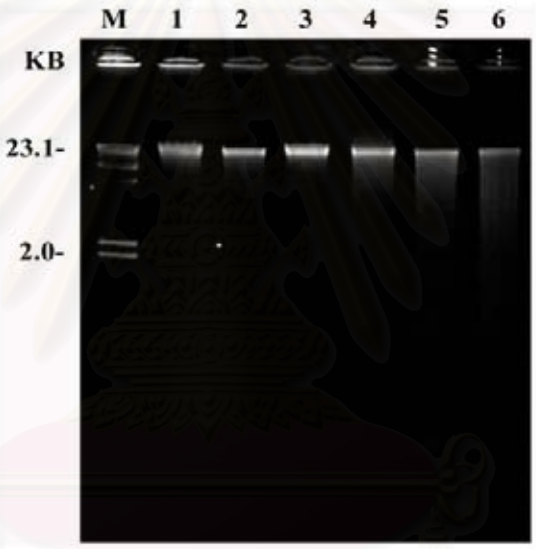

Figure 3.1 A 1% agarose gel showing the quantity of genomic DNA extracted from the foot tissue of *H. asinina* (lanes 1-6). Lane M is λ-*Hind* III DNA marker

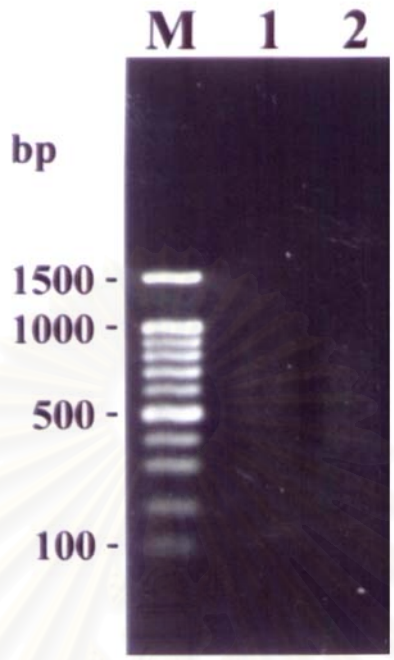

Figure 3.2 A 1.8% agarose gel showing preamplification of bulked genomic DNA of fast-growing (FG1, lane 1, N=10) and slow-growing (SG1, lane 2, N=10) of *H. asinina*. A 100 bp DNA ladder (lane M) is used as the DNA standard.

3.2.1 First screening of candidate growth-related marker in *H. asinina*

Selective amplifications were carried out with the first primer set which contained 64 primer combinations (E_{+3}-1-8 and M_{+3}-1-8). The products of the selective amplification were first electrophoretically analyzed by agarose gel (Fig. 3.3). The selective amplifications that showed several bands of PCR products were then further analyzed by 4.5% denaturing polyacrylamide gel electrophoresis to obtain a high resolution of AFLP pattern. The candidates of growth-related markers were selected from observation of differences that were clearly found in one, but not in the other (Fig 3.4). A total of 40 fast-growing and 43 slow-growing related AFLP

markers from 44 primer combinations were found from the screening of 2 bulked DNA (FG1 and SG1) of *H. asinina* (Table3.1).

3.2.2 Second screening of candidate growth-related markers in *H. asinina*

To reduce false positive AFLP markers from the first screening, polymorphic AFLP primer combinations that yielded the candidates of fast-growing and slow-growing related markers were further screened with 4 bulked DNA. Each bulked DNA contained pool genomic DNA from 10 individuals. The first sample set will now be referred as FG1 and SG1 and the second sample set as FG2 and SG2. AFLP patterns from 44 primer combinations were compared between FG1, FG2 and SG1, SG2. DNA bands that appeared only in FG1, FG2 but not SG1, SG2 and vice versa were chosen as candidate growth-related markers. Consequently, a large number of false positive markers from the first screening were reduced. The number of candidate markers was significantly reduced from 40 and 44 to 2 and 7 for fast growing and slow growing, respectively (Fig.3.5, Table 3.1).

3.2.3 Third screening of candidate growth-related markers in *H. asinina*

To confirm the candidate growth-related markers from the second screening, polymorphic AFLP primer combinations yielded candidate fast-growing or slow-growing-related markers were further screened again with 6 bulked DNA (FG1, SG1, FG2, SG2 and the additional third sample set; FG3 and SG3). Unfortunately, no growth-related AFLP markers were found after three round screening using the first set of primers (E_{+3}-1-8 and M_{+3}-1-8) (Fig.3.6). Therefore, the second set of primers, which contained 112 primer combinations (E_{+3}-1-14 and M_{+3}-9-16), was further used to screen for the candidate growth-related markers with 6 bulked genomic DNA (FG1, SG1, FG2, SG2, FG3 and SG3). From the AFLP patterns, one candidate of fast-growing related AFLP marker with size 225 bp was found from primers E_{+3}-3 + M_{+3}-12. Overall, one candidate of fast-growing related AFLP marker was found from screening various DNA bulked of fast-growing and slow-growing *H. asinina* by using 176 primer combinations. The results were summarized in Table 3.1.

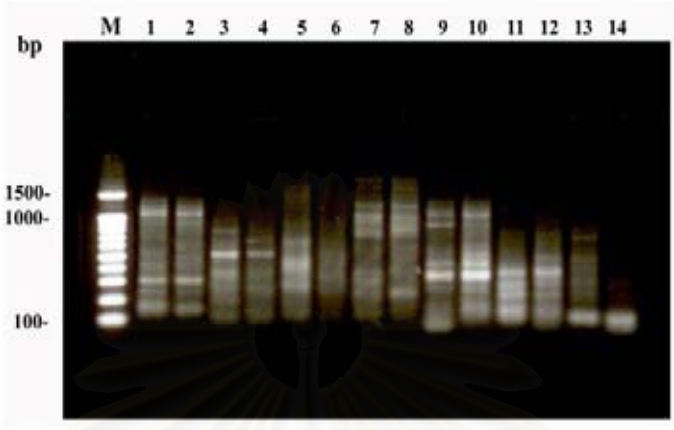

Figure 3.3 A 1.8% agarose showing the selective amplification products of slow-growing (SG) and fast-growing (FG) *H. asinina* using various primer combinations:

Lanes 1-2 : *Eco* RI_{+3}-1 *Mse*I_{+3}-5 Lanes 3-4 : *Eco* RI_{+3}-1 *Mse*I_{+3}-6

Lanes 5-6 : *Eco* RI_{+3}-1 *Mse*I_{+3}-7 Lanes 7-8 : *Eco* RI_{+3}-1 *Mse*I_{+3}-8

Lanes 9-10 : *Eco* RI_{+3}-2 *Mse*I_{+3}-5 Lanes 11-12 : *Eco* RI_{+3}-2 *Mse*I_{+3}-6

Lanes 13-14: *Eco* RI_{+3}-2 *Mse*I_{+3}-7 Lane M : a 100 bp DNA ladder

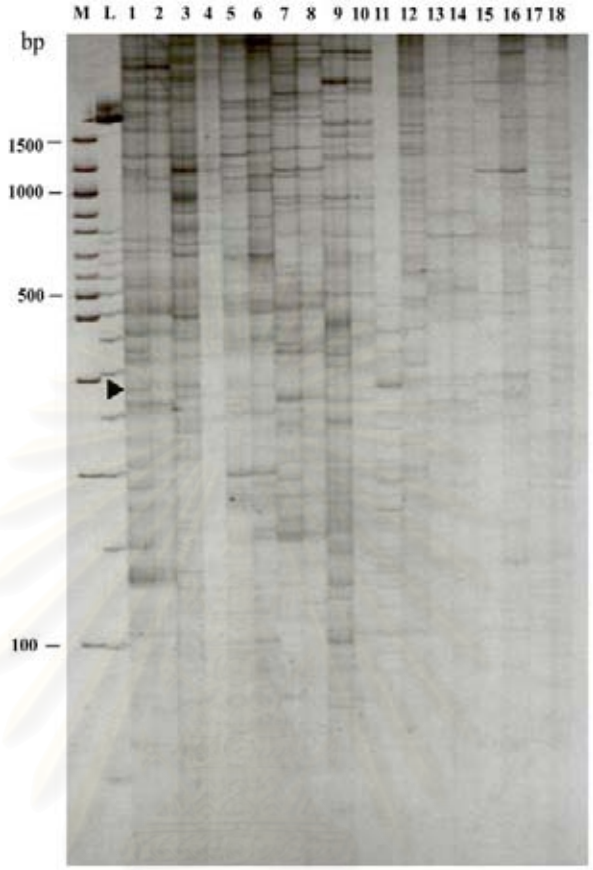

Figure 3.4 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of SG1 and FG1 of *H. asinina*, by using various primer combinations:

SG1 = lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 FG1 = lanes 2, 4, 6, 8, 10, 12, 14, 16, 18

Lanes 1-2: *Eco* RI_{+3}-3 *Mse*I_{+3}-7 Lanes 3-4: *Eco* RI_{+3}-3 *Mse*I_{+3}-8

Lanes 5-6: *Eco* RI_{+3}-4 *Mse*I_{+3}-1 Lanes 7-8: *Eco* RI_{+3}-4 *Mse*I_{+3}-2

Lanes 9-10: *Eco* RI_{+3}-4 *Mse*I_{+3}-3 Lanes 11-12: *Eco* RI_{+3}-4 *Mse*I_{+3}-4

Lanes 13-14: *Eco* RI_{+3}-6 *Mse*I_{+3}-7 Lanes 15-16: *Eco* RI_{+3}-6 *Mse*I_{+3}-8

Lanes 17-18: *Eco* RI_{+3}-7 *Mse*I_{+3}-1 Lanes M, L: a 100 bp, a 50 bp DNA standards (Arrowhead indicates a candidate growth-related marker)

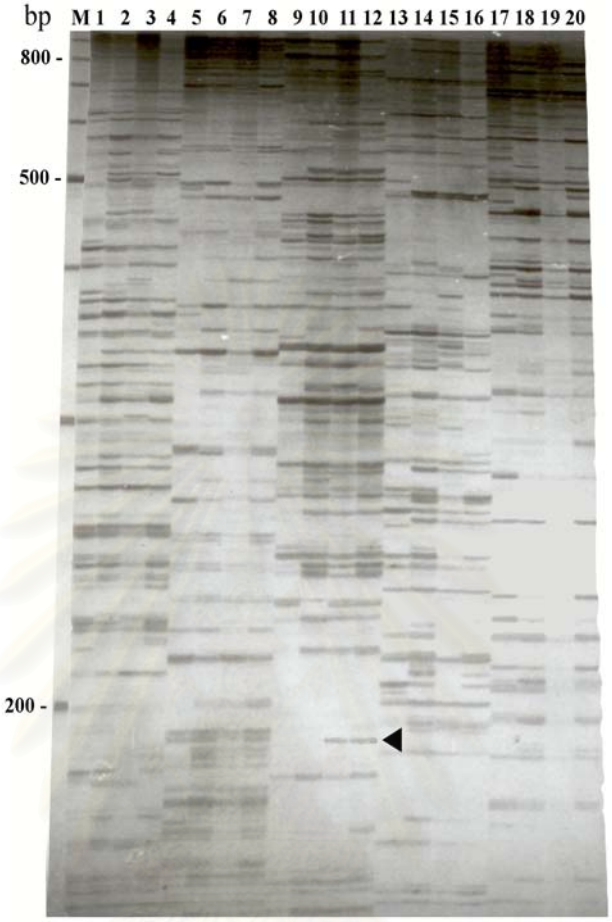

Figure 3.5 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of SG1, SG2, FG1 and FG2 of *H. asinina*, using various primer combinations:

SG1 = lanes 1, 5, 9, 13, 17 SG2 = lanes 2, 6, 10, 14, 18

FG1 = lanes 3, 7, 11, 15, 19 FG2 = lanes 4, 8, 12, 16, 20

Lanes 1-4: *Eco* RI_{+3}-1 *Mse*I_{+3}-7 Lanes 5-8: *Eco* RI_{+3}-1 *Mse*I_{+3}-8

Lanes 9-12: *Eco* RI_{+3}-2 *Mse*I_{+3}-5 Lanes 13-16: *Eco* RI_{+3}-2 *Mse*I_{+3}-6

Lanes 17-20: *Eco* RI_{+3}-2 *Mse*I_{+3}-7 Lane M: a 100 bp DNA standard

(Arrowhead indicates a candidate growth-related marker)

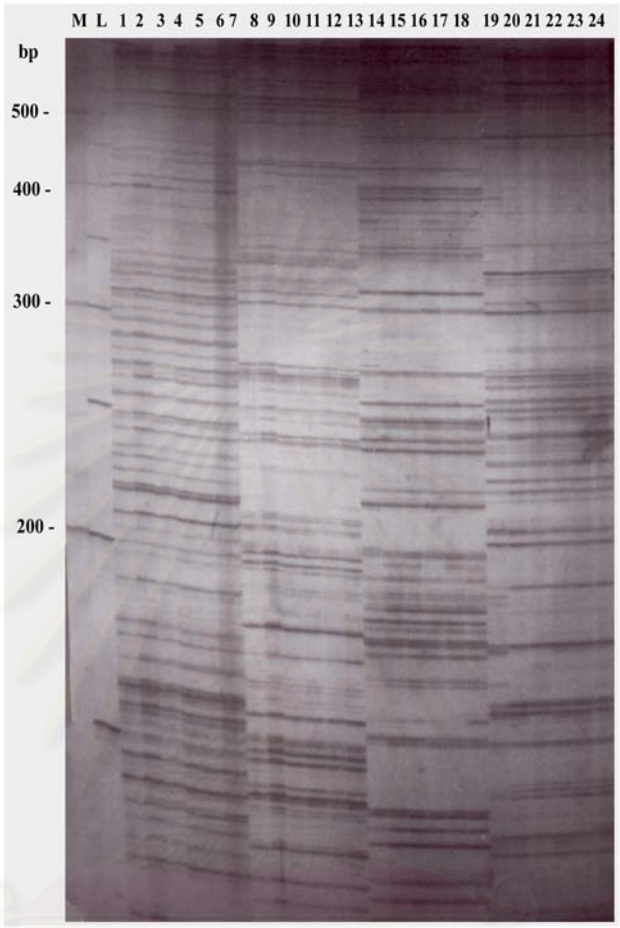

Figure 3.6 A 4.5% denaturing polyacrylamide gel showing AFLP pattern of SG1, SG2, SG3, FG1, FG2 and FG3 of *H. asinina,* using various primer combinations:

SG1 = lanes 1, 7, 13, 19 SG2 = lanes 2, 8, 14, 20

SG3 = lanes 3, 9, 15, 21 FG1 = lanes 4, 10, 16, 22

FG2 = lanes 5, 11, 17, 23 FG3 = lanes 6, 12, 18, 24

Lanes 1-6: *Eco* RI_{+3}-3 *Mse*I_{+3}-3 Lanes 7-12: *Eco* RI_{+3}-3 *Mse*I_{+3}-8

Lanes 13-18: *Eco* RI_{+3}-4 *Mse*I_{+3}-7 Lanes 19-24: *Eco* RI_{+3}-8 *Mse*I_{+3}-3

Lane M: 100 bp DNA standard Lane L: 50 bp DNA standard

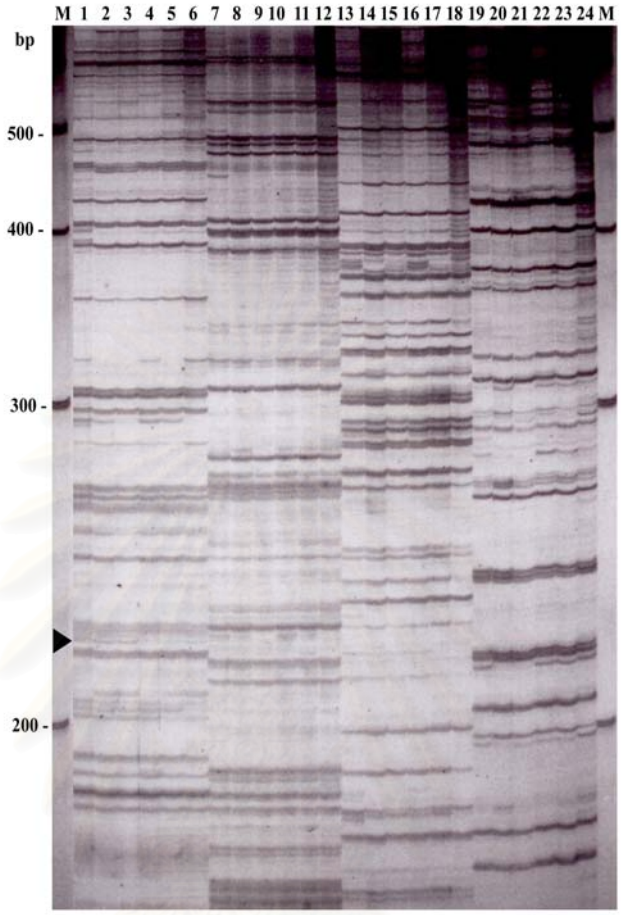

Figure 3.7 A 4.5% denaturing polyacrylamide gel showing AFLP patterns of SG1, SG2, SG3, FG1, FG2 and FG3 of *H. asinina*, using various primer combinations:

SG1 = lanes 1, 7, 13, 19 SG2 = lanes 2, 8, 14, 20

SG3 = lanes 3, 9, 15, 21 FG1 = lanes 4, 10, 16, 22

FG2 = lanes 5, 11, 17, 23 FG3 = lanes 6, 12, 18, 24

Lanes 1-6: *Eco* RI_{+3}-3 *Mse*I_{+3}-12 Lanes 7-12: *Eco* RI_{+3}-3 *Mse*I_{+3}-13

Lanes 13-18: *Eco* RI_{+3}-3 *Mse*I_{+3}-14 Lanes 19-24: *Eco* RI_{+3}-3 *Mse*I_{+3}-15

Lane M: a 100 bp DNA standard Lane L: a 50 bp DNA standard

(Arrowhead indicates a candidate growth-related marker)

Table 3.1 Candidate growth-related markers of *H. asinina* generated by AFLP analysis of 2, 4 and 6 bulked DNA using combinations of primers E_{+3}-1-14 + M_{+3}-1-16.

Primer combinations	2 bulked DNA (bp)		4 bulked DNA (bp)		6 bulked DNA (bp)	
	SG1	FG1	SG2	FG2	SG3	FG3
E_{+3}-1+ M_{+3}-1	280, 510	-		-	-	-
E_{+3}-1+ M_{+3}-2	150, 230	-	230	-	-	-
E_{+3}-1+ M_{+3}-3	140	-	-	-	-	-
E_{+3}-1+ M_{+3}-4	-	500	-	-	-	-
E_{+3}-1+ M_{+3}-5	210, 220	690, 1,500	-	-	-	-
E_{+3}-1+ M_{+3}-6	340	200, 270, 1,300		-	-	-
E_{+3}-2+ M_{+3}-2	255, 490	-	255	180	-	-
E_{+3}-2+ M_{+3}-3	150	-	-	-	-	-
E_{+3}-2+ M_{+3}-4	-	210	-	-	-	-
E_{+3}-2+ M_{+3}-5	-	150, 220	-	190	-	-
E_{+3}-3+ M_{+3}-2	800	-	-	-	-	-
E_{+3}-3+ M_{+3}-3	-	940	400, 470	-	-	-
E_{+3}-3+ M_{+3}-5	-	360	-	-	-	-
E_{+3}-3+ M_{+3}-6	660, 1,150	270, 320, 1,200	-	-	-	-
E_{+3}-3+ M_{+3}-7	380	-	-	-	-	-
E_{+3}-4+ M_{+3}-2	230, 270, 290	220	-	-	-	-
E_{+3}-4+ M_{+3}-3	-	190	-	-	-	-
E_{+3}-4+ M_{+3}-4	200	-	-	-	-	-
E_{+3}-4+ M_{+3}-6	-	330, 440	-	-	-	-
E_{+3}-4+ M_{+3}-7	250, 480	-	360	-	-	-
E_{+3}-4+ M_{+3}-8	350	-	-	-	-	-
E_{+3}-5+ M_{+3}-2	170, 190	160	-	-	-	-
E_{+3}-5+ M_{+3}-3	165, 200, 220	-	-	-	-	-
E_{+3}-5+ M_{+3}-4	200, 1,200	-	-	-	-	-
E_{+3}-5+ M_{+3}-5	-	150	-	-	-	-
E_{+3}-5+ M_{+3}-6	-	160, 290	-	-	-	-

Table 3.1 (continued)

Primer combinations	2 bulked DNA (bp)		4 bulked DNA (bp)		6 bulked DNA (bp	
	SG1	FG1	SG2	FG2	SG3	FG3
E_{+3}-5+ M_{+3}-7	-	340	-	-	-	-
E_{+3}-5+ M_{+3}-8	440	-	-	-	-	-
E_{+3}-6+ M_{+3}-1	-	410	-	-	-	-
E_{+3}-6+ M_{+3}-2	150	170	-	-	-	-
E_{+3}-6+ M_{+3}-3	-	155	-	-	-	-
E_{+3}-6+ M_{+3}-6	660	250	-	-	-	-
E_{+3}-6+ M_{+3}-7	700	-	-	-	-	-
E_{+3}-7+ M_{+3}-2	140	155, 170, 192	-	-	-	-
E_{+3}-7+ M_{+3}-3	-	250	-	-	-	-
E_{+3}-7+ M_{+3}-4	-	190	-	-	-	-
E_{+3}-7+ M_{+3}-5	185	450	-	-	-	-
E_{+3}-7+ M_{+3}-6	170, 300	165, 250	-	-	-	-
E_{+3}-7+ M_{+3}-8	200	-	-	-	-	-
E_{+3}-8+ M_{+3}-2	140, 185	130, 150	-	-	-	-
E_{+3}-8+ M_{+3}-3	170, 200	300	450	-	-	-
E_{+3}-8+ M_{+3}-5	-	1,200		-	-	-
E_{+3}-8+ M_{+3}-7	-	1,500		-	-	-
E_{+3}-8+ M_{+3}-8	1,500	-	480	-	-	-
E_{+3}-3+ M_{+3}-12	-	-	-	-	-	**225**
Total	**43**	**40**	**7**	**2**	**0**	**1**

3.2.4 Cloning and characterization of candidate growth-related markers

To further characterize the candidate of growth-related marker, the AFLP fragment showing growth specificity in the third screening, which constituted of a 225 bp fragment from E_{+3}-3 + M_{+3}-12 primers (as called FGE3M12), was gel-eluted and reamplified (Fig 3.8). The reamplified PCR product was successfully cloned into pGEM-Teasy vector and transformed into XL1 blue cells. Colony PCR was performed to evaluate the insert size of randomly selected recombinant clones. A product with expected size of approximately 400 bp (225 bp + 180 bp of the vector) was observed (Fig. 3.9). Recombinant clone containing a FGE3M12 fragment was subjected to automated nucleotide sequencing (Fig. 3.10).

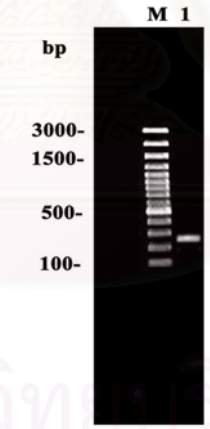

Figure 3.8 A 1.5% agarose gel showing the reamplification of the candidate fast-growing related AFLP fragment (FGE3M12, lane 1). Lane M is a 100 bp DNA ladder.

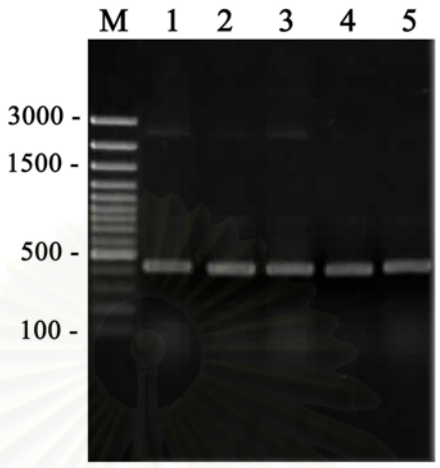

Figure 3.9 A 1.5% agarose gel showing colony PCR product of recombinant plasmids containing the candidate growth-related AFLP marker, FGE3M12 (lanes 1-5). Lane M is a 100 bp DNA ladder.

Homology searches using BLAST*N* and BLAST*X* programs were performed against the NCBI GenBank Database. The sequence of FGE3M13 did not show significant matching to the sequences previously deposited in the GenBank (E-value cut off of 10^{-4}). Therefore, it was regarded as an unknown sequence.

GATGAGTCCTGAGTAACGGTGTCCCAGCAATACTGACTTCACATATTGTACCCAAAT
GAAGGTTCGAACCCGGGTCTTTGGCGTGGCGCGCTTCATCCACCAGGCTACCCCACC
GCGCCTATTCCTGGTATTGACAAGTACGTCAAATGCATTCCTTCACGTGGATACGTC
AGATACGTTCACTTAGGTTAGTCTTCGGTTTGGA**TGTGAATTGGTACGCAGTCA**

Figure 3.10 Nucleotide sequence of a fast-growing related marker FGE3M12. Sequence and position of original AFLP marker was illustrated in boldface. Sequence and the position of the forward and those complementary with the reverse primer were underlined.

3.2.5 Development of a growth-related Sequence Characterized Amplified Region (SCAR) marker in *H. asinina*

To develop a rapid and specific method for detection of fast-growing *H. asinina*, an AFLP marker was converted to a SCAR marker. This SCAR marker was derived from the sequence of the candidate fast-growing related AFLP marker, FGE3M12. The forward and reverse primers were selected from the sequence of FGE3M12 (Fig. 3.10) which resulted in a SCAR marker of 183 bp. Growth specificity was tested on the genomic DNA of fast growing (N=9) and slow growing (N=9) *H. asinina*. However, the SCAR marker was observed in both fast growing *H. asinina* as well as slow growing abalone which deemed that the conversion of growth-related AFLP marker to growth-related SCAR marker was unsuccessful (Fig. 3.11).

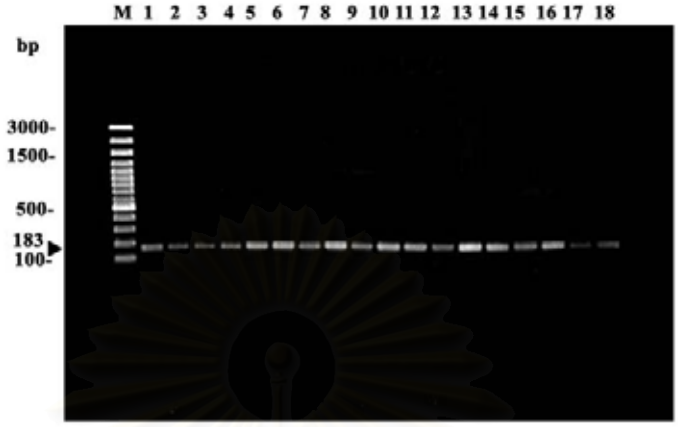

Figure 3.11 A 1.5% agarose gel showing PCR products of a SCAR marker, derived from the candidate growth-related AFLP marker (FGE3M12), tested on genomic DNA of fast growing (lanes 1-9) and slow growing (lanes 10-18) *H. asinina*. Lane M is a 100 bp DNA ladder.

3.2.6 Examination of polymorphism with Single-strand conformation polymorphisms (SSCP) analysis

SSCP analysis was then performed to identify whether amplification product, which was developed as a SCAR marker, contained single nucleotide polymorphism (SNP) for fast-growing and slow-growing individuals of *H. asinina.* The amplification products of a SCAR marker from fast-growing *H. asinina* were run on a 17.5% non-denaturing polyacrylamide gel and stained with 0.1% silver nitrate as described in 2.10. Different banding patterns were observed among individuals but no specific DNA band was found between fast-growing and slow-growing *H. asinina*. The result

of SSCP analysis showed that the SCAR marker was polymorphic, but not growth linked (Fig. 3.12).

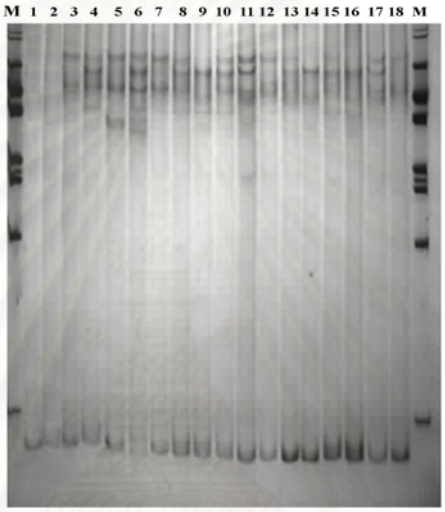

Figure 3.12 A 17.5% silver stained non-denaturing polyacrylamide gel showing SSCP pattern of the amplification product of a SCAR marker derived from the FGE3M12 fragment tested on genomic DNA of fast-growing (lanes 3-10) and slow-growing (lanes 11-18) *H. asinina*. Lane M is a 100 bp DNA ladder. Lanes 1 and 2 are the non-denatured PCR products (double stranded DNA control).

3.3 RNA extraction

Total RNA was extracted from haemocyte of fast-growing and slow-growing abalone *H. asinina* by the Trizol reagent. The OD_{260}/OD_{280} ratio of total RNA prepared by this method was 1.5-1.8, indicating acceptable quality of total RNA used in this study.

The quality of total RNA was monitored by running on 1% agarose-formaldehyde gel. RNA samples presented a dominant band size of about 1.9 kb corresponding to 18S rRNA (Fig. 3.13).

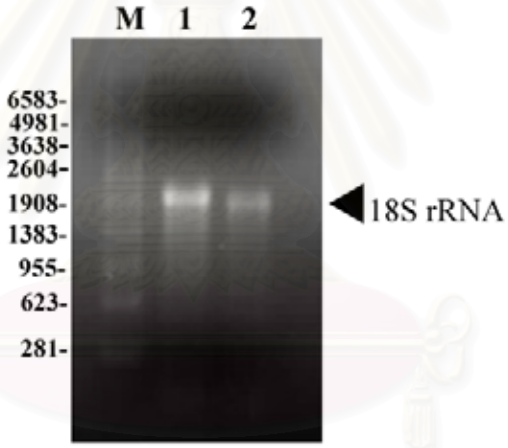

Figure 3.13 Total RNA from haemocytes of *H. asinina* (lanes 1-2) electrophoresed on 1% formaldehyde agarose gel. Lane M: a RNA marker.

3.4 RAP-PCR analysis

RAP-PCR was carried out to isolate various types of markers' expression in fast-growing and slow-growing *H. asinina*. The first strand cDNA template was synthesized from pooled total RNA of the fast growing (N=5) and the slow growing (N=3) with decanucleotide primers (UBC 101, UBC 119 and OPA 01; Fig. 3.14). The first strand-cDNA template was subjected to low-stringency PCR using the single synthesizing primers or combination of each synthesizing primer with one of the 17 primers available (Table 2.5). Overall, RAP-PCR was performed using 43 different primer pairs.

The amplification products were first analyzed by using agarose gel electrophoresis (Fig. 3.15). Successful amplification products were further analyzed on denaturing polyacrylamde gel electrophoresis (Fig. 3.16).

Of the 43 combination primers, only two RAP-PCR markers that indicated differential expression between the 2 bulked were found using primers UBC101/UBC 191. Both the amplified 210 bp and 260 bp fragments were found in only slow growing *H. asinina*.

Figure 3.14 A 1% agarose gel showing the first strand cDNA from pooled total RNA of fast-growing (lanes 1, 2 and 3; N=5) and slow-growing (lanes 4, 5 and 6; N=3) *H. asinina*, using different primers: primer UBC101 (lanes 1, 4), primer UBP119 (lanes 2, 5) and primer OPA 01 (lanes 3, 6). Lane M is a 100 bp DNA ladder.

Figure 3.15 A 1.8% agarose gel showing RAP-PCR products generated from the first strand cDNA of pooled total RNA of fast-growing (lanes 1, 3, 5, 7, 9) and slow-growing (lanes 2, 4, 6, 8, 10) *H. asinina* using different primers.

Lanes 1-2 : UBC 101/UBC 191 Lanes 3-4 : UBC 101/UBC 273

Lanes 5-6 : UBC 101/UBC 217 Lanes 7-8 : UBC 119/UBC 128

Lanes 9-10 : UBC 119/UBC138 Lane M : a 100 bp DNA standard

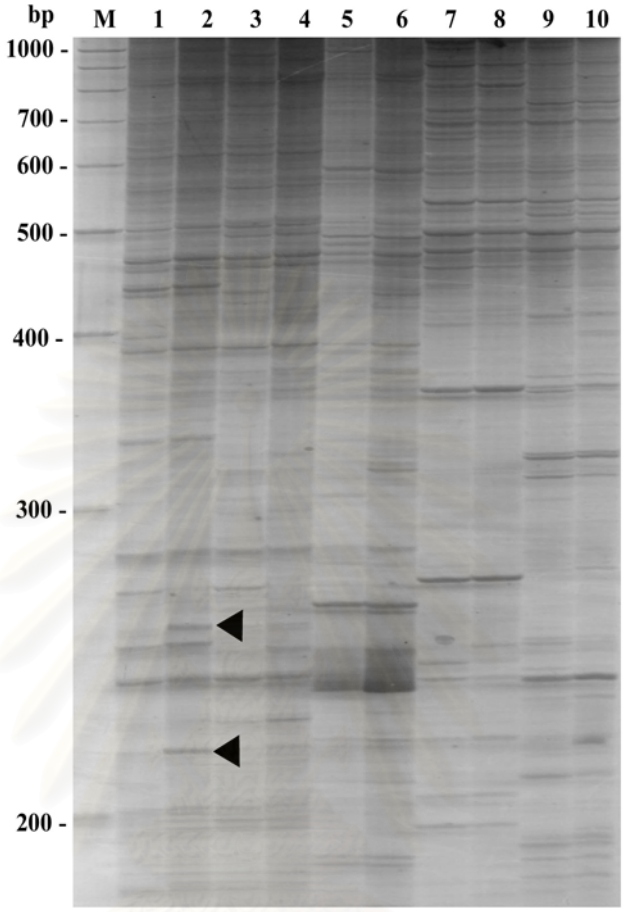

Figure 3.16 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns from pooled total RNA of fast-growing (lanes 1, 3, 5, 7, 9) and slow-growing (lanes 2, 4, 6, 8, 10) *H. asinina* using different primers.

Lanes 1-2: UBC 101/UBC 191 Lanes 3-4: UBC 101/UBC 273

Lanes 5-6: UBC 101/UBC 217 Lanes 7-8: UBC 119/UBC 128

Lanes 9-10: UBC 119/UBC138 Lane M: a 100 bp DNA standards

(Arrowhead indicates candidate growth-related markers)

3.4.1 Cloning and characterization of candidate growth-related expression markers in *H. asinina*

The two candidates of growth-related expression markers, the 210 bp (called SGRAP1) and 260 bp (called SGRAP2) fragments were excised, eluted out of the gel and reamplified with the same combination primers of UBC 101/UBC 191 (Fig. 3.17).

The reamplified PCR products were successfully cloned. Colony PCR was performed to evaluate the insert size of recombinant plasmids (+180 bp of the vector, Fig. 3.18, Fig. 3.19). The recombinant plasmids containing SGRAP1 and SGRAP2 were subjected to automated DNA sequencing (Fig. 3.20, Fig. 3.21). The sequence was searched for homology to the data in GenBank. Blast results of both slow-growing related expression markers are shown in Fig. 3.22, Fig. 3.23 and Table 3.2. The SGRAP1 fragment showed significant match to ADP/ATP carrier protein (E-value=10^{-17}) while the SGRAP2 fragment was homologues to inosine triphosphatase (E-value=10^{-7}).

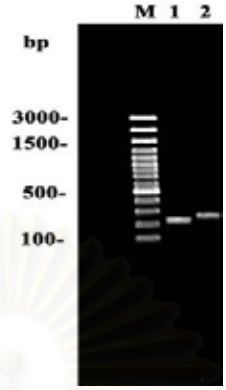

Figure 3.17 A 1.5 % agarose gel showing the reamplification of 2 candidate growth–related RAP-PCR markers (SGRAP1, lane 1 and SGRAP2, lane 2) using primers UBC 101/ UBC 191. Lane M is a 100 bp DNA ladder.

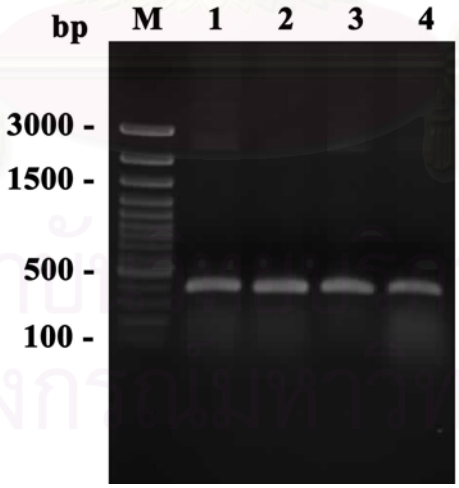

Figure 3.18 A 1.5% agarose gel showing colony PCR products of the recombinant clones containing the SGRAP1 fragment (lanes 1-4). Lane M is a 100 bp DNA ladder.

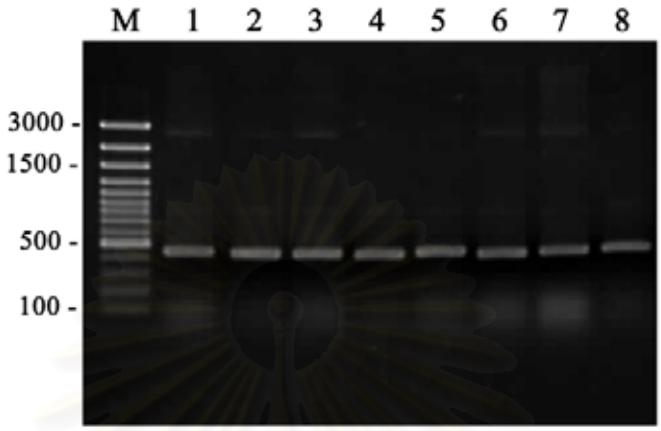

Figure 3.19 A 1.5% agarose gel showing colony PCR products of recombinant clones containing the SGRAP2 fragment (lanes 1-8). Lane M is a 100 bp DNA ladder.

GCGCCTGGAGGATCGTATCAATAGGGTAGGACAGCAATCCAGCACTGATGGTTACAG
TGTAGCCCAACATAAATGACAGAATCAAGCTGTTACTGTCACCAAGGAGAACTGGCT
TAAGGGTGTCGTAAAAGCCAAAGTAGCATCCTCTGTGGACAATGATGCCCACGCAGG
AGATGACGAAACCTCTGTACAGTCCAAC**AAAGCCATCG**

Figure 3.20 Nucleotide sequence of the slow-growing related expression marker, SGRAP1. Sequence and position of original RAP-PCR marker was illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were underlined.

GCGCCTGGAGTTCCCTGATACTCTGGCAAGTCAATGTCCTGTCTCTTGACCTTATAT
GGAAAGTCAGGTCCAAGAATTTGTACAAATTCTTCCAGCTTCTTCTTGTTTCCTGTG
ACAAGTACGATCTGTCTGGCCATGTTCTTCATCCACACAAAAGCTTTGTGAGATAAG
TTTTTAGCAAGTCCATAGCTTTTCCACGATGGGAAATAGTGTTTTTCACACTTTTGT
CCATTTCAGCATATGTCTTATC**AAAGCCATCG**

Figure 3.21 Nucleotide sequence of the slow-growing related expression marker, SGRAP2. Sequence and position of original RAP-PCR marker was illustrated in boldface. Sequence and position of the forward and those complementary with the reverse primer were underlined.

```
                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value


gi|21593729|gb|AAM65696.1|  ADP,ATP carrier-like protein [Ar...    90   2e-17
gi|26451091|dbj|BAC42650.1|  putative ADP,ATP carrier [Arabi...    90   2e-17
gi|12580863|emb|CAC27140.1|  ADP, ATP carrier protein precur...    89   4e-17
gi|3220183|gb|AAC23561.1|  ADP/ATP carrier [Trypanosoma bruc...    87   1e-16
gi|995929|gb|AAA75627.1|  rhodesiense ADP/ATP carrier              87   1e-16
gi|29468114|gb|AAO85399.1|  putative hydrogenosomal ADP/ATP ...    87   2e-16
gi|51340032|gb|AAU00712.1|  ATP/ADP translocase [Leishmania ...    87   2e-16
gi|18110|emb|CAA46311.1|  mitochondrial ADP/ATP translocator...    86   2e-16


gi|21593729|gb|AAM65696.1|

ADP,ATP carrier-like protein [Arabidopsis thaliana]
Length = 379

 Score = 90.1 bits (222), Expect = 2e-17
 Identities = 43/65 (66%), Positives = 53/65 (81%), Gaps = 2/65 (3%)
 Frame = -1

Query: 189 VGLYRGFVISCVGIIVHRGCYFGFYDTLKPVLLGD--SNSLILSFMLGYTVTISAGLLSY 16
           VGLYRGF ISCVGI+V+RG YFG YD+LKPV+L D   +S + SF+LG+ +TI AGL SY
Sbjct: 244 VGLYRGFNISCVGIVVYRGLYFGLYDSLKPVVLVDGLQDSFLASFLLGWGITIGAGLASY 303

Query: 15  PIDTI 1
           PIDT+
Sbjct: 304 PIDTV 308
```

Figure 3.22 The BlastX showing a significant match to the sequence in the GenBank database of the deduced amino acid sequence of the slow growing-related expression marker, SGRAP1 marker.

```
                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value


gi|31982664|ref|NP_080198.2|  inosine triphosphatase [Mus mu...    57   2e-07
gi|56203883|emb|CAC16798.3|  GD:ITPA [Homo sapiens] >gi|5620...    55   5e-07
gi|13398328|gb|AAK21848.1|  inosine triphosphate pyrophospha...    55   5e-07
gi|27732417|ref|XP_230604.1|  similar to inosine triphosphat...    55   6e-07
gi|12842364|dbj|BAB25571.1|  unnamed protein product [Mus mu...    54   1e-06
gi|50751047|ref|XP_422234.1|  PREDICTED: similar to Inosine ...    53   2e-06


gi|39573554|dbj|BAD04065.1|
inosine triphosphate pyrophosphatase [Mus musculus]
          Length = 198

 Score = 57.0 bits (136), Expect = 2e-07
 Identities = 26/42 (61%), Positives = 33/42 (78%)
 Frame = -3

Query: 127 MARQIVLVTGNKKKLEEFVQILGPDFPYKVKRQDIDLPEYQG 2
           + ++IV VTGN KKLEE +QILG +FP  ++ Q IDLPEYQG
Sbjct: 6   VGKKIVFVTGNAKKLEEVIQILGDNFPCTLEAQKIDLPEYQG 47


 Score = 37.7 bits (86), Expect = 0.098
 Identities = 17/30 (56%), Positives = 23/30 (76%)
 Frame = -1

Query: 240 DKTYAEMDKSVKNTISHRGKAMDLLKTYLT 151
           ++TYAEM KS KNTISHR +A+  L+ Y +
Sbjct: 161 EQTYAEMPKSEKNTISHRFRALHKLQEYFS 190
```

Figure 3.23 The BlastX results showing a significant match to the sequence in the GenBank database of the deduced amino acid sequence of the slow growing-related expression marker, SGRAP2 marker.

Table 3.2 Blast results of the sequences from the slow growing-related expression markers identified by RAP-PCR analysis.

Clone	Gene homologue	Closest species	E-value
SGRAP1	ADP, ATP carrier protein	*Arabidopsis thaliana*	10^{-17}
SGRAP2	Inosine triphosphatase	*Mus musculas*	10^{-7}

Based on the sequence of the two slow growing-related RAP-PCR markers, two primer pairs were designed for RT-PCR analysis (Table 3.3) to further confirm differential expression of the RAP-PCR markers.

Table 3.3 Primers, sequences, annealing temperature and expected size of primers from the SGRAP1 and SGRAP2 fragments.

Primer	Sequence	Tm (^{o}C)	Expected size (bp)
SGRAP1-F	5′-GAT CGT ATC AAT AGG GTA GG-3′	58	167
SGRAP1-R	5′-TGG ACT GTA CAG AGG TTT CG-3′	60	
SGRAP2-F	5′-CCC TGA TAC TCT GGC AAG-3′	56	179
SGRAP2-R	5′-CTT GCC AGA GTA TCA GGG-3′	56	

3.5 Semi-quantitative RT-PCR

3.5.1 Determination of the optimal $MgCl_2$ concentration

Determination of optimal concentration for each primer set was performed by using different $MgCl_2$ concentration (1-3 mM) with the standard RT-PCR reaction. The PCR products were run on agarose gel and the concentration of $MgCl_2$ that gave the highest yields for each product was chosen. The SGRAP1 and SGRAP2 markers gave the highest yield at 2.5 mM and 1.5 mM $MgCl_2$, respectively. An internal control, β-actin, showed the highest amplification product at 1.0 mM $MgCl_2$ concentration (Fig 3.24).

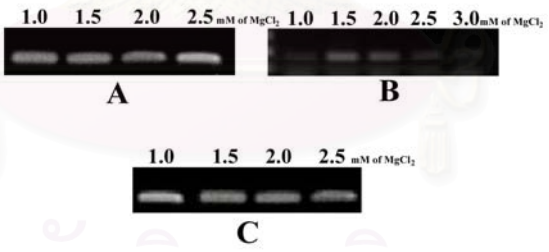

Figure 3.24 Determination of the optimal $MgCl_2$ concentration of SGRAP1 (A), SGRAP2 (B) and β-actin (C) for PCR amplification by varying concentration of $MgCl_2$ from 1-3 mM.

3.5.2 Determination of cycling parameter

To semi-quantitative the transcripts by PCR, it is necessary to select the most proper number of amplification cycles, before reaching the process of plateau amplification phase. The amplification product showing a sharp DNA band on agarose gel could then be correctly quantified. In this experiment, numbers of cycles were determined in the range of 20-36 cycles. The number of cycles that gave the highest yield before the product reached a plateau phase was chosen. The appropriate number of cycles for SGRAP1 and SGRAP2 was 24 and 28, respectively, whereas that of β-actin reached a plateau of amplification after 22 cycles (Fig. 3.25). Therefore, amplification cycles of 22, 24 and 28 were selected and further investigated for each RAP-PCR marker transcript.

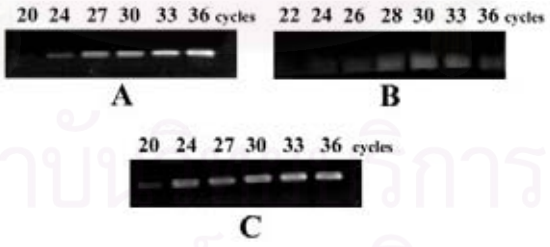

Figure 3.25 Determination of the optimal cycling number of semi-quantitative RT-PCR of SGRAP1 (A), SGRAP2 (B) and β-actin (C) by varying numbers of amplification cycles from 20-36 cycles.

3.5.3 Identification of growth-related expression marker transcripts in *H. asinina*

The optimized conditions as described above were used in this process of RT-PCR analysis. The cDNAs of fast-growing (N=5) and slow-growing (N=5) *H. asinina* were used as the templates. From the results, two developed RAP-PCR markers, SGRAP1 and SGRAP2 showed differences in the transcriptional level. The level of expression of SGRAP1 in slow-growing *H. asinina* was statistically significant than that of the fast-growing ones ($p < 0.05$). Likewise, the level of expression of SGRAP2 in slow-growing *H. asinina* was clearly higher than that of the fast-growing ones (Fig. 3.26)

Figure 3.26 RT-PCR of the first strand cDNA synthesized from total RNA of fast growing and slow growing *H. asinina*. Internal control is β-actin (220 bp).

Panel A, primer SGRAP1-F/R, Lanes 1-5: fast-growing *H. asinina*

Lanes 6-10: slow-growing *H. asinina*

Panel B, primer SGRAP2-F/R Lanes 1-5: slow-growing *H. asinina*

Lanes 6-10: fast-growing *H. asinina*

3.6 Identification of insulin-related peptides homologue in *H. asinina*

Another approach to identify growth-related marker in *H. asinina* was performed by identifying an insulin-related peptide homologue by PCR amplification. Amino acid sequences of molluscan insulin-related peptides were retrieved from the GenBank database and aligned using Clustal X program.

An alignment of the amino acid sequences suggested that insulin-related peptides of gastropod *Lymnaea stagnalis* and *Aplysia californica* had the conserved region as shown in Fig. 3.27. The conservative region was chosen to design degenerated primers. The primers deemed to further amplify cDNA from haemocytes of *H. asinina* for determination of insulin-related peptides homologue. The interesting band (as called IGFH), approximately 200 bp, resulting from PCR amplification by degenerated primers (Fig 3.28), was excised from the gel and cloned into pGEM-Teasy vector and transformed into XL1 blue cells. Colony PCR was performed to evaluate the insert size of randomly-selected recombinant clones. A product with expected size of approximately 380 bp (200 bp + 180 bp of the vector) was observed (Fig. 3.29). The colony PCR products were digested with restriction enzymes (*Alu* I and *Rsa* I) to ensure the unique insert DNA fragment (Figs. 3.30, 3.31). The recombinant plasmids showed different digested pattern indicating different inserted fragments of the recombinant clones. Two recombinant plasmids that showed different pattern were chosen for further analysis (clones 3 and 7, Figs. 3.30, 3.31). The two recombinant plasmid DNAs were extracted and subjected to automated nucleotide sequencing. Sequence alignment by Clustal X showed almost identical sequences (Fig. 3.32). Homology searches using BLAST*N* and BLAST*X* programs were performed against the NCBI GenBank Database. The sequence of this clone did not show significant matching to insulin-like

peptide as expected and it did not match any sequence previously deposited in the GenBank (E-value cut off of 10^{-4}). Therefore, it was regarded as an unknown sequence. However, two primers (IGFH-F and IGFH-R) were designed based on the IGFH sequence for RT-PCR amplification of fast-growing and slow-growing *H. asinina*.

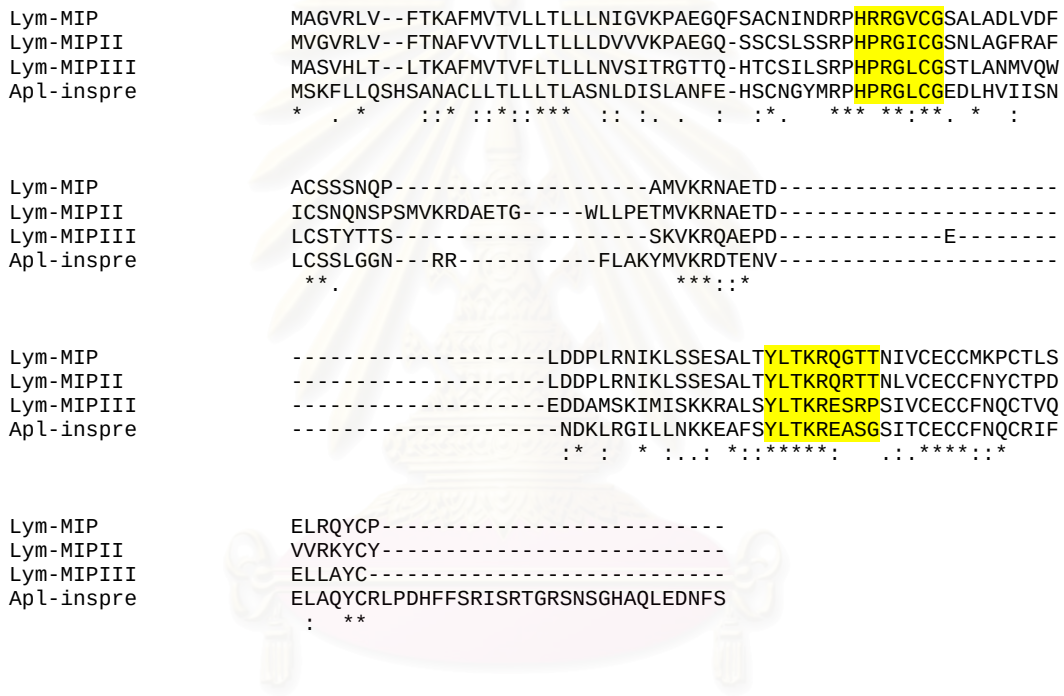

Figure 3.27 Multiple alignments of the amino acid sequences of insulin-like peptide genes from *Lymnaea stagnalis* and *Alypsia californica* by Clustal X program. Two conserved regions shown in shaded boxes were selected to design two degenerated primers.

3.6.1 RT-PCR analysis

From determination of the optimal PCR condition, it was found that $MgCl_2$ concentration gave the highest yield at 1 mM $MgCl_2$ and the cycle number of 28 cycles was most appropriate for the PCR amplification (Fig. 3.33). The cDNAs of fast-growing (N=5) and slow-growing (N=5) *H. asinina* were used as the templates for RT-PCR analysis. From the results, PCR products of fast-growing and slow-growing *H. asinina* did not show significant differences in the transcriptional level (Fig 3.34).

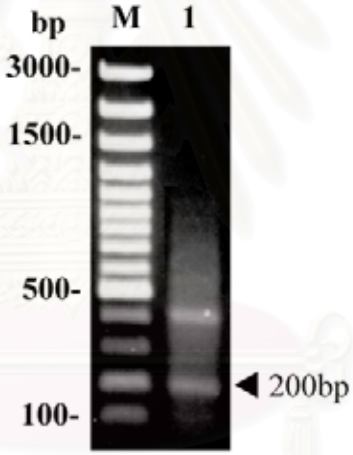

Figure 3.28 A 1.5 % agarose gel showing the PCR amplification of cDNA from haemocyte of *H. asinina* using the two degenerated primers (lane 1). Lane M is a 100 bp DNA ladder.

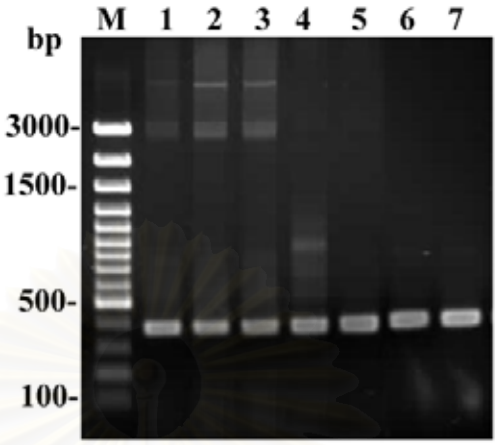

Figure 3.29 A 1.5% agarose gel showing colony PCR products of the IGFH clones (lanes 1-7; clones 1-7 respectively). Lane M is a 100 bp DNA ladder.

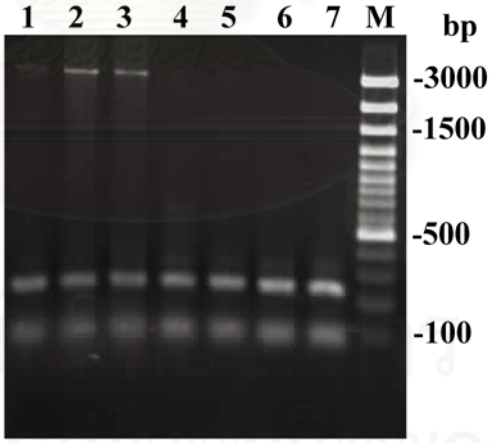

Figure 3.30 A 1.5% agarose gel showing digestion patterns of colony PCR products with *Rsa* I (lanes 1-7; clones 1-7 respectively). Lane M is a 100 bp DNA ladder.

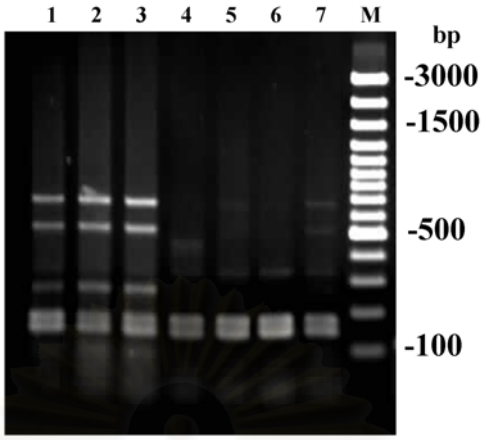

Figure 3.31 A 1.5% agarose gel showing digestion patterns of colony PCR products with *Alu* I (lanes 1-7; clones 1-7 respectively). Lane M is a 100 bp DNA ladder. (clone 3 and clone 7 represent each pattern, which was later extracted and sequenced)

```
IGFH7           ACATCGCCGTGGGGTGTGTGGAAGGAAGGTAAAAAGTGTCACGCGACACGCTATCACACA
IGFH3           ACATCGCCGTGGGGTCTGCGGAAGGAAGGTAAAAAGTGTCACGCGACACGCTATCACACA
                *************** ** ****************************************

IGFH7           ATTAACCATGGAACTCAACACTATGACAGCATTAGCACAGCTTCAGTCTGCTCAAGGCAG
IGFH3           ATCAACCATGAAACTCAACACTATGACAGCATTAGCACAGCTTCAGTCTGCTCAAGGCAG
                ** ******* **************************************************

IGFH7           AATATTTTTATCACAAGATTTTCAATGGCAACGAGTCCGTCCGTCAAACAAACATTATAC
IGFH3           AATATTTTTATCACAAGATTTTCAATGGCAACTAGTCCGTCCGTCAAACAAACATTATAC
                ******************************** ****************************

IGFH7           TATCTGACCAAGACACAAGGGACAA
IGFH3           TATTTGACCAAGACACAAGGGACAA
                *** **********************
```

Figure 3.32 Multiple sequence alignment of clones IGFH3 and IGFH7 from two degenerated primers using Clustal X program. Positions of original primers were illustrated in boldface. Sequence and position of the forward primers and those complementary with the reverse primer were shaded boxes.

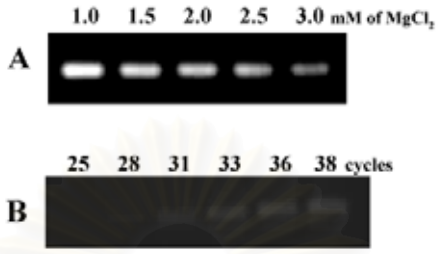

Figure 3.33 Determination of the optimal $MgCl_2$ concentration from 1-3 mM (A) and determination of the optimal cycling number from 25-38 cycles (B) for amplification of IGFH fragment.

Figure 3.34 RT-PCR of the first strand cDNA synthesized from total RNA of fast-growing (lanes 1-5) and slow-growing (lanes 6-10) *H. asinina*. Internal control is β-actin (220 bp).

3.7 Identification of 3´-end of insulin-related peptides homologue in *H. asinina*

To further characterize the IGFH fragment, we amplified its 3´ end by using the IGFH-F primer and oligo (dT_{20}) primer. The interesting band, approximately 200 bp (Fig. 3.35), was excised from the gel and cloned into pGEM-Teasy vector and transformed into XL1 blue cells. Colony PCR was performed to evaluate the insert size of randomly-selected recombinant clones. A product with expected size of approximately 400 bp (220 bp + 180 bp of the vector) was observed (Fig. 3.36). All clones randomly chosen to perform colony PCR were extracted and checked fragment size using *Eco* RI (Fig. 3.37) and one clone was sequenced as the clones' representative (Fig. 3.38). The sequence was searched for homology to the data in GenBank. However, the blast result of this clone did not show significant match to insulin-related peptides but it showed a significant match to NADH dehydrogenase subunit 5 (*Haliotis rubra*, E-value=10^{-6}) (Fig 3.39).

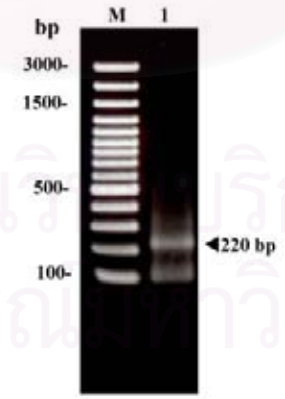

Figure 3.35 A 1.5 % agarose gel showing the PCR amplification of cDNA from haemocyte of *H. asinina* using IGFH-F and oligo (dT_{20}) primer (lane 1). Lane M is a 100 bp DNA ladder.

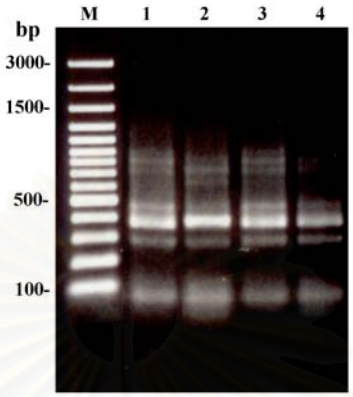

Figure 3.36 A 1.5% agarose gel showing colony PCR products of the recombinant clones carrying a 220 insert fragment (lanes 1-4). Lane M is a 100 bp DNA ladder.

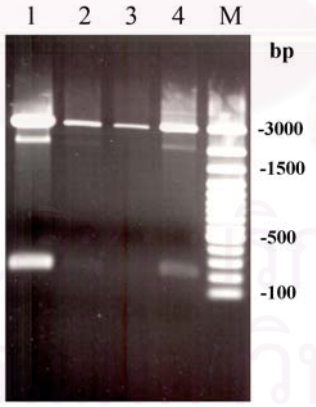

Figure 3.37 A 1.5% agarose gel showing the digestion product of the recombinant plasmids (lanes 1-4) with *Eco* RI. Lane M is a 100 bp DNA ladder.

AGGAAGGTAAAAAGTGTCACGCGGTAGGGAGTTTTTTCAGCAGAAACTAATTAGTTTTTT
AGTTATAATAGTTGTTTGATCCGCTTTTTTGATTTTGTTTTGGGTTGTGATGTAAGTGACTG
ATTGACTAAAAAAAAAAAAAAAAAAAA

Figure 3.38 Nucleotide sequence of representative clone corresponding to 3´end of the IGFH.

```
                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value

gi|49146565|ref|YP_026073.1|  NADH dehydrogenase subunit 5 [...    52   5e-06


gi|49146565|ref|YP_026073.1|  NADH dehydrogenase subunit 5 [Haliotis rubra]
Length = 580

 Score = 52.0 bits (123), Expect = 5e-06
 Identities = 26/32 (81%), Positives = 29/32 (90%)
 Frame = +2

Query: 17  SRGREFFQQKLISFLVIIVV*SAFLILFWVVM 112
           SRG EFFQQKLISFLV++VV SA LILFWVV+
Sbjct: 549 SRGSEFFQQKLISFLVMMVVWSAVLILFWVVV 580
```

Figure 3.39 The BlastX result showing a significant match to the sequence in the Genbank database of amino acid sequence of the clone corresponding to the 3´ end of the IGFH.

CHAPTER IV

DISCUSSION

Abalone has been dramatically increasing its commercial importance due to its popularity among consumers all over the world. However, today, the supply does not considerably suit the demand because abalone usually takes enormous time to be at market size, mostly more than a year. Abalone is a slow growing gastropod. Consequently, improved growth rates would result in considerable cost saving for the abalone aquaculture industry. However, no study has been ever conducted before concerning the growth performance of Thai abalone but there has been some researches dealing with other aspects as presented in the Introduction and in the next paragraph.

Most recently, Tang et al. (2005) used RAPD and microsatellite analyses to determine genetic heterogeneity of the tropical abalone (*H. asinina*) in Thai waters. One hundred and thirteen polymorphic RAPD fragments were generated. Geographic heterogeneity and Fst analyses revealed population differentiation between *H. asinina* from the Gulf of Thailand and the Andaman Sea ($p < 0.0001$). Three microsatellite loci indicated relatively high genetic diversity in *H. asinina* (total number of alleles = 26, 5, 23 and observed heterozygosity = 0.84, 0.42 and 0.33, respectively). Significant population differentiation was also found between samples from different coastal regions ($p < 0.0001$). Therefore, they concluded that the gene pool of natural *H.*

asinina in coastal Thai waters can be genetically divided to 2 different populations; the Gulf of Thailand and the Andaman Sea.

Identification of growth-related marker of *Haliotis asinina* at the genomic DNA level using AFLP analysis

In the past decade, various methods have been developed for the identification and typing of prokaryotic and eukaryotic organisms at the DNA level. These methods differ in their taxonomic range, discriminatory power, reproducibility, and ease of interpretation and standardization. One of the newest and most promising methods is amplified-fragment length polymorphism (AFLP) analysis. This method combines universal applicability with high powers of discrimination and reproducibility. An increasing number, more than 1200 up to now, of reports describe the use of AFLP analysis for plant and animal genetic mapping, medical diagnostics, phylogenetic studies, and microbial typing. Also, the method has also been employed in various aquacultural developments, such as of channel catfish *Ictalurus punctatus*, blue catfish *I. furcatus* (Liu et al., 1998), Kuruma prawn *Penaeus japonicus* (Moore et al., 1999), rainbow trout *Oncorhynchus mykiss* (Nakamura et al., 2001), black tiger shrimp *Penaeus monodon* (Wilson et al., 2002), Pacific oyster *Crassostrea gigas* (Li and Guo, 2004).

In addition, AFLP is frequently cited as a fast and reliable method to scan the genome in search of specific polymorphisms. It is also found that the principle of the technique allows the screening of a very high number of loci for polymorphism and the detection of a greater number of polymorphic DNA fragments than any other PCR-based detection systems (Vos et al., 1995).

In this study, we have not used only AFLP technique but also bulked segregant analysis to identify markers linked to growth trait in *Haliotis asinina*. Bulked segregant analysis (BSA) has been shown to be an efficient way to generate a number of markers linked to important agronomic traits (Michelmore et al., 1991). This technique has been used in conjunction with RAPD in a number of cases (Delourme et al., 1994; Jourden et al., 1996). However, in the present study AFLP technique was employed along with the BSA as it offers the advantage of analyzing a large number of markers in a single experiment and is highly reproducible (Powell et al., 1996).

So far, as it should have been clearly seen that this potential method is a very effective and powerful way to project through any experiments. However, there exist some difficulties needed to overcome before reaching the final pleasurable result. Indeed, many AFLP markers are redundant and hence too expensive and too laborious for large-scale single locus screenings. Due to this, there is a strong need to convert specific AFLP markers into single locus PCR markers, such as sequence characterized amplified region (SCAR) markers, for this marker technique is easy to use, less laborious and inexpensive for simple locus assays. Though marker conversion seems technically easy, some hurdles also need to be taken, as the detail of the hurdles and their solutions are to be clearly discussed in the next part of this chapter.

In this study, from a total of 176 primer pairs used in screening 6 bulked DNA samples, only one candidate growth marker was found. The AFLP marker was converted into a more convenient marker. The major reason why the AFLP marker needed to be converted have been discussed above. An additional technical reason is that the resulting markers from AFLP analysis are always dominant in nature.

As mentioned, for ease of use AFLP marker needed to be converted into simple SCAR marker, namely FGE3M12. This methodology involves characterization of the linked marker and the desire of locus-specific primers (Paran and Michelmore, 1993). The conversion of a linked marker to SCAR has been applied successfully in a number of studies involving RAPD markers (Naqvi and Chattoo, 1996; Lahogue et al., 1998; Barret et al., 1998) and AFLP markers (Adam-Blondon et al., 1998; Bradeen and Simon, 1998).

The AFLP marker was converted to a SCAR marker and primers were designed based on internal AFLP fragments. However, it was occurred that the resulting SCAR marker was not successful in discriminating fast and slow growing *H. asinina,* as first expected. PCR amplification of genomic DNA did not reveal any different polymorphism between the two groups of *H. asinina.* This phenomenon has ever observed in many studies.

Thamrungtanakit (2004) combined bulked segregant analysis (BSA) and AFLP for isolation of sex specific AFLP marker in *Penaeus monodon.* Candidate sex specific AFLP marker obtained when converted to SCAR marker did not retain the original sex specificity. Likewise, Preechaphol (2004) determined sex AFLP markers in giant fresh water prawn *Macrobrachium rosenbergii* and found the same circumstance. As we can learn from the two cases, AFLP markers when converted to SCAR marker changed their expression. The specificity did not retain after being converted. Therefore, polymorphism of the PCR product was further analyzed by using SSCP analysis.

The polymorphism identification through SSCP is a possible effective way to find genetic markers useful for improved selection of agricultural populations, namely

when applied to candidate genes associated with quantitative genetic variation in traits of economic importance. Also, it is a powerful method for identifying sequence variation in amplified DNA (Bonifácio et al., 2001). The technique is based on electrophoretic detection of conformational changes in single-stranded DNA molecules resulting from point mutations or other forms of small nucleotide changes (Xie et al., 2002). So far, many researches have been successfully conducted via this technique. Fornzler et al. (1998) successfully mapped gene in zebrafish by using SSCP analysis. Additionally, Fernández et al. (2002) differentiated genes of the clam species *Ruditapes decussatus* (grooved carpet shell) and *Venerupis pullastra* (pullet carpet shell). It was achieved based on PCR-SSCP analysis. A short fragment (150 bp) of the α-actin gene was amplified by PCR. Amplicons were denatured to obtain single-stranded DNA, electrophoresed on a non-denaturing polyacrylamide gel and visualised by silver staining for detection of SSCPs. Species-specific DNA band patterns were obtained for *R. decussatus* and *V. pullastra*, allowing clear differentiation of the two clam species. Moreover, Rakus et al. (2003) detected polymorphism of major histocompatibility complex class II B genes in different lines of the common carp (*Cyprinus carpio*). They examined the polymorphism of the major histocompatibility complex (MHC) gene class II *B* in nine carp lines. Polymerase chain reaction was used to amplify *Cyca-DAB* gene fragments comprising part of exon 1, complete intron 1 and almost complete exon 2. Exon 2 encodes for the β_1 domain which is the most polymorphic fragment of MHC class II molecules. SSCP was applied to detect different MHC class II *B* haplotypes. The analysis successfully revealed the presence of seven different haplotypes occurring with various frequencies.

However, the result in this study showed that the PCR product of each *H. asinina* gave insignificantly different polymorphism. In other words, the polymorphism found was not growth linked.

As described, the SCAR marker did not succeed in discriminating fast and slow growing *H. asinina*. It is to be discussed that there are two possible reasons to explain the phenomena. The first reason is that the marker found might be the false positive marker. The other is that the SCAR marker may actually be the growth-related marker but polymorphism of the positive AFLP marker comes up to be at either side of the ends of the restriction cutting site (*Eco* RI or *Mse* I). If the latter case happens to be the real occurrence, it is needed to know the information about the sequence flanking the AFLP band and then design a new primer by basing it on the sequence flanking mentioned and direct amplification of genomic DNA.

As suggested, in solving the problem, PCR-based genome-walk analysis should be further carried out. The primary application of genome-walk analysis is to rapidly clone the promoter and other upstream regulatory elements in genes which cDNA sequence was previously available. This approach can be practically applied for identification of single nucleotide polymorphisms (SNPs). Sasazaki et al. (2004) developed breed identification markers derived from AFLPs in beef cattle. They employed PCR-walking analysis to determine both flanking sites of AFLP fragment and converted them into single nucleotide polymorphism markers. Then, they designed primers based on the flanking sites. The newly-designed primers were employed in testing genomic DNA of two breeds (namely, Japanese black and Holstein) of cattle. PCR products were sequenced and identified the mutation by comparing between the two breeds. To identify the mutations that caused AFLP

polymorphisms, the sequence information of the genomic DNA flanking AFLP fragment was needed. At last, this study identified six mutations responsible for the original AFLP polymorphism. Two were insertion/deletion in AFLP fragment; four were SNPs at the restriction sites (two in *Eco* RI and two in *Taq* I).

Clearly then, the technique PCR-walk analysis and the assumptions about the false positive markers mentioned earlier should be a reasonable explanation about the phenomena. Also, the method of PCR-walk analysis should be the best way possible to solve the problem of SCAR marker. Therefore, it can be concluded that the assumption we discussed about should be an interesting method for more experiments in the future.

Identification of growth-related expression markers by RAP-PCR

Identification of candidate genes influencing any important trait can be approached through an analysis of complementary DNA (cDNA), copy of messenger RNA (mRNA). The identification enabled us to understand biological responses and genetic improvement. Over the last few years, several techniques have been developed to analyze differential gene expression. RNA-fingerprinting by arbitrarily primed PCR (RAP-PCR) has been proven to be an efficient and reliable technique for examining differentially regulated genes (Neumann et al., 2002). RAP-PCR technique is based on the synthesis of cDNA using arbitrary primers and PCR amplification. The technique was applied to identify the characteristics of genes in many important aquaculture studies.

Boag et al. (2000) isolated and characterized sex-specific transcripts from *Oesophagostomum dentatum* by RNA arbitrarily-primed PCR. The study investigated

sex-specific gene expression in the nodule worm using the technique of RAP-PCR. Northern blot analysis proved ten ESTs to be expressed exclusively in males, while two were expressed solely in female stages. The two female-specific ESTs had similarity to vitellogenin-5 and endonuclease III from *Caenorhabditis elegans*. The remaining ESTs had no similarity to any nucleic acid or protein sequences contained in the databases. The isolation and characterization of sex-specific ESTs from *Oesophagostomum dentatum* provides a unique opportunity for studying the reproductive biology of parasitic nematodes at the molecular level, with a view toward novel approaches for parasite control.

Rodius et al. (2004) used RAP-PCR to detect the induction of gene expression in freshwater bivalves (*Unio tumidus*) transplanted into the Moselle River. They detected changes in gene expression in organisms for which only minimal genomic information is available. In this study, RAP-PCR was used to detect modification of mRNA expression in *U. tumidus*. A signal was obtained only with the cDNAs from animals exposed at the downstream station, confirming that the variation detected by RAP-PCR corresponds to an increase of gene expression. Chemical analysis of sediments from the control and river sites indicated that the levels of several potential pollutants were similar at the three locations and below currently accepted pollution thresholds. Their results indicate that RAP-PCR is a sensitive technique that can be applied in field studies to identify modifications in the gene expression of bioindicator species. This approach can complement chemical, biochemical and population studies to assess the impact of human activity on the ecological quality of aquatic systems.

Preehaphol (2004) examined genes specifically expressed in each male morphotype and females of *M. rosenbergii* by employing RAP-PCR. Fourteen

transcripts were specifically expressed in males whereas 7 transcripts were restrictively expressed in females. In addition, 4, 4 and 6 transcripts were specifically expressed in blue-claw, orange-claw and small males, respectively. A 340 bp band was observed in small male *M. rosenbergii* when analyzed by UBC428 disregarding any combined primer. The marker can be used as a morphotype-specific expression marker of the small male *M. rosenbergii*. Additional primers will be tested for isolation of genes specifically expressed in other male morphotypes of *M. rosenbergii*.

Therefore, RAP-PCR was employed in this study to identify candidate growth differential expression marker. In the process of RAP-PCR analysis, RNA sample was prepared from haemocytes of *H. asinina* because the haemocytes are a possible source to find and study growth factor (Lebel et al., 1996, Serpentini et al., 2000). By comparative analysis, two slow-growing related expression markers were identified (SGRAP1 and SGRAP2) through screening with 43 primer combinations. The SGRAP1 fragment showed a significant match to ADP/ATP carrier protein, while the SGRAP2 fragment was homologue to inosine triphosphatase.

The differential expressions of both genes were confirmed by using semiquantitative RT-PCR. Consequently, it successfully showed differences in the transcriptional level. The level of expression of SGRAP1 in slow-growing *H. asinina* was statistically significant than that of the fast-growing ones ($p<0.05$). Likewise, the level of expression of SGRAP2 in slow-growing *H. asinina* was clearly higher than that of the fast-growing ones. In summary, RAP-PCR analysis identified two slow-growing related expression markers, namely ADP/ATP carrier protein and inosine triphosphatase, both of which involve metabolic pathways. The ADP/ATP carrier

(ADP/ATP translocase, ADP/ATP translocator protein, adenine nucleotide translocase) is the most abundant protein in mitochondria and catalyzes the exchange of adenine nucleotide across the mitochondria inner membrane. This exchange is essential to the transfer of energy from oxidative phosphorylation to extramitochondrial processes (Battini et al., 1987; Kunji, 2004). As for inosine triphophatase (ITP-ase), its function is to catalyze the pyrophosphohydrolysis of inosine triphosphate (ITP) to inosine monophosphate. Inosine monophosphate (IMP) synthesized by the de novo or salvage purine pathways occupies a pivotal position in purine metabolism. In nucleated cells, IMP is converted to ATP via adenosine monophosphate (AMP) or to guanosine triphosphate (GTP) via guanosine monophosphate (GMP). However, IMP may also be phosphorylated to ITP. The putative role of ITPase is to recycle purines trapped in the form of ITP and to protect the cell from the accumulation of “rogue” nucleotides, such as ITP, dITP or xanthosine triphosphate (XTP), that may be incorporated into RNA and DNA (Sumi et al., 2002).

Besides, we tried to search for an insulin-like peptide from *H. asinina* using degenerated primers designed from conserved region of molluscan insulin-like peptides. Insulin-related peptides are present in a wide variety of invertebrates. In insect and mollusk species, there is evidence that insulin peptides play, as in vertebrate organisms, a major role in development and metabolism. In the gastropods, molluscan insulin-like peptides (MIPs) from the *Lymnaea stagnalis* (Smit et al., 1988, 1998) and insulin precursor from *Aplysia californica* (Floyd et al., 1999) have been characterized. Both gastropods were found possessing the typical conserved structure (Gricourt et al., 2003). That is the reason that the invertebrates were chosen to design

degenerated primers. In the past, it has been known that molluscs possess growth hormone (GH) -like molecules since Lubet (1971) worked with the gastropod *Crepidula fornicata*. Later, Geraerts (1976) characterized a growth hormone produced by neurosecretory cells in the cerebral ganglia of *Lymnaea stagnalis*. This hormone was subsequently shown to stimulate shell growth (Dogterom et al., 1979; Dogterom and Jentjens, 1980) and to influence certain metabolic pathways (Dogterom, 1980; Dogterom and Robles, 1980). The series of genes coding for molluscan isulin-like peptide (MIP) in *L. stagnalis* has been identified (Smit et al., 1992). There is also evidence to suggest that gastropods possess a somatostatin-like growth-promoting molecule (Grimm-Jorgensen, 1983a; Marchand et al., 1989). Insulin precursor from *Aplysia californica* (Floyd et al., 1999) had also been characterized and sequenced. Clearly then, growth-regulating substances in gastropods are being actively researched.

In this study, the degenerated primers were designed from insulin-like peptides of the gastropod molluscs, *L. stagnalis*, and *A. californica* to identify insulin-related peptides homologue in *H. asinina*. However, the homologue in *H. asinina* was not found as expected.

In summary, identification of growth-related markers of *H. asinina* in the DNA level by AFLP analysis was not achieved. In the cDNA level, differential expression of markers using RAP-PCR identified two slow-growing related expression markers, ADP/ATP carrier protein and inosine triphosphatase. Both genes involve metabolic pathways. Additionally, the certain homologue was not successfully found in identification of insulin like peptide homologue. However, RAP-PCR markers obtained might be successfully applied to determine *H. asinina* in

the hatchery from where we collected the samples, not universal, though. Even if the results are not directly related to growth, it can be a basement for more researches on this Thai topical abalone *Haliotis asinina.*

CHAPTER V

CONCLUSIONS

1. One candidate of fast-growing related AFLP markers (FGE3M12) was identified from screening 176 primer combinations with 6 bulks (FG1, FG2, FG3, SG1, SG2, SG3) genomic DNA of fast growing and slow growing of *H. asinina*.

2. One SCAR marker developed from growth-related AFLP fragment generated the positive amplification product in both fast and slow growing of *H. asinina*. Therefore, development of a SCAR marker was unsuccessful.

3. SSCP analysis of amplification product, which was developed as a SCAR marker showed that the SCAR marker was polymorphic, but not growth linked.

4. Two slow-growing related expression markers were identified (SGRAP1 and SGRAP2) through screening with 43 primer combinations. The SGRAP1 fragment showed a significant match to ADP/ATP carrier protein, while the SGRAP2 fragment was homologue to inosine triphosphatase.

5. Semiquantitative RT-PCR confirmed the differential expressions of SGRAP1 and SGRAP2 markers. It showed differences in the transcriptional level. The level of expression of SGRAP1 in slow-growing *H. asinina* was statistically significant than that of the fast-growing ones ($p<0.05$). The level of expression of SGRAP2 in slow-growing *H. asinina* was higher than that of the fast-growing ones.

6. The degenerated primers were designed from insulin like peptides of the gastropod mollusks, *L. stagnalis* and *A. californica* to identify insulin-related peptides homologue in *H. asinina*. However, the homologue in *H. asinina* was not identified.

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APPENDICES

Appendix A

Chemicals for preparation of polyacrylamide gel and silver staining

1. **4.5% Denaturing acrylamide solution, 500 ml**

Acrylamide	21.375 g
Bis-acrylamide	1.125 g
7M urea	210 g

2. **40% acrylamide solution (crosslink = 37.5:1), 500 ml**

Acrylamide	194.80 g
Bis-acrylamide	5.19 g

3. **Bind silane**

95% ethanol	995 µl
Bind silane	4µl
Acetic acid	5µl

4. **Fix/stop solution (10% glacial acetic acid), 2 liters**

Glacial acetic acid	200 ml
Deionized water	1800 ml

5. **Staining solution, 1.5 liters**

$AgNO_3$	1.5 g
37% formaldehyde	2.25 ml

6. **Developing solution, 3 liters**

$NaCO_3$	90 g
37% formaldehyde	5 ml
Sodium thiosulfate (10mg/ml)	600 µl

Appendix B

Primer combinations for AFLP analysis

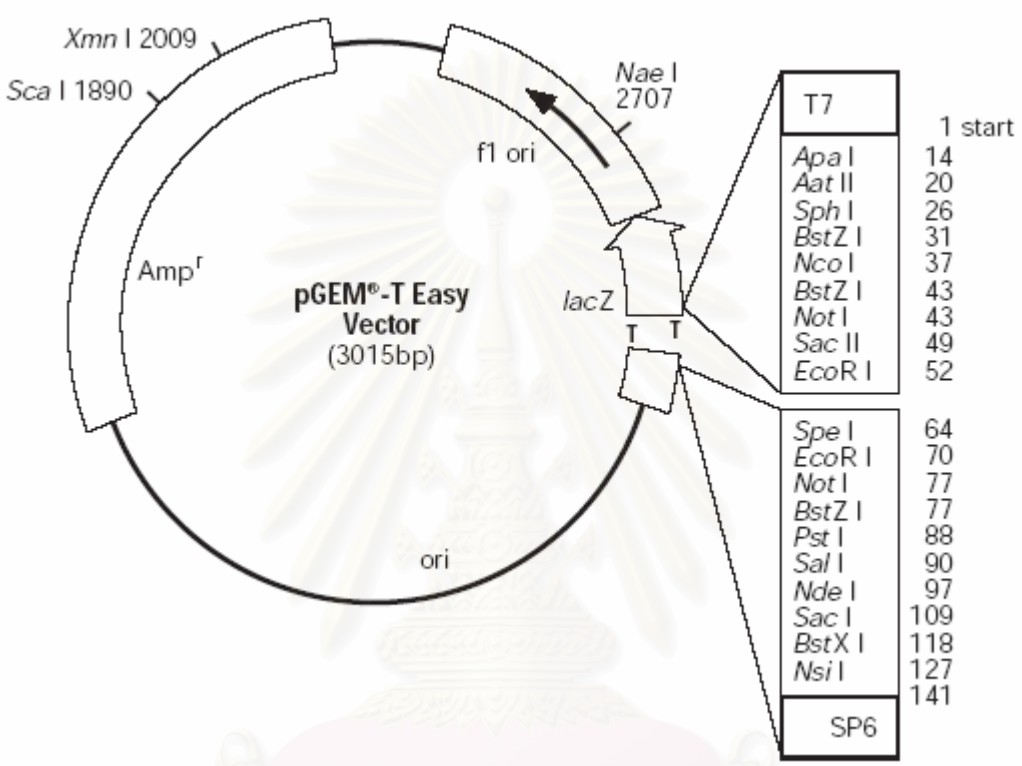
*Eco*RI$_{+3}$ / *Mse*I$_{+3}$	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	✓	✓	✓	✓	✓	✓	✓	✓								
2	✓	✓	✓	✓	✓	✓	✓	✓								
3	✓	✓	✓	✓	✓	✓	✓	✓								
4	✓	✓	✓	✓	✓	✓	✓	✓								
5	✓	✓	✓	✓	✓	✓	✓	✓								
6	✓	✓	✓	✓	✓	✓	✓	✓								
7	✓	✓	✓	✓	✓	✓	✓	✓								
8	✓	✓	✓	✓	✓	✓	✓	✓								
9	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
10	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
11	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
12	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
13	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
14	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

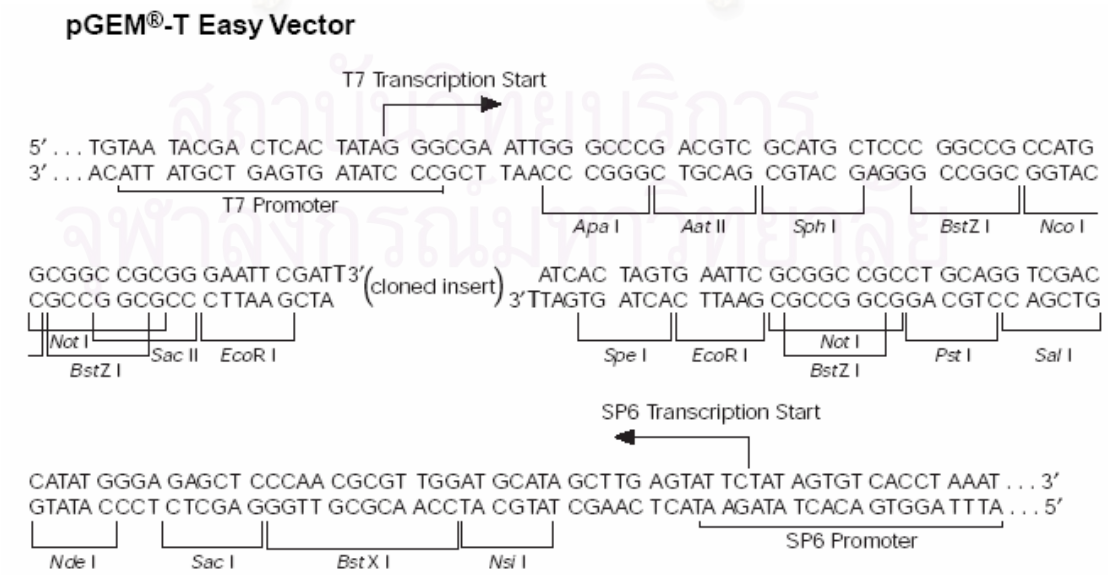
***First primer set for screening candidate growth markers in boldface**

****Second primer set for screening candidate growth markers in regular face**

Appendix C

Restriction mapping of pGEM®T-easy Vector





BIOGRAPHY

Miss Thidarat Klongtruajroke was born on January 13, 1979. She graduated with the Bachelor of Science from the Department of Biochemistry at Chulalongkorn University in 2000. She has studied for the degree of Master of Science at the department of Biotechnology, Chulalongkorn University since 2001.