ลักษณะความแตกต่างของเปลือกและความแปรผันทางพันธุกรรมของ
หอยทากยักษ์แอฟริกา *Achatin fulica* (Bowdich, 1822)
ที่พบในประเทศไทย

นายธนพันธุ์ ปัทมานนท์

SHELL MORPHOLOGICAL DIFFERENCES AND

GENETIC VARIATION OF THE GIANT AFRICAN

SNAIL *ACHATINA FULICA* (BOWDICH, 1822)

IN THAILAND

Mr. Tanapan Pattamarnon

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Environmental Biology
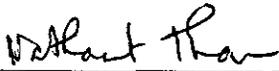
Suranaree University of Technology

Academic Year 2004

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IN THAILAND

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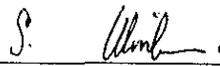
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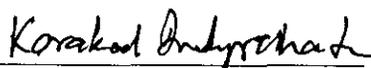(Dr. Nathawut Thanee)

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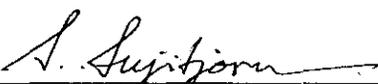
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ธนพันธุ์ ปัทมานนท์ : ลักษณะความแตกต่างของเปลือกและความแปรผันทางพันธุกรรม
ของหอยทากยักษ์แอฟริกา *Achatina fulica* (Bowdich, 1822) ที่พบในประเทศไทย (SHELL
MORPHOLOGICAL DIFFERENCES AND GENETIC VARIATION OF

THE GIANT AFRICAN SNAIL *ACHATINA FULICA* (BOWDICH, 1822) IN
THAILAND) อาจารย์ที่ปรึกษา : อาจารย์ ดร.พอล เจ โกรดิ, 226 หน้า.
ISBN 974-533-435-9

เก็บตัวอย่างหอยทากยักษ์แอฟริกา (*Achatina fulica*) (Gastropoda: Achatinidae) จำนวน
215 ตัว จากพื้นที่ต่างๆ ในประเทศไทย 9 แห่ง และประเทศมาเลเซีย 1 แห่ง รวม 10 แห่ง ทำการ
วิเคราะห์ลักษณะทางสัณฐานวิทยา และความหลากหลายทางพันธุกรรมโดยวิธี polymerase chain
reaction restriction fragment length polymorphism (PCR-RFLP) กับยีน cytochrome oxidase
subunit I (COI_{710}) ที่มีความยาว 710 ลำดับเบส วิธี single strand conformational polymorphism
(SSCP) กับยีน COI_{215} ที่มีความยาว 215 ลำดับเบส และยีน PMX และวิธี randomly amplified
polymorphism DNA (RAPD) ผลการวิเคราะห์ด้วย 3 วิธีแรก พบว่า หอยทากยักษ์แอฟริกาใน
ประเทศไทยมีความหลากหลายทางพันธุกรรมต่ำ และไม่พบความแตกต่างทางพันธุกรรม แต่ผลการ
วิเคราะห์ด้วยวิธี PCR-RAPD โดยใช้ไพรเมอร์ 4 ชนิด ได้แก่ OPA02 OPA17 OPB11 และ OPZ09
ให้แถบ ดีเอ็นเอ ทั้งสิ้น 22 รูปแบบ โดยมีแถบดีเอ็นเอ ทั้งหมด จำนวน 117 แถบ ที่มีความยาว 200-
1,700 ลำดับเบส โดยมีจำนวน 72 แถบ คิดเป็นร้อยละ 61.54 ที่ให้ผลเป็น polymorphic ค่าระยะห่าง
ทางพันธุกรรม (genetic distance) เฉลี่ยของทุกไพรเมอร์ มีค่าระหว่าง 0.0317-0.1378. พบแถบดีเอ็น
เอ 14 แถบ จากการวิเคราะห์ด้วย ไพรเมอร์ OPB11 ที่มีความแตกต่างทางพันธุกรรมอย่างมีนัยสำคัญ
($P<0.01$) ในกลุ่มตัวอย่างหอยทั้งหมดที่ทำการศึกษา เมื่อพิจารณาในระดับกลุ่มประชากร พบว่า
ไพรเมอร์ OPB11 ให้ผลว่า หอยทากที่จังหวัดสงขลามีความแตกต่างจากหอยทากจากแหล่งอื่นอย่าง
มีนัยสำคัญ ($P<0.001$) ซึ่งผลนี้ ชี้ให้เห็นว่า gene pool ของหอยทากยักษ์แอฟริกาในประเทศไทยได้
มีการแบ่งแยกการผสมพันธุ์แล้ว ผลจากการสร้างสายสัมพันธ์ทางวิวัฒนาการแบบ neighbor-joining
ด้วยข้อมูลของ RAPD สามารถอธิบายเส้นทางการกระจายของหอยทากยักษ์แอฟริกาในประเทศ
ไทยได้ โดยผลชี้ให้เห็นว่าหอยทากยักษ์แอฟริกาได้ถูกนำเข้าจากประเทศมาเลเซียมาที่ จังหวัด
สงขลาเป็นแห่งแรก ก่อนที่จะกระจายไปส่วนอื่นของประเทศไทย และหอยทากยักษ์แอฟริกา ที่
จังหวัดนครราชสีมา อาจถูกนำมาโดยตรงจากประเทศมาเลเซีย

จากการศึกษาการแสดงออกของยีนที่เกี่ยวเนื่องกับความเครียดโดยวิธี RNA arbitrary
primed polymerase chain reaction (RAP-PCR) ในหอยทดลองสามกลุ่ม คือ กลุ่มหอยปกติ กลุ่มกึ่ง
จำศีลฤดูร้อน (partial aestivation) และกลุ่มจำศีลฤดูร้อน (full aestivation) ได้ทำการ โคลน

(clone) และหาลำดับเบสจากแถบดีเอ็นเอที่แสดงออกต่างกัน จำนวน 7 แถบ มีเพียง 3 แถบ ที่ตั้งชื่อว่า AFRAP2/228410 AFRAP9/138350 และ AFRAP9/138470 ที่สามารถนำมาศึกษาระดับ การแสดงออกของยีนได้ นอกจากนั้นยังศึกษาระดับการแสดงออกของยีน achacin (ยีนสร้าง โปรตีนที่มีคุณสมบัติเป็น antibacterial peptide) ผลการศึกษาด้วยวิธี semiquantitative PCR พบ ระดับของการแสดงออกที่แตกต่างอย่างมีนัยสำคัญ ระหว่างหอยปกติ หอยกึ่งจำศีลฤดูร้อนและหอย จำศีลฤดูร้อน ($P<0.05$) การเปลี่ยนแปลงระดับการแสดงออกของยีนที่เกี่ยวเนื่องกับความเครียด ใน หอยทากยักษ์แอฟริกา อาจส่งผลต่อการเปลี่ยนแปลงทางสรีระของหอย และอาจเป็นสาเหตุของการ ตาย เมื่อหอยอยู่ในสภาวะจำศีลฤดูร้อนต่อเนื่องเป็นเวลานาน

สาขาวิชาชีววิทยา
ปีการศึกษา 2547

ลายมือชื่อนักศึกษา _Tanapan Pattam_
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TANAPAN PATTAMARNON : SHELL MORPHOLOGICAL DIFFERENCES AND GENETIC VARIATION OF THE GIANT AFRICAN SNAIL *ACHATINA FULICA* (BOWDICH, 1822) IN THAILAND. THESIS ADVISOR : PAUL J. GROTE, Ph.D. 226 PP. ISBN 974-533-435-9

GENETIC VARIATION/ *ACHATINA FULICA*/ MORPHOMETRIC/ STRESS RELATED GENE EXPRESSION

The giant African snail, *Achatina fulica*, (Gastropoda: Achatinidae) was collected from 10 geographic locations in Thailand and Malaysia (N=215) and analyzed by morphometrics of the shell, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of a 710 bp fragment of cytochrome oxidase subunit I (COI_{710}), single strand conformational polymorphism (SSCP) of COI_{215} and PMX and randomly amplified polymorphic DNA (RAPD). Results from the first techniques revealed limited genetic diversity and a lack of genetic heterogeneity of *A. fulica* in Thailand. In contrast, RAPD-PCR using four primers (OPA02, OPA17, OPB11, and OPZ09) generated 117 scorable bands (200-1700 bp), 72 of which (61.54%) were polymorphic. Twenty-two RAPD patterns (4, 6, 6, and 6 patterns, respectively) were observed. The average genetic distance across all primers ranged from 0.0317-0.1378. Fourteen fragments generated from OPB11 illustrated significant genetic heterogeneity across all samples (P<0.01). At the population level, OPB11 revealed significant genetic differences between Songkhla and each of the remaining samples (P<0.001). This indicated that the *A. fulica* gene pool in Thailand is reproductively isolated. A neighbor-joining tree based on RAPD analysis implied a possible route of distribution of *A. fulica* in Thailand. Genetic heterogeneity and

phylogenetic analysis suggested that *A. fulica* may have been initially introduced to Songkhla before subsequent distribution to other places in Thailand anthropologically. In addition, the phylogenetic tree suggested that *A. fulica* in Nakhon Ratchasima may have been directly introduced from Malaysia.

Stress-related transcripts were isolated from normal, partially aestivated and fully aestivated *A. fulica*. RNA arbitrary prime polymerase chain reaction (RAP-PCR) was used to determine the expression levels. Seven differentially displayed RAP bands were cloned and sequenced, three of which (AFRAP2/228410, AFRAP9/138350 and AFRAP9/138470) were successfully amplified. Expression levels of these transcripts and an achacin gene (an antibacterial peptide previously reported in *A. fulica*) were semiquantitatively examined. Results indicated significant differences in expression levels of these transcripts between normal and stressed snails ($P<0.05$). Fluctuations of gene expression in response to stress conditions of *A. fulica* may cause serious physiological changes resulting in high mortality of snails if the aestivation process is prolonged.

School of Biology   Student's Signature _____

Academic Year 2004   Advisor's Signature _____

Co-advisor's Signature _____

Co-advisor's Signature _____

ACKNOWLEDGMENTS

Tanapan Pattamarnon

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LIST OF ABBREVIATIONS

bp	=	base pair
°C	=	degree Celcius
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine triphosphate
dTTP	=	deoxythymidine triphosphate
DNA	=	deoxyribonucleic acid
HCl	=	hydrochloric acid
IPTG	=	isopropyl-thiogalactoside
Kb	=	kilobase
M	=	Molar
$MgCl_2$	=	magnesium chloride
mg	=	Milligram
ml	=	Millilitre
mM	=	Millimolar
ng	=	Nanogram
OD	=	optical density
PCR	=	polymerase chain reaction
RNA	=	Ribonucleic acid
RNase A	=	ribonuclease A
rpm	=	revolution per minute

LIST OF ABBREVIATIONS (Continued)

SDS	=	sodium dodecyl sulfate
Tris	=	tris (hydroxyl methyl) aminomethane
μg	=	Microgram
μl	=	Microliter
μM	=	Micromolar
UV	=	ultraviolet

CHAPTER I

INTRODUCTION

The giant African snail, *Achatina fulica* (Bowdich, 1822), is a large terrestrial snail with relatively long, narrow conical shell. The snail originally inhabited the eastern coast of Africa (Mead, 1961). It has been anthropologically transported to other areas in Africa and colonized in Ethiopia, Ghana, Kenya, the Ivory Coast, Morocco, Mozambique, Somalia, and Tanzania (Venette and Larson, 2004). Currently, *A. fulica* is distributed in all of sub-Saharan Africa (Raut and Barker, 2002).

The giant African snail has been intentionally and accidentally introduced to many parts of the world for different purposes (e.g. as medicine and food) (Raut and Barker, 2002). During the nineteen century, *A. fulica* dispersed eastward across the Indian Ocean from Africa to India, Sri Lanka, and then reaching Southeast Asia passed to China, Taiwan, Japan, Hawaii, and the South Pacific islands (Mead, 1961) (Figure 1.1). The giant African snail is found abundantly in areas having tropical climates with warm, mild year-round temperatures and high humidity (Venette and Larson, 2004).

As in other non-native species, *A. fulica* populations generally increase dramatically after introductions (Crooks and Soule, 1996; Craze, and Mauremootoo, 2002). The giant African snail has caused damage to plants and is, therefore, a significant pest of agricultural crops. (Smith and Fowler, 2003). It is also an

intermediate host for the rat lung worm (*Angiostrongylus cantonensis*) and the related *Angiostrongylus costarecensis* that causes eosinophelic meningitis in humans. As a result, this snail species has been listed as one of the 100 worst invasive species and reported to be the most damaging land snail in the world (Venette and Larson, 2004).
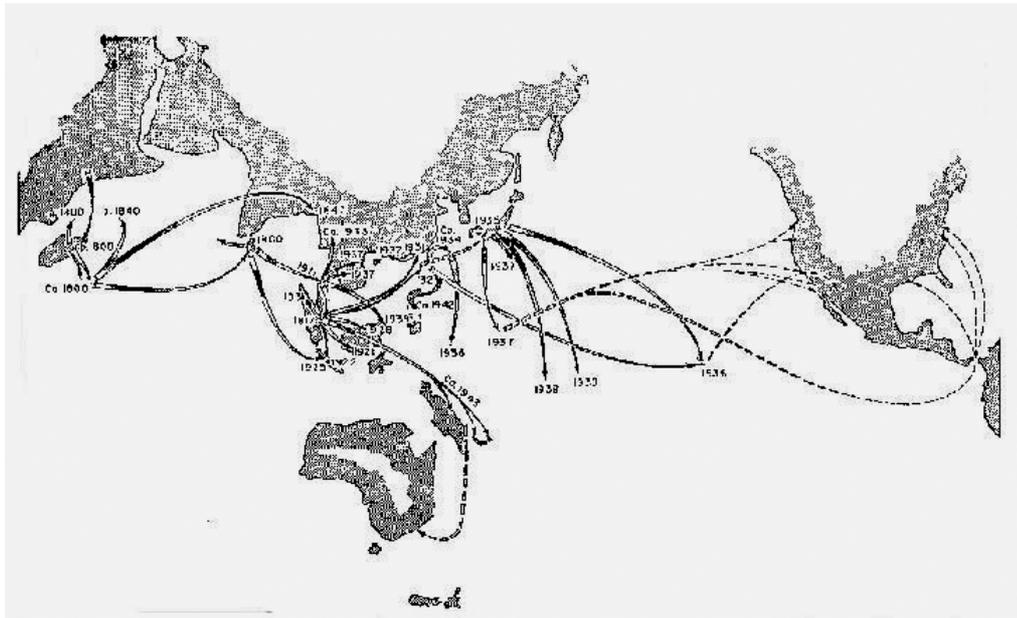
Figure 1.1 Distribution of the giant African snail, *A. fulica* (Bowdich, 1822), from its original place, East Africa, showing its movement eastward across the Indian Ocean to Asia, the South Pacific and to America. Broken lines indicate interception; solid lines indicate establishment (Mead, 1961).

The giant African snail might have been first introduced into Thailand from Malaysia in 1937 (ศักดิ์ศิริ เกิดปรีดี, 2499, Saksiri Kerdpreedee, 1956). Living specimens from Thailand were obtained in 1938 by Boettger (1951) quoted in Mead (1961). Therefore, Mead (1961) concluded that the first giant African snails entered Thailand from infested areas in Malaysia at least by 1937 for duck feeding by Chinese duck raisers. This information was concordant with the estimates of Abbott (1949)

and Rees (1951). However, ศักดิ์ศิริ เกิดปรีดี (2499) and โชติ สุวัตถิ (2505) (Chote Suvatthi, 1962) reported that the snail was introduced into peninsular Thailand (e.g. Songkhla, Trang and Nakhon Si Thammarat) in 1934.

The mode of dispersion of the giant African snail in Thailand is not clearly known. In 1939, five years after the first presumable entry, the snail population increased dramatically; it was commonly found along forest edges and in modified forest and plantation habitats and caused serious damage to many kinds of plants. The snails were reported to be a significant pest of agricultural crop at Pattani and adjacent areas in 1939 (ศักดิ์ศิริ เกิดปรีดี, 2499). The eradication program was initially carried out in 1939 by the Ministry of Agriculture and continually performed for several years. The giant African snail, however, still dispersed to Bangkok in 1956 (พิสิษฐ์ เสพสวัสดิ์, 2510; Pisit Sepsawat, 1967) or 1957 (Mead, 1961) and to Chanthaburi in 1958 (สวาท รัตนวรพันธุ์ และประจง สุดโต, 2518; Sawart Ratanaworobhan and Prajong Sudto, 1975).

Snails infested northern Thailand regions such as Chiang Mai in 1964, Lampang in 1966 and Phrae in 1971 from unknown origin (สวาท รัตนวรพันธุ์ และประจง สุดโต, 2518). The establishment of the giant African snail at Mae Mo district, Lampang occurred in 1969. An officer of the Electric Generating Authority of Thailand brought a couple of living snails from Lampang Municipality to his home at Mae Mo district. After a few days he released those snails into his backyard. A few years later, a large number of snails had been found along with scores of eggs in Mae Mo district and the surrounding areas. By 1972, the giant African snail had caused a great deal of damage to vegetables, ornamental plants, trees and shrubs in the residential areas of the mine and surrounding villages (สวาท รัตนวรพันธุ์ และ ประจง

สุดโต, 2518).

During 1934 to 1985, the giant African snail still spread to several other parts of Thailand. The Ministry of Agriculture promoted the control and eradication programs of this species. The programs included both physical and chemical controls of the snail. Subsequently, a biological control program for *A. fulica* was performed by The National Biological Control Research Center. Between 1973-1976 where two species of predaceous snails, a rosy wolfsnail (*Eugrandina rosea*) and a carnivorous snail (*Gonaxis quadrilateralis*), were introduced from Hawaii. After pre-release testing, they were released into infested areas at Muak Lek district, Saraburi province and Pak Chong district, Nakhon Ratchasima province but the program has not been conclusive (บรรพต ณ ป้อมเพชร และโกศล เจริญสม, 2521; Banpot Napompeth and Kosol Charernsom, 1971). However, Cowie (2001) suggested that the use of snails as biocontrol agents against other snails might be inappropriate and unsuccessful. In the case of the predatory snail *E. rosea* that was widely used (along with other species) against *A. fulica* on Pacific and Indian Ocean islands, it has been reported that *E. rosea* has seriously interfered with population of endemic island species of snails.

In Africa, the giant African snail is utilized as food by local people. Snail meat is an expensive food and in French is called "escargot". In China and Taiwan, a number of factories produce frozen and canned products from the giant African snail meat which have been exported to France and the USA under the trade name "Achatine Escargot" (Upatham, Kruatrachue, and Baidikul, 1988).

In Thailand and Malaysia, the giant African snail has been collected and used as the source of calcium and protein for poultry feeding (Mead, 1961). At present, this snail seems to be less important because the pest status is no longer existent compared

to that of the last two decades because of significant reduction of the giant African snail which may have resulted from several uncertain factors. The main factors affecting survival of the snail populations may be the environmental has become unsuitable for the snail because of human activities and unusual climate such as drought and flood. The remaining factors may be predation by the introduced predaceous snails and natural enemies including rats, mongooses, monitor lizards, coconut crabs, birds, insects such as ants, flies, termites, and the Lampyrid beetle or glow worm (จินดา จันทร์อ่อน, 2516; Jinda Chan-on, 1973). Other factors including,

sterility, exposure to sunlight, traumatic breaks, diseases, parasites, starvation and genetic problems may also play important roles on the reduction of this snail (Mead, 1961). Additionally, the establishment of snail meat product factories is also a major factor because snails were heavily collected as raw materials of snail canned or frozen products for exportation (Upatham, Kruatrachue, and Baidikul, 1988).

The global spread of this snail has been significantly assisted anthropologically. According to the history of distribution of the giant African snail in Thailand, most of snails were accidentally moved across geographic locations as a hitchhiker on agricultural products or transportation container. For invasive species, there are three phases for invasion processes: (1) arrival and establishment, (2) spread and (3) some sort of equilibrium (Williamson, 1996). In the case of the introduced giant African snail in Thailand, it may have reached the equilibrium phase because the snail populations continuously decline. The population reduction of *A. fulica* is commonly found in all infested area in many countries (Mead, 1961).

Tomiyama and Nakane (1993) studied the dispersal patterns of the giant African snail by radio tracking. They found that juveniles dispersed longer distances

than young adults and adults. The most active juvenile moved 500 meters in six months while young adults and adults moved within narrow areas. Therefore, each snail population may be reproductively isolated owing to limited gene flow between groups unless there is movement anthropomorphically. It has been suggested that genetic drift is involved in the establishment and in outbreaks of introduced species. Genetic variation of *A. fulica* in geographically different locations in Thailand may be promoted through effects of genetic drift in population of small effective population size such as are found in the giant African snail. Accordingly, variation of *A. fulica* based on shell morphology and genetics was studied. The basic information from this study would imply the genetic status of introduced *A. fulica* in Thailand which is useful for establishment of the appropriate management programs in this species.

Objectives of this thesis

1. To examine variation and differentiation in shell morphology of *A. fulica* from different geographic locations in Thailand using morphometric analysis.

2. To determine intraspecific genetic variability, geographical heterogeneity, and distribution patterns of *A. fulica* in Thailand based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), randomly amplified polymorphism DNA-polymerase chain reaction (RAPD-PCR), and single strand conformational polymorphism (SSCP).

3. To investigate expression patterns of stress-related genes in the collar tissue of *A. fulica* during the normal and stress conditions using RNA arbitrarily primed (RAP) PCR.

CHAPTER II

LITERATURE REVIEW

Taxonomy and external morphology of *Achatina fulica*

Taxonomy

The taxonomic placement of the giant African snail is as follows;

Phylum Mollusca

    Class Gastropoda

        Subclass Pulmonata

            Order Stylommatophora

                Suborder Sigmurethra

                    Family Achatinidae

                        Genus *Achatina*

                            Species *Achatina fulica*

Common name: Giant African snail, giant African land snail or

Escargot géante (France)

Scientific name: *Achatina fulica* (Bowdich, 1822)

External morphology

Shell

The shell is an external hard part covering the soft parts and internal

organs. When fully grown shell size may be up to 20 cm (8 inches) in length and 12 cm

(almost 5 inches) in maximum diameter. Generally there are seven to nine whorls and rarely as many as ten whorls. The shape of shell is conical with a moderately swollen body whorl and a sharply conical spire, which is distinctly narrowed but scarcely drawn out at the apex. The outline varies greatly from very slender to moderately obese, the broader specimens tending to be shorter for the same number of whorls. All whorls are decidedly convex, due to the broadly impressed sutures. The aperture is relatively short, even in the broadest specimens, being always shorter than the spire, often considerably so. The outer lip is usually sharp and thin, rarely somewhat thickened or even slightly expanded in very old specimens; it is very convex, evenly curved into a regular semi-ellipse, and inserted on the body-whorl at a sharp, open angle, the upper part of the body whorl being scarcely or not flattened behind the lip. The columella is generally concave; lesser concaved columella tend to be somewhat twisted (Figure 2.1). The broader shells tend to have a more concave columella. (Bequaert, 1950). Shell coloration may be variable due to environmental conditions and diet, generally it is reddish-brown with light yellowish, vertical (axial) streaks or a light coffee color on almost part of the body whorl in some specimens. The colors fade with age in the earliest whorls appearing lighter or less intense, becoming darker and more vibrant nearest the body whorl (Robinson, 2004) (Figure 2.2A, B).
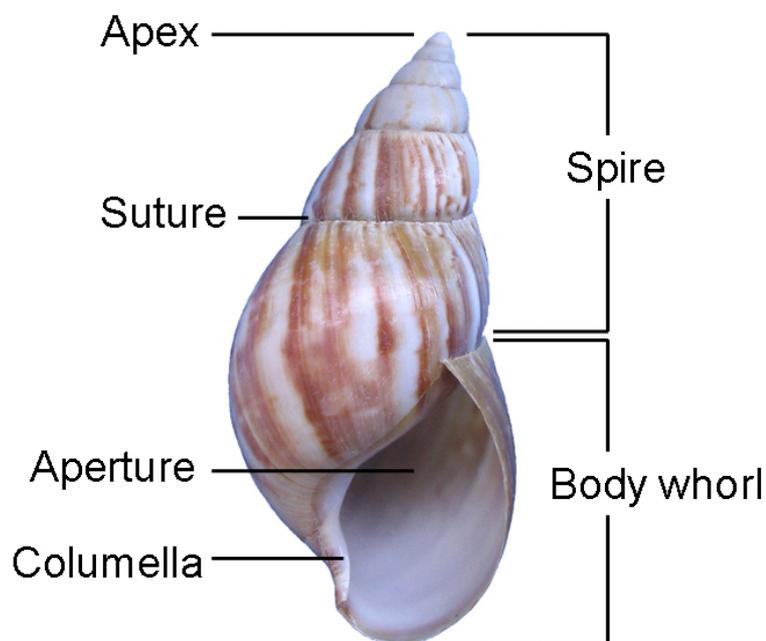
Figure 2.1 Shell morphology of *A. fulica* (Bowdich, 1822)

The egg is broadly ellipsoidal, 5 to 5.5 mm by 4 to 4.5 mm, white or slightly yellowish, with a very thin and brittle calcareous shell. The newly hatched nepionic shell is 5 to 5.5 mm long and about 4.5 mm wide, of 2 nearly smooth whorls, without any granulation or decussation and with only the weakest traces of vertical wrinkles. In young shells, the periostracum of the early post nepionic whorls shows in addition to the decussation a superficial, microscopic crisscross texture, as if a finely woven cloth had been pressed onto the surface; but no trace of this remains on older shells. In the largest full grown adult shells, of 7 to 9 whorls, the body whorl is nearly even, the growth striae being very low or superficial, except below the suture where they form short, strong folds, very lightly crenulating the irregular sutural line. When the periostracum is fresh and intact, the terminal whorls appear not only smooth, but also glossy. The ground color and markings vary greatly. When present in the adult, the darker markings appear almost at once on the first post-nepionic whorl as faint,

vertical, straight, pale-brown streaks; in very young shells, these streaks stop at the periphery, forming there slightly deflected spots.

Figure 2.2 Shell of *A. fulica* (Bowdich,1822). A: Dorsal side, B: Ventral side C: Living snail

Soft parts and internal organs

Soft parts of *A. fulica* consist of head, foot, mantle and mantle cavity, and visceral mass. Visceral mass is a combine digestive system and reproductive system and is completely covered by the shell. General anatomy of soft parts and internal organs of *A. fulica* was shown in Figure 2.3.

Head

The head is at the anterior end of the animal and is well developed. It bears the mouth at its anterior end. The mouth is flanked by large fleshy lips. The head bears two pairs of retractile sensory tentacles at its anterior dorsal corners. The more dorsal and posterior cephalic tentacles are the larger and each bears an eye at its tip. The much smaller, more ventral and anterior oral tentacles have no eyes (Figure 2.2C) The common gonopore of the hermaphroditic reproductive system is located on

the right side of the head (Figure 2.4A) It is slightly posterior to the right cephalic tentacle. It is normally small and difficult to see but may be large and conspicuous if some of the reproductive apparatus, such as the penis, is protruding from it.

Foot

The foot is a long, wide, muscular organ with a smooth flat sole forming its ventral surface. The sole fits against the substratum and creeps along it. A mucus-secreting pedal gland opens between the head and the foot and secretes lubricating mucus onto the substratum in the path of the foot. This mucus creates the familiar slime trail of terrestrial snails and slugs.

Mantle and mantle cavity

The mantle is the body wall of the dorsal surface of the visceral mass. It encloses the mantle cavity and secretes the shell. The mantle is folded to form a deep recess, the mantle cavity, immediately posterior to the head on the dorsal side of the animal. The mantle cavity of pulmonates is the lung, a large air space enclosed by the folded mantle. The edge anterior of the mantle of *A. fulica* forms a conspicuous mantle skirt, or collar. In life specimen the skirt fits around the lip of the aperture of the shell and seals it. The fold of mantle is the roof of the lung. The unfolded dorsal body wall is its floor. The opening of the mantle cavity, called a pneumostome, is a passage way for breathing air into and out of the cavity. (Figure 2.4B)
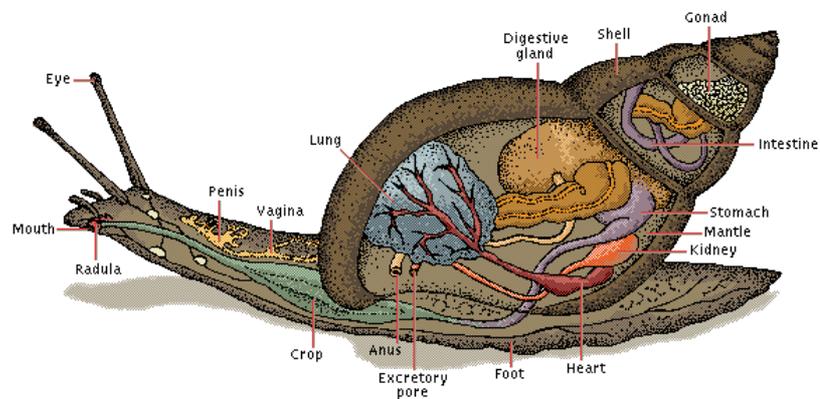
Figure 2.3 Anatomy of *A. fulica* (from Microsoft Encarta®, www, 2005)

Available: http://encarta.msn.com/media_461556103/Snail_Anatomy.html
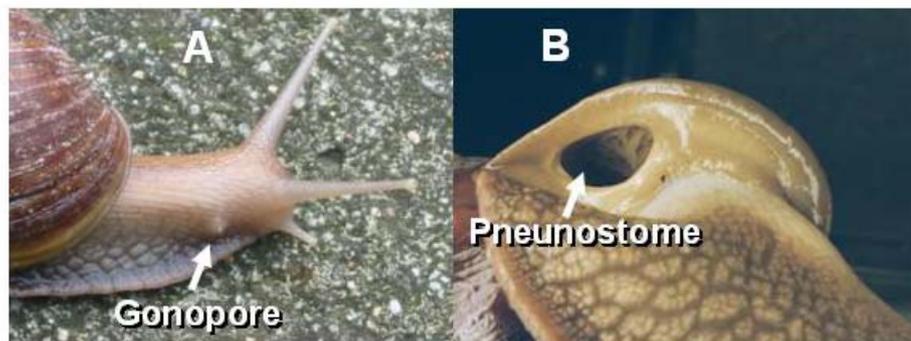
Figure 2.4   Gonopore and pneumostome of *A. fulica*: (A) gonopore indicated by an arrow (B) pneumostome located on the right side of the collar

Visceral mass

It is the large, coiled mass of tissue sitting on the dorsal surface of the foot, posterior to the head. It consists of almost part of digestive system and reproductive system.

Digestive system

The mouth opens into a small anterior buccal cavity. Most of the interior of the buccal cavity is the pharynx. The radula (an organ which bears rows of

numerous tiny chitinous teeth) lies on the floor of the pharynx. The radula is situated on the dorsal surface of the connective tissue, odontophore, which contributes to the floor of the pharynx. Many muscles of the buccal mass operate the radula and odontophore.

A short and narrow esophagus exits the posterior end of the pharynx passes through the nerve ring, turns to the left and widens to become the anterior end of the crop (a large, thin-walled storage organ extending the entire length of the hemocoel). A pair of salivary glands partially covers the walls of the anterior end of the crop. Each gland drains to the pharynx via a long salivary duct that passes through the nerve ring following the crop posteriorly, passes the origin of the columellar muscle. In this region it is obscured by the large, white albumen gland of the reproductive system and the two dark brown digestive ceca. The right digestive cecum is in the upper, small whorls of the visceral mass and, in fact, accounts for most of the volume of the upper whorls. The ovotestis is also in the upper whorls located along the inside curve of the spiral. The albumen gland is also present in the spiral of the visceral mass but is lower down, in the middle region and base of the visceral mass. The left digestive cecum lies at the base of the visceral mass and extends into the mantle roof to lie against the kidney.

Between the two digestive ceca the crop expands to become the stomach into which the digestive ceca open.. The stomach narrows to become the intestine which is also embedded in the left digestive cecum. It makes a large loop as it passes through the gland. The intestine emerges from the digestive cecum as the rectum. The rectum runs anteriorly along the right side of the mantle cavity to open at the anus.

Reproductive system

A. fulica is hermaphroditic with internal fertilization. The protandric (male organs develop before female organs) ovotestis is located at the top of the visceral mass high in the spire. It is embedded in the digestive cecum and is pale creamy-white (in live specimen) or dark brown (preserved specimen). In preserved specimens the ovotestis is darker than the digestive cecum whereas the opposite is true for fresh materials. The degree of pigmentation of gonads depends on the reproductive conditions of the individual.

The albumen gland secretes a nutritive material around the fertilized egg. Its size varies with changing reproductive conditions. A small, but conspicuous white (in live specimen), convoluted the hermaphroditic duct extends from the ovotestis to the albumen gland. Sperm are stored in the hermaphroditic duct which functions as a seminal vesicle. Fertilization occurs in a fertilization chamber embedded in the albumen gland (at the point where the hermaphroditic duct, albumen gland duct and spermoviduct join). Anteriorly the albumen gland is continuous with a very thick and conspicuous spermoviduct or common gonoducts. This is a combined sperm duct and oviduct running side by side.

The spermoviduct nears the head, it divides into a separate oviduct and sperm duct. The oviduct secretes a calcareous shell around the fertilized egg as it passes downstream. The oviduct joins the distal end of the spermathecal duct to form the short vagina. A pair of large, branched mucous glands arises from vagina. The mucus glands secrete mucus for lubrication. The sperm duct exits the spermoviduct as a discrete independent duct and turns medially, passes over the base of the vagina and under the right cephalic tentacle retractor muscle, then expands in diameter to become the penis. During copulation, the penis is inserted into the gonopore and vagina and

delivers sperm to the partner. At the point where the penis begins to expand, it is joined by a long, slender, blind diverticulum, the flagellum. Sperm from the sperm duct are packaged into spermatophores in the flagellum. The penis joins the vagina to form a short common chamber which opens to the exterior through the common gonopore locate on the right side of the head.

Biology of *A. fulica*

The giant African snail (*A. fulica*) is highly adaptable to a wide range of environments. Snails reach sexual maturity within less than a year (assuming no delays due to hibernation or aestivation), and can live for up to 9 years, although 3-5 years is more normal (Tomiyama, 1993)

Tomiyama (1994, 1996) described the reproductive biology of *A. fulica* that reciprocal copulation is typically lasts for 6-8 hours to produce viable eggs. Eggs are laid in clutches of 10-400 eggs within 8-20 days of copulation, usually in nests excavated in the soil, but sometimes among leaves and stones on the ground surface. Repeated egg-laying can occur from a single mating, as the sperm is stored in each snail. The frequency of egg laying depends on the local climate, particularly according to the duration and frequency of the rainy seasons; periods of drought will prevent or delay feeding activity and reproduction. Tomiyama (1993) reported that individual snail can lay up to 300 eggs/year in Hong Kong, up to 500 eggs/year in Sri Lanka and well over 1,000 eggs/year in Hawaii and Calcutta.

Upon emerging from the eggs, the hatchlings consume their eggshells as well as unhatched siblings and surrounding organic detritus. They remain underground for 5-15 days. Once out of the nest, the young snails continue to feed on organic detritus and preferred host plants, remaining fairly close to the nest for a couple of weeks.

After this time, they will range further a field, each individual ultimately establishing a home range within 2 months, feeding primarily on plants at night and returning to roost before dawn (Tomiyama, 1993)

The giant African snail prefers environments that are rich in calcium carbonate, such as limestone, marl, and built up areas where there is an abundance of cement or concrete. In these calcium-rich areas, the shells of adults tend to be thicker and opaque. Juveniles generally have a thinner, more translucent shell and are more brittle. Animals whose shells measure between 5 and 30 mm in length appear to cause the most damage to plants. Food preferences of A. fulica depend on the availability of plant species. Larger snails continue to feed on plant materials, but also become increasingly detritus-feeders as they age. Shell coloration may be variable due to environmental conditions and diet. Generally, it is reddish-brown with light yellowish, vertical (axial) streaks. The two shell colors are not distinct from each other and are somewhat streaked or smudged in appearance. Another shell color variation resembles a light coffee color. The colors fade with age in the earliest whorls appearing lighter or less intense, becoming darker and more vibrant nearest the body whorl (Rees, 1951; Mead, 1961).

Movement and dispersal of *A. fulica*

Natural spread

A snail can move from one place to another place by creeping on the substrate with its foot pad but the movement is extremely slow. Tomiya and Nakane (1993) studied the dispersal pattern and rate of natural spread of *A. fulica* by radio tracking in the Pacific Islands. They found that in suitable conditions (high humidity, precipitation, abundant hosts, high population density), juveniles dispersed longer

than young adults and old adults. The active juveniles can naturally disperse at an estimated rate of 125 meters per month or about 500 meters in six months while young adults and old adults moved within a narrower scale. Other factors such as temperature, precipitation, available hosts, and physical substrates affect the activity, the rate of natural spread and also the rate of daily movement of snails (Robinson, 2002). Raut and Barker (2002) suggested that *A. fulica* is tolerant to a wide variety of environmental conditions. It evidently needs a combination of a constantly high temperature year round and much humidity at least during part of the year, the drier months being spent in dormant aestivation. The world distribution of *A. fulica* is shown in Appendix

Human-assisted spread

Human activities both intentional and unintentional are important factors causing snail to disperse widely, as escapees from snail-farming operations as snail intentionally spread by individual as "hitch-hikers" on various shipping containers, etc. USDA (www, 2004) report that the giant African snail is routinely intercepted by the Animal and Plant Inspection Service (APHIS) in baggage of international travelers, on and in shipping containers, and in plant shipments from the Hawaiian Islands, Guam and other Pacific Island groups. Those places are reported as an endemic area of *A. fulica* infestation.

Pest significances of *A. fulica*

Crops affected by *A. fulica* include coffee in Tanzania. Outside Africa, various economically important crops; amararanth, banana and plantain, various beans and peas, carambola and bilimbi, breadfruit and jackfruit, eggplant/aubergine, various

cultivars of *Brassica* (cabbage, broccoli, cauliflower, etc.), radishes, cacao, carrot, cassava, castor, chilies and peppers, citrus, coffee, cotton, corm, cotton, eucalyptus, fig, pumpkins and melons, cucumber, jute, kokko, mahogany, mulberries, okra, onions, palm nuts, passionfruit, potatoes, rubber, shishu, soursop, spinach, sunflower, sweet potato, taro, tea, teak, tobacco, tomato, vanilla and yam, were significantly affected by the giant African snail (Raut, 1982).

A wide variety of horticultural and medicinal plants have also been destroyed by the giant African snail. In most parts of the world, the amount of damage is greatest when *A. fulica* is first established. The snails during this period are usually very large and their populations increase exponentially. After this period, the populations reach a stable population phase prior to reduction of the snail ultimately (Raut, 1991).

Venette and Larson (2004) reported that *A. fulica* has a remarkably broad host range. Apparently, the snail (especially younger snails) prefers soft textured banana (*Musa* sp.), bean *(Beta vulgaris)* and marigold (*Tagetes patula*) (Raut and Ghara, 1989). As the snail matures, dietary preferences broaden to a larger variety of plants (Singh and Roy, 1979), including brinjal (*Solanum melongena*), cabbage and cauliflower (*Brassica oleracea vars capitata* and *botrytis*), Lady's finger (*Abelmoschus esculentus*), sponge gourd (*Luffa cylindrica)*, pumpkin (*Cucurbita pepo*), papaya (*Carica papaya*), cucumber (*Cucumis sativus*) and peas (*Pisum sativum*) (Thakur, 1998).

Epidemiology and public health significances

The giant African snail is recognized as one of the intermediate hosts of *Angiostrongylus cantonensis*, which is known as the rat lung worm causing

eosinophilic meningitis worldwide. This disease is endemic in Southeast Asia and the Pacific regions. The typical clinical symptom is acute meningitis with an eosinophilic pleocytosis frequently accompanied by encephalopathy (Punyagupta, Bunnag, Juttijudata, and Rosen, 1970). *A. cantonensis* is widely found in many country including China, India, Thailand, Malaysia, Indonesia, Vietnam, Taiwan, Japan, and Cuba (Crook, Fulton, and Supanwong, 1968). Yi, Chen, Chen E.R., Hsieh, and Shih (1975) reported 27 cases of angiostrongyliasis in Taiwan while 19 angiostrongyliasis cases in Thailand were also reported (Punyagupta, Jittijudata, and Bunnag, 1975). Tsai, Liu, and Kunin (2001) reported that two outbreaks of central nervous system infection with *A. cantonensis* occurred in Kaoshiung, Taiwan, during 1998 and 1999 among 17 Thai laborers who ate raw snails.

The giant African snail is usually infected with *A. cantonensis* by ingestion of parasite larvae in rat feces or direct penetration of the parasite via the foot pad of snails (Crook, Fulton, and Supanwong, 1968). The parasites are found in various organs of the snail hosts (85% in mantle 11% in kidney and 4% in other organs) (Brockelman, Chusatayanond, and Baidikul, 1976). The parasite passes to humans through eating raw or improperly cooked snail meat infection of *A. cantonensis* is usually associated with high level of eosinophils and menigoencephalitis. In Thai patients, almost all of the cases were found in northeastern people (Chotmonkol and Tiamkao, 1992).

Population decline in *A. fulica*

Population decline is the typical phenomenon found among *A. fulica* populations in many infested areas and was first reported by Green (1910). A preliminary survey in Sri Lanka found that the extinction or near extinction of *A fulica*

after well colonization has occurred in some infested areas. Mead (1961) reported the concomitantly progressive decline in numbers and the average size of each snail. The true giant forms (e.g. 4-6 inches of shell length) are rarely found in old established populations. The appearing forms ranges from 3-4.5 inches. Several scientists have given explanations about population decline in *A. fulica* but the exact reasons have not yet known. Nevertheless, Mead (1961) concluded causes of population decline in *A. fulica* as follows:

Population senility. Some authors have attributed the decline in the population of the giant African snail to mythical population senility but there was not clearly evidences for this hypothesis.

Sterility. This physiological process may result in a population decline through reduced fecundity and reproductive capacity of *A. fulica*. Mead (1961) reported correlations between gigantism and sterility in mollusks.

Starvation. Chamberlin (1962) pointed out from ecological study of *A. fulica* that starvation was the main cause for mortality of the giant African snail in the Tinian Island. Nutrition deficiencies, including the lack of calcium and other trace elements in the soil environment would result in high mortality rate in this species.

Exposure to direct sunlight. This phenomenon was observed in some infested areas which severe overcrowding of snail population.

Traumatic breaks. The shell was spontaneously broken for which Mead (1961) suggested genetic, pathological or nutritional factors that may cause thin and brittle shells which in turn were more prone to fatal or traumatic breaks.

Predators. Natural enemies of *A. fulica* such as rat, mongoose, otter, varanus monitor, some birds and some insect for example flies, glow worm may cause severe reduction of *A. fulica* in a particular area.

Genetics.   Mead and Kondo (1949) indicated that a genetic rather than ecologic was responsible for the reduction phenomenon in several populations of *A. fulica*. The malformed specimens which were found to account for about one third of the entire Saipan population at Marina Islands have been interpreted as indubitable signs of population degeneration and decline. The gigantic form showed signs of being reproductively sterile.

Diseases.   Mead (1957) suggested that disease is probably the main factor for the severe reduction of in number of *A. fulica* because an identical symptom of leukodermia (destruction of melanophore in the dermis) was found in snails collected from different infested areas. The transmission of disease can result from physical contact and feeding habits of the snails.

Studies of genetic variation of organisms by morphometric analysis

Morphometric is the quantitative study of patterns of covariance with shape (Bookstein 1991). Many morphological attributes of biological form are obviously reflected the evolutionary processes. Therefore, morphological variation has been traditionally used to infer phylogenetic relationships (MacLeod, 1999). Morphometric analysis can be used in taxonomic studies for discrimination or differentiation in both intraspecific and interspecific levels. Dickinson (1987) classified morphometric, normally used in his laboratory, into three types; traditional, landmark-based and outline-based morphometric, respectively.

Traditional morphometric (Marcus, Corti, Loy, Naylor, and Slice, 1996)

This type of morphometric analysis comprises data (linear measurements, angles, areas, etc.) and methods (resemblance functions and/or ordination,

classification) that have been employed in order to capture and summarize information on shape variation by sampling shape in an *ad hoc* kind of the way that has no necessary connection to the objects being analyzed. Sometimes, ratios of measurements have been used by some workers, either as additional descriptors together with the original or other measurements, or to provide a single index of some aspect of shape that is considered important.

Landmark-based morphometric (Stone, 1998)

Landmark-based morphometric analysis is based on the use of specific points on a biological structure, its image or outline that can be located according to some rule and so can be considered homologous across a sample of the same kind of structure. Landmark-based methods are frequently referred to as "geometric morphometric" (Bookstein 1986). Landmarks may be defined operationally as relocatable coordinate positions on an object in a two-dimensional or three-dimensional Euclidean measurement space (a space where distances between two points are defined as Euclidean distances in some system of coordinates.).

Bookstein (1991) identified three classes of biological landmarks discrete juxtapositions of structures or tissues (Type 1), maxima of curvature (Type 2), or extrema (Type 3). This classification focuses attention on the amount of information necessary to identify or relocate the landmark.

The type 1 landmark require the most biological information to identify and may occur at any point on or within a form so long as that form is composed of different structures or tissue types. While this landmark is constrained to exist on the boundaries (= outlines) of the structural components or tissue-defined regions, their locations are not determined by any characteristics of the overall

boundary or outline.

The type 2 landmark is constrained to lie on the boundaries of single structures or regions and is defined by the nature of the curving surface of that boundary. Therefore the type 2 landmark is located relative to the distribution of adjacent boundary coordinates.

The type 3 landmark represents those coordinate locations on the single structures (irrespective of whether the structure is composed of various substructures or regions) that represent the extremes of the structure's boundaries. Like the type 2 landmark, these points are constrained to lie on the object s outline (MacLeod, 1995)

Outline-based morphometric

This method is used when some or all of the objects under study lack landmarks. Although in the past there have been acrimonious exchanges regarding the value of doing so, one can use methods based on the closed outline of objects with the considerable success (Bookstein, 1991). However, MacLeod (1999) defined the outline-based morphometric to be a type of landmark-based morphometric.

Data collection and analysis

Data used for morphometric analysis is dependent upon the types of morphometric studies. They may be numerical values of measurement or pictures with numerical values or outline of shape. Data is usually analyzed by multivariate analysis using different methods, for example, principal component analysis (PCA), principal coordinates, canonical discriminate analysis (CDA), cluster analysis, multivariate analysis of variance (MANOVA), etc. Advantages and disadvantages among different methods have been discussed and are still controversial. Only cluster

analysis which is used in this thesis, will therefore be discussed.

Cluster analysis

Cluster analysis is a multivariate procedure for detecting natural groupings in data. There are two different types of cluster analysis; hierarchical and K-Means clustering, respectively. The former procedure comprises hierarchical linkage methods whereas the latter splits a set of objects into a selected number of groups by maximizing between-cluster variation and minimizing within-cluster variation.

The hierarchical clustering routinely produces a 'dendrogram' showing how data points can be clustered. Three different algorithms can be used for analysis:

Unweighted pair-group method using an arithmetic average (UPGMA) where clusters are joined based on the average distance between all members in the two groups.

Single linkage (nearest neighbour) method where clusters are joined based on the smallest distance between the two groups.

Ward's method where clusters are joined such that increase in within-group variance is minimized.

One method is not necessarily better than the other even though a single linkage method is not recommended in some circumstances. Nevertheless, it may be useful to compare dendrograms given by the different algorithms in order to informally assess the robustness of the groupings (SYSTAT 11, Computer Program, 2004).

The K-means clustering splits a set of objects into a selected number of groups by maximizing between-cluster variation relative to within-cluster variation. It is similar to performing a one-way analysis of variance (ANOVA) where the groups are unknown and the largest F value is sought by reassigning members to each group. The

K-means starts with one cluster and splits it into two clusters by picking the farthest case from the center as a seed for a second round of clustering and assigning each case to the nearest center. It continues splitting one of the clusters into two (and reassigning cases) until the within-groups sum of squares can no longer be reduced.

Several commercially available computer packages using for multivariate analysis can be applied to morphometric analysis. They include SPSS (SPSS Inc.), SYSTAT (SYSTAT Software Inc.) and Image Analyzer Pro® (MediaCybermatics Inc.). Additionally, freeware written for the specific research purposes are also available including PADWIN, PAST, MorphoSys, TpsRelw, and TpsSplin

Molecular studies and molecular markers

Classes of DNA

Comparative examination of DNA sequences across different taxa show that there are particular classes of sequences commonly found in many organisms. DNA can then be classified in many ways according to its function, its structure and its locations. Many of these categories overlap, with a particular segment of DNA falling into several categories at once (Figure 2.5).

coding | non-coding

nuclear DNA

introns
regulatory
regions

exons

non-repetitive
repetitive

rDNA
histones

mini satellite DNA
microsatellite DNA
non-tandem repeats
(SINEs and LINEs)

mtDNA

ND genes
cyt b
tRNAs

D-loop
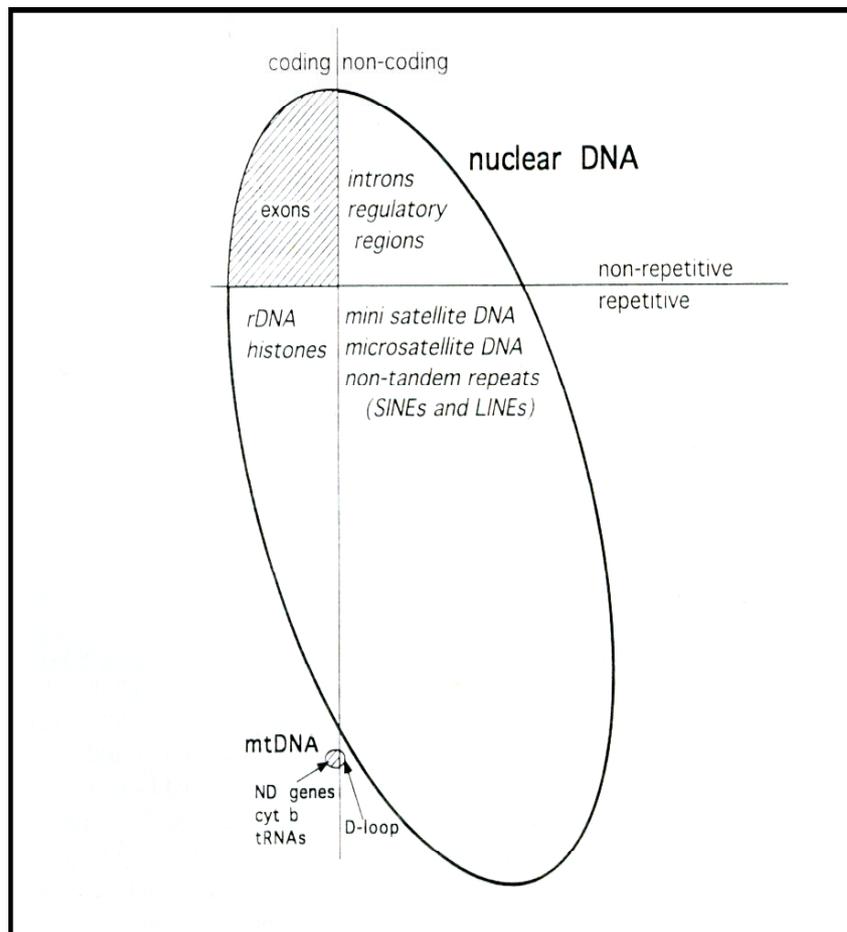
Figure 2.5 Schematic representation of some classes of DNA sequences (Park , 1994).

In animal cells, DNA can be classified as nuclear DNA (chromosomal DNA) and mitochondrial DNA (extrachromosomal DNA or organelle DNA). In this sense, types of DNA are classified according to locations of genes (of DNA sequences). Alternatively, DNA segments can be classified as coding (genes) and non-coding DNA (intervening sequences, intergenic sequences, minisatellites, microsatellites and non-tandem repeats. In addition, DNA can be classified as repetitive and non-repetitive sequences.

Nuclear DNA markers

Nuclear DNA is generally composed of non-repetitive (single copy nuclear DNA, intergenic sequences and pseudogenes) and repetitive sequences (nuclear ribosomal DNA, satellites, minisatellites and microsatellites). Approximately 70% of the mammalian nuclear genome contains non-repetitive DNA (Avise, 1994). The remaining part is DNA that is repeated anywhere in the genome from a few to thousands of copies of various sequences.

Generally, DNA markers from Single copy nuclear (scn)DNA and microsatellites are regarded as co-dominant markers because homozygotes can be differentiated from heterozygotes and therefore, observed and expected heterozygosity can be estimated and compared. Hardy-Weinberg disequilibrium within a particular population at a given locus can be tested. On the other hand, nuclear DNA markers based on multi-locus fingerprinting, RAPD-PCR and AFLP are treated as dominant markers.

Mitochondrial DNA markers

The animal mitochondrial genome is a closed circular and double stranded DNA molecule contained in mitochondria. There are up to several thousand copies of the mitochondrial genome per cell. This extra-chromosomal DNA is about 15700-19500 base pairs (bp) in length (Brown, 1983).

The vertebrate mitochondrial genome is composed of 13 protein coding genes, 2 genes coding for ribosomal RNAs (12S and 16S ribosomal rRNA), 22 transfer RNA (tRNA) coding genes, and the control region (D loop) containing an initiation site for replication and transcription. The genes that code for proteins are subunits 1, 2, 3, 4, 4L, 5 and 6 of NADH dehydrogenase (ND), cytochrome b, three subunits of

cytochrome c oxidase (CO I, II and III), and two subunits of ATP synthetase (ATPase 6 and 8).

Unlike the nuclear genome, the genes in the animal mitochondrial genome do not contain intervening sequences (introns) and they are usually separated by less than 10 bp. Genome organization of mtDNA genes is generally different in every phylum, which has been studied (Figure 2.6).

Patterns of animal mitochondrial genome evolution are composed of substitutions (transitions and transversions) which predominate over genome size polymorphism (deletions and insertions). Transitions are substitutions from one purine base to another purine or from one pyrimidine to the other pyrimidine. On the other hand, transversions are substitutions of purines to pyrimidines and *vice versa.* Theoretically, expected transversional changes should be two times higher than observed transversions if mutation occurs randomly. However, transitional mutations are accumulated more frequently than expected (Nei, 1987) especially in the mitochondrial genome in which transitions out-number transversions.

Generally, substitutions are common mutations and are much more frequent than either deletions or insertions or rearrangements. Therefore, the point mutations can be easily evaluated by loss and gain of restriction sites which are obtained from the RFLP data. Deletions and insertions have also been reported. These kinds of mutation usually occur at the control region (D-loop) and intergenic spacers. The displacement loop (D-loop) generally has a higher substitution rate than any other mitochondrial gene. The evolution rate of this region is between two to five times faster than that of the mitochondrial protein coding genes. The control region also has the highest frequency of length mutations at the population level. It is usually primarily responsible for the mtDNA length polymorphism in vertebrates.

Considering organelle segregation, animal mtDNA is a haploid and non-recombinant molecule reflecting, generally, only one type of mtDNA in an organism. Therefore, intra-individual variation (heteroplasmy) would be expected to be very rare. However, mtDNA heteroplasmy has been reported in certain species for instance, in mice.

Mitochondrial DNA evolves 5-10 times faster than single copy nuclear DNA (Brown, Mathew, and Wilson, 1979). This important property provides the magnifying ability to distinguish and identify the differences between populations and between closely related taxa.

Figure 2.6 An example of animal mitochondrial DNA (invertebrate, mosquito,

Anopheles gambiae), showing genome organization (Beard, Hamm, and Collins,

1993).

Molecular approaches used in this study

Molecular genetic approaches are useful and can be applied for genetic studies of various organisms. Several molecular approaches were used for identification of molecular markers at genomic DNA and cDNA levels in various species. Several molecular techniques have been applied for studies of genetic diversity in the giant African snail (*A. fulica*). The former level includes restriction fragment length polymorphism-polymerase chain reaction (PCR-RFLP), randomly amplified polymorphic DNA (RAPD) and single strand conformational polymorphism (SSCP). In addition, DNA sequencing was used for characterization of nucleotide sequences of interesting genes (or DNA fragments).

Polymerase chain reaction (PCR)

The introduction of the polymerase chain reaction (PCR) has revolutionized molecular biological researches. PCR is a technique for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (Figure 2.7). The use of PCR for molecular genetic studies in organisms is increased dramatically. This technique has facilitated the analysis of sequence variation and has enabled a new PCR-based technique to be developed for wider applications. The basic knowledge of a particular region from a few taxa (conserved but, specific sequences) permits the amplification of the same DNA sequences from distantly related species.

The PCR technique involves three steps

(a) Denaturation of double stranded DNA by heating.

(b) Annealing of extension primers to the target sites.

(c) Primer extension, in which strands complementary to the region

between the flanking primers are synthesized under the influence of a thermostable DNA polymerase (usually *Taq* polymerase).

The reaction is carried out repeatedly through steps a-c. Initially, synthesis of amplified products will go beyond the sequence complementary to the other primer binding site. This happens at both primer annealing sites on both DNA strands resulting in a synthesis of longer but complete products (product with primer at only one end).

In the subsequent cycle, the sample and newly synthesized strands serve as DNA templates. The amount of DNA in the amplified region (which has its end defined by the position of primers) will increase exponentially whereas longer sequences from the starting DNA accumulate in a linear fashion. These resulting products form only a small fraction of the total PCR amplification products.
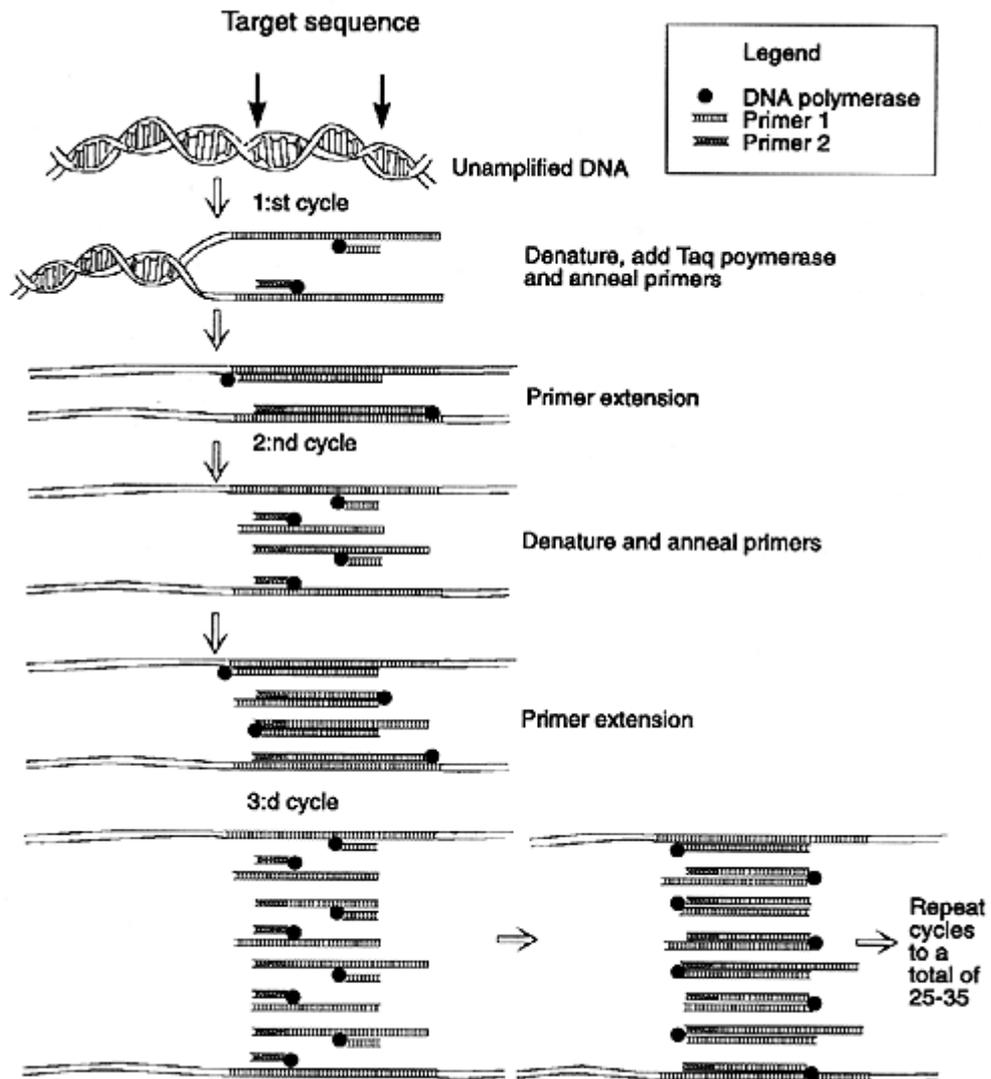
Figure 2.7 General illustration of the polymerase chain reaction for amplifying DNA.

(Modified from Palumbi, *et al*., 1991)

Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis is used for indirect evaluation of genetic variation at the DNA level. Variation in restriction enzyme cleavage sites generates size differences of the resulting fragments. Therefore, RFLP represents polymorphism of restricted DNA fragments. The assumption of the RFLP method is that if two samples share particular size fragments, they must also share flanking restriction sites.

For a conventional RFLP approach, genomic DNA of each individual is cut using one or more restriction endonucleases. The digested DNA fragments are electrophoresed through the agarose gel and transferred to a supporting membrane (nylon or nitrocellulose) before hybridizing with a specific radiolabeled DNA probe. Results from restriction analysis can be visualized by autoradiography.

The conventional RFLP analysis has some limitations. It requires fairly large amounts of genomic DNA (5-10 μg of genomic DNA for detection of single copy genes) and cloned probes that should be specific to organisms under investigation. The conventional method is time-consuming, particularly when dealing with a large number of specimens in population genetic studies. Therefore, it has been replaced by PCR-based methods, for example; PCR-RFLP, amplified fragment length polymorphism (AFLP), single-stranded conformational polymorphism (SSCP), and microsatellites.

PCR-RFLP is a technique for genetic studies based on amplification of a specific target fragment in vitro followed by restriction analysis of the amplification product with informative restriction endonucleases. Since a large number of copies of a particular target DNA fragment are produced from a small amount of starting DNA template within a short period of time, simple detection methods by ethidium bromide

and/or silver staining are sensitive enough to determine polymorphism of the investigated DNA fragments obviating the need to use a radioisotope-based approach for detection.

The animal mtDNA is probably the most popular portion of the animal genome used for population and evolutionary studies. With incorporation of PCR, the tedious step of mtDNA purification is eliminated. PCR-RFLP analysis can then be applied to fresh, frozen, and ethanol-preserved specimens. Sample sizes can be increased to as much as those used for allozyme analysis. Additionally, mtDNA is haploid; therefore, scoring of data is straightforward. Lost and gain of restriction sites among different restriction profiles can then be readily determined.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD-PCR (Williams, Kubelik, Livak, Rafalski, and Tingey, 1990; Welsh and McClelland, 1990) is conceptually simple technique for examination of genetic diversity of organisms. The amplification protocol differs from that of the standard PCR (Erlich, 1989) in that only a single random oligonucleotide primer is employed. Therefore, prior knowledge of the genome subjected to analysis is not required.

Typically a short oligonucleotide primer (e.g. 10-12 mers) with the G+C content more than 50% is used resulting in a high probability to amplify part of the genome containing several priming sites close to one another that are in an inverted orientation. The technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length. The amplification products are resolved by electrophoresis (Figure 2.8)

Several advantages of RAPD-PCR are reported. These include universal sets of primers which can be used on a variety of species with different genome sizes, a

large number of available primers, no radioactive probes, and a quick and simple protocol (Hawland and Arnau, 1994). Nevertheless, there are some disadvantages of the RAPD-PCR approach for population genetics and taxonomic studies. Identical electrophoresed fragments may not be produced in different laboratories because RAPD-PCR is snsitive to slight changes in amplification conditions. The RAPD-PCR amplified fragment may reflect either a homologous (AA) or heterozygous (Aa) situation. Only the absence of a fragment reveals the an genotype. This disadvantage of RAPD-PCR results in an inability to estimate heterozygosity and the actual status of interested alleles because homozygosity can not be dissociated from heterozygosity (Weising, Hybom, Wolff and Meyer, 1995).

DNA sequencing

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined region. There are two general methods for sequencing of DNA segments: the "chemical cleavage" procedure (Maxam and Gilbert, 1977) and the "chain termination" procedure (Sanger, Nicklen, and Coulson, 1977). Nevertheless, the latter method is more popular because the chemical cleavage procedure requires the use of several hazardous substances.

DNA sequencing is the most optimal method for several genetic applications. This technique provides high resolution, facilitating interpretation. DNA fragments generated from PCR can be directly sequenced or, alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library.
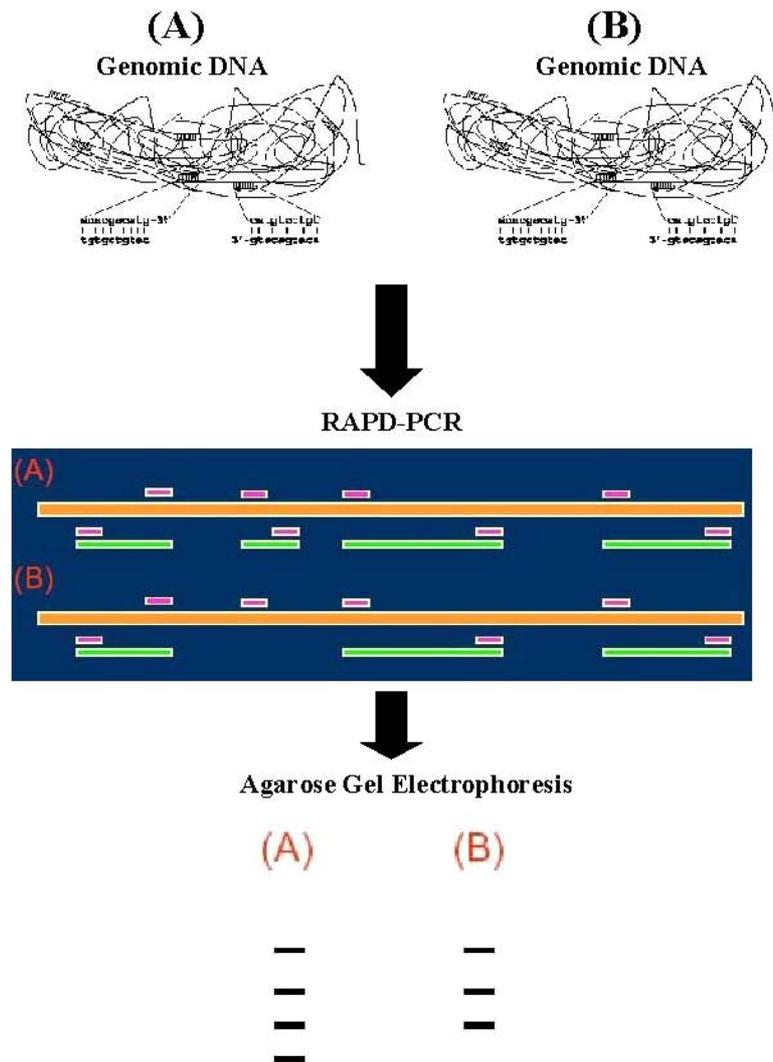
Figure 2.8 Schematic presentation of RAPD-PCR.

(Modified from: Pacific Agri-Food Research Centre, Canada, www, 2003) Available:

http://res2.agr.ca/parc-crapac/summerland/progs/biotech/bakkeren/RAPDdia_e.htm

The enzymatic sequencing approach has presently been developed as an automated method (Figure 2.9). DNA sequences can be detected using a fluorescence-based system following labeling with a fluorescent dye. PCR allow the possibility to isolate homologous DNA sequences from any organism of interest with unprecedented speed. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

DNA sequencing is particularly suitable for population genetic studies of various species. The DNA sequence of each individual is directly compared. Levels of genetic diversity between individuals within geographic samples, between geographic samples, and between species can be reliably examined. In addition, DNA sequencing can be used in coupled with RAPD and AFLP markers to convert dominant markers to sequence-characterized amplified region (SCAR) markers that are co-dominantly segregated.
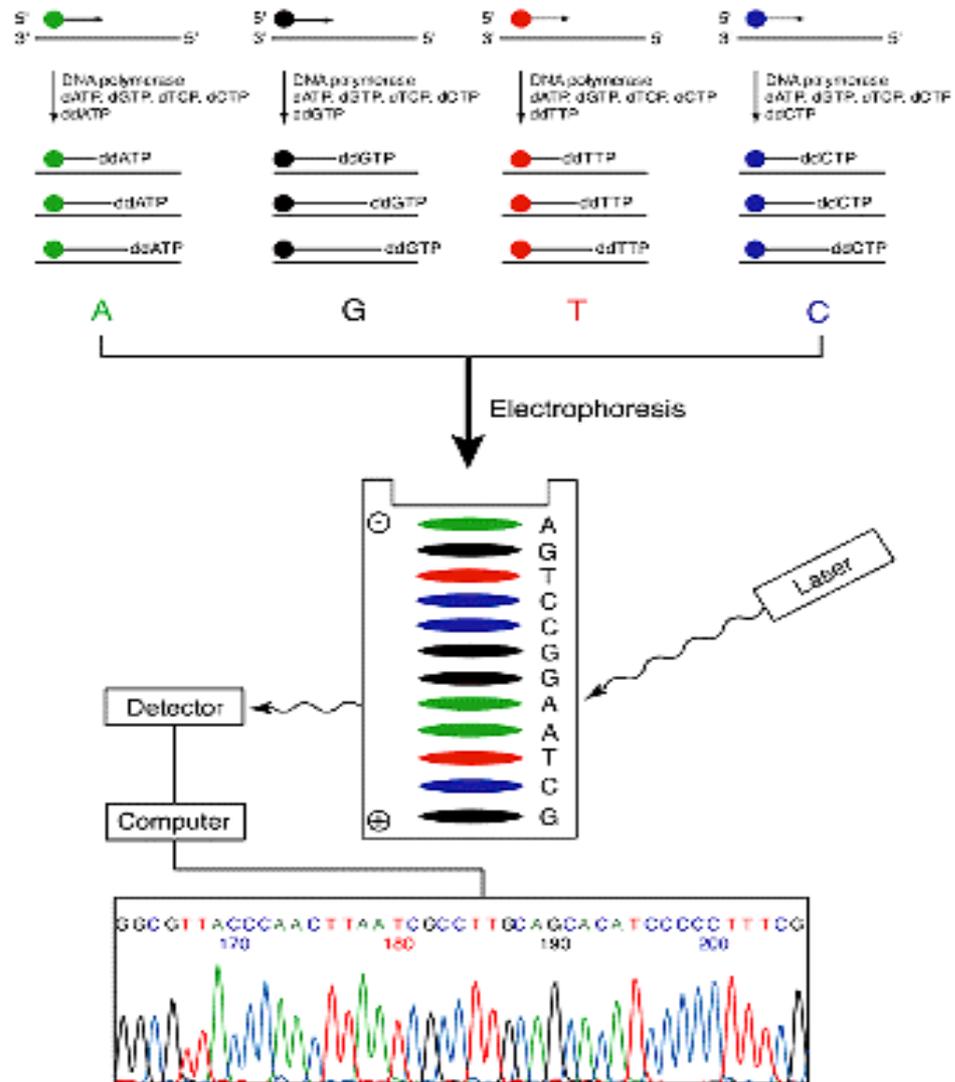
Figure 2.9 A schematic diagram illustrating principles of Automated DNA sequencing.

(Modified from: Jakubowski, www, 2002) Available:

http://employees.csbsju.edu/hjakubowski/classes/ch331/dna/oldnalanguage.html)

Single-stranded conformation polymorphism (SSCP) analysis

SSCP analysis is one of the effective techniques used for the detection of mutations and variation of the DNA due to single nucleotide polymorphism (SNP) and/or insertions/deletions (indels). SSCP relies on the principle that the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is dependent on its structure and size (Orita, Iwahana, Kanazawa, Hayashi, and Sekiya, 1989; Hayashi, 1991).

Single-stranded molecules take on secondary and tertiary structures (conformations) due to base pairing between nucleotides within each strand. These conformations depend on the length of the strand, and the location and number of regions of base pairing. They also depend on the primary sequence of the molecule, such that a nucleotide change at a particular position can alter its conformation. Hence, molecules differing in their conformations (e.g., due to a single nucleotide change) can be separated in low cross-link non-denaturing polyacrylamide gels (with or without glycerol supplementation) (Figure 2.10). The method has effectively been used to analyze point mutations of small amplicons (100–400 bp) (Hayashi, 1992).

SSCP is simple, and sensitive enough to detect one or a few base differences in the sequence of short DNA fragments (Hayashi, 1992). The disadvantage of SSCP is that it requires nucleotide sequence data for the design of the specific primers. Moreover, reproducibility of the technique seems to be problematic because SSCP patterns are affected by temperature and degree of cross-linking. Additionally, multiallelic patterns of some nuclear DNA markers may complicate the precise estimation of allele frequencies from the SSCP patterns.
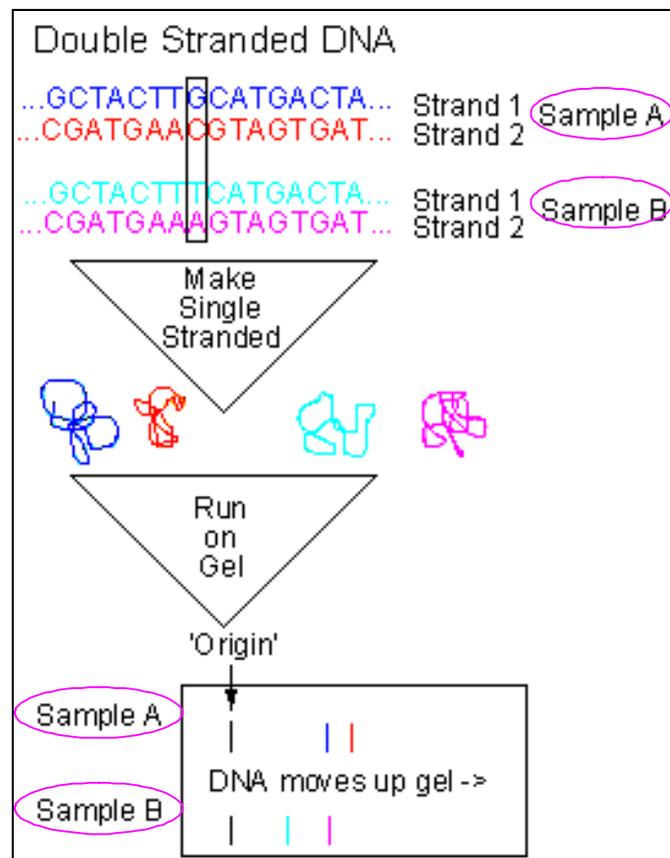
Figure 2.10 A schematic diagram illustrating principles of SSCP analysis

(From: Australian museum, evolutionary biology unit, www, 2003)

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

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a comparable method of conventional PCR but the first strand cDNA template rather than genomic DNA was used as the template in the amplification reaction (Figure. 2.11). It is a direct method for examination of gene expression of known sequence transcripts in the target species. Alternatively, RT-PCR can also be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene-specific primers from the original species and the first

strand cDNA of the interesting species as the template. The amplified product is further characterized by cloning and sequencing.

RNA arbitrary-primed (RAP-PCR)

RAP-PCR is a comparable method of conventional randomly amplified polymorphic DNA (RNA) but the first strand cDNA template rather than genomic DNA was used as the template in the amplification reaction. RAPD-PCR was established in 1999 (Williams *et al.*, 1990; Welsh and McClelland, 1990).

The amplification conditions in RAPD differ from the standard PCR in that only single random primer (usually 10 mer with GC content usually at least 50%) is employed. RAPD is utilized to amplified target DNA on the basis that the nuclear genome may contain several priming sites close to one another that are located in an inverted orientation. Accordingly, the primer is utilized to scan genome for the small inverted sequences resulting in amplification of DNA segments of variable length.

Subsequently, RNA fingerprinting by arbitrary primed PCR (RAP-PCR) (Welsh *et al.*, 1992) was introduced. The technique required reverse transcription of the target total RNA (or mRNA) to the first strand cDNA (by oligo dT or short and long random nucleotides). The synthesized cDNA was included as the template in the PCR reaction composing of the single primer or a combination of primers. The amplification products are size-fractionated through agarose or denaturing polyacylamide gels and detected by either radiolabeled or non-radiolabeled (ethidium bromide or silver staining) detection methods.

The intensity of RAP-PCR bands produced from different experimental samples is considered. Bands that are present in one sample and absent in another or bands that exhibit large differences in the intensity across the experimental treatments

should represent potentially differentially expressed mRNA transcripts and require further characterization. The fragments can be cloned and sequenced. The expression levels of interesting bands are then examined using specific primers
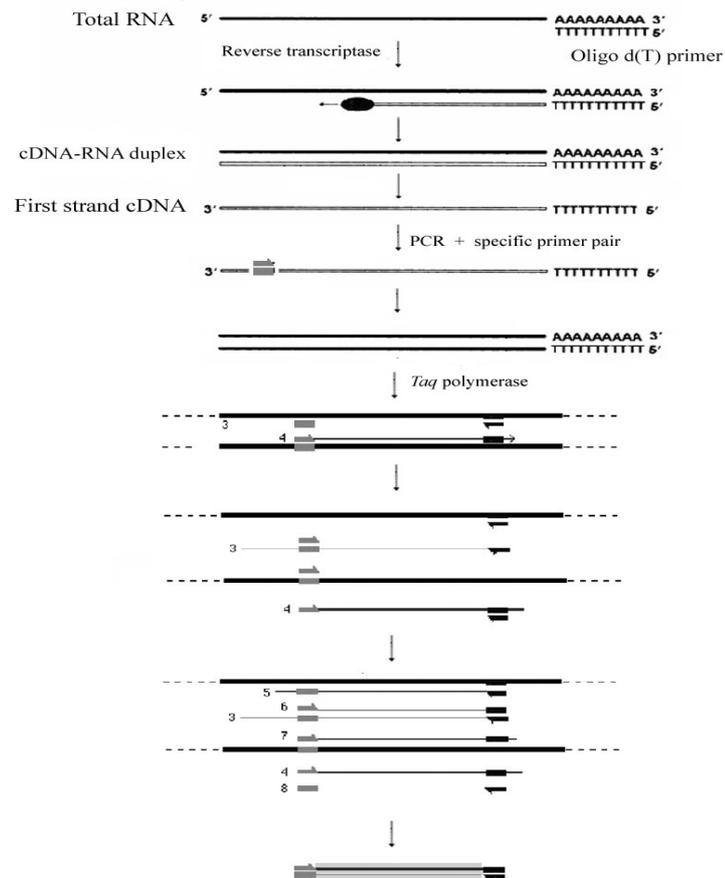
Figure 2.11 Overall concepts of the RT-PCR procedure. During first-strand cDNA synthesis an oligo d(T) primer anneals and extends from sites present within the mRNA. Second strand cDNA synthesis primed by the 18-25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* polymerase. These DNA fragments serve as the template for PCR amplification.

(Modified from: Ibelgaufts, www, 2002)

Available: http://www.copewithcytokines.de/cope.cgi?008220

Morphometric and molecular genetic studies in mollusks

There are a few publications about morphometric and molecular studies in mollusks, especially in the land snails. Almost all publication usually concerned species which are significant in public health or have high economic values.

McShane, Schiel, Mercer, and Murray (1994) studied relationships between the shell length and other parameters (shell height, shell width, shell weight, foot weight, dry foot weight, and total weight) in the black abalone (*Haliotis iris*) from different localities in New Zealand. The mean shell length varied among 61 localities explaining that more than 70% of the variation in the other parameters co-varied with the shell length. Significant sources of variation in the mean shell length included latitude (sea surface temperature) and relative exposure. Variation in all morphometric parameters occurred among localities but such variation, although significant, was generally not large (<10% of mean values). Although the spatial scales examined covered several hundred kilometers, the largest morphometric variation shown was between neighboring localities (200 meters apart). This indicated that morphometric variation occurred over small spatial scales.

Chiu, Chen, Lee, and Chen, (2002) used thirteen characters of the shell and the operculum to discriminate morphotype variations in the viviparid snail (*Cipangopaludina chinensis*) in Taiwan This species is one of the most widely distributed freshwater gastropods in Asia and exhibits intraspecific variations of shell morphology. A total of 251 individuals of *C. chinensis* collected from 5 geographically different samples (4 samples from Taiwan, and 1 sample from Korea) were morphometrically analyzed. Data were statistically analyzed by multidimensional scaling (MDS) and canonical discriminate analysis (CDA). Two

morphotypes (a tall-spired and a short-spired form) were found among the 5 populations. MDS indicated that snails with the shorter shell spire from two locations (Chutzuhu and Laumay) were morphologically related. In addition, CDA suggested that spire height is the most important character contributing to variation between these populations.

Conchological and anatomical characters are commonly used for identification of land gastropod species. Three putative conchologically different morphotypes of *Cochlicopa* in central Europe (*C. nitens*, *C. lubrica* and *C. lubricella*) were genetically analyzed. Snails previously classified as *C. lubricella* type I on shell morphology are genetically indistinguishable from *C. lubrica*, whereas *C. lubricella* type II constituted a distinct group. Molecular data indicated the shortcoming of shell morphometric characteristics for determining taxonomy of these species (Armbruster, 1995).

Stothard *et al*. (2002) differentiated the freshwater snails within the *Bulinus africanus* group (*Bulinus nasutus* and *B. globosus*) from East Africa using shell morphology (by morphometric analysis) and molecular data (by DNA sequences of cytochrome oxidase subunit 1, COI) studies. Identification of populations of *B. nasutus* and *B. globosus* by shell characteristics was unreliable and UPGMA cluster analysis failed to differentiate species of these snails. In contrast, single nucleotide polymorphism (SNP) of COI was used to develop a novel multiplexed *SNaP*shot^{TM}. Seven geographic samples were typed with 4 *SNaP*shot^{TM} primers and validation within shell morphology was compared to reference specimens from Zanzibar. Four locations were found to contain *B. nasutus* and two locations were found to contain *B. globosus*. A mixed population containing both *B. nasutus* and *B. globosus* was found at Kinango. The assay could be further broadened for

identification of other snail intermediate host species.

Some freshwater snails of the genus *Lymnaea* (Basommatophora, Lymnaeidae) act as the intermediate hosts of the liver fluke (*Fasciola hepatica* Linnaeus, 1758) that causes fascioliasis in animals and humans. However, species identification of *Lymnaea* snails is complicated by several factors including the dissection process, intra and interspecific similarity and variability of morphological characters. Using the classical morphology method, among the following species, *L. viatrix*, *L columella*, *L. diaphana*, *L. cousini*, *L. rupestris*, and *L plicata* could not be clearly distinguished. But, only *L. viatrix*, *L. columellar* and *L. diaphana* in South America have been found to be natural hosts of *F. hepatica.* PCR-RFLP of the first and second internal transcribed spacers (ITS1 and ITS2) of rDNA and the mitochondrial 16S ribosomal gene (16S rDNA) were used to differentiate *L. columella*, *L. viatrix*, and *L. diaphana* from Brazil, Argentina, and Uruguay as well as to verify whether the molecular results were concordant with those of classical morphological methods. PCR-RFLP analysis of the ITS1, ITS2, and 16S using 12 restriction enzymes revealed characteristic patterns for *L. columella* and *L. diaphana* which were concordant with the classical morphology. On the other hand, 1-6 restriction profiles were generated in *L. viatrix* populations while the morphological approach provided fixed species pattern results (Carvalho *et al.*, 2004).

Taxonomy of *Biomphalaria* snails is problematic due to high intra-specific variability but high interspecific similarity in anatomical and morphological characters among some species Molecular taxonomy has been applied to solve several problems which cannot be concluded by classical taxonomy. PCR-RFLP analysis of the ITS region of rDNA and a part of mitochondrial COI were used for identification

of several *Biomphalaria* species from Brazil and some other regions of South America. ITS2 (460 bp) of *Biomphalaria glabrata*, *B. tenagophila* and *B. straminea* (intermediate hosts of *Schistosoma mansoni*) in Brazil were amplified and sequenced. Nucleotide sequences of ITS2 were used for identification of restriction enzymes providing species-specific digestion patterns in these species. Four restriction enzymes were used and *Hpa* II provided simple species-specific profiles easily visualized in polyacrylamide gels. PCR-RFLP analysis of ITS2 with *Hpa*II is an auxiliary tool for morphological clarification and taxonomic and phylogenetic studies of neotropical planorbids (Vidigal *et al.*, 2004).

Genetic diversity and species-diagnostic markers of five oysters in Thailand; *Crassostrea belcheri* (Sowerby, 1871), *C. iredalei* (Faustino, 1932)*, Saccostrea cucullata* (Born, 1778), *S. forskali* (Gmelin, 1791) and *Striostrea (Parastriostrea) mytiloides* (Lamarck, 1819), were investigated by randomly amplified polymorphic DNA (RAPD) analysis. A total of 135, 127, 108, 131 and 122 genotypes were observed from primers OPA09, OPB01, OPB08 UBC 210 and UBC220, respectively. Two hundred and fifty-four reproducible and polymorphic fragments (200-2,500 bp in length) were generated across the five investigated species. The average number of bands per primer varied between 12.4 to 32.2. The percentage of polymorphic bands within *Crassostrea* (53.23%-77.67%) was lower than that of *Saccostrea* and *Striostrea* oysters (86.21%-99.36%). Nine, four and two species-specific markers were found in *C. belcheri, C. iredalei*, and *S. cucullata*, respectively. The mean of a ratio between the number of genotypes generated by each primer and the number of investigated specimens of *C. belcheri* (0.58) was lower than that of the remaining species (0.90-1.00). Genetic distances between pairs of oyster samples were between

0.105-0.811. A neighbor-joining tree indicated distant relationships between *Crassostrea* and *Saccostrea* oysters but closer relationships were observed between the latter and *S. mytiloides*, a representative species of *Striostrea* oysters (Klinbunga *et al.*, 2002). On the basis of RAPD analysis described above, species-specific markers were found in C *belcheri, C.iredalei, and S. cucullata* but not in S. *forskali and S. mytiloides.* Three *C. belcheri* specific RAPD fragments were cloned and sequenced. A primer set was designed from each of the recombinant clones (pPACB1, pPACB2, and pPACB3). The polymerase chain reaction product showed expected sizes of 536, 600 and 500 bp, respectively, with the sensitivity of detection approximately 30 pg of C. *belcheri* total DNA template. Specificity of pPACB1 was examined against 135 individuals of indigenous oyster species in Thailand and against outgroup references *S. commercialis* (*N*=12) and *Perna viridis (N=12)*. Results indicated the species-specific nature of primers developed from pPACB1. This primer set can be used for broodstock selection and determination of *C. belcheri* larvae to assist the selective breeding program for this commercially important species. (Klinbunga *et al.*, 2000).

Popongviwat (2001) examined genetic diversity and identified species-specific markers of three abalone species in Thailand, *Haliotis asinina*, *H. ovina,* and *H. varia* using RAPD analysis. Five decanucleotide primers (OPB11, UBC101, UBC195, UBC197 and UBC271) were selected from one hundred and thirty primers screened for genetic analysis of abalone in Thailand. High levels of genetic diversity between three abalone species were observed when UBC101 and OPB11 were used. The average similarity index within samples of *H. asinina, H. ovina,* and *H. varia* were 0.7927-0.8496, 0.6010-0.7032, and 0.5259-0.6102, respectively. Genetic differences

within 3 species were 0.2995, 0.4328, and 0.4295 for *H. asinina, H. ovina*, and *H. varia,* respectively. The average genetic distance of *H. asinina* in the Gulf of Thailand was 0.0243, which indicates that they are closely related. Large genetic distances were observed between each of the Gulf of Thailand and the Andaman and the Philippine samples. A neighbor-joining tree constructed from the average genetic distance between paired geographic samples indicated phylogenetically clear separation between investigated abalone species (using two primers) and geographic samples of *H. asinina* (using five primers). Species-specific RAPD markers were found in *H. asinina* (1700 and 320 bp from UBC101, 1030 and 650 bp from UBC195, 1450, and 750 bp from UBC197, and 680 bp from UBC271, respectively). In addition, a population-specific marker was observed in the Philippine sample (380 bp from UBC101) and the Talibong Island sample (880 bp from UBC271).

Genetic diversity and molecular diagnostic markers of the introduced golden apple snail (*Pomacea canaliculata* (Lamarck, 1801) and four native apple snails, *Pila ampullacea, P. angelica, P pesmei,* and *P. polita* in Thailand were studied by PCR-RFLP of COI and RAPD analyses. Twenty-one mitochondrial composite haplotypes showing non-overlapped distribution between species were found. Geographic heterogeneity and population differentiation analyses indicated significant genetic differences among species ($P<0.0001$) and within *P. pesmei* ($P<0.0001$) and *P. angelica* ($P<0.0004$), but not in *Pomacea canaliculata* ($P>0.0012$) *P. ampullacea* ($P=0.0824$-1.000) and *P. polita* ($P=1.000$). Neighbor-joining trees between pair of composite haplotypes, geographic samples, and species revealed clear differentiation of each snail species and also indicated that *P. angelica,* and *P. pesmei* are closely related phylogenetically as appears from their morphology. In addition, 16S rDNA of

representative individuals of each apple snail was cloned and sequenced. Species-specific PCR for detection of *Pomacea canaliculata* was successfully developed (N=131 and 82 for the target and non-target species respectively) with the sensitivity of detection approximately 50 pg of *Pomacea canaliculata* DNA template. Amplification of genomic DNA (50 pg and 25 ng respectively) isolated from fertilized eggs and juveniles (1, 7 and 15 days after hatching) of *Pomacea canaliculata* also suggested, that discrimination between *P. canallivulata* and *Pila* species could be carried out since the early stages of development (Thaewnon-ngiw, 2003)

Two hundred and two polymorphic fragments (180–1500 bp in length) were generated overall across the investigated samples (*N*=254) based on RAPD-PCR using primers OPA07, OPA10 and UBC122. The percentage of polymorphism bands were   98.86%, 94.56%, 90.91%, 96.94%, and 92.51% for *Pomacea canalicullata, P. angelica, P. ampullarcea, P. angelica, P. pesmei* and *P. polita* respectively. These indicated high genetic polymorphism of these taxa. Neighbor-joining trees between pairs of geographic samples within species suggested intraspecific population subdivision within *P. angelica, P. pesmei*, and *P. polit*a. Candidate species-specific markers found in *Pomacea canaliculata* (360 bp OPB10), *P. ampullacea* (640 bp OPA07), *P. angelica* (380 bp UBC122), and *Pila* species (430 bp, OPA07) were cloned and sequenced. A 259 bp SCAR marker was found in 95% of *Pila* apple snails (N=163), but not in *Pomacea canaliculata* (N=30). Therefore, this marker could be used in coupling with *Pomacea canalicullata* specific marker to differentiate the introduced and native apple snail in Thailand (Thaewnon-ngiw, 2003).

Land snails of the genus *Partula* in the Pacific Ocean have given excellent

opportunities to study adaptation and speciation. Morphological studies of *Partula* land snails from the Society Islands of French Polynesia have shown that large differences occur in shell shape, color and banding patterns between nearby populations, even in the absence of any obvious geographical barriers to the movement of snails or environmental gradients. The snails elsewhere exhibited relative uniformity over large geographic distances. SSCP analysis was carried out (16S1-TGACTGTGCAAAGGTAGC; 16S2-CTGGCTTACGCCGGTC) for examination of genetic diversity of the snails. Haldane's test and Fisher's exact test were used to examine genetic heterogeneity in the entire data and pairwise comparisons between pairs of populations, respectively. The relative frequencies of two mitochondrial haplotypes changed abruptly over small distances, seemingly independent of the environments. Although the transition roughly coincides with clines in the frequencies of some morphological characteristics, it appears to be unrelated to others. It is likely that many of the differences accumulated while populations were isolated from one another, through the effects of random genetic drift and selection. Isolation of populations may have occurred as a result of demographic changes or the process of colonization if occasional long-distance migrants establish populations ahead of the main invading front. Current genetic drift, even without restrictions to gene flow, may contribute to genetic patchiness on a small scale, although it is likely that characteristics such as shell colors and banding patterns are influenced by selection (Goodacre, 2001).

CHAPTER III

MATERIALS AND METHODS

Sample collection

Two hundred and fifteen individuals of the giant African snail (*A fulica*) were collected from 9 geographically different locations in Thailand and 1 location in Malaysia (Table 3.1 and Fig. 3.1). Specimens were weighed, marked, and given numbers individually. Live snails were anesthetized by keeping at -80°C for 2 hours before being subjected to DNA extraction. Alternatively, specimens were kept in a -80°C freezer until further required.
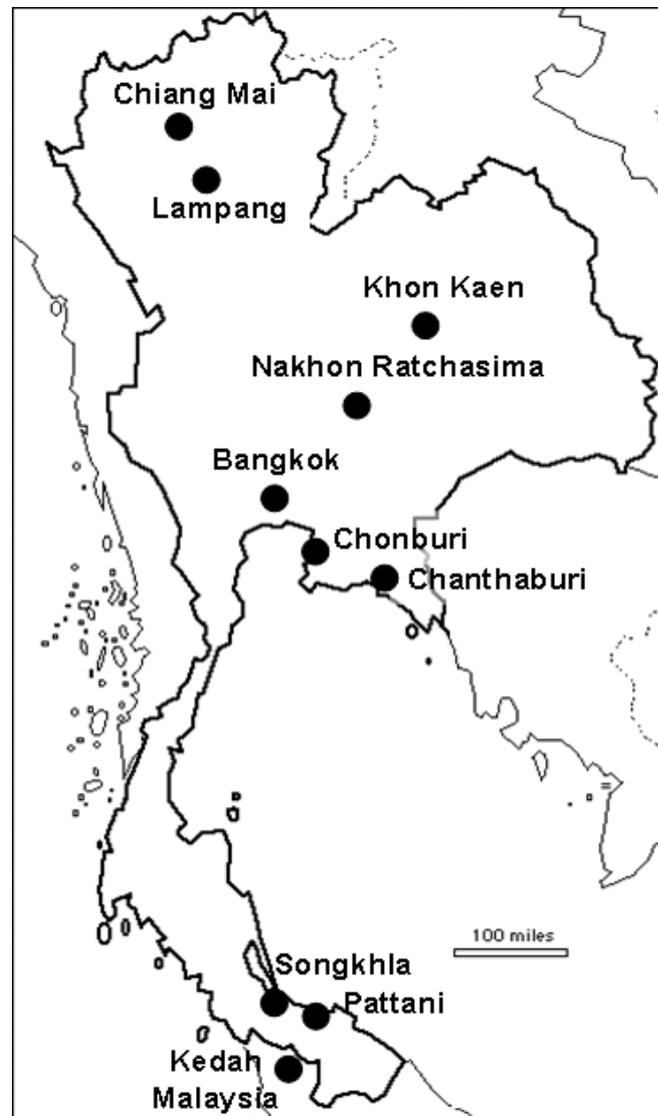
Figure 3.1 Map indicating 10 different geographical locations (9 locations in Thailand and 1 location in Malaysia) for sample collection of *A. fulica* used in this study.

Table 3.1 Sampling collection and sample sizes of *A. fulica* used in this study

Locations	Abbreviation	No. of collected individuals (*N*)	No. of investigated individuals			
			Morphometric (*N*)	PCR-RFLP (*N*)	SSCP (*N*)	RAPD (*N*)
Bangkok	BKK	20	20	9	20	20
Chonburi	CBR	20	20	6	20	20
Chanthaburi	CTB	20	20	5	20	20
Chiang Mai	CHM	20	20	8	20	20
Lampang	LAP	20	20	5	20	20
Nakhon Ratchasima	NRM	26	26	6	26	26
Khon Kaen	KOK	20	20	5	20	20
Songkhla	SKA	33	33	8	33	30
Pattani	PAT	25	25	8	25	25
Malaysia	MAL	11	11	5	11	11
Total		**215**	**215**	**65**	**207**	**215**

Morphometric analysis

Six shell characters adapted from Kristensen and Christensen (1989), Stothard *et al*. (2002) and Chiu *et al*. (2002) were measured using vernier calipers along imaginary straight lines (Fig. 3.2). The total shell length (LT) was measured along an axis passing through the apex to the bottom of the shell. The spire length (LS) was measured from the beginning of the first suture to the apex of the shell. The total shell width (WT) is the maximum width perpendicular to the shell length. The aperture length (LA) is the distance from the beginning of the first suture to the bottom of the shell. The aperture width (WA) is the maximum diameter of the shell aperture. The length of diagonal aperture/suture (LD) was measured from the first suture across diagonally to the opposite side of second suture. The unit of the measurement is expressed in millimeters with 2 positions after the decimal point.

All numerical data were subjected to cluster analysis. To avoid bias from different weights of numeric values in each character, all data were standardized to Z scores before subjected to multivariate analysis. The analyses were carried out using SYSTAT 11 program (SYSTAT Software Inc. USA). The criteria of analysis were K-mean clustering with Euclidian distance matrix and five predetermined groups.
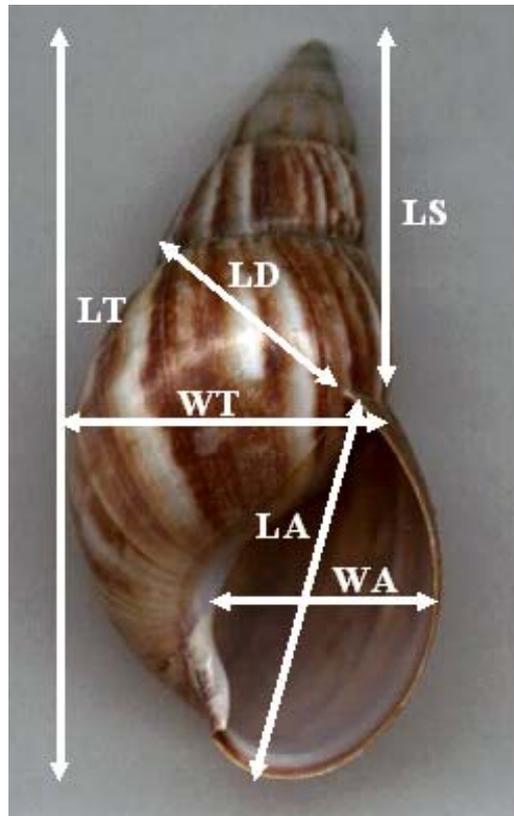
Figure 3.2 The six continuous variables measured on the shell of the giant African snail *A. fulica* (Bowdich, 1822) LT = total length of shell, LA = length of aperture, WT = width of shell, WA = width of aperture, LS = length of spire, LD = length of diagonal aperture/suture (adapted from Kristensen and Christensen, 1989 and Chiu *et al.*, 2002).

DNA extraction

Extraction of genomic DNA

Genomic DNA was extracted from a piece of foot muscle of each *A. fulica* individual using a phenol-chloroform-proteinase K method (Sirawut Klinbunga *et al*., 1999). A piece of foot muscle tissue was dissected out from a frozen snail placed in a prechilled microcentrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle. SDS (10%) and RNase A (10 mg/ml) solutions were added to a final concentration of 1.0% (w/v) and 100 µg/ml, respectively. The resulting mixture was then incubated at $37^{\circ}C$ for 1 hour. At the end of the incubation period, a proteinase K solution (10 mg/ml) was added to the final concentration of 200 µg/ml and further incubated at 55°C for 3-4 hours.

An equal volume of buffer-equilibrated phenol was added and gently mixed for 15 minutes. The solution was centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube. This extraction process was then repeated once with phenol:chloroform : isoamylalcohol (25:24:1) and twice with chloroform : isoamylalcohol (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. DNA was precipitated by an addition of two volume of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at $-20^{\circ}C$ for 2 hours. 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% ethanol (5 minutes and brief washes, respectively). After centrifugation, the supernatant was removed. The DNA pellet was

air-dried and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37°C for 1-2 hours and kept at 4°C until further needed.

PCR-RFLP analysis

Screening of primers

Universal primers for amplification of the large subunit of nuclear ribosomal DNA LSU 1-3, LSU 2-4, 18S rDNA, 16S mitochondrial DNA, and COI, which were previously used for population genetic studies of several species were used to test for the amplification success against a few individuals of *A. fulica* (Table 3.2). Primers for amplification of the COI gene segment were selected for population genetic studies of *A. fulica*.

Table 3.2 Primer and primer sequences screened for population genetic studies of *A. fulica* in Thailand

Primer	Sequence	Reference
LSU 1-3	F: 5' –GGGTTGTTTGGGAATGCAGC-3' R: 5'- GTTAGACTCCTTGGTCCGTG-3'	Wade, Mordan, and Clarke (2001)
LSU 2-4	F: 5'-CTAGCTGCGAGAATTAATGTGA-3' R: 5'-ACTTTCCCTCACGGTACTTG-3'	Wade, Mordan, and Clarke (2001)
18S rDNA	F: 5'-TGGATCCGGGCAAGTCTGGTGCC-3' R: 5'-TGAAGTCAAGGGCATCACAGACC-3'	Siludjai (2000)
16S rDNA	F: 5'-CGCCTGTTTAACAAAAACAT-3' R: 5'-CCGGTCTGAACTCAGATCATGT-3'	Plumbi *et al.* (1991)
COI	F: 5'-GGTCAACAAATCATAAAGATATTGG-3' R: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer *et al.* (1994)

Polymerase chain reaction (PCR) of COI

The COI gene segment of each snail was amplified by PCR in a 50 µl reaction volume containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1% Triton X-100, 2 mM MgCl$_2$, 100 µM of each dNTP (dATP, dGTP, dCTP, and dTTP), 0.5 mM of each primer, 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes, Finland), and 50 ng of genomic DNA template. PCR was performed in a thermal cycler (OmmiGene, Hybaid, England) with predenaturing at 94°C for 3 minutes followed by 10 cycles of denaturation at 94°C for 1 minute, annealing at 48°C for 1 minute and extension at 72 ^{o}C for 1 minute, and additional 35 cycles of amplification using and annealing temperature of 55°C for 1 minute in each cycle. The final extension was performed at 72°C for 7 minutes

Agarose gel electrophoresis

An appropriate amount of agarose (SeaKem® LE Agarose, Cambrex Corporation) was weighed and mixed with an appropriate volume of 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.3). The gel slurry was boiled in a microwave oven to complete solubilization, and allowed to cool to approximately 55°C before poured into the gel mold. A comb was inserted. The gel was left to solidify at room temperature for 30-45 minutes. When necessary, the comb was carefully removed. The agarose gel was submerged in a chamber containing enough 1X TBE buffer to cover the gel by approximately 0.5 cm. Appropriate volumes of PCR products were mixed with one-fourth the volume of the 10X loading dye (0.25% bromophenol blue and 25% Ficoll (Sigma chemical, USA) in water) and loaded into the well. A 100 bp DNA ladder (SibEnzyme Ltd. Russia) was used as the standard DNA marker

Electrophoresis was carried out at 5-6 volts/cm until bromophenol blue moved approximately one-half the distance of the gel in normal electrophoresis or three-fourth of the gel in PCR-RFLP and RAPD procedures. The electrophoresed gel was stained with an ethidium bromide solution (1 µg/ml) for 10 minutes and destained in running tapwater to remove unbound ethidium bromide from the gel. DNA fragments were visualized under a UV transilluminator (UVP, USA) and photographed through a red filter using SLR Camera (Pentax K1000, Japan) with Fomapan Classic 100 film. The exposure time was 10-18 seconds.

To determine the amplification success of COI, five microliters of the amplified product were electrophoresed through 1% agarose gel. Samples showing positive results were subjected to restriction enzyme analysis.

Restriction analysis of COI

The amplified COI gene segment was screened against 17 restriction endonucleases obtained from Boehringer Mannheim, Germany; *Acs* I (A/GAATTT/C) as well as Promega Corporation Medison, Wisconsin, USA; *Alu* I (AGCT), *Bam* HI (GGATCC), *Cla* I (ATCGAT), *Dde* I (CTNAG), *Dra* I (TTTAAA), *Eco* RI (GATTC), *Hae* III (GGCC), *Hin*d III (AAGCTT), *Hin*f I (GANTC), *Kpn* I (GGTACC), *Mbo* I (GATC), *Nde* I (CATATG), *Rsa* I (GTAC), *Ssp* I (AATAAT), *Taq* I (TCGA), and Vsp I (ATTAAT). The digestion was performed in a final volume of 50 µl reaction mixture containing 1X restriction enzyme buffer, 4 mM spermidine trihydrochloride, 0.1 µg/ml BSA, 3 units of each restriction endonuclease, 6-10 µl of the amplification product, and the appropriate amount of sterile deionized water.

The reaction mixture was incubated at 37°C overnight except for *Taq* I in which the reaction was incubated at 65°C, instead. One-tenth volume of 3 Molar of sodium acetate (pH 5.2) was added into the digestion mixture. The digested DNA was precipitated by the addition of 2.5 volume of ice-cold absolute ethanol and incubated at -80°C for at least 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at room temperature and briefly washed with 70% ethanol. The DNA pellet was air-dried and resuspended in 10 µl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The DNA solution was incubated at 37°C for 1-2 hours for complete solubilization. The digested DNA was mixed with an appropriate volume of 10X loading dye (0.25% bromophenol blue and 25% Ficoll in water) and electrophoretically analyzed through 2% agarose gel at 5-6 volts/cm for 2-3 hours.

RAPD-PCR analysis

Screening of primers for RAPD analysis

One hundred and one primers (consisted 88 of decanucleotides primers from Operon Biotechnologies and University of British Columbia, Vancouver, British Columbia, Canada, 7 of minisatellites, and 6 of microsatellites) (Table 3.3) were screened for the amplification success against 3 representative individuals of *A. fulica.*

RAPD PCR was carried out in a final volume of 25 µl containing 10 mM Tris-HCl; pH8.8, 50 mM KCl, 1% Triton X-100, 2 mM MgCl$_2$, 100 µM of each dNTP (dATP, dGTP, dCTP and dTTP), 0.4 mM of each primer, 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes, Finland) and 50 ng of DNA template. PCR was performed in a thermal cycler (OmmiGene, Hybaid) including predenaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 10 seconds, annealing at 36°C for 30 seconds and extension at 90°C for 90 seconds. The final extension was performed at 72°C for 5 minutes. Four Operon primers (OPA02, OPA17, OPB11, and OPZ09) that gave reproducible results were selected for analysis of genetic diversity of *A. fulica*.

After amplification, 5 µl of the amplified product were electrophoresed through a 1% agarose gel to determine whether the reaction was successfully amplified. Samples showing positive results were subjected to electrophoretic analysis as soon as possible.

SSCP analysis

Two genes were selected for SSCP analysis across all the *A. fulica* samples. The COI (mitochondrial gene) and an unknown gene containing a stretch of nucleotides specifically found in chromosome X of mammals and isolated from

complementary (c) DNA of the giant tiger shrimp, *Penaeus monodon* (nuclear gene), (hereafter, called the PMX gene) were used in this study.

Table 3.3 RAPD primers and their sequences initially screened by this study

Primer	Sequence (5'-3')
UBC101	GCGCCTGGAG
UBC102	GGTGGGGACT
UBC104	GGGCAATGAT
UBC105	CTCGGGTGGG
UBC106	CGTCTGCCCG
UBC107	CTGTCCCTTT
UBC108	GTATTGCCCT
UBC110	TAGCCCGCTT
UBC112	GCTTGTGAAC
UBC115	TTCCGCGGGC
UBC116	TACGATGACG
UBC117	TTAGCGGTCT
UBC117	TTAGCGGTCT
UBC119	ATTGGGCGAT
UBC120	GAATTCCCC
UBC128	GCATATTCCG
UBC129	GCGGTATAGT
UBC133	GGAAACCTCT
UBC135	AAGCTGCGAG
UBC137	GGTCTCTCCC
UBC141	ATCCTGTTCG
UBC145	TGTCGGTTGC

Table 3.3 (continued)

Primer	Sequence (5'-3')
UBC146	ATGTGTTGCG
UBC149	AGCAGCGTGG
UBC150	GAAGGCTCTG
UBC153	GAGTCACGAG
UBC158	TAGCCGTGGC
UBC159	GAGCCCGTAG
UBC160	CGATTCAGAG
UBC169	ACGACGTAGG
UBC174	AACGGGCAGC
UBC175	TGGTGCTGAT
UBC191	CGATGGCTTT
UBC196	CTCCTCCCCC
UBC200	TCGGGATATG
UBC201	CTGGGGATTT
UBC202	GAGCACTTAC
UBC203	CACGGCGAGT
UBC204	TTCGGGCCGT
UBC217	ACAGGTAGAC
UBC259	GGTACGTACT
UBC262	CGCCCCCAGT
UBC268	AGGCCGCTTA
UBC273	AATGTCGCCA
UBC299	TGTCAGCGGT
UBC428	GGCTGCGGTA
UBC456	GCGGAGGTCC
UBC457	CGACGCCCTG
UBC 460	ACTGAC CGGC

Table 3.3 (continued)

Primer	Sequence (5'-3')
OPA01	CAGGCCCTTC
OPA02	TGCCGAGCTG
OPA03	AGTCAGCCAC
OPA04	AATCGGGCTG
OPA05	AGGGGTCTTG
OPA06	GGTCCCTGAC
OPA07	GAAACGGGTG
OPA08	GTGACGTAGG
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG
OPA11	CAATCGCCGT
OPA12	TCGGCGATAG
OPA13	CAGCACCCAC
OPA14	TCTGTGCTGG
OPA15	TTCCGAACCC
OPA16	AGCCAGCGAA
OPA17	GACCGCTTGT
OPA18	AGGTGACCGT
OPA19	CAAACGTCGG
OPA19	CAAACGTCGG
OPA20	GTTGCGATCC
OPB01	GTTTCGCTCC
OPB02	TGATCCCTGG
OPB03	CATCCCCCTG
OPB04	GGACTGGAGT
OPB05	TGCGCCCTTC
OPB06	TGCTCTGCCC
OPB07	GGTGACGCAG

Table 3.3 (continued)

Primer	Sequence (5'-3')
OPB08	GTCCACACGG
OPB09	TGGGGGACTC
OPB10	CTGCTGGGAC
OPB11	GTAGACCCGT
OPB12	CCTTGACGCA
OPB13	TTCCCCCGCT
OPB14	TCCGCTCTGG
OPB15	GGAGGGTGTT
OPM09	GTCTTGCGGA
OPZ09	CACCCCAGTC
Minissatellite and Microsatellite primers	
INS	ACAGGGGTGTGGGG
YNZ22	CTCTGGGTGTCGTGC
M13	GAGGGTGGNGGNTCT
YN73	CCCGTGGGGCCGCCG
PER I	GACNGGNACNGG
HRU18	ACCCGGCGCTTATTAGAG
HRU33	CCCAAGGTCCCCAAGGTCAGGGAGGCG
$(CA)_8$	CACACACACACACACA
$(CT)_9$	CTCTCTCTCTCTCTCT
$(CAC)_8$	CACCACCACCAC CACCACCACCAC
$(GTG)_8$	GTGGTGGTGGTGGTGGTGGTGGTG
$(GATA)_4$	GATAGATAGATAGATA
$(GACA)_4$	GACAGACAGACAGACA

The expected product (approximately 150 bp) of the PMX gene allowed direct application of heterospecific primers. On the other hand, the amplification product of the COI gene segment of *A. fulica* was approximately 710 bp. This fragment length is

not appropriate for SSCP analysis, which requires approximately 100-300 bp fragments. As a result, the amplified 710 bp fragment was cloned and sequenced. Primers providing a suitable range of the amplification products were designed and genotyped across all the specimens.

Cloning of amplified COI

Preparation of COI gene fragment

A 710 bp fragment of COI of *A. fulica* was amplified by primers LCO1490 and HCO2198 (Folmer *et al*., 1994) using conditions previously described for PCR-RFLP analysis. The COI fragment was fractionated through an agarose gel in duplicate. One was run side-by-side with a 100 bp DNA marker (usually on the left). The other was loaded into another well (usually on the right) of the gel. After electrophoresis, agarose gel containing the DNA standard and its proximal DNA sample was cut and stained with ethidium bromide (0.5 μg/ml). The position of the non-stained target DNA fragment was then referenced without exposure to the UV.

The DNA fragment was excised from the gel with a sterile razor blade. DNA was eluted from the agarose gel using a QIAquick gel Extraction kit (QIAGEN, Germany) according to the protocol recommended by the manufacturer. The excised gel was transferred into a microcentrifuge tube and weighed. Three gel volumes of the QG buffer were added. The mixture was incubated at 50°C for 10 minutes with brief vortexing every 2-3 minutes. The mixture was applied to the QIAquick spin column placed into an Eppendorf tube and centrifuged at 13,000 rpm for 1 minute at room temperature. The flow-through was discarded and 0.75 ml of the PE buffer was added. The QIAquick spin column was then centrifuged at 13,000 rpm for 1 minute at room

temperature. After the flow-through was discarded, the column was further centrifuged at room temperature for an additional 1 minute at 13,000 rpm to remove the trace amount of the washing buffer. The column was then placed into a new microcentrifuge tube and 30 µl of the EB buffer (10 mM Tris-HCl, pH 8.5) was added to the center of the QIAquick membrane. The column was incubated at room temperature for 1 minute before centrifuging at 13,000 rpm for 1 minute. The eluted sample was stored at -20°C until further required.

Ligation of the COI fragment to pGEM® -T easy vector

DNA fragments were ligated to the pGEM®-T Easy vector (Promega Corporation Madison, Wisconsin, USA) in a 10 µl reaction volume containing 5 µl of 2X Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, and 10% polyethylene glycol; MW 8000), 3 Weiss units of T4 DNA ligase, 25 ng of pGEM®-T Easy vector and 50 ng of DNA insert. The reaction mixture was incubated at 4°C overnight before transforming to competent *Escherichia coli* cells strain JM109 (recAl supE44 endAl hsdR17 gyrA96 relAl thi 8(lac- pro AB) F' (traD 36 proAB+ lacq lacZ 8 Ml 5)

Transformation of ligation products to *E. coli* host cells

Preparation of competent cells

A single colony of *E. coli* strain JM109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract, and 0.5% NaCl, pH 7.0) with vigorous shaking at 37°C overnight. The starting culture was then inoculated into 50 ml of LB broth and the culture was continued at 37°C with vigorous shaking to an OD_{600} of 0.5-0.8 was reached. The cells were briefly chilled on ice for 10 minutes,

and recovered by centrifugation at 2,700 g for 10 minutes at 4°C. The pellets were resuspended in 30 ml of ice-cold MgCl$_2$/CaCl$_2$ solution (80 mM MgCl$_2$ and 20 mM CaCl$_2$) and centrifuged as above. After resuspension in 2 ml ice-cold 0.1 M CaCl$_2$, the concentrated cell suspension was divided to 200 µl aliquots. The competent cells were used immediately or, alternatively, stored at –80°C for subsequently used.

Transformation

The competent cells were thawed on ice for 5 minutes. Two to four microlitres of the ligation mixture were added and gently mixed by pipetting and left on ice for 30 minutes. The transformation reaction was then heat-shocked in a 42°C water bath for 45 seconds without shocking. The reaction tube was immediately placed in ice for 2-3 minutes. The mixture was removed from the tube and added to a new tube containing 1 ml of prewarmed SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCI, 2.5 mM KC1, 10 mM MgCl$_2$, 10 mM MgS0$_4$, and 20 mM glucose). The cell suspension was incubated with shaking at 37°C for 90 minutes. The mixture was centrifuged for 20 seconds at room temperature, gently resuspended in 100 µl of SOC medium and spread onto a selective LB agar plate containing 50 µg/ml of ampicillin, 25 pg/ml of IPTG, and 20 pg/ml of X-gal and further incubated at 37°C overnight (Sambrook, Fritsch and Maniatis, 1989). The recombinant clones containing inserted DNA were white whereas those without inserted DNA were blue.

Examination of the insert sizes by colony PCR and restriction of the amplification products

Colony PCR was performed in a 25 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KC1, 0.1% Triton X-100, 100 mM of each dNTP, 2

mM MgCl$_2$, 0.1 μM each of pUCl (5'-CCGGCTCGTATGTTGTGTGGA-3') and pUC2 (5'-GTGGTG CAAGGCGATTAAGTTGG-3'), and 0.5 unit of Dynazyme™ DNA Polymerase. An interesting colony was picked by a pipette tip and served as the template in the reaction.

PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute and extension at 72°C for 1.5 minutes. The final extension was carried out at the same temperature for 7 minutes. The colony PCR products were electrophoresed though a 1.2% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

For examination of digestion patterns, the colony PCR product containing an insert was separately digested with *Hind* III and *Rsa* I in a final volume of 15 μl reaction containing l0X buffer (6 mM Tris-HCl, 6 mM MgCl$_2$, 50 mM NaCl, and 1 mM DDT, pH 7.5 for *Hind* III or 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 5 μl of the colony PCR product. The reaction mixture was incubated at 37°C overnight before electrophoresed though a 1.2% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

Isolation of recombinant plasmid DNA

A white colony was inoculated into a sterile tube containing 3 ml of LB broth supplemented with 50 μg/ml of ampicillin and incubated with shaking (200 rpm) at 37°C overnight. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN, Germany). The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 1 minute. The supernatant was carefully

decanted. The pellets was resuspended in 250 µl of the PI buffer. Subsequently, 250 µl of the P2 buffer was added. The tube was gently inverted for 4-6 times. Then, 350 µl of the N3 buffer was added. The tube was thoroughly but gently inverted 4-6 times and centrifuged at 13,000 rpm for 10 minutes. The supernatant was carefully collected and applied to the QIAprep column and centrifuged at 13,000 rpm for 1 minute. The flow-though solution was discarded. The QIAprep spin column was washed by adding 500 µl of the PB buffer and centrifuging at 13,000 rpm for 1 minute. The flow-though solution was discarded. The PE buffer (750 µl) was added and centrifuged as above. After the flow-though 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 by the addition of 50 µl of the EB buffer (10 mM Tris-HCl, pH 8.5) or ultrapure water to the center of the QIAprep column and leaving for 1 minute before centrifuging at 13,000 rpm for 1 minute. The concentration of extracted plasmid DNA was spectrophotometrically measured.

DNA sequencing

The recombinant clones were unidirectionally sequenced using the M13 forward primer (5'-TTTTCCCAGTCACGAC-3') on an automatic sequencer at the Bioservice Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC). Nucleotide sequences were blasted against data in the GenBank (http://www.ncbi.nlm.nih.gov) using BLASTN (nucleotide similarity). Significant similarity was considered when the probability (E) value was $<10^{-4}$.

Primer design

Two pairs of primers (AFCOI215-F/R and AFCOIXXX-F/R) were designed from a 710 bp COI sequence using the Primer Premier 5.0 software (PREMIER Biosoft International). The criteria for primer designing were 1) PCR product length was 150-300 bp, 2) primers were 18-25 bases in length, 3) melting temperature was 55-70°C, Tm = 2 (A+T) + 4 (G+C), 4) base distribution of the primers were random, avoiding polypurine and polypyrimidine tracts, 5) the difference of the melting temperature of any primer pair did not exceed 5°C, and 6) ΔG of annealing between pairs of primers and the secondary structure of each primer was not lower than -5. The primers were ordered and purchased from Biosynthesis (USA).

Amplification of COI$_{215}$ and PMX

A 215 bp fragment of COI (COI$_{215}$) was amplified from each snail using newly designed primers (AFCOI215-F: 5'-CTTTACATTTGGGCTGGTG-3,' and AFCOI 215-R: 5'-GAAAGATGTGTTGAAGTTACGAT-3'). The PCR was performed in a final volume of 50 μl containing 10 mM Tris-HCl; pH 8.8, 50 mM KCl, 1% Triton X-100, 2mM MgCl$_2$, 100 μM of each dNTP (dATP, dGTP, dCTP, and dTTP), 0.5 mM of each primer, 0.75 unit of DyNAzyme^{TM} II DNA Polymerase, and 50 ng of DNA template. PCR was performed in a thermal cycler (OmmiGene, Hybaid) with predenaturing at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 7 minutes.

Recipes for amplification of the PMX homologue (220 bp) were identical to those for COI$_{215}$ with the exception that DyNAzymeTM II DNA Polymerase was reduced to 0.5 units. The PCR profiles for the PMX homologue were predenaturation

at 94°C for 3 minutes followed by 5 cycles of denaturation at 94°C for 45 seconds, annealing at 45°C for 1 minute and extension at 72°C for 1 minute, and 35 additional cycles of amplification using an annealing temperature of 53°C. The final extension was performed at 72°C for 7 minutes.

Preparation of glass plate

The glass plate was thoroughly wiped with 2 ml of 95% commercial grade ethanol in one direction with a tissue. 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 95% ethanol and 10 µl of 5% glacial acetic acid) and left for approximately 10-15 minutes. Excess binding solution was removed with a piece of tissue. The long glass plate was further cleaned with 95% ethanol 3 times. The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetra- sitoxone). The cleaned glass plates were assembled with a pair of 0.4 mm spacers and a pair of the gel clamps.

The 15-20% low crosslink (37.5:1 acrylamide:bis-acrylamide, containing 1x TBE gel buffer equivalent to 2.66 % crosslink) non-denaturing polyacrylamide gels were prepared by dilution of a 40% stock solution to the required gel concentration. The acrylamide gel solution (30-40 ml) may be mixed with glycerol (5% or 10% concentration), if desired, and 240 µl of 10% APS and 24 µl of TEMED. The analytical comb was inserted into the prepared gel and polymerization was allowed for 4 hours or overnight.

For SSCP analysis, 8 µl of the amplified COI$_{215}$ and PMX product were mixed with four additional volume of the denaturing gel-loading solution (98% formamide,

0.025% bromophenol blue, 0.025% xylene cyanol, and 10 mM NaOH). The mixture was heated at 95°C for 5 min and immediately placed on ice for 2-3 minutes. Electrophoresis was carried out at 200 V for 12-116 at 4°C. As a control, the non-denatured COI$_{215}$ or PMX was also included in the gel.

Silver staining

The gel plates were carefully separated. The glass plate with the gel was placed in a plastic tray containing 1.5 liters of the fix/stop solution and agitated for 25-30 minutes (40 minutes for RAP-PCR gels). Then the gel was washed 3 times by shaking in deionized water for 3 minutes each. The gel was lifted out from the tray between each wash and the washed water was allowed to drain out of the gel for 10-20 seconds. The gel was transferred to 0.1% silver nitrate solution (1.5 g of silver nitrate and 2.25 ml of 37% formaldehyde in 1.5 liter deionized water) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 liter of deionized water with shaking and immediately placed in the tray containing 1.5 liter of the chilled developing solution (90 g of sodium carbonate in 3 liters of deionized water and 2.25 ml of 37% formaldehyde and 300 µl of a 10mg/ml sodium thiosulfate solution were added before used). This step is crucial and the time taken to soak the gel in the water and transfer it to the chilled developing solution should be no longer than 5-10 seconds. The gel was well agitated until the first bands were visible (usually 1.5-2 minutes). The gel was then transferred to another tray containing 1.5 liter of chilled developer and shaken until bands from every lane were observed (usually 2-3 minutes). One liter of the fix/stop solution was directly added to the developing solution and shaking was continued for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was left at room temperature.

Data analysis

Nucleotide sequences of the amplified COI fragments (710 and 215 bp) and RAP-PCR fragments were compared with those previously deposited in the GenBank using BLASTN and BLASTX (available at http://www. ncbi/nlm/nih.gov). Significant probabilities of matched nucleotides/proteins were considered when the E-value was $<10^{-4}$.

RAPD and SSCP fragments are treated as independent characters. Sizes of the bands were estimated by comparing with a 100 bp ladder and recorded in a binary matrix to represent the presence (1) or the absence (2) of a particular band.

The modified Roger's genetic distance between geographic samples was calculated (Wright, 1978). Pairwise genetic distance was then subjected to phylogenetic reconstruction using a neigh-joining approach (Saitou and Nei, 1987) using Neighbor in PHYLIP (Felsenstein, 1993). The NJ tree was appropriately illustrated using TREEVIEW.

Genetic heterogeneity between pairs of geographic samples was examined using with the Markov chain approach (Gua and Thompson, 1982). F_{ST} statistics (θ, Weir and Cockerham, 1984)) between pairs of samples were analyzed using TFPGA (available at http://www.public.asu.edu/~mmille8/tfpga.htm).

Identification and characterization of stress-related genes in the collar tissue of the giant African snail (*Achatina fulica*)

Experimental snails

Thirty-six individuals of wild giant African snail (*A. fulica*) were collected from Pattani and separated to 3 groups ($N = 12$ for each group) including the control where snails were kept in the laboratory conditions and normally fed. The partial

aestivation group where snails were maintained in dry and starved conditions for 5 days (snails completely withdrew their footpad and head into the shell but did not produced an epiphragm). The full aestivation group where snails were maintained in dry and starved conditions for 15 days until the snails withdrew their footpad and head deeply into their shells and produced an epiphragm to close the shell aperture completely.

Total RNA extraction

A piece of collar tissues was immediately placed in mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI REAGENT (1 ml/50-100 mg tissue) and homogenized. Additional 500 µl of TRI REAGENT were added. The homogenate and left for 5 minutes, before adding 0.2 ml of chloroform. The homogenate was vortexed for 15 seconds and left at room temperature for 2-15 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase (inclusively containing RNA) was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture was left at room temperature for 10-15 minutes and centrifuged at 12,000g for 10 minutes at 4-25°C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4°C. The ethanol was removed. The RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated water for immediate use. Alternatively, the RNA pellet was kept under absolute ethanol in a -80°C freezer for long storage.
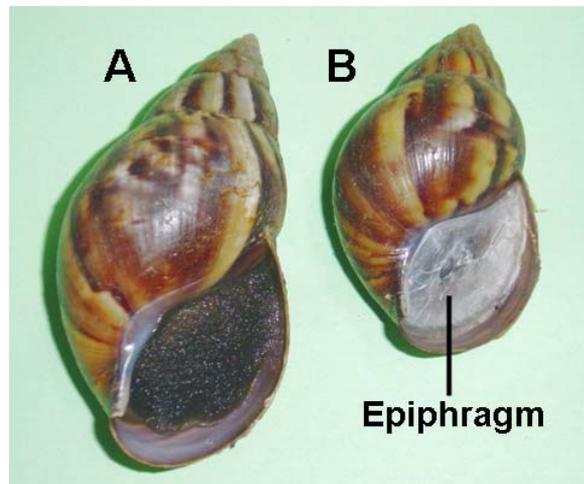
Figure 3.3 Experimental snails used for isolation and characterization of stress-related genes of *A. fulica*. The partially aestivated snail (A) completely withdrew footpad and head into the shell aperture but did not produce an epiphragm whereas the fully aestivated snail (B).where the snail withdrew footpad and head deeply into its shell and produced an epiphragm completely cover the shell aperture

Measuring concentrations of extracted total RNA by spectrophotometry

The concentration of extracted RNA samples was estimated by measuring the optical density at 260 nm. An OD_{260} of 1.0 corresponds to a concentration of 40 µg/ml of single stranded RNA (Sambrook *et al.*, 1989). Therefore, the concentration of total RNA samples were estimated in µg/ml by using the following equation,

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

The purity of RNA samples can be evaluated from a ratio of OD_{260}/OD_{280}. The ratio of appropriately purified RNA was 2.0 (Sambrook *et al.*, 1989).

Synthesis of the first strand cDNA

The first strand cDNA was synthesized from 1 µg of total RNA extracted from the collar tissue of *A. fulica* using an ImProm-II^{TM} Reverse Transcription System Kit (Promega Corporation Madison, Wisconsin, USA Corporation). Total RNA was combined with 0.5 µg of oligo (dT$_{18}$) for typical RT-PCR or OPA02 and OPZ09 for RAP-PCR (see below) and appropriates DEPC-treated water in a final volume of 5 µl. The reaction was incubated at 70°C for 5 minutes and immediately placed on ice for 5 minutes. After that, 5X-reaction buffer, MgCl$_2$, dNTP mix and RNasin were added to the final concentrations of 1X, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of an ImProm-II^{TM} reverse transcriptase was added and gently mixed by pipetting. The reaction mixture was incubated at 25°C for 5 minutes and at 42°C for 90 minutes. The reaction was incubated at 70°C for 15 minutes to terminate reverse transcriptase activity. Concentration and rough quality of the first stranded cDNA was spectrophotometrically examined (OD$_{260}$ and OD$_{260}$/OD$_{280}$) and electrophoretically analyzed (1.0% agarose gel). The first stranded cDNA was five-fold diluted for further used as thr template of RT-PCR analysis.

Examination of the expression level of the gene encoding an antibacterial peptide in *A. fulica*

The cDNA sequence of an antibacterial peptide, achacin was retrieved from the GenBank (Accession no. X64584 S46142). A pair of primers (ABPAF406-F: 5'-TTTACGACTCACTTGCCTAACG-3'and ABPAF406-R-5'-TACCTTCTGGCGT ACCCACTTT-3') were designed.

For RT-PCR, it is necessary to include the positive control (usually a housekeeping gene exhibiting relatively constant expression levels across treated

conditions of the experiment) in the experiment. One of the common house keeping gene used as the control in RT-PCR is β-actin. A 315 bp segment of β-actin was successfully amplified by using the heterospecific primers Actin1: 5'-GGTATCCTCACCCTCAAGTA-3' and Actin 2 : 5'-AAGAGCGAAACCTTCA TAGA-3' initially designed from the β-actin cDNA of the giant tiger shrimp (*Peneaus monodon*) against the first strand cDNA template of *A. fulica*. The amplified β-actin of *A. fulica* was cloned and sequenced. A new primer pair (ACTINAF-F: 5'-GCTTGCTGATCCACATCTGCT-3' and ATJNAF-R: 5'-GAGTCTGCGGGTATCC ACGA-3') generating a 217 bp fragment was designed and used for the control reaction.

Achacin and β-actin were separately amplified in a final volume of 50 μl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1% Triton X-100, 2 mM MgCl$_2$, 100 μM of each dNTP (dATP, dGTP, dCTP, and dTTP), 0.5 mM of each primer, 1 unit of DyNAzyme^{TM} II DNA Polymerase (FINNZYMES, Finland), and 750 ng of the cDNA template. The PCR was performed in a thermal cycler (OmmiGene, Hybaid) with predenaturing at 94°C for 3 minutes followed by 20, 25, 30, 35 and 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 7 minutes. The resulting products were electrophoretically analyzed through 1.2% agarose gels.

RNA arbitrary primed-polymerase chain reaction (RAP-PCR)

Thirty decanucleotide primers of the University of British Columbia and Operon Biotechnologies primers (UBC 101, UBC 119 UBC 120, UBC 128, UBC 135, UBC 138, UBC 158, UBC 159, UBC 160, UBC 174, UBC 191, UBC 217, UBC 222

UBC 228 UBC 263, UBC 268, UBC 273, UBC 299, UBC 428, UBC 456, UBC 457, UBC 459, OPA 01. OPA 02, OPA 07, OPA 08, OPA 16, OPA 17, OPB 11 and OPZ 09) were screened for identification of stage-specific and/or differential expression RAP-PCR fragments.

An equal amount of total RNA from 3 individuals of each treatment was pooled to the final amount of 1.5 μg and subjected to the first strand cDNA synthesis using an ImProm- II^{TM} Reverse Transcription System Kit. Nevertheless, 1 μM of an arbitrary primer (OPA 02 and OPZ 09) rather than oligo d(T) was used as the synthesizing primer. The resulting product was spectrophotometrically measured.

One microgram of the first strand cDNA was used as a template for PCR amplification in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25°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™ DNA Polymerase, 1 μM each of the first arbitrary primer (used for the first strand cDNA synthesis) and the second arbitrary primer (the first primer or one of the thirty primers described previously).

PCR was performed by predenaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 36°C for 60 seconds and extension at 72°C for 90 seconds. The final extension was carried out for 7 minutes at 72°C. Five microlitres of the amplification product were electrophoretically analyzed to verify whether the amplification was successful.

Denaturating polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels (4.5%) were 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 plate sandwich was held at a 45 degree angle on the bottom corner. The acrylamide solution was then gently injected into one side of the assembled plates using 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 required, the spring clips and the sealing tapes were carefully removed. The top of the gel was rinsed with 1X TBE. The sharkstooth comb was rinsed with water.

The gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was securely clamped with the integral gel clamps along the sides of the sequencing apparatus. The upper and lower buffer chambers were filled with approximately 300 ml of 1x TBE. The sharkstooth comb was reinserted into the gel until the teeth just touched the surface of the gel. Six microlitres of the acrylamide gel loading dye (98% formamide, 200 µl EDTA, 0.25% bromophenol blue and 0.25% xylenecyanol) were loaded into each well. The gel was prerun at 30-40 W for 20 minutes.

Six microliters of the amplification products were mixed with 3 µl of the loading buffer and heated at 95°C for 5 minutes before snap cooling 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 (xylenecyanol moved out from the gel for approximately 30 minutes).

Elution of DNA from polyacrylamide gels

RAP-PCR fragments exhibiting presence/absence and/or differential expression patterns 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 ultrapure water was added and incubated overnight at 37°C. These eluted RAP products were kept at 4°C for immediate use or at -20°C for subsequently used.

Cloning and characterization of eluted RAP-PCR fragments

The eluted RAP-PCR fragments were reamplified as described previously with the exception that 0.2 µM of each primer and 0.2 µM of dNTPs were used. The amplification conditions consisted of 1 cycle of 94°C for 3 minutes, followed by 25-35 cycles additional at 94°C for 30 seconds, 36°C for 1 minute, and 72°C for 1.30 minute. The final extension was performed at 72°C for 7 minutes. The reamplified product was electrophoretically analyzed through a 1.5-1.75 % agarose gel at 7.5 volts/cm for approximately 1 hour.

The reamplified DNA fragment was excised from the agarose gel, eluted, cloned, and sequenced. The sequenced DNA fragments were BLAST against previously deposited sequences in the Genbank (http://www.ncbi.nlm.nih.gov/Genbank) using BLASTN and BLASTX. Significant similarity was considered when the E value was less than 10^{-4}.

Three primer pairs were designed from nucleotide sequences of RAP-PCR fragments using Primer Premier 5.0 program and subjected to semi-quantitative PCR against the first strand cDNA of all treated snails (Table 3.4).

Table 3.4 Sequences and melting temperature of primers and sizes of the expected amplification product of candidate stress differential expression marker from RAP-PCR analysis

Primer	Sequence (5'-3')	Tm (^{o}C)	Expected size (bp)
1. AFRAP2/228410F	GAGCAGGTGGATGAGGAAGA	55.0	310
AFRAP2/228410R	CCGATGGGAGAAGCAATAGA	50.0	
2. AFRAP9/138350F	GCACACCGACCTTTTCTAACT	56.4	203
AFRAP9/138350R	CCGTGGCTTTATTGTTTCAG	56.1	
3. AFRAP9/138410F	ACAGTAAGCCAGCAACAAAAG	55.2	184
AFRAP2/138410R	TGAATGGGACATAGTGGAGATA	54.7	

Semiquantitative RT-PCR of stress related transcripts

The standard amplification used for amplification of achacin and β-actin was initially performed PCR parameters were further adjusted to measure the amplification product semiquantitatively.

Opimization of the first strand cDNA template

The optimal concentration of the first strand cDNA template between 250, 350, 450, 550, 650, 750, 850 and 1,000 ng were examined in a 50 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1% Triton X-100, 2 mM MgCl$_2$, 100 µM of each dNTP (dATP, dGTP, dCTP and dTTP), 0.5 mM of each primer, 1 unit of DyNAzyme^{TM} II DNA Polymerase

PCR was performed in a thermal cycler (OmmiGene, Hybaid) with predenaturing at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for

30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 7 minutes. The resulting products were electrophoretically analyzed through 1.2% agarose gels. The template concentration that gave product specificity and clear results were selected for further optimization of PCR conditions.

Determination of the optimal primer concentration

The optimal primer concentration for each transcript (0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4 μM) was examined using the standard PCR condition described previously. The resulting product was electrophoretically analyzed. The primer concentration that gave product specificity and clear results were selected for further optimization of PCR conditions.

Determination of the optimal annealing temperature

The optimal annealing temperature for amplification of each transcript (calculated Tm with 1-2°C increase and decrease within the range of 3°C) was examined using the standard PCR conditions and the optimal primer and template concentrations obtained. The temperature that gave the highest specificity was chosen.

Determination of the optimal number of amplification cycles

The PCR amplifications were carried out at 22, 24, 26, 28, 30, 32 and 34 cycles using the optimal concentration of primers and template and the optimal annealing temperature. The amplification product (5 μl) was analyzed by agarose gel electrophoresis. Density of the electrophoresed bands was plotted against the number of cycles. The number of cycles that still provided the PCR product in the exponential rage (did not reach a plateau level of amplification) was chosen.

Table 3.5 PCR profiles and conditions used for semi-quantitative analysis of stress-related transcripts found from RAP-PCR analysis

Primer	cDNA template (ng)	MgCl$_2$ (mM)	Primer (µM)	PCR condition
AFRAP2/228	750	1.5	0.15	94°C, 3 min; 1 cycle followed by 94°C, 30 sec; 56°C, 60 sec and 72°C, 45 sec for 40 cycles and 72°C, 7 min; 1 cycle
AFRAP9/158-1	750	1.5	0.20	94°C, 3 min; 1 cycle followed by 94°C, 30 sec; 53°C, 60 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min; 1 cycle
AFRAP9/158-2	750	1.5	0.20	94°C, 3 min; 1 cycle followed by 94°C, 30 sec; 53°C, 60 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min; 1 cycle

Gel electrophoresis and semiquantitative analysis

The ratio between the target transcript and β-actin of each snail in different treatment of *A. fulica* was determined. Five microliters of the PCR product were analyzed by 1.2% agarose gel, which were electrophoresed at 6 volts/cm for 1 hours. The gel was stained with 1.0 μg/ml ethidium bromide for 5 minutes and destained in the running tap water for 10 minutes. The intensity of target and control bands was quantifiedly from glossy prints of the gels using the Gel-Pro Analyzer version 3.1.0 (Media Cybermatics USA).

Data analysis

The expression level of each transcript in the collar tissue of each individual of *A. fulica* was normalized by that of β-actin. Significantly different expression levels between different groups (normal, partial aestivation, and full aestivation groups) were tested using one way analysis of variance (ANOVA) and Duncan's new multiple range test.

CHAPTER IV

RESULTS

Morphometric analysis

Linear measurement of six shell characters of each snail was carried out (N = 215). Detailed information of all numerical measurement values is shown in Table 4.2. The data were standardized to eliminate possible errors, if any, before being subjected to multivariate analysis using a K-mean clustering method. Investigated *A. fulica* samples were allocated to 5 hierarchical groups on the basis of phylogenetic analysis of *A. fulica* in this study.

All investigated individuals were regarded as adults with different sizes. The average total shell length (LT), the length of aperture (LA) the spire length (LS), the width of aperture (WA) the total shell width (WT) and the diagonal distances of body whorl (LD) was 78.34, 42.96, 38.15, 22.52, 38.57, 38.57 mm. and 27.20 mm., respectively (Table 4.1).

The results of K-mean clustering showed the LT had highest *F*-ratio (951.918) following by the LS (720.570), the LD (306.993), the WT (267.903), the WA (126.652), and the LA (111.112) (Table 4.2). Notably, the *F*-ratio from this analysis should not be used for testing statistical significances because the clusters are formed to characterize differences. In addition, the K-mean method maximizes between SS and minimizes within SS resulting in high *F*-ratio value than that from the typical *F*-statistics (e.g. in one way analysis of variance). Nevertheless, relative

discrimination values (log *F*-ratio values) can be used to compare the power of different variables. Results indicated that the LT (relative discrimination value = 2.979) and the LS (2.858) were more powerful in discrimination of snail groups than the LD (2.487) and the WT (2.428) and the WA (2.103) and the LA (2.046), respectively.

Morphometric analysis did not differentiate *A. fulica* in this study according to original geographic locations. The clustering profile plots suggested comparable power of morphometric variables of the shell of *A. fulica* with the exception of large snails where the WA may a provide different point of view. The K-mean clustering analysis allocated individuals from different samples into a single cluster.

Two large (1 and 2) and medium (4 and 5) and a small cluster were obtained. The first cluster was composed of individuals from all groups. Snails from this cluster were those exhibiting variables slightly lower than the average value of each variable. The second cluster contained the highest number of members. Snails exhibiting all variables greater than the average values from all samples were allocated into this cluster. The third cluster contained only 5 members (2 individuals from Songkhla and Pattani and a single individual from Chanthaburi). All individuals were large sized snails. The fourth and the fifth clusters were composed of 54 and 22 individuals, respectively. All variables of members of the former were slightly lower than those of the first cluster whereas those of the latter were greater than variables in the second cluster, respectively (Table 4.3 and Fig. 4.1).

Table 4.1 Summary of 6 morphometric analysis of 6 shell characters of *A. fulica*. The range (1st row) and the average with the

standard variation (2nd row) of variable in each column are illustrated

Location	*N*	LT (mm.)	LS (mm.)	LA (mm.)	WA (mm.)	WT (mm)	LD (mm)
BKK	20	70.00 - 89.50	36.50 – 49.50	39.00 – 44.00	21.00 – 25.00	35.00 – 43.00	25.50 – 32.00
		80.45 ± 7.176	44.25 ± 4.26	39.25 ± 3.37	22.70 ± 1.16	36.93 ± 2.40	28.10 ± 2.09
CRB	20	64.00 – 92.00	33.00 – 51.00	33.00 – 43.00	20.00 – 27.00	34.00 – 44.50	23.50 – 33.00
		76.73 ± 8.95	42.00 ± 5.66	37.28 ± 3.22	22.28 ± 1.90	38.50 ± 2.92	26.83 ± 2.50
CTB	20	69.00 – 99.00	36.50 – 62.50	35.00 – 45.00	21.50 – 27.00	36.00 – 49.00	24.50 – 33.00
		82.75 ± 8.89	45.92 ± 6.35	40.07 ±3.18	23.70 ± 1.97	41.27 ± 4.04	28.50 ± 2.96
NRM	26	65.00 - 90.00	33.00 - 50.00	32.00 – 44.00	19.00 – 26.00	32.50 – 44.50	22.50 – 31.00
		75.36 ± 8.13	41.03 ± 5.44	37.17 ± 3.51	21.90 ± 1.61	37.19 ± 3.18	26.28 ± 2.45
KOK	20	65.00 – 88.00	33.00 – 50.00	32.00 – 44.00	19.00 – 24.50	32.50 – 42.50	22.50 – 31.00
		76.37 ± 8.04	41.62 ± 5.53	37.42 ± 3.32	21.95 ± 3.16	37.32 ± 3.16	26.47 ± 2.35
CHM	20	68.50 – 90.50	35.00 – 90.50	33.50 – 44.00	19.50 – 26.00	34.00 – 44.50	23.50 – 32.00
		76.87 ± 7.91	41.87 ± 5.49	37.47 ± 3.31	22.12 ± 1.74	37.62 ± 3.132	26.57 ± 2.56
LAP	20	68.00 – 88.00	35.00 – 50.00	33.00 – 44.00	20.00 – 25.00	31.00 – 43.00	23.00 – 31.00
		76.97 ± 7.14	41.37 ± 5.34	37.30 ± 3.20	22.325 ± 1.44	37.20 ± 12.74	26.15 ± 2.45
SKA	33	62.00 – 115.00	32.50 – 68.00	31.00 – 49.00	17.50 – 27.00	31.50 – 49.00	22.50 – 37.00
		78.24 ± 12.94	43.16 ± 8.55	38.04 ± 4.77	2.34 ± 5.49	38.28 ± 4.00	27.48 ± 3.76
PAT	25	70.00 – 108.00	36.50 – 67.00	34.00 – 48.00	21.50 – 27.00	35.50 – 51.50	24.50 – 36.00
		82.84 ± 10.40	46.04 ± 7.82	39.82 ± 3.72	24.04 ± 1.79	41.26 ± 4.50	28.62 ± 3.36
MAL	11	70.00 – 84.00	36.50 – 48.00	33.00 – 42.50	21.00 – 23.00	36.00 – 39.50	25.00 -28.00
		75.40 ± 5.34	41.22 ± 3.84	37.36 ± 2.73	21.90 ± 0.58	37.66 ± 1.18	26.13 ± 1.05
TOTAL	**215**	**62.00 – 115.00**	**32.50 – 68.00**	**31.00 – 49.00**	**17.50 – 27.00**	**31.00 – 51.50**	**22.50 – 37.00**
		78.34 ± 9.37	**42.96 ± 6.42**	**38.15 ± 3.69**	**22.52 ± 1.86**	**38.57 ± 3.71**	**27.20 ± 2.88**

Table 4.2 Statistic summary of morphometric variable used for K- mean clustering

analysis of *A. fulica* in this study

Character	Between cluster sum of squares	df	Within cluster sum of squares	df	*F*-ratio[a]
LT	17826.188	4	983.147	210	951.918
LS	8223.078	4	599.125	210	720.570
LA	1978.355	4	934.769	210	111.112
WA	528.718	4	219.166	210	126.652
WT	2462.283	4	482.525	210	267.903
LD	1517.404	4	259.496	210	306.993
Total	32536.026	24	3478.228	1260	

[a] *F*-ratio = (Between cluster SS/df)/(Within cluster SS/df)

Table 4.3 Results of K-mean cluster analysis of *A. fulica* showing distributions of the

members of each cluster across overall investigated samples

Cluster no.	*N*	Members of cluster									
		BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
1	65	8	5	5	5	4	8	7	8	8	7
2	69	9	7	6	7	7	7	7	9	6	3
3	5	-	-	1	-	-	-	-	2	2	-
4	54	1	6	2	7	8	10	6	11	2	1
5	22	2	2	6	1	1	1	-	3	6	-
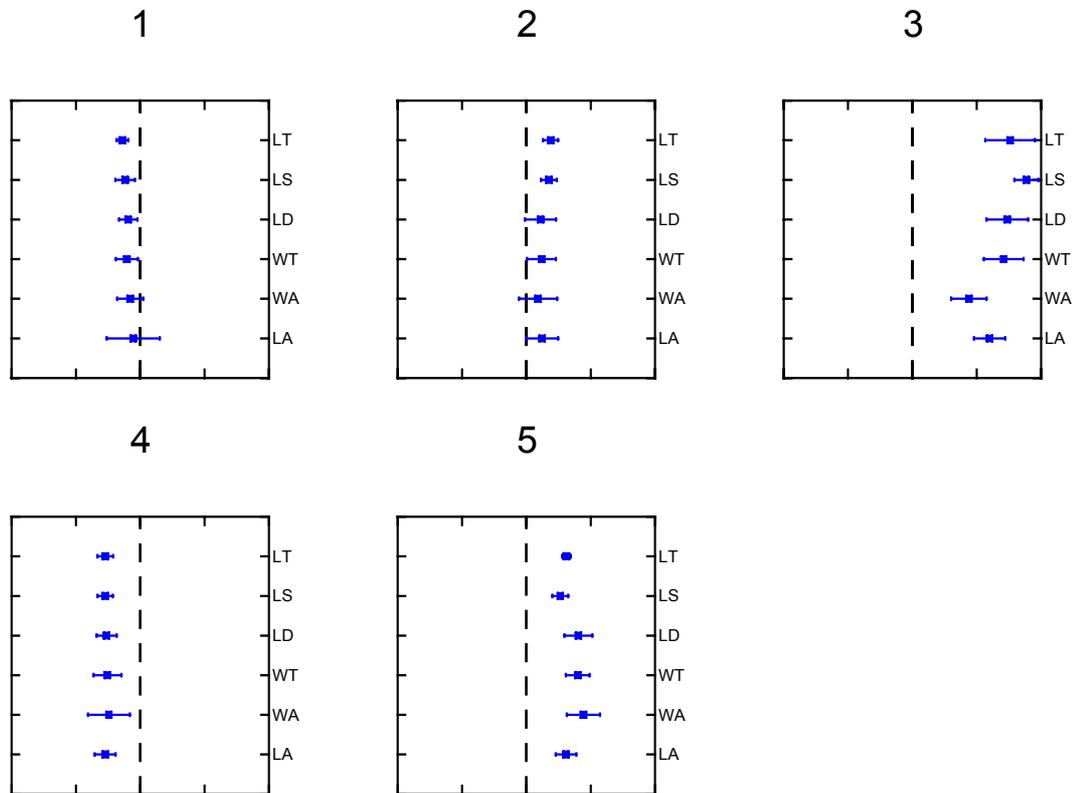
Figure 4.1 Cluster profile plots from K-mean cluster analysis of *A. fulica* in this study.

Six variable characters were plot by means ± one standard deviation. Broken line on

the middle of each cluster is average value (means) of each variable character from

shell morphometric analysis.

DNA extraction

The quality and quantity of genomic DNA extracted from the foot muscle of *A. fulica* were determined by agarose gel electrophoresis and UV spectrophotometry respectively. High molecular weight (>23.1 kb) DNA was primarily observed along with slightly sheared DNA (Fig. 4.2). An OD_{260}/OD_{280} ratio was 1.4-2.0. DNA samples showing the ratio much lower then 1.8 was possibly contaminated with residual protein or phenol. In contrast, those with the ratio greater than 2.0 may be contaminated with RNA. The former samples were further digested with proteinase K (100 μg/ml) in the presence of 0.5% SDS at 37°C for 1-2 hours before extracted once with buffer equilibrated phenol and once with chloroform-isoamyl alcohol (24 :1). Purified genomic DNA was recovered by ethanol precipitation.

PCR-RFLP analysis

Screening of primers

Six sets of primer including the universal primers for 16S rDNA and COI and a primer pair designed from nucleotide sequence of COI of the scallop (JS-1/JS-2) from mitochondrial DNA and the universal primers of the large subunit (LSU) of ribosomal DNA; LSU1/LSU3 and LSU2/LSU4 from nuclear DNA were screened for the amplification success against representative individual of *A. fulica.* The JS-1/JS-2 and 16S rDNA primers did not give the amplification product whereas LSU1/LSU3, LSU2/LSU4, 18S rDNA and COI yielded the amplification products of approximately 600 bp, 650 bp, 600 bp and 710 bp respectively (Fig. 4.2 and Table 4.1). Based on the primary assumption that mitochondrial DNA genes evolve faster than do nuclear genes, COI was selected and further tested for the amplification success using the

larger sample size. The clear amplification product (710 bp in length) was consistently observed. Neither interspecific length polymorphism nor intraspecific length heteroplasmy was found in the amplified COI gene region. Therefore the COI gene segment was chosen for population genetic studies of *A. fulica*.
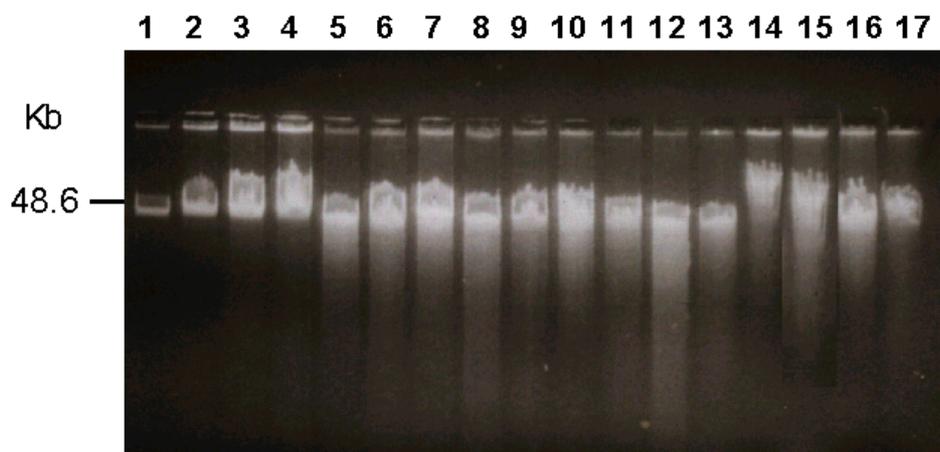
Figure 4.2 A 1.0% ethidium bromide stained agarose gel showing genomic DNA extracted from the foot muscle of *A. fulica* (lanes 5-17). Genomic DNA was electrophoretically analyzed at 100 volts for 45 minutes. Lanes 1-4 were 50, 100, 200 and 300 ng of undigested λ DNA, respectively.

Restriction analysis of the amplified COI of *A. fulica*

The amplified COI segment of individual *A. fulica* was digested with 16 restriction endonucleases including *Alu* I, *Acs* I, *Bam* HI, *Cla* I, *Dde* I, *Dra* I, *Eco* RI, *Hae* III, *Hind* III, *Kpn* I *Mbo* I, *Rsa* I, *Nde* I, *Ssp* I *Taq* I and *Vsp* I. Five restriction endonucleases (*Alu* I, *Dde* I, *Hin*d III, *Mbo* I and *Rsa* I) digested the amplified COI of *A. fulica* (Table 4.5). Nevertheless, *Mbo* I is an expensive enzyme. Additionally, *Hind* III is a hexanucleotide recognizing enzyme that may not be informative when tested across all the snails.
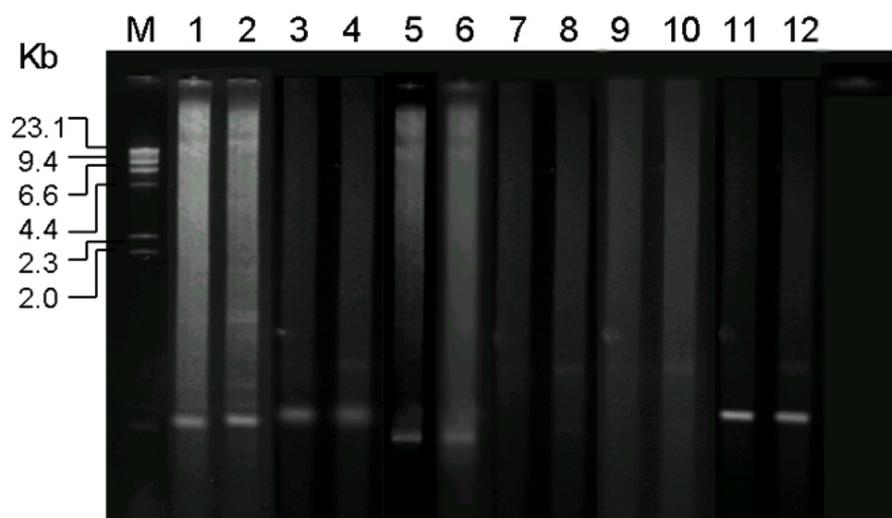
Figure 4.3 Five microliters of the PCR products resulted from amplification of genomic DNA of *A. fulica* using LSU1/LSU3 (lanes 1-2), LSU3/LSU4 (lanes 3-4), 18S rDNA (lanes 5-6), JS-1/JS-2 (lanes 7-8), 16S rDNA (lanes 9-10), and COI (lanes 11-12). The PCR products were electrophoretically analyzed through a 1.0% agarose gel. Lane M was λ-*Hin*d III marker.

Table 4.4 Screening of primers for PCR-RFLP analysis of *A. fulica*

Primer	Amplification results
LSU1/LSU3	Approximately 600 bp
LSU2/LSU4	Approximately 650 bp
18S rDNA	Approximately 600 bp fragment
JS-1/JS-2	No amplification product
16SrDNA F/R	No amplification product
COI	Approximately 710 bp fragment

As a result, only *Alu* I, *Dde* I and *Rsa* I were preliminary used for PCR-RFLP analysis of COI of each *A. fulica* (N = 65). All tested enzymes provided a monomorphic restriction profile (pattern A: 340 bp, 150 bp, 130 bp and 90 bp from *Alu* I; pattern A: 400 bp and 310 bp from *Dde* I and pattern A: 370 bp and 340 bp from *Rsa* I). Therefore, a mitotype of all snails was AAA (arranged from the restriction pattern of *Alu* I, *Dde* I and *Rsa* I, respectively) implying a lack of mtDNA polymorphism in this species based on (Table 4.5). PCR-RFLP of COI of *A. fulica* was not carried on and was replaced by a more sensitive approach.

Table 4.5 Results from digestion of the COI gene segment (710 bp) with various restriction enzymes

Restriction endonuclease	Restriction pattern (bp)
Alu I	A: 340, 150, 130 and 90
Acs I	dnc
Bam HI	dnc
Cla I	dnc
Dde I	A: 400, 310
Dra I	dnc
Eco RI	dnc
Hae III	dnc
Hind III	360, 350
Kpn I	dnc
Mbo I	A: 380, 330
Rsa I	A: 370, 340
Nde I	dnc
Ssp I	dnc
Taq I	dnc
Vsp I	Dnc

dnc = did not cut
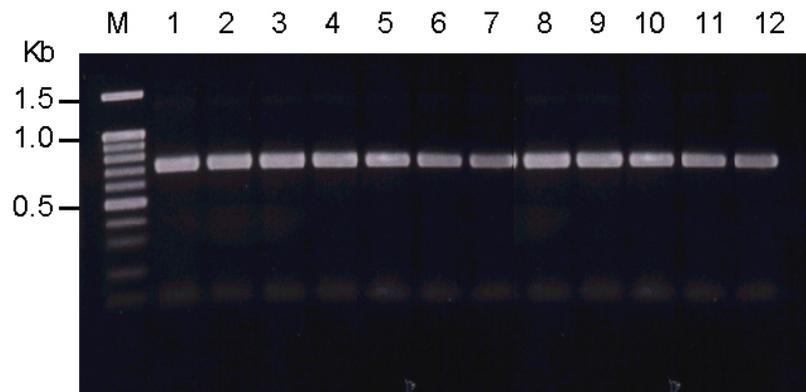
Figure 4.4 Five microliters of the amplified COI gene segment (710 bp) of *A. fulica* from Songkhla (lanes 1-3), Chiang Mai (lanes 4-6), Bangkok (lanes 7-9) and Nakhon Ratchasima (lanes 10-12) were electrophoresed through a 1.2% agarose gel and stained with ethidium bromide. A 100 bp DNA ladder (lane M) was used as a marker.
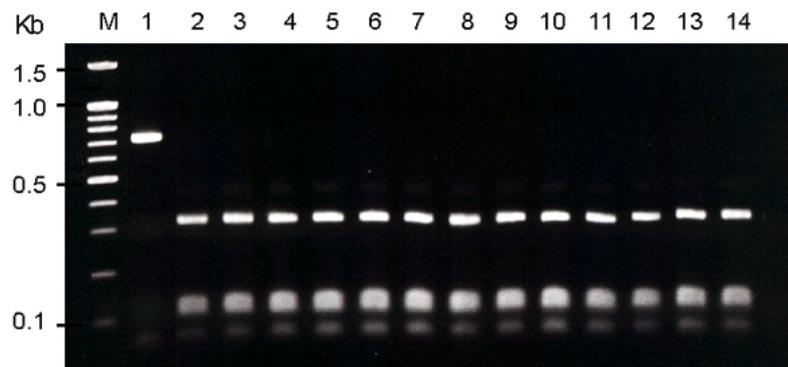
Figure 4.5 Restriction analysis of COI of *A. fulica* COI from Songkhla (lanes 2-5), Bangkok (lanes 6-9 and Chiang Mai (lanes 10-14) with *Alu* I. A monomorphic pattern (340 bp, 150 bp, 130 bp and 90 bp) was observed with this enzyme digestion. Lanes M and 1 are a 100 bp DNA marker and the undigested PCR product, respectively.
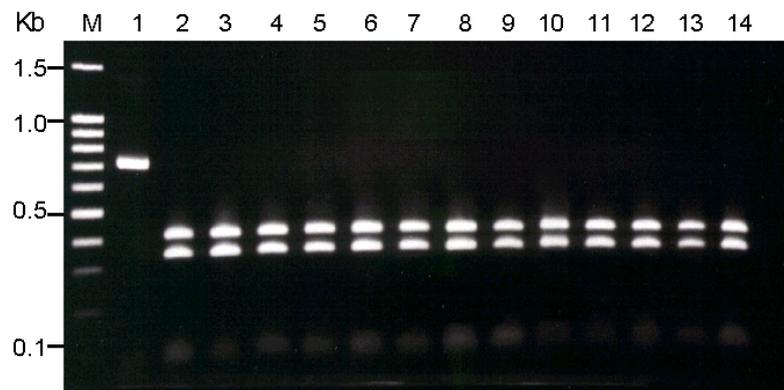
Figure 4.6 Restriction analysis of COI of *A. fulica* from Pattani (lanes 2-5), Chonburi (lanes 6-10) and Khon Kaen (lanes 10-14) with *Dde* I. A monomorphic pattern (400 bp and 310 bp) was observed with this enzyme digestion. Lanes M and 1 are a 100 bp DNA marker and the undigested PCR product, respectively.

Figure 4.7 Restriction analysis of COI of *A. fulica* COI from Chanthaburi (lanes 2-5), Bangkok (lanes 6-9) and Lampang (lanes 10-14) with *Rsa* I. A monomorphic pattern (370 bp and 340 bp) was observed with this enzyme digestion. Lanes M and 1 are a 100 bp DNA marker and the undigested PCR product, respectively.

SSCP analysis

Selected of genes

PCR-RFLP did not reveal polymorphism of the mitochondrial COI gene across all investigated individuals. One possibility was that PCR-RFLP may not be sensitive enough to detect variability of the introduced species like *A. fulica*. SSCP which is suitable to screen mutations in DNA segments was then applied. Two genes, one from the mitochondrial genome (COI) and the other from the nuclear genome (PMX), were then used.

The optimal fragment size of DNA for SSCP analysis was between 100-300 bp. The amplified PMX suits this requirement and could be directly analyzed. Nevertheless, the fragment size of COI amplified from universal primers was much greater than that required by this technique. Therefore, the amplified 710 bp COI fragment needed to be cloned and sequenced. New primers generating the appropriate fragment length could be designed. The amplification product was then subjected to the analysis.

Cloning of amplified COI and primer design

The actual size of the COI gene segment amplified using universal COI primer (LCO1490 and HCO2198 were 677 bp in length. Two pair of primers which were expected to provide the amplification product of 215 bp (AFCOI215) and 260 bp (AFCOI260 bp) was designed (Fig. 4.8).

The designed primers were tested for amplification success with representative individuals of *A. fulica*. The AFCOI215 primer gave a single band of the amplification product according to expectation (215 bp in size further called COI_{215}) without nonspecific amplification product (Figure 4.9). In contrast, AFCOI260

primers did not yield the amplification when amplified at the annealing temperature of 55°C. A touchdown PCR was applied (initial annealing at 60°C and lowering 1°C for each cycle until 55°C, see materials and methods) and successfully generated a 260 bp product from genomic DNA of the same individuals (Figures 4.10 and 4.11). Nevertheless, AFCOI215 primers consistently gave a 215 bp amplification product. The amplification success of this primer pair was 100%. This amplification COI fragment was then subjected to SSCP analysis.

Specificity of AFCOI215 primers

The important property of AFCOI215 primers was their species-specificity. The universal COI primer (LCO1490 and HCO2148) was tested and successfully amplified genomic DNA of various mollusks including the introduced golden apple snail (*Pomacea canaliculata*), 4 species of Thai native apple snails (*Pila ampullacea, Pila pesmei, Pila polita and Pila angelica*) and 3 species of Thai oysters (*Crassostrea belcheri, Crassostrea iredalei and Saccostrea cucullata*). In contrast, AFCOI215 only provided positive amplification in *A. fulica*. This indicated the species-specific nature of the developed marker.

PMX gene and primers

PMX primers were designed from cDNA of the giant tiger shrimp (*P. monodon*). This cDNA was classified as an unknown transcript containing short nucleotides specifically found in the X or Y chromosome of human. This primer pair easily amplified a 197 bp product in *A. fulica* (Figure 4.12). Like AFCOI215, PMX primers also yielded 100% amplification success in *A. fulica*. Therefore, optimal conditions for analysis of the amplified COI and PMX homologues were examined.

Optimization of SSCP condition

Although SSCP is a simple and sensitive approach for detecting point mutations in investigated DNA segments, conditions (gel concentrations, temperature and the percentage of glycerol) for operating nondenaturing electrophoresis should be optimized for consistent results. Different gel concentrations (10%, 15% and 20%), crosslink (49:1 and 37.5:1 of acrylamide: bis-acrylamide) and glycerol (0%, 5%, or 10%) were carefully tested for SSCP analysis of the amplified COI and PMX. Results indicated that 15% polyacrylamide gel (37.5:1) supplemented with 5% (COI) or 10% glycerol (PMX) gives consistent patterns. The gel was usually electrophoresed at 200 volts for 9-12 hours (COI) or 14-16 hours (PMX) at 4°C.

SSCP patterns of COI215 and PMX of each snail was examined across all individuals ($N = 207$). A total of 5 and 3 patterns were observed, respectively. Genetic distance between pairs of samples was calculated. The genetic distance estimated from COI polymorphism was 0.0000-0.0802 whereas that of PMX polymorphism was 0.0000-0.0621. The average genetic distances from both genes ranged from 0.0000-0.0716. This reflected low genetic diversity of *A. fulica* in the present study.

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AATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGG
AATTCGATTGGTCAACAAATCATAAAGATATTGGTACCTTATACATAATTTTTGGTGTATGATGTGGGATAGTT
GGCACAGGCTTGTCACTCTTAATTCGGTTAGAGCTTGGAACAGTGGGAACCTTAACTGATGATCACTTTTTCAA
CGTGGTTGTAACTGCGCATGCTTTTGTCATAATTTTTTTTATGGTTATACCAATTATAATTGGCGGATTTGGAA
ACTGGATGGTCCCAATACTTATTGGTGCTCCTGATATAAGATTTCCACGAATAAATAATATAAGGTTTTGACTT
TTACCACCTTCATTTACTTTGTTAATCTGTTCAAGTATAAGTGGAAGGAGGGGCTGGAACTGGGTGGACTGTGT
ACCCGCCCTTAAGTTCTTGCTTAGGACACAGAGGGGCTTCAGTTGATTTAGCTATTTTTTCTTTACATTTGGGC
TGGTGTATCCTCAATTTTAGGGGCAATCAATTTTATTACGACGGTGTATAATATACGAGCCTCTGGGCTAACTA
TAGAACGGGTAAGCTTATTCGTGGGGGTCAATTTTAGTTACAGTGTTCCTACTATTGGTTATCATTGCCAGTTC
TAGCGGGGGCAATTACTATACTATTAACGGATCGTAACTTCAACACATCTTTCTTTGACCCAGCTGGTGATTTT
TTGGTCACCCTGAAGTTTAAAATCCTAGAATTCGCGGCCGCCT
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Figure 4.8 Nucleotide sequence of COI fragment (710 bp) of *A. fulica* amplified using primers LCO1490 and HCO2198 (bold). Two pairs of primers were designed. Sequences of the newly designed (1ˢᵗ pair) AFCOI215 forward primer and those complementary to the AFCOI215 reverse primer are illustrated in bold-italics and underlined. Sequences of the (2ⁿᵈ pair) AFCOI260 forward primer and those complementary to the AFCOI260 reverse primer are bold and underlined.
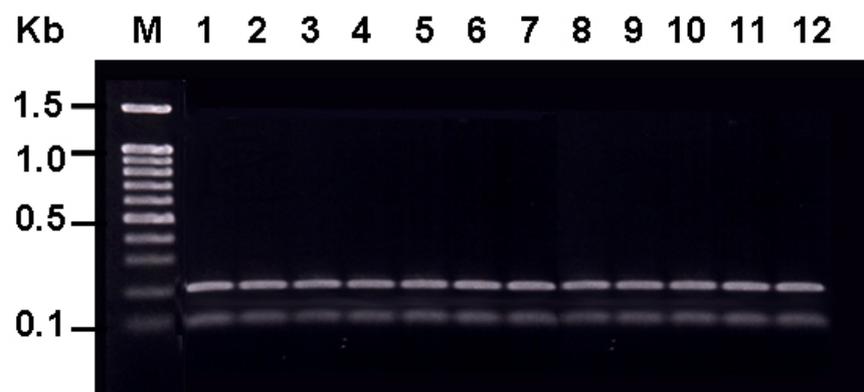
Figure 4.9 A 1.2% agarose gel illustrating the amplification product of AFCOI215 primers (215 bp) using genomic DNA of 12 snails collecting from Songkhla (lanes 1-3), Bangkok (lanes 4-6), Chiang Mai (lanes 7-9) and Khon Kaen (lanes 10-12).
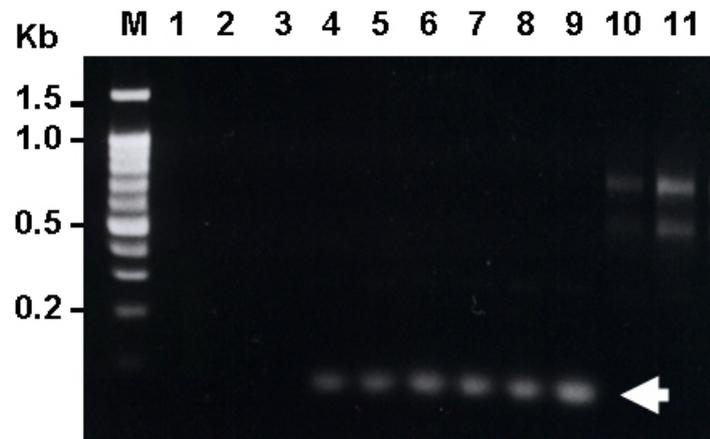
Figure 4.10 Results from amplification of genomic DNA of 11 individuals of *A. fulica* (lanes 1-11) at the annealing temperature of 55°C for 40 cycles. No amplification products were observed. Lane M is a 100 bp DNA marker. An arrow indicated the primer dimer.
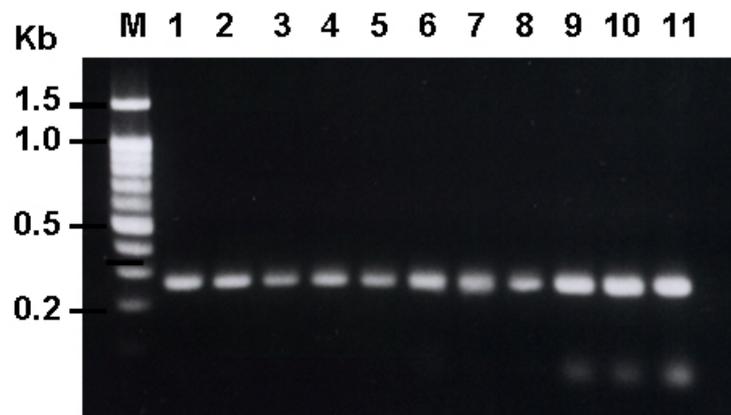
Figure 4.11 Results from amplification of genomic DNA of 11 individuals of *A. fulica* (lanes 1-11) using the touchdown PCR conditions. The expected product (260 bp) was observed in all tested individuals. A 100 bp DNA ladder (lane M) was included as a marker.
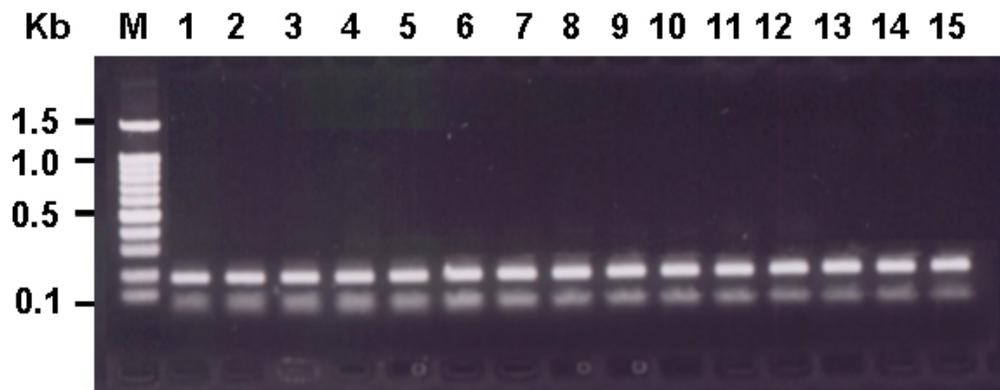
Figure 4.12 A 1.2% agarose gel illustrating the amplification product of PMX (197 bp) using genomic DNA of 12 snails collecting from Songkhla (lanes 1-5), Bangkok (lanes 6-10) and Chiang Mai (lane 11-15).
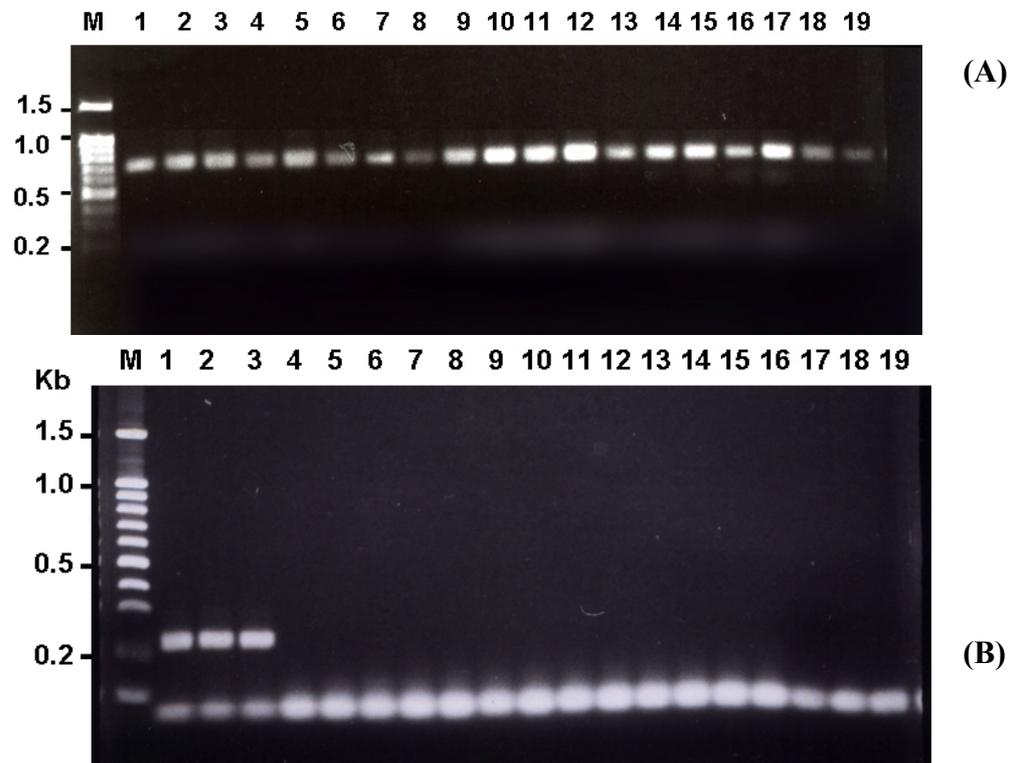
Figure 4.13 Agarose gel electrophoresis showing specificity of AFCOI215 to *A. fulica*. Genomic DNA of *A. fulica* (lanes 1-3), *Pomacea canaliculata* (lanes 4-5), *Pila ampullaceal* (lanes 6-7), *Pila pesmei* (lanes 8-9), *Pila polita* (lanes 10-11), *Pila angelica* (lanes 12-13), *Crassostrea belcheri* (lanes 14-15), *Crassostrea iredalei* (lanes 16-17) and *Saccostrea cucullata* (lanes 18-19) was amplified by PCR using LCO1490 and HCO2198 (A) and AFCOI215 (B).
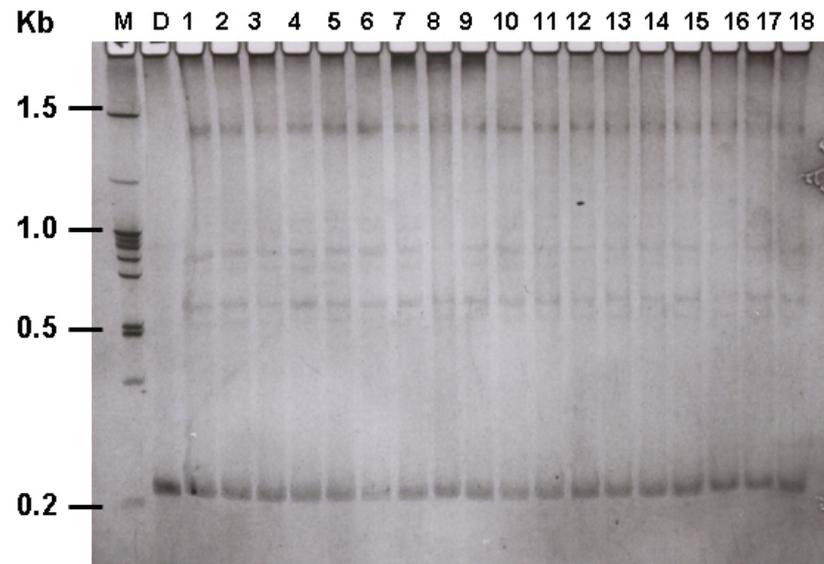
Figure 4.14 SSCP patterns of COI_{215} amplified using AFCOI215 primers electrophoresed through a 10% nondenaturing polyacrylamide gel (49:1 crosslink) and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. The gel has high background with unclear single-stranded bands.
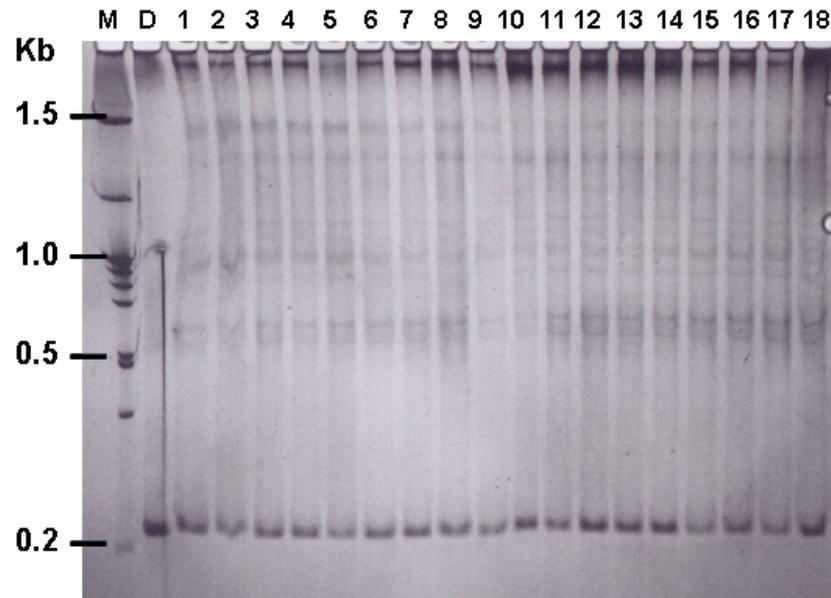
Figure 4.15 SSCP patterns of COI_{215} amplified using AFCOI215 primers electrophoresed through a 10% nondenaturing polyacrylamide gel (49:1 crosslink) supplemented with 5% glycerol and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. Single-stranded bands were unclear.
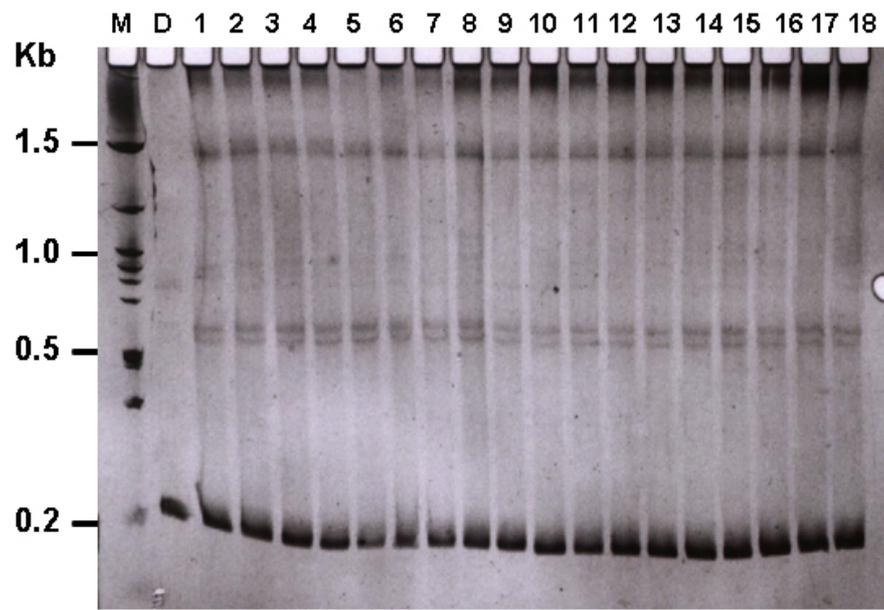
Figure 4.16 SSCP patterns of COI_{215} amplified using AFCOI215 primers electrophoresed through a 10% nondenaturing polyacrylamide gel (49: 1 crosslink) supplemented with 10% glycerol and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. Single-stranded bands were unclear.
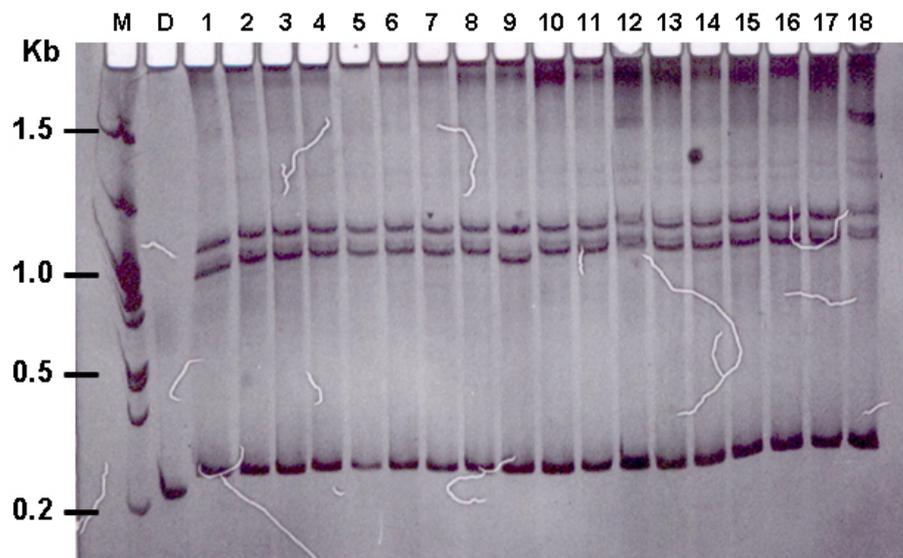
Figure 4.17 SSCP patterns of COI_{215} amplified using AFCOI215 primers electrophoresed through a 10% nondenaturing polyacrylamide gel (37.5:1 crosslink) and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. Clear single-stranded bands were observed.
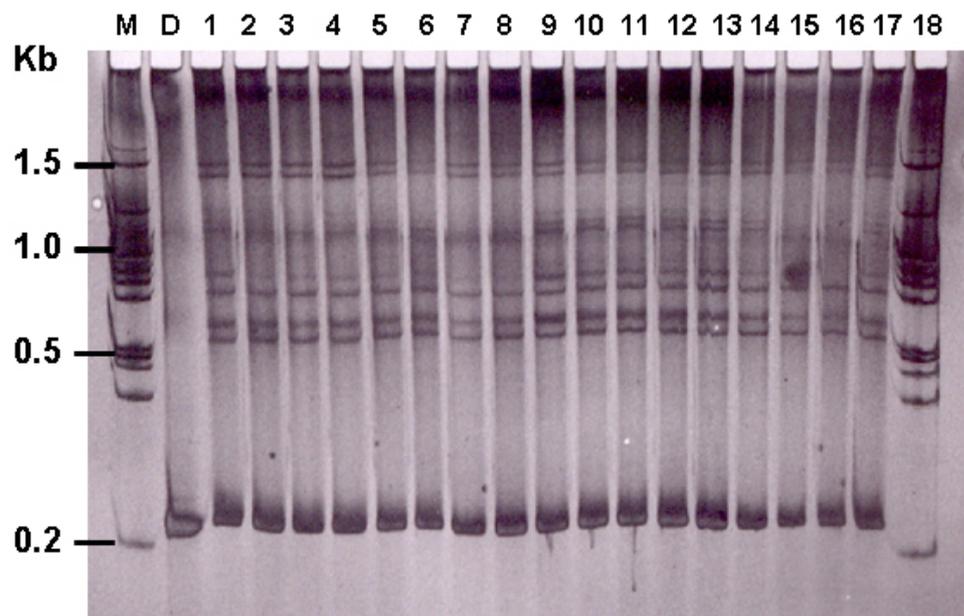
Figure 4.18 SSCP patterns of COI_{215} amplified using AFCOI215 primers electrophoresed through a 10% nondenaturing polyacrylamide gel (37.5:1 crosslink) supplemented with 5% glycerol and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. Single-stranded bands were reasonably clear.
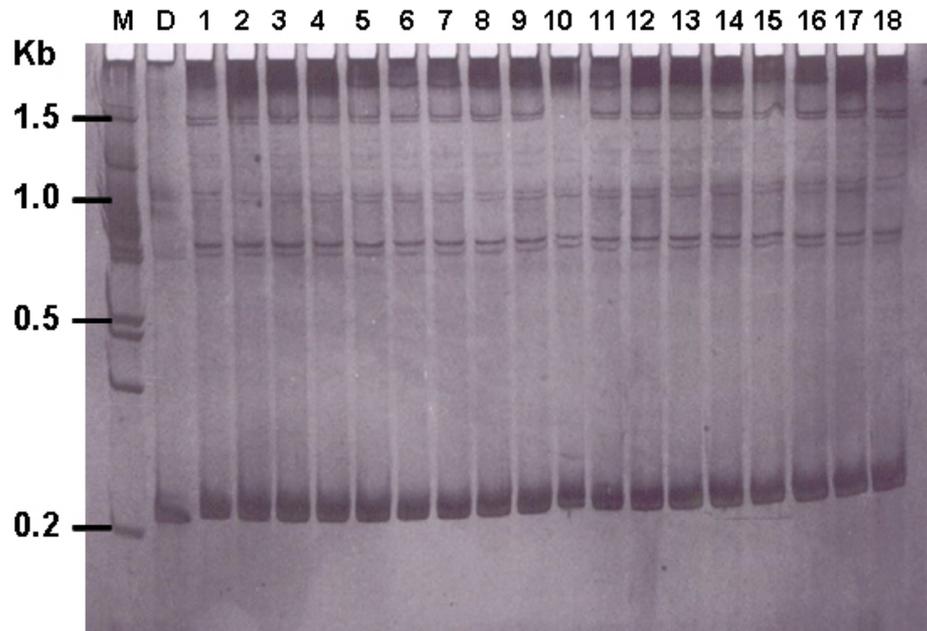
Figure 4.19 SSCP patterns of COI_{215} amplified using AFCOI215 primers electrophoresed through a 15% nondenaturing polyacrylamide gel (37.5:1 crosslink) supplemented with 5% glycerol and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. Sharp SSCP bands were consistently observed.
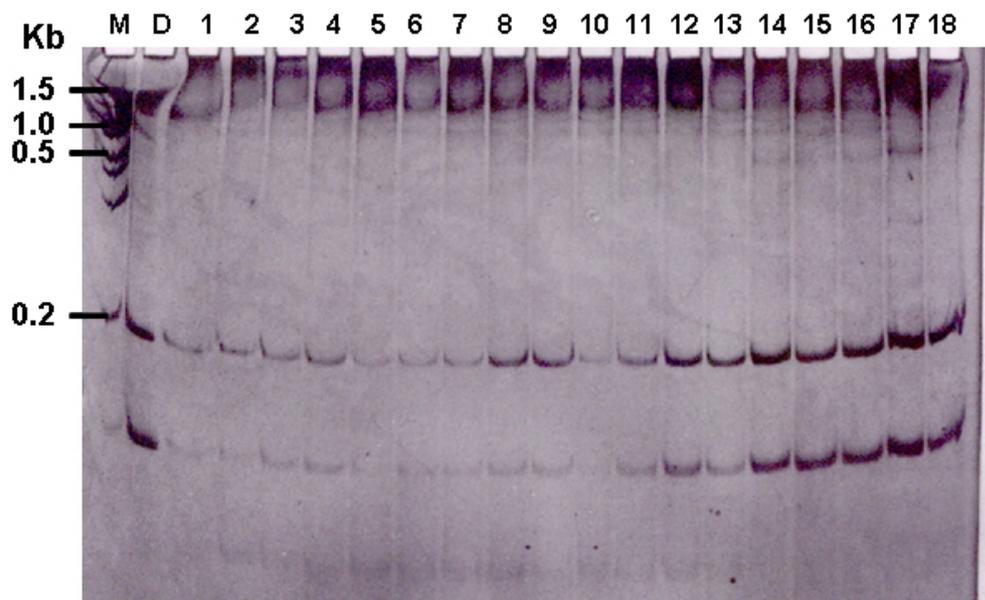
Figure 4.20 SSCP patterns of PMX (197 bp) electrophoresed through a 15% nondenaturing polyacrylamide gel (37.5:1 crosslink) and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. The SSCP bands were not well fractionated and unclear.
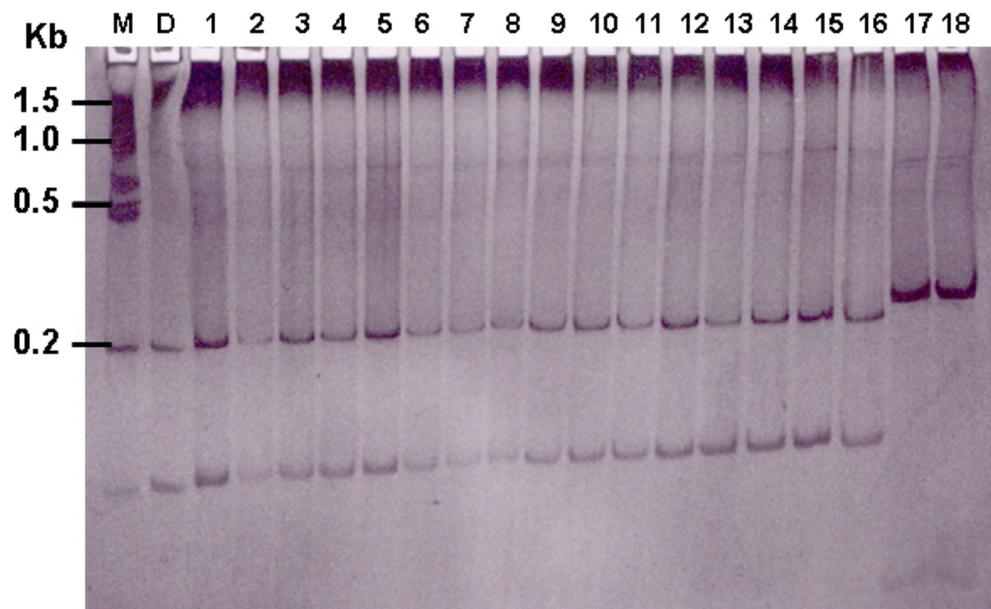
Figure 4.21 SSCP patterns of PMX (197 bp) electrophoresed through a 15% nondenaturing polyacrylamide gel (37.5:1 crosslink) supplemented with 5% glycerol and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. The SSCP bands were unclear.
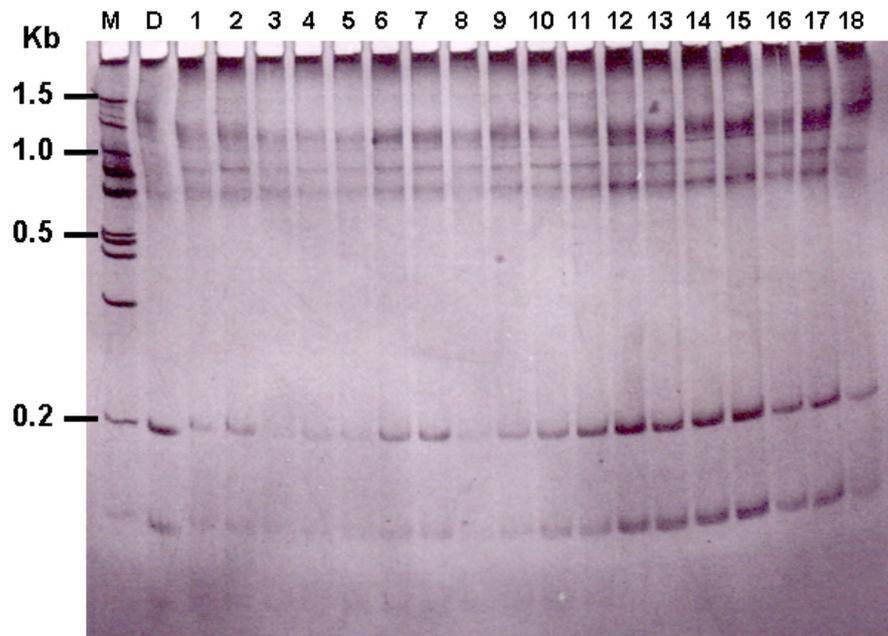
Figure 4.22 SSCP patterns of PMX (197 bp) electrophoresed through a 20% nondenaturing polyacrylamide gel (37.5:1 crosslink) and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. The SSCP bands were well separated but unclear.
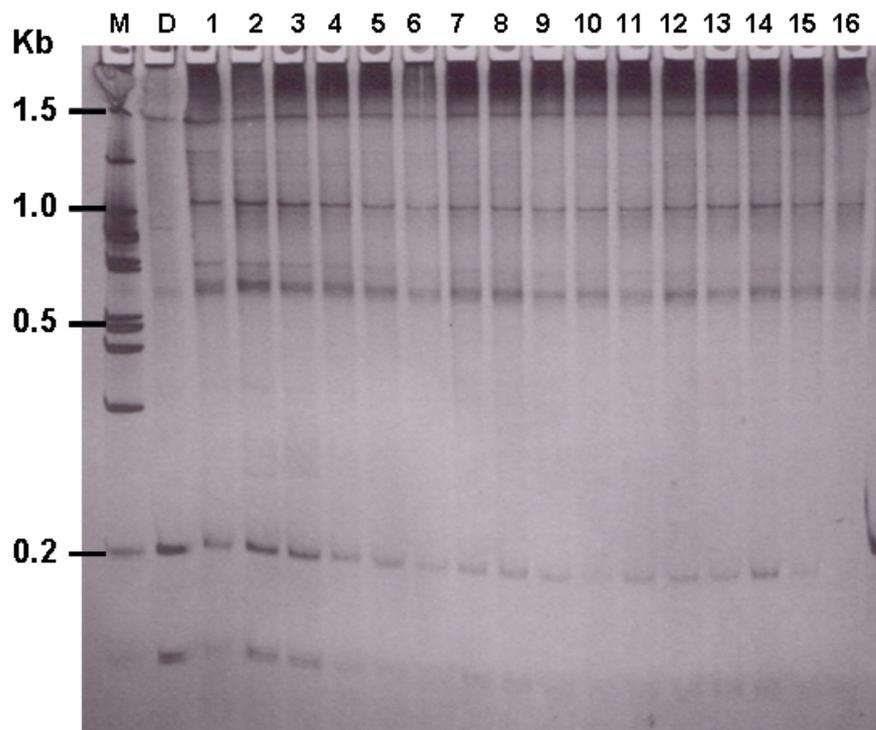
Figure 4.23 SSCP patterns of PMX (197 bp) electrophoresed through a 15% nondenaturing polyacrylamide gel (37.5:1 crosslink) supplemented with 10% glycerol and silver stained. Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively. The SSCP bands were not well separated.
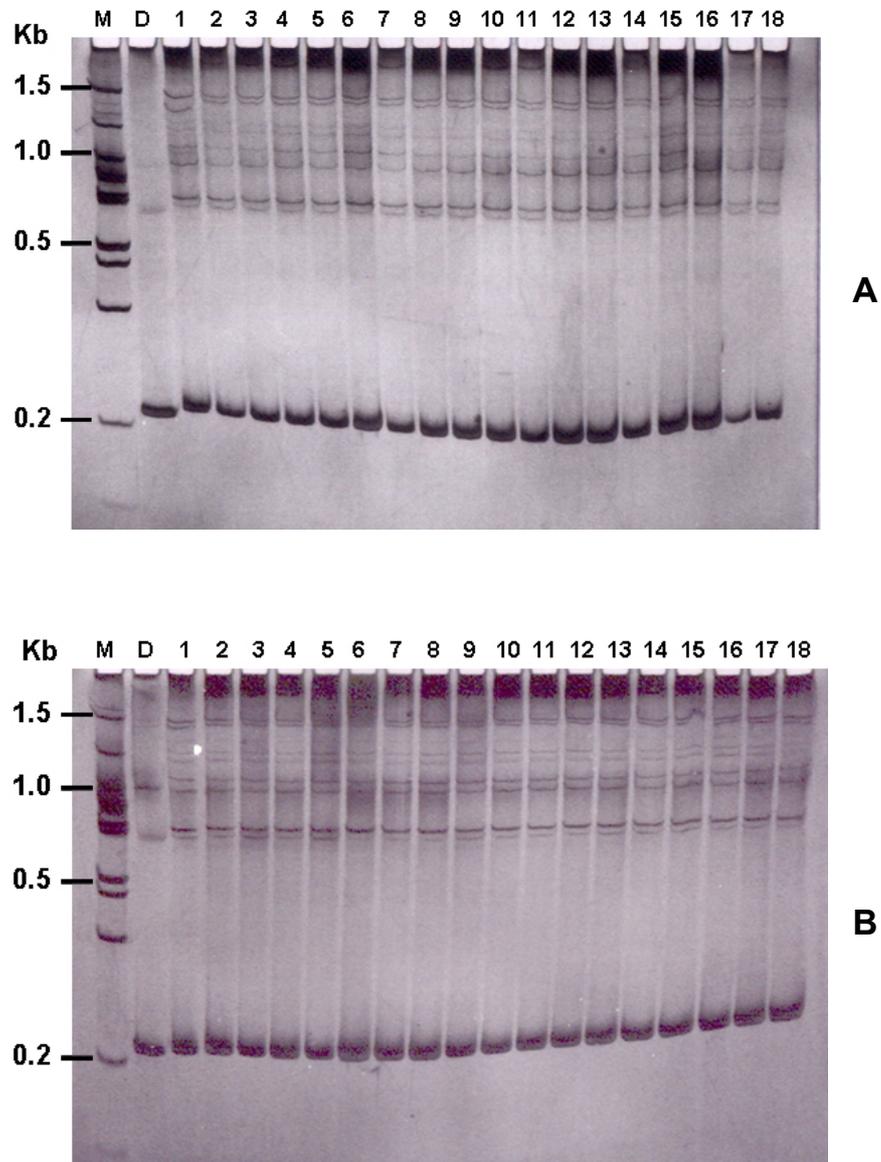
Figure 4.24 Examples of SSCP patterns of COI$_{215}$ of *A. fulica* from Songkhla (lanes 1-9, panel A), Bangkok (lanes 10-18, panel B), Chiang Mai (lanes 1-9, panel B) and Nakhon Ratchasima (lanes 10-18, panel B). Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively.
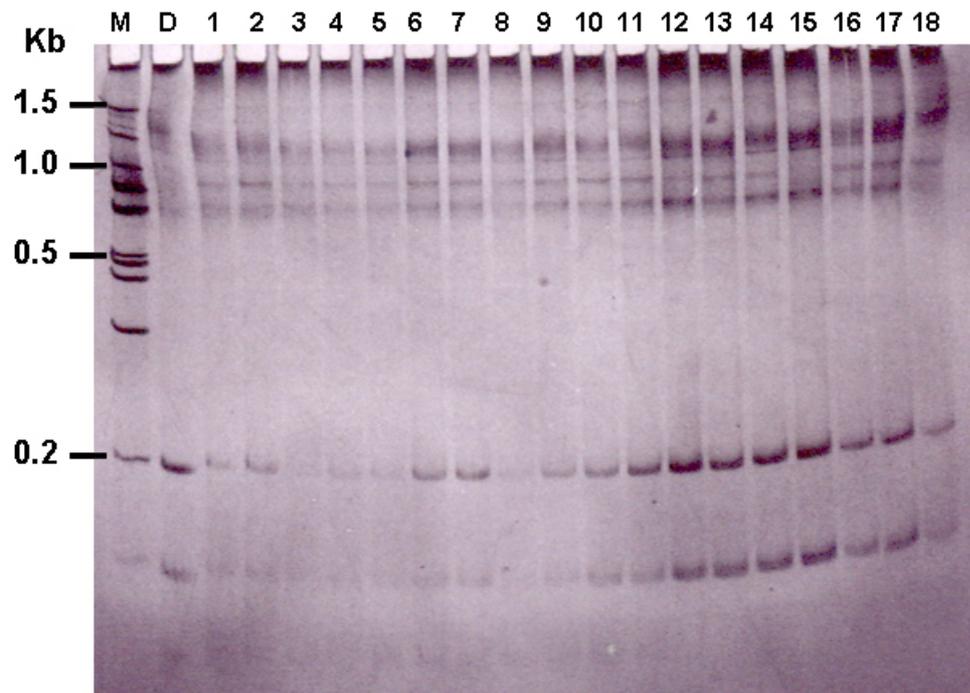
Figure 4.25 Examples of SSCP patterns of PMX (197 bp) of *A. fulica* from Pattani (lanes 1-9) and Lampang (lanes 10-18). Lane M and D are a 100 bp DNA ladder and the nondenatured PCR product, respectively.

A neighbor-joining tree constructed from the average pairwise genetic distance of COI and PMX allocated investigated samples to 3 groupings; group I (Songkhla, Pattani, Khon Kaen, Chiang Mai and Chanthaburi), group II (Bangkok and Nakhon Ratchasima) and group III (Chonburi and Lampang). As typically found in introduced species, the phylogenetic tree did not reflect genetic differentiation of *A. fulica* according to geographic origins. Moreover, the tree did not imply the possible spread of *A. fulica* in Thailand as phylogenetic influences are contradictory to the previous report.

Each SSCP band was treated as a locus and subjected to genetic heterogeneity analysis and F_{ST} statistics (θ). The former did not reveal significant differences of allele frequencies across overall individuals suggesting a lack of genetic heterogeneity of *A. fulica* ($P > 0.05$). In contrast, F_{ST} statistics (θ) revealed genetic differences of *A. fulica* at 2 loci (700 bp and 920 bp) of COI. The gene flow level at these loci was 0.9542 and 0.5895, respectively (Table 4.9).

Considering at the population level, no genetic heterogeneity was observed between pairs of sample using SSCP analysis of COI_{215}, PMX or the combine data of both gene segments (Tables 4.10 -4.12).

Table 4.6 Pairwise modified Roger's genetic distances (Wright, 1978) between *A. fulica* samples resulted from SSCP analysis of

the COI_{215} gene segment

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0248	-								
CTB	0.0497	0.0248	-							
CHM	0.0497	0.0248	0.0000	-						
LAP	0.0248	0.0000	0.0248	0.0248	-					
NRM	0.0248	0.0000	0.0248	0.0248	0.0000	-				
KOK	0.0497	0.0248	0.0000	0.0000	0.0248	0.0248	-			
SKA	0.0715	0.0467	0.0219	0.0219	0.0467	0.0467	0.0219	-		
PAT	0.0693	0.0444	0.0248	0.0248	0.0444	0.0444	0.0248	0.0080	-	
MAL	0.0583	0.0335	0.0583	0.0583	0.0335	0.0335	0.0583	0.0802	0.0779	-

Table 4.7 Pairwise modified Roger's genetic distances (Wright, 1978) between *A. fulica* samples resulted from SSCP analysis of the PMX gene segment

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0273	-								
CTB	0.0273	0.0000	-							
CHM	0.0273	0.0000	0.0000	-						
LAP	0.0273	0.0000	0.0000	0.0000	-					
NRM	0.0144	0.0129	0.0129	0.0129	0.0129	-				
KOK	0.0000	0.0273	0.0273	0.0273	0.0273	0.0144	-			
SKA	0.0240	0.0204	0.0204	0.0204	0.0204	0.0255	0.0240	-		
PAT	0.0071	0.0344	0.0344	0.0344	0.0344	0.0216	0.0071	0.0305	-	
MAL	0.0592	0.0556	0.0556	0.0556	0.0556	0.0607	0.0592	0.0352	0.0621	-

Table 4.8 Average pairwise modified Roger's genetic distances (Wright, 1978) between *A. fulica* samples resulted from SSCP analysis of the COI and PMX gene segments

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0258	-								
CTB	0.0407	0.0149	-							
CHM	0.0407	0.0149	0.0000	-						
LAP	0.0258	0.0000	0.0149	0.0149	-					
NRM	0.0207	0.0051	0.0201	0.0201	0.0051	-				
KOK	0.0298	0.0258	0.0109	0.0109	0.0258	0.0207	-			
SKA	0.0525	0.0362	0.0213	0.0213	0.0362	0.0382	0.0227	-		
PAT	0.0444	0.0404	0.0287	0.0287	0.0404	0.0353	0.0178	0.0170	-	
MAL	0.0587	0.0424	0.0573	0.0573	0.0424	0.0444	0.0587	0.0622	0.0716	-
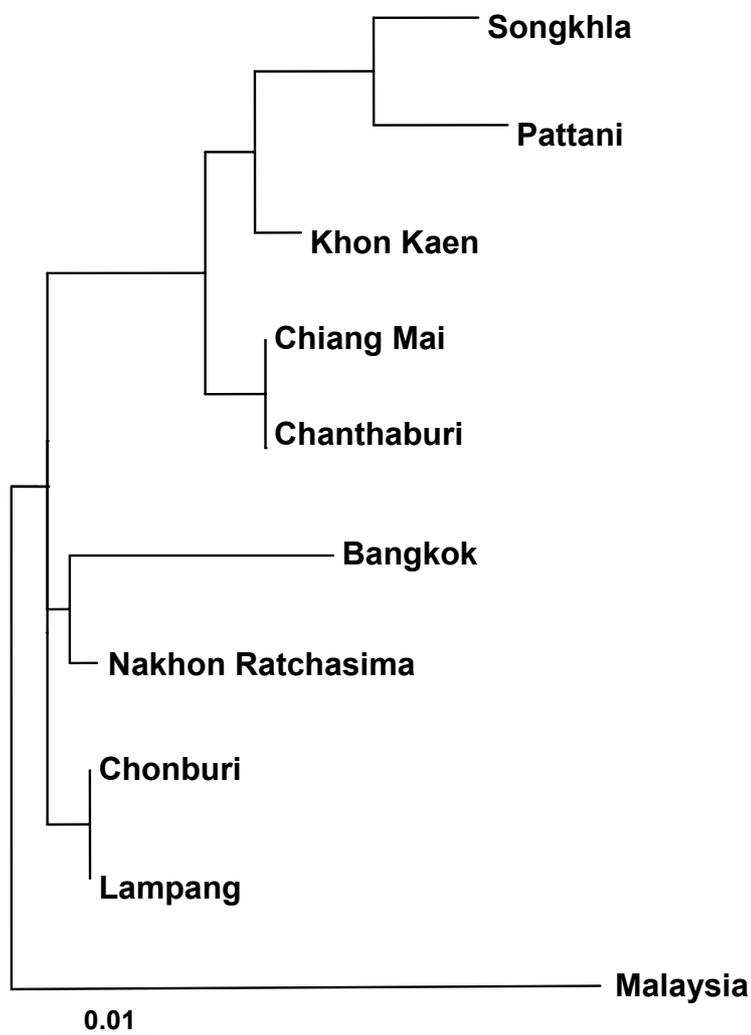
Figure 4.26 A neighbor-joining tree illustrating genetic relationships of *A. fulica* in this study. The phylogenetic tree was constructed from the average genetic distances of COI_{215} and PMX gene segments analyzed by SSCP.

Table 4.9 Genetic heterogeneity test and F_{ST} statistics (θ) of SSCP bands of COI and PMX gene segments across overall investigated individuals of *A. fulica*

Band size (bp) [a] (Locus)	Genetic heterogeneity (P-value)	F-statistics (θ)	N_em
COI$_{215}$			
600 (1)	1.0000	undefined	undefined
700 (2)	0.6576	0.2076*	0.9542
920 (3)	0.0458	0.2978*	0.5895
950 (4)	0.9058	0.1083	2.0584
1000 (5)	1.0000	undefined	undefined
1100 (6)	0.9604	0.1375	1.5682
1400 (7)	1.0000	undefined	undefined
1500 (8)	1.0000	undefined	undefined
1700 (9)	1.0000	undefined	undefined
PMX			
650 (10)	0.9996	-0.0168	very large
700 (11)	0.9570	0.0150	16.4167
800 (12)	1.0000	undefined	undefined
900 (13)	1.0000	undefined	undefined
950 (14)	1.0000	undefined	undefined
1100 (15)	1.0000	undefined	undefined

* = Frequency of SSCP bands with significant differentiation ($P < 0.01$)

[a] = Sizes appeared in nondenaturing gels (not the actual sizes)

95% CI with $P < 0.05$ for overall population = 0.0019-0.1670, Overall geographic heterogeneity for overall population is $P = 1.0000$, F-statistic (θ) for overall loci = 0.0434 resulted in an estimate overall gene flow = 5.51038 individuals per generation.

Table 4.10 Pairwise genetic heterogeneity between different samples of *A. fulica* resulted from SSCP analysis of COI_{215}

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000^{ns}	-								
CTB	1.0000^{ns}	1.0000^{ns}	-							
CHM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-						
LAP	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-					
NRM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-				
KOK	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-			
SKA	0.9999^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-		
PAT	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-	
MAL	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-

ns = not significant,   * = significant at $P < 0.05$

Table 4.11 Pairwise genetic heterogeneity between different samples of *A. fulica* resulted from SSCP analysis of the

PMX gene segment

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000^{ns}	-								
CTB	1.0000^{ns}	1.0000^{ns}	-							
CHM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-						
LAP	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-					
NRM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-				
KOK	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-			
SKA	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-		
PAT	1.0000^{ns}	0.9997^{ns}	0.9997^{ns}	0.9997^{ns}	0.9997^{ns}	0.9999^{ns}	1.0000^{ns}	0.9987^{ns}	-	
MAL	0.9999^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	0.9999^{ns}	1.0000^{ns}	1.0000^{ns}	-

ns = not significant,   * = significant at $P < 0.05$

Table 4.12 Pairwise genetic heterogeneity between different samples of *A. fulica* resulted from SSCP analysis of COI (215 bp) and PMX gene segment

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000^{ns}	-								
CTB	1.0000^{ns}	1.0000^{ns}	-							
CHM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-						
LAP	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-					
NRM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-				
KOK	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-			
SKA	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-		
PAT	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-	
MAL	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-

ns = not significant, * = significant at $P < 0.05$

RAPD analysis

Primer screening

Eighty-eight decanucleotide, seven minisatellite and six microsatellite primers were tested for the amplification success against three representative individuals of *A. fulica*. Seventy-two primers were successfully amplified in *A. fulica* genomic DNA (Table 4.13). Four primers (OPA02, OPA17, OPB11 and OPZ09) that provided consistent results were selected for further analysis using larger sample size of *A. fulica*.

RAPD patterns

Four, six, six and six RAPD patterns were observed from examination of overall snail individuals using OPA02, OPA17, OPB11, and OPZ09, respectively. A total of 117 RAPD fragments ranging from 200-1,700 bp in length were generated. The average number of RAPD bands per primer was 29.25. The number of scorable bands across investigated samples of *A. fulica* was 34, 27, 30 and 26 bands for OPA02, OPA17, OPB11 and OPZ09 respectively. A total of 72 polymorphic bands (found in less than 95% of overall investigated specimens) accounting for 61.54% were observed. Forty-five monomorphic bands (found in ≥ 95% of overall specimens) accounting for 38.46% were found. The polymorphic bands found in each respective primer were 17 bands (50%), 15 (55.56%), 21 (70%) and 19 (73.07%) respectively (Table 4.14).

RAPD polymorphism resulting from OPA02

Thirty-four scorable RAPD fragments, ranging from 220-1600 bp, were generated when analyzed overall population of *A. fulica* (*N*=207) with OPA02 (Figure

4.27). Seventeen fragments (50%), were monomorphic for which 13 bands (1,480, 1,200, 1,150, 820, 780, 720, 600, 550, 520, 450, 400, 380 and 250 bp) were found in all specimens (100%). Other 4 fragments (920, 680, 620 and 500 bp) were monomorphic and all found in 95.81% of investigated specimens, respectively. Eighteen fragments (50%) generating by this primer were polymorphic. These consisted of 1,600, 1,550, 1,500, 1,450, 1,400, 1,250, 1,050, 980, 900, 880, 650, 580, 480, 350, 320, 280 and 220 bp fragments. (Table 4.15).

Table 4.13 Primer, nucleotide sequences and the amplification success of RAPD primers initially screened in this study

Primer	Nucleotide sequence	Amplification success
UBC101	GCGCCTGGAG	+
UBC102	GGTGGGGACT	+
UBC104	GGGCAATGAT	-
UBC105	CTCGGGTGGG	-
UBC106	CGTCTGCCCG	-
UBC107	CTGTCCCTTT	-
UBC108	GTATTGCCCT	+
UBC110	TAGCCCGCTT	-
UBC112	GCTTGTGAAC	-
UBC115	TTCCGCGGGC	+
UBC116	TACGATGACG	+
UBC117	TTAGCGGTCT	+
UBC119	ATTGGGCGAT	++
UBC120	GAATTTCCCC	+
UBC128	GCATATTCCG	+
UBC129	GCGGTATAGT	-
UBC133	GGAAACCTCT	-
UBC135	AAGCTGCGAG	+
UBC137	GGTCTCTCCC	+
UBC141	ATCCTGTTCG	+
UBC145	TGTCGGTTGC	+
UBC146	ATGTGTTGCG	+
UBC149	AGCAGCGTGG	-
UBC150	GAAGGCTCTG	-
UBC153	GAGTCACGAG	++
UBC158	TAGCCGTGGC	++

Table 4.13 (continued)

Primer	Nucleotide sequence	Amplification success
UBC159	GAGCCCGTAG	++
UBC160	CGATTCAGAG	-
UBC169	ACGACGTAGG	++
UBC174	AACGGGCAGC	-
UBC175	TGGTGCTGAT	+
UBC176	CAAGGGAGGT	+
UBC177	TCAGGCAGTC	+
UBC191	CGATGGCTTT	+
UBC196	CTCCTCCCCC	+
UBC200	TCGGGATATG	++
UBC201	CTGGGGATTT	+
UBC202	GAGCACTTAC	+
UBC204	TTCGGGCCGT	+
UBC217	ACAGGTAGAC	+
UBC222	AAGCCTCCCC	++
UBC259	GGTACGTACT	-
UBC262	CGCCCCCAGT	++
UBC268	AGGCCGCTTA	-
UBC273	AATGTCGCCA	-
UBC299	TGTCAGCGGT	+
UBC428	GGCTGCGGTA	+
UBC429	AAACCTGGAC	+
UBC456	GCGGAGGTCC	+
UBC457	CGACGCCCTG	++
UBC460	ACTGACCGGC	+
OPA01	CAGGCCCTTC	++
OPA02	**TGCCGAGCTG**	+++
OPA03	AGTCAGCCAC	+

Table 4.13 (continued)

Primer	Nucleotide sequence	Amplification success
OPA04	AATCGGGCTG	+
OPA05	AGGGGTCTTG	+
OPA06	GGTCCCTGAC	++
OPA07	GAAACGGGTG	++
OPA08	GTGACGTAGG	++
OPA09	GGGTAACGCC	++
OPA10	GTGATCGCAG	++
OPA11	CAATCGCCGT	++
OPA12	TCGGCGATAG	++
OPA13	CAGCACCCAC	+
OPA14	TCTGTGCTGG	++
OPA15	TTCCGAACCC	++
OPA16	AGCCAGCGAA	+
OPA17	**GACCGCTTGT**	+++
OPA18	AGGTGACCGT	++
OPA19	CAAACGTCGG	+
OPA20	GTTGCGATCC	++
OPB01	GTTTCGCTCC	++
OPB02	TGATCCCTGG	++
OPB03	CATCCCCCTG	++
OPB04	GGACTGGAGT	+
OPB05	TGCGCCCTTC	++
OPB06	TGCTCTGCCC	++
OPB07	GGTGACGCAG	++
OPB08	GTCCACACGG	++
OPB09	TGGGGGACTC	+
OPB10	CTGCTGGGAC	++
OPB11	**GTAGACCCGT**	+++

Table 4.13 (continued)

Primer	Nucleotide sequence	Amplification success
OPB12	CCTTGACGCA	++
OPB13	TTCCCCCGCT	++
OPB14	TCCGCTCTGG	+
OPB15	GGAGGGTGTT	++
OPM09	GTCTTGCGGA	+
OPZ09	**CACCCCAGTC**	+++
Minisatellite set		
INS	ACAGGGGTGTGGGG	-
YNZ22	CTCTGGGTGTCGTGC	-
M13	GAGGGTGGNGGNTCT	-
YN73	CCCGTGGGGCCGCCG	-
PER I	GACNGGNACNGG	-
HRU18	ACCCGGCGCTTATTAGAG	-
HRU33	CCCAAGGTCCCCAAGGTCAGGGAGGCG	-
Microsatellite set		
$(CA)_8$	CACACACACACACACA	-
$(CT)_9$	CTCTCTCTCTCTCTCT	-
$(CAC)_8$	CACCACCACCAC CACCACCACCAC	-
$(GTG)_8$	GTGGTGGTGGTG GTGGTGGTGGTG	-
$(GATA)_4$	GATAGATAGATAGATA	-
$(GACA)_4$	GACAGACAGACAGACA	-

- = amplification was not successful.

+ = amplification was successful but the amplification bands were faint or complex.

++ = amplification was successful. The amplification bands were intense but primers

were not selected for population genetic studies.

+++ = amplification was successful. The amplification bands were intense and

primers were selected for further population genetic studies.
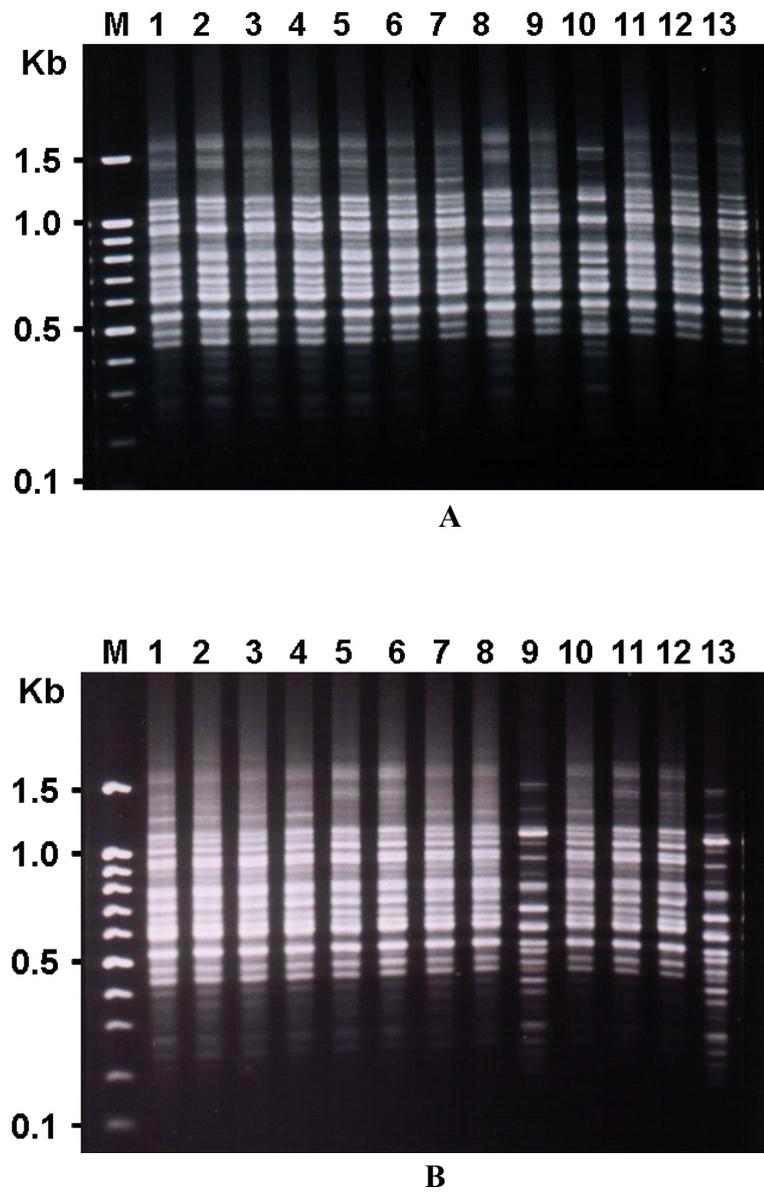
Figure 4.27 Examples of RAPD pattern of A. *fulica* from Songkhla (lanes 1-7; panel

A), Nakhon Ratchasima (lanes 8-13; panel A), Bangkok (lanes 1-8; panel B) and

Pattani (lanes 9-13; panel B) using the primer OPA02. Lanes M are a 100 bp marker.
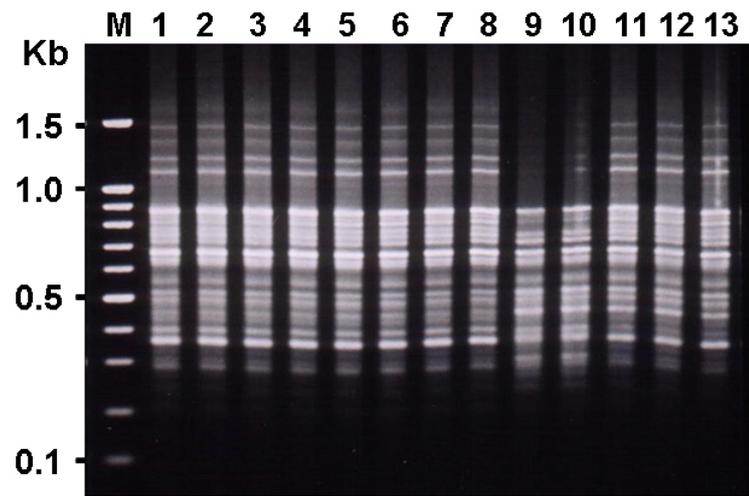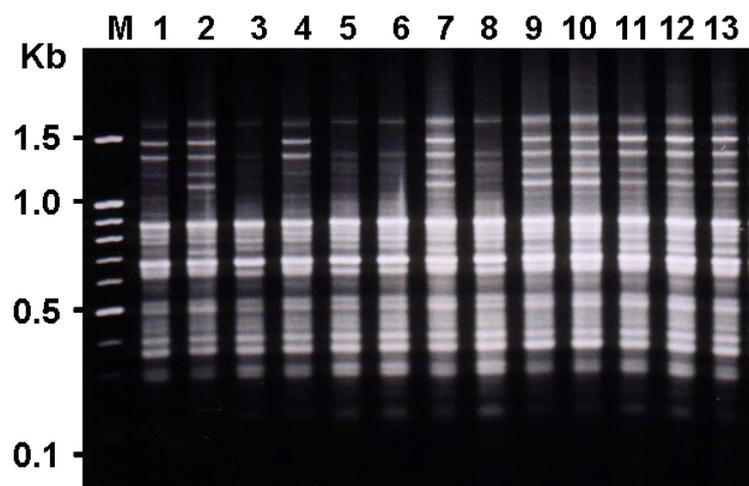
Figure 4.28 Examples of RAPD patterns of A. *fulica* from Chiang Mai (lanes 1-8; panel A), Chonburi (lanes 9-13; panel A), Malaysia (lanes 1-6; panel B) and Khon Kaen (lanes 9-13; panel B) using the primer OPA17. Lanes M are a 100 bp ladder.
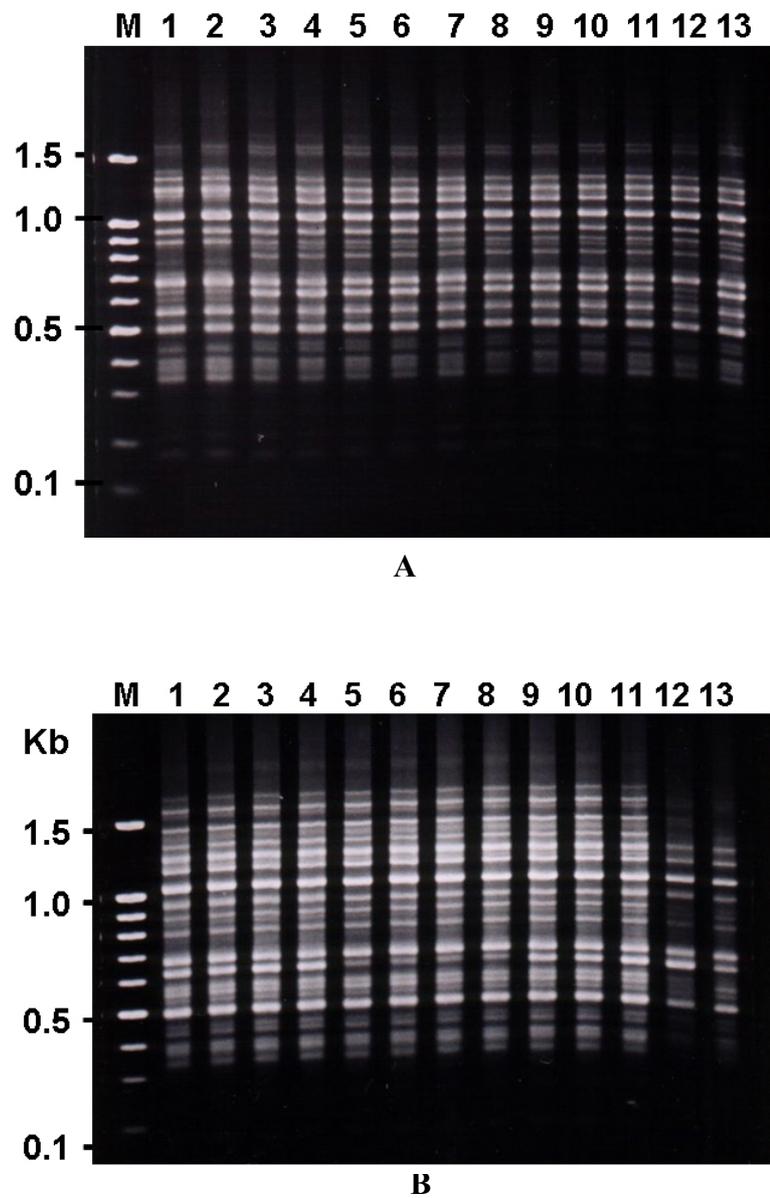
Figure 4.29 Examples of RAPD patterns of *A. fulica* from Pattani (lanes 1-7; panel A),

Bangkok (lanes 8-13; panel A), Songkhla (lanes 1-7; panel B) and Lampang (lanes 9-

13; panel B) using the primer OPB11. Lanes M are a 100 bp ladder.
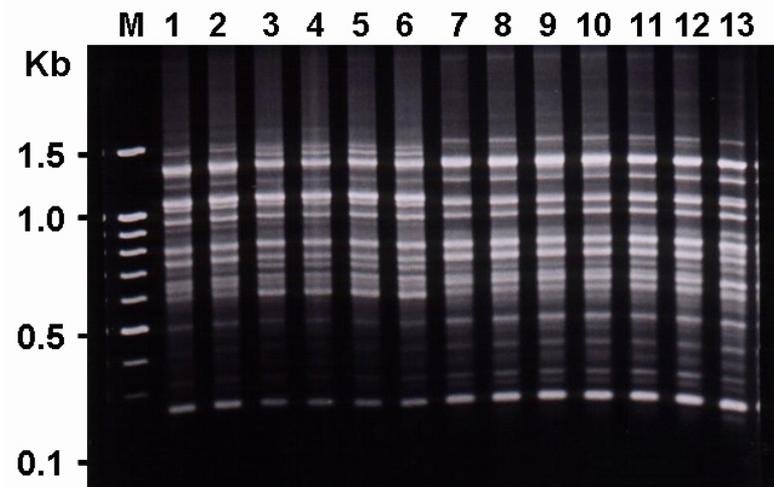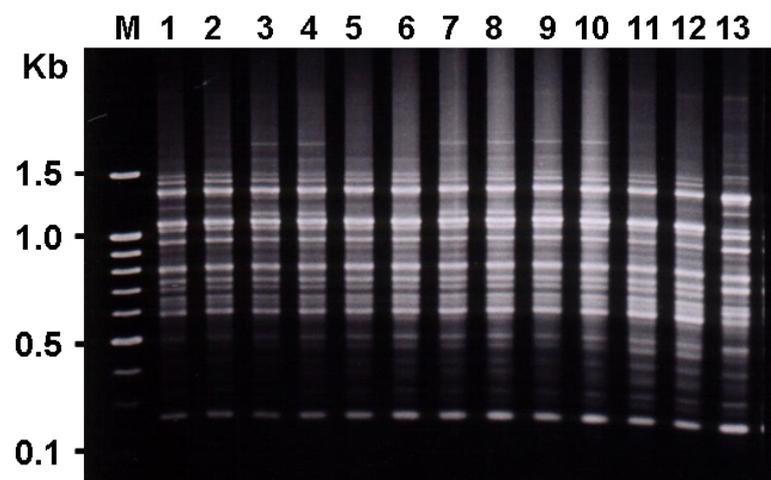
Figure 4.30 Examples of RAPD patterns of A. *fulica* from Songkhla (lanes 1-6; panel A), Bangkok (lanes 7-13; panel A), Chanthaburi (lanes 1-7; panel B) and Chiang Mai (lanes 8-13; panel B) using the primer OPZ09. Lanes M are a 100 bp ladder.

RAPD polymorphism resulting from OPA17

Twenty-seven scorable RAPD fragments ranging from 250-1600 bp were generated from amplification of genomic DNA of *A. fulica* (N = 207) with OPA17 (Figure 4.28). Twelve fragments accounting for 44.44% of overall bands were monomorphic, ten of which (880, 780, 680, 620, 500, 420, 400, 350, 300 and 280 bp) were fixed in all investigated specimens (100%). The remaining two fragments (700 and 520 bp) were monomorphic and found in 97.21% of overall specimens for both fragments. Fifteen fragments (1,600, 1,480, 1,400, 1,50, 1,100, 1,000, 980, 920, 820, 800, 720, 550, 480, 320 and 250 bp) accounting for 55.56% of overall RAPD bands generated by OPA17 were polymorphic (Table 4.16).

RAPD polymorphism resulting from OPB11

OPB11 gave thirty scorable RAPD fragments ranging from 200-1,700 bp in length (Fig. 4.29). Nine fragments (30%) were monomorphic, four of which (1,250, 950, 560 and 500 bp) were found in overall snail specimen (100%). The remaining five fragments (1,300, 1,050, 620, 400 and 380 bp) were all found in 96.74% of investigated specimens. A total of 21 polymorphic fragments (1,700, 1,600, 1,550, 1,450, 1,400, 1,200, 1,150, 1,100, 880, 850, 800, 700, 680, 600, 580, 520, 460, 420, 350, 250 and 200 bp) accounting for 70% of scored fragments were polymorphic (Table 4.17).

RAPD patterns of OPZ09

Twenty-six scorable RAPD fragments ranging from 250-1,700 bp were generated from amplification of genomic DNA of *A. fulica* with this primer (Figure 4.30). Seven fragments composing of 1,400, 950, 620, 600 and 250 bp in length accounting for 29.92% of overall fragments were monomorphic, five of which were

fixed when overall specimens were genetically examined. The remaining two fragments with five fragments (750 and 680 bp) were found in 96.74% of overall samples. Nineteen polymorphic fragments. (1,600, 1,500, 1,480, 1,420, 1,250, 1,100, 1,000, 900, 850, 800, 700, 550, 450, 400, 350, 320, and 300 bp in length) accounting for 73.07% of all fragments were observed (Table 4.18).

Table 4.14 Sequences of RAPD primers, size and number of amplified bands and the percentage of polymorphic and monomorphic bands results from RAPD analysis of *A. fulica* in Thailand using RAPD primers OPA02, OPA17, OPB11 and OPZ09.

Primers	Sequences	Size-range (bp)	No. of RAPD bands	Polymorphic bands (%)	Monomorphic bands (%)
OPA02	TGCCGAGCTG	220-1,600	34	17 (50.00%)	17 (50.00%)
OPA17	GACCGCTTGT	250-1,600	27	15 (55.56%)	12 (44.44%)
OPB11	GTAGACCCGT	200-1,700	30	21 (70.00%)	9 (30.00%)
OPZ09	CACCCCAGTC	250-1,700	26	19 (73.07%)	7 (29.92%)
Total		**200-1,700**	**117**	**72 (61.54%)**	**45 (39.46%)**

*RAPD bands found in less than 95% of all specimens were considered to be polymorphic bands.

Table 4.15 Frequencies of 34 RAPD bands generated from OPA02 found in 10 different samples of *A. fulica*

Size (bp)	Frequencies (%)									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
220	55.00	55.00	55.00	50.00	40.00	60.87	35.00	58.62	50.00	45.45
250	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
280	50.00	45.00	45.00	50.00	60.00	43.48	70.00	51.72	58.33	54.55
320	85.00	95.00	95.00	85.00	95.00	82.61	85.00	79.31	75.00	90.91
350	65.00	60.00	60.00	65.00	45.00	73.91	45.00	68.97	66.67	54.55
380	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
400	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
450	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
480	65.00	60.00	60.00	65.00	45.00	73.91	45.00	66.97	66.67	54.56
500	95.00	100.00	100.00	100.00	100.00	95.65	95.00	89.66	91.67	100.00
520	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
550	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
580	5.00	0.00	0.00	0.00	0.00	4.35	5.00	10.34	8.33	0.00
600	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 4.15 (continued)

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
620	95.00	100.00	100.00	100.00	100.00	95.65	95.00	89.66	91.67	100.00
650	5.00	0.00	0.00	0.00	0.00	4.35	5.00	10.34	8.33	0.00
680	95.00	100.00	100.00	100.00	100.00	95.65	95.00	89.66	91.67	100.00
720	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
780	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
820	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
880	85.00	95.00	95.00	85.00	95.00	82.61	85.00	79.31	75.00	90.91
900	85.00	95.00	95.00	85.00	95.00	82.61	85.00	79.31	75.00	90.91
920	95.00	100.00	100.00	100.00	100.00	95.65	95.00	89.66	91.67	100.00
980	50.00	55.00	55.00	50.00	40.00	56.52	30	48.28	41.67	45.45
1,050	65.00	60.00	60.00	65.00	45.00	73.91	45.00	68.97	66.67	54.55
1,150	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1,200	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1,250	55.00	55.00	55.00	50.00	40.00	60.87	35	58.62	58.33	54.55

Table 4.15 (continued)

Size (bp)	Frequencies (%)									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kean	Songkhla	Pattani	Malaysia
1,400	60.00	60.00	60.00	65.00	45.00	69.57	40.00	58.62	58.33	54.55
1,450	85.00	95.00	95.00	85.00	95.00	82.61	85.00	79.31	75.00	90.91
1,480	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1,500	35.00	40.00	40.00	35.00	55.00	26.09	55.00	31.03	33.33	45.45
1,550	50.00	55.00	55.00	50.00	40.00	56.52	30.00	48.28	41.67	45.45
1,600	35.00	40.00	40.00	35.00	55.00	25.09	55.00	31.03	33.33	45.45

Table 4.16 Frequencies of 27 RAPD bands generated from OPA17 found in 10 different samples of *A. fulica*

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
250	5.00	10.00	10.00	10.00	0.00	8.70	5.00	24.14	20.33	9.10
280	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
300	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
320	50.00	55.00	65.00	45.00	35.00	60.87	50.00	51.72	54.17	54.50
350	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
400	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
420	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
480	25.00	25.00	30.00	25.00	15.00	34.78	20.00	37.93	33.33	27.30
500	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
520	100.00	100.00	100.00	100.00	100.00	91.30	100.00	96.55	95.83	90.90
550	50.00	55.00	65.00	45.00	35.00	69.57	50.00	55.17	58.33	63.60
620	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 4.16 (continued)

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
680	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
700	100.00	100.00	100.00	100.00	100.00	91.30	100.00	96.56	95.83	90.9
720	50.00	55.00	65.00	45.00	35.00	60.87	50.00	51.72	54.17	54.50
780	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
800	50.00	55.00	65.00	45.00	35.00	69.57	50.00	55.17	58.33	63.60
820	50.00	55.00	65.00	45.00	35.00	69.57	50.00	55.17	58.33	63.60
880	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
920	50.00	55.00	65.00	45.00	35.00	69.57	50.00	44.83	50.00	63.60
980	50.00	55.00	65.00	45.00	35.00	69.57	50.00	44.83	50.00	63.60
1,000	50.00	55.00	65.00	45.00	35.00	69.57	50.00	44.83	50.00	63.60
1,100	70.00	60.00	65.00	70.00	80.00	47.83	65.00	55.17	50.00	45.50
1,250	70.00	60.00	65.00	70.00	80.00	47.83	65.00	55.17	50.00	45.50
1,400	100.00	100.00	100.00	100.00	100.00	91.30	100.00	86.21	87.50	90.90
1,480	70.00	60.00	55.00	70.00	80.00	47.83	65.00	55.17	50.00	45.50
1,600	75.00	70.00	65.00	80.00	80.00	56.52	70.00	68.97	62.50	54.50

Table 4.17 Frequencies of 34 RAPD bands generated from OPB11 found in 10 different samples of *A. fulica*

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
200	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.34	8.33	0.00
250	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.34	8.33	0.00
350	100.00	100.00	100.00	100.00	100.00	100.00	100.00	75.86	79.17	100.00
380	100.00	100.00	100.00	100.00	100.00	100.00	100.00	86.21	87.50	100.00
400	100.00	100.00	100.00	100.00	100.00	100.00	100.00	86.21	91.67	100.00
420	100.00	100.00	100.00	100.00	100.00	100.00	100.00	75.86	79.17	100.00
460	60.00	60.00	60.00	45.00	55.00	65.00	65.00	48.28	54.17	45.45
500	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
520	80.00	75.00	75.00	75.00	65.00	78.26	70.00	75.86	75.00	54.56
560	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
580	80.00	75.00	75.00	75.00	65.00	78.26	70.00	72.41	70.83	54.56
600	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.34	8.33	0.00

Table 4.17 (continued)

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
620	100.00	100.00	100.00	100.00	100.00	100.00	100.00	89.66	91.57	100.00
680	80.00	75.00	75.00	75.00	65.00	78.26	70.00	86.21	91.57	100.00
700	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.34	8.33	0.00
800	80.00	75.00	75.00	75.00	65.00	78.25	70.00	75.86	75.00	54.56
850	80.00	75.00	75.00	75.00	65.00	78.25	70.00	62.07	75.00	54.56
880	100.00	100.00	100.00	100.00	100.00	100.00	100.00	75.86	79.17	100.00
950	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1,050	100.00	100.00	100.00	100.00	100.00	100.00	100.00	89.66	91.67	100.00
1,100	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.34	8.33	0.00
1,150	80.00	75.00	75.00	75.00	65.00	78.25	70.00	75.86	75.00	54.56
1,200	80.00	75.00	75.00	75.00	65.00	78.25	70.00	62.07	62.50	54.55
1,250	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 4.17 (continued)

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
1,300	100.00	100.00	100.00	100.00	100.00	100.00	100.00	89.66	91.57	100.00
1,400	100.00	100.00	100.00	100.00	100.00	100.00	100.00	75.86	79.17	100.00
1,450	60.00	60.00	60.00	45.00	55.00	52.17	60.00	37.96	37.50	45.45
1,550	60.00	60.00	60.00	45.00	55.00	66.22	65.00	48.28	54.17	45.45
1,600	80.00	85.00	85.00	70.00	90.00	86.95	95.00	62.07	70.83	90.91
1,700	80.00	75.00	75.00	75.00	65.00	78.26	70.00	62.07	62.50	54.56

Table 4.18 Frequencies of 26 RAPD bands generated from OPZ09 found in 10 different samples of *A. fulica*

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
250	100.00	100.00	100.00	100.00	10a0.00	100.00	100.00	100.00	100.00	100.00
300	50.00	35.00	35.00	40.00	45.00	39.13	40.00	27.59	45.83	63.64
320	100.00	90.00	95.00	100.00	100.00	91.30	95.00	82.76	83.33	90.91
350	50.00	35.00	35.00	40.00	45.00	39.13	40.00	27.59	45.83	63.64
400	50.00	45.00	40.00	40.00	45.00	47.83	45.00	34.48	54.17	63.64
450	75.00	60.00	55.00	65.00	70.00	60.67	70.00	58.62	70.83	100.00
500	100.00	90.00	95.00	100.00	100.00	91.30	95.00	93.10	91.67	100.00
550	75.00	60.00	55.00	65.00	70.00	60.87	70.00	48.28	62.50	90.91
600	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
620	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
680	100.00	100.00	100.00	100.00	100.00	100.00	100.00	89.66	91.67	90.91
700	100.00	90.00	95.00	90.00	100.00	91.20	95	86.21	83.33	100.00

Table 4.18 (continued)

Size (bp)	Frequencies									
	Bangkok	Chonburi	Chantha buri	Chiang Mai	Lampang	Nakhon Ratcha sima	Khon Kaen	Songkhla	Pattani	Malaysia
750	100.00	100.00	100.00	100.00	100.00	100.00	100.00	89.66	91.67	90.91
800	75.00	75.00	80.00	75.00	75.00	78.26	70.00	58.62	75.00	63.64
850	75.00	60.00	55.00	65.00	70.00	60.87	70.00	58.62	70.83	100.00
900	50.00	65.00	65.00	60.00	55.00	60.87	60.00	72.41	54.17	36.36
950	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1,000	50.00	55.00	60.00	60.00	55.00	52.17	55.00	48.28	29.17	27.27
1,250	100.00	90.00	95.00	100.00	100.000	52.17	55.00	65.52	45.83	36.36
1,400	100.00	100.00	100.00	100.00	100.00	91.30	95.00	93.10	91.67	100.00
1,420	50.00	55.00	60.00	50.00	55.00	100.00	100.00	100.00	100.00	100.00
1,480	100.00	100.00	100.00	90.00	100.00	100.00	100.00	82.76	83.33	90.91
1,500	75.00	60.00	55.00	75.00	70.00	60.87	70.00	55.17	70.83	90.91
1,600	25.00	25.00	20.00	25.00	25.00	21.74	30.00	20.69	16.67	27.27
1,700	25.00	25.00	20.00	25.00	25.00	21.74	30.00	20.69	16.67	27.27

Genetic distance between different samples of *A. fulica* analyzed by RAPD analysis

Genetic distances between different samples of *A. fulica* resulted from RAPD analysis using primers OPA02, OPA17, OPB11 and OPZ09 were calculated using the modified Roger's method (Wright, 1978).

Pairwise genetic distance between *A. fulica* samples from OPA02 ranged from 0.0117-0.1004 (Table 4.19). The lowest genetic distance was 0.0117 found between the Chonburi and the Chanthaburi samples. The greatest genetic distance was 0.1004 found *A. fulica* collected from Nakhon Ratchasima and Pattani

Low genetic distances were found between different *A. fulica* samples (0.0113-0.1512) using primer OPA17 (Table 4.20). The lowest genetic distance was found between samples from Bangkok and Khon Kaen (0.0113). The Nakhon Ratchasima and Lanpang samples exhibited the highest genetic distance (0.1512) analyzed by this primer.

OPB11 showed greater genetic distance among pairs of sample than did OPA02 and OPA17. Genetic distances estimated from RAPD patterns of this primer ranged from 0.0237-0.2439 (Table 4.21). The lowest genetic distance was 0.0237 found between the the Chonburi and the Songkhla. The greatest genetic distance was found between *A. fulica* from Songkhla and Chanthaburi (0.2439). Large genetic distances were found between *A. fulica* from Songkhla and each of the remaining samples (0.1368-0.2439).

Genetic distances between *A. fulica* samples using primer OPZ09 ranged from 0.0152-0.2177 (Table 4.22). The giant African snail from Chonburi and Lampang showed the lowest genetic distance by this informative primer (0.0152). The greatest

genetic distance (0.2177) was found between the Songkhla and the Chiang Mai samples. Like the results from OPB11, large genetic distances were found between *A. fulica* from Songkhla and each of the remaining samples (0.1177-0.2177).

Table 4.19 Pairwise modified Roger's genetic distances (Wright, 1978) between different samples of *A. fulica* resulted from RAPD analysis using OPA02

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0611	-								
CTB	0.0557	0.0117	-							
CHM	0.0405	0.0293	0.0176	-						
LAP	0.0837	0.0226	0.0344	0.0519	-					
NRM	0.0661	0.0654	0.0537	0.0361	0.0881	-				
KOK	0.0743	0.0517	0.0634	0.0592	0.0291	0.0847	-			
SKA	0.0269	0.0730	0.0719	0.0551	0.0847	0.0770	0.0752	-		
PAT	0.0348	0.0901	0.0905	0.0753	0.0986	0.1004	0.0880	0.0243	-	
MAL	0.0572	0.0144	0.0261	0.0254	0.0263	0.0615	0.0373	0.0608	0.0787	-

Table 4.20 Pairwise modified Roger's genetic distances (Wright, 1978) between different samples of *A. fulica* resulting from RAPD analysis using OPA17

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0246	-								
CTB	0.0872	0.0647	-							
CHM	0.0159	0.0190	0.0752	-						
LAP	0.0455	0.0680	0.1327	0.0575	-					
NRM	0.1057	0.0831	0.0602	0.0938	0.1512	-				
KOK	0.0113	0.0172	0.0819	0.0272	0.0508	0.1004	-			
SKA	0.0575	0.0596	0.1170	0.0683	0.0849	0.0729	0.0540	-		
PAT	0.0595	0.0483	0.0901	0.0602	0.1018	0.0559	0.0542	0.0293	-	
MAL	0.0880	0.0660	0.0762	0.0803	0.1335	0.0202	0.0827	0.0585	0.0414	-

Table 4.21 Pairwise modified Roger's genetic distances (Wright, 1978) between different samples of *A. fulica* resulting from RAPD analysis using OPB11

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0264	-								
CTB	0.0502	0.0237	-							
CHM	0.0283	0.0548	0.0785	-						
LAP	0.0467	0.0692	0.0929	0.0392	-					
NRM	0.0345	0.0569	0.0807	0.0272	0.0136	-				
KOK	0.0303	0.0417	0.0654	0.0305	0.0332	0.0240	-			
SKA	0.1998	0.2201	0.2439	0.1816	0.1810	0.1849	0.2038	-		
PAT	0.1302	0.1566	0.1803	0.1108	0.1256	0.1242	0.1379	0.1368	-	
MAL	0.0760	0.0984	0.1222	0.0550	0.0293	0.0415	0.0616	0.1872	0.1233	-

Table 4.22 Pairwise modified Roger's genetic distances (Wright, 1978) between different samples of *A. fulica* resulting from RAPD analysis using OPZ09

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0361	-								
CTB	0.0861	0.0939	-							
CHM	0.0463	0.0824	0.1224	-						
LAP	0.0393	0.0152	0.1012	0.0796	-					
NRM	0.0659	0.0677	0.1168	0.1055	0.0684	-				
KOK	0.0442	0.0765	0.0558	0.0748	0.0736	0.0855	-			
SKA	0.1774	0.1643	0.1066	0.2177	0.1724	0.1265	0.1429	-		
PAT	0.0609	0.0828	0.0936	0.1028	0.0884	0.0232	0.0842	0.1177	-	
MAL	0.1412	0.1773	0.1728	0.1092	0.1770	0.1096	0.1549	0.1889	0.1000	-

Table 4.23 Average pairwise modified Roger's genetic distances (Wright, 1978) between different samples of *A. fulica* resulting from RAPD analysis using four primers OPA02, OPA17, OPB11, and OPZ09.

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0412	-								
CTB	0.0674	0.0474	-							
CHM	0.0317	0.0469	0.0698	-						
LAP	0.0573	0.0423	0.0869	0.0561	-					
NRM	0.0815	0.0801	0.0890	0.0745	0.0875	-				
KOK	0.0410	0.0472	0.0653	0.0474	0.0464	0.0863	-			
SKA	0.1138	0.1311	0.1378	0.1283	0.1279	0.1229	0.1213	-		
PAT	0.0743	0.0993	0.1118	0.0882	0.1078	0.0786	0.0956	0.0805	-	
MAL	0.0865	0.0857	0.0949	0.0643	0.0854	0.0691	0.0814	0.1232	0.0837	-

The average genetic distance across all primers ranged from 0.0317 (Bangkok and Chiang Mai) to 0.1378 (Chanthaburi and Songkhla) implying low levels of genetic differentiation of this species (Table 4.23).

The average genetic distances across all RAPD primers were subjected to phylogenetic reconstruction using a neighbor-joining (NJ) approach. The topology of the N-J tree did not differentiate *A. fulica* samples according to geographic locations but implied possible routes of the introduction of *A. fulica* from Malaysia to Thailand.

The phylogenetic relationship of *A. fulica* based on RAPD analysis showed that the Nakhon Ratchasima sample exhibited phylogenetically close relationship with *A. fulica* from Malaysia. As a result, it was possible that the limited number of *A. fulica* from Malaysia may have been directly transferred to Nakhon Ratchasima anthropologically.

Phylogenetic relationships of the remaining samples give a clear distribution of *A. fulica* in Thailand. The giant African snail may have introduced to peninsular Thailand (Songkhla and Pattani) before being anthropologically transferred to other places. Based on the NJ tree constructed from RAPD data, *A. fulica* from peninsular Thailand may have been transferred to Bangkok and Chiang Mai. Subsequently, the giant African snail may have introduced to other places from either Bangkok or Chiang Mai.

For dominant markers generated from RAPD, a particular fragment is treated as a locus. Genetic heterogeneity of the introduced *A. fulica* was examined using the exact test. *F*-statistics (θ) were also calculated for subsequent estimation of the gene flow at different RAPD loci.

Primers OPA02 did not yield fragments exhibiting genetic heterogeneity across all samples ($P>0.05$). On the other hand, one fragment (locus) from OPA17 (1,400 bp), fourteen fragments (loci) from OPB11 (1,400, 1,300, 1,100, 1,050, 880, 700, 620, 600, 420, 400, 380 and 350, 250 and 200 bp), and four loci from OPZ09 (1,250, 700, 500 and 320 bp) revealed genetic heterogeneity in all investigated samples (Table 4.24).

Disregarding non-significant loci, low (<1) and moderate (<5 exchanged individuals per generation) gene flow levels were observed at those loci. Therefore, these RAPD loci can be used for population genetic studies of *A. fulica*.

At the population level, no genetic heterogeneity was found from RAPD analysis using OPA01 and OPA17. Nevertheless, results from OPB11 revealed significant genetic differences between *A. fulica* from Songkhla and each of the remaining samples ($P<0.05$). Two comparisons from RAPD analysis using OPZ09 (Songkhla-Chiang Mai and Songkhla-Lampang) were also genetically significant differences ($P<0.05$). These circumstances support that *A. fulica* may have first been introduced to Songkhla. This sample presently displays the control diversity of *A. fulica* in Thailand. Results also indicated that RAPD analysis is more powerful than PCR-RFLP (COI) and SSCP (COI and PMX) analyses.
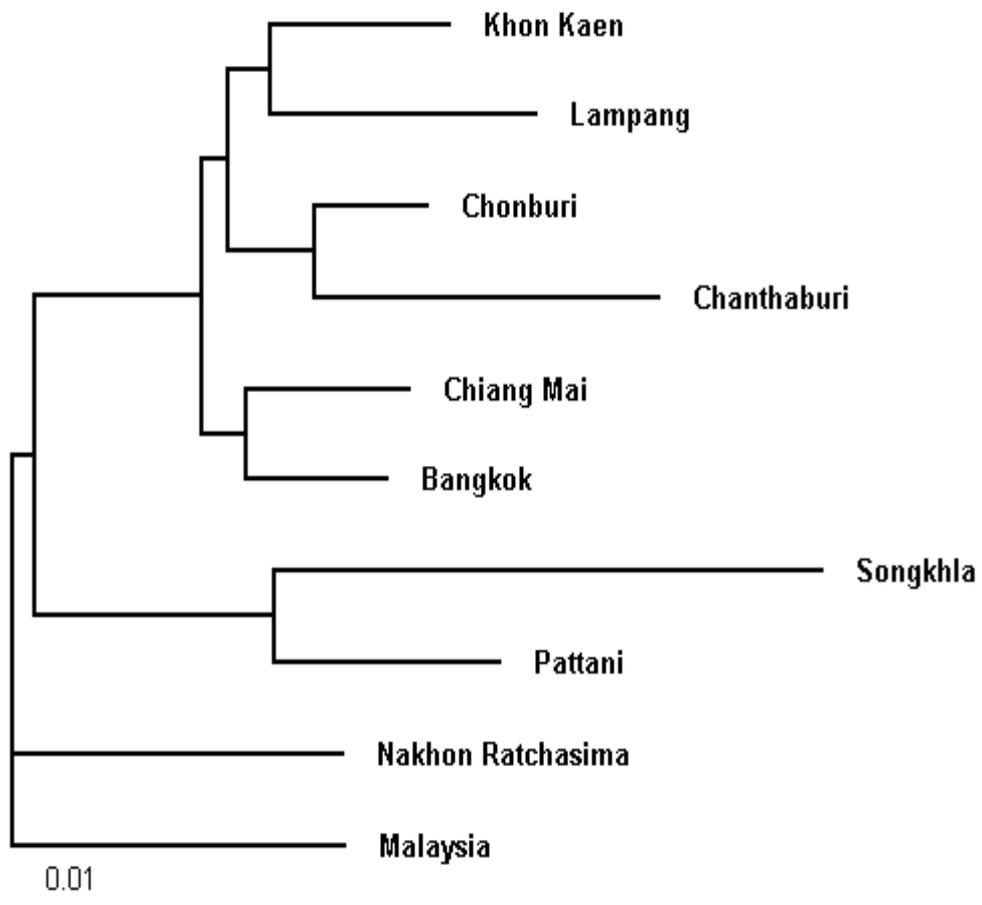
Figure 4.31 A neighbor-joining tree illustrating genetic relationships of 10 samples of *A. fulica*. The phylogenetic tree was constructed from the average modified Roger's genetic distances resulting from RAPD analysis using OPA02, OPA17, OPB11, and OPZ09 primers.

Table 4.24 Population differentiation of *A. fulica* resulted from RAPD analysis using four primers OPA02, OPA17, OPB11 and OPZ09. An analysis using geographic heterogeneity (exact test) and *F*-statistic

Size (bp)	Geographic heterogeneity (*P*-value)	*F*-statistic (θ)	N_em
OPA 02			
1,600 (1)	0.4768	0.0026	95.9038
1,550 (2)	0.5496	0.0011	227.0227
1,500 (3)	0.4040	0.0026	95.9038
1,480 (4)	1.0000	undefined	undefined
1,450 (5)	0.4626	0.0278	8.7422
1,400 (6)	0.5526	0.0044	56.5681
1,250 (7)	0.6500	-.0020	very large
1,200 (8)	1.0000	undefined	undefined
1,150 (9)	1.0000	undefined	undefined
1,050 (10)	0.4920	0.0063	39.4321
980 (11)	0.6046	0.0011	227.0227
920 (12)	0.5040	0.1683	1.2354
900 (13)	0.4716	0.0278	8.7428
880 (14)	0.4626	0.0278	8.7428
820 (15)	1.0000	undefined	undefined
780 (16)	1.0000	undefined	Undefined
720 (17)	1.0000	Undefined	Undefined
680 (18)	0.5398	0.1683	1.2354
650 (19)	0.5292	0.0017	146.8088
620 (20)	0.5366	0.1683	1.2354
600 (21)	1.0000	Undefined	undefined
580 (22)	0.5484	0.0017	146.8088
550 (23)	1.0000	Undefined	undefined

Table 4.24 (continued)

Size (bp)	Geographic heterogeneity (*P*-value)	*F*-statistic (θ)	N_em
520 (24)	1.0000	undefined	undefined
500 (25)	0.5276	0.1683	1.2354
480 (26)	0.4882	0.0063	39.4325
450 (27)	1.0000	undefined	undefined
400 (28)	1.0000	undefined	undefined
380 (29)	1.0000	undefined	undefined
350 (30)	0.5130	0.0063	39.4325
320 (31)	0.3988	0.0278	8.7428
280 (32)	0.5280	0.0014	178.3246
250 (33)	1.0000	undefined	undefined
220 (34)	0.7324	-0.0020	very large
OPA 17			
1,600 (35)	0.7610	-0.0009	very large
1,480 (36)	0.1974	0.0168	14.6309
1,400 (37)	**0.0402***	**0.2459***	**0.7666**
1,250 (38)	0.3566	0.0131	18.8339
1,100 (39)	0.3756	0.0131	18.8339
1,000 (40)	0.1606	0.0180	13.6388
980 (41)	0.2416	0.0180	13.6388
920 (42)	0.2400	0.0180	13.6388
880 (43)	1.0000	undefined	undefined
820 (44)	0.2924	0.0123	276.96
800 (45)	0.2904	0.0123	276.96
780 (46)	1.0000	undefined	undefined
720 (47)	0.3920	0.0093	26.6331
700 (48)	0.3858	0.1711	1.2111

Table 4.24 (continued)

Size (bp)	Geographic heterogeneity (*P*-value)	*F*-statistic (θ)	N_em
680 (49)	1.0000	undefined	undefined
620 (50)	1.0000	undefined	undefined
550 (51)	0.3150	0.0123	20.0752
520 (52)	0.1512	0.1942	1.2111
500 (53)	1.0000	undefined	undefined
480 (54)	0.6216	-0.0042	very large
420 (55)	1.0000	undefined	undefined
400 (56)	1.0000	undefined	undefined
350 (57)	1.0000	undefined	undefined
320 (58)	0.3414	0.0093	26.6333
300 (59)	0.8544	0.1876	1.0826
280 (60)	1.0000	undefined	undefined
250 (61)	0.2310	0.0066	37.6287
OPB 11			
1,700 (62)	0.1472	0.0304	7.9736
1,600 (63)	0.0478*	0.0543	4.3540
1,550 (64)	0.2660	0.0170	14.4558
1,450 (65)	0.0722	0.0240	10.1666
1,400 (66)	**< 0.0001***	**0.4159***	**0.3511**
1,300 (67)	**< 0.0001***	**0.4584***	**0.2953**
1,250 (68)	1.0000	undefined	undefined
1,200 (69)	0.1148	0.0321	7.5381
1,150 (70)	0.3458	0.0212	11.5424
1,100 (71)	**0.0002***	**0.0811**	**2.8326**
1,050 (72)	**< 0.0001***	**0.4584***	**0.2953**
950 (73)	1.0000	undefined	undefined

Table 4.24 (continued)

Size (bp)	Geographic heterogeneity (*P*-value)	*F*-statistic (θ)	N_em
880 (74)	**0.0000***	**0.4159***	**0.3511**
850 (75)	0.1276	0.0321	7.5381
800 (76)	0.2974	0.0212	11.5428
700 (77)	**< 0.0000***	**0.0811***	**2.8326**
680 (78)	0.3060	0.0158	15.5727
620 (79)	**< 0.0001***	**0.4584***	**0.2954**
600 (80)	**< 0.0001***	**0.0811**	**2.8326**
580 (81)	0.1506	0.0275	8.8409
560 (82)	1.0000	undefined	undefined
520 (63)	0.3200	0.0212	11.5428
500 (84)	1.0000	undefined	undefined
460 (85)	0.1964	0.0170	14.4558
420 (86)	**< 0.0001***	**0.4259***	**0.3511**
400 (87)	**< 0.0001***	**0.4584***	**0.2953**
380 (88)	**< 0.0001***	**0.3461***	**0.4723**
350 (89)	**< 0.0001***	**0.3463***	**0.4719**
250 (90)	**< 0.0001***	**0.0976***	**2.3115**
200 (91)	**< 0.0001***	**0.0976***	**2.3115**
OPZ 09			
1,700 (92)	0.6814	-.0062	undefined
1,600 (93)	0.7368	-.0062	undefined
1,500 (94)	0.1398	0.0414	5.7886
1,480 (95)	0.2694	0.2007*	0.9956
1,420 (96)	0.2366	0.0142*	17.3556
1,400 (97)	0.8404	0.1876*	1.0826

Table 4.24 (continued)

Size (bp)	Geographic heterogeneity (*P*-value)	*F*-statistic (θ)	N_em
1,250 (98)	**< 0.0001***	**0.3429***	**0.4750**
1,100 (99)	0.5848	0.0006	416.416
1,000 (100)	0.2208	0.0142	17.3556
950 (101)	0.8280	0.1876*	1.0826
900 (102)	0.8082	-.0039	undefined
850 (103)	0.2034	0.0765	3.0179
800 (104)	0.9126	-.0067	undefined
750 (105)	0.1718	0.2007*	0.9956
700 (106)	**< 0.0001***	**0.3429***	**0.4790**
680 (107)	0.2120	0.2007*	0.9956
620 (108)	1.0000	undefined	undefined
600 (109)	1.0000	undefined	undefined
550 (110)	0.0954	0.0393	26.6317
500 (111)	**< 0.0001***	**0.0429**	**0.4790**
450 (112)	0.1278	0.0765	3.0179
400 (113)	0.5682	-.0003	undefined
350 (114)	0.7480	-.0058	undefined
320 (115)	**< 0.0001***	**0.2509***	**0.7464**
300 (116)	0.8466	-.0058	undefined
250 (117)	1.0000	undefined	Undefined

F-statistics (θ) for overall loci = 0.0628, an overall N_em = 3.7309

* = *P-value* of RAPD bands with significant differences ($P < 0.01$)

= 95% CI with $P < 0.05$ for overall population is 0.0450 – 0.0846

Table 4.25 Pairwise genetic heterogeneity between different samples of *A. fulica* resulting from RAPD analysis using OPA02

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000 ns	-								
CTB	1.0000 ns	1.0000 ns	-							
CHM	1.0000 ns	1.0000 ns	1.0000 ns	-						
LAP	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	-					
NRM	1.0000 ns	1.0000 ns	0.9998 ns	1.0000 ns	0.5938 ns	-				
KOK	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	0.2576 ns	-			
SKA	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	0.9981 ns	1.0000 ns	0.9998 ns	-		
PAT	1.0000 ns	1.0000 ns	0.9994 ns	0.9993 ns	0.9896 ns	0.9965 ns	0.9993 ns	1.0000 ns	-	
MAL	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	1.0000 ns	-

ns = not significant,　　*　significant at $P < 0.05$

Table 4.26 Pairwise genetic heterogeneity between different samples of *A. fulica* resulting from RAPD analysis using OPA17

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000 ^{ns}	-								
CTB	0.9786 ^{ns}	1.0000 ^{ns}	-							
CHM	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	-						
LAP	1.0000 ^{ns}	0.9995 ^{ns}	0.0125 ^{ns}	0.9999 ^{ns}	-					
NRM	0.9011 ^{ns}	0.9990 ^{ns}	1.0000 ^{ns}	0.9830 ^{ns}	0.0046 ^{ns}	-				
KOK	1.0000 ^{ns}	1.0000 ^{ns}	0.9843 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	0.9127 ^{ns}	-			
SKA	1.0000 ^{ns}	1.0000 ^{ns}	0.4547 ^{ns}	1.0000 ^{ns}	0.9915 ^{ns}	0.9468 ^{ns}	1.0000 ^{ns}	-		
PAT	1.0000 ^{ns}	1.0000 ^{ns}	0.9690 ^{ns}	1.0000 ^{ns}	0.7817 ^{ns}	0.9996 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	-	
MAL	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	0.5705 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	-

ns = not significant,   * significant at $P < 0.05$

Table 4.27 Pairwise genetic heterogeneity between different samples of *A. fulica* resulting from RAPD analysis using OPB11

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000^{ns}	-								
CTB	1.0000^{ns}	1.0000^{ns}	-							
CHM	1.0000^{ns}	1.0000^{ns}	0.9997^{ns}	-						
LAP	1.0000^{ns}	1.0000^{ns}	0.9838^{ns}	1.0000^{ns}	-					
NRM	1.0000^{ns}	1.0000^{ns}	0.9847^{ns}	1.0000^{ns}	1.0000^{ns}	-				
KOK	1.0000^{ns}	1.0000^{ns}	0.9999^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-			
SKA	**< 0.0001***	**< 0.0001***	**< 0.0001***	**0.0004***	**0.0004***	**< 0.0001***	**< 0.0001***	-		
PAT	0.4647^{ns}	0.0565^{ns}	**0.0029***	0.9682^{ns}	0.7847^{ns}	0.8607^{ns}	0.4289^{ns}	**0.0005***	-	
MAL	0.9996^{ns}	0.9105^{ns}	0.6347^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	0.3195^{ns}	0.9947^{ns}	-

ns = not significant,   * significant at $P < 0.05$

Table 4.28 Pairwise genetic heterogeneity between different samples of *A. fulica* resulting from RAPD analysis using OPZ09

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000 ^{ns}	-								
CTB	1.0000 ^{ns}	0.9946 ^{ns}	-							
CHM	1.0000 ^{ns}	0.9389 ^{ns}	0.9536 ^{ns}	-						
LAP	1.0000 ^{ns}	1.0000 ^{ns}	0.9981 ^{ns}	0.9978 ^{ns}	-					
NRM	1.0000 ^{ns}	1.0000 ^{ns}	0.9799 ^{ns}	0.9917 ^{ns}	1.0000 ^{ns}	-				
KOK	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	-			
SKA	0.2330 ^{ns}	0.318 ^{ns}	0.9186 ^{ns}	**< 0.0001***	**0.0072***	0.1115 ^{ns}	0.1941 ^{ns}	-		
PAT	1.0000 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	0.9905 ^{ns}	0.9999 ^{ns}	1.0000 ^{ns}	1.0000 ^{ns}	0.2939 ^{ns}	-	
MAL	0.9739 ^{ns}	0.4042 ^{ns}	0.9208 ^{ns}	1.0000 ^{ns}	0.2574 ^{ns}	0.9848 ^{ns}	0.9954 ^{ns}	0.1466 ^{ns}	0.9985 ^{ns}	-

ns = not significant,　　 * significant at $P < 0.05$

Table 4.29 Pairwise genetic heterogeneity between different samples of *A. fulica* resulting from RAPD analysis using OPA02, OPA17, OPB11 and OPZ09

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000^{ns}	-								
CTB	1.0000^{ns}	1.0000^{ns}	-							
CHM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-						
LAP	1.0000^{ns}	1.0000^{ns}	0.9999^{ns}	1.0000^{ns}	-					
NRM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	0.9999^{ns}	-				
KOK	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-			
SKA	0.8266^{ns}	0.1238^{ns}	0.0105^{ns}	0.2378^{ns}	$0..2197^{ns}$	0.0889^{ns}	0.4129^{ns}	-		
PAT	1.0000^{ns}	1.0000^{ns}	0.9987^{ns}	1.0000^{ns}	0.9982^{ns}	1.0000^{ns}	1.0000^{ns}	0.9719^{ns}	-	
MAL	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	0.9999^{ns}	1.0000^{ns}	-

ns = not significant,   * significant at $P < 0.05$

Genetic diversity of *A. fulica* using combined data from RAPD and SSCP analysis.

Data of both SSCP (less informative) and RAPD (more informative) analysis were combined and subjected to identical data analysis as for each technique. Genetic distances between pairs of investigated samples were 0.032-0.1210.

The N-J tree constructed from the average genetic distances between pairs of samples revealed comparable topology to that from RAPD analysis. Identical conclusions could be drawn from the phylogenetic tree that *A. fulica* was first introduced to peninsular Thailand and subsequently spread to other places in Thailand. Pairwise genetic heterogeneity analysis revealed a lack of heterogeneity when both data sets were combined.

Table 4.30 The average modified Roger's genetic distances between different *A. fulica* samples resulting from RAPD and SSCP analysis

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	0.0389	-								
CTB	0.0638	0.0437	-							
CHM	0.0322	0.0433	0.0619	-						
LAP	0.0532	0.0375	0.0787	0.0514	-					
NRM	0.0744	0.0718	0.0815	0.0686	0.0785	-				
KOK	0.0385	0.0430	0.0608	0.0450	0.0423	0.0769	-			
SKA	0.1080	0.1210	0.1252	0.1168	0.1182	0.1139	0.1125	-		
PAT	0.0714	0.0925	0.1022	0.0813	0.1001	0.0733	0.0885	0.0741	-	
MAL	0.0839	0.0808	0.0906	0.0635	0.0805	0.0662	0.0772	0.1168	0.0829	-
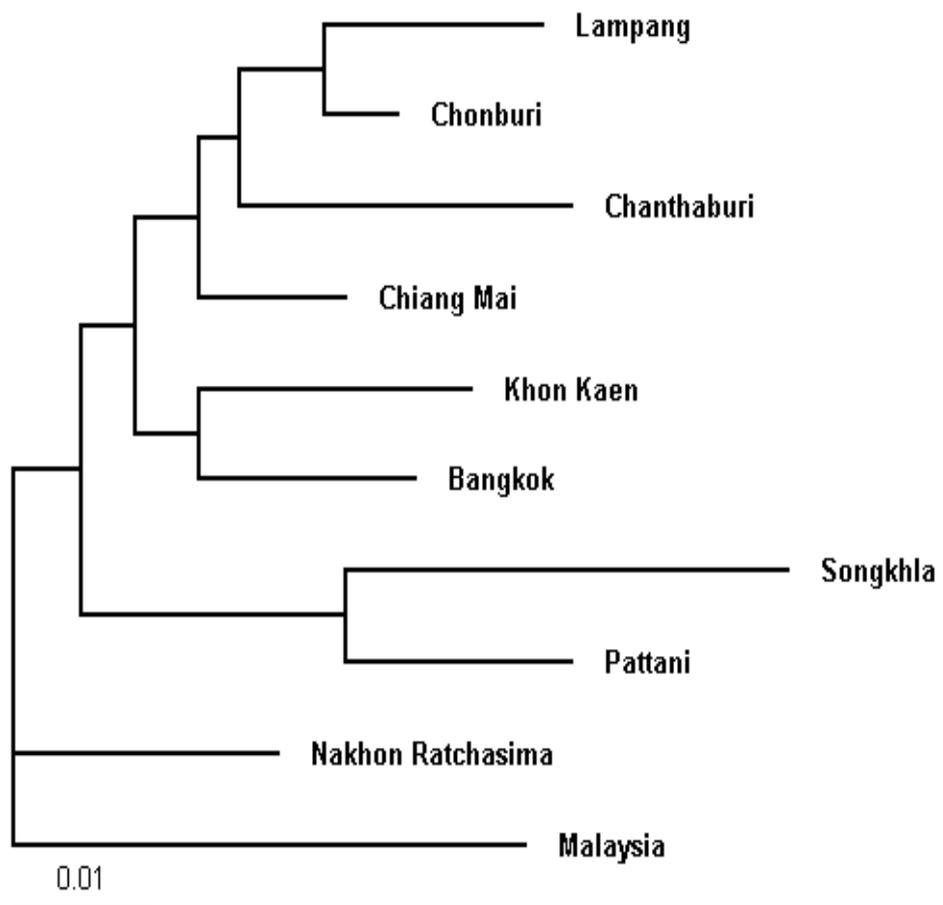
Figure 4.32 Neighbor-joining tree constructed from genetic distances resulted from RAPD and SSCP analysis.

Table 4.31 Pairwise genetic heterogeneity analysis between different samples of *A. fulica* resulting from RAPD and SSCP analysis

	BKK	CBR	CTB	CHM	LAP	NRM	KOK	SKA	PAT	MAL
BKK	-									
CBR	1.0000^{ns}	-								
CTB	1.0000^{ns}	1.0000^{ns}	-							
CHM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-						
LAP	1.0000^{ns}	1.0000^{ns}	$1.00^{ns}00$	1.0000^{ns}	-					
NRM	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-				
KOK	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-			
SKA	0.9690^{ns}	0.4852^{ns}	0.1309^{ns}	0.6609^{ns}	0.6160^{ns}	0.3850^{ns}	0.8294^{ns}	-		
PAT	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	0.9985^{ns}	-	
MAL	1.0000^{ns}	1.0000^{ns}	1.0000	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	1.0000^{ns}	-

ns = not significant,   * significant at $P < 0.05$

Isolation and characterization of stress-related genes in the collar tissue of *Achatina fulica*.

Determination of expression levels of a gene encoding an antibacterial peptide, achacin, in collar of normal and aestivated snails.

The complete cDNA of achacin previously isolated and deposited in the GenBank (Accession no. X64584 S46142) was retrieved. A pair of primers was designed to generate the expected amplification product of 406 bp in length (Figure 4.33). An approximately 400 bp amplification product was obtained from amplification of genomic DNA of *A. fulica* using heterospecific primers from *P. monodon*. This fragment was cloned and sequenced. The actual size of β-actin in *A. fulica* was 395 bp. More specific primers were designed (Figure 4.34) to generate a product of 271 bp to be used as the control for semiquantitative analysis.

RT-PCR of the first strand cDNA of a single individual of normal, partial aestivation, and full aestivation snails gave the correct amplification product for both primers pairs (Fig. 4.35). The optimal conditions (e.g, concentration of template and the number of amplification cycles) for semi-quantitative PCR of achacin were further calibrated. Using 750 ng of the first strand cDNA template and the amplification profiles consisting of $94^{o}C$ for 30 seconds, $55^{o}C$ for 1 minute, and $72^{o}C$ for 1 minute for 30 cycles (achacin) or 26 cycles (β-actin), and the final extension at $72^{o}C$ for 7 minutes provided the amplification product that still did not reach a plateau of amplification.

Semi-quantitative RT-PCR was then carried out against 12 individuals of each experimental group. The expression level of achacin and β-actin of each snail was examined (Figure 4.36). After electrophoresis, the relative expression level (a ratio

between achacin and β-actin) was calculated and indicated that normal snails expressed an achacin transcript at the highest level (1.2371-1.4005). The expression levels of this transcript was dramatically decreased after stressing the snails for 5 days (0.8496-1.0428) before the expression level was increased but to a lesser extant than for the normal conditions in the full aestivation snails (1.0738-1.1770) (Table 4.32).

The average expression level of the achacin gene of respective snail groups was 1.3391 ± 0.05, 0.9909 ± 0.05 and 1.1859 ± 0.18. Result from statistical analysis indicated significant differences of the expression levels among all treated groups ($P<0.05$). This indicated that stress (dry) conditions affected expression of an achacin gene.

```
CAGTTTTCTAGCCTGTTGTAAAGCTTCACCCCAAAGCCGAAATGCTGCTTTTAAACTCAG
CGCTGTTTATTCTGTGTCTTGTCTGCTGGCTTCCAGGAACAAGTTCATCGCGTGTTTTAA
CCAGAAGAGAAGGTCCCCAGTGCAGTCGCTCGGTAGACGTTGCTGTGGTCGGGGCTGGAC
CTTCAGGAACCTATTCTGCTTACAAACTTCGGAATAAGGGACAAACTGTCGAATTGTTTG
AGTACTCCAACAGAATTGGAGGACGTTTGTTTACGACTCACTTGCCTAACGTCCCCGATC
TTAATTTGGAATCTGGTGGGATGAGGTACTTCAAAAACCACCACAAAATATTTGGGGTTC
TGGTCAAGGAGTTAAACCTGAGCAATAAAGAATTCACGGAAGGTTTTGGGAAACCTGGAA
GAACAAGGTTCTTCGCACGGGGAAAGAGCCTGACTTTAGAAGAAATGACCAGTGGAGATG
TGCCTTACAACCTGAGCACAGAAGAAAAGGCAAATCAAGCAAACCTTGCCGGATACTATC
TTAAGAAACTGACGGGATTTGACGGGGAGGTTCTTACAATACCTCAGGCAAACAAACTGG
AGGTGGACGATGGCAGGAAGCTTTACCAACTTACAGTGGACGAAGCTCTAGACAAAGTGG
GTACGCCAGAAGGTAAAGAATTTCTCAAAGCTTTTTCCACTGGAAACACTGAATTCATTG
AAGGCGTAAGCGCTGTCAACTATTTCCTAGTAGAACTGGGTGAAAGAGAAGAGGAGATCC
TTACCTTGACTGATGGAATGAGTGCATTACCACAGGCGCTTGCCGATGCTTTCTTAAAGT
CAAGCACAAGCCACGCCTTAACGCTGAACAGAAAGTTGCAATCCTTATCAAAAACAGACA
ACGGTCTCTATCTACTAGAGTTTCTAGAAACAAACACGCATGAAGGCTACACGGAAGAAA
GTAACATTACAGACCTTGTATGCGCCCGCAAGGTTATACTGGCAATACCTCAGTCTGCTT
TGATACATCTTGACTGGAAGCCATTACGATCTGAAACCGTCAATGAAGCCTTCAATGCGG
TAAAATTTATACCAACAAGTAAAGTTTTTCTGACATTTCCCACAGCCTGGTGGCTGAGTG
ACGCCGTGAAAAATCCTGCCTTCGTCGTTAAATCGACCTCTCCTTTCAATCAGATGTACG
ACTGGAAGTCATCAAACGTGACCGGAGATGCTGCCATGATTGCCAGCTATGCCGATACCT
CCGATACAAAATTTCAAGAGAATCTGAACTCAAAGGTGAACTGATTCCAGGATCTGCTC
CTGGTGCCAACAGAGTTACGGTCGCTCTCAAGGAGGAACTACTTAGTCAACTTTCTCAGG
CTTATGGAATAGAGCGCAGTGATATTCCGGAACCGAAAAGCGGAACATCCCAGTTTTGGT
CGAGCTACCCTTTTGAAGGAGACTGGACCGTATGGAAGGCAGGATACCACTGTGAATACA
CACAGTACATCATAGAACGACCATCTCTCATTGACGATGTGTTTGTTGTAGGATCAGACC
ACGTGAACTGCATCGAAAACGCCTGGACGGAGTCAGCTTTTCTCAGTGTAGAGAATGTTT
TTGAGAAGTATTTCTGATAAATTATCTACACCTGTCACTGGTTTCTTCTTTACCAGAAAT
GATTATTCTATACTCTAGCCAGAGCTTAGTGTTTTTCTTTATATTTTCTTTTTTTTTCCGT
TTTCTTCTTCTCATCCAGTCTAAAGCTGGTATACAGAGTGTTTATGAAGCGCATTTGTGT
TTCCTCAGAGGTAGTTAAATTGTTCCTTTTTGTTGCAAACATGCACCGAAGTGTCATTTT
GGTTTGACTGTAGGTAATAAAGCAATGCACATCAAACTACCAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 4.33 The full length cDNA sequence of achacin gene from *A. fulica*

(Accession no. X64584 S46142). Positions of the forward (AFCOI406F) primer and

those complementary to the reverse (AFCOI406R) primer are illustrated in bold and

underlined.

```
CATTCATGCTGGATCGGCATAACCGGAATTGATGGTCCAGATTCGTCGTACTCCTGGTATCC
TCACCCTCAAGTACGTGCTTGCTGATCCACATCTGCTGGAAGGTGGAGAGCGAGGCCAGGAT
GGAGCCGCCGATCCATACGGAGTACTTGCGCTCGGGCGGGGCAATGATCTTGATCTTCATGG
TGGAGGGGGCGAGGGCAGTGATCTCCTTCTGCATCCTGTCGGCGATGCCAGGGTACATGGTG
GTGCCTCCGGACAGCACGGTGTTGGCGTACAGATCTTTACGGATGTCGACGTCGCACTTCAT
GATGGAGTTGTAGGTGGTCTCGTGGATACCCGCAGACTCCTCTATGAAGGTTTCGCTCTTAT
ACCCAGGAATGAGGGCTGGAACA
```

Figure 4.34 Nucleotide sequence of β-actin of *A. fulica*. Positions of the forward (AFActin-F) and those complementary to the reverse (AFActin-R) primers are illustrated in bold and underlined. The expected amplification fragment is 271 bp in length.
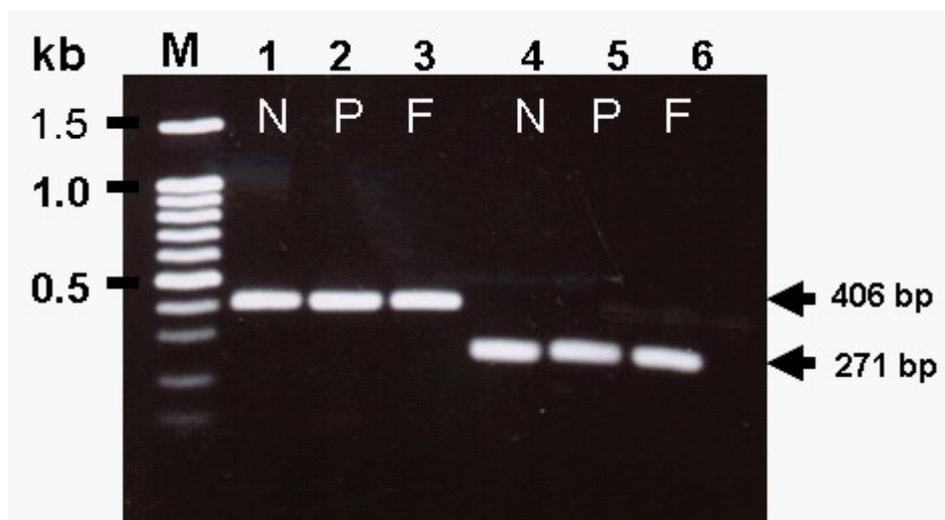
Figure 4.35 Agarose gel electrophoresis showing the amplification results of an achacin gene in snails (lanes 1-3) and the control β-actin (lanes 4-6) using the first strand cDNA of normal (N), partial (P) and full (F) aestivation as the template for 26 cycles. A 100 bp DNA marker (lane M) was included as the marker.
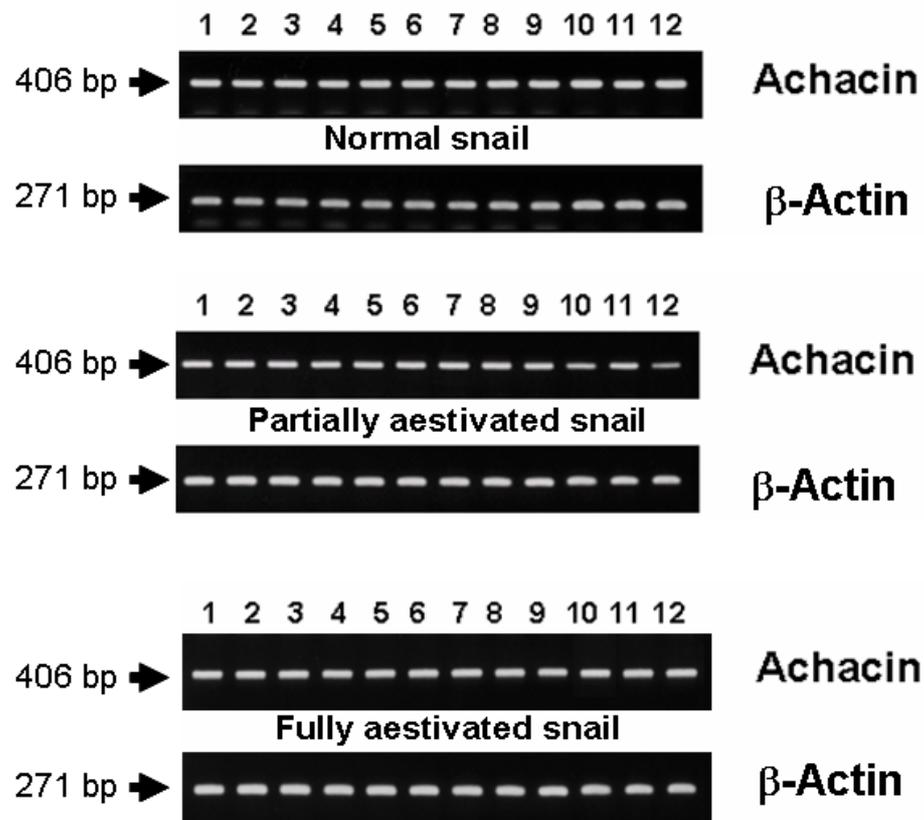
Figure 4.36 Expression level of achacin and β-actin transcript in three experimental snail group (normal, $N = 12$; partial aestivation, $N = 12$, and full aestivation, $N = 12$). The relative expression level (achacin / β-actin) of different snail groups were statistically analyzed.

Table 4.32 The relative expression levels of achacin and β-actin genes in each individual of normal, partially aestivated and fully aestivated snails analyzed by semi-quantitative PCR.

Normal Snail

Intensity	Snail 1	Snail 2	Snail 3	Snail 4	Snail 5	Snail 6	Snail 7	Snail 8	Snail 9	Snail 10	Snail 11	Snail 12
Achacin	170.17	169.94	171.1	169.04	172.56	173.23	173.21	177.46	177.48	182.19	180.65	185.9
Beta Actin	126.42	123.54	123.23	126.67	123.21	125.5	124.35	134.42	132.85	147.27	146.4	139.85
Ratio	**1.3461**	**1.3756**	**1.3885**	**1.3345**	**1.4005**	**1.3803**	**1.3929**	**1.3202**	**1.3359**	**1.2371**	**1.2339**	**1.3293**

Partially Aestivated Snail

Intensity	Snail 13	Snail 14	Snail 15	Snail 16	Snail 17	Snail 18	Snail 19	Snail 20	Snail 21	Snail 22	Snail 23	Snail 24
Achacin	188.96	189.61	189.35	189.5	187.98	190.63	192.15	191.07	190.87	176.33	185.98	151.65
Beta Actin	191.87	191.85	189.87	188.46	188.33	188.15	185.54	185.59	193.46	182.24	178.35	178.5
Ratio	**0.9848**	**0.9883**	**0.9973**	**1.0055**	**0.9981**	**1.0132**	**1.0356**	**1.0295**	**0.9866**	**0.9676**	**1.0428**	**0.8496**

Fully Aestivated Snail

Intensity	Snail 25	Snail 26	Snail 27	Snail 28	Snail 29	Snail 30	Snail 31	Snail 32	Snail 33	Snail 34	Snail 35	Snail 36
Achacin	199.29	197.69	199.94	194.65	189.46	192.48	195.73	198.25	192.19	198.25	197.98	202.46
Beta Actin	171.72	167.96	170.13	169.17	165.56	168.13	169.95	174.18	174.56	184.63	182.88	177.1
Ratio	**1.1606**	**1.1770**	**1.1752**	**1.1506**	**1.1444**	**1.1448**	**1.1517**	**1.1382**	**1.1010**	**1.0738**	**1.0826**	**1.1432**

Identification and characterization of differentially expressed genes in relative to stress by RAP-PCR.

Genes exhibiting stress-related expression patterns among different groups of A. fulica were identified by RAP-PCR analysis (30 primer combinations). Interesting RAP-PCR markers were those present or absent in at least one group of snails and those which exhibited differential expression patterns in at least one group of *A. fulica*.

Several markers were identified after being electrophoretically analyzed by 4.5% denaturing polyacylamide gels (Figures 4.37-4.39). The markers having expression patterns described above were scored and recorded for further characterization (Table 4.33). Nevertheless, fragments which are smaller than 200 bp in length were not considered because nucleotide sequences of small fragment may not allow appropriate positions for primer designation.

Seven differentially expressed (some fragments were absent in at least one group of snails) RAP-PCR bands consisting of 350 bp, 470 bp and 680 bp fragments from OPZ09 + UBC138, a 370 bp fragment from OPZ09 + UBC459, 410 bp, 500 bp and 520 bp fragments from OPA02 + UBC228 were cloned and sequenced. Nucleotide sequences of these transcripts were blasted against data in the GenBank. All cloned fragments did not reveal significant similarity with previously deposited sequences in the database (e-values $>10^{-4}$) (Table 4.34). Therefore, they were regarded as newly isolated unknown transcripts in *A. fulica*.

A primer pair was designed from each transcript (Fig. 4.40). RT-PCR was carried out to confirm the expression patterns of these markers. Only three primers AFRAP9/158370, AFRAP9/135470, and AFRAP2/228410, generated the expected product when tested with the first strand cDNA of a representative individual from

different treated groups of *A. fulica*. In contrast, AFRAP9138680, AFRA2/228500, AFRAP2/228510 gave nonspecific amplification products and AFRAP9/459370 did not generate the PCR product. RT-PCR conditions (annealing temperature, concentrations of template, $MgCl_2$ concentrations) were extensively calibrated but a single expected band was not obtained. Therefore, only three RAP-PCR derived markers (AFRAP9/158370, AFRAP9/135470 and AFRAP2/228410) were subjected to semi-quantitative PCR analysis.
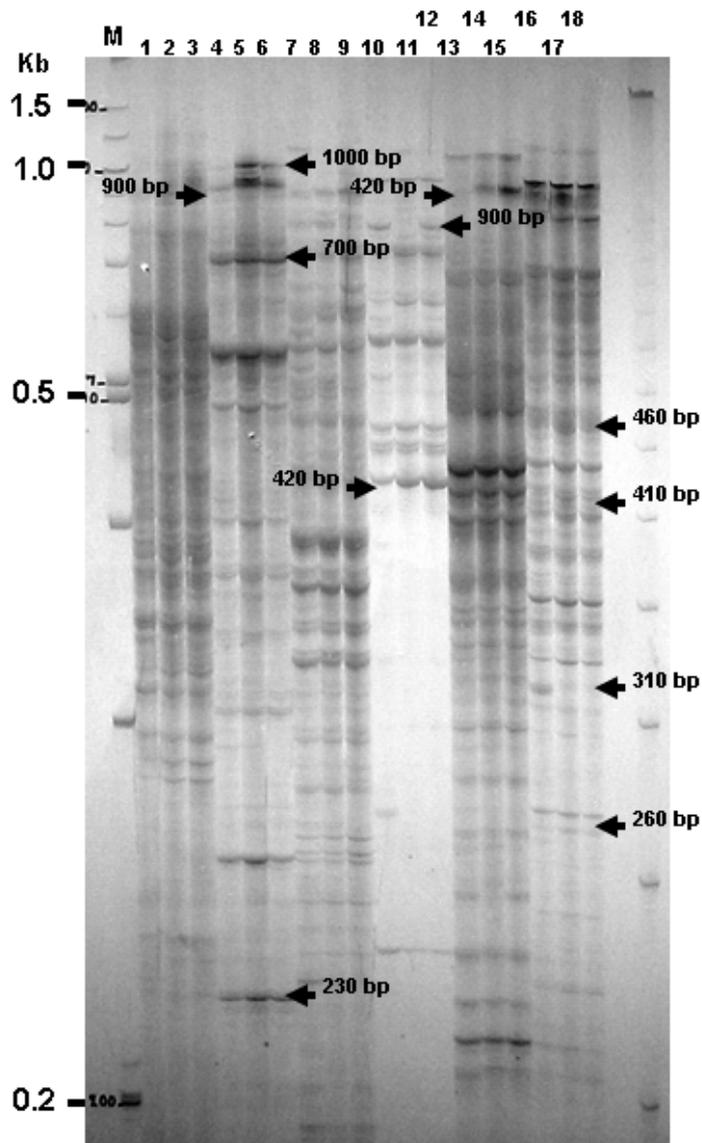
Figure 4.37 Denaturing polyacrylamide gel electrophoresis of RAP-PCR products of normal (lanes 1, 4, 7, 10, 13, and 16), partial aestivation (lanes 2, 5, 8, 11, 14 and 17) and full aestivation (lanes 3, 6, 9, 12, 15 and 18) generated from OPZ09 + UBC101 (lanes 1-3), OPZ09 + UBC119 (lanes 4-6), OPZ09 + UBC122 (lanes 7-9), OPZ09 + UBC128 (lanes 10-12), OPZ09 + UBC135 (lanes 13-15) and OPZ09 + UBC138 (lanes 16-18). A 100 bp ladder (lane M) was included as a DNA marker. Arrows indicated presence/absence and differential expressed RAP-PCR fragments found in this gel.
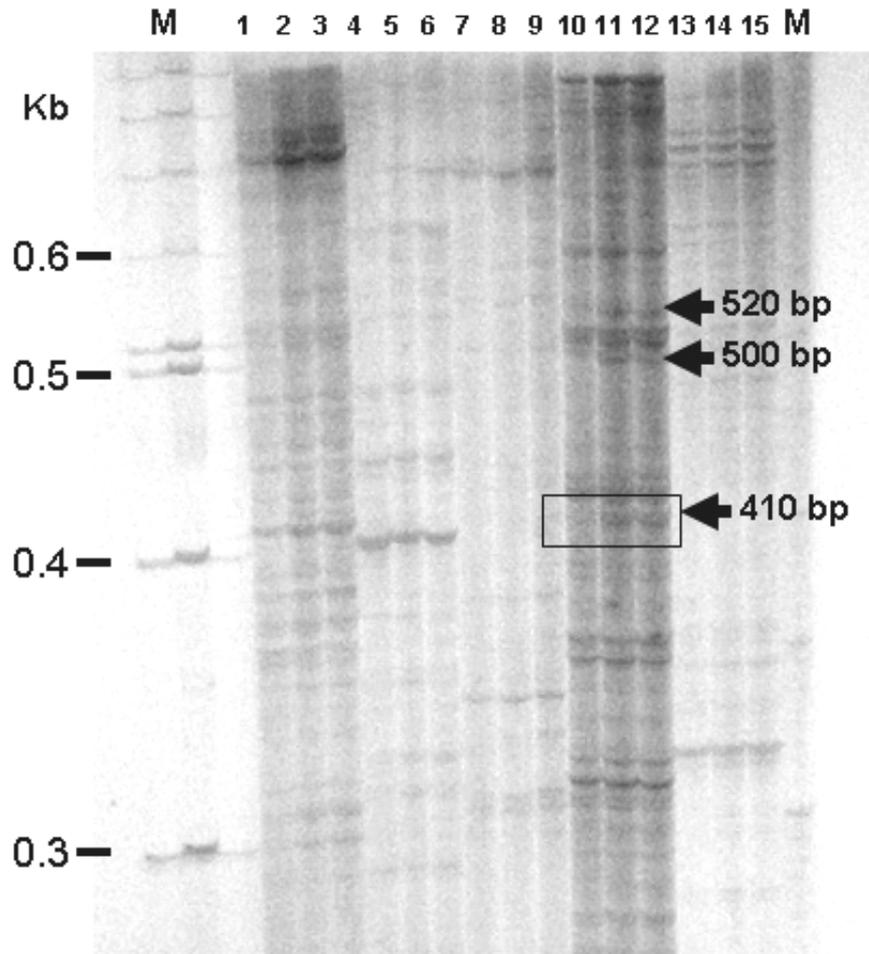
Figure 4.38 Denaturing polyacrylamide gel electrophoresis of RAP-PCR products of normal (lanes 1, 4, 7, 10 and 13), partial aestivation (lanes 2, 5, 8, 11 and 14) and full aestivation (lanes 3, 6, 9, 12 and 15) generated from OPA02 + UBC174 (lanes 1-3), OPA02 + UBC191 (lanes 4-6), OPA02 + UBC217 (lanes 7-9), OPA02 + UBC228 (lanes 10-12) and OPA02 + UBC263 (lanes 13-15). A 100 bp ladder (lane M) was included as a DNA marker. Arrows indicated RAP-PCR fragments that were cloned and sequenced. A 410 bp band was subjected to semiquantitative PCR analysis.
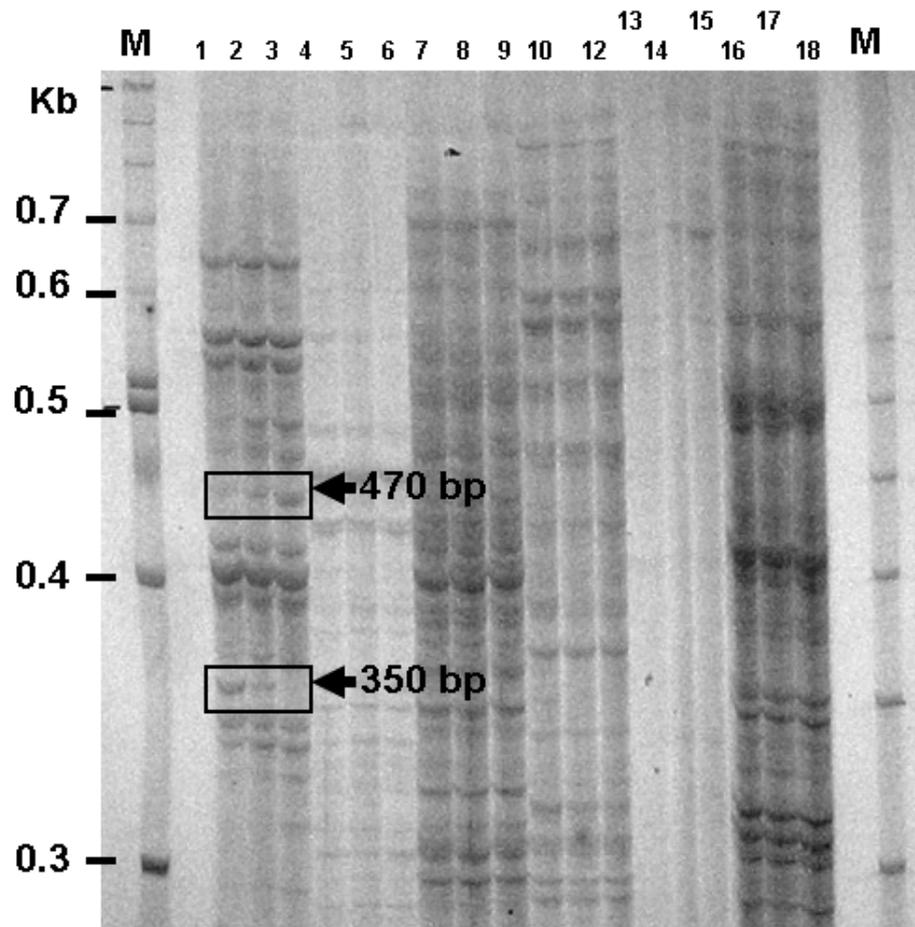
Figure 4.39 Denaturing polyacrylamide gel electrophoresis of RAP-PCR products of normal (lanes 1, 4, 7, 10, 13 and 16), partial aestivation (lanes 2, 5, 8, 11, 14 and 17) and full aestivation (lanes 3, 6, 9, 12, 15 and 18) generated from OPZ09 + UBC138 (lanes 1-3), OPZ09 + UBC191 (lanes 4-6), OPZ09 + UBC200 (lanes 7-9), OPZ09 + UBC217 (lanes 10-12), OPZ09 + UBC263 (lanes 13-15) and OPZ09 + UBC228 (lanes 16-18). A 100 bp ladder (lane M) was included as a DNA marker. Arrows indicated RAP-PCR fragments that were cloned, sequenced and subjected to semi-quantitative PCR analysis.

Table 4.33 RAP-PCR fragments illustrating differential expression patterns between different groups of *A. fulica*

Primer combination	Size (bp)	Band intensity		
		Normal snails	Partial aestivation	Full aestivation
OPZ09 + UBC119	230	+	+++	+
	700	+	+++	+++
	900	+	+++	+
	1000	-	++	+
OPZ09 + UBC128	410	+	++	+++
	780	+	-	+
OPZ09 + UBC135	900	-	+	+++
OPZ09 + UBC138	**350***	+++	+	-
	470*	+	++	+++
	680*	+	++	+++
OPZ09 + UBC158	260	-	++	+++
	310	+++	-	-
	410	+	++	+++
	780	+	-	+
OPZ09 + UBC299	300	+++	+	+
	350	+++	-	-
OPZ09 + UBC459	370*	++	-	-
OPZ09 + OPA09	380	-	++	++
	390	+++	+	+
OPA02 + UBC228	**410***	-	++	++
	500*	-	++	++
	520*	-	++	++
	800	+	++	+++
OPA02 + UBC128	410	-	++	++
	610	-	++	++
OPA02 + UBC217	790	+++	-	-
	690	+	++	+++

* RAP bands that were cloned and characterized. RAP-PCR fragments successfully subjected to semi-quantitative PCR analysis are illustrated in boldface.

AFRAP2/228410 expressed in all stages of the experimental snail but the expression level of partial aestivation snails (0.9667-1.0652) was greater than that of full aestivation (0.5491-0.7531) and normal (0.4016-0.8083) snails, respectively. Statistically significant differences between the expression level of partial aestivation and normal snails ($P<0.05$) and partial aestivation and full aestivation snails ($P>0.05$) but not between normal and full aestivation snails ($P>0.05$) were observed (Table 4.35 and Fig. 4.41).

The expression level of AFRAP9/138350 dramatically dropped from the normal snails (0.4489-0.7531) to no expression in partial aestivation (0.0000) and the low expression level in full aestivation (0.1411-0.3432). The expression patterns of this transcript examined by RAP-PCR and RT-PCR were concordant. The expression level of each treated groups of *A. fulica* was significant statistically ($P<0.05$) (Table 4.36 and Figure 4.42).

No expression of AFRAP9/138470 was observed in the normal snails. In contrast, the expression level of this transcript was positively related with the stress time as the expression level of partial aestivation snails was greater than that of the normal snails but lower than that of full aestivation snails (0.1867-0.3066). Like AFRAP9/138350, the expression patterns of AFRAP9/138470 examined by RAP-PCR and RT-PCR were concordant confirming differential expression of this transcript. The expression level of different experimental groups of *A. fulica* was significant statistically ($P< 0.05$) (Table 4.37 and Figure 4.43).

Table 4.34 Characterization of stress related gene of *A. fulica*

Combination primer	Length of sequences (bp)	Name of new designed primer	Expected PCR Product (bp)	BLAST X	BLAST N	Amplification result
OPZ09 + UBC138	350	AFRAP9/138350	203	unknown	unknown	203 bp
OPZ09 + UBC138	470	AFRAP9/138470	184	unknown	unknown	184 bp
OPZ09 + UBC138	680	AFRAP9/138680	377	unknown	unknown	Nonspecific products
OPZ09 + UBC459	370	AFRAP9/459370	224	unknown	unknown	No product
OPA02 + UBC228	410	AFRAP2/228410	310	unknown	unknown	310 bp
OPA02 + UBC228	500	AFRAP2/228500	551	unknown	unknown	Nonspecific products
OPA02 + UBC228	510	AFRAP2/228510	339	unknown	unknown	Nonspecific products

A. AFRAP2/228410

GGTACCTTATACATAATTTGGTGTATGATGTGGGATAGTTGGCACAG**TGAATGGGAC
ATAGTGGAGATA**GCTTGTCACTCTTAATCGGTTAGAGCTTGGAACAGTGGAACCTTA
ACTGATGATCACTTCAACGTGGTTGTAACTGCGCATGCTTTTGTCATAATTTGGTTA
TACCAATTATAATTGGCGGATTTGGAAACTGGATGGTCCCAATACTTATTGGTGCTC
CTGATATAAGATTTCCACGAATAAATAATATAAGGTTTTGACTTACCACCTTCATTA
CTTGTTAATCTGTTCAAGATAAGTGGAAGGAGGGGCTGGAACTGGGTGGACT**TCTAT
TGCTTCTCCCATCGG**GTGTACCCGCCCTTAAGTTCTTGCTTAGGACACAGAGGGGCT
TCAGTTGATTTAG

B. AFRAP9/138350

CACCCCAGTCCACACAGGGAAAAAACTAAGTTCTAAAAACAAGGCTCTCTCTATCAC
ATTTTCTATAGACAATCTCACAGCTTGACAAAAACACGTCTACACAGTTTCACGTTC
AAACTAAGACTGACTTTCCTAACGGCAAGCTTTT**GCACACCGACCTTTTCTAACT**GA
CGTGTTTTGCACACGGACTTGAGTTGTAGACTACAAAAACACACTACTAGTTAATAC
CCAAATACTCTCTATGCCGAATACACAAATACTCTCTGTACCAGAATTGCTATTCTG
TTACACAAGTCTGTCTGCCACTTAATTGCCATGTACTCCCCCATGC**CTGAAACAATA
AAGCCACGG**CTAAA

C. AFRAP9/138470

AGCCGTGGCGGATAGAAGATACAGAATACAACACATTCACAATTGCTGATGGGTAGTTCAAA
CATGCACAGGACTGTGCTATGTCATTAATCCAACTGAAAGGATAATTCACATTGAAATAATG
ATCTAAAGTACAGTACAGT**ACAGTAAGCCAGCAACAAAAG**CGTTTATTATGACTATCAAAAC
ATGTATATTATAGGTTATATATGTACCATTACACACTTGGCTGAACATTCACCTTGTTTACA
TGAGACACAGGACATGACACAGACATGTTCCATTGAGGGAAGCTGTTTAGACTGTCA**TATCT
CCACTATGTCCCATTCA**TCAATCAGAATTCTGTACTCTATTACAACATTCTGGGACAAAAAC
ATTGTCTGTTTTGGCAATTATCAGCCCATAGCTGACAAGCTAGTGTTAGGGCTGGTGAGCCA
CGGCTAAA

Figure 4.40 Nucleotide sequences of RAP-PCR fragments exhibiting differential expression patterns and subjected to semi-quantitative PCR analysis. Positions of the forward primers and those complementary to the reverse primers are underline and boldfaced.
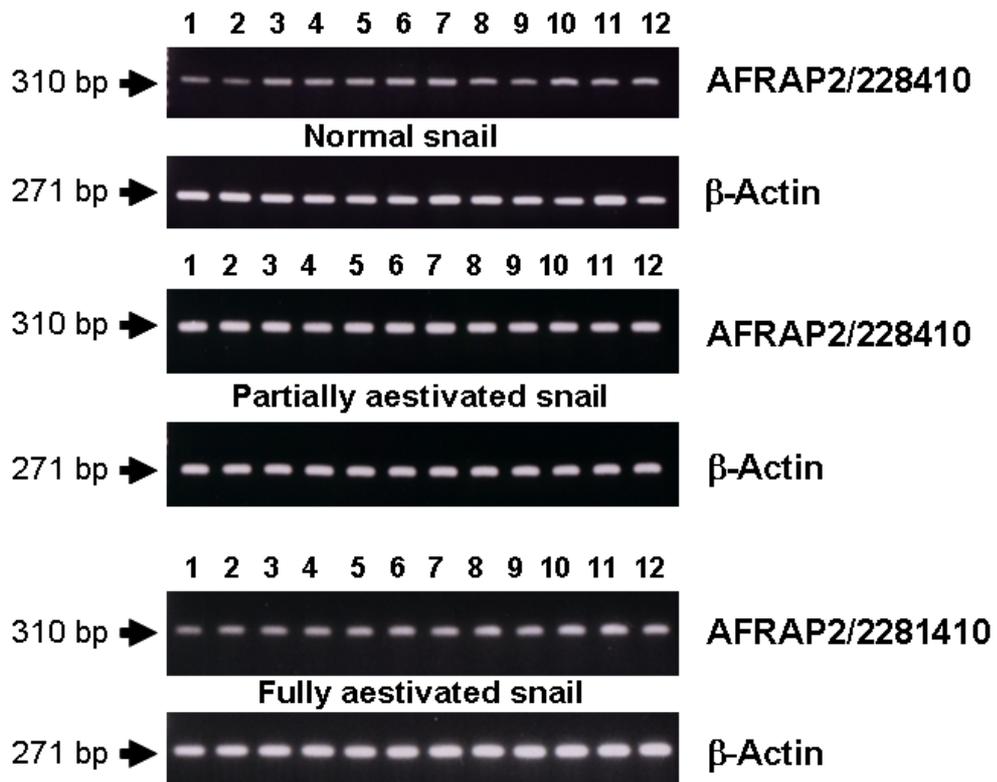
Figure 4.41 Expression level of AFRAP2/228410 and β-actin transcript in three experimental snail group (normal, *N* = 12; partial aestivation, *N* = 12 and full aestivation, *N* = 12). The relative expression level (AFRAP2/228410 /β-actin) of different snail groups were statistically analyzed.

Table 4.35 The relative expression levels of AFRAP2228410 and β-actin genes in each individual of normal, partial aestivation and full aestivation snails analyzed by semi-quantitative PCR

Normal snails

Intensity	Snail 1	Snail 2	Snail 3	Snail 4	Snail 5	Snail 6	Snail 7	Snail 8	Snail 9	Snail 10	Snail 11	Snail 12
AFRAP2228410	95.211	75.789	129.08	124.57	127.95	139.72	142.11	126.78	107.18	138.82	129.78	127.87
Actin	188.45	184.59	186.84	182.43	171.75	172.86	182.16	174.64	168.82	167.9	180.62	162.48
Ratio	**0.5052**	**0.4106**	**0.6909**	**0.6828**	**0.7450**	**0.8083**	**0.7801**	**0.7260**	**0.6349**	**0.8268**	**0.7185**	**0.7870**

Partial estivated snails

Intensity	Snail 13	Snail 14	Snail 15	Snail 16	Snail 17	Snail 18	Snail 19	Snail 20	Snail 21	Snail 22	Snail 23	Snail 24
AFRAP2228410	183.41	189.1	180.35	173.86	177.89	176.02	184.02	177.35	178.73	173.93	174.84	181.52
Actin	179.21	177.53	175.13	179.84	176.18	172.9	176.63	175.55	175.57	171.46	173.56	173.7
Ratio	**1.0234**	**1.0652**	**1.0298**	**0.9667**	**1.0097**	**1.0180**	**1.0418**	**1.0103**	**1.0180**	**1.0144**	**1.0074**	**1.0450**

Fully estivated snails

Intensity	Snail 25	Snail 26	Snail 27	Snail 28	Snail 29	Snail 30	Snail 31	Snail 32	Snail 33	Snail 34	Snail 35	Snail 36
AFRAP2228410	90.912	105.95	110.5	114.92	117.58	125.84	124.2	134.22	126.07	140.24	146.11	130.1
Actin	165.56	169.47	167.25	166.59	163.34	167.05	166.54	166.05	164.58	166.05	165.46	166.5
Ratio	**0.5491**	**0.6252**	**0.6607**	**0.6898**	**0.7198**	**0.7533**	**0.7458**	**0.8083**	**0.7660**	**0.8446**	**0.8831**	**0.7814**
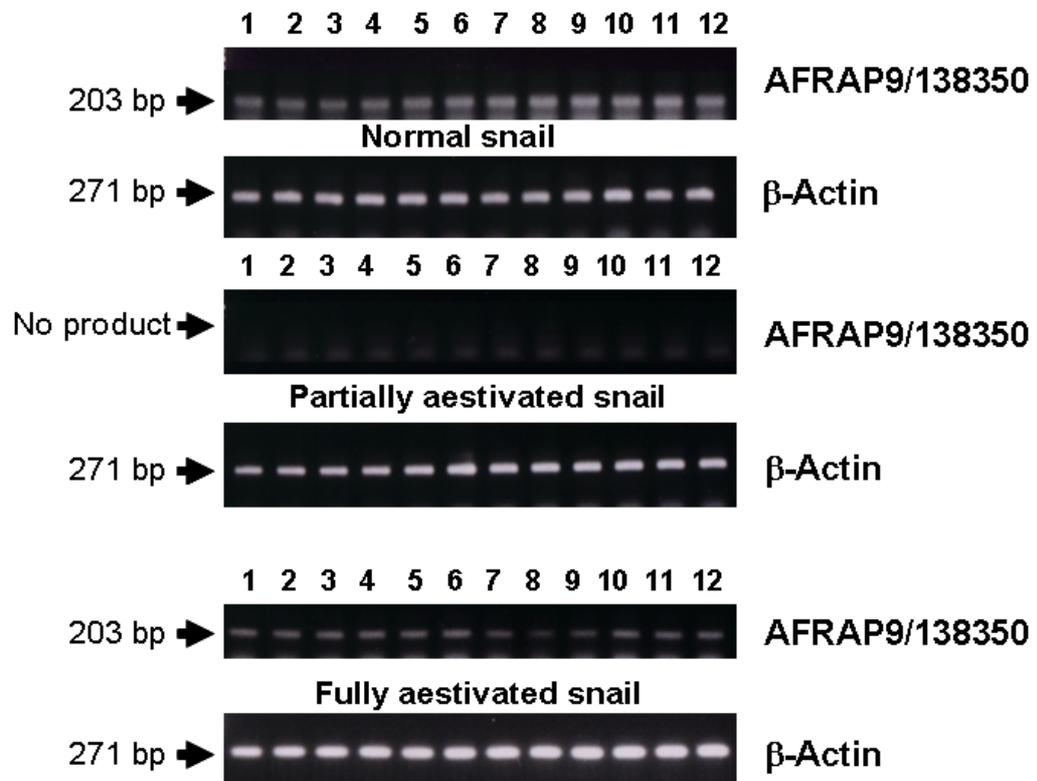
Figure 4.42 Expression level of AFRAP9/138350 and β-actin transcript in three experimental snail group (normal, *N*=12; partial aestivation, *N*=12 and full aestivation, *N*=12). The relative expression level (AFRAP9/138350/β-actin) of different snail groups were statistically analyzed.

Table 4.36 The relative expression levels of AFRAP9/138350 and β-actin genes in each individual of normal, partial aestivation and full aestivation snails analyzed by semi-quantitative PCR

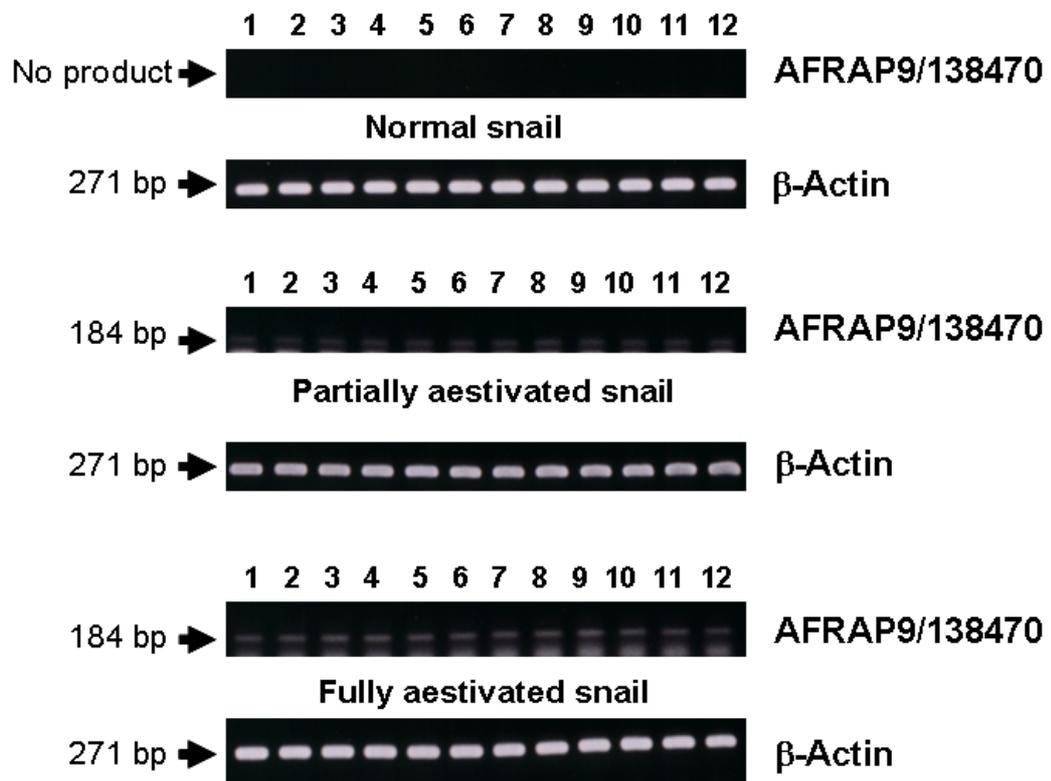**Normal snails**

Intensity	Snail 1	Snail 2	Snail 3	Snail 4	Snail 5	Snail 6	Snail 7	Snail 8	Snail 9	Snail 10	Snail 11	Snail 12
AFRAP91581	71.538	68.169	67.921	79.356	85.999	95.234	103.03	109.55	114.19	110.23	116.56	100.17
Actin	140.54	151.85	150.78	163.07	159.54	156.85	145.61	148.89	151.63	168.8	148.04	162.29
Ratio	**0.5090**	**0.4489**	**0.4505**	**0.4866**	**0.5390**	**0.6072**	**0.7076**	**0.7358**	**0.7531**	**0.6530**	**0.7874**	**0.6172**

Partially aestivated snails

Intensity	Snail 13	Snail 14	Snail 15	Snail 16	Snail 17	Snail 18	Snail 19	Snail 20	Snail 21	Snail 22	Snail 23	Snail 24
AFRAP91581	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actin	130.9	146.7	152.04	152.54	164.35	172.83	167.3	168.31	163.65	164.4	161.73	159.75
Ratio	**0.000**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

Fully aestivated snails

Intensity	Snail 25	Snail 26	Snail 27	Snail 28	Snail 29	Snail 30	Snail 31	Snail 32	Snail 33	Snail 34	Snail 35	Snail 36
AFRAP91581	48.424	52.051	35.661	58.373	54.579	66.038	34.228	25.82	39.281	65.163	59.512	57.688
Actin	186.85	186.68	188.14	183.73	180.75	184.9	184.86	183	183.92	189.87	186.14	180.92
Ratio	**0.2592**	**0.2788**	**0.1895**	**0.3177**	**0.3020**	**0.3572**	**0.1852**	**0.1411**	**0.2136**	**0.3432**	**0.3197**	**0.3189**

Figure 4.43 Expression level of AFRAP9/138470 and β-actin transcript in three experimental snail group (normal, *N*=12; partial aestivation, *N*=12 and full aestivation, *N*=12). The relative expression level (AFRAP9/138350/β-actin) of different snail groups were statistically analyzed.

Table 4.37 The relative expression levels of AFRAP9/138470 and β-actin genes in each individual of normal, partial aestivation and full aestivation snails analyzed by semi-quantitative PCR

Normal snails

Intensity	Snail 1	Snail 2	Snail 3	Snail 4	Snail 5	Snail 6	Snail 7	Snail 8	Snail 9	Snail 10	Snail 11	Snail 12
AFRAP9/138470	0.00	0.00	0.00	0.00	0.00	0.00	00.00	0.00	0.00	0.00	0.00	0.00
Actin	190.89	192.61	192.89	192.67	190.95	189.6	189.83	188.85	177.5	184.88	192.22	192.56
Ratio	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

Partially aestivated snails

Intensity	Snail 13	Snail 14	Snail 15	Snail 16	Snail 17	Snail 18	Snail 19	Snail 20	Snail 21	Snail 22	Snail 23	Snail 24
AFRAP9/138470	10.41	9.9386	10.086	11.292	12.227	11.634	12.481	14.535	11.971	12.006	12.323	11.37
Actin	154.04	157.3	151.25	157.32	169.47	162.24	156.76	161.34	155.05	156.85	153.05	148.46
Ratio	**0.0676**	**0.0632**	**0.0667**	**0.0718**	**0.0721**	**0.0717**	**0.0796**	**0.0901**	**0.0772**	**0.0765**	**0.0805**	**0.0766**

Fully aestivated snails

Intensity	Snail 25	Snail 26	Snail 27	Snail 28	Snail 29	Snail 30	Snail 31	Snail 32	Snail 33	Snail 34	Snail 35	Snail 36
AFRAP9/138470	41.082	43.491	51.375	52.427	47.064	45.815	46.063	48.53	54.645	45.334	40.019	34.644
Actin	191.41	188.93	184.24	181.55	181.72	181.08	178.95	180.91	178.2	184.18	185.56	185.52
Ratio	**0.2146**	**0.2302**	**0.2788**	**0.2888**	**0.2590**	**0.2530**	**0.2574**	**0.2683**	**0.3066**	**0.2461**	**0.2157**	**0.1867**

Table 4.38 The average relative expression level of achacin and RAP-PCR-derived markers of different groups of *A. fulica*

Genes / Marker	Normal snail	Partially aestivated snail	Fully aestivated snail
Achacin	1.3391 ± 0.05^a	0.9909 ± 0.05^b	1.1859 ± 0.18^c
AFRAP2/228410	0.6923 ± 0.12^a	1.0230 ± 0.02^b	0.7120 ± 0.08^a
AFRAP9/138350	0.6076 ± 0.12^a	0.0000	0.2683 ± 0.07^c
AFRAP9/138470	0.0000	0.1341 ± 0.21^b	0.2499 ± 0.03^c

*Different superscripts indicated significant differences in expression levels at $P < 0.05$
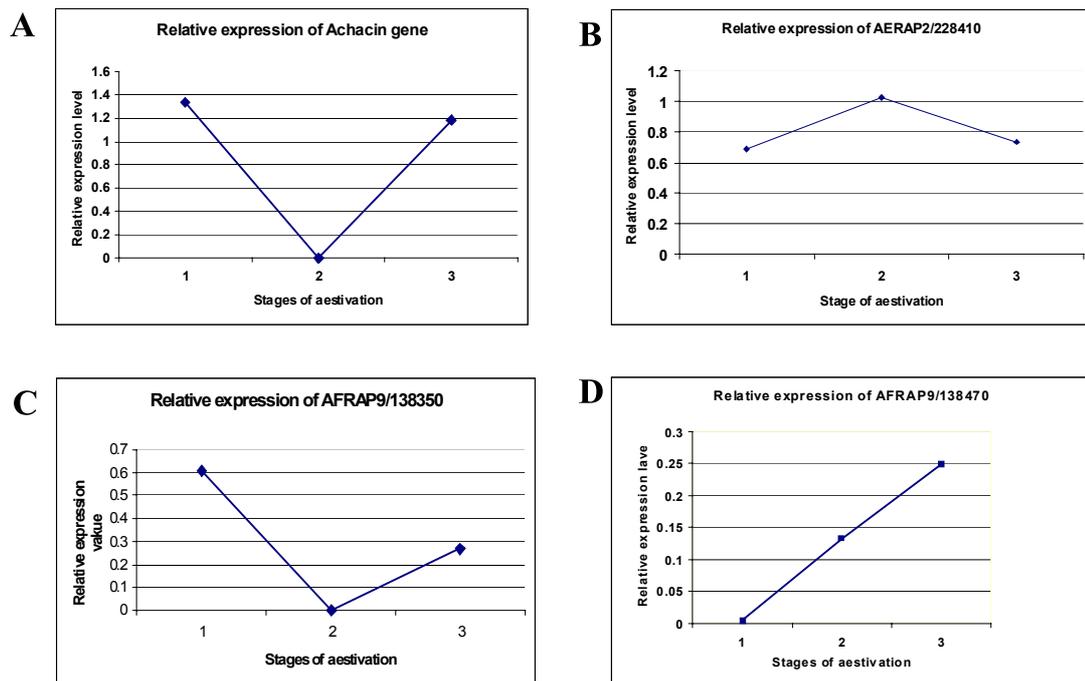
Figure 4.44 Mean plots illustrating relative expression level (ordinate) of achacin (A), AFRAP2/228410 (B), AFRAP9/138350 (C) and AFRAP9/138470 (D) of normal (1), partial aestivation (2) and full aestivation (3) groups of *A. fulica* (abscissa).

CHAPTER V

DISCUSSION

Genetic diversity of *A. fulica* in Thailand

The giant African snail (*A. fulica*) was not endemic but was introduced from Malaysia to Thailand approximately 70 years ago. During the initial stages of invasion, *A. fulica* rapidly increased in number and became one of the most serious pests of various crop species in Thailand. Nevertheless, the number of *A. fulica* rapidly declined 20 years since introduction (Mead, 1961). It was suggested that genetic problems (founder effects, genetic drift, and bottleneck effects) might be one of several important factors involving the population decline in *A. fulica* (Mead, 1961).

Information on sizes of populations and levels of genetic diversity of interesting species is important for the development of the appropriate natural resources management and conservation schemes for those taxa. There have been no reports on morphological and genetic variability of the giant African snail in Thailand; therefore, the basic information, which is not available at present, should be investigated extensively.

Land snails, including *A. fulica*, typically live in discrete populations, often isolated from one another with low dispersal ability (Denny, 1980; Fearnley, 1993). This suggests that land snails are prone to effects of population differentiation with reduced gene exchanges between demes, leading presumably to strong local

differentiation (Schilthuizen and Lombaerts, 1994; Pfenninger Bahl and Streit, 1 996). Moreover, habitat fragmentation and instability of human-disturbed environments may impose severe restrictions on gene flow and increase random genetic drift. Extinction and recolonization dynamics in local populations may also modify the distribution of genetic variability, leading to a decrease or an increase of variation among populations (Schilthuizen and Lombaerts, 1994; Ruckelshaus, 1998; Arnaud, Madec, Bellido and Guiller, 1999). Furthermore, some studies on intraspecifc genetic variation over heterogeneous habitats suggest that genetic differentiation can result from specific habitat-related effects at local or microgeographic scales (Johannesson and Tatarenko, 1997).

Although numerical taxonomy has been criticized by several classical taxonomists, morphometrics has widely been used to differentiate species within a species complex (Chiu, Chen, Lee and Chen, 2002) morphometrically analyzed the shell and operculum of the viviparid snail, *Cipangopaludina chinensis*, originating from 5 geographically different locations in Taiwan (*N*=251). The analyses demonstrated 2 morphotypes (a tall-spired form and a short-spired form) and also indicated that snails with the shorter shell spire in two study areas were morphologically related. Different growth rates in the spire contributed to the major dfference in the shell shapes between different snail morphotypes.

Morphometric analysis of *A. fulica* was carried out using 6 morphological characters (LT, LS, LD, WT, WA and LA), but biogeographic differentiation was not observed in this introduced species because specimens from different locations randomly clustered together.

Molecular biological approaches were then applied for genetic analysis of

A. fulica. Several possible techniques could be applied for genotyping of *A. fulica*. Nevertheless, PCR-RFLP analysis is a promising approach to be utilized for population genetics and systematic studies in this taxon. This simple genetic approach has been successfully used to identify the existence of three populations of the honey bee, *Apis cerana*, in Thailand (Sihanatavong, Sitipraneed and Klinbunga, 1999), to investigate genetic differences between the introduced golden apple snail (*Pomacea canaliculata*) and the local Thai species (*Pila ampullacea*, *P. angelica*, *P. pesmei*, and *P. polita*) (Thaewnon-ngiw, 2003), and to determine population genetics and phylogeny of the black tiger prawn, *Peneus monodon*, in Thailand (Siludjai, 2000). Universal primers for both nuclear and mitochondrial DNA genes are available and easy to access.

Since mtDNA is haploid and transmitted maternally, the effective population size estimated from mtDNA is generally smaller than that estimated from nuclear DNA markers such as allozymes and single copy nuclear DNA (Birky, Furest, and Maruyama*.,* 1989). This increases its sensitivity to inbreeding and bottleneck effects compared to nuclear DNA markers (Ward and Grewe, 1994; O'Connell *et al.*, 1998). Therefore, (mitochondrial) COI rather than nuclear rDNA (18S rDNA, LSU1/3, and LSU2/4) was subjected to PCR-RFLP analysis using 3 restriction enzymes (*Alu* I, *Dde* I and *Rsa* I).

It was generally accepted that colonization of invasive species in new areas would be associated with population bottlenecks that reduce within-population genetic diversity and increase genetic differentiation among populations (Williamson, 1996; Neil, Andrew, David and Ted, 2000).However, only one mitotype was found across all investigated individuals. This suggested 2 possible circumstances on the use

of PCR-RFLP of COI in *A. fulica*. The first possibility was that PCR-RFLP was unfortunately not sensitive enough to detect genetic variability in *A. fulica*. The other possibility was that a lack of COI polymorphism was a consequence of population genetic phenomena (e.g. founder effects and/or strong genetic drift) in this species.

Klinbunga *et al.* (2003) examined genetic diversity of oysters; *Crassostrea belcheri* (Sowerby, 1871), *C. iredalei* (Faustino, 1932), *Saccostrea cucullata* (Born, 1778), *S. forskali* (Gmelin, 1791), and *Striostrea (Parastriostrea) mytiloides* (Lamarck, 1819) (Ostreoida, Mollusca) were analysed by PCR-RFLP of 16S rDNA with *Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I and *Taq* I, 18S rDNA with *Hin*f I, and COI with *Acs* I, *Dde* I and *Mbo* I. A total of 41 mitotypes (3, 3, 5, 23 and 10 mitotypes, respectively) were observed. Species-diagnostic markers were specifically found in *C. belcheri*, *C. iredalei*, and *S. cucullata*, but not in *S. forskali* and *Striostrea mytiloides*, where common composite haplotypes were shared between those species.

Thaewnon-ngiw (2003) studied genetic diversity and species-diagnostic markers in the introduced apple snail, *Pomacea canaliculata*, and in the native Thai apple snails, *Pila ampullacea*, *P. angelica*, *P. pesme,* and *P. polita*. The snails were investigated by restriction analysis of COI, as reported for the first time. Twenty-one mitotypes (6, 3, 4, 7 and 1 mitotypes, in the respective species) showing non-overlapping distributions among species were found. Genetic heterogeneity analysis indicated significant differences between species ($P<0.0001$) and within *P. pesmei* ($P<0.0001$) and *P. angelica* ($P<0.0004$). No such heterogeneity was observed in *Pomacea canaliculata* ($P>0.0036$ as modified by the Bonferroni procedure), *P. ampullacea* ($P = 0.0824$-1.000) and *P. polita* ($P = 1.0000$). A neighbor-joining tree based on genetic distance between pairs of mitotypes differentiated all species and

indicated that *P. angelica* and *P. pesmei* are closely related phylogenetically.

Results from both previous publications implied reasonable genetic polymorphism of COI (which was amplified from genomic DNA of *A. fulica* using the same universal primers) for determination of gene diversity and differentiation at both inter- and intraspecific levels. Therefore, SSCP analysis of COI polymorphism, which is more sensitive than PCR-RFLP, was then applied. Additionally, a homologue of the PMX gene was included in the analysis.

SSCP requires fragments smaller than 300 bp for effective analysis of point mutations in the DNA segments. A 710 bp COI fragment was then cloned and sequenced. A new primer pair generating the amplification product of 215 bp (AFCOI215F and AFCOI215R) was designed and gave 100% amplification success across all specimens. Species-specificity was also observed from the newly developed primers as they only provided the amplified product in *A. fulica* but not in other molluscs including apple snails (*Pomacea canaliculata, Pila ampullacea, P. pesmei, P. polita and P. angelica*) and oysters (*Crassostrea belcheri, C. iredalei. and Saccostrea cucullata*), where the original COI primers (LCO1490 and HCO2198) successfully amplified COI from all the above-mentioned species.

It has been reported that *A. fulica* is heavily infected by *Angiostrogylus cantonensis*, which causes eosinophilic meningocephalitis and brain damage in humans. As a result, species-specific markers are useful to detect the species origin of snails (or tissues) consumed by the patients. This DNA marker can be applied to evaluate the prevalence of infection of *A. cantonensis* in *A. fulica* and apple snails (*Pomacea canaliculata, P. ampullacea, P. polita, P. angelica* and *P. pesmei*) in Thailand.

Only 5 and 3 SSCP patterns were observed from COI and PMX, respectively. Similar levels of modified Roger's genetic distance were found for pairs of samples for both (mitochondrial) COI and (nuclear) PMX. Theoretically, COI should exhibit much greater genetic distances than the coding sequence PMX. As a result, the limited diversity of *A. fulica* in this study is suspected to have resulted from population parameters rather than sensitivity of the molecular techniques.

Two SSCP fragments revealed significant F_{ST} stastistics (theta) across all samples (P<0.01). Nevertheless, these fragments did not show significant allele distribution frequencies between pairs of samples when analyzed by the exact test (no genetic heterogeneity, P>0.05). At the population level, no population differentiation of *A. fulica* was observed (P>0.05).

Although SSCP may detect 60-90% of point mutations examined by DNA sequencing (Dean and Milligan, 1998), analysis of polymorphism of COI by DNA sequencing may still not resolve the problems. Based on the fact that the giant African snail was introduced to Thailand approximately 70 year ago, DNA segments that can be used to infer differentiation of these snails should exhibit a mutation rate of greater than 10^{-3} per site per year per evolutionary line,assuming neutral mutations with T = D/2α, where T = time since the shared ancestor, D = genetic distance between comparisons, and α = the neutral mutation rate of a gene under investigation.

RAPD analysis is the simple method widely used to scan different parts of the genome of organisms. In this case, RAPD-PCR was used to identify DNA segments exhibiting high evolutionary rates using 4 different primers (OPA02, OPA17, OPB11, and OPZ09).

Although a large number of scorable RAPD fragments (117) were obtained using 4 selected primers, only 22 RAPD patterns were observed from 207 individuals. The percentage of polymorphic bands ranged from 50%-73.07%.

Tassanakajon, Pongsomboon, Jarayabhand, Klinbunga, and Boonsaeng, (1998) examined genetic variation in wild black tiger shrimp, *Penaeus monodon.* Specimens were collected from five geographically separated locations (Satun-Trang, Phangnga, and Medan in the Andaman Sea and Chumphon and Trad in the Gulf of Thailand). A total of 100 *P. monodon* individuals were investigated using seven arbitrarily selected primers. Fifty-eight (72.5%) of eighty reproducible RAPD fragments ranging in size from 200 to 2,200 bp were polymorphic. RAPD analysis yielded a total of 252 genotypes. A Monte Carlo analysis illustrated geographic heterogeneity in genotype frequencies within this species, suggesting that genetic population structure does exist in this taxon ($P<0.001$).

Thaewnon-ngiw *et al.* (2003) studied the genetic diversity of the introduced golden apple snail, *Pomacea canaliculata*, and four native apple snails, *Pila ampullacea*, *P. angelica*, *P. pesmei* and *P. polita* in Thailand by RAPD analysis. Two hundred and two polymorphic fragments (180-1,500 bp in length) were generated across all investigated samples (N=254) using three informative primers (OPA07, OPB10, and UBC122). The percentages of polymorphic bands were 98.86%, 94.56%, 90.91%, 96.94% and 95.51% for *Pomacea canaliculata, P. ampullacea, P. angelica, P. pesmei* and *P. polita*, respectively.

The low number of RAPD patterns and the low percentage of polymorphic RAPD fragments found in *A. fulica* compared with those found in the introduced golden apple snails and other local species described above further confirmed the

status of limited genetic diversity of the introduced giant African snail.

Inter and intrapopulation genetic diversity can enhance adaptation to a particular habitat and also expand the boundary of colonization and distribution, enabling a species to survive in a wide variety of conditions (Williamson, 1996). The limited genetic diversity of *A. fulica* in Thailand was possibly related to environmental versatility.

Three primers (OPA02, OPA17, and OPZ09) provided identical results and indicated that the gene pool of *A. fulica* in Thailand was panmictic (P>0.05). Nevertheless, OPB11 generated interesting results on genetic diversity and population differentiation of *A. fulica* in this study. Six polymorphic patterns were observed, similar to the numbers for OPA17 and OPZ09. The percentage of polymorphic bands was 70.00%, which was comparable with that of OPZ09 (73.07%). Nevertheless, 14 of 21 polymorphic bands generated by this primer revealed genetic heterogeneity across all samples (P = 0.001 for a 1,110 bp fragment, and P < 0.0001 for the remaining fragments), indicating the existence of population genetic differentiation of *A. fulica* in Thailand. The F_{ST} statistics of these fragments indicated that *A. fulica* is a low or moderate gene flow species. Moreover, significant genetic differentiation was observed between pairs of samples based on the exact test (P<0.001).

Wright (1978) suggested that F_{ST} values between 0.00-0.05 indicated low degrees of population differentiation whereas the values of 0.05-0.15 indicated a medium degree of differentiation. High genetic differentiation is considered for F_{ST} values of 0.15-0.25. With the relationship of $N_{e}m = 1- F_{ST})/(4\ F_{ST})$, the value greater than 0.25 suggests very low gene flow (<0.7 individuals per generation). At this level, the gene flow is not strong enough to homogenize population differentiation and loss

of genetic differentiation caused by genetic drift (Slatkin, 1987; Slatkin and Barton, 1989).

The gene flow of 14 informative fragments from OPB11 over all samples was 0.2953-2.8326 individuals per generation, indicating that *A. fulica* exhibits low-moderate gene flow levels. Results from this study supported the hypothesis that limited numbers of *A. fulica* founders should have been introduced to Thailand or, alternatively, introduced snails were pedigree relationships reflected the limitation of genetic diversity of *A. fulica*. A lack of genetic differentiation based on morphometrics, PCR-RFLP of COI, SSCP of COI_{215} and PMX, and RAPD analysis using OPA02, OPA17 and OPZ09 may have resulted from the use of slowly-moderately evolving DNA fragments that cannot differentiate a species that has been introduced for approximately 70 years like *A. fulica*.

Moreover, very high population densities and urbanization, intensive agricultural activities and extensive road networks have turned the agricultural areas into strongly isolated areas. It is expected that the giant African snails bound to these habitats with limited dispersal ability would likely experience strong effects of genetic drift causing a loss of genetic diversity in each geographic sample.

Although *A. fulica* is expected to be a low-medium gene flow species, anthropological transfer of *A. fulica* to different places in Thailand may homogenize the effects of genetic drift preventing genetic differentiation between geographically different samples of this species. In addition, the time since the first introduction of the giant African snail to Thailand was approximately 70 years which may not be enough time for accumulation of significant genetic heterogeneity.

Results from population genetic studies of *A. fulica* in this study indicated that the limited genetic diversity of the giant African snail was not a feature of the

molecular approaches used in this study but rather is caused by population and biological parameters (e.g., limited numbers of founders and/or pedigree individuals introduced to Thailand and an operation of strong genetic drift of *A. fulica*.

Meads (1961) concluded that the first snails had entered Thailand from infested areas in Malaysia by 1937. That observation corresponded with the estimations reported by Abbott (1949) and Rees (1951). However, ศักดิ์ศิริ เกิดปรีดี(2499) (Saksiri Kerdpreedee, 1956) and โชติ สุวัติถิ (2505) (Chote Suvatti, 1962) reported that *A . fulica* was introduced from Malaysia to some areas in Peninsular Thailand (Songkhla, Trang and Nakhon Si Thammarat) in 1934 by Chinese duck raisers. The giant African snail was found in Bangkok in 1956 (พิสิษฐ์ เสพสวัสดิ์, 2510) (Pisit sepsawat, 1967) or 1957 (Mead, 1961), Chanthaburi in 1958, Chiang Mai in 1964, Lampang in 1966, and Phrae in 1971 (สวาท รัตนวรพันธุ์ และ ประจง สุดโต, 2518) (Sawart Ratanaworobhan and Prajong Sudto, 1975).

Disregarding the Nakhon Ratchasima sample, a neighbor-joining tree from RAPD analysis indicated that *A. fulica* was probably first introduced to Songkhla and Pattani and subsequently spread to Bangkok and other places, respectively. Like other introduced species, anthropological transfer of *A. fulica* prevents the ability to identify a phylogeography of this species.

Isolation and characterization of stress-related transcripts

The giant African snail responds to various stress conditions (drought, starvation, and temperature changes) by aestivation. Nevertheless, no information about expression of stress-related genes is available for this species at present. Therefore, differentially expressed transcripts in the collar tissue of *A. fulica* in

response to dry conditions were identified and characterized using RAP-PCR. RT-PCR was carried out to evaluate the expression level of RAP-PCR derived markers.

Expression of an achacin (an antimicrobial peptide) gene was also determined. Host defenses in invertebrates relies on innate, non-adaptive mechanisms (Allen, 1983). Antimicrobial peptides (AMPs) are generally small cationic molecules that play an important role in the innate immune defense against bacterial and fungal pathogens (Boman, 1995) but some also exhibit antiviral or antiparasitic activity (Hancock and Diamond, 2000; Kansawa *et al*., 2004) and even antitumor activity (Cruciani, Barker, Zasloff, Chen and Colamonici, 1991). Achacin (and its cDNA) was the first AMP isolated from *A. fulica* (Obara *et al*., 1992; Satake *et al*., 1999).

The giant African snails exhibited full aestivation when the epiphragm was produced and completely enclosed the shell aperture (after approximately 15 days under stressed conditions). Therefore, the stage when the snails withdraw into the shell but did not produce an epiphragm was counted as the partial aestivation stage.

Several stress-related transcripts were isolated from different groups (normal, partial aestivation, and full aestivation) of *A. fulica*. Seven transcripts were cloned and sequenced. Nucleotide sequence comparisons indicated that they were newly isolated transcripts, and they were thus regarded as unknown genes.

A pair of primers was designed for each transcript, but only 3 markers (AFRAP2/228410, AFRAP9/138350, and AFRAP9/138470) were successfully amplified and subjected to quantitative RT-PCR analysis. Different expression patterns of these transcripts were observed. The expression levels of both achacin and AFRAP9/138350 were significantly decreased at the partial aestivation stage ($P<0.05$). These levels then increased back to the normal level for the former

(achacin) ($P>0.05$), but increased to a lesser level for the latter ($P<0.05$) at the full aestivation stage. The expression level of AFRAP2/228410 was up-regulated at the partial aestivation stage ($P<0.05$) prior to reduction to the normal level in the full aestivation snails ($P>0.05$). AFRAP9/138470 was up-regulated for both partial and full aestivation stages ($P<0.05$).

Likewise, Doungpunta (2004) isolated 7 stress-related fragments from hemocytes of the normal and heat-treated *P. monodon* by RAP-PCR analysis. It was found that 4 and 3 fragments were up- and down-regulated in response to the temperature, respectively. Four genes (RAP12, RAP16, RAP21 and RAP58) were cloned and sequenced. The expression level of these unknown transcripts was heat-induced.

Theoretically, severe environmental changes cause adaptive responses of organisms. Fluctuations of gene expression in response to stress conditions of *A. fulica* may cause serious physiological changes resulting in high mortality of snails If the aestivation process is prolonged.

In conclusion, morphometrics and several molecular techniques were used to estimate levels of genetic diversity or to identify population differentiation of *A. fulica* in Thailand and are first reported in this study. The ability to identify genetic heterogeneity of *A. fulica* is crucial for the construction of genetically based management programs in this species. Stress-related genes identified by this study illustrated alteration of gene expression due to environmental stress. Identical techniques can be applied for isolation and characterization of genes controlling important physiological and biological processes in this species as well as in other economically important species effectively.

CHAPTER VI

CONCLUSIONS

1. Morphometric analysis of *A. fulica* collected from 10 different locations in Thailand and Malaysia (N=215) using the K-mean clustering method did not reveal geographic differentiation of this taxon.

2. PCR-RFLP of COI (710 bp) with *Alu* I, *Dde* I, and *Rsa* I generated only one mitotype (AAA) across overall samples (N=207) implying a lack of COI polymorphism in *A. fulica*.

3. Eight and three patterns were found from SSCP analysis of COI215 and PMX of *A. fulica* (N = 207), respectively. A lack of genetic heterogeneity was observed between pairs of samples ($P>0.05$). The average genetic distance of COI215 and PMX ranged from 0.0000-0.0716. A neighbor-joining tree did not show a phylogeography of *A. fulica* in Thailand.

4. Twenty-two patterns were observed in RAPD analysis using OPA02, OPA17, OPB11, and OPZ09 primers (4, 6, 6, and 6 patterns, respectively). A total of 117 scorable bands (200-1,700 bp) were generated, 72 of which (61.54%) were polymorphic. The average genetic distance across all primers ranged from 0.0317-0.1378.

5. The primer OPB11 gave 14 RAPD fragments exhibiting significant genetic differentiation across all samples ($P<0.01$ for both the exact test and *F*-statistics). At the population level, OPB11 revealed significant genetic differences between the

Songkhla and each of the remaining samples ($P<0.001$) indicating that the gene pool of *A. fulica* in Thailand was not panmictic.

6. A neighbor-joining tree based on RAPD analysis implied a possible route of distribution of *A. fulica* in Thailand. Genetic heterogeneity and phylogenetic analysis suggested that *A. fulica* may have been initially introduced to Songkhla before subsequent anthropological distribution to other places in Thailand. In addition, *A. fulica* in Nakhon Ratchasima may be directly introduced from Malaysia.

7. Seven stress-related transcripts were isolated from different groups of *A. fulica* by RAP-PCR. Three of these unknown genes (AFRAP2/228410, AFRAP9/138350, and AFRAP9/138470) were successfully amplified and subjected to quantitative RT-PCR analysis.

8. The expression levels of a gene coding for achacin and of AFRAP2/228410, AFRAP9/138350, and AFRAP9/138470 genes in normal and stressed snails (partial and full aestivation) were significantly different ($P<0.05$) implying that environmental stress altered expression patterns of these genes

9. Population genetic studies of *A. fulica* at the macrogeographic scale using RAPD and additional approaches (microsatellite and amplified fragment length polymorphism: AFLP) should be carried out for evaluation of genetic diversity of this species more accurately.

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