POPULATION GENETIC STUDY OF THE MELON FLY PARASITOID, *PSYTTALIA FLETCHERI* SILVESTRI (HYMENOPTERA: BRACONIDAE) USING MICROSATELLITE MARKERS

SOMJIT TINKRATHOK

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……………………………………..
Miss Somjit Tinkrathok
Candidate

…………………………...…...……...…
Assoc. Prof. Sangvorn Kitthawee, Ph.D.
(Entomology)
Major-Advisor

……..……………………...............……
Asst. Prof. Suksiri Vichasri-Grams,
Dr. rer nat (Molecular Biology)
Co-Advisor

………………………………...………
Assoc. Prof. Mathurose Ponglikitmongkol,
Ph.D. (Molecular Biology)
Co-Advisor

…………………………...………………
Dr. Jeerapun Worapong,
Ph.D. (Plant Pathology)
Co-advisor

…………………………………
Prof. M. R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies

…………………………………………...
Prof. Pornsawan Visoottiviseth,
Ph.D.
Chair
Doctor of Philosophy Program in Biology
Faculty of Science

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on
7 June, 2007

……………………………………..
Miss Somjit Tinkrathok
Candidate

……………………..…..……..……
Assoc. Prof. Surin Peyachoknagul,
Dr. Agr.(Plant Molecular Biology)
Chair

…………………………..…...……..…
Assoc. Prof. Sangvorn Kitthawee,
Ph.D. (Entomology)
Member

……..…………………..............……
Asst. Prof. Suksiri Vichasri-Grams,
Dr. rer nat (Molecular Biology)
Member

…………………………...…………
Dr. Jeerapun Worapong,
Ph.D. (Plant Pathology)
Member

……………………………...………
Assoc. Prof. Mathurose Ponglikitmongkol,
Ph.D. (Molecular Biology)
Member

………………………………………
Prof. M. R. Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

…………………………………………...
Prof. Amaret Bhumiratana, Ph.D.
Dean
Faculty of Science
Mahidol University

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Somjit Tinkrathok

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SOMJIT TINKRATHOK 4436039 SCBI/D

Ph. D. (BIOLOGY)

THESIS ADVISORS: SANGVORN KITTHAWEE, Ph.D., SUKSIRI VICHASRI GRAMS, Dr. rer nat, MATHUROSE PONGLIKITMONGKOL, Ph.D., JEERAPUN WORAPONG, Ph.D.

ABSTRACT

Psyttalia fletcheri is an important endoparasitic wasp of the melon fly. It has been used as a biological control agent against the melon fly for nearly a century but biodiversity and population genetic information on this species is still not well understood. In this study, fourteen microsatellite loci were isolated using 5′ anchored PCR and enrichment methods. Two loci failed to amplify and five out of the remaining twelve amplifiable loci were found to be polymorphic among investigated samples in Thailand from Kanchanaburi, Chanthaburi, Nonthaburi, Nakhon Ratchasima, Chumphon, Phitsanulok, Uttaradit-A and Uttaradit-B. The mean number of alleles per locus ranged from 3.8 to 8.6 alleles. The mean observed heterozygosities across all loci for each population ranged from 0.224 to 0.412. Heterozygote deficiencies were observed in all populations, especially in Uttaradit-A, Nakhon Ratchasima, and Uttaradit-B populations where F_{IS} estimates were 0.352, 0.358 and 0.490, respectively. These values indicated a high level of inbreeding within those populations. Interestingly, both estimates, F_{ST} and R_{ST}, showed the significant genetic differentiation among populations, $\theta = 0.125$ and $\rho = 0.208$, respectively. This result is related to the estimates of pairwise F_{ST} between populations that were significantly greater than zero. Only two pairwise F_{ST} values between Uttaradit-A and B populations ($F_{ST} = 0.091$) and between Kanchanaburi and Phitsanulok ($F_{ST} = 0.024$) showed non significant population differentiation. The relationships between pairwise F_{ST} values and geographical distance among investigated populations were not significant. Nei's genetic distances and identities among populations were then calculated. These values indicated high levels of genetic identities among populations ranging from 88% to 98%, not including Uttaradit-A and B populations which were collected from different hosts. The genetic relationships among investigated populations were analyzed by constructing a UPGMA cladogram on the basis of Nei's genetic distances. The cladogram illustrated two distinctive clades: the Uttaradit-A population was separated into one clade by itself whereas the others were together within another clade. However, it was found that populations of Uttaradit-B seem to separate from the others in the latter clade. The genetic diversity in Uttaradit populations may be explained by geographical distribution and host-plant or host fly preferences or both. Results of this study can provide some information about genetic differentiation in the investigated populations of *P. fletcheri*. Further studies on genetic differentiation within this species over a larger geographical area may provide a clearer conclusion.

KEY WORDS: MELON FLY PARASITOID, PSYTTALIA, MICROSATELLITES,
POPULATION GENETICS

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การศึกษาพันธุศาสตร์ประชากรของแตนเบียนของแมลงวันแตงชนิด *PSYTTALIA FLETCHERI* SILVESTRI
(HYMENOPTERA: BRACONIDAE) โดยใช้ไมโครแซทเทิลไลท์มาร์คเกอร์
(POPULATION GENETIC STUDY OF THE MELON FLY PARASITOID, *PSYTTALIA FLETCHERI*
SILVESTRI (HYMENOPTERA: BRACONIDAE) USING MICROSATELLITE MARKERS)

สมจิตต์ ทินกระโทก 4436039 SCBI/D

ปร.ด. (ชีววิทยา)
คณะกรรมการควบคุมวิทยานิพนธ์: สังวรณ์ กิจทวี, Ph.D., สุขศิริ วิชาศรี กรามส์, Dr. rer nat, มธุรส พงษ์ลิขิตมงคล, Ph.D., จีรพันธ์ วรพงษ์, Ph.D.

บทคัดย่อ

Psyttalia fletcheri เป็นแตนเบียนชนิดหนึ่งที่มีความสำคัญต่อการควบคุมประชากรของแมลงวันแตงโดยชีววิธี ได้มีการนำแตนเบียนชนิดนี้ไปทดลองใช้ในการควบคุมประชากรของแมลงวันแตงเป็นระยะเวลานาน แต่ความรู้พื้นฐานในด้านความหลากหลายทางชีววิทยาและพันธุศาสตร์ประชากรของแมลงเบียนชนิดนี้ยังไม่เป็นที่ทราบที่แน่ชัด ในการ ศึกษาครั้งนี้ได้มีการพัฒนาไมโครแซทเทิลไลท์มาร์คเกอร์จำนวน 14 ตำแหน่ง (loci) โดยใช้วิธี 5′ anchored PCR and enrichment พบว่ามีจำนวน 2 ตำแหน่ง ที่ไม่สามารถเพิ่มปริมาณดีเอ็นเอโดยวิธีพีซีอาร์ และจำนวน 5 ตำแหน่งที่มีความหลากหลายในการทดสอบกับประชากรของแมลงเบียนที่นำมาศึกษาจากพื้นที่ต่าง ๆ ในประเทศไทย ดังต่อไปนี้ กาญจนบุรี จันทบุรี นนทบุรี นครราชสีมา ชุมพร พิษณุโลก อุตรดิตถ์-A และ อุตรดิตถ์-B ค่าเฉลี่ยของจำนวนอัลลีลต่อตำแหน่งที่พบในแต่ละประชากรอยู่ระหว่าง 3.8 ถึง 8.6 อัลลีล ในขณะที่ค่าเฉลี่ยของ observed heterozygosity ในแต่ละประชากรอยู่ระหว่าง 0.224 ถึง 0.412 นอกจากนี้ค่า F_{IS} (inbreeding coefficient) ของทุกประชากรที่นำมาศึกษายังแสดงให้เห็นถึงภาวะขาดแคลน heterozygotes ซึ่งชี้ให้เห็นถึงระดับ inbreeding ในประชากรมีค่อนข้างสูง โดยเฉพาะในประชากรจาก อุตรดิตถ์-A นครราชสีมา และ อุตรดิตถ์-B ซึ่งมีค่า F_{IS} = 0.352, 0.358 และ 0.490 ตามลำดับ จากการประมาณค่าเฉลี่ย F_{ST} ($\theta = 0.125$) และ R_{ST} ($\rho = 0.208$) พบว่ามีความแตกต่างทางพันธุกรรมระหว่างประชากรที่นำมาศึกษาอย่างมีนัยสำคัญ ซึ่งสอดคล้องกับค่า pairwise F_{ST} เมื่อเปรียบเทียบความแตกต่างทางพันธุกรรมระหว่างประชากรแต่ละคู่ มีจำนวนสองคู่เท่านั้นที่ไม่พบความแตกต่างอย่างมีนัยสำคัญคือ ระหว่างอุตรดิตถ์-A และ อุตรดิตถ์-B (F_{ST} = 0.091) และ ระหว่างกาญจนบุรี และ พิษณุโลก (F_{ST} = 0.024) นอกจากนี้ยังพบว่าความแตกต่างทางพันธุกรรมระหว่างประชากรไม่ขึ้นอยู่กับระยะทาง (กิโลเมตร) ของแต่ละตัวอย่างประชากรที่นำมาศึกษา จากการคำนวณค่า Nei’s genetic identities ระหว่างประชากร พบว่าประชากรที่นำมาศึกษามีความใกล้ชิดทางพันธุกรรมสูง คือมีค่าระหว่าง 88% ถึง 98% โดยไม่รวมประชากรจาก อุตรดิตถ์-A และ อุตรดิตถ์-B เมื่อสร้าง UPGMA cladogram จากค่า genetic distances พบว่า ประชากรจากอุตรดิตถ์-A ถูกแยกออกไป จากประชากรอื่นอย่างชัดเจน ส่วนประชากรที่เหลือทั้งหมดถูกรวมอยู่ในกลุ่มเดียวกัน โดยที่ประชากรจากอุตรดิตถ์-B มีแนวโน้มที่จะแยกห่างจากประชาการอื่น ๆ ในกลุ่มเดียวกัน อธิบายได้ว่าประชากรของ *P. fletcheri* จาก อุตรดิตถ์ ถูกแยกออกมาจากประชากรอื่น ๆ อาจเนื่องมาจากสภาพทางภูมิศาสตร์ และ ความจำเพาะต่อชนิดของพืชหรือแมลงอาศัย (host-plant or host fly) หรือทั้งสอง ผลการศึกษาครั้งนี้สามารถอธิบายถึงโครงสร้างและความแตกต่างทางพันธุกรรมของประชากร *P. fletcheri* ที่นำมาศึกษา อย่างไรก็ตามการขยายพื้นที่ในการศึกษาประชากรของแมลงเบียนชนิดนี้ให้ครอบคลุมมากขึ้นในอนาคตอาจช่วยให้อธิบายและสรุปผลในด้านความแตกต่างทางพันธุกรรมในประชากรได้ชัดเจนมากขึ้น

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LIST OF ABBREVIATIONS

A	adenine
A_{260}	absorbance at 260 nanometer
ATP	adenosine triphosphate
APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
C	cytosine
ddH_2O	deionized water
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
et al.	et alii (Latin), and others
G	guanine
g	gram
HCl	hydrogen chloride
i.e.	id est (that is)
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
KCl	potassium chloride
LB	Luria broth
M	molar
mg	milligram
min	minute
µJ	microJoule
ml	milliliter
mm	millimeter

LIST OF ABBREVIATION (CONT.)

mM	millimolar
mRNA	messenger ribonucleic acid
NBT/BCIP	Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
NCBI	National Center for Biotechnology Information
NEB	New England Bio laboratory
nm	nanometer
nM	nanomolar
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	second
SSC	sodium chloride-sodium citrate buffer
SSCP	Single-Stranded Conformational Polymorphism
T	thymine
Ta	annealing temperature
TB	terrific Broth
TE	tris-ethylenediamine tetraacetic acid
TEMED	N,N,N´,N´-tetramethylethylenediamine
Tm	melting temperature
TBE	tris-borate-ethylenediamine tetraacetic acid
U	unit
UV	ultraviolet
USDA	United States Department of Agriculture
V	voltage
W	watt
w/v	weight by volume

LIST OF ABBREVIATION (CONT.)

X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
µg	microgram
µl	microliter
µm	micromolar

CHAPTER I

INTRODUCTION

The melon fly, *Bactrocera cucurbitae* (Coquillett), an indigenous species of pest in Tropical Asia, is now the pest that causes serious damage to many species of fruits and vegetables especially cucurbits throughout Asia, Oceania, Indo-Pacific and some part of Africa (Chinajariyawong et al., 2000; Koyama et al., 2004; Dhillon et al., 2005). These fruits and vegetables are, for example, bitter gourd (*Momodica charantia*), cucumber (*Cucumis sativas*), muskmelon (*Cucumis melo*), snake gourd (*Trichosanthes anguina*), ivy gourd (*Coccinia grandis*) including non cucurbits such as tomato, chilies, mango, papaya, string bean, okra, and guava. In Thailand, melon fly is a very serious pest of cucurbit productions (Chinajariyawong et al., 2000). Therefore, it was identified as one of five important pest of agriculture in South East Asia (Waterhouse, 1993). To control this important pest, the integrated strategies such as chemical sprays, fruit bagging, trapping, field sanitation, male sterile releasing, and its natural enemies are required (DeBach & Rosen, 1991; Chinajariyawong et al., 2000; Dhillon et al., 2005).

Psyttalia fletcheri (Silvestri) is an endoparasitoid (Hymenoptera: Braconidae) that has been reported to be a major parasitoid of melon fly. It was released and quickly became established as an effective biological control agent (Nishida, 1955; Clausen et al., 1965). Of all the parasitoids used for melon fly control, *P. fletcheri*, which was introduced from India in 1916, was the most effective control agent (Fullaway, 1920; Clausen et al., 1965). The average parasitization rates ranged from 5-44%, depending on the season (Newell et al., 1952). In Hawaii, the natural enemies of fruit flies were surveyed globally for effectiveness, introduced into a controlled setting and then released it into the environment in order to control the heavy infestation of fruit flies, including *B. cucurbitae* (Clausen et al., 1965). In Thailand, *P. fletcheri* was found as a dominant parasitoid in cucurbits that had been attacked by melon flies in every part of country (Chinajariyawong et al., 2000). Nonetheless, *P. fletcheri* has not been used as a biological control agent to control the melon fly in

Thailand since biodiversity and population genetic information of this species is still poorly described and clarified.

Several genetic markers have been used for evaluating genetic variations in organisms. For example, allozymes, RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SSCP (Single-Strand Conformational Polymorphism) and SSR (Simple Sequence Repeats) or microsatellite markers (Loxdale & Lushai, 1998; Caterino et al., 2000; Avise, 2004). Of all the above genetic markers, Tautz (1989) noted that the greatest frequency of variations found in microsatellite markers. Moreover, microsatellites were not only showed extensive length polymorphisms between different individuals of the same species; they were also found higher incidences of replicability, co-dominance and require smaller amounts of DNA for purposes of analysis (Litt & Luty, 1989; Tautz, 1989; Weber & May, 1989). From these reasons, microsatellite markers become the most popular genetic markers used for population genetic analysis (Avise, 2004).

Microsatellite markers of several hymenopterans have been developed for studying genetic variations within and among populations, for example, honey bee (*Apis mellifera*) which a large amount of genetic variation was detected by using seven microsatellite loci (Estoup et al., 1995), thirteen microsatellite loci for the fig wasp, genus *Pegoscapus*, were developed and those microsatellite makers can be used to distinguish the cryptic *Pegoscapus* species (Molbo et al., 2002). In addition, a number of microsatellite markers of parasitic wasps, such as, *Diaeretiella rapae* (Hymenopetera: Braconidae), *Aphidus ervi* (Hymenopetera: Braconidae) were developed and successful for population genetic analysis (Baker et al., 2003; Hufbauer et al., 2004). However, at the time of this study, there were no reports found in either DNA databases or related scientific journals about the microsatellite markers and population genetic study of melon fly parasitoid, *P. fletcheri*.

Thereby, analysis of the population genetic structure of melon fly parasitoid, *P. fletcheri* from different localities of Thailand using microsatellite markers was performed to reveal genetic variations within and between populations of *P. fletcheri*. Microsatellite markers of *P. fletcheri* would be firstly elucidated here in this research.

CHAPTER II

SPECIFIC RESEARCH QUESTIONS AND OBJECTIVES

2.1 Specific research questions

The researcher sought to answer the following questions:

(1) What are the characteristics of microsatellites in *P. fletcheri*?

(2) What methodologies and procedures can be used to detect genetic variations within and between populations?

(3) How much genetic variation appears in natural populations of *P. fletcheri* in Thailand?

2.2 Objectives

This research posed the following specific objectives:

(1) To develop microsatellite markers of *P. fletcheri*.

(2) To determine genetic variations within and between populations of *P. fletcheri* in Thailand using microsatellite markers.

CHAPTER III
LITERATURE REVIEW

3.1 Description of fruit fly parasitoids

Parasitoids are insects that parasitize other insects. The parasitic process takes place when the female parasitoids attack their host and lay eggs in or on the host body using highly specialized ovipositors. Subsequently, parasitoid larvae develop by feeding on the host body and then pupate inside or outside the host, eventually killing the host (Godfray, 1994). The adult stage of the parasitoid is free living; that is, the insect requires food such as honeydew, nectar or pollen. Some parasitoid adults feed on the fluid of the host body, while others require only fresh water (DeBach & Rosen, 1991; Godfray, 1994).

Taxonomically, parasitoids make up a very large group of insects — including diobiont parasitoids, which live outside the host, and koinobiont parasitoids that are further subdivided into endoparasitoids, which develop inside of the host, and ectoparasitoids, which develop outside the host body.

Parasitoids of the Tephritid fruit fly that are a particularly important for fruit fly control programs. Over 100 species of fruit fly parasitoids have been recorded as parasitoids in the family Braconidae, subfamily Opiinae (Wharton, 2007) — fruit fly parasitoids that are important natural enemies of the fruit flies. Generally, each species of fruit fly parasitoids has a different host preference. For example, Oriental fruit fly parasitoid is the parasitoid that prefers to attack the Oriental fruit fly, *Bactrocera dorsalis* (Hendel); Mediterranean fruit fly parasitoid is the parasitoid that prefers to attack the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann); and melon fly parasitoid is the parasitoid that is prone to attack the melon fly, *Bactrocera cucurbitae* (Coquillett) as its major host fly (Wharton & Giltrap, 1983).

Fruit fly parasitoids have a different manner for attacking their host. Fruit fly parasitoids are koinobiont endoparasitoids — parasitoids that allow the host to continue its development and often do not kill or consume the host until the host is about to either pupate or become an adult. Generally, fruit fly parasitoids prefer to attack their host at the egg or larval stage and then emerge as adult from the host puparium (Wharton, 2007). Parasitoids that prefer to attack their host at the egg stage are called egg parasitoids; whereas, parasitoids that prefer to attack their host at the larval stage are called larval parasitoids. Additionally, parasitoids can also be categorized according to the number of parasitoids that are successfully developed per individual host. Parasitoids that developed singly within an individual host are known as solitary parasitoids; whereas, parasitoids that develop in numbers ranging from two to many individuals per one host are called gregarious parasitoids (DeBach & Rosen, 1991; Godfray, 1994). However, superparasitism — parasitism of the same host by two or more individuals of parasitoids but generally only one parasitoid can survive. Many of the fruit fly parasitoids that have been used as biological control agent against fruit flies are solitary fruit fly parasitoids because they are the most effective for host elimination because only one parasitoid is needed to kill one host.

3.2 Classification of fruit flies and parasitoids

3.2.1 Taxonomy of fruit flies

Fruit flies are insect pests that belong to the family Tephritidae. The literature has identified over 4,000 recognized species, grouped in an estimated 500 genera. Approximately 70 species have been defined as economically important agricultural pests (e.g., Mediterranean fruit fly (*C. capitata*), Oriental fruit fly (*B. dorsalis*), the melon fly (*B. cucurbitae*), the olive fruit fly (*B. oleae*), Queensland fruit fly (*B. tryoni*), and the peach fruit fly (*B. zonata*) (White & Elson-Harris, 1992). Fruit flies are classified as follows:

Kingdom: Animalia (Animals)
  Phylum: Arthropoda (Arthropods)
    Class: Insecta (Insects)
      Order: Diptera (Flies)
        Superfamily: Tephritoidea
          Family: Tephritidae (Fruit flies)

3.2.2 Taxonomy of parasitoids

The major groups of parasitoids belong to the order Hymenoptera that are the most biologically diverse group of insects with approximately 150,000 described species (Gaston, 1991; LaSalle & Gauld, 1991). Approximately 50,000 species of parasitoids have been identified and a lot are waiting to be discovered and identified (Pennacchio & Strand, 2006).

Hymenopterans have been classified into two suborders, the Symphyta and the Apocrita. The Symphyta contains a few species of parasitoids whereas the Apocrita is dichotomized into two major divisions, the Parasitica and the Aculeata. Most Aculeata are predators and pollen collectors. Only few species in this division are characterized as parasitoids. As its name implies, almost of the Parasitica are parasitoids. The Parasitica are further subdivided into seven superfamilies and 30 families (Godfray, 1994) (Table 3.1).

Psyttalia fletcheri Silvestri is a fruit fly larval endoparasitoid of the family Braconidae. Before 1987 most of the species of *Psyttalia* were placed in the genus *Opius* and *Psyttalia* was treated as a subgenus of *Opius* (Fischer, 1972). The *Psyttalia* was elevated to the generic range by Wharton (1987). In summary, the melon fly parasitoid is classified as follows:

- Kingdom: Animalia
  - Phylum: Arthropoda
    - Class: Insecta
      - Order: Hymenoptera
        - Suborder: Apocrita
          - Family: Braconidae
            - Subfamily: Opiinae
              - Genus: *Psyttalia*
                - Species: *fletcheri* Silvestri
              - Common name: Melon fly parasitoid

Table 3.1 Superfamilies and families of Hymenoptera containing parasitoids and the estimated number of described species found in the world fauna (Godfray, 1994).

Suborder	Division	Superfamily	Family	Estimated described species
Symphyta		Orussoidea	Orussidae	75
Apocrita	Aculeata	Chrysidoidea	Dryinidae	850
			Bethylidae	2000
			Chrysididae	3000
		Vespoidea	Tiphiidae	1500
			Pompilidae	4000
	Parasitica	Trigonalyodae	Trigonalyidae	70
		Evanoidea	Evaniidae	400
			Aulacidae	150
			Gasteruptiidae	500
		Cynipoidea	Ibaliidae	9
			Figitidae	125
			Eucoilidae	1000
			Charipidae	1200
		Chalcidoidea	Leucospidae	139
			Chalcididae	1500
			Eurytomidae	1100
			Torymidae	1500
			Agaonidae (Fig wasp)	800
			Eucharitidae	350
			Pteromalidae	3100
			Signiphoridae	75
			Encyrtidae	>3000
			Aphelinidae	900
			Eulophidae	>3000
			Trichogrammatidae	532
		Proctotrupoidae	Proctotrupidae	334
			Daipriidae	2028
			Scellionidae	2768
			Platygastridae	987

Table 3.1 Superfamilies and families of Hymenoptera containing parasitoids and the estimated number of described species found in the world fauna (Godfray, 1994). (continued)

Suborder	Division	Superfamily	Family	Estimated described species
		Ceraphronoidea	Megaspilidae Ceaphornidae	250
		Ichneumonoidea	Ichneumonidae	15000
			Braconidae	10000

There were approximately 50 species are categorized as members of the genus *Psyttalia* (Fischer, 1972; Fischer, 1987; Wharton, 1987). Seven species of this genus were recorded in Thailand by Chinajariyawong et al. (2000). Three of them were identified as *P. fletcheri, P. incisi, and P. makii* and another four species were classified as *Psyttalia* sp. 1, *Psyttalia* sp. nr (near) *fletcheri*, *Psyttalia* sp. nr *incisi* and *Psyttalia* sp. nr *makii*. These species are similar in morphological characters but their host preferences are different, for example, the host preference of *P. fletcheri* is the melon fly, *B. cucurbitae*, whereas *B. dorsalis* is the major host of *P. incisi* and *P. makii* (Wharton & Gilstrap, 1983).

3.3 Life history and biology of melon fly parasitoid

3.3.1 Life cycles of melon fly parasitoid

Melon fly parasitoid, *P. fletcheri* is hypermetamorphosis insect that has four distinctive stages of metamorphosis, egg, larva, pupa and adult as same as complete metamorphosis insects but there are two or more different stage of larva. The life cycle of melon fly parasitoid starts when the adult female lays eggs into the third instar larva of its host. Within 37-40 hours after implantation, the egg hatches into a larva, passes through four stages of instar, pupates, and then emerges as adult within 4-8 days, whereupon, the host is killed (Willard, 1920; Bautista et al., 2000). Adult females of melon fly parasitoid (Fig 3.1) live as free living and are ready to mate as soon as they emerge as adults.

3.3.2 Genetic system of melon fly parasitoid

The genetic system of melon fly parasitoid is classified as haplodiploid as same as other hymenopterans. Male develops from an unfertilized egg and it has haploid genome (n) whereas female develops from a fertilized egg, therefore it has diploid genome (2n) (Lester & Selander, 1979; Cook, 1993). Thus, the genetic variability among individual insects in the order Hymenoptera is significantly lower than what is found among other insects, particularly eusocial insects that result from inbreeding (Graur, 1985). However, the diploid male of some hymenopterans can occasionally be found either under laboratory condition or wild condition (Periquet et al., 1993; Holloway et al., 1999).

Figure 3.1 Adult female of melon fly parasitoid, *P*. *fletcheri* Silvestri (Bauer, 2006).

3.4 Melon fly biology and distribution

3.4.1 Host plants of melon fly

Melon fly, *B. cucurbitae* is one of a serious pest that has the wide range of host plants. Over 125 plant species are vulnerable to infestation and damage, particularly cucurbits (Weems, 1964). Typically, the hosts of melon fly are young fruits of the Cucurbitaceous but receptacles, stalks, vines and stems are also known to be attacked. Numerous species of plants beyond the Cucurbitaceae family were found to be alternative hosts for melon fly (Clausen et al., 1965; Meksongsee et al., 1988; Chinajariyawong et al., 2000). The adaptation of the melon fly to alternative hosts has significantly contributed to the economic importance of this pest. For example, many species of cucurbits such as cucumber (*Cucumis sativus*), zucchini (*Cucubita pepo*), bitter melon (*Minordica charantia*), ivy gourd (*Coccinia grandis*), including some of non-cucurbits, have been destroyed by melon fly that results in significant economic losses world wide (Nishida, 1956; Messing et al., 1996; Chinajariyawong et al., 2000).

3.4.2 Melon fly life cycle

The life cycle of melon fly consists of four distinctive stages, egg, larva, pupa and adult (Fig. 3.2). The development of melon fly is hypermetamorphosis that there are three larva stages. The female fly lays a number of eggs, approximately 1-40 eggs per clutch that are able to penetrate the skin of fruits with the use of its ovipositor (Fletcher, 1989). One female may lay over 1000 eggs during her life time (Anonymous, 2000). Approximately 24 hours after oviposition, the eggs hatch into larvae and the larvae feed on the flesh of fruit and cause damage to the infested fruits. The melon fly larvae grow in size by molting which classified into 3 stages, first instar, second instar and third instar. The last stage of the larva goes to the pupal stage by twitching out of the fruit and tunneling into the soil for protection until it develops for 7 days into the adult fly. The adult female begin to mate and lay eggs about 2 weeks after emergence from pupa (Hollingsworth & Allwood, 2000). Adults are long life, typically for five months in the tropics and up to fifteen months in cool climate (Anonymous, 2000).

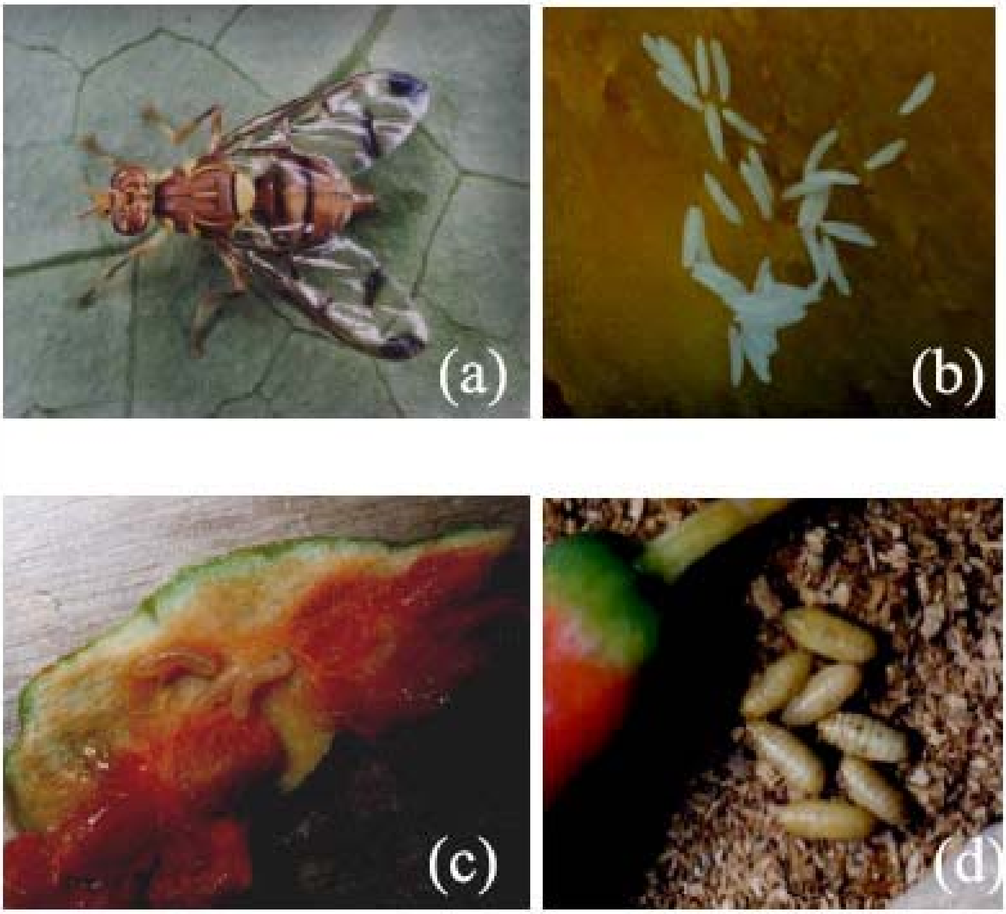

Figure 3.2 Four stages of melon fly, *B. cucurbitae*, (a) adult female, (b) eggs, (c) larvae, and (d) pupae (Hollingsworth & Allwood, 2000).

3.4.3 The origin and distribution of melon fly

Melon fly, *B. cucurbitae*, an indigenous species of Tropical Asia, but has now spreads throughout the world (Hollingsworth & Allwood, 2000). Based on the Technical Bulletin of the United States Department of Agriculture (USDA) (Clausen et al., 1965), melon fly has been recorded as a major pest of cucurbit plants, not only in Tropical Asia but in many other areas of the world such as Hawaii, Africa, Indo-Australia and Pacific Islands. Introduction of melon fly to new geographic regions has occurred accidentally by the transportation of fruits which carried the fruit fly eggs or larvae (Clausen et al., 1965). Recent fruit fly surveys and research has revealed the presences of melon fly in virtually every part of Thailand and has been the major cause of damage to cucurbit production in both the corporate agricultural sector as well as among small family farms (Meksaongsee et al., 1988; Chinajariyawong et al., 2000).

3.4.4 Strategies to control melon fly

Eradication of melon fly and others fruit flies from the corporate agricultural and small family farms sectors is very big issue that requires an integrated pest management (IPM) program (Meksaongsee et al., 1988; DeBach & Rosen, 1991; Dhillon et al., 2005). For example, many of the following strategies have been used to control the pest population: pesticide use, sterile insect technique, trapping, fruit wrapping, crop rotations, sanitation, and biological control. The biological control strategy using natural enemies to control insect pest is a classical way and is now widely used in many areas of the world because it does not cause environmental pollution nor pose health risks to human (DeBach & Rosen, 1991). However, this strategy needs to be integrated with other biological knowledge and practice. The application of biological control agents needs to be understood in relation to their biological impact to ecology, behavior and genetic background. Such knowledge is helpful in order to manage their application in ways that maximize high efficiency of pest controls.

3.5 Melon fly parasitoid as biological control agent

One of natural enemies of melon fly is a parasitic wasp, *P. fletcheri* — a native species of Indo Pacific. It was introduced to Hawaii from India for melon fly control since 1916 (Fullaway, 1920; Nishida, 1956; Wharton & Gilstrap, 1983). Once it had become widely established and deployed throughout the Hawaiian Islands, the melon fly parasitoid contributed to the reduction of *B. cucurbitae* populations (Nishida, 1956). In addition, *P. fletcheri* was also introduced from Hawaii to the other Pacific Islands, such as Guam, Solomon Islands for melon fly control (Anonymous, 2004). In Thailand, *P. fletcheri* has been found to be the most common parasitoid among various kinds of cucurbit fruits, including some non-cucurbits (Clausen et al., 1965; Meksongsee et al., 1988; Chinajariyawong et al., 2000). From these reports, *P. fletcheri* has the potential to be an effective biological control agent in the future if the basic knowledge is developed on how to use it as a melon fly control agent.

3.6 Molecular markers for population genetic studies

Various molecular biology techniques have been developed for using as genetic markers, starting with the protein electrophoresis method that applied to allozyme and isozyme systems (Lewontin & Hubby, 1966). As a result of the discovery of DNA restriction enzyme (Meselson & Yuan, 1968), the next technique that became popular in the late 1970s and 1980s was the RFLP (Restriction Fragment Length Polymorphisms) method (Avise, 2004). At same time, mtDNA (mitochondrial DNA) polymorphism became as dominant as allozyme studies. In the 1990s, The discovery of the PCR (Polymerase Chain Reaction) technique, a revolutionary technique which lead to the rapid development of many other molecular techniques such as DNA sequencing (Sanger et al., 1977), RAPD (Random Amplified Polymorphic DNA), SSCP (Single-Strand Conformational Polymorphisms), AFLP (Amplified Fragment Length Polymorphisms), STR (Short Tandem Repeat) or microsatellites (Avise, 2004).

Presently, there are many molecular markers used for a wide range of insect studies, depending on the specific research focus, cost, time, application, and so on (Loxdale & Lushai, 1998; Behura, 2006). The following passages describe samples of genetic markers that have been used for population genetic studies in insects, especially in hymenopterans.

3.6.1 Allozymes/Isozymes

Allozyme is a term that represents products of different alleles at a specific locus of the given enzyme. Though the enzymes are produced at different loci, their function is similar. Hence, these enzymes are called isozymes.

Allozymes/isozymes are the most popular protein markers that can be separated on starch gel or polyacrylamide gel electrophoresis by the variation of charge-size of the enzyme (Loxdale & Lushai, 1998; Avise, 2004). Protein markers have been widely used for over a decade because they are inexpensive, co-dominant, and relatively simple to use as a method to investigate genetic variations of various organisms such as small insects like wasps, ants and aphids (Loxdale & Lushai, 1998). The genetic variability within and among populations of social wasps, solitary wasps and fruit fly parasitoids was estimated using allozyme markers and the results of such studies has revealed that heterozygosity were significantly low (Lester & Selander, 1979; Kitthawee et al., 1999). One reason that scientists have found less genetic variations among hymenopterans than among the other insects is because the genetic system of hymenopterans are haplodiploid that causes inbreeding and/or parthenogenesis (Lester & Selander, 1979; Cook, 1993; Butcher et al., 2000).

3.6.2 RFLP (Restriction Fragment Length Polymorphism)

The RFLP is a molecular technique that has been used to observe genetic diversity of various organisms. The RFLP analysis is based on two molecular techniques: the restriction endonuclease digestion and the hybridization. The different lengths of DNA fragments derived from restriction enzyme digestion and those DNA fragments can be separated and compared to the DNA sized standard by agarose gel, electrophoresis then transferred DNA fragments to filter membrane and hybridized with DNA probe. The polymorphism of restriction fragment lengths of each DNA sample revealed by mutations of the DNA at a recognition sites resulting of loss or creation the new recognition sites of restriction enzymes. The RFLP technique can be used as genetic markers for linkage map analysis, phylogenetic analysis, and the determination of genetic diversity among species and among individuals of the same species. RFLP can also be used for segregation analysis of progenies because it is stable and reproducible. However, one major drawback of the RFLP technique relates to the high quality and quantity of DNA needed to achieve discernible results, in addition to high cost and labor requirements. Thus, this maker is not appropriate for small organisms such as ants, aphid, parasitoids and so on (Dowling et al., 1996; Brettschneider, 2001; Avise, 2004).

3.6.3 RAPD (Random Amplified Polymorphic DNA)

The RAPD (Random Amplified Polymorphic DNA) is a dominant marker that has been widely used for genetic diversity screening since 1990 (Avise, 2004). Genetic variations are detected by noting the presence or absence of random amplified polymorphic DNA that are generated by PCR using arbitrary primers (e.g., 10-12 bp). The principle that lies behind this method involves random priming with the target DNA and the DNA that lies between the priming sites should be amplified (Williams et al., 1990). If the sequences of DNA within the PCR priming sites are changed or mutates, then the patterns of amplification products of the given primer also change. However, there are many other factors that can affect the amplification of DNA, in addition to changing the DNA priming sites (e.g., concentration of primer and template, PCR reagents and annealing temperature). Additionally, another defect of RAPD relates, in some case, to its poor reproducibility (Penner et al., 1993; Perez et al., 1998). For these reasons, the use of RAPD has decreased in recent years (Avise, 2004).

3.6.4 AFLP (Amplified Fragment Length Polymorphism)

The AFLP method is a modification of RFLP analysis. It was developed by Vos et al., (1995). This technique is based on selected PCR amplification of restriction fragments of digested genomic DNA. The polymorphisms are shown as presence and absence of restriction fragments rather than length differences; therefore, they appear as dominant marker as one would find in RAPD but are more reliable and reproducible. AFLP analysis begin with total genomic DNA digestion and ligation with oligonucleotide adapters, selective amplification of restriction fragments concludes with gel electrophoresis of amplified fragments (Vos et al., 1995). Both RAPD and AFLP techniques do not require the information of DNA sequences and are suitable for the study of genetic diversity of various organisms such as plants, small insects, and animals (Tracey et al., 2004).

3.6.5 DNA sequencing

In recent years, the numbers of documented nucleotide sequences have increased exponentially. At the beginning of the year 2003, nearly 25 million sequences, up from 115,000 taxa, were deposited into the GenBank — an open access, annotated collection of all publicly available nucleotide sequences and their protein translations (Avise, 2004). The nucleotides in each position of DNA sequence alignment have been used to characterize a particular state for DNA sequencing analysis. The changes of nucleotides at a given position can be conversed into the quantitative measurement of genetic distance (Hillis et al., 1996). The explosion of DNA sequencing information has led to many approaches to genetic research such as ribosomal RNA gene (rRNA), mitochondrial DNA (mtDNA) sequencing analysis and so on. The most frequently used DNA sequencing analyses for population genetic studies are non-coding region DNA (e.g. Internal Transcribed Spacer I (ITS1) and Internal Transcribed Spacer II (ITS2) of nuclear rRNA genes) (Wu et al., 2004; Thanwisai et al., 2006). The advantage of using nucleotide sequencing analyses is that it provides detailed genetic information. Moreover, the silent mutations can be detected by nucleotide sequence alignments, whereas other techniques cannot detect silent mutations. Notwithstanding the advantage of nucleotide sequencing is not the best method for studying genetic variations at the individual level of population that involves many loci and large numbers of individual of organisms are required (Avise, 2004).

3.6.6 Animal mitochondrial genome

Animal mitochondrial DNA (mtDNA) is an extrachromosomal DNA, typically closed circular, double stranded molecule, ranging in size length from 15-20 kb. Only the cnidarians classed Cubozoa, Scphozoa and Hydrozoa have been found to have a linear mtDNA molecule (Bridge et al., 1992). With few exceptions, all mitochondrial genomes compose of 37 genes coding for 22 tRNAs, 2 rRNAs, and 13 mRNAs and specify the synthesis of proteins involved in the electron transport system and oxidative phosphorylation (Boore, 1999; Avise, 2004). The evolution of the mitochondrial genome is independent of the nuclear genome (Moritz et al., 1987) and is known to have a high mutation rate when compared to the nuclear genome but the gene order of mtDNA is relatively conserved (Boore, 1999). Because of its compact size, it is possible to sequence the entire genome. At present, several complete mitochondrial genomes of insects have been sequenced, for example honey bee, *Apis mellifera* (Crozier & Crozier, 1993), *Anopheles gambae* (Beard et al., 1993), *Drosophila yacuba* (Clary & Wolstenholme, 1985) and Mediterranean fruit fly, *Ceratitis capitata* (Spanos et al., 2000). In addition, the partial mtDNA sequences of numerous insect species are also being documented in notable DNA databases. These available sequences have substantially contributed to taxonomies and population studies (Simon et al., 1994). Universal primers for mtDNA amplification have been designed and are being used across the entire insect mtDNA (Loxdale & Lushai, 1998). Many population genetic studies of insects, such as parasitic wasp (*Ahidus ervi*), blowflies (*Lucilia spp*), and Oriental Fruit Fly, *Bactrocera dorsalis* (Stevens et al., 2002; Hufbauer et al., 2004; Shi et al., 2005) were conducted using differentiation of mtDNA sequences. Recently, 2041 bp of mtDNA, comprising 528 bp of the 3′ end of 16S rRNA, 841 and 596 bp of COI and COII segment, respectively have been used as genetic markers for studying genetic differentiation of aphid parasitic wasp, *Diaeretiella rapae* which were collected from different hosts. It has been found unique 42 haplotypes but the association between mtDNA haplotypes and host species were not found in either the ancestral range or part of the introduced range (Baer el al., 2004). Additionally, the 1249 bp of mtDNA sequences have been used to examine the population genetics of native and introduced aphid parasitoid, *Aphidius ervi* (Hymenoptera: Braconidae) that was introduced to North America from Western Europe. One of two common haplotypes was shared among native and introduced populations (Hufbauer et al., 2004). However, the problems of using mtDNA as a marker in population, phylogeographic and phylogenetic studies have been reported due to the effect of inherited symbionts that can reduce or increase the diversity of mtDNA, especially in arthropods. For this reason, mtDNA is not the best methodology for studying the recent history or evolution of arthropods (Hurst & Jiggins, 2005).

3.6.7 SSCP (Single-Strand Conformational Polymorphism)

SSCP is a rapid and sensitive method for genetic variation detection of denatured PCR products, ~300 bp in length, through neutral polyacrylamide gel electrophoresis analysis (Orita et al., 1989). This type of polymorphism is generated from a single-stranded DNA that typically folds back upon itself to form a complex of three-dimensional structure. Even the presence of a single nucleotide substitution may results in an altered conformation that affects the electrophoretic mobility of the neutral polyacrylamide gel, particularly under special conditions that do not allow the analyzed DNA fragment to renature (Hartl, 2000; Avise, 2004). For these reasons, the SSCP technique becomes widely used for distinguishing genetic variation in any region of the genome, for example RAPD-SSCP of parasitic wasp, *Bracon hebeto*r and *Aedes aegypti* (Antolin et al., 1996), 16S rRNA and Cytochrome oxidase (i.e., COI and COII) of the aphid parasitic wasp, *Diaeretiella rapae* (Baer et al., 2004), partial ND1 gene (882 bp) and partial cytochrome b gene (750 bp) of red wood ant, *Formica lugubris* (Gyllenstrand & Seppa, 2003). and nuclear genome (Boge et al., 1994; Borsa & Coustau, 1996). In addition, a large number of individual can be rapidly screened for genetic variation rather inexpensively even if each haplotype requires sequencing.

3.6.8 Microsatellites

Microsatellites are known as Simple Sequence Repeats (SSR) (Tautz & Renz, 1984) or Short Tandem Repeats (STRs) (Edwards et al., 1991) and are composed of tandemly repeated motif (approximately 1-6 bp). Typically the entire sequence of the repeat array is less than 100 bp in length (Tautz, 1989). The numbers of base pairs in each repeat motif are used to classify the types of microsatellites to mono-, di-, tri-, tetra-nucleotide repeats that composed of one, two, three and four base pairs in each repeat motif, respectively. For example, (A/T)n, (CA/GT)n, (CAG/GTC)n, (AATT/TTAA)n where “n” is the number of repeats.

Microsatellite loci are found in all organisms but are more frequently found in eukaryotic genomes and, to a lesser degree, in prokaryotic and eubacterial genomes (Tautz & Renz, 1984; Litt & Luty, 1989; Weber & May, 1989). Tautz (1989) reported that every 10 kb of eukaryotic DNA sequences, at least one simple sequence stretch can be expected to find. The most common microsatellites found in all organisms are poly (A)/ poly (T). However, they are not effectively used as DNA markers because they are not stable during the polymerase chain reactions; moreover, it is difficult to determine the size of the alleles (Beckmann & Weber, 1992). From microsatellites surveys of human and rat, Beckmann and Weber (1992) showed that CA/GT repeats are the most abundant dinucleotide repeats, as well as in *Drosophila melanogaster* (Schug et al., 1998). While GA/CT and AT/TA repeats are more common than CA/TG in plants whereas AT/TA repeats are common in yeast (Stallings, 1992; Lagercrantz et al., 1993). However, the types and abundance of microsatellites loci are apparently different in each particular organism. For example, Fagerberg et al. (2001) reported that microsatellite loci are not abundant in the hard tick, *Ixodes scapullaris* and the yellow fever mosquito, *Aedes aegypti* but abundance in the genome of *Anopheles* spp. (Lanzaro et al., 1995; Rongnoparut et al., 1996; Sinkins et al., 2000). In the insect microsatellite database (InSatDb) reported that 3.4% of honey bee, *Apis mellifera* genome (228.45 Mb) is microsatellites whereas microsatellites of *Drosophila melanogaster* are found only 1.56% of its genome (Nagaraju et al., 2006). However, there were no recorded about the abundance of microsatellites in the genome of fruit fly parasitoids.

Even though microsatellites are found in all organisms and widely disperse throughout the genome, their function remains unclear. In some cases of human genetic disorders were associated with the number of microsatellite repeats such as fragile X syndrome involving to the CGG repeats that can exceed 200 repeat count, that contrast to the normal range of 6-50 repeats (Fu et al., 1991; Verkerk et al., 1991; Ashley & Sherman, 1995).

Generally, microsatellite core repeats are flanked by conserved sequences; hence, it is possible to design specific primers for amplification of the locus of interest. The polymorphism of a microsatellite locus is defined by the difference of allele size and the different size corresponds to the variable number of tandem repeats. The variable number of repeats or different allele can be specified by sequencing polyacrylamide gel electrophoresis system that are visualized either by silver staining (Klinkicht & Tautz, 1992) or by autoradiography in which a base pair difference can be detected. One last method worth mentioning is automated sequencing techniques that are rapid and the large number of sample can be detected but this method requires

5′ end labeled primers with fluorescence dye, expensive instruments and software to detect alleles.

Since the emergence of microsatellite DNA markers in the late 1980s, this methodology has become one of the most popular DNA marker technique as evidenced by the huge number of microsatellites catalogued electronically (i.e., GenBank, Genome Database, GDB) or documented in the Primer Note of Molecular Ecology — a publication that has been documenting new microsatellite primers since the year 2001. In fact, the genomes of many organisms have now been completed and are available inform several genome databases: for example, human, rat, pig, and several insects such as *Drosophila*, honey bee and mosquito (Adams et al., 2000; Robert et al., 2002; Consortium, 2006). These DNA databases are useful sources of microsatellite markers for the organisms that were mentioned above. On the other hand, there are many other species such as biological control insects and fruit fly parasitoids which we lack sufficient knowledge related to their DNA sequences and microsatellite loci.

Microsatellite markers can be obtained in several ways. The easiest way is to search for DNA sequences containing microsatellite regions from the DNA database as mentioned above. However, this method is useful in as much as there is sufficient data housed in these databases specific to the organism being studied, such as human, rat and animals with economic and commercial value like pigs and chickens (Beckmann & Weber, 1992; Moran, 1993; Dib et al., 1996). If there is insufficient DNA data on the species of interest, new microsatellite markers need to be discovered by screening many thousands of clones of genomic DNA libraries. This process has many major drawbacks in that it is time consuming, expensive, labor intensive and has many technical requirements (Zane et al., 2002). Beyond these limitations, there were many techniques that have been developed to deal with the problems of isolating microsatellites. For example, enrichment methods have been developed to increase the number of clones for those microsatellites with low counts of the target genome (Ostrander et al., 1992; Kandpal et al., 1994; Fagerberg et al., 2001). Fisher et al. (1996) reported another method to isolate microsatellites without library screening using 5′ anchored primers containing microsatellite repeats. This method locates the

DNA region containing microsatellite repeats as same as appear in the designed anchored primers.

Because of high polymorphism, selectively neutral, codominant and a small amount of DNA are required; microsatellite markers have become very powerful tools for studying the genetic variation within and between populations (Avise, 2004). Moreover, microsatellites are also used as DNA markers for genome mapping, linkage analysis (Georges et al., 1993; Rohrer et al., 1994; Ron et al., 1994; Taramino & Tingey, 1996; Ferdig & Su, 2000; Nyanjom et al., 2003; Solignac et al., 2004) and are used for forensic DNA analysis (Pestoni et al., 1995; Poetsch et al., 2001).

3.7 Microsatellite evolution and mutation model

Microsatellite mutation rates found in *in vivo* systems are frequently about 10^{-2} events per locus per generation in *E. coli* (Levinson & Gutman, 1987) and 10^{-4} to 10^{-5} in yeast (Henderson & Petes, 1992; Strand et al., 1993). The low mutation rates were found in *Drosophila* at around 5 x 10^{-6} (Schug et al., 1997). Relative to higher organisms like human, the mutation rate can be estimated by pedigree analyses to be around 10^{-3} per locus per generation (Weber & Wong, 1993).

3.7.1 Mutational mechanisms of microsatellites

The mechanism of microsatellite instability at a locus has focused on the two major mechanisms (Eisen, 1999). The first mechanism is referred to as Slipped-Strand Mispairing (SSM) (Schlotterer & Tautz, 1992; Levinson & Gutman, 1987). The mutation process involves an irregular DNA replication process by which there is a misalignment between the DNA strands containing the microsatellite repeats. The unpaired nucleotides loop occurs in one or more repeat sequences then followed by replication or repair, which can lead to an insertions or deletions of one or several of the short repeat units. The second mechanism that presumes to potentially alter the lengths of microsatellites is Unequal Crossing-Over (UCO) (Hancock, 1999). Unequal crossing-over occurs at the earlier stage of meiosis when each chromosome forms identical strands of DNA that join together at the specific area called the centromere. The identical strands are known as sister chromatids where the unequal crossing-over take placed. Unequal crossing-over can be explained by a two-step process. The first step involves a misalignment of a sister chromatid and the second step entails a crossing-over at a specific region of strand overlap. The exchange of DNA strand can occur both in between chromatids in the same chromosome (sister chromatid) and between chromosomes. This process gives rise to a deletion in one DNA molecule and an insertion in the other and can increase the variation of alleles at a microsatellite locus in term of expansion and contraction in tandemly repeat array; as well as give rise to the homogenization of variants within an array of microsatellite repeats (Smith, 1976; Dover, 1982). Unequal crossing-over tends to occur in long tandemly repeated sequences where the recombination machinery cannot easily determine the correct alignment between two strands.

However, the mutation of microsatellite repeats not only results from the SSM and UCO but it is also dependent on other process that can alter the structure of the repeated array. The overall expansion of repeat sequences can also be influenced by the degree to which SSM and UCO events are basically biased toward insertions or deletions and by the (unknown) selective forces that may act to retain or eliminate the repetitive DNA sequence (Levinson & Gutman, 1987)

The variable numbers of microsatellite repeats have been used to specify the alleles at a given locus. Researchers have found that the longer the length of the microsatellite sequence, the higher the mutation rates — especially uninterrupted repeated arrays or perfect repeat sequences (Weber, 1990). However, most of microsatellite loci that have been tested for polymorphism are not characterized at the sequence level; therefore, the status of interruption of the locus is unknown (Hancock, 1999).

3.7.2 Theoretical mutation models

Microsatellites are known as predominant makers used for many applications in evolutionary genetic studies (Bowcock et al., 1994; Estoup et al., 1995a; Paetkau et al., 1995). To use these markers, the mutational events that shape microsatellite evolution are necessary to be understood. Several empirical and theoretical models have been used to examine the mutational processes of microsatellites that are necessary for an accurate estimation of population parameters (Estoup & Cornuet, 1999). The two classical models have been utilized for microsatellite loci; the simplest model is the Stepwise Mutation Model (SMM) that was introduced to population genetic theory by Ohta and Kimura (1973). Originally, this model was utilized with population containing N (i.e. the effective population size), diploid individuals, randomly mating populations, equilibrium distribution of allelic frequencies and selectively neutral alleles. The SMM assumes that the repeat number of microsatellite locus changes by only one repeat number with the resulting mutation having an equal probability of loss or gain (Kimura & Ohta, 1978). The entire sequence of allelic states can be illustrated by the integers (e.g., A_{-1}, A_0, A_1, etc.). The movement of allele changes state by which mutation can occur either one step in the positive direction or one step in the negative direction within the allele space (Kimura & Ohta, 1978). If the mutation rate is ν, then the probabilities of allelic change by gaining or losing a repeat is $\nu/2$, and the probability of no mutation is $1-\nu$.

A considerable amount of research has been conducted that employed the SMM model (Shriver et al., 1993; Valdes et al., 1993; Kimmel et al., 1996; Fu & Chakraborty, 1998). Through trial studies, Levinson and Gutman (1987) found that the slipped strand mispairing mutation mechanism is relevant to the SMM model. One working assumption of the SMM model relates to the possibility that allele may mutate towards allele states which already exist in the population. In this case the same state of alleles is not necessarily identical to its descent.

By contrast, another theoretical model assumes that each mutation can randomly give rise to any new allele that was not previously present in the population. This theoretical model, known as the Infinite Allele Model (IAM) (Crow & Kimura, 1964), has been used to study the mechanism of maintenance of protein polymorphism. Another microsatellite mutation model that corresponds to SMM was introduced by Di Rienzo et al. (1994) and is called the Two Phase Model (TPM). This model assumes that the allelic state gains or loses an absolute number of X repeat units with equal probability for the symmetrical mutation model. For example, if mutations occur, the probability of one step mutation (one-phase) is P_{SMM}, and the probability of multi-step mutations (two-phase) is $1-P_{SMM}$, in this case, the variable X follows a geometric distribution defined as $P(X=x) = \alpha(1-\alpha)^{x-1}$ specified by its variance $\sigma^2 = (1-\alpha)/ \alpha^2$. The latter term allows more than a single repeat unit change at each step of mutation (Estoup et al., 2002). However, the results of their study showed that there were no upper or lower limits of SMM, TPM and IAM on the number of repeat unit in an allele (Estoup & Cornuet, 1999).

The K-allele model (KAM) (Crow & Kimura, 1970), a classical model, could be also considered as a microsatellite mutation model, even though it is rarely cited in literature. Under this model, there are exactly K possible allelic states, and many alleles have constant probability [$\mu/(K-1)$] of mutating towards any of the other $K-1$ allelic states. The IAM corresponds to the KAM in which K is infinite. All of these microsatellite mutation models, except IAM, hypothesize that any allele can mutate towards an allelic state that previously existed in the population and thus generate size homoplasy — identical alleles that do not necessarily share a common ancestor (Estoup et al., 2002).

3.8 Statistical analyses of microsatellite variations

Microsatellites, the most applicable molecular makers, are widely used for a number of analyses such as genetic mapping, linkage analyses, paternal and kinship identification (Murray, 1996). Furthermore, microsatellite variations provide very useful data for population genetic studies and help to answer questions such as the amount of hybridization between closely related species and the level of variation within and among population. In addition, they can be used to estimate effective population size (Allen et al., 1995), migration rate, degree of population subdivision (Weir & Cockerham, 1984; Slatkin, 1995; Rousset, 1996) and genetic distance (Nei, 1972).

Currently, there are several statistical methodologies that have been specifically developed for population level analysis for example, estimates of the degree of population subdivision (e.g. F_{ST} or θ (Weir & Cockerham, 1984), R_{ST} (Slatkin, 1995), ρ_{ST} (Rousset, 1996)), genetic distance (e.g., Nei´s D_S (Nei, 1972), D_{SW} (Shriver et al., 1993)). However the most common use for population analysis based on microsatellite system is F statistics, which was developed by Wright (1965). Many published studies related to population structures have used F statistics which takes into consideration the estimation of inbreeding coefficients within and among subpopulations (i.e. F_{IS} and F_{ST} respectively) and within the entire population (F_{IT})

(Weir & Cockerham, 1984; Cockerham & Weir, 1993; Slatkin, 1995), including gene flow (N_m), under the island model that was introduced by Wright (1931), $F_{\mathrm{ST}} \approx 1/(4N_{\mathrm{m}}+1)$ (Wright, 1951; Weir & Hill, 2002). To use these estimators, the classes of microsatellite mutation model such as IAM, SMM/TPM have to be considered. For example, the F-statistics based on SMM model for estimating population differentiation is R_{ST} which,

$$R_{\mathrm{ST}} = \frac{\overline{S} - S_w}{\overline{S}},$$

where S_{w} and $\overline{S}$ are the average sum of squares for allele size difference within a subpopulation and for the entire population respectively (Slatkin, 1995).

Under the assumption of IAM model, F-statistics can be estimated from a ratio of the observed to expected heterozygosity where,

$$F_{\mathrm{IS}} = \frac{\overline{H_S} - H_I}{\overline{H_S}} \ ,$$

where $\overline{H_S}$ is the average of expected heterozygosity estimated from each subpopulation by,

$$H_{\mathrm{S}} = 1 - \sum_{i=1}^{k} p_i^2 \ ,$$

and where H_{I} is the average observed heterozygosity,

$$H_{\mathrm{I}} = \sum_{i=1}^{k} \frac{H_i}{k} \text{ for } k \text{ subpopulations.}$$

However, before advancing the methodology of statistical analysis of data for population substructure, the three main assumptions need to be examined. First, the selective neutrality of each locus should be analyzed; second, the presence of null allele (i.e. alleles which are not detected via PCR analysis) should be identified; finally, each locus should be tested for the independent assortment before combining the data from all loci (Murray, 1996). One way that researchers can detect the presence of selection is by comparing the observed genotype frequencies to those expected from the Hardy-Weinberg equilibrium methodology. In addition, observed and expected heterozygosity can be also compared (Edwards et al., 1992).

An unbiased estimation of heterozygosity (*He*) is

$$He = \frac{n(1 - \sum_{i-1}^{k} p_i^2}{n - 1},$$

where n is the sample size and p_i is the frequency of ith allele.

Nevertheless, statistical applications that have been used to assess microsatellite population data up to this point in time have not yielded definitive results. Hence, greater numbers of loci and variation in the mutation model of microsatellites (i.e., in relation to their structural features) are needed with more powerful statistical test (Estoup & Cornuet, 1999).

3.9 Genetic variability in natural populations of parasitoids

There were considerable researches have been conducted to examine genetic variability in natural populations of parasitoids which are important insects of biological control program. For example, Kitthawee et al. (1999) used allozyme electrophoresis to investigated genetic variation in natural populations of Oriental fruit fly parasitoid, *Diachasmimorpha longicaudata* in Thailand. They found that heterozygosity of each parasitoid population was relatively low. Owen et al. (1992) reported similar results in bumble bees (Hymenoptera: Apidae), and found a significantly lower mean heterozygosity than the other hymenoptera. When compared to the other insects, hymenopterans typically have less genetic variation. For example, the mean expected heterozygosity (± standard error) of ten species of solitary wasps was found to be 0.048(± 0.005), whereas the mean expected heterozygosity of thirty-one species of *Drosophila* was 0.135(± 0.011) (Graur, 1985).

In recent years, use of protein polymorphism for population genetic studies has declined since the advent of Polymerase Chain Reaction (PCR), automate DNA sequencing, and the advancement of molecular biotechnology coupled with higher powered computers for computation. As a result of these advancements, new molecular maker tools have evolved such as mtDNA genes, rRNA genes, RADP, AFLP, and microsatellites (Caterino et al., 2000; Behura, 2006). Edwards and Hoy (1993) investigated the polymorphism of two parasitoids, *Trioxys pallidus* and *Diglyphus begini*, using the RAPD technique. One hundred twenty and twenty-five of ten base arbitrary primers were tested using *T. pallidus* and *D. begini* respecively. As expected, the estimated gene diversity (± standard deviation) of the selected colony (0.233±0.057) of *T. pallidus* was significantly lower ($P < 0.05$) than that of the field-collected population (0.341±0.030). Interestingly, the estimated gene diversity of laboratory-maintained *D. begini* colony (0.378±0.053) was found to be similar to the value calculated for field-collected *T. pallidus* (0.341±0.030) and significantly greater ($P < 0.05$) than the gene diversity of the selected *T. pallidus* laboratory colony (0.233±0.057). However, it is impossible to conclude whether the *D. begini* colony maintained its diversity better in the laboratory or whether its diversity dropped from the higher level of diversity in the field without any information on the field populations of *D. begini*. The findings from this study also showed that the level of genetic variation detected using the RAPD technique was greater than the variation typically detected in Hymenoptera using allozyme analysis.

Vaughn and Antolin (1998) used the combination of two molecular techniques, (i.e., RAPD-PCR and SSCP) to investigate the population structure of the aphid parasitoid wasp *Diaeretiella rapae* (Hymenoptera: Braconidae: Aphidiidae) in an environment where two aphid hosts were available for oviposition. In this study RAPD, which is typically a dominant marker, was used in conjugation with SSCP analysis where the marker can be visualized as codominant marker. They found 11 codominant and 34 dominant RADP polymorphisms that conformed to Mendelian segregation patterns. Additionally, three analog estimates values (i.e., F_{ST} (Wright, 1951), θ (Weir & Cockerham, 1984), and F_{ST} (Lynch & Milligan, 1994)) of genetic differentiation were similar in this study for female and male combination. The average of those estimates were 0.067, 0.065 and, 0.064 respectively. The effective migration rates (N_m) between the populations ranged from 1.2-1.6 per year, indicating a relatively low dispersal rate. Genetic distances between populations were also calculated and the results showed that the genetic differentiation of populations were less than 1 km from each other. The results also indicated that *D. rapae* populations were genetically subdivided on a small spatial scale that corresponded to host-use patterns.

Genetic variation and founder effects in *D. rapae* populations that originated from Eastern Australia were also investigated in Western Australia using microsatellite markers (Baker et al., 2003). The results of this study provided information about the evolutionary processes of species within the genera, as well as information about the demographic history of particular species populations (Luikart, England, 1999). In this study, several statistical tests have been used to estimate genetic variation among population (e.g. ANOVA, F_{ST}, R_{ST}). For instance, when compared to the populations from the Old World the results of a microsatellite analysis showed low allelic length and low allele frequency variation, revealing that these individual wasps experienced a significant founder effect.

Recent population genetics studies of an introduced pea aphid parasitoid, *Aphidus ervi*, a native species of Eurasia, was examined by using five microsatellite loci and SSCP of mtDNA (i.e., CO I and CO II) (Hufbauer et al., 2004). The microsatellites data showed not significant different heterozygosity. These results suggested that only mild bottleneck occurred in spite of the large number of individual wasps (1000 individual wasps) that were introduced. Moreover, they found one of two common haplotypes was shared between native and introduced population of *A. ervi*. In contrast, de León et al. (2004) reported most of the ISSR-PCR markers showed no band sharing between the Texas and California populations of egg parasitoids of the glassy-winged sharpshooter, *Gonatocerus morrilli* (Howard). Such results are not typically found unless the populations are reproductively isolated. In addition, another molecular maker, ITS-2, showed different sizes of DNA fragments of 865 and 1099 bp for California and Texas populations, respectively. These intriguing observations strongly suggest that *G. morrilli* may exist in nature as a species complex. However, more research is needed to confirm these results.

As noted above, numerous articles reported findings related to population genetic studies of parasitic wasps, but only a few population genetic studies of fruit fly parasitoids were found — particularly melon fly parasitoid. Notwithstanding, some articles about population genetic studies indicated that molecular markers can be useful tools for investigating a diverse array of target populations.

CHAPTER IV

MATERIALS AND METHODS

4.1 Sample collections

Melon fly parasitoids, *P. fletcheri* were sampled from the different localities of Thailand (Fig. 4.1). Infested fruits especially ripening ivy gourd (*Coccinia grandis*) and other cucurbit fruits (e.g., wild bitter gourd, luffa, cucumber and snake gourd) including non cucurbits such as rose apple (*Eugenia javanica*) and guava (*Psidium guajava*) were collected and brought to the insectary. Fruit fly larvae were allowed to feed on flesh of fruit until they develop into pupa state. The parasitized larvae will emerge as parasitoids, whereas non-parasitized larvae will emerge as fruit flies. Fruit flies and parasitoids were identified using White and Elson-Harris (1992), and Wharton and Gilstrap (1983) key, respectively. Life specimens were stored at −80°C for further DNA extractions.

The numbers of parasitoids which were used for population genetic study are differed according to their locality (Table 4.1), that dependent upon the abundance of parasitoid found in each collecting field.

Figure 4.1 The local map of melon fly parasitoid sample collections in Thailand. (CP = Chumphon, CT = Chanthaburi, KN = Kanchanaburi, NB = Nonthaburi, NR = Nakhon Ratchasima, PS = Phitsanulok, UD = Uttaradit).

Table 4.1 Melon fly parasitoid samples collected from different localities of Thailand, host plants and host flies.

Province (code)	Number of female parasitoid	Host fly (host plant)	Collection time
Kanchanaburi (KN)	64	*B. cucurbitae* (*C. grandis*)	Sept-Dec 2005
Chanthaburi (CT)	63	*B. cucurbitae* (*C. grandis*)	Dec 2005
Nonthaburi (NB)	79	*B. cucurbitae* (*C. grandis*)	Aug-Sept 2005
Nakhon Ratchasima (NR)	60	*B. cucurbitae* (*C. grandis*)	Dec 2005
Chumphon (CP)	19	*B. cucurbitae* (*C. grandis*)	Jul-Sept 2005
Phitsanulok (PS)	44	*B. cucurbitae* (*C. grandis*)	Sept-Oct 2002
Uttaradit-A (UD-A)	14	*B. correcta* *B. dorsalis* (*E. javanica*)	June 2003
Uttaradit-B (UD-B)	11	*B. correcta* *B. dorsalis* (*P. guajava*)	June 2003

4.2 Genomic DNA isolation of adult parasitic wasp

Melon fly parasitoid genomic DNA was isolated using a method developed by Rick Lifton at Standford University. Adult female and male parasitoids using for microsatellite marker development were provided by Don McInnis of USDA Facility at Manoa, Honolulu, Hawaii. Both sexes of adult parasitic wasps were cleaned by soaking in 70% ethanol and rinsing twice with ddH$_2$O. The head part of each parasitoid was cut off before grinding in 500 µl of Lifton grind buffer (0.2 M sucrose, 0.05 M EDTA, 0.1 M Tris, pH 9.0, and 0.5% SDS). Five to ten wasps were placed in each 1.5 ml cleaned microcentrifuge tube and then homogenized on ice with micro-pastel. The homogenate was filtrated through 1 ml syringe packed with sterile 100% polyester fiber to get rid of the debris. The homogenate was treated with 3 µl of 20 mg/ml of Proteinase K and incubated at 65°C for one hr. After that procedure, 75 µl of cold 8 M potassium acetate was added in each sample tube and immediately put on ice for at least one hr. The cell debris and protein pellet was precipitated by centrifugation at 10,000 rpm for 15 min. The supernatant was transferred to a new tube and treated with 1 µl of 10 mg/ml RNase A and then incubated at room temperature for 15 min. Subsequently, the sample was extracted once with phenol-choloform-isoamyl alcohol (25:24:1 v/v) (PCI) and once with chloroform-isoamyl alcohol (24:1 v/v). The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 1 volume of cold isopropyl alcohol. Sample tube was converted gently and subsequently incubated at −20°C 16-18 hr or overnight. The DNA pellet was precipitated by centrifugation at 13,500 rpm for 25 min at 4°C and washed once with 500 µl of 70% clod ethanol then centrifuged with the same speed at 4°C for 15 min. The alcohol was drained well and DNA pellet was dried using a vacuum pump for 10 min or air dried at the room temperature for 30 min. The DNA pellet was resuspended in 50-100 µl ddH$_2$O or TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) depending on the quantity of pellets. For individual wasp DNA isolation, the DNA pellet sample was resuspended in only 30 µl of double distilled water by using the same protocols as above. The quality and quantity of isolated DNA samples were measured by taking 1 µl for measuring on 0.8% agarose gel electrophoresis in 0.5X TBE buffer (45 mM

Tris-borate, 1 mM EDTA) with a known quantity of DNA standard marker (0.5 µg *Hin*dIII-digested lambda DNA) which used for the DNA sample quantity estimation.

Isolation of genomic DNA of individual wasp for population genetic study was also performed with the same protocol but it is slightly different. The whole body of parasitoid was used to extract DNA and there was no filtration of homogenate after sample grinding.

4.3 Agarose gel electrophoresis

Genomic DNA samples were quantified and qualified by 0.8% agarose gel electrophoresis in 0.5X TBE comprised with 0.5 µg/ml ethidium bromide. After well polymerized, the gel was pre-run for 30 min at 100 V to eliminate excess ethidium bromide using Mupid-mini gel electrophoresis chamber (a product of Japan). Two microliters of DNA (each sample mixed well with 1 µl of 6X loading dye (0.25% Bromophenol bule, 0.25% Xylene cyanol, 30% Glycerol) and 3 µl of dH_2O) were subsequently loaded into individual wells. Then the loaded gel was electrophoresed at 100 V for 45 min or until the dye front (i.e. Bromophenol bule, Fluka) migrated to three-fourth of the gel length. Standard DNA markers such as *Hin*dIII-digested lambda DNA, 100 bp ladder was also loaded in the same gel for computing the concentration and sizing of samples. The image of DNA samples were visualized by exposure to UV light and gel photos were taken by using hand-held photo documentation camera (GelCam) with black and white film (Polaroid).

4.4 Restriction enzyme digestion and purification of genomic DNA

Approximately 5 µg of genomic DNA was digested with single and double restriction enzymes, *Sau* 3A and *Alu* I (Roche). The restriction reaction was carried out in a 100 µl volume consisting of 1X supplied reaction buffer, 75 µl of genomic DNA, and 10 U of each restriction enzyme. All reaction tubes were incubated in a 37°C air incubator for at least 1.5 hr up to overnight. Restriction DNA fragments were separated by 0.8% agarose gel electrophoresis with 0.5X TBE buffer and stained with ethidium bromide. DNA fragment size 300 bp to 1000 bp was isolated from agarose gel and purified by using Geneclean Spin Kit-Isolation (Bio101, Inc). Purified restriction DNA fragments were used for further microsatellite development.

4.5 Microsatellite marker development using 5′ anchored PCR technique

There were two techniques that were used for microsatellite marker development in this study. The first technique was the 5′ anchored PCR developed by Fisher et al. (1996) and the second technique was a modified enrichment technique (Ostrander et al., 1992; Kandpal et al., 1994; Kijas et al., 1994; Hamilton et al., 1999).

4.5.1 Primer design and PCR amplification for microsatellite isolation

Development of microsatellite markers from *P. fletcheri* was initiated by using the 5´anchored PCR method (Fisher et al., 1996). Ten degenerate microsatellite primers or 5´anchored primers, in conjunction with microsatellite repeats (e.g., $(TG)_6$, $(TC)_6$, $(GC)_6$, $(AT)_6$, $(CAA)_4$ (Table 4.2), were designed for amplification of microsatellite repeat regions in the genomic DNA of melon fly parasitoid. The primers consisted of two parts. Part one was comprised of the 5´ anchor that contained seven variable nucleotides and part two was made up of 6 and 4 repeats of dinucleotide and trinucleotide repeats respectively (e.g., the anchored primer for screening (TC)n repeats, 5´ KKVRVRVCTCTCTCTCTCT 3´). The two nucleotides at 5´ end of anchor primer were designed to anneal to any nucleotide.

The letter K represents G and T, with G pairing with C or T, and T pairing with A or G. The next five nucleotides were designed to prohibit pairing with the complementary base of microsatellite repeats, the degenerate sequence VRVRV did not pair with GA repeats (Fisher et al., 1996).

Table 4.2 The sequences of 5´ anchored microsatellite primers for microsatellite isolation (H = A, C, T; K = G, T; R = A, G; V = A, C, G; Y = C, T).

primers	Sequences 5´ to 3´	Length (base)	Tm (°C)	Ta (°C)
PAT_6	KKRYRYRATATATATATAT	19	46	42
PTA_6	KKYRYRYTATATATATATA	19	46	42
PGC_6	KKRVRRRGCGCGCGCGCGC	19	72	57
PCG_6	KKVRVRVCGCGCGCGCGCG	19	73	57
PCT_6[a]	KKVRVRVCTCTCTCTCTCT	19	60	55
PTC_6[a]	KKRVRVRTCTCTCTCTCTC	19	60	55
PGT_6[b]	KKHVHVHGTGTGTGTGTGT	19	59	55
PTG_6[b]	KKVHVHVTGTGTGTGTGTG	19	59	55
$PAAC_4$	KKYRYYRAACAACAACAAC	19	55	50
$PCAA_4$	KKYYRYYCAACAACAACAA	19	55	50

[a] Primer designed by Fisher et al. (1996)

[b] Primer designed by Hayden and Sharp (2001)

The amplification reaction was carried out in a PCR thermal cyclear (GeneAmp 2700, Perkin-Elmer) with a total volume of 25 µl that was made from 3 mM $MgCl_2$, 20-30 ng of genomic DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, 1X PCR reaction buffer (Applied Biosystems) and 1 U of AmpliTaq DNA polymerase. The PCR amplification was initiated with a denaturing step at 94°C for 2 min and followed by 35 cycles consisting of 1 min at 94°C for denaturation, 30 sec at 42-57°C (depended on melting temperature of each primer) for annealing and 1 min at 72°C for extension step, and finalized with an extension step at 72°C for 5 min. Digested genomic DNA with *Sau*3A and *Alu*I restriction enzymes were also used as DNA template for amplification of microsatellite using 5′ anchored primers.

4.5.2. Cloning of microsatellite amplification products

Amplification products that resulted from each set of degenerate primers were cleaned using a GeneClean Spin Kit (Bio101, Inc) and the cleaned amplified product was ligated to the cloning site of pCR2.1-TOPO vector (Invitrogen) by using 4 µl of fresh PCR product with 1 µl TOPO vector and 1 µl salt solution provided by the manufacturer and then incubated at room temperature for 5 min. The ligation can be stored at −20°C or be allowed to continue the transformation process.

Bacterial transformations were performed using a heat shock technique. The bacterial competent cell used in this technique was *E. coli* DH5α (Invitrogen) which was stored at −80°C. Competent cells were taken from cold storage (i.e., −80°C) and thawed on ice. After being thawed, 2 µl of ligation reaction was added immediately into the 50 µl competent cells tube, gently mixed by taping the tube, incubated on ice for 30 min and heat shocked at 42°C for 30 sec and then immediately put on ice for 2 min before adding the whole volume of transformed cells into 250 µl S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 0.4% glucose, 10 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 5 mM $MgSO_4$), which was prepared in 15 ml sterile capped tube and incubated at 37°C for 1 hr in a horizontally shaking water bath at the speed of 200 rpm. Lastly, 50 µl of transformed cells and the remaining concentrate volume of transformed cells were plated onto a 1.5% selective LB agar supplemented with 0.04 mg/ml X-gal (Sigma) and 0.1 mg/ml ampicillin (Sigma) and then incubated up side down at 37°C for 16-18 hr.

4.5.3 Colony selection and plasmid isolation

The well isolated pure white colonies were picked up and re-streaked on the fresh agar plate with the same media; then single colonies were picked up for analysis of insertion fragments. Each clone was amplified in order to check the size of the insert by using the PCR method with universal primers, M13 forward and M13 reverse primers — both primers flank the cloning site, that allows to amplify if that colony containing inserted DNA.

Interested clones containing inserted DNA fragments were selected to culture in 2 ml of TB growth media (Terrific Broth, Bio101, Inc) supplemented with 0.1 mg/ml ampicillin and then incubated at 37°C in a horizontal shaking water bath for 14-16 hr at the speed of 200 rpm. Fresh bacterial cultures were separated into 2 parts, one part (850 µl) was taken out for bacterial clone preservation and the rest was used for plasmid DNA isolation. Plasmid DNA isolation was performed by using the Qaiprep® Spin Miniprep Kit (QAIGEN) following the manufacturer's protocol. Recombinant plasmid DNA was eluted with 35 µl of ddH_2O for further DNA sequencing analysis.

4.5.4 Inserted DNA fragment detection

All isolated recombinant plasmids were amplified using M13 forward and reverse primers to determine the insert size of each clone before sequencing. According to DNA fragment (PCR product) that was ligated to plasmid vector, pCR2.1-TOPO (Fig. 4.2) was inserted at the cloning size between the M13 forward and reverse sequence that allowed the insert fragment to be identified by PCR.

To identify the insert DNA, the amplification reaction was carried out in a 25 µl total volume made from 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.25 µM of each M13 forward and reverse primers, 5 ng of recombinant plastmid (bacterial clone culture can be direct amplified instead of plasmid) and 1 U AmpliTaq DNA polymerase (Applied Biosystems, USA). PCR was performed using standard method for amplification using M13 primers at the 50°C annealing temperature. The appearance of a PCR product meant that the clone contains insert DNA. If no PCR product appeared, then it signified that the clone contained self ligation of plasmid without an insert DNA.

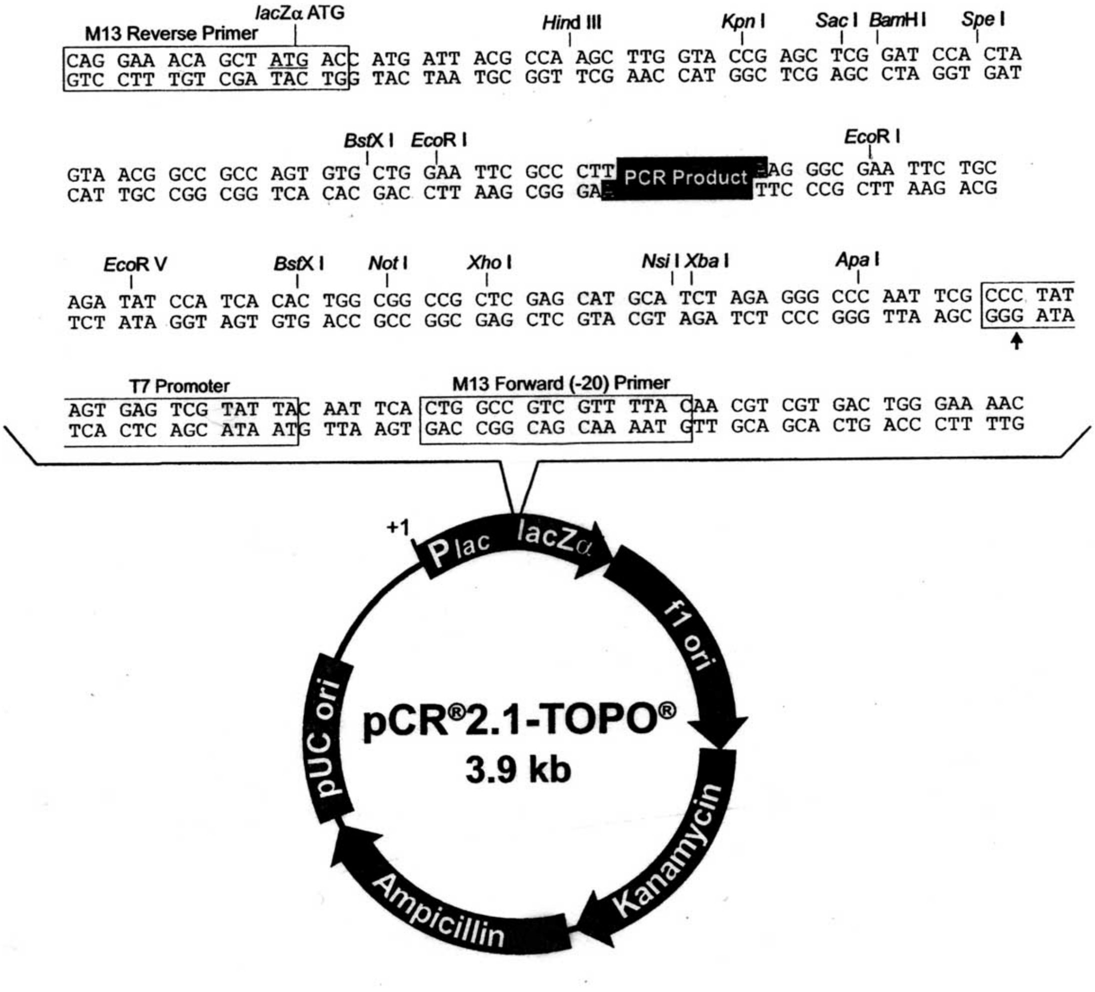

Figure 4.2 Structure of cloning vector (pCR 2.1-TOPO) with M13 forward and reverse priming sites flanking insert DNA fragment (Invitrogen, life technologies).

4.6 Microsatellite marker development using modification of enrichment method

The microsatellite enrichment method used in this study was modified from the microsatellite enrichment method (Ostrander et al., 1992; Kandpal et al., 1994; Kijas et al., 1994; Hamilton et al., 1999). Ostrander et al. (1992) reported that this approach increased the probability of finding microsatellite repeats (e.g. (CA)n, (AG)n repeats) with approximately 50% of the positive clones.

4.6.1 Genomic DNA restriction enzyme digestion

Restriction enzyme digestion was carried out in a 1.5 ml microcentrifuge tube. An approximate amount of 5-10 µg of *P. fletcheri* genomic DNA was digested with combination of three restriction enzymes, *Hae*III, *Rsa*I and *Alu*I (NEB, Inc). These enzymes restricted to four base pair recognition sites. The digestion reactions were carried out in 150 µl total volume with 1X NEB buffer 2 and 1X BSA (NEB), and 20 U of each restriction enzymes. The digestion reaction was incubated overnight at 37°C for complete digestion, and then incubated at 65°C for 20 min to inactivate enzyme activities. The digested genomic DNA was cleaned with GeneClean Spin Kit (Bio 101, Inc.) according to the manufacturer's protocol. Then cleaned digested genomic DNA was electrophoresed on a 1.5% agarose gel in 0.5X TBE in order to examine the size range of digested DNA and estimate the quantity. The processed DNA was used for further analysis.

4.6.2 Ligation of digested DNA fragments to adaptors

Restriction fragments, with an approximate size range between 200 to 1000 bp, were ligated to double strand DNA adaptor created from *Bam*HI-*Xmn*I (C strand) non-palindromic adaptor (5' dGATCCGAACCCCTTCG 3') and non palindromic adaptor complement (G strand) (5' dCGAAGGGGTTCG 3') (NEB, Inc.). Both strands were complemented with each other by combining 20 µl of 50 µM of each strand, 5 µl 10X buffer (250 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$) and 5 µl of ddH_2O that was added and heated in a 95°C water bath for 5 min. The adaptor tube was left to slowly cool down at room temperature for 30 min. This pre-annealed adaptor solution contained 20 ρM/µl adaptor and was stored at –20 °C for future use. The adaptor can ligate to a blunt end or cohesive end. The ligation reactions were carried out with 3 µl pre-annealed adaptor, 3 µl 10X NEB buffer 2, 0.3 µl 100X BSA

(NEB, Inc.), 3 μl 10mM ATP, 20 U *Xmn*I, 250 U T4 DNA ligase (NEB, Inc.) and 10 μl of blunt end restricted genomic DNA fragments, then followed by adding ddH_2O with the volume up to 30 μl and incubated at 16°C overnight. The following day, the ligation reaction was heated up to 65°C for 20 min to inactivate enzyme activities.

4.6.3 Amplification of adaptor ligated genomic DNA fragments

Two microliters of the ligation reaction were taken for PCR amplification by using the NEB short primer (5′CGAACCCCTTCG3′). The PCR reaction was performed in total volume 25 μl with 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM each of dNTP, 0.4 μM NEB short primer, 2 μl of ligation solution and 1 U AmpliTaq DNA polymerase (Applied Biosystems, USA). The PCR amplification was performed with a denaturing step at 94°C for 2 min, followed by 25 cycles of 94°C for 1 min, annealing step at 40°C for 1 min and extension step at 72°C for 1 min, and finally, another extension step for 5 min and hold at 8°C. The PCR products were examined on a 1.5% agarose gel in 0.5X TBE buffer.

4.6.4 Oligonucleotide DIG-ddUTP probe labeling

Oligonucleotide microsatellite probes (Table 4.3) were labeled using the DIG-Oligonucleotide 3′-End Labelling Kit, 2nd Generation (Roche). The labeling reactions were carried out in a 10 μl volume consisting of 100 ρmol of oligonucleotide, 1X buffer, 5 mM $CoCl_2$, 0.05 mM DIG-ddUTP, and 20 U of Terminal transferase. Those chemicals in the reaction were supplied by manufacturer except oligonucleotide. Then up to 10 μl of ddH_2O was added, mixed and briefly centrifuged. The reactions were incubated at 37°C for 15 min and then placed on ice. The reaction was stopped by adding 2 μl 0.2 M EDTA (pH 8.0). The control-oligonucleotide labeling reaction was also performed. The efficiency of the labeling reactions was determined by using a comparison with the control-oligonucleotide according to the manufacturer's protocol.

Table 4.3 Sequences of oligonucleotide microsatellite probes.

Microsatellite probes	Oligonucleotide Sequences (5′ to 3′)	Length (bp)	Tm (°C)
$(TC)_{15}$	TCTCTCTCTCTCTCTCTCTCTCTCTCTCTC	30	69
$(TG)_{15}$	TGTGTGTGTGTGTGTGTGTGTGTGTGTGTG	30	69
$(AAT)_8$	AATAATAATAATAATAATAATAAT	24	44
$(ACC)_8$	ACCACCACCACCACCACCACCACC	24	71
$(CAA)_8$	CAACAACAACAACAACAACAACAA	24	58
$(TGA)_8$	TGATGATGATGATGATGATGATGA	24	58

4.6.5 Microsatellite survey using slot blot hybridization analysis

According to the lack of microsatellite knowledge in the genome of *P. fletcheri*, the hybridizations of *P. fletcheri* genomic DNA were conducted for microsatellite survey using oligonucleotide microsatellite labeled probes (Table 4.3). This technique was performed in a 10 µl volume consisting of ddH_2O, genomic DNA and 1 µl of 3 N NaOH. Then the sample was incubated in a 65°C water bath for 30 min in order to denature the DNA, after which the sample was quickly chilled on ice. The denatured DNA was blotted onto a nylon membrane (Osmonics) using vacuum slot blot apparatus (Minifold1, Schleicher & Schuell, Geramany). DNA blotted membrane was put on the clean filter paper to air dry for 10 min and exposed to 120,000 µJ UV using a UV Stratalinker 1800 (Stratagene). The UV crosslinked membrane was pre-hybridized for 1 hr at 42°C in the solution containing 2% blocking reagent (Roche), 0.1% SDS, 5X SSC, 0.04% N-lauroylsacosinate, and 50% deionized formamide; the labeled probe was resuspended in the same formula. Hybridization of the membrane with DIG-labeled probe was carried out at 42°C for 16-18 hr or overnight. After hybridization, the membrane was washed twice with 2X SSC and 0.1% SDS for 5 min each at the room temperature. Subsequently to stringency washes which were performed two times with 0.5X SSC and 0.1% SDS for 15 min each at the different temperature, depending on the melting temperature of each probe and the stringency required. In this section, washing was performed at 55°C for $(AAT)_8$, $(CAA)_8$ and $(TGA)_8$ probes and 65°C for $(TC)_{15}$ and $(TG)_{15}$ probes. Every step of washing and detection were carried out with gentle agitation using a rotor shaker (Hoefer Scientific Instrument).

The colorimetric detection of probe molecules was performed following the manufacturer’s protocol for the DIG High Prime DNA Labeling and Detection Kit (Roche). Subsequently to stringency washes, the membrane was rinsed with washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% (w/v) Tween 20; adjust to pH 7.5 with solid NaOH) for 2 min and then incubated in 1X blocking buffer (10X blocking solution diluted in maleic acid buffer) for 30 min at room temperature. The blocking solution was removed and the Anti-DIG-AP antibody conjugate (Roche), diluted 1: 10,000 in 1X blocking buffer was added to the membrane and incubated for 30 min at room temperature. The membrane was then washed twice in washing buffer for 15 min each, and then the membrane was subsequently equilibrated in detection buffer (0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min. The Colorimetric detection was performed by adding 10 ml fresh solution of NBT/BCIP (0.4 mg/ml nitro blue tetrazolium chloride (NBT), 0.19 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate (BCIP), 0.1 M Tris, pH 9.5, 50 mM magnesium sulphate). The color reaction was developed in a few minutes and the reaction was stopped by TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).

4.6.6 Hybridization of ligated genomic DNA fragments using DIG labeled microsatellite probes

Hybridizations of amplified ligated genomic DNA, mentioned in section 4.6.3, using DIG-labeled oligonucleotide microsatellite probes were performed in a 100 μl volume consisting of 40 μl amplified ligated genomic DNA, 2 μl DIG-labeled microsatellite probe and 58 μl 6X SSC buffer. The reaction tubes were placed at 95°C for 15 min to denature the genomic DNA and the reactions were subsequently incubated for 16 to 18 hr at 65°C for $(GT)_{15}$, $(TC)_{15}$ and $(ACC)_8$ probes and 50°C for $(TGA)_8$ probes. This step, the DNA fragments containing microsatellite repeats were expected to hybridize with the given labeled microsatellite probes. The hybridization mixture was used for further capturing step.

4.6.7 Microsatellite DNA capturing using Anti-digoxigenin magnetic particles

Anti-digoxigenin magnetic particles or beads were used to capture the microsatellite DNA fragments form the hybridization mixture consisting of the DNA fragments hybridized with DIG-labeled microsatellite probe, free DNA fragments and free DNA probes. The capturing step was performed using 35 μl of Anti-digoxigenin Magnetic Particles (10 μg/μl, Roche) which were prior equilibrated three times in 100 μl of TEN100 buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and separated for 1 min using a magnet, after which TEN100 was removed from the magnetic beads. In order to capture the DNA fragment containing the microsatellite repeats, 150 μl of hybridization buffer (6X SSC, 0.1% SDS) was added to the magnetic beads and then 100 μl of hybridization mixture was added and incubated at room temperature for 30 min with a constant gentle agitation. At this step, the hybridized DNA fragments were captured by magnetic beads coated with Anti-DIG. Unbounded DNA fragments were eliminated by using high stringency washing steps. The bead-probe-DNA complex was separated by applying a magnet and removing the hybridization solution. The washing step was then carried out 2 times with 200 µl of 2X SSC with 0.1% SDS, incubated at room temperature for 5 min and then magnetized for 1 min before removing the washing solution. The procedure was followed by two subsequent washing using 200 µl of 1X SSC with 0.1% SDS at 42°C for 10 min. Each probe was then washed with the same washing buffer at melting temperature minus 5°C before leaving the washed tubes at room temperature for 2 min. After that, a magnet was applied to remove the residual of washing solution. The final wash was performed with the same solution; additionally, each tube was washed and then incubated at the melting temperature minus 5°C for 5 min, followed by a rinse with 1X SSC (without SDS) and then the solution was removed before engaging the eluting step.

Captured DNA fragments were eluted using 60 µl of a preheated TE buffer and incubated at 95°C for 10 min. Then the eluting tube was placed to the magnet and eluted DNA fragments which were dissolved in the TE buffer were quickly transferred to a new tube. Another 60 µl of TE buffer was added to the dried magnetic beads and then stored at −20°C as a backed up sample.

The eluted DNA fragments that were expected to be microsatellite enriched DNA were amplified to increase the copy number of DNA containing microsatellite repeats. Additionally, the amplified products were cloned into the plasmid vector by using a TOPO-TA Cloning kit (Invitrogen). Positive clones were then selected and screened with microsatellite probes by slot blot hybridization and/or multiple PCR primer amplification using M13 forward, M13 reverse primers and 5′ anchored primer containing an interesting microsatellite repeats. The clones containing microsatellite are expected to see positive signals or to see the extra fragment from multiple primer amplification.

4.7 DNA sequencing and analysis

Positive clones were selected and sequenced at the Greenwood Molecular Biology Facility (GMBF) on the campus of the University of Hawaii at Manoa, Honolulu, Hawaii using an ABI Prism BigDye® Terminator (Applied Biosystems 377XL DNA Sequencers).

All DNA sequences were analyzed for microsatellite repeats by using the Tandem Repeats Finder (TRF) program, version 3.21 (Benson, 1999). Microsatellite primers were designed from microsatellite flanking sequences by using Primer 3 (Rozen & Skaletsky, 2000) and PrimerSelect software (DNASTAR, Inc.).

4.8 Optimization of PCR conditions

Polymerase chain reaction condition of microsatellite amplifications were optimized using specific primers — designed from flanking DNA sequences of each microsatellite locus. PCR reactions were carried out in 0.2 ml PCR thin-wall tubes (Axygen) in 15 µl of the total volume with 5-10 ng of genomic DNA of individual parasitoid wasp, 1X PCR reaction buffer (75 mM Tris-HCl, pH 8.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 1.5-2 mM $MgCl_2$, 1 µl 2 mM of each dNTP, 0.5 µl of 10 µM of each primer, 0.5 U of *Taq* DNA polymerase (Fermentas) and supplemented with PCR enhancer (e.g. 1X MasterAmp PCR enhancer with betaine, Epicentre and 0.1 mg/ml BSA, Sigma) if needed. The amplification reactions were conducted in PCR Thermal Cycler (GeneAmp® PCR System 9700, Applied Biosystems) and Peltier Thermal Cycler MJ Research PTC-200. The amplification parameters for the PCR of each microsatellite locus were as follows: an initial denaturing step at 95°C for 2 min; 25-30 cycles of denaturation at 94°C for 30 sec, annealing at 50-65°C for 30 sec and extension at 72°C for 30 sec; and finally 30 min at 72°C. The PCR products were analyzed through electrophoresis with 2% agarose gel in 0.5X TBE buffer and ethidium bromide (0.5 µg/ml). Then the reaction was run at 100 V for 30 min or until the dye front reached 2/3 of gel length.

4.9 Sequencing gel electrophoresis for microsatellite genotyping

Genomic DNA of individual wasp collected from natural population was used as the DNA template for microsatellite amplification. The PCR products of each microsatellite locus were analyzed using sequencing electrophoresis set (Sequi-Gen GT sequencing Cell, BIO-RAD). The procedure of sequencing polyacrylamide gel preparation was followed the manual provided by manufacture (BIO-RAD). A bind-silane glass plate was prepared by cleaning it once with dH_2O using dust free tissue paper and once with 95% ethanol, then the entire glass plate was coated with 1 ml of bind-silane solution (8 ml absolute ethanol, 200 μl glacial acetic acid, 1.8 ml ddH_2O and 10 μl bind-silane, Sigma) and the treated glass plate was allowed to air dry in the dust free environment for approximately 3 hr. Another glass plate called the repel silane glass plate was prepared by cleaning with 95% ethanol and dH_2O respectively and then the entire surface of glass plate was treated with anti rain drops liquid (Glaco, automobile product of Japan) that is similar function as repel silane. Repel silane treated plate was air dried for 15 min before cleaning the plate again to remove excess Glaco using 95% ethanol and dH_2O followed by drying the plate for 5 min. The two glass plates were assembled using the sequencing apparatus with a 0.4 mm spacer. Then 50 ml of 6% polyacrylamide gel (19:1 acrylamide: bisacrylamide) with 7 M urea in 1X TBE buffer was prepared and 400 μl of 10% APS and 40 μl TEMED (N, N, N′, N′-tetramethylethylenediamine) were added to denatured polyacrylamide solution just before injecting the solution into the assembled glass plates using 60 ml syringe — avoiding the creation of bubbles during injection. The shark tooth comb was placed on top of the gel with the tooth up. Then the polyacrylamide plate was left at room temperature to polymerize for at least 3 hr. After the plate was well polymerized, 350 ml and 500 ml of 1X TBE buffer was added to the lower and upper tank respectively and the shark tooth was inverted and put deep into the gel approximately 2 mm between the glass plates. Then the acrylamide gel plate was pre-run at 50 – 60 W (~ 1600 V) until the temperature of glass plate reached 50°C.

Five microliters of amplified microsatellite DNA samples were mixed with 10 μl of SSRP loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) and then denatured at 94°C for 5 min and immediately placed on ice before loading. Each well was well flushed with a 1X TBE buffer to remove excess urea before applying the samples. Four to five microliters of denatured DNA samples with loading dye were loaded in each well and 10 base pair ladder standard DNA size maker was also loaded in the same gel. The samples were run at constant power (40-50W, ~1600 V and ~25 mA) for approximately 1.5 hr or until the front dye reached the bottom of the gel. The microsatellite alleles were visualized by silver staining. Stained glassed plates were air dried and photographs have been taken for further microsatellite genotyping.

4.10 Silver staining

After electrophoresis, the glass plates were separated from each other. Polyacrylamide gel was stuck to the bind-silane treated plate which this plate must be handled without touching the gel. The gel plate was stained with a silver staining method developed by Benbouza et al. (2006). The gel plate was placed in a fixing solution (10% ethanol and 0.5% acetic acid) for 5 min and then stained with silver stain (1.5 g silver nitrate, 1.5 ml formaldehyde in 1000 ml ddH_2O) for 10 min, rinsed with dH_2O and placed into developer solution (15 g NaOH and 2 ml of 37% formaldehyde in 1000 ml ddH_2O) for 5-10 min or until microsatellite bands appeared as desired. Then the silver stain developing was stopped by soaking the plate in the fixing solution for 2 min. Every steps of staining were performed with constant gentle agitation on the horizontal shaker (The belly Dancer, Stovall LifeScience, Inc). Then the stained plate was air dried before being photographed or scanned for further analysis.

4.11 Population genetic analysis

Microsatellite alleles of each locus were scored and analyzed only in sampled female parasitoids. Population genetic analysis was performed using POPGENE version 1.32 (Yeh et al., 1997), GENEPOP version 3.4 (Raymond & Rousset, 1995) and FSTAT version 2.9.3 (Goudet, 2001).

Allele frequencies, mean number of alleles per locus, observed heterozygosity (H_o), expected heterozygosity (H_e) and The deviation from Hardy−Weinberg equilibrium was estimated using Chi-square (χ^2) test. The F-statistic parameters (e.g. F_{IS}, F_{IT} , F_{ST} and R_{ST} (ρ), analog of F_{ST}) were estimated under the assumption of Weir and Cockerham (1984) (F_{ST} or θ) and Rousset (1996) (R_{ST} or ρ). These assumptions were calculated on the basis of SMM. Genetic differentiation between populations was estimated by pairwise F_{ST}, these values were also used for finding correlation between genetic differentiation and geographical distance (km) by scattered plot. Moreover, the genetic identity (*I*) and Nei's genetic distance (*D*) (Nei, 1972) were calculated and the *D* values were used to construct the cladogram of parasitoid population samples.

CHAPTER V

RESULTS

5.1 Microsatellite DNA isolation

Microsatellites of *P. fletcheri* were isolated for the purpose of using them as genetic markers for population genetic study in this species. Two techniques were used to isolate microsatellite DNA in the genome of *P. fletcheri.* The first technique was the 5′anchored PCR (Fisher et.al., 1996) and the second technique was a modification of microsatellite enrichment (Ostrander et al., 1992; Kandpal et al., 1994; Kijas et al., 1994; Hamilton et al., 1999).

5.1.1. Microsatellite markers development using 5′anchored PCR

Microsatellite PCR amplification using PCT_6/PTC_6 and PGT_6/ PTG_6 primers at the 3.0 to 4.0 mM $MgCl_2$ yield a number of PCR amplification products with the size ranging from 300 bp to more than 1500 bp (Fig 5.1-5.2). In contrast, there were no PCR amplification products from the PAT_6/PTA_6 and PCG_6/PGC_6 primers. The complementary of primer themselves must be considered by this case. Whereas the $PAAC_4$ and $PCAA_4$ primers resulted only few bands of PCR amplification products. These results indicated that TG and TC microsatellite repeats were commonly found in the genome of *P. fletcheri*.

Furthermore, the results of PCR amplification reactions using PTG_6 primer with digested genomic DNA template showed greater amounts of amplification products than using intact genomic DNA templates (Fig 5.3).

Only 107 recombinant clones were obtained from every class of amplification products with the size of inserts ranging from 250 to 1600 bp. Fifty-one clones resulted from the cloning of PCT_6/PTC_6 amplification products, whereas 36 and 20 clones were derived respectively from PGT_6/PTG_6 and $PAAC_4/PCAA_4$, (Table 5.1).

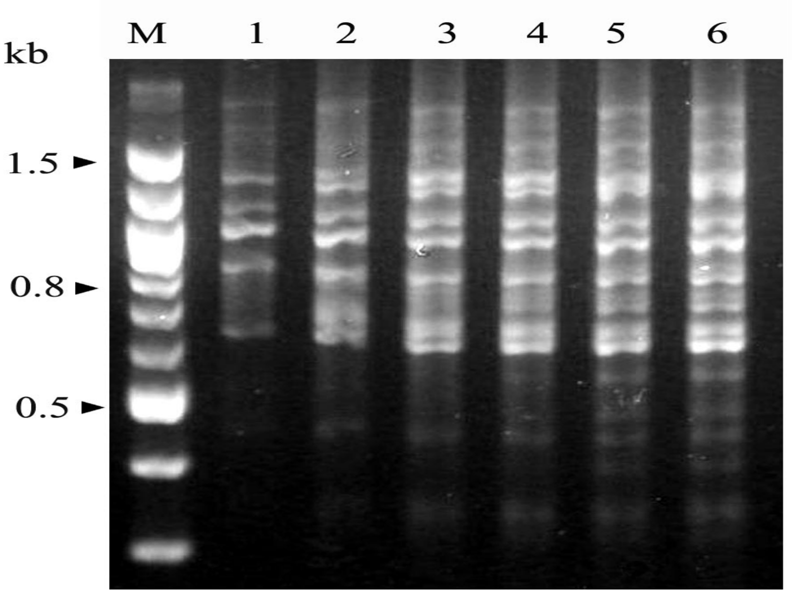

Figure 5.1 PCR profiles of microsatellite amplification using PCT_6 primer with variable concentrations of $MgCl_2$. Lane M, 100 bp standard DNA marker; lanes 1-6, the PCR products using 1.5 , 2.0, 2.5, 3.0, 3.5 and 4.0 mM $MgCl_2$, respectively.

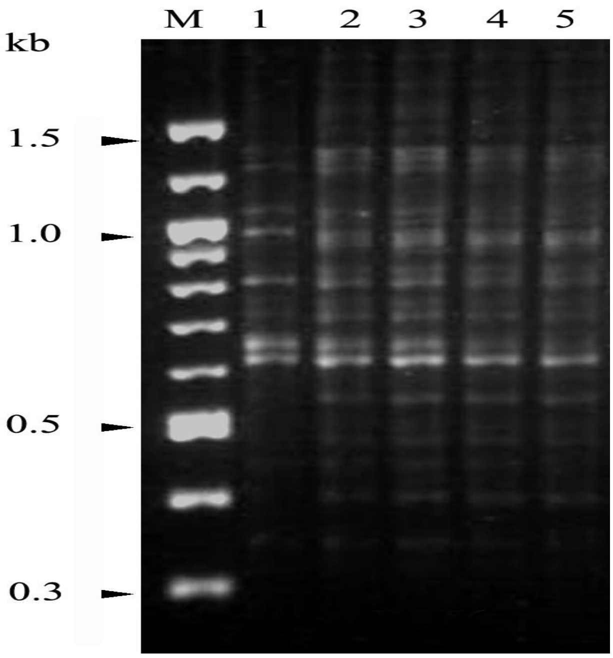

Figure 5.2 PCR profiles produced from different amount of genomic DNA using PCT_6 primer. Lane M, 100 bp standard DNA marker; lanes 1-5, PCR products using DNA temple, 250, 50, 25, 12.5 and 5 ng, respectively.

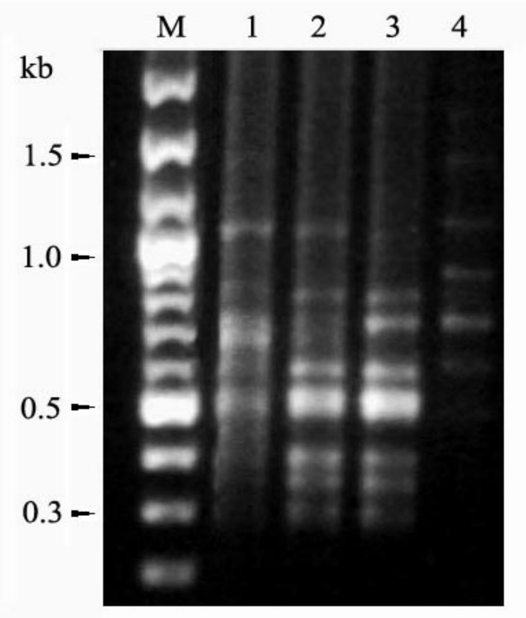

Figure 5.3 PCR profiles generated from PTG$_6$ primers using digested genomic DNA and intact genomic DNA of *P. fletcheri*. Lane M, 100 bp standard DNA marker; lanes 1-3, PCR products generated from digested genomic DNA using *Alu* I, *Sau* 3A and combination of *Alu* I and *Sau* 3A, respectively; lane 4, PCR products generated from undigested genomic DNA.

Table 5.1 The numbers of microsatellite clones obtained from different degenerate primers, size of insert DNA, the number of sequenced clones and CG content.

Primers	Number of recombinant clones	Size of insert DNA (bp)	Number of sequenced clones	%G+C (average)
PCT6/PTC6	51	350 – 1600	5	42.05
PGT6/PTG6	36	250 – 1200	15	41.77
PAAC4/PCAA4	20	600 – 1600	3	44.21
Total	107	250 – 1600	23	(42.68)

Twenty-three clones were selected to sequence and most of those clones were not only contained microsatellite at both ends of the DNA sequences derived from different 5′anchored microsatellite primers, but the repeated DNA at the internal sequences were also observed. However, each of microsatellite repeats found in every sequenced DNA did not exceed 10 repeats (Table 5.2). Moreover the percentages of GC and AT content for all DNA sequences were calculated by using the EditSeq program (DNASTAR) and resulted in an average AT content that was higher than the GC content by approximately 15% in the partial genomic DNA of *P fletcheri*.

Seven sequenced clones were selected to design primers for the microsatellite maker evaluation (Table 5.3). The melting temperature (Tm) of each primer was roughly calculated based on the equation, Tm (°C) = [4 x (G+C)] + [2 x (A+T)].

This study is the first time for microsatellite isolation in *P. fletcheri*. The 5′ anchored PCR method (Fisher, et. al., 1996) — a simple method that was used for preliminary research in surveying the microsatellite of this parasitic wasp. By using this technique, researcher anticipated a low copy number of microsatellites in the genome of this insect species. Therefore, a microsatellite enrichment technique was employed in order to increase the probability to find informative microsatellite markers that might lead to further studies and a better understanding of the genetic structure of *P. fletcheri* populations.

Table 5.2 Microsatellites obtained from the 5' anchored PCR technique.

Clone	5′ terminal repeat	Internal repeat	3′ terminal repeat
1	$(TC)_7$	$(C)_5G\ (C)_8G(C)_4$	$GA(GG)_2(GA)_7$
2	$(CT)_6\ (G)_5\ (A)_3\ T\ (A)_4\ (ATT)_2$	$(ACAATTGA)_2$	$(GA)_2CAGG(GA)_6$
3*	$(TC)_6\ ATT\ (GC)_6$	$(AT)_3TCTT(AT)_4\ A_5$	$(GA)_6$
4	$(TC)_6\ (TGG)_2$	$(T)_8$	$(GA)_6(AG)_2$
5	$(TC)_7$	$(A)_9\ C\ (A)_7\ (T)_2\ (TA)_2\ (GA)_2(A)_3\ (C)_3$, $(AAT)_3\ (A)_8$	$(AG)_6$
6*	$(GT)_7$	$(GA)_4\ CG\ (CA)_2\ A(CA)_2$	$(CA)_6$
7	$(GT)_6$		$(CA)_6$
8*	$(GT)_7(CA)_2$	$(TA)_3$, $(TA)_2\ TTT\ (TA)_6$	$(CA)_7$
9	$(TG)_7$	$(T)_9$, $(T)_4(C)_4\ (TTTC)_2$	$(AC)_5$
10	$(TG)_7$		$(AC)_7$
11	$(GT)_6$	$(TTG)(T)_9(CTT)_2$, $(T)_7$, $(A)_{10}$	N/A
12*	$(TG)_7GGGAGA(GGGA)_3\ AGAGGG$		$(CA)_6$
13*	$(TG)_8$	$(T)_8$, $(T)_8$, $(A)_6$, $(TCA)_4$	$(CA)_6$
14	$(GT)_6$		$(AC)_6$
15	$(GT)_6$	$(TGA)_4$	$(AC)_7$
16*	$(GT)_9$	$(AT)_5(A)_4(AT)_2ACC(AT)_3CT(AT)_2$	$(AC)_6$
17	$(GT)_6$	$(GA)_2(A)_7GG(A)_9GGGAGGGGA$, $(AG)_3$	$(CA)_8$
18	$(GT)_6GCGT(G)_{12}$, $(A)_9T(GA)_3$		$(AC)_7$
19	$(GT)_6$		(AC)7
20	$(AAC)_4TCAAC$	$ATT(ATTTTT)_3TT$	$(TGG)_4(TCG)_2$
21	$(AAC)_2TAC(AAC)$		$(TTG)_4$
22*	$(CAA)_4(CAC)_3$	$(T)_5(G)_4(T)_2(G)_7$	$(GTT)_4$

* Selected clones for microsatellite primer design

Table 5.3 Characteristic of each microsatellite locus obtained from 5′anchored PCR technique, primer sequences, melting temperature (Tm) and expected PCR product length.

Clone	Locus	Repeat type	Primer sequence 5′ to 3′	Tm (°C)	Product length (bp)
3	Pfgc12	$(TA)_4AAGA(TA)_3$ N_{33} $(GC)_6$	F: GCGGGTCTCTCTCTCTCAT R: TTTCGGCTCGACAAGAGAAT	60 58	118
6	Pfga01	$(GA)_4CG(CA)_2A$ $(CA)_2$	F: CCAACACAGCACAACGTCTT R: GGATCGTGATTTTCGCACTT	60 58	153
8	Pfta06	$(GT)_6$, $(TA)_6$	F: CTGCAGGATGGGAGACTC R: KKHVHVHGTGTGTGTGTGT	58 60	198
12	Pftg17	$(TG)_7GG(GA)_2$ $(GGGA)_3$	F: KKHVHVHGTGTGTGTGTGT R: GGATAAGGGGTGAGAAATTG	56 62	185
13	Pftca01	$(GT)_6$, $(TCA)_4$	F: CATCGGTGTTTGAGGAAAAAA R: KKHVHVHGTGTGTGTGTGT	58 62	177
16	Pftg26	$(GT)_9N_{10}$ $(AT)_5$	F: KKHVHVHGTGTGTGTGTGT R: GGAGACTCAGTCATTCCCAAT	62 62	186
22	Pfcaa5	$(CAA)_4(CAC)_3$	F: GTCCGTCCAACAACAACAA R: TGGACTGCTGGACTCGAT	56 56	226

5.1.2 Microsatellites isolation using a modified enrichment technique

The outcome of slot blot hybridization by using different DIG-labeled oligonucleotide microsatellite probes (i.e., $(TC)_{15}$, $(TG)_{15}$, $(AAT)_8$, , $(CAA)_8$, $(TGA)_8$), the $(TG)_{15}$ and the $(TC)_{15}$ resulted in the stronger and weaker hybridization signals, respectively (Fig. 5.4). In contrast, there was no hybridization signal from $(AAT)_8$ probes whereas other probes such as $(CAA)_8$, $(TGA)_8$ (data not shown) and $(ACC)_8$ showed relatively weak signals. This preliminary result indicates that di-nucleotide (TG/AC) and (TC/AG) repeats are common in the genome of *P. fletcheri* that are relevant to results derived from microsatellite isolation using 5′anchored PCR.

Fifty-one positive clones obtained from microsatellite enriched library were sequenced. All of sequenced clones contain microsatellite repeats but most of them are shorter than 10 repeats. Notwithstanding, two clones that were obtained from the TG enriched library contained an uninterrupted repeat motif at the 20 and 48 TG repeats. As a result, out of 51 sequenced clones, 25 clones contained identical sequences especially clones obtained from the TG enriched library that yielded three sets of identical sequences (i.e., 10, 3, and 2) while the other clones (i.e., obtained from a different enriched library such as TC, TGA, CAA, ACC) were also found to have identical sequences (Table 5.4). However, for the purposes of this research, seven clones were chosen to design primers for microsatellite marker evaluation (Table 5.5).

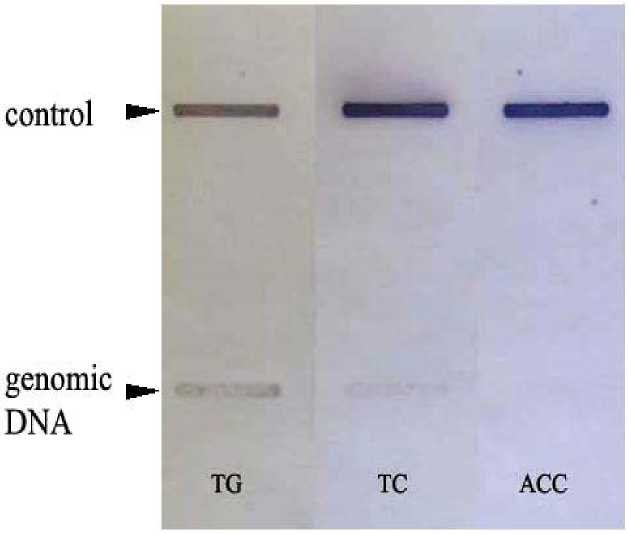

Figure 5.4 Slot blot hybridization signals of *P. fletcheri* genomic DNA using $(TG)_{15}$, $(TC)_{15}$ and $(ACC)_8$ DIG labeling probes; control is oligonucleotide microsatellite repeats (i.e., TG, TC and ACC repeat).

Table 5.4 The numbers of sequenced clones, number of clone containing microsatellites, redundant clones, and average of GC/AT content in the partial genome of *P. fletcheri*.

Probe	Number of sequenced clone	Number of clones containing microsatellites (> 10 repeats)	Number of redundant clone	% G+C (average)
$(TG)_{15}$	20	2	15	39.83
$(TC)_{15}$	11	0	4	38.74
$(TGA)_8$	6	0	2	49.41
$(CAA)_8$	9	0	2	43.22
$(ACC)_8$	5	0	2	41.84
Total	51	2	25	(42.61)

Table 5.5 Microsatellites loci obtained from modification of microsatellite enrichment method.

Clone	Locus	Repeat type	Primer sequence 5′ to 3′	Tm (°C)	Product length (bp)
TGA02	Pfat02	$(AT)_7$	F: AGTCTCAAACCCGACGAGAA R: AATGAAACAGCGAGGATGGA	60 58	212
TGA06	Pftga06	$(TGACGA)_2(TGA)_3$ $TACTGA(TGT)_2$ $TGATAA(TGA)_6$	F: TCAGCGGGATGACGATGAC R: ATGACGATTTTCGATCTGTCTGAC	60 68	159
TGA12	Pftc12	$(TC)_3GG(TC)_5$	F: CCGCATGAGGCAGTAAAGAA R: GGCACTTCCTTGTCGCTTTA	60 60	180
TG01	Pftg18	$(GT)_{48}$	F: CCCAGGAGGAGTGCGTGTG R: GAGGGTGGAGGGATTCTGCT	64 64	236
TG15	Pftg15	$(GT)_{20}(GCGC)GTG$ T(GC)GT	F: TCAAGGTCCTGCAGTTCTCAG R: TCACTCTGTTGCAGCATAGGACTC	60 68	136
TG108	Pftg108	(TA)5T(CA)2T (TA)3	F: ACGCCGGCTGACTCCTG R: GTAAAGACCGAAATAACACAAG	58 60	183
TC38	Pftc38	(GA)5TA(GA)AT (GA)TCC(GA	F: GATCTCGTGCAGCAAACAGA R: GTGGTCCCAAAAACAACACC	60 60	216

5.2 Optimum PCR conditions of each microsatellite locus amplification

Each of microsatellite locus amplification using the genomic DNA of the individual parasitoid was optimized under the general condition (Table 5.6). Only two loci (e.g., Pftg15 and Pftg18) that generally resulted in nonspecific products and stutter DNA bands were optimized by using a special condition. In order to reduce nonspecific PCR products and stutter bands or shadow bands, the following steps were utilized: increased the annealing temperature at the first three cycles of amplification and then reduced to lower temperature for the next cycles, and the PCR reaction was performed only 25 cycles including the long final extension step for 30 minutes at 65°C. Additionally, a PCR enhancer (i.e., 1X masterAmp PCR enhancer, Epicentre) was also found to be useful for reducing stutter bands. However, of the fourteen loci, two loci (i.e., Pfcaa5 and Pftc38) failed to amplify.

Table 5.6 Optimum PCR conditions of each microsatellite locus obtained from 5′ anchored PCR and enrichment method.

Locus	Repeat motif	Number of allele	MgCl (mM)	PCR additive	Ta (°C)	Size range (bp)	PCR cycle
Pfgc12	(TA)$_4$AAGA(TA)$_3$ N$_{33}$ (GC)$_6$	1	1.5	no	55	118	35
Pfga01	(GA)$_4$CG(CA)$_2$A (CA)$_2$	1	1.5	no	55	153	35
Pfta06	(GT)$_6$, (TA)$_6$	1	1.5	BSA	55	198	35
Pftg17	(TG)$_7$GG(GA)$_2$ (GGGA)$_3$	1	1.5	no	55	185	35
Pftca01*	(GT)$_6$, (TCA)$_4$	2	1.5	BSA	55	179-181	35
Pftg26*	(GT)$_9$N$_{10}$ (AT)$_5$	4	1.5	BSA	50	186-192	35
Pfcaa5	(CAA)$_4$(CAC)$_3$	–	–	–	–	–	–
Pfat02	(AT)$_7$	1	1.5	no		212	35
Pftga06[a]	(TGACGA)$_2$(TGA)$_3$ TACTGA(TGT)$_2$ TGATAA(TGA)$_6$	1	1.5	no	60	159	35
Pftc12*	(TC)$_3$GG(TC)$_5$	13	1.5	Betaine	55	180-220	35
Pftg15[a]*	(GT)$_{20}$	15	1.5	Betaine	62	124-158	28
Pftg18*	(TG)$_{48}$	35	1.5	Betaine	62	184-278	28
Pftg108	(TA)5T(CA)2T (TA)3	2	2	no	55	181-183	35
Pftc38	(GA)5	–	–	–	–	–	–

[a] locus come from identical clones

* polymorphic locus

5.3 Microsatellite Polymorphism

Amplified PCR products of each microsatellite locus using genomic DNA of individual parasitic wasp were analyzed by sequencing polyacrylamide gel electrophoresis system with a silver stain. Of the 12 amplifiable loci, only six polymorphic loci (i.e., Pftg26, Pftca01, Pftc12, Pftg15, Pftg18, and Pftg108) were observed (Table 5.7) but only five loci were used as genetic markers for *P. fletcheri* population genetic study. Moreover, these microsatellite loci can be applicable to population genetic study of the other species of *Psyttalia* such as *P. incisi*.

5.3.1 Allele frequencies and heterozygosity

Pftg108 locus consists of two alleles (Fig 5.5) but it was excluded as a genetic marker because the predominant allele frequency exceeded 0.95 when tested among the sampled populations. The allele frequencies, heterozygosity of each polymorphic microsatellite locus including the number of allele at a locus found in each population were recorded in the Table 5.8

The Pftca01 is another microsatellite locus that has only two alleles (i.e., A (179 bp) and B (181 bp) (Fig 5.6). The frequency of the predominant allele (181 bp) across all of the sampled population is also high (0.928) but it is acceptable range for use as a genetic marker. The observed heterozygosity at this locus in all populations except Nonthaburi and Nakhon Ratchasima populations which was found to be fixed at allele B (181bp) is ranging from 0.0526 in the Chumphon population to 0.4062 in the Kanchanaburi population. The expected heterozygosity, not including Nonthaburi and Nakhon Ratchasima populations is ranging between 0.0512 in the Chumphon population to 0.3512 in the Uttaradit-B population (Table 5.8) that the observed heterozygosity in this population is only 0.0909. In the Uttaradit-A population, the observed and expected heterozygosity is 0.1429 and 0.3367, respectively.

Table 5.7 Polymorphic loci of microsatellite makers derived from *P. fletcheri* samples, number of allele, frequency of predominant allele, number of individual females.

Locus	Repeat motif in original clone	Number of allele	Frequency of predominant allele	Number of individual females	Allele size range (bp)
Pftg108	(TA)5T(CA)2T (TA)3	2	0.9956	225	181-183
Pftca01	$(GT)_6$, $(TCA)_4$	2	0.9280	354	179-181
Pftg26	$(GT)_9N_{10}(AT)_5$	4	0.9259	351	186-192
Pftc12	$(TC)_3GG(TC)_5$	13	0.7274	354	180-220
Pftg15[a]	$(GT)_{20}$	15	0.7286	350	124-158
Pftg18	$(TG)_{48}$	35	0.1272	334	184-278

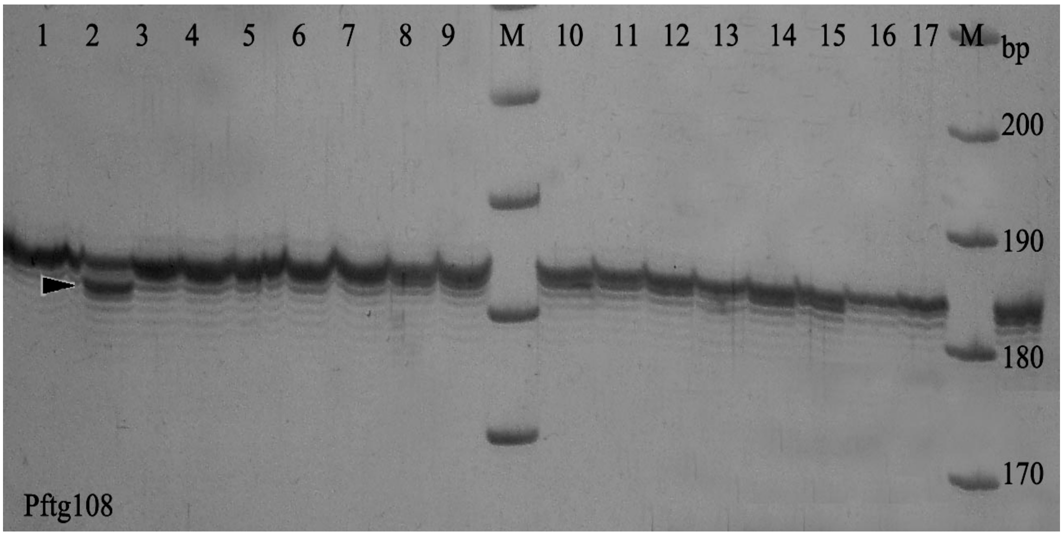

Figure 5.5 Photograph of silver stained polyacrylamide gel of Pftg108 locus from 17 individual wasps of Kanchanaburi population. Lane M, 10 bp DNA ladder; lane 2, polymorphic allele found in a single female of the sampled population; lane 1 and lanes 3-17, monomorphic allele.

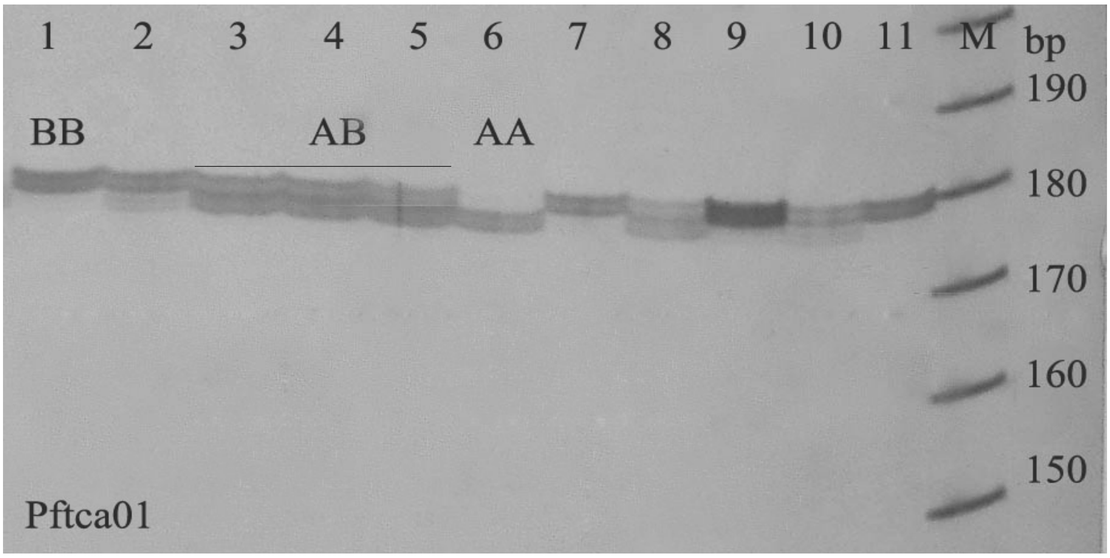

Figure 5.6 Photograph of silver stained polyacrylamide gel of polymorphic alleles at the Pftca01 locus from 11 individual wasps of Phitsanulok population. Lane M, 10 bp DNA ladder; lanes 1, 2, 7, 9 and 11, homozygous genotype (BB); lanes 3, 4, 5, 8 and 10, heterozygous genotype (AB); lane 6, homozygous genotype (AA).

Table 5.8 Allele frequencies and heterozygosity of each microsatellite locus in *P. fletcheri* sampled populations from different localities in Thailand.

	Population							
Locus	KN	CT	NB	NR	CP	PS	UD-A	UD-B
Pftca01	*n=64*	*n=63*	*n=75*	*n=60*	*n=19*	*n=44*	*n=14*	*n=11*
A (179)	0.2031	0.0317	0.0000	0.0000	0.0263	0.1023	0.2143	0.2273
B (181)	0.7969	0.9683	1.0000	1.0000	0.9737	0.8977	0.7857	0.7727
Na	2	2	1	1	2	2	2	2
He	0.3237	0.0615	0.0000	0.0000	0.0512	0.1836	0.3367	0.3512
Ho	0.4062	0.0635	0.0000	0.0000	0.0526	0.1591	0.1429	0.0909
Pftg26	*n=64*	*n=63*	*n=79*	*n=60*	*n=19*	*n=44*	*n=13*	*n=9*
A (186)	0.0234	0.0556	0.0000	0.0083	0.1053	0.0000	0.8462	0.3333
B (188)	0.9766	0.9365	0.9557	0.9917	0.8684	1.0000	0.1538	0.6667
C (190)	0.0000	0.0079	0.0443	0.0000	0.0000	0.0000	0.0000	0.0000
D (192)	0.0000	0.0000	0.0000	0.0000	0.0263	0.0000	0.0000	0.0000
Na	2	3	2	2	3	1	2	2
He	0.0458	0.1198	0.0847	0.0165	0.2341	0.0000	0.2604	0.4444
Ho	0.0469	0.1111	0.0886	0.0167	0.2632	0.0000	0.0000	0.0000
Pftc12	*n=64*	*n=63*	*n=79*	*n=60*	*n=19*	*n=44*	*n=14*	*n=11*
A (180)	0.3047	0.1905	0.1266	0.1417	0.4737	0.1591	0.0000	0.0000
B (182)	0.0000	0.0000	0.0000	0.0250	0.0000	0.0000	0.1429	0.0909
C (184)	0.6953	0.7381	0.8734	0.8333	0.3947	0.7841	0.0000	0.2273
D (186)	0.0000	0.0714	0.0000	0.0000	0.1316	0.0114	0.0714	0.1364
E (188)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2500	0.1364
F (190)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2500	0.0909
G (192)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1429	0.1818
H (194)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0714	0.0000
I (198)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0455
J (204)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0455
K(206)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0455
L (208)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0714	0.0000
M (220)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0455	0.0000	0.0000
Na	2	3	2	3	3	4	7	9
He	0.4237	0.4138	0.2211	0.2849	0.6801	0.2384	0.8189	0.8554
Ho	0.4219	0.3968	0.2278	0.2167	0.5789	0.2273	0.7857	0.9091

Table 5.8 Allele frequencies and heterozygosity of each microsatellite locus in *P. fletcheri* sampled populations from different localities in Thailand. (Continued)

	Population							
Locus	**KN**	**CT**	**NB**	**NR**	**CP**	**PS**	**UD-A**	**UD-B**
Pftg15	*n=64*	*n=63*	*n=75*	*n=60*	*n=19*	*n=44*	*n=14*	*n=11*
A (124)	0.0703	0.1190	0.0067	0.0250	0.0789	0.0341	0.0357	0.0000
B (126)	0.0078	0.0000	0.0000	0.0000	0.0000	0.0114	0.0000	0.0000
C (128)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0114	0.0000	0.0000
D (130)	0.0000	0.0079	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
E (132)	0.0000	0.0238	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F (134)	0.0000	0.0079	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G (138)	0.0859	0.0476	0.0000	0.1500	0.0000	0.0341	0.1429	0.0000
H (140)	0.0078	0.0079	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
I (142)	0.0859	0.1587	0.0000	0.0417	0.0263	0.1136	0.0357	0.0000
J (144)	0.6328	0.5635	0.9533	0.6583	0.8158	0.7159	0.7857	0.9091
K (146)	0.0469	0.0556	0.0067	0.1000	0.0526	0.0227	0.0000	0.0000
L (148)	0.0156	0.0000	0.0000	0.0250	0.0000	0.0000	0.0000	0.0000
M (150)	0.0469	0.0079	0.0333	0.0000	0.0263	0.0341	0.0000	0.0000
N (152)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0227	0.0000	0.0000
O (158)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0909
Na	9	10	4	6	5	9	4	2
He	0.5751	0.6369	0.0900	0.5311	0.3241	0.4698	0.3597	0.1653
Ho	0.4219	0.4127	0.0800	0.3667	0.3158	0.4318	0.1429	0.0000
Pftg18	*n=63*	*n=62*	*n=78*	*n=56*	*n=19*	*n=44*	*n=9*	*n=3*
A (184)	0.0000	0.0081	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
B (186)	0.0000	0.0242	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
C (190)	0.0000	0.0000	0.0000	0.0179	0.0000	0.0000	0.0000	0.0000
D (202)	0.0000	0.0242	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
E (210)	0.0159	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F (212)	0.0079	0.0565	0.0000	0.0000	0.0000	0.0227	0.0000	0.0000
G (214)	0.0079	0.0000	0.0128	0.0179	0.0000	0.0000	0.0000	0.0000
H (216)	0.0000	0.0000	0.0000	0.0804	0.0000	0.0000	0.0000	0.0000
I (220)	0.0000	0.0000	0.0000	0.0179	0.0000	0.0455	0.0000	0.0000
J (222)	0.0000	0.0161	0.0000	0.0000	0.0000	0.0227	0.0000	0.0000

Table 5.8 Allele frequencies and heterozygosity of each microsatellite locus in *P. fletcheri* sampled populations from different localities in Thailand. (Continued)

	Population							
Locus	KN	CT	NB	NR	CP	PS	UD-A	UD-B
Pftg18	*n=63*	*n=62*	*n=78*	*n=56*	*n=19*	*n=44*	*n=9*	*n=3*
K (224)	0.0159	0.0242	0.5064	0.0000	0.0000	0.0114	0.0000	0.0000
L (226)	0.0238	0.0403	0.0000	0.0714	0.0000	0.0000	0.0000	0.0000
M (228)	0.0238	0.0565	0.0256	0.0000	0.0000	0.0000	0.0000	0.0000
N (230)	0.0714	0.0806	0.0705	0.1964	0.3421	0.0114	0.0000	0.0000
O (232)	0.0397	0.0403	0.0064	0.0893	0.0526	0.0682	0.0000	0.0000
P (234)	0.0794	0.0806	0.0000	0.1518	0.0526	0.0568	0.0000	0.0000
Q (236)	0.0635	0.0323	0.0000	0.1339	0.0000	0.2500	0.0000	0.0000
R (238)	0.0714	0.0806	0.0064	0.0179	0.3158	0.0341	0.0000	0.0000
S (240)	0.0635	0.0484	0.0064	0.0357	0.0000	0.0795	0.0000	0.0000
T (242)	0.0635	0.0484	0.0064	0.0179	0.3158	0.0341	0.0000	0.0000
U (244)	0.0873	0.0565	0.0064	0.0089	0.0000	0.1023	0.0556	0.0000
V (246)	0.0714	0.0323	0.1603	0.0000	0.0000	0.0682	0.0000	0.0000
W (248)	0.0476	0.0161	0.0705	0.0446	0.0000	0.0000	0.0556	0.0000
X (250)	0.0794	0.0726	0.0769	0.0089	0.1579	0.0114	0.0000	0.0000
Y (252)	0.0556	0.0484	0.0256	0.0000	0.0263	0.0568	0.0556	0.0000
Z (254)	0.0794	0.0323	0.0192	0.0089	0.0000	0.0682	0.0556	0.1667
a (256)	0.0238	0.0161	0.0000	0.0179	0.0000	0.0341	0.0000	0.3333
b (258)	0.0000	0.0242	0.0000	0.0179	0.0000	0.0000	0.0000	0.0000
c (260)	0.0000	0.0161	0.0000	0.0357	0.0000	0.0227	0.0000	0.0000
d (262)	0.0079	0.0000	0.0000	0.0089	0.0000	0.0000	0.1111	0.3333
e (264)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1111	0.0000
f (266)	0.0000	0.0242	0.0000	0.0000	0.0000	0.0000	0.0000	0.1667
g (268)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2222	0.0000
h (270)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1111	0.0000
i (278)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2222	0.0000
Na	21	25	14	20	7	18	9	4
He	0.9368	0.9482	0.7000	0.8938	0.7493	0.8928	0.8519	0.7222
Ho	0.7619	0.6935	0.7308	0.5179	0.5263	0.7500	0.8889	0.3333

n = number of individual female parasitoid
He = Nei's (1973) expected heterozygosity
Na = Number of alleles

The Pftg26 locus consists of four alleles with the size range between 186-192 bp (Fig 5.7-5.8). Allele B (188 bp) is the most common allele observed among all of the sampled populations. Moreover, it is also found to be a predominant allele among all sampled populations except the Uttaradit-A population (Table 5.8) (i.e., the dominant allele was not B (188 bp) but rather A (186 bp)) (Fig 5.8). However, allele A was not found in Nonthaburi and Phitsanulok populations. This locus was found to be fixed at Allele B among all individual in Phitsanulok populations. The Nakhon Ratchasima population was likewise found to be almost fixed at the frequency 0.991 (Table 5.8). Only one individual wasp showed a heterozygous genotype of AB while all others were noted as the same genotype, BB. Interestingly, allele D (192 bp) was specific to the Chumphon population, whereas allele C (190 bp) was observed only in Nonthaburi and Chanthaburi populations. The highest observed heterozygosity (i.e., 0.2632) was observed in Chumphon population, while the lowest observed heterozygosity (i.e., 0.000) was found in Phitsanulok population that was fixed at allele B. In the Uttaradit-A and Uttaradit-B populations, the expected heterozygosity is relatively high at 0.2604 and 0.4444, respectively, but none of heterozygotes were observed in these two populations (Table 5.8).

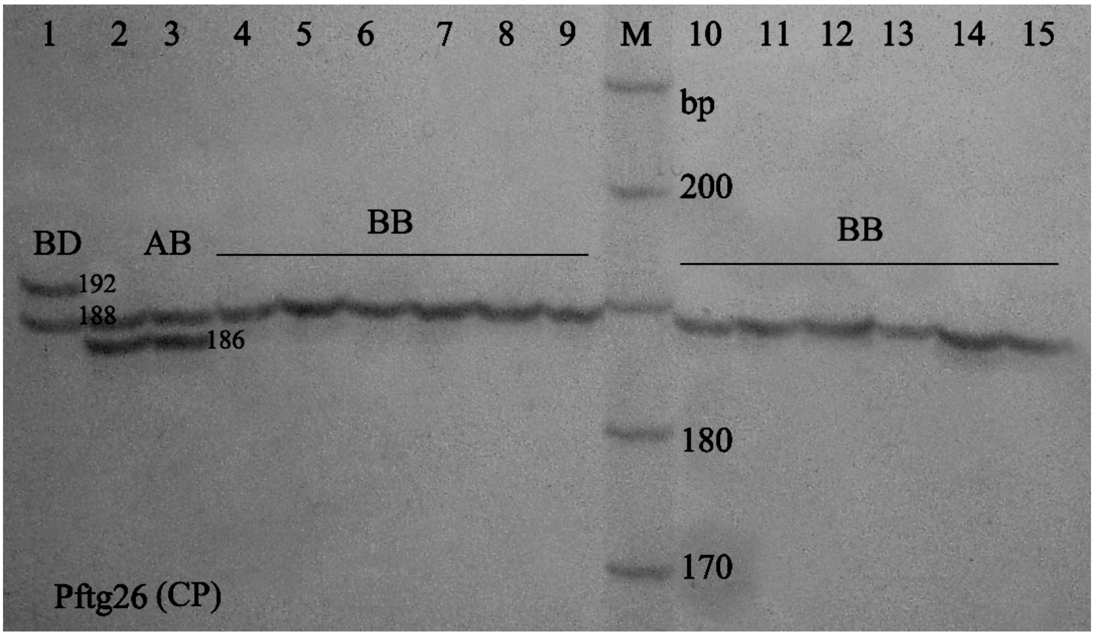

Figure 5.7 Photograph of silver stained polyacrylamide gel of the Pftg26 locus from 15 individual wasps of Chumphon population. Lane M, 10 bp DNA ladder; lane 1, heterozygous genotype (BD) found in a single female of the whole population; lanes 2-3, heterozygous genotype (AB); lanes 4-15, homozygous genotype (BB).

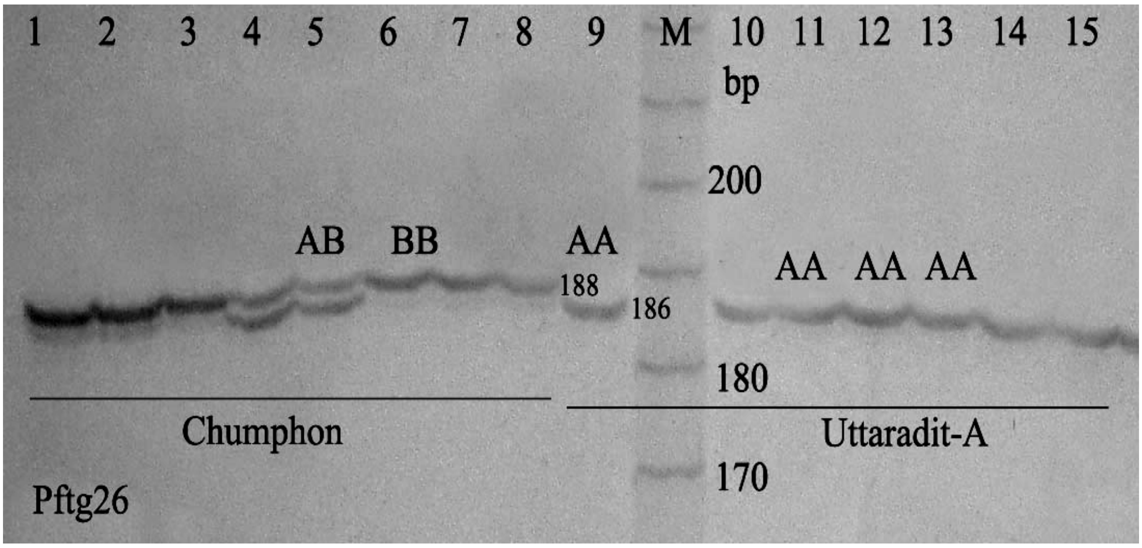

Figure 5.8 Photograph of silver stained polyacrylamide gel of the Pftg26 locus showing two different alleles from eight individual wasps and seven individual wasps of sampled Chumphon and Uttaradit populations, respectively. Lane M, 10 bp DNA ladder; lanes 1-3 and 7-8, homozygous genotype (BB); lanes 4-5, heterozygous genotype (AB); lanes 9-15, homozygous genotype (AA).

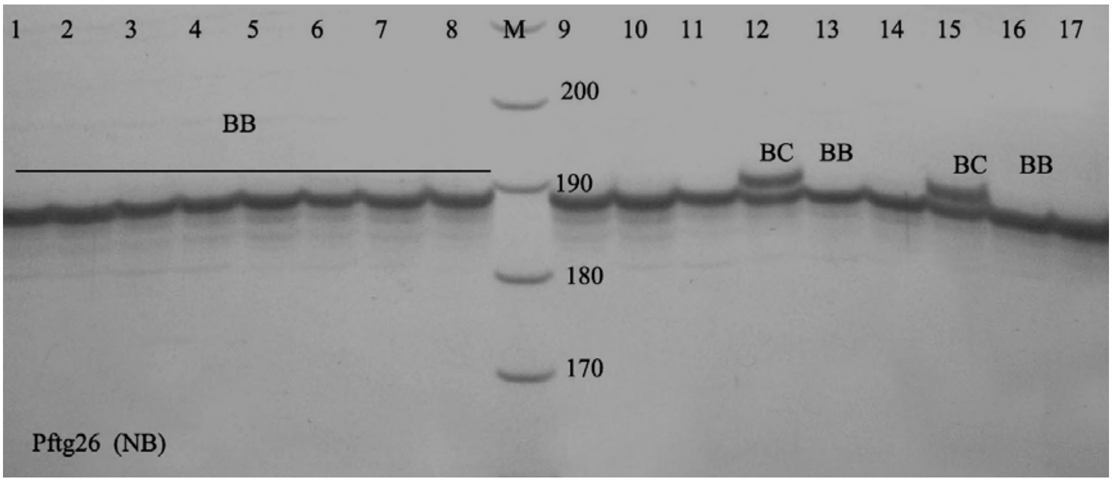

Figure 5.9 Photograph of silver stained polyacrylamide gel of the Pftg26 locus from 17 individual wasps of Nonthaburi population. Lane M, 10 bp DNA ladder; lanes 1-11, 13-14, and 16-17, homozygous genotype, BB; lanes 12 and 15, heterozygous genotype (BC).

The Pftc12 locus consists of 13 alleles with the size ranging from 180-220 bp. Allele C (184 bp) was observed as a predominant allele with the high frequency (0.7274) calculated across all populations. This allele was observed in all population except the Uttaradit-A population — the number of individual parasitoid collecting from this site was relativey small compared to the number of alleles that were observed in this population. There were only 2-4 alleles found in all populations, excluding the populations from Uttaradit-A and Uttaradit-B. Among the Kanchanaburi and Nonthaburi populations, only two alleles (i.e. A (180 bp) and C (184 bp) were observed (Fig 5.10), whereas the alleles A, C, and D (186 bp) were found among the Chanthaburi, Phitsanulok and Chumphon populations. Interestingly, there was a large allele size (M, 220 bp) found only in the Chumphon population (Fig 5.11). Furthermore, allele B (182 bp), were observed only among the Nakhon Ratchasima (Fig 5.12) Uttaradit-A and Uttaradit-B (Fig 5.13) populations. Another noteworthy observation related to the very high diversity (i.e., in terms of the allele number at Pftc12 locus) found among the small sampled populations of Uttaradit-A and Uttaradit-B with 7 am 9 alleles, respectively. Moreover the observed heterozygosity of these two populations is also relatively high — that is 0.7857 in Uttaradit-A and 0.8554 in Uttaradit-B (Fig 5.13, Table 5.8). Not including Uttaradit-A and B, observed heterozygosity of all populations is ranged between 0.2167 and 0.5789 whereas the expected heterozygosity is ranged between 0.2211 and 0.6801.

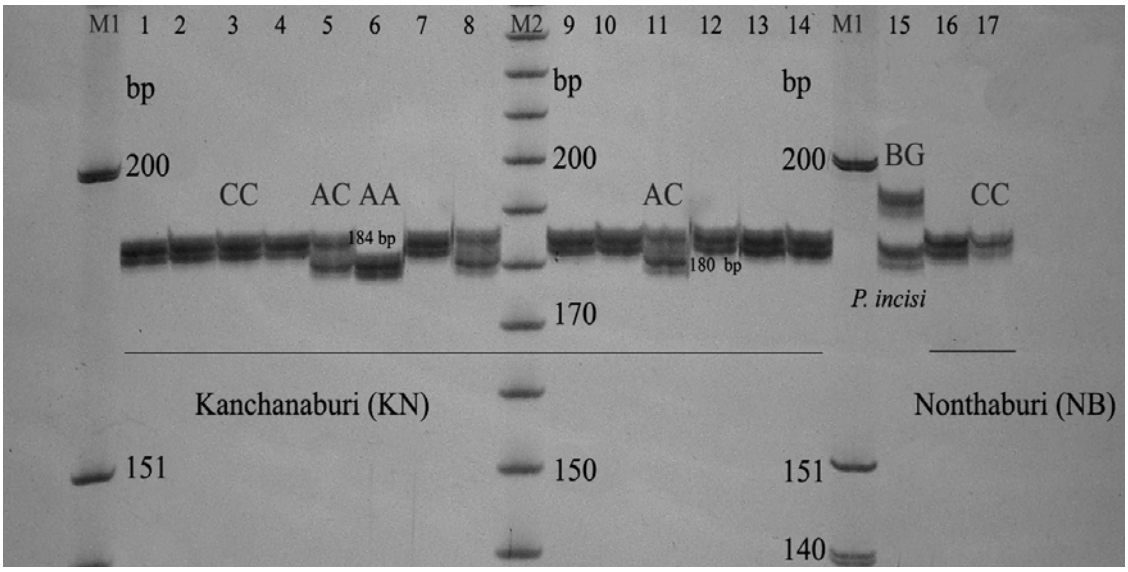

Figure 5.10 Photograph of silver stained polyacrylamide gel of the Pftc12 locus from 14 and 2 individual wasps of Kanchanaburi, Nonthaburi population, respectively and one individual wasp of *P. incisi*. Lane M1, Phi174/*Hin*fI DNA ladder; lane M2, 10 bp DNA ladder; lanes 1-14, homozygous and heterozygous genotype of individual wasp of Kanchanaburi; lane 15, genotype of an individual of *P. incisi*; lanes 16-17, homozygous genotype of two individual of Nonthaburi population.

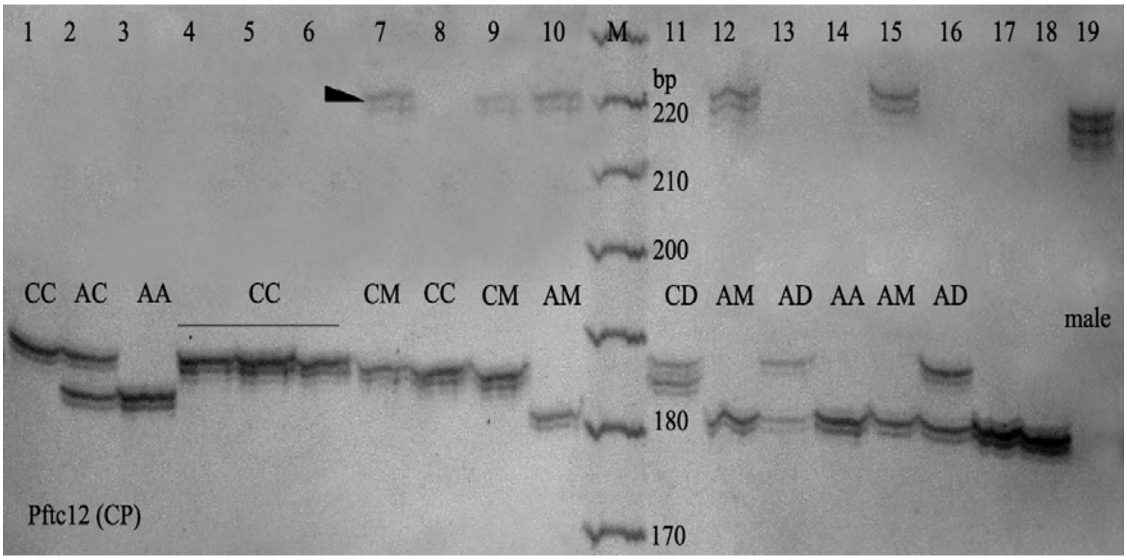

Figure 5.11 Photograph of silver stained polyacrylamide gel of the Pftc12 locus showing four different alleles (i.e., A, C, D and M, arrowed) in Chumphon population. Lane M, 10 bp DNA ladder; lanes 1-18, variable genotypes of each individual female; lane 19, individual male showing a specific allele (i.e.,M) found only in this population.

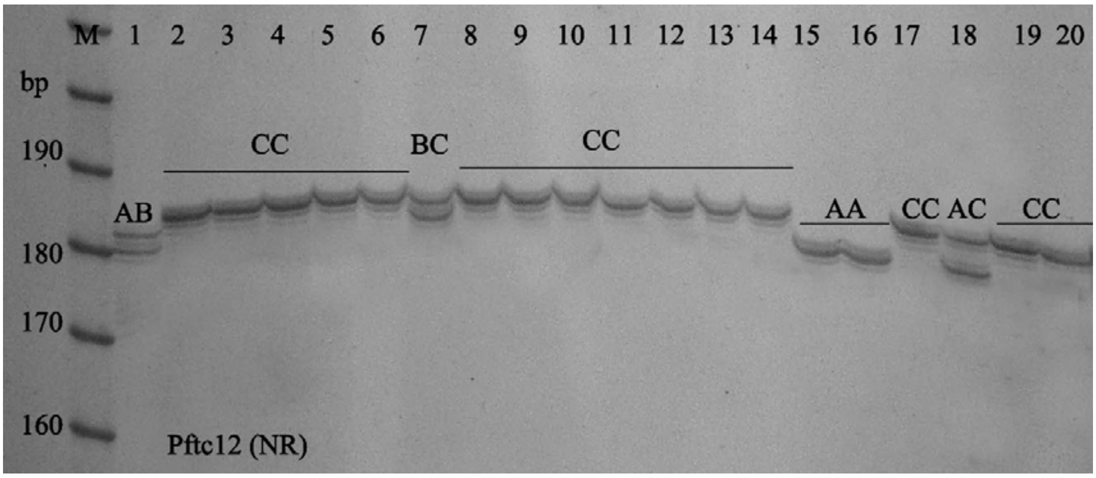

Figure 5.12 Photograph of silver stained polyacrylamide gel of the Pftc12 locus showing three different alleles in Nakhon Ratchasima population. Lane M, 10 bp DNA ladder; lanes 1-20, genotypes of 20 individual wasps of the Nakhon Ratchasima population.

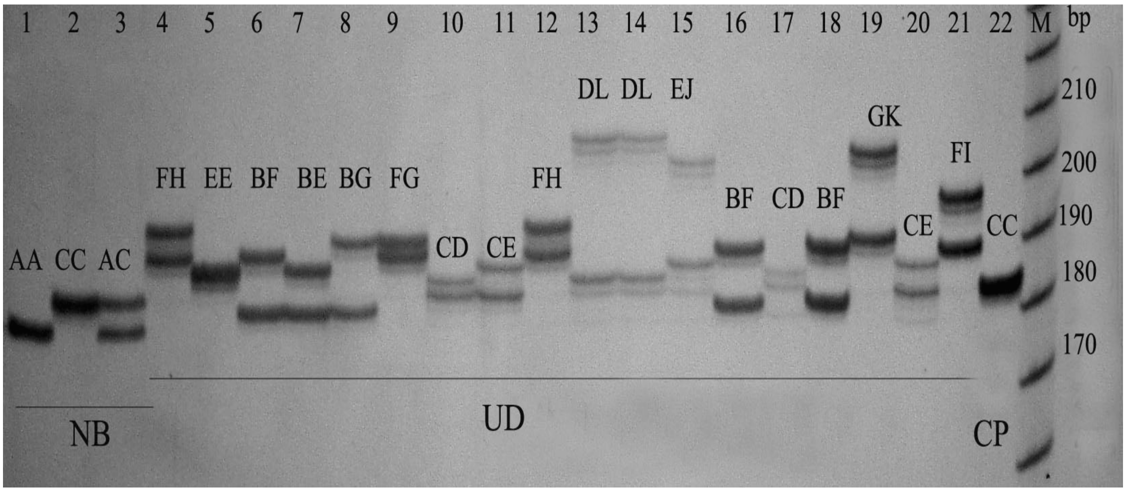

Figure 5.13 Photograph of silver stained polyacrylamide gel of the Pftc12 locus showing high diversity of allelic range found in Uttaradit-A and B. Lane M, 10 bp DNA ladder; lanes 1-3, known genotype samples from three individual wasps of the Nonthaburi population used for sizing; lanes 4-21, genotypes of 18 individual wasps of Uttaradit-A and B population showing high diversity of alleles; lane 22, known genotype (CC) of an individual wasp of Chumphon used for sizing.

At the Pftg15 locus, 15 alleles were observed with the sizes ranging from 124-158 bp (Fig 5.14-5.16). Allele J (144 bp) were observed as the predominant allele in all sampled populations. The highest and the lowest frequencies of this allele were found in the Nonthaburi (0.9533) and Chanthaburi populations (0.5635), respectively.

The allelic diversity of Pftg15 that was observed in each population was found from 2 to 10 alleles. The highest allelic diversity was observed in the Chanthaburi population and the lowest allelic diversity was observed in Uttaradit-B, whereas nine alleles were observed in Kanchanaburi and Phitsanulok populations. Six alleles (i.e., A, G, I, J, K and M) were shared among those three populations. Six and five alleles were observed in Nakhon Ratchasima and Chumphon populations, respectively while only three alleles (i.e., A, I and J) were shared among the two populations (Table 5.8).

Remarkably, the homozygous genotype, JJ (144/144 bp) was observed as having the highest number of genotype among all of the sampled populations, especially in Nonthaburi and Uttaradit-A. Four alleles (i.e., C, D, N and O) were observed as having alleles specific to some populations such as allele C (128 bp) and N (152 bp) were observed only in Phitsanulok population. Allele D (130 bp) and O (158 bp) were found only in the Chanthaburi and Uttaradit-B populations, respectively.

The observed heterozygosity of all populations is ranging from 0.0000 (i.e., Uttaradit-B) to 0.4318 (i.e., Phitsanulok). The observed heterozygosity of Nonthaburi and Uttaradit-A populations is relatively low at 0.0800 and 0.1429 respectively, compared to the others. The expected heterozygosity of all populations is ranging from 0.0900 (in Nonthaburi) to 0.6369 (in Chanthaburi).

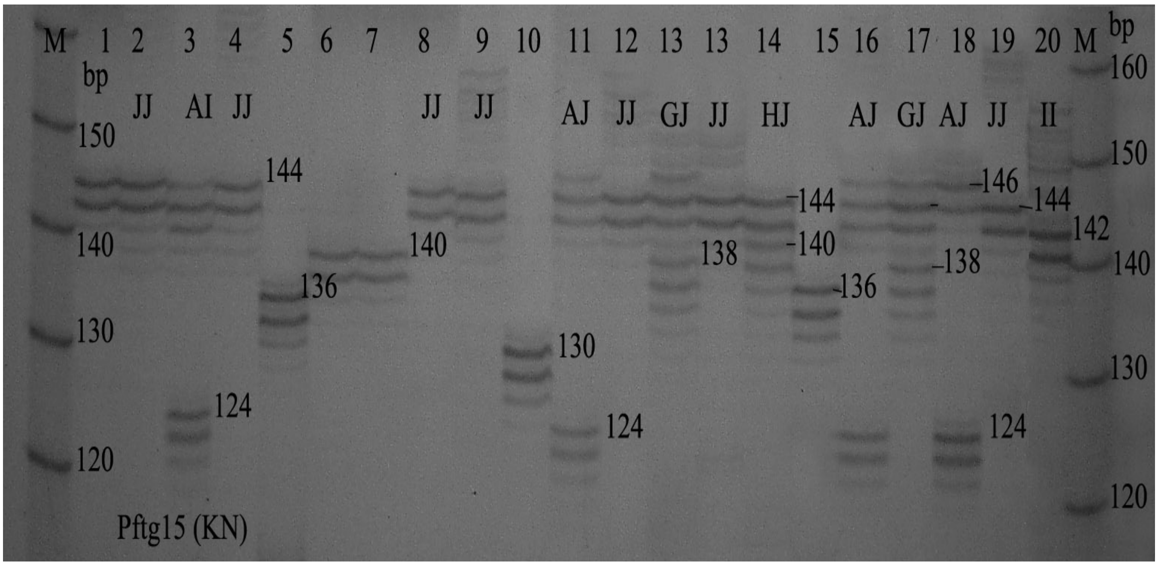

Figure 5.14 Photograph of silver stained polyacrylamide gel of the Pftg15 locus showing variable genotypes of each individual female parasitoid from the Kanchanaburi population. Lane M, 10 bp DNA ladder; lanes 1-4, 6-9, 11-14, 16-20, the genotype of each individual female parasitoid; lanes5, 10, 15, known microsatellite sequenced clone of pftg15 locus.

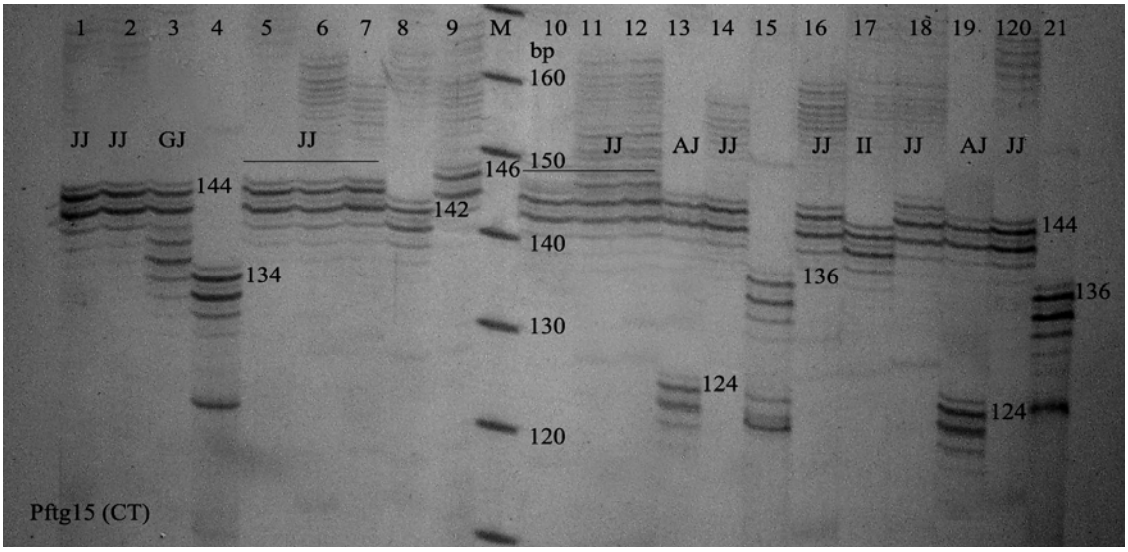

Figure 5.15 Photograph of silver stained polyacrylamide gel of the Pftg15 locus showing variable genotypes of each individual female parasitoid of Chanthaburi population. Lane M, 10 bp DNA ladder; lanes 1-3, 5-14, 16-20, show the genotypes of each individual female parasitoid; lanes 4, 15 and 21, show known microsatellite sequenced clone of pftg15 locus.

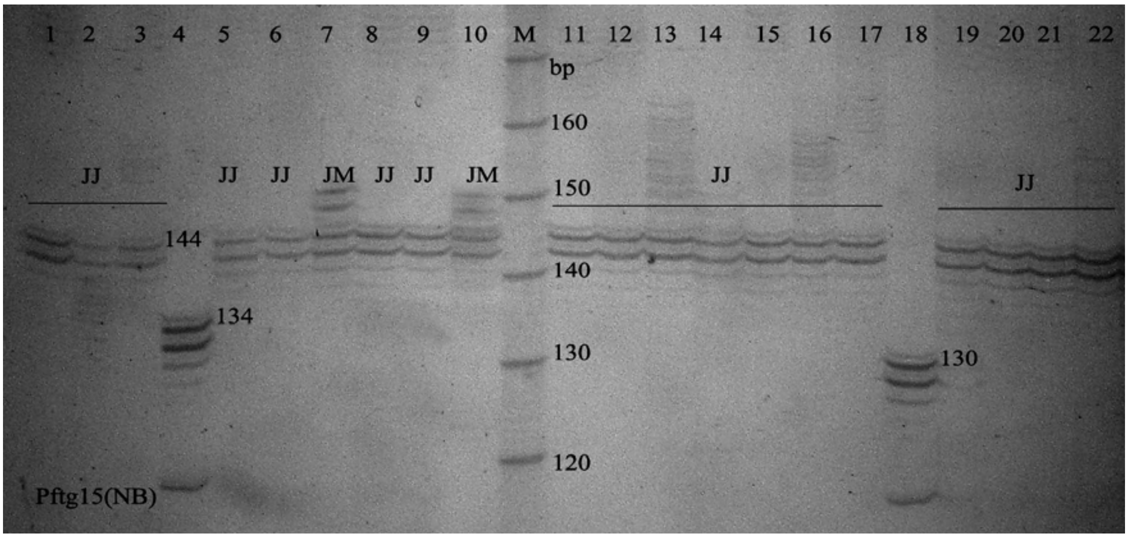

Figure 5.16 Photograph of silver stained polyacrylamide gel of the Pftg15 locus showing the high frequency of homozygous (JJ) genotypes of female parasitoid in Nonthaburi population. Lane M, 10 bp DNA ladder; lanes 1-3, 5-17, 19-22, show the genotypes of each individual female parasitoid; lanes 4 and 18, show known microsatellite sequenced clone of pftg15 locus.

The Pftg18 is the locus that has the highest allelic diversity among all of the microsatellite loci used in this study. This locus consists of 35 alleles with sizes ranging from 184 to 278 bp (Fig 5.17-5.19). An allelic distribution of this locus was calculated across all sample populations and was found to have had the lowest (0.0015) and the highest frequency (0.1272) observed at allele A (184 bp) and allele K (224 bp), respectively (Fig 5.21).

The allele with the highest counts (i.e., 25 alleles) among investigated populations was observed in the Chanthaburi population and the lesser numbers (i.e., 21, 20, 18, 14 alleles) were observed among Kanchanaburi, Nakhon Ratchasima, Phitsanulok and Nonthaburi populations, respectively. In contrast, low numbers of allele count were found in three other populations: Chumphon, Uttaradit-A and B with counts of 7, 9 and 4 alleles, respectively. However, the sampled numbers of individual female populations were also low, especially among sampled population of Uttaradit A and B (e.g., 14 and 11, respectively), in addition to the fact that many samples were not be able to score.

If the populations from Uttaradit A and B are excluded, then the common alleles that were observed in all populations are as followings: N (230 bp), O (232 bp), R (238 bp), T (242 bp) and X (250 bp). Additionally, Z (254 bp) was found among all populations except Chumphon. Even though the allele, K (224 bp) were not found among all populations, it was found to have the highest frequency calculated across all populations (Fig 5.21). Interestingly, most of the alleles that were observed in Uttaradit-A and B populations were large in size ranging from 244 to 278 bp. However, a larger sample would be needed to examine how much diversity of allele at a locus appearing in these two populations.

Several alleles were observed as having alleles for populations that were specific to geographic regions. For example, allele A (184 bp), B (186 bp) and D (202 bp) were observed in Chanthaburi population only, C (190 bp) and H (216) were observed only in Nakhon Ratchasima and allele e (264 bp), g (268 bp), h (270 bp) and i (278) bp) were observed in the population of Uttaradit-A only. Furthermore, allele a (256 bp) was observed only in the Uttaradit-B population and allele E (210 bp) was observed in the Kanchanaburi population only.

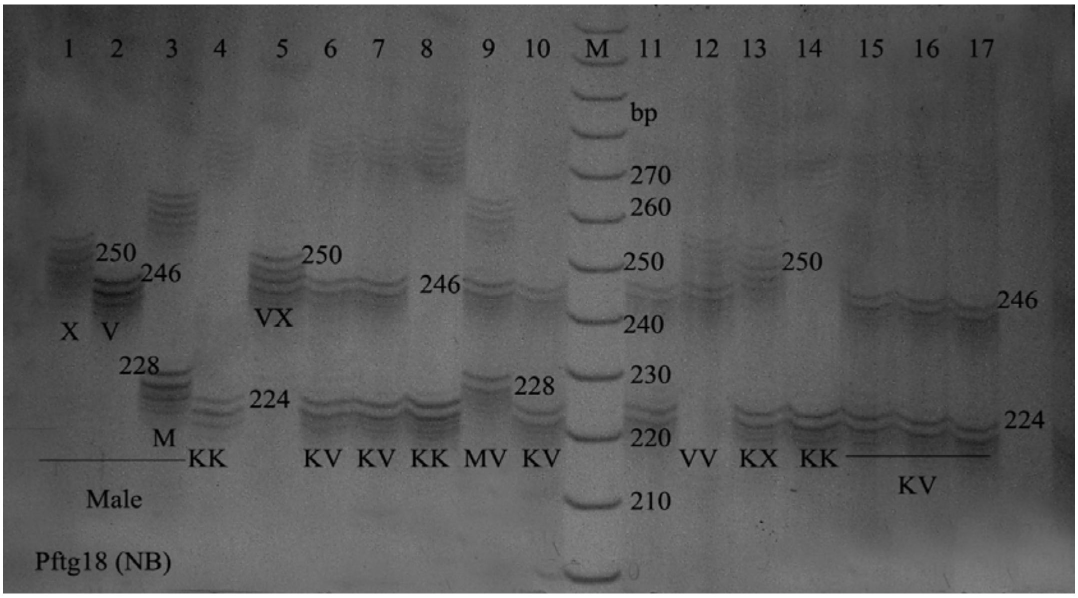

Figure 5.17 Photograph of silver stained polyacrylamide gel of the Pftg18 locus showing the variable genotypes of female parasitoid in Nonthaburi population. Lane M, 10 bp DNA ladder; lanes 1-3, variable genotypes of male parasitoids; lanes 4-17, variable genotypes of each individual female parasitoid.

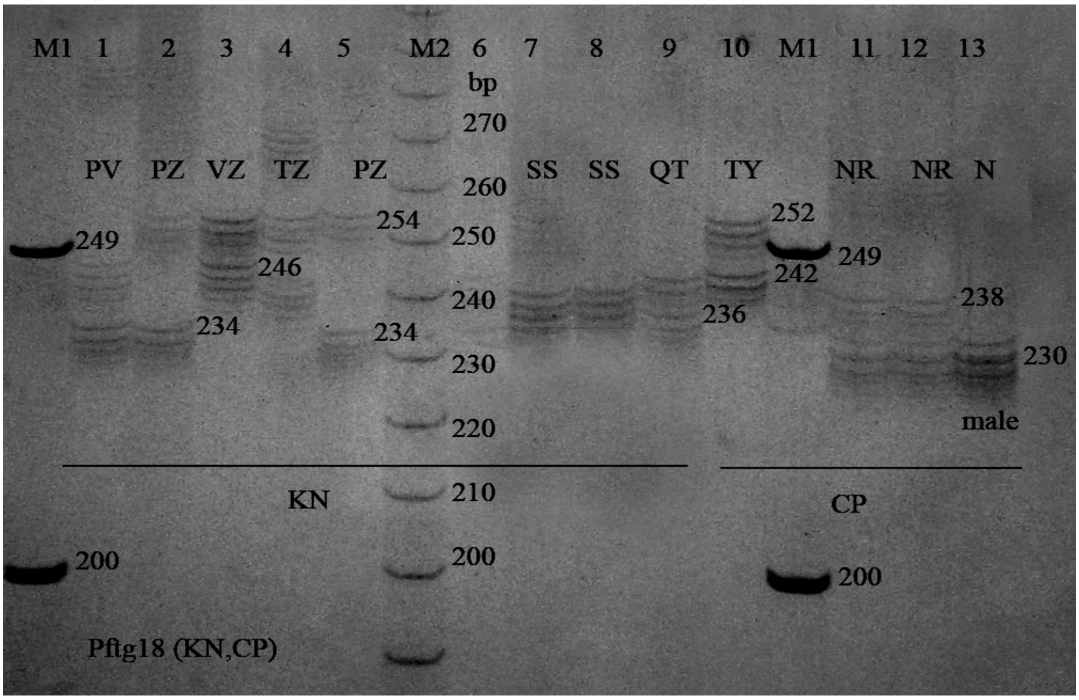

Figure 5.18 Photograph of silver stained polyacrylamide gel of the Pftg18 locus showing the different genotypes of parasitoid sample in Kanchanaburi and Chumphon populations. Lane M1, Phi174/HinfI DNA ladder; lane M2, 10 bp DNA ladder; lanes 1-12, genotypes of each individual female parasitoid; lane 13, male parasitoid genotype (haploid) at allele size 230 bp or allele N.

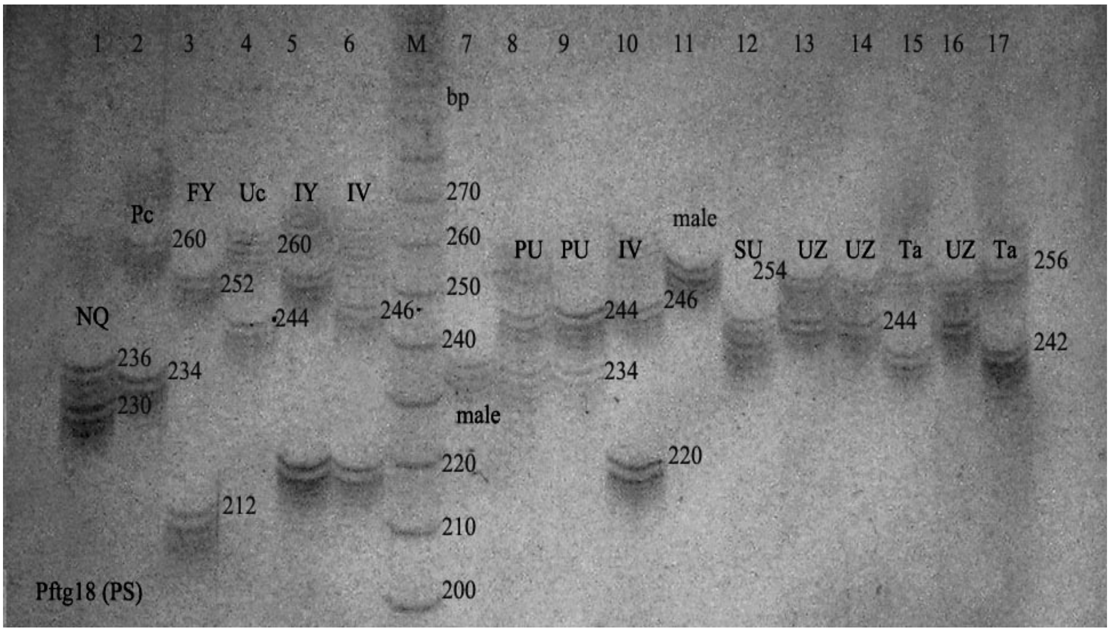

Figure 5.19 Photograph of silver stained polyacrylamide gel of the Pftg18 locus show the different genotypes of parasitoid sample in Phitsanulok populations. Lane M, 10 bp DNA ladder; lanes 1-6, 8-10 and 12-17, variable genotypes of individual female parasitoid samples; lanes 7 and 11, different genotypes of male parasitoid (haploid).

Observed and expected heterozygosity at the Pftg18 locus of all populations were uniformly high. The highest heterozygosity (i.e., 0.8889) was observed in the Uttaradit-A population, while the lowest heterozygosity (i.e., 0.3333) was observed in the Uttaradit-B population — the expected heterozygosity in this population was 0.7222. The expected heterozygosity of all population ranged from 0.7000 to 0.9482. However, among all sampled populations, most of the expected heterozygosity was higher than the observed heterozygosity.

5.3.2 Distributions of allele frequencies

Across all sampled populations, the distributions of allele frequencies of each locus were demonstrated in Fig 5.20 and Fig 5.21. The predominant alleles among four loci (i.e., Pftca01, Pftc12, Pftg26 and Pftg15) showed relatively high frequencies (0.7274 – 0.9280) compared to other alleles at the same locus. The predominant allele frequency of the Pftg18 locus was only 0.1272; however, it was relatively high when compared to other alleles at the same locus. In addition, most of the allele size differences of 2 bp (one unit of microsatellite repeat motif) were observed in every locus. This phenomenon may be because the allelic state changed according to the Stepwise Mutation Model (SSM). Notwithstanding this change, there were three gaps found between the allele size 194 and 198 bp, 198 and 204 bp, and 208 and 220 bp in the Pftc12 locus: one gap was discovered between 152 and 158 bp in the Pftg15 locus and five gaps were found between 186 and 190 bp, 190 and 202 bp, 202 and 210 bp, 216 and 220 bp, and 270 and 278 bp at the Pftg18 locus.

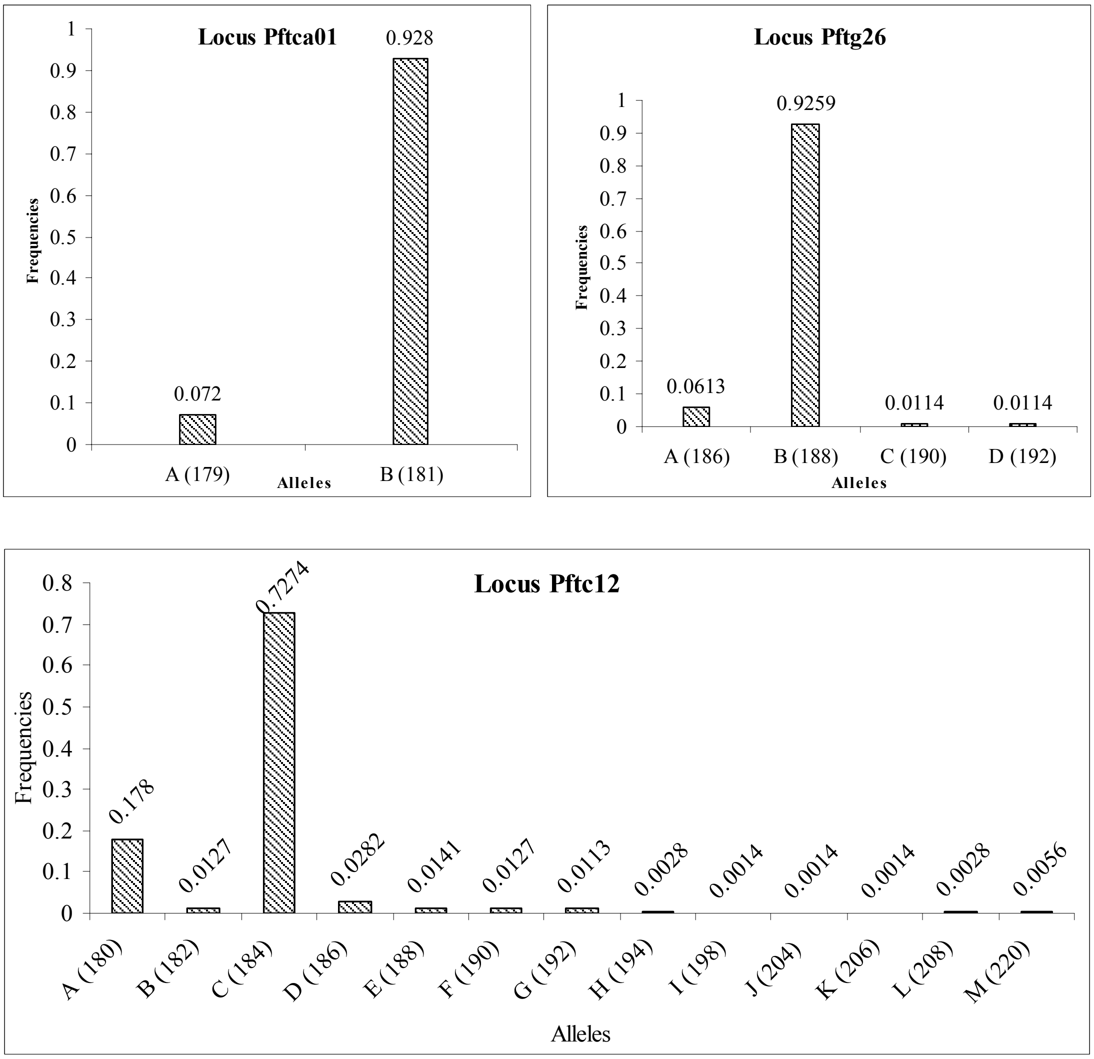

Figure 5.20 Allele distribution of the Pftca01, Pftg26 and Pftc12 loci cross all sampled populations.

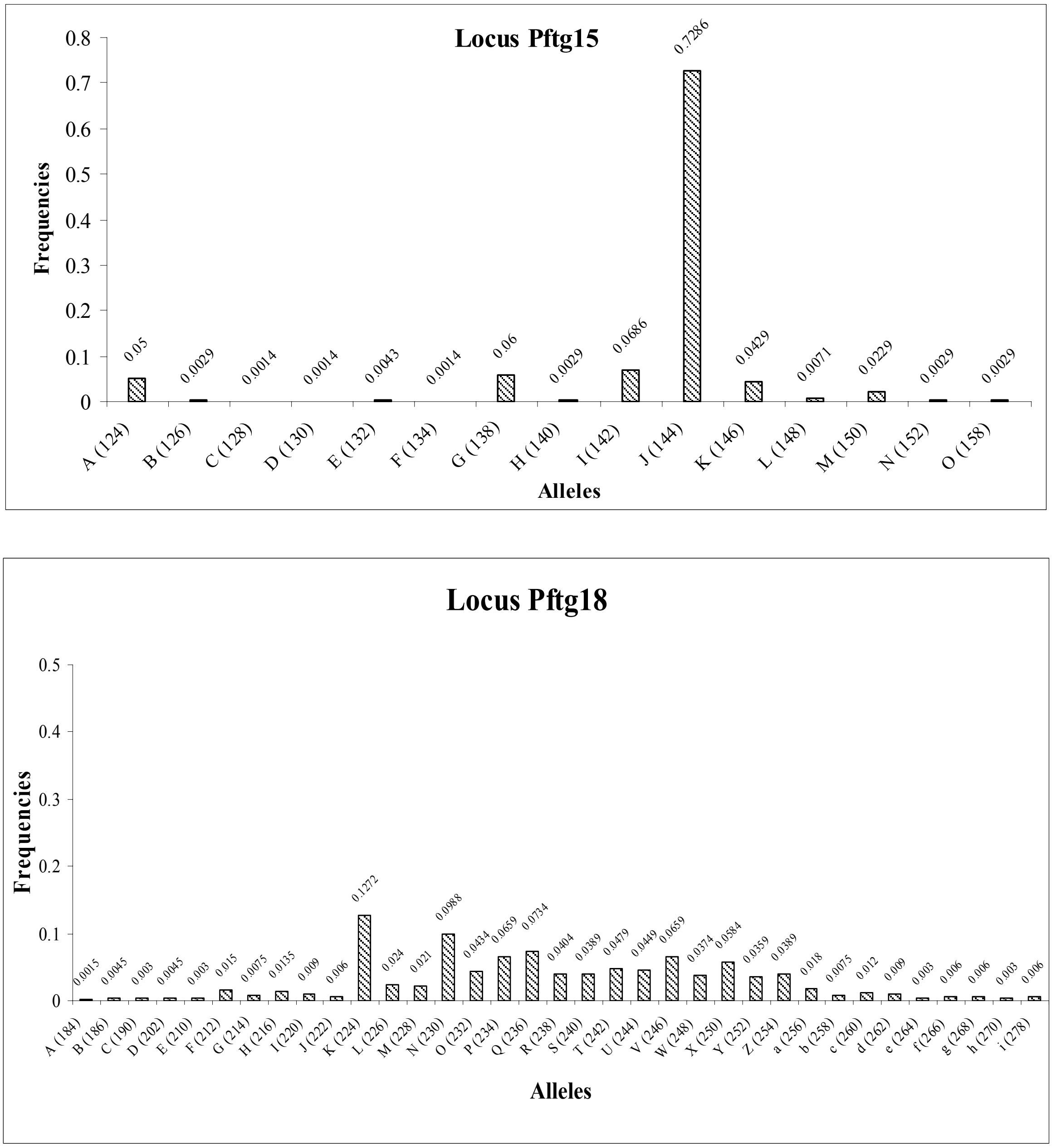

Figure 5.21 Allele distribution of the Pftg15 and Pftg18 loci across all sampled populations.

5.4 Genetic variability in female parasitoid samples from different localities in Thailand

Genetic variability was detected at the five respective microsatellite loci among samples of female parasitoid populations taken from different localities in Thailand. All populations showed signs that the loci were 80-100% polymorphic. However, among the Nonthaburi and Nakhon Ratchasima populations, there were no signs of polymorphisms at the Pftca01 locus, nor were there signs of polymorphism in the Phitsanulok population at the Pftg26 locus. All populations showed high levels of genetic diversity with mean number of allele per locus that ranged from 3.8 in Uttaradit-B to 8.6 in the Chanthaburi population. The mean expected and observed heterozygosity ranged from 0.2191 to 0.5255 and from 0.2236 to 0.4118, respectively (Table 5.9). The mean expected heterozygosity (*He*) was generally higher than the observed heterozygosity (*Ho*). Additionally, only the Nonthaburi population, with observed heterozygosity of 0.2254, was found to be higher than the expected heterozygosity (i.e., 0.2191).

5.5 Chi-square (χ^2) test for Hardy-Weinberg Equilibrium (HWE)

The Chi-square tests for HWE at each locus among all sampled populations were computed using Levene's (1949) algorithm (Table 5.10). At the Pftca01 locus, two sampled populations (i.e., Uttaradit-A (P = 0.0169) and Uttaradit-B (P = 0.006)) showed significant departure from HWE. At the Pftg26 locus, three sampled populations (i.e., Chanthaburi, Uttaradit-A and Uttaradit-B) showed significant deviation from HWE ($P < 0.01$). At the Pftc12 locus, with exception of Uttaradit-A population, all populations were conformed to HWE. In contrast, at the Pftg15 and Pftg18 loci, most of the sampled populations showed signs of significant departure from HWE ($P < 0.01$), except the Chumphon and Phitsanulok populations for pftg15, and the Nonthaburi, Uttaradit-A and B populations for Pftg18 locus.

Table 5.9 Genetic variability of different sampled populations in Thailand over five microsatellite loci.

Population	Percentage of polymorphic loci	Mean number of alleles per polymorphic locus (± SE)	Mean heterozygosity	
			He (± SE)	*Ho* (± SE)
Kanchanaburi	100	7.2 (04031)	0.4610 (0.0132)	0.4118 (0.0184)
Chanthaburi	100	8.6 (0.5474)	0.4361 (0.0210)	0.3355 (0.0201)
Nonthaburi	80	4.6 (0.2749)	0.2191 (0.0143)	0.2254 (0.0148)
Nakhon Ratchasima	80	6.4 (0.4534)	0.3453 (0.0219)	0.2236 (0.0130)
Chumphon	100	4.2 (0.1949)	0.4078 (0.0307)	0.3474 (0.0218)
Phitsanulok	80	6.6 (0.4787)	0.3569 (0.0231)	0.3136 (0.0195)
Uttaradit-A	100	4.8 (0.3875)	0.5255 (0.0357)	0.3921 (0.0515)
Uttaradit-B	100	3.8 (0.4472)	0.5077 (0.0417)	0.2667 (0.0573)
All			0.4213 (0.0131)	0.3057 (0.0176)

He = Nei's (1973) expected heterozygosity

Table 5.10 Chi-square (χ^2) and *P*-value (in parenthesis) of different sampled population of melon fly parasitoid in Thailand.

Locus	Chi-square (χ^2) value of sampled populations							
	KN	CT	NB	NR	CP	PS	UD-A	UD-B
Pftca01	3.9750	0.0504	0.0000	0.0000	0.0000	1.0084	5.7065*	7.5294**
	(0.0462)	(0.8224)	N/A	N/A	(1.0000)	(0.3153)	(0.0169)	(0.0061)
Pftg26	0.0244	17.092**	0.1446	0.0000	0.3409	0.0000	16.762**	10.473**
	(0.8759)	(0.0007)	(0.7037)	N/A	(0.9522)	N/A	(0.0000)	(0.0012)
Pftc12	0.0096	0.8392	0.0473	5.3372	9.6736	0.3991	47.473**	49.200
	(0.9221)	(0.8401)	(0.8278)	(0.1487)	(0.1391)	(0.9404)	(0.0008)	(0.0702)
Pftg15	80.952**	91.135**	149.08**	76.097**	18.318	17.884	18.143**	21.053**
	(0.0000)	(0.0000)	(0.0000)	(0.0000)	(0.0500)	(0.9950)	(0.0059)	(0.0000)
Pftg18	441.95**	686.86**	90.511	636.53**	67.583**	262.70**	46.250	12.000
	(0.0000)	(0.0000)	(0.4947)	(0.0000)	(0.0000)	(0.0000)	(0.1178)	(0.0620)

χ^2 = 0.000 (N/A) meant that locus was fixed at one allele

* significant departure Hardy-Weinberg equilibrium ($P \leq 0.05$)

** highly significant departure from Hardy-Weinberg equilibrium ($P < 0.01$)

5.6 Fixation index (F_{IS})

The fixation index (F_{IS}), a measurement of heterozygote deficiency or excess, was computed by following the procedures adapted by Weir and Cockerham (1984) by using FSTAT, version 2.9.3 (Goudet, 2001). Fixation index (F_{IS}) values can range from –1 to (i.e., excess heterozygotes to 1 (i.e., deficit heterozygotes). If F_{IS} equal to zero indicates random mating in certain population.

The fixation indices in this population genetic study were calculated separately for each microsatellite locus among eight populations. The values of the fixation index for each population were recorded in Table 5.11. Fixation index at the Pftca01 locus revealed significant heterozygote excess in the Kanchanaburi population (F_{IS} = – 0.248, P = 0.04). In Chanthaburi population, heterozygote excess was not significant (F_{IS} = – 0.025, P = 0.952), whereas F_{IS} = 0 was observed in the Chumphon population.

At the pftg26 locus, the negative values of F_{IS} were found among three populations (i.e., Kanchanaburi, Nonthaburi and Chumphon) but all values were not significant different from zero. F_{IS} was equal to 0.000 in the Nakhon Ratchasima population, while the F_{IS} of the Uttaradit-A and B population was equal to + 1.000 that mean heterozygotes are absent in these two populations. Additionally, at the Pftg15 locus, the deficiency of heterozygotes was observed among all populations (F_{IS} > 0), particularly among the Uttaradit-B, (F_{IS} = 1.00, $P < 0.01$) and Uttaradit-A (F_{IS} = + 0.626, $P < 0.01$) populations. Likewise, other populations (e.g., Chanthaburi, Nakhon Ratchasima and Kanchanaburi) also showed evidence of significant heterozygote deficiencies.

At the pftc12 locus, the Nonthaburi and Uttaradit-B populations showed non significant negative fixation index values (i.e. F_{IS} = – 0.024 and – 0.015, respectively), whereas the other populations showed positive fixation index values that ranged from + 0.012 in the Kanchanaburi population to + 0.247 in the Nakhon Ratchasima population. However, none of any population showed significant homozygous excess at this locus.

At the Pftg18 locus, the Nonthaburi population showed slight excess heterozygotes with a negative fixation index value (i.e., F_{IS} = – 0.036, P = 0.3100).

Table 5.11 Fixation indices (F_{IS}) and P-value (in parenthesis) of each microsatellite locus among different sampled populations of melon fly parasitoid in Thailand.

Locus	Fixation indices (F_{IS}) value of sampled populations							
	KN	CT	NB	NR	CP	PS	UD-A	UD-B
Pftca01	– 0.248* (0.0428)	– 0.025 (0.9500)	***** (N/A)	***** (N/A)	0.000 (1.000)	+ 0.145 (0.3728)	+ 0.600 (0.0638)	+ 0.762* (0.0370)
Pftg26	– 0.016 (0.9770)	+ 0.081 (0.2135)	– 0.040 (0.8685)	0.0000 (1.000)	– 0.098 (0.7368)	***** (N/A)	+ 1.000** (0.0050)	+ 1.000** (0.0050)
Pftc12	+ 0.012 (0.5738)	+ 0.049 (0.3650)	– 0.024 (0.6560)	+ 0.247* (0.0263)	+ 0.175 (0.1728)	+ 0.058 (0.5073)	+ 0.153 (0.1580)	– 0.015 (0.7120)
Pftg15	+ 0.274** (0.0003)	+ 0.359** (0.0003)	+ 0.117 (0.1318)	+ 0.317** (0.0003)	+ 0.053 (0.4935)	+ 0.092 (0.2098)	+ 0.626** (0.0050)	+ 1.000* (0.0503)
Pftg18	+ 0.194** (0.0003)	+ 0.276** (0.0003)	– 0.036 (0.3100)	+ 0.428** (0.0003)	+ 0.322* (0.0108)	+ 0.171** (0.0025)	+ 0.015 (0.7803)	+ 0.667 (0.0695)
All	+ 0.114** (0.0005)	+ 0.238** (0.0003)	– 0.022 (0.3598)	+ 0.358** (0.0003)	+ 0.174* (0.018)	+ 0.133** (0.0025)	+ 0.352** (0.0003)	+ 0.490** (0.0003)

* $P < 0.05$, ** $P < 0.01$

N/A = that population was fixed at given locus

In contrast, significant heterozygote deficiencies were observed among all populations, except Nonthaburi and Uttaradit-A populations, with positive fixation index values that ranged from 0.171 to 0.428, whereas Uttaradit-B shows non significant heterozygote deficiency even though the thigh value of F_{IS} was observed (+ 0.667, $P > 0.05$) that may cause by the F_{IS} was calculated from only 3 individual female wasps. Fixation index estimates across all loci were positive values in all populations except the Nonthaburi population which shows non significant negative value ($F_{IS} = -0.022$, $P = 0.3598$). The highly significant levels of excess homozygote were observed in the Uttaradit-B, Nakhon Ratchasima and Uttaradit-A populations (i.e., $F_{IS} = +0.490$, +0.358 and +0.352, respectively).

5.7 Genetic differentiation

5.7.1 Pairwise F_{ST}

Pairwise F_{ST} was calculated by using a weighted analysis of variance (Cockerham, 1973; Weir and Cockerham, 1984). The pairwise F_{ST} was calculated separately for each pair of populations, (Table 5.12) and resulted in a range from 0.0153 to 0.5150. By comparisons, all pairwise F_{ST} estimates between populations were significantly greater than zero ($P < 0.05$). Only two pairwise F_{ST} between the Uttaradit-A and B populations ($F_{ST} = 0.091$) and between Kanchanaburi and Phitsanulok populations ($F_{ST} = 0.024$) showed non significant population differentiation. Other populations (i.e., except Uttaradit-B) showed a significantly higher range from 0.2799 (between Uttaradit-A and Chumphon) to 0.5150 (between Uttaradit-A and Nonthaburi); whereas by comparison, the pairwise F_{ST} value between Uttaradit-B and the other populations ranged from 0.0913 (between Uttaradit-A and B) to 0.2380 (between Uttaradit-B and Nonthaburi). One pairwise F_{ST} (i.e., between Chumphon and Nonthaburi) was also relatively high compared to the other pairwise (i.e., excluding Uttaradit-A and B populations). The pairwise F_{ST} value among eight populations of melon fly parasitoid (Table 5.12) revealed high levels of genetic differentiation in the Uttaradit-A and B populations. Moreover, the pairwise F_{ST} values of the Nonthaburi population, as compared to the other populations, also showed moderate levels of genetic differentiation.

Table 5.12 Pairwise F_{ST} over five microsatellite loci of different localities of female parasitoid populations in Thailand.

Population	KN	CT	NB	NR	CP	PS	UD-A
CT	0.0153*	—					
NB	0.1382*	0.1321*	—				
NR	0.0405*	0.0229*	0.1459*	—			
CP	0.0732*	0.0774*	0.2440*	0.1175*	—		
PS	0.0241^{ns}	0.0253*	0.1371*	0.0289*	0.1425*	—	
UD-A	0.3136*	0.3221*	0.5150*	0.3953*	0.2799*	0.3840*	—
UD-B	0.1295*	0.1435*	0.3280*	0.2015*	0.1202*	0.1837*	0.0913^{ns}

* = significant different ($P < 0.05$)

The scatter plot below illustrates the level of genetic differentiation (based on pairwise F_{ST}) and geographical distance (km) among eight investigated populations. The outcome revealed no significant correlation (r = 0.0961, $P = 0.6265$) between geographical distance and genetic differentiation (Fig 5.22).

In order to calculate *F*-statistics, estimates of a single locus, including all loci across eight populations were computed using the software FSTAT, version 2.9.3 (Goudet, 2001). The estimates of inbreeding coefficient (*f*) showed a positive value in every locus that ranged from 0.040 at the Pftca01 locus to 0.287 at the Pftg15, where the mean value of the *f* estimated from all loci across all population was showed significant positive value ($f = +0.191$, $P < 0.05$) (Table 5.13). This result suggested a non-random mating and heterozygosity deficit or inbreeding within the population. The value of *F*, the measure of inbreeding over all loci and over all populations, was 0.292 and showed evidence of a population structure. The two overall estimations of population differentiation θ and ρ (i.e., $\theta = 0.125$, $\rho = 208$) (Table 5.13) showed significant greater than zero that revealed significant genetic differentiation among populations of the melon fly parasitoid in Thailand. The estimated value of ρ over all loci and over all populations was greater than the value of θ. This result suggest that the estimation of genetic differentiation by using allele size difference (ρ) among individuals maybe more accurate than using an allele frequency (θ).

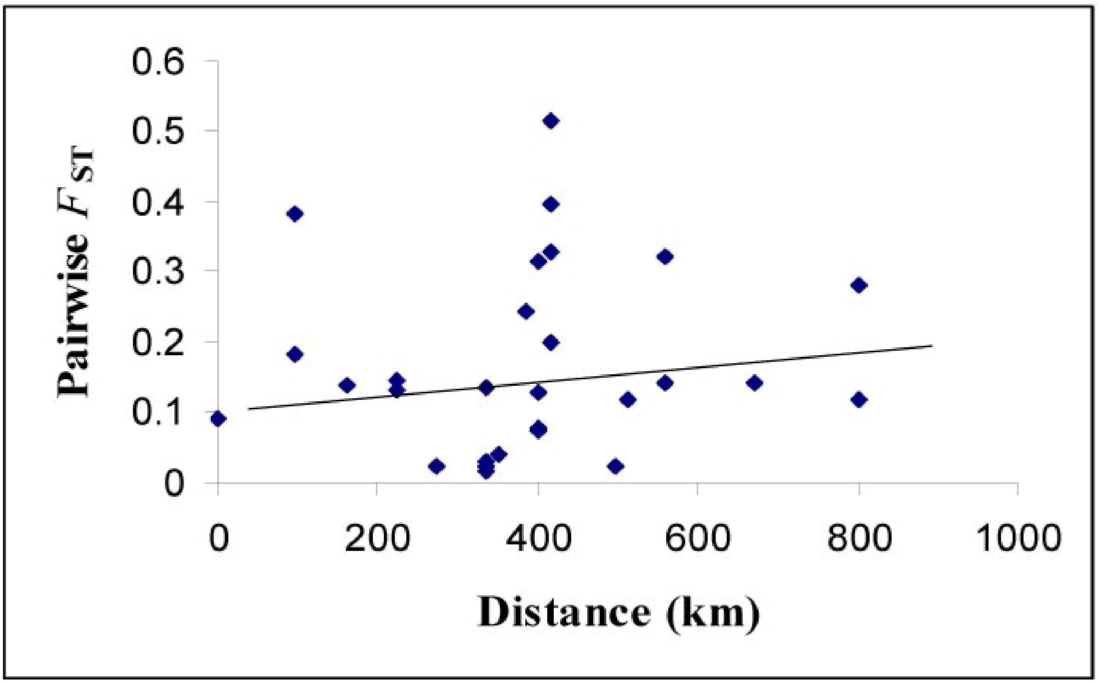

Figure 5.22 Relationships between pairwise F_{ST} and geographical distance among investigated populations of the melon fly parasitoid in Thailand.
(correlation, r = 0.0961, $P = 0.6265$)

Table 5.13 Single locus and over all locus estimates of Wright's *F*-statistics and genetic differentiation for the eight populations of the melon fly parasitoid in Thailand.

Locus	F_{IS} (f)$^{+}$	F_{IT} (F)$^{+}$	F_{ST} (θ)$^{+}$	R_{ST} (ρ)$^{++}$
Pftca01	0.040	0.151	0.115	0.115
Pftg26	0.244	0.561	0.420	0.407
Pftc12	0.071	0.222	0.162	0.293
Pftg15	0.287	0.331	0.062	0.056
Pftg18	0.211	0.284	0.094	0.222
Mean	0.191*	0.292*	0.125*	0.208*§
Upper bound**	0.256	0.371	0.225	N/A
Lower bound	0.078	0.239	0.083	N/A

f , inbreeding coefficient; *F*, over all inbreeding coefficient,

θ, fixation index; ρ, population differentiation based on stepwise mutation model

+, Weir & Cockerham (1984) estimations of F_{IS} (f), F_{IT} (F) and F_{ST} (θ);

++, over all samples estimated following Rousset (1996)

§, value over loci (weighted)

**95% confidence interval bounds on the estimates over all loci

* = significant different ($P < 0.05$)

5.7.2 Genetic identity (*I*) and genetic distance (*D*)

The values of genetic identity and genetic distance were estimated using Nei's unbiased measures (Nei, 1972). The genetic identity (*I*) and genetic distance (*D*) values among eight sampled populations of female parasitoid from different localities over five microsatellite loci were recorded in the Table 5.14. The values of genetic identity among populations were relatively high and ranged from 0.5362 (between Uttaradit-A and Nakhon Ratchasima) to 0.9805 (between Nakhon Ratchasima and Chanthaburi). The lowest value of genetic distance (i.e., 0.0197) was observed between the Nakhon Ratchasima and Chanthaburi populations while the highest value was found between the Uttaradit-A and Nakhon Ratchasima populations. Both observed values (i.e., *I* and *D*) were inversely related to each other (e.g., when the identity value (*I*) between given populations was high, the distance value (*D*) was low). When compared to other populations, the genetic identity values of the Uttaradit-A and Uttaradit-B populations showed uniformly low identity that ranged from 0.5312 to 0.6221 and from 0.7881 to 0.8207, respectively. Moreover, the genetic identity values among the other population pairs were noticeably higher than 0.9 — with the exception of the genetic identity values between the Chumphon and Nonthaburi populations, and the Chumphon and Phitsanulok populations (e.g., 0.8807 and 0.8855, respectively). The high level of genetic distances that was observed between the Uttaradit-A and other populations ranged from 0.4746 to 0.6232.

Table 5.14 Nei's unbiased measures of genetic identity (*I*, above diagonal) and genetic distance (*D*, below diagonal) Nei (1972) between different sampled populations based on five microsatellite loci.

Population	KN	CT	NB	NR	CP	PS	UD-A	UD-B
KN	—	0.9799	0.9315	0.9688	0.9220	0.9784	0.5421	0.8068
CT	0.0204	—	0.9361	0.9805	0.9212	0.9767	0.5536	0.7982
NB	0.0710	0.0660	—	0.9346	0.8807	0.9401	0.5549	0.7994
NR	0.0317	0.0197	0.0677	—	0.9096	0.9769	0.5362	0.7881
CP	0.0813	0.0821	0.1271	0.0948	—	0.8855	0.6221	0.8207
PS	0.0218	0.0236	0.0618	0.0234	0.1216	—	0.5312	0.8016
UD-A	0.6124	0.5914	0.5890	0.6232	0.4746	0.6326	—	0.7890
UD-B	0.2147	0.2254	0.2239	0.2381	0.1976	0.2212	0.2370	—

Nei's (1972) genetic distance was used to construct a cladogram by using UPGMA which was modified from NEIGHBOR procedure of PHYLIP version 3.5 (Fig. 5.23). Eight sampled population were clearly formed into two distinctive clades. All populations except Uttaradit-A were grouped together in order to the genetic distance among them was dramatically low. Whereas the population of Uttaradit-A was separated into another clade by itself. Remarkably, the Uttaradit-B population seems to be separated from the others in the latter clade.

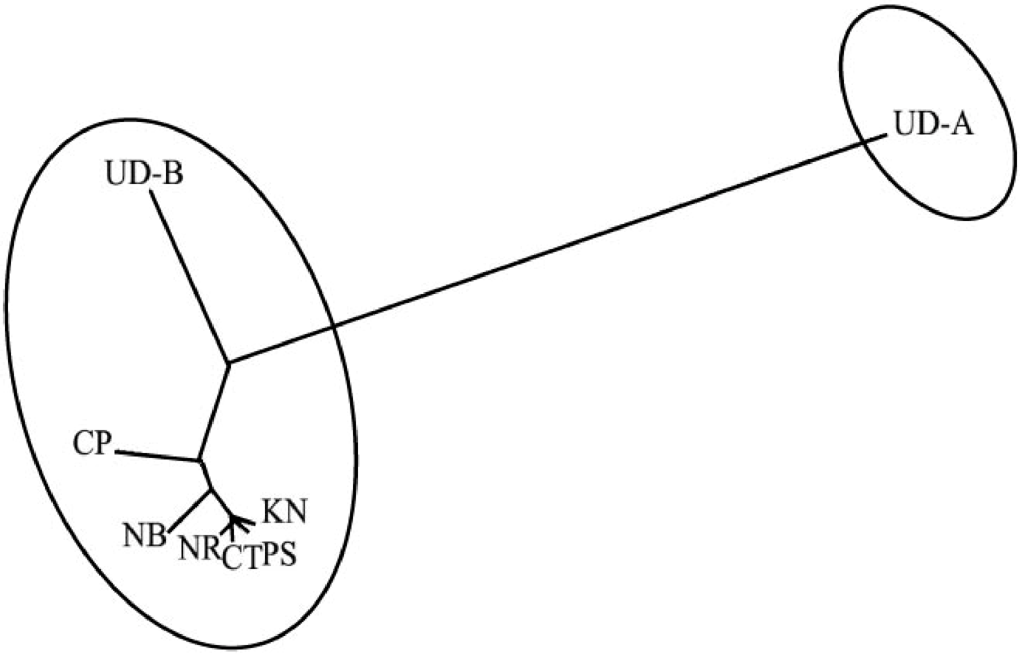

Figure 5.23 A UPGMA unrooted cladogram based on Nei's genetic distance showing genetic relationships among 8 populations of the melon fly parasitoid, *P. fletcheri* from different localities in Thailand (CP, Chumphon; CT, Chanthaburi; KN, Kanchanaburi; NB, Nonthaburi; NR, Nakhon Ratchasima; PS, Phitsanulok and UD-A and B, Uttaradit population A and population B).

CHAPTER VI
DISCUSSION

6.1 Microsatellites of the melon fly parasitoid, *P. fletcheri*

Microsatellites have proved to be valuable markers in insect population genetic studies, e.g. Hymenoptera, aphids, mosquitoes, moths and butterflies (Bogdanowicz et al., 2000; Hughes & Queller, 1993; Lehmann et al., 1997; Palo et al., 1995; Sunnucks & Hales, 1996), and paternity studies in Hymenoptera (Estoup et al., 1995b). Because microsatellites have a highly polymorphic rate, co-dominant and easily scored, they have become the most popular marker for population genetic studies, not only in insects but also various taxa of organism. However, a major drawback to using microsatellite marker lies with the need to isolated new markers, particularly from species that are being examined for the first time. This process resulted in added expense, time and technical requirements (Zane et al., 2002). The numbers of clones containing microsatellites that were obtained by means of traditional methods have been reported to range from 12% to less than 0.04% (Zane et al., 2002). Beyond these limitations, a number of techniques have been developed to deal with the problem of microsattelites isolation and have also increased the number of clone containing microsatellites.

This is the first time for microsatellite isolation in the melon fly parasitoid, *P. fletcheri* that was followed the simple technique (i.e., 5′ anchored PCR) developed by Fisher et al., (1996) to isolate unknown microsatellite data of this species. Di-nucleotides (i.e., TG/AC and TC/AG) repeats were found to be a major repeat motifs in *P. fletcheri*, similar to what was found among the others insect genomes such as *Drosophila melanogaster* (Schug et al., 1998), including human and rat genomes (Beckmann & Weber, 1992). Amplification of *P. fletcheri* genomic DNA yielded PCR products that contained at least two microsatellites (one on each end of amplicon) and which retained their original repeat length (i.e., six repeats for di-nucleotide repeats and four repeats for tri-nucleotide repeats) in each degenerated microsatellite primers. However, the number of microsatellite repeats that were found in some clones exceeded the original repeat length. Internal microsatellite repeats were also found in some clones; however, most of their lengths were not longer than six repeats in di-nucleotides and four repeats in tri-nucleotides. Generally, it was difficult to find polymorphism in the short repeats that ranged from six to nine repeat lengths. In this study, three polymorphic loci, Pftc12, Pftg26 and Pftca01 obtained from 5′ anchored PCR method were contained short microsatellite repeats (i.e., $(TC)_3(GG)(TC)_5$, $(GT)_9N_{10}(AT)_5$ and $(GT)_6(TCA)_4$, respectively) in the original sequenced clones. Polymorphic loci containing short repeat length (i.e. $(AT)_9$, $(GT)_9$ $(GA)_4$) was also found in other insects — for example, the common wasp, *Vespula vulgaris* (Daly et al., 2002), as well as polymorphic loci of the short repeat motifs (i.e. $(CA)_5$, $(TC)_5$ and $(CTG)_2(TTG)_3$ (Scott et al., 2004) being observed in *Helicovera armigera* (Lepidoptera: Noctuidae). The probability of finding polymorphic microsatellite loci from *P. fletcheri* could be increased if larger numbers of clones were screened and sequenced. This study noted that some microsatellite loci that were developed from 5′ anchored PCR method yielded non specific PCR products. For instance, one locus (i.e. Pfcaa5) was failed to amplify. This study conjectured that the failure may have been attributed to the changing of DNA sequences at the priming site and directly related to the fact that the genomic DNA of *P. fletcheri* used for microsatellite marker development in this study came from the different sources. Microsatellite marker development was conducted in Hawaii and the source of genomic DNA used for microsatellite isolation was isolated from introduced species from India to Hawaii. And those markers were then used as genetic markers for studying the populations of *P. fletcheri* that is an indigenous species of Thailand.

Nevertheless, the 5′ anchored PCR method suitable technique for microsatellite surveys in laboratories because only basic instruments are required. Another method that was employed to isolate microsatellite of *P. fletcheri* was taken from a modified enrichment method used by others (Ostrander et al., 1992; Kijas et al., 1994; Hamilton et al., 1999). This approach increased the probability of finding microsatellite repeats (e.g. (CA)n, (AG)n repeats) with approximately 50% of the results being positive clones (Ostrander et al.,1992). Therefore, this technique was chosen for purposes of isolating microsatellites of *P. fletcheri* in instances where the arthropod genome may not be abundantly found (Fagerberg et al., 2001).

Six different types of microsatellite repeats (i.e., (TG), (TC), (AAT), (CAA), (ACC) and (TGA)) were expected to be found in the genome of *P. fletcheri* because those same types of microsatellite repeats have been found in other species of insects such as *Venturia canescens* (Hymenoptera: Ichneumonidae) (Butcher et al., 2000), honeybee, *A. mellifera* and bumblebee, *B. terrestris* (Estoup et al., 1993) and aphid parasitoid, *A. ervi* (Hymenoptera: Braconidae) (Hufbauer et al., 2001). Zane et al., (2002) reviewed that the enrichment efficiency noted in his study ranged from 20% to 90% in a large variety of taxa, using di-, tri- and tetra-nucleotide probes. By contrast, not many positive clones were obtained from enrichment method for microsatellite isolation in *P. fletcheri*. Most of the sequenced clones showed signs of short microsatellites that were less than ten repeats in length. Only two clones contained long length of complete or perfect TG repeats (i.e., $(TG)_{20}$ and $(TG)_{48}$). Moreover, many repetitive sequences were found in the different clones that may have been caused by biased PCR amplification of enriched DNA fragments before cloning (Zane et al., 2002).

However the enriched efficiency level can be affected by two main factors. First, the salt concentration used in the washing buffer may have affected the enrichment efficiency level as evidenced by a 30% reduction when the hybridization washing step was changed from 5 times with 2.0X SSC to 3 times with 0.5X SSC at the 65°C of washing. Second, the temperature of washing step may have also affected the enrichment efficiency level as observed at 71°C when the efficiency declined from 50% at 50°C to 10% at the low stringency wash (2.0X SSC) and declined to 0% at the high stringency wash (0.5X SSC) (Cordeiro et al., 1999). Notwithstanding, these results were in stark contrast to outcomes published by Ma et al. (1996) who reported non-variance of the enrichment level in libraries created with 65°C and 45°C wash temperatures. This finding suggests that at the low salt concentration, the wash temperature may not effect to the efficiency level of the enrichment.

This study identified and further analysis seven microsatellite loci that were obtained from the enrichment method described above. One of the seven microsatellite loci (Pftc38) failed to amplify, three other loci (i.e. Pftc12, Pftg15 and Pftg18) showed polymorphic alleles in natural population tests (predominant allele < 95%), while the last three loci (Pfat02, Pftga06 and Pftg108) showed no signs of polymorphism even though the two allele found in the Pftg108 showed the predominant allele above 95%. The Pftg15 and Pftg18 loci contained the long repeat arrays of di-nucleotide at 20 and 48 repeat motifs, respectively. The variable numbers of repeats at a given locus were specified as variable allele numbers. The longer repeat length showed evidence of higher mutation rates, especially uninterrupted repeated arrays (Weber, 1990). This result supported the notion that the locus, Pftg15 and Pftg18 showed high variable numbers of alleles with 15 and 35 alleles respectively. On the other hand, the microsatellite loci (i.e., Pftg26, Pftca01 and Pftc12) that carried the short length of repeats showed lower variable allele numbers, similar to data reported by Daly et al. (2002); Hufbauer et al. (2001).

6.2 Genetic variation in natural population of *P. fletcheri*

Genetic variation among haplodiploid insects was considered low compared to other insects that have been studied. For example, the expected mean allozyme heterozygosity of ten species of solitary wasps was only 0.048 (Graur, 1985), 0.119-0.358 at 12 loci in the six populations of fruit fly parasitoid, *D. longicaudata* (Kitthawee et al., 1999) and 0.024-0.067 at four loci in the six populations of solitary parasitic wasp, *Opius juglandis* (Lester & Selander, 1979). However, the lack of genetic variation was not found among haplodiploid insects when using microsatellite markers because of the high mutation rate of these markers. The expected mean of the microsatellite heterozygosity was 0.614 at seven loci in honeybee, *A. mellifera* (Estoup et al., 1995a), 0.729 at three loci in three species of ants (Hedrick & Parker, 1997) and 0.280-0.641 at four loci in the four populations of aphid parasitoid, *D. rapae* (Baker et al., 2003).

Genetic variation of the melon fly parasitoid, *P. fletcheri* populations was first investigated using five novel microsatellite markers. The expected mean heterozygosity at five loci among eight natural populations ranged from 0.2191 to 0.5255. The lowest genetic variation was found in the Nonthaburi population (He = 0.2191, Ho = 0.2254), where there was no significant deviation from the HWE nor any signs of homozygote excess ($F_{IS} = -0.022$, $P = 0.6638$). This phenomenon may have several plausible explanations that account for the reduction in genetic variation within the population: parthenogenesis and allele fixation — the latter was observed at the Pftca01 locus in this population. Moreover, the target population may have been subjected to a bottleneck due to environmental disturbance that changed host abundance (Lester & Selander, 1977), inbreeding and/or the relatively small isolated habitat within the urban areas that restricted gene flow. By contrast, the highest genetic variation within the natural populations was found in the Kanchanaburi population (Ho = 0.4118). This may related to the high mean number of alleles per locus in this population (Lowe et al., 2004), which was 7.2 (± SE 0.403).

A significant amount of heterozygote deficiency was observed by Kitthawee et al. (1999) in the natural populations of the Oriental fruit fly parasitoid, *D. longicaudata* which was investigated from the data of protein polymorphism. In my study, heterozygote deficiencies were also found in all populations, especially in Uttaradit-A ($F_{IS} = + 0.352$, $P < 0.01$), Nakhon Ratchasima ($F_{IS} = + 0.358$, $P < 0.01$ and Uttaradit-B populations. ($F_{IS} = + 0.490$, $P < 0.01$). Lester and Selander (1979) noted that heterozygote deficiency was typically found among haplodiploid insects. Moreover, other causal factors (e.g., inbreeding, allele dropped out or null allele and natural selection against heterozygote) may have contributed to the heterozygote deficiency. However, in this study and among the large sample tests, there was no evidence of null alleles.

In this study, estimates of pairwise F_{ST} between populations resulted in observations of significant genetic differentiation among investigated populations of *P. fletcheri* from the different localities in Thailand. Only two pairwise F_{ST} values between the Uttaradit-A and B populations ($F_{ST} = 0.091$) and between Kanchanaburi and Phitsanulok populations ($F_{ST} = 0.024$) showed non significant genetic differentiation. By contrast, the populations with high level of genetic differentiation should have low level of gene flow between them. The parameter for gene flow (Nm) was derived by Wright (1931), $Nm = 0.025 (1–F_{ST}) / (F_{ST})$. Therefore, the level of gene flow between Uttaradit-A and other populations was limited that may explained by host plant or host fly preference. However, the comparisons between the populations which were collected from the same host plant (*C. grandis*) and host fly (*B. cucurbitae*), the pairwise F_{ST} value between populations of Nonthaburi and Chumphon ($F_{ST} = 0.244$) was higher than the others. The geographical distance between these two populations plus small isolated habitat of Nonthaburi population may explain the limited gene flow.

The *F*-statistics estimates can ranged from 0 (no genetic differentiation) to 1 (fixation of alternative alleles), both estimates (i.e., θ and ρ) of multilocus and across all populations of the melon fly parasitoid in Thailand, the ρ or R_{ST} estimate ($\rho = 0.208$) and the θ or F_{ST} ($\theta = 0.125$) showed significant greater than zero. This result indicated genetic differentiation among populations of *P. fletcheri* in Thailand. Baker et al. (2003) reported the two estimates (i.e., θ and ρ) of aphid parasitoid, *D. rapae* in Western Australia populations by using four microsatellite loci; both estimates showed the strong level of genetic differentiation ($\theta = 0.424$ and $\rho = 0.685$). Theoretically, the population differentiation is more accurately estimated by ρ, because this value was estimated under the assumption of the SMM that is better account for the high mutation rate of microsatellite markers. In contrast, θ was estimated under the assumption of the IAM that usually underestimates population differentiation when using microsatellite makers (Slatkin, 1995; Hedrick, 1999).

The genetic differentiation among natural populations of *D. longicaudata* estimated by F_{ST} based on protein polymorphism was relatively low ($F_{ST} = 0.064$) (Kitthawee et al., 1999) when compare to F_{ST} estimate among populations of *P. fletcheri* in this study. However, it is difficult to compare the level of genetic differentiation among populations of these two fruit fly parasitoids (i.e., *D. longicaudata* and *P. fletcheri*) which were estimated from the different types of genetic markers. Relatively lack of studies that have investigated genetic variation in natural population of fruit fly parasitoids using microsatellites, however there were some population genetic studies of the other insect parasitoids. Fore example, the population genetics of the coexisting butterfly parasitoids *Cotesia melitaearum* and *Hyposoter horticlola* were analyzed using nine and four microsatellite loci, respectively. The overall F_{ST} value for *C. melitaearum* ($F_{ST} = 0.378$) was substantially greater than F_{ST} value for *H. horticlola* ($F_{ST} = 0.063$). The results indicated higher degree of the genetic differentiation in *C. melitaearum* populations than the *H. horticlola* populations. Both species showed significant relationship between pairwise F_{ST} and geographical distance (km) (Kankare et al., 2005). In contrast, the relationships between pairwise F_{ST} values and geographical distance (km) among the populations of *P. fletcheri* were not significant in this study.

Genetic differentiation among investigated populations was also estimated by Nei's genetic distance (*D*) and identity (*I*). These estimates revealed high levels of genetic identities among populations ranging from 88% to 98%, not including Uttaradit-A and Uttaradit B populations which were collected from different hosts. The UPGMA unrooted cladogram that was constructed from Nei's genetic distance illustrated two distinctive clades (Fig 5.23). The Uttaradit-A population was separated into one clade by itself whereas the others were grouped together within another clade. However, it was found that the population of Uttaradit-B seem to separate from the others in the latter clade. The genetic diversity in Uttaradit populations maybe explained by the geographical distribution and host plant or host fly preferences or both. Most populations which were collected from the same host plant (*C. grandis*) and the same host fly (*B. cucurbitae*) showed high levels genetic identities (> 90%), whereas the genetic identity between Chumphon and Nonthaburi populations (i.e., 88%) was lower than those values. This value relevant to the pairwise F_{ST} value between the two populations as aforementioned that may confirmed that the limitation of gene flow between Chumphon and Nonthaburi populations.

Brussard et al. (1985) noted that the Nei's genetic identities for insects at various taxonomic levels manifested considerable overlap between taxonomic levels. Among different populations of the same species, the genetic identities ranged from 88% to 100%, subspecies ranged from 77% to 99% and sibling species ranged from 56% to 94%, respectively. Thus, populations that were collected from different host plant and host fly (e.g., Uttaradit-A — host plant, *E. javanica*, Uttaradit-B — host plant, *P. guajava* and other populations — host plant, *C. grandis*) showed low level of genetic identity. The genetic identities Between Uttaradit-A and other populations ranged from 53% to 62%, and between Uttaradit-B and other populations ranged from 79% to 82%. As the genetic identity values, the Uttaradit-A and Uttaradit-B populations may classify as sibling species and subspecies, respectively even though they were similar in morphology. Additionally, high allelic diversity at the Pftc12 locus was found in the small numbers of individuals in the Uttaradit-A (n=14) and Uttaradit-B populations (n=11) at seven and nine alleles, respectively; whereas only

2-4 alleles were found among the other populations. Extremely high allelic diversity at the Pftc12 locus, specific only to these two populations, tended to be an informative genetic marker that differentiated the sibling species of the genus *Psyttalia*; however, a confirmed biological species was required. A single microsatellite marker developed from other parasitoids was also found to be a useful tool in differentiating various strains of *Trichogramma cacoeciae* that were collected from different insect host (i.e., carob moth, *Ectomyelois ceratoniae* and grapevine moth, *Lobesia botrana*), and can also be used as an identification and monitoring tool in pest management programs (Pizzol et al., 2005).

This is the first study of population genetic of *P. fletcheri* using microsatellite makers. The results obtained from this study may provide some genetic information of the investigated populations. However, further study on genetic diversity within this species over the larger geographical area may provide more information and clearer conclusion.

CHAPTER VII
CONCLUSIONS

In this study, the main objective was to measure genetic variations within and between populations of *P. fletcheri* in Thailand using microsatellite markers. However, there were no any microsatellite markers were developed for interested species or related species. Therefore another objective, to develop genetic marker (i.e. microsatellites) of *P. fletcheri* had to be projected. The results suggested that the novel microsatellite markers develop in this study are being effective markers that can be used to evaluate the genetic variation within and among population of either *P. fletcheri* populations or *P. incisi* populations. The results of this study can be concluded as follows:

1. There were fourteen microsatellite loci were developed using 5′ anchored PCR and enrichment methods. Two loci were failed to amplify. Five loci (i.e. Pftca01, Pftg26, Pftc12, Pftg15, and Pftg18) out of twelve amplifiable loci were found polymorphism among sampled populations.

2. Significant heterozygote deficiencies were found in all populations. This result reveals inbreeding within population, especially in the population of Uttaradit-A, Uttaradit-B and Nakhon Ratchasima.

3. There was significant genetic differentiation among investigated populations of *P. fletcheri* which was estimated from both θ (F_{ST}) and ρ (R_{ST}) across five microsatellite loci and across eight populations. Only two population pairs showed non significant different for estimates of pairwise F_{ST} (i.e., between Uttaradit A and B populations and between Phitsanulok and Kanchanaburi populations). This indicated that the limitation of gene flow among those populations.

4. The relationship between genetic differentiation and geographical distance (km) among investigated populations was not significant.

5. High genetic identities were observed among all populations ranging from 88% - 98% not including Uttaradit A and B populations that were collected from different hosts. The UPGMA cladogram constructed from genetic distance between

populations illustrate two distinctive clades. One clade represent Uttaradit-A population and another clade represent all others population including Uttaradit-B that seem to separate from the others in the latter clade. The genetic diversity of Uttaradit population may explained by geographical distribution and host plant or host fly or both.

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APPENDIX

APPENDIX A

Reagents and preparations

Lifton grind buffer / homogenized buffer

0.2 M sucrose
0.05 M EDTA
0.1 M Tris, pH 9.0
0.5% SDS

TE buffer

10 mM Tris-Cl, pH 8.0,
1 mM EDTA

TEN100 buffer

10 mM Tris-HCl, pH 7.5
1 mM EDTA
100 mM NaCl

5X TBE running buffer stock

450 mM Tris-base
450 mM Boric acid
10 mM EDTA, pH 8.0
For the working solution, dilute to 1X, or 0.5 X concentrations
0.5X (45 mM Tris-borate, 1 mM EDTA)

Ethidium bromide stock solution (10 mg/ml)

Dissolve 100 mg of ethidium bromide in 10 ml of dH2O. Wrap tube in aluminum foil and store at 4 °C.

Caution: Ethidium bromide is extremely mutagenic. Wear gloves, eye protection, and a lab coat when working with it.

Sample loading dye (6X)

30% Glycerol
0.25% Bromophenol blue
0.25% Xylene cynanole FF
Store at 4 °C.

SSRP loading dye

10 mM NaOH
95% formamide
0.05% bromophenol blue
0.05% xylene cyanol

Maleic acid buffer

0.1 M maleic acid
0.15 M NaCl
Adjust to pH 7.5 with 1 M solid NaOH

20X SSC (stock solution)

3 M NaCl
0.2 M sodium citrate
Adjust to pH 7.0 with 1 M HCl

Washing Buffer

0.1 M maleic acid
0.15 M NaCl
0.3% (w/v) Tween 20
Adjust to pH 7.5 with solid NaOH

Blocking stock solution (10X)

Dissolves blocking reagent (Boehringer DIG RNA labeling and detection kit) in maleic acid buffer (10% w/v) by constantly stirring on a heating block (65 °C) or heat in microwave, autoclave and store at 4 °C.

Working solution, 1X, prepared by diluting the stock solution 1:10 in maleic acid buffer.

Detection buffer

0.1 M Tris-HCl
0.1 M NaCl
50 mM $MgCl_2$, pH 9.5

The Colorimetric detection solution (always prepare fresh)

0.4 mg/ml nitro blue tetrazolium chloride (NBT)
0.19 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate (BCIP)
0.1 M Tris, pH 9.5
50 mM magnesium sulphate

Antibiotic stock solution

Ampicillin stock solution (100 mg/ml in dH_2O)
store at –20 °C

LB broth (1000 ml)

10 g tryptone
5 g yeast extract
5 g NaCl
Autoclave and store at room temperature

LB agar plates (1000 ml)

10 g tryptone

5 g yeast extract

5 g NaCl

15 g agar

Autoclave and store at 4 °C

S.O.C. medium

2% tryptone

0.5% yeast Extract

0.4% glucose

10 mM NaCl

2.5 mM KCl

5 mM $MgCl_2$

5 mM $MgSO_4$

$MgSO_4$ (1 M stock solution)

24.65 g $MgSO_4.7H_2O$

Dissolves in 1000 ml dH_2O and Sterilized by filtration

6% denaturing polyacrylamide gel in 7 M Urea (100 ml)

40% (19:1) AccuGel	15 ml
10X TBE	10 ml

210 g Urea

Add ddH_2O up to 100 ml and filtrate through Watman filter paper

Bind silane working solution

Ethanol	8 ml
Gracial acetic acid	200 µl
Bind silane	10 µl
ddH_2O	1.8 ml

Staining solutions

Fixing and stopping solution: 10% ethanol and 0.5% acetic acid

Silver staining solution: 1.5 g silver nitrate, 1.5 ml 37% formaldehyde in 1000 ml ddH_2O

Developing solution: 15 g NaOH and 2 ml of 37% formaldehyde in 1000 ml ddH_2O

APPENDIX B

DNA sequences

Locus	Microsatellite sequences
Pftg108	> Pftg108 CCGTTTACAGTGGACTGACGCCGGCTGACTCCTGATTGGCTACGGCAGATGCACCTAT CCTATATATATATCACATTATATACAATCAGAACCAGTCGTTACAGCGTGTCGCGCGA ATATGAAAGGTAAAAGTCACTTGAAATTAAATTGAATGATAGAGTCCATTCCTGTGGA AAACCTTGTGTTATTTCGGTCTTTACAACCAAGTTACATGGACCAAAAAATAAAAATC ACCGATGTGAAAATTCACGTGATATTGTGTGGTTGCACAAGGTCTTTGTGATGAAAAA ACACACTTTTTTGTTCTTTCTTTTTTTCTTTCTATCCATGGATTTTCCATTTGTATTG
Pftca-01	> Pftca01 GGAATGTGTGTGTGTGTGTGGATAAATTGAAACAAGTTAACTTCTACTTTTTTTTATA CAAACCATTTCCACTTTGTCACTTTTGATAATTACAATAAAAACGCGCGTACTCGATA TTTAATTTCATTCAAATTTTTTTTCTACGTTTATTTATCTCGTTATTAATTAGAATAA TTTCACATCGGTGTTTGAGGAAAAAATAAAGTAATTAGAATGTGAAGAGGATGTTTGC TTCGAGCGTTATTAAATGAATTAATTATTAATGAATATTGAGATTAATCGTAGGCGAA TTTTTAGTCATCATCATCAATTCATTGACTACGTGTAATCGGTGTTTGACACACACAC ACTCGGTC
Pftg26	> Pftg26 GGTGTGTGTGTGTGTGTGTGTAAACTGGTGACATATATATATAAAATATACCATATATCT ATATGTATACATAATTCACAGTTAGTGAAAAAATATCAATTTTAGTCATCCAATTTTC TTCAAGACAAGAATCATTCAATATCCAACAAAAGTAAAATATTACCATTATTGGGAAT GACTGAGTCTCCCATCCTGCAGGGATATACAATTCTCTTACTGAGCTGAGACCATCGG AGCTTGACTGAAAAAAGTGTGGGAGGGAGACTGAGTGCAAAAAATTGTCTTCTTTTCC AACCTCATTAAAGAGTAGTGTTCAATTACTGGATTTGTTGTGTATTGGGCTACACATA TGTGCTCGGTATCAAGGCATTTTTGTAGGATAAATGTGACACACACACACTCGCACC
Pftc12	> Pftc12 TGCCTCGGACCCCCTCACCAACCGCGTATCCACCACCTACCCCTGCCTCACCCGTCTC TCCGCATGAGGCAGTAAAGAAAGATACCGTGAGATCCCGAGACAACACCCATATTGCC AAAGCTTTTTTTGTTTCTCAATCTCTCGGTCTCTCTCTCTACACTTCTACTCCATCGG AGACTGGATAGATTTTTCTCTGCGGACTTAATCTAAGCCTCAGAATAAAGCGACAAGG AAGTGCCGAGTATCTTCTCCTTTCCATTTGATTTTCTCTCTCACTCTTTGCACTTTTT TATCCCTTGGAGGAGAGTTTTTCGAGGACTGAGCACACTTCTCCTCTTGTTTTCTGAA TTCATCTCAAACTTTTGGAATCAACTTTCAAACTGTATCTGAAGAAGAGTGAAAGAGC
Pftg15	> Pftg15 TCAAGGTCCTGCAGTTCTCAGGAGGAGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTGTGTGTGCGCGTGTGCGTGAGACACTGGATTTTTAGTGCCACGATTTGGAAGGAGT CCTATGCTGCAACAGAGTGATTTAATAAGGTAACGTGATTTCTATTTGCCGATTATTC TTGCCCCGTTTTTGGTAGTTTCGTGAGAGCATCGGTATGCTCATTTATATCGCGAAAT AGGTCGGGTAGAAACGAAATCGCGTGT
pftg18	> Pftg18 GCAAGGTCCTGCAGTTCCCAGGAGGAGTGCGTGTGTGTGTGTGTGTGTGTGTGTGTGTG TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG TGTGTGTGTGTGATCACTTTTTTTTCGTTCACGCTCCCGGACGAACGCGCAGTGCGATC GGATTTTTTTTCATCGCAATCGATAGATATTATTGAGGACAAGATCGGATTAgTTTTG GAGCAGAATCCCTCCACCCTCTGCTGAGTTTTTTCGGAAGGAAGGGTTGATAGGGAAT AATATCAATCATTCAGTCGTTCAATCTTTTACGTTCACGTTCCCGGACGAACGCGCAG

Underlined sequences are primers

BIOGRAPHY

NAME	Miss Somjit Tinkrathok
DATE OF BIRTH	5 February 1968
PLACE OF BIRTH	Nakhon Ratchasima, Thailand
INSTITUTION ATTENDED	Kasetsart University, 1986-1990: Bachelor of Science (Biology) Kasetsart University, 1991-1994: Master of Science (Genetics) Mahidol University, 2001-2007: Doctor of Philosophy (Biology)
SCHOLARSHIPS	A scholarship under the Commission on Higher Education Staff Development Project, Thailand, June 2001-May 2004
POSITION & OFFICE	A lecturer, 1994-2000 Assistant Professor, 2001- present Department of Biology, Naresuan University
HOME ADDRESS	1 Moo 1 Thumbon Ban Mai Amphur Nong-Boon-Maak Nakhon Ratchasima, Thailand 30410
E-MAIL	somjitt@hotmail.com, tinkrathok@yahoo.com