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TITLE: Molecular Diversity of Tomato Thrips, *Ceratothripoides claratris* (Shumshur) (Thysanoptera: Thripidae) Populations Found in Thailand Using PCR-SSCP

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THESIS

MOLECULAR DIVERSITY OF TOMATO THRIPS, *CERATOTHRIPOIDES CLARATRIS* (SHUMSHUR) (THYSANOPTERA: THRIPIDAE) POPULATIONS FOUND IN THAILAND USING PCR-SSCP

URAKORN THAKAEW

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Urakorn Thakaew 2010: Molecular Diversity of Tomato Thrips, *Ceratothripoides claratris* (Shumshur) (Thysanoptera: Thripidae) Populations Found in Thailand Using PCR-SSCP. Master of Science (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Professor Tipvadee Attathom, Ph.D. 99 pages.

Thrips, *Ceratothripoides claratris* ranks among the most noxious insects attacking several field and greenhouse cultivated crops in Thailand. Varieties or biotypes of thrips can not be differentiated morphologically. This study aimed to determine genetic variation among and within *C. claratris* populations in Thailand using DNA-based analysis. Based on sequences from other insects, available from GeneBank/EBML database, degenerate primers were designed for 9 genes of insect: 3 mitochondrial genes Cytochrome oxidase I (*COI*), ATP synthase subunits 6 (*ATP6*) and NADH dehydrogenase (*NADH*); 6 nuclear genes Phosphoenolpyruvate carboxykinase (*PEPCK*), Elongation factor1α (*EF1α*), Opsin (*OPS*), Alcohol dehydrogenase (*ADH*), Dopa decarboxylase (*DDC*) and Arginine kinase (*ARGK*). The thrips mitochondrial gene *COI* and the nuclear genes *EF1α* and *ARGK* genes were amplified and cloned using the designed degenerate primers. The obtained thrips DNA sequences of each gene were used to develop locus specific primers. The developed specific primer sets and the published primers for the internal transcribed spacer *(ITS*) could successfully amplify specific DNA fragments from *C. claratris* in all populations studied. Intraspecific variation of twenty-six accessions of *C. claratris* collected from different areas in Thailand was determined by molecular analysis using SSCP techniques. SSCP analysis showed little polymorphism of specific amplified products. The amplified *EF1α* gene product was apparently monomorphic and only 5, 3 and 7 banding patterns were detected for *COI*, *ITS* and *ARGK* loci. UPGMA cluster analysis of all populations distinguished 15 groups at 0.77 of similarity coefficient, using polymorphic bands pattern generated from 4 primer amplifications separated on poly acrylamide gel. All tested *COI*, *ARGK* and *ITS* genes have considerable potential to be used as DNA markers for population analysis in thrips.

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Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

ADH	=	Alcohol dehydrogenase
ARGK	=	Arginine kinase
ATP6	=	ATP synthase subunits 6
BLAST	=	Basic Local Alignment Search Tool
bp	=	base pair
COI	=	Cytochrom oxidase I
DDC	=	Dopa decarboxylase
DNA	=	deoxyribo nucleic acid
dNTP	=	deoxy nucleotide triphosphase
EF1α	=	Elongation factor 1α
F	=	forward
g	=	gram
HCl	=	hydrochloric acid
IPTG	=	isopropyl-β-D-thiogalactopyranoside
ITS	=	Internal transcribed spacer
KCl	=	potassium chloride
M	=	Molar
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	milimeter
mM	=	millimolar
$MgCl_2$	=	Magnesium chloride
mtDNA	=	mitochondrial DNA
NAD5	=	NADH dehydrogenase subunit 5
ng	=	nanogram
OPS	=	Opsin
PCR	=	polymerase chain reaction
PEPCK	=	Phosphoenolpyruvate carboxykinase
R	=	reverse
rpm	=	rotation per minute

LIST OF ABBREVIATIONS (Continued)

sec	=	second
ssDNA	=	single-stranded DNA
SSCP	=	Single Strand Conformation Polymorphism
µg	=	microgram
µl	=	microlitre
µM	=	micro molar

Molecular Diversity of Tomato Thrips, *Ceratothripoides claratris* (Shumshur) (Thysanoptera: Thripidae) Populations Found in Thailand Using PCR-SSCP

INTRODUCTION

Tomato, *Lycopersicon esculentum* (Mill) (Solanaceae), is native to tropical South America (Taylor, 1986). All tomato varieties in Europe and Asia originated from seeds obtained in South America by Spanish and Portuguese merchants during the 16th century (Villareal, 1978). Thereafter, in less than a century tomato has become a major world food crop (FAO, 2000). It is an important source of lycopene and vitamin C (Madhavi and Salunkhe, 1998). In Thailand, tomatoes are grown in all regions of the country but major production areas are found in the central and north-eastern regions. They are consumed as fresh fruits and, in addition, processed for export as canned fruits, concentrated juice, and dried fruits, and generating export incomes of over one billion Thai Baht annually (Anonymous, 2004). In 2002 world production of tomatoes was estimated at 108 million metric tons of which 242,000 metric tons were produced in Thailand (FAO, 2004).

Surveys for tomato pests in field and protected cultivation in several provinces in Thailand indicated that whitefly, thrips, aphids, mealy bug, leaf hopper, American bollworm, beet armyworm, leaf miner, diamond back moth and flea beetle were important pests of tomato. The American bollworm, whitefly, and thrips were the most frequently found and cause severe damage on tomato. Beneficial insects found in tomato field were parasitic wasp, predatory Hemipteran and Dipteran, ant lion nymph and ant. In protected cultivation, thrips is considered significantly important insect pest for tomato.

Thrips (Thysanoptera: Thripidae) are polyphagous insects and most of them are worldwide serious pests of vegetables. Crop damage is caused directly by mechanical damage through feeding and oviposition and indirectly by transmitting tospoviruses (Tommasini and Maini, 1995). Within the pest complex of tomatoes in

Asia, thrips are some of the most destructive pest. *Thrips palmi* Karny, *Scirtothrips dorsalis* Hood, *Megalurothrips usitatus* Bagnall, *T. tabaci* Lindeman, *T. flavus* Schrank, *Frankliniella occidentalis* (Pergande) and *F. schultzei* (Trybom) are the most commonly occurring thrips species on tomatoes in Southeast Asia (Bansiddhi and Poonchaisri, 1991; Bernardo, 1991; Chang, 1991). However, in Thailand the thrips fauna on tomatoes are not well documented. So far only *T. flavus* and *Ceratothripoides claratris* (Shumsher) have been recorded on tomatoes (Bansiddhi and Poonchaisri, 1991; Murai *et al*., 2000; Rodmui, 2002).

Ceratothripoides claratris is a key pest attacking tomatoes and the predominant thrips species on field and greenhouse-grown tomatoes in Thailand, especially in the central plain area (Murai *et al*., 2000; Rodmui, 2002). Apart from Thailand, *C. claratris* has also been recorded in India (Jangvitaya, 1993) and Malaysia (S. Okajima cited in Murai *et al*., 2000). The closely related *C. dianthi* (Priesner) is one of the most harmful thrips species found in greenhouse in southern Italy (Marullo, 1998). Both larvae and adults of *C. claratris* damage tomatoes by voraciously feeding on the foliage, stems and fruits; in addition, oviposition by females on fruits leads to scarification and malformation of tomatoes (Murai *et al*., 2000; Premachandra *et al*., 2004). Moreover, *C. claratris* is apparently vectoring *Tomato necrotic spot* virus (TNSV) (Premachandra *et al*., 2004) and is vectoring a yet to be identified tospovirus of the serogroup IV, serologically and genetically closely resembling the recently described *Capsicum* chlorosis virus (CaCV) (McMichael *et al*., 2002), which causes severe losses in tomato production in Thailand (Premachandra *et al*., 2004).

The lack of basis behavioral and ecological data seriously hampers the development of new non-chemical control methods for thrips on tomato. The effective and sustainable control strategy can be designed only when thrips species and biotypes are clarified and the interactions between thrips, their host plant and their natural enemies are thoroughly investigated. This study aimed to differentiate tomato thrips populations in Thailand and determined their genetic relatedness which is necessary data in forming effective thrips control strategy.

Objectives

The objectives of this study were 1) to develop specific primers for tomato thrips, *Ceratothripoides claratris* and 2) to explore for effective DNA markers for differentiation of tomato thrips, *Ceratothripoides claratris* populations in Thailand and to study genetic diversity and determining genetic relationships among and within *Ceratothripoides claratris* populations in Thailand, using Polymerase Chain Reaction - Single Strand Conformation Polymorphism- (PCR-SSCP).

Literature Review

Thrips biology and morphology

Thrips belong to the order Thysanoptera and about 5000 species exist in the world (Fenemore, 1984). Some species of this order, such as those of the family Thripidae (Ross *et al*., 1982) are especially destructive. Thrips possess very short piercing mouthparts (Lewis, 1973; Fenemore, 1984) compared to Hemiptera, and can pierce only superficial cells of the plant surface which eventually coalesce giving a bleached appearance. In addition, there is often distortion of growth in response to the insect's injected saliva (Fenemore, 1984).

Both adult males and females, together with the first and second nymphal instars, often exist simultaneously on flowers, but not prepupal or pupal stages (Vijayasegaran, 1986). The adults of both sexes possess wings and fly readily and are also visible to the naked eye. Both nymphal stage I and II, however, are wingless, pale white to transparent and barely visible to the naked eye. Parthenogenetic reproduction is common among thrips and males are rare in some species. Thrips provide a link between insects with incomplete metamorphosis and those that display complete metamorphosis (Fenemore, 1984).

Life cycle of *C. claratris*. had been studied by Rodmui (2002). *C. claratris* has six developmental stages, egg, first (L1) and second instar larva (L2), prepupa, pupa and the adult. Female *C. claratris* inserts the bean-shaped egg (average size 0.13 mm) into the plant tissue (Murai *et al*., 2000). The newly laid eggs are pale white in color. Immediately before hatching the red pigmented eyes appear. The small white first instar larva starts to feed on the plant tissue right after emergence. The average size of a first instar larva is approximately 0.10 and 0.40 mm in width and length, respectively. The second instar larva is considerably bigger in size (width and length 0.16 and 0.8 mm, respectively) (Rodmui, 2002). The late second instar larva drops from the foliage to the soil or leaf litter, where it develop into the non-moving and non-feeding prepupa and pupa. The pupa stage is in the soil or on leaf litter. The adult is dark brown in color (Jangvitaya, 1993; Mound and Kibby, 1998; Rodmui, 2002).

The average size of the female is approximately 0.20 mm in width and 1 mm in length. With 0.18 and 0.80 mm in width and length, respectively, Male is normally smaller than female (Rodmui, 2002).

Figure 1 Life cycle of *Ceratothripoides claratris* (Shumshur) which consists of 6 stages.

a Eggs
b First instar larva
c Second instar larva
d Prepupa
e Pupa
f Adult

Source: Rodmui (2002)

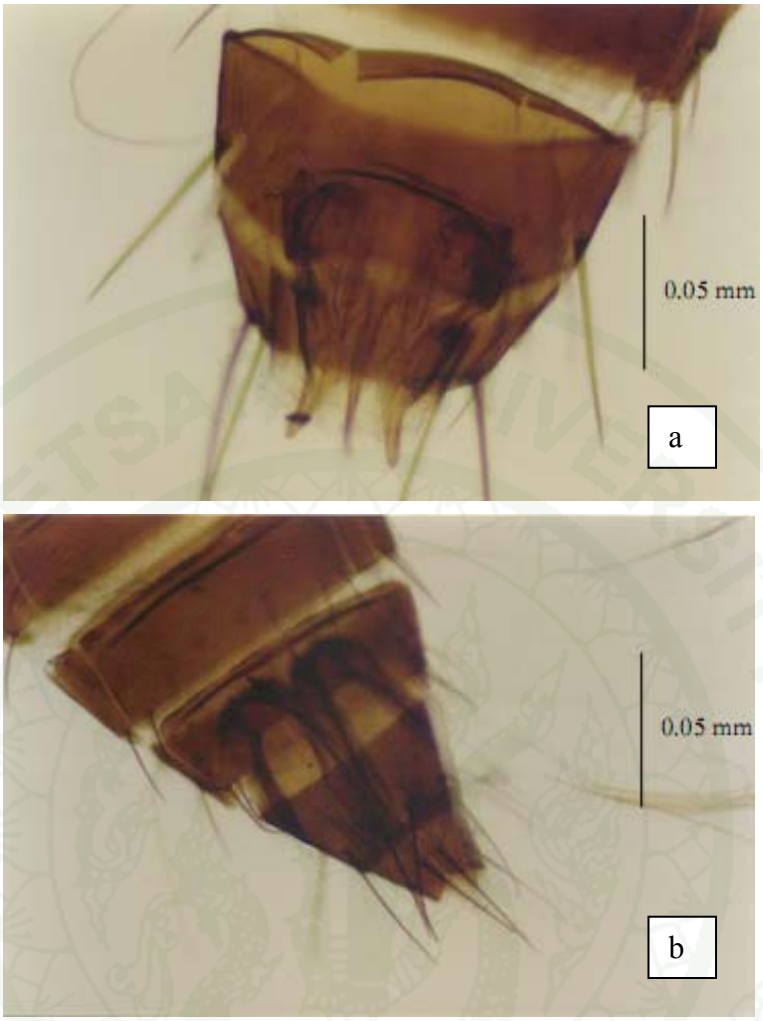

Figure 2 Abdomens of male and female of *Ceratothripoides claratris* (Shumshur), the female was generally larger than the male and had saw-like ovipositor.

a Male

b Female

Source: Rodmui (2002)

Economic important of thrips

Thrips (Thysanoptera: Thripidae) are polyphagous insects and most of them are worldwide serious pests of vegetables. Crop damage is caused directly by mechanical damage through feeding and oviposition and indirectly by transmitting plant diseases (Tommasini and Maini, 1995). Feeding of larvae and adults as well as oviposition by female *C. claratris* cause physical damage and in severe infestations tomato plants start to dry out. Dark spots of excrements are often visible on the leaves. At high population densities, thrips also feed and oviposit on young fruits, causing scarification and malformation *C. claratris* is competent for the tospovirus, capsicum chlorosis virus (CaCV). Since 2002, these tospovirus infections were observed in the greenhouse, causing serious problems on tomato plants. Disease outbreaks were always associated with severe *C. claratris* infestations.

The direct damage of *C. claratris* coupled with its virus transmission lead to high losses in tomato production in Thailand (Murai *et al*., 2000; Rodmui, 2002). Presently, the predominant plant protection strategy in the vegetables in Asia is chemical control. Between 1980 and 1999 the quantity of pesticides imported to Thailand has increased from 9,855 to 33,969 tons, at an annual growth rate of 6.7% (Anonymous, 2002). This heavy use of pesticides resulted in emerging problems such as pest resistance (Talekar and Shelton, 1993; Williams and Dennehy, 1996), resurgence, deleterious effect on natural enemies, contamination of water sources, and direct health hazards to both farmers and consumers (Saha, 1993).

Host plant and natural enemies

Thrips are polyphagous insects and serious pests of vegetable crops. They have been reported on tomato, pepper, eggplant, watermelon, muskmelon, cucumber, garlic and potato in low-elevation areas (Bernardo, 1991).

In addition to tomatoes *C. claratris* has been recorded on egg plants, *Solanum melongena* L. (Solanaceae), cucumbers, *Cucumis sativus* L., pumpkins, *Curcurbita moschata* (Duch.) (both Cucurbitaceae), *Vigna sinensis* (L.) Savex Hass and *V. unguilulata* (L.) Walp (both Fabaceae), and bird peper, *Capsicum annuum* L. (Solanaceae) (Jangvitaya, 1993; Mound and Kibby, 1998). Apart from Thailand, *C. claratris* has also been recorded in India (Jangvitaya, 1993) and Malaysia (S. Okajima cited in Murai *et al*., 2000). The closely related *C. dianthi* (Priesner) is one of the most harmful thrips species found in greenhouses in southern Italy (Marullo, 1998).

Since thrips are minute insects, many of their predators, in particular the more prey specific ones are small arthropods. The location of thrips, especially the immature stages that are fixed upon plant leaves also limits the kind of predators to those that occur on foliage. The natural enemies of thrips are *Mallada basalis* (Walker) (Chandish and Singh, 1999; Kabissa *et al*., 1996), *Wollastoniella rotunda* Yasunaga & Miyamoto (Rattanaka, 2003), *Orius* sp., American sphecid wasp, larvae of chrysopid and coniopterygid, and many mesostigmatid and trombidiid mites (Lewis, 1973). In addition to parasitoids and predators, pathogens are also natural enemies of thrips. Several of them were found to cause fatal diseases to thrips especially the entomophathogenic fungi. It is well known that fungi invade insect via external cuticle and not be ingested to initiate disease. This makes them prime candidates for use against plant sucking insects including thrips. The fungi, *Paecillomyces fumosoroseus* and *Beauveria bassiana* were used effectively for controlling *C. claratris* (Attathom *et al*., 2003). It is belived that there are still numerous other species of fungi that can serve as potential biological agents for controlling thrips as well as for other plant sucking insect species.

Genetic studies in insects and thrips

Methods based on molecular techniques are in many cases suitable for identification of insects. Since molecular techniques are based on the genotypic characteristics of an organism, which are stable, they can be used to identify all life

stages. Furthermore, the specimens do not need to be intact or alive, as is the case for isozyme eletrophoresis that also has been used for identification of insect groups (Menken & Ulenberg, 1987), including thrips (Reboredo *et al*., 2003). Though molecular methods like PCR and sequencing were introduced in entomology as tools in phylogenetic studies to generate sequences data, they are now also used as diagnostic tools (Caterino *et al*., 2000). The most extensively used methods in diagnostic entomology are PCR and RFLP analysis. Several thrips can be identified by PCR-RFLP on the mitochondrial cytochrome oxidase I gene (COI) (Brunner *et al*., 2002) and ITS sequences (Moritz *et al*., 2000; Toda & Komazaki, 2002), respectively.

For species identification and molecular diversity studies, researchers have employed various molecular markers and methods, (Barr *et al*., 2005), cytochrome b, 16S rRNA, 18S rRNA, 28S rRNA, 5.8S rRNA (Rokas *et al*., 2002; Kjer, 2004), microsatellites (Kim & Sappington, 2005), real time PCR (Kox *et al*., 2005), polymerase chain reaction-restriction fragment polymorphism (Toda & Komazaki, 2002), random polymorphic DNA (Bayar *et al*., 2001; Gyulai *et al*., 2001), internal transcribed spacers (Moritz *et al*., 2001; Toda & Kamazaki, 2002), mitochondrial cytochrome oxidase (mtCOI) (Brunner *et al*., 2002; Frey, 2004), etc., in a number of organisms.

DNA markers have been applied to species discrimination using such methods as randomly amplified polymorphic DNA (RAPD) (Black *et al*., 1992), PCR-single strand conformational polymorphism (PCR-SSCP) (Clapp *et al*., 2000) and microsatellite polymorphism (Figueroaet *et al*., 1999). In addition to these methods, PCR and restriction fragment length polymorphism (PCR-RFLP) analysis has been used for species identification in nematodes (Orui, 1996), mites (Fenton *et al*., 1995) and insects (Tuda *et al*., 1995; Roehrdanz, 1997; Brown *et al*., 1999; Honda *et al*., 1999).

About 5,200 species of Thysanoptera (thrips) are currently recognized worldwide. They are divided into two suborders, the Terebrantia about 2,100 species and the Tubulifera about 3,100 species. Identification of Thysanoptera is often difficult.

Several polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) protocols have been developed in order to separate selected groups of thrips species, including *Thrips palmi* (Brunner *et al*., 2002; Toda and Komazaki, 2002). The inter and intra specific polymorphism of thrips was highlighted.

Using random amplified polymorphic DNA (RAPD) (Bayar *et al*., 2002).

Gillings *et al*. (1995) used RAPD-PCR technique for identify thrips (*Aleurodothrips fasciapennis* and *Frankliniella occidenalis*) by amplify in ITS (internal transcribed spacer) region of the ribosomalDNA. The technique has the advantage of combining highly conserved sequences in the 18S and 5.8S rDNA regions with variable sequences in the ITS regions at species level.

Moritz *et al*. (2000) developed a method based on the polymerase chain reaction (PCR) which uses internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) of the ribosomal deoxyribonucleic acid (DNA). This diagnostic method utilizes of 5 restriction enzymes (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I) that identifies thrips. To identify several species of thrips in the Thripidae family including *Frankliniella occidentalis* and *Echinothrips americanus*, Moritz *et al*. (2000)

The same method, ITS2-RFLP had been employed to identify nine thrips species found in Japan (Toda *et al*. 2002)

Frankliniella intonsa (Tybom) was analyzed molecularly using RAPD-PCR method by Gyulai *et al*. (2002) They used one male and one female of thrips that selected from different host plant namely *Medicago sativa* and *Trifolium pretense* in

Valko and Nagykovacsi in Hungary. Fifteen primers were utilized and primer OPQ14 showed of *F. intonsa*.

Kox *et al*. (2005) used Real-time PCR assay for identification of *thrips palmi*. They have developed a real-time PCR assay based on TaqMan. Primers and probe were selected within the mitochondrial cytochrome oxidase I (COI) gene.

Molecular markers

Genetic polymorphism is defined as the simultaneous occurrence of two or more discontinuous variants (or genotypes) of a trait in the same population. Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is expensive and laborious. A wide variety of techniques have, therefore, been developed in the past few years for visualizing DNA sequence polymorphism.

The term DNA-fingerprinting was introduced for the first time by Jeffrey *et al*. (1985) to describe bar-code-like DNA fragment patterns generated by multilocus probes after electrophoretic separation of genomic DNA fragments. The emerging patterns make up a unique feature of the individual and are currently considered to be the ultimate tool for biological individualization. Recently, the term DNA fingerprinting/profiling is used to describe the combined use of several single locus detection systems and is being used as versatile tools for investigating various aspects of plant and animal genomes. These include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, plant breeding, and diagnostics.

Types and description of molecular markers

Restriction fragment length polymorphism (RFLP) is a variation in the DNA sequence of a genome that can be detected by breaking the DNA into fragments with restriction enzymes and analyzing their size by gel electrophoresis. RFLP analysis can identify some differences in sequence when they occur in a restriction enzyme

recognition site. Analysis of RFLP variation was an important tool in genome mapping, localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing (Saiki *et al*., 1985)**.**

Random Amplification of Polymorphic DNA (RAPD) is a type of PCR reaction, but the segments of DNA that are amplified are random. The PCR is performed using genomic DNA template and a single arbitrary, short primer (8-12 nucleotides), expecting that some fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction (Williams *et al.,* 1990).

Variable Number Tandem Repeats (or VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length between individuals. Each variant acts as an inherited allele, allowing them to be used for personal or parental identification. Their analysis is useful in genetics and biology research, forensics, and DNA fingerprinting (Jeffrey *et al.,* 1985).

Amplified fragment length polymorphism PCR (AFLP) is a PCR-based method used in genetics research and DNA fingerprinting. It has many advantages when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and microsatellites. AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques (Mueller and Wolfenbarger, 1999), but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (Meudt and Clarke 2007). As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi, and plants, where much is still unknown about the genomic makeup of various organisms.

Microsatellites, or Simple Sequence Repeats (SSRs), are polymorphic loci present in nuclear and organellar DNA that consist of repeating units of 1-6 base pairs in length (Turnpenny and Ellard 2005). They are typically neutral, co-dominant and are used as molecular markers which have wide-ranging applications in the field of genetics, including kinship and population studies. However, most microsatellite loci are located in genomic regions of unknown function which are generally highly polymorphic. Null alleles, where some sequence variants do not amplify in PCR, are a major concern for microsatellite scoring and could lead to erroneous population genetic analysis (Chapuis and Estoup, 2006)

DNA markers

Ribosomal RNA genes PCR-based were the first nuclear genes used to study evolution because these markers are highly conserved (multi copy), and therefore is currently used in molecular systematic studies. However, the ribosomal RNA gene evolves slowly which may not quickly enough for use in studies of population-level analyses (Strand *et al*, 1997). The other nuclear DNA markers has been developed for amplifying nuclear genes based on detection of nucleotide sequence variation within introns of a few specific protein-coding genes (Stephen and Baker, 1994 and Strand *et al.*, 1997), because intron provides more variation than exon. Several nuclear DNA-based markers commonly are used to study population structure and systematics (Morton *et al.,* 1996), Strand *et al.* (1997), Cummings and Michael (1998), Figge *et al*. (1999) and Wall, 2002). There are several advantages of nuclear genes in evolutionary information. Nuclear gene markers can be used for phylogenetic reconstruction, for better phylogenetic resolution with shallow phylogenetic levels than other markers, for testing result derived from other genomes and from morphology, and for identification biological phenomena such as reticulation and convergence (Wall, 2002).

mtDNA is a powerful subject for evolutionary studies, and has been used to study population structure and gene flow, hybridization, biogeography, and phylogenetic relationships (Avise *et al*, 1987). The small size, relatively rapid rate of

evolutionary change, and maternal inheritance of mtDNA make it suitable for examining population history and evolution among closely related taxa (Gray, 1989; Lansman *et al*., 1981; Simon *et al*., 1991). Molecular studies of mtDNA have employed both RFLPs and sequencing of specific regions of the mtDNA following cloning or amplification by the polymerase chain reaction (Satta and Takahata, 1990; Pashley and Ke, 1992). Within mitochondria, there are regions that diverge rapidly, while other regions are highly conserved, making the different regions suitable for analysis of different taxonomic levels (Simon *et al*., 1991; Liu and Beckenbach, 1992; Tamura, 1992). Interestingly, insect mtDNA evolves more slowly than human mtDNA and human mtDNA evolves five to ten times faster than nuclear DNA (Powell *et al*., 1986).

The mitochondrial genome of insects is a circular DNA molecule of ca 18.5 kb in size (Hoy 1994). In general, each mitochondrion is believed to contain between 2-10 copies of the genome. Single cells contain up to several hundred mitochondrial and consequently, several thousand copies of mtDNA genes may occur in one cell (Lightowlers *et al*., 1997; Scheffler, 2000). This provides optimal conditions for PCR-based analyses and is an important reason for the attractiveness of mtDNA in molecular diagnostics.

DNA Cloning

A nucleotide sequence is called degenerate if one or more of its positions can be occupied by more than one of the four nucleotides (Kwok *et al*., 1994). The total number of different oligos in the resulting mixture is known as the degeneracy of the primer. Such primers are widely used in screening genomic DNA to identify homologues of already partially known genes. Degenerate primers are used to amplify conserved sequences of a gene or gene from the genome of an organism. Degenerate primer can generally be used when there is evidence of highly conserved regions or motifs of amino acids that can be designed into degenerate primers; these regions may be conserved between species.

Molecular cloning refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it *in vivo*. Cloning is frequently employed to amplify DNA fragments containing genes, but it can be used to amplify any DNA sequence such as promoters, non-coding sequences and randomly fragmented DNA. It is utilised in a wide array of biological experiments and practical applications such as large scale protein production. In essence, in order to amplify any DNA sequence in a living organism that sequence must be linked to an origin of replication, a sequence element capable of directing the propagation of its self and any linked sequence. In practice, however, a number of other features are desired. A variety of specialized cloning vectors have been developed that allow protein expression, tagging, single stranded RNA and DNA production and a host of other manipulations. Cloning of any DNA fragment essentially involves four steps: fragmentation, ligation, transfection, and screening/selection. Although these steps are invariable among cloning procedures a number of alternative routes can be selected, these are summarised as a 'cloning strategy'. Initially, the DNA of interest needs to be isolated to provide a relevant DNA segment of suitable size. Subsequently, a ligation procedure is employed whereby the isolated fragment is inserted into a vector. The vector (which is frequently circular) is linearised by means of restriction enzymes, and incubated with the fragment of interest under appropriate conditions with an enzyme called DNA ligase. Following ligation the vector with the insert of interest is recircularized and can be transfected into cells. A number of alternative techniques are available, such as chemical sensitivation of cells, electroporation and biolistics. Finally, the transfected cells are cultured. As the aforementioned procedures are of particularly low efficiency, there is a need to identify the cells that have been successfully transfected with the vector construct containing the desired insertion sequence in the required orientation. Modern cloning vectors include selectable antibiotic resistance markers, which allow only cells in which the vector has been transfected, too grow. Additionally, the cloning vectors may contain colour selection markers which provide blue/white screening (α-factor complementation) on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present in the cells obtained. Further investigation of the resulting colonies is required to confirm

that cloning was successful. This may be accomplished by means of PCR, restriction fragment analysis and/or DNA sequencing**.**

Detection of polymorphisms

Single-Strand Conformation Polymorphism (SSCP)

First announced in 1989 as a new means of detecting DNA polymorphisms, or sequence variations, SSCP analysis offers an inexpensive, convenient, and sensitive method for determining genetic variation (Sunnucks *et al*., 2000). SSCP is a technique for detection of polymorphism of PCR products that have been amplified using specific primers and are then separated by electrophoresis on a nondenaturing polyacrylamide gel. The separation of different alleles is based on subtle differences in sequence (often a single base pair) which results in a different secondary structure and a measurable difference in mobility through the gel matrix (Orita *et al*., 1989). The mobility of double-stranded DNA in gel electrophoresis is dependent on strand size and length but is relatively independent of the particular nucleotide sequence. The mobility of single strands, however, is noticeably affected by very small changes in sequence, possibly one changed nucleotide out of several hundred. Small changes are noticeable because of the relatively unstable nature of single-stranded DNA. In the absence of a complementary strand, the single strand will undergo intrastrand base pairing to some extent, resulting in loops and folds that give the single strand a unique 3D structure. A single nucleotide change could dramatically affect the fragment's mobility through a gel by altering the intrastrand base pairing and its resulting 3D conformation. Like restriction fragment length polymorphisms (RFLPs), SSCPs are allelic variants of inherited, genetic traits that can be used as genetic markers. Unlike RFLP analysis, however, SSCP analysis can detect DNA polymorphisms and mutations at multiple places in DNA fragments (Orita *et al*., 1989). As a mutation scanning technique, though, SSCP is more often used to analyze the polymorphisms at single loci, especially when used for medical diagnoses (Sunnucks *et al*., 2000).

Single strand conformation polymorphism (SSCP), is a powerful and rapid technique for gene analysis particularly for detection of point mutations and typing of DNA polymorphism (Orita *et al.,* 1989). SSCP can identify heterozygosity of DNA fragments of the same length and can even detect mutations of a few nucleotide bases as the mobility of the single-stranded DNA conformation changes depending on the exact DNA sequence. To overcome problems of reannealing and complex banding patterns, an improved technique called asymmetric-PCR SSCP was developed (Ainsworth *et al.,* 1991), wherein the denaturation step was eliminated and a large-sized sample could be loaded for gel electrophoresis, making it a potential tool for high throughput DNA polymorphism. It was found useful in the detection of heritable human diseases. In plants and animal, however, it is not well developed although its application in discriminating progenies can be exploited, once suitable primers are designed for agronomically important traits (Fukuoka *et al.,* 1994).

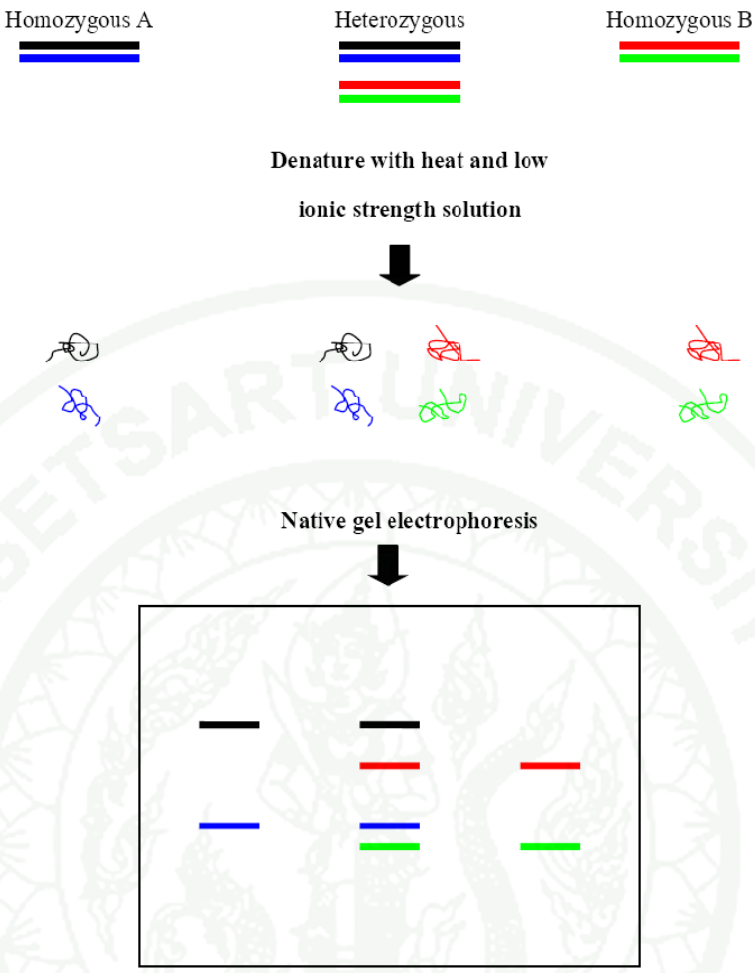

Figure 3 Diagram illustrating the principle of Single-Strand DNA Conformation Polymorphism (SSCP).

Materials and Methods

1. Thrips collection

Thrips populations were collected from major tomato growing provinces in different geographical regions in Thailand. Thirty-two adult thrips were collected from each of 26 locations (Table 3) making a total of 832 samples. The collected adult thrips were examined and morphologically identified under stereo microscope to confirm the species of *Ceratothripoides claratris*. All samples were stored in 70% ethanol at -20 ºC until use.

2. DNA Extraction

DNA was extracted from individual adult thrips collected from each location. The DNA extraction was performed as follows: individual thrips were crushed in 10 µl of commercial PCR buffer with 1% Tween 20 and 1µg/µl of Proteinase K (QIAGEN). The DNA lysate was then incubated at 60 ºC for 1 hour and then incubated at 95°C for 10 min to inactivate proteinase K, centrifued and supernatants was transferred to new tube. 1-2 µl of lysate DNA solutions were used as template for PCR amplification.

3. Design degenerate primers

The degenerate primers of nine genes of interest namely: Cytochrome oxidase I (*COI*), ATP synthase subunits 6 (*ATP6*), NADH dehydrogenase (*NADH*), Phosphoenolpyruvate carboxykinase (*PEPCK*), Elongation factor1α (*EF1α*), Opsin (*OPS*), Alcohol dehydrogenase (*ADH*), Dopa decarboxylase (*DDC*) and Arginine kinase (*ARGK*) genes were designed. The nucleotide sequences of these nine genes were obtained from the GenBank database at NCBI (http://ncbi.nlm.nih.gov/). To be able to amplify those genes from thrips genome, the primers were designed and aligned using the ClustalW and GENEDOC program. Proposed oligos were further modified manually. For the calculation of the molecular weight, annealing

temperatures and secondary structures like dimmers or hairpins, the Fast-PCR software (Kalendar, 2005) was used. Degenerate primers were synthesized for PCR corresponding to conserved regions in genes or gene families of interest, with at least one intron between primer sites. More than one primer set was designed for some genes. Primers were synthesized by Ocimum Biosolutions (India) and by Isogen Life Science (Netherlands).

Primer pairs were designed to cover the following properties: (1) All conserved regions, at least 21 bases with 50% of G and C content. (2) Annealing temperature between 50-65°C with a maximum 4°C difference between the two primers of any single pair. (3) No primer dimer formation, either with itself or any other member of the pair. (4) The length of the target DNA sequences should not be longer than 2000 bases pairs.

Universal degenerate code (Table 1) was used for the description of primer sequences.

Table 1 Universal degenerate code.

Index	Base (s)
R	A/G
Y	C/T
H	A/C/T
N	A/C/G/T

4. PCR amplification for testing the designed degenerate primers

Candidate genes were PCR amplified from genomic DNA isolated from adult thrips using each degenerated primer pair. Each PCR amplification reaction in 10 µl volume contained 2 µl of template DNA, 10 pmol of each of the primers, 200 µM of each dNTPs (Promega), 1x PCR buffer (NH_4SO_4, 2mM $MgCl_2$ and 0.5U of Taq DNA polymerase (Fermentas). Amplification was performed on a T1 Thermocycler

(Biometra) or on a PTC100 (MJ Research). Cycling started with an initial 94°C for 3 min, then followed by 35 cycles of 94°C for 45 sec, the appropriate annealing temperature for 45 sec, 72°C for 2 min and final extension at 72°C for 5 min. The amplification products were checked by 1% agarose gel electrophoresis for presence of fragments corresponding to the expected sizes. Selected PCR products were cloned for sequencing.

5. Cloning and sequencing of PCR fragments

PCR products of the targeted gene were purified using the MinElute PCR Purification Kit (Qiagen, Germany) and ligated into plasmids using the pGEM easy vector (Promega, USA). For a single ligation 1 to 5 ng PCR product were mixed with 0.2 µl pGEM easy vector, 0.2 U T4 ligase and 2 µl rapid ligation buffer (all reagents provided within the kit). A final volume of 4 µl was set with sterile water and the reaction incubated over night at 4°C.

Ligated DNA was split into two aliquots of 2 µl, transferred in 1.5 ml Eppendorf tubes and placed on ice. Meanwhile an aliquot of competent *E. coli* (*Escherichia coli* 'DH10B') cells was thawed on ice, mixed gently and 50 µl cell suspensions were placed into each tube using pre-chilled pipette tips. The ligated plasmid was transformed into competent cells by electroporation using MicroPulser^{TM} (BIO-RAD, USA). 950 µl SOC broth were added to each tube and the cells grown for 1 hr at 37°C. Afterwards the cells were pelleted by centrifugation at 2500 rpm for 5 min in a chilled centrifuge and resuspended in 100 µl LB broth. The suspension was plated onto Petri dishes with LB (Luria-Bertani) medium agar containing 100 µg/ml of antibiotic (ampicilin) added by mixing of 100 µl IPTG (100 mM) and 20 µl X-gal (50 mg/ml). The plates were grown at 37°C over night. The Blue-White colony selection was used to identify transformants. Individual colonies were picked for direct PCR amplification to check the presence of insert using T7 or SP6 primers. The remain of the same single colony was grown overnight in an incubator shaker at 37°C, 150 rpm in 5 ml of LB medium broth with 100 µg/ml of ampicilin. Plasmids were

extracted from cultured cells using QIAprep Spin Miniprep kit (Qiagen, Germany) or GeneAid kit following the manufacturers' procedures.

Sequencing of PCR products of each gene was performed using automated sequencing machines of Macrogen Inc (Korea) and 1st BASE Pte Ltd. (Malaysia).

6. Design of specific primers

Sequences of Cytochrom oxidase I (COI), Elongation factor1α (EF1α) and Arginine kinase (ARGK) genes were obtained. No sequences were obtained from all positive clones of each of these genes, Phosphoenolpyruvate carboxykinase (PEPCK), Opsin (OPS), Alcohol dehydrogenase (ADH) and Dopa decarboxylase (DDC).

The obtained sequences were then compared with sequences of the COI, EF1α and ARGK genes from other insect species to check whether the correct gene had been obtained. Specific primers were designed to amplify the candidate gene from thrips. The specific primer sets targeted the introns of the genes because a higher level of polymorphism would be expected compared to exons. Based on sequence data, specific primer for *C. claratris* COI, EF1α and ARGK genes were designed and used for PCR-SSCP analysis.

In this study, fragment of internal transcribed spacer (ITS) gene was also amplified using the published specific primer (Ruman-Jones *et al*., 2006) and this primer was used for PCR-SSCP analysis.

7. Polymerase chain reaction – Single stand conformation polymorphism (PCR-SSCP) for polymorphism detection

PCR amplification

To detect polymorphism, genomic DNA samples extracted from the collected *C. claratris* were amplified by using the specific primer in 96-well plates. The total

reaction mixture of 15 µl contained 200 µM dNTPs (Promega), 2.5 mM $MgCl_2$ (Fermentas), 5 pmole of each primers, 1x PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 0.3 unit of *Taq* DNA polymerase (Fermentas) and 20 ng of genomic DNA template. Amplification was carried out at 94°C for 3 min, followed by 35 cycles of 45 sec at 94°C, 45 sec at the appropriate annealing temperature, 90 sec at 72°C, and a final extension at 72°C for 5 min. PCR products were checked for quality and size by electrophoresis on 1% agarose gel using 1xTBE buffer at 100 V for 20 min. The PCR products were then stained with ethidium bromide and photographed under ultraviolet light. PCR products were subsequently used for SSCP analysis.

Single stand conformation polymorphism (SSCP)

SSCP was performed by denaturing the double-stranded DNA products as follows: 1.5 µl of each PCR product were added to 3.5 µl loading dye (95% formamide, 10 mM NaOH, 0.025% xylene cyanole and 0.025% bromophenol blue). The mixture was denatured at 98°C for 10 min and immediately placed on ice water to stabilize single strands. 2 µl of these samples were loaded on 30 cm × 40 cm × 0.4 mm polyacrylamide gel using non-denaturing conditions (Single-Strand DNA Polymorphism, Orita *et al*., 1989). The polyacrylamide gel composed of 12 ml Sequagel MD solution (National Diagnostics, U.S.A.), 7.2 ml 5X TBE (10.8% Tris, 5.5% Boric, 0.02M EDTA pH8), 40 ml distilled water, 50 µl NNNN-Tetramethylene diamine (TEMED), 500 µl 10% APS (Ammonium peroxide sulphate). The samples were subjected to electrophoresis using a Hoefer SQ3 vertical electrophoresis apparatus (Amersham Pharmacia Biotech, U.S.A.), in 0.5X TBE buffer operated at 300 mA, 10 Watt in refrigerator at 4°C. The running time depended on the size of DNA fragment being analyzed (Table 2). The SSCP patterns were visualized by silver staining.

Silver staining

The gels on glass plates were covered with fix solution (10% acetic acid) and shook gently on orbital shaker (ArmaLab, U.S.A.) for 30 min, and washed 3 times, 10

min each with reverse osmosis water. Silver staining was carried out for 30 min on shaker using 1% silver nitrate (Fisher Scientific Ltd., UK) added with 1.5 ml/l of 37% formaldehyde. After staining, the gel was rinsed with distilled water and DNA bands were developed using chilled developing solution, containing 50g/l sodium bicarbonate (Riedel-de Haën, GmbH), 1.5 ml/l of 37% formaldehyde, and 1mg/l sodium thiosulphate. The gel was agitated well until all bands become visible. The developing reaction was terminated by adding stop solution (10% acetic acid) directly to the developing solution and the gel was incubated for 5 min. The gels were then washed thoroughly with deionized water for 15 min, then left to air dry. Band pattern was analyzed to determine genetic variation of thrips, *Ceratothripoides claratris* populations.

Table 2 Conditions for SSCP analysis of PCR amplified gene products.

DNA region	Watt	Running time (hr)
Cytochrom oxidase I (COI)	10	16.30
Internal transcribed spacer (ITS)	10	16.30
Elongation factor1α (EF1α)	10	14
Arginine kinase (Argk)	10	11

8. Cluster analysis

All bands revealed by each primer set were visually recorded as binary data by 1 (present) or 0 (absent). The binary data was analyzed with the computer program NTSYSpc version 2.02 (Rohlf, 1993). An unweighted pair group arithmetic mean method (UPGMA) cluster analysis was performed using the JACCARD's similarity coefficient. The dendrogram was generated with the tree option (TREE) and cophenetic value distance matrix was derived from dendrogram with a COPH program in NTSYSpc. The cophenetic value distance matrix was compared for correlation with the original matrix with the MXCOMP NTSYS program. Bootstrap values were calculated with 1000 replicates by winboot program (Yap and Nelson, 1996).

RESULTS AND DISCUSSION

1. Thrips collection

Thrips, *Ceratothripoides claratris* populations were collected from major tomato cultivated areas in different geographical regions of Thailand. Figure 1 is map of Thailand highlighted 26 provinces which were sites of collection for tomato thrips populations. A total of 26 populations of thrips were collected from tomato plantations in 26 provinces in the north, northeast, central and west of Thailand (Table 3). The 26 provinces were as followed:

Northern region: Chiang Mai, Chiang Rai, Lampang, Lamphun and Phayao.

Central region: Ang Thong, Chai Nat, Kamphaeng Phet, Nakhon Pathom, Nakhon Sawan, Phichit, Sing Buri and Uthai Thani.

North-Eastern region: Amnat Charoen, Buri Ram, Mukdahan, Nakhon Ratchasima, Nong Khai, Sakon Nakhon, Ubon Ratchathani and Yasothon.

Western region: Kanchanaburi, Phetchaburi, Prachuap Khiri Khan, Ratchaburi and Tak.

At least 32 individual adult thrips were collected in each location for DNA analysis.

The collected thrips populations were given the accession number to ease further experiments (Table 3). The missing accession numbers were the populations in which number of collected individual thrips were not enough for the experiment (less than 32). It was noticed that on cloudy or rainy day, *C. claratris* population were seldomly found, providing inadequate number of thrips for the collection. Thrips normally prefers warm and sunny day.

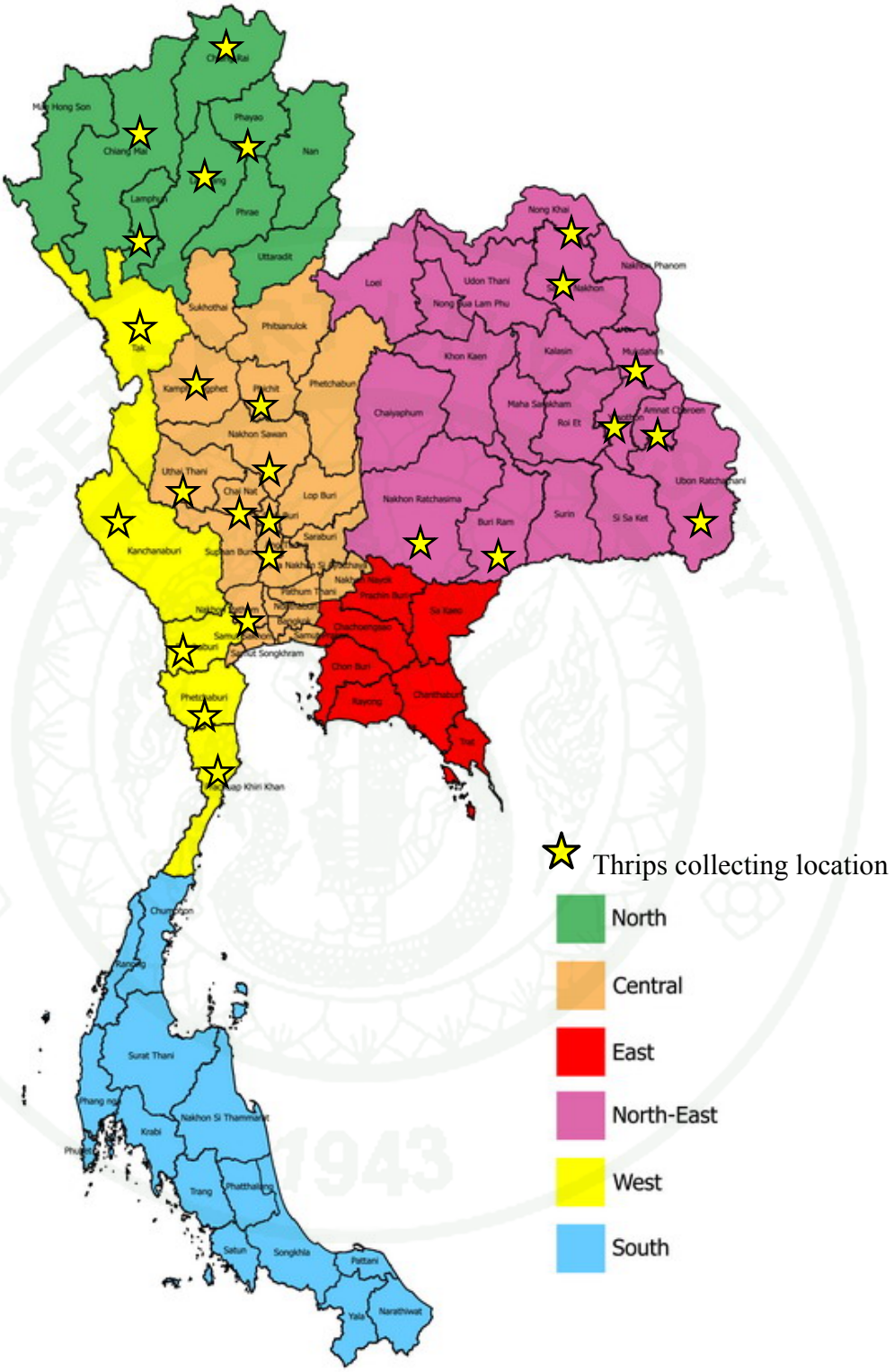

Figure 4 Map of Thailand indicated sites of thrips population collection in different geographical regions.

Source: www. Map of thailand.org

Table 3 Sites of collection of tomato thrips, *Ceratothripoides claratris* in different regions of Thailand and the designated accession number for each thrips population.

Site of collection		Accession no.[1/]
Region	Province	
Northern	Chiang Rai	P19
	Chiang Mai	P20
	Phayao	P22
	Lamphun	P23
	Lampang	P24
Central	Nakhon Pathom	P14
	Nakhon Sawan	P25
	Phichit	P26
	Kamphaeng Phet	P27
	Uthai Thani	P28
	Chai Nat	P29
	Sing Buri	P30
	Ang Thong	P31
North-Eastern	Nong Khai	P1
	Sakon Nakhon	P2
	Mukdahan	P4
	Amnat Charoen	P5
	Ubon Ratchathani	P6
	Buri Ram	P8
	Nakhon Ratchasima	P9
	Yasothon	P10
Western	Ratchaburi	P15
	Phetchaburi	P16
	Prachuap Khiri Khan	P17
	Kanchanaburi	P18
	Tak	P21

[1/] P = Thrips population

Missing accession numbers were thrips populations in which the collected number were less than 32 individuals and were excluded from this study.

2. Design of degenerate primer

Eighteen degenerate primer pairs for the Cytochrom oxidase I, ATP synthase subunits 6, NADH dehydrogenase, Phosphoenolpyruvate carboxykinase, Elongation factor1α, Opsin, Alcohol dehydrogenase, Dopa decarboxylase and Arginine kinase genes were designed. The primer sequences are listed in Table 4. For all genes, DNA sequences were retrieved from the NCBI (http://ncbi.nlm.nih.gov/). Nucleotide sequences were first aligned with the GeneDoc program and manually edited. Then primer sequences were designed from the highly conserved regions. The alignments of the variable part of each sequence are shown in figures 5-13. Sizes of each of the gene fragments including exons and introns are shown in Table 5.

The results demonstrated that sequences from other insects can provide enough information to design primers that amplify DNA from the studied insect. This is encouraging for the prospect of applying this thesis protocol to study of hitherto unstudied insects.

To design primers specific to certain genes in any particular insect, it is suggested to search for the reported sequence of each gene from other insects as much as possible. More information can provide the better selecteion for appropriate conserved region and degenerate base. In addition, one should try explore for reported information for many kinds of gene in order to obtain varieties of information for further study.

Table 4 Sequences of degenerate primers.

Gene name	Degenerate Primer (5'→3')	Primer pair for PCR amplification	Number of intron in PCR product
Cytochrom oxidase I (COXI)	F1) ATT AGG AGC HCC HGA YAT AGC ATT	F1+R1	-
	R1) CAG GCA AGA TTA AAA TAT AAA CTT CTG		
	F2) CAG TTA TAC CTA TTA TAA TTG G	F2+R2	-
	R2) GCT ATT ATT GCA TAA ATT ATT CC		
	F3) CCT ATT ATA ATT GGA GGA TTY GG	F3+R3	-
	R3) GAG GCA AGA TTA AAA TAT AAA CTT CTG		
ATP synthase subunits 6 (ATP6)	F) ATA GCT AAT ATA GTT TCA GGA CA	F+R	-
NADH dehydrogenase (NADH)	R) GCT TTA TTA TTC ATA TGT GCT GG		
Phosphoenolpyruvate Carboxykinase (PEPCK)	F1) GAG GGT TGG CTT GCT GAR CAY ATG	F1+R1	2
	R1) CAT TAT CAC TTT GCC CTT GTG YTC NGC	F1+R2	2
	F2) GAG GGT TGG CTT GCC GAR CAY ATG	F2+R1	2
	R2) CAG GCT GGA TCG ATG ATN GGR CAY TG	F2+R2	2
Elongation factor1α (EF1α)	F1) AAG ATG CCC TGG TTC AAG GGN TGG	F1+R1	2
	R1) CCG TTG GAG ATC TGR CCA GGR TG		
Opsin (OPS)	F1) TGG ACA ATG TGT ATG ATT GCN TTY GA	F1+R1	1
	R1) TGT TCA CGC ATT GCY TTY TCR TG		
Alcohol dehydrogenase (ADH)	F1) GCT GGT CTG GGT GGC ATT GGN CTN GA	F1+R1	2
	R1) ACA CGT GGC TCT ACR TCN ARC CA		
Dopa decarboxylase (DDC)	F1) CCA GCT TGT ACT GAR CTN GAR GT	F1+R1	2
	R1) CCC AGG AGA CCA GCT CGC TCN ACN GA	F1+R2	3
	F2) CAT GTG GAT GCT GCN TAY GCN GG	F1+R3	4
	R2) CAT CCA TTT GTG TGG RTT RAA RTT	F2+R2	1
	R3) TAC AGT GGA TCT ACR TTR AAN GC	F2+R3	2
Arginine kinase (Argk)	F1) GAT CCC ATC ATT GAR GAY TAY CA	F1+R1	1
	R1) TCC ATC TCY TTG TAC TGN GCY TC	F1+R2	1
	R2) TCA CCC TCC TTG AAC AGR AAR TG		

F=Forward primer, R=Reverse primer, H=(A/C/T), N=(A/G/C/T), R=(A/G), Y=(C/T)

Table 5 Size of characterized gene fragments including exon and intron.

Gene	Name	Coding region (exon)	Intron length (bp)	Total fragment length (bp)
COI	Cytochrom oxidase I	528	0	528
ATP6	ATP synthase subunits 6	500	0	500
NADH	NADH dehydrogenase	500	0	500
PEPCK	Phosphoenolpyruvate Carboxykinase	236	493+104	833
EF1α	Elongation factor1α	448	456+78	982
OPS	Opsin	337	362	699
ADH	Alcohol dehydrogenase	571	75+68	714
DDC	Dopa decarboxylase	280	844+147	1271
DDC	Dopa decarboxylase	590	844+147+191	1772
DDC	Dopa decarboxylase	670	844+147+191+94	1946
DDC	Dopa decarboxylase	110	191	301
DDC	Dopa decarboxylase	190	191+94	475
ARGK	Arginine kinase	199	305	504
ARGK	Arginine kinase	328	305	633

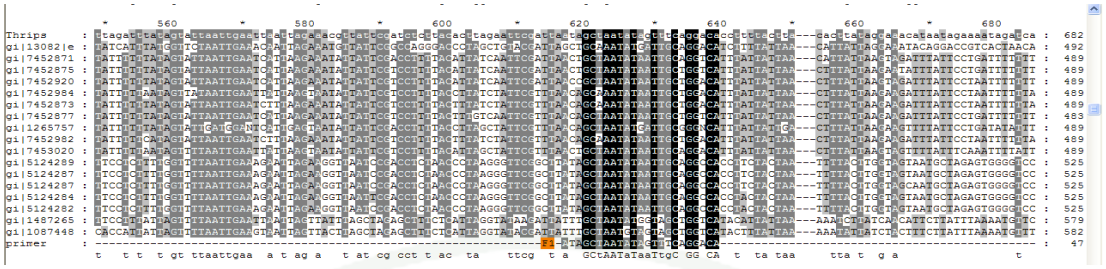

Figure 5 Sequences alignment of ATP synthase subunits 6 gene (*ATP6*) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

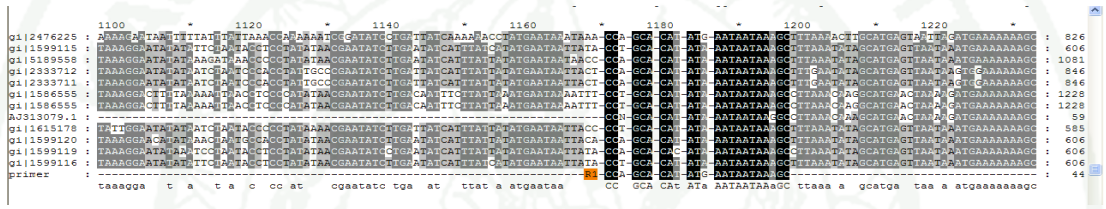

Figure 6 Sequences alignment of NADH dehydrogenase gene (*NADH*) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

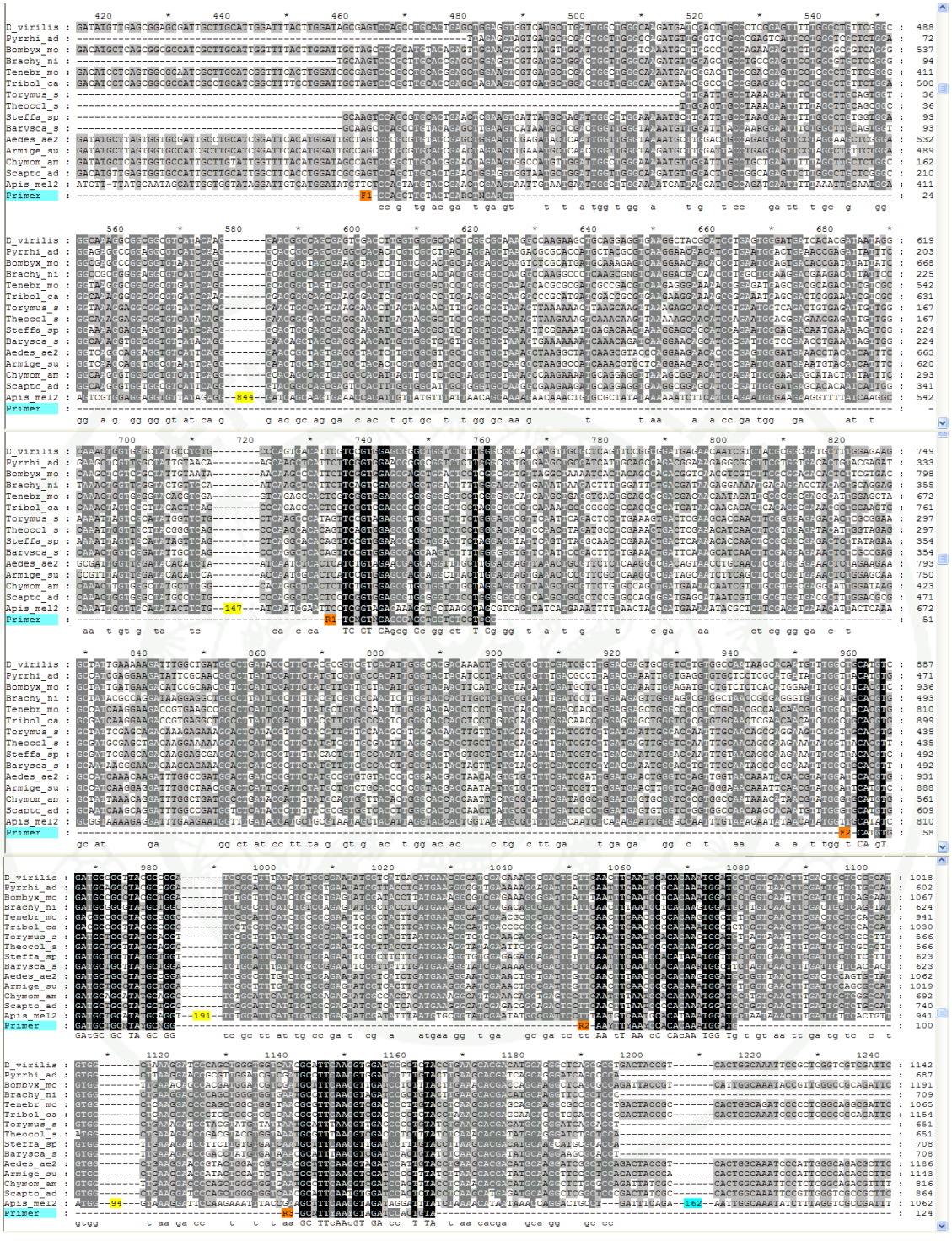

Figure 7 Sequences alignment of Dopa decarboxylase gene (*DDC*) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

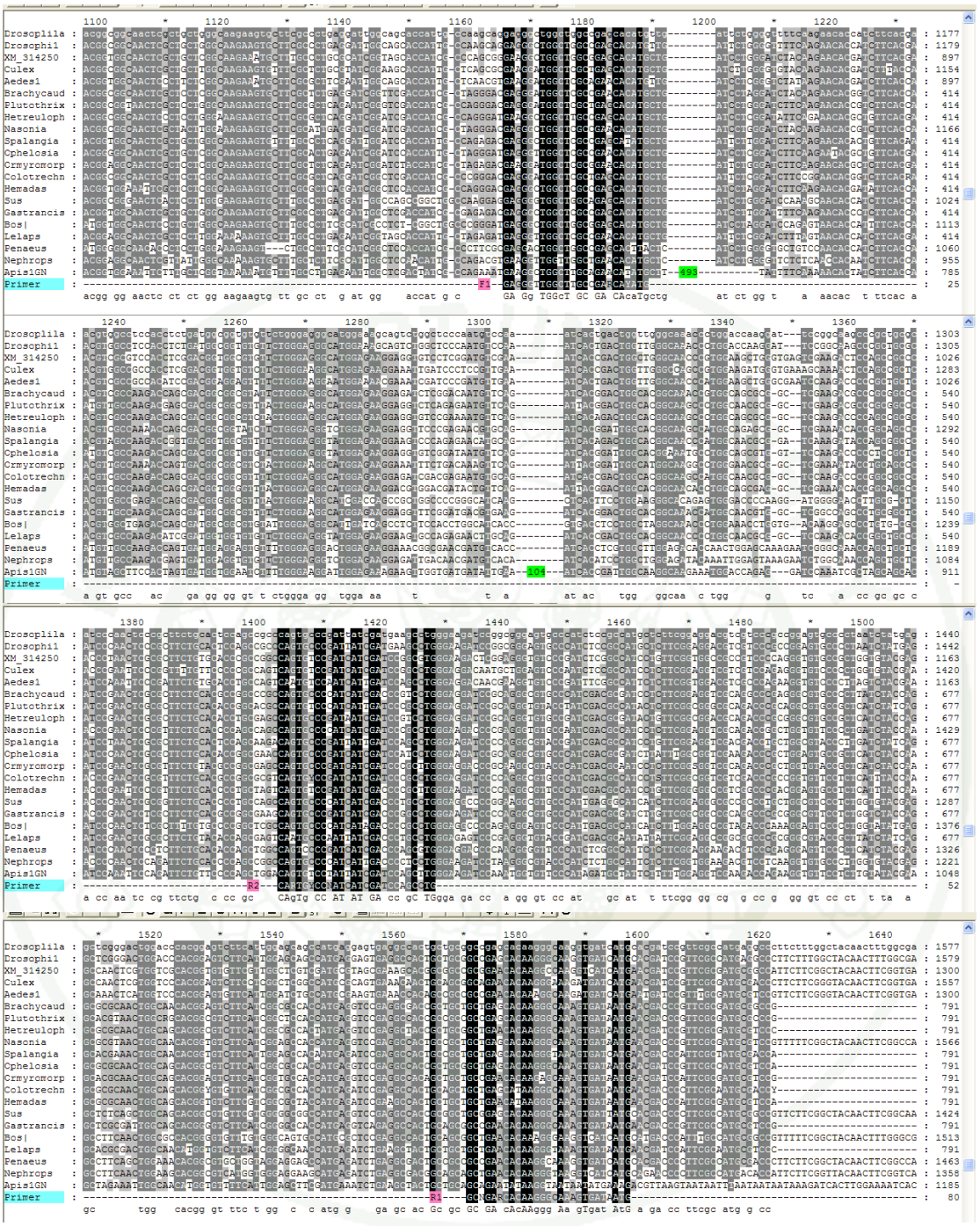

Figure 8 Sequences alignment of Phosphoenolpyruvate Carboxykinase gene (*PEPCK*) of various insect species by GeneDoc program.The degenerated primers were designed in conserved region.

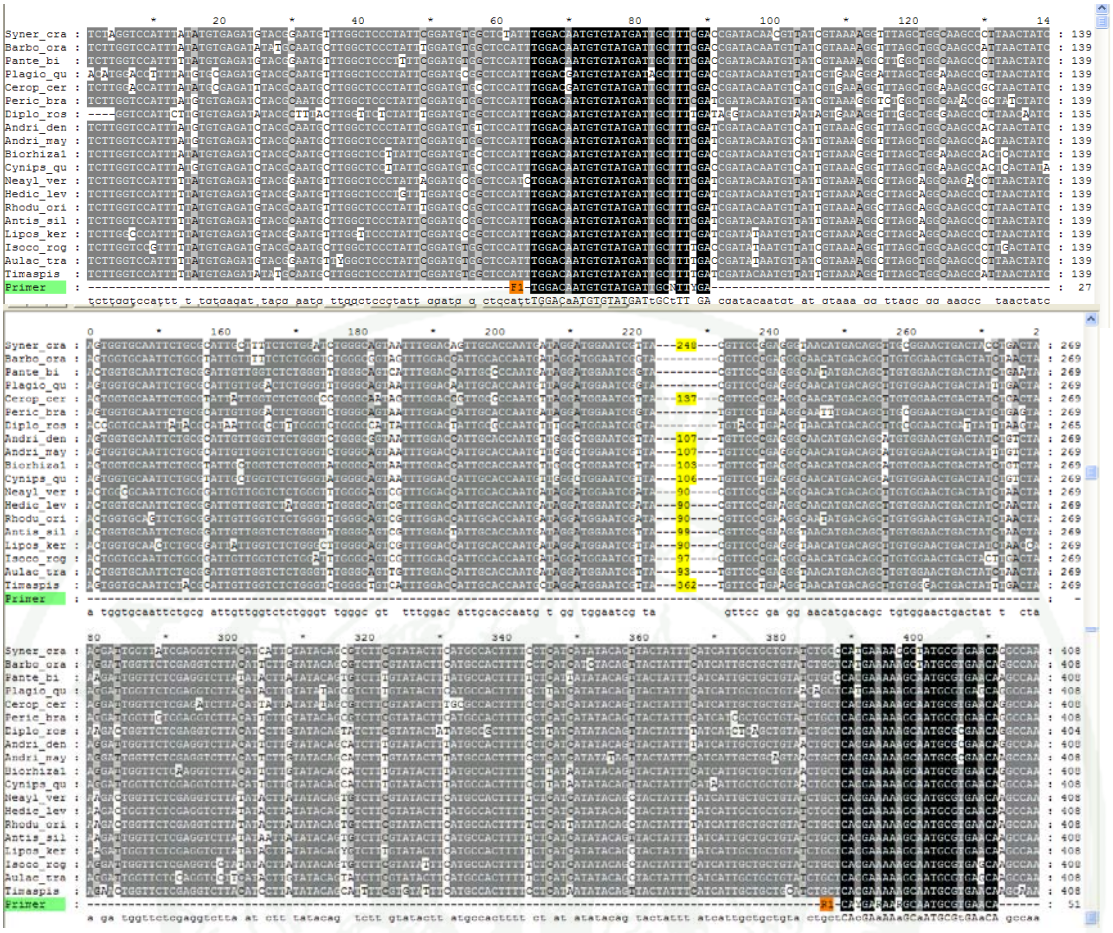

Figure 9 Sequences alignment of Opsin gene (*OPS*) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

Figure 10 Sequences alignment of Cytochrom oxidase I gene (*COI*) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

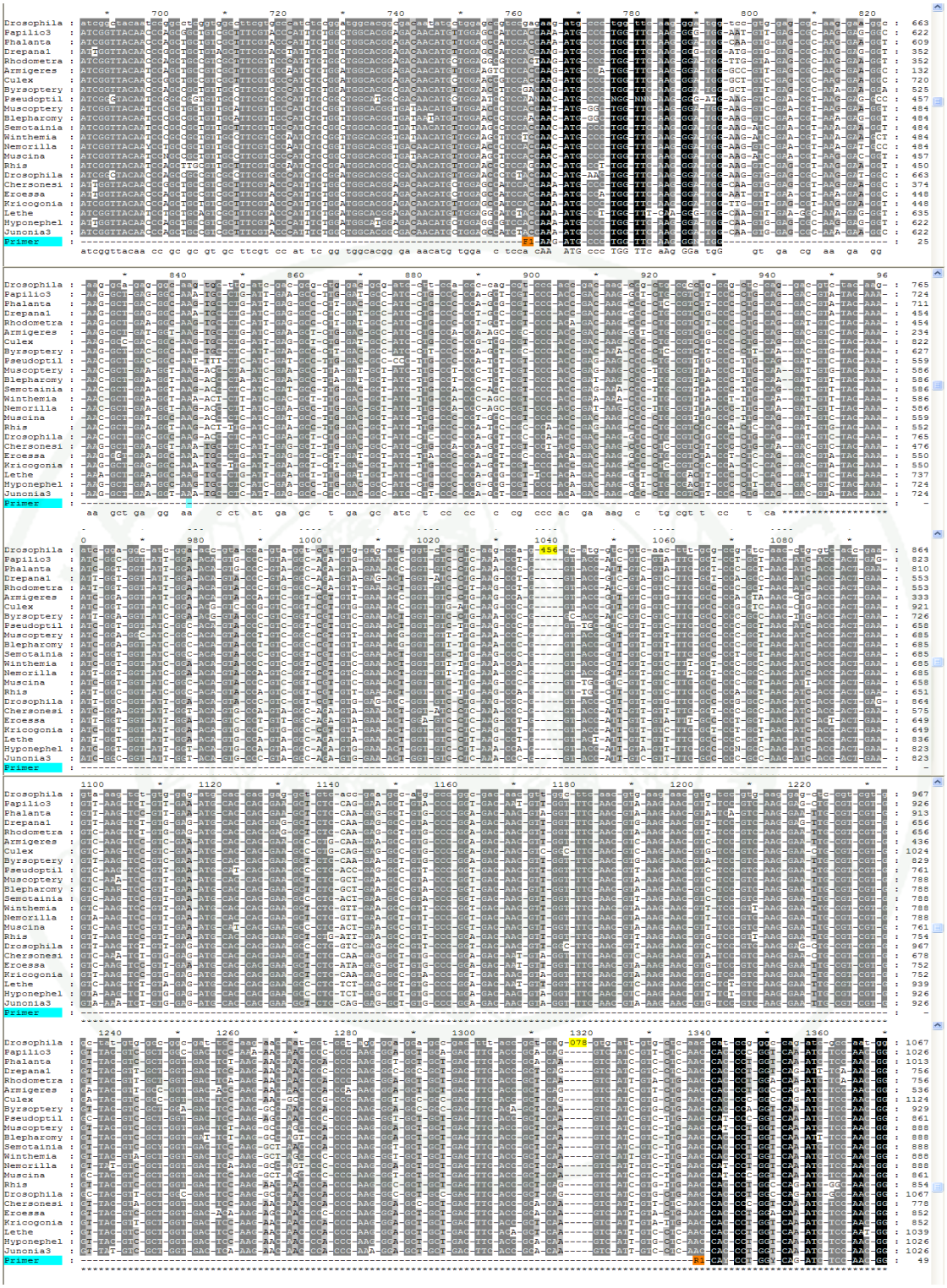

Figure 11 Sequences alignment of Elongation factor 1α gene (EF1α) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

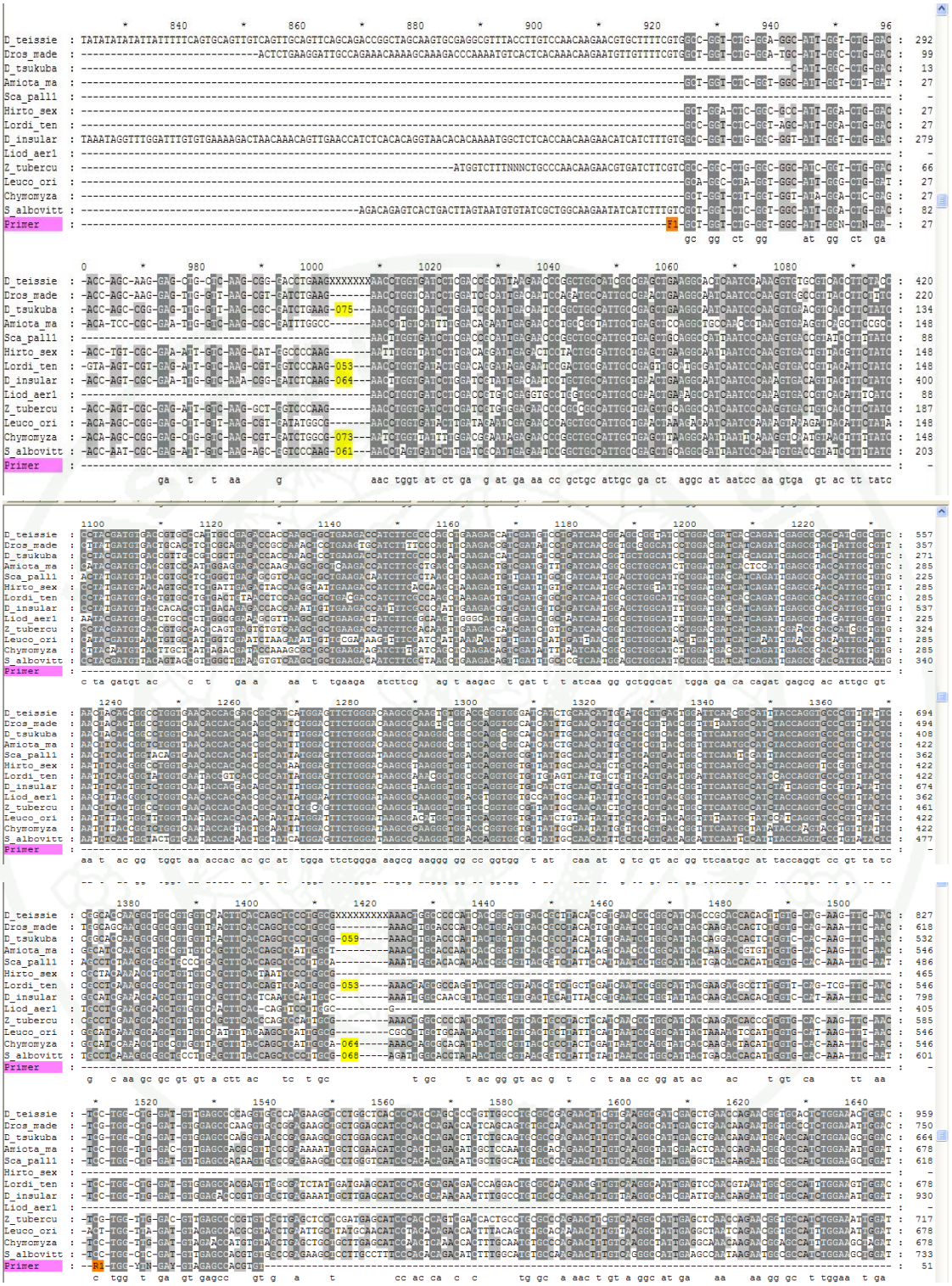

Figure 12 Sequences alignment of Alcohol dehydrogenase gene (*ADH*) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

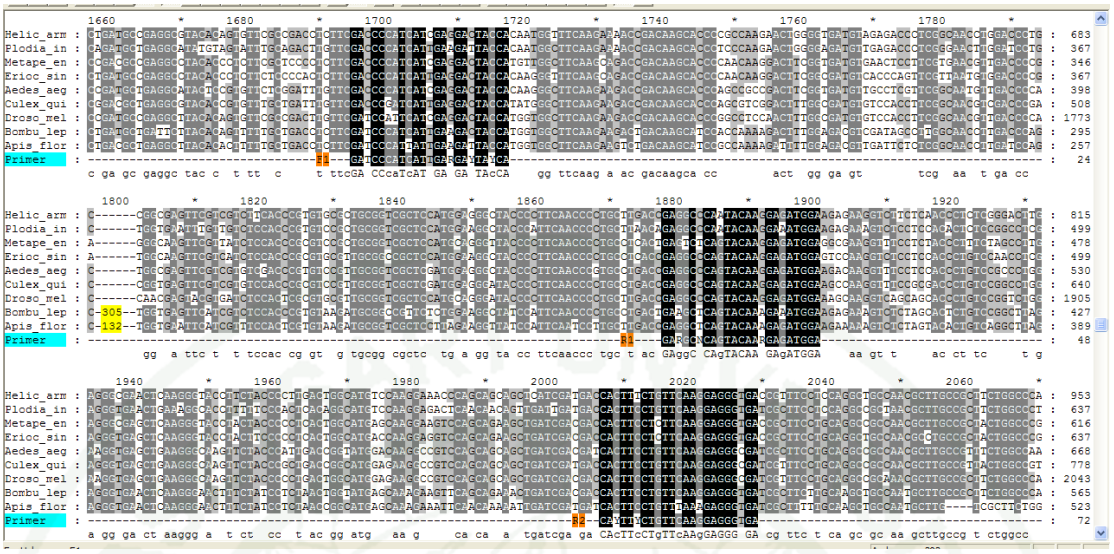

Figure 13 Sequences alignment of Arginine kinase gene (ARGK) of various insect species by GeneDoc program. The degenerated primers were designed in conserved region.

3. Polymerase chain reaction (PCR) using degenerate primers

Eighteen degenerate primer pairs were initially screened to identify the appropriate primers that consistently revealed well amplified products. Among them, seventeen degenerate primer pairs gave PCR products.

Nine genes fragments were amplified by polymerase chain reaction using degenerate primer sets. The amplifications were successful across seven genes: Cytochrom oxidase I (*COI*), Phosphoenolpyruvate carboxykinase (*PEPCK*), Elongation factor1α (*EF1α*), Opsin (*OPS*), Alcohol dehydrogenase (*ADH*), Dopa decarboxylase (*DDC*) and Arginine kinase (*ARGK*). Only two genes: ATP synthase subunits 6 (*ATP6*) and NADH dehydrogenase (*NADH*) generated unclearly interpretable PCR products (Figure 14).

The PCR reactions using Cytochrom oxidase I (*COI*), Phosphoenolpyruvate carboxykinase (*PEPCK*), Elongation factor1α (*EF1α*), Opsin (*OPS*), Alcohol dehydrogenase (*ADH*), Dopa decarboxylase (*DDC*) and Arginine kinase (*ARGK*) primer set amplified a fragment in all collected thrips populations, producing a single band on agarose gel. These PCR products were selected for cloning. The positive clones indicated by blue-white colony selection were sent for sequencing. The DNA sequences obtained from the clones were used to redesign primer set specific for certain genes of *Ceratothripoides claratris*.

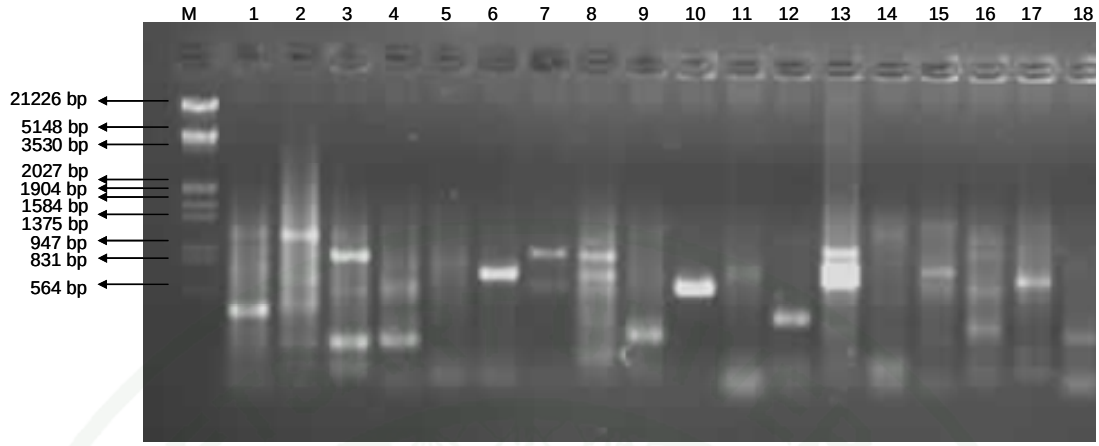

Figure 14 PCR products of nine genes using the designed degenerate primer pairs.

Lane M = Standard size (λ/Hind III+EcoRI)
Lane 1 = COI F1+R1
Lane 2 = COI F2+R2
Lane 3 = COI F3+R3
Lane 4 = PEPCK F1+R1
Lane 5 = ATP6+NADH
Land 6 = PEPCK F1+R2
Lane 7 = PEPCK F2+R1
Lane 8 = PEPCK F2+R2
Lane 9 = EF1α F1+R1
Lane 10 = OPS F1+R1
Lane 11 = ADH F1+R1
Lane 12 = DDC F1+R1
Lane 13 = DDC F1+R2
Lane 14 = DDC F1+R3
Lane 15 = DDC F2+R2
Lane 16 = DDC F2+R3
Lane 17 = ARGK F1+R1
Lane 18 = ARGK F1+R2

4. Cloning and sequencing of PCR fragments

From blue-white colony selection, the positive clones (white colony) were obtained, eight clones of *COI*, seven clones of *PEPCK*, seven clones of *EF1α*, one clone of *OPS*, one clone of *ADH*, six clones of *DDC* and thirty-one clones of *ARGK* were selected for DNA sequencing.

Two sequences from eight positive clones of *COI* gene, four sequences from seven positive clones of *EF1α* gene and three sequences from thirty-one positive clones of *ARGK* gene, were obtained. The sequences of *COI*, *EF1α* and *ARGK* genes from *C. claratris* clones were compared to GenBank databases using BLAST for verification of gene identity. These DNA sequences were selected to design specific primer. No sequences were obtained from the clones of Phosphoenolpyruvate carboxykinase, Opsin, Alcohol dehydrogenase and Dopa decarboxylase genes (Table 6). Sequences of the clones of *COI*, *EF1α* and *ARGK* genes are presented in Figure 15-23.

After alignment with *COI* gene from other insect species, the two obtained *COI* gene sequences namely: Thrips_COI_Thr-10_T7 and Thrips_COI_Thr-11_T7 were both found to be 529 bp in length without intron.

The four obtained *EF1α* gene sequences namely: Thr_EF1-4-1_T7, Thr_EF1-4-3_T7, Thr_EF1-4-9_SP6 and Thr_EF1-4-7_T7, were 459, 459, 437 and 515 bp in length respectively and two intron of size 78 bp and 456 bp is present.

The obtained *ARGK* gene sequences namely: ARGK_2B-Thr-2_T7, ARGK_2B-Thr-6_T7 and ARGK_5E-Thr-3_T7, were 327, 409 and 455 bp in length including one intron of size 81 bp.

All above mentioned sequences were used to design specific primer sets for *Ceratothripoides claratris*.

After sequencing analysis, it was found that some positive clones of each gene showed no homology with the expected genes. This may be due to non-specific bands that could also be amplified when using the degenerate primer in PCR reaction.

Table 6 Numbers of positive clone from blue-white colony selection and numbers of sequence obtained from each PCR amplified gene using the designed degenerate primer pairs.

Gene	Number of positive clones	Numbers of obtained sequence
Cytochrom oxidase I (*COI)*	8	2
Phosphoenolpyruvate Carboxykinase (*PEPCK*)	**7**	-
Elongation factor1α (*EF1α*)	7	4
Opsin (*OPS*)	1	-
Alcohol dehydrogenase (*ADH*)	1	-
Dopa decarboxylase (*DDC*)	6	-
Arginine kinase (*ARGK*)	31	3

CCT-ATT-ATA-ATT-GGA-GGA-TTT--GGA-AAC-TGA-CTT-GTA-CCT-TTA-ATA-TTA-GAA-GCA-CCA-GAT-ATA-ATT-TAT-CCA-CGA-ATA-AAC-AAT-ATA-AGA-TTT-TGA-TTA-TTA-CCT-CCA-TCT-TTA-AGG-CTT-ATA-ATT-ATA-AGA-TTA-TCA-AAA-GAA-GGT-ACA-GGT-ACA-GGT-TGA-ACA-GTA-TAT-CCA-CCT-TTA-TCT-ACT-TTT-TAT-CAT-GCA-AGA-ATT-AGG-GTA-GAT-TAC-ACA-ATT-TTT-TCT-CTT-CAT-TTA-GCA-GGA-ATC-TCA-TCA-ATT-ATA-GGA-GCT-TTG-AAT-TTT-ATT-TCC-ACT-ATT-TTT-AAT-ATA-TTT-CCA-AAA-AAA-CTA-AAG-CAA-GAT-AAA-GTT-TAT-TTA-TTT-GTT-TGA-TCA-GTT-AAT-TTA-ACA-GCA-GTA-TTA-TTG-TTA-TTA-TCA-TTG-CCA-GTA-TTA-GCA-GGA-GCA-ATT-ACA-ATA-CTT-TTG-ACA-GAC-CGA-AAC-TTA-AAC-ACT-ACC-TTT-TTT-GAT-CCT-GGA-GGA-GGA-GGA-GAT-CCA-GTT-TTA-TAC-CAA-CAC-TTA-TTT-TGA-TTT-TTT-GGA-CAC-CCA-GAA-GTT-TAT-ATT-TTA-ATC-TTG-CCTC

Figure 15 Sequences of 529 bp from clone Thrip_COI_Thr-10_T7 of Cytochrom oxidase I (*COI*) gene of the collected *Ceratothripoides claratris*.

CCT-ATT-ATA-ATT-GGA-GGA-TTT-GGA-AAC-TGA-CTT-GTA-CCT-TTA-ATA-TTA-GAA-GCA-CCA-GAT-ATA-ATT-TAT-CCA-CGA-ATA-AAC-AAT-ATA-AGA-TTT-TGA-TTA-TTA-CCA-CCA-TCT-TTA-AGG-CTT-ATA-ATT-ATA-AGA-TTA-TCA-AAA-GAA-GGT-ACA-GGT-ACA-GGT-TGA-ACA-GTA-TAT-CCA-CCT-TTA-TCT-ACT-TTT-TAT-CAT-GCA-AGA-ATT-AGG-GTA-GAT-TAC-ACA-ATT-TTT-TCT-CTT-CAT-TTA-GCA-GGA-ATC-TCA-TCA-ATT-ATA-GGA-GCT-TTG-AAT-TTT-ATT-TCC-ACT-ATT-TTT-AAT-ATA-TTT-CCA-AAA-AAA-CTA-AAG-CAA-GAT-AAA-GTT-TAT-TTA-TTT-GTT-TGA-TCA-GTT-AAT-TTA-ACA-GCA-GTA-TTA-TTG-TTA-TTA-TCA-TTG-CCA-GTA-TTA-GCA-GGA-GCA-ATT-ACA-ATA-CTT-TTG-ACG-GAC-CGA-AAC-TTA-AAC-ACT-ACC-TTT-TTT-GAT-CCT-GGA-GGA-GGA-GGA-GAT-CCA-GTT-TTA-TAC-CAA-CAC-TTA-TTT-TGA-TTT-TTT-GGA-CAC-CCA-GAA-GTT-TAT-ATT-TTA-ATC-TTG-CCTC

Figure 16 Sequences of 529 bp from clone Thrip_COI_Thr-11_T7 of Cytochrom oxidase I (*COI*) gene of the collected *Ceratothripoides claratris.*

AAG-ATG-CCC-TGG-TTC-AAG-GGG-TGG-GCC-ATC-GAG-CGC-AAG-GAA-
GGC-AAG-GCT-GAC-GGC-AAG-TGC-CTG-ATT-GAG-GCC-CTC-GAT-GCT-
ATC-CTG-CCC-CCC-TCG-CGG-CCT-ACC-GAC-AAG-GCT-CTT-CGC-CTG-
CCC-CTC-CAG-GAT-GTG-TAC-AAG-ATT-GGC-GGT-ATT-GGC-ACT-GTC-
CCT-GTT-GGC-CGT-GTG-GAG-ACC-GGT-CTT-CTG-AAG-CCT-GGT-ATG-
GTT-GTG-ACC-TTC-GCT-CCT-GCC-AAC-CTG-ACC-ACT-GAA-GTC-AAG-
TCA-GTT-GAA-ATG-CAC-CAC-GAG-GCC-CTT-CAG-GAA-GCT-GTA-CCC-
GGC-GAC-AAT-GTT-GGC-TTC-AAC-ATC-AAG-AAC-GTG-TCT-GTT-AAG-
GAG-TTG-CGT-CGT-GGT-TAC-GTT-GCT-AGC-GAC-TCT-AAG-AAC-AAT-
CCT-GCC-AAG-GGT-GCT-GCT-GAT-TTC-ACT-GCT-CAA-GTC-ATT-GTC-
CTA-AAC-CAT-CCT-GGC-CAG-ATC-TCC-AAC-GGA-ATC-ACT-AGT

Figure 17 Sequences of 459 bp from clone Thr_EF1-4-1_T7 of Elongation factor1α (*EF1α*) gene of the collected *Ceratothripoides claratris.*

AAG-ATG-CCC-TGG-TTC-AAG-GGT-TGG-GCC-ATC-GAG-CGC-AAG-GAA-
GGC-AAG-GCT-GAT-GGC-AAG-TGC-CTG-ATT-GAG-GCC-CTC-GAT-GCT-
ATC-CTG-CCC-CCC-TCG-CGG-CCT-ACC-GAC-AAG-GCT-CTT-CGC-CTG-
CCC-CTC-CAG-GAT-GTG-TAC-AAG-ATT-GGC-GGT-ATT-GGC-ACT-GTC-
TCT-GTT-GGC-CGT-GTG-GAG-ACC-GGT-CTT-CTG-AAG-CCT-GGT-ATG-
GTT-GTG-ACC-TTC-GCT-CCT-GCC-AAC-CTG-ACC-ACT-GAA-GTC-AAG-
TCC-GTT-GAA-ATG-CAC-CAC-GAG-GCC-CTT-CAG-GAA-GCT-GTA-CCC-
GGC-GAC-AAT-GTT-GGC-TTC-AAC-ATC-AAG-AAC-GTG-TCT-GTT-AAG-
GAG-TTG-CGT-CGT-GGT-TAC-GTT-GCT-AGC-GAC-CCT-AAG-AAC-AAT-
CCT-GCC-AAG-GGT-GCT-GCT-GAT-TTC-ACT-GCT-CAA-GTC-ATT-GTC-
CTA-AAC-CAC-CCT-GGC-CAG-ATC-TCC-AAC-GGA-ATC-ACT-AGT

Figure 18 Sequences of 459 bp from clone Thr_EF1-4-3_T7 of Elongation factor1α (*EF1α*) gene of the collected *Ceratothripoides claratris*.

AAG-ATG-CCC-TGG-TTC-AAG-GGT-TGG-GCC-GTG-GAC-CGG-AAG-GAG-GGC-AAG-GCT-GAG-GGC-AAG-TGC-CTG-ATC-GAG-GCC-CTC-GAC-GCC-ATC-TTG-CCG-CCC-AGC-CGG-CCC-ACT-GAC-AAG-CCC-CTC-CGCCTG-CCT-CTC-CAG-GAC-GTG-TAC-AAG-ATC-GGT-GGT-ATC-GGA-ACC-GTC-CCC-GTC-GGC-CGT-GTG-GAG-ACT-GGT-CTG-CTC-AAG-CCC-GGT-ATG-GTC-GTC-ACT-TTC-GCC-CCT-GCC-AAC-CTG-ACC-ACT-GAA-GTC-AAG-TCC-GTG-GAG-ATG-CAC-CAC-GAG-GCC-CTC-ACC-GAG-GCC-GTT-CCC-GGC-GAC-AAC-GTC-GGC-TTC-AAC-ATC-AAG-AAC-GTG-TCC-GTC-AAG-GAG-CTG-CGT-CGT-GGC-TAC-GTC-GCT-GGT-GAC-TCC-AAG-AAC-GCT-CCC-CCG-AGG-GGT-GCT-GCT-GAC-TTC-ACC-GCC-CAG-GTC-ATT-GTG-CTG-AAC-CAC-CCT-GGC-CA

Figure 19 Sequences of 437 bp from clone Thr_EF1-4-9_SP6 of Elongation factor1α (*EF1α*) gene of the collected *Ceratothripoides claratris.*

AAG-ATG-CCC-TGG-TTC-AAG-GGT-TGG-GCC-GTG-GAC-CGG-AAG-GAG-GGC-AAG-GCT-GAG-GGC-AAG-TGC-CTG-ATC-GAG-GCC-CTC-GAC-GCC-ATC-TTG-CCG-CCC-AGC-CGG-CCC-ACT-GAC-AAG-CCC-CTC-CGCCTG-CCT-CTC-CAGGTAGGCCCTTC-GAA-ACG-GCG-GGT-TTC-CCA-ATC-CGC-AGC-AGC-GAT-TTG-TCA-TTA-CTT-AAC-TAA-TTA-ATT-TGG-AAA-CAG-GACGTGTAC-AAG-ATC-GGT-GGT-ATC-GGA-ACC-GTC-CCC-GTC-GGC-CGT-GTG-GAG-ACT-GGT-CTG-CTC-AAG-CCC-GGT-ATG-GTC-GTC-ACT-TTC-GCC-CCT-GCC-AAC-CTG-ACC-ACT-GAA-GTC-AAG-TCC-GTG-GAG-ATG-CAC-CAC-GAG-GCC-CTC-ACC-GAG-GCC-GTT-CCC-GGC-GAC-AAC-GTC-GGC-TTC-AAC-ATC-AAG-AAC-GTG-TCC-GTC-AAG-GAG-CTG-CGT-CGTGGCTACGTC-GCT-GGT-GAC-TCC-AAG-AAC-GCT-CCC-CCG-AGG-GGT-GCT-GCT-GAC-TTC-ACC-GCC-CAG-GTC-ATT-GTG-CTG-AAC-CAC-CCT-GGC-CAG

Figure 20 Sequences of 515 bp from clone Thr_EF1-4-7_T7 of Elongation factor1α (*EF1α*) gene of the collected *Ceratothripoides claratris*.

G-ATC-CCA-TCA-TTG-AGG-ATT-ATC-ACA-AGG-GCT-TCA-AGA-AGA-CAG-ACA-AGC-ACC-CTC-CCA-AGA-ACT-GGG-GCG-ATG-TCG-AGA-CCC-TCT-CTG-ATG-TGG-ACC-CCA-C-0-81---CG-GCG-AGT-ACG-TCG-TGT-CGA-CCC-GTG-TCC-GTT-GCG-GCC-GCT-CCA-TGG-AGG-GCT-ACC-CCT-TCA-ACC-CTT-GCC-TGA-CCG-AGG-CCC-AGT-ACG-CCG-AGA-TGC-AGG-ACA-AGG-TGT-CCT-CCA-CCC-TGT-CTG-GCC-TGG-AGG-GCG-AGC-TCA-AGG-GCA-CCT-ACT-ACC-CCC-TCA-AGG-GCA-TGA-CCA-AGG-AGG-TGC-AGC-AGA-AGC-TGA-TCG-ACG-ACC-ATT-TCC-TGT-TCA-AGG-AGG-TG

Figure 21 Sequences of 327 bp from clone AGRK_2B-Thr-2_T7 of Arginine kinase (*ARGK*) gene of the collected *Ceratothripoides claratris*.

G-ATC-CCA-TCA-TTG-AGG-ATT-ATC-ACA-AGG-GCT-TCA-AGA-AGA-CAG-ACA-AGC-ACC-CTC-CCA-AGA-ACT-GGG-GCG-ATG-TCG-AGA-CCC-TCT-CTG-ATG-TGG-ACC-CCA-CCG-TGC-GTG-TCT-TTA-CCG-CCG-TGC-CTG-CGT-GTG-TCC-TGC-GAC-GTG-GTG-TGT-CCT-GAC-GTT-GGC-GTG-TCC-TTG-TTT-GCT-TGG-CAG-GGC-GAG-TAC-GTC-GTG-TCG-ACC-CGT-GTC-CGT-TGC-GGC-CGC-TCC-ATG-GAG-GGC-TAC-CCC-TTC-AAC-CCT-TGC-CTG-ACC-GAG-GCC-CAG-TAC-GCC-GAG-ATG-CAG-GAC-AAG-GTG-TCC-TCC-ACC-CTG-TCT-GGC-CTG-GAG-GGC-GAG-CTC-AAG-GGC-ACC-TAC-TAC-CCC-CTC-AAG-GGC-ATG-ACC-AAG-GAG-GTG-CAG-CAG-AAG-CTG-ATC-GAC-GAC-CAT-TTC-CTG-TTC-AAG-GAG-GTG

Figure 22 Sequences of 409 bp from clone AGRK_2B-Thr-6_T7 of Arginine kinase (*ARGK*) gene of the collected *Ceratothripoides claratris.*

G-ATC-CCA-TCA-TTG-AGG-AT--ATC-ACA-AGG-GCT-TCA-AGA-AGA-CAG-ACA-AGC-ACC-CTC-CCA-AGA-ACT-GGG-GCG-ATG-TCG-AGA-CCC-TCT-CTG-ATG-TGG-ACC-CCA-CCG-TGC-GTG-TCT-TTA-CCG-CCG-TGC-CTG-CGT-GTG-TCC-TGC-GAC-GTG-GTG-TGT-CCT-GAC-GTT-GGC-GTG-TCC-TTG-TTT-GCT-TGG-CAG-GGC-GAG-TAC-GTC-GTG-TCG-ACC-CGT-GTC-CGT-TGC-GGC-CGC-TCC-ATG-GAG-GGC-TAC-CCC-TTC-AAC-CCT-TGC-CTG-ACC-GAG-GCC-CAG-TAC-GCC-GAG-ATG-CAG-GAC-AAG-GTG-TCC-TCC-ACC-CTG-TCT-GGC-CTG-GAG-GGC-GAG-CTC-AAG-GGC-ACC-TAC-TAC-CCC-CTC-AAG-GGC-ATG-ACC-AAG-GAG-GTG-CAG-CAG-AAG-CTG-ATC-GAC-GAC-CAC-TTC-CTG-TTC-AAG-GAG-GGT-GAA-TCG-AAT-TCC-CGC-GGC-CGC-CAT-GGC-GGC-CGG-AGC-ATG-CAG

Figure 23 Sequences of 455 bp from clone AGRK_5E-Thr-3_T7 of Arginine kinase (*ARGK*) gene of the collected *Ceratothripoides claratris*.

5. Design of specific primers

Based on the obtained sequences of Cytochrom oxidase I (*COI*), Elongation factor1α (*EF1α*) and Arginine kinase (*ARGK*), specific primers were designed flanking the introns or extending into the introns in such a way that different loci could be amplified individually for each gene. All of the target sequences had introns, except Cytochrom oxidase I sequence. The sequences of *COI*, *EF1*α and *ARGK* genes from *C. claratris* clones were compared to GenBank databases using BLAST for verification of gene identity. Figure 24-26 are the partial alignment of *COI*, *EF1*α and *ARGK* sequences from *C. claratris* clones with some sequence of *COI*, *EF1*α and *ARGK* genes from other insect species for the design of specific primer. The designed *ARGK* specific primer for *C. claratris* had one intron. The designed *EF1*α specific primer for *C. claratris* had two introns. Table 7 showed the sequences of the *C. claratris* specific primers that were designed from sequences obtained from this study and the published primer of internal transcribed spacer (*ITS*) gene. They were used for PCR-SSCP analysis of *C. claratris*. Eight hundred and thirty-two samples of *C. claratris* collected from 26 provinces in the country were screened for polymorphism.

Table 7 Sequence of the specific primers

Primer name	Primer sequence	Reference
Cytochrom oxidase I (CerCOI)	F) TTG GAA ACT GAC TTG TAC CTT T	Present study
	R) GAG GCA AGA TTA AAA TAT AAA CTT CTG	Present study
Internal transcribed spacer (ITS)	F) TGT GAA CTG CAG GAC ACA TG	Ruman-Jones *et al*. 2006
	R) GTT RGT TTC TTT TCC TC	Ruman-Jones *et al*. 2006
Elongation factor1α (CerEF1α)	F) GAC GCC ATC TTG CCG CCC AG	Present study
	R) TCG CCG GGA ACA GCT TCG GT	Present study
Arginine kinase (CerARGK)	F) CAA GCA CCC TCC CAA GAA CTG G	Present study
	R) CAC CTC CTT GGT CAT GCC CT	Present study

Once a particular band has been sequenced, it is possible to design new primers that are specific to that band in the gene of interest. This study has successfully designed primers specific to the COI, EF1α and ARGK genes and revealed the promising application of these primers for molecular analysis of population differentiation. Results from the present study indicated that several loci would be quite useful in studies of diversity species of thrips. It is hoped that these primers will enable the application of genetic investigation in other studies of the Order Thysanoptera.

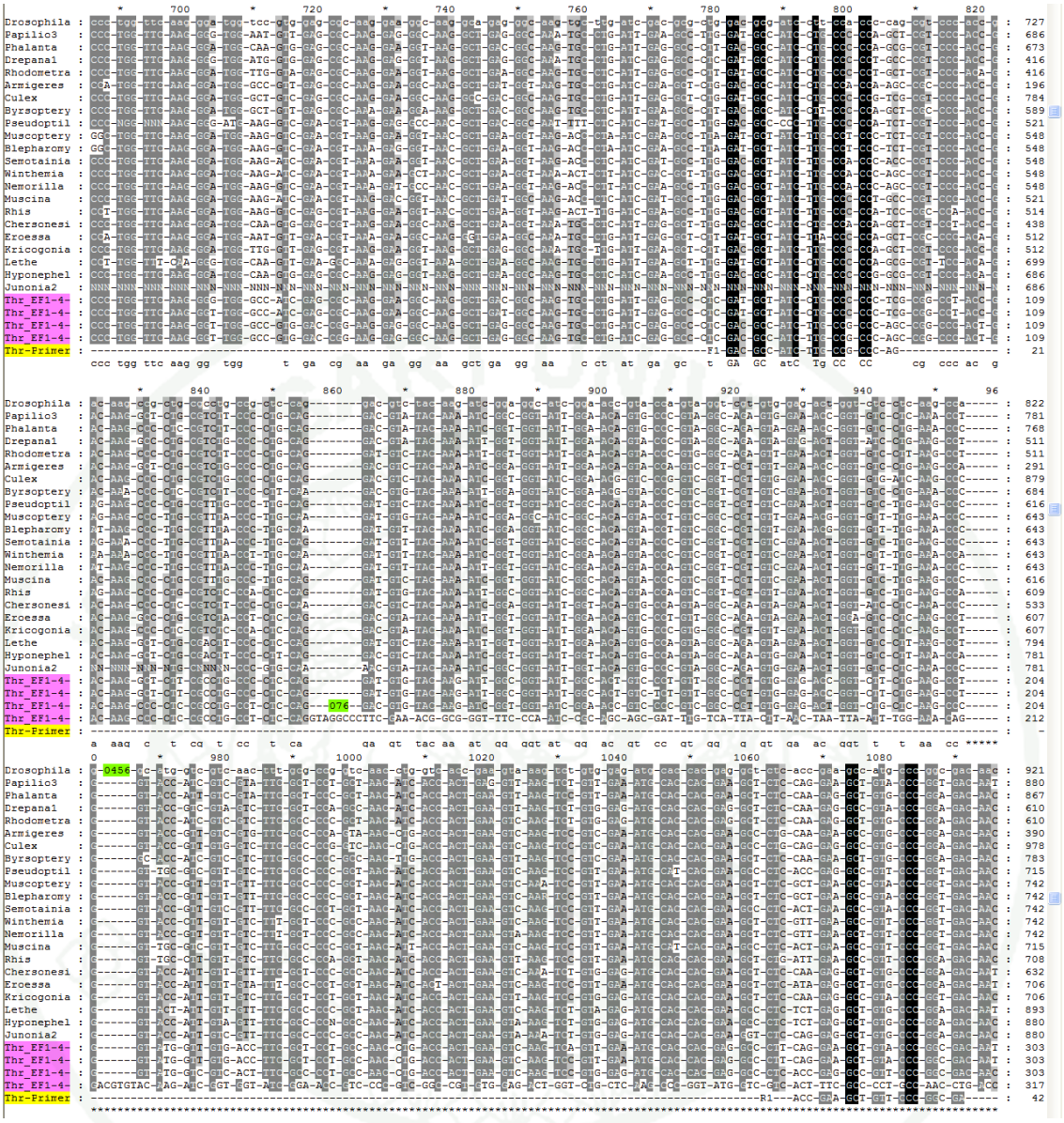

Figure 24 Sequences alignment of Elongation factor 1α gene (*EF1α*) of various insect species by GeneDoc program. The specific primers were designed in conserved region.

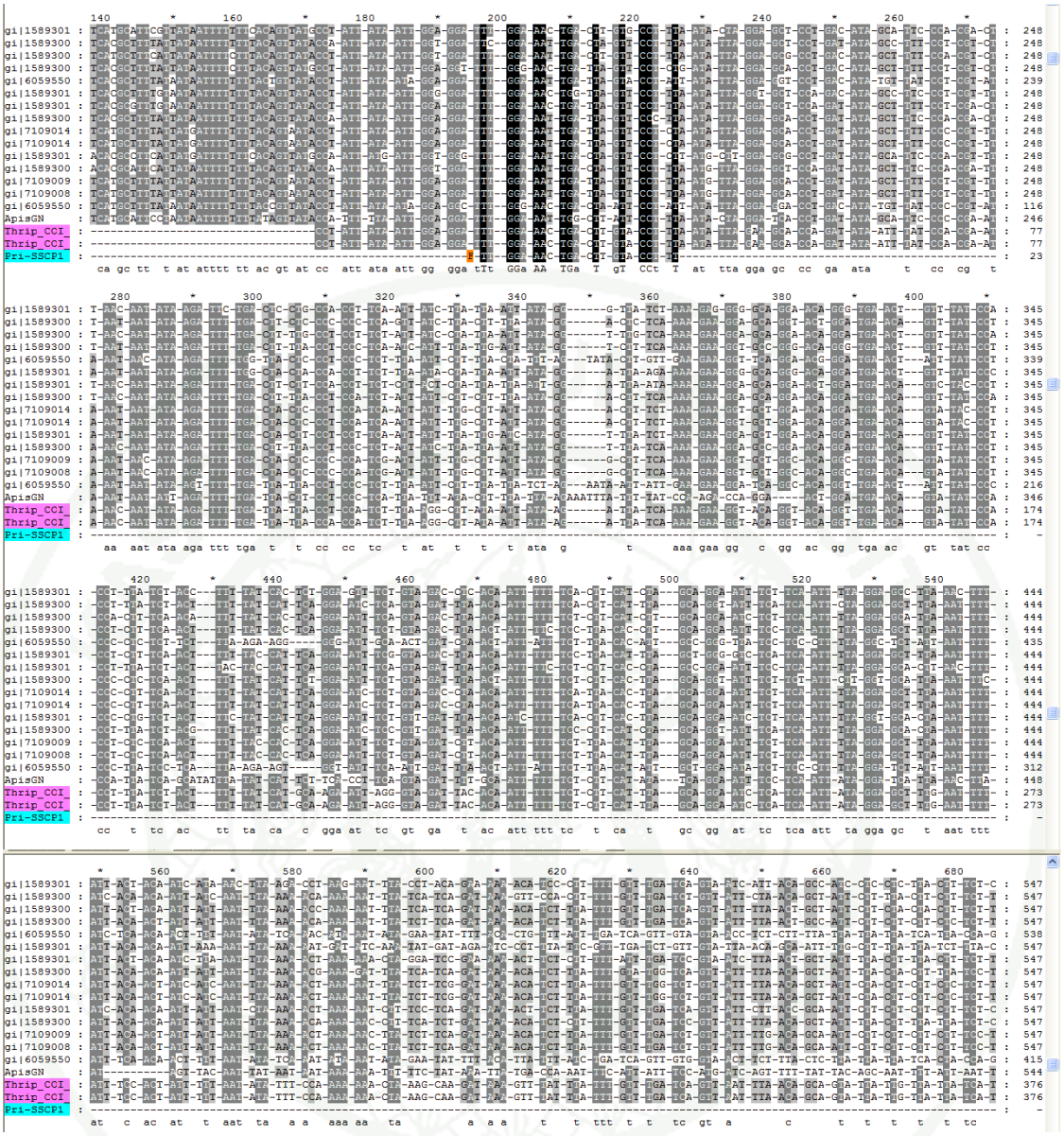

Figure 25 Sequences alignment of Cytochrom oxidase I gene (*COI*) of various insect species by GeneDoc program. The specific primers were designed in conserved region.

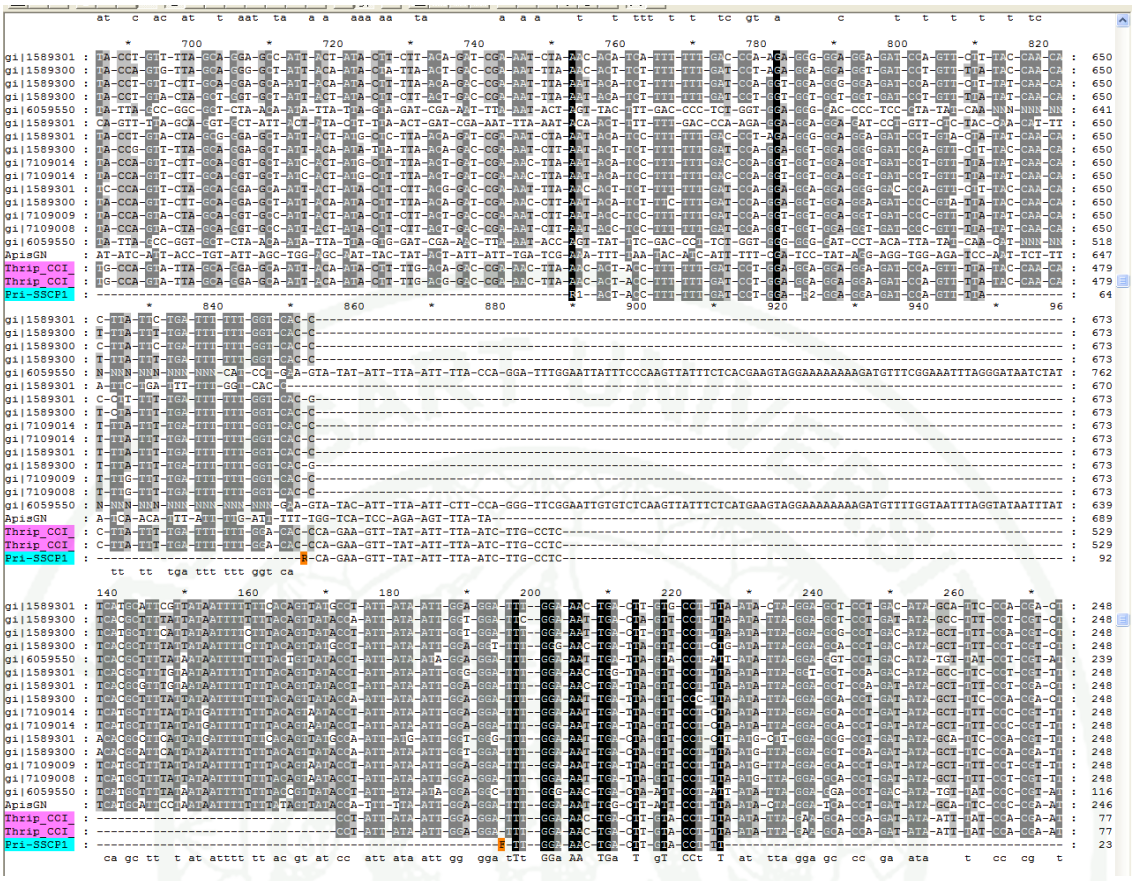

Figure 25 (Continued)

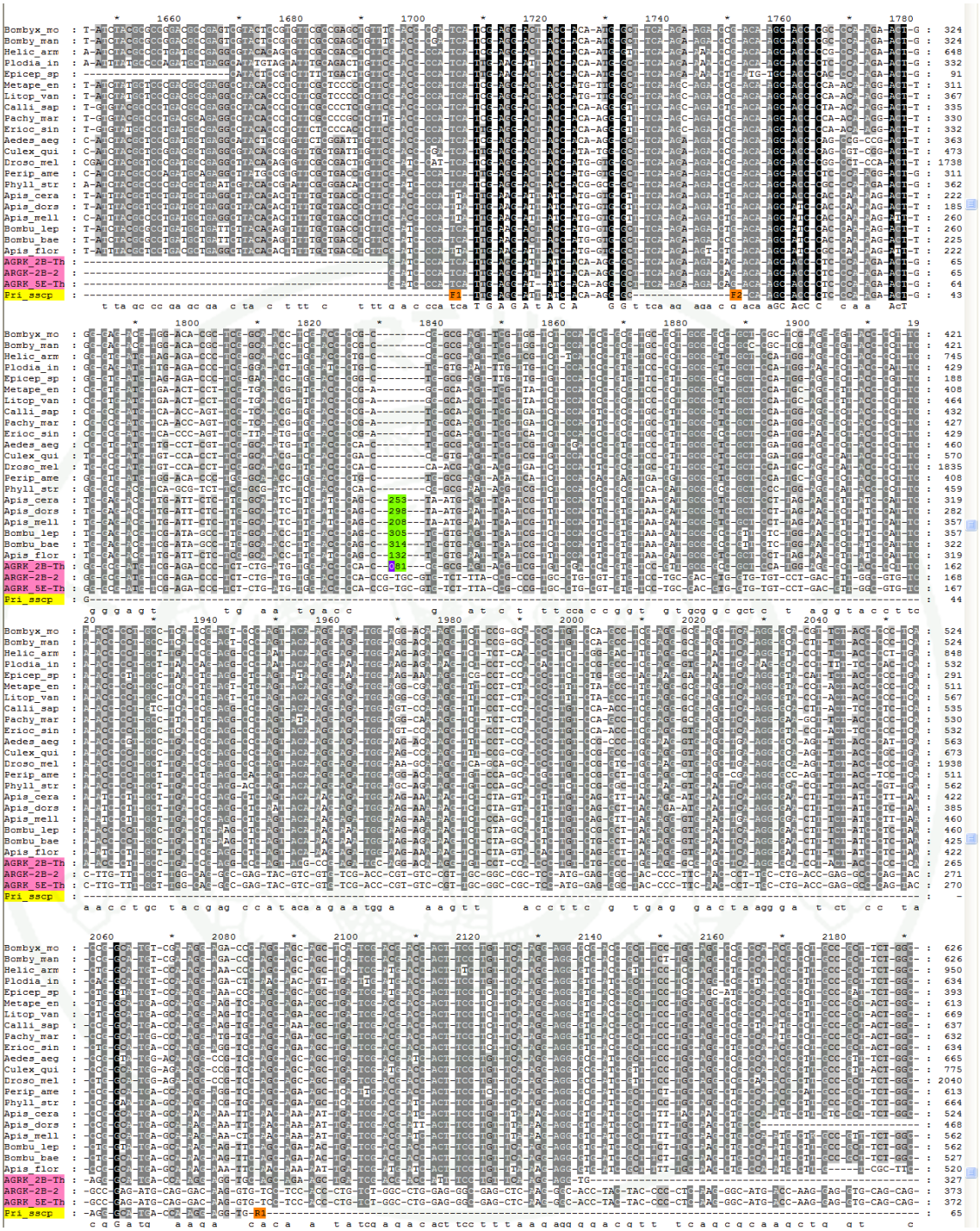

Figure 26 Sequences alignment of Arginine kinase gene (*ARGK*) of various insect species by GeneDoc program. The specific primers were designed in conserved region.

6. Polymerase chain reaction – Single stand conformation polymorphism (PCR-SSCP) for polymorphism detection

Eight hundred and thirty-two samples of *C. claratris* collected from 26 sites around the country were assayed using PCR-SSCP and alleles at four gene loci (*COI*, *ITS*, *ARGK* and *EF1α*) were identified. The specific primer of *COI*, *ITS, ARGK* and *EF1α* genes successfully amplified a fragment from all *C.claratris* populations. On agarose gel, a single band of approximate 550, 500, 300 and 450 bp were visible respectively. Different alleles could not be distinguished. However, using SSCP, several alleles could be identified in all populations. All amplified DNA fragments were denatured and electrophoresed on non-denaturing polyacrylamide gel (SSCP).

The PCR amplification of Cytochrome oxidase I [*COI* (529 bp)], Internal transcribed spacer [*ITS* (500 bp)], Arginine kinase [*ARGK* (327 bp)] and Elongation factor1α [*EF1α* (437 bp)] genes of all tested populations of *C. claratris* obtained from PCR reaction using primer previously published and primers designed in this study produced single band. The appropriate annealing temperature of each specific primer was 55°C, 58°C, 55°C and 59°C respectively. The PCR-SSCP assay was used to detect the presence of any polymorphism at these genes among collected populations of *C. claratris*.

The CerCOI specific primers successfully amplified a fragment of COI gene from all thrips populations. On agarose gel, only a single band of approximate 550 bp was observed, different alleles could not be distinguished (Figure27). However, using SSCP analysis different alleles could be identified in all thrips populations. Figure 28 showed different alleles from all collected thrips populations demonstrated by SSCP analysis.

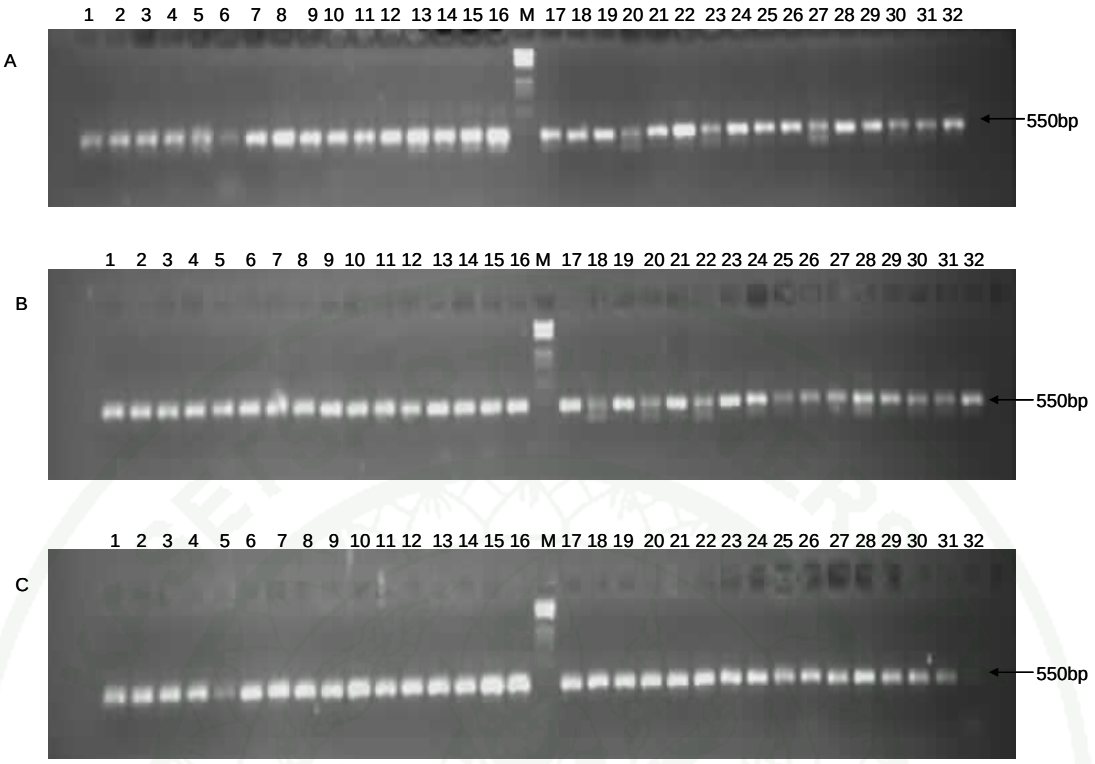

Figure 27 PCR products of 32 individuals of *Ceratothripoides claratris* samples on agarose gel using CerCOI specific primer set compared with the standard size (M; λ/HindIII+EcoRI). The approximate size of each PCR amplified fragment is 550 bp.

A) Population collected from Nong Khai province
B) Population collected from Nakhon Pathom province
C) Population collected from Tak province

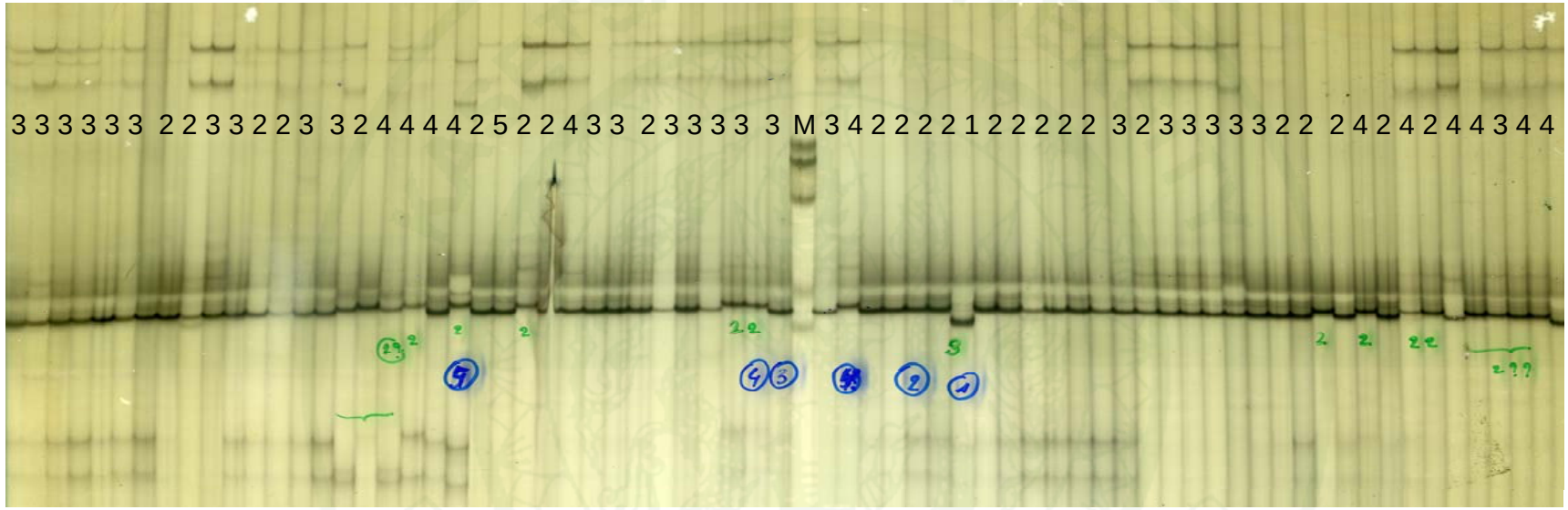

Figure 28 SSCP patterns of COI gene of each accession of all collected *Ceratothripoides claratris* populations using CerCOI specific primer. Five different banding patterns were detected. M= the standard size (λ/HindIII+EcoRI)

The ITS specific primers successfully amplified a fragment of ITS gene from all thrips populations. On agarose gel, only a single band of approximate 500 bp was observed, different alleles could not be distinguished (Figure29). However, using SSCP analysis different alleles could be identified in all thrips populations. Figure 30 showed different alleles from all collected thrips populations demonstrated by SSCP analysis.

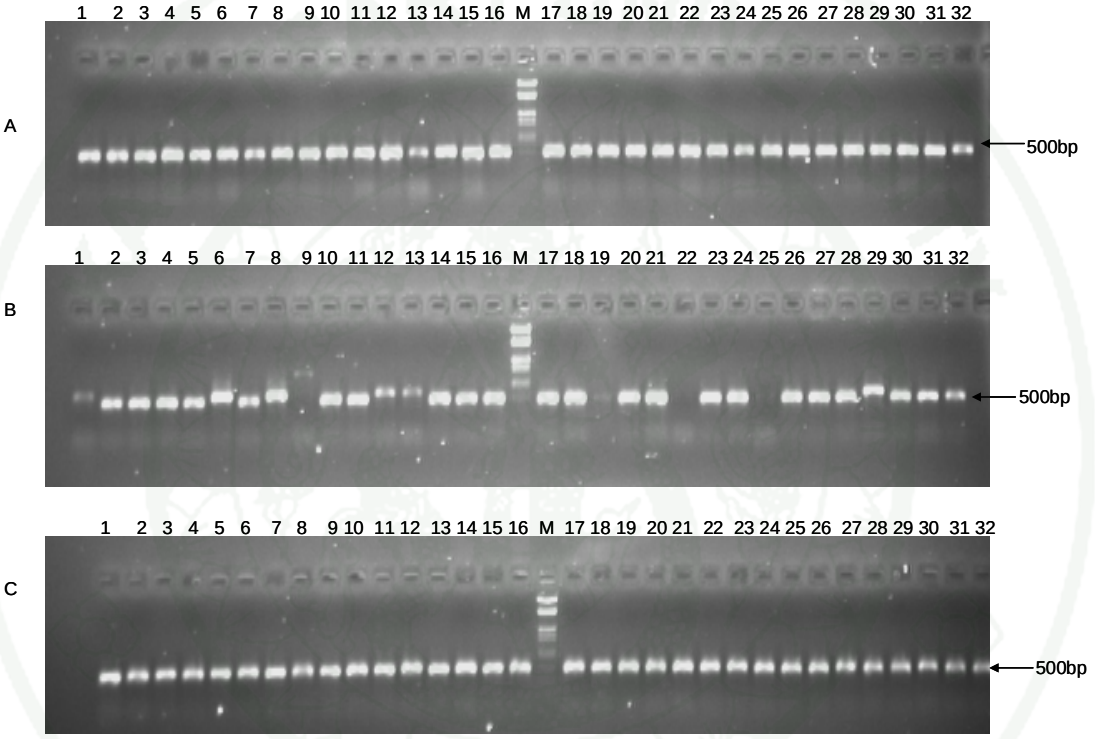

Figure 29 PCR products of 32 individuals of *Ceratothripoides claratris* samples on agarose gel using CerITS specific primer set compared with the standard size (M; λ/HindIII+EcoRI). The approximate size of each PCR amplified fragment is 500 bp.

A) Population collected from Nong Khai province
B) Population collected from Nakhon Pathom province
C) Population collected from Tak province

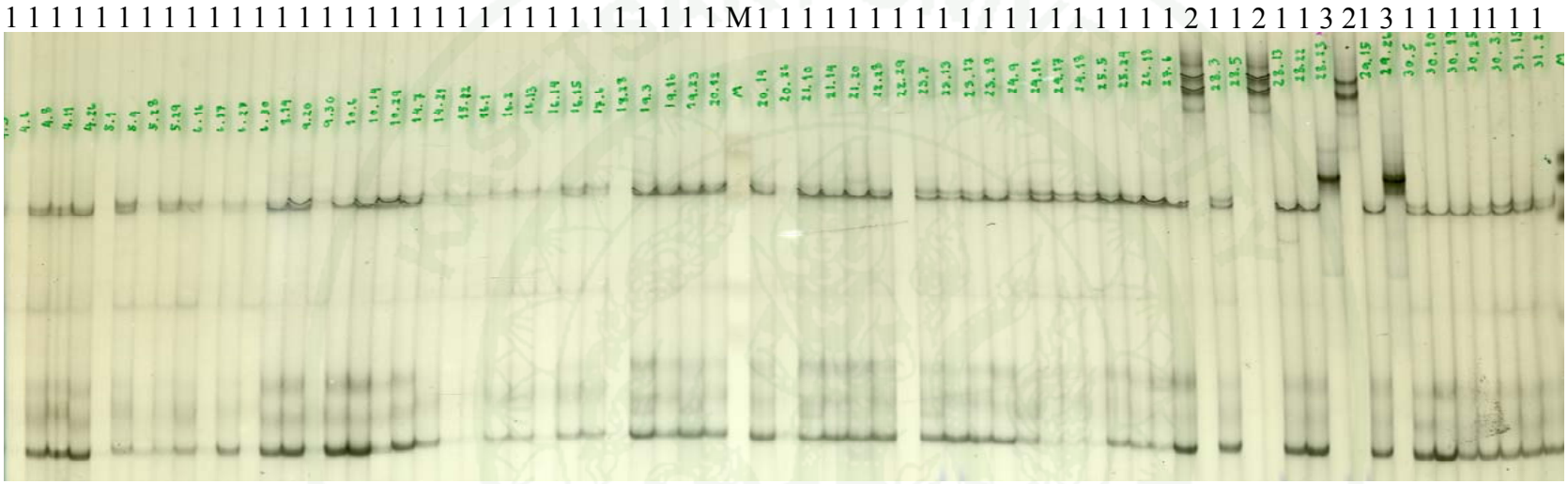

Figure 30 SSCP patterns of ITS gene of each accession of all collected *Ceratothripoides claratris* populations using CerITS specific primer. Three different banding patterns were detected. M= the standard size (λ/HindIII+EcoRI)

The CerARGK specific primers successfully amplified a fragment of ARGK gene from all thrips populations. On agarose gel, only a single band of approximate 300 bp was observed, different alleles could not be distinguished (Figure31). However, using SSCP analysis different alleles could be identified in all thrips populations. Figure 32 showed different alleles from all collected thrips populations demonstrated by SSCP analysis.

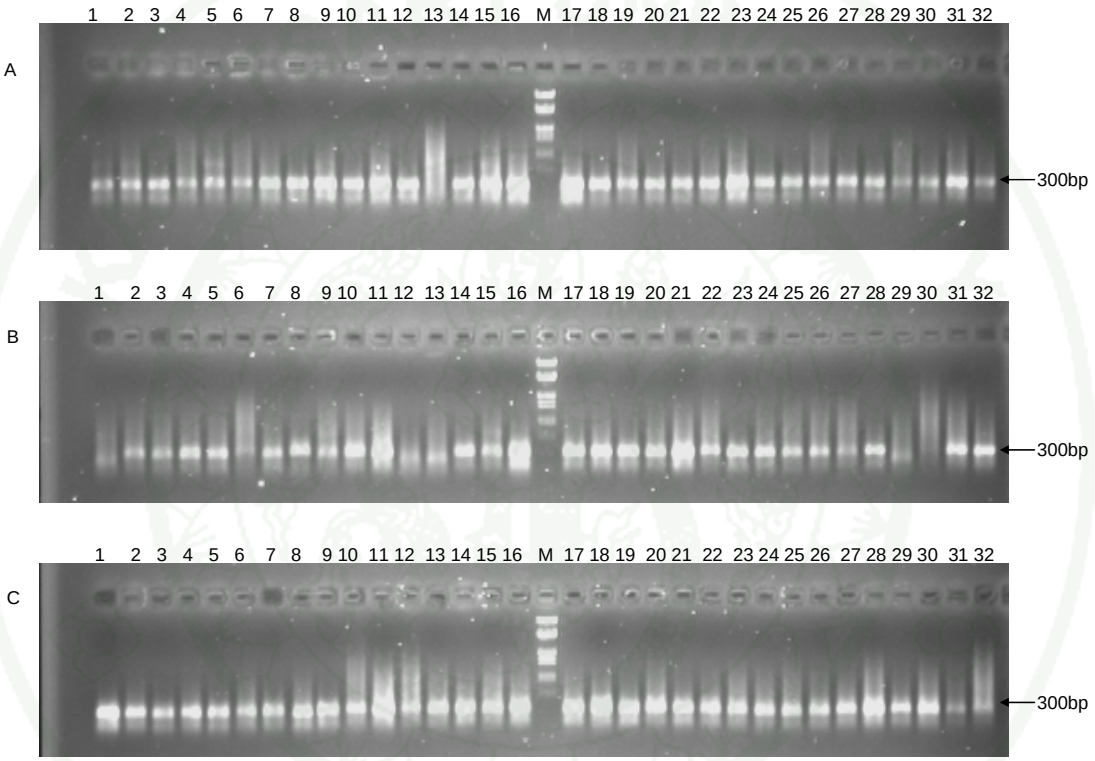

Figure 31 PCR products of 32 individuals of *Ceratothripoides claratris* samples on agarose gel using CerARGK specific primer set compared with the standard size (M; λ/HindIII+EcoRI). The approximate size of each PCR amplified fragment is 300 bp.

A) Population collected from Nong Khai province
B) Population collected from Nakhon Pathom province
C) Population collected from Tak province

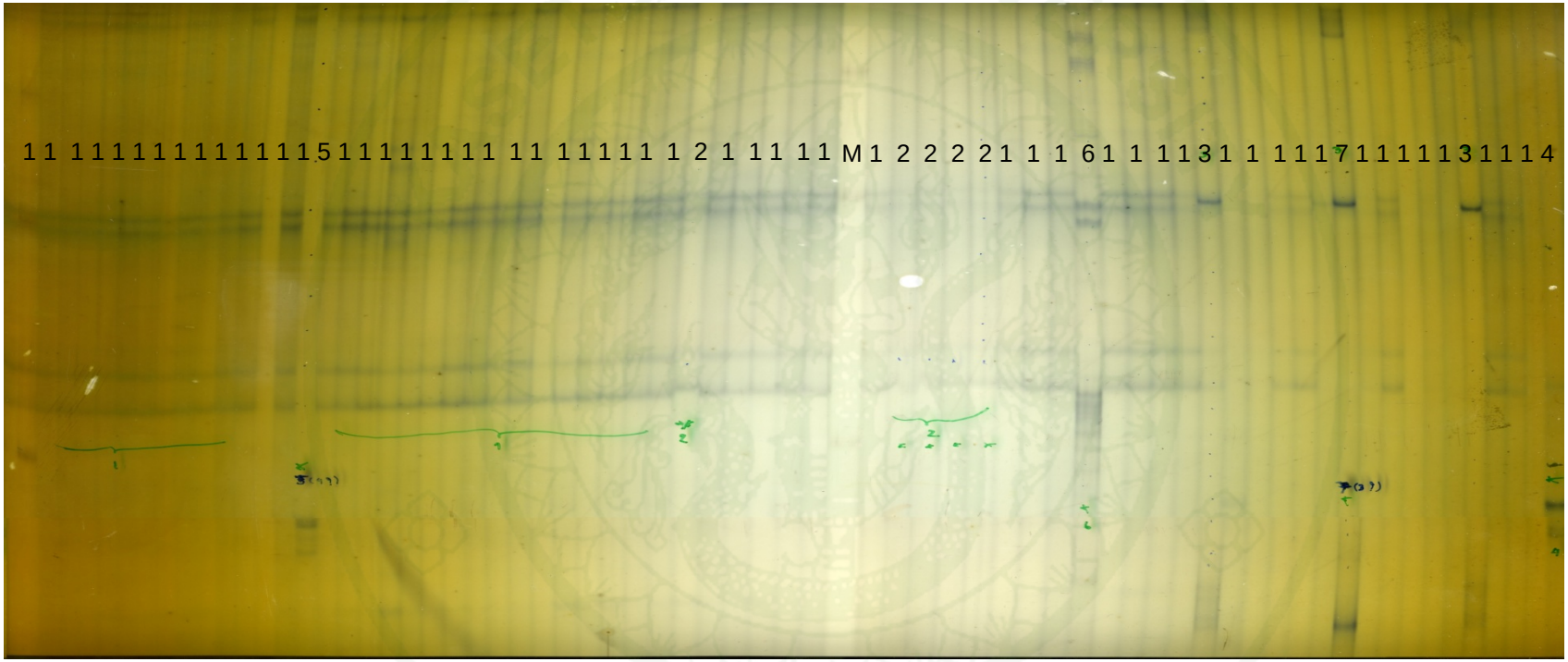

Figure 32 SSCP patterns of ARGK gene of each accession of all collected *Ceratothripoides claratris* populations using CerARGK specific primer. Seven different types of banding pattern were detected. M= the standard size (λ/HindIII+EcoRI)

The CerEF1α specific primers successfully amplified a fragment of EF1α gene from all thrips populations. On agarose gel, only a single band of approximate 450 bp was observed, different alleles could not be distinguished (Figure33). However, using SSCP analysis different alleles could be identified in all thrips populations. Figure 34 showed different alleles from all collected thrips populations demonstrated by SSCP analysis.

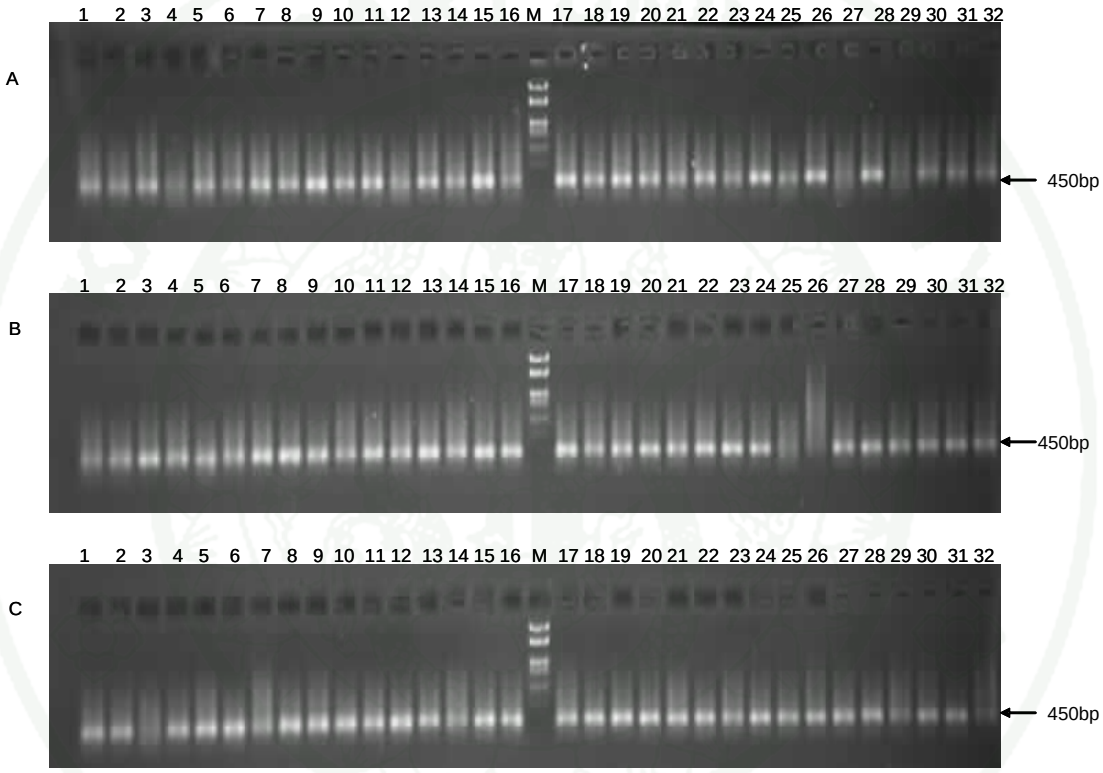

Figure 33 PCR products of 32 individuals of *Ceratothripoides claratris* samples on agarose gel using CerEF1α specific primer set compared with the standard size (M; λ/HindIII+EcoRI). The approximate size of each PCR amplified fragment is 450 bp.

A) Population collected from Nong Khai province
B) Population collected from Nakhon Pathom province
C) Population collected from Tak province

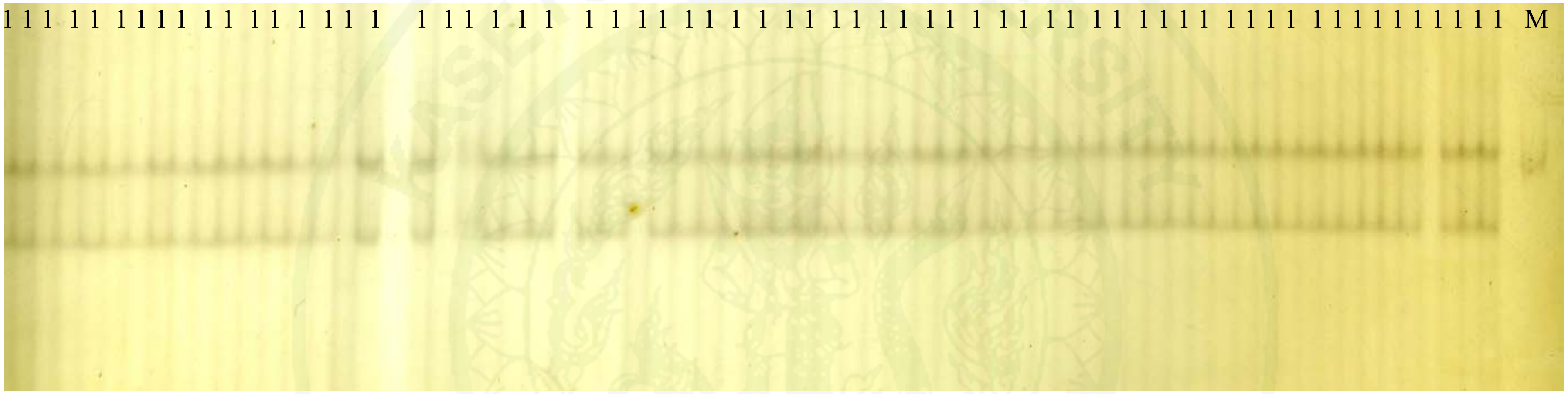

Figure 34 SSCP patterns of EF1α gene of each accession of all collected *Ceratothripoides claratris* populations using CerEF1α specific primer. Only one banding patterns was detected. M= the standard size (λ/HindIII+EcoRI)

The PCR-SSCP analysis indicated that all of the amplified *EF1α* gene fragments gave an identical band pattern. Therefore, this gene can not be used to differentiate *C. claratris* populations from different locations. However, the PCR-SSCP analysis of *COI*, *ITS* and *ARGK* gene fragments revealed 5, 3 and 7 different band patterns respectively. For the mitochondrial *COI* marker, 88% of the individuals had one haplotype and 11% had an alternate haplotype while just a few individuals were observed for the other 3 haplotypes. Similarly for the nuclear *ITS* and *ARGK* loci, one allele was extremely common, while only some isolated individuals had the alternate alleles. The *COI* locus combined with the nuclear genes can therefore be used to clarify *C. claratris* populations collected from different locations.

The SSCP patterns of *COI* fragment of the gene are illustrated in Figure 28. Five alleles were observed. Three different SSCP patterns were identified for the *ITS* fragment (Figure 30) Seven different SSCP patterns for the *ARGK* locus (Figure 32) and only one SSCP pattern was detected for the *EF1α* fragment (Figure 34).

The SSCP assays indicated a low genetic diversity in *C. claratris* collected from different locations in Thailand. The SSCP markers from four specific loci in the present study may not be suitable for estimating genetic similarity between very closely related accessions of *C. claratris*. In order to be useful for genetic diversity research, the gene specific primer must contain regions where the sequence varies at high rate among populations. Therefore, *C. claratris* collections should be analyzed with a larger number of loci and more accessions from broader geographic range to obtain duplicated genetic diversity estimation in this thrips.

Generally, SSCP is capable of detecting single nucleotide mutation but SSCP markers using specific primers in the present study may not be suitable for estimating genetic similarity between very closely related populations of *C. claratris*. Numbers of SSCP pattern of genes of *C. claratris* collected from different locations in Thailand are listed in table 8.

The PCR-SSCP analysis of COI gene grouped the 26 *C. claratris* populations into 8 clusters, designated as Group A-Group H.

Group A comprised of five *C. claratris* populations. They were *C. claratris* collected from Nong Khai, Sakon Nakhon, Prachuap Khiri Khan, Lamphun and Lampang.

Group B comprised of five *C. claratris* populations. They were *C. claratris* collected from Mukdahan, Amnat Charoen, Ubon Ratchathani, Ratchaburi and Phayao.

Group C comprised of five *C. claratris* populations. They were *C. claratris* collected from Buri Ram, Phichit, Kamphaeng Phet, Ang Thong and Kanchanaburi.

Group D comprised of *C. claratris* collected from Nakhon Ratchasima.

Group E comprised of four *C. claratris* populations. They were *C. claratris* collected from Yasothon, Nakhon Sawan, Tak and Chiang Rai.

Group F comprised of three *C. claratris* populations. They were *C. claratris* collected from Nakhon Pathom, Uthai Thani and Phetchaburi.

Group G comprised of two *C. claratris* populations. They were *C. claratris* collected from Chai Nat and Sing Buri.

Group H comprised of *C. claratris* collected from Chiang Mai.

The PCR-SSCP analysis of ITS gene grouped the 26 *C. claratris* populations into 3 clusters, designated as Group A-Group C.

Group A comprised of twenty-three *C. claratris* populations.They were *C. claratris* collected from Nong Khai, Sakon Nakhon, Mukdahan, Amnat Charoen, Ubon Ratchathani, Buri Ram, Nakhon Ratchasima, Yasothon, Nakhon Pathom, Nakhon Sawan, Phichit, Sing Buri, Ang Thong, Ratchaburi, Phetchaburi, Prachuap Khiri Khan, Kanchanaburi, Tak, Chiang Rai, Chiang Mai, Phayao, Lamphun and Lampang.

Group B comprised of *C. claratris* collected from Kamphaeng Phet.

Group C comprised of two *C. claratris* populations. They were *C. claratris* collected from Uthai Thani and Chai Nat.

The PCR-SSCP analysis of ARGK gene grouped the 26 *C. claratris* populations into 7 clusters, designated as Group A-Group G.

Group A comprised of eightteen *C. claratris* populations. They were *C. claratris* collected from Nong Khai, Sakon Nakhon, Mukdahan, Amnat Charoen, Ubon Ratchathani, Nakhon Ratchasima, Yasothon, Nakhon Pathom, Nakhon Sawan, Sing Buri, Ang Thong, Ratchaburi, Phetchaburi, Prachuap Khiri Khan, Kanchanaburi, Tak, Chiang Rai and Phayao.

Group B comprised of *C. claratris* collected from Buri Ram.

Group C comprised of *C. claratris* collected from Phichit.

Group D comprised of *C. claratris* collected from Kamphaeng Phet.

Group E comprised of *C. claratris* collected from Uthai Thani.

Group F comprised of *C. claratris* collected from Chai Nat.

Group G comprised of three *C. claratris* populations. They were *C. claratris* collected from Chiang Mai, Lamphun and Lampang.

The EF1α gene grouped the 26 *C. claratris* populations into only one clusters.

The PCR-SSCP analysis revealed that heterozygosity was very low among the collected *C. claratris* populations which indicated that *C. claratris* populations are very closely related. And Thai *C. claratris* found in different provinces may be common in origin.

This study demonstrated that the SSCP markers from the four specific primers may not be applicable for variability stydy on this insect species. Primers specific to any certain gene must contain regions where the sequence varies at high rate among populations. Eventhough, the PCR-SSCP analysis of the *C. claratris* populations presented here could demonstrate the existence of polymorphism among populations, it is recommended that PCR-SSCP analysis will provide better genetic diversity estimation of this insect species when greater number of primers and more accessions from broader geographic range are employed.

However, the PCR-SSCP detects only the presence of the nucleotide sequence , not type of mutations. For this regard, PCR-SSCP analysis should be supported by DNA sequencing, and cumulative data concerning the relationship between the phenotype and type of mutations are required. Additionally, these genetic marker will offer the most powerful insights when they are combined with demographic or behavioral data when available.

Table 8 Numbers of SSCP pattern of genes of *Ceratothripoides claratris* collected from different locations in Thailand and the designated population group**.**

Region	Province	SSCP pattern of each marker gene							
		COI	group	*ITS*	group	*ARGK*	group	*EF1α*	group
Northern	Chiang Rai	2*	E	1*	A	1*	A	1*	A
	Chiang Mai	1,2	H	1	A	2	G	1	A
	Phayao	2,3	B	1	A	1	A	1	A
	Lamphun	3	B	1	A	2	G	1	A
	Lampang	3	A	1	A	2	G	1	A
Central	Nakhon-Pathom	3,4	F	1	A	1	A	1	A
	Nakhon Sawan	2	E	1	A	1	A	1	A
	Phichit	2,4	C	1	A	6	C	1	A
	Kamphaeng-Phet	2,4	C	2	B	3	D	1	A
	Uthai Thani	3,4	F	2,3	C	7	E	1	A
	Chai Nat	4	G	2,3	C	3,4	F	1	A
	Sing Buri	4	G	1	A	1	A	1	A
	Ang Thong	2,4	C	1	A	1	A	1	A
North-Eastern	Nong Khai	3	A	1	A	1	A	1	A
	Sakon Nakhon	3	A	1	A	1	A	1	A
	Mukdahan	2,3	B	1	A	1	A	1	A
	Amnat-Charoen	2,3	B	1	A	1	A	1	A
	Ubon-Ratchathani	2,3	B	1	A	1	A	1	A
	Buri Ram	2,4	C	1	A	5	B	1	A
	Nakhon-Ratchasima	2,4,5	D	1	A	1	A	1	A
	Yasothon	2	E	1	A	1	A	1	A
Western	Ratchaburi	2,3	B	1	A	1	A	1	A
	Phetchaburi	3,4	F	1	A	1	A	1	A
	Prachuap-Khiri- Khan	3	A	1	A	1	A	1	A
	Kanchanaburi	2,4	C	1	A	1	A	1	A
	Tak	2	E	1	A	1	A	1	A

*** Type of polymorphism pattern**

7. Cluster analysis

A total of fifteen banding patterns were scored according to their presence and absence of shared polymorphism bands across the *COI* and three nuclear loci. One hundred and forty-one thrips representing each of the multilocus genotypes found in each of the 32 *C. claratris* populations were selected for further clustering analysis. All polymorphic bands were used to construct the dendrogram by the unweighted pair-group method with an arithmetic average algorithm (UPGMA) and the computational analysis was done by NTSYSpc program (Rohlf, 1993). Based on the constructed dendrogram using a 0.77 similarity level, the total thrips, *C. claratris* populations could be clustered into 15 groups (Figure 35). Group A contained the most common genotype including most of the accessions from Northern, Central, North-Eastern and Western regions. Group B and K included only Northern region. Group C and J included only North-Eastern region. Group D, E, F, G, H, I, L, M, N and O included only Central region. Group E consisted of three regions that were Central, North-Eastern and Western regions. The cophenetic correlation (r) was 0.98408. The compositions of each group were described in Table 9.

Group A comprised of twenty-five *C. claratris* populations. They were *C. claratris* collected from Nong Khai, Sakon Nakhon, Mukdahan, Nakhon Sawan, Amnat Charoen, Lampang, Lamphun, Phayao, Ubon Ratchathani, Chiang Rai, Buri Ram, Prachuap Khiri Khan, Phetchaburi, Ratchaburi, Yasothon, Chiang Mai, Tak, Uthai Thani, Kamphaeng Phet, Phichit, Nakhon Pathom, Sing Buri, Nakhon Ratchasima, Ang Thong and Kanchanaburi.

Group B comprised of *C. claratris* collected from Chiang Mai.

Group C comprised of *C. claratris* collected from Amnat Charoen.

Group D comprised of two *C. claratris* populations. They were *C. claratris* collected from Buri Ram and Uthai Thani.

Group E comprised of eleven *C. claratris* populations. They were *C. claratris*

from collected Buri Ram, Kanchanaburi, Phetchaburi, Ang Thong, Sing Buri, Chai Nat, Uthai Thani, Kamphaeng Phet, Phichit, Nakhon Pathom and Nakhon Ratchasima.

Group F comprised of *C. claratris* collected from Uthai Thani.
Group G comprised of *C. claratris* collected from Chai Nat.
Group H comprised of *C. claratris* collected from Uthai Thani.
Group I comprised of *C. claratris* collected from Uthai Thani.
Group J comprised of *C. claratris* collected from Buri Ram.

Group K comprised of three *C. claratris* populations. They were *C. claratris* collected from Chiang Mai, Lampang and Lamphun.

Group L comprised of *C. claratris* collected from Phichit.
Group M comprised of *C. claratris* collected from Kamphaeng Phet.
Group N comprised of *C. claratris* collected from Chai Nat.
Group O comprised of *C. claratris* collected from Uthai Thani.

Results from this study indicate that the thrips population in Thailand is highly homogenous with about 85% of all specimens assayed belonging to a single multilocus haplotype. Still some interesting patterns were observed. All individuals of the Pichit population had the alternate *COI* genotype, while this genotype also occurred at a low frequency in the surrounding populations and in Northeastern Thailand. Although very rare, 5 out of 6 individuals having the *ARGK* allele 2 were from Northern Thailand (Chiangmai, Lamphun and Lampang) and allele 4 was found only in Chainat, UthaiThani and Kamphaengphet, three neighbouring provinces in Central Thailand.

Results from this study could bring the conclusion that the *C. claratris* collected from different geographical regions in Thailand are genetically closely related. Populations from different regions were clustered in the same group for example *C. claratris* collected from the North, Central, North-East and West were

clustered in cluster A. Therefore, it could be suggested that *C. claratris* populations in Thailand may have originated from the same place. In its evolutionary history, *C. claratris* was introduced to Thailand from abroad and may have dispersed forming new populations maintaining a high genetic similarity compared to the parental population. The two observed *COI* haplotypes may point to independent introductions. Furthermore, ecological differences may have an effect on the extent of gene flow between populations. Factors such as availability of alternative host plants, agricultural practicing, geographic and climatic conditions in each location may contribute to conservation or loss of genetic variability among *C. claratris* populations in Thailand. We feel that evaluation in this cluster analysis is ambiguous. Therefore population characterization by detecting the variation at the level of DNA sequences may be more reliable. The results obtained in this study should be confirmed by repeating PCR-SSCP analysis on additional loci in which more polymorphism can be generated leading to better distinctions. However, more polymorphisms can be generated by using more primers. Therefore, the clear differentiation among tomato thrips, *C. claratris* populations in Thailand could be overcome by using additional markers generated by more primers and more accessions from broader geographic range for genetic diversity estimation in this species of thrips.

Table 9 Clusters of *Ceratothripoides claratris* collected from different locations in Thailand**.**

Region	Province	Groups
Northern	Chiang Rai	A
	Chiang Mai	A,B,K
	Phayao	A
	Lamphun	A,K
	Lampang	A,K
Central	Nakhon Pathom	A,E
	Nakhon Sawan	A
	Phichit	A,E,L
	Kamphaeng Phet	A,E,M
	Uthai Thani	A,D,E,F,H,I,O
	Chai Nat	E,G,N
	Sing Buri	A,E
	Ang Thong	A,E
North-Eastern	Nong Khai	A
	Sakon Nakhon	A
	Mukdahan	A
	Amnat Charoen	A,C
	Ubon Ratchathani	A
	Buri Ram	A,E,J
	Nakhon Ratchasima	A,E
	Yasothon	A
Western	Ratchaburi	A
	Phetchaburi	A,E
	Prachuap Khiri Khan	A
	Kanchanaburi	A,E
	Tak	A

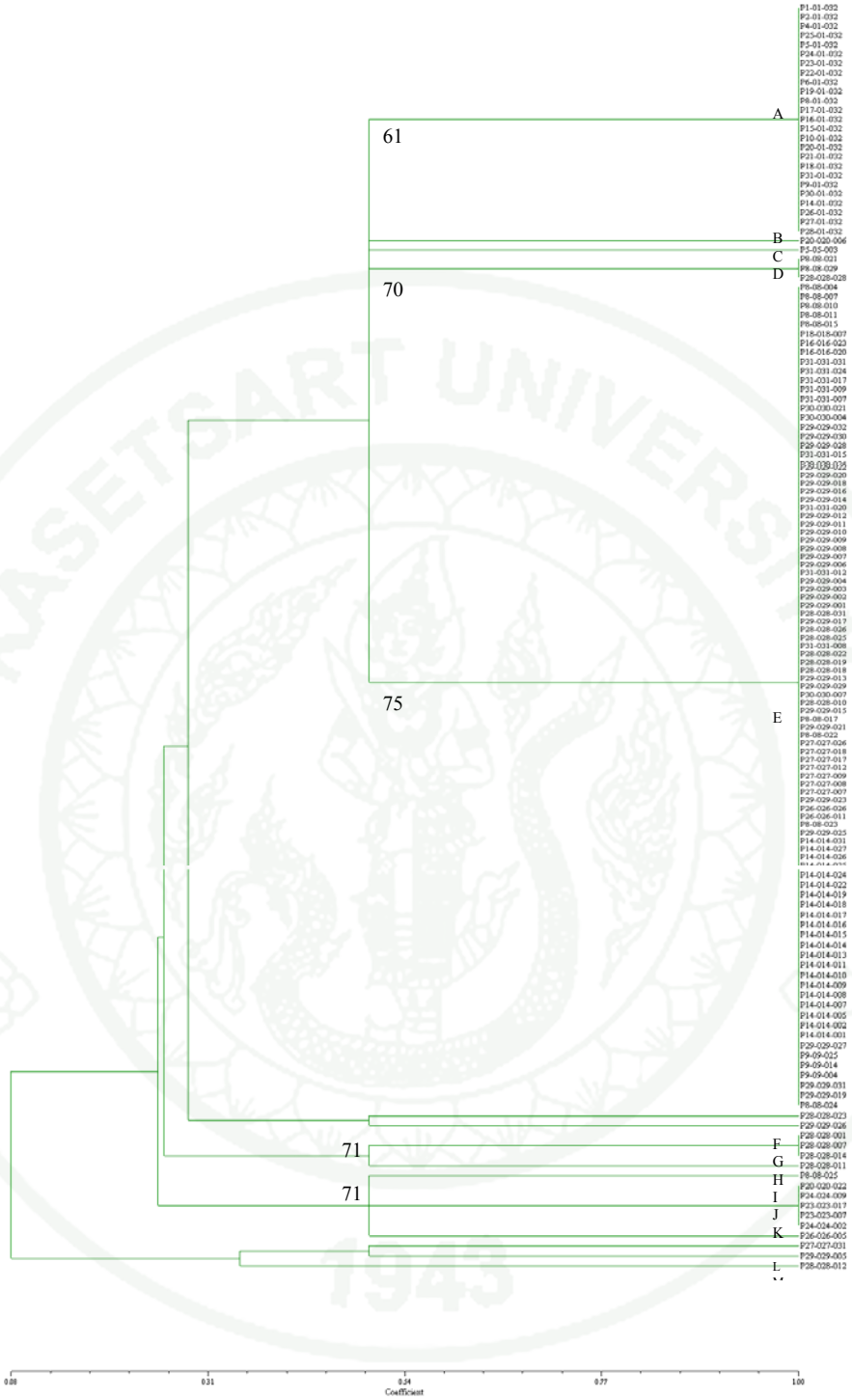

Figure 35 Phenetic dendrogram of genetic diversity of *Ceratothripoides. claratris* based on the binary matrix of polymorphic bands, using the UPGMA algorithm and Jaccard's similarity coefficient (NTSYS program). Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branch.

CONCLUSION

Ceratothripoides claratris (Shumsher) is a key pest attacking tomatoes and is the predominant thrips species on field and greenhouse-grown tomatoes in Thailand. Information on genetic variation in population is important for species and biotypes clarification which enable the developing of the effective and sustainable control strategy. This study is aimed at verifying the *Ceratothripoides claratris* diversity in Thailand by studying the genetic polymorphism among and within populations of this species of thrips using the highly efficient molecular techniques, PCR-SSCP analysis.

Although no sequence information was available for the particular target species, sufficient information on related species was stored in public databases allowing the design of PCR amplification primers. All nine primer sets (*COI*, *ATP6*, *NADH*, *PEPCK*, *EF1α*, *OPS*, *ADH*, *DDC* and *ARGK*) were designed corresponding to conserved sequence regions in other insect species. Seven genes namely: *COI*, *PEPCK*, *EF1α*, *OPS*, *ADH*, *DDC* and *ARGK* provided PCR amplified fragments of thrips genome. These PCR products were selected for cloning and sequencing. Only three genes *COI*, *EF1α* and *ARGK*, sequences were obtained and could successfully amplify a fragment of the thrips genome. The obtained sequences of these genes were used to design specific primers.

The three specific primer sets: CerCOI, CerEF1α, CerARGK were designed from obtained thrips genome sequences. The specific primer set for ITS gene was obtained from the report of Ruman-Jones *et al*. (2006). With PCR amplification, the specific primers amplified a single locus of *COI*, *EF1α*, *ARGK* and *ITS* genes from the *C. claratris* genome. When using SSCP analysis, polymorphism was detected in *COI*, *ARGK* and *ITS* genes except the *EF1α* gene. Even though the PCR-SSCP technique sometimes can reveal single nucleotide substitutions, this study revealed limited genetic polymorphisms among *C. claratris* populations collected from different locations in Thailand. Still, different alleles in each population were distinguished and counted.

Thrips, *Ceratothripoides claratris* populations in Thailand were analyzed by PCR-SSCP of *COI*, *EF1α*, *ARGK* and *ITS* genes. Results indicated that Thai thrips, *Ceratothripoides claratris* populations could be differentiated by the analysis of some genes using molecular technique which showed polymorphism within and among populations. This may suggest that thrips populations collected from different geographical regions of Thailand are more than one group.

The result of this study demonstrated the potential of the PCR-SSCP analysis for genetic diversity identification and this technique can be applied for estimating the genetic variability among closely related insects that are difficult to detect by other techniques. The approach of using PCR-SSCP analysis for population differentiation will be more clearly and accurate when using additional primers targeting mitochondrial and nuclear genes. This approach provides more information available for the study of genetic diversity of insects. Therefore, DNA based markers developed from mitochondrial and nuclear genes have the potential for use in population genetic studies in *Ceratothripoides claratris*.

Data from this study may form the basis for the future biotype identification of *Ceratothripoides claratris* populations in Thailand and thereby could provide useful information to broaden the scope of an effective and sustainable control strategy for *Ceratothripoides claratris*.

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APPENDICES

APPENDIX A

Buffers, reagents and medium

1. TE buffer

10 mM Tris HCl (pH 8.0)
1 mM Na_2-EDTA (pH 8.0)

2. TE buffer + RNase A

TE buffer 960 ml
RNase A (2 mg/ml) 40 µl
Store solution at 4C.

3. Solution for agarose gel electrophoresis

3.1 Tris-acetate (TAE)

1x TAE buffer was used as an electrophoresis buffer throughout the study. The working solution of 1x TAE buffer was prepared from stock solution of 50x TAE buffer, as follows:

Tris-base 242 g
Glacial acetic acid 57.5 ml
0.5 M EDTA (pH 8.0) 100 ml

The volume of the solution was adjusted to 1 litter with distilled water and sterilized by autoclaving for 15 min at 121 °C.

3.2 Tris-borate (TBE)

0.5x TBE buffer was used as an electrophoresis buffer throughout the study. The working solution of 0.5x TBE buffer was prepared from stock solution of 5x TAE buffer, as follows:

Tris-base 54.0 g
Boric acid 27.5 ml
0.5 M EDTA (pH 8.0) 20.0 ml

The volume of the solution was adjusted to 1 litter with distilled water and sterilized by autoclaving for 15 min at 121 °C.

3.3 Gel loading buffer

Bromophenol blue 0.025 g
Glycerol 3.0 ml

4. Ethidium bromide solution (10 mg/ml)

The ethidium bromide solution was prepared by dissolving 1 g of ethidium bromide in 100 ml of distilled water. The solution was stored in light-tight container at room temperature.

5. Formamide loading buffer

80%(w/v) deionized formamide
10 mM EDTA (pH 8.0)
1 mg/ml xylene cyanol FF
1 mg/ml bromophenol blue

6. Luria-Bertani medium (LB medium)

Tryptone 10.0 g
Yeast extract 5.0 g
NaCl 5.0 g

The pH was adjusted to 7.0 with 5N NaOH (~0.2 ml). The volume of the solution was adjusted to 1 liter with distilled water. Sterilization was achieved by autoclaving for 15 min at 121 °C. For agar plate, agar (15g/l) was added to the liquid media before autoclaving.

7. Ampicillin solution

Ampicillin stock solution (50 mg/ml) was prepared by dissolving 50 mg of ampicillin in 1 ml of distilled water, sterilizing by filtration through a 0.22 micron disposable filter. The solution was stored at 4 °C

APPENDIX B

Techniques

1. Agarose Gel Electrophoresis

The 1.5% agarose was prepared by adding 1.5 g of agarose powder to 100 ml of 1x TAE buffer. The slurry was heated in a microwave oven until the agarose was completely dissolved. The warm agarose solution was cooled to 45-55 °C and then poured into the mold which had a comb placed about 0.5-1.0 mm above the mold. The gel was between 3-5 mm thick and no air bubbles were allowed. The gel was allowed to set at room temperature for 35-45 min. After that the comb was removed and the gel was ready for samples loading. The gel was then placed in the electrophoresis tank and the 1x TAE buffer (Appendix A) was added to cover the gel to a depth of about 1 mm. The DNA samples were mixed with gel loading buffer in the ratio of 3: 1 (DNA: gel loading buffer) and added into the slots of the submerged gel. After that the electrical leads were attached to the electrophoresis tank and the voltage was applied. The DNA migrated toward the anode. The gel was run until the gel loading buffer has migrated to the appropriate distance through the gel. The gel was then stained in 0.5 mg/ml ethidium bromide (EtBr) solution and examined under an UV-transilluminator to visualize DNA bands.

2. Single-Strand Conformation Polymorphism (SSCP) Analysis

Sample preparation

1. 150-300 ng of amplified DNA (usually about 10% of the total PCR volume) can be loaded per well. Aliquot proper amount of sample into separate tubes and add an equal volume of 2 x SSCP gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA).

2. Prior to loading, denature the samples at 95 °C for 5 min and then place on ice.

Gel electrophoresis

Note: every primer set will require different conditions. Important variables includes run temperature (generally between 4 °C and room temp, although cooler is usually better; first testing 10 °C is recommended) and whether or not glycerol is added to the gel.

3. Silver Staining of DNA in Polyacrylamide Gels

Highly sensitive detection of nucleic acids in the nanogram range has been achieved by the specific chemical reduction of silver ions. The methods for silver-staining nucleic acids employ either a histologically derived procedure that uses ammoniacal solutions of silver or a photochemically derived reaction in which silver binds to nucleic acid bases and is then selectively reduced by chemical agents or light. These silver staining protocols can be as sensitive as radioisotopic methods. However, they are complex and time consuming and require the preparation and handling of several solutions. In an attempt to simplify the routine use of silver stains to detect nucleic acids, Bassam *et. al*. 1991 has optimized the photochemically derived silver stain originally introduced by Merril *et. al*. 1984 for protein staining and later applied to nucleic acids which uses formaldehyde to selectively reduce silver ions to metallic silver under alkaline conditions.

Several steps were omitted and others modified. Image development was in the presence of sodium thiosulfate as suggested by Blum *et. al* 1987 but instead using higher concentrations of formaldehyde. This procedure is highly sensitive, avoids unspecific background staining without loss of contrast, uses less silver and no oxidizing pretreatments, and stains complex mixtures of DNA resolved in polyacrylamide gels bound to polyester backing film. The originally suggested concentration of $AgNO_3$ (12 mM) can be halved provided the concentration of formaldehyde is increased at least two- to threefold during image development. Reduction of silver by formaldehyde is concentration dependent. Optimal band intensity occurs at about 0.00555% (by volume) formaldehyde. Thiosulfate dissolves

insoluble silver ions from the gel surface which in turn decreases nonspecific staining. A concentration of 4 μM thiosulfate is sufficient to reduce nonspecific background staining without noticeably affecting DNA image development.

Gels polymerized onto polyester backing films are handled easily during staining, and, when dried, produce a permanent record. However, the polyester film layer has the disadvantage of decreasing the surface area of the gel in contact with the liquid, slowing diffusion of solutes in and out the gel matrix. This lengthens the time required for image development during staining which can substantially increase background staining. The limit of detection upon visual inspection of double-stranded DNA was approximately 1 pg/ml. This is about 1,000 to 10,000 times more sensitive than ethidium-bromide staining.

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