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Molecular diversity of tomato thrips, *Ceratothripoides claratris* (Shumshur) (Thysanoptera: Thripidae) populations found in Thailand using PCR-SSCP

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The thrips mitochondrial gene Cytochrome Oxidase I (*COI*), the nuclear genes Elongation Factor1α (*EF1α*) and Arginine Kinase (*ARGK*) genes were amplified and cloned using degenerate primers designed based on sequences from other insect species. The obtained thrips DNA sequences of each gene were used to develop locus specific primers. The specific primer sets that were developed and the published primers for the internal transcribed spacer (ITS) could successfully amplify specific DNA fragments from *Ceratothripoides 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 0.75 of similarity coefficient with 0.98408 cophenetic correlation, using polymorphic bands pattern generated from 4 primer amplifications separated on polyacrylamide gel.

Key words: Ceratothripoides claratris, polymorphism, diversity, SSCP

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Introduction

Ceratothripoides claratris (Shumsher) is a major pest in tomato production 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). 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 a vector for tomato necrotic spot virus (TNSV) (Premachandra *et al*., 2004).

To date little information is available on the genetic diversity of thrips in Thailand. No molecular studies have assessed genetic diversity among populations of *C. claratris*. The study of genetic diversity is important for understanding species diversity, phylogenetic patterns and evolutionary processes. In pest species, this is also important for the development of the effective and sustainable control strategy. No biotype differentiation has been reported in *C. claratris*. Morphological characterization alone does not provide sufficient information to fully understand the genetic diversity within a species. At present a number of DNA-based molecular marker techniques are available and widely used to provide information about the diversity of genotypes in plants and animals such as RFLPs (restriction fragments length polymorphism) (Botstein et al., 1980), AFLPs (amplified fragments length polymorphism) (Vos *et al*., 1995), RAPDs (random amplified polymorphic DNA) (Williams *et al*., 1990; Kazan *et al*., 1993) and SSCP (single-strand conformation polymorphism) (Orita *et al*., 1989).

Single strand conformation polymorphism (SSCP) electrophoresis enables one to detect polymorphism in a DNA fragment due to as little as a single base substitution (Orita *et al*., 1989). The differentiation of DNA fragments of the same length differing in their sequences is based on a sequence dependent mobility shift of single-stranded DNA during electrophoresis. Examples of the use of SSCP analysis in taxonomic investigations have been reported. In most of these studies (Hiss *et al*., 1994; Tokue *et al*., 1995; Travis & Keim, 1995; Walsh *et al*., 1995; Stothard *et al*., 1998; Koekemoer *et al*., 1999) single gene loci were used, though multiple gene loci have also been investigated (Ohsako *et al*., 1996; Nakamura *et al*., 1998). The objectives of this study were 1) to develop specific primers for tomato thrips, *C. claratris* and 2) to study genetic diversity and determining genetic relationships among and

within *C. claratris* populations in Thailand, using Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP).

Materials and methods

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 (Table1) making a total of 832 samples. The adult thrips were examined and morphologically identified under a stereo microscope. All samples were stored in 70% ethanol at -20 ºC until use.

DNA Extraction

DNA was extracted by crushing individual adult thrips 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. Centrifugation and transfer of supernatants to new tube.

Design of degenerate primers

PCR primer pairs for Cytochrome oxidase I (*COI*), Elongation factor1α (*EF1α*) and Arginine kinase (*ARGK*) genes were designed based on information of DNA sequences obtained from other insect species. Sequences from different insect species were retrieved from publicly accessible DNA databases (GeneBank) by keyword and BLAST searching. The sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalW/index.html). The resulting alignments were improved by visual inspection using the GeneDoc program.

The degenerated primer pairs were used to amplify the target genes. 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. After amplification, the reaction products were electrophoresed on 1% agarose gels. The reproducible PCRs were selected for cloning using the pGEM -T Easy

vector (Promega, USA). Individual clones were picked, and plasmid DNA was purified and sent for sequencing.

Table 1. Sites of collection of tomato thrips, *Ceratothripoides claratris* in different regions of Thailand.

Site of collection Region	Province	Accession no.[1/]
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
	PrachuapKhiri 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.

Design of specific primers

The DNA sequences obtained from the cloned fragments were integrated in the alignments containing the sequences of other insect species. The intron-

exon boundaries (GT-AG) were determined in the *C. claratris* sequences. The conserved region of *C. claratris* sequences were chosen to design specific primers. In addition, the published specific primer pair (Ruman-Jones *et al*., 2006) for the amplification of the internal transcribed spacer (ITS) gene was also used for PCR-SSCP analysis.

PCR-SSCP

To detect polymorphism, genomic DNA samples of the collected *C. claratris* were amplified by using the specific primer pairs in 96-well plates. A 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 amplified products from four specific primers were analyzed using the SSCP method. 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). 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 length of running time depended on the size of the DNA fragment being analyzed (Table 2). The SSCP patterns were visualized by silver staining.

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).

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

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

Results and discussion

Design of degenerate primers

Sequences coding for COI, EF1α and ARGK genes representing various insect species were obtained from GenBank databases. The retrieved sequences consisted of partial and complete cDNAs, EST and genomic DNA fragments. The sequence alignment allowed the design of primer set of general usability among insects. The primer sequences of each gene are shown in Table 3.

The PCR reactions using COI, EF1α and ARGK primer set amplified a fragment in all collected thrips populations, producing a single band on agarose gel. Two sequences from eight positive clones of COI gene, four sequences from seven positive clones of EF1*α* gene and three sequences from thirty-one positive clones of ARGK gene, were obtained. 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. After alignment with COI gene from other insect species, the two obtained COI gene sequences were both found to be 529 bp in length without intron. The four obtained EF1*α* gene sequences 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 were 327, 409 and 455 bp in length including one intron of size 81 bp.

Design of specific primers

Based on the obtained sequences of COI, EF1α and 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 COI sequence. The designed ARGK specific primer for *C. claratris* had one intron. The designed EF1α specific primer for *C. claratris* had two introns. The sequences of the *C. claratris* specific primer that were designed from sequences and the published primer of ITS gene are shown in Table 4.

Table 3. Sequences of degenerate primers.

Gene name	Degenerate Primer (5'→3')
Cytochrome oxidase I(COI)	Forward: CCT ATT ATA ATT GGA GGA TTY GG Reverse: GAG GCA AGA TTA AAA TAT AAA CTT CTG
Elongation factor1α (EF1α)	Forward: AAG ATG CCC TGG TTC AAG GGN TGG Reverse: CCG TTG GAG ATC TGR CCA GGR TG
Arginine kinase (Argk)	Forward: GAT CCC ATC ATT GAR GAY TAY CA Reverse: TCA CCC TCC TTG AAC AGR AAR TG

H=(A/C/T), N=(A/G/C/T), R=(A/G), Y=(C/T)

Table 4. Sequence of the specific primers.

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

PCR-SSCP

The specific primers of *COI, EF1α, ARGK* and ITS genes successfully amplified a fragment from all *C. claratris* populations. On agarose gel, a single band of approximate 550, 400, 300 and 500 bp were visible respectively. Different alleles could not be distinguished. However, using SSCP, several alleles could be identified in all populations.

The PCR-SSCP analysis indicated that all of the amplified *EF1α* gene fragments gave an identical band pattern. Therefore this gene cannot 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, five alleles were observed. Three different SSCP patterns were identified for the ITS fragment and seven different SSCP patterns for the *ARGK* locus.

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.

Even though the SSCP-PCR 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 could be distinguished and counted. This may suggest that thrips populations collected from different geographical regions of Thailand are more than one group. This approach provides more information available for the study of genetic diversity of insects.

Cluster analysis

A total 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 (Fig. 1). Cluster A contained the most common genotype including most of the accessions from Northern, Central, North-Eastern and Western regions. Cluster B and K included only Northern region. Cluster C and J

included only North-Eastern region. Cluster D, E, F, G, H, I, L, M, N and O included only Central region. Cluster 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 5.

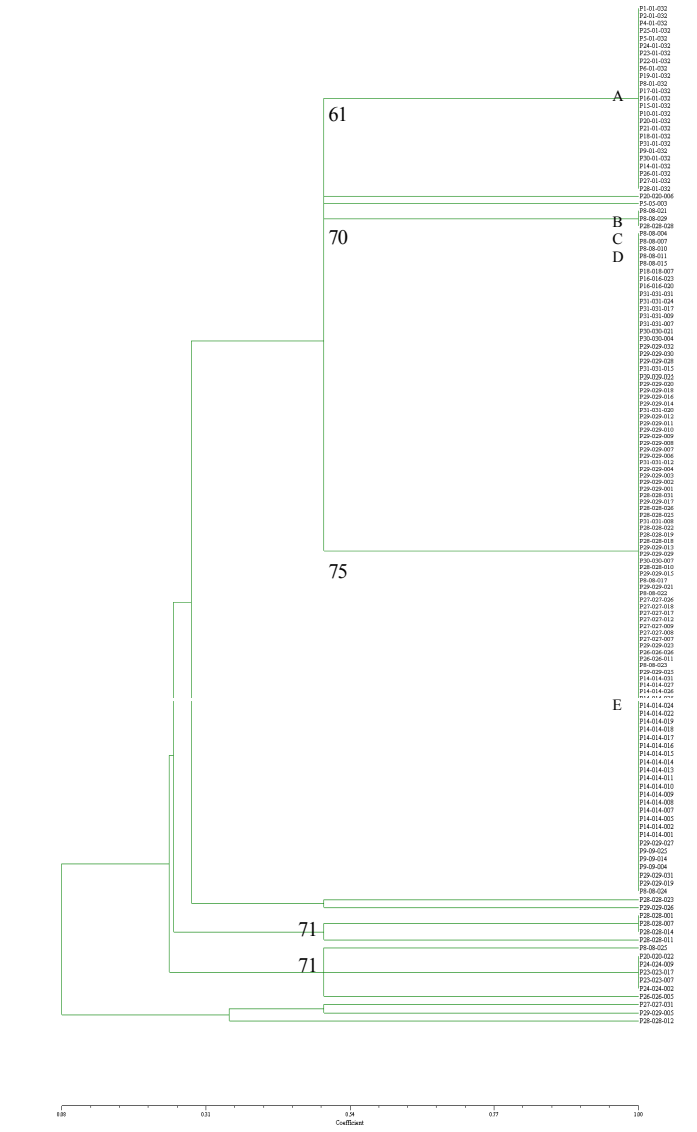

Fig. 1. Phenetic dendrogram of genetic diversity of *Ceratothripoides claratris* based on the binary matrix of polymorphic bands, using the UPGMA algorithm and Jaccard's similarity coefficient (NTSYS program). Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branch.

Table 5. Clustered of *Ceratothripoides claratris* collected from different locations in Thailand.

Groups	Province
A	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
B	Chiang Mai
C	Amnat Charoen
D	Buri Ram and Uthai Thani
E	Buri Ram, Kanchanaburi, Phetchaburi, Ang Thong, Sing Buri, Chai Nat, Uthai Thani, Kamphaeng Phet, Phichit, Nakhon Pathom and Nakhon Ratchasima
F	Uthai Thani
G	Chai Nat
H	Uthai Thani
I	Uthai Thani
J	Buri Ram
K	Chiang Mai, Lampang and Lamphun
L	Phichit
M	Kamphaeng Phet
N	Chai Nat
O	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.

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. 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.

Therefore, DNA based markers developed from nuclear and mitochondrial genes have the potential for use in population genetic studies in *C. claratris* and our data may form the basis for the future biotype identification of *C. claratris* populations in Thailand and thereby could provide useful information to broaden the scope of an effective and sustainable control strategy for *C. claratris*.

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