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GENETIC VARIATION IN THE BEE MITE *Tropilaelaps* spp. REVEALED BY SEQUENCING THE ITS REGION AND BY RAPD ANALYSIS.

Miss Warisa Tangjingjai

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ความหลากหลายทางสายพันธุ์ของไรผึ้งในจีนัส *Tropilaelaps* ซึ่งมี 2 สปีชีส์ คือ *T. clareae* และ *T. koenigerum* ในประเทศไทยได้ถูกตรวจสอบด้วยการหาลำดับเบสตรงบริเวณ อินเทอร์นอลทรานสไครบสเปเซอร์ (ITS) ของนิวเคลียร์ไรโบโซมัลดีเอ็นเอ (nrDNA) และการใช้เทคนิค PCR-RAPD จากการหาลำดับเบสของ ITS ที่เพิ่มปริมาณโดย PCR ของตัวอย่างไรผึ้งทั้งสองสปีชีส์ซึ่งอยู่ตามภูมิภาคต่างๆ โดยในไร *T. clareae* ได้หาลำดับเบสของ ITS จากไร 20 ตัวอย่างซึ่งมีความยาวของสายนิวคลีโอไทด์เท่ากับ 519 เบส ส่วนในไร *T. koenigerum* ได้หาลำดับเบสของ ITS จากไร 5 ตัวอย่างซึ่งมีความยาวของนิวคลีโอไทด์เท่ากับ 520 เบส พบว่าไรที่อยู่ในสปีชีส์เดียวกันไม่มีความแตกต่างของลำดับเบสดังกล่าว แต่เมื่อนำลำดับเบสของ ITS จากไร *T. claerae* มาเรียงเปรียบเทียบกับลำดับที่ได้จากไร *T. koenigerum* พบว่ามีความแตกต่างกันของลำดับเบส โดยมี point mutation เกิดขึ้น 19 ตำแหน่ง นอกจากนี้ยังมี gap อีก 7 ตำแหน่งที่เกิดจากการลดหรือการเพิ่ม และจากการใช้โปรแกรม Kimura' s two parameter วิเคราะห์ข้อมูลของการเปรียบเทียบลำดับเบสระหว่างไรทั้งสองสปีชีส์ พบว่ามีความแตกต่างของลำดับเบสเท่ากับ 3.79 % นอกจากนี้พบว่าลำดับเบสของ ITS ของไร *T. koenigerum* เท่านั้นที่มีนิวคลีโอไทด์จำนวน 5 เบสเพิ่มเข้ามา และในไร *T. clareae* มีบริเวณตัดจำเพาะของเอนไซม์ *Rsa*I 2 ตำแหน่งในขณะที่ในไร *T. koenigerum* มีบริเวณตัดจำเพาะของเอนไซม์ *Rsa*I เพียง 1 ตำแหน่ง จากข้อมูลนี้สามารถนำไปใช้เป็นวิธีแยกไรสองสปีชีส์ออกจากกันได้

จากการวิเคราะห์ด้วยเทคนิค PCR-RAPD ใน 16 กลุ่มตัวอย่าง ของไร *T. clareae* ซึ่งแบ่งเป็นไรปรสิตในผึ้งหลวง (*A. dorsata*) 8 กลุ่ม และปรสิตในผึ้งพันธุ์ (*A. mellifera*) 8 กลุ่ม จากแหล่งต่าง ๆ และ 2 กลุ่มตัวอย่างของไร *T. koenigerum* จากสมุทรสาครและจันทบุรี โดยเลือกใช้ 3 primer คือ OPA07, OPA11 และ OPA12 ซึ่งจะให้รูปแบบของแถบดีเอ็นเอจำนวน 60, 58 และ 35 รูปแบบตามลำดับ เมื่อคำนวณค่าเฉลี่ยของ genetic distance ที่ได้จาก 3 primer แล้วนำมาสร้างความสัมพันธ์เชิงวิวัฒนาการโดยวิธี UPGMA สามารถแยกไร 2 สปีชีส์ออกจากกันได้อย่างชัดเจน ในขณะเดียวกันสามารถแยกไร *T. clareae* ได้เป็น 2 กลุ่ม โดยแบ่งเป็นกลุ่มไรปรสิตในผึ้งพันธุ์ และ 2 ตัวอย่างที่เป็นปรสิตในผึ้งหลวง (E2D และ N2D) ส่วนอีกกลุ่มหนึ่งเป็นปรสิตในผึ้งหลวงทั้งหมด

ภาควิชา .....

สาขาวิชา หลักสูตรเทคโนโลยีทางชีวภาพ

ปีการศึกษา 2541

ลายมือชื่อนิสิต .....

ลายมือชื่ออาจารย์ที่ปรึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

C827322 : MAJOR BIOTECHNOLOGY
KEY WORD: : *Tropilaelaps* spp. , RAPD-PCR/GENETIC VARIATION/ ITS SEQUENCING

WARISA TANGJINGJAI: GENETIC VARIATION IN THE BEE MITE ,*Tropilaelaps* spp. REVEALED BY SEQUENCING THE ITS REGION AND BY RAPD ANALYSIS.
THESIS ADVISOR: ASST. PROF. PATCHARA VERAKALASA Ph.D.
THESIS CO-ADVISOR: ASSOC. PROF. CHARIYA LEKPRAYOON
ASSOC. PROF. SIRIPORN SITIPRANEED Ph.D. 116 pp. ISBN 974-639-716-8

Level of genetic variation of sibling mite species, *Tropilaelaps clareae* and *T. koenigerum* in Thailand was investigated by sequencing of PCR-amplified internal transcribed spacer (ITS) region of nuclear ribosomal DNA and RAPD-PCR analysis.

No sequence polymorphisms in the amplified ITS region were observed among twenty individuals of *T. clareae* and among five representatives of *T. koenigerum*. At an interspecific level, nineteen point mutations constituting of ten transitions and nine transversions were found. Moreover, seven gaps resulted from insertions/deletions were observed. The estimated sequence divergence between *T. clareae* and *T. koenigerum* was 3.79 %. A 5 bp insertion found in *T. clareae* could be used to dissociate this species from *T. koenigerum*. Nevertheless, restriction analysis of an amplified ITS region with *Rsa* I can also rapidly detected this interspecific variation.

RAPD analysis of one hundred and twenty-five of *T. clareae* (16 samples) and sixteen individuals of *T. koenigerum* (2 samples) using primers OPA07, OPA11 and OPA12 indicated high genetic polymorphisms in these two species. A total of 153 genotypes was found from all three RAPD primers (60, 58, and 35 patterns from OPA7, OPA11, and OPA12, respectively). The genetic distance among pairs of *T. clareae* samples was 0.081-0.2314 while that of *T. koenigerum* was 0.0289. Using RAPD analysis, the genetic distance between these species was estimated to be 0.8464. No evidences of interspecific hybridization were observed.

A UPGMA phylogeny indicated large distance between *T. clareae* and *T. koenigerum* and monophyletic status of both taxa. For *T. clareae*, all investigated samples could be allocated into two different groups, consisting of that contained all *T. clareae* from *A. mellifera* host and two samples from *A. dorsata* (E2D and N1D) and another group consisted of all remaining *T. clareae* from *A. dorsata* host.

ภาควิชา..... ลายมือชื่อนิสิต.....

สาขาวิชา หลักสูตรเทคโนโลยีทางชีวภาพ ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2541 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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LIST OF ABBREVIATIONS

A, T, G, C	=	nucleotide containing the bases adenine, thymine, guanine, and cytosine, respectively
bp	=	base pair
°C	=	degree Celsius
cm	=	centimetre
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleoside triphosphates (dATP,dTTP, dGTP, dCTP)
ddNTPs	=	dideoxyribonucleoside triphosphates (ddATP,ddTTP, ddGTP, ddCTP)
EDTA	=	ethylenediamine tetra acetic acid
HC1	=	hydrochloric acid
kb	=	kilobase
KC1	=	potassium cholride
1rRNA	=	large subunit ribosoma1 RNA
$MgC1_2$	=	magnesium chloride
ml	=	millilitre
mM	=	millimolar
mt DNA	=	mitochondrial DNA
ng	=	nanogram
PCR	=	polymerase chain reaction
RFLP	=	restriction fragment length polymorphism
SDS	=	sodium dodecyl sulfate
srRNA	=	small subunit ribosomal RNA

TEMED = N,N,N′,N′-tetramethylethylenediamine

Tris = tris(hydroxy methyl) aminomethane

μg = microgram

μl = microlitre

μM = micromolar

UV = ultraviolet

V = volt

W = watt

CHAPTER I

Introduction

1.1 Biology of Bee Mites

All bee mites are large obligate external parasites fed and reproduced in the honey bee brood, principally during late larval and pupal stages of the hosts inside the sealed brood cells. Newly developed adults, along with the maternal mites, leave the cells with the emerging bees. Although the hosts survive, they are weaker leading to their shorter life span (De Jong, 1990).

Several honey bee associated mite species are found in Asia. These are composed of members of Varroidae including *Varroa jacobsoni, V. underwoodi, Euvarroa sinhai* and *E. wongsirii*, and Laelapidae including *Tropilaelaps clareae* and *T. koenigerum*. At present, these parasites associate with six *Apis* taxa (Table 1.1). Although horizontal infection of these mites can occurred in the European honey bee (*A. mellifera*), more host/parasite specific infection was observed in the native honey bee species (*A. dorsata, A. cerana, A. koshevnikovi, A. mellifera, A. florea, A. andreniformis* and *A. laboriosa*) (Wongsiri et al., 1995).

Among all bee mites, *V. jacobsoni* and *T. clareae* are possibly the most important parasitic taxa as they are more destructive than others. The values loss from these parasitic species are significant to beekeepers, as they severely attack *A. mellifera* brood.

Table 1.1 Honey bee mites and host associations.

Mite Species	Host Species	Host Ecology & Behavior
Varroidae		
V. jacobsoni	*Apis cerana* *A. koschevnikovi* *A. mellifera*	Construct multi-comb nests inside cavities in trees or caves. *A. cerana* can be kept in man-made hives; colonies are smaller in size; has distinct shorter drone production seasons
V. underwoodi	*A. cerana*	
E. sinhai *E. wongsirii*	*A. florea* *A. andreniformis*	Build single-comb nests attached to small tree branches or twigs in dense vegetation; small colonies; frequently short distance vegetation and migration; high swarming rates; drone cells larger than workers.
Laelapidae		
T. clareae	*A. dorsata* *A. laboriosa* (*A. mellifera*)	Build single-comb nests in the open; combs suspended under large branches of tall trees, roofs of buildings, or rock overhangs, large colonies, obligatory seasonal migration over long distance.
T. koenigerum	*A. dorsata* *A. laboriosa*	

(Delfinado-Baker and Peng, 1995)

Tropilaelaps is an old world tropical genus of the family Laelapidae. It is proposed that *T. clareae* and *T. koenigerum*, have evolved from their presumbly ancestor within the Hypoaspidinae and become parasites of the honey bee brood (Eickwork, 1988). Previously, biology of *T. clareae* was not well understood. After *A. mellifera* was introduced to Asia for commercial honey production purposes, it was found that *T. clareae* could also infect this honey bee species. Unfortunately, *A. mellifera* is highly susceptible to *T. clareae* resulting in a significant loss of the honey production (De Jong, 1990).

Therefore, *T. clareae* is one of the most serious pest of cultured honey bee in Asia (Burgett, Akratanakul and Morse, 1983; Delfinado-Baker, Underwood and Baker, 1985). This parasite was firstly described in the Phillipines by Delfinado and Baker (1961). It firmly attached to dead larvae, pupae and adults of *A. mellifera* (Bhardwaj, 1968; Krantz and Kitprasert, 1990). Subsequently, it was also found to be a parasite on *A. dorsata* (Bharadwaj, 1968) and found in *A. mellifera* only in the geographic areas where *A. dorsata* is overlapping distributed to. Based on the basic information that *T. clareae* distributes in tropical and subtropical areas, *A. dorsata* rather than *A. mellifera* should be a native host for this parasite (Burgett, Akratanakul and Morse, 1983; Kumar, Kumar and Bhala, 1993). Interestingly, *T. clareae* is more harmful to the exotic species like *A. mellifera* than the native host, *A. dorsata* (Eickwork, 1988).

Tropilaelaps spp., can be morphologically classified as follows, (Krantz, 1978).

Phylum Arthropoda

Class Arachnida

Subclass Acari

Order Parasitiformes

Suborder Gamasida

Family Laelapidae

Genus *Tropilaelaps*

species *clareae*

koenigerum

Scientific name : *Tropilaelaps clareae* Delfinado and Baker, 1961

Tropilaelaps koenigerum Delfinado-Baker and Baker, 1982

Morphologically, the external features of the respiratory system or more specifically, the peritremes and stigmata, have been taxonomically used for classification and systematics in the subclass Acari (Krantz, 1978). Thus, the middle position of the stigmata, which dorsolaterally open near coxae III or IV (Fig 1.1), is called gamasid which is the morphologically specific markers for members of suborder Gamasida. In most gamasid mites, each stigma has an elongated peritreme formed by sclerotized groove usually extends forward along

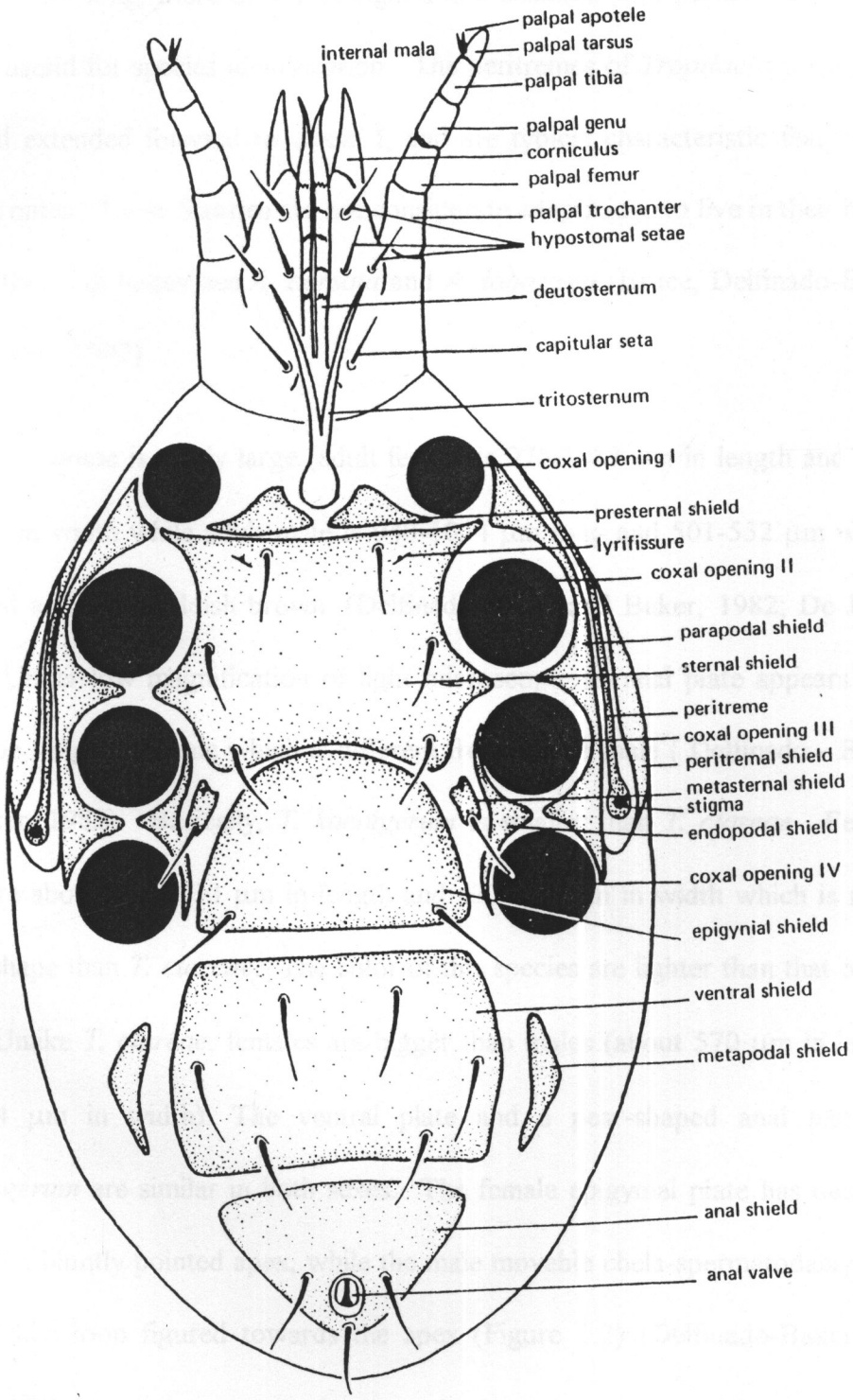

Fig. 1.1 General morphology of gamasid mites (ventral view) after Kantz, 1978

the lateral margin and occasionally reaches to or slightly beyond coxae I. The peritremes are long, more or less straight and associated with peritrematic shields that are useful for species identification. The peritremes of *Tropilaelaps* spp., are long and extended forward to coxae I, and are typical characteristic features of gamasid mites. These features are not considerate adaptations to live in their hosts, such as the giant honey bee *A. dorsata* and *A. laboriosa* (Bruce, Delfinado-Baker and Vincent, 1997).

T. clareae is fairly large (adult female is 976-1083 μm in length and 528-581 μm in width while adult male is 940-1054 μm long and 501-552 μm wide), elongated and light-reddish brown. (Delfinado-Baker and Baker, 1982; De Jong, 1990). Under low magnification of light microscope, an anal plate appears as a horseshoe shape in female which is different from that in male (Delfinado - Baker and Baker, 1982). Basically, *T. koenigerum* is smaller than *T. clareae*. Female adults are about 684-713 μm in length and 433-436 μm in width which is more oval in shape than *T. clareae*. The color of this species are lighter than that of the other. Unlike *T. clareae*, females are bigger than males (about 570 μm in length and 364 μm in width). The ventral plate and a pear-shaped anal plate of *T. koenigerum* are similar in both sexes. The female epigynial plate has declivus sides and a bluntly pointed apex, while the male movable chela-spermatodactyl has a pigtail like loop figured towards the apex (Figure 1.2) (Delfinado-Baker and Baker, 1982).

The data concerning life cycle of *T. clareae* is only available on *A. mellifera* host and are unfortunately incomplete. Although no data for

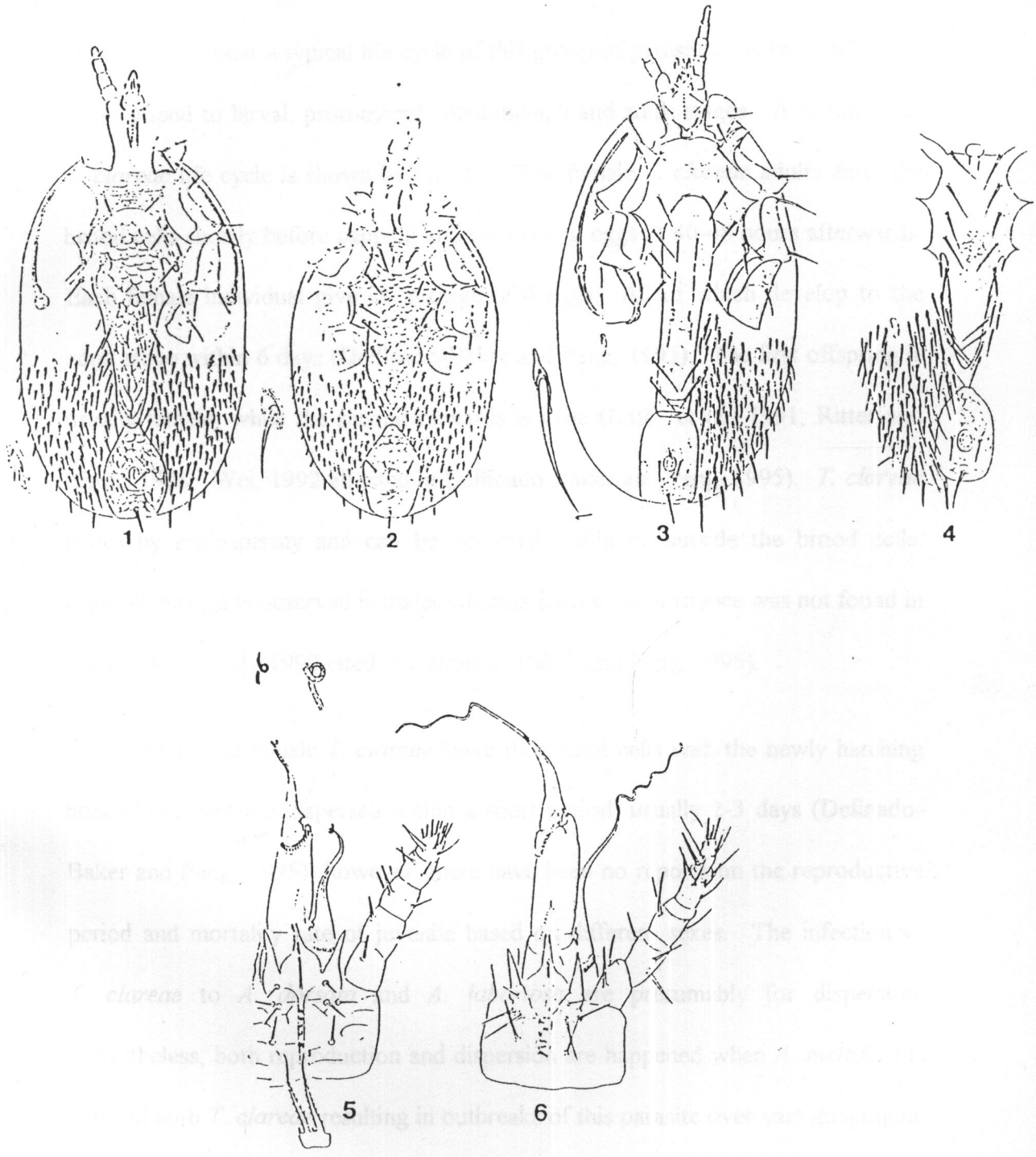

Fig 1.2 *T. koenigerum* :(1) female venter, (2) male venter, (5) male gnathosoma and close-up of movable chela-spermatodactyl ; *T. clareae* : (3) female venter, (4) male venter, (6) male gnathosoma showing movable chela-spermatodactyl (Delfinado-Baker and Baker, 1982).

developmental biology of male *T. clareae* has been reported, the female mites of this species exhibit a typical life cycle of this group of parasites. After hatching, it is developed to larval, protonymph, deutonymph and adult stages. A summary of *T. clareae* life cycle is shown by Fig. 1.3. The female *T. clareae* adults enter the brood cells shortly before capping and start laying eggs at 40-48 hours afterwards. Each female individual give an average of 4 eggs. All of which develop to the adult stage within 6 days (Delfinado-Baker and Peng, 1995). The first offspring is usually female, while the second onwards is male (Rath et al., 1991; Ritter and Ritter, 1988; Wei, 1992 all cited in Delfinado-Baker and Peng, 1995). *T. clareae* mates by podospermy and can be occurred inside or outside the brood cells. Multiple mating is observed in males whereas such a circumstance was not found in female (Rath et al., 1990 cited in Delfinado-Baker and Peng, 1995).

Male and female *T. clareae* leave their natal cells with the newly hatching honey bees, and are dispersed within a short period, usually 2-3 days (Definado-Baker and Peng, 1995); however, there have been no reports on the reproductive period and mortality rate of juvenile based on different sexes. The infection of *T. clareae* to *A. dorsata* and *A. laboriosa* are presumably for dispersion. Nevertheless, both reproduction and dispersion are happened when *A. mellifera* is infected with *T. clareae* resulting in outbreaks of this parasite over vast geographic areas (Wongsiri et al., 1995; Delfinado-Baker, Rath and Boecking, 1992). In *T. koenigerum* there have been no reports on the life cycle of this taxon.

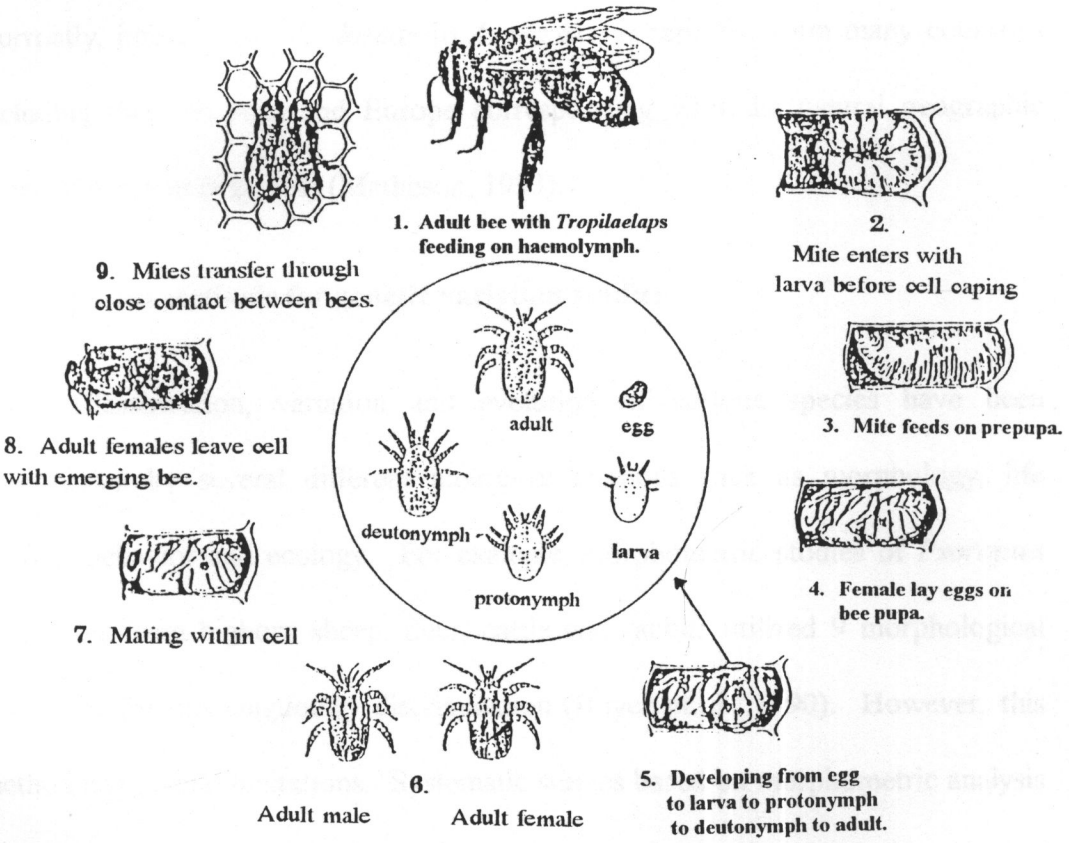

Fig 1.3 Life cycle of *T. clareae* (Wongsiri, 1989).

Distribution of *T. clareae* is associated with that of its natural host, *A. dorsata*. Historically, repeated introductions of *A. mellifera* to Asia have allowed consistently contact between these bee species. In 1961, it was reported that *A. mellifera* in the Philippines were seriously infested by *T. clareae*. Currently, infestion of *T. clareae* to *A. dorsata* is reported from many countries including those in Asia and Europe corresponding with the natural geographic range of the host (Fig. 1.4) (Matheson, 1993).

1.2 Classical methods for genetic variation studies

Classification, variation and evolution of various species have been investigated by several different biological methods such as morphology, life history, behavior and ecology. For example, morphometric studies of *Psoroptes* spp. mites from bighorn sheep, deer, cattle and rabbits utilized 9 morphological characters for unambiguously discrimination (Boyce et al., 1990). However, this method has several limitations. Systematic studies based on morphometric analysis require a large number of samples and experienced scientists to decide whether investigated characters are informative (Rinderer, 1986). More importantly, some morphometric characters are often environmentally influenced therefore populations of a particular species may be misclassified due to ecological variants. Accordingly, the classical method has been increasingly confirmed by molecular techniques, based on protein or DNA polymorphisms (Weising et al., 1995).

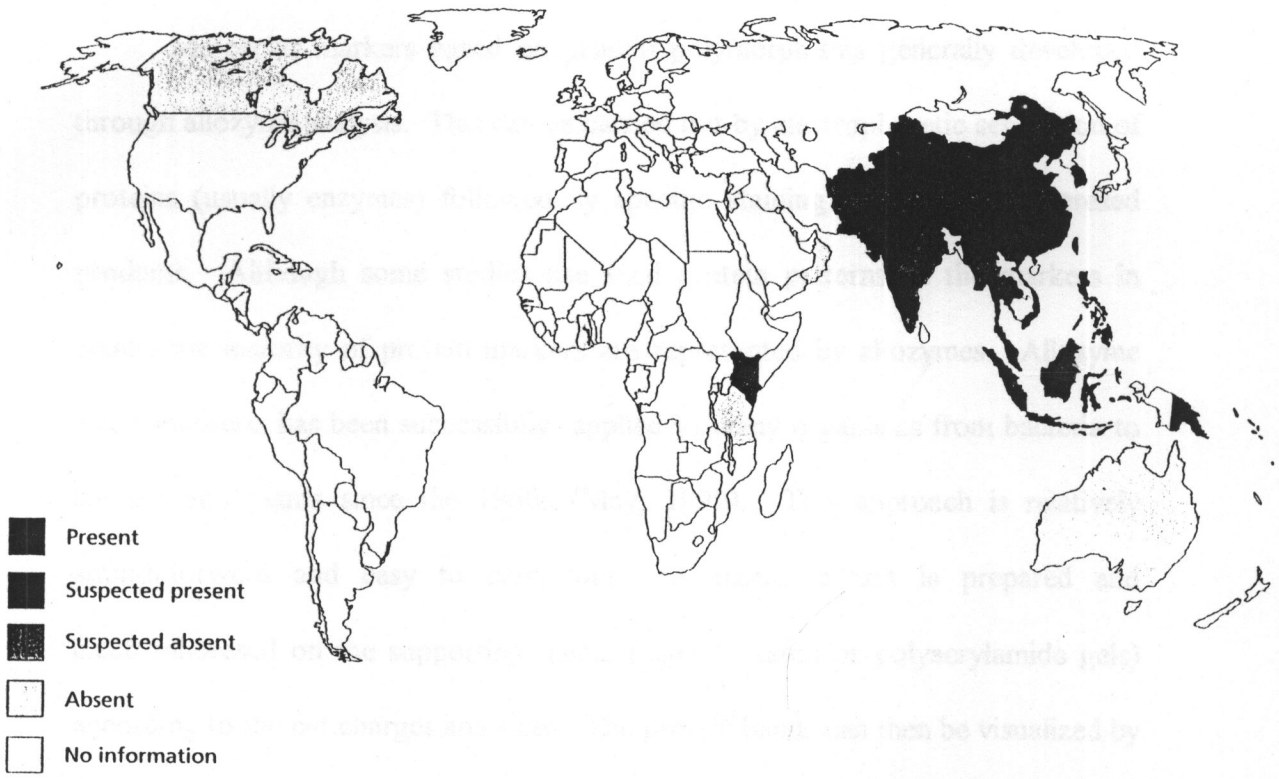

Fig. 1.4 Worldwide distribution of *Tropilaelaps* (Matheson, 1993).

1.3 Molecular techniques for genetic variation studies

1.3.1 Allozyme technique

Molecular markers based on protein polymorphisms generally developed through allozyme analysis. This can be carried out by electrophoretic separation of proteins (usually enzymes) followed by specific staining of the electrophoresed products. Although some studies use seed protein patterns as the markers in plants, the majority of protein markers are represented by allozymes. Allozyme electrophoresis has been successfully applied to many organisms from bacteria to animals and plants since the 1960s (May, 1992). This approach is relatively straightforward and easy to carry out. A tissue extract is prepared and electrophoresed on the supporting media (usually starch or polyacrylamide gels) according to the net charges and sizes. The protein bands can then be visualized by specific histochemical stains of investigated enzymes. Once the electrophoresed gel are stained, the status of homo- or heterozygosity at such a locus can be examined. The number of band is reflected from configuration of the enzyme molecule (mono, di or tetramers coupling with homo or heterozygotic states). The positions of polymorphic bands are genetically informative (Weising et al., 1995).

Analysis of protein polymorphisms in mites have been mainly reported in studies of inter- and intraspecific levels. For example, genetic divergence between the green and red forms of the two-spotted spider mite was examined using malate dehydrogenase (*MDH**) and further analyzed by polyacrylaminde gel electrophoresis. The *MDH** specific allele for each forms of the mites was identified (Goka et al., 1996). Theoretically, the allozyme approach is a

reasonably powerful technique as a large number of individuals can be determined in a limited period of time. However, it has some limitations. For instance, synonymous mutation is not able to be detected. Likewise, nucleotide substitutions changing one non-polar amino acid to another do not alter the electrophoretic mobility of the protein. Lacks of allozyme variability (17 gene loci, from 14 allozyme systems) among populations of *V. jacobsoni* composing of 12 apiaries from European countries and one from China were reported (Biasiolo, 1992). As described previously, allozymes underestimate levels of genetic variation due to its low ability to detect polymorphic loci. Therefore, allozyme analysis may not be an appropriate technique for evaluation of genetic variability in *T. clareae* and *T. koenigerum*.

1.3.2 DNA-based techniques

Analysis of polymorphisms at the DNA level is the direct approach to study genetic variation at both inter- and intraspecific levels. Theoretically, various DNA-based techniques, having different sensitivity of detection are available but the most important factor is to select the most appropriate technique (e.g. reasonable sensitivity, cost-effective, less time consuming) to answer a particular problem.

Nuclear DNA, mitochondrial DNA, and nuclear ribosomal DNA have been commonly employed in genetic variation studies. The following discussion describes some of the attributes of mitochondrial and nuclear ribosomal DNA.

A. Mitochondrial DNA

Genetic polymorphisms using analysis of mitochondrial DNA (mtDNA) have been employed. Since 1979 in most cases, it is more powerful than that of allozymes for determination of population structure, biogeogrophy and phylogenetic relationships (Avise et al., 1987). Due mainly to its small genome size, rapid rate of evolutionary changes and maternal inheritance, mtDNA is also suitable for examining history and evolution among closely related taxa (Gray, 1989; Lansman et al., 1981; Simon et al., 1991 all cited in Hoy, 1994). Studies of mtDNA polymorphisms have been reported using RFLP and/or DNA sequencing (Navajas et al, 1996). The former can be carried out from the entire mtDNA or alternatively, from PCR-amplified mtDNA segments followed by restriction analysis of such amplification band . The latter is suitable for small fragments which can be amplified by DNA cloning or by PCR followed by DNA sequencing (Satta and Tukahara 1996; Pashley and Ke 1992 all cited in Hoy, 1994).

mtDNA has a number of positive properties which is suitable for evolutionary and systematic studies. These are (1) maternal inheritance, (2) general conservation of gene order and composition within the same phylum, (3) a rapid rate of sequence divergence, and (4) small size and abundance resulting in its easy isolation (Hoy, 1994). Analysis of mtDNA in various taxa causes better understanding of this extrachromosomal DNA. It was subsequently found that the universal genetic code system is not valid for some genetic codes. Navajas et al. (1996) investigated genetic variation of phytophagous mites using sequence analysis of cytochrome oxidase subunit I (CO I, 340 bp) in twenty phytophagous

mite species. High genetic variation levels of this A+T rich region were observed which occurred through synonymous transitions. Moreover, it was also found that the genetic codes in phylophagous mites were apparently similar to those in insects.

B. Nuclear ribosomal DNA.

Ribosomes are a major component of cells involving in translation of messenger RNAs into proteins. Ribosomes consist of ribosomal RNA (rRNA) and proteins and can be dissociated into a large and a small subunits. The ribosomal DNA is frequently used to examine interspecifically evolutionary relationships among various taxa because they are universally present in all organisms having the protein synthesizing system. The rDNA is versatile to be used for detection of polymorphisms at different levels because this moderately repettitive region contains both conserved (e.g. 18S and 28S) and more variable regions (e.g. ITS, IGS).

In eukaryotes, the nuclear ribosomal genes encoding the 18S (small subunit) and 28S (large subunit) rRNAs are clustered as arrays of tandem repeats located in the nucleolar organizing regions of the chromosomes (Fig. 1.5). There are approximately 100 to 500 copies of rDNA repeated transcription units found in most animals (Hoy, 1994). The repeated transcription unit is composed of part of the promoter region, external transcribed spacer (ETS), an 18S rDNA coding region, an internal noncoding transcribed spacer (ITS), a 28S rRNA coding region, and an intergenic nontranscribed spacer (IGS).

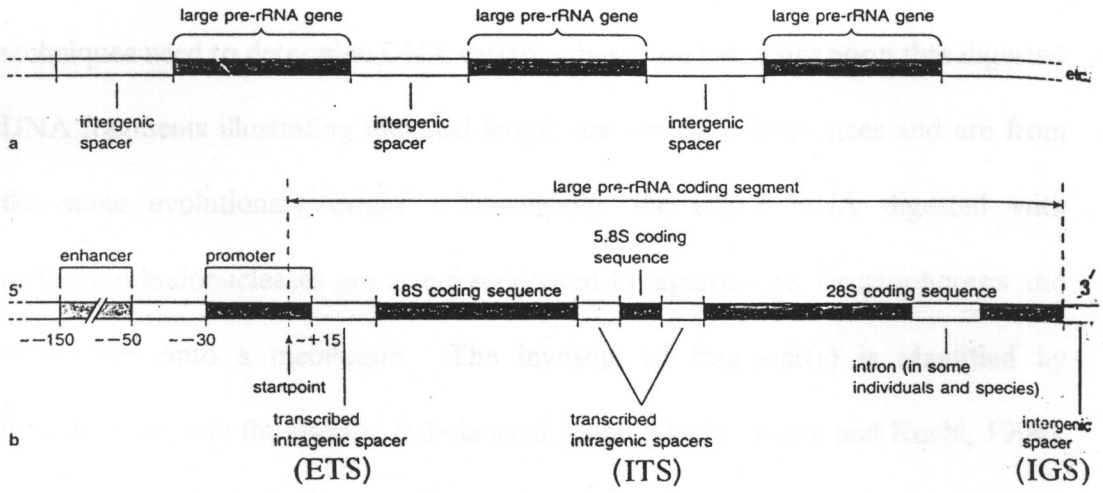

Fig. 1.5 Large pre- rRNA genes and their spacer: (a) The pattern of tandemly repetitive repeats of the large pre-rRNA genes, (b) the arrangement of coding sequences and internal transcribed spacer (Wolfe, 1993).

Different portions of the rDNA repeated unit evolve with significantly different rates (Kuperus and Chapco 1994; Navajas et al. 1995; Sappal et al. 1995). Thus, evolutionary studies may employ different segments of the unit depending on the taxonomic levels of organisms under investigation. In general, a high degree of polymorphism has been found in the noncoding segments of the repeat unit (ETS, ITS, IGS). Thus, population and species diagnostic markers in sibling species have been studied using these segments (McLain et al., 1995; Norris et al., 1996; Porter and Collins 1991).

1.3.2.2 Molecular techniques based on DNA analysis

A. Restriction fragment length polymorphism analysis (RFLP)

Restriction fragment length polymorphisms (RFLP) is one of several techniques used to determine DNA variation based on the assumption that digested DNA fragments illustrating identical length are similar in sequences and are from the same evolutionary origin. Technically, the target DNA digested with restriction endonucleases are size-fractionated by agarose gel electrophoresis and transferred onto a membrane. The investigated fragment(s) is identified by hybridization with the specific radiolabeled probe (Davis, Batteg and Kuehl, 1994). An example of RFLPs in mite is the identification of species-diagnostic markers between three sibling species of genus *Panonychus* (*P. mori, P. citri* and *P. ulmi*) and *Tetranychus urticae* through Southern hybridization with rDNA probes (Osakabe and Sakagami, 1994).

B. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a new approach applied for population genetic and systematic studies. This technique allows short DNA fragment (usually smaller than 2 kb) to be amplified *in vitro*. To amplify a particular DNA fragment, two single-stranded complementary primers are designed to a specific motif of the DNA template. The activity of a thermostable DNA polymerase in the suitable buffer system and thermo-cycling (denaturation, annealing and polymerization steps) results in exponential amplification of a given DNA fragment between the primer sites (Weising et al., 1995).

The ability to amplify interested DNA (RT-PCR for RNAs) through PCR opens the new approach for various biological disciplines (e.g. systematics, evolution, ecology). For instance, studies of bee mites using RFLP had been formerly limited by the amount of required DNA as typical RFLP needs at least 2 μg of genomic DNA for each analysis. Nevertheless, approximately 10-50 ng of genomic DNA is more than sufficient to be used as the template for the amplification reaction. This makes several molecular biological studies possible in tiny organisms like *T. clareae* and *T. koenigerum*.

C. PCR-RFLPs

This technique has been, and still is, the most common approach used for determination of genetic diversity at various taxonomic levels. After the DNA of interest is amplified by PCR, an aliquot of the amplification reaction is then simply digested with each restriction endonuclease. The resulting DNA fragments are electrophoresed through the appropriate gel medium (agarose or polyacrylamide) and visualized under the UV light after ethidium bromide staining. The most important advantage of this technique is that hybridization of labeled DNA probes to the target restricted DNA is obviated. Furthermore, the technique *per se* is much simpler than conventional RFLP approach.

D. Randomly amplified polymorphic DNA (RAPD) PCR.

RAPD-PCR was developed by Williams et al. (1990) who demonstrated that genomic DNA from a distantly related group of organisms could be amplified using a single short primer (9 or 10 nucleotides long) composed of an arbitrary

oligonucleotide sequence. The primer can be randomly designed without any prior knowledge of the sequence information of organisms under investigation. The only limitation is that the primers should have at least 50 % G+C content and should not contain palindromic sequences. Different random primers use with the same genomic DNA produce different numbers and sizes of DNA fragments (Ellsworth et al., 1993; Kernodle et al., 1993). After amplification, the amplified DNA patterns can be conveniently determined by agarose gel electrophoresis.

In insect, RAPD-PCR has been increasingly used for several applications. These include identification of molecular markers in populations (Tasanakajorn et al., 1995; Rao, Bhat and Toey 1996), determination of paternity in dragonflies (Hardrys et al., 1993), and communally breeding beetles (Scoot et al., 1992; Scoot and Williams, 1993), an analysis of population genetic structure and genetic variation (Richner et al., 1997; Shankaranarayanan et al., 1997 and Swoboda et al., 1997).

Based on various applications described above, RAPD-PCR may be useful for determination of genetic differences in *T. clareae* and *T. koenigerum* at individual, population or species levels because the number of primers used are unlimited and the primers which are suitable for such different taxonomic levels can be chosen. RAPD-PCR is particularly useful for species having limited genetic information or for organisms which have not been genetically investigated before.

de Guzman et al. (1997) examined genetic variation in *V. jacobsoni* from the United States, Russia, Morocco, Germany, Italy, Spain, Portugal, Japan, Brazil and Puerto Rico using RAPD analysis. The results showed that all *V. jacobsoni*

collected from the United States had the same RAPD pattern to that of those originated from Russia, Morocco, Germany, Italy, Spain, and Portugal (called the Russian pattern). Nevertheless, this pattern was different from that found in Japan, Brazil, and Puerto Rico (called the Japanese pattern). The OPP-03 and OPP-07 generated 422 bp and 766 bp fragments which common in samples carrying the Russian pattern but were not observed in those of the Japanese pattern. Two bands located at 675 bp and 412 bp were found in all individuals from the United States and Europe. These results suggested that *V. jacobsoni* of the United State was historically originated from Russia through Europe, while that of Brazil and Puerto Rico are probably originated from Japan. The two different patterns were widespread over vast geographic areas by introduction of the host species.

Edwards et al. (1997) employed RAPD-PCR to discriminate three *Typlodromalus* spp. (*T. limonicus*, *T. manihoti* and *T. tenuiscutus*). Five of eight RAPD-PCR primers could be used to distinguish these three species unambiguously. The genetic distances within-species (0.072-0.186) were much lower than that of between-species (0.407-0.656) illustrating the effective ability to identify cryptic mite species using this approach.

E. DNA Sequencing

The most direct method for determination of polymorphisms at the DNA level is sequencing of the interested orthologous DNA fragment of related organisms. DNA sequencing provides highly informative data and can be used for different discriminatory power by choosing appropriate regions of the genome.

Theoretically, DNA sequencing can be carried out using either chemical or enzymatic methods. The enzymatic method is currently more popular and is based on the ability of DNA polymerase to extend the primer until a chain-terminating nucleotide (each of ddNTPs) is incorporated to the newly synthesizing chain. The sequencing reaction is performed as a set of four separate reactions, each of which contains all four deoxyribonucleoside triphosphates (dNTPs) supplemented with a limiting amount of each dideoxyribonucleoside triphosphate (ddNTP) per reaction. Because ddNTPs lack the 3′ OH group necessary for chain elongation, the growing oligonucleotide is selectively terminated at G, A, T or C, depending on the respective dideoxy analog in the reaction. The resulting fragments are separated according to size by high resolution denaturing polyacrylamide gel electrophoresis (Sanger et al., 1977).

More recently, the sequencing approach has been greatly facilitated by PCR. The investigated DNA segment was amplified using a pair of primers. The PCR product can then be sequence directly or alternatively after cloning (Hoelzel and Green, 1992). In various taxa, the ITS of nuclear rDNA sequence are useful for evolutionary and systematics studies (Tang et al., 1996; Paskewitz, Wesson and Collins 1993; Vogler and DeSalle 1994; Kollipara et al., 1997). The rDNA primers are available in several organisms and seem to be universal for example a pair of primers originally developed from the fungal ITS also worked well in insects (White et al., 1990).

A phylogenetic relationship among members of *Ixodes* sepecies in the family Acari was recently reported based on sequence divergence of the ITS

region of rDNA (Wesson et al., 1993). Morphologically, *Ixodes pucificus* and *I. dammine* are taxonomically complicated and are not able to unambiguously distinguish. As a result, direct sequencing of PCR-amplified ITS segment was carried out in three *Ixodes* species including *I. pucificus* (from California and Arizona), *I. scapularis* (from Georgia and North Carolina), and *I. dammini* (from Maryland, Massachusetts, New Jersey, New York and Wisconsin). Variation of nucleotide sequences was observed in these taxa at intra-individuals within a population, inter-individuals from different geographic origin of a species and interspecific levels. DNA polymorphisms resulted from small deletions and insertions were typical in the ITS region. Homogenization of rDNA multigene arrays for sequence variants by concerted evolution seemed to occur at a relatively rapid rate. It was also found that numerous polymorphic sites found in *I. pacificus* and *I. dammine*, facilitating a possible use of these sequences to assess relationship among sibling species.

Based on the maximum parsimony and two distance methods (unweighted pair-group with arithmetic averages and neighbor-joining), sequence variation in ITS1 and ITS2 suggested that *I. scapularis* and *I. dammini* are closely related species reflected from high similarly in ITS sequences of individuals from geographically isolated locations. Evidently, individuals from geographically separated samples of *I. pacificus* were intraspecifically less related and clearly different genetically to those of *I. scapularis/dammini*. Likewise, McLain et al. (1995) reported population genetic structure of *I. scapularis* from eastern seaboard of the United States by determination of the ITS sequence. Twenty sequences were obtained from individuals originating from different localities; 10

of which were from the southeast (Georgia and Florida), seven were from the middle east (North Carolina and Maryland) and the rest were from the northeast (Massachusetts, New Jersey and New York). Phylogeny based on neighbor-joining and the maximum parsimony methods allocated most of the Southeastern and the middle Eastern individual together but fail to cluster those from the northeast.

Fenton at al. (1997) studied genetic variation in eriophyid mites by analysis of DNA sequences of PCR-amplified ITS. A total of 7 species composing of *Cecidophyopsis ribis*, *C. grossulariae*, *C. spicata*, *C. alpina*, *C. aurea*, *C. grossulariea* and *Phylocoptes gracillis* were investigated. It was found that 92-99 % of ITS1 sequences from different *Cecidophyopsis* spp. was similar. Inter-specific differences between *Cecidophyopsis* were found in seventeen simple sequence repeats (SSRs), fourteen point mutations and two deletions. No intra-specific variation in SSRs was observed.

1.4 Aims of this thesis

Prior to the present research, there have been no reports on genetic variation studies concerning two species of *Tropilaelaps* (*T. clareae* and *T. koenigerum*). Thus, the objectives of this thesis are to discriminate these two species by sequencing the ITS_1- ITS_2 region and to determine intraspecific genetic variation of *T. clareae* from different geographic locations within a species and between different hosts using RAPD approach.

CHAPTER II

Materials and Methods

2.1 Instruments

1. Autoclave HA-30 (Hirayama Manufacturing Co., Japan)
2. Automatic pipette P2, P20, P100 (Gilson Medical Electronics S.A., France)
3. Camera Pentax super A (Asahi Opt. Co., Japan)
4. Electonic balance Alsep EY220A (A&D Co. Ltd., Japan)
5. Electrophoresis apparatus
    5.1 Horizontal agarose gel electrophoresis apparatus (9x12 cm. gel)
    5.2 Vertical gel electrophoresis apparatus for DNA sequencing (Hoefer, England)
6. -20 °C freezer (Krungthai Ltd., Thailand)
7. -80 °C freezer (Krungthai Ltd., Thailand)
8. Gel dryer 583 (Bio-RAD Laboratories, USA)
9. High speed microcentrifuge MC- 15A (Tomy Seiko, Japan)
10. Heating block BD 17016-26 (Sybron Thermolyne Co., USA)
11. Incubator BM-600 (Memmert Gamblt, Germany)
12. Light box 2859 SHADON (Shandon Scientific Co., Ltd., England)
13. Magnetic stirrer M21/1 (Franz Morat KG GambH, Germany)
14. Microcentrifuge Force 6 (Denver Instrument company, USA)
15. Power supplies (Bio-RAD Laboratories, USA)

15.1 Power PAC 300

15.2 Power PAC 3000

16. Shaking water bath O1PF623 (New Brunswick Scientific Co. Inc., USA)
17. Standard film cassette: Ranex Regular Screen Eastman (Eastman Kodak company, USA)
18. Thermal Cycler:GeneAmp PCR system 2400 (Perkin Elmer Cetus, USA)
19. UV transilluminator 2011 Maccrovue (SanGabriel California, USA)
20. Vortex genic K550-G (Scientific Industries Inc., USA)

2.2 Inventory Supplies

1. Black and white film (TriX-pan 400, Eastman Kodak Company Ltd., USA)
2. Whatman 3 mm. filter paper (Whatman Internation Ltd., England)
3. Hyper-film MP (Amersham International, England)
4. 0.5 ml and 1.5 ml microcentrifuge tubes (Axygen Hayward, USA)
5. 30X40 cm glass plates (Axygen Hayward, USA)
6. Pipette tips (Axygen Hayward USA)
7. 0.2 ml thin-wall microcentrifuge tubes (Axygen Hayward, USA)

2.3 Chemical reagents

Name	Company	Country
Absolute ethanol	Merck	Germany
Acrylamide	Merck	Germany
Agarose gel	FMC Bioproducts	USA
- Metaphor Agarose		
- SeaKem LE Agarose		
Ammonium persulfate	Merck	Germany
Boric acid	Merck	Germany
Bromophenol blue	Sigma	USA
Chelex® 100 resin	Bio-RAD	USA
Developer	Kodak	USA
Ethylenediamine tetraacetic acid disodium salt dihydrate(Na_2EDTA)	Fluka	Switzerland
Ethidium bromide	Sigma	USA
Ficoll 400	Sigma	USA
Fixer	Kodak	USA
GeneAmp PCR core reagents	Perkin Elmer	USA
- Ampli*Taq* DNA polymerase (5U/μl)		
- 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)		
- 25mM $MgCl_2$ solution		
- 10 mM dNTPs (dATP, dCTP, dGTP and dTTP)		

Name	Company	Country
N,N′-methylene-bis-acrylamide	Sigma	USA
N,N,N′,N′-tetramethylenediamine (TEMED)	Sigma	USA
Geneclean II kit	Bio 101	USA
- Glassmilk		
- 6 M Sodium iodide		
- TBE modifier		
- New wash concentrate		
OmniBase™ DNA cycle sequencing system	Promega	USA
- 5x DNA sequencing buffer (250 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$)		
- d/ddNTP nucleotide mixes		
- DNA sequencing stop solution (10 mM NaOH, 95 % formamide, 0.05% bromophenol blue, 0.05 % xylene cyanol)		
- 10 u/µl T4 polynucleotide kinase		
- 10X T4 polynucleotide kinase buffer (500 mMTris-HCl pH 7.5, 100mM $MgCl_2$, 50 mM DTT, 10 mM spermidine)		
- 200 ng/µl pGEM3zf(+) control DNA		
- 24 mer pUC/M13 forward primer		
Potassium acetate	Merck	Germany
Potassium chloride	Merck	Germany

Name	Companpy	County
Sodium acetate	Merck	Germany
Sodium dodecyl sulfate (SDS)	Sigma	USA
Tris-(hydroxy methyl)-aminomethane	Fluka	Switzerland
Urea	Fluka	Switzerland
Xylene cyanol FF	Sigma	USA
100 base pair DNA ladder	Promega	USA

2.4 Oligonucleotide primers

Oligonucleotides used for PCR were purchased from Bioservice Unit, National Center for Genetic Engineering and Biotechnology, National Science and Biotechnology Development Agency, Thailand or from Biosynthesis, Inc., USA The primer sequences are shown in Table 2.1 and 2.2 .

2.5 Radioisotope

[γ-^{32}P] ATP specific activity 3,000 Ci/mmol (Amersham, England)

2.6 Sample collections

Two parasitic mite species, *T. clareae* and *T. koenigerum* were collected from the geographic ranges of these taxa. The former can be found in two hosts including *A. dorsata* and *A. mellifera* and widely distributed whereas the latter is host-specific to *A. dorsata*. Therefore, *T. clareae* from *A. dorsata* was sampled from Lumpang, Nakhonratchasima, Chanthaburi, Trad, Samutsakhorn, Samutsongkhram, Prachuab Khiri Khan and Chumporn while *T. clareae* from *A. mellifera* was collected from Chiang Mai, Uttaradit, Pisanuloks, Khon Kaen,

Udon Thani, Chanthaburi, Bangkok and Chumporn. Individuals of *T. koenigerum* was collected from Samut Songkhram and Chanthaburi.

An individual of the parasites was separately placed in a 0.5 ml microcentrifuge tube and immediately transferred to a tank containing liquid nitrogen until further required. A number of specimens was approximately 15-50 individuals per location.

Table 2.1 Primer sequences used in PCR and sequencing of an amplified ITS region of ribosomal DNA.

Name	Sequence (5′ to 3′)	Tm (°C)	
ITS5[1]	GGAAGTAAAAGTCGTACAAGG	63	PCR/Sequencing
ITS4[1]	TCCTCCGCTTATTGATATGC	58	PCR/Sequencing
inITS5[2]	TCGTATGTATTCCATTCGTA	54	Internal primer used for sequencing
inITS4[2]	CATAGACACAAGGCATCCAT	58	Internal primer used for sequencing

[1]After White et al., 1990.

[2]Primers designed in this study.

Table 2.2 The sequences of all random primers primirily used for screening of informative primers for *T. clareae* and *T. koenigerum*.

Primer	Sequences (5′ to 3′)
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-13	CAGCACCCAC
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-17	GACCGCTTGT
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC

Table 2.3 The number of specimen being sequenced and the geographic origin of specimens used in this study.

Abbreviation	Sampling Area	Host	Number of specimens being sequenced
N2M	Bupphachat Beefarm, Muang, Uttaradit	*A. mellifera*	2
N1M	Supha Beefarm, Maerim, Chiang Mai	*A. mellifera*	1
N1D	Thungkweng Market, Hang chat, Lumpang	*A. dorsata*	2
NE1M	Preservation and Bee Culture Center 3, Khon Kaen	*A. mellifera*	1
NE2M	Phupan Beefarm, Muang, Udon Thani	*A. mellifera*	1
NE1D	Nongnunnak, Nakhon Ratchasima	*A. dorsata*	1
C2M	Naraesaun University, Phisanulok	*A. mellifera*	1
C1M	Chulalongkorn University, Bangkok	*A. mellifera*	1
C1D	Samroy Yod, Prachuap Khiri Khan	*A. dorsata*	1
C2D	Krathumban, Samutt Sakorn	*A. dorsata*	1
E1M	Preservation and Bee Culture Center 4 Chanthaburi	*A. mellifera*	2
E3D	Muang, Trat	*A. dorsata*	2
S1M	Preservation and Bee Culture Center 5 Chumphon	*A. mellifera*	1
S1D	Sawi, Chumphon	*A. dorsata*	3
TKC	Krathumban, Samutt Sakorn	*A. dorsata*	3
TKE	Soy Down, Chanthaburi	*A. dorsata*	2

2.7 DNA extraction

Total DNA was individually extracted from each specimen using the modification of the method of Walsh et al. (1991). When needed, an individual mite was removed from a storage tank, placed in a microcentrifuge tube (1.5 ml) containing 35 µl of 5% chelex and homogenized with a micropestle for a few strokes. The homogenate was gently vortexed at low speed (3-4 degree) for 30 seconds and incubated at 55 °C for at least 3 hours. The mixture was then incubated at 95-100 °C for 7 minutes before centrifugation at 8,000x*g* for 10 minutes at room temperature. The supernatant was carefully transferred to a new sterile microcentrifuge tube. The DNA solution was stored at 4 °C and utilized as the template for PCR amplification.

2.8 PCR amplification

2.8.1 Amplification of the internal transcribed spacer region (ITS)

The ITS of *T. clareae* and *T. koenigerum* was amplified by PCR using a pair of universal primers previously successful used for amplification of the ITS in fungi (White et al., 1990). PCR was carried out in a 25 µl reaction mixture constituting 4 µl of DNA template, 200mM each of dNTPS (dATP, dCTP, dGTP and dTTP), 2.5 mM of $MgCl_2$, 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 0.2 µM each of the forward and reverse primers and 0.6 unit of *Taq* DNA polymerase.

The reaction mixture was firstly predenatured at 94 °C for 1 minute followed by 35 cycles consisting of a denaturation at 92 °C for 1 minute, an

annealing at 52 °C for 1 minute and an extension at 72 °C for 2 minutes. The final extension was performed at 72 °C for 7 minutes. After amplification, the resulting product was examined by agarose gel electrophoresis and further purified for sequencing.

2.8.2 Random amplified polymorphic DNA analysis of *T. clareae* and *T. koenigerum*

Four microliters of total DNA extraction from each individual mite was used as DNA template for PCR in a total volume of 25 µl. An appropriate amount of other constituents composing of 100 mM each of dNTPs (dATP, dCTP, dGTP and dTTP), 2.0 mM of $MgCl_2$, 1x PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl), 0.2 µM of a particular primer and 1.0 unit of *Taq* DNA polymerase was added.

The thermo-cycler profile (Perkin Elmer Model 2400) was composed of predenatured at 94 °C for 1 minute followed by 40 cycles of a 92 °C denaturation for 30 second, a 36 °C annealing for 45 second and a 72 °C extension for 2 minutes. The final extension was carried out at 72 °C for 5 minute. The amplification products were electrophoretically analyzed by appropriate concentrations of agarose gels.

2.9 Agarose gel electrophoresis

An appropriate amount of agarose (SaeKem LE or Metaphor) was weighed out and mixed with Tris-Borate-EDTA buffer (TBE; 89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3) to make the desired gel concentration (1.5% for SeaKem LE and 1.8% Metaphor for detection of ITS

and RAPD-PCR products, respectively). The dissolved agarose was heated until complete solubilization and cooled at room temperature to 50 °C before poured into a gel mould in which a comb was already inserted. When the gel had solidified, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1x TBE buffer that cover the gel for approximately 0.5 cm.

An appropriate amount of ITS or RAPD-PCR amplified DNA was mixed with one-fifth volume of the loading dye buffer (15% ficoll 400, 0.25% bromophenol blue and 0.25% xylene cyanol FF) before carefully loaded into the well. Three microlitters (390 ng) of a 100 bp ladder was used as a DNA standard. Electrophoresis was operated at 100 volts until bromophenol blue moved to approximately 3 cm (for ITS) and 0.5 cm (for RAPD) from the bottom of the gel. The electrophoresed gel was stained by immersed in a 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide in distilled water for 30 minutes. DNA fragments were visualized under a UV transilluminator and photographed through a red filter using Kodak Tri-X-pan 400.

2.10 Preparation of DNA template for DNA sequencing

The amplified ITS fragment was size-fractionated through a 1.5% agarose gel prepared in 1x TBE buffer and electrophoretically analyzed side by side with a 100 bp ladder. After electrophoresis was completed, the lane corresponding to the DNA marker was excised and stained with ethidium bromide as described above. The ITS fragment could also be excised by comparing its molecular length

corresponding to that of the ladder and placed into a pre-weighed 1.5 ml microcentrifuge tube, one-half volume of TBE modifier and 4.5 volumes of 6M sodium iodide were added. The gel was dissolved by inversion of the tube for several times and further incubated at 55 °C for 10 minutes. Five microliters of glass milk was added to the gel solution. The suspension was incubated on ice for 5 minutes. The glass milk was pelleted by centrifugation at 8000x*g* for 30 seconds. After the supernatant was removed and discarded, 250 µl of a new wash solution was added. The pellet was resuspended and centrifuged at the same speed for 30 seconds. This process was further carried twice. The ITS amplified DNA was recovered from the washed pellet with an addition of the suitable amount of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubated at 55 °C for 5 minutes. The solution was centrifuged at 5000x *g* for 30 seconds, the supernatant containing the eluted DNA was collected and used for direct-sequencing.

2.11 DNA Sequencing

OmniBase™ DNA cycle sequencing system and OmniBase™ sequencing enzyme mixes (Promega) were used for direct-sequencing of the amplified ITS of *T. clareae* and *T. koenigerum*. In this sequencing system, a ^{32}P end-labeled primer was annealed to the DNA template and extended by the DNA polymerase activity in the presence of four deoxyribonucleside triphosphates (dNTPs). The reaction also contained one of four dideoxyribonucleoside triphosphates (ddNTPs), which terminated elongation reaction when being incorporated into the DNA synthesizing chain. After sequencing, the products were electrophoretically separated on a high-resolution denaturing polyacrylamide gel. The results were

visualized by autoradiography. Using the external primers, approximately 200 bp of the sequences could be unambiguously determined, as a result; internal primers were designed to obtained the 3′ downstream sequences of the ITS.

2.11.1 End-labeling of the primer.

The primer was 5′-end-labeled with [γ-^{32}P] ATP (specific activity 3000 Ci/mmol) using the forward activity of T_4 polynucleotide kinase. The labeling reaction mixture in a total volume of 10 µl was set up. This composed of 6 sets of double-stranded sequencing reactions. Each of which contained 10 pmole of a particular primer, 10 pmole of [γ-^{32}P] ATP, 1 µl of 10x buffer (500 mM Tris-HCl pH 7.5, 100 mM DTT, 50 mM spermine) and 5 unit of T_4 polynucleotide kinase. The reaction was gently mixed by pipetting and incubated at 37 °C for 10 minutes. The labeling reaction was terminated by incubated at 90 °C for 2 minutes. The reaction mixture was briefly centrifuged and stored at -20 °C until further needed.

2.11.2 Extension-termination reactions.

The template/primer mix for each of four sequencing reactions was prepared in a 0.5 ml microcentrifuge tube by adding 2 ng of DNA template, 5 µl of 5x sequencing buffer (250 mM Tris-HCl pH 9.0, 10mM $MgCl_2$), 1.5 µl of end-labeled primer (2.11.1) and appropriate amount of nuclease-free water making the final volume up to 10 µl. Finally, 10 units of OmniBase™ Sequencing enzyme was added. The solution was gently mixed by pipetting. Four microliters of each

reaction was added to a 0.5 ml microcentrifuge tube containing 2 µl of appropriate d/ddNTP termination mix and briefly mixed before subjected to cycle sequencing in a thermo-cycler. The cycle sequencing reaction was initially denatured at 94 °C for 1 minute followed by typical PCR steps of a 95 °C denaturation for 30 seconds, a 44 °C annealing for 45 seconds and a 70 °C extension for 60 seconds for 30 cycles. At the end of the cycle-sequencing reaction, 3 µl of a stop solution was added to each sequencing reaction. Prior to loading of these samples on a denaturing sequencing gel, the reactions were heated at 70 °C for 2 minutes and snap-chilled on ice.

2.11.3 Polyacrylamide gel electrophoresis

A pair of glass plates were carefully cleaned with soap and water before thoroughly rinsed with deionized water, 70% ethanol and left to air-dry. The shorter plate was siliconized with a glass coating solution, (Rain-X, Unellco Co., USA). The glass plates were assembled with spacers (0.4 mM thickness). The side and bottom edges of the plates were sealed with plastic tape.

Sixty milliliters of 6% denaturing acrylamide gel (in 1X TBE, 8M urea, 0.084 M acrylamide, 2 mM bis-acrylamide) was prepared in a 100 ml beaker. To initiate gel polymerization, 280 µl of 10% ammonium persulphate and 60 µl of TEMED (N,N,N′,N′-tetramethylethylenediamine) was added to the acrylamide mixture and gently mixed. The resulting solution was immediately poured into the gel apparatus with a short plate face-up. The gel mixture was slowly poured between the glass plates, with a smooth flow rate to prevent any air bubbles. The angle of glass plates was adjusted to allow the gel solution flowed down slowly at

one side. When the gel solution reached the top of the short plate, a 0.4 mm thick shark tooth comb was inserted to approximately 5 mM in depth. The polymerization process were allowed to be completed for 2-3 hours.

When required, the sealing tape was removed. The gel was cleaned to remove spilled acrylamide solution from plate surfaces with water. The gel mould was placed to a sequencing chamber. An enough amount of 1x TBE was added to the upper and lower chamber allowing the gel plates submerged for 2 - 3 cm in the buffer. The comb was removed. The wells were immediately flushed out with a long-stem Pasteur pipette using the buffer in the upper reservoir to remove undesired small pieces of polyacrylamide. A comb was reinserted into the gel. The gel was pre-run at constant power of 35 watts (1.2 kilovolts) for 30 minutes. Prior to loading of the samples, the gel wells were carefully rinsed with the buffer to remove any residual urea. The heat-danatured samples were loaded. The gel was electrophoresed at the same constant power for 2-3 hours.

2.11.4 Autoradiography

After electrophoresis, the glass plates were removed from the electrophoretic apparatus and placed under the running tap water until surfaces of both plates were cooled. The sealing tape and spacers were removed. The shorter glass plate was prized apart using either scissors or a spatula, therefore the gel was remained on the longer glass plate. The gel was transferred to a piece of pre-cut Whatman 3 MM filter paper by laying the paper on top of gel and slowly lower the remaining of the filter paper to cover the entire polyacrylamide gel. The paper was slowly peeled back. At this stage the gel should stick with the filter paper. The 3 MM filter paper containing the sequencing gel was covered with

cling-film and dried at 80 °C for 30-60 minutes in a gel dryer. The dried gel was then placed in an X-ray cassette. In the dark room, an autoradiography film was placed on the dried sequencing gel. The cassette was kept in a -80 °C freezer for 16-24 hours. When required, it was removed from a freezer and left to thaw out to room temperature for approximately 30 minutes. The exposed film was developed and fixed according to the manufacture's instruction. The sequences obtained were visualized manually.

2.12 Statistical analysis of genetic variation

2.12.1 DNA sequence analysis

The sequences obtained from the amplified ITS of *T. clareae* in *A. dorsata* and *A. mellifera* and *T. koenigerum* in *A. dorsata* hosts were aligned using Clustal W. The genetic distance between sequences (*d*) can be calculated by Kimura's two parameter method using the formula;

$$d = (1/2)\ln(a) + (1/4)\ln(b)$$

where a = 1/(1-2P-Q and b = 1/(1-2Q); P and Q represent the proportion of nucleotide differences resulted from transitional and tranvertional mutatations, respectively.

Practically the genetic distance of investigated sequences was routinely calculated using DNAdist in Phylip 3.56c.

2.12.2 RAPD analysis

The reproducible and well resolvable bands with the molecular length between 265-2040 bp were scored from photographes of the gels. The band intensity differences due to homo- and heterozygotic states were not considered.

Accordingly, the presence (1) and absence (0) of an amplified fragment was treated in a dominant fashion.

The similarity coefficiency between a pair of individuals was calculated using the formula:

$$S_{xy}=2n_{xy}/n_x+n_y,$$

where; n_x and n_y represent the number of scorable fragments from individual x and y, respectively.

n_{xy} is the number of fragments shared by both individuals.

Similarity index within a population (S) is calculated as the average of S_{xy} across all pairwise comparisons between individuals within such a population.

Genetic similarity between populations with a correction for within population similarity is:

$$S_{aij} = 1+S'_{ij}-0.5\ (S_i+S_j),$$

where S_i and S_j represent the S estimates for population i and j, respectively

S'_{ij} is the average similarity between random pairs of individuals across populations i and j (Lynch 1990).

S_{aij} was then converted to the genetic distance (D_{ij}) using the equation:

$$D_{ij} = 1- S_{aij}$$ (Lynch 1991).

2.12.3 Phylogenetic construction

A phenogram based on the distance approach was constructed using the unweight pair-group method with arithmetic average (UPGMA) implemented in Phylip version 3.57c (Felsenstein, 1991).

CHAPTER III

Results

3.1 DNA extraction

The quantity of isolated genomic DNA of *T. clareae* and *T. koenigerum* could not be spectrophotometrically measured due to the limited amount of DNA obtained. Electrophoretic analysis revealed that extracted total DNA was greatly degraded, therefore the DNA concentration of each sample could not be compared with the standard DNA marker (Fig 3.1).

3.2 Testing range of DNA template for PCR amplification of the ITS region

To overcome difficulties to quantify the DNA template, two fold increases in volume (from 1-10 µl) of extracted DNA was served as the DNA template in the PCR amplification of the ITS region as described in Fig.3.2. The ITS PCR product approximately 600 bp in size was initailly observed at 1 µl DNA template. The resulting product was gradually increased until 4 µl of DNA . At the concentration of template greater than this, smeared PCR product was usually observed. As a result, amplification of the ITS region was basically carried out using 4 µl of extracted *T. clareae* and *T. koenigerum* DNA. If smeared PCR product was observed after electrophoresis, the amount of DNA template used in the amplification reactions was then reduced.

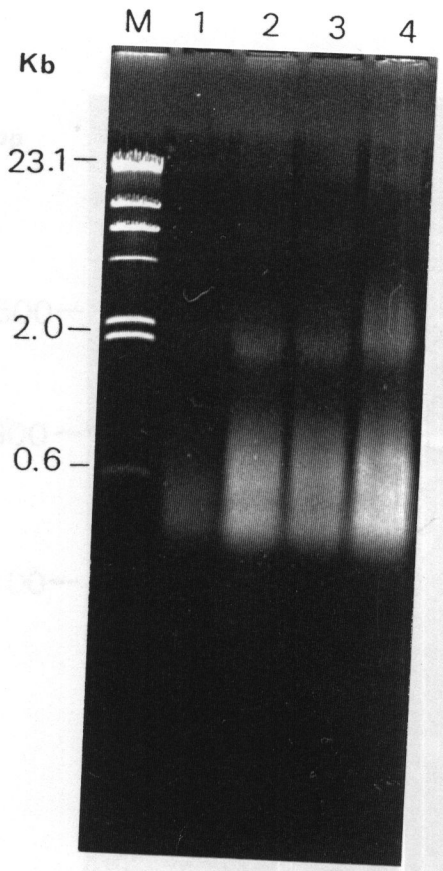

Fig. 3.1 Agarose gel electrophoresis showing the quality of DNA extracted from *Tropilealaps* mites.

lane M = DNA marker (λ/HindIII)

lanes 1-4 = Total DNA from 1, 3, 5 and 10 mite individuals, respectively.

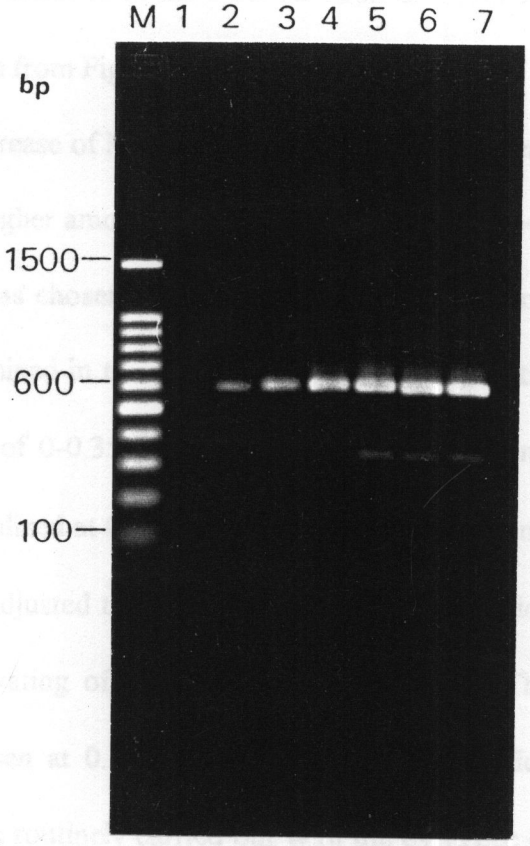

Fig. 3.2 Agarose gel electrophoresis showing the PCR-amplified ITS product (600bp) using different amount of the DNA template at a constant $MgCl_2$ (3.5 mM) and primer concentration (0.10 μM).

lane M = A 100 bp DNA ladder

lanes 1-7 = The resulting ITS product using 0, 1, 2, 4, 6 and 8 μl of DNA template, respectively.

3.3 Optimization of PCR condition for amplification of the ITS region in *T. clareae* and *T. koenigerum*.

To evaluate the most appropriate amount of $MgCl_2$ for amplification of the ITS region from *T. clareae*, a series of 0-4 mM $MgCl_2$ concentration with a 0.5 mM increment was included in the PCR reaction at a primer concentration of 0.2 μM. As can be seen from Fig.3.3, the amplified product was firstly appeared at 0.5 mM $MgCl_2$. An increase of $MgCl_2$ concentration to be higher than 1.5 mM did not yield significantly higher amount of the amplified product, therefore an optimal $MgCl_2$ concentration was chosen at this concentration. The most optimal primer concentration was examined in the same manner, the concentration of primer was varied in the range of 0-0.35 μM, with a 0.5 μM increment. The amplified product was firstly visualized at 0.05 μM of each primer and dramatically increased when the primer were adjusted to 0.10 μM. Higher concentrations of the primers did not resulted in elevating of the PCR product (Fig.3.4). The optimal primer concentration was chosen at 0.10 μM. Therefore, an amplification of the ITS region in *T. clareae* was routinely carried out with the existence of 1.5 mM $MgCl_2$ and 0.10 μM each of the primers.

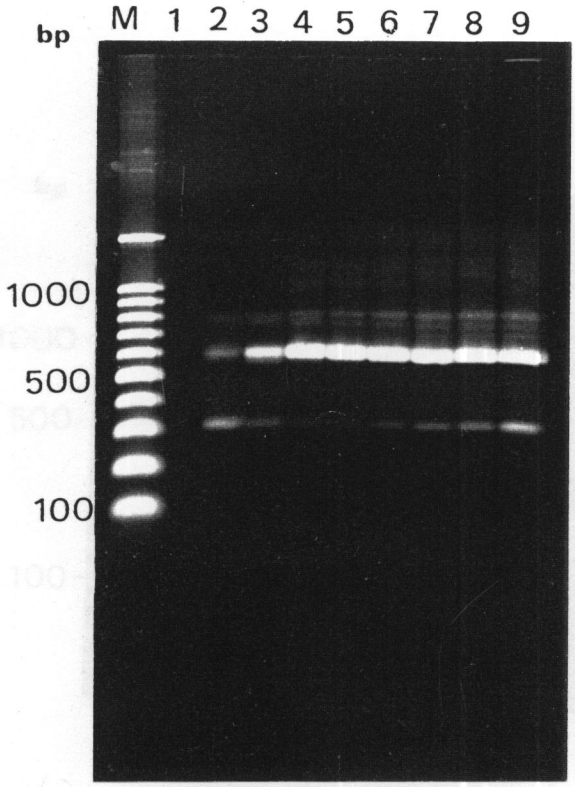

Fig. 3.3 Agarose gel electrophoresis showing the results from optimization of $MgCl_2$ concentration used for amplification of the ITS region at a constant primer concentration of 0.2 μM.

lane M = A 100 bp DNA ladder

lanes 1-9 = The PCR-amplified ITS product resulted from using 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0,3.5 and 4.0 mM of $MgCl_2$ in the PCR, respectively.

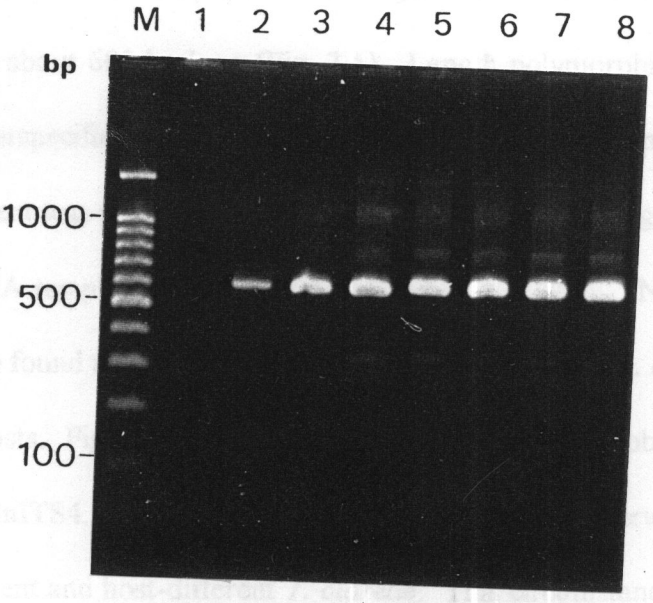

Fig 3.4 Agarose gel electrophoresis showing the results from optimization of primer concentrations used for amplification of the ITS region at 1.5 mM $MgCl_2$ concentration.

lane M = A 100 bp ladder

lanes 1-8 = The PCR-amplified ITS product when 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 μM of primers was included in the reaction.

3.4 Preparation of DNA template and direct sequencing

Amplification of an ITS region of twenty *T. clareae* individuals originating from different geographic locations and host species (*A. dorsata* or *A. mellifera*) and 5 individuals of *T. koenigerum* from geographically distinct samples were performed. The size of PCR-amplified ITS region of both *T. clareae* and *T. koenigerum* were about 600 bp long (Fig. 3.5). Length polymorphisms at both intraspecific and interspecific levels were not observed. After electrophoresis, the amplified ITS fragment was purified with approximately 80% yield (Fig. 3.6). This double stranded DNA template was subjected to cycle-sequencing. No sequence polymorphisms were found for all *T. clareae* investigated both from *A. dorsata* and from *A. mellifera* hosts. Fig. 3.7 showed the partial ITS sequences obtained from the internal primer, inITS4. No sequence polymorphisms were observed between geographically different and host-different *T. clareae*. This circumstance was also observed in all *T. koenigerum* investigated. Fig. 3.8 showed an autoradiogram illustrating interspecific polymorphisms of an amplified ITS region of *T. clareae* and *T. koenigerum.*

The average base compositions for an ITS region of both species were 25%A, 30%T, 25%G and 20%C. A sequence from each species (519 bp for *T. clareae* and 520 bp from *T. koenigerum*) was aligned by using Clustal W (Fig 3.9). The sequence similarity of the ITS region was 94.2 %. A total of 19 point mutations was illustrated between these sequences. There were 10 transitional mutations (5 of A↔G and T↔C) and 9 transversional mutations. Differences due to insertions/deletions was also observed (number of gaps = 7). Interestingly, the ITS region of *T. koenigerum* consistently contained a sequence of TTCTC which

was not found in that of *T. clareae*. The genetic distance (sequence divergence) between these two taxa calculated using Kimura's two parameter approach was 3.79 %.

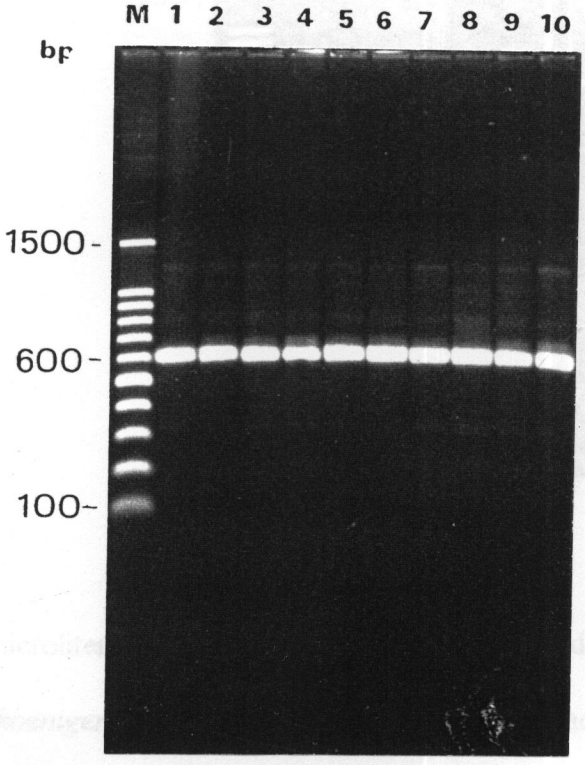

Fig 3.5 The ITS region amplified by PCR and electrophoresed through a 1.5% agarose gel.

lane M = A 100 bp DNA ladder

lanes 1-5 = The PCR-amplified ITS region of *T. clareae*

lanes 6-10 = The PCR-amplified ITS region of *T. koenigerum*

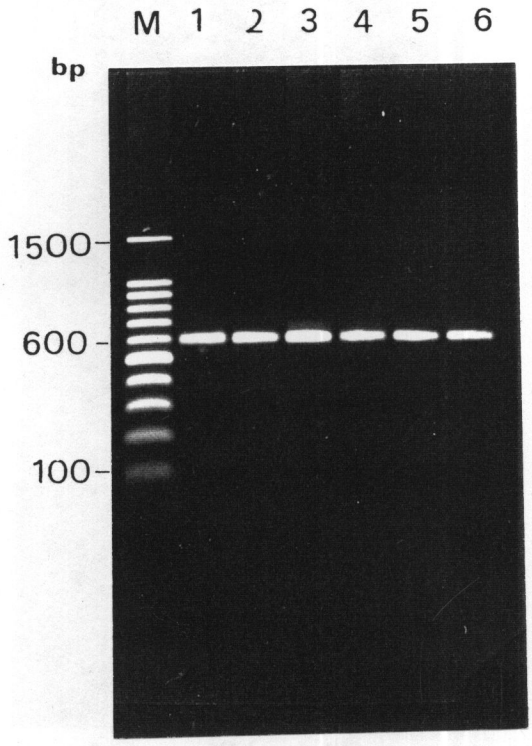

Fig 3.6 Five microliters of the PCR products from each individual of *T. clareae* or *T. koenigerum* was electrophoretically size-fractionated through a 1.5% agarose gel. The purified ITS DNA was recovered using a GeneClean II Kit (Bio101).

lane M = A 100 bp DNA ladder

lanes 1-3 = The ITS DNA amplified through PCR

lanes 4-6 = The ITS DNA recovered using GeneClean.

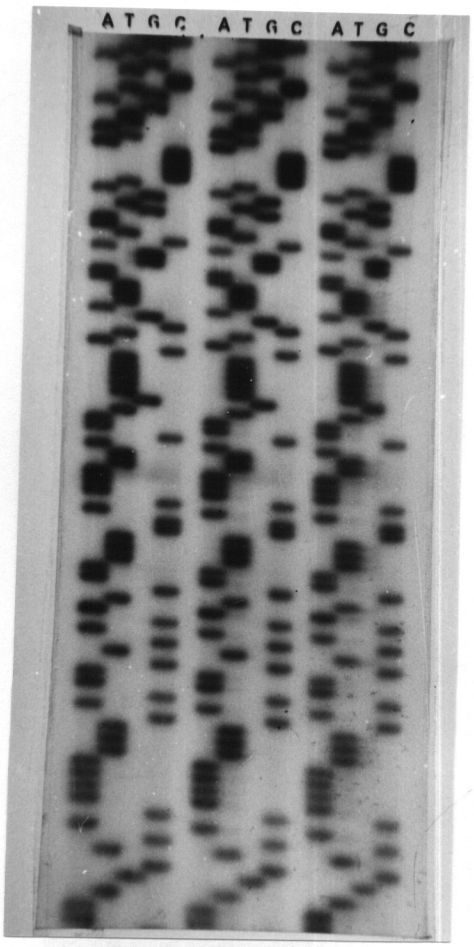

Fig 3.7 An autoradiogram showing partial ITS sequence obtained from using the primer inITS4. No sequence polymorphisms were observed between geographically different (ND and SD) and host-different *T. clareae*

Abbreviation ND = Geographic origin from the North, *A. dorsata* is the host.

NM = Geographic origin from the North, *A. mellifera* is the host.

SD = Geographic origin from the South, *A. dorsata* is the host.

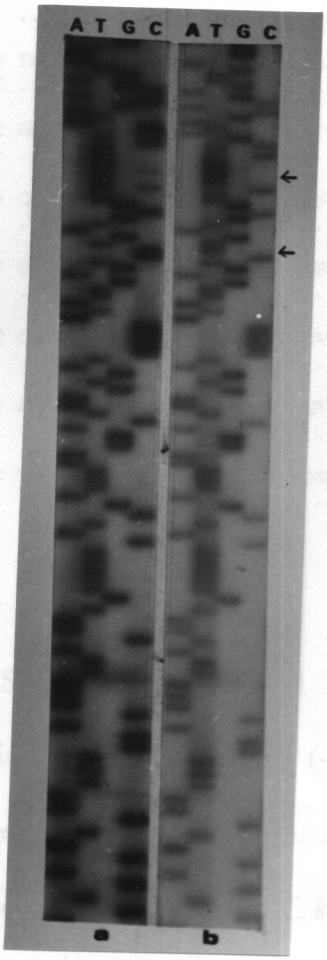

Fig. 3.8 An autoradiogram illustrating interspecific polymorphisms (arrows) of an amplified ITS region of *T. clareae* (a) and *T. koenigerum* (b).

```
                                                                        60
T. clareae     CGTAGTGAAC CTGCGGAAGG ATCATTACTG TCGCAAAGTC CATTCACTCC GTCGGCGAGC
T. koenigerum ..........  ..........  ..........  ....G.....  ..........  ..........
                                                                       120
               GAGTGGTGCT CGAATGATGT TCTAACCCTC TC-----CGC GGAGGCGACG GGAGAGGCAT
               ..........  .........C  ..........  ..TTCTC...  .......G..  ..........
                                                                       180
               CTGTGCCCAG TATCGTATGT ATTCCATTCG TATTGCGATC TGACTTCGGC TGTGAAGTTA
               .........C  ..........  ....G.....  ...C.....A .A........  ..........
                                                                       240
               GGCGCGCGTC GCCGGTGCGT CCGGTTTGAC ATGCTTTTCC ATTTAACTCG TGCTATGGAG
               .........-  .......T..  .........-  -.........  C..C......  ......C...
                                                                       300
               AAAAGAAGAA CGCATCAGGA CTCAATATGG GGGATCACTT AGTCCTTAAA TCGATGAAAA
               ...--.....  .......A..  ..........  ..........  ..........  ..........
                                                                       360
               ACATTGTAAT TTGTGGAAAT TGATGTGAGT TGTGAAATTT TGTGAGCATT GTGTTTTTGA
               .....A....  ..........  ..........  ..........  ..........  ..........
                                                                       420
               ATGAAAATTT CAGCATGGAT GCCTTGTGTC TATGCTACAC TTGTTTCAGT ATATAACTCG
               ..........  ..........  ..........  ..........  ..........  ..........
                                                                       480
               TAGTATATGT ACTTACTATT GCCGT-ACGC AATGGTATAA AATCTCCACG GTCACGAGAG
               ..C....A..  ...G......  .....T.T..  ..........  ......G...  ..........
                                                          525
               TGATGCTGCC TGCTCAAGTT GACGTGTATC TGAAATCAAG TGTGA
               ..........  ..........  ..........  ..........  .....
```

Fig. 3.9 Sequence for ITS from *T. clareae* and *T. koenigerum* after alignment. Dots indicate identities, and dashes (---) are gaps introduced to located homologous nucleotide position. An insertion of 5 bp (TTCTC) at the nucleotide position 93-97 is *T. koenigerum*-specific. Two sites of GTAC sequence in *T. clareae* (starting from nucleotide sites 428 and 444) whereas in *T. koenigerum* found one site (starting from nucleotide site 444 that same in *T. clareae*) that are recognition site of *Rsa*I.

3.5 Determination of inter- and intraspecific genetic variation of *T. clareae* and *T. koenigerum* using RAPD analysis.

Since the intraspecific diversity of *T. clareae* or *T. koenigerum* could not be detected by sequencing of the ITS region, an alternative method based on RAPD approach was then carried out. A total of 20 octanucleotide primers were screened whether they could be used to examine the genetic variation within and between these species. Based on the sequencing results, these taxa were closely related so only *T. clareae* was initially used during screening for the suitable RAPD primers. It was found that only OPA08 did not yield the successful amplification results (Fig. 3.10). Three primers (OPA07, OPA11 and OPA12) were selected for further studies on genetic variation of *T. clareae* and *T. koenigerum* on the basis of consistent, repeatable and easily scorable results of these primers.

The most optimal $MgCl_2$ concentration for each random primer was carefully examined as described previously. The most optimal $MgCl_2$ concentration for all selected primers was 2.0 mM (Fig. 3.11). This concentration resulted in consistent and repeatable results across investigated individual.

Analysis of sixteen and two colonies (principally 8 individuals per colony) of *T. clareae* and *T. koenigerum* using OPA07, OPA11 and OPA12 indicated both inter- and intraspecific polymorphisms. Examples of RAPD banding patterns generated by OPA07, OPA11 and OPA12 are shown in Fig. 3.12-3.17. A total of 86 fragment were unambiguously scored (265-2200 bp in size). Of these, fifty-one (36 polymorphic bands) and twenty-two fragments (3 polymorphic bands)

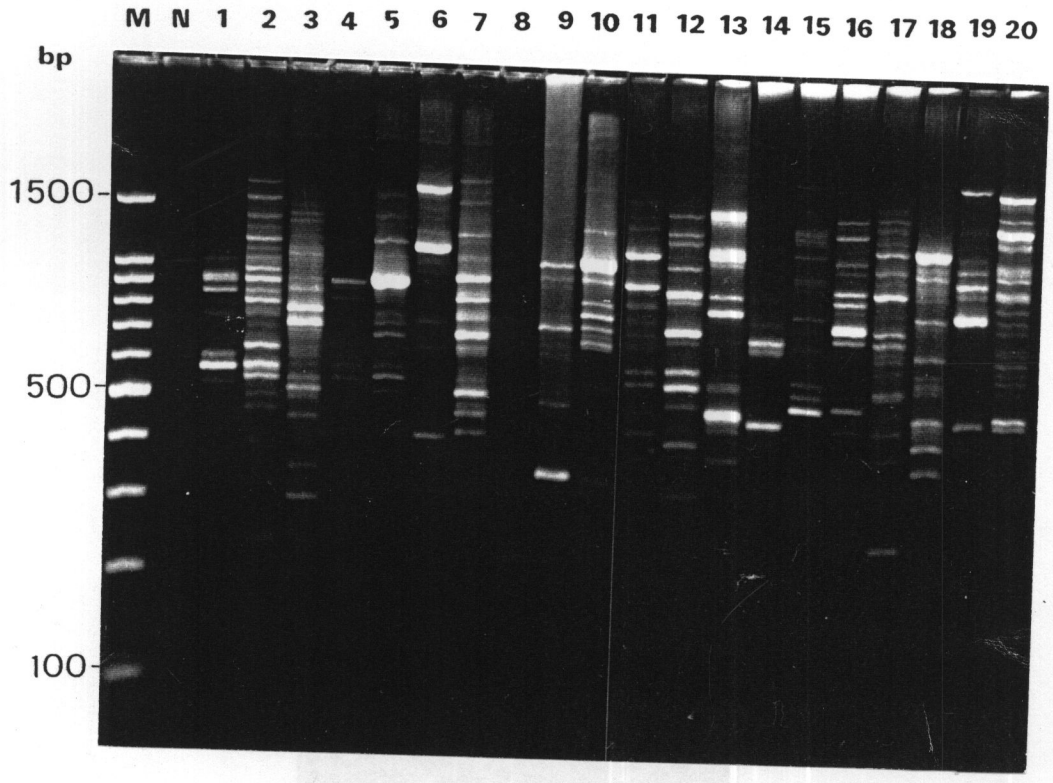

Fig 3.10 Ten microliters of each RAPD reaction was electrophoretically separated through a 1.8% Metaphor agarose gel.

lane M = A 100 bp DNA ladder

lane N = Negative control

lanes 1-20 = Banding patterns of *T. clareae* resulted from RAPD-PCR using primer OPA01-OPA20, respectively.

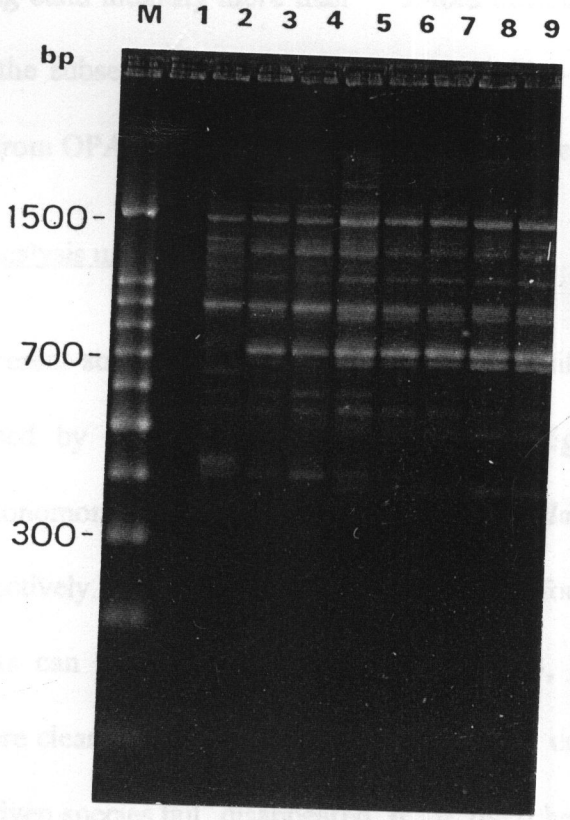

Fig 3.11 Optimization of $MgCl_2$ concentration for RAPD-PCR assay using the primer OPA07 (at 0.4 μM primer concentration).

lane M = A 100 bp DNA ladder

lane 1-9 = Amplification patterns resulted from including of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM $MgCl_2$ in the RAPD reaction, respectively

were found in *T. clareae* and *T. konigerum*, respectively. Only thirteen fragments were shared by both species (Table 3.1 and 3.2).

The RAPD patterns of all investigated individuals are shown in Appendix A. Only reproducible bands were scored for presence (1) or absence (0). RAPD fragments having band intensity more than 2-fold difference did not score and omitted from the subsequent analysis. One hundred fifty-two RAPD genotypes were observed from OPA07, OPA11 and OPA12, respectively.

RAPD analysis using OPA07 primer

In the present study, a polymorphic band was classified to be polymorphic if it is possessed by less than 95% of overall investigated specimens. The percentage of monomorphic and polymorphic bands in *T. clareae* were found 8.8% and 50%, respectively.Eigth monomorphic bands were found in *T. koenigerum* (Table 3.2). As can be seen from Fig. 3.12 and 3.13, inter and intraspecific polymorphic were clearly observed. In this study, a band carried by individuals of all colony in a given species but disappeared from the other was regarded as a species-specific band. For *T. clareae*, 4 fragments (1980, 1210, 850 and 640 bp) were species-specific while a total of 8 species-specific fragments were observed in *T. koenigerum* (2040, 2000, 1550, 1375, 1195, 1110, 610 and 430 bp). The 950 bp, 880 bp and 760 bp fragments were found in both taxa and may be useful as genus specific RAPD markers. A 1360 bp fragment was only observed in the C1D sample. The percentage of RAPD bands observed in each sample (colony) of *T. clareae* and *T. koenigerum* can be illustrated by Table 3.3 (A).

A total of 60 detected RAPD genotypes was observed when *T. careae* and *T. koenigerum* individuals were amplified using OPA07. Genotypes distribution frequencies resulted from RAPD banding patterns are shown in Table 3.4 (A). Interestingly, no shared genotypes were observed at interspecific level.

RAPD analysis using OPA11 primer

Five species-specific bands (1650 bp, 1140 bp, 920 bp,540 bp and 430 bp fragments) were found in *T. clareae* whereas four specific RAPD fragments (1490, 1440, 1300 and 810 bp) were observed in *T. koenigerum* (Fig. 3.14 and 3.15). The RAPD fragment found in all groups was not observed. The 910 bp and 640 bp fragments were found in *T. koenigerum* but observed in relatively low frequencies in *T. clareae* (0.8 % and 19.2 % of investigated individuals). The percentage of RAPD bands observed in each sample (colony) of *T. clareae* and *T. koenigerum* can be illustrated by Table 3.3 (B). Fifty-eight genotypes were observed from 141 specimens investigated (Table3.4B). The interspecifically shared genotype was not observed between these taxa. Interestingly, a 1210 bp appeared in almost all *T. clareae* samples in *A. mellifera* host (83.9% of all individuals) but it was found in 25.4% of *T. clareae* sample in *A. dorsata* host (Table 3.3B).

RAPD analysis using OPA12 primer

The results from this primer clearly indicated interspecific differences between *T. clareae* and *T. koenigerum* (Fig. 3.16 and 3.17). A total of seven fragment (1490 bp, 1360 bp, 1250 bp, 900 bp, 720 bp 595 bp and 545 bp) was specific to the former. Four of these (900 bp, 720 bp, 595 bp and 545 bp)

were fixed and found in all investigated *T. clareae* individuals (Table 3.4C). Eight *T. koenigerum*-specific fragments were completely fixed. Therefore these and four RAPD fragments from *T. clareae* can be used for unambiguous dissociation of these two species easily. A 1250 bp RAPD fragment was fixed in *T. koenigerum* and found in almost all of the *T. clareae* mite. Therefore, it may be served with relatively high confidence as a genus-specific RAPD marker.

Genotype distribution frequencies of RAPD patterns using OPA12 are shown in Table 3.4C. The lowest number of genotype (35) was found from this primer compared to 60 and 58 patterns resulted from OPA07 and OPA11, respectively.

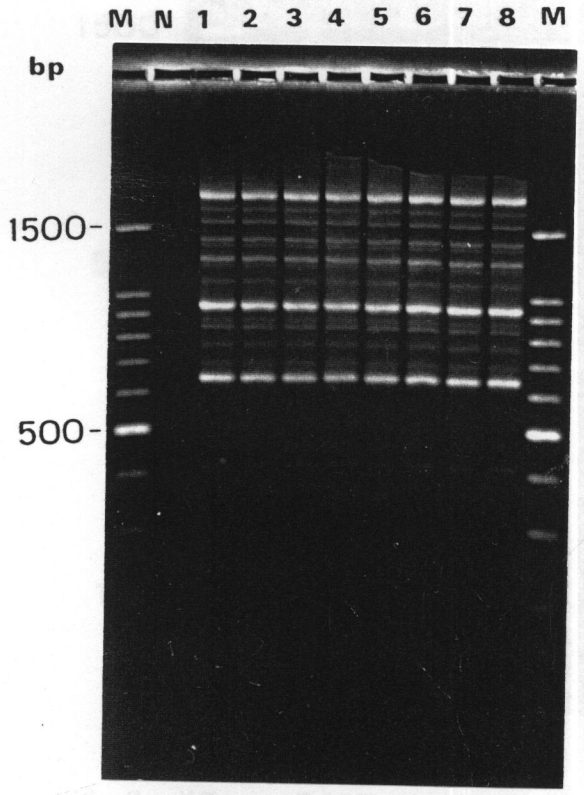

Fig. 3.12 RAPD patterns of *T. clareae* generated from OPA07. Ten microliters of the PCR products was electrophoretically separated through a 1.8% Metaphor agarose gel.

lane M = A 100 bp DNA ladder

lane N = Negative control

lanes 1-8 = RAPD banding patterns of *T. clareae* individuals from the same colony.

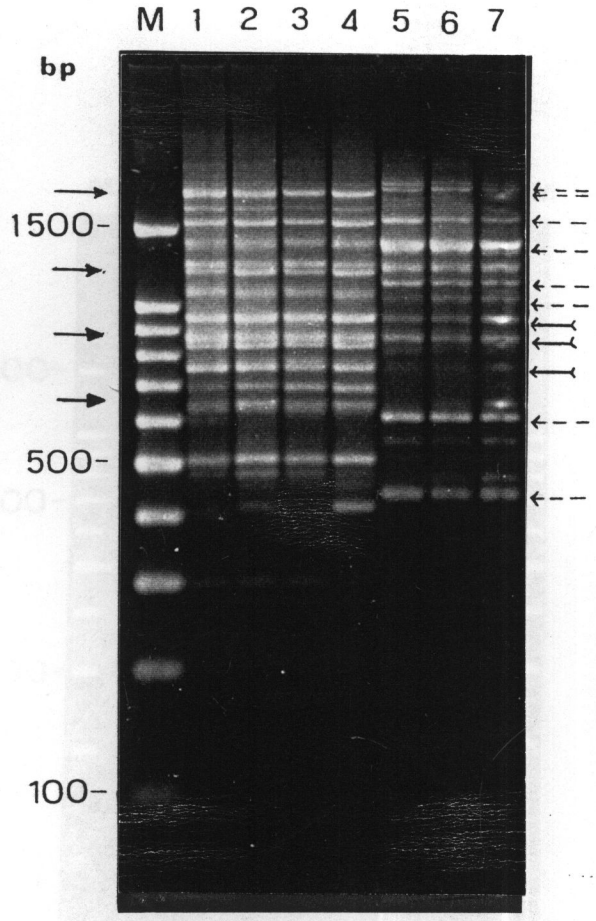

Fig 3.13 Interspecifically different RAPD banding patterns between *T. clareae* and *T. koenigerum* were observed. Ten microliters of RAPD-amplified products using OPA07 was loaded into a 1.8% Metaphor agarose gel and electrophoretically analyzed.

lane M = A 100 bp DNA ladder

lanes 1-4 = RAPD banding patterns of four *T. clareae* individuals

lanes 5-7 = RAPD banding patterns of three *T. koenigeum* individuals.

⟶ = showing species specific band of *T. clareae*,

- - → = showing species specific band of *T. koenigerum*

⟼ = showing genus specific band .

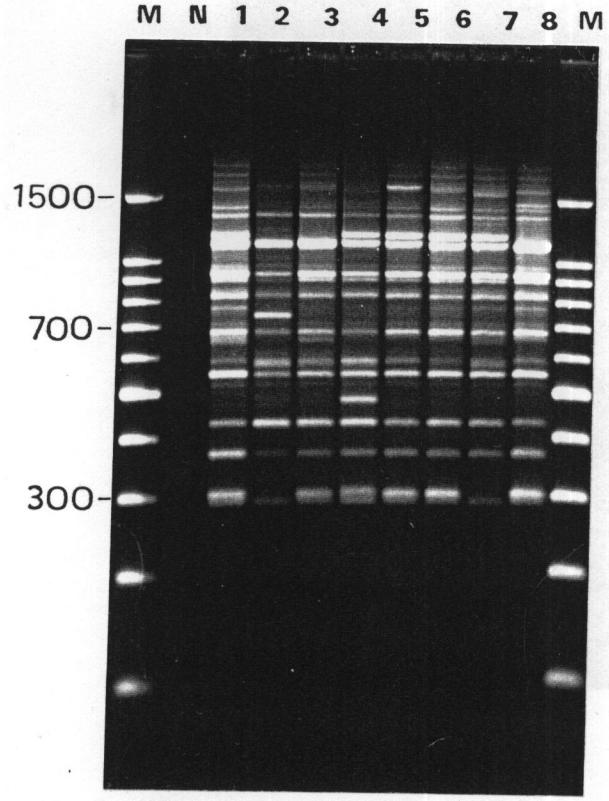

Fig. 3.14 RAPD patterns of *T. clareae* generated from OPA11. Ten microliters of the resulting product were loaded into a 1.8 % Metaphor agarose gel and electrophoretically analyzed.

lane M = A 100 bp DNA ladder

lane N = Negative control

lanes 1-8 = RAPD banding patterns of eight individuals of *T. clareae* originating from the same colony.

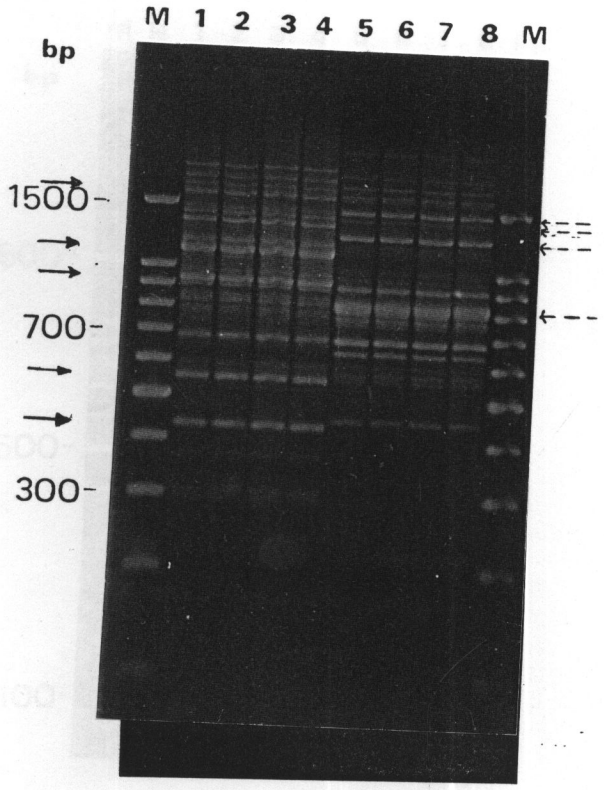

Fig 3.15 Interspecifically different RAPD banding patterns between *T. clareae* and *T. koenigerum* were observed. Ten microliters of RAPD-amplified products using OPA11 was loaded into a 1.8% Metaphor agarose gel and electrophoretically analyzed.

lane M = A 100 bp DNA ladder

lanes 1-4 = RAPD banding patterns of 4 representatives of *T. clareae*

lanes 5-8 = RAPD banding patterns of 4 representatives of *T. koenigerum*

→ = showing species specific band of *T. clareae*,

--→ = showing species specific band of *T. koenigerum*

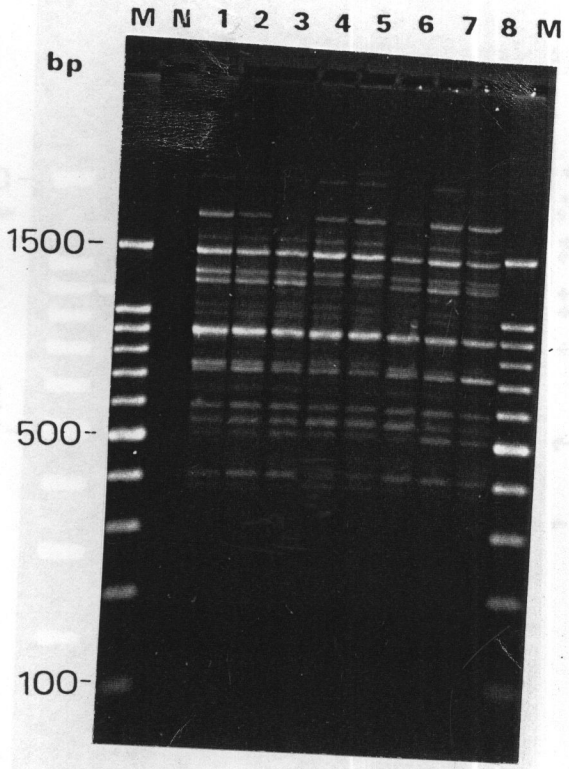

Fig. 3.16 RAPD banding patterns of *T. clareae* generated from OPA12. Ten microliters of the resulting product were loaded into a 1.8 % Metaphor agarose gel and electrophoretically analyzed.

lane M = A 100 bp DNA ladder

lane N = Negative control

lanes 1-8 = RAPD banding patterns of eight individuals of *T. clareae* originating from the same colony.

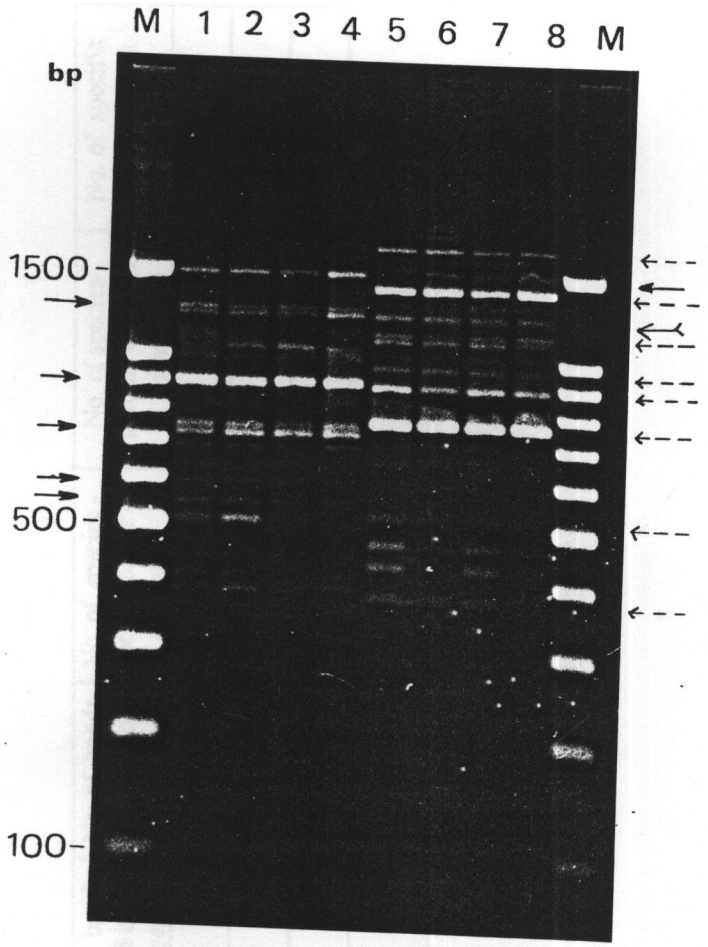

Fig 3.17 Interspecifically different RAPD patterns between *T. clareae* and *T. koenigerum* were observed. Ten microliters of RAPD-amplified products using OPA12 was loaded into a 1.8% Metaphor agarose gel and electrophoretically analyzed.

lane M = A 100 bp DNA ladder

lanes 1-4 = RAPD banding patterns of 4 representatives of *T. clareae*

lanes 5-8 = RAPD banding patterns of 4 representatives of *T. koenigeum*

→ = showing species specific band of *T. clareae*,

--→ = showing species specific band of *T. koenigerum*

⤚ = showing genus specific band .

Table 3.1 Number of amplified bands in RAPD analysis of *Tropilaelaps clareae* and *T. koenigeum*

Primer No.	No. of amplified bands	No. of bands found in *T. clareae*	No. of bands found in *T. koenigerum*	No. of bands found in both species	No. of species - specific bands of *T. clareae*	No. of species-specific bands of *T. koenigerum*	No. of specific bands of both species
OPA07	34	20	8	6	4	8	3
OPA11	26	18	5	3	5	4	-
OPA12	26	13	9	4	7	8	1

Table 3.2 Total number of bands, percentage of polymorphic and monomorphic bands (in brackets) found in *T. clareae* and *T. koenigerum*

Primer	No. of amplified bands	No. of bands found in *T. clareae*		No. of bands found in *T. koenigerum*		No. of bands found in both species	
		No. of monomorphic bands	No. of polymorphic bands	No. of monomorphic bands	No. of polymorphic bands	No. of monomorphic bands	No. of polymorphic bands
OPA07	34	3 (8.8%)	17 (50%)	8 (23.5%)	-	3 (8.8%)	3 (8.8%)
OPA11	26	6 (23%)	16 (46.2%)	3 (11.5%)	2 (7.7%)	-	3 (11.5%)
OPA12	26	5 (19.2%)	8 (30.8%)	1 (3.8%)	1 (3.8%)	1 (3.8%)	3 (11.5%)

Table 3.3 The percentage of RAPD fragments (in base pairs) within each colony (n=8 except C2D, n=7 and C1M, n=6) of *T. clareae* and *T. koenigerum*.

A. Primer OPA07

	C1D	C2D	C3D	E1D	E2D	N1D	S1D	S2D	N1M	N2M	NE1M	NE2M	C1M	C2M	E1M	S1M	TKC	TKE
2040	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
2020	0	0	0	100	12.5	0	0	0	0	0	0	0	50	0	0	50	0	0
2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1980	100	100	100	100	100	100	100	100	100	75	100	50	100	100	100	50	0	0
1710	100	87.5	0	100	12.5	0	100	100	12.5	0	0	0	100	100	100	62.5	0	0
1550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1530	100	100	0	100	12.5	0	100	100	87.5	0	100	0	100	100	62.5	50	0	0
1390	100	100	100	100	100	100	100	87.5	100	87.5	100	50	100	0	100	87.5	0	0
1375	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1360	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1210	100	100	100	100	100	100	100	100	25	100	100	100	100	100	100	100	0	0
1195	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1180	0	0	25	62.5	87.5	87.5	100	87.5	75	87.5	100	100	100	87.5	0	75	0	0
1160	0	0	0	12.5	25	12.5	0	0	0	0	0	0	0	12.5	0	25	0	0
1110	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1070	100	100	0	100	100	0	100	100	0	0	0	0	100	50	0	25	0	0
1050	0	0	0	0	12.5	0	0	0	0	0	0	0	0	0	0	0	100	100
950	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	62.5	100
880	100	100	100	100	100	87.5	100	100	87.5	87.5	100	100	100	100	100	100	100	100
850	100	100	100	100	37.5	75	100	100	100	37.5	100	100	100	100	100	100	0	0
780	0	0	0	0	0	0	0	12.5	0	0	0	0	0	0	0	0	0	0
760	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
690	100	100	100	100	0	12.5	100	100	100	87.5	100	0	100	100	100	100	0	0
640	100	87.5	100	100	100	100	100	75	100	100	100	100	100	100	100	100	0	0
620	0	0	0	62.5	0	0	100	62.5	0	0	25	0	83.33	25	0	0	0	0
610	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
590	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	12.5	0	0
550	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	12.5	100	100
510	100	100	100	100	0	0	100	100	0	0	0	0	100	100	100	50	0	0
480	0	0	0	87.5	0	0	100	12.5	87.5	25	0	0	16.67	12.5	0	0	0	12.5
460	100	100	100	12.5	100	100	50	0	100	100	100	100	83.33	100	100	100	0	0
430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
410	100	0	0	0	0	0	0	25	87.5	0	0	100	100	100	0	87.5	0	0
405	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12.5	0	0

Table 3.3 (continue)
B. Primer OPA11

	C1D	C2D	C3D	E1D	E2D	N1D	S1D	S2D	N1M	N2M	NE1M	NE2M	C1M	C2M	E1M	S1M	TKC	TKE
1650	75	100	87.5	100	100	100	100	100	100	87.5	37.5	75	100	100	50	100	0	0
1490	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	75	100
1440	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	87.5	100
1300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1210	0	28.75	12.5	87.5	75	0	0	0	87.5	62.5	100	75	100	100	62.5	87.5	0	0
1140	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
920	100	100	100	100	100	100	100	100	100	100	100	87.5	100	100	100	100	0	0
910	0	0	0	0	0	0	0	0	0	0	0	12.5	0	0	0	0	100	100
820	100	100	0	100	100	0	100	100	100	100	100	75	100	100	100	100	0	0
810	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
770	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	75	0
740	0	0	0	0	0	0	0	0	100	0	37.5	12.5	100	0	12.5	0	0	0
720	0	0	0	0	0	0	0	0	0	62.5	12.5	12.5	0	0	0	0	0	0
680	100	100	100	100	100	100	100	100	75	100	87.5	87.5	0	100	100	100	0	0
640	12.5	28.57	0	0	0	100	0	37.5	100	0	12.5	12.5	0	0	0	0	100	100
590	87.5	0	0	0	37.5	100	100	100	87.5	100	87.5	87.5	100	100	100	37.5	0	0
580	0	0	25	0	0	0	0	0	12.5	0	12.5	25	0	12.5	0	0	0	0
540	100	100	100	100	100	100	100	100	100	100	87.5	100	100	100	100	100	0	0
480	25	0	0	0	0	0	0	0	75	62.5	50	37.5	100	100	12.5	0	0	0
450	0	0	0	0	0	0	0	0	0	100	12.5	12.5	0	0	0	0	75	100
430	100	100	100	100	100	100	100	100	100	87.5	100	100	100	87.5	100	87.5	0	0
395	0	0	0	0	0	0	0	0	87.5	0	0	0	0	0	0	0	0	0
370	100	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
305	100	100	37.5	87.5	75	87.5	100	50	0	0	50	37.5	33.33	75	75	50	0	0
290	87.5	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
265	0	0	0	0	0	0	0	0	0	100	0	0	100	100	0	0	0	0

Table 3.3 (continue)

C. OPA12 Primer

	C1D	C2D	C3D	E1D	E2D	N1D	S1D	S2D	N1M	N2M	NE1M	NE2M	C1M	C2M	E1M	S1M	TKC	TKE
1670	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1490	100	100	75	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
1420	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1360	100	100	75	100	100	100	87.5	100	100	50	100	100	66.67	100	75	100	0	0
1250	100	100	75	100	100	100	87.5	100	100	75	100	100	100	100	75	100	100	100
1140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
1120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	100	100
1050	100	0	100	12.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
970	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
900	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
895	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
760	0	12.5	0	0	0	0	0	0	25	0	0	0	16.67	25	0	0	100	100
740	100	87.5	0	100	37.5	75	0	0	62.5	62.5	100	75	100	100	0	100	0	0
720	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
680	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0
660	0	100	0	100	100	0	0	0	62.5	0	75	50	0	100	0	100	0	0
595	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
545	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0
510	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
505	0	87.5	25	62.5	87.5	100	0	0	100	100	100	50	100	100	100	100	0	0
480	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
420	0	0	0	0	0	0	0	0	0	50	0	0	33.33	62.5	100	0	100	100
410	0	100	100	100	100	100	100	100	100	75	100	100	100	100	100	100	0	0
390	100	0	100	0	0	0	0	0	100	75	0	0	100	100	25	100	0	0
385	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
310	0	0	100	0	0	0	0	0	100	62.5	0	0	0	0	100	75	0	0

Table 3.4 Genotype distribution frequencies of RAPD patterns of *T. clareae* and *T. koenigerum*.

A. Primer OPA07

sample	Genotype																																	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI
C1D	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2D	0	5	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C3D	0	0	0	0	6	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	2	0	0	0	0	
C2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	3	1	
E1D	0	0	0	0	0	0	1	1	1	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
E2D	0	0	0	0	0	0	0	0	0	0	0	5	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
E1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
N1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
N1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
N2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
NE1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
NE2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
S1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0	0	0	0	0	
S2D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	1	1	1	0	0	0	0	0	0	0	
S1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
TKC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
TKE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Table 3.4 (continue)

Primer OPA 07 (continue)

sample	Genotype																								
	AJ	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX	AY	AZ	BA	BB	BC	BD	BE	BF	BG	BH
C1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C3D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E2D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E1M	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N1M	0	0	4	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N2M	0	0	0	0	0	0	0	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE1M	0	0	0	0	0	0	0	0	0	0	0	2	6	0	0	0	0	0	0	0	0	0	0	0	0
NE2M	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0	0	0
S1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S2D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2	0	0	0	
TKC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	5	0	
TKE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	1	

Table 3.4 (continue)

B. Primer OPA11

sample	Genotype																																		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI
C1D	5	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2D	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C3D	0	0	0	0	0	0	0	0	1	3	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	0	0	0
C2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	4	1	0	0	0	0	0	0	0
E1D	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E2D	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E1M	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0
N1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1
N2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE2M	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S1D	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S2D	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0
S1M	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TKC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TKE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3.4 (continue)
B. Primer OPA11 (continue)

sample	Genotype																						
	AJ	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX	AY	AX	BA	BB	BC	BD	BE	BF
C1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C3D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E2D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N1M	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N2M	0	0	0	1	1	2	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE1M	0	0	0	0	0	0	0	0	0	2	2	2	1	0	0	0	0	0	0	0	0	0	0
NE2M	0	0	0	0	0	0	0	0	0	0	1	0	0	1	2	1	1	0	0	0	0	0	0
S1D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S2D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S1M	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
TKC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	1	1
TKE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0

Table 3.4 (continue)
C. Primer OPA12

sample	Genotype																																		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI
C1D	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2D	0	6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C3D	0	0	0	5	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0
E1D	0	4	3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E2D	0	3	0	0	0	0	0	0	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	2	0	0	0	0	0	0	0	0	0	0
N1D	0	0	0	0	0	0	0	0	0	0	2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	2	1	0	0	0	0	0	0	0
N2M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	0	1	2	1	1	0	0	0
NE1M	0	6	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NE2M	0	2	1	0	0	0	0	0	0	1	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
S1D	0	0	0	0	0	0	0	0	0	0	0	0	7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S2D	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S1M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0
TKC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0
TKE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8

Estimation of genetic relatedness within and between *Tropilaelaps* species was carried out. Presumably, individuals from the same colony was regarded as the same group, therefore, one hundred and twenty-five investigated individuals were grouped to 16 samples of *T. clareae* and 2 samples of *T. koenigerum*. For *T. clareae*, the similarity index within each group was between 0.8055-1.000 (OPA07), 0.8359 - 1.000 (OPA11) and 0.8251 - 1.000 (OPA12) (Table 3.5). The average similarity within each group was 0.8717-0.9823. Individuals from S1D possessed the highest genetic similarity within group whereas those from N2M showed the lowest within-group similarity. Apparently, the mean similarity index of *T. clareae* in the *A. dorsata* host was higher than that from the *A. mellifera* host (0.9567 in the former compared to 0.9241 in the latter). Genetic polymorphisms were not observed in *T. koenigerum* when OPA12 was employed. Nevertheless, lower similarity indices were observed with other primers. The mean similarity within each group was 0.9559 and 0.9969 for TKC and TKE, respectively.

Similarity index between group of *T. clareae* from OPA07 was 0.5829-0.9870 whereas that between a pair of TKC and TKE was 0.9600. Interspecific similarity ranged from 0.1749-0.2875. These values were dramatically lower than between group similarity of each species.

The similarity index both within and between species resulted from RAPD analysis using OPA11 is shown by Table 3.6B. The interspecific similarity between a pair of representative groups of *T. clareae* and *T. koenigerum* was 0.0000 to 0.1113. Within each species, the similarity between pairs of samples was

0.5835-0.9582 in *T. clareae* while that between TKC and TKE was 0.9023. Regarding the host species, *T. clareae* from *A. dorsata* showed higher similarity index (0.9582-0.9870) than did *T. clareae* from *A. mellifera* (0.7747-0.9018).

Like OPA07 and OPA11, large similarity values resulted from the primer OPA12 were observed intraspecifically (0.6543-0.9820 for *T. clareae* and 0.9600 for *T. koenigerum*) whereas significant lower similarity was found at interspecific level (0.0621-0.1515). Considering the host species, the ranges of similarity of *T. clareae* were not largely different (0.6943-0.9821 and 0.7736-0.9489) for A. *dorsata* and *A. mellifera* host species, respectively.

The mean similarity index between pairs of samples across all primers was illustrated by Table 3.6D. Higher intraspecific similarity levels were typically observed than interspecific similarity level. Genetic distance was converted from similarity values and showed the same trend (Dij = 1-Sij).

In *T. clareae*, the mean genetic distance between pairs of samples across all investigated primer ranged from 0.0181 to 0.2013 while that between TKC and TKE was 0.0289. Considering the host species, genetic distance of *T. clareae* within *A. mellifera* was 0.0538-0.1446.

Table 3.5 Within colony similarity of sixteen and two of *T. clareae* and *T. koenigerum*

Samples	OPA07	OPA11	OPA12	average all primers
C1D	1.0000	0.9231	1.0000	0.9744
C2D	0.9787	0.9516	0.9625	0.9643
C3D	0.9814	0.9054	0.9140	0.9336
C1M	0.9538	0.9802	0.9382	0.9574
C2M	0.9423	0.9661	0.9626	0.9570
E1D	0.9367	0.9760	0.9636	0.9588
E2D	0.9061	0.9355	0.9614	0.9343
E1M	0.9786	0.9081	0.9340	0.9402
N1D	0.9255	0.9881	0.9774	0.9637
N1M	0.9249	0.9273	0.9392	0.9305
N2M	0.8740	0.9159	0.8251	0.8717
NE1M	0.9829	0.8359	0.9796	0.9328
NE2M	0.9429	0.8359	0.9182	0.8990
S1D	0.9827	1.0000	0.9643	0.9823
S2D	0.9175	0.9080	1.0000	0.9418
S1M	0.8055	0.9239	0.9829	0.9041
TKC	0.9781	0.8895	1.0000	0.9559
TKE	0.9907	1.0000	1.0000	0.9969

Table 3.6 Similarity index with a correction of within-colony similarity effect (upper diagonal) and genetic distance(below diagonal) for each pairwise comparison of *T. clareae* and *T koenigerum* samples.

A. Primer OPA07

	C1D7	C2D7	C3D7	C1M7	C2M7	E1D7	E2D7	E1M7	N1D7	N1M7	N2M7	NE1M7	NE2M7	S1D7	S2D7	S1M7	TKC7	TKE7
C1D7	-	0.9288	0.8075	0.8907	0.8746	0.8158	0.7113	0.8818	0.6782	0.7168	0.6746	0.7789	0.6904	0.8305	0.8403	0.8050	0.1829	0.2060
C2D7	0.0665	-	0.8700	0.8732	0.8543	0.8502	0.7643	0.9363	0.7319	0.7026	0.7277	0.8365	0.6601	0.8741	0.8728	0.7918	0.1988	0.2237
C3D7	0.1831	0.1100	-	0.7760	0.7828	0.7414	0.7736	0.9221	0.8520	0.7208	0.8464	0.8723	0.7733	0.7741	0.7601	0.7980	0.2192	0.2463
C1M7	0.0863	0.0931	0.1915	-	0.9870	0.8797	0.7326	0.8322	0.6981	0.7323	0.6946	0.8146	0.7186	0.9111	0.8940	0.8235	0.1749	0.1984
C2M7	0.0965	0.1062	0.1790	0.0510	-	0.7935	0.6881	0.8458	0.6925	0.7279	0.7000	0.8074	0.7546	0.8439	0.8427	0.8152	0.1905	0.2155
E1D7	0.1526	0.1075	0.2176	0.0656	0.1460	-	0.6849	0.8072	0.6417	0.6719	0.6525	0.7568	0.5829	0.9200	0.8780	0.7420	0.1799	0.2098
E2D7	0.2417	0.1781	0.1710	0.1974	0.2362	0.2366	-	0.7250	0.8658	0.6719	0.8070	0.8067	0.7847	0.6603	0.7021	0.7102	0.2315	0.2587
E1M7	0.1075	0.0424	0.0578	0.1340	0.1147	0.1505	0.2174	-	0.7808	0.7187	0.7762	0.8541	0.7505	0.8319	0.8249	0.8102	0.2073	0.2331
N1D7	0.2846	0.2202	0.1015	0.2415	0.2424	0.2894	0.0501	0.1713	-	0.7005	0.8678	0.8738	0.8567	0.6935	0.6657	0.7401	0.2220	0.2509
N1M7	0.2456	0.2492	0.2324	0.2071	0.2057	0.2590	0.2590	0.2331	0.2248	-	0.7126	0.7994	0.7072	0.7204	0.6633	0.7368	0.2570	0.2875
N2M7	0.2624	0.1986	0.0813	0.2174	0.2081	0.2528	0.0830	0.1501	0.0320	0.1868	-	0.8670	0.7933	0.7072	0.6647	0.7451	0.2206	0.2519
NE1M7	0.2125	0.1443	0.1099	0.1538	0.1552	0.2012	0.1378	0.1266	0.0804	0.1545	0.0614	-	0.8069	0.8168	0.7923	0.8073	0.2104	0.2366
NE2M7	0.2811	0.3006	0.1889	0.2297	0.1880	0.3569	0.1398	0.2557	0.0775	0.2267	0.1151	0.1560	-	0.6391	0.6315	0.7761	0.2317	0.2600
S1D7	0.1608	0.1066	0.2079	0.0571	0.1186	0.0397	0.2190	0.1487	0.2606	0.2334	0.2211	0.1660	0.3237	-	0.9157	0.7500	0.1798	0.2108
S2D7	0.1184	0.0752	0.1893	0.0416	0.0871	0.0491	0.2106	0.1231	0.2558	0.2579	0.2310	0.1579	0.2987	0.0344	-	0.7473	0.1921	0.2174
S1M7	0.0978	0.1003	0.0954	0.0561	0.0587	0.1291	0.1629	0.0818	0.1254	0.1284	0.0946	0.0869	0.0980	0.1440	0.1142	-	0.2074	0.2319
TKC7	0.8064	0.7799	0.7607	0.7913	0.7699	0.7778	0.7109	0.7712	0.7300	0.6948	0.7057	0.7703	0.7290	0.8008	0.7559	0.6864	-	0.9804
TKE7	0.7893	0.7611	0.7398	0.7738	0.7510	0.7539	0.6897	0.7515	0.7073	0.6703	0.6804	0.7502	0.7068	0.7759	0.7367	0.6662	0.0043	-

Table 3.6 (continue)

B. Primer OPA11

	C1D11	C2D11	C3D11	C1M11	C2M11	E1D11	E2D11	E1M11	N1D11	N1M11	N2M11	NE1M11	NE2M11	S1D11	S2D11	S1M11	TKC11	TKE11
C1D11	-	0.8608	0.7450	0.7477	0.8403	0.8777	0.8901	0.8989	0.8721	0.7712	0.8023	0.8190	0.8197	0.9582	0.9183	0.8645	0.0155	0.0156
C2D11	0.0765	-	0.7309	0.6644	0.7635	0.8979	0.8771	0.8141	0.7977	0.7014	0.7159	0.7420	0.7472	0.8761	0.8399	0.8531	0.0325	0.0327
C3D11	0.1693	0.1976	-	0.5835	0.6710	0.7949	0.7837	0.7246	0.7783	0.6480	0.6654	0.6610	0.7011	0.7779	0.7594	0.7714	0.0000	0.0000
C1M11	0.2039	0.3015	0.3592	-	0.8953	0.7607	0.7735	0.7859	0.6846	0.8654	0.8476	0.8072	0.7753	0.7667	0.7539	0.7747	0.0000	0.0000
C2M11	0.1043	0.1953	0.2647	0.0778	-	0.8409	0.8568	0.8581	0.7737	0.8240	0.9018	0.8419	0.8263	0.8583	0.8284	0.8515	0.0000	0.0000
E1D11	0.0719	0.0659	0.1458	0.2174	0.1221	-	0.9536	0.8861	0.8104	0.7933	0.7966	0.8358	0.8300	0.9080	0.8680	0.9424	0.0000	0.0000
E2D11	0.0391	0.0664	0.1367	0.1844	0.0940	0.0021	-	0.8989	0.8293	0.8069	0.8173	0.8434	0.8418	0.9247	0.8911	0.9339	0.0000	0.0000
E1M11	0.0167	0.1158	0.1822	0.1583	0.0790	0.0561	0.0229	-	0.8319	0.8108	0.8243	0.8643	0.8455	0.9263	0.8929	0.8873	0.0000	0.0000
N1D11	0.0832	0.1722	0.1684	0.2995	0.2034	0.1716	0.1325	0.1162	-	0.7125	0.7462	0.7543	0.7897	0.9026	0.9070	0.8016	0.1113	0.1119
N1M11	0.1539	0.2381	0.2683	0.0883	0.1227	0.1584	0.1244	0.1069	0.2425	-	0.8024	0.8215	0.7992	0.7941	0.7951	0.8140	0.0000	0.0000
N2M11	0.1172	0.2197	0.2453	0.1005	0.0392	0.1494	0.1084	0.0877	0.2058	0.1192	-	0.8064	0.8035	0.8269	0.8280	0.8233	0.0000	0.0000
NE1M11	0.0606	0.1518	0.2097	0.1009	0.0591	0.0702	0.0423	0.0078	0.1577	0.0601	0.0696	-	0.8206	0.8326	0.8171	0.8420	0.0217	0.0250
NE2M11	0.0380	0.1248	0.1478	0.1110	0.0529	0.0542	0.0221	0.0047	0.1006	0.0607	0.0506	0.0000	-	0.8447	0.8341	0.8398	0.0360	0.0395
S1D11	0.0033	0.0997	0.1748	0.2235	0.1248	0.0800	0.0430	0.0278	0.0915	0.1695	0.1311	0.0853	0.0514	-	0.9545	0.8998	0.0000	0.0000
S2D11	0.0172	0.1099	0.1673	0.2102	0.1287	0.0940	0.0506	0.0351	0.0610	0.1425	0.1040	0.0748	0.0361	0.0195	-	0.8779	0.0531	0.0534
S1M11	0.0590	0.0847	0.1433	0.1774	0.0935	0.0076	0.0000	0.0288	0.1499	0.1116	0.0967	0.0379	0.0183	0.0622	0.0580	-	0.0000	0.0000
TKC11	0.8906	0.8880	0.8974	0.9349	0.9278	0.9327	0.9125	0.8988	0.8275	0.9084	0.9084	0.8410	0.8050	0.9447	0.8657	0.9067	-	0.9023
TKE11	0.9459	0.9431	0.9527	0.9901	0.9830	0.9880	0.9677	0.9540	0.8821	0.9636	0.9586	0.8930	0.8567	1.0000	0.9206	0.9620	0.0424	-

Table 3.6 (continue)

C. Primer OPA12

	C1D12	C2D12	C3D12	C1M12	C2M12	E1D12	E2D12	E1M12	N1D12	N1M12	N2M12	NE1M12	NE2M12	S1D12	S2D12	S1M12	TKC12	TKE12
C1D12	-	0.7548	0.8003	0.8193	0.7874	0.7832	0.7278	0.6543	0.7842	0.7662	0.7337	0.7714	0.7851	0.7587	0.7587	0.7915	0.0870	0.0909
C2D12	0.2278	-	0.6943	0.8564	0.9082	0.9637	0.9547	0.7679	0.9236	0.8608	0.7772	0.9705	0.9284	0.8309	0.8480	0.9102	0.0942	0.0983
C3D12	0.1567	0.2453	-	0.7587	0.7240	0.7039	0.7156	0.7898	0.7361	0.8234	0.7548	0.7016	0.7246	0.7679	0.7780	0.7917	0.0621	0.0648
C1M12	0.1498	0.0953	0.1674	-	0.9080	0.8479	0.8320	0.8096	0.9005	0.8731	0.8567	0.8830	0.8517	0.7880	0.8005	0.8927	0.1224	0.1276
C2M12	0.1939	0.0557	0.2143	0.0424	-	0.8997	0.8862	0.7968	0.8621	0.8908	0.8221	0.9103	0.8604	0.7508	0.7671	0.9374	0.1439	0.1497
E1D12	0.1986	0.0007	0.2349	0.1030	0.0634	-	0.9440	0.7489	0.9137	0.8475	0.7647	0.9644	0.9315	0.8373	0.8544	0.9043	0.0843	0.0880
E2D12	0.2529	0.0086	0.2220	0.1178	0.0758	0.0185	-	0.7910	0.9149	0.8547	0.7717	0.9509	0.9202	0.8606	0.8779	0.8907	0.0861	0.0900
E1M12	0.3127	0.1817	0.1342	0.1265	0.1515	0.1999	0.1567	-	0.8227	0.8427	0.8493	0.7846	0.7736	0.7826	0.7923	0.8114	0.1452	0.1515
N1D12	0.2045	0.0477	0.2096	0.0573	0.1080	0.0568	0.0545	0.1330	-	0.8509	0.8257	0.9512	0.9283	0.8845	0.9020	0.8666	0.0879	0.0920
N1M12	0.2034	0.0914	0.1630	0.0656	0.0601	0.1039	0.0959	0.0938	0.1074	-	0.8368	0.8669	0.8322	0.7650	0.7815	0.9489	0.0985	0.1025
N2M12	0.1788	0.1180	0.1148	0.0250	0.0718	0.1297	0.1215	0.0303	0.0756	0.0454	-	0.8012	0.7750	0.7414	0.7474	0.8337	0.1105	0.1152
NE1M12	0.2184	0.0019	0.2452	0.0759	0.0608	0.0073	0.0196	0.1722	0.0273	0.0925	0.1012	-	0.9369	0.8367	0.8538	0.9148	0.0842	0.0879
NE2M12	0.1740	0.0133	0.1915	0.0765	0.0801	0.0094	0.0196	0.1525	0.0195	0.0965	0.0966	0.0120	-	0.8867	0.9042	0.8649	0.0881	0.0921
S1D12	0.2235	0.1339	0.1713	0.1632	0.2127	0.1267	0.1022	0.1666	0.0863	0.1867	0.1534	0.1352	0.0545	-	0.9821	0.7550	0.0833	0.0875
S2D12	0.2235	0.1347	0.1790	0.1686	0.2142	0.1274	0.1028	0.1747	0.0868	0.1881	0.1652	0.1360	0.0550	0.0000	-	0.7714	0.0952	0.1000
S1M12	0.1999	0.0638	0.1567	0.0678	0.0354	0.0690	0.0814	0.1470	0.1136	0.0121	0.0703	0.0664	0.0857	0.2185	0.2200	-	0.0777	0.0808
TKC12	0.9130	0.8884	0.8949	0.8467	0.8374	0.8975	0.8964	0.8218	0.9008	0.8711	0.8020	0.9056	0.8710	0.8988	0.9048	0.9137	-	0.9600
TKE12	0.9091	0.8843	0.8922	0.8415	0.8316	0.8938	0.8907	0.8155	0.8967	0.8670	0.7974	0.9019	0.8670	0.8964	0.9000	0.9106	0.0400	-

Table3.6 (continue)

D. overall primer

	C1D	C2D	C3D	C1M	C2M	E1D	E2D	E1M	N1D	N1M	N2M	NE1M	NE2M	S1D	S2D	S1M	TKC	TKE
C1D	-	0.8481	0.7843	0.8192	0.8341	0.8256	0.7764	0.8117	0.7783	0.7514	0.7369	0.7898	0.7651	0.8491	0.8391	0.8203	0.0951	0.1042
C2D	0.1236	-	0.7651	0.7980	0.8420	0.9039	0.8654	0.8394	0.8177	0.7549	0.7403	0.8497	0.7786	0.8604	0.8536	0.8517	0.1085	0.1182
C3D	0.1697	0.1843	-	0.7061	0.7259	0.7466	0.7576	0.8122	0.7888	0.7307	0.7555	0.7450	0.7330	0.7733	0.7658	0.7870	0.0938	0.1037
C1M	0.1467	0.1633	0.2314	-	0.9301	0.8294	0.7794	0.8092	0.7611	0.8236	0.7996	0.8349	0.7819	0.8219	0.8161	0.8303	0.0991	0.1087
C2M	0.1216	0.1191	0.2193	0.0571	-	0.8447	0.8104	0.8336	0.7761	0.8142	0.8080	0.8532	0.8138	0.8177	0.8127	0.8680	0.1115	0.1217
E1D	0.1410	0.0580	0.1994	0.1287	0.1105	-	0.8608	0.8141	0.7886	0.7709	0.7379	0.8523	0.7815	0.8884	0.8668	0.8629	0.0881	0.0993
E2D	0.1779	0.0840	0.1763	0.1665	0.1353	0.0857	-	0.8050	0.8700	0.7778	0.7987	0.8670	0.8489	0.8152	0.8237	0.8449	0.1059	0.1162
E1M	0.1456	0.1133	0.1247	0.1396	0.1151	0.1355	0.1323	-	0.8118	0.7907	0.8166	0.8343	0.7899	0.8469	0.8367	0.8363	0.1175	0.1282
N1D	0.1908	0.1467	0.1598	0.1994	0.1843	0.1726	0.0790	0.1402	-	0.7546	0.8132	0.8597	0.8576	0.8269	0.8249	0.8028	0.1410	0.1516
N1M	0.2010	0.1929	0.2013	0.1203	0.1295	0.1739	0.1598	0.1446	0.1916	-	0.7839	0.8293	0.7795	0.7598	0.7466	0.8332	0.1185	0.1300
N2M	0.1861	0.1788	0.1471	0.1143	0.1064	0.1773	0.1043	0.0894	0.1045	0.1171	-	0.8243	0.7906	0.7585	0.7467	0.8007	0.1104	0.1224
NE1M	0.1638	0.0993	0.1883	0.1102	0.0917	0.0929	0.0666	0.1022	0.0885	0.1024	0.0774	-	0.8548	0.8257	0.8211	0.8547	0.1054	0.1165
NE2M	0.1644	0.1462	0.1761	0.1391	0.1070	0.1402	0.0605	0.1376	0.0659	0.1280	0.0874	0.0538	-	0.7902	0.7899	0.8269	0.1186	0.1305
S1D	0.1292	0.1134	0.1847	0.1479	0.1520	0.0821	0.1214	0.1144	0.1461	0.1965	0.1685	0.1288	0.1432	-	0.9508	0.8016	0.0877	0.0994
S2D	0.1197	0.1066	0.1785	0.1401	0.1433	0.0902	0.1213	0.1110	0.1345	0.1962	0.1667	0.1229	0.1299	0.0181	-	0.7989	0.1135	0.1236
S1M	0.1189	0.0829	0.1318	0.1004	0.0625	0.0686	0.0814	0.0859	0.1290	0.0840	0.0870	0.0637	0.0673	0.1416	0.1307	-	0.0950	0.1042
TKC	0.8700	0.8521	0.8510	0.8576	0.8450	0.8693	0.8399	0.8306	0.8194	0.8248	0.8052	0.8390	0.8017	0.8814	0.8421	0.8356	-	0.9476
TKE	0.8814	0.8628	0.8613	0.8685	0.8552	0.8786	0.8494	0.8403	0.8287	0.8336	0.8121	0.8484	0.8102	0.8908	0.8524	0.8463	0.0289	-

The mean genetic distance of all pairwise comparisons was used for phylogenetic reconstruction using unweighted pair-group method using an arithmetic average (UPGMA). The dendrogram indicate large separation of congeneric species like *T. clareae* and *T. koenigerum* (Fig.3.18). Nevertheless, phylogeographic separation was not observed among 16 samples of *T. clareae* (Fig.3.19-3.20). Based on this dendrogram, two lineages of *T. clareae* was observed. The first group contained all samples in the *A. mellifera* host and those of E1D and N1D (from *A. dorsata*). The other group consisted of all remaining *T. clareae* that uses *A. dorsata* as a host. This indicated closer relationships of such a parasite from the same rather than from different host species.

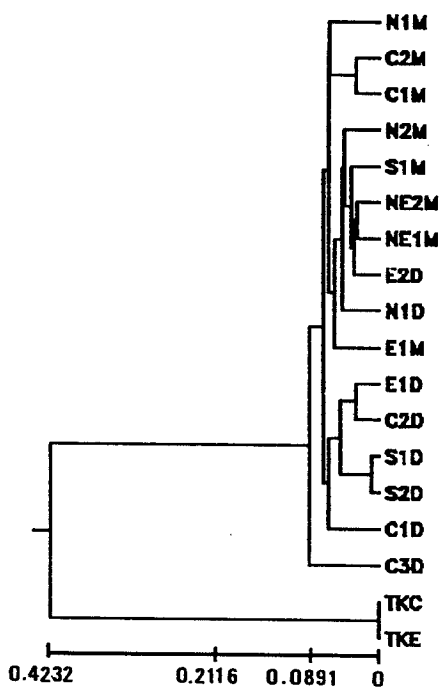

Fig 3.18 UPGMA dendrogram showing relationships among 16 groups of *T. clareae* and 2 groups of *T. koenigerum* based on genetic distance shown in Table 3.6 D.

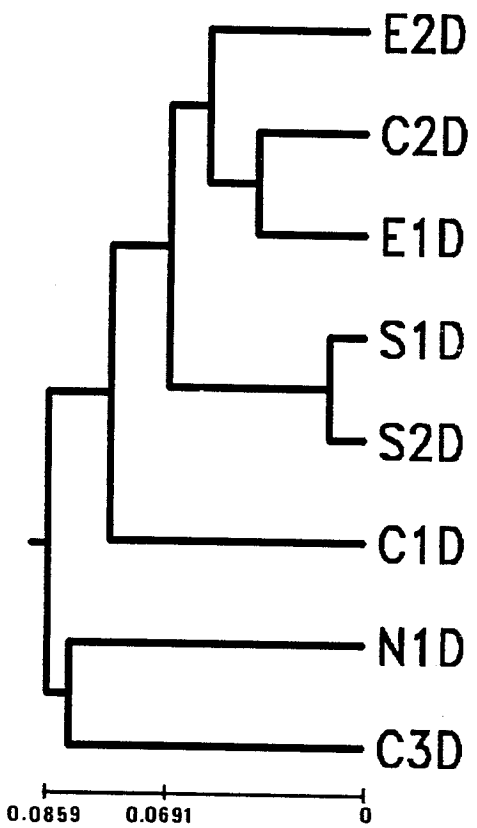

Fig 3.19 UPGMA dendrogram showing the relationships among 8 groups of *T. clareae* having *A. dorsata* host.

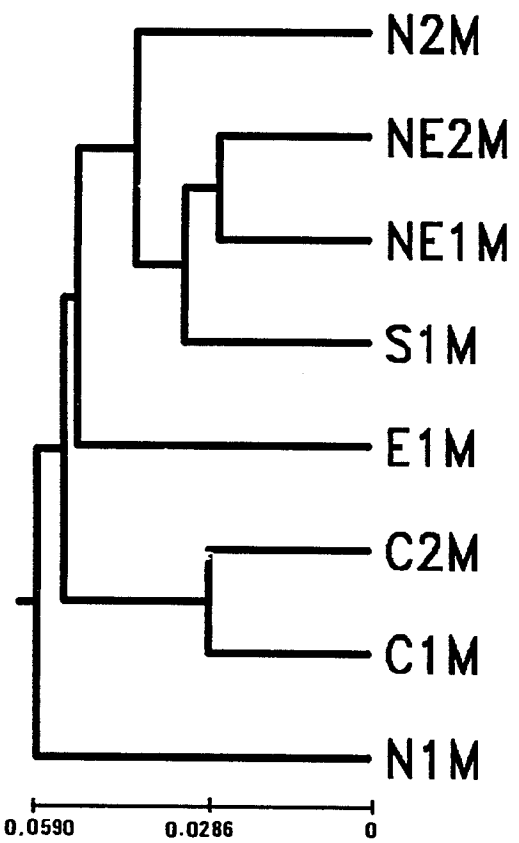

Fig 3.20 UPGMA dendrogram showing the relationships among 8 groups of *T. clareae* having *A. mellifera* host.

CHAPTER IV

Discussion

Several genetic studies, particularly diversity at inter- and intraspecific levels in *Apis* species have been reported (Smith and Brown, 1988, Hall and Smith, 1991, Moritz et al., 1994). Nevertheless, such population genetic studies have not been reported in honey bee-associated mite species like *T. clareae* and *T. koenigerum*.

Originally, *T. clareae* and *T. koenigerum* were found in *A. dorsata* and *A. laboriosa*. After the introduction of *A. mellifera* to Asia since the last three decades, it was found that *T. clareae* can also infect *A. mellifera*. Indicating transitional infection from *A. dorsata* to *A. mellifera* and *vice versa*. At present, *T. clareae* has become the most important pest for the European honey bee, *A. mellifera* (Wongsiri et al., 1995).

Basically, population genetic studies at DNA level of both bee mites and the honey bee *per se* was limited due mainly to insufficient amount of DNA required by classical molecular approaches (e.g. allozymes, hybridization-based RFLP) therefore analysis of genetic polymorphisms in these taxa were not practical except for mtDNA markers. After the polymerase chain reaction (PCR) has been introduced, it is much more convenient to study other genetic markers (PCR-RFLP, RAPD, microsatellite markers) in these species without the requirement for large amount of DNA.

The most disadvantage problem of this study was resulted from sampling strategy. A large number of colonies representing accurate geographic origins

were required. However, it was difficult to collect *T. clareae* and *T. koenigerum* in *A. dorsata* throughout the geographic ranges of the host because of the aggressive behavior of *A. dorsata*. Additionally, *A. dorsata* usually constructs the hives under large branches of tall trees, roofs of buildings or rock overhangs. This was difficult, or in some cases not possible, to sample the specimens (Delfinado-Baker and Peng, 1995). Difficulties for sampling of *A. mellifera* was that this species was introduced and exchangeable between farms, therefore geographic origin of *A. mellifera* in Thailand is not accurate.

In the present study, genomic DNA of *T. clareae* and *T. koenigerum* was extracted using Chelex®. This DNA isolation method is simple and rapid. More importantly, it does not require organic solvent extraction therefore problems from dealing with hazardous substances are eliminated. The only disadvantage of DNA isolated by Chelex® is that sheared DNA is obtained. Although this is not crucial for typical PCR (i.e. amplification of an ITS region), the quality of isolated DNA seems to be the most important factor to obtain reliable results from RAPD-PCR. At this point it was required to compromise this disadvantage by using primers that provided highly consistent results among replication.

It has been reported that the polymorphisms (either sequence or length polymorphisms) at an ITS region are useful for population and/or systematic studies of several closely related taxa (Porter and Collins, 1991; Hsiao et al., 1994; McLain et al., 1995 and Sappal et al., 1995). Based on the fact that the entire intervening ITS region (ITS1 and ITS2) flanked with a highly conserved 5.8S rDNA covers approximately 2-3 kb (Wesson et al.,1993), it is possible to study

genetic diversity between *T. clareae* and *T. koenigerum* by direct sequencing of PCR-amplified ITS region.

Part of the amplified intervening ITS1 and ITS2 region of *T. clareae* and *T. koenigerum* was about 600 bp. The length of this amplified fragments was comparable to that of grasses, 588-603 bp (Hsiao et al., 1994). This indicates interspecific length polymorphisms of the ITS region when amplified with the same primers.

High similarity (94.2%) between the amplified product from each of both species indicated their homologous locus. There were 19 point mutational differences between these two taxa. These consisted of 10 transitional and 9 transvertional substitutions. Sequence differences due to deletions and/or insertions were also observed. All of these corresponded to 3.7% sequence divergence between *T. clareas* and *T. koenigerum*. Apparently, this polymorphic level was comparable to that between *Anopheles freebori* and *A. occidentalis* (3.6%) inferred from the ITS2 sequences but approximately two fold lower than that between *A. hersi* and *A. occidentalis* (Porter and Collin, 1991).

It should be emphasized that a 5 bp insertion observed in all investigated *T. koenigerum* was not observed in *T. clareae* individuals. This can be used to confirm the different taxonomic status between these two taxa. In spite of the fact that no commercial restriction endonucleases recognizing this sequence is available, RFLP analysis of amplified ITS could not be directly deduced using the characteristic of this insertion. However, RFLP analysis can still distinguish the two mite species when amplified ITS DNA is restricted with *Rsa* I (a tetrameric restriction enzyme which recognizes a GTAC sequence). Based on the obtained

sequences, three digested ITS fragments would be observed from *T. clareae* whereas in *T. koenigerum* has one recognizes restriction site for this enzyme in the amplified ITS1-ITS2 region. This is the real advantage of the present study as PCR-RFLP of the ITS region is convenient, fast and cost-effective particularly when the taxonomic status of a given *Tropilaelaps* individual need to be examined unambiguously.

No intraspecific polymorphism was observed in both *T. clareae* and *T. koenginerum*, therefore sequencing of the amplified ITS1-ITS2 could not be used to determine genetic variation at this level. Zhuo et al.(1994) reported no genetic variability between the amplified ITS sequences of 11 lake trout (*Salvelinus namaycush*) individuals from five different populations.

On the basis of DNA sequences of *T. clareae* and *T. koenigerum*, it could be concluded that interspecific cross between these sibling mite species should not be happened as indicated by monophyletic status of each taxa.

An alternative method used for genetic diversity analysis of these two species in this study was RAPD. This technique has been increasingly used for determination of genetic variability in various taxa. RAPD is particularly useful for rapid detection of divergence and for identification of DNA markers (e.g. individual-, population-, species- or genus-specific) between investigated taxa (Hadrys et al. 1992).

Swoboda and Bhalla (1997) used this technique to determine inter- and intraspecific variation of wild and cultivated forms of fan flower, *Scaevola* spp.. Large genetic differences among these species were found from RAPD analysis

suggesting the possibility to apply this approach for breeding programs of these taxa at both intra- and interspecific levels.

The copepod sea louse (*Lepeophtheirus salmonis*) is one of the important horizontally transmitting ectoparasites in salmonids. A drastic decrease in number of the sea trout (*Salmo trutter*) stock in Scotland and Ireland since 1989 has been reported. It is thought that an outbreak of *L. salmonis* originated from poor management roles of inshore salmon farms causes this circumstance. Nevertheless, the previous data based on allozymes did not reveal population subdivisions in *L. salmonis*. Genetic variability of *L. salmonis* from the Atlantic salmon (*S. salar*), rainbow trout (*Onchorhynchus mykiss*) and sea trout (*S. trutta*) host species among the coasts of Scotland was further examined using six informative RAPD primers for sixteen samples (wild and farmed *S. salar*, wild *S. trutta* and farmed *O. mykiss*). The results indicated homogeneity between wild *S. solar* and *S. trutta* but highly significant differentiation was found between native and farmed salmonid species. Moreover, spatial (geographic) and temporal (time) genetic differences of *L. salmonis* from the farms were also observed. More importantly, some *L. salmonis* individuals from the west coast of Scotland possessed the farm markers indicating the possibility of their farm origin (Todd et al., 1997).

It was not surprised that genetic difference between *T. clareae* and *T. koenigerum* was greater than that of within each species. This result was consonant to that from DNA sequencing data. At intraspecific level, a total of one hundred and forty-two genotypes were found from 125 *T. clareae* individuals using three informative RAPD primers whereas only ten genotypes were observed in sixteen individuals of *T. koenigerum*. It should be noted that the genotypes shared between species were not observed.

Regarding this information and that from genetic similarity (or distance), it clearly indicated that *T. clareae* showed greater intraspecific polymorphisms than did *T. koenigerum*.

The percentage of polymorphic bands using three primers (OPA7, OPA11 and OPA12) in *T. clareae* were higher (50%, 46.2% and 30.8%) than those of *T. koenigerum* (0%, 7.7% and 3.8%). Disregarding the sample sizes, this parameter also implied limited genetic variability in *T. koenigerum*. Several useful RAPD markers were also found in both taxa. For example, the number of RAPD fragments specifically found in *T. clareae* was 4 from OPA07, 5 from OPA11 and 7 from OPA12. Comparably, 8, 4 and 8 RAPD fragments were also species-specific to *T. koenigerum*. Moreover, the 980 bp, 880 bp, 760 bp (using OPA07) and 1250 bp (using OPA12) fragments may be useful as genus-specific RAPD markers because they were found in almost all individuals of both taxa.

The presence or absence of scorable RAPD fragments were compared between individuals within a particular sample (colonies), between all individuals from all possible comparisons of a pairs of samples (colonies) representing by S and Sij, respectively. Thus, if S is higher than Sij, it indicates higher level of within group similarity than between group similarity (Lynch, 1990). Theoretically, similarity index estimated from RAPD can be seriously biased because co-migrating bands may not be a homologous allele of a particular locus (Narang et al., 1994). To minimize this effect, the bands having the equal molecular length but their intensity was greater than 2 times differences were regarded to be non-homologous. Such bands were not included in the data analysis.

Considering the host species, the average similarity index of *T. clareae* in *A. mellifera* (0.9241) was lesser than that of *T. clareae* in *A. dorsata* (0.9567) implying higher genetic distance of this parasite within the former host than the latter host species. It has been reported that *T. clareae* can interchangeably switch the host species in both directions therefore this difference may not be typical.

The mean genetic distances of overall primers for all possible pairwise comparisons were subjected to phylogenetic reconstruction (using UPGMA). It indicated large genetic differences between congenerically sibling species like *T. clareae* and *T. koenigerum* (Fig.3.18). Nevertheless, geographically specific patterns were neither observed among all samples of *T. clareae* in *A. mellifera* nor that in *A. dorsata* (Fig.3.19). The reason to explain this was that *T. clareae* in *A. mellifera* have been concurrently moved across vast geographic locations within Thailand accordingly, genetic differentiation of *T. clareae* has been disturbed. The reason for lack of phylogeographical differentiation in *A. dorsata* may be resulted from its ability to migrate with the long distance. High gene flow level in this species may homogenize the intraspecific differentiation. It is premature at this stage to conclude this circumstance unless a further study using other molecular markers from single copy nuclear DNA (scnDNA) or mtDNA is carried out.

The dendrogram indicated monophyletic status of both species. This information supported that there have been no cross-fertilization between these taxa. Within *T. clareae*, it was able to separate all samples to two group. The first group contained all *T. clareae* individuals in *A. mellifera* and those of the samples E2D and N1D (from *A. dorsata*). The second group was composed of the remaining *T. clareae* in the *A. dorsata* host. This information essentially indicated

closer evolutionary relationships of such a parasite from the same rather than from different host species.

Why were E2D and N1D allocated into a group of *T. clareae* in the *A. mellifera* host? This may be a consequence of sampling errors caused by insufficient number of investigated individual within a colony. Nevertheless, it should not be overlooked this signal as *T. clareae* from *A. mellifera* may interspecifically switch to *A. dorsata* and the historical circumstance was incidentally traced by the RAPD results.

RAPD are useful for not only determination of genetic variation and population structure in various organisms but also for identification of markers linked to biologically important phenotypes e.g. such linked to resistance to diseases (Lewis et al, 1997). Bai et al., (1997) screened seven hundred and fifty-six arbitrary primers for identification of RAPD markers linked to common bacterial resistance genes in a bean plant (*Phaseolus vulgaris*) while introgression of nematode (*Meloidogyne arenaria*) resistance genes in *Arachis hypogaea* from *A. cardenasii* can be identified by RAPD analysis of a F_2 population derived from the cross between these two species (Garcia et al., 1996).

When the reference population of a species under investigation is available, RAPD is also an important approach that can be used for construction of a genome map. This application is widely used for mapping of plant genomes at present (Marillia and Scoles, 1996).

Regarding the convenience and flexibility of RAPD, it can be concluded that RAPD is suitable to be used for population genetic studies of various taxa. In the present study, it was indicated that when this technique was used with caution,

it could detect intraspecific genetic variation of *T. clareae* and *T. koenigerum* for which sequencing of the amplified ITS region did not reveal polymorphisms at such level.

CHAPTER V

Conclusions

1. Interspecific genetic variation between *T. clareae* and *T. koenigerum* were found at the ITS1-ITS2 region in nuclear ribosomal DNA. A total of nineteen point mutations (ten transitional and nine transversional mutations) and seven insertions/deletions were observed.

2. On the basis of the ITS sequences, sequence divergence between these two taxa was 3.79%. However, intraspecific polymorphisms were not observed in these species.

3. Randomly Amplified Polymorphic DNA (RAPD) analysis of one hundred and twenty- five individual of *T. clareae* and *T. koenigerum* with primers OPA07, OPA11 and OPA12 yielded a total of one hundred and fifty-two genotypes.

4. A UPGMA dendrogram indicated monophyletic status of both *T. clareae* and *T. koenigerum*. It allocated all but E2D and N1D *T. clareae* samples into two different groups depending on the host species implying closer related of *T. clareae* originating from the same than between different hosts.

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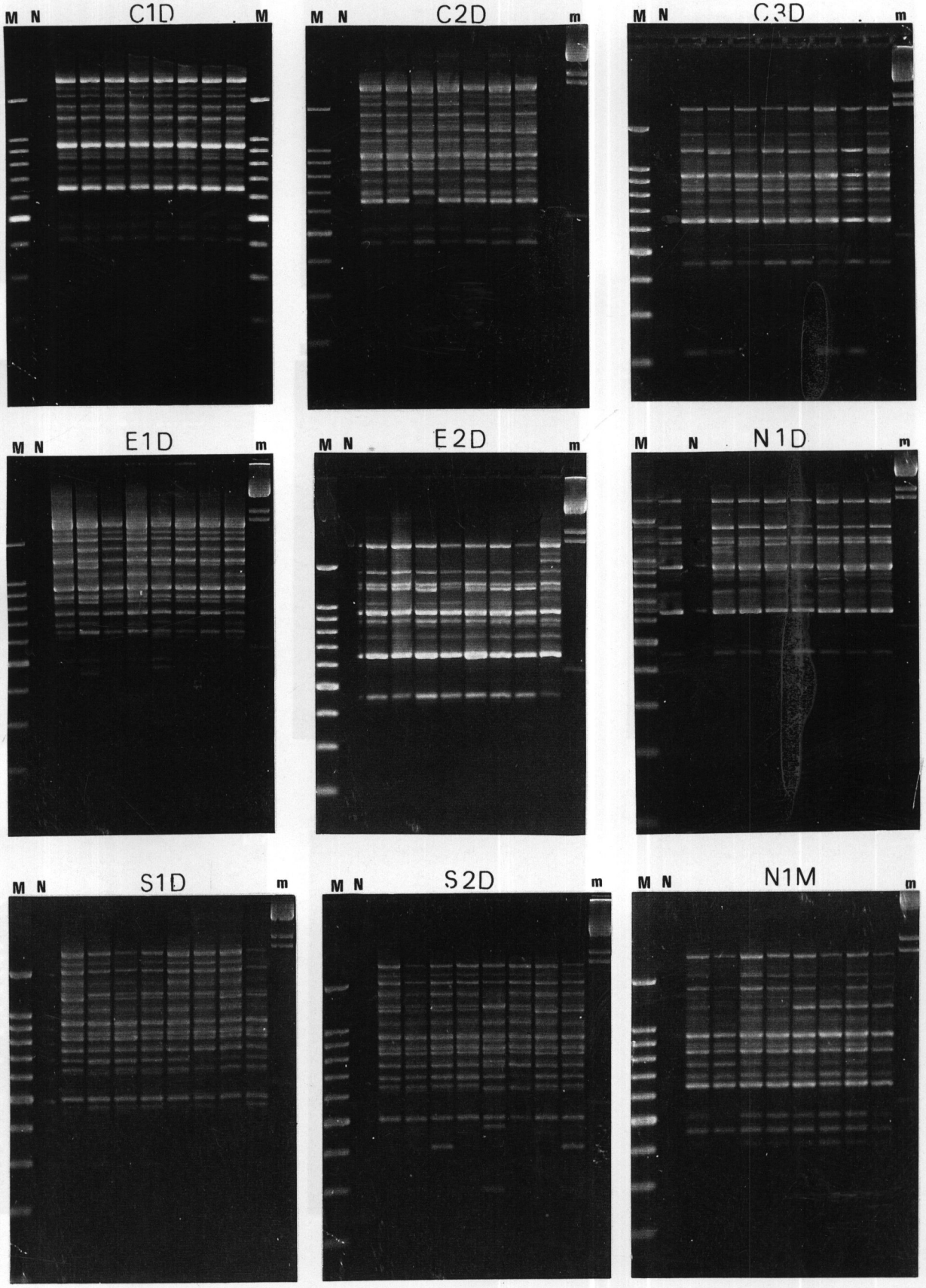
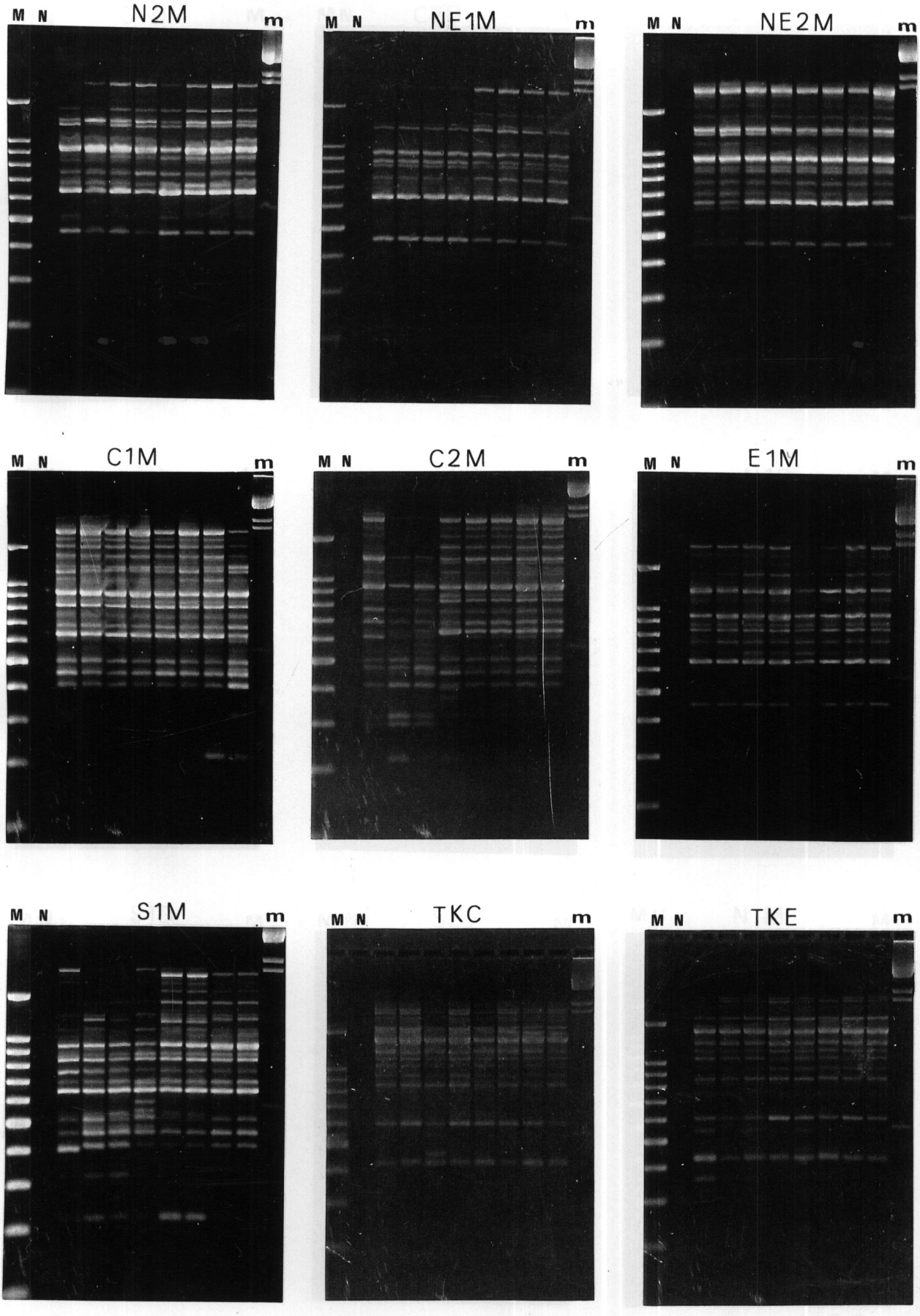
APPENDIX A

RAPD-PCR patterns using primer OPA07, OPA11 and OPA12 for each group of 16 groups of *T. clareae* and 2 groups of *T. koenigerum.*

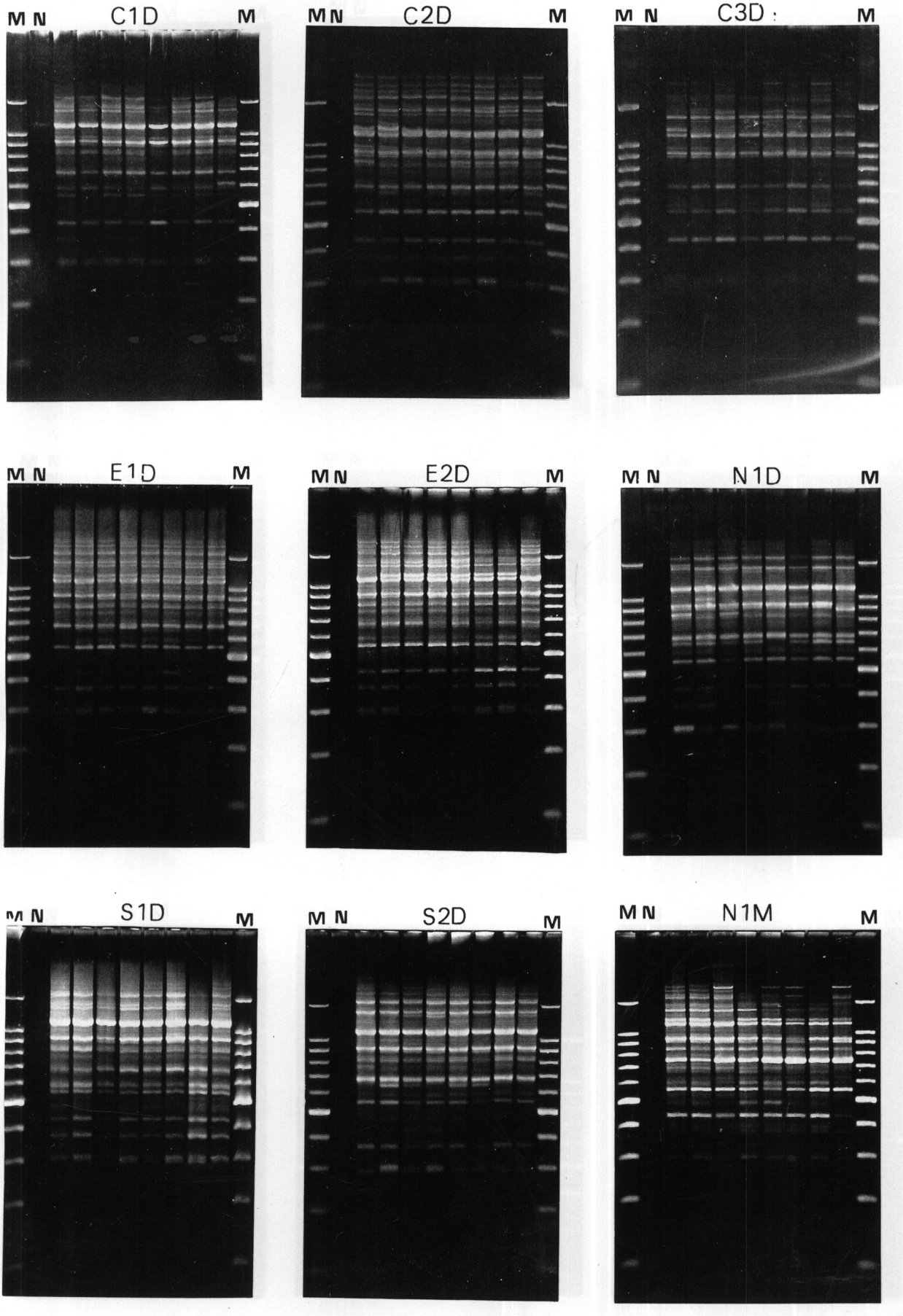
Primer OPA07



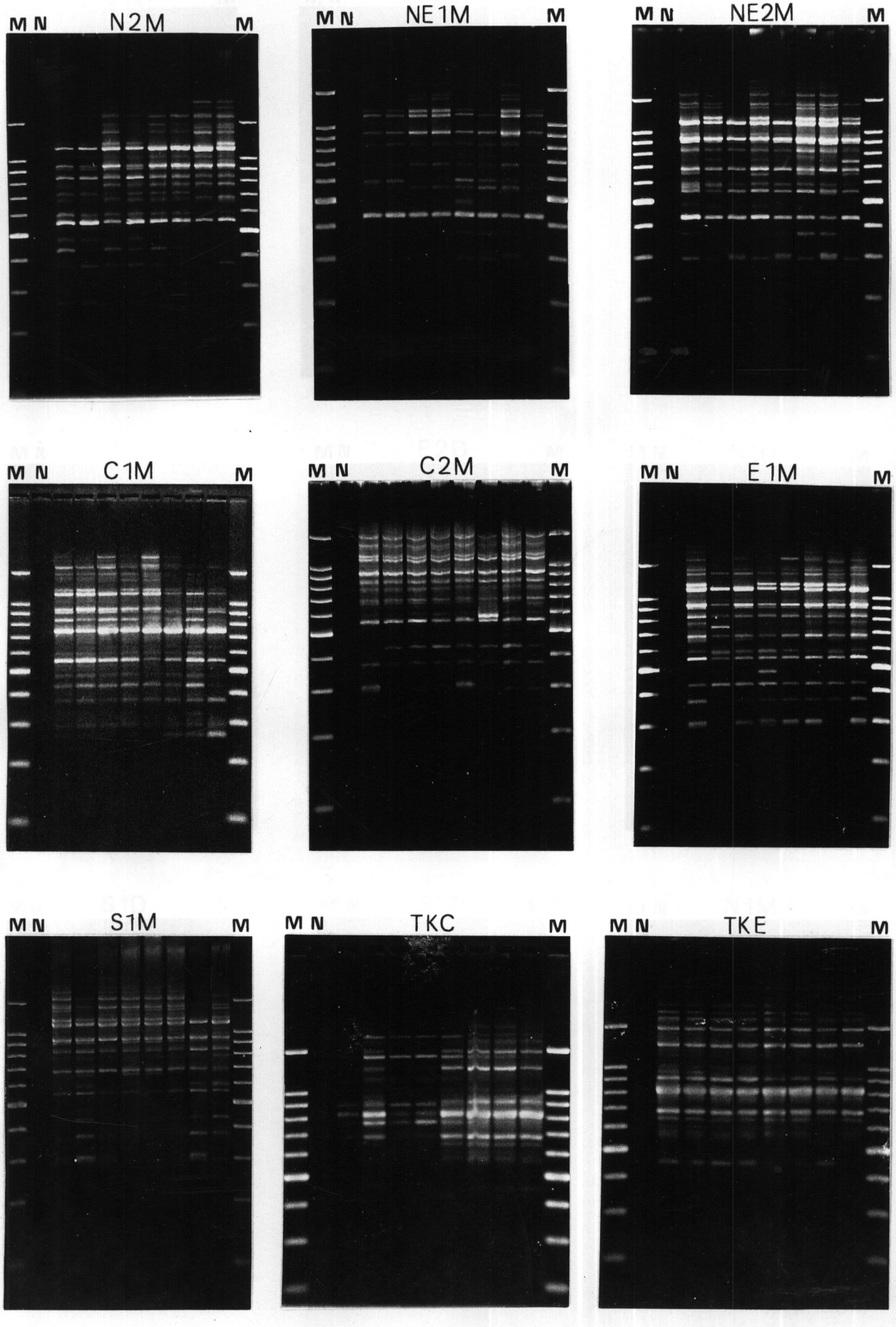
Primer OPA07 (continue)



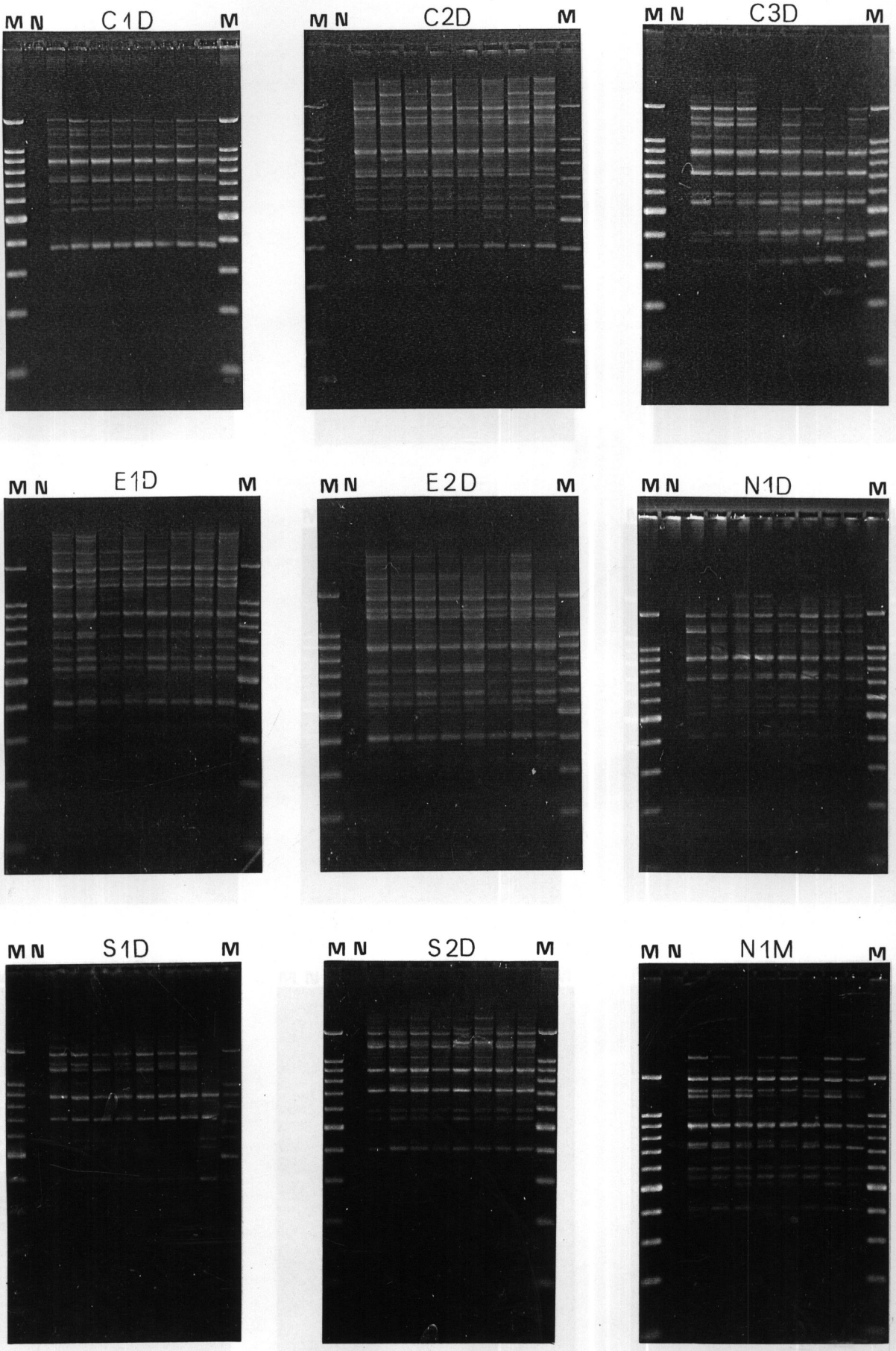
Primer OPA11



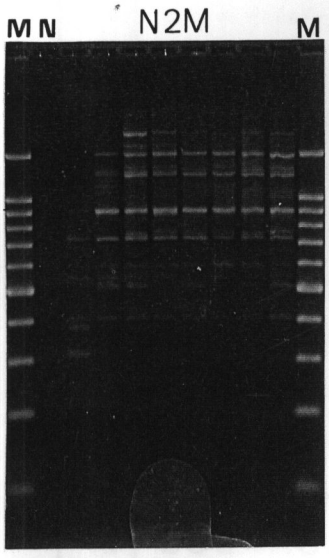
Primer OPA11 (continue)

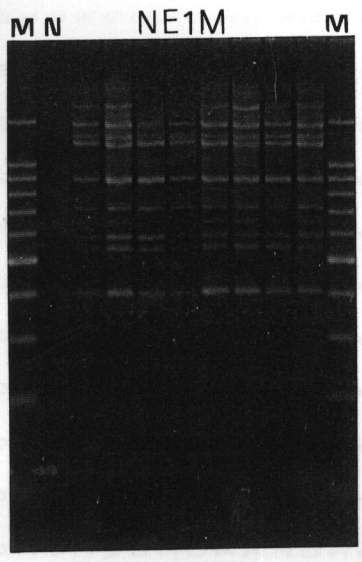


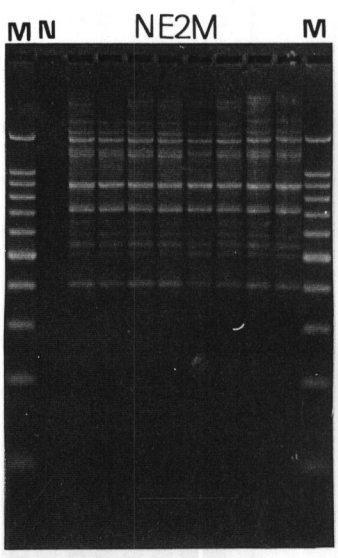
Primer OPA12

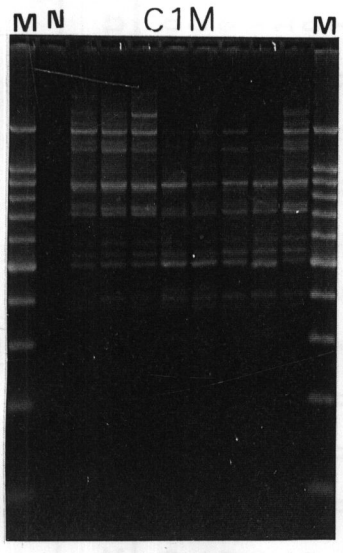


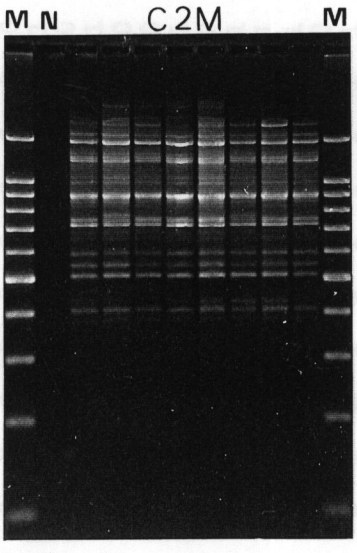
Primer OPA12 (continue)

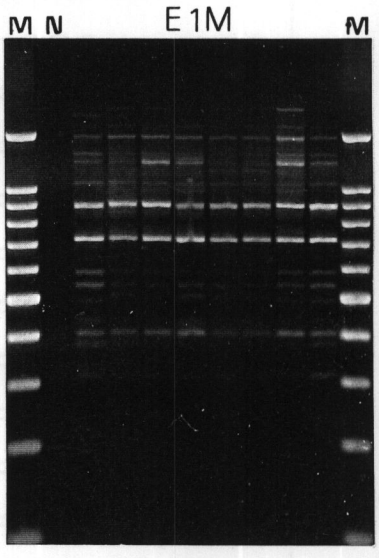


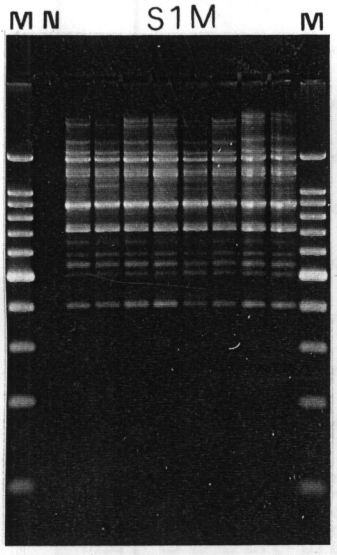


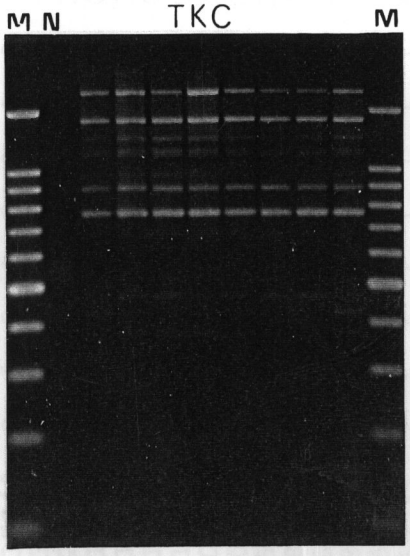


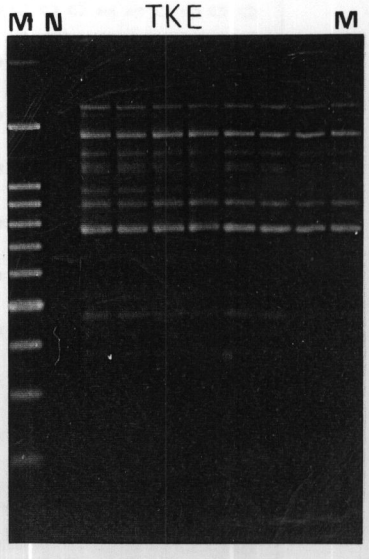












APPENDIX B

Genotype for each primer in 16 sample groups of *T. clereae* and 2 of *T. koenigerum*.

OPA07 primer

size (bp)	Genotype																																		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ
2040	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2020	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1980	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1710	1	1	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1530	1	1	1	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1390	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0	1
1375	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1360	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1210	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1195	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1180	0	0	0	0	0	1	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0
1160	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
1110	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1070	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
1050	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
950	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
880	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
850	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
780	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
760	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
960	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
640	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
620	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1
610	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1
590	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
510	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
480	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	1	1	1	1	1	1	1
460	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0
430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
410	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1
405	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0

OPA07 primer (Continue)

Size(bp)	Genotype																							
	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX	AY	AZ	BA	BB	BC	BD	BE	BF	BG	BH
2040	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
2020	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0
2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
1980	1	1	1	1	1	1	0	1	1	0	1	1	0	1	0	0	0	0	1	1	1	0	0	0
1710	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0
1550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
1530	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0
1390	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	0
1375	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
1360	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1210	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
1995	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
1180	0	1	0	0	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	0	0	0
1160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
1110	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
1070	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
1050	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
950	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
880	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
850	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0
780	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
760	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
690	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0
640	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
620	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
610	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
590	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
550	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
510	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0
480	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
460	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
410	0	1	0	1	1	1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	0	0	0
405	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0

OPA11 primer

size(bp)	Genotype																																		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ
1650	1	0	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1
1490	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1440	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1210	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	0	1	0	1
1140	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
920	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
910	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
820	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
810	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
770	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
740	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
720	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
680	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
640	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1
590	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1
580	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	1
540	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0
480	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
450	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	0	0	1	0	1
430	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
395	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
370	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
305	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
290	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1	1	0	1	0	0	0
265	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0

OPA11 primer (Continue)

Size (bp)	Genotype																					
	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX	AY	AZ	BA	BB	BC	BD	BE	BF
1650	1	1	0	1	1	1	1	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0
1490	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1
1440	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
1300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
1210	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0
1140	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
920	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0
910	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1
820	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0
810	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
770	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
740	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0
720	0	0	1	1	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
680	0	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	0	0	0
640	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1	1	1
590	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0
580	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0
540	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0	0
480	1	0	1	1	0	1	1	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0
450	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	1
430	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
395	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
370	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
305	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0
290	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
265	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

OPA12 primer

Size(bp)	Genotype																																
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI
1670	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1490	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
1420	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1360	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	0
1250	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1	1
1140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
1050	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
970	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
900	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
895	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
760	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	1	1
740	1	1	1	0	0	0	0	1	0	0	0	1	0	0	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	1	0	1	1	0	0
720	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
680	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
660	0	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
595	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
545	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
510	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
505	0	1	0	0	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
480	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
420	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0	0	1	1
410	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	0
390	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	0	0
385	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
310	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	1	0	0	0

Biography

Miss Varisa Tangjingjai was born on April 1, 1972 in Prachuap Khiri Khan. She graduated with the degree of Bachelor of Science from Department of Biology at Chulalongkorn University in 1994. In 1995, she has studies Master degree of Science at the program of Biotechnology, Chulalongkorn University.