# 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.