CHAPTER IV

RESULTS

4.1 Total DNA extraction

Total DNA was extracted from the thorax of each worker. The extracted DNA was dissolved in 60 µl, 50 µl, 40 µl, 30 µl, and 20 µl of TE buffer for *T. thoracica*, *T. collina*, *T. terminata*, *T. laeviceps*, and *T. fuscobalteata* respectively. High molecular weight DNA of all species are about 23.1 kb in length, slightly sheared DNA were observed (Figure 4.1). The amount of DNA extracted was estimated by agarose gel electrophoresis compared with the λ*Hin*d III standard DNA marker. Usually, about 25 ng/µl was obtained per individual specimen.

4.2 PCR amplification and optimization of PCR condition

The PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Many amounts of copies can be amplified from a few amount of DNA template. The mitochondrial analysis in this study was based on the 16S rRNA and PCR technique was used to amplify the amount of DNA.

PCR condition was followed the protocol of Costa *et al.* (2003), with a few modifications. DNA templates were amplified by polymerase chain reaction on thermocycler performing 40 amplification cycles (denaturation 94 °c, 1 min ; annealing 42 °c, 1.30 min and extension 64 °c, 1.30 min), followed by a final extension step at 72 °c for 5 min. PCR amplifications

were carried out in 25 µl total reaction volume using 2.5 µl of reaction buffer, 4 µl of dNTPs mixture (final concentration 100 μM each), 0.5 μl of each primer (final concentration 0.2 μM each), 0.04 unit of RED *Teq* DNA polymerase, 1 μl of DNA template, and sterile water.

PCR product after agarose gel electrophoresis showed that non specific bands and primer dimer bands were not observed (Figure 4.2). The approximated size of PCR product of 16S rRNA gene was 550 bp. No difference in length of PCR products was found in all samples.

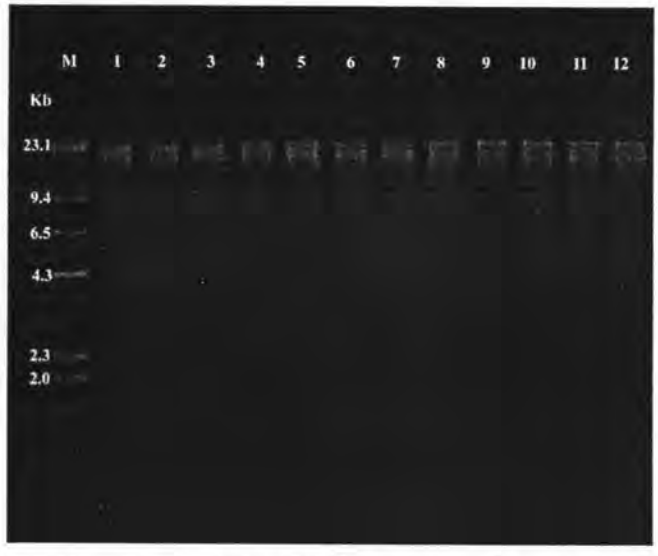

Figure 4.1 High molecular weight DNA extracted from thorax of all species.
Lane M: λ*Hin*d III standard DNA marker
Lane 1-12: Total DNA from each individual of stingless bees

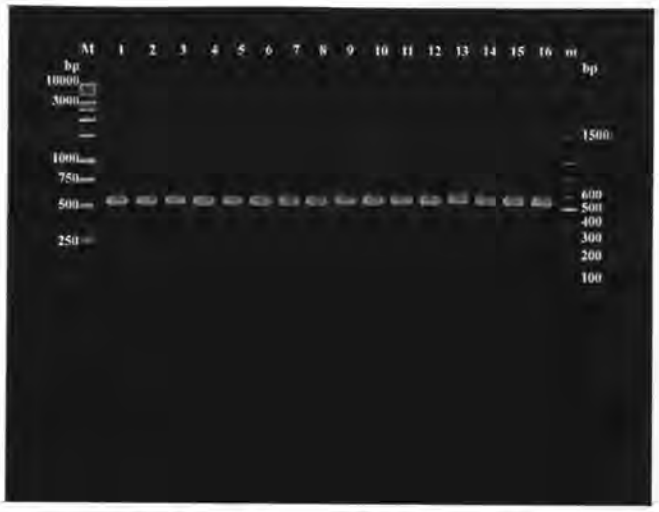

Figure 4.2 PCR amplified products of 16S rRNA gene of mtDNA of all species.

Lane M: 1 kb DNA ladder

Lane m: 100 bp DNA ladder

Lane 1-16: PCR amplified products from each individual of stingless bees

4.3 The PCR-RFLP in 16S rRNA gene of mtDNA of five species (*T. collina*, *T. fuscobalteata*, *T. laeviceps*, *T. terminata*, and *T. thoracica*)

PCR products of 16S rRNA gene of mtDNA were preliminary investigated with 12 restriction enzyme; *Afl* II, *Ase* I, *Bam*H I, *Dra* I, *Eco*R V, *Hinf* I, *Hpy*188 III, *Mse* I, *Pac* I, *Rsa* I, *Sau*3A I, and *Ssp* I. It was found that *Afl* II, *Bam*H I, *Eco*R V, and *Hinf* I could not digest the PCR products. Whereas, *Dra* I, *Hpy*188 III, and *Ssp* I could digest and were selected to digest all PCR products of five species.

Approximately 5 μl of PCR products of all samples were digested at 37 % with 10 units of *Dra* I, 5 units of *Hpy*188 III, and 10 units of *Ssp* I. The incubation time was 18 hours.

4.3.1 The PCR-RFLP in 16S rRNA gene of mtDNA of *T. collina*

The digestion of *Dra* I, *Hpy*188 III, and *Ssp* I showed polymorphism 3 patterns (250, 180, 130, 120, 115, 70, 50, 35, and 30 bp), 4 patterns (415, 255, 250, 220, 165, 135, 65, and 30 bp), and 4 patterns (350, 290, 225, 135, 125, 85, 65, and 40 bp), respectively.

Figure 4.3, 4.4, and 4.5 showed the restriction patterns, the size and number of restriction fragments when cut the PCR products with *Dra* I, *Hpy*188 III, and *Ssp* I respectively.

Locations and restriction patterns of all samples of *T. collina* in Thailand after digested with *Dra* I, *Hpy*188 III, and *Ssp* I are shown in Appendix II and Appendix III.

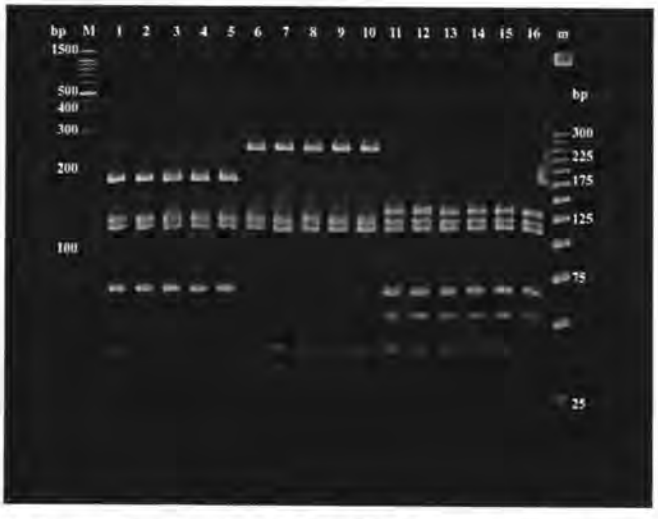

Figure 4.3 Restriction patterns in 16S rRNA gene of mtDNA of *T. collina* after digested with *Dra* I.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-5: Pattern A
Lane 6-10: Pattern B
Lane 11-16: Pattern C

Figure 4.4 Restriction patterns in 16S rRNA gene of mtDNA of *T. collina* after digested with *Hpy*188 III.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-4: Pattern A
Lane 5-8: Pattern B
Lane 9-12: Pattern C
Lane 13-16: Pattern D

Figure 4.5 Restriction patterns in 16S rRNA gene of mtDNA of *T. collina* after digested with *Ssp* I.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-4: Pattern A
Lane 5-8: Pattern B
Lane 9-12: Pattern C
Lane 13-16: Pattern D

4.3.2 The PCR-RFLP in 16S rRNA gene of mtDNA of *T. fuscobalteata*

Dra I is only one restriction enzyme which showed polymorphism in *T. fuscobalteata* (Figure 4.6). Two patterns from the total 7 bands (200, 165, 120, 120, 45, 35, and 30 bp) were found. Only one colony from Uttaradit Province (Northern Thailand) has different pattern from the others.

The digestion of *Hpy*188 III showed 2 bands of 415 and 135 bp and *Ssp* I showed 2 bands of 295 and 255 bp. The variation could not be detected from the results of *Hpy*188 III and *Ssp* I digestion.

Figure 4.7 and 4.8 showed the restriction patterns, the size and number of restriction fragments when cut the PCR products with *Hpy*188 III and *Ssp* I respectively.

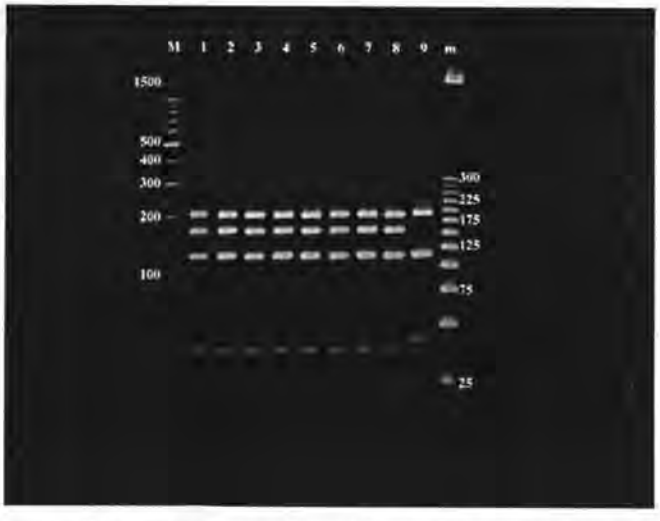

Figure 4.6 Restriction patterns in 16S rRNA gene of mtDNA of *T. fuscobalteata* after digested with *Dra* I.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-8: Pattern A
Lane 9: Pattern B

Figure 4.7 Restriction pattern in 16S rRNA gene of mtDNA of *T. fuscobalteata* after digested with *Hpy*188 III.

Lane M: 100 bp DNA ladder

Lane m: 25 bp DNA ladder

Lane 1-16: Samples of *T. fuscobalteata* after digested with *Hpy*188 III

Figure 4.8 Restriction pattern in 16S rRNA gene of mtDNA of *T. fuscobalteata* after digested with *Ssp* I.

Lane M: 100 bp DNA ladder

Lane m: 25 bp DNA ladder

Lane 1-16: Samples of *T. fuscobalteata* after digested with *Ssp* I

4.3.3 The PCR-RFLP in 16S rRNA gene of mtDNA of *T. laeviceps*

The digestion of *Dra* I, *Hpy*188 III, and *Ssp* I showed polymorphism 4 patterns (400, 365, 330, 295, 120, 70, 35, and 30 bp), 2 patterns (415, 365, 135, and 50 bp), and 3 patterns (415, 290, 210, 135, 125, and 80 bp) respectively.

Figure 4.9, 4.10, and 4.11 showed the restriction patterns, the size and number of restriction fragments when cut the PCR product with *Dra* I, *Hpy*188 III, and *Ssp* I respectively.

Locations and restriction patterns of all samples of *T. laeviceps* in Thailand after digested with *Dra* I, *Hpy*188 III, and *Ssp* I are shown in Appendix II and Appendix III.

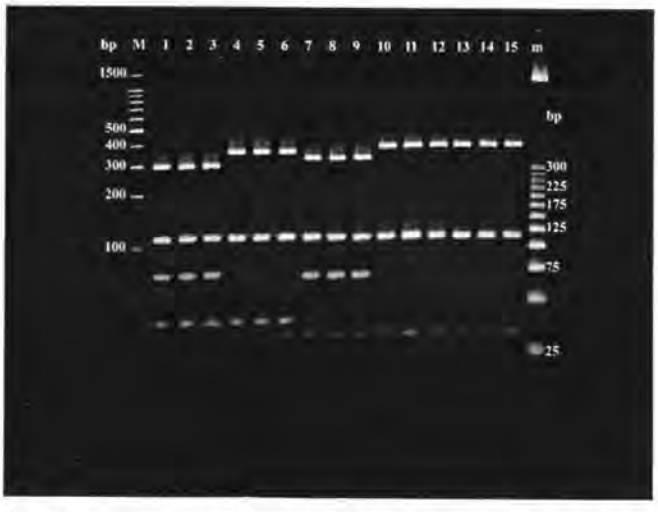

Figure 4.9 Restriction patterns in 16S rRNA gene of mtDNA of *T. laeviceps* after digested with *Dra* I.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-3: Pattern A
Lane 4-6: Pattern B
Lane 7-9: Pattern C
Lane 10-15: Pattern D

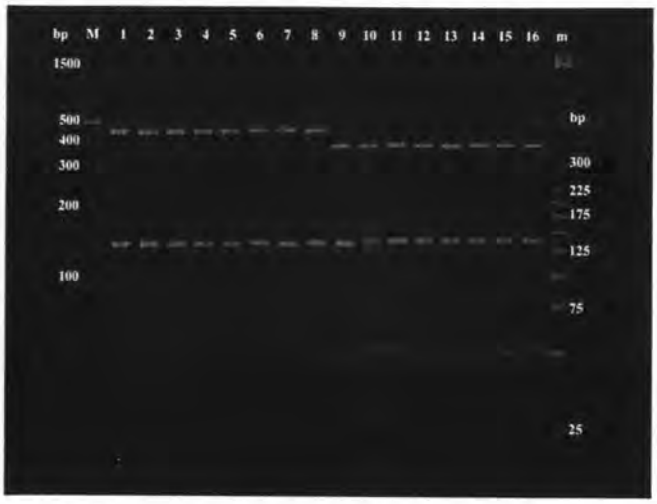

Figure 4.10 Restriction patterns in 16S rRNA gene of mtDNA of *T. laeviceps* after digested with *Hpy*188 III.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-8: Pattern A
Lane 9-16: Pattern B

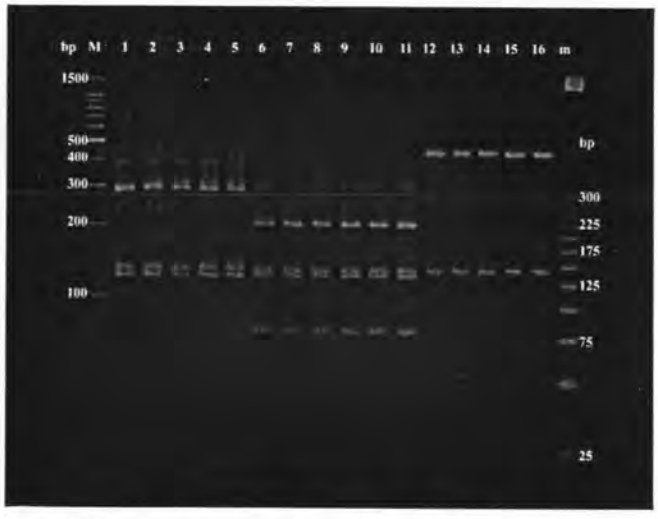

Figure 4.11 Restriction patterns in 16S rRNA gene of mtDNA of *T. laeviceps* after digested with *Ssp* I.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-5: Pattern A
Lane 6-11: Pattern B
Lane 12-16: Pattern C

4.3.4 The PCR-RFLP in 16S rRNA gene of mtDNA of *T. terminata*

Dra I is only one restriction enzyme which showed polymorphism (Figure 4.12). Two patterns from the total 7 bands (295, 285, 120, 75, 70, 35, and 30 bp) were found. Locations and restriction patterns of all samples of *T. terminata* in Thailand after digested with *Dra* I are shown in Appendix II and Appendix III.

The digestion of *Hpy*188 III, and *Ssp* I showed 2 bands (415 and 135 bp), and 3 bands (290, 135, and 125 bp), respectively. No different pattern was found among *T. terminata* samples of Thailand when detected with *Hpy*188 III, and *Ssp* I. Figure 4.13 and 4.14 showed the restriction patterns, the size and number of restriction fragments when cut the PCR products with *Hpy*188 III, and *Ssp* I respectively.

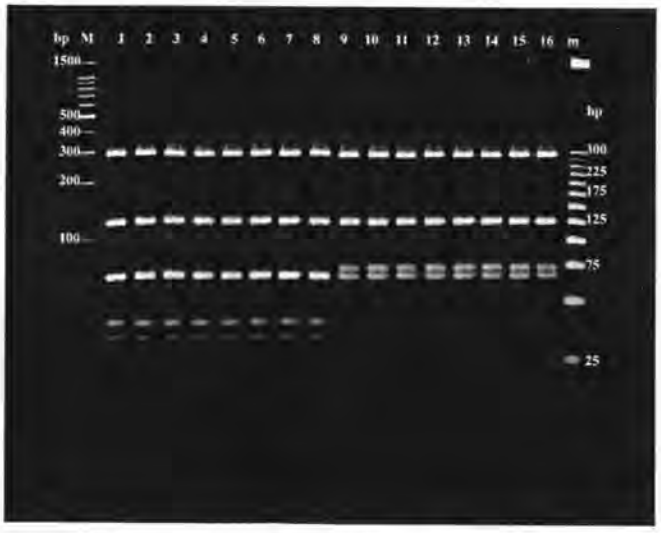

Figure 4.12 Restriction patterns in 16S rRNA gene of mtDNA of *T. terminata* after digested with *Dra* I.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-8: Pattern A
Lane 9-16: Pattern B

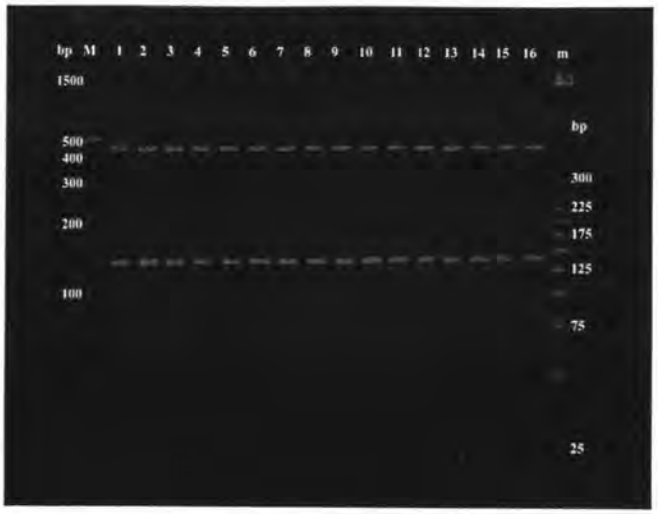

Figure 4.13 Restriction pattern in 16S rRNA gene of mtDNA of *T. terminata* after digested with *Hpy*188 III.

Lane M: 100 bp DNA ladder

Lane m: 25 bp DNA ladder

Lane 1-16: Samples of *T. terminata* after digested with *Hpy*188 III

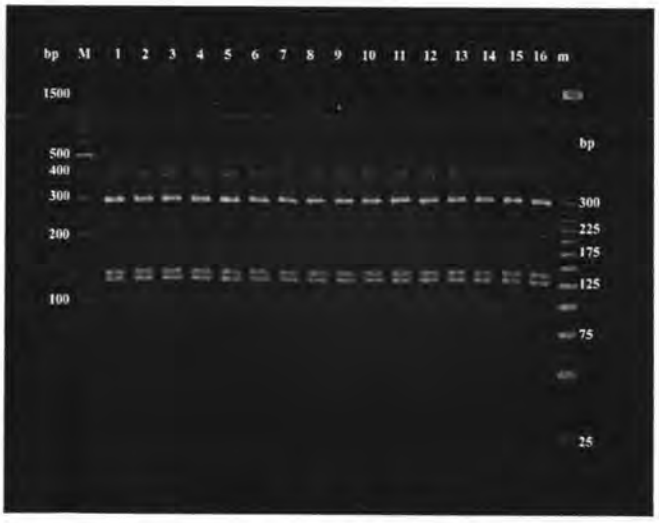

Figure 4.14 Restriction pattern in 16S rRNA gene of mtDNA of *T. terminata* after digested with *Ssp* I.

Lane M: 100 bp DNA ladder

Lane m: 25 bp DNA ladder

Lane 1-16: Samples of *T. terminata* after digested with *Ssp* I

4.3.5 The PCR-RFLP in 16S rRNA gene of mtDNA of *T. thoracica*

Two different patterns (Figure 4.15) were found in the 16S rRNA gene when digested the PCR products with *Dra* I. The different pattern was detected from a colony of *T. thoracica* in Kanchanaburi Province (Western Thailand).

Figure 4.16 showed the number and pattern of restriction fragments when digested with *Hpy*188 III. A pattern of 2 bands; 415 and 135 bp was presented among populations.

The *Ssp* I digestion showed 3 fragments; 290, 135, and 125 bp (Figure 4.17). No variation among populations was detected.

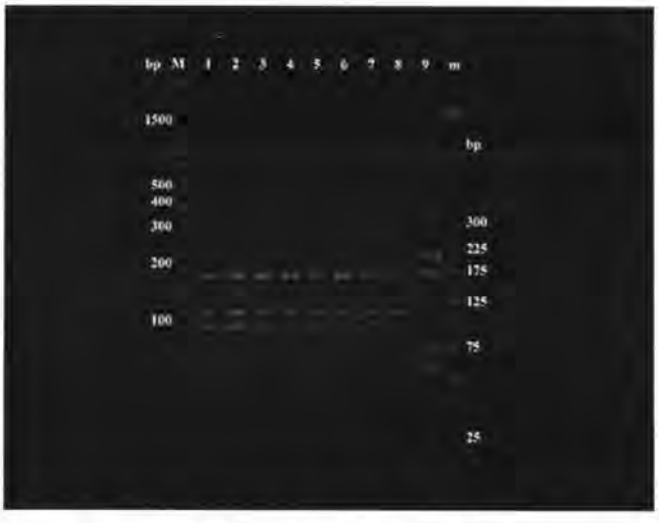

Figure 4.15 Restriction patterns in 16S rRNA gene of mtDNA of *T. thoracica* after digested with *Dra* I.

Lane M: 100 bp DNA ladder
Lane m: 25 bp DNA ladder
Lane 1-8: Pattern A
Lane 9: Pattern B

Figure 4.16 Restriction pattern in 16S rRNA gene of mtDNA of *T. thoracica* after digested with *Hpy*188 III.

Lane M: 100 bp DNA ladder

Lane m: 25 bp DNA ladder

Lane 1-16: Samples of *T. thoracica* after digested with *Hpy*188 III

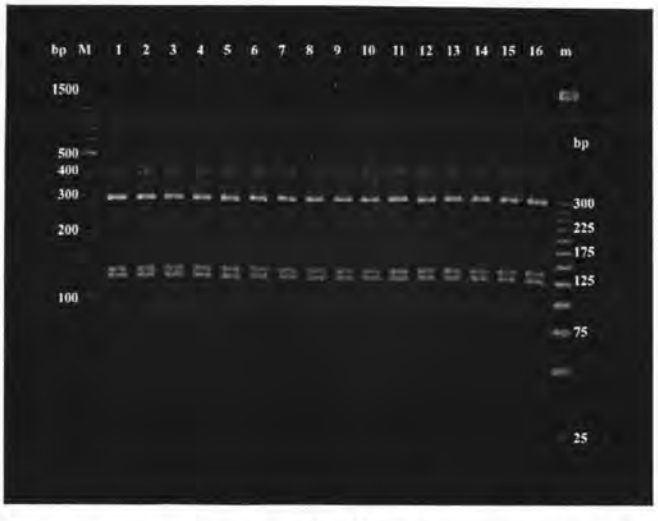

Figure 4.17 Restriction pattern in 16S rRNA gene of mtDNA of *T. thoracica* after digested with *Ssp* I.

Lane M: 100 bp DNA ladder

Lane m: 25 bp DNA ladder

Lane 1-16: Samples of *T. thoracica* after digested with *Ssp* I

4.4 PCR-RFLP analysis of mtDNA of five species of stingless bees in Thailand

4.4.1 Genetic diversity among five species of *Trigona* in Thailand

The distribution of 25 haplotypes among geographic regions when digested with 3 restriction enzymes (*Dra* I, *Hpy*188 III, and *Ssp* I) indicated genetic diversity of each stingless bee species in Thailand.

Genetic distance (*D*) estimated between pair of *Trigona* (five species) haplotypes ranged from 0.00000 [*T. laeviceps* (C 061-063 and S 116-118, 131) and *T. terminata* (C 022-023, E 024-028, and N 001-008)] to 0.082844 [*T. collina* (S 193-198, 214-224) and *T. fuscobalteata* (N 004)] (Table 5).

From genetic distance values could be constructed dendrogram, which separated all stingless bee samples into five groups. The first group was a cluster of *T. terminata* samples; the second group consisted of *T. laeviceps* and *T. terminata* samples; the third group was a cluster of *T. thoracica* samples; the forth group was a cluster of *T. collina* samples; and the last group was a cluster of *T. fuscobalteata* samples. In addition, the same subfamily species, *Melipona compressipes*, it was selected for outgroups (Figure 4.18).

The high genetic variations were found in *T. collina* and *T. laeviceps* which revealed 10 different haplotypes in each species. On the other hand, *T. fuscobalteata*, *T. terminata*, and *T. thoracica* showed low genetic diversity that 2 different haplotypes were found in each species.

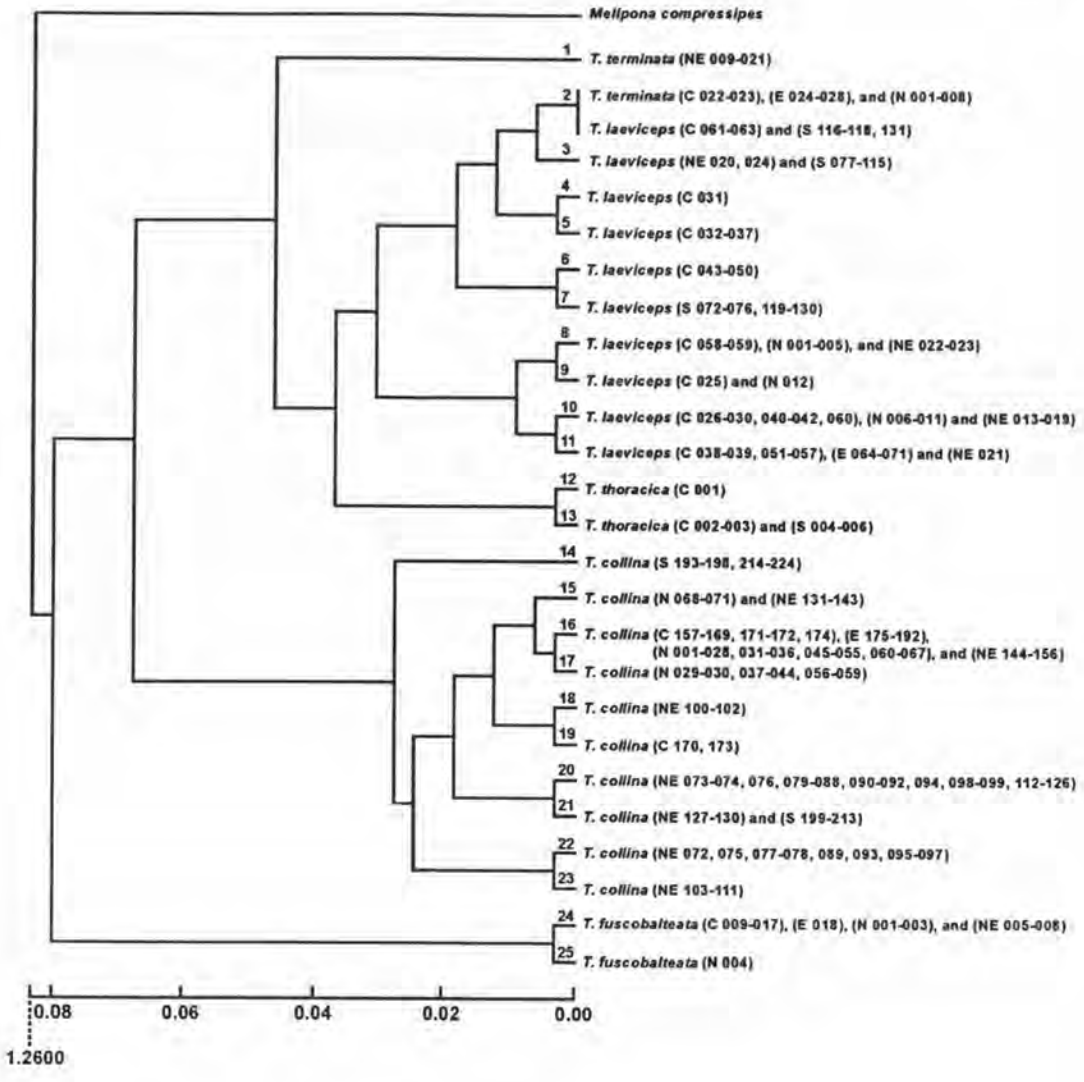

Figure 4.18 Dendrogram of relationships among five species of *Trigona* in Thailand using genetic distance data of PCR-RFLP (3 enzymes) at 16S rRNA gene of mtDNA. (C = Central, E = Eastern, N = Northern, NE = Northeast, and S = Southern).

The distribution of *Dra* I, *Hpy*188 III, and *Ssp* I digestion from *Trigona* 16S rRNA gene was shown in Table 4. Twenty-five haplotypes were generated from their distribution frequencies.

Table 4 Geographic distribution of 25 haplotypes of five species of *Trigona* in Thailand (abbreviation see Figure 4.18).

haplotype	Geographic distribution frequency (no. of individual)					Total
	North	Northeast	East	Central	South	
1	0	0.100(13)	0	0	0	0.032(13)
2	0.084(8)	0	0.156(5)	0.070(5)	0.050(4)	0.054(22)
3	0	0.016(2)	0	0	0.488(39)	0.101(41)
4	0	0	0	0.014(1)	0	0.002(1)
5	0	0	0	0.085(6)	0	0.015(6)
6	0	0	0	0.113(8)	0	0.020(8)
7	0	0	0	0	0.212(17)	0.042(17)
8	0.053(5)	0.016(2)	0	0.028(2)	0	0.022(9)
9	0.010(1)	0	0	0.014(1)	0	0.005(2)
10	0.063(6)	0.054(7)	0	0.127(9)	0	0.054(22)
11	0	0.008(1)	0.250(8)	0.127(9)	0	0.044(18)
12	0	0	0	0.014(1)	0	0.002(1)
13	0	0	0	0.028(2)	0.038(3)	0.012(5)
14	0	0	0	0	0.212(17)	0.042(17)
15	0.042(4)	0.100(13)	0	0	0	0.042(17)
16	0.558(53)	0.100(13)	0.563(18)	0.225(16)	0	0.246(100)
17	0.147(14)	0	0	0	0	0.034(14)
18	0	0.023(3)	0	0	0	0.007(3)
19	0	0	0	0.028(2)	0	0.005(2)
20	0	0.264(34)	0	0	0	0.084(34)
21	0	0.147(19)	0	0	0	0.047(19)
22	0	0.070(9)	0	0	0	0.022(9)
23	0	0.070(9)	0	0	0	0.022(9)
24	0.032(3)	0.031(4)	0.031(1)	0.127(9)	0	0.042(17)
25	0.010(1)	0	0	0	0	0.002(1)
Total	1.000(95)	1.000(129)	1.000(32)	1.000(71)	1.000(80)	1.000(407)

Genetic diversity among *T. collina* samples

PCR-RFLP analysis of 16S rRNA gene revealed high diversity among populations of *T. collina*. Ten haplotypes from 224 samples were found when digested with 3 restriction enzymes (Figure 4.20). Genetic distance among samples ranged from 0.006638-0.041682 (mean = 0.02416).

The genetic distance values were used to construct a UPGMA dendrogram. All samples of *T. collina* were divided into three major clusters. The first one was the cluster of the most Southern samples (S 193-198 and 214-224), the second cluster consists of Central (C 157-174), Northern (N 001-071), East (E 175-192) and Northeast samples (NE, 073-074, 076, 079-088, 090-092, 094, 098-102, and 112-156), and some samples of South (S 199-213), and the last one was a cluster of the most Northeast samples (NE 072, 075, 077-078, 089, 093, 095-097, and 103-111) (Figure 4.19).

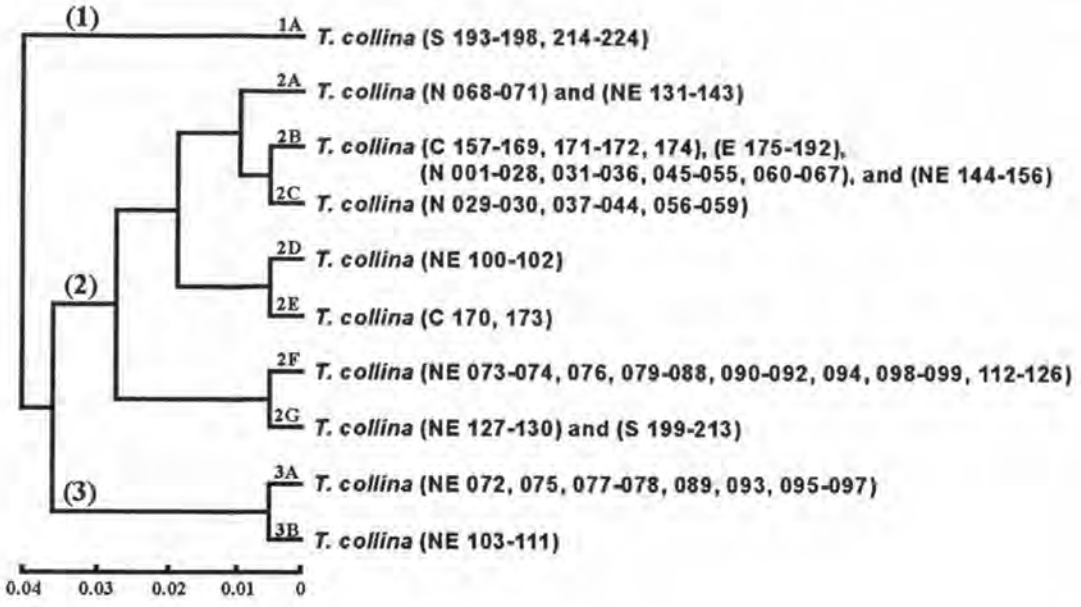

Figure 4.19 Dendrogram of relationships among *T. collina* populations in Thailand using genetic distance data of PCR-RFLP (3 enzymes) at 16S rRNA gene of mtDNA. (C = Central, E = Eastern, N = Northern, NE = Northeast, and S = Southern).

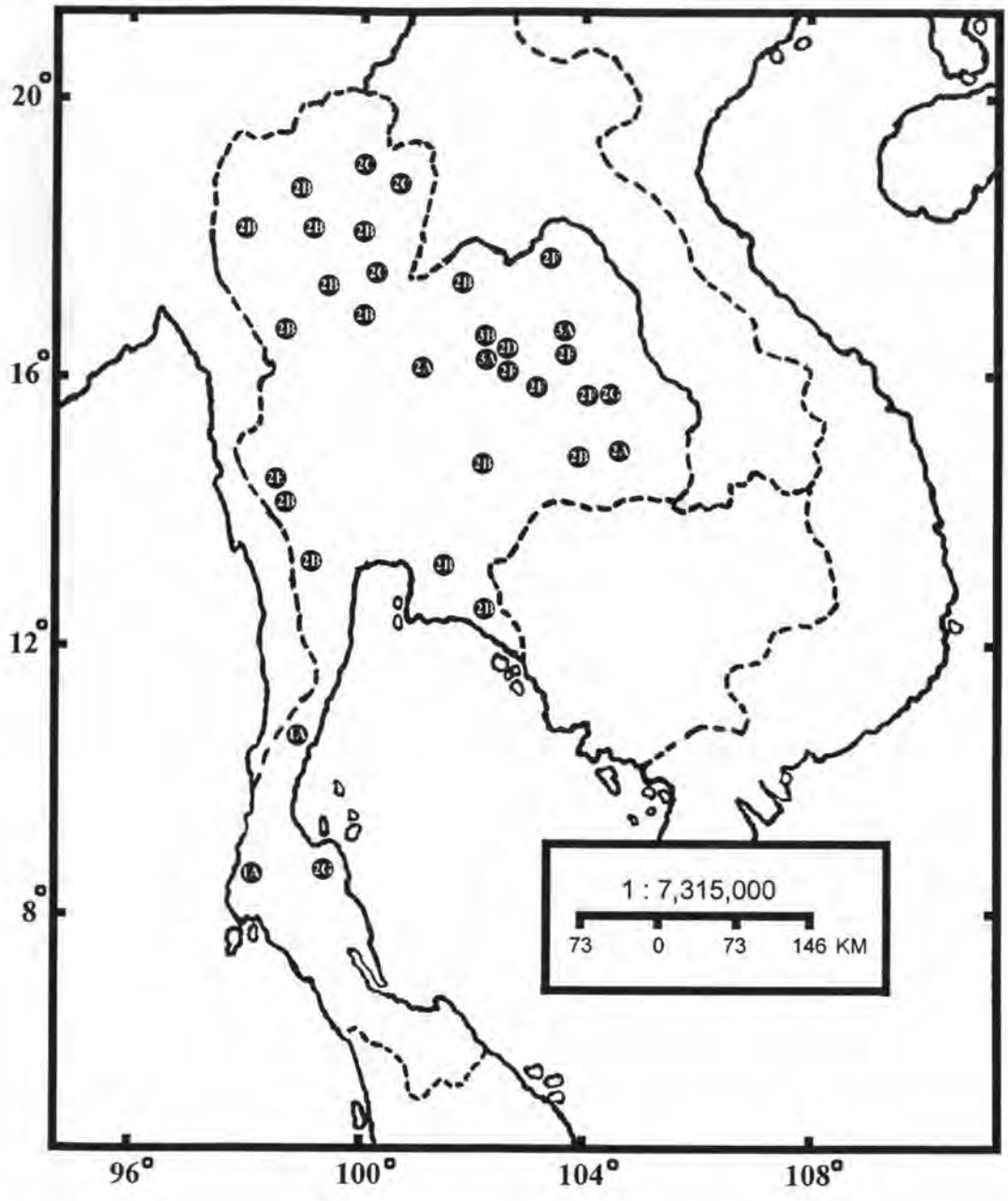

Figure 4.20 Distribution of mtDNA haplotypes of *T. collina* in Thailand resulted from digestion of 16S rRNA gene with 3 restriction enzymes (abbreviation see Figure 4.19).

Genetic diversity among *T. fuscobalteata* samples

When digested with 3 restriction enzymes, 2 haplotypes were found from 18 samples (Figure 4.22). The cluster using UPGMA analysis could divide samples of this species from different locations into two groups (Figure 4.21). First group was a cluster of Central (C 009-017), East (E 018), Northern (001-003), and Northeast (NE 005-008) samples. The second was the sample from Northern (N 004 = Uttaradit Province) which separated from the others. Genetic distance among groups was 0.009752.

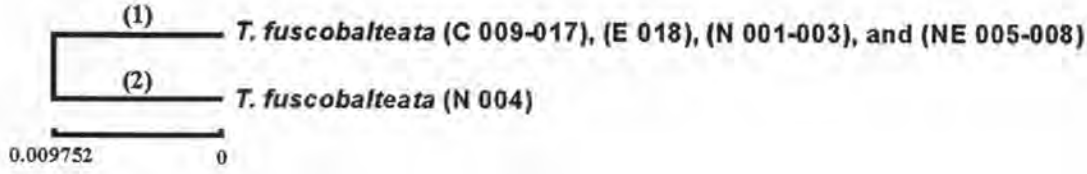

Figure 4.21 Dendrogram of relationships among *T. fuscobalteata* populations in Thailand using genetic distance data of PCR-RFLP (3 enzymes) at 16S rRNA gene of mtDNA. (C = Central, E = Eastern, N = Northern, and NE = Northeast).

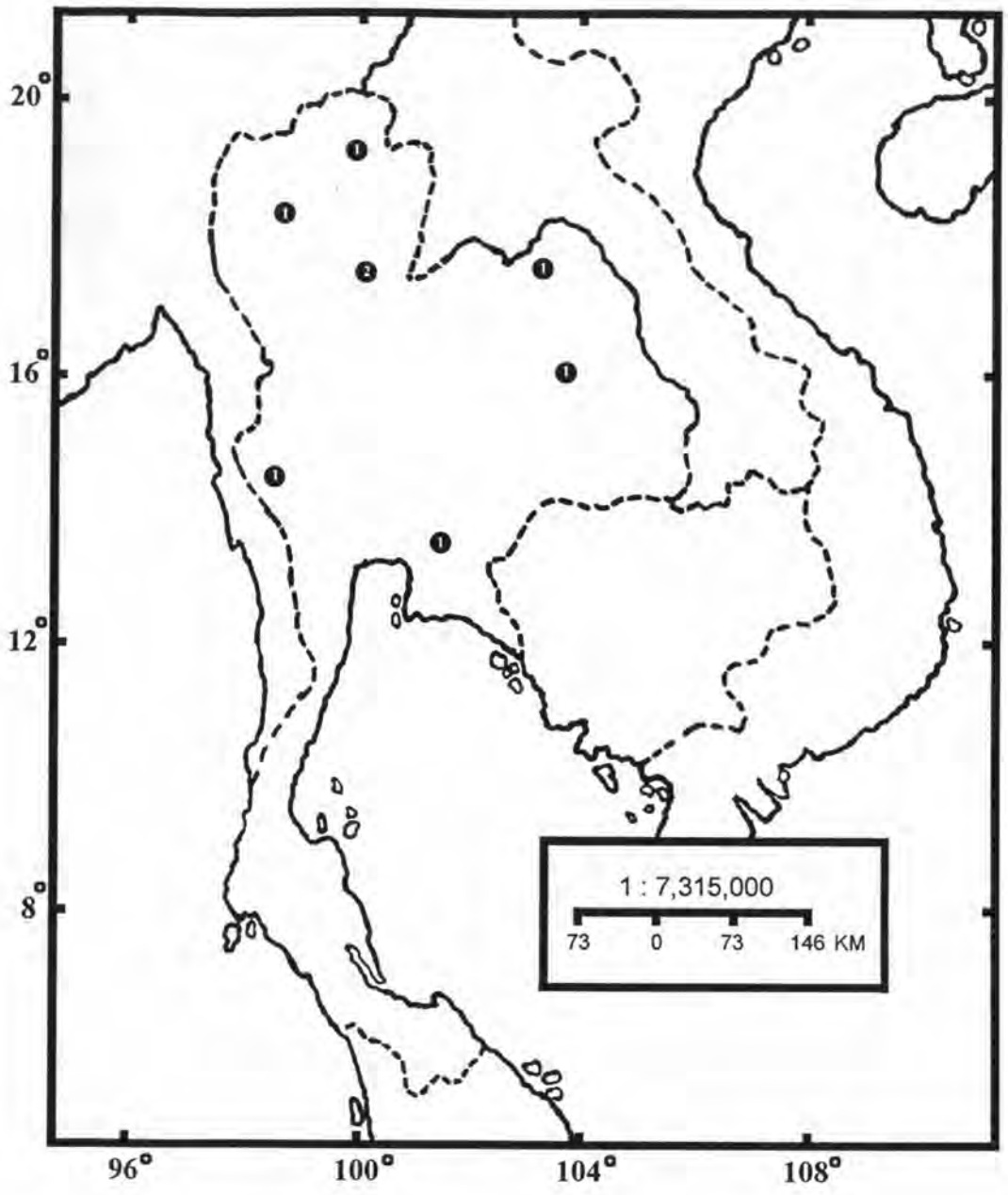

Figure 4.22 Distribution of mtDNA haplotypes of *T. fuscobalteata* in Thailand resulted from digestion of 16S rRNA gene with 3 restriction enzymes (abbreviation see Figure 4.21).

Genetic diversity among *T. laeviceps* samples

PCR-RFLP analysis of 16S rRNA gene revealed high diversity among samples. There are 10 haplotypes from 131 samples when digested with 3 restriction enzymes (Figure 4.24). Genetic distance among populations ranged from 0.008727-0.049354 (mean = 0.02904).

From genetic distance values, that all samples of this species could divided into two clusters. The first cluster consists of Central (C 031-037, 043-050, and 061-063), some part of Northeast (NE 020, 024), Southern (S 072-130), and Phuket Island populations (S 131) (Central to Southern region). The second consists of Central (C 025-030, 038-042, and 051-060), East (E 064-071), Northern (N 001 and 006-012), and most Northeast populations (NE 013-019 and 021-023) (Northern to Central region) (Figure 4.23).

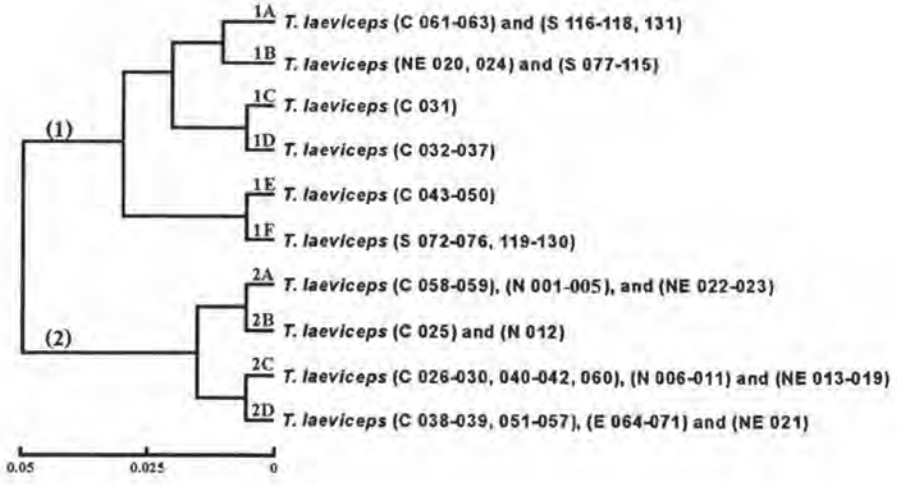

Figure 4.23 Dendrogram of relationships among *T. laeviceps* populations in Thailand using genetic distance data of PCR-RFLP (3 enzymes) at 16S rRNA gene of mtDNA. (C = Central, E = Eastern, N = Northern, NE = Northeast, and S = Southern).

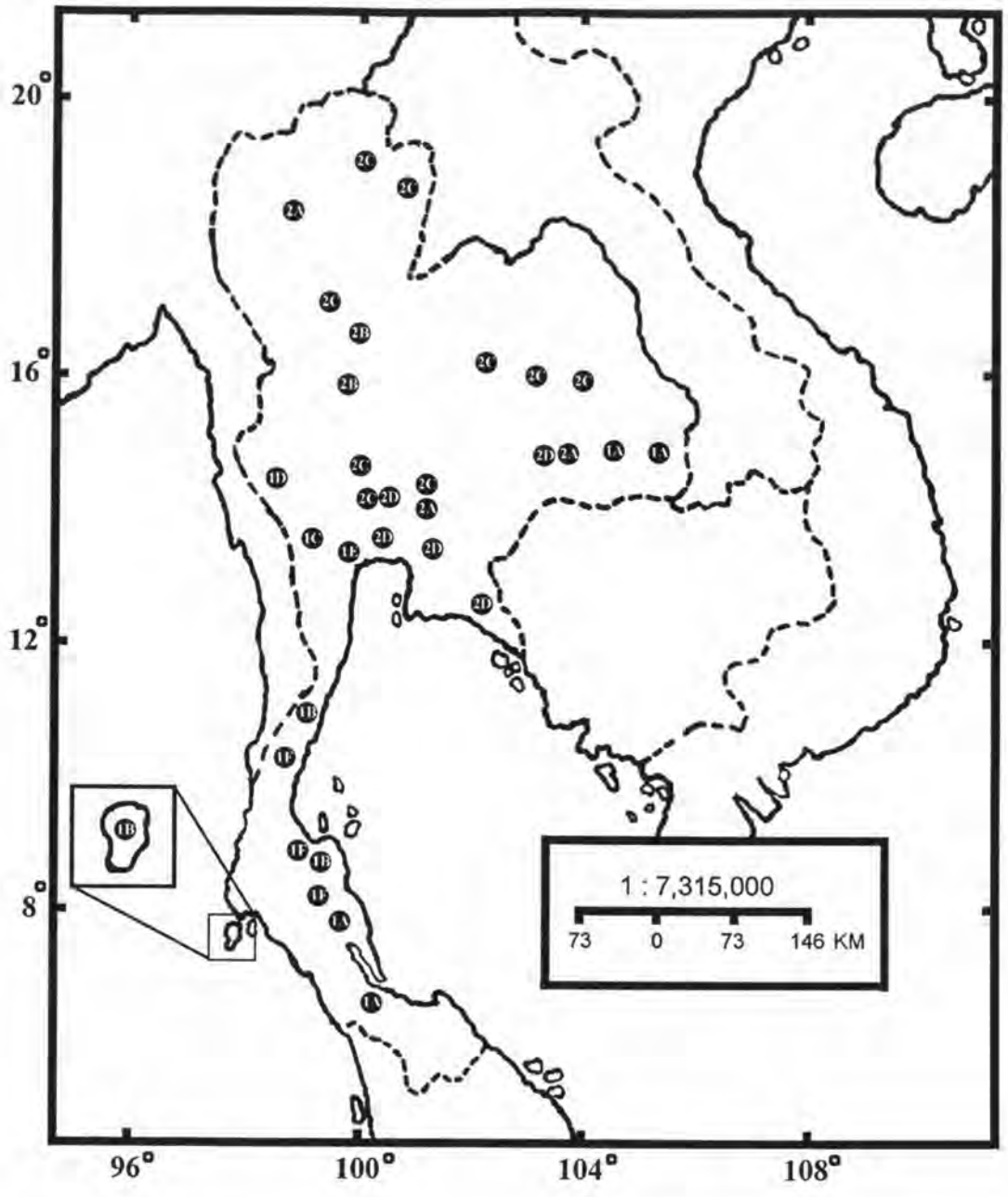

Figure 4.24 Distribution of mtDNA haplotypes of *T. laeviceps* in Thailand resulted from digestion of 16S rRNA gene with 3 restriction enzymes (abbreviation see Figure 4.23).

Genetic diversity among *T. terminata* samples

PCR-RFLP analysis of 16S rRNA gene revealed 2 haplotypes from 28 samples (Figure 4.26). The genetic distance between clusters was 0.0176, indicated that high genetic differentiation between their groups. The cluster using genetic distance by UPGMA analysis (3 restriction enzymes) could be divided all sample of this species into two clusters, the first one is a cluster of Northeast (NE 009-021) and the second cluster consists of Central (C 022-023), East (E 024-028), and Northern samples (N 001-008) (Figure 4.25).

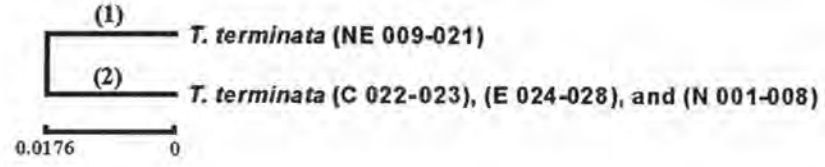

Figure 4.25 Dendrogram of relationships among *T. terminata* populations in Thailand using genetic distance data of PCR-RFLP (3 enzymes) at 16S rRNA gene of mtDNA. (C = Central, E = Eastern, N = Northern, and NE = Northeast).

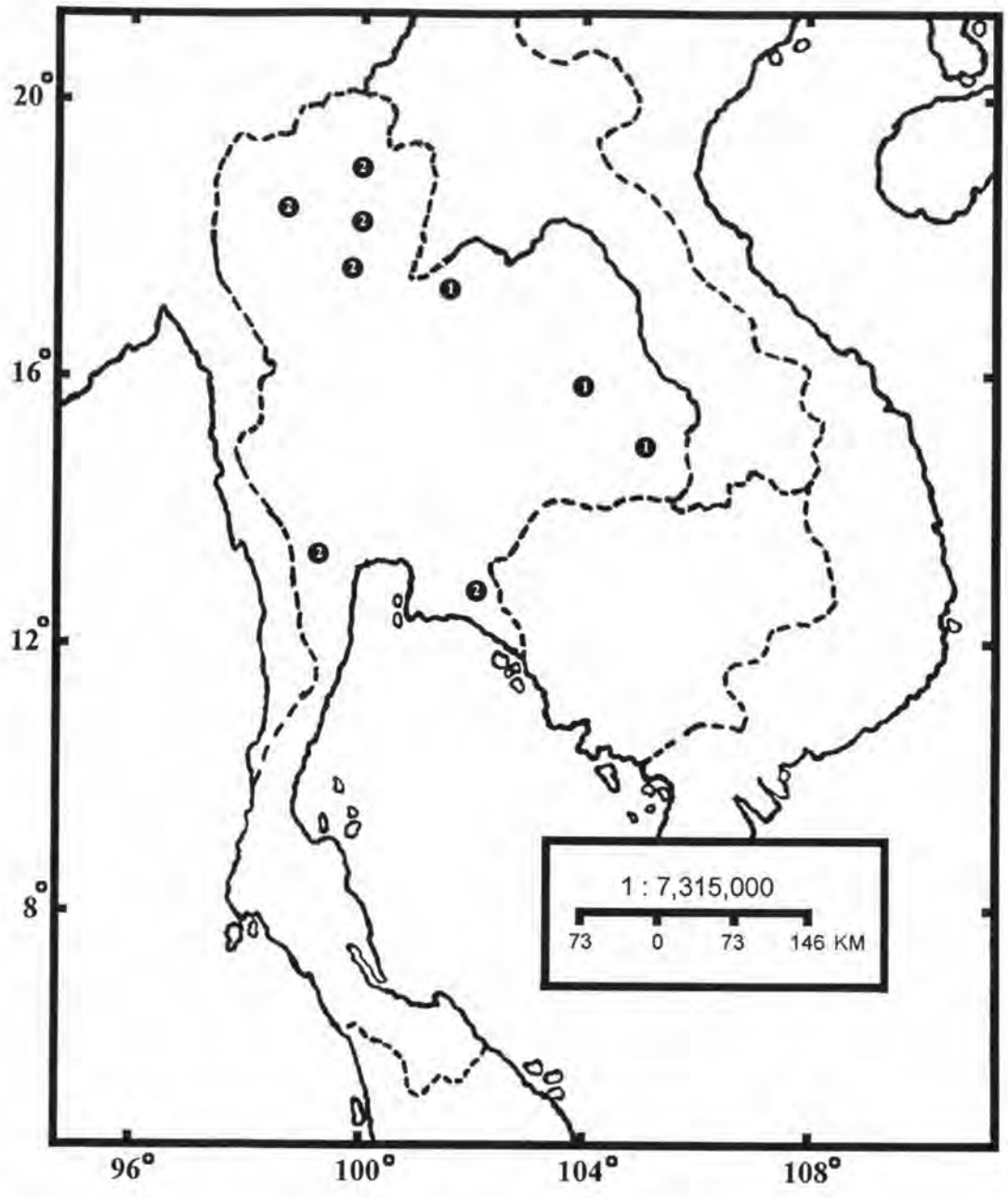

Figure 4.26 Distribution of mtDNA haplotypes of *T. terminata* in Thailand resulted from digestion of 16S rRNA gene with 3 restriction enzymes (abbreviation see Figure 4.25).

Genetic diversity among *T. thoracica* samples

From PCR-RFLP analysis, there are 2 haplotypes from all six samples (Figure 4.28). The genetic distance was 0.007897. The cluster groups produced from genetic distance value could be separated them into two groups, the first groups was Central (C 001 = Kanchanaburi Province) sample and the last groups consisted of Central (C 002-003) and Southern (S 004-006) samples (Figure 4.27).

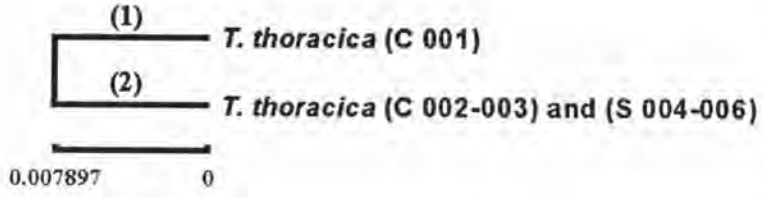

Figure 4.27 Dendrogram of relationships among *T. thoracica* populations in Thailand using genetic distance data of PCR-RFLP (3 enzymes) at 16S rRNA gene of mtDNA. (C = Central and S = Southern).

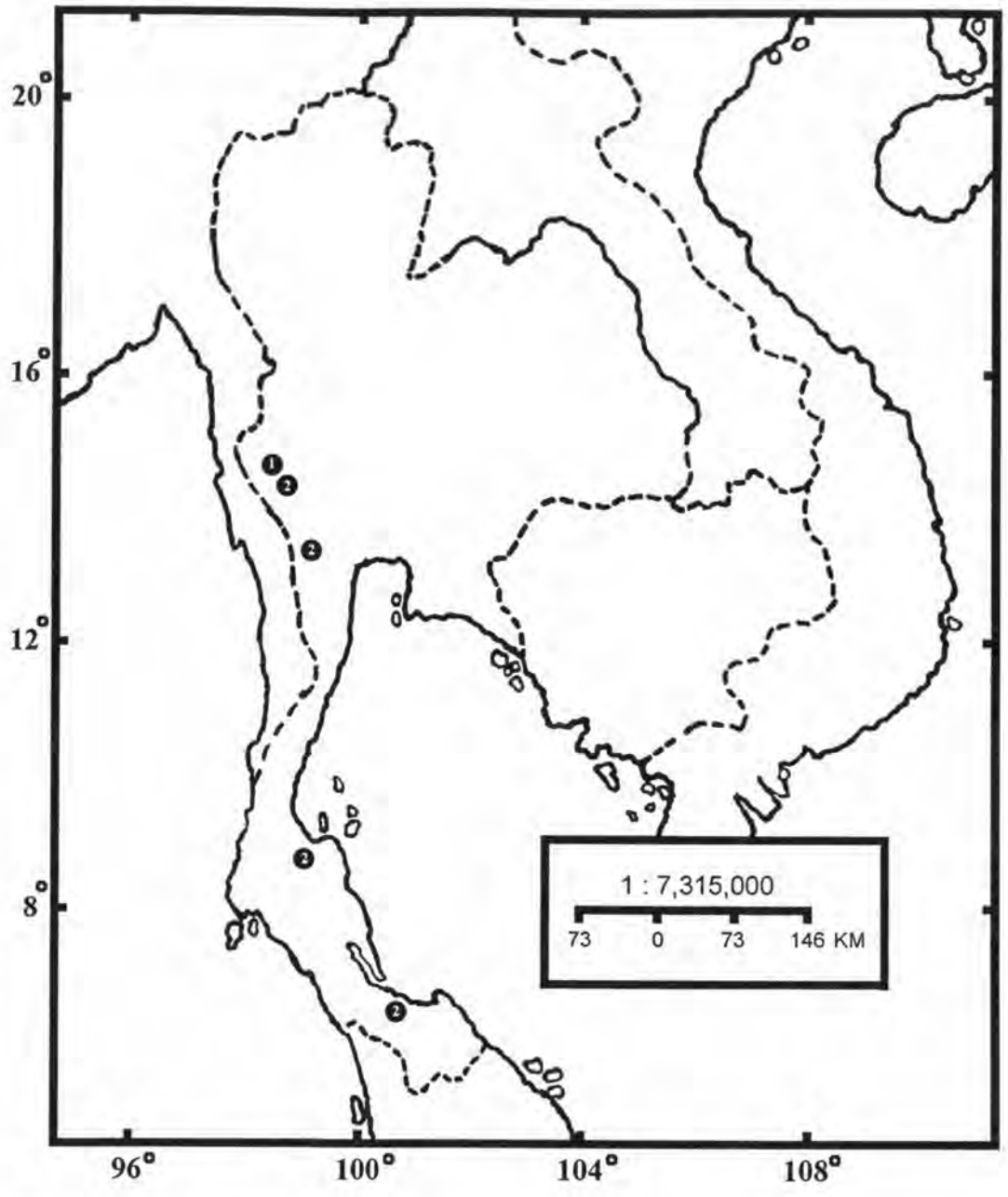

Figure 4.28 Distribution of mtDNA haplotypes of *T. thoracica* in Thailand resulted from digestion of 16S rRNA gene with 3 restriction enzymes (abbreviation see Figure 4.27).

4.4.2 Multidimensional scaling model (MDS)

The multidimensional scaling was employed to provide a graphical summary of the species-level results because of the very large number of taxa. MDS explores similarity relationships in Euclidian space and has the advantage of permitting genetically intermediate taxa to remain spatially intermediate, rather than forcing them to cluster into a pseudogroup as in hierarchical methods (Lessa, 1990). The MDS model of genetic distance values revealed clearly distinguished among three species (red = *T. collina*, green = *T. fuscobalteata*, blue = *T. thoracica*). Exception of *T. laeviceps* group (sky), they overlap with *T. terminata* group (violet) (Figure 4.29 and 4.30).

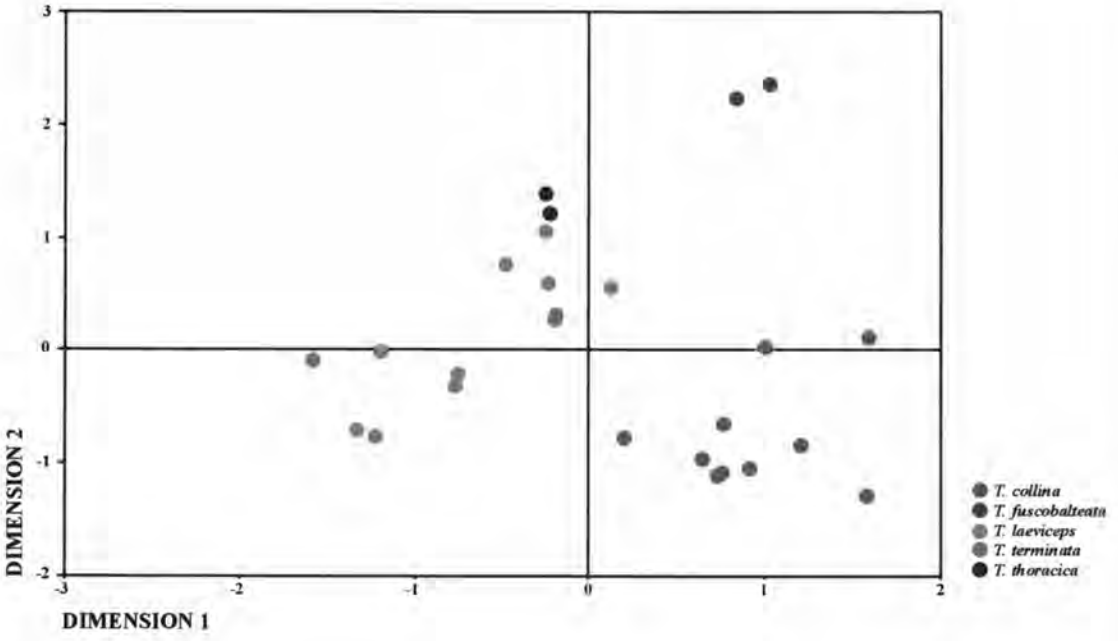

Figure 4.29 Multidimensional scaling model (two dimensions) generated from genetic distance matrices of PCR-RFLP data.

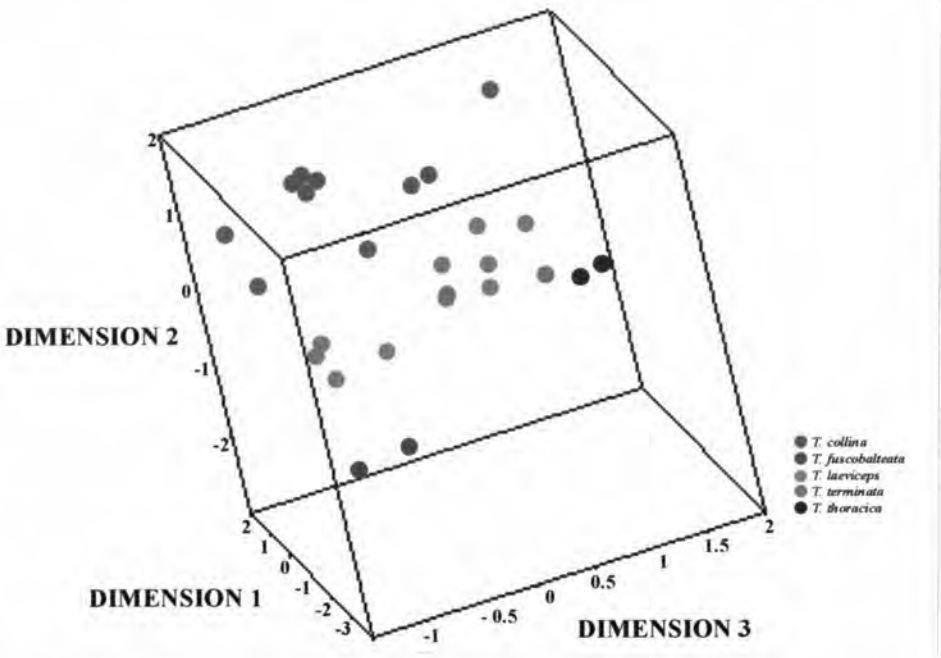

Figure 4.30 Multidimensional scaling model (three dimensions) generated from genetic distance matrices of PCR-RFLP data.

Table 5 Genetic distance between pairs of haplotypes from digestion patterns of 16S rRNA gene with three restriction enzymes (*Dra* I, *Hpy*188 III, and *Ssp* I).