CHAPTER 4

RESULTS AND DISCUSSION

4.1 mtDNA D-loop sequence analyses

After sequencing, the result is shown an electrophoregram, which is saved as an ABI-Trace data-file. Double peaks or conflicts between forward and reverse strands were always treated in the same way. In case of clear double peaks, or conflicts between forward and reverse strands, the sequences were considered to be possible pseudogenes and eliminated from the analyses. In most cases double peaks were not only found in single positions, but were more numerous. In sequences where single loci were disturbed by noise, the higher peak was chosen as the correct nucleotide base, if the signal from both the forward and reverse sequence was identical. If conflicts could not be resolved in this fashion, sequences were eliminated from the population studies. As gaps and length variants were not to be expected in the studied mitochondrial sequences, gaps were removed manually after rechecking the electrophoregram in SeqMan for the individuals and positions in question. As unresolved positions may or may not host a new haplotype, population studies could only be done with those sequences where all sites were clearly resolved. Sequencing did not result in the same quality and length of all sequences. For the final alignment, a compromise was made between using the maximal number of readable positions and the maximal number of individuals.

The complete sequence from 55 samples were found, each sample was sequenced using eight primers by the primer walking strategy to achieve a complete full-length double strand nucleotide sequence. Primer D-loop2F and D-loop3R were used to confirm the sequence at the repeat motif region (Figure 4.1).

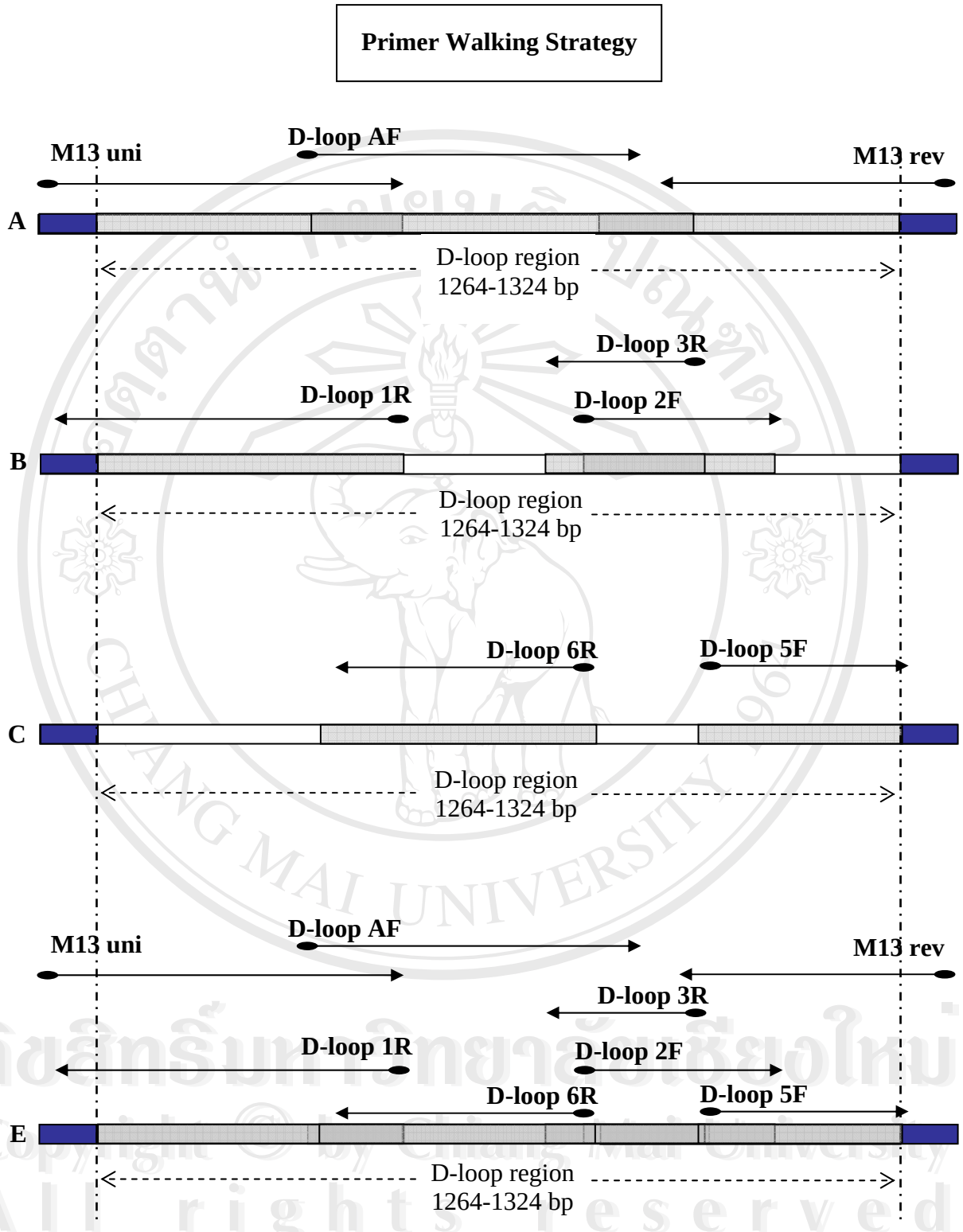

Figure 4.1 The result of primer walking strategy. The bar represents a segment of target DNA (shaded) flanked by vector sequences containing primer-binding sites (solid).

Ursing and Arnason (1998) describe the D-loop region between positions 15434 to 16675 of the porcine mtDNA D-loop reference sequence. The length of this region depends on the variable number tandem repeat (VNTR) or heteroplasmic repetitive sequence that located at the conserved sequence blocks (CSB) between CSB-1 and CSB-2 (Figure 4.3). In our samples, nucleotide sequence lengths of 1264 to 1324 bp were found, depending on the structure of repeat motif CGTGCGTACA. The results confirm observations made by Ghivizzani *et al.* (1993) and Lunt *et al.* (1998). The range of the repeat number was between 22-28 units and the frequency was 2, 7, 8, 18, 15, 4 and 1, respectively. The frequency histrogram revealing difference in the distribution profile of the porcine mtDNA VNTR is showing Figure 4.2.

However, in the majority of research project on the porcine mtDNA D-loop, the tandem repeat motif was not included in the analysis because the number of repeats was too variable within individuals indicating a high degree of heteroplasmy. Thus, this heteroplasmic region is eliminated before the multiple sequence alignment, except for the first unit at position 16146 (Okumura *et al*., 2001; Watanabe *et al*., 2001).

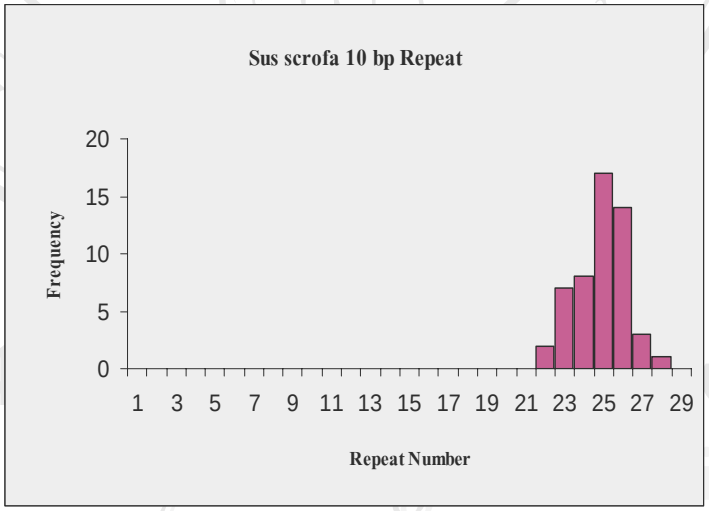

Figure 4.2 The frequency histrogram revealing difference in distribution profile of porcine mtDNA VNTR.

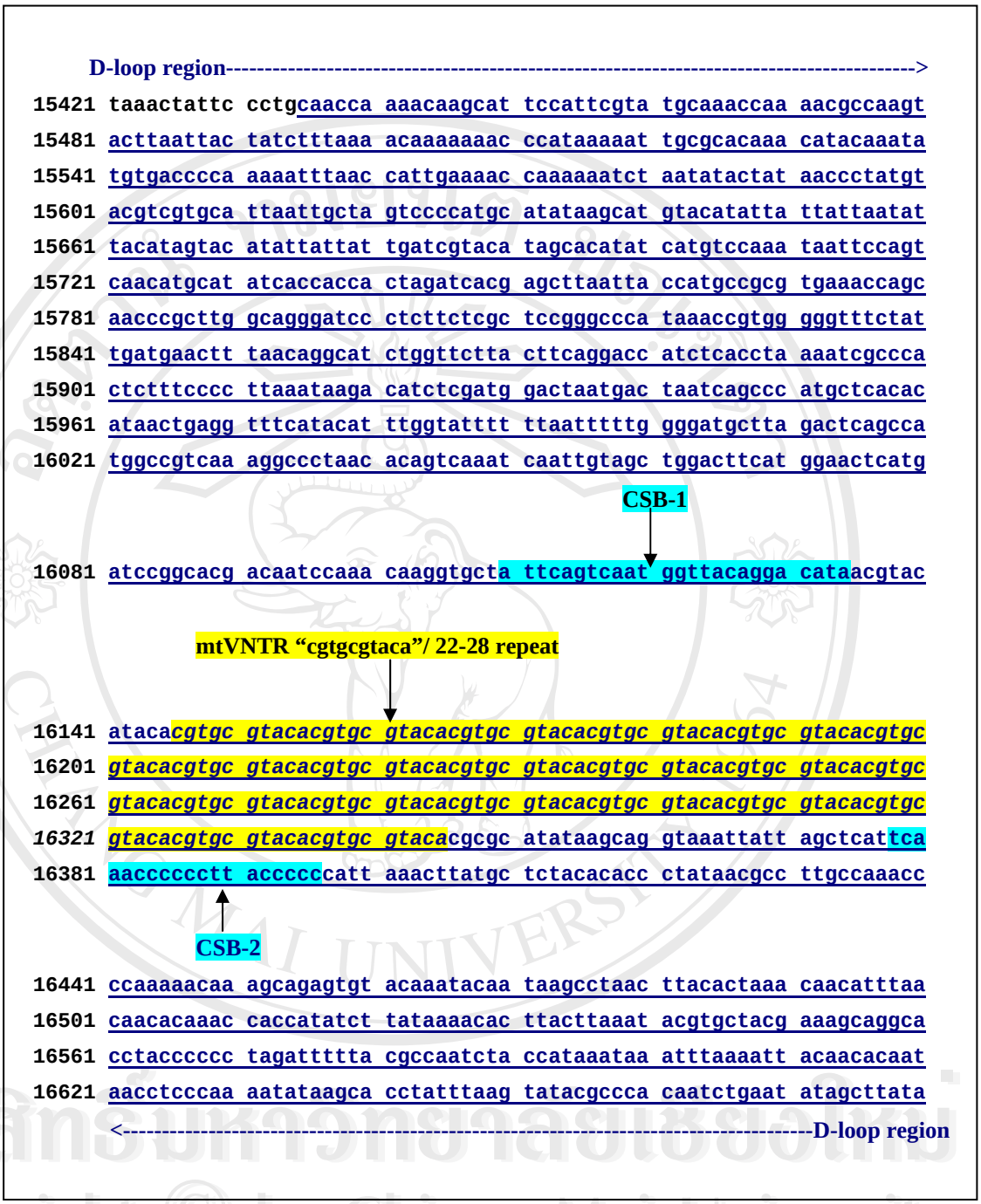

Figure 4.3 Nucleotide sequence of the porcine mtDNA D-loop region and the mtVNTR (Ursing and Arnason, 1998**).**

4.2 Haplotypes of Thai pigs

A 1044 bp long mtDNA D-loop region sequence obtained from 55 Thai pigs was analyzed. After exclusion of the repeat motif, 14 haplotypes were found. The mtDNA haplotypes investigations among the Thai pigs are shown in Table 4.1 and Figure 4.4. Haplotypes 1 to 12 are typical for Thai native, haplotype 13 and 14 are only found in Thai wild boars. The multiple sequence alignment of 14 Thai pigs haplotypes is displayed in Appendix B.

Table 4.1 mtDNA haplotypes of Thai native pigs from several locations in the Northern area and Thai wild boars.

Haplotype	Breeds	Locality (Amphur/Province)	n
1	Thai native pig	Muang/ Mae Hongson	4
2	Thai native pig	Jhom Thong/ Chiang Mai	4
		Chiang San/ Chiang Rai	2
3	Thai native pig	Tung Hua-chang/ Lamphun	1
		Viang Chai/ Chiang Rai	3
		Chiang San/ Chiang Rai	2
4	Thai native pig	Chiang San/ Chiang Rai	2
5	Thai native pig	Chiang San/ Chiang Rai	1
6	Thai native pig	Chiang San/ Chiang Rai	1
7	Thai native pig	Jhom Thong/ Chiang Mai	2
8	Thai native pig	Jhom Thong/ Chiang Mai	1
9	Thai native pig	Jhom Thong/ Chiang Mai	3
10	Thai native pig	Fang/ Chiang Mai	4
11	Thai native pig	Jhom Thong/ Chiang Mai	2
12	Thai native pig	Chiang Dao/ Chiang Mai	16
		Muang/ Mae Hongson	2
13	Thai wild boar	San Sai/ Chiang Mai	1
14	Thai wild boar	San Sai/ Chiang Mai	4
Total			55

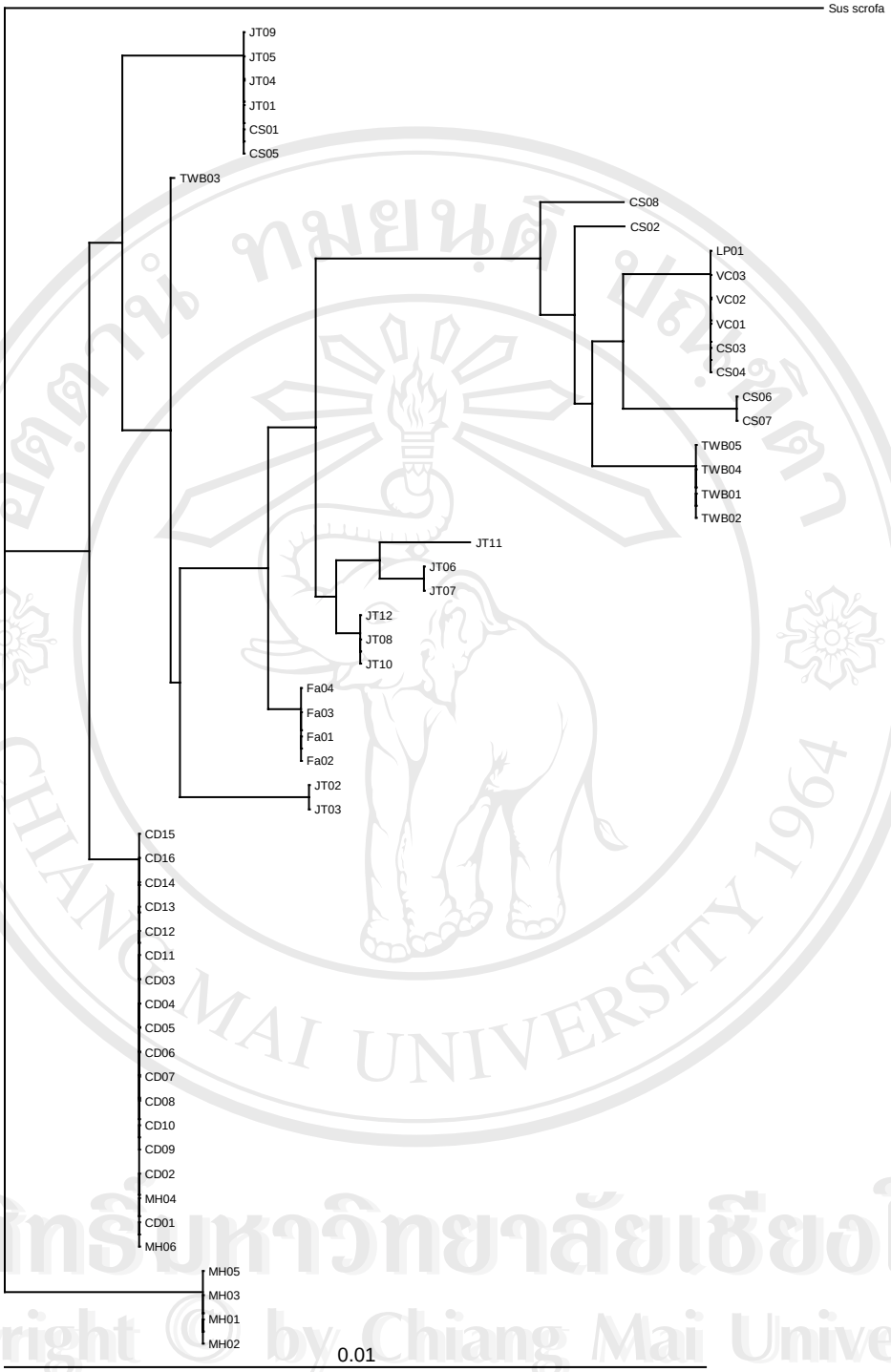

Figure 4.4 Neighbor-joining phylogenetic trees of mtDNA D-loop haplotypes from Thai pigs in Northern Thailand. The corresponding regional sequence of the *Sus scrofa* (GenBank Accession No. AJ002189) was used as an outgroups.

4.3 Nucleotide sequence variations

The mtDNA D-loop sequence comparisons between Thai pig haplotypes and further porcine mtDNA sequences taken from GeneBank were done in reference to the sequence of *Sus scrofa* (Figure 4.5) (Ursing and Arnason, 1998; GenBank Accession No. AJ002189). A total of 32 mtDNA haplotypes were compared, nucleotide substitutions were assessed using 12 haplotypes of Thai native pigs (haplotypes 1-12), 2 haplotypes of Thai wild boars (haplotypes 13 and 14), 7 haplotypes of European indigenous pigs (*Sus scrofa*, HS, LW, PT, DR, LR, BS), 6 haplotypes of Asian indigenous pigs (CJ, JH, TC, MS, SM, MC), 2 haplotypes of European wild boars (EWR1, EWB2), and 3 haplotypes of Asian wild boars (RWB, JWB, KWB).

The sequence alignment of the total mtDNA D-loop region without the VNTR (1044 bp) has revealed nucleotide variations at 54 positions (including gaps) from 1047 positions (percentage of polymorphisms: 5.16%; Figure 4.6 A). For Thai native pigs and Thai wild boars, 33 polymorphic sites compared with *Sus scrofa* have been detected, (represented 3.15%; Figure 4.6 B) Within groups comparisons displayed 19 polymorphic sites, representing variation of 1.85% (Figure 4.6 C). Three of the 54 position represented insertion/ deletion of single nucleotide base pairs (at positions 131, 144 and 274). At position 131, G was only found in *Sus scrofa*, HS, LW, PT, DR, LR, BS, and EWB1 and can be regarded as specific character for European pig breeds. Moreover, A at position 144 was only found in HS as well as C at position 274 specifically for the *Sus scrofa* reference sequence. The remaining 51 variable positions were 49 nucleotide transition and 2 transversions (25:1 ratio of transition: transversion) indicating a strong transitional bias that is common in mammalian mitochondrial evolution (Brown *et al.*, 1979; Watanabe *et al.*, 2001). Unique specific sequences for Thai pigs and other Asian pigs were found at positions 109, 136, 146, 159, 961, and 1018 (Figure 4.5). It is interesting that some of these variations are partly also found in the haplotypes of the European wild boar. However it is possible that this might come from a nucleotide substitution bias back to the common ancestor (Gongora *et al.*, 2004).

Figure 4.5 Types of nucleotide substitutions at the variable site in the mtDNA D-loop haplotype. Positions are according to the *Sus scrofa* reference (Ursing and Arnason, 1998; GenBank Accession No. AJ002189). The symbol (.) denotes congruence with haplotype *Sus scrofa*; (-) denotes a deletion or insertion.

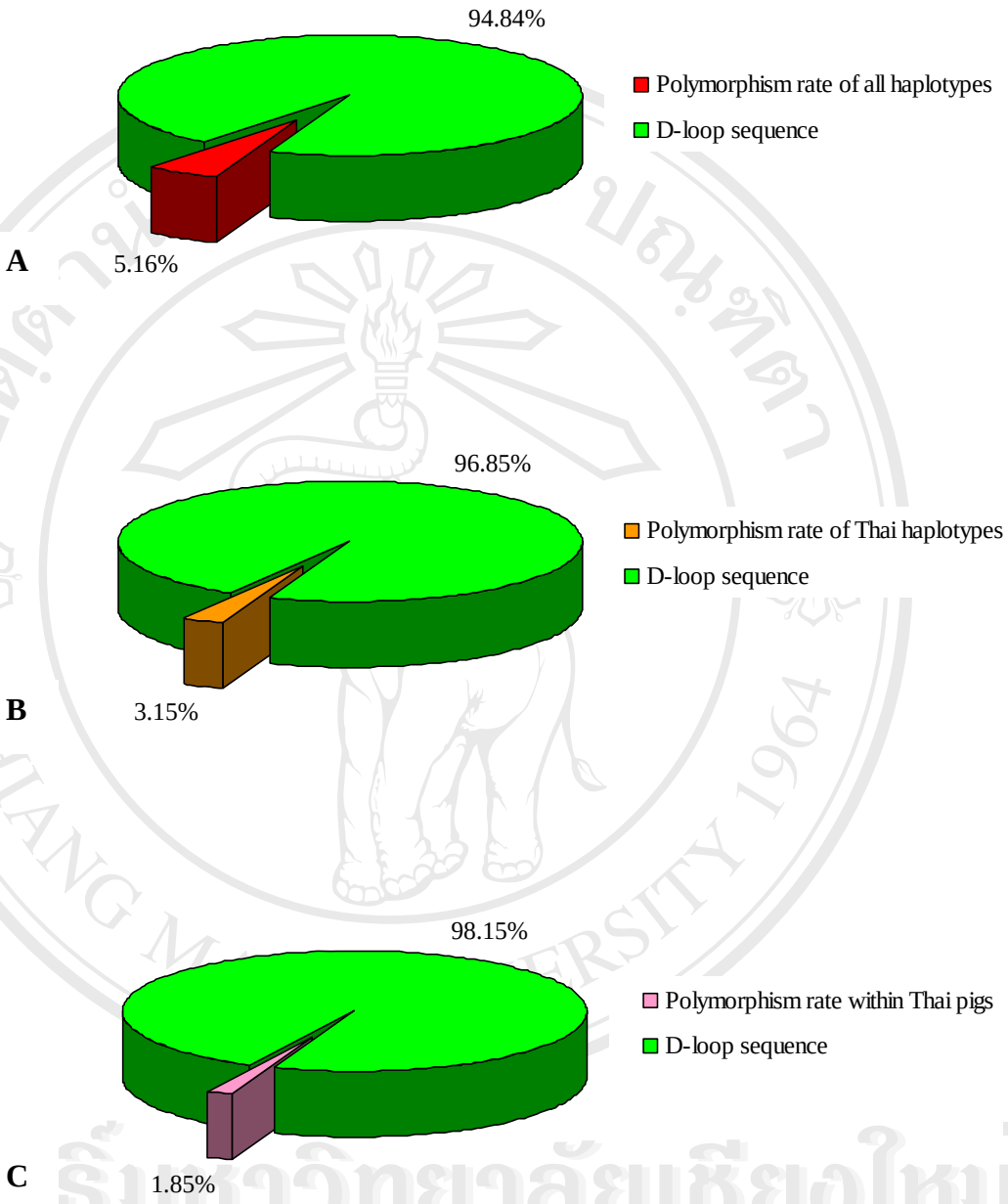

Figure 4.6 Comparison of polymorphism rate in D-loop sequences. Polymorphisms in A refer to all haplotypes, polymorphisms in B compare Thai haplotypes with others (*Sus scrofa*), polymorphisms in C within Thai pigs.

4.4 Phylogenetic analyses

The results of the Kimura 2-parameter method (Kimura, 1980) was used to calculate the number of nucleotide substitution per site between all pairs of sequences, and Phylogenetic trees were subsequently constructed using the neighbour-joining method. The Warthog (*Phacochoerus aethiopicus*) sequence was also determined and used as an outgroup (Figure 4.7). In each phylogeny, the mtDNA D-loop sequence analysis showed that the indigenous Thai pigs were grouped to the other Asian breeds and they were distinctly different from the European breed, there were classified as two major groups i.e. European groups (EU) and Asian groups (AS) (Figure 4.8). In the EU group, EWB1 showed the highest genetic distance compared with the others European breeds. In the AS group, the wild boar from Korea and Japan showed the highest genetic distance compared with the other Asian breeds. In the case of Thai wild boars (haplotype 12) it was found that they have a genetic similarity with Thai native pigs from Lamphun and Chiang Rai Provinces (haplotypes 3, 4, 5, 6).

Moreover, Thai native pig haplotype 1 from Mae Hong-son Province displayed a high genetic similarity with the wild boar haplotype RWB from Ryukyu Islands, located between Korea and Japan (Okumura *et al.*, 2001). Thai native pigs with others haplotypes showed a high genetic similarity with other Asian native breeds e.g. haplotypes 7, 8, 9, 10 were grouped to the Moncai (MC) pig from Vietnam and to the Sasuma (SM) from Japan. The last group of Thai native pigs (haplotypes 2, 11, 12, 13) clusters with Meishan (MS) and Tong Cheng (TC) from China. This situation might be the result of immigration events in the former times or of the transfer of haplotypes that probably arose from the 18th and 19th century common interbreeding of Asian stocks (Kijas and Andersson, 2001). In addition, Okumura *et al.* (2001) stated that European domestic pigs (*Sus scrofa scrofa*) were cross bred with Chinese (*Sus scrofa moupinensis*) and Indian pigs (*Sus scrofa cristatus*) and that these modified breeds, together with others, were carried on the 18th and early 19th century voyages to South-east Asia and beyond.

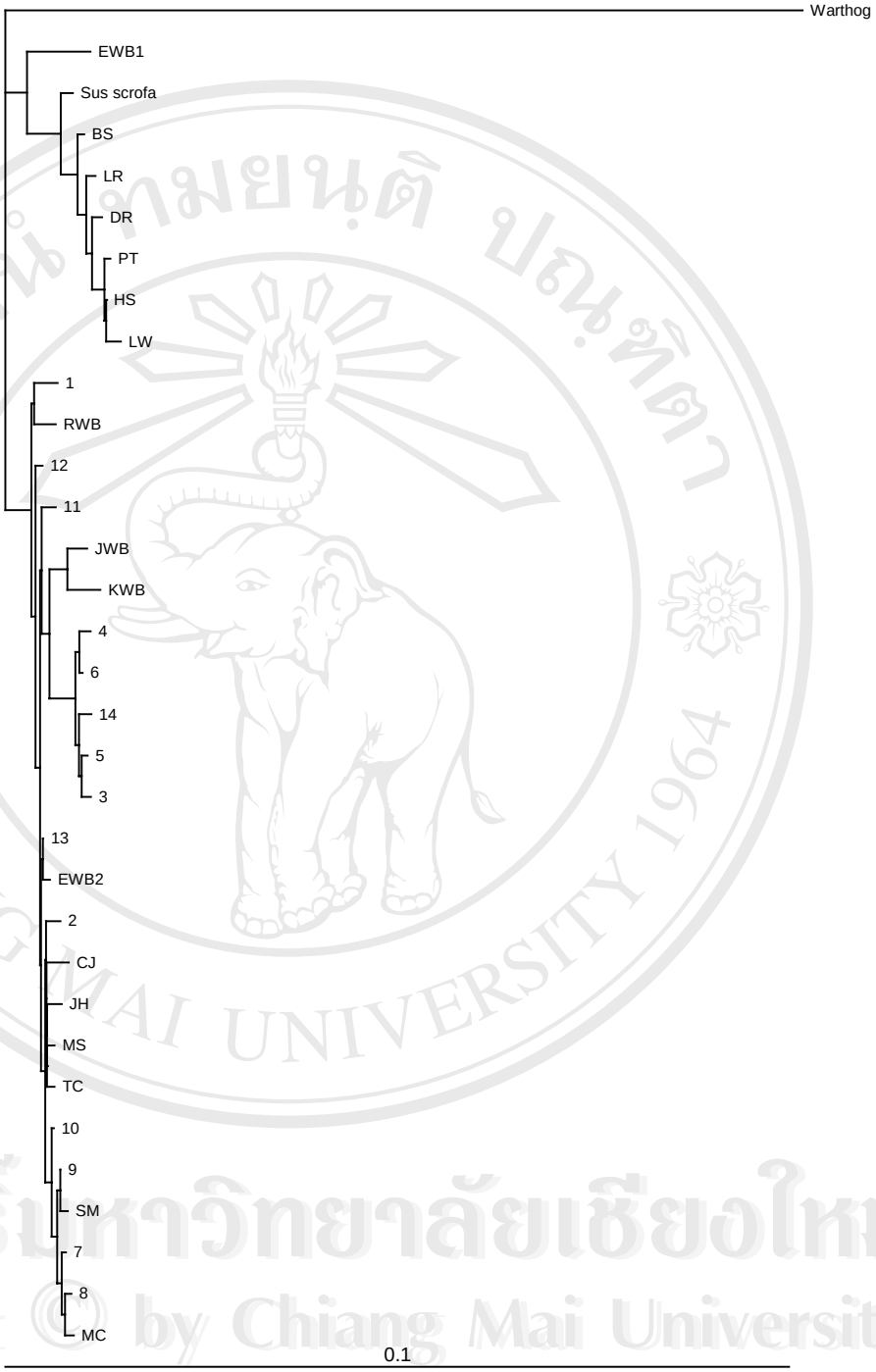

Figure 4.7 Neighbor-joining phylogenetic trees of mtDNA D-loop haplotypes from Thai pigs in Northern Thailand and European and Asian domestic pigs and wild boars. The corresponding regional sequence of the Warthog (*Phacochoerus aethiopicus*; GenBank Accession No. AB046876) was used as an outgroup.

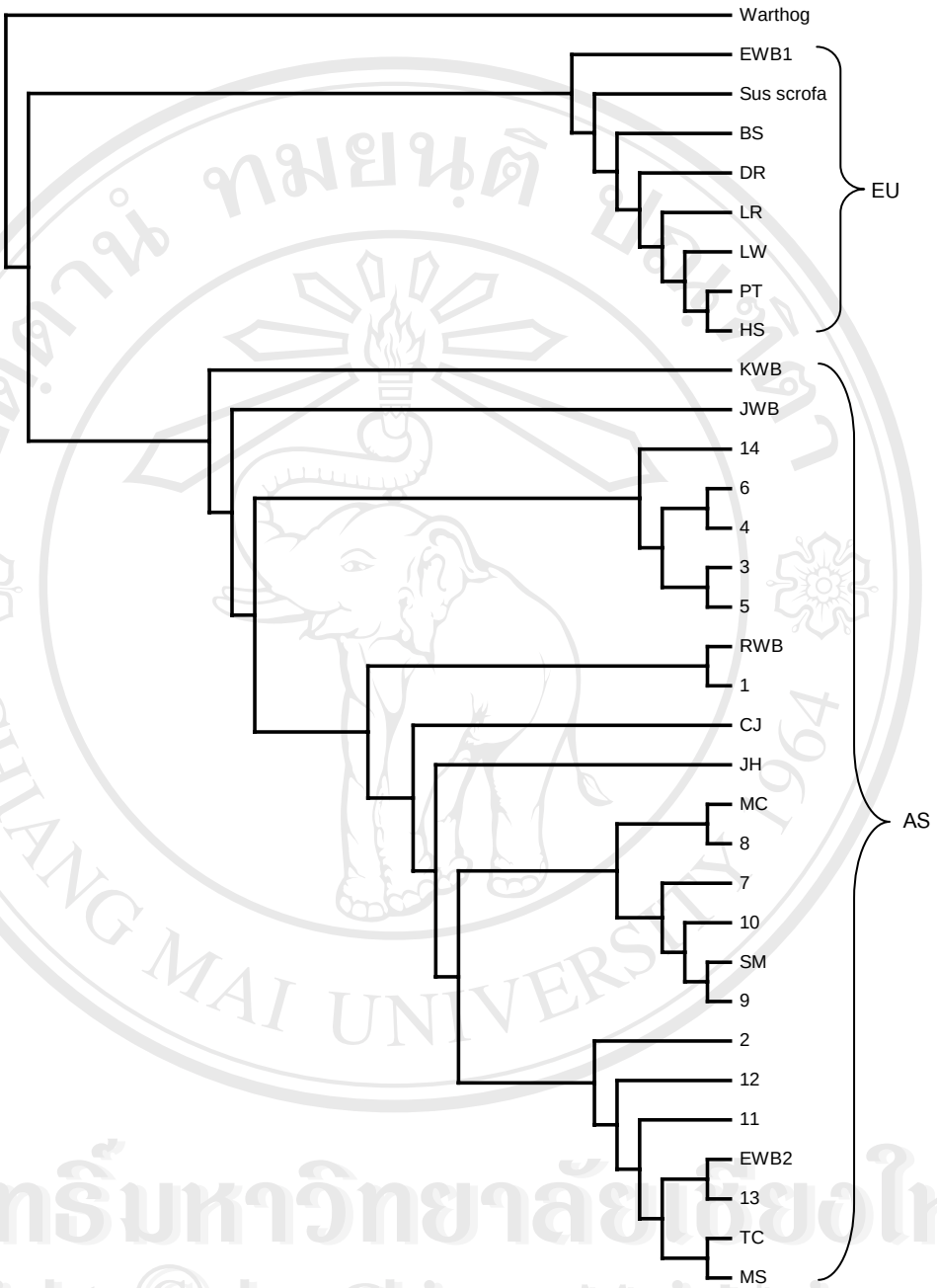

Figure 4.8 The neighbor-joining dendrogram; EU is the European group and AS is the Asian group.

However, one putative European wild boar sequence (haplotype EWB2; Accession No.AB059651) has none of the European motif nucleotides and clusters with the Asian group. This result in concordance with the study of Okumura *et al.* (2001) and Gongora *et al.* (2004) who found that some European pig breeds have a high genetic similarity with Asian pig breeds and are at the same time distinctly different from the same European breed. They assumed this result that may reflect a data entry error, or a misnamed sample (Gongora *et al.*, 2004). Alternatively, this result may arise from the difference sequence length studied, whether there is outgroup comparison or not, and the low level of bootstrap support between groups (Okumura *et al.*, 2001). Therefore, this study can not discuss whether the gene flow occurred from Asian mitochondrial types into European wild boars and domestic pigs, or the Asian type distributed widely around the Eurasian continent from Asia to Northern Europe.