# Chapter 4

# Results and Discussions

## 4.1 Morphometric analysis

All specimens were measured for 5 parameters and weight as well as were identified for their age level and sex. The data were shown in Table 1. Results comparing the two subspecies were obtained using analysis of variance

The wing length, tarsometatarsus length, base of beak to nape length, third digit length showed no difference between two groups. But the beak length showed a significant different between two groups (Beak length mean= 1.410, F value=10.06 at $p<0.05$). See Appendix III.

The beak length compared between two subspecies showed the difference, but all specimens are caged birds in captivity, so the result might have some bias due to their changed feeding behavior from their wild habitat, or differences in husbandry between the two sample groups.

Morphometric analysis using more characters should have been done for a larger number of animal both in the wild and captive for differentiation of their morphology, and might have produced more significant taxonomic differences.

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Table 1. Morphometric data of two subspecies (G=*Gallus gallus gallus*, S=G. g. *spadiceus*)

| No. | Wgt | BL | WL | TM | HD | TD | AGE | SEX |
|---|---|---|---|---|---|---|---|---|
| G1 | 0.85 | 1.2 | 29.0 | 7.7 | 5.7 | 5.3 | A | F |
| G2 | 1.20 | 1.1 | 36.8 | 9.2 | 6.2 | 4.1 | A | M |
| G3 | 1.30 | 1.3 | 33.7 | 8.5 | 6.8 | 5.5 | A | M |
| G4 | 0.67 | 1.2 | 29.5 | 7.4 | 6.0 | 4.6 | A | F |
| G5 | 0.84 | 1.5 | 30.2 | 7.8 | 5.8 | 5.0 | A | F |
| G6 | 0.79 | 1.2 | 32.2 | 7.6 | 5.8 | 4.2 | A | F |
| G7 | 0.66 | 1.3 | 30.5 | 7.5 | 5.8 | 5.0 | A | F |
| G8 | 0.77 | 1.4 | 30.5 | 7.7 | 5.7 | 4.7 | A | F |
| G9 | 1.20 | 1.5 | 35.7 | 8.9 | 6.4 | 4.9 | A | M |
| G10 | 1.30 | 1.4 | 36.7 | 9.3 | 6.3 | 5.3 | A | M |

| No. | Wgt | BL | WL | TM | HD | TD | AGE | SEX |
|---|---|---|---|---|---|---|---|---|
| S83 | 0.70 | 1.4 | 30.4 | 7.0 | 5.5 | 5.0 | A | F |
| S84 | 1.20 | 1.7 | 38.0 | 8.9 | 6.2 | 5.4 | A | M |
| S85 | 1.10 | 1.6 | 36.3 | 9.1 | 6.4 | 5.6 | A | M |
| S86 | 0.84 | 1.4 | 29.5 | 7.2 | 5.2 | 5.0 | A | F |
| S87 | 1.10 | 1.6 | 35.7 | 8.7 | 6.0 | 5.7 | A | M |
| S88 | 0.93 | 1.6 | 34.5 | 7.2 | 5.7 | 4.9 | A | F |
| S89 | 0.75 | 1.2 | 27.5 | 7.3 | 5.8 | 5.1 | J | F |
| S90 | 0.73 | 1.5 | 28.0 | 7.0 | 5.8 | 5.2 | J | F |
| S91 | 0.68 | 1.5 | 29.0 | 6.7 | 5.3 | 4.6 | J | F |
| S92 | 1.05 | 1.6 | 32.2 | 8.9 | 6.4 | 5.3 | J | M |

Wgt = Weight (kg)
BL = Beak length (cm)
WL = Wing length (cm)
TM = Tarsometatarsus length (cm)
HD = Head (Basement of upper beak to occipital, cm)
TD = Third digit length (cm)
A = Adult M= Male
J = Juvenile F= Female

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## 4.2 Protocol used for DNA extraction.

2 protocols for mtDNA extraction were used; first the Chelex extraction and second, the classical phenol-chloroform extraction. Ethidium bromide stained agarose gel (Figure 4.1) shows the DNA extraced from Chelex compare to phenol-chloroform extracted product. Chelex ectracted product can not be observed on the gel while concentrated Phenol-Choroform extracted product gave a clear signal of DNA quantity obtained from the extraction.

The comparative result was also observed by the first PCR product on 2% agarose gel electrophoresis. Using the Chelex extract to add PCR mixture as template DNA gave no signal from PCR. Total of 20 samples has been tried and double extraction trials were done.

PCR signals were successfully obtained using phenol-chloroform extracts as an alternative template in PCR mixture. One microliter of Bovine Serum Albumin (BSA) needs to be added as an inhibitors terminator(Cooper, 1994) to all PCR mixtures, otherwise no signals can be obtained.

Cooper (1994) mentioned that the Heme and cytochrome from blood are the major PCR inhibitors. Meckvichai (1997) succeeded in using Chelex extracts for cytochrome b gene amplification but it could not be used for D-loop amplification in this study.

A concentrated purified phenol-chloroform extract is recommended to use as a template DNA for chicken D-loop amplification if sample used are bloodstains.

### 4.3 DNA amplification from D–loop region

Optimization of PCR condition, using the universal cytochrome b primer for amplification of the some samples of Chelex extracted product and the positive bands were obtained. The PCR mixture and cycles was described in chapter 2.

Using the Chelex extracted solution as template DNA for the PCR reaction gave all negative results when using D-loop primer for amplification.

The alternative phenol-chloroform extracted products were used as an improved substitution. These samples gave positive signals of the D-loop amplified product (Figure 4.2 a and b) but Bovine Serum Albumin (BSA, Sigma) was needed for every PCR reaction tube as inhibitor terminators.

PCR primers was a chicken specifically designed and could amplify the entire 1254 bp fragment, the whole D-loop region.

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Figure4.1 Ethidium bromide stained agarose gel (1.5%) show the DNA extraced from Chelex compare to phenol-chloroform extracted product. M=$\phi$X174 DNA marker, Lane 1= Chelex ectracted product can not be observed on the gel, Lane 2= Phenol-Choroform extracted product (concentrated) gave a clear signal of DNA quantity obtained.

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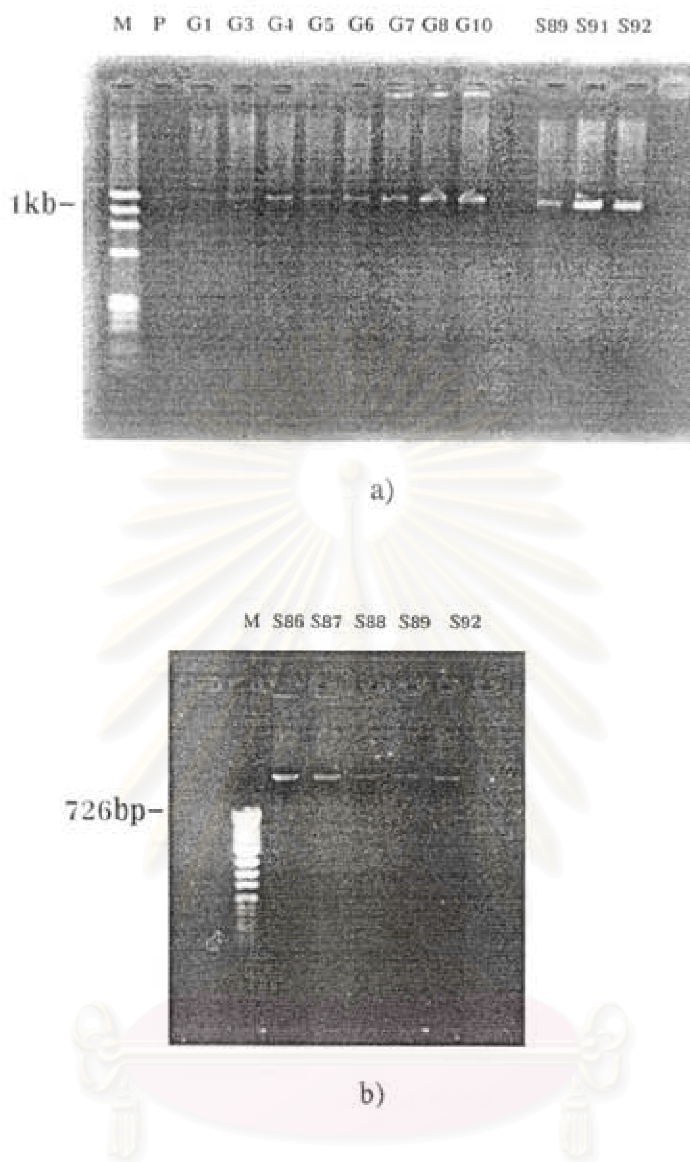


Figure 4.2 Ethidium bromide stained gel showing the positive PCR signal of amplified Chicken D-loop gene product.

a) M=∅X174 Hae III DNA marker, G1, 3, 4,5, 6, 7, 8 and 10= *Gallus gallus gallus* samples, S89, 91, 92= *G. g. spadicues* samples, P = positive control.

b) M=∅X174 Hinf I DNA marker, S86, 87, 88, 89 and 92= *G. g. spadiceus* samples.

Figure 4.3 The exposed X-ray film from $^{32}$P-labelled chicken D-loop DNA sequence.

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### 4.4 Second PCR generating ss-DNA

For manual DNA sequencing using chain-terminating inhibitor (Sanger, 1977), it is better to generate the single-stranded DNA from the first PCR product. Gyllensten (1988) described the protocol for asymmetric PCR that it could generate the single-stranded DNA for the sequencing. The result is not shown here.

### 4.5 DNA sequence analysis

225 bp sequence from 16 samples, 6 *Gallus gallus spadiceus* and 10 *G. g. gallus*, were analyzed. The obtained sequences were mitochondrial D-loop L-chains (base position L41-L323 compare to *Gallus domesticus* by Desjardins and Morais, 1990 with 3 bases missing). The nucleotide composition of the D-loop sequence were reported by Desjardins and Morais (1990) as; A=26.7, G=13.3, C=26.3 and T=33.7.

There are 5 sites of variation in *G. g. gallus* and 15 sites in *G. g. spadiceus* which means the genetic variation in the observed area of *G. g. spadiceus* is three times higher than that of *G. g. gallus.*

Within subspecies *G. g. gallus* , 5 transitions (TS) were found. There is no transvertional (TV) nucleotide substitution in this subspecies. The genetic distance within this subspecies= 0.0000-0.0225. Sequence divergence varies from 0 to 2.25%. Fumihito et al (1994) reported the variation of D-loop region of 2 *G. g. gallus* from Thailand that there are 1.25 % sequence divergence.

Within subspecies *G. g. spadiceus*, 10 transitions and 5 transversions were found. The TS: TV ratio is 2:1. The sequence number S84 and S85 showed 100% homology along 225 bp alignment so they were treated into the same taxa when using for the parsimonious analysis. The genetic distance within this subspecies=

I17538016

0.0000-0.0654. Sequence divergence varies from 0 to 6.54% while Fumihito et al (1994) reported 4.25 % from one sample of *G. g. spadiceus* from Thailand.

There are 9 sites variable between two subspecies. The TS: TV ratio is 8:1. The genetic distances between two subspecies= 0.0134-0.0800. Genetic distances between groups' seem to be larger than that of within each group.

Using the published Japanese quail (*Coturnix coturnix japonica*) as an outgroup reference, 20 variation site were found after aligning the sequences by CLUSTAL V in PHYLIP program version 3.57c (Felsenstein, 1993). The obtained sequences showed the CCC base triplets, which are shown underlined in Figure 4.4, that are similar to the sequences published by Fumihito et al. (1994) but were missed from domestic chicken (*Gallus domesticus*) D-loop that was published by Desjardins and Morais (1990).

All sequences were analyzed for their phylogenetic relationship using the genetic distance data (Kimura's 2 parameters) shown in Table 3.2. The tree was constructed with neighbor-joining method using PHYLIP program version 3.572c and was shown in Figure 4.6. The samples were divided in two groups. One with all the *G. g. spadiceus* (S84, 85, 86, 87, 88 AND 92) and *G. g. gallus* number G9, and another with 9 *G. g. gallus* samples (G 1, 2, 3, 4, 5, 6, 7, 8 and 10)

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CLUSTAL V multiple sequence alignment

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JAPQU           CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTCGATACATTT
1994            CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTCGATACATTT
S84             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
S85             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
S86             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGGGCATACATTT
S87             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGGGCATACATTT
S88             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGGGCATACATTT
S92             CCCCTTTCCCCCCCGGGGGGGGTATACTATGCATAATCGGGCATACATTT
G1              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G2              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G3              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G4              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G5              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G6              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G7              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G8              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G9              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G10             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
                ************** ************************   ********

JAPQU           ATATTCCACATATACTATGGTACCGGTAATATATATTATATACGTACTAA
1994            ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
S84             ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
S85             ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
S86             ATATACCACATATATTATGGCACCGGTAATATATACTATATATGCACTAA
S87             ATATACCACATATATTATGGCACCGGTAATATATACTATATATGCACTAA
S88             ATATACCACATATATTATGGCACCGGTAATATATACTATATATGCACTAA
S92             ATATATCCCATATATTATGGCACCGATAATATATACTATATTGCCACTAA
G1              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G2              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G3              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G4              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G5              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G6              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G7              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G8              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G9              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G10             ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
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JAPQU     ACCCATTATATGTATACGGGCATTA-CATATATTCCCCATTTCTCCCCAT
1994      ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S84       ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S85       ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S86       ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S87       ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S88       ACCCATTATATGTATACGGGCATTAAATTATATTCCACATTTCTCCCAAT
S92       ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G1        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G2        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G3        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G4        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G5        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G6        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G7        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G8        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G9        ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G10       ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
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JAPQU     GTACATTA-GTGCATGCTCCAAGACATA----------------------
1994      GTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGAC
S84       GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCTCATAGAC
S85       GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCTCATAGAC
S86       GTCCATACTATGCATGATCCAAGACATACTCATTCACCCTTCTCATAGAC
S87       GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTTCTCATAGAC
S88       GCCCATTCTATGCATGATCCAAGACATACTCATTCACCTCTCTCATAGAC
S92       GTCCATTCTATGCATGATCTAAGACATACTCGTTCACCCTTCTCATAGAC
G1        GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G2        GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G3        GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G4        GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G5        GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G6        GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G7        GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G8        GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G9        GTCCATTCTATGCATGATCCAAGACATACCCATTCACCCTCCTCATGGAC
G10       GTCCATTCCATGCATGATCCAAGACATACTCATTCACCCTCCTCATAGAC
          *  ***    ****** ** * ******
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JAPQU      -------AACCA-TAC--GTTCACC
1994       AGCTCCAAACCACTACCAAGTCACC
S84        AGCTCCAAACCACTACCAAGTCACC
S85        AGCTCCAAACCACTACCAAGTCACC
S86        AGCTCCAAACCACTACCAAGTCACC
S87        AGCTCCAAACCACTACCAAGTCACC
S88        AGCTCCAAACCACTACCAAGTCACC
S92        AGCTCCAAACCACTACCAAGTCACC
G1         AGCTCCAAACCACTACCAAGTCACC
G2         AGCTCCAAACCACTACCAAGCCACC
G3         AGCTCTAAACCACTACCAAGCCACC
G4         AGCTCTAAACCACTACCAAGCCACC
G5         AGCTCTAAACCACTACCAAGCCACC
G6         AGCTCTAAACCACTACCAAGCCACC
G7         AGCTCTAAACCACTACCAAGCCACC
G8         AGCTCCAAACCACTACCAAGTCACC
G9         AGCTCCAAACCACTACCAAGTCACC
G10        AGCTCTAAACCACTACCAAGCCACC
                  ***** ***     ****
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Figure 4.4 L-chain sequences of 225 bases of the mitochondrial control region from *G. g. gallus* and *G. g. spadiceus* using the published Japanese quail ( JAPQU, *Coturnix coturnix japonica*) as an outgroup. 1994 is Thai Red junglefowl published sequence by Fumihito et al., 1994. CCC base triplets underlined in the first section were bases missed in two previous publications by Desjardins and Morais 1990 but similar to the sequence by Fumihito el al, 1994. The stars under the blocks mark the homology of nucleotides.

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From the DNA sequence analysis, it is shown that *Gallus gallus spadiceus* samples have higher divergence in their nucleotide sequence than that of *G. g. gallus*. Using the assumption that mutation occur through time of evolution, it is possible that the *G. g. gallus* might have evolved from *G. g. spadiceus*.

The wider range of distribution and more number of population and individuals may cause the higher genetic variation in *G. g. spadiceus* even small number of samples were collected.

From tree drawn by neighbor-joining method, sample number G9 that fall into the same group with other subspecies might have had an evidence of genetic hybridization with *G. g. spadiceus* but need confirmation by nuclear DNA profile.

The most parsimonious tree showed the distinctive two groups with the unclear polytomies, which are S84, S85, G1, G2, G8 and G9. These samples might have common sequence characters in the observed 225 bp even their morphology are different. The sequence number S84 and S85 that showed 100 % homology might have common ancestor.

Regarding the conservation genetics, the animal number G8 and G9 may not be appropriate to use as a parent stock for the *gallus* lineage production since their DNA profile were closed to their *spadiceus* counterparts. All other sample would be a good stock and should have been preserved.

Table 2. Estimated genetic distances among 16 samples, published sequence (1994) and outgroup (Japq, *Coturnix coturnix japonica*) obtained from 225 bp of D-loop sequence.

| | Japq | 1994 | S84 | S85 | S86 | S87 | S88 | S92 | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Japq | - | | | | | | | | | | | | | | | | | |
| 1994 | 0.0844 | - | | | | | | | | | | | | | | | | |
| S84 | 0.0910 | 0.0181 | - | | | | | | | | | | | | | | | |
| S85 | 0.0910 | 0.0181 | 0.0000 | - | | | | | | | | | | | | | | |
| S86 | 0.1160 | 0.0414 | 0.0226 | 0.0226 | - | | | | | | | | | | | | | |
| S87 | 0.1094 | 0.0366 | 0.0180 | 0.0180 | 0.0045 | - | | | | | | | | | | | | |
| S88 | 0.1160 | 0.0604 | 0.0411 | 0.0411 | 0.0272 | 0.0226 | - | | | | | | | | | | | |
| S92 | 0.1556 | 0.0805 | 0.0605 | 0.0605 | 0.0462 | 0.0414 | 0.0654 | - | | | | | | | | | | |
| G1 | 0.0910 | 0.0135 | 0.0045 | 0.0045 | 0.0272 | 0.0226 | 0.0458 | 0.0654 | - | | | | | | | | | |
| G2 | 0.0970 | 0.0181 | 0.0089 | 0.0089 | 0.0318 | 0.0271 | 0.0505 | 0.0702 | 0.0045 | - | | | | | | | | |
| G3 | 0.0970 | 0.0226 | 0.0134 | 0.0134 | 0.0365 | 0.0317 | 0.0552 | 0.0751 | 0.0089 | 0.0045 | - | | | | | | | |
| G4 | 0.0970 | 0.0365 | 0.0180 | 0.0180 | 0.0411 | 0.0364 | 0.0600 | 0.0800 | 0.0225 | 0.0180 | 0.0134 | - | | | | | | |
| G5 | 0.0970 | 0.0365 | 0.0180 | 0.0180 | 0.0411 | 0.0364 | 0.0600 | 0.0800 | 0.0225 | 0.0180 | 0.0134 | 0.0000 | - | | | | | |
| G6 | 0.0970 | 0.0365 | 0.0180 | 0.0180 | 0.0411 | 0.0364 | 0.0600 | 0.0800 | 0.0225 | 0.0180 | 0.0134 | 0.0000 | 0.0000 | - | | | | |
| G7 | 0.0970 | 0.0365 | 0.0180 | 0.0180 | 0.0411 | 0.0364 | 0.0600 | 0.0800 | 0.0225 | 0.0180 | 0.0134 | 0.0000 | 0.0000 | 0.0000 | - | | | |
| G8 | 0.0910 | 0.0135 | 0.0045 | 0.0045 | 0.0272 | 0.0226 | 0.0458 | 0.0654 | 0.0000 | 0.0045 | 0.0089 | 0.0225 | 0.0225 | 0.0225 | 0.0225 | - | | |
| G9 | 0.0910 | 0.0272 | 0.0089 | 0.0089 | 0.0318 | 0.0271 | 0.0505 | 0.0702 | 0.0134 | 0.0180 | 0.0225 | 0.0180 | 0.0180 | 0.0180 | 0.0180 | 0.0134 | - | |
| G10 | 0.0970 | 0.0318 | 0.0134 | 0.0134 | 0.0365 | 0.0317 | 0.0552 | 0.0751 | 0.0180 | 0.0134 | 0.0089 | 0.0045 | 0.0045 | 0.0045 | 0.0045 | 0.0180 | 0.0225 | - |

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Figure 4.6 The phylogenetic tree inferred from genetic distance data using neighbor-joining method (PHYLIP version 3.572c.) shows the separation of two groups.

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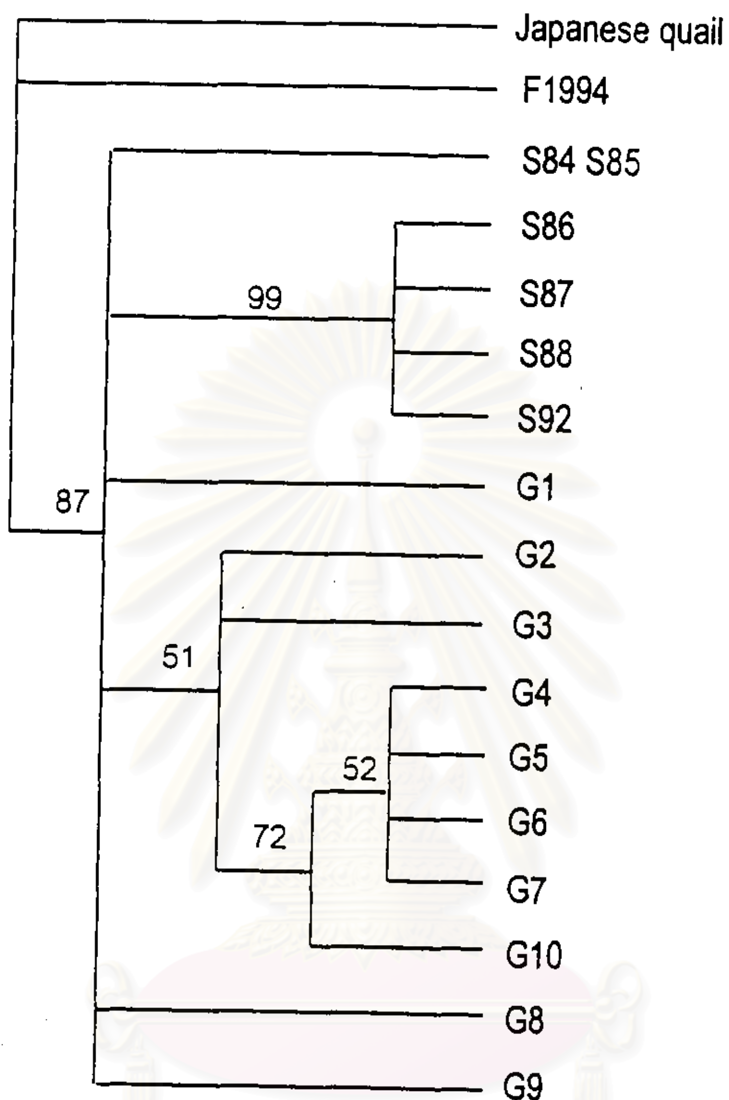


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Figure 4.7 The most parsimonious tree drawn from PAUP version 3.0 shows the separation of two group with some polytomies which are not support by 50% bootstrapping criteria.