Analysis of Genetic Relationship amongs Indonesian Native Chicken Breeds based on 335 D-loop Sequences

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ABSTRAK

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Studi keragaman genetik dan filogeni ayam lokal Indonesia dilakukan berdasarkan sekuen D-loop DNA mitokondria (DNAmt). Segmen hypervariabel I (HVI) pada D-loop DNA mitokondria di amplifikasi PCR, kemudian disekuen. Sejumlah 335 individu ayam lokal yang berasal dari pulau Sumatera dan Jawa dan 3 individu ayam Hutan Hijau (*Gallus varius*) diambil sampelnya. Populasi ayam lokal Indonesia adalah Pelung Sembawa, PL (n=18), Pelung Cianjur, PLC (n=29) and Arab Silver, ARS (n=30), Cemani, CM (n=32), Gaok, GA (n=7), Kedu Hitam, KDH (n=11), Wareng, T & TW (n=10), Cemani, CMP (n=2), Kedu, KD (n=26), Kedu Putih, KDP (n=15), Sentul Jatiwangi, STJ (n=27), Ayam Kate, KT (n=29), Ayam Sentul, STC (n=15), Arab Golden, ARG (n=26), Ayam Merawang, MR (n=28), Kedu Putih Jatiwangi, KDPJ (n=6) and Kapas, KPS (n=21). Sedangkan ayam Hutan Hijau terdiri dari 3 individu dari pulau Flores (FL5 and FL57) dan 1 individu (BD42) dari pulau Sumbawa. Sekuen sepanjang 530 pada panjang basa pertama digunakan untuk analisis dalam penelitian ini. Hasil analisis terhadap 335 sekuen ditemukan 82 haplotipe yang diidentifikasi dari 78 tempat polimorfik. Terdiri dari 79 haplotipe ditemukan di ayam lokal ter identifikasi pada 57 tempat polimorfik, sedangkan 3 haplotipe berasal dari ayam Hutan Hijau. Analisis filogeni menunjukkan bahwa ayam lokal Indonesia dapat digolongkan menjadi 5 clade (clade I, II, IIIc, IIId and IV) dari 7 clade (clade I, II, IIIa, IIIb, IIIc, IIId and IV) yang sebelumnya telah teridentifikasi pada ayam lokal di Asia. Terdapat 3 haplotipe (CM10, CM32 dan STC12) yang menyimpang dari referensi 7 clade yang tersedia. Haplotipe STC12 masuk dalam clade yang sama dengan *Gallus gallus gallus* (GenBank dengan No. akses AB007720). Ketika CM10 (sama dengan CM14), CM32 dan STC12 dihilangkan, 77 haplotipe ayam lokal diidentifikasi dari 53 tempat polimorfik. Sedangkan semua individu ayam hutan hijau termasuk dalam satu clade tersendiri. Clade I mempunyai 3 haplotipe, clade II mempunyai 52 haplotipe, clade IIIc mempunyai 1 haplotipe (ARS30) clade IIId mempunyai 9 haplotipe, dan dan clade IV mempunyai 11 haplotipe. Hubungan filogenetik antar populasi ayam tidak berkorelasi dengan lokasi geografis. Hasil analisis variasi molekuler (AMOVA) menunjukkan bahwa variasi genetik antar individu ayam didalam suatu populasi adalah sebesar 67,42% sedangkan variasi genetik antar populasi rumpun ayam sebesar 32,58%.

Kata Kunci: Ayam Lokal, Ayam Hutan Hijau, D-Loop DNA Mitokondria, HV 1, Clade, Haplotipe, Filogeni, Variasi Genetik

ABSTRACT

SULANDARI, S., M.S. ARIFIN ZEIN and T. SARTIKA. 2008. Analysis of genetic relationship among Indonesian native chicken breeds based on 335 D-loop sequences. *JITV* 13(4): 294-306.

The Mitochondrial DNA (mtDNA) D-loop segment was PCR amplified and subsequently sequenced for a total of 335 individuals from Indonesian native chicken. The individuals were drawn from sixteen populations of native chicken and three individuals of green jungle fowls (*Gallus varius*). Indonesian native chicken populations were: Pelung Sembawa, PL (n = 18), Pelung Cianjur, PLC (n = 29) and Arab Silver, ARS (n=30), Cemani, CM (n = 32), Gaok, GA (n = 7), Kedu Hitam, KDH (n = 11), Wareng, T & TW (n = 10), Cemani, CMP (n = 2), Kedu, KD (n=26), Kedu Putih, KDP (n = 15), Sentul Jatiwangi, STJ (n = 27), Ayam Kate, KT (n = 29), Ayam Sentul, STC (n = 15), Arab Golden, ARG (n = 26), Ayam Merawang, MR (n = 28), Kedu Putih Jatiwangi, KDPJ (n=6) and Kapas, KPS (n = 21). Green jungle fowls were: two individuals from Flores island (FL5 and FL57) and one individual (BD42) from Sumbawa island. The sequences of the first 530 nucleotides were used for analysis. Eighty two haplotypes were identified from 78 polymorphic sites for the 335 individuals. Seventy nine haplotypes were identified in native chicken from 57 polymorphic sites while three were of jungle fowls. Phylogenetic analysis indicates that Indonesian native chicken can be grouped into five clades (Clade I, II, IIIc, IIId and IV) of the previously identified seven clades (Clade I, II, IIIa, IIIb, IIIc, IIId and IV) in Asian domestic chicken. Haplotypes CM10 and CM32 fall to a different category while STC12 is also on its own. Interestingly STC12 clusters together with *Gallus gallus gallus* (GenBank accession No.

AB007720). When CM10 (same as CM14), CM32 and STC12 were removed, 77 haplotypes of domestic chicken were identified from 53 polymorphic sites. All the green jungle fowls are clustered to one clade of their own. The clades of domestic chicken are: Clade I which has three haplotypes, Clade II has 52 haplotypes, Clade IIIc has one haplotype (represented by ARS30), Clade IIId has nine haplotypes while Clade IV has eleven haplotypes. The phylogenetic relationship between chicken populations has no link to the geographic locations. Analysis of molecular variance showed that the genetic variation within populations was 67.42% while 32.58% accounted for the genetic differentiation between populations.

Key Words: Native Chiken, Green Jungle Fowls, D-Loop DNA Mitochondria, HV-1, Clade, Haplotype, Phylogenetic, Genetic Variation

INTRODUCTION

Chicken is by far the most widely distributed of all livestock species in Indonesia. It plays a very significant role as a source of income and high quality protein to the rural households. Chicken rearing is relatively cheap so even the poor smallholders can afford to keep it. Native chicken appears to possess enormous genetic diversity especially with regard to the adaptive traits, the ability to survive harsh conditions and under minimum feeding regimes. The Indonesian native chicken breeds apparently have species physical characteristic which differenciate them into at least 31 breeds or distinct groups of local chicken namely: Kampung, Pelung, Sentul, Wareng, Lamba, Ciparage, Banten, Nagrak, Rintik/Walik, Siem, Kedu Hitam, Kedu Putih, Cemani, Sedayu, Olagan, Nusa Penida, Merawang atau Merawas, Sumatra, Balenggek, Melayu, Nunukan, Tolaki, Maleo, Jepun, Ayunai, Tukung, Bangkok, Brugo, Bekisar, Cangehgar/Cukir/Alas, and Kasintu (NATAAMIJAYA, 1996 and 2000). Most of the native chickens in Indonesia are raised under extensive traditional system where they are free to scavenge around farmer's house during the day and sleep wherever they like to such as: at trees, hollows and even inside the villager's houses.

Nevertheless, the genetic potential in almost all chicken lines has not much revealed. As studied from the reports on the result of study conducted in the period of 25 years, apparently only around 25% of the number of breeds of Indonesian native chickens used for research activities, amongst all: Pelung, Sentul, Kedu, Merawang, Cemani, and Kampung chicken. So there is no date/information about the genetic variety, which be able to reveal the molecular genetic analysis on the entire Indonesian native chickens. If there is information, but up to now, the molecular genetic study on the native chicken in Indonesia is still partial and most likely said uncomprehensive. Beside that, the genetic information obtained only from several chicken breeds.

With the development of the PCR technology, DNA polymorphisms have become the markers of choice for molecular based survey of genetic variation. Currently the two most popular classes of markers in livestock genetic characterization studies are mtDNA sequences particularly the sequence of the hypervariable region of the D-loop or control region, and autosomal microsatellite loci (SUNNUCKS, 2000). Genetic markers play a major role in investigation of evolutionary, population and conservation genetic questions. MtDNA is used in livestock genetic studies to produce phylogenetic trees at several taxonomic levels. Knowledge on the distribution of chicken genetic diversity in Indonesia would be useful in optimizing both conservation and utilization strategies for indigenous chicken genetic resources. In the past, attempts have been made to characterize local chicken using morphological traits (such as plumage colour, feathering pattern, etc.) which have limited utility in the study of genetic variation. Mitochondrial DNA (mtDNA) sequences have successfully been used to determine genetic diversity in Asian chicken (NIU *et al*., 2002; LIU *et al*., 2004).

Mitochondria have extranuclear DNA called mtDNA which carry genetic information needed for the mitochondria metabolism. Chicken mtDNA has 16,775 base pairs (Desjardins and Morais, 1990). MtDNA is highly polymorphic compared to nuclear DNA, evolutionary rate being 5 to 10 times faster than the nuclear genome (BROWN *et al*., 1982). Different regions of the mtDNA evolve at different rates (SACCONE *et al*., 1991), making it a marker of choice for studying genetic diversity within as well as between species. The displacement (D)-loop region is non-coding and evolves much faster than other regions of the mtDNA genome. Since D-loop shows variation within species, it can be used to detect evolution and geographic patterns of diversity in livestock species. The understanding of phylogeography will elucidate the demographic history, origin and population expansion of livestock species. The high rate of evolution and ability to detect differentiation between domestic lineages make the use of D-loop the approach of choice for livestock genetic studies. Networks (BANDELT *et al*., 1999) to overcome the problem of parallel mutations and lineage exchange between divergent populations have supplemented phylogenetic trees.

The overall objective was to use sequences of the D-loop hypervariable 1 (HV 1) segment of the mtDNA as a molecular marker to study genetic diversity and relationship of Indonesian native chicken breeds.

MATERIALS AND METHODS

Sampling

Blood samples of 335 individuals were collected from 16 populations of Indonesian native chicken breeds (Pelung Sembawa, Pelung Cianjur, Arab Silver, Gaok, Kedu Hitam, Wareng, Cemani, Kedu, Kedu Putih, Sentul Jatiwangi, Kate, Sentul Ciamis, Arab Golden, Merawang, Kedu Putih Jatiwangi, and Kapas), and 3 individuals of green jungle fowls (*Gallus varius*) from 2 populations (Flores and Sumabawa Island) were used in this study (See Table 2). Published reference sequence assembled from KOMIYAMA *et al*., 2003 (GenBank accession number AB098668) was also included in this analysis.

DNA amplification and sequencing

The primers used to amplify the hypervariable 1 (HV1) segment were L16750 (5′-AGGACTACGGCTTGAAAAGC-3′) as forward primer and CR1b (5'-CCATACACGCAAACCGTCTC -3') as reverse primer. The PCR reactions were performed in a 30 µl reaction volume containing 2.5 mM of each dNTPs, 14 pmol of each primer, 1.5 mM MgCl2, 1 × PCR buffer comprising 10 mM Tris-HCl (pH 8.3) and 50 mM KCl, and 1.25 U Taq DNA polymerase. PCR amplifications were carried out on a GeneAmp® PCR system 9700 (Applied Biosystems, USA) thermal cycler. The reaction profile was: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 1 min. The last cycle was followed by a final extension step at 72°C for 10 min. PCR products were electrophoresed on a 2.0% (w/v) agarose gel stained with ethidium bromide in a 1 × TAE buffer at 100 volts for 1 hour.

PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH, Germany) according to the manufacturer's protocol. Direct sequencing of HV1 segment of the D-loop region was performed using two internal primers CR-for (5′-TCTATATTCCACATTTCTC-3′) and CR-rev (5′-GCGAGCATAACCAAATGG-3′). Sequencing was done using the BigDye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and the purified sequencing products were electrophoresed on an ABI 3730 XL automated capillary DNA sequencer (Applied Biosystems, USA).

Data analysis

MtDNA sequences for the first 530 nucleotides of D-loop were aligned using the program ClustalX 1.83 (Thompson *et al*., 1997; available at ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX). The polymorphic sites were identified by realigning the sequences to a reference using computer program MacClade 4.0 (Maddison and Maddison, 2000; available at http://ag.arizona.edu/macclade/macclade.html). Phylogenetic analyses were conducted using the program MEGA version 3.0 (KUMAR *et al*., 2004; available at http://www.megasoftware.net/). Maternal genetic differentiation was quantified using hierarchical analysis of molecular variance (AMOVA) (EXCOFFIER *et al*., 1992; http://anthro.unige.ch/arlequin).

RESULTS AND DISCUSSION

Mitochondrial DNA (MtDNA) polymorphism

MtDNA D-loop sequences were obtained for a total of 335 chicken samples. A partial mtDNA D-loop, HV 1 domain with the first 530 bases was considered for this study. Eighty two (82) haplotypes were identified from 78 polymorphic sites (Figure 1), consisting of seventy nine (79) found in Indonesian native chickens while three (3) identified in jungle fowls. When alignment gaps were excluded and haplotypes CM10, CM32 and STC12 removed, the number of haplotypes reduced to 77 haplotypes of native chicken was defined by 53 variation sites.

The variable positions of the 82 haplotypes found in 480 partial D-loop sequences of native chicken are shown in Figure 1, i.e. higher variability was observed between nucleotides 167 and 397 with only six polymorphic sites within the first 166 nucleotides.

It is demonstrated in Figure 1 that vertically oriented numbers indicate the site position and the sequences shown are only the variable sites. Dots (.) indicate identity with the reference sequence (GenBank accession number AB098668) (KOMIYAMA *et al*., 2003), different base letters denote substitution while dashes (-) refer to insertion or deletion.

Phylogenetic analysis

Phylogenetic tree of haplotypes

Phylogenetic analyses of the 77 haplotypes defined in Indonesian native chicken illustrated the evolutionary relationships as well as their genetic diversity. Neighbour-joining (a distance based method for constructing phylogeny) identifies closest pairs of taxonomic units (neighbours) by distances between them and connects them through a single node (SAITOU and NEI, 1987). Two D-loop sequences of Gallus from GenBank were included as outgroups (*Gallus gallus gallus* and *Gallus gallus bankiva*). The phylogenetic tree constructed for all the 77 haplotypes is shown in Figure 2.

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          0000011111111222222222222222222222222222222222222233333333333333333333444444
          1223636677899011111222222223333334444455556667889999900000112234455666999133447
          8691830717839324789012456890347892367803691580011268923689050202745037169705379

Ref       TCCATTGTTAACTCAATACTCATCACCTCCAGAGTTAACCTACCATTAAATCTCATCTCTATCAATTTCTCTGCGGCCA
CM10      ........................T....G...C.....C......G..C.......TC......C.T.T.....-..
CM32      ........................T....G...C.....C......G..C.......TC......C.T.T.A...-..
ARS18     ..................................................................C....-..
KD7       ..................................................................CA...-..
KPS8      ..............................................C...................C....-..
KD10      ..............................................C.....................-..
KT7       ............................................GC.....................-..
ARG26     ..............................................C..................T.....-..
ARG1      ..............................................C.....................A...-..
ARG2      ..............................................C...........T..........-..
ARG5      ..............................................C...........T.........A...-..
ARS30     .......C......G........T..........C............C........C...........A...-..
PLC4      ............G...................CC....C.T....G..C....C.CTC.............-..
T1        ............G...................CC....C.T....G..C....C.CTC....G........-..
PL10      ............G...................CC...GC.T....G..C....C.CTC.............-..
MR7       ............G...................CC....C.T....G.......C.CTC.............-..
GA3       ............G...................CC....C.....G..C....C.CTC.............-..
KT28      ............G...................CC....C.T....G..C....C.CTC..........A.....-..
KD4       ............G...................CC....C.T..C.G..C....C.CTC.............-..
PLC6      ............G...................CC....C.T....G..C....C.CTC...........A...-..
CM25      .G..........G...................CC....C.T....G..C....C.CTC...........A...-..
KT8       ............G...................CC....C.T....G..C.....CTC...........A...-..
PL16      ............G...................CC....C.T....G..C....C.CTC...........A.A.-..
STJ15     ............G...................C.T...C.T....G..C....C.CTC.............-..
STJ20     ............G...................C.T...C.T....G..C....C.CTC...........A...-..
STC14     ............G.................A..C.....C.....G..C....C.CTC...........A...-..
PLC8      ............G...................CC....C.T....G..C..T.C.CTC...........A...-..
STJ2      ............G...................C.....C.T....G..C..T.C.CTC.............-..
STJ12     ............G...................C.....C.T....G..C..T.C.CTC...........A...-..
PL7       ............G...................CC....C.T....G..C..T.C.CTC.............-..
KD9       ............G...................CC....C.T....G..C..T.C.CTC......C.....-..
STJ9      ............G...................CC....C.T....G..C..T.C.CTC.............T-..
PLC20     ............G.....T.............CC....C.T....G..C....C.CTC.............-..
STJ8      ............G.....T.............CC....C.T....G..C....C.CTC...........A...-..
STJ28     ............G...................CC......T....G..C....C.CTC...........A...-..
STJ29     ............G...................CC......T....G..C....C.CTC.............-..
KDP7      ..T.........G...................CC....C.T..C.G..C....C.CTC...........A...-..
STJ10     ..T.........G...................CC....C.T....G..C....C.CTC...........A...-..
CM31      ..T.........G...................CC....C.T....G..C....C.CTC.............-..
PLC9      ............G...................CC....C.T....G..C....C.CTC...G.........-..
ARS26     ............G....C..............CC....C.T....G..C....C.CTC...G.........-..
PL11      ............G...................CC....C.T....G..C....C.CTC...G......T.....-..
PLC19     ............G...................CC....C.T....G..C....C.CTC...G........A...-..
KPS3      ............G...................CC....C.T....G..C....C..TC...G........A...-..
PLC23     ...T........G...................CC....C.T....G..C....C.CTC...G.........A.-..
PLC24     ...T........G...................CC....C.T....G..C....C.CTC...G........A...-..
PLC2      ............G...................CC....C.T....G..C....C..TC.............-..
KDH2      ............G...................CC....C.T.G..G..C....C..TC.............-..
PL13      ............G...................CC..A.C.T....G..C....C..TC...........A.-..
PL18      ............G...................CC....C.T....G..C....C..TC...........AC-..
ARS13     ............G...................CC....C.T....G.......C..TC.........T.....-..
PLC1      ............G...................CC....C.T....G.......C..TC.........T....C-..
PLC7      ............G...................CC....C.T....G.......C..TC.........T.A...-..
KDP12     ............G...................CC....C.T...CG.......CT.TC.........T.A...-..
CM5       ............G.......T...........CC....C.T....G..C....C..TC.........T.A...-..
KDH6      ............G........T..........CC....C.T....G..C....C..TC.........T.....-..
CM29      ............G...................CC....C.T....G..C....C..TC.........T.....-..
PLC3      ............G...................CC....C.T....G..C....C..TC...........A...-..
KPS6      ....A........G...................CC....C.T....G..C....C..TC...........A...-..
KPS26     ............G...................CC...AC.T....G..C....C..TC...........A...-..
CMP1      ...........C.G...................CC....C.T....G..C....C..TC...G.........-..
KT19      ...........C.G...................CC....C.T....G..C....C..TC.............-..
KPS5      ............G...................CC....C.T....G..C....C..TC...G.........-..
KDP2      ............G...................CC....C.....G..G..C....C..TC.............-..
ARS1      ............G.C.................CC....C.T.......C.......TC.............-T.
KPS4      ............G.C..........T.....CC....C.T.......C.......TC.............-T.
T7        ............G.C....G...........CC....C.T.......C.......TC..T...........-T.
KPS29     ............G.C.................CC....C.T.......C.......TC...G.........-T.
ARS5      ...........A..G.C.................CC....C.T.......C.......TC...........A...-T.
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          0000011111111222222222222222222222222222222222222233333333333333333333444444
          1223636677899011111222222223333334444455556667889999900000112234455666999133447
          8691830717839324789012456890347892367803691580011268923689050202745037169705379

ARS29     ..........A..G.C.................CC....C.T.......C.......TC.................-T.
TW2       ............G.C.................C....C.T.......C.......TC............A...-T.
TW6       ............G.C.................C....C.T.......C.......TC................-T.
KDPJ4     ............G.C.................CC....C.T.......C.......TC............A...-T.
ARS14     ............C.G.C.................CC....C.T.......C.......TC............A...-T.
ARS19     ............C.G.C.................CC....C.T.......C.......TC................-T.
CM33      ............G..................ACC....C......G..C.....T.TC........TC..A...-..
CM34      ............G..................ACC....C......G..C.....T.TC........TC......-..
KT18      ............G..................ACC....C......G..C.......TC...G...CTC......-..
STC12     ........C.......................CC....C.T..............TC.A...C..T.T..T..-T.
BD42      C.....ACCCCACA.T.-TA.GCGT.TA.T.TCAC..T..AGTT....CCATAAGC..A.G.....C..ATCA...-.T
FL57      C.....ACCCCACA.T.-TA.GCGT.TA.T.TCAC..T..AGTT....CCATAAGC..A.G.....C..ATCA...-..
FL5       C....CACCCCACA.T.-TA.GCGT.TA...TCAC..T..AGTT....CCATAAGC..A.G.....C..ATCA...-..
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Figure 1. Nucleotide polymorphisms observed in D-loop domain of 335 chicken sequences

Phylogenetic analysis shows that Indonesian native chicken can be grouped into five clades (Clade I, II, IIIc, IIId and IV) of the previously identified as seven clades (Clade I, II, IIIa, IIIb, IIIc, IIId and IV) in Asian domestic chicken. All the haplotypes and their grouping to various clades are shown in Table 1. Most of the haplotypes were grouped to Clade II which contributed 67% of the haplotypes.

None of the samples in this study belonged to Clades IIIa and IIIb. There are unassigned clade found in 4 samples (CM10, CM14, CM32 and STC12) and the samples were not included in this analysis. Interestingly STC12 clusters together with *Gallus gallus gallus* (GenBank acc. No. AB007720). Most of the samples belong to clade IIId are Arab Golden chickens (See Table 1), so it is though there was Chinese influence. According to BJORNSTAD (personal communication), Chinese domestic chicken samples are grouped in to Clade IIId dominantly.

Phylogenetic relationship between chicken populations

A neighbour-joining tree (Figure 3) was constructed using pairwise genetic distances calculated with Kimura two parameter model.

The tree shows that the phylogenetic relationship between populations is not linked to their geographical locations. Nevertheless, chicken populations from Southern Sumatera are more closely related to those from Western Java than are to Central Java. Pelung chicken collected from South Sumatera and Pelung collected in Cianjur have close phylogenetic relationship (Figure 3), suggested that Pelung chickens from South Sumatera might be originally from Cianjur which known as popular place for Pelung chickens. The sampling locations of the chicken populations are shown in Table 2.

Network analysis

Median joining networks were drawn to illustrate the genetic diversity of Indonesian chicken. The networks were colour coded to differentiate between different clades (Figure 4).

Figure 4 was presents that area of each circle is proportional to the frequency of the corresponding haplotype. Different classes of haplotypes are distinguished by use of colour codes. White circle denotes clade I, blue circles denotes clade II, yellow circles refer to clade IV while colours light green and dark green denote Clades IIIc and IIId respectively. Indonesian native chicken breeds is different from indigenous chicken in other Asia regions because dominantly enters clade II. The number of haplotypes in the clade II is 52, so (Figure 4) it seems the number of blue color circle is 52. DNA sequence of haplotype CM21 has the most high frequency, i.e. seen from blue color circle with the biggest form. This haplotype CM21 is related to the most number to other haplotypes. On the contrary, if a haplotype has a low frequency, it is indicated with a small circle.

The red dots illustrate median vectors (mv), produced by the network software, representing putative intermediate haplotypes that have not been found or sampled. The numbers between the haplotype nodes refer to the positions of nucleotide mutations compared to reference sequence (GenBank accession number AB098668).

Analysis of molecular variance (AMOVA)

Maternal genetic variation within populations, between populations within geographic groups and between geographic groups was further quantified using a hierarchical analysis of molecular variance (AMOVA; EXCOFFIER *et al*., 1992). Analyses of molecular

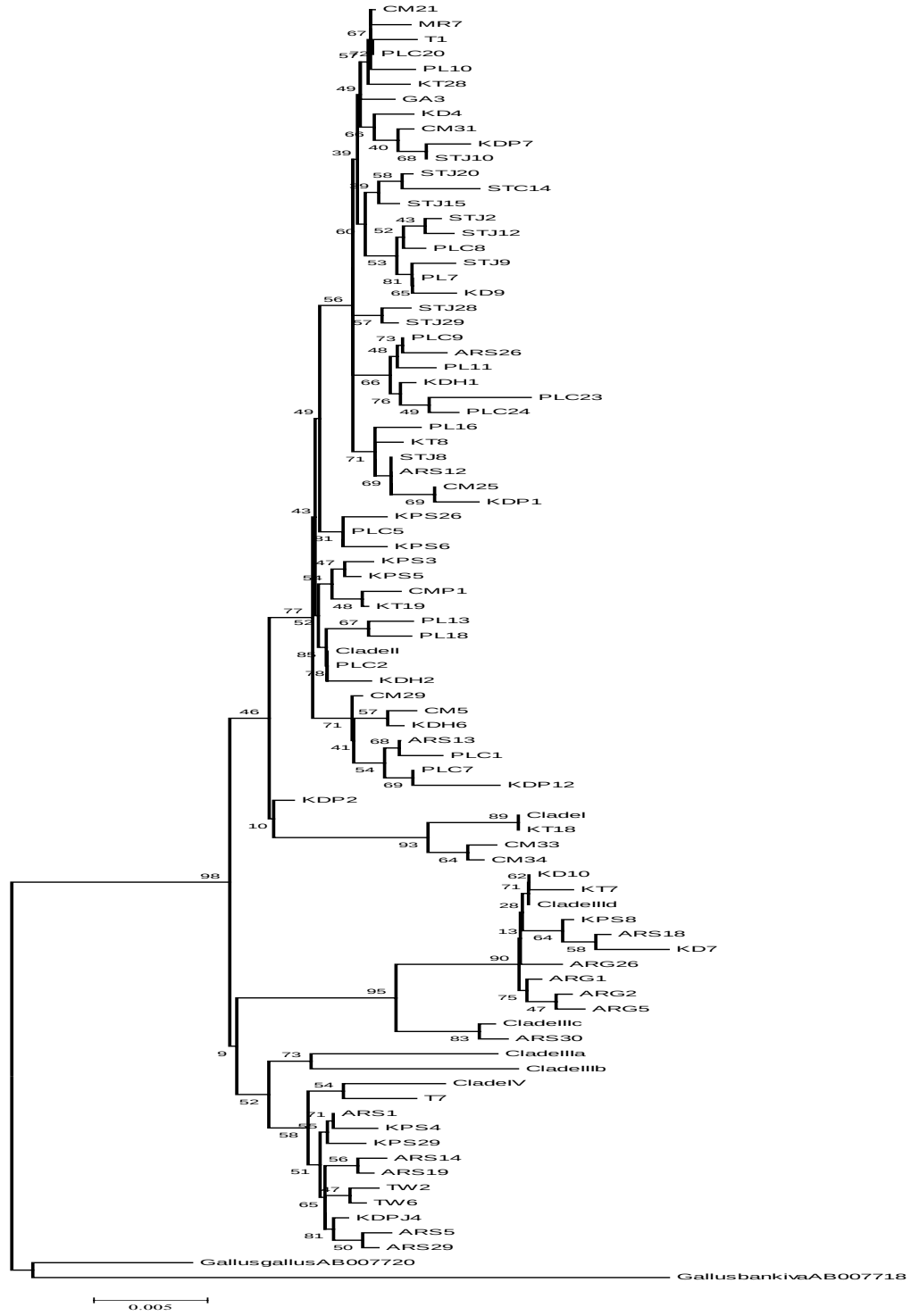


Figure 2. Neighbour-joining tree reconstructed using MEGA 3.1 software from 77 haplotypes identified for Indonesian native chicken. Two haplotypes of the genus *Gallus* retrieved from GenBank; *Gallus gallus gallus* (GenBank accession number AB007720) and *Gallus gallus bankiva* (GenBank accession number AB007718) and seven clade reference haplotypes (Clade I, II, IIIa, IIIb, IIIc, IIId and IV) based on BJORNSTAD *et al*. (2005). The numbers at the nodes represent the percentage bootstrap values for interior branches after 1000 replications

Table 1. Indonesian chicken haplotype groups

Clade	Haplotype	Individuals with this kind of haplotype	No
Clade I	CM33	CM33	1
	CM34	CM34	2
	KT18	KT18, KT27	3
Clade II	T1	T1, T6	4
	PL10	PL10	5
	KD4	KD4,KD12	6
	CM21	PLC4, PLC15, PL3, PL15, PL14, KPS24, KPS16, KPS14, KD8, KD18, ARS3, ARS2, KD14, KD29, KD28, KD26, KD25, TW7, MR25, MR24, MR23, MR22,KDH12, KDH11, KDH8, KDH7, ARS6, ARS9, MR16, STC10, ARS11, MR14, MR12, STC17, STC13, MR30, MR29, STC4, MR10, MR9, MR3, MR2, KT31, KT30, KDH3, KT23, KT22, GA4, GA1, CM35, CM9, CM11,KT14, KT13, KT12, KT11, KT10, KT9, CM30, CM23, PLC4, CM20, CM17, CM16, CM1, CM4, CM3, KT5, KT4, KT3, KT2, KT1, ARS21, STJ16, KDP8, KDP14, KDP13, KDP11, KDP4, KDP16	7
	STJ28	STJ28, KDPJ3	8
	STJ29	STJ29	9
	KDP7	KDP7	10
	STJ10	STJ10	11
	PLC20	PLC20, KT15, KT16, KPS30	12
	STJ8	STJ8	13
	STJ15	STJ15	14
	STJ20	STJ20	15
	STC14	STC14, STC15	16
	STJ2	STJ2, STJ5, STC11	17
	STJ12	STJ12	18
	PL7	PL7, STJ23, PL12, ARS7, ARS22, KDH5, MR27	19
	PLC8	PLC8, PL2, PL6, PL9, PL20, KDPJ6,KDP5,MR11,MR18, STJ24,STJ27	20
	KD9	KD9	21
	CM31	CM31	22
	PLC9	PLC9, GA6, GA7, GA8, KD27, KT20, KT26, PLC18	23
	CM25	CM25, KDP1	24
	KT8	KT8	25
	PL16	PL16	26
	STJ9	STJ9	27
	CMP1	CMP1	28
	PLC19	PLC19, PLC21, PLC30, KDH1, KD2	29
	KPS3	KPS3	30
	PLC23	PLC23	31
	PLC24	PLC24	32
	ARS26	ARS26	33
	PL11	PL11	34
	GA3	GA3, GA10	35
	KDP2	KDP2, KDP6	36
	PLC2	PLC2, ARS8, ARS10, ARS17, ARS20, ARS27, CM7, CM12, CM13, CM19, KDH4, KD1, KD15, KD19, STJ25, KT25, KT32, STC3, STC5, STC6, STC8, KPS13, KPS22, PL17, CMP2,ARG30, MR6, MR26, MR28, KDPJ2, KPS9	37
	KDH2	KDH2, KD17, KDP15	38
	PL13	PL13	39
	PL18	PL18	40
	ARS13	ARS13, CM2, STC1, KPS25	41
	PLC1	PLC1	42

Table 1. (Continue)

Clade	Haplotype	Individuals with this kind of haplotype	No
	PLC7	PLC7, PLC22, CM26	43
	KDP12	KDP12	44
	CM5	CM5, CM6	45
	KDH6	KDH6, KD6	46
	CM29	CM29, KT21	47
	PLC3	PLC3, PLC5, PLC10, PLC11, PLC13, PLC14, PLC16, PLC25,ARS15, CM22,KD24, STJ3, STJ17, STJ18, STJ19, STJ21, STJ22, STC7, MR1,KDPJ1, KDPJ9	48
	KPS6	KPS6	49
	KPS26	KPS26	50
	KT19	KT19	51
	KPS5	KPS5	52
	MR7	MR7, MR17	53
	KT28	KT28, KT29	54
	PLC6	PLC6, PLC12, PLC17, PLC26, PLC27, PLC29, PL5, PL8, PL19, ARS12, ARS23,CM8,CM15, CM18, KDH9, KD5, KDP10, STJ13, STJ14, STJ26, STJ30, STJ31, MR4, MR19, KPS2, KPS23	55
Clade IIIc	ARS30	ARS30, STC16,ARG28	56
Clade IIId	ARS18	ARS18,ARS28,KD3,KD11,KD23,KPS27, KD30, KDP3, STJ4	57
	KD7	KD7	58
	KPS8	KPS8	59
	KT7	KT7	60
	ARG26	ARG26	61
	ARG1	ARG1, ARG4, ARG10	62
	ARG2	ARG2, ARG3, ARG6, ARG12, ARG18, ARG19, ARG23, ARG24, ARG25	63
	ARG5	ARG5, ARG7, ARG8, ARG9, ARG14, ARG15, ARG16, ARG17, ARG27, ARG29	64
	KD10	KD10, ARG13,MR5,MR15,KPS1,KPS10	65
Clade IV	T7	T7,TW5	66
	ARS14	ARS14,ARS16	67
	ARS19	ARS19, KD13, MR8, MR13, KPS11	68
	TW6	TW6	69
	ARS1	ARS1, ARS4, T2, T3, TW1	70
	KPS4	KPS4	71
	KPS29	KPS29	72
	ARS5	ARS5,ARS24,ARS25	73
	ARS29	ARS29	74
	TW2	TW2	75
	KDPJ4	KDPJ4	76
Unassigned clade	CM10	CM10, CM14	77
	CM32	CM32	78
	STC12	STC12	79
Clade of Jungle fowls	FL5	FL5	80
	FL57	FL57	81
	BD42	BD42	82

Variance was based on Kimura-2-parameter distances. The calculations were performed based on 1000 permutations using the computer software Arlequin version 3.01 (SCHNEIDER *et al*., 2005), and the results are presented in Table 3. The analysis was done to give more insight to how the genetic variation is distributed between individuals within populations, between populations within groups and between groups.

When the 16 populations were defined into two geographic groups (South Sumatera and Java), the genetic variation between individuals within populations was 65.49%, that occurring between

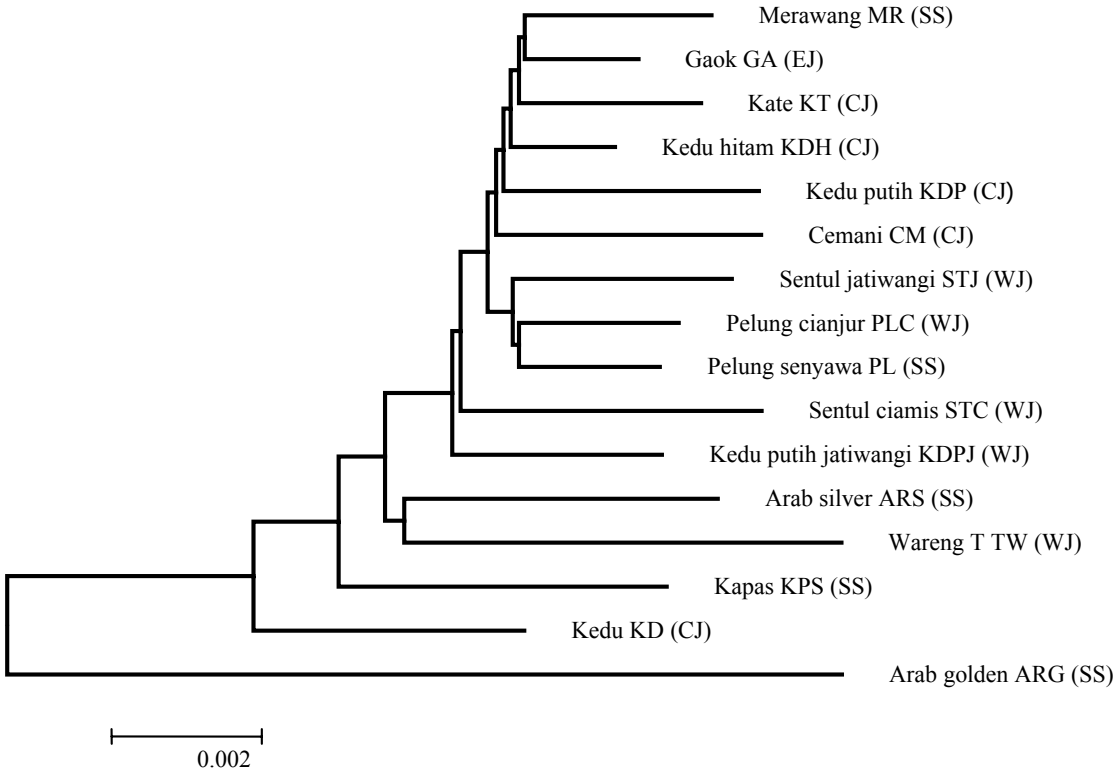


Figure 3. Neighbour-joining tree constructed from 16 populations of Indonesian chicken based on mtDNA D-loop sequences. Calculations of genetic distances were based on kimura-2-parameter distance matrix and this was implemented by use of computer program MEGA version 3.1 (KUMAR *et al*., 2004)

Table 2. Information of Indonesian chicken samples sequenced

Popu lation	Breed name	No. of sequences	Sampling location
1	Pelung Cianjur (PLC)	29	Cianjur, West Java
2	Pelung Sembawa (PL)	18	Sembawa, South Sumatera
3	Arab Silver (ARS)	30	Sembawa, South Sumatera
4	Cemani (CM)	32	Kedu, Central Java
5	Gaok (GA)	7	Bangkalan, Madura island, East Java
6	Kedu Hitam (KDH)	11	Maron, Temanggung, Central Java
7	Wareng (T and TW)	10	Tangerang, Banten Province
8	Kedu (KD)	26	Maron, Temanggung, Central Java
9	Kedu Putih (KDP)	15	Maron, Temanggung, Central Java
10	Sentul Jatiwangi (STJ)	27	Jatiwangi, West Java
11	Kate (KT)	29	Yogyakarta, Central Java
12	Sentul Ciamis (STC)	15	Ciamis, West Java
13	Arab Golden (ARG)	26	Sembawa, South Sumatera
14	Merawang (MR)	28	Sembawa, South Sumatera
15	Kedu Putih Jatiwangi (KDPJ)	6	Jatiwangi, West Java
16	Kapas (KPS)	21	Sembawa, South Sumatera
17	Cemani Sembawa (CMP)	2	Sembawa, South Sumatera
18	Gallus varius (FL)	2	Flores Island
19	Gallus varius (BD)	1	Sumbawa Island

Note: Analysis of phylogenetic relationship between chicken populations was conducted for 16 populations (population no. 1-16)

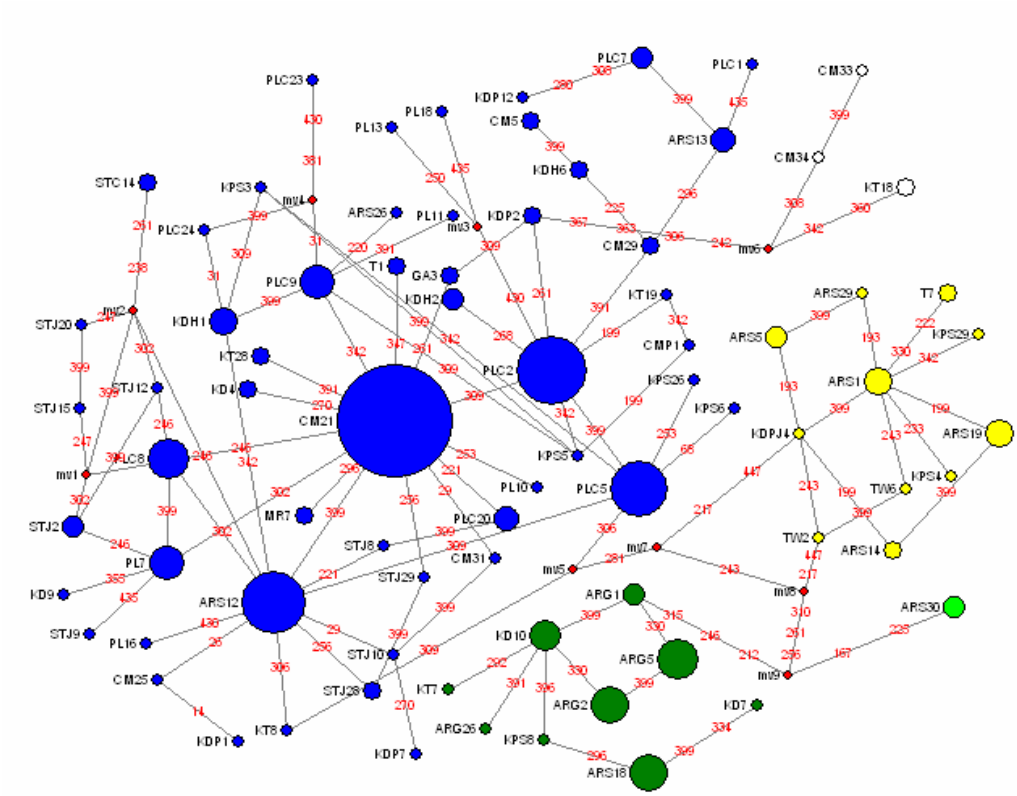


Figure 4. Median-joining network (ε = 0) for the 77 haplotypes of Indonesian domestic chicken based on the polymorphic sites of the mitochondrial D-loop region

Populations within groups were 28.69% while the genetic variation between groups accounted for 5.82%. The low genetic differentiation resulting from the geographical groups suggests that Indonesian domestic chickens have not been subdivided across the regions hence this implies that the breeding females may have been exchanged. A higher proportion of chicken populations have a common maternal origin. Considering all the 16 populations as a single group, the genetic variation within populations was 67.42% while 32.58% accounted for the genetic differentiation between populations within the group.

Population diversity

The DNA sequence diversity indices were determined to elucidate the extent of genetic variability in all the 16 chicken populations. The analysis included all domestic chicken except CMP1, CMP2, CM10, CM14, CM32 and STC12. The diversity indices calculated for the 326 sequences are presented in Table 4. The highest number of haplotypes was found in Sentul chickens collected from Jatiwangi (H = 16) and the lowest number was detected in the Gaok chicken population (H = 3). The gene (haplotype) diversity was highest in Kapas chicken population collected from South Sumatera (Hd = 0.96190) while the lowest in Kate chicken population (Hd = 0.72906). The average haplotype diversity over all populations was approximately 0.91619 for the 326 chicken D-loop sequences.

The nucleotide diversity, π is more suitable parameter than haplotype diversity to estimate the genetic diversity in populations. The former addresses both the frequency of haplotype and nucleotides were different between haplotypes. The average nucleotide diversity over all populations was estimated for the 326 D-loop sequences to be 0.00901 nucleotide substitutions per site. The highest nucleotide diversity was found in Kapas (π = 0.01078) and Kedu (π = 0.01100) populations, and the lowest was in Gaok chicken population.

Clade distribution in Indonesia chicken populations

Site of collection samples in this study was from 2 islands, Sumatera and Java. Generally, clade distribution of Indonesian native chicken breeds were dominated by clade II (blue colour), except for population of Arab Golden (ARG) which dominated by green colour (clade IIId) and Wareng (T&TW) chickens which dominated by yellow colour (clade IV). Furthe

Table 3. Analysis of molecular variance (AMOVA) based on D-loop sequences of Indonesian chicken

Samples	Variation (%)				
	No. of groups	No. of populations	Within populations	Among populations within groups	Among groups
All 16 chicken populations	2	16	65.49	28.69	5.82
All 16 chicken populations	1	16	67.42	32.58	-

Table 4. MtDNA diversity indices in Indonesian chicken populations based on D-loop sequences

Region	Population	N	S	H	Hd	Π
South sumatera	Pelung Sembawa (PL)	18	9	10	0.91503	0.00403
	Arab Silver(ARS)	30	23	14	0.92414	0.00964
	Arab Golden(ARG)	26	14	7	0.74154	0.00389
	Merawang(MR)	28	16	9	0.77249	0.00556
	Kapas(KPS)	21	22	15	0.96190	0.01078
West Java	Pelung Cianjur(PLC)	29	11	12	0.87931	0.00407
	Wareng(TTW)	10	10	6	0.88889	0.00746
	Sentul Jatiwangi(STJ)	27	18	16	0.92308	0.00545
	Sentul Ciamis(STC)	14	15	7	0.85714	0.00641
	Kedu Putih Jatiwangi(KDPJ)	6	8	5	0.93333	0.00541
Central Java	Kate(KT)	29	20	10	0.72906	0.00494
	Kedu hitam(KDH)	11	7	7	0.81818	0.00316
	Kedu putih(KDP)	15	22	9	0.84762	0.00744
	Cemani(CM)	29	13	12	0.81281	0.00461
	Kedu(KD)	26	24	14	0.90462	0.01100
East Java	Gaok(GA)	7	2	3	0.76190	0.00198
Total		326	51	76	0.91619	0.00901

N, Number of sequences used; S, Number of segregating sites; H, Number of haplotypes; Hd, Haplotype diversity; π, Nucleotide diversity

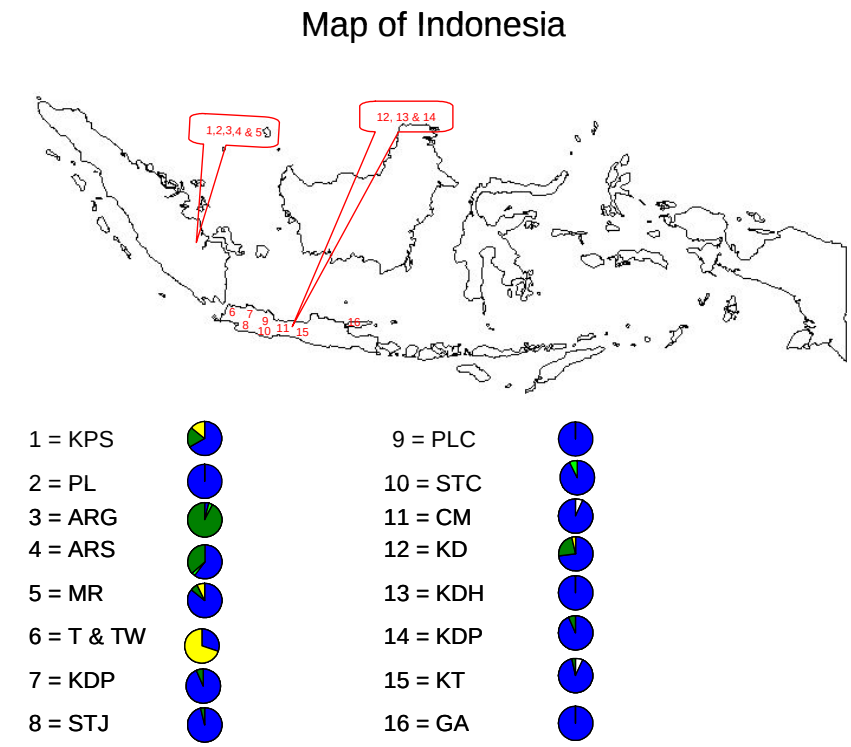
study is needed to analyze more Indonesian native chicken breeds from other islands in Indonesia.

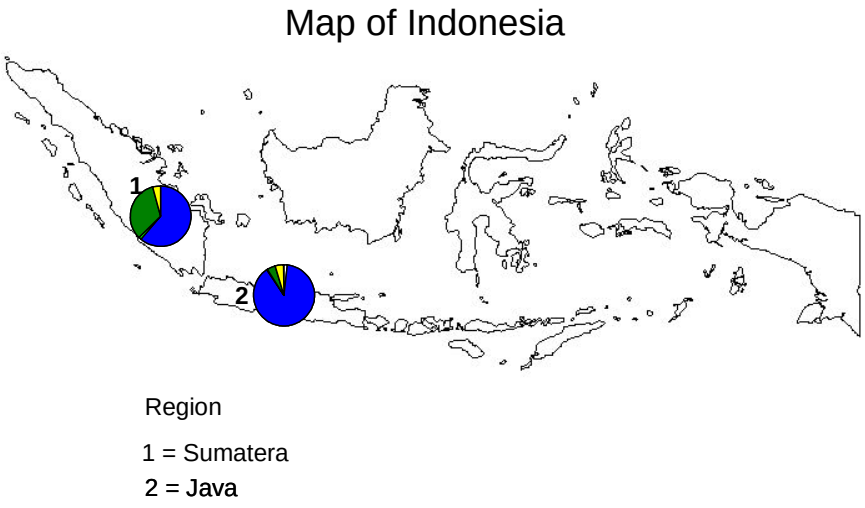
CONCLUSIONS

This study has proved that mtDNA and more specifically D-loop HV 1 segment is powerful molecular tool in resolving phylogenetic relationships within species and also understanding the genetic diversity.

The phylogenetic relationship between chicken populations has no link to the geographic locations.

Analysis of molecular variance showed that the genetic variation within populations was 67.42% while 32.58% accounted for the genetic differentiation between populations.





RFEFERENCES

BANDELT, H.J., P. FORSTER and A. ROHL. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16: 37-38.

BROWN, W.M., E.M. PRAGER, A. WANG and A.C. WILSON. 1982. Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *J. Mol. Biol.* 212: 599-634.

DESJARDINS, P. and R. MORAIS. 1990. Sequence and gene organization of the chicken mitochondrial genome. A novel gene order in higher vertebrates. *J. Mol. Biol.* 212: 599-

EXCOFFIER, L., P. SMOUSE and J. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.

KOMIYAMA, T., K. IKEO and T. GOJOBORI. 2003. Where is the origin of the Japanese gamecocks? *Gene* 317: 195-202.

KUMAR, S., K. TAMURA and M. NEI 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief. Bioinform*. 5: 150-163

LIU, Z.G., C.Z. LEI, J. LUO, C. DING, G.H. CHEN, H. CHANG, K.H. WANG, X.X. LIU, X.Y. ZHANG, X.J. XIAO and S.L. WU. 2004. Genetic variability of mtDNA sequences in Chinese Native Chicken Breeds. *Asian-Aust. J. Anim. Sci.* 17: 903-909.

MADDISON, D.R. and W.P. MADDISON 2000. MacClade 4: Analysis of phylogeny and character Evolution, 4.0. Sinauer Associates, Sunderland, MA.

NATAAMIJAYA, A.G, 2000. The native chicken of Indonesia. *Bul. Plasma Nutfah* 6: 1-6.

NATAAMIJAYA, A.G., S.N. JARMANI and T. SARTIKA. 1996. Konsep strategi penanganan pelestarian plasma nutfah pertanian secara ex-situ ternak ayam buras. Agricultural Germ Plasm Use and Preservation Project, Bogor.

NIU, D., Y. FU, J. LUO, H. RUAN, X.P. YU, G. CHEN and Y.P. ZHANG. 2002. The origin and genetic diversity of Chinese native chicken breeds. *Biochem. Gen.* 40: 163-174.

SACCONE, C., G. PESOLE and E. SBISA. 1991. The main regulatory region of mammalian mitochondrial DNA: structure-function model and evolutionary pattern. *J. Mol. Evol*. 33: 83-91.

SAITOU, N. and M. NEI. 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4: 406-425.

SCHNEIDER, S., D. ROESSLI and L. EXCOFFIER. 2000. Arlequin ver. 2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

SUNNUCKS, P. 2000. Efficient genetic markers for population biology. *Trends Ecol. Evol.* 15: 199-203.

THOMPSON, J.D., T.J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN and D.G. HIGGINS. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuc. Acids Res*. 25: 4876-4882.