Analysis of Comparative Phylogenetic Story by Using Autosomal Markers and Mitochondrial Sequences

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Abstract: The current study aims at the molecular assessment of genetic diversity within and between Vietnamese local chicken populations. On average, a total of 32 individuals per Vietnamese local chicken population was randomly sampled. Nine Vietnamese chickens breeds and 2 The exotic chicken breeds originating from China were used. The DNA polymorphism was assessed using a set of 29 microsatellite markers recommended by FAO. A fragment of 455 bp from the mtDNA D-loop region was amplified. The results showed that at the autosomal level, the Vietnamese local chicken breeds from different agro-ecological zones represent genetically distinct populations. The northern breeds are clearly separated from breed of the South Central Coast and from breed of the Mekong Delta. The Vietnamese local chicken breeds are highly polymorphic and originated from eight maternal lineages. These lineages are present across the country. Two chicken breeds of Chinese origin, Tam Hoang and Luong Phuong, kept in the National Institute of Animal Sciences are genetically distinct from the Vietnamese local breeds. The Vietnamese chicken breeds are genetically separated from the Chinese chicken gene pool.

Keywords: Vietnamese Chicken Breed, Genetic Diversity, Microsatellite, mtDNA

1. Introduction

Microsatellites and mitochondrial DNA (mtDNA) sequences have already proved to be useful for assessing genetic variability, while single nucleotide polymorphisms (SNPs) are becoming more and more popular due to their very high density and availability of high throughput genotyping techniques. Microsatellites are tandem repeats in the genomic DNA with very short (1-5bp) simple sequence motifs, and hence they are autosomally inherited. They are considered to be evenly distributed in the genome [30]. Unlike microsatellite markers, mtDNA is maternally inherited. The mtDNA is a circular molecule of 16,785 bp in size [5]. The displacement loop (D-loop) region of the mtDNA contains elements that control the replication of the molecule and is highly polymorphic.

A combination of these two markers is a complementary approach that combines the highly polymorphic microsatellites whose high mutation rates allow for small-scale resolution of more recent demographic events with mtDNA which shed light on phylogeographic events dating further back in time [11]. An assessment of genetic structure based on these two markers with different modes of inheritance provides more insights into the evolutionary forces shaping genetic diversity.

Eleven Vietnamese local chicken breeds have been reported [17] but the definition of these breeds is not fully standardized. It appears unlikely that comprehensive survey based on large scale phenotypic characterisation can be achieved considering the wide range of local chicken breeds and the diversity of local production systems. Therefore, the assessment of genetic relationships within and between populations using different molecular markers is a useful prerequisite for the development of effective conservation programs. The current study aims at the molecular assessment of genetic diversity within and between Vietnamese local chicken populations.

2. Materials and Methods

This work was carried out in 11 villages of four agro
ecological zones located in both the northern and southern part of Vietnam. In this study, a set of nine Vietnamese local chicken breeds and two exotic breeds of Chinese origin kept in Vietnam, were studied.

On average, a total of 32 individuals per Vietnamese local chicken population was randomly sampled where on average one male and one female per household were used (Table 1).

### Table 1. Information of blood samples.

| Breed | Agro-ecological zone | Study area | No of blood samples |
|-------|----------------------|------------|---------------------|
| Vietnamese | H'Mong | Northwest | Mai Son, Son La | 31 |
|          | Mia     | Red River Delta | Duong Lam, Son Tay | 32 |
|          | Ho      |            | Hoai Duc, Ha Tay | 32 |
|          | Ri      |            | Thuan Thanh, Bac Ninh | 32 |
|          | Dong Tao |            | Khoai Chau, Hung Yen | 32 |
|          | Te      |            | Ba Vi, Ha Tay NIAS | 32 |
|          | Choi    | South Central Coast | Ninh Hoa, Khanh Hoa | 33 |
|          | Ac      |            | Tan An, Long An | 32 |
|          | Tau Vang |            |                  | 33 |
| Chinese  | Luong Phuong | Mekong Delta | NIAS | 32 |
|          | Tam Hoang |            |                  | 32 |

Key: NIAS = National Institute of Animal Sciences.

The exotic chicken breeds originating from China have been kept as conservation flocks at the National Institute of Animal Husbandry since 1995 and 2003, respectively. Here, 32 (16 males and 15 -17 females) per each breed were selected. Altogether, a total of 353 individuals were sampled. The DNA polymorphism was assessed using a set of 29 microsatellite markers recommended by [13].

### 2.1. Microsatellite Variability

Observed and expected heterozygosity and inbreeding coefficients (F<sub>IS</sub>) for each population were calculated. STRUCTURE analysis [26] was used to cluster individuals to 2 ≤K≤9 assumed clusters with 100 runs for each K value. This K values are used based on a assumption that each Vietnamese chicken breed was genetically different from the others. Comparisons of 100 runs were done by using SIMCOEFF [28]. Solutions with a similarity higher than 95% were considered as identical. The most frequent solution was visualised using DISTRUCT [29].

To determine the clustering that best classifies, the ΔK statistics was applied as [7]. Phylogenetic network analysis was done by transforming the kinship matrix [6] into a distance matrix and used as input for the SPLITSTREE software [14].

### 2.2. Mitochondrial Variability

A fragment of 455 bp from the mtDNA D-loop region was amplified using primers mtGlu-F (5'-GGCTTGAAAAGCCATGTTG-3') and mtGlu-R (5' - CCAAAAAAGAGAAAGGAACC-3'). Due to their circular nature, these primers are positioned at bases 16739–16775 (forward primer) and 649–668 (reverse primer) of the complete mtDNA sequence of domestic chickens (X52392, Desjardins and Morais, 1990). PCR amplifications and sequencing were done as described by [23]. To align DNA sequences, Align IR software was used (LICOR Inc. Nebraska, USA). The list of sequences used in this study and the corresponding Gen Bank accession numbers are provided in Table 2.

### Table 2. Haplotype names and accession numbers of chicken mtDNA sequences used in this study.

| Haplotype name | Accession number | Reference |
|----------------|------------------|-----------|
| A1 – A9        | GU564361 - GU564369 | This study |
| B1 – B8        | GU564370 - GU564377 | This study |
| C              | GU564378         | This study |
| D1 – D5        | GU564379- GU564383 | This study |
| E1 – E9        | GU564384- GU564392 | This study |
| F              | GU564393         | This study |
| G1 – G2        | GU564394- GU564395 | This study |
| H1 – I2        | GU564396- GU564397 | This study |
| Liu_A1         | AB114069         | Liu et al. (2006) haplotype A1 |
| Liu_B1         | AB007744         | Liu et al. (2006) haplotype B1 |
| Liu_C1         | AB114070         | Liu et al. (2006) haplotype C1 |
| Liu_D1         | AY588636         | Liu et al. (2006) haplotype D1 |
| Liu_E1         | AB114076         | Liu et al. (2006) haplotype E1 |
| Liu_F1         | AF512285         | Liu et al. (2006) haplotype F1 |
| Liu_G1         | AF512288         | Liu et al. (2006) haplotype G1 |
| Liu_H1         | D82904           | Liu et al. (2006) haplotype H1 |
| Liu_I1         | AB009434         | Liu et al. (2006) haplotype I1 |
| Oka_D6         | AB268535         | Oka et al. (2007) haplotype D6 |
| Oka_G1         | AB268545         | Oka et al. (2007) haplotype G1 |
| Oka_F1         | AB268543         | Oka et al. (2007) haplotype F1 |
The position and number of polymorphic sites as well as corresponding haplotypes were calculated using MEGA v. 3.1 [16]. The distribution of haplotypes in the samples was computed using TCS v. 1.21 [4]. Median joining networks of haplotypes were constructed following the algorithm of [2] and using NETWORK v. 4.5.1.0 (http://www.fluxus-engineering.com/sharenet.htm). As reference, network analysis was used first to create a skeleton which was based on the most frequent haplotypes of the nine clades of Liu's network [20] and the three additional clades (D, G and F) of [25]. This skeleton assigns clades to suggested regions of domestication in chickens, which were Yunnan and/or surrounding areas (Liu’s clades A, B, F and G), South and Southwest China and/or surrounding areas and Southeast Asia (Liu’s clade C, D, H, I and Oka’s clade D, F, G), and the Indian subcontinent (Liu’s clade E). Nomenclatures of the nine clades reported by [20] were used as reference for the clade notation in this study. The sequences used for alignment consisted of 455 bp. Various networks were constructed by using different epsilon (ε) values ranging from zero to 20. There were no considerable differences among the different networks except a slight increase in the network connections where clades joined. The median network presented used an epsilon value of 5. The haplotype and nucleotide diversities of breeds were computed using ARLEQUIN v. 3.1 [8].

To analyse if mtDNA clades also differed at the autosomal level, the data obtained from genotyping 29 microsatellite markers was used. These individuals were labelled according to their clade affiliation based on mtDNA sequences. The microsatellite genotyping data were used in the Bayesian model-based clustering as implemented in STRUCTURE v.2.3.1 to cluster individuals to a varying number of K clusters (2 ≤ K ≤ 8) [26]. Runs within each K-value showing a similarity coefficient of 0.95 and higher were considered as identical.

3. Results and Discussions

3.1. Microsatellite Variability

3.1.1. Genetic Diversity

Expected heterozygosity of Vietnamese local chicken breeds varied considerably ranging from 0.573 (± 0.035) in Dong Tao chicken to 0.696 (± 0.021) in Tau Vang chicken (Table 3).

| Breeds          | Abbreviation | n  | HE ± SD      | Ho ± SD  | FIS |
|-----------------|--------------|----|--------------|----------|-----|
| H’Mong          | HM2_VN       | 31 | 0.657 ± 0.028| 0.633 ± 0.016 | 0.038 |
| Mia             | DT_VN        | 32 | 0.646 ± 0.033| 0.610 ± 0.016 | 0.058 |
| Ri              | Ho_VN        | 32 | 0.648 ± 0.031| 0.606 ± 0.016 | 0.065 |
| Ho              | Mia_VN       | 32 | 0.618 ± 0.034| 0.564 ± 0.016 | 0.088 |
| Dong Tao        | Ri_VN        | 32 | 0.573 ± 0.035| 0.548 ± 0.016 | 0.046 |
| Te              | Te_VN        | 32 | 0.635 ± 0.029| 0.595 ± 0.016 | 0.065 |
| Choi            | Choi_VN      | 33 | 0.623 ± 0.035| 0.645 ± 0.016 | -0.027 |
| AC              | TV_VN        | 32 | 0.610 ± 0.033| 0.608 ± 0.016 | 0.003 |
| Tau Vang        | Ac_VN        | 33 | 0.696 ± 0.021| 0.563 ± 0.016 | 0.193 |
| Luong Phuong    | TH_VN        | 32 | 0.680 ± 0.023| 0.657 ± 0.017 | 0.034 |
| Tam Hoang       | LP_VN        | 32 | 0.627 ± 0.023| 0.606 ± 0.016 | 0.033 |

The observed heterozygosity of the Tau Vang chickens is much lower than expected one (0.563 ± 0.016) resulting in high Fis estimate in this population. This result indicates that a level high of inbreeding occurred in this breed. Similar observations were made in some other breeds as well (Ho, Te and Ri), while in Choi chicken an excess of heterozygosity was found indicating that mating between closely related chicken has avoided in this breed.

Analysing a wide range of chicken populations originating from various continents and management systems, [13] found mean allele numbers and expected heterozygosity estimates per population varying from 2.30 to 6.72 and 0.28 to 0.67, respectively, with the Vietnamese H’mong breed being the most variable one. [3] reported that the mean expected heterozygosity of Vietnamese chickens in the Ha Giang province was 0.62, while the corresponding values for Red Jungle Fowl, Chinese and commercial breeds were 0.60, 0.47 and 0.40, respectively. Compared to other breeds from other countries, Vietnamese chickens represent higher expected heterozygosity [18], [27], [1], [10]. [1] at investigating the genetic diversity, relationship and population structure of 110 local Swedish chickens showed that expected heterozygosity ranged from 0.231 to 0.515. [18] researched on South African chicken and showed that the expected heterozygosity ranged from 0.510.03 to 0.620. An assessment of genetic diversity of six Egyptian local chicken strains indicated that expected heterozygosity varied from 0.4170 to 0.660 [27].

3.1.2. Genetic Structure and Genetic Difference

Analyzing the rate of change in log likelihood of the population structure from K = 2 to K= 9 suggested an optimal clustering at K = 6. This result is in agreement with the results from the pairwise comparison of runs for individual values of K using SIMCOEFF. The results of the STRUCTURE analysis are depicted in Figure 1.
At $K = 6$ the following pattern is found: There are four clearly distinct clusters: the two exotic breeds of Chinese origin form one cluster, and Ac, Choi, and Dong Tao breed formed individual clusters, respectively. Less clearly distinct are the clusters formed by the H’mong and the Te breeds. All other breeds are mixtures of the basic clusters, where the red river delta breeds (Mia, Ri and Ho) are mixtures of the H’mong- and Dong Tao-type, while the Tau Vang breed in

**Figure 1.** Clustering of Vietnamese chicken populations (Number in parenthesis is the number of identical solution at 95% threshold).

**Figure 2.** Phylogenetic network of 11 studied populations.
the Mekong delta has a strong influence both of local Vietnamese breeds and of breeds with Chinese origin. This structure is also reflected in the phylogenetic network (Figure 2), in which the distinct clusters found in the STRUCTURE analysis also form clearly distinct branches, while the ‘mixed’ populations are positioned close to the central node, indicating similar distances to the distinct clusters.

### 3.2. Mitochondrial Variability

#### 3.2.1. Within-Population Diversity

Eight clades (A-G and I) were formed by 37 haplotypes. Total of haplotypes per breed is shown in Table 4.

| Breed      | Agro ecological zone | Study area            | n   | No. of Polymorphic sites | No. of Haplotypes | Haplotype diversity (±SD) |
|------------|----------------------|-----------------------|-----|--------------------------|-------------------|--------------------------|
| Tam Hoang  | Northwest            | Mai Son, Son La       | 20  | 23                       | 6                 | 0.778 ± 0.055            |
| Luong Phuong | Red River Delta     | Duong Lam, Son Tay    | 20  | 10                       | 7                 | 0.737 ± 0.094            |
| Ho         | Red River Delta      | Hoai Duc, Ha Tay      | 20  | 22                       | 12                | 0.911 ± 0.045            |
| Dong Tao   | Red River Delta      | Thuan Thanh, Bac Ninh | 20  | 8                        | 4                 | 0.615 ± 0.105            |
| Te         | Red River Delta      | Khoai Chau, Hung Yen  | 20  | 20                       | 7                 | 0.768 ± 0.080            |
| Choi       | South Central Coast  | Ba Vi, Ha TayNIAS     | 20  | 14                       | 5                 | 0.716 ± 0.086            |
| Ac         | South Central Coast  | Ninh Hoa, Khanh Hoa   | 19  | 15                       | 4                 | 0.754 ± 0.053            |
| Tau Vang   | Mekong Delta         | Tan An, Long An       | 21  | 13                       | 5                 | 0.767 ± 0.053            |
| Tam Hoang  | Mekong Delta         |                         | 20  | 24                       | 13                | 0.942 ± 0.034            |
| Tam Hoang  | (imported from China)| NIAS                 | 21  | 19                       | 8                 | 0.852 ± 0.053            |
| Total      |                      |                       | 222 | 43                       | 37                | 0.849 ± 0.184            |

The lowest haplotype diversity (0.62 ± 0.105) was estimated in the Ho breed, while the highest corresponding value (0.942 ± 0.034) was observed in the Tau Vang breed.

Although the majority of the Vietnamese chicken breeds in this study were assigned to clade A and B, the Vietnamese breeds were found to be highly polymorphic in the mtDNA D-loop region. Estimates of haplotype diversity ranged from 0.62 to 0.94 in this study and were higher than reported previously. [23] found the haplotype diversity ranging from 0.61 to 0.73 and from 0.27 to 0.78 in Chinese chickens and purebred lines, respectively. [21] pointed out three of 12 Chinese breeds with only one haplotype. A recent studies on 4 Chinese native breeds and 7 Tibetan chicken breeds showed that the haplotype diversity were 0.916 and 0.925, respectively [12], [33]. The high degree of diversity of the Vietnamese breeds is in agreement with previous reports showing high diversity at the autosomal level analysing microsatellites [13] [3].

To explore the genetic diversity of Chinese indigenous chicken breeds, a 585 bp fragment of the mitochondrial DNA (mtDNA) region was sequenced in 102 birds from the Xichuan black-bone chicken, Yunyang black-bone chicken and Lushi chicken. In addition, 30 mtDNA D-loop sequences of Silkie fowls were downloaded from NCBI. The mtDNA D-loop sequence polymorphism and maternal origin of 4 chicken breeds were analysed in this study. 2. The results showed that a total of 33 mutation sites and 28 haplotypes were detected in the 4 chicken breeds. Three clusters were formed in 4 Chinese native chickens and 12 reference breeds. Both the Xichuan black-bone chicken and Yunyang black-bone chicken were grouped into one cluster. Four haplogroups (A, B, C and E) emerged in the median-joining network in these breeds. 3. It was concluded that these 4 Chinese chicken breeds had high genetic diversity. The phylogenetic tree and median network profiles showed that Chinese native chickens and its neighbouring countries had at least two maternal origins, one from Yunnan, China and another from Southeast Asia or its surrounding area.

#### 3.2.2. Breed Distribution Within Clades

The Vietnamese local chickens were found in all eight clades (Figure 3). The distribution of the Vietnamese breeds into clades was not related to their geographical distribution. The most frequent clades A and B included all nine Vietnamese breeds. A considerable proportion of Vietnamese local chickens belonged to clade D while only a small number of Vietnamese chickens was assigned to the five remaining clades (C, E, F, G and I). In contrast, the majority (76%) of Chinese chickens were found in clade E whereas no Chinese chickens were observed in clades D, G and I.

The majority of the Vietnamese local chickens carried mtDNA haplotypes that clustered in clades A and B. Based on the skeleton of supposed regions of domestication, this finding suggests the existence of two maternal lineages dominating in the Vietnamese local chickens which presumably originate from Yunnan and surrounding regions in China [20]. Fourteen percent of Vietnamese chickens were found in clade D indicating that this clade also contributed considerably to the Vietnamese local chickens. [20] and [25] suggested that this clade has its root in Southeast, South and Southwest China and/or surrounding areas (i.e. Vietnam, Burma, Thailand, and India). This finding would be in agreement with historical records of human immigration from southern China to Vietnam. Yuè people are inhabitants in the Southeastern coast of China and are the ancestors of the Cantonese, i.e. Guangzhou and Guangxi Southern Chinese people. By the 3rd century B. C., Yuè people emigrated from Southern China to the Red River Delta of Vietnam and mixed with the indigenous Van Lang Vietnamese population [31]. Additionally, Southern Chinese people from Yunnan, Guangzhou and Guangxi Provinces
arrived at the North of Vietnam and moved to the South from the 17th to the 19th century A. C. [24]. Descriptions of immigration always state that people of a family moved together with their animals which could result in the introduction of chickens from Southern China into the North and the South of Vietnam. While Yunnan, South and Southwest China might be seen as region of origin of the Vietnamese chicken breeds, the majority of individuals of Chinese breeds in this study were not assigned to these maternal lineages. This finding indicates that two Chinese breeds kept at NIAS do not represent the breeds of Yunnan, South and Southwest China.

The high proportion of haplotype D1 found in the Choi chickens is in agreement with findings of [20], who reported that clade D mainly consisted of game birds. On the other hand, the clustering of the remaining Choi chickens in clades A and B is consistent with the study of [25] who found game birds assigned to their clades B and E. Consequently, our findings suggest that the game breed Choi is a mixture of multiple maternal lineages.

A small number of Vietnamese chickens distributed in clades C, F, G and I indicate that these clades have little contribution to Vietnamese chickens. A small portion (2%) of Vietnamese local chickens was observed in clade E, originating from the Indian subcontinent [20], which otherwise harboured mainly the Chinese chickens studied. Vietnamese local chickens in this clade included the Ri and Tau Vang breeds. This observation may indicate a possible exchange of genetic material between the Ri and Chinese chickens due to the wide distribution of the Ri chickens, while the Chinese origin of the Tau Vang breed [19] is known and explains the distribution of this breed in both Vietnamese and Chinese clades. This finding is also in agreement with the analysis at the autosomal level in which the Tau Vang breed showed clear admixture between the Chinese and Vietnamese gene pools.

### 3.3. Relationship Between mtDNA and Autosomal Genetic Structure

The results of the STRUCTURE analysis from $K = 2$ to $K = 6$ are shown in Figure 2. The repeatability, i.e. the number of runs giving result with similarity coefficient 0.95, varied from 34 to 100 from $K = 2$ to $K = 6$, while no identical runs were found at $K = 7$ and 8 (data not shown). For all $K$ values, the mtDNA defined clade E was found as a pure cluster at the autosomal level while the other seven mtDNA defined clades were mixed to different degrees (Figure 4).
Comparing results of phylogenetic relationship using mtDNA polymorphism and autosomal microsatellites it becomes obvious, that the Chinese breeds cluster together and are separated from the Vietnamese local breeds using both genetic marker systems, indicating a clear genetic differentiation between them and the Vietnamese breeds. Although [32] assumed that the Chinese chickens from NIAS have introgressed into local Vietnamese chickens, our results do not support this hypothesis, except for the Ri and Tau Vang chickens. In contrast to microsatellite analyses, which found that clustering of Vietnamese local breeds using microsatellites has a relationship to their geographical distribution, no sub-structuring was found between the Vietnamese local breeds at the mtDNA level.

In microsatellite analyses, clustering of Vietnamese local breeds using microsatellites has a relationship to their geographical distribution but there is no sub-structuring found between the Vietnamese local breeds at the mtDNA level. The different results obtained in both types of markers could be due to the different mode of inheritance. Microsatellites are highly polymorphic markers with their locus specificity, abundance and random distribution over the genome, co-dominant inheritance [34]. Unlike microsatellite markers, mtDNA is maternally inherited. MtDNA is used to infer regions of domestication and to identify the number of maternal lineages and their geographic origins [9]. Unlike autosomal genetic markers, mtDNA transferred from mother to offspring is not rearranged due to recombination and less affected by gene drift [15]. In addition, mtDNA has a lower mutation rate than microsatellite as argued by [11].

4. Conclusion

At the autosomal level, the Vietnamese local chicken breeds from different agro-ecological zones represent genetically distinct populations. The northern breeds are clearly separated from breed of the South Central Coast and from breed of the Mekong Delta. The Vietnamese local chicken breeds are highly polymorphic and originated from eight maternal lineages. These lineages are present across the country. Two chicken breeds of Chinese origin, Tam Hoang and Luong Phuong, kept in the National Institute of Animal Sciences are genetically distinct from the Vietnamese local breeds. The Vietnamese chicken breeds are genetically separated from the Chinese chicken gene pool.

In contrast to microsatellite analyses, which found that clustering of Vietnamese local breeds using microsatellites has a relationship to their geographical distribution, no sub-structuring was found between the Vietnamese local breeds at the mtDNA level.

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