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Genetic diversity analysis using microsatellite markers in indigenous Peruvidai chicken

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Abstract
The present study was the first report on Indigenous Peruvidai chicken ecotypes in Tamil Nadu state, India in order to untangle the genetic diversity of within Peruvidai chicken population. Six microsatellite loci recommended by the Food and Agriculture Organization (FAO) were used. Thirty birds were sampled from five different districts in western parts of Tamil Nadu. DNA was collected and PCR was carried out to amplify the target regions. Sequencing was carried out and the data were analyzed for genetic diversity indicators with Cervus 3.0.7 and GenAlEx 6.5 and Popgene software programmes. A total number of 112 alleles were detected with an average value of 3.7 alleles per locus. The Peruvidai chicken of Tiruppur and Dharmapuri districts were most distant with a Nei's genetic distance value of 0.30 and Erode and Dharmapuri districts were least distant with a value of 0.165. Erode and Salem pair had Nei's genetic identity value of 0.842, which is highest among all pairs of ecotypes. Mean Fixation indices' (FST, FIS, and FIT) values were 0.092, 0.115 and 0.182, respectively, across all 20 loci investigated. The mean number of polymorphic information content observed was 0.626. The results of this study indicated that the studied populations were genetically differentiated and polymorphic in nature.

Keywords: Peruvidai chicken, ecotypes, microsatellite marker, genetic diversity, conservation

1. Introduction
Chicken are the most profuse among the poultry and consumed by human beings worldwide (Liu *et al.,* 2006) [1] it was reflected globally by the fastest and strong growth of poultry production as a commercial sector (Aboe *et al*., 2006) [2]. In the recent centuries poultry industry is mostly dominated by producing commercial hybrids to get maximum economic return with short span of time. More over the industry is mainly focused to exploit the maximum genetic potential for commercial use such as for more egg production in layers and high body weight gain in broilers with less input. This resulted in selection of fewer chicken breed which cater and fulfill their target area and further the industry maintain the homogeneity among these commercially produced hybrids. This in turn reduces the genetic diversity and converged to focus their attention on a particular poultry germplasm or breed and neglecting the indigenous chicken breeds. Local or indigenous ecotypes are the repository of possessing more genetic diversity with various genotypes for adaptation to local climate and this will serve as a source material for the breeder and for researchers (Todano *et al.,* 2007) [3]. As per the FAO (2017) [4] report the loss of indigenous chicken breed will become a serious problem in developing countries and results in loss of indigenous poultry germ plasm and its genetic diversity. In order to maintain the genetic diversity the steps were initiated by Food and Agricultural Organization (FAO) to give a limelight about the erosion of indigenous genetic diversity worldwide (Weigend & Romanov, 2002) [5]. Heterozygosity and or gene diversity is one of the valuable tool for measuring the genetic variation of certain populations (Bao *et al*., 2008) [6], reflecting the genetic variation of different populations at different loci. Maintain the genetic diversity helps in balanced ecological and soil water environment (Liao *et al*., 2016) [7]. Genetic diversity can be assessed in poultry with numerous molecular markers and software tools but among all, microsatellites are the marker of choice by many researchers (Das *et al.,* 2015) [8]. Since these markers are randomly distributed throughout the genome, highly polymorphic and ideal for decoding the genetic variability.

Indian chicken ecotypes consisted of diverse phenotypes of almost many centuries of natural selection and reared by small marginal and smallholder farmers across distinct agro-ecological regions. (Rudresh *et al*., 2015) [9]. Among the native chicken breeds / ecotypes in India, the "Peruvidai" is very much popular among the farmers in western part of Tamil Nadu and there is a growing interest in rearing of these birds. Peruvidai chicken is hardy in nature, ability to thrive under adverse climatic conditions, known for their meat and egg quality with desirable taste and flavor along with the fighting quality of cocks (Kumaravel *et al*., 2021) [10]. Despite the importance of native chicken in tribal / rural areas, information is lacking on their genetic makeup with respect to genetic variability, genetic relationships, performance, adaptability and resistance to many diseases (Rudresh *et al*., 2015) [9]. In this current scenario present study was taken up in identifying the genetic diversity within the population of Peruvidai indigenous chicken ecotypes with appropriate microsatellite marker panel in western part of Tamil Nadu.

2. Materials and Methods

2.1 Experimental birds

Indigenous Peruvidai chicken ecotypes belonging to Dharmapuri, Erode, Namakkal, Salem and Tiruppur districts of Tamil Nadu State, India were chosen for the present study (Figure 1). A total 30 randomly chosen adult birds from the above districts were used for the present study.

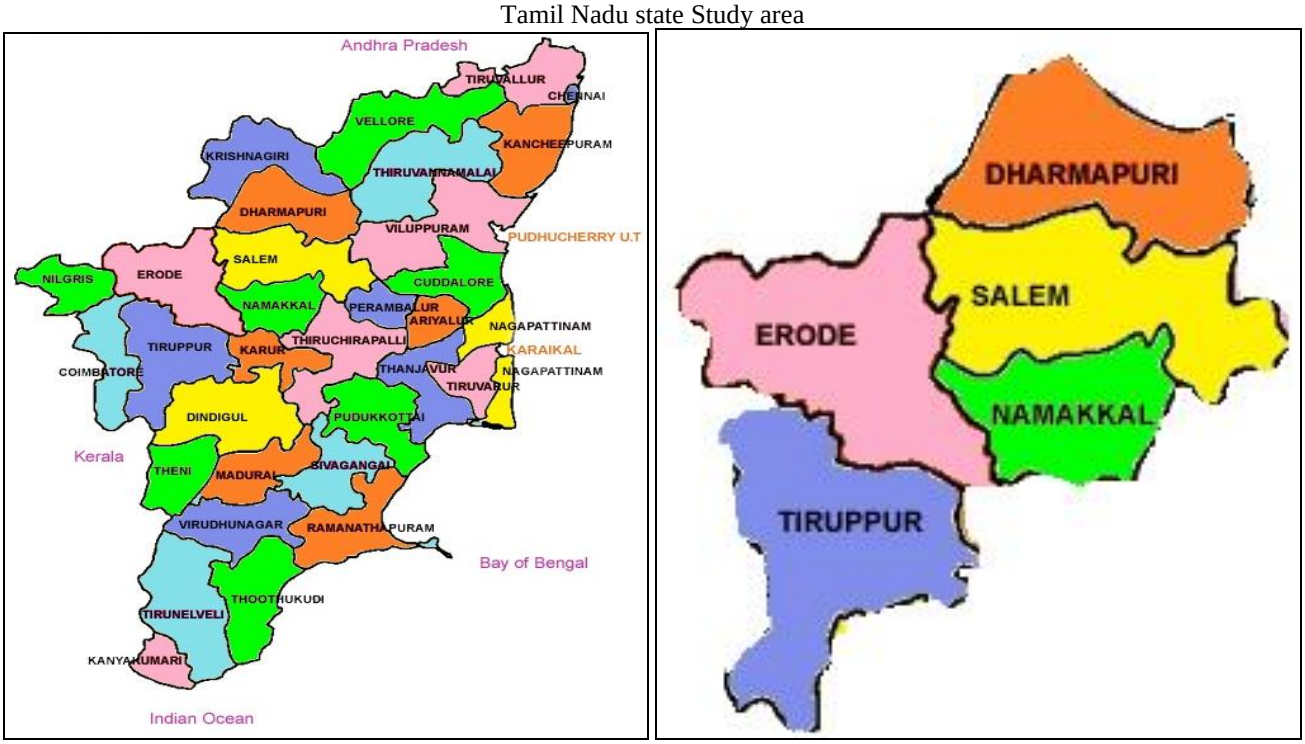

Fig 1: Map showing the study area (Western part of Tamil Nadu)

2.2 Sample collection and DNA Isolation

Blood samples (0.5-2.0 ml per bird) were aseptically collected into the vacutainers containing EDTA (5.4 mg) from the wing vein. Utmost care was taken that the samples were taken from unrelated Peruvidai chicken birds to ensure that the samples were randomly selected and it represents the population. High molecular weight genomic DNA was isolated as per standard protocol of phenol chloroform-iso amyl alcohol extraction (Sam brook and Russell 2001) [11]. The quality of DNA was assessed through 0.7% horizontal Agarose gel electrophoresis. The purity and concentration of DNA were determined through NanoDrop® spectrophotometer. DNA of good quality having intact band without smearing and satisfactory purity were used for further analysis. The final DNA concentration of 50 ng of DNA per µl was prepared and used for further PCR reaction.

2.3 Microsatellite markers and primers

A total of six microsatellite primer sets specific for chicken were used in the study as recommended by FAO, United Nations, Rome, Italy (FAO, 2011) [12]. The details of microsatellite primers were given in Table 1. Only forward primers of each pair were labelled with one of the four fluorophore i.e. FAM and TAM which were synthesized by Bio serve Biotechnologies (India) Pvt. Ltd., Hyderabad, Telangana. The reverse primers were kept unlabelled.

Table 1: Primer sequence, annealing temperature and PCR product size of chicken microsatellite markers

Sl. No.	Primer name	Primer sequence in 5'-3' orientation	5' labelling with fluorochrome	Annealing temperature (°C)	PCR product size
1.	ADL0278	F-CCAGCAGTCTACCTTCCTAT R-TGTCATCCAAGAACAGTGTG	FAM	60.0	114-126
2.	LEI0166	F-CTCCTGCCCTTAGCTACGCA R-TATCCCCTGGCTGGGAGTTT	FAM	60.0	354-370
3.	MCW0014	F-TATTGGCTCTAGGAACTGTC R-GAAATGAAGGTAAGACTAGC	FAM	58.0	164-182
4.	MCW0034	F-TGCACGCACTTACATACTTAGAGA	TAM	60.0	212-246

		R-TGTCCTTCCAATTACATTCATGGG			
5.	MCW0067	F-GCACTACTGTGTGCTGCAGTTT R-GAGATGTAGTTGCCACATTCCGAC	FAM	60.0	176-186
6.	MCW0104	F-TAGCACAACTCAAGCTGTGAG R-AGACTTGCACAGCTGTGTACC	FAM	60.0	190-234

2.4 PCR amplification of microsatellite loci: Each 25 µl PCR reaction mix for each DNA sample and microsatellite locus was prepared with 12.5 µl of 2 X PCR Master mix (Ampliqon Red PCR Master mix), 0.5 µl of DNA Template (50ng), 0.5 µl of each Forward and Reverse Primers and11.0 µl of nuclease free water. The PCR aamplification was carried out in programmable thermal cycler (Eppendorff, USA) using PCR program consisting of initial Denaturation at 94 °C for 5 min, followed by 30 cycles of (i) Denaturation at 94 °C for 1 min, (ii) Annealing at optimized temperature for 30 seconds and; (iii) Extension at 72 °C for 45 seconds, followed by a final extension at 72 °C for 5 minutes and 4 °C forever for Final holding. Amplified PCR products were checked on 1.5% horizontal submarine Agarose gel electrophoresis and their final resolution was confirmed running with molecular size marker (50 bp DNA ladder- Promega). The PCR products were genotyped using automatic sequencer at Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka, India. The results were obtained using gene mapper V4.1. (Applied Bio system, USA)

2.5 Statistical analysis: The allele data were subjected to the Excel Microsatellite Tool Kit and GenAlex 6 (Peakall and Smouse 2012) [13] for estimating various parameters. Allele frequency (Af), mean number of alleles (Na) effective number of alleles (Ne), percentage of polymorphic loci, heterozygosity (H), polymorphism information content (PIC), genetic distance, genetic identity and departure from H-W equilibrium were studied.

3. Results and Discussion

3.1 The microsatellite polymorphism evaluated by the number of alleles analyzed and described in Table 2

Table 2: Summary of Heterozygosity information over all loci for each population

Ecotypes	Value	Na	Ne	I	Ho	He	uHe	F
Namakkal	Mean	3.500	2.574	1.036	0.528	0.586	0.639	0.085
	SE	0.428	0.306	0.117	0.125	0.044	0.048	0.231
Salem	Mean	3.667	2.936	1.122	0.583	0.620	0.677	0.002
	SE	0.422	0.374	0.141	0.160	0.062	0.067	0.250
Erode	Mean	3.667	2.629	1.062	0.528	0.593	0.646	0.072
	SE	0.422	0.292	0.118	0.117	0.050	0.054	0.199
Tiruppur	Mean	4.000	2.982	1.202	0.611	0.655	0.715	0.057
	SE	0.365	0.223	0.072	0.141	0.026	0.028	0.204
Dharmapuri	Mean	3.833	2.830	1.063	0.444	0.560	0.611	0.178
	SE	0.601	0.453	0.223	0.119	0.116	0.126	0.156
Grand Mean and SE over Loci and Pops								
		Na	Ne	I	Ho	He	uHe	F
Total	Mean	3.733	2.790	1.097	0.539	0.603	0.658	0.075
	SE	0.191	0.144	0.061	0.056	0.028	0.031	0.089

Genetic variation was analysed with the effective number of alleles in the population (Ne), and it is mostly smaller than the observed number in experiments because of the big differences in allele frequencies in domestic animals. In the experimental study, high effective number of alleles (Greater than 4) indicates that the sample size was adequate, reflecting the efficiency of the used set of loci and its richness of genetic information (Soltan *et al.*, 2018) [14]. In this study the mean Ne obtained was 2.79, which may be due to selection of less number of markers and the sample size. But the obtained results suggest that the gene pool among all the districts were similar and can have the potential to use further in breeding strategy.

The results of F- Statistics and Polymorphic Information Content (PIC) are analyzed and described in Table 3.

Table 3: F-Statistics and Estimates of Nm over All Populations for each Locus and polymorphic information content.

Locus	F_{IS}	F_{IT}	F_{ST}	Nm	PIC
ADL0278	0.381	0.434	0.086	2.669	0.729
LEI0166	-0.263	-0.204	0.047	5.053	0.510
MCW0014	1.000	1.000	0.164	1.277	0.615
MCW0034	-0.114	-0.021	0.084	2.743	0.682
MCW0067	-0.184	-0.088	0.081	2.844	0.627
MCW0104	-0.132	-0.028	0.092	2.477	0.595
Mean	0.115	0.182	0.092	2.844	0.6265
SE	0.200	0.186	0.016	0.500	0.012

From the obtained results it was noticed that the mean polymorphic information content was 0.6265. Microsatellites showing PIC values higher than 0.5 indicate that more genetic information can be provided by SSR loci (Botstein *et al*., 1980) [15]. Genetically, a microsatellite marker with a PIC > 0.7 is considered as an ideal one for genetic studies (Bai *et al*., 2016) [16]. In the present study, ADL0278 microsatellite loci showed PIC values of over 0.7, reflecting the possibility of using these loci as candidate genes for the future genetic studies in Peruvidai chicken.

Relatively high mean pair wise F_{ST} value was observed between the Peruvidai chicken in five different districts. The population genetic differentiation coefficient (F_{ST}) was 0.092, which indicated that the genetic variation among populations accounted for 9.20% of the total genetic variation. It indicated that the genetic variation among population covered is small proportion of the total genetic variation and that there was a slight differentiation between the districts investigated. For the interpretation of F_{ST}, it has been suggested that a value lying in the range 0-0.05 indicates little genetic differentiation. The value between 0.05 and 0.15, indicates moderate differentiation and the value between 0.15 and 0.25 indicates great differentiation; and values above 0.25, very great genetic differentiation. Balloux *et al.* (2002) [17] elaborated that a F_{ST} of 0.05 will generally be considered as reasonably low, and investigators may interpret that structuring between sub populations is weak. Present study showed moderate genetic differentiation between five districts of Peruvidai chicken, since all the selected areas are sharing the geographical borders with each other.

The inbreeding coefficient (F_{IS}) of the five different populations of Peruvidai chicken was 0.115. The F_{IS} represents a degree of nonrandom mating which shows the deviation from Hardy-Weinberg equilibrium. (Tadano *et al*., 2007) [3] Positive number for F_{IS} means deviation from Hardy-Weinberg equilibrium. Two markers, ADL0278 and MCW0014 in all the five districts showed positive numbers and in other four markers the negative sign of F_{IS} represents that random mating is followed. Further it indicates that the allele was well fixed in all loci.

3.2 Pair wise Nei Unbiased Genetic Distance and Nei Unbiased Genetic identity: The pair wise Nei Unbiased Genetic Distance (below diagonal) and Nei Unbiased Genetic identity (above diagonal) was given in Table 4 (Nei., 1987) [18]. The results showed that the Peruvidai chicken of Tiruppur and Dharmapuri districts were most distant with a Nei's genetic distance value of 0.301; Erode and Dharmapuri districts were least distant with a value of 0.165; Erode and Salem pair had Nei's genetic identity value of 0.842. The more distant identity may be due to the geographical locations.

Table 4: Pairwise Population Matrix of Nei Unbiased Genetic Distance and Nei Unbiased Genetic identitiy.

Namakkal	Salem	Erode	Tiruppur	Dharmapuri	Districts
0.000	0.817	0.817	0.811	0.819	Namakkal
0.202	0.000	0.842	0.793	0.785	Salem
0.202	0.172	0.000	0.812	0.848	Erode
0.209	0.232	0.208	0.000	0.740	Tiruppur
0.200	0.243	0.165	0.301	0.000	Dharmapuri

4. Conclusion

Overall, the microsatellite marker ADL0278 with PIC value of more than seven may be further exploited for genetical studies for genetic variation in Peruvidai chicken. The richness of genetic diversity through number of effective alleles indicates that further exploratory study required with more no of microsatellite markers. F_{IS} value indicates that random mating is practiced in the selected areas with few alleles were fixed. As diversity exists in Peruvidai chicken ecotypes, which may be further exploited for genetic improvement. The results of microsatellite analysis study clearly indicated that all the six microsatellite markers are suitable tools to investigate questions of genetic variability, gene flow and the mating system in Peruvidai chicken ecotypes. Further research is needed to evaluate genetic variations with large number of samples for conservation strategy.

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6. Conflict of interest: No potential conflict of interest relevant to this article was reported.

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8. References

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