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RESEARCH ARTICLE

Microsatellite Marker Based Genetic Diversity among Four Varieties of Pakistani Aseel Chicken

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ABSTRACT

Indian Aseel chicken (*Gallus gallus*) is traditionally used as a favorite game bird all over the world. Bird fighting communities of Pakistan are the major source of its conservation and there are at least four distinctively recognized varieties of Aseel chicken based upon selective breeding, geographical location and color patterns. A pioneering study on genetic diversity of these varieties namely Lakha (n=17), Mushki (n=19), Mianwali (n=19) and Peshawari (n=13) was undertaken using FAO recommended 10 microsatellite loci. A total of 91 alleles were observed in 4 varieties of Aseel chicken with an average of 9.1 alleles per locus. Number of alleles varied between 4 to 8 in Lakha, 4 to 9 in Mushki, 3 to 10 in Mianwali and 3 to 7 in Pashawari. Mean polymorphic information content values were 0.67, 0.69, 0.71 and 0.65 in individual varieties, respectively. Mean observed and expected heterozygosity index values of 0.3941 and 0.7376 were recorded in Lakha, 0.4105 and 0.7468 for Mushki, 0.4105 and 0.7718 Mianwali and 0.3692 and 0.7191 for Peshawari. Mean Fixation index (*Fst*) value was calculated as 0.1264. Highest Nei's standard genetic distance (*Ds*) value of 1.0735 was observed between Mushki and Peshawari, whereas its value was minimum (0.3533) between Lakha and Mushki. This report describes genetic diversity of Aseel chicken in Pakistan and provides foundation data to initiate extensive and more comprehensive studies on indigenous chicken genetic resource conservation and its future utilization in commercial breeding programs.

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INTRODUCTION

Apparently the chicken was domesticated by the Aryan people in the Indus Valley civilization during 2500–2000 B.C. (West and Zhou, 1989; Crawford, 1995). Different varieties of poultry contribute to the Animal Genetic Resource (AnGR) of Pakistan. Phenotypic and genotypic data is essential for the characterization of indigenous AnGR for the effective conservation of useful gene pool for future generations. The Aseel chicken is important as a game bird both in Pakistan and the world over. Due to its unique aggressive behavior and meat value, it is especially bred by cock fighter communities in different rural areas of Pakistan.

Aseel has four varieties depending upon its place of origin and phenotypic characteristics. Lakha Cheena (mottled) Aseel found abundantly throughout the Punjab including Gujranwala, Sialkot, Faisalabad, Multan, Sargodha, Mianwali, Khushab and Rawalpindi districts, has reddish brown plumage with white and black mottling. This variety lays brown shelled eggs. The body weight ranges 3.0-3.8 kg in males and 2.5-3.2 kg in females in local scavenging conditions. Mushki Aseel is heavy weight and native of Mianwali, Khushab, Jauharabad, Sargodha, Talagang, Fatehjung and Bhakkar districts of the Punjab province. Mushki Aseel has a black plumage and has black pigmentation in beak and shanks. Body weight ranges 3.0-3.5 kg in males and 2.5-3.0 kg in females. Mianwali Aseel is medium weight variety available in Mianwali, Khushab, Jauharabad, Sargodha, Kalabagh and Bhakkar districts of

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the Punjab Province. This variety possesses a dark brown plumage. Early growth rate of this variety is better than the other varieties. The body weight ranges 2.5-3.0 kg in males and 2.0-2.2 in females. It exhibits good resistance against a number of diseases and is heat resistant. Peshawari Aseel is slightly light in weight and located in Peshawar, Mardan and Nowshera districts of Khyber Pakhtoonkhwa province. The body weight ranges 2.8-3.2 kg in males and 2.2-2.5 in females. The Peshawari Aseel has a wheaten colored plumage.

Different genetic marker have been used for evaluation of genetic variability in poultry including DNA fingerprinting (Dunnington *et al.*, 1994; Mafeni *et al.*, 1997), RAPD (Smith *et al.*, 1996) and microsatellites (Crooijmans *et al.*, 1995; Vanhala *et al.*, 1998; Wimmers *et al.*, 2000). Microsatellites are the latest molecular markers used in gene marker studies for their co-dominant, highly polymorphic nature, availability throughout the genome so the microsatellites are identified as reliable markers in chicken. (Romanov and Weigend, 2001; Zhang *et al.*, 2002; Hillel *et al.*, 2003). Very little information on genetic makeup, genetic variability and differentiation of the local Aseel varieties is available. The present study is the preliminary report on Aseel chicken of Pakistan to evaluate the genetic variability among four different varieties.

MATERIALS AND METHODS

Selection of birds and DNA extraction: Birds of four varieties of Aseel chicken kept at the Indigenous Chicken Genetic Resource Conservation Centre at the Department of Poultry Production, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, Pakistan were sampled for this study. Blood samples (3 mL) were collected from 68 (Lakha 17, Mushki 19, Mianwali 19 and Peshawari 13) unrelated birds aseptically and DNA extraction was carried out in Molecular Biology and Genomics Lab. of University of Veterinary and Animal Sciences, Lahore by the Sambrook and Russel (2001) protocol and the DNA was stored at -20°C until ready for PCR amplification.

Microsatellite markers: A set of ten FAO (MoDAD programme) recommended microsatellite markers (ADL23, ADL102, ADL136, ADL158, ADL 171, ADL176, MCW5, MCW7, MCW41, MCW59 (Crooijmans *et al.*, 1996; Groenen *et al.*, 1997; Cheng *et al.*, 1995; Horbanczuk *et al.*, 2007) were selected for this study and were supplied by Gene Link, USA (Table 1).

PCR Amplification and PAGE: Avian DNA amplification involved initial denaturation at 95°C (5 min), followed by 35 cycles of denaturation at 94°C (1 min), primer annealing at temperature range between 62°C to 52°C (Touch down protocol) (1 min), extension at 72°C (1 min) and final extension at 72°C (7 min). All markers were optimized for amplification by polymerase chain reaction (PCR) using BioRad thermal cycler. A volume of 25 µL of reaction mixture was prepared using 100 ng genomic DNA, 10 pM of forward and reverse primers, 0.2 mM dNTP, 10 mM Tris HCl, 2.0 mM MgCl₂ along with 0.5 unit of Taq DNA polymerase (Fermentas,

Thermo Sci. USA) and double distilled de-ionized water. Optimal conditions for PCR amplification were empirically determined to produce PCR product for DNA marker. The PCR products were electrophoresed on 12% non denaturing polyacrylamide gel in 1X TAE buffer at 250 volts for 4 hours.

Genotyping and statistical analysis: Each PCR product was genotyped and PAGE data was analyzed to workout standard parameter of genetic diversity in four varieties of Pakistani Aseel chicken. Standard ladder DNA marker was used to estimate allele size, whereas polymorphic information content (PIC), Matching Probability, Power of exclusion and Power of discrimination values were calculated using Power Stat 2.1 software. POPGENE 1.31 computer package (<http://www.ualberta.ca/~fych/fych>, Yeh and Yong, 1999) to workout the values of observed and expected heterozygosity, observed and effective number of alleles, F- statistics, Shannon's information index and Nei's genetic distance.

RESULTS AND DISCUSSION

Genetic variations: All 10 loci were found polymorphic in four Aseel varieties of Pakistan under study. The analysis of data revealed that the observed number of alleles varied from 4 to 8 in Lakha 4 to 9 in Mushki, 3 to 10 in Mianwali and 3 to 7 in Pashawari. Numbers of alleles per locus ranged from 6 (MCW059) to 14 (ADL136) with mean number of loci 9.1 ± 2.18 in all microsatellite loci. This study indicated slightly higher number of alleles when compared to the findings of Parmar *et al.* (2007) and Alipanah *et al.* (2011), whereas the findings of Pandey *et al.* (2003) in Indian Aseel (3 to 9 alleles) were similar to this study. Mean numbers of effective alleles in all populations were 6 ± 1.48 compared to 3.09 as reported by Pandey *et al.* (2003). The highest over all PIC value was found in locus MCW5 (0.88) while lowest in locus ADL102 (0.71) for all four varieties (Table 2). The average, PIC values for all loci among four varieties were calculated to be 0.67, 0.69, 0.71 and 0.65 for Lakha (Cheena), Mushki, Mianwali and Peshawari respectively (Tables 3, 4, 5, 6). These PIC values are slightly higher to those reported by Pandey *et al.* (2003) and comparable with Tibetan chicken (0.71) by Kong *et al.* (2010) and Brazilian Chicken (0.73) as reported by Clementino *et al.* (2010). Overall high PIC value indicates that the particular locus is highly informative which may be used to resolve queries of forensic nature and help to evaluate the genetic diversity of different breeds of poultry.

Heterozygosity of markers: Overall expected heterozygosity (He) value for all microsatellite loci among all four varieties was found with 0.8329 (Table 2) which is on the higher side when compared to 0.701 as reported by Parmar *et al.* (2007) in indigenous Kadaknath breed of poultry and 0.62 in Vietnamese chickens by Berthouly *et al.* (2010). However Pirany *et al.* (2007) reported it as 0.78 in some Indian chicken populations which is closer to current study. The average values of observed and expected heterozygosity of all varieties of Aseel chicken varied between 0.3941 and 0.7376 for Lakha, 0.4105 and

Table 1: Microsatellite markers information

Marker	5'-3' Sequence	Repeat motif	Ch. #	Gene bank #	Ta (°C)
ADL23	FP: CTTCTATCCTGGGCTTCTGA RP: CCTGGCTGTGTATGTGTTGC	(CA)5(CG)4(CA)9	5	L23905	62-52
ADLI02	FP: TTCCACCTTTCTTTTTTATT RP: GCTCCACTCCCTTCTAACCC	(GT)18	30	G01547	58-48
ADLI36	FP: TGTCAGCCCATCGTATCAC RP: CCACCTCCTCCTCTGTTCA	(TG)10TC(TG)10	6	G01561	62-52
ADLI58	FP: TGGCATGGTTGAGGAATACA RP: TAGGTGCTGCACTGGAAATC	(CA)12	C30, E29	G01582	62-52
ADLI71	FP: ACAGGATTCTTGAGATTTTT RP: GGTCTTAGCAGTGTGTTGTT	(TG)18	E43	G01593	58-48
ADLI76	FP: TTGTGGATTCTGGTGGTAGC RP: TTCTCCGTAACACTCGTCA	(GT)12	E6	G01598	62-52
MCW5	FP: ACCTCTGCTGCAAATAAATTGC RP: TCACTTTAGCTCCATCAGGATTCA	(TG)14	C11	-	62-52
MCW7	FP: AGCAAAGAAGTGTCTCTGTTTCAT RP: ACCCTGCAAAGTGGGAGGTTCTCA	(TG)5	I	-	62-52
MCW41	FP: CCCATGTGCTGAATAAATTGGG RP: CCAGATTCTCAATAAATGGCAG	-	C3	-	62-52
MCW59	FP: AAGTGCCTTTGCTATCCTGATTGG RP: AACTCCTATTGTGCAGCAGCTTAT	(AG)22	CIE2	-	62-52

Ch. #, Chromosome no; Ta, Annealing temperature

Table 2: Population genetic values of all markers in all Aseel varieties

Marker	na	ne	I	Ho	He	Fis	Fit	Fst	Nm	Matching probability	Power of Discrimination	Power of Exclusion	PIC
ADL23	8	5.4082	1.8353	0.3235	0.8211	0.5081	0.6137	0.2148	0.914	0.132	0.868	0.074	0.79
ADLI02	8	3.7762	1.5525	0.2647	0.7406	0.5999	0.6522	0.1309	1.6601	0.195	0.805	0.061	0.71
ADLI36	14	7.8639	2.3116	0.7941	0.8793	0.0307	0.0937	0.0650	3.5973	0.103	0.897	0.588	0.86
ADLI58	8	4.6754	1.7515	0.2794	0.7919	0.5448	0.6401	0.2094	0.9439	0.191	0.809	0.055	0.75
ADLI71	9	6.1003	1.9623	0.3676	0.8423	0.5268	0.5553	0.0603	3.8934	0.124	0.876	0.095	0.82
ADLI76	10	5.9626	1.9857	0.1029	0.8385	0.8684	0.8860	0.1336	1.6217	0.155	0.845	0.009	0.8
MCW5	11	8.7659	2.2550	0.4118	0.8925	0.4749	0.5277	0.1004	2.2397	0.061	0.939	0.121	0.88
MCW7	9	7.1524	2.0651	0.7500	0.8666	0.0963	0.1433	0.0520	4.5584	0.097	0.903	0.561	0.86
MCW41	8	5.6390	1.8408	0.0294	0.8288	0.9613	0.9681	0.1756	1.1737	0.176	0.824	0.001	0.8
MCW59	6	5.5845	1.7538	0.6618	0.8270	0.0456	0.1766	0.1373	1.5713	0.127	0.873	0.393	0.8
Mean	9.1	6.0928	1.9314	0.3985	0.8329	0.4505	0.5199	0.1264	1.7277				
(St. Dev.)	(2.1833)	(1.4806)	(0.2348)	(0.2607)	(0.0439)								

na, Observed no. of alleles; ne, effective no. of alleles; I, Shannon's index; Ho, observed heterozygosity; He, expected heterozygosity; Fis, Inbreeding coefficient; Nm: Gene flow estimated from $F_{st} = 0.25(1 - F_{st})/F_{st}$.**Table 3:** Values for each of microsatellite marker used on Lakha (Cheena) Aseel

Marker	na	ne	I	Ho	He	Matching probability	Power of Discrimination	Power of Exclusion	PIC
ADI23	4	2.4286	1.0910	0.3529	0.6061	0.419	0.581	0.088	0.54
ADLI02	4	2.4701	1.0321	0.2353	0.6132	0.329	0.671	0.040	0.52
ADLI36	8	5.898	1.8942	0.7647	0.8556	0.253	0.747	0.535	0.81
ADLI58	5	3.5901	1.4175	0.1765	0.7433	0.253	0.747	0.024	0.68
ADLI71	5	3.3218	1.3502	0.4706	0.7201	0.225	0.775	0.163	0.65
ADLI76	6	3.7051	1.5011	0.1176	0.7522	0.273	0.727	0.011	0.68
MCW5	8	5.8980	1.8880	0.4706	0.8556	0.107	0.893	0.163	0.81
MCW7	7	5.3519	1.7565	0.6471	0.8378	0.232	0.768	0.351	0.79
MCW41	5	3.0421	1.2997	0.0000	0.6916	0.329	0.671		0.62
MCW59	4	3.1243	1.1907	0.7059	0.7005	0.260	0.740	0.437	0.61
Average	5.6	3.8830	1.4421	0.3941	0.7376				0.67
(St. Dev.)	(1.5776)	(1.3377)	(0.3144)	(0.2617)	(0.0911)				

na, Observed no. of alleles; ne, effective no. of alleles; I, Shannon's index; Ho, observed heterozygosity; He, expected heterozygosity.

Table 4: Values for each of microsatellite marker used on Mushki Aseel

Marker	na	ne	I	Ho	He	Matching probability	Power of Discrimination	Power of Exclusion	PIC
ADL23	8	3.8201	1.6428	0.4737	0.7582	0.269	0.731	0.165	0.71
ADLI02	5	2.7245	1.1568	0.1053	0.6501	0.296	0.704	0.019	0.59
ADLI36	9	6.7477	2.0225	0.7368	0.8748	0.158	0.842	0.488	0.83
ADLI58	5	1.8656	0.9399	0.2632	0.4765	0.490	0.510	0.049	0.43
ADLI71	7	5.3881	1.7959	0.4211	0.8364	0.186	0.814	0.127	0.78
ADLI76	6	3.5567	1.4556	0.0526	0.7383	0.263	0.737	0.003	0.67
MCW5	8	5.049	1.7917	0.5263	0.8236	0.152	0.848	0.212	0.77
MCW7	8	6.3333	1.9465	0.8947	0.8649	0.158	0.842	0.785	0.82
MCW41	5	3.5049	1.3699	0.1053	0.7340	0.274	0.726	0.009	0.66
MCW59	4	3.2523	1.2676	0.5263	0.7112	0.258	0.742	0.266	0.65
Average	6.5	4.2242	1.5389	0.4105	0.7468				0.69
(St. Dev.)	(1.7159)	(1.5893)	(0.3582)	(0.2805)	(0.1194)				

na, Observed no. of alleles; ne, effective no. of alleles; I, Shannon's index; Ho, observed heterozygosity; He, expected heterozygosity.

Table 5: Values for each of microsatellite marker used in Mianwali Aseel

Marker	na	ne	I	Ho	He	Matching probability	Power of Discrimination	Power of Exclusion	PIC
ADL23	8	3.0209	1.4362	0.2632	0.6871	0.285	0.715	0.049	0.63
ADL102	6	4.0791	1.5415	0.5263	0.7752	0.169	0.831	0.266	0.74
ADL136	10	7.1485	2.0895	0.9474	0.8834	0.175	0.825	0.893	0.84
ADL158	7	3.1255	1.5070	0.3158	0.6984	0.319	0.681	0.070	0.65
ADL171	7	5.2319	1.7949	0.2105	0.8307	0.191	0.809	0.033	0.78
ADL176	6	5.1571	1.7084	0.2105	0.8279	0.169	0.831	0.033	0.78
MCW5	7	4.4845	1.7247	0.2105	0.7980	0.191	0.809	0.033	0.75
MCW7	6	4.9452	1.6944	0.7895	0.8193	0.252	0.748	0.580	0.77
MCW41	3	2.7557	1.0566	0.0000	0.6543	0.363	0.637		0.57
MCW59	5	3.6281	1.3875	0.6316	0.7440	0.263	0.737	0.331	0.68
Average values	6.5	4.3577	1.5941	0.4105	0.7718				0.71
(St. Dev.)	(1.841)	(1.3348)	(0.278)	(0.3006)	(0.0739)				

na, Observed no. of alleles; ne, effective no. of alleles; I, Shannon's index; Ho, observed heterozygosity; He, expected heterozygosity.

Table 6: Values for each of microsatellite marker used on Peshawari Aseel

Marker	na	ne	I	Ho	He	Matching probability	Power of Discrimination	Power of Exclusion	PIC
ADL23	5	2.1392	1.0656	0.1538	0.5538	0.420	0.580	0.018	0.5
ADL102	4	2.3151	0.9611	0.1538	0.5908	0.373	0.627	0.018	0.48
ADL136	7	3.3137	1.4807	0.6923	0.7262	0.337	0.663	0.416	0.66
ADL158	6	2.7705	1.2968	0.3846	0.6646	0.361	0.639	0.105	0.6
ADL171	7	5.5410	1.8316	0.3846	0.8523	0.207	0.793	0.105	0.8
ADL176	3	2.7705	1.0579	0.0000	0.6646	0.361	0.639		0.57
MCW5	7	4.3333	1.6696	0.4615	0.8000	0.183	0.817	0.156	0.74
MCW7	6	5.2000	1.7141	0.6154	0.8400	0.160	0.840	0.543	0.83
MCW41	4	3.3137	1.2659	0.0000	0.7262	0.302	0.698		0.64
MCW59	4	3.8851	1.3714	0.8462	0.7723	0.373	0.627	0.687	0.69
Average values	5.3	3.5582	1.3715	0.3692	0.7191				0.65
(St. Dev.)	(1.494)	(1.1669)	(0.2994)	(0.2919)	(0.1009)				

na, Observed no. of alleles; ne, effective no. of alleles; I, Shannon's index; Ho, observed heterozygosity; He, expected heterozygosity.

**Fig. 1:** Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5**Table 7:** Genetic diversity in all four varieties of Aseel

Strain	markers	na	ne	Ho	He	I
Lakha	10	5.6	3.883	0.3941	0.7376	1.4421
Mushki	10	6.5	4.2242	0.4105	0.7468	1.5389
Mianwali	10	6.5	4.3577	0.4105	0.7718	1.5941
Peshawari	10	5.3	3.5582	0.3692	0.7191	1.3715

na, Observed no. of alleles; ne, effective no. of alleles; Ho, observed heterozygosity; He, expected heterozygosity; I, Shannon's index.

Table 8: Nei's (1978) genetic identity and distance among different varieties of Aseel

	Lakha	Mushki	Mianwali	Peshawari
Lakha	-	0.7023	0.3835	0.5027
Mushki	0.3533	-	0.5233	0.3418
Mianwali	0.9585	0.6476	-	0.6793
Peshawari	0.6878	1.0735	0.3868	-

Above diagonal, Nei's (1978) genetic identity; below diagonal, genetic distance.

0.7468 for Mushki, 0.4105 and 0.7718 for Mianwali, 0.3692 and 0.7191 in Peshawari (Table 7). These results are indicative of relatively higher degree of heterozygosity in Pakistani Aseel varieties. Mianwali presented highest. Expected heterozygosity based genetic variability, followed by Mushki, Lakha and Peshawari which demonstrates low level of inbreeding in these populations.

Genetic distances: Pairwise Nei's genetic distances (Nei, 1978) was used to estimate evolutionary divergence among four varieties of Aseel chicken. Dendrogram of all four

varieties is shown in Fig 1. Highest genetic distance was observed between Mushki and Peshawari (1.0735), consequently yielding least genetic identity (0.3418). Lakha and Mushki on the other hand demonstrated least genetic distance value (0.3533) therefore showing highest genetic similarity (0.7023; Table 8). This information is important to devise effective breeding strategies for genetic improvement of these varieties depending upon the nature of market demand for higher growth rate, free range poultry meat, free range eggs and to breed them pure for taking advantage of heterosis in economic traits for between and within indigenous chicken populations of Pakistan.

Conclusions: The genetic diversity analysis of Aseel chicken varieties revealed their close genetic relationships and provided basic information for future detailed studies to preserve this important part of animal genetic resource of Pakistan. Microsatellite markers can effectively be used for genetic characterization of chicken.

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