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A PANEL OF MICROSATELLITE MARKERS FOR GENETIC DIVERSITY AND PARENTAGE ANALYSIS OF DOG BREEDS IN PAKISTAN

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ABSTRACT

A molecular genetics tool comprised of a panel of 15 microsatellite markers was developed and used to investigate parentage and breed characterization of two most kept breeds of dogs including German shepherd and Labrador retriever in Pakistan. Blood samples of 20 dog families (10 from each breed) were collected from Army dog Breeding Training Center and School, Rawalpindi, Pakistan and Kennel Club of Pakistan. Genomic DNA was extracted by standard inorganic protocol. Microsatellite markers with high Polymorphism Information Content (PIC) and He (Hetrozygosity) values were selected and optimized into four multiplexes. Amplification reactions were followed by genotyping in 7% non-denaturing polyacrylamide gel electrophoresis (PAGE). Parentage analysis of 20 families using this panel of microsatellite markers was 100% successful. Average values of Polymorphism Information Content (PIC), Heterozygosity (He) and Combined Power of Exclusion (CPE) combined for both of the breeds were found to be 0.724, 0.6345 and 0.9998 respectively. Moreover, deviation from Hardy-Weinberg equilibrium equation was observed moderately for both dog breeds. Allelic frequencies for majority of the microsatellite markers between both dog breeds were clearly distinct. This study demonstrated the panel of 15 microsatellite markers could effectively validate parentage and breed characterization in dogs.

Key words: Characterization, Parentage analysis, Microsatellite markers, Dog breeds, Pakistan.

INTRODUCTION

Dog is the most ancient and successfully domesticated mammal on the earth (Turnbell and Reed, 1974). Modern breeds of dog were domesticated from wolves (Vila *et al*., 1997). Almost 300 hundred breeds of dog exsit on the earth today. Dog is the most kept pet animal and no other mammal has enjoyed such a close relationship with human being (Parker *et al*., 2004). In Pakistan, German shepherd (GS) and Labrador retriever (LR) are major pet dog breeds. Most of the animals among populations of these breeds are kept without genetically tested pedigree records. Profiling based upon DNA testing to determine parentage, individual identity and breed characterization are becoming dire need due to huge involvement of these two breeds as pet animals in Pakistan now days.

However, owners of the pet dogs are conscious and require confirmation that their animals should be genetically purebred. No significant data is available to represent the population molecular genetics of GS and LR existing in Pakistan on the basis of which DNA testing can be done with greater reliability. Effect of different native breeding regimes on allelic frequencies of these populations is also a question mark and emphasizes the need to understand the present genetic structure of these breeds in Pakistan. Application of microsatellite markers as a most effective tool to investigate and establish the phylogenetic structures of the populations based upon the genetic pool of the populations. Breeding regimes of the being followed by the organisms and constituents of the populations has been validated times and again. Parameters of molecular genetics in the population structures, such as Polymorphism, Heterozygosity, Homozygosity, Gene flow, Allelic frequencies and Power of exclusion, are crucial to be determined for a set of microsatellite marker to be used to establish its reliability for breed characterization, DNA fingerprinting for parentage analysis and individual identity. These parameters detected much of variations within and among different breeds depending upon the microsatellite loci selected for such kind of studies.

Microsatellite markers, also known as Short Tandem Repeats (STRs), are specific motif (2-6 bp) based repetitive DNA sequences widely spreading throughout the whole genome (Hammond *et al.*, 1994). Many microsatellite markers have been reported can be used for DNA fingerprinting in dogs (Fredholm and Wintero, 1996, Ichikawa *et al.*, 2001, Irion *et al*., 2003, DeNise *et al*., 2004, Oishi *et al.*, 2005). A novel panel of 15 microsatellite markers consisting of fourteen di repeat and one tetra repeat markers was selected for analyzing genetic diversity and population structure of GS and LR

for the first time in Pakistan. The main aim of this study was to use this panel of highly polymorphic microsatellite markers on genetic samples of GS and LR populations in Pakistan to establish its validity for parentage analysis, individual identity and breed characterization.

MATERIALS AND METHODS

Sampling and DNA Extraction: Blood samples of 20 families (46 samples) of German shepherd and Labrador retriever breeds (10 families per breed) were taken from Army Dog Center, Rawalpindi and Lahore kennel club, Pakistan. Each family consisted of father, mother and their pup. Genomic DNA was extracted from blood samples following standard inorganic method (Sambrook and Russel, 2001). After DNA quantification, concentration of all the DNA samples was brought to same level i.e. 50 ng/µL.

Microsatellite markers: Microsatellite markers (REN04M22, REN02K21a, REN01E5a, REN41D20b, REN105L03, REN42M07, REN49F22b, REN162C04, REN45F03b, REN67C18a, REN47J11b, REN42N13b, REN47D17, FH2054 and AHTk211) having high PIC values were selected (Jouquand *et al.,* 2000; Ji *et al.,* 2007). Two microsatellite markers "FH2054 and AHTk211" were selected from ISAG recommended panel of microsatellite markers, while the rest of 13 markers were new to be used for parentage analysis and breed characterization. Specific primers for each marker were designed using software "Primer3" (URL: *frodo.wi.mit.edu*).

Amplification reactions: Primers of each microsatellite marker were optimized for successful amplification and were grouped into four multiplexes (Dayton *et al.,* 2009) according to their annealing temperature and product size specifications. Amplification reaction was carried out for all DNA samples with each microsatellite multiplex in thermocycler (BIO-RAD). Amplification reaction consisted volume of 25 µL containing DNA (50ng/µL), $MgCl_2$ (2.5 mM), amonium persulphate buffer (10x), dNTPs (25 mM), DNA Taq polymerase (05U, Fermentas, California, USA), forward and reverse primers (10 pM each) and double distilled water. PCR was carried out using initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 94° C for 1 min, annealing at the 52-55°C (as optimized per microsatellite multiplex) for 45 seconds and extension at 72° C for 1 min, followed by final extension at 72 °C for 10 min. Amplified products were incubated at 4°C.

Genotyping: PCR products were subjected to 7% non-denaturing Polyacrylamide Gel Electrophoresis for genotyping (Wang *et al.,* 2003). 3 µL PCR product along with 2 µL of loading dye was loaded in the gel and were run at 120 volts for 8 hours in PAGE unit of Major Science, model no. MV-20DSYS.

Statistical analysis: Allelic data was analyzed to calculate Polymorphic Information Content, Hetrozygosity, Power of exclusion, Hardy-Weinberg equation, Allelic frequencies, Fis, Fst, Fit and Gene flow values by using software "POPGEN 3.2 and POWER STAT".

RESULTS

Average number of effective alleles observed for both breeds was 5.23 per locus, with range of values from 1.4 (microsatellite REN49F22b) to 9.9 (microsatellite REN47J11b), having these values of 3.25 and 3.33 for GS and LR respectively. Wright's fixation index (Fis) averaged as -0.5864 for GS and -0.501867 for LR, showing excess of heterozygosity. Average of values of heterozygosity was found to be 0.6345 for both of the dog breeds with observed heterozygosity values as 0.7420 for GS while 0.6754 for LR population. PIC value, combined for both of the dog breeds, averaged as 0.724. The PIC values ranged from 0.17 (microsatellite REN49F22b) to 0.88 (microsatellite REN41D20b) with an average of 0.558 in GS, while it ranged from 0.36 (microsatellite AHTk211) to 0.76 (microsatellite REN45F03b) with an average of 0.614 in LR. Values of Power of exclusion ranged from 0.065 to 0.999 in GS and these were in range of 0.014 to 0.999 in LR. Combined Power of Exclusion (CPE) reached the value of 0.9998. Parentage analysis performed was 100% successful for all microsatellite markers in both populations.

A moderate trend of deviation from Hardy-Weinberg equilibrium equation was observed in both populations. Eight out of fifteen markers showed deviation from Hardy-Weingberg equilibrium equation in GS, while this trend was shown by seven markers in LR. Genetic distance between German shepherd and Labrador retriever was found to be 0.9775. Allelic frequencies of the majority of the microsatellite markers were having very distinct patterns of distribution between both tested dog breeds. Mean Fis value was observed as 0.1169; Fst value was found to be 0.1524 and gene flow value (Nm) was calculated as 1.39.

Table 1. Number of effective alleles, polymorphism information content, power of exclusion, Differentiation in subpopulations, Gene Flow and Average Heterozygosity of different loci.

Locus	Allele Range	German Shepherd								Labrador Retriever							
		Na	Ne	Na	Ne	Na	Ne	Na	Ne	Na	Ne	Na	Ne	Na	Ne	Na	Ne
REN04M22	7-8	4	2.44	0.5652	0.6029	0.52	0.251	0.33099	0.0417	6	4.50	0.5217	0.7952	0.75	0.207	0.05804	0.3293
REN02K21a	6-10	7	5.24	0.7826	0.8261	0.78	0.648	0.02282	-0.0210	3	1.98	0.5217	0.5053	0.41	0.207	1.00000	-0.0554
REN01E5a	13-16	6	3.75	0.8261	0.7498	0.69	0.648	0.40796	-0.1263	6	4.54	0.7391	0.7971	0.75	0.491	0.13441	0.0521
REN41D20b	17-26	12	8.82	1.0000	0.9063	0.88	0.912	0.03275	-0.1279	6	2.58	0.5217	0.6261	0.58	0.207	0.21176	0.1481
REN105L03	18-17	5	3.14	0.5652	0.6966	0.63	0.251	0.18057	0.1706	5	3.07	0.7391	0.6889	0.62	0.491	0.74526	-0.0968
REN42M07	16-13	4	2.19	0.6522	0.5556	0.48	0.358	0.05820	-0.2000	6	3.88	0.9130	0.7430	0.69	0.822	0.16803	-0.2306
FH2054	21-26	3	2.39	0.8696	0.5952	0.52	0.734	0.00481	-0.4935	4	3.56	0.9130	0.7353	0.67	0.822	0.55252	-0.2694
REN49F22b	7-6	2	1.24	0.2174	0.1981	0.17	0.035	1.00000	-0.1220	3	1.71	0.4347	0.4251	0.37	0.137	0.14352	-0.0455
REN162C04	7-11	4	2.80	0.5217	0.6570	0.64	0.472	0.29353	0.1882	3	2.87	0.3913	0.6657	0.58	0.109	0.00433	0.3991
AHTk211	10-7	4	2.77	0.6956	0.6531	0.57	0.422	0.76796	-0.0888	3	1.65	0.3043	0.4009	0.36	0.109	0.12984	0.2241
REN45F03b	66-59	4	2.71	1.0000	0.6454	0.57	0.999	0.00000	-0.5838	6	4.79	1.0000	0.8087	0.76	1.000	0.00000	-0.2640
REN67C18a	49-37	2	2.00	1.0000	0.5111	0.38	0.999	0.00000	-1.0000	5	3.98	0.9565	0.7652	0.73	0.912	0.00003	-0.2778
REN47J11b	33-26	7	5.48	1.0000	0.8356	0.80	0.999	0.00058	-0.2231	6	4.54	1.0000	0.7971	0.74	0.999	0.00033	-0.2824
REN42N13b	13-27	4	1.65	0.3913	0.4029	0.31	0.065	0.79190	0.0072	3	2.04	0.1739	0.5217	0.47	0.014	0.00031	0.6593
REN47D17	26-14	3	2.17	1.0000	0.5517	0.43	0.999	0.00000	-0.8529	6	4.20	1.0000	0.7787	0.73	0.999	0.00709	-0.3127
Mean		4.73	3.25	0.7420	0.7391	0.558			-0.5864	4.73	3.33	0.6754	0.6754	0.614			-0.5018

Ne = Number of effective alleles
PIC = Polymorphism Information Content
PE = Power of Exclusion
F_{st} = Differentiation in subpopulations
Na = Total number of alleles
Ho = Heterozygosity observed
He = Heterozygosity expected

Table: 2. (Above diagonal = Genetic Identity, Below diagonal = Genetic Distance)

Pop. ID	German shepherd	Labrador retriever
German shepherd	****	0.3763
Labrador retriever	0.9775	****

Table: 3 Summary of F-Statistics and Gene Flow for All Loci among two Populations

Locus	Fis	Fit	Fst	Nm
REN04M22	0.2053	0.3501	0.1823	1.1217
REN02K21a	-0.0341	0.1840	0.2109	0.9355
REN01E5a	-0.0344	0.0868	0.1172	1.8835
REN41D20b	-0.0151	0.1269	0.1399	1.5368
REN105L03	0.0377	0.1508	0.1175	1.8770
REN42M07	-0.2176	0.0472	0.2175	0.8995
FH2054	-0.3696	-0.0799	0.2116	0.9317
REN49F22b	-0.0698	-0.0095	0.0563	4.1883
REN162C04	0.2944	0.4456	0.2143	0.9163
AHTk211	0.0302	0.1560	0.1296	1.6785
REN45F03b	-0.4060	-0.1687	0.1687	1.2316
REN67C18a	-0.5670	-0.4350	0.0842	2.7181
REN47J11b	-0.2521	-0.1119	0.1119	1.9836
REN42N13b	0.3751	0.3876	0.0200	12.2692
REN47D17	-0.5367	-0.2709	0.1730	1.1953
Mean	-0.1169	0.0533	0.1524	1.3900

Nm= Gene Flow, Fst= Differentiation in subpopulations, Fis= Wright's Fixation Index (Measure of heterozygote excess or deficiency), Fit= Inbreeding coefficient of an individual

Table. 4 AMOVA Population Variation

Source of Variation	d.f	Sum of squares	Variance components	Percentage of variation
Among Populations	1	78.674	1.60464 Va	24.82
Within Populations	90	437.435	4.86039 Vb	75.18
Total	91			
Fixation Index (Fst)		0.24820		

DISCUSSION

A panel comprising of 15 microsatellite markers, grouped into four multiplexes, was used successfully to investigate and validate the characterization and parentage confirmation of individuals of two dog breeds including German shepherd and Labrador retriever breeds of dogs in Pakistan. Analyzed data showed mean PIC value combined for both of the breeds as 0.724. This value showed the better worth of this panel of microsatellite markers as compared to DeNise *et al.,* (2004), who worked on two panels of microsatellite markers revealing PIC values of 0.61 and 0.53.

Results of present study showed excess of heterozygosity for this panel of microsatellite markers with a value of 0.6354, which corresponds to the value of heterozygosity "0.618" found by Irion *et al.,* 2003. Combined power of exclusion calculated on the basis of allelic data of both of the breeds was found as 0.9998 using 15 microsatellite markers in the present study. This CPE value is same as the value 0.999, reported by Ji *et al*., (2007), who conducted validation studies on panel of 22 microsatellite markers for parentage analysis.

According to results of this panel of microsatellite markers, genetic distance between German shepherd and Labrador retriever was 0.9775 (Table 2) which is much better value to conclude genetic distance between these two breeds as compared to Zajic and Simpson 1999, who worked on characterization of German shepherd, Labrador retriever breeds of dogs and found genetic distance of "0.273". This difference of values may have been aroused due to difference in structures of gene pools and sample size from both dog populations, and panel of microsatellite markers used. Value of subpopulation differentiation "Fst" was found to be 0.1524 (Table 3) which corresponds to the value of

0.154, reported by Kim *et al.*, (2001), who worked on genetic structures of the Asian dog population. Different Studies on microsatellites, mitochondrial and Y-chromosomal DNA diversity were conducted world widely to designate the origination of dogs (Pang *et al*., 2009; Ardalan *et al*., 2011; Ding *et al*., 2012; Erdogan *et al*., 2013). Genetic variations regarding dog origination, domestication and early evolutionary history were also reported (Brown *et al*., 2011; Freedman *et al*., 2014).

A moderate number of the microsatellite markers deviated from Hardy-Weinberg equilibrium equation ($P < 0.05$) which showed that there was moderate stress of forces such as selection, suppression and migration involved in establishment of these breeds as according to Savolainen *et al.*, (2002), in the populations of this region. Obeying of rest of the markers to the Hardy-Weingberg equation showed the freedom of breeding patterns in these populations. Both of the dog breed populations showed to comprise a very distinct structure of gene pool as value of Gene flow (Nm) for this panel of microsatellite markers was found to be 1.39 which is a non-significant value in order to establish common sharing of the genetic pool between both breeds of dogs investigated.

Conclusion: Only 2 of the microsatellite markers (AHTk211 and FH2054) were taken from ISAG recommended panel of microsatellite markers. Rest of the microsatellite markers were used for parentage analysis and breed characterization in dogs for the first time. Issues of determination of parameters of molecular genetics involved in investigation of parentage, individual identity and breed characterization of dogs were successfully subjected in this study. These results indicated these genetic parameters from this panel of 15 microsatellite markers developed in present study was found to be very effective, efficient and valid for the said purposes.

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