A PANEL OF MICROSATELITE 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 (Heterozygosity) 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 exist 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), MgCl₂ (2.5 mM), ammonium 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 L PCR product along with 2 L 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, Heterozygosity, 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-Weinberg 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.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele Range</th>
<th>German Shepherd</th>
<th>Labrador Retriever</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na</td>
<td>Ne</td>
<td>Na</td>
</tr>
<tr>
<td>REN04M22</td>
<td>7-8</td>
<td>4</td>
<td>2.44</td>
</tr>
<tr>
<td>REN02K21a</td>
<td>6-10</td>
<td>7</td>
<td>5.24</td>
</tr>
<tr>
<td>REN01E5a</td>
<td>13-16</td>
<td>6</td>
<td>3.75</td>
</tr>
<tr>
<td>REN41D20b</td>
<td>17-26</td>
<td>12</td>
<td>8.82</td>
</tr>
<tr>
<td>REN105L03</td>
<td>18-17</td>
<td>5</td>
<td>3.14</td>
</tr>
<tr>
<td>REN42M07</td>
<td>16-13</td>
<td>4</td>
<td>2.19</td>
</tr>
<tr>
<td>FH2054</td>
<td>21-26</td>
<td>3</td>
<td>2.39</td>
</tr>
<tr>
<td>REN49F22b</td>
<td>7-6</td>
<td>2</td>
<td>1.24</td>
</tr>
<tr>
<td>REN162C04</td>
<td>7-11</td>
<td>4</td>
<td>2.80</td>
</tr>
<tr>
<td>AHTk211</td>
<td>10-7</td>
<td>4</td>
<td>2.77</td>
</tr>
<tr>
<td>REN45F03b</td>
<td>66-59</td>
<td>4</td>
<td>2.71</td>
</tr>
<tr>
<td>REN67C18a</td>
<td>49-37</td>
<td>2</td>
<td>2.00</td>
</tr>
<tr>
<td>REN47J11b</td>
<td>33-26</td>
<td>7</td>
<td>5.48</td>
</tr>
<tr>
<td>REN42N13b</td>
<td>13-27</td>
<td>4</td>
<td>1.65</td>
</tr>
<tr>
<td>REN47D17</td>
<td>26-14</td>
<td>3</td>
<td>2.17</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>4.73</td>
<td>3.25</td>
<td>0.7420</td>
</tr>
</tbody>
</table>

Ne = Number of effective alleles  
PIC = Polymorphism Information Content  
PE = Power of Exclusion  
Fst = Differentiation in subpopulations  
Na = Total number of alleles  
Ho = Heterozygosity observed  
He = Heterozygosity expected
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-Weinberg 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.

**REFERENCES**

Ardalan, A., C.F.C. Kluetsch, Ai-bing Zhang, M.
Erdogan, Uhlén, M. Houshmand, C. Tepeli, S.
Comprehensive study of mtDNA among
Southwest Asian dogs contradicts independent
domestication of wolf, but implies dog–wolf
hybridization. Ecology and Evolution 3: 373–
385.

Bannasch, K.D. Ahrens, J.T. Wu, M. Okon,
B.N. Sacks (2011) Phylogenetic Distinctiveness of
Middle Eastern and Southeast Asian Village
Dog Y Chromosomes Illuminates Dog Origins.
PLoS ONE 6(12): e28496. doi:10.1371/journal.
pone.0028496

Developmental validation of short tandem repeat
reagent kit for forensic DNA profiling of canine

DeNise, S., E. Johnston, J. Halverson, K. Marshall, D.
Rosenfeld, S. McKenna, T. Sharp and J.
parentage verification and probability of match
for identity in American kennel club breeds
using 17 canine microsatellite markers. Anim.
Genet. 35:14-17.

Ding, Z.L., M. Oskarsson, A. Ardalan, H. Angleby, L.G.
Dahlgren, C. Tepeli, E. Kirkeness, P. Savolainen
in Southern East Asia is supported by analysis of

Erdogan, M., C. Tepeli, B. Brengi, M. D. Akbulut, C.
Uğuz, P. Savolainen, C. Öz beyaz (2013).
Genetic variability among native dog breeds in

resolution of parentage in dogs by amplification

Freedman, A.H., Gronau I., Schweizer R.M., Ortega-Del
Vecchyo D., Han E. et al. (2014). Genome
Sequencing Highlights the Dynamic Early
doi:10.1371/journal.pgen.1004016

tandem repeat loci for use in personal

parentage testing based on microsatellite

Eggleston, S. S. Hughes and N. C. Pedersen
(2003). Analysis of Genetic Variation in 28 Dog
Breed Populations with 100 Microsatellite

dogs parentage testing by using 22 ISAG
microsatellite markers. Korean J. Vet. Res. 47

and characterization of a set of 100 tri- and
dinucleotide microsatellites in the canine

Genetic variability in East Asian dogs using
microsatellite loci analysis. J. Hered. 92: 398-
403.

Pang, J.F., C. Kluetsch, X.J. Zou, A. Zhang, L.Y. Luo, H.
Angleby, A. Ardalan, C. Ekström, A. Sköl len mo,


