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Microsatellite DNA Analysis of Genetic Diversity and Parentage Testing in Popular Dog Breeds in India

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ABSTRACT

Background: Microsatellite DNA sequencing has emerged as an important method for determining genetic variety and parentage in domesticated species, including popular dog breeds. The role of microsatellite markers helps in understanding the genetic landscape of dog breeds in India, where the growing popularity of particular breeds has generated worries about inbreeding and loss of genetic variety.

Methods: For the parentage testing in canine microsatellite length, polymorphism markers were used to check the efficacy of the markers. In the current study 5 fluorescently labeled 12 SSR markers were used to check the use of the markers in popular owned-dog breeds (Labrador, German Shepherd, Pug, Mudhol Hound, Tibetan Mastiff, Gaddi dog, Beagle, Belgian Malinois, Pointer, and Cane Corso). The number of alleles, heterozygosity, polymorphism information content and probability of exclusion were determined for all the markers to check the effectiveness of the markers.

Result: The analysis utilized a panel of microsatellite markers to evaluate genetic variation among several breeds including Punjab, Haryana, Himachal Pradesh and Karnataka. The mean number of alleles per locus ranged from 5 to 29 and the effective number ranged from 3.6 to 15.2. The expected heterozygosity was greater than 0.73. The population inbreeding coefficient (FIS) demonstrated that there was no inbreeding in the breeds studied. The polymorphism information content and the probability of the exclusion values were greater than 0.65. The combined probability of exclusion for all the breeds was (2.82E-12) 0.99999995. The findings revealed that the 12 particular microsatellite markers chosen for paternity testing demonstrated substantial exclusion probability for determining parentage.

Key words: Canine, Microsatellite, Parentage determination, Probability of exclusion.

INTRODUCTION

Dogs have coexisted with humans for thousands of years and have been used as guard animals to herd livestock, hunt and protect homes, as well as companion animals (Pedersen *et al.,* 2015; Wayne and von Holdt, 2012; Larson *et al.,* 2012). *Canis lupus*, the wolf, is estimated to have given rise to dogs roughly 100,000 years ago. Since prehistoric times, the dog species has evolved through stringent selection (for desirable traits) which has ultimately led to the evolution of more than 350 distinct breeds of dogs worldwide. Scientific breeding of dogs is now a popular practice to entice dog owners and buyers by stamping on desirable traits of purebred dogs (Haq *et al.,* 2024). This although increases the price, also necessitates molecular testing of parentage to verify the claim of parentage of the animals.

Molecular markers can identify the degree of genetic relatedness between animals, making parentage and individual identification easier. Microsatellites are tracts composed of short tandem repeats (STRs) or simple sequence repeats (SSRs) of DNA patterns ranging between one to six nucleotides, with repeats of 5 to 50 times (Vieira *et al.,* 2016). Repeat sequences are distributed ubiquitously in the genome, highly variable and have been demonstrated to be effective tools in genome mapping (Oudet *et al.,* 1991). Microsatellites have been effectively used to determine the molecular signatures or DNA fingerprints of individuals (humans and animals), to determine parentage, to build pedigree, to select animals through marker-assisted selection for genetic improvement through selective breeding, *etc.* The use of microsatellites as molecular markers for animal identification and parentage verification generated highly accurate and effective results (Linacre *et al.,* 2011).

Identification of breed-specific molecular signatures benefits dog owners and breeders and helps characterize the dog germplasm maintained in India (both foreign and

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indigenous) (Singh 2022; Raja *et al.,* 2017). Parentage determination using microsatellite and SNP marker panels (Kalbfleisch *et al.,* 2018; Heaton *et al.,* 2014; Yu *et al.,* 2015; Flanagan and Jones, 2019) have been reported for different species. Relevant literature reports the associated prospects and challenges with parentage determination in humans and animals (Stark and Delatycki 2014; Chan *et al.,* 2014; Goswami, 2015). Minimal works have reported on applications of SSR markers for parentage determination in dogs (Kaur *et al.,* 2025; Hollinshead *et al.,* 2020), especially in India (Sowmyashree *et al.,* 2022). The present research has been designed to investigate the informative microsatellite markers for parentage testing in canines. A Ph.D. thesis has been submitted from our lab on parentage determination in cattle and buffalo using microsatellites as well as SNP markers (Singh 2021) and relevant literature was published and presented (Rana *et al.,* 2025; Singh 2022; Mukhopadhyay and Singh 2021). This work aims to create and standardize a set of SSR primers to validate and verify parentages in dogs using the most popular dog breeds maintained in India.

MATERIALS AND METHODS

Experimental animal selection and DNA extraction

The experimental animals were selected based on trio and duo relationship to assess the informativeness of the markers for parentage determination, belonging to ten divergent germplasm, namely, (Labrador (Abbrevi*a*ted as Lab), German Shepherd (GS), Pug, Mudhol Hound (MH), Tibetan Mastiff (TMS), Beagle, Belgian Malinois (BM), Pointer and Cane Corso (CC)) breeds and Gaddi dogs. The animals were available from dog owners and breeders belonging to four Indian states: Punjab, Himachal Pradesh, Haryana and Maharastra (Table 1). Two ml of peripheral blood was collected aseptically with an anticoagulant (0.5 M EDTA). Genomic DNA was extracted using the commercially available kit and Phenol:Chloroform: Isoamyl alcohol (PCI) method (with modification of Sambrook and Russel 2001). Samples collected from distant places were stored at -20° and transported to the lab maintaining a cold chain. The quality and quantity of the extracted DNA were then measured with a NanoDrop (Thermo Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively.

SSR-marker selection

Initially, 15 microsatellite markers (5′ fluorescent labeled with FAM, HEX, or TAMRA) (Table 2) were selected based on the higher polymorphism information content (PIC) and observed heterozygosity (He) from reported primers (Yuzbasiyan-Gurkan *et al.,* 1997; Kukekova *et al.,* 2004). The primers were custom synthesized and the SSR length

Table 1: Family orientation (Sire/Dam/Offspring) and breed detail of the experimental animals.

Sire	Dam	Offspring	Trio-Id	Duo-Id	Breed
1	2	3	T1	--	Labrador
7	8	9	T2	--	Labrador
16	17	18	T3	--	German shepherd
25	26	27	T4	--	German shepherd
40	41	42	T5	--	Pug
47	48	46	T6	--	Mudhol hound
50	51	49	T7	--	Mudhol hound
54	53	52	T8	--	Mudhol Hound
57	56	55	T9	--	Mudhol hound
60	59	58	T10	--	Mudhol hound
64	65	66	T11	--	Tibetan mastiff
67	68	69	T12	--	Gaddi
67	68	70	T13	--	Gaddi
67	68	71	T14	--	Gaddi
82	83	84	T15	--	Belgian malinois
93	94	95	T16	--	Cane corso
13	NA	15	--	D1	Pug
22	NA	24	--	D2	German shepherd
31	NA	33	--	D3	Pug
NA	85	86	--	D5	Beagle
NA	87	88	--	D6	Pointer
NA	87	89	--	D7	Pointer
61	62	NA	--	D4	Gaddi*

* For parentage assignment testing.

Table 2: Detail of the 5' labeled simple sequence repeat primers.

Loci	Forward_Primer (5' to 3') sequence (and length)	Reverese_Primer (5' to 3') sequence (and length)	Allele_size	TM (°C)	Dye
NPPM10	GTGGACCATGTGACTCTTGA (20)	TTTGTGTGATGCCACTACAGTAAG (24)	176-182	58	6-FAM
NPPM244	GTCACTTAATAGGATGATTTCTTGG (25)	CTAAAACCTGGATTGTCTAATTTGT (25)	315-338	58	6-FAM
NPPM30	AGGACTATTTCACGCCTTGTTG (22)	ATTCCCACCTCAGTGATTACAG (22)	276-286	58	HEX
NPPM769	TGGTAGCCACAGAAGCATTG (20)	TTGGATTAAGTGTGTAGTCCTGAGC (25)	218-238	58	TAMRA
NPPM855*	TGAGTTTTTGGTCCCCTCCA (20)	CTCTGGTCCAGCAGTTGAAAC (21)	226-238	58	TAMRA
NPPM858	CAGTTTGCTACCTTTTGTGTAATCA (25)	CTCACCCATTGTAGTCTCTGTCTTC (25)	187-204	58	HEX
NPPM905	TCCAGAGTCACAACTTCAGAAAC (23)	GCTAGATTGCTGCCCTTTACTC (22)	201-221	58	HEX
NPPM930	TCTTTACCCTTCTGGAAAATGAG (23)	GTGATTGAACACGCAAGGGAT (21)	247-262	58	TAMRA
NPPM981*	GAACATCTTCCTTCTTCCACTG (22)	TCCTAGAGACCTGGGATGAAGT (22)	318-328	58	HEX
PEZ11	ATTCTCTGCCTCTCCCTTTG (20)	TGTGGATAATCTCTTCTGTC (20)	121-173	55	6-FAM
PEZ12	GTAGATTAGATCTCAGGCAG (20)	TAGGTCCTGGTAGGGTGTGG (20)	266-313	58	TAMRA
PEZ16	GCTCTTTGTAAAATGACCTG (20)	GTGGGAATCGTCCTAAAACCC (21)	281-317	58	6-FAM
PEZ17	CTAAGGGACTGAACTTCTCC (20)	GTGGAACCTGCTTAAGATTC (20)	199-227	58	HEX
PEZ22*	TGGGGAGATCTACAGACCAC (20)	CTAATGTGTCTCTCAAGCCG (20)	171-189	55	6-FAM
UOR4107	TGACCCTTCTACAACTCGGG (20)	TGTGACCAGTCACTGCTTCC (20)	220-232	58	TARMA

*Results were obtained for 12 primer pairs.

polymorphism was done from Biologia Research India. Pvt. Ltd, Karnal, India.

Analysis of SSR-length polymorphism results

The genotypic data were first manually checked for inconsistencies using Microsoft Office Excel 2007. The Peak Scanner™ Software v1.0 and GeneMapper® Software were used to perform the analysis of *.fsa files. The Windows OS-based stand-alone Peak Scanner™ Software (v1.0) (https://peak-scanner-software.software.informer.com/1.0/) was used to accurately identify the correct peaks and fragment sizes vis-s-*via* functional annotation (*viz.* labeling, merging and splitting) of peaks and further the peak data was feed in Microsoft Excel for the genetic analysis parameters. The descriptive statistics based on genotyping data were obtained using the Genetic Analysis in Excel (GenAlEx) tool v. 6.5 (Peakall and Smouse, 2012). The number of alleles per locus (Na), the effective number of alleles (Ne) and the fixation index (F) expected homozygosity and heterozygosity (Levene 1949) and expected heterozygosity (Nei 1973).

The Hardy-weinberg equilibrium test was carried out with the help of the POPGENE computer program (Raymond and Rousset, 1995), which was used to estimate F-statistics (the global mean inbreeding coefficient [FIT], the average inbreeding coefficient of an individual concerning the local subpopulation [FIS] and the average inbreeding coefficient of subpopulations relative to the total population [FST]) for each locus, the pairwise FST Allelic occurrence, Genic Variation Statistics for All Locations Molecular Evolutionary Genetics, Summary of Heterozygosity Statistics. The exclusion probability (Jamieson and Taylor 1997) and the polymorphism information content (Botstein *et al.,* 1980) were calculated by using PARFEX v1.0 EXCEL™ tool and Cervus 3.0.7 software (https://cervus.software.informer.com/download/). The probability of exclusion or power of exclusion (PE) is a priori statistic that determines the likelihood for a sample to be representative of a population (Zhou *et al.,* 2017).

The genetic parameters were obtained using the following formula:

Polymorphism informative content (PIC) for co-dominant markers =

$$PIC_i = 1 - \sum P^2i - (\sum P^2i)\ 2 + \sum P^4i$$

Where,

n = Number of alleles.

pi and pj = Allele frequencies in population i and j respectively (Botstein *et al.,* 1980).

Heterozygosity (He) = H = $1 - \sum h = \sum 2pq$

Where,

h = Homozygosity.

p and q = Frequencies of two alleles of a locus.

$$\text{Homozygosity (Ho)} = h = \sum i\ (pi)^2$$

Where,
pi = Frequency of i^{th} allele of a locus.

Probability of exclusion (PE) = PE = h^2 (1-2hH^2)

Where,
h = Frequency of heterozygotes.
H = Frequency of homozygotes.

Likelihood ratio for parentage assignment =

$$L(H_1,H_2)|= \frac{P(D|H_1)}{P(D|H_2)}$$

Where,
H_1 = The first hypothesis stating the agreement that the candidate parental pair is the true parental pair.
H_2 = Hypothesis stating that the alternative candidate parental pair is the true parental pair.
D = Data in the form of offspring and parental genotypes.

RESULTS AND DISCUSSION

Genetic diversity of microsatellites

Out of 15 SSR markers used 12 markers were amplified for the samples under study. The results obtained have been presented based on the output of these 12 markers. Table 3 shows the sample size, observed number of alleles, effective number of alleles and microsatellite loci of the experimental samples. The number of alleles per SSR locus (Na) ranged from 5 (NPPM10) to 29 (PEZ12), with a mean of 15.4167 (±8.2402 s.e.). The number of effective alleles per locus (Ne) varied from 3.6140 (NPPM10) to 15.2178 (PEZ16), with a mean value of 7.9664 (±4.2066 s.e.). The mean value of Shannon's Information index (I) was 2.1804 (± 0.5581 s.e.).

Measures of heterozygosity statistics

The average expected heterozygosity (Ave_Exp_Het) across all loci was 0.85 (Table 4). The observed heterozygosity (Obs_Het) average was 0.80. The expected heterozygosity was found to be relatively high for all the markers as all the markers have heterozygosity of more than 0.5. All 12 markers were found to be highly polymorphic and can be used for the genetic studies of the dogs.

Therefore, the Mean (± SEM) observed heterozygosity, averaged over loci, was 0.8020 ± 0.1345, which was lower than the expected heterozygosity.

The population inbreeding coefficient (F_{IS}) ranged from -0.1400 (NPPM10) to 0.2274 (NPPM930). The Fis value was positive in a few markers, indicating the in-breeding of the population. The F_{ST} values of all the loci was 0.0000 which indicated there was no genetic subdivision. The genetic variation existed within dogs (Table 5).

Table 3: Genetic parameters of the 12 microsatellite loci obtained from dog populations.

Locus	Sample size	na*	ne*	I*
NPPM30	114	9	5.61	1.86
NPPM769	114	20	13.59	2.76
PEZII	114	25	13.74	2.85
NPPM905	114	10	4.25	1.69
NPPM930	114	13	7.29	2.16
NPPM10	114	5	3.61	1.36
PEZ12	114	29	10.36	2.80
PEZ17	114	10	5.51	1.88
PEZ16	114	28	15.22	3.02
UOR4107	104	11	4.26	1.73
NPPM244	114	17	7.75	2.34
NPPM858	114	8	4.40	1.71
Mean	113	15.42	7.97	2.18
St. Dev		8.24	4.21	0.56

Note: Na = Number of alleles; Ne = Number of effective alleles; I = Shannon's Information index.

Table 4: Measures of genetic variation (heterozygosity statistics for all loci) in dog population.

Locus	Sample size	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Avg_Het
NPPM30	114	0.12	0.88	0.17	0.83	0.82	0.82
NPPM769	114	0.04	0.96	0.07	0.93	0.93	0.93
PEZII	114	0.00	1.00	0.06	0.94	0.93	0.93
NPPM905	114	0.26	0.74	0.23	0.77	0.76	0.76
NPPM930	114	0.33	0.67	0.13	0.87	0.86	0.86
NPPM10	114	0.18	0.82	0.27	0.73	0.72	0.72
PEZ12	114	0.04	0.96	0.09	0.91	0.90	0.90
PEZ17	114	0.23	0.77	0.17	0.83	0.82	0.82
PEZ16	114	0.25	0.75	0.06	0.94	0.93	0.93
UOR4107	104	0.29	0.71	0.23	0.77	0.77	0.77
NPPM244	114	0.46	0.54	0.12	0.88	0.87	0.87
NPPM858	114	0.19	0.81	0.22	0.78	0.77	0.77
Mean	113	0.20	0.80	0.15	0.85	0.84	0.84
St. Dev		0.13	0.13	0.07	0.07	0.07	0.07

Note: Obs_Hom = Observed homozygosity; Exp_Hom = Expected homozygosity; Obs_Het = Observed heterozygosity; Exp_Het = Expected heterozygosity.

Table 5: Summary of the F-statistics and gene flow among dog populations.

Locus	Sample size	Fis	Fit	Fst	Nm*
NPPM769	114	-0.042	-0.042	0.000	****
PEZll	114	-0.079	-0.079	0.000	****
NPPM905	114	0.036	0.036	0.000	****
NPPM930	114	0.227	0.227	0.000	****
NPPMlO	114	-0.140	-0.140	0.000	****
PEZ12	114	-0.068	-0.068	0.000	****
PEZ17	114	0.057	0.057	0.000	****
PEZ16	114	0.193	0.193	0.000	****
UOR4107	104	0.070	0.070	0.000	****
NPPM244	114	0.376	0.376	0.000	****
NPPM858	114	-0.044	-0.044	0.000	****
Mean	113	0.046	0.050	0.000	1000

Nm = Gene flow estimated from F_{ST} = 0.25 (1 - F_{ST})/F_{ST}.

Table 6: Summary of the chi-square and hardy weinberg test.

Locus	DF	Chi-Sq	Probability	Significance
NPPM30	36	142.027	0.000	***
NPPM769	190	324.874	0.000	***
PEZ11	300	368.802	0.004	***
NPPM905	45	162.047	0.000	***
NPPM930	78	165.012	0.000	***
NPPM10	10	29.441	0.001	***
PEZ12	406	399.108	0.587	ns
PEZ17	45	60.104	0.065	ns
PEZ16	378	565.516	0.000	***
UOR4107	55	80.343	0.015	*
NPPM244	136	274.682	0.000	***
NPPM858	28	34.536	0.184	ns

ns= Not significant; * P<0.05, ** P<0.01, *** P<0.001.

Hardy-weinberg test

The results of HWE tests of the 12 microsatellite loci indicated UOR4107 shows significant differences (P>0.05) and NPPM30, NPPM769, NPPM905, NPPM930, PEZ16, NPPM244 are statistically significant (P>0.001) (Table 6). The de*via*tion from the Hardy Weinberg can be due to the non-random mating or due to some evolutionary processes. The Ewens-Watterson test of neutrality differentiates between the observed and expected hemizygosities in a sample under a neutral model (Table 7). The mean values, corresponding standard errors of the means, lower and upper limits at 95% level of confidence have been adumbrated for each SSR markers.

The observed F for the markers lies between the upper and the lower limit of 95% which depicted those markers were not under any selection pressure or associated with any of the quantitative traits. Thus, these markers can be used for the parentage identification in dogs.

Polymorphism information content and probability of exclusion

Polymorphism information content (PIC) and the probability of exclusion are indeed important measures in genetic research of microsatellites. PIC and probability of exclusion were used to assess the informativeness of a genetic marker. High PIC values suggest that a marker is highly informative and can discriminate well between alleles, making it useful for various applications such as genetic diversity and parentage studies (Serrote *et al.,* 2020). The use of quantitative genotypes for statistical assignment of parentage has been discussed by Hamilton (2021). Parentage assignment using genotyping by sequencing data has been recently reported by Whalen *et al.* (2019) All the markers in the study were highly polymorphic, as all had a PIC value of more than 0.673. Probability of exclusion represents the marker's average capability to eliminate one parent when the genotype of that parent is unknown, to confirm the parent's contribution to the offspring's genotype when the offspring's genotype is either known or unknown, or to exclude both potential parent pairs when determining offspring parentage. The exclusion probability (Table 8) for all the markers values greater than 0.658 which depicts that all the markers were highly informative which can help to achieve the 99.9% success rate for the parentage studies as the combined exclusion probability (CPE) values of 0.99999995.

A total of 12 microsatellite loci were found and analyzed after being combined into four multiplex PCR reaction systems and genotyped in two multiplex loading systems. Because of the high variability of these microsatellite loci, very precise genotyping panels could be utilized for individual genotyping, parentage verification and individual identification. The total diversity structure was found to be quite strong and it corresponded with the use of the varieties and the breeding program's tactics based on parental group pairings. All of these findings highlight the significance

Table 7: Ewens-Watterson Test for Neutrality for the simple sequence repeat markers.

Locus	n	k	Obs.F	Min F	Max F	Mean	SE	L95	U95
NPPM30	114	9	0.178	0.111	0.870	0.310	0.013	0.169	0.617
NPPM769	114	20	0.074	0.050	0.722	0.131	0.002	0.082	0.237
PEZ11	114	25	0.073	0.040	0.668	0.100	0.001	0.066	0.168
NPPM905	114	10	0.236	0.100	0.855	0.279	0.010	0.156	0.541
NPPM930	114	13	0.137	0.077	0.812	0.215	0.006	0.127	0.422
NPPM10	114	5	0.277	0.200	0.932	0.507	0.028	0.265	0.866
PEZ12	114	29	0.097	0.035	0.629	0.082	0.001	0.056	0.140
PEZ17	114	10	0.181	0.100	0.855	0.277	0.009	0.161	0.519
PEZ16	114	28	0.066	0.036	0.639	0.086	0.001	0.058	0.145
UOR4107	104	11	0.235	0.091	0.826	0.251	0.007	0.143	0.471
NPPM244	114	17	0.129	0.059	0.759	0.157	0.003	0.097	0.291
NPPM858	114	8	0.227	0.125	0.885	0.346	0.014	0.188	0.648

Table 8: Polymorphism information content (PIC) and probability of exclusion (PE) of the markers.

Marker	Polymorphism information content	Exclusion probability
PEZ16	0.932	0.972
NPPM769	0.926	0.966
PEZ11	0.916	0.959
PEZ12	0.893	0.944
NPPM930	0.854	0.895
NPPM244	0.831	0.872
PEZ17	0.811	0.841
NPPM30	0.797	0.824
NPPM858	0.765	0.794
UOR4107	0.754	0.777
NPPM905	0.753	0.782
NPPM10	0.673	0.658

and necessity of maintaining these genotypes in germplasm repositories.

CONCLUSION

In conclusion, the results of analyzing the dog populations in India using 12 new microsatellite markers revealed their average anticipated heterozygosity and observation heterozygosity. As a result, these microsatellite markers are highly applicable to the populations studied. These findings suggest that the microsatellite markers have acceptable resolution when used to detect variations between dog breeds. Furthermore, power exclusion will be employed as a strong tool for paternity testing.

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Future perspectives

In the future, the microsatellites identified in this work could be used to assess dog population structure, history and diversity, hence assisting in the genetic improvement of Indian dog breeds. To overcome outstanding identifying issues, more phenotypic and passport data checks are required.

Author contributions

Ygeshwar Sandhu: Did the lab work and sample collection; Bhawanpreet Kaur: manuscript writing; Manpreet Kaur: Data analysis; Yatish H.M.: Sample collection from Karnataka; C.S. Mukhopadhyay:PI of the project, guidance, proofreading.

All authors contributed to the manuscript revision and read and approved the present version.

Ethics approval and consent to participate

Permission from the Institutional Animal Ethics Committee (IAEC) was obtained (IAEC/2020/200-219, 14.12.2020).

Consent for publication

Obtained from the Office of the Director of Research, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

Research involving human participants and/or animals

Animals with IAEC Permission from the Institutional Animal Ethics Committee (IAEC) was obtained (IAEC/2020/200-219, 14.12.2020).

Informed consent

NA.

Conflict of interest

The authors declare no conflict of interest.

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