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Genetic Diversity and Phylogenetic Relationship Analysis of two Rabbit Breeds by Microsatellite Markers

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ABSTRACT

Soviet Chinchilla (SC) and Californian White (CW) breeds of rabbits maintained in the Rabbit research Centre, college of Veterinary Science, Rajendranagar, Hyderabad were utilized for the present study. Rabbit specific microsatellite primers utilized were successfully amplified in both the breeds by PCR and these primers are highly polymorphic and informative with number of alleles ranged from 4 to 11 in SC and 6 to 10 in CW. A total of 199 alleles (102 in SC and 97 in CW) were observed across the amplified loci. The overall mean of observed, expected and unbiased expected heterozygosity values were 0.681, 0.842 and 0.872 in SC and 0.665, 0.849 and 0.880 in CW, respectively. The mean inbreeding coefficient was 0.194 in SC and 0.221 in CW. Only 1 locus showed negative inbreeding coefficient in both the breeds. The mean PIC was 0.822 and 0.831 in SC and CW breeds respectively. The overall mean F_{IS}, F_{IT} and F_{ST} values were 0.208, 0.238 and 0.040 respectively. At two loci SAT07 and SAT16 moderate and high degree of differentiation was observed between the two breeds and all the remaining 10 loci have the F_{ST} values less than 0.05 suggesting that differentiation did not exist between the two breeds at these loci. The sufficiently high mean values of observed number of alleles, observed heterozygosity and PIC for various microsatellites in the present study supported their suitability for genetic diversity and phylogenetic relationship studies.

Keywords: Soviet Chinchilla, Californian White, Microsatellite primers, Heterozygosity, PIC

Molecular markers revealing the polymorphism at the DNA level are now key players in Animal Genetics. They serve as a useful tool to explore and understand the genetic architecture of the individuals. Recent advances in molecular techniques for genetic characterization of livestock involving molecular markers such as RFLP, microsatellites and SNPs have made it easy to explore the Genetics of livestock. Microsatellites are tandem repeats of short DNA sequences with high polymorphism and co-dominant in inheritance. In the recent past, microsatellites have become more popular in investigations including their extensive use in the construction of genetic maps (Knapik *et al*., 1998; Cregan *et al.,* 1999) and human diseases (Mahadevan *et al*., 1992; Stallings, 1994; O'Donnell and Warren, 2002). The commercial broiler rabbits maintained in India were imported few decades back from their native temperate regions of the world. These breeds maintained in the tropical environment need to be evaluated and characterized to improve their production performance. Estimation of genetic variation within and among the breeds is a basic tool for selection. Diversity analysis using microsatellite markers allows the estimation of genetic diversity within breeds and provides additional information for the design and interpretation of the breeding programmes.

The present investigation was undertaken with the objective to study the genetic diversity and phylogenetic relationship between two rabbit breeds by utilizing microsatellite markers.

MATERIALS AND METHODS

Genomic DNA isolated from 30 rabbits was utilized for analysis by using 12 rabbit specific and 8 interspecies

microsatellite markers. The markers chosen from published literature were detailed in Table 1.

The population structure, genetic variability and genetic distance were estimated and tested using suitable statistical methods. The allele data were subjected to the Excel Microsatellite Tool kit (Park, 2001) and GenAlex 6.1 (Peakall and Smouse, 2006) for estimating various parameters.

About 3-6 ml blood from each rabbit was collected into vacutainer tubes containing 2.7% EDTA. Genomic DNA was isolated from collected blood following standard Phenol-Chloroform method (Sambrook and Russel, 2001).

The genomic DNA quantity was estimated by using Nano drop equipment (JENWAY Genova Nano) and the quality by electrophoresis on 0.8% agarose gels. DNA purity was indicated by the ratio of optical absorbance at 260 and 280 nm. PCR was carried out using thermal cycler (Eppendorf) with final reaction volume of 12.5 µl, the alleles resolved on 8% polyacrylamide gel (PAGE) and silver staining was used to stain the gel. The bands visualized under UV light

Table 1: Information of utilized microsatellite loci

Sl. No	Locus	Primer sequence (5'→ 3')	Annealing Temp (°C)
		(a) Rabbit Specific Primers	
1	SAT02	F : GCTCTCCTTTGGCATACTCC R : GCTTTGGATAGGCCCAGATC	59.5
2	SAT03	F : GGAGAGTGAATCAGTGGGTG R : GAGGGAAAGAGAGAGACAGG	58.5
3	SAT04	F : GGCCAGTGTCCTTACATTTGG R : TGTTGCAGCGAATTGGGG	68.5
4	SAT05	F : GCTTCTGGCTTCAACCTGAC R : CTTAGGGTGCAGAATTATAAGAG	61.0
5	SAT07	F : GTAACCACCCATGCACACTC R : GCACAATACCTGGGATGTAG	55.9
6	SAT08	F : CAGACCCGGCAGTTGCAGAG R : GGGAGAGAGGGATGGAGGTATG	60.5
7	SAT12	F : CTTGAGTTTTAAATTCGGGC R : GTTTGGATGCTATCTCAGTCC	55.5
8	SAT13	F : CAGTTTTGAAGGACACCTGC R : GCCTCTACCTTTGTGGGG	51.9
9	SAT16	F : AATCAGCCTCTATGAATTCCC R : AATGCTACATGGTAACCAGGC	51.9
10	SOL30	F: CCCGAGCCCCAGATATTGTTACCA R: TGCAGCACTTCATAGTCTCAGGTC	55.5
11	SOL33	F : GAAGGCTCTGAGATCTAGAT R : GGGCCAATAGGTACTGATCCATGT	57.7
12	SOL44	F : GGCCCTAGTCTGACTCTGATTG R : GGTGGGGCGGCGGGTCTGAAAC	60.5

(b) Inter species primers			
13	BM3205	F : TCTTGCTTCCTTCCAAATCTC R : TGCCCTTATTTTAACAGTCTGC	54.0
14	ETH225	F : GATCACCTTGCCACTATTTCCT R : ACATGACAGCCAGCTGCTACT	55.5
15	ILSTS005	F : GGAAGCAATGAAATCTATAGCC R : TGTTCTGTGAGTTTGTAAGC	55.0
16	ILSTS011	F : GCTTGCTACATGGAAAGTGC R : CTAAAATGCAGAGCCCTACC	55.0
17	ILSTS017	F : GTCCCTAAAATCGAAATGCC R : GCATCTCTATAACCTGTTCC	55.0
18	ILSTS019	F : AAGGGACCTCATGTAGAAGC R : ACTTTTGGACCCTGTAGTGC	55.0
19	ILSTS033	F: TATTAGAGTGGCTCAGTGCC R: ATGCAGACAGTTTTAAGAGGG	55.0
20	TGLA227	F :CGAATTCCAAATCTGTTAATTTGT R :ACAGACAGAAACTCAATGAAAGA	55.0

Table 2: Parameters analyzed at various microsatellite loci studied

Locus	N	Na	Ne	I	Ho	He	uHe	F_{IS}	PIC	OR
(a) Soviet Chinchilla										
SAT02	15	8	7.143	2.021	0.667	0.860	0.890	0.225	0.844	0.633
SAT03	14	8	6.031	1.937	0.714	0.834	0.865	0.144	0.815	0.749
SAT04	14	9	5.765	1.939	0.714	0.827	0.857	0.136	0.806	0.761
SAT05	15	8	6.522	1.960	0.800	0.847	0.876	0.055	0.828	0.896
SAT07	15	11	7.031	2.138	0.400	0.858	0.887	0.534	0.843	0.304
SAT08	14	8	6.222	1.930	0.643	0.839	0.870	0.234	0.819	0.621
SAT12	14	7	5.227	1.756	0.429	0.809	0.839	0.470	0.781	0.361
SAT13	15	9	7.895	2.130	0.600	0.873	0.903	0.313	0.860	0.523
SAT16	15	4	3.600	1.329	0.600	0.722	0.747	0.169	0.672	0.711
SOL30	15	8	7.031	2.009	0.800	0.858	0.887	0.067	0.841	0.874
SOL33	15	11	9.783	2.332	0.800	0.898	0.929	0.109	0.889	0.804
SOL44	15	11	8.491	2.251	1.000	0.882	0.913	-0.134	0.871	1.308
Mean	14.667	8.5	6.728	1.978	0.681	0.842	0.872	0.194	0.822	0.712
(b) Californian White										
SAT02	15	8	7.143	2.016	0.733	0.860	0.890	0.147	0.844	0.743
SAT03	14	8	6.222	1.921	0.643	0.839	0.870	0.234	0.819	0.621
SAT04	14	9	6.125	1.986	0.643	0.837	0.868	0.232	0.818	0.624
SAT05	15	7	6.716	1.925	0.533	0.851	0.880	0.373	0.833	0.456
SAT07	14	8	6.644	1.971	0.571	0.849	0.881	0.327	0.831	0.507
SAT08	14	8	7.259	2.032	0.786	0.862	0.894	0.089	0.847	0.837
SAT12	15	6	4.945	1.687	0.400	0.798	0.825	0.499	0.769	0.335

SAT13	15	10	8.491	2.222	0.600	0.882	0.913	0.320	0.871	0.515
SAT16	15	5	4.592	1.567	0.467	0.782	0.809	0.403	0.748	0.425
SOL30	15	9	8.182	2.148	0.867	0.878	0.908	0.013	0.865	0.975
SOL33	15	10	8.654	2.214	0.733	0.884	0.915	0.171	0.873	0.708
SOL44	13	9	7.511	2.088	1.000	0.867	0.902	-0.154	0.852	1.363
Mean	14.500	8.083	6.874	1.981	0.665	0.849	0.880	0.221	0.831	0.676

N- Sample size, Na- Mean number of alleles, Ne- Effective number of alleles, I- Shannon's Information Index, Observed Ho- heterozygosity, He- Expected heterozygosity, uHe- Unbiased Expected heterozygosity, PIC-Polymorphism Information content, F_{IS}- Fixation Indices and OR- Outcrossing rates.

using gel documentation system against 50 bp ladder as a standard scale. The types of bands and their genotypes were documented and utilized for analysis of various parameters and phylogenetic relationship among the two rabbit breeds.

RESULTS AND DISCUSSION

Genotyping of the individual rabbit at various loci was done based on the presence or absence of a particular allele. The presence of two alleles of similar length (bp) at a locus was considered as homozygous, while that with dissimilar length was considered heterozygous. Among the 20 loci studied, the eight inter species did not show any amplification but the 12 rabbit specific loci were amplified successfully revealing that the primers designed for cattle, buffaloes, sheep and goats utilized in the present study were not amplifiable in the two rabbit breeds studied. The sample size, mean number of alleles, effective number of alleles, Shannon's information index, observed, expected and unbiased expected heterozygosity, Polymorphism information content, fixation indices and out crossing rate obtained in the present study were summarized in Table 2. The fixation indices of the two breeds at each locus are presented in Table 3 and χ^2 values for testing HWE at various loci are given in Table 4.

All the rabbit specific loci in both the breeds were amplified for 15 samples except SAT03, SAT04, SAT08 and SAT12 in Soviet Chinchilla breed and SAT03, SAT04, SAT07 and SAT08 in Californian White breed; and SOL44 amplified in 13 samples of CW. A total of 199 alleles (102 in Soviet Chinchilla and 97 in Californian White) were amplified in the two breeds studied.

All the 12 loci amplified in this study were found to be 100% polymorphic in both the breeds, which correspond to the findings of Surridge *et al.,*1999 in European wild rabbits, Wu Xin-Sheng *et al.,* 2008 in Angora rabbits, Grimal *et al.,* 2012 in Egyptian and Spanish rabbits and Thimmayya *et al.,* 2012 in pygmy rabbits. Chantry *et al.,* 2006 reported that only 81% of the utilized microsatellites were polymorphic in European rabbits, which were lower than the findings in present study. The overall mean number of alleles in SC and CW were 8.500 and 8.083, respectively. The mean Number of alleles per locus recorded in the present study was higher than the range of 3-7 reported in the literature (Van Haeringes, 1996; Mougel *et al*., 1997; Zhu *et al*., 2005 and Wu Xin-Sheng *et al*., 2008). Slightly higher number of alleles (8-17) was reported by Surridge *et al.,* 1999.

Table 3: Fixation indices of the two breeds at each locus

Locus	Fixation indices		
	F_{IS}	F_{IT}	F_{ST}
SAT02	0.186	0.216	0.037
SAT03	0.189	0.223	0.042
SAT04	0.184	0.212	0.034
SAT05	0.215	0.239	0.031
SAT07	0.431	0.468	0.065
SAT08	0.160	0.172	0.014
SAT12	0.484	0.507	0.043
SAT13	0.316	0.329	0.019
SAT16	0.291	0.391	0.141
SOL30	0.040	0.052	0.013
SOL33	0.140	0.147	0.008
SOL44	-0.143	-0.105	0.033
Mean	0.208	0.238	0.040

The mean number of alleles (MNA) observed over a range of loci for different breeds are also known as the allelic diversity and it is an important parameter of genetic

Table 4: χ^2 values for testing HWE at various loci

	Soviet Chinchilla				Californian White			
Locus	**d.f**	**Chi-Square value**	**Probability**	**Significance**	**d.f**	**Chi-Square value**	**Probability**	**Significance**
SAT02	28	31.500	0.295	ns	28	27.533	0.489	ns
SAT03	28	44.858	0.023	*	28	37.333	0.112	ns
SAT04	36	44.139	0.165	ns	36	53.690	0.029	*
SAT05	28	50.110	0.006	**	21	26.442	0.190	ns
SAT07	55	89.124	0.002	**	28	38.111	0.096	ns
SAT08	28	33.818	0.207	ns	28	21.778	0.791	ns
SAT12	21	31.844	0.061	ns	15	35.659	0.002	**
SAT13	36	52.083	0.040	*	45	94.583	0.000	***
SAT16	6	13.517	0.036	*	10	22.144	0.014	*
SOL30	28	37.183	0.115	ns	36	43.883	0.172	ns
SOL33	55	57.917	0.368	ns	45	43.292	0.545	ns
SOL44	55	54.167	0.506	ns	36	55.467	0.020	*

ns = not significant; * significant at p<0.05; ** significant at p<0.01; *** significant at p<0.001

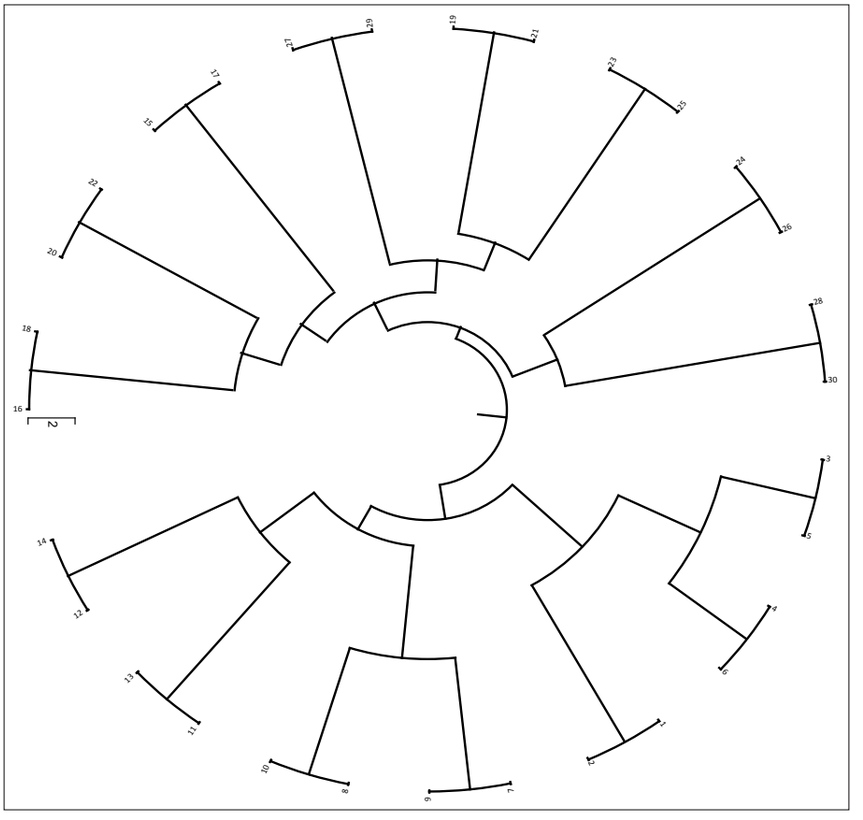

Fig. 1: Dendrogram showing the phylogenetic relationships among the two rabbit breeds

variation (Frankham *et al.*, 2002). For estimation of genetic diversity between breeds or genetic groups at least 5 different alleles per locus were required (FAO, 2004). In the present study, except for SAT16 loci (Na = 4) in Soviet Chinchilla rabbits all the loci in both the breeds showed a minimum of 5 alleles per locus, indicating that sufficient number alleles were amplified to estimate the genetic diversity among these two genetic groups. The MNA per locus observed in the present study was similar to the reports of Burton *et al.,* 2002 in hare but higher when compared with findings of Wu Xin-Sheng *et al.,* 2008, Mougel *et al.,* 1997, Zhu *et al.,* 2005, Andersson *et al.,* 1999 and lower to the findings of Surridge *et al.,* 1997 and Wu *et al.,* 2010. The mean effective number of alleles were 6.728 and 6.874 in SC and CW breeds, respectively, which were slightly higher (6.625 ± 0.498) than the finding of Wu *et al.,* 2010.

The expected heterozygosity is one of the indices used to assay the genetic variation of each population. Genetic variability of a population is usually measured by the average heterozygosity per locus, while the gene differences between two populations may be measured by the genetic distance proposed by Nei, 1972. Mean expected heterozygosity in Soviet Chinchilla breed was 0.842 and Californian White breed was 0.849, which indicated that genetic diversity in both the breeds in the present study was high. The mean value of expected heterozygosity values of both the breeds was comparable with the findings of Wu *et al.* (2010) in American Rex Rabbit and higher than the findings of Thimmayya *et al.* (2012) in pygmy rabbits, Queney *et al.* (2001) and Larbi *et al.* (2014) in European rabbit and Wu Xin-Sheng *et al.* (2008) in Angora rabbits. The Unbiased expected heterozygosity ranged from 0.747 to 0.913 in SC and CW it ranged from 0.809 to 0.915. The mean outcrossing rates in the present study were found to be 0.712 and 0.676 in SC and CW, respectively.

Botstein *et al.* (1980) first reported that PIC index can be used to evaluate the level of gene variation. When PIC > 0.5, the locus was of high diversity, when PIC < 0.25, the locus was of low diversity and the locus was of intermediate diversity, when PIC ranges between 0.25 and 0.5. Each locus has the PIC value greater than 0.5 in both the breeds, which indicated the presence of high diversity and highly informative nature of all the loci amplified. The PIC values obtained was comparable with reports of Wu Xin-Sheng *et al.* (2008), Zhu *et al.* (2005), Wu *et al.* (2010) and Han *et al.* (2005) in various rabbit breeds.

Wright (1951) developed three F-statistics for testing the genetic differentiation among subpopulations and also to summarize the genetic structure of a population and its subpopulations. The negative values of F_{IS} and F_{IT} at SOL44 locus indicated the occurrence of heterozygote genotypes at a proportion higher than the homozygous genotypes at that locus and the remaining loci have positive values of F_{IS} indicating the deficit of heterozygotes in both the breeds. At the SAT07 locus, F_{ST} value was 0.065, which indicated that moderate differentiation is observed between the two breeds at that locus and SAT16 locus showed F_{ST} value as 0.141 indicating the high degree of differentiation between the two breeds at that locus. The mean FST value was 0.04; this level of differentiation is lesser than the reports of Grimal *et al.,* 2012 in Egyptian and Spanish breeds, Larbi *et al.,* 2014 in Tunisian populations and Abdel-Kafy *et al.,* 2016 in Middle-Egypt region native rabbit populations.

The Nei's genetic distance between two breeds obtained in the present study was 0.611 and the genetic identity estimate was 0.543. The breeds were tested for departure from Hardy-Weinberg equilibrium at all the loci studied. In SC breed 5 loci (SAT03, SAT05, SAT07, SAT13 and SAT16) and 5 loci (SAT04, SAT12, SAT13, SAT16 and SOL44) in CW were found to be deviating significantly from Hardy-Weinberg equilibrium, which was an indication of selection at these loci. The dendrogram (Fig. 1) obtained in the present study revealed that the two breeds were identified as two separate clusters and departed from each other showing considerable genetic distance among them. But there is slight intermixing of germplasm between the breeds regarding one rabbit. The phylogeny of 15th rabbit of Soviet Chinchilla was found blended. This might be due to the accidental crossing between the two breeds during previous generations.

CONCLUSION

The present study showed high mean observed number of alleles, observed heterozygosity and PIC for the 12 microsatellites in both the breeds. This indicated the suitability of these microsatellites for genetic diversity studies in rabbits. High degree of differentiation between the two breeds was observed at SAT16 loci and moderate differentiation was observed at SAT07 loci. All the other

loci have the F_{ST} values less than 0.05, suggesting that differentiation did not exist between these two breeds at the loci studied.

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