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A study on genetic characteristics of Flemish giant and APAU fawn rabbits through DNA fingerprinting

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Abstract
The present study was undertaken for assessment of genetic diversity in Flemish Giant and APAU Fawn rabbits through DNA fingerprinting using a total of 20 microsatellite markers 12 (rabbit specific primers) and 8(cross species primers). All 12 rabbit specific markers showed amplification but cross species markers did not show any amplification. The mean number of alleles ranged from 4(sat 3) to 12 (sat 5) in Flemish Giant, whereas it ranged from 5 (sat 3) to 11 (sat 8) in APAU Fawn with a total of 189 alleles across the 20 loci. The overall mean observed heterozygosity was found to be 0.348 in Flemish Giant and 0.394 in APAU Fawn. The overall mean expected heterozygosity was found to be 0.828 in Flemish Giant and 0.813 in APAU Fawn. The overall mean unbiased expected heterozygosity was found to be 0.873 in Flemish Giant and 0.858 in APAU Fawn. The overall mean PIC was found to be 0.805 in Flemish Giant and 0.785 in APAU Fawn. All the 12 primers which amplified were found to be polymorphic and highly informative. The overall mean inbreeding coefficient (F) of Flemish Giant was found to be 0.599 while in APAU Fawn it was 0.523. The mean F_{IS}, F_{ST} and F_{IT} obtained in the present study was 0.561, 0.070 and 0.583 respectively. Out of 20 microsatellite marker primers studied, 8 markers showed significant deviation from Hardy – Weinberg equilibrium which might be due to heterozygote deficiency.

Keywords: genetic diversity, pcr, microsatellite markers, apau fawn, Flemish giant rabbits

Introduction
Rabbits exhibit exceptional phenotypic diversity; are of great commercial value and serve as important animal models in biomedical research (Carneiro *et al.*, 2011) [1]. Understanding the genetic structure of these domestic species provides a window into the process of domestication and motivates the design of studies aimed at making links between genotype and phenotype. Characterization of these genetic resources will serve as essential pre requisite for the identification, effective management and utilization of rabbit, which will facilitate their conservation. Genetic variation should be taken into account to guide the conservation programs, while highly polymorphic microsatellite DNA marker provide a good tool to assess the genetic variation (Tautz and Renz, 1984, Estoup *et al*., 1993 and Weber and Wrong, 1993) [2, 3, 4]. Knowledge of genetic variation within and among various breeds is required to meet future demands. There is a clear need for the development of molecular tools in this species for fundamental research in order to localize traits with economic interest and to undertake the marker assisted selection. Microsatellite markers are able to express genetic information accurately for its excellence of stability, accuracy and security.

Materials and Method
A total of 20 animals from two breeds namely Flemish Giant and APAU Fawn (each 10) breeds maintained at Rabbit Research Centre, Department of Animal Genetics and Breeding, College of Veterinary Science, Rajendranagar were utilized for the present study.10 ml of blood was collected from each animal aseptically through heart puncture into vacutainer tubes containing EDTA (2.7%) as anticoagulant.

Genomic DNA and PCR amplification of different allelic segments
The genomic DNA was isolated through standard phenol: chloroform extraction method (Sambrook and Russell, 2001) [5] and the quantity and quality evaluated by UV spectrophotometer and electrophoresis on 0.8% agarose gels. The Polymerase chain reaction (PCR) amplification of all 20 microsatellite loci was carried out in 0.2 ml capacity eppendorf tubes, using thermal cycler (Eppendorf) containing master mixture of 12.5µl which includes 1µl (100 ng) of genomic DNA, 1.25µl of 10X Taq Buffer, 0.25µl of dNTPs (10mM/µl), 0.75µl

of Forward- Primer (100 pM/µl), 0.75µl of Reverse- Primer (100 pM/µl), 0.75µl of $MgCl_2$ (25 pM/µl), 0.5 U of Taq Polymerase (1 unit/µl) and 7.25µl of Autoclaved MilliQ water was added to make up final volume. The PCR reactions conditions for the amplification of microsatellite marker primers were initial denaturation at 95 ºC for 5 min, followed by 34 cycles of 1 min at 94 ºC,30 sec at optimal annealing temperatures, 30 sec at 72 ºC and final extension step at 72 ºC for 5 min. Amplified segments were resolved on 8% Polyacrylamide gel with standard 50 bp ladder and stained with Silver staining and visualized on Geldoc system.

The statistical analysis of the data generated from the DNA fingerprint patterns for each of the loci studied were utilized for the calculation of the following parameters**.**

Percentage of Polymorphic Loci (P) was calculated by using the formula

$$P = \frac{\text{Number of polymorphic loci observed}}{\text{Total number of loci studied}} \times 100$$

Heterozygosity (H) was calculated by using the formula given by Nei, (1973) as:

$$H = 1 - \Sigma P_i^2$$

Where, P_i = frequency of i^{th} allele

$$\text{Observed heterozygosity} = \frac{\text{Number of heterozygotes}}{\text{Total number of individuals studied}} \times 100$$

$$\text{Expected heterozygosity} = 1 - \Sigma P_j^2$$

Polymorphism Information Content (PIC) was calculated by using the formula (Nei, 1978) as:

$$PIC = 1 - \Sigma^{k}_{i=1} P_i^2 - \Sigma^{k-1}_{i=1} \Sigma^{k}_{j=1} 2P_i^2 P_j^2$$

Where,
P_i = frequency of i^{th} allele
P_j =frequency of j^{th} allele
i and j = number of allele

F-Statistics (F_{IS}, F_{ST}, F_{IT})

F_{IS} = Indicates the reduction in heterozygosity of an individual due to non-random mating.

F_{ST} = measured as coefficient of gene differentiation. Lower F_{ST} value indicates higher relationship between the breeds.

F_{IT}= measures the reduction in heterozygosity of an individual relative to the total population.

All are measured by Standard F-STAT programme (Goudet, 1995)

Outcrossing Rate

The outcrossing rate was calculated as

$$\text{Outcrossing rate} = \frac{1 - F_{IS}}{1 + F_{IS}}$$

Where, F_{IS} is coefficient of inbreeding

Test for Hardy-Weinberg Equilibrium

The deviation of the population from Hardy-Weinberg equilibrium is an indication of intensity of external factors and this was tested by chi-square test using the formula (Snedecor and Cochran, 1989) [9].

$$\chi^2 = \Sigma^{k}_{i=1} \left(\frac{(O - E)^2}{E} \right)$$

Where, O = Observed frequency
E = Expected frequency
k = Number of genotypes

Results and Discussion

The sample size, mean number of alleles, effective number of alleles, Shannon's information index, observed heterozygosity, expected and unbiased expected heterozygosity, polymorphism information content, and outcrossing rates obtained in the present study population wise, locus wise are detailed in Table 1. An animal was genotyped either as a homozygote (based on the presence of single band) or a heterozygote (based on presence of double bands) with respect to particular microsatellite loci. The genomic DNA was amplified with 12 rabbit specific markers (sat2, sat3, sat4, sat5, sat7, sat8, sat12, sat13, sat16, sol30, sol33 and sol44) and 8 inter species markers (BM3205, ETH225, ILSTS005, ILSTS011, ILSTS017, ILSTS019, ILSTS033 and TGLA227). All 12 rabbit specific markers showed amplification but inter species markers of cattle buffaloes, sheep and goats did not show any amplification. The population differentiation was examined by fixation indices $F_{IS,}$ $F_{IT,}$ F_{ST} by using F- stat program detailed in Table 2. Chi- square test was used to test whether the population was under Hardy - Weinberg equilibrium

In the present study, 20 markers generated a total of 189 alleles with an average mean number of alleles 8.50 and 7.25 in Flemish Giant and APAU Fawn respectively. The overall average number of alleles per locus of 7.87 obtained in the present study was in line with the recommendation by FAO suggesting at least 5 different alleles per locus for estimation of genetic diversity. Frankham *et al*. (2002) [10] pointed out that the average number of alleles per locus known as allele diversity is an important parameter of genetic diversity. However, one marker (Sat3) revealed low allelic diversity with less than five alleles (4.00) in Flemish Giant.

The overall means for observed heterozygosity in Flemish Giant and APAU Fawn were 0.348 and 0.394, whereas the expected heterozygosity was 0.827 and 0.855, respectively. Most of the loci showed observed heterozygosity less than expected heterozygosity. The lowest observed heterozygosity (0.00) was found for Sat3, Sat12, Sat16 and Sol30 in Flemish Giant and Sat3, Sol33 in APAU Fawn. The lowest observed heterozygosity was due to the presence of more homozygote individuals for the samples analyzed. The highest expected heterozygosity was 0.900 (Sol33) in Flemish Giant and 0.885 (Sat4 and Sat5) in APAU Fawn, which reflects the existence of variation in the breed. The high expected heterozygosity values observed in the present study indicate higher amount of genetic variability reflecting the existence of within breed genetic diversity that can be exploited in the population of Flemish Giant. The overall observed heterozygosity less than expected heterozygosity might be due to the fact that samples collected from the rabbits which were maintained as closed populations over many generations even though samples selected were from unrelated individuals and also due to the presence of more homozygous individuals (less variation in

the breed) in the samples analyzed. The results obtained in the present study were on par with the values of Grimal *et al.* (2012) [11] in four Egyptian and one Spanish rabbit populations and Surridge *et al.* (1999) [12] in European wild rabbits.

The overall mean PIC was found to be 0.805 in Flemish Giant and 0.785 in APAU Fawn. The PIC values for all the loci were found to be more than 0.50 indicating that these markers are highly informative for characterization of Flemish Giant and APAU Fawn rabbits. The PIC values observed in most of the loci are comparable with the reported values of Zhu *et al.* (2004) [13] in Vc-I, Vc-II, Japanese White, Qingzilan and New Zealand White rabbit breeds.

The mean heterozygosis deficit (F_{IS}) obtained in the present study was 0.561. Coefficient of coancestry (F_{ST}) calculated pair wise for both the breeds was 0.070, whereas the overall variation of individuals compared to total population (F_{IT}) was 0.583. Wright (1978) [14] reported that if the F_{ST} value of groups between 0 and 0.05 suggested that differentiation did not exist in subgroup, moderate differentiation if the value of F_{ST} between 0.05 and 0.15, a high degree of differentiation if F_{ST} value were ranged from 0.15 to 0.25.

The χ^2 test for Hardy – Weinberg equilibrium revealed that there was a significant deviation of observed allele frequencies from expected for 8 number of loci in both Flemish Giant and APAU Fawn except for loci Sol33 in Flemish Giant and Sat8, Sat13, Sol33 in APAU Fawn. This indicated that both populations were not in equilibrium status which could be due to long term selective breeding being practiced in these populations for the higher body weights in Flemish Giant and higher body weights and fawn fur colour in APAU Fawn. The small sample size of (20 animals) could be a reason for the deviations from Hardy – Weinberg Equilibrium. The deviation from the equilibrium frequency might be due to the null alleles, nonrandom mating, high selection pressure, random genetic drift, smaller population size etc.

The loci in Flemish Giant population Sat2, Sat3, Sat4, Sat5, Sat7, Sat8, Sat12, Sat13, Sat16, Sol30, and Sol44 were shown significant difference for Chi-square calculated value indicating the deviation from Hardy - Weinberg equilibrium. In APAU Fawn breed except the locus Sat8, Sat13 and Sol33 all others showed significant difference ($P \leq 0.01$). Majority of the loci studied shown significant departure from Hardy - Weinberg equilibrium in Flemish Giant (11) and APAU Fawn (9) genetic groups.

Table 1: Number of animals (N), Mean number of alleles (N_a), Effective number of alleles (N_e), Shannon's Information Index (I), Observed heterozygosity (H_o), Expected heterozygosity (H_e) and Unbiased Expected heterozygosity (uH_e), Polymorphism Information content (PIC) and Outcrossing rates of various microsatellite loci studied

Population	Locus	N	N_a	N_e	I	H_o	H_e	uH_e	PIC	Outcrossing rate
FG	Sat2	10	11.000	9.091	2.303	0.200	0.890	0.937	0.880	0.126
FG	Sat3	9	4.000	3.000	1.215	0.000	0.667	0.706	0.607	0.000
FG	Sat4	10	7.000	5.556	1.834	0.200	0.820	0.863	0.797	0.138
FG	Sat5	10	11.000	9.091	2.303	0.600	0.890	0.937	0.880	0.508
FG	Sat7	10	9.000	5.882	1.973	0.200	0.830	0.874	0.811	0.137
FG	Sat8	10	9.000	6.452	2.042	0.500	0.845	0.889	0.829	0.420
FG	Sat12	9	6.000	4.765	1.677	0.000	0.790	0.837	0.761	0.000
FG	Sat13	10	11.000	8.333	2.250	0.800	0.880	0.926	0.868	0.833
FG	Sat16	9	5.000	3.522	1.427	0.000	0.716	0.758	0.677	0.000
FG	Sol30	10	8.000	7.143	2.025	0.000	0.860	0.905	0.844	0.000
FG	Sol33	10	11.000	10.000	2.346	0.900	0.900	0.947	0.891	1.000
FG	Sol44	9	10.000	6.231	2.058	0.778	0.840	0.889	0.822	0.862
	Mean	9.66	8.500	6.589	1.954	0.348	0.827	0.872	0.805	0.335
FN	Sat2	10	8.000	4.545	1.791	0.200	0.780	0.821	0.758	0.146
FN	Sat3	10	5.000	4.167	1.505	0.000	0.760	0.800	0.720	0.000
FN	Sat4	10	10.000	8.696	2.233	0.200	0.885	0.932	0.874	0.127
FN	Sat5	10	8.000	7.407	2.033	0.900	0.865	0.911	0.849	1.034
FN	Sat7	9	6.000	4.765	1.677	0.222	0.790	0.837	0.761	0.163
FN	Sat 8	10	11.000	8.333	2.250	0.700	0.880	0.926	0.868	0.659
FN	Sat12	10	6.000	4.444	1.617	0.200	0.775	0.816	0.741	0.148
FN	Sat13	8	6.000	4.267	1.576	0.875	0.766	0.817	0.728	1.333
FN	Sat16	10	6.000	5.405	1.735	0.100	0.815	0.858	0.788	0.064
FN	Sol30	10	6.000	4.545	1.643	0.000	0.780	0.821	0.748	0.000
FN	Sol33	10	9.000	5.714	1.947	1.000	0.825	0.868	0.804	1.538
FN	Sol44	9	6.000	5.226	1.721	0.333	0.809	0.856	0.781	0.259
	Mean	9.66	7.250	5.626	1.811	0.394	0.811	0.855	0.785	0.455

Note: FG indicates Flemish Giant and FN indicates APAU Fawn

Table 2: Estimates of fixation indices at different loci

locus	F_{IS}	F_{IT}	F_{ST}
Sat 2	0.760	0.782	0.090
Sat 3	1.000	1.000	0.167
Sat 4	0.765	0.778	0.054
Sat 5	0.145	0.195	0.058
Sat 7	0.739	0.759	0.077
Sat 8	0.304	0.331	0.039
Sat 12	0.872	0.879	0.054
Sat 13	-0.018	0.038	0.055
Sat 16	0.935	0.939	0.071
Sol 30	1.000	1.000	0.099
Sol 33	-0.101	-0.064	0.034
Sol 44	0.326	0.354	0.041
Overall Mean	0.561	0.583	0.070
SE	0.117	0.112	0.011

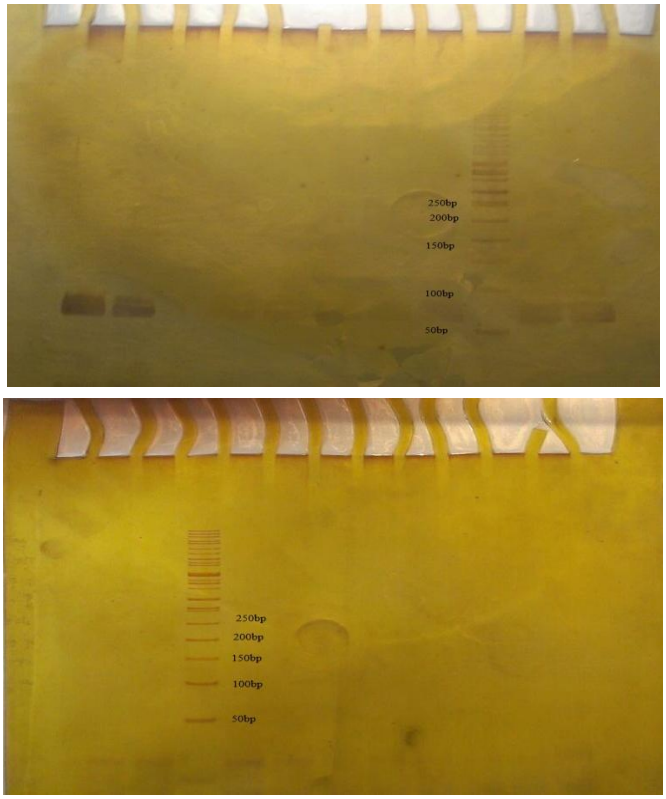

Fig 1: PAGE image showing no amplification of inter species microsatellite marker ILSTS005 in Flemish Giant (top) and APAU Fawn (Bottom).

Conclusion

Based on the present study, the results suggested that all 12 rabbit specific markers showed amplification but 8 cross species markers did not show any amplification. Majority of the loci studied shown significant departure from Hardy - Weinberg equilibrium in Flemish Giant (11) and APAU Fawn (9) genetic groups. The protocols standardized for the isolation of genomic DNA and PCR amplification in Flemish Giant and APAU Fawn rabbits were found to be satisfactory and enabled the assessment of genetic diversity in both the genetic groups.

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