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EVALUATION OF SIX CAMEL BREEDS FOR HETEROZYGOCITY THROUGH RESTRICTION FRAGMENT LENGTH POLYMORPHISM

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ABSTRACT

In the camel tyrosinase gene, a restriction site provoked by the T variant was used in a special restriction fragment length polymorphism analysis (PCR-RFLP) for genotyping of animals from six different Pakistani camel breeds (Marecha, Dhatti, Larri, Kohi, Campbelpuri and Sakrai). For this purpose, four new primer pairs were designed for sequencing the coding region of exon 1 of the tyrosinase gene. PCR reactions were carried out in a total volume of 25 µl using 100 ng genomic DNA to amplify a 474 bp fragment at 56°C. A SNP (T/C) at 200 bps was found and exploited with a Dde I restriction enzyme that resulted in three different genotypes i.e. TT, TC and CC in each studied camel breed. Significant differences in the genotype frequency between the breeds were recorded. The Sakrai breed showed a distinctly higher frequency of heterozygous animals compared to Marecha, Dhatti, Larri and Kohi breeds. Our new designed primers could be used for genotype screening of other camel breeds. However, for understanding the contribution of tyrosinase gene and its antagonist i.e. agouti in the coat colour production, complete sequence of the gene is imperative.

Key words: Camel, genotyping, RFLP, tyrosinase.

INTRODUCTION

Restriction endonuleases recognize the sequence in the genome and cleave the DNA at the particular sequence or its recognition site. Such enzymes can detect difference between alleles with and without mutation. Restriction endonucleases have become an indispensable tool for the manipulation of DNA (Szalay *et al*., 1979). Dde I has been purified and characterized from a sulfate-reducing, anaerobic bacterium, Desulfovibrio desulfuricans, Norway strain (Makula and Meagher, 1980).

The tyrosinase gene (TYR) or C locus has long been implicated in the coat colour determination. This gene is of interest in farm animals because of its role in coat colour production. It has been shown to cause a range of dilution phenotypes including complete albinism in cattle, humans, mice and chickens (Schmidtz *et al*., 2001). Tyrosinase gene has been reported to be consisting of 5 exons and 4 introns in mammals (Giebel *et al.,* 1991).

Traditionally, camels have been classified according to their functions. They have also been classified according to their habitat e.g. riverine, desert and mountain (Wilson, 1997). Camels have been further categorised into conventional types comparable to those applied to cattle i.e. beef, dairy, dual purpose and racing (Wardeh *et al.,* 1991). Many modern classifications have advanced little beyond these concepts and are usually simply tribal or location names (Hoste *et al.*, 1985), with little attempt to assign the quantitative production parameters, which are very important for breed description. Objective of the present study was to screen the camels genotypically that have been so far classified on the basis of performance and tribal ownership.

MATERIALS AND METHODS

A total of 157 camels belonging to six different Pakistani breeds Viz. Marecha (n= 26), Dhatti (n= 24), Larri (n= 27), Kohi (n= 30), Campbelpuri (n= 25) and Sakrai (n= 25) were included in this study. These animals belonged to different ecological zones of Pakistan and presented a high variation in their coat colour (Table 1). Genomic DNA was isolated from their hairs using NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's protocol.

PCR amplification and sequence analysis

Four primer pairs were designed for a part of the coding sequence of exon 1 of the tyrosinase gene. The raw sequence based on four PCRs using primer pairs

Table 1: Breed type and coat colour of the experimental camels

S. No.	Breed	Type	Coat colour
1.	Marecha	Riverine	Brown and dark brown
2.	Dhatti	Riverine	Light and dark fawn
3.	Larri	Riverine	Reddish or brown
4.	Kohi	Mountain	Creamy white or dark brown
5.	Sakrai	Riverine	Reddish brown
6.	Campbelpuri	Mountain	Dark brown to blackish

designed from homolog regions of cattle (Schmutz *et al*., 2004), horse (Wagner and Reissmann, 2000), human (Gotoh *et al*., 2004) and mouse (Lavado *et al*., 2005) (GenBank accession numbers NM18100, AF2556101, AF252540, NM000372 and NM011661, respectively) were used to amplify a main part of the coding region of exon 1 of tyrosinase gene of dromedary camel (dTYR) in 5′ and 3′ direction. The first primer pair D-TY-A up 5′ > AGC CTG TGC CTC CTC CAA GAA < 3′ and D-TY-A low 5′ > TGC ATC CAT ACA AAG AAG TCA TAA < 3′ yielded a 474 bp fragment. Second pair C1-A11 up 5′ > AAT GCT CCT GGC TGT TTT GTA <3′ and C1-A11 Low 5′ > CTG CCA GGA GGA GAA GGA TGC T <3′ was used to amplify 819 bp fragment, third pair C1 up 5′ > TGC CTG CTG TGG AGT TTC<3′ and C1 Low 5′ > GCC GAA GCC CTG GTG GAT G <3′ amplified 516 bp fragment and fourth pair KC1 up5′ > CCA GCT CTC AGG CAG GGG T < 3′ and KC1 Low 5′ > GAC TCT TCT TGT TGC TGT GGG A < 3′ yielded 516 bp fragment. The amplified fragments of these primer pairs overlapped and resulted in a 770 bp total sequence. PCR reactions were carried out using thermocycler (Biometra, Germany) in a total volume of 25 µl containing 2.5 mM, $MgCl_2$, 0.2 mM dNTP 1U Taq DNA Polymerase (Genaxxon, Germany) 0.2 µM of forward and reverse primer and 100 ng genomic DNA. After an initial denaturation with 94°C for 2 minutes, 35 cycles were done each consisting with 94°C for 1 minute, annealing at 56°C (primer pairs D-TY-A up, D-TY-A low; C1-A11 up, C1-A11 Low and C1 up and C1 low), 60°C (KC1 up and KC1 low) for 30 seconds and extension at 72°C for 40 seconds. The final step lasted for 10 minutes at 72°C. The PCR amplified fragments were excided from 2% agarose gel and purified using Gene Clean II Kit (Q BIO gene, Canada). Each fragment was sequenced in both directions using BigDye Terminator v1.1 Cycle Sequencing chemistry on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). All sequence alignments and distance calculations were made by Lasergene software (DNAStar, USA).

PCR-RFLP analysis

PCR reactions for a Dde I restriction were carried out in a total volume of 25 µl with the primer pair D-TY-A up and D-TY-A low to amplify a 474 bp fragment at 56°C. PCR product (15 µl) was mixed with master mix comprising 2.0 µl buffer, 0.2 µl bovine serum albumin (BSA), 0.2 µl Dde I enzyme (2 U, Promega Madison WI, USA) and 2.6 µl water and incubated at 37°C for 3 hours. After incubation, 5 µl loading dye buffer was added and electrophoresed for 2 hours, as previously described (Shah *et al*., 2006).

Statistical analysis

Frequencies of different genotype animals in each breed were computed. Chi square test was used to ascertain the magnitude of variation in these frequencies between breeds.

RESULTS AND DISCUSSION

A part of exon 1 (779 bp) of tyrosinase gene of dromedary camel (dTYR) was amplified using four primer pairs namely C1A11up, C1A11 low, C1 up, C1 low, D-TY-A up, D-TY-A low, KC1 up, KC1 low. Primer pair D-TY-A up and D-TY-A low amplified a 474 bp fragment that contained a T/C variation and created a Dde I restriction site. Since there was a permanent restriction site, the whole restriction resulted in 392 bp + 82 bp homozygous TT, 392 bp + 82 bp + 53 bp heterozygous TC and 339 bp + 82 bp + 53 bp homozygous CC genotypes. An example is shown in Plate 1, where nucleotide "CC" created a restriction point producing fragments of 135 bp + 339 bp.

PCR-Dde I restriction was used to analyze the genotype variation at tyrosinase gene in camels included in the present study. Sakrai breed possessed a significantly ($P<0.05$) higher number of homozygous with restriction (TT) and heterozygous (CT) animals than other five breeds. It also showed a significantly lower number of homozygous without restriction (CC) animals than that of Marecha, Dhatti, Larri and Kohi breeds (Table 2). No significant difference was observed among Marecha, Dhatti, Larri, Kohi and Campbelpuri breeds. Number of animals used and frequencies of three genotype animals i.e. TT, CT and CC among each breed is also presented in Table 2.

Table 2: Breed differences and frequency of the different genotype animals detected through Dde I restriction (Tyrosinase gene)

Breed	No.	Ma	Dh	La	Ko	Ca	Sa	Genotype frequency		
								TT	CT	CC
Ma	26	--	0.92	0.59	0.58	2.64	8.20*	0.192	0.462	0.346
Dh	24		--	0.09	0.07	5.13	11.62*	0.208	0.334	0.458
La	27			--	0.02	5.01	11.58*	0.185	0.370	0.445
Ko	30				--	4.80	11.28*	0.200	0.367	0.433
Ca	25					--	2.06	0.320	0.520	0.160
Sa	25						--	0.400	0.560	0.040

Ma = Marecha, Dh = Dhatti, La = Larri, Ko = Kohi, Ca = Campbelpuri and Sa = Sakrai;
TT = homozygous with restriction, CT = heterozygous and CC = homozygous without restriction
* = significant at P<0.05.

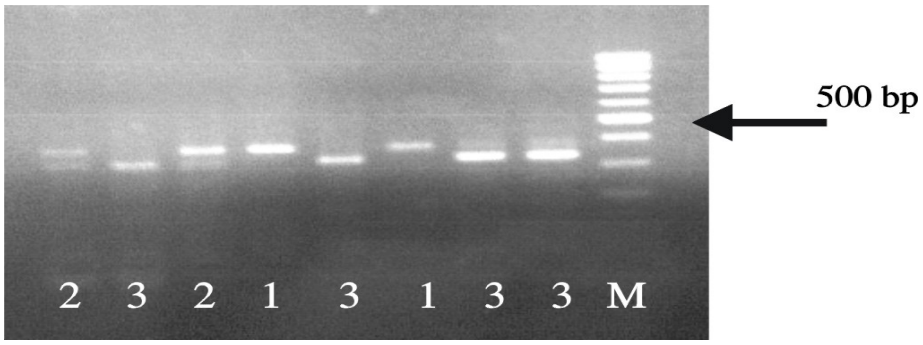

Plate 1: Different genotype camels screened with restriction fragment length polymorphism (RFLP) with Dde I, on 2% agarose gel; 1 (TT), 2 (CT), 3 (CC) genotypes and M (Marker).

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-1                                                                    a
 1 atggctccttgg ctgttttgtta[1] ctggcctgcctg tggagtctcdc[3]agacctccgc tggccatttc
61 cctcgtaggcct gtgcctcccttc caaagaagc[5]ttg atggagaagg aatgctgccc gcngtgggag
121 ggtgacggga gtccctgtgg [ccagctcttca ggcaggggtt][7] cctgtcagga catcaatctg
181 tccaaggcac cacctggacn tcagttcccc ttcacagggg tggatgaccg ggaatcttgg
241 ccctctgtct tttataacag gacctgccag tgctttgaca acttcatggg attcaactgt
301 ggaaattgca agtttggctt ccggggaccc aactgcagag agaggcgact tttggtgaga
361 agaaacatct ttgatttgag tgtcccagag aagaacaaat ttcttgccta cctcacttta
421 gccaaacata ccaccagccc agactacgtc atccccacgg gcacctatgg ccaaatgaat
481 aatggatcaa cacccatgtt caatgacatc aacgttttatg acccctttgt atggatgcau[6]
541 tattatgtgt caagggacac gctgcttggg gggtctgaaa tctggaaaga cattgatttt
601 gctcatgaag caccaggctt cctgccttgg cata[gcactcc tcttgctgct gtgggga[8]acaa
661 gaaatccaga agctgacagg ggatgagaac ttcactattc catactggga ctggcgagat
721 gcagacaact gtgaaatttg tacagatgag tacatgggag ggcgcgaccc cacaaatgcgu[4]
781 [aacttactcca gcccaggcatc[4] ctccttctccc tcctgggcag[2]
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Fig. 1: Coding sequence of camel Tyrosinase gene (black letters) with primers position (C1A11up[1], C1A11 low[2]; C1 up[3], C1 low[4]; D-TY-A up[5], D-TY-A low[6]; KC1 up[7], KC1 low[8]) and restriction site Dde I (box). Bold letters show the horse sequence.

About 150 different TYR mutations have been characterized in humans (Oetting and King, 1999), cattle (Schmutz *et al*., 2004), rabbit (Aigner *et al*., 2000) and mice (Beermann *et al*., 2004). This gene was of interest in farm animals because of its role in coat colour. In this study, a 779 bp fragment was sequenced in the exon 1 of the camel tyrosinase gene (Fig. 1).

By sequencing camels of different breeds, a single nucleotide polymorphism (C/T) on position 200 after ATG causing an amino acid substitution (Pro/Leu) was detected (Fig. 1). A restriction site (Dde I) provoked by the "C" variant was used as special restriction fragment length polymorphism (PCR-RFLP) for genotype screening of studied camel breeds. The Sakrai breed showed higher frequency ($P<0.05$) of homozygote without restriction (TT = 0.40) than the Marecha, Dhatti, Larri and Kohi breeds.

For genotype screening, the use of restriction enzymes is an easy and reliable method even though there is a small fragment of the gene with a SNP. Coat colour has been a well established character of world famous breeds of livestock. However, for understanding the contribution of tyrosinase gene and its antagonist i.e. agouti in the coat colour production complete sequence is imperative.

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