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Identification of SNPs in PRNP gene in Karnah sheep breed of India

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ABSTRACT

Single-strand conformation polymorphism (SSCP) of ovine PRNP coding region exon-3 was detected in 50 blood samples from Karnah sheep (*Ovis aries*) breed of North temperate Himalayan regions of India. PCR amplicons developed by using PrP forward primers (5'-caa ggt ggt agc cac agt ca-3') and reverse primers (5'-cca cca ctc gct cca tta tc-3') of approximate size of 358 bp were generated for PrP gene exon-3 partial CD's covering 352 to 700 nucleotide positions. A total of 5 variants A, B, C, D and E were observed. SNP at codon 136, 154 and 171 are AA and VV with frequencies of 90.91 and 9.09%, RR and RH genotypes were present in 90.91 and 9.09%, QQ and QR were having frequencies of 54.55 and 45.45% respectively. The generated data will help for the detection of SNP markers related to disease resistance or susceptibility in India.

Key words: Karnah Sheep, Prion protein gene, Single strand conformation polymorphism, Sheep, SNP

Scrapie, a contagious prion disease of sheep and goat (Prusiner 1998), is largely controlled by 3 polymorphic amino acid positions (136, 154 and 171) of the ovine prion protein gene (Hunter 1997). The prion diseases can affect both humans and animals and it is invariably fatal due to the neurological damage it causes. Apart from nursing care, there is no vaccine or medicine that prevent the disease and. However, they are rare neurodegenerative disorders affecting only one in a million populations. Despite this, remarkable attention has been focused on this disease due to their unique biology and also because of the fears that an epidemic of a newly recognized bovine prion disease among domesticated animals could pose a threat to public health through dietary exposure to infected tissues (Collinge *et al*. 1991, Ridley and Baker 1995). In sheep, variation in the PrP gene was identified at a number of codons, but polymorphism at 3 codons (136, 154, and 171) has reported linkages, with the incidence of scrapie (Baylis and Goldman 2004). The association between *ovine* PrP polymorphism at codons 136, 154, and 171 and scrapie susceptibility is the basis for the marker-assisted breeding for decreased scrapie susceptibility now underway in many countries (Tranulis *et al*. 2002). Various PCR-based approaches have been used to determine ovine PrP sequences at codons 136, 154, and 171. In addition, identification of the novel haplotypes $A_{136}H_{154}R_{171}$ and VRR in sheep (Kutzer *et al*. 2002) makes the results from the current typing methods difficult or sometimes impossible to interpret. PCR coupled with single-strand conformational polymorphism (SSCP) allows for the accurate identification of ovine PrP genotypic variants which can detect molecules differing by as little as a single base substitution.

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MATERIALS AND METHODS

Animals and sample collection: Purebred Karnah sheep from the farmers' flocks from Himalayan regions were included in this study. The average flock size ranged between 15 and 100. Blood samples (52) were collected randomly from Karnah sheep in its native tract in and around Karnah Taluka, by picking up not more than 2 animals from each flock and not more than 2 flocks from each village.

DNA extraction and PCR amplification: Approximately 10 ml blood from each animal was collected aseptically and brought to laboratory at 4°C. Genomic DNA was isolated using standard procedure (Sambrook *et al*. 1989). After checking the quality and quantity DNA was diluted to a final concentration of 50 ng/ml and stored at 4°C. PCR primers (5'-tct tac gtg ggc att tga tg-3', 5'-tga ctg tgg cta cca cct tg-3', 5'-caa ggt ggt agc cac agt ca-3', 5'-cca cca ctc gct cca tta tc-3', 5'-gat aat gga gcg agt ggt gg-3', 5'-gct tgt cat ttc cca gtg ct-3') were designed to amplify 3 480, 358 and 310 bp fragment of exon-3 of the ovine PrNP gene covering the part of intron-2, complete exon-3 and part of intron-3. PCR was

carried out on about 50 ng genomic DNA, 0.5 IU Taq DNA polymerase, and 1× reaction buffer in a 25 ml reaction volume. Amplifications were performed in a MJ PTC 100 thermal cycler programmed for an initial denaturation step at 94°C for 2 min, followed by 32 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 30s, followed by a final extension step at 72°C for 5 min. Amplified products were visualized by electrophoresis in 1% LE agarose gel, using 1×TBE buffer.

Single strand conformational polymorphism analysis: An aliquot of 0.7 ml of each amplified product was mixed with 7 ml of loading dye. The PCR products were denatured at 85°C for 12 min in thermo cycler, followed by rapid cooling. The samples were loaded on 12% acrylamide: bisacrylamide gels containing 0.2% glycerol. Electrophoresis was performed using Protean-II xi cells, at 385 V for 225 min at 4°C in 1% TBE buffer. The gels were stained with silver nitrate using a standard protocol (Bassam *et al*.1991). The gels were dried on gel dryer.

PCR cleanup and sequencing: The PCR-SSCP amplicons were identified for sequencing based on different haplotypes observed on gels for genomic DNA products generated by (forward 5'-CAAGGTGGTAGCCACAGTCA-3' and reverse 5'-CCACCACTCGCTCCATTATC-3') primers as our aim was to screen the SNPs in this region having nucleotides 136,154 and 171 which are considered important for scrapie disease susceptibility/resistance. Each variant from this region was used for sequencing in duplicate. Further, the same animal's amplicons for fragment I generated by primers (forward 5'-tct tac gtg ggc att tga tg-3' and reverse 5'-tga ctg tgg cta cca cct tg-3') and fragment –III amplified by primers (forward 5'-gat aat gga gcg agt ggt gg-3' and reverse 5'-gct tgt cat ttc cca gtg ct-3' were selected, so that complete exon 3 of the PRNP gene can be re-constructed based on alignment of sequences. PCR products in 96 well plates were treated with shrimp alkaline phosphatase (1 unit/ml) and Exonuclease III (10 units/ml) using a volume equaling 5% of the PCR product, incubated at 37°C for 30 min followed by 80°C for 10 min. Out of this 1–5 ml PCR product was used in sequencing. Both forward and reverse primers were used in all sequencing reactions. PCR products were loaded in to a 96 wells, format and analyzed on ABI 370 DNA analyzer. All sequences were analyzed using DNA Star (version 4.0). The SNPs were identified in assembled sequences. The amino-acid and codon identification was done using online sequin software. The SSCP alleles were identified using the nomenclature followed by Zhou *et al*. (2005) and compared with amino acid of the codon. All data were manually checked for accuracy at codon where polymorphism was observed. In alignment of sequenced chromatographs, the ambiguous letter of nucleotides or N especially in the beginning of the sequences was ignored and the validity of results was not compromised.

RESULTS AND DISCUSSION

Farmers owning Karnah sheep in Karnah sub division of Kuppwada district of Jammu and Kashmir were interviewed. The farmers were told about the symptoms of scratching, uncoordinated movements of infected sheep. All the farmers under study informed that they have never observed any symptoms detailed to them for the incidence of the scrapie. Further the veterinary staff of the respective areas also confirmed that neither of the sheep in the sampled flocks showed any symptom of scrapie nor they have received any case of clinical scrapie alive or carcass for postmortem showing any prevalence of the BSE disease in sheep of these areas.

PCR-SSCP profile: PCR product amplicons developed by using PrP forward primers (5'-caa ggt ggt agc cac agt ca-3') and reverse primers (5'-cca cca ctc gct cca tta tc-3') of approximate size of 358 bp were generated for PrP gene exon-3 partial CD's covering 352 to 700 nucleotide positions in different breeds. The SSCP amplimers were resolved on non-denaturing gel.

PCR-SSCP variant: The PCR-SSCP band patterns of PrP gene partial CD's of exon-3 are shown in Fig. 1. A total of 5 variants A, B, C, D, and E were observed (Table 1). Variant 'A' had the highest frequency represented by 78.73% animals. Variant 'D' was observed in one animal with genotypic frequency of 2.12%.

DNA sequence analysis of PrP gene: PCR-SSCP products

Table 1.

Variants in group	Breed	Frequency (%)
A	Kar 1, Kar 2, Kar 3, Kar 6, Kar 8, Kar 9, Kar 10	78.73
B	Kar 4	8.52
C	Kar 5	4.25
D	Kar 7	2.12
E	Kar 11	6.38

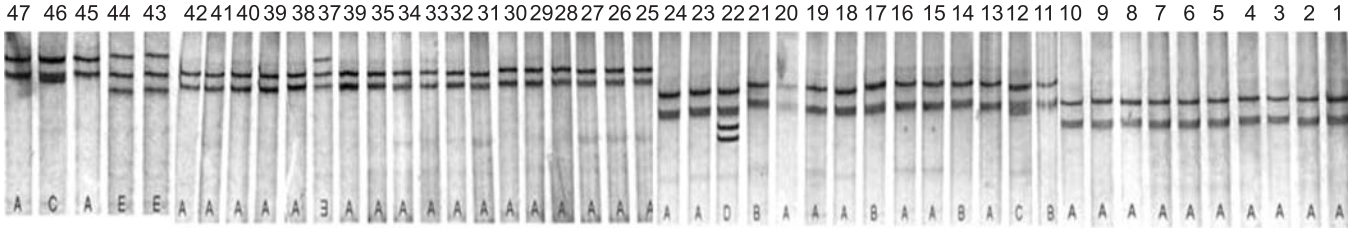
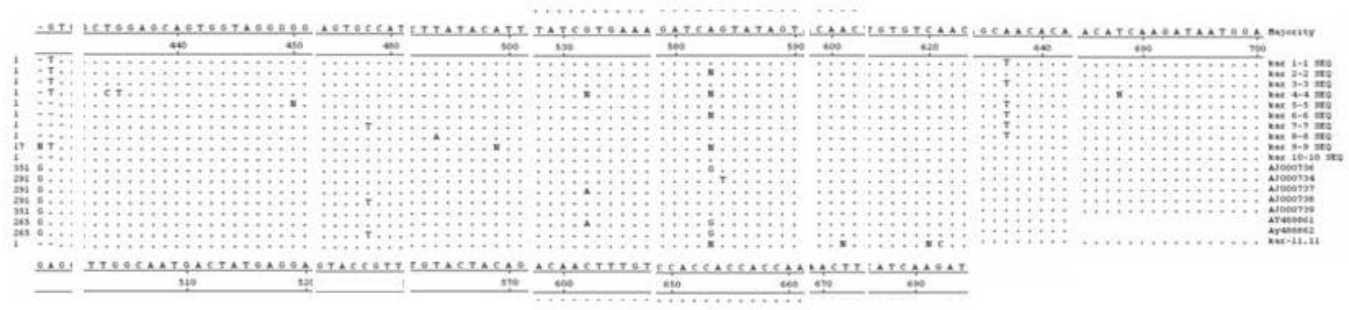


Fig. 1. SSCP gel showing different band pattern (variants) in Karnah sheep.

Table 2. Nucleotide sequence comparison of PrP gene (Exon-3) in Karnah sheep breed



generated by forward primer and reverse primer were sequenced separately. After checking the PCR amplification, purified products were sequenced on ABI-3100 automated sequencer using Hi-DiTM formamide terminator chemistry.

Sequence analysis of Karnah sheep breed: The alignment of 11 nucleotide sequence of Karnah sheep breed PCR - SSCP variants and known gene bank sequences are presented in Table 2. The sequences of this breed were completely aligned with known PrP exon-3 sequences from nucleotide positions 352–700. However, the nucleotide sequences substitution at respective positions in Karnah sequences vis a vis with gene bank sequences are represented by change nucleotide at respective positions. Fig. 2 shows a phylogenic tree of Karnah sheep breed PrP gene exon-3 partial CD's in comparison with known gene bank sequence developed by CLUSTAL-W module of Megalign DNA Star software. Our sequences were distinctly placed in comparison to gene bank sequences. In Karnah sheep breed sequence 9 and 10 are closely related and followed by sequence no 11 while other sequences were placed far distinctly apart from each other with in breed.

Protein translation of PrP gene sequences: The projected translation of genetic codes formed by the triplet of PrP nucleotides were developed using edit sequence module of DNA Star Version 4 software. The amino acid sequence of DNA in these breeds were aligned with amino acid sequence

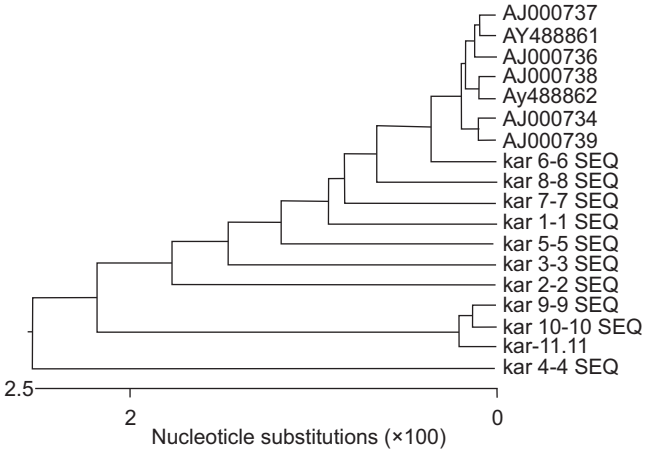


Fig. 2. Phylogenic tree of Karnah sheep PrP nucleotide sequence in comparison with gene bank sequences.

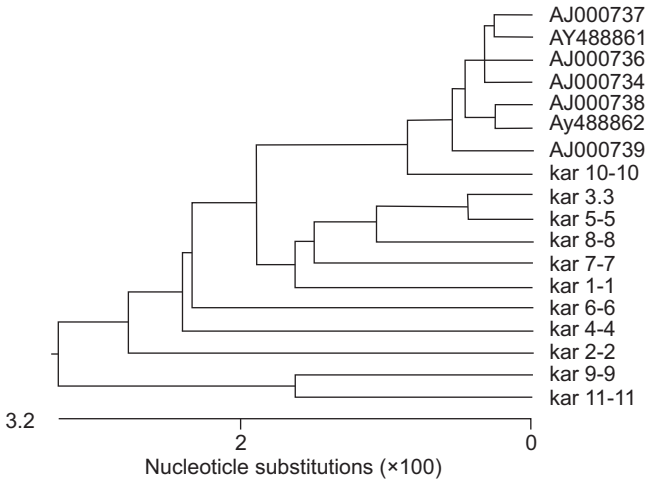


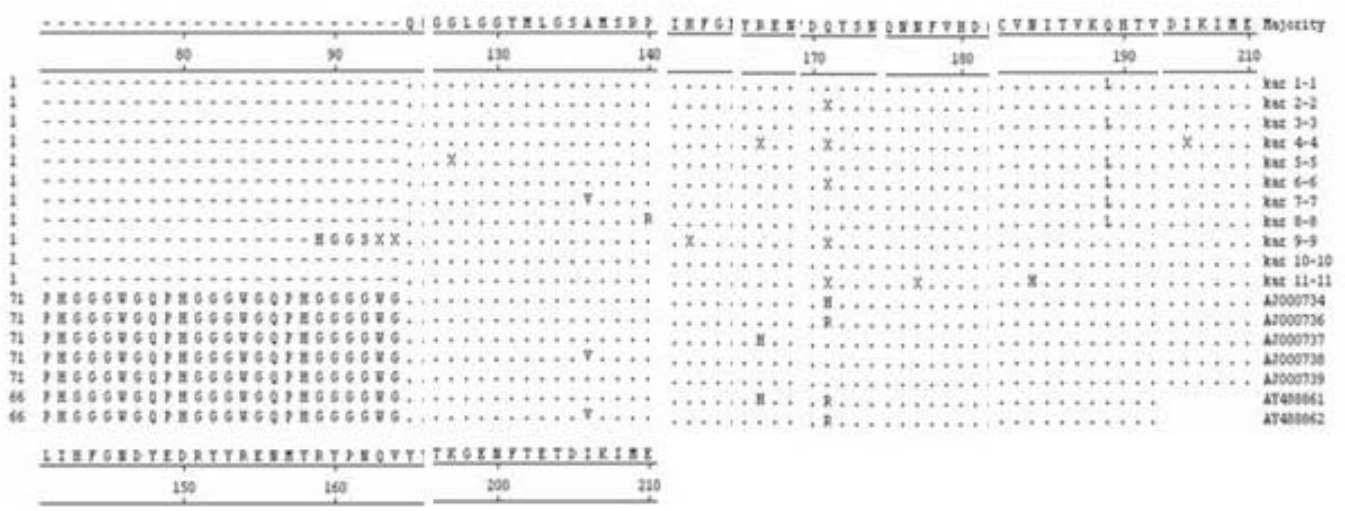
Fig. 3. Phylogenic tree of amino acid sequence (PrP gene Exon-3) in Karnah sheep compared with known gene bank sequence.

of known gene bank accessions from codon number 95 to 210 of complete protein sequences of PrP gene exon-3. The align sequences with similar amino acid pattern has been depicted by dots while altered amino acid are shown by respective symbols.

Protein sequence of PrP gene: The amino acid sequence of Karnah sheep breed translated from PrP gene exon-3 DNA sequences are presented in Table 3 along with amino acid sequences based on gene bank amino acids sequences of PrP gene exon-3 complete CDs'. Our sequences are fully aligned with that of gene bank sequences except at some codon where altered amino acids have been shown. Fig. 3. shows the phylogenic tree of projected amino acid sequences based on Karnah sheep PrP gene exon-3 protein sequences in comparison to that of ovine PrP sequences of gene bank accessions. Majority of Karnah sheep amino acid sequences are distinctly placed in comparison to that of gene bank sequence forming separate clusters except variant no. 10, which is closely aligned with that of gene bank sequence.

Single nucleotide polymorphism in PrP gene: We resolved the DNA fragment of PrP gene exon-3 partial CDs that includes the important codons 136, 154 and 171 for the susceptibility/resistance against the scrapie disease in sheep. We delineated the single nucleotide polymorphism in DNA

Table 3. Amino acid sequence comparison of PrP gene (Exon-3) in Karnah sheep breed



sequences varying from nucleotide positions 352 to 700. The SNP sites were identified in the DNA sequences of different sheep breeds in comparison to respective positions of complete CDs' of ovine PRNP gene exon-3 based on NCBI gene bank accessions.

Out of 750 nucleotide positions 14 showed changes in nucleotide sequences which were resulted into change in amino Acids. Out of these 14 nucleotide positions, 6 (450, 499, 532, 583, 601 and 688) were heterozygous in different breeds. In rest of the nucleotide positions i.e. 434, 435, 478, 490, 494, 620, 621 and 637 were homozygous (Table 4).

SNPs at codons (136, 154 and 171) having association with scrapie disease: At codon 136 two genotypes AA and VV were observed with frequencies of 90.91 and 9.09%. These data are consistent with the observations at V_{136} which is considered a rare allele in sheep population. These findings are in agreement with the earlier reports based on large scale scrapie surveillance in US sheep populations (O'Rourke *et al*. 1996, Stephens, 1998, De Siliva *et al.* 2003). The prevalence of this allele in indigenous sheep is indicative of the susceptibility of indigenous sheep to scrapie; however no report has so far been published where any animal carrying this allele contracted the disease. This could have been either due to small sample size or due to the other management practices that could be important in prevention of onset of disease conditions. The known polymorphism of argenine (R) and histidine (H) were also observed in this study at codon 154. At this codon RR and RH genotypes were present in 90.91 and 9.09% animals. Whereas in Karnah sheep breed the frequencies of 'QQ' and 'QR' genotypes were 54.55 and 45.45%, respectively. 'Q' allele at 171 loci alone has been reported to be positively correlated with naturally occurring incidence of scrapie in sheep (Hunter *et al.* 1994, De Silva *et al*. 2003). In present study, majority of sheep breeds surveyed

Table 4. SNP in PrP gene (Exon-3) in Karnah sheep breed

Nucleotide position	SNP	Homozygous/ Heterozygous	Type (AA→AA)	Breed
434	T/C	Homozygous	Synonymous	Kar 4
435	G/T	Homozygous	Synonymous	Kar 4
450	G/A	Heterozygous	Non synonymous (G→S)	Kar 5
478	C/T	Homozygous	Non synonymous (A→V)	Kar 7
490	C/G	Homozygous	Synonymous	Kar 8
494	T/A	Homozygous	Non synonymous (P→R)	Kar 8
499	A/G	Heterozygous	Non synonymous (H→R)	Kar 9
532	G/A	Heterozygous	Non synonymous (R→H)	Kar 4
583	A/G	Heterozygous	Non synonymous (Q→R)	Kar 2, 4, 6, 9, 11
601	A/G	Heterozygous	Non synonymous (N→S)	Kar 11
620	C/N	Homozygous	Synonymous	Kar 11
621	A/C	Homozygous	Non synonymous (N→H)	Kar 11
637	A/T	Homozygous	Non synonymous (Q→L)	Kar 1, 3, 5, 6, 7, 8
688	T/C	Heterozygous	Non synonymous (I→L)	Kar 4

posses the 'Q' allele in homozygous condition at locus 171 but none of the animal showed any clinical symptoms of scrapie so far. Zlotnik and Katiyar (1961) documented that 4 animals died because of scrapie infection. However, their genotypes were not studied using molecular markers. These results suggest that either some mutation or polymorphism at other codons or loci is protecting the animals from getting infected with prion protein or it is the managemental practices of sheep rearing solely on range grazing without any concentrate supplements probably prevents the onset of scrapie disease symptoms. These results have important bearing on prevention of scrapie disease in sheep globally through traditional management of sheep on forages. It has been very well documented even in BSE outbreaks in cattle population of United Kingdom that only the animals fed on animal protein showed the symptoms of mad cow syndrome.

SNPs at other codons in indigenous sheep breeds: The frequencies of GG and GS genotypes at codon 127 in Karnah sheep were 90.91 and 9.01%. At codon 140 genotypes PP and RR were observed with frequencies of 90.91 and 9.09%. This was also a new SNP, which was observed in this indigenous breed as no earlier studies reported this polymorphism. At codon 143, A/G nucleotide substitution at 499 position resulted into the SNP with amino acid change from H (histidine) → R (arginine) in 9.09% animals of Karnah sheep breed. This polymorphism was reported earlier by De Silva *et al.* (2003) in US sheep breeds. It is possible that these alleles present in Karnah sheep breed because of immigration through some degree crossing with exotic sheep breeds like Rambouillet imported from USA. The frequencies of NN and HH genotypes at codon 184 were 90.91 and 9.09%, respectively. The frequencies of QQ and QL genotype were 45.45 and 54.55% at codon 189 in Karnah sheep breed. These are new SNPs which were observed in this indigenous breed as no earlier studies reported this polymorphism. Our results are in confirmation with the earlier reports of PCR-SSCP variants in PrP gene at exon-3 in other breeds (Zhao *et al.* 2005). However they reported 9 PCR-SSCP variants in 400 sheep from 6 different breeds, e.g. Awassi, Borderdale, Corriedale, Finnish Landrace, Merino and Romney. We found that unlike earlier methods, e.g. PCR-RFLP, which determine PrP genotype by detecting the nucleotide sequence at specific positions, PCR-SSCP technique profiles an extended region of exon-3, the key exon in determining PrP genotype. This endows the PCR-SSCP method with several technical advantages over the other methods; first it is able to determine the A/V_{136}-H/R_{154}-H/Q/R/K_{171} haplotype, which is considered important in determining scrapie susceptibility (Baylis and Goldmann 2004). Second, the resultant SSCP profiles are uncomplicated and can be easily interpreted either manually or automatically with a gel reader. Third, the technique allows for the visualization of individual amplimers, and this can readily identify erroneous results, including the presence of contaminating DNA sequences or unexpected amplification products. Fourth, because the PCR-SSCP technique scans the whole sequence of the gene region amplified, it has the potential to identify unknown or new allelic variations. Finally, the PCR-SSCP approach is less expensive, particularly, when large numbers of samples require analysis.

In total 10 SNPs have been observed in this study including the principal SNPs at 136, 154 and 171 codons. The significance of the polymorphism at other codons in prevention of scrapie disease susceptibility in indigenous breed cannot be ruled out because sheep carrying known susceptible alleles at 136 AA/VV, at 154 RR and at 171 QQ did not show any symptoms of naturally occurring scrapie. Similar views were also held by Westaway *et al.* (1994) and Hunter (1996). Further these results carry specific significance in confirming the concept of Hunter *et al.* (1994), where it has been suggested that despite the positions of susceptible alleles in animal may not show the symptoms of naturally occurring scrapie because additional factors might be involved in control of scrapie including genotype × environment interactions. In this case, our results are of special significance and can have a reaching consequences in prevention of scrapie disease symptoms in sheep globally. Before, this concept is applied globally, more detailed genetic survey in larger population is warranted by molecular markers using high throughput screening protocols.

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