

# Mitochondrial haplotypes reveal a strong genetic structure for three Indian sheep breeds

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## Summary

This survey represents the first characterization of mitochondrial DNA diversity within three breeds of Indian sheep (two strains of the Deccani breed, as well as the Bannur and Garole breeds) from different geographic regions and with divergent phenotypic characteristics. A 1061-bp fragment of the mitochondrial genome spanning the control region, a portion of the 12S rRNA gene and the complete phenyl tRNA gene, was sequenced from 73 animals and compared with the corresponding published sequence from European and Asian breeds and the European Mouflon (*Ovis musimon*). Analysis of all 156 sequences revealed 73 haplotypes, 52 of which belonged to the Indian breeds. The three Indian breeds had no haplotypes in common, but one Indian haplotype was shared with European and other Asian breeds. The highest nucleotide and haplotype diversity was observed in the Bannur breed (0.00355 and 0.981 respectively), while the minimum was in the Sangamneri strain of the Deccani breed (0.00167 and 0.882 respectively). All 52 Indian haplotypes belonged to mitochondrial lineage A. Therefore, these Indian sheep are distinct from other Asian and European breeds studied so far. The relationships among the haplotypes showed strong breed structure and almost no introgression among these Indian breeds, consistent with Indian sheep husbandry, which discourages genetic exchange between breeds. These results have implications for the conservation of India's ovine biodiversity and suggest a common origin for the breeds investigated.

**Keywords** genetic diversity, Indian sheep, mitochondrial DNA, mitochondrial lineage, phylogeographic distribution, sheep domestication.

## Introduction

The domestic sheep is an important livestock species in many developing countries. In India, livestock represent an integral part of the farming system and rural economy. Traditionally, Indian sheep are reared by various communities such as Dhangar in Maharashtra, Guddi in Himachal Pradesh and Raika in Rajasthan, whose traditional occupation is sheep breeding (Karve 1961). These communities follow strict rules for the migration, distribution and conservation of resources. Sheep are reared under the transhumance system of management, wherein migration is determined by the availability of fodder, the farming system and the climatic conditions. Movement usually does not

occur between states, and the migratory route of each shepherd community is often fixed. Such rigid management and the reluctance of sheep herding communities to give up their traditional practices and customs have likely been responsible for the maintenance of sheep breed diversity in India (Acharya 1982).

In the present study, we have analysed three economically important Indian sheep breeds, which have very different phenotypic characteristics and geographic ranges. Garole are tiny (approximately 14 kg adult weight), prolific sheep from the swampy delta region of the Ganga River, known as Sunderban, West Bengal (Ghalsasi & Nimbkar 1993; Fahmy & Mason 1996). It is a coarse-wool sheep breed, highly resistant to various diseases (foot rot, gastrointestinal diseases) and parasites, and is well adapted for grazing in wet conditions. It is mainly reared for meat, although the meat is considered tough and is priced 15–20% lower than that of other breeds (Sodhi *et al.* 2003). The other two breeds, Deccani and Bannur, are both from the southern peninsular region of India, which has the

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largest sheep population (20.5 million) and accounts for 51.4% of the total sheep population of the country (Acharya 1982). The Deccani is a medium-sized coarse-wool breed that is reared mainly for meat. Animals of this breed can survive in the semi-arid environment of the Maharashtra state. Rams are horned and ewes are polled. Its fleece is extremely coarse, hairy and open. The breed is very well adapted for long migration, poor nutrition and drought, and is resistant to tropical diseases. Five strains of Deccani, viz. Kolhapuri, Lonand, Madgyal, Sangamneri and Sangola, have been reported (Ghanekar 1983). The breed is predominantly black or black with white markings; however, white and brown/fawn animals are also found, particularly in Lonand and Sangamneri. Two of the five strains of Deccani (Lonand and Sangamneri) were examined as part of this work. The third breed, Bannur, is a relatively small, meat sheep from Karnataka state, and is favoured for its body conformation. It is white in colour, but in some cases, the face is light brown and this colour may extend to the neck. Its ears are long, leafy and drooping, and its coat is extremely coarse and hairy. A large percentage of animals carry wattles, and both sexes are polled (Acharya 1982).

To date, there have not been an extensive evaluation of the genetic diversity existing within Indian sheep. A recent examination of three Indian breeds using a set of 11 autosomal microsatellites (Mukesh *et al.* 2006) revealed high levels of allelic diversity. Such studies are important to document the population structure, priority for conservation and likely origin of Indian sheep. The sequence variation existing within the mitochondrial DNA (mtDNA) has proven particularly useful for addressing such questions across a number of livestock species. The observation that mtDNA haplotypes group together in distinct clades has revealed insights into the history of domestication in cattle (Loftus *et al.* 1994; Bradley *et al.* 1996), pigs (Giuffra *et al.* 2000), goats (Luikart *et al.* 2001) and horses (Vila *et al.* 2001). Domestic sheep have a global distribution and a growing number of genetic surveys have been conducted, which report on the haplotypes and mix of mtDNA lineages observed within the indigenous breeds. A general finding has been the presence of multiple mtDNA lineages within breeds, independent of their geographic location or phenotype. A survey of 19 Chinese breeds found that 15 contained a mixture of haplotypes from lineages A, B and C (Chen *et al.* 2006). An investigation of five Turkish breeds reported the same result (Pedrosa *et al.* 2005), while an examination of a range of European breeds found the presence of both lineage types A and B occurring at differing frequencies (Meadows *et al.* 2005; Pereira *et al.* 2006). The existence of multiple mtDNA lineages and their mixing within breeds has been interpreted as evidence for multiple domestication events and subsequent human-mediated introgression between domestic sheep populations. To date, no mtDNA sequence has been reported from any Indian breed; however, based on three PCR-RFLP tests, Meadows

*et al.* (2005) reported preliminary evidence suggesting that Garole animals carried the clade A sequence.

The objective of this study was to investigate the genetic structure of three Indian sheep breeds using mtDNA sequence variation. The genetic relationship among the breeds was examined and compared with other European and Asian populations to begin a detailed documentation of India's ovine biodiversity.

## Materials and methods

### Sample collection and DNA isolation

Blood samples of 73 Indian sheep were collected from local populations of Garole ( $n = 18$ , from eight flocks, 24 South Parganas district, West Bengal), two strains of Deccani (Sangamneri:  $n = 18$ , from 10 flocks, Ahmadnagar district, Maharashtra; Lonand:  $n = 16$ , from 12 flocks, Pune district, Maharashtra) and Bannur ( $n = 21$ , from 16 flocks, Karnataka; Table 1 and Fig. 1). Care was taken to collect samples from unrelated individuals based on information provided by the shepherds. DNA was isolated from the samples using the method by Miller *et al.* (1988) with minor modifications.

### PCR amplification and mtDNA sequencing

The mtDNA, which spans the mitochondrial control region (CR), part of the 12S rRNA coding region (*MT-RNR1*) and the tRNA<sup>Phe</sup> (*MT-TF*) gene, was sequenced using primers designed from the complete ovine mtDNA sequence (AF010406; Hiendleder *et al.* 1998). Primers mtCR-F2 (5'-AACTGCTTGACCGTACATAGTA-3') and mtCR-R1 (5'-AGAAGGGTATAAAGCACCGCC-3') were used to amplify the 1246-bp region of the mtDNA (Meadows *et al.* 2005). The resulting PCR products were bidirectionally sequenced using the DYEnamic ET sequencing kit and the MegaBACE 1000 DNA Analysis System (Amersham Biosciences).

### Data analysis

The DNA sequence traces were edited manually by inspecting electrophorograms using CHROMAS LITE 2.01 (<http://www.technelysium.com.au>). The sequences from 73 Indian sheep (EF056393–EF056465) and published sequences ( $n = 83$ ; AY879343, AY879347–AY879387, AY879409–AY879432, AY879436–AY879439, AY879442–AY879447, AY879451–AY879457) representing four European breeds (Tyrolean mountain sheep,  $n = 18$ ; Tyrolean stone sheep,  $n = 13$ ; Forest sheep,  $n = 12$ ; Carylthian sheep,  $n = 18$ ), the European Mouflon ( $n = 4$ ) and an Asian breed, Javanese Thin Tail ( $n = 18$ ), were aligned using CLUSTALX 1.83 (Thompson *et al.* 1997). The alignments were imported in MEGA 3.1 (Kumar *et al.* 2004), trimmed to a minimum of 1061 bp as described by Meadows *et al.* (2005)

**Table 1** Estimates of genetic diversity within the sheep populations.

Breed/strain	<i>n</i>	Hn <sup>1</sup>	P sites <sup>2</sup>	M sites <sup>3</sup>	$\pi$ <sup>4</sup>	Hd <sup>5</sup>	<i>D</i> <sup>6</sup>
Indian populations							
Bannur	21	18	17	17	0.00355	0.981	3.762
Garole	18	13	12	13	0.00221	0.941	2.340
Deccani (Lonand)	16	10	10	10	0.00204	0.900	2.167
Deccani (Sangamneri)	18	11	9	9	0.00167	0.882	1.771
Population summaries <sup>7</sup>							
All animals	156	73	71	75	0.00620	0.962	6.566
Indian animals	73	52	33	36	0.00394	0.984	4.168
Asian animals	91	57	37	41	0.00512	0.972	5.427
European animals	65	19	21	21	0.00327	0.857	3.467
Type 'A' animals	86	55	35	38	0.00371	0.977	3.927
Type 'B' animals	70	18	16	16	0.00160	0.845	1.696

<sup>1</sup>Observed haplotypes (Hn).

<sup>2</sup>Phylogenetically informative sites (P sites).

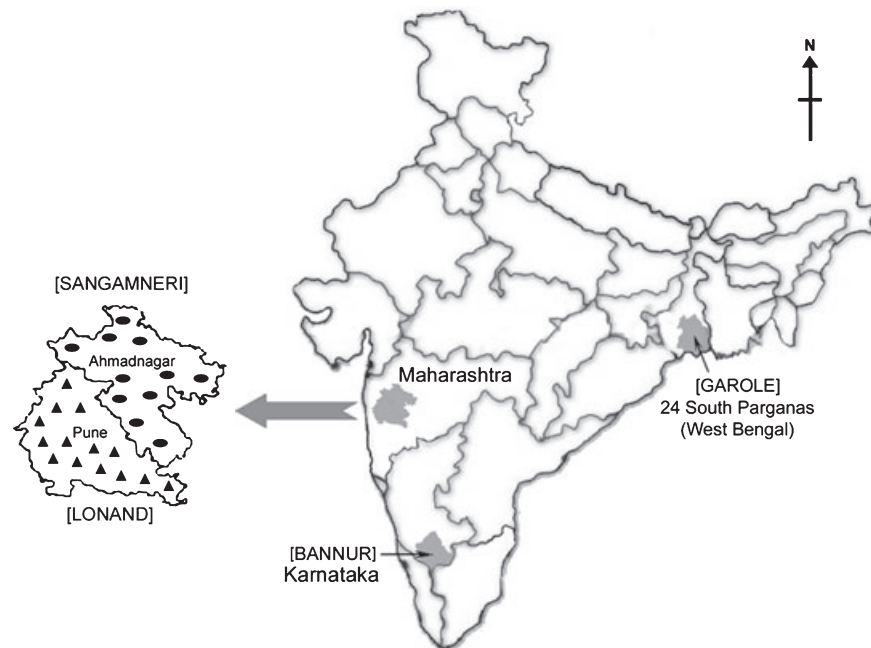
<sup>3</sup>Mutations (M sites).

<sup>4</sup>Population nucleotide diversity ( $\pi$ ).

<sup>5</sup>Haplotype diversity (Hd).

<sup>6</sup>Average number of nucleotide differences (including indels) between haplotypes (*D*).

<sup>7</sup>Summary statistics were calculated following separation of the total data set into either geographical origin (Indian, Asian or European) or mitochondrial lineage (A or B).

**Figure 1** Geographic distribution of the Deccani (two strains, Sangamneri and Lonand), Bannur and Garole breeds in India.

and inspected for the presence of singletons (polymorphic sites appearing in only one animal) and parsimony-informative sites (which appear in more than one animal). For the current analysis, the singletons were replaced with the consensus base, as they were not considered phylogenetically informative. However, singletons remain in the sequences submitted to GenBank. Diversity parameters such as nucleotide diversity ( $\pi$ ) and haplotype diversity (Hd) were calcu-

lated using DNASP 4.10 (Rozas *et al.* 2003). Similarly, sequence divergence between the two mitochondrial lineages (A and B) and between the two geographic regions (Asia and Europe) were calculated in terms of the average number of nucleotide differences between the haplotypes (*D*) and the average number of nucleotide substitutions per site between the populations (*K*) using DNASP 4.10. Genetic distance was estimated using Kimura's 2-parameter method, and a

neighbour-joining haplotype tree was constructed using MEGA 3.1. The median-joining network (Bandelt *et al.* 1999) was calculated to investigate the relationship between haplotypes using NETWORK 4.1.1.2 (<http://www.fluxus-engineering.com>). Nucleotide weighing ( $w$ ) was set to reflect the difference in mutational frequency between transversions ( $w = 20$ ) and transitions ( $w = 10$ ) before a conservative network (with  $\varepsilon = 0$ ) was generated using mtDNA sequence from 91 Asian animals (73 Indian and 18 Javanese Thin Tail).

## Results

### mtDNA sequence variation

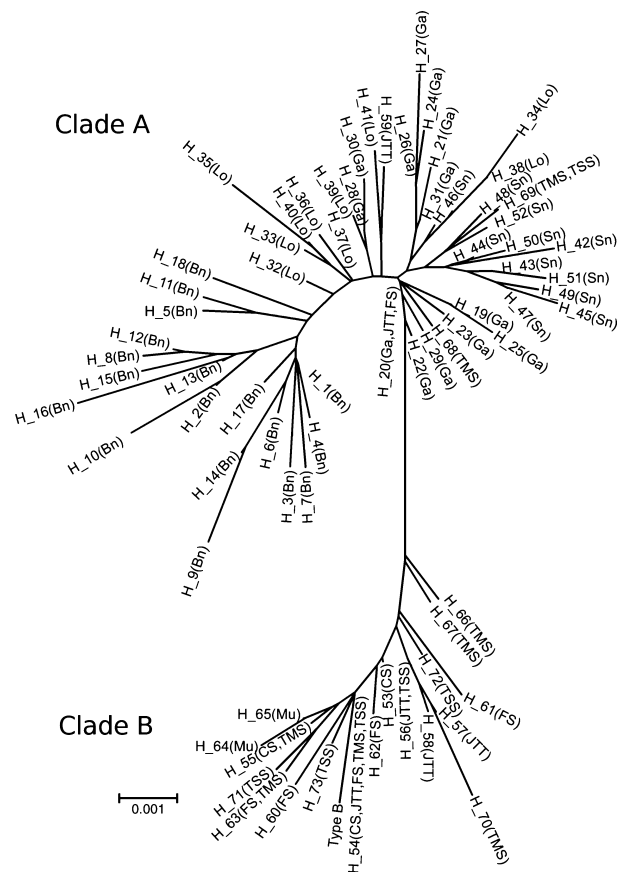
Breed-specific estimates of genetic diversity (Table 1) indicated that Bannur contained the highest and Sangamneri the lowest nucleotide diversity ( $\pi$ ) within the Indian populations. Indian breeds contained variable numbers of polymorphic sites (9–17), although the nucleotide diversity ( $\pi$ ), average number of differences ( $D$ ) and haplotype diversity ( $H_d$ ) were broadly similar (Table 1). As expected, analysis between the Asian and European breeds revealed high sequence diversity ( $K = 0.231$ ,  $D = 9.498$ ), which was slightly less than that observed between the two (A and B) mtDNA lineages ( $K = 0.268$ ,  $D = 10.993$ ; Table 2). Published sequences spanning the same mtDNA segment from 83 animals from other Asian and European sheep were used to facilitate comparison against the characterized haplotypes. Analysis of the combined data set of 156 sequences revealed 71 positions with nucleotide substitutions. Most (41) were phylogenetically informative while the remainder (30) were singletons. In addition to nucleotide substitutions resulting in alleles AF010406.1:m.401A and m.402A in Garole and Sangamneri, as well as allele m.422C in seven animals of Bannur, two indels were observed. Both indels were T insertions: the first was observed in a published European and Asian sequence (Meadows *et al.* 2005) and the second appeared fixed in the Bannur and Lonand breeds (see positions 16473 and 448 respectively in Table S1).

### mtDNA haplotypes within Indian sheep

The 41 phylogenetically informative substitutions and two indels defined 73 mtDNA haplotypes, 52 of which were

observed in Indian animals. The frequency and breed membership of each haplotype are presented in Table S1. Interestingly, there were no haplotypes common across Indian breeds. Even the two strains of Deccani, the Lonand and Sangamneri, which are in geographic proximity (Fig. 1), did not contain a common haplotype among the 34 animals sequenced. The only haplotype that was found in an Indian animal and another breed was H\_20, which was observed in four Garole, three Javanese Thin Tail and two European animals. There were five other haplotypes that were shared among the non-Indian breeds while 67 were unique (Table S1).

The haplotype structure was examined by calculation of mismatch distribution (pairwise combinations of sequence differences), which showed two distinct peaks (data not shown). This is consistent with the presence of the two major mtDNA lineages, A and B (Meadows *et al.* 2005). To determine the clade membership of each haplotype, a neighbour-joining tree was constructed (Fig. 2) using the



**Figure 2** Neighbour-joining tree of 73 mitochondrial sheep haplotypes found within 156 Asian and European sheep including Mouflon, a wild sheep. Breed names are abbreviated as Bannur (Bn), Garole (Ga), Lonand (Lo), Sangamneri (Sn), Javanese Thin Tail (JTT), Forest sheep (FS), Carynthian sheep (CS), Tyrolean mountain sheep (TMS) Tyrolean stone sheep (TSS) and Mouflon (Mu). The Type 'B' sequence refers to the reference mitochondrial sequence (AF010406; Hiendleder *et al.* 1998).

**Table 2** Sequence divergence between the populations.

Populations	$K^1$	$D^2$	Shared mutations
Asian/European	0.231	9.498	17
Type A/Type B	0.268	10.993	11

<sup>1</sup>Average number of nucleotide differences between the populations.

<sup>2</sup>Average number of nucleotide substitutions per site between the populations.

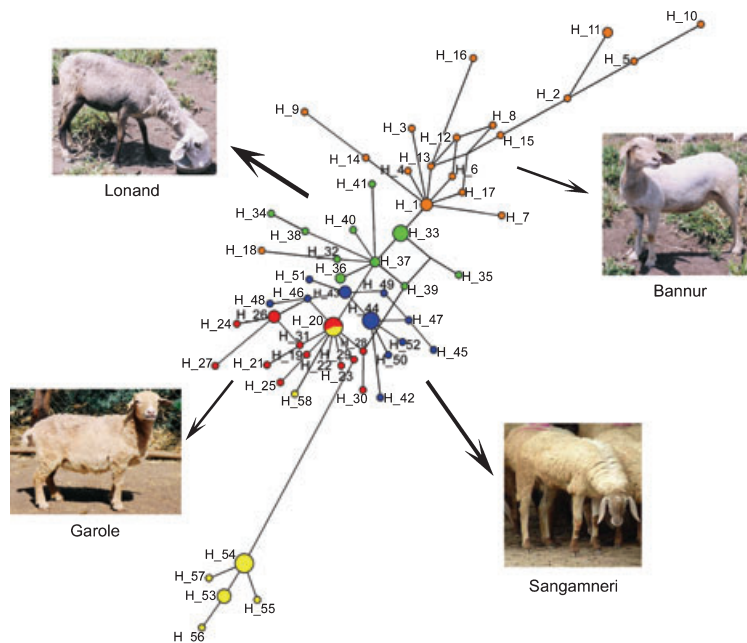
73 haplotypes. The tree contained two distinct branches: clades A and B. All the 73 Indian sheep carried a haplotype, which clustered with previously characterized clade A sequences, while most of the European breeds carried clade B sequences. Clade C sequences, reported at low frequency in Asian, Middle Eastern and European breeds (Guo *et al.* 2005; Pedrosa *et al.* 2005; Pereira *et al.* 2006), were not observed in the Indian sheep analysed. Within the clade A branch, four clusters were observed, which loosely discriminated the breeds (Fig. 2). All haplotypes of the Bannur breed formed a distinct cluster, while those of the Lonand and Sangamneri strains were predominant in two other clusters. Two Garole haplotypes and one Javanese Thin Tail haplotype shared the Lonand cluster, whereas a few of the Garole (five), Lonand (two) and European (one) haplotypes shared the Sangamneri cluster. The fourth cluster included haplotypes mostly from the Garole breed, along with the shared H<sub>20</sub> haplotype and one haplotype from the Tyrolean mountain sheep (a European breed).

To further investigate the possibility of substructure existing within the clade A, the 58 mtDNA haplotypes from Asian individuals were used to construct a median-joining network (Fig. 3). For each haplotype, clear correspondence was evident between the regions of the network and the breed of origin. This was most obvious for the 18 unique Bannur haplotypes, which were interconnected to each other by 21 links and to the rest of the network via a single connection. Similarly, the Deccani haplotypes were predominantly located in the middle of the network along with the Garole. All but the H<sub>58</sub> Javanese Thin Tail haplotypes were separated from the Indian haplotypes (Fig. 3).

## Discussion

The exclusive presence of a single mtDNA lineage in the analysed breeds characterizes these Indian sheep breeds separately from other populations sampled to date. This finding may arise from high levels of relatedness and a low effective population size ( $N_e$ ) within the animals sequenced. Sequence analysis of the mtDNA from Indian sheep showed high genetic diversity within the breeds. The haplotype diversity in the analysed breeds was not only higher than that in other European breeds, but also the haplotypes in the Indian breeds were due to mutations mostly at positions different from the mutational positions observed within the European breeds and the other Asian breed, Javanese Thin Tail (Table S1). However, considering multiple sampling of phenotypically distinct breeds and moderate-to-high levels of both observed nucleotide ( $\pi$ ) and haplotype (Hd) diversity (Table 1), low  $N_e$  appears unlikely. The alternative explanation is a single origin for the three Indian breeds and a lack of subsequent introgression from European breeds expected to carry the Type B mtDNA lineage (Meadows *et al.* 2005; Pereira *et al.* 2006). However, more Indian sheep breeds need to be analysed to confirm these findings.

The relationship between the Indian sheep mtDNA haplotypes was further examined using a median-joining network (Fig. 3). Three striking differences were found when compared to a similar analysis of Chinese breed Type A sequences (Chen *et al.* 2006). The Indian network had no clearly defined central haplotype, revealed a lack of haplotype sharing between Indian populations and displayed very strong breed structure. This is in direct contrast to the Chinese sheep haplotype network, which had a single



**Figure 3** Median-joining network ( $\epsilon = 0$ ) of the mitochondrial haplotypes observed in the Asian domestic sheep breeds: Lonand (green), Bannur (orange), Garole (red), Sangamneri (blue) and Javanese Thin Tail sheep (yellow). The areas of the circles are proportional to the frequencies of the samples.

high-frequency central haplotype, extensive haplotype sharing and weak breed structure (Chen *et al.* 2006; Fig. 2). The network for Indian sheep suggests a history for these breeds defined by very low levels of interbreeding between populations and total reproductive isolation. This is entirely consistent with Indian sheep husbandry, which contains long-standing and strong cultural and traditional barriers and discourages genetic exchange between the breeds. In addition to the unique animal husbandry practice, the breeds under investigation have undergone selection in different climates and in different geographic regions of the subcontinent. Together, the result is a strong genetic structure with mtDNA haplotypes that are diagnostic of particular breeds. This has relevance to considerations of bioconservation, as each of the three breeds tested appear to be genetically, as well as phenotypically, distinct. The genetic structure appears to extend even below the breed level. Lonand and Sangamneri, the two strains of Deccani, did not share a single haplotype and occupied distinct but neighbouring sections of the haplotype network (Fig. 3), indicating that they are genetically separate. This suggests that these strains might actually be different breeds rather than strains of the Deccani breed. However, this needs to be further analysed using nuclear markers and phenotypic studies. Among the Indian haplotypes, only one haplotype (H\_20) was shared by four Garole, two Javanese Thin Tail and two European animals (Table S1). The Garole and Javanese Thin Tail have previously been shown to share the same mutation, which underpins the *FecB* prolificacy phenotype (Davis *et al.* 2002). H\_20, therefore, appears to be the maternal signature of this genetic link. While it cannot be directly inferred from the present molecular data, the transport of Garoles south into the Indonesian archipelago is the most likely direction of this migration (Davis *et al.* 2002).

A number of questions remain to be answered concerning the number, timing and location of domestication events that gave rise to modern domestic sheep. The global existence of at least five mtDNA lineages may have arisen following a single domestication event, which sampled a heterogeneous ancestral population. Alternatively, each mtDNA lineage might be the result of biologically, geographically and/or temporally distinct events. The finding in this study that multiple breeds from the Indian subcontinent contain a single mtDNA lineage provides evidence for the second hypothesis. Each breed appears to be derived from the same domestication event, but the breeds appear to have diversified because of climatic conditions and selection pressure for specific needs. Each breed has subsequently remained free of introgression. As a result, the Indian breeds examined in this study contain a strong genetic structure, which remains uncomplicated by the influence of mixture and interbreeding. Analysis of additional Indian breeds is required to determine if this is a general phenomenon of Indian sheep breeds.

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### Supplementary Material

The following supplementary material is available for this article online from <http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2007.01636.x>

**Table S1** Haplotype definitions of Asian and European sheep populations.

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