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Further evidence for paternal inheritance of mitochondrial DNA in the sheep (*Ovis aries*)

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The mitochondrial DNA of 172 sheep from 48 families were typed by using PCR-RFLP, direct amplification of the repeated sequence domain and sequencing analysis. The mitochondrial DNA from three lambs in two half-sib families were found to show paternal inheritance. Our findings provide direct evidence of paternal inheritance of mitochondria DNA in sheep. A total of 12 highly polymorphic microsatellite markers, which mapped on different chromosomes, were employed to type the sheep population to confirm family relationships. Possible mechanisms of paternal inheritance are discussed.
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Introduction

Mitochondrial DNA (mtDNA) is the only extranuclear genome in the animal cytoplasm, and exists as multiple copies with a high mutation rate. Mammalian mtDNAs include 37 genes, including 13 polypeptide-encoding genes, 22 tRNA genes and two rRNA genes, which encode essential components of oxidative phosphorylation (OXPHOS) in mitochondrial inner membrane, generating cellular energy in the main form of adenosine triphosphate (ATP). In recent years, the structure and function of mtDNAs have been a popular area of research in molecular evolution, classification, population genetic analysis, the identification of relatives, forensic science, aging, disease diagnosis, apoptosis and the analysis of quantitative trait loci (QTL) (Albuquerque *et al*, 1998; Wallace, 1999; Gray *et al*, 1999; Sutovsky and Schatten, 2000).

Animal mtDNA has been thought to be strictly following maternal inheritance. A typical experiment was the study of the mtDNA RFLP of the reciprocal hybrid lines between horse (*Equus caballus*) and donkey (*Equus asinus*). The mules' mtDNA patterns were identical to those of the horse, while the hinnies exhibited a pattern identical to the donkey (Hutchison *et al*, 1974). However, evidence for occasional paternal inheritance has been reported in animals including the fruit fly *Drosophila melanogaster* (Kondo *et al*, 1992), human *Homo sapiens* (Schwartz and Vissing, 2002), mouse *Mus musculus* (Gyllensten *et al*, 1991; Shitara *et al*, 1998), cattle *Bos Taurus* (Steinborn *et al*, 1998), *Lepidopteran* insects (Lansman *et al*, 1983) and honeybee *Apis mellifera L* (Meusel and Moritz, 1993). In the marine mussel *Mytilus edulis*, the paternal mtDNA was proven to be more extensive (Sutherland *et al*, 1998).

The complete mtDNA sequence has been determined in sheep (Hiendleder *et al*, 1998). The displacement loop region (D-loop) is the noncoding control region of the mtDNA, and plays an important role in replication and transcription. Within the D-loop there is a tandem repeated sequence region with a motif, 75 bp in length, sited between the Pro-tRNA gene and a conserved sequence block 1 (CSB 1) (Zardoya *et al*, 1995). Paternal inheritance in an ovine family (named Pmt family) was reported by Zhao *et al* (2001), but the pedigree of Pmt family was not checked. In this work, we traced the ewe in the Pmt family and found the ewe mated with a randomly selected ram produced another family, which again showed paternal mtDNA inheritance. To confirm the paternal inheritance in these families, we have investigated not only by checking DNA polymorphisms in mtDNA D-loop (within the non-coding region) and in COI gene (within the coding region) but also by the direct sequencing of the repeated region in the mtDNA D-loop. Subsequently, the pedigree of the Pmt families was also checked with 12 highly polymorphic microsatellite markers to confirm blood relationship. Our work has provided further solid evidence for paternal inheritance of mtDNA in the sheep.

Materials and methods

DNA samples

Blood samples from 172 sheep of 48 hybrid families produced by the Dorset breed (male line) crossed to the Small-tail Han breed (female line, a Chinese local breed) were collected and preserved in ACD buffer until DNA purification with SDS, proteinase K and phenol. DNA was isolated from the whole blood using standard SDS/proteinase K methods and phenol/chloroform extractions (Sambrook *et al*, 1989).

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Analysis of mtDNA

In all, 50 μl of each PCR mixture containing 100 ng of total DNA, 50 pmol of each primer, 50 μl of 10 × *Taq* polymerase buffer solution and 1 U of *Taq* polymerase for PCR was incubated in the thermal cycler (PE Amp2400, Peking–Elmer, USA). The primer sequences and products yielded are listed in Table 1, and the primers locations in the mtDNA D-loop are illustrated in Figure 1. Products were separated in 1% agarose gels in 1 × TAE, pH 8.0. The excised fragments were purified with the Geneclean kit (Biolab LTD) and inserted in the pGem-T Easy vector (Promega, USA), following the protocols of the manufacturers. Clones were purified with the Geneclean kit and sequenced by an ABI 377 DNA sequencer. For each fragment, sequences were based on the analysis of two independent clones.

PCR-RFLP analysis

The PCR products cut from agarose gels were purified by Geneclean kit (Biolab LTD). Purified PCR product (5 μl) was mixed with 2 U restriction enzymes and 1.2 μl 10-fold buffer solution, and ddH_2O was added until the total volume reached 12 μl. The mixture was incubated at 37°C for 4 h.

Microsatellite genotyping

In order to confirm the paternity or maternity of each family, 12 highly polymorphic microsatellite markers distributed on nine chromosomes were scored using the method of Crawford *et al*, 1995). The nucleotide sequences of the primers are shown in Table 2.

Table 1 The primer nucleotide sequences and yielded products of mtDNA

Primer names	*Primer sequences*	*Yielded products*	*Fragment length of PCR products/bp*
P1F P4R	5′-CAACACCCAAAGCTGAAGTTC-3′ 5′-CTAGGCATTTTCAGTGCCTTGC-3′	D-loop	963+75*n*
P2F P3R	5′-TTATGTCTGTCTTGAATGTGCT-3′ 5′-CCCTACATGCTTCAGTGAAGTG-3′	Repeated sequence region	40+75*n*
COIF COIR	5′-GCAGAGTTTGAAGCTGCT-3 5′-AGCTGACGTGAAGTAAGC-3′	Partial COI Partial $tRNA^{Cys}$ $tRNA^{Tyr}$	1053

n: repeats number.

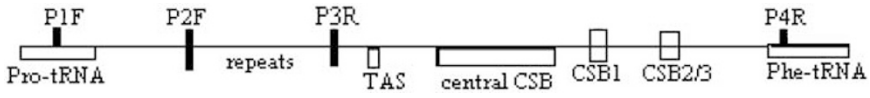

Figure 1 Structure of sheep mtDNA D-loop and the location of the primers. CSB, conserved sequence block; TAS, termination-associated sequence.

Table 2 The information of the nucleotide sequences of the primers

Microsatellite	*Primer nt sequence*	*Het. (%)*	*Chro.*	*Size (bp)*	*Accession no.*
OarAE101	F: ttcttatagatgcactcaagctagg R: taagaaatatatttgaaaaaactgtatctccc	75	6	99–123	L13692
OarHH55	F: gttattccatattctttcctccatcataagc R: ccacacagcgcaactaaaacccagc	64	6	119–139	L13693
OarHH35	F: aattgcattcagtatctttaacatctggc R: atgaaaatataaagagaatgaaccacacgg	87	4	117–155	L12554
IL2RA	F: agcagaggtacaggtggtaagca R: gatatgccttggagaaggtagcgtat	70	13	172–192	[a]
MT2	F: tgaggaggattcgttaccgtctgag R: ccgaggggcagcggattaag	79	14	149–227	[a]
CSSM31	F: ccaagtttagtacttgtaagtaga R: gactctctagcactttatctgtgt	79	23	172	U03838
MAF4	F: aaaggtcttgactctgtgtcgg R: tccacggggtctcaaagagtcg	86	1	157–221	M61729
OARAE64	F: tgcaagaagggcagaccttggag R: cagaccactctcttccctccacg	89	7	122–158	L13869
OARCP49	F: cagacacggcttagcaactaaacgc R: gtggggatgaatattccttcataagg	86	17	85–107	U15702
OARHH30	F: ctcagtctcaactttgttcctctatagc R: gaaagctaaggctgaacattgtgccc	79	2	103–117	U08877
BMS2508	F: tttctgggattacaaaatgctc R: tttcttaggggagtgttgattc	67	6	92–118	G18959
BM143	F: acctgggaagcctccatatc R: ctgcaggcagattctttatcg	83	6	98–120	G18387

[a]http://www.roslin.ac.uk/.

Results

PCR-RFLP analysis of the mtDNA D-loop region

The PCR products from D-loop regions with the primers P1F and P4R were screened for polymorphism with digestion of 11 restriction enzymes (Table 3). RFLPs were detected and used in the evaluation of the genetic variation of sheep populations (data not shown here). Two half-sib families (named the Pmt family) produced within 3 years of each other were found to show paternal inheritance, which means that the mtDNA patterns of the progeny was identical to their father's (Figure 2, Figure 3, Figure 5). These two families share the same mother (named M) but two different fathers (named F1 and F2), and one family has only one lamb (called P1) while the other has three lambs (called P2, P3 and P4, respectively). The PCR-RFLPs patterns of the mtDNA with the primer pair P1F and P4R in the Pmt family were distinguishable between the father's mtDNA (named P type) and the mother's mtDNA (named M type) (Figure 2). The mtDNA of the progeny P1, P2 and P3 were from the father's side but that of P4 was from the mother's side. We suspected that the polymorphism was the result of repeat number variation. The primers P2F and P3R were designed to assess this interpretation. The PCR products were of two different lengths. One was 340 bp in size (40bp + 75bp × 4), derived from the father, and the other was 265 bp (40bp + 75bp × 3), derived from the mother. The amplification confirmed our interpretation of the RFCP pattern, and also the paternal origin of the mtDNA in three of the lambs (Figure 3). The PCR products of two types were sequenced, and the nucleotide sequence data indicate that the paternal type has four copies of the repeat motif, while the maternal type possessed only three copies of repeat motif (Figure 4).

Table 3 PCR-RFLP of mtDNA D-loop in Pmt family

Enzymes	*Molecular size of restriction fragments (bp)*	
	Type I	*Type II*
*Acc*I	641, 622	536, 622
*Alu*I	625, 332, 265, 41	550, 332, 265, 41
*Apa*I	691, 572	616, 572
*Bam*HI	668, 595	593, 595
*Ban*II	691, 572	616, 572
*Dpn*I	618, 593, 52	543, 593, 52
*Dde*I	679, 362, 207, 15	604, 362, 207, 15
*Hae*III	689, 357, 144, 63, 10	614, 357, 144, 63, 10
*Hap*II	685, 229, 349	610, 229, 349
*Hinc*II	669, 594	669, 519
*Taq*I	799, 464	724, 464

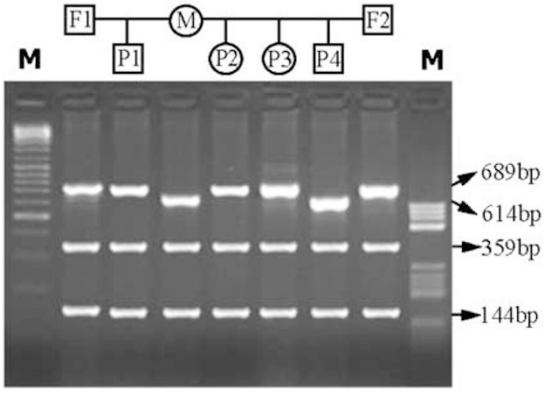

Figure 2 Patterns of mtDNA D-loop digested by *Hae*III in Pmt family showing paternal inheritance of sheep mtDNAs. M lane is the 1 kb ladder marker.

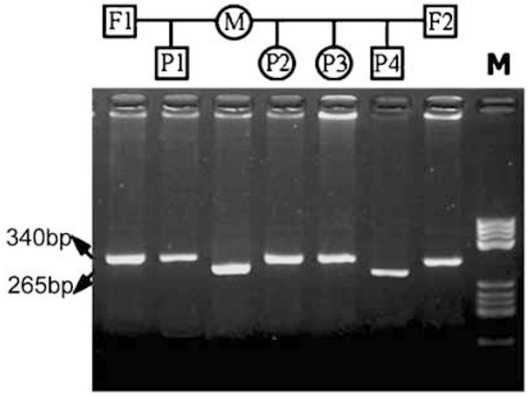

Figure 3 Amplification of the repeated region in Pmt family showing paternal inheritance of sheep mtDNAs. M lane is the 1 kb ladder marker.

Sequence analysis of mtDNA coding region

The CO I gene of mtDNA in Pmt family was also amplified with the primers COI F and COI R. When the PCR products were cut with *Hinf* I enzyme, PCR-RFLPs were obtained, which again showed that three lambs followed paternal inheritance (Figure 5). The restriction site alteration was confirmed by direct sequencing of the PCR products (Figure 5).

Microsatellite analysis

A total of 12 microsatellite markers were genotyped, which are highly polymorphic and follow strict Mendelian inheritance (Figure 6). The genotyping clearly confirmed the Pmt family's consanguineous relationships (Table 4).

Discussion

Reports of paternal inheritance of animal mtDNA have drawn close attention and suspicion. The accumulating evidence for the phenomenon has become relatively extensive however. There are two possible mechanisms that could explain the transmission of paternal mtDNA inheritance. One is paternal leakage of mtDNA. Labeled mitochondria from mouse spermatids could be 80% in the one-cell zygote but fell to 25% in two-cell, 9% in four-cell and approximately 1% in eight-cell or late stages (Cummins *et al*, 1998). Paternal mtDNA can enter eggs with sperm, and might be maintained at low level in the fertilized eggs. The other possible mechanism is recombination of mtDNA into the nucleus. In several species, including human, chimpanzee and cats (*Felis*), mitochondrial genes have been incorporated into the nuclear genome, known as *Numt* DNA (Lopez *et al*, 1994; Wallace *et al*, 1997). Usually Numts have acted as pseudogenes in

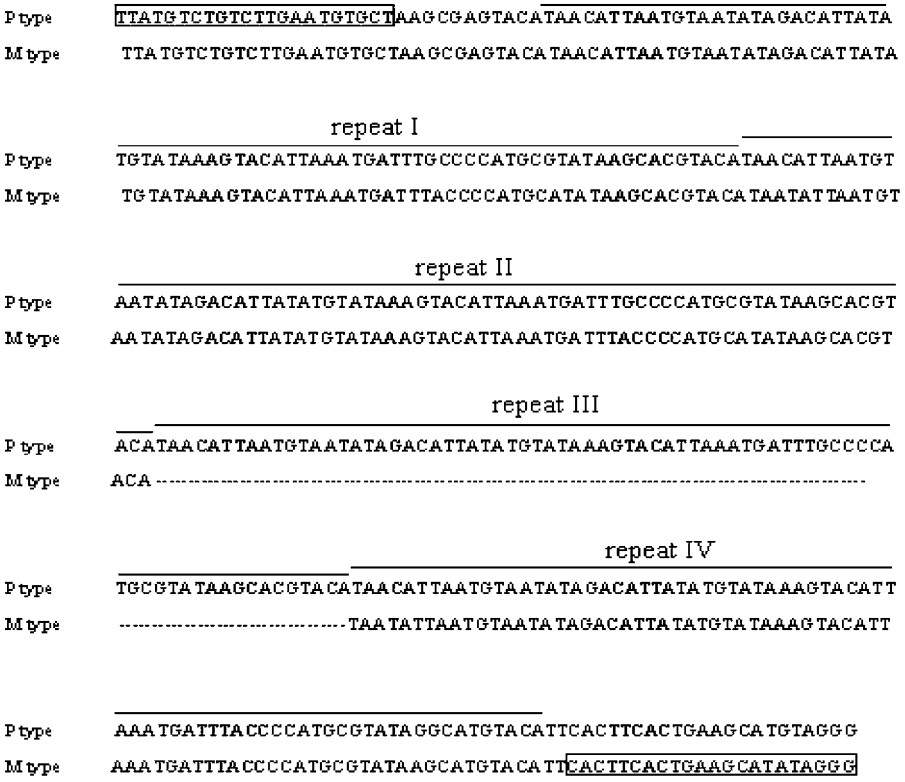

Figure 4 Nucleotide sequence comparison of the repeated region in D-loop between the M-type and the P-type sheep. Dashes indicate deleted base pairs and the primer is in the box.

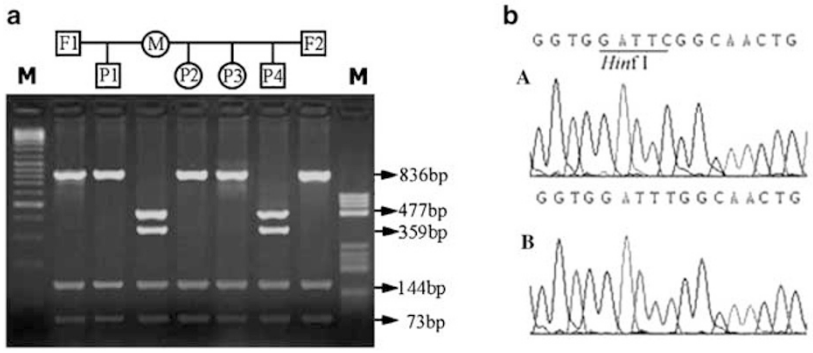

Figure 5 Polymorphic cleavage site of Hinf I in the sheep. CO I gene reveals paternal inheritance. M1 is the 1 kb ladder marker. M2 is the PBR322/HaeIII marker. A and B represent the type A and type B of ovine mtDNA, respectively.

nuclear genome, but there is a possibility of interaction between nuclear and mitochondrial DNA, and it is conceivable that Numts could be transferred back to the mtDNA. It has been reported that the extensive recombination occurred between mtDNAs (Hagelberg *et al*, 1999). Awadalla *et al* (1999) suggest that such recombination most likely happens between paternal and maternal mtDNAs.

We report here the paternal inheritance of ovine mtDNA in two related hybrid families produced by Dorset crossed to Small Tail Han sheep. The phenomena was identified by PCR-RFLPs in the D-loop region and the CO I gene, by further amplification and direct sequencing. No heteroplasmy of mtDNA has been detected from these sheep although this has been reported in other animals such as bat, horse, seal, rabbit and pig. Both maternal and paternal mtDNAs were found in different offspring. We suggest that both parent's mtDNAs might be present at an early stage but that only one survives to later development. However, the mechanism for such a process remains to be elucidated.

The occasional paternal inheritance seen suggests that the effects of paternal mtDNA should be considered as a genetic factor that could influence evolution, disease development and production traits in animals.

Table 4 The genotypes of each marker locus in Pm families

Microsatellite	*F1*	*P1*	*M*	*P2*	*P3*	*P4*	*F2*
CSSM31	178/178	178/178	178/178	172/178	172/178	172/178	172/172
MT2	150/150	150/150	150/150	147/150	147/150	147/150	147/147
IL2RA	178/192	178/192	178/192	178/192	178/192	178/192	178/178
OARAE64	130/130	130/130	130/130	124/130	124/130	124/130	124/124
MAF4	221/221	221/221	221/221	205/221	205/221	205/221	205/205
OARCP49	107/107	107/107	107/107	95/107	95/107	95/107	95/95
OARHH30	113/117	117/117	117/117	103/117	103/117	103/117	103/103
OARAE101	103/103	97/103	97/97	97/109	97/109	97/109	109/109
BM143	106/110	106/106	106/110	102/110	110/110	110/110	102/110
BMS2508	100/112	100/112	94/112	110/112	94/100	94/100	100/110
OARHH35	127/127	127/137	137/137	127/137	137/137	137137	127/137
OARHH55	124/148	124/148	148/148	124/148	148/148	124/148	124/148

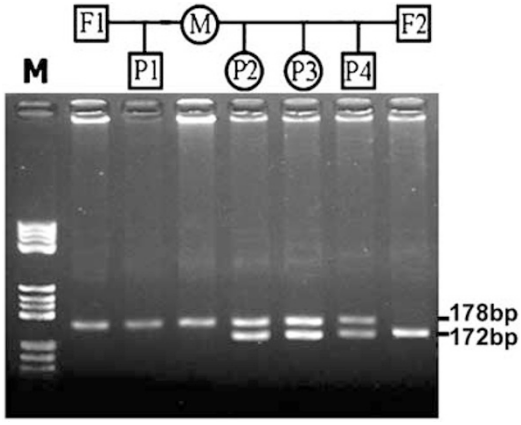

Figure 6 Representative genotyping of the microsatellite marker CSSM31 in the Pmt sheep family. M is the 1 kb ladder marker.

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