

Molecular analysis of wild and domestic sheep questions current nomenclature and provides evidence for domestication from two different subspecies

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Complete mitochondrial DNA (mtDNA) control regions (CR) were sequenced and analysed in order to investigate wild sheep taxonomy and the origin of domestic sheep (*Ovis aries*). The dataset for phylogenetic analyses includes 63 unique CR sequences from wild sheep of the mouflon (*O. musimon*, *O. orientalis*), urial (*O. vignei*), argali (*O. ammon*) and bighorn (*O. canadensis*) groups, and from domestic sheep of Asia, Europe and New Zealand. Domestic sheep occurred in two clearly separated branches with mouflon (*O. musimon*) mixed into one of the domestic sheep clusters. Genetic distances and molecular datings based on *O. canadensis* CR and mtDNA protein-coding sequences provide strong evidence for domestications from two mouflon subspecies. Other wild sheep sequences are in two additional well-separated branches. *Ovis ammon collium* and *O. ammon nigrimontana* are joined with a specimen from the transkaspiian Ust-Urt plateau currently named *O. vignei arkal*. *Ovis ammon ammon*, *O. ammon darwini* and *O. vignei bochariensis* represent a separate clade and the earliest divergence from the mouflon group. Therefore, *O. musimon*, *O. vignei bochariensis* and Ust-Urt sheep are not members of a 'moufloniform' or *O. orientalis* species, but belong to different clades. Furthermore, Ust-Urt sheep could be a hybrid population or an *O. ammon* subspecies closely related to *O. ammon nigrimontana*.

Keywords: *Ovis*; mitochondrial DNA; domestication; molecular dating; taxonomy; conservation genetics

1. INTRODUCTION

Domestic animals have played an important role in human history, but the origins of most livestock species are not well understood. Mitochondrial (mt) sequences have been used to study the origin of cattle (Loftus *et al.* 1994), swine (Giuffra *et al.* 2000), horse (Vila *et al.* 2001) and goat (Luikart *et al.* 2001). For these species, the number of presumed wild progenitors is limited. For the domestic sheep (*Ovis aries*), however, a large number of wild and possibly ancestral species and subspecies exist (Ryder 1984). Accordingly, several Eurasian wild sheep of the highly polymorphic genus *Ovis* have been proposed as ancestors of domestic sheep or are believed to have contributed to specific breeds (Reed 1960; Zeuner 1963). The taxonomy of these wild sheep is confused and controversial (Geist 1991a), hindering unequivocal identification and classification for conservation management (Geist 1991b) of this important genetic resource for a major agricultural species.

Based on morphological data, numerous wild sheep

classifications and revisions have been proposed during the last two centuries (summarized in Nadler *et al.* 1973a). A basic difference lies in the number of species recognized. Some authors follow the classification of Tsalkin (1951), proposing two (*O. ammon*, *O. nivicola/canadensis*) or even a single polymorphic species (*O. ammon*) for Eurasian and North American sheep. Other classifications follow Lydekker (1913) and Nasonov (1923) and discriminate between four to nine species. In a more recent classification based on chromosome numbers, *n* and geographical distribution of wild sheep, Nadler *et al.* (1973a) recognize four groups of sheep. These are mouflon (*O. musimon/orientalis*, $2n = 54$), urial (*O. vignei*, $2n = 58$), argali (*O. ammon*, $2n = 56$) and amphiberian dall (*O. dalli*, $2n = 54$), bighorn (*O. canadensis*, $2n = 54$) and snow (*O. nivicola*) sheep. However, Siberian snow sheep were later shown to have a karyotype of $2n = 52$ (Korobytsina *et al.* 1974). Geist (1991a) recognizes six species for the genus *Ovis*: the primitive *vignei*, the paedomorphic *orientalis*, the hypermorphic *ammon* and three pachyercine species, *nivicola*, *dalli* and *canadensis*. Despite the differences in chromosome number, different species of the genus *Ovis* can hybridize in captivity (Vorontsov *et al.* 1972; Nadler *et al.* 1973b) and natural habitats where

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ranges overlap (Nadler *et al.* 1971; Valdez *et al.* 1978), and produce fertile offspring. Accordingly, mouflon/urial hybrid zones with individuals displaying intermediate chromosome numbers of 55 to 57 are observed in northern (Nadler *et al.* 1971) and southeastern Iran (Valdez *et al.* 1978). These data have been interpreted in support of a single 'moufloniform' (*O. orientalis*) species, comprising mouflon and urial populations (Valdez *et al.* 1978; Valdez 1982). The International Union for the Conservation of Nature and Natural Resources (IUCN) has essentially adopted this classification of only three species (*O. orientalis*, *O. ammon* and *O. nivicola*) of Eurasian wild sheep (Shackleton *et al.* 1997).

Three major groups of Eurasian wild sheep, mouflon, urial and argali, have been proposed as ancestors of the domestic sheep or are believed to have contributed to specific breeds. Reed (1960) suggested that archaeological evidence pointed to Asiatic mouflon (*O. orientalis*) as the ancestor of domestic sheep. According to Zeuner (1963), however, the urial (*O. vignei*) was the first domesticate in the Aralo-Caspian basin and domestic forms subsequently spread throughout the Middle East and into Europe. Another line of domesticated sheep was believed to be derived from mouflon (*O. musimon* or *O. orientalis*) stock, which was brought into Europe and mixed with the urial derivatives. According to this view, the domestic sheep of southeast Asia are derived from urial, but argali (*O. ammon*) alleles are supposed to have been introduced repeatedly into these lines (Zeuner 1963).

In accordance with the domestication hypothesis of Zeuner (1963), Wood & Phua (1996) identified two major mitochondrial DNA (mtDNA)-types among New Zealand sheep based on sequence comparisons of the mtDNA control region (CR), but the origin or relationship to wild sheep had not been investigated. Phylogenetic analyses on mtDNA restriction fragment length polymorphisms (RFLP) of Eurasian breeds (Hiendleder *et al.* 1998a) and CR sequence data of New Zealand sheep (Hiendleder *et al.* 1999) demonstrated two different maternal origins among modern sheep breeds. However, although the RFLP analyses showed a close relationship between some *O. aries* and mouflon (*O. musimon*) haplotypes, the data suggested an additional wild ancestor different from the proposed urial (*O. vignei*) and argali (*O. ammon*) sheep (Hiendleder *et al.* 1998a).

Complete CR sequences of *O. aries* reported to date are from European breeds (Zardoya *et al.* 1995; Hiendleder *et al.* 1998a) or breeds of European origin (Wood & Phua 1996) and just two complete CR sequences of wild sheep, mouflon (*O. musimon*) and urial (*O. vignei bochariensis*), have been determined (Hiendleder *et al.* 1998a). Phylogenetic analyses have only been performed on a subset of the available domestic sheep CR sequence data (Hiendleder *et al.* 1999). In the present study we have produced additional CR sequences that represent domestic sheep from west and central Asia, and eight wild sheep populations of the mouflon (*O. musimon*), urial (*O. vignei*), argali (*O. ammon*) and bighorn (*Ovis canadensis*) groups. The complete *Ovis* CR database has been used for phylogenetic analyses in order to investigate the origin and evolution of domestic sheep and to help clarify the taxonomy of the genus *Ovis*. New sequences from 2.3 kb of *O. canadensis canadensis* mtDNA protein-

coding regions were used to calibrate divergence times among the different sheep lineages.

2. MATERIAL AND METHODS

DNA of wild and domestic sheep (table 1) was extracted from blood, skin or liver samples by standard procedures (Sambrook *et al.* 1989). Two primers, tRNA-Phe (5'-TCATCTAGG-CATTTTCAGTG-3') and tRNA-Pro (5'-CTCACCATCAACCCCAAAGC-3') were designed from the ovine reference sequence, *O. aries* [AF010406] (Hiendleder *et al.* 1998b), to amplify the complete mtDNA CR, using *Pwo* polymerase (Hybaid-AGS, Heidelberg, Germany). The PCR products were purified from agarose gels and cloned into pCR-TOPO-BluntII (Invitrogen, Groningen, The Netherlands). Both strands of plasmid inserts were sequenced by standard procedures on a LICOR 4200 automated sequencer (MWG Biotech, Ebersberg, Germany). *Ovis ammon ammon* and *O. ammon darwini* CR was amplified using *Taq* polymerase (Hybaid-AGS), gel purified and cloned into pCR2.1-TOPO (Invitrogen). Complete CR sequences of three clones each, derived from independent PCR reactions, were determined using ³⁵S sequencing reactions and an additional internal primer (DL3R 5'-TAGATGAGATGGCCCTGA-3') according to standard procedures (Sambrook *et al.* 1989). In case of nucleotide (nt) variation among clones from the same individual, the consensus sequence was reported. In cases where the PCR produced several bands due to heteroplasmy (figure 1), the dominant fragment regardless of its size was cloned. CR nt sequence data reported in this paper have been submitted to GenBank with accession numbers AF242347, AF242348 and AY091486-AY091500. Users of these data are kindly requested to refer to the present publication.

Other complete CR sequences of the domestic sheep (L29055, Zardoya *et al.* (1995); Z35228-Z35268 and Z35293, Wood & Phua (1996); AF039578 and AF039579, Hiendleder *et al.* (1998a)) of the Rasa Aragonesa, Merino, Romney, Coopworth, Perendale × Romney and Merinolandschaf breeds, one *O. musimon* (AF039577, Hiendleder *et al.* (1998a)) and one *O. vignei bochariensis* (AF039580, Hiendleder *et al.* (1998a)) were obtained from GenBank.

The CR sequences were aligned manually. The number of repetitive sequence elements was reduced to the two repeats found in *O. canadensis*. In all 66 sequences, the 5' and 3' copies were retained. Maximum likelihood (ML) trees, distances and support values were calculated using the TREE-PUZZLE program v. 4.0 package (Strimmer & von Haeseler 1996). The Tamura-Nei (TN-93) model of nt evolution (Tamura & Nei 1993) with the assumption of rate heterogeneity among sites was used for the analysis of nt sequence data. In order to confirm the presence of major clusters identified by the ML analysis, the sequences were also analysed using maximum parsimony (Fitch 1971) and neighbour joining (Saitou & Nei 1987) as implemented in the PAUP* program package (Swofford 1998).

PCR primers (OatGL 5'-AGTACAAGTACTTCCAA-3' and OatLH 5'-AATTTTTTGGTTCCTAAGA-3') were used to amplify a 2.3 kb region (nt positions 9435-11713) of the mt genome of the bighorn sheep, *O. canadensis canadensis*. The primers were designed from the complete mtDNA sequence of the domestic sheep, *O. aries* [AF010406] (Hiendleder *et al.* 1998b). The PCR products were twice precipitated with ethanol and sequenced directly by cycle sequencing using fluorescent dye-labelled primers, and analysed on a LICOR 4000L automated sequencer (AH diagnostics, Skärholmen, Sweden and MWG

Table 1. Origin of wild and domestic sheep sampled for mitochondrial DNA control region sequences.

name ^a	breed ^b	number of specimens	country/region
<i>Ovis canadensis canadensis</i>	n.a.	1	Canada/Rocky Mountains
<i>Ovis musimon</i>	n.a.	2	Germany/Hessen
<i>Ovis vignei bochariensis</i>	n.a.	2	Turkmenistan/southeast
<i>Ovis vignei arkal</i>	n.a.	1	Kazakhstan/Ust-Urt
<i>Ovis ammon ammon</i>	n.a.	1	Mongolia/Altai
<i>Ovis ammon darvini</i>	n.a.	1	Mongolia/Gobi-Altai
<i>Ovis ammon nigrimontana</i>	n.a.	2	Kazakhstan/Kara-Tau
<i>Ovis ammon collium</i>	n.a.	1	Kazakhstan/Karaganda
<i>Ovis aries</i>	Edilbey	1	Kazakhstan/Alma-Ata
<i>Ovis aries</i>	Astrachan	1	Kazakhstan/Tschimkent
<i>Ovis aries</i>	Gizarr	1	Tadjikistan
<i>Ovis aries</i>	Awassi	1	Syria
<i>Ovis aries</i>	Kivircik	1	Turkey/Aegean
<i>Ovis aries</i>	Daglic	1	Turkey/west Turkey
<i>Ovis aries</i>	Akkaraman	1	Turkey/central Anatolia

^a Nomenclature is according to Vorontsov *et al.* (1972).

^b n.a., not applicable.

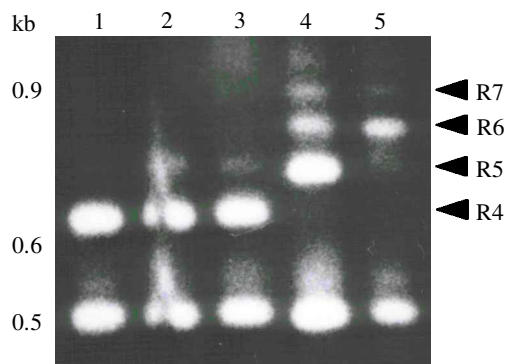


Figure 1. 1.3% agarose gel showing heteroplasmic length variation in sheep CR. PCR amplified complete CR were digested with *Bam*HI to visualize an invariant 3' fragment and variable length 5' fragments containing from 4 to 7 copies (R4–R7) of the sheep 75/76 nt tandem-repeat motif (arrows). (1) Homoplasmic *Ovis aries*, (2) heteroplasmic *O. aries*, (3) heteroplasmic *O. musimon*, (4) heteroplasmic *O. ammon collium* and (5) heteroplasmic *O. ammon nigrimontana*.

Biotech, Munich Germany). This sequence has been deposited at GenBank with accession number AJ409303.

The protein-coding genes of NADH3, NADH4L and NADH4 of the bighorn sheep, *O. canadensis* (this study), cow, *Bos taurus* [J01394] (Anderson *et al.* 1982), domestic sheep, *O. aries* [AF010406] (Hiendleder *et al.* 1998b), pig, *Sus scrofa* (Ursing & Arnason 1998a), alpaca, *Lama pacos* [Y19184] (Ursing *et al.* 2000), fin whale, *Balaenoptera physalus* [X61145] and blue whale, *B. musculus* [X72204] (Arnason & Gullberg 1993), the hippopotamus, *Hippopotamus amphibius* [AJ010957] (Ursing & Arnason 1998b), horse, *Equus caballus* [X79547] (Xu & Arnason 1994) and the donkey, *E. asinus* [X97337] (Xu *et al.* 1996) were aligned by eye. No gaps needed to be included. To this dataset the alignment of the partial Cyt *b* gene of *O. canadensis* [U17859] (Groves & Shields 1996) to the Cyt *b* of the species mentioned above was added. After removal of gaps produced by adding the partial Cyt *b* alignment, 999 amino-acid (aa) sites remained for analysis. Distance and support values

were calculated by the TREE PUZZLE v. 4.0 program (Strimmer & von Haeseler 1996).

3. RESULTS

(a) General characteristics of ovine control regions

The lengths of the ovine mt CR sequences range from 1031 nt (*O. canadensis canadensis*) to 1333 nt (*O. ammon nigrimontana*). This length variability is caused by different copy numbers of a 75 nt tandem-repeat element in the *Ovis* CR (Hiendleder *et al.* 1998a,b). Repeat numbers vary from two in *O. canadensis canadensis*, to four in all but one *O. aries*, *O. musimon*, *O. vignei bochariensis*, five in *O. aries* Astrachan, *O. vignei arkal*, *O. ammon collium*, *O. ammon ammon*, and a maximum of six in both *O. ammon nigrimontana*. In some wild and domestic sheep samples, evidence for heteroplasmy (Hiendleder *et al.* 1998b), i.e. a variable number of repeat units within individuals, was observed (figure 1). In this case, the predominating band had been cloned, as it represents the majority of the mtDNA population within an individual. All *O. aries* sequences and the *O. vignei bochariensis* sequence obtained from GenBank had four copies of the tandem-repeat element, except for the *O. musimon* sequence, which contained five copies. The sequence variation among repeats of an individual was much smaller than between individuals. This allowed trimming of the number of repeats in all of the sequences to two, the lowest observed number of repeats in this dataset, and includes the sequence data of the repetitive elements for analysis.

The nt frequency in *Ovis* CRs standardized to two tandem-repeat elements is 31.9% adenine, 24.0% cytosine, 14.6% guanine and 29.5% thymine. These values are expected for mammalian CR sequences. Apart from repeat copy-number length differences, only three insertion/deletion events (indels) of more than a single nt were detected. All of the indels occurred within the first 180 nt of the CR. A four nt deletion was found in four *O. aries* sequences from GenBank, and an insertion of eight and a deletion of six nt are present in *O. canadensis canadensis* (figure 2). The tandem-repeat element exhibits

an insertion in a subset of the newly produced sequences. In *O. ammon collium*, *O. ammon nigrimontana* and the specimen designated *O. vignei arkal*, a 'T' insertion leads to a 76 nt sequence in all but the 5'-most repeat units (figure 3a).

Within each tandem repeat, mirror symmetrical and thus stem-loop structure-forming octamer sequences (Hiendleder *et al.* 1998b) are highly conserved. But the potential recognition site for H-strand arrest is variable among sheep. Nt variation in proposed ovine homologues (Wood & Phua 1996; Hiendleder *et al.* 1998b) of TAS-A and CSB 2 + 3 is low, but it is considerable in CSB 1 (figure 3a-d). By contrast, L- and H-strand promoters and the origin of H-strand replication are completely conserved (not shown).

(b) *Sheep phylogeny and molecular dating*

The initial alignment contained 63 unique sequences with a length of 1045 nt (figure 2). Only 13 indel events were observed. Indels were excluded prior to phylogenetic analyses because the placement of indels is sometimes ambiguous and the evolution of gaps is not implemented in most molecular evolutionary models. After removal of gaps and ambiguous sites around gaps, 1008 nt with 205 variable positions (20.3%) in 61 unique sequences remained for analysis. All phylogenetic reconstruction methods found the same major clusters as shown in figure 4a,b. The trees are shown unrooted in order to illustrate the distinctness of the major clades. However, when CR sequences of the domestic goat, *Capra hircus* (Takada *et al.* 1997) and cow, *B. taurus/indicus* (Loftus *et al.* 1994), were included as outgroups in the initial alignment, 862 characters remain for phylogenetic analyses after excluding gaps. The root of the sheep tree is then placed by all analysis methods along the branch leading to the bighorn sheep (*O. canadensis*). Wild and domestic sheep occurred in four clearly separated branches. Two of the branches contain distinct clusters of domestic sheep. The relationships within each of the clusters of the domestic sheep remain unresolved and thus form star-like patterns. One of these clusters encompasses all three representatives of the mouflon (*O. musimon*). Another branch groups *O. ammon collium*, *O. ammon nigrimontana* and a specimen from the transkaspien Ust-Urt plateau, as a clearly separated clade. *O. ammon ammon*, *O. ammon darwini* and *O. vignei bochariensis* occurred as the earliest split from the most distant bighorn (*O. canadensis canadensis*). However, the branching pattern remains unresolved.

The maximum distance among the CR sequences is ca. 11% and is observed between the bighorn and all other sheep. Distances are lower for other groups of sheep. Especially within the clusters of domestic sheep, only a few substitutions are observed. The low distances and the high transition/transversion ratio (table 2) indicate a limited amount of randomization in the sequence data, making them suitable for estimating divergence times. These can be calculated after the substitution rate among the sheep has been estimated. In order to calibrate the substitution rate, a well-established reference point like the A/C-60 can be used. The A/C-60 reference for calibrating substitution rates is based on the assumption that an artiodactylan family, the Ruminantia, and Cetacea diverged at 60 million years before the present (MyrBP) (Arnason &

Gullberg 1996). This date is supported by the palaeontology of whales and has been corroborated by the use of other calibration points for the molecular clock within mammals (Arnason *et al.* 2000). However, CR sequences evolve too rapidly and can not be directly utilized for comparisons between sheep, cattle and whales. Instead, the deepest divergence among sheep has been dated on the basis of protein-coding sequences.

The ML distance between the domestic sheep (*O. aries*) and the bighorn (*O. canadensis*) protein-coding genes has been calculated to 0.01508 using the mtREV-24 model of aa sequence evolution. The number of absolute aa differences is 15. The average ML distance between the cow and the two sheep is 0.0804. The absolute difference is 76 aa substitutions. Based on the A/C-60 reference and the data from complete mt genomes, a cow-sheep split of 30 MyrBP has been estimated (Hiendleder *et al.* 1998b). Thus, domestic sheep and bighorn sheep have then diverged at 30 MyrBP \times (0.01508/0.0804), which equals 5.63 MyrBP. This value has then been used as a calibration point in order to calculate divergence times within the genus *Ovis* from distances of the CR sequences. Distances were calculated under the TN-93 model of sequence evolution. The transition/transversion parameter has been estimated by TREE-PUZZLE from the dataset to 21.7 and the pyrimidine/purine transition parameter to 0.82. Furthermore, a gamma model (Yang 1994) of rate heterogeneity (Γ) with four classes of variable sites and one class of invariable sites (*I*), resulting in an alpha parameter of 0.86 as estimated by TREE-PUZZLE, has been taken into account. Table 2 summarizes the distances and divergence times of well-separated lineages.

4. DISCUSSION

The taxonomy of wild sheep based on morphological traits such as horn shape and pelage characteristics is often confused (Geist 1991a). Hybrid zones between species (Nadler *et al.* 1971, 1973a; Valdez *et al.* 1978) further complicate the systematics of Eurasian wild sheep. Mitochondrial DNA sequence data are therefore expected to help clarify wild sheep taxonomy. Phylogenetic reconstructions placed haplotypes from the argali subspecies *O. ammon collium*, *O. ammon nigrimontana* and *O. ammon ammon*, *O. ammon darwini* in two well-separated branches (figure 4a). This is consistent with geographical origins of the subspecies (table 1) and current IUCN systematics and nomenclature for *O. ammon* ssp. (Shackleton *et al.* 1997). The Altai argali (*O. ammon ammon*) and the Gobi argali (*O. ammon darwini*) show much similarity, and it has been speculated that size differences could be due to differences in productivity of their ranges (Geist 1991a). Our molecular dating has estimated the time of divergence for *O. canadensis* from all other sheep of the present study at 5.63 MyrBP. Based on this estimate, divergence time for *O. ammon ammon* and *O. ammon darwini* haplotypes was calculated at equal to or more than 1.32 MyrBP (table 2). This is consistent with a subspecies status based on estimates of speciation duration (1.2–3.2 MyrBP) for mammals during the Pliocene/Pleistocene (Avice *et al.* 1998).

Genetic distance and divergence time for the Altai argali (*O. ammon ammon*) and Gobi argali (*O. ammon darwini*)

Table 2. Number of transitions and transversions observed between mtDNA control region sequences of major sheep lineages and derived distances and divergence times.

comparison ^a	transitions ($n \pm \text{s.e.}$)	transversions ($n \pm \text{s.e.}$)	distance ^b (TN93 + \bar{I} + I)	divergence (MyrBP)	distance ^b (TN93)	divergence (MyrBP)
<i>Ovis canadensis canadensis</i> vs. other sheep	86.2 \pm 4.2	12.8 \pm 0.5	<0.205>	<5.63>	<0.109>	<5.63>
<i>Ovis ammon collium</i> / <i>Ovis ammon nigrimontanum</i> <i>Ovis vignei</i> arkal vs. <i>Ovis aries</i>	62.9 \pm 5.7	3.1 \pm 0.5	0.117 \pm 0.007	3.21 \pm 0.20	0.070 \pm 0.004	3.62 \pm 0.21
<i>Ovis ammon ammon</i> / <i>Ovis ammon darwini</i> <i>Ovis vignei bochariensis</i> vs. <i>Ovis aries</i>	55.8 \pm 5.5	3.6 \pm 0.9	0.103 \pm 0.008	2.83 \pm 0.22	0.063 \pm 0.005	3.25 \pm 0.26
<i>Ovis ammon nigrimontanum</i> / <i>Ovis vignei arkal</i> vs. <i>Ovis ammon collium</i>	31.6 \pm 1.2	1.0 \pm 0.0	0.056 \pm 0.002	1.54 \pm 0.05	0.033 \pm 0.001	1.70 \pm 0.05
<i>Ovis ammon ammon</i> vs. <i>Ovis ammon darwini</i>	31.0 \pm n.a. ^c	0.0 \pm n.a. ^c	0.048 \pm n.a. ^c	1.32 \pm n.a. ^c	0.032 \pm n.a. ^c	1.65 \pm n.a. ^c
<i>Ovis aries</i> cluster A vs. <i>Ovis aries</i> cluster B	34.4 \pm 2.5	0.5 \pm 0.8	0.056 \pm 0.003	1.54 \pm 0.08	0.036 \pm 0.002	1.86 \pm 0.10
<i>Ovis aries</i> cluster B '64' vs. rest cluster B	8.9 \pm 2.2	1.4 \pm 0.7	0.016 \pm 0.002	0.43 \pm 0.05	0.011 \pm 0.002	0.57 \pm 0.10
<i>Ovis musimon</i> vs. rest <i>Ovis aries</i> cluster B '64'	9.0 \pm 2.0	0.8 \pm 0.9	0.014 \pm 0.004	0.38 \pm 0.11	0.009 \pm 0.003	0.46 \pm 0.15
<i>Ovis aries</i> cluster A '88' vs. rest cluster A	9.6 \pm 2.1	0.2 \pm 0.6	0.011 \pm 0.005	0.30 \pm 0.14	0.010 \pm 0.001	0.52 \pm 0.05
<i>Ovis aries</i> cluster A '73' vs. rest cluster A	5.8 \pm 3.5	0.3 \pm 0.6	0.010 \pm 0.004	0.27 \pm 0.11	0.008 \pm 0.002	0.41 \pm 0.10

^a Subclusters within *Ovis aries* clusters A and B are labelled according to their bootstrap values as shown in figure 4b.^b Distances were calculated assuming a TN93 model of sequence evolution, plus a gamma (\bar{I}) model of rate heterogeneity with four classes of variable sites, plus one class of invariable sites (I) and assuming rate homogeneity.^c n.a., not applicable.

[illegible]

Figure 2. (First part of figure opposite) Complete alignment of variable nt positions in mt control region sequences of the genus *Ovis*. Dots show identity with the first sequence, deletions/insertions are represented by dashes. Nucleotide numbering is according to the complete mt genome of the domestic sheep (Hiendleder *et al.* 1998b). Insertions relative to the numbering of the reference sequence are indicated by alphabetical suffixes. Only two 75/76 nt tandem repeat sequences as found in *O. canadensis* have been retained. The presence or absence of two to four additional copies of the tandem repeat sequence is indicated by a corresponding number of asterisks/dashes.

Kara Tau argali (*O. ammon nigrimontana*) (figure 4a; table 2). Until now, sheep of this region have been placed in the urial group and, accordingly, named *O. vignei arkal*. However, with regard to neck ruff, saddle patch and horn curl (Valdez 1982), Ust-Urt sheep show phenotypic similarities with *O. vignei severtzovi* that occurs further to the southeast in the Kizil-Kum desert and Nura-Tau range (Shackleton *et al.* 1997). The status of *O. vignei severtzovi* has long been controversial, and, based on a chromosome number of $2n = 56$ (Bunch *et al.* 1998), it was recently reclassified as an argali, *O. ammon severtzovi*. This raises

(d)

(q)

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(a)

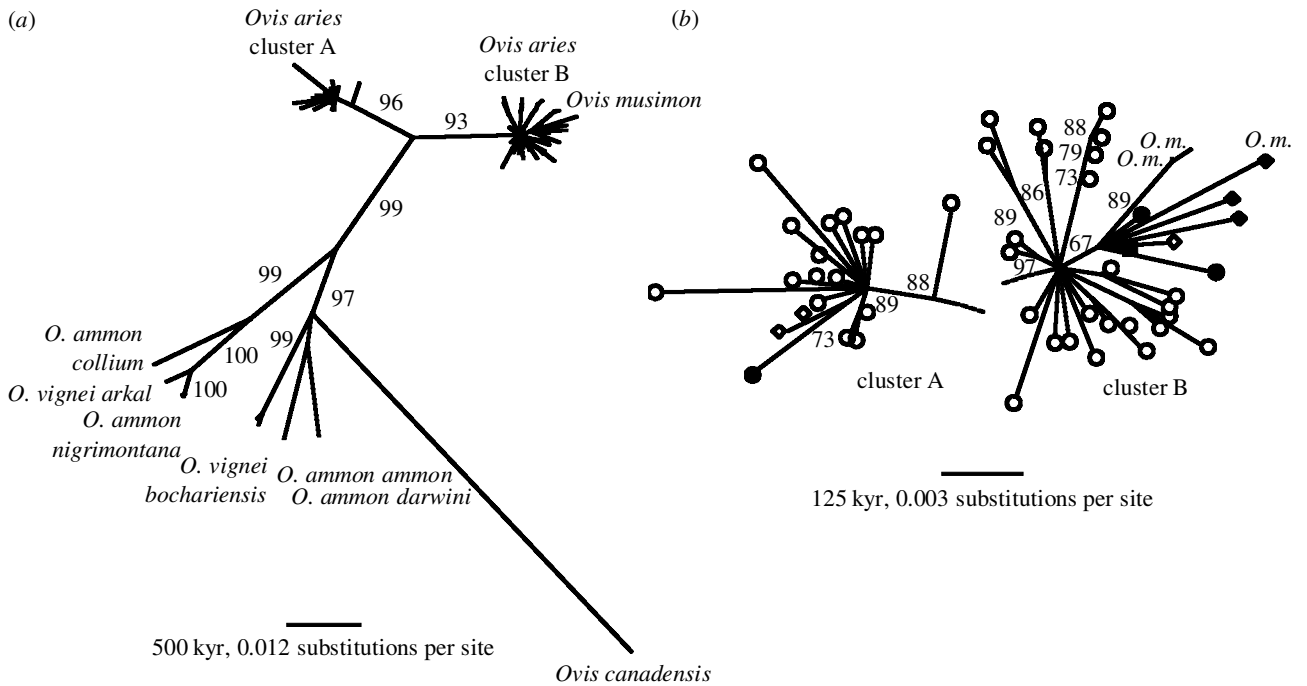


Figure 4. Phylogenetic relationships among wild and domestic forms of the genus *Ovis*. (a) Maximum likelihood tree of mtDNA CR sequences of all 61 unique sequences as reconstructed by TREE-PUZZLE v. 4.0. Branches with less than 50% quartet puzzling (QP) support have been collapsed. (b) Fourfold enlargement of *O. aries* and *O. aries/O. musimon* clusters A and B. QP support values are reported on the nodes. (Origin of *O. aries*: open circle, New Zealand; filled circle, Europe; filled square, Near East (Syria); filled diamond, Asia Minor (Turkey); open diamond, Central Asia (Kazakhstan, Tadjikistan); *O. m.*, *Ovis musimon*.)

the possibility that Ust-Urt sheep also belong to the argali group. Another explanation would be a hybrid origin of Ust-Urt sheep due to overlapping ranges of urial and argali, like the hybrid zones of mouflon and urial in Iran (Nadler *et al.* 1971; Valdez *et al.* 1978), as the former range of Ust-Urt sheep could have merged with the range of *O. ammon severtzovi* (Nadler *et al.* 1973a). Unfortunately, chromosome data of Ust-Urt sheep that could help to clarify this question are not available.

The Ust-Urt sheep haplotype is clearly separated from haplotypes obtained for the Bukhara urial (*O. vignei bochariensis*), a true urial with $2n = 58$ (Nadler *et al.* 1973a), which is found in southeastern Turkmenistan, southern Uzbekistan and Tadjikistan. The *bochariensis* urial specimen is placed in a branch with *O. ammon ammon*, *O. ammon darwini* and *O. canadensis canadensis*. Nadler *et al.* (1973a) believed that urial sheep with $2n = 58$ were the most primitive form, ancestral to all other sheep with lower chromosome numbers, considering that fusion rather than fission is the predominant mechanism for changes in diploid number in cattle, sheep and goats. The

mtDNA-based phylogenetic reconstructions show that *O. vignei bochariensis* haplotypes are indeed basal to mouflon/domestic sheep and the *O. ammon collium/O. ammon nigrimontana*/Ust-Urt sheep branch. However, branching order in the *O. canadensis canadensis/O. ammon ammon/O. ammon darwini/O. vignei bochariensis* branch is not completely resolved. The molecular data on *O. musimon*, *O. vignei bochariensis* and Ust-Urt wild sheep clearly show that they cannot, as was done previously (Valdez *et al.* 1978; Valdez 1982; Shackleton *et al.* 1997), be lumped into a single 'moufloniform' (*O. orientalis*) branch of sheep. Instead, they are members of three distinct clades and, considering the estimated distances and divergence times, could be different species. Similarly, the CR distances between the two clusters with argali sheep haplotypes are comparable with those between well-established species like that of harbour and grey seal (Arnason *et al.* 1993) or pygmy and common chimp (Arnason *et al.* 1996). This and the resulting divergence times indicate that the two argali matrilineages are in the process of speciation or already belong to different species.

In domestic animals, bi- or multiphyletic origins are not uncommon. Two distinct mtDNA lineages, an Asian and European clade, have been identified in cattle (Loftus *et al.* 1994; Bradley *et al.* 1996) and swine (Giuffra *et al.* 2000). In the dog (Tsuda *et al.* 1997; Vila *et al.* 1997), horse (Vila *et al.* 2001) and goat (Luikart *et al.* 2001), several clusters implying multiple integrations of matrilineages have been described. The degree of sequence divergence between the two major mt lineages in bovines has led to the hypothesis of independent domestication from two different subspecies of *Bos primigenius*, giving rise to

Figure 3. (Opposite) Alignment of proposed regulatory elements (Wood & Phua 1996; Hiendleder *et al.* 1998a,b) in *Ovis* CR. (a) The 3' tandem repeat sequence of 75 or 76 nt with putative D-loop strand termination site (dotted line box) and octamer sequences (solid boxes) that form the stem of predicted 39 nt secondary structures (-8.2 kcal mol^{-1}). The loop region of these structures is overlined. (b) TAS-A element. (c) CSB 1. (d) CSB 2 + 3. Nt numbering above sequence elements is according to the complete mt genome of the domestic sheep (Hiendleder *et al.* 1998b).

the *B. taurus* and *B. indicus* forms of cattle (Loftus *et al.* 1994; Bradley *et al.* 1996; Troy *et al.* 2001). Similar results have been reported for the domestic pig, where Asian and European subspecies of wild boar, *Sus scrofa*, are thought to have been domesticated (Giuffra *et al.* 2000). Phylogeographic analysis of goat mtDNA has revealed three major lineages, with one lineage occurring only in eastern and southern Asia. These results, combined with recent archaeological findings, also point to centres of origin in Asia, as well as in the near East (Luikart *et al.* 2001). Previous analyses of a large number of sheep samples from Europe and a limited number of samples from central Asia have led to the proposal of European and Asian domestic sheep mtDNA clades (Hiendleder *et al.* 1998a). Domestic sheep samples from central Asia and the near East that have been investigated in the present study support this view, the near Eastern samples clustering with the European clade. The relatively high incidence of haplotype A in New Zealand breeds is presumably a relic of the fat-tailed sheep from India, which were the first sheep in Australasia (Ryder 1984), because a male-mediated upgrading of fat-tailed sheep with rams of European breeds leaves mtDNA unaffected. As in other major farm-animal species (MacHugh & Bradley 2001), the two well-separated *O. aries* mtDNA lineages A and B (figure 4a,b) detected among individuals from sheep breeds with different geographical origins suggest domestication from two distinct wild populations. Alternatively, domestic sheep could have originated from a single polymorphic ancestral population, where two major haplotypes became fixed through 'lineage sorting' (Avise *et al.* 1987). Although the latter possibility cannot be completely dismissed with the current dataset, the first alternative seems more likely, considering the estimated divergence times for clusters A and B, which are far in excess of the ca. 11 000 years (Ryder 1984) of domestication history, and the striking similarities in branching patterns observed in cattle, pig and water buffalo mtDNA (MacHugh & Bradley 2001). Estimates on the divergence time for clusters A and B (equal to or more than 1.54 MyrBP) are similar to the differences found between argali subspecies (table 2). The star-like expansion of domestic sheep CR sequences indicates that only a very limited amount of genetic variation was introduced from the ancestral matriline in each of the domestication events. Only a small number of substitutions have then accumulated in the ca. 11 000 years (Ryder 1984) of further breeding. These do not allow further differentiation of the domestic sheep based on mtDNA data using the current approach and thus lead to the unresolved branching pattern and short branches within the two clusters of domestic sheep.

The present data confirm previous results on two major mtDNA lineages in domestic sheep obtained by simple sequence comparisons (Wood & Phua 1996), phylogenetic analyses on datasets limited in resolution (Hiendleder *et al.* 1998a), or geographical sampling (Hiendleder *et al.* 1999). The *O. aries* cluster B comprises all mouflon (*O. musimon*) specimens in a subcluster separate from New Zealand *O. aries*. However, support for the subset that also encompasses *O. aries* from Germany, Turkey, Syria and Kazakhstan is only 64%. The position of *O. musimon* is in agreement with the view of Zeuner (1963) that one line of domesticated sheep derived from

the mouflon (*O. musimon*/*O. orientalis*) stock, which was subsequently brought into Europe. Although European mouflon (*O. musimon*) is now considered a neolithic feral domesticate introduced to Corsica and Sardinia ca. 6000 BC (Poplin 1979; Vigne 1999), it should nevertheless closely resemble wild sheep ancestral to both *O. aries* and *O. musimon* in cluster B. Likely candidates for truly wild ancestors of cluster B are mouflon populations found in Turkey and western Iran. These sheep are currently referred to as *O. orientalis anatolica* and *O. orientalis gmelini*, although their subspecies status is debatable. The other major *O. aries* mtDNA branch with haplogroup cluster A does not, as expected from the domestication hypothesis of Zeuner (1963), contain urial (*O. vignei* spp.) or any other investigated wild sheep sequences. The urial (*O. vignei bochariensis*) considered in phylogenetic reconstructions is placed in a different branch (figure 4a) and shows distances and divergence times (table 2) incompatible with any contribution to domestic sheep matriline. Furthermore, CR data from another sampled urial population (*O. vignei cycloceros*) are as much diverged from *O. aries* as *O. vignei bochariensis* (data not shown). This excludes urial (*O. vignei* spp.) as a matriline source for cluster A of domestic sheep. As tree topology (figure 4a), distances and divergence times (table 2) also exclude argali (*O. ammon* spp.) as maternal ancestors of *O. aries*, the origin of haplogroup A remains unknown. Considering the probable subspecies relationship of the founders of both clusters of domestic sheep matriline, mouflon (*O. orientalis*) populations of the eastern mouflon range are probable candidates.

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