

Mitogenomic Meta-Analysis Identifies Two Phases of Migration in the History of Eastern Eurasian Sheep

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Abstract

Despite much attention, history of sheep (*Ovis aries*) evolution, including its dating, demographic trajectory and geographic spread, remains controversial. To address these questions, we generated 45 complete and 875 partial mitogenomic sequences, and performed a meta-analysis of these and published ovine mitochondrial DNA sequences ($n = 3,229$) across Eurasia. We inferred that *O. orientalis* and *O. musimon* share the most recent female ancestor with *O. aries* at approximately 0.790 Ma (95% CI: 0.637–0.934 Ma) during the Middle Pleistocene, substantially predating the domestication event (~8–11 ka). By reconstructing historical variations in effective population size, we found evidence of a rapid population increase approximately 20–60 ka, immediately before the Last Glacial Maximum. Analyses of lineage expansions showed two sheep migratory waves at approximately 4.5–6.8 ka (lineages A and B: ~6.4–6.8 ka; C: ~4.5 ka) across eastern Eurasia, which could have been influenced by prehistoric West–East commercial trade and deliberate mating of domestic and wild sheep, respectively. A continent-scale examination of lineage diversity and approximate Bayesian computation analyses indicated that the Mongolian Plateau region was a secondary center of dispersal, acting as a “transportation hub” in eastern Eurasia: Sheep from the Middle Eastern domestication center were inferred to have migrated through the Caucasus and Central Asia, and arrived in North and Southwest China (lineages A, B, and C) and the Indian subcontinent

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(lineages B and C) through this region. Our results provide new insights into sheep domestication, particularly with respect to origins and migrations to and from eastern Eurasia.

Key words: wild ancestor, domestication, gene flow, mitogenome, *Ovis aries*, meta-analysis, colonization simulation.

Introduction

As one of the first animals ever domesticated, sheep (*Ovis aries*) have played an important role in human society and have spread almost globally, following human migrations (Colledge et al. 2005; Chessa et al. 2009). Early evidence implied that modern sheep breeds were first domesticated from Asian mouflon (*O. orientalis*) in the Fertile Crescent approximately 8–11 thousand years ago (ka) (Ryder 1984). Following domestication, as many as 1,400 sheep breeds have been developed from their wild ancestors after long-term natural and intense artificial selection (Scherf 2000). During this process, human activities have played a significant role in determining the patterns of gene flow among breeds and populations (e.g., Warmuth et al. 2012). Thus, an examination of continent-wide genetic variability among modern native sheep breeds can provide a comprehensive, in-depth understanding of their genetic origins and dispersal, as well as insight into the impact of human activities on sheep throughout history.

In recent decades, remarkable analytical advances in paleontological and molecular genetics have transformed our understanding of the origins and regional expansion of domestic sheep (Poplin 1979; Hiendleder, Mainz, et al. 1998; Pedrosa et al. 2005; Chessa et al. 2009; Meadows et al. 2007, 2011; Kijas et al. 2009, 2012; Demirci et al. 2013). Morphological change and demographic analysis implied that sheep were likely brought under domestication in a region that stretches from northern Zagros to southeastern Anatolia, approximately 10.5–11 ka or perhaps even earlier (Peters et al. 2005). In addition, a recent investigation on endogenous retroviral sequences revealed a remarkable secondary population expansion of improved domestic sheep, most likely out of Southwest Asia (i.e., the Middle East; Chessa et al. 2009). Mitochondrial DNA (mtDNA) sequence analyses have identified a general phenomenon of multiple maternal lineages (i.e., A, B, C, D, and E), some with specific geographic ranges, implying multiple maternal origins and possibly independent domestication events in sheep (Wood and Phua 1996; Hiendleder, Mainz, et al. 1998; Guo et al. 2005; Pedrosa et al. 2005; Tapio et al. 2006; Meadows et al. 2007; Singh et al. 2013).

Estimates from complete and/or partial mtDNA sequences have enabled various divergence time estimates between domestic and wild sheep as well as among the five major maternal lineages of *O. aries* (e.g., Hiendleder, Mainz, et al. 1998; Pedrosa et al. 2005; Chen et al. 2006; Meadows et al. 2011). In general, the estimated divergence times among the five major lineages have been much earlier than the domestication period inferred from archeological evidence (Bar-Yosef and Meadow 1995; Zeder 2008). For example, the divergence time between the two most common lineages (i.e., A and B) was estimated to be as early as 1.6–1.7 Ma based on cytochrome b (*Cyt-b*) sequences (Hiendleder, Mainz, et al. 1998). In addition,

Pedrosa et al. (2005) and Chen et al. (2006) suggested the divergence time of lineage C from lineages A and B to be approximately 0.42–0.76 Ma and approximately 0.45–0.75 Ma from the analysis of control region and *Cyt-b* sequences, respectively. However, a more recent study (Meadows et al. 2011) using 12 protein-coding genes from complete mitogenomes implied more recent divergence between the lineages: For example, 0.590 ± 0.17 Ma between A and B and 0.26 ± 0.09 Ma between C and E.

So far, most ovine mtDNA investigations have only focused on one or two segments within *Cyt-b* gene and the control region (including the hypervariable region; e.g., Pedrosa et al. 2005); nevertheless, high levels of recurrent mutations observed in the short segment within control region in many mammal species may bias dating estimates (e.g., Achilli et al. 2009, 2012; see also the reviews in Torroni et al. 2006; Taberlet et al. 2008). Moreover, previous sheep mtDNA studies have merely included breeds at a regional (e.g., Pedrosa et al. 2005; Chen et al. 2006; Wang et al. 2006; Meadows et al. 2007) or subcontinental scale (e.g., Tapio et al. 2006), whereas maternal lineages of domestic sheep, particularly for breeds in Southwest, Central, East and South Asia, including the Caucasus, Iran, Pakistan, Nepal, Indonesia, Mongolia, China, and India, have been largely excluded from integrated analyses. In addition, the divergence scenarios have not been fully evaluated based on complete mitogenomes either, which could have provided refined phylogenies of maternal lineages and robust estimations of genetic variability and divergence time in domestic animals (see the review in Wang et al. 2014). Therefore, although these early mtDNA studies have provided useful insights into the history of sheep domestication in Eurasia, answers to some basic questions surrounding the domestication process are far from being settled. For example, phylogenetic relationships among wild and domestic sheep (e.g., Hiendleder, Lewalski, et al. 1998; Meadows et al. 2007), divergence times between the major maternal lineages (e.g., Pedrosa et al. 2005; Zeder 2008; Meadows et al. 2011), demographic history and population recolonization (Dobney and Larson 2006; Zeder 2008), and origins of different mtDNA lineages (Tapio et al. 2006; Meadows et al. 2007; Demirci et al. 2013; Singh et al. 2013), as well as the continent-wide patterns of gene flow from the postulated Middle Eastern domestication center to Central, East and South Asia (see, e.g., Tapio et al. 2006, 2010; Cai et al. 2007, 2011) remain provisional or unaddressed.

The main objective of our study was to better understand the domestication and expansion of *O. aries* across Eurasia through a meta-analysis of complete and partial ovine mitogenomic sequences. More specifically, we aimed to refine and challenge existing paradigms on the wild origin, lineage divergence, demographic history and population recolonization of modern sheep, particularly the breeds present in eastern Eurasia. For these purposes, we sequenced the complete

mitogenomes of 45 individuals (including *O. orientalis*, *O. vignei*, and 42 native breeds of *O. aries*) and the control region of a total of 875 animals (including 51 native breeds) from eastern Eurasia (fig. 1 and supplementary tables S1 and S2, Supplementary Material online). Together with the sequences retrieved from GenBank, we analyzed 85 complete mitogenomes of domestic sheep including each of the 5 lineages and 10 complete mitogenomes of *O. orientalis*, *O. musimon*, *O. vignei*, *O. ammon*, and *O. canadensis* using phylogenetics, molecular-dating, and demographic-reconstruction approaches. Full control region and *Cyt-b* sequences of seven extant wild sheep species (*O. orientalis*, *O. musimon*, *O. vignei*, *O. ammon*, *O. canadensis*, *O. dalli*, and *O. nivicola*) were also included in phylogenetic reconstructions. Furthermore, we carried out a meta-analysis and a simulation of colonization (e.g., approximate Bayesian computation, ABC) of mtDNA sequences, including 547 partial *Cyt-b* and 1,470

partial control region sequences published previously (supplementary tables S2 and S3, Supplementary Material online), from native sheep breeds across Eurasia. We tried to address these questions and test two hypotheses on domestication and migrations of sheep distributed particularly in eastern Eurasia. One is the more recent origin and dispersal of lineage C when compared with those of the two widely distributed lineages A and B (Bruford 2005; Tapio et al. 2006). Another is that the arrival of some Indian sheep from the Middle Eastern domestication center could be through the Mongolian Plateau region, where archeological remains showed an early presence of domestic sheep in ancient history (e.g., Kuo et al. 1999; see also Yang et al. 2015). Our results could help researchers better understand the demographic forces and human practice associated with animal domestication and migration in history (e.g., Hodges 1999; Larson et al. 2007, 2010; Larson and Burger 2013).

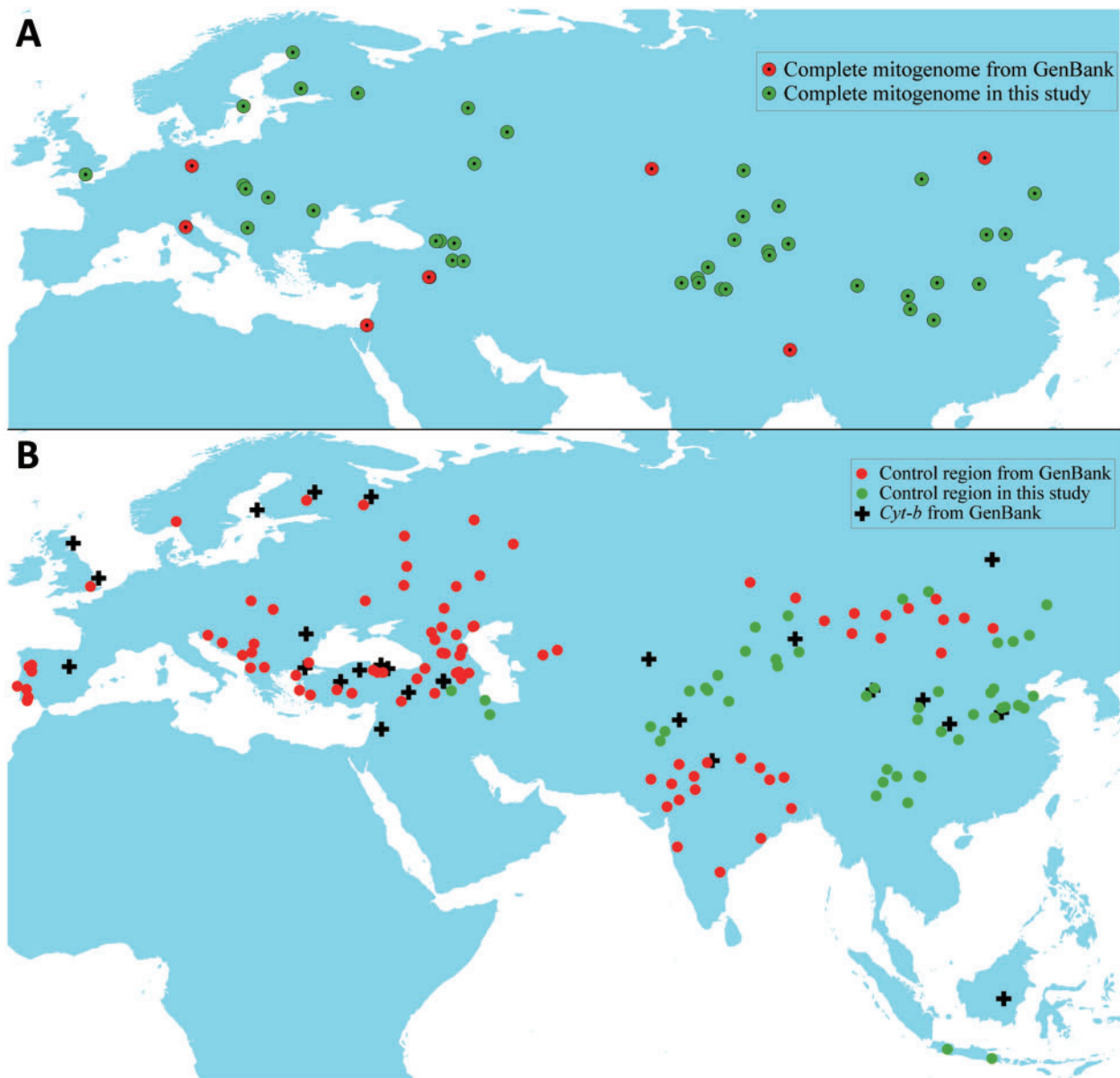


FIG. 1. Geographic distribution of the samples in this and early ovine mtDNA studies.

Results

Geographic Patterns of mtDNA Variation

The 45 complete domestic (GenBank accession numbers KF938317–KF938359) and wild (KF938360–KF938361) sheep mitogenomes (supplementary table S1, Supplementary Material online) sequenced in this study showed considerable sequence variability as well as variation in diversity among different regions (supplementary table S4 and fig. S1, Supplementary Material online). Also, we detected a large number of variable sites in the integrated data of

partial *Cyt-b* and control region (supplementary tables S2 and S3, Supplementary Material online). Full description of the complete mitogenome and partial mtDNA sequence variations is in supplementary information S1, Supplementary Material online.

All control region and *Cyt-b* sequences analyzed in this study can be assigned to the five previously defined lineages (supplementary tables S2 and S3; see also supplementary figs. S2 and S3, Supplementary Material online). The two partial mtDNA fragments displayed similar geographic patterns (fig. 2B and C). For control region sequences, lineages A and

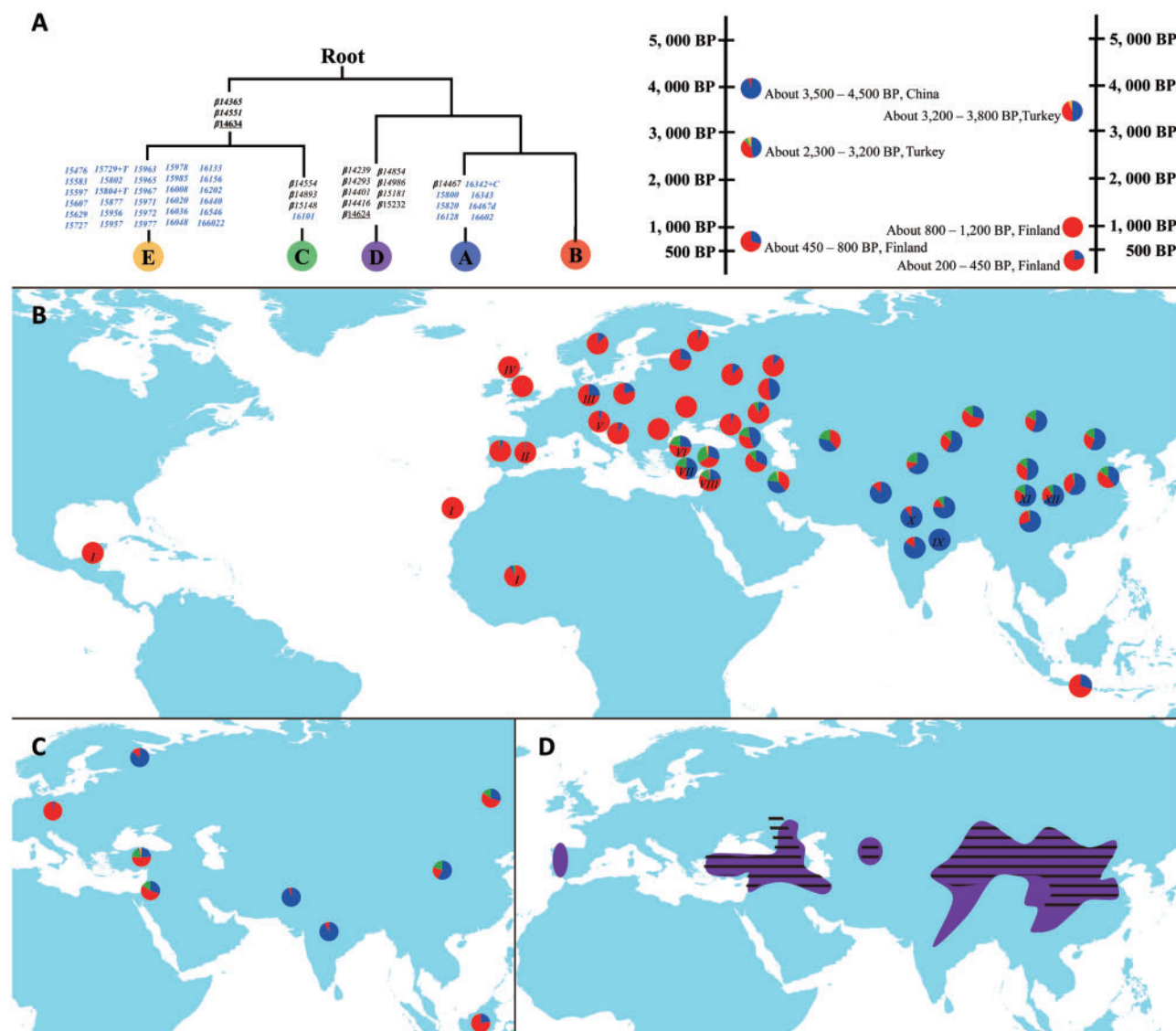


Fig. 2. Geographic distribution of the five major maternal lineages across Eurasia based on sequences obtained in this study and retrieved from GenBank. (A) Phylogenetic tree inferred from partial control region sequences (left) and lineage composition of sheep in different geographic regions at different time points (right) based on ancient specimens (Cai et al. 2007, 2011; Demirci et al. 2013; Niemi et al. 2013); (B) lineage frequency distribution of partial control region sequences; previously reported lineage frequencies in 12 regions (I–XII) are detailed in supplementary table S17, Supplementary Material online; (C) lineage frequency distribution of partial *Cyt-b* sequences; (D) geographic distribution of fat-tailed native sheep breeds (regions with black lines) and lineage C (region colored in purple). Pie plots show the proportions of the five distinct lineages (A–E) of domestic sheep in the different geographic regions (for the details of the geographic regions, see supplementary tables S5 and S6, Supplementary Material online). In the phylogenetic tree, diagnostic mutations are shown on the branches and are named according to their nucleotide positions relative to the reference sequence AF010406; amino acid replacements are underlined and synonymous replacements are marked in black. Control region mutations (15,437–16,616 bp) are shown in blue. Insertions are indicated by a “+” after the position number and followed by the type of inserted nucleotide(s). Mutations with prefix “β” indicate identical variable sites found in Meadows et al. (2007), which are used to define the five major lineages.

B were the most common and most widely distributed, with a mean combined frequency of approximately 89% (fig. 2B and C). Lineage A was extremely frequent (~77%) in the Indian subcontinent, although its frequency was less than 10% in Europe. In contrast, lineage B was found mostly in Europe, with its highest frequency (> 90%) in Southwest Europe (fig. 2B and C). Lineage C occurred mainly in the Middle East, the Caspian Sea region, North China, and the Mongolian Plateau, with a mean frequency of approximately 18% (fig. 2B), whereas a few haplotypes of lineage C were also found in the Iberian Peninsula, India, Nepal, and Southwest China. A majority of the breeds harboring lineage C were fat-tailed (including fat-rump; 73.1%), higher than the proportion of fat-tailed breeds having lineage A (50.8%) or B (44.8%) (supplementary tables S5 and S6, Supplementary Material online). In addition, we found a significantly higher mean frequency of lineage C in fat-tailed breeds than in short-tailed breeds (fat-tailed: $f_C = 0-0.50$, mean $f_C = 0.19$; short-tailed: $f_C = 0-0.40$, mean $f_C = 0.05$; two-sample Kolmogorov–Smirnov test: $P < 0.01$; supplementary fig. S4, Supplementary Material online). Of the total 149 breeds studied here, 66 are fat-tailed, 78 harbor lineage C, and 57 are fat-tailed sheep carrying lineage C. Compared with the overlap expected by chance, there is a large and significant excess of breeds that are fat-tailed harboring lineages C (lineage C: observed $n = 57$, expected by chance $n = 34.65$, $P < 0.001$; supplementary fig. S5; Supplementary Material online). Lineages D and E accounted for approximately 1% of the total samples and were only found in the Middle East (see fig. 2B and C).

A synthetic map across Eurasia showed that the breeds in the Mongolian Plateau region had the highest genetic variability (π) of control region in Asia (fig. 3A; supplementary table S7, Supplementary Material online). For lineages A and B, a relatively high level of nucleotide diversity was found in the Indian subcontinent (fig. 3B and C). In addition, the synthetic map revealed the highest level of lineage C variability in the breeds of North China, even higher than that of the breeds in the Middle East (fig. 3D), the presumed domestication center of modern sheep (Ryder 1984).

Phylogenetic Relationships

Phylogenetic relationships inferred from all the 95 complete *Ovis* mitogenomes (supplementary table S1, Supplementary Material online) are shown in figure 4. The 85 complete mitogenomes of *O. aries* were assigned to five major lineages (fig. 4). *Ovis vignei*, *O. ammon*, and *O. canadensis* clustered into three independent clades separated from *O. aries*, whereas *O. canadensis* showed the largest divergence. The clade of *O. musimon* and *O. orientalis* was closely related to *O. aries*. In the phylogenetic trees built from the full control region, *Cyt-b*, and protein gene sequences of the complete mitogenomes, the four branches of wild sheep agreed with the topology inferred from the complete mitogenomes, but domestic sheep sequences formed an unresolved group rather than the five major lineages (supplementary figs. S6–S9, Supplementary Material online). Additional phylogenetic trees obtained with the full control region and *Cyt-b* sequences of wild and domestic sheep (supplementary figs.

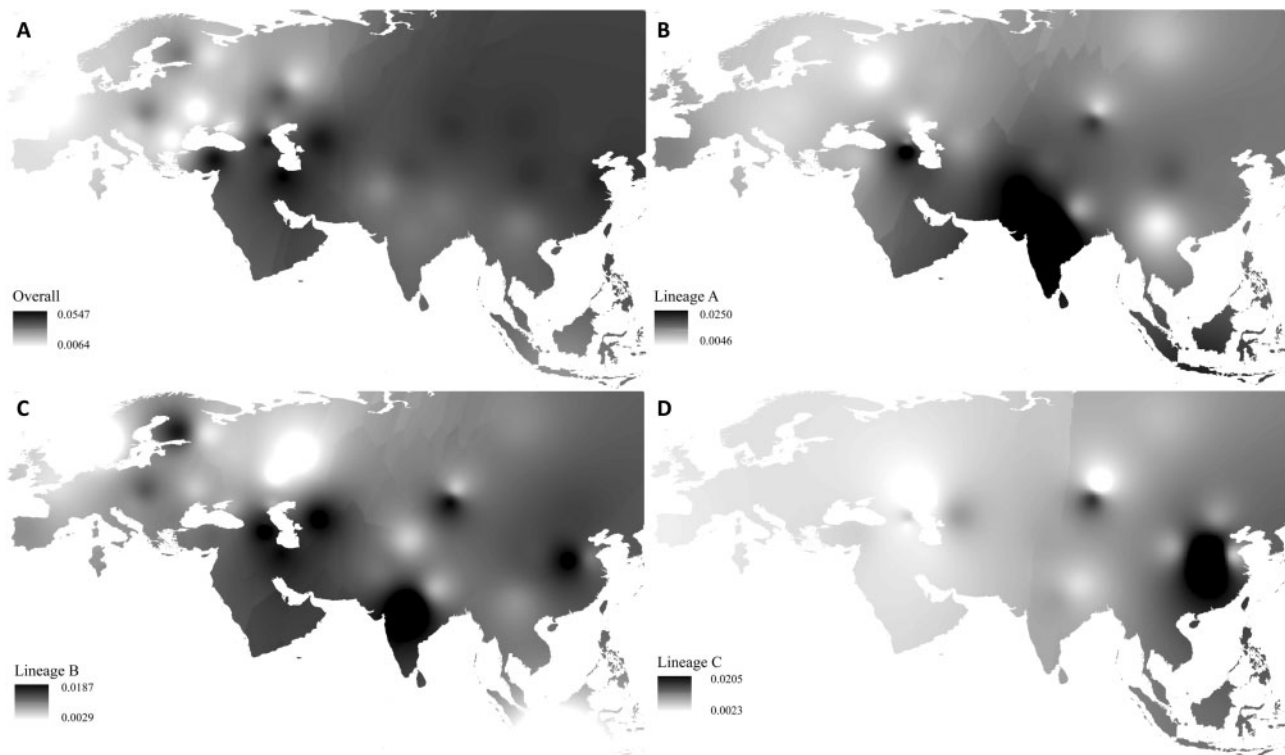


Fig. 3. Synthetic maps illustrating geographic variation of nucleotide variability for the total lineages and lineages A, B, and C. (A) The total lineages, (B) lineage A, (C) lineage B, and (D) lineage C.

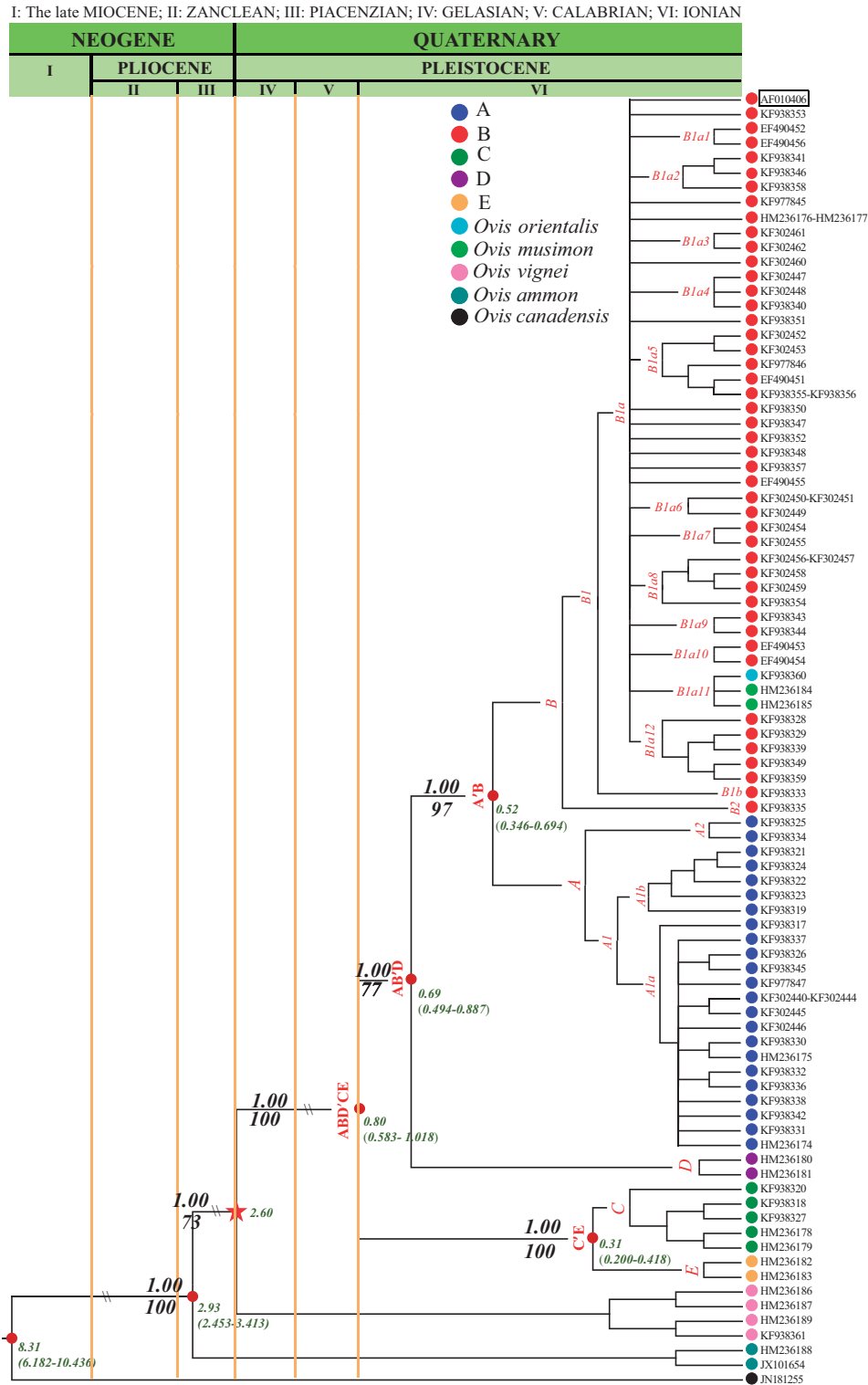


Fig. 4. Phylogeny of domestic and wild sheep inferred from a total of 95 complete mitogenomes (supplementary table S1, Supplementary Material online) using BI and ML methods with posterior probability (the first value) and bootstrap values (the second value) on the nodes, respectively. Divergence times for the lineages (Ma) were estimated only based on the 61 complete mitogenomes of native domestic sheep breeds and wild sheep species (see supplementary table S1, Supplementary Material online).

S10 and S11 and tables S8 and S9, Supplementary Material online) showed different topologies from that inferred from the complete mitogenomes (fig. 4 and supplementary fig. S6, Supplementary Material online). Specifically, instead of

showing close relationships only to lineage B as inferred from the complete mitogenomes (fig. 4), the haplotypes of *O. musimon* and *O. orientalis* clustered with lineages A, B, and C of *O. aries* control region sequences (supplementary fig. S10,

Supplementary Material online), and they even shared some *Cyt-b* haplotypes of lineages A, B, C, and E (supplementary fig. S11, Supplementary Material online).

The reduced median network analysis of partial control region sequences showed several major radiating nodes at a few mutation steps within lineages A and B. Different contributions of breeds to different regions were evident, but none of the major nodes consisted of apparent region-specific haplotypes (supplementary fig. S2, Supplementary Material online). In addition, analysis of molecular variance and pairwise-population F_{ST} values indicated genetic differentiation between European and Asian breeds, whereas considerable maternal gene flow was found among the breeds within Asia and Europe, respectively (supplementary figs. S12–S13 and tables S10 and S11, Supplementary Material online).

Selective Pressure on Different Lineages

The log-likelihood values ($\ln L$) under the one-, two-, three- and four-ratio models were $\ln L = -18,462.81$, $-18,454.79$, $-18,419.46$ and $-18,413.27$, respectively (table 1). The ω ratio differed between the branches under the same model and varied for the same branches under different models (table 1). The likelihood ratio tests (LRTs) revealed that the differences between two models for all the pairwise comparisons were significant ($P < 0.01$) and that the four-ratio model (free-ratio model) best fit the data, which indicated different ω ratios among the lineages. Mean ω values for the lineages were $\omega_A = 0.0457$, $\omega_B = 0.0775$, $\omega_D = 0.0494$, and $\omega_{C+E} = 0.0496$ (supplementary fig. S14, Supplementary Material online); note that these values are all much lower than 1. This observation indicates that the maternal lineages (A, B, D, and C + E) have been under strong but variable intensity of purifying selection: Purifying selection on amino acid changes in lineage B has been slightly weaker than that on the other lineages. Thus, divergence time estimation (see below) based on the protein-coding genes would be biased. Instead, using the synonymous sites might be a better choice for divergence time estimation.

Divergence Times for the Nodes

The estimated divergence times within the comprehensive evolutionary framework of the Cetartiodactyla are shown in supplementary figure S15, Supplementary Material online. The *O. vignei/O. aries* split, which is the calibration point applied to estimate the divergence times between extant *O. aries* lineages, was 2.6 ± 0.9 Ma. That time is far earlier than the most recent common ancestor (TMRCA) of domestic sheep (~ 0.79 Ma; 95% CI: 0.64–0.93 Ma; table 2), and even older than the *O. ammon/O. aries* split (2.13 ± 0.29 Ma) estimated by Meadows et al. (2011). The *Capra/Ovis* split was estimated to be 14.7 ± 2.1 Ma (supplementary fig. S15, Supplementary Material online), and is much older than the date based on the ungulate fossil record (~ 5.00 – 7.0 Ma; Luikart et al. 2001). Using the calibration point, we obtained a substitution rate of 0.70×10^{-8} substitutions per nucleotide/year for complete mitogenome, 3.12×10^{-8} substitutions per nucleotide/year for control region, and 0.49×10^{-8} per nucleotide/year for *Cyt-b* without partitions.

The divergence times for each node were mostly concordant under global and local clock models when estimated from the complete mitogenomes, the synonymous mutations or the third-codon positions (table 2). The earliest split was estimated to be approximately 0.73–0.93 Ma for the divergence of C and E from A, B, and D (see the node 4 in table 2), whereas the most recent split was between lineages C and E at approximately 0.29–0.36 Ma (see the node 1 in table 2), greatly predating sheep domestication (~ 8 – 11 ka; Ryder 1984). The time to TMRCA of the two most common lineages (A and B) was estimated to be approximately 0.50–0.53 Ma (see node 2 in table 2). Under the relaxed molecular clock, we also obtained similar estimates of divergence times for the nodes based on different data sets (i.e., complete mitogenomes, synonymous, and third-codon positions; see table 2). However, divergence times for nodes 2, 3, and 4 estimated from synonymous mutations by the Bayesian Inference (BI) approach were significantly ($P < 0.05$) higher than those by the global and local maximum likelihood (ML)

Table 1. Number of Parameters Fitted, dN/dS Ratios, Log-Likelihood Scores, and Their Differences under Different Models.

Model	p	$\ln L$	ω	Models Compared	$2\Delta \ln L$	
A: One ω ratio	ω^0	102	$-18,462.81$	$\omega^0 = 0.0563$		
B: Two ω ratios	ω^B	103	$-18,454.79$	$\omega^B = 0.0702$		
	ω^0		$\omega^0 = 0.0436$	A versus B	16.04**	
C: Three ω ratios	ω^A	104	$-18,419.46$	$\omega^A = 0.0447$	A versus C	86.70**
	ω^B		$\omega^B = 0.0744$	A versus D	99.08**	
	ω^0		$\omega^0 = 0.0486$	B versus C	70.66**	
D: Four ω ratios	ω^A	105	$-18,413.27$	$\omega^A = 0.0457$	B versus D	83.04**
	ω^B		$\omega^B = 0.0775$	C versus D	12.38**	
	ω^D		$\omega^D = 0.0494$			
	ω^0		$\omega^0 = 0.0496$			

NOTE.— p , number of parameters in the model; $\ln L$, log-likelihood score; ω , the dN/dS ratio for the branches; ω_A , ω_B , and ω_D are the dN/dS ratios for branches lineages A, B, and D, respectively (see supplementary fig. S14, Supplementary Material online); ω_0 is the background dN/dS ratio for the rest branch(es); $2\Delta \ln L$, twice the log-likelihood difference of the models compared.

**Very significant ($P < 0.01$).

Table 2. Divergence Time Estimated by the Sequences of Complete Mitogenomes and the Protein-Coding Genes (synonymous mutation and the third-codon position) Using ML and BI Methods.

Method	Data Set	Model	Node	Node 1 ($T_{C/E}$) ^{Ma}	Node 2 ($T_{A/B}$) ^{Ma}	Node 3 ($T_{AB/D}$) ^{Ma}	Node 4 ($T_{ABD/CE}$) ^{Ma}	Node 5 ($T_{Ovaries/Ovignai}$) ^{Ma}	$T_{Ovaries/Oammon}$ ^{Ma}	$T_{Ovaries/Ocamaderis}$ ^{Ma}
ML	Mitogenome	Global	Time	0.36 (0.278–0.439)	0.51 (0.402–0.616)	0.74 (0.613–0.867)	0.88 (0.743–1.013)	2.60	3.00 (2.673–3.323)	7.72 (6.567–8.883)
		Local	95%(CI)	0.34 (0.276–0.472)	0.53 (0.397–0.668)	0.78 (0.600–0.956)	0.93 (0.721–1.131)	2.60	3.06 (2.697–3.419)	8.15 (6.635–9.664)
	Synonymous	Global	Time	0.31 (0.217–0.405)	0.52 (0.373–0.661)	0.68 (0.536–0.829)	0.79 (0.637–0.934)	2.60	2.92 (2.535–3.312)	8.36 (6.441–10.286)
		Local	95%(CI)	0.31 (0.200–0.418)	0.52 (0.346–0.694)	0.69 (0.494–0.887)	0.80 (0.583–1.018)	2.60	2.93 (2.453–3.413)	8.31 (6.182–10.436)
	Third codon	Global	Time	0.29 (0.190–0.390)	0.50 (0.361–0.639)	0.64 (0.497–0.783)	0.73 (0.579–0.881)	2.60	2.81 (2.418–3.202)	7.47 (6.157–8.783)
BI	Mitogenome	Relaxed-molecular clock	Time	0.29 (0.190–0.390)	0.50 (0.359–0.636)	0.64 (0.498–0.783)	0.73 (0.581–0.882)	2.60	2.81 (2.414–3.200)	7.47 (6.158–8.786)
		Median	95%HPD	0.35 (0.130–0.641)	0.55 (0.266–0.913)	0.85 (0.390–1.413)	0.92 (0.464–1.498)	2.60	2.68 (2.462–3.031)	6.13 (2.464–11.618)
	Synonymous	Global	Time	0.41 (0.147–0.772)	0.61 (0.291–1.013)	0.96 (0.478–1.604)	1.06 (0.541–1.716)	2.60	2.62 (2.458–3.338)	5.89 (5.456–11.598)
		Local	95%(CI)	0.36 (0.142–0.656)	0.57 (0.27–0.912)	0.87 (0.421–1.428)	0.94 (0.472–1.496)	2.60	2.62 (2.461–3.083)	6.49 (2.478–12.647)
	Third codon	Global	Time	0.36 (0.142–0.656)	0.57 (0.27–0.912)	0.87 (0.421–1.428)	0.94 (0.472–1.496)	2.60	2.62 (2.461–3.083)	6.49 (2.478–12.647)

NOTE.—“—” not available.

approaches, respectively (supplementary fig. S16, Supplementary Material online).

Prehistoric Population Expansions

Bayesian skyline plot (BSP) reconstructions of historical population expansions using the complete mitogenomes revealed the profile of predomestic change in N_e over large time scales. Based on the estimated TMRCA for the lineages (~0.79 Ma) from complete mitogenomes, the ovine lineages showed a steep increase in N_e at approximately 20–60 ka (supplementary fig. S17, Supplementary Material online). A prehistoric steep increase in N_e was also identified in the simulations of the partial *Cyt-b* and control region sequences (supplementary fig. S18, Supplementary Material online). However, population growth was found to have occurred at approximately 50–300 ka, much earlier than the time obtained from simulations of the complete mitogenomes (~20–60 ka; supplementary fig. S17, Supplementary Material online).

Postdomestic Lineage Expansions

A synthetic map constructed with the use of interpolated λ_1 values, the eigenvalues for the first multidimensional scaling (MDS) plot dimension, allows us to examine the gradients of colonization out of the sheep domestication center that peak in the Middle East (fig. 5A; supplementary table S12, Supplementary Material online). λ_1 explains 69.3% of the total variation. We observe a significant correlation between the λ_1 eigenvalues of Asian populations and their geographic distances from the domestication center (lineages A, B, and C of Central and East Asian populations: $r = 0.201$; $P < 0.05$; lineage A of Arabian and Indian populations: $r = 0.547$; $P < 0.01$; see fig. 5C and D). This suggests that the major colonization process of the Middle Eastern sheep to eastern Eurasia (including Mongolia, China, and India) was through the Caucasus and Central Asia. The interpolation map of the λ_2 eigenvalues suggests that the second MDS dimension could represent genetic influence from the Mongolian Plateau region in China and the Indian subcontinent (fig. 5B). λ_2 explains 27.3% of the total variation. Its ranking shows the Mongolian Plateau region at one extreme, whereas the Indian subcontinent at the other extreme. This is supported by a strong and significant correlation observed between geographic distances from the putative region of initial colonization (i.e., the Mongolian Plateau region) and λ_2 values across eastern Eurasian populations ($r = 0.372$; $P < 0.01$; fig. 5E). Eigenvalues λ_1 and λ_2 for all the populations are shown in supplementary table S12, Supplementary Material online.

The star-like median-joining networks (supplementary fig. S2, Supplementary Material online) and mismatch distributions (supplementary fig. S19, Supplementary Material online) revealed genetic signatures of postdomestic demographic population expansions in lineages A, B, and C. The inference was corroborated by F_s (Fu 1997), Tajima's D (1989), and scaled effective population size statistics ($2Nu$; N represents the effective population size and u denotes the mutation rate). Both Fu's F_s and Tajima's D statistics showed

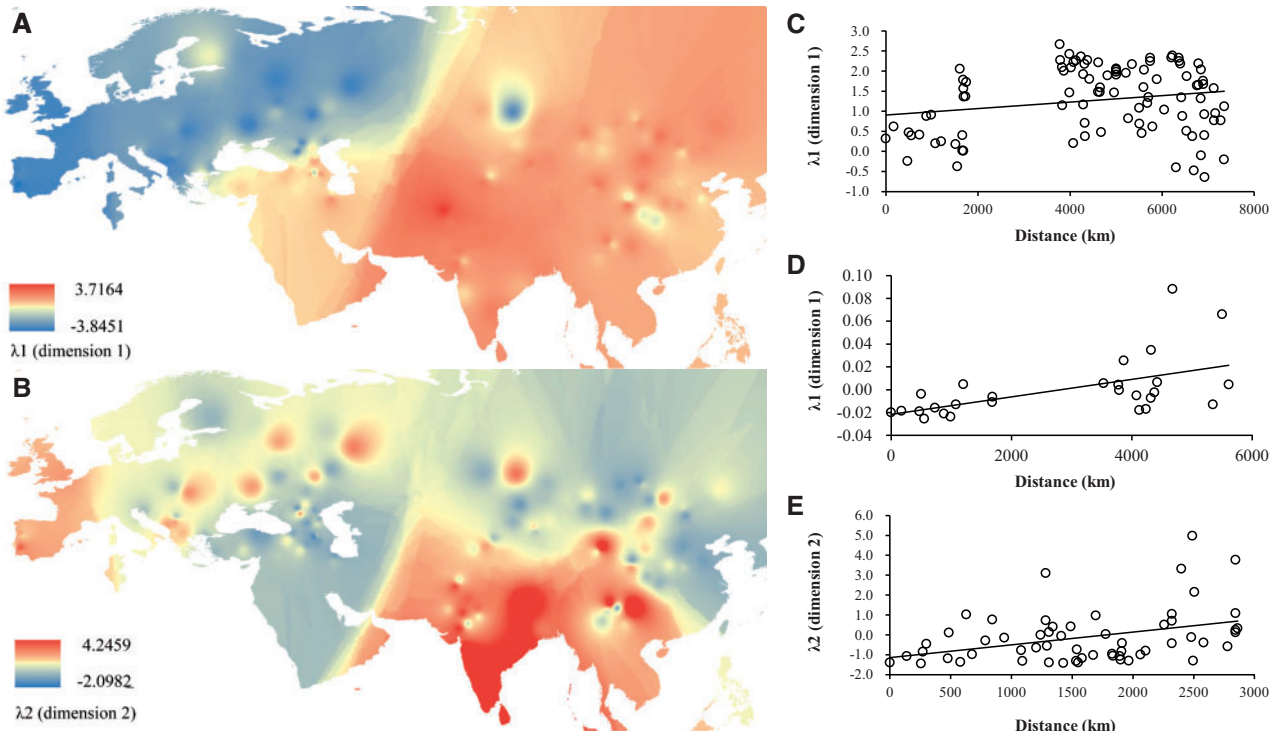


Fig. 5. Synthetic maps illustrating geographic variation of eigenvalues (λ) for the first two MDS dimensions (λ_1 and λ_2) and regression of λ versus geographic distance from the putative original site of colonization process. (A) synthetic map for λ_1 , (B) synthetic map for λ_2 , (C) regression of λ_1 versus geographic distances from the domestication center of sheep (represented by the geographic distance from the Kilis province of Turkey, where ancient domestic sheep are located; Demirci et al. 2013) for Asian populations ($r = 0.201$; $P < 0.05$); (D) regression of λ_1 (based on lineage A only) versus geographic distances from the domestication center of sheep (represented by the geographic distance from the Kilis province of Turkey, where ancient domestic sheep are located; Demirci et al. 2013) for sheep populations from the Indian subcontinent ($r = 0.547$; $P < 0.01$); and (E) regression of λ_2 versus geographic distances from a putative “transportation hub” of the Mongolian Plateau region (represented by the geographic distance from the northernmost population [Transbaikalian Finewool] sampled) for eastern Eurasian (including China, Mongolia, and India) populations ($r = 0.372$; $P < 0.01$).

significant ($P_F < 0.001$; $P_D < 0.001$; table 3) departures from neutrality in the three lineages. Additionally, the observed mismatch distributions of the lineages were fitted to the sudden population expansion models with very low values of the sum of squared deviation ($SSD \leq 0.005$; table 3) statistic and Harpending’s Raggedness index (Harpending 1994; $r_H = 0.021\text{--}0.031$; $P_R < 0.5$; table 3). Furthermore, the estimated pre- and postexpansion scaled effective population sizes ($2Nu$) indicated an increase in the effective population size for each of the lineages (A: 0.0–77.81; B: 0.04–16.63, and C: 0.00–15.77; table 3). The postdomestic expansion time expressed in twice the number of generations multiplied by the mutation rate ($\tau = 2 ut$) was found to be 6.443 ka (90% CI = 4.279–7.569 ka), 6.811 ka (90% CI: 3.502–9.706 ka), and 4.549 ka (90% CI = 2.402–6.652 ka) for lineages A, B, and C, respectively, when assuming an initial expansion (i.e., lineage A involving European sheep; Tapio et al. 2006) to equal 9 ka (table 3 and fig. 6). Separate analyses for the four major geographic areas (the Middle East, India, East Asia, and Europe) resulted in wider confidence intervals than those for the combined analysis and showed somewhat different estimates of τ (table 3). In particular, the expansion time for lineage C in the Middle East (3.910 ka; 90% CI: 2.818–5.120 ka) was more recent than that in East Asia (4.967 ka; 90% CI: 1.893–7.842 ka), whereas relatively earlier expansions in the Middle East

were inferred for lineages A and B (table 3). Additionally, we found much later expansions of lineages A and B in India (lineage A: 4.033 ka; 90% CI: 1.517–23.100 ka; lineage B: 3.393 ka; 90% CI: 0.961–16.311 ka) than those in East Asia (lineage A: 5.877 ka; 90% CI: 5.216–6.681 ka; lineage B: 7.008 ka; 90% CI: 3.030–16.348 ka), respectively.

ABC analyses based on the control region sequences identified an optimal model for each of the five sets of candidate colonization models (lineage A first-step, lineage A second-step, lineage B first-step, lineage B second-step, and lineage C; supplementary information S4, Supplementary Material online). The optimal models exhibited much higher posterior probability and nonoverlapped 95% CIs as compared with other candidate models (table 4). These optimal models indicated that 1) lineage A first colonized from the Middle East to the Mongolian Plateau region and the Indian subcontinent separately, and later from the Mongolian Plateau region to North China, and then to Southwest China (fig. 6); 2) lineage B first colonized from the Middle East to the Mongolian Plateau region, and then from the Mongolian Plateau region to North and Southwest China and the Indian subcontinent separately (fig. 6); and 3) Lineage C first colonized from the Middle East to the Mongolian Plateau region, and later from the Mongolian Plateau region to North China, and then to the Indian subcontinent (e.g., Nepal) (fig. 6).

Table 3. Sudden Expansion Model Parameters Estimated from the Distribution of Pairwise Differences between Sequences within mtDNA Lineages and Neutrality Tests for Different Lineages.

Lineages	Areas	τ (90% CI)	θ_0	Θ_1	SSD	P_{SSD}	r_H	P_R	ka (90% CI)	Tajima's D		Fu's F_S	
										D	P_D	F_S	P_F
A	All	2.62 (1.740–3.078)	0.00	77.81	0.005	0.044	0.030	0.548	6.443 (4.279–7.569)	-2.06	0.000	-25.09	0.000
	India	1.64 (0.617–9.394)	4.95	99,999.00	0.008	0.498	0.011	0.471	4.033(1.517–23.100)	-1.08	0.103	-24.68	0.000
	East Asia ^a	2.39 (2.121–2.717)	0.01	99,999.00	0.000	0.437	0.039	0.246	5.877 (5.216–6.681)	-2.33	0.000	-26.50	0.000
	Middle East	2.50 (1.969–3.004)	0.00	99,999.00	0.006	0.072	0.053	0.103	6.148 (4.842–7.387)	-2.16	0.001	-26.97	0.000
	Europe	3.66 (2.061–5.852)	0.53	41.25	0.005	0.541	0.028	0.558	9.000 (5.068–14.390)	-1.04	0.129	-4.89	0.011
B	All	2.77 (1.424–3.947)	0.04	16.63	0.001	0.783	0.021	0.759	6.811 (3.502–9.706)	-2.24	0.000	-25.48	0.000
	India	1.38 (0.391–6.633)	2.54	99,999.00	0.006	0.474	0.020	0.594	3.393 (0.961–16.311)	-0.83	0.190	-12.88	0.001
	East Asia ^a	2.85 (1.232–6.648)	1.03	10.45	0.002	0.743	0.012	0.893	7.008 (3.030–16.348)	-1.93	0.004	-26.14	0.000
	Middle East	3.00 (1.621–3.949)	0.00	32.66	0.001	0.673	0.023	0.685	7.377 (3.986–9.711)	-2.28	0.000	-26.66	0.000
	Europe	2.85 (1.232–6.648)	1.03	10.45	0.002	0.743	0.012	0.893	6.000 (5.383–6.711)	-1.93	0.004	-26.14	0.000
C	All	1.85 (0.977–2.705)	0.00	15.77	0.000	0.944	0.031	0.784	4.549 (2.402–6.652)	-2.41	0.000	-27.22	0.000
	East Asia ^a	2.02 (0.770–3.189)	0.00	11.08	0.002	0.675	0.030	0.780	4.967 (1.893–7.842)	-2.02	0.004	-24.70	0.000
	Middle East	1.59 (1.146–2.082)	0.00	99,999.00	0.000	0.829	0.052	0.406	3.910 (2.818–5.120)	-2.24	0.001	-27.33	0.000

NOTE.— $\tau = 2ut$, u is the mutation rate for the haplotypes, t is the time in generations; 90% CI, 90% confidence interval (CI) obtained by the parametric bootstrapping with 1,000 resampling; $\theta_0 = 2uN_0$, N_0 is the initial effective population size; $\theta_1 = 2uN_1$, N_1 is the final effective population size; SSD is the sum of square deviations (SSD) between the observed and the expected mismatch; P_{SSD} , P_R , P_D , and P_F are the corresponding significance values for SSD, r_H , Tajima's D (Tajima 1989), and Fu's F_S (Fu 1997); r_H , Harpending's Raggedness index (Harpending 1994).

^aEast Asia includes the regions of Northern China and the Mongolian Plateau (for details, see supplementary table S5, Supplementary Material online).

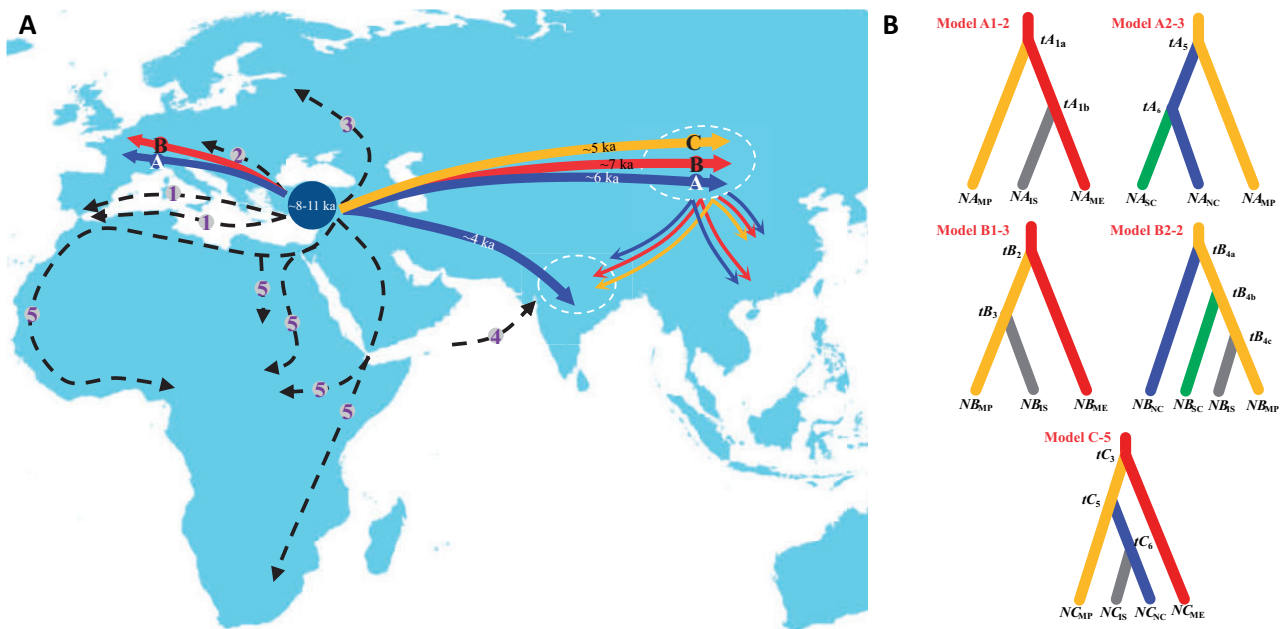


FIG. 6. (A) The major migratory events and routes of the major sheep lineages across Eurasia (lineage A, in blue; lineage B, in red; and lineage C, in yellow). Migration routes reported in previous studies have also been included: (1) The Mediterranean routes (Ryder 1984), (2) the Danubian route (Ryder 1984), (3) the northern Europe route (Tapio et al. 2006), (4) the ancient sea trade route to the Indian subcontinent (Singh et al. 2013), and (5) routes of introduction and spread of sheep pastoralism in Africa (Muigai and Hanotte 2013); (B) the optimal population colonization models for A/B/C lineages in DIYABC v.2.0.4 (Cornuet et al. 2014) (supplementary information S5, Supplementary Material online). Colors in the two figures are irrelevant between each other.

Taken together, our modeling results provided strong quantitative evidences supporting that the Mongolian Plateau region was acted as a “transportation hub” for sheep migration in Asia.

Discussion

In this study, we sequenced the largest number of complete ovine mitogenomes and control region sequences in sheep to

date. We also performed the first meta-analysis of complete and partial mitogenomic sequences of wild and domestic sheep across Eurasia. Our results clarify the domestication and history of sheep distributed particularly in eastern Eurasia regarding their origins, lineage divergence, demographic history, population recolonization, and evolutionary forces shaping the sheep mitogenomes. Below, we discuss these issues in more details and relate our findings to those

Table 4. Results of Model Choice for the Colonization Scenarios of Sheep Lineages A, B, and C Tested in the ABC Analyses.

Scenarios	Posterior Probability	95% Confidence Intervals
The models of lineage A in the first step		
A1-1	0.120	0.015 – 0.224
A1-2	0.842	0.822 – 0.862
A1-3	0.038	0.020 – 0.056
The models of lineage A in the second step		
A2-1	0.215	0.168 – 0.263
A2-2	0.060	0.016 – 0.105
A2-3	0.725	0.707 – 0.742
The models of lineage B in the first step		
B1-1	0.253	0.245 – 0.261
B1-2	0.258	0.245 – 0.272
B1-3	0.489	0.481 – 0.497
The models of lineage B in the second step		
B2-1	0.043	0.038 – 0.048
B2-2	0.939	0.933 – 0.945
B2-3	0.004	0.003 – 0.004
B2-4	0.005	0.005 – 0.006
B2-5	0.009	0.008 – 0.010
The models of lineage C		
C-1	0.158	0.149 – 0.167
C-2	0.079	0.069 – 0.088
C-3	0.207	0.200 – 0.215
C-4	0.138	0.128 – 0.147
C-5	0.418	0.407 – 0.430

NOTE.—The best-supported model with the highest posterior probability is indicated in italics.

that have emerged from early mtDNA studies on the ovine species.

Phylogenies

Phylogenetic analyses of complete mitogenomes showed a high resolution among wild sheep as well as among the major lineages of domestic sheep (fig. 4). The complete mitogenomes of *O. orientalis* and *O. musimon* formed a monophyletic group (fig. 4) that was incorporated within lineage B of domestic sheep. However, the analysis of full control region and *Cyt-b* fragments showed that *O. orientalis* is also closely related to other lineages (e.g., lineages A, C, and E) of *O. aries* (supplementary figs. S10 and S11, Supplementary Material online). This difference could be ascribed to the small number ($n = 3$) of *O. musimon* and *O. orientalis* complete mitogenomes available in this study (fig. 4).

Full control region and *Cyt-b* fragments from the complete mitogenomes produced similar phylogenies with fully resolved phylogenetic relationships of wild sheep, but they failed to define the phylogenetic relationships among the major lineages of domestic sheep (supplementary figs. S7 and S8, Supplementary Material online). Our results suggest that partial fragments of the complete mitogenomes would be problematic when making phylogenetic inferences about

domestic sheep. This problem arises due to diagnostic substitutions located elsewhere in the mitogenome (fig. 4; supplementary table S13, Supplementary Material online). Thus, the diagnostic substitutions for species and lineages presented here (fig. 4; supplementary table S13, Supplementary Material online) can serve as an important resource for maternal genetic differentiation between domestic and wild sheep as well as between the lineages within domestic sheep. Also, they might be helpful for addressing certain conflicts described above in future.

Origins and Migrations of Lineage C

Lineage C showed a restricted distribution in semidesert and steppe regions, 30–45°N. Given the limited geographic range, lineage C in domestic sheep may represent a recent genetic introgression from wild ancestors, rather than an independent domestication event. This hypothesis is supported by at least three lines of evidence: 1) lower mean frequency of lineage C (~10.36%) across the global breeds, and absence or extremely low frequency of lineage C (e.g., due to a recent expansion process; fig. 2B) in native breeds in Europe, the Indian subcontinent (including India and Pakistan), Southwest China, South Asia (including Indonesia), and Africa; 2) the extensive human practice of mating domestic ewes and wild rams documented in Central Asia and North China (Carruthers 1949; Aniwashi et al. 2007); and 3) lineage C shows an even earlier population expansion in East Asia (4.967 ka; 90% CI: 1.893–7.842 ka) than that in the presumed sheep domestication center of the Middle East (3.910 ka; 90% CI: 2.818–5.120 ka). High frequency and genetic variability (h and π) of lineage C were observed in breeds of northern central China (figs. 2B and 4D). These observations suggest that North China could be one of the origin regions for lineage C sheep, where sheep farming has been dated to approximately 5–8 ka at an early Neolithic site (Chen 1990; Cai et al. 2011; Yang et al. 2015). Nevertheless, we note that lineage C was not detected in earlier ancient DNA analyses of archaeological sheep remains (~3.5–4.5 ka; $n = 8$, Cai et al. 2007; $n = 14$, Cai et al. 2011; Yang et al. 2015; see also fig. 2A), which could be due to sampling effects.

Interestingly, lineage C co-occurs with indigenous fat-tailed breeds in Eurasia (fig. 2D), and our results suggest that the prevalence of lineage C could be probably linked to geographic range of fat-tailed breeds (see also Bruford 2005; Tapio et al. 2006), as deserving further investigations. Fat-tailed sheep was first recorded on an ancient Uruk II (~5 ka) and Ur stone vessel and mosaics in Iraq (~4.4 ka) by archaeological evidence (Ryder 1983; see also Muigai and Hanotte 2013). However, we observed very few or not at all lineage C in sheep from Pakistan, India, Southwest China, and Indonesia (fig. 2, supplementary tables S2 and S3, Supplementary Material online). These observations, together with the colonization scenarios of lineage C reconstructed using ABC, suggested that lineage C first colonized from the Middle East to the Mongolian Plateau region through the Caucasus, east of the Caspian Sea and Central Asia, then

dispersed to North China and the Qinghai-Tibetan Plateau, and finally arrived in Nepal.

Our results imply that the five maternal lineages evolved at different times before domestication (table 2). Zeder et al. (2006) suggested that independent domestication events in domestic animals might represent the introduction of founders that were subsequently submerged in the recruitment of local wild animals. Larson and Burger (2013) recently proposed that hybridization between local wilds and introduced domestic populations was common in a wide range of plant and animal species. Here, the extremely low occurrence of lineages D and E in domestic sheep and their restricted geographical distributions indicate that these lineages most likely represent additional two introgression events rather than independent domestication events.

Population Expansions and Migrations in Eastern Eurasia

We observed both prehistoric (~20–60 ka) and postdomestication (~3.5–9 ka) demographic expansions for the three major lineages (A, B, and C). Thus, the wild ancestors of domestic sheep underwent a major population expansion before the Last Glacial Maximum (~19.0–26.5 ka). Results of the BSP inferences on demographics should be interpreted with caution due to the confounding effect of population structure (e.g., Grant et al. 2012) and uncertainty in time estimation (e.g., Heller et al. 2013; see also the review by Ho and Shapiro 2011). Nevertheless, the worldwide collection of samples from a large number of populations and the application of multiple approaches in molecular clock calibration should minimize these effects.

We observed high levels of diversity (π) for lineage B in the breeds from Central Asia, with a decreasing gradient to the east (fig. 3C). This geographic pattern could be interpreted as the genetic introgression of Middle Eastern (rather than European) lineage B sheep into East Asian breeds through the Caucasus (Tapio et al. 2006) and Central Asia (fig. 6). This explanation is supported by the similar expansion time of lineage B sheep in the Middle East and East Asia but a later expansion of lineage B in Europe (the Middle East: 7.377 ka, 90% CI: 3.986–9.711 ka; East Asia: 7.008 ka, 90% CI: 3.030–16.348 ka; Europe: 6.000 ka, 90% CI: 5.383–6.711 ka; table 3). We also observed a high level of nucleotide diversity (π) for lineage A in the Caucasus, following a west–east decreasing gradient. In addition, decrease with geographic distance eastwards for the λ_1 eigenvalues is significant across East Asian (including China and Mongolia) populations with its peak in the Middle Eastern domestication center ($r = 0.201$; $P < 0.05$; fig. 5A and C). Moreover, East Asian breeds showed closer genetic relationships with the breeds in Central Asia, Caucasus, and Turkey than those in Europe, as indicated by the MDS plot and pairwise genetic differentiation (supplementary figs. S12 and S13 and table S12, Supplementary Material online). Thus, the maternal lineages in the Middle Eastern sheep may have been introduced to East Asia through the Caucasus and Central Asia by commercial trade between the West and East (e.g., Cai et al. 2011). Recent archeological

studies in North China suggest that the West–East trade contact could be traced back to the prehistoric period through the “Bronze Road” across the vast Eurasian landmass approximately 4.5–5.3 ka (Yi 2004). Also, at this time millet was transported from its domestication site in North China to the Caucasus region approximately 7 ka (Hunt et al. 2008; Jones and Liu 2009). However, regression analysis (fig. 5C) and Bayesian simulations (fig. 6B) of lineage A identified its south-eastwards migration from the Middle East to the Indian subcontinent through Arabia (and/or the Arabian Sea; see, e.g., Singh et al. 2013), which could be due to ancient overland (and/or maritime) trade (e.g., the Silk Road) between Arabia and India dated as early as approximately 4.5 ka (e.g., Gauri 2013).

We found high levels of genetic variability in sheep breeds distributed in North China and the Mongolian Plateau (fig. 3A), with a decreasing gradient to the southwest. This finding, together with the extreme ranking of λ_2 eigenvalues in these regions (fig. 5B), suggests that the Mongolian Plateau region could be a secondary center of dispersal serving as a “transportation hub” in eastern Eurasia, and sheep have migrated to China (lineages A, B, and C) and the Indian subcontinent (lineages B and C) from the Middle East through this region (fig. 6). This suggestion is corroborated by several pieces of evidence: 1) Bayesian coalescence-based simulations identified two phases of migration in eastern Eurasian sheep: The Middle Eastern sheep first arrived in the Mongolian Plateau region, and then dispersed to other regions including North and Southwest China (lineages A, B, and C; fig. 6) and the Indian subcontinent (lineages B and C; fig. 6); 2) a high and significant correlation was obtained for the southwest dispersal ($r = 0.372$; $P < 0.01$; fig. 5E); 3) the expansion of lineage B in the Indian subcontinent was much later than in East Asia (table 4), and lineage C was observed in a few samples from the Indian subcontinent; 4) several animal domestications/early Holocene animal management (e.g., pig, Larson et al. 2010; cattle, Zhang et al. 2013; chicken, Xiang et al. 2014) and crop cultivation events (e.g., millet, Lu et al. 2009; Yang et al. 2012) took place in the middle and lower reaches of the Yellow River in North China; 5) archeological remains showed an early presence of domestic sheep in Inner Mongolia and northern China during the period of Hongshan Culture (~4.2–6.5 ka) (Yang et al. 2015), and archeological sites in Tibet dating to 3.6–5.2 ka are marked by the presence of sheep bones (Chen et al. 2015); and 6) there were several popular ancient trade routes between China and India, by both land (through the Southern Silk Road route and the Tibet–Nepal and Burma–India routes) and sea 3–4 ka (Pe 1959; Shaha 1970). Analyses of genome-wide single nucleotide polymorphisms (SNPs) (~50 K) in a representative set of 22 native Asian sheep breeds suggest two similar colonization processes of eastern Eurasian sheep domestication as those obtained from mtDNA variability (for the statistical analyses and results of SNPs, see supplementary information S2, table S14, and figs. S20–S23, Supplementary Material online). Also, by using the TreeMix algorithm in the program TreeMix v.1.12 (Pickrell and Pritchard 2012), analyses of the SNP data set reveal strong gene flows between the native breeds

of different geographic regions and forward and backward movements as well (supplementary information S3 and fig. S24; Supplementary Material online).

Multiple Forces Acting on the Maternal Genetic Make-up of Eastern Eurasian Sheep

Frequent animal exchange, particularly of breeding ewes, between the breeds in different regions through ancient trade routes (e.g., the Silk Road) could have contributed to the geographic patterns observed in this study (figs. 2 and 3). In addition to the initial population expansion during the Neolithic, sheep maternal lineages could also have spread to East Asia and the Indian subcontinent through the Mongolian invasions. Overall, our findings suggest strong historical human-mediated gene flow between breeds across Eurasia (supplementary figs. S12, S13, and S20–S24, Supplementary Material online; see also, e.g., Kijas et al. 2009, 2012). This hypothesis is also compatible with the high level of genetic admixture revealed by a recent genome-wide analysis of sheep breeds across the world (Kijas et al. 2012).

In addition to the ancient haplotypes (e.g., a1, b1, and c1), we also detected several derived predominant haplotypes (e.g., a2 and b2) composing apparent substructures within the lineages (supplementary fig. S2, Supplementary Material online). During the initial population expansion, a variety of new haplotypes might have appeared in different regions, resulting from local evolutionary dynamics, for example, selective constraints imposed by the regional environments (Zhang et al. 2013). In the Indian subcontinent, we found high levels of nucleotide diversity of lineages A and B (fig. 3B and C). Because the expansion times of lineages A and B in the Middle East greatly predate those in India, this observation implies that some new haplotypes could have arisen under secondary domestication in India (Singh et al. 2013), driven by local population dynamics.

The ω ratios ($\omega = dN/dS$; dN, nonsynonymous substitutions; dS, synonymous substitutions; see Materials and Methods) of lineages A–E ranged from 0.0457 to 0.0775 and were lower than those of other domestic animals such as goat ($\omega = 0.123$ – 0.387 ; Nomura et al. 2013), dog ($\omega = 0.183$; Björnerfeldt et al. 2006), and yak ($\omega = 0.231$; Wang et al. 2011). It is reasonable to expect a stronger purifying pressure on sheep than some other domestic animals, as the sheep have often been subject of highly industrialized agricultural practice. Widely variable pastoral environments, such as highland plateau, areas of high precipitations (Joost et al. 2007), and dry desert regions, may have also maintained relatively intense selection pressures on sheep throughout history (e.g., Lv et al. 2014). The low ω values and significant differences between them (table 2) suggest that severe selective pressures have continued to operate on these lineages.

Approaches for Estimating Divergence Time

The use of molecular clock methods to infer date of evolutionary divergence events should be cautioned because a diverse range of available molecular clock methods and models

can yield different estimates of ages (e.g., the reviews in Kumar 2005; Ho 2014; Ho and Duchêne 2014). Here, the divergences between domestic lineages dated by BI were much earlier than those by ML (table 2). The difference was consistent with that in an early study on complete mitogenomes from modern horses using external calibration points (Achilli et al. 2012), but contrasted with that of a study on human mtDNA using internal calibration points (Pereira et al. 2010). Achilli et al. (2012) suggested that the BI method behaves better when inferring the ages of nodes for any given lineages using internal calibration points. However, ML should be a more appropriate method by which to estimate the divergence time without a fossil record as internal calibration point. Nomura et al. (2013) argued that the fossil record in general does not always point to the “real” divergence time, but rather that the split of two lineages was older than the age of the first fossil record. In this study, we used a paleontological calibration point (i.e., the divergence time of 2.6 Ma between *O. aries* and *O. vignei*) applied to archeological timeframes. Early studies indicated that the paleontological calibration is more reasonable and if this younger fossil age had been used as the calibration point, divergence times would have been grossly underestimated (e.g., Nomura et al. 2013; Jiang et al. 2014). Although the approach has been successful in dating interspecies divergence based on mtDNA (e.g., Nomura et al. 2013) and gene sequences (e.g., Jiang et al. 2014), we should be also aware of the uncertainties from such analyses associated with the impact of different timescales (e.g., the reviews in Ho et al. 2011; Ho and Duchêne 2014). The local clock, a more complex model than the global clock model, allows for different molecular clocks for the branches. Because there was genetic differentiation between wild and domestic sheep (p distance = 0.025, data not shown), the evolutionary times estimated under the local clock model with an assumption of different molecular clocks for domestic and wild sheep should be more reasonable (table 2).

Also, several biological and methodological factors can affect time estimation, including natural selection and accurate characterizations of time-dependent molecular evolutionary rate and timescale (e.g., Ho et al. 2011; Ho and Duchêne 2014). We estimated that the oldest split for lineages C and E from A, B, and D in domestic sheep occurred at approximately 0.790 Ma (95% CI: 0.634–0.934 Ma) based on synonymous substitutions, which is more recent than that estimated using a small number of complete mitogenomes in an early study (0.920 Ma, $n = 16$; Meadows et al. 2011). This may be because molecular clocks at synonymous sites display less time dependence and should be closer to the neutral molecular clock than those at nonsynonymous sites (e.g., the reviews in Kumar 2005; Ho et al. 2011; Ho 2014). Our detection of strong purifying selection on the protein-coding regions of the *O. aries* lineages (supplementary fig. S14, Supplementary Material online) also suggests that the divergence time estimation based on complete mitogenomes could be biased (see also the reviews in Ho et al. 2011; Ho and Duchêne 2014). Instead, using only synonymous sites might be a better choice for divergence time estimation.

Moreover, a variety of molecular evolution rates for different components of mtDNA may also have contributed to the bias in the estimation of divergence time using complete mitogenomes (Torrioni et al. 2006; Taberlet et al. 2008; Achilli et al. 2009; see also the reviews in Ho and Shapiro 2011; Ho 2014; Ho and Duchêne 2014). Similar differences were also observed in the mtDNA studies of other domestic animals, such as horse (e.g., Lippold et al. 2011; Achilli et al. 2012) and goat (e.g., Naderi et al. 2008; Nomura et al. 2013).

Conclusions

In conclusion, the first meta-analysis of complete and partial mtDNA sequences of wild and domestic sheep throughout Eurasia reveals that 1) the most common female ancestor of the five *O. aries* lineages lived at approximately 0.79 Ma (95% CI: 0.637–0.934 Ma) during the Middle Pleistocene, being much earlier than their domestication event (~8–11 ka); 2) the ancestor of *O. aries* experienced a rapid increase in N_e immediately before the Last Glacial Maximum (~19.5–26 ka); 3) two migratory waves (the first wave: lineages A and B; the second wave: lineage C) at approximately 4.5–6.8 ka along different routes created the original maternal genetic make-ups of modern sheep across Eurasia, with the influence of prehistoric human activities, and North China could be an origin region for lineage C sheep; 4) the Mongolian Plateau region was a secondary zone/center of sheep dispersal as a “transportation hub” in eastern Eurasia: Sheep from the Middle Eastern domestication center were inferred to have migrated through Caucasus and Central Asia, and arrived in North and Southwest China (lineages A, B, and C) and the Indian subcontinent (lineages B and C) through this region; and 5) lineage A was inferred to have been introduced into the Indian subcontinent from the Middle East through Arabia. The results of this study significantly improve our understanding of sheep domestication across Eurasia, particularly the dispersal to and from eastern Eurasia.

Materials and Methods

Samples, DNA Extraction, mtDNA Sequencing, and Sequence Quality Trimming

Complete mitogenomes of 45 animals representing 42 modern native breeds (*O. aries*) from Azerbaijan, Moldova, Serbia, Ukraine, Russia, Kazakhstan, Poland, Finland, China, and United Kingdom and two wild species (*O. orientalis* and *O. vignei*) from Kazakhstan were sequenced (fig. 1 and supplementary table S1, Supplementary Material online). In addition, the control region of 875 animals representing 51 eastern Eurasian (including 41 Chinese, 3 Iranian, 3 Pakistani, 2 Indonesian, and 2 Siberian [the Republic of Buryatia, Russia]) native breeds was also sequenced (fig. 1 and supplementary tables S2, Supplementary Material online). In all the cases, pedigree information and knowledge of local herdsmen were used to ensure that animals were purebred and unrelated. Summary of sample information, including species/breed names and codes, sites with their geographic coordinates, and sample sizes are detailed in supplementary tables S1 (complete mitogenomes) and S2 (control region),

Supplementary Material online. Genomic DNA was extracted from whole blood or marginal ear tissue using a standard phenol–chloroform method (Sambrook and Russell 2001).

Twenty-one primer pairs, including 18 used in Meadows et al. (2011) and three new pairs (supplementary table S15, Supplementary Material online) designed from the *O. aries* complete mitogenome reference sequence AF010406 (Hiendleder, Mainz, et al. 1998) in this study, were used to amplify the complete mitogenome of domestic and wild sheep. Polymerase chain reaction (PCR) products from the 43 domestic and two wild individuals were produced following Meadows et al. (2007). In addition, a pair of primers, forward primer MSD-F 5'-ACAACACGGACTTCCCCTC-3' (map positions 15522–15541 bp of AF010406) and reverse primer MSD-R 5'-CCAAGCATCCCCAAAAATTA-3' (map positions 16299–16318 bp of AF010406), was designed to amplify a 749-bp fragment of the control region. PCR amplification was conducted in 20 μ l containing 30 ng of total DNA, 0.4 μ M of each primer, and 2 \times Es Taq Master Mix (ComWin Biotech, Beijing, China) with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 1 min at 72°C, and a final 10-min extension at 72°C. All products were sequenced directly in the forward and reverse directions using the amplification primers on an ABI 3730 capillary sequencer (Applied Biosystems, Life Technologies, NY). Homologous sequences of control region in Indonesian, Iranian, Mongolian, Nepalese, and Pakistani sheep were obtained following Luo et al. (2005).

All the reads were assessed manually and aligned using CLUSTAL_X v.2.1 (Larkin et al. 2007). Complete mitogenomes were assembled and aligned to AF010406 using SeqMan II software in the Lasergene package v.12 (DNASTAR, Inc., Madison), and they were further trimmed to exclude the 75/76 bp tandem repeats (Lancioni et al. 2013) from bp 15650 to 15905. After trimming, the complete mitogenomes were fractionated into four portions, including the concatenations of tRNA, rRNA, 13 protein-coding genes with the ND6 gene readjusted to the same reading direction as the other genes (e.g., Peng and Zhang 2011), and the control region.

mtDNA Data Sets and Sequence Alignment

Fifty complete mitogenomic sequences were retrieved from GenBank, including 42 from *O. aries*, 2 from *O. musimon*, 3 from *O. vignei*, 2 from *O. ammon*, and 1 from *O. canadensis* (supplementary table S1, Supplementary Material online). In addition, a total of 2,017 partial *O. aries* mtDNA sequences (1,470 partial control region sequences from 98 breeds and 547 partial *Cyt-b* sequences from 50 breeds; supplementary tables S2 and S3, Supplementary Material online) of native Eurasian breeds (covering the Indian subcontinent, the Middle East, the Caucasus, Southwest Asia, Central Asia, Mongolia, and Europe) were extracted from GenBank. We also collected full control region (number of sequences = 346; supplementary table S8, Supplementary Material online) and *Cyt-b* (number of sequences = 271; supplementary table S9, Supplementary Material online) sequences of domestic and

wild sheep (including *O. orientalis*, *O. musimon*, *O. vignei*, *O. ammon*, *O. canadensis*, *O. dalli*, and *O. nivalis*) from GenBank. All sequences were screened for quality using the approach of Yao et al. (2009) and Shi et al. (2014), and problematic sequences were discarded in downstream analysis.

After quality trimming and filtering, all the complete and partial mitogenomic fragments obtained in this study were compared with the respective sequences collected from GenBank using the nucleotide BLAST (Basic Local Alignment Search Tool) program (Altschul et al. 1997). Common fragments of 506 bp (map positions 14453–14958 of AF010406) for *Cyt-b* and 292 bp (15541–15654 and 15955–16132 of AF010406) for control region were aligned and edited for analysis using ClustalX v.2.1 (Larkin et al. 2007).

Sequence Variation and Phylogenetic Analyses

Measures of sequence variation, including number and proportion of substitutions (s), transition/transversion ratio, and nucleotide (π) and haplotype diversity (h), were computed using the program DnaSP v.5.1 (Rozas et al. 2003). Estimates of π and h were corrected for sample size using rarefaction. The five major maternal lineages (A–E) of *O. aries* are defined by specific diagnostic mutations common to the individual lineages (e.g., Meadows et al. 2007, 2011), and the lineage frequencies in each breed/population (or group of breeds/populations) were estimated by counting.

Pairwise-population fixation index (F_{ST}) values (Reynolds et al. 1983) and analysis of molecular variation were calculated based on the 2,345 partial control region sequences across Eurasia using Arlequin v.3.5 (Excoffier and Lischer 2010). The obtained genetic distances were used to draw MDS plots in the R package v. 3.1.2 (R Core Team 2014), and eigenvalues (λ) for the first two dimensions (dimensions 1 and 2) were calculated in each breed/population. To visualize the geographic distribution patterns of π and λ , interpolation maps were constructed using the ArcMap program in ArcGIS v.10.0 software. The inverse distance weighted option with a power of 2 was selected for the interpolation of the surface (e.g., Hanotte et al. 2002). Regression of λ_1 versus geographic distance from the domestication center of sheep (represented by the geographic distance from the Killis province of Turkey, where ancient domestic sheep are located; Demirci et al. 2013), and λ_2 versus geographic distance from a putative “transportation hub” of the Mongolian Plateau region (represented by the geographic distance from the northernmost population [Transbaikalian Finewool] sampled) were conducted separately for populations through various migration routes using SPSS v. 18.0.

Phylogenetic relationships among haplotypes were inferred from the complete mitogenomes using the BI approach within MrBayes v.3.2.2 (Ronquist et al. 2011) and the ML approach within PhyML v.3.0 (Guindon and Gascuel 2003). Hierarchical LRTs were implemented to select a best-fit model of nucleotide substitution in the phylogenetic analysis using the program jModelTest v.2.1.4 (Darriba et al. 2012). Out of 88 candidate models, the best-fit models under the Bayesian information criterion,

HKY85 + I + G, HKY + G + I, HKY + G, and TN93 + H + G (for details of the models, see Hasegawa et al. 1985; Tamura and Nei 1993; Posada 2008), were selected for the data sets of protein-coding genes, control region, *Cyt-b*, and complete sequences of the mitogenomes, respectively. Bootstrap support values for the ML analysis were generated with 1,000 replicates (Felsenstein 1985). The BI analysis was run with four simultaneous Markov chains for 20 million generations, starting from a random tree. The sampling frequency was set to 1/1,000, and the first 20% of trees obtained were discarded as a burn-in. In addition, we reconstructed phylogenetic trees based on the full control region (1,105–1,333 bp) and *Cyt-b* sequences (1,063–1,143 bp) of wild and domestic sheep (supplementary tables S7 and S8, Supplementary Material online). Phylogenetic trees were inferred using the BI, ML, maximum parsimony (MP), and neighbor-joining methods with the HKY + G model as described in Rezaei et al. (2010). We also constructed median-joining networks from the full control region and *Cyt-b* sequences of domestic and wild sheep (including the hybrids), and attempted to assign each *Cyt-b* or control region sequence to the specific lineages defined previously using diagnostic mutations and the near-matching strategy (e.g., Wu et al. 2007; Achilli et al. 2012). Similarly, median-joining networks (Bandelt et al. 1999) were built based on the partial control region or *Cyt-b* sequences separately.

Detection of Signatures of Selection on Protein-Coding Genes

Signatures of selection on the protein-coding genes of the five lineages were detected using the branch-site models of ω ($\omega = dN/dS$; dN , nonsynonymous substitutions; dS , synonymous substitutions) implemented in the CODEML program of the package PAML v.4.7 (Yang 2007). ω is an indicator of natural selection. Values of $\omega = 1$, $\omega < 1$ and $\omega > 1$ indicate neutral evolution, purifying and diversifying positive selection, respectively (Goldman and Yang 1994; Crandall et al. 1999). Values of ω were estimated for the codons of different branches (lineages A–E) that were assumed to have evolved independently of one another. Four codon-substitution models were used to investigate the signature of selection. The phylogenetic tree for protein-coding genes was constructed using the MP method as implemented in MEGA v.5.2.2 (Tamura et al. 2011).

First, we tested the simplest branch, which assumed the same ω ratio for all the branches in the entire tree. Second, we assumed a two-ratio model that assigned a ω ratio for the tested branch and a background ω ratio for all the other branches. Third, we tested the three-ratio model, which allowed two different ω ratios on the two tested branches and a background ω ratio for the other branches. Finally, we adopted a free-ratio model, which assumed a different ω ratio for each of the four branches in the phylogenetic tree. Log-likelihood scores ($\ln L$) evaluated the quality of the fit of the tested data to the conditions of the model; a higher log-likelihood value (close to zero) was a “better”-fitting model (Yang 1998). LRTs were further employed to compare

the fit of the models to the data by comparing twice the log-likelihood difference ($2\Delta\ln L$) to a χ^2 distribution with degrees of freedom equal to the difference in the number of parameters between the two models (Yang 1998).

Divergence Time Estimation

Phylogenetic relationships within the genus *Ovis* (see Results) inferred in previous analyses were used to estimate the divergence times between the major *O. aries* lineages using PAML v.4.7 (Yang 2007) and BEAST v.1.7.5 (Drummond and Rambaut 2007). Due to the lack of an exact fossil record between *Ovis* species for the calibration, we used a comprehensive evolutionary framework (see Nomura et al. 2013; Jiang et al. 2014) to estimate the divergence time between *O. aries* and *O. vignei*. A phylogenetic tree including 24 species (supplementary table S16, Supplementary Material online) was inferred based on the 13 mtDNA protein-coding genes using the GTR + I + G model in MrBayes v.3.2.2 (Ronquist et al. 2011). The divergence times were estimated based on five fossil calibration points (18.3–28.5 Ma between Bovinae and Caprinae, 52–58 Ma between Cetacea and hippopotamus, > 34.1 Ma between baleen and toothed whales, 42.8–63.8 Ma between Caniformia and Feliformia, and 62.3–71.2 Ma between Carnivora and Perissodactyla; see Nomura et al. 2013; Jiang et al. 2014). We applied the obtained *O. aries/O. vignei* divergence time (2.6 Ma; see Results) and three models to estimate the divergence times between the five *O. aries* mtDNA lineages. Global and local clock models were implemented using the ML in PAML v.4.7 (Yang 2007) and the uncorrelated relaxed-clock model was implemented using BEAST v.1.7.5 (Drummond and Rambaut 2007).

We only considered the 61 complete mitogenomes of wild sheep species and native breeds of domestic sheep (supplementary table S1; Supplementary Material online) and applied three strategies in the ML analysis: One considered the complete mitogenomes under the TN93 model, the second considered the synonymous mutations under the HKY85 model, and the third considered only the third-codon positions under the HKY85 model. Similarly, Bayesian Markov chain Monte Carlo (MCMC) analysis of molecular sequences was performed by applying the three strategies in the program BEAST v.1.7.5 (Drummond and Rambaut 2007). Parameters of prior distributions, including models of nucleotide substitution and the divergence time between *O. aries* and *O. vignei*, were set the same as in the ML analyses described above. Three independent runs were performed with 50 million iterations. Samples were drawn every 5,000 MCMC steps, with the first 25% samples discarded as burn-in. The results of the three independent runs were combined using the LogCombiner program (available at <http://beast.bio.ed.ac.uk/LogCombiner>, last accessed October 16, 2014) from BEAST v.1.7.5 (Drummond and Rambaut 2007). Convergence was confirmed by effective sampling size (ESS) greater than 200 using the program Tracer v.1.5 (Drummond and Rambaut 2007; available at <http://beast.bio.ed.ac.uk/Tracer>, last accessed December 26, 2014).

BI of Population Expansions

Based on the divergence time of internal nodes estimated above and the 51 complete mitogenomes of native domestic sheep breeds, we reconstructed the change in N_e of *O. aries* through time using BSPs (Drummond et al. 2005). The analyses were also performed on the partial *Cyt-b* and control region sequences. We ran three independent chains in each analysis using BEAST v.1.7.5 (Drummond and Rambaut 2007), with 50 million generations (after discarding the first 10% of sampled generations as burn-in) and samples drawn every 5,000 steps. We applied the HKY85 and TN93 models under relaxed-clock model for complete mitogenomes and partial *Cyt-b* sequences, respectively. In the analysis of control region sequences, we set similar parameter values of 200 million generations (after discarding the first 10% of sampled generations as burn-in) with samples drawn every 2,000 steps under the HKY85 and relaxed-clock models. The combination of three independent results and checks of convergence were performed following the same procedures as described above.

Signatures of population expansions were examined using Arlequin v.3.5 (Excoffier and Lischer 2010). First, the observed and expected mismatch distributions of pairwise differences between haplotypes were compared using Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) tests of neutrality. Furthermore, we estimated the parameters for the sudden population expansion model (Rogers 1995), and the fit of the data to the sudden population expansion model was tested. Harpending's raggedness index (r_{Hj} ; Harpending 1994) of the observed mismatch distribution was also calculated. P values of the SSDs test to evaluate the fit and significance for the parameters (r_{Hj} , D , and F_s) were determined with 1,000 coalescent simulations using Arlequin v.3.5 (Excoffier and Lischer 2010). All the partial control region sequences were included in the calculations.

Further, to corroborate our inference that the Mongolian Plateau region serves as a "transportation hub" in eastern Eurasia (see Results), we distinguished several candidate colonization scenarios for the three main *O. aries* lineages (A, B, and C) using the ABC (Beaumont et al. 2002) procedure in DIYABC v.2.0.4 (Cornuet et al. 2014). By incorporating all the *O. aries* control region sequences of a 292-bp-long hypervariable fragment, we tested six, eight, and five colonization models regarding potential migration routes from the Middle Eastern domestication center to different regions in eastern Eurasia (e.g., the Mongolian Plateau region, North China, Southwest China, and the Indian subcontinent) for the lineages A, B, and C, respectively (supplementary fig. S25, Supplementary Material online). Detailed information about the ABC analyses including the candidate colonization models tested was provided in supplementary information S4, Supplementary Material online.

Supplementary Material

Supplementary information S1–S4, figures S1–S25, and tables S1–S19 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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