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Original Research

Ancient mitochondrial genome depicts sheep maternal dispersal and migration in Eastern Asia

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ABSTRACT

Sheep have been one of the most important groups of animals since ancient times. However, the knowledge of their migration routes and genetic relationships is still poorly understood. To investigate sheep maternal migration histories alongside Eurasian communications routes, in this study, we obtain mitochondrial genomes (mitogenomes) from 17 sheep remains in 6 Chinese sites and 1 Uzbekistan site dated 4429–3100 years before present (BP). By obtaining the mitogenomes from the sheep (4429–3556 BP) found in the Tongtian Cave site in Xinjiang, Altai region of northwest China, our results support the emergence of haplogroup C sheep in Xinjiang as early as 4429–3556 BP. The combined phylogenetic analyses with extant ancient and modern sheep mitogenomes suggest that the Uzbekistan-Altai region may have been a migration hub for early sheep in eastern Asia. At least two migration events have taken place for sheep crossing Eurasia to China, one passing by Uzbekistan and Northwest China to the middle and lower reaches of the Yellow River at approximately 4000 BP and another following the Altai region to middle Inner Mongolia from 4429 BP to 2500 BP. Overall, this study provides further evidence for early sheep utilization and migration patterns in Eastern Asia.

Introduction

Sheep (*Ovis aries*), one of the earliest domesticated animals (Chessa et al., 2009), have provided meat, wool, milk, and other products for thousands of years (Wang, 2017). The history of the origin, domestication, and migration of sheep reflects the development of human society. Sheep may have been domesticated from the Asian mouflon (*Ovis orientalis*) in the Fertile Crescent 11,000–8000 years before present (BP) (Ryder, 1984) and were genetically influenced by wild relatives such as argali (*Ovis ammon*) and European mouflon (*Ovis musimon*) during the early domestication process (Barbato et al., 2017; Zhao et al., 2017). Analyses of ancient DNA (aDNA) from Neolithic Anatolian sheep remains suggest that early sheep in the putative domestication center experienced multiple domestication episodes at approximately 8000 BP, implying multiple and parallel sheep domestication processes in various regions by different Neolithic communities (Yurtman et al., 2021). Over the next few thousand years, sheep gradually spread from the early domestication center to Asia, Europe, and Africa by different routes, and various breeds were formed under artificial and natural selection processes (Tapio et al., 2006; Muigai and Hanotte, 2013; Zhao et al., 2017; Sassi-Zaidy et al., 2022). The Eurasian Steppe stretches across the Eurasian continent, from Hungary and the Mediterranean in the west to Mongolia and Northeast China in the east, providing a natural passageway for sheep to spread on the grasslands. For thousands of years, the thriving agricultural civilization and economic exchanges between the east and west along the Eurasian Steppe greatly influenced the early sheep dispersal and migration patterns.

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Previously, archaeological and biomolecular evidence of Neolithic sheep remains from southern Kyrgyzstan demonstrated the emergence of food-producing economies in interior Central Asia as early as 8000 BP (Taylor et al., 2021). Then, a mitogenome study of modern *Ovis* species revealed that two waves of sheep migration occurred in East Eurasia approximately 6800–4500 BP driven by prehistoric West-East commercial trade (Lv et al., 2015). Recently, a third expansion of domestic sheep from the center of domestication to Central and Eastern Asia was proposed, which was deemed to be influenced by the excellent fat storage capacity of fat-tailed sheep in extreme climates (Deng et al., 2020). Studying the history of sheep domestication and migration is helpful in reflecting the development of human society and provides a reference for the conservation and utilization of sheep genetic resources worldwide.

It is widely accepted that sheep have five dominant maternal haplogroups (A, B, C, D, and E) with geographical distribution patterns (Meadows et al., 2005, 2007; Lv et al., 2015). Haplogroup A sheep are mainly distributed in Asia, haplogroup B sheep are mostly found in Europe (Meadows et al., 2005), and haplogroup C sheep are mainly distributed in the Middle East, the Caspian Sea region, and Northern China (Lv et al., 2015). For modern Chinese native sheep breeds, haplogroups A, B, and C are the dominant lineages; nevertheless, different sheep populations from different regions exhibit various lineage constitutions. For instance, haplogroup A accounts for approximately 60% of modern Chinese sheep (Zhao et al., 2017), but it is much more common and widely distributed in northern populations (Zhao et al., 2013, 2017; Lv et al., 2015). Both haplogroups B and C are less dominant in modern Chinese sheep (Zhao et al., 2017). In general, haplogroup B is widely distributed in northern populations (Zhao et al., 2017), while haplogroup C is more common in sheep populations from the Tibetan Plateau but less common in those from the Southwest China mountainous region (Zhao et al., 2013). Moreover, different migration routes have been proposed for haplogroups A, B, and C sheep in Eastern Asia, from the putative domestication center to North and Southwest China as well as the Indian subcontinent through the Mongolian Plateau as a "transportation hub" in East Eurasia approximately 4500–6800 BP (Lv et al., 2015). The aDNA analyses on 4200–2500 BP sheep remains from North China suggested the dominance of haplogroup A (95.5%) and a small proportion of haplogroup B (4.5%) in early Chinese sheep populations during the Bronze Age and a close relationship to modern Chinese sheep (Cai et al., 2007, 2010, 2011). Meanwhile, sheep bones dating from ~4000 BP were unearthed in the Inner Mongolia-Gansu-Qinghai area (Xie, 1975; Ben, 1979; Huang, 1996; Yuan, 2001; Guo, 2012; Wang et al., 2017, 2020) and provided potential evidence for earlier sheep introduction and utilization along with the thriving communications among nomadic tribes 4000 BP (Jeong et al., 2020; He et al., 2023). However, limited research has been employed to investigate the lineage origins of early sheep populations in Northwest China and their genetic contributions to modern sheep.

In this study, ancient sheep remains from seven sites in China and Uzbekistan, representing typical sheep populations along the early migration route from Central Asia to East Asia through the Altai region, were collected and sequenced to obtain their mitogenomes. Early sheep populations were intensively used in the regions covering most of these sites (Qiu, 1996; Cai, 2002; Yuan et al., 2007b; Lv, 2009; Brunson et al., 2016). Among them, the Sapalli Tepe site is located on the right bank of the Amu Darya River, northwest of Ammuz city, Uzbekistan, which represents the Oxus civilization of the Late Bronze Age (3719–3519 BP) in southern Central Asia (Dani, 1992). Excavated artifacts such as pottery and bone tools indicate that the region was in a period of settled agriculture (Hemphill, 1999). The Tongtian Cave site, located in the water-rich Altai region of Xinjiang Province, was an important hub for ancient communication and exchange between West and East Asians in Northwest China (Yu et al., 2018). According to the archaeobotanical, palynological and anthracological radiocarbon data, the Tongtian Cave site was dated between 5200 and 3200 calibrated years BP (Zhou et al., 2020). However, most of the sheep bones and teeth were mainly excavated from layers 2 and 4 (Yu et al., 2018), which were 4429–3556 BP referring to the age of naked barley and common wheat from the same layers (Zhou et al., 2020). The abundant sheep remains and the earliest barley and wheat at the Tongtian Cave site (Zhou et al., 2020) suggest active sheep husbandry and provide clues to potential migration, communication, and diffusion between Western and Eastern Asia. The middle and lower reaches of the Yellow River in China covered five sites in this study, which were flourishing areas for animal husbandry 4350–3100 BP (Yuan, 2008; Dong et al., 2021). In detail, the Erlitou site was the earliest capital city of the Xia Dynasty (3800–3500 BP) in China (Zhang et al., 2008), and large amounts of animal remains, including sheep, pigs, cattle, and dogs, were unearthed (Li, 2004). The Wadian, Wangchenggang, Guchengzhai, and Taosi sites are representative of the Longshan culture in the Central Plains of China (Nu, 2013; Yuan et al., 2020). Large numbers of animal remains, such as cattle, pigs, and sheep, have been unearthed at these sites, reflecting the well-developed farm animal rearing practices in the Central Plains at that time (Qiu, 1996; Cai, 2002; Lv, 2009; Larson et al., 2010; Brunson et al., 2016). By yielding mitogenomes from ancient sheep remains and combining analysis with extant ancient and modern datasets from sheep worldwide, this study aimed to explore the origins and lineage structures of sheep in Eastern Asia and to delineate the migration patterns of early sheep populations to Eastern Asia 4429–3100 BP.

Results

aDNA extraction and sequencing

After primary assessment and pretreatment, all samples were treated following an experimental workflow for ancient DNA, which included DNA extraction, double-stranded library preparation, and mitogenome capture sequencing. Seventeen samples yielded high-quality DNA and were pooled and captured. A total of 20 Mb–2679 Mb of clean data were obtained for each sample. All the mock extractions and libraries were negative.

Mitogenome assembly

A total of 17 mitogenome sequences were obtained with greater than 80% coverage rates. Among them, six samples had 100% coverage rates, and five samples had 90%–99.99% coverage rates (Table 1). All samples exhibited 2%–37% C–T postmortem damage rates at the 5′ ends of reads (Figs. S1 and S2; Table 1), suggesting the authenticity of the obtained aDNA and the reliability of the downstream analysis.

Species identification and haplogroup determination

Three methods were applied for the species identification of the ancient remains. First, when mapping the qualified sequencing reads to the sheep and goat mitogenome references, all 17 ancient samples yielded higher mapping rates against the sheep mitogenome reference sequence than against the goat mitogenome reference sequence (Table S1). Second, all samples showed greater numbers of reads when assigned to sheep than to goat according to the MTaxi results (Table S1). Third, by combining the ancient mitogenome sequences with the 35 complete mitogenome sequences of the genera *Ovis* (31) and *Capra* (4) (Table S2), all 17 ancient samples were in the

Table 1
Sequencing information for the 17 ancient mitogenome sequences.

Site	Sample	Age (BP)	Damage pattern				Mapping quality	Endogenous ancient DNA content	Average coverage	>1× coverage	>3× coverage	>5× coverage
			1st base 3′	2nd base 3′	1st base 5′	2nd base 5′						
Erlitou site (ELT)	ELG2Y	3800–3500	0.2507	0.1887	0.2147	0.1806	35.86	0.0176%	23.0997	99.79%	99.09%	98.61%
	ELS2		0.2616	0.2339	0.1531	0.1535	36.09	0.0310%	127.0521	100.00%	100.00%	100.00%
	ELS3		0.1852	0.1552	0.1647	0.1480	36.00	0.0009%	0.0557	100.00%	100.00%	99.99%
	ELT2		0.2073	0.1449	0.0939	0.1319	34.25	0.0038%	5.6014	89.29%	59.70%	33.81%
Guchengzhai site (GCZ)	GCZ1Y	4100–3150	0.2980	0.1569	0.2312	0.1471	33.11	0.0117%	2.5219	99.93%	99.42%	99.20%
Sapalli Tepe site (SPL)	SPL1	3719–3519	0.2593	0.1210	0.1275	0.1325	34.29	0.0017%	2.5044	86.16%	45.38%	20.44%
	SPL3		0.2740	0.2273	0.1575	0.1283	14.61	0.0003%	0.7185	88.92%	55.27%	32.86%
	SPL9		0.2304	0.1511	0.1546	0.1177	36.40	0.0080%	57.4394	100.00%	100.00%	100.00%
	SPLA		0.1060	0.1250	0.0410	0.0816	13.22	0.0419%	1.8639	87.69%	44.19%	15.15%
	SPLB		0.3136	0.2335	0.1806	0.1347	36.39	0.2298%	23.2714	99.40%	98.42%	97.64%
Taosi site (TS)	TS1Y	4350–3850	0.3313	0.2410	0.2532	0.2117	36.59	0.0078%	35.9224	100.00%	99.50%	99.19%
Tongtian Cave site (TTD)	TTD1	4429–3556	0.0876	0.1168	0.0520	0.1101	36.18	0.0126%	27.4720	99.78%	98.71%	98.28%
	TTD3		0.0658	0.0977	0.0480	0.0962	36.38	0.0117%	229.3335	100.00%	99.99%	99.99%
	TTD7		0.0235	0.0247	0.0211	0.0219	36.61	0.0080%	320.8628	99.98%	99.98%	99.97%
	TTD8		0.0244	0.0444	0.0250	0.1538	33.79	0.0014%	1.7171	82.55%	23.92%	3.32%
Wangchenggang site (WCG)	WCG1Y	3550–3100	0.2360	0.1753	0.2104	0.1586	34.92	0.0196%	5.9132	100.00%	99.98%	99.95%
Wadian site (WD)	WD1S	4150–3950	0.4225	0.3217	0.3762	0.2661	35.53	0.0329%	22.3568	98.51%	97.00%	95.83%

sheep clade (Fig. 1), suggesting that these samples originated from sheep.

According to the SNPs of the 17 ancient sequences relative to the reference sequence AF010406, 15 of the 17 samples could be divided into a specific haplogroup (Tables 2 and S3). By constructing a maximum likelihood (ML) tree and performing a principal component analysis (PCA) plot with 315 modern sheep mitochondrial DNA (mtDNA) sequences (Figs. S3 and S4; Tables 2 and S4), the haplogroups of 17 ancient samples were determined (Table 2). As a result, nine samples, including all four samples from Erlitou, one from Wangchenggang, one from Wadian, one from Tongtian Cave, and two from Sapalli Tepe, were determined to belong to haplogroup A; four samples, including one from Guchengzhai, one from Taosi, and two from Sapalli Tepe, were determined to belong to haplogroup B; and four samples, including three from Tongtian Cave and one from Sapalli Tepe, were determined to belong to haplogroup C.

Population and demographic analyses

To determine the genetic relationships between ancient samples and modern sheep from different regions, PCA analyses were deployed for haplogroups A, B, and C (Fig. 2). The general view and local magnification of the PCA plots for the different haplogroups revealed that most of the ancient sheep samples of haplogroup A (except SPL3 and ELT2) were genetically close to modern sheep from various regions in China, while the ancient sheep of haplogroup B were genetically close to modern sheep from Tibet, Inner Mongolia, and East China, while most of the ancient haplogroup C sheep (except SPLA) were genetically close to modern sheep from North China, Inner Mongolia, and Xinjiang.

Population genetic analyses showed that ancient populations from different regions had similar haplotype diversity (Table S5). Nevertheless, the Sapalli Tepe site had the highest nucleotide diversity (0.0852 ± 0.0521), followed by sites in the middle and lower reaches of the Yellow River (0.0414 ± 0.0230), while the Tongtian Cave site in Xinjiang had the lowest nucleotide diversity (0.0362 ± 0.0241). By deploying the F_{ST} (pairwise population fixation index) value to estimate population divergence (Fig. S5; Table S6), the ancient samples from the middle and lower reaches of the Yellow River showed close genetic relationships with the ancient sheep from the Sapalli Tepe site (0.0776) and modern sheep from different regions of China (−0.1712–0.0640) and the Middle East (0.0676) but diverged from the ancient population from the Tongtian Cave site (0.3899) and modern sheep from Africa (0.4452) and Europe (0.2782). The ancient samples from the Tongtian Cave site had a relatively close genetic relationship with modern sheep from the Middle East (0.1361) but were divergent from the ancient population from the middle and lower reaches of the Yellow River (0.3899) and modern sheep from Xinjiang (0.2895), Central and East Asia (0.3173), South Asia (0.3165), Europe (0.5974), East China (0.4987), and Southwest China (0.3791). In addition to being genetically close to ancient sheep from the middle and lower reaches of the Yellow River, the ancient population from the Sapalli Tepe site was close to modern sheep from most regions of the world, except for sheep from modern Africa (0.2889), Europe (0.2375), and Xinjiang in China (0.2099).

Divergence time estimation

RelTime-ML and Yule model trees were constructed to estimate the divergence times among different haplogroups (Table S7). Based on the RelTime-ML tree analysis, haplogroup C sheep were suggested to have diverged from haplogroups A and B 0.58 million BP (Ma), and then the divergence between haplogroups A and B occurred 0.49 Ma. Meanwhile, the Yule model tree suggested that the estimated divergence time of haplogroup C from haplogroups A and B

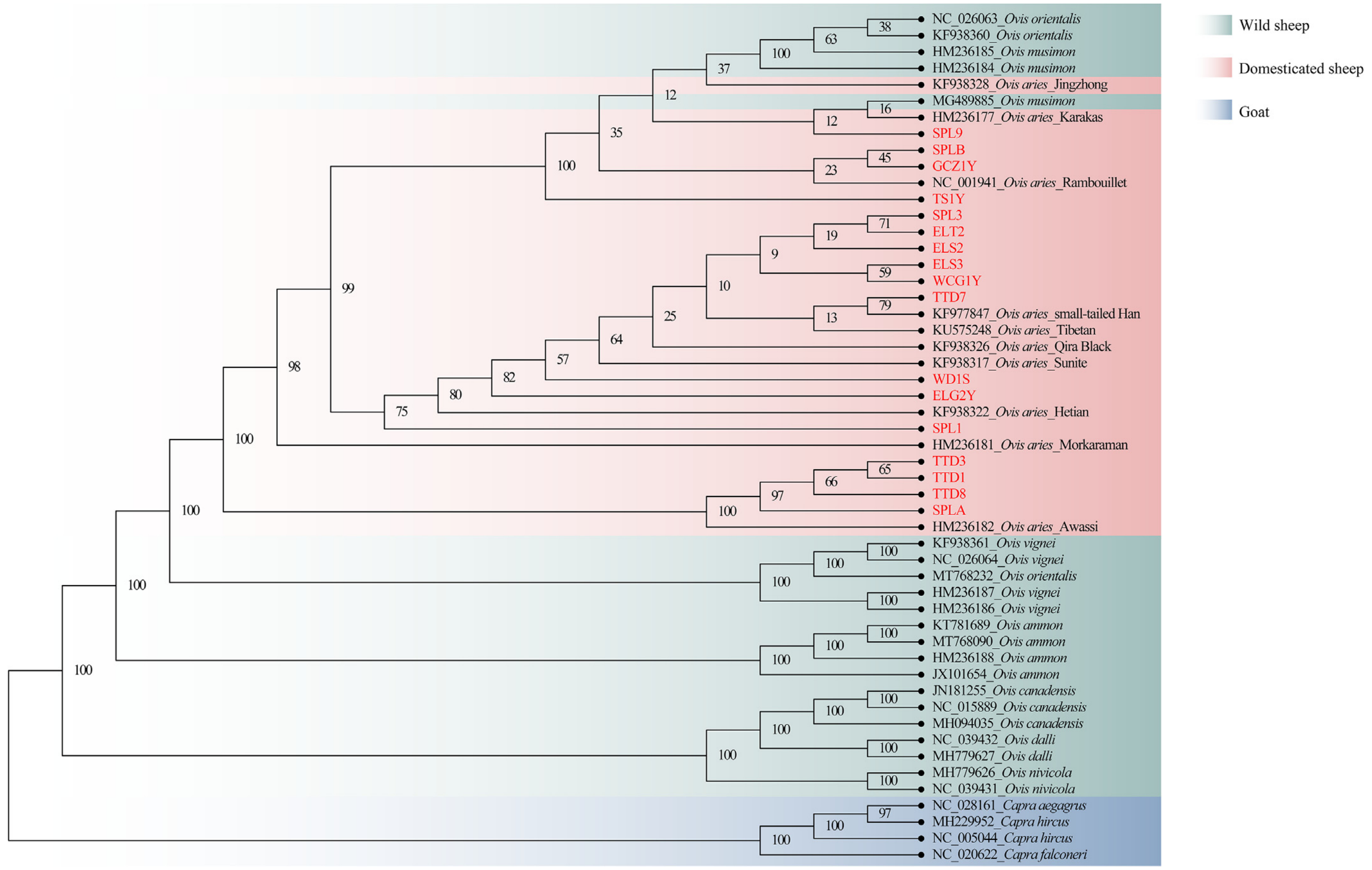

Fig. 1. Phylogenetic tree identifying the species of 17 ancient samples by combining with 35 modern mitogenomes including genera *Ovis* and *Capra* using CIPRES Science Gateway, with bootstrap values shown at the nodes. The ancient samples are highlighted in red.

was 0.80 Ma, and the divergence time between A and B was 0.60 Ma (Fig. 3). Therefore, our dataset showed that haplogroup C sheep may have diverged from haplogroups A and B 0.58 Ma–0.80 Ma, and haplogroups A and B sheep diverged 0.49 Ma–0.60 Ma (Fig. 3).

Analysis with published ancient sequences

By combining analyses of our dataset with 22 extant mtDNA D-loop fragments extracted from ~2500 BP Inner Mongolia sheep remains (Han et al., 2009) (Table S8) and modern sequences, the phylogenetic tree revealed that 9 samples, including SPL1, SPL3, TTD7, ELS2, ELS3, ELT2, ELG2Y, WD1S, and WCG1Y, were clustered with the ancient Inner Mongolian haplogroup A sheep (Fig. S6), four samples (SPL9, SPLB, TS1Y, and GCZ1Y) were clustered with the ancient Inner Mongolian haplogroup B sheep, while the remaining four samples (TTD1, TTD3, TTD8, and SPLA) from the Tongtian Cave and Sapalli Tepe sites were clustered with ancient Inner Mongolian haplogroup C sheep and several modern sheep (Fig. S6). In addition,

Table 2
Haplogroup determinations for the ancient samples.

Region	Site	ID	SNPs	ML tree	PCA plots
The middle and lower reaches of the Yellow River	Erlitou, China	ELG2Y	A	A	A
		ELS2	A	A	A
		ELS3	A	A	A
		ELT2	A	A	A
	Guchengzhai, China	GCZ1Y	B	B	B
	Taosi, China	TS1Y	B	B	B
	Wangchenggang, China	WCG1Y	A	A	A
	Wadian, China	WD1S	A	A	A
Northwest Frontier of China/Altai	Tongtian Cave, China	TTD1	C	C	C
		TTD3	C	C	C
		TTD7	ND	A	A
		TTD8	C	C	C
Uzbekistan/Central Asia	Sapalli Tepe, Uzbekistan	SPL1	A	A	A
		SPL3	A	A	A
		SPL9	B	B	B
		SPLA	ND	ND	C
		SPLB	B	B	B

ND, not determined.

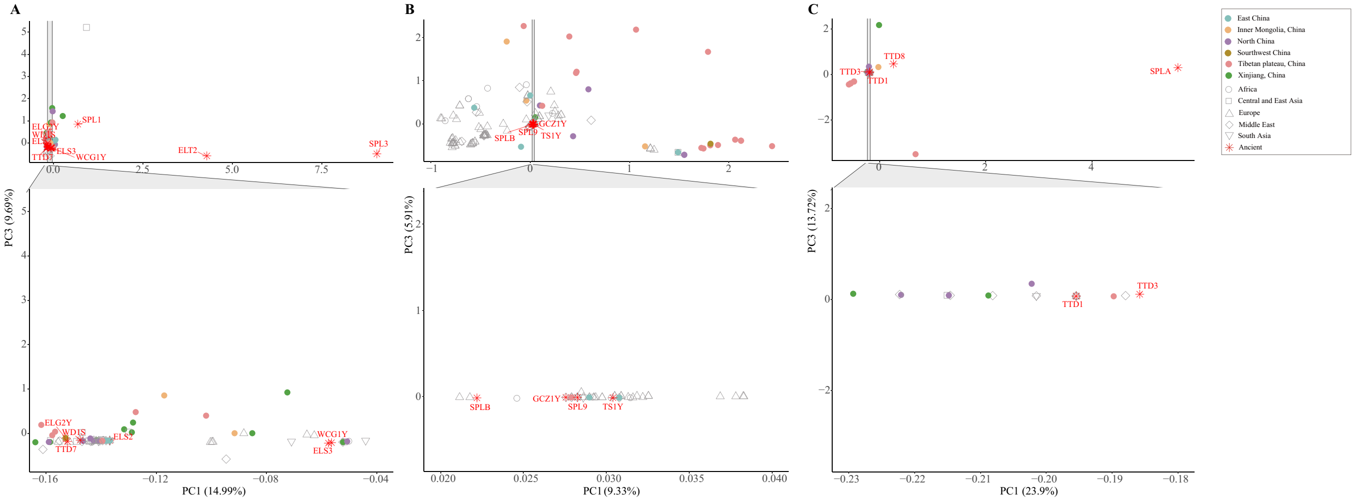

Fig. 2. Principal component analysis of 17 ancient samples and relative haplogroups of modern sheep.

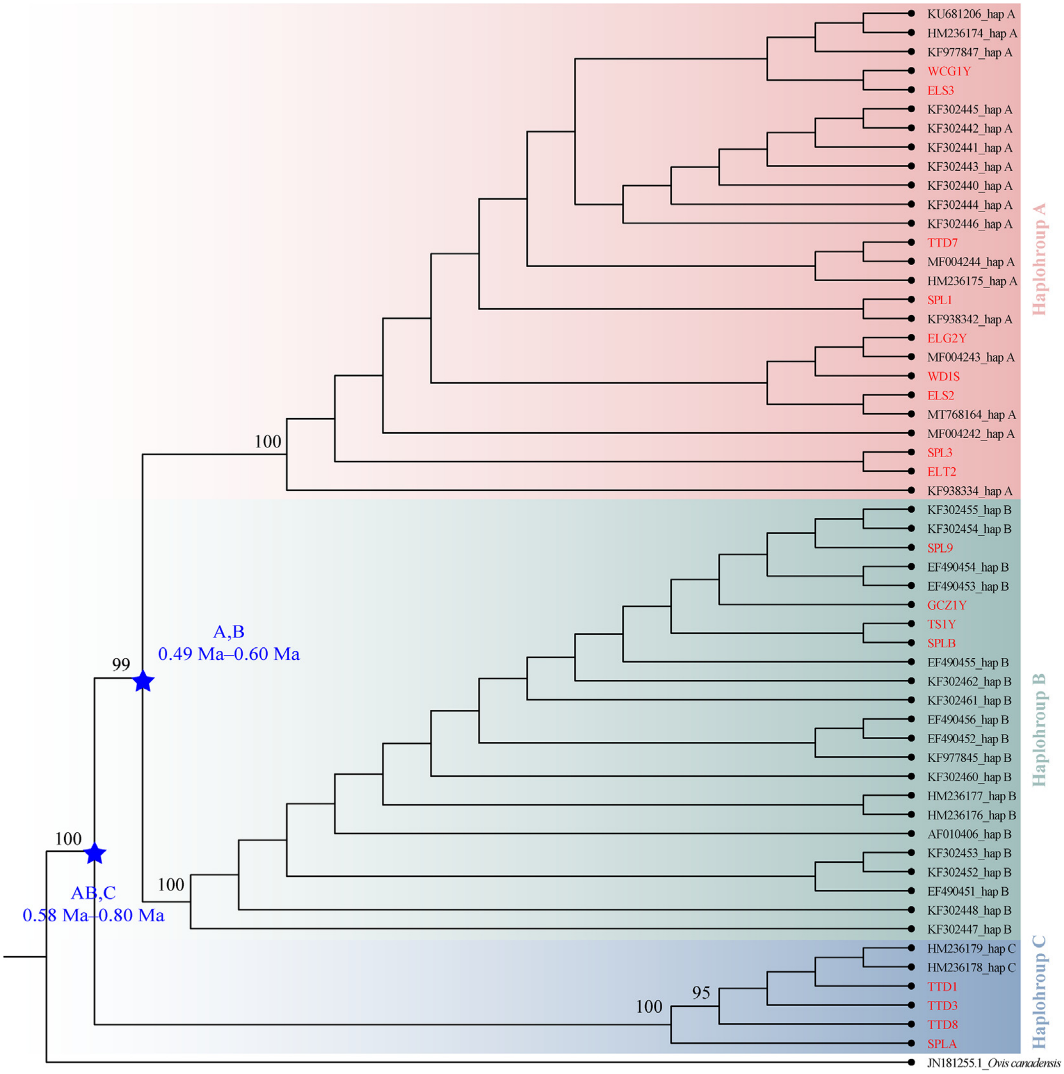

Fig. 3. Divergence time estimations for sheep from haplogroups A, B, and C from 17 ancient mitogenome sequences and 39 modern sheep mitogenome sequences, with posterior probability shown on the nodes. The ancient samples are highlighted in red. The blue stars and letters on the nodes represent the divergence times of the different haplogroups.

by combining 11 published D-loop fragments from ~2000 BP southern Altai sheep remains (Dymova et al., 2017) (Table S8) and modern sequences, a Bayesian tree was constructed for haplogroup A and B samples to elucidate the phylogenetic relationships between the ancient sheep populations from Uzbekistan, northwest China, middle and lower reaches of the Yellow River, and the younger southern Altai sheep (Fig. S7). The results revealed that 7 samples, including WD1S, SPL1, TTD7, WCG1Y, ELS2, ELS3, and ELG2Y, were genetically close to the ~2000 BP haplogroup A sheep from southern Altai, while 4 samples (TS1Y, SPL9, SPLB, and GCZ1Y) were genetically related to the ~2000 BP haplogroup B Altai sheep.

Discussion

In this study, 17 ancient sheep mitogenome sequences with a greater than 80% coverage rate were obtained from 7 sites in China and Uzbekistan (Table 1). The sites involved in this study are representative and of great significance to address the route of early sheep populations from putative domestication centers to Asia, especially within China. In particular, the Sapalli Tepe site in Uzbekistan represents an area of settled agricultural culture during the Late Bronze Age, where thriving economic and cultural exchanges between the East and West took place for thousands of years (Ren, 2014). The Tongtian Cave site, located in the Altai region of northwestern China and central Eurasia, was on the routes early agricultural society residents used to carry crops into the Altay region in Xinjiang and later into the Yellow River Basin (Zhou et al., 2020). The joint study of ancient sheep mitogenomes from Uzbekistan, Northwest China, and the middle and lower reaches of the Yellow River in China could facilitate the study of early routes of sheep migration across Eurasian communications.

It has been proposed that North China may be one of the origins of the world's haplogroup C sheep population (Lv et al., 2015), of which the divergence times from haplogroups A and B date back to 0.58 Ma–0.80 Ma (Fig. 3). In this study, three of four ancient samples from Tongtian Cave in Northwest China were found to belong to haplogroup C (Table 2). According to the direct radiocarbon dates on the charcoal and organic material excavated from the same layers as the ancient sheep remains (Zhou et al., 2020), the age of the Tongtian Cave sheep was dated to 4429–3556 BP. Based on the evidence from archaeological materials excavated over China, the earliest domesticated sheep population in China has been suggested to have been distributed in Northwest China at approximately 5000 BP (Ben, 1979; Institute of Archaeology, 1999). Therefore, our results provide evidence for the emergence of haplogroup C sheep as early as 4429–3556 BP at Tongtian Cave and support the assertion that the ancient sheep population at Tongtian Cave may have been the earliest local C haplogroup sheep in China. In addition to the samples from Tongtian Cave, one of the five samples from the Sapalli Tepe site was also determined as haplogroup C. However, the Tongtian Cave haplogroup C population and the Sapalli Tepe haplogroup C sheep showed a relatively distant genetic relationship (Fig. 2), implying that the 4429–3556 BP haplogroup C sheep in Xinjiang may have existed as an early independent population.

The Tongtian Cave site is located in the Altai Mountains, where a small number of sheep populations may have arrived and lived. Therefore, geographic isolation may have occurred at the Tongtian Cave site, which would have resulted in low nucleotide diversity (Table S5) and limited genetic communication with other populations (Table S6; Fig. S5). Despite this possibility, sheep from both haplogroups A and C from Tongtian Cave have been observed to be genetically close to or share a recent common ancestor with ancient sheep from southern Altai and Inner Mongolia (Figs. S6 and S7). Meanwhile, the ancient Sapalli Tepe sheep population harbored the dominant haplogroups A, B, and C (Table 2), representing the ancient sheep in Central Asia with the highest nucleotide and haplotype diversity (π and Hd) compared with ancient populations from the other two regions (Table S5). Moreover, the ancient Sapalli Tepe sheep were close to modern sheep from most regions of the world (Table S6). Therefore, these results illustrated that the Uzbekistan-Altai region might have been a transportation hub for early sheep migration to eastern Asia, in addition to the previously reported Mongolian Plateau as a sheep migration hub (Lv et al., 2015).

The ancient haplogroup C sheep were also found to be genetically close to modern sheep from North China, Inner Mongolia, and Xinjiang (Fig. 2), suggesting that haplogroup C sheep from the Tongtian Cave site may have contributed genetically to modern Chinese sheep or may have come from the same early ancestor. In addition, a close phylogenetic relationship between Tongtian Cave haplogroup C sheep and several ~2500 BP Inner Mongolia sheep was observed (Fig. S6), suggesting putative genetic communications or hereditary transmission events among early domestic sheep from Northwest China to Inner Mongolia ~2500 BP. Interestingly, the geographic distribution of modern C haplogroup sheep populations was found to be similar to the geographic distribution of fat-tailed sheep populations in China (Lv et al., 2015). This is consistent with the claim that sheep may have, on the one hand, traveled through northern Kazakhstan to the Altai with herdsmen in the western steppe and, on the other hand, spread northward through the inner Asian mountain corridor and farther east into Mongolia and China (Hermes et al., 2020). Therefore, we infer a possible migration wave in which the fat-tailed haplogroup C sheep populations were able to adapt to severe climates (Atti et al., 2004; Mwacharo et al., 2017; Xu et al., 2023) and migrate to Inner Mongolia 2500 BP, where a harsh climate change occurred, transitioning from warm and wet to cold and dry (Zhang, 2022).

With its close geographic distance from the sheep domestication center and the abundant number of Neolithic sheep remains excavated, Central Asia has been deemed one of the most important distribution centers for sheep expansion in eastern Eurasia (Legge, 1992; Hermes et al., 2020; Taylor et al., 2021; Nishiaki et al., 2022). Our data showed that the ancient Sapalli Tepe sheep may share the same early ancestor with the younger domestic populations in southern Altai, Inner Mongolia, and North China as well as modern sheep from various regions in China (Table S6; Figs. S5–S7). A recent study revealed that humans in Central Asia and Xinjiang had frequent interactions in the inner Asian mountain corridor during the Bronze Age (Kumar et al., 2022). One major exchange and spread route for an early productive economic system, such as one dealing in sheep, goats, cattle, and barley connecting East and Central Asia, has been proposed to have run across the oases in southern Asia between Iran, Central Asia, and the Taklamakan Basin of Xinjiang and eventually into northern China (Christian, 2000; Kuzmina, 2008; Spengler and Willcox, 2013). Together with the archaeological evidence of plentiful sheep remains dating from ~4000 BP that were unearthed alongside the area range from the Inner Mongolia-Gansu-Qinghai regions to the North China Plain (Xie, 1975; Ben, 1979; Huang, 1996; Yuan, 2001; Yuan et al., 2007a; Guo, 2012; Wang et al., 2017, 2020), our results well support the possibility of a sheep migration route from Uzbekistan to the middle and lower reaches of the Yellow River through northwestern China along with the presence of thriving communication among nomadic tribes 4000 BP (Jeong et al., 2020; He et al., 2023).

Materials and methods

Samples

A total of 35 ancient animal samples were collected from seven archaeological sites, including 6 archaeological sites from China dated at 4429–3100 BP and 1 site called Sapalli Tepe in Uzbekistan dating from 3719–3519 BP. Five sites (Taosi, Wadian, Wangchenggang, Guchengzhai, and Erlitou) from China were located in the middle and lower reaches of the Yellow River and belonged to the Longshan and Erlitou cultural periods. The sixth site, the Tongtian Cave Site, was located in the Altai territory of Xinjiang, where several stone tools, bones and teeth of sheep, wheat, and naked barley (Zhou et al., 2020) had been found, strongly suggesting early human activity and cultural transmission in this region. A wealth of relics was unearthed from the Sapalli Tepe site in Uzbekistan, including pottery, stone tools, bronze, and bone vessels, representing a settled agricultural culture in the area during the Late Bronze Age (Ren, 2014). Detailed sample information is presented in Table S9.

DNA extraction

All aDNA experiments were conducted in the Ancient DNA Laboratories of China Agricultural University and Foshan University. The equipment was soaked in 5% (v/v) sodium hypochlorite solution and washed with 75% (v/v) alcohol and then exposed to ultraviolet radiation for 1 h. Soil adhering to the surface of ancient samples was carefully removed with a drill. The ancient samples were then washed with 5% (v/v) sodium hypochlorite solution, followed by double-distilled water, and subjected to ultraviolet irradiation for 30 min. The washed samples were ground into a fine-grained powder using an automatic quick-grinding machine. Then, 50 mg–100 mg of sample powder was subjected to DNA extraction according to the previous method (Dabney et al., 2013; Dabney and Meyer, 2019) under the following modified conditions: 1) a setting of 45°C as the lysis temperature; and 2) 90 mM as the sodium acetate concentration in the binding buffer. By incubating 25 μL of double-distilled water in the column membrane twice for 10 min using the MinElute polymerase chain reaction (PCR) purification Kit (QIAGEN, Hilden, Germany), a final volume of 50 μL of DNA was obtained. Several mock extractions and library preparations were carried out alongside the samples in the same manner to monitor for contamination.

Mitogenome capture sequencing

For each sample, 50 ng of input DNA was used to prepare the library with the VAHTS Universal Plus DNA Library Prep Kit for MGI (Vazyme Biotech Co., Ltd, China). The qualified libraries were then subjected to the subsequent mitogenome capture. The complete mitogenomes of sheep (*Ovis aries*) were obtained using long-range PCR amplification and then broken into short fragments as capture baits in the prepared DNA library. Mitogenome enrichment was performed by pooling four or five different libraries equally in a total of 1 ng DNA using the xGen Hybridization and Wash Kit (Integrated DNA Technologies, Inc., USA). The enriched libraries were cyclized using the VAHTS Circularization Kit for MGI (Vazyme Biotech Co., Ltd.) and sequenced using the PE100 MGI platform.

Data filtering and alignment

Read quality was assessed using FastQC v0.11.9 (Simon, 2010). Adapters and reads shorter than 25 bp and reads with a Phred quality score lower than 30 were filtered and then merged into single sequences using fastp (Chen et al., 2018). Alignments were generated using the BWA aln (Li and Durbin, 2009) against sheep (NC_001941.1) and goat (NC_005044.2) mitogenome reference sequences from the NCBI database. The resulting bam files were sorted and filtered with a mapping quality threshold <20 using Samtools v1.9 (Li, 2011). Duplicate reads were removed using MarkDuplicates of Picard v2.22.9 (http://broadinstitute.github.io/picard/). The respective bam files from mixed capture sequencing data of the same sample were merged using Samtools v1.9 (Li, 2011). Endogenous aDNA content, average coverage, and mapping quality were obtained using Qualimap v2.2.2 (Okonechnikov et al., 2016). Variant detection was performed on the bam files using UnifiedGenotyper in Genome Analysis Tool Kit (GATK) v3.5 (DePristo et al., 2011). The authenticity of the retrieved mapped reads was evaluated using mapDamage 2.0 (Jónsson et al., 2013).

Mitogenome assembly

A total of 17 ancient samples with more than 80% coverage against the sheep mitogenome reference were used for mitogenome assembly. Bcftools (Li, 2011) and UnifiedGenotyper in GATK v3.5 (DePristo et al., 2011) were used to generate complete sequences based on mutation sites against the reference mitogenome. Mapping Iterative Assembler (MIA, v1.0) (Green et al., 2008) was used to generate complete sequences based on the bam files. The final, assembled mitogenome sequences were defined based on the alignment of the two generated sequences. An SNP was only adopted as a true SNP when its depth exceeded 2/3 of the total sequencing depth according to the visualization of the bam files using the Integrative Genomics Viewer (IGV) (Thorvaldsdottir et al., 2013).

Species identification

Three methods were performed to identify the species sequence data present in the ancient samples. First, the mapping rates generated from sequenced reads aligned to sheep (NC_001941.1) and goat (NC_005044.2) mitogenome reference sequences specifically using BWA aln (Li and Durbin, 2009) were compared. Second, bam files were assigned to sheep and goat mitogenome references using the comparative tool MTaxi (Atağ et al., 2023). Third, CIPRES Science Gateway was used to construct an ML tree (model: GTRGAMMA, bootstrap iterations: 1000, -f a, -x 12,345, -p 12,345) (Miller et al., 2015). The species of the 17 ancient samples were identified by combining the results with the analyses of 35 modern, complete mitogenome sequences of the genera *Ovis* (31) and *Capra* (4) (Table S2).

Phylogenetic and demographic analyses

The haplogroups of the 17 ancient mitogenome sequences were inferred according to the variant sites with the reference sequence, AF_010406, and finally determined by another ML tree and PCA using 315 extant sheep mitogenome sequences (Table S4). The ML tree was calculated using CIPRES Science Gateway, and the PCA was performed using the R package "adegenet" (Jombart, 2008).

Based on the geographic distribution, the origins of the ancient samples were divided into three regions: 1) the middle and lower reaches of the Yellow River, including the Guchengzhai, Erlitou, Taosi, Wangchenggang, and Wadian sites; 2) the northwest frontier of China/Altai, including the Tongtian Cave site; and 3) Uzbekistan/Central Asia, including the Sapalli Tepe site. The areas where the 315 modern sheep breeds were sampled were divided into 13 regions, including Central Asia, Europe, and the Middle East. After that, demographic analyses were performed using PCA plots for haplogroup A, B, and C samples using the R package "adegenet" (Jombart, 2008) and using the F_{ST} calculation of Arlequin v3.5 (Excoffier and Lischer, 2010). Population genetic indices, including Nh (number of haplotypes), Hd (haplotype diversity), and π (nucleotide diversity) of ancient mitogenome sequence variation from different regions, were calculated using DnaSP v5.1 (Librado and Rozas, 2009).

Divergence time estimation

Two methods for tree construction, RelTime-ML from MEGA 7 (Kumar et al., 2016) and Yule model from BEAST v1.7.5 (Drummond et al., 2012), were used separately to calculate divergence times for sheep haplogroups A, B, and C. Both methods used the same dataset, including the 38 modern sheep mitogenome sequences (Table S7), the outgroup (*Ovis canadensis*), and the nucleotide substitution model (GTR + I + G) inferred from jModelTest 2.1.1 (Darriba et al., 2012). For the BEAST method, the divergence times were set between the A and B haplogroups as 0.346 Ma–0.694 Ma, and those between the C and A and B haplogroups were set as 0.583 Ma–1.108 Ma, as inferred from (Lv et al., 2015). Fossil calibration points were set as the ages of the archaeological sites. Samples were drawn for every 1000 Bayesian Markov chain Monte Carlo (MCMC) steps under 100 million iterations, and the first 10% of samples were discarded as burn-in. Convergence was confirmed with an effective sampling size (ESS) greater than 200 using the program Tracer v.1.7 (Rambaut et al., 2018). For the MEGA method, a RelTime-ML time tree with 1000 bootstraps was constructed using the same dataset and divergence times.

Analysis of published ancient sequences

Two Bayesian trees were constructed using modern sheep mtDNA sequences and different published ancient sheep mtDNA sequence datasets (Table S8) by MrBayes 3.2 (Ronquist et al., 2012) using the best-fit nucleotide substitution model selected with jModelTest 2.1.0 (Darriba et al., 2012). MCMC analyses were run with 20, 000, 000 generations and sampled every 2000 generations.

Data availability

The mitogenomes generated in the study are deposited in China National GeneBank Database (CNGBdb), with project accession number CNP0004148.

CRediT authorship contribution statement

Liu Yang: Methodology, Visualization, Writing - Original draft, Writing - Review & Editing. **Xing Zhang**: Methodology, Writing - Review & Editing. **Yaning Hu** and **Piao Zhu**: Methodology. **Hua Li**: Analysis. **Zhenyu Peng**: Methodology. **Hai Xiang**: Conceptualization, Writing - Review & Editing, Funding acquisition. **Xinying Zhou**: Resources, Supervision. **Xingbo Zhao**: Conceptualization, Resources, Funding acquisition.

Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgg.2023.06.002.

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