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Ancient DNA analysis of elite nomadic warrior from Chinge-Tey I funerary commemorative complex in the "Valley of the Kings", Tuva

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Abstract

Background In the I[st] millennium BC bearers of the Scythian-type nomadic cultures inhabited the steppes of Eurasia, from Northern China to the Carpathians. According to archaeological data, the origin of nomadic life style and economy can be traced to the eastern part of this steppe "corridor", primarily to the territory of the present-day Republic of Tuva in Russia. Here, in the Turan-Uyuk Basin, also known as the "Valley of the Kings", some of the earliest known Scythian-type archaeological sites called Arzhan-1, Arzhan-2, Chinge-Tey I, Tunnug 1 were studied. Each of them is a large-scale funerary commemorative complex with burials of tribal nomadic leaders, surrounded by graves of supposed members of their families or associates. All these people belonged to the societies which are associated with the earliest nomadic cultures in Asia. Representatives of similar cultures will later be known and described as the Scythians/the Saka in Assyrian, Achaemenid, and Greek sources. Arzhan 2 and Chinge-Tey I elite level sites as well as ordinary pastoralist burials of the early-Scythian period in Tuva are attributed to the Aldy-Bel archaeological culture of the Early Iron Age (8th– 6th century BC). Taking the first step to shed light on the genetic origin of Aldy-Bel elites, we carried out a comparative genome-wide analysis of an elite level person buried in grave 9 at Chinge-Tey I (7th– 6th centuries BC) and two published earlier genomes of individuals, whose burials (graves 14 and 22) accompanied the 'royal couple' (grave 5) at Arzhan-2. This study aims also at checking a hypothesis of genetic kinship between human individuals buried in the large-scale burial complexes of the "Valley of the Kings" and brings up the issue of possible dynastic connections of local elites, buried under different kurgans of the valley.

Results First, ancient DNA analysis of an elite nomadic warrior from Chinge-Tey I has been carried out, thus a third wide-genome dataset for Aldy-Bel culture– one of the earliest nomadic cultures in Asia, is presented in this study. Second, we undertook a comparative analysis of genome-wide data of three mentioned Aldy-Bel culture representatives and individuals of the other Bronze and Early Iron Age population groups of Asia to estimate their

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possible genetic connections. Then, kinship analysis was undertaken for these three Aldy-Bel culture individuals. Finally, mitochondrial and Y-chromosome haplogroups of Chinge-Tey princely person were compared to those of other Aldy-Bel culture representatives and to individuals of subsequent Scythian-type Uyuk-Sagly culture in Tuva.

Conclusion (1) Generating the third wide-genome of the enabled us to undertake its comparison with two other genomes of Aldy-Bel culture representatives (Arzhan-2, graves 14 and 22) and with other Bronze and Early Iron Age population groups in Asia to trace the origin and genetic connection of Aldy-Bel population, representing one of the earliest Scythian-type nomadic group. (2) The results obtained show that the princely individual from Chinge-Tey I and two 'king's associates' from Arzhan-2 were genetically close to nomads of simultaneous Tasmola culture in Eastern and Central Kazakhstan and pastoralists buried in the Early Iron Age cemeteries of present-day Xinjiang (first of all, Abusanteer archaeological site). Aldy-Bel culture representatives appeared also close to individuals of the Middle Bronze Age Okunevo culture in the Minusinsk Basin. Besides, Aldy-Bel pastoralists turned out genetically close to nomads of the subsequent Uyuk-Sagly culture in Mongolia (5th – 3rd centuries BC). (3) Ancient DNA kinship analyses, undertaken for three Aldy-Bel culture individuals pointed out to the absence of their tribe kinship. (4) On the other hand, Chinge-Tey warrior's mitochondrial haplogroup G was previously described in two (graves 14 and 5) individuals from Arzhan-2, including a female individual from the "royal" tomb 5. This result provided a possibility of maternal kinship among this so called 'queen' from Arzhan-2 and the princely person from Chinge-Tey I. This possibility supported a hypothesis of their family ties suggested on archaeological materials. Y-chromosome haplogroup Q1b1, revealed for the princely person, was widely distributed among local people of Aldy-Bel and subsequent Uyuk-Sagly cultures.

Keywords Southern Siberia, Tuva, Early Iron age, Chinge-Tey I, Arzhan-2, Aldy-Bel culture, Nomads, Elite burial, Ancient DNA, Scythian period

Background

During the Ist millennium BC numerous groups of mounted pastoralists and nomadic warriors, conventionally called under the united name of the Scythians, inhabited the Eurasian steppes and lower mountain ranges, stretching from Northern China to the Carpathians. The Scythians proper were nomadic tribes of the Northern Black Sea steppe, described by Greek historian Herodutus in the 5th century BC. The name Scythians (in line with the name Saka of the Achaemenid sources) was later conventionally spread to the other nomadic groups of the Eurasian steppe. Archaeologists, although, tend to replace it by more correct term "Scythian-type nomads", also for the easternmost groups, inhabited Inner and Central Asia (Tuva, Altay, Kazakhstan, Xinjiang) and South Siberia (The Minusinsk Basin), who lived far from the writing civilizations. Accordingly, different archaeological cultures associated to these groups are conventionally called Scythian-type ones and their historical period correlates more or less to the Early Iron Age.

What these different tribes had in common was their special mobile pastoral economy and mounted life-style, a set of believes and social organization, as far as they can be reconstructed by archaeological studies. However, these multiple groups differed from each other and modern archaeology often studies them individually [1].

These horse-riding warriors and mobile pastoralists were the earliest nomads of Eurasia, forerunners of the Huns, the Turks and the Mongols. One of the theories about their origin, considered by archaeologists, was based on Herodotus account that "Nomad Scythians, who inhabiting Asia, entered the north Caucasus from the east" [1]. In 1970-s, when elite level kurgan named Arzhan with the earliest known Scythian-type objects was excavated in Tuva, this semi-mythical idea received a strong confirmation. Since then, numerous archaeological data have testified that the origin of the nomadic life-style, economy and traditions traces back to the easternmost part of the Eurasian steppe "corridor" and primarily to the territories of the present-day Eastern Kazakhstan and the Republic of Tuva in Russia [2, 3].

The steppe-mountainous region of Tuva lies in the heart of Inner Asia, in the Upper Yenisey Basin. In the early Ist millennium BC the climate and pastures here were especially favorable for horse-breeding [2, 3]. It's home to the oldest studied burial sites of the nomads, with the largest and architecturally sophisticated funerary commemorative complexes of their elites situated in the Turan-Uyuk Basin of Tuva, also known as the "Valley of the Kings" (Fig. 1A-B) [4].

Four largest complexes are Arzhan-1, Arzhan-2, Tunnug 1, and Chinge-Tey I which form a huge chain of sites along the whole valley which was a burial place of the highest rank nomadic leaders. The first two sites were excavated in 1970-s and 2000-s, whereas two latter are still under excavations [5, 6, 7, 8]. Arzhan-1 and Tunnug 1 date back to the 9–early 8th centuries BC– a transitional Bronze to Iron Age period or the earliest chronological horizon of the forming nomadic culture. Finds made here illustrate the beginnings of the 'Animal Style' and

earliest examples of the riding horse equipment [7, 9]. Late Bronze Age period in Tuva (late IInd– early Ist millennium BC) saw a mosaic of peoples and cultures. Little finds and no genome-wide data are so far available for these groups which made it difficult to trace the origin of the earliest nomadic cultures in this core region [10].

Arzhan-2 and Chinge-Tey I are of the next period, 7th – 6th centuries BC, associated to the Aldy-Bel (further– AB) archaeological culture of the next, early nomadic or early Scythian period (Fig. 1B). They are located at a distance of twenty kilometers to each other. Sites of both periods are crucial to understand the origin and early development of the nomadic culture and its bearers in the Asian steppe.

Examination of numerous artifacts, such as gold jewelry, weapons, horse gear, and burial garments indicates that during the first half of the Ist millennium BC nomadic tribes of Tuva had close interactions with the populations of present-day Kazakhstan, Northwestern China (Xinjiang), and possibly even more distant regions [11, 12]. Archaeological data suggest that AB culture bearers could come to Tuva from Kazakhstan as a small group and took a dominant position among local people of the Late Bronze Age [10, 13].

At Chinge-Tey I (7th– 6th centuries BC) eight graves of young warriors were studied at the periphery of the burial complex. They are considered as to accompany nomadic leaders, buried in the center of the mound, to 'another world', providing, apparently, a symbolic protection of the central burials [11, 12]. In 2022, researchers from the South Siberian Archaeological Expedition of the State Hermitage Museum uncovered a burial of a male (grave 9) in the central part of Chinge-Tey I burial complex (52.034675, 93.477008) (Fig. 1C-D). His tomb was situated at a depth of 4 m beneath a comprehensive stone construction. This male individual, who was buried with a number of artistic gold objects and an intricately decorated weapon, belonged to the elite stratum of a nomadic society. The unusual long robe and unique glass bowl, likely of Assyrian origin, suggest some Western connections of this princely person or his elder relatives [14]. A preliminary analyses of the materials allows us to date the grave to the first half of the 6th century BC [9].

Up to now only two genome-wide datasets were published for the AB culture representatives, despite their crucial role for the issue of the oldest nomadic groups' origin. These two individuals are from the graves 14 and 22, accompanying the so to say 'king and queen' couple at Arzhan-2 (grave 5) [15]. Based on their genome datasets, it has been suggested that these two individuals had genetic affinities to synchronous eastern populations of Central Asia (Kazakhstan and Mongolia) [15].

Besides, several ancient DNA (aDNA) studies, focusing on mtDNA and Y-chromosomal markers, have also been carried out for the AB culture individuals (Supplementary Table 1) [15, 16, 17]. Mitochondrial DNA haplogroups A4, A8, A11, C4, C5b1, G, G2a, H, T1a, U4a3, U5a1d2b, U5a1f1 and Y1 were identified for individuals buried at Arzhan-2 [15]. Similarly, individuals from AB culture sites of 'ordinary' social level had mitochondrial haplogroups C4d and HV6 (Eki-Ottug-2) and C4a1a3 (Bai-Dag-8) [17].

Dominant Y-chromosome markers among AB culture bearers correspond to haplogroup R1a (16: Arzhan-2, Eki-Ottug 1 and 2, Bai-Dag 6 and 8), which is also widely presented in South and West Asia, as well as Central and Eastern Europe [18]. Less common among studied AB culture individuals is Y-chromosome haplogroup Q1b. All the same it was identified for the other individuals from Eki-Ottug 1 (Q1b1a3), Bay-Dag 6 (Q1b1a3) and Bay-Dag 8 (Q1b1a) (please, see Supplementary Table 1) [16, 17].

In this study we generated genome-wide data for a princely person excavated in Chinge-Tey I grave 9, which provided the third genome-wide dataset for AB culture. Our second purpose was estimating possible kinship of these AB culture people, buried in two elite level sites of the 'Valley of Kings'. Then we carried out the comparative analysis of these three genome-wide data those of individuals from adjacent regions of the same chronological time frame as well as more ancient populations from the eastern part of Central Asia. The aim was to estimate genetic affinity between population groups of these regions which might have contributed to the early nomadic population of Tuva. In the end we compared available mitochondrial and Y-chromosome datasets of AB culture representatives, both from elite-level sites of Chinge-Tey I and Arzhan-2 and ordinary burials from the other valleys (Eki-Ottug 1–2, Bai-dag 6, 8) to get an idea of their diversity and dominated haplogroups. Together with archaeological facts and observations, results obtained in this and similar studies should lead us to understanding of the origin of the earliest nomads and their elites in Tuva as well as of social structure of local Scythian-type nomadic societies. Moreover, further studies of individuals buried in elite level complexes of the "Valley of the Kings" could shed light on their possible genetic affinities or even kinship with nomadic leaders of the period from the other parts of Central Asia.

Materials and methods

Remains of the buried person and AMS date of the grave

Skeletal remains of the buried person was unearthed from intact grave 9 in the central part of the Chinge-Tey I funerary commemorative complex (Fig. 1) [9]. According to osteological data the skeleton belonged to a male of 20–25-year-old. Morphology of his skull exhibited similarities to the other series of skulls of the AB culture

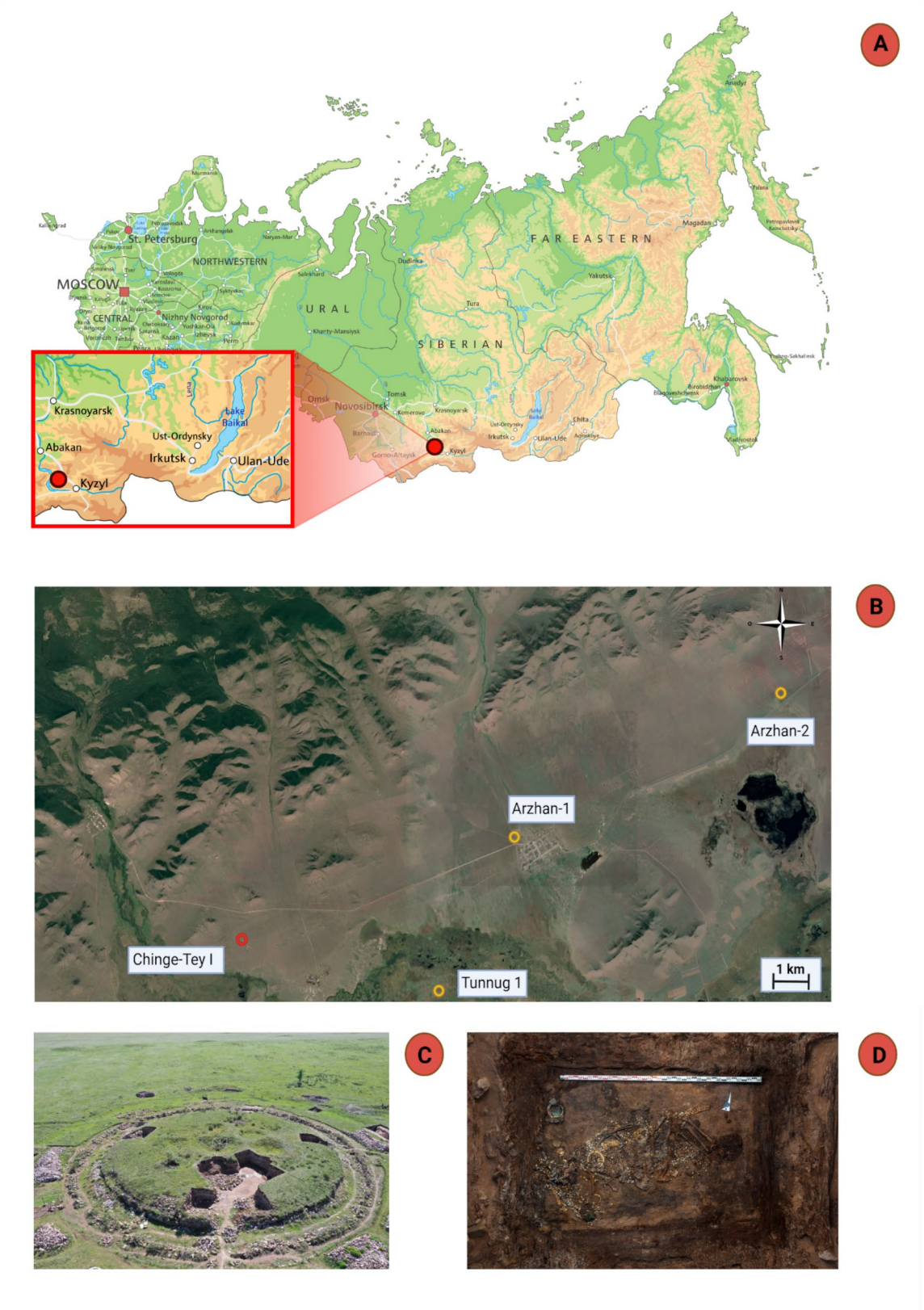

Fig. 1 (See legend on next page.)

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Fig. 1 Chinge-Tey I funerary commemorative complex location and excavation. (**A**) Geographical map showing the Chinge-Tey I in the Republic of Tuva of Russia (red circle) where the bioarchaeological material of elite nomadic warrior was collected; (**B**) Map of the "Valley of the Kings", Republic of Tuva of Russia. The elite funerary commemorative complexes marked by yellow (Arzhan-1, Arzhan-2, Tunnug 1) and red (Chinge-Tey I) rings. (**C**) Aerial view of the Chinge-Tey I in the Republic of Tuva of Russia during the fieldwork in 2022. (**D**) General view of the burial in grave 9 in the Chinge-Tey I (photo by Nikiforov V.I.)

bearers which suggests he was a reliable 'biological' representative of the group. Of special importance is the fact that a group of male skulls from Arzhan-2 graves appeared the closest to Chinge-Tey princely person's skull [19]. AMS date obtained in the ^{14}Chrono Centre for Climate, the Environment and Chronology of Queen's University Belfast (human bone) provided a date from the first quarter of the 8th calBC to the middle of the 6th calBC (UBA-49955) (Supplementary Table 2). Such a wide interval is due to the so-called Hallstatt Plateau on the calibration curve, which is a general problem for the samples of the period [20].

Ancient DNA extraction, DNA library preparation, and genome sequencing

Human bioarchaeological material was genetically analyzed in the present study on the basis of a quadripartite agreement between the State Hermitage Museum, European University at St. Petersburg, Genotek Ltd., and Grotex LLC (19-05-2022). Two molar teeth were used for meticulous sampling and grinding to small fragments in the extra-clean room DNA facilities of Genotek Ltd. (Moscow, Russia). The aDNA facility is separated from the post-PCR laboratory, and all the laboratory equipment has never been used in the other rooms of the laboratory. The aDNA facility has a positive pressure system with HEPA filters and ultraviolet-C light sources; it has limited access, and employees have to suit up in one-time full-body coveralls, gloves, face masks, and overshoes. The aDNA facility is divided into two zones: one for DNA extraction and another for DNA library setup.

aDNA was extracted from the bone powder using a phenol-chloroform extraction method with prior demineralization as previously described in literature [21, 22]. Several independent aDNA extractions were performed. Human teeth were cleaned from the dust with a scalpel, then polished with an engraver to remove the surface layer. Further, a fragment of polished bone was sawed out with a diamond disc and washed consistently with water, 1% chlorine solution, and 96% ethanol, and dried then. After that, the bone fragment was ground into splinters and incubated in 5 ml of ethylenediaminetetraacetic acid solution (EDTA 0.5 M, pH 8.0). Bone fragments were incubated overnight at room temperature in a shaker between 25 and 30 °C. After demineralization, the fragments of the bone were washed with ultra-pure water, and after that, about 0.2 g of these splinters were placed in Eppendorf microtubes with a capacity of 1.5 mL. Each of the samples was added to 600 µL of extraction buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA; 2% SDS, pH 8.0), 60 µL of Proteinase K, and 40 µL of dithiothreitol (DTT), having been incubated overnight at 56 °C. After the incubation period, 600 µL of phenol/chloroform (1/1, v/v) was added to each of the samples, which were subjected to vigorous vortexing for 5 min. Then, the samples were subjected to centrifugation for 7 min at 12,000 rpm for the formation of the supernatant (aqueous phase), which separated the DNA from the proteins. About 500 µL of the aqueous phase (without disturbing the interphase) of each sample was transferred into Eppendorf microtubes with a capacity of 1.5 mL. Then it was added 60 µL of 3 M sodium acetate (pH 5.2), and 600 µL of 100% 2-propanol, mixed by turning the tube, and incubated at -20 °C for 20 min. After that, all samples were centrifuged for 10 min at 13,200 rpm, the supernatant was carefully removed, and the residue was washed once with 1 ml of 80% ethanol, dried on air, and dissolved in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of extracted DNA was measured by Qubit 3.0 (Thermo Fisher Scientific, USA).

A custom Illumina library preparation method, including preliminary uracil DNA glycosylase, UDG (New England Biolabs, USA) treatment, was used. This method includes several steps:

- Uracil DNA glycosylase (UDG) treatment, blunting, and end-repair of the DNA fragments;
- DNA library cleanup;
- Adapter mix preparation and adapter ligation;
- DNA library cleanup;
- Filling gaps with a large fragment of *Bst* polymerase;
- Polymerase chain reaction of DNA library;
- DNA library cleanup.

The amplified DNA libraries were quantified using a High Sensitivity DNA Kit on a 2100 Bioanalyzer instrument (Agilent Technologies, USA). Multiple negative controls were used during the aDNA extraction and DNA library amplification. The DNA of all researchers involved in this study was taken to analyze potential contamination of the DNA extracts. The results showed that the mtDNA and Y-chromosome haplogroups identified for the Chinge-Tey I individual did not match those of any researchers. Whole-genome sequencing (WGS) was performed on NovaSeq6000 sequencing system (Illumina, USA) with single-end reads of 150 bp length.

Sanger sequencing of hypervariable region I (HVRI) and coding part of mitochondrial genome and their analysis

SNPs without strong statistical support, which were necessary for mtDNA haplogroup identification, were validated using Sanger sequencing. DNA primers, which were previously proposed by Sampietro and colleagues [23] and developed in this study (Supplementary Table 3), were used to amplify the HVRI fragment and fragments of the coding part of the mitochondrial genome; the same SNPs were previously used for mitochondrial haplogroup identification in the study by Unterländer and colleagues [15]. The obtained amplicons were sequenced using the ABI 3730xl platform (Thermo Fisher Scientific, USA).

Bioinformatics and statistical analyses of ancient DNA sequencing data

The single-end DNA reads were obtained in FASTQ format and processed using the PALEOMIX (v1.3.2) pipeline [24]. The AdapterRemoval v2 tool [25] was used for the DNA library adapter filtration and for the elimination of low-quality bases (Ns or BaseQ < 5). DNA reads shorter than 25 bp or that contained more than 30 bp of missing data were trimmed out (Supplementary Table 4). BWA-MEM v0.6.2, with option "FilterUnmappedReads: yes", human reference genome (GRCh37), and filtered reads from the previous step were implicated in the mapping procedure. PCR duplicates were excluded from the SAM files after the mapping stage using the Picard v1.128 tool (https://broadinstitute.github.io/picard). Mapping statistics and the percentage of endogenous DNA reads are presented in Supplementary Table 4. Pseudo-haploid calls (61,115) were generated for randomly selected reads using pileupCaller (https://github.com/stschiff/sequenceTools). 1240k Reference Panel was used as a reference database [26].

We used only DNA reads with a base and mapping quality of ≥30. mtDNA haplogroup identification was carried out using the Haplocheck tool [27] with the default parameters (--level 0.01, --mapQ 30, --baseQ 20, --deletions). MtDNA tree Build 17 (18 Feb 2016) database was also used for mtDNA haplogroup identification. Mitochondrial DNA contamination test with a Bayesian approach implementation (contamMix v1.0-11) was performed as previously described by Fu with colleagues [28]. An additional *schmutzi* contamination test was carried out [29].

DNA-based sex identification was also carried out based on a method previously developed by Skoglund and colleagues [30]. Y-chromosome contamination test was performed using the 1000 Genome Reference Panel and the 1240k Reference Panel as the reference databases using the hapCon tool [31]. We also launched contamination.R code with the "Method 2" parameter from ANGSD software [32] for male X-chromosome contamination check. Methods of moments (MoM) and maximum likelihood (ML) estimates were applied for this analysis. ISOGG web-service v15.73 (http://www.isogg.org/tree) and SNPs that were found in Y-chromosome were used for Y-chromosome haplogroup identification.

The Allen Ancient DNA Resource (AADR) database v54.1 [26] was used for genome-wide comparative study. We combined SNPs that were obtained for elite nomadic warrior from Chinge-Tey I with data for modern and ancient populations from the AADR database. We used a genomic dataset for ancient human individuals from the time-preceded, time-synchronous, and time-subsequent archaeological cultures to describe their genetic affinities with Chinge-Tey I individual and on the other hand, we included genome data for two AB individuals that were previously found in Arzhan-2 [15]. Several Neolithic and Bronze Age datasets from Eastern Eurasia, India, Turkey, and the North Caucasus were added to the analyses as outgroup for detailed clustering of the individuals studied. Finally, modern and ancient human individuals were genotyped at 61,115 common sites.

Principal component (PCA), admixture, and kinship analyses

Principal component analysis (PCA) for modern and ancient human populations from the AADR dataset [26] and for Chinge-Tey I individual was carried out using the smartpca tool from the EIGENSOFT package [33]. Default parameters (lsqproject: YES; shrinkmode: YES; numoutlieriter: 0) were used for this type of analysis.

We included genomic datasets of bearers corresponded to several Neolithic, Bronze Age, and Iron Age archaeological cultures and archaeological sites from the territory of present-day Russia (North Caucasus, Siberia), Kazakhstan, Kyrgyzstan, China, Mongolia, India, and Turkey into PCA analysis among them: AB (including Chinge-Tey I individual), Uyuk-Sagly, Afanasievo, Okunevo, Sintashta, Tagar, and Tasmola archaeological cultures. Ancient individuals from the synchronous Xinjiang (Zhagunluke, Jierzankale, Dongtalede, Songshugou, and Abusanteer) and Mongolia archaeological sites were also included in the analysis (Supplementary Dataset 1: for Fig. 2 (S1); Supplementary Dataset 1: for Fig. S2). In the manuscript, only PCA for ancient individuals was selected (Fig. 2) for greater visibility. A PCA plot showing all ancient individuals marked using Master IDs from the AADR database is shown in Fig. S1. A combined PCA plot for modern and ancient human individuals is presented in Fig. S2.

Admixture analysis with the ADMIXTURE tool (v.1.23) [33] was carried out after dataset filtration in PLINK (parameters: indep-pairwise 200 25 0,4) [34]. The smallest cross-validation error was produced for the number of ancestral populations (K) equal to 12. The ancient

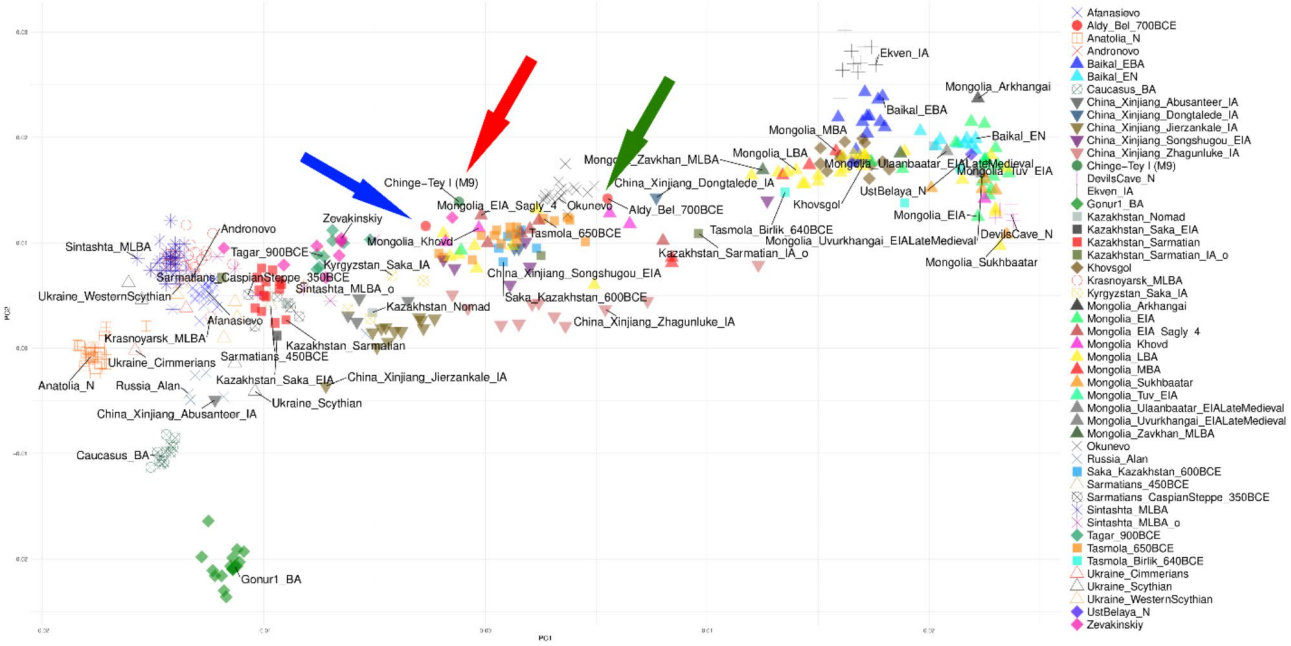

Fig. 2 Principal component analysis (PCA) of the elite nomadic warrior from Chinge-Tey I grave 9 and two other Aldy-Bel culture bearers from Arzhan-2, graves 14 and 22 in comparison to 415 preceded and synchronous human individuals (Supplementary Dataset 1: for Fig. 2) which were projected on the modern individuals inhabiting Eurasia. The Chinge-Tey I individual, grave 9 analyzed in this study is shown by red arrow. The Arzhan-2 individual, grave 14 is marked by blue arrow. The Arzhan-2 individual, grave 22 is marked with green arrow

populations, which have possible historical connections to the AB culture, as well as populations that showed the least genetic distances from the AB culture bearers excavated from Arzhan-2 and Chinge-Tey I archaeological complexes (based on PCA), were used for ADMIXTURE analysis (Supplementary Dataset 1: for Fig. 3(S3)). Projection to the modern human populations (HO, Human Origins panel containing 412 thousand SNPs) was performed in all PCA and ADMIXTURE plots that were constructed in this study (Supplementary Dataset 1: for Projection).

Kinship analysis was performed using READ software using the methodology previously described by Wang and colleagues [35]. Genomic datasets of two AB culture bearers (Arzhan-2, graves 14 and 22) and other ancient individuals (including Tasmola culture bearers) were selected based on previous (PCA and ADMIXTURE) analyses. The final list of individuals used in kinship analysis is included in Supplementary (Supplementary Dataset 1: for READ).

f3-statistics analysis was performed in several ways. In one of them we assumed that the AB culture representatives had a local genetic substrate of the Mid Bronze Age Okunevo culture bearers, with the traces of genomic introgression of Central Asian nomads, including ancient people groups related to individuals buried in archaeological sites from Xinjiang as well as Zevakinskiy burial mound, Tasmola culture bearers and the Scytho-Siberian mobile groups of Central Eurasia (marked as Sakas in AADR dataset)– all three from the Early Iron Age burials in Kazakhstan (approximately 1000–600 BCE) (Supplementary Dataset 11: F3-stat). On the other hand, we looked for the genetic affinity of AB culture bearers with time-preceded and time-synchronous groups of the Late Bronze and Early Iron Age archeological sites in adjacent Mongolia (including burials of Uyuk-Sagly culture). Yoruba population genotypes were used as an outgroup in this analysis, which is a common practice in population studies of non-African populations (Supplementary Dataset 1: F3(out)-stat). The qp3Pop tool with default parameters [33] was used for *f3-statistics* analysis.

We also used qpAdm tool from ADMIXTOOLS (v.1.23) package [33] and the same population groups (Supplementary Dataset 1: qpAdm) to show detailed genetic relationships between ancient people inhabited South Siberian and Central Asian region during the Bronze and Early Iron Ages.

Results and discussion

The total number of DNA reads generated for the elite nomadic warrior from Chinge-Tey I was 841,339,208. The endogenous DNA content in the dataset was 0.7%. The average mitochondrial and nuclear genome coverage was 1.22× and 0.21×, respectively. The detailed statistics of mapping is presented in Supplementary Table 4. The contamination tests based on mitochondrial DNA (excluding *schmutzi* test, which considers deamination patterns and is not sufficient for UDG-treated DNA)

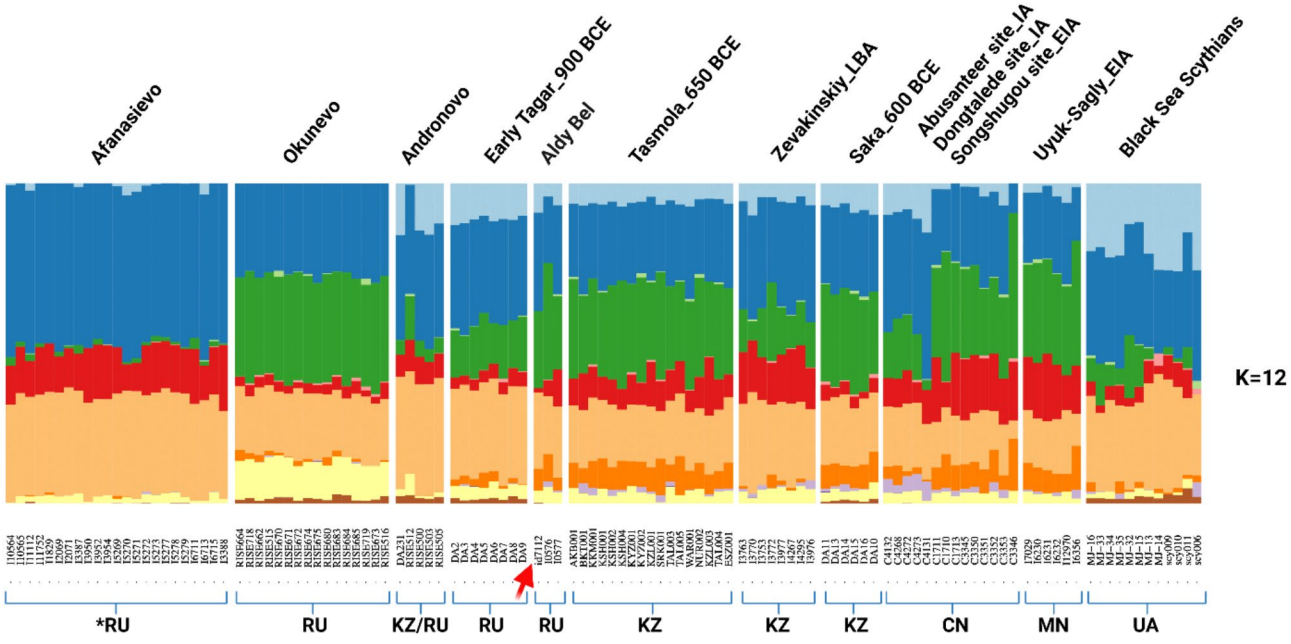

Fig. 3 ADMIXTURE profiles (K = 12) of the three Aldy-Bel culture bearers: Chinge-Tey I (grave 9, id7112, marked by red arrow), Arzhan-2, grave 22 (I0576), Arzhan-2, grave 14 (I0577), and human individuals from archaeological sites of the Afanasievo, Okunevo, Andronovo, Tagar, Uyuk-Sagly, Tasmola cultures, and ancient human individuals from synchronous archaeological sites in present-day Kazakhstan (Zevakinskiy_LBA, Saka_Kazakhstan_600 BCE), Northwestern China (Xinjang_Abusanteer_IA, Xinjang_Dongtalede_IA, Xinjang_Songshugou_EIA), and Ukraine (Black Sea Scythians and Cimmerians). * - present-day territories (countries): RU– Russia; KZ– Kazakhstan; CN– China; UA– Ukraine

and X-chromosome data showed that the genomic dataset al.most met the requirements for aDNA analysis (Supplementary Tables 5–6). The contamination levels obtained using the ANGSD tool are near the threshold values and are suitable for further analysis. The methods of moments estimate varied from 4.27% (Method 2) to 8.06% (Method 1). On another hand, hapCon estimates were approximately 11% for our data (Supplementary Table 6). In the previous ancient DNA studies genomic datasets of male individuals with contamination above 10% in either ANGSD or hapCon considered as contaminated [36]. However, the 1% excess (hapCon) allows us to carefully use the genomic data.

Only two mitochondrial DNA SNPs (14766T and 14783 C) were called in the WGS dataset due to rigorous data filtration and low genome coverage. Other positions in mtDNA that did not have strong statistical support (4833G, 6688T, 9540 C, 16223T) were validated using Sanger sequencing (Supplementary Table 3). The same positions were previously used for mitochondrial haplogroup identification for AB individuals from Arzhan-2 archaeological complex [15]. Mitochondrial DNA analysis based on NGS data, Sanger sequencing, and the Haplocheck tool indicated that elite nomadic warrior from Chinge-Tey I inherited mitochondrial haplogroup G based on polymorphisms in their mitochondrial genome (Supplementary Dataset 2: mtDNA SNPs). The same haplogroup was previously described for two individuals from the Arzhan-2, graves 5 and 14. Noteworthy, one of them was a woman from "royal" grave 5 [15]. According to technological analysis, gold decorations associated to her burial and ones from the Chinge-Tey grave 9 could have been made identically by one and the same craftsman [37]. Taken into account the time difference of the burials, one can suggest that the warrior could inherit these valuable decorations as family heirlooms. The absence of wide-genome data for the Arzhan-2 'queen' makes it so far impossible to undertake kinship analysis of these two high social level individuals. Nonetheless, the fact that they share mitochondrial haplogroup G is in line with the abovementioned archaeological hypothesis. Mitochondrial haplogroup G itself is rarely presented in published ancient individuals; however, its subclade G2a is frequent among ancient and modern people of northern East Asia and Central Asia [38, 39, 40, 41]. G2a mtDNA haplogroup was also described in another AB individual from Arzhan-2 (grave 25) [15], and its subclade G2a1g was found in an individual from Eki-Ottug 1 burial complex, kurgan 12 (time-subsequent Uyuk-Sagly culture) [17].

Y-chromosome haplogroup identification showed that Chinge-Tey I princely warrior inherited Q1b1 (Q-L476) haplogroup (Y-chromosome SNPs are presented in Supplementary Dataset 2: Y-chromosome SNPs). Individuals of the same period with this haplogroup were identified previously in AB archeological sites Bai-Dag 6 (kurgan

2, grave 1), Bai-Dag 8 (kurgan 1, grave 3), Eki-Ottug 1 (kurgan 3), and in several human individuals from burials of Uyuk-Sagly culture (Eki-Ottug 1) [16, 17, 42]. Q1b1 Y-chromosome haplogroup was also identified for the bearers of subsequent Uyuk-Sagly culture, excavated from the Chandman cemetery in present-day northern-west Mongolia, adjacent to south Tuva (grave 47, burials 2–3; grave 48, burial 6; grave 53, burial 2; grave 58, burial 2) [43].

The fact that haplogroup Q is absolutely dominated in the early Uyuk-Sagly culture burials [16] led us to supposition that princely burial at Chinge-Tey I, grave 9, although made according to the AB traditions, might already reflect a process of a new population appearing in the area inhabited by the AB group. This suggestion coincides to archaeological observation that some finds in grave 9 were of new cultural tradition, alien for the AB culture, but common for the succeeding Uyuk-Sagly culture [9].

The SNP dataset from the nuclear genome of Chinge-Tey I individual obtained in this study was merged with specially selected SNP datasets from ancient and modern human individuals from the AADR database (Supplementary Dataset 1). Among ancient individuals, we mostly selected those whose cranial and skeletal remains were found in archaeological sites of time-preceded, time-synchronous, and time-subsequent cultures from a certain region. Ancient individuals, bearers of Afanasievo, Okunevo, Tagar, and Uyuk-Sagly archaeological cultures from the Altay-Sayan region were analyzed together with bearers of Sintashta and Tasmola archaeological cultures from South Ural and Kazakhstan. SNP datasets belonged to human individuals from the Mongolian archaeological sites and especially the synchronous Xinjiang archaeological sites (Zhagunluke, Jierzankale, Dongtalede, Songshugou, and Abusanteer) were also included in the study in view of possible cultural exchange between South Siberia, Central Asia and Xinjiang [44]. This merged dataset was applied to principal component analysis (PCA), ADMIXTURE, and kinship analyses to describe the genetic origin and genetic affinities of elite nomadic warrior from Chinge-Tey I.

Based on the PCA plot, we clearly showed that the Chinge-Tey I individual and two individuals from Arzhan-2 have genetic affinities to bearers of synchronous Tasmola culture, subsequent Uyuk-Sagly culture (samples from Mongolia), and individuals buried in the Early Iron Age Xinjiang archaeological sites: Songshugou, Dongtalede, Abusanteer, and Zhagunluke (Fig. 2). These results are consistent with some conclusions of archaeologists and physical anthropologists: there are evidences of cultural and technological exchange between Xinjiang and Tuva during the Early Iron Age [14, 45, 46]. Besides, bioarchaeological similarity of Tasmola and AB culture individuals has been observed [47] as well as important analogies in artifacts placed in the burial complexes of both cultures [48, 49].

Interestingly, Tasmola culture individuals have significant genetic diversity; e.g., ancient individuals from Taldy II, Karashoky I, and Kyzylshilik burial grounds, Nurken II, and Kyzyl barrows have significant genetic distances from those buried in Birlik barrows. Similar genetic pattern is observed for the AB culture bearers. One of them (Arzhan-2, grave 22, I0576) is placed relatively far away from two others: an individual from Arzhan-2, grave 14, (I0577) and the warrior from Chinge-Tey I, grave 9. However, the last two have different parentally inherited markers: mtDNA A8a haplogroup for Arzhan-2, grave 14 individual vs. G haplogroup for Chinge-Tey I individual; and Y-chromosome R1a1a1b haplogroup for Arzhan-2, grave 14 individual vs. Q1b1 haplogroup for Chinge-Tey I individual.

A comparable picture was obtained from the ADMIXTURE analysis. Three AB culture bearers under discussion share a similar genetic profile with representatives of the Middle Bronze Age Okunevo culture and individuals from Tasmola and synchronous sampling group (approximately dated as 600 BCE) from Eastern and Central Kazakhstan (DA10-DA16 Master IDs in the AADR database) and marked in general as 'Saka' from Kazakhstan [38]. AB culture representatives are also genetically close to people from synchronous archaeological sites from Xinjiang, mostly Songshugou, Dongtalede, Abusanteer, and Zhagunluke (Fig. 3; Fig. S3). They are also close to bearers of the subsequent Uyuk-Sagly culture from present-day Mongolia.

We also carried out a kinship analysis between three available genome data of AB culture bearers and several human individuals found in time-synchronous archaeological sites that have high genetic affinities based on PCA plots (Fig. 2; Fig. S1; Fig. S2) to Chinge-Tey I individual (Supplementary Dataset 1: for READ). However, we found no kinship between them (Fig. S4). One can suggest this is due to the elite level of the former vs. ordinary state of all other used individuals.

Archaeological materials allow hypothesis that AB culture bearers came to the present-day Tuva from the territory of modern Kazakhstan. It could be a group of newcomers who took a dominant position over the local Late Bronze Age population [2]. On the other hand, according to archaeological data too, AB culture people could have partly inherited from another late Bronze Age group, represented by the sites of Arzhan-1 and Tunnug 1– those of the earliest Scythian-type, pre-Aldy Bel horizon. Their ancestry is believed to come from the Bronze Age Mongolia and Kazakhstan [10]. Recent archaeological studies revealed also significant technological

innovations as contribution to the AB culture from people and cultures of modern day Xinjiang [3].

Keeping in mind these archaeological observations and hypotheses, and based on the results of haplogroup identification, PCA and ADMIXTURE analysis described above, we estimated possibility of contacts between AB culture bearers and representatives of the Bronze and Early Iron Age cultures from the mentioned regions at genomic level.

f3-statistics of the form F3(X, Y; target) with the AB culture bearers as target showed the absence of significantly negative Z scores (Supplementary Dataset 3: F3-stats). Nevertheless, *f3-statistics* of the form F3(X, Aldy Bel; outgroup) with X as the Bronze– Early Iron Ages people from Mongolian cemeteries and the Yoruba people as outgroup demonstrated high level of genetic relationships between AB culture nomads and pastoralists buried in preceding, synchronous and succeeding (Uyuk-Sagly culture) sites in present-day Mongolia (Supplementary Dataset 3: F3(out)-stat).

The genetic relationship between the three AB culture bearers from Chinge-Tey I (id7112, grave 9), Arzhan-2 (I0576, grave 22) and Arzhan-2 (I0577, grave 14), and the Bronze and Early Iron Age individuals from the adjacent steppe, was also assessed using 3-source qpAdm analysis. In this approach we fixed Okunevo culture people (Russia_BA_Okunevo.SG) as main possible genetic substrate, and used AB culture bearers as the target. AB culture bearers shared their genetic ancestry with: (source1) South Siberian Okunevo culture individuals (Russia_BA_Okunevo.SG), (source2) Bronze Age pastoralists from various archaeological sites in present-day Mongolia, and (source3) individuals from Zevakinskiy burial mound in Eastern Kazakhstan (transitional period from the Late Bronze to the Early Iron Age), Abusanteer archaeological site in Western Xinjiang (Early Iron Age), and Iron Age bearers of Sarmatian culture in Western Kazakhstan. It is worth noting that ancient people buried in Abusanteer seem to have close genetic relationships with the AB culture bearers (reliable groups are marked by green in Supplementary Dataset 3: qpAdm). We will be able to estimate this DNA study result when archaeological materials from this site are entirely published.

The main area of the Okunevo culture sites was the Minusinsk Basin, whereas the Okunevo-type sites in Tuva are rare. The future of this population is unknown. Judging by the result obtained, it could take part in the formation of the AB population of Tuva. It's worth noting that archaeological observations place the origin of some images of the early-Scythian animal style in the artistic tradition of Okunevo culture [50, 51, 52].

Archaeological materials from Zevakinsky burial mound in Eastern Kazakhstan, dated back to the transitional period from the Late Bronze to the Early Iron Age, are also consistent with the conception of this area as one of the ancestral regions for AB culture [3].

Conclusion

Aldy-Bel archaeological culture in the present-day Republic of Tuva, Russia held one of the key positions in the early history of the so-called Scythian-type cultures in Asia. The bearers of AB culture left a number of archaeological sites across the Republic of Tuva. The "Valley of the Kings" the only place here where elite-level funerary commemorative complexes of the earliest nomads are located and studied.

Despite the significance of AB culture in the formation of the early-Scythian World in the Asiatic steppes, genetic origin and diversity of its population and kinship system as to how it was reflected in the funeral sites, hasn't been yet well studied. A few studies that have been published to date have mostly used mtDNA or Y-chromosome markers [16, 17, 42]; two of them have been published in Russian only [16, 42]. Until recently, two low coverage nuclear genome datasets of AB culture bearers have been available in public databases only. These two individuals are from periphery burials of Arzhan-2 and it's still unclear whether they represent genetic ancestry of the local elite or not.

This study presents the first nuclear genome dataset of an elite nomadic warrior from the Chinge-Tey I funerary commemorative complex of the "Valley of the Kings", the third one for AB culture as a whole. Despite the levels of exogenous DNA contamination being close to the threshold and only one new ancient human genome being studied, the dataset can now be used in studies devoting to formation and interconnections of the early Scythian-type Asian cultures. As a dataset for the elite level individual, it will be important for estimating certain kinship between the leaders of different nomadic groups over the wide steppe area and their possible dynastic connections.

Then, we undertook a comparative analysis of this genome with genomic datasets of two other AB culture bearers from another elite burial mound in the same valley, the time-preceded Arzhan-2 [12]. With this we got an idea about genomic diversity of three people, especially between the ones buried in one and the same site as 'guards' of the royal couple. aDNA analysis revealed no kinship between them, but their possible kinship to the Arzhan-2 'royal couple' should be estimated once wide-genome datasets of the latter are obtained. Wide-genome data of the other people buried in Arzhan-2 and Chinge-Tey I 'periphery' graves should be generated too for obtaining the whole picture of the society members buried under the elite–level complexes.

The three datasets have also been compared to nuclear genomes of the time-preceded and time-synchronous individuals from adjacent regions of Mongolia,

Kazakhstan, Northwestern China (Xinjiang) and the Minusinsk Basin. These regions are important from archaeological perspective as possible ancestral lands for population groups which contributed to the formation of the AB culture as well as less known cultures of the preceding, pre-Aldy Bel horizon in Tuva. Comparative genome analysis shows that AB culture bearers had certain genetic affinities with following population groups:

- individuals of Okunevo culture of the early II millennium BC, whose probable contribution to the early Scythian-type cultures of Central Asia has been suggested by archaeologists [50, 51];
- people from time-synchronous nomad burials in Kazakhstan and Xinjiang– the main regions archaeologically considered as ancestral lands for the migration to Tuva and formation of AB culture and its elite stratum [3, 45];
- Representatives of the succeeding Uyuk-Sagly culture.

These results, together with previously published archaeological, bioarchaeological, and stable isotope data confirm potential connections and common genetic ancestry between AB culture individuals and those of abovementioned cultures of Central Asia [4, 12, 44, 47, 48, 49].

To reach our purposes with the lack of wide-genome data, we used also mtDNA and Y-chromosome haplogroup data. Elite nomadic warrior from Chinge-Tey I has mitochondrial haplogroup G which was previously identified in two individuals from Arzhan-2 (graves 5, 14), including a female individual from the "royal" burial [15]. The latter was previously suggested as the elder relative of the Chinge-Tey princely person. The fact of their shared mitochondrial haplogroup could serve for possible confirmation of this hypothesis [14].

Chinge-Tey I individual also has Q1b1 Y- haplogroup, which was previously observed in AB culture bearers buried in ordinary archaeological sites. It possibly demonstrates common ancestry of this local group regardless the social position of its representatives [16, 17, 42]. The same Q1b1 haplogroup refers also to several individuals from the Uyuk-Sagly culture burials in both Tuva and Northwestern Mongolia [43]. This can be explained in two ways: either Uyuk-Sagly nomads could inherit Q1b1 from the AB group, or the new population group could appear in Tuva quite early, at the time when AB culture representatives still inhabited the region. More DNA analyses of both groups are extremely needed for better understanding of this and other mentioned issues.

Given the mosaic picture of different Late Bronze and Early Iron Age cultures in Central Asia in a whole and Tuva in particular, this study allowed us building a plan for the next study.

DNA study of human remains from the Late Bronze Age burials of Tuva, those of the so called Mongun-Taiga culture are necessary to estimate its genetic contribution to the early Scythian-type cultures and horizons. Of special interest are individuals from the sites of Pre-Aldy Bel horizon in Tuva [3, 10], both from the 'Valley of the Kings' and beyond. The former are elite- and high-level complexes Arzhan-1, Tunnug-1 and Arzhan 5 [7, 9]. The latter are Dogee-Baary II, mound 40 and Badanka IV in the different valleys of the Altai-Sayan mountains. Human remains from Arzan-1 are unavailable, but those from the other sites are of particular importance and we plan to use them in our next research. Broad genomes of the "royal" couple from Arzhan-2, grave 5, and all surrounding people are of particular need.

We suggest that close collaboration between archaeologists, physical anthropologists, and paleogeneticists on these materials would allow more accurate purposes to be addressed and clearer results to be obtained on historical, demographic, and genetic processes during the formation of the early nomadic groups in Asia.

Abbreviations

PCR	Polymerase chain reaction
WGS	Whole-genome sequencing
mtDNA	Mitochondrial DNA
SNP	Single nucleotide polymorphism
CRS	Cambridge reference sequence
PCA	Principal component analysis
AB culture	Aldy-Bel culture

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions

AN, AR, AI, KC, and VI conceived and designed experiments; NA, EU, and KC performed sampling; NPL (Nikolay Plotnikov) and AK extracted ancient DNA; AI performed library preparation and sequencing; NS and EB designed PCR primers and performed Sanger sequencing; AN, EV, FS, DK, XS, OP, NPO (Nadezhda Pogodina), and AR analyzed the data, AN, NS, SP, AR, and KC wrote original draft. All authors read and approved the final manuscript.

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Data availability
All mitochondrial Sanger sequences, Illumina raw DNA reads for elite nomadic warrior from Chinge-Tey I barrow are publicly available at the NCBI BioProject: PRJNA1093285.

Declarations

Ethics approval and consent to participate
This study was approved by the Genotek Ethics Committee (approval number, 2022-11) and followed the tenets of the 1964 Declaration of Helsinki. This study was conducted under the ethics guidelines for ancient DNA research: Alpaslan-Roodenberg S, Anthony D, Babiker H, Bánffy E, Booth T, Capone P et al.: Ethics of DNA research on human remains: five globally applicable guidelines. Nature 2021; 599: 41–46.

Consent for publication
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Competing interests
The authors declare no competing interests.

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