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# Three Thousand Years of Continuity in the Maternal Lineages of Ancient Sheep (*Ovis aries*) in Estonia

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## Abstract

Although sheep (*Ovis aries*) have been one of the most exploited domestic animals in Estonia since the Late Bronze Age, relatively little is known about their genetic history. Here, we explore temporal changes in Estonian sheep populations and their mitochondrial genetic diversity over the last 3000 years. We target a 558 base pair fragment of the mitochondrial hypervariable region in 115 ancient sheep from 71 sites in Estonia (c. 1200 BC – AD 1900s), 19 ancient samples from Latvia, Russia, Poland and Greece (6800 BC – AD 1700), as well as 44 samples of modern Kihnu native sheep breed. Our analyses revealed: (1) 49 mitochondrial haplotypes, associated with sheep haplogroups A and B; (2) high haplotype diversity in Estonian ancient sheep; (3) continuity in mtDNA haplotypes through time; (4) possible population expansion during the first centuries of the Middle Ages (associated with the establishment of the new power regime related to 13<sup>th</sup> century crusades); (5) significant difference in genetic diversity between ancient populations and modern native sheep, in agreement with the beginning of large-scale breeding in the 19<sup>th</sup> century and population decline in local sheep. Overall, our results suggest that in spite of the observed fluctuations in ancient sheep populations, and changes in the natural and historical conditions, the utilisation of local sheep has been constant in the territory of Estonia, displaying matrilineal continuity from the Middle Bronze Age through the Modern Period, and into modern native sheep.

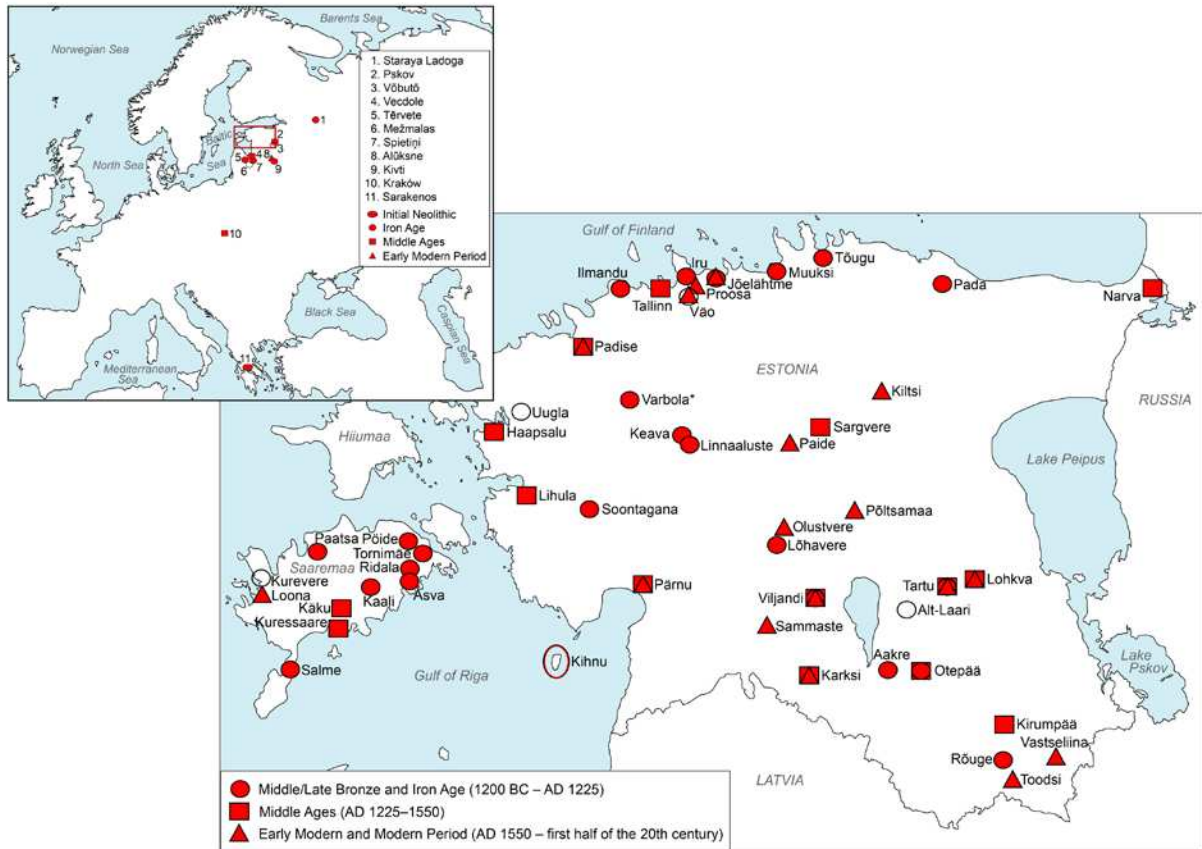
Keywords: *Ovis aries*, aDNA, mtDNA, genetic diversity, ancient lineage, Estonia

# Introduction

## Background

Sheep (*Ovis aries*) were domesticated around 11 000 years ago in the area of the Fertile Crescent [1]. It has been suggested that sheep were initially reared for meat and milk (so-called ‘primitive’ sheep populations [3]) and only later for wool [2]. It has been demonstrated that early movements of domesticated sheep out of the Middle East and into Europe included predominantly ‘primitive populations’ while more recent movements included sheep with markedly improved wool production [3]. The latter were dispersed in several migratory episodes across Eurasia and Africa, where they gradually replaced majority of primitive sheep populations and provided the foundation for many modern breeds [3].

As the wild ancestors of domestic sheep are absent in northern Europe, the first zooarchaeological evidence for sheep is thought to be associated with the introduction of domestic animals. In Estonia, the first zooarchaeological evidence for domestic sheep is a bone awl of sheep metacarpal bone recovered from a Late Neolithic Corded Ware (2900–1800 BC) burial, dated by associated finds to the beginning of that period, 2900–2700 BC [4]. This period likely marks the first steps of animal husbandry in the territory of present Estonia, although evidence for livestock husbandry remains sparse even into the Early (1800–1200 BC) and Middle (1200–850 BC) Bronze Age period. Nevertheless, by the Late Bronze Age (850–500 BC) animal husbandry had developed substantially, evidenced by abundant remains of cattle, sheep, goat, horse and pig in the archaeological record of settlement sites like Asva on Saaremaa Island [5] (Fig 1). The subsequent Early Iron Age (500 BC – AD 550) yields little osteological evidence for animal husbandry, which could be attributed both to the mixed nature of archaeological deposits and the dispersed settlement pattern characteristic of this period (making it difficult to locate sites on landscape). From the Late Iron Age (AD 550–1225) onwards, animal remains, especially of domesticates, again become numerous and are hereafter abundant in the archaeological deposits.



**Fig 1. Geographic origin of ancient sheep samples analysed in this study.** Samples ( $n = 134$ ) come from Estonia ( $n = 115$ ), Latvia ( $n = 7$ ), Poland ( $n = 3$ ), Russia ( $n = 7$ ), and Greece ( $n = 2$ ). The location of Kihnu Island in the Gulf of Riga for which the native sheep breed is named, is circled; the samples of Kihnu sheep ( $n = 44$ ) were collected from the mainland population in south-western Estonia. Site marks on map can indicate several samples from one site; colourless marks indicate sites without any successful samples (for details see S1 Table).

The time of the crusades in Estonia (AD 1208–1227) which marks the transition from the prehistoric to the historic era and the beginning of the Middle Ages (AD 1225–1550), brought significant changes in power structures and settlement. These decades are illustrated by the Chronicle of Henry of Livonia, where he describes ‘countless’ numbers of sheep or ‘other livestock’ besides cattle, oxen and horses, that were taken from the local inhabitants during the campaigns [6]. Medieval zooarchaeological material, however, comes predominantly from urban deposits; rural assemblages tend to be more rarely excavated and/or poorly preserved. Urban assemblages are dominated by domestic mammals like cattle, sheep, goat, and pig, while the exploitation of wild game is practically non-existent, in

comparison to prehistoric times, *e.g.* [7]. Widening trade (*e.g.* through the German Hanse), the development of towns, and growing human populations all influenced animal husbandry, supposedly increasing the amount of livestock. The local livestock populations may have been augmented by imported individuals, although this practice would have been rather exceptional and occurred only in wealthy manors as known from the later periods.

The Middle Ages came to an end with the Livonian war (AD 1558–1583). Well dated zooarchaeological material from the 17<sup>th</sup> and 18<sup>th</sup> centuries is scanty, firstly, because of limited archaeological interest in the Early Modern Period (AD 1550–1800) the faunal remains are often not collected during the fieldwork, and secondly, it is difficult to archaeologically distinguish the layers of this period from more recent ones. The same issues apply to the following Modern Period (AD 1800 – first half of the 20<sup>th</sup> century) which also marks the beginning of large-scale breeding. The wide use of Merino sheep after the Middle Ages is believed to have been the most influential factor in the development of modern breeds [8]. In Italy, Spanish Merino rams were used to improve local sheep as early as in 1435 [9], although in northern part of Europe it started much later: in Sweden from 1723 [2] and in Finland the import of ‘Spanish’ sheep with finer wool has been recorded from the 16<sup>th</sup> century [10]. In Estonia, the first documents of foreign ‘English’ sheep breeds are known from the 1670s; around the same time some imported breeds have also been recorded in Latvia [11]. The first written documents referring to local Estonian sheep appear at the end of the 18<sup>th</sup> century (1794), where small sheep with coarse wool, long slender legs and relatively short tails have been described [12]. At the same time, first attempts were made to improve local sheep with Spanish Merinos [12]. In 1824, the true breeding of fine-wool sheep in Estonia began in wealthier manors, with extensive improvement of local sheep first with Merino and then with other breeds like Shropshire and Cheviot; the latter two were used to develop the modern-day Estonian breeds, namely the Estonian Blackhead and Estonian Whitehead, respectively [12–15]. Among peasants, breed improvement started later, in the second half of the 19<sup>th</sup> or in the beginning of the 20<sup>th</sup> century, and at a more limited scale [12].

‘Native’ sheep survived in peripheral areas of Estonia in spite the introgression of improved and imported breeds, and the neglect of local aboriginal sheep under more recent Soviet influence (*i.e.*, during the period of collective and state farming in 1950s–1991). These sheep are now being actively revived, based predominantly on a relict population from Kihnu Island in the Riga Bay (Fig 1). The ‘Kihnu native sheep’ breed (Fig 2) was finally accepted as a licensed breed in January 2016 (Veterinary and Food Board, Estonia). Based on

microsatellite analysis, Kihnu sheep have been shown to be genetically distinct from modern breeds and also from other primitive northern European native breeds (Tapio *et al.*, in prep.), and ancient mitochondrial DNA (mtDNA) analyses have suggested a link between Kihnu and archaeological sheep populations in Estonia [16]. Similar circumstances of indigenous populations surviving on the peripheries have occurred in other parts of Europe, *e.g.* [17] and have been suggested to potentially represent descendants of the first migratory waves of primitive populations out of the Fertile Crescent [3].



**Fig 2. Kihnu native sheep.** Note the different colours of the coarse wool (white, grey, black), slender un-woolly legs, and horns on both males (in front, on left) and females (behind, on right). Both males and females can also be polled or scarred (the light grey ram in front has scarred horns). Photo: Eve Rannamäe (April 2016).

## Aims of the Study

Analyses of archaeological sheep remains in Estonia have primarily relied on morphological methods, providing information about overall size and body type, as well as the consumption and utilisation of sheep and their by-products, *e.g.* [7,18–24]. Additionally,

studies of archaeological textiles have revealed information about the local wool, which was two-layered, semi-coarse and variable in fibre coarseness, *e.g.* [25]. However, uneven representation of osteological material from different time periods, insufficient dating, and only partial analysis of many bone assemblages has made it difficult to draw wider conclusions about the development of sheep populations. Only in recent years molecular methods have been applied to study the genetic diversity of maternal lineages among Estonian ancient sheep. Mitochondrial DNA analysis of 31 ancient sheep remains dated *c.* 800 BC to AD 1700 demonstrated the presence of haplogroups A and B from the Late Bronze Age onwards, revealed several unique haplotypes and suggested higher mtDNA haplotype diversity during the Middle Ages as compared to the preceding and following periods [16].

Here, we build on previous ancient DNA (aDNA) studies by increasing both the targeted sequence length, and the number of samples from different archaeological contexts to more fully investigate the temporal fluctuations of sheep populations in Estonia, and explore the degree of continuity in maternal lineages from the Middle Bronze Age to recent breeding in the Modern Period.

Our first aim is to investigate the hypothesis of a shift in mitochondrial genetic diversity during the transition from the prehistoric to the historic period, *i.e.*, from the Late Iron Age to the Middle Ages. This hypothesis is based in part on the preliminary ancient mtDNA results published by [16], but also on osteological evidence for an increase in sheep consumption, and a decrease in withers height in domestic livestock during the Middle Ages [7]. Our second aim is to establish the degree of continuity between present-day populations of Kihnu sheep and local populations dating back to the Bronze Age. For example, are there any chronological changes in the maternal genetic diversity in archaeological sites where sheep husbandry can be traced through several centuries? To this end, we analysed mtDNA from 115 archaeological sheep remains (including 28 samples first reported in [16] for which we sequenced additional fragments of mtDNA) recovered from 71 archaeological sites in Estonia and dated from around 1200 BC to AD 1900s (Fig 1; S1 Table).

To provide comparative ancient material from neighbouring northern, eastern and southern areas, and from the area closer to the domestication centre, we also extracted mtDNA from 19 ancient sheep from 11 archaeological sites in Latvia, Russia, Poland, and Greece, dated from around 6800 BC to AD 1700 (Fig 1; S1 Table). We complemented this dataset with previously published sequences from Finland to assess the affinities of maternal sheep lineages between Estonia and Finland through time.



Our results provide additional basis for subsequent studies on maternal genetic variation in sheep and contribute to the understanding of the history of sheep husbandry not only in Estonia but also in broader areas in the north-eastern part of Europe.

## Materials and Methods

### Ancient Samples and Archaeological Sites

Samples ( $n = 134$ ) of archaeological sheep bones were obtained from archaeological collections in Estonia (71 sites;  $n = 115$ ), Latvia (6 sites;  $n = 7$ ), Russia (3 sites;  $n = 7$ ), Poland (1 site;  $n = 3$ ), and Greece (1 site;  $n = 2$ ) (Fig 1; S1 Table). Sampling permission was obtained from each institution housing the zooarchaeological collections (listed in S1 Table).

Estonian samples come from both urban and rural contexts – towns, castles, manors, monasteries, hillforts, settlement sites, field remains and burials – dating from the Middle Bronze Age to the 20<sup>th</sup> century. The majority of the samples come from the mainland with fewer from the Saaremaa Island. Micro-regions yielding sheep remains over a long chronological span were preferentially targeted to identify temporal changes within a site or region, including Viljandi-Karksi, Tartu-Lohkva and Saaremaa Island. All samples were dated based on archaeological context or radiocarbon dating (S1 Table and S2 Text). Although sample selection aimed for homogeneous geographical and temporal coverage, it was constrained by standard archaeological contingencies of preservation and accessibility, plus the limitations of taxonomical identification of sheep remains.

Regions outside Estonia were not systematically sampled, but included primarily as comparative data. Most samples were contemporaneous with the Estonian data-set: Polish and Russian samples come from medieval towns Kraków, Pskov and Staraya Ladoga, of which the latter is concurrent with the Estonian Late Iron Age; Latvian samples come from settlement sites, hillforts, and castles – and represent time periods from the Iron Age to Early Modern Period. Initial Neolithic samples from Sarakenos cave in Greece precede the Estonian oldest samples by five thousand years, making them the oldest in the dataset (S1 Table).

Because of the abundance of bones in the faunal assemblages compared to teeth, as well as the increased ability to distinguish sheep from goats based on bone elements, most samples were bones, with only one tooth selected. For samples from the same archaeological deposit it was confirmed that each individual was sampled only once, by (a) morphological characteristics such as overall size and degree of epiphyseal fusion of long bones (used to

estimate age at death of the animal [26]), and (b) single nuclear polymorphisms (SNPs) in mtDNA sequences.

All Estonian, Latvian, Russian, Polish, and Greek specimens are reported here for the first time, except 28 Estonian samples that were already included in a pilot study by [16], where a 213 base pair (bp) long fragment of the mitochondrial D-loop was targeted (fragments 1–2); in the current study we extended these sequences (fragments 3–5; S1 and S2 Table).

## Ancient DNA Extraction

Sample preparation and DNA extraction of archaeological sheep bones were conducted in the dedicated aDNA laboratory at the University of Tartu. We followed the silica spin column protocol first described by [27] and amended by [10] with slight modifications (S1 Text). Briefly, 0.5 ml of bone powder was incubated overnight in an extraction buffer of 0.45M EDTA pH 8.0, 1M of Urea, and 200 µg of proteinase K at 55°C. The resulting supernatant was concentrated using Amicon Ultra-4 30kDa Centrifugal Filter Units (Merck Millipore, Darmstadt, Germany) and purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). A total of 150 µl of DNA solution was eluted from each sample for subsequent polymerase chain reaction (PCR) amplification.

A 599 bp fragment of mtDNA hypervariable D-loop control region corresponding to positions 15957–16556 in GenBank accession NC001941 [28], was amplified using five sheep-specific primer pairs yielding overlapping sequences [10] (S2 Table). PCR amplification was carried out with HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) in 25 µl reaction mix using 3 µl DNA extract, 0.2 µM of each primer, and 0.25 units (U) of Uracil DNA Glycosylase (Sigma-Aldrich). PCR conditions were: 37 °C for 10 min; 95 °C for 15 min; 55 three-step cycles of 94 °C for 30 s, 58 °C for 40 s and 72 °C for 1 min; and final extension of 72 °C for 10 min. Each DNA extract was amplified at least twice. All samples with successful PCR products were sequenced in both directions to achieve a minimum of two identical amplicons. PCR product purification and sequencing was performed following [29], and using the same primers as for the initial PCR.

## Authenticity of the Ancient DNA Results

Sample preparation and DNA extraction followed strict protocols for in-laboratory contamination control and detection [30–32]: (1) samples were prepared and extracted in a laboratory dedicated to aDNA; (2) pre-PCR and post-PCR analyses were conducted by different people in different laboratories located in separate buildings; (3) blank extracts and negative controls were incorporated into extractions and PCRs; (4) multiple haplotypes were observed, including within extraction batches that suggests a lack of cross-sample contamination; (5) repeated extractions of 28 bone samples were performed independently in two aDNA laboratories: at the University of Tartu, Estonia and at the Natural Resources Institute Finland, Finland using the same protocols and primer sets; repeated extractions of seven bone samples were performed at the University of Tartu, Estonia (S1 Table); all repeat PCR amplifications yielded the same sequences; (6) possible C→T and G→A substitutions [33–34] were avoided by using Uracil DNA Glycosylase in the PCR reaction; (7) only reproducible results were included in the analyses.

## Modern Samples of Kihnu Native Sheep

Previously collected blood samples of Kihnu sheep ( $n = 44$ ) were kindly provided by veterinarian Anneli Ärmpalu-Idvand (Kihnu Native Sheep Society; no permits and ethical approval were required for the sampling). DNA was extracted in the University of Tartu using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. A 1451 bp fragment of mtDNA control region was amplified with one primer pair (S2 Table). PCR was performed in 20  $\mu$ l containing 20–80 ng of DNA, 0.5  $\mu$ M of each primer, 1x Advantage-2 PCR Buffer (BD Biosciences, Franklin Lakes, NJ, USA), 0.2 mM dNTP (Thermo Fisher Scientific, Waltham, US) and 0.5x Advantage-2 Polymerase Mix (BD Biosciences). Cycling parameters were: 95 °C for 1 min, followed by 10 touch-down cycles at 95 °C for 30 s, 60 °C for 30 s (temperature was reduced by 0.5 °C in each cycle), 68 °C for 2 min, followed by 25 cycles: 95 °C for 30 s, 55 °C for 30 s and 68 °C for 2 min. The final extension was 5 min at 68 °C. PCR product purification and sequencing procedures followed [35]. Sequencing was carried out for both DNA strands using the same primers as for the initial PCR.

## Sequence Analysis

The obtained ancient sequences were edited using Geneious v.6.1.7. [36, <http://www.geneious.com>]. Multiple alignments of the ancient and modern sequences were conducted using ClustalW (cost matrix IUB, gap open cost 10, gap extend cost 0.2) through Geneious. After the removal of primer sequences, the alignment was truncated to 559 bp (region 15978–16536 in GenBank accession NC001941).

The number of haplotypes ( $h$ ), haplotype ( $Hd$ ) and nucleotide diversities ( $\pi$ ), and Tajima's D values ( $D$ ) for each cohort were calculated with DnaSP v.5.10 [37]. Median-joining networks of the obtained D-loop haplotypes were produced through Network v.4.6.1.3. [38, [www.fluxus-engineering.com](http://www.fluxus-engineering.com)] with default values.

We assessed the continuity of haplotypes through time using a three-dimensional statistical parsimony network through TempNet [39] implemented through the R package v.3.1.2 [40]. We calculated the phylogenetic relationship between temporal cohorts, pairwise population fixation index ( $F_{ST}$ ) values [41] and analysis of molecular variation (AMOVA) using Arlequin v.3.5.1.2 [42].

The analysis was approached from two perspectives. Our primary focus was on Estonian material in order to assess the presence, continuity, and changes of unique and shared haplotypes through time. This was assessed by calculating the genetic diversity values and assessing the haplotype continuity for four temporal cohorts ( $n = 86$ ; alignment length 559 bp): (1) Middle/Late Bronze and Iron Age (hereafter Bronze/Iron Age;  $n = 28$ ), (2) Middle Ages ( $n = 39$ ), (3) Early Modern and Modern Period ( $n = 19$ ), and (4) Kihnu sheep ( $n = 44$ ). Note that two samples that could not be assigned to either the Iron Age or Middle Ages (51OaVar1 and 67OaOte3; S1 Table), were omitted from temporal analysis. To further study the haplotype continuity within particular archaeological region, samples from Viljandi-Karksi ( $n = 21$ ), Tartu-Lohkva ( $n = 12$ ), and Saaremaa Island ( $n = 10$ ) were analysed separately by a three-dimensional statistical parsimony network.

The secondary focus of the analysis was the comparison of Estonian ancient and modern data with other regions in Europe. A median-joining network was calculated with the Latvian, Russian, Polish and Greek ancient samples of this study ( $n = 14$ ), and available sequences from GenBank – Finnish ancient ( $n = 26$ ) and modern ( $n = 32$ ) sheep sequences by [10]. Although the study sought to compare the Estonian data to previously published ancient sequences from neighbouring regions, *e.g.* [10,43–46], only those of [10] (samples from AD

800–1800) targeted homologous regions to our study. This final dataset was made up of 204 sequences trimmed to 523 bp.

## Results and Discussion

### PCR Amplification and DNA Preservation of Ancient Samples

In total, PCR amplification of all five primer sets was successful for 102 of the 134 sampled archaeological sheep specimens dating from *c.* 6800 BC to AD 1900s (76% success rate). Amplification failed completely for 22 samples, including two specimens reported in [16]; and was only partially successful (418–509 bp) for 10 samples – these were omitted from further analyses. All full and partial sequences were submitted to GenBank (accession nos. KP052793–KP052807 and KP052809–KP052815 for the updated 22 sequences reported first in [16]; KU670230–KU670319 for the 90 sequences newly reported in this study; and KX056139–KX056146 for the eight haplotypes of the 44 Kihnu sheep sequences; S1 Table).

No significant differences in the preservation of DNA were detected in temporal or spatial cohorts (S1 and S3 Tables). All successfully amplified ancient samples were consistent with morphological species identification of *Ovis aries*; no other species (*e.g.* domestic goat, *Capra hircus*) were identified. In general, the relatively high success rate for DNA amplification can be attributed to the recent antiquity of the samples, the correct osteological identification of species, as well as the taphonomic and climate conditions conducive to DNA preservation in Estonian ground.

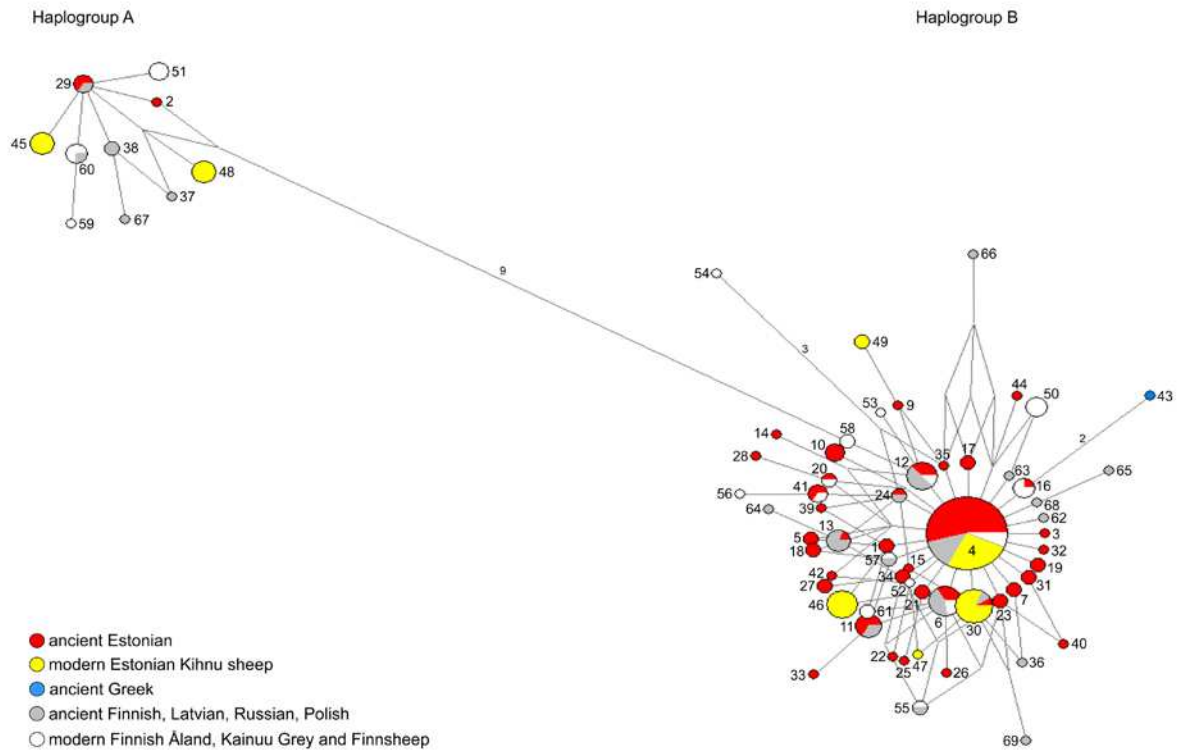
### European Network

Surveys of ovine mtDNA variability have supported a broad genetic base during domestication [8]. Variation of hypervariable D-loop region of the mtDNA has defined at least five lineages (A–E) within modern breeds. Of these, haplogroups A and B are with wide global dispersal and located in every studied region, first documented by [47]. Haplogroup C is more common in Asia, the Fertile Crescent, Caucasus and Iberian Peninsula, *e.g.* [48–50]. Haplogroups D and E are the most recent discoveries and restricted to Middle East, Caucasus and Turkey, *e.g.* [49,51–52]. All five haplogroups have also been detected in ancient populations [10,16,43–45,53–58].

Previous studies on modern sheep genetics have shown that genetic diversity indices of the first domestic populations in the initial region of domestication have been very high

and decreased with geographic distance from the centre [59–61]. This association is not always strong, which may be due to the extensive introgression of Merinos after the Middle Ages, resulting in extensive haplotype sharing of the modern breeds [8]. However, ancient DNA studies in the north-eastern Baltic Sea region have confirmed the tendency for decreasing genetic diversity with increasing physical distance from the domestication centre for cattle [62] and for sheep [16]. A recent study by [49] has also suggested strong historical human-mediated gene flow between breeds across eastern Eurasia, and proposed that sheep have spread with two different migratory waves: lineages A and B at *c.* 6400–6800 years ago, and lineage C at *c.* 4500 years ago.

Latvian, Russian, Polish and Greek specimens were incorporated into the present study to provide a wider context for ancient sheep in Europe, in addition to ancient and modern Finnish samples from GenBank [10]. In total, 49 *Ovis aries* mtDNA haplotypes were defined by 40 variable sites (S5 Table) within the complete ancient and modern dataset. Following the standard classification of the sheep mtDNA haplogroups [52], 15 sequences were assigned to haplogroup A (10%) and 131 to haplogroup B (90%). In general, a high level of maternal diversity was observed, with 51% of the haplotypes being found in only one individual. A median-joining network of this larger dataset clearly cluster the sequences into these two haplogroups, where the central haplotype *H4* (Fig 3; S1 Fig; S6 Table) appears to be the founder of the lineage, from which the other haplotypes radiate with relatively few mutation steps. Some region-specific haplotypes become apparent, like (a) haplotypes represented by Kihnu sheep, (b) haplotype associated with the oldest sample in the dataset – the Greek sheep, and (c) haplotypes containing modern Finnish sheep breeds.



**Fig 3. The median-joining network of 523 bp mtDNA D-loop haplotypes depicting the relationships between Estonian, Latvian, Russian, Polish, Greek, and Finnish ancient and modern sheep.** The samples in the network are: Estonian ( $n = 88$ ), Latvian ( $n = 5$ ), Russian ( $n = 6$ ), Polish ( $n = 2$ ), and Greek ( $n = 1$ ) ancient, and Estonian modern Kihnu sheep ( $n = 44$ ) of our study; and Finnish ancient ( $n = 26$ ) and modern ( $n = 32$ ) samples from [10]. The numbers of the haplotypes are according to S6 Table. The size of the given node is proportional to the number of samples represented in a haplotype, with the smallest node representing a single individual. Branch length is proportional to the mutational distance; only mutational distances greater than 1 are indicated. For an elaborated time-period specific network see S1 Fig.

The Sarakenos sheep from the Initial Neolithic Greece (1230aSara2), which age was determined on the basis of a series of radiocarbon dates of charcoal to *c.* 6800 BC, is one of the earliest known sheep specimens in that region [2,63–64], and represents a very early stage of domesticated sheep dispersal into Europe. The Sarakenos cave is a site where sheep and goats seem to have appeared suddenly and have immediately dominated the faunal assemblage, being clearly a food waste, possibly by Neolithic shepherds [65]. In our network

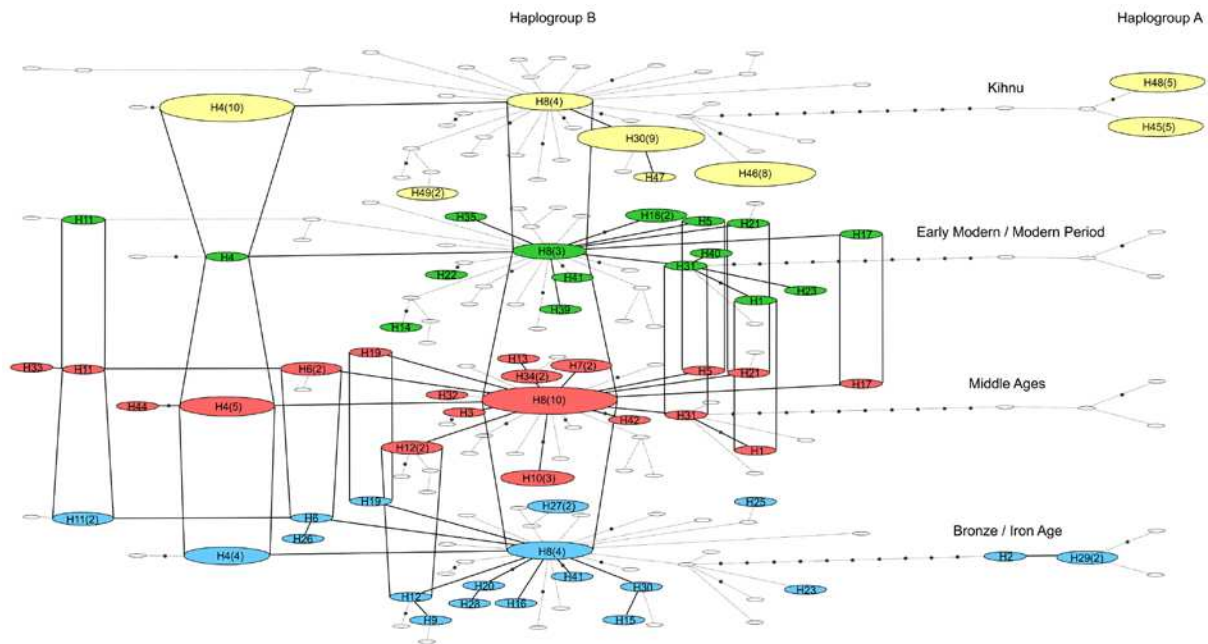
the Sarakenos sheep has a unique haplotype (*H43*) which is one of the most distant from the central haplotype in haplogroup B.

Comparisons between Estonian and Finnish sheep reveal 10 shared haplotypes, while 20 haplotypes are specific to Finland and 33 to Estonia (Fig 3; S1 Fig; S6 Table). In haplogroup A one post-medieval (= early modern) Finnish sample is shared with two Estonian Iron Age samples. In haplogroup B, in addition to the central haplotype, ancient Estonian from all time periods are shared with ancient Finnish sequences, as well as with modern Finnsheep. A large proportion of Kihnu sheep belong to the most abundant haplotype *H4*, sharing common ancestry with sheep from broad geographical and temporal distribution. Kihnu sheep have common ancestry also with medieval Russian and the Iron Age Estonian sheep (*H30*). However, there are also several haplotypes which are unique to Kihnu sheep (*H45–H49*). Interestingly, none of the Estonian samples share haplotypes with other modern Finnish breeds such as Kainuu Grey and Åland sheep. Likewise, none of the Finnish sheep (either ancient or modern), share haplotypes with the Estonian Kihnu sheep (except in the central haplotype of haplogroup B). In summary, Estonian ancient sheep show an affinity to eastern, southern, and northern European ancient sheep, as well as to native Finnsheep, while the Kihnu, Kainuu Grey and Åland native breeds appear to be genetically more distinct.

## Mitochondrial Diversity and Continuity in Estonian Sheep

Among the ancient and modern Estonian specimens, 45 mtDNA haplotypes were observed (47% unique haplotypes), again dominated by haplogroup B ( $n = 119$ , 90%), with a lower frequency of haplogroup A ( $n = 13$ , 10%). The three-dimensional network outlines the central structure of the haplotypes of Estonian sheep through time (Fig 4). Two of the haplotypes, *H4* and *H8*, are continuous through all four periods. Other haplotypes disappear or emerge in time, and some, due to sampling effect, are discontinuous between the periods. This extensive continuity of core mtDNA haplotypes may be result of husbandry practices favouring the maintenance of female animals, who were kept in large numbers for population reproduction and milk production. Comparison of the maternal lineages and the shared haplotypes from as early as the Middle Bronze Age strongly suggest the affinity between Kihnu sheep and haplotypes characteristic to ancient sheep.

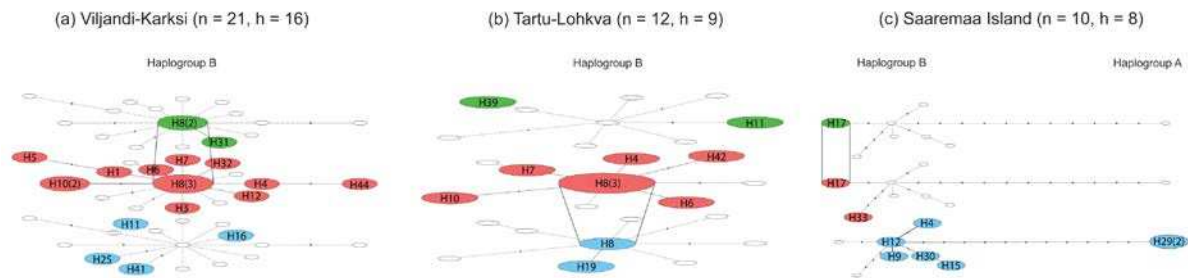




**Fig 4. Three-dimensional network of Estonian ancient and modern Kihnu native sheep from four time periods.** A three-dimensional statistical parsimony network was calculated using TempNet based on 559 bp mitochondrial D-loop haplotypes of Estonian sheep (total  $n = 86$ ,  $h = 45$ ) dating to the Bronze/Iron Age (blue), Middle Ages (red), and Early Modern / Modern Period (green), as well as Kihnu sheep ( $n = 44$ ,  $h = 8$ ; yellow). The size of the given node is proportional to the number of samples represented in a haplotype, with the smallest node representing a single individual; number of samples greater than one is indicated in the parentheses. Branch length is not proportional to the mutational distance; mutational distances greater than 1 are indicated with black dots. Colourless nodes denote haplotypes absent within the time period.

None of the continuous lineages (*e.g.* *H4*, *H8*, *H11* in Fig 4) appear to be region-specific. Therefore, we attempted to test whether we would detect continuity in the selected micro-regions. Two Teutonic Order related sites – Viljandi (castle, town and suburb) and Karksi (castle) – formed a dual power center with close hinterlands during the Middle Ages [66] and were of special interest in this study, as zooarchaeological studies at these sites have shown changing consumption patterns, and a decrease in the wither's height of the main livestock during the Middle Ages, *e.g.* [7,18–19,24]. The temporal haplotype network of Viljandi-Karksi region (Fig 5a) reveals that no prehistoric haplotypes continued into medieval period. Interestingly, the same pattern is seen for Saaremaa Island (Fig 5c), but not for Tartu-

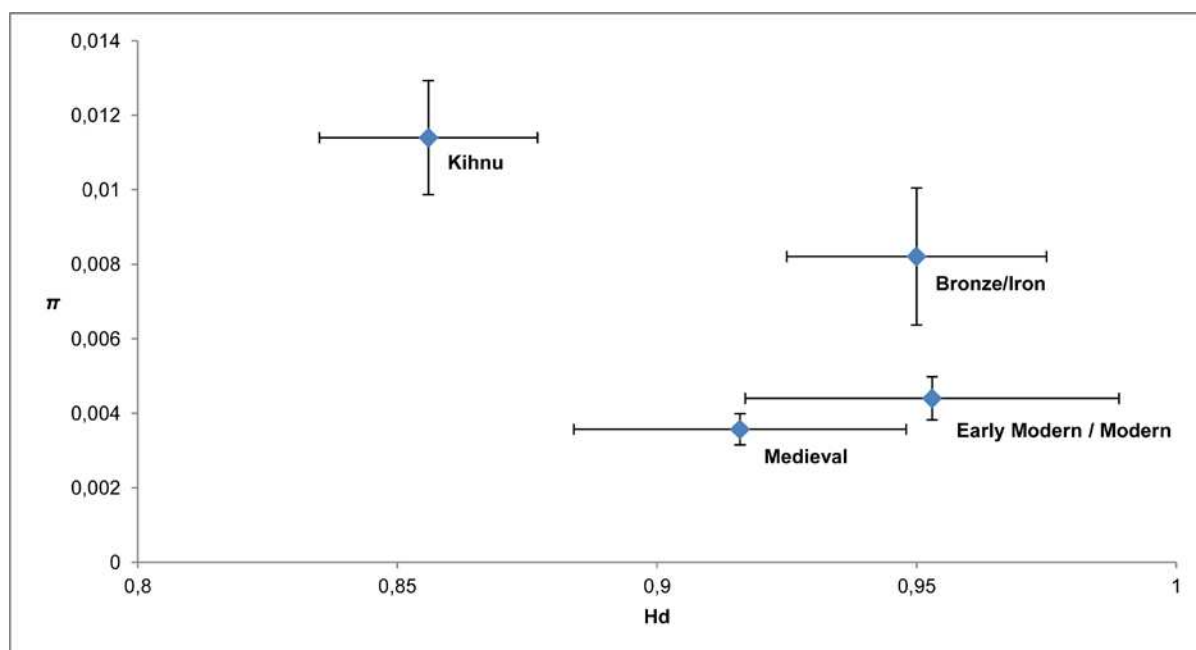
Lohkva micro-region (Fig 5b). However, since the sample sizes are small here, caution should be taken not to over interpret the results.



**Fig 5. Three-dimensional network of Estonian ancient sheep from three micro-regions through three time periods.** A three-dimensional statistical parsimony network was calculated using TempNet based on 559 bp mitochondrial D-loop haplotypes of Estonian sheep from three micro-regions: (a) Viljandi-Karksi, (b) Tartu-Lohkva and (c) Saaremaa Island dating to the Bronze/Iron Age (blue), Middle Ages (red), and Early Modern / Modern Period (green). The size of the given node is proportional to the number of samples represented in a haplotype, with the smallest node representing a single individual; number of samples greater than one is indicated in the parentheses. Branch length is not proportional to the mutational distance; mutational distances greater than 1 are indicated with black dots. Colourless nodes denote haplotypes absent within the time period.

We expected to observe significant fluctuations in the genetic diversity of ancient sheep mtDNA in Estonia from Bronze Age to Modern Period, as the animal husbandry would have followed the expansions and declines of livestock populations – for example due to human migrations, wars or ecological changes. In particular, our expectations were that medieval populations would display significantly higher mitochondrial diversity compared to other time cohorts, as suggested in [16]. The opposite pattern, however, was observed in our study. The medieval cohort displayed the lowest genetic diversity indices within the ancient cohorts, whereas the highest haplotype diversity was observed within the Early Modern / Modern Period. Contemporary Kihnu sheep are characterized by the lowest haplotype diversity and the highest nucleotide diversity. Overall, the comparison of haplotype and nucleotide diversity indices revealed no significant differences among the temporal cohorts (Fig 6; Table 1). The general homogeneity among time periods may reflect the hardiness and continuity of sheep populations through time. Although famines, plagues and murrains were

often devastating to the populations of both human [67–68] and livestock, it has been argued that the recovery of livestock after these events was rather rapid [69]. Primitive and hardy sheep are usually more likely to survive harsh climatic conditions, including cold winters with only dry leaves for feed [70]. However, this pattern in genetic diversity among temporal populations may also reflect the difficulty in identifying short-term events in zooarchaeological remains due to a lack of resolution in the archaeological stratigraphy.



**Fig 6. Scatterplot diagram for nucleotide ( $\pi$ ) and haplotype ( $Hd$ ) diversities of the Estonian ancient and modern Kihnu native sheep by time period.** Calculations are based on 559 bp mtDNA D-loop sequences from four temporal cohorts of Bronze/Iron Age ( $n = 28$ ), Middle Ages ( $n = 39$ ), Early Modern / Modern Period ( $n = 19$ ) and Kihnu sheep ( $n = 44$ ). Error bars indicate standard deviation (Table 1).

**Table 1. Statistics for four temporal cohorts of Estonian ancient and modern Kihnu native sheep by time period.**

Time cohort	$n$	$S$		$h$		$Hd$		$\pi \pm SD$	$D$
		(gaps not con.)	(gaps con.)	(gaps not con.)	(gaps con.)	with standard deviation SD (gaps not con.)	(gaps con.)		
Bronze/Iron Age	28	26	27	18	19	$0.950 \pm 0.025$	0,96032	$0.00821 \pm 0.00184$	-1.13699
Middle Ages	39	21	21	20	20	$0.916 \pm 0.032$	0,91633	$0.00357 \pm 0.00042$	-2.00057*

<b>Early Modern / Modern Period</b>	<b>19</b>	15	<b>16</b>	14	<b>16</b>	<b>0.953 ± 0.036</b>	0,97661	<b>0.00440 ± 0.00058</b>	<b>-1.59901</b>
<b>Kihnu</b>	<b>44</b>	21	<b>21</b>	8	<b>8</b>	<b>0.856 ± 0.021</b>	0,85624	<b>0.01140 ± 0.00153</b>	<b>1.03872</b>

The calculations are based on 559 bp mtDNA D-loop sequences. Results are presented according to whether gaps were not considered (*gaps not con.*) or considered as the fifth state (*gaps con.*) in the calculations. Number of samples ( $n$ ), number of variable sites ( $S$ ), number of haplotypes ( $h$ ), haplotype diversity ( $Hd$ ), nucleotide diversity with standard deviation ( $\pi \pm SD$ ), and Tajima's D ( $D$ ) are shown. Values used in the subsequent analyses and discussion are shown bold.

\* Statistical significance at  $p < 0.05$ .

## Population Expansion in the Middle Ages

Although the medieval period is not characterised by increasing genetic diversity indices, interestingly, Tajima's D values seem to suggest a population expansion during this period ( $D = -2.00057$ ,  $p < 0.05$ ; Table 1; [71]). To verify this result, we eliminated the possible bias caused by uneven length of the temporal cohorts, as Bronze/Iron Age (2225 years) yields samples from far longer period than the Middle Ages (325 years). We decreased the number of sequences only to the ones from the later centuries of the Late Iron Age (AD 900–1225), and also divided the medieval period into two – the earlier (AD 1225–1400) and the later (AD 1400–1550) part. We then compared the genetic diversities and Tajima's D between those groups. The results confirmed a potential population expansion in the first period of the Middle Ages ( $D = -2.12072$ ,  $p < 0.01$ ), but showed no significant changes between the last centuries of the Late Iron Age and the first half of the Middle Ages ( $\chi^2 = 13.371$ ,  $p = 0.4975$   $df = 14$ ) or between the earlier and later part of the Middle Ages ( $\chi^2 = 12.504$ ,  $p = 0.5659$ ,  $df = 14$ ).

These results correlate with the increasing utilisation of livestock seen in the medieval osteological data. Archaeological and zooarchaeological data suggest that as human population expanded, so did the demand for agricultural products, resulting in subsequent expansion of local livestock populations. The establishment of new power structures starting from the 13<sup>th</sup> century created a degree of social polarisation between the colonists and the colonised, arguably with a stronger impact on the local society than the crusades [72–73]. Nevertheless, as much of the local population – the farmers (and their livestock) – remained intact, the economic basis remained consistent, with new power centres dependent on indigenous population and their farming [72–73].

Livestock expansion could be associated with the expanding trade as well, but assessing the degree of livestock trade during the Middle Ages is complicated, largely due to a lack of documentary evidence. During the Middle Ages sheep products, wool and textiles particularly, were very important in western Europe [2]. However, in Livonia there are no traces or documentation of medieval wool trade, instead the textiles were imported from western Europe, and in the rural areas local wool in the textile manufacture prevailed all through the Middle Ages [25]. Furthermore, land-routes between the Hanseatic towns in western Europe and Livonia were not favoured for trade, instead sea-routes were more common [74] – by which an extensive sheep trade seems unlikely (and not supported by written documentation). The occasional import of breeding rams, however, could be speculated to have occurred also in medieval Livonia, as known from other countries, *e.g.* [9–10], but these individuals would likely have had a limited effect on the local gene pool [8,10], and certainly would not be visible in the maternal lineages. Although colonizers may have brought in new livestock, in Livonia the subsequent development of sheep populations was most probably autonomic, especially in the first centuries after the conquest (see also [2]).

Overall, the mitochondrial genetic results obtained in this study support the historic and archaeological evidence for population expansion of local sheep, rather than significant introduction of imported animals during the medieval period.

## **Modern Kihnu Native Sheep Compared to Ancient Populations**

Our results suggest that following the Middle Ages, the indigenous sheep population in Estonia continued to expand until they became part of the large-scale breeding process in the 19<sup>th</sup> and 20<sup>th</sup> century. Due to decrease in the use of local sheep and mixing them with modern imported breeds, local sheep were driven to almost extinction. This population decline was a gradual process which reached its low point during the last decades of the 20<sup>th</sup> century and is now reflected in the genetic diversity estimates of the Kihnu sheep analysed in this study. As revealed by the genetic differentiation estimates shown in Table 2 (and S4 Table), Kihnu sheep are significantly different than the ancient sheep cohorts, while no significant difference is observed amongst ancient cohorts. What might account for the genetic distinctiveness of the Kihnu sheep? First, the decrease in population size during the last (two) hundred years may be reflected in the relatively high value of Tajima's D in Kihnu

sheep ( $D = 1.03872$ ,  $p > 0.10$ , not significant), as one possible interpretation for this high value could be recent population contraction [71]. Secondly, Kihnu sheep samples in this study are all descended from a population from the Kihnu Island, recently acknowledged as a native breed that is currently distributed across Estonia. This relatively small founding population, however, could have caused genetic drift, which has resulted in somewhat lower haplotype diversity (0.856 in Kihnu sheep) compared to the Early Modern / Modern cohort (0.953). However, Kihnu sheep have considerably higher nucleotide diversity ( $\pi = 0.01140$ ) compared to the ancient cohorts. The latter coincides with the high number of novel haplotypes among Kihnu sheep and might reflect the accumulation of mutations during population development [54].

**Table 2. Genetic differentiation estimates between four temporal cohorts of Estonian ancient and modern Kihnu native sheep.**

	Bronze/Iron Age	Middle Ages	Early Modern / Modern Period
Bronze/Iron Age ( $n = 28$ )	-	-	-
Middle Ages ( $n = 39$ )	$\chi^2 = 35.843$ $p = 0.2929$ ( $df = 32$ )	-	-
Early Modern / Modern Period ( $n = 19$ )	$\chi^2 = 29.640$ $p = 0.4322$ ( $df = 29$ )	$\chi^2 = 30.121$ $p = 0.3087$ ( $df = 27$ )	-
Kihnu ( $n = 44$ )	$\chi^2 = 47.775$ $p = 0.0018^*$ ( $df = 23$ )	$\chi^2 = 58.148$ $p = 0.0002^{**}$ ( $df = 25$ )	$\chi^2 = 50.545$ $p = 0.0003^{**}$ ( $df = 21$ )

Calculations were made in DnaSP by Pearson's chi-squared test ( $\chi^2$ ). The statistical significance ( $p$ ) and the degrees of freedom ( $df$ ) are shown.

\* Statistical significance at  $0.001 < p < 0.01$ .

\*\* Statistical significance at  $p < 0.001$ .

We recognize a potential bias in our grouping of temporal cohort, as Kihnu sheep represent a contemporaneous 'living population', while the ancient samples are drawn from a group of individuals living over several centuries, and thus may have artificially inflated diversity values. Also, the time periods used in the analyses are not of the same duration: 2225 years for Bronze/Iron Age, 325 years for medieval and 450 years for Early Modern / Modern Period. To test the possible bias for the longest, prehistoric cohort, five of the oldest samples from the Bronze and Early Iron Age were left out and statistical significances for genetic differences were re-calculated only with sequences from the Late Iron Age ( $n = 22$ ;

period length 700 years). Using this shortened time span, the Late Iron Age still displayed no significant differences with any of the populations except for the Kihnu sheep ( $\chi^2 = 43.851$   $p = 0.0024$ ,  $df = 21$ ).

Although we recognize that mtDNA genetic diversity indices are only one part of the genetic puzzle, this study provides the basis for future analyses based on Y-chromosome, or nuclear SNPs to assess the extent of genetic diversity loss within this valuable native breed.

## Conclusions

In this study, we sought to extract mtDNA from archaeological sheep remains and establish a baseline for genetic diversity in ancient sheep in Estonia. Our results point to excellent mtDNA preservation of osteological remains in various archaeological sites in Estonia, within mitochondrial lineages falling into the haplogroups (A and B) observed in other parts of north-eastern Europe.

Based on previous ancient DNA and osteological data, we expected to observe temporal fluctuation in sheep genetic diversity, particularly within the medieval period. Instead, we identified relative stability in genetic diversity and continuity in the ancient matriline from Middle Bronze Age to modern sheep, with no obvious interruptions, introgressions or imports from outside regions and in spite of the introduction of new breeds from the 18<sup>th</sup> century onwards. Therefore, it seems that sheep husbandry in the Estonian area has been persistent and relatively autochthonous. Even if human-mediated animal movements did occur, these did not affect the majority of the sheep population, as demonstrated by a lack of significant genetic differentiation between the ancient populations. Nevertheless, some changes in maternal genetic diversity were observed in the first half of the Middle Ages when sheep populations seem to have expanded due to the growing human populations and their need for subsistence. Further investigation is needed to determine the extent to which changes in the animals' physical size is related to this population expansion or to other environmental and climatic changes. We also explored three micro-regions to examine sheep husbandry in detail, supporting the changes seen in the 13<sup>th</sup> century, as predominantly new haplotypes were observed in medieval Viljandi-Karksi, Tartu-Lohkva and Saaremaa Island compared to the Late Iron Age. Due to relatively small sample sizes, questions of continuity at a micro-scale remain open.

A significant change was recorded in the modern Kihnu native sheep, which had gone through a population decrease during the last centuries, resulting in lower haplotype diversity and high rate of novel haplotypes compared to the ancient populations. Nevertheless, our results suggest a clear connection between the earliest studied sheep and Kihnu native breed, and induce further research on their history and position among the northern European native sheep breeds.

As studies of short mitochondrial DNA fragments have limitations, phenotypic and Y-chromosome DNA needs to be studied, especially by improved aDNA extraction and high-throughput sequencing. Further genetic, archaeological, and historical studies of modern and ancient sheep in Estonia and neighbouring regions would expand the understanding of both the early stages of the first domesticates in the region as well as the subsequent development of the populations.

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**S1 Table. Sample data.** Ancient sheep samples used in this study, with relevant archaeological and genetic data. Samples are ordered by time period.

SAMPLE INFORMATION				DATING			SEQUENCING				
Sample ID	Archaeological site, year of excavation, collection code <sup>1</sup>	Region (micro-region <sup>2</sup> )	Skeletal element <sup>3</sup>	Dating by archaeological context	Dating by AMS <sup>14</sup> C	Time period <sup>4</sup>	No of extractions	Amplification success	Sequence length (bp)	Haplo-group	GenBank accession number <sup>5</sup>
88OaSara1	Sarakenos cave, Greece (no code given)	Greece	atlas	c. 6800 BC [1]	-	Initial Neolithic	1	-	-	-	-
123OaSara2	Sarakenos cave, Greece (no code given)	Greece	atlas	c. 6800 BC [1]	-	Initial Neolithic	1	+	558	B	KU670303
33OaJoe1	Jöelähtme stone-cist grave 1983 (AI 5306)	Estonia	humerus	1200–800 BC [2]	-	Middle Bronze Age	2**	-	-	-	-
1OaRid1	Ridala settlement site 1961 (AI 4261)	Estonia	humerus	800–600 BC [3]	-	Late Bronze Age	2*	-	213 [4]	B [4]	KP052792 [4]
13OaRid2	Ridala settlement site 1961 (AI 4261)	Estonia (c)	metatarsus	800–600 BC [3]	-	Late Bronze Age	2(*)	+	558	B	KP052801
18OaAsva1	Asva settlement site 1965 (AI 4366)	Estonia (c)	metacarpus	800–600 BC [3]	786–522 BC (95.4%) (2505 ± 30 BP) Lab no: Poz-58805 [4]	Late Bronze Age	2(*)	+	558	B	KP052805
104OaAsva2	Asva settlement site 2013 (AI 7065)	Estonia	mandible	800–600 BC [3]	-	Late Bronze Age	1	-	-	-	-
105OaAsva3	Asva settlement site 2013 (AI 7065)	Estonia (c)	mandible	800–600 BC [3]	-	Late Bronze Age	1	+	558	A	KU670286
35OaKaa1	Kaali settlement site 1977 (AI 5043)	Estonia	proximal phalanx	700–500 BC [3,5–6]	-	Late Bronze Age / Early Iron Age	2**	±	418	B	KU670236
48OaKaa3	Kaali settlement site 1977 (AI 5043)	Estonia	proximal phalanx	700–500 BC [3,5–6]	-	Late Bronze Age / Early Iron Age	1	±	418	B	KU670243
36OaVao1	Väo Jaani stone-cist grave 1982 (AI 5220)	Estonia	metatarsus	500–250 BC [3,7]	-	Early Iron Age	2**	-	-	-	-
55OaVao4	Väo Jaani stone-cist grave 1982 (AI 5220)	Estonia	metatarsus	500–250 BC [3,7]	-	Early Iron Age	1	-	-	-	-
57OaTou1	Tõugu II <i>tarand</i> grave 1994 (AI 6003)	Estonia	humerus	250 BC – AD 50 [3]	362–169 BC (95.4%) (2182 ± 31 BP) Lab no: SUERC-55405	Early Iron Age	1	+	558	B	KU670250
61OaAlt1	Alt-Laari settlement site 2008 (TÜ 1695)	Estonia	mandible	AD 50–550 [8]	-	Early Iron Age	1	-	-	-	-
74OaTou2	Tõugu II <i>tarand</i> grave 1995 (AI 6003)	Estonia	radius	500 BC – AD 50 [3]	-	Early Iron Age	1	-	-	-	-
92OaSpie1	Spietiņi settlement site 1963 (VI 65)	Latvia	humerus	AD 0–300 [9]	-	Early Iron Age	1	±	418	B	KU670276
93OaKiv1	Kiviti settlement site 1958 (VI 37)	Latvia	calcaneus	AD 0–600 [10]	AD 125–258 (90.8%) AD 285–287 (0.2%) AD 296–322 (4.3%) (1817 ± 29 BP) Lab no: SUERC-55406	Early Iron Age	1	+	558	A	KU670277
97OaTou3	Tõugu II <i>tarand</i> grave 1993 (AI 6003)	Estonia	proximal phalanx	End of Pre-Roman Iron Age [3]	-	Early Iron Age	1	-	-	-	-
107OaMuuk1	Muuksi stone-cist grave 1976 (AI 4980)	Estonia	proximal phalanx	500 BC – AD 550 [3]	AD 180–185 (0.4%) AD 214–386 (95.0%) (1754 ± 31 BP) Lab no: SUERC-55411	Early Iron Age	1	+	558	B	KU670288
23OaRou1	Rõuge settlement site 1959 (AI 4100)	Estonia	metatarsus	AD 500–1000 [11]	AD 666–821 (93.2%) AD 842–860 (2.2%) (1265 ± 30 BP) Lab no: Poz-58804 [4]	Late Iron Age	2(*)	+	558	B	KP052809
25OaSal1	Salme boat-grave 2008 (SM 10601)	Estonia (c)	humerus	AD 700 [12]	-	Late Iron Age	2(*)	+	558	B	KU670233
50aPada1	Pada settlement site 1981 (AI 5200)	Estonia	metatarsus	AD 700–1100 [13]	-	Late Iron Age	2(*)	+	558	A	KP052794
70aSoon1	Soontagana hillfort 1968 (PäMu 2/A 2434)	Estonia	metatarsus	AD 900–1200 [14]	-	Late Iron Age	2(*)	+	558	B	KP052796
31OaKivi2	Viljandi settlement site 1999 (VM 10742)	Estonia (a)	radius	AD 1000–1225 [15]	-	Late Iron Age	2(*)	+	558	B	KP052815
30OaKea1	Keava hillfort 2001 (TÜ 1026;419)	Estonia	metatarsus	AD 1000–1300 [16]	-	Late Iron Age	2(*)	+	558	B	KP052814
16OaLoh1	Lõhavere hillfort 1940 (AI 3578;1824)	Estonia	metatarsus	AD 1200–1300 [14]	-	Late Iron Age	2(*)	+	558	B	KP052804
44OaIru1	Iru hillfort 1985 (AI 5302)	Estonia	humerus	Late Iron Age [17]	-	Late Iron Age	1	+	558	B	KU670239
45OaOte2	Otepää settlement site 1989 (AI 5907)	Estonia	humerus	AD 900–1400 [18]	AD 1184–1275 (95.4%) (800 ± 30 BP) Lab no: Poz-61915	Late Iron Age <sup>5</sup>	1	+	558	B	KU670240

49OaIru2	Iru hillfort 1986 (AI 5302)	Estonia	metatarsus	Late Iron Age [17]	-	Late Iron Age	1	+	559	B	KU670244
52OaSuu2	Viljandi settlement site 1999 (VM 10741)	Estonia (a)	tibia	AD 1150–1225 [15]	-	Late Iron Age	1	+	558	B	KU670247
53OaIru3	Iru hillfort 1986 (AI 5302)	Estonia	proximal phalanx	Late Iron Age [17]	-	Late Iron Age	1	+	558	B	KU670248
56OaJoe4	Jöelähtme stone-cist grave 1983 (AI 5306)	Estonia	humerus	1200–800 BC [2]	AD 691–749 (24.7%) AD 762–885 (70.7%) (1224 ± 28 BP) Lab no: SUERC-55404	Late Iron Age	1	+	558	B	KU670249
58OaTor1	Tornimäe settlement site 2004 (AI 6688)	Estonia (c)	metatarsus	AD 800–1050 [19]	-	Late Iron Age	1	+	558	B	KU670251
59OaLin1	Linnaaluste III settlement site 2002 (TÜ 1115:123)	Estonia	metatarsus	AD 700–1100 [20]	-	Late Iron Age	1	+	558	B	KU670252
60OaAak1	Aakre Kivivare hillfort 2011 (TÜ 1928)	Estonia	mandible	AD 550–1050 [21]	-	Late Iron Age	1	-	-	-	-
62OaPaa2	Pada I hillfort 1983 (AI 5249)	Estonia	radius	AD 1000–1200 [22]	-	Late Iron Age	1	+	558	B	KU670253
63OaVar2	Varbola Jaanilinn hillfort 1977 (AI 4783)	Estonia	talus	AD 1100–1300 [23]	-	Late Iron Age	1	-	-	-	-
64OaPoi1	Pöide hillfort 1993 (SM 1460)	Estonia (c)	humerus	AD 700–900 / 1100–1300 [24]	-	Late Iron Age	1	+	558	A	KU670254
66OaUug1	Uugla settlement site 2006 (AM A 1026)	Estonia	medial phalanx	AD 1000–1300 [25]	-	Late Iron Age	1	-	-	-	-
71OaKurev1	Kurevere stone grave, year unknown (AI 1394)	Estonia	humerus	Iron Age [3,26]	-	Iron Age	1	-	-	-	-
75OaAak2	Aakre Kivivare hillfort 2011 (TÜ 1928)	Estonia	tooth	AD 800–1050 [21]	-	Late Iron Age	1	±	509	A	KU670260
90OaTer1	Tervete hillfort 1958 (VI 24e)	Latvia	metacarpus	AD 1000–1200 [27]	-	Late Iron Age	1	+	558	A	KU670274
91OaTart2	Tartu town 2011 (TM A 194)	Estonia (b)	mandible	Iron Age [28]	-	Iron Age	1	+	558	B	KU670275
94OaMez1	Mežmalas hillfort 1963 (VI 80)	Latvia	humerus	AD 600–900 [29]	-	Late Iron Age	1	-	-	-	-
106OaIlm1	Ilmandu III <i>tarand</i> grave 1994 (AI 6009)	Estonia	humerus	500 BC – AD 550 [3]	AD 1039–1110 (46.8%) AD 1115–1207 (46.8%) (902 ± 26 BP) Lab no: SUERC-55410	Late Iron Age	1	+	558	B	KU670287
108OaVob1	Vöbutõ settlement site 2013 (no code given)	Russia	humerus	AD 1100–1200 [30]	-	Late Iron Age	1	-	-	-	-
109OaMusu1	Viljandi settlement site 2004 (VM 10952)	Estonia (a)	humerus	Viking Age [31]	-	Late Iron Age	1	+	558	B	KU670289
118OaMusu2	Viljandi settlement site 2004 (VM 10952)	Estonia (a)	humerus	Iron Age [31]	AD 1033–1191 (93.7%) AD 1198–1205 (1.7%) (910 ± 31 BP) Lab no: SUERC-55412	Late Iron Age	1	+	558	B	KU670298
119OaRus1	Staraya Ladoga 2010 (no code given)	Russia	metacarpus	AD 870–880 [32]	-	Late Iron Age	1	+	558	B	KU670299
120OaRus2	Staraya Ladoga 2011 (no code given)	Russia	metacarpus	AD 1000–1300 [32]	-	Late Iron Age	1	+	558	B	KU670300
121OaRus3	Staraya Ladoga 2011 (no code given)	Russia	metatarsus	AD 800–1100 [32]	-	Late Iron Age	1	+	558	B	KU670301
133OaKurev2	Kurevere stone grave, year unknown (AI 1394)	Estonia	talus	Iron Age [3,26]	-	Iron Age	2**	-	-	-	-
139OaIlm2	Ilmandu III <i>tarand</i> grave 1994 (AI 6009)	Estonia	proximal phalanx	Iron Age [3]	-	Iron Age	1	-	-	-	-
140OaJak3	Tartu town 2011 (TM A 188)	Estonia (b)	metatarsus	AD 1000–1100 [33]	-	Late Iron Age	1	+	558	B	KU670316
65OaPaa1	Paatsa hillfort 1963 (AI 4337)	Estonia (c)	radius	Late Iron Age [34]	-	Late Iron Age	1	+	558	B	KU670255
51OaVar1	Varbola Jaanilinn hillfort 1941 (no code given)	Estonia	humerus	Late Iron Age / Middle Ages [14]	-	Late Iron Age / Middle Ages	1	+	558	B	KU670246
67OaOte3	Otepää hillfort 1961 (AI 4036)	Estonia	metatarsus	AD 1000–1400 [35]	-	Late Iron Age / Middle Ages	1	+	558	B	KU670256
4OaJaan1	Viljandi town 1990 (VM 10258)	Estonia (a)	metatarsus	AD 1250–1300 [36]	-	Middle Ages	2(*)	+	558	B	KP052793
6OaPost1	Viljandi town 2001 (VM 10872)	Estonia (a)	metatarsus	AD 1400–1550 [37]	-	Middle Ages	2(*)	+	558	B	KP052795
9OaSpo1	Viljandi town 1999 (VM 11090)	Estonia (a)	metacarpus	AD 1300–1550 [38]	-	Middle Ages	2(*)	+	558	B	KU670230
10OaHuv1	Viljandi town 1991 (no code given)	Estonia (a)	metacarpus	AD 1225–1350 [39]	-	Middle Ages	2(*)	+	558	B	KP052798
11OaLoss1	Viljandi town 2001 (VM 10848)	Estonia (a)	metatarsus	c. AD 1500 [40]	-	Middle Ages	2*	+	558	B	KP052799
12OaOrdu2	Viljandi castle 2003 (VM 10922)	Estonia (a)	calcaneus	AD 1250–1350 [41]	-	Middle Ages	2(*)	+	558	B	KP052800
14OaJaan2	Viljandi town 1990 (VM 10258)	Estonia (a)	metacarpus	AD 1250–1300 [36]	-	Middle Ages	2*	+	558	B	KP052802

150aSpo2	Viljandi town 1999 (VM 11090)	Estonia (a)	metatarsus	AD 1300–1550 [38]	-	Middle Ages	2(*)	+	558	B	KP052803
170aOte1	Otepää hillfort 1962 (AI 4036)	Estonia	radius	Middle Ages [42]	-	Middle Ages	2(*)	+	558	B	KU670231
190aTart1	Tartu town 2011 (TM A 194)	Estonia (b)	humerus	Middle Ages [28]	-	Middle Ages	2(*)	+	558	B	KP052806
200aTal1	Tallinn town 2008 (AI 6917)	Estonia	metatarsus	AD 1250–1400 [43]	-	Middle Ages	2(*)	+	558	B	KU670232
210aPar1	Pärnu town 1992 (PäMu 14640/A 2509)	Estonia	cranium	AD 1400–1550 [44]	-	Middle Ages	2(*)	+	558	B	KP052807
220aPar2	Pärnu town 1992 (PäMu 14640/A 2509)	Estonia	cranium	AD 1400–1550 [44]	-	Middle Ages	2*	-	213 [4]	A [4]	KP052808 [4]
270aLut1	Tartu town 2009 (TM A 178)	Estonia (b)	metatarsus	AD 1250–1400 [45]	-	Middle Ages	2(*)	+	558	B	KP052811
280aJak1	Tartu town 2010 (TM A 188)	Estonia (b)	radius	AD 1300–1500 [46]	-	Middle Ages	2(*)	+	558	B	KP052812
290aJak2	Tartu town 2011 (TM A 188)	Estonia (b)	metacarpus	AD 1250–1500 [33]	-	Middle Ages	2(*)	+	558	B	KP052813
380aKir1	Kirumpää castle 2005 (TÜ 1433)	Estonia	tibia	AD 1250–1400 [47]	-	Middle Ages	2**	-	-	-	-
430aLih1	Lihula town 2012 (AM A 1121)	Estonia	radius	AD 1250–1400 [48]	-	Middle Ages	1	+	558	B	KU670238
500aKir2	Kirumpää settlement site 2005 (TÜ 1434)	Estonia	radius	AD 1250–1700 [47]	AD 1294–1406 (95.4%) (610 ± 35 BP) Lab no: Poz-61910	Middle Ages	1	+	558	B	KU670245
680aLohk1	Lohkva settlement site 2012 (TÜ 2004)	Estonia	metatarsus	Middle Ages [49]	-	Middle Ages	1	±	418	B	KU670257
690aHaa1	Haapsalu town 2002 (HM 8914)	Estonia	metacarpus	Middle Ages [50]	-	Middle Ages	1	+	558	B	KU670258
720aLih2	Lihula town 2012 (AM A 1121)	Estonia	mandible	Middle Ages [48]	-	Middle Ages	1	-	-	-	-
730aKir4	Kirumpää settlement site 2005 (TÜ 1434)	Estonia	mandible	AD 1250–1700 [47]	-	Middle Ages	1	-	-	-	-
760aKar1	Karksi castle 2012 (TÜ 1929)	Estonia (a)	humerus	AD 1250–1300 [51]	-	Middle Ages	1	+	558	B	KU670261
770aVilMu1	Viljandi town 1995 (VM 10942)	Estonia (a)	metatarsus	AD 1250 – beginning of 1300s [52]	-	Middle Ages	1	+	558	B	KU670262
780aKures1	Kuressaare castle 2012 (SM 10663)	Estonia (c)	humerus	Middle of AD 1300s [53]	-	Middle Ages	1	+	558	B	KU670263
790aEka1	Tallinn town 2012 (AI 7032:B55)	Estonia	metatarsus	AD 1300–1500 [54]	-	Middle Ages	1	+	558	B	KU670264
800aKak1	Käku smithy site 2012 (AI 6845)	Estonia (c)	metacarpus	Middle of AD 1400s [55]	-	Middle Ages	1	+	558	B	KU670265
820aSarg1	Sargvere settlement site 2007 (TÜ 1574)	Estonia	radius	AD 1530–1550 [56]	-	Middle Ages	1	+	558	B	KU670267
830aPadi1	Padise monastery 2010–2011 (no code given)	Estonia	radius	AD 1300–1500 [57]	-	Middle Ages	1	+	558	B	KU670268
860aSau1	Tallinn town 1998 (AI 6332)	Estonia	metatarsus	Middle Ages [58]	-	Middle Ages	1	+	558	B	KU670271
870aNar1	Narva town 2005 (NLM 2420)	Estonia	radius	End of AD 1200s – beginning of 1600s [59]	-	Middle Ages	1	+	558	B	KU670272
950aVec1	Vecdole castle 1967 (VI 123)	Latvia	metatarsus	AD 1250–1350 [27]	-	Middle Ages	1	+	558	B	KU670278
960aPih1	Pskov town 2013 (no code given)	Russia	tibia	AD 1300–1500 [60]	-	Middle Ages	1	+	558	A	KU670279
980aEka2	Tallinn town 2012 (AI 7032:B-63)	Estonia	humerus	AD 1400–1450 [54]	-	Middle Ages	1	±	469	A	KU670280
1020aHar1	Tallinn town 1989 (AI 6176)	Estonia	cranium	Middle Ages [61]	-	Middle Ages	1	+	558	B	KU670284
1030aRoos1	Tallinn town 1996 (AI 6109)	Estonia	cranium	AD 1300–1500 [62]	-	Middle Ages	1	+	558	B	KU670285
1100aBot1	Tartu town 1989 (TM 2032)	Estonia (b)	metatarsus	End of AD 1200s – beginning of 1300s [63]	-	Middle Ages	1	+	558	B	KU670290
1110aLatte1	Tartu town 1987 (TM A 108)	Estonia (b)	humerus	AD 1300–1400 [64]	-	Middle Ages	1	+	558	B	KU670291
1120aLatte2	Tartu town 1987 (TM A 108)	Estonia (b)	metatarsus	AD 1400–1500 [64]	-	Middle Ages	1	+	558	B	KU670292
1130aJaana3	Viljandi town 1991 (VM 10324)	Estonia (a)	metacarpus	AD 1500–1550 [36]	-	Middle Ages	1	+	558	B	KU670293
1160aKraK1	Kraków town 2006 (1822/06)	Poland	radius	AD 1250–1350 [65]	-	Middle Ages	1	+	558	B	KU670296
1170aKraK2	Kraków town 2005 (138/05)	Poland	mandible	AD 1000–1200 [65]	-	Middle Ages	1	±	418	B	KU670297
1220aPih2	Pskov town 2013 (no code given)	Russia	humerus	AD 1400–1500 [60]	-	Middle Ages	1	+	558	B	KU670302

125OaPar3	Pärnu town 1992 (PäMu 14640/A 2509)	Estonia	cranium	Middle Ages [44]	-	Middle Ages	1	±	509	B	KU670305
126OaPar4	Pärnu town 2002 (PäMu A 2570)	Estonia	humerus	AD 1300–1600 [66]	-	Middle Ages	1	±	509	A	KU670306
130OaEka3	Tallinn town 2012 (AI 7032:B-60)	Estonia	metacarpus	AD 1500–1550 [54]	-	Middle Ages	1	+	558	B	KU670310
131OaKura1	Tartu town 1999 (TM A 191)	Estonia (b)	metatarsus	Middle Ages [67]	-	Middle Ages	1	+	558	B	KU670311
132OaSau2	Tallinn town 1998 (AI 6332)	Estonia	metatarsus	Middle Ages [58]	-	Middle Ages	1	+	558	B	KU670312
128OaPih3	Pskov town 2013 (no code given)	Russia	metatarsus	End of AD 1300s – beginning of 1400s [60]	-	Middle Ages	1	+	558	B	KU670308
136OaKar4	Karksi castle 2011 (TÜ 1929)	Estonia (a)	metatarsus	AD 1300–1400 [68]	-	Middle Ages	1	+	558	B	KU670313
137OaKar5	Karksi castle 2012 (TÜ 1929)	Estonia (a)	radius	AD 1200–1300 [51]	-	Middle Ages	1	+	558	B	KU670314
138OaKra3	Kraków town 2005 (105/05)	Poland	metacarpus	AD 1250–1300 [65]	-	Middle Ages	1	+	558	B	KU670315
143OaKar7	Karksi castle 2011 (TÜ 1929)	Estonia (a)	metatarsus	AD 1400–1500 [68]	-	Middle Ages	1	+	558	B	KU670319
80aVas1	Vastseliina castle 2005 (TÜ 1435)	Estonia	metatarsus	AD 1500–1700 [69]	-	Early Modern Period	2(*)	+	558	B	KP052797
81OaPol1	Põltsamaa castle 1998 (TÜ 714)	Estonia	humerus	End of AD 1500s [70]	-	Early Modern Period	1	+	558	B	KU670266
84OaKil1	Kiltsi manor 2008 (RM 6782/A 160)	Estonia	radius	End of AD 1500s – beginning of 1600s [71]	-	Early Modern Period	1	+	558	B	KU670269
85OaPai1	Paide castle 2011 (TÜ 1924)	Estonia	humerus	AD 1550 – beginning of 1600s [72]	-	Early Modern Period	1	+	558	B	KU670270
89OaAlu1	Alūksne castle 1982 (VI 231)	Latvia	metatarsus	AD 1500–1700 [27]	-	Early Modern Period	1	+	558	B	KU670273
99OaLohk2	Lohkva settlement site 2012 (TÜ 2004)	Estonia (b)	metacarpus	Beginning of Early Modern Period [49]	-	Early Modern Period	1	+	558	B	KU670281
100OaPadi2	Padise monastery 2010–2011 (no code given)	Estonia	humerus	Time of the Livonian War (1558–1583) [57]	-	Early Modern Period	1	+	558	B	KU670282
101OaPadi3	Padise monastery 2010–2011 (no code given)	Estonia	cranium	AD 1600s [57]	-	Early Modern Period	1	+	559	B	KU670283
114OaJaana4	Viljandi town 1991 (VM 10324)	Estonia (a)	metatarsus	AD 1550 – beginning of 1600s [36]	-	Early Modern Period	1	+	558	B	KU670294
115OaVilKv1	Viljandi town 1997 (VM 10589)	Estonia (a)	humerus	AD 1500–1900 [73]	-	Early Modern Period	1	+	558	B	KU670295
124OaOlu2	Olustvere settlement site 1982 (AI 4998)	Estonia	mandible	Early Modern Period [74]	-	Early Modern Period	1	+	558	B	KU670304
127OaPar5	Pärnu town 2002 (PäMu A 2570)	Estonia	metatarsus	AD 1650 – beginning of 1700s [66]	-	Early Modern Period	1	+	559	B	KU670307
129OaAlu2	Alūksne castle 1978 (VI 231)	Latvia	metacarpus	AD 1500–1700 [27]	-	Early Modern Period	1	+	558	B	KU670309
135OaKar3	Karksi castle 2011 (TÜ 1929)	Estonia	humerus	AD 1550 – beginning of 1600s [68]	-	Early Modern Period	1	-	-	-	-
141OaJak4	Tartu town 2011 (TM A 188)	Estonia (b)	humerus	AD 1600–1700 [33]	-	Early Modern Period	1	+	558	B	KU670317
142OaKar6	Karksi castle 2011 (TÜ 1929)	Estonia (a)	metatarsus	AD 1550 – beginning of 1600s [68]	-	Early Modern Period	1	+	558	B	KU670318
24OaOlu1	Olustvere settlement site 1982 (AI 4998)	Estonia	metatarsus	-	AD 1516–1595 (35.0%) AD 1617–1670 (49.9%) AD 1780–1799 (9.2%) AD 1945–1953 (1.3%) (265 ± 30 BP) Lab no: Poz-58806 [4]	Early Modern / Modern Period	2(*)	+	558	B	KP052810
32OaLoo1	Kihelkonna Loona settlement site 1956 (AI 4129)	Estonia (c)	metatarsus	-	AD 1650–1695 (20.2%) AD 1726–1814 (52.5%) AD 1838–1843 (0.5%) AD 1853–1868 (1.5%) AD 1917–... (20.8%) (185 ± 30 BP) Lab no: Poz-61907	Early Modern / Modern Period	2**	+	558	B	KU670234

34OaPro1	Proosa field remains 2009 (TÜ 1770)	Estonia	mandible	1000–500 BC [75]	AD 1644–1700 (21.9%) AD 1720–1819 (47.9%) AD 1832–1880 (6.8%) AD 1915–... (18.9%) (190 ± 40 BP) Lab no: Poz-61908	Early Modern / Modern Period	1	+	558	B	KU670235
37OaSam1	Sammaste stone grave 1989 (VM 10234)	Estonia	humerus	Iron Age [76]	AD 1681–1738 (27.3%) AD 1757–1762 (0.7%) AD 1803–1937 (67.4%) (105 ± 30 BP) Lab no: Poz-61909	Early Modern / Modern Period	1	+	558	B	KU670237
46OaJoe3	Jõelähtme stone-cist grave 1983 (AI 5306)	Estonia	humerus	1200–800 BC [2]	AD 1650–1695 (20.2%) AD 1726–1814 (52.5%) AD 1838–1843 (0.5%) AD 1853–1868 (1.5%) AD 1917–... (20.8%) (185 ± 30 BP) Lab no: Poz-61913	Early Modern / Modern Period	1	+	558	B	KU670241
47OaVao3	Väo Jaani stone-cist grave 1982 (AI 5220)	Estonia	metatarsus	Early Pre-Roman Iron Age [3,7]	AD 1682–1736 (27.1%) AD 1805–1935 (68.3%) (100 ± 30 BP) Lab no: Poz-61914	Early Modern / Modern Period	1	+	558	B	KU670242
70OaToo1	Toodsi Liidva settlement site 2010 (TÜ 1868)	Estonia	metatarsus	-	AD 1957–1963 (21.0%) AD 1974–1985 (74.4%) (130.72 ± 0.55 pMC) Lab no: Poz-61912	Modern Period	2**	±	469	A	KU670259

<sup>1</sup> **Samples were provided by the following institutions:**

Estonia: University of Tartu (TÜ), Tallinn University (AI), Estonian History Museum (AM), Museum of Viljandi (VM), Tartu City Museum (TM), Saaremaa Museum (SM), Pärnu Museum (PäMu), Virumaa Museum (RM), Narva Museum (NLM), and Läänemaa Museum (HM).

All necessary permits were obtained for the described study, which complied with all relevant regulations:

sampling protocols no 4–13, 15–24, 26–50, 52–58 for the samples held in the collections of TÜ (including samples from AI, VM, TM, PäMu and HM);

sampling protocols no 175–232 for samples held in AI (including samples from AM, SM and PäMu).

No permits were required for the samples held in TM, NLM and RM.

Latvia: Latvian National Museum of History (no permits were required for the described study).

Russia: Archaeological Center of Pskov Region, Zoological Institute of Russian Academy of Sciences (no permits were required for the described study).

Poland: Polish Academy of Sciences (no permits were required for the described study).

Greece: Aegean University Rhodes (no permits were required for the described study).

<sup>2</sup> **Micro-regions yielding sheep remains over a long chronological span were preferentially targeted to identify temporal changes within a site or region, including:**

(a) Viljandi-Karksi – Viljandi town and its surroundings with Late Iron Age, medieval and early modern deposits, to which the nearby Karksi castle was added, with its exceptionally good stratigraphy from the 13th to 17th century;

(b) Tartu-Lohkva – Tartu town with adjacent Lohkva settlement site with samples from the Late Iron Age to the 17th century;

(c) Saaremaa Island as a geographically separated region with samples dating from the Late Bronze Age up to the 19th century.

<sup>3</sup> **Most samples were bones, while one was of a tooth:**

humeri (n = 31) and metapodii (n = 56) were preferred, but crania (n = 6), mandibles (n = 10), atlases (n = 2), radii (n = 14), tibiae (n = 3), calcanea (n = 2), tali (n = 2), and phalanges (n = 7) were also chosen as these could be distinguished from goats with more confidence.

Taxonomical identification was assigned with the help of the anatomical reference collection of the Department of Archaeology in the University of Tartu, and using the bone atlas by [77].

<sup>4</sup> The Early Modern Period in the current study corresponds to the Post-Medieval period in [4].

<sup>5</sup> **GenBank accession numbers:**

KP052793–KP052807 and KP052809–KP052815 are for the updated 22 sequences reported first in [4]. Two sequences – 1OaRid1 and 22OaPar2 – are reported in [4], but were unsuccessful with rest of the primer pairs used in this study.

KU670230–KU670319 are for the 90 sequences newly reported in this study.

<sup>5</sup> Based on <sup>14</sup>C dating the sample 45OaOte2 falls in between the Late Iron Age and Middle Ages, but is analysed in the Bronze/Iron Age cohort.

\* Samples extracted twice: at the Natural Resources Institute Finland, Finland and the University of Tartu, Estonia.

(\*) Samples extracted twice: at the Natural Resources Institute Finland, Finland and the University of Tartu, Estonia, but amplified with different primer pairs.

\*\* Samples extracted twice at the University of Tartu, Estonia.

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**S2 Table. List of primers.** List of primers used in this study, with fragment length, nucleotide position of initiation of amplification according to GenBank accession NC001941 [1], and success rate of each primer.

	Primer pair	Fragment length	Start position [NC001941]	Success rate	References
Ancient	Fragment 1 For 5'GTTTCACTGAAGCATGTAGGG3' Rev 5'CATGGTGAACAAGCTCGTGA3'	116 bp	15957	88%	[2]
	Fragment 2 For 5'TCAACATGCGTATCCTGTCC3' Rev 5'ATGGCCCTGAAGAAAGAACC3'	164 bp	16027	84%	[2]
	Fragment 3 For 5'CCCATTAAGTGGGGTAA3' Rev 5'AATACCAAATGCATGACACCA3'	172 bp	16124	91%	[2]
	Fragment 4 For 5'TCAGCCCATGCCTAACATAA3' Rev 5'TGAGGATGCTCAAGATGCAG3'	143 bp	16252	93%	[2]
	Fragment 5 For 5'CGGAGCATGAATTGTAGCTG3' Rev 5'GTATTGAGGGCGGATAAAT3'	208 bp	16348	88%	[2]
Modern	LA1 For 5'CAACCTCCTAAAATGAAGACAAG Rev 5'GTATAAGTCTATTGAAAGTTAACAGGA	1451 bp	15280	100%	This study

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**S3 Table. Success rate of ancient samples.** The success rate of all ancient samples analysed in the current study ( $n = 134$ ) by (a) region, (b) time period, and (c) conjoined cohorts of region and time period. To examine the possible effect of soil and bedrock conditions on aDNA preservation, comparison was made for the three ancient time periods and geographical regions of northern, western, and southern Estonia, plus the samples from outside Estonia. No significant differences in the preservation of DNA were detected in temporal or spatial cohorts.

	No of samples	Successful with all primer pairs	Success rate	No of partial samples	Total success rate (with all primer pairs + partial)	Unsuccessful samples
<b>a) Region</b>						
Northern Estonia	37	29	78%	1	81%	7
Southern Estonia	57	46	81%	5	90%	6
Western Estonia	21	13	62%	2	71%	6
Latvia, Russia, Poland, Greece	19	14	74%	2	84%	3
<b>Total:</b>	<b>134</b>	<b>102</b>	<b>76%</b>	<b>10</b>	<b>84%</b>	<b>22</b>
<b>b) Time period</b>						
Neolithic / Bronze / Iron Age	55	34	62%	4	69%	17
Iron Age / Middle Ages <sup>1</sup>	2	2	100%	-	100%	-
Middle Ages	54	45	83%	5	93%	4
Early Modern / Modern Period	23	21	91%	1	96%	1
<b>Total:</b>	<b>134</b>	<b>102</b>	<b>76%</b>	<b>10</b>	<b>84%</b>	<b>22</b>

<sup>1</sup>Not applicable for temporal analysis.

<b>c) Success rate (all primer pairs) for every region by time period <sup>2</sup></b>			
	Neolithic / Bronze / Iron Age	Middle Ages	Early Modern / Modern Period
Northern Estonia ( $n = 29$ )	61%	91%	100%
Southern Estonia ( $n = 46$ )	75%	81%	85%
Western Estonia ( $n = 13$ )	53%	80%	100%
Latvia, Russia, Poland, Greece ( $n = 14$ )	60%	86%	100%

<sup>2</sup>At  $p < 0.05$  the differences between preservation of different time and geographical cohorts are not significant ( $\chi^2 = 6.0353, p = 0.419244$ ).

**S4 Table. Population pairwise  $F_{ST}$  of four temporal cohorts of Estonian ancient and modern Kihnu native sheep.** Assessment of genetic differentiation of the Estonian ancient ( $n = 86$ ) and modern ( $n = 44$ ) sheep cohorts using pairwise  $F_{ST}$  values shows significant genetic differentiation between Kihnu and medieval, and Kihnu and early modern / modern sheep, as well as between the Bronze/Iron Age and medieval sheep populations. This supports the population expansion discussed in the main text, and the distinctiveness of Kihnu sheep compared to the ancient populations.

	<b>Bronze/Iron Age</b>	<b>Middle Ages</b>	<b>Early Modern / Modern Period</b>	<b>Kihnu</b>
<b>Bronze/Iron Age (<math>n = 28</math>)</b>	0	-	-	-
<b>Middle Ages (<math>n = 39</math>)</b>	0.03080*	0	-	-
<b>Early Modern / Modern Period (<math>n = 19</math>)</b>	0.01620	0.01333	0	-
<b>Kihnu (<math>n = 44</math>)</b>	0.02529	0.13773**	0.09663*	0

\* Statistical significance at  $0.01 < p < 0.05$ .

\*\* Statistical significance at  $p < 0.001$ .

Statistical significance was estimated using a permutation simulation with 10 000 permutations.



H_36	.....T.....T.....CC.....T.-..	1	B	90OaTer1
H_37	CG.....G.ACC.TGGT...TT.CC...C..A.-..	1	A	930aKivt1
H_38	CG.....GCACC.TGGT...TT.CC...C..A.-..	1	A	960aPih1
H_39	.....A.C...T.....CC.....-..	1	B	990aLohk2
H_40	C.....T.....CC.....	1	B	1010aPadi3
H_41	.....A.....T.....CC...T.....-..	2	B	1090aMusul 1240aOlu2
H_42	...T.....T.....CCC.....-..	1	B	1120aLatte2
H_43	.G.G...A.....T.....CC.....-..	1	B	1230aSara2
H_44	.....T.....GCC...C...-A	1	B	1360aKar4
H_45	CG.....GCACC.TG.T...TT.CC...TC..A.-..	5	A	LA11 LA15 LA18 LA22 LA29
H_46	.....C...T.....CC.....C-..	8	B	LA16 LA20 LA28 LA31 LA32 LA34 LA35 LA42
H_47	.....T.....CCC.....T.-..	1	B	LA23
H_48	CG...T...G.ACC.TG.T...TT.CC...C..A.-..	5	A	LA27 LA39 LA43 LA44 LA45
H_49	..G.....TG.....CC.....A.-..	2	B	LA33 LA38

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**S6 Table. Haplotype data for ancient and modern samples of this study, and for comparative samples from Finland.** Mitochondrial DNA haplotype data for the median-joining network of 523 bp mtDNA D-loop haplotypes presented in Fig 3 (main text): Estonian ( $n = 88$ ), Latvian ( $n = 5$ ), Russian ( $n = 6$ ), Polish ( $n = 2$ ) and Greek ( $n = 1$ ) ancient, and Estonian modern Kihnu sheep ( $n = 44$ ) samples of our study; and Finnish ancient ( $n = 26$ ) and modern ( $n = 32$ ) samples (JX484017–JX484025, JX484035–484057, JX484111–JX484136; [1]). Note that because of the shorter alignment length used in the median-joining networks in Fig 3 and S1 Fig (523 bp compared to the longer alignment of 559 used in the rest of the study), one distinctive mutation recorded in the 559 bp alignment that separated haplotypes *H4* and *H8*, has been merged (see also S5 Table). Therefore, in the current table and in the networks presented in Fig 3 and S1 Fig, the central haplotype in haplogroup B is named *H4*, and *H8* is missing from the list.

<i>h</i>	<i>n</i>	<i>H</i>	<i>individuals</i>
H_1	2	B	40aJaan1 1000aPadi2
H_2	1	A	50aPada1
H_3	1	B	60aPost1
H_4	52	B	70aSoon1 110aLoss1 120aOrdu2 160aLoh1 230aRoul 270aLut1 280aJak1 300aKeal 370aSaml 450aOte2 500aKir2 570aToul 580aTor1 670aOte3 760aKar1 820aSarg1 850aPail 860aSaul 870aNar1 890aAlul 910aTart2 1020aHar1 1100aBot1 1110aLatte1 1130aJaan3 1140aJaan4 1190aRus1 1200aRus2 1210aRus3 1220aPih2 1300aEka3 1320aSau2 1420aKar6 JX484035 JX484042 JX484046 JX484125 JX484130 LA1 LA2 LA3 LA4 LA5 LA6 LA7 LA8 LA9 LA10 LA19 LA24 LA25 LA37
H_5	2	B	80aVas1 1430aKar7
H_6	9	B	90aSpo1 290aJak2 1060aIlml 1160aKrak1 1290aAlu2 JX484036 JX484037 JX484132 JX484136
H_7	2	B	100aHuv1 1310aKural
H_9	1	B	130aRid2
H_10	3	B	140aJaan2 150aSpo2 190aTart1
H_11	6	B	170aOte1 620aPada2 1180aMusu2 1410aJak4 JX484119 JX484134
H_12	8	B	180aAsva1 210aPar1 950aVec1 1370aKar5 1380aKrak3 JX484048 JX484117 JX484131
H_13	5	B	200aTal1 JX484124 JX484126 JX484127 JX484128
H_14	1	B	240aOlu1
H_15	1	B	250aSall
H_16	4	B	310aKivi2 JX484039 JX484043 JX484044
H_17	2	B	320aLoo1 800aKak1
H_18	2	B	340aPro1 840aKil1
H_19	2	B	430aLih1 1400aJak3
H_20	2	B	440aIru1 JX484047
H_21	2	B	460aJoe3 1030aRoos1
H_22	1	B	470aVao3
H_23	2	B	490aIru2 1270aPar5
H_24	2	B	510aVar1 JX484129
H_25	1	B	520aSuu2
H_26	1	B	530aIru3
H_27	2	B	560aJoe4 1070aMuuk1
H_28	1	B	590aLin1
H_29	3	A	640aPoi1 1050aAsva3 JX484135
H_30	11	B	650aPaa1 1280aPih3 LA13 LA14 LA17 LA26 LA30 LA40 LA41 LA46 LA47
H_31	2	B	69Haa1 1150aVilKv1
H_32	1	B	770aVilMul
H_33	1	B	780aKures1
H_34	2	B	790aEka1 830aPadi1
H_35	1	B	810aPol1
H_36	1	B	900aTer1
H_37	1	A	930aKivt1
H_38	2	A	960aPih1 JX484116
H_39	1	B	990aLohk2
H_40	1	B	1010aPadi3
H_41	3	B	1090aMusu1 1240aOlu2 JX484045
H_42	1	B	1120aLatte2
H_43	1	B	1230aSara2

H_44	1	B	1360aKar4
H_45	5	A	LA11 LA15 LA18 LA22 LA29
H_46	8	B	LA16 LA20 LA28 LA31 LA32 LA34 LA35 LA42
H_47	1	B	LA23
H_48	5	A	LA27 LA39 LA43 LA44 LA45
H_49	2	B	LA33 LA38
H_50	4	B	JX484017 JX484018 JX484024 JX484025
H_51	3	A	JX484019 JX484020 JX484021
H_52	1	B	JX484022
H_53	1	B	JX484023
H_54	1	B	JX484038
H_55	2	B	JX484040 JX484112
H_56	1	B	JX484041
H_57	2	B	JX484049 JX484114
H_58	2	B	JX484050 JX484051
H_59	1	A	JX484052
H_60	4	A	JX484053 JX484054 JX484057 JX484111
H_61	2	B	JX484055 JX484056
H_62	1	B	JX484113
H_63	1	B	JX484115
H_64	1	B	JX484118
H_65	1	B	JX484120
H_66	1	B	JX484121
H_67	1	A	JX484122
H_68	1	B	JX484123
H_69	1	B	JX484133

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1. Niemi M, Bläuer A, Iso-Touru T, Nyström V, Harjula J, Taavitsainen JP, et al. Mitochondrial DNA and Y-chromosomal diversity in ancient populations of domestic sheep (*Ovis aries*) in Finland: comparison with contemporary sheep breeds. *Genet Sel Evol.* 2013;45(2). doi:10.1186/1297-9686-45-2

**S1 Text. Sampling and DNA extraction protocol.** Bone samples were prepared in a separate room dedicated for sampling archaeological specimens, using separate equipment. Non-disposable equipment (*e.g.* drill bits) was decontaminated between the samples, and latex gloves and protective clothing were worn when handling the specimens. The protocol followed in the study was first described by [1] and amended by [2] with slight modifications.

1. Remove the outer surface of the bone with a drill. Drill 0.5 ml bone powder and divide it into three 2 ml tubes.
2. To dissolve the bone powder, add 900  $\mu$ l 0.45M EDTA pH 8.0, 100 $\mu$ l 1M urea and 200  $\mu$ g Proteinase K. Incubate overnight in an end-over-end shaker at 55 °C.
3. Centrifuge 2000 rpm for 5 min. To concentrate the sample, transfer three supernatants in to a single Amicon Ultra-4 30kDa Centrifugal Filter Unit (Merck Millipore, Darmstadt, Germany) and centrifuge 4000 rpm for 8–15 min. The preferred concentrated volume is 200–250  $\mu$ l (max 300  $\mu$ l). Move the concentrate into a 2 ml tube.
4. To bind the DNA, add 5 volumes QIAquick (Qiagen, Hilden, Germany) PB-buffer. Vortex and spin down. Load 650  $\mu$ l of the concentrate onto a QIAquick column and centrifuge 13 000 rpm for 1 min. Discard the flowthrough. Repeat the process until all of the extract has passed through the column.
5. To wash the DNA, add 650  $\mu$ l QIAquick PE-buffer and centrifuge 13 000 rpm for 1 min. Discard the flowthrough. To dry the column completely, centrifuge 13 000 rpm for 1 min. Discard the flowthrough.
6. To elute the DNA, load 75  $\mu$ l QIAquick EB-buffer directly onto the column. Incubate 3 min and centrifuge 13 000 rpm for 1 min. Repeat the process. The total volume of the DNA extract is 150  $\mu$ l.

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**S2 Text. Radiocarbon dating.** The results in this study rely to a certain extent on the accurate dating of the archaeological sheep remains. The majority of samples were dated based on archaeological context, that is, through the associated finds and site stratigraphy. As the bones were recovered from a variety of sites (settlements, hillforts, cemeteries) and excavated at different times and under different conditions, the documentation and therefore the precision of contextual dating could vary. Nevertheless, care was taken to sample the bones with an applicable context. For most of the samples with unclear context radiocarbon dating was conducted (S1 Table).

Fourteen samples were radiocarbon dated by AMS in the Poznań Radiocarbon Laboratory (all calibrations according to IntCal13 atmospheric curve [1]; OxCal v4.2.3 [2]; r:5) and SUERC Radiocarbon Dating Laboratory (all calibrations according to IntCal13 atmospheric curve [1]; OxCal v.4.1.7 [3]; r:5). For three samples the datings have previously been reported in [4].

Some of the dating results were somewhat surprising, as seven samples produced dates significantly later than those based on the archaeological context: five of those come from Middle Bronze and Early Iron Age stone graves, which have complicated contextual data as well as poor documentation; and other two derive from a settlement site and field remains, which also can yield chronologically mixed archaeological material. These results raise awareness of the complexity of archaeological contexts and should be noted when working on and interpreting the past faunal remains.

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