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CHALLENGING DNA SAMPLES ARE VALUABLE SOURCES FOR GENETIC INFORMATION OF POPULATIONS AND INDIVIDUALS

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Dedicated to Minna Ruokonen

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29.8.2020 Matti Heino

Abbreviations

AD	*anno Domini*
aDNA	ancient DNA
BP	Before Present
bp	base pairs
cal	calibrated years
COI	cytochrome c oxidase I gene
CytB	mitochondrially encoded cytochrome B gene
FS	foetal side of a placenta
mtDNA	mitochondrial DNA
MS	maternal side of a placenta
ND2	mitochondrially encoded NADH dehydrogenase 2 gene
ND6	mitochondrially encoded NADH dehydrogenase 6 gene
Numt	nuclear mitochondrial DNA
OP	orange particles found on the maternal side of a placenta
SNP	single nucleotide polymorphism
UC	umbilical cord

Original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I Valtonen, M., Heino, M., Aspi, J., Buuri, H., Kokkonen, T., Kunnasranta, M., Palo, J. U. & Nyman, T. (2015). Genetic monitoring of a critically-endangered seal population based on field-collected placentas. *Annales Zoologici Fennici, 52*(1-2), 51-65. doi:10.5735/086.052.0205

II Heino, M., Granroth, J., Aspi, J., & Pihlström, H. (2019). A previously undescribed Javan tiger *Panthera tigris sondaica* specimen, and other old and rare tiger specimens in the Finnish museum of natural history. *Mammal Study, 44*(1). https://doi.org/10.3106/ms2018-0036

III Honka, J., Heino, M., Kvist, L., Askeyev, I., Shaymuratova, D., Askeyev, O. V., Askeyev, A. O., Heikkinen, M. E., Searle, J. B. & Aspi, J. (2018). Over a thousand years of evolutionary history of domestic geese from russian archaeological sites, analysed using ancient DNA. *Genes, 9*(7), 367. doi:10.3390/genes9070367

IV Salmi, A., & Heino, M. (2019). Tangled worlds: The Swedish, the Sámi, and the reindeer. *International Journal of Historical Archaeology,* 1-23. doi:10.1007/s10761-018-0465-2

V Heino, M., Askeyev, I., Shaymuratova (Galimova), D., Askeyev, O., Askeyev, A., van der Valk, T., Pečnerová, P., Dalén, L., Aspi, J. (2019). 4000-year-old reindeer mitogenomes from the Volga-Kama region reveal continuity among the forest reindeer in northeastern part of European Russia. *Arheologiâ evrazijskih stepej, 4(179-190).*

Author contributions

Article	I	II	III	IV	V
Original idea	MK	HP, JG	IA, DS, OA, AA, JA, LK, MTH, JH	AKS	IA, MTH, DS, OA, AA, JA
Sample collection	TK, MK, MV	HP, JG	IA, DS, OA, AA	AKS	IA, DS, OA, AA
Laboratory work	MTH, MV, HB	MTH, HP, JG	JH, MTH	AKS, MTH	MTH, PP
Data analyses	MV, MTH, TN	MTH, HP, JG	JH	AKS, MTH	MTH, TvdV
Manuscript preparation	MV, TN, JP, MTH, JA, MK, TK	HP, MTH, JA, JG	JH, MTH, LK, IA, DS, OA, AA, MEH, JS, JA	AKS, MTH	MTH, IA, DS, OA, AA, TvdV, PP, LD, JA

Matti T. Heino (MTH), Jouni Aspi (JA), Igor Askeyev (IA), Dilyara Shaymuratova (DS), Oleg Askeyev (OA), Arthur Askeyev (AA), Henry Pihlstöm (HP), Janne Granroth (JG), Anna-Kaisa Salmi (AKS), Mia Valtonen (MV), Johanna Honka (JH), Mervi Kunnasranta (MK), Tommi Nyman (TN), Laura Kvist (LK), Tom van der Valk (TvdV), Patrícia Pečnerová (PP), Tuomo Kokkonen (TK), Hanna Buuri (HB), Jukka Palo (JP), Marja E. Heikkinen (MEH), Jeremy Searle (JS), Love Dalén (LD)

Contents

1 Introduction

DNA studies have traditionally targeted samples that originate from modern individuals. This approach has many caveats. Studies on modern individuals provides only a very limited and possibly wrong picture of evolution. This is because certain assumptions (e.g. about the mutation rate) are needed to infer the past processes, and these assumptions often contain uncertainty. Additionally, more recent evolutionary processes may mask those that have happened earlier. Targeting DNA from historical and ancient samples therefore offers a better alternative to study the past, because by comparing long-dead individuals to modern and other ancient individuals, one can make direct observations of evolution and demographic processes. Ancient DNA has proven to be especially helpful for studies on domestication (see e.g. Gaunitz et al., 2018; Skoglund, Ersmark, Palkopoulou, & Dalén, 2015), phylogeography (Doan et al., 2017), human migration (see e.g. Haak et al., 2015; Saag et al., 2019), bioarchaeology (see e.g. Schroeder et al., 2019), past environmental communities (see e.g. Willerslev et al., 2014), disease epidemics (see e.g. Rascovan et al., 2019) and historical trade routes (see e.g. Star et al., 2017).

In addition to showing a potentially distorted picture of the past, studying samples originating from living organisms may be problematic also because acquiring the sample is often harmful for the study individuals. Especially, research on endangered species and populations should preferably use methods that avoid interfering with the study individuals. It is sometimes possible to obtain DNA samples from living individuals without harming them, utilizing so-called non-invasive samples that originate from DNA which the individual has left behind in the environment. Such samples can be, for example, hair samples from hair traps (Rovang et al., 2015), shed feathers (Horváth et al., 2005), fecal samples (Ramon-Laca et al., 2015) or snow tracks (Dalén et al., 2007). More and more genetic monitoring is done using environmental DNA samples, for example by extracting DNA from water samples or soil (Thomsen & Willerslev, 2015, Taberlet et al., 2018). Even though environmental DNA is commonly used to investigate which taxa are present or absent in the environment, in some cases even population-level information of the study species can be obtained (Sigsgaard et al., 2016).

At the beginning of my PhD, the intention was to study purely ancient DNA of reindeer and seals. Confessedly, none of the projects that were initially in my PhD plan ended up in this thesis. Some of these original projects grew considerably larger than originally planned and therefore they are still work in progress. Some

where superseded by interesting side projects that developed during the PhD. The studies on ancient Russian reindeer and geese started when Igor and Oleg Askeyev and Dilyara Shaymuratova from the Institute of Problems in Ecology and Mineral Wealth of the Tatarstan Academy of Sciences contacted us regarding opportunities for collaboration. The study on historical tiger specimens on the other hand developed after Henry Pihlström and Janne Granroth from the Finnish museum of natural history re-discovered poorly documented tiger specimens from their museum collections. Henry and Janne asked if we could use DNA analyses to try to gain information on the likely geographic origin of these specimens. Despite these and other changes in my original plan, the major theme in my research remained as studies on degraded DNA, and the already previously done study on the Saimaa seal placentas fitted within this theme.

1.1 Ancient DNA

Ancient DNA can be loosely described as any DNA that is extracted from biological material that has not been preserved specifically for DNA research. The term is however more commonly used regarding DNA from ancient or historical samples. The samples in question usually derive from bones, but also hair, sediments, old wood, eggshells etc. can be used as sources. Due to exposure to environmental factors such as humidity, radiation, and acidity, ancient DNA is commonly highly fragmented and affected by post-mortem changes (e.g. Thomas & Gilbert, 2006).
The research field of ancient DNA is relatively young but has provided some ground-breaking insights on evolution and population history of several species. First ancient DNA studies were conducted in mid 1980s, when Svante Pääbo reported results of a 2,400 year old mummy from Egypt (Pääbo, 1985) and Higushi and coworkers presented results from an extinct quagga (Higushi et al., 1984). The early ancient DNA studies commonly targeted mitochondrial DNA sequences. The reason for this was that there are multiple copies of mitochondria per cell, making its recovery therefore more likely than recovering nuclear sequences from old, usually highly degraded samples. Even though mitochondrial DNA remains a valuable marker documenting maternal lineages, recent advances in sequencing technology and data interpretation (Hofreiter et al., 2015) have made it easier to target nuclear DNA as well. The field of ancient DNA has especially benefited from newly developed sequencing (and statistical) methods. Especially the technique called shotgun sequencing has had a large impact on ancient DNA studies, because

the technique can utilize fragmented DNA, which is typical for ancient DNA samples.

In addition to fragmentation, ancient DNA is also characterized by postmortem changes such as cross-links between DNA strands and deamination. Cross-links may hinder amplification of ancient DNA, a step usually required in ancient DNA analysis. Deamination on the other hand causes misincorporation of bases during the amplification step. Even though these modifications and fragmentation complicate the analysis of ancient DNA, they can also be used to distinguish authentic ancient DNA from contaminating modern DNA (Skoglund et al., 2014). Contamination from modern DNA or cross-contamination from other ancient samples are common problems when working with ancient DNA. Depending on study species, modern DNA is often more prevalent in the environment and may shadow the authentic ancient DNA of a sample. This may cause modern DNA to be interpreted as ancient, and lead to wrong conclusions. In order to minimize the possibility of contamination, sample collection should preferably be done in a sterile manner. After bringing the samples to the laboratory, certain procedures should be applied, as exemplified already by Cooper and Poinar (2000). These include performing the contamination prone steps of DNA extraction in laboratory facilities that are dedicated to work on ancient DNA. These need to be separated from laboratories that handle modern DNA and/or amplified DNA samples. Even if strict measures to prevent contamination are followed throughout the sample collection and genetic data production, it is still possible that the data will suffer from contamination. The level of contamination can be estimated using statistical methods, and the sequences likely resulting from contamination can be removed before data analysis. Finally, it is important to use common sense and evaluate if the data and results make sense in the light of what is already known about the study system.

1.2 Modern DNA

As the word implies, modern DNA derives from modern or relatively recent biological material. Taken that the samples have not degraded due to unfavourable storage conditions, these samples commonly contain more high-quality DNA than ancient samples, i.e. DNA is not heavily fragmented and is more abundant and therefore easier to study. However, this is not always the case. Researchers may also have to use biological material that has been exposed to elements that are unfavourable for DNA survival. Such material may come from e.g. long-dead

carcasses (predated or naturally died animals, road-kills) or samples that have been noninvasively collected as explained above.

Samples may also include inhibitors, which may prohibit extraction and/or amplification of DNA (a step usually required for downstream genetic analyses). Inhibition is caused by substances that interfere with chemicals used in DNA amplification and may derive from the tissue in itself (for example keratin in hair) or from the environment (soil humus). Many DNA extraction protocols take into account the possible presence on inhibiting substances by incorporating chemicals and steps that remove as much as possible of these substances (e.g. Schrader et al., 2012).

1.3 Domesticated study species

Domestication of animal and plant species has been one of the greatest advancements in the human history, enabling the development of large human communities and civilizations. Domestication is the process where animal or plant population is adapting to human control. This commonly happens through artificial selection where humans choose which individuals get to reproduce based on their favourable characteristics.

Biological traits of species dictates much of the domestication process. For example, generation interval affects how quickly species responds to artificial selection. The shorter the interval, more quickly the species may respond genetically to selection. For this reason, domestication of mammal species happens usually slowly and gradually, while for example in the case of certain plant species, domestication may happen quickly. Different domestic species are in different stages of domestication. For some species, such as for reindeer, the difference between domestic and wild populations is small, while for some species, the domestic and wild populations are clearly diverged.

Domestication may lead to genetic bottlenecks, first when only a certain portion of the wild population is subjected for domestication, and second, when this population is then subjected for increasing artificial selection through time (Zeder et al., 2006). On the other hand, sometimes human selection may increase genetic variation on certain loci. For example, many domestic mammal species such as horse have more coat colour variation than their wild relatives.

Ancient DNA can be used to obtain important knowledge about domestication. It can for example be used to pinpoint geographical locations where the domestication process of certain species has started (see e.g. Røed et al., 2008),

inform about the timing of domestication (see e.g. Skoglund et al., 2015) and evolution of the domesticated species through time (see e.g. Fages et al., 2019).

1.3.1 Goose

The European domestic goose (*Anser anser*) has been domesticated from the wild greylag goose (Shi, Wang, Zeng, & Qiu, 2006; Wang et al., 2010). Its domestication process has been proposed to have followed the prey-pathway, according to which it was hunted for meat before being domesticated (Larson & Fuller, 2014). However, the domestication history of the European domestic goose is still largely unknown. Historical records indicate that geese were used already by the ancient Egyptians, Romans and Mesopotamians (Zeuner, 1963) and it has been proposed that geese were domesticated around 3000 BCE either in south-eastern Europe (Crawford, 1984) or in Egypt (Zeuner, 1963). Domesticated geese were used for certain in Egypt and in Europe around 1550–1150 BCE (Zeuner, 1963). By the 1st century BCE, the Romans had already several different breeds of geese (Albarella, 2005) and in the Medieval Period, peasants commonly kept large flocks of geese (Albarella, 2005). The European domestic goose was probably introduced into Scandinavia during the Early Iron Age (400 BCE–550 CE), as indicated by archaeological evidence (Tyrberg, 2002).

Goose domestication has previously been studied using mitochondrial DNA analysis of exclusively modern geese demonstrating that modern domestic geese were derived from a limited genetic base (Heikkinen et al., 2015, Fig. 1). However, in that study, it was not possible to interpret if the observed low diversity of modern breeds was due to domestication or if it was due to foundation of the breeds some hundreds of years ago.

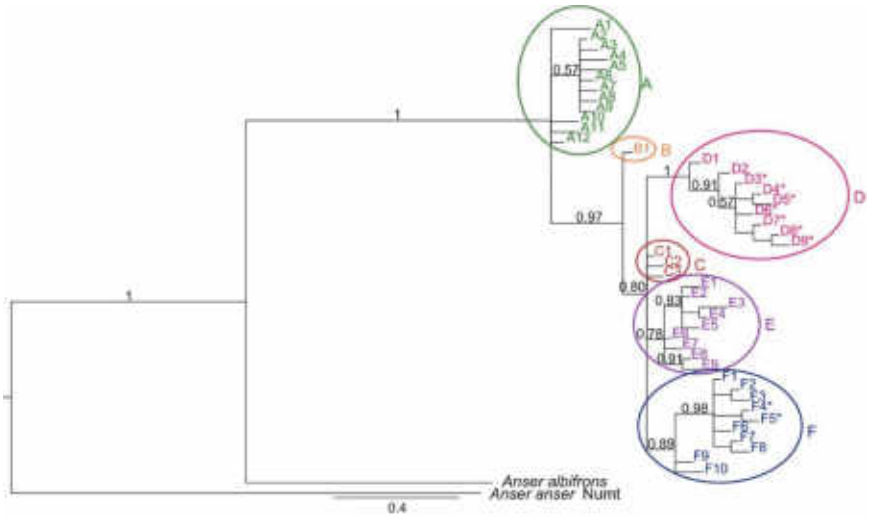

Fig. 1. Bayesian phylogeny showing the relationships of mitochondrial haplotypes of wild graylag (*Anser anser*) and European domestic goose. Asterisk indicates haplotypes that are shared between wild graylag and domestic goose. Posterior support values above 0.5 are shown. Six main haplogroups (A-F) are indicated. Outgroups: *Anser anser* Numt = graylag nuclear mitochondrial DNA insert, *Anser albifrons* = greater white-fronted goose. (Reprinted, with permission, from Heikkinen et al. 2015 ©John Wiley and Sons)

1.3.2 On reindeer domestication

Domestic reindeer differs from many other domesticated animal species in that the human control over the domestic herds is more relaxed than with many other domestic species, such as for example horses, pigs and chickens, which are kept in strong control over the whole lifespan of the individual. Domestic reindeer are therefore commonly referred as semi-domestic. Additionally, the semi-domestic reindeer herds commonly live in close proximity with wild herds leading to intermixing between the wild and semi-domestic herds. In Fennoscandia, semi-domestic reindeer populations have been mixing with wild Norwegian mountain reindeer populations, which are today restricted to south-central Scandinavian mountains, and with wild Finnish forest reindeer population in eastern Finland.

Not much is known about the early history of reindeer domestication. Some scholars have suggested that some form of reindeer herd control by humans has

already taken place during the Late Pleistocene (e.g. Patte, 1958). Other researchers have however refuted some of these views (e.g. Weinstock, 2000). The earliest definite descriptions of the use of semi-domestic reindeer come from written records from China from 499 AD in which a monk named Huei Shen described a people living possibly around the area of Baikal who, for example, milked reindeer and used them for pulling sledges (Laufer, 1917). The first written documents describing domestic reindeer in Fennoscandia come from an account of Norwegian chieftain Ohthere of Hålogaland (Ottar) who travelled to England and met King Alfred the Great in 890 AD. Ohthere said that he owned 600 reindeer of which six were used as decoys to lure and catch wild reindeer.

Røed et al. (2008) have shown that the reindeer has likely been domesticated at least twice by the people living in different regions of Eurasia, and that some wild populations, such as the Finnish forest reindeer, have not contributed much if any ancestry to the domestic herds. The mitochondrial lineages that at present are the dominating lineages among the Fennoscandian domestic reindeer appeared in southern (Røed, Flagstad, Bjørnstad, & Hufthammer, 2011; Røed et al., 2014) and northern Norway (Bjørnstad, Flagstad, Hufthammer, & Røed, 2012; Røed, Bjørklund, & Olsen, 2018) only about 500 years ago. This timing coincides with a change in reindeer pastoralism from people having small herds towards keeping larger herds. The origin of the two main Fennoscandian semi-domestic lineages remain elusive, but recently an origin east of Fennoscandia was tentatively suggested (Røed, Bjørklund, & Olsen, 2018).

1.4 On the tiger subspecies

Tigers have traditionally been divided into eight Recent subspecies: the Bengal or Indian tiger *P. t. tigris*, the Caspian tiger *P. t. virgata*, the Amur or Siberian tiger *P. t. altaica*, the South China tiger *P. t. amoyensis*, the Indochinese tiger *P. t. corbetti*, the Sumatran tiger *P. t. sumatrae*, the Javan tiger *P. t. sondaica*, and the Balinese tiger *P. t. balica* (Mazák, 1981; Mazák, 2013). Additionally, Luo et al. (2004, 2008, 2010) have recognized a ninth subspecies, the Malayan tiger *P. t. jacksoni*. Tiger taxonomy is however controversial subject, and other authors have suggested that only two or perhaps three subspecies should be recognized (Kitchener, 1999; Kitchener & Yamaguchi, 2010; Kitchener et al., 2017; Kitchener & Dugmore, 2000; Wentzel et al., 1999; Wilting et al., 2015).

Of the traditionally recognized subspecies, many have become extinct during the last 100 years. The Balinese tiger went extinct during the 1930's (Seidensticker,

1987). The Caspian and Javan tigers likely went extinct in the 1970's (Can, 2004; Seidensticker & Suyono, 1980; Seidensticker, 1987. The South China tiger went extinct in the wild in the early 1990's (Tilson, Defu, Muntifering, & Nyhus, 2004), but persists in captivity. The wild populations of the other remaining tiger subspecies are also threatened by extinction. Amur, Bengal, and Sumatran tigers are however relatively numerous in captivity (Luo et al., 2008).

1.5 Saimaa ringed seal

Lake Saimaa, which is the largest lake complex in Finland, harbours a ringed seal subspecies (*Pusa hispida saimensis*) (Fig. 2), which is thought to be derived from the Baltic Sea ringed seal (*Pusa hispida botnica*). The subspecies has been thought to have been landlocked in Saimaa since the postglacial land uplift separated part of the original population into the lake about 9000 years ago. Both nuclear and mitochondrial DNA of modern Saimaa ringed seal has been previously studied (Nyman et al., 2014; Valtonen et al., 2012). Based on these studies, Saimaa ringed seal in not however genetically particularly close to the modern Baltic ringed seal, suggesting that the histories of the ringed seal populations in the Baltic region may be more complex than generally thought.

Fig. 2. Saimaa ringed seal. Photo: Mia Valtonen.

The subspecies experienced a dramatic decline in population size from a possible 1000 individuals (Kokko et al., 1999) at the beginning of the 20th century due to hunting and fishing, and was on the verge of extinction in 1980s, with only about 120 individuals left. Since then, the population size has increased, with an estimate of 380-400 individuals in 2019 (http://www.metsa.fi/saimaannorppa/hyljekanta2019). Like other ringed seal subspecies, Saimaa ringed seal prefers giving birth in snow lairs that they dig from under the ice (Sipilä, 2003). Because the Saimaa ringed seal does not eat its placenta, after the breeding season, placentas can usually be located from the bottom of the lake from close proximity of the lair.

2 Genetic markers used in the study

Mitochondrial DNA was used as a genetic marker in all of the articles. Mitochondria are cell organelles, which produce most of the energy required by cells using aerobic respiration. Mitochondria derive from phagocytosised bacteria, which were absorbed early in the evolution of eukaryotes (Martin & Mentel, 2010). Mitochondria are usually transmitted maternally, (but see Kondo et al., 1990; Kvist, Martens, Nazarenko, & Orell, 2003; Luo et al., 2018; Skibinski, Gallagher, & Beynon, 1994), so their sequences are used to track the maternal history of individuals. Mitochondria have their own small circular genome that encodes components of the respiratory chain complexes (Fig. 3.) In animals, the size of the mitochondrial genome is around 16 kilo bases. Sequences of the mitochondria are commonly used in evolutionary studies to infer relationships of populations and individuals. Animal mitochondrial genomes commonly consists of 37 genes and other regions, including highly variable non-coding control region, which plays a role in replication and transcription of the mitochondrial DNA (Boore, 1999). Because of its high mutation rate, the control region is especially useful in studying relationships of populations within a species. The high mutation rate can also sometimes be problematic due to homoplasy where different lineages mutate to same state irrespective of their evolutionary relationship. Therefore, sequencing more slowly evolving regions/genes may sometimes be more desirable. Mitochondrial DNA is especially useful in aDNA research because it is found in up to thousands copies per cell, making it more likely for the mitochondrial DNA to survive over time compared to nuclear sequences, which are only found in two copies per each cell in diploid organisms.

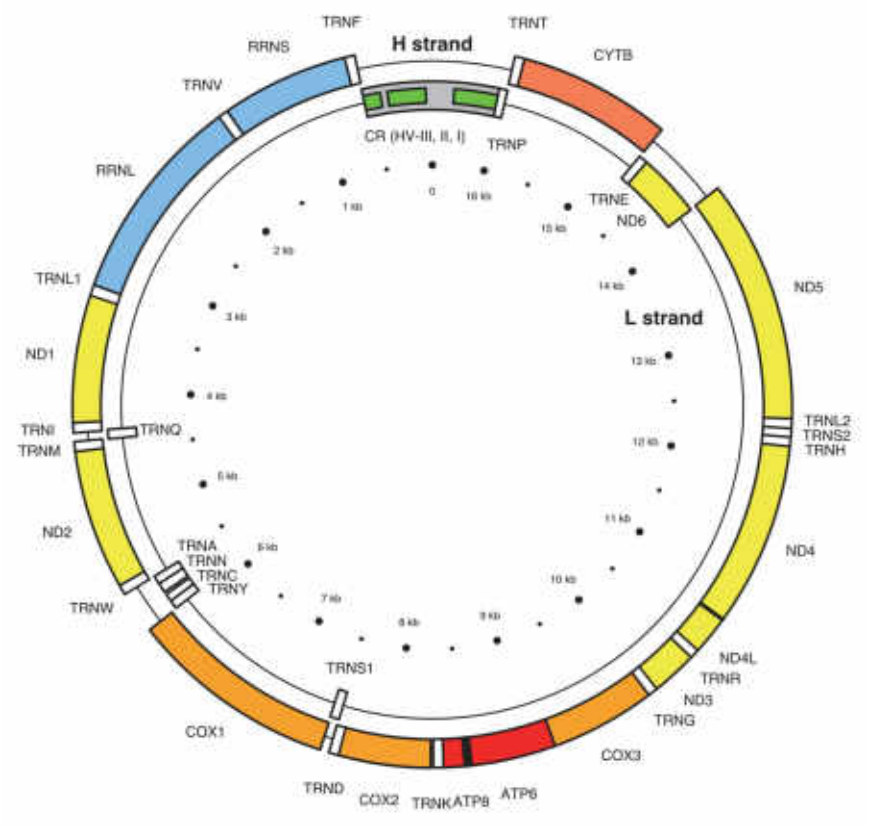

Fig. 3. Schematic picture of the human mitochondrial genome. Genes encoded on the heavy (H) and light (L) strand are shown. Species studied in this thesis (reindeer, tiger, ringed seal and domestic goose) have the same genes in the same order as human, with the exception that goose has ND6 and CytB genes in the opposite order. Lengths of the mitochondrial genomes of the studied species are: reindeer; 16362 bp, tiger; 16990 bp, ringed seal; 16754 bp, goose; 16738 bp. Picture: Emmanuel Douzery, CC BY-SA 4.0.

Because mtDNA alone has a very limited power to distinguish individuals from each other, in subproject I, we also studied nuclear microsatellite loci. Microsatellites are regions of repetitive DNA that are abundant in eukaryote genomes. A microsatellite may consist of tandem repeats of one to six nucleotides. The number of repeats in each loci is highly variable between individuals, which is why microsatellites are used in individual identification and forensics. The number

of repeats is commonly considered to evolve neutrally through insertions and deletions of single repeats at a time (Ellegren, 2004).

Mitochondria comprises only a part of the genetic material of an individual. Furthermore, as mitochondria is transmitted maternally, it can be used to study the maternal history of the study species. Therefore, results from studies that focus exclusively on mitochondria may give somewhat limited picture on the evolution and population history of the study species. Targeting nuclear loci would however have been difficult and out of the scope in many of the subprojects due to following reasons: 1) When these studies were conducted, genome-wide data was only available for one the study species, the tiger. Therefore, in order to make comparisons to modern populations, we would also have needed to generate data from modern samples. 2) There would have been available microsatellite data from modern reindeer populations (Røed et al., 2008). However, DNA in ancient samples is usually too degraded for microsatellite studies. 3) Therefore, the best option to generate nuclear data would have been to shotgun sequence the samples. In shotgun sequencing, sequence data is generated randomly across the genome. 4) Generating adequate amount of shotgun sequencing data would have been more costly.

2.1 Aims of the study

The aim in my research was to use challenging and unconventional samples to study the population history of the study species. The samples in question were either ancient (or historical), or non-invasively collected modern samples.

In subproject I, the utility of non-invasively collected Saimaa ringed seal placentas for genetic identification of individuals and measures of population level genetic parameters was evaluated. Because the Saimaa ringed seal is highly endangered and the genetic monitoring of the subspecies has so far relied on randomly found dead individuals, obtaining useful genetic data from placentas would be highly beneficial. Placentas are more numerous and more easily obtainable from the environment than any other types of tissue. Thus, the resolution of the monitoring would increase if they provided useful information. Because a placenta is composed of tissues that derive from both the mother and the pup (which would increase their utility), I attempted to genotype both the mother and the pup by sampling tissue from different spots on the placentas.

Natural history collections offer an enormous opportunity to study present and past biological diversity. Unfortunately, especially in the past, collected samples were often not carefully curated in the sense that their origin and other contextual

information was not included with the sample or may have been lost in time. The situation may have been exacerbated if the collections have been moved to different facilities, during which some records may have been lost. In article II I attempted to identify the subspecies and geographical origin of some tiger samples located at the Finnish Natural history museum for which no curated information was available. The aim therefore was to increase the scientific value of this tiger collection and present it to the wider public.

In article III we aimed to shed further light on the domestication of the goose by DNA sequencing goose remains from Russian archaeological sites. Special interest was to investigate if domestic goose haplotypes were present in the archaeological material, and to determine whether there had happened changes in the genetic diversity through time.

In article IV, my goal was to understand what kind of role reindeer had in the encounters of the Sámi and the Swedes during the Middle Ages and early modern times in northern Fennoscandia. Historically reindeer may have played an important role on these northern areas by comprising products for trading in addition to its use in transport. We utilized ancient DNA, stable isotopes and zooarchaeology on reindeer remains that originated from marketplaces, towns and agrarian settlements. Stable isotopes were used to study if the reindeer had been fed by humans, and aDNA analysis were used to establish whether the reindeer in question had been wild or domestic.

In article V I studied how 4000-year-old reindeer samples from the forest region of Tatarstan are genetically related to modern populations. As reindeer has gone extinct from Tatarstan, it was of interest to study if closely related lineages still prevail in some other regions.

3 Material and methods

3.1 Samples

3.1.1 Saimaa ringed seal placental samples

The studied placentas were collected by my collaborators. Most were collected from nest sites after the ice had melted in May (Auttila et al., 2014, Figs. 4, 5, 6), but some were collected already during the annual seal census counts. Population size of the Saimaa ringed seal is estimated annually by counting the number of found nests. Because the Saimaa ringed seal builds its nest in a snow pile close to the shore, after the ice has melted, placentas are commonly visible from the water surface and can be collected for example by using a long stick (Fig. 4). Between 2009 and 2011 a total of 59 placentas were collected, which is a considerable sample size taken the small population size on the Saimaa ringed seal. Most of the placentas were at least partly decomposed, evaluated by eye. Tissue samples were obtained from four different parts of each intact placenta: maternal (i.e., uterine) side (MS); foetal (i.e., membrane) side (FS); umbilical cord, or in absence of it, a vein (UC); and orange particles (OP), which are bilirubine-containing particles found on the maternal side of the placenta (van den Broeck, 1904) (Fig. 6). Additionally, blood samples were obtained from four placentas that looked exceptionally fresh. The samples were randomized and subjected to DNA extraction.

Fig. 4. Mia Valtonen picking up a Saimaa ringed seal placenta. Photo: Juha Taskinen.

Fig. 5. Saimaa ringed seal placenta. Photo: Mia Valtonen.

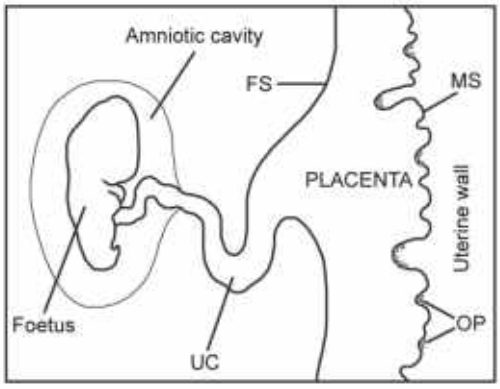

Fig. 6. Structure of the placenta, and different sampling spots used in this study: MS = maternal side, FS = foetal side, UC = umbilical cord, OP = orange particles. (Reprinted,

with permission, from Valtonen et al. 2015 ©Finnish Zoological and Botanical Publishing Board 2015)

Additional tissue samples were obtained from five dead pups, of which the natal site, and therefore the corresponding placenta were known. These included three that were found as stillborn and two that had accidentally been caught and died in fishing nets. The genotypes obtained from these pups could be compared to the genotypes that were obtained from different sampling spots of their placentas, and thus used to pinpoint the sampling spot on placenta that gives the most reliable pup genotype.

3.1.2 Tiger samples

Recently, during inventory of the collections of the Finnish Museum of Natural History, seven poorly documented tiger *(Panthera tigris*) samples were re-discovered. The samples originate from the 19th and 20th centuries and have very little background information about the subspecies or geographical origin. This is because during that time, specimens of taxa that were not native to Finland were commonly purchased from traders or obtained from foreign scientists. In order to gain information on the likely sub-species status and geographical origin of these samples, and therefore to increase the scientific value of these specimens, two skeletons and five pelts were sampled for teeth, bone, skin, hair or footpads (Fig. 7).

Fig. 7. Skull of UN 2485, one of the studied tiger specimens. Photo: Janne Granroth.

3.1.3 Archaeological geese samples

We sampled a total of 67 goose bones from Russian archaeological sites spanning the time between the 4th and 18th centuries. The bones were classified as belonging to domestic geese based on morphology. The study sites are located west from the Ural mountains in Tatarstan, Saratov, Chivash, Nizhny Novgorod, Leningrad and Pskov regions (Fig. 8). Most of the samples originate from Tatarstan (N = 51).

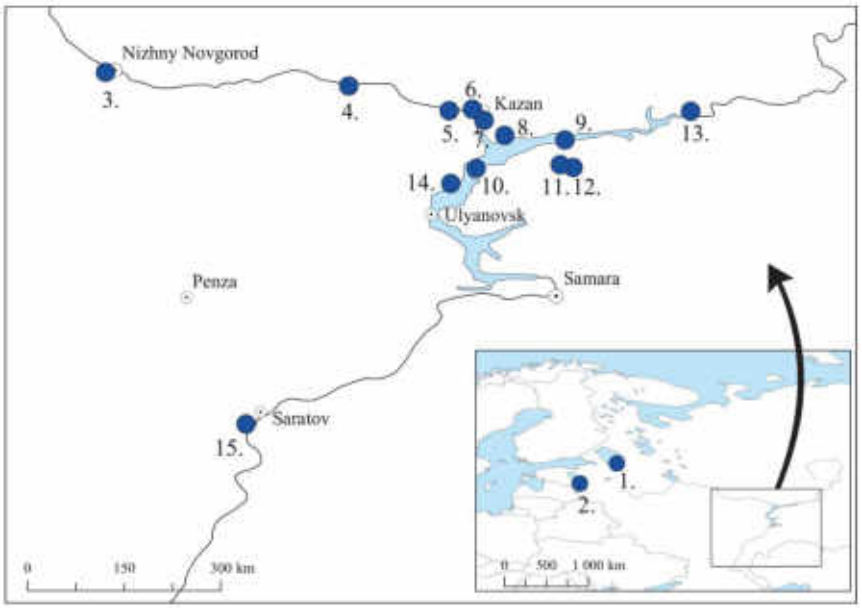

Fig. 8. Archaeological sites for the studied domestic geese from 4th–18th century: 1. Staraya Ladoga (Leningrad Region), 2. Pskov city (Pskov Region), 3. Nizhny Novgorod Kremlin (Nizhny Novgorod Region), 4. Chebosakry city (Chuvash Republic), 5. Sviyazhsk (Tatarstan Republic), 6. Kazan Kremlin (Tatarstan Republic), 7. Kazan State University, Kazan city (Tatarstan Republic), 8. Imenkov hillfort (Tatarstan Republic), 9. Ostolopovskoe settlement (Tatarstan Republic), 10. Bulgar (Tatarstan Republic), 11. Toretskoe settlement (Tatarstan Republic), 12. Bilyarsk (Tatarstan Republic), 13. Elabuga hillfort (Tatarstan Republic), 14. Tetyushkoe II hillfort (Tatarstan Republic), and 15. Bagaevskoe settlement (Saratov Region). (Reprinted from Honka et al., 2018, CC BY 4.0)

3.1.4 Archaeological reindeer samples from Finland

The samples for DNA analysis of reindeer originated from two archaeological sites, Oravaisensaari (N =2), and Ylikylä (N = 2), located in the southern part of Finnish Lapland (Article IV, p. 264, Fig. 1). Samples have been directly radiocarbon dated to between 1401-1797 calibrated years (cal) AD. It was not known whether the bones presented wild or domestic reindeer.

3.1.5 Ancient reindeer samples from Tatarstan

We subjected six reindeer samples for DNA analysis from the archaeological site Pestrechinskaya II. The site is located in present day Tatarstan and dated to around 4000 cal BP.

3.2 Laboratory methods

Mitochondrial DNA was targeted in all sub-projects. In article I, we studied also nuclear microsatellite markers. The targeted regions of each specific work are listed in table 1. Because it was expected that the DNA in the samples would be highly fragmented, we amplified short DNA fragments between 100-150 base pairs in each article II-V.

Table 1. Overview of taxons and targeted regions studied in each article.

Taxon	Markers	Article
Saimaa ringed seal	11 autosomal microsatelllite loci, mtDNA control region	I
Tiger	mtDNA regions ND2, COI, ND6, and CytB	II
Goose	mtDNA control region	III
Reindeer	mtDNA control region	IV
Reindeer	Whole mitochondrial genome	V

DNA work on historical and ancient bone samples, prior to amplification, was performed in ancient DNA laboratories either at the Centre for Material Analysis, University of Oulu, Finland or at the Swedish Museum of Natural History, Stockholm, Sweden. Procedures that minimize contamination and maximize the possibility of obtaining genetic data from the samples were followed (Fig. 9). First, the outer layer of the sampling location on each bone was polished off to get rid of as much of surface contaminants as possible. Then approximately 50 mg of bone powder was obtained by drilling inside the bone to provide bone powder from which the DNA was then extracted, using a modified version of the silica-column protocol first described by Yang, Eng, Waye, Dudar, & Saunders (1998) and later modified by Gamba et al. (2014; 2016) or alternatively a protocol outlined in Ersmark et al. (2015) which is a modified version of the protocol C in Yang et al. (1998).

Fig. 9. Working in the aDNA laboratory at the Centre for Material Analysis, University of Oulu. Photo: Pekka Moilanen.

Preparation of amplification mixtures for articles II, III, IV and V was also performed in the clean room to prevent contamination. All other steps of the protocols after that were performed in a regular molecular genetic lab. For article I, all laboratory work was performed at the molecular biology laboratory of the Ecology and Genetics Research Unit of the University of Oulu using standard protocols. The targeted DNA regions were amplified by PCR to provide enough template for subsequent sequencing reactions and microsatellite fragment length detection. PCR profiles for the amplifications can be found from the original papers (sub-projects I-V). Each reaction was replicated at least once to identify possible post-mortem changes and other sequencing artefacts.

The success of each amplification reaction was assessed using agarose gel electrophoresis, after which the successful reactions were cleaned using Exonuclease I - Alkaline Phosphatase-method, and sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequencing reactions were run on 3130xl Genetic Analyzer (Applied Biosystems).

3.2.1 Specific laboratory methods used in article V

DNA extracts were turned into Illumina sequencing libraries. The specific protocol can be found from the subproject V. Libraries were then sequenced together with other ancient reindeer and moose libraries on one Illumina MiSeq and HiSeq lane.

3.3 Data analyses

3.3.1 Initial processing of raw data

In article I, the chromatograms from the fragment length analysis were turned into genotypes using GENEMAPPER ver. 4.0 (Applied Biosystems). In articles II, III, IV and V, the mtDNA reads were inspected, edited and assembled into consensus sequences using CodonCode Aligner (Version 4.0.4, CodonCode Corporation).

3.3.2 Data validation

MICRO-CHECKER ver. 2.2.3 (Van Oosterhout et al., 2004), FreeNA (Chapuis & Estoup, 2007) and MICRODROP ver. 1.01 (Wang et al., 2012) were used to identify possible genotyping errors (i.e., stuttering, allelic dropout and null alleles) in the microsatellite data in article I.

3.3.3 Reference data

Reference data were incorporated in the analyses in all of the subproject. In subproject I, Saimaa ringed seal mtDNA data from Valtonen et al. (2012) and microsatellite data from Valtonen et al. (2014) were used. In subproject II, concerning the genetic identification of tiger specimens, I compared the genotypes of the study individuals with the genotypes obtained from Buddhakosai et al. (2016), Driscoll et al. (2009), Kitpipit, Tobe, & Linacre (2012), Luo et al. (2004), Sun et al. (2015), Wilting et al. (2015) and Xue et al. (2015). In subproject III where we studied ancient goose samples from Russia, data from Heikkinen et al. (2015), Honka et al. (2017), Lomakina et al. (2015), Ruokonen et al. (2000), Ruokonen et al. (2008) and Wang et al. (2010) were used. In subproject IV on the archaeological reindeer from Finland, I used data from Bjørnstad & Røed (2010), Bjørnstad et al. (2012), Røed et al. (2011) and Røed et al. (2008). Finally, in subproject V where I studied the genetic relatedness of ancient reindeer from Tatarstan to modern

populations, data from Røed et al. (2008), Kholodova et al. (2011), Baranova et al. (2012), Kvie et al. (2016a and 2016b), Korolev et al. (2017) and Ju et al. (2016) was used.

3.3.4 Genetic diversity, structure and isolation-by-distance

For the microsatellite data in article I, ARLEQUIN ver. 3.5.1.2 (Excoffier & Lischer, 2010) was used to calculate genetic diversity indices, GENEPOP ver. 4.1.3 (Rousset, 2008) was used to test departures from Hardy-Weinberg equilibrium, and SPAGEDI ver. 1.3 (Hardy & Vekemans, 2009) was used to test for the presence of isolation by distance.

Genetic diversity indices for the mtDNA sequence data in article I were calculated using ARLEQUIN and in article III using DnaSP v.5 (Librado & Rozas, 2009). ARLEQUIN was further used to calculate analysis of molecular variance (AMOVA) (Excoffier et al., 1992) and genetic distances between temporal groups in article III.

3.3.5 Phylogenetic analyses

MrBayes (Version 3.2, Ronquist et al., 2012) was used to build a Bayesian phylogenetic tree in article II and VI. TempNet (Prost & Anderson, 2011) was used to draw a temporal statistical parsimony network in article III, and PopART (Version 1.7, http://popart.otago.ac.nz) was used to build a Median-Joining network in articles III, IV and V.

3.3.6 Individual identification and kinship analyses

In article I, GENALEX ver. 6.41 (Peakall & Smouse, 2006, 2012) was used to calculate the probability if identity (PI) and the probability of exclusion (PE). PI is a likelihood that two randomly chosen individuals have the same genotype across all studied loci. In other words, it measures how reliably individuals can be differentiated from other individuals. PE measures how reliably any given individual can be ruled out as a parent of any other individual. It therefore estimates the power and utility of the microsatellite data in parentage analysis. In this article, COLONY ver. 2.0.4.1 (Jones & Wang, 2010) was further used to investigate the adequacy of microsatellite panels for inferring sibship and parentage.

Sequences of ancient reindeer from Tatarstan obtained in article V were BLAST searched against the GenBank database to infer the closest species matches.

3.3.7 Other statistical methods used in article I

χ^2-test for homogeneity was used to test whether the four main placental sampling spots differed with respect to overall genotyping success. ANOVA in SPSS Statistics 19 (IBM) was used to test the effect of the quality of the placenta on amplification success of UC samples, which were observed to yield the pups' genotypes. To see if placentas can be used to get population-level genetic parameters, the diversity and differentiation estimates obtained from the placentas were compared with the reference data. This was done by calculating an exact G-test in GENEPOP to test differences in allele frequencies between placentas and the reference data, and by calculating the Spearman's rankorder correlation in SPSS to test correlation between mtDNA haplotype frequencies in the two datasets.

4 Results and discussion

4.1 Saimaa ringed seal placentas as tools for population monitoring

It was clear from the microsatellite chromatograms that many samples contained DNA from more than one individual: More than two alleles at one locus, indicating a mixture of the mother's and pup's DNA, were detected most often in MS (maternal side) samples and least frequently in UC samples (34.6% and 3.6%, respectively). This was expected because placentas are chimeras containing tissue from both the mother and the pup. This however complicated the handling, analysis and interpretation of the results. For forensic researchers it is common to obtain DNA samples from crime scene that are mixtures of more than one individual. Usually however, the investigators have knowledge about the genotypes of victim/s and suspect/s of the crime to which the results from the DNA mixtures can be compared to. Using this analogy for the study on the Saimaa seal placentas, we didn't have either knowledge about the "victims" nor the "suspects" genotype before we obtained genotypes directly from some of the pups (from which we also had placentas) and could compare these genotypes with those obtained from different spots on the placenta. When comparing the genotypes of the five reference pups to those of their corresponding placentas, UC samples were the only ones that produced completely matching multi-locus genotypes. After this discovery, genotypes from the UC samples were taken to represent the genotypes of the pups, and all the following analysis on genetic diversity, individual identification relatedness etc. used these genotypes.

Microsatellite diversity was very low but observed (HO) and expected (HE) heterozygosities corresponded closely with estimates obtained previously using conventional samples. MtDNA haplotype frequencies obtained from placentas correlated strongly with haplotype frequencies of reference dataset. Due to low genetic variability in the studied markers, reliable estimates for relatedness of individuals were not obtained.

I was not able to reliably solve genotypes of the mothers. I tried to assess which spot on the placenta had most genetic differences compared to the genotype obtained from the umbilical cord of the same placenta. As the umbilical cord gave the pups genotype, reasoning was that the genotype most dissimilar to the pup's genotype would be closest to mother's genotype. This exercise however did not

provide consistent results and is not reported in the published article. Further insights could possibly be attained, if one would obtain tissue samples from some of the mothers and compare the genotypes to those obtained from the placentas of the same individuals.

Overall, when using the genotypes obtained from the umbilical cord, the work showed that the Saimaa ringed seal placentas collected at nest sites are useful samples for population monitoring and can be used to identify individuals. This is important because the placentas that remain after nesting are so called non-invasive samples and collecting them does not harm the individuals. Furthermore, genetic research so far on this elusive species has used samples obtained mainly from stillborn, by-caught or stranded seals. These samples present individuals which no longer belong to the population, whereas placental samples present individuals that still remain and possibly breed in the population. This is a clear advantage regarding population monitoring. Additionally, placentas are more abundantly available than individuals found dead, increasing the number of available samples and therefore the resolution of population monitoring. Because other seal species also leave their placentas after giving birth, this method could be extended also for these species.

Genotyping more nuclear loci than was done in this study could possibly result in data that could be used to obtain reliable estimates of relatedness between samples. This could be done either by shotgun sequencing the samples or typing them with a SNP panel. The upcoming *de-novo* genome of the Saimaa ringed seal will facilitate both of these approaches (https://www.saimaaringedseal.org/index.html). Furthermore, studying tissue samples from some of the mother's as an addition to their placenta's might help to figure out to extract the mother's genotype from the placenta.

4.2 Origin and subspecies status of the tiger samples in the Finnish Museum of Natural History

Five of the tiger samples could be identified by subspecies with reasonable confidence (Table 2). The genotyping results strongly suggested that the suspected Javan tiger UN 2485 is indeed an actual a Javan tiger (Fig. 10). Sample UN 365, which according to documentation originates from "India ost" (i.e., the East Indies, meaning present-day Southeast Asia), had a Sunda Island specific haplotype. It was however not possible to discern from which of the specific islands this individual originates from, because it had a haplotype that has been observed on all of the

main Sunda Islands. One would need to identify more SNPs that differentiate between tigers originating from these islands to better assess from where UN 365 originates.

Table 2. Inferred subspecies of the study samples.

Study sample	Inferred subspecies of the study sample
UN 2166	South China tiger
UN 365	Sumatran / Javan / Balinese tiger
UN 378	Bengal tiger
UN 2137	Amur tiger
UN 2485	Javan tiger
UN 2390	South China tiger
UN 2484	Malayan tiger

Specimen UN 378, and an individual without locality data but originating from the nineteenth century, had a haplotype which is specific to the Bengal tiger, therefore strongly suggesting that this individual is a Bengal tiger. The haplotypes of the two tigers known to originate from China were consistent with their locality: UN 2137 was identified as an Amur tiger, and UN 2166 as a South China tiger. The haplotype of the specimen UN 2390 was unique, but closest to one South China tiger haplotype. This individual therefore might represent South China tiger genetic diversity which has disappeared from the current population, or which previous studies had not captured. Sample UN 2484 also had a haplotype that so far has only been observed among Malayan tiger. UN 2484 is therefore most probably a Malayan tiger.

Overall, the research revealed that the samples harbor a surprisingly high subspecies diversity and include rare specimens such as the two Sunda Island tigers, of which one could be further identified as an extinct Javan tiger. This newly obtained data significantly increased the scientific value of the tiger collection of the Finnish Museum of Natural History and presented to the wider scientific community previously unknown rare tiger specimens.

Fig. 10. Specimen UN 365 that was determined to have originated from the Sunda Islands. The resolution of the genetic analysis was not enough to determine whether the individual is Sumatran, Javan or Balinese tiger, but the stripe pattern is *sondaica*-like suggesting that the individual may be a Javan tiger. Photo: Henry Pihlström. (Reprinted, with permission, from Heino et al., 2019 ©The Mammal Society of Japan)

4.3 History of domestic goose in Russia

46 out of the 67 studied samples produced reliable results. Samples belonged to eight haplotypes, of which three have not been observed before. The haplotypes fell into three different lineages: haplogroup D, haplogroup F, and the lineage constituting the taiga bean goose (*Anser fabalis fabalis*). The majority of known domestic goose haplotypes belong to haplogroup D and the rest to the F-haplogroup together with wild graylag goose haplotypes. The temporal statistical parsimony network together with the haplotype assignments of the studied samples is shown in Fig 11.

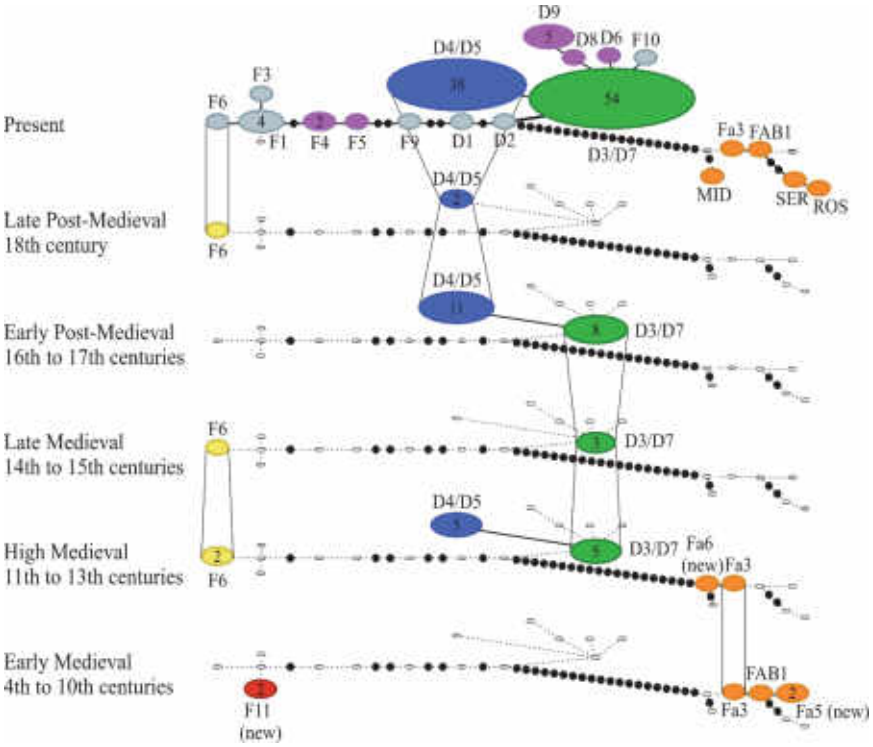

Fig. 11. Temporal statistical parsimony network of the historical goose samples from Russia and modern greylag goose (*Anser anser*) haplogroups D and F. Domestic haplotypes D3 and D7 are in green, D4–D5 in blue, and D6, D8–D9, and F4–F5 in purple. Wild greylag goose is in grey. Selected modern bean goose (*Anser fabalis*) haplotypes are in orange. MID, SER, and ROS indicate subspecies *Anser fabalis middendorffii*, *Anser fabalis serrirostris*, and *Anser fabalis rossicus*, respectively. FAB and Fa indicate *Answer fabalis fabalis*. Sizes of the ellipses are proportional to haplotype frequencies. Numbers inside ellipses indicate the number of samples having the haplotype (for haplotypes with more than one sample). White ellipses indicate missing haplotypes and black dots mutations. (Reprinted from Honka et al., 2018, CC BY 4.0)

The network shows that the typical domestic goose haplogroup D appears in the material in the studied sites between the 11th to 13th centuries and it's presence continues to the present day. Interestingly, haplotypes typical for the taiga bean goose were observed in the oldest studied material. It remains unclear whether these individuals present misidentified wild taiga bean geese or possibly domesticated taiga bean geese.

According to the nucleotide and haplotype diversity estimates, genetic diversity was highest during the High and Late Medieval Periods. This pattern however needs to be taken with caution, as the sample sizes varied between time periods.

4.4 Archaeological reindeer of northern Finland

The archaeological reindeer samples dated between 1400-1800 cal AD from northern Finland were not closely related to present-day domestic reindeer from Fennoscandia nor to the domestic reindeer from northern Fennoscandia originating from the twentieth century (Fig. 12). Instead, they were most closely related to present-day Finnish forest reindeer and ancient reindeer from Finnmark, mostly from the time period between ca. 3400 and 500 BCE. Based on size of the reindeer remains, on vegetation history and on genetic factors, Bjørnstad et al. (2012) have argued that the reindeer from Finnmark from the time period between ca. 3400 and 500 BCE may have been forest reindeer. Based on the above, it's likely that individuals studied in this work have been forest reindeer. This interpretation is in line also with what is known on the historical distribution of forest reindeer in Finland (Luukko 1954:111; Virrankoski 1973:271–272; Lundmark 1982:161). Because forest reindeer have not been shown to have contributed significant genetic ancestry to the present-day domestic reindeer in Fennoscandia (Røed et al., 2008), it is most parsimonious to conclude that the study samples most likely originated from wild individuals.

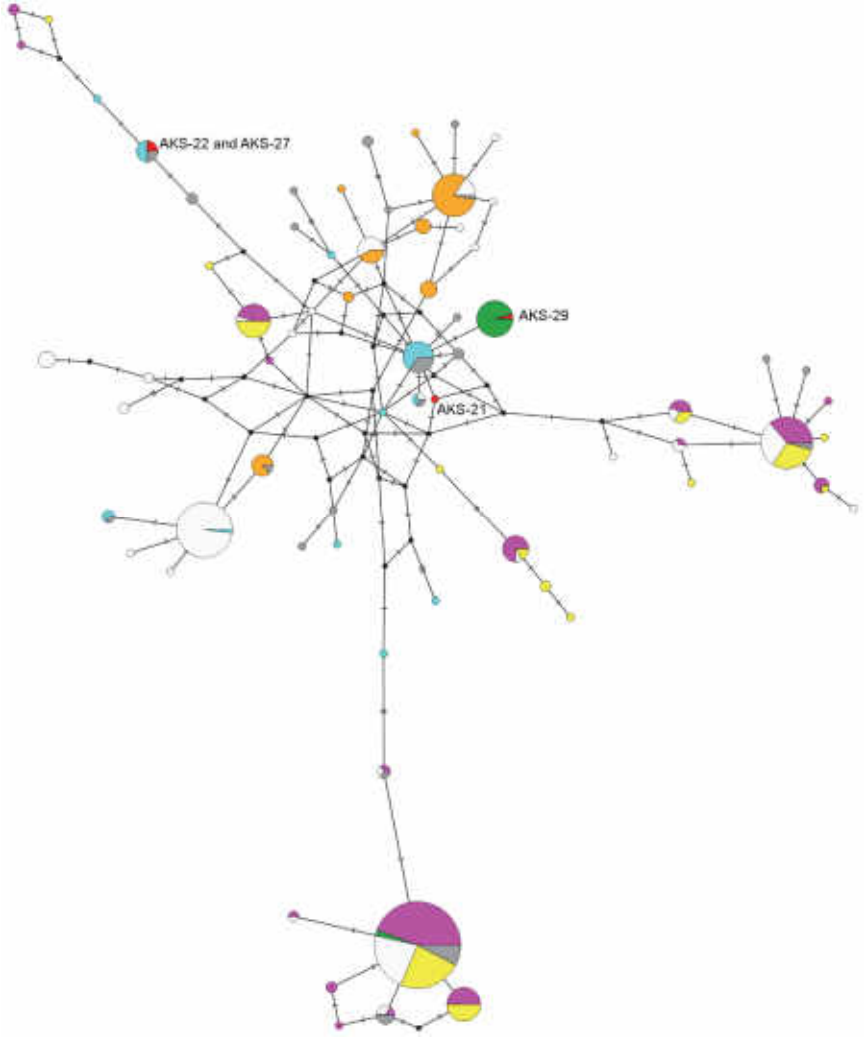

Fig. 12. Haplotype network of the studied samples and historical and modern reference data. Study samples are in red, modern domestic reindeer from Fennoscandia in pink, domestic reindeer from the early twentieth century from northern Fennoscandia in yellow, modern wild Norwegian mountain reindeer in white, modern Finnish forest reindeer in green, reindeer from Hardangervidda from the time period between ca. 1210–1310 AD in orange, reindeer from Finnmark from the time period between ca. 3400–500 BC in turquoise, and reindeer from Finnmark from the time period between ca. 100–1750

AD in grey. Sizes of the circles correspond to observed haplotype frequencies. Hatch marks indicate mutations, and black circles indicate median vectors. (Reprinted, with permission, from Salmi & Heino 2019 ©Springer Nature)

Unfortunately, there is no old reindeer bones for which it would be known that they present domestic or wild reindeer. We are therefore relying on modern data in the inferences of which genetic lineages are domestic and which are wild. This is problematic because modern populations do not necessarily reflect all the diversity that has been present in historical populations. For example, it is possible that the historical domestic populations included lineages that have since been lost from the domestic populations (for example as in the case of Prezwalski horse), so our interpretations are not on such solid ground. Rather, it is possible that before large-scale reindeer pastoralism spread over Fennoscandia, there were other local domestic lineages. It would be crucial therefore to study how much wild and domestic reindeer differ by their nuclear DNA and then investigate historical material for these possible differences. *De novo* genome and some re-sequenced genomes have recently been published (Weldenegodguad et al., 2020), but there isn't yet exact studies on the possible genome-wide genetic differences between wild and domestic individuals. Conducting this kind of study first on modern samples would facilitate the research also on ancient samples.

4.5 Genetic relatedness of the 4000-year-old reindeer from Tatarstan with modern populations

For all other samples except P13, 98-99% of the mitochondrial genome sequence was resolved at 3X coverage. Because at the time of the writing the article, only one mitogenome sequence of a modern individual was available in GenBank, most of the comparisons were based on mtDNA control region data, since a lot more reference data was available regarding this marker. Based on this marker, genetic relatedness between the historical reindeer of Tatarstan and the present-day wild populations of the northeastern part of European Russia was observed. The 4000-year-old reindeer from Tatarstan shared haplotypes especially with modern reindeer from the taiga zone of the northeastern part of European Russia, which implies that there is genetic continuity between these populations. However, as this study was conducted only on mitochondrial DNA, it provides only a very limited picture on the genetic relationships of the reindeer.

The phylogenetic tree that shows the haplogroup affiliations of the study samples is shown in Fig.5 (subproject V, page 186). The most interesting

observation from the tree is that four of the study samples are situated in relatively basal positions of haplogroup II. This haplogroup, together with haplogroup Ib, is the dominating haplogroup of Fennoscandian domestic reindeer (Flagstad and Røed, 2003; Røed et al., 2008; Kvie et al., 2016b). It was originally thought to have originated in Europe during the Late Pleistocene, south of the Fennoscandian ice sheet, but these results suggest that it may have originated east of Fennoscandia. The Fennoscandian domestic reindeer, however, likely has not directly originated from the population presented by the Pestrechinskaya II as the haplotypes observed in this material are not particularly close to the Fennoscandian haplotypes.

5 Conclusions

My research shows that valuable individual and population level genetic information can be obtained from unconventional biological samples. Each sub-project was successful in the sense that new relevant data and conclusions could be obtained.

In subproject I, we investigated the usability of non-invasively collected placentas of Saimaa ringed seal for individual identification. We were able to obtain pups genotypes from the placentas, and these genotypes could be used to calculate population level genetic parameters. Reliable estimates of relatedness of individuals were however not obtained, as the genetic variability in the studied microsatellite markers was too low in the population. Increasing the number of markers for example by typing single nucleotide polymorphism (SNPs) across the nuclear genome might provide enough resolution to obtain reliable estimates of relatedness.

In subproject II, I determined to which likely subspecies the historical tiger specimens belonged to, and from which likely geographical area they originate. Five of the samples could be reliably identified by subspecies. Subspecies diversity was surprisingly high and included rare specimens such as two Sunda Island tigers, of which one could be further identified as an extinct Javan tiger. As relatively few known Javan tiger specimens exist in the natural history collections in the world, the specimen identified in this study may in the future provide important additional information on this species. Additional genetic data obtained from this and other Javan tiger specimens could for example be used to study population level genetic changes in species that is going towards extinction.

Subproject III investigated the history of the domestic goose in Russia by studying specimens from archaeological sites. Analyses showed that the typical domestic goose haplotypes were present in the material from the 11th century onwards. Surprisingly, also bean goose haplotypes were observed among the studied material. The domestic/wild status of these individuals remains uncertain.

In subproject IV, I studied mtDNA from reindeer material originating from archaeological sites in Finland. As the studied specimens were genetically related to the present-day Finnish forest reindeer, a tentative conclusion is that they present wild reindeer. However, caution is needed, as the modern-day diversity of Fennoscandian domestic reindeer may not accurately present the historical diversity. I am at the moment studying archaeological reindeer samples from

additional sites in Fennoscandia in order to determine when large-scale reindeer pastoralism started and from which region did it originate.

In subproject V, the studied 4000-year-old reindeer samples from Tatarstan shared haplotypes especially with modern reindeer from the taiga zone of the northeastern part of European Russia, implying genetic continuity between these populations. Interestingly also a connection to the major Fennoscandian domestic haplogroup was observed, but the importance of this observation needs to be validated in future studies. The data generated in this study will be used in a large-scale investigation on the phylogeography of mitochondrial lineages of reindeer through the last Ice Age, which we are conducting at the moment together with my collaborators.

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I Valtonen, M., Heino, M., Aspi, J., Buuri, H., Kokkonen, T., Kunnasranta, M., Palo, J. U. & Nyman, T. (2015). Genetic monitoring of a critically-endangered seal population based on field-collected placentas. *Annales Zoologici Fennici, 52*(1-2), 51-65. doi:10.5735/086.052.0205

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Genetic monitoring of a critically-endangered seal population based on field-collected placentas

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Genetic analyses of non-invasively collected samples are increasingly being used in the monitoring of wildlife populations and individuals. This study is the first describing the use of placentas as non-invasive genetic samples from a natural population. We collected 66 placentas from birth-lair sites of Saimaa ringed seals (*Phoca hispida saimensis*) after the breeding seasons, with the aim of obtaining DNA from both the pup and the mother. Umbilical cord samples proved to yield the pup genotypes, but mothers could not be genotyped with confidence. Comparisons with existing mtDNA and microsatellite reference data sets showed that placentas can be used for inferring population-level genetic parameters. Our microsatellite panel provided sufficient resolution for genetic identification of individuals but, due to the extremely low variability of the population, parentage and sibship could not be inferred reliably. Field-collected placentas could provide means for genetic monitoring of many other seal species as well.

Introduction

Genetic analyses of non-invasively collected samples, such as hair and faeces, are being increasingly utilized in wildlife research, management, and conservation. Applications of these methods include, for example, identification of species or individuals, and estimation of home range size, gene flow, local population size, and individual reproductive success (Waits & Paetkau 2005, Schwartz *et al*. 2007). Non-invasive approaches have become more or less established practice in studies on large terrestrial mammals, which are hard to find and capture (Arandjelovic *et al*. 2011, Kopatz *et al*. 2012, Davoli *et al*. 2013). In marine mammals, the collection of non-invasive samples is often more challenging, but shed skin (Swanson *et al*. 2006, Baker *et al*. 2013, Martinez-Bakker *et al*. 2013), faeces (Parsons *et al*. 2006, Valqui *et al*. 2010),

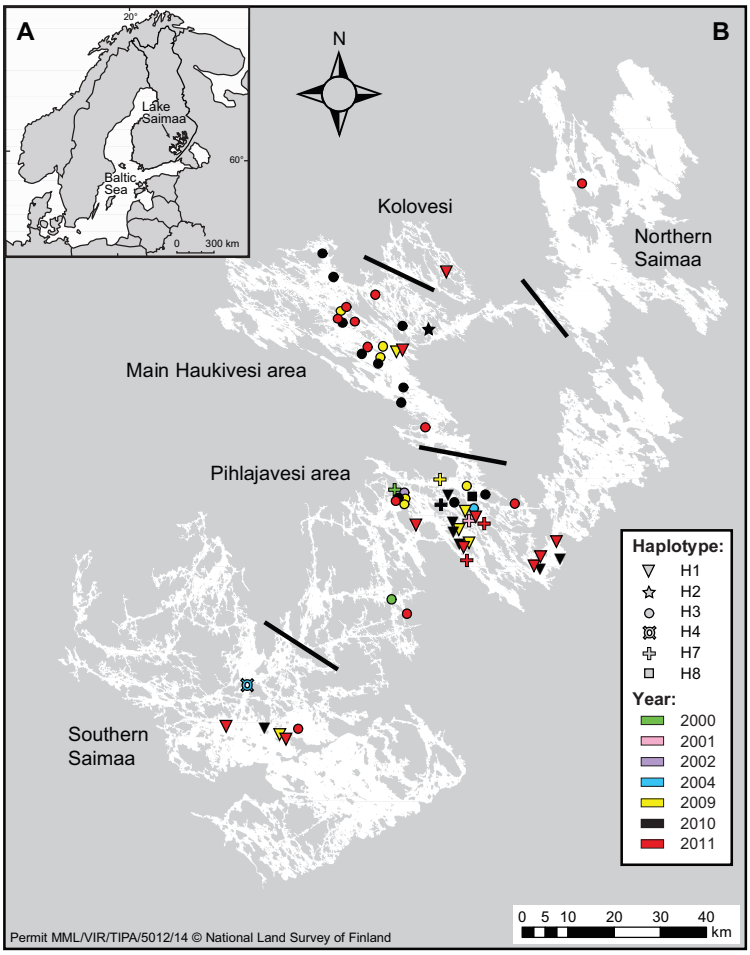

Fig. 1. (**A**) Location of Lake Saimaa in Finland, and (**B**) collection sites of Saimaa ringed seal placentas. Different symbols denote different mtDNA haplotypes and colours the year of collection.

and even environmental DNA from the water column (Foote *et al*. 2012) have been successfully used as a source of genetic information.

Here, we describe how field-collected placentas can be used for non-invasive genetic monitoring. The target population was the landlocked Saimaa ringed seal (*Phoca hispida saimensis*). This endemic subspecies inhabits Lake Saimaa in southeastern Finland (Fig. 1A). The population of currently circa 300 seals is threatened mainly by high mortality of juveniles due to entanglement in fishing gear and by climate change and, therefore, is classified as critically endangered (Rassi *et al*. 2010, Kovacs *et al*. 2012). Given the still-precarious situation of the subspecies, there is a clear need for continuous monitoring of the population, which could be substantially enhanced by remotely collected genetic samples. It has been estimated that around 70%–80% of the adult females give birth annually (Sipilä 2003), and that some 50–60 pups are born each spring. Placentas are relatively easy to collect post-partum: females give birth to a single pup in subnivean lairs dug into snowdrifts formed along shores of islands and islets (Sipilä 2003) and, after the breeding season, the placenta can often be found from the bottom of the lake within a few meters from the lair. Preservation of the tissue, and DNA, is extended by the low temperature of the water during and right after the ice-covered season.

Ideally, placentas could provide a unique opportunity for obtaining the DNA and genotypes of two different individuals from a single

sample, because mammalian placentas are composed of both foetal and maternal tissue. Like other carnivorans, pinnipeds have an endotheliochorial placenta (Stewart & Stewart 2009) with extensive intermingling of uterine and chorionic tissues, which causes a part of the uterine component to be torn away along with the placenta at birth. In the case of the Saimaa ringed seal, identification of females based on shed placentas could potentially be used to infer individual-level breeding-site fidelity and reproductive success, and could also lead to more accurate population-size estimates. Similarly, genotyping pups using placentas could yield information on long-term dispersal patterns and survival probabilities, if the individuals are recaptured and genotyped later.

The main aims of this study were to (1) investigate whether it is possible to identify seal mothers and/or offspring based on genetic profiling of field-collected placentas, and (2) find out the optimal spot for extracting DNA of each of these individuals from the placentas. We also wanted to (3) evaluate whether placentas could be used as a source of information in genotype-based mark–recapture and kinship studies, and (4) examine the utility of placentas in inferring population-genetic parameters of the Saimaa ringed seal using both nuclear (microsatellites) and mitochondrial (mtDNA control-region sequences) markers.

Material and methods

Sample collection and handling

Lake Saimaa is a large (ca. 4400 km^2) and shallow lake (mean depth 12 m, max. 85 m) with over 13 000 islands (Kuusisto 1999). Birth lairs are identified during annual lair censuses conducted throughout the lake during each April (Metsähallitus 2014). A few placentas used in this study were found from collapsed lairs during the censuses, but most placentas were collected by scuba diving from birth-lair sites after ice break-up in May, some 2–3 months after the birth of pups (*see* Auttila *et al*. 2014).

Placentas were searched for at birth-lair sites in the main breeding areas (Fig. 1B) in 2009 and 2010 (28 and 46 sites, respectively), and from all known sites in 2011 (50 sites). A total of 59 placentas were recovered (n_{2009} = 13, n_{2010} = 21, n_{2011} = 25), meaning that a placenta was found from 48% of the inspected lair sites. An unusual visual observation of nursed seal twins in 2009 was confirmed by our finding of two placentas only one meter apart on the lake bottom below a nearby birth-lair site (*see* below). In addition, seven placentas that had been collected in 2000–2006 and deposited in a tissue bank maintained by the University of Eastern Finland and Parks & Wildlife Finland were genotyped. All placentas were stored at –20 °C.

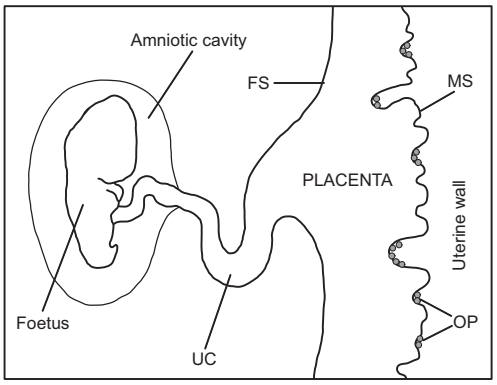

Fig. 2. Structure of the placenta, and different sampling spots used in this study: MS = maternal side, FS = foetal side, UC = umbilical cord, OP = orange particles.

The state of decomposition of each placenta was assessed visually using the following three-stage ordinal scale: (1) fresh, (2) partly decomposed and (3) decomposed. Of the 66 placentas sampled (Appendix), nearly half were classified as partly decomposed (48%), while 23% were categorized as fresh, and 29% as decomposed.

In order to identify the placental sampling spots from which the mother's and pup's DNA could be extracted separately, samples were taken from four different parts of each intact placenta (Fig. 2): maternal (i.e., uterine) side (MS); foetal (i.e., membrane) side (FS); umbilical cord, or in absence of it, a vein (UC); and orange particles (OP), which are bilirubine-containing particles found on the maternal side of the placenta (Van den Broeck 1904). We were able to take all four subsamples from 58 intact placentas (Appendix). For eight placentas with only shreds

of the membrane left, only an FS sample was taken. An additional blood sample (BS) was collected from four very fresh placentas that had been collected frozen on ice in April in 2000, 2001 and 2009. Altogether 244 tissue samples from 66 placentas were analysed, and for the laboratory analysis each sample was given a random number in order to prevent subjective interpretation of genotypes (i.e., samples were genotyped blind).

Samples from five pups (three stillborn and two by-caught pups with known natal sites) for which the corresponding placenta was available were used as reference samples. These samples were used to determine which of the sampling spots was optimal for obtaining the pup's genotype, and which for obtaining the mother's genotype.

Laboratory analyses

Total genomic DNA was extracted using the DNeasy Blood and Tissue Kits (Qiagen) according to the manufacturer's protocol. Each sample was genotyped at eleven microsatellite loci originally developed for other pinnipeds (annealing temperature (°C) and number of PCR cycles in parentheses): *Hg3.6* (58, 40), *Hg4.2* (61, 40), *Hg6.1* (58, 40), *Hg8.9* (58, 45), *Hg8.10* (53, 40), *SGPv9* (60, 40) (Allen *et al*. 1995), *Hgdii* (58, 40) (Allen *et al*. 1995, Twiss *et al*. 2006), *Hl15* (53, 43) (Davis *et al*. 2002), *SGPv10* (55, 40), *SGPv11* (55, 40) and *SGPv16* (51, 43) (Goodman 1997). These loci have previously been used in genetic analyses of the Saimaa ringed seal (*see* Valtonen *et al*. 2014), but the existing laboratory protocols were modified in order to optimise amplification success for placental DNA: PCR reactions contained 1 μl template DNA, 0.4 μM each primer, 0.6 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 1X PCR buffer, 1.75 mM $MgCl_2$, 0.2 mM of each dNTP (Finnzymes), and 1 mg ml^{-1} BSA (Thermo Scientific) in a total reaction volume of 10 μl. Reactions were performed under the following conditions: 95 °C for 10 min followed by 40–45 cycles of 95 °C for 30 s, 51–61 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were run on an ABI 3730 DNA Analyzer (Perkin Elmer Applied Biosystems), and genotypes were inferred using GENEMAPPER ver. 4.0 (Applied Biosystems).

MICRO-CHECKER ver. 2.2.3 (Van Oosterhout *et al*. 2004) was used for identifying possible genotyping errors (i.e., stuttering, allelic dropout, and null alleles) for UC samples, which were found to yield the pups' genotypes (*see* Results), with Bonferroni-adjusted 95% confidence intervals. FreeNA (Chapuis & Estoup 2007) was used to estimate the frequency of null alleles at each locus. In order to determine whether the data set was affected by allelic dropout due to poor sample quality, we used MICRO-DROP ver. 1.01 (Wang *et al*. 2012) to test for a correlation between the amount of missing data and homozygosity across individuals and loci. The mean error rate per locus and observed error rate per multilocus genotype were calculated by replicated genotyping of a subset of the samples: PCR was repeated three times for each locus for the UC sampling spots of the placentas of the twin individuals, and for the four UC samples for which a corresponding reference pup was available. In addition, a 704 bp-fragment from the 5´ domain of the mitochondrial control region was sequenced for all placentas as described by Valtonen *et al*. (2012).

Data analysis

A χ^2-test for homogeneity was used to test whether the four main placental sampling spots (MS, FS, UC, OP) differed with respect to overall genotyping success: a full 11-locus genotype *vs*. more than two alleles at any of the loci (suggesting mixture of pup's and mother's DNA), or an otherwise unclear genotype at any locus (e.g., locus did not amplify at all, or the signal of the shorter allele was weaker than that of the longer allele, which could indicate genotype mixture).

We compared the genotypes obtained from the different sampling spots (MS, FS, UC, OP, BS) within each placenta to investigate whether the mother's and/or pup's genotype could be inferred. Data from the five reference pups were used to determine the placental sampling spots that produced the best match with the corre-

sponding pup's genotype. PCR was repeated three times for all these placental samples, in order to acquire reliable genotyping results for comparison with the "correct" pup's genotype. As no reference samples of mothers were available, the optimal sampling spot for obtaining the mother's DNA was assessed indirectly, i.e., by comparing the multilocus genotypes of the reference pups with different placental sampling spots, in order to determine which ones produced clearly incompatible results.

The effect of the quality of the placenta (state of decomposition) on amplification success, measured as the number of loci successfully amplified from UC samples, which were found to yield the pups' genotypes (*see* Results), was tested using one-way ANOVA in SPSS Statistics ver. 19 (IBM).

Genetic diversity and isolation-by-distance

Population-level genetic parameters were estimated based on the UC samples, because these were found to yield the pups' genotypes (*see* Results). We estimated the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, and Wright's inbreeding coefficients (F_{IS}) using ARLEQUIN ver. 3.5.1.2 (Excoffier & Lischer 2010). GENEPOP ver. 4.1.3 (Rousset 2008) was used to test for departures from Hardy-Weinberg equilibrium and for the presence of linkage disequilibrium between pairs of microsatellite loci. Haplotype (h) and nucleotide (π) diversities for the mtDNA control-region sequence data set were estimated using ARLEQUIN.

The presence of an isolation-by-distance pattern among placentas was tested by contrasting pairwise microsatellite- and mtDNA-based genetic relatedness with geographical distance on a logarithmic scale in SPAGEDI ver. 1.3 (Hardy & Vekemans 2009). We chose the kinship coefficient of Loiselle *et al*. (1995) as a pairwise estimator of genetic relatedness, as it is considered suitable for data sets containing rare alleles, and does not assume Hardy-Weinberg equilibrium (Vekemans & Hardy 2004). Ten spatial distance classes were created using the equal-frequency method, which produces distance intervals with uneven distances, but with roughly equal numbers of pairwise comparisons. The mean kinship coefficient of each distance class, as well as the overall regression slope, were tested for a significant departure from zero by 10 000 permutations, and standard errors were estimated by jackknifing over loci.

The utility of placentas for estimating population-level genetic parameters was evaluated by contrasting the aforementioned diversity and differentiation estimates with reference values obtained in previous genetic analyses of Saimaa ringed seals: Valtonen *et al*. (2012) sequenced the mtDNA control-region from 203 dead individuals (stillborns, weaned pups, and adults) and 12 placentas, and Valtonen *et al*. (2014) analyzed variation at 17 microsatellite loci in 172 seals. The placental samples used by Valtonen *et al*. (2012) were excluded from the mtDNA reference data (but included in our present data set), and the microsatellite data set of Valtonen *et al*. (2014) was in our main comparisons reduced to include the same 11 loci as our placental data. To correct for unequal sample sizes when comparing microsatellite allelic richness (A_R) and mtDNA haplotype richness (a), the reference microsatellite and mtDNA data sets were rarefied in HP-RARE (Kalinowski 2005) based on the numbers of placentas. Differences in microsatellite allele frequencies between placentas and the 11-locus reference data set were tested in GENEPOP using an exact G-test. Additionally, differences in allele frequencies were assessed between placentas and a subset of the reference data (n = 65) spanning the same time period as the placental samples (the 2000s). The correlation between mtDNA haplotype frequencies in the placental and reference data sets was tested using Spearman's rank-order correlation in SPSS.

Identification of individuals and kinship analyses

To evaluate whether our panel of 11 microsatellite loci allows reliable identification of Saimaa ringed seal individuals, we estimated the probability of identity (PI, i.e., the probability that two randomly chosen individuals have identical

multilocus genotypes) as well as the corresponding value for siblings (PI_{SIB}) using GENALEX ver. 6.41 (Peakall & Smouse 2006, 2012). For comparison, we calculated these probabilities also for the reference data set of Valtonen *et al.* (2014) using both 11 and 17 loci. For this and further analyses, missing data were not allowed at any loci; after exclusion of individuals with incomplete genotypes, the 11- and 17-locus reference data sets comprised 171 and 168 individuals, respectively.

GENALEX was also used to calculate the probability of exclusion (PE) in the placental and reference data sets, in order to estimate the power and utility of the 11- and 17-locus microsatellite panels in parentage analysis. PE was estimated for three alternative scenarios: PE_1 = probability for excluding a putative parent when the other parent is known; PE_2 = probability of exclusion of a putative parent when the other parent is unknown; PE_3 = probability of excluding two putative parents (Jamieson & Taylor 1997).

In order to determine the optimal number of loci for identification of Saimaa ringed seal individuals (*see* Waits & Paetkau 2005), we used MM-DIST (Kalinowski *et al.* 2006) to compute expected and observed mismatch distributions for placentas as well as for the 11- and 17-locus reference data sets. MM-DIST calculates probability distributions for genotypic differences, i.e., the numbers of loci that differ among individuals in a population. The program creates the observed distribution for the studied data set, as well as expected distributions for individuals with different degrees of relatedness: unrelated individuals, fullsibs, and parent–offspring pairs. The congruence between observed and expected MM-distributions, as well as between observed distributions estimated for the placental and 11-locus reference data sets, were tested using a χ^2-test for goodness of fit. In order to meet the test's assumption of all expected frequencies exceeding 1, the following mismatch categories were combined: for placentas, the two lowest and three highest categories; for the 11-locus reference data, the two lowest categories; for the 17-locus data, the three lowest and three highest categories; and for the comparison of observed MM-distributions in the placental and 11-locus reference data sets, the two lowest and two highest categories.

The adequacy of the 11- and 17-locus marker systems for inferring sibship and parentage was further studied using COLONY ver. 2.0.4.1 (Jones & Wang 2010). First, we inferred full- and half-sibs from the data on placentas alone; in this analysis, the aforementioned placentas of the confirmed twins were used as an additional reference to determine whether they could be recognised as siblings. Second, we introduced potential adult fathers ($n = 6$) and mothers ($n = 4$) from the reference data set into the analysis of placentas, in order to assign parentage and sibship simultaneously. Finally, we analysed the full microsatellite reference data to infer sibship and parentage based on 17 loci. Three independent replicate runs were carried out with each data set to ensure convergence. Allele frequencies were estimated for each data set by COLONY, polygamy was assumed for both sexes, and no sibship prior was used. For placentas alone, we used the full-likelihood method with high precision and 'long' run length. Otherwise similar settings were used for the analysis involving placentas and potential parents, but run length was set to 'medium'. For the large reference data with 17 loci, the pairwise-likelihood score method and 'very long' run length were chosen.

Results

Genotyping

Genotyping success varied among different placental sampling spots (Table 1). Full, unambiguous 11-locus genotypes were obtained for 51% of UC (umbilical cord/vein) samples, but for only 0%–12% of the other sampling spots. More than two alleles at one locus, indicating a mixture of the mother's and pup's DNA, were detected most often in MS (maternal side) samples and least frequently in UC samples (34.5% and 3.6%, respectively). A χ^2-test of homogeneity showed a highly significant difference in genotyping success among MS, FS, UC, and OP sampling spots ($\chi^2 = 56.45$, df = 6, $p < 0.001$).

When comparing the genotypes of the five reference pups with those of their corresponding

Table 1. Genotyping success at 11 microsatellite loci for each placental sampling spot. MS = maternal side, FS = foetal side, UC = umbilical cord/vein, OP = orange particles, BS = blood sample.

	MS		FS		UC		OP		BS		n_{total}
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	
Unambiguous genotype	5	8.6	8	12.1	28	50.9	7	12.1	0	0	48
More than 2 alleles	20	34.5	9	13.6	2	3.6	8	13.8	0	0	39
Unclear genotype	33	56.9	49	74.2	25	45.5	43	74.1	4	100	154
n_{total}	58		66		55		58		4		

placentas, UC samples were the only ones that produced completely matching multilocus consensus genotypes (Table 2). None of the remaining main sampling spots (MS, FS, OP) yielded a genotype that was both unambiguous and clearly different from the pup's genotype, indicating that the mothers' genotypes could not be reliably determined.

The MICRO-CHECKER analysis suggested the presence of null alleles for three loci (*Hg8.9*, *SGPv11* and *SGPv16*), but estimated null-allele frequencies were low ($r \leq 0.08$) at each locus, and the result is likely caused by the presence of population structure (Valtonen *et al.* 2014). Scoring errors suggested for *Hg8.9* were ruled out by rechecking the data independently by two researchers. Further, no significant correlation between the amount of missing data and homozygosity was found either across individuals ($r = 0.163$, $p = 0.123$) or loci ($r = –0.247$, $p = 0.738$). The estimated mean error rate per locus was 0.036, and the observed error rate per multilocus genotype 0.313. Degree of decomposition had no effect on amplification success in UC samples (one-way ANOVA: $F_{2,52} = 0.609$, $p = 0.548$).

Comparison of population-genetic parameters estimated from the placental and reference data sets

Based on the above, UC samples were taken to represent the genotypes of the pups. Unclear UC genotypes (i.e., longer allele producing a taller peak than the shorter allele, *see* above) were accepted as a true genotype, but samples with missing data or more than two alleles at any locus were discarded; hence, further analyses were conducted with the 47 placentas that had full UC genotypes at 11 microsatellite loci.

Population-level microsatellite diversity estimated on the basis of placentas was very low, but observed (H_O) and expected (H_E) heterozygosities corresponded closely with estimates obtained for the reference data sets of 11 and 17 loci (Table 3). Allelic richness (A_R) was slightly higher in the 11-locus reference data set than in the placental samples even after correcting for sample-size differences by rarefaction (Table 3), which was, however, expected given that the individuals in the reference data set represent a longer period and a wider geographic area, and that some placentas may represent siblings due to the fact that they had been collected from the same birth-lair sites during consecutive springs (*see* below).

F_{IS} values estimated for the pooled placental sample as well as for the two regional subsamples did not depart from zero at $p \leq 0.05$ (Table 3), but the pooled data set was not in Hardy-Weinberg equilibrium (Fisher's exact test over all loci, $p = 0.006$). However, when the Main Haukivesi ($n = 17$) and Pihlajavesi areas

Table 2. Congruence (i.e., percentage of full matches at 11 loci) between consensus genotypes of different placental sampling spots and those of corresponding reference pups (note that only an FS sample could be obtained from the placenta of Pup 3). MS = maternal side, FS = foetal side, UC = umbilical cord/vein, OP = orange particles.

	MS	FS	UC	OP
Pup 1	64	55	100	45
Pup 2	73	91	100	45
Pup 3	–	45	–	–
Pup 4	64	45	100	55
Pup 5	82	73	100	27

(n = 24; *see* Fig. 1B) were analysed separately, deviations from Hardy-Weinberg equilibrium were found neither over all loci nor at any individual locus after a sequential Bonferroni correction.

Significant linkage disequilibrium remained between loci *Hg8.9* and *Hl15* even after a sequential Bonferroni correction; this most likely reflects differentiation between the Pihlajavesi and Main Haukivesi areas. Allele frequencies in placentas and the 11-locus reference data set differed significantly (χ^2 = 41.03, df = 22, p = 0.008), but the difference disappeared after reducing the reference data to represent the same time span as the placentas (χ^2 = 23.17, df = 22, p = 0.392).

Six mtDNA haplotypes were detected in the 63 placentas successfully analysed for mtDNA variation (Fig. 1B; *see* Valtonen *et al*. 2012 for details of haplotypes). Haplotypes H1 and H3 dominated the sample with relative frequencies of 36.5% and 49.2%, respectively, while H7 comprised 9.5% of the haplotypes, and H2, H4, and H8 were found in a single placenta each. When considering all haplotypes (H1–H8) found by Valtonen *et al*. (2012), placental haplotype frequencies correlated strongly with estimates derived from the mtDNA reference data set (Spearman's ρ = 0.805, p = 0.016). Estimated haplotype (h ± SD) and nucleotide (π ± SD) diversities as well as haplotypic richness (a) were very low (0.625 ± 0.037, 0.005 ± 0.005, and 6.00, respectively), but similar to those observed for the reference data set (0.649 ± 0.021, 0.005 ± 0.005 and 6.69, respectively; the estimate of a in the reference data set was obtained by rarefaction to n = 63).

A weak but statistically significant negative relationship between relatedness and spatial distance between pairs of placentas was detected using both microsatellites (b = –0.025, p = 0.009; Fig. 3A) and mtDNA control-region sequences (b = –0.128, p < 0.001: Fig. 3B). These results corresponded well with the previous study of Valtonen *et al*. (2014).

Identification of individuals, and kinship analyses

The probabilities of identity (i.e., probability that two individuals share the same multilocus genotype; PI for randomly chosen individuals and PI_{SIB} for siblings), were very low, but slightly higher in placentas than in the 11-locus reference data set (Table 4). With six additional loci (17 loci in total), PI_{SIB} values were an order and PI values two orders of magnitude lower. The estimated probabilities of exclusion were sufficiently high ($p \geq 0.99$) for excluding two puta-

Table 3. Estimates of genetic diversity in the Saimaa ringed seal population based upon analyses of 11 microsatellite loci in UC samples of placentas, and 11 and 17 loci in the reference data set. Average total number of alleles (N_A), allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) are given. Allelic richness (A_R) estimates in the reference data sets were obtained by rarefaction to the sample size (47, 17, or 24) in the corresponding sample in the placental data set. $P_{(HWE)}$ values indicating significant deviations from Hardy-Weinberg equilibrium are set in boldface.

Sample	n	N_A ± SD	A_R	H_O ± SD	H_E ± SD	F_{IS}	$P_{(HWE)}$
Placentas							
Total sample	47	2.91 ± 2.12	2.91	0.33 ± 0.24	0.35 ± 0.23	0.057^{ns}	**0.006**
Main Haukivesi area	17	2.64 ± 1.57	2.64	0.35 ± 0.27	0.39 ± 0.27	0.100^{ns}	0.396
Pihlajavesi area	24	2.60 ± 1.35	2.45	0.35 ± 0.23	0.34 ± 0.22	–0.020^{ns}	0.987
11-locus reference data							
Total sample	172	3.73 ± 4.10	3.38	0.35 ± 0.22	0.38 ± 0.24	0.073***	< **0.001**
Main Haukivesi area	79	3.45 ± 3.56	2.90	0.38 ± 0.24	0.40 ± 0.25	0.038^{ns}	< **0.001**
Pihlajavesi area	43	2.55 ± 1.63	2.35	0.32 ± 0.25	0.30 ± 0.23	–0.065^{ns}	0.280
17-locus reference data							
Total sample	172	3.47 ± 3.32	3.14	0.33 ± 0.21	0.36 ± 0.22	0.075***	< **0.001**
Main Haukivesi area	79	3.18 ± 2.92	2.70	0.34 ± 0.23	0.35 ± 0.24	0.024^{ns}	< **0.001**
Pihlajavesi area	43	2.59 ± 1.42	2.43	0.31 ± 0.25	0.30 ± 0.23	–0.034^{ns}	0.144

ns = not significant, *** p < 0.001.

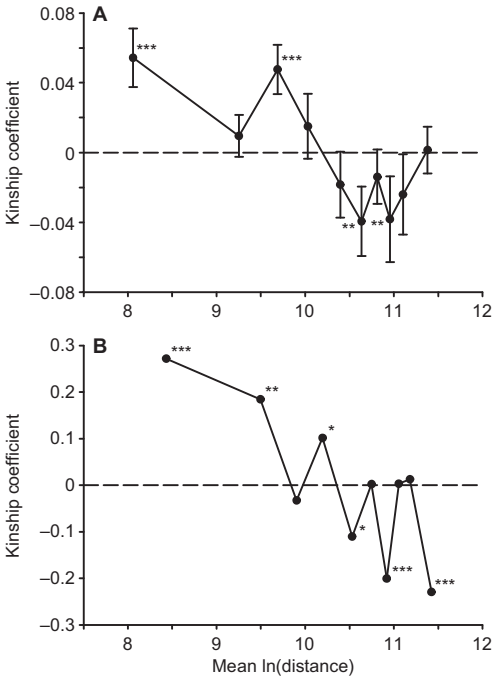

Fig. 3. Average Loiselle's kinship coefficient plotted against logarithmic distance between pairs of Saimaa ringed seal placentas. The plots are based on (**A**) 11 microsatellite loci, and (**B**) mtDNA haplotypes. Asterisks denote distance classes that differ significantly from mean kinship: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

tive parents (PE_3) for both reference data sets, but not for placentas. Moreover, none of the data sets provided enough resolution for excluding one parent (PE_1 and PE_2; Table 4).

Table 4. Probabilities of identity and exclusion estimated for Saimaa ringed seal placentas genotyped at 11 loci, as well as for the 11- and 17-locus reference data sets. PI = probability of identity for unrelated individuals, PI_{SIB} = probability of identity for siblings, PE_1 = probability for excluding a putative parent when the other parent is known, PE_2 = probability for excluding a putative parent when the other parent is unknown, PE_3 = probability of excluding two putative parents.

	Placentas	Reference data set	
		11 loci	17 loci
PI	2.098×10^{-4}	4.581×10^{-5}	4.817×10^{-7}
PI_{SIB}	1.695×10^{-2}	1.010×10^{-2}	1.193×10^{-3}
PE_1	0.878	0.930	0.978
PE_2	0.638	0.734	0.839
PE_3	0.972	0.990	0.999

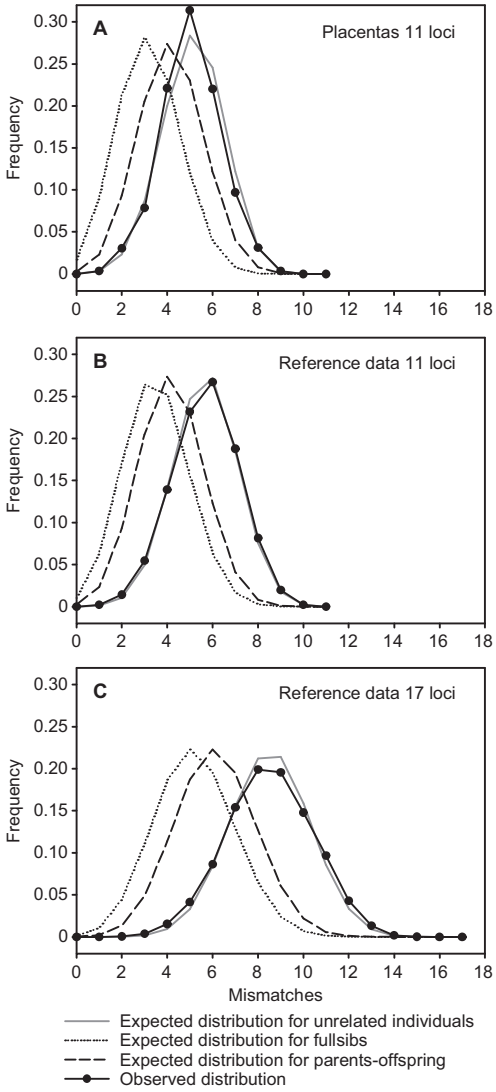

Fig. 4. Mismatch distributions among genotypes of Saimaa ringed seal placentas based on (**A**) 11 microsatellite loci, and within the reference data set based on (**B**) 11 loci and (**C**) 17 loci.

Mismatch distributions estimated for placentas and the 11- and 17-locus reference data sets were fairly consistent with the expected distributions of unrelated individuals (Fig. 4). Observed and expected distributions did not differ in the case of placentas ($\chi^2 = 9.85$, df = 8, $p = 0.276$), but a highly significant deviation was found in both reference data sets ($\chi^2 = 30.39$, df = 10,

$p < 0.001$ for the 11-locus reference data set, and $\chi^2 = 111.43$, df = 13, $p < 0.001$ for the 17-locus data set). Deviations from expectation are, however, not pronounced (Fig. 4B and C), and are probably explained by spatial genetic structure in the population (*see* above) and the relatively large sample sizes in the reference data sets. The observed mismatch distributions of placentas and the 11-locus reference data set differed significantly ($\chi^2 = 206.26$, df = 9, $p < 0.001$), because the distribution based on placental genotypes is slightly shifted towards smaller numbers (Fig. 4A and B).

No pairs of individuals with fully matching multilocus genotypes were found within any of the data sets. There were four pairs with a single mismatch (1MM-pairs) in the placental and 30 in the 11-locus reference data, whereas none were observed in the 17-locus data set. The number of 2MM-pairs was 33 for placentas, while 206 and 8 pairs were present in the 11- and 17-locus reference data sets, respectively. However, when the placental and 11-locus reference data sets were combined, we found five UC samples having full match with a genotype present in the reference data set; however, the matching reference individuals had died 3–30 years before collection of the placentas.

In COLONY analyses, replicate runs of each data set produced different results, and the analyses generally did not seem to have enough power to resolve relationships. The results suggested parentage of individuals that had died before the birth of suggested offspring, and also produced unrealistically numerous full- and half-sibling pairs. Moreover, the aforementioned placentas of seal twins were not recognised as siblings.

Discussion

The ecology of the critically endangered Saimaa ringed seal is well known as a result of three decades of intensive field research and telemetry studies (e.g., Hyvärinen *et al*. 1995, Rautio *et al*. 2009, Niemi *et al*. 2012). Nevertheless, as the population density is low, and the animal itself elusive and difficult to capture, all aspects of its ecology and behaviour cannot be studied using traditional monitoring approaches. Recent population-genetic analyses have demonstrated very low genetic variability in the population (Palo *et al*. 2003, Valtonen *et al*. 2012, Martinez-Bakker *et al*. 2013, Valtonen *et al*. 2014), and have revealed clear spatial structuring among the main breeding areas, indicating limited movement of Saimaa ringed seals and especially females (Valtonen *et al*. 2012, 2014). However, those analyses were mainly based on stillborn, by-caught, and stranded seals, of which only about 25 are recovered each year (Metsähallitus 2014). In addition, the samples were "retrospective" in the sense that the individuals were dead and, hence, removed from the population.

Under these circumstances, placentas collected from breeding sites could provide a highly useful source for non-invasive genetic sampling of live individuals: because of the thorough springtime breeding-site inspections conducted by Parks & Wildlife Finland (the authority responsible for monitoring and conservation of the Saimaa ringed seal), placentas of nearly half of the pups born each year can be recovered with a reasonable effort. Compared with other non-invasive samples such as hair and faeces, placentas provide a large amount of DNA, as well as the theoretical prospect of genotyping both the offspring and its mother from a single sample (cf. Stewart & Stewart 2009). As our analyses show, the pup's multilocus genotype can be reliably inferred from umbilical cord samples. Unfortunately, we did not succeed in genotyping the mothers, most likely due to mixture of maternal and offspring tissues and, therefore, genotypes, on the maternal side of the placenta.

Despite the limitation concerning the identification of breeding females, our results demonstrate that genetic tags obtained from umbilical cords could be used for studies on postnatal movements and long-term survival of Saimaa ringed seal individuals. Such studies could be conducted by comparing placental multilocus genotypes to tags that are later obtained from, for example, hair samples collected from haul-out sites, from live seals captured during telemetry studies, or from by-caught or stranded carcasses.

The extremely low microsatellite diversity of the Saimaa ringed seal ($H_E = 0.36$; Valtonen *et al*. 2014) poses a clear challenge for genetically-based tagging (cf. Waits & Paetkau

2005). However, sufficient resolution for individual identification is provided by our panel of 17 microsatellite loci: the estimated probability of identity for unrelated individuals ($PI = 4.8 \times 10^{-7}$) is lower, and that for siblings ($PI_{SIB} = 1.2 \times 10^{-3}$) very close, to the conservative threshold values (1×10^{-6} and 1×10^{-3}, respectively) recommended by McKelvey and Schwartz (2004). In the current population of approximately 300 seals (Metsähallitus 2014), our figures translate to 0.0001 expected full genotype matches for unrelated individuals and 0.36 matches for siblings, although the true numbers may be slightly higher due to the pronounced spatial genetic differentiation in the population found by Valtonen *et al*. (2012, 2014), i.e., because seals within each region tend to be more closely related and, thus, have more similar genotypes than individuals on average in the population. PI and PI_{SIB} values are higher for the 11-locus placental and reference data sets, and numbers of 1MM- and 2MM-pairs in their mismatch distributions (Fig. 4A and B) exceed the recommendations of Waits and Paetkau (2005) (< 1–2 1MM- and < 10 2MM-pairs in the data). Nevertheless, as pointed out by Waits and Paetkau (2005), the number of loci required also depends on the number of individuals to be compared, so even the 11-locus panel could be sufficient for spatially and/or temporally restricted surveys of the focal population.

Although our marker system allows genetic identification of individuals, the low population-level variability means that the limits of even the full 17-locus panel are reached when attempting to infer parentage or sibship among the sampled individuals and/or placentas. This is especially seen in the kinship analyses in COLONY, in which the output of all runs suggested implausible or impossible parentage and sibship. The unfortunate consequence of this is that one of our main goals could not be reached, because pups (placentas) collected from the same site during different springs could not be confidently determined to be siblings. At least some sibling pairs are likely to be present in our data set, because placentas having identical mtDNA haplotypes were collected from the same or closely located birth-lair sites in different years (Fig. 1B), but this could also be explained by the generally low level of mtDNA variation and the presence of an isolation-by-distance pattern within the lake (Fig. 3, and Valtonen *et al*. 2014). Importantly, however, the analytical impediments that follow from lack of marker resolution can be overcome by applying new, genome-scan based methods (e.g., Tokarska *et al*. 2009), so field-collected placentas undoubtedly can also be applied for studying breeding-site fidelity of females in the near future.

Interestingly, our results demonstrate that key population-level diversity and differentiation indices can be estimated based on placental samples. Both microsatellite and mtDNA diversities of placentas corresponded closely with previous results derived from large reference data sets comprising individuals found dead during a time span of 30 years (Valtonen *et al*. 2012, 2014), especially after applying rarefaction to correct for differences in sample size. No differences in microsatellite allele frequencies were detected between placentas and a subset of the reference data representing the same time span, the 2000s, and mtDNA haplotype frequencies in placentas and the reference data set were likewise highly correlated. Also the isolation-by-distance patterns of placentas were very similar to the findings of Valtonen *et al*. (2014).

Conclusions and further prospects

This study is the first describing the utility of placentas in non-invasive genetic monitoring of a natural population. Saimaa ringed seal placentas are relatively easily collected from birth-lair sites, and comparisons with existing reference data sets demonstrate that placentas can be used for estimating standard population-genetic parameters in separate breeding areas or within the whole lake. Genotyping pre-dispersal juveniles from umbilical cord samples provides a unique opportunity for tracking individuals from their natal site to later recapture(s) or death, yielding information on their dispersal patterns and long-term survival prospects. In addition, determining the pups' gender from umbilical cord samples using genetic markers (Curtis *et al*. 2007) would yield information on the sex ratio within the population and in different breeding

areas. Unfortunately, even our 17-locus microsatellite panel does not provide enough discriminatory power for pedigree construction and kinship analyses, due to the fact that this small population with extremely low genetic diversity and significant structuring (Valtonen *et al*. 2012, 2014) is inevitably inbred, but such analyses will undoubtedly be enabled by next-generation sequencing and genotyping technologies in the near future.

As our results show, high-quality DNA can be obtained from placentas, but wider application of our method is to some degree restricted by postnatal consumption of the placenta (placentophagia) by females in most mammalian species (Kristal *et al*. 2012). However, the order Pinnipedia is one of the few exceptions here, and genetic monitoring based on placental samples could be used, for example, for the Ladoga ringed seal (*P. h. ladogensis*), which inhabits Lake Ladoga in northwestern Russia, and which has breeding habits that closely resemble those of the Saimaa ringed seal (Kunnasranta *et al*. 2001). In addition, many other pinnipeds, such as grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*), and all otariids, typically give birth on land and regularly at the same locations (Riedman 1990), which offers a good opportunity for collection of placentas also from these species.

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Appendix. Collection information on the Saimaa ringed seal placentas included in this study. Collection date and location, samples taken (MS = maternal side; FS = foetal side; UC = umbilical cord/ vein; OP = orange particles; BS = blood) and quality (1 = fresh; 2 = partly decomposed; 3 = decomposed) of each placenta are given.

ID	Code	Collection date	Location	Samples	Quality
I-18	I-18_P_y2000	14 Apr. 2000	Pihlajavesi area	MS, FS, UC, OP, BS	1
I-19	I-19_P_y2000	17 Apr. 2000	Pihlajavesi area	MS, FS, UC, OP	2
I-20	I-20_P_y2001	12 Apr. 2001	Pihlajavesi area	MS, FS, UC, OP, BS	1
I-21	I-21_P_y2002	22 Apr. 2002	Pihlajavesi area	MS, FS, UC, OP	2
I-22	I-22_S_y2004	14 Apr. 2004	Southern Saimaa	FS	2
I-23	I-23_P_y2004	6 Apr. 2004	Pihlajavesi area	MS, FS, UC, OP	2
I-24	I-24_S_y2006	2006	Southern Saimaa	FS	2
I-26	I-26_S_y2009	18 Apr. 2009	Southern Saimaa	MS, FS, UC, OP, BS	1
I-27	I-27_H_y2009	19 Apr. 2009	Main Haukivesi area	MS, FS, UC, OP, BS	1
I-28	I-28_P_y2009	25 Apr. 2009	Pihlajavesi area	MS, FS, UC, OP	2
I-29	I-29_H_y2009	19 May 2009	Main Haukivesi area	MS, FS, UC, OP	3
I-30	I-30_H_y2009	19 May 2009	Main Haukivesi area	MS, FS, UC, OP	3
I-31	I-31_H_y2009	19 May 2009	Main Haukivesi area	MS, FS, UC, OP	3
I-32	I-32_P_y2009	28 May 2009	Pihlajavesi area	MS, FS, UC, OP	2
I-33A	I-33A_P_y2009	28 May 2009	Pihlajavesi area	MS, FS, UC, OP	3
I-33B	I-33B_P_y2009	28 May 2009	Pihlajavesi area	MS, FS, UC, OP	2
I-34	I-34_P_y2009	28 May 2009	Pihlajavesi area	MS, FS, UC, OP	2
I-35	I-35_P_y2009	28 May 2009	Pihlajavesi area	FS	3
I-36	I-36_P_y2009	29 May 2009	Pihlajavesi area	MS, FS, UC, OP	3
I-37	I-37_P_y2009	29 May 2009	Pihlajavesi area	MS, FS, UC, OP	3
I-38	I-38_P_y2010	14 May 2010	Pihlajavesi area	MS, FS, OP	2
I-39	I-39_P_y2010	13 May 2010	Pihlajavesi area	MS, FS, UC, OP	1
I-40	I-40_P_y2010	13 May 2010	Pihlajavesi area	MS, FS, UC, OP	3
I-41	I-41_P_y2010	13 May 2010	Pihlajavesi area	MS, FS, UC, OP	2
I-42	I-42_P_y2010	11 May 2010	Pihlajavesi area	MS, FS, UC, OP	1
I-43	I-43_P_y2010	12 May 2010	Pihlajavesi area	MS, FS, UC, OP	1
I-44	I-44_P_y2010	13 May 2010	Pihlajavesi area	MS, FS, UC, OP	2
I-45	I-45_P_y2010	13 May 2010	Pihlajavesi area	MS, FS, OP	3
I-46	I-46_P_y2010	13 May 2010	Pihlajavesi area	MS, FS, UC, OP	2
I-47	I-47_P_y2010	12 May 2010	Pihlajavesi area	MS, FS, UC, OP	3
I-48	I-48_P_y2010	13 May 2010	Pihlajavesi area	MS, FS, UC, OP	3
I-49	I-49_S_y2010	27 May 2010	Southern Saimaa	MS, FS, UC, OP	3
I-50	I-50_H_y2010	10 May 2010	Main Haukivesi area	MS, FS, UC, OP	3
I-51	I-51_H_y2010	3 May 2010	Main Haukivesi area	MS, FS, UC, OP	3
I-52	I-52_H_y2010	3 May 2010	Main Haukivesi area	MS, FS, UC, OP	2
I-53	I-53_H_y2010	12 May 2010	Main Haukivesi area	MS, FS, UC, OP	2
I-54	I-54_H_y2010	6 May 2010	Main Haukivesi area	MS, FS, UC, OP	1
I-55	I-55_H_y2010	12 May 2010	Main Haukivesi area	MS, FS, UC, OP	1
I-56	I-56_H_y2010	15 May 2010	Main Haukivesi area	MS, FS, UC, OP	2
I-57	I-57_H_y2010	12 May 2010	Main Haukivesi area	MS, FS, UC, OP	1
I-58	I-58_H_y2010	12 May 2010	Main Haukivesi area	MS, FS, UC, OP	2

continued

Appendix. Continued.

ID	Code	Collection date	Location	Samples	Quality
I-59	I-59_N_y2011	19 May 2011	Northern Saimaa	MS, FS, UC, OP	2
I-60	I-60_K_y2011	23 May 2011	Kolovesi	MS, FS, UC, OP	2
I-61	I-61_H_y2011	12 May 2011	Main Haukivesi area	FS	3
I-62	I-62_H_y2011	12 May 2011	Main Haukivesi area	MS, FS, UC, OP	3
I-63	I-63_H_y2011	16 May 2011	Main Haukivesi area	MS, FS, UC, OP	2
I-64	I-64_H_y2011	12 May 2011	Main Haukivesi area	MS, FS, UC, OP	2
I-65	I-65_H_y2011	13 May 2011	Main Haukivesi area	MS, FS, UC, OP	1
I-66	I-66_P_y2011	9 May 2011	Pihlajavesi area	MS, FS, UC, OP	1
I-67	I-67_P_y2011	9 May 2011	Pihlajavesi area	FS	2
I-68	I-68_P_y2011	10 May 2011	Pihlajavesi area	MS, FS, UC, OP	2
I-69	I-69_P_y2011	10 May 2011	Pihlajavesi area	MS, FS, UC, OP	2
I-70	I-70_P_y2011	10 May 2011	Pihlajavesi area	FS	2
I-71	I-71_P_y2011	11 May 2011	Pihlajavesi area	MS, FS, UC, OP	1
I-73	I-73_P_y2011	11 May 2011	Pihlajavesi area	MS, FS, UC, OP	1
I-74	I-74_P_y2011	11 May 2011	Pihlajavesi area	MS, FS, UC, OP	2
I-75	I-75_P_y2011	12 May 2011	Pihlajavesi area	MS, FS, UC, OP	2
I-76	I-76_P_y2011	12 May 2011	Pihlajavesi area	MS, FS, UC, OP	2
I-77	I-77_P_y2011	12 May 2011	Pihlajavesi area	MS, FS, OP	2
I-78	I-78_P_y2011	12 May 2011	Pihlajavesi area	MS, FS, UC, OP	3
I-79	I-79_S_y2011	7 May 2011	Southern Saimaa	MS, FS, UC, OP	3
I-82	I-82_S_y2011	8 May 2011	Southern Saimaa	FS	2
I-84	I-84_S_y2011	8 May 2011	Southern Saimaa	MS, FS, UC, OP	3
I-85	I-85_H_y2011	23 Apr. 2011	Main Haukivesi area	MS, FS, UC, OP	1
I-86	I-86_H_y2011	20 Apr. 2011	Main Haukivesi area	FS	2
I-87	I-87_H_y2011	2011	Main Haukivesi area	MS, FS, UC, OP	2

II

A previously undescribed Javan tiger *Panthera tigris sondaica* specimen, and other old, rare tiger specimens in the Finnish Museum of Natural History

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Running title: Javan tiger specimen in the FMNH

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Abstract. We describe a specimen of the extinct Javan tiger *Panthera tigris sondaica* in the Finnish Museum of Natural History LUOMUS (FMNH) in Helsinki, Finland. This specimen has not previously been described in the literature. It consists of the complete skeleton of a subadult individual collected in the nineteenth century, supposedly in Java. We confirmed the specimen's identity as a Javan tiger with a DNA analysis, an identification which was supported by a morphological examination. In addition to this Javan tiger specimen, we also subjected a few other old, wild-collected tiger specimens in the collections of the FMNH to DNA analysis. Notable results of these analyses were the identification of two twentieth-century flat skin specimens of the South China tiger *P. t. amoyensis*, which still survives in captivity but is extinct in the wild, and a probable Malayan tiger *P. t. jacksoni* skull specimen. Results of a DNA analysis of one further nineteenth-century specimen, a mounted skin of a juvenile, were inconclusive beyond establishing that it originates from the Sunda Islands; however, certain features of this specimen's pelage suggest that it, too, may be a Javan tiger.

Key words: extinction, haplotypes, mtDNA, Sunda Islands.

The tiger *Panthera tigris*, one of the most recognizable and charismatic of extant mammals, has in recent decades and centuries become increasingly rare due to human persecution and habitat destruction. In the long run the tiger's continued survival in the wild is dependent on the implementation of efficient conservation measures (Dinerstein et al. 2007).

The tiger has traditionally been divided into eight Recent subspecies: the Bengal or Indian tiger *P. t. tigris*, the Caspian tiger *P. t. virgata*, the Amur or Siberian tiger *P. t. altaica*, the South China tiger *P. t. amoyensis*, the Indochinese tiger *P. t. corbetti*, the Sumatran tiger *P. t. sumatrae*, the Javan tiger *P. t. sondaica*, and the Balinese tiger *P. t. balica* (Mazák 1981, 2013). A ninth subspecies, the Malayan tiger *P. t. jacksoni*, was recognized by Luo et al. (2004, 2008, 2010). Of these putative subspecies, the Balinese tiger became extinct in the 1930's (Seidensticker 1987). The Caspian tiger probably became extinct in the early 1970's (Can 2004), whereas the Javan tiger apparently disappeared in the 1970's (Seidensticker and Suyono 1980; Seidensticker 1987). The South China tiger became extinct in the wild in the early 1990's (Tilson et al. 2004), but persists in captivity. The remaining wild populations of the other tiger subspecies are also threatened with extinction; however, Amur, Bengal, and Sumatran tigers are relatively numerous in captivity (Luo et al. 2008).

It should be noted that tiger taxonomy is a fairly controversial subject. Some authors have suggested that the differences between various tiger taxa are sufficiently great to justify even species-level distinctions (Cracraft et al. 1998; Mazák and Groves 2006). In contrast, other authors have suggested that the number of "traditionally" recognized tiger taxa is too high and that only two or perhaps three subspecies should be recognized (Kitchener 1999; Wentzel et al. 1999; Kitchener and Dugmore 2000; Kitchener and Yamaguchi 2010; Wilting et al. 2015; Kitchener et al. 2017). In this paper, we recognize nine tiger subspecies, *sensu* Luo et al. (2004). However, we do this for the sake of convenience and clarity of communication rather than as an explicit taxonomic statement.

Recently, Yamaguchi et al. (2013) did a global survey of Javan, Balinese, and Caspian tiger specimens held in natural history museums. The authors identified 88 specimens of Javan tigers in various collections in Europe and Asia (as well as one specimen that was assigned by the authors as either a Javan or a Balinese tiger). Here, we report the existence of a further Javan tiger specimen, overlooked by Yamaguchi et al. (2013). This specimen, a complete mounted skeleton, has been housed in the Finnish Museum of Natural History LUOMUS (FMNH) in Helsinki, Finland, since the nineteenth century. It has been on public display from time to time, but has not been properly described in the scientific literature. In addition to bringing attention to this neglected

specimen, we present the results of morphological and molecular investigations of the taxonomic identities of a few other old, poorly documented tiger specimens in the collections of the FMNH.

Materials and methods

History and morphological description of the specimen

The nucleus of the FMNH osteological collection was formed in the mid-nineteenth century by Evert Julius Bonsdorff (1810–1898), a Professor of Anatomy and Physiology at the University of Helsinki. Bonsdorff collected several hundred mammalian specimens from all around the world (Palmén 1878). Many of the specimens were purchased from professional animal dealers rather than collected by trained naturalists; thus, their locality data are often vague.

Bonsdorff's collection includes a mounted tiger skeleton, which has the FMNH catalogue number UN 2485 (Fig. 1). According to the original label, the specimen was collected in 1857. The specimen was placed on public display shortly thereafter and is mentioned in a nineteenth-century museum catalogue (Palmén 1878). In this catalogue it is also stated that the specimen's place of origin is "Java". In the late 1990's, UN 2485 was taken off public display and placed in storage, where it remained until 2018, when the mounted skeleton was taken apart and reassembled. UN 2485 was put back on public display at the FMNH in August 2018.

Fig. 1. UN 2485, *Panthera tigris sondaica*. Note: the photograph was taken in May 2018, after the skeleton had been cleaned and re-assembled. Photo: Janne Granroth.

UN 2485 is subadult, and the epiphyseal plates of its limb bones are not ossified. Its sex is not recorded, but it is apparently a male. This is suggested by the relatively long and large canines, as well as other cranial dimensions. For instance, the greatest length of the skull is 311 mm and the condylobasal length is 283 mm. Thus, even though the individual was not fully adult, its skull size was already outside of the recorded range of female Javan tigers and within that of males (Mazák 1981, 2013; Mazák and Groves 2006). See Table 1 for further details. According to Mazák and Groves (2006), cranial morphology reliably distinguishes the Javan tiger from Sumatran and mainland tigers (but not from Balinese tigers). Particularly diagnostic, in their view, are the nasal bones, which should be long and narrow, and the occipital plane, which should be "obviously" narrow in a Javan tiger (Mazák and Groves 2006: p. 281; cf. Fig. 1 in Yamaguchi et al. 2013, and Plate I in Pocock 1929). In both these respects, the morphology of UN 2485 is consistent with its identification as the Javan tiger. Interestingly, UN 2485 has small "ramps" on the upper orbital edges, which are usually not present in Javan tigers (Yamaguchi et al. 2013). However, the specimen's concave mandible shape (cf. profile type 1, Fig. 2 in Yamaguchi et al. 2013) is typically *sondaica*-like.

Table 1. Selected skull measurements of UN 2485, compared with data from the literature[1]

		Mazák and Groves (2006)				Mazák (1981, 2013)			
dimension	UN 2485	♂, min.	♂, max.	♀, min.	♀, max.	♂, min.	♂, max.	♀, min.	♀, max.
Greatest skull length	311.0	306.0	338.0	260.0	292.0	306.0	349.0	270.0	292.0
Condylobasal length	283.0	263.0	299.6	234.0	262.0	269.0	303.0	241.2	262.0
Mastoid width	123.7	114.0	131.0	101.5	115.0	N/A	N/A	N/A	N/A
Zygomatic width	209.0	198.0	243.7	166.0	200.0	198.0	246.0	181.0	209.0
Rostral width	93.92	88.40	99.40	75.50	86.00	88.40	99.40	76.70	86.50
Nasal length	102.8	100.4	110.0	84.00	94.50				
Interorbital width	60.46	56.00	66.50	47.00	59.00				
Postorbital width	58.91	50.80	61.80	50.00	56.50				
Occipital height	95.10	91.00	102.0	77.00	86.00				
Mandible length	207.0	198.0	226.0	172.0	197.3				
Mandible height	100.4	96.00	117.5	79.00	97.00				
Upper carnassial length	34.49	31.89	36.00	30.00	32.50				
Lower carnassial length	24.88	23.50	27.00	21.00	24.00				

[1] Measurements are in millimeters.

Genetic analyses

In addition to a morphological examination, we performed mitochondrial DNA (mtDNA) analyses to establish the taxonomic identity of UN 2485. Six other tiger specimens in the FMNH collections were also subjected to mtDNA analyses. These specimens consist of one skull, two mounted skins, and three flat skins (Table 2). The specimens were selected because they have vague or missing locality data, and were not identified to subspecies level. It is known, however, that all these specimens are old; the two youngest specimens are from the late 1950's and 1960, respectively, and all others were collected more than a century ago. Furthermore, they almost certainly represent wild, rather than captive, animals.

Table 2. The FMNH tiger specimens analysed in this study

FMNH collection number	specimen type	locality data	date of collection	
UN 365	mounted skin	Southeast Asia[1]	1857	juvenile, ♀
UN 378	mounted skin		19th century	
UN 2137	flat skin	China[2]	before 1956–1957	
UN 2166	flat skin	China[2]	1960	juvenile
UN 2390	flat skin		before 1912	
UN 2484	skull	India	1900	
UN 2485	mounted skeleton	Java	1857	

[1] "India ost", i.e., "East Indies" in the original label.

[2] Presented as a gift to the Finnish Embassy in Beijing by the Government of the People's Republic of China.

The steps preceding PCR amplification were conducted in sterile room facilities at the Center of Microscopy and Nanotechnology at the University of Oulu, following established ancient DNA research protocols to prevent contamination. DNA was extracted from tooth, bone, footpad, skin, and hair samples. The outermost surface of each hard tissue sample was removed, after which a powder sample was obtained by drilling. DNA extraction followed the protocol originally described by Yang et al. (1998), and modified by Gamba et al. (2014, 2016). Double digestion, however, was not used. Negative controls were included in extractions and PCR amplifications to monitor possible contamination.

The samples were analysed by typing single-nucleotide polymorphisms (SNPs) in four mtDNA regions (ND2, COI, ND6, and CytB), which have been shown to be diagnostic of the various tiger subspecies by previous studies (Luo et al. 2004; Driscoll et al. 2009; Kitpipit et al. 2012; Sun et al. 2015; Wilting et al. 2015; Xue et al. 2015; Buddhakosai et al. 2016). The used PCR primers are shown in Table 3. Each fragment was amplified and sequenced a minimum of two times to identify mis-incorporated bases that result from post-mortem DNA damage. PCR reactions were performed in a 25 µl volume of 1× PCR Buffer (QIAGEN), 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTPs, 1 mg/ml BSA, 2 units of HotStarTaq DNA Polymerase (QIAGEN), and 2 µl of DNA extract. The PCR cycling protocol follows Xue et al. (2015), except that we used 95°C in the initial denaturation, and 55 additional cycles after the touchdown step. Each successful PCR reaction was purified by using Exonuclease I and Shrimp Alkaline Phosphatase. BigDye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific) was used for the sequencing reactions. Each fragment was sequenced in both directions, and the reactions were analysed with ABI 3730 DNA Analyzer (Applied Biosystems). Chromatograms were inspected and edited using

the program CodonCode Aligner (Version 4.0.4, CodonCode Corporation). Sequences obtained in this study have been deposited in GenBank under the accession numbers MH290767–MH290787.

Table 3. Primer pairs used in this study

Primer name	Sequence	PCR length (bp)	Reference
ND6-1F	TAACTATACAGTGCTGCAATTCCT	229	Driscoll et al. (2009)
ND6-1R	CTATGGCTACTGAGCCCTACC		Driscoll et al. (2009)
ND6-shortF	TAGCAAGCAGTCAACAACTC	100	This study
ND6-1R	CTATGGCTACTGAGCCCTACC		Driscoll et al. (2009)
CytbbF	CCCTCAGGAATGGTGTCC	156	Driscoll et al. (2009)
CytbbR	GGCGGGGATGTAGTTATCA		Driscoll et al. (2009)
ND2bF	TATCACAAACATGAAACAAAACG	185	Driscoll et al. (2009)
ND2bR	GTATAGGTTAAGTAGTGCTGTTATG		Driscoll et al. (2009)
CO1F	GCTGATTGGCCACTCTTCAC	190	Driscoll et al. (2009)
CO1R	ACTCCTATTGACAAGACGTAGTGGA		Driscoll et al. (2009)

We compared the obtained genetic profiles to the profiles reported in previous studies (Luo et al. 2004; Driscoll et al. 2009; Kitpipit et al. 2012; Sun et al. 2015; Wilting et al. 2015; Xue et al. 2015; Buddhakosai et al. 2016). The sequences were aligned in the program MEGA7 (Kumar et al. 2016), using the ClustalW algorithm (Higgins et al. 1994), and a table of the variable positions was compiled.

We also built a Bayesian phylogenetic tree by using otherwise the same data set as above, except that a leopard *Panthera pardus* mtDNA sequence (accession no. EF551002, Wei et al. 2011) was added as an outgroup. Also, haplotype SUMx was removed from the analysis due to it having missing data on a polymorphic site. Sequences were trimmed to equal length, and the data set was reduced into haplotypes. We included the 138 bp COI region that was sequenced only from the study sample UN 2137, other study samples having missing data in this region. jModelTest (Version 2.1.4, Guindon and Gascuel 2003; Darriba et al. 2012) was used to determine the most optimal nucleotide substitution model according to the Bayesian Information Criterion. For this analysis, sample UN 365 was removed from the data set due to it having lots of missing data. The most optimal model, HKY+I, was used in the subsequent run on MrBayes (Version 3.2, Ronquist et al. 2012). The analysis was run for 2 500 000 generations saving every 1000 sample. The first 250 000 samples were discarded as burn-in. The 50 percent majority rule tree was visualized with FigTree (Version 1.4, Available at http://tree.bio.ed.ac.uk/software/figtree/, Accessed 28 May 2018).

Results

The mtDNA results are shown in Table 4, and the phylogenetic tree showing the relationships of the study samples with the reference data is depicted in Fig. 2. The nucleotide positions discussed in the following text refer to the nucleotide positions in the domestic cat *Felis catus* mitochondrial genome (Lopez et al. 1996).

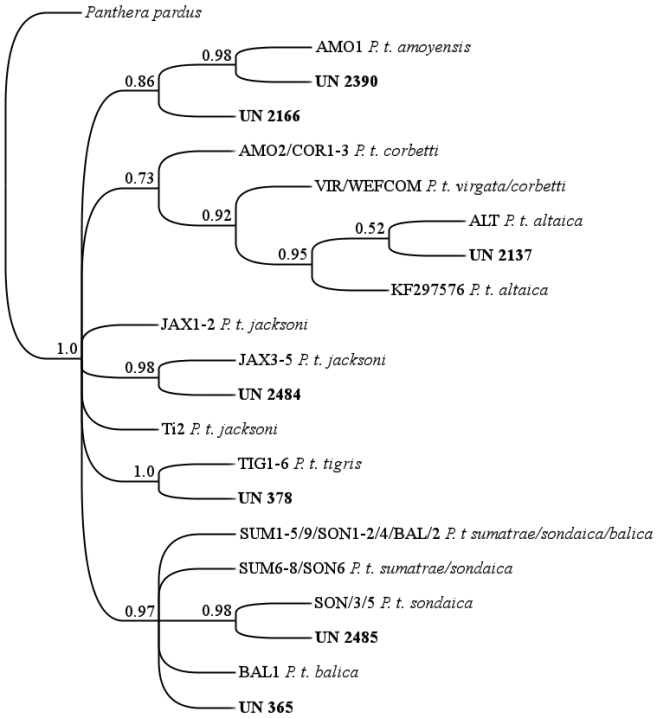

Fig. 2. Bayesian 50 percent majority rule tree showing the phylogenetic relationships of the study samples (in bold) with the reference data. Posterior probability values are shown above branches.

The suspected Javan tiger UN 2485 had two SNPs which are diagnostic of Sunda Islands tigers (T on position 5608 and G on position 15743). This specimen further has an SNP which has only been found in Javan tiger specimens (G on position 14698). Thus, the genotyping results strongly suggest that UN 2485 is a Javan tiger *Panthera tigris sondaica*.

Table 4. Comparison of the genetic profiles of the FMNH *Panthera tigris* study samples with reference data

			Region on the mitochondrial genome	ND2				COI		ND6						CytB		
			nucleotide position[a]	5 5 1 5	5 1 8	5 3 3	5 6 0 8	7 2 8 7	7 3 0 4	1 4 4 1	1 4 5 7	1 4 5 2	1 4 6 8	1 4 6 8	1 4 7 1	1 5 6 1	1 5 7 3	1 5 7 6
	Sample no		**Inferred subspecies of the study samples**															
STUDY SAMPLES	UN 365		Sumatran / Javan / Balinese tiger	?	?	?	?	?	?	?	?	?	?	—	—	—	G	—
	UN 378		Bengal tiger	—	G	A	—	?	?	T	—	—	T	—	—	—	—	—
	UN 2137		Amur tiger	—	G	—	—	C	—	T	—	—	—	—	A	—	—	C
	UN 2166		South China tiger	—	G	—	—	?	?	—	—	—	—	—	—	—	—	—
	UN 2390		South China tiger	—	—	—	—	?	?	—	—	—	—	—	—	—	—	—
	UN 2484		Malayan tiger	G	G	—	—	?	?	T	—	—	—	—	—	—	—	—
	UN 2485		Javan tiger	—	G	—	T	?	?	T	—	—	—	G	—	—	G	—
	Common name	**Scientific name**	**Haplotype[b]**															
REFERENCE DATA	South China tiger	*P. t. amoyensis*	AMO1[2]	A	A	G	C	T	G	C	A	C	C	A	G	C	A	T
	Indochinese tiger	*P. t. corbetti*	AMO2 (CORa)[1], COR1-3[31]	—	G	—	—	—	—	T	—	—	—	—	—	—	—	C
	Malayan tiger	*P. t. jacksoni*	COR4 (JAX1)[11], COR5 (JAX2)[1]	—	G	—	—	—	—	T	—	—	—	—	—	T	—	—
	Malayan tiger	*P. t. jacksoni*	COR6 (JAX3)[2], COR7 (JAX4)[5], COR8 (JAX5)[3]	G	G	—	—	—	—	T	—	—	—	—	—	—	—	—
	Malayan tiger	*P. t. jacksoni*	Ti2[1]	—	G	—	—	—	—	T	—	—	—	—	—	—	—	—
	Bengal tiger	*P. t. tigris*	TIG1-6[15]	—	G	A	—	—	—	T	—	—	T	—	—	—	—	—
	Caspian / Indochinese tiger	*P. t. virgata / corbetti*	PTV-2 (VIR)[17], WEFCOM[1]	—	G	—	—	—	—	T	—	—	—	—	A	—	—	C
	Amur tiger	*P. t. altaica*	ALT[13]	—	G	—	—	C	—	T	—	—	—	—	A	—	—	C
	Amur tiger	*P. t. altaica*	KF297576[1]	—	G	—	—	C	—	T	—	T	—	—	A	—	—	C
	Sumatran / Javan / Balinese tiger	*P. t. sumatrae / sondaica / balica*	SUM1-5[11], SUM9[1], SON1-2[3], SON4[1], BAL[2], BAL2[2]	—	G	—	T	—	—	T	—	—	—	—	—	—	G	—
	Sumatran tiger	*P. t. sumatrae*	SUMx[1]	—	G	—	?	—	—	T	—	—	—	—	—	—	G	—
	Sumatran / Javan tiger	*P. t. sumatrae / sondaica*	SUM6-8[5], SON6[1]	—	G	—	T	—	A	T	—	—	—	—	—	—	G	—
	Javan tiger	*P. t. sondaica*	SON[9], SON3[6], SON5[1]	—	G	—	T	—	—	T	—	—	—	G	—	—	G	—
	Balinese tiger	*P. t. balica*	BAL1[1]	—	G	—	T	—	—	T	G	—	—	—	—	—	G	—

[a] Nucleotide position on the domestic cat (*Felis catus*) mitochondrial genome (Lopez et al. 1996).

[b] Reference haplotypes are from Luo et al. (2004), Driscoll et al. (2009), Kitpipit et al. (2012), Sun et al. (2015), Wilting et al. (2015), Xue et al. (2015), and Buddhakosai et al. (2016).

Haplotypes that are on the same line form only one haplotype when observing the sequence regions studied here.

Number of individuals in the reference data carrying each haplotype is indicated with superscript.

— indicates identical base to haplotype AMO1.

? Indicates unknown base.

Grey color indicates subspecies specific SNPs according to Luo et al. (2004), Xue et al. (2015), and Wilting et al. (2015).

UN 365 (Fig. 3) is a mounted juvenile female specimen, approximately the size of an adult leopard, that was obtained via the Amsterdam-based animal dealer G. A. Frank (1809–1880) from "India ost" (i.e., the East Indies, meaning present-day Southeast Asia). The DNA isolated from this specimen was so degraded that we were not able to obtain full sequences from all targeted regions. We were, however, able to establish that specimen UN 365 lacked the Javan tiger-specific SNP (G on position 14698; see above). On the other hand, this specimen did possess one of the Sunda Islands tiger-diagnostic SNPs (G on position 15743). Thus, the mtDNA haplotype of the specimen strongly suggests that UN 365 originates from one of the Sunda Islands (see Discussion).

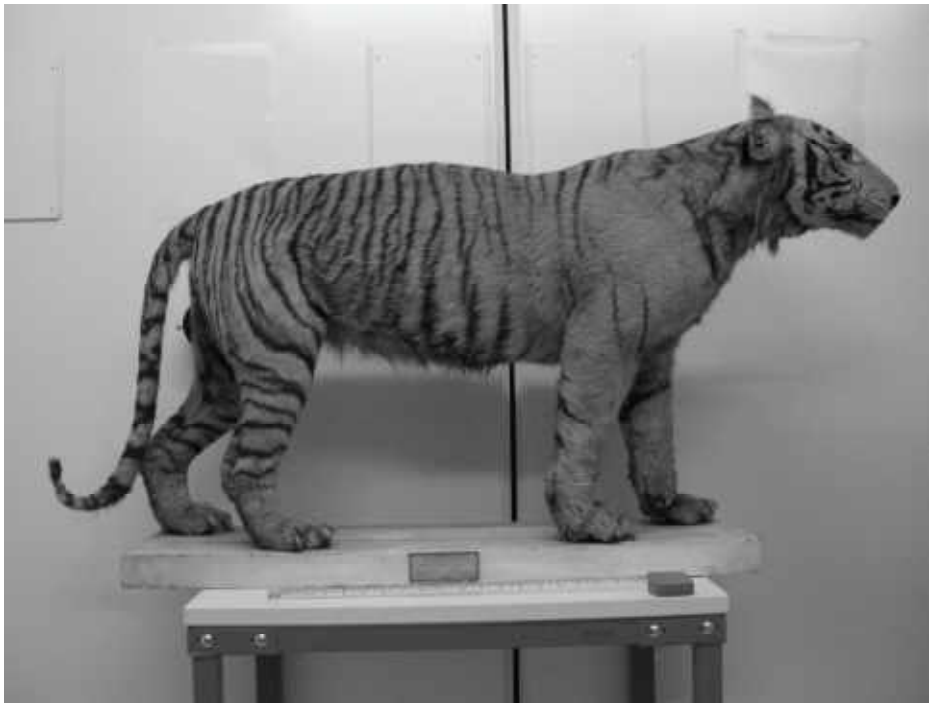

Fig. 3. Mounted juvenile specimen UN 365, as seen from the right. Length of exposed part of tape measure 50 cm. Photo: Henry Pihlström.

Specimen UN 378 is a mounted adult with no locality data. There is also no collection date for this specimen, but it is certainly from the nineteenth century. UN 378 had two SNPs which are diagnostic of Bengal tiger (A on position 5533 and T on position 14618). The results therefore strongly suggest that UN 378 is a Bengal tiger *Panthera tigris tigris*.

UN 2137 is a flat skin from China; this specimen, which lacks exact locality data, was donated to the Finnish Embassy in Beijing in the 1950's by the Government of the People's Republic of China. This individual had an SNP that has been observed only among Amur, Caspian and Indochinese tigers (A on position 14711). All Amur and Caspian tigers in the reference data share this SNP, but not all Indochinese tigers do. UN 2137 further had an SNP that is diagnostic of Amur tigers (C on position 7287). The results therefore strongly suggest that UN 2137 is an Amur tiger *Panthera tigris altaica.*

UN 2166 is a flat skin of a tiger cub, which, like UN 2137, was a gift presented to Finnish diplomats in China in 1960. DNA extracted from specimen UN 2166 had two SNPs which are diagnostic of the South China tiger (A on position 5518 and C on position 14591). The results therefore strongly suggest that UN 2166 is a South China tiger *Panthera tigris amoyensis*.

UN 2390 (Fig. 4) is a flat trophy skin, collected prior to 1912. It had an SNP which is diagnostic of the South China tiger *Panthera tigris amoyensis* (C on position 14591). On the other hand, this specimen did not have another SNP, which is also supposedly diagnostic of the South China tiger (A on position 5518). Nevertheless, when looking at the haplotype on the whole (Fig. 2), UN 2390 seems to be closest to the South China tiger haplotype AMO1, which suggests that UN 2390 is a South China tiger. It is possible that this specimen represents South China tiger genetic diversity which has disappeared from the current population, and which previous studies have not yet captured.

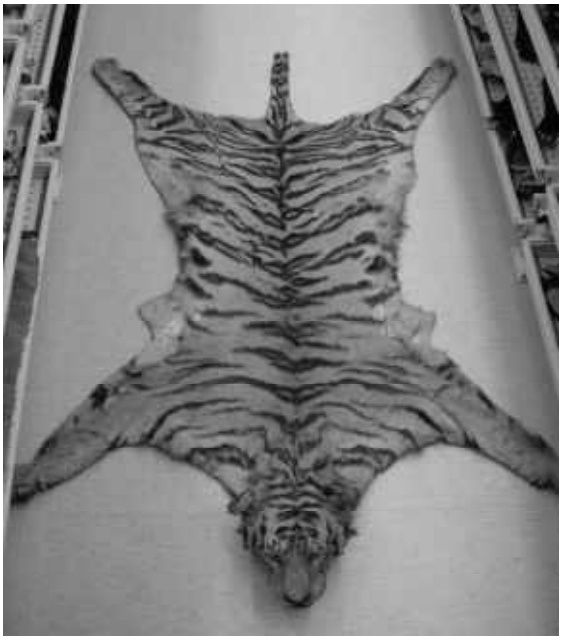

Fig. 4. UN 2390, *Panthera tigris amoyensis*. The distal part of the tail is missing. Photo: Henry Pihlström.

Finally, UN 2484 is a skull collected in "India" in 1900. It had a haplotype which in the reference data has only been found in the Malayan tiger. This haplotype differs from all other haplotypes by having G on the position 5515. The results therefore suggest that UN 2484 is a Malayan tiger *Panthera tigris jacksoni*.

Discussion

Javan tigers have been kept in zoological parks in the past but no captive individuals remain today. Despite occasional rumoured recent sightings, it is unfortunately virtually certain that the Javan tiger is also extinct in the wild (Seidensticker and Suyono 1980; Seidensticker 1986, 1987). Thus, the only remaining way to study Javan tigers is to investigate preserved specimens in natural history museum collections. Yamaguchi et al. (2013) located fewer than 90 Javan tiger specimens in museums worldwide. However, as the current study shows, previously undocumented

or unidentified Javan tiger specimens may still remain to be discovered in natural history museums or in private collections around the world.

In recent decades, molecular genetics has become a powerful tool for taxonomically identifying old museum specimens, complementing traditional morphology-based studies. Although DNA degrades over time, it is often possible to recover it from the tissues of decades-old or even centuries-old specimens (Wandeler et al. 2007; Burrell et al. 2015). In the case of tigers, both mitochondrial genomes (Kitpipit and Linacre 2012; Sun et al. 2015) and whole-genome sequences (Cho et al. 2013) are nowadays available for reference. In the current study, by using genetic markers we have been able to establish taxonomic identities of specimens completely or almost completely lacking useful provenance data, which it would hardly have been possible to identify only morphologically.

A few of the FMNH specimens showed unexpected kinds of genetic diversity. For example, based on its haplotypes the mounted nineteenth-century specimen UN 378 appears to be a Bengal tiger, i.e., *Panthera tigris tigris*. However, we note that an unpublished data set in GenBank (Wajjwalku et al., accession numbers KC879287–KC879297) of tigers presumably originating from Thailand includes three haplotypes that are very similar to the Bengal tiger haplotypes TIG1–6. One of these is, in fact, identical to the haplotype formed by TIG1, 2, 4, 5, and 6. Based on genetic variation of modern tiger populations, northern Indochina and southern China have been proposed as the centre of origin of the Pleistocene radiation of tigers (Luo et al. 2004; Buddhakosai et al. 2016). It is therefore possible that populations in this area still retain some ancestral haplotypes that have since become fixated in the Bengal tiger. The fact that UN 378 has specifically the haplotype that is found in Bengal tigers but not the other haplotypes that are found in Thailand, suggests that this individual more likely originates from India than from Thailand.

The three flat skin specimens investigated in the present study turned out to represent two different subspecies. UN 2137, originating from China in the 1950's, is an Amur tiger, i.e., a tiger taxon that still survives in the wild. The two other FMNH flat skin specimens, UN 2390 and UN 2166, are also of Chinese origin but represent the South China tiger. At the time when these two specimens were collected, this tiger taxon was still to be found in the wild (Tilson et al. 2004). Today, however, the South China tiger only survives in captivity.

The DNA samples taken from specimen UN 2484, a skull collected in 1900, suggest that it is a Malayan tiger. However, according to the original label UN 2484 is originally from "India", whereas the Malayan tiger has only been recorded from Peninsular Malaysia and from southern

Thailand (Luo et al. 2004; Buddhakosai et al. 2016). Assuming that this is not a case of accidental mislabelling, there are two possible explanations for this unexpected result. The unknown author of the original label may have had a different geographical concept of "India" in mind than a modern-day museum worker would have (e.g., using "India" to refer to the southern parts of Asia in general as opposed to just the Indian Peninsula). Alternatively, the skull may indeed have been collected from India in the modern geographical sense, but the specimen, which hails from an era when Asia's tiger populations were not only larger than today but also more interconnected, may represent a genetic lineage that has since disappeared from the modern Bengal tiger population (cf. Mondol et al. 2013). The skull of specimen UN 2484 is notably large; indeed, its dimensions exceed those recorded for Indochinese tigers *Panthera tigris corbetti*, but fall within the range of male Bengal tigers (Mazák 1981, 2013). Because of its large size UN 2484 is almost certainly a male. Selected skull measurements of this specimen are presented in Table 5. These are compared to those of male Indochinese tigers and Bengal tigers, but not to Malayan tigers as skull measurements of undoubted representatives of this taxon have, to our knowledge, unfortunately never been published. Thus, its large size notwithstanding, we cannot rule out the possibility that UN 2484 could be a Malayan tiger. The question of the Malayan tiger's size range requires additional morphological data in order to be settled.

Table 5. Selected skull measurements of UN 2484, compared with data on Indochinese tigers from Mazák and Groves (2006) and on Bengal tigers from Mazák (2013)[1]

		P. t. corbetti		*P. t. tigris*	
dimension	UN 2484	♂, min.	♂, max.	♂, min.	♂, max.
Greatest skull length	370.0	294.2	365.0	329.0	378.0
Condylobasal length	320.0	266.0	312.5	288.5	334.7
Mastoid width	139.5	115.0	140.0	N/A	N/A
Zygomatic width	255.0	184.6	247.4	222.4	264.8
Rostral width	102.6	85.50	101.9	90.80	106.0
Nasal length	136.1	101.0	121.0	N/A	N/A
Interorbital width	86.55	52.00	75.00	N/A	N/A
Postorbital width	62.89	53.10	68.50	N/A	N/A
Mandible length	230.0	193.0	233.0	N/A	N/A

[1] Measurements are in millimeters.

There remains one old FMNH tiger specimen that we were not able to fully identify: UN 365, the mounted skin of a juvenile collected in Southeast Asia ("India ost") in 1857. As noted, the mtDNA haplotype of this tiger shows that it is from one of the Sunda Islands, but does not allow for pinpointing whether it is from Sumatra, Java, or Bali. The specimen's morphology may, however,

offer some clues. The individual's pelage base colour is rather dark, which is a trait shared by all Sunda Islands tigers (Schroeter 1981), but the stripes are narrow and notably few in the shoulder/flank regions (Fig. 3). An absence of stripes in this region is more typical of Javan than of either Sumatran or Balinese tigers (Mazák 2013). However, there is disagreement concerning the utility of stripe patterns in tiger subspecies identification, and some authors have suggested that these characters show too much inter-subspecies overlap to be conclusive (e.g., Kitchener 1999). Thus, it is unfortunately not currently possible to pinpoint a more exact origin for this specimen. Future studies targeting nuclear DNA may yet uncover variation that separates these three tiger taxa. For the time being, however, we may only conclude that UN 365 is "possibly" a Javan tiger.

Contemporary genetic data and coalescent simulations suggest that modern tiger populations retain only a fraction of their former genetic diversity, and that effective population sizes are much lower than they have historically been (Mondol et al. 2013; Singh et al. 2015). Present-day DNA samples alone, however, do not tell the whole story of a species' recent genetic history (Jansson et al. 2014). Proposed inferences are indirect, which means that there is the possibility of introducing errors when inferring process from pattern. Different demographic histories can leave similar genetic signatures in contemporary populations and can also mask one another (Spurgin et al. 2014). Therefore, samples from different eras are required in order to detect possible temporal genetic changes. The study of DNA from museum specimens can address these issues. For example, by comparing DNA samples taken from extant and historical Bengal tiger populations, Mondol et al. (2013) were able to show that there has been a significant decrease in genetic diversity within this subspecies. As the Bengal tiger is the most numerous of the surviving tiger subspecies, it is probable that the genetic diversity has decreased to an even greater extent in the other tiger subspecies in historical times (cf. Singh 2017).

As this study shows, other undocumented specimens of Javan tigers (as well as specimens of other rare/extinct tiger taxa) may still remain in natural history collections around the world. Even when locality data are incomplete or missing, it may still be possible to identify such specimens by morphological and molecular methods. We hope that this study will stimulate the search for and the discovery of additional specimens representing lost tiger populations.

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III

genes

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Article

Over a Thousand Years of Evolutionary History of Domestic Geese from Russian Archaeological Sites, Analysed Using Ancient DNA

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Abstract: The European domestic goose is a widely farmed species known to have descended from the wild greylag goose (*Anser anser*). However, the evolutionary history of this domesticate is still poorly known. Ancient DNA studies have been useful for many species, but there has been little such work on geese. We have studied temporal genetic variation among domestic goose specimens excavated from Russian archaeological sites (4th–18th centuries) using a 204 base pair fragment of the mitochondrial control region. Specimens fell into three different genetic clades: the domestic D-haplogroup, the F-haplogroup that includes both wild and domestic geese, and a clade comprising another species, the taiga bean goose. Most of the subfossil geese carried typical domestic D-haplotypes. The domestication status of the geese carrying F-haplotypes is less certain, as the haplotypes identified were not present among modern domestic geese and could represent wild geese (misclassified as domestics), introgression from wild geese, or local domestication events. The bones of taiga bean goose were most probably misidentified as domestic goose but the domestication of bean goose or hybridization with domestic goose is also possible. Samples from the 4th to 10th century were clearly differentiated from the later time periods due to a haplotype that was found only in this early period, but otherwise no temporal or geographical variation in haplotype frequencies was apparent.

Keywords: greylag goose; *Anser anser*; mitochondrial DNA; control region; D-loop; domestication; Medieval Period

1. Introduction

The European domestic goose (*Anser anser*) is one of the few domesticated animals whose evolutionary and domestication history is still largely unknown. As a "minor" domesticate, it is rarely mentioned or discussed in historical documents [1]. Although undoubtedly not as economically or numerically important as the domestic chicken, the domestic goose is quite widely farmed to provide a source of meat, liver (foie gras), eggs, feathers, and down. Historical records indicate that the use of geese, e.g., the fattening of goose for the table and force-feeding, has been known since Egyptian times [2]. The Romans utilized geese extensively for their eggs and meat and also practiced

force-feeding to enlarge the livers [1,2]. The feathers of geese were plucked by Romans to be used in cushions and upholstery and quills were utilized for writing since the 5th century CE (CE: Common Era; BCE: Before Common Era) [2]. Geese have also been used as guards due to their loud cackling [2]. In addition to their economical use, geese have had a religious significance in certain cultures, e.g., Roman Egypt, Asia Minor, Greece and Roman Italy [1].

The European domestic goose is descended from wild greylag goose (*A. anser*) [3,4] and based on its pink bill coloration more likely from the eastern subspecies (*Anser anser rubrirostris*) than the nominate western subspecies (*Anser anser anser*) [5]. The domestication process of the greylag goose followed the prey pathway, in which the species was first being hunted before more intensive herd-management started [6]. The goose is easy to domesticate from goslings and has a natural tendency to gather fat for migration, which has been exploited to make the wild goose too heavy to fly [2]. Geese have also been domesticated in southeast Asia but derived from another species, the swan goose (*Anser cygnoid*), and the domesticate is known as the Chinese goose [3,7–10]. The European domestic goose and Chinese domestic goose can readily hybridize with each other [7,11], and the European domestic goose is known also to hybridize with its wild counterpart [2,11]. Hereon, use of the term "domestic geese" relates to European domestic geese unless otherwise stated.

It has been proposed that geese were domesticated around 3000 BCE in southeastern Europe [7], possibly in Greece [2] (for a review see [1]), but reliable evidence of domestic geese comes from a much later period (8th century BCE) in *The Odyssey* [2]. Another potential domestication site is in Egypt during the Old Kingdom (2686–1991 BCE) due to iconographic evidence of goose exploitation, but this scenario for the original domestication event has been considered less likely [2]. Geese were also herded by ancient Mesopotamians for food and sacrifices and depicted in Mesopotamian art from the early Dynastic Period (2900–2350 BCE) onwards [2]. Certainly, fully domesticated geese were present during the New Kingdom times in Egypt (1552–1151 BCE) and contemporaneously in Europe [2], and goose husbandry involving several varieties was well established by the Romans by the 1st century BCE [1]. In the Medieval Period, goose husbandry was at its peak with large flocks kept by peasants [1]. Archaeological evidence of the domestic goose in northern Europe indicates that it was probably introduced into Scandinavia during the Early Iron Age (400 BCE–550 CE), and domesticated geese definitely appeared there by the Late Iron Age onwards (550–1060 CE) [12].

Mitochondrial DNA (mtDNA) analysis of modern geese has been employed to make inferences about domestication and has demonstrated that modern domestic geese were derived from a limited genetic base [11]. However, it was not possible to interpret if the observed low diversity dated back to the time of domestication or if it was of a more recent origin [11], perhaps originating with the creation of the modern breeds some hundreds of years ago. Analysis of archaeological and museum samples from different time periods, but of the same geographical area, may make it possible to parse out the genetic signature of domestication and the formation of modern breeds [13].

Goose bones found from archaeological contexts often lack suitable morphological criteria to distinguish domestic individuals from their similarly sized wild forms [1] and usually the classification of goose bones to domestic and wild forms is not even attempted. Ancient DNA (aDNA) studies could overcome the limitation of identification of wild and domestic goose bones in archaeological contexts but there have been very few studies on aDNA in geese. In a recent aDNA and domestication review, goose was not even mentioned [13] although there was a small-scale study by Barnes et al. [14] to separate wild and domestic geese at archaeological sites in the UK. Ancient DNA studies on other goose species have also been scarce and focused mainly on species identification [15–17] or for studying genetic diversity [18].

A large collection of domestic goose bones became available from 15 archaeological sites in Russia, providing an unprecedented opportunity to apply aDNA analysis to domestic geese over a wide timescale. Unlike many previous studies, comparative skeletal collections were used to classify goose bones as wild or domesticated (see [19]). Earlier published sources [20–22] were also followed to determine criteria for the separation of domestic and greylag goose bones. The samples apparently

cover the whole history of domestic geese in the Middle Volga Region spanning from the onset of Medieval Period (4th–5th centuries) to the 18th century (Table S1). None of the previous goose aDNA studies have used such a wide temporal scale or studied domestic goose in such a northern location. These aspects are of interest but also aDNA time-series like this can potentially provide insights into the overall domestication history of the domestic goose.

In our study, we not only analyzed the temporal genetic variability among the Russian domestic geese from different time-periods, but we also compared the genetic diversity between the ancient samples and modern domestic goose breeds. We used a part of the mtDNA control region as our genetic marker because the high substitution rate in this non-coding region makes it very valuable in a species which otherwise has low sequence variability [23]. Also, mtDNA preservation is better than for nuclear DNA in ancient samples because of the higher copy numbers in cells. In addition, a large reference database of modern samples exists for mtDNA. To gain greater knowledge on the origin and evolution of the European domestic goose, the aims of our study were (1) to establish if modern domestic goose haplotypes were present in the Russian archaeological samples, (2) to determine if there is temporal change in haplotype proportions in samples from different historical periods, and (3) to estimate temporal fluctuations in genetic diversity in those periods.

2. Materials and Methods

2.1. Sample Material

Subfossil goose bones (n = 67) classified as domestic geese were derived from 4th–18th century CE Russian archaeological sites in Tatarstan Republic (n = 51), Saratov Region (n = 1), Chuvash Republic (n = 4), Nizhny Novgorod Region (n = 3), Leningrad Region (n = 5), and Pskov Region (n = 3) (Figure 1, Table S1). The archaeological context and identification of bird remains from the Volga Region (Tatarstan, Saratov, Chuvash, and Nizhny Novgorod) were previously described in [19,24], and the site descriptions from the Leningrad and Pskov Regions in [25].

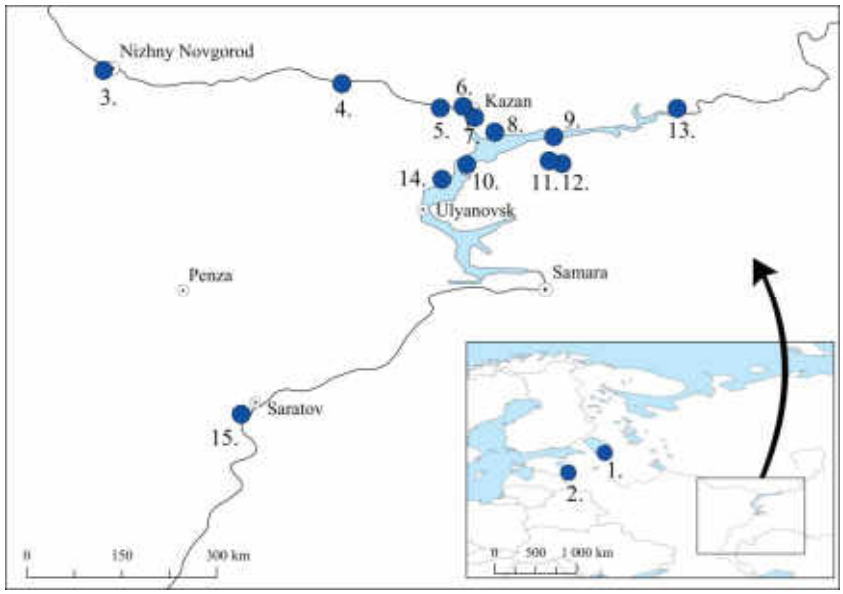

Figure 1. Archaeological locations for subfossil domestic geese from 4th–18th century CE (Common Era): 1. Staraya Ladoga (Leningrad Region), 2. Pskov city (Pskov Region), 3. Nizhny Novgorod Kremlin

(Nizhny Novgorod Region), 4. Chebosakry city (Chuvash Republic), 5. Sviyazhsk (Tatarstan Republic), 6. Kazan Kremlin (Tatarstan Republic), 7. Kazan State University, Kazan city (Tatarstan Republic), 8. Imenkov hillfort (Tatarstan Republic), 9. Ostolopovskoe settlement (Tatarstan Republic), 10. Bulgar (Tatarstan Republic), 11. Toretskoe settlement (Tatarstan Republic), 12. Bilyarsk (Tatarstan Republic), 13. Elabuga hillfort (Tatarstan Republic), 14. Tetyushkoe II hillfort (Tatarstan Republic), and 15. Bagaevskoe settlement (Saratov Region).

2.2. DNA Extraction and Amplification

For each goose bone fragment sampled, the outer layer of the bone was removed using a drill (Dremel, Breda, The Netherlands), and subsequently about 50–150 mg of bone powder was collected, depending on the size of the fragment. DNA extractions were performed using a silica spin column–based protocol originally published by Yang et al. [26] as modified in [27,28] with slight modifications. Samples were first pre-lysed in 1 mL of lysis buffer (EDTA 0.45 M, *N*-layrylsarcosyl 0.5% and proteinase K 0.25 mg/mL), incubating samples for 50 min at 55 °C under rotation. The lysis solution was removed by centrifuging for 2 min at 11,300 *g*, and the supernatant was discarded. Then, 1 mL of fresh lysis buffer was added to the pelleted bone powder and samples were incubated for 1 h at 55 °C and then overnight at 37 °C under rotation. The second lysis fraction was centrifuged for 2 min at 12,000 *g* and used for DNA extraction as the second fraction should be enriched in endogenous DNA [28–32].

The supernatant from the second lysis fraction was mixed with 3 mL of 10 mM Tris-EDTA buffer and concentrated with Amicon® Ultra-4 Centrifugal Filter Unit 30 kDA (Merck Millipore, Darmstadt, Germany) to 250 µL by centrifuging for 20 min at 1000 *g*. The flow-through was discarded, 3 mL of 10 mM Tris-EDTA buffer was added, and samples were concentrated to a final volume of 100 µL by centrifuging for 20–30 min at 1000 *g*. DNA was extracted from the concentrate using MinElute PCR purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions using the centrifugal speeds from [28] with the exception of eluting the DNA to 50 µL of preheated (60 °C) EB buffer with 0.05% final concentration of TWEEN® 20 (Sigma-Aldrich, Saint Louis, Missouri, USA). The column was left to stand for 5 min, centrifuged at 12,000 *g* and this step was repeated, yielding 100 µL of eluted DNA that was stored in −20 °C.

We designed primers that amplify the hypervariable portion of the mitochondrial control region domain I for the geese studied and which contain mismatches to Numts (nuclear sequences of mitochondrial origin [33], for review see [34]). Precautions to avoid amplification of Numts in geese have been previously published in [23]. Because of the Numt involving the control region, we were not able to obtain primer pairs that would amplify overlapping fragments as would normally be preferred. Instead, we used primer pairs AdCR1-F/AdCR1-R (AdCR1-F: 5′CCCCATACACGTA CATACTATAG, AdCR1-R: 5′GTTGGGTGTTGTGGGGTG) and AdCR2-F/AdCR2-R (AdCR2-F: 5′TGAATGCTCTAGGACCACAC, AdCR2-R: 5′CGACTAATAAATCCATCTGATAC) that overlap only by their primer sequence (Figure 2). After the removal of the primer sequences, the sequences consisted of two fragments that could be subsequently concatenated. The primer pair AdCR1-F/AdCR1-R amplified 123 base pairs (bp) and the primer pair AdCR2-F/AdCR2-R amplified 111 bp resulting in a 204 bp concatenated sequence. The haplotype sequences are available in GenBank with accession numbers MH491822-MH491827. We performed PCR in 25 µL reaction volumes using 1× PCR buffer (HotStarTaq, Qiagen), 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 mg/mL BSA (Bovine serum albumin), KAPA Taq HotStart polymerase (Kapa Biosystems, Wilmington, Massachusetts, USA) and 2 µL of extracted DNA. The thermal profile consisted of 95 °C for 10 min, followed by 55 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s with a final extension of 72 °C for 7 min. Each DNA fragment was amplified at least twice. We used BigDye Terminator v.3.1 (Applied Biosystems, Foster City, California, USA) for sequencing with the PCR-primers and the reactions were run on an ABI 3730 (Applied Biosystems). Sequences were manually edited using the program

CodonCode Aligner v.4.0.4. (CodonCode Corporation,, Centerville, Massachusetts, USA) and aligned using the ClustalW algorithm [35] implemented in the CodonCode Aligner.

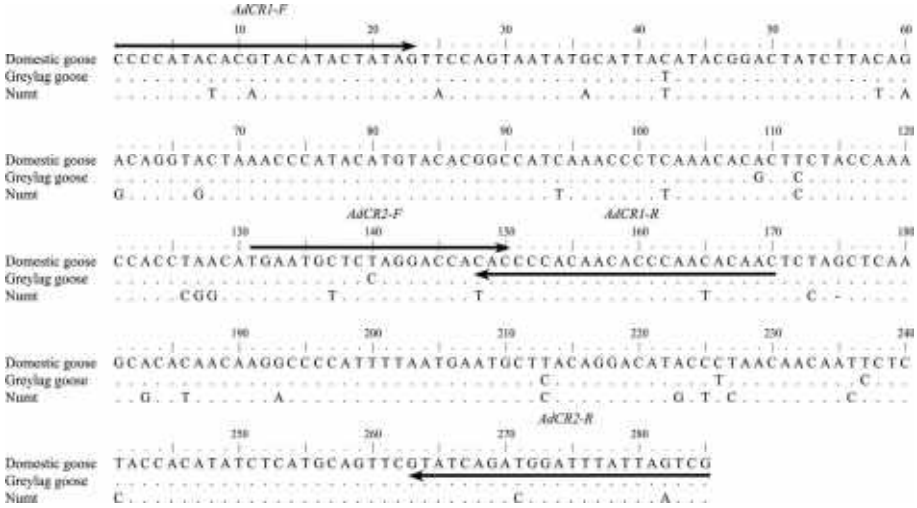

Figure 2. Alignment of domestic goose (GenBank accession number: GQ120441), greylag goose (AF159961) and Numt (nuclear sequence of mitochondrial origin; AF159970) sequences of the hypervariable part of the mitochondrial control region domain I (285 bp). The designed primer pairs AdCR1 and AdCR2 are shown as black arrows. The primer sequences were removed, and the remaining two fragments were concatenated for later analysis.

2.3. Authentication

Several measures were followed to avoid contamination of the ancient samples [36]. The DNA extractions and the pre-PCR work were carried out at the Center of Microscopy and Nanotechnology at the University of Oulu, Finland in dedicated aDNA clean room facilities that were physically separated from the modern DNA and the post-PCR facilities. Further, the bone drilling was performed within a separate room from the DNA extraction and PCR preparation. The PCR-reaction setup was performed in a separate dedicated UV sterilizing PCR workstation (Peqlab, Fareham, United Kingdom). Negative controls were used in the extractions and the PCRs and the amplifications were performed at least twice for each sample to identify post-mortem base modifications. For final analyses, we only accepted sequences that were sequenced successfully at least twice from both strands. In addition, amplification of longer fragment (170 bp including the primers) showed a large decrease in amplification success, in line with the fragmentary nature of ancient DNA.

2.4. Sequence Analyses

We used the program BioEdit 7.2.5 [37] to align our sequences with those obtained from GenBank and the modern sequences from [11]. The Heikkinen et al. [11] sequences included greylag goose haplotypes classified into six different clades (A–F) and individual domestic goose sequences (n = 101) from the clades D and F. Haplogroup D consists of majority of known domestic goose haplotypes (D3–D9) while the rest of the domestic goose haplotypes are found within the F-haplogroup (F4–F5) along with wild greylag goose haplotypes (Figure 3). The domestic haplogroup D contains also wild greylag geese that have most probably a recent hybrid origin between wild and domestic geese [11]. A variety of domestic goose breeds are listed in Heikkinen et al. [11]. We obtained GenBank sequences

of wild greylags (AF159961–AF159963 [23], KT276333–KT276355 [38] and EU601724–EU601734 [39]), domestic geese (GQ120441 [4]) and bean geese (*Anser fabalis*; EU186807, EU186812, EU186810, EU186805 [40] and MH491808 [41]). We constructed a median-joining network [42] implemented in the program PopART [43] using an ε value of zero. Following Bensasson et al. [44] and Heikkinen et al. [11], we used the Numt sequence AF159970 [23] as an outgroup for the network. We also constructed a temporal statistical parsimony network for the ancient samples, the modern haplotype groups that contain the domestic geese (D- and F-haplogroups), and the modern bean goose sequences using the TempNet [45] R-script [46].

We estimated the genetic diversity of the domestic geese for different historical time periods. We calculated the number of haplotypes (H), haplotype (h) and nucleotide (π) diversities, Tajima's D and Fu's Fs in each temporal group using DnaSP v.5 [47]. We tested if the diversity estimates h and π differed between these historical time periods and 'the Present Period' using one tailed t-tests (Welch's t-test). The presence of genetic structure among the temporal samples was investigated using analysis of molecular variance (AMOVA [48]) as implemented in Arlequin 3.5.1.3 [49]. We used this software also to estimate genetic distance among the three temporal groups with pairwise Φ_{ST} using the Kimura 2-parameter genetic distance [50] and tested for significance with 10,000 permutations. We chose the Kimura 2-parameter substitution model according to AIC (Akaike Information Criteria; 1279) and BIC (Bayesian Information Criterion; 3570) values in the MEGA7 program [51]. The best-supported substitution model was the HKY [52], but this model is not implemented in the Arlequin program, so the second best-supported model was used. We applied a sequential Bonferroni correction [53] to the Φ-statistics.

3. Results

3.1. Mitochondrial DNA Haplotypes

In total, we obtained sequences from 46 of the 67 archaeological bones sampled. The overall sequencing success rate was 76%. The sequencing failed especially for the oldest (4th–8th century) samples with the success rate of only 36% for these samples. Twenty-one individuals were excluded as follows: PCR amplification failed completely for eight samples, was only sporadically successful for another eight samples, and five samples were possible duplicates of already sequenced individuals.

Eight haplotypes were found in our study, of which three (F11, Fa5, and Fa6) were new, and the remaining five haplotypes were previously described. The new haplotypes can be attributed to previously described lineages, and we used the existing nomenclature [11,41] to name the haplotypes. We found 30 variable sites among the ancient geese (Table S2). The haplotypes found in our study belonged to three different lineages shown in Figure 3: haplogroup D, haplogroup F, and the taiga bean goose (*Anser fabalis fabalis*). Haplogroup D contains the majority of known domestic goose haplotypes while the F-haplogroup harbors the rest of the known domestic goose haplotypes along with wild greylag goose haplotypes (see Section 2 above). These haplogroups were well-separated in the haplotype network (Figure 3), although some caution in interpretation is necessary. This network is based on a short control region sequence (204 bp) and has misplaced some haplotypes classified on the basis of the whole control region (1249 bp [11]), most notably A1 and F10. Sequences classified as D4 and D5 based on 1249 bp could not be separated on the basis of the 204 bp sequence that we studied, and so are designated D4/D5; likewise, for sequences D3 and D7.

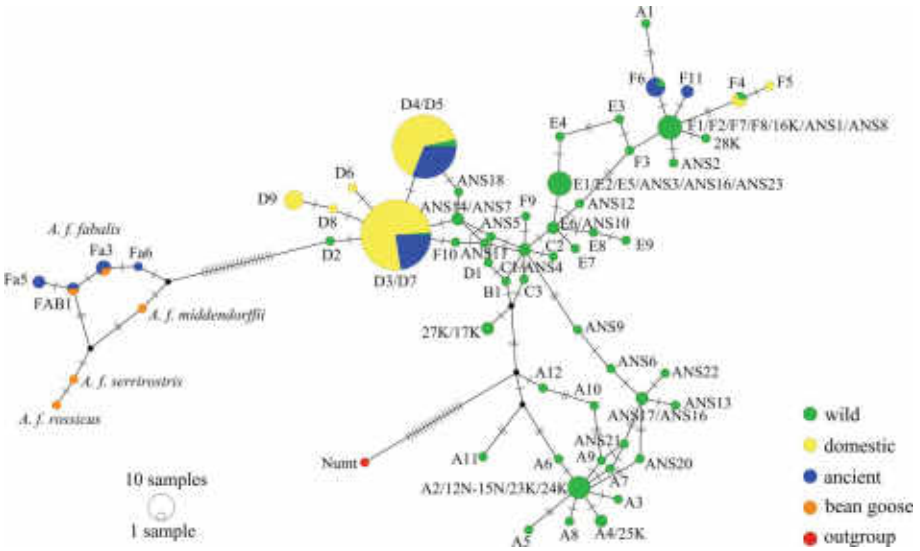

Figure 3. Median-joining haplotype network of the concatenated hypervariable part of the mitochondrial control region (204 bp) of subfossil goose samples (ancient), modern domestic goose samples (domestic), modern wild greylag goose *Anser anser* haplotypes (wild), bean goose *Anser fabalis* haplotypes (bean goose), and nuclear sequence of mitochondrial origin, Numt (outgroup) sequences. Forward slashes between haplotype names denote haplotypes that differ over the whole control region sequence (1249 bp) but cannot be distinguished in the 204 bp sequence analyzed here. Circle area is proportional to the frequency of each haplotype and mutational steps between the haplotypes are indicated by tick marks across branches.

Sixteen of the ancient geese carried the D3/D7 haplotype, while 18 individuals carried the D4/D5 haplotype (Figure 3, Table S1), representing domestic geese. Both the D3/D7 and D4/D5 haplotypes appeared during the High Medieval Period from the 11th century onward, and their presence continued until the present day (Figure 4). These haplotypes were about equally common in the ancient samples except in the Late Medieval Period (14th to 15th century) the D4/D5 haplotype was absent and in the Late Post-Medieval Period (18th century) the D3/D7 haplotype was absent. However, the samples sizes were low for these periods. During the Present Period D3/D7 (n = 38) and D4/D5 (n = 54) are the most common haplotypes among the domestic geese. In addition, a few wild greylag geese harbor D3/D7 and D4/D5 haplotypes in the Present Period (Figure 3). Twenty-five of the ancient D-haplogroup samples were attributed to domestic goose based on bone morphology, but for the remainder, there was uncertainty whether the bones were from domestic or wild birds (Table S1).

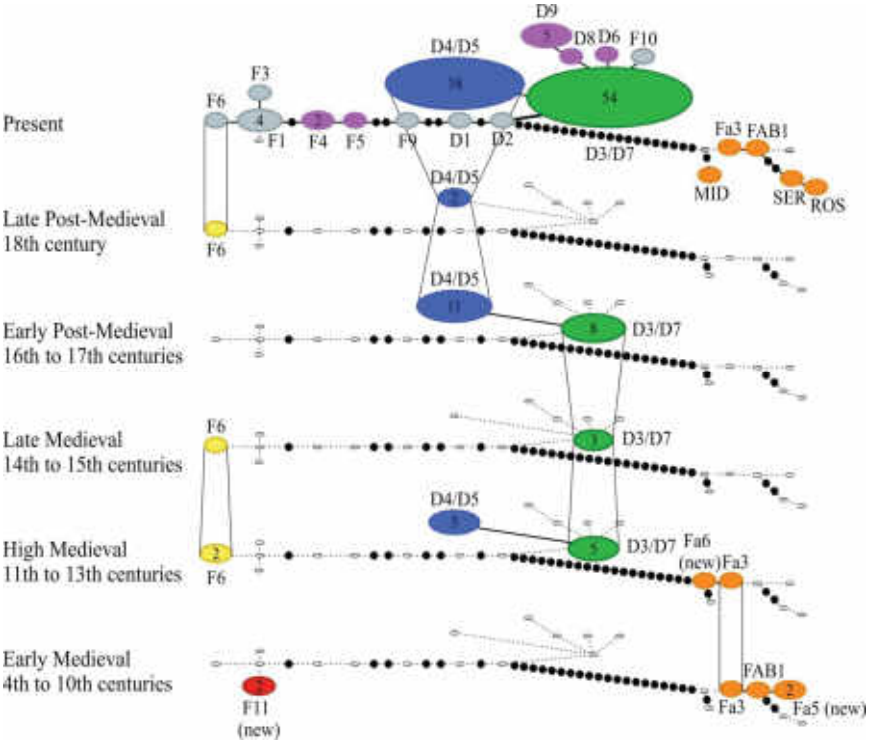

Figure 4. Temporal statistical parsimony network of the concatenated hypervariable part of the mitochondrial control region (204 bp) of subfossil goose samples from Russia from the 4th to 18th century CE and modern greylag goose (*Anser anser*) haplogroups D and F. These groups contain the known domestic goose haplotypes D3 and D7 (green), D4–D5 (blue), and D6, D8–D9, and F4–F5 (purple), and the wild greylag goose in grey color in the depiction for the Present Period. Selected modern bean goose (*Anser fabalis*) haplotypes are also shown separated by at least 21 mutational steps from the others (orange color). MID, SER, and ROS denote the subspecies *Anser fabalis middendorffii*, *Anser fabalis serrirostris*, and *Anser fabalis rossicus*, respectively, while FAB and Fa denote *Anser fabalis fabalis* haplotypes. The size of each ellipse is proportional to the frequency of each haplotype and the number of individuals greater than one are indicated with a number within the ellipse. Small white ellipses indicate haplotypes absent in that time period. Number of black dots +1 connecting haplotypes equals to nucleotide differences.

Six individuals carried haplotypes belonging to the F-haplogroup (Figure 3), with four individuals having haplotype F6 that is associated with contemporary individuals of the wild greylag goose from Lake Kulykol, Kazakhstan [11]. The remaining two individuals possessed a previously undescribed haplotype F11 that was only present in the Early Medieval temporal group (4th to 10th century; Figure 4). Based on the morphological identification, the bones containing the F11 haplotype were assigned to either domestic or domestic/wild greylag (Table S1). Thus, whether this haplotype represents wild greylag or true domestic goose is uncertain. The haplotype F6 appeared in the fossil record during the High Medieval Period (11th to 13th century) and was present in all of the time periods except for Early Post-Medieval Period (16th to 17th century). Half of the subfossil bones carrying F6 haplotypes were identified as domestic and half as domestic or wild greylag goose based on morphology (Table S1).

Six samples were of a different species—the taiga bean goose (*Anser fabalis fabalis*). One of these had an FAB1 haplotype that has been found in Norway, Sweden, Finland, western Russia, and western Siberia (Table S1 in [40]). Two had a haplotype Fa3 that has been identified as the most common haplotype among the taiga bean geese hunted in Finland [41]. Two other previously undescribed taiga bean goose haplotypes Fa5 and Fa6 were also detected, these were closely related to haplotypes FAB1 and Fa3 (Figure 3). The taiga bean geese were restricted to the two earliest time periods: the Early Medieval (4th to 10th century) and the High Medieval (11th to 13th century; Figure 4). Almost all of the taiga bean goose bones had been identified as domestic geese based on bone morphology, only two bones had been considered doubtful, representing either domestic or wild greylag goose (Table S1) but not the bean goose.

Geographically, the haplotype distribution did not show clear trends (Figure 5). In three sampling sites (Nizhny Novgorod, Kazan Kremlin, and Elabuga hillfort), both D3/D7 and D4/D5 haplotypes were present, but in most of the sites, only one of these two main haplotypes were found. Haplotype F6 was present in four geographically close sampling sites: Kazan city, Bulgar and Ostolopovskoe and Toretskoe settlements. Haplotype F11 was restricted to the Imenkov hillfort and the Tetyushkoe II hillfort. The Staraya Ladoga archaeological site contained only taiga bean geese, and three sites (Bulgar, Bilyarsk, and Tetyushkoe II hillfort) in the Middle Volga Region contained taiga bean geese along with the domestic geese.

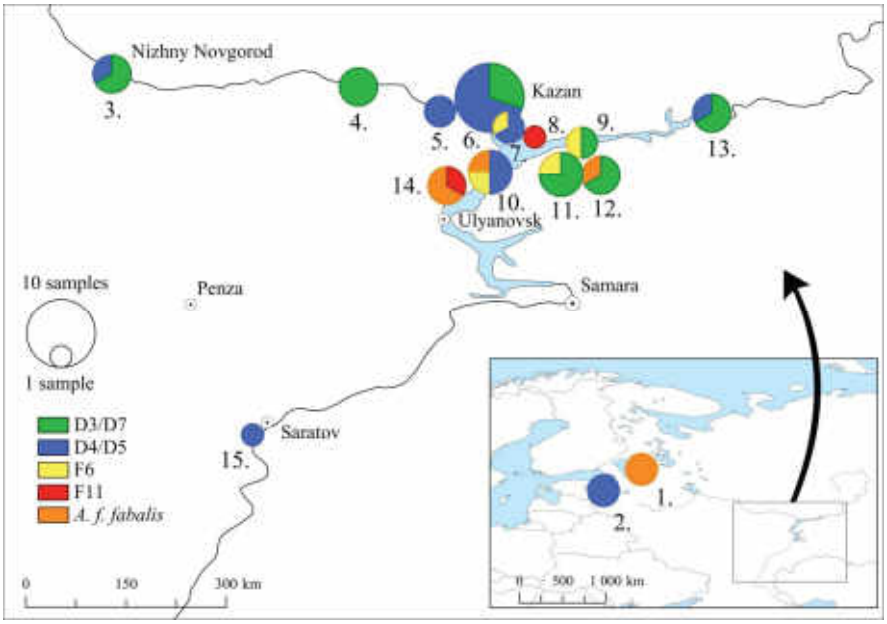

Figure 5. Haplotype frequencies in ancient goose samples from archaeological sites based on the concatenated hypervariable part of the mitochondrial control region (204 bp). The sampling locations are as follows: 1. Staraya Ladoga (Leningrad Region), 2. Pskov city (Pskov Region), 3. Nizhny Novgorod Kremlin (Nizhny Novgorod Region), 4. Chebosakry city (Chuvash Republic). 5. Sviyazhsk (Tatarstan Republic), 6. Kazan Kremlin (Tatarstan Republic), 7. Kazan State University, Kazan city (Tatarstan Republic), 8. Imenkov hillfort (Tatarstan Republic), 9. Ostolopovskoe settlement (Tatarstan Republic), 10. Bulgar (Tatarstan Republic), 11. Toretskoe settlement (Tatarstan Republic), 12. Bilyarsk (Tatarstan Republic), 13. Elabuga hillfort (Tatarstan Republic), 14. Tetyushkoe II hillfort (Tatarstan Republic), and 15. Bagaevskoe settlement (Saratov Region).

3.2. Genetic Diversity

Genetic diversity of the domestic geese was compared between different time periods. The bean goose haplotypes were excluded from this analysis as exclusively *A. anser* haplotypes are found in modern domestic geese [11], and it is their derivation that was the focus of this study (although the recovery of bean goose haplotypes is of considerable interest and is considered in the Discussion; see below). The groupings compared were the High and Late Medieval Periods (11th–15th centuries CE), the Post-Medieval Period (16th–18th centuries CE) and the Present Period (Table 1). The combining together of the two Medieval Periods and the two Post-Medieval Periods was to increase sample sizes. The Early Medieval Period was not combined with the other Medieval Periods as it would have resulted in an extremely long time period (4th–15th centuries CE), with a heterogeneity that may introduce bias. In addition, the sample size for the Early Medieval Period was small (n = 2). The number of haplotypes was the highest in the Present Period, but that is not surprising given the large sample size. The nucleotide and haplotype diversities were the highest in the Medieval Period ($p < 0.001$; Table 1), but given the long time period of samples, this should be viewed with caution. The very high nucleotide diversity in the Medieval Period relates to the relatively high frequency of haplotype F6 individuals (Figure 4). Tajima's D and Fu's Fs were not significant for any of the temporal groups.

Table 1. Population summary statistics for the concatenated hypervariable part of the mitochondrial DNA (mtDNA) control region (204 bp) of the domestic goose comparing the Present Period, the Post-Medieval Period (16th–18th centuries) and the High and Late Medieval Periods (11th–15th centuries). Sample size (n), number of haplotypes (H), haplotype (h), and nucleotide diversity (π) with standard deviations (SD) and Tajima's D and Fu's Fs for testing population expansion. Statistically significant values of comparisons with the Present Period are indicated.

Period	n	H	h (SD)	π (SD)	D	Fs
Present	102	7	0.584 (0.030)	0.0056 (0.0012)	−1.207	−0.753
Post-Medieval	22	3	0.541 * (0.068)	0.0056 (0.0028)	−1.573	1.652
High and Late Medieval	16	3	0.658 ** (0.075)	0.0134 † (0.0042)	0.482	3.924

* t = 2.89, d.f. = 23, $p < 0.01$; ** t = 3.90, d.f. = 16, $p < 0.001$; † t = 7.38, d.f. = 15, $p < 0.001$.

We used AMOVA to partition the genetic diversity among the different temporal groups. The among group variation explained 6.2% of the total genetic variation, while the within group variation explained 93.8%. The pairwise Φ_{ST} value (0.11, p = 0.02) was high and statistically significant between the Present and the High and Late Medieval groups, indicating that these groups are the most differentiated (Table 2).

Table 2. Pairwise Φ_{ST} values for the concatenated hypervariable part of the mitochondrial DNA (mtDNA) control region (204 bp) of the domestic goose between the Present Period, the Post-Medieval Period (16th–18th centuries) and the High and Late Medieval Periods (11th–15th centuries). Statistically significant values ($p < 0.05$) after Bonferroni correction indicated with an asterisk.

Time Period	Present (n = 102)	Post-Medieval (n = 22)
Post-Medieval (n = 22)	0.026	
High and Late Medieval (n = 16)	0.105 *	0.064

4. Discussion

In this study, we have identified three genetic lineages among Medieval and Post-Medieval domestic goose samples from Russian archaeological sites: the main domestic D-haplogroup, F-haplogroup, and taiga bean goose haplotypes. We used the modern breeds from Heikkinen et al. [11]

as representatives of current variation among modern domestic geese. In Russia, the domestic goose breeds have either European origin or the local geese have been crossbred with European or Chinese breeds [54]. Most of our samples were excavated from the Middle Volga Region that is currently dominated by 10 breeds of domestic geese, which all belong to or are in one way or another connected with European breeds of geese [55]. In addition, some breeds are crossed with or have a direct relationship with Chinese domestic goose breeds [55]. Thus, the breeds in Heikkinen et al. [11] should represent well the variation also in contemporary Russia. No Chinese ancestry was detected in our samples, so presumably the crossbreeding of these geese is a more recent phenomenon. However, we cannot detect if the European geese have been mated with Chinese goose ganders with maternally inherited mtDNA.

The most common mitochondrial haplotypes among the modern domestic goose D3 and D4 (D3: $n = 53$ and D4: $n = 33$ [4,11]) were also the most common haplotypes in these Russian archaeological sites. However, the sequenced fragment of the mtDNA control region could not differentiate between haplotypes D3 and D7 or D4 and D5, thus haplotypes D5 and D7 might actually be present in the ancient sample. In any case, the haplotypes D3/D7 and D4/D5 appear to be domestic haplotypes, as among modern specimens, they are restricted to domestic geese, except for a few wild individuals in Scotland (haplotype D4), The Netherlands (haplotype D3) [11], and Norway (haplotype ANS19 in [38], which is a partial sequence of D5 in Heikkinen et al. [11] and also in Wang et al. [4]), which probably have a hybrid (wild × domestic) origin. This presence of apparently "domestic" haplotypes in the archaeological record is of interest for domestication history. During the domestication process, the first genetic bottleneck occurs during the early phase of domestication when a subset of a population is selected for domestication [56,57]. Illustrations from the Old Kingdom Egypt show already diverse coloration in geese and during Roman times, several goose varieties were recognized, such as mottled and white types [1]. The breeds Embden, Toulouse, Sebastopol, and the swan goose derivatives "Chinese" and "African" were known prior to the mid-19th century [5,7,58] and could have served as a basis for modern breeds. The second bottleneck occurs with origin of modern breeds when certain desirable traits are selected for [56,57]. As our samples pre-date the modern breeds, which were mostly formed due to intensive selective breeding within the past 200 years [59,60], it is possible that the dominance of D3/D7 and D4/D5 is due to the first domestication bottleneck. We did not detect other domestic haplotypes (D6, D8, D9, F4 or F5) found only in domestics in the survey of Heikkinen et al. [11] suggesting that these haplotypes could be of a more recent origin. This could imply that the modern breeds are derived from a limited gene pool that possibly traces back to the original domestication event.

Considering the F-haplogroup, there could be a variety of explanations for its presence in the ancient samples. In modern samples this haplogroup was found in non-breed Turkish domestic geese, wild greylag geese from Iran and Kazakhstan and wild greylag geese from The Netherlands and Denmark that are most probably descendants of introduced eastern *Anser anser rubrirostris* geese [11]. First, all ancient samples belonging to the F-haplogroup could in fact be hunted wild greylag geese that were misidentified as domestic geese based on bone morphology. Second, individuals belonging to the F-haplogroup could be the descendants of hybridization between the domestic and wild geese. In this case, a wild goose of the F-haplogroup must have mated with a domestic gander as mtDNA is maternally inherited. However, this mating between adult geese seems unlikely as geese tend to mate for life and females are philopatric to their natal sites [61]. It is also possible that wild goslings or eggs were collected and raised, providing opportunities for hybridization. Goslings can readily imprint to humans, a feature that has substantially helped in taming wild geese. In Eurasia, it has been common to collect eggs and goslings and further raise them in captivity partly, for companionship and partly as a source of food [62–73] (see Text S1). Incorporation of wild forms into the domestic gene pool has been practiced with several other domestic species as well (pig [74,75], cattle [76], horse [77,78], donkey [79], and dromedary [80]). Third, the F-haplotypes could represent an independent domestication event of the local eastern greylag geese, as this haplogroup seems to be more typical for the eastern *Anser anser*

rubrirostris geese [11]. This would imply that the goose was domesticated at least twice; however, this scenario does not have the support of any other evidence pointing to several domestication events.

Considering the individual haplotypes within the F-haplogroup, the F11 haplotype was only found from the Early Medieval Period (4th to 8th century) in the Middle Volga Region. These samples have been associated with an ethnic group called the Imenkov culture. This culture had a distinctive ethnic composition, economic activity, and, in particular, the use of domestic animals, which distinguished them from subsequent cultures, which were an ethnic combination of Turkic and Finno-Ugric [81]. This change in the ethnic composition in the Middle Volga Region could explain why the goose haplotypes from the Early Medieval Period differed from the later time periods. These bones are from immature or subadult birds, suggesting they were domestic rather than wild [19] and thus possibly representing an extinct lineage of domestic geese. This would imply that the first domesticated geese appeared in the Middle Volga Region with the onset of the Medieval Period (4th–10th centuries). However, it cannot be ruled out that these individuals were goslings collected from the wild. Overall, the first morphologically identified domestic geese appeared in the archaeological record in the European part of Russia and Ukraine in 500 BCE–300 CE and several settlements harbored domestic geese during the Medieval times [19,21,24,82–89] (see Text S1). In western Siberia, the domestic goose appeared much later (16th–17th century) [90,91].

Haplotype F6 was present in all temporal groups except in the Early Medieval Period (4th to 10th century) and the Early Post-Medieval Period (16th to 17th century). From the Present Period, it has only been found from one modern wild greylag goose from Kazakhstan [11]. This haplotype could have originated from wild hunted greylag geese or from a domestic goose lineage that has either gone extinct or has remained undetected in previous studies. It might be possible to find F-haplotypes from some local domestic goose breeds, such as the domestic geese bred by Udmurts and Maris that had several greylag goose characteristics [92] (see Text S1), the Shadrin breed that originates from local wild and domestic geese [51] (see Text S1) or the Javakhetian or Bogdanovski breed from Georgia that has been claimed to have descendent directly from local wild geese [54]. The domestic origin of the subfossil geese carrying haplotype F6 is the most supported alternative, because this haplotype was found in almost all temporal groups from the 11th century onward with no other F-haplotypes. A more varied selection of F-haplotypes would have been expected if the geese would have been hunted individuals. The absence of F6 in the Early Post-Medieval Period could be due to sampling effect or that the popularity of the domestic lineage of geese carrying this haplotype had decreased.

The genetic diversity was the highest during the High and Late Medieval Period according to the haplotype and nucleotide diversity estimates, probably due to high number of F-haplotypes. However, our sample sizes were not equal among the temporal groups, nor were the time periods the same length, all aspects that could bias our results. Although goose bones are reported to be more frequent in archaeological sites during the 13th–14th centuries [1,19], we did not detect signs of population growth in any of the temporal groups according to Tajima's *D* and Fu's *Fs*. The pairwise Φ_{ST} values showed an increase in genetic differentiation over time. The preceding time periods were genetically similar as the pairwise Φ_{ST} values were low and non-significant but statistically significant values were detected between the temporarily most separated groups, namely the amalgamated High and Late Medieval and the Present Period. Overall, the genetic differentiation was gradual and detected only over longer time spans.

The presence of taiga bean geese among the subfossil samples was unexpected as all the bones were classified as domestic or domestic/wild greylag goose based on the morphology and no other species were selected for this study. The taiga bean goose bones can either be from wild hunted individuals misidentified as domestic goose, from domesticated bean geese or from domestic geese hybridized with bean geese. The similarly sized goose species are difficult to identify by species as has been noted also in a previous study [14], especially from fragmented or poorly preserved material. This most probably explains the presence of taiga bean geese in our sample. Bean geese migrate through the Middle Volga Region during the spring and autumn migrations [93] and have

done so in the past as well [94–99] (see Text S1). It is possible, but very unlikely, that the bean goose was domesticated at least to a certain degree which made the bones morphologically more similar to the typical domestic goose. No evidence exists for this, as only the greater white-fronted goose (*Anser albifrons*) and the Canada goose (*Branta canadensis*) have been domesticated on experimental basis in ancient or historical times [2,7]. The hybridization of the domestic goose and the bean goose is plausible since hybrids between these species have been observed at least in captivity [100]. Again, only wild greater white-fronted geese have been known to be bred with domestic geese, to create a breed called Pskov bald (see Text S1). All the taiga bean goose fossils are from the earliest time periods, the Early and High Medieval Period (4th to 13th centuries), which could indicate that wildfowling was more common during this era or that the two species are easier to discriminate from the more recent samples.

Our study provides the basis for aDNA analysis of domestic goose also over larger geographic areas and broader time frames. The analysis of ancient samples from the area of possible goose domestication including Egypt, ancient Mesopotamia, Turkey and Greece and nearer the time when domestication would have occurred would greatly help pinpoint the timing and location of the domestication event(s). However, possible problems may arise with DNA preservation in hot and humid conditions of the Mediterranean region [101]. An additional benefit of further sampling of both domestic and wild geese is the information it would provide on changes in goose husbandry versus wildfowling and on the economic activities of people.

5. Conclusions

We traced over a thousand years of evolutionary history of the European domestic goose through the Medieval and the Post-Medieval Periods in Russian archaeological sites, especially in the Middle Volga Region. We identified three genetic lineages among the samples: D-haplogroup, F-haplogroup, and the taiga bean goose. We found that geese of the typical domestic goose haplogroup D were present at least from the High Medieval Period (11th century CE) onward. However, the origin of the geese carrying the F-haplotypes is less certain, as the haplotypes found are not present among modern domestic geese. Surprisingly, we also found bean goose haplotypes, even from goose bones classified as "domestic."

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/9/7/367/s1, Table S1. List of the successfully analyzed subfossil goose samples from Russia; Table S2. Variable nucleotide sites; Text S1. Archaeological and historical context of the samples.

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IV

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Tangled Worlds: The Swedish, the Sámi, and the Reindeer

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Abstract Reindeer pastoralism developed among the indigenous Sámi of northern Fennoscandia, but the established colonial relationship with Sweden brought on an expanded use of reindeer. Tradesmen, priests, and officials of Swedish origin benefited from domesticated reindeer in many ways – trading reindeer products and using reindeer as transport during winter trips to marketplaces. Reindeer were, therefore, in many ways focal in the encounters between the Sámi and the Swedish. In this paper, we use zooarchaeology, stable isotope analysis, and ancient DNA analysis to interpret reindeer remains from towns, marketplaces, and agrarian settlements in medieval and early modern northern Fennoscandia. We argue that reindeer played important roles in contacts and encounters. The Sámi, the Swedish, and the reindeer formed a multispecies community. The exploration of the relationships in this multispecies community captures the complexity of human and human-animal relationships in colonial encounters. Moreover, it emphasizes the importance and agency of animals in colonial histories.

Keywords Sámi archaeology · Fennoscandia · Reindeer · Zooarchaeology · Stable isotope analysis · Ancient DNA analysis

Introduction

Reindeer are ubiquitous in northern Fennoscandia. Wild and later domesticated reindeer have played important roles in the subsistence, lifeways, and cosmology of northern peoples from the Stone Age onwards (Helskog 2011). Reindeer remains are

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also common at archaeological sites in Fennoscandia, including medieval and early modern urban and agrarian sites (Backe 1995; Vretemark 1995; Puputti 2010; Salmi 2011; Salmi and Kuokkanen 2014; Vretemark 2014). However, their origin and domestication status has remained a mystery because of the presence of two reindeer subspecies, the co-occurrence of wild reindeer hunting and reindeer pastoralism, and the methodological difficulties in identifying different types of reindeer.

This paper addresses these problems by means of zooarchaeological, stable isotope, and ancient DNA (aDNA) analyses of reindeer remains. We combine faunal analyses from seven archaeological sites in present-day northern Finland and Sweden with a small sample of stable isotope and aDNA data to look at the different kinds of reindeer present at these sites. Morphological analysis and age profiles are usually not indicative of reindeer domestication status in faunal assemblages from northern Fennoscandia due to reasons related to reindeer biology and the variability of reindeer herding practices. However, the anatomical distribution of reindeer remains can be used to infer whether the animals were slaughtered locally or whether people mainly bought reindeer meat as meat cuts. Stable isotopes (^{15}N and ^{13}C) may indicate reindeer feeding practices. Ancient DNA is used to infer whether the animals were wild or domesticated. We targeted the mitochondrial control region, because as mitochondria are present in multiple copies per cell, mitochondrial DNA is more likely to be recovered from degraded specimens. Additionally, mitochondrial control region sequences have previously been used to identify possible wild and domestic individuals (Bjørnstad et al. 2012; Røed et al. 2008, 2012, 2014), whereas at present, no nuclear DNA markers are known that are suitable for degraded DNA and could be used for this purpose.

After this, the paper expands into an exploration of reindeer as a nexus of colonial relationships between the Swedish and the indigenous Sámi. Medieval and early modern northern Fennoscandia was a melting pot of cultures, languages, and lifeways, as well as an area increasingly enmeshed in colonial relationships. Reindeer pastoralism developed among the Sámi from the Late Iron Age onwards (Bjørklund 2013). Later, in the medieval and early modern periods, some tradesmen, officials, and priests also owned the reindeer they used for winter transportation (Kortesalmi 2008:63-64, 73-81; Mäntylä 1971:109). Reindeer products such as meat, skins, and crafts were important trade goods in the trade between the Sámi and the growing agrarian and urban populations in the northern parts of Fennoscandia (Luukko 1954:396–397; Virrankoski 1973:453–454). Moreover, the use of reindeer as a means of transportation in the vast northern landscape without roads was common to both tradesmen and Sámi reindeer herders. The paper presents a detailed look into the different types of reindeer present at these sites, as well as the range of social and multi-species relationships that were linked to the presence of these animals. It also allows the examination of animals as historical actors along with humans in a colonial world (DeJohn Anderson 2006).

The Swedish and the Sámi

From the thirteenth century onwards, the Swedish crown, as well as the state of Novgorod, took an increased interest in the northern areas. According to the historical record, trade and settlement politics and Christianization were the

main tools used to attach local societies to the state. More specifically, parishes, marketplaces, and towns were established, churches were built, and rights to appropriate land for farming and tax breaks were promised to farmers settling in the northern river valleys on both sides of the Gulf of Bothnia (Vahtola 1991: 184; Wallerström 1995a:52–63). Recent research has revealed, however, that local populations had considerable impact on and agency in the colonization process, acting as mediators and negotiating the terms with the crown and its officials – in fact, the cooperation of local societies was essential for the successful Swedish colonization of the area (Bergman and Edlund 2016; Kuusela et al. 2016).

In the late medieval and early modern periods (ca. 1400–1700 CE), the area that later became northern Sweden and present-day northern Finland was inhabited by the indigenous Sámi, who practiced a mixed livelihood of reindeer herding, fishing, hunting, and gathering, as well as by an agrarian society using a variety of wild resources. The identity and cultural practices of the agrarian population were the results of an amalgamation of identities of local Iron Age societies and settlers from southern Fennoscandia and Karelia (in present-day eastern Finland and southeastern Russia) (Bergman and Edlund 2016; Kuusela et al. 2016; Ylimaunu et al. 2014). These societies mixed and interacted in many ways. For instance, the *birkarls,* an organization of powerful merchant-farmers residing on the western coast of the Gulf of Bothnia and in the southern parts of the Tornio River valley, acted as middlemen in the trade between the Sámi and local farmers, and eventually the king of Sweden. They also collected taxes (Bergman and Edlund 2016; Vahtola 1991:218–224). Officials of the crown and priests resided in coastal parishes and towns, performing official and business duties with the Sámi and other inland populations on their winter trips to the north (Kortesalmi 2008: 66–73).

The Swedish state effectively colonized the Sámi and their lands. However, the process was slow. It included multiple types of interaction between societies, and the day-to-day interactions between people of different ethnicities were complicated. Late medieval and early modern northern Fennoscandia was a melting pot where people of different origins met and created new hybridized cultural practices (e.g., Kuusela et al. 2016; Olsen et al. 2011; Salmi et al. 2014; Ylimaunu et al. 2014). Moreover, the Swedish and the Sámi were linked by economic and familial ties (Bergman and Edlund 2016; Vahtola 1991:190). They also shared social customs, subsistence activities, lifestyles, and material culture (Bergman and Edlund 2016; Salmi et al. 2014; Vahtola 1991:190).

In the seventeenth century, a clearer colonial agenda and an ideology with clearly unequal power relations emerged. Colonialism refers to a situation of unequal power relations between peoples where one is able to exploit the other based on political, economic, or ideological differences (e.g., Piñón 2002:114; Reindhard 2001). The way the Swedish crown acted in the Sámi land is consistent with the characteristics of colonialism (Lehtola 2015). In the seventeenth century, the crown began to control and use the northern resources by concentrating trade in certain towns and marketplaces and by establishing mines and other related production sites (Lindmark 2013; Luukko 1954:196–199, 204; Vahtola 1987, 2005; Wallerström 1983:44, 50). An agrarian and sedentary lifestyle became seen as the proper Swedish way to live (Lindmark 2013;

Nurmi 2009). Moreover, the crown attempted to replace the traditional Sámi social organization, the *siida*, by Swedish administration (Lehtola 2015). Christianization of the Sámi also intensified from the seventeenth century onwards (Kylli 2012).

People and Reindeer in Northern Fennoscandia

Mainland northern Fennoscandia is home to two reindeer subspecies, mountain reindeer (*Rangifer tarandus tarandus*) and forest reindeer (*R.t. fennicus*). Wild mountain reindeer were previously extant in the whole mountain area of northern Fennoscandia and forest reindeer all over the taiga zone of northern Finland (Helle 1982: 13). Wild reindeer hunting was an important source of livelihood for the different populations inhabiting the area from the Stone Age onwards (e.g., Myrvoll et al. 2011). It continued until the seventeenth century and even later in spite of the decreasing numbers of wild reindeer and the onset of reindeer pastoralism (Enbuske 1995:171, 350; Kortesalmi 2008:23-24; Luukko 1954:111; Myrvoll et al. 2011; Tegengren 1952; Virrankoski 1973:271–272).

The domesticated reindeer of Scandinavia were domesticated from the wild mountain reindeer (Røed et al. 2008). The transition to reindeer pastoralism probably began between the eighth and eleventh century (Bergman et al. 2013; Bjørklund 2013). It was a focal means of livelihood around the sixteenth century in the mountain areas of Sweden and Norway (e.g., Bergman et al. 2013; Bjørnstad et al. 2012; Bjørklund 2013). From then on, reindeer pastoralism spread and became a major source of livelihood, as well as the basis for social organization, in many areas (Bergman et al. 2013; Bjørklund 2013; Bjørnstad et al. 2012; Hansen and Olsen 2014: 195–206; Mulk 2009; Sommerseth 2011; Wallerström 2000). It has to be noted, though, that the transition to pastoralism occurred at different times in different areas (Tegengren 1952).

In addition to the Sámi, domesticated reindeer were important to other groups as well. The *birkarls,* merchants in towns, crown officials, and priests owned reindeer that they used for pulling sleighs during winter voyages to marketplaces. Sámi reindeer herders took care of these reindeer (Kortesalmi 2008:44-52, 73-81; Mäntylä 1971:109). The agrarian population in northeastern Finland adopted reindeer husbandry from the Sámi from the late seventeenth century onward (Kortesalmi 2008:137–174).

Archaeological Material

The archaeological sites are farm sites, urban sites, and marketplaces situated in present-day northern Sweden and Finland (Fig. 1). Faunal analyses (Table 1) from these sites have been previously reported or published. In this paper, we rely on osteological reports and published data (Backe 1995; Ohtonen 1984; Puputti 2010; Salmi 2011, 2017; Salmi and Kuokkanen 2014; Vretemark 1995, 2014). Samples for stable isotope and aDNA analyses were taken from the assemblages from Oulu, Tornio, Oravaisensaari, and Ylikylä (Table 2).

Oulu

The town of Oulu was founded on the mouth of the Oulujoki River in 1605. A number of archaeological excavations have taken place in the town. The faunal material analyzed derives from the excavations conducted in Pikisaari, Byströmin talo, Virastotalo, Lyseo, Kajaaninkatu, and Franzenin puisto. The locations were residential plots that belonged to various social groups, such as merchants and in some cases craftsmen, along with their families. The faunal remains from these excavations date from the early seventeenth century to the turn of the eighteenth and nineteenth centuries (Salmi and Kuokkanen 2014).

Björsbyn

Björsbyn was a village situated to the north of the town of Luleå and partially excavated in 1921. Two foundations of timber-framed houses with two fireplaces and the remains of a cellar were excavated. Archaeological evidence suggests that the houses were inhabited in the fifteenth and sixteenth centuries (Liedgren and Bergman 2015).

Gamla Kyrkby

In the medieval period, the archaeological site of Gamla Kyrkby was a marketplace situated on the mouth of the Piteå River. Archaeological excavations were conducted at the site in the late 1960s and early 1970s. During the excavations, a number of building remains along with a large artifact assemblage indicative of a wide trade network were

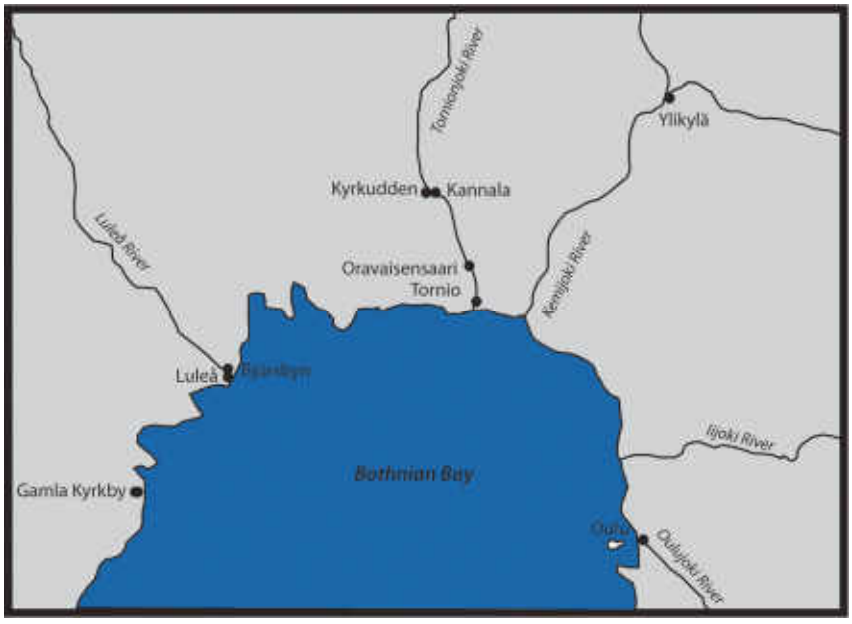

Fig. 1 Map of northern Fennoscandia and the archaeological sites mentioned in the paper

Table 1 Species frequencies as NISP (Number if Identified Specimens) and MNI (Minimum Number of Individuals) (in brackets). In some cases, only NISP or MNI data is presented due to missing information in the osteological reports

NISP/(MNI)	Oravaisensaari	Ylikylä	Tornio	Oulu	Kyrkudden	Gamla Kyrkby	Björsbyn
Cattle (Bos taurus)	94	52 (2)	1904 (27)	1293 (46)	(24)	2428	403
Sheep or goat (Ovis aries/Capra hircus)	40	3 (1)	782 (23)	474 (25)	(4)	1196	87
Pig (Sus scrofa domesticus)	32		282 (13)	227 (17)	(1)	497	36
Reindeer (Rangifer tarandus)	54	26 (2)	85 (10)	95 (5)	(8)	76	22
Elk (Alces alces)					(1)	123	
Horse (Equus caballus)	1		7 (2)	5 (1)	(3)	41	
Arctic hare (Lepus timidus)	7	1 (1)	239 (17)	52 (7)	(1)		
Beaver (Castor fiber)	1				(1)		
Red squirrel (Sciurus vulgaris)			3 (1)	1 (1)			1
Red fox (Vulpes vulpes)			16 (2)	9 (1)			
Dog (Canis familiaris)						1	
Ermine (Mustela erminea)				3 (1)			
Brown bear (Ursus arctos)	5						
Seal (Phocidae)	1		91 (3)	42 (9)		797	166
Wood grouse (Tetrao urogallus)	11	2 (2)	326 (17)	158 (21)	(1)		4
Black grouse (Tetrao tetrix)	2	1 (1)	73 (12)	79 (19)		24	
Willow grouse (Lagopus lagopus)	2		133 (18)	54 (13)		4	
Hazelhen (Bonasa bonasia)	1		7 (3)	26 (8)			
Chicken (Gallus domesticus)	1			7 (3)			
Black-throated loon (Gavia arctica)					(1)		
Goosander (Mergus merganser)			5 (1)	2 (1)			
Red-breasted merganser (M. serrator)			11 (2)				

Table 1 (continued)

NISP/(MNI)	Oravaisensaari	Ylikylä	Tornio	Oulu	Kyrkudden	Gamla Kyrkby	Björsbyn
Long-tailed duck (Clangula hyemalis)			1 (1)				
Greater scaup (Aythya marila)			2 (1)				
Tufted duck (Aythya fuligula)			2 (1)				
Greylag goose (Anser anser)			13 (5)			2	
Lesser white-fronted goose (Anser erythropus)			3 (1)				1
Bean goose (Anser fabalis)			24 (4)				
Whooper swan (Cygnus cygnus)			30 (5)	1 (1)			
Eurasian sparrowhawk (Accipiter nisus)						1	
Pike (Esox lucius)	9				(3)	34	
Perch (Perca fluviatilis)	5				(5)	17	3
Bream (Abramis brama)	2					6	
Cod (Gadus morhua)							1
Burbot (Lota lota)						1	
European whitefish (Coregonus lavaretus)							1
Pike-perch (Sander lucioperca)		4 (1)					
Salmon or trout (Salmo sp.)	8			1			1
Total NISP (MNI)	276	89 (10)	4049 (169)	2529 (179)	(53)	5248	726
% reindeer bones NISP (MNI)	20	29 (20)	2 (6)	4 (3)	(15)	1	3

Table 2 Samples taken for stable isotope and aDNA analysis and the results of stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$). Stable isotope data from Lahtinen and Salmi (2018) (1–4), Oinonen 2017a, 2017b (5–9). Age estimations for long bone epiphyseal fusion Takken-Beijersbergen and Hufthammer (2012)

Sample No.	Sample ID	GenBank no.	Site	Skeletal element	Age (months)	dC13(‰)	dN15(‰)	% C	% N	C/N	Dating ID	BP	cal AD/*Context date*
1	OFRA-1-D		Oulu	Tibia dist	>18–30	−19.6	5.5	43.0	15	3.4			*Seventeenth century*
2	TOKE-4023		Tornio	Humerus dist	> 6–15	−21.2	3	43.3	13	4.0			*1621–1660*
3	TOKE-SY22P		Tornio	Humerus dist	>6–15	−19.0	4.1	44.6	16	3.3			*1621–1660*
4	ROTI-1458		Ylikylä	Humerus	>6–15	−20.0	3.9	44.9	15	3.5			*1449–1706*
5	AKS-21	MH010859	Ylikylä	Metatarsal	>18–30	−19.5	5.6	41.3	16	3.1	Hela 4056	261 ± 21	1527–1797
6	AKS-22	MH010860	Ylikylä	Tibia dist	>18–30	−19.1	5.4	41.4	15	3.2	Hela 4057	440 ± 21	1426–1471
7	AKS-27	MH010861	Oravaisensaari	Ulna prox	>42–48	−19.7	2.7	39.3	15	3.2	Hela 4062	445 ± 22	1425–1467
8	AKS-29	MH010862	Oravaisensaari	Antler		−21.6	5.3	37.2	14	3.2	Hela 4064	514 ± 21	1401–1441
9	AKS-30		Oravaisensaari	Metatarsal prox		−18.5	2.0	35.8	13	3.1	Hela 4065	375 ± 22	1448–1629

investigated. Archaeological evidence suggests that the site was used in the fourteenth and fifteenth centuries (Wallerström 1995b).

Tornio

The town of Tornio was founded on a former marketplace in 1621 (Mäntylä 1971:33–36). Archaeological excavations were conducted in Tornio throughout the 1990s and 2000s. Plots in different areas of the town were excavated and archaeological remains dating from the seventeenth to the nineteenth century were discovered. The faunal materials covered in this paper derive from excavations at the sites of Keskikatu, Westring, Purra and Aho, Aspio and Viippola, and Välikatu. They date from 1621 to ca. 1800 (Puputti 2010). The cultural layers investigated in these excavations represent residential plots inhabited by merchants, with the exception of the richest ones, whose households were located in areas that have not yet been investigated (Mäntylä 1971:125–126,244; Ylimaunu 2007:18–19).

Oravaisensaari

Oravaisensaari is an island located in the Tornionjoki River a few kilometers upstream from the river mouth. The island was part of the village of Vojakkala, which was one of the largest villages in the river valley (Koivunen 1991:142; Vahtola 1991:241). Historical records indicate that there was agrarian settlement at the site from the sixteenth century onward (Vahtola 1991:241). The village of Vojakkala was home to a number of *birkarl* families throughout the sixteenth and early seventeenth centuries (Vahtola 1991:241).

In 1973–74 and 1980, archaeological excavations were conducted in the area where the house of *birkarl* Niilo Niilonpoika Oravainen was supposedly located in the late sixteenth century (Koivunen 1991:142). In the excavations, the identity of the farm's inhabitants remained unconfirmed, but building remains and other finds, including animal remains, were unearthed. The building remains and most of the finds dated from the fifteenth to the seventeenth century (Koivunen 1991:142–145).

Kyrkudden

Kyrkudden is located on the western shore of the Tornionjoki River. During the excavation, a cemetery dating from the eleventh to the seventeenth century was investigated. In addition to the cemetery, there were remains of a marketplace that was established in the fourteenth century. The marketplace remained in use until the seventeenth century (Wallerström 1995b). The faunal remains derive from the marketplace (Vretemark 1995).

Ylikylä

The oldest agrarian settlement in the Rovaniemi parish was probably located in the village of Ylikylä (Paavola 1995). The village is located on the bank of the Ounasjoki River. Historical records of settlement at the site date to the late medieval period, possibly to the mid-fifteenth century (Kostet and Närhi 1979; Paavola 1985).

The site was excavated in 1978–79 and 1982–83. During the excavations, building remains and other finds dating from the late fourteenth to the seventeenth century were discovered (Paavola 1995). The faunal remains analyzed originate from contexts interpreted as remains of fireplaces dating from ca. 1400–1700 (Salmi 2011).

Zooarchaeological Analysis

Due to morphological similarity and overlapping size, it is often impossible to tell apart wild forest reindeer, wild mountain reindeer, and domesticated reindeer based on fragmentary archaeological remains. Moreover, the identification of hunting or herding cultures is complicated by the various hunting and herding strategies employed (Hambleton and Rowley-Conwy 1997; Jomppanen and Näkkäläjärvi 2000; Korhonen 2008:137; Lahti 2006; Luukko 1954:162-164; Myrvoll et al. 2011; Vuorela 1975:57-58). Therefore, the analysis of age profiles provides inconclusive results on the domestication status of the animals. However, the analysis of skeletal frequencies can be used to obtain information on meat supply networks.

The anatomical distribution of reindeer remains is presented as the number of identified specimens (NISP), minimum number of elements (MNE), and modified anatomical units (MAU) (Table 3). The underlying assumption is that unequal skeletal frequencies may indicate meat cut trade. Here, the amounts of upper limb bones and vertebrae on one hand, and cranium and extremities on the other, are analyzed based on NISP counts. Percentages based on MNE counts – that circumvent the problem of different numbers of bone elements in the cranium and extremities in comparison with the upper limbs and vertebrae – are presented for Ylikylä, Oravaisensaari, Tornio, and Oulu. For Gamla Kyrkby, Kyrkudden, and Björsbyn, MNE counts could not be calculated due to missing information in the publications or osteological reports. Therefore, we rely on NISP-based anatomical distributions for these sites and compare them to the NISP-based values from the other sites. It is assumed that although virtually all body parts of reindeer could be used for food preparation (Soppela 2000), the meat-poor cuts, including cranial bones and extremities, were rarely traded.

The MNEs of meatier body parts (upper limb bones and vertebrae) and less meaty body parts (extremities and cranium) are roughly equal between the Ylikylä and Oravaisensaari assemblages (Table 3; Fig. 2). This suggests that animals were slaughtered and consumed locally. When using NISP counts for these two sites, the percentages of meatier body parts are lower, ca. 20–40%, due to the high degree of fragmentation and high number of cranial bones in the mammalian skeleton. The NISP-based distributions from Gamla Kyrkby and Kyrkudden are similar to those from Oravaisensaari and Ylikylä, suggesting that a similar interpretation of animals being butchered and consumed locally is likely to be valid. On the contrary, in Oulu, Tornio, and Björsbyn, meaty body parts are clearly more abundant. This points towards the trade of meat cuts. However, it should be noted that the sample sizes of some of the assemblages were very small. Furthermore, the different quantification methods used due to the lack of data in some of the osteological reports complicated the comparison of anatomical distributions of reindeer remains from different sites.

Table 3 Skeletal frequencies as NISP, MNE and MAU. In some cases, only NISP data is presented due to missing information in the osteological reports. The data from Gamla Kyrkby is missing due to missing sample sizes in percentage calculations in the osteological report

Anatomical element	Oulu*			Tornio			Oravaisensaari			Ylikylä			Björsbyn	Kyrkudden
	NISP	MNE	MAU	NISP	MNE	MAU	NISP	MNE	MAU	NISP	MNE	MAU	NISP	NISP
Cranium	8	1	1.00	5	2	1.00	25	5	5.00	7	2	2.00	4	7
Antler							7	1	0.50					5
Scapula	4	4	2.00	8	4	2.00							2	
Humerus	13	11	5.50	17	16	8.00	6	4	2.00	1	1	0.50	3	1
Radioulna	10	6	3.00	3	3	1.50	4	3	1.50	3	2	1.00		
Pelvis	10	3	3.00	3	2	1.00	6	3	1.50				3	3
Femur	10	6	3.00	11	7	3.50	3	2	1.00				2	3
Tibia	11	9	4.50	1	1	0.50	5	4	2.00				2	
Metacarpus										1	1	0.50	2	
Metatarsus							4	4	2.00	5	2	1.00	4	
Metapodial I/IV	4	2	1.00											
Carpals	14	6	3.00	4	2	1.00								
Tarsals	10	6	3.00	1	1	0.50	3	3	1.50	1	1	0.50		
Phalanges	4	1	0.04	14	3	0.13	6	1	0.04	3	1	0.04		3

*Excluding the Virastotalo assemblage, information not available, $N = 26$

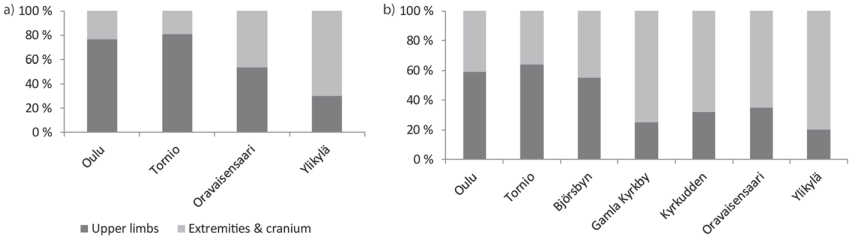

Fig. 2 Percentages of cranial bones and extremities vs. upper limb bones as (**a**) MNE and (**b**) NISP. Only NISP counts are presented for some of the sites due to missing information in the osteological reports

In sum, zooarchaeological data suggests that reindeer were slaughtered locally in Oravaisensaari, Ylikylä, Gamla Kyrkby, and Kyrkudden, whereas in Oulu, Tornio, and Björsbyn, the reindeer remains probably originate from meat cuts.

Ancient DNA Analysis

Ancient DNA analyses were made on four samples (Table 2: samples 5–8) with the aim of finding out whether the animals were wild or domesticated. All laboratory work prior to PCR cycling was conducted in a dedicated ancient DNA laboratory located at the Center of Microscopy and Nanotechnology at the University of Oulu. Stringent measures were followed in order to prevent contamination. First, the outer layer of the bones was removed using a drill bit, after which bone powder was obtained by drilling inside the bone. DNA was extracted from the bone powder using the protocol described by Yang et al. (1998) with the modifications proposed by Gamba et al. (2014) and Gamba et al. (2016), with the exception that double digestion was not performed. Blank extraction controls were included in the batch to monitor contamination.

PCR was used to amplify part of the mitochondrial control region in three overlapping fragments. The PCR primers used are shown in Table 4. PCR reactions were performed in volumes of 25 μl with 1X PCR Buffer (QIAGEN), 2.5 mM MgCl2, 0.2 μM of each primer, 0.2 mM dNTPs, 1 mg/ml BSA, 2 units of HotStarTaq DNA Polymerase (QIAGEN), and 2 μl of DNA extract. The cycling protocol consisted of initial denaturation at 95 °C for 10 min, followed by cycling between denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s, so that the annealing

Table 4 Used PCR primers

Primer pair name	Sequence	PCR length (bp)	Reference
RangD	Forward: TATAATAGTACATTAAAYTAYATRCCCC	141	This study
	Reverse: GGGGRCGGGATACGCATGTTG		
RangE	Forward: GTACATRGCACATTRRGTCAAATC	114	This study
	Reverse: GGGATCCCTGCCAAGCGGGTTG		
RangF	Forward: CAACATGCGTATCCCGYCCCC	130	This study
	Reverse: AATTCATTAAATAGCTACCCCCAC		

temperature was lowered from the initial 58 °C by one °C in every cycle until an annealing temperature of 50 °C was reached. After this, the cycling was continued for 55 cycles with the annealing temperature of 50 °C, and the PCR was ended with final extension at 72 °C for 7 min. In order to identify possible misincorporated bases resulting from post-mortem DNA damage, each PCR was replicated. Negative controls were included in PCR. The PCR reactions were purified using Exonuclease I and Shrimp Alkaline Phosphatase, after which sequencing reactions were performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Sequencing products were run on ABI 3730 DNA Analyzer (Applied Biosystems). DNA sequences were inspected and edited using the CodonCode Aligner program (Version 4.0.4, CodonCode Corporation). We were able to replicate all PCR fragments except one fragment for sample 8. In this case, the corresponding PCR primer pair did not amplify a second time. None of the extraction or PCR blanks amplified in any PCRs. The sequences from this study are deposited in GenBank under the accession numbers MH010859-MH010862.

We compared the obtained consensus sequences to a large reference data set consisting of modern (Røed et al. 2008) and historical (Bjørnstad and Røed 2010; Bjørnstad et al. 2012; Røed et al. 2011) reindeer from Fennoscandia. Sequences were aligned in MEGA7 (Kumar et al. 2016) using the ClustalW algorithm and truncated to equal lengths. The final data set was 183 bp in length and consisted of 488 sequences. PopART (Version 1.7, http://popart.otago.ac.nz) was used to build a Median-Joining network (Bandelt et al. 1999).

As seen in the network (Fig. 3), the study samples are most closely affiliated with ancient reindeer from Finnmark in Norway and modern Finnish forest reindeer. They are not closely related to modern domestic reindeer from Fennoscandia or the domestic reindeer of the twentieth century from northern Fennoscandia. More specific affiliations of the study samples are described here. Sample 5 (Table 2) is located in the central part of the network with a unique haplotype, which is one mutation away from ancient reindeer from Finnmark and modern Finnish forest reindeer. Samples six and seven share a haplotype with ancient reindeer from Finnmark mostly from the period between ca. 3400 and 500 BCE. Sample eight has a haplotype typical of modern Finnish forest reindeer. Sample eight had one base Y (C or T) in a position that has variation in the data set. We therefore also built a Median-Joining network without this sample. The resulting network (not shown) is similar to that in Fig. 3, except for samples six and seven, which have haplotypes that differ from each other by one mutation. Both of them, however, share their haplotype with ancient reindeer from Finnmark.

The close relationship of the study samples and the ancient samples from Finnmark is interesting. Based on the size of the archaeological reindeer from Gressbakken in Finnmark (2000–1500 BCE), on their genetic proximity to modern Finnish forest reindeer, and on the fluctuating vegetation history, Bjørnstad et al. (2012) hypothesized that these reindeer may have been forest reindeer. When this is taken into consideration, along with the backcast distribution of forest reindeer in Finland in historical times (Lundmark 1982:161; Luukko 1954:111; Virrankoski 1973:271–272) and the relatedness to modern Finnish forest reindeer, the most parsimonious interpretation for the status of the reindeer studied here is that they are forest reindeer. Because Finnish forest reindeer are not known to have provided a significant genetic contribution to the modern Fennoscandian domestic pool (Røed et al. 2008), the genetic results suggest that these

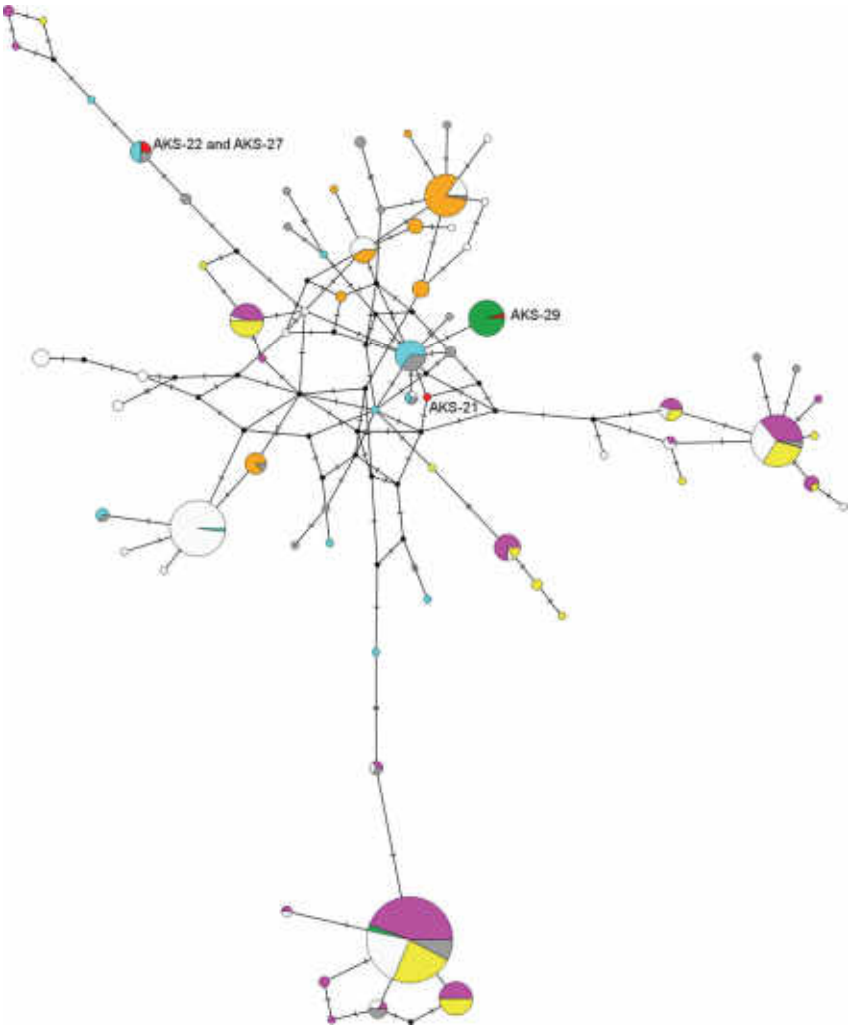

Fig. 3 Median-Joining network of the study samples and the historical and modern reference samples. The samples are color-coded as follows: study samples in red, modern domestic reindeer from Fennoscandia in pink, domestic reindeer from the early twentieth century from northern Fennoscandia in yellow, modern Finnish forest reindeer in green, modern wild Norwegian mountain reindeer in white, reindeer from Hardangervidda from the period between ca. 1210 and 1310 CE in orange, reindeer from Finnmark from the period between ca. 3400 and 500 BCE in turquoise, and reindeer from Finnmark from the period between ca. 100 and 1750 CE in grey. The sizes of the circles correspond to the observed frequency of each haplotype. Mutations are shown as hatch marks and median vectors as black circles

individuals were wild. This interpretation, however, is not without caveats. Firstly, modern and early-twentieth-century domestic populations do not necessarily represent all the genetic diversity of historical domestic reindeer in northern Europe. The genetic composition of Fennoscandian wild and domestic herds has changed significantly during historical times (Bjørnstad and Røed 2010; Bjørnstad et al. 2012; Røed et al. 2011; 2014).

Secondly, domestic and wild reindeer readily interbreed. This sometimes makes it difficult to use maternally inherited mitochondrial DNA to distinguish with full certainty between wild, domestic, tundra, and forest reindeer individuals. Because of these issues, more powerful genetic analyses, such as those based on ancient genome-wide data, are warranted to more securely identify between historical wild and domestic individuals. In sum, the aDNA suggests that the individuals analyzed were wild, but more detailed data would be needed to show this conclusively.

Stable Isotope Analysis

Stable isotope analysis can be used to assess a number of factors ranging from mobility to diet and weaning age. Here, the analyses of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are used to evaluate human influence on reindeer diet. $\delta^{13}C$ is indicative of terrestrial/marine components in the diet, as well as environmental factors, whereas $\delta^{15}N$ can be used to assess the trophic level of the individual (Eriksson 2013). Analysis of samples 1–4 is described in detail in Lahtinen and Salmi (2018). Samples 5–8 were radiocarbon dated and analyzed for stable isotopes at the Laboratory of Chronology at the University of Helsinki. Collagen was extracted using a modified Longin (1971) method. Radiocarbon analysis was conducted using an AMS instrument and stable isotope analysis using the EA-IRMS (Elemental Analyzer-IRMS; NC 2500 + Thermo Finnigan Delta Plus Advantage) instrument (Oinonen 2017a, b). Dating results are presented in Table 2.

The mean $\delta^{13}C$ value in the reindeer bone samples was −19.8‰, with a range from −19‰ to −21.6‰ and a standard deviation of 1.0 (Table 2; Fig. 4), which is within the expected range for reindeer on a terrestrial, lichen-rich diet (Drucker et al. 2010; Salmi and Fjellström n.d.).

The mean $\delta^{15}N$ value in the reindeer bone samples was 4.2‰, ranging from 2.0‰ to 5.6‰. The standard deviation was 1.4 (see Table 2). The bone $\delta^{15}N$ values of reindeer with lichen-rich diets and not influenced by humans are ca. 1.4–3.1‰ (Drucker et al. 2001; Fjellström 2011; Salmi and Fjellström n.d.). We have preliminary evidence

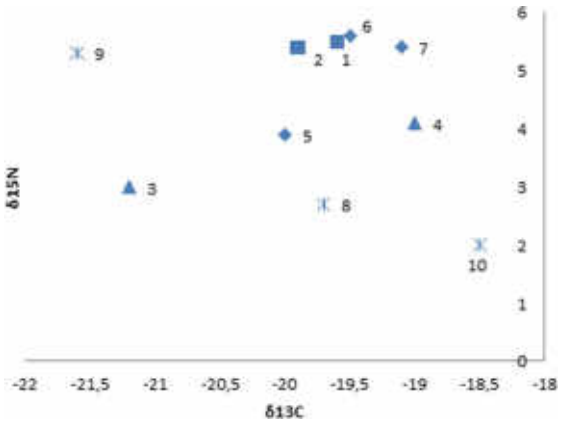

Fig. 4 Scatterplot of $\delta^{13}C$ and $\delta^{15}N$. ■ Oulu, ▲ Tornio, ◊ Ylikylä, * Oravaisensaari

suggesting that reindeer feeding, specifically supplementary winter feeding with twigs and grasses that have higher $\delta^{15}N$ values than lichen, which comprises the natural winter diet, elevates $\delta^{15}N$ values in reindeer (Salmi and Fjellström n.d.). The $\delta^{15}N$ values are also elevated in young reindeer up to the age of ca. one year. Lactation, which continues up to the age of ca. 6–7 months (Nieminen and Pietilä 1999:116), elevates nitrogen values because the calf is in effect feeding on its mother (Eriksson 2013). After weaning, $\delta^{15}N$ values stay elevated up to the age of ca. one year due to the recycling of body protein in the winter (Barboza and Parker 2006; Nieminen 1994; Parker et al. 2005). Starvation elevates $\delta^{15}N$ because muscle proteins are consumed for energy (Eriksson 2013).

Six samples had elevated $\delta^{15}N$ values, indicating young age, starvation, or a human-influenced diet. Young age cannot be ruled out as an explanation for three of these (3–4, 8). In the assemblage from Oulu, one individual with an elevated $\delta^{15}N$ value was over 1.5 years of age (1). In Ylikylä, two adult individuals had elevated $\delta^{15}N$ values (6–7). The individuals with a $\delta^{15}N$ within a normal reindeer range can derive either from wild reindeer or domesticated reindeer that were not given supplementary fodder. In sum, the isotope analyses did not yield conclusive indication of the presence or absence of human influence on reindeer diet.

Discussion: Tangled Worlds

In Ylikylä, Oravaisensaari, Gamla Kyrkby, and Kyrkudden, faunal data point towards local butchery and consumption of reindeer. According to the historical records, the farmers settling in the Ylikylä and Oravaisensaari areas owned reindeer used for transportation purposes (Enbuske 1995:171, 350; Kortesalmi 2008: 44–52). In the Ylikylä assemblage, two reindeer had elevated $\delta^{15}N$ values, possibly indicating a human-influenced diet. However, the aDNA evidence points towards wild reindeer both in Ylikylä and Oravaisensaari. Moreover, wild reindeer hunting was practiced in or near these areas up to the seventeenth century (Enbuske 1995:171, 350; Kortesalmi 2008:44; Lundmark 1982:161; Luukko 1954:111; Virrankoski 1973:271–272), supporting the interpretation of the aDNA evidence as being indicative of wild forest reindeer. The faunal remains from Kyrkudden and Gamla Kyrkby derive from cultural layers associated with marketplaces (Wallerström 1995b:78, 178–180). The reindeer from these assemblages may derive from domesticated reindeer that were slaughtered for consumption or sale at the marketplace (Wallerström 1995b:78). Marketplaces were meeting grounds for Swedish tradesmen and Sámi.

In the assemblages from Oulu, Tornio, and Björsbyn, meaty body parts were clearly more abundant. This probably means that the people in these places mostly bought the reindeer meat cuts they consumed. The merchants of Tornio owned reindeer that they used as draft animals on winter trade trips, but the relatively low number of reindeer bones in the urban faunal assemblage and their uneven skeletal profile suggest that most of the reindeer remains originated from meat cuts bought from the Sámi. In the assemblage from Oulu, one individual had an elevated $\delta^{15}N$ value, possibly indicating human influence on its diet. If that was the case, its meat was most likely sold to the consumer from Oulu by a reindeer herder, based on the fact that the population in the area did not herd or own reindeer themselves (Kortesalmi 2008:40).

This ambiguous interpretation of the data illustrates well the methodological challenges in interpreting reindeer remains from northern Fennoscandia. However, the complex and contradictory data can also be interpreted as evidence of the complexity of the relationships people at each site had with reindeer. There were many types of reindeer around: domesticated herds, transport reindeer, wild reindeer living in different ecological niches. Moreover, people engaged with the animals in different ways: as merchandise, wild game, property, companions, and helpers on winter journeys.

The environment and its animals are usually situated as the subjects of appropriation, disruption, and destruction in colonial contacts between Europeans and natives in different parts of the globe (e.g., Crosby 1986). Indeed, the colonization of the Sámi lands has been described as environmental colonialism, where the natural resources of marginal areas are exploited for the benefit of others (Åström 1978:113; Massa 1994). The utilization of the natural resources of former colonies continues up to today (Huggan and Tiffin 2007). Also disputes about land use in Sámi areas continue today. For example, the mining industry and its effects on the environment and animals are debated in northern Fennoscandia (Ojala and Nordin 2015).

However, the roles of the environment and animals have probably been more complex and multifaceted in past colonial encounters. Animals have acted in different roles in colonial contexts. There were symbolic associations between certain ethnicities and animal species. Sometimes animals played active roles in the contacts between ethnic groups (DeJohn Anderson 2006). Recent work on multispecies ethnography (Kirksey and Helmreich 2010) and archaeology (Pilaar Birch 2018) has emphasized that humans and animals are bound together within multispecies webs consisting of several species and that the lives of the organisms bound in these webs are shaped by political, economic, and cultural forces (Domanska 2018; Haraway 2008:165, 216; Kirksey and Helmreich 2010). In northern Fennoscandia, the encounters, relationships, and practices that connected the Sámi and the Swedish often had to do with reindeer, which shows how these animals acted as mediators, facilitators, and active agents in colonial contacts. It also shows that the Sámi, the Swedish, and the reindeer formed a web that can be described as a multispecies community, a web of interacting organisms.

Archaeological and historical data suggest that reindeer were central in the trade relationships between the Sámi and the Swedish in many ways. The development of reindeer pastoralism has been linked to the growing need to produce reindeer products for the global market and for paying the taxes exerted by the emerging nation-states (e.g., Wallerström 2000), although reasons internal to Sámi society contributed to the development of reindeer pastoralism (e.g., Bjørklund 2013). Among the Swedish, reindeer hides were an important export item in international trade. Other reindeer products, such as bone and antler crafts and meat, were bought and used by the Swedish urban and agrarian populations (Luukko 1954:396–397; Virrankoski 1973:453–454). The reindeer remains discovered in the archaeological assemblages from the towns of Tornio and Oulu, the village of Björsbyn, and the marketplaces of Gamla Kyrkby and Kyrkudden are probably indications of such trade.

Tradesmen, officials, and priests also needed a means of transport in the northern landscape without roads, and they used reindeer for that purpose (Fig. 5). Historical data indicates that although tradesmen, officials, and priests sometimes owned the reindeer they used for traction, the animals were in fact kept in the care of Sámi

Fig. 5 Explorers and scientists travelling in northern Fennoscandia engaged with transport reindeer. Réginald Outhier travelled to Lapland with the expedition of Pierre-Louis Maupertuis, occasionally rode on reindeer sleighs, and documented the journey (Outhier 1975 [1744])

reindeer herders when not used for trips up north (Kortesalmi 2008:44-52; Mäntylä 1971:109). Sámi guides, reindeer drivers, and other helping hands were present during these trips (Kortesalmi 2008:103–108). The *birkarls* usually hired women to take care of their reindeer, probably because of the division of labor in the nomadic reindeer herding practiced in Tornio Lapland. The female reindeer keepers (*lappekonner* or *renekonor* in Swedish) took care of a *birkarl*'s reindeer and also engaged in their business activities. In many instances, there was a sexual relationship between the reindeer keeper and the *birkarl* (Kortesalmi 2008:49–52). Both women and men are mentioned as reindeer keepers for the officials and priests (Kortesalmi 2008:75), and the drivers were in sometimes poor people, women, or children less than ten years old (Kortesalmi 2008:101).

These arrangements add another dimension to the interactions between the Sámi, the Swedish, and the reindeer. The tradesmen, priests, and officials were dependent on the Sámi reindeer keepers, drivers, and guides and their knowledge of the animals and the landscape. Therefore, there were different types of ties between the Sámi reindeer keepers, drivers, and guides, ranging from close relations between the *birkarls* and their reindeer keepers to one-time employment. In addition, there were also close relationships between the Sámi, the Swedish, and the draught reindeer. Draught reindeer were usually bulls that were castrated and trained for draught use. In such a relationship, a close companionship often forms between the human and animal partners, crossing species boundaries (Argent 2010; Vuojala-Magga 2010). Training a reindeer and working with it creates deep bonds and trust between the animal and the human. These deep bonds are based on trust, knowing the reindeer, and reacting to each other's feelings (Vuojala-Magga 2010). Also cohabitation and care tend to create mutual relationships between humans and individual animals (Oma 2010). Ethnographic

evidence also suggests that the Sámi understood reindeer as persons who were capable of communication, emotions, intentional action, and meaningful relationships with people. Although reindeer personhood was different from human personhood, they lived in the same environment as people and shared moments of communication and reciprocity with them (Helander-Renvall 2010). Therefore, there were personal bonds between the Sámi reindeer drivers and herders, tradesmen, officials, priests, and the reindeer with which they worked.

Conclusion

The identification of wild and domesticated reindeer in the archaeological record of northern Fennoscandia is complex. In addition to biological reasons, the problems in identifying different types of reindeer in the faunal assemblages stem from the multiple types of relationships people had with these animals – meat and hide production, hunting, transport, and ownership. Some of these relationships can be traced through zooarchaeology, aDNA analysis, and stable isotope analysis, as well as through contextual historical data.

We have traced the complex relationships between the indigenous Sámi, the Swedish, and the reindeer in late medieval and early modern Fennoscandia. Reindeer were central in the relationships between these societies in many ways – they acted as collaborators and mediators, as well as objects of ownership and trade. Reindeer pastoralism developed among the Sámi, but tradesmen, priests, and officials of Swedish origin benefited from domesticated reindeer in many ways – trading reindeer products and using reindeer for transport during winter trips in the northern landscape. The use of draught reindeer and Sámi reindeer herders to take care of them further tied together the Sámi, the Swedish, and the reindeer. From the late seventeenth century onwards, farmers also adopted reindeer husbandry from the Sámi, which illustrates well how both parties of a colonial encounter undergo cultural changes.

Recent research has shown that the interactions and power relations between Late Iron Age and medieval societies in northern Fennoscandia were complex, multifaceted and shifting (Bergman and Edlund 2016; Kuusela et al. 2016; Ylimaunu et al. 2014). We have attempted to show that also animals, especially reindeer, played important roles in the contacts and encounters between these societies. Together, the Sámi, the Swedish, and the reindeer formed a multispecies community, a web of interacting organisms. In the northern landscape, cooperation with reindeer was essential for carrying out a range of activities, including trade and professional responsibilities. Close-knit relationships formed between humans and the reindeer they were dependent on and worked with. Reindeer were active agents in shaping the relationships between the Sámi and the Swedish. The history of colonial contact between the Sámi and the Swedish is therefore also the history of reindeer.

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V

CHAPTER 5

4000-YEAR-OLD REINDEER MITOGENOMES FROM THE VOLGA-KAMA REGION REVEAL CONTINUITY AMONG THE FOREST REINDEER IN NORTHEASTERN PART OF EUROPEAN RUSSIA

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Introduction

Of the three main ecotypes of reindeer in Eurasia, especially the forest reindeer has suffered due to human over hunting and habitat fragmentation. At present, the Eurasian forest reindeer is found in multiple regional subpopulations in European Russia, Finland and Asia, many of which are endangered (Gunn 2016). In historical times however the range of the forest reindeer has been larger and probably more continuous. Reindeer *Rangifer tarandus* L., 1758 is known in the Middle Volga region from the Middle Pleistocene (located in Tunguz) (Alekseeva, 1990). In the second half of the Late Pleistocene (Würm) on the territory of the Middle Volga region, reindeer was a common species of periglacial forest-steppe landscapes (Turubanova, 2002; Petrova, 2009). In the early Holocene and the first half of the middle Holocene, according to the number of bone remains from archaeological sites, its number in the territory of Tatarstan was significantly lower than in Würm (Petrenko, 1984, 2007; Askeyev et al., 2009). In the second half of the middle Holocene (Subboreal period) and at the beginning of the late Holocene (SubAtlantic–1), reindeer were widely distributed throughout Tatarstan, and its populations size was the largest during the entire Holocene period (Zbrueva, 1937; Petrenko, 1984, 2007; Gasilin, 2009; Askeyev et al., 2009). According to archaeozological data in the 4th-7th centuries and the 10th-17th centuries AD the reindeer lived throughout the territory of Tatarstan, its bones were diagnosed on 10 archaeological sites (Petrenko, 1984, 2007; Askeyev et al., 2016). In the 18th century - the first half of the 19th century, reindeer continued to be found in all large forest areas both north of the Volga and Kama rivers, and on some large woodlands south of these rivers (Eversmann, 1840; Kirikov, 1960, 1966). At the end of the 19th – beginning of the 20th centuries, this species was very rarely found in the northern and northeastern regions of Tatarstan (Bogdanov, 1871; Kirikov, 1960, 1966). The last reliable data on the findings of reindeer in Tatarstan fall on the twenties of the 20th century (Bashkirov, Grigoriev, 1931; Kirikov, 1960, 1966). In order to study the faith of these southernly distributed reindeer from the boreal forest regions of the Volga-Kama region, we obtained genetic data from 4000-year-old reindeer samples from Tatarstan and compared it with data from modern Eurasian populations. We also compared the body size estimates of the reindeer with estimates obtained from other ancient sites in Russia (see Appendix 2).

Material and methods

Samples and DNA extraction

We subjected six samples from the Pestrechinskaya II site to DNA analysis (Table 1). The samples consisted of post-cranial skeletal parts and teeth. All genetic work prior the sequencing library amplifications was conducted in ancient DNA laboratory located at the Swedish Museum of Natural History. Around 50 mg of bone powder was obtained from each sample by drilling inside the bone. DNA was then extracted using the protocol outlined in Ersmark et al. (2015). This protocol is a modified version of the protocol C in Yang et al. (1998).

Library preparation, mitochondrial genome capture and sequencing

Uracil-DNA-glycosylase (UDG) treated sequencing libraries were built according to step (g) Library preparation: full uracil–DNA–glycosylase treatment (III) as in Rohland et al. (2015), which is based on the methods described in Meyer and Kircher (2010) and Kircher et al. (2012). The six amplified libraries of the reindeer from the Pestrechinskaya II site were pooled together with five other ancient reindeer

libraries in equimolar ratios. Each library had a unique barcode combination. The pool was then subjected to mitogenome capture as described in Maricic et al. (2010) using deer-specific bates. After the capture, the pool was turned into a complete sequencing library by PCR, using indexing primers as in Meyer and Kircher (2010). The quality and concentration of the purified library pool was quantifiedon a 2100 Bioanalyzer (Agilent), and the pool was combined with other capture pools that had different barcode combinations and indexes into a single pool in equimolar concentrations. The final pool was sequenced on one Illumina MiSeq lane with a 2x151bp setup and on one HiSeq lane with 2x126bp setup.

Data processing

Fastq-data from both runs was merged and demultiplexed based on the unique sample barcodes (custom python script), removing reads with an incorrect barcode pairing (~1% of reads). We then removed sequencing adapters using Trimmomatic (Bolger et al. 2014) and subsequently merged the reads with AdapterRemovalV2 (Schubert et al. 2016). The first and the last 7 base pairs of each read were removed as these represent the barcodes. Merged reads where then mapped to the reindeer mitogenome reference (GenBank accession number KM506758, Ju et al. 2016) using bwa aln (Li and Durbin 2009), excluding reads below 15 base pairs. During the mapping, the human mitogenome (hg19 and PhiX genome (NC_001422) reference sequences were used as decoys. We then removed duplicates (samtools rmdup, Li et al. 2009). Mitogenomes were constructed by calling the major allele at each site covered by at least three independent reads and above 90% of reads agreeing on the major allele.

Mitogenome sequence phylogeny

The consensus sequences with at least 3x coverage were used in the following analyses. We included a published mitogenome of an Aoluguya reindeer (GenBank accession number KM506758, Ju et al. 2016), and aligned the sequences using MAFFT online version 7 (https://mafft.cbrc.jp/alignment/server/, Katoh et al. 2002; Katoh and Standley 2013; Katoh et al. 2017). In order to infer phylogenetic relationships among the study samples, we then built a Bayesian phylogenetic tree using MrBayes

Table 5-1.

Reindeer samples analyses in the study

Таблица 5-1.

Образцы северного оленя, проанализированные в данном исследовании.

DNA sample code	Sample no	Bone	Archaeological lable (in English and Russian)
P3	3	humerus	Pestrechinskaya II site 2013, Digging 1, plot G/5, layer 9, sector B, 12.08.13, p. 57 (Пестречинская II стоянка 2013, Р.1, уч.Г/5, пласт 9, сектор Б, 12.08.13, стр. 57)
P5	5	metatar-sus	Pestrechinskaya II site 2013, Digging 1, plot B/9, layer 11, without location (Пестречинская II стоянка 2013, Р.1, уч.Б/9, пласт 11, б/м)
P10	10	humerus	Pestrechinskaya II site 2013, Digging 1, plot G/5, layer 8, out clusters of bones, 11.08.13 (Пестречинская II стоянка 2013, Р.1, уч.Г/5, пласт 8, вне скопления костей, 11.08.13)
P13	13	metatar-sus	Pestrechinskaya II site 2013, Digging 1, plot V 4,5,6, abreast layers 8 – 9, bones from a landslide outcrop and scree (Пестречинская II стоянка 2013, Р.1, уч. В 4, 5, 6, уровень пласта 8-9, кости из обнажения оползня и осыпи
P17	17	teeth	Pestrechinskaya II site 2013, Digging 1, plot B/10, layer 10 (Пестречинская II стоянка 2013, Р.1, уч.Б/10, пласт 10)
P20	20	phalanx1	Pestrechinskaya II site 2013, Digging 1, plot G/5, layers 9, depth 162,5 cm, 12.08.13 (Пестречинская II стоянка 2013, Р.1, уч.Г/5, пласт 9, гл. - 162,5 см, 12.08.2013)

version 3.2 (Ronquist et al. 2012), running the analysis for 2,500,000 generations and saving every 1000th sample. HKY+I substitution model was used in the run, as this was inferred as the most optimal according to jModelTest version 2.1.4 (Guindon and Gascuel 2003; Darriba et al. 2012) that could be used in MrBayes. The first 250,000 samples were discarded as burn-in, and a 50 percent majority rule tree was visualized using FigTree version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

MtDNA control region haplotype sharing

Due to the limited number of complete mitogenomic sequences for comparative purposes, we made further analyses using only the control region, from which there is more reference data available. First we studied possible haplotype sharing between the historical reindeer from Tatarstan and present day populations. We included a large number of sequences representing both wild and domestic Eurasian reindeer diversity (Røed et al. 2008; Kholodova et al. 2011; Baranova et al. 2012; Kvie et al. 2016a; Kvie et al. 2016b; Korolev et al. 2017), aligned these together with our sequences, and truncated the dataset to 179 base pairs in order to accommodate all the sequences. We then identified shared haplotypes within the dataset using PopART version 1.7 (http://popart.otago.ac.nz).

MtDNA control region haplogroup affiliations

In order to identify to which mtDNA control region haplogroup each sample belonged to, we made a phylogenetic tree with representative haplotypes of each haplogroup from Kvie et al. (2016b). This was done with BEAST version 1.10.4 (Suchard et al. 2018) using tip dates (Drummond et al. 2002), HKY+gamma+invariant sites as a substitution model with 4 gamma categories, strict clock and GMRF Bayesian Skyride (Minin et al. 2008) as a tree prior. The analysis was run for 100000000 iterations logging parameters every 10000 iterations. Maximum clade credibility tree was built after discarding the first 10000000 states as burnin. The tree was visualized with FigTree version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Results and discussion

Mitogenome sequence phylogeny

98-99% of the sequence was resolved at 3X coverage, for all samples except P13, which was resolved to 87%. These sequences have been submitted in GenBank under the accession numbers MK608014-MK608019. Each sample has a unique haplotype. The general relationships of the mitogenomes are shown in Figure 5-1. All groupings have a high support. Samples P20 and P17 group together and further form a group with a modern Aoluguya reindeer from China. Samples P3 and P10 are closely related to each other, and together group with P13. Sample P5 is basal to this latter group.

MtDNA control region haplotype sharing

Because this part of the analysis is based on very short sequences, the results should be interpreted with some caution. We, however, observed mtDNA continuity between the historical reindeer of the Volga-Kama region and present day wild populations of the northeastern part of the European Russia: Sample P13 had the same haplotype as some wild reindeer from Cispolar Urals and Taimyr. Samples P3 and P10 shared a haplotype with wild reindeer from Mezen and Peza–Kosminsk regions. Sample P5 shared a haplotype with wild reindeer from Cispolar Urals as did the sample P17. Sample P20 had a unique haplotype. All in all, the historical reindeer from Tatarstan shared haplotypes especially with modern reindeer from the taiga zone of the northeastern part of European Russia, implying genetic relatedness between these populations. It is also worth noting that we didn't observe any haplotype sharing with Eurasian domestic reindeer nor the Finnish forest reindeer.

MtDNA control region haplogroup affiliations

As seen from the Figure 2, samples P5, P13, P3 and P10 take basal positions in haplogroup II.

Based on mitochondrial control region data, this haplogroup is at present mostly found in western parts of the reindeer distribution in Eurasia, and is especially common among the semi-domestic reindeer of Fennoscandia, where together with haplogroup Ib, it is the dominant haplogroup (Flagstad and Røed 2003; Røed et al. 2008; Kvie et al. 2016b). The fact that a lot of basal diversity regarding this haplogroup is observed among the ancient reindeer from the Pestrechinskaya II site, might suggest that this haplogroup has its origin east of Fennoscandia. The haplogroup II haplotypes observed among the ancient reindeer from the Pestrechinskaya II site are not however particularly closely related to the haplotypes observed among the Fennoscandian semi-domestic reindeer, which together with the absence of haplogroup Ib in Pestrechinskaya II

site may suggest that the Fennoscandian domestic reindeer lineages have probably not directly originated from the population presented by the Pestrechinskaya II site. Samples P17 and P20 are placed on the base of haplogroup If, but without statistical support due to the low resolution on the deeper nodes in haplogroup I.

Conclusions

Our results suggest that there is genetic continuity between the historical reindeer from the Volga-Kama region and present day wild reindeer from northeastern part of the European Russia, especially from the taiga zone. Even though our sample size was rather small, we further observed surprisingly lot of basal diversity within mitochondrial haplogroup II, and this finding may have significance regarding the deep history of this haplogroup.

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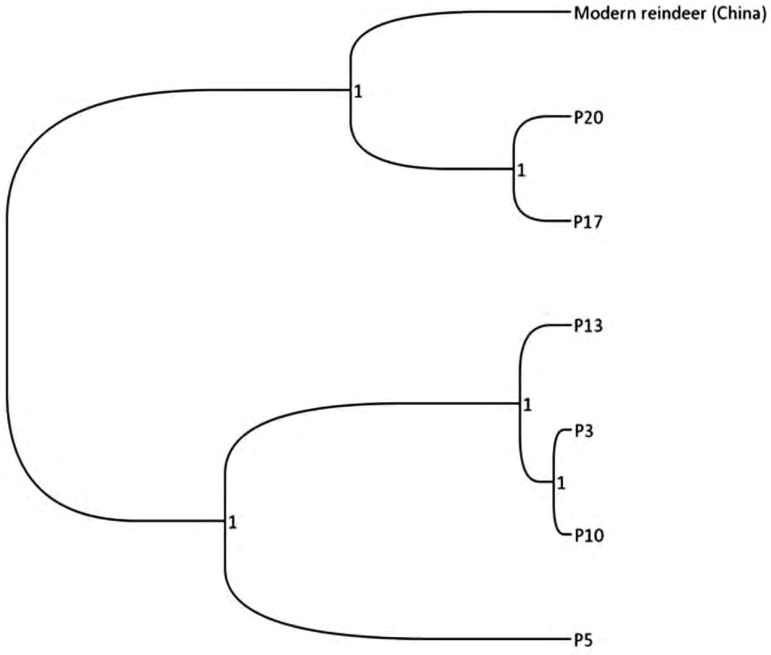


Fig. 5-1. Bayesian phylogenetic tree depicting the relationships of the study samples and a modern Aoluguya reindeer from China.

Рис. 5-1. Байесовское филогенетическое дерево, изображающее взаимоотношения исследованных образцов и современного северного оленя Аолугуя из Китая.

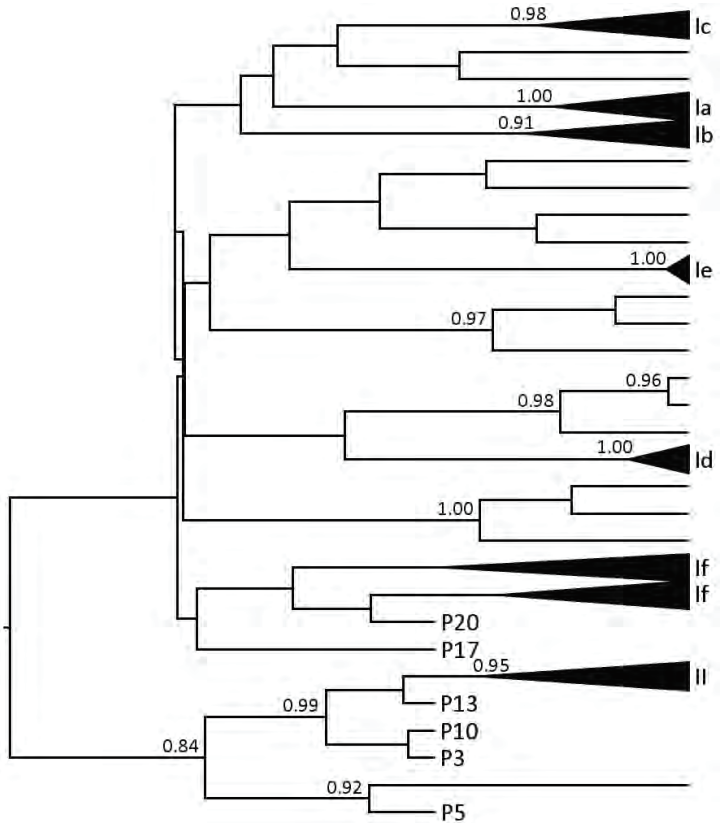


Fig. 5-2. Bayesian phylogenetic tree showing the mitochondrial control region haplogroup affiliations of the study samples. Posterior probabilities above 0.80 are shown above the nodes. The branches that are unlabelled, represent undefined mtDNA control region haplogroups.

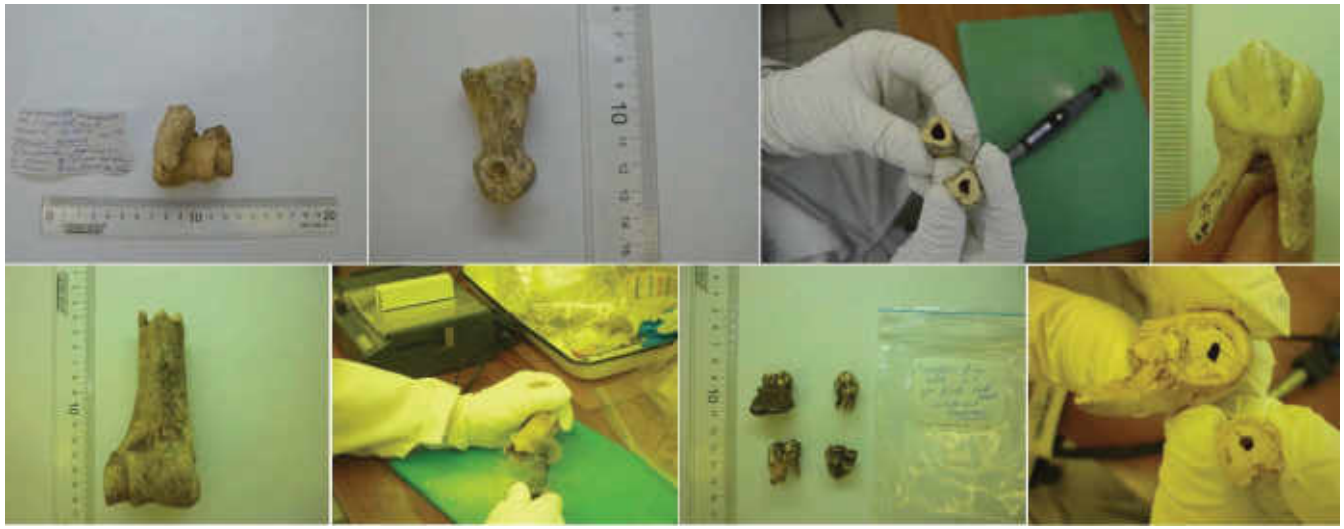
Рис. 5-2. Байесовское филогенетическое дерево, показывающее принадлежность гаплогруппы митохондриального контрольного региона исследуемых образцов. Условные вероятности выше 0,80 показаны над узлами. Ветви, которые не имеют метки, представляют собой неопределенные гаплогруппы контрольного региона мтДНК.

APPENDIX 1.

SELECTION, ETIQUETTE AND EXAMPLE OF PREPARATION OF SAMPLES OF ANCIENT BONES OF THE REINDEER OF TATARSTAN REPUBLIC

(LABORATORY BIOMONITORING OF THE INSTITUTE OF PROBLEMS IN ECOLOGY AND MINERAL WEALTH, TATARSTAN ACADEMY OF SCIENCES, KAZAN, RUSSIA)

Fig. 5-3.



APPENDIX 2.

The size of the reindeer from Holocene time in the Russia

As for the dimensional variability of reindeers in the Holocene, very little research is done mainly on the measurement of bones from individual archaeological sites without significant comparative aspects with similar osteological collections. I. Tsalkin's scientific publications (1961, 1962) provide data on the measurement of the bones of reindeer from a number of archaeological sites in the forest zone of the Upper Volga region dating back to the period of the beginning of the first millennium AD. A fairly extensive database on the size of the bones of the reindeer from the Holocene of the Urals and Western Siberia is given in the articles and PhD thesis of P. Kosintsev (1991, 1992, 1997a, b, 2009), (Razhev, Kosintsev, Ulitko, 2005) and the thesis PhD of O. Bachura (2006). They conclude that in the Holocene the reindeers of the Urals had large bones of the skeleton compared to the late Pleistocene and apparently belonged to the forest form. At the same time, in the late Holocene of Western Siberia P. Kosintsev (1997a, b) and at the end of the Middle Holocene of the Polar Urals (Kosintsev, 2009), based on comparatively large osteological material, concludes that reindeers in the forest-tundra and the northern taiga belt were very similar to the representatives modern tundra subspecies *Rangifer tarandus tarandus*. Interesting results with the use of statistical methods of research on reindeer osteology and osteometry of the early Holocene frozen site in the Siberian High Arctic (study on the Zhokhov site faunal remains, De Long Islands LE-3534: LE-3529 Reindeer bone fragments 8050±70, LE-3536 Reindeer antler 8610±220, Reindeer bone fragments 7810±180, GIN-6400 Reindeer humerus 7930±40) were received (Pitulko, Kasparov, 1998; Pitulko et al., 2015). Comparison of the size of the reindeer bones from the island of Zhokhov with the similar sizes of the reindeer of the late Pleistocene of the Urals and Transbaikalia and with modern tundra populations was made: the reindeer of the early Holocene of the island of Zhokhov were somewhat larger than the late Pleistocene Ural reindeer and much larger than the Transbaikalian ones, while they were almost identical to the representatives modern tundra reindeer (*Rangifer tarandus tarandus*), however, the reindeer of the island of Zhokhov were much more graceful (Pitulko, Kasparov, 1998).

On the basis of the algorithm proposed by Weinstock (1997a, b, 2000, 2006), we made a preliminary calculations of the Variability Size Index (VSI). The VSI is calculated according to this formula: VSI= (x – m /2xs) × 50; where x represents the actual measurement for which the index is being calculated, m is the arithmetical mean of the standard population for the dimension in question and s is the standard deviation of the standard population for that dimension. For all the VSI's of a bone fragment, the mean is calculated and used further. Combining all the individual 'mean VSI's' from a site ensures that the sites can be compared to each other. VSI calculated based on the data of osteometric studies of Holocene reindeer from Russia (see Fig. 5-4) : 1. Early Holocene - island of Zhokhov, The Novosibirsk Islands (76º08'N 152º43'E (Pitulko, Kasparov, 1998; Pitulko et al., 2015); 2.Yanganape 2 (Layer 3. 3320 ± 50 BP, CO AN - 3930; (67º42'N 67 ° 51'E)), the Polar Urals (Kosintsev, 2009); 3. As a standard population, of the reindeers data from Ust'-Poluysk site (Salekhard, 66º56'N 66º56'E) were used (the tundra-forest zone, the end of the first millennium BC - the beginning of the first millennium AD) (Kosintsev, 1997a); 4.Vermulegan 1 (15th-16th centuries AD) (65º47'N 64º04'E) (Kosintsev, 1997b); 5. The settlements of the Upper Volga region (the beginning of the first millennium AD) (Tsalkin, 1961, 1962); 6. Pestrechinskaya II site (end of the Middle Holocene 3700 BP), Republic of Tatarstan (55º72'N. 49 ° 63'E) (Askeyev I. V. personal data); 7. Grotto Bobylek, Middle Ural (56º23'N. 57 ° 37'E) (1743±110 BP – IEPA – 139a, 1713±110 BP – IEPA – 140a), (Razhev, Kosintsev, Ulitko, 2005).

Based on the results of the calculation of the Variability Size Index (VSI), it can be concluded that the reindeer of the forest belt of Eastern Europe in the Holocene were very large (Pestrechinskaya II site – VSI = 69,6 and settlements of the Upper Volga region - VSI = 52,64) and should refer to a large forest reindeer form similar to the *Rangifer tarandus fennicus* Lonnberg, 1908 (modern forest reindeer is VIS = 46,65. Calculations are performed according to osteometric data from Sokolov, Chernyavsky, 1962) and middle size (Grotto Bobylek – VSI = 14,45). Eduard Friedrich von Eversmann also drew attention to the very large sizes of taiga reindeer from Kazan province compared to semi-domestic reindeer from Siberia (Eversmann, 1840). Professor E. F. von Eversmann was the

first zoologist who not only saw, but also carried out measurements (8 specimens) of reindeer from the taiga forests of the Volga – Ural region. The reindeer of the Holocene tundra and forest-tundra, as well as the northern part of the taiga zone of Western Siberia, were approximately of the similar size (VSI = 1,95; -0,94; 0,2 (standart population) and in their size should be referred to the tundra form (The modern tundra reindeer is VIS = 4,93. Calculations are performed according to osteometric data from Kuzmina, 1971). The reindeer of the early Holocene from the Island of Zhokhov according to the results of VSI (-5,49) were not large - High Arctic ecotype, that's probably consistent with the fact that they lived on the northern edge of its range, and the population had an insular character. Thus, the VSI -method applied to reindeer showed the existence of three main ecotypes of reindeer on the territory of Russia in the Holocene: tundra reindeer, boreal forest reindeer and High Arctic reindeer. In addition, this method is very effective for determining the assessment of the climatic parameters of the existence of different populations in ecotypes of reindeer. Reindeer body size variability could be used as a reliable proxy for environmental conditions during Holocene.

Fig.5-4. Representation of reindeer body size and mean VSIs from Holocene sites of the Russia.
Standard population (3) from Ust'-Poluysk site.
Рис. 5-4. Изображение размеров тела и среднего VSIs северных оленей из голоценовых местонахождений России. Стандартная популяция (3) из Усть – Полуйского городища.

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