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Lynx presence in Roman times in the lower germanic *Limes* region: The case of Alphen aan den Rijn

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ABSTRACT

In 2001 and 2002 an excavation conducted in the town of Alphen aan den Rijn in the Netherlands revealed the vestiges of a Roman fort, *Castellum Albaniana*, situated along the historical Rhine delta and used for centuries during the Roman occupation (41 AD – 275 CE). Among the animal bones retrieved from the surrounding defensive ditches, remains of *Lynx lynx* bones were found. Lynx is currently not native to the Netherlands but might have been in historical times and it could have been transported to the *Limes* region by the Romans or caught in the direct surroundings of the *castellum*. In the present study, we describe the retrieved lynx bones initially identified based on morphology. We performed ancient DNA amplification, sequencing and alignment to confirm species identification and to determine the haplotype. Previous haplotyping of lynx from various sites reported by other studies has shown that lynx distribution in Europe during Roman times was very different from its current distribution. DNA analysis of cytochrome oxidase I and cytochrome *B* confirmed the identification of the animal species as *Lynx lynx*. Sequencing of the mitochondrial control region revealed that the animal carried a DNA haplotype, different from those from North Sea fossil lynx remains, but comparable to a haplotype found in southern France. Analysis of stable isotope of the bone materials, to determine the region where the animal lived, suggests the provenance of the animal from a region which comprises southern and central Europe including a part of the Netherlands.

1. Introduction

In the years 2001–2002, the remains of a Roman fort, *Castellum Albaniana*, were unearthed during an archaeological excavation in the Dutch town of Alphen aan den Rijn, situated along the historical Rhine delta in the Netherlands (Polak et al., 2004). It was one of around 20 known forts in the Netherlands that formed part of the Rhine *Limes*, the northwestern frontier of the Roman Empire from ca. 40 – 270 CE (ibid.). The *Castellum Albaniana* was used for several centuries during the Roman occupation. The chronology of the *castellum* can be divided into three distinctive periods, identified stratigraphically and dendrochronologically: a first *castellum* period was marked by the presence of a wooden construction (41–69 AD) which was followed by a specific distinctive wooden construction in the second period (until the middle of the second century) and followed by the third period marked by the presence of various stone constructions (most likely ending around 275 CE). At the end of the third century, the fort was abandoned by the Romans.

During the excavation several animal bones were found in the defensive ditch surrounding the *castellum*. Amongst these, bones of a lynx were also unearthed. Lynx remains are not commonly found in historical periods and reports on their findings are scarce. The lynx remains found at Alphen aan den Rijn concern postcranial remains most likely in anatomical position. These remains are subject to the current study.

Eurasian lynx is not native to the Netherlands at the present time, but

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was in ancient times. There have been few reports of findings of lynx remains in the Netherlands. The oldest remains known in archaeological material are those retrieved at the Neolithic site of Schipluiden (3,650–2,050 cal. BC), where four molars were found. At the Roman fort of Valkenburg (1st century CE), a complete skull was discovered (Bree and Clason, 1971; Kooijmans and Jongste, 2006). These finds were unfortunately not available for research. So far no postcranial remains of lynx have been retrieved from archaeological sites in the Netherlands other than those found at Alphen aan den Rijn.

It is assumed that the Romans considered the lynx to be an exotic animal. According to Pliny the Elder the lynx would have been an animal from Ethiopia (Pliny the Elder, *Naturalis Historia*, book VIII 30(72) [1st cent. AD] (Rackham et al., 1997). The same Pliny, however, writes that the Gauls were acquainted with an animal similar to a wolf but with spots like a leopard (Pliny *Naturalis Historia*, book VII 28)(70) (Rackham, 1997). It is known that large quantities of wild animals were procured by the Romans from different parts of the Provinces for the spectacles (*venationes*) in Rome and other large towns of the Empire. Special soldiers in the army, most notably *venatores* and *vestigiatores*, had the task to hunt for wild animals (Epplett, 2001). Furs of wild carnivores, like wolf and lion, were used for the legionaries' uniforms like those of the *signiferi* (Webster, 1969, Junkelmann, 1986, Davies, 1989). Descriptions of lynx in ancient texts or pictographical representations, however, are scarce. Pliny reports the display of a lynx by Pompey (Pompejus Magnus) in his *ludi* in 55 BC (Pliny the Elder, *Naturalis Historia*, book VIII 28 (70) (Rackham et al., 1997). This event is, however, disputed and it is not sure whether it ever occurred (Keller, 1909).

The natural habitat for a Eurasian lynx is forest or open mountainous terrain. Extensive studies by Schmidt et al. (2011), Rueness et al. (2014), Rodríguez-Varela et al. (2016) and recently by Lucena-Perez et al. (2020) have documented lynx populations in Europe in ancient and more recent times. Lynx, which lives a solitary and withdrawn life, was not rare in Central Europe through the Pleistocene era until the Weichselian glaciation, as attested by the numerous findings, but one of the most common animal species (Charles, 1997; Sommer and Benecke, 2006). However, precise distribution patterns of lynx in later prehistoric

and historic times in Europe are not clear (Breitenmoser, 1998, Niedziałkowska et al., 2006). Hilly or open terrain could be found in the more eastern and southern parts of the Netherlands, but not in the Rhine delta. The region where the *castellum* was located was a swamp-like area and it was situated in the delta of the river Rhine, which was huge in Roman times and had a different course from the present one (Manuel, 2013). Pleistocene bone materials presumed to be from wild animals, amongst others lynx, were retrieved from the North Sea region, which, until approximately 8,000 before present, was an open-land mass called Doggerland, that connected the British islands to the European continent (Fitch et al., 2007). Doggerland used to be a more open and forest-like area and is a rich source for various fossil remains derived from megafauna animals (Kolfschoten and Laban, 1995). Previously it was already shown that lynx remains were found in the United Kingdom that dated back to very early Mediaeval times (Hetherington et al., 2006). More recently, Pleistocene lynx skeletal remains (a jaw, tooth and vertebra) were also found on the Sand Motor in the North Sea region (Bakker, 2018; Langeveld and Liscaljet, 2019). Lynx of latest Mesolithic age was reported from Steetley Cave, Notthinghamshire, UK, by Bramwell et al. (1984). Locations mentioned in this study can be seen in Fig. 1.

The aim of this study was to present the zooarchaeological remains found at Alphen aan den Rijn and to determine genetically whether the retrieved lynx skeletal remains belonged to a *Lynx lynx* (Eurasian lynx) or to another species, like the closely related *Lynx pardinus* (Iberian lynx), or even to another genus. We used the established and recently adjusted ancient DNA isolation methods by Rohland and Hofreiter (2007), Rohland et al. (2018) and performed Q-PCR and melting curve analysis for identification. Subsequently, we investigated the haplotype of the animal by mitochondrial control region sequencing. We compared the obtained haplotypes derived from two lynx specimens (a jaw and a tooth) from the North Sea region, as well as published lynx haplotypes to the lynx haplotype from the Alphen aan den Rijn excavation. Next to the genetic analyses, strontium isotope analysis was conducted with the aim to gain insight into its provenance.
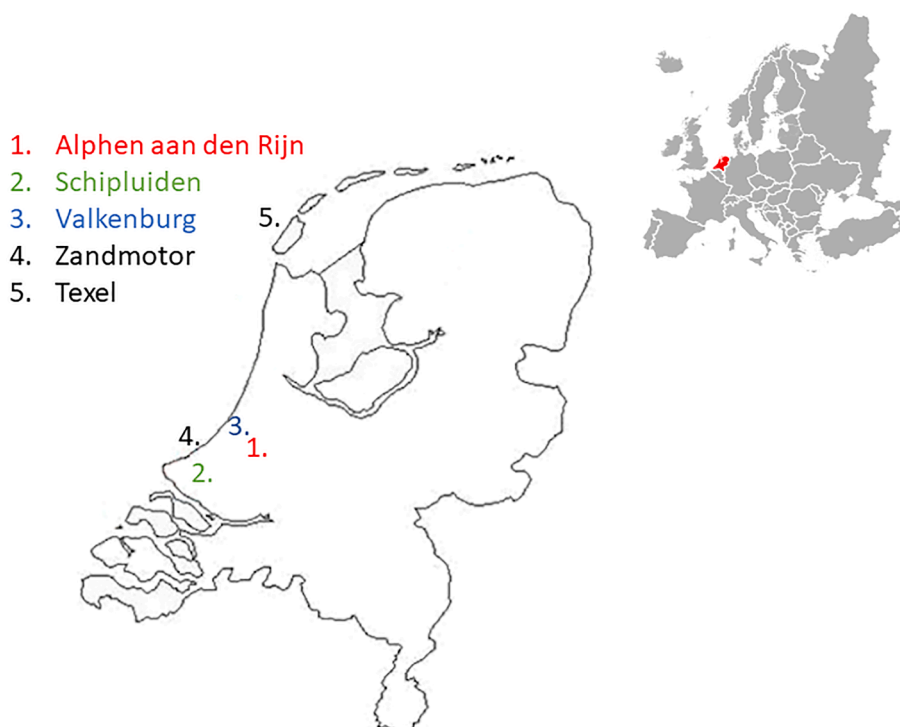

1. Alphen aan den Rijn
2. Schipluiden
3. Valkenburg
4. Zandmotor
5. Texel

Fig. 1. Map of the Netherlands with locations of the sites of the lynx remains analyzed in this study.

Table 1

Total of mammal remains found at the *castellum* of Alphen aan den Rijn (all three periods). The table contains an overview of the mammal remains analyzed. It is important to note that this is not a complete listing of all the bone material recovered during excavation, but rather the material that came from known contexts. The number represents the total number of identified specimens (NISP) per species.

Species	Number	Percentage
Bos taurus - cattle	204	49.3
Equus caballus - horse	2	0.5
Sus domesticus - pig	25	6.0
Ovicaprids – sheep/goats	22	5.3
Ovis aries - sheep	3	0.7
Capra hircus - goat	1	0.2
Canis familiaris - dog	5	1.2
Felis catus - cat	1	0.2
Homo sapiens - human	1	0.2
Lynx lynx - lynx	7	1.7
Cervus elaphus – red deer	1	0.2
Large mammal	61	14.7
Medium mammal	15	3.6
Indeterminate	66	15.9
Total mammals	**414**	
Anas sp. - duck	5	
Pisces - fish (undertermined)	1	
Mytilus edulis (common blue mussel)	2	
Total other categories	**8**	

2. The excavation site and zooarchaeology

2.1. The archaeozoological assemblage from Alphen aan den Rijn

The zooarchaeological research revealed predominantly the presence of domestic animals, amongst others consisting of pig, sheep, horse and goat remains. The materials from the 2001–2002 campaigns were hand-collected and over 400 kg of bones were analyzed and the only wild animal found, besides lynx, was a red deer (table 1). The archaeozoological assemblage was not sieved, hence skeletal remains from small mammals, birds and fish are scarce or absent. Of the materials analyzed, 414 of the bone fragments came from mammals. A statistically insignificant number was derived from bird, fish and shell remains (see also Table 1).

The lynx bones most likely belonged to one animal. An overview of all the identified skeletal elements can be found in Table 2. The bones were found in the defensive ditch of the *castellum* (trench 40) which was used for dumping of materials from butchering activities. Seven skeletal elements were recovered: right and left femurs, a patella, two pelvic halves and two sacral vertebrae (Fig. 2). The left femur and the patella were both complete; the right femur is preserved for 75%. The distal end of the bone was not recovered. The left femur was also broken three quarters down the shaft, but both proximal and distal ends were recovered, and could be reassembled. The morphometrical resemblance

of the two femora, and the matched articulation of one of them with the pelvis leaves no doubts that the bones belong to one individual. The sacral vertebrae were also preserved for 75%, missing only some of the most fragile processes. Each pelvis half was about 50% preserved, the right pelvis was represented by an ilium and the left by the ischium. In both cases, at least part of the acetabulum was preserved. These bones were identified as Eurasian lynx (*Lynx lynx*) on the base of morphological criteria. The species identification was supported by comparison to known *Lynx lynx* bones in the collection of Naturalis Biodiversity Center in Leiden.

The bones were all recovered in the lowest layer of the filling from defensive ditch 1, a location that clearly remained stratigraphically undisturbed and dated to the first phase (Period I) of the *castellum* (Rien Polak, personal communication) and where predominantly remains of domestic animals were found. These were, in contrast to the lynx bones, highly fragmented and bearing signs of butchering marks, a clear sign of human consumption. It is known from historical sources and excavations that lynx was not a consumed animal (Davies, 1971). In addition, lynx remains from this period in the Netherlands are very rare: only one complete skull (*cranium + mandibula*) from the Roman fort of Valkenburg has so far been documented (Bree and Clason, 1971). This skull was found in the right half of the *praetentura* of *castellum* 1 in Valkenburg and dated to the early forties of the 1st century CE. This specimen came from a young (subadult) individual, possibly a male. Its size was compared with other archaeological remains from Northern Europe which showed a similar size to prehistoric remains. Indirect evidence for the presence of lynx seems to be represented by prints left on a *tegula* (roof tile) from Voorburg-Arentsburg, the Roman town of *Forum Hadriani*, (Gazenbeek, 2014). The finds might indicate that lynx could have roamed freely in the tile production area, which may not have been in the same region as *Forum Hadriani* according to Gazenbeek (2014). The remains from Valkenburg were cautiously interpreted as the result of an animal caught in the vicinity of the fort.

The bones of the lynx from Alphen aan den Rijn did not show any signs of butchery indicating that the animal was not consumed. It can, however, not be ruled out that the animal was skinned for its fur as the bones that would show evidence of skinning were not recovered. No clear signs of butchering marks were reported for the Roman skull from Valkenburg either, however, notches were found on the forehead of the skull which were tentatively interpreted as butchering marks, indicating that only the fur/skin, attached to the head, was brought to the fort; see also Bree and Clason, page 134 (1971).

Signs of pathology were not observed on the bones of lynx from Alphen aan den Rijn and there were no signs of gnawing or burning. We presume the animal was an adult as the epiphyses of the femurs were completely fused, as were the acetabula. Strongly developed bone structure at the location of muscle attachments around the epiphyses and the acetabula would indicate that the animal might have been of advanced age.

Table 2

Total fragments of elements for lynx (*Lynx lynx*) from Alphen aan den Rijn.

Skeletal element	Total	completeness	marks		Measurements* (mm)
Femur left	1	Complete	No signs of butchering of gnawing	Proximal and distal epiphyses fused	GL = 222,47 BP = 41,69 SD = 13,97 DC = 19,37 BD = 36,03
Femur right	1	Partially preserved	No signs of butchering of gnawing	Proximal part and diaphysis present Proximal epiphysis fused	BP = 41,85 SD = 13,90 DC = 19,72
Patella	1	Complete	No signs of butchering of gnawing		GL = 29,68 GB = 17,96
Pelvis right	1	Partial	No signs of butchering of gnawing	Ilium Acetabulum fused	GL= (155,03) LAR = 25,54
Pelvis left	1	Partial	No signs of butchering of gnawing	ischium	
Sacral vertebrae	2	Almost complete	No signs of butchering of gnawing	Part of the processes missing	
total	**7**				

* According the guidelines by von den Driesch (1976).

Fig. 2. Photographs of the lynx bones found at the *castellum* excavation site in Alphen aan den Rijn, the Netherlands: photograph *A* shows the femur and pelvis specimens, photograph *B* shows the patella and the vertebrae. Photograph *C*, prehistoric lynx mandibula found on the island of Texel, the Netherlands and *D*, photograph of sampling of the prehistoric lynx tooth found on the Sand Motor, the Netherlands.

3. Materials and methods

3.1. DNA

3.1.1. Samples for DNA analyses

Materials included in the study were obtained from sources and locations mentioned in Table 3 and in Fig. 1. Lynx bone specimens were kindly donated by fossil collectors Hidde Bakker (mandibula), Dick Duineveld (tooth) and Henk Mulder (vertebra) and morphological identifications were performed by Charlie Schouwenburg and Dick Mol (Natuurhistorisch Museum Rotterdam). Reference bone (femur) and lynx fecal material were donated by Bram Langeveld (Natuurhistorisch Museum Rotterdam) and Emile Prins (GaiaZoo), respectively, and most likely originate from Norwegian lynx populations (personal communications).

Table 3
Materials used in this study.

Species	Specimen	identification number	Age (years)	Collecting location
Lynx lynx	femur	ALP0149-2592 [1]	± 2000	Alphen aan den Rijn, the Netherlands
Lynx lynx	femur	NMR9990-3210 [2]	recent	Blijdorp Zoo, The Netherlands
Lynx lynx	mandibula	HB40TX [3]	unknown	Texel, The Netherlands
Lynx lynx	tooth	DDLL1 [4]	unknown	Sand Motor, The Netherlands
Lynx lynx	vertebra	NMR-3192 [2]	unknown	Sand Motor, The Netherlands
Lynx lynx	Feaces	GZLL1 [4]	recent	GaiaZoo, The Netherlands

Specimens deposited in collections of [1]Department of Archeology, University of Amsterdam, [2]Natural History Museum Rotterdam (Langeveld and Liscaljet, 2019), [3]Private collection Hidde Bakker (Bakker, 2018), [4]University of Applied Sciences Leiden.

3.1.2. DNA isolations

Sampling of recent and ancient materials was performed in the dedicated ancient DNA facility of Naturalis Biodiversity Center. Bone materials were cleaned using 0.5% hypochlorite and 70% ethanol and sampled using a Parkside 4 mm cordless drill. Subsequently, materials were treated according to the method described by Rohland and Hofreiter (2007) and according to the updated protocol by Rohland et al. (2018). Briefly, samples were treated with 1 ml lysis buffer containing 0.5 M EDTA pH 8.0, 0.5% v/v Tween-20 (Sigma-Aldrich) and 250 ng/µl proteinase K (Qiagen) overnight at 37 °C under constant rotation. After lysis, materials were spun down at $16,400 \times g$ and the lysate was bound to silica spin columns (Qiagen) in the presence of binding buffer containing 5 M guanidine hydrochloride (VWR), 40% v/v 2-isopropanol, 0.12 M sodium acetate and 0.05% v/v Tween-20 (Sigma-Aldrich). Incubations were directly added to Qiagen DNA spin columns and procedures were followed essentially as described by the supplier. DNA isolations from fecal material were done using a QIAamp DNA stool kit (Qiagen) as described by the manufacturer. All isolated DNA materials were quantitated using a NanoDrop One instrument (Thermo Fisher) and stored at −20 °C for subsequent PCR amplifications and further analysis. All standard chemicals used for DNA extractions were reagent grade (Sigma-Aldrich).

3.1.3. DNA amplifications and sequencing

Amplifications of COI and control regions were done using a CFX96 touch instrument and CFX Maestro analysis software (Bio-Rad). Primers to amplify a part of the cytochrome oxidase I gene were designed with Geneious Prime software versions 2019 (Biomatters). As forward primer and reverse primer we used 5′- ACGTRGTAGCCCAYTTCCAC and 5′- RCGTGAAGTGYATTTTTGCCCA, 1115F and 1245R, respectively. For the control region and cytochrome *B* amplifcations primers were used as described (Rodríguez-Varela et al., 2016). Cycling was essentially performed as described (Horn et al., 2016). In brief, cycling for 40 cycles included an initial denaturation step for two minutes at 95°, a denaturation step for 15 s at 95 °C, an annealing step at 50 °C for 15 s and an elongation step at 72 °C for 30 s. After amplification, melting

characteristics of the amplicons were determined by gradually increasing the temperature from 60 °C to 95 °C (10 s, increment 0.5 °C per cycle). Products identified by melting characteristics were also analyzed on a 1.5% agarose gel. In case of low DNA yields, Q-PCR methods were adjusted by changing the annealing temperature to 47 °C, prolonging the cycling steps to 30 s and extending the cycling to 50 times.

After cleanup of the DNA samples using a QIAquick PCR purification kit (Qiagen), samples were quantitated on a NanoDrop One instrument (Thermo Fisher) and run on a 1.5% agarose gel. Subsequently samples were sent to BaseClear for Sanger sequencing both forward and reverse strands and some samples were sequenced in-house. Results were analyzed using Geneious Prime software version 2019.1.3 (Biomatters). Alignments of sequences were performed using the built-in Geneious global alignment tool with free end gaps algorithm allowing for 65% similarity. Sequences obtained were compared to sequences deposited in NCBI GenBank and described by Rueness et al. (2014) and Rodríguez-Varela et al. (2015). A deposited sequence, KJ210009 (Rodríguez-Varela et al., 2015) of Iberian lynx was used as an outgroup in the analysis. A phenetic tree was created by using the Neighbor-Joining tree build method and the Tamura-Nei algorithm with *Lynx pardinus* as outgroup. Bootstrap analyses were performed with 1000 iterations.

3.1.4. Haplotype analysis

After sequence analysis and alignment in Geneious, haplotypes were analyzed using Population Analysis with Reticulate Trees PopART version 1.7 (Leigh et al., 2015). Alignments were imported in PopART in Nexus file format. Traits were attributed to the sequences as described (Leigh et al., 2015) and subsequently minimum spanning networks were created to visualize the relationship between the haplotypes (Bandelt et al., 1999).

3.2. Strontium ratio determinations

Despite the fact that no dental elements were recovered, is was decided to execute Sr isotope analysis on one of the bone samples. The high susceptibility of archaeological bone to diagenetic alterations prevents research into the geological origin of the possible lynx (see for instance Hedges, 2002; Kohn et al., 1999; Nelson et al., 1986). The obtained $^{87}Sr/^{86}Sr$ ratio will to a greater or lesser extent be influenced by diagenetic Sr from the burial environment; the biogenetic Sr isotope composition may even be completely replaced by a diagenetic signal. However, if the generated $^{87}Sr/^{86}Sr$ ratio is incompatible with the expected local Sr isotope signature for the Alphen aan den Rijn region, a non-local origin of the animal may be assumed. Hence, although the available material does not allow to determine a possible geological provenance, the obtained Sr isotope data may provide an additional line of evidence to support the DNA results and archaeological interpretation of the find.

A bone sample from the femur was taken using a new circle cutter on a Dremel device. Next, using an acid-cleaned diamond tipped bur, the discoloured outer surface of the bone sample was discarded and circa 10 mg of bone powder was sampled and collected in hydrochloric acid precleaned 2 ml polyethylene Eppendorf centrifuge tubes and transferred to the US Federal Standard Class 100 clean laboratory facility at the Vrije Universiteit Amsterdam for Sr purification. The bone sample was leached in 500 μl 5 M acetic acid followed by one Milli-Q rinse. The leachate was collected in an acid cleaned Savillex 7 ml beaker and dried down overnight. The leachate and the bone samples were dissolved in 500 μl 3 M HNO₃. Strontium column extraction was performed following the published protocol (Kootker et al., 2016a). Half of the bone and leachate samples were loaded on annealed Re filaments. The isotope compositions were measured using a Thermo Scientific Triton Plus instrument. The strontium ratios were determined using a static routine and were corrected for mass fractionation to $^{86}Sr/^{88}Sr$ ratio of 0.1194. All measurements were referenced to the within-run value of the

NBS987 standard (0.710251 ± 0.000012, 2SD, N = 5). The total procedural blanks (N = 2) contained a negligible amount of 26.1 and 26.9 pg strontium respectively.

4. Results

4.1. DNA isolations

Ancient as well as recent lynx skeletal remains were first prepared for DNA isolations. Photographs of the lynx skeletal remains from Alphen aan den Rijn and the jaw found on the island of Texel are shown in Fig. 2. The input for isolation ranged between 68 and 140 mg bone and tooth material. DNA was obtained from finely ground bone and tooth materials ranging between 2 ng/μl up to 20 ng/μl. The lynx bone from Alphen aan den Rijn yielded 18 ng/μl, indicating that the DNA was readily obtained also from older material. The very old materials, jaw and tooth, yielded 20 and 10 ng/μl, respectively (data not shown). Interestingly, $OD_{260/280}$ ratios were better using the recently described method by Rohland, specifically designed for small DNA fragments, than obtained by the earlier method described by the same authors (2007, 2018). Ratios were 1.7–1.9 for the recently published method, whereas ratios were around 1.4–1.5 for the isolations by the earlier method. We have used samples with ratios closest to 1.8 for further PCR amplifications and DNA sequencing. A representative amplification of ancient DNA is shown in Fig. 3A. In order to optimize the amplification of control region sequences from very old materials which was not always successful
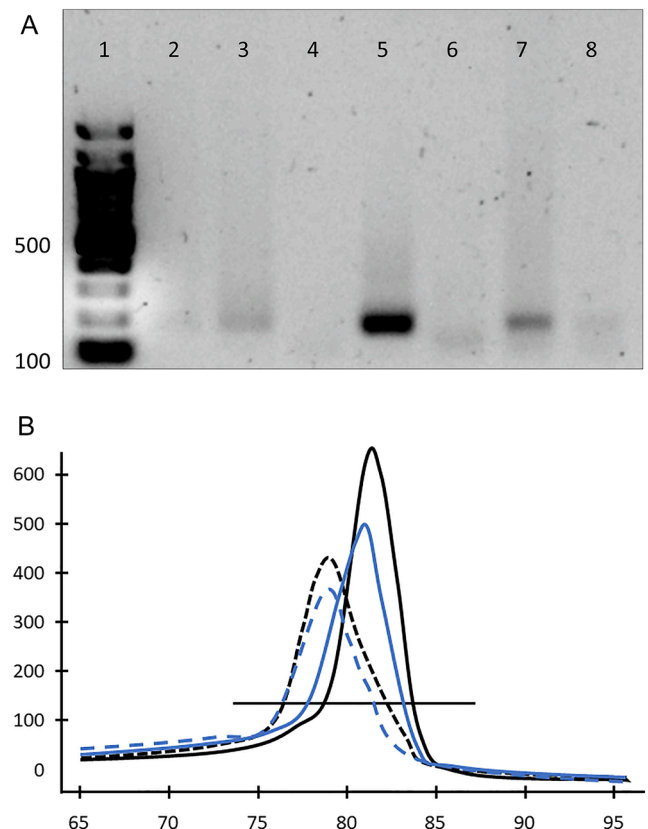
Fig. 3. *A,* PCR amplification of the DL5R region of the mitochondrial control region. Lane 1, molecular standard; lanes 2 and 3, recent lynx bone extractions; lane 4, non-template control; lane 5, lynx bone from the Alphen aan den Rijn excavation site; lane 6, ancient lynx tooth, first extraction; lane 7, ancient lynx tooth, second extraction; lane 8, ancient lynx jaw. *B,* Melting curve analysis of lynx bone DNA. Black and blue solid lines represent the Alphen aan den Rijn specimen and the Sand motor tooth specimen, respectively, and striped black and blue lines represent non template controls for both samples indicative of a primer dimer product. Threshold is set at the black horizontal line.

using PCR, DNA was amplified in a Q-PCR instrument and melting temperatures (*Tm*) of specific products were determined. Specific control region amplicons demonstrated *Tm* values of 81.5 °C, whereas non-specific products were observed in controls at 79 °C (Fig. 3B). After specific amplification, all obtained products were run on an agarose gel, isolated and cleaned using Qiaquick gel extraction kit. DNA fragments yielded the expected size and were subsequently sequenced.

4.2. Sequencing of mitochondrial gene regions

Sequencing of the cytochrome oxidase I gene, cytochrome *B* and control region parts revealed sequence differences in the various samples. The results of sequencing of the cytochrome oxidase I gene are shown in Fig. 4. Full control region, cytochrome Oxidase I and cytochrome *B* sequences obtained are shown in Fig. 6. Reference sequences

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1: GCAGTATTCGCTATTATGGGAGGCTTTGTCCATTGANTCCCCCTATTCTCAGGGTATACCCTTGATAATACTTGGGCAAAAATNCACTTC
2: GCAGTATTCGCTATTATGGGAGGCTTTGTCCATTGATTCCCCCTATTCTCAGGGTATACCCTTGATAATACTTGGGCAAAAATTCACTTC
3: GCAGTATTCGCTATTATGGGGGGCTTTGTTCATTGATTCCCCCTATTCTCAGGGTATACCCTTGATAATACTTGGGCAAAAATTCACTTC
4: GCAGTGTTCGCTATTATGGGAGGCTTTGTTCATTGATTCCCCCTATTCTCAGGATATACCCTTGATAATACTTGGGCAAAAATTCACTTC
5: GCAGTATTCGCTATCATGGGAGGCTTTGTCCACTGGTTCCCCCTATTCTCAGGGTATACTCTGGATGATACTTGGGCAAAAATCCACTTC
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1: *Lynx lynx* Alphen aan den Rijn Roman excavation

2: *Lynx lynx* KP202283

3: *Lynx canadensis* KP202281

4: *Lynx pardinus* KX911410

5: *Lynx rufus* KP2024285

Fig. 4. Cytochrome oxidase I alignment of the Alphen aan den Rijn lynx sequence obtained in this study compared to GenBank lynx sequences as indicated.

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 1:  CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACACACAATGCAAAA
 2:  CCCCCCATAAAATAAAGTAAGTAAAAACCCCCTATCACCATGACCCTAAACATACAATGCAAAA
 3:  CCCCCCATAAAATAAAGTAAGTAAAAACCCCCTATCACCATGACCCTAAACATACAATGCAAAA
 4:  CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACACACAATGCAAAA
 5:  CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACACACAATGCAAAA
 6:  CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACATACAATGCAAAA
 7:  CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACACACAATGCAAAA
 8:  CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACATACAATGCAAAA
 9:  CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACATACAATGTAAAA
10: CCCCCCCCAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACATACAATGCAAAA
11: CCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACATACAATGTAAAA
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1: *Lynx lynx*, femur, Alphen aan den Rijn, Roman excavation site, the Netherlands

2: *Lynx lynx*, tooth, Sand Motor, the Netherlands

3: *Lynx lynx*, jaw, Texel, the Netherlands

4: *Lynx lynx* KP748522, Sima Pagolusieta, Spain, Hjerk Nord, Denmark

5: *Lynx lynx* KJ210010, Coulet des Roches, France

6: *Lynx lynx* KJ210011, Grotta della Madonna, Arene Candide; KJ210012, Grotta della Madonna; KJ210013, Arene Candide, Italy

7: *Lynx lynx* KP748523, Cueva de los Cinchos, Spain

8: *Lynx lynx* KP748524, Rascano, Spain, Hjerk Nord, Denmark

9: *Lynx lynx* KP748525, Hjerk Nord, Denmark

10: *Lynx lynx* KP748526, Serpenteko, Spain

11: *Lynx lynx* fecal sample, GaiaZoo, the Netherlands

Fig. 5. Control region (DL5R part) alignment of the various lynx sequences obtained in this study compared to GenBank lynx sequences as indicated.

were obtained from NCBI GenBank (complete mitochondrial genomic sequences of *Lynx lynx*, KP202283, *Lynx canadensis*, KP202281, *Lynx pardinus*, KX911410, *Lynx rufus*, KP202285) and aligned to the newly generated sequences generated in this study. As shown in the figure, the newly obtained DNA sequences all aligned with *Lynx lynx*, confirming the assumption based on morphological inspection that the species was a Eurasian lynx (*Lynx lynx*). Since these sequence data and data from cytochrome *B* sequencing analyses were not discriminatory for haplotyping (data not shown), additional sequencing of specific parts of the control region on the mitochondrial genome was performed. We performed sequencing on the DL5R and LI2F regions (named after the primers used), as done by others on ancient and recent lynx materials previously and we aligned our data to published data by Rodríguez-Varela et al. (2015) and by Rueness et al. (2014). We determined sequences spanning a region of 136 base pairs maximally in the DL5R region and 148 base pairs maximally in the LI2F regions of the different samples (see also Fig. 6). A part of the alignment of the DL5R region, which was most illustrative for the observed differences, is shown in Fig. 5. Specific differences were found at four different locations in the analyzed DL5R region, as can be seen in the figure. Since the LI2F region did not demonstrate variable sites we did not further use it for haplotyping. The haplotype determined from the lynx remains from Alphen aan den Rijn is different from the haplotype from the very old North Sea coastal samples (*Lynx lynx* tooth and jaw) and the haplotypes from the KP748525 and KP748526 sequences. In addition, the haplotype does not match with the haplotype from the fecal lynx material. The haplotype of the ancient vertebra specimen (NMR-3192) could not be determined since the sequence data were of low quality and highly fragmentary; the readable sequence was in a non-variable part of the control region. We can conclude from these data that the haplotype of the Lynx sample from the Roman excavation site of Alphen aan den Rijn completely matches with an ancient haplotype retrieved from France and an ancient haplotype found in Spain (KJ210010, Rodríguez-Varela et al., 2015). This haplotype has been found in Denmark as well according to a published report (Rueness et al., 2014). The reference materials obtained from the Netherlands did not fully match with the haplotype of the specimens found at the excavation site. As the sequences do not align to other ancient sequences found previously at Arene Candide or Grotta della Madonna in northern Italy either (Rodríguez-Varela et al., 2015), the specimen from Alphen aan den Rijn has more affinity with a southwestern group (including France and Spain) than a north-eastern group including the Northern sea and more northern European regions. Since our sequence data were limited, the data from our experiments were matched to a region in the DNA that is identical for both KJ210011, KJ210012 and KJ210013 sequences as determined by Rodríguez-Varela et al. (2015) (Fig. 6).

With the obtained sequence information we performed a phenetic analysis. The results are depicted in Fig. 7, in which a tree is shown in panel A and a median joining network in panel B. The Alphen aan den Rijn sample clusters with haplotypes in a clade that is clearly different from the clade in which the Dutch samples from the North Sea coast are found.

4.3. Strontium ratio analyses

The bone sample exhibited an $^{87}Sr/^{86}Sr$ ratio of 0.708821; the leachate Sr isotope composition was a little more radiogenic, i.e. 0.709530. Although the difference between the two can be considered significant, both ratios are compatible with the Alphen aan den Rijn region (zones B and C in Kootker et al., 2016b). Consequently, the obtained Sr isotope ratios do not contribute to a further interpretation or contextualisation of the lynx in Roman Alphen aan den Rijn. The data are most probably representative of the diagenetic Sr signature of the local burial environment and are not indicative of a non-local origin of the animal.

5. Discussion

In this study we have analyzed bone remains from Eurasian lynx (*Lynx lynx*), found at the Roman *Castellum* excavation site in Alphen aan den Rijn (the Netherlands). We used a combination of PCR and Q-PCR; the latter method does present an added value to regular PCR as it appeared that success rates of amplification of ancient DNA were higher using Q-PCR instrumentation and it includes the melting curve step for rapid identification of desired products. After determination of melting temperature for specific products, ancient DNA samples were rerun on standard agarose gels to ascertain the proper product length which formed a second confirmation of the required product. We recommend including melting curve analysis in ancient DNA analysis as in our hands it appears to be a sensitive and fast method to determine the presence of specific amplicons. Subsequently, DNA was isolated from gel, purified and sequenced. In the isolation procedures we used the updated ancient DNA method described by Rohland et al. (2018) that has proven to be very effective. This method facilitates the isolation of DNA and is especially suited for more degraded DNA in order to perform subsequent sequencing. The original method described by Rohland and Hofreiter (2007) was used in our procedures as well and yielded reasonable amounts of DNA material from the ancient jaw, tooth and vertebra from the North Sea Sand Motor region.

DNA sequences form the COI gene and control region sequences confirmed the previous morphological identification of the bones to *Lynx lynx*. Analyzed prehistoric specimens found in the North Sea region in the Netherlands showed different haplotypes compared to the Roman lynx bone from the excavation site. Although our sampling was limited, we tentatively conclude that the lynx found in the surrounding of the *castellum* at Alphen was not a native individual. The haplotypes, based on the control region sequences, of the lynx from the Alphen aan den Rijn excavation site and the haplotypes of the tooth and jaw from the North Sea regions are different on two nucleotide positions. Interestingly, the North Sea samples are identical and they are different from the haplotypes described by Rueness et al. (2014) and Rodríguez-Varela et al. (2015). The differences between the sequences of the North Sea samples and those described in the paper by Rueness et al. (2014) may be a result of the age difference, since the North Sea samples are most likely Pleistocene specimens, however, they do compare well to the samples described by Rodríguez-Varela et al. (2015), which were late Pleistocene/early Holocene dated. Both jaw and tooth samples demonstrate identical control region sequences. We sequenced the proximal 5′ end of the control region as well as the distal 3′ end of the region, each part consisting of approximately 70 base pairs. As the proximal 5′ part was of high quality and was sufficiently informative, we conclude that there is sufficient difference between the samples. This would mean that the sequence from the specimen from Alphen aan den Rijn does not match with sequences from prehistoric samples found in the Netherlands. It seems likely that it does not descend from the prehistoric lynx population and therefore belongs to a different population. It is possible, however, that the native prehistoric lynx population in the area surrounding Alphen aan den Rijn became extinct by Roman times.

The question remains how the lynx bones found at Alphen aan den Rijn ended up in the defensive ditch of the *castellum*. As the bones of lynx from Alphen aan den Rijn appear to belong to a more southern haplotype it is tempting to conclude that the animal was brought by Roman soldiers with a southern origin. Few references to lynx are present for the Roman times, as this animal was not very familiar or not commonly exploited by the Romans. Keller (1909) mentioned that a lynx was used in the Roman circus in 55 BC but this author also indicates doubts on whether this actually occurred. It is improbable to think that this animal was hunted for its meat, or even for ludic shows. It might be assumed that it was hunted or kept, maybe as a mascot, for its fur and skin as in the case of wolf or lion skins used as part of a military costume. Following this assumption we would expect to find bones from the skull and feet, which is not the case for the remains of lynx found at Alphen

Control region sequence ancient *Lynx lynx* (Alphen aan den Rijn) identification number ALPO149-2592

TGTGCTTGCCCAGTATGTCTTTACCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACAC
ACAATGCAAAATTAACCTACCAACCACTGGCCCGCAAATCCCATGAATATTAAGCATGTANNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NTAGTGCTTAATCGTGCATTATACCTTGCTCTAGAACAGTCCTTCATGGACCTCAACTGTCCAGAGGAAGCTTAA
TCACCTGGTCTCGAGAAACCAGCAATCCTTGCTCGAACGTGTACCTCTTCTCGCTCCGGGTCATAGCTGTTTCC

Control region sequence *Lynx lynx* (GaiaZoo) identification number GGLL1

TGTGCTTGCCCAGTATGTCTTTACCCCCCATAAAATAAACTAAGTAAAAACCCCCTATCACCATGACCCTAAACAT
ACAATGTAAAATTAACCTACCAACCACTGGCCCGCAAATCCCATGAATATTAAGCATGTANNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NTAGTGCTTAATCGTGCATTATACCTTGCTCTAGAACAGTCCTTCATGGACCTCAACTGTCCAGAGGAAGCTTAA
TCACCTGGTCTCGAGAAACCAGCAATCCTTGCTCGAACGTGTACCTCTTCTCGCTCCGGGTCATAGCTGTTTCC

Control region sequence ancient *Lynx lynx* (Texel) identification number HB40TX

TGTGCTTGCCCAGTATGTCTTTACCCCCCATAAAATAAAGTAAGTAAAAACCCCCTATCACCATGACCCTAAACAT
ACAATGCAAAATTAACCTACCAACCACTGGCCCGCAAATCCCATGAATATTAAGCATGTA

Control region sequence ancient *Lynx lynx* (Sand Motor) identification number DDLL1

TGTGCTTGCCCAGTATGTCTTTACCCCCCATAAAATAAAGTAAGTAAAAACCCCCTATCACCATGTCCGTAAACAT
ACAATGCAAAATTAACCTACCAACCACTGGCCCGCAAATCCCATGAATATTAAGCATGTA

Control region sequence ancient *Lynx lynx* (Sand Motor) identification number NMR-3192

CTGAATGGAAAATTAACCTACYAACCACTGGCCCGCAAATCCCATGAATATTAAGCATGTANNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNAATCACCKGGTCTCGAGAAACCAGCAATCCTTGCTCGAACGTGTACCTCTTCTCGCTCCGGGTCATAGCTGTT
TCCKAA

Cytochrome oxidase subunit 1 sequence ancient *Lynx lynx* (Alphen aan den Rijn) identification number ALPO149-2592

TACGTGGTAGCCCATTTCCACTATGTCCTGTCAATGGGGGCAGTATTCGCTATTATGGGAGGCTTTGTCCATTGG
TTCCCCCTATTCTCAGGGTATACCCTTGATAATACTTGGGCAAAAATNCACTTCACG

Cytochrome B sequence ancient *Lynx lynx* (Alphen aan den Rijn) identification number ALPO149-2592

CCATCGTTGTATTTCAACTACAAGAACTTAATGACCAACATTCGAAAATCACACCCCCTCATCAAAATTATTAACC
ACTCATTCATCGACCTGCCCGCCCCATCCAATATTTCAGCATGGTGNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNAATCCTACAGATCCTCACCGGCCTTTTCCTAGCCATACATTACACATCAGACACAACAACCGCCTTCTCA
TCGGTCGCCCATATCTGCCGCGACGTTAACTACGGCTGAATC

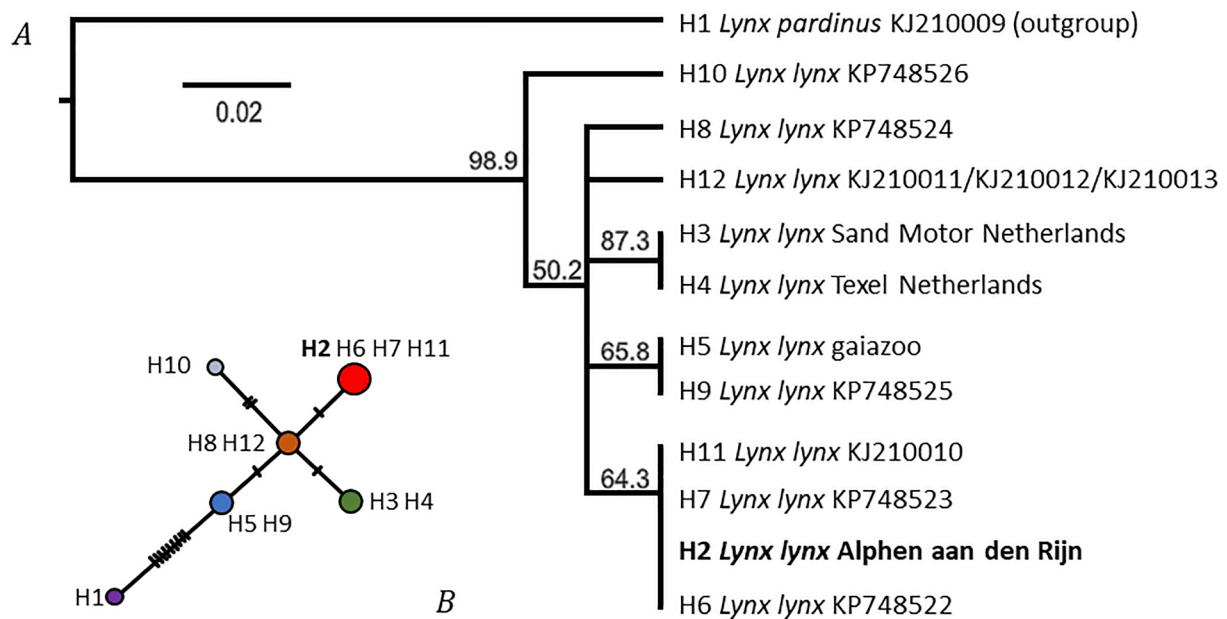**Fig. 6.** Full DNA sequence information retrieved in this study.

Fig. 7. *A*, phenetic tree of the retrieved haplotypes; *B*, minimal spanning network of retrieved haplotypes in this study and haplotypes obtained from GenBank. The red circle indicates the Alphen aan den Rijn lynx haplotype; the other colors represent the various other haplotypes obtained in the study and retrieved from GenBank, as indicated.

aan den Rijn. The presence of prehistoric remains of lynx from Schipluiden was explained by Kooijmans and Jongste (2006) by import of skins from outside the region and the results of the current study indicate that the specimen from Alphen aan den Rijn might be considered an 'exotic' animal. It is tempting to assume that also the lynx from Alphen aan den Rijn was transported to this area by the Roman soldiers from a more southern origin.

6. Conclusion

DNA analysis and zooarchaeological research confirms identification of the lynx remains found in a Roman *castellum* in Alphen aan den Rijn as Eurasian lynx (*Lynx lynx*). The results of the DNA haplotype analysis show that the lynx remains found in the *Limes* region are different from fossil material of Eurasian lynx from the North Sea region and match with a haplotype found in Southern Europe.

CRediT authorship contribution statement

Ivo R. Horn: Conceptualization, Investigation, Data curation, Writing - original draft. **Aviva Shuman:** Conceptualization, Investigation, Writing - original draft. **Irene Chan:** Investigation. **Cornelis H. Maliepaard:** Investigation. **Lisette M. Kootker:** Investigation, Methodology, Writing - review & editing. **Marcel Eurlings:** Investigation. **Roderick Bakker:** Investigation, Writing - review & editing. **Barbara Gravendeel:** Writing - review & editing. **Chiara Cavallo:** Conceptualization, Investigation, Writing - original draft.

Declaration of Competing Interest

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

Sequence data deposition

DNA control region sequences are available at GenBank under accession numbers MW328716, MW328717, MW328718, MW328719 and MW328720, cytochrome oxidase subunit 1 under accession number MW328714 and cytochrome b under number 328715.

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