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10.1177/0959683614540953

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### Multiproxy study of the last meal of a mid-Holocene Oyogos Yar horse, Sakha Republic, Russia

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<td>Complete List of Authors:</td>
<td>Gravendeel, Barbara; Naturalis Biodiversity Center, Protopopov, Albert; Academy of Science, Bull, Ian; University of Bristol, School of Chemistry Duijm, Elza; Naturalis Biodiversity Center, Gill, Fiona; University of Leeds, School of Earth and Environment Nieman, Aline; Naturalis Biodiversity Center, Rudaya, Natalia; Institute of Archaeology and Ethnography, Centre of Cenozoic Geochronology Tikhonov, Alexei; Russian Academy of Sciences, Zoological Institute Trofimova, Svetlana; Ural Branch Russian Academy of Sciences, Laboratory for Paleocology van Reenen, Guido; IBED-UvA, Paleoeckology Vos, Rutger; Naturalis Biodiversity Center, Zhilich, Snezhana; Institute of Archaeology and Ethnography, Centre of Cenozoic Geochronology van Geel, Bas; IBED-UvA, Paleocology;</td>
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Abstract

The last meal of a horse that lived in the northern part of the Yakutia-Sakha Republic (Russia) ca 5400 years ago was studied using pollen, spores, botanical macroremains, lipid composition and ancient DNA in order to reconstruct its components. Pollen of Poaceae was superabundant, but this may be due to over-representation as a consequence of grazed inflorescences of grasses. We evaluate the palaeo-
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macrofossils and chemical data show what the animal had eaten. Pollen grains and the
aDNA record also give information about taxa that occurred elsewhere in the
landscape. The combined data point to an open landscape of a coastal tundra
dominated by graminoids (Poaceae, Cyperaceae) with a limited amount of Birch and
Alder.

Introduction

The contents of the stomach and intestines of frozen animals are an important source
of information about the food choice of animals and the palaeo-environment where
these animals were living (Ukrainev, 1979; 1993; 2013; van Geel et al., 2008,
2011a,b; Guthrie, 1990; 2001; Lazarev, 2008). In 2010 a frozen horse (Fig. 1a,b) was
found in the area called Oyogos Yar, in the Ust-Yana region of the Sakha Republic
(formerly Yakutia; 72° 40'49.42"N, 142° 50'38.33"E; Boeskorov et al., 2013). The
Yukagir horse was radiocarbon dated 4630 ± 35 BP (GrA-54020). After calibration
(http://www.calpal-online.de/) this date corresponds to a period between ca. 5442 and
5326 calendar years BP and therefore the carcass is among the youngest wild horse
fossils in North-east Siberia. For the history and extinction chronology of horses and
other large herbivores in the Arctic we refer to Sher et al. (2005) and Guthrie (2006).
Oyogos Yar is one of the richest areas for Quaternary fossils in the northern part of
the Sakha Republic. It is located on the mainland coast of the Dmitri Laptev Strait and
stretches more than 100 km, from Cape Svyatoi Nos in the west to the mouth of the
Kondratieva River in the east. The Yedoma deposits of the Oyogos Yar’s northern
slope reach up to 40-50 m above sea level. The icy deposits in the area include the
marine isotope stage 3 and mainly stage 2 (coldest part of the last ice age;
Schirrmeister et al., 2013). The western part of Oyogos Yar is composed of lake-
alluvial silt sediments reaching 5-7 m a.s.l., which are contemporary with the
sediments of the Olyor Formation (Lower Kolyma River basin, Lower Pleistocene).
For present day climatic conditions, soils, vegetation cover (among which grassy
tundra in dry coastal areas) and other landscape characteristics we refer to Smith et al.

The partial carcass was found trapped in a thermokarst pit and buried in Late
Pleistocene deposits. It represents the remains of an adult female horse about 5 years
old. The frozen corpse is represented by the well-preserved head with neck (detached
from the body), and the hind part of the body with hind legs and tail and internal
organs in the abdominal cavity. The front legs are missing. Body measurements
showed that the height of the horse in the hind quarters was about 130 cm. Thus, the
new finding relates to the “undersized” horses, similar to the Lena horse (*Equus
lenensis* Russ.), which inhabited Eastern Siberia and became extinct in the Holocene
(Gromov and Baranova, 1981). The scarcity of mummified horse remains determines
the high scientific and museum value of the new discovery.

We sampled and studied material from the Yukagir horse colon in order to reconstruct
the species composition of its last meal and the palaeovegetation in the area where the
animal was living. The colon contents were subsampled to provide separate aliquots
of material for each analytical procedure. We followed a multi-proxy approach,
including the analysis of microfossils (pollen, spores), macroremains (including
epidermis and moss remains), chemistry and ancient plant DNA.

**Methods**

*Microfossils and macroremains*
The preparation of a subsample for the study of microfossils in Russia was as follows: after thawing, the sample was sieved through a sieve with a mesh of 250 µm to remove large particles. Subsequently the material was treated with 10% hydrochloric acid and 10% potassium hydroxide, and then washed with distilled water and centrifuged. After sieving (meshes of 7 µm), the material on the sieve was put in a tube and glycerin was added. The microfossil analysis was conducted with 400x magnification. The preparation of a subsample for microfossil analysis in the Netherlands was according to Faegri and Iversen (1989) and Moore et al. (1991), but without HF treatment, and the analysis was worked out with 400x and 1000x magnifications. Identifications of microfossils are based on Moore et al. (1991), Beug (2004) and a pollen reference collection. The identification of fungi was based on van Geel and Aptroot (2006) and Cugny et al. (2010). Macrofossils were prepared according to Mauquoy and van Geel (2007). Mosses were identified using Landwehr (1984), Lawton (1971), Nyholm (1968), Smith (1978), Siebel and During (2006), The Plant List (2012), and Touw and Rubers (1989).

Lipids

Lipids were extracted from freeze-dried, ground colon contents using the methodology of McCartney et al. (2013) to optimize recovery of the dialkyl glycerol ether archaeol (2,3-di-O-phytanyl-sn-glycerol) if present. Briefly, 6.92 µg of internal standard, 1,2-di-O-rac-hexadecyl glycerol (Santa Cruz Biotechnology Inc., CA), was added to each sample before lipid extraction and the total lipid extract was obtained using an extraction procedure modified from Bligh and Dyer (1959). Acid methanolysis was used to cleave polar head groups from archaeol. Silica column chromatography was used to separate the total lipid extract into an apolar fraction and a fraction containing predominantly hydroxyl group-bearing components. For this
latter fraction, analytes were derivatised to their respective trimethylsilyl (TMS) ethers by adding 50 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and 50 µl pyridine to the sample and heating at 70 °C for 1 h. Samples were dissolved in ethyl acetate prior to analysis by gas chromatography/mass spectrometry (GC/MS).

GC/MS was conducted using a Trace 1300 GC coupled to an ISQ MS (Thermo Scientific, Hemel Hempstead, UK), equipped with a non-polar fused silica capillary column (CPSil-5CB, 50 m x 0.32 mm x 0.12 mm, Agilent J&W). The following temperature program was used: initial temperature 40 °C, rising to 130°C at 20°C min⁻¹, then rising to 300°C at 4°C min⁻¹, holding at 300°C for 25 min. The ion source was maintained at 300 °C and the transfer line at 300 °C. The emission current was set to 50 µA and the electron energy to 70 eV. The analyzer was set to scan m/z 50-650 with a scan cycle time of 0.6s.

**DNA extraction**

The outer layer of the intestinal samples was carefully removed with a scalpel to prevent contaminants in the extractions and inner parts of the same freeze-dried, ground colon contents sampled for chemical and morphological analyses were sampled for DNA extraction. Each sample was ground to fine powder in liquid nitrogen with a mortar and pestle. Ca. 100 mg was used for a CTAB extraction (Doyle and Doyle, 1987). A freshly prepared CTAB buffer (2% CTAB, 2% PVP, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1.42 M NaCl, 2% 2-mercaptoethanol) was added to the ground samples before incubation for 1 hour at 65 °C under agitation. DNA was subsequently extracted using chloroform:isoamyl alcohol (24:1), precipitated with ice-cold isopropyl alcohol and re-suspended in 1×TE buffer. The suspension was
then re-precipitated with NH₄ acetate and pure ethanol at -20 °C for 30 minutes, washed twice in 76% ethanol 10 mM NH₄ acetate and the resulting pellet was air dried and re-suspended in 1×TE buffer. Subsequently, aliquots of each extraction were further purified using Promega PCR purification columns. All extractions were carried out in the special ancient DNA facility of Leiden University following established protocols to avoid contamination (Cooper and Poinar, 2001).

**PCR amplification**

Amplifications of the plastid *rbcL* DNA barcoding marker were performed using forward primer Z1aF and reverse primer 19bR (Hofreiter et al. 2000). Amplification of the plastid intergenic *trnL-trnF* spacer was performed using forward primer E and reverse primer F (Taberlet et al. 1991). Primers were labeled for sequencing with IonExpress labels. The PCR was carried out in 25µl reactions containing 1U Phire hot start II DNA polymerase, Phire reaction buffer, 1mM MgCl₂, 0.1mg/ml BSA, 1% DMSO, 0.05 mM dNTPs, and 0.4 µM of each primer. Amplifications were performed using a 5 min activation step at 98 °C, followed by 40 cycles at 98 °C for 5 s, 55 °C for 20 s and 72 °C for 60 s, and a concluding step at 72 °C for 5 min.

**Ion Torrent sequencing**

Primer dimer and other contaminants were removed by using Ampure XP beads (Agencourt) to which the PCR products were bound. The beads were washed with 150 µl 70% EtOH twice and resuspended in 20 µl TE buffer. Cleaned PCR products were quantified using an Agilent 2100 Bioanalyzer DNA High sensitivity chip. An equimolar pool was prepared of the amplicon libraries at the highest possible concentration. This equimolar pool was diluted according to the calculated template dilution factor to target 10-30% of all positive Ion Sphere Particles. Template
preparation and enrichment was carried out with the Ion One Touch 200 Template kit with use of the Ion One Touch System, according to the manufacturers protocol. The quality control of the Ion one touch 200 Ion Sphere Particles was done with the Ion Sphere Quality Control Kit using a Life Qubit 2.0. The Enriched Ion Spheres were prepared for sequencing on a Personal Genome Machine (PGM) with the Ion PGM 200 Sequencing kit as described in the protocol and deposited on an Ion-314-chip (520 cycles per run) in three consecutive loading cycles for one sequencing run.

Data analysis

Reads obtained from Ion Torrent sequencing were trimmed with CLC Workbench Genomics (version 4.5) for primers and MID label tags with a custom script. Only reads with a length of at least 100 bp and a mean quality score of Q20 or higher were selected for further analysis. Reads were clustered into Operational Taxonomic Units (OTU’s) defined by a sequence similarity of at least 97% using the Octopus pipeline CD-HIT (Li and Godzik, 2006). Singletons were omitted. Representative consensus sequences of each cluster were blasted against NCBI GenBank data for taxonomic identification up to family and sometimes genus level.

Results

Microfossils

Table 1 shows the results of the microfossil analysis. Percentages are based on the pollen sum, which is the total of recorded pollen grains per sample. Non-pollen microfossils were excluded from the sum but their frequencies are expressed as percentages on that pollen sum. A cluster of pollen grains of Poaceae (Fig. 1d) was
recorded as a single grain. The ascospore cells of the dung-inhabiting Sporormiella-type and the ascospores of the coprophilous Sordaria-type (van Geel and Aptroot, 2006) show relatively high percentages. Delitschia ascospores also point to the occurrence of feces as a substrate. No fungal fruit-bodies were found during macrofossil analysis, so ascospores were ingested by chance, together with the grazed herbaceous vegetation. Clasterosporium caricinum is an indicator for the local occurrence of Carex (van Geel and Aptroot, 2006). A newly recorded dark-brown ascospore type (38-53 x 20-33 µm, including the light-brown velum) was named as Type 815 (Fig. 1e). Those spores show a pore with a thickened wall at one end. The other end is flattened, with a pore. Considering their morphology, these spores may well represent one of the dung-inhabiting Sordariales (Lundqvist, 1972). Newly recorded globose spores, 18-26 µm in diameter with an irregularly placed, dense pattern of rounded appendages (0.3-1 µm in diameter) were named as Type 816 (Fig. 1f). These spores probably have a Bryophyte origin. The microfossil sample contained many multicellular rhizoids fragments with oblique septa as occurring in Bryum (Fig. 2d).

**Macrofossils**

The investigated sample consisted of debris of vegetative plant remains. In total 45 ml of vegetation debris was investigated. The plant remains were fragmented with different degrees of decomposition and therefore estimation of mass or volume fraction of the individual categories was impossible. The plant remains mainly consisted of vegetative remains of Cyperaceae (Fig. 1g-m) and, to a lesser extend of Poaceae (Fig. 1c). Two fragmented Poaceae spikelets were found. The following bryophytes could be identified: Plagiommium cf. ellipticum, cf. Rhizommium...
pseudopunctatum, Polytrichastrum alpinum (Fig. 2a,b), Campylium cf. stellatum (Fig. 2c) and Sphagnum sp. (see Table 1 for summary of results).

Ancient DNA

Taxa with 97% certainty or higher retrieved encompass genera within the Cyperaceae (Carex and Eriophorum), Cornaceae (Cornus), Ericaceae (Pyrola), Poaceae (Agrostis, Anthoxanthum, Avena, Catabrosa, Dactylis, Festuca), Ranunculaceae (Caltha, Ficaria), Rosaceae (Comarum), Salicaceae (Salix), Saxifragaceae (Saxifraga) and Polytrichaceae (Polytrichum and Polytrichadelphus) (see Table 1 for more details).

Lipids

Figure 3 shows the \(n\)-alkane and \(n\)-alkanol distributions of the horse intestinal tract contents. \(n\)-alkanes range from \(C_{24}\) to \(C_{33}\) with a strong odd over even predominance, maximising at \(C_{29}\). \(n\)-alkanols range from \(C_{22}\) to \(C_{28}\), maximising at \(n\)-\(C_{26}\). This distribution of \(n\)-alkanes and \(n\)-alkanols is consistent with a major input of higher plant organic matter with the predominance of the \(C_{26}\) \(n\)-alkanol indicating a significant contribution from Poaceae (Dove and Mayes, 1996; Maffei, 1996; van Bergen et al., 1997; Bughalo et al., 2004; Killops and Killops, 2005).

Figure 4 shows a partial gas chromatogram of the alcohol fraction isolated from the horse intestinal tract contents. The presence of a suite of 5\(\beta\)-stanol components (\(C_{27}\) to \(C_{29}\)) confirms that this is digested matter, since these compounds are uniquely formed in the digestive tract by biohydrogenation of unsaturated sterols by digestive tract bacteria (Murtaugh and Bunch, 1967). The predominance of the \(C_{29}\) 5\(\beta\)-stanols
(stigmastanol and epistigmastanol) and the occurrence of the phytosterol sitosterol is consistent with an herbivorous diet (Leeming et al., 1996; Bull et al., 2002).

No archaeol was detected in the horse intestinal tract alcohol fraction. This is consistent with previous studies on faecal lipids of modern herbivorous mammals (Gill et al., 2010) in which archaeol, attributed to digestive tract methanogenic archaea, was detected in the faeces of foregut fermenters, but not hindgut fermenters, including horses.

Discussion

Palaeovegetation - Based on the pollen record it seems that Poaceae (grasses) were a major component in the vegetation. But the presence of a cluster of pollen grains of Poaceae shows that the pollen spectra can be strongly biased by the food selection of the animal and by the fact that inflorescences - if still full of not yet released pollen grains - may result in over-representation in pollen spectra. Pollen grains ingested during the growing season will mainly represent the taxa that were flowering when the animal collected its food and therefore the pollen record of intestinal contents may well be strongly seasonally dependent (compare present day ‘pollen calendars’ showing the different flowering periods of taxa).

The macrofossil record supports the conclusion based on pollen, as epidermis fragments of Poaceae were observed, but the vegetative remains of Cyperaceae were more common than those of Poaceae. The mosses probably were ingested by chance, together with the monocots. The identified moss species indicate moist or wet habitats (fens, marshes or along streams) and calcareous soils. Based on the study of ancient
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DNA we have additional data about plant taxa that played a role in the vegetation where the horse grazed its last meal. *Caltha, Carex, Comarum* and *Eriophorum* may well have formed part of the vegetation in moist areas in the landscape, while *Agrostis, Avena, Dactylis* and *Festuca* may have grown on dry soils.

The age of the Yukagir horse is about 5400 calendar years before present according to radiocarbon dating. Pollen diagrams from lake deposits allow us to compare the vegetation record from the horse with the regional historical vegetation development. The middle Holocene vegetation near the Laptev Sea coast is characterized by tundra vegetation similar to the modern vegetation (Andreev et al., 2011). According to Smith et al. (1995) nowadays upland areas along the Arctic coast support grassy steppe-like tundra ecosystems, in very dry climatic conditions with precipitation lower than 200 mm per year.

Apart from climatic factors, like temperature and precipitation, grazing herbivores may also have had an impact on the species composition of past vegetation. Olofsson (2006; see also Zimov, 2005) found that increased reindeer grazing pressure in northern Norway stimulated grassland species at the expense of dwarf shrub vegetation. Poaceae are 'adapted' to grazing as their growing points are just above the soil surface. Grazing does not do any harm to grasses; in fact grazing stimulates their growth.

*Comparison of methods* - The methods used (microfossils, macroremains, aDNA and chemistry) show agreements and differences in the results and we realize that these methods are not equal in their palaeo-environmental indicator value. Macrofossils and lipid data mainly tell us about the food choice of the animal, but the pollen and aDNA records of taxa do not necessarily all point to the composition of the last meal of the
horse. Ancient DNA may mainly come from the ingested plant species, but pollen grains may also be a source of the DNA-recorded taxa. Wind-pollinated taxa produce high amounts of pollen that is transported all over the region and that may stick to vegetation. This pollen may be ingested by chance, so not intentionally, together with the food plants. DNA barcodes can be retrieved from pollen and in this way species, not forming a part of the regular diet, may have been recorded. The pollen of insect-pollinated taxa is less common (produced in relatively low amounts) but may also be deposited on grazed plants. Grazing animals also may ingest some of the litter on top of the soil and in this way pollen from throughout the flowering season may be ingested. A total of 18 different plant families were identified based on our integrated study of pollen, Ancient DNA and macroremains. The pollen study retrieved most of the families (11 of which 6 were not detected by Ancient DNA or macroremains). The Ancient DNA study revealed 9 families (of which 3 families were not detected by the other methods) and the macroremains indicated that 6 families were part of the palaeo-environment (2 of these families were not detected by the other methods; Table 1). We advocate a multiproxy approach for reconstruction of palaeoenvironments and palaeodiets to identify as many families as possible.

In lake sediments and peat deposits, the frequency changes of spores of coprophilous fungi reflect changes of the population densities of mammals (e.g., Innes et al., 2013). Individual samples from the intestines of herbivores can deliver different, but important information. Based on the presence of fungal fruit-bodies in intestinal mammoth samples, van Geel et al. (2008; 2011a,b) concluded that coprophagy played a role in the behavior of mammoths. The frequency of spores of coprophilous fungi in the intestinal sample from the horse does not show more than the presence of faeces in the area where the horse lived. For climatic conditions as
derived from the fossil record in northern Siberia we refer to Giterman et al. (1982),
Andreev et al. (2011) and to Andreev and Tarasov (2013).

Conclusions

Four methods applied to intestinal material of a middle-Holocene horse point to a diet of mainly Cyperaceae and Poaceae. Macrofossils and chemistry mainly reflect the ingested plants. The taxa detected by ancient DNA and pollen analysis may partly reflect species that were not actively grazed, but were producing pollen elsewhere in the landscape.

Acknowledgements

Annemarie Philip prepared the microfossil samples and Jan van Arkel made the pictures of microfossils and macroremains. Three anonymous reviewers provided useful suggestions that greatly improved the initial version of this manuscript.

References


http://mc.manuscriptcentral.com/holocene


Figure 1: remains of the frozen Yakutian horse and various plant remains from its colon. 1a: hind part of the horse body. 1b: head. 1c: epidermis Poaceae. 1d: cluster of Poaceae pollen. 1e: Type 815 ascospores. 1f: Type 816 (Bryophyte?) spores. 1g-m: various types of cyperaceous epidermis with spiny leaf margins (1g-j) and papillae (1h-j). In: unidentified broken seed.

Figure 2: moss remains from the colon of the Yakutian horse. 2a,b: cross section through leaf of Polytrichastrum alpinum. 2c: leaves of Campylium cf. stellatum. 2d: multicellular rhizoids with obliques septa (cf. Bryum).

Figure 3: n-alkyl lipids from horse intestinal tract contents

Figure 4: Partial gas chromatogram of the alcohol fraction isolated from the horse intestinal tract contents. Trivial names are given in brackets.

Table 1: Microfossil spectra, macrofossil and Ancient DNA data. NL: analysis by BvG; RU: analysis by NAR, SST and SVZ. Non-pollen palynomorphs were recorded in the NL sample only. Observations that were made after finishing the counting procedure have been indicated with +.
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<td>Anthoxanthum sp.</td>
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<td>Avena sp.</td>
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<td>Catabrosa sp.</td>
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<td>Dactylis sp.</td>
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<td>Caltha sp.</td>
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<td>Picaria sp.</td>
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<td>JN893736</td>
<td>126</td>
<td>10^-56</td>
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<td>JN891054</td>
<td>126</td>
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<td>Saxifragaceae</td>
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**Cryptogams:**

- Amblystegiaceae: Campylium cf. Stellatum +
- Mniaceae: Plagiomnium cf. ellipticum +
- Rhizomnium cf. pseudopunctatum +

**Polytrichaceae:**

- Polytrichum sp. rbcL (8535) DQ645987 115 1e-50
- Polytrichadelphus sp. rbcL (13333) AF478207 130 2e-53
- Polytrichastrum alpinum +

**Sphagnaceae:**

- Sphagnum sp. +

**Fungi:**

- Delitschiaceae: Delitschia +
- Magnaporthaceae: Clasterosporium +
- Sporormiaceae: Sporormiella- 7.8 type (T.HdV-113)
- Sordariaceae: Sordaria-type 1.4 (T.HdV-55)
- Indet: Type 815 0.5
- Type 816 1.4
Figure 1: remains of the frozen Yakutian horse and various plant remains from its colon. 1a: hind part of the horse body. 1b: head. 1c: epidermis Poaceae. 1d: cluster of Poaceae pollen. 1e: Type 815 ascospores. 1f: Type 816 (Bryophyte?) spores. 1g-m: various types of cyperaceous epidermis with spiny leaf margins (1g-j) and papillae (1h-j). 1n: unidentified broken seed.

297x420mm (300 x 300 DPI)
Figure 2: moss remains from the colon of the Yakutian horse. 2a,b: cross section through leaf of Polytrichastrum alpinum. 2c: leaves of Campylium cf. stellatum. 2d: multicellular rhizoids with obliques septa (cf. Bryum).

297x420mm (300 x 300 DPI)
Figure 3: n-alkyl lipids from horse intestinal tract contents
190x254mm (96 x 96 DPI)