The Holocene

Multiproxy study of the last meal of a mid-Holocene Yakutian horse

Journal:	*The Holocene*
Manuscript ID:	HOL-13-0195
Manuscript Type:	Paper
Date Submitted by the Author:	16-Dec-2013
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Keywords:	Yakutia, mid-Holocene, horse, last meal, ancient DNA, lipids, pollen and spores, macrofossils
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Abstract

The last meal of a horse that lived in northern Yakutia 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 inflorescenses of grasses. We evaluate the palaeo-environmental indicator value of the different methods applied. Botanical 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 with a limited amount of Birch and Alder shrubs and support the hypothesis that horses changed their diet from browsing to grazing concurrent with the spread of savanna and steppe grasslands.

Introduction

The Equidae are considered one of the prime examples for studying mammalian evolution due to their excellent fossil record. Their major adaptive radiation occurred during the early Miocene. Significant changes in dental morphology from low-crowned to high-crowned teeth occurred together with major changes in spreads of grasslands requiring a change in diet from predominantly browsing on woody plants to grazing on grasses and sedges. However, little evidence has yet been available to test this hypothesis (Wang et al. 1994). 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 (Ukraintseva 1979, 1993; van Geel et al., 2008, 2011a,b; Guthrie, 1990; Lazarev, 2008). In 2010 a frozen horse (Fig. 1a,b) was found in the area caled Oyogossky Yar, in the Ust-Yana region of Yakutia (72^{o} 40’49.42’’N, 142 ^{o} 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. Oyagossky Yar is one of the richest areas for Quaternary fossils in northern Yakutia. It is located on the mainland coast of the Dmitri Laptev Strait and stretches more than 100 km, from Cape Saint Nose (Svyatoi Nos) in the west to the mouth of the Kondratieva River in the east. The Edoma deposits of the Oyagossky Yar’s northern slope reach up to 40-50 m above sea level. The icy deposits in the area include the entire second half of the Pleistocene. The western part of Oyagossky Yar is composed of lake-alluvial silt sediments reaching 5-7 m a.s.l., which are contemporary with the sediments of the Olersky Formation (Lower Kolyma River basin, Lower Pleistocene). The partial

carcass 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. 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, in order to test the hypothesis that horses shifted their diet during evolution to become predominant grazers. 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 samples was carefully removed with a scalpel to prevent contaminants in the extractions. 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; 2000).

PCR amplification

Amplifications of the plastid *rbc*L 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 $MgCl_2$, 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

With the use of Ampure XP beads from Agencourt primer dimer was removed from the PCR products generated. 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 ISPs. 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). Only reads with a length of at least 100 bp 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. Representative consensus sequences of each cluster were blasted against NCBI GenBank data for taxonomic identification up to family 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 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 a 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: *Plagiomnium cf. ellipticum*, cf. *Rhizomnium 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 100% certainty retrieved encompass genera within the Cyperaceae (*Carex* and *Eriophorum*), Myricaceae (*Comptonia*), Poaceae (*Poa*), Ranunculaceae (*Caltha*) and Salicaceae (*Salix*). Matches found between Ion Torrent reads obtained and reference data in NCBI GenBank were less (88-96%) for *Agrostis* (Poaceae) and *Menyanthes* (Menyanthaceae) (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} alkanol indicating a significant contribution from Poaceae (Dove & Mayes, 1996; Maffei, 1996; van Bergen et al., 1997; Bughalo et al., 2004; Killops & Killops, 2005).

Figure 4 show a partial gas chromatogram of the alcohol fraction isolated from the horse intestinal tract contents. The presence of a suite of 5β-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} 5β-stanols (stigmastanol and epistigmastanol) and the occurrence of the phytosterol sitosterol is consistent with an herbivorous diet (Leeming et al., 1997; 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 inflorescenses - 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 represent mainly 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 DNA we have additional data about plant taxa that played a role in the vegetation where the horse grazed its last meal. *Carex*, *Eriophorum, Caltha* and *Menyanthes* may well have formed part of the vegetation in moist areas in the landscape, while *Agrostis* and *Poa* may have grown on dry soils. Uncertainties in the identifications based on aDNA are expressed as 'percentages query coverage'. Retrieval of *Menyanthes* (93% coverage) and *Agrostis* (96% coverage), although supported with short reads only, is likely correct as the present distribution of these taxa is congruent with the area where the horse was found. We reject any possibility of contamination as we included extraction and amplification blanks in all analyses.

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 the development of tundra with a vegetation similar to the modern (Andreev et al., 2011). Apart from climatic factors, like temperature and precipitation, grazing herbivores may also have had an impact on the species composition of the vegetation. Olofsson (2006) 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. Based on observations in 'Pleistocene Park' in northern Yakutia, Zimov (2005) developed a theory about the disappearance of the mammoth steppe at the beginning of the Holocene. At that time mammoths and other large herbivores disappeared and in northern Siberia the grasslands of the mammoth steppe were replaced by mossy tundra and forest tundra. The only herbivores to survive were reindeer that mainly grazed on lichens, and moose that fed on willows. Many authors (e.g. Guthrie, 2001) consider the change of landscape and vegetation at the start of the Holocene as the effect of climate change from dry to humid conditions, but according to Zimov, Pleistocene animals would have maintained their own grassland ecosystem unless hunting humans acted as ecosystem terminators. Zimov suggests that herbivore populations were decimated by human hunting. The present climate in northeastern Siberia is not humid but rather is characteristic of an arid steppe and according to Zimov the northern grasslands would have remained viable in the Holocene if the great herds of Pleistocene animals had remained in place to maintain the landscape. If we accept Zimov's argument that the pasture landscapes were destroyed because herbivore populations were decimated by human hunting, then it stands to reason that

those landscapes can be reconstituted by the return of appropriate herbivore communities.

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 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 may be ingested by chance, together with the food plants. 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 16 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 7 families (of which 2 families were not detected by the other methods) and the macroremains indicated that 6 families were part of the palaoe-environment (2 of these families were not detected by the other methods; Table 1). We advocate a multiproxy approach for reconstruction of palaeo-environments 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. Considering the potential bias in the pollen record and the availability of only one horse meal we cannot make firm conclusions about climatic conditions. For climatic conditions as derived from the fossil record in northern Siberia we refer to Andreev et al. (2011) and to Andreev and Tarasov (2013).

Conclusions

Four methods to study 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. The combined data collected support the hypothesis that horses changed their diet from predominantly browsing on woody plants to grazing on grasses and sedges concurrent with the spread of savanna grasslands.

Acknowledgements

Annemarie Philip prepared the microfossil samples and Jan van Arkel made the pictures of microfossils and macroremains.

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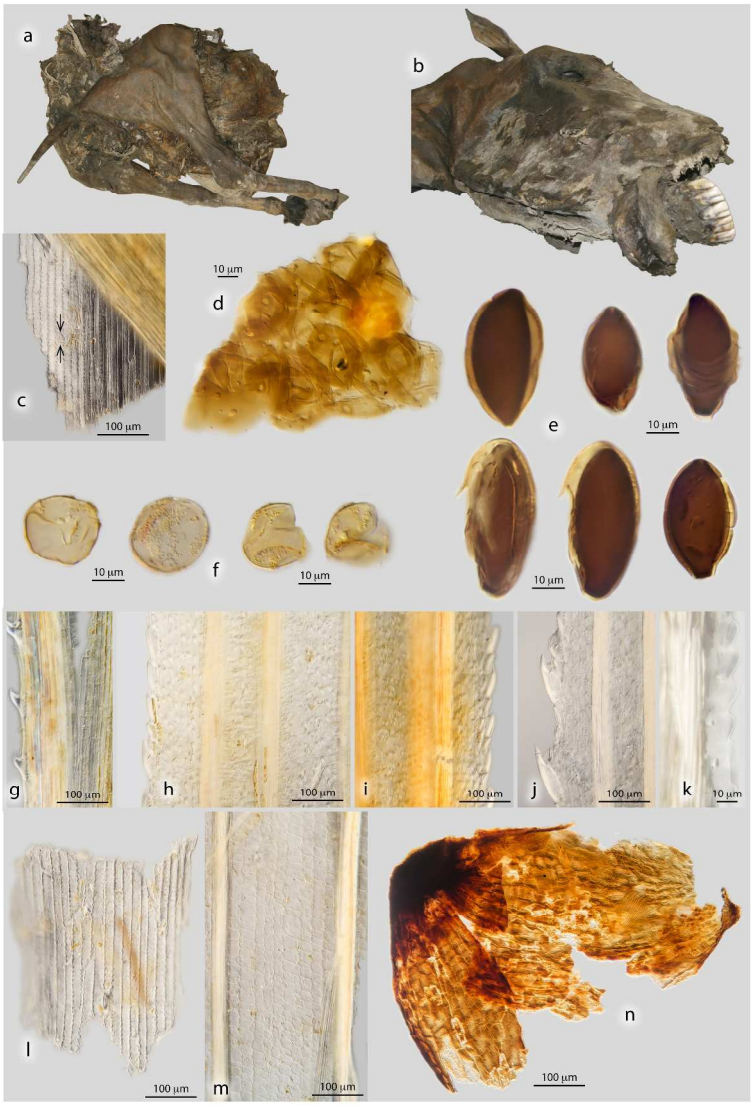
Figure 1: remains of the frozen Yakutian horse and various plant remains from its colon. 1a: hind part of the horse body. 1b: head. 1c: epidermis Poaceae. 1d: cluster of Poaceae pollen. 1e: Type 815 ascospores. 1f: Type 816 (Bryophyte?) spores. 1g-m: various types of cyperaceous epidermis with spiny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

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: 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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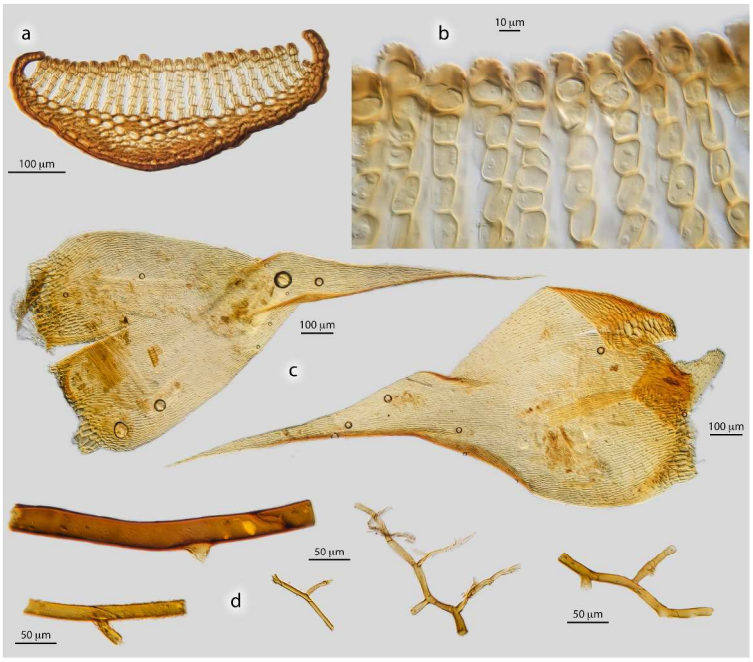
297x420mm (300 x 300 DPI)



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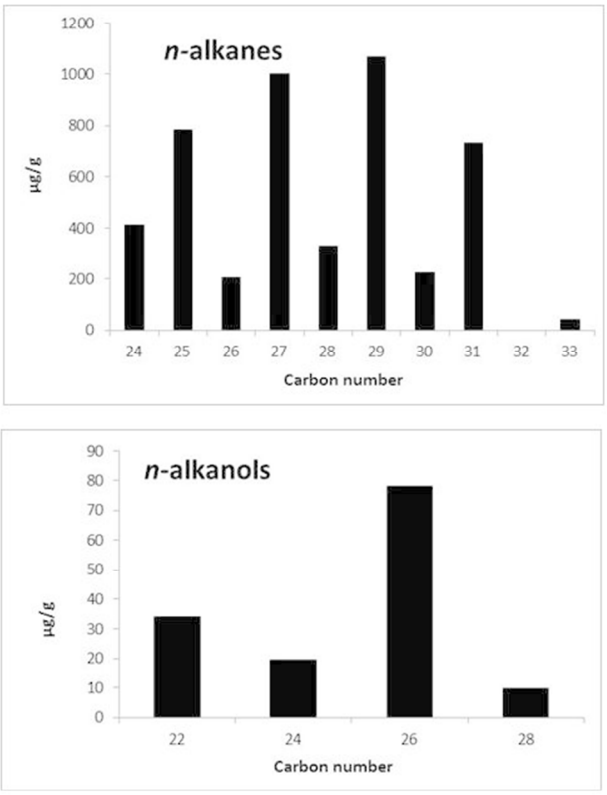

Figure 3: n-alkyl lipids from horse intestinal tract contents
190x254mm (96 x 96 DPI)

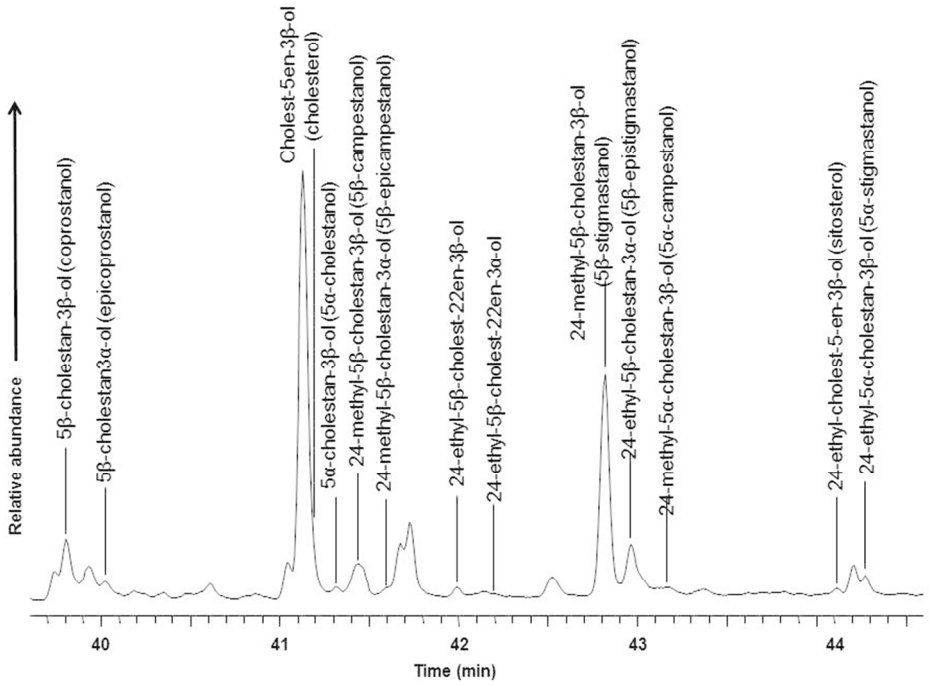

Figure 4: Partial gas chromatogram of the alcohol fraction isolated from the horse intestinal tract contents. Trivial names are given in brackets.
254x190mm (96 x 96 DPI)

Table 1.

Family/order	Genus/species	Pollensum (NL) 422 %	Pollensum (RU) 311 %	DNA barcoding marker	Most similar GenBank accession	Query cover (bp)	E value	Macroremains vegetative	Macroremains fertile (spikelets)
Phanerogams:									
Apiaceae	indet	0.2	-						
Asteraceae	Tubiliflorae	0.5	0.6						
	Artemisia sp.	+							
Betulaceae	*Alnus* sp.	1.7							
	Betula sp.	2.4							
	Betula sect. *Nanae*		0.6						
Cyperaceae	indet	2.6	4.5					+	
	Carex sp.			*trnL*	JN873698	100	3e-40		
	Eriophorum sp.			*trnL*	JN644736	100	5e-54		
Ericales	indet	0.2	0.3						
Indet			0.3						
Menyanthaceae	*Menyanthes* sp.			*rbcL*	JN965669	93	1e-17		
Myricaceae	*Comptonia* sp.			*rbcL*	DQ310505	100	0		
Papaveraceae	*Papaver rhoeas* -type	+							
Pinaceae	indet		0.3						
	Abies sp.		0.3						
	Pinus subgenus *Diploxylon*		0.3						
Plantaginaceae	*Plantago* sp.		0.3						
Poaceae	indet	91.5	91.6	*trn*L	HM590235	100	6e-42	+	+
	Agrostis sp.			*rbcL*	EF115543	96	6e-65		
	Poa sp.			*trnL*	JN030974	100	2e-36		
Ranunculaceae	indet	0.2	0.3						
	Caltha sp.			*rbcL; trnL*	AY365367	100	1e-37		
Salicaceae	*Salix* sp.	0.7		*trnL*	JN873698	100	3e-40		

Cryptogams:								
Amblystegiaceae	*Campylium* cf. *stellatum*							+
Mniaceae	*Plagiomnium* cf. *ellipticum*							+
	Rhizomnium cf. *pseudopunctatum*							+
Polypodiophyta	indet		0.6					
Polytrichaceae	indet			*trnL*	AF545029	94	9e-17	
	Polytrichastrum alpinum							+
Sphagnaceae	*Sphagnum* sp.		+					+
Fungi:								
Delitschiaceae	*Delitschia* (Type TM-023)	+						
Magnaporthaceae	*Clasterosporium caricinum* (T. 126)	+						
Sporormiaceae	*Sporormiella*-type (T. HdV-113)	7.8						
Sordariaceae	*Sordaria*-type (T. HdV-55)	1.4						
Indet	Type 815	0.5						
	Type 816	1.4						