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ABSTRACT

Intestinal samples from the one-month-old Siberian mammoth calf 'Lyuba' were studied using light microscopy and ancient DNA to reconstruct its palaeo-environment and diet. The palynological record indicates a 'mammoth steppe'. At least some pollen of arboreal taxa was reworked, and thus the presence of trees on the landscape is uncertain. In addition to visual comparison of 11 microfossil spectra, a PCA analysis contributed to diet reconstruction. This yielded two clusters: one of samples from the small intestine and the other of large-intestine samples, indicating compositional differences in food remains along the intestinal tract, possibly reflecting different episodes of ingestion. Based on observed morphological damage we conclude that the cyperaceous plant remains and some remains of dwarf willows were originally eaten by a mature mammoth, most likely Lyuba's mother. The mammoth calf probably unintentionally swallowed well-preserved mosses and mineral particles while eating fecal material deposited on a soil surface covered with mosses. Coprophagy may have been a common habit for mammoths, and we therefore propose that fecal material should not be used to infer season of death of mammoths. DNA sequences of *trn*L and *rbc*L genes amplified from ancient DNA extracted from intestinal samples confirmed and supplemented plant identifications based on microfossils and macro-remains. Results from different extraction methods and barcoding markers complemented each other and show the value of longer protocols in addition to fast and commercially available extraction kits.

1. Introduction

In May 2007, a frozen, female woolly mammoth calf (*Mammuthus primigenius* Blumenbach, 1799; Fig. 1,1) was discovered by reindeer herders along the Yuribei River on the Yamal Peninsula (68°38′N; 71°40′E; Kosintsev et al., 2010; Fisher et al., in press) and acquired by the Shemanovskiy Museum and Exhibition Center in Salekhard, Yamalo-Nenets Autonomous Okrug. Its bone collagen was AMS-dated at 41,910+550/−450 BP, and intestinal material was dated at 41,700 +700/−550 BP (Kosintsev et al., 2010). The mammoth calf, now known as 'Lyuba', thus lived during the relatively mild Middle Pleniglacial of the Last Ice Age (Marine Isotope Stage 3).

The calf appears outwardly to be in nearly perfect condition, although the "shrunken" cross-sections of her limbs show that she has lost significant water mass, and dissections documented multiple instances of internal postmortem alteration (Fisher et al., in press). Lyuba weighed ca. 50 kg when discovered; she is 85 cm high and 115 cm from trunk to tail. Based on counts of postnatal daily growth increments in dentin, Rountrey et al. (in press) determined her age at death to be about one month. Isotope series from her teeth suggest she was born in spring (climatically late winter), probably before new plant growth had begun (Rountrey et al., in press). Despite the potential lack of high-quality plant foods available to her mother at this time, abundant subcutaneous fat indicates

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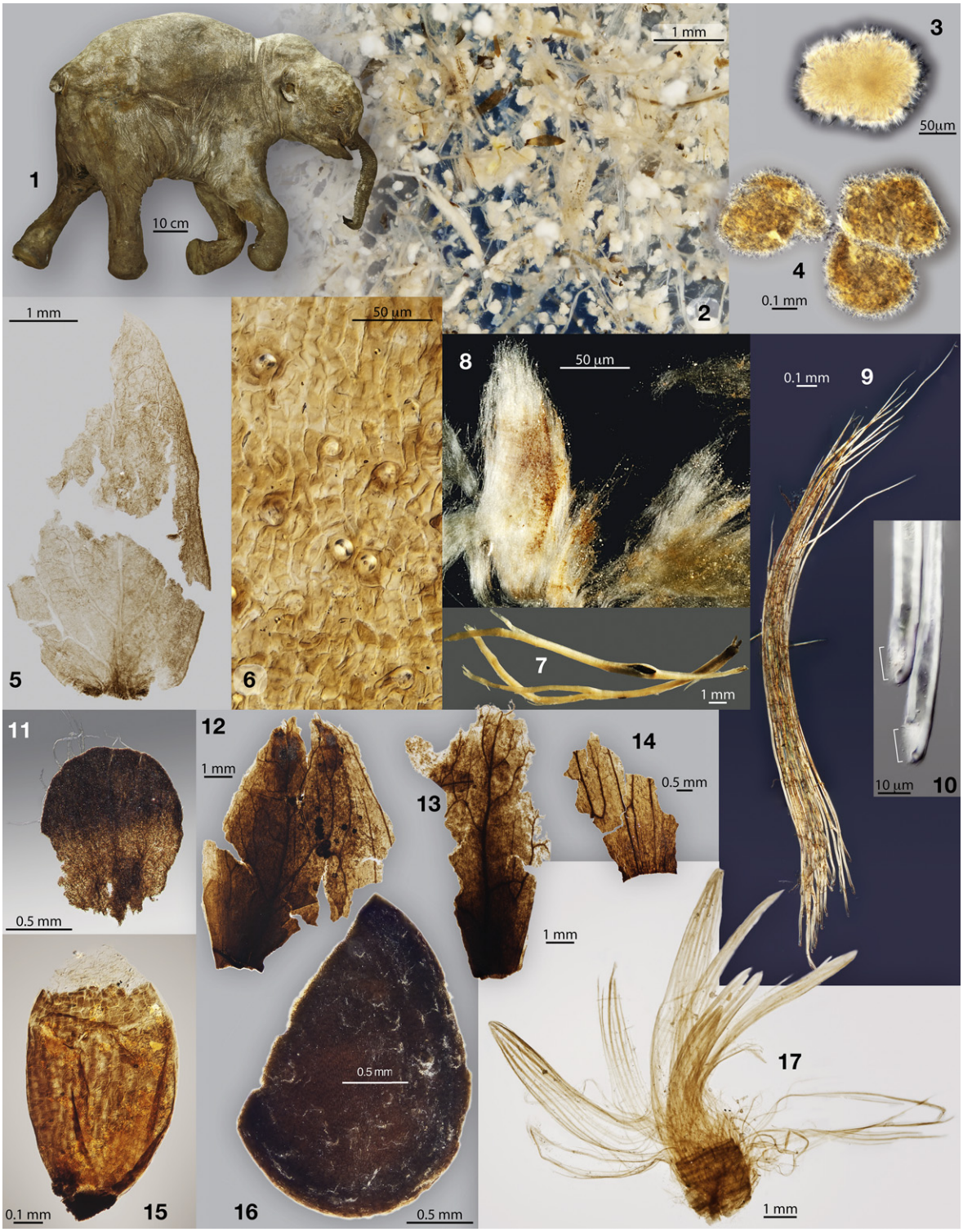

Fig. 1. 1: baby mammoth Lyuba; 2: particles of milk-residue (white dots) between plant remains; 3 and 4: milk particles; 5: leaf fragment of *Salix*; 6: *Salix*, epidermis; 7: *Salix*, twigs without bark; 8 and 9: separate bundles of hairs from bud scales or leaves of *Salix*; 10: *Salix,* two leaf hairs, showing asymmetrical basal points of attachment, which keep hairs parallel to the stem axis where they cover leaves or bud scales; 11: *Salix*, leaf or bud scale; 12–14: unidentified leaf or bud scale fragments; 15: cf. *Juncus*, seed; 16: *Menyanthes trifoliata*, incomplete seed; 17: unidentified young plant.

that this mammoth calf was well nourished. At one month of age, Lyuba's nutritional requirements would likely have been met solely by her mother's milk (judging from observations on African elephants; Lee and Moss, 1986; Moss, 1992), and the lack of significant wear on the dP2s (the only teeth that had erupted and come into occlusion) indicates that she would have been incapable of efficiently masticating vegetation. However, plant remains are abundant in some areas of her intestines.

We studied botanical microfossils, macroremains and ancient DNA present in samples from Lyuba's small and large intestines. Contrary to the description of Kosintsev et al. (in press), Lyuba's stomach and small intestine were not empty. Our objective was to

reconstruct both the palaeo-environment and diet of this mammoth calf and compare the results to those of similar studies on fecal material of mature mammoths by van Geel et al. (2008, 2011 and references there in). A chemical study of samples from Lyuba's intestines will be published separately.

2. Methods

Material was separated for analysis of microfossils and macroremains from fourteen intestinal samples. Macrofossil samples were prepared according to Mauquoy and van Geel (2007). For microfossil analysis, sub-samples of ca. 1 cc were treated with KOH and subsequently acetolysed according to Faegri and Iversen (1989). For the separation of organic material from mineral particles, a bromoform-ethanol mixture (specific gravity 2) was used. The microfossils were embedded in glycerine jelly, and microscope slides were sealed with paraffin. Pollen was identified using Beug (2004), Moore et al. (1991) and a pollen reference collection. Non-pollen palynomorphs (NPP) were recorded in addition to pollen grains. Type numbers for NPP refer to illustrations and descriptions given by van Geel (1978), van Geel et al. (1981, 1989), Pals et al. (1980), and van Geel and Aptroot (2006).

A Detrended Correspondence Analysis (DCA) and Principal Components Analysis (PCA) were carried out on the log-transformed counts of pollen, spores, fungi and other microfossils to determine if there were compositional differences in the microfossil assemblages along the intestinal tract. The PCA was carried out on the covariance matrix of the log-transformed microfossil data. As the log of zero is undefined, a value of one was added to all counts before the log-transformation. The ordination analyses were done using CANOCO 4.5 (ter Braak and Smilauer, 2002).

Ancient DNA – Subsamples for ancient DNA analysis were taken from two samples (Table 1). DNA extractions were carried out using three different methods: CTAB (Doyle and Doyle, 1987), a silica-based protocol (Höss and Pääbo, 1993), and the DNeasy plant miniprep protocol (QIAGEN). All extractions were carried out in the special Ancient DNA facility of Leiden University, which provided a work area physically separated from general molecular laboratories as required for established ancient DNA protocols (Cooper and Poinar, 2000). All extractions included a control blank. Some of the results reported here were replicated at the molecular laboratories of IBED at the University of Amsterdam.

For the first method, approximately 100 mg of sample was ground to fine powder in liquid nitrogen with a mortar and pestle. A total of 1 ml of 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, and the samples were subsequently incubated under agitation for 1 h at 65 °C. DNA was extracted twice using 450 μl of chloroform:isoamyl alcohol (24:1). Pellets were precipitated with ice-cold isopropyl alcohol. Dried pellets were resuspended in TE buffer and left overnight at 4 °C. The next day, 2.5 volumes of pure ethanol and NH_4-acetate were added to a final concentration of 2.5 M, and the suspension was left to reprecipitate at −20 °C for 30 min. The resulting pellets were washed twice in 76% ethanol 10 mM NH_4-acetate, air dried, and dissolved in TE.

For the second protocol, 100–200 mg of sample was ground with a mixer mill. An extraction buffer (0.45 M EDTA, pH 8.0 with 0.25 mg/ml proteinase K) was added, and the samples were then incubated under rotation overnight at room temperature in the dark. The next day, the samples were centrifuged for 2 min at 4000 rpm, after which 8 ml of a silica buffer (5 M GuSCN, 0.05 Tris, pH 8.0, 0.025 M NaCl, 40 μl dissolved silica) was added. The samples were again rotated in the dark for 3 h and then centrifuged for 1 min at 4000 rpm. The resulting pellets were washed with a buffer (51% pure ethanol, 125 mM NaCl, 10 mM Tris, 1 mM EDTA), air dried, and resuspended in TE buffer.

For the third method, the protocol provided by QIAGEN was followed, except that an additional 25 μl of proteinase K was added to buffer AP1 to optimize lysis.

All DNA extracts were further purified using PCR Wizard purification columns (Promega) and diluted 1:10. PCR reactions were carried out in 20-μl volumes containing 2.5 mM dNTPs, 10 mM primers, 5× PCR buffer, Phire Hot Start DNA polymerase (Finnzymes), and 2 μl of template. Primers used were Z1aF and 19bR (Hofreiter et al., 2000), amplifying a 157-bp fragment of the chloroplast *rbc*L gene, and trnL g and trnL h (Taberlet et al., 2007), amplifying a 73–124-bp fragment from the chloroplast *trn*L intron. The PCR program started with an activation step of 5 min at 98 °C, followed by 35 cycles of 5 s at 98 °C, 20 s at 63–64 °C and 30 s at 72 °C, and a final step of 1 min at 72 °C. Blunt-end PCR products obtained were tailed and then cloned using the TOPO TA Cloning kit (Invitrogen). From each plate, 5–10 clones were sequenced both in forward and reverse orientation on an ABI 3730xl (Applied Biosystems) at Macrogen, Amsterdam. Electropherograms were visually checked and contigged using Sequencher version 4.10.1 (GeneCodes). The consensus sequences were then compared with data in the NCBI GenBank using Blast Search. Identifications were accepted only if the query coverage was 100%. As Blast searches only give a measure of confidence of sequence similarity rather than taxonomic assignment, we applied a statistical evaluation of the Blast results (Munch et al., 2008a, 2008b). The closest match in the first ten hits was determined by a bootstrap analysis with 1000 replicates using PAUP* version 4.0b.10. The identification of unknown DNA sequences can only be as good as the reference databases used. Errors can occur when databases are incomplete or include data of wrongly identified organisms (Munch et al., 2008a, 2008b). The reference database used here (NCBI GenBank) is prone to these problems, so we took care to base our identifications on multiple hits derived from different sources.

3. Results

3.1. Microfossils and macroremains

Results of the analysis of pollen, spores and macrofossils are given in Tables 2 and 3, and Figs. 1–4 show a selection of macrofossils and microfossils. Samples SIN1, STO1, and STO2 (throughout the text, STO indicates samples from the stomach, SIN indicates samples from the small intestine, and LIN indicates samples from the large intestine) were too poor in pollen and macrofossils to

Table 1
Overview of samples analyzed with the different methods employed in this study (+, data were collected; –, no data were collected). Samples SIN1 to SIN7 were taken from the small intestine; samples LIN1 to LIN5 were from the large intestine. Samples SIN1 (small intestine), and STO1 and STO2 (stomach) had very few microfossils and were not analyzed. Chemistry is not covered in the present study.

Sample	SIN2	SIN3	SIN4	SIN5	SIN6	SIN7	LINI	LIN2	LIN3	LIN4	LIN5
Microfossils	+	+	+	+	+	+	+	+	+	+	+
Macroremains	+	+	+	+	+	+	+	+	+	+	+
aDNA	+	–	–	–	–	–	+	–	–	–	–
Chemistry	+	–	–	–	–	–	+	–	–	–	–

Table 2
Microfossil record of 11 samples from Lyuba's intestines. Samples SIN2 to SIN7 are from small intestines. Samples LIN1 to LIN5 are from large intestines.

Lyuba microfossils	SIN 2		SIN 3		SIN 4		SIN 5		SIN 6		SIN 7		LIN 1		LIN 2		LIN 3		LIN 4		LIN 5		Total	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n total	% total
Pollen and spores																								
cf. *Aesculus hippocastanum*		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Alnus	11	2.8	4	1.2	7	2.2	6	1.5	4	1.2	1	0.3	1	0.3	3	0.9	4	1.1	6	1.8		0.0	47	1.22
Betula	54	13.5	49	15.3	46	14.4	51	12.8	53	16.3	13	3.6	9	2.4	34	10.5	28	8.0	29	8.8	11	3.2	377	9.80
Carpinus		0.0	2	0.6	3	0.9	2	0.5	1	0.3		0.0		0.0		0.0		0.0	1	0.3		0.0	9	0.23
Corylus	3	0.8	1	0.3	3	0.9		0.0	4	1.2		0.0	1	0.3	1	0.3	3	0.9		0.0		0.0	16	0.42
Ilex		0.0	1	0.3	2	0.6		0.0	1	0.3		0.0		0.0		0.0	2	0.6	2	0.6		0.0	8	0.21
cf. *Juqlans*		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Juniperus		0.0	1	0.3	1	0.3		0.0		0.0		0.0		0.0	2	0.6	1	0.3	1	0.3		0.0	6	0.16
Picea	7	1.8	12	3.7	9	2.8	14	3.5	7	2.1	1	0.3	1	0.3	4	1.2	7	2.0	3	0.9	2	0.6	67	1.74
Pinus	53	13.3	49	15.3	33	10.3	25	6.3	42	12.9	10	2.8	11	2.9	15	4.6	19	5.4	16	4.8	10	2.9	283	7.35
cf. *Populus*		0.0	1	0.3		0.0		0.0		0.0		0.0	6	1.6		0.0		0.0		0.0	1	0.3	8	0.21
Quercus		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.3		0.0	1	0.3		0.0	2	0.05
Salix	10	2.5	3	0.9	4	1.3	4	1.0	13	4.0	5	1.4	4	1.0	4	1.2	4	1.1	1	0.3	3	0.9	55	1.43
Ulmus	1	0.3	4	1.2		0.0	1	0.3	2	0.6	1	0.3		0.0	2	0.6	1	0.3		0.0		0.0	12	0.31
Apiaceae	1	0.3		0.0	3	0.9	1	0.3		0.0		0.0	6	1.6	5	1.5	15	4.3	6	1.8	14	4.1	51	1.33
Artemisia	15	3.8	26	8.1	13	4.1	6	1.5	16	4.9	6	1.7	9	2.4	17	5.2	11	3.2	21	6.3	14	4.1	154	4.00
Asteraceae liguliflorae	2	0.5		0.0		0.0		0.0	1	0.3		0.0		0.0	1	0.3		0.0		0.0		0.0	4	0.10
Asteraceae tubuliflorae	2	0.5	4	1.2	5	1.6	3	0.8	2	0.6	1	0.3	11	2.9	7	2.2	13	3.7	31	9.4	20	5.9	99	2.57
Brassicaceae	1	0.3	1	0.3	1	0.3	1	0.3		0.0	1	0.3		0.0	2	0.6	2	0.6	1	0.3	3	0.9	13	0.34
Caryophyllaceae	3	0.8	1	0.3	1	0.3		0.0	2	0.6	1	0.3		0.0	1	0.3	1	0.3	3	0.9	4	1.2	17	0.44
Chenopodiaceae	6	1.5	2	0.6	5	1.6		0.0	6	1.8		0.0		0.0	3	0.9	1	0.3	4	1.2	1	0.3	28	0.73
Cyperaceae	93	23.3	64	19.9	73	22.9	47	11.8	25	7.7	5	1.4	156	40.8	40	12.3	40	11.5	49	14.8	37	10.9	629	16.35
Ericales	5	1.3	1	0.3		0.0	2	0.5	2	0.6	1	0.3		0.0	3	0.9	1	0.3	1	0.3		0.0	16	0.42
Fabaceae		0.0		0.0	1	0.3		0.0	1	0.3		0.0	11	2.9	2	0.6	2	0.6	2	0.6	12	3.5	31	0.81
Filipendula ulmaria	2	0.5		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	2	0.05
Helianthemum		0.0		0.0	1	0.3	2	0.5		0.0		0.0		0.0	2	0.6		0.0		0.0		0.0	5	0.13
Lysimachia		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Menyanthes	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.3		0.0		0.0	2	0.05
Papaver		0.0		0.0	1	0.3		0.0	1	0.3	2	0.6	1	0.3	3	0.9	13	3.7	8	2.4	1	0.3	30	0.78
Parnassia	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
cf. *Pedicularis*		0.0		0.0		0.0		0.0	1	0.3		0.0		0.0		0.0	10	2.9	7	2.1	2	0.6	20	0.52
Plantago spec.	2	0.5		0.0	1	0.3		0.0	1	0.3		0.0		0.0	1	0.3		0.0		0.0		0.0	5	0.13
Poaceae	120	30.1	93	29.0	98	30.7	231	57.9	136	41.7	309	86.6	153	40.1	161	49.5	155	44.4	130	39.3	191	56.2	1777	46.18
Polemonium		0.0		0.0		0.0	1	0.3		0.0		0.0		0.0	1	0.3	1	0.3		0.0	1	0.3	4	0.10
Polygonum bistorta	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Polygonum persicaria type		0.0		0.0		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Potentilla type		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.3		0.0		0.0		0.0	1	0.03
Ranunculaceae	1	0.3		0.0	2	0.6	2	0.5	1	0.3		0.0	1	0.3	1	0.3	5	1.4	4	1.2	4	1.2	21	0.55
Rosaceae undif.	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Rumex acetosella type		0.0		0.0	1	0.3		0.0	1	0.3		0.0		0.0	2	0.6		0.0		0.0	2	0.6	6	0.16
Sparganium	2	0.5		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	3	0.08
Thalictrum		0.0		0.0	1	0.3		0.0	2	0.6		0.0		0.0	1	0.3	4	1.1	2	0.6	3	0.9	13	0.34
cf. *Typha angustifolia*		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Valeriana	1	0.3	1	0.3		0.0		0.0		0.0		0.0	1	0.3	5	1.5	5	1.4	2	0.6	4	1.2	19	0.49
Pollen sum	399	100.0	321	100.0	319	100.0	399	100.0	326	100.0	357	100.0	382	100.0	325	100.0	349	100.0	331	100.0	340	100.0	3848	100.00
cf. Equisetum	1	0.3	1	0.3	2	0.6	2	0.5	3	0.9	1	0.3		0.0		0.0		0.0		0.0		0.0	10	0.26
Monolete psilate	14	3.5	11	3.4	13	4.1	9	2.3	12	3.7	1	0.3		0.0	4	1.2	6	1.7	4	1.2	2	0.6	76	1.98
Monolete verrucate		0.0	2	0.6	1	0.3		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0	4	0.10
Osmunda		0.0		0.0		0.0		0.0		0.0	1	0.3		0.0		0.0	1	0.3	1	0.3	1	0.3	4	0.10
Sphagnum	3	0.8	1	0.3	3	0.9	8	2.0		0.0		0.0	1	0.3		0.0	3	0.9	1	0.3		0.0	20	0.52
Trilete undiff.		0.0		0.0		0.0		0.0		0.0		0.0		0.0	2	0.6		0.0	2	0.6	1	0.3	5	0.13
Fungi																								
Sordaria-type (T.55). ascospores	5	1.3	1	0.3	2	0.6	1	0.3	1	0.3	19	5.3	10	2.6	7	2.2	4	1.1	3	0.9	7	2.1	60	1.56

Cercophora-type (T.112)		0.0		0.0		0.0		0.0		0.0	1	0.3		0.0		0.0		0.0	1	0.3	2	0.05
Sporormiella (T.114). ascospore cells	27	6.8	16	5.0	24	7.5	20	5.0	31	9.5	13	3.6	71	18.6	139	42.8	64	18.3	53	16.0	104	30.6	562	14.60
Gaeumannomyces (T.126)	36	9.0	30	9.3	42	13.2	21	5.3	28	8.6	9	2.5	42	11.0	69	21.2	42	12.0	25	7.6	100	29.4	444	11.54
Coniochaeta cf. *ligniaria* (T.172)	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03
Podospora type (T. 368). ascospores	1	0.3		0.0		0.0		0.0		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0	2	0.05
Urocystis		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.3	101	29.7	102	2.65
Algae																								
Botryococcus	11	2.8	12	3.7	9	2.8	14	3.5	11	3.4	1	0.3	4	1.0	14	4.3	13	3.7	13	3.9	11	3.2	113	2.94
Pediastrum	37	9.3	36	11.2	23	7.2	18	4.5	8	2.5	1	0.3	21	5.5	72	22.2	53	15.2	44	13.3	19	5.6	332	8.63
Spirogyra. smooth spores (T.130)	1	0.3	3	0.9		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0	1	0.3		0.0	6	0.16
Mougeotia laetevirens type (T.373)		0.0		0.0		0.0	2	0.5	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0	3	0.08
Type 128A algal spores	3	0.8	3	0.9	1	0.3	3	0.8	9	2.8	1	0.3		0.0	4	1.2	7	2.0	6	1.8	5	1.5	42	1.09
Type 225	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	3	0.9		0.0	4	0.10
Other																								
Freshwater sponges, needles (T.220)	7	1.8	1	0.3	8	2.5	3	0.8	12	3.7	1	0.3	1	0.3	1	0.3		0.0		0.0		0.0	34	0.88
Hystrichosphaeridae	1	0.3		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0	2	0.05
Aquilapollenites-'complex'	2	0.5	2	0.6	3	0.9	1	0.3		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0	9	0.23
Assulina (T.32)		0.0	1	0.3		0.0		0.0	1	0.3		0.0	5	1.3		0.0		0.0	1	0.3		0.3	8	0.21
Pre-Quaternary microfossils		0.0	4	1.2		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	4	0.10
Volcanic ash (tephra)		0.0		0.0	1	0.3		0.0		0.0		0.0		0.0		0.0		0.0		0.0		0.0	1	0.03

Table 3
Macrofossil record of Lyuba's intestinal samples.

	Small intestine	Large intestine
Sand	+	+
Milk	+	−
Vivianite	−	+
Charcoal	−	+
Carex, vegetative remains	+	+
Poaceae, vegetative remains	−	+
Salix, vegetative remains	+	+
Menyanthes, seed	−	+
cf. *Juncus*, seeds	+	−
cf. Abietinella abietina	+	−
Aulacomnium turgidum	+	+
Drepanocladus aduncus	+	+
Hylocomium splendens	+	+
Polytrichum juniperinum	+	+
Sarmentypnum sarmentosum	+	+
Thuidium delicatulum	+	+
Sporormiella, fruit-body	−	+
Oribatida	+	+
Daphnia, ephippia	+	+

analyze. Most pollen spectra are dominated by Poaceae and Cyperaceae, indicating an open landscape. A lump of Poaceae pollen was found in sample SIN5 (small intestine), probably indicating that an unripe inflorescence was included in that sample. Other herbaceous taxa in the pollen record are *Artemisia*, other Asteraceae, Apiaceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Ericales, Fabaceae, *Helianthemum*, *Menyanthes*, *Papaver*, *Plantago*, *Polemonium*, Ranunculaceae, *Rumex*, *Thalictrum*, *Valeriana*, *Sparganium* and *Typha*. Ferns are represented by monolete psilate spores. Tree pollen is represented by *Pinus*, *Picea*, *Betula*, *Alnus* and low values of *Carpinus, Corylus* and *Ulmus*. Pollen grains of *Populus*, *Ilex*, cf. *Juglans* and *Juniperus* occur rarely.

In addition to pollen, the non-pollen palynomorphs (including fungal spores) can be used as environmental indicators. Many hyphopodia of *Gaeumannomyces* were recorded (Fig. 4, 41). This is a parasitic fungus that was recognized to be a *Carex* indicator in Weichselian and Holocene peat deposits (Pals et al., 1980; van Geel et al., 1989; Ran, 1990). *Sporormiella* is a common dung-inhabiting fungus (Davis, 1987), and *Sporormiella* ascospore cells, together with ascospores of the coprophilous *Sordaria*-type (van Geel and Aptroot, 2006), indicate the availability of dung on the landscape where Lyuba lived. Two fruit-bodies of *Sporormiella*-type with spores inside were found, one in the pollen slide of sample LIN4 (from the large intestine) and one in macrofossil sample LIN5 (Fig. 4, 42–44). The algae *Pediastrum* and *Botryococcus*, the algal spore Type 128A, and freshwater sponge spicules are indicative of open water.

In five microfossil samples from the small intestine, rare but well-preserved pollen grains (Fig 4, 49) of the Cretaceous-Tertiary *Aquilapollenites* complex were recorded (Jardine and Harrington, 2008; Nichols et al., 2010; identified by G.F.W. Herngreen, Utrecht). In combination with pollen of the thermophilous taxa *Aesculus*, *Carpinus*, *Corylus*, *Ulmus*, *Ilex* and cf. *Juglans* (all of which were rare), this indicates that the microfossil spectra contain some reworked material (see discussion below).

The DCA yielded a gradient length of 1.6 for the first axis. This represents a relatively short gradient (i.e., samples tend to show high similarity in microfossil content), and warrants use of a Principal Components Analysis (PCA) to detect patterns in microfossil composition (ter Braak and Smilauer, 2002). The configuration of the samples in the PCA ordination diagram (Fig. 5A) shows two clusters; one cluster (samples SIN2, 3, 4, 5 and 6) is from the small intestine and the other cluster represents large-intestine samples (LIN2, 3 and 4). Outliers (SIN7, LIN1, and LIN5) are discussed below. The loadings (Fig. 5B) indicate how microfossil variables contribute to PCA axes one and two.

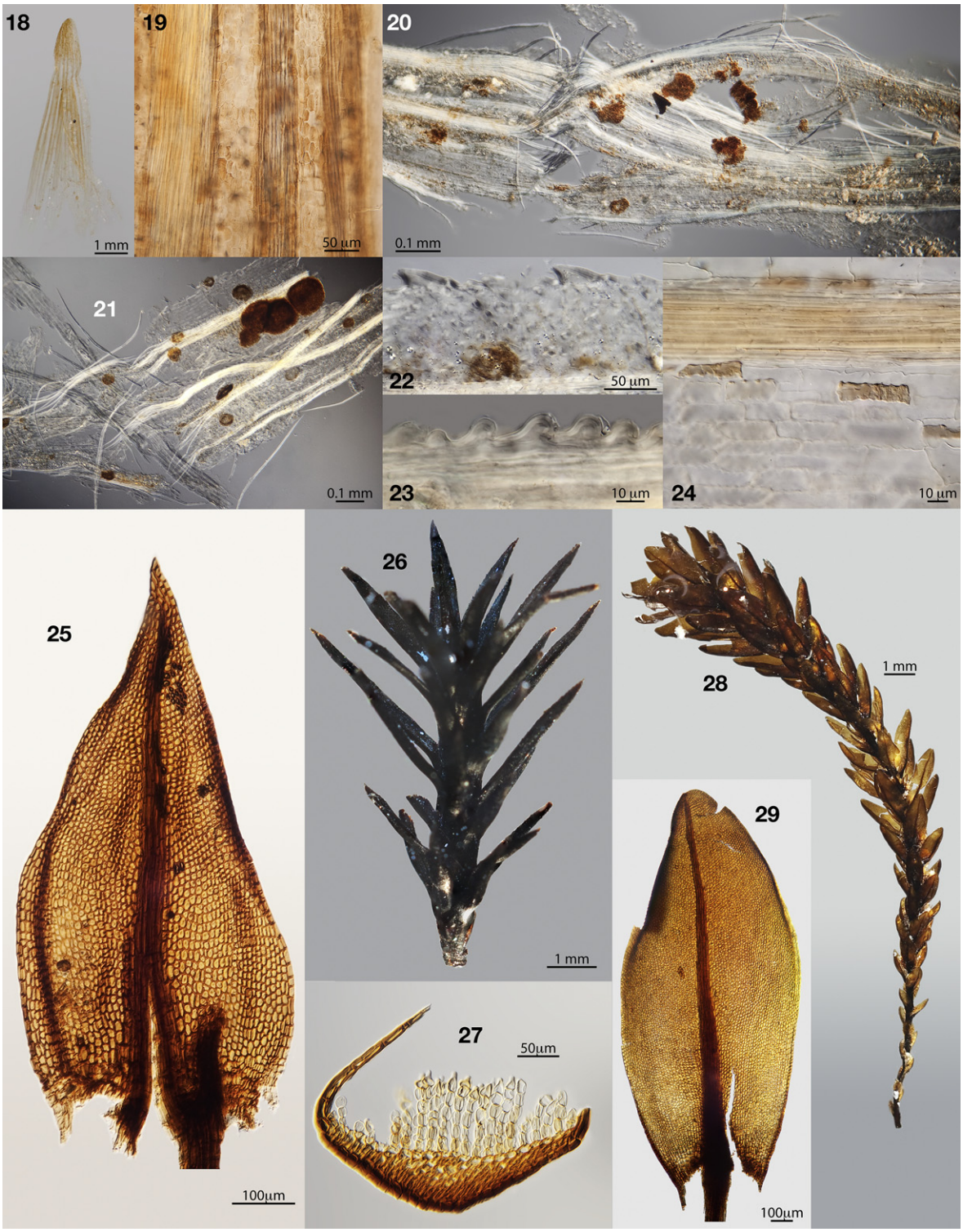

Fig. 2. 18 and 19: details of 17 (see Fig. 1); 20 and 21: Cyperaceous epidermis tissue showing mastication damage; 22: Spiny cyperaceous leaf margin; 23: papillate cyperaceous epidermis in lateral view; 24: overview of cyperaceous epidermis; 25: cf. *Abietinella abietina*, stem leaf; 26: *Polytrichum juniperinum*; 27: Cross section of leaf *P. juniperinum*; 28 and 29: *Aulacomnium turgidum*.

The total amount of material available for macrofossil analysis was only ca. 180 ml (sub-samples for microfossil analysis were also searched for macrofossils), and therefore only the total macrofossil record of the sub-samples is shown (Table 3). Remains of dwarf *Salix* species were recorded (including a twig without bark; Fig. 1, 5–7), and many bundles of bud-covering hairs of *Salix* were observed (Fig. 1, 8–10). Most of the macrofossil material consisted of cyperaceous vegetative remains, especially fragments of epidermal tissue. These cyperaceous epidermis remains were damaged in a way that we never observe in samples from peat deposits: many fragments showed torsion and other damage that suggested they had been masticated (Fig. 2, 20 and 21). This

Fig. 3. 30: *Thuidium delicatulum*; 31: *T. delicatulum*, stem leaf; 32: *T. delicatulum*, branch leaves; 33 and 34: *Sarmentypnum sarmentosum*; 35 and 36: *Bryum* sp.; 37 and 38: *Drepanocladus aduncus*.

damage was in sharp contrast to the excellent preservation of the mosses (Fig. 2, 25–29; Fig. 3, 30–38; Fig. 4, 39 and 40).

3.2. Ancient DNA

Overall, 35 DNA sequences were matched with data in NCBI GenBank (Table 4). These DNA sequences collectively represent seven families, classified within six plant orders. Five of these families, classified within three plant orders (indicated with an asterisk in Table 4) were found in a replicate experiment carried out in Amsterdam. The DNA sequences confirm results obtained from analysis of microfossils and macroremains and complement them in cases where remains had become unrecognizable due to masticatory processes (Hofreiter et al., 2000; van Geel et al., 2008,

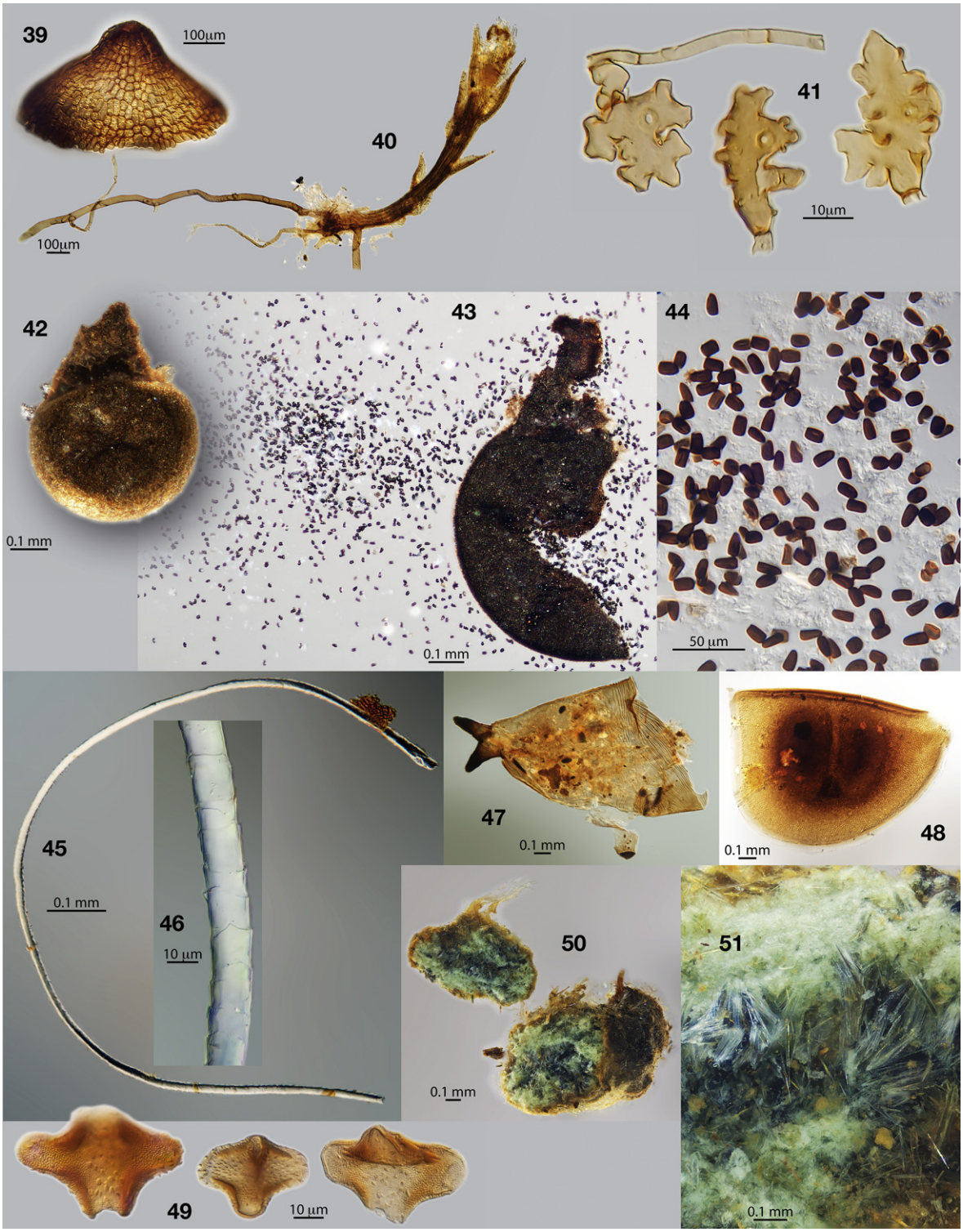

Fig. 4. 39: calyptra of moss; 40: cf. *Bryum*, young shoot with multicellular rhizoids with oblique septa; 41: hyphopodia of *Gaeumannomyces*; 42: fungal fruit-body; 43: same fruit-body as 42, after squash, showing *Sporormiella*-type ascospore cells; 44: detail of 43; 45 and 46: unidentified mammal hair (from microfossil sample SIN 7); 47: unidentified zoological remains; 48: *Daphnia*, ephippium; 49: Pollen of pre-Quaternary *Aquilapollenites*; 50 and 51: Vivianite clusters showing crystalline structure.

2011). Ranunculaceae was the only family identified using *rbc*L sequences; all others were identified based on *trn*L sequences. Asteraceae, Betulaceae, Polygonaceae and Ranunculaceae could not be identified to a lower taxonomic level than family. Salicaceae and Linaceae could be specified further to the generic level (*Salix* was also recognized among both microfossils and macroremains, but *Linum* was not recognized in either). Cyperaceae was the only family in which multiple genera could be distinguished: *Carex* and *Eriophorum*, which could be specified to species level (*Carex diluta* and *E. angustifolium*). The genus *Carex* was recognized among the macroremains, but only the family level could be resolved from pollen. It is notable that *C. diluta* is the only species identified from all three extraction methods. Most identifications were based on sequences amplified from a single extraction method only.

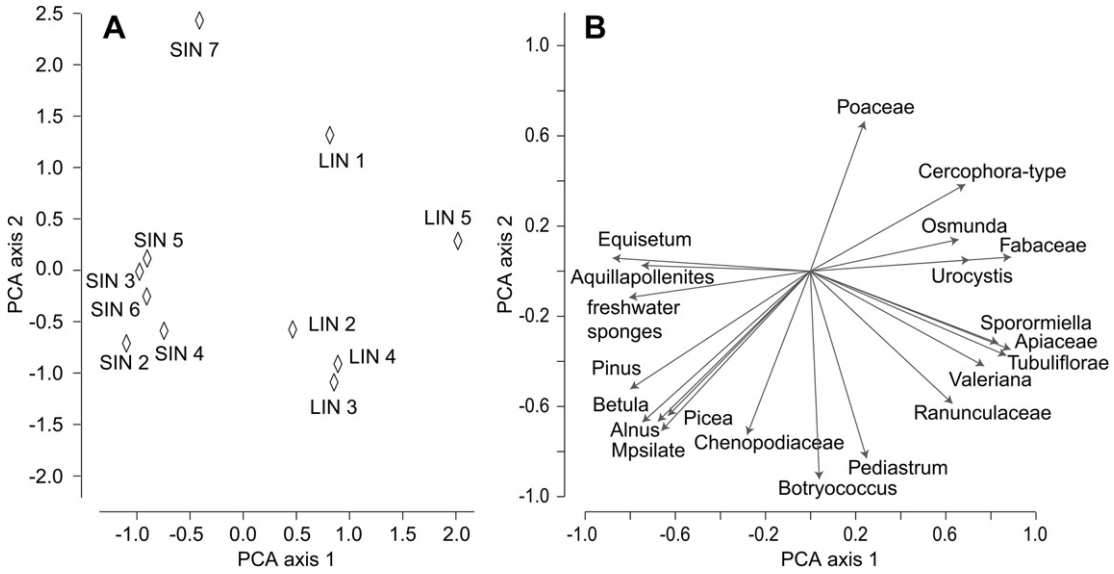

Fig. 5. Ordination diagrams showing the sample scores (A) and the loadings (B) derived from a PCA of the covariance matrix of the log-transformed microfossil data. The first axis explained 33% and the second axis 20% of the variation observed. To avoid clutter, only the highest loadings (>0.6) for axis 1 or axis 2 are shown.

3.3. Mineral components

Sample LIN4 contained vivianite (Fig 4, 51), a hydrated iron phosphate that is whitish gray but turns blue when oxidized. Vivianite frequently occurs where zoological remains, normally low in iron but high in phosphates, are buried in damp sediment that is relatively rich in iron but poor in phosphate. Microbial activity in anaerobic, waterlogged conditions plays an important role in vivianite formation (McGowan and Prangnell, 2006), and Fisher et al. (in press) propose involvement of lactic-acid-producing bacteria in mobilizing phosphate from Lyuba's bones, permitting subsequent vivianite formation. We observed sand-sized grains of quartz and other minerals in some samples (especially SIN7, from the small intestine), but we did not subject these to further analysis. Except for the vivianite noted above, none of these sediment grains are "precipitates" (contra Kosintsev et al., 2010; in press).

4. Discussion and conclusions

4.1. Palaeo-environment

Dominance of Poaceae, Cyperaceae, *Artemisia* and other plants characteristic of open environments (e.g., *Helianthemum* and *Polemonium*) represents the typical profile of the mammoth steppe that occurs so often in northern pollen spectra of the last Ice Age (Guthrie, 1990; 2001). The pollen of arboreal taxa such as *Pinus*, *Picea*, *Betula* and *Alnus* may indicate that these trees formed part of the vegetation, but thermophilous taxa like *Corylus*, *Carpinus* and *Ulmus* suggest long-distance transport or redeposition of Quaternary interglacial or older material (e.g., *Aquilapollenites*!). If this interpretation is correct, then some of the pollen of *Pinus*, *Picea* and *Betula* may also have been transported from far away or may be older, redeposited material.

4.2. Diet

Given Lyuba's age and the lack of occlusal attrition on her teeth (Rountrey et al., in press), her main food source was probably milk (Fig. 1, 2–4), but this immediately raises the question of how to interpret the macroscopic plant remains in her intestine. Fisher et al. (in press) noted the presence of sheared monocot stems and leaves in a size range that suggested mastication by a mature mammoth. We now add to this the sheared and twisted cyperaceous epidermal material, the *Salix* twig without bark, and the bundles of bud-covering hairs of *Salix*, all of which we interpret as indicative of mastication. Again, Lyuba's dentition seems incapable of producing this deformation.

The resolution of this conundrum proposed by Fisher et al. (in press) is that Lyuba must have ingested fecal material from an older mammoth. As in young elephants, this behavior would have helped her build up an intestinal flora of bacteria that would have been useful later, as her normal diet began to include more plant material. In young elephants, this behavior typically involves ingestion of fresh feces (e.g., Leggett, 2004), frequently from the

Table 4
Plant identifications based on ancient DNA amplified and sequenced from intestinal material of Lyuba. The n stands for the number of clones sequenced.

Order	Family	Genus/species	Bootstrap support (%)	Gene (n)		Extraction		
				*trn*L	*rbc*L	CTAB	Silica	DNeasy
Asterales	Asteraceae*	Indet.	100	1	–	–	1	–
Caryophyllales	Polygonaceae	Indet.	100	1	–	–	1	–
Fagales	Betulaceae	Indet.	100	1	–	–	–	1
Malpighiales	Salicaceae	*Salix*	100	6	–	2	–	4
	Linaceae	*Linum*	63	1	–	–	–	1
Poales	Cyperaceae	*Carex diluta*	64	17	–	11	1	5
		Eriophorum angustifolium	62	1	–	1	–	–
		E. sp.	100	2	–	2	–	–
Ranunculales	Ranunculaceae	Indet.	100	–	5	–	–	5

mother, and we suspect that Lyuba would have followed the same pattern.

In contrast to the markedly distorted cyperaceous plant remains in Lyuba's intestine, moss remains in the same samples were extremely well-preserved, showing no evidence of mastication. A plausible hypothesis that would reconcile these differences, and also explain other details of the composition of intestinal samples, is that Lyuba could have ingested feces that had been deposited by an adult mammoth on a moss-covered soil surface. While ingesting fecal material, the mosses could have been unintentionally ingested by Lyuba, without mastication, along with sandy sediment from the substrate. This soil or sediment could have also included the pre-Quaternary pollen taxa and even interglacial arboreal pollen derived from the thermophilous taxa noted above. We propose a second possible agent for introduction of reworked material below, but Lyuba herself is one candidate.

An especially interesting addition to this picture derives from our observation of fruit-bodies of *Sporormiella*-type with spores inside. These fruit-bodies do not form until at least one week of post-defecation exposure (Krug et al., 2004). Thus, if Lyuba ingested fresh dung, as argued above, the fruit-bodies must have already been present within the dung of the older individual – provisionally identified here as Lyuba's mother. In this case, Lyuba's *mother* must have eaten mammoth dung exposed on the landscape for a minimum of one week. Other cases of mammoth coprophagy are now well documented (van Geel et al., 2008, 2011), but they involve animals that, in retrospect at least, were close to death. Lyuba's mother thus presents (indirectly, through Lyuba herself) the first evidence for coprophagy by a mammoth that was not, as far as we know, on the verge of dying. That is, although we do not know Lyuba's mother's fate, there is no reason to think that she was moribund when she displayed coprophagous behavior; indeed, Lyuba's excellent condition suggests that both she and her mother were healthy. Lyuba and her mother thus provide evidence suggesting that coprophagy may have been a normal part of mammoth behavior.

Clementz et al. (2009) and Rountrey (2009) have suggested that coprophagy (implied as routine behavior) may be part of the explanation for unusually enriched $\delta^{15}N$ values observed in mammoths. Mammoths consistently have higher $\delta^{15}N$ than other associated herbivores, including those assumed to have similar digestive physiology and diet such as woolly rhinoceros and horse (Bocherens, 2003). Herbivore dung is enriched in ^{15}N over diet by about 0.5–3‰ (Sponheimer et al., 2003). Thus, consistent inclusion of dung as part of the diet could lead to enriched tissue $\delta^{15}N$. However, the magnitude of the difference in $\delta^{15}N$ between mammoths and other monogastric herbivores is around 2.8‰ (Bocherens, 2003). Explaining such a difference in values by coprophagy alone would require that mammoths almost exclusively consumed dung (assuming similar diet-tissue enrichments for vegetation and dung – to our knowledge, diet-tissue enrichment for a dung diet has not been studied), which seems unlikely. Coprophagy may account for some of the observed enrichment, and this study, along with others (van Geel et al., 2008, 2011), suggests coprophagy was common, but some other factor (e.g., increased nitrogen recycling; Bocherens, 2003) probably contributed as well.

We have considered the possibility that Lyuba herself ingested old dung, and thus directly acquired the fungal fruit-bodies in question rather than "inheriting" them through her mother's dung. However, a bolus of old dung from the landscape would probably have been larger than Lyuba's mouth, and at the season of her death, may or may not have thawed after over-wintering in a frozen state. It may thus have been hard for a mammoth this young to ingest. We cannot rule out this possibility, but we consider it more likely that Lyuba's mother ingested the old dung.

Regardless of who ate old dung, an adult mammoth's trunk-mediated drinking behavior represents a second means by which sediment grains might have entered the "dung stream." In addition, we consider this the most likely means of inclusion of the freshwater sponge spicules, *Daphnia* ephippia, reworked pollen grains, and algae, including diatoms, that Kosintsev et al. (2010, in press) observed and interpreted as material that Lyuba either acquired by drinking or during drowning. As for drinking, Lyuba may not have needed to drink water at all, as she would have been nursing frequently. The possibility that Lyuba acquired these items during drowning is taken up below.

As discussed above, behaviors of both Lyuba and her mother (or another adult) probably account for the materials discovered in Lyuba's intestine. In turn, each individual's contribution probably consists of materials from multiple ingestion events. To help reconstruct such a series of events, we would like to have obtained a well-controlled proximodistal sequence of samples. However, conditions of sampling permitted us only to attribute samples to small or large intestine (distinguished by diameter). Pollen in the samples we obtained must reflect to some extent the diversity and abundance of ingested plants, but the assemblage could be biased by direct ingestion of inflorescences (as suggested by the lump of Poaceae pollen in sample SIN5). In addition, our pollen spectra probably reflect the pollen rain at locations where Lyuba's mother fed. In Yamal today, dry conditions on ridge tops occur in close proximity to wetland vegetation in valleys, on a landscape of rather low relief overall. A similar juxtaposition of disparate habitats probably characterized Pleistocene Yamal. A final source of heterogeneity is that Lyuba's mother's coprophagy could have combined micro- and macrofossils representing one time of year with food materials and pollen rain typical of another time of year – and if she consumed more than one dung bolus during the period just before Lyuba's death, this effect might have been further compounded.

Principal Components Analysis (PCA) of the microfossil record yielded two clusters. One includes small-intestine samples, and the other consists of large-intestine samples, indicating differences in ingested plant remains along the intestinal tract. Each sample is to some degree unique, but the clustering suggests that we could be dealing with at least two distinguishable events of coprophagy by Lyuba, an earlier occasion represented in the large intestine and a later occasion represented in the small intestine. Most samples from the small intestine contained spores of *Equisetum* and some pollen of the pre-Quaternary *Aquilapollenites* (absent in large-intestine samples). Small-intestine samples had relatively low percentages of *Sporormiella* (3.6–9.5%), while samples from the large intestine show *Sporormiella* percentages between 16.0 and 42.8%. Differences between samples from the small and the large intestine could reflect differences in the season of production or location of origin of ingested dung, but given the variety of possible contributions to the dung stream, we cannot reconstruct such events precisely. Observed differences do not, however, require any changes in Lyuba's own behavior, because coprophagy is the only way to account for the amount of masticated vegetation in the small and large intestine.

There were three outliers in the PCA: sample LIN1 (large intestine) contained a relatively high percentage of Cyperaceae and Fabaceae; sample LIN5 (large intestine) also showed a high Fabaceae percentage in combination with abundant Asteraceae tubuliflorae and *Urocystis* spores (for illustrations of spores, see Vanky, 1994; van Geel et al., 2011); and sample SIN7 (small intestine) was dominated by Poaceae pollen and showed relatively high levels of *Sordaria*-type ascospores. We do not have ecological explanations for the different microfossil compositions of the outliers, apart from the variable contingencies of where and when food was consumed.

As proposed above, the extremely well-preserved mosses may represent the site or sites where Lyuba ingested mammoth dung. The ecological preferences of the moss species are quite different, suggesting that Lyuba collected dung from different habitats. *Abietinella abietina* is a calciphile species of dry habitats. *Aulacomnium turgidum* also prefers calcareous habitats (dry to moist organic soils). *Drepanocladus aduncus* is often found submerged or emergent in shallow water. *Sarmentypnum sarmentosum* is often found beside lakes and streams, on rocks and in fens, sometimes submerged. *Drepanocaldus aduncus* occurs in moist to wet (often submerged), ususally calcareous habitats. *Hylocomium splendens* and *Thuidium delicatulum* occur on various substrates.

Kosintsev et al. (2010, in press) report on an investigation that paralleled ours and interpreted the presence of spores of *Sordaria*-type and *Sporormiella*-type in Lyuba's intestinal material as indicative of coprophagy (although they propose another interpretation later). However, in our view, basing such an inference on spores alone is not justified. Any sample from the mammoth steppe may contain such spores (see, e.g., Davis, 1987; van Geel et al., 2007). Wind-dispersed ascospores of coprophilous fungi, some of which have sticky appendages, would have been present on plants that were eaten, and even without direct ingestion (of plants with attached spores), airborne spores can attach to mucus and sputum in the nose and mouth and thus enter the alimentary tract. Only the presence of fruit-bodies of coprophilous fungi in intestinal samples, or within fecal boli, can be treated as compelling fungal evidence of coprophagy (Aptroot and van Geel, 2006; van Geel et al., 2008, 2011).

The analysis of Kosintsev et al. (2010) also differs from ours in interpreting plant remains in Lyuba's intestine as indicative of her season of death, which they interpret as late summer/early autumn. One scenario by which this might work is if the seasonally diagnostic plant remains they recognize – phytoliths and seeds – were eaten by Lyuba's mother and soon after were ingested by Lyuba via coprophagy. However, this position ignores the possibility, discussed above, that Lyuba's mother could have eaten old fecal boli that might indeed have been originally defecated in late summer/autumn, but could have been "recycled" later during the following winter or spring, only reaching Lyuba at a later time. Secondly, because plants in Lyuba's environment would have shown limited, if any, growth from autumn through late spring, Lyuba's mother could have ingested phytoliths and seeds indicative of autumn at any time during the following seasons, giving the observation little power for resolving season of death. Another interpretation Kosintsev et al. (2010) propose is that Lyuba herself, in an early venture into herbivory (without coprophagy), ingested seasonally diagnostic phytoliths and seeds shortly before her death. We regard this as unlikely for a one-month-old mammoth, but it also has the consequence of implying a summer season of birth, which would not take best advantage of the short arctic growing season. Finally, Kosintsev et al. (2010) propose that the diagnostic phytoliths and seeds could have been acquired by Lyuba from "bottom sediments" in the location where she died, either by oral intake associated with drowning or by perimortem uptake via the anus. However, phytoliths and seeds could have had a long residence-time in "bottom sediments" giving this approach even lower resolving power than others. Moreover, the oral route was refuted by Fisher et al. (in press) because it fails to explain the absence of plant material in Lyuba's stomach, where we observed milk-residue, but no significant plant material. The rectal route for this third option is without any precedent known to us and seems especially unlikely as an explanation for presence of such material in the *small* intestine. Even in their most recent statement, Kosintsev et al. (in press) do not offer a clear or compelling defense of any of these options.

The most surprising discovery in Lyuba's intestinal contents was an ulna of vole (*Microtus* sp.; Kosintsev et al., in press). The hair we observed during macrofossil analysis (Fig. 4, 45–46) is of a size consistent with a small mammal such as a vole, but we have not yet confirmed such an identification. We also found another hair (slightly longer, but similar in thickness), during preliminary examination of samples for assessment of evidence of mastication. Unfortunately, there are many scenarios under which a hair from a small mammal (or a single bone) might have have been ingested by Lyuba's mother when feeding on vegetation (and passed to Lyuba through dung) or ingested by Lyuba when eating dung. We therefore refrain from proposing any particular interpretation until these observations are replicated in other cases. The diameter of the hair and its cuticle scale pattern suggest that it is not mammoth hair or contaminant human hair.

4.3. Ancient DNA extractions and barcoding markers used

In this study, three different DNA extraction methods were used. The CTAB method dissolves plant cell membranes and forms complexes with DNA molecules (Rogers and Bendich, 1985). These complexes can be separated from proteins, lipids and other PCR inhibitors by a series of washing steps. With this protocol, we identified four different plant taxa in Lyuba's intestines. The silica-based and QIAGEN Dneasy protocols rely on adsorption of DNA by a silica membrane. In sufficiently salty conditions, silica selectively adsorbs DNA molecules by means of hydrogen bonds, leaving proteins, lipids and other PCR inhibitors in the aquatic phase. Precipitation of silica with ethanol-based washing steps removes these PCR inhibitors (Melzak et al., 1996; Cady et al., 2003). With these different adsorption protocols, three and five plant taxa, respectively, could be identified in Lyuba's intestines. The amount of successful amplifications from all three methods was quite similar, and the methods complemented each other. This shows that it is beneficial to try multiple protocols to obtain more results in ancient DNA studies. Considerably more DNA sequences could be amplified using the *trn*L primers than the *rbc*L primers. The identifications made with both markers did not overlap though. This shows that identifications made using different barcoding markers can complement each other. We therefore recommend use of multiple markers in ancient DNA studies of diet and palaeo-environment, in addition to chemical and paleoecological methods.

5. Conclusions

Lyuba is a remarkable source of data relevant to studies of mammoth behavior and environment. We have shown evidence through analyses of intestinal contents that young mammoth calves, like young elephants, engaged in coprophagy. Furthermore, the presence of fungal fruit-bodies in Lyuba's large intestine suggests either that Lyuba's mother was also coprophagous, or that Lyuba ingested both fresh and old dung. The observation that old dung was ingested means that the state of plant remains in the intestines may not be reflective of the season of death. Furthermore, the assemblages present in the intestines may not represent the typical plant taxa eaten by mammoths during the season in which Lyuba actually died. Given accumulating evidence that mammoths routinely engaged in coprophagy, including ingestion of old dung, we suggest caution when attempting to determine seasonal dietary preferences or season of death from mammoth intestinal contents.

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