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Original Investigation

Comparison of genetic and morphological characters in fossil teeth of grey voles from the Russian Far East (Rodentia: Cricetidae: *Alexandromys*)

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ABSTRACT

Far-Eastern grey voles of the genus *Alexandromys* are a group of 12 species distributed exclusively in the Eastern Palearctic. Morphologically they are hard to be distinguished due to interspecific overlapping of several characters. In recent material of *Alexandromys* morphometric analyses of teeth (in particular the first lower molar) proved to be a good tool for species identification, although there are overlaps in several measurements. In subfossil and fossil material, preliminary analyses indicated considerable variation in tooth morphology and, in comparison to extant species, the presence of additional morphologically ambiguous forms. In the present study we tested in subfossil and fossil teeth of the genus *Alexandromys* from Russian Far East whether taxonomic classification based on morphology is in concordance with species determination based on DNA analysis. We also asked whether the morphologically ambiguous forms could be assigned to described species by means of DNA sequence analysis. The material originated from Medvezhyi Klyk Cave Deposits (Primorskyi Krai, Russia) and had an age of 2000–40,000 years. The results showed that the success rate in PCR amplification was ∼50% and it was possible to obtain PCR products of >400 bp. The concordance between taxonomic assignment based on morphology and genetics was high. Out of 17 samples that were successful in the sequence analysis, only one yielded a classification that contradicts morphology and one specimen could not be classified since the delivered sequence most probably is a numt (nuclear copy of mitochondrial origin). Although only one of the four unidentified teeth in our sample was successful in the DNA analysis and allowed taxonomic assignment, our results suggest that genetic species determination could be a valuable tool to classify subfossil and fossil teeth in Arvicolinae.

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Introduction

Starting already in the 1980s (e.g., Higuchi et al., 1984; Pääbo, 1989) the analysis of ancient DNA (aDNA) has gained more and more importance in addressing evolutionary questions. Meanwhile a plethora of studies on extinct taxa were published, e.g., on ratite birds (e.g., Cooper et al., 1992; Cooper et al., 2001; Mitchell et al., 2014), on mammoths (e.g., Hagelberg et al., 1994; Yang et al., 1996; Debruyne et al., 2003; Krause et al., 2005; Poinar et al., 2006; Rogaev et al., 2006;), or on Neandertals (e.g., Krings et al., 1997; Green et al., 2008;), to name but a few.

Yet, linking genetic data of extant organisms with those of fossil material or even extinct taxa to address phylogenetic, phylogeographic or population genetic questions remains an important challenge for many organism groups. In many cases this will not be feasible in the near future (or possibly never), e.g., taxa that got extinct too long ago. Yet, for several taxonomic groups, there might be a chance, e.g., to test by molecular genetic analyses whether evolutionary hypotheses proposed on fossil specimens are in accordance with the molecular genetic reconstruction of phylogenetic relationships based on extant species. In addition, extremely interesting questions concern how and how fast haplotype diversities

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and distribution change over time. A pioneer study in this respect was performed by Leonard et al. (2000), where mitochondrial (mt) DNA sequence variation was analysed in permafrost-preserved brown bear (*Ursus arctos*) specimens (dated 14,000–42,000 years old). Specimens conserved in permafrost are certainly an optimal source of aDNA which mostly is exceptionally well preserved. For other fossil material (e.g., teeth or bones) not preserved in permafrost, phylogeographic or population genetic studies are so far scarce.

For example, Hadly et al. (2004) used aDNA (maximum radiocarbon dated age 2860 ± 70 years before present (BP)) of *Microtus montanus* and *Thomomys talpoides* to compare two independent estimates of population size (ecological and genetic) and to test the results by serial coalescent simulations. Genetic diversities of extant and ancient populations were compared in the studies of Chan et al. (2005) and Hadly et al. (2003) based on aDNA from subfossil teeth of *Ctenomys sociabilis* dating back to 10,000 years BP. These studies revealed a striking decline in genetic diversity suggesting a population bottleneck that should have occurred before 1000 years BP. In another study, investigations of aDNA from subfossil teeth of *Microtus longicaudus* (up to 2400 years BP) indicated that the genetic patterns were similar to those of modern populations (Spaeth et al., 2009). More recently, Martínková et al. (2013) used recent samples as well as teeth of an age of up to ~4500 years BP of *Microtus arvalis* to trace the colonization of the Orkney archipelago by this species.

To address phylogeographic questions by linking fossil specimens and extant taxa, congruence of taxonomic classification is an important issue. In some taxonomic groups this is not a trivial question, e.g., in voles of the genus *Alexandromys* Ognev, 1914 (Abramson and Lissovsky, 2012) distributed in the Russian Far East. Despite detailed morphological descriptions and identification keys (Golenishchev, 1982; Gromov and Polyakov, 1977) most of the 12 species are still difficult to determine (Pozdniakov, 1993; Sheremetyeva et al., 2009; Voyta and Golenishchev, 2007, 2008; Voyta et al., 2011). Voyta et al. (2013) performed morphometric analyses on variation of first lower molar (m1) of Russian Far-Eastern grey voles to test whether the shape of m1 can be used for correct species identification in the genus *Alexandromys*. The results indicated that the shape of enamel loops of m1 (shape of occlusal surface) can be used for species identification, but not for phylogenetic analyses due to the high variability in some species (*A. maximowiczii*, *A. middendorffii*, *A. oeconomus*; examples provided in Fig. 1). For example, there is a broad confluent dentine field between the anterior cap and triangle 5 in *A. oeconomus* which was found in 97% specimens from extant populations. However this confluence sometimes appears in other species, too. Yet, the study of Voyta et al. (2013) was based on material from extant populations and the question remained whether the same characters are suitable to classify subfossil and fossil teeth. Preliminary morphological analyses on teeth originating from Medvezhyi Klyk Cave Deposits (Primorskyi Krai, Russia; estimated age from ~2,000 to 40,000 years BP) indicated considerable variation in tooth morphology and the presence of additional, morphologically ambiguous forms (Voyta et al., in prep).

In the present pilot study fossil teeth of representatives of *Alexandromys* were analysed to assess whether molecular genetic methods (i.e., aDNA analysis) of these teeth can be used for species assignment. A set of fossil teeth from the Medvezhyi Klyk Cave material was selected and investigated by PCR experiments designed to amplify various sections of the mt control region (CR). The Medvezhyi Klyk Cave Deposits provided huge material allowing to sacrifice a considerable number of teeth for this study. The following questions were addressed: (1) Does the genetic species determination depart from the morphology-based classification? (2) Can the morphologically ambiguous forms be assigned to described species by means of DNA sequence analysis? (3) What is the expected success rate, i.e., can subfossil and fossil teeth be routinely used for DNA analyses? (4) Up to which size can PCR products be expected?

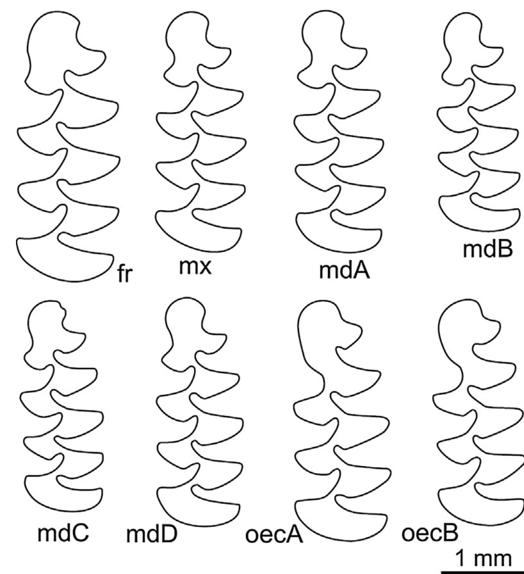

Fig. 1. Typical m1 morphotypes of three *Alexandromys* species as determined by Voyta et al. (2013): fr – *A. fortis*; mx – *A. maximowiczii*; mdA–D – different variants of *A. middendorffii*; oecA–B – different variants of *A. oeconomus*.

Material and methods

Samples

The material investigated in this study comprises subfossil and fossil teeth originating from Medvezhyi Klyk cave deposits (Primorskyi Krai, Russia) which are part of the collections of the Institute of Biology and Soil Science (IBSS; Russian Academy of Sciences, Vladivostok, Russia). The 13 lithological layers recognized in the Medvezhyi Klyk cave comprise a total excavation depth of 5.3 m (Tiunov et al., 2015). Sediments were selected during excavation with a conditional horizon of 5–10 cm. According to the radiocarbon dating of a humerus of a brown bear found in layer 7 (1.08–1.18 m) the estimated age of this layer is 13,790–14,200 years (GIN-13479) (see Tiunov et al. (2015)). Estimates for the other layers range from 11,000–45,000 years BP.

We accept the taxonomy of Abramson and Lissovsky (2012) and use in the following the genus name *Alexandromys* instead of *Microtus. Alexandromys* forms a monophyletic group in the gene tree presented in Bannikova et al. (2010). The following species of Far-Eastern grey voles were identified within the material of the Medvezhyi Klyk cave deposits (Voyta et al. in prep): *A. fortis* (Büchner, 1889), *A. maximowiczii* (Schrenk, 1858), *A. middendorffii* (Poljakov, 1881), *A. mongolicus* (Radde, 1861), and *A. oeconomus* (Pallas, 1776). In addition some morphologically atypical forms could not be unambiguously classified (data not shown): *A.* ex gr. *maximowiczii* (*A.* cf. *maximowiczii* or *A.* cf. *middendorffii* or a still unknown taxon), *A. maximowiczii-middendorffii* (by shape of anteroconid m1 intermediate between the two species), *Alexandromys* indet 1 (teeth too much destroyed, not classified), *Alexandromys* indet 2 (completely atypical, cannot be assigned to any extant species). For the present study a set of 36 molars m1 of grey voles were selected which were analysed genetically in addition to the morphological investigation (Table 1). These specimens comprised: *A. fortis*, *A. maximowiczii*, *A. oeconomus*, and *A.* ex gr. *maximowiczii*, *Alexandromys* indet 2.

Table 1
Measurements (in mm) of subfossil and fossil teeth (m1) of Far-Eastern grey voles from Medvezhyi Klyk Cave Deposits, Sikhote-Alin Mts., Primorskyi Krai, Russia. Landmark distances *a*, *b* and *c* are depicted in Fig. 2. kya = thousand years ago.

Sample Nr.	Species	*a*	*b*	*c*	Lithological layer/Estimated age BP
050-IBSS	*A. maximowiczii*	1.47	1.02	0.81	3–7/2–5 kya
051-IBSS	*A. fortis*	1.72	1.37	1.01	3–7/2–5 kya
052-IBSS	*A. fortis*	1.84	1.41	1.11	3–7/2–5 kya
059-IBSS	*A. maximowiczii*	1.52	1.11	0.86	3–7/2–5 kya
071-IBSS	*A. indet*	1.44	1.05	0.91	3–7/2–5 kya
072-IBSS	*A. fortis*	1.57	1.26	0.98	3–7/2–5 kya
089-IBSS	*A. fortis*	1.70	1.28	1.03	3–7/2–5 kya
091-IBSS	*A. fortis*	1.98	1.49	1.32	3–7/2–5 kya
100-IBSS	*A. maximowiczii*	1.62	1.05	0.92	3–7/2–5 kya
104-IBSS	*A. fortis*	1.79	1.26	1.07	3–7/2–5 kya
130-IBSS	*A. oeconomus*	1.49	1.08	0.70	3–7/2–5 kya
155-IBSS	*A. indet*	1.34	1.01	0.80	6–8/5–10 kya
156-IBSS	*A. indet*	1.19	0.96	0.69	6–8/5–10 kya
158-IBSS	*A. fortis*	1.62	1.34	0.93	6–8/5–10 kya
166-IBSS	*A. fortis*	1.70	1.28	0.96	6–8/5–10 kya
169-IBSS	*A. maximowiczii*	1.33	1.02	0.84	6–8/5–10 kya
170-IBSS	*A. maximowiczii*	1.39	1.08	0.87	6–8/5–10 kya
180-IBSS	*A. fortis*	1.71	1.29	1.01	6–8/5–10 kya
225-IBSS	*A. fortis*	1.62	1.24	1.02	6–8/5–10 kya
226-IBSS	*A. fortis*	1.62	1.23	1.03	6–8/5–10 kya
381-IBSS	*A.* ex gr. *maximowiczii*	1.53	0.90	0.72	6–8/5–10 kya
385-IBSS	*A. maximowiczii*	1.27	1.01	0.84	6–8/5–10 kya
387-IBSS	*A. fortis*	1.34	1.04	0.81	6–8/5–10 kya
395-IBSS	*A. fortis*	1.44	1.06	0.89	6–8/5–10 kya
462-IBSS	*A. maximowiczii*	1.55	1.02	0.91	11/>30 kya
460-IBSS	*A. maximowiczii*	1.47	1.04	0.77	11/>30 kya
491-IBSS	*A. fortis*	1.62	1.36	0.96	11/>30 kya
502-IBSS	*A. fortis*	1.69	1.30	0.93	11/>30 kya
616-IBSS	*A. fortis*	2.06	1.37	1.17	11/>30 kya
613-IBSS	*A. fortis*	1.55	1.21	0.95	11/>30 kya
713-IBSS	*A. fortis*	1.88	1.36	1.12	11/>30 kya
755-IBSS	*A. fortis*	1.75	1.26	0.97	12/>40 kya
800-IBSS	*A. fortis*	1.48	1.18	0.89	12/>40 kya
707-IBSS	*A. maximowiczii*	1.35	0.95	0.78	11/>30 kya
756-IBSS	*A. maximowiczii*	1.41	1.11	0.94	12/>40 kya
799-IBSS	*A. maximowiczii*	1.43	1.07	0.87	12/>40 kya

Morphological investigation of molars (measurements and occlusal surface shape) was carried out as described in Voyta et al. (2013). The occlusal surface of first lower molars (m1) was drawn using a binocular microscope Leica MZ 6. The shape of each occlusal surface was analysed with 19 two-dimensional Cartesian landmarks. The analyses were performed on average values of three repetitions of landmarking. Centroid size, Procrustes coordinates and relative warps were calculated using the software MorphoJ (Klingenberg, 2011). Consensus configurations of each species samples were obtained with the software TPS (tpsSuper by F. James Rohlf, Version 2.00; Rohlf and Slice, 1990). Four linear measurements (landmark distances *a*, *b*, and *c*; see Fig. 2) were determined with the program IMP 6 (Sheets, 2001) and compared to mean values obtained from recent material.

DNA analysis

All DNA extractions were performed in a clean room and obeying all standard routines for working with aDNA, e.g., regularly overnight UV irradiation of the lab, treatment of work surfaces with DNA Exitus (AppliChem, Darmstadt, Germany) and/or 10% sodium hypochlorite, extra UV irradiation of tubes and other equipment such as the zirconium oxide balls used for grinding of the teeth, etc. All post-PCR work was performed in a separate laboratory. For DNA extraction teeth were pulverized with a Retsch MM400 grinding mill for 2 min at 30 Hz using 2 ml Eppendorf Safe-Lock tubes and 10 zirconium oxide balls (2 mm diameter). DNA was extracted with the *Promega* DNA IQ^{TM} System – Tissue and Hair Extraction Kit (*Promega Corporation*, Madison, USA) according to the manufacturer's instructions for DNA extraction from bone. Final elution of DNA solution was in 30 μl. The genetic markers used comprise various sections of different lengths of the mt control region (CR). This highly variable noncoding sequence has been used in our previous studies (Haring et al., 2000; Haring et al., 2011; Sheremetyeva et al., 2009) and allows clear species

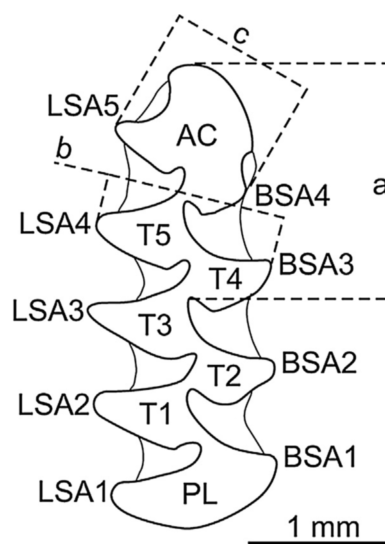

Fig. 2. Terms of morphology of m1 and measurements frame on the tooth outline of *A. fortis* (N. 089–IBSS); "Tn" – triangles of occlusal surfaces; "AC" – anterior cap; "BSAn" – buccal salient angles; "LSAn" – lingual salient angles (terminology of the occlusal surface follows Van der Meulen (1973)); "a" – anteroconid length; "b" – anteroconid width; "c" – AC width; PL – posterior loop.

Table 2
Primers and primer combinations.

Primers	Sequence (5′–3′)	Reference
Pro+	5′-ACCATCAGCACCCAAAGCTG-3′	Haring et al. (2000)
mico2a−	5′-ATACATGCTTATATGCTAGGGG-3′	This study
mico3b−	5′-GGGGTATGAGGATTATAC-3′	This study
mico3c−	5′-GTAAATAGAGCATTAATTAAA-3′	This study
mico15−	5′-ACCAGATGCCTGATAAAGT-3′	This study
mico2b−	5′-TACAATACATGCTTATATGCTA-3′	This study
mico5a+	5′-GGATATTAAGATCAATTATTTA-3′	This study

determination. The variety of tested PCR primers available for this taxonomic group should minimize the potential danger of misinterpretation of results due to suboptimal primer binding. Partial sequences of the mt CR were amplified with different combinations of two forward and five reverse primers (designed for PCR products of different sizes in *Alexandromys* spp.) listed in Table 2. The various PCR products ranged from 123 to 417 bp in length (Table 3). PCR was performed with Amplitaq Gold 360 DNA-Polymerase (together with the buffer provided by the manufacturer) in 25 μl 0.5 μM of each primer, 0.2 mM of each dNTP (Invitrogen, Carlsbad, CA, USA) and with 2 μl DNA solution. If PCR was unsuccessful, varying amounts of template DNA were used in subsequent PCR reactions. Thermal cycling conditions: 94 °C for 3 min; 45 cycles of 94 °C for 30 s, 45–62 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C for 10 min. Negative controls were performed to screen for contaminated reagents: (i) control extractions (without DNA) instead of template; (ii) reaction with A.d. instead of template. PCR products were extracted from agarose gels with the QIAquick Gel Extraction Kit (Qiagen, Inc.) and either sequenced directly or (in case of only weak bands) cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) prior to sequencing. Sequencing (both directions) was performed at LGC Genomics (Berlin, Germany). For direct sequencing the PCR primers were used, while cloned PCR products were sequenced with M13 universal primes.

For taxonomic assignment sequences were compared with our data set of various *Microtus* spp. using the software BioEdit (Hall, 1999) and subjected to BLAST searches in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The CR sequences are suitable to distinguish the species analysed, e.g., even in the 84 bp-sequence (from the smallest 123 bp PCR fragment; primers Pro+/mico2a−) there are four characteristic substitutions differentiating *A. fortis* from *A. maxomiwiczii*. In the highly variable 181 bp-section obtained with primers Pro+/mico3c− (222 bp fragment) there are up to 23 variable positions differentiating *A. fortis* from *A. maxomiwiczii* (Supplementary data; alignment). Sequences obtained in the present study are deposited in GenBank under the accession numbers: KT438867–KT438874. Sequences <200 bp cannot be deposited in GenBank, thus, these sequences can be retrieved from the alignment in the Supplementary data.

Table 3
Primer combinations.

	Fragment length (bp)
Pro+/mico2a−	123
Pro+/mico3b−	247
Pro+/mico2b−	247
Pro+/mico3c−	222
mico5a+/mico15−	233
Pro+/mico2a−	123
Pro+/mico15−	417

Table 4
Measurements (in mm) of m1 of extant Far-Eastern grey voles of the genus *Alexandromys* as determined by Voyta et al. (2013) with material from the collections of the Zoological Institute (St. Petersburg), and of the IBSS (Vladivostok). Mean values, standard error of mean, standard deviation and range of landmark distances *a*, *b*, and *c* (see Fig. 2).

	a	*b*	*c*
A. fortis (n = 55)	1.75 ± 0.02/0.16 1.43–2.13	1.27 ± 0.01/0.09 1.07–1.47	1.04 ± 0.01/0.11 0.83–1.30
A. maximowiczii (n = 55)	1.52 ± 0.014/0.10 1.33–1.79	1.06 ± 0.009/0.07 0.92–1.22	0.86 ± 0.009/0.07 0.61–1.02
A. oeconomus (n = 62)	1.47 ± 0.02/0.17 1.22–2.01	1.13 ± 0.007/0.62 1.00–1.28	0.80 ± 0.008/0.06 0.64–0.97

Results

Morphological analysis

Morphological terms and measurements of m1 are depicted in Fig. 2. Fig. 3 shows the outlines of each specimen while measurements are listed in Table 1 together with the taxonomic assignment based on morphology and age estimates. For most specimens morphological classification was straightforward and most teeth lie within the size limits of the species as established from recent material (Voyta et al., 2013) (Table 4). Three of the specimens were classified as *A. indet*. Two of them (#071, #155) have an atypical asymmetric anterior cap (AC) of the anteroconid with a disproportionately developed antero-lingual part, the lingual insure of AC being very shallow. The third one (#156) displays a lingual part of AC similar to *A. oeconomus*, but a very complicated buccal side (not found in *A. oeconomus*). These variants were not found so far in extant populations. One specimen (#130) assigned to *A. oeconomus* had an atypical shape found very rarely (<1%; Voyta et al., unpubl.) in extant natural populations. In some cases, where the shape of occlusal surfaces of m1 could not be assigned clearly to either *A. fortis* or *A. maximowiczii* (Pozdniakov, 1993; Voyta et al., 2013), classification was mainly based on size; Teeth of *A. maximowiczii* are on average smaller (e.g., #462). One specimen (#381) with an atypical shape of anteroconid shows at best weak similarity to *A. maximowiczii*, but could even belong to a so far unknown (extinct?) species. It was classified as *A.* ex gr. *maximowiczii*. To summarize, the material analysed morphologically in the present study were assigned to the following species/morphological forms: *A. fortis* (20 specimens), *A. maximowiczii* (11), *A. oeconomus* (1), *A.* ex gr. *maximowiczii* (1), and *A. indet* (3) (see Table 5).

Genetic analysis

Results of the genetic analysis are summarized in Table 5. From 20 out of the 36 *Alexandromys* spp. samples we yielded PCR products of the expected sizes (using various primer combinations; Table 3). In the positive samples one to four different PCR products were obtained using the various primer combinations. Repetition of the same PCR provided usually the same results, i.e., the same individuals yielded repeatedly positive or negative results, respectively. Nevertheless, Table 5 indicates that PCR success is to some extent stochastic and there was not a single specimen yielding positive results with all primer combinations. Out of the positive samples, only one (#616) allowed amplification of the longest section of 417 bp. From 17 samples at least one PCR product could be sequenced (Table 5), while three individuals (#71, #130, #800) allowed occasionally amplification of PCR products of very low amounts visible only as faint bands in the agarose gel, which failed in the sequencing reaction. Repeated trials to amplify (or re-amplify) further PCR products in those three samples failed until the DNA was used up.

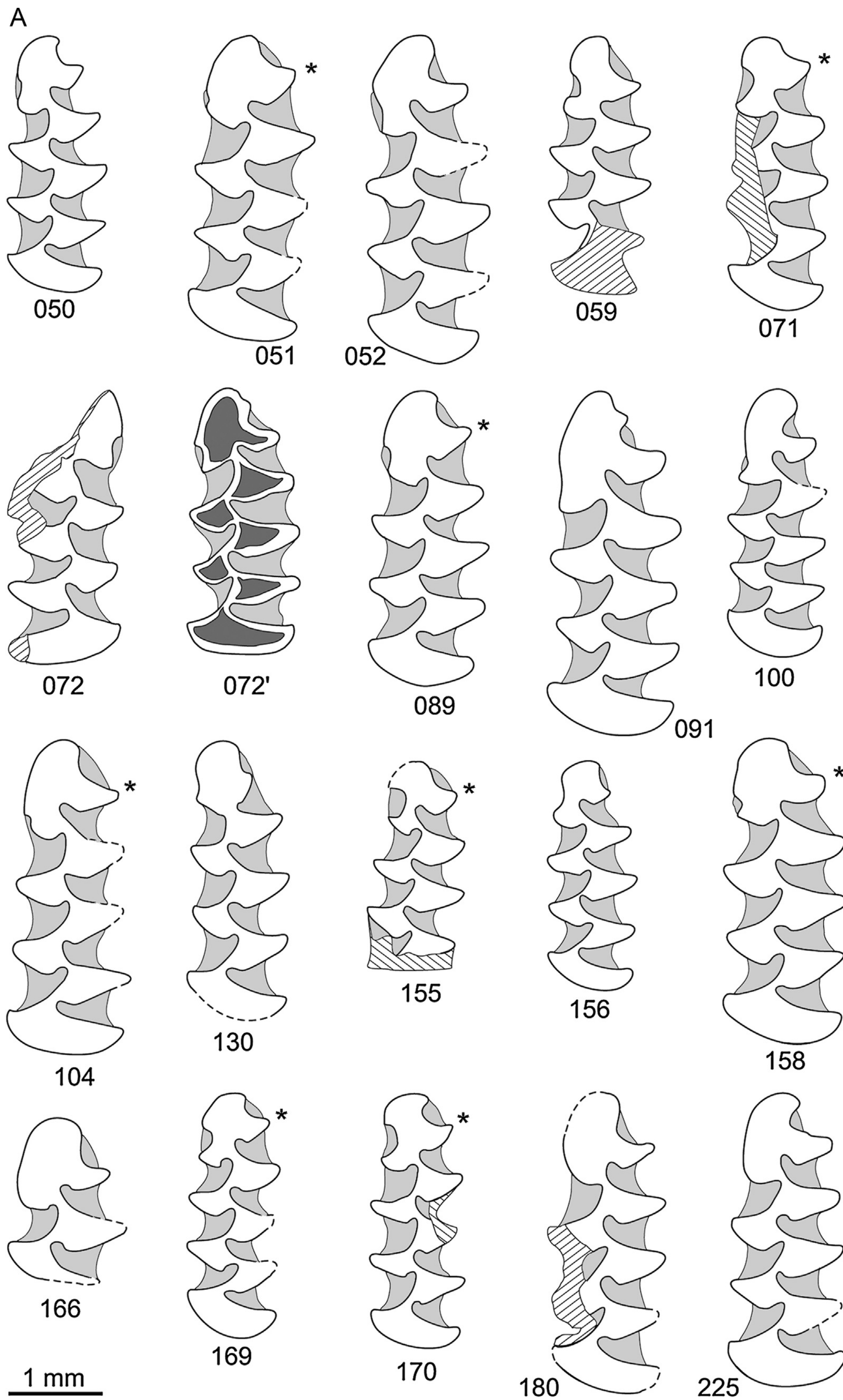

Fig. 3. (A) Outlines of specimens 050–225. Teeth are presented in occlusal and right view. Teeth that have been photographed from the left side are mirrored and are indicated by asterisks. Specimen #072 was broken on the occlusal side (no anteroconid shape present), therefore, it is depicted from the root side in addition (072′). (B) Outlines of specimens #226–800. Teeth are presented in occlusal and right view. Teeth that have been photographed from the left side are mirrored and are indicated by asterisks.

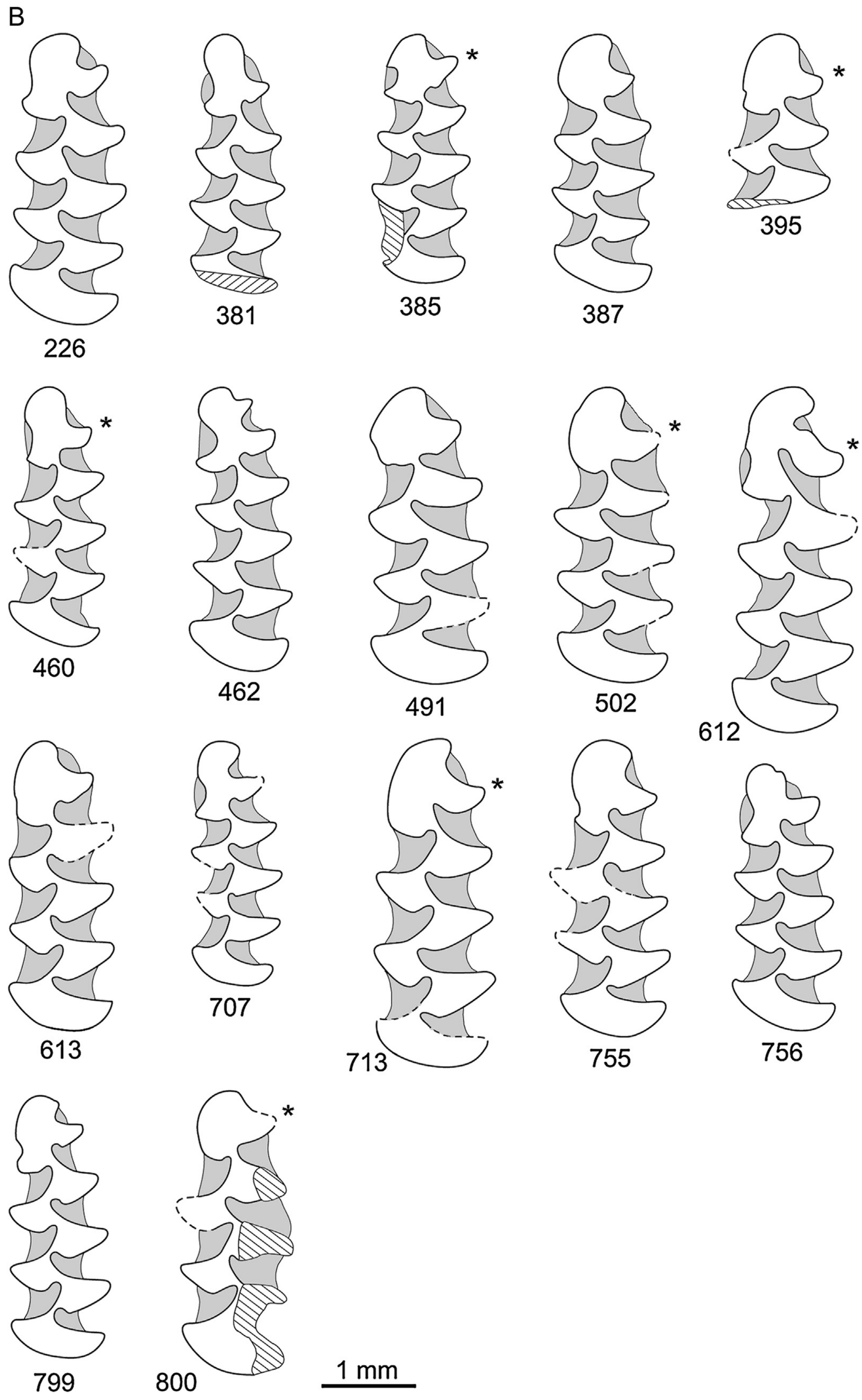

Fig. 3. (*Continued*).

Table 5
Results of PCR experiments. Primer combinations and expected fragment sizes are indicated. Empty cells indicate unsuccessful PCR amplification. "PCR" = successful PCR amplification without sequencing; "Seq" = PCR product sequenced; "Seq-c" = PCR product cloned and sequenced. kya = thousand years ago.

Nr.	Morphological classification	Age estimate BP (kya)	Genetic classification	Pro+/2a– 123 bp	Pro+/mico3c– 222 bp	mico5a+/mico15– 233 bp	Pro+/mico15– 417 bp	Pro+/mico3b– 247 bp	Pro+/mico2b– 247 bp
051	*A. fortis*	2–5	*A. fortis*	PCR	Seq	Seq		PCR	
052	*A. fortis*	2–5	*A. fortis*	PCR	Seq	Seq			
072	*A. fortis*	2–5	*A. fortis*	PCR	Seq	Seq			
089	*A. fortis*	2–5	*A. fortis*	PCR	Seq	PCR			PCR
091	*A. fortis*	2–5	*A. fortis*	Seq-c					PCR
104	*A. fortis*	2–5	*A. fortis*	Seq-c					PCR
158	*A. fortis*	5–10							
166	*A. fortis*	5–10							
180	*A. fortis*	5–10	*A. fortis*	Seq-c					
225	*A. fortis*	5–10	*A. fortis*	Seq-c					
226	*A. fortis*	5–10	*A. maximowiczii*	Seq-c					
387	*A. fortis*	5–10							
395	*A. fortis*	5–10							
491	*A. fortis*	>30	*A. fortis*	Seq-c					
502	*A. fortis*	>30	*Apodemus*	Seq-c					
613	*A. fortis*	>30	*A. fortis*		Seq	Seq			
616	*A. fortis*	>30	*A. fortis*		PCR	PCR	Seq	PCR	
713	*A. fortis*	>30	*A. fortis*	PCR	Seq-c	Seq-c			
755	*A. fortis*	>40	*A. fortis*	PCR	Seq-c	Seq-c			
800	*A. fortis*	>40		PCR					
050	*A. maximowiczii*	2–5							
059	*A. maximowiczii*	2–5							
100	*A. maximowiczii*	2–5		PCR					
169	*A. maximowiczii*	5–10							
170	*A. maximowiczii*	5–10	*A. maximowiczii*	Seq-c					
385	*A. maximowiczii*	5–10							
460	*A. maximowiczii*	>30							
462	*A. maximowiczii*	>30							
707	*A. maximowiczii*	>30							
756	*A. maximowiczii*	>40							
799	*A. maximowiczii*	>40							
130	*A. oeconomus*	2–5		PCR					
381	*A.* ex gr. *maximowiczii*	5–10							
071	*A. indet*	2–5			PCR				
155	*A. indet*	5–10	*A. maximowiczii*	Seq-c	PCR	Seq		Seq	
156	*A. indet*	5–10							

Up to three PCR products of each positive specimen were sequenced (Table 5). From six samples we could retrieve a section covering ~417 bp by combining the sequences of PCR products yielded with the two primer pairs Pro+/mico3c– and mico 5a+/mico15– (with a 4 bp sequence that remained undetermined due to primer overlap). From one individual (#155) a 373 bp-section was obtained by combining the fragments amplified with the primers Pro+/mico3b– and 5a+/mico15–. From one sample (#089) only the first of these two sections (222 bp fragment, sequence: 161 bp) could be determined. From eight samples we could amplify the smallest fragment only (126 bp; the information in this short section is very limited but allows species assignment).

Taxonomic classification and sequence diversity

Sequences were compared with sequences derived from our phylogenetic study (Haring et al., 2011) as well as compared to sequences in GenBank by BLAST search. The alignment in Supplement 1 shows the sequences together with some reference sequences. Sequences obtained from one individual always provided consistent species assignment. Reproducibility of species assignment was high: in eight out of 17 specimens more than one sequence could be determined yielding the same assignment in all cases.

Among the six *M. fortis* samples of which the 123 bp fragment was sequenced, one sample (#91) differed by one substitution from the *M. fortis* reference sequence and one (#225) by two substitutions. These three substitutions were not found in any *M. fortis* as presently available in GenBank. Among seven *M. fortis* fragments of 222 bp the sequences differ by altogether five single substitutions (three of them C/T ambiguities) as well as a dinucleotide difference which was found in four individuals (TA vs. CG). Both variants (TA vs. CG; positions 174, 175 in the alignment in the Supplementary Material) are found in extant populations too, while the single base substitutions were not found in any published sequence so far. Among the eight variable sites found in the six *A. fortis* sequences of the 233 bp fragment, five are found in published sequences from extant specimens.

Finally, the sequence of the 417 bp fragment obtained from sample #616 differed by two substitutions from the consensus sequence of published *A. fortis* and both substitutions are found in extant individuals, too.

Concerning *A. maximowiczii*, the sequence obtained from sample #155 (247 bp-fragment) has one substitution not found in any extant sample of *A. maximowiczii*, but in several samples of other taxa (*A. gromovi*, *A. oeconomus*, *A. sachalinensis*). The other seven positions differing from the *A. maximowiczii* reference sequence are substitutions frequently found in various combinations in extant samples of *A. maximowiczii*. Comparing the two sequences of sample #155 covering the 5′ part of the whole section analysed (obtained with Pro+/mico2a– and Pro+/mico3b–), there is one difference. This substitution is found in the Pro+/mico2a– sequence which was obtained by cloning and, therefore, probably a PCR error (see Discussion). The sequence of the 123 bp fragment of sample #170 is identical to the reference sequence. Details of sequence variation can be found in the Supplementary alignment file.

To summarize, in 15 out of 17 samples the taxonomic determination based on morphology was in accordance with the

mitochondrial haplotype. Sample #226 (morphologically assigned to *A. maximowiczii*) yielded a sequence of *A. fortis*. Furthermore, sample #502 yielded a sequence that proved to be most similar to *Apodemus* sp. in the BLAST search instead of the expected species *A. fortis*.

Discussion

Genetic species determination

This pilot study gives evidence that subfossil and fossil teeth are promising material for aDNA analyses, although there is a considerable dropout rate. The main results of the investigation are: (1) The success rate in PCR amplification of the material investigated is acceptable: 56% including the samples from which only PCR products could be obtained; 47% if only samples that were successfully sequenced are considered. (2) While it was even possible to obtain PCR products of >400 bp, in 11 cases only the smallest fragment of 123 bp could be obtained. (3) The concordance between taxonomic assignment based on morphology and genetics is high. (4) Species assignment by DNA analysis was highly reproducible.

Concordance between genetic and morphological assignment

Our results show that taxonomic classification is feasible even with aDNA from material dated at >40,000 years BP and proved to be concordant with morphology-based classification. Yet, DNA-based classification appears as a good complementary tool especially in those cases where morphological differentiation is ambiguous or fails. Especially among Far-Eastern grey voles the species pairs *A. maximowiczii* vs. *A. fortis* and, *A. maximowiczii* vs. *A. middendorffii* (Voyta et al., 2013) often display overlapping morphotypes of m1. Thus, the assumption that the latter one is present in the studied cave deposits was doubtful. The genetic analysis does not provide any hint for the occurrence of *A. middendorffii*. Unfortunately, out of those four specimens that could not be classified unambiguously by their morphological features, only one was genetically determined (#155: *A. maximowiczii*). Given the fact that the sample size of this pilot study was quite small final conclusions on presence or absence of taxa in the deposits would be premature.

In only two out of 17 samples the genetic classification departed from the morphology-based assignment. Concerning specimen #226 (morphologically assigned to *A. maximowiczii*) its genetic classification as *A. fortis* could be explained in two ways: (1) Genetics wrong: Although all precautions were taken to avoid contamination and all negative controls were ok, contamination can never be ruled out. (2) Morphology wrong: An aberrant large form of m1 resembling in shape *A. maximowiczii*. Unfortunately, a re-sampling of the same tooth is not possible with the method applied. Therefore, it is necessary to test and establish more preservative DNA extraction methods allowing to keep as much material of the teeth intact (see below).

The second case of discordance (#502) is most probably due to accidental amplification of a "numt" (nuclear copy of a mt sequence that has been transferred to the nuclear (nc) genome; Lopez et al. (1994); for review see e.g., in Hazkani-Covo et al. (2010)). One can be sure that the DNA based assignment (genus *Apodemus*) is wrong as the hypsodont teeth of voles cannot be confused with the brachyodont teeth of murids. The nc genomes of most species contain numts of many mt genes. Although these nc copies are not functional, they change slowly over time in comparison to the mt genes as the evolutionary rate in the nc genome is much slower due to the fact that DNA replication in the nc genome is more accurate. Therefore, (1) the DNA sequences of mt and nc paralogs diverge over time and (2), in an interspecific comparison between a numt in one species and the corresponding mt gene in another species, a much lower distance is expected than in an orthologous comparison of the functional mt genes. The fact that the sequence we have amplified in this sample is at minimum 5% different from all *Apodemus* sequences in GenBank (and no identical or more similar sequences were found), can be considered as a strong indication that it might be derived from a numt and not from a contamination.

Limitations and concluding remarks

In the present study we could show that subfossil and fossil teeth of up to 40,000 years age allowed PCR amplification of fragments up to 417 bp. Our results based on the genus *Alexandromys* serve as an example for a general applicability of DNA analyses of subfossil and fossil teeth. We found good concordance between taxonomic assignment based on morphology and genetics. Thus, genetic species determination can be a valuable tool to classify problematic fossil specimens with ambiguous morphology. Such investigations could contribute to phylogenetic studies and might be even used for population genetic issues. The success rate in PCR amplification is around 50%, calling for further methodological improvement, such as establishment of preservative DNA extraction methods and primer optimization. As a matter of fact, the shortness of obtainable sequences puts severe limitations on the possibilities of bioinformatic analyses. However, in most cases the sequence information will allow assignment to genetic lineages detected in extant taxa (and established with fresh tissue) as well as detection of new haplotypes or haplogroups. In any case, much effort is needed to obtain longer sequences to obtain more specific assignment (e.g., to certain clades, haplogroups or subspecies). For this task it is crucial that primers are conserved enough to bind in all possible taxa (i.e., that they bind in well conserved regions) and – on the other hand – that they amplify variable sections. A potential problem when dealing with short sequences that are combined to longer ones is that the overlap must be long enough to ensure critical evaluation and to exclude the possibility that some of the sequences are derived from contaminations. In addition, haplotype occurrence or the disappearance of haplotypes over time might add important information to our knowledge of Pleistocene distribution ranges (as e.g., in Martínková et al. (2013)).

A critical point is to evaluate whether the observed sequence variation is partly due to PCR errors and how to distinguish real substitutions from artefacts. In the present study there is one substitution that most reasonably can be interpreted as such an artificial difference, while many other substitutions were either confirmed by overlapping fragments or involve positions that are variable in extant populations.

The procedure applied in the present analysis is clearly suboptimal under a conservatory aspect. The destructive method of DNA extraction was used here, because the main question was whether the DNA in the samples is of sufficient quality at all. Therefore, we decided to sacrifice complete teeth. Yet for future analyses, the next step will be to test whether more preservative DNA extraction methods provide equally successful results for such old samples. Whether the "non-invasive" method of complete demineralization described by Hasan et al. (2014) could be adapted for such investigations remains to be tested.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mambio.2015.08.001.

References

Abramson, N., Lissovsky, A.A., 2012. Subfamily Arvicolinae. In: Pavlinov, I.Y., Lissovsky, A.A. (Eds.), The Mammals of Russia: A Taxonomic and Geographic Reference. Archive of Zoological Museum of MSU. KMK Scientific Press, Moscow, pp. 220–276.

Bannikova, A.A., Lebedev, V.S., Lissovsky, A.A., Matrosova, V., Abramson, N.I., Obolenskaya, E.V., Tesakov, A.S., 2010. Molecular phylogeny and evolution of the Asian lineage of vole genus *Microtus* (Rodentia: Arvicolinae) inferred from mitochondrial cytochrome b sequence. Biol. J. Linn. Soc. 99, 595–613.

Chan, Y.L., Lacey, E.A., Pearson, O.P., Hadly, E.A., 2005. Ancient DNA reveals Holocene loss of genetic diversity in a South American rodent. Biol. Lett. 1, 423–426.

Cooper, A., Lalueza-Fox, C., Anderson, S., Rambaut, A., Austin, J., Ward, R., 2001. Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. Nature 409, 704–707.

Cooper, A., Mourer-Chauviré, C., Chambers, G.K., von Haeseler, A., Wilson, A.C., Pääbo, S., 1992. Independent origins of New Zealand moas and kiwis. Proc. Natl. Acad. Sci. 89, 8741–8744.

Debruyne, R., Barriel, V., Tassy, P., 2003. Mitochondrial cytochrome *b* of the Lyakhov mammoth (Proboscidea, Mammalia): new data and phylogenetic analyses of Elephantidae. Mol. Phylogenet. Evol. 26, 421–434.

Golenishchev, F.N., (Ph.D. Thesis) 1982. Grey voles (subgenus *Microtus*, species-group "maximowiczii") of East Siberia and Far East. Zoologicheskii Institut AN SSSR, pp. 23.

Green, R.E., Malaspinas, A.-S., Krause, J., Briggs, A.W., Johnson, P.L., Uhler, C., Meyer, M., Good, J.M., Maricic, T., Stenzel, U., 2008. A complete Neandertal mitochondrial genome sequence determined by high-throughput sequencing. Cell 134, 416–426.

Gromov, I., Polyakov, I.Y., 1977. Voles (Microtinae), Fauna of the USSR. Mammals 3.

Hadly, E.A., Ramakrishnan, U., Chan, Y.L., Van Tuinen, M., O'Keefe, K., Spaeth, P.A., Conroy, C.J., 2004. Genetic response to climatic change: insights from ancient DNA and phylochronology. PLoS Biol. 2, e290.

Hadly, E.A., van Tuinen, M., Chan, Y., Heiman, K., 2003. Ancient DNA evidence of prolonged population persistence with negligible genetic diversity in an endemic tuco-tuco (*Ctenomys sociabilis*). J. Mammal. 84, 403–417.

Hagelberg, E., Thomas, M.G., Cook, C.E.J., Sher, A.V., Baryshnikov, G.F., Lister, A.M., 1994. DNA from ancient mammoth bones. Nature 370, 333–334.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Haring, E., Herzig-Straschil, B., Spitzenberger, F., 2000. Phylogenetic analysis of Alpine voles of the *Microtus* multiplex complex using the mitochondrial control region. J. Zool. Syst. Evol. Res. 38, 231–238.

Haring, E., Sheremetyeva, I.N., Kryukov, A.P., 2011. Phylogeny of Palearctic vole species (genus *Microtus*, Rodentia) based on mitochondrial sequences. Mamm. Biol. 76, 258–267.

Hasan, M.M., Hossain, T., Majumder, A.K., Momtaz, P., Sharmin, T., Sufian, A., Akhteruzzaman, S., 2014. An efficient DNA extraction method from bone and tooth samples by complete demineralization followed by the use of silica-based columns. Dhaka Univ. J. Biol. Sci. 23, 101–107.

Hazkani-Covo, E., Zeller, R.M., Martin, W., 2010. Molecular poltergeists: mitochondrial DNA copies (numts) in sequenced nuclear genomes. PLoS Genet. 6, e1000834.

Higuchi, R., Bowman, B., Freiberger, M., Ryder, O.A., Wilson, A.C., 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature 312, 282–284.

Klingenberg, C.P., 2011. MorphoJ: an integrated software package for geometric morphometrics. Mol. Ecol. Resour. 11, 353–357.

Krause, J., Dear, P.H., Pollack, J.L., Slatkin, M., Spriggs, H., Barnes, I., Lister, A.M., Ebersberger, I., Pääbo, S., Hofreiter, M., 2005. Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. Nature 439, 724–727.

Krings, M., Stone, A., Schmitz, R.W., Krainitzki, H., Stoneking, M., Pääbo, S., 1997. Neandertal DNA sequences and the origin of modern humans. Cell 90, 19–30.

Leonard, J.A., Wayne, R.K., Cooper, A., 2000. Population genetics of Ice Age brown bears. Proc. Natl. Acad. Sci. 97, 1651–1654.

Lopez, J.V., Yuhki, N., Masuda, R., Modi, W., O'Brien, S.J., 1994. Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. J. Mol. Evol. 39, 174–190.

Martínková, N., Barnett, R., Cucchi, T., Struchen, R., Pascal, M., Pascal, M., Fischer, M.C., Higham, T., Brace, S., Ho, S.Y., Quéré, J.-P., O'Higgins, P., Excoffier, L., Heckel, G., Hoelzel, A.R., Dobney, K.M., Searle, J.B., 2013. Divergent evolutionary processes associated with colonization of offshore islands. Mol. Ecol. 22, 5205–5220.

Mitchell, K.J., Llamas, B., Soubrier, J., Rawlence, N.J., Worthy, T.H., Wood, J., Lee, M.S., Cooper, A., 2014. Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution. Science 344, 898–900.

Pääbo, S., 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc. Natl. Acad. Sci. U.S.A. 86, 1939–1943.

Poinar, H.N., Schwarz, C., Qi, J., Shapiro, B., MacPhee, R.D.E., Buigues, B., Tikhonov, A., Huson, D.H., Tomsho, L.P., Auch, A., Rampp, M., Miller, W., Schuster, S.C., 2006. Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. Science 311, 392–394.

Pozdniakov, A.A., 1993. Morphotypical variation of molar teeth occlusal surface of the grey voles of species-group maximowiczii (Rodentia, Cricetidae): an experience of quantitative statistic analysis. Zool. Zh. 72, 114–125.

Rohlf, F.G., Slice, D.E., 1990. Extension of the Procrustes method for the optimal superimposition of landmarks. Syst. Zool. 39, 40–59.

Rogaev, E.I., Moliaka, Y.K., Malyarchuk, B.A., Kondrashov, F.A., Derenko, M.V., Chumakov, I., Grigorenko, A.P., 2006. Complete mitochondrial genome and phylogeny of Pleistocene mammoth *Mammuthus primigenius*. PLoS Biol. 4, e73.

Sheets, H.D., 2001. IMP Software Series. Canisius College, Buffalo, Available from: http://www.canisius.edu/~sheets/morphsoft.html (downloaded on 13 April 2015).

Sheremetyeva, I.N., Kartavtseva, I.V., Voyta, L.L., Kryukov, A.P., Haring, E., 2009. Morphometric analysis of intraspecific variation in *Microtus maximowiczii* (Rodentia, Cricetidae) in relation to chromosomal differentiation with reinstatement of *Microtus gromovi* Vorontsov, Boeskorov, Lyapunova et Revin, 1988, stat. nov.*. J. Zool. Syst. Evol. Res. 47, 42–48.

Spaeth, P.A., Van Tuinen, M., Chan, Y.L., Terca, D., Hadly, E.A., 2009. Phylogeography of *Microtus longicaudus* in the tectonically and glacially dynamic central Rocky Mountains. J. Mammal. 90, 571–584.

Tiunov, M.P., Golenishchev, F.N., Voyta, L.L., 2015. The first finding of Mimomys in the Russian Far East. Acta Palaeontol. Pol., http://dx.doi.org/10.4202/app.00082.2014.

Van der Meulen, A.J., 1973. Middle Pleistocene smaller mammals from the Monte Peglia (Orvieto, Italy), with special reference to the phylogeny of *Microtus* (Arvicolidae, Rodentia). Quaternaria 17, 1–144.

Voyta, L.L., Golenishchev, F.N., 2007. Geometric morphometry of molars in three species of the common field vole, the subgenus *Alexandromys* (Rodentia: Cricetidae: Microtus). In: Rozhnov, V.V., Tembotova, F.A. (Eds.), Mammals of Mountain Territories. KMK Scientific Press, Moscow, pp. 79–84.

Voyta, L.L., Golenishchev, F.N., 2008. Analysis of variation of the molars in gray voles *Microtus* Schrank, 1798 (Rodentia: Cricetidae: Arvicolinae) using methods of geometric morphometrics. In: 11th International Conference "Rodens et Spatium" on Rodent Biology, Moscow. KMK Sci Press, Myshkin, Russia, pp. 149–150.

Voyta, L.L., Golenishchev, F.N., Tiunov, M.P., 2013. Analysis of shape and size variation of the first lower molar in the Far-Eastern grey voles of genus *Alexandromys* (Rodentia: Cricetidae) from Russian fauna using geometric morphometrics. Russ. J. Theriol. 12, 19–32.

Voyta, L.L., Golenishchev, F.N., Tiunov, M.P., Panasenko, V.E., 2011. Some restrictions of the morphometric methods as applied to classification of fossil remains *Sorex* (Eulipotyphla: Soricidae) and *Microtus* (Rodentia: Cricetida). In: VIth European Congress of Mammalogy, Paris. Universite Pierre et Marie Curie, Paris, France, pp. 19–32.

Yang, H., Golenberg, E.M., Shoshani, J., 1996. Phylogenetic resolution within the Elephantidae using fossil DNA sequence from the American mastodon (*Mammut americanum*) as an outgroup. Proc. Natl. Acad. Sci. U.S.A. 93, 1190–1194.