

Morphometric and genetic analyses of diversity of the Lena horse (*Equus lenensis* Russanov, 1968; Mammalia: Equidae)

Natalia N. Spasskaya*, Igor Ya. Pavlinov, Fedor S. Sharko,
Eugenia S. Boulygina, Svetlana V. Tsygankova, Artem V. Nedoluzhko,
Gennady G. Boeskorov, Evgeny N. Mashchenko

ABSTRACT. A pilot study of *E. lenensis* was carried out based on a small sample using a) standard morphometrics of the axial skull, mandible, the upper and lower cheek teeth; b) geometric morphometrics of the enamel crown patterns of the 1st upper and lower molars; c) molecular phylogenetic analysis with the complete mitochondrial genome sequencing. A certain morphological heterogeneity of *E. lenensis* by mostly dental and partly cranial characters was revealed. The study shows a necessity to reconsider species allocation of some records of Pleistocene horses in North East Siberia, and to carry out large-scale comprehensive revision of these materials using new approaches.

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KEY WORDS: *Equus lenensis*, Pleistocene, North East Eurasia, morphology, geometric morphometrics.

Natalia N. Spasskaya [equusnns@mail.ru], I.Ya. Pavlinov [ipvl2008@mail.ru] Zoological Museum of Lomonosov Moscow State University, Bolshaya Nikitskaya Str. 6, 125009 Moscow, Russia; Fedor S. Sharko [fedosic@gmail.com] Institute of Bioengineering, Research Center of Biotechnology of the Russian Academy of Sciences, 119071 Moscow, Russia; National Research Centre “Kurchatov Institute”, Akademika Kurchatova Sq. 1, 123098 Moscow, Russia; Eugenia S. Boulygina, Svetlana V. Tsygankova, National Research Centre “Kurchatov Institute”, Akademika Kurchatova Sq. 1, 123098 Moscow, Russia; Artem V. Nedoluzhko [nedoluzhko@gmail.com], Faculty of Biosciences and Aquaculture, Nord University, 8049 Bodø, Norway; Gennady G. Boeskorov [gboeskorov@mail.ru], Academy of Sciences of Sakha (Yakutia) Republic, Prospect Lenina 33, 677007 Yakutsk, Russia; Evgeny N. Mashchenko [evmash@mail.ru], Borissiak Paleontological Institute Russian Academy of Sciences, Profsojuznaya St., 123, 117647 Moscow, Russia; Academy of Sciences of Sakha (Yakutia) Republic, Prospect Lenina 33, 677007 Yakutsk, Russia.

Морфометрический и генетический анализ изменчивости ленской лошади (*Equus lenensis* Russanov, 1968; Mammalia: Equidae)

Н.Н. Спасская*, И.Я. Павлинов, Ф.С. Шарко, Е.С. Булыгина,
С.В. Цыганкова, А.В. Недолужко, Г.Г. Боекоров, Е.Н. Мащенко

РЕЗЮМЕ. Пилотное (на небольшой выборке) исследование *E. lenensis* проведено с применением а) стандартной морфометрии осевого черепа, нижней челюсти верхних и нижних щёчных зубов; б) геометрической морфометрии структуры зубной коронки 1-го верхнего и нижнего коренных; в) молекулярно-филогенетического анализа на основе секвенирования полного митохондриального генома. Выявлена определённая морфологическая разнородность *E. lenensis* по пропорциям черепа и зубов и по структуры зубной коронки коренных. Исследование показало необходимость пересмотра видовой принадлежности некоторых находок плейстоценовых лошадей Северо-Восточной Сибири и проведения широкомасштабной комплексной ревизии этих материалов с использованием новых подходов.

КЛЮЧЕВЫЕ СЛОВА: *Equus lenensis*, плейстоцен, Северо-Восточная Евразия, морфология, геометрическая морфометрия.

* Corresponding author

Introduction

During the Pleistocene and the beginning of the Holocene, the horses (genus *Equus*) were among most abundant members of the megafauna in North East Eurasia; currently, they account for about 27 percent of all the remains of large mammals that were excavated and/or thawed from permafrost (Lazarev, 2008). Therefore, detailed exploration of their taxonomic diversity based on diversity of data and contemporary methodical approaches is essential for understanding temporal dynamics of faunal complexes of that region. However, information on their species diversity and geographical distribution is still very incomplete. Several forms were described (Rusanov, 1968), which were recognized subsequently as distinct species (Lazarev, 1980) supposedly having been replacing each other in time: the Early Pleistocene *E. coliemensis* Lazarev, 1980 and *E. nordostensis* Rusanov, 1968, the Middle Pleistocene *E. orientalis* Rusanov, 1968, and Late Pleistocene *E. lenensis* Rusanov, 1968). The ranges of rather local *E. coliemensis* and *E. nordostensis* partially overlapped, and *E. orientalis* and *E. lenensis* seemed to be distributed much wider than their predecessors (Lazarev, 1980).

Unfortunately, a definitive analysis of taxonomic status of the above species and their distribution in both time and space is quite problematic because their diagnostic characters are not discrete, and geological ages of their type specimens were not identified precisely in most cases, save for *E. lenensis* (Belolyubsky *et al.*, 2008). In the species delimitation of the Pleistocene horses in North East Siberia, the experts were usually guided by the general stratigraphy of the respective localities and, to a large extent, by general size of the horses decreasing in the order of *E. nordostensis* — *E. coliemensis* — *E. orientalis* — *E. lenensis*. At present time, all remains of the fossil horses from the Late Pleistocene and the Early Holocene of North East Siberia are usually classified as *E. lenensis*. However, a preliminary visual examination of the materials (primarily skulls) indicated their apparent morphological heterogeneity. This provided a certain reason to make the following assumptions.

From the one hand, a geographically widespread and long existing species, currently identified as *E. lenensis*, was either extremely variable geographically or actually represented a flock of several close allospecies. From the other hand, several horse species could occur sympatrically in the region under consideration during the Pleistocene. This supposition presumes that the Pleistocene species of horses of North East Siberia could be distributed much wider both in time span and geographical distribution than it is currently believed. With respect to *E. lenensis*, it was known to have persisted not only until the early Holocene, but also up to the historical time in some local refugia (Kuznetsova *et al.*, 2001; Schirmermeister *et al.*, 2002). Besides, it seems plausible to suppose that some species of the North American caballoid horses could have spread locally to

North East Siberia during the Late Pleistocene, which might be especially true for *E. lambei* Hay, 1917. At last, a Pleistocene horse morphologically different from *E. lenensis* discovered in the region recently (Spasskaya *et al.*, 2012) is to be mentioned.

These considerations make it quite reasonable to initiate a thorough exploration of the diversity of the Pleistocene horses in North East Siberian and eventually in a more widespread region.

Herewith, provided are the most important results of our pilot study of morphological and genetic differentiation of *E. lenensis* and several other morphologically similar Pleistocene to Holocene horses. Our main task was to analyze heterogeneity of the materials identified as *E. lenensis* using a combination of several quantitative methods, including standard and geometric morphometrics of craniodental characters and mitochondrial DNA sequencing.

Material and methods

A pilot status of our study was caused by a limited amount of the material involved in it (Appendix 1; the specimens IDs provided in the text are also given there). In addition to our main object *E. lenensis*, the materials on the following Pleistocene horse species were included in the sample: *E. latipes* Gromova, 1949; *E. uralensis* Kuzmina, 1975; *E. dalianensis* Zhou, Sun, Xu, Li, 1985, and *E. lambei*. Besides; limited data on the modern *E. c. przewalskii* Poljakov, 1881 and several Siberian native breeds of *E. ferus caballus* Linnaeus, 1758 were also included to widen the factual basis of our comparisons. The species allocation of the studied specimens was adopted according to the original identifications by their collectors. The individual age of all specimens was identified as more than 5 years based on standard criteria of the teeth (Dyurst, 1936). The sets of species and specimens, for which respective morphometric materials were available, were differed because of the specifics of the fossil materials. Therefore, the following six blocks of morphometric data were analyzed separately: linear measurements of the axial skull and mandible, linear measurements of the upper and lower cheek teeth, the crown enamel patterns of the 1st upper and lower molars.

For a number of specimens of *E. lenensis*, radiocarbon age was determined (five of them were dated in 2019), the range varied from 20 000 to more than 47 000 BP (see Appendix 1).

The materials studied on each species, together with their sources and designations adopted herewith, are indicated in the Appendix 1.

For the morphometric analyses, the entire sample was arranged as follows. To identify taxonomic differences by the skull and dental measurements, three main groups were separated: *E. lenensis*, *E. przewalskii*, and *E. f. caballus*, each included 6 to 15 specimens. The subsamples designed for the analysis of dental crown patterns, included also a few specimens of *E. lambei* and single specimens per each of *E. nordostensis*,

E. coliemensis, *E. orientalis*, *E. latipes*, *E. uralensis*, and *E. dalianensis*. As the morphometric data were processed independently within each of the above-indicated six blocks, the overall number of the analyzed items exceeds the real number of individuals in our sample, as it is characterized in the Appendix 1.

For the present analysis of the mitochondrial genome, a specimen of *E. lenensis* (PIN No 3913/70) was sequenced because of peculiarities of its lower molars crown pattern: its double knot shape was similar to that of *E. lambei*. A complete mitochondrial genome obtained previously for another specimen of *E. lenensis* (PIN 301/1, MN187571; Nedoluzhko *et al.*, 2020) was included in this study. The following specimens were also used for the phylogenetic reconstruction based on the coding sequences (CDS) of the mitochondrial genome: two specimens of *E. cf. lambei* from the Yukon Peninsula (KT168318.2; KT168322.2); several specimens of *Equus* (*Equus*) sp. from the Yukon Peninsula (KT757763.1), from the Taimyr Peninsula (CGG10022; CGG10023), from the middle reaches of the river Yana (KT368725.1); the specimens of the modern horse *E. f. caballus* (KT368726.1; NC_001640.1) and *E. przewalskii* (NC_024030.1); specimens of *Equus kiang* Moorcroft, 1841 (HM118851.1) and *Equus asinus* Linnaeus, 1758 (KX683425) were constituted an outgroup for the rooting of molecular phylogenetic tree.

For the standard morphometric analyses, we used 40 linear measurements of the axial skull, 15 measurements of the mandible, 24 measurements of the upper cheek teeth, 31 measurements of the lower cheek teeth, all taken according to the standard schemes (Eisenmann *et al.*, 1988) by a standard caliper from the museum specimens. In 8 specimens of *E. lenensis* with partially damaged skulls, the empty cells in the tables with measurements were filled with the average values of respective characters calculated for this species; the total number of such substitutions ranged from 2 to 9 cells per 24 to 31 characters in total.

For the geometric morphometric analyses, the outline points were used to describe the enamel crown patterns of the 1st upper and lower molars (M1 and m1) (on this method, see Mitteroecker & Gunz, 2009; Zelditch *et al.*, 2012; Vasiliev *et al.*, 2018). These outlines were set on the photos taken either from the museum specimens or, in the case of teeth of several extinct taxa, from their pictures in respective publications (listed in the Appendix 1). The general outlines of the teeth were described by 100 points each, and the fosses outlines on the upper molars were described by 50 points each. Terminology for the elements of the dental crowns was adopted after Eisenmann *et al.* (1988).

Since the samples characterized by different sets of characters did not coincide, morphometric analyses was performed independently for each of the six data blocks indicated above. In the case of cranial and dental standard characteristics, the original linear measurements were used as variables in all comparisons. In the case of the dental crown patterns, first, x-y-coordinates of the outline points were

transformed into coefficients of the Fourier rows (or ellipses) by the Elliptical Fourier Analysis (EFA), with 20 harmonics calculated, and second, principal components (PCs) were extracted from these coefficients to be used as variables in the comparisons. An effectiveness of describing the tooth crown outlines this way was shown in a number of studies (Renaud *et al.*, 2009; Labonne *et al.*, 2014).

We estimated contributions of the species and age differences to the overall morphological disparity in the sample using one-way analysis of variance (ANOVA). The contribution in question was measured as a total ratio of the sums of squares corresponding to the explained and random variances for all variables in the given analysis (on this measure, see Pavlinov *et al.*, 2008; Pavlinov, 2011). The ANOVA was also used to evaluate individual contributions of the particular variables to the species differences by the *F* test, these estimates were used for selection of the variables for the subsequent discriminant function analysis (DFA).

Overall disparity structure in the sample, for each of the data block, was analyzed by the multidimensional scaling (MDS) based on the matrices of correlation distances between the specimens. The analyses were based on visual estimations of distributions of the specimens in the spaces of the 1st and 2d MDS axes.

The differences between the specimens with respect to their supposed taxonomic differentiation by the morphometric variables were explored using DFA. For each of the data block, we included all variables with the significance level (*p*) of the *F* criterion not exceeding 0.4, as it was estimated preliminary by ANOVA, with no additional selection of those variables by the DFA algorithm being applied. This approach allowed us to maximize the differences being analyzed, with the threshold set empirically based on the minimal (*F* to remove) criterion built into the DFA. The differences in question were evaluated by Mahalanobis distances (DM); their values were considered within each of the data blocks because of the differences in number of variables in them. The above three species were used as a teaching sample, combined distributions of all specimens were then analyzed in the spaces of the 1st and 2d canonical variables (CVs) obtained by the DFA for the respective data blocks.

Computations on the morphometric data were performed using the following programs. Outline points were set in the tpsDig2 (Rohlf, 2015). The EFA and PCs extraction were performed in the PAST (Hammer *et al.*, 2001). Most of the other calculations were performed in the software package Statistica for Windows (StatSoft, 2014).

Ancient DNA (aDNA) extraction from the bone powder was conducted in aDNA facilities of the National Research Center “Kurchatov Institute” (Moscow, Russia), using the methodology described by L. Orlando with colleagues (Orlando *et al.*, 2013). DNA library for sequencing was constructed using Ovation Ultralow Library System V2 (NuGEN, USA), according the manufacturer’s protocol. The final library was quantified by a high-sensitivity chip on a 2100

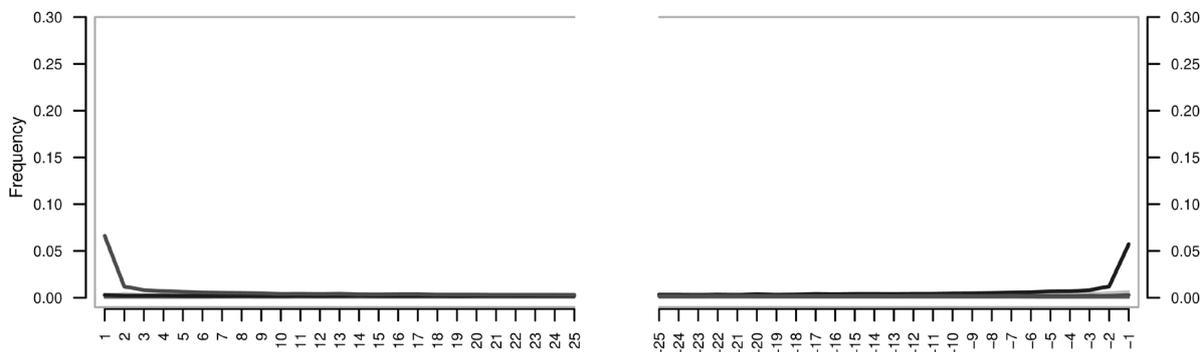


Fig. 1. Contamination test of DNA from the specimen of *E. lenensis* (PIN 3913/70) by the modern genetic material.

Bioanalyser instrument (Agilent Technologies, USA) and was sequenced on the Illumina NovaSeq6000 (Illumina, USA) platform with 150 base pairs paired-end reads.

In total, 183 397 565 paired-end reads were generated for PIN № 3913/70 *E. cf. lenensis* DNA-library. Illumina reads were filtered by PALEOMIX pipeline (Schubert *et al.*, 2014) with mapDamage2 tool (Jonsson *et al.*, 2013). This pipeline allows to perform reads quality filtering, adapter trimming, mapping to reference genome, and ancient DNA postmortem cytosine deamination marks identification (Schubert *et al.*, 2014). *E. f. caballus* reference mitochondrial genome sequence (NC_001640.1, assembly EquCab3.0) and Bowtie 2 aligner under the “very-sensitive” and “rescale” options were used for analysis. Only PALEOMIX filtered reads (586) that contained specific to ancient DNA postmortem cytosine deamination patterns were used for *E. lenensis* (PIN № 3913/70) whole mitochondrial DNA assembly (MN187576). The model postmortem DNA damage from nucleotide misincorporation patterns is presented in Fig. 1. The phylogenetic relationships were reconstructed using Maximum likelihood analysis was conducted using RAxML (Stamatakis *et al.*, 2008).

Results

Analyses of the cranial and dental measurements

Interspecific differences in the linear measurements revealed by ANOVA are most significant for the cheek teeth, especially the upper ones: the ratio of the explained variance equals to 40.4% for the upper and to 23.7% for the lower teeth, while it equals to 20.6% for the axial skull and only to 17.2% for the mandible. These figures indicated that conclusions about species allocations of the Pleistocene and modern horses, based on the cranial (especially mandibular) measurements can be very unreliable.

Comparison of the three main groups (*E. lenensis*, *E. przewalskii*, *E. f. caballus*) by the linear measurements of their axial skulls showed the following (Fig. 2). In the space of the first two MDS axes (Fig. 2A), *E. lenensis* overlapped largely with *E. f. caballus*, while *E. przewalskii*

took a separate position, though without an evident hiatus. Among *E. lenensis*, the specimens GM DPMGI 1715, DPMGI 5059, IPM 2437 took a somewhat remote position relative to others. The DFA did not provide a clear separation of these three groups (Fig. 2B); with this, *E. lenensis* and *E. przewalskii* appeared to be most distinct (DM = 114.8), while the differences between the former and *E. f. caballus* were minimal (DM = 32.0).

The results of comparison of these groups by the mandible characters were somewhat different (Fig. 2C, D). In the space of the first two MDS axes (Fig. 2C), *E. lenensis* occupied an intermediate position between *E. przewalskii* and *E. f. caballus*, with the specimens GM DPMGI 6730 and PIN 3491/70 grouping with the former and the specimens GM DPMGI 3750 and IPM 2539 tending to be close to the latter. The DFA did not reveal any separation of these groups (Fig. 2D); contrary to the analysis of the axial skull, in the given case the differences are greatest between *E. przewalskii* and *E. f. caballus* (DM = 20.6), while the differences of *E. lenensis* from them are less (DM = 7.8–11.3).

Comparison of *E. lenensis*, *E. przewalskii*, and *E. f. caballus* by their cheek teeth measurements showed the following (Fig. 3). In the space of the first two MDS axes defined by the upper teeth (Fig. 3A), they were distributed without an apparent hiatus: *E. lenensis* and *E. f. caballus* form a combined cloud, with a compact cloud of *E. przewalskii* adjacent to them. The overlapping zone of these groups included the specimen of *E. orientalis* (GM DPMGI 4612) and one of two *E. coliemensis* (KU-12), while the type specimen of the latter (GM DPMGI 1741) took a remote position. The group of *E. lenensis* showed a clear heterogeneity: GM DPMGI 3607 was situated close to *E. przewalskii*, while GM DPMGI 3715 and PIN 4223/72 were distinct. The DFA clearly distinguished the three main groups, with *E. lenensis* being the least specific (Fig. 3B); the DMs between it and two other groups were 81.0–89.8, while between *E. f. caballus* and *E. przewalskii* it was 165.6. A separate position was occupied by *E. orientalis* and the type specimen of *E. coliemensis*, with the latter’s second specimen (KU-12) belonging evidently to *E. lenensis*.

The results by MDS for the lower cheek teeth (Fig. 3C) were in general similar to those for the upper ones;

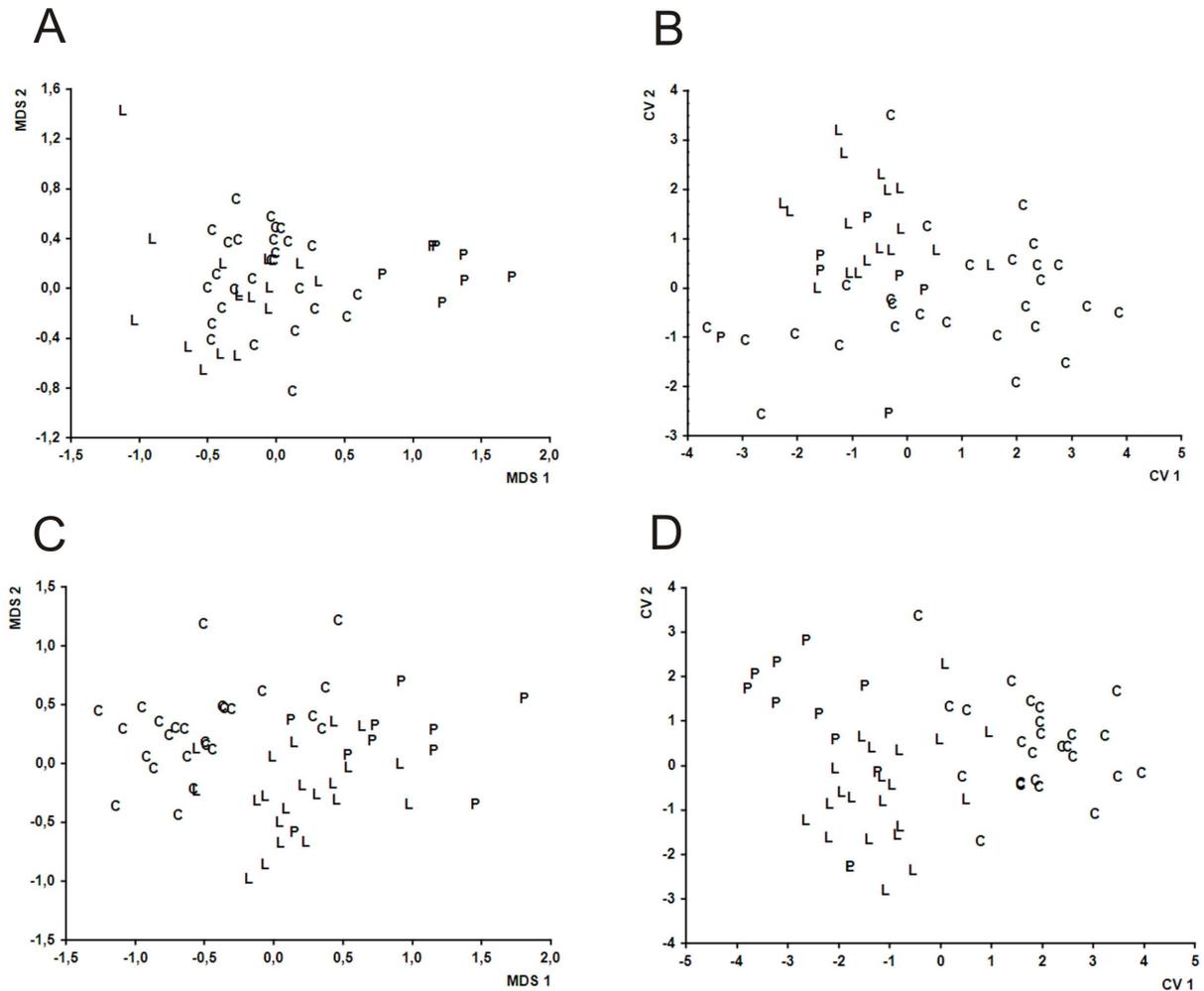


Fig. 2. The result of analyses of the horses based on the measurements of the axial skull (A, B) and mandible (C, D): distribution of specimens along the first two axes of the multidimensional scaling MDS 1, MDS 2 (A, B) and the first two canonical variables CV 1, CV 2 (C, D) is shown. Designations of species: C — *E. f. caballus*, L — *E. lenensis*, P — *E. przewalskii*.

E. lenensis overlapped significantly with *E. f. caballus* and even to a greater extent with *E. przewalskii*. However, several specimens of *E. lenensis* (PIN 301/133, PIN 301/135, PIN 3491/70) appeared to be rather specific with respect to the rest. The specimens of *E. coliemensis*, *E. orientalis*, and *E. nordostensis* were distributed within the general cloud. The DFA clearly separated the main three groups (Fig. 3D), but unlike for the upper teeth, here *E. lenensis* was the most specific: the DMs between it and two other groups were 418.8–555.2, and between *E. f. caballus* and *E. przewalskii* it was 238.2. As to the *E. coliemensis*, its type specimen occupied a quite separate position, with another one being grouped with *E. lenensis*. At last, two specimens of *E. orientalis* were separated both from each other and from other horses, though both were situated close to *E. lenensis*.

The craniodental measurements most correlated with the 1st MDS axis and thus most significant for the uncovering heterogeneity of *E. lenensis* are listed in the Appendix 2.

The results of analyses of differences among Pleistocene and modern horses by cranial and dental measurements, exposed above, can be summarized as follows.

1. In general, the dental traits were shown by ANOVA to have a greater diagnostic value for the species discriminations than the cranial ones, with the upper dentition measurements appeared to be the most and the mandibular once the least significant.

2. Significant distinctness of *E. przewalskii* with respect to *E. lenensis* and *E. f. caballus* (native breeds) by craniodental measurements was shown.

3. A group of specimens identified as *E. lenensis* was distinguished as clearly separated by the DFA, but this result should not be taken for a decisive evidence of its morphological compactness. This is because it is a principal function of DFA to make a priori classified groups as distinct and compact as possible, which is especially true for the small samples, as in our case. With this respect, quite important seemed

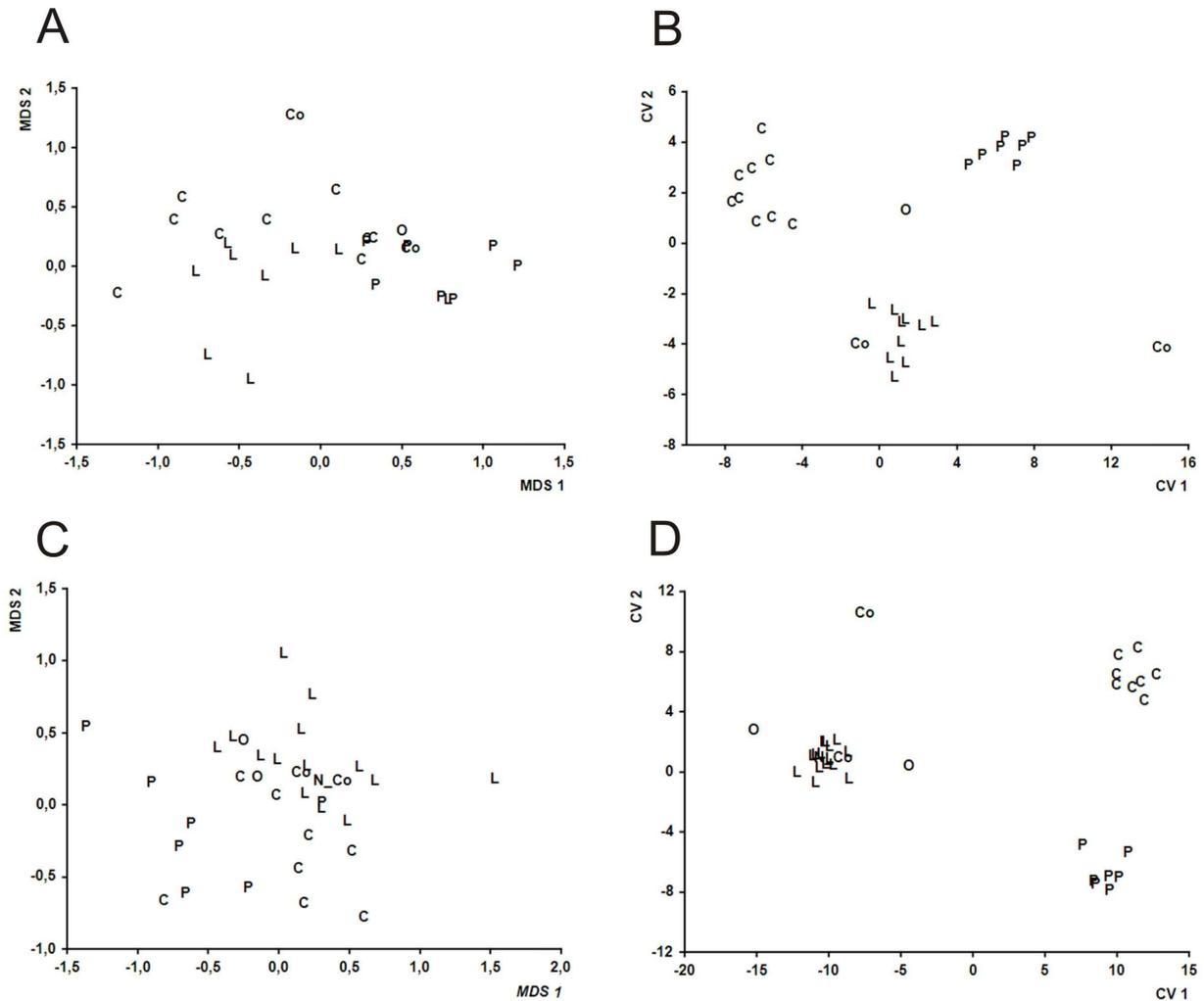


Fig. 3. The result of the analyses of the diversity of horses based on the measurements of the upper (A, B) and lower (C, D) cheek teeth: distribution of specimens along the first two axes of the multidimensional scaling MDS 1, MDS 2 (A, C) and the first two canonical variables CV 1, CV 2 (B, D). Designations of the species of the three main groups as in Fig. 2, designations of other species: Co — *E. coliemensis*, N — *E. nordostensis*, O — *E. orientalis*.

to be results of the MDS that showed this group to be heterogeneous by forming a rather loose “cloud”, with several, though vague subgroups distinguishable in it. Craniodental measurements most significant for uncovering heterogeneity of *E. lenensis* were identified for subsequent analysis on a more extensive material.

4. The similarity relations of the specimens identified previously as members of distinct horse species — *E. coliemensis*, *E. orientalis*, and *E. nordostensis* — did not agree with any of their previous taxonomic allocations. For example, the specimen GM DPMGI 648 had been first designated as a paratype of *E. orientalis* by Rusanov (1968), allocated subsequently to *E. coliemensis* by Lazarev (1980) and then to *E. nordostensis* by Belolyubsky *et al.* (2008); according to the results of our analyses, it was grouped with *E. lenensis*. The specimen KU-12 was previously allocated to *E. coliemensis*, but it was shown above to differ significantly from the latter’s type specimen and to group with *E. lenensis*.

Analyses of the dental crown patterns

The results of the analyses of species and age differences by ANOVA were as follows. As in the case of linear measurements, the portion of species differences in the overall disparity of the crown patterns was higher for the upper tooth than for the lower, 50.1% and 31.2%, respectively. The portion of age differences is significantly less, 9.0% and 6.0% for upper and lower tooth, respectively.

The regions of the greatest variability in the enamel structure of the crown of the 1st molars were identified (Fig. 4). On the upper tooth (Fig. 4A), they were located between mesostyle and metastyle on the external part of the enamel outline, along the anterior and posterior edges of the protocone, as well as in region of pli paraconule in the prefossette. On the lower tooth (Fig. 4B), shapes of the inner part of preflexid, the entoconid, and ectoflexid appeared to be most variable.

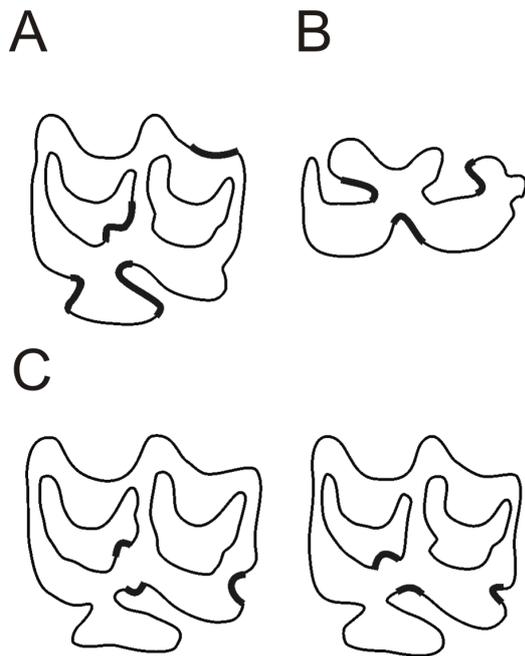


Fig. 4. Enamel crown patterns of the upper (A) and lower (B) first molars in the horses described by the outline points method; the consensus configurations of the crown patterns in the subgroups of *E. lenensis* (C). Bold lines indicate the areas with the greatest contributions to the overall differences.

Distribution of the specimens in the space of the 1st and 2d MDS axes for the upper molar had the following features (Fig. 5A). The groups of *E. przewalskii* and *E. lambei* were quite compact, while *E. lenensis* and *E. f. caballus* were more dispersed, and *E. f. caballus* overlapped significantly with *E. przewalskii* and partly with *E. lenensis*. The latter's specimens appeared to be divided into four small subgroups, with some falling into *E. przewalskii*, some into *E. lambei*, several being combined with *E. coliemensis*, others united with specimens of *E. uralensis* and *E. f. caballus*. Single specimens of *E. latipes*, *E. uralensis*, and *E. dalianensis* fallen into a common cloud with *E. przewalskii*, several with *E. f. caballus* and with one subgroup of *E. lenensis*. According to the DFA (Fig. 5B), the specimens of *E. lenensis*, *E. f. caballus* and *E. przewalskii* formed virtually a single cloud, with the specimen of *E. latipes* falling into it. A small group of *E. lambei* was clearly separated from this cloud. The specimens of each of *E. coliemensis* and *E. uralensis* took isolated positions, while *E. dalianensis* was placed between *E. lenensis* and *E. lambei*.

Distribution of the *E. lenensis* specimens, taken separately, in the space of the 1st and 2d MDS axes for the upper molar indicated their dividing into two subgroups (Fig. 6). One of the latter included the specimens GM DPMGI 33 (type specimen), GM DPMGI 33/82, GM DPMGI 1715, GM DPMGI 3607, another included the specimens GM DPMGI 3750, PIN 301/1, PIN 4223/72,

ZIN 28825, IAM F-195, IAM F-2530. The consensus configurations of the tooth crown pattern calculated for these subgroups (Fig. 4B) indicated that they differed from each other by the shape of hypocone, inner portion of the prefossette, and inner portion of the postprotoconal groove.

Distribution of all the specimens in the space of the 1st and 2d MDS axes for the lower molar was a kind of a vague cloud, with *E. lenensis* being decomposed into two groups (Fig. 5B). According to the DFA (Fig. 5G), *E. przewalskii* formed a rather compact group adjoining a relatively compact group of *E. lenensis*, the latter incorporating also *E. uralensis*. The distribution of *E. f. caballus* turned out to be very dispersed, with some of them fitting into *E. lenensis*. Among the remaining species, the position of *E. orientalis* was most distinct. It is to be noticed that initially supposed similarity of the specimen PIN 3913/70 of *E. lenensis* to *E. lambei* by its lower molar crown pattern (see Introduction) was not approved by the DFA.

The most significant results of the analyses of the crown patterns of the M1 and m1 can be summarized as follows:

1. Age-related variation (very worn teeth not included) of the tooth crown patterns was shown to be rather low, so it might be possible not take it in consideration in the explorations of species differentiation of the Pleistocene and modern horses;
2. Taxonomic differences in the crown pattern were shown to be more significant for the upper molar than for the lower;
3. *E. lenensis* and *E. lambei* were shown to be distinct in some dental features revealed by the DFA and less so in the dental features revealed by the MDS;
4. *E. lenensis* was shown to be morphologically heterogeneous by the upper molar crown patterns. According to the DFA, it could be divided into four subgroups combining with some other horse species. According to the MDS, when considered separately, it could be divided into two subgroups;
5. The areas of the enamel outlines most affecting the overall disparity of the dental crown patterns, as revealed by the geometric morphometrics, did not coincide with the crown elements traditionally used for delimitation of the Pleistocene horse species. For the upper molars, the most attention was usually paid to the shape, size and inclination of the protocone relative to the axis of the tooth, and to the width of the inner (postprotocol) valley. For the lower molars, differences in the shape of the so called double knot, length and shape of preflexide and postflexid, and the location of isthmus were traditionally considered the most pronounced.

Analysis of the mitochondrial genomes

This study represents the comparative analysis of two whole mitochondrial genomes of *E. lenensis*. The mitochondrial genome of the specimen of *E. lenensis* (PIN № 301/1, MN187571) specimen has been described previously (Nedoluzhko *et al.*, 2020). The mitochondrial

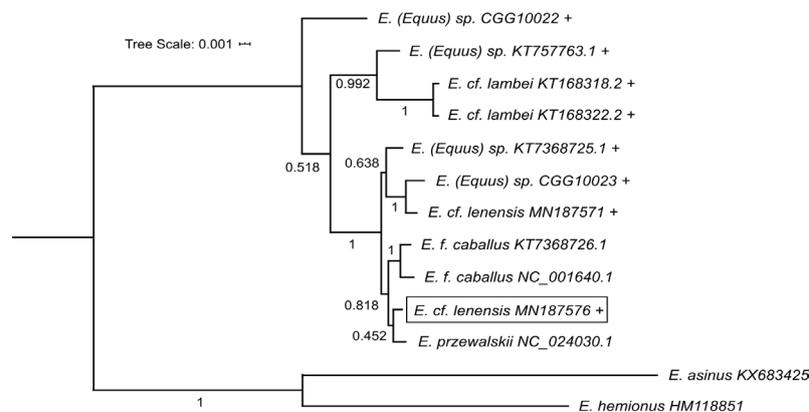


Fig. 7. Maximum likelihood phylogenetic tree of *E. lenensis* and several other species based on their CDS. Pleistocene specimens are marked with crosses, *E. lenensis* specimen PIN 3913/70 is highlighted with a rectangle. Figures on the branches indicate bootstrap support.

genome of the *E. lenensis* (PIN № 3913/70) specimen, that had specific traits in enamel structure of the crown of the lower molars, was analyzed for first time (MN187576).

Results of our phylogenetic analysis of the CDS of the mitochondrial DNA sequences of *E. lenensis* (MN187571 and MN187576) together with *E. lambei* and three other Pleistocene horses (Fig. 7) did not show any clear subdivision of the former into any subgroups compatible with those revealed by the morphometric analyses. This result might indicate either a genetic uniformity of this entire group or a poor resolution of this marker for discriminating its subgroups. We suppose that a more in-depth paleogenomic analyses of the nuclear DNA would allow us to explore the history of *E. lenensis* and related taxa of the Beringia region.

Discussion

Taxonomic structure of the Pleistocene caballoid horses of North East Eurasia is still very debatable. Morphological heterogeneity of the materials on them was repeatedly shown, but lack of a precisely dated large enough sample did not allow to revise already recognized and to describe new taxa (Vangengeim, 1961; Sher, 1971). Validity of some species, which descriptions were based on very limited number of specimens from different localities was actively disputed, which was true, in particular, for *E. orientalis* (Sher, 1971). Morphological evidence for acknowledging species status of *E. lenensis* was presented by B.S. Rusanov (1968), P.A. Lazarev (1980; Lazarev & Tomskaya, 1987), I.E. Kuzmina (1997). P.A. Lazarev (1980, Tables 1, 3–5) divided the cranial material on *E. lenensis* he studied into two geographically defined groups from Western and East Yakutia; their average sizes were somewhat

different, but this point was not discussed in his work. Subsequently, the issues of intraspecific morphological variation of *E. lenensis*, including its possible correlation with geographical factors, were not discussed. However, even a cursory examination of limited materials on this species indicated certain differences in sizes and proportions of the skull, dentition, shape of the 1st molars, etc. An analysis of all such materials was complicated by the lack of clear morphological diagnostic features for the Late Pleistocene horses of North East Siberia, as well by the lack of a representative sample of the AMS-dated material.

Our pilot study, although based on a quite limited data, makes it possible to point out some important results to be taken in consideration in the further research, they are briefly discussed below.

It was shown that the species delineation of the Siberian Pleistocene horses, as they were identified in previous studied, by cranial measurements only was not reliable enough. Instead, linear measurements and enamel patterns of the cheek teeth, especially upper ones, appeared to be much more informative for this purpose. Due to this, previous species allocation of a number of specimens studied by us was shown to be incorrect, so its reconsideration based on the dental characters is needed.

Disagreement between the dental crown elements used traditionally for delimitation of the horse species and revealed by the geometric morphometrics deserves clarification. One probable cause is that in the formalized approach we used, the mutual displacements of all parts of the teeth are ascribed an equal weight and evaluated simultaneously, regardless of whether the variations are intraspecific or interspecific. In contrast, in the traditional studies, experts used to analyze these two levels of variation separately based on a priori species allocation. However, the differences they used to interpret taxonomically significant might in fact reflect

but some aspects of the morphological disparity not directly related to the taxonomic diversity. Therefore, further exploration of more dental materials, based on an independent reliable method of species delimitation, is needed to uncover those dental crown elements that actually allow to discriminate species.

Morphological heterogeneity of *E. lenensis*, together with some other close species, revealed by us was not clear-cut; separate analyses of the data blocks with isolated axial skulls, mandibles, and teeth provided different groupings of the specimens allocated to it. An evident cause of this inconsistency is that these morphological structures are but weakly correlated and thus yield different similarity relations. That was probably why we failed to uncover any evident geographical factors correlated with such kind of morphological heterogeneity. Therefore, in order to make the latter more explicit, the next step of exploration of the Late Pleistocene horses of North East Siberia should incorporate the data on as many complete specimens as possible.

All these inconsistencies, both “outer” (with previous studies) and “inner” (within our study), clearly indicate that an extensive development (more materials) of further explorations in caballoids should be completed with an “intensive” one (more advanced multivariate statistical methods). With this, special attention is to be paid to a correct combination of different methodologies allowing for cross-validation of the results they provide. For instance, the MDS carries out an analysis of the structure of diversity without prior recognition of any groups; besides, it’s being based on a decomposition of distance matrix makes it more appropriate for the exploration of small samples, unlike nowadays more popular principal component analysis (PCA) based on correlation matrix. As to the DFA, it allows to analyze diagnostic features of priority defined groups, to evaluate posterior probabilities of allocation of the specimens to them, and to compare results of different prior groupings to see which one is most adequate to the structure of the overall diversity revealed by the MDS.

Molecular methods may have good prospects for taxonomic studies of the Pleistocene materials on the horses (Weinstock *et al.*, 2005; Orlando *et al.*, 2008, 2013; Librado *et al.*, 2016). Work with the mitochondrial genomes from North East Siberia is just at its beginning (our study, as well as Nedoluzhko *et al.*, 2020). Full nuclear genomes have been obtained so far only for three specimens, one was a Holocene horse from the Yana River basin — Batagai, radiocarbon age $4\,400 \pm 35$ uncal. yBP (Gr 50842) (Librado *et al.*, 2015); other two were Pleistocene horses from Taimyr: CGG10022 — $42\,692 \pm 891$ BP (UBA-16478); CGG10023 — $16\,099 \pm 192$ BP (UBA-16479) (Schubert *et al.*, 2014b). However, morphological descriptions of respective materials on them have not been made and, accordingly, it is not possible to allocate these genetically dated specimens to known species. As to our materials, they also do not allow to discuss an issue of concordance between

genetic and morphometric data with respect to the species delineation. Thus, there is currently no any adequate conjoint morpho-genetic analysis of the taxonomic diversity of the caballoid horses of North East Eurasia.

A similar conclusion is true for the Pleistocene horses of North America, where 10 to 40 species were recognized dependent on different data and methods (Azzaroli, 1998). Genetic studies (mitochondrial control region HVR1) have questioned such a rich species diversity of those horses. Based on the genetic and morphological (metapodial bones) data, only two lines were identified, namely non-caballoid stilt-legged and caballoid stout-legged horses, each probably represented by few species (Weinstock *et al.*, 2005; Heintzman *et al.*, 2017). In the light of new data, debatable appeared to be not only validity of some species but also their belonging to the group of caballoid horses, which may be true, in particular, for *E. lambei* (Burke & Cinq-Mars, 1996). An integrated approach using genetic analysis in conjunction with standard and geometric morphometric analyses showed a great perspective of such synthesis, making it possible to clarify taxonomic status and relationships of various forms in the genus *Equus* (Barrón-Ortiz *et al.*, 2017).

We did not touch in this study any particular questions concerning species systematics of the Siberian horses because of an evident shortage of the studied materials. However, our results indicated that the future taxonomic studies based on the analyses of more specimens, including the type material, should not limit themselves to the traditional skeletal materials but include the dental crown patterns, as well. With this, special attention should be paid to the taxonomic differentiation of the populations now allocated to the *E. lenensis* and its most close allies.

Conclusions

The results presented in this paper demonstrated a good perspective for using new characters and methods to solve certain taxonomic problems in groups of the Pleistocene and modern horses of North East Eurasia. This pilot research revealed the following most important points.

Using geometric morphometrics, the most variable areas of the molar crown patterns were identified, which differ from those traditionally used in the caballoid taxonomy. A possibility of incorporating them in the taxonomic studies of the horses will need further examination based on more materials and adequate methods.

The use of nuclear genomes is needed for a better understanding of the level of genetic variation and phylogenetic relationships of Pleistocene horses in North East Eurasia and North West North America.

A certain heterogeneity of the *E. lenensis* group in dental and partly cranial features was confirmed. But a small sample of material did not allow us a more accurate description of the identified groups and to associate them with certain spatial or temporal factors.

A necessity of reconsidering species allocation of some materials and, in the future, a large-scale comprehensive revision of the Pleistocene horses of the North-East of Siberia was shown.

Over the past 40 years, an extensive new paleontological material has been accumulated and its amount continues to grow, partly due to global climate change. The need for a comprehensive large-scale taxonomic revision of the genus *Equus* of North East Eurasia and North West North America is obvious. It should be based on a joint analysis of more extensive both morphological and genetic materials, in the first case using a set of the modern morphometric methods.

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Appendix 1. Craniodental materials on the Pleistocene and modern *Equus* used in this research (species listed alphabetically).

E. coliemensis: GM DPMGI lower tooththrow No 1721, upper tooththrow No 1741 (type specimen *E. coliemensis* Lazarev, 1980), KU — upper tooththrow (fig. 12).

E. dalianensis: KU — upper and lower tooththrows (fig. 44).

E. f. caballus: Mongol bread (complete skulls): ZIN — Nos 5231, 18055, 18056, 18057, 18058, 18059, 20137; ZMMU — Nos S-110476, S-110478, S-134563, S-163569, S-190395; UB — Nos 1, 2; Yakut bread (complete skulls): GM — Nos 4, 5, 8, 16, 33, 4693, 1931-32, 1932-34; ZIN Nos 4051, 15245, 31168; ZMMU — Nos S-181390, S-186091, S-186096, S-186097, S-186098; Tuva bread (axial skulls): ZMMU — Nos S-186099, S-197459, S-197460, S-197463.

E. lambei: HA — upper and lower tooththrows (figs. 16, 17, 24).

E. latipes: KU — upper and lower tooththrows (fig. 19).

E. lenensis: GM DPMGI — axial skull with tooththrows Nos 33 (type specimen of *E. lenensis* Russanov, 1968), 33/82, 1715, 3715, 3607, 3750, 5059, 6730; mandible with tooththrow Nos 3750, 4826, 6730; IAM —

axial skull with tooththrows Nos F-195, F-254, F-2431, F-2437, F-2442, F-2530; mandible with tooththrow Nos F-649, F-2379, F-2436, F-2438, F-2539, F-2540, F-2541, F-2542, F-2543; KU — upper and lower tooththrows (fig. 39); PIN — axial skull with tooththrows 301/533, 301/1, 4223/72; mandible with tooththrow Nos 3491/611, 3491/630, 3913/70, 301/133, 301/135, 301/540; ZIN — axial skull with tooththrows No 19031.

E. nordostensis: GM DPMGI — upper tooththrow No 4659, lower tooththrow Nos 92, 648, 6435.

E. orientalis: GM DPMGI — upper tooththrow No 4612, lower tooththrow Nos 152, 564, 1732, 3600.

E. przewalskii (complete skulls): AN — Nos 288/120, 374, 378, 1032, 1035, 1154, 1195, 2412; TGU — Nos 3525, 3526; ZIN — Nos 5212, 5213, 5214, 5216, 5218, 27089; ZMMU — Nos S-1772, S-115391, S-133806, S-187005.

E. uralensis: KU — upper and lower tooththrows (figs. 28, 29).

Specimens with radiocarbon age determined:

Specimens <i>E. lenensis</i>	BP*	¹⁴ C, uncal. BP (1σ)	cal. BP Radiocarbon Calibration Program CALIB REV7.1.0**	Comments
GM DPMGI No 33	33 560±250–230			CrA-43065 (Kuznetsova & Plicht, 2009)
GM DPMGI No 3750	26 340±140			CrA-43060 (Kuznetsova & Plicht, 2009)
GM DPMGI No 3607	2 310±80			LU-1084 (Belolybsky <i>et al.</i> , 2008)
PIN No 301/1		21 105±55	68.3 (1 sigma) 25356–25566 1.000 95.4 (2 sigma) 25240–25642 1.000 Median Probability: 25456	IGAN/CIR UG6966
PIN No 301/533		19 795±50	68.3 (1 sigma) 23724–23943 1.000 95.4 (2 sigma) 23616–24043 1.000 Median Probability: 23833	IGAN/CIR UG6969
PIN No 3491/630		39 272±147	68.3 (1 sigma) 42861–43166 1.000 95.4 (2 sigma) 42714–43324 1.000 Median Probability: 43017	IGAN/CIR UG6971
PIN No 3913/70		<46 230		IGAN/CIR UG6967
PIN No 3491/611		<46 615		IGAN/CIR UG6968

* without specifying the type of date.

** Reimer *et al.*, 2016.

Collection IDs:
AN — Research Museum, Biosphere Reserve “Askania Nova”;
GM DPMGI — Geological Museum of the Diamond and Precious Metals, Geology Institute SB RAS;

HA — Harington & Clulow (1973);
IAM — Ice Age Museum, Moscow, Russia;
KU — Kuzmina (1997);
PIN — Paleontological Institute RAS;
TGU — Zoological Museum, Tomsk State University;

UB — State Agrarian Institute, Mongolia;
 ZIN — Zoological Institute RAS;
 ZMMU — Zoological Museum, Lomonosov Moscow State University.

Designations:

IGAN/CIR — Institute of Geography, Russian Academy of Sciences, Moscow, Russia / Center for Applied Isotope Studies, University of Georgia, Athens, USA;

CrA — Centre for Isotope Research, Groningen University, Groningen, Netherlands;

LU — Laboratory of geomorphological and paleogeographic studies of the Polar Regions and Oceans V.P. Köppen, Leningradsky University (now — Saint Petersburg State University), Saint Petersburg, Russia.

Appendix 2. Craniodental measurements of *E. lenensis* most significant for analysis of its heterogeneity (see text for explanation).

No	Measurements	<i>n</i>	M	min	max	SD
1	Palathal breadth at P2	18	67.7	59.2	79.3	4.3
2	Choan width (maximum)	16	41.4	35.4	48.2	3.5
3	Mastoid width	17	118.8	110.5	129.5	4.6
4	Bazygomatic breadth	17	202.9	188.8	212.5	6.9
5	Occipital height	18	60.7	52.4	67.4	4.6
6	Mandibule length	16	445.3	413.0	490.0	19.8
7	Lower cheek teeth total length	17	176.8	162.2	198.5	10.7
8	Lower cheek teeth premolar length	17	89.2	80.0	97.0	6.1
9	Mandible symphyse length	17	89.3	74.5	111.5	9.9
10	Mandible height at ascending ramus	16	207.7	186.2	222.5	9.5
11	Mandible height at condyle	15	222.7	201.2	237.0	10.7
12	Breadth P2	16	25.9	22.0	30.8	2.3
13	Length protocone P2	8	10.1	7.0	12.2	1.8
14	Breadth P3	18	28.0	22.4	33.0	2.5
15	Length protocone P3	18	13.5	11.2	16.4	1.4
16	Breadth protocone P3	9	5.0	4.0	6.9	0.8
17	Length M1	18	24.1	20.4	30.2	2.1
18	Breadth M2	18	26.1	22.0	29.5	1.7
19	Breadth protocone M2	9	5.1	3.5	8.0	1.4
20	Breadth protocone M3	8	4.3	2.7	6.7	1.1
21	Breadth p2	17	16.3	14.0	19.3	1.4
22	Length double knot p2	8	14.9	11.5	17.0	1.8
23	Length postflexid p2	8	14.8	10.2	17.0	2.2
24	Length p3	18	28.1	24.5	31.0	1.5
25	Length double knot p3	9	15.6	13.0	18.3	1.6
26	Length postflexid p3	9	13.6	7.4	16.3	2.6
27	Length double knot p4	9	15.1	12.9	16.5	1.5
28	Length double knot m1	9	13.3	11.5	14.9	1.0
29	Length double knot m2	9	13.7	12.4	16.2	1.2
30	Length double knot m3	18	33.2	29.0	41.0	2.9

Designations: upper and lower molars: M1 and m1; upper and lower premolars: P1 and p1.