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Article:
Meyer, Matthias, Palkopoulou, Eleftheria, Baleka, Sina et al. (18 more authors) (2017) Palaeogenomes of Eurasian straight-tusked elephants challenge the current view of elephant evolution. eLife. e25413. pp. 1-14. ISSN 2050-084X

https://doi.org/10.7554/eLife.25413

Palaeogenomes of Eurasian straight-tusked elephants challenge the current view of elephant evolution

Tracking no: 24-01-2017-SR-eLife-25413R1

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Abstract:
The straight-tusked elephants *Palaeoloxodon* spp. were widespread across Eurasia during the Pleistocene. Phylogenetic reconstructions using morphological traits have grouped them with Asian elephants (*Elephas maximus*), and many paleontologists place *Palaeoloxodon* within *Elephas*. Here we report the recovery of full mitochondrial genomes from four and partial nuclear genomes from two *P. antiquus* fossils. These fossils were collected at two sites in Germany, Neumark-Nord and Weimar-Ehringsdorf, and likely date to interglacial periods ~120 and ~244 thousand years ago, respectively. Unexpectedly, nuclear and mitochondrial DNA analyses suggest that *P. antiquus* was a close relative of extant African forest elephants (*Loxodonta cyclotis*). Species previously referred to *Palaeoloxodon* are thus most parsimoniously explained as having diverged from the lineage of *Loxodonta*, indicating that *Loxodonta* has not been constrained to Africa. Our results demonstrate that the current picture of elephant evolution is in need of substantial revision.

Impact statement: DNA sequences from the Middle Pleistocene reveal that the extinct Eurasian straight-tusked elephants were closely related to today's African forest elephants (Loxodonta cyclotis) in Africa.

Competing interests: No competing interests declared

D, Hofreiter M, 2017, http://www.ebi.ac.uk/ena, PRJEB18563; Mitochondrial DNA sequences from 4 Palaeoloxodon antiquus fossils: Meyer M, Baleka S, Stiller M, Nagel S, Nickel B, Schüler T, Hofreiter M, 2017, www.ncbi.nlm.nih.gov/genbank/, KY499555 - KY499558 Reporting Standards: N/A

Ethics:
Human Subjects: No Animal Subjects: No

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Palaeogenomes of Eurasian straight-tusked elephants challenge the current view of elephant evolution

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Abstract

The straight-tusked elephants *Palaeoloxodon* spp*.* were widespread across Eurasia during the Pleistocene. Phylogenetic reconstructions using morphological traits have grouped them with Asian elephants (*Elephas maximus*), and many paleontologists place *Palaeoloxodon* within *Elephas*. Here we report the recovery of full mitochondrial genomes from four and partial nuclear genomes from two *P. antiquus* fossils. These fossils were collected at two sites in Germany, Neumark-Nord and Weimar-Ehringsdorf, and likely date to interglacial periods ~120 and ~244 thousand years ago, respectively. Unexpectedly, nuclear and mitochondrial DNA analyses suggest that *P. antiquus* was a close relative of extant African forest elephants (*Loxodonta cyclotis*). Species previously referred to *Palaeoloxodon* are thus most parsimoniously explained as having diverged from the lineage of *Loxodonta*, indicating that *Loxodonta* has not been constrained to Africa. Our results demonstrate that the current picture of elephant evolution is in need of substantial revision.

43 Main text

44 **Introduction**

45 In the late Miocene in Africa, the last of several major radiations within Proboscidea
46 gave rise to the family Elephantidae, which comprises living elephants and their extinct
47 relatives including mammoths (genus *Mammuthus*) and various dwarf elephant species
48 from Mediterranean islands. The three living elephant species (the African savanna
49 elephant, *Loxodonta africana*, the African forest elephant, *L. cyclotis* and the Asian
50 elephant, *Elephas maximus*), represent the last remnants of this family and of the
51 formerly much more widely distributed and species-rich order Proboscidea. Apart from
52 mammoths, the elephant genus with the most abundant fossil record in Eurasia is
53 *Palaeoloxodon* (straight-tusked elephants; Figure 1), which appears in Eurasia around
54 0.75 million years ago (Ma) (Lister 2015). Based on morphological analyses,
55 *Palaeoloxodon* is widely accepted as being more closely related to the extant Asian
56 elephant than to mammoths or extant African elephants (Shoshani et al. 2007, Todd
57 2010), and is often subsumed into the genus *Elephas* (Maglio 1973, Sanders et al. 2010).
58 Across its range from Western Europe to Japan, *Palaeoloxodon* probably comprised
59 several species (Shoshani et al. 2007), and based on morphological comparisons all of
60 them are considered to be derived from the African *Palaeoloxodon* (or *Elephas*) *recki*
61 (Maglio 1973, Saegusa and Gilbert 2008), which was the predominant proboscidean
62 lineage in Africa during the Pliocene and Pleistocene but went extinct around 100
63 thousand years ago (ka) (Owen-Smith 2013). Straight-tusked elephants may have
64 survived in mainland Eurasia until around 35 ka, although the youngest reliably dated
65 remains are from the last interglacial, 115-130 ka (Stuart 2005).

66   Recent technological progress has pushed back the temporal limit of ancient DNA
67 research, enabling, for example, recovery of a low coverage genome of a ~700,000 year-
68 old horse preserved in permafrost (Orlando et al. 2013). For more temperate regions,
69 however, evidence of DNA preservation reaching far beyond the last glacial period is still
70 limited to a single locality, Sima de los Huesos in Spain, where DNA has been recovered
71 from ~430 ka old hominin and bear remains (Dabney et al. 2013, Meyer et al. 2016).
72 While genetic analyses of the extinct interglacial fauna remain a challenging
73 undertaking, recent advances in ancient DNA extraction (Dabney et al. 2013) and

74 sequencing library construction (Meyer et al. 2012) have improved access to highly
75 degraded DNA.

76 **Results and Discussion**

77 To better understand the evolutionary relationships between the extinct straight-tusked
78 elephants and other elephant species, we attempted DNA extraction and sequencing
79 from several *P. antiquus* fossils, four of which we investigated in-depth. Three of these,
80 which were all unambiguously assigned to *P. antiquus* based on their morphology, were
81 from Neumark-Nord (NN) 1 in Germany, a fossil-rich site that has been proposed to date
82 to MIS 5e (~ 120 ka) or MIS 7 (~ 244 ka) or both (Mania 2010, Schüler 2010, Penkman
83 2010). This site has yielded one of the largest collections of *P. antiquus* remains known
84 to date. The fourth fossil was recovered during recent active mining in the travertine
85 deposits of Weimar-Ehringsdorf (WE), Germany, a quarry that has for more than a
86 century yielded a rich collection of fossils representing a typical European interglacial
87 fauna (Kahlke 1975). Weimar-Ehringsdorf is best known for the discovery of
88 Neanderthal remains in the early 20th century, and the assemblage is dated to MIS 7
89 (Mallick and Frank 2002). The *Paleoloxodon* bone fragment from Weimar-Ehringsdorf is
90 morphologically undiagnostic with respect to species. However, it was found in the
91 Lower Travertine, which was dated to ~ 233 ka (Schüler 2003) and where *P. antiquus* is
92 the only elephantid found so far. We performed DNA extraction, library preparation,
93 hybridization capture and high-throughput sequencing on all four fossils (Figure 2 –
94 source data 1) and obtained full mitochondrial genome sequences for all of them (Figure
95 2 – figure supplement 1). All sequences show short fragment lengths (Figure 2 – figure
96 supplement 2) and signals of cytosine deamination compatible with the old age of the
97 specimens (Figure 2 – figure supplement 3).

98   We inferred a phylogeny using the four *Paleoloxodon* mitochondrial genomes and
99 mitochondrial genomes from 16 *M. primigenius*, two *E. maximus* and 13 *Loxodonta*
100 individuals. The latter were chosen for a diversity of haplotypes, including forest
101 elephant derived ('F-clade') haplotypes as well as 'S-clade' haplotypes found only among
102 savanna elephants (Debruyne 2005). For calibration, we used an estimated divergence
103 of the African elephant lineage from that of Asian elephants and mammoths of 6.6-8.6
104 Ma (Rohland et al. 2007). Surprisingly, *P. antiquus* did not cluster with *E. maximus*, as
105 hypothesized from morphological analyses. Instead, it fell within the mito-genetic

4

106   diversity of extant *L. cyclotis*, with very high statistical support (Figure 2). The four
107   straight-tusked elephants did not cluster together within this mitochondrial clade, but
108   formed two separate lineages that share a common ancestor with an extant *L. cyclotis*
109   lineage 0.7-1.6 Ma (NN) and 1.5-3.0 Ma (WE) ago, respectively. However, mitochondrial
110   DNA represents a single, maternally inherited locus and does not reflect the full
111   evolutionary history of populations or species. Furthermore, the transfer of
112   mitochondrial DNA between hybridizing species is not unusual when gene flow is
113   strongly male-mediated (Petit and Excoffier 2009, Li et al. 2016, Cahill et al. 2013), as is
114   the case with elephants. For example, mitochondrial sequences of the F-clade have also
115   been found in some *L. africana* individuals (Debruyne 2005) despite the very substantial
116   divergence of their nuclear genomes (Roca, Georgiadis, and O'Brien 2005, Rohland et al.
117   2010), a pattern that has been attributed to mitochondrial gene flow from forest to
118   savanna elephants (Roca, Georgiadis, and O'Brien 2005).

119   We therefore performed shotgun sequencing of DNA libraries prepared from the
120   two best-preserved NN individuals (a petrous bone and a molar) to recover nuclear DNA
121   sequences. When mapped to the *L. africana* reference genome, 39% and 28% of the
122   sequence reads generated from these specimens were identified as elephant,
123   respectively (Figure 2 – source data 1). A neighbor-joining phylogenetic tree based on
124   ~770M (petrous) and ~210M (molar) base pairs of *P. antiquus* nuclear DNA placed *P.*
125   *antiquus* and *L. cyclotis* as sister taxa to the exclusion of *L. africana* (Figure 2). A tree
126   with identical topology was obtained using coding sequences only and a maximum
127   likelihood approach (Figure 2 – figure supplement 4). Despite the high sequence error
128   rates associated with the low coverage genomes generated from the *P. antiquus*
129   specimens, all nodes in the nuclear trees show maximal bootstrap support. The
130   mitochondrial and nuclear phylogenies thus support a sister group relationship between
131   *P. antiquus* and *L. cyclotis*.

132   Despite their geographical proximity, the WE and NN specimens are found in
133   different positions in the mitochondrial tree. Given that the three NN specimens show
134   highly similar mitogenome sequences, we considered whether the sites date to different
135   interglacials. Electron Spin Resonance (ESR) dating of tooth enamel has been applied to
136   both sites, suggesting an age of ~ 117 ka (range 97 – 142 ka (Schüler 2010)) for the NN1
137   layers from which our samples originate and of 233 ka (range 216 – 250 ka (Schüler
138   2003)) for the WE specimen. In order to better estimate the age of the NN1 site, we used

139 amino acid racemization of snail opercula (Penkman et al. 2011), including samples
140 from the continental Eemian type-site of Amersfoort (Zagwijn 1961) (Figure 2 – source
141 data 1), which is correlated with MIS 5e (Cleveringa et al. 2000). The NN1 opercula show
142 similar (perhaps slightly lower) levels of biomolecular degradation compared to
143 Amersfoort, suggesting an Eemian age for NN1 (Figure 2 – figure supplement 5).
144 Importantly, NN1 shows very similar levels of amino acid racemization in intra-
145 crystalline protein decomposition as a second site at Neumark-Nord (NN2), indicating
146 that both sites are of the same age. Considerable evidence supports an Eemian age for
147 NN2, including palaeomagnetic data that shows a correlation with the MIS 5e Blake
148 event and thermoluminescence dating of flint to ~126 ± 6 ka (Sier et al. 2011). These
149 results therefore indicate an Eemian age also for NN1. Since the WE specimen likely
150 dates to the previous interglacial, this suggests that the very different mitogenomes
151 between WE and NN1 may reflect the contraction and re-expansion of the range of *P.*
152 *antiquus* across glacial cycles.

153   Our results have implications both for understanding elephant evolutionary
154 history and for the use of morphological data to decipher phylogenetic relationships
155 among elephants. The combination of strongly supported mitochondrial and nuclear
156 DNA phylogenies clearly demonstrate that *Palaeoloxodon antiquus* is more closely
157 related to *Loxodonta* than to *Elephas* (Figure 3), suggesting that *Elephas antiquus* should
158 not be used synonymously for *Paleoloxodon antiquus* when referring to the taxon. The
159 new phylogeny suggests a remarkable degree of evolutionary transformation, from an
160 ancestor that possessed the features of the cranium and dentition of *Loxodonta*, shared
161 by both *L. africana* and *L. cyclotis* (Maglio 1973, Sanders et al. 2010), to a descendant
162 that is highly similar to *Elephas* (*sensu stricto*, the lineage of the Asian elephant) in many
163 morphological features. However, it should be noted that currently available genomic
164 data from elephantids only allow for reconstructing the broad picture of elephant
165 evolution. More complex evolutionary scenarios are conceivable, which might explain
166 the presence of some *Elephas*-like traits in *P. antiquus*. These could for example involve
167 gene flow, as has been shown for *L. africana* and *L. cyclotis* based on mitochondrial
168 evidence (Roca, Georgiadis, and O'Brien 2005). In addition, the very large effective
169 population size of the forest elephants (Rohland et al. 2010) could have allowed the
170 retention of ancestral traits by incomplete lineage sorting.

In summary, the molecular results presented here urge for a re-examination of morphology across the Elephantidae. This is especially important as the fossil record for elephants dates back several million years, well beyond the survival of ancient DNA. If, for example, *P. recki*, which was the most abundant Pleistocene elephant species in Africa, is indeed ancestral to *P. antiquus* and thus also represents a member of the *Loxodonta* lineage, the interpretation of the fossil record of elephantids in Africa is in strong need of revision. Furthermore, in contrast to the genera *Mammuthus* and *Elephas*, which also had their origin in Africa, the lineage of *Loxodonta* is generally assumed never to have left Africa. Although Osborn (1942) placed *Palaeoloxodon* in the Loxodontinae on the basis of several cranial characters, later authors (Shoshani et al. 2007, Todd 2010) have rejected this placement in favour of a placement in Elephantinae, restricting *Loxodonta* (and Loxodontinae) geographically to Africa. However, our data reveal that the *Loxodonta* lineage (as *Paleoloxodon*) also colonized the Eurasian continent. Last, the finding that *L. africana* is genetically more distant from *L. cyclotis* than is *P. antiquus* strongly supports previous evidence that urged recognition of *L. cyclotis* and *L. africana* as distinct species and underlines the importance of conservation efforts directed towards African forest elephants.

Material and Methods

Sampling, DNA extraction and library preparation

In January 2014, a fragment of an elephant long bone was discovered during work at the Ehringsdorf quarries. The specimen was removed from the lower travertine (~3m above the basis and 2.5 m below the Pariser horizon), which has been dated by micro probe U/Th-series dating of primary travertine (16) and ESR dating of tooth enamel (Schüler 2003) to ~ 233 ka. A piece of the bone (inventory number 14/18-1) was transferred to the ancient DNA laboratory at the MPI-EVAN in Leipzig. Ten extracts were prepared using between 36 and 53 mg of bone (totaling 425 mg) material following the method of Dabney et al. 2013 (Dabney et al. 2013). From these extracts, 30 libraries were prepared using single-stranded library preparation (Gansauge and Meyer 2013) with input volumes of 4, 8 and 12 µl DNA extract (of 25 µl extract volume), respectively. The number of library molecules was determined by digital droplet PCR using the QX200 system (Bio-Rad) with EvaGreen chemistry (QX200 ddPCR EvaGreen Supermix, Bio-Rad) and primers IS7 and IS8 (Meyer and Kircher 2010) following the manufacturer's instructions (Figure 2 – source data 1). Libraries were then amplified using AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific) (Dabney and Meyer 2012) and labeled with two sample-specific indices (Kircher, Sawyer, and Meyer 2012).

Ancient DNA work on the Neumark-Nord specimens was carried out in the ancient DNA laboratory at the University of Potsdam. Initially, 8 specimens were screened for the presence of elephant DNA of which the two best preserved ones (individual 23, Landesmuseum Halle museum inventory number HK 2007:25.285,117; a tusk fragment [NEU2A] and a fragmentary upper jaw [NEU8B]; inventory number HK 92:990) were selected for further analyses. In addition, in 2013, the petrous bone of individual 30 (NEPEC; inventory number HK 2007:25:280 = E15.1.96) was sampled and also used in the analysis. Fourteen DNA extracts were prepared from individual 30, and 6 from each of the two other specimens, using approximately 50 mg of bone powder in each extraction. DNA extraction and library preparation were performed as described above but including *Archaeoglobus fulgidus* uracil-DNA glycosylase in library preparation (Gansauge and Meyer 2013), which removes the majority of uracils that are typically present in ancient DNA fragments. In addition, to maximize yields in library preparation, two extracts (25 µl each) from each specimen were combined and 40 µl were used as input for library preparation. Reaction volumes in steps 1-3 of the protocol were

221 doubled to accommodate larger input volumes of extract. Optimal amplification cycle
222 numbers were established using qPCR (PikoReal Real-Time PCR system, Thermo Fisher
223 Scientific) with primers IS7 and IS8 (Gansauge and Meyer 2013). Libraries were then
224 amplified and labeled with one sample-specific index. After purification (MinElute PCR
225 purification kit, Qiagen), the different libraries for each sample were pooled.

226 ***Enrichment and sequencing of mtDNA***

227 52-mer capture probes for the enrichment of mtDNA sequences from elephants
228 were designed using the published mtDNA genome sequences of African forest elephant
229 (NC_020759), Asian elephant (NC_005129), African savanna elephant (NC_000934) and
230 the mastodon (NC_009574), with one probe starting at each position in these genomes.
231 Probes containing simple repeats longer than 24bp (repetition of the same 1-8 bp
232 sequence motif) were removed. Single-stranded biotinylated DNA probes were
233 generated as described elsewhere (Fu et al. 2013) and used for two successive rounds of
234 hybridization capture following a bead-based protocol (Maricic, Whitten, and Pääbo
235 2010). Enriched libraries were pooled and sequenced on one lane of an Illumina
236 HiSeq2500 in paired-end mode (2x 76 cycles plus 2x 7 cycles index reads; Weimar-
237 Ehringsdorf libraries) or on an Illumina NextSeq 500 (2x 76 cycles plus 1x 8 cycles index
238 read; Neumark-Nord libraries).

239 ***Mitochondrial sequence data processing and consensus calling***

240 Sequences were assigned to their source library requiring perfect matches to one
241 of the expected indices or index pairs and overlap-merged to reconstruct full-length
242 molecule sequences (Renaud, Stenzel, and Kelso 2014). Due to the different properties
243 of the data obtained from Weimar-Ehringsdorf and Neumark-Nord with regard to
244 sequence length distribution and damage patterns (Figure 2 – figure supplements 2 and
245 3), two different strategies were used for mapping and consensus calling. To minimize
246 the loss of alignments due to the high frequencies of damaged-induced substitutions in
247 the Weimar-Ehringsdorf data, mapping to the *L. cyclotis* mtDNA genome (JN673264)
248 was performed as previously described for the Sima de los Huesos mtDNA assemblies
249 (Dabney et al. 2013), using BWA and allowing up to five C to T substitutions but not
250 more than three of other types. The sequences from Neumark-Nord were mapped with
251 'ancient' parameters as described elsewhere (Meyer et al. 2012). PCR duplicates were
252 removed with bam-rmdup (Stenzel (2014) Biohazard, available from

253   https://bitbucket.org/ustenzel/biohazard) by calling a consensus from sequences with
254   identical alignment start and end coordinates. Sequences shorter than 30 bp were
255   discarded. An overview of the DNA extracts, libraries and sequences generated in this
256   study is provided in Figure 2 – source data 1.

257   When visually inspecting the Weimar-Ehringsdorf sequence alignments, we
258   identified several regions in the mitochondrial genome where more than one sequence
259   variant was present. Based on BLAST searches on a subset of these sequences, we found
260   that they derived from present-day human or microbial contamination. We thus aligned
261   all sequences to the identified contaminant genomes (GenBank accession nos.
262   NC_012920, AF365635 and CP008889) and removed sequences that showed a greater
263   similarity to one of the contaminants than to the African forest elephant mtDNA. No
264   removal of contaminant sequences was necessary for the Neumark-Nord samples. To
265   minimize the impact of damage-derived C to T substitutions on consensus calling, all T
266   occurring in the first and last three positions of the Weimar-Ehringsdorf sequences were
267   substituted by N. Next, a position-based tabular output was generated from the
268   alignment files using the 'mpileup' function of SAMtools (Li et al. 2009). This file was
269   used to call the consensus at positions with minimum sequence coverage of 3 if the
270   sequences were in at least 67.0% agreement. At three positions in the mtDNA genome
271   (positions 384, 8467, 8469) with low consensus support, we spotted obvious alignment
272   errors in one or all specimens and determined the consensus base manually. Apart from
273   a ~500 bp stretch of repetitive sequence in the D-loop, which cannot be reconstructed
274   with short DNA fragments, only 4 positions remain undetermined in the Weimar-
275   Ehringsdorf sequence and even fewer (between none and 3) in the Neumark-Nord
276   sequences.

277   ***MtDNA phylogenetic reconstructions***

278   We estimated mitochondrial phylogenies using the software BEAST (Drummond
279   et al. 2012) v 1.8.2 and a data set including 31 complete mitochondrial genomes
280   (GenBank accession nos.; *L. cyclotis*: JN673264, JN673263, KJ557424, KJ557423,
281   KY616976, KY616979, KY616978; *L. africana:* WA4020, KR0014, KR0138, NC000934,
282   DQ316069, AB443879; *E. maximus*: NC005129, DQ316068; *M. primigenius*: DQ316067,
283   NC007596, EU155210, EU153449, EU153455, EU153456, EU153458, EU153445,
284   EU153446, EU153447, EU153448, EU153452, EU153453, EU153454, JF912200; *M.*

285   *columbi*: JF912199). For three of the *L. cyclotis* mitogenomes (KY616976, KY616979 and

286   KY616978), only partial mitochondrial sequences were previously published. Full

287   genome sequences were obtained using previously collected samples (Ishida et al. 2013)

288   and the amplification and sequencing strategy detailed by (Brandt et al. 2012), except

289   that additional primers were used in sequencing (Figure 2 - source data 1). The

290   complete mitochondrial genome sequences were partitioned prior to analysis into four

291   partitions, representing concatenated genes (with ND6 reversed), tRNAs, rRNAs, and the

292   control region, and analyses were performed with and without the control region

293   fragment. All BEAST analyses were performed assuming the flexible skygrid coalescent

294   model (Gill et al. 2013) and the uncorrelated lognormal relaxed molecular clock

295   (Drummond et al. 2006). We calibrated the molecular clock using the ages of ancient tips

296   and a lognormal prior with a mean of 7.6 million years and standard deviation of

297   500,000 years for the divergence of the *Loxodonta* and *Elephas/Mammuthus* lineages

298   (Rohland et al. 2007). Ages of the ancient samples were sampled from normal

299   distributions derived from stratigraphic and previously estimated radiometric dates:

300   Neumark-Nord: 142-92 ka (Schüler 2010); Ehringsdorf 250-216 ka (Mallick and Frank

301   2002). Separate evolutionary rates and models of nucleotide substitution, as estimated

302   using jModelTest (Posada 2008), were estimated for each partition in the alignment. We

303   ran two MCMC chains for 60 million iterations each, with trees and model parameters

304   sampled every 6 thousand iterations. Chain convergence and parameter sampling were

305   examined by eye using Tracer v 1.6 (Rambaut A, Suchard MA, Xie D & Drummond AJ

306   (2014) Tracer v1.6, available from http://beast.bio.ed.ac.uk/Tracer). The first 10% of

307   samples were discarded from each run after which the two runs were combined. Trees

308   were summarized and maximum clade credibility (MCC) trees identified using

309   TreeAnnotator v 1.8.2, which is distributed as part of the BEAST package. MCC trees

310   were   edited   and   annotated   using   FigTree   v1.4.2

311   (http://tree.bio.ed.ac.uk/software/figtree/).

312   ***Shotgun sequencing of nuclear DNA***

313   Libraries from the three Neumark-Nord samples and another sample (not

314   included in this study) were pooled in equimolar concentrations and shotgun-sequenced

315   on a NextSeq 500 (2x 76bp cycles) at Harvard Medical School. Following determination

316   of endogenous content and complexity in each library, two of them (NEPEC from the

317   petrous bone and NEU2A from the molar fragment) were chosen for additional

11

318   sequencing and were pooled together with another sample (not included in this study)

319   for one additional run on Illumina's NextSeq 500.

320   ***Nuclear sequence data processing***

321   Sequences were assigned to their source library according to their index allowing

322   for one mismatch. Adapters were trimmed and paired-end sequences were merged with

323   SeqPrep 1.1 (https://github.com/jstjohn/SeqPrep) using default parameters but with a

324   modification in the source code to retain the best base quality scores in the merged

325   region. Merged sequences shorter than 30bp were discarded. Alignment to the African

326   savanna   elephant   reference   genome   (loxAfr4;   downloaded   from

327   ftp://ftp.broadinstitute.org/distribution/assemblies/mammals/elephant/loxAfr4/) was

328   performed with BWA's version 0.7.8 (Li and Durbin 2009) using 'ancient' parameters

329   and SAMtools' v.0.1.19 'samse' command (Li et al. 2009). A custom script was used to

330   remove duplicates, which takes into account the alignment coordinates of both ends of

331   the sorted sequences and their orientation.

332   From the first sequencing run, 47% and 35% of the sequences from the libraries

333   from the petrous bone and the molar fragment (NEPEC and NEU2A, respectively)

334   aligned to the reference genome while only 0.5% of the sequences from the third library

335   (NEU8B) aligned to the reference genome. The high percentage of mapped sequences in

336   the petrous sample is consistent with previous reports on the superior DNA

337   preservation in this part of the skeleton (Gamba et al. 2014). Following the second

338   sequencing run, the total endogenous content of the first two libraries was estimated to

339   39% and 28%, with an average sequence length of 39bp and 38bp, respectively (Figure

340   2 – source data 1). The average depth of coverage was 0.65-fold for NEPEC and 0.14-fold

341   for NEU2A. Both of them showed low frequencies of C to T to substitutions at the 5' and

342   3' end, which are characteristic for *Afu* UDG-treated single-stranded libraries (Gansauge

343   and Meyer 2013), except for in CpG context, where deamination of 5-methylcytosine

344   leaves thymine and not uracil (Figure 2 – figure supplement 3).

345   We also processed sequencing data from an African forest elephant (SL0001) that

346   was sequenced to high-coverage at the Broad Institute and re-processed sequencing

347   data of an Asian elephant from (Lynch et al. 2015). We trimmed adapters with SeqPrep

348   1.1 using default parameters and aligned paired-end reads to loxAfr4 using BWA's 'aln'

349   algorithm and SAMtools' 'sampe' command. Duplicate reads were removed with

350 SAMtools's 'rmdup'. Moreover, we used the high-coverage genome of a woolly mammoth
351 (Wrangel) from (Palkopoulou et al. 2015). The woolly mammoth alignments were re-
352 processed for removal of duplicate reads with the custom script mentioned above.

Nuclear DNA phylogenetic reconstruction

354   To determine the phylogenetic relationships between the two *P. antiquus*
355 specimens and other members of the *Elephantidae* family, we called pseudo-haploid
356 consensus sequences for all autosomes of the two *P. antiquus* samples (~770 and ~210
357 million sites, respectively). Sites with base quality below 30 and reads with mapping
358 quality below 30 were filtered out. To exclude post-mortem damage-derived C to T
359 substitutions, we trimmed 2bp from the ends of all reads. We included regions of the
360 loxAfr4 genome for which at least 90% of all possible 35-mers do not find a match at
361 another position allowing for up to one mismatch, similar to the mappability filter
362 described in (Prüfer et al. 2014). We used a majority-allele calling rule that required at
363 least one read aligned at each position of the genome. Using the same approach, we
364 called sequences for an Asian elephant (Uno (Lynch et al. 2015)), a woolly mammoth
365 (Wrangel (Palkopoulou et al. 2015)) and an African forest elephant (SL0001; Broad
366 Institute). We also used the reference sequence loxAfr4, as an African savanna elephant.
367 We estimated the number of differences per base-pair for pairwise comparisons of all
368 sequences and constructed a distance matrix, from which we built a Neighbor-joining
369 (NJ) tree using PHYLIP version 3.696 (Felsenstein 2005). To obtain support values for
370 the nodes of the tree, we performed a bootstrap analysis (100 replicates) by splitting all
371 autosomes in blocks of 5Mb and randomly sampling blocks with replacement and built a
372 majority-rule consensus tree.

373   We also extracted coding DNA sites (CDS) of protein-coding genes using the
374 Ensembl 87 release for the loxAfr3 genome (downloaded from
375 http://www.ensembl.org/) from each elephant genome sequence. CDS mapping to
376 unknown chromosomes as well as CDS containing partial codons were excluded,
377 resulting in a total of 86,212 CDS. Multiple sequence alignments were generated for each
378 gene using MAFFT –ginsi (Katoh et al. 2002, Katoh and Standley 2013) with 1,000
379 iterations, which were concatenated into a single fasta file. Maximum likelihood
380 phylogenetic analysis was performed with RAxML v8.2 (Stamatakis 2014) with the
381 GTRGAMMA model of nucleotide substitutions and 100 bootstrap trees. The resulting

382 phylogeny is identical in its topology to that of the NJ tree with 100% bootstrap support
383 (Figure 2 – figure supplement 4).

384 ***Dating the specimens***

385   Amino acid racemization (AAR) analyses were undertaken on the intra-
386 crystalline protein from four individual *Bithynia tentaculata* opercula from the Eemian
387 type-site, Amersfoort (Cleveringa et al. 2000): Amersfoort-1, upper depth 27.71, lower
388 depth 28.50 (NEaar 2982-3, 3972 & 4681) and compared with previously published
389 data from a single horizon at Neumark-Nord 1 (15.5.87/2, Schluffmudde, 25 cm under
390 Anmoor = surface of the lower shore area; NEaar 5698-5703 (Penkman 2010)) and
391 several horizons from Neumark-Nord 2 (Sier et al. 2011). All samples were prepared
392 using procedures of isolating the intra-crystalline protein by bleaching (Penkman et al.
393 2008). Two subsamples were then taken from each shell; one fraction was directly
394 demineralised and the free amino acids analysed (referred to as the 'free' amino acids,
395 FAA, F), and the second was treated to release the peptide-bound amino acids, thus
396 yielding the 'total' amino acid concentration, referred to as the 'total hydrolysable amino
397 acid fraction (THAA, H*). Samples were analysed in duplicate by RP-HPLC. During
398 preparative hydrolysis, both asparagine and glutamine undergo rapid irreversible
399 deamination to aspartic acid and glutamic acid respectively (Hill 1965). It is therefore
400 not possible to distinguish between the acidic amino acids and their derivatives and they
401 are reported together as Asx and Glx respectively. The D/L values of aspartic
402 acid/asparagine, glutamic acid/glutamine, alanine and valine (D/L Asx, Glx, Ala, Val) are
403 then assessed to provide an overall estimate of intra-crystalline protein decomposition
404 (Penkman et al. 2011).

405
406

Figure and data legends

408

409   **Figure 1:** *Palaeoloxodon antiquus*, geographic range based on fossil finds (after Pushkina
410   2007). White dots indicate the locations of Weimar-Ehringsdorf and Neumark-Nord.

411

412   **Figure 2**: Phylogenetic trees relating the mitochondrial and nuclear sequences of *P.*
413   *antiquus* (NN and WE) to other elephantids. **(A)** Maximum clade credibility (MCC) tree
414   resulting from a BEAST (Drummond et al. 2012) analysis of 35 complete mitochondrial
415   genomes using 15,447 sites. Node bars and numbers show the 95% highest posterior
416   density estimates for node ages and clade support, respectively. Mitochondrial
417   partitioning scheme and molecular and coalescent models are described in 'Materials
418   and methods'. (**B)** Pairwise-distance Neighbor-joining tree from between 210 million
419   and 2.5 billion base pairs of nuclear shotgun sequence data. Bootstrap support values
420   from 100 replicates are shown inside nodes. Summary statistics of the underlying
421   sequence data are available in Figure 2 – source data 1.

422

423   **Figure 3:** A revised tree of phylogenetic relationships among elephantids, color-coded
424   by their presumed geographical range.

425

426   **Figure 2 -figure supplement 1:** Sequence coverage of the NN and WE mitochondrial
427   genomes.

428

429   **Figure 2 – figure supplement 2:** DNA fragment size distribution inferred from full-
430   length mtDNA sequences.

431

432   **Figure 2 – figure supplement 3**: Frequency of C to T substitutions for each position in
433   the sequence alignments. **(A)** Substitution frequencies in mitochondrial alignments.
434   Substitution frequencies are depressed in the Neumark-Nord libraries due treatment
435   with uracil-DNA-glycosylase (UDG). **(B)** In nuclear sequence alignments, the

436   deamination signal could be partly restored by limiting analysis to cytosines in CpG
437   content. Since the majority of cytosines in CpG dinucleotides are methylated in
438   mammalian genomes, deamination leaves thymines, which are not excised by UDG.

439

440   **Figure 2 – figure supplement 4:** Maximum likelihood tree from concatenated nuclear
441   protein-coding sequences with bootstrap support values shown inside nodes.

442

443   **Figure 2 - figure supplement 5:** Amino acid racemization data. D/L values of Asx, Glx,
444   Ala and Val for the free amino acid (FAA, panels on the left) and total hydrolysable
445   amino acid (THAA, panels on the right) fraction of bleached *Bithynia tentaculata*
446   opercula from Amersfoort, Neumark-Nord 1 and 2. Ranges for samples from UK sites
447   correlated with MIS 5e and MIS 7 are indicative only, as effective diagenetic
448   temperatures are likely to have differed significantly between Britain and continental
449   Europe. The boundary of the box closest to zero indicates the 25th percentile, the
450   dashed line within the box marks the mean and the boundary of the box farthest from
451   zero indicates the 75th percentile. The 10th and 90th percentiles are represented by
452   lines above and below the boxes. The results of each duplicate analysis are included in
453   order to provide a statistically significant sample size.

454

455   **Figure 2 – source data 1**
456   This spreadsheet contains summary statistics of all sequence data generated in this
457   study, the sequences of PCR primers used for reconstructing mtDNA sequences of extant
458   elephants, as well as amino acid racemization data on opercula of *Bithynia tentaculata*
459   from   Amersfoort.

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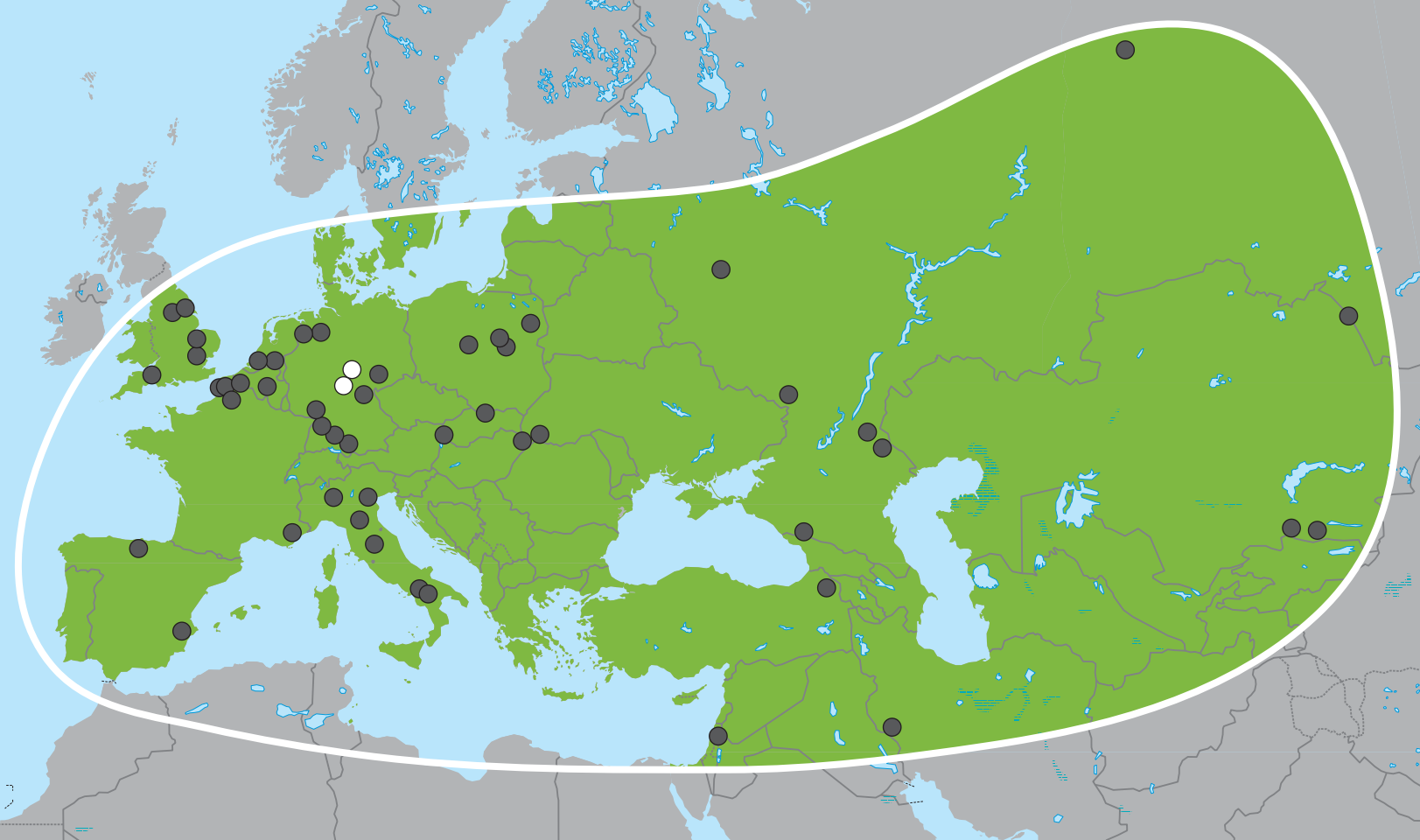601   *Geochronology* 3 (1-2):2-25. doi: 10.1016/j.quageo.2007.07.001.
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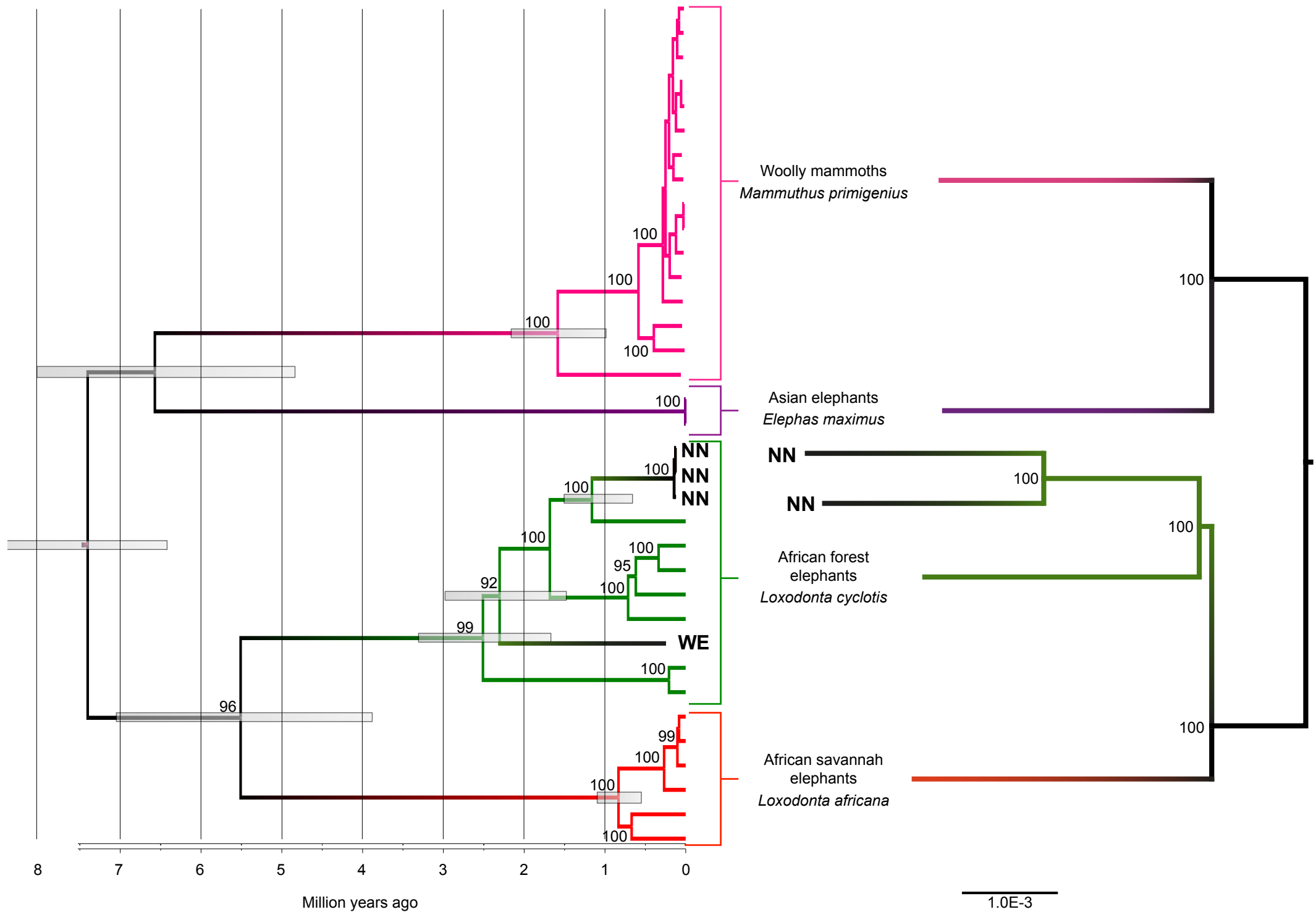608   doi: 10.1093/molbev/msn083.
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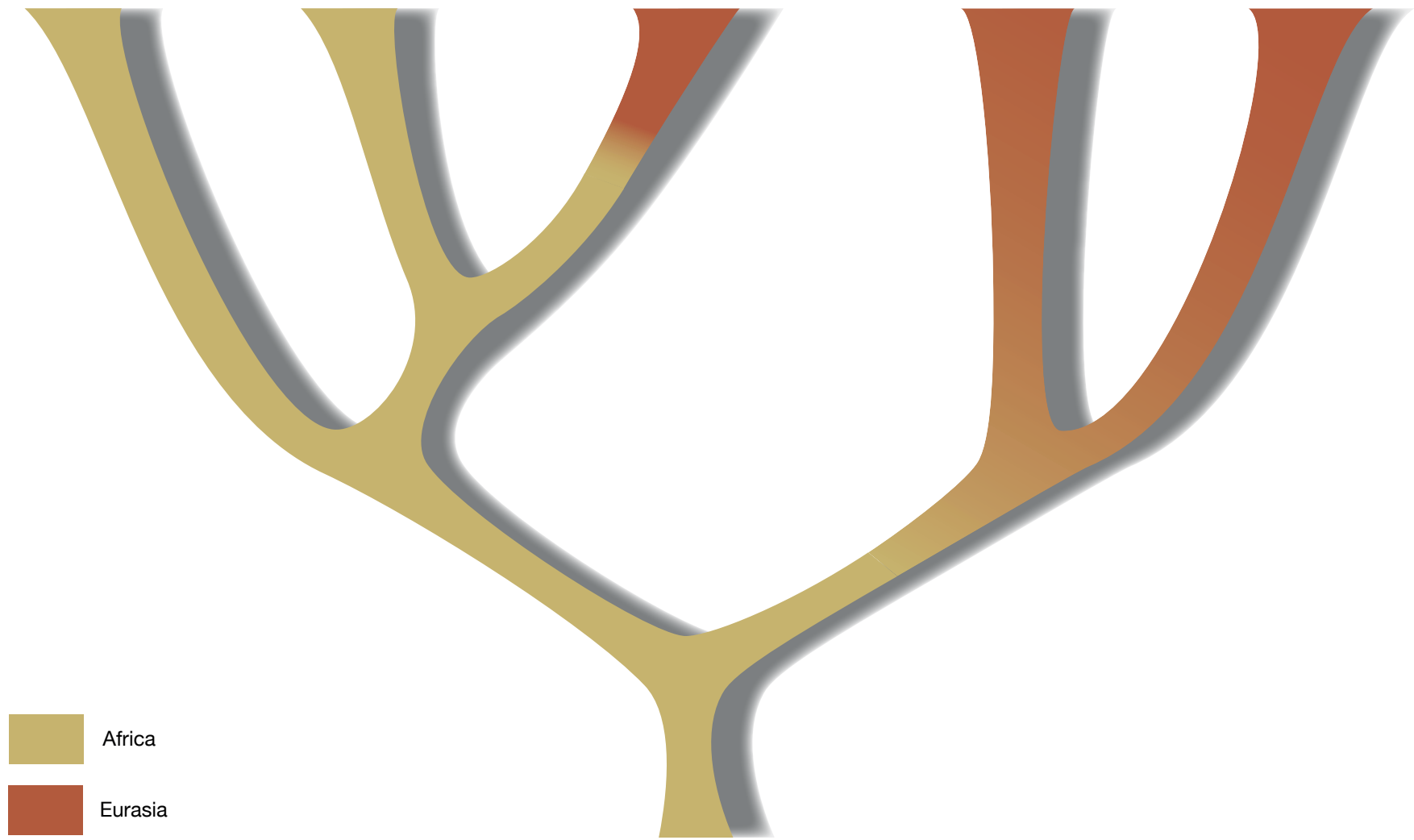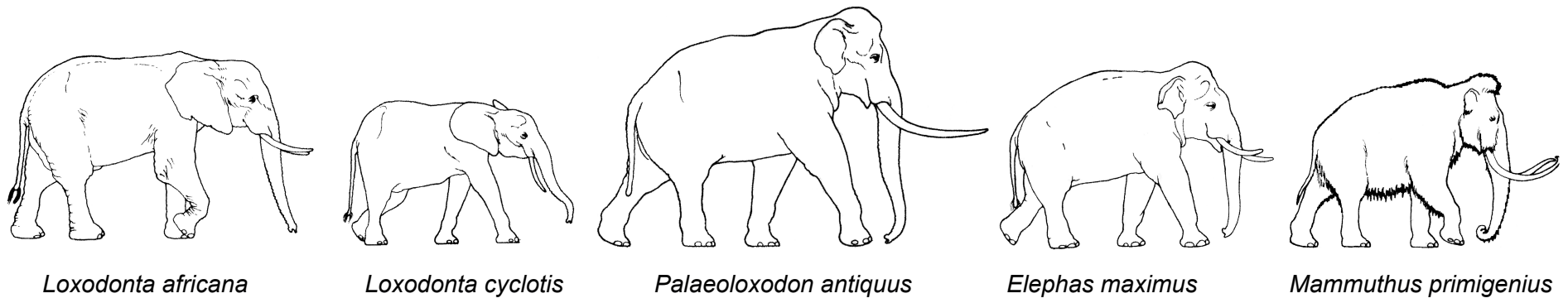617   10.1038/nature12886.

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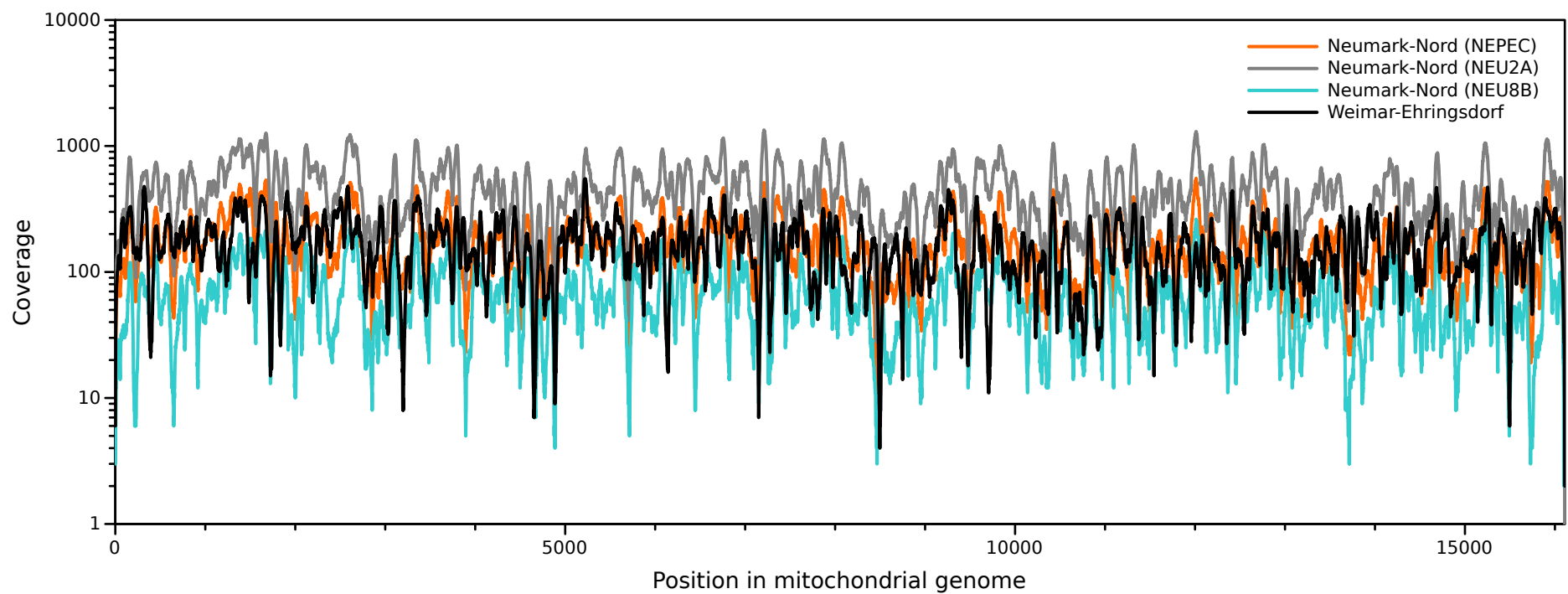663   Geologische Stichting, Nieuwe Serie* 14:15-45.
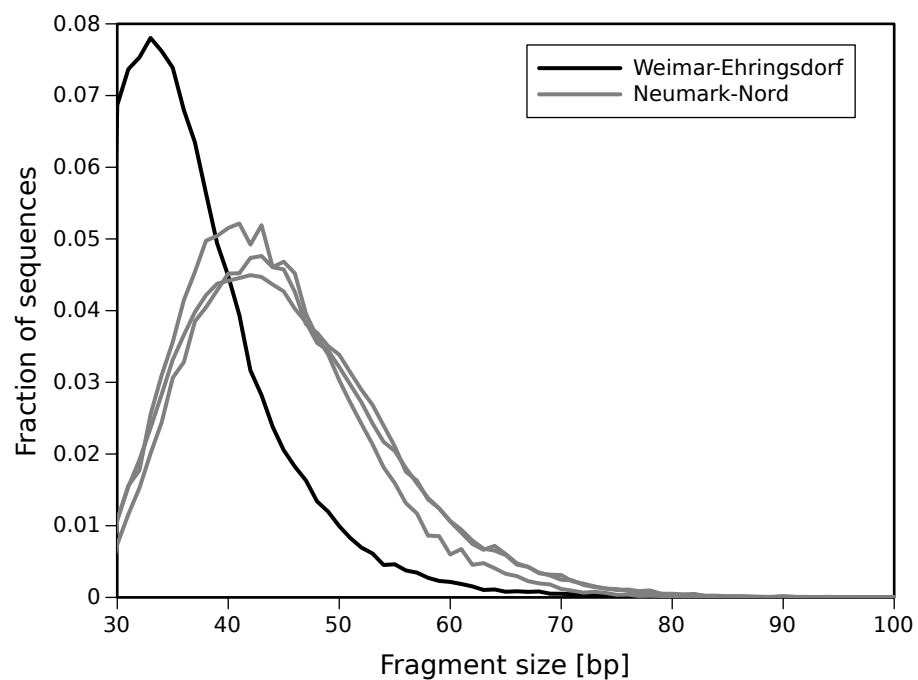
mtDNA

nuDNA

Woolly mammoths
Mammuthus primigenius

Asian elephants
Elephas maximus

NN
NN
NN

African forest
elephants
Loxodonta cyclotis

WE

African savannah
elephants
Loxodonta africana

Million years ago

1.0E-3

Loxodonta africana   *Loxodonta cyclotis*   *Palaeoloxodon antiquus*   *Elephas maximus*   *Mammuthus primigenius*

Africa

Eurasia

A

Weimar-Ehringsdorf
Neumark-Nord

B

NEPEC
NEU2A

Loxodonta cyclotis

100

Paleoloxodon antiquus NEU2A

100

100

Paleoloxodon antiquus NEPEC

Loxodonta africana

Mammuthus primigenius

100

Elephas maximus

6.0E-4