Genomic disintegration in woolly mammoths on Wrangel island

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Research Article

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Abstract

Woolly mammoths (*Mammuthus primigenius*) populated Siberia, Beringea, and North America during the pleistocene and early holocene. Recent breakthroughs in ancient DNA sequencing have allowed for complete genome sequencing for two specimens of woolly mammoths (Palkopoulou et al. 2015). One mammoth specimen is from a mainland population 45,000 years ago when mammoths were plentiful. The second, a 4300 yr old specimen, is derived from an isolated population on Wrangel island where mammoths subsisted with small effective population size more than 43-fold lower than previous populations. These extreme differences in effective population size offer a rare opportunity to test nearly neutral models of genome architecture evolution within a single species. Using these previously published mammoth sequences, we identify deletions, retrogenes, and non-functionalizing point mutations. In the Wrangel island mammoth, we identify a greater number of deletions, a larger proportion of deletions affecting gene sequences, a greater number of candidate retrogenes, and an increased number of premature stop codons. This accumulation of detrimental mutations is consistent with genomic meltdown in response to low effective population sizes in the dwindling mammoth population on Wrangel island. In addition, we observe high rates of loss of olfactory receptors, either because these loci are non-essential or because they were favored by divergent selective pressures in island environments. Finally, at the locus of *FOXQ1* we observe two independent loss-of-function mutations, which would confer a satin coat phenotype in this island woolly mammoth.
Introduction

Wooly mammoths (*Mammuthus primigenius*) were among the most populous large herbivores in North America, Siberia, and Beringia during the Pleistocene and early Holocene ([Stuart et al. 2004](#)). However, warming climates and human predation led to extinction on the mainland roughly 10,000 years ago ([Nogués-Bravo et al. 2008](#)). Lone isolated island populations persisted out of human reach until roughly 3,700 years ago when the species finally went extinct ([Vartanyan et al. 2008](#)). Recently, two complete high-quality high-coverage genomes were produced for two woolly mammoths ([Palkopoulou et al. 2015](#)). One specimen is derived from the Siberian mainland at Oimyakon, dated to 45,000 years ago ([Palkopoulou et al. 2015](#)). This sample comes from a time when mammoth populations were plentiful, with estimated effective population size of $N_e = 13,000$ individuals ([Palkopoulou et al. 2015](#)). The second specimen is from Wrangel Island off the north Siberian coast ([Palkopoulou et al. 2015](#)). This sample from 4,300 years ago represents one of the last known mammoth specimens. This individual comes from a small population estimated to contain roughly 300 individuals ([Palkopoulou et al. 2015](#)). These two specimens offer the rare chance to explore the ways the genome responds to pre-extinction population dynamics.

Nearly neutral theories of genome evolution predict that small population sizes will lead to an accumulation of detrimental variation in the genome ([Lynch 2007](#)). Such explanations have previously been invoked to explain genome content and genome size differences across multiple species ([Lynch 2006](#)). Yet, within-species comparisons of how genomes are changed by small effective population sizes remain necessarily rare. These mammoth specimens offer the unique opportunity for within-species comparative genomics under a 43-fold reduction in population size. This comparison offers a major advantage as it will be free from confounding biological variables that are present in cross species comparisons. If nearly neutral dynamics lead to an excess of detrimental variation, we should observe an excess of harmful mutations in pre-extinction mammoths from Wrangel Island.

We use these two ancient DNA sequences to identify retrogenes, deletions, premature stop codons, and point mutations found in the Wrangel Island and Oimyakon mammoths. We identify an excess of putatively detrimental mutations, with an excess of stop codons, an excess of deletions, an increase in the proportion of deletions affecting gene sequences, an increase in non-synonymous substitutions relative to synonymous substitutions, and an excess of retrogenes, reflecting increased transposable element activity. These data bear the signature of genomic meltdown in small populations, an empirical proof of principle for nearly-neutral genome evolution. They furthermore paint a dire portrait of detrimental variants collecting in pre-extinction genomes, a warning for continued efforts to protect current endangered species with small population sizes.
Results

Excess of amino acid substitutions and stop codons

We identified all SNPs in each mammoth genome using GATK (McKenna et al. 2010) and identified all non-synonymous and synonymous changes relative to the L. africana reference genome. We observe a significant increase in the number of heterozygous non-synonymous changes relative to synonymous changes in the Wrangel island genome compared with Oimyakon ($\chi^2 = 68.799$, $df = 1$, $P < 2.2 \times 10^{-16}$; Table S1). There is also a significant increase in the number of homozygous mutations at non-synonymous sites relative to synonymous sites ($\chi^2 = 9.96$, $df = 1$, $P < 0.0016$; Table S1). We further observe an excess of premature stop codons in the genome of the Wrangel Island mammoth, with 1.8X as many genes affected. There are 503 premature stop codons in the Oimyakon genome (adjusting for a 30% false negative rate at heterozygous sites) compared with 819 in the Wrangel island genome (Figure 1). There is a significant excess of olfactory genes that appear to be pseudogenized with an EASE enrichment score of 9.1 (Table S2) (Huang, Sherman and Lempicki 2009a;b). We observe 85 truncated olfactory receptors and 3 vomeronasal receptors as well as multiple signal transduction peptides compared with 44 olfactory receptors and 2 vomeronasal receptors pseudogenized in the mainland mammoth.

Deletions

We identify 13,318 deletions over 1 kb long in the Wrangel island genome, 8833 (correcting for a 3.48% false negative rate) in the Oimyakon genome (Table 1). There is a significant difference in the size distribution of deletions identified in the two mammoth samples, with a mean of 1423 bp in Oimyakon and 1324 bp in the Wrangel mammoth (Wilcoxon $W = 60252000$, $P < 2.2e-16$; Figure 2). No significant difference is observed between the Wrangel island mammoth down sampled sequence data ($\bar{x} = 1314$ bp, $W = 6683700$, $P = 0.7683$) suggesting that the observed decrease in size is not due to differences in coverage. Some 396 genes have deleted exons in the Wrangel Island mammoth compared to only 192 in Oimyakon (Table 1), a significant excess of genes deleted compared to expectations based on the number of deletions ($\chi^2 = 9.6878$, $df = 1$, $P = 0.001855$). Among these deleted genes, 12 in the mainland mammoth are homozygous compared to 17 homozygous exon deletions in the Wrangel Island Mammoth. A total of 6 homozygous mutations are shared between both mammoths. Gene functions for affected genes in the Oimyakon mammoth include steroid metabolism, cytochromes, drug metabolism, endopeptidases, apoptosis, keratin formation, ADP-ribose transferases, cytoskeleton, and alternative splicing (Table S3). Gene functions overrepresented among deletions in the Wrangel Island mammoth include only drug metabolism, zinc finger proteins, and cytochromes (Table S3).

Among the genes deleted in the Wrangel Island mammoth, several have phenotypes of interest in other organisms. We observe a hemizygous deletion in riboflavin kinase. Homozygous knockouts of riboflavin kinase, essential for B2 utilization/FAD synthesis, are
embryonic lethal in mice (Yazdanpanah et al. 2009). We also observe a deletion in TCTP, another embryonic lethal. The mammoth also carries a deletion in NSUN2, a gene involved in brain development associated with NSUN2 cognitive impairment in humans and fruit flies (Abbasi-Moheb et al. 2012). Several genes that are wholly or partially deleted may be expected to cause dominant, detrimental phenotypes. The Wrangel mammoth is hemizygous for a deletion in Spef1. Spef1 is necessary for sperm flagella structure (Chan et al. 2005). Heterozygous Spef1 mutants cause fishhook shaped sperm and partial sterility in mice (Olds-Clarke and Johnson 1993). If the phenotype in mammoths reflects the known partially dominant phenotypes in mice, this mammoth is likely to be partially sterile. The Wrangel Island specimen additionally has a hemizygous deletion in FOXC2. Missense mutations in FOXC2 in humans are associated with a dominant phenotype of lymphedemadistichiasis syndrome, associated with swelling of the limbs, aberrant eyelashes, and in some cases cleft palate (Connell, Brice and Mortimer 2008). Homozygous knockout mutants in mice develop abnormal lymphatic systems, consistent with the symptoms of lymphedema (Petrova et al. 2004). Finally, We identify a hemizygous deletion in the Wrangel island mammoth that would remove the entire gene sequence at the FOXQ1 locus. The alternative haplotype carries a frameshift mutation that disrupts the FOXQ1 functional domain. FOXQ1 knock-outs in mice are associated with the satin coat phenotype, which results in translucent fur but normal pigmentation due abnormal development of the inner medulla of hairs (Hong et al. 2001), to with two independent mutations producing this phenotype (Hong et al. 2001). FOXQ1 also regulates mucin secretion in the GI tract, a case of pleiotropic functions from a single gene (Verzi et al. 2008). If the phenotype in elephantids matches the phenotype exhibited in mice, this mammoth would have translucent hairs and a shiny satin coat, caused by two independently formed knock-out alleles at the same locus.

Retrogene formation

Retrogene formation can serve as a proxy for retrotransposon activity. We observe 1.3X more retrogenes formed in the Wrangel island mammoth. We identify retrogenes that display exon-exon junction reads in genomic DNA. The Wrangel Island mammoth has 2853 candidate retrogenes, in comparison with 2130 in the Oimyakon mammoth (Table 1). This excess of retrogenes is consistent with increased retroelement activity in the Wrangel Island lineage. During retrogene formation, highly expressed genes, especially those expressed in the germline, are expected to contribute to new retrogenes. To determine the types of loci that had been copied by retrotransposons, we performed a gene ontology analysis using DAVID (Huang, Sherman and Lempicki 2009a,b). Functional categories overrepresented among candidate retrogenes include genes involved in transcription, translation, cell division/cytoskeleton, post translational modification, ubiquitination, and chaperones for protein folding (Table S4-S5). All of these are expected to be highly expressed during cell divisions or constitutively expressed, consistent with expectations that highly expressed genes will be overrepresented. Gene ontologies represented are similar for both mammoths (Table S4-S5).
Genomic effects of demography

Under nearly-neutral theory of genome evolution, detrimental mutations should accumulate in small populations as selection becomes less efficient ([Lynch 2007](#)). This increase in non-neutral amino acid changes and premature stop codons is consistent with reduced efficacy of selection in small populations. We attempted to test this theory for silent and amino acid replacement substitutions whose mutation rates and selection coefficients are well modeled in the literature. Under nearly neutral theory, population level variation for non-synonymous amino acid changes should accelerate toward parity with population level variation at synonymous sites.

Given the decreased population size on Wrangel Island, we expect to observe an accumulation of detrimental changes that would increase heterozygosity at non-synonymous sites ($H_N$) relative to synonymous sites ($H_S$) in the island mammoth. Heterozygosity depends directly on effective population sizes. We observe $H_S = 0.00130$ in the Wrangel Island mammoth, which is 80% of $H_S = 0.00161$ observed in the Oimyakon mammoth (Table 2). To determine whether such results are consistent with theory, we fitted a model using PSMC inferred population sizes for the Wrangel island mammoth, based on decay of heterozygosity of $(1 - 1/2N)^tH_0$. The observed reduction is directly consistent theoretical expectations that $H_S = 0.00131$.

At non-synonymous sites, however, there are no closed-form solutions for reduced population sizes. We observe $H_N = 0.000490$ in the Wrangel Island Mammoth, 95% of $H_N = 0.000506$ in the Oimyakon mammoth (Table 2). To determine whether such results could be caused by accumulation of nearly-neutral variation, we simulated population trajectories estimated using PSMC. We were able to qualitatively confirm results that population trajectories from PSMC with previously described mutation rates and selection coefficients can lead to an accumulation of detrimental alleles in populations. However, the magnitude of the effects is difficult to fit precisely. The simulations show a mean $H_S = 0.00141$ and $H_N = 0.000379$ in Oimyakon and $H_S = 0.00120$ and $H_N = 0.000330$ for the Wrangel Island Mammoth (Figure [SI](#)). In simulations, we observe a reduction to 86% of previous levels, with simulated $H_N/H_S = 0.299$ in the Wrangel Island Mammoth. These numbers are less than empirical observations of $H_N/H_S = 0.370$ (Table 2).

Several possibilities might explain the observed disparity between simulations and data. The simulations may be particularly sensitive to perturbations from PSMC population levels or time intervals. Similarly, selection coefficients that differ from the gamma distribution previously estimated for humans might lead to greater or lesser changes in small populations. Additionally, an acceleration in generation time on Wrangel Island is conceivable, especially given the reduced size of Wrangel Island. Finally, positive selection altering nucleotide variation on the island or the mainland could influence diversity levels.
Discussion

Nearly neutral theories of genome evolution

Nearly-neutral theories of genome evolution have attempted to explain the accumulation of genome architecture changes across taxa (Lynch 2007). Under such models, mutations with selection coefficients less than the nearly neutral threshold will accumulate in genomes over time. Here, we test this hypothesis using data from a woolly mammoth sample from just prior to extinction. We observe a stark excess of retrogenes, deletions, amino acid substitutions, and premature stop codons in woolly mammoths on Wrangel Island. We further observe hemizygous mutations that based on phenotypes in mice and humans would be expected to have dominant phenotypes of lymphedema, and partial sterility. Given the long period of isolation and extreme population sizes observed in pre-extinction mammoths on Wrangel Island, it is expected that genomes would deteriorate over time. These results offer empirical proof of nearly-neutral theory of genetic changes, that under small effective population sizes, detrimental mutations can accumulate in genomes.

We observe two independent loss-of-function mutations in the Wrangel Island mammoth at the locus of FOXQ1. One mutation removes the entire gene sequence via a deletion, while the other produces a premature stop codon in the CDS. Based on phenotypes observed in mouse models, these two independent mutations would result in a satin fur coat, as well as gastric irritation (Verzi et al. 2008). Many phenotypic screens search for homozygous mutations as causative genetic variants that could produce disease. More recently, it has been proposed that the causative genetic variation for disease phenotypes may be heterozygous non-complementing detrimental mutations (Thornton, Foran and Long 2013). These data offer one case study of phenotypic change through independent non-functionalizing mutations in a single individual, empirical support for independent non-functionalizing mutations as the basis of phenotypic change.

Island specific changes

One of the two specimens comes from Wrangel Island, off the northern coast of Siberia. This mammoth population had been separated from the mainland population for at least 6000 years after all mainland mammoths had died off. Prior to extinction, some level of geographic differentiation combined with differing selective pressures led to phenotypic differentiation on Wrangel island. One possible explanation for the poor fit of simulations is that generation time may have decreased. Previous work suggested a very high mutation rate for woolly mammoths based on comparisons between island and mainland mammoths. It is possible that an acceleration in generation times could cause the accumulation of more mutations over time, and that the real mutation rate is similar to humans \((1 - 2 \times 10^{-8})\) (Scally and Durbin 2012) rather than \(3.8 \times 10^{-8}\) (Palkopoulou et al. 2015). Such changes would be consistent with island dwarfism being correlated with shorter generation times, and would explain the unusually high mutation rate estimate for mammoths based on branch shortening observed in (Palkopoulou et al. 2015).
We observe large numbers of pseudogenized olfactory receptors in the Island mammoth. Olfactory receptors evolve rapidly in many mammals, with high rates of gain and loss (Nei, Niimura and Nozawa 2008). The Wrangel island mammoth has massive excess even compared to the mainland mammoth. Wrangel island had different flora compared to the mainland, with peat and sedges rather than grasslands that characterized the mainland (Lozhkin et al. 2001). It is possible that island habitats created new selective pressures that resulted in selection against some olfactory receptors. Such evolutionary change would echo gain and loss of olfactory receptors in island Drosophila (Stensmyr, Dekker and Hansson 2003). It is equally possible that olfactory receptors are not essential and as such they are more likely to fall within the nearly neutral range Nei, Niimura and Nozawa (2008). Either of these hypotheses could explain the current data.

**Implications for conservation genetics**

Many factors contributed to the demise of woolly mammoths in prehistoric times. Climate change led to receding grasslands as forests grew in Beringea and North America. Human predation placed a strain on already struggling populations. Unlike many cases of island invasion, Wrangel Island mammoths would not have continuous migration to replenish variation after mainland populations went extinct. Under such circumstances, detrimental variation would quickly accumulate on the islands. The genetic load observed in these island mammoths, with the excess of deletions, especially genes for sterility, disease, and recessive lethals may also have limited survival of these struggling pre-extinction populations.

Many modern day species, including elephants, are threatened or endangered. Asiatic cheetahs are estimated to have fewer than 100 individuals in the wild (Hunter et al. 2007). Pandas are estimated to have 1600 individuals living in highly fragmented territories (Wang, Xu and Ouyang 2009). Mountain Gorilla population sizes have been estimated as roughly 300 individuals, similar to what as been shown for pre-extinction mammoths (Guschanski et al. 2009). If nearly neutral dynamics of genome evolution affect contemporary endangered species, detrimental variation would be expected in these genomes. With single nucleotide changes, recovered populations can purge detrimental variation in hundreds to thousands of generations, returning to normal genetic loads (Balick et al. 2015). However, with homozygous deletions, it is difficult to see how genomes could recover quickly. The realm of compensatory or back mutations to reproduce deleted gene sequences will be limited or impossible. Thus we might expect genomes affected by genomic meltdown to show lasting, irreversible repercussions that will prevent population recovery.

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presented in Lynch et al. 2015.

Supporting Data Files

Methods

We used previously aligned bam files from ERR852028 (Oimyakon) and ERR855944 (Wrangel) (Table S6) (Palkopoulou et al. 2015). We were not able to use two other mammoth sequences are publicly available, M4 and M25 from Lynch et al. (Lynch et al. 2015). These sequences display abnormal PSMC results (Figure S2), high heterozygosity (Figure S3), and many SNPs with asymmetrical read support (Figure S4). The unrealistically high heterozygosity as well as abnormal heterozygote calls raise concerns with respect to sequence quality. For further description, please see Supporting Information.

Synonymous and nonsynonymous substitutions

We used the GATK pipeline (McKenna et al. 2010) v3.4-0-g7e26428 to identify SNPs in the aligned sequence files for the Oimyakon and Wrangel Island mammoths. We identified and realigned all indel spanning reads according to the standard GATK pipeline. We then identified all SNPs using the Unified Genotyper, with output mode set to emit all sites. We used all CDS annotations from cDNA annotations from L. africana r3.7 and liftover coordinates to identify SNPs within coding sequences. We identified all stop codons, synonymous substitutions, and non-synonymous substitutions for the Wrangel Island and Oimyakon mammoths at heterozygous and homozygous sites.

Retrogenes

We aligned all reads from the mammoth genome sequencing projects ERR852028 (Oimyakon) and ERR855944 (Wrangel) (Table S6) against elephant cDNA annotations from L. africana r3.7. Sequences were aligned using bwa 0.7.12-r1044 (Li and Durbin 2009), with parameters set according to (Palkopoulou et al. 2015) bwa aln -l 16500 -o 2 -n 0.01 in order to account for alignments of damaged ancient DNA. We then collected all reads that map to exon-exon boundaries with at least 10 bp of overhang. Reads were then filtered against aligned genomic bam files produced by Palkopoulou et al (Palkopoulou et al. 2015), discarding all exon-exon junction reads that have an alignment with equal or better alignments in the genomic DNA file. We then retained all putative retrogenes that showed signs of loss for two or more introns, using only cases with 3 or more exon-exon junction reads.
**Deletions**

We calculated coverage depth using samtools \[ \text{Li et al.} 2009 \] with a quality cutoff of -q 20. We then implemented change point analysis \[ \text{Yao} 1988 \] in 20 kb windows. We allowed for a maximum of one CNV tract per window, with minimum of 1 kb and maximum of 10 kb (half the window size) with a 100 bp step size. We did not attempt to identify deletions smaller than 1 kb due to general concerns of ancient DNA sequence quality as well as stochastic variation in coverage. We then excluded all sequences with long tracts of ’N’s in the reference genome, where reads are not expected to align. To determine the effects that coverage differences would have on deletions, we downsampled the sequence file for the Wrangel Island mammoth using samtools, using chromosome 1 as a test set. We observe a reduction in the number of deletions for chromosome 1 from 1035 deletions to 999 deletions, resulting in an estimated false negative rate of 3.65% at reduced coverage.

**Demography**

We identified SNPs that differentiate Mammoth genomes from the reference using samtools mpileup (options -C50 -q30 -Q30), and bcftools 1.2 consensus caller (bcftools call -c). The resulting vcf was converted to fastq file using bcftools vcf2fq.pl with a minimum depth of 3 reads and a maximum depth of twice the mean coverage for each genome. Sequences were then converted to psmc fasta format using fq2psmcfa provided by psmc 0.6.5-r67. We then ran psmc with 25 iterations (-N25), an initial ration of $\frac{\theta}{\rho}$ of 5 (-r5), and parameters 64 atomic time intervals and 28 free parameters (-p ”4+25*2+4+6”) as was done in previous analysis of woolly mammoths \[ \text{Palkopoulou et al.} 2015 \]. Effective population sizes and coalescence times were rescaled using previously estimated mutation rates of $3.8 \times 10^{-8}$. Using the population size estimates from PSMC, we calculated the expected reduction in heterozygosity at synonymous sites according to $(1 - \frac{1}{2N})^t$ for each time period in PSMC output. We compared the number of deletions, number of premature stop codons, proportion affecting gene sequences, and number of putative retrogenes between the two mammoth genomes using chi squared tests.

**Simulations**

To determine expectations of sequence evolution at non-synonymous sites under population crash, we ran simulations using SLiM v. 2.0 population genetic software \[ \text{Messer} 2013 \]. We modeled two classes of sites: neutral and detrimental. For detrimental mutations we used a gamma distributed DFE with a mean of -0.043 and a shape parameter of 0.23 as estimated for humans \[ \text{Eyre-Walker, Woolfit and Phelps} 2006 \], assuming a dominance coefficient of 0.5 and free recombination across sites. Mutation rates were set as $3.8 \times 10^{-8}$ based on previously published estimates \[ \text{Palkopoulou et al.} 2015 \]. The trajectory of population sizes was simulated according to estimates from PSMC, omitting the initial and final time points from PSMC, which are often subject to runaway behavior. We then simulated the accumulation of $H_N/H_S$ in the Wrangel Island Mammoths. Simulations were run with a
burn-in of 100,000 generations. We simulated 50 replicates of haplotypes 100 sites for each mutation class.

**Gene Ontology**

To gather a portrait of functional categories captured by deletions, retrogenes, and stop codons, we identified all mouse orthologs based on ENSEMBL annotations for *L. africana* 3.7 for affected gene sequences. We then used DAVID gene ontology analysis with the clustering threshold set to ‘Low’ (http://david.ncifcrf.gov/) Accessed April 2016) (Huang, Sherman and Lempicki 2009a,b). Tables S2-S5 include all functions overrepresented at an EASE enrichment cutoff of 1.5. Full gene ontology data is included in Supplementary Information.
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Table 1: Mutations Identified in Mammoth Genomes

| Mutation                  | Oimyakon | Wrangel |
|---------------------------|----------|---------|
| Deletions                 | 8833\(^1\) | 13318   |
| Retrogenes                | 2130     | 2853    |
| Genes with exons deleted  | 199\(^1\) | 396     |
| Stop Codons               | 503\(^2\) | 819     |

\(^1\) Corrected for false negative rate of 3.48%

\(^2\) Corrected for false negative rate of 30% at heterozygous sites established by Palkopoulou et al 2015.
Table 2: Non-synonymous and Synonymous Heterozygosity

|        | Wrangel | Oimyakon¹ |
|--------|---------|-----------|
| \(H_S\) | 0.00130 | 0.00161   |
| \(H_N\) | 0.000490| 0.000506  |
| \(H_N/H_S\) | 0.370    | 0.314     |

¹ Oimyakon corrected for false negative rate of 30% established by Palkopoulou et al 2015.
Figure 1: Excess of putatively detrimental mutations in the Wrangel Island Genome. A) Deletions B) Genes deleted C) Retrogenes D) Premature stop codons. Numbers shown are corrected for false negative rates of 30% for heterozygous SNPs and 3.48% for deletions in the lower coverage Oimyakon mammoth.
Figure 2: eCDF for the size distribution of deletions in the Oimyakon and Wrangel Island genomes. There is a significant reduction in the size of deletions identified in the Wrangel Island Genome.
Supplementary Information

Analysis of samples M4 and M25

We aligned all major runs in the SRA for two *M. primigenius* specimens previously published, M4 and M25 (Table S6, Lynch et al. 2015). As a comparison for sequence quality, we also aligned and analyzed reads for one female *E. maximus* specimen sequenced and processed in the same study. The *E. maximus* sample, previously labeled in the SRA as “Uno”, is from Maya, a former resident of the San Diego Zoo wild-born in Assam, India, North American Studbook Number 223, Local ID #141002 (O. Ryder, personal communication). Previously published sequences for all three elephantids were aligned to the *L. africana* r.4.0 reference genome using bwa 0.7.12-r1044 (Li and Durbin 2009), with parameters set according to Palkopoulou et al. (2015) bwa aln -l 16500 -o 2 -n 0.01. Indels were identified and realigned using GATK as defined above. We then generated all SNPs using samtools mpileup (-C50 -u -g) and consensus fastq was generated using bcftools consensus caller (bcftools call -c) and bcftools vcf2fq.pl with a minimum depth threshold of 3 reads and a maximum depth of twice the mean coverage for each genome. Resulting fastq files were converted to psmcfa using the PSMC toolkit (Li and Durbin 2011). We then ran PSMC (Li and Durbin 2011) exactly as described in Palkopoulou et al. (2015), with 64 time intervals, (-p "4+25*2+4+6").

Demographic inference for mammoth samples from Oimyakon and Wrangel Island (Palkopoulou et al. 2015) show $N_e \leq 25,000$ (Figure S2). Analysis of samples M25 and M4 suggests $N_e$ in the range of $10^{10}$-$10^{11}$ over the history of woolly mammoths (Figure S2), a result that is inconsistent with estimates based on mtDNA (Barnes et al. 2007) or habitat availability (Nogués-Bravo et al. 2008). Demographic inference for Maya the elephant yields $N_e < 20,000$, with a bottleneck event roughly 200,000 years ago.

Given the inconsistencies in the M4 and M25 results, we examined heterozygosity data more directly for each of the samples, using chromosome 1 as an example dataset. We calculated heterozygosity for 10 kb windows in each mammoth and elephant sample. M4 and M25 both display high heterozygosity. We observe 30 heterozygous sites per 10 kb window in M4, and 38 heterozygous sites per 10 kb window in M25. These numbers are 2-3 fold higher than the observed mean of 11-14 sites per 10 kb window in Wrangel, Oimyakon, and Maya (Table S7, Figure S3). The abnormally high heterozygosity is likely to explain abnormal estimates of $N_e$ from PSMC. We then examined support for heterozygous SNP calls, using the first 5000 SNPs on chromosome 1 as a test set. If sites are truly heterozygous, there should be symmetrical support for each base by site. We identified sites with significantly skewed support in a binomial test. Mammoth specimens M4 and M25 from Lynch et al. 2015 have an excess of SNPs with significantly asymmetrical support compared to the Oimyakon and Wrangel mammoths, as well as Maya the elephant (Table S8, Figure S4A-S4E). There is a greater number of asymmetric sites that favor the reference allele than the non-reference allele in both M4 and M25 (Table S8, Figure S4A-S4B). Such asymmetry would be expected if some other elephantid DNA had contaminated these two samples. Multiple mammoths were sequenced in the lab, only some of which have been
published (http://mammoth.psu.edu/moreThanOne.html; accessed June 18, 2016). We are currently unable to examine all potential sources of contamination. These results left us concerned for the quality of the sequences. Hence, we did not include the two mammoth specimens M4 and M25 in the current genomic analysis of deletions, retrogenes, stop codons, or amino acid substitutions.
Table S1: Non-synonymous and synonymous sites

|                | Wrangel | Oimyakon¹ |
|----------------|---------|-----------|
| Heterozygous   | Non-synonymous | 12784    | 9445     |
|                | Synonymous             | 10231    | 8913     |
| Homozygous     | Non-synonymous          | 16149    | 13447    |
|                | Synonymous               | 21842    | 18950    |

¹ Raw numbers, without correction for changes in coverage.
Table S2: DAVID Gene ontology for premature stop codons in the Wrangel Island Mammoth

| Specimen | Function       | EASE score |
|----------|----------------|------------|
| Oimyakon | Olfactory receptors | 4.1        |
| Wrangel | Olfactory receptors | 9.1        |
|          | Ankyrin domains  | 1.6        |
Table S3: DAVID Gene ontology for deleted exons

| Specimen | Function                     | EASE score |
|----------|------------------------------|------------|
| Oimyakon | Steroid metabolism          | 3.1        |
|          | Cytochromes                  | 2.7        |
|          | Drug metabolism              | 2.7        |
|          | endopeptidase                | 2.6        |
|          | DAPIN apoptosis               | 2.2        |
|          | Keratin                      | 2.0        |
|          | ADP-ribose transferase       | 1.9        |
|          | Cytoskeleton                 | 1.9        |
|          | Alternative splicing         | 1.5        |
| Wrangel  | Drug metabolism              | 3.1        |
|          | Zinc finger                  | 2.4        |
|          | Cytochromes                  | 1.8        |
Table S4: DAVID Gene ontology for retrogenes in the Oimyakon Mammoth

| Function                        | EASE score |
|---------------------------------|------------|
| Ribosome                        | 6.3        |
| Post translational modification  | 4.4        |
| Lipoproteins                    | 3.4        |
| Spliceosome                     | 3.1        |
| RNA binding                     | 2.6        |
| Lipoprotein metabolism          | 2.2        |
| Nucleolus                       | 2.0        |
| Glutamine metabolism            | 1.9        |
| Aspartate metabolism            | 1.8        |
| Starch and drug metabolism      | 1.7        |
| Proteasome                      | 1.6        |
| Translation initiation          | 1.6        |
Table S5: DAVID Gene ontology for retrogenes in the Wrangel Island Mammoth

| Function                  | EASE score |
|---------------------------|------------|
| Ribosome                  | 8.3        |
| Ubl conjugation           | 6.8        |
| Spliceosome               | 4.3        |
| Translation initiation    | 2.8        |
| Lipoprotein               | 2.6        |
| Nuclear body              | 2.3        |
| Cytoskeleton              | 2.0        |
| Aminoacylation            | 1.8        |
| HEAT elongation           | 1.6        |
| RNA splicing              | 1.6        |
Table S6: SRA and ENA Identifiers for Mammoth and Elephant Sequence Data

| Specimen | Database ID   |
|----------|--------------|
| Oimyakon | ERR852028    |
| Wrangel  | ERR855944    |
| Maya     | SRX1015606   |
|          | SRX1015608   |
| M4       | SRX1015711   |
|          | SRX1015712   |
|          | SRX1015714   |
|          | SRX1015715   |
|          | SRX1015717   |
|          | SRX1015679   |
|          | SRX1015671   |
|          | SRX1015640   |
|          | SRX1015634   |
|          | SRX1015625   |
| M25      | SRX1015733   |
|          | SRX1015732   |
|          | SRX1015729   |
|          | SRX1015727   |
|          | SRX1015726   |
Table S7: Heterozygous sites per 10 kb

| Hets | Specimen |
|------|----------|
| 12   | Wrangel  |
| 14   | Oimyakon |
| 11   | Maya     |
| 30   | M4       |
| 38   | M25      |
Table S8: Asymmetrical Support

| Asymm SNPs | Favor Ref | Favor Alt | Specimen   |
|------------|-----------|-----------|------------|
| 498        | 166       | 332       | Wrangel    |
| 217        | 59        | 158       | Oimyakon   |
| 377        | 240       | 137       | Maya       |
| 1355       | 1179      | 176       | M4         |
| 2383       | 1859      | 524       | M25        |
Figure S1: Simulations for heterozygosity at synonymous and non-synonymous sites for the Oimyakon and Wrangel Island mammoths. Empirical values for the genome wide average are shown in blue.
Figure S2: PSMC results for four woolly mammoths and one elephant. M4 and M25 both display effective population sizes of $10^{10}$ or higher.
Figure S3: Heterozygosity for mammoth and elephant samples.
Figure S4: Asymmetric SNPs out of 5000 representative SNPs on chromosome 1.