2012

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Original Publication Citation

Ishida, Y., Roca, A. L., Fratpietro, S., & Greenwood, A. D. (2012). Successful genotyping of microsatellites in the woolly mammoth. *Journal of Heredity, 103*(3), 459-464. doi:10.1093/jhered/esr139

Journal of Heredity 2012:103(3):459–464
doi:10.1093/jhered/esr139
Advance Access publication January 10, 2012

Successful Genotyping of Microsatellites in the Woolly Mammoth

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Genetic analyses using ancient DNA from Pleistocene and early Holocene fossils have largely relied on mitochondrial DNA (mtDNA) sequences. Among woolly mammoths, *Mammuthus primigenius*, mtDNA analyses have identified 2 distinct clades (I and II) that diverged 1–2 Ma. Here, we establish that microsatellite markers can be effective on Pleistocene samples, successfully genotyping woolly mammoth specimens at 2 loci. Although significant differentiation at the 2 microsatellite loci was not detected between 16 clade I and 4 clade II woolly mammoths, our results demonstrate that the nuclear population structure of Pleistocene species can be examined using fast-evolving nuclear microsatellite markers.

Key words: *ancient DNA,* Mammuthus primigenius*, short tandem repeats, speciation*

Genetic studies employing ancient DNA have been conducted on Pleistocene fossils from a variety of species, including woolly mammoths (Barnes et al. 2007; Debruyne et al. 2008; Gilbert et al. 2008). While often highly informative, mitochondrial DNA (mtDNA) records the evolutionary history of only the maternal lineage, which may provide an incomplete picture for species in which dispersal differs substantially between males and females (Roca et al. 2005, 2007; Petit and Excoffier 2009). For example, among elephants, gene flow between core social groups (herds) is male mediated because females do not typically migrate between herds (Poole 1989; Nyakaana and Arctander 1999; Archie et al. 2008). Sex differences in dispersal and in the variance of reproductive success may be responsible for the dissimilar phylogeographic patterns sometimes detected between mtDNA and nuclear loci in elephants (Hoelzer 1997; Roca et al. 2005, 2007; Hedrick 2007; Petit and Excoffier 2009; Rohland et al. 2010; Ishida et al. 2011).

Analyses of mtDNA from large numbers of temporally and geographically diverse woolly mammoth fossils have demonstrated the presence of multiple mtDNA lineages (Barnes et al. 2007; Debruyne et al. 2008; Gilbert et al. 2008), with the most basal split occurring between mtDNA clades I and II, which diverged 1–2 Ma (Gilbert et al. 2008). The deep split detected in woolly mammoth mtDNA may reflect speciation events, migrations, habitat changes, or glacial cycles (Barnes et al. 2007; Debruyne et al. 2008; Gilbert et al. 2008; Miller et al. 2008). Alternatively, because the social structure of woolly mammoths was likely similar to that of extant African elephants (Haynes 1991), the deeply divergent clades of mammoth mtDNA may be consequent to sex differences in dispersal and in the variance of reproductive success, believed responsible for the dissimilar phylogeographic patterns detected for mtDNA and nuclear loci in African elephants (Hoelzer 1997; Hedrick 2007; Rohland et al. 2010; Enk et al. 2011; Ishida et al. 2011).

The use of nuclear DNA markers can provide a more complete picture of population structure among elephants than the use of mtDNA alone (Ishida et al. 2011). Unlike mtDNA, nuclear genetic patterns reflect the contributions of maternal and paternal lineages. Nuclear markers, such as microsatellites, have been developed for extant elephants. To examine their utility in woolly mammoths, we tested elephant microsatellite markers on woolly mammoths. We successfully genotyped 20 woolly mammoths at 2 short tandem repeat (STR) loci, and compared microsatellite patterns between specimens belonging to each of the 2 highly divergent mammoth mtDNA clades, to determine whether microsatellite loci support the separation of mammoths into divergent groups concordant with the deep separation detected using mtDNA.

Materials and Methods

Mammoth Samples and Ancient DNA Extraction

Woolly mammoth samples were kindly provided by 3 collections. Mammoths provided by the American Museum of Natural History were from Alaska, the Taimyr Peninsula (Russia), and Wrangel Island (Russia) and were designated AMNH, AMNH_Tai, and AMNH_WRA, respectively.

Table 1 Mammoth sample information

Museum number	Locality and sample type	Age[a]	Sample provider[b]
AMNH493	Alaska, locality unkown, dentin	NA	RM
AMNH8460	Engineer Creek, Alaska, dentin	13 775 ± 145 bp	RM
AMNH_JARKOV	Lake Taimyr	20 380 ± 140 bp	RM
AMNH_RM103	Lake Taimyr	NA	RM
AMNH_RM23	Lake Taimyr	NA	RM
AMNH_RM24	"Hook" mammoth, Kruchok, r humerus	20 550 ± 70 bp	RM
AMNH_RM27	Pilot Site, Arilakh, l humerus	43 130 ± 1280 bp	RM
AMNH_RM4	L. Taimyr, Baskura Peninsula, r humerus	26 080 ± 170 bp	RM
AMNH_WRA_SP5	Wrangel Island	NA	RM
AMNH_WRA_SP6	Wrangel Island	4590 ± 50 bp	RM
WMM_BOE1	Bolshoy Lyakhovsky Island, mandible	NA	GB
WMM_BOE4	Kolyma River, skull	Kargin interglacial	GB
WMM_BOE5	Alazeya River, mandible	NA	GB
WMM_BOE7	Indigirka River basin, skull	NA	GB
WMM_BOE10	Adycha River, Ulakhan Sullar, rib	NA	GB
WMM_BOE16	Indigirka River basin, tooth fragment	11–13 000	GB
WMM_BOE20	Vilyui River, Namskeya Terasa, mandible	NA	GB
PI_DUB1835	NA	NA	ID
PI_DUB3067	NA	NA	ID
PI_yakutia	NA	NA	ID

[a] NA indicates locality or dating data not available.

[b] RM: Ross DE MacPhee, American Museum of Natural History; GB: Gennady Boeskorov, Mammoth Museum, Yakutsk; and ID: Irena Dubrovo, Paleontological Institute, Moscow.

Samples provided by the World Mammoth Museum in Yakutsk were designated WMM; samples from the Paleontological Institute, Moscow were designated PI. Localities and known ages of samples are listed in Table 1. Ancient DNA extractions of the samples and all preamplification work were performed in the Paleo-DNA Laboratory, an accredited forensics "Clean Lab" at Thunder Bay, Canada, dedicated to research using ancient and degraded DNA. We followed procedures established for ancient DNA (Greenwood and Paabo 1999; Paabo et al. 2004; Willerslev and Cooper 2005; Roca et al. 2009), as detailed in Supplementary Data.

PCR Amplification and Sequencing

For STR loci, PCR primers were tagged for fluorescence detection (Boutin-Ganache et al. 2001). PCR amplification used a touchdown protocol (see Supplementary Data). Microsatellite products were separated on an Applied Biosystems (ABI) 3100 Genetic Analyzer using a GeneScan-350 ROX size standard (ABI) and Hi-Di Formamide (ABI). Results were analyzed with GeneMapper ID v3.2 (ABI). For 7 of the mammoth samples, microsatellite scoring was conducted independently by 2 workers (Y.I. and S.F.) with identical outcomes.

To improve amplification success rate with ancient DNA, we tested 8 primer pairs designed to produce shorter amplicon sizes at microsatellite loci (Ishida et al. forthcoming). These had been developed to improve amplification success for DNA extracted from African elephant dung samples (and were named by adding "s" for "short" to the end of the original locus designation). Only 2, FH60s and MS04s, worked consistently among mammoth samples, the others either failing to amplify or working on only few samples. In African elephants, these 2 loci are not in linkage disequilibrium (Comstock et al. 2002). Mammoth microsatellites FH60s and MS04s were sequenced to confirm the identity of the locus for samples WMM_BOE16 and WMM_BOE10.

For mtDNA, PCR used forward primer 5′-GCTCTA-CAAGCAATACTTTATAATCG-3′ and reverse primer 5′-AAATTGGGCTGATTTTCCTG-3′. The PCR and sequencing reactions for woolly mammoth DNA followed previously established procedures (Greenwood and Paabo 1999; Roca et al. 2009), with details included as Supplementary Data. Sequences of mtDNA and of microsatellites were too short to deposit in Genbank and can also be found in the Supplementary Data.

Phylogenetic Analyses

Mitochondrial sequence data were aligned using ClustalX version 2.0 (http://www.clustal.org/) (Larkin et al. 2007), and alignments were inspected visually. Phylogenetic relationships among mtDNA haplotypes were assessed using 4 approaches implemented in PAUP* 4.0b10 (Altivec) (Swofford 2002), as detailed in Supplementary Data.

Statistical Analyses

Genetic diversity was estimated using the Excel microsatellite toolkit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/) (Park 2001) and ARLEQUIN 3.1.1 (Excoffier and Lischer 2010). Population differentiation between clade I and clade II mammoth microsatellite alleles for each locus and across 2 loci was tested by Fisher's exact probability test based on 1000 steps in Markov chain algorithms in GENEPOP (http://genepop.curtin.edu.au/) (Rousset 2008). Exact tests of Hardy–Weinberg equilibrium using a Markov

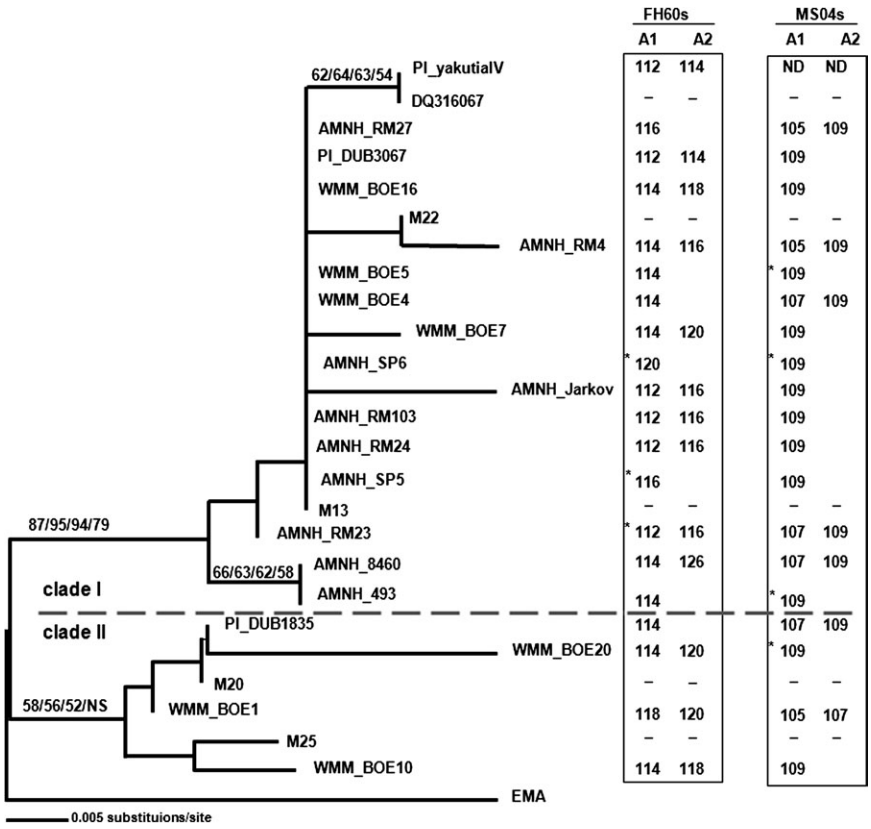

Figure 1. Mitochondrial and nuclear analyses of mammoth samples. Left panel shows a phylogenetic tree inferred using 60 bp of the mitochondrial NADH dehydrogenase subunit 4 (*NADH4*) sequenced from 20 mammoth samples, along with sequences from GenBank (M13, M20, M22, and M25) (Gilbert et al. 2008); clade I and clade II mtDNAs were identified. The tree was rooted with an Asian elephant sequence (GenBank accession AJ428946) designated EMA. The neighbor joining (NJ) tree is shown, with bootstrap values for (left to right) maximum parsimony, NJ, minimum evolution, and maximum likelihood methods; NS indicates not supported. Right panel shows allele sizes at 2 microsatellite loci, FH60s and MS04s, corresponding to each mammoth listed directly to the left in the phylogeny. One allele size (A1) is listed for homozygotes; 2 allele sizes (A1 and A2) are listed for heterozygotes. Genotypes for which the number of successful replications was less than the minimum recommended (3 for heterozygotes and 4 for homozygotes) are indicated by an asterisk (*); ND indicates that alleles were not detected.

chain (forecasted chain length: 1 000 000) were performed with ARLEQUIN 3.1.1 (Excoffier and Lischer 2010). F_{st} between clade I and clade II mammoths was estimated using microsatellite data in GENEPOP (http://genepop.curtin.edu.au/) (Rousset 2008).

Results

Two primer pairs (FH60s and MSO4s; the latter renamed from the African elephant microsatellite LafMS04) (Nyakaana and Arctander 1998; Fernando et al. 2001) yielded results across our 20 mammoth samples which, compared with other tested microsatellite markers (see Supplementary Data), were consistent, unambiguous, and polymorphic. An example of the microsatellite profile of mammoth DNA for FH60s and MS04s is shown in Supplementary Figure 1. The identity of the 2 loci in mammoths was confirmed by sequencing. Heterozygotes could be identified when both alleles were present in one PCR or when single alleles of different sizes were amplified in separate PCR replicates. Allelic dropouts were calculated as the proportion of PCRs on identified heterozygotes that amplified only 1 of the 2 alleles (Allentoft et al. 2011). Calculated allelic dropout ratios were 0.59 for FH60s and 0.47 for MS04s. This is high but comparable to the dropout rates of 0.36–0.70 (average 0.53) observed for microsatellite loci in the most analogous previous ancient DNA study, conducted on moa specimens (Allentoft et al. 2011). For FH60s and MS04s, we attempted to confirm heterozygosity among mammoths by scoring the results of 3 independent PCR amplifications. Homozygotes were confirmed using at least 4 independent amplifications, as has been previously suggested for forensic and ancient DNA (Allentoft et al. 2011). Successful amplifications for each sample ranged from 3 to 8 (average 5.55) for FH60s and from 2 to 9 (average 5.47) for MS04s.

For microsatellite FH60s, 13 of 20 mammoths were heterozygous and a total of 6 alleles were detected across the

Table 2 Observed (H_o) and expected (H_e) heterozygosity at 2 microsatellite loci in mammoths

Locus	H_o	Clade I (Ho)	Clade II (H_o)	H_e	Clade I (H_e)	Clade II (H_e)
FH60s	0.65	0.63	0.75	0.76	0.76	0.71
MS04s	0.37	0.33	0.50	0.36	0.30	0.61

mammoths (Figure 1). Allele sizes (112, 114, 116, 118, 120, and 126) were largely consistent with stepwise mutation of a 2-bp repeat. For MS04s, among 19 successful samples, 7 individuals were heterozygous and a total of 3 alleles were identified. Allele sizes (105, 107, and 109) were consistent with stepwise mutation of a 2-bp repeat. As would be expected, rare alleles were not found to be homozygous: For locus FH60s, only the 2 most common alleles, 114 and 116, were homozygous in any mammoths; for locus MS04s, the relatively rarer alleles 105 and 107 were detected only as heterozygotes.

We established that the specimens used for microsatellite genotyping (below) include 16 clade I and 4 clade II mammoths (Barnes et al. 2007; Debruyne et al. 2008; Gilbert et al. 2008) (Figure 1). For the 2 microsatellite loci, levels of observed and expected heterozygosity were similar whether within-clade or across all mammoths (Table 2). Both loci were found to be in Hardy–Weinberg equilibrium when all samples were included ($P > 0.05$); this result is notable because specimens from even a single locale could reflect allele changes that can occur locally over long spans of time. Although the data were limited to variation at only 2 microsatellite loci across 20 individuals, we looked for evidence of nuclear differentiation between mammoths carrying clade I and those with clade II mtDNA. For both loci, alleles observed among the 4 clade II mammoths were also found in clade I mammoths (Figure 2). F_{st} values between clade I and clade II mammoths did not support strong differentiation at either nuclear locus: F_{st} was 0.043 for FH60s, 0.019 for MS04s, and 0.036 for the 2 loci combined. For MS04s, the distribution of alleles was similar across mammoths in the 2 clades (Figure 2), and differentiation between the clades was not statistically supported (Fisher's exact test, $P = 0.616$). For FH60s, the distribution of microsatellite alleles across mammoths in the 2 mtDNA clades (Figure 2) appeared to be somewhat less similar than for MS04s, although differentiation between the clades did not reach statistical significance for FH60s ($P = 0.052$). The combined loci did not demonstrate differentiation ($P = 0.142$), and additional STR genotyping would be required to reach strong conclusions about woolly mammoth nuclear genetic differentiation (Koskinen et al. 2004).

Discussion

Although microsatellites have been characterized in extinct animals, such as moas (Allentoft et al. 2009, 2011), this is the first attempt to our knowledge at microsatellite analysis on a collection of Pleistocene fossils. We demonstrated that microsatellite genotyping methods can be modified to accommodate Pleistocene fossils across time spans comparable to those for which mtDNA diversity can be examined. Only two microsatellite markers out of a larger set tested (see Supplementary Data) worked consistently in this study. This is similar to results reported for moa microsatellites: of 89 primer pairs tested in moas (including some developed in related species), only 5 loci proved effective for genotyping (Allentoft et al. 2011).

With highly degraded Pleistocene DNA, nuclear amplicon lengths must be limited. Allelic dropout may be especially high in ancient DNA due not only to selective amplification of short alleles (Wattier et al. 1998) but also to stochastic effects in which the low copy number of DNA can lead to only one of the alleles in a heterozygote being randomly amplified in the earlier steps of PCR (Taberlet

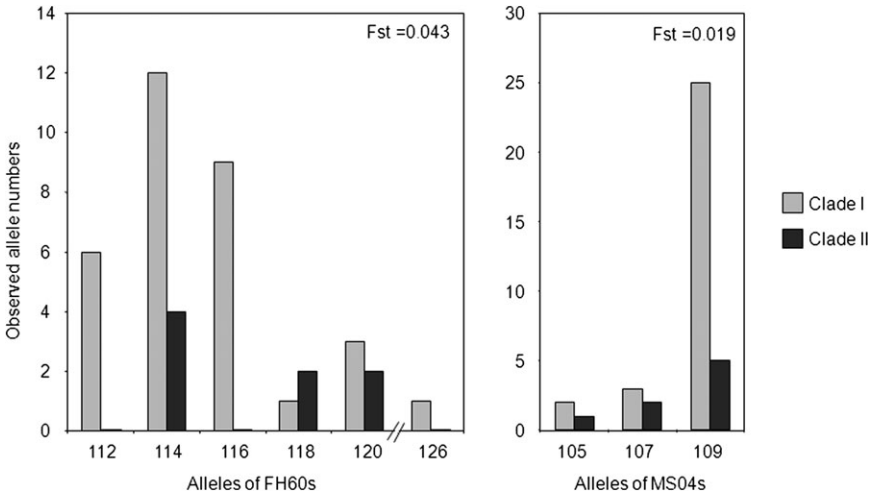

Figure 2. Allelic counts at 2 microsatellite loci in woolly mammoths. Totals are for mammoths carrying mtDNA clade I (light shading) or clade II (dark shading), with F_{st} between clade I and clade II mammoths indicated for each locus.

et al. 1996). For the 2 successful microsatellite loci, calculated dropout rates were high, 0.59 and 0.47 for microsatellite loci FH60s and MS04s, respectively, emphasizing the requirement for replicate PCRs to accurately genotype samples. After several replicates, mammoths were successfully genotyped and revealed substantial heterozygosity, in line with expected values (Table 2).

The distinctive mammoth mtDNA clades uncovered by previous studies may at least in part reflect the matrilocal and matrilineal structure of elephantid social groups (Roca et al. 2005, 2007; Enk et al. 2011). We found that mammoth genotypes at 2 microsatellite loci did not reveal significant differentiation between clade I and clade II mammoths, although any conclusion must be qualified because the data set was comprised of 2 loci and the sample size for Clade II mammoths was limited. Our results nonetheless demonstrate that it is possible to generate a more complete picture of population structure for Pleistocene species using fast-evolving nuclear microsatellite markers.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Funding

Thomas F. and Kate Miller Jeffress Memorial Trust (J-855).

Acknowledgments

The authors wish to thank Michele Tisdale and Shannon Hensley for laboratory support. We thank Ross MacPhee of the American Museum of Natural History; Gennady Boeskorov of the Institute for Diamond and Precious Metals Geology, Siberian Branch of Russian Academy of Sciences, Yakutsk; and Irina Dubrovo of the Paleontological Institute, Moscow, for providing mammoth samples and information about dates and localities.

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Received September 15, 2011; Revised November 1, 2011; Accepted November 16, 2011

Corresponding Editor: William Murphy