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Population-level genotyping of coat colour polymorphism in woolly mammoth (*Mammuthus primigenius*)

Claire Workman [a], Love Dalen [b], Sergey Vartanyan [c], Beth Shapiro [d], Pavel Kosintsev [e], Andrei Sher [f,1], Anders Gotherstrom [g], Ian Barnes [a,*]

[a] School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, England, UK
[b] Molecular Systematics Laboratory, Swedish Museum of Natural History, SE-104 05, Stockholm, Sweden
[c] Department of Geography, Herzen University, nab. Moyki, 48, St. Petersburg, 191186, Russia
[d] Department of Biology, The Pennsylvania State University, 208 Mueller Laboratory, University Park, PA 16802, USA
[e] Institute of Plant and Animal Ecology, Russian Academy of Sciences, 202 8 Marta Street, 620144 Ekaterinburg, Russia
[f] Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences (RAS), 33 Leninskiy Prospect, 119071 Moscow, Russia
[g] Department of Evolutionary Biology (EGS), Uppsala University, SE-752 36 Uppsala, Sweden

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ABSTRACT

Patterns in the spatial or temporal distribution of genotypes may be indicative of natural selection. Previous work on the woolly mammoth melanocortin-1 receptor (*Mc1r*) gene identified three polymorphic positions that suggest Pleistocene populations may have harboured both light- and dark-haired mammoths (Rompler et al., 2006, 313: 62). Here, we extend this work and present the first population-level analysis of a functional gene in an extinct species. We genotyped the *Mc1r* gene in 47 woolly mammoth samples excavated from sites across the central portion of the woolly mammoths' former range to examine the extent of variation of this polymorphism through time and across space. Only one individual was found to be heterozygous, indicating that the frequency of the 'light' mutant allele was very low. We conclude that light-coloured woolly mammoths would have been very rare, and may even have been non-existent if the 'light' mutant allele was strongly selected against in its homozygotic form. With the increasing availability of large-scale sequencing technologies, population-level datasets capable of identifying local adaptation will become increasingly attainable.

1. Introduction

The distribution of phenotypic variation within and between populations is of fundamental importance to our understanding of the evolutionary process. Any heritable variation exhibited may be due to the effects of genetic drift; however it could also be due to natural selection (Stearns and Hoekstra, 2001). Whilst changes in phenotype through time and space may be indicative of selection, identifying the genetic basis for those changes is challenging. The fitness advantage that the alleles of interest confer to the animal must be determinable, and preferably quantifiable. Furthermore, the molecular pathways underlying a particular trait are often complex and poorly understood. Despite these difficulties, there are a number of well-supported examples where genetic polymorphism has been shown to cause phenotypic variation among populations under differential selection (Hedrick, 2006).

The ability to study the genetics of polymorphic traits has been important in understanding the evolution of these traits, in terms of identifying cases of convergent evolution, examining the type of selection that maintains the polymorphism, as well as in establishing past species histories. Systems where a phenotypic variant can be confidently derived from genotype are particularly interesting in palaeontology where information about traits such as appearance, behaviour and diet would otherwise be inaccessible by analysis of traditional data types. One such system is language, where analysis of Neanderthal remains has identified sequence similarities in the FOXP2 gene with modern-day humans, which suggest similarities in the development of language and speech (Lai et al., 2001; Krause et al., 2007). Hair colour is another such system. Ancient DNA sequencing of fossil horses indicates an increase in coat colour variation shortly after the beginnings of horse domestication (Ludwig et al., 2009), and analysis of the Neanderthal *Mc1r*

* Corresponding author. Tel.: +44 (0)1784 443562.
E-mail address: ian.barnes@rhul.ac.uk (I. Barnes).
[1] Deceased.

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gene has led to the proposal of red-haired, pale-skinned individuals (Lalueza-Fox et al., 2007). Furthermore, previous ancient DNA work on the *Mc1r* gene in woolly mammoths identified three non-synonymous polymorphic positions that defined two allelic variants of mammoth *Mc1r*; allele 1 (Thr^{21}–Arg^{67}–Arg^{301}) and allele 2 (Ala^{21}–Cys^{67}–Ser^{301}). Functional testing showed partial loss of function with allele 2, which, at least in its homozygotic state, might have been sufficient to result in an individual with lighter coat colour. These findings suggested a light/dark coat colour polymorphism in populations of Pleistocene woolly mammoth (Rompler et al., 2006). Hair found both loose and on frozen carcasses of woolly mammoths had already suggested coat colour variation, with a range in colouration from blonde to orange to black, although it has also been proposed that this variation is due to post-mortem diagenetic changes to hair chemistry (Lister and Bahn, 2007).

However, none of the studies described above have examined the degree of polymorphism on a population level. This omission makes it difficult to examine the degree of phenotypic variation among geographical regions, and to identify changes in allele frequencies through time caused by genetic drift or natural selection. In this case, the study by Rompler and colleagues raised an obvious question: how was the coat colour polymorphism maintained in this species? One possibility is that coat colour was an adaptive trait, and that any temporal changes observed were correlated with known past climate-induced changes in the environment, or that spatial variation was due to differences among habitats. Alternatively, if the phenotypic variation was functionally unimportant then the effects of genetic drift may have caused the less common morph to rise to high frequency when population size was small (Stearns and Hoekstra, 2001).

In this study we examine the evidence of temporal or spatial structure in the distribution of coat colour polymorphism in the woolly mammoth. Using 108 samples from different geographical regions across Russia and North America, we sequenced short fragments of the *Mc1r* gene where the three polymorphic positions are located.

2. Materials and methods

2.1. Samples

The samples used in this study came from North America ($n = 12$) and different geographical regions of Russia; Vologda Oblast ($n = 2$), Taimyr ($n = 26$), mainland Yakutia ($n = 20$), the New Siberian Islands ($n = 19$), and Chukotka ($n = 29$) (Fig. 1). While the samples from the Chukotka and Taimyr regions were previously unanalysed, the remaining samples included in this study had yielded either long fragments of mitochondrial DNA or nuclear DNA during previous analyses (Barnes et al., 2007; Gilbert et al., 2008; Dalén et al., unpublished data; Supplementary Table S1).

2.2. Ancient DNA extraction

DNA extractions and PCR setups were carried out in a dedicated ancient DNA laboratory that was physically separated from any post-PCR DNA handling. Bone, teeth and tusk samples were cleaned by mechanical removal of the surface before extraction commenced. Subsequently, each sample was ground to powder with a drill and then incubated overnight under rotation at 56 °C in extraction buffer (0.45 M EDTA pH 8.0, 0.1 M urea and 100 μg proteinase K). The samples were then extracted using the silica-binding approach (Yang et al., 1998). Extractions were carried out using batches of 12 samples; each batch contained at least one extraction control blank. Finally, Tween-20 mixture was added to the extracts to a final concentration of 0.05%.

2.3. Ancient mitochondrial DNA amplification

Amplification of the terminal 23 bp of tRNA-Pro and the first part (363 bp) of the hypervariable portion of the control region was carried out firstly to ensure the newly extracted mammoth samples from the Chukotka and Taimyr regions contained endogenous DNA, and secondly for the purposes of haplogroup assignment. The following primers were used in the amplification: mam_15393F

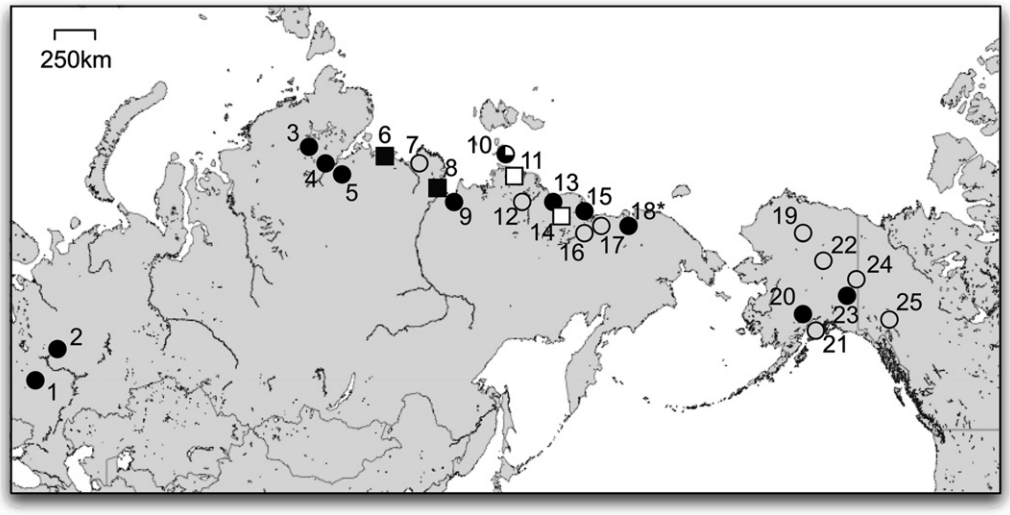

Fig. 1. Map of geographical distribution of samples used in this study along with the four samples analysed by Rompler et al. (2006), which are shown as squares. The colour of the symbols indicates the frequency of individuals with heterozygote genotypes (white) and individuals homozygotic for the 'dark' allele (black) at each site. Grey circles indicate sites which failed to yield amplification products. Sites of sampled *Mammuthus primigenius*: Vologda Oblast – 1. Tula, 2. Kineshma; Taimyr Peninsula – 3. Peshnaya, 4. Khatanga; 5. Taimyr (Kuehlkohn Lake); Yakutia - 6. Olenek-Anabar interflow, 7. Nagym, 8. Bykovsky Peninsula, 9. Buor-Khaya Peninsula, 10. Bolshoy Lyakhovsky Island, 11. Oyagosskiy Yar, 12, 13. Indirka Lowland, 14. River Kolopatkaya, 15. Bolshaya Chukochya River, 16, 17. Kolyma Lowland; 18. Chukotka; Alaska - 19. Sixtymile Creek, 20. Gold Hill, 21. Seward, 22. Fairbanks Creek, 23. Lost Chicken Creek, 24. Dome Creek, 25. Canada (Cold Run Creek). *$n = 9$; number of sites from which samples were found within 1° longitude and latitude of one another.

and mam_15780R. The primers were the same as those used to amplify part of a 741 base pair long fragment of mitochondrial DNA by Barnes and colleagues (Barnes et al., 2007).

PCR amplifications of the mitochondrial fragment were performed in 25 μL reactions with 2 μL of extract and 1U Platinum *Taq* Hi-Fidelity Polymerase (Invitrogen, Carlsbad, CA). PCR control blanks were used in each amplification; each control contained the reaction components but with no added DNA. PCR amplicons were visualised on agarose gels stained by ethidium bromide. Samples were sequenced using an ABI3730XL machine by Macrogen (Seoul, Korea).

2.4. Phylogenetic analyses

A previous study had proposed that the two primary mitochondrial clades found in our study area might represent sympatric species (Gilbert et al., 2008). In order to test the possibility that these two haplogroups might demonstrate differentiation at the *Mc1r* locus, we assigned haplogroup status by sequence comparison to previously published individuals from both clades, via construction of a neighbour-joining tree under an HKY85 model of substitution (Hasegawa et al., 1985) using PAUP*4.0b10 (Swofford, 2002).

2.5. Ancient nuclear DNA amplification

Each of the three polymorphic positions previously identified (Rompler et al., 2006) was sequenced using single nucleotide polymorphism (SNP) typing. PCR reactions were designed to produce very short fragments (SNP A – 48 bp; SNP B – 50 bp; SNP C – 60 bp including primers) of the *Mc1r* gene where these three polymorphic positions are located.

In order to design the PCR primers two mammoth *Mc1r* sequences available in the GenBank Database were used (accession numbers DQ648860 and DQ648859; Rompler et al., 2006). Each primer pair was aligned with human, cow and mouse *Mc1r* sequences. In doing so it was found that it was not possible to discriminate between cow and mammoth when amplifying the region around SNP B. Since cow DNA is a common contaminant in PCR reagents (Leonard et al., 2007), the reverse primer was extended to amplify a 64 bp fragment that included a diagnostic base for cow.

Amplifications of the three polymorphic positions within the *Mc1r* gene were performed in 50 μL reactions with 2 μL of extract and 1U HotstarTaq Polymerase (Qiagen, Sussex, UK). Biotinylated reverse primers were used in the amplification, as pyrosequencing requires biotinylated products. At least one PCR control blank was used in each batch of amplifications.

PCR reaction conditions were the same for the amplification of both the mitochondrial fragment and the three nuclear polymorphic positions; annealing temperatures however, differed between primers (Supplementary Table 2). Reaction conditions were as follows: 94 °C for 4 min, 55 repetitions of denaturation at 94 °C for 30 s, annealing at between 52 °C and 57 °C for 30 s (depending on the primer being used), extension at 68 °C for 30 s, final elongation at 68 °C for 6 min and finally cooled to 10 °C for 1 min. PCR amplicons were visualised on agarose gels stained by ethidium bromide.

2.6. Pyrosequencing

The short fragments of the *Mc1r* gene were sequenced using pyrosequencing technology. PCR products were prepared by immobilizing 30–45 μl of the biotinylated product on streptavidin-coated Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) by incubating at room temperature for 10 min in 1X PSQ binding buffer (5 mM Tris–HCl, 1 M NaCl, 0.5 mM EDTA, 0.05% Tween-20 [pH 7.6]). The binding buffer was replaced with denaturation solution by removing all liquid using a vacuum; the sample was then incubated for 1 min and washed twice with 150 μl Washing buffer. The sequencing primer was added in a 55 μl volume of 35 μM primer in 1X annealing buffer (20 mM Tris–Acetate, 5 mM $MgAc_2$ [pH 7.6]) and heated for 2 min at 80 °C. Pyrosequencing (Ronaghi et al., 1998) was carried out on a PSQTM 96 MA pyrosequencer using the SNP software and SNP reagent kit (Biotage, Uppsala, Sweden) according to the manufacturers instructions. The PSQTM 96 MA SNP software (Biotage, Uppsala, Sweden) was used to determine the nucleotide dispensation order, as well as the SNP-genotype and surrounding sequence and, in the contamination assay, to quantify the respective alleles.

3. Results

3.1. Target mitochondrial fragment

Out of the 55 woolly mammoth samples from the Chukotka and Taimyr regions analysed in this study 37 were successfully sequenced for the targeted mitochondrial fragment (Supplementary Table 1). No contamination was detected in any of the extraction or PCR control blanks used.

3.2. Phylogenetic analysis

One of the 37 newly extracted woolly mammoth samples belonged to clade 2, with the remaining 36 samples falling into clade 1. These results are concordant with previous work that has identified clade 2 individuals as rare (c. 7% of all individuals analysed to date). The clade 2 samples observed in the studies by Barnes et al. (2007) and Gilbert et al. (2008) were from the region between the Lena and Kolyma rivers. An additional study however, found one clade 2 individual in the Taimyr Peninsula (Debruyne et al., 2008).

3.3. Mc1r polymorphisms

Of the 108 woolly mammoth samples used in the study 47 yielded sequences for all three targeted polymorphic positions (Supplementary Table 3.). Contamination was not detected in any of the extraction or PCR control blanks. One individual out of the 47 samples was heterozygous for the three positions. The remaining 46 samples were homozygous for the wild-type allele (allele 1). It is well known that apparently homozygous genotypes can be artefactual results of a process known as allelic dropout, where one of the alleles in a heterozygous individual fails to amplify during PCR. Conversely, observations of heterozygous SNP-genotypes can result from misincorporation during PCR. To confirm the obtained results, we therefore conducted a second amplification and genotyping for a subset of samples from Siberia ($n = 25$), for each of the three SNP positions ($n = 75$). Individuals that were homozygous during the first genotyping ($n = 24$) were observed as homozygous also during replication. This, together with the observation that all homozygous genotypes were scored for the same allele in both replicates, as well as the sensitivity of pyrosequencing analysis, which can detect weak alleles down to 5% strength compared to the dominant allele, indicates that the lack of heterozygosity in these samples is not the result of allelic dropout during the PCR process. For the sample identified as heterozygous during the initial analysis, however, we recovered both alleles, for all three SNPs, also during the second genotyping.

4. Discussion

This study confirms the presence of the allele for light fur colour in Pleistocene populations of the woolly mammoth, but suggests that it occurred at very low frequency (c. 0.01). Of 47 individuals successfully tested, the mutant allele was found only once. This sample, found on Bolshoy Lyakhovsky Island and dated to >55600 uncal BP, was interestingly identified both as a relatively small individual (by A.S.), and to fall within mitochondrial clade 2, a group previously identified as an exclusively Siberian population.

Further investigation of other samples failed to support any association between the blonde allele, and geographical location, skeletal phenotype or mitochondrially-determined population affiliation. Three other samples from this location were genotyped but no other carriers of this mutant allele were detected. Two further samples of relatively small individuals were also genotyped to investigate whether this mutant allele was particularly abundant in smaller mammoths, but both samples were homozygous for the wild-type allele (allele 1). Finally, a total of eight clade 2 individuals were screened, but no other mutant alleles were detected. Furthermore, an analysis of other samples taken across part of the woolly mammoths' range failed to find the mutant allele, and we thus conclude that it must have persisted within the population at a low frequency.

In different species of animals melanocortin-1 receptor dysfunction caused by loss of function mutations within the *Mc1r* gene results in either yellow or red hair (Rees, 2003). In the woolly mammoth the second ($Arg^{67}Cys$) substitution has been identified as the main cause of melanocortin-1 receptor dysfunction (Rompler et al., 2006). A light-coloured subspecies of the beach mouse has been found with an arginine to cysteine substitution at the homologous position of their *Mc1r* gene (Hoekstra et al., 2006), supporting the proposal that any woolly mammoths that were homozygous for the 'light' allele would have had pale hair rather than darker, red hair (Rompler et al., 2006) as is observed in humans as a result of MC1R dysfunction (Frandberg et al., 1998; Rees, 2000). On the basis of the pattern observed in other taxa (e.g. rock pocket mice, Nachman et al., 2003; Kermode bears, Ritland et al., 2001; red fox, Våge et al., 1997) it is likely that mammoths identified as heterozygous in the *Mc1r* gene would have had dark hair, as the wild-type 'dark' allele is typically dominant over the mutant 'light' allele. In order to observe light-coloured hair, an individual would thus need to be homozygous for the mutant allele. Given the low mutant allele frequency suggested by this study, it is unlikely that many, if any, light-haired mammoths existed. We would therefore suggest that the colour differences observed in permafrost-preserved mammoth hair relate primarily to diagenesis, and are unrepresentative of the living animal.

Earlier work done by Rompler et al. (2006) found that two out of the four samples they genotyped were heterozygous individuals; the other two were homozygous for the wild-type allele (allele 1). The first heterozygous mammoth was recovered from the River Kolopatkaya in the Kolyma Lowland area of Yakutia, and dated to 42,960 radiocarbon years before present. We genotyped three samples found within 300 km of this sample but we did not detect the mutant allele in any of them. The second heterozygous sample (Rompler et al., 2006) was found in Oyagosskiy Yar on mainland Yakutia, approximately 120 km away from where our heterozygous sample was found, and has been dated to 40,700 radiocarbon years before present. The heterozygous individual found in our study was dated as older than 55,600 radiocarbon years before present. The small number of heterozygous mammoths that have been found to date ($n = 3$) makes it difficult to evaluate whether there was any spatial or temporal structure in the distribution of *Mc1r* alleles. However, the close proximity of the heterozygous individuals on Bolshoy Lyakhovsky Island and Oyagosskiy Yar is noteworthy (Fig. 1). During the last Ice Age, what are now the New Siberian Islands were connected to the mainland due to a decrease in sea level. The occurrence of two heterozygous mammoths in relatively close proximity to one another could indicate the presence of a population of woolly mammoths harbouring this mutant allele. Other woolly mammoth remains that could provide evidence for this may now lie underneath the Dmitry Laptev Strait, which covers the land bridge that once connected the New Siberian Islands to the mainland.

The work by Rompler and colleagues suggested a particularly high frequency of the mutant 'light' allele in Pleistocene woolly mammoth populations (c. 0.25), and as a consequence that the occurrence of light-coloured mammoths in the population would be highly likely. However, the genotyping of many more samples, from a much larger area of the woolly mammoth's former range, and the detection of only one individual carrying this mutant allele suggests it was extremely rare. Even the results of the two studies combined give a frequency of the mutant allele at 0.03. One interpretation is that this allele arose fairly recently, and did not have time to penetrate the population to any significant degree. However the pattern of mutations in this allele, consisting of three nonsynonymous substitutions, is compatible with significant antiquity. It is, therefore, more likely that it conferred no significant advantage to its carrier, as alleles that confer a fitness benefit to an organism in comparison with other alleles of the same gene will typically increase within the population (Stearns and Hoekstra, 2001). Conversely, recessive deleterious alleles can persist at low frequencies in a population even if the homozygous phenotypes (e.g. light-coloured mammoths) are strongly selected against.

The possibility that some spatial or temporal structure exists for adaptive loci in mammoth cannot be rejected from these data, and the current rapid increase in sequencing availability make it likely that large-scale analysis will be feasible in the near future. Future work could include genotyping additional samples from Bolshoy Lyakhovsky Island, Oyagosskiy Yar and the Kolyma Lowland, where the three heterozygous individuals were found. If further sequencing of additional mammoth samples does reveal a localised distribution of higher frequency light-coloured genotypes, we can foresee two possible explanations to the existence of mammoths with light-coloured fur; crypsis or thermal adaptation. Differences in coat colour observed in other organisms such as mice have been proposed as an adaptation driven by predation from visual hunters (e.g. Hoekstra et al., 2004). While adult mammoths are unlikely to have been sufficiently preyed upon to drive selection for coat colour camouflage, it may have provided some protection for neonates. However, in this case any benefit would only be seasonal; unlike the habitat of rock pocket and beach mice whose environment is uniform across the year, mammoth would most likely have experienced both spatial and temporal variation in their habitat (Guthrie, 2001). Alternatively, light coat colour may have been beneficial to woolly mammoths living in particularly cold and windy environments, where light coat colour is thermally advantageous due to the greater radiative heat loads acquired through increased penetration of radiation into light versus dark coats (Walsberg et al., 1978).

In conclusion, this study represents the first population-level study of a functional gene in an extinct species. We find that although Pleistocene populations did harbour individuals with both the wild-type and mutant alleles, the frequency of the mutant allele was low enough to suggest that light-coloured woolly mammoths probably were very rare, or even non-existent. Whilst the data presented here fail to support evidence for regional adaptation in mammoth, this approach is clearly one that in the future will demonstrate features of extinct fauna that would otherwise remain obscure.

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Appendix. Supplementary material

Supplementary data associated with the article can be found in online version at doi:10.1016/j.quascirev.2010.08.020.

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