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Twenty-five thousand years of fluctuating selection on leopard complex spotting and congenital night blindness in horses

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Leopard complex spotting is inherited by the incompletely dominant locus, *LP*, which also causes congenital stationary night blindness in homozygous horses. We investigated an associated single nucleotide polymorphism in the *TRPM1* gene in 96 archaeological bones from 31 localities from Late Pleistocene (approx. 17 000 YBP) to medieval times. The first genetic evidence of *LP* spotting in Europe dates back to the Pleistocene. We tested for temporal changes in the *LP* associated allele frequency and estimated coefficients of selection by means of approximate Bayesian computation analyses. Our results show that at least some of the observed frequency changes are congruent with shifts in artificial selection pressure for the leopard complex spotting phenotype. In early domestic horses from Kirklareli–Kanligecit (Turkey) dating to 2700–2200 BC, a remarkably high number of leopard spotted horses (six of 10 individuals) was detected including one adult homozygote. However, *LP* seems to have largely disappeared during the late Bronze Age, suggesting selection against this phenotype in early domestic horses. During the Iron Age, *LP* reappeared, probably by reintroduction into the domestic gene pool from wild animals. This picture of alternating selective regimes might explain how genetic diversity was maintained in domestic animals despite selection for specific traits at different times.

1. Introduction

Leopard complex spotting has been a popular coat colour phenotype of domestic horses since ancient times (figure 1). In Europe, horses with these phenotypes were most likely highly regarded, as they are depicted carrying royalty and noblemen from early medieval times onwards [3]. Today, leopard complex spotting phenotypes are frequently found in a range of breeds from Asia (Altai Horses, Mongolian Pony), America (Appaloosa, American Miniature Horse, Colorado Ranger Horse, Falabella, Pony of the Americas, Spanish Mustang) and Europe (British Spotted Pony, Knabstrupper, Noriker, Karabaier). Leopard complex spotting (*LP*) is inherited in an autosomal incomplete dominant mode. Despite its popularity, the underlying genetics of this phenotype was, for a long time, unknown. *LP* was mapped to horse chromosome 1
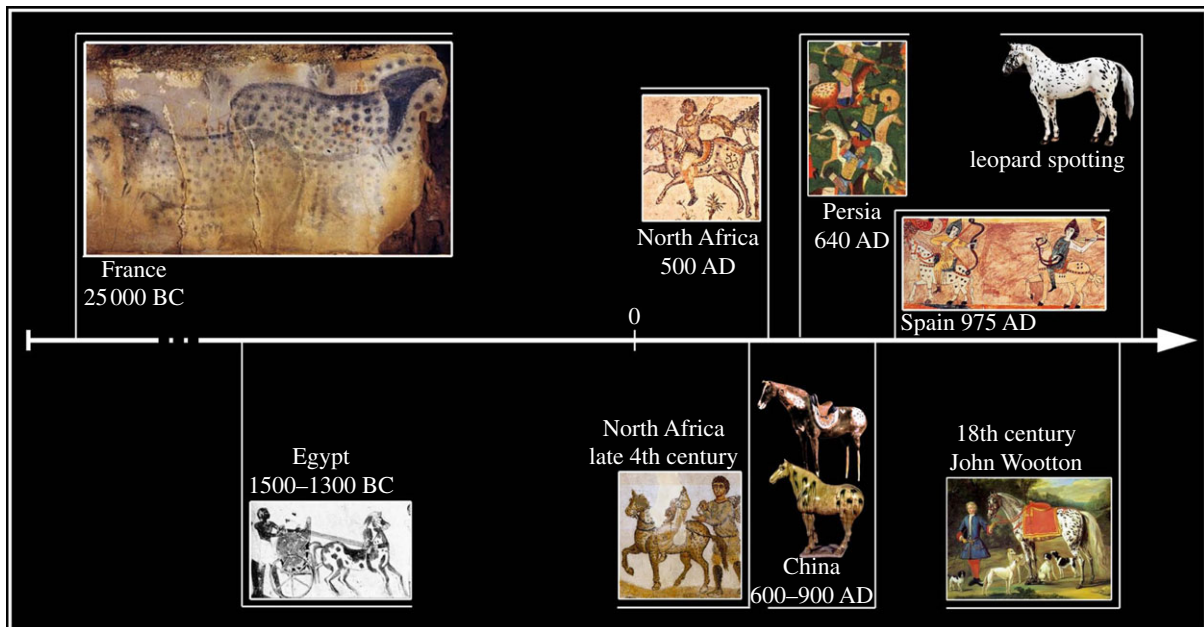
Figure 1. Examples of leopard complex horses in human artefacts and culture. Pictures from left to right: (*a*) the panel of the dappled horses—'Le panneau des Chevaux ponctués', Cabrerets, Lot, France (Photo from P. Cabrol, Centre de Préhistoire du Pech Merle). (*b*) There are several examples of spotted horses in the art of ancient Egypt dating from 1500 to 1300 BC (http://www.spanishjennet.org/history.shtml). (*c*) The mosaics from North Africa are from the *Dominus Iulius* at Carthage [1]. (*d*) This Persian plateau was passed from conqueror to conqueror until the arrival of the Muslims from the south in 640 AD. Persian art objects from that time to the present show spotted horses, suggesting that spotted horses were common in Persia since before the Muslim conquest (picture from http://www.spanishjennet.org/history.shtml). (*e*) Chinese horse sculptures dating to 600–900 AD. (*f*) The mosaic (Spain 975 AD) is from the *Beato de Gerona Codex*, dating to 975 and attributed to the *Abad Domenicus* [2]. (*g*) The famous eighteenth century painting from John Wootton titled 'Lady Conaway's Spanish Jennet' is owned by the Marquees of Hertford. (*h*) Modern Knabstrupper horse from the famous horse breeding farm 'Aus der schützenden Hand' (Werpeloh, Germany) showing leopard complex spotting (Photo: Thomas Hackmann). (Online version in colour.)

and several associated single nucleotide polymorphisms (SNPs) in *transient receptor potential cation channel subfamily M, member 1* (*TRPM1*) have been identified [4–7]. Recently, a 1378 bp long terminal repeat (LTR) of an endogenous retrovirus insertion in intron 1 of the *TRPM1* gene has been identified as the cause of leopard complex spotting [8].

Homozygosity for *LP* has also been associated with congenital night blindness (CSNB) in several horse breeds (e.g. Appaloosa and Miniature) [8–12]. In addition to other senses (e.g. olfaction), vision is important for communication, localization and characterization of prey, orientation and predator avoidance [13]. Therefore, individuals affected by CSNB are more susceptible to predation than unaffected individuals in natural environments. Under domestic conditions, affected individuals are known to be nervous, apprehensive and sometimes difficult to handle in dim or dark environments [14]. For these reasons, it seems likely that homozygotes were selected against in wild populations and potentially also in early domestic populations. Recently, genetic evidence for the leopard complex spotted phenotype has been found in pre-domestic Pleistocene and Holocene populations [8,15]. Although these genetic data are supported by Palaeolithic cave paintings where dappled horses are portrayed (figure 1), the intention of the Palaeolithic artists is still under discussion [16,17]. Here, we extend these analyses to early domestic horses by typing a SNP in the *TRPM1* gene (ECA1 : 108,249,293 C > T) that was found to be associated with both *LP* and CSNB in Appaloosa horses [7,8]. We typed this SNP in 96 horses from 19 localities from Siberia, China, Middle and Eastern Europe, and the Iberian Peninsula dating from the Late Pleistocene to medieval times and analyse the pattern of allelic fluctuation discovered.

2. Material and methods

(a) Samples
Overall, we included 96 horse (*Equus caballus*) bone and tooth specimens from 31 different localities from Siberia, Middle and Eastern Europe, China and from the Iberian Peninsula (figure 2 and the electronic supplementary material, table S1). These samples cover a time span from the Late Pleistocene to medieval times and are all dated either by archaeological context or ^{14}C dates (electronic supplementary material, table S2). Except those from Kirklareli–Kanligecit (*n* = 10) and Chicha (*n* = 6), all samples were previously genotyped for eight other coat colour loci [18] (electronic supplementary material, table S3).

(b) Ancient DNA extraction and amplification
Approximately 250 mg of bone material was used per extraction. External surfaces of bones were removed by abrasion to minimize environmental contamination. Each sample was ground to powder with a freezer mill and incubated in 0.45 M EDTA (pH 8.0) and 0.25 mg ml^{-1} proteinase K overnight at room temperature under rotation. After centrifugation for 5 min at 4000 r.p.m. in a Universal 320 centrifuge (Hettich), DNA was purified from the supernatant using a silica-based method as previously described [19,20].

LP primers were designed based on one of the associated SNPs previously reported [7,10] and added to our primer set for detecting coat colour SNPs. Amplifications were performed in two steps using multiplex PCR combined with a singleplex PCR as previously described [18,21]. PCR products varied in length between 52 and 78 bp (including primers; see the electronic supplementary material, table S4). Four microlitres of extract were used for each multiplex PCR. Negative extraction controls and negative PCR controls were used in each PCR. Amplification products were visualized on agarose gels.

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Figure 2. Geographical origin of samples (1. Maliy Lyakhovsky Isl., North Siberia; 2. Bol'shoy Lyakhovsky Isl., North Siberia; 3. Oyagosskiy Yar, Kondrat'evo R., mouth, Siberia; 4. Kotel'niy Isl., Anisiy Cape, Siberia; 5. Fengtai, Qinghai; 6. Tartas1, West Siberia; 7. Denisova-Pescera, Siberia (Altai); 8. Chicha, West Siberia; 9. Om-1, Siberia (Altai); 10. Arzan1, South Siberia (Tuva); 11. Arzan2, South Siberia (Tuva); 12. Barsucij Log, South Siberia (Tuva); 13. Olon-Kurin-Gol 10, Siberia (Mongolia); 14. Barsucij Log, South Siberia (Tuva); 15. Petersfels, South Germany; 16. Kniegrotte, Germany (Thuringia); 17. Span-Koba, Ukraine (Peninsula Crimea); 18. Mayaki, Ukraine; 19. Molyukhov Bugor, Ukraine; 20. Pietrele, Romania; 21. Vitanesti, Romania; 22. Cascioarele, Romania; 23. Garbovat, Romania; 24. Kirklareli – Kanligecit, Turkey; 25. Lori-Berd, North Armenia; 26. Shirakavan, Armenia; 27. Miciurin, Moldova; 28. Atxoste, Spain; 29. Cueva Fosca-Valencia-Cartellon, Spain; 30. Cueva Rubia-Valmayor/Madrid, Spain; 31. El Acequion, Spain; 32. Soto de Medinilla-Valladolid, Spain; 33. Mucientes-Valladolid, Spain).

(c) Authentication

DNA sampling, extractions and pre-PCR preparations were carried out in laboratories dedicated to ancient DNA at the Leibniz Institute for Zoo and Wildlife Research (Ludwig Laboratory) in Berlin, Germany, following the standard procedures to avoid contamination. Independent replications were carried out in a different laboratory at the University of York (Hofreiter Laboratory), UK for seven bones positive for the SNP allele associated with *LP*.

(d) Mutation analysis

LP locus: in order to avoid false-positive detection of mutation owing to postmortem DNA degradation (cytosine deamination), we used two different primer pairs for sequencing both strands to detect the point mutation in the *TRPMI* gene. These primers are designed for the pyrosequencing technology.

(i) Other coat colours gene

As previously described, we used a set of eight SNPs in six genes for detecting basic colours (bay, black and chestnut), diluted phenotypes (silver and cream) and spotted or painted phenotypes (overo, tobiano and sabino) in addition to leopard spotting. Primers are listed in the electronic supplementary material, table S3.

(ii) Pyrosequencing

Biotinylated PCR products were prepared at the PyroMark vacuum prep workstation according to the manufacturer's instructions. Amplicons for each SNP were sequenced using pyrosequencing technology on a PSQ 96MA (Biotage). The SNPs were identified using PSQ 96MA and automatically edited by the PSQ 96MA SNP software. The results for the colour determination are summarized in electronic supplementary material, table S3 and in detail for leopard in electronic supplementary material, table S5.

(e) Allelic dropout

The probability (P) of a false heterozygote individual is calculated after n replicates as: $P = K(K/2)^{n-1}$, where K is the observed number of allelic dropouts divided by all heterozygous individuals. For all genes, we did a minimum of four replications except for *ASIP* and *TRPM1* (six replications minimum) which reduced the risk of non-detection of a heterozygote individual to an average of 0.3% ($p = 0.0078$ for *KIT13*, 0.0015 for *KIT16* and *MATP*, and 0.00012 for *MC1R*).

(f) Estimating the allele frequency of missed alleles

We computed the upper bound of the allele frequency of a coat colour allele that was present in the pre-domestic population, but not observed in our samples assuming a binomial distribution. Given n samples, we calculated the likelihood that we did not detect a colour allele with a frequency of less than or equal to 5% (electronic supplementary material, table S6). This likelihood was taken into account for the simulation analysis (see §2g below).

(g) Testing temporal changes and estimating selection coefficients

We investigated the temporal change in the frequency of the *LP* allele between each consecutive pair of samples by means of a Bayesian simulation test [22], in order to determine the periods when the changes observed cannot be attributed to genetic drift and sampling error alone. Considering that selection pressure owing to CSNB could be present, this test was also applied with weak negative selection (selection coefficient of −0.01) exclusively on *LP* homozygotes. This additional test was performed in order to appreciate how significant the frequency changes would be in those periods when horses shifted from their natural environment to captivity. Afterwards, an approximate Bayesian computation (ABC) [23] analysis was employed for obtaining the joint posterior probability distribution of two parameters: (i) a coefficient of selection owing to CSNB, which affected the survival of the *LP* homozygotes and (ii) a coefficient of artificial selection that increased/decreased *LP* alleles in the effective population (as if it affected the reproductive success). Both selection coefficients were simulated from uniform priors (−1.0 to 1.0), and no assumptions regarding selection were taken; the estimates of selection coefficients were fully determined by the fit of the simulations in relation to the observed allele frequencies. The employed summary statistic (SuS) was the Euclidean distance between allele frequencies, and rejection was stated whenever the simulated

SuS was different from the observed one, so the posterior is exact and no regression adjustments were necessary (as in reference [24]). As Eurasian horse populations have increased since pre-domestication times [25], the census population size before the Copper Age was set to 10 000 diploid individuals and after those times (3600 BC) an exponential population growth was set, which resulted in a population of 80 000 individuals by the last sampling time (late Iron Age). A variable number of simulations (between 500 000 and 10 million) were run in order to yield at least 10 000 non-rejected iterations per comparison. Owing to the patchy nature of our sampling, the entire analysis was also repeated only with the samples from Eastern Europe, in order to rule out that an overspread sampling is a source of bias (if our results were too reliant on differences observed among samples too separated to be assumed as belonging to a single panmictic population, then the Eastern European analysis should disagree with the overall analysis). Additional runs were performed for optimization of the runs, to check the robustness of the results to variation of fixed parameters and for exploring alternative scenarios.

The program used was a modification of the software TAFT v. 2.3 [22] where initial allele frequencies were simulated from a Dirichlet distribution conditional on the observed frequencies. Considering a Wright−Fisher population model, the intermediate generations had a multinomial distribution, but the sampling and the separating of the effective population from the total population had a hypergeometrical distribution, which is more appropriate [26].

3. Results

Overall, genotypes from 96 ancient samples were considered for *LP* (*TRPM1* SNP ECA1 : 108,249,293 C > T) as well as for eight other coat colour SNPs in six genes (*MC1R*, *ASIP*, *SILV*, *MATP*, *EDNRB*, *KIT*). These SNPs included the basic colours (bay, black, chestnut), dilution phenotypes (cream, silver) and white spottings (in addition to *LP* also tobiano, sabino and overo; electronic supplementary material, tables S3 S5 and S7). The *LP* associated allele was discovered in eight early domestic horses. In addition to *LP*, the tobiano and sabino alleles were previously identified in eight and three of the early domestic samples, respectively [18].

Our results show that the presence of leopard complex spotted horses continues into early domestic times (figure 3). At the archaeological site of Kirklareli−Kanligecit (Turkish-Thrace) dating to the early Bronze Age (2700−2200 BC), six of 10 horses investigated shared the *LP* associated SNP including one homozygous individual. Compared with the pre-domestic European horses examined in this study, the difference in the *LP* frequency (from 0.0556 to 0.350) represents a statistically significant increase ($p = 0.0151$) that becomes even more significant ($p = 0.0019$) if a small negative selection coefficient (-0.01) is introduced to account for CSNB, which is thought to be caused by the same mutation [8,10−12]. This test was used only to emphasize how significant the difference would be under the likely scenario that CSNB affected reproductive success of carriers, even to a minuscule extent. Consequently, our estimate of an artificial selection coefficient accounting for the observed increase in *LP* frequency, as obtained by the ABC analysis, is positive and high (0.3652, 95% CI = 0.0−0.85). The high positive value for the estimated selection coefficient suggests that the horses from Kirklareli−Kanligecit were domestic horses. This conclusion is further supported by their archaeological context
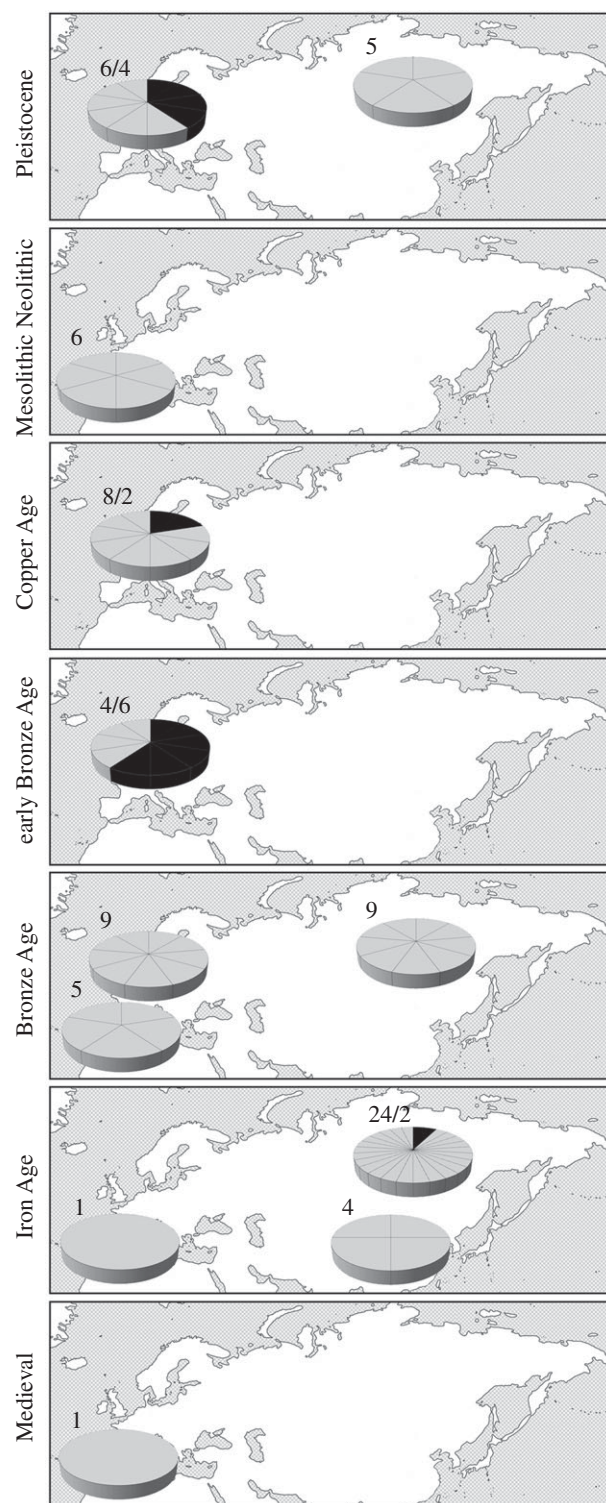

Figure 3. Distribution of the *LP*-allele during archaeological times in different regions.

and the fact that there is no archaeological evidence for wild horses in Turkish-Thrace during the Mid Holocene [27].

LP is not present in our domestic horse samples dating to the middle and late Bronze Age. This difference is also significant ($p = 0.0081$), whereas the estimate of the artificial selection coefficient becomes negative (figure 4; electronic supplementary material, table S8 and text S9). The phenotype reappears in our sample set during the early Iron Age, with the first evidence originating from a single sample from the fortified settlement of Chicha (1400−1300 BC), one of the most important archaeological sites of the transitional period between the late Bronze Age and the early Iron Age
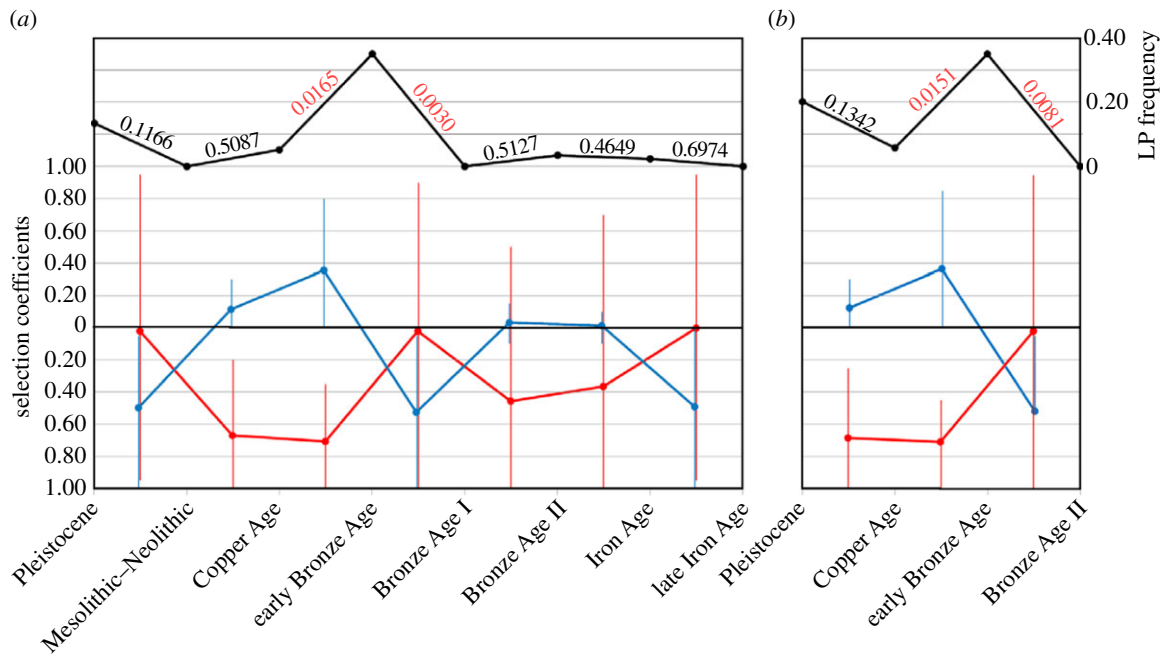
Figure 4. Changes in the allele frequency of *LP* (upper graph, scale at right) and estimated selection coefficients (*s*) obtained by ABC (bottom graph left scale) for the overall sampling (*a*) and for Eastern European samples (*b*). The blue line shows the estimated selection coefficient for artificial selection on leopard complex spotting, the red line the estimates for selection caused by CSNB, and the black line the changes in the frequency of the *LP* allele. The vertical lines in blue or red indicate the 95% probability credible intervals for the selection coefficient estimates. The numbers above the black line indicate significance of the changes in the allele frequency of *LP*, with red values showing statistically significant values. (Online version in colour.)

in the forest steppe region of western Siberia. We also found evidence for another leopard complex spotted horse from Arjan (800 BC), an Iron Age Scythian grave from the Tuva region in western Siberia (figure 3). The horses from Chicha are morphologically separated into two groups: mainly large and strongly built horses in period 1 where wild species dominate the faunal assemblage, and smaller horses in period 2 where domestic animals are clearly prevailing [28]. The Chicha bones analysed here represent both large, probably wild horses from period 1 (CIC1, CIC2, CIC3) and small, probably domestic horses from period 2 (CIC4, CIC6, CIC8). Based on morphological traits of the skeleton, the *LP* horse from Chicha was likely a wild-caught animal, suggesting that this phenotype could have been reintroduced into the domestic gene pool from a wild population. Although apparently present in the domestic gene pool during the Iron Age, with only two of 33 individuals, leopard complex spotted horses seem to remain rather rare during this period.

4. Discussion

Here, we show that *LP* is not only the most common spotting pattern identified among the 96 ancient samples analysed so far, but also the only one which has so far been found in both pre-domestic and early domestic horses. Considering the archaeological record [27], the high allele frequency of *LP* and the positive selection coefficient for the *LP* allele found for the horses from Kirklareli–Kanligecit (Turkey), these horses almost certainly belonged to a domestic population. Given that no wild horse remains have been reported from this region from the time to which the investigated samples date [27], it is reasonable to assume that these horses were not only domesticated, but also imported to this region.

Previous studies on the population structure of pre-domestic horses indicate that they existed as a panmictic population ranging from the French Pyrenees to Siberia, with a second isolated subpopulation inhabiting the Iberian Peninsula since glacial times [25,29]. Given such a population structure, it is not unlikely that leopard spotted horses were also present in Siberia. However, until now, this phenotype has only been found in pre-domestic horses from Western and Eastern Europe.

It has previously been suggested that the genetic diversity of domestic animal species has been augmented through backcrossing with their wild ancestors [29], and a replacement of the original domestic mtDNA genomes with those from local wild boar populations has been demonstrated in early domestic pigs from Europe [30]. After their initial domestication, probably around 3500 BC in the North Caspian region [31], domestic horses were distributed very rapidly all over Eurasia, allowing incorporating of local genetic diversity of the respective wild populations into the domestic gene pool. For example, the high mtDNA diversity found in domestic horses is explained by assimilation of local wild-caught mares into the domestic gene pool [25,32]. It is therefore plausible that the geographical origin of the *LP* allele in the domestic horses from Kirklareli–Kanligecit lies in Europe. Assuming that the horse from Chicha indeed represented a wild-caught horse, the *LP* allele in domestic horses may have originated from multiple interbreeding of domestic horses with local wild-caught mares. Our results also add further evidence that horses already displayed a high diversity of coat colour phenotypes during the earliest stages of the domestication process.

Although we did not find any evidence for it, we cannot exclude that *LP* persisted at low frequency in the domestic population during the late Bronze Age. Considering only the Bronze Age samples in our dataset, the probability that the

LP allele frequency was lower than 0.15 is 95%, and 99% for a frequency below 0.22. Given these numbers and considering the patchy set of samples, it is of course possible that the *LP* allele had been retained in part of the Bronze Age domestic horse population at low frequency and thence reintroduced into others. However, when analysing archaeological materials, sampling is often patchy because of the nature of the archaeological record. In our sample set, the phenotype reappears around 1400 BC in a horse sample from Chicha (West Siberia). Although, based on its skeletal morphology, this horse probably represents a wild horse [28], a second *LP* positive Iron Age sample, originating from a Scythian grave dating to 800 BC, clearly represents a domestic animal [21,33]. Together, these results are thus ambiguous on the origin of the *LP* allele in Iron Age and later domestic horse populations.

As we could only analyse horses from a single early Bronze Age site, we cannot draw any conclusions whether the high frequency of the *LP* allele observed at Kirklareli–Kanligecit was a general feature of domestic horses at this time or specific to the breeding practices and population of this specific site. However, the *LP* phenotype seems likely to have been rare or even absent from the gene pool of domestic horses later during the Bronze Age, as we found no evidence for it in later Bronze Age horses. It is possible that once farmers became aware of the difficulties of rearing night blind animals, which may have been easy targets for predation and theft as well as difficult to handle under low light conditions, they selected against them. This argument is supported by two facts: (i) genetic drift alone is unlikely to explain the absence of the *LP* allele during the late Bronze Age ($p = 0.0081$; see the electronic supplementary information); and (ii) the estimated selection coefficient in addition to negative selection owing to CSNB was also strongly negative for this time period (-0.5176, 95% CI $= -1.0$ to -0.05). Both factors argue for active human-mediated selection against the *LP* phenotype during the later Bronze Age, in the opposite direction of the selection coefficient suggested for the early Bronze Age. However,

LP was almost certainly selected for again at some point after the Iron Age, suggesting another swing in the selective preferences of horse breeders with regard to phenotype. Our modelling results further support this notion as the major shifts in allele frequency at the *TRPMI* gene over time almost certainly reflect changes in the selective regime (figure 4), owing to a combination of the negative effect of CSNB and fluctuating artificial selection on the colour phenotype. Thus, the phenotypic and breeding preferences of early horse breeders seem to have changed over time, just as the preferences of animal breeders change today.

We did not investigate any medieval samples, but leopard complex spotted phenotypes become increasingly abundant in texts and iconographies from this time onward, indicating its increased prestigious value. From early medieval times onwards, there are many paintings showing noblemen on such horses [3]. There is also a documented increase in the frequency of the leopard complex phenotype in the breed Noriker during the Baroque Age, which was achieved by crossbreeding with Spanish horses [34], with the goal to breed exotic coloured gala horses. During the following centuries, the *LP* phenotype went out of fashion and became very rare again, along with all other white spotted phenotypes [34]. Today, breeding efforts have intensified again in favour of white spotted phenotypes owing to a growing interest in their restoration, and *LP* phenotypes are found in many breeds testifying to their popularity [35]. Assuming that this picture of alternating selective regimes holds also true for other loci, it might explain how genetic diversity was maintained in domestic populations at all times despite extensive selection for specific traits during certain time periods.

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