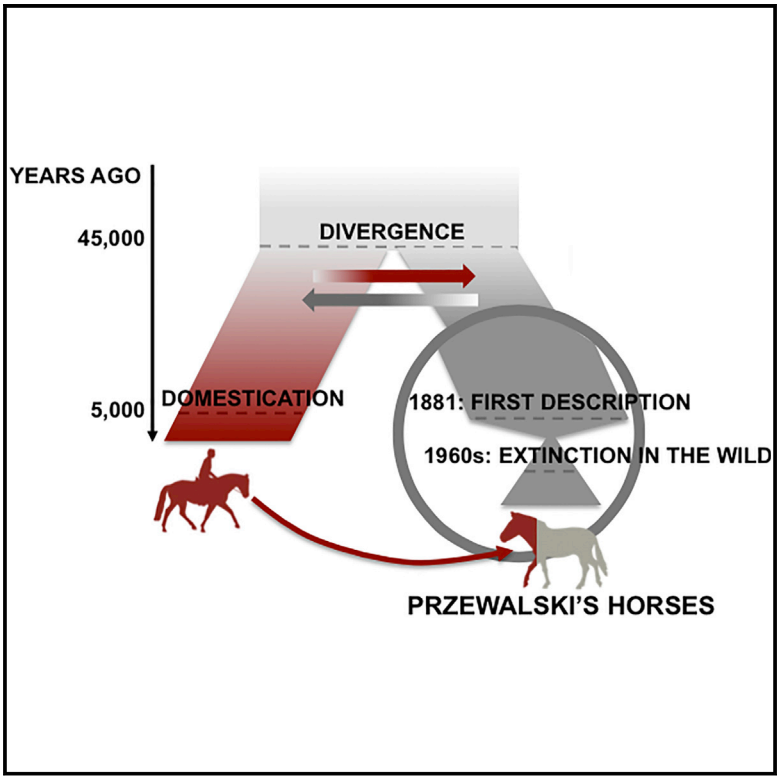
Report

Current Biology

Evolutionary Genomics and Conservation of the Endangered Przewalski's Horse

Graphical Abstract



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In Brief

Der Sarkissian et al. characterized complete genomes of modern and historical Przewalski's horses, a wild and endangered population distinct from domesticated horses. Comparative analyses revealed specific signatures of selection, increased inbreeding, and variable introgression from domesticated horses in the last 110 years in captivity.

Highlights

- Complete genomes were sequenced for 21 domesticated and 17 Przewalski's horses
- Selection signatures and variants specific to Przewalski's horses were detected
- Domesticated and Przewalski's horse ancestors mixed post-divergence ~45,000 years ago
- Captivity increased inbreeding and domestic introgression in Przewalski's horses

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CellPress

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Evolutionary Genomics and Conservation of the Endangered Przewalski's Horse

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SUMMARY

Przewalski's horses (PHs, *Equus ferus ssp. przewalskii*) were discovered in the Asian steppes in the 1870s and represent the last remaining true wild horses. PHs became extinct in the wild in the 1960s but survived in captivity, thanks to major conservation efforts. The current population is still endangered, with just 2,109 individuals, one-quarter of which are in Chinese and Mongolian reintroduction reserves [1]. These horses descend from a founding population of 12 wild-caught PHs and possibly up to four domesticated individuals [2–4]. With a stocky build, an erect mane, and stripped and short legs, they are phenotypically and behaviorally distinct from domesticated horses (DHs, *Equus caballus*). Here, we sequenced the complete genomes of 11 PHs, representing all founding lineages, and five historical specimens dated to 1878–1929 CE, including the Holotype. These were compared to the hitherto-most-extensive genome dataset characterized for horses, comprising 21 new genomes. We found that loci showing the most genetic differentiation with DHs were enriched in genes involved in metabolism, cardiac disorders, muscle contraction, reproduction, behavior, and signaling pathways. We also show that DH and PH populations split ~45,000 years ago and have remained connected by gene-flow thereafter. Finally, we monitor the genomic impact of ~110 years of captivity, revealing reduced heterozygosity, increased inbreeding, and variable introgression of domestic alleles, ranging from non-detectable to as much as 31.1%. This, together with the identification of ancestry informative markers and corrections to the International Studbook, establishes a framework for evaluating the persistence of genetic variation in future reintroduced populations.

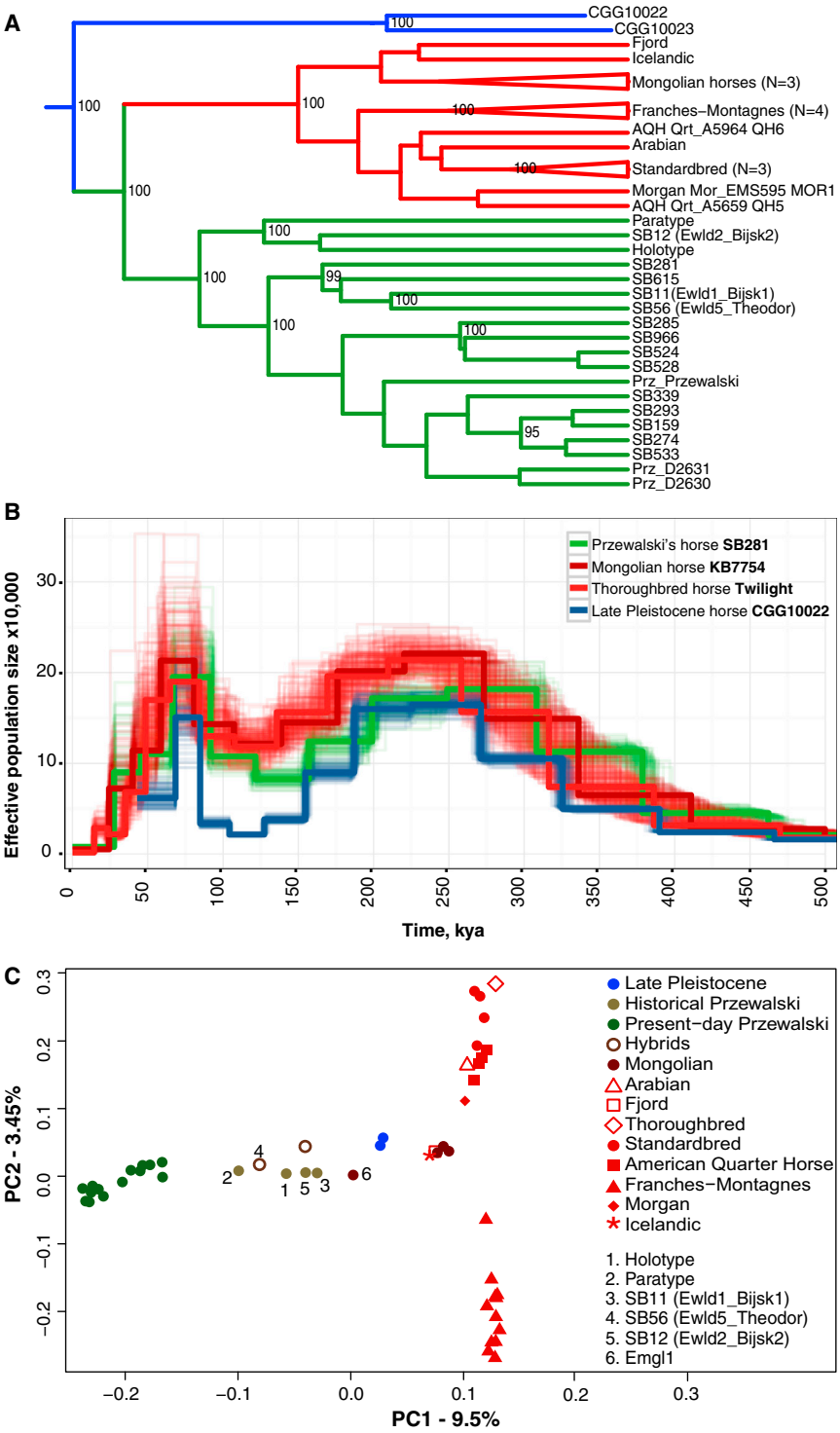

Figure 1. Genomic Structure among Late Pleistocene Horses in Blue, DHs in Red, and PHs in Green

(A) Exome-based ML tree. Nodes show bootstrap support ≥ 95%. AQH, American Quarter Horse.

(B) PSMC profiles. Thin lines represent 100 bootstrap replicates. kya, thousand years ago.

(C) PCA based on genotype likelihoods.

See also Figures S1 and S2 and Tables S1 and S2.

RESULTS AND DISCUSSION

Despite their additional chromosomal pair (2n = 66 versus 2n = 64 [5]), PHs can successfully reproduce with DHs, resulting in fully viable and fertile offspring. The extent to which those horses admixed in the past is, however, contentious. Mitochondrial DNA (mtDNA) [6–8], the X [9–11] and Y [11–13] chromosomes, and a limited number of autosomal SNPs [14] have supported admixture, with PHs appearing within the genetic variability of DHs, in line with possible domestic contributions listed in the International Studbook [2–4]. Conversely, ~54,000 SNPs [15] and one complete PH genome [16] indicated that PH and DH populations probably diverged ~38,000–72,000 years ago [17], with no sign of admixture [16].

To elucidate the evolutionary history of PHs, we used the Illumina paired-end technology and generated whole-genome sequences of 33 living horses, including 11 PHs representing all pedigree founding lineages (18.0–23.4× average depth-of-coverage), one F1 PH × DH hybrid (22.8×), and 21 horses from five domestic breeds (7.7–22.6×). These complement an existing dataset of seven DH and three PH genomes [16, 18]. We also sequenced the genomes of five historical specimens: two PH pedigree founders from the early 1900s (SB11/Ewld1_Bijsk1 and SB12/Ewld2_Bijsk2, each at 0.9×), one hybrid (SB56/Ewld5_Theodor, 4.3×), as well as the PH Holotype (1.4×) and one Paratype specimen (3.7×), both from the late 19th century (Table S1). DNA fragmentation and nucleotide mis-incorporation patterns, indicative of post-mortem damage [17, 19], supported the authenticity of our ancient genomes (Figure S1). We limited the impact of nucleotide mis-incorporations on downstream analyses by enzymatically treating ancient DNA extracts [20] and/or by downscaling base quality scores at damaged positions [14]. The genomes of the Holotype, Paratype, and SB11/Ewld1_Bijsk1 specimens showed error rates lower than those of the other historical horses (0.099%–0.258% versus 0.463%–0.622% errors/base) but similar to those of Late Pleistocene horses (0.103%–0.207% [21]). In comparison, error rates in modern horses were 0.018%–0.040% in DHs and 0.036%–0.051% in PHs (Table S1).

Phylogenetic analyses identified three main mtDNA lineages and two Y chromosome haplotypes in PHs. PHs represent a

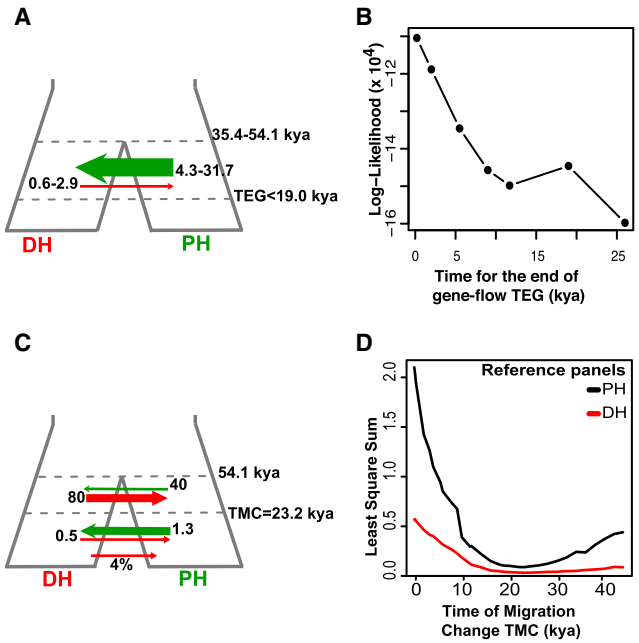

Figure 2. DH and PH Demographic Models Explored in Dadi and Projection Analyses

(A) Best two-population model supported by dadi.

(B) Dadi model Log(likelihood) for varying starting dates of isolation between DHs (Franches-Montagnes) and PHs (TEG).

(C) Best two-population model supported by projection analyses.

(D) Model LSS when considering varying dates for the change in migration dynamics (TMC) for the genome projections based on the DH (Franches-Montagnes, red) and PH (black) reference panels.

See also Table S3.

paraphyletic assemblage within DHs in the mtDNA tree [6–8] and exhibit Y chromosome haplotypes not found in any other domesticated stallion, nor in a ~16,000-year-old horse [21] (Figures S2A and S2B). The maternal and paternal lineages are in agreement with the recorded PH pedigree, except for SB528 and SB285, respectively. An exome-based maximum-likelihood (ML) phylogeny revealed DHs and PHs as two reciprocally monophyletic clades distinct from Late Pleistocene horses (Figure 1A) [21], in accordance with the population tree reconstructed in TreeMix using 198,932 SNPs (Figure S2C). These results are consistent with Pairwise Sequential Markovian Coalescent (PSMC) demographic trajectories ([21]; Figure 1B), indicating an early divergence (~150,000 years ago) for the population of Late Pleistocene horses and synchronized DH and PH profiles until their most recent history. This also reflects the clusters identified in the STRUCTURE analyses of 12 autosomal STRs (Figure S2D) and principal-component analyses based on genome-wide SNP calls or genotype likelihoods (Figure 1C, S2E, and S2F). The latter show no overlap for historical and modern PHs along the first principal component, potentially as a consequence of captivity. Altogether, our results support the two Late Pleistocene horses analyzed, DHs, and PHs as three populations that diverged ~150,000 years ago [16, 21]. The discrepancy between the phylogenies inferred from autosomes, the Y chromosome, and mtDNA could reflect the recruitment of only few stallions during the domestication process, the co-segregation of mtDNA haplogroups in the ancestral populations of DHs and PHs, and/or maternally driven gene-flow post-divergence [10–12, 22, 23].

We then aimed at identifying the genetic variants underlying the striking phenotypical differences between DHs and PHs. We found 509 copy-number variants (CNVs) in PHs, encompassing a total number of 1.65 Mb, 219 kb of which were previously reported in [24]. These contained 233 genes, including genes encoding proteins associated with glycolsphingolipid metabolic process, scarring, decreased corneal thickness, and the sulfuric ester hydrolase activity, which is key for the bone and cartilage matrix composition [25]. We also extracted 101 exonic ancestry informative markers for PHs, of which 53 represent non-synonymous mutations in genes involved in cardiac activity (*CACNA1D*, *ITGA10*), protein digestion, and absorption (*COL18A1*, *COL15A1*); disorders in muscles, ligaments, and the tissues surrounding muscles, blood vessels, and nerves (*PALMD*, *MCPH1*); diseases of the sebaceous glands (*PALMD*, *MCPH1*); and musculoskeletal and craniofacial abnormalities (*MCPH1*, *SETBP1*) (Table S2). We further sought for regions maximizing the genetic differentiation between DHs and PHs, using the F_{ST} fixation index. These contained 874 genes showing significant enrichment in signaling pathways (chemokine, kit receptor, and the inhibitor of DNA binding), in the glycogene and glycerophospholipid metabolisms, and in pathways involved in striated muscle contraction, heart and metabolic diseases, and mood disorders (Table S2). We finally used SweeD [26] to identify 76 protein-coding candidates for selective sweeps within PHs, including *MCR2*, which encodes one receptor of the ACTH stress hormone. Candidate genes showed enrichment for energetic and metabolic pathways, the cardiovascular system, muscular contraction, and immunity. Two genes, *CACNA1D* and *PLA2G1B*, supported a significant enrichment for the gonadotropin-releasing hormone signaling pathway, which is essential in sexual behavior and aggressiveness [27], possibly in relation to the temper of PHs (Table S2). Overall, our analyses unveil some potential drivers of the marked anatomical, physiological, and behavioral differences between DHs and PHs, some of which might have been enhanced during domestication.

We next reconstructed the past population history of DHs and PHs. Using dadi [28], our best-fit comparison consistently supported models involving asymmetrical gene flow after divergence (~35,000–54,000 years ago, in agreement with [16, 21]), almost entirely from PHs into DHs (Figure 2A; Table S3). We also noticed that the fit could be substantially improved when considering that migration stopped within recent times, i.e., as early as the last 200 years, suggesting that genetic restocking from wild animals was likely common until recently (Figure 2B; Table S3). Using genome projections [29], the best model was recovered by accounting for a recent 4% migration pulse from DHs and two epochs with asymmetrical gene flow, first from DHs into PHs, then reversed (Figure 2C; Table S3). The best fit was obtained for migration changing during the Last Glacial Maximum (~23,000 years ago; Figure 2D), when rates decreased ~31- to 160-fold. This change in the direction and magnitude of migration could reflect a combination of sexual behavior and demographic factors, as the detected contribution of DHs was higher when populations were much larger

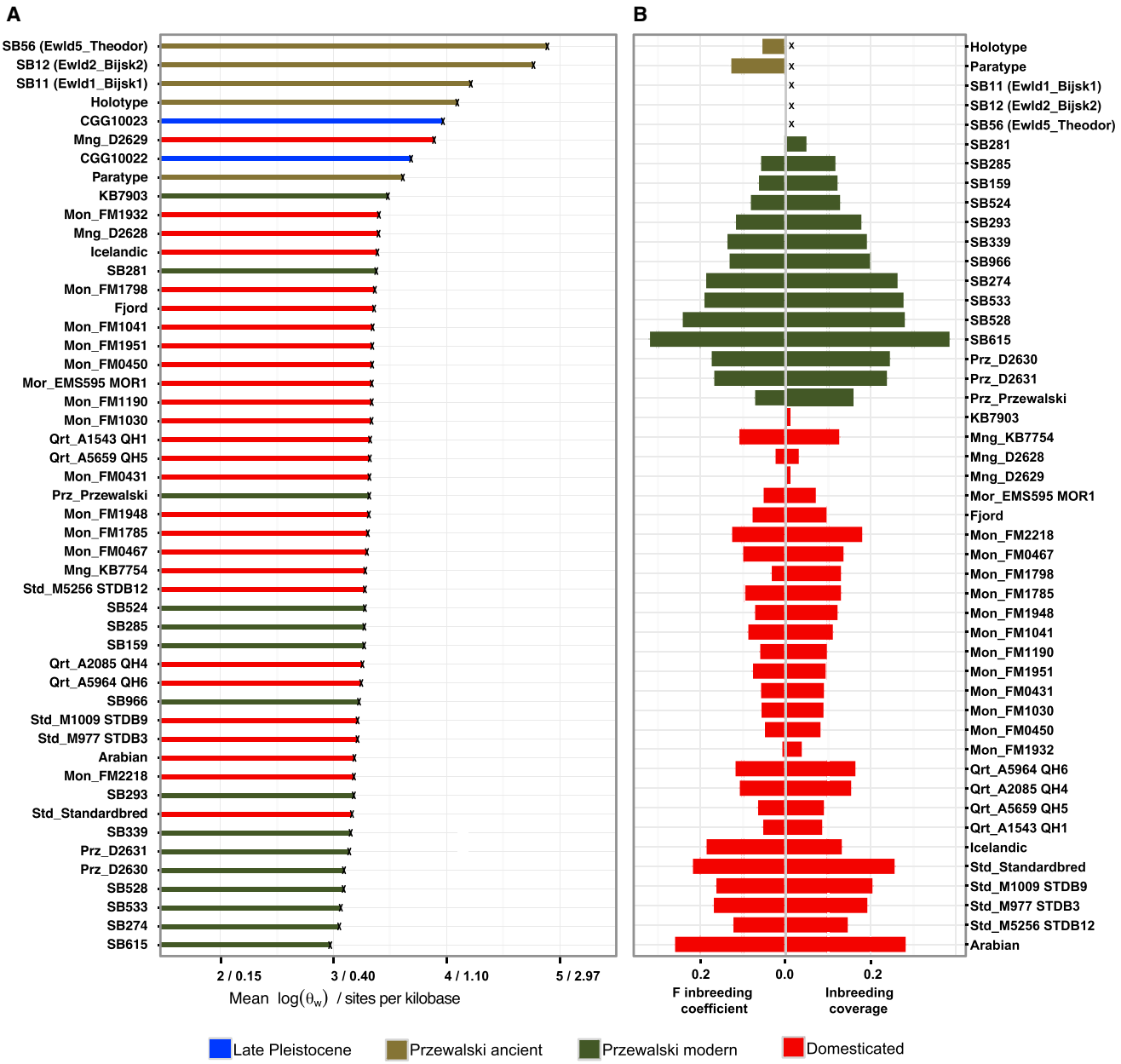

Figure 3. Heterozygosity and Inbreeding Estimates

(A) Average genome-wide heterozygosity for autosomes, disregarding transitions.

(B) FHMM inbreeding coefficient and inbreeding coverage across modern and historical PHs and DHs. X denotes not estimated.

(Figure 1B). Altogether, our analyses suggest connection by gene flow after divergence and prior to horse domestication.

We then evaluated the genetic impact of the bottleneck associated with ~110 years of captivity in PHs. Their average heterozygosity (0.39–0.59 heterozygous sites per kilobase) was comparable to that in other endangered mammalian species (e.g., 0.12–0.65 in [30, 31]) but lower than in DHs (0.40–0.98) and historical PHs (0.74–2.35; Mann-Whitney test, $p \leq 1.88 \times 10^{-3}$; Figure 3A). Another consequence of the bottleneck is the higher inbreeding coverage, i.e., the proportion of mostly homozygous blocks [21, 32], in PHs (0.052–0.388) than in modern DHs (0.006–0.285; two-sample t test, $p = 6.81 \times 10^{-3}$). Additionally, the inbreeding coefficient of historical PHs, as estimated using a novel method accommodating data uncertainty (FHMM, Supplemental Experimental Procedures), was lower than in modern PHs ($p = 4.43 \times 10^{-2}$) (Figure 3B; Table S1). Altogether, this demonstrates that captivity has significantly impacted the genetic diversity of PHs, reducing heterozygosity and increasing inbreeding.

We investigated admixture with DHs as another potential consequence of captivity. Using NGSAdmix [33], eight clusters recapitulated DH breeds and their differentiation from PHs

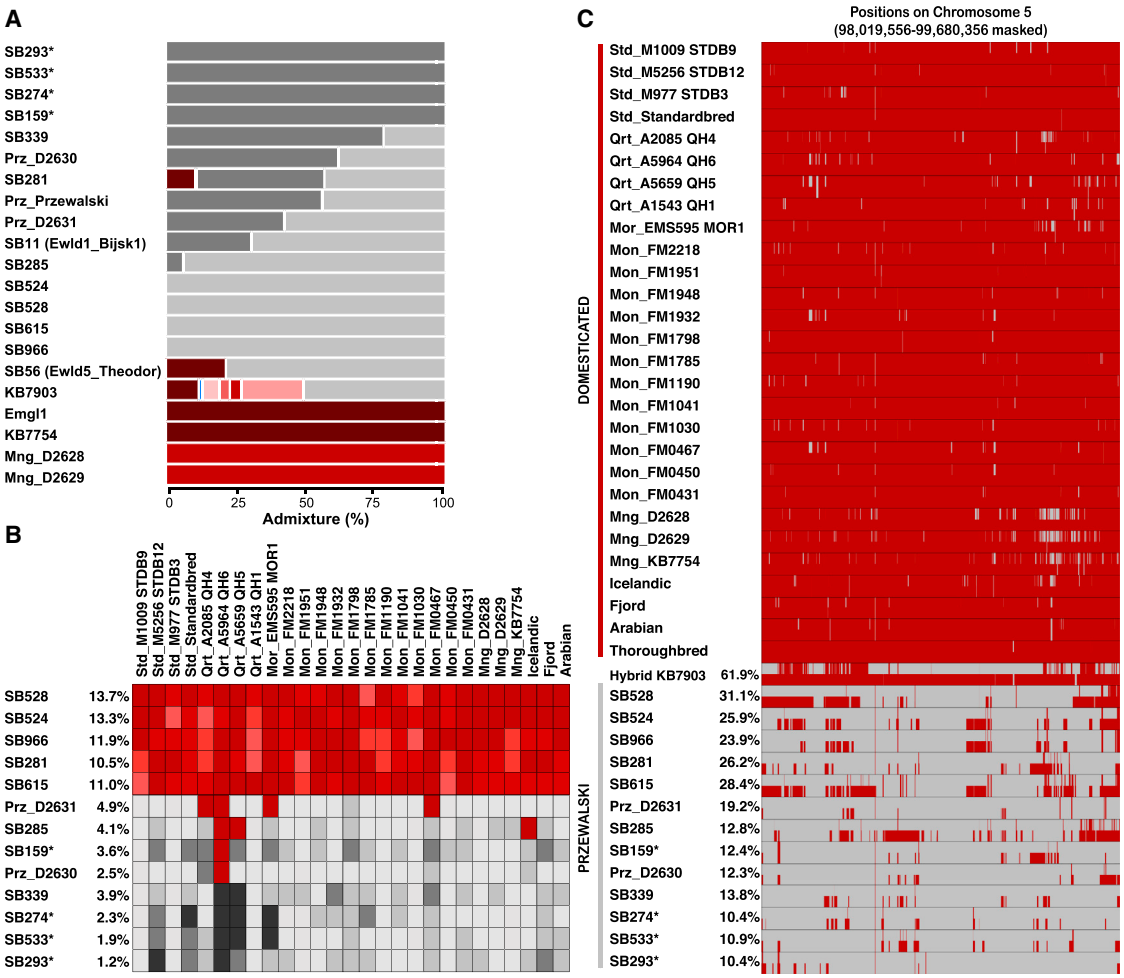

Figure 4. DH Introgression into PHs

(A) Genetic components estimated in NGSAdmix (eight clusters). The gray and red gradients represent the PH and DH components, respectively.

(B) D statistic heatmaps. The tree topology (Outgroup,(PH,(DH,Paratype))) is tested for each DH and PH combination, disregarding transitions. Darker red colors indicate the admixture component in significant tests; darker gray shades indicate non-significant tests. The proportion of DH introgression within each PH genome, estimated by the average of f_4 ratios involving Franches-Montagnes horses, is indicated next to the PH sample labels.

(C) Chromosome painting of DH (red) and PH (gray) ancestry blocks identified along chromosome 5.

Asterisk denotes A-line PHs. See also Figure S3 and Tables S4 and S5.

(Figures 4A and S3A). Although inconsistent with the pedigree describing the historical specimen SB56/Ewld5_Theodor as the F1 offspring of pure PH (SB11/Ewld1_Bjisk1) and DH (Emgl1) parents, this horse displayed, respectively, 79% and 21% of PH and DH components, providing clear evidence of introgression into the early descent of the PH founders.

We sought further admixture evidence using the f_3 statistics in (PH,DH;PH) tests [34] and found significant evidence of DH ancestry within modern PHs (Table S4). This was confirmed using D statistics [35] (Figures S3B–S3D), as topologies in the form of (Outgroup,(DH,(PH,PH))) were rejected in a large proportion of the tests. Taking advantage of the genome sequence of the Paratype specimen and (Outgroup,(DH,(PH,Paratype))) trees, we identified three groups of PHs with increasing levels of domestic ancestry (Figure 4B; Table S4): (1) SB274, SB293, SB339, and SB533 were found to be no closer to DHs than the Paratype; (2) SB281, SB524, SB528, SB615, and SB966 showed a significant genetic contribution from all the DHs tested (representing 10.5%–13.7% of their genome); (3) whereas SB159, SB285, Prz_D2630, Prz_D2631, and Prz_Przewalski showed a significant genetic contribution (2.5%–4.9%) from a minority of the DHs tested.

We next mapped admixture blocks along the genome using LAMP [36] and the whole panel of DH and modern PH genomes (except Prz_Przewalski, showing coverage artifacts confounding LAMP). A first group (SB281, SB524, SB528, SB615, and SB966), showed LAMP-admixture proportions of 23.9%–31.1%, with introgression tracts unevenly scattered across the genome. The remaining PHs exhibited 10.4%–19.2% admixture, corresponding to shorter tracts and earlier admixture events (Figure 4C). Interestingly, we found a derived allele associated with increasing wither height at *ZFAT* in SB524, SB528, and SB966, in a region likely introgressed from DHs (Table S5). Overall, these results confirm the DH introgression depicted in the

International Studbook, with individuals tested for the A line (SB159, SB274, SB293, and SB533) considered as the pedigree's purest line, virtually devoid of admixture [37]. We should, however, caution that, despite being significantly correlated (Pearson's correlation coefficients ≥ 0.98, $p \leq 7.94 \times 10^{-6}$), admixture coefficients estimated using LAMP and D statistics were likely overestimated. The DH ancestry for the KB7903 hybrid is indeed ~62% in LAMP, instead of the expected ~50%, and the higher error rate of the Paratype genome inflates allele sharing—hence, D statistics—between modern PHs and DHs.

In addition, to provide the most extensive genomic resource for horses classified as "endangered" on the IUCN Red List [1], our study fulfills the goal of the IUCN/SSC Equid Specialist Group conservation program [38] by identifying hybrids, AIMs, and candidate alleles maximizing the adaptation of PHs to their native environment. In the future, cost-effective genome-wide analyses targeting these markers should help minimize the contribution of domestic ancestry and limit loss of genetic diversity in a closed breeding program, with the ultimate objective of maintaining a viable population, once reintroduced in the wild.

EXPERIMENTAL PROCEDURES

Samples and methods are described in Supplemental Experimental Procedures. Material was shipped in compliance with CITES regulation for endangered species, following Materials Transfer Agreements between Copenhagen and San Diego (Biomaterial Request BR2013045) and Certificate of Scientific Exchange (COSE) in place at both institutions (12US50818A/9 San Diego; DK003 Natural History Museum of Denmark, Copenhagen). An extended Supplementary Material can be found at: http://geogenetics.ku.dk/publications/si-przewalski.

ACCESSION NUMBERS

The accession number for the sequences reported in this paper is European Nucleotide Archive: PRJEB10098.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.08.032.

AUTHOR CONTRIBUTIONS

W.Z., O.A.R., and L.O. conceived the project, and L.O. designed the study. N.I.A., R.S., A.T., O.A.R., M.M., S.R., and T.L. provided samples. C.D., L.E., G.K.B., S.P., C.S., M.N., V.J., M.M., and L.O. performed laboratory analyses. C.D., L.E., M. Schubert, M.A.Y., P.L., M.F., H.J., G.K.B., A.A., F.G.V., B.P., A.G., A.F., B.L.-G., T.M.B., M. Slatkin, and L.O. analyzed genomic data. G.K.B., A.S.O., K.M., C.F., C.L.G., A.L., T.S.P., E.W., T.M.B., O.A.R., M.M., S.R., T.L., and L.O. provided reagents and material. L.O. wrote the paper with input from all co-authors. C.D., P.L., M. Schubert, M.F., M.A.Y., and L.O. wrote the supplementary information, with input from C.G.

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