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# Analysis of horse genomes provides insight into the diversification and adaptive evolution of karyotype

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**Karyotypic diversification is more prominent in *Equus* species than in other mammals. Here, using next generation sequencing technology, we generated and de novo assembled quality genomes sequences for a male wild horse (Przewalski's horse) and a male domestic horse (Mongolian horse), with about 93-fold and 91-fold coverage, respectively. Portion of Y chromosome from wild horse assemblies (3 M bp) and Mongolian horse (2 M bp) were also sequenced and de novo assembled. We confirmed a Robertsonian translocation event through the wild horse's chromosomes 23 and 24, which contained sequences that were highly homologous with those on the domestic horse's chromosome 5. The four main types of rearrangement, insertion of unknown origin, inserted duplication, inversion, and relocation, are not evenly distributed on all the chromosomes, and some chromosomes, such as the X chromosome, contain more rearrangements than others, and the number of inversions is far less than the number of insertions and relocations in the horse genome. Furthermore, we discovered the percentages of LINE\_L1 and LTR\_ERV1 are significantly increased in rearrangement regions. The analysis results of the two representative *Equus* species genomes improved our knowledge of *Equus* chromosome rearrangement and karyotype evolution.**

Horses are recognized as extremely successful domestic animals. Humans in many parts of the world have relied on them for thousands of years<sup>1</sup>. The genus *Equus* originated on the North American continent and migrated 2.6 million years ago over the Bering Strait during the Ice Age<sup>2</sup>. Horses, donkeys, and zebras evolved from the same ancestor. The speciation events were accomplished through acute chromosomal rearrangements, with the rearrangement rate ranging from 2.9 to 22.2 per million years, which is significantly higher than in other mammals<sup>3-9</sup>. *Equus* species possess widely varying diploid chromosome numbers, from 2n = 32 (Mountain zebra) to 66 (Przewalski's horse). Przewalski's horse has a different chromosome number than domestic horses because of a Robertsonian translocation, resulting in one pair of metacentric chromosomes (ECA5) split into two pairs of acrocentric chromosomes<sup>10-12</sup> (EPR23 and EPR24). Although the offspring produced from a cross between Przewalski's horse and a domestic horse had 65 chromosomes, it was fertile<sup>11,13</sup>, unlike the mule (2n = 63, offspring of male donkey and female horse) and the hinny (2n = 63, offspring of male horse and female donkey), which are sterile.

Przewalski's horse ("wild horse" hereafter) is the only wild horse species surviving in the world today<sup>14</sup>. Because of environmental change and human activities, this species dropped to only 12 individuals in the middle of the last century. Today, the number has increased to approximately 2000, located in the field or in zoos, but all of them are descendants of those 12 ancestors<sup>15</sup>. This event dramatically reduced the genetic variation of the wild horse, which could reduce the ability of the species to adapt to environment change. Severe genetic bottlenecks have also occurred with European bison<sup>16</sup>, northern elephant seals<sup>17</sup> and cheetahs<sup>18</sup>. Therefore, the wild horse is not only a valuable wildlife resource but also a promising model for the study of population genetics. The Mongolian horse is an ancient horse breed that has been an integral part of the culture of nomadic pastoralists in North Asia. The Mongolian horse has a large population with abundant genetic diversity. This ancient breed has influenced other



Northern European horse breeds<sup>19</sup>. It has acquired many special abilities and attributes, such as endurance and disease resistance, and is well adapted to its harsh conditions—a cold, arid climate and poor grazing opportunities<sup>20</sup>. Dramatic chromosomal rearrangement in the horse is a notable feature in comparison to other mammals, and this makes the horse an ideal model for studying chromosomal evolution.

In this study, we obtained quality whole-genome sequences of a male wild horse and a male Mongolian horse using next-generation sequencing technology. The genome sequences of the two representative *Equus* species would improve the genomic maps of the horse. Importantly, based on this, we will focus on karyotypic diversification and explore the genetic mechanisms and evolution rules through analysis of comparative genomics, further uncovering the genetic mechanisms of chromosomal evolution for *Equus* species.

## Results

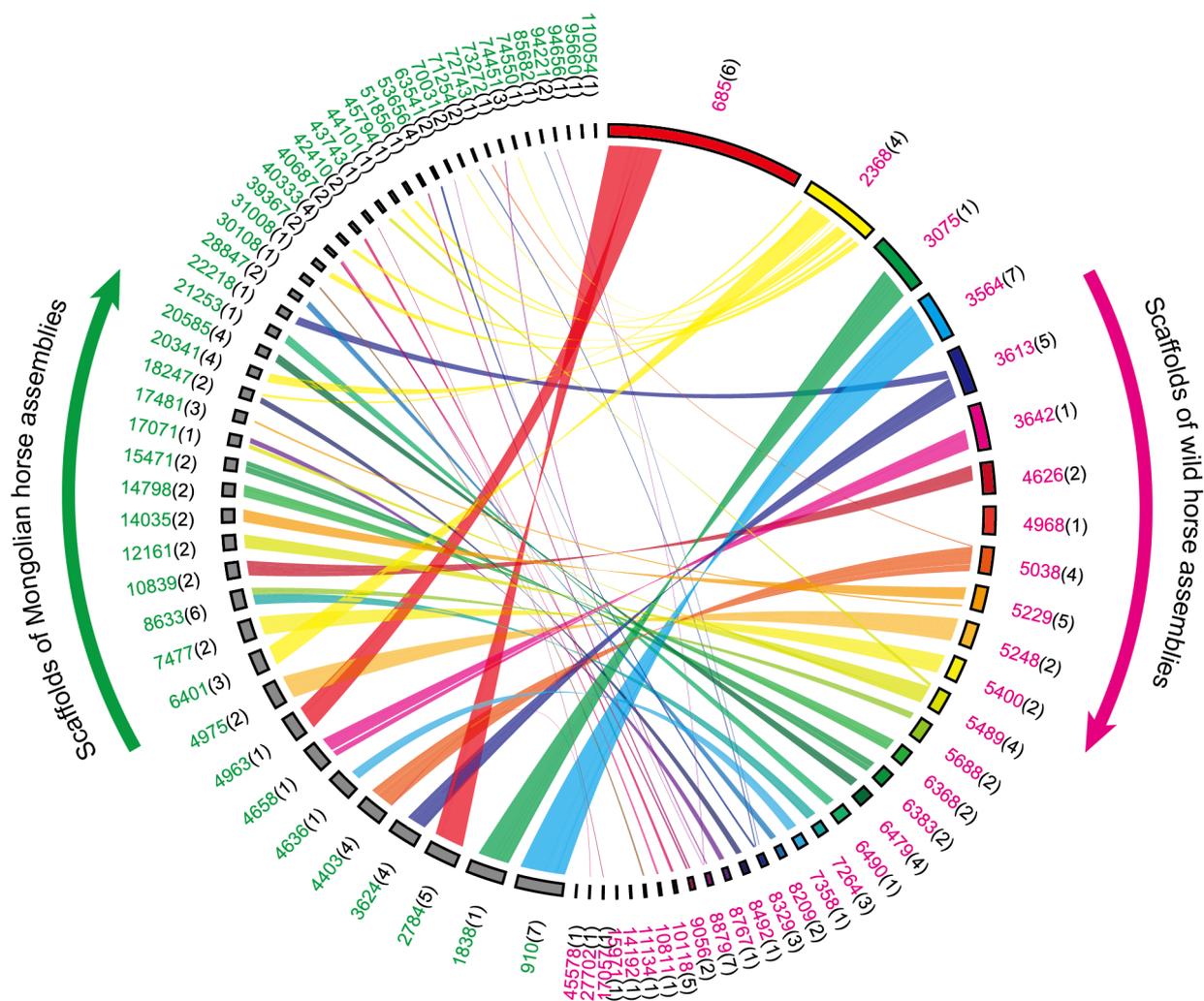
**Genome sequencing and assembly.** The wild horse and Mongolian horse genomes were sequenced using the Illumina HiSeq platform. A paired-end library (500 bp) and two mate-paired libraries (3 kb, and 8 kb) were constructed for both the wild horse and Mongolian horse. In total, we generated 231.21 Gb and 224.17 Gb of usable sequences for the wild horse and Mongolian horse. The sequence depth was 93× and 91×, respectively (Supplementary Table S1). The

sequencing error rates were 0.000575 and 0.000507 for the wild horse and Mongolian horse, respectively (Supplementary Table S2). After assembly, both the wild horse and Mongolian horse generated the same length of genome sequences (2.38 Gb) (Supplementary Table S3).

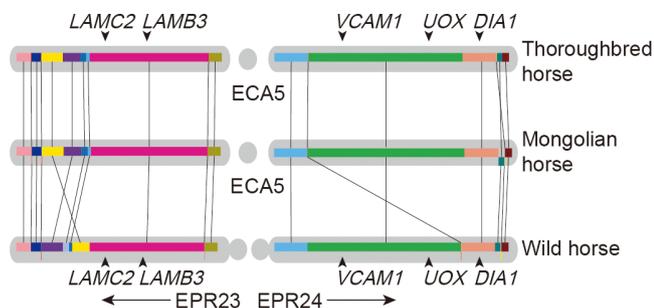
We checked 248 core eukaryotic genes<sup>21</sup> in our two assemblies, and found the completeness was comparable with that of published genomes sequences<sup>22–26</sup> (Supplementary Table S4). We did not detect any misassemblies<sup>27</sup> when comparing our genome assemblies with the wild horse and Mongolian horse sequences available in Genbank. (Supplementary Table S5).

We assembled Y chromosome of wild horse and Mongolian horse (Fig. 1). In previous studies<sup>28</sup>, 127 markers on horse Y chromosome were reported, and in this study, 87 markers and 103 markers could be detected in wild horse and Mongolian horse assemblies, respectively (Supplementary Table S6, 7). Thus, 34 scaffolds (3,018,288 bp) of wild horse and 48 scaffolds (1,971,029 bp) of Mongolian horse were identified originated from Y chromosome. The length of collinearity regions between wild horse and Mongolian horse was around 1.74 Mbp.

To improve gene prediction accuracy, eight types of tissue samples (heart, liver, spleen, lung, kidney, brain, spinal cord and muscle) from a female Mongolian horse were used to construct cDNA libraries. The RNA-seq was performed using the 454 FLX+ platform, and 853,978 reads were obtained with an average length of 458 bp



**Figure 1 | Scaffolds of Y chromosome of wild horse and Mongolian horse.** Thirty-four scaffolds of wild horse and 48 scaffolds of Mongolian horse are shown in this figure, and collinearity regions are linked. Numbers located outside of the brackets are the scaffolds ID of wild horse (carmine) and Mongolian horse (green). Numbers located inside of the brackets represent count of markers detected in the scaffolds.



**Figure 2 | Synteny analysis.** Microsynteny between chromosome 5 of domesticated horses (ECA5) and chromosomes 23 and 24 of wild horses (EPR23, EPR24). Locally Collinear Blocks (LCBs) are marked with the same color and connected by straight lines. The probes (*LAMC2*, *LAMB3*, *VCAM1*, *UOX*, *DIA1*), which are used for FISH, are also detected in this figure.

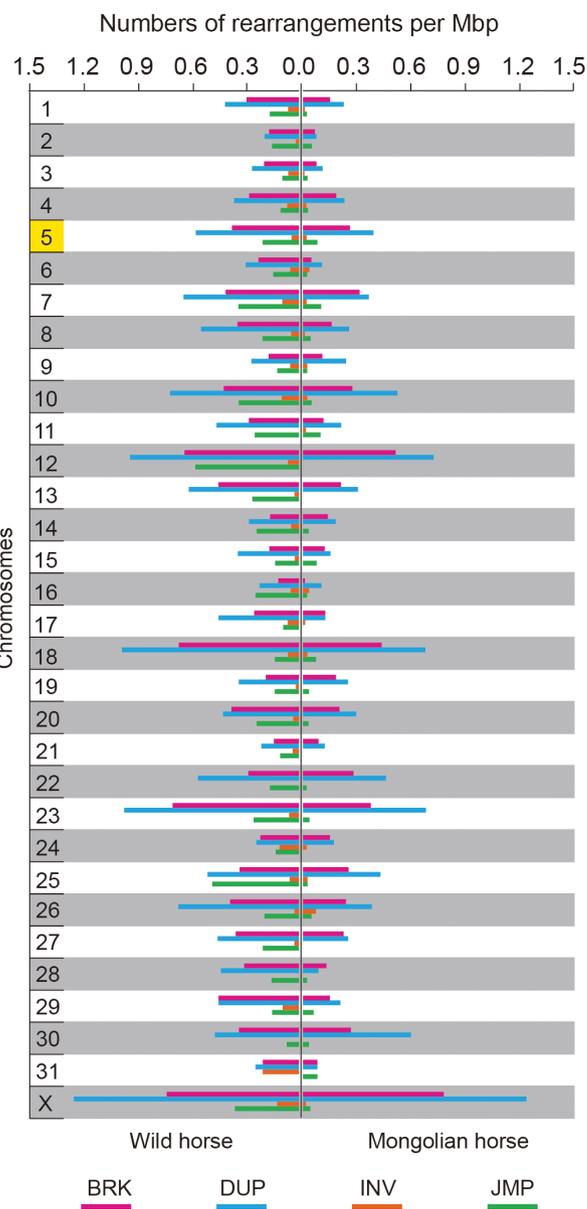
(Supplementary Fig. S1). From these transcriptome data, aided by homology-based gene prediction methods, we estimated that the horse genome contained 20,000 to 21,000 protein-coding genes.

**Synteny analysis.** Robertsonian translocation, which is also called whole-arm translocation or centric-fusion translocation, is a common form of chromosomal rearrangement. Previous studies based on fluorescence in situ hybridization (FISH) results indicate that chromosomes 23 and 24 of the wild horse are homologous with chromosome 5 of the domestic horse. After assembling the wild horse and Mongolian horse genome, we masked out all repetitive sequences and found that EPR23 and 24 of the wild horse and ECA5 of the Mongolian horse could be aligned to the chromosome 5 of the reference genome<sup>29</sup>. The five probes (*LAMC2*, *LAMB3*, *VCAM1*, *UOX* and *DIA1*), which were used in FISH mapping in previous research to confirm that EPR23, 24 is homologous with ECA5<sup>12</sup>, were also identified in both the wild horse and domestic horse genome (Fig. 2).

To study the relationship between Robertsonian translocation and local rearrangement, we performed whole genome synteny analysis. We compared wild horse genome and Mongolian horse genome to the Thoroughbred horse genome, respectively. Collinearity region between Mongolian horse and Thoroughbred horse (2.25 Gbp) was slightly longer than that between wild horse and Thoroughbred horse (2.23 Gbp). 124 Mbp (5.51%) of wild horse genome and 76 Mbp (3.34%) of Mongolian horse genome could not align to Thoroughbred horse genome. Four types of rearrangement, BRK (insertion of unknown origin), DUP (inserted duplication), INV (inversion), and JMP (relocation), were identified (Supplementary Table S8, 9).

Since artifactual mis-joins of assemblies could be counted as rearrangements, we attempted to estimate the correct rate of these rearrangements breakpoints. We remapped the usable reads to the genomes assemblies of wild horse and Mongolian horse, respectively. Then we checked the number of mapped reads in the breakpoint of each type of rearrangements. If the number was less than three, we considered the assembly was incorrect (Supplementary Fig. S2), otherwise correct (Supplementary Fig. S3). We counted 100 breakpoints for each type of rearrangement, and calculated the correct rate. The correct rates of INV (92%) and JMP (82%) were higher than those for BRK (76%) and DUP (58%) in assemblies of wild horse. In Mongolian horse, the correct rates were similar with those of wild horse (Supplementary Table S10).

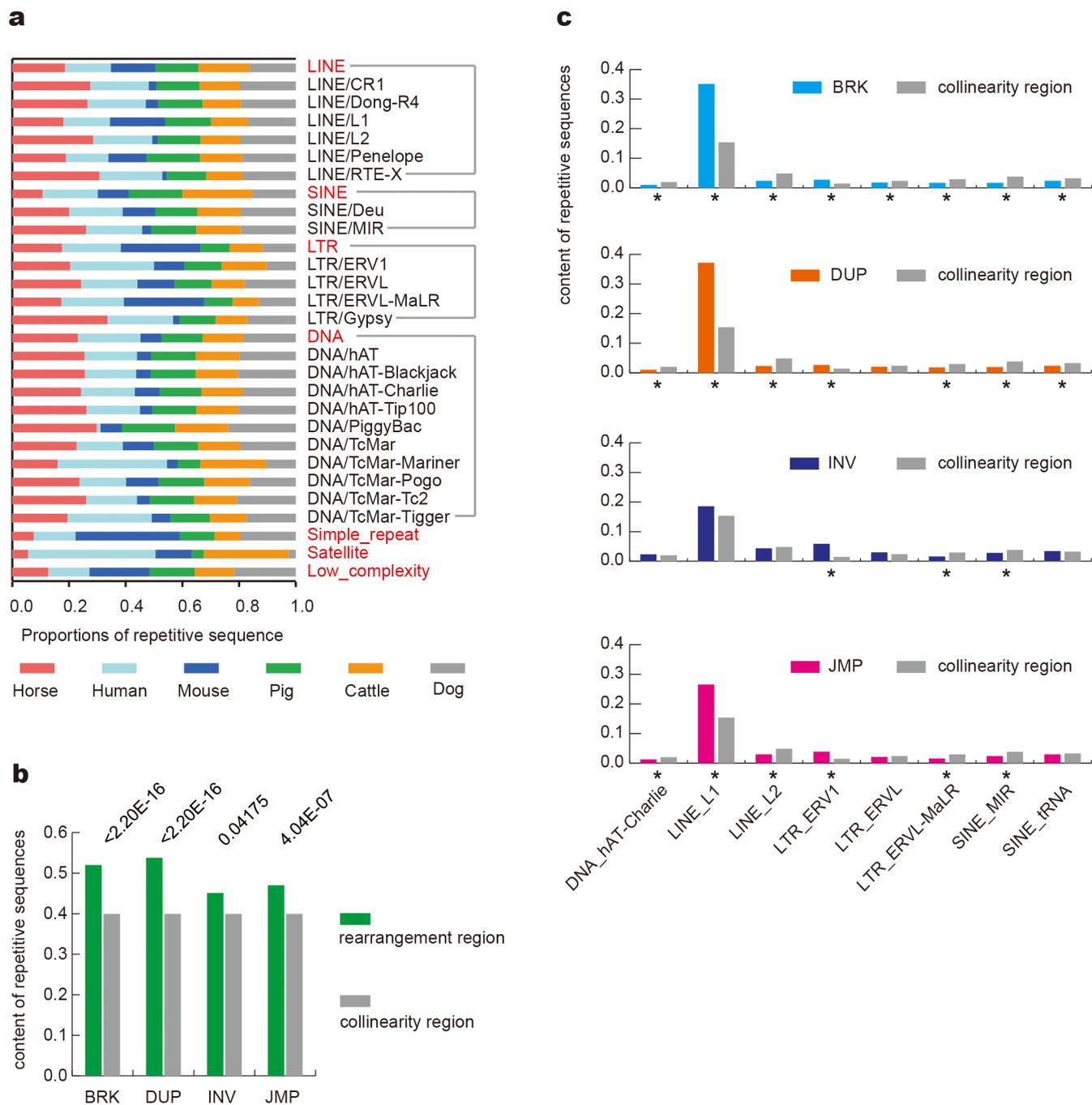
The potential rearrangement sites were investigated for potential synapomorphies. We counted the rearrangement events in two situations: (1) Assume genome sequences of Thoroughbred horse and Mongolian horse were consensus, and identify rearrangements in wild horse; (2) Assume genome sequences of Thoroughbred horse



**Figure 3 | Local rearrangements in the wild horse and Mongolian horse.** Chromosome 5 of domestic horse had undergone Robertsonian translocation (marked as yellow). Thoroughbred horse genome was used as the reference, so the chromosome undergone Robertsonian translocation was also chromosome 5 for wild horse in this figure. BRK: insertion of unknown origin; DUP: inserted duplication; INV: inversion; JMP: relocation.

and wild horse were consensus, and identify rearrangements in Mongolian horse. We found that rearrangement events in the first situation were dramatically more than that in the second (Supplementary Fig. S4). This result was consistent with phylogeny.

The numbers of rearrangements on each chromosome were counted (Supplementary Table S11). Chromosome 5 does not have a greater number of local rearrangements compared with the other chromosomes, although chromosome 5 had undergone Robertsonian translocation (Fig. 3). We noticed that the number of inversions is far less than that of insertions and relocations in the horse genome. Some chromosomes, including the X chromosome, contain more local rearrangements than others. Local rearrangements in the genome of wild horse are more numerous than in that of the Mongolian horse.



**Figure 4 | Analysis of repetitive sequences.** (a) The proportions of repetitive sequences among six species of mammals. Seven common repetitive sequences are marked in red, and the subclasses are marked in black. (b) The content of repetitive sequences is significantly increased in rearrangement regions compared with the collinearity region. The “p-value” is shown on the top. (c) Some repetitive sequences representing content greater than 0.5% of the genome. The content of repetitive sequences significantly increased in BRK/DUP/INV/JMP regions compared with the collinearity region. “\*” p-value < 0.05.

**Repetitive sequences.** Repetitive sequences comprise approximately 50% of the mammal genomes<sup>30</sup> and are associated with syntenic breakpoints and chromosomal fragility<sup>31–33</sup>. Repetitive sequences of six species of mammals (horses<sup>29</sup>, humans<sup>30</sup>, mouse<sup>34</sup>, dogs<sup>35</sup>, cattle<sup>36</sup> and pigs<sup>37</sup>) were examined in this study (Fig. 4a). Seven common repetitive sequences were identified: short interspersed repeated sequences (SINE), long interspersed repeated sequences (LINE), long terminal repeated (LTR), DNA elements, satellites, simple repeats and low complexity. Broadly, the analysis of these sequences indicated that 41.4% of the horse genome sequences are repetitive sequences, which is comparable to the percentages in

humans (46.8%), mouse (42.5%), dogs (40.0%), cattle (47.1%), and pigs (39.1%). LINES comprise 22.6% of the horse genome, which is more than in humans (19.7%), mouse (19.1%), dogs (19.8%), cattle (21.9%), and pigs (18.4%). SINEs can be found in 7.3% of the horse genome, less than in the human (13.4%), mouse (7.5%), dog (10.6%), cattle (17.0%), and pig (13.0%) genomes (Supplementary Fig. S5).

The distribution of repetitive sequences in each chromosome was also examined. The results indicated that each chromosome contains a similar proportion of repetitive sequences, except the X chromosome, which contains a higher proportion of repetitive sequences than autosomes in the six species.



Using those rearrangement regions of the wild horse genome, we studied the association between rearrangement and repetitive sequences. We found some types of repetitive sequences were significantly increased in the rearrangement regions (Fig. 4b, Supplementary Table S12). This result is consistent with previous findings<sup>31–32</sup>. Interestingly, the proportions of LINE\_L1 and LTR\_ERV1 increased, but the proportions of LINE\_L2 and several other repetitive sequences decreased (Fig. 4c, Supplementary Table S13 to S16). This result suggests that LINE\_L1 and LTR\_ERV1 may play a more important role in chromosome rearrangement.

**Heterozygosity analysis.** We identified 1,280,203 and 2,203,945 heterozygous SNPs (within an individual) in the genomes of the wild horse and Mongolian horse (Supplementary Table S17 to S19). Small indels were also identified in the genomes of the wild horse and Mongolian horse (Supplementary Table S20). The heterozygosity rates were  $0.52 \times 10^{-3}$  and  $0.89 \times 10^{-3}$  in the wild horse and Mongolian horse, respectively. The heterozygosity of the wild horse is considerably lower than that of the Mongolian horse.

SNPs were not evenly distributed among the wild horse chromosomes but were evenly distributed in the Mongolian horse chromosomes (Fig. 5a). We explored the heterozygosity rates of different regions using sliding windows of 50 kb with a step size of 10 kb. The number of sliding windows with high heterozygosity rate in Mongolian horse genome was considerable greater than that in wild horse genome (Supplementary Fig. S6). Another interesting phenomenon found in the genome of the wild horse was that heterozygous SNPs were completely excluded in many large regions (Fig. 5b). The sequence coverage of those regions in the wild horse was the same as in the Mongolian horse (Fig. 5c, Supplementary Fig. S7).

In the wild horse, there is a total length of 1287 M of homozygous regions (there are no SNP in wild horse, and there are more than 0.8SNP/Kbp in Mongolian horse) and 58 homozygous regions were larger than 1 Mbp (Supplementary Table S21). A total of 4508 genes were located in those homozygous regions. Enrichment analysis indicated that these genes were enriched for specific functional categories of olfactory transduction ( $n = 118$ ,  $p\_value = 8.90e-12$ ), regulation of cell proliferation ( $n = 11$ ,  $p\_value = 1.70e-02$ ), calcium ion binding ( $n = 9$ ,  $p\_value = 1.70e-02$ ) and others.

## Discussion

In the past decades, researchers have studied chromosomal rearrangement using different conventional methods such as chromosome banding and FISH. However, many local rearrangements are extremely difficult to detect. Here, we sequenced and de novo assembled the homologous chromosomes that had undergone Robertsonian translocation. Our study indicated that Robertsonian translocation did not increase local rearrangements. These findings indicated that Robertsonian translocation and local rearrangements may be caused by different mechanisms. From our results, inversions are rarer than insertions and relocation, suggesting that insertions and relocation may play a more important role in shaping the genome.

Some studies have demonstrated that repetitive sequences are associated with syntenic breakpoints and chromosomal fragility<sup>31,32</sup>. This study did not reveal significant differences in repetitive sequences among different species and different chromosomes (except the X chromosome). Different strategies of genome sequencing (clone by clone and whole genome shot-gun) may impact the actual content of repetitive sequences in the genomes. Our results suggest that chromosomal local rearrangements are highly associated with repetitive sequences. However, these repetitive sequences did not contribute equally to rearrangement. LINE\_L1 and LTR\_ERV1 may play a more important role than other repetitive sequences.

In the middle of the last century, the population of the wild horse dropped to only 12 individuals. The genetic bottleneck and inbreeding caused by this event may be the reason for the many more homozygous regions in the wild horse genome. One interesting result is that heterozygous SNPs are completely excluded from chromosome 26 of the wild horse. It was the largest fragment without heterozygous SNPs. As sequencing coverage of EPR26 is similar to other autosomes, we confirmed that there was a pair of chromosome 26 in this individual (Supplementary Fig. S8). Another explanation could be that this pair of chromosome 26 was present because of uniparental isodisomy<sup>38–40</sup>. We also sequenced a short region (~700 bp) in chromosome 26 of several other wild horse samples using the Sanger method and found 6 SNPs, indicating that this region is heterozygous in some other wild horses.

The analysis results of the two representative *Equus* species improved the genomic maps of the horse. It also revealed the unique aspects of the chromosomal rearrangement and improved our understanding of chromosomal evolution in mammals implicating *Equus* is thus a promising model to explore the Karyotypic instability. These analysis and discoveries would benefit studies of mammal karyotypic evolution and chromosomal rearrangement, and studies of human disease caused by chromosome aberration.

## Methods

**Sampling and genome sequencing.** Protocols used for this experiment were consistent with those approved by the Institutional Animal Care and Use Committee at Inner Mongolia Agricultural University. For sequencing, a male wild horse was selected from the “YE MA International Group” of Xinjiang, China, and a Mongolian horse was selected from the Xilingol League of Inner Mongolia, China. DNA was extracted from ear tissue and peripheral blood cells. Illumina HiSeq 2000 was used to sequence the genomes of wild horse and Mongolian horse using a shotgun strategy. A pair-end library (500 bp, standard genomic library and sequenced using paired-end reads) and two mate-pair libraries (3 kb, and 8 kb) were constructed for each horse. The length of reads was 101 bp for pair-end library and two mate-pair libraries. Library preparation and sequencing followed the manufacturer’s instructions, and sequence reads were collected from the Illumina data processing pipeline.

**Data filtering.** The following types of reads were filtered out: (1) reads with more than 3 unidentified nucleotides, (2) reads with average phred quality below Q30, and (3) reads with unidentified nucleotides in the first 50 nucleotides.

**Genome assembly.** The genome sequences of the wild horse and Mongolian horse were assembled with short reads using SOAPdenovo<sup>41</sup>. We first assembled the short reads of the pair-end library (500 bp) into contigs using sequence overlap information. Then, we used the information of the mate-pair libraries (3 kb and 8 kb) to join the contigs into scaffolds. Finally, “Gapcloser” (<http://soap.genomics.org.cn/soapdenovo.html>) was used to close the gaps inside the scaffolds.

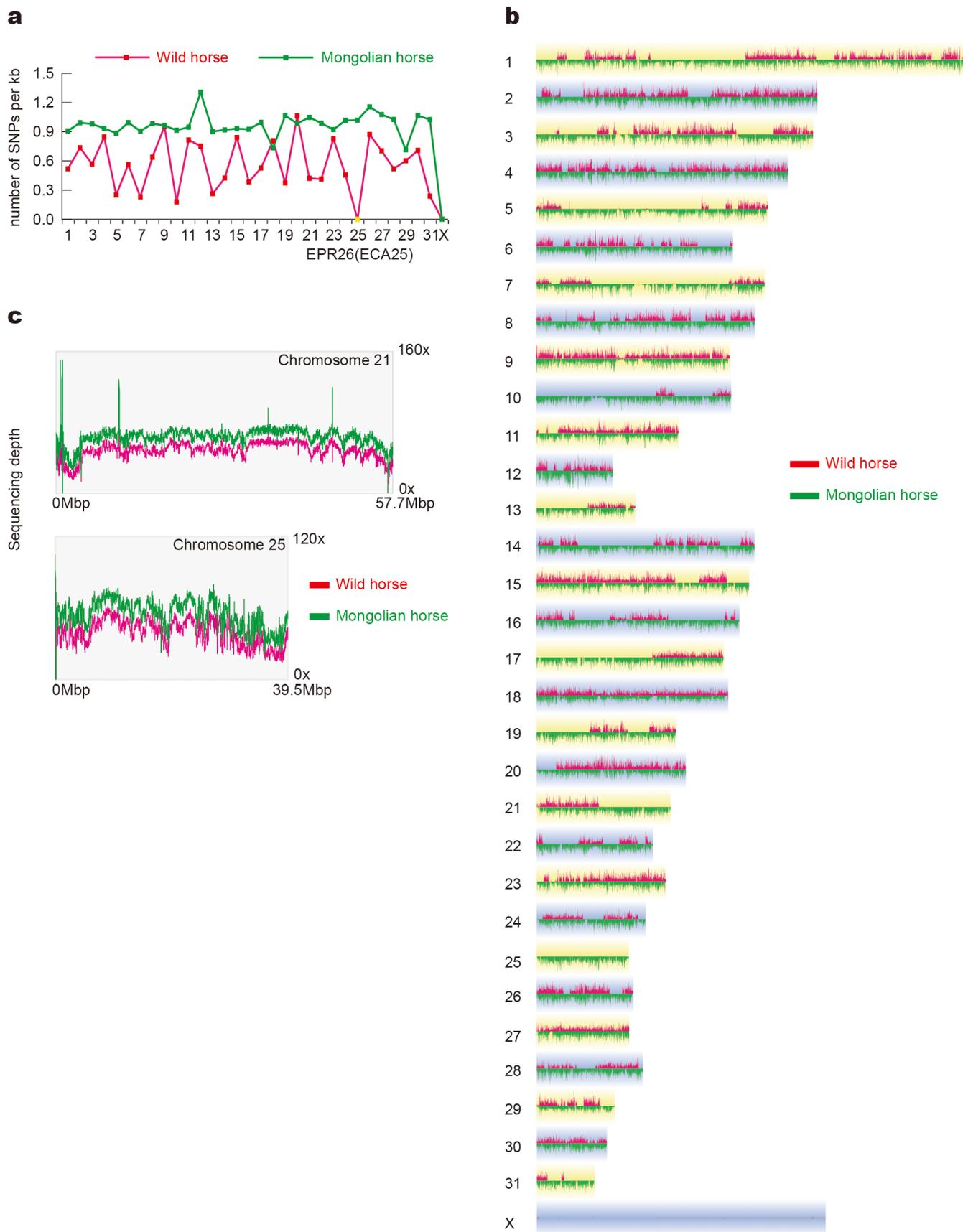
**Genome annotation.** For protein-coding gene annotation, ab initio prediction was performed by MAKER<sup>42</sup>. We generated cDNA data from multiple RNA sources. Using an oligoDT-based approach, cDNA libraries were constructed from eight types of tissue samples (heart, liver, spleen, lung, kidney, brain, spinal cord and muscle) from a female Mongolian horse. The library was sequenced using a Roche 454 FLX+ platform.

**Estimated sequencing error rate.** Data from the X chromosome was used to estimate the sequencing error rate, which is hemizygotic in males. The calculations were not influenced by heterozygous SNPs<sup>43</sup>. All qualified reads from the wild horse and Mongolian horse were mapped to the X chromosome of *Equus caballus*, and all repetitive and low complexity regions were excluded. At each nucleotide position, the predominant call was assumed to be true, and all others were considered to be errors.

**Synteny analysis.** We used the MAUVE program<sup>44</sup> to construct the synteny map for chromosome 5 of domestic horses (Thoroughbred and Mongolian horse) and chromosomes 23 and 24 of the wild horse. We masked out all repetitive sequences, and unique sequences were preserved. Then, we used the Mauve Contig Mover (MCM) to order the draft genomes of the wild horse and Mongolian horse relative to the Thoroughbred horse genome (Equcab 2.0). The synteny analysis used progressiveMAUVE.

We used MUMmer<sup>45</sup> to perform the synteny analysis for the whole genomes of the wild horse and Mongolian horse, in addition to the reference genome. Four types of rearrangements (BRK, DUP, INV, JMP) were identified using the “nucmer” module. The parameter was Options “-c 800 -g300 -l 100”.

**Repetitive sequence analysis.** We screened DNA sequences for interspersed repeats and low complexity DNA sequences using RepeatMasker (<http://www.repeatmasker>).



**Figure 5 | Effect of genetic bottleneck on genome landscape.** (a) The SNPs distribution of each chromosome in the wild horse and Mongolian horse. For the Mongolian horse, the SNP distribution of each autosome is similar, but for the wild horse, the SNP distribution among the autosomes is different, and there are no SNPs on EPR26 (ECA25 in this figure). (b) Contrast of heterozygous SNPs between the wild horse and Mongolian horse. (c) The sequencing depths of chromosome 21 and 25.



org/) in the collinearity and rearrangement regions. Collinearity regions, which were larger than 100 kb, were used for following analysis, and rearrangement loci plus 2 kb extended flanking regions were treated as rearrangement regions. The “T-test” was performed using R software.

**SNP calling and heterozygosity rate estimation.** We utilized the BWA program<sup>46</sup> to map the usable reads from the pair-end libraries (500 bp) of the wild horse and Mongolian horse to the genome sequences of Thoroughbred horse (Equcab 2.0). The parameters chosen for mapping were as follows: seed length of 32, and the maximum occurrences for extending a long deletion of 10. Duplicated reads were removed by SAMtools<sup>47</sup>. SNPs and InDels were called using the Genome Analysis Toolkit<sup>48</sup> according to the guidelines as described.

The heterozygosity rate was estimated as the density of heterozygous SNPs for the whole genome. For the estimation of local heterozygosity rate, sliding windows of 50 kb with 80% overlap between adjacent windows were used to scan the genome.

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## Author contributions

M.D., H.M., Ya.Z., Z.S. designed and managed the project. J.H., W.Y., Ya.Z., H.M. designed and performed the genome assembly and analyses. J.H., H.M., Ya.Z., M.D. wrote the paper. D.D., D.B., J.H., Yi.Z., H.H., H.B., Y.B. collected samples and prepared the nucleic acid samples. Yi.Z., W.S., B.L., D.B., L.Y., B.J., Q.Z., Y.G., J.W., W.B., A.L., Yu.Z., L.Z., Z.Z., W.Z. performed the genomes sequencing.

## Additional information

**Data Access** The Whole Genome Shotgun project has been deposited in DDBJ/EMBL/GenBank as project accession PRJNA200657 and PRJNA200654 of wild horse and Mongolian horse, respectively. The genome assembly of wild horse has been deposited at DDBJ/EMBL/GenBank under the accession ATBW00000000 and this version described in this paper is version ATBW01000000. The genome assembly of Mongolian horse has been deposited at DDBJ/EMBL/GenBank under the accession ATDM00000000 and the version described in this paper is version ATDM01000000. Transcript sequencing data have been deposited under Short Read Archive (SRA) accession SRR1014663.

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