Molecular Cellular Level and Genetic Material of Donkeys

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Lack of archaeological and whole-genome diversity data has restricted current knowledge of the evolutionary history of donkeys. With the advancement of science and technology, the discovery of archaeological evidence, the development of molecular genetics, and the improvement of whole-genome sequencing technology, the in-depth understanding of the origin and domestication of donkeys has been enhanced. The donkey is an equine animal of the order Odd-toed ungulates. Among common livestock animals, it occupies an important place in the history of human transportation. It is often used as a pack animal for long-distance transportation due to its good packability, durability, and traction, thereby greatly facilitating commerce among regions. With the advancement of modern technology, mechanization of agriculture, and rapid development of transportation, the service value of donkeys has decreased. In economically developed regions (such as Italy and America), donkeys are more often bred as ornamental pets. However, in fast-growing developing countries (such as China), donkeys are more often used as a source of meat for mass breeding because of their rich intra-muscular fat and superior taste. Donkey milk is being sold as a commodity for the treatment of infants, the sick and the elderly who are physically weak. Donkey milk is rich in nutrients and is whey-protein milk, which is the closest to human milk and can be used as a substitute for breast milk, and has various functions such as regulating immunity and promoting growth. The scale of donkey breeding and the quality of donkey breeds are also decreasing in developed areas; however, the size of donkey inventories continues to slowly increase in developing areas.

donkey bioarcheology gene

1. Origin of the Donkey and Archaeological Findings

1.1. Linguistic Evidence on the Origins of the Donkey

The early history of the African donkey is arduous to explore due to the lack of direct archaeological data. The wild relatives of the donkey have been hunted to extinction. Therefore, gathering detailed data to track the genetic information of their ancestors is difficult. Another strategy for filling this historical gap is the use of linguistics. Donkey and donkey terminologies have been recorded in various African and Near Eastern languages. Compiling these terms and tracing the connections could suggest some hypotheses about the domestication process and its pathways of transmission. These connections could be combined with modern ethnographic data to reconstruct the prehistory of the African donkey [1][2].

Linguistic evidence suggested that various branches of West Asian-African languages appear to have fairly distinctive wild-ass vocabularies. The main distributions are as follows: #kuur, which is widely distributed in Africa; #harre Ethiopian; #d-q-r Cushitic; #aɣyul Berber; and #aʒḍ Berber. In most cases, people were sufficiently familiar with the wild ass to name the creature in pre-domestication times. A comprehensive analysis revealed that the root #k-r is common in Central Africa, and it appears to have been transmitted to the Lake Chad region from the Cushitic region of the Horn of Africa (thus subsumed under the Nilo-Saharan languages). These findings prove that donkeys have been described in various languages of different tribes at different times, leading to the hypothesis that donkeys may have been domesticated many times on the Sahara periphery [3][4].

1.2. Archaeological Discovery of the Domestic Donkey

In the early years of human society, small city states proliferated, and the political, economic, social, and ritual natures of cities experienced an extensive change. Production specialization, large-scale long-distance transportation, and the extensive increase in the scale of warfare led to an increasingly important role of donkeys in the exchange between regions. Goods, such as copper and other products, were increasingly transported. Development that transformed the scale of the economic system benefited the domestic asset owners, creating a new class of merchants, and donkeys became progressively valuable. As donkeys began to be widely used in the Near East, “donkey caravans” were formed to specialize in the transportation of goods. Donkeys spread as a totem associated with worship among merchants and herders, who occupied specialized positions in the growing complexity of the social structure of urban societies, to secure their social status. An intact fossilized donkey bone was found at a site in Israel. The results of the burial excavation and analysis were used to integrate zooarchaeological, architectural, stratigraphic, and typological analyses of this bioarcheological deposit and identified donkey burial as a ritual deposit, clarifying the importance of this taxon to the religious and economic spheres of the Near East Early Bronze Age (EBA). If the results are confirmed, these findings will open a new gateway to understanding donkey burials scattered throughout the region [5][6].

1.3. Archeopathological Study of the Domestic Donkey

The domestication process in donkeys may be slower and not as linear as previously supposed. Species–specific indicators of the early stages of domestication need to be identified to characterize the length and course of this complex process. Unfortunately, the earliest stages of donkey domestication are when they most resemble their wild ancestors, making them difficult to distinguish. The recently discovered skeleton and concurrent studies of modern African wild ass and donkey metacarpals were intended to elaborate the analysis of the original skeleton and develop markers for the domestication process of the donkey. Skeletal fragments show a tendency for Egyptian donkeys to decrease in size over time; thus, skeletologically, large donkeys are usually considered wild and small donkeys are usually considered domestic [7]. Morphological evidence of load-carrying is displayed on contemporaneous burial remains from the same region, providing powerful support for domestication [8]. The well-preserved integral skeleton provides a morphological context for each bone that isolated skeletal fragments from previous archaeological findings lack, and a particular opportunity to apply metacarpal indicators and new paleopathological methods to distinguish hunted wild asses from domesticated ones [4][9].

2. Research Findings at the Molecular Cellular Level

2.1. Chromosomes

Horses, donkeys, and zebras belong to the genus *Equus*, which was formed approximately 4–4.5 million years ago. Although the equine fossil record represents a textbook example of evolution, the sequence of events that led to the existence of species diversity to date remains unclear. The entire genome and the genomes of surviving equine species were sequenced, the genetic material composition of specific genealogies were deciphering, and the complex history of the formation of equine species was revealed. Surprisingly, multiple examples of hybridization have been found throughout the genus *Equus* despite extremely different chromosomal structures, in contrast to theories that promoted chromosomal incompatibilities as a driving factor in the origin of the equine species [10][11].

Six indexed DNA libraries from the Somali wild ass (SOM), Onager (ONA), Tibetan kiang (KIA), Grevy's (GRE), mountain (HAR), and plains (BOE) zebras were prepared successfully. The genome sets of all extant zebras and donkeys of the genus are currently completely represented. In addition, museum specimens were used to characterize the genome of extinct quagga zebra (which became extinct in the early 1900s) [12]. Combined with the horse genome data from previous studies [13], this completes the genomes of all extant species of the genus. The analysis was performed through simulation experiments by scanning all genomes. The results showed that the earliest species formation gene flow occurred in North America. The ancestors of today's donkeys and zebras dispersed between 2.1 and 3.4 million years into the American continent, eventually experiencing major population expansions and collapses that coincide with past climate change events. Evidence of gene flow involving three contemporary equine species was also found despite the chromosome numbers ranging from 16 pairs to 31 pairs. These findings challenged the notion that the accumulation of chromosomal rearrangements drove complete reproductive isolation and promoted equids as a fundamental model for understanding the interplay between chromosomal structure, gene flow, and eventual species formation [14].

2.2. Fluorescence In-Situ Hybridization (FISH)

Chromosome banding and new molecular cytogenetic techniques, particularly FISH, using chromosome painting probes, were used early on to understand the phylogenetic and systemic relationships of species and provide insights into the possible mechanisms of species formation [15]. Comparative detection was performed on the sequences of telomeres, meristematic granules, and kernel composition regions in domestic horses (*Equus caballus*) and domestic donkeys (*Equus asinus*) by using primed in-situ DNA synthesis (PRINS) and FISH. The findings did not reveal any additional sites in horses and donkeys but confirmed the differences in signal intensity and frequency between the individual chromosome pairs in the two species. As in horses, no interstitial telomeric sites (ITSs) were detected in the donkey genome through PRINS analysis, possibly due to the multiple chromosomal rearrangements or the gradual loss of repetitive sequences that occurred during evolution after divergence from a common ancestor. On the contrary, another hypothesis indicated that these sequences are present in a very low copy number, hence not detected [16][17].

The presence of constitutive alkaline disruption sites (ALSs) in donkey (*E. asinus*) and stallion (*E. caballus*) spermatozoa was investigated using DNA breakage detection–FISH and comet assay. ALSs in the sperm of donkey was 1.3 times greater than in stallion and the length of the comet tail obtained in donkey sperm was 1.6 times longer than that observed in horse ($p < 0.05$). The difference is significant between these two species. The results suggested that ALS represents a species-specific issue in mammalian species related to chromatin organization in sperm and somatic cells, and it may diverge even at short phylogenetic distances [18].

Within the karyotype of the domestic donkey, heterochromatic bands of non-centric chromosomes have been described in sub-centric and telomeric positions. By using FISH, changes in the intensity and distribution of fluorescent signals were observed after in-situ hybridization with two DNA probes containing fragments of the two major equine satellite DNA families. Chromosome 1 has heterochromatic bands in the proximal regions of the long and short arms, and the number and distribution of large clusters of satellite DNA could define at least nine polymorphic variants constituting sexual heterochromatin that could not be detected by the C-banding method alone [19].

3. Research on Genetic Material

3.1. Ancient DNA

A low-coverage draft genome sequence was acquired from a horse bone recovered from a permafrost site in the Yukon Territory, Canada, dated to approximately 560,000 to 780,000 years ago [20]. Comparative genomics revealed that the *Equus* lineage that gave rise to all contemporary horses, zebras, and donkeys originated approximately 4–4.5 million years ago, which is much earlier than previously suspected. These data supported the contention that Przewalski horses, an endangered subspecies native to the Mongolian steppe, represent the last surviving population of wild horses.

The available genetic data from donkeys revealed two distinct mitochondrial DNA (mtDNA) haplotypes, indicating two distinct domestication events that occurred in Northeastern Africa approximately 5000–7000 years ago. The absence of a clear phylogeographic structure in domestic donkey haplotypes and the paucity of information on the genetic composition of African wild ass ancestors resulted in difficulty determining the feral ancestry and geographic origin of domestic mitochondrial branches. The analysis of ancient archaeological and historical museum samples provided genetic information on the historical Nubian wild ass (*Equus africanus africanus*), Somali wild ass (*Equus africanus somaliensis*), and ancient donkeys. The results indicated that the donkeys of clade 1 have a long history in the Sahara, and their ancestors were Nubian wild asses that were crossed with domestic donkeys over a long period of time by introducing several maternal haplotypes from the wild asses; the gene flow of this clade is continuous. A relative of the Somali wild ass is the ancestor of clade 2 (probably extinct). The Somali wild ass, on the other hand, belongs to clade 3, which is highly separated from clades 1 and 2, indicating that the Somali wild ass is not the ancestor of these two clades. The low variation and large sample size of the Somali wild ass made it unlikely that other lineages could be identified [21]. The very ancient merging period of the evolutionary branch reflects a long period before donkey domestication, suggesting that the extensive genetic structure, fragmentation, and geographic isolation of mitochondrial variation in wild asses may have preceded domestication. The results illustrated the complexity of animal domestication and valuably contributed to the debate on the variation, phylogeny, and management of the extant but critically endangered African wild ass. Most of the available mitochondrial diversity samples have been obtained from captive-bred Somali wild-ass populations, but further research of the DNA and chromosomes of extant populations requires additional specimens for DNA analysis.

In 2022, Todd et al. constructed a comprehensive genomic panel of 207 modern and 31 ancient donkeys and 15 wild equids in order to elucidate the domestication history of donkeys, uncovering a robust phylogeographic structure of modern donkeys [22]. The findings support a single domestication of donkeys in Africa around 7000 years ago, followed by further expansion across that continent and Eurasia and eventual return to Africa. The latest findings are not consistent with previous studies, with differences in the number of domestications of donkeys. In addition, researchers have discovered a previously unknown genetic lineage in the Levant (2200 years ago), a finding that adds to the ancestry of the Asian donkey.

Evolutionary processes, including selection, could be indirectly inferred from the patterns of genomic variation in contemporary populations or species. Sequencing ancient DNA from samples with time intervals could provide insights into the past selection processes, as time-series data could directly quantify population parameters collected before, during, and after selection-driven genetic changes [23]. Incorporating temporal sampling and generation of ancient genomic datasets in the context of evolutionary biology and using some emerging techniques that have not been widely used by evolutionary biologists more accurately restore the true population data of the species throughout its history. However, these same data have limitations, and they may be influenced by post-mortem damage, fragmentation, low coverage, and typically low sample sizes.

3.2. Microsatellite Markers

Within the framework of varietal protection, genetic characteristics are important for the integrity of the variety, and they are a prerequisite for the treatment of genetic resources. In the last decades, the use of molecular markers has played an important role in the analysis of genetic diversity and genetics. Among the different types of molecular markers, microsatellite markers are widely used due to their ease of PCR amplification and ability to analyze large amounts of genetic variation (allelic variation at each locus). Microsatellite markers are simple sequence repeats, usually consisting of 1–6 nucleotide repeats, which are abundant and distributed throughout the genome. Microsatellite markers are highly polymorphic, species-specific, and co-dominant compared with other molecular markers, and thus have become increasingly important genetic markers for genetic diversity, population genetics, and disease diagnosis. These advantages validate the continued use of microsatellite markers in different studies to quantify genetic variation within and between species for the conservation management of animal populations [24][25].

Studies on the genetic characteristics of donkey breeds is scarce, and they are mainly concentrated on Mediterranean and Asian breeds. In 2006, 24 pairs of microsatellites from the horse genome were used to amplify the genomes of eight local donkey breeds in China. The results indicated that the microsatellites of similar species are conserved and could be used to analyze the genetic diversity of donkeys. Researchers also needed to have a deeper understanding of the genetic information and genetic relationships within and among breeds to obtain more accurate and generalized conclusions, which could be more effectively applied to the conservation and further utilization of donkey breeds [26][27][28]. In terms of average allele number, the genetic variability observed in three Sicilian donkey breeds should be lower than in five Spanish and three Croatian breeds but higher than in the Italian Amiata donkey [29]. However, the expected heterozygosity was lower than that of the European breeds and the eight Chinese donkey breeds mentioned above. Considering that the breed Pantesco is undergoing genetic recovery, the actual numbers are sparse, thus making it an endangered breed with low genetic variability. Molecular characterization of Sicilian varieties revealed a high degree of internal structure, evidence that could be largely attributed to the Pantesco structure, which is clearly distinct from Ragusano and Grigio Siciliano; meanwhile, the significant divergence of the Pantesco structure seems to be the hallmark of the proper genetic management program undertaken thus far [30][31].

In the past decade, researchers in various countries around the world, such as the United States [32], India [33], South Korea [34], Ethiopia [35], Turkey [36], and Serbia [37] have also been using microsatellite markers to experimentally test local donkey breeds. However, with the exception of China and Italy, these studies have not formed a coherent research system, partly related to the endangered population of local donkey breeds, the weak emphasis on donkey science, and the lack of research efforts to protect local donkey breeds. The Mongolian wild ass [38] and the Kiang (*Equus kiang*) in China, and the Pantesco, Ragusano , and Grigio Siciliano in Italy [31], and Banat donkey in Serbia [37] are protected as endangered species, and their population size has been increased to some extent.

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