

Ancient DNA unravels species identification from Laosicheng site, Hunan Province, China, and provides insights into maternal genetic history of East Asian leopards

DEAR EDITOR,

The Laosicheng site in Yongshun County, Hunan Province, China, plays an important role in elucidating the animal population composition of the region prior to the middle Qing Dynasty (approximately 300 years ago). This site has yielded a substantial number of animal bones, 11 960 of which have been identified to the species level, serving as direct evidence of ancient animal populations (Hunan Provincial Institute of Cultural Relics and Archaeology, 2018). Notably, two mammalian skulls from this site, designated as Laosicheng1: 10YLG10 (101) and Laosicheng2: 10YLG10 (134), have garnered particular attention, with Laosicheng2 selected as the cover image for the above publication. Initially, these skulls were classified as Canids, with Laosicheng1 identified as a dog and Laosicheng2 identified as a wolf (Hunan Provincial Institute of Cultural Relics and Archaeology, 2018). However, the accuracy of these classifications has been called into question. In this context, a comprehensive analysis was undertaken, involving precise cranial morphological examination and mitochondrial genome (mitogenome) sequencing of the two Laosicheng samples using micro-computed tomography (micro-CT) and ancient DNA (aDNA) techniques to facilitate taxonomic reassessment. The results revealed that both skulls are attributable to leopards (*Panthera pardus japonensis*). Furthermore, phylogenetic analysis using data from this study and global leopard datasets, with a focus on East Asia, strongly suggested that East Asian leopards (*P. p. orientalis*, *P. p. japonensis*, and *P. p. delacouri*) belong to three distinct populations. This insight significantly advances our understanding of the maternal genetic history of East Asian leopards.

Initially, micro-CT scans (Institute of High Energy Physics, Chinese Academy of Sciences, China) were performed on the two samples to obtain precise cranial morphological data for species identification. This process also ensured the preservation of the original morphological characteristics of the skull before proceeding to genetic analysis. To describe the skull morphology, 22 skull measurements (Supplementary Figure S1 and Table S1) were taken from 3D models, with the average values and standard deviations (SD) of these measurements shown in Supplementary Table S1. Skull size

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was generally similar to that of *P. pardus* and *P. uncia*, but conspicuously larger than that of *Neofelis* and Felini species. Notably, the auditory bulla of the skulls was inflated, with a relatively small ectotympanic chamber, a feature distinct from *P. uncia*. Furthermore, the mastoid process was relatively weak, and the distant postorbital constriction supported a closer affinity to *P. pardus* than to *P. uncia*. For accurate species identification, photographs displaying the dorsal view of skulls from 23 extant Felidae species, including 15 Felini species, six Pantherini species, and the two Laosicheng samples, were collected and used for principal component analysis (PCA) employing a variance-covariance matrix of eight landmark measurements (Supplementary Tables S1–S3). Results demonstrated that the Laosicheng samples formed a loose cluster, positioned between the Felini and Pantherini species (Supplementary Figure S2), without providing a definitive species identification. Although the morphological evidence suggested that the two samples belonged to the family Felidae, characterized by distinctive features (e.g., auditory bulla and mastoid process) (Supplementary Figure S3), the damaged state of the skulls presented challenges in specific species determination. Therefore, we attempted to recover endogenous aDNA sequences for a more accurate identification.

Previous studies have established the effectiveness of aDNA analysis in the taxonomic identification of ancient animals, with genetic data also allowing for a more comprehensive understanding of population genetic history (Zhu et al., 2022). Here, high-quality mitogenome sequences were successfully obtained for the Laosicheng samples (Supplementary Figures S4). Utilizing a dataset combining the two Laosicheng samples with 114 other individuals representing all genera of the family Felidae (Supplementary Table S4), pairwise genetic distance analysis indicated that the Laosicheng samples share a closer genetic affiliation with the *Panthera* genus than any other ($F_{ST} < 0.01$) (Supplementary Figures S5, S6 and Table S5). Further genetic distance comparisons between the two Laosicheng samples and 79 individuals within the *Panthera* genus (including *P. pardus* ($n=50$), *P. leo* ($n=5$), *P. uncia* ($n=15$), *P. onca* ($n=4$), and *P. tigris* ($n=5$)) (Supplementary Figures S5, S6 and Table S5) placed the Laosicheng samples closest to

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P. pardus (Supplementary Figures S5, S6 and Table S5), with both Laosicheng individuals being genetically closest to *P. p. japonensis* than any other subspecies (Figure 1B; Supplementary Figure S6 and Table S5). To verify species identification and gain further insights into the maternal genetic history of East Asian leopards, the same dataset was used to construct phylogenetic trees (Supplementary Figures S7, S8 and Table S4). The resulting Bayesian and maximum-likelihood trees were consistent with the genetic distance results. Based on these findings, we propose the classification of the two Laosicheng samples as leopards, specifically *P. p. japonensis*.

All *P. pardus* species individuals were categorized into two groups (Eurasia and Africa), revealing a deep bifurcation (~769 thousand years ago (ka), 95% CI: 518–1 000 ka) (Figure 1C) between the Eurasian and African mitochondrial lineages, similar to the findings of Paijmans et al. (2021) and

supporting Africa as the most likely place of origin (Supplementary Figures S7–S9). The Asian population of *P. pardus* was divided into three main lineages, including the North group represented by *P. p. orientalis*, Central group represented by *P. p. japonensis*, and South group represented by *P. p. delacouri*. The Central group included one sample (GenBank accession number: MK043027) sourced from a zoo at Daejeon O-World amusement park in Korea that was taxonomically specified as *P. p. orientalis*, but was found to contain approximately 30% genetic admixture from the North-Chinese leopard (*P. p. japonensis*) (marked in Figure 1C with **P. p. orientalis_Korea*) (Kim et al., 2016). Additionally, another sample (GenBank accession number: MG932393) collected from Shanxi Province, China, was only identified as *P. pardus*, with no further subspecies information, but which clustered with *P. p. japonensis* in the phylogenetic tree (marked in Figure 1C with **P. p. japonensis_Shanxi*) (Ren et al., 2019).

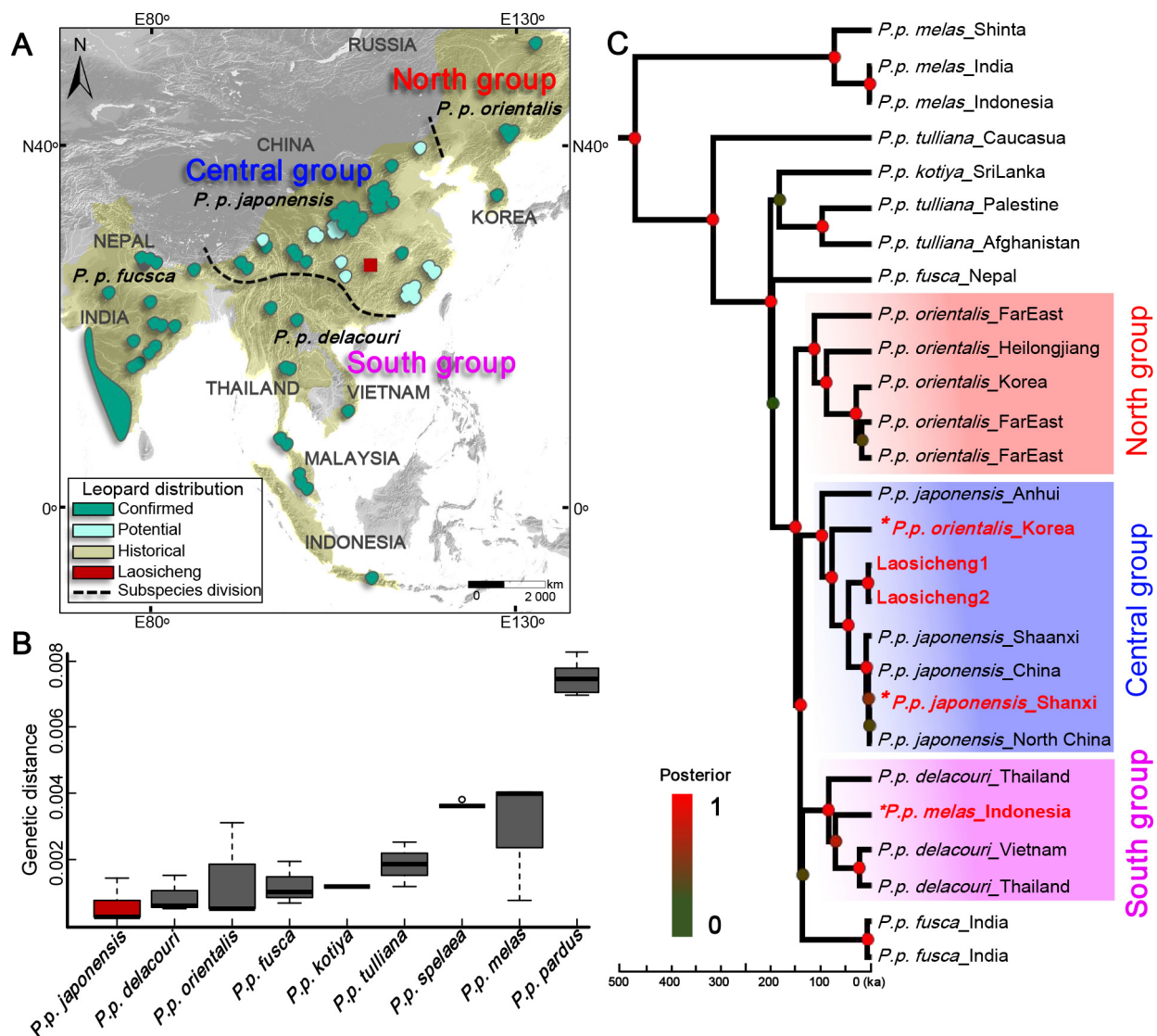


Figure 1 Morphological and genetic analysis of Laosicheng individuals (*Panthera pardus*)

A: Geographic distribution of three primary leopard populations in East Asia based on Laguardia et al. (2017), Kitchener et al. (2017), and this study. Dark green shaded area represents confirmed distribution of leopards, light green shaded area represents potential distribution area. Black dashed line represents boundary between three groups (North, Central, and South). Laosicheng is marked with red square. B: Boxplot of genetic distances across 50 individuals covering nine subspecies of *P. pardus*. C: Partial tree is based on selected individuals from global populations (full tree shown in Supplementary Figure S7). Laosicheng samples and three samples of interest are shown in red. Posterior values are marked with dots in varying colors, ranging from green to red, reflecting posterior support values from 0 to 1.

The South group included an Indonesian sample (GenBank accession number: MH588627) (marked in Figure 1C with **P. p. melas_Indonesia*) closely related to *P. p. delacouri*, which may be attributed to historical exchanges of live animals or acquisition of hunting trophies between the Southeast Asian islands and the mainland (Pajmans et al., 2018) (Figure 1A, C). The two Laosicheng samples exhibited the highest genetic correlation with each other and were grouped with individuals from the Central group (Figure 1C), aligning with previous results showing similarity with *P. p. japonensis*.

Three East Eurasian populations of leopard (*P. p. orientalis*, *P. p. japonensis*, and *P. p. delacouri*) exhibited a continuous distribution, with significant correlations between genetic patterns and geographical locations (Figure 1A, C; Supplementary Figures S7–S9 and Table S6). Given the lack of clear biogeographical barriers and minimal molecular differences between continental Asian leopards and Javan leopards (Wilting et al., 2016), the International Union for the Conservation of Nature (IUCN) Cat Specialist Group, comprising 22 experts from the Cat Classification Task Force, has proposed that *P. p. japonensis* be merged with *P. p. orientalis*, and possibly *P. p. delacouri* with *P. p. orientalis*, based on molecular evidence from only 724 bp concatenated mitochondrial sequences (Kitchener et al., 2017; Wilting et al., 2016). However, complete mitogenomes are significantly more informative and reliable than partial sequences (Zhu et al., 2022). Notably, based on complete mitogenomes, our phylogenetic trees distinctly separated *P. p. orientalis*, *P. p. japonensis*, and *P. p. delacouri* into North, Central, and South groups, respectively (posterior value=1) (Figure 1C; Supplementary Figures S7, S8). This finding was further corroborated by molecular variance analysis (AMOVA) ($F_{ct}=37.72\%$, $P=0.100$) and phylogenetic network analysis (Supplementary Figure S9 and Table S6). Divergence of these three clades was estimated to have occurred approximately 155 ka (95% CI: 102–215 ka) (Figure 1C). Previous microsatellite analysis has also indicated significant genetic divergence between *P. p. orientalis* and *P. p. japonensis* (Wang, 2019). Thus, despite the absence of a clear biogeographical barrier, we conclude that they can be accurately considered as three distinct (sub)populations.

The two Laosicheng samples clustered with individuals from the Central group, consistent with their place of origin. More than 19 leopards have been found at the Laosicheng site (including the two identified in this study) (Hunan Provincial Institute of Cultural Relics and Archaeology, 2018), indicating that approximately 300–600 years ago, there was a relatively significant leopard population in the surrounding area. This phenomenon differs greatly from the current distribution of leopards in central China (Laguardia et al., 2017). Bayesian skyline plot analysis indicated that the effective population size of leopards has consistently declined over the past 30 000 years (Supplementary Figure S10). However, we speculate that intensified human activities over the past few centuries have greatly contributed to the decline of leopard populations. As concluded in previous research, major threats to leopard populations include human-wildlife conflict, prey decrease, habitat loss and degradation, and hunting/poaching (Laguardia et al., 2017).

In summary, our study resolved the controversial identification of mammalian remains from the Laosicheng site, determining that the two skulls in question should be recognized as leopards (*P. p. japonensis*). This finding not

only clarifies the species identification but also provides novel insights into the maternal genetic history of East Asian leopards, markedly diverging from the existing genetic framework. The skull measurements obtained from the two Laosicheng specimens acquired in this study have bridged a gap in the digital morphological model and genetic data of ancient leopards in China, exemplifying the successful integration of morphological and molecular data for the accurate identification of mammalian remains from archaeological sites. Animal remains are essential for regional zoological research, and for the validation of taxonomic and antiquity attributions. Our research illustrates the efficacy of genetic methodologies in resolving identification challenges associated with mammalian remains from archaeological sites and highlights the potency of these approaches in elucidating the evolutionary history of animals.

DATA AVAILABILITY

The data reported in this paper were deposited in GenBase of the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences/China National Center for Bioinformation under accession numbers C_AA055152.1 and C_AA055153.1 (<https://ngdc.cnbc.ac.cn/genbase/>), in the NCBI GenBank database under accession numbers OR871436 and OR871437, and in the Science Data Bank under <https://doi.org/10.57760/sciencedb.14194>.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

M.Z., Q.M.F., and L.H.M. designed the study. L.H.M. conducted the archaeological excavations. M.Z. collected and drilled samples. M.Z., Y.X.Z., Y.M.H., and Q.G.J.Z. performed CT scanning, CT data collection, and morphological character discussion. M.Z., Q.Y.D., R.W.Y., F.L., X.T.F., and P.C. performed the DNA experiments. M.Z., C.H.W., and Y.X.Z. performed data processing and analysis and wrote the manuscript. All authors read and approved the final version of the manuscript.

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