Early Holocene chicken domestication in northern China

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Chickens represent by far the most important poultry species, yet the number, locations, and timings of their domestication have remained controversial for more than a century. Here we report ancient mitochondrial DNA sequences from the earliest archaeological chicken bones from China, dating back to ∼10,000 B.P. The results clearly show that all investigated bones, including the oldest from the Nanzhuangtou site, are derived from the genus *Gallus*, rather than any other related genus, such as *Phasianus*. Our analyses also suggest that northern China represents one region of the earliest chicken domestication, possibly dating as early as 10,000 y B.P. Similar to the evidence from pig domestication, our results suggest that these early domesticated chickens contributed to the gene pool of modern chicken populations. Moreover, our results support the idea that multiple members of the genus *Gallus*, specifically *Gallus gallus* and *Gallus sonneratii* contributed to the gene pool of the modern domestic chicken. Our results provide further support for the growing evidence of an early mixed agricultural complex in northern China.

ancient DNA | chicken | domestication | species origin

In his epochal work on domestication, Darwin suggested that domestic chicken (*Gallus gallus domesticus*) originated from red jungle fowl (*Gallus gallus gallus*) ∼4,000 y B.P. in the Indus Valley (1). However, more recent evidence, based on both mitochondrial (mt) and nuclear DNA (2–4), refutes a monophyletic origin of *G. g. domesticus*. Analyses of large-scale mtDNA datasets (5) strongly suggest that chickens were domesticated multiple times in different parts of Asia, including regions in South Asia, Southwest China, and Southeast Asia. Although some of the earliest chicken bones have been discovered in northern China, dating to over 10,000 B.P. at the Nanzhuangtou site and to over 7,000 B.P. at several other sites (e.g., Cishan and Peiligang), northern China has not yet been suggested as a center of chicken domestication for two main reasons. First, it is unclear if the discovered bones really represent domesticated rather than wild members of the genus *Gallus* (6), and second, northern China is currently a semiarid steppe, and therefore does not provide suitable habitat for jungle fowl, the wild ancestor of domestic chicken. However, abundant remains of tropical animal and plant species excavated at the Cishan and Nanzhuangtou sites show that northern China was much warmer and more humid, with much more extensive forest coverage during the early Holocene (7, 8), providing a potentially suitable habitat for jungle fowl at this time. Moreover, previous studies have revealed northern China as a center for both early pig domestication (9) and the earliest millet domestication (10, 11) already by 10,000 B.P., showing that agriculture existed in this region at the time to which the earliest chicken bones date.

Previous studies (9, 12, 13) have shown that ancient DNA analyses can be informative with regard to determining the places of domestication for a species. The time, region, and pattern of chicken domestication in particular regions over the world have also been worked out using ancient DNA analysis (14–17). However, the oldest chicken sequences analyzed to date are only around 4,000 y old, substantially postdating the beginning of chicken domestication.

Therefore, we chose 39 ancient chicken bones from three archaeological sites in the area of the Yellow River (Cishan, Nanzhuangtou, and Wangyin), representing the earliest sites for chicken bones both in northern China and worldwide, and one younger archaeological site in the middle area of the Yangtze River (Jiuliandun Chu Tombs) for ancient DNA analyses (Fig. 1 and Table 1). Details for all chicken bones and archaeological sites can be found in Table S1.

Results and Discussion

Isolated bones from different genera of the Galliformes are difficult to ascertain to genus level using morphological analyses alone. Therefore, we chose 39 presumed chicken bones from four Chinese archaeological sites for ancient DNA extraction and PCR amplification of a 159-bp fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene (for primers, see Table S2). We were able to amplify this fragment for 13 specimens and compared genetic distances using the 13 obtained sequences and 196 homologous sequences from six

Significance

Ancient DNA analysis is a powerful tool to reveal the geographical origins of domesticated species. Here we obtained ancient mtDNA sequences from the earliest archaeological chicken bones from northern China as early as 10,000 y ago. Combined analyses of our ancient sequences with a large dataset of published modern and ancient chicken mtDNA sequences suggest that northern China was likely one of several regions of chicken domestication and provide further insights into the process of human-mediated spread of chickens across the globe. Our results not only suggest that the oldest archaeological chicken bones recovered so far are indeed from ancestors of domestic chickens, but also provide further evidence for one of the earliest, mixed agricultural complexes in the world.

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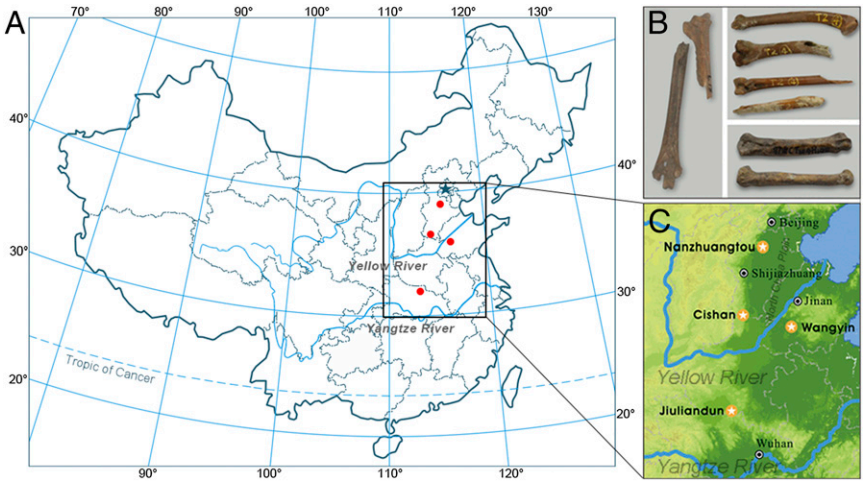

Fig. 1. Examples of ancient chicken bones and localities of all investigated samples. (*A*) Map of China showing the location of the four archaeological sites (red dots). (*B*) Typical ancient chicken bones unearthed in northern China. (*C*) Close-up of the Yellow River area from which the samples originate. The sites are marked with yellow dots with white asterisks.

Galliformes genera, including *Gallus*, *Phasianus*, *Alectoris*, *Lophura*, *Tetraophasis*, and *Syrmaticus* (Table S3). The results clearly show that the ancient sequences are closer to the genus *Gallus* than to any other genus (Fig. S1), identifying the bones as originating from the genus *Gallus* rather than from any other genus within the Galliformes.

A subset of eight samples representing the same four archaeological sites (Cishan, Nanzhuangtou, Wangyin, and Jiuliandun Chu Tombs) also yielded a 326-bp fragment of the mitochondrial control region, assembled in two fragments. We aligned these sequences with 10 published ancient chicken sequences (Table S4) and 1,001 extant published sequences from four *Gallus* species (Table S5). We identified a total of 293 haplotypes, including 266 haplotypes of red jungle fowl and modern domestic chickens, 13 haplotypes in three other *Gallus* species (*Gallus varius*, *Gallus sonneratii*, and *Gallus lafayetii*), 9 unique ancient haplotypes, 1 haplotype shared by modern domestic chickens and *G. sonneratii*, 3 haplotypes shared by modern chickens and ancient specimens, and 1 haplotype shared by modern domestic chickens, *G. sonneratii* and ancient specimens (Table S5).

An unrooted Bayesian phylogenetic tree using these 293 haplotypes (Fig. S2) reveals eight divergent clades (A–H) (Table S5). The median-joining network analysis shows a picture highly consistent with the Bayesian tree reconstruction (Fig. 2). Among the eight major clades, clade H (number of haplotypes: N1 = 13; number of individuals: N2 = 20) is more distantly related to all other clades, and consists of three distinct subclades, which correspond to *G. lafayetii*, *G. sonneratii*, and *G. varius* sequences, respectively. Ancient specimens across the world were distributed to dominant clades A (N1 = 58; N2 = 343), C (N1 = 77; N2 = 194), and F (N1 = 53; N2 = 273), falling within *G. gallus* (domestic chickens and red jungle fowls) from all over the world. Clade B (N1 = 29; N2 = 93) and clade E (N1 = 39; N2 = 72) consist of red jungle fowls and domestic chickens without any specific geographic affiliation. Clade D (N1 = 18; N2 = 18) and clade G (N1 = 6; N2 = 6) both only contain red jungle fowls; however, individuals in clade D were all *Gallus gallus murghi* from India, whereas clade G individuals were other red jungle fowl subspecies from the Malayan Archipelago.

We determined the ages of the archaeological chicken bones by direct accelerator mass spectrometry radiocarbon dating, as well as association with stratigraphic and contextual archaeological evidence (Table 1). Cultural deposits from which the chicken bones originated at the Cishan site and Nanzhangtou site yielded calibrated radiocarbon dates of ∼7,400 B.P. and ∼10,000 B.P., respectively, and both sites lack later deposits. The Wangyin site was clearly accumulated during two contiguous periods, and the ages of the archaeological remains ranged from 4,500–3,500 B.P. The Jiuliandun Chu Tombs represent a site of hermetic cemeteries from the Eastern Zhou Dynasty, and the excavated chicken bones were from the 10th century B.C. (Table 1 and Table S1). Direct radiocarbon dating of samples CS1 and NZT1, from the Cishan site and Nanzhuangtou site, respectively, yielded calibrated dates of ∼7,900 and 10,400 y, confirming the Neolithic context of these samples (Table S6).

The eight ancient sequences from China represent eight different haplotypes, and the 10 published ancient sequences from Chile, Spain, and Hawaii represent another five haplotypes

Table 1. Summary of ancient samples analyzed

Location	Sample size	Age (archaeological context, B.P.)	Source
Nanzhuangtou site, Hebei, China	22	10,465–10,430 direct/10,500–9,700 associated	Present study
Cishan site, Hebei, China	7	7,960–7,845 direct/7,500–7,300 associated	
Wangyin site, Shandong, China	6	4,500–3,500 associated dates	
Jiuliandun Chu tombs, Hubei, China	4	3,000–2,300 associated dates	
Albarracin, Spain	1	1,450–1,000	Storey et al. (15)
Valduno, Spain	1	After 1,000	
La Cartuja, Spain	1	350–280	
Lualaʻi, Waimea, Hawaiʻi	1	After 1,000	
Pelekane site, Hawaiʻi	1	After 1,000	
Puu Lanai Ranch site, Hawaiʻi	2	After 1,000	
El Arenal 1, Chile	2	Cal. 622 ± 35 B.P. and Cal. 506 ± 30 B.P., respectively	Storey et al. (14, 15, 18)
Hanga Hahave, Easter Island	1	Prehistoric and context of classic, Ahu-Moai Period Crematoria	

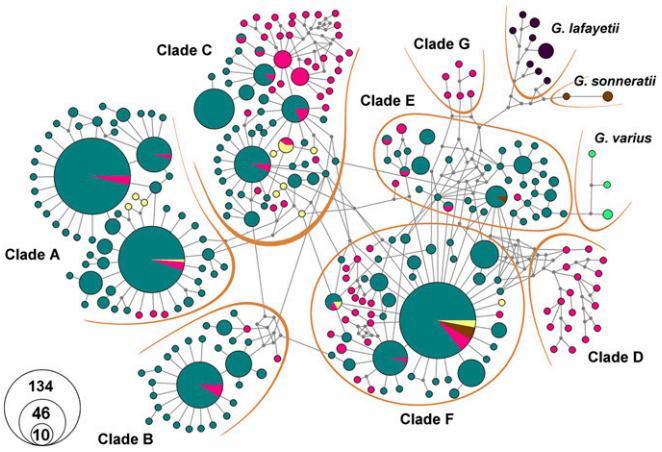

Fig. 2. Haplotype distribution illustrated by median-joining network analysis. Each haplotype is represented by a circle, with the area of the circle proportional to the haplotype's frequency. Different colors indicate samples originating from different species, with deep green indicating domestic chicken breeds and red indicating red jungle fowls, yellow ancient chicken specimens, purple *G. lafayetii*, brown *G. sonneratii*, and light green *G. varius*. Haplotypes inferred by the network analysis—but not observed in the dataset—are indicated by small gray dots.

(Table S4). All ancient sequences could be assigned to one of the modern haplogroups (Fig. 2). The three oldest samples (NZT1, NZT2, and NZT3) from the Nanzhuangtou site (~10,400 B.P.) and one sample (WY2) from the Beixin cultural layer (about 4,500 B.P) at the Wangyin site fall into clade A, with NZT1 belonging to the dominant modern haplotype A46, whereas NZT2, NZT3, and WY2 represent different unique haplotypes. WY1, the other ancient sample from the Dawenkou cultural layer (4,300–3,500 B.P.) at the Wangyin site, represents a unique haplotype within clade C, whereas the sequences obtained from the Jiuliandun Chu Tombs (3,000–2,300 B.P.) were found to represent unique haplotypes (C74 and C75) also within clade C, which also includes two unique haplotypes obtained from samples (PAQH1 and HWIP2) from Hanga Hahave on the Easter Island (prehistoric and context of classic, Ahu-Moai Period Crematoria) (14) and Pelekane sites in Hawaii (after 1,000 B.P.), respectively. The remaining three ancient samples (HWIW2, HWIR1, and HWIR2), respectively from Luala'i of Waimea (after 1,000 B.P.) and Puu Lanai Ranch sites (after 1,000 B.P.), both in Hawaii, share the modern haplotype H88, which also belongs to clade C. The only sequence obtained from the Cishan site (~7,900 B.P.) was found to belong to modern haplotype F40, within clade F. Similarly, two ~600-y samples (CHLA1 and CHLA4) from the El Arenal 1 site in Chile (Cal. 622 ± 35 B.P. and Cal. 506 ± 30 B.P., respectively) (18) and two samples (ESVA1 and ESLC1) from Valduno (after 1,000 B.P.) and La Cartuja (350–280 B.P.) in Spain were shown to carry the dominant haplotype F27 within clade F. Finally, a unique haplotype from a sample (ESAL3) from Albarracin in Spain (1,450–1,000 B.P.) also belongs to clade F.

To further investigate the phylogeographic signal of the dataset, we investigated the modern domestic chicken haplotype composition of different geographical regions (Fig. 3 and Table 2). We defined the geographic areas as northern Asia (samples mainly from northern China, South Korea, and Japan), Southeast Asian Mainland (southern China, Vietnam, Myanmar, Thailand, and Laos), South Asia (Nepal, India, and Sri Lanka), Southeast Asian Islands (Indonesia and Philippines), Eurasia (Turkey and Iran), East Africa (Kenya and Madagascar), West Africa (Ghana), and America (United States). The geographical distribution of haplogroups revealed substantial phylogeographic structure in modern chicken breeds (Fig. 3 and Table 2). We also

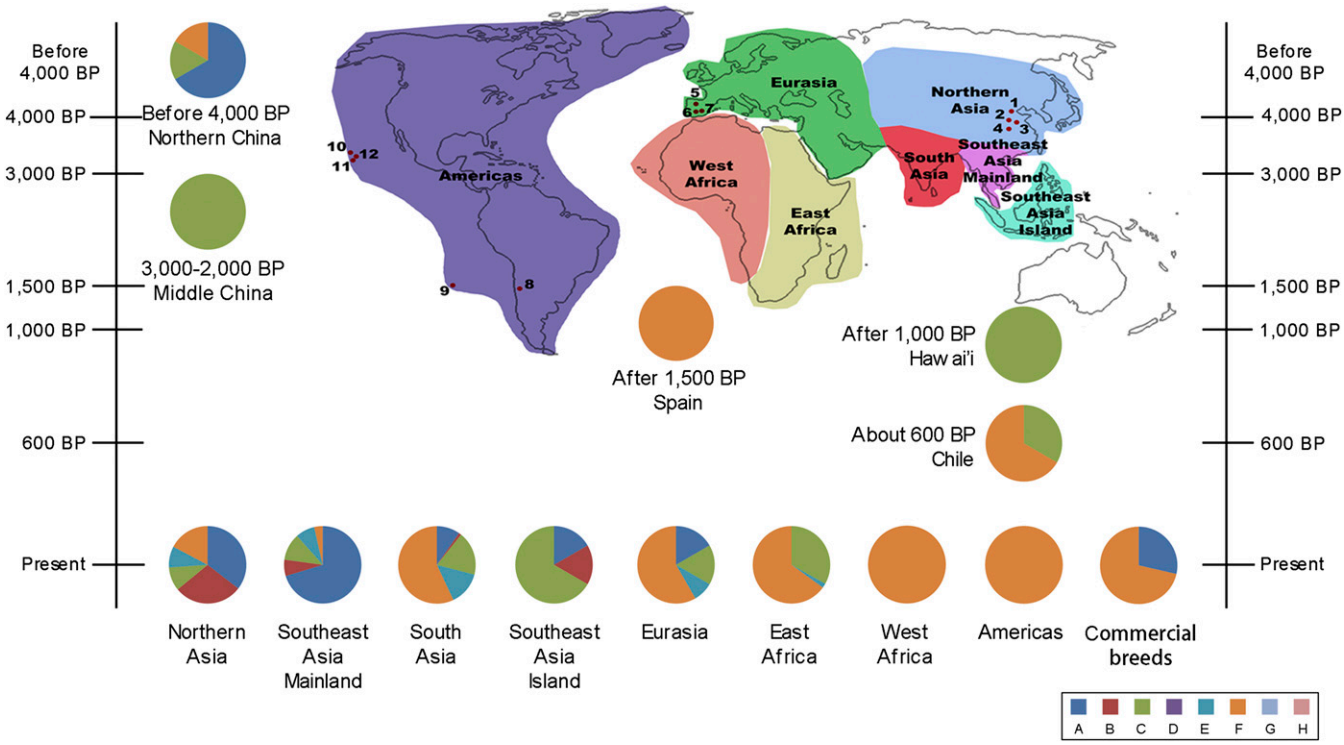

Fig. 3. Haplotype composition of domestic chicken in different geographic areas at different time points. Haplotype composition for each region and each period are indicated by corresponding pie charts. The inset shows the geographical areas defined. Numbers 1–12 show the provenance of ancient specimens, corresponding to the Nanzhuangtou site, Cishan site, Wangyin site, and Jiuliandun Chu Tombs in China (1–4); Valduno, La Cartuja, and Albarracin in Spain (5–7); El Arenal 1 and Hanga Hahave, Easter Island (8–9); and Puu Lanai Ranch Site, Luala'i, and Waimea and Pelekane site in Hawaii (10–12).

Table 2. Haplotype composition of the defined geographic regions based on 1,019 control region sequences

Species	Geographical area	A	B	C	D	E	F	G	H	Total
Domestic chickens	Northern Asia	73	62	16	0	18	35	0	0	204
	South Asia	9	1	17	0	13	53	0	0	93
	Southeast Asia Mainland	234	23	38	0	30	12	0	0	337
	Southeast Asia Island	1	1	4	0	0	0	0	0	6
	Eurasia	2	0	2	0	1	7	0	0	12
	East Africa	0	0	53	0	3	104	0	0	160
	West Africa	0	0	0	0	0	10	0	0	10
	Americas	0	0	0	0	0	2	0	0	2
	Commercial breed	2	0	0	0	0	5	0	0	7
Red jungle fowls	Northern Asia	3	4	0	0	0	9	0	0	16
	South Asia	2	0	39	18	0	18	0	0	77
	Southeast Asia Mainland	6	1	7	0	5	0	0	0	19
	Southeast Asia Island	0	0	5	0	0	0	5	0	10
	Region unkown	7	1	5	0	1	5	1	0	20
G. sonneratii, *G. lafayetii* and *G. varius*		0	0	0	0	1	7	0	20	28
Ancient chickens	Chile	0	0	1	0	0	2	0	0	3
	China	4	0	3	0	0	1	0	0	8
	Spain	0	0	0	0	0	3	0	0	3
	USA: Hawai'i	0	0	4	0	0	0	0	0	4
Total		343	93	194	18	72	273	6	20	1,019

In the Species column, green represents species other than *G. gallus*, and yellow represents ancient chickens.

visualized haplotype compositions of different geographical areas in chronological order. The dominating haplogroups A, C, and F among modern chicken are all already present in the Yellow River area in northern China earlier than 4,500 y ago. The fact that NZT1, dating to ~10,400 y, belongs to the dominant modern haplotype A46 further confirms long-term genetic continuity between the ancient *Gallus* specimens from northern China and modern domestic chicken populations.

Altogether, the ancient sequences show considerable genetic diversity and our results are consistent with the middle and lower stream of the Yellow River in northern China as a site for chicken domestication, although we caution that ancient DNA sequences > 4,500 y from other regions are lacking.

Based on morphological data (19), Cishan has been regarded as the site that yielded the oldest chicken bones, dating to more than 7,000 y before present. In contrast, whether domestic chicken were present at Nanzhuangtou—which dates to an even earlier age—has been disputed (20–22), partially based on the belief that the original human inhabitants did not have the agrotechnical skills to breed chickens. Moreover, because of its semiarid climate and lack of habitat for red jungle fowl, northern China has so far not been taken into account as a possible location for chicken domestication. Thus, although the earliest chicken bones were unearthed in northern China, most researchers insisted that chickens were exclusively domesticated in South and Southeast Asia (2, 15, 23). However, massive environmental changes are well documented for the North China Plain during the past 10,000 y, including changes in temperature, humidity, climate, and hydrogeologic conditions, with corresponding changes in flora and fauna (24–26). Abundant remains of tropical animal and plant species excavated at the Cishan and Nanzhuangtou sites testify that the North China Plain was warmer and more humid, with much larger forest cover during the early Holocene (7, 8). Thus, at that time, northern China represented a suitable habitat for jungle fowl. Moreover, recent evidence suggests that the earliest cultivation of foxtail millets (10, 11), as well as early pig domestication (9), took place in this area, and excavations of the Nanzhuangtou site revealed evidence for early domesticated dogs (21). Therefore, there is no reason to consider the domestication of chicken in this region as unrealistic.

However, in contrast to the bones from Cishan, the status of the Galliformes bones from Nanzhuangtou is controversial, and they have been suggested to represent wild jungle fowl or even pheasant (genus *Phasianus*) bones (20–22). Our analyses clearly show that they belong to the genus *Gallus*. Thus, if they are interpreted as wild jungle fowl, it would only underscore the argument that the environment at this time was different enough to provide a habitat for wild jungle fowl populations, which could have been the basis for domestic chicken populations.

Ultimately, it is—based on genetic analyses alone—of course impossible to prove that the chicken bones analyzed represent domestic rather than wild chicken populations. However, taking into account that: (*i*) they were retrieved from archaeological contexts representing transitional (Nanzhuangtou) and agricultural (Cishan) societies; (*ii*) chicken bones are present across several thousand years in the archaeological record of northern China; (*iii*) these findings predate archaeological chicken remains from any other region by several thousands of years; and (*iv*) all major modern chicken haplogroups and also one of the most common haplotypes are represented in our ancient DNA sequences, we argue that the genetic analyses presented here support the up to ~10,000-y-old *Gallus* bones from Nanzhuangtou and Cishan being the remains of a population ancestral to at least some of modern chicken mtDNA diversity. Whether the earliest samples represent hunted wild jungle fowl or indeed the remains of an early domesticated chicken population cannot be determined from the current data, but is in our view also of limited importance to the understanding of the overall domestication process.

Several animal domestications and crop cultivations have taken place in the middle and lower reaches of the Yellow River, and their descendants were eventually dispersed by humans to many other regions (27). Moreover, the archaeological evidence of chicken in South and Southeast Asia is substantially younger than that in northern China. However, the results of previous archaeological research (28), as well as the process of domestication itself, suggest that the earliest investigated cultures were just undertaking the initial stages of the domestication process and it is unlikely that the chickens at this early stage of domestication were spread to southern Asia. Furthermore, the human cultures of the Yangtze River basin and the Indus-Ganges Valley were contemporaneous with those of the Yellow River reaches, and many important animal and plant domestications now seem to have taken place independently and contemporaneously (29–32). Thus, the presumption that southern Asian chickens were introduced from northern China would be an overinterpretation of the data. Rather, the geographical distribution of chicken

haplotypes suggests that three broad regions, including northern China, South Asia, and Southeast Asia, should be considered as the initial regions for chicken domestication. This conclusion is further supported by the fact that, although appearing early, haplogroup C is a minor component in current northern Asia and Southeast Asia Mainland chickens, but a major one in South Asia and Southeast Asia Island individuals, and also abundantly occurs in South and Southeast Asian red jungle fowls (Table 2), suggesting parallel domestication in multiple areas.

In contrast to the large haplogroup diversity in Asian regions, genetic diversity declines in chicken populations both east- and westward, with increasing distance from those three proposed regions of chicken domestication. Eventually, with sufficient distance, both modern and ancient chicken populations become fixed for a single haplogroup, mostly dominant haplogroup F, except for ancient populations from Chile and Hawaii, which were fixed for haplogroup C (Fig. 3), although one has to caution that the numbers of ancient samples investigated are low for these areas.

Combined phylogenetic analyses on modern and ancient DNA sequences from all over the world (15) have supported the hypothesis of multiple maternal chicken origins in South and Southeast Asia. Our results now add northern China as another center of chicken domestication within Asia.

Materials and Methods

Sample Detail Information. We used 39 ancient chicken bones from four Chinese archaeological sites (detailed information in Table S1) for ancient DNA analysis. Seven specimens were from the Cishan site (36°34′511 ″N, 114°06′720 ″E), a Neolithic site that is located in Wu'an county of Hebei Province, China, in the middle Yellow River region between the Loess Plateau and the North China Plain at an elevation of 260–270 m above sea level. The Cishan site is a prototypical site of the Cishan culture that represents a Neolithic phase culture covered by a number of archaeological sites in the middle Yellow River basin of northern China. Two radiocarbon dates of excavated charcoal from two pits yielded uncalibrated ages of 7,355 ± 100 B.P. and 7,235 ± 105 B.P., respectively (7). Archaeological excavations revealed evidence of domesticated pigs, dogs, and chickens, as well as barley and millet farming. Cishan represents one of the oldest sites in the world to have evidence for domesticated chickens and pigs. There are dozens of chicken bones unearthed in the Cishan site. The mean length of the tarsometatarsus of these remains is slightly larger than that of modern jungle fowls, but smaller than in modern chicken (19). We collected six chicken tibia specimens and one metatarsus specimen from one pit for ancient DNA analyses.

Another 22 specimens originated from the Nanzhuangtou site (39°6′40 ″N, 115°39′25 ″E), which is an early Neolithic Yellow River site near Lake Baiyangdian in Xushui County, at 21 m above sea level, located at the foot of the Taihang Mountains at the western border of the North Chinese plain. Samples from the cultural deposits from which the chicken bones originate yielded uncalibrated radiocarbon dates ranging from 10,500–9,700 y B.P. (20). The Nanzhuangtou site has been excavated three times, in the years 1986, 1987, and 1997. In addition to a number of stone tools and millet seeds, large numbers of faunal bones were uncovered and archaeological woods, leaves, and seeds were found scattered throughout the cultural deposits, suggesting that by this time agriculture had been already relatively well developed in this transitional society. We collected 22 ancient chicken bones from the Nanzhuangtou site, including 1 tibia, 1 tarsometatarsus, 6 humeri, and 14 femurs. The bones were all unearthed from the fourth and sixth layer during the excavations in 1986 and 1987. The charcoal pieces that were radiocarbon dated to 10,500–9,700 y B.P. were also from these layers. The species status of the Galliform remains from Nanzhuangtou has been controversial with one report listing them as *Gallus spec* (20) and another one as *Phasianus spec* (21).

Six specimens were from the Neolithic Wangyin site (35°27′N, 116°46′E), situated 12.5 km south of Yanzhou city proper, southern Shandong province, China, in the lower Yellow River valley. Through six excavations from 1975 to 1978, a total of 143 pits in four districts (Middle, North, South, and West districts) were excavated. The Wangyin site consists of two cultural layers, where the bottom belongs to the late Beixin culture, radiocarbon dated to 4,500 B.P., and the upper stratum belongs to the early Dawenkou culture, going back to approximately 4,300–3,500 B.P. according to ^{14}C-dating (33). Numerous faunal remains, including chicken, pig, cattle, dog, cat, tiger, and even Chinese alligator bones were recovered from this site. Pig remains were discovered in all districts and from many (more than half) of the pits, suggesting the beginning of large-scale pig production. Although chicken bones were not found widely (much less than pig remains), the successive findings of chicken bones between Bexin culture layers and Dawenkou culture layers confirms long-lasting chicken breeding in this geographical region. The findings of Chinese alligator bones point to vast water areas with lush grass and dense forests cover, suggesting that the environment was similar to the current situation in the Yangtze River valley. For our study, one tarsometatarsus from the Bexin Cultural phase and five tarsometatarsi from the Dawenkou Culture phase were collected for ancient DNA analyses.

Finally, four chicken bones were from the Jiuliandun Chu Tombs, an archaeological site of the Chu Kingdom in the Warring States period of the Eastern Zhou Dynasty (3,000–2,300 B.P.), located in Wudian Town in Zaoyang city, Hubei province, in the middle area of the Yangtze River (34). Tomb no. 1 and Tomb no. 2 had a complex internal structure and were the largest ones among all cemeteries. A large number of funerary objects were discovered in theses tombs, including several horse-and-chariot burial pits. In this archaeological site, four chicken left humeri representing four individuals from two pits of Tomb no. 1 were collected.

Ancient DNA Extraction. All pre-PCR work was conducted in a physically isolated laboratory dedicated to ancient DNA analysis at China Agricultural University. Ancient chicken bones were prepared by cautiously cleaning the adhering soils from the outside and interior surfaces using abrasive paper, and then washing them with 5% (vol/vol) sodium hypochlorite solution followed by double-distilled water and drying under UV-irradiation. After that, 200–500 mg of bone powder was generated by drilling into the bones. DNA extraction was performed using QIAamp DNA Investigator (Qiagen) and Amicon Ultra-4 (Millipore). DNA extraction followed the QIAamp DNA Investigator handbook for purification of total DNA from bones and teeth. Amicon Ultra-4 (Millipore) filters were used to concentrate ancient DNA to a volume of ∼50 μL. Several mock extractions were carried out alongside in the same manner to monitor for contamination.

A total of five samples representing various haplotypes were sent to the Ancient DNA Laboratory at the Research Center for Chinese Frontier Archeology at Jilin University for independent replication. DNA was extracted using a modified ancient DNA extraction technique after the protocol proposed by Rohland and Hofreiter (35) in the replication experiment.

Amplification and Sequencing of Ancient DNA. We used loop-mediated PCR (L-PCR) followed by a specific singleplex PCR amplification and Sanger sequencing to obtain the targeted ancient chicken DNA sequences. L-PCR is designed to efficiently enrich the target copy number using loop-mediated isothermal amplification primer sets (36); subsequent singleplex PCR then allows generating a specific amplicon that can then be sequenced.

Nuclear insertions of mtDNAs (NUMTs) were cautiously considered by investigating modern chicken genome sequences and amplification of NUMTs was avoided by careful primer design. The chicken mitochondrial genome sequence (accession no.: NC_001323) was used to perform similarity searches against the latest database of the draft sequence of the chicken genome (Gallus_gallus-4.0) by NCBI/BLAST/nucleotide blast (blast.ncbi.nlm.nih.gov/Blast.cgi). The parameter for the maximum expectation value in searches was e = 10^{-4} to recover hits that were biologically significant (37) and no filters were used during searches.

All L-PCR primers were designed by online loop-mediated isothermal amplification primer designing software (primerexplorer.jp/e/). The *COI* primers were degenerate by design to allow amplification of both *Gallus* (NC_001323) and *Phasianus* (NC_015526) sequences. Meanwhile, primers for control region were designed using the published sequence of *G. gallus* (NC_001323). All primer sequences are available in Table S2.

L-PCR was setup using 25-μL volumes containing 1 U AmpliTaq Gold polymerase (Applied Biosystems), 1× PCR buffer with 3 mM Mg^{2+}, 2 mM dNTPs, 1 μM forward and reverse inner primers, 0.2 μM forward and reverse outer primers, and 3 μL DNA extract. Moreover, 1 U Uracil-*N*-glycosylase (Sigma) was added to eliminate uracil from ancient DNA templates. For specific singleplex PCR, primers were 0.5 μM each and 0.3μL L-PCR product was added as DNA template; all other ingredients were identical to those in L-PCR. Several blank controls were set up at all amplifications. None of our blanks showed amplification products of the expected size.

L-PCR used the following cycling conditions: 37 °C for 10 min, 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 61/65/62 °C (for LCOI, LCR1, and LCR2 reactions, respectively) for 40 s, 72 °C for 30 s, and a final extension of 10 min at 72 °C. Secondary PCR was under the following cycling conditions: 37 °C for 10 min, 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 61/59/59 °C (for *COI*, CR1, and CR2 reactions, respectively) for 30 s, 72 °C for 30 s, and a final extension of 10 min at 72 °C. Amplifications of the extraction blank controls and PCR blank controls were performed in all experiments to monitor contaminations.

PCR products were purified using the QIAquick PCR purification kit (Qiagen). Sequencing was carried out on an ABI 3730XL automated DNA sequencer (Applied Biosystems) using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing kit.

Radiocarbon Dating Analyses. All samples used in the present study were from well-defined archaeological contexts. Nevertheless, the chicken bones from the Cishan and Nanzhuangtou sites that yielded DNA sequences were sent for radiocarbon dating using direct accelerator mass spectrometry at Beta Analytic to provide further support of their age.

Phylogenetic Analysis. The selected 159-bp sequence of *COI* was aligned as the anchor and the organism was defined to *Galliformes* (taxid: 8976) to obtain homologous sequences from GenBank (Table S3). The genetic distance within and between populations was computed using Arlequin 3.5.1.2 (38). We compared genetic distances using the 13 obtained sequences and 196 homologous sequences from 6 Galliformes genera (Table S3), including *Gallus* (four species: *G. gallus*, *G. varius*, *G. sonneratii*, and *G. lafayetii*; total number of sequences, $n = 147$), *Phasianus* (two species: *Phasianus colchicus* and *Phasianus versicolor*; $n = 18$), *Alectoris* (three species: *Alectoris chukar*, *Alectoris rufa*, and *Alectoris melanocephala*; $n = 15$), *Lophura* (three species: *Lophura ignita*, *Lophura nycthemera*, and *Lophura diardi*; $n = 5$), *Tetraophasis* (two species: *Tetraophasis szechenyii* and *Tetraophasis obscurus*; $n = 6$), and *Syrmaticus* (two species: *Syrmaticus ellioti* and *Syrmaticus humiae*; $n = 5$). The results clearly showed that for this DNA fragment, the ancient sequences are closer to the genus *Gallus* than to any other genus (Fig. S1).

The assembled 326-bp sequences of the mitochondrial control region were analyzed along with all available *Gallus* sequences from GenBank, including ancient chicken sequences (Table S4), modern domestic chicken sequences and sequences of four wild *Gallus* species (*G. gallus*, *G. varius*, *G. sonneratii*, and *G. lafayetii*) (Table S5). These sequences were aligned using MUSCLE (39) in MEGA 5.05 (40); FaBOX (users-birc.au.dk/biopv/php/fabox/) was then used to identify haplotypes (41), exported as aligned FASTA files, and converted into Nexus format by using Forcon 1.0 (42) for subsequent network analysis.

BEAST v1.7.4 was used for phylogenetic analysis of the relationship between ancient samples and modern *Gallus* species (43). Conversion of the former aligned NEXUS file into a BEAST XML input file was done using the program BEAUti (Bayesian Evolutionary Analysis Utility). For this analysis, the GTR substitution model with γ-distributed rates was identified by jModelTest 2.1.1 (44) as the most appropriate DNA subsitution model. A Yule model as a simple model of speciation that is generally more appropriate when considering sequences from different species was chosen as the tree prior. The length of MCMC was set to 10,000,000. The program TreeAnnotator v1.7.4 was used to summarize the results with discarding the first 10% as burn-in and to find the best supported phylogenetic tree. Finally, the tree was depicted using FigTree v1.4.0. To further elucidate the differences among the varying haplotypes, Median-joining networks (45) were reconstructed using Network 4.6.1.0 (www.fluxus-engineering.com/index.htm).

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