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.

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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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 (Juliandun 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
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 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 murphi from India, whereas clade G individuals were other red jungle fowl subspecies from the Malay 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 Nanzhuangtou 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 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 Pelekanes 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.), 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
they have been suggested to represent wild jungle fowl or even the Galliformes bones from Nanzhuangtou is controversial, and domestication of chicken in this region as unrealistic. Therefore, there is no reason to consider the observations of the Nanzhuangtou site revealed evidence for early dog domestication (21). Thus, although the earliest pig domestication (9), took place in this area, and excavations of the Nanzhuangtou site revealed evidence for early dog domestication (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 domestifications 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 G is a minor component in current northern Asia and the domestication haplogroups C and E are predominant in all of the other regions.

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 between ancient chicken populations both east- and westward, the number of haplotypes diminishes for a single haplogroup, mostly dominant haplogroup F, except for ancient populations from the Philippines and Sumatra, which are fixed for haplogroup C (Fig. 3), although one has to caution that the numbers of ancient samples investigated are low for these areas.

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 (39°34′51″ 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 chickens. 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°56′47″ N, 115°51′38″ E), a Neolithic site in Wudian Town of Zaoyang county, located in the northern end of the middle Yellow River valley. 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. A large number of funerary objects were discovered in these 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 carefully 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 QAQ and contaminiation.

A total of five samples representing various haplotypes were sent to the Ancient DNA Laboratory at the Research Center for Chinese Frontier Archaeology 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 designing. 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 sequences were used for further analyses.

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 COI and COI-2 genes. We used 25-μL volumes containing 1 U AmpliTaQ Gold polymerase (Applied Biosystems), 1x PCR buffer with 3 mM Mg2+, 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 0.3 μL L-PCR product was added as DNA template; all other ingredients were identical to those in L-PCR. All primer sequences are available in Table S2.

L-PCR was setup using 25-μL volumes containing 1 U AmpliTaQ Gold polymerase (Applied Biosystems), 1x PCR buffer with 3 mM Mg2+, 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 0.3 μL L-PCR product was added as DNA template; all other ingredients were identical to those in L-PCR. L-PCR products were sequenced using standard priming reactions, and the successively obtained sequences were aligned using sequest. Although chicken bones were not found widely (much less than pig remains), the successive findings of chicken bones between Beixin culture layers and Dawenkou culture layers confirm long-lasting chicken breeding in this geographic region. The findings in the Dawenkou culture layers confirm long-lasting chicken breeding in this geographic region.
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. lafayetti) (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 ModelTest 2.1.1 (44) as the most appropriate DNA substitution model. A Yule model as a simple model of speciation that is generally more appropriate when con- sidering sequences from different species was chosen as the tree prior. The length of MCCM 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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Fig. S1. Average number of pairwise differences between aDNA and six Galliformes genera. Blue color below diagonal indicates the net number of nucleotide differences (Nei’s distance) between genera, orange on diagonal indicates the pairwise differences within genera and green above diagonal indicates the pairwise differences between genera. Different depths of a color indicate varying degrees of divergence, with deeper colors indicating greater divergence.

Fig. S2. Unrooted Bayesian consensus phylogenetic tree of 293 unique haplotypes. Combined phylogenetic analysis of four Gallus species and ancient specimens resulted in eight divergent clades (A–H). Ancient specimens were distributed to dominant clades A, C, and F, falling within Gallus gallus, whereas Gallus lafayetii, Gallus sonneratii, and Gallus varius formed three relatively autocephalous subclades of clade H and showed remote relationship with all other clades. The insert figure further illuminates the phylogenetic relationship among these eight clades.
Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)
Table S5 (DOCX)
Table S6 (DOCX)