BIOLOGICAL SCIENCES

Establishing the validity of domestication genes using DNA from ancient chickens

Linus Girdland Flink[a,b,c*], Richard Allen[a], Ross Barnett[a], Helena Malmström[d], Joris Peters[e], Jonas Eriksson[f], Leif Andersson[f,g], Keith Dobney[h], Greger Larson[a]

[a] Durham Evolution and Ancient DNA, Department of Archaeology, Durham University, South Road, Durham, DH1 3LE, UK
[b] School of Biological and Biomedical Sciences, Durham University, South Road, Durham, DH1 3LE, UK
[c*] Earth Sciences, Natural History Museum, Cromwell Road, London, SW7 5BD
[d] Dept. of Evolutionary biology, EBC, Uppsala University, Norbyägen 18D, 75236 Uppsala, Sweden
[e] Dept. of Veterinary Sciences, Institute of Palaeoanatomy, Domestication Research and the History of Veterinary Medicine, Ludwig Maximilian University Munich, and State Collection of Anthropology and Palaeoanatomy, Kaulbachstr. 37, 80539, Munich, Germany
[f] Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, SE-75123 Uppsala, Sweden
[g] Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Box 7022, SE-75007 Uppsala, Sweden
[h] Dept. of Archaeology, University of Aberdeen, St. Mary's, Elphinstone Road, Aberdeen, AB24 3UF, Scotland

Corresponding author: Greger Larson, greger.larson@durham.ac.uk Tel: 0044 (0)191 334 1574

Significance statement
Recent studies have begun to identify the genetic basis for numerous traits that differentiate modern domestic species from their wild counterparts. In both plants and animals, genes that underlie traits that are ubiquitous in modern breeds (referred to as domestication genes) are often presumed to have been selected early during the domestication process. Here, by typing genetic variability in ancient European chickens over the past 2,000 years, we show that a mutation though to be crucial during chicken domestication had not yet been subjected to strong human-mediated selection. This result suggests that the temporal origins of mutations found only in domestic populations cannot be deduced using modern populations alone.

Key words: selective sweep, breed formation, animal domestication, Gallus gallus,

Abstract
Modern domestic plants and animals are subject to strong human driven selection for desired phenotypic traits and behavior. Large-scale genetic studies of modern domestic populations and their wild relatives have revealed not only the genetic mechanisms underlying specific phenotypic traits, but also allowed for the identification of candidate domestication genes. The relative importance of these genes at the outset of domestication depends on the assumption that robust inferences about the past can be made on the basis of modern data alone. A growing body of evidence from ancient DNA (aDNA) studies, however, has revealed that ancient and even historic populations bear little resemblance to their modern counterparts. Here, we test the temporal context of selection on specific loci known to differentiate domestic chickens from their wild ancestors. We extracted DNA from 81 ancient chickens representing 12 European archaeological sites dated from approximately 280 BC to the 18th century AD. We targeted three unlinked genetic loci: the mitochondrial control region, a gene associated with yellow skin color (*BCDO2*), and a putative domestication gene possibly linked to photoperiod and reproduction (*TSHR*). Our results reveal significant variability in both nuclear genes suggesting that the commonality of yellow skin in western

breeds and the near fixation of *TSHR* in all modern chickens took place only in the past 500 years. In addition, mitochondrial variation has increased as a result of recent admixture with exotic breeds. We conclude by emphasizing the perils of inferring the past from data generated from modern animals alone.

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Introduction

The resolution afforded by multiple genetic loci and more recently, complete genomes has led to an increased understanding of the pattern and process of plant and animal domestication (1, 2). More specifically, genetic analyses have uncovered selective sweeps, Quantitative Trait Loci, and even causative mutations underlying a wide range of behavioral and morphological traits, some of which define specific breeds, and others that differentiate domestic plants and animals from their wild ancestors (1, 3, 4).

Because many of these traits are present in either single or relatively few closely related modern breeds, the earliest occurrence of specific phenotypes (and the underlying causative mutations) are presumed to have occurred well after the initial domestication process. These are referred to (at least in the plant genetic literature) as improvement genes (2). In animals, these include hairlessness in Mexican and Peruvian dogs (5), dorsal hair ridges in Vietnamese, Thai and Rhodesian Ridgebacks (6), excessive skin folds in western Shar-Peis (7), double muscling in two cattle breeds (8), and a curly coat mutation found in Selkirk Rex cats (9), none of which are thought to have been present during early domestication.

Some causative mutations, however, underlie traits found in numerous, distantly related breeds. Alleles that are fixed in domestic variants and are often presumed to have been under selection at the outset of domestication are referred to by both the plant (2) and animal (3) domestication literature as domestication loci. In some cases, including Grey coloring (10) and altered gaits in horse breeds (11), brachycephaly in dogs (12), and muscle growth in pigs (13), no hypotheses were proposed for the timeframe of first appearance of these traits. In others, however, the commonality of both small size (14, 15) and chondrodysplasia (16) across modern dog breeds, and the widespread occurrence of pea-combs in chickens (17), however, led the authors of these studies to suggest that the genetic mutations underlying these characteristics were selected for during the early stages of the domestication process. More recently, a whole-genome re-sequencing study compared variation in 14 unrelated dog breeds and wolves, and identified 36 regions potentially targeted during early domestication including 10 genes that allowed dogs to better digest starches compared with wolves (18). Because increased amylase activity was ubiquitous in dogs but absent in wolves, the authors concluded that this change must have occurred when early dogs began adapting to a starch rich diet provided by humans.

Recent studies have also identified mutations in domestic chickens that are rare or completely absent from their primary ancestor, the Red Junglefowl (*Gallus gallus*) (19). Based on archaeological bones identified in Neolithic sites in the Yellow River basin, chickens were thought to have been domesticated as early as 6000 BC (20). This conclusion has recently been questioned, however, since bones presumed to originate from chickens in the original faunal analysis (21, 22) have been shown to be pheasants (23, 24). As a result, a re-evaluation of all the early finds is necessary to establish the chronology and geography of chicken domestication.

Genes that differentiate modern domestic chickens from Red Junglefowl include those that underlie the *yellow skin* phenotype present in the vast majority of western, commercial chicken breeds as well as numerous geographically restricted and fancy breeds. Yellow skin is caused by a recessive allele of the *BCDO2* gene (25). *BCDO2* encodes the beta-carotene dioxygenase 2 enzyme that cleaves colorful carotenoids into colorless apocarotenoids (26).

While the expression of the dominant allele in skin tissue results in white skin color, the recessive allele possesses one or more cis-acting and tissue-specific regulatory mutations that inhibit expression of *BCDO2* in skin tissue. Provided that sufficient carotenoids are available in the diet, the recessive allele reduces carotenoid cleavage and allows them to be deposited in skin tissue leading to yellow skin (25). This recessive *BCDO2* allele was likely acquired millennia after chicken domestication through its later hybridization with the Grey Junglefowl (GJF, *Gallus sonneratii*) present in South Asia (25). Red and Grey Junglefowl are known to hybridize in contact zones in the Indian sub-continent (27, 28) and it is possible that domestic poultry engaged in the same behavior after they were introduced from Southeast Asia. Given the ubiquity and genomic signatures of strong human-driven selection of the *yellow skin* trait in modern, western commercial chickens (29), Eriksson et al. (25) suggested that this trait was favored by humans after chickens acquired the trait in South Asia, but before the first wave of domesticated chickens arrived in Europe between 900-700 BC (30, 31).

In addition, a recent analysis of pooled wild and domestic chicken samples revealed strong selection signatures across a number of loci as well as a missense mutation in the thyroid stimulating hormone receptor (*TSHR*), a locus possibly linked to shifts in seasonal mating (29). Given its ubiquity in domestic breeds (264 out of 271 birds representing 36 global populations were homozygous for the sweep allele and the remaining seven were heterozygous), and the general absence of the derived allele in Red Junglefowl, the authors of that study concluded that the *TSHR* locus may have played a crucial role during chicken domestication (29).

The assumption that patterns of modern DNA variability can be used as a proxy for the recent and even distant past can be valid. For example, a study of allelic variation in the coat color gene *MC1R* across wild boar and domestic pigs concluded that, given the fixation of multiple, consecutive non-synonymous mutations found in domestic pigs, selection for novel, variable coat colors likely began during the early phases of domestication (32). The general suggestion that coat color change took place soon after animal domestication has been supported by an ancient DNA study of archaeological horse remains that demonstrated firstly that pre-domestic horses possessed one of only two colors. The same study also revealed a dramatic increase in variability amongst eight coat color loci in horses dated to the late Early Bronze Age (~2000-1800 BC) (33), more than one thousand years after horses are thought to have been domesticated (33, 34).

Most ancient DNA studies, however, have revealed the hazards of assuming that modern genetic variation can be used to draw conclusions about past population variability. For instance, a mutation in the *NAM-BI* gene, associated with increased grain size in wheat, was thought on the basis of fixation in modern cultivars to have been selected for during the early phases of the domestication process (35). A genetic survey of 19th century historical seeds, however, revealed that the fixation of the modern, sweep allele occurred only recently during crop improvement (36). A similar study of ancient maize concluded that, although two genes (*tb1* and *pbf*) had been fixed for the domestic variant by ~4000 years ago, one gene (*su1*) still possessed significant variability as long ago as ~2000 years ago (37). Likewise, a number of studies of ancient mitochondrial DNA have shown that lineage replacement (often rapid and geographically widespread) was common amongst both domestic (38, 39) and wild (40, 41) populations. These studies demonstrated that conclusions drawn from modern datasets regarding past population dynamics (including the early stages of domestication) require testing through direct observation.

Here, we investigate whether the *TSHR* gene was selected for during the early stages of chicken domestication (29), and if early poultry keepers favored the BCDO2 gene that underlies yellow skin in chickens soon after it was acquired from the Grey Junglefowl (25, 29). In order to do so, we attempted to genotype SNPs linked with the sweep alleles in both *TSHR* and *BCDO2* in 81 ancient European chickens dating to approximately 280 BC to the

18th century AD (Table S1, Supplemental Text). If *TSHR* played a critical role during the domestication process, all the samples analyzed here should have been fixed for the derived *TSHR* allele, as observed in worldwide modern populations (29). Likewise, if *BCDO2* was acquired, favored and maintained soon after its introgression from Grey Junglefowl, the *yellow skin* phenotype should have been expressed in a significant proportion of the ancient European populations. Lastly, we assess the hypothesis that the presence of mitochondrial DNA (mtDNA) control region (CR) haplogroups A-D reflects evidence for the recent introduction of East Asian chickens into the European gene pool (42).

Results

For each ancient individual, we attempted to amplify a 58bp fragment surrounding the candidate missense (Gly>Arg) SNP in the *TSHR* gene (29), a 51bp fragment surrounding a SNP in the *BCDO2* gene associated with the *yellow skin* allele (SNP B in Table 1 of (25)), and a 201bp fragment of the mtDNA CR (43). Overall, 56/81 (69%) ancient chicken remains provided reproducible results for at least one out of three loci (Figure 1, Table S1, Figure S1). We observed allelic drop out in a number of heterozygous specimens, for both *TSHR* and *BCDO2*. However, the probability of falsely assigning a heterozygous individual as a homozygote after five replications was estimated to be <0.01 and external replication on 12 samples yielded identical mtDNA CR and *TSHR* sequences (Supplementary Text).

Amongst the 45 specimens from whom the *TSHR* locus was successfully genotyped, nine individuals were homozygous for the derived (domestic) sweep allele, 14 were homozygous for the wild type allele, and 22 specimens were heterozygous (Figure 1, Table S1). The results of a Fisher exact revealed that the sweep allele was significantly less frequent in the ancient sample than in modern chickens ($p<0.0001$). A binomial probability test demonstrated that observing these frequencies among the ancient samples assuming the frequencies of the modern samples (Figure S1; Table S2) (29) is very unlikely ($p<0.001$). In addition, a χ^2 test on *TSHR* genotype frequencies derived from the 2nd-3rd century AD Künzing Roman population (Figure 1, Table S1; S3) revealed that the observed frequencies are consistent with Hardy-Weinberg equilibrium ($p>0.95$, $\chi^2=0.004$, df=1) (though one group had fewer than the recommended minimum number of expected genotypes/individuals).

Of the 26 specimens successfully genotyped for the *BCDO2* locus, 20 were homozygous for the white skin allele (found in Red Junglefowl) one individual was homozygous for the yellow skin allele (derived from the Grey Junglefowl), and five individuals were heterozygous (Figure 1, Table S1). Because only one functional copy of the *BCDO2* gene is necessary to effectively cleave carotenoids, the *yellow skin* phenotype can only be expressed in chickens that are homozygous for the yellow skin allele and consume sufficient carotenoids in their diet. Of the 26 successfully genotyped individuals, only one chicken from the site of Altenburg in Germany (dated to Iron Age La Tène D, ca. 150-15 BC; Figure 1, Table S1) was capable of expressing the *yellow skin* phenotype. Because genotype/phenotype frequencies reported previously were selected on the basis of their phenotypes (25), we did not carry out statistical comparisons of allele frequencies between ancient and modern populations.

The targeted mtDNA CR fragment was successfully sequenced in 39 individuals (Table S1). The topology of a Maximum likelihood tree constructed from an alignment of 201bp haplotypes matched the Neighbor-Joining tree generated by Liu et al. (19), suggesting that 201bp is sufficient for recovering the major clades present in the chicken mitochondrial tree (Figure 2, Figure S2) (43, 44). We identified a total of three haplotypes among the ancient specimens, all of which clustered within the E clade on the chicken mitochondrial tree (19, 45) (Figure 2, Table S4). The E3 (n=1) and E6 (N=2) haplotypes (19) were present only in Medieval and post-Medieval chickens from England (Table S1, S4), whereas the remaining 36 individuals possessed a 201bp haplotype corresponding to haplotypes E1, E5, E12, E15 or E16 described using a 519bp fragment (Table S4) (19). Considering only the presence or absence of specific haplogroups, there is a significant difference in haplogroup frequencies

between the ancient and modern datasets (Fisher exact test, $p<0.005$, Table S5). A binomial probability distribution revealed that, assuming the frequency reported for modern European chickens (Table S5; 15% of modern European chickens possess haplotypes from clades A-D), the probability of observing only the E haplogroup in 44 ancient specimens (the novel 39 sequences combined with previously published data, Table S5) is <0.002.

DISCUSSION

The *TSHR* domestication locus

The locus encoding the thyroid stimulating hormone receptor (*TSHR*) on chromosome 5 in domestic chickens has recently been shown to have undergone a massive selective sweep (29). A non-conservative amino-acid substitution (a missense mutation Gly558Arg) was identified as a potential candidate causal, and target mutation for the selective sweep. Though the function (and corresponding phenotype) associated with the derived allele remains unknown, it is possible that this gene variant affects photoperiod control and the absence of strict seasonal reproduction, a trait commonly found in domestic animals but rare or absent among their wild relatives (25, 29). Because 264 out of 271 modern birds representing 36 globally distributed populations were homozygous for the derived sweep allele (the seven remaining were heterozygous), and because the Red Junglefowl individuals that also possessed the mutation were thought have acquired it from domestic chickens, Rubin et al. (29) suggested that *TSHR* was a domestication locus. If this selective sweep occurred during the early phase of domestication, all ancient chickens that post-date their domestication and located outside the natural distribution range of Red Junglefowl (eliminating the potential for backcrossing with wild birds) should also possess the derived allele.

The results presented here, however, demonstrate that though the derived allele was present in European chickens dated to approximately 280 BC to the 18th century AD, it was only found on 44% of the typed chromosomes and the wild type allele persisted at intermediate frequencies until at least the 16th-18th century AD (Figure 1, Table S1; S2). These results suggest that the *TSHR* mutation was neither a prerequisite, nor critical in the immediate aftermath of chicken domestication. While the strength of the selection pressure that drove the sweep haplotype in modern populations is not in doubt (29), the data presented here suggest that the fixation of the derived allele in European chickens was much closer to the present and certainly within the last 500 years, possibly commensurate with the improvement of farmyard animals that began during the industrial revolution (27).

In one scenario, the sweep allele was fixed in Western Europe and spread across the world with newly synthesized commercial breeds that emerged during the 2nd half of the 19th century (27). This hypothesis seems overly simplistic, however, given that the *TSHR* allele was found to be fixed (or nearly so) in modern Egyptian Fayoumi chickens and Silkie, Cochin and Hua-Tung chickens from China (29) that are unlikely to have been strongly influenced by European commercial breeds. It is therefore possible that the *TSHR* sweep allele became fixed in populations originating outside of Europe well before the creation of modern breeds, and that these birds in turn replaced the initial populations introduced into Europe. This narrative is supported by the observation that the Classical Greek chickens were the only population analyzed in this study that were homozygous for the sweep allele (Figure 1, Table S1). Though the earliest Central European chickens that arrived north of the Alps ~600-400 BC likely descended from founder populations that were present in 8th-5th century BC Greek settlements on the Mediterranean coast, the 1st century BC chicken population at Kassope included in this study could have arrived as part of a secondary introduction of chickens. During the Achaemenid (550-330 BC) and Hellenistic (323-31 BC) periods, the Aegean region, a commercial hub that integrated most of the Near and Middle East including the Indus Valley (46), could have been responsible for the introduction of chicken populations that had undergone a selective sweep at the *TSHR* locus. Crucially, however, none of the Greek individuals possessed the introgressed Grey Jungle fowl *BCDO2* allele (Figure 1) suggesting these two loci have been selected for and fixed at different times and places.

The *BCDO2* yellow skin locus

The study that discovered the *TSHR* sweep (29) also confirmed a selective sweep encompassing the *BCDO2* locus. Unlike *TSHR*, however, *BCDO2* is only fixed in a limited number of (often commercial and geographically widespread) breeds (25). The *yellow skin* phenotype was previously shown to result from the presence of a *BCDO2* allele (allowing carotenoids to accumulate in the skin) that domestic chickens acquired not from their primary ancestor, the Red Junglefowl, but from introgression between domestic chickens and Grey Junglefowl populations indigenous to South Asia (25).

The data presented here reveal that though the *yellow skin* allele was present on 13% of chromosomes in ancient European chickens, only a single ancient bird was homozygous for the GJF allele and was therefore the only one capable of expressing yellow skin (Table S1, S2). This is consistent with 17th-19th century records suggesting that a number of widespread, prolific and economically important breeds raised in western and southern Europe, including the Dorking, Houdan, Sultan, Spanish or B/W Bantams clearly had white legs, while the Hamburgh, Polish, Turkish, and Crève Cœur breeds had leg colors ranging between slate blue to dark leaden-blue (27, 47). Some 17th century European breeds including the Padua did possess yellow legs (47) and the trait was explicitly mentioned in relation to heavy, fast-growing types including Cochin, Brahma and Malay breeds imported by sea (and therefore named Captain's birds) from the Far East into Europe and the United States during the 1820s-1850s (27). It is therefore possible that the increase in the yellow skin phenotype only took place after recent introductions of foreign birds to Europe and the breed formation process that consequently led to the creation of modern, widespread commercial broiler and egg-laying breeds. Tegetmeier (27), for instance, noted the remarkable rapid growth and great size attained by crossbred birds produced by mating Cochins with the large traditional French Crève Cœur, La Flèche and Houdan breeds. The ubiquity of the *yellow skin* phenotype in commercial, modern, and some rare, geographically restricted breeds can therefore be explained by rapid, worldwide spread of newly synthesized commercial chickens. This suggestion is further supported by the fact that the *yellow skin* phenotype appears infrequently or is completely absent in rare breeds such as the Friesian Fowl, Houdan, and Westfälischer Totleger (25).

The mitochondrial control region

The mtDNA control region is a widely used locus in chicken genetic studies and has frequently been used to investigate domestication, admixture and migration (19, 44, 45, 48). Though previous studies have concluded that modern European chickens (and Western commercial breeds) primarily possess mitochondrial haplotypes belonging to the E clade (19, 45), additional studies (42, 49) have demonstrated that haplogroups A-D are also present in a wide variety of breeds, most likely as a result of the intentional importation and hybridization of East Asian breeds into Europe during breed formation and breed improvement over the past 500 years (42).

Though a recent study of complete mtDNA chicken genomes revealed the existence of 14 mitochondrial clades found worldwide (45), all 39 ancient chickens sequenced in this study, and five archaeological Spanish chickens typed in a previous study (44), possessed exclusively E-clade haplotypes (Table S1; S5). These results firstly demonstrate that chickens initially introduced to Europe possessed a small fraction of the variability present in Southeast Asia where chickens were originally domesticated. Secondly, all haplotypes belonging to other haplogroups therefore represent recent introductions. Interestingly, because all but three ancient European chickens possessed a single haplotype (probably corresponding to the common E1 haplotype) (19) (Table S4), the presence of haplotypes E3 and E6 in Medieval and post-Medieval contexts from the UK may imply secondary introductions.

Lastly, while the nuclear loci typed in this study show a dramatic reduction in variability between ancient and modern populations, the mitochondrial signatures reveal an initial uniformity followed by an increase in haplogroup diversity in modern birds. These seemingly divergent genetic patterns reflect different human goals over the last two centuries including intensive selection for traits associated with behavior, production (*TSHR*) and skin color (*BCDO2*), but also for phenotypic variability in fancy breeds achieved through the importation of East Asian varieties (42).

Conclusions

A variety of genetic techniques and analytical approaches have led to the identification of selective sweeps and causative mutations that differentiate populations of domestic plants and animals from their modern wild counterparts. The ubiquity of sweep alleles across numerous breeds has often been used as an argument to infer their ancient origins, and as an argument that the SNPs and associated traits were selected for during the early phases of domestication (4, 14, 17, 18, 35). The patterns of allelic diversity presented here, however, challenge the hypothesis that modern variation can be directly mapped onto the past. The allelic variability within *TSHR* in ancient chickens instead demonstrates that this locus was not crucial for the early development of domestic chickens, and that the fixation of the derived allele, at least in European chickens, took place only in the past few hundred years. Similarly, though the *yellow skin* allele was present in ancient European chickens, the phenotype was rare, and the fixation of the *BCDO2* allele in numerous modern chicken breeds was likely also a recent occurrence.

The historical and ancient patterns of genetic variation for each of these three loci reveal the dangers of extrapolating modern patterns of fixation directly into the deep past. In the two nuclear genes, the reduction in variability was more likely coincident with the more recent breed formation process that was instigated during the industrial revolution (27) and the subsequent development of modern commercial chicken breeds. This pattern demonstrates how easy it is to underestimate the potential of alleles to become ubiquitous through initial strong selection leading to fixation, followed by geographic proliferation through human-assisted migration. The process of recent breed formation, coupled with strong directed selection and admixture with exotic breeds, have radically shaped the gene pools of modern domestic plants and animals, often distorting their deeper genetic history (50). As a result, hypotheses regarding past population dynamics drawn solely from modern datasets require verification through direct observation. This is especially true where zooarcheological or historical records suggest recent origins for some traits. For example, though conclusions drawn from the genetic variability in modern samples suggest that small size (14, 15) and chondrodysplasia (51) in dogs, and pea-combs in chickens (17) occurred early during domestication, small dogs (< 30 cm shoulder height) did not appear in the Eurasian archaeological record until after the first millennium BC (52), the first evidence for chondrodysplasia is found in Egyptian tomb art dating to the late 3rd millennium BC (53), and the peacomb trait in chickens was still rare in mid-19th century English fowl (27).

These lines of evidence alone do not undermine claims for much earlier appearances of these, and other domestication-related traits, but they do suggest that modern genetic data should be considered within a broader context that includes an appreciation for the potential of rapid and widespread demographic shifts. Future studies that investigate ancient genetic variability in loci known to underlie behavioral, dietary, and phenotypic differences between wild and domestic plants and animals will help to reveal the first appearance and timings of selection pressures. These data will lead to the creation of a significantly more robust geographic and temporal interpretative framework to more fully understand the early patterns and processes of domestication.

MATERIALS AND METHODS

Ancient chicken specimens were selected to represent three geographical locations and two

major time bins (1: Late Iron Age, Late Hellenistic and Roman contexts, dating to approximately 280 BC-5th century AD, and 2: Medieval and post-Medieval contexts, dating to approximately the 10th-18th century AD, Table S1). The majority of chicken bones come from closed contexts that have been dated either through stratigraphic (cultural) association or direct radiocarbon dating (Supplementary Text).

DNA extraction was performed in dedicated ancient DNA laboratories at Durham University and Uppsala University following stringent laboratory procedures according to commonly applied guidelines (54, 55). The work was carried out by lab personal wearing protective lab coats and over-shoes, or coveralls and dedicated lab clogs, double pairs of gloves (with the outer pairs of gloves changed in between every step of the preparation/extraction procedure). All equipment and work surfaces are routinely cleaned before and after each use with a dilute solution of bleach (5-10% active sodium hypochlorite) followed by rinsing with ddH_2O and ethanol (70%- 99%). A strict one-way system for entering the labs is in use in order to avoid carry-over of post-PCR contaminants.

DNA extractions were performed as previously (39) (see Supplementary Materials for a detailed account of experimental procedures). To authenticate the results, twelve chickens were independently replicated for the CR and *TSHR* SNP at Uppsala University (Table S1). CR sequences were amplified using previously published PCR primers GG144F-GG387R (43), while PCR and sequencing primers for *TSHR* and *BCDO2* were designed in PSQ Assay Design (Qiagen) (Table S6). CR sequences were Sanger Sequenced at the DNA sequencing facility at Durham University. *TSHR* and BCDO2 PCR amplicons were genotyped on the Q24 (Qiagen) pyrosequencer in the Archaeology department at Durham University, UK, or on a PSQ™ 96MA (Biotage) pyrosequencer at Uppsala University. Full PCR cycling conditions and sequencing methods are presented in detail in the Supplementary Text.

MtDNA CR sequence data was analyzed and assembled in Geneious v.5.4 (56) and manually edited in Se-Al (http://tree.bio.ed.ac.uk/software/seal/). Reference sequences were compiled from previous publications (19, 42) and aligned with the novel sequences deposited into GenBank (XXX-XXXX).

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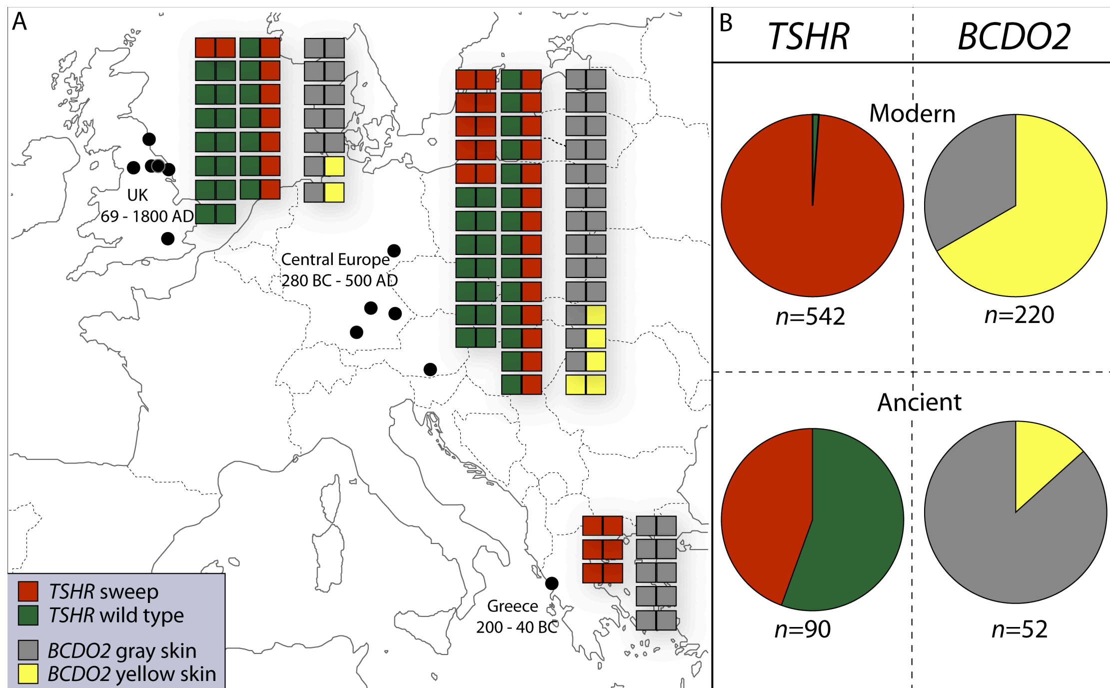
Figure 1
Panel *A* depicts a map showing the locations and chronology of archaeological sites (black dots) from three different European regions where ancient chicken remains analyzed in this study were excavated (see also Supp Text, Figure S3). The pairs of colored boxes adjacent to each region show which of two alleles of two nuclear loci (explained in the legend) were present in each genotyped individual (Table S1). Panel *B* depicts pie charts that show the differing genotype frequencies of the pooled ancient samples (from 290 BC to the 18th century AD) below and the modern populations above. Genotype frequencies found in modern chicken populations at the *TSHR* locus were derived from Rubin et al. (29) and at the *BCDO2* locus from Eriksson et al. (25).

Figure 2
Panel *A* depicts two pie charts showing differences in the frequency of mtDNA haplogroups in ancient and modern European chicken populations. The ancient sample consists of specimens sequenced in this study (n=39) and from a previous publication (n=5; Storey et al.

2012). The colors in panel *A* correspond to haplogroups depicted in a phylogenetic tree in panel B. Panel *B* depicts a phylogenetic tree constructed using haplotypes defined using a 519bp mtDNA fragment defined by Liu et al. (19). Colorless clades possess haplotypes not found in modern or ancient European samples and clades other than E present in modern chickens were likely introduced recently from East Asia. The tree was constructed using the Maximum Likelihood (ML). A more detailed ML tree, including detailed description of methods and results (including nodal support values), is shown in Figure S2.



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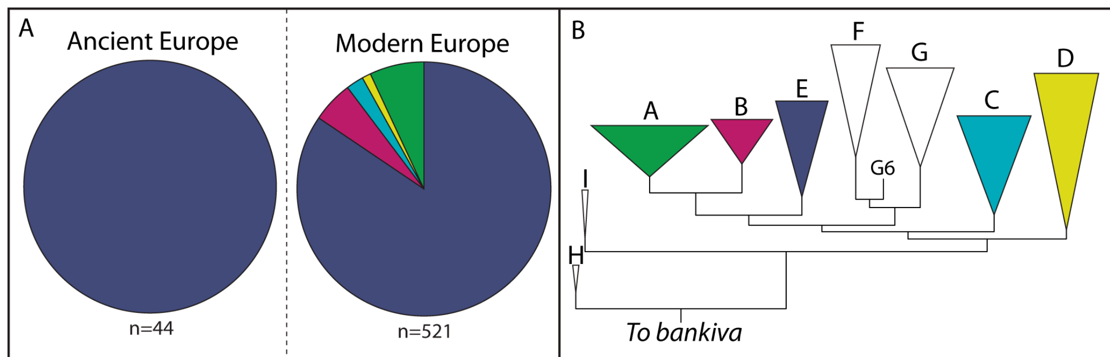

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Supplementary Materials

Archaeological specimens

81 ancient chicken bones from 12 archaeological sites, representing three major European locations (UK, Central Europe and Greece) and two major time bins (1: Late Iron Age La Tène C and D, Late Hellenistic/Early Roman to Roman, and 2: medieval and post-medieval periods) were extracted for DNA (Table S1). The majority of specimens were dated by means of stratigraphic/contextual evidence to between 280 BC to the 18th century AD (Figure 1; S3, Table S1). A single specimen from Kassope (Greece) was directly radiocarbon dated (Beta Analytic Inc., Miami, USA) to 2150-1990 cal. BP (2 σ calibration).

The chicken bones selected for this ancient DNA study originate from securely dated archaeological contexts in Greece (Kassope), Germany (Altenburg-Rheinau, Epfach, Künzing, Manching), Austria (Magdalensberg), Arbeia (South Shields Roman Fort) and various other UK contexts (Table S1). For German and Austrian contexts, given the problems with intrusive finds originating from later occupation phases, we only sampled faunal assemblages from (areas in) sites occupied for a limited period of time and with no overlaying building structures or archaeological strata.

Although our sampled specimens are relatively modern and geographically distant relative to the domestication centers in SE Asia, which date to as early as 6000 BC (1) (though see Main Text for a discussion on issues relating to *Gallus* finds in some early contexts), they represent among the earliest, if not the earliest, introduction to Europe. The first wave of introduction likely arrived around 900-700 BC in Greece and on the Iberian Peninsula (by Phoenician sea trade). The central European chicken was likely derived from the early Greek founder population which was distributed throughout the northern Mediterranean, and from there, introduced in Central Europe across the Alps around 600 – 400 BC. Zooarchaeological evidence for the presence of chicken in Central Europe (Germany, Austria, and Switzerland) dates to the Hallstatt C-D period ca. 800 - 475 BC. One major mechanism of introduction consisted of trade with ancient Greek settlement colonies in the coastal regions of the northwestern Mediterranean. During the following La Tène period (475-30/1 BC) *Gallus* bones

remain rare in archaeological contexts with frequencies increasing slightly during its later phases. After the Roman conquest poultry keeping became comparably rapidly and widely established in the north-western Provinces of the Roman Empire, but not so in regions outside the area of influence of Rome, where it only became economically important in medieval times (2). We can therefore safely assume that these Late Iron Age/Early Roman birds descend from early *Gallus* introduced into Europe. For the broadly contemporaneous birds from Classical Greece, however, this is not necessarily the case, considering the fact that in the meantime 'Greece' had been part of the Persian, Greek and Roman Empire with trade connections to Mesopotamia and India (3). SE Europe may therefore have witnessed multiple introductions from the Near East.

Description of key archaeological sites

Kassope, Greece: Ancient Greek city founded 350 BC and abandoned 30 BC (3). Friedl (4) presented an analysis of the fauna. The chicken remains studied here originate from house 3, occupied during the 2nd and 1st century BC.

Magdalensberg, Austria: Trading settlement founded during the second third of the 1st century BC and inhabited until about 50 AD (5). The bird remains including numerous chicken bones excavated at this site have been identified by Dräger (6).

Altenburg-Rheinau, Germany: Celtic oppidum occupied between ca. 150 and 15 BC (7). Excavations on the eastern peninsula during the 1970s produced the chicken remains, which have been analyzed by Moser (8).

Epfach, Germany: Roman military station and settlement, occupied with some interruptions from the 1st until the beginning of the 5th century AD (9). Chicken remains have been found throughout the sequence (10).

Künzing, Germany: The *Gallus* finds originate from the Mithras sanctuary (*Mithraeum*) near the Roman settlement. This sanctuary was in use from the 2nd century AD until the second half of the 3rd century AD (11). The birds represent offerings to the God Mithras. Excavations produced an assemblage of > 7,500 chicken remains (12).

Manching, Germany: Celtic oppidum inhabited between ca. 300 BC until 50/30 BC, probably the largest city north of the Alps during this period. After abandonment, some small-scale Roman occupation took place, probably in form of a *mansio* or perhaps *vicus*, lasting until the middle of the 3rd century AD (Sievers 2010). The chicken remains form part of a huge faunal assemblage (> 350,000) constituting a most useful source of information about late La Tène animal husbandry in Central Europe (13).

UK

Arbeia, South Shields Roman Fort, England

A Roman fort built at the mouth of the River Tyne in the late second century AD, which was converted into a supply base for food in the early second century AD. After a large fire in the late third or early fourth century AD, the fort was redesigned and rebuilt, and continued in use into the early fifth century AD (14).

The remaining chicken remains, from the north of England, which were utilized in this study came from two archaeological sites in York (Spurriergate and St Saviourgate) (15), located within the core of the medieval city; from a site in Beverley, East Riding of Yorkshire (16), and from South England and an excavation in East London (recovered from excavations in preparation for the construction of the Docklands Light Railways) (17).

The vertebrate material from Spurriergate was recovered from excavations in a former car park and beneath several 1960's buildings that were subsequently demolished prior to the archaeological interventions. The chicken bones were retrieved mainly from fills of rubbish pits and dump layers associated with occupation of Anglian/Anglo-Scandinavian, medieval and post-medieval date. The excavations at St Saviourgate largely revealed pits containing refuse of a mixed nature, from primary butchery waste to household rubbish. The chicken bones were recovered from pit fills of late medieval date.

Chicken remains from Beverley, East Riding of Yorkshire, were recovered from excavations at the site of the former Picture Playhouse and Swimming Pool in the

heart of the medieval town at the north side of Saturday Market. This had been a market area of Beverley since the 12th century, becoming known as the Corn Market by the 14th century and as Saturday Market by the 16th. The site was the location of a meat market by the 18th century, and quite probably much earlier, with an arcaded butchers' shambles built in 1753 and a fish shambles built behind the butchers' market in 1777. All of the contexts from which the fowl bones came were pit fills or ground raising deposits of medieval and post-medieval date (15, 16).

Ancient DNA laboratories and experimental setup

DNA extractions and PCR amplifications were performed in a dedicated ancient DNA laboratory in the Department of Archaeology (Durham Evolution and Ancient DNA-DEAD) at Durham University, UK. We followed strict laboratory procedures according commonly used guidelines (18, 19). All equipment and work surfaces were cleaned before and after each use with a dilute solution of bleach (10% active sodium hypochlorite) followed by ddH_2O and ethanol (99%). Pipettes and plastic racks were UV-irradiated in a dedicated cross-linker (at <15 cm for at least 30 min at 254nm wavelength) prior to and after use. Pre- and post-PCR laboratories are physically isolated; access to the pre-PCR laboratories is restricted to Ancient DNA lab users only and access is also prohibited if the lab user had entered post-PCR areas the same day. Ancient DNA lab users wear clean lab coats, double layer of gloves (nitrile and latex) and over-shoes, to avoid introducing contaminants from post-PCR areas.

Independent replication of 12 ancient specimens was performed in a dedicated ancient DNA laboratory at EBC, Uppsala University, Sweden. Lab work follows commonly used guidelines (18, 19). Lab users wear coveralls, facemasks, dedicated lab clogs and double layers of gloves. The lab is equipped with positive air pressure and UV lamps and is routinely deep cleaned with bleach (1%-5% active sodium hypochlorite), ddH_2O and dilute ethanol (70%).

Ancient DNA extraction

The ancient chicken bones were prepared for DNA extraction (one DNA extract/specimen) by removing an approximately one-millimeter layer of outer bone surface by abrasion using a Dremel drill with clean, one-time-use cut-off wheels

(Dremel no 409). A subsection of the bone was subsequently isolated and pulverized in a Micro-Dismembrator (Sartorious-Stedim Biotech), followed by collection in 15mL Grainer tubes. Milling containers and grinding balls were subsequently suspended and cleaned in 1% virkon, and rinsed in absolute (99%) ethanol.

50-100 mg bone powder/specimen was digested in 0.425M EDTA (pH 8), 0.05% SDS, 0.05M Tris-HCI and 400µg Proteinase K, and incubated overnight on a rotator at 50°C until fully dissolved. The DNA extraction master mix, excluding Proteinase K, was UV-irradiated at 254 nm for an hour in a cross linker prior to use. Once dissolved overnight, 2mL of solution was concentrated in a Millipore Amicon Ultra-4 30KDa MWCO to a final volume of 100µL. The concentrated DNA extract was purified using the QIAquick PCR Purification Kit (Qiagen) following manufacturers recommendations, except that the final elution step was performed twice (2 x 50µL) to produce a final volume of 100µL. One in five DNA extractions were blank, negative controls containing only extraction buffer and Proteinase K.

During replication in Uppsala, the ancient specimens were UV irradiated at 1J/cm2 per side (254nm wave length) and 1mm of the surface was removed prior to powderization. DNA was extracted as in Svensson et al. (20). 40 to 80mg bone powder was incubated in 1 mL of 0.5M EDTA, pH 8, 1M Urea and 100µg Proteinase K for 22 hours at 38°C together with 4 negative, blank controls. An additional 100µg Proteinase K was then added and the samples were incubated for 3 more hours at 55°C. DNA was further extracted using Qiagen PCR Purification Kit and finally eluted in 100µL Elution Buffer (Qiagen).

PCR amplification

A 201 base pair (bp) mitochondrial control region (CR) fragment (21, 22) and one SNP in each of two autosomal nuclear loci (*BCDO2* and *TSHR*) were targeted for PCR amplification (Table S6). PCR setup was performed in a fume hood in a dedicated facility adjacent to the dedicated ancient DNA extraction facility. The PCR setup facility is subject to positive air pressure that minimizes the risk of introducing contaminant DNA. One in eight PCR reactions were negative controls. In addition, one positive control (a modern Gray junglefowl, GJF) was included for each round of

PCR amplifications. In order to avoid contaminating the aDNA PCR reactions with modern GJF DNA, the modern positive control was stored in the dedicated PCR/post-PCR room and added to the reaction tubes whence placed on the thermal cycler. PCRs were visualized on a 1-2% agarose gel using GelRed and UV illumination. PCR products were purified using ExoSAP-IT (USB Affymetrix) and stored at -20°C prior to sequencing.

Independent replication of CR sequences and the *TSHR* SNP was performed in Uppsala with slight modifications to the PCR protocol: addition of RSA (rabbit serum albumine) was used instead of BSA (bovine serum albumine) and Smart Taq (Naxo) was used instead of Taq Gold (see below). Apart from following the PCR cycling conditions described below, replicate PCRs for the CR sequence were also performed following Storey et al. (22).

MtDNA CR (201bp)

PCRs were setup in 25μL reactions using 1U Taq GOLD (Applied Biosystems), 1x Gold buffer (Applied Biosystems), 2.5mM $MgCl_2$, 0.5μg/μL BSA (bovine serum albumine), 200μM of each dNTP, 0.4μM of each forward and reverse primers, and 2-5μL of aDNA extract. One in eight PCR reactions were negative blank controls. PCR cycling conditions were 95°C for 5min, 50 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, followed by 72°C for 10 min.

BCDO2

PCRs were setup in 25μL reactions using 1.0-1.25U Taq GOLD (Applied Biosystems), 1x Gold buffer (Applied Biosystems), 2.5mM $MgCl_2$, 0.5μg/μL BSA (bovine serum albumine), 1M betaine, 200μM of each dNTP, 0.4μM of each primer, and 2-5μL of aDNA extract. One in eight PCR reactions were negative blank controls. PCR cycling conditions were 95°C for 5min, 50 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec, followed by 72°C for 10 min.

TSHR

PCRs were setup in 25µL reactions using 1.0-1.25U Taq GOLD (Applied Biosystems), 1x Gold buffer (Applied Biosystems), 2.5mM $MgCl_2$, 0.5µg/µL BSA (bovine serum albumine), 200µM of each dNTP, 0.6µM of the biotinylated forward primer and 0.8µM of the reverse primer, and 2-5µL of aDNA extract. One in eight PCR reactions were negative blank controls. PCR cycling conditions were 95°C for 5min, 50 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, followed by 72°C for 10 min.

DNA sequencing, genotyping and data analysis

Sanger sequencing was performed on the Applied Biosystems 3730 DNA Analyzer at the DNA Sequencing Service at the School of Biological and Biomedical Sciences (Durham University). Trace files were manually inspected using 4Peaks (Mekentosj) or Geneious v.5.4 (23) and built into contigs by hand in Se-Al (http://tree.bio.ed.ac.uk/software/seal/) or automatically in Geneious v.5.4 using the assembly function (default parameters). At least two (but usually three) independent PCR replicates/sequences per sample were performed in order to ensure authenticity. Sporadic non-replicable post-mortem DNA template damage was observed in several sequences (C->T transitions). This type of damage often results from deamination through hydrolysis resulting in the conversion of cytosine bases to uracil (alternatively hydroxyuracil) or adenine to hypoxanthine. Uracil is read as thymine and hypoxanthine as guanine by DNA polymerases during PCR amplification and subsequently induces the common Type 2 (C/T or A/G) damage (19, 24, 25).

Pyrosequencing was performed in-house at the Archaeology department in Durham using the PyroMark Q24 (Qiagen) following manufacturers guidelines, and using Qiagen Q24 sequencing reagent kits. Results/sequences/genotypes were analyzed in the PyroMark Q24 software (Qiagen) using modified settings: accepted peak deviation and minimum peak heights were set to less strict to account for low signal intensity and slight deviations in peak heights (which, if observed, could be the result of Type-2 C->U deamination/error). Dispensation order was automatically generated using the PyroMark Q24 software (Qiagen, and see Table S6).

In order to account for allelic dropout that is common in ancient DNA studies (20), each SNP/genotype was confirmed by repeated genotyping from 2-8 independent PCRs (at least two independent replications for heterozygous specimens that did not show evidence of allelic dropout, but up to eight replications in for homozygous specimens but also heterozygous specimens for which we repeatedly observed allelic dropout). The probability of falsely assigning a heterozygous individual as homozygous was calculated as follows: P(false homozygote)=$K*(K/2)^{n-1}$, where n is the number of replicates and K is the observed number of allelic dropouts divided by the total number of genotypings of heterozygous individuals (20, 26).

Fisher exact test, as implemented in R v.2.15.2 (27), was used to test for differences in allele (and mtDNA CR haplotype) frequencies between modern and ancient populations. In addition, binomial probability distributions (the *pdbinom, dbinom* and *gbinom* functions in R v.2.15.2) were used to examine differences in allele frequencies between ancient and modern populations and to authenticate the ancient DNA results, assuming that putative contamination would reflect genotype frequencies in modern populations (Figure S1; Table S2 and S3).

In Uppsala, the TSHR SNP was genotyped on a PSQ™ 96MA (Biotage. Uppsala), using pyrosequencing™ technology and the SNP software and SNP reagent kit (Biotage, Uppsala). Sample preparation was performed according to Pyrosequencing instructions using 25µl of PCR product.

Phylogenetic reconstruction

An alignment of previously published mtDNA CR haplotypes (28, 29) was used as a reference to identify haplotypes (Table S4 depict unique E-clade haplotypes). Modern reference and ancient DNA consensus sequences were aligned in Geneious v.5.4 (23) using MAFFT (30). Phylogentic analysis was performed using PhyML v.3.0 (31) as implemented in Geneious v.5.4 (23). The nucleotide substitution model was estimated in using MrModeltest 2.3 (32) and PAUP* 4.0 (33) as implemented in MrMtGui (34). The best-fit model using both aLRT statistics and AIC was HKY+I+G. Nodal support values were estimated through bootstrapping. A *Gallus gallus bankiva* sequence (AB007718) was used as outgroup.

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Figure legends

Figure S1.

A figure depicting the number of expected TSHR wild type alleles in a sample of 89 given the frequencies reported previously (35) (Table S2), and the expected number of non-E clade haplotypes in a sample of 44 given the frequency reported previously for modern populations (28, 29, 36) (Table S5). The green stars depict the empirical, observed frequencies (Table S1-S3).

Figure S2.

A: A Maximum Likelihood tree constructed using the haplotypes reported by Liu et al. (2006). Numbers indicate bootstrap support values. B: A Maximum Likelihood tree constructed using the 201bp mtDNA CR haplotypes of Liu et al. (29) amplified for the ancient European specimens (Supplementary Text, Table S4; S6).

Figure S3.

A map depicting archaeological sites, the number of sampled specimens and DNA retrieval success rate (success indicated as at least one reproducible genotype/specimen, Table S1).