RESEARCH PAPER

Genetic Insight into an Extinct Population of Asian Elephants (*Elephas maximus*) in the Near East

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The current range of the Asian elephant is fragmented and restricted to southern Asia. Its historical range was far wider and extended from Anatolia and the Levant to Central China. The fossil record from these peripheral populations is scant and we know little of their relationship to modern Asian elephants. To gain a first insight to the genetic affinity of an E. maximus population that once inhabited Turkey we sequenced ca. 570 bp mtDNA from four individuals dating to ~3500 cal. BP. We show that these elephants carried a rare haplotype previously only observed in one modern elephant from Thailand. These results clarify the taxonomic identity of specimens with indeterminate morphologies and show that this ancient population groups within extant genetic variation. By placing the age of the common ancestor of this haplotype in the interval 3.7–58.7 kya (mean = 23.5 kya) we show that range-wide connectivity occurred at some time or times since the start of MIS 3, ~57 kya, probably reflecting range and population expansion during a favourable climatic episode. The genetic data do not distinguish natural versus anthropogenic origin of the Near Eastern Bronze Age population, but together with archaeological and paleoclimatic data they allow the possibility of a natural westward expansion around that time.

Keywords: Asian elephant; *Elephas maximus*; ancient DNA

1. Introduction

The Asian elephant (*Elephas maximus*, L. 1758) is an endangered species whose current distribution extends from the Indian subcontinent to Borneo (**Figure 1A**). Its range was far wider during the Pleistocene and early Holocene, extending from Anatolia and the Levant in the west, along a narrow land corridor by the southern Asian coastline, to the Indian subcontinent and as far eastward as southern and central China (Santiapillai and Jackson 1990, Sukumar 2011) (**Figure 1A**). Since the fossil record is scant, often comprising single fragmented teeth or long bones that can be confused with other elephantid species, their affinities and distributional change are poorly understood (Turvey *et al.* 2013). Another possibly complicating factor is human translocation of living elephants from their core distribution (e.g. India) as part of emerging trade networks and long-range war campaigns (Collon 1977, Albenda 2008).

To gain insight to the *E. maximus* population that once inhabited the Near East we sequenced mitochondrial DNA (mtDNA) extracted from four ancient teeth excavated at Kahramanmaraş (Gavur Lake Swamp) in south-central Anatolia (**Figure 1A, Table 1**). The elephant remains have been directly ¹⁴C dated to approximately 3500 BP (ca. 1500 BCE) and represent one of the largest assemblages of Holocene *E. maximus* in the region (Albayrak and Lister 2012). The dates place the remains within the Late Bronze Age, when the region around Kahramanmaraş was likely an area of influence of both the Mittani and Hittite Kingdoms (Alaura 2016). The collection is primarily made up of loose teeth but also includes partial skulls and post-cranial elements (**Figure 2**). Although most specimens show strong morphological affinity to modern *E. maximus*, a number of molars could not be identified with certainty as *E. maximus* compared to the Pleistocene species *Palaeoloxodon antiquus* (straight-tusked elephant).

Here we demonstrate the phylogenetic position of the elephant teeth (determinate and indeterminate) in relation to the known mitochondrial phylogeography of modern Asian elephant populations (Vidya, Sukumar and Melnick 2009). Further, by estimating mtDNA coalescence times we discuss the position of the Turkish remains in the broader historical context of *E. maximus*.

2. Methods

We extracted DNA from nine elephantid teeth from Kahramanmaraş and sequenced 579 bp mitochondrial DNA, including 574 bp of the fragment analysed in Vidya, Sukumar and Melnick (2009), comprising the C-terminal of cyt-b, t-RNA$_{Thr}$, t-RNA$_{Pro}$ and the hypervariable left

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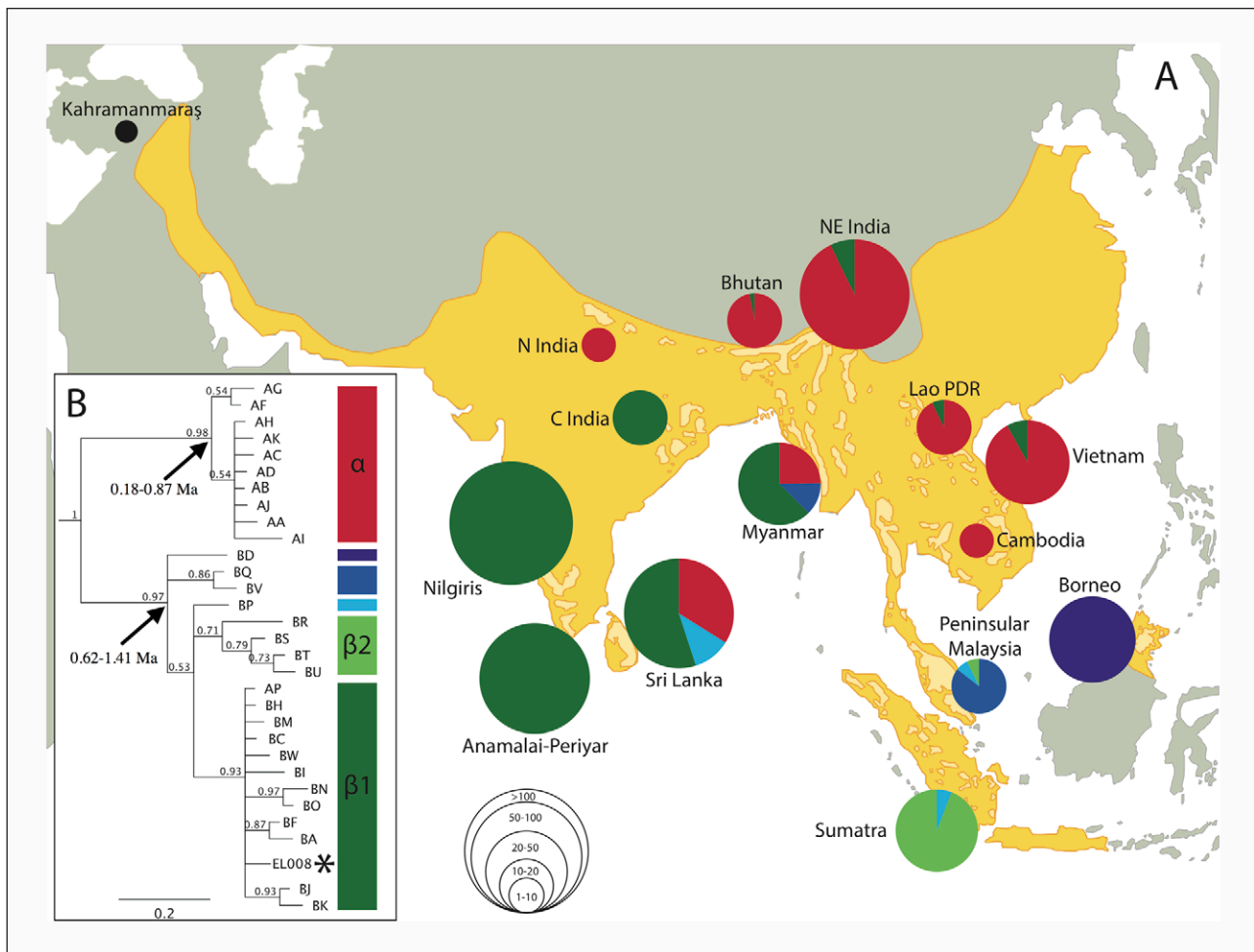Corresponding author: Adrian M. Lister (a.lister@nhm.ac.uk)

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Figure 1: (A) A map depicting the present-day (light yellow) and ancient (dark yellow) range of Elephas maximus (Olivier 1978, Choudhury et al. 2008), the location of Kahramanmaraş, and the relative proportion of mtDNA haplotypes based on data published in Vidya, Sukumar and Melnick (2009). **(B)** A Bayesian phylogenetic tree produced in MrBayes of haplotypes analysed in Vidya, Sukumar and Melnick (2009), but restricted to the 570 base pairs sequenced for this study. Numbers show posterior support values for the nodes. Arrows indicate node age estimates (see Results). Nomenclature is reported as in Vidya, Sukumar and Melnick (2009). The asterisk denotes the haplotype observed in the Kahramanmaraş elephants, here represented by sample EL008 (Table 1).

Table 1: A table summarizing the specimens analysed in this study, from Albayrak and Lister (2012). M = upper molar, m = lower molar.

Sample ID	NHM ID	Species (morphology)	Element	DNA
46GGB05	EL001	cf. *Elephas maximus*	m3	No
46GGB04	EL002	*Elephas maximus*	M3	No
46GGBX	EL003	*Elephas maximus*	M3	No
46GGB02	EL004	*Elephas maximus*	M3	Yes
46GGB20	EL005	cf. *Elephas maximus*	M2	No
46GGB01	EL006	cf. *Elephas maximus*	m3	Yes
46GGB09	EL007	indet.	m2	Yes
46GGB03	EL008	indet.	m3	Yes
46GGB07	EL009	cf. *Elephas maximus*	m1	No

domain of the control region. The sampling, DNA extraction and all pre-PCR work were carried out in a dedicated ancient DNA laboratory at the Natural History Museum, London, following strict protocols and recommendations (Gilbert *et al.* 2005). No work on other elephantid DNA had taken place previously in the ancient DNA laboratory.

2.1. DNA extraction
Teeth chosen for DNA extraction were decontaminated first by applying a 1% dilute solution of bleach (sodium hypochlorite) on and around the surface area targeted for dentine excision, followed by UV irradiation at 254 nm for 20 min at a distance of <10 cm. The outer surface

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Figure 2: A partially reconstructed skull of Elephas maximus from Gavur Lake Swamp (MTA Natural History Museum, Ankara).

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Table 2: PCR primers designed for this study.

Name	Sequence 5'–3'	Length bp	Fragment	Nucleotide positions in reference to NC_005129.2 (with primers/without primers)
P1F	CATGAATTGGCAGYCAACCA	20	1	15,154–15,265 (103 bp)/15,174–15,243 (63 bp)
P1R	CCTGCARTTGGTAGGAAAGC	20	1	
P2F	CTTCTCCATTATTCTAGCTTTCC	23	2	15,221–15,336 (116 bp)/15,244–15,314 (71 bp)
P2R	TGGYTTTCATTTATGGYTTACA	22	2	
P3F	CATCAAGTAACCCCTATAGTATAAGAC	27	3	15,275–15,391 (117 bp)/15,302–15,369 (68 bp)
P3R	TTTTGGGTATTGATAGCGAGGT	22	3	
P4F	AAGGGTATTCAGGGAAGAGG	20	4	15,343–15,449 (107 bp)/15,363–15,428 (66 bp)
P4R	GCACGATRTACATAGCGGATT	21	4	
P5F	CCTCGCTATCAATACCCAAAA	21	5	15,371–15,472 (102 bp)/15,392–15,452 (61 bp)
P5R	ATGTATGGGGACGAGCATTT	20	5	
P6F	TAAATGCTCGTCCCCATACA	20	6	15,452–15,552 (101 bp)/15,472–15,530 (59 bp)
P6R	CATGGGGTAAATAATGTGATGC	22	6	
P7F	CCATACYATGTATAATCGTGCATCA	25	7	15,512–15,629 (118 bp)/15,537–15,622 (86 bp)
P7R	TCCATGARCTARAACATRACCTGTG	25	7	
P8F	TCAATGTGTYRAGTCATATTYBTG	24	8	15,575–15,685 (111 bp)/15,599–15,664 (66 bp)
P8R	CGATCAAGAGCTTTAATGTGC	21	8	
P9F	TCATGGATATTRTTYRCCTACGA	23	9	15,623–15,741 (119 bp)/15,646–15,721 (76 bp)
P9R	AACCGTTGGAGGTGATATGC	20	9	
P10F	AAGCTCTTGATCGTRCATAGC	21	10	15,673–15,776 (104 bp)/15,694–15,756 (63 bp)
P10R	GTTGATGGTTTCTCGGAGGT	20	10	

(~1 mm) was removed by abrasion and tooth powder was obtained using a Dremel drill at the lowest possible rpm setting. We obtained a total of 30–60 mg powder per specimen.

DNA was extracted and purified following a modified version of a silica-binding protocol (Yang *et al.* 1998, Ottoni *et al.* 2013). Tooth powder was suspended and digested overnight at constant rotation at 50°C in 2 mL of extraction buffer consisting of 0.425 M EDTA (pH8), 1 mM Tris–HCl (pH8), 0.05% w/v SDS, and 0.33 mg/ml Proteinase K. The digested solution was concentrated to ≤100 μL using Amicon Ultra-4 centrifugal filter units with a molecular weight cut-off of 30 kDa (Amicon® Ultra, Millipore). We isolated and purified the DNA on silica columns (QIAquick® PCR purification kit, Qiagen) following the manufacturer's protocol and eluted in 100 μL EB buffer. One negative extraction control was processed alongside the nine tooth samples.

2.2. PCR amplification
A series of novel PCR primers were designed in Primer3Plus (Untergasser *et al.* 2012) to amplify short (101–119 bp) and mostly overlapping mtDNA fragments (**Table 2**). Singleplex PCRs were carried out in 25 μL reactions using 1.0 U Smart-Taq Hot DNA Polymerase and 1X Smart-Taq Buffer with $(NH_4)_2SO_4$ (Naxo), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 mg/mL final concentration of RSA, and 0.2 μM of each primer. The cycling conditions were 95°C for 15 min followed by 55 cycles of 94°C for 20 sec, 55–57°C for 20 sec, 72°C for 20 sec, and a final extension for 5 min at 72°C. We obtained two PCR amplified products/fragment/specimen (amplified at different times) and included one negative PCR control for every eight PCRs.

2.3. DNA sequencing
PCR products were purified using the QIAquick® PCR purification kit (Qiagen), pooled into equimolar ratios following quantification on a Qubit® (Invitrogen), and constructed into barcoded sequencing libraries using the TruSeq Nano DNA sample prep kit (v2, Illumina) following manufacturer's recommendations (but omitting initial shearing) at the sequencing facility at the Natural History Museum, London. Sequencing was performed in-house on the Illumina MiSeq platform the using MiSeq Reagent Kits v2 at ~10% of a full sequencing run. Libraries were de-multiplexed on the MiSeq platform and exported in FastQ format. FastQ files were trimmed in Geneious V7 (Kearse *et al.* 2012) first by removing regions with more than a 5% chance of an error per base, and secondly by retaining regions with no more than 2 bases with a quality of ≤30. Sequences were aligned against a reference *E. maximus* sequence (Genbank accession AY245813), and consensus sequences were called using majority consensus and highest cumulative quality scores. Bases with a quality of ≤30 were ignored and called as Ns. Base coverage ranged from ca. 1000–30,000.

2.4. Phylogenetic analysis
We downloaded reference data from two *Loxodonta cyclotis* sequences (JQ438507 and KJ557424), two *Loxodonta africana* sequences (JQ438674 and JQ438767) (Ishida *et al.* 2013, Finch *et al.* 2014), as well as two *Mammuthus primigenius* sequences (GU984769 and KC427898) (Nyström *et al.* 2010, Palkopoulou *et al.* 2013) and aligned them with the Kahramanmaraş sequences using MAFFT v7.017 (Katoh *et al.* 2002). We then aligned and pruned the DNA sequences to previously published *Elephas maximus* data (Fernando *et al.* 2000, Fernando *et al.* 2003, Vidya, Sukumar and Melnick 2009). Of the 579 bp

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amplified from the Kahramanmaraş samples we used 570 bp for the phylogenetic analysis described below. However, the Kahramanmaraş sequence data includes 32 bp missing data resulting from the difficulty in designing PCR primers to amplify short and fully overlapping amplicons.

We first assessed the phylogenetic position of the ancient Turkish specimens using MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001) and a non-partitioned data set of unique haplotypes (n = 38 including *Loxodonta* and *Mammuthus*; n = 32 *Elephas*). We ran three heated chains (temperature = 0.4) over 10,000,000 generations with a subsampling frequency of 10,000 and a burn-in length of 1,000,000 generations. The consensus tree was visualized in Geneious 7 (Kearse *et al.* 2012). The nucleotide substitution model for the phylogenetic analysis of the non-partitioned dataset in MrBayes was HKY+I+G, which was the best-fitting model for all partitions but one in the Partition Finder analysis described below.

We then estimated the age of the common ancestor of the node defining the β1-subclade in which we observe the Kahramanmaraş haplotype using BEAST 2.1.3 (Bouckaert *et al.* 2014). Nucleotides were first grouped into five different partitions: the 1st, 2nd, and 3rd codon positions respectively for cyt-b; the tRNA$_{Thr}$ and tRNA$_{Pro}$; and the control region. Nucleotide substitution models were estimated for each partition using the Bayesian Information Criterion in Partition Finder (Lanfear *et al.* 2012). The best-fit model for 1st and 3rd codon positions of the cyt-b gene, the tRNA$_{Thr}$/tRNA$_{Pro}$, and the control region was HKY+I+G, and the K80 model for the 2nd codon position in cyt-b. The BEAST analysis included all DNA sequences compiled for this study: *Loxodonta* (n = 4), *Mammuthus* (n = 2), Kahramanmaraş (n = 4), and *Elephas maximus* (n = 534).

We used tip dating on ancient DNA sequences as follows:

· GU984769 (4.8 kyr) (Nyström *et al.* 2010).
· KC427898 (33.7 kyr) (Palkopoulou *et al.* 2013).
· Kahramanmaraş elephants (3.5 kyr) (Albayrak and Lister 2012).

To obtain new MCRA estimates we used the following node age calibrations based on mitogenome divergence times estimated previously using a 'narrow range fossil calibration' (Brandt *et al.* 2012):

· Root (normal distribution, mean = 6.81 My, σ = 0.5 My),
· *Loxodonta* (normal distribution, mean = 5.51 My, σ = 0.7 My),
· *Elephas* and *Mammuthus* (normal distribution, mean = 6.01 My, σ = 0.7 My),
· *Elephas* (normal distribution, mean = 1.35 My, σ = 0.25 My).

We then placed uniform priors on the following clades and estimated their MRCA:

· *Elephas* β clade (lower = 0.0 My, upper = Infinity)
· *Elephas* α clade (lower = 0.0 My, upper = Infinity)
· Kahramanmaraş 'clade' (lower = 0.0035 My, upper = Infinity).

We used a strict clock model under a constant population size prior. The clock rate prior was set to default (uniform distribution ranging from 0 – infinity). Three runs of 30,000,000 generations were completed, reaching effective sample sizes (ESS) of >100 (though the vast majority were >200) for each parameter. Trees and trace files were sampled/logged every 3000 generations. The trace files were also visually inspected using Tracer V1.6 (Rambaut, Suchard and Drummond 2013) to ensure proper mixing and that the Markov chain had reached stationary distribution.

3. Results and Discussion

We successfully amplified and sequenced DNA from four specimens, yielding a success rate of *ca.* 44% (4/9), consistent with previously published data on *ca.* 120 bp mtDNA fragments sequenced from ancient pigs from Near East dating to approximately 3500 cal. BP (Ottoni *et al.* 2013). We furthermore observed damaged bases (C > T or G > A transitions) across all sequenced DNA fragments, consistent with cytosine deamination to uracil which is a common type of damage in ancient DNA molecules (Gilbert *et al.* 2003). The negative extraction and PCR controls did not produce PCR amplified products. These observations support the authenticity of our data.

3.1. Genetic affinity of the Kahramanmaraş elephants

The mtDNA phylogeny of extant *E. maximus* consists of two highly divergent clades (α and β). The four ancient elephants from Kahramanmaraş all carried an mtDNA haplotype that groups in the β1 clade of the major β clade of *E. maximus* (**Figure 1B**). A comparison with previously published data on NCBI GenBank shows that the Kahramanmaraş sequences are identical to only a single modern elephant from Thailand (Unpublished GenBank accession JQ287727). While this haplotype appears to be rare among extant populations, the β1 sub-clade is present across their range (except Peninsular Malaysia, Borneo and Sumatra), being particularly abundant in South and Central India – and varies in frequency from 3.2–100% (Vidya, Sukumar and Melnick 2009). The lack of a clear phylogeographic structure means that it would be problematic attempting to 'link' the Kahramanmaraş elephants to any specific modern population. We therefore conclude only that they group within present-day variation.

The α and β clades are estimated to share a most recent common ancestor (MRCA) some 1.35 [0.9–1.84] Ma (Brandt *et al.* 2012). The internal MRCAs for these clades have been estimated to 0.86 Ma (0.52–1.21 Ma) and 1.58 Ma (1.28–1.86 Ma) respectively (Vidya, Sukumar and Melnick 2009). Our analysis in BEAST provides somewhat younger dates and places the MRCA of the α-clade at 0.49 Ma (0.18–0.87 Ma) and the β-clade at 0.99 Ma (0.62–1.41 Ma). However, the MRCA for all *E. maximus* as well as the MRCA of the α and β clades are still older than the earliest morphologically distinct *E. maximus* fossils, which date to *ca.* 0.2–0.1 Ma (Maglio 1973, Lister *et al.* 2013), implying that the present-day mtDNA gene pool consists of lineages that originated in earlier, ancestral *Elephas* species

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(Vidya, Sukumar and Melnick 2009). Morphology as well as geographic and temporal distribution suggests that *E. hysudricus* is a likely ancestor of *E. maximus* (Maglio 1973, Lister *et al.* 2013). However, the deep divergences of the α and β clades might in theory reflect genetic contribution from more than one species, such as *E. hysudrindicus* or even *P. namadicus*, in addition to *E. hysudricus* (Lister *et al.* 2013). This possibility is congruent with a recent paper (Meyer *et al.* 2017) that analysed mitogenomes and partial autosomal genomes of *P. antiquus* and suggested possible historical gene flow between that species and *E. maximus.* Similarly, historical gene flow between African savanna and forest elephants (*L. africana* and *L. cyclotis*) explains the observation that some savanna elephants carry the deeply divergent 'F-clade' mitochondrial haplotypes and not 'S-clade' haplotypes, despite the former being characteristic of forest elephants while the latter is private to savanna elephants (Roca, Georgiadis and O'Brien 2004).

By estimating that the haplotype carried by the Kahramanmaraş elephants and a modern Thai elephant shared a common ancestor that lived 3.7–58.7 kyr ago (95% HPD; mean 23.5 kyr) we can conclude that it very likely belonged to a population of *E. maximus*. This implies that range-wide population connectivity, such as gene flow or migration, has taken place at some time or times since the start of MIS 3 some 57 kya, likely reflecting range and population expansion that led to the establishment of the Bronze Age southwest Asian population.

3.2. Molecular taxonomy and tooth morphology

Deraniyagala (1950) defined a subspecies, *E. maximus asurus*, for the Near Eastern elephants, but their supposed characteristics were based on doubtful interpretation of Bronze Age illustrations, aside from the suggestion, based on rather few bone measurements, that the animals were of unusually large size (Deraniyagala 1955, Pfälzner 2013). While the majority of molars from Kahramanmaraş are of typical *E. maximus* morphology, a few are more ambiguous (Albayrak and Lister 2012) (**Table 1**). The sequencing results allow us to confirm the taxonomic identity of one tooth (46GGB01) identified as likely *E. maximus* but of unusual occlusal morphology, and two (46GGB03 and 46GGB09) whose specific identity was indeterminate between *E. maximus* and the *P. antiquus*. All are confirmed as belonging to the lineage of extant *E. maximus.* In light of the new genetic data and the morphological homogeneity of most of the assemblage (Albayrak and Lister 2012), it is parsimonious to conclude that the Kahramanmaraş population represents a population of *E. maximus* that harboured greater variation in dental morphology than extant populations (Albayrak and Lister 2012). Our mtDNA evidence provides no indication of genetic divergence from East Asian populations, but further modern and ancient genetic and morphological data – preferably including nuclear DNA sequences – are required to resolve this and other questions concerning the complex evolutionary history of *E. maximus* (Meyer *et al.* 2017).

3.3. Status of the Near-Eastern Asian elephant population

The sharing of a haplotype between the Turkish fossils and a modern individual from the Far East supports the hypothesis that the Near-Eastern elephants were the western-most expansion of the core population of India and Southeast Asia. It leaves open the possibility that the Turkish population was established only shortly before its Bronze Age date. However, the estimated age of the common ancestor of the Kahramanmaraş haplotype, extending to 58.7 kyr at its 95% lower bound, means that the Near Eastern populations could alternatively have been established, or at least separated from other populations in southern Asia, as long ago as MIS 3, assuming that the Turkish population and their direct ancestors were isolated without gene flow. The split time between the population ancestral to the Kahramanmaraş elephants and the eastern core populations could in theory have been even longer in the presence of gene flow. The genetic data do not distinguish between these possibilities, nor therefore between natural range expansion and human introduction of elephants into the Near East. The latter has been posited mainly due to the lack of well-dated skeletal remains of elephants before 3500 BP, or ivory (which could have been humanly transported) before 4000 BP (Çakirlar and Ikram 2016). The use of captive elephants likely did not begin in India until ca. 4600 BP (Sukumar 2011), and deliberate long-distance movement of elephants has been thought impracticable in the Bronze Age (Pfälzner 2013). Although there are historical/pictorial sources referring to the movement of live elephants in the Bronze and Iron Ages in southwest Asia (Collon 1977), the westward movement of elephants from India for war or other purposes is first clearly recorded around ca. 2600 BP (Sukumar 2011). Of considerable significance is that the Kahramanmaraş assemblage, although not systematically excavated, appears very likely to represent a wild-living population. It comprises various elements of the skeleton of multiple individuals, some of them probably associated, with no cut marks, artefacts, or other sign of human activity (Alaura 2016, Yar *et al.* 2016) in stark contrast to Near-Eastern archaeological sites with occasional elephant remains (Çakirlar and Ikram 2016). This suggests a naturally established population, or conceivably a wild-living population founded by humanly-introduced animals.

Combining these data suggests that the most likely scenario is the westward expansion of the elephants' range, presumably during favourable climatic episode(s), i.e. with sufficient warmth and moisture to provide the required vegetation and drinking water to support the animals along the route. These requirements would have been necessary whether the population arrived by natural expansion or human agency. Rowe *et al.* (2012) show cyclicity of wet and dry episodes in Turkey extending back into the Late Pleistocene, as do Rajagopalan *et al.* (1997) for the Indian subcontinent. Corresponding cyclical variation in the range of mammal species, including elephants, is likely. More recent studies show that the climate of the Near and Middle East underwent a shift from wetter

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conditions in the early Holocene to drier conditions from around 6500 BP, but that this was interrupted by an interval of moister conditions between around 5000–3500 BP (Finné *et al.* 2011). For example, Migowski et al. (2006) indicate a wet phase in the Levant *ca.* 5600–3500 BP, based on Dead Sea lake level changes. A variety of data from Lake Van in southeastern Turkey and Soreq Cave in Israel suggests that a moister climate prevailed in the region during the Chalcolithic and Early Bronze Age, although the region became significantly more arid from around 2000 cal. BC (*ca.* 4000 BP) (Bar-Matthews et al. 1999, Wick, Lemcke and Sturm 2003). Together, these data suggests that wetter climatic periods suitable for the immigration of elephants did prevail in the region at intervals during the Holocene, although the general climatic trend is one of increasing aridity.

Cyclical expansion and contraction of range is also supported by earlier fossil records of *Elephas* in the Near East, where the earliest dated finds are an Early Pleistocene molar tooth of *Elephas* sp. from Evron Quarry in Israel (Tchernov *et al.* 1994), and five Middle Pleistocene teeth of *Elephas* cf. *hysudricus* from Ma'ayan Baruch in Israel and 'Ain Soda in Jordan (Lister *et al.* 2013).

Data Accessibility Statement
The consensus DNA sequences are publically available on GenBank (accession numbers MF 314177-MF 314180).

Competing Interests
The authors have no competing interests to declare.

Author contributions
LGF designed the study, acquired and analysed data, and co-wrote the manuscript. EA provided samples and contextual information, and critically revised the manuscript. AML conceived the study and co-wrote the manuscript.

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