Ancient *Mycobacterium leprae* genomes from the mediaeval sites of Chichester and Raunds in England

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Abstract

We examined six skeletons from mediaeval contexts from two sites in England for the presence of *Mycobacterium leprae* DNA, each of the skeletons displaying osteological indicators of leprosy. Polymerase chain reactions directed at the species-specific RLEP multicopy sequence produced positive results with three skeletons, these being among those with the clearest osteological signs of leprosy. Following in-solution hybridization capture, sufficient sequence reads were obtained to cover >70% of the *M. leprae* genomes from these three skeletons, with a mean read depth of 4–10×. Two skeletons from a mediaeval hospital in Chichester, UK, dating to the 14th–17th centuries AD, contained *M. leprae* strains of subtype 3I, which has previously been reported in mediaeval England. The third skeleton, from a churchyard cemetery at Raunds Furnells, UK, dating to the 10th to mid-12th centuries AD, carried subtype 3K, which has been recorded at 7th–13th century AD sites in Turkey, Hungary and Denmark, but not previously in Britain. We suggest that travellers to the Holy Land might have been responsible for the transmission of subtype 3K from southeast Europe to Britain.

Keywords: Ancient DNA, Leprosy, Mediaeval England, *Mycobacterium leprae*, Palaeopathology

1. Introduction

Leprosy is a slowly progressive, chronic granulomatous disease caused by *Mycobacterium leprae* (Hansen, 1874) and potentially, in a minority of cases, by the more recently characterised agent described as *Mycobacterium lepromatosis* (Han et al., 2008). The primary symptoms are granulomas of the skin, peripheral nerves and respiratory tract, but sometimes the eyes, skeleton and nasal cartilage are also affected (Britton and Lockwood, 2004). The bacilli accumulate in the extremities of the body, invading the Schwann cells causing nerve damage followed by a gradual sensory loss and eventually leading to deformities and disabilities (Masaki et al., 2013). A multi-drug regime comprising dapsone, rifampicin and clofazimine has been used successfully to treat 16 million leprosy patients over the last twenty years, but new infections are frequent with 210,671 leprosy cases reported in 2017 (World Health Organisation, 2018). With the highest incidence of new cases occurring in northeast South America, central Africa and the Indian subcontinent, leprosy is classified as a 'neglected tropical disease' (Lenk et al., 2018). Although the disease itself is curable, leprosy-related deformities and disabilities are irreversible, especially when treatment has been delayed (Britton and Lockwood, 2004). Some 2–3 million people worldwide display post-leprosy disfigurements, and many are subject to the social discrimination referred to as leprosy stigma, which in the past was driven by misunderstandings regarding transmission of the disease, and which still persists today in some parts of the world (Grzybowski et al., 2016).

Leprosy is one of the oldest diseases known to humankind. Although ambiguous, textual references to skin diseases in the Indian *Atharva Veda* and *Laws of Manu* (2000–1500 BC) (Bloomfield, 2004) and the Egyptian Ebers papyrus (1550 BC) (Hulse, 1972) have been identified as leprosy, and there are more recent accounts of the disease dating from the 6th century BC to 1st century AD from India (Bhishagratna, 1996), China (McLeod and Yates, 1981; Leung, 2008), Greece (Pinhasi et al., 2005) and Rome (Roberts and Manchester, 2010). Additional evidence is provided by

palaeopathological examination of archaeological skeletons for the osteological manifestations of the disease that can be observed in the hands, feet, facial bones, tibiae and fibulae of affected skeletons (Ortner, 2003). The oldest skeleton displaying such lesions dates to 2000 BC, from Rajasthan in northwest India (Robbins et al., 2009), in accordance with the Indian textual references from the same period. It has been suggested that the disease was brought to Europe and Northern Africa by the armies of Alexander the Great, with their return from the Indian campaign in 327–326 C (Roberts and Manchester, 2010). There is skeletal evidence of leprosy in Egypt at 200 BC (Dzierzykray-Rogalski, 1980) and in Western Europe from the 4th century AD (Reader, 1974). However, the disease appears to have been uncommon in Europe until the Mediaeval period, when skeletons displaying lesions become more abundant (Roberts and Manchester, 2010). In Britain, the prevalence of leprosy peaks in the 13th century AD and then declines during the 15th century AD before becoming uncommon again from the 16th century AD onwards (for a review of the osteological evidence for Britain, see Roberts, 2002), possibly because of improved social conditions combined with the development of enhanced resistance to the disease among the human population (Schuenemann et al., 2013). The decline is mirrored in continental Europe (Bennike, 2002), although the disease persisted in some parts of Norway and elsewhere until the 19th century AD (Boldsen, 2001).

About 5% of leprosy cases develop skeletal changes, and the lesions used in osteological assessment of the disease can be ambiguous. An important adjunct to palaeopathological analysis has therefore been provided by the detection and sequencing of *M. leprae* DNA, which is sometimes preserved in archaeological skeletons displaying osteological lesions and has also occasionally been detected in skeletons free from such lesions (Donoghue et al., 2017). Initially, ancient DNA typing was used mainly to support osteological identifications of leprosy (Rafi et al., 1994; Taylor et al., 2000, 2006; Donoghue et al., 2001, 2005, 2015; Inskip et al., 2015), but with increasing knowledge of genomic diversity among extant *M. leprae* strains it has become possible to contextualise ancient DNA data within an evolutionary scheme for the bacterium (Schuenemann et al., 2018). The *M. leprae* genome is 3.27 Mb, substantially smaller than the 4.42 Mb genome of *Mycobacterium tuberculosis*, and contains relatively high number of pseudogenes, indicative of reductive evolution (Singh and Cole, 2011). Different strains show high sequence similarity, with only a small number of variations in the form of indels (short insertions or deletions) and single nucleotide polymorphisms (SNPs) (Monot et al., 2009). The SNP variations were initially used to divide modern isolates into four main types and 16 subtypes called 1A–1D, 2E–2H, 3I–3M and 4N–4P. With the addition of more sequences, this classification has become elaborated into a phylogenetic scheme comprising six main branches, with branches 1 and 2 corresponding to types 1 and 2, respectively, branch 3 to subtype 3I, branch 4 to the type 4 strains and also subtypes 3L and 3M, and branches 5 and 0 to different variants of subtype 3K (Schuenemann et al., 2013, 2018). Among modern isolates, variants display geographical partitioning with branch 1 associated with South and East Asia, branch 2 with south and Southwest Asia, branch 3 with Central and North America, branch 4 with West Africa and South America, and branches 5 and 0 with East Asia (Monot et al., 2009; Schuenemann et al., 2013). However, these present-day distributions do not reflect the full complexity of *M. leprae* distribution in

the past, especially in mediaeval Europe where subtypes within branches 2, 3, 4 and 0 have been identified in skeletons dating from the 5th–14th centuries AD (Singh and Cole, 2011).

Although *M. leprae* aDNA has been reported from a number of British sites (reviewed by Donoghue et al., 2017), sufficient data for subtype identification has only been obtained from six skeletons from the St Mary Magdalen *leprosarium* in Winchester (Schuenemann et al., 2013; Taylor et al., 2013; Mendum et al., 2014; Roffey et al., 2017) and one skeleton from a cemetery in Great Chesterford, Essex (Schuenemann et al., 2018). Three of the Winchester skeletons yielded subtype 3I and the other three, as well as the Great Chesterford sample, were subtype 2F. To extend the geographical range of our knowledge of ancient *M. leprae* subtypes in Britain, we carried out a biomolecular examination of six skeletons from two sites from mediaeval England (Fig. 1), each displaying pathological lesions indicative of leprosy though with varying degrees of ambiguity. We report *M. leprae* genome sequences for three of these skeletons. Two of the genomes correspond to subtype 3I, previously known in Britain, but the third is novel to Britain and highlights the role that individual mobility might have played in adding complexity to the phylogeography of *M. leprae* in mediaeval Europe.

Fig. 1. Locations of the sites from which skeletal samples were obtained.

2. Material and methods

2.1 Skeletons

Samples were selected, with permission, from the collection of the Biological Anthropological Research Centre, University of Bradford, UK, based on various criteria. First, skeletons that clearly show pathological rhinomaxillary changes indicative of leprosy were identified. Given that these changes are pathognomonic for lepromatous leprosy, bilateral and symmetrical non-specific changes in the lower limbs and feet of those skeletons are hypothesized to be associated with the disease as

well. Additionally, skeletons were sought that showed non-specific lesions in the lower limbs and feet as commonly seen in leprosy but where no rhinomaxillary alterations could be recorded, either because they were not present or because they could not be observed due to the state of preservation of the skeleton. The distribution of these lesions made the differential diagnosis of leprosy for these skeletons likely. The decision about which skeletal element and, in case of bilateral skeletal involvement, which side of the body would be sampled, was based on whether or not destruction was justifiable given the importance of the specimens for future studies.

Based on these criteria, samples were taken from six skeletons from two sites (Table 1, Supplementary Note). Skeletons C21, C35, C48 and C227 were excavated in 1989 from a cemetery that had belonged to the Hospital of St James and St Mary Magdalene, Chichester, UK. The original hospital was founded c.1118 AD and housed leprosy sufferers until being dissolved in 1442 AD and converted to an almshouse when the prevalence of leprosy declined in the UK (Magilton et al., 2008). Based on osteological data, C21 was a young adult male, C35 an adult of indeterminate sex, C48 a mature adult male, and C227 an adult, probable male. All four skeletons were dated to the 14th–17th century AD, based on historical documents and associated pottery (Magilton et al., 2008). Skeletons R5046 and R5256 were males of 17–25 and 25–35 years at age of death, respectively, from the late Anglo-Saxon churchyard cemetery at Raunds Furnells, Northamptonshire, UK, excavated during 1977–1985. Stratigraphical analyses and radiocarbon dating suggested that the churchyard cemetery was in use from the mid-10th until mid-12th centuries (Boddington, 1996).

Table 1
Details of skeletons and samples that were taken.

Site	Skeleton	Sex, age at death	Leprosy indicators[a]			Elements sampled[a]
			Rhinomaxillary changes	Sub-periosteal new bone formation	Other changes	
Chichester	C21	Young adult male	Yes	Yes	Yes	Tibia, metatarsus
	C35	Adult, indeterminate sex	No	Yes	Yes	Tibia
	C48	Mature adult	Yes	Yes	Yes	Tibia, fibula
	C227	Male(?) adult	Yes	Yes	Yes	Calcaneus, phalanx
Raunds	R5046	Male, 17–25 years	Yes	Yes	Yes	Fibula
	R5256	Male 25–35 years	No	Yes	Yes	Tibia, fibula, new bone formation

[a] For details of lesions and elements sampled, see Supplementary Note (summary of archaeological sites, pathological lesions of skeletons, and elements that were sampled) and Supplementary Table 1 (detailed osteological report).

Skeletons C21, C48 and C227 from Chichester and the Raunds skeleton R5046 displayed rhinomaxillary changes and other lesions indicative of leprosy (Table 1, Supplementary Note, Supplementary Table 1). The fourth Chichester skeleton, C35, did not show the typical rhinomaxillary

changes (the viscerocranium was absent) but had infective destruction of the talonavicular and tibiotalar joints, an infection of the right calcaneus indicated by the presence of a draining sinus, dorsal tarsal bars and sub-periosteal new bone formation on the tibiae, fibulae and feet. Individual R5256 from Raunds also did not show the typical rhinomaxillary changes but had extensive sub-periosteal new bone formation on other skeletal elements. Although initially reported as a case of possible leprosy (Powell, 1996: 123), this pattern of bone formation is not specific to leprosy, and a non-leprous diagnosis has been suggested (Craig and Buckberry, 2010).

2.2 Ancient DNA regime

DNA extractions, PCRs and Illumina library preparations were performed in two physically separated laboratories within the specialized ancient DNA research facility at the University of Manchester. Each laboratory was supplied with ultra-filtered air under positive displacement. After each use, benches and equipment were decontaminated by UV irradiation and by cleaning with 5% hypochlorite acid, 70% ethanol and DNA Away (Molecular Bioproducts). Small equipment, plasticware and UV-stable reagents were decontaminated by UV irradiation (254 nm, 120,000 mJ cm^{-2} for 2×5 min, with 180° rotation between the two exposures) before use. Aqueous solutions were similarly irradiated for 15 min. Personnel wore a disposable forensic suit, face mask, hair net, goggles, two layers of gloves and disposable shoe covers at all times. DNA extractions were accompanied by two blanks (normal extraction but without skeletal material) per five samples and every set of 5–7 PCRs was accompanied by at least two blanks (set up with water rather than DNA extract).

2.3 DNA extraction, PCR and sequencing

Bone samples were taken using a hacksaw or electronic drill by personnel wearing protective clothing, including forensic suits, hair nets, face masks and two pairs of sterile gloves. Samples were placed in sterile plastic bags and stored under dry and cool conditions and transferred to the ancient DNA facility. The bone surfaces were decontaminated by mechanical removal of the outer 1–2 mm of each sample, followed by UV irradiation (254 nm, 120,000 mJ cm^{-2}) for 2×5 min, with 180° rotation between the two exposures (Bouwman et al., 2006). Bone samples were then placed in a DNA-free plastic bag wrapped in a sterile piece of aluminium foil and crushed into fine powder. DNA was extracted from 0.2 g of bone powder by standard methods (method D of Bouwman and Brown, 2002; Dabney et al., 2013).

An initial screening for presence of *M. leprae* DNA was carried out by hemi-nested PCR directed at the RLEP repetitive element, in 50 µl reactions comprising 2.5 µl of DNA extract or 1.0 µl of first round PCR product, 1× AmpliTaq Gold PCR Master Mix (ThermoFisher Scientific), 2 mM $MgCl_2$, 200 µM dNTPs, 200 ng each primer, 1% bovine serum albumin and 1.25 units AmpliTaq Gold DNA polymerase (ThermoFisher Scientific). The primers for the first PCR (forward: 5´–CACCTGATGTTATCCCTTGC–3´; reverse: 5´–ATCATCGATGCACTGTTCAC–3) amplified a 133 bp fragment, and the second PCR (forward: 5´– CATTTCTGCCGCTGGTATC –3´; reverse as for first PCR) amplified a 111 bp fragment. Cycling conditions were 7 min at 95°C, followed by 35 cycles each consisting of 1 min at 56°C, 1 min at 72°C, 1 min at 94°C, and a final cycle at 56°C for 1 min and

72°C for 10 min. PCR products were analysed by agarose gel electrophoresis and directly purified using the QIAquick PCR product purification kit (Qiagen) prior to Sanger sequencing (GATC Biotech, Cologne).

Dual-indexed libraries for Illumina sequencing were prepared from positive samples. No DNA fragmentation step was performed as ancient DNA is already highly degraded. Library preparation included a blunt-end repair step but no A-tailing, followed by purification using the MinElute PCR purification kit (Qiagen), with elution in 20 µl. Subsequent adapter ligation was performed using p5 and p7 adapters at a concentration of 0.2 µM (Meyer and Kircher, 2010). Nicks from the previous step were filled in with Bst polymerase before quantification by qPCR (Roche LightCycler 480) and fluorimetry (Qubit 2.0) to determine the number of cycles required for the subsequent indexing PCR. Sample-specific barcodes were added by double-indexing (Kircher et al., 2012), using KAPA HiFi Uracil+ (Kapa Biosystems). Samples were then pooled in equimolar ratios and sequenced from both ends in a single flow cell (Illumina HiSeq 4000). As well as shotgun sequencing, samples were also sequenced after enrichment by in-solution hybridization capture (MYcroarray) according to the manufacturer's instructions for degraded samples. RNA baits were transcribed from 80-mer oligonucleotides complementary to the *M. leprae* TN genome to give an array with 2× tiling density. Sequence data are curated at the European Nucleotide Archive under study accession number PRJEB31393.

2.4 Data analysis

Raw sequencing data were pre-processed with AdapterRemoval 2.1 (Schubert et al., 2016) to remove adapter sequence remnants, trim low quality bases and merge paired-end reads. Reads of at least 25 bp which formed pairs with at least 11 bp overlap, and non-overlapping pair mates of >25 bp, were retained in separate files. The paired-end reads were then mapped to the *M. leprae* TN genome with BWA 0.7.12 (Li and Durbin, 2009). The alignments were cleaned by soft clipping, sorted based on coordinate with Picard Tools (http://broadinstitute.github.io/picard), and mapped reads with a quality score of at least 20 extracted using SAMtools 0.1.19 (Li et al., 2009). Read duplicates were removed using the MarkDuplicates option in Picard Tools. The mapped reads with duplicates removed were converted to Fasta files and tested by BLAST (Altschul et al., 1990) with the outputs visualised using MEGAN 6 (Huson et al., 2016). Base quality score recalibration was performed with GATK 3.6 (McKenna et al., 2010) using the non-human genome method. The recalibrated alignments containing the reads that mapped to *M. leprae* and were verified by BLAST were visualized using Geneious 8.1.9 (Kearse et al., 2012). Polymorphisms were considered genuine if supported by at least 5× coverage and a variant frequency of at least 80%.

3. Results

Samples (Table 1) were screened for the presence of *M. leprae* DNA by hemi-nested polymerase chain reactions (PCRs) directed at the multicopy RLEP element, which is believed to be specific to this species (Braet et al., 2018) and has previously been used for detection of *M. leprae* ancient DNA (Donoghue et al., 2017). The first-round PCRs provided products of the correct size, as judged by

agarose gel electrophoresis, for the two samples (tibia and metatarsus) taken from skeleton C21, the two samples (tibia and fibula) from C48, and the single sample (fibula) from R5046 (Table 2). The second-round PCRs provided bands of the expected sizes from the same samples, and no others. The results were replicated with a second set of PCRs on the same extracts. None of negative controls (extraction blanks and PCR blanks) revealed amplification products. Direct sequencing of the PCR products verified their identity as genuine RLEP amplicons.

Table 2

Results of RLEP PCRs.

Skeleton	Sampled element	PCR results[a]
C 21	Tibia	+,+
	Metatarsus	+,+
C 35	Tibia	–,–
C 48	Tibia	+,+
	Fibula	+,+
C227	Calcaneus	–,–
	Phalanx	–,–
R5046	Fibula	+,+
R5256	Tibia	–,–
	Fibula	–,–
	New bone formation	–,–

[a] Result of first hemi-nested PCR, result of second hemi-nested PCR.

Samples from the three positive skeletons – C21, C48 and R5046 – were further studied by Illumina sequencing. Shotgun sequencing was attempted for all three samples but less than 0.001% of the reads mapped to the *M. leprae* TN genome, which was insufficient for genome analysis. In-solution hybridization capture was therefore used to enrich the samples for *M. leprae* sequences. Enrichment dramatically increased the numbers of reads mapping to the reference genome (Supplementary Table 2), with >70% of the genome covered for each of the samples and a mean read depth of 4–10×. The data enabled the ancient strains to be assigned to *M. leprae* genotypes (Table 3) (Monot et al., 2009), revealing that C21 and C48 belong to subtype 3I and R5046 to subtype 3K.

Table 3

Genotype assignments.

Skeleton	SNP position[a]	Type	SNP position[a]	Subtype

	14,676	1,642,875	2,935,685		413,902	1,133,492	2,312,059	3,267,975	
C21	C	T	C	3	G	T	C	G	I
C48	C	T	C	3	G	T	C	G	I
R5046	C	T	C	3	G	G	G	G	K

[a] SNP positions according to the *M. leprae* TN genome sequence.

Comparisons between different modern strains of *M. leprae* have revealed a total of 215 polymorphic sites (Monot et al., 2009). These sites were examined in the ancient *M. leprae* genomes to determine whether the SNP version that was present was the same as in the *M. leprae* TN reference sequence, or was the alternative SNP version present in some other modern genomes (Supplementary Table 3). Of the three ancient genomes, R5046 was the most greatly diverged from *M. leprae* TN, with 119 of the 183 SNPs (65.0%) that were covered by the ancient sequence displaying the version not present in the reference genome. In comparison, 53.1% and 56.1% of the SNPs covered in the C21 and C48 genomes, respectively, had the non-reference version. The greater dissimilarity between R5046 and *M. leprae* TN reflects the greater phylogenetic distance between subtype 3K and subtype 1A, to which TN belongs (Schuenemann et al., 2018). An additional 41 sites, comprising 30 SNPs and 11 indels, were specific to the three ancient genomes reported here (Supplementary Table 4). Of the 30 SNPs, 18 were present only in the R5046 genome, and seven and five were unique to C21 and C48, respectively. None of the 30 SNPs were present in all three ancient genomes. Of the eleven indels, five were specific to R5046 and six were present in all three samples, four of the latter in pseudogenes.

4. Discussion

We report the results of analyses of seven skeletons from two mediaeval sites in England, each of the skeletons displaying osteological indicators of leprosy, though with different degrees of ambiguity. We identified *M. leprae* DNA in three skeletons and following enrichment by in-solution hybridization obtained sufficient sequence data to assign skeletons C21 and C48 to subtype 3I and skeleton R5046 to subtype 3K. Although C21 and C48 came from the same cemetery, and were curated together for 25 years prior to DNA analysis, we can be confident that both contain endogenous *M. leprae* DNA (as opposed to cross-contamination between the skeletons or contamination from a single external source) as their *M. leprae* genome sequences are non-identical.

Each of the three skeletons that produced positive results had extensive osteological indications of leprosy, including rhinomaxillary changes, sub-periosteal new bone formation, and other characteristic lesions on various skeletal elements. Of the samples that produced negative results, C227 had a pathological condition most likely indicative of leprosy, in particular pencilling of the fifth metatarsal with complete resorption of the head and distal part of the diaphysis in the right foot as well as a slight pitting of the palate. However, this skeleton displayed relatively poor physical preservation, indicating that the failure to detect *M. leprae* DNA was possibly due to biomolecular degradation before the skeleton was excavated. Skeletons C35 and R5256 did not display rhinomaxillary changes,

weakening the diagnosis of leprosy in both cases. The pathology of the feet of skeleton C35 did support a diagnosis of leprosy. R5256 had lesions characteristic of leprosy, but displayed new bone formation on the *ossa coxae* and left scapula, skeletal elements that are not usually involved in leprosy infection, possibly suggesting a systemic condition other than leprosy. Overall, the results confirm those of other groups (Rafi et al., 1994; Taylor et al, 2000, 2006, 2013; Donoghue et al., 2001, 2005, 2015; Inskip et al., 2015) by emphasising the value of ancient DNA analysis as a means of providing independent support to palaeopathological identifications for leprosy.

M. leprae strains previously reported from mediaeval Britain and Ireland have been assigned to subtypes 2F and 3I (Taylor et al., 2013, 2018; Schuenemann et al., 2013, 2018; Mendum et al., 2014), the latter corresponding to branch 3 in the more recent phylogenetic classification (Schuenemann et al., 2018). The discovery of subtype 3I in two skeletons from Chichester, dating to the 14th–18th centuries AD, is therefore consistent with the results of these previous studies. Subtype 3K, however, has not previously been reported in Britain. In modern *M. leprae*, this subtype is associated with East Asia, in particular Japan, China, the Philippines and New Caledonia (Schuenemann et al., 2018). Among ancient specimens it has been detected in a Turkish skeleton from the 8th–9th centuries AD (Erdal, 2004), three skeletons from Hungary, from the 7th–10th centuries AD (Pálfi et al., 2002; Molnár et al., 2006; Schuenemann et al., 2018), and another from 11th–13th century AD Denmark (Schuenemann et al., 2018). The R5046 skeleton is from a similar period (10th to mid-12th centuries AD) as are these other European detections, but is the most westerly in location, and hence the most distant from the modern distribution of the subtype. The distribution pattern raises the intriguing possibility that the individual represented by skeleton R5046 did not contract leprosy in Britain but instead had travelled to continental Europe and/or Asia and contracted the disease there. It has previously been suggested that human mobility along the Silk Route was responsible for bringing subtype 3K to Eastern Europe from its supposed centre of origin in East Asia (Monot et al., 2009). During the Anglo-Saxon period, up until the 10th century AD, there was also extensive travel between Britain and continental Europe, especially of educated clerics who taught and held religious positions in various European countries (Palmer, 2009). One of the routes taken by travellers to reach the Holy Land from West and Central Europe began in Vienna and passed along the Danube and the *Via Diagonalis* to Constantinople, traversing Hungary, Serbia and Bulgaria (The Way to Jerusalem, 2018). It is therefore possible that leprosy of subtype 3K was transmitted to Britain and other parts of Western Europe by people who had travelled to the Holy Land and back via this route.

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