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The Distribution and Origins of Ancient Leprosy

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Abstract

Human leprosy is primarily caused by *Mycobacterium leprae*, but also by the related '*M. lepromatosis*'. Ancient leprosy can be recognised in archaeological materials by the paleopathology associated with multi-bacillary or lepromatous forms of the disease. Whole *M. leprae* genomes have been obtained from human skeletons, and diagnostic aDNA fragments have been recovered. The derived *M. leprae* phylogenies, based on single nucleotide polymorphisms, mirror past human migrations, as *M. leprae* is usually an obligate pathogen. The detection of *M. leprae* in historical leprosy cases is assisted by the hydrophobic *M. leprae* cell envelope, which is composed of unusual lipids that can be used as specific biomarkers. Lipid biomarkers are more stable than aDNA and can be detected directly without amplification. Indigenous human leprosy is extinct in Western Europe, but recently, both *M. leprae* and '*M. lepromatosis*' were found in British red squirrels. Leprosy may also be found in nine-banded armadillos (*Dasypus novemcinctus*) where it can cause a zoonotic human infection. Certain leprosy-like diseases, caused by uncultivable species in cats, for example, may be related to *M. leprae*. The closest extant relatives of leprosy bacilli are probably members of the *M. haemophilum* taxon, emerging pathogens with genomic and lipid biomarker similarities.

Keywords: ancient DNA, lipid biomarkers, genotyping, leprosy, paleopathology, evolution

1. Introduction

Leprosy (Hansen's disease) is a chronic infectious disease that has been recognised over millennia. In the majority of human cases, it is caused by *Mycobacterium leprae*, but recently a

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related organism, '*M. lepromatosis*', has also been implicated [1] and appears to cause diffuse lepromatous leprosy (DLL). Both organisms are obligate pathogens that are uncultivable in cell-free growth media. Although '*M. lepromatosis*' has been the subject of many recent publications [2–5], there is still discussion about whether it is a distinct species [6]; currently, it is a name without standing in nomenclature (http://www.bacterio.net/-nonvalid.html). Leprosy is primarily a disease of peripheral nerves and skin, but it also affects bones. The genomes of *M. leprae* and '*M. lepromatosis*' have been sequenced, and it is clear that they diverged from a common ancestor many millennia ago [7, 8]. The genome of '*M. lepromatosis*' confirms a close but distinct relationship with *M. leprae*, and both organisms can also cause disease in animals, such as armadillos and squirrels [9–12]. The closest ancestors of these leprosy bacilli are probably relatives of *M. haemophilum* that has genomic and lipid biomarker similarities [13–16].

Initially, ancient leprosy was recognised by the paleopathology associated with multi-bacillary or lepromatous forms of the disease [17, 18]. Leprosy causes skeletal changes in the rhinomaxillary area, including pitting and perforation in the palate, resorption of the nasal spine and the maxilla leading to loss of the upper teeth. The tubular bones of the hands and feet are frequently involved. In the tibia and fibula, inflammatory periostitis can be recognised; the metatarsals and metacarpals are often resorbed so these small bones develop a pencil shape. In sub-adult individuals afflicted with multibacillary leprosy, the development of the secondary dentition can be affected, leading to a rare condition, *leprogenic odontodysplasia* (LO), where the incisor teeth exhibit a characteristic root constriction [19]. Intriguingly, this has been seen only in archaeological cases and not in a clinical setting. Cases have been described from medieval Denmark [20] and in four individual medieval inhumations from the St. Mary Magdalen, Winchester leprosarium [21]. Subtle skeletal changes like grooving on the volar surfaces of the proximal phalanges may also accompany paucibacillary forms of leprosy that cause digital contracture or loss of pain sensation [22].

Suspected leprosy cases can be confirmed by the detection of *M. leprae* ancient DNA (aDNA) [23, 24] and further characterised by repetitive DNA sequences and genotyping [25, 26]. The aDNA detection of *M. leprae* in historical cases is probably assisted by the protective presence of unusual lipids in the *M. leprae* cell envelope. These lipids can be used as specific biomarkers; they are more stable than aDNA and can be directly detected without amplification (*vide infra*). Lipid biomarkers have been used to confirm aDNA findings [21, 27–29]. However, due to their stability, lipid biomarkers can also confirm a diagnosis of leprosy initially based on paleopathology, even in the absence of aDNA [30].

2. Causes and distribution of modern leprosy

2.1. Mycobacterium leprae

M. leprae, the main cause of leprosy in humans, is a slow-growing intracellular *Mycobacterium* and the average incubation period of the disease is about 5 years, although symptoms may occur within 1 year or up to 20 years after infection [31]. Leprosy mainly affects the skin, peripheral nerves, the mucosa of the upper respiratory tract and the eyes, as *M. leprae* has a tropism for

Schwann cells in nerves and macrophages in the skin [32]. The infection is transmitted by direct contact with untreated cases or healthy carriers or via infectious aerosols [33]. The clinical presentation of leprosy depends upon the cell-mediated immune (CMI) response to infection. If the host has an effective CMI response, few lesions develop, and there are only scanty bacilli in the tissues. However, some patients are anergic to M. leprae, so develop lepromatous leprosy with ineffective antibodies, a high bacterial load and multiple lesions. The clinical presentation of leprosy in a patient can vary over time, so there are borderline leprosy types where the immune response is unstable. It can show a wide range of clinical presentations from tuberculoid leprosy (TT) through borderline forms: borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) to lepromatous leprosy (LL) [34]. A recent World Health Organization classification scheme recognises a simplified two-category system of either paucibacillary or multibacillary forms of leprosy [35]. The histopathology of skin lesions varies from compact granulomas to diffuse infiltration of dermis, which largely depend upon the immune status of the patient and may not be in agreement with the clinical diagnosis [36, 37]. The mycobacterial antigens can activate a chronic inflammatory response that is exacerbated by pro-inflammatory cytokines. Therefore, in late stages of leprosy, there may be no *M. leprae* bacilli in the tissues, but residual mycobacterial antigens can drive an inflammatory response that causes neurological damage [38].

2.2. 'Mycobacterium lepromatosis'

'M. lepromatosis' appears to have a tropism for endothelial cells and can give rise to vasculitis and necrotic erythema. It seems to be less common than M. leprae and was initially believed to be geographically restricted to patients from Mexico and the Caribbean, where it was identified in patients suffering from diffuse lepromatous leprosy (DLL) [1, 39-41]. It was subsequently recognised in Brazil, Myanmar, Canada and Singapore and in mixed infections with M. leprae [3, 4]. Symptoms, characteristic of 'Lucio's phenomenon', have been associated with 'M. lepromatosis' [1, 40, 42]. A case of two Mexican siblings infected with 'M. lepromatosis' indicates facile transmission [5, 6]. However, 'M. lepromatosis' has recently been found in the wild Eurasian red squirrel, Sciurus vulgaris, in the British Isles, from England, Scotland and Wales [11, 12]. In addition, M. leprae was found in red squirrels on the Isle of Wight and Brownsea Island, close to the south coast of England [43, 44]. This was very surprising, as although indigenous leprosy was prevalent in the human population of the British Isles in the first millennium (CE), it is now believed to be extinct. In these modern squirrels, the macroscopic signs and histopathology were characteristic of lepromatous leprosy, but no pathological differences were noted between infections caused by 'M. lepromatosis' or M. leprae [12, 45]. The strain of 'M. lepromatosis' in British wild squirrels is genetically distinguishable from Mexican strains found in modern day humans, and it appears that these strains diverged from a common ancestor about 26,000 years ago [12]. However, the M. leprae strain found in British red squirrels is similar to a strain found in human remains from a mediaeval leprosy hospital in Winchester [46], only 70 km from the Isle of Wight and Brownsea Island. One suggestion is that, in the past, humans may have been infected through direct contact with red squirrels as these were prized for their meat and fur [12]. They were also kept as pets, as is evident from various illustrated medieval manuscripts and art, for example 'A Lady with a Squirrel and a Starling' by Holbein the Younger (painted ca. 1526–1528, National Portrait Gallery, London).

2.3. Nature and distribution of M. leprae genotypes

Major collaborative studies based on single nucleotide polymorphism (SNP) typing have established that modern *M. leprae* consists of four distinct genotypes that are associated with different human populations [47]. It is believed that the ancestral precursor of *M. leprae* experienced an evolutionary bottleneck and thereafter developed independently in different human populations [26, 48]. In Europe, indigenous leprosy is now largely extinct, so a further study also looked at *M. leprae* from archaeological cases using aDNA methods [26]. This identified SNP type 3 cases from various European countries for the first time, including Denmark, Hungary, Croatia, Turkey and Britain. Some cases provided subtypes I, M or K. Genotype 3 strains were also found from Roman Egypt and by others in medieval Central Europe [30, 49]. Later studies also reported SNP type 2 strains for the first time in medieval cases from Winchester, UK [21] and from Sweden [50, 51]. Archaeological remains from Japan yielded a SNP type 1 from that country [52]. Several of the robust cases were subsequently amplified by whole genome sequencing (WGS) [46, 53].

Monot et al. [26] also recognised sub-genotypes from extant cases, thereby enabling more precise associations between *M. leprae*, geographical location and present human populations ranging from China [54] to South America [55]. In a detailed study of modern M. leprae that included SNP typing, variable-number-tandem-repeat (VNTR) analysis and WGS, Truman et al. [9] examined 50 patients with leprosy and 33 wild armadillos (Dasypus novemcinctus) in the United States, together with reference strains from other parts of the world. Seven M. leprae SNP types were detected. The SNP type for some patients with possible exposure by foreign residence was typical of *M. leprae* from foreign locations. The most abundant SNP type was 3I that is generally associated with historical northwest European or American populations. The SNP sub-type 3I-1 strains, with one copy of an 11-bp indel (indel_17915) had ancestral bases, but all other *M. leprae* strains have two copies. Type 3I-2 strains, a development of the ancestral 3I-1 strains, similarly have only one copy of indel ML_17915 and can be identified by base C at position 1527056 instead of base G present in type 3I-1 isolates [9]. These 3I-2 strains were found in all armadillos and most of the indigenous patients so the authors concluded that armadillos act as a reservoir for *M. leprae* and that there is zoonotic spread of leprosy in the Southern United States. As the disease was not present in the New World before European contact, it is assumed that the spread of the disease was linked to human migrations and that armadillos acquired leprosy from human cases [45, 56].

2.4. Transmission of leprosy

Recently it was realised that the enhanced hydrophobicity of tubercle bacilli is a key factor in aerosol transmission [57, 58]. Since it is becoming established that aerosol transmission is a prime mode for the spread of leprosy bacilli [33, 59], the transmissibility of the different manifestations of *M. leprae* should be considered. In a detailed study [33], it was demonstrated that MB/LL cases provided more transmissible bacilli than PB/TT patients. It would be of great interest to compare the relative cell envelope surface lipid composition of LL and TT leprosy bacilli to explore the possibility that the hydrophobicity of LL forms is enhanced or otherwise. It may also be possible to determine directly the relative hydrophobicity of *M. leprae* in biopsy

material, using micro fluorescence methods [60]. The evasion of airways epithelial clearance [33, 59] may be encouraged by enhanced hydrophobicity of infective agents.

3. Recognition, diagnosis and spread of ancient leprosy

3.1. Pathology and recognition of ancient leprosy

Leprosy is primarily a disease of the peripheral nervous system. In the past, the disease would run its natural course, resulting in both specific and nonspecific bony changes plus paleopathology due to secondary infections following nerve damage [17, 18, 61]. Ancient leprosy is typically recognised by the presentation known as *facies leprosa* or rhinomaxillary syndrome, in which the nasopharynx is remodelled, the nasal spine and palate are resorbed, and eventually also the maxilla, leading to loss of the upper teeth. There are changes to the tubular bones of the hands and feet including osteoporosis caused by disuse, pitting and perforation. The long bones of the lower leg also show paleopathology associated with inflammatory periostitis [30, 62–64].

M. leprae ancient DNA (aDNA) was first detected in skeletal remains with typical leprosy paleopathology soon after the introduction of PCR [23]. Subsequently, many further paleopathological cases of leprosy were confirmed by *M. leprae* aDNA from across Europe and the Middle East [24–27, 30, 49–51, 64–69]. Specific *M. leprae* short DNA sequences were targeted as ancient DNA (aDNA) becomes highly fragmented over time [70]. *M. leprae* aDNA amplification has confirmed leprosy and enabled genotyping of isolates from Europe, Byzantine Turkey and Roman Egypt (**Table 1**). As additional methodologies were developed, different *M. leprae* strains were distinguished by microsatellite analysis based on aDNA repetitive sequences [27, 71] and now whole *M. leprae* genomes have been obtained from historical human skeletons [46, 53]. The results of aDNA amplification studies, WGS and lipid biomarker detection are summarised in **Table 1**.

3.2. The potential of lipid biomarkers

The detection of *M. leprae* in historical leprosy cases is assisted by the *M. leprae* cell envelope, which is composed of unusual lipids some of which can be used as specific biomarkers (**Figures 1–3**). The mycolic acids of *M. leprae* are restricted to homologous α - and ketomycolates [79, 80], whose major components are shown in **Figure 1**.

Characteristic mycocerosic acids are components of both phthiocerol dimycocerosate waxes (PDIMs) (**Figure 2**) [81–83] and so-called phenolic glycolipids (PGLs) (**Figure 3**) [82–85]. *M. leprae* mycocerosates unusually include major amounts of a C_{34} component, accompanied by small proportions of a C_{33} acid (**Figure 2**). *M. haemophilum* produces a PGL with the same two internal sugars (3-O-Me-rhamnose and 2,3-di-O-Me-rhamnose), but in reversed order and with different linkages (**Figure 3**). Besra et al. [13] concluded that this mycocerosate profile was essentially the same, thereby revealing a close phylogenetic link between *M. leprae* and *M. haemophilum* for the first time.

	24.1			NT . 1	
Century (CE); location: cases	M. leprae		M. leprae genotype	Notes	Publications
	DNA	Lipids			
1st; Israel, Akeldema, Himmon valley: SC1	+				Matheson et al. [69]
1st–4th; Uzbekistan Devkesken 6: 5b	+	+	3L		Taylor et al. [27]
4th; Egypt, Dakhleh Oasis, Kellis 2: B116 and 7 other samples			3K/L/M (B116)		Donoghue et al. [72]; Monot et al. [26]
4th–7th; Israel, Jerusalem: HZ	+				Spigelman and Donoghue [67]
5th–6th; United Kingdom, Great Chesterfield: GC96	+	+	3I-1 (variant)	MTB –	Inskip et al. [73]
6th–7th; Israel, monastery on River Jordan: AR	+				Rafi et al. [23]
6th–8th; Italy, Morrione: T68, T108	1/2 + (T108)				Donoghue et al. [30]
7th; Hungary, Szeged- Kiskundorozsma- Daruhalom dűlő II: KD271, KD517, KD518	3/3+	KD517+	3K (KD271)	KD517 lipids+ and MTB+	Minnikin et al. [29]; Lee et al. [28]; Donoghue et al. [30]
7th; Italy, Vicenne: T18, T31, T144	1/3+ (T18)	2/2+ (T18, T144)		DNA- lipids+ (T144)	Donoghue et al. [30]
7th–8th; Hungary, Szentes-Kistőke: SK11	+				Donoghue et al. [30]
7th–9th; Hungary, Bélmegyer-Csömöki domb: 22	+	+		MTB lipid+	Donoghue et al. [30]; Molnár et al. [74]
7th–9th; Hungary, Szarvas Grexa, Téglagyár: SG-38		ĊŃ			Minnikin et al. [29]; Donoghue et al. [30]
8th–9th; Turkey, Kovuklukaya: 9/1, 11/2, 20/1, 24/1	3/4+ (11/2–)	1/3+ (24/1+)	3K (20/1)		Minnikin et al. [29]; Donoghue et al. [30]
8th–9th; Croatia, Radasinovci: 2A, 3A	+				Watson et al. [49]
8th–9th; Austria, Zwölfaxing: 70, 88	2/2+			MTB DNA+ (88)	Donoghue et al. [30]
9th–10th; Czech Republic, Prušánky: 188	+		3M		Donoghue et al. [30]

Century (CE); location: cases	M. leprae		<i>M. leprae</i> genotype	Notes	Publications
	DNA	Lipids			
10th; Hungary, Hajdúdorog-Gyúlás: HG-56	+	+			Minnikin et al. [29]; Donoghue et al. [30]
10th; Hungary, Sárrétudvari-Hízóföld: S237 10th–11th; UK, Norwich: 11287, 11503, 11784			3	Palate+ Toe-	Haas et al. [65] Watson et al. [49]
10th–11th; Hungary, Püspökladány- Eperjesvölgy 11, 222, 429, 503	+	+ (503)	3K (222) 3M (503)	222 and 503 MTB+	Donoghue et al. [30]
10th–12th; UK, Wharram Percy: G708	+		3		Taylor and Donoghue [71]
11th; Sweden, Björned: A4	+	+		MTB+	Donoghue et al. [72]; Minnikin et al. [29]
10th–12th; Sweden, Sigtuna: 10, 32H, 3077, 3092V, 3093F, 3159Hsin, 3320V, 3401H, F13320, S10V3	7/10+		2F (3092 and 3077) 3I (3093)	WGS	Economou et al. [50, 51]; Schuenemann et al. [46]
11th; Hungary, Felgyő, Kettőshalmi-dűlő: 2467, 3658	1/2+			3658+	Donoghue et al. [30]
11th; Hungary, Lászlófalva-Szentkirály: 79	+			MTB+	Donoghue et al. [30]
11th–12th; UK, Orkney: CC4	+				Taylor et al. [66]
9th–13th; UK, Winchester: Sk2, Sk7, Sk19 Sk8, Sk14, Sk27 Sk18			3I-1 2F	WGS Sk18 (weak)	Schuenemann et al. [46]; Taylor et al. [21] Roffey <i>et al.</i> 2017 [75]
11th–14th: Denmark.	1/5+	+	2F (Refshale16)	Refshale	Schuenemann et al.
Refshale:2, 16, 26, 32, 36	1 -		()	16+	[46]
12th; Spain, Seville: A43, A120	+				Montiel et al. [76]
12th; Czech Republic, Žatec: AO9611, AO9731	+				Likovsky et al. [77]
12th–14th; Poland, Suraz: A1	+				Donoghue et al. [70]; Witas et al. [78]



Table 1. Detection of ancient leprosy using aDNA and lipid biomarkers.

M. leprae mycolic acids



Figure 1. Mycolic acids of *M. leprae*. The main $C_{78} \alpha$ -mycolate and C_{83} ketomycolate are shown; additional homologous components are also present.

The lipid composition of '*M. lepromatosis*' remains to be determined, but limited information is available for *M. haemophilum*. In addition to α - and ketomycolates, *M. haemophilum* appears to have methoxymycolates, on thin-layer chromatography of extracts [86], but in a previous study, the patterns were unclear with material being degraded by acid methanolysis [87]. A gas chromatographic profile of *M. haemophilum* fatty acids [86] displayed an essentially typical mycobacterial profile, including tuberculostearic acid. The analysis was not extended to search for the unusual mycocerosic acids found previously in *M. haemophilum* (**Figure 2**) [13]. The only novel component was an incompletely characterised monounsaturated 2-methylbranched C₂₅ fatty acid and an enhanced proportion of C₂₂ docosanoic acid was noted as being



Phthiocerol dimycocerosates (PDIMs)

Figure 2. Phthiocerol dimycocerosates (PDIMs) of *M. leprae*. The C_{33} and C_{34} mycocerosates are diagnostic components for *M. leprae* and *M. haemophilum*, but C_{29} , C_{30} and C_{32} acids are shared with members of the *M. tuberculosis* complex [13, 81, 82].

similar to that found in *M. leprae* in a previous study [88]. However, an analysis of three *M. leprae* isolates did not record unusually enhanced proportions of docosanoic acid [80], nor did an additional analysis of *M. haemophilum* fatty acids [89]. It is interesting to compare the profile of uncharacterised fatty acids from *M. haemophilum* in an older study [87] with the more recent study [86]. An unusual large peak, labelled 19A, in the first analysis [87] could possibly correspond to the minor branched C_{25} acid in the later analysis [86]. This unusual C_{25} acid is a potentially valuable biomarker for *M. haemophilum* so its structure and cellular location should be investigated.

The biomarker potential of *M. leprae* lipids has been harnessed by fluorescence high performance liquid chromatography (HPLC) of pyrenebutyric acid (PBA) esters of mycolic acid pentafluorobenzyl (PFB) esters [90] and negative-ion chemical-ionisation gas-chromatography mass-spectrometry (NI-CI GC-MS) of mycocerosate PFB esters [91, 92]. Mycolate HPLC is exemplified in **Figure 4** for standard *M. leprae* and an extract of a skeleton (Sk2) from a mediaeval leprosy hospital near Winchester, UK [21]. Fluorescent mycolate derivatives are recognised by reverse-phase HPLC (**Figure 4A**), collected and analysed by normal phase HPLC to separate the α - and ketomycolate classes (**Figure 4B**). Reverse-phase HPLC provides the size and overall composition of the α -mycolates (**Figure 4C**) and ketomycolates (**Figure 4D**) for comparison with standard *M. leprae*.



Glycosyl phenolphthiocerol dimycocerosates (phenolic glycolipids, PGLs)

Figure 3. Phenolic glycolipids of *M. leprae* and *M. haemophilum*. The common phenolphthiocerol unit is attached to distinctive trisaccharides that share particular diagnostic sugars, 3-O-Me-rhamnose and 2,3-di-O-Me-rhamnose [13].

Selected ion monitoring NI-CI GC-MS analyses of mycocerosate PFB esters from Winchester skeleton Sk2 [21] and standard *M. leprae* are shown in **Figure 5**. There is good correspondence between the Sk2 extract and the standard; the Sk2 profile is unpublished work (O.Y-C. Lee, H.H.T. Wu, G.M. Taylor, K. Tucker, R. Butler, S. Roffey, P. Marter, D.E. Minnikin, G.S. Besra, G.R. Stewart, manuscript in preparation). In summary (**Table 1**), aDNA analysis with occasional lipid biomarker support has been successful in characterising ancient leprosy [21, 27, 29].

3.3. Distribution and phylogeny of ancient leprosy

Further, aDNA studies based on *M. leprae* sub-genotypes have given valuable information about the distribution of the disease in different human populations in the past [26]. The earliest known case of leprosy recognised by both skeletal paleopathology and aDNA, was from the early first millennium CE from the Ustyurt Plateau, Uzbekistan [93], with radiocarbon dating that suggests a date between the first and third centuries CE [94]. The *M. leprae*



Figure 4. Mycolic acid profiles of Winchester skeleton Sk2. (A) Total mycolates, reverse phase HPLC; (B) collected total mycolates (MAs), normal phase; (C) collected α -mycolates, reverse phase; (D) Collected ketomycolates, reverse phase [21].

aDNA from this location was found to be of sub-genotype 3L [27] and the variable number tandem repeat analysis identified a unique aDNA profile [71]. Sub-genotyping has revealed that in historical Europe, there are clear differences between the leprosy found in human



Figure 5. Mycocerosic acid profiles of Winchester skeleton Sk2. Selected ion monitoring NI-CI GC-MS of mycocerosic acid pentafluorobenzyl ester from A, Sk2 and B, *M. leprae* standard.

populations from central and southern Europe, compared with northwest Europe (**Table 1** and **Figure 6**). In Scandinavia and the British Isles, there are examples of *M. leprae* genotypes 2F and 3I [21, 46, 53, 75]. In historical northwest Europe, 3I-1 sub-genotypes were common, but in Hungary, Byzantine Turkey and the Czech Republic, sub-genotypes 3K and 3M were found [30]. It is believed that these differences reflect past human population movements. In northwest Europe, people travelled from Siberia and the Arctic, whereas central Europe was colonised by successive migrations from central Asia *via* ancient routes, such as the so-called Silk Road. WGS of the 3K subtype shows that it belongs to the earliest lineage of extant *M. leprae*, now termed branch 0 [46], and therefore carries characteristics of the most recent common ancestor (MRCA), not found in other groups. The distribution of the various European sub-genotypes is summarised in **Figure 6** and their phylogenetic relationship in **Figure 7**. It would be informative to have more data points for the Mediterranean basin and major countries, such as Spain, France and Germany.



Figure 6. Geographical distribution of ancient leprosy sub-genotypes in the European area. Three type 3 strains are included where sub-typing was not determined.

3.4. Co-infection of leprosy and tuberculosis

Leprosy was a significant problem in Scandinavia until a century ago, leading to the identification of the leprosy bacillus by Hansen in 1871 [95], although publication was delayed due to the inevitable unsuccessful attempts at culture. In Central Europe, however, leprosy was prevalent in the first millennium CE, but a subsequent decline appeared to coincide with the upsurge of tuberculosis. Support for a period of overlap between leprosy and tuberculosis has been provided by a number of clear archaeological examples of dual infection, from first century AD Israel, fourth to fifth century Roman Egypt, seventh to eleventh century Hungary, eighth to ninth century Austria to tenth to thirteenth century Sweden [30, 72]. In one particular case, it was possible to use quantitative lipid biomarker analysis to estimate the relative amount of leprosy and tuberculosis infection [28–30]. Mathematical modelling to explore the epidemiological consequences of dual infection concluded that the disappearance of leprosy could indeed be explained by *M. leprae/M. tuberculosis* co-infections [96]. This may explain the present absence of indigenous human leprosy in Europe. Currently characterised *M. leprae/M. tuberculosis* co-infections are summarised in **Table 2**.



Figure 7. A phylogeny of selected *M. leprae* strains. The phylogeny was derived from an alignment of genomic SNPs [46]; ancient strains are denoted in bold. Phylogenies were generated in MEGA7 [105], using Maximum Likelihood methods. Phylogenies based on Neighbour Joining methods generated similar dendrograms. The scale represents the number of substitutions per site. Bootstrap values were determined from 500 replicates. '*M. lepromatosis*' was used as an out-group (not shown). Subtypes are indicated in brackets.

Authors	Year	Region	Century (CE)	Methods and comments	
Nuorala et al. [97]	2004	Sweden	10th-13th	PCR: <u>ML</u> RLEP 129/99 bp; <u>MTB</u> 123 bp/92 bp. Nested products sequenced	
Donoghue et al. [72]	2005	Egypt	4th–5th	PCR : <u>ML</u> RLEP 129/99 bp; <u>MTB</u> 123 bp/92 bp	
		Hungary	10th-16th		
		Israel	1st		
		Sweden	10th-13th		
Matheson et al. [69]	2009	Israel	1st	PCR: <u>ML</u> RLEP 129/99 bp; <u>MTB</u> IS6110 123/92 bp	
Minnikin et al. [29]	2011	Hungary	7th	PCR : Not re-tested; Lipids : mycolates and mycocerosates indicate relative disease load for Kiskundorozsma- Daruhalom dűlő II Grave KD517	
Minnikin et al. [29]	2011	Hungary	15th	PCR : Not re-tested; Lipids : MTB methoxymycolates and ML ketomycolates for Szombathely Grave 6	
Molnár et al. [74]	2015	Hungary	7th–9th	PCR: <u>MTB</u> IS6110 123/92 bp; IS1081 113 bp; <u>ML</u> not tested; Lipids : mycolates, mycolipenate and mycocerosates for Bélmegyer-Csömöki domb Grave 22	
Donoghue et al. [30]	2015	Central and Eastern Europe	Various 6th–11th	PCR : <u>ML</u> RLEP 129/99 bp; 111 bp; 80 bp and probe; RepLep 66 bp and probe; SNP typing indicates migratory patterns into Europe. Coinfections suggest role of MTB in decline of European leprosy	

Table 2. aDNA and lipid biomarker detection of ancient M. leprae and M. tuberculosis complex co-infections.

4. Origins and evolution of leprosy

4.1. Genomics of modern leprosy

Whole genome sequencing has revealed large numbers of pseudogenes in both *M. leprae* and '*M. lepromatosis*' [7, 8, 98–100]. These genomic studies revealed that both *M. leprae* and '*M. lepromatosis*' have undergone a reductive evolution in which extensive recombination events have occurred between dispersed repetitive sequences, leading to less than half of their genomes containing functional genes. In a preliminary study [7], it was indicated that the genome of '*M. lepromatosis*' (~3.22 Mb) was 1.6% smaller than that (~3.27 Mb) of *M. leprae* [98, 99]. A comprehensive parallel study gave a similar genome size of ~3.21 for '*M. lepromatosis*' [8]. Functional comparisons revealed that whereas *M. leprae* has a defective *heme* pathway, '*M. lepromatosis*' lacked several genes needed for amino acid synthesis [8]. It is apparent that '*M. lepromatosis*' is the closest known mycobacterial taxon to the established species of *M. leprae rae*. Phylogenetic analysis indicates that '*M. lepromatosis*' and *M. leprae* diverged from a most recent common ancestor (MRCA) about 13.9 million years ago [8].

4.2. Evolutionary origins of leprosy bacilli

The deep origins of mycobacterial disease remain to be clearly defined [3, 47, 98, 99]. In contrast to tuberculosis, which appears to stretch back hundreds of thousands of years [57, 58], the earliest manifestations of human leprosy are found in skeletal remains only about 4000 years old [101]. However, the older participation of animal hosts cannot be ruled out, as it is increasingly evident that Pleistocene megafauna may have had a major involvement in tuberculosis evolution [58]. A possible ancestral organism to the organisms that cause leprosy may have been more like modern M. haemophilum, an emerging pathogen with a variety of possible natural reservoirs. The first significant link identified between M. leprae and M. haemophilum was established a quarter of a century ago in a study of the so-called 'phenolic glycolipids' (PGLs) [13]. As shown in Figure 3, the similarity in the oligosaccharide composition of the PGLs was striking and the mycocerosate profile (Figure 2) almost identical. This early key observation was subsequently reinforced by taxonomic studies that showed a close association of M. leprae and M. haemophilum [14, 15, 102]. Again, in studies comparing M. leprae and 'M. lepromatosis', M. haemophilum was the nearest neighbour [8, 39], as illustrated in Figure 8. The recent determination of a full genome (~4.23 Mb) for M. haemophilum confirmed the close link [16], as shown in Figure 8. M. haemophilum is consistently placed outside of the M. leprae/M. lepromatosis group but between M. leprae and other mycobacteria such as the M. tuberculosis complex. It was suggested that the reductive evolution of M. leprae and 'M. lepromatosis' was not shared with the most recent common ancestor but started after the divergence of M. haemophilum from both taxa [16]. The relatedness of M. haemophilum, M. leprae, 'M. lepromatosis' and related taxa is shown in Figure 8.

4.3. Animal and environmental sources of leprosy ancestors

To assess the involvement of ancient relatives of *M. haemophilum* in the evolution of leprosy bacilli, it is necessary to consider the ecological, environmental and animal host preferences of



Figure 8. A phylogeny of *M. leprae* strains and other mycobacterial species. Genomic sequence coding for DnaN [103] from illustrative mycobacterial species was aligned with Clustal Omega [104] and their phylogeny inferred with MEGA7 [105] using the Maximum Likelihood methods and the Hasegawa-Kishino-Yano model with possible invariant sites. Phylogenies consistent with this interpretation were obtained with Neighbour-joining methods and when concatenated amino acid sequence of conserved proteins was used in the alignment. Bootstrap values are derived from 500 replicates.

this taxon. *M. haemophilum* is slow growing, requires iron supplementation and prefers a low growth temperature of 30°C. The first description of *M. haemophilum* was as a pathogen causing skin infections, particularly not only in immunocompromised patients [106, 107], but also in healthy children [108]. In a range of children, a variety of other clinical manifestations were encountered [15]. In two instances, *M. haemophilum* infections mimicked the appearance of leprosy [109, 110] and a co-infection of *M. leprae* and *M. haemophilum* has been reported [111]. Also, animal infections are common, with zebra fish (*Danio rerio*) being particularly susceptible [15]. More recently, a heavily infected leatherback sea turtle (*Dermochelys coriacea*) was found [112]. Infection of a haemophilum can move freely in a variety of environments, but it does not give a clear indication whether there is a particular zoonotic host in which the evolution of *M. haemophilum* may have occurred.

As noted previously, both *M. leprae* and '*M. lepromatosis*' can cause disease in squirrels [11, 12, 43, 44]. The presence of leprosy in armadillos is long established [9, 10, 114, 115] and, indeed, the armadillo was a prime source of material for early studies of the leprosy bacillus [79–81, 83, 84]. It is apparent that infected armadillos can spread leprosy to the human population [9, 10]. However, the leprosy introduced into the Americas by human migration was passed on to indigenous armadillos [46] so they can be eliminated as an environmental evolutionary source. The involvement of squirrels in the UK is more intriguing as it is difficult to envisage how

the diseases can have been contracted from human sources. A direct evolutionary pathway from ancient squirrel-like animals to humans is unlikely, but it is possible that squirrels are representative of other animals that may have acted as environmental reservoirs. In the case of '*M. lepromatosis*', a geographical association between patients and Mexican field rats (*Rattus rattus*) suggests a possible environmental reservoir [8].

4.4. Animal diseases resembling leprosy

Cases of tuberculoid nodular thelitis in both cattle [116] and goats [117] appear to be caused by uncultivable acid-fast species related to M. leprae and 'M. lepromatosis'. However, the interrelationships between these agents, infecting cattle and goats, need to be defined more precisely before the disease can be considered as a true variety of leprosy. A complex scenario is emerging regarding the status of infections categorised as 'feline leprosy' [118-121]. After many early reports of diverse manifestations of cat leprosy, a definitive study clarified the scene [122]. It was apparent that the rat leprosy bacillus, M. lepraemurium, made a contribution to disease, but the influence of a novel uncultivable Mycobacterium, whose closest relative was M. malmoense, was noted. In a follow-up study [123], it was observed that younger cats were susceptible to M. lepraemurium, but more mature felines typically harboured the novel uncultivable agent. In an interesting development, PCR amplification of 16S rRNA sequences, from the uncultured feline agent AJ294740-6, showed that the greatest nucleotide identity was shared with M. leprae and M. haemophilum, as well as M. malmoense; indeed a specific additional nucleotide correlated with only with *M. leprae* [124]. This particular taxon, expressed in cases from eastern Australia, New Zealand and possibly Canada, has been provisionally labelled 'M. lepraefelis' [121]. Three North American feline infections appeared to be caused by another uncultivable agent with close 16S rRNA relatedness to M. leprae and more distant affinity to M. haemophilum, among other species [125]. Initially labelled 'M. visibilis', but more properly 'M. visibile', this taxon remains uncharacterised and unfortunately unavailable for further study [120]. In a limited area of southeast Australia, studies of feline leprosy have revealed the presence of *M. lepraemurium* and an uncultivable novel agent, labelled 'M. tarwinense'. This agent was indicated to be a fastidious member of the M. simiae complex [120, 126] so it does not appear to have a direct relationship with M. leprae or 'M. lepromatosis'.

4.5. Overall interrelationships of leprosy affiliates

The precise interrelationships between all the bacterial taxa causing leprosy-like diseases require further study. It is clear that *M. leprae* or '*M. lepromatosis*' cause human leprosy and the same agents can routinely infect armadillos and squirrels. The apparent affinities of the feline leprosy taxon, labelled '*M. lepraefelis*', with *M. leprae* and *M. haemophilum* must be explored. The agents causing tuberculoid nodular thelitis in cattle and goats appear to have an affinity with established leprosy bacilli and this should be thoroughly investigated. In view of present uncertainties, it is premature to consider any concept of an *M. leprae* complex, as has been discussed [6, 8, 118, 127].

The possible origins and interrelationships of all agents causing leprosy-like disease are summarised in **Figure 9**. The phylogeny of *M. haemophilum* with *M. leprae* and *'M. lepromatosis'* indicates a deep common ancestor for all three taxa [16]; this ancestor is provisionally labelled



Figure 9. Origins and interrelationships of agents causing leprosy-like disease. Proposed relationships requiring further study are indicated (?). 'PROTOLEP' represents a prototype taxon with the specific type of outer membrane lipids expressed in *M. haemophilum*, *M. leprae* and possibly '*M. lepromatosis*'. *M. simiae* complex (1) represents species (*M. florentinum*, *M. interjectum*, *M. sherrissii*, *M. triplex*) apparently expressing genes for PDIM synthesis; *M. simiae* complex (2) includes the remaining species [128].

'PROTOLEP' in **Figure 9**. This hypothetical taxon is considered to incorporate characteristic cell envelope lipids, such as the C_{34} mycocerosates found in *M. leprae* and *M. haemophilum* (**Figure 2**). Sensitive lipid biomarker analysis has the potential to help identify the uncultivable agents causing feline leprosy ('*M. lepraefelis*', '*M. visibile*') and tuberculoid nodular thelitis in cattle and goats (**Figure 9**). It is an open question whether these agents have any affinity with *M. leprae*, '*M. lepraemurium* and '*M. tarwinense*' [119, 120, 126] (**Figure 9**) are distinct. '*M. tarwinense*' appears to be an affiliate of the *M. simiae* complex, which appeared to have little phylogeny with *M. leprae* and related taxa until detailed genomic characterisation of nontuberculous mycobacteria indicated that particular *M. simiae* complex members (*M. florentinum*, *M. interjectum*, *M. sherrissii*, *M. triplex*) apparently have genes for PDIM synthesis (**Figure 9**) [128]. It would be of interest to discover if there is any similarity between the proven PDIMs of *M. leprae* and those suggested to be expressed by these members of the *M. simiae* complex.

5. Conclusions

An understanding of the origins and spread of leprosy depends on establishing detailed knowledge of the ancient genotypes and their correlation with modern disease. The overall scenario has been expanded by the recent characterisation of the distinct modern clade, currently labelled '*M. lepromatosis*'. The availability of a full genome for '*M. lepromatosis*' is allowing specific probes to be developed to search for ancient expression of this biotype. Ongoing research is demonstrating that subtle lipid biomarker differences may be of value in distinguishing '*M. lepromatosis*' from *M. leprae*. The overall picture for the global development of leprosy suggests that the ancient disease evolved into a number of recognisable clades in Africa/Eurasia. It is clear that leprosy was introduced into the Americas by human migration, and the disease was passed on to indigenous armadillos. The deeper origins of leprosy appear to be inextricably linked to relatives of the environmental taxon *M. haemophilum*. Diseases in cats, cattle and goats, with affiliations and resemblances to leprosy, require detailed investigation.

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