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1 **Title: Red squirrels in the British Isles are infected with leprosy**

2 **bacilli**

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31

32 **Abstract: Leprosy, caused by infection with *Mycobacterium leprae* or the recently**
33 **discovered *Mycobacterium lepromatosis*, was once endemic in humans in the**
34 **British Isles. UK red squirrels (*Sciurus vulgaris*) have increasingly been observed**
35 **with leprosy-like lesions on the head and limbs. Using genomics, histopathology**
36 **and serology we found *M. lepromatosis* in squirrels from England, Ireland and**
37 **Scotland, and *M. leprae* in squirrels from Brownsea Island, England. Infection**
38 **was detected in overtly diseased and seemingly healthy animals. Phylogenetic**
39 **comparisons of British and Irish *M. lepromatosis* with two Mexican strains from**
40 **humans showed they diverged from a common ancestor around 27,000 years ago**
41 **whereas the *M. leprae* strain is closest to one that circulated in Medieval England.**
42 **Red squirrels are thus a reservoir for leprosy in the British Isles.**

43

44 **One Sentence Summary:** Diseased British and Irish red squirrels are infected with
45 two different bacteria that cause leprosy in humans and represent a potential zoonotic
46 threat.

47

48 **Main text - 1827 words**

49 **Main text, legends, etc. ~ 2355 words without references**

50

51 **Main Text:** Often considered a disease of the past, leprosy remains a public health problem
52 in certain low and middle-income countries with ~220,000 new cases reported annually (1).
53 Leprosy was rife in Europe in the Middle Ages but disappeared during the 15th-16th centuries
54 probably because of social segregation, other infectious diseases such as plague or changes in
55 host immunity (2–5). Today, all British clinical cases occur in individuals with a history of
56 residence in a leprosy endemic country (6). The disease manifests in different forms, ranging
57 from multibacillary, or lepromatous, to paucibacillary, or tuberculoid, depending on the
58 immunogenetics of the host (4). In all forms, skin lesions are accompanied by peripheral nerve
59 damage, which causes sensory loss and may lead to deformities.

60 It was generally accepted that leprosy resulted solely from inter-human transmission
61 of *M. leprae* but in recent years compelling evidence emerged from the southern USA for
62 zoonotic cases following exposure to infected nine-banded armadillos (*Dasypus novemcinctus*)
63 (7–9). Furthermore, *M. leprae* was considered to be the sole causative agent of leprosy until
64 2008 when a new species, *M. lepromatosis*, was identified in patients with diffuse lepromatous
65 leprosy (DLL) (10). Such cases were primarily associated with Mexico and the Caribbean
66 region (11). Comparison of the genome sequences of *M. lepromatosis* and *M. leprae* revealed
67 that despite separating millions of years ago, the two genomes are remarkably similar in their
68 size, organization and (pseudo)gene content, but show only 88% sequence identity (11).

69 The Eurasian red squirrel *Sciurus vulgaris* is a widespread Palearctic species found
70 from Ireland in the West to Kamchatka in the East (12, 13). However, in the United Kingdom
71 (UK) the *S. vulgaris* population of ~140,000 is severely threatened by habitat loss, squirrel
72 poxvirus infection and competition with >2.5 million grey squirrels, *Sciurus carolinensis*,
73 introduced from North America (14, 15). Due to their endangered status, red squirrels are now

74 protected (16). Recent detection of mycobacterial infection in red squirrels was reported in
75 Scotland, with lesions and histopathology characteristic of DLL and evidence for *M.*
76 *lepromatosis* being the etiological agent (17). Similarly affected squirrels were observed on
77 the Isle of Wight and Brownsea Island in Southern England (18) and observations of squirrel
78 leprosy in Scotland are increasing (Fig. 1). Here, we investigated these cases using 70 red
79 squirrel cadavers from the UK, with or without disease signs, 40 cadavers from Ireland, where
80 no sightings of squirrels with leprosy signs have been reported, and four Scottish grey squirrel
81 cadavers.

82 A differential PCR screen was implemented to detect *M. leprae* and *M. lepromatosis*
83 DNA (11). A total of 172 tissue samples from 13 animals with and 101 without leprosy features
84 were analyzed (tables S1, S2, (19)). Six Scottish squirrels (two without clinical signs (17)), two
85 from Ireland (no clinical signs), and one from the Isle of Wight, England, (18) contained *M.*
86 *lepromatosis*, in several tissue samples from different anatomical sites, whereas all 25 red
87 squirrels (17 without clinical signs) tested from Brownsea Island were infected with *M. leprae*
88 (Fig. 1, table S3). No cases of co-infection were observed (table S3). From the combined
89 results, we concluded that 21% (21/101; 95%CI 13-30%) of the squirrels without clinical signs
90 and all of the animals with clinical signs (13/13) harbored leprosy bacilli.

91 Serological tests were performed on nine diseased and 14 healthy red squirrels from
92 Scotland and England, and the four grey squirrels. The greys were all sero-negative whereas
93 13/23 blood samples from red squirrels contained antibodies for the leprosy-specific antigen,
94 phenolic glycolipid-1 (20) (table S4, (19)). Serology is useful to confirm the disease and predict
95 infection in live animals but cannot be used for species identification as both *M. leprae* and *M.*
96 *lepromatosis* produce this cell wall antigen (11).

97 Diseased Scottish squirrels, infected with *M. lepromatosis*, displayed a range of
98 macroscopic lesions including alopecia, extensive swelling of the snout, lips, eyelids, the ear
99 pinnae and limb extremities (Figs. 1, 2A, S1, tables S2, S5, (19)). Histopathological
100 examination of four such squirrels (Fig. 2B) revealed granulomatous dermatitis, sheets of
101 epithelioid macrophages and large numbers of acid-fast bacilli (AFB). There was neural

102 involvement with the presence of AFB in nerve endings; neuritis was patchy and more
103 frequently perineural (Fig. 2C). Inflammation was not focused exclusively around nerves and
104 was mostly dermal. There were no signs of vasculitis, but AFB were present intravascularly
105 (Fig. 2C). Similar lesions were observed in eight squirrels from Brownsea Island infected with
106 *M. leprae*, although these animals also harbored numerous AFB in the spleen (Fig. 2C).
107 Overall, the macroscopic signs and histopathology were characteristic of lepromatous leprosy
108 (Figs. 2A, B, Figs. S2, S3). From post-mortem inspection of diseased squirrels it was not
109 possible to distinguish between infection with *M. lepromatosis* or *M. leprae*, as in human
110 leprosy (11, 21, 22).

111 To obtain deeper insight into the strains responsible and to perform phylogenetic
112 analyses we used a variety of DNA enrichment techniques (table S6) prior to Illumina
113 sequencing since neither *M. leprae* nor *M. lepromatosis* can be cultured (19). Sufficient
114 sequence coverage of *M. lepromatosis* genomes from seven squirrels was obtained (table S7).
115 In parallel, we sequenced an additional genome of *M. lepromatosis*, PI-02, from a PGL-1-
116 seropositive patient from Sinaloa, Mexico (tables S1, S4). The resultant sequence reads were
117 mapped against the reference *M. lepromatosis* genome sequence from a patient from
118 Monterrey, Mexico (11) to identify polymorphisms. Consistent with previous *M. leprae*
119 genome comparisons (9, 11, 23), there was an exceptionally high level of sequence
120 conservation between *M. lepromatosis* strains (99.99% identity) despite their different
121 geographic origins. The two Mexican patient isolates differed by only seven single nucleotide
122 polymorphisms (SNPs) whereas the number of SNPs in the six British and Irish strains ranged
123 from one to 17 on pairwise comparisons (table S8). Overall, there are roughly 400 SNPs that
124 distinguish *M. lepromatosis* strains from Mexico and the British Isles (table S8). Clustering of
125 Mexican and British *M. lepromatosis* strains into two distinct lineages was supported by
126 maximum parsimony (Fig. S4) and neighbor joining (Fig. S5) phylogenetic reconstructions.
127 Based on the *M. leprae* mutation rate (19) and using the Bayesian inference software, BEAST
128 (24), we estimated that the British Isles and Mexican strains diverged from their most recent
129 common ancestor around 27,000 years ago whereas the Irish and UK strains diverged as

130 recently as 200 years ago (Fig. 3A). The latter estimate is consistent with the date of the first
131 campaign to reintroduce the red squirrel into Ireland from England between 1820 – 1856,
132 following its extinction in the 17th century (12, 25). This suggests that these animals may
133 already have been infected with *M. lepromatosis* when they were reintroduced.

134 Finding *M. leprae* in red squirrels in the UK was unexpected, since leprosy was
135 eradicated from the British Isles several centuries ago, thus demonstrating that a pathogen can
136 persist in the environment long after its clearance from the human reservoir. Furthermore, this
137 is only the second report of *M. leprae* in non-primate species. From Bayesian and maximum
138 parsimony analysis (Fig. 3B, fig. S4A) we note that the two closest relatives to the strain of *M.*
139 *leprae* found on Brownsea Island were both from medieval Europe. Intriguingly, one of these
140 (SK2) originated from the skeletal remains of a leprosy victim buried about 730 years ago in
141 Winchester, a city situated a mere 70 km from Brownsea Island (Fig. 1). Like SK2, the
142 Brownsea Island strain of *M. leprae* belongs to sequence type 3I, which forms a distinct *M.*
143 *leprae* branch (Fig. 3B) (3) and is now endemic in wild armadillos in the Southern USA (9).
144 Thus, *M. leprae* with this particular sequence type is capable of infecting at least three different
145 hosts: humans, red squirrels and armadillos.

146 Since there were no obvious genomic polymorphisms restricted to the *M. leprae* 3I
147 type that might account for this broad host range (tables S9, S10) we explored the possibility
148 that these three species might share a major susceptibility gene and focused on *TLR1*. This
149 candidate gene, encoding the surface-exposed Toll-like receptor 1 (TLR1) displayed on
150 various epithelial and immune cells, is known to be associated with susceptibility to leprosy
151 (Fig. 4A). A dysfunctional *TLR1* allele encoding an I602S variant with an altered
152 transmembrane domain is prevalent in Caucasians and is associated with a decreased risk for
153 leprosy (5, 26). By contrast, the *TLR1* N248S variant is associated with an increased risk of
154 leprosy in humans. This mutation is located in the ninth repeat of the extracellular leucine-rich
155 repeat (LRR) region of TLR1 (27). Furthermore, in nine-banded armadillos an R627G change
156 in TLR1 (close to the Toll/Interleukin receptor (TIR) domain, Fig. 4A), seemingly confers
157 resistance to leprosy (28). Using PCR the coding exon of *TLR1* was amplified and sequenced

158 from 58 red (with or without lesions) and three grey squirrels (tables S11, S12, S14 (19)). On
159 comparison of the sequences and TLR1 alignments (table S13) no polymorphisms were
160 observed at the same sites associated with leprosy in humans and armadillos. However, in
161 some red squirrels, two distinct polymorphic sites exist: a single SNP leading to a S494N
162 mutation in the nineteenth repeat of the LRR region and a cluster of linked mutations that
163 produce S657N, L660V and N662C variants in helix 1 of the TIR domain (Fig. 4B). These
164 mutations were found less frequently in squirrels infected with leprosy bacilli compared to
165 healthy animals suggesting that they may confer protection (OR: 5.77, 95% CI: 1.42 - 23.41,
166 $p=0.01$ for 494N and OR: 4.89, 95% CI: 0.98 - 24.53, $p=0.05$ for 657N-660V-662C).

167 It is unclear whether leprosy is contributing to the demise of the red squirrel population
168 or how these animals became infected with *M. lepromatosis* or *M. leprae*. Since *M.*
169 *lepromatosis* has only recently been discovered as a human pathogen (10), and there are few
170 detailed case reports (10, 11, 21, 29), further investigation is required to establish its relative
171 prevalence in wildlife compared to humans. *M. leprae* was long considered to be an obligate
172 human pathogen that was introduced to the Americas by European settlers, prior to
173 anthroponotic infection of armadillos, since there are no human skeletal remains with signs of
174 leprosy from the pre-Columbian era (9). The discovery that the strain of *M. leprae* in red
175 squirrels on Brownsea Island today is essentially the same as one that circulated in medieval
176 England and Denmark, and highly related to the extant North American armadillo strain, raises
177 the possibility of a second anthroponotic introduction in Europe. If this were the case, it must
178 have occurred several centuries ago as leprosy became increasingly scarce in the British Isles
179 after the 17th century (3). It is also conceivable that humans may have been infected through
180 contact with red squirrels bearing *M. leprae* as these animals were prized for their fur and meat
181 in former times (30). Our findings demonstrate that further surveys of animal reservoirs of
182 leprosy bacilli are warranted, since zoonotic infection from such reservoirs may contribute to
183 the inexplicably stubborn plateau in the incidence of the human leprosy epidemic despite
184 effective and widespread treatment with multidrug therapy (1).

185

186 **References and Notes**

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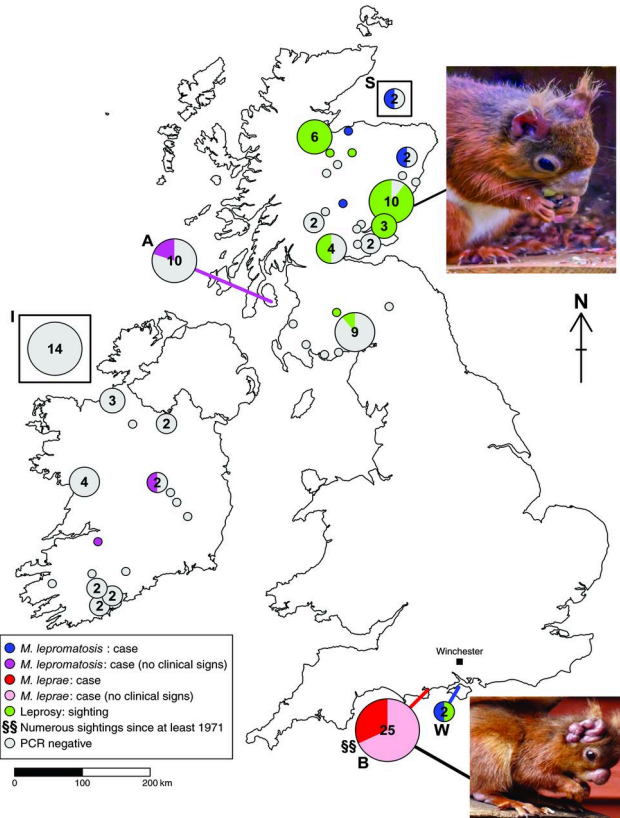
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298 sequence read files were deposited in Sequence Read Archive (SRA) of the National Center for
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302 representative TLR1 sequences at GenBank under accession numbers KX388139, KX388140
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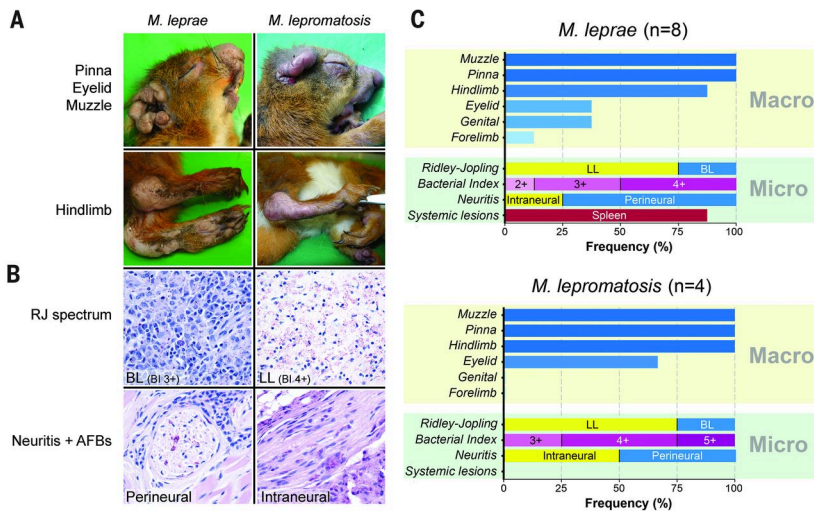
309

310 **Fig. 1. Squirrel sampling sites in the British Isles.** Pie charts indicate the location of sites
311 where squirrels were sighted or found and color-coded as indicated in the box, numbers within
312 circles indicate different animals tested where $N > 1$. Boxed circles refer to squirrels of
313 unknown location: I, Ireland; S, Scotland. A, Isle of Arran; B, Brownsea Island; W, Isle of
314 Wight. The figure was drawn in R (v3.2.23 © 2015 The R Foundation for Statistical
315 Computing) with the package *maps* (v3.1.0) using the *mapdata* (v2.2-6) “worldHiresMapEnv”
316 and the package *plotrix* (v3.6-2) for pie charts.



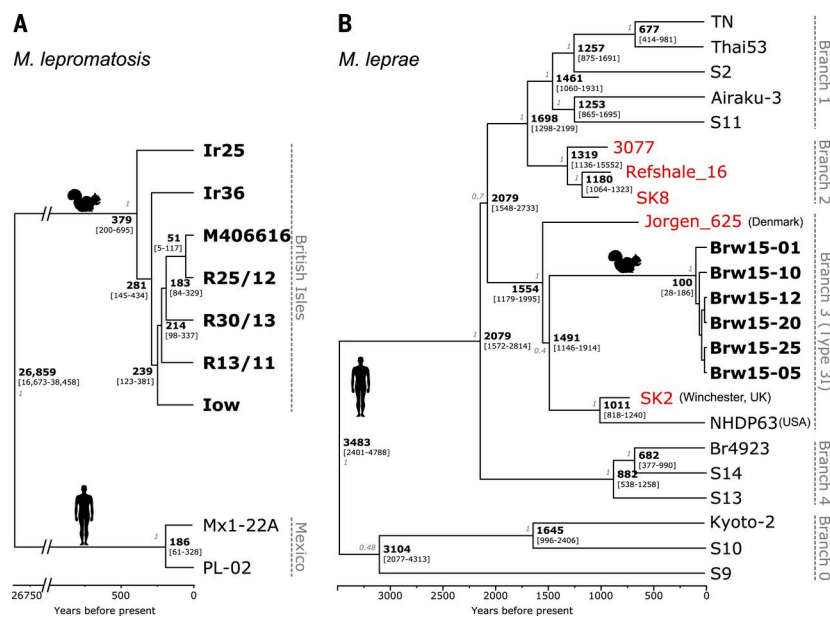
317

318 **Fig. 2. Gross histopathological features of red squirrels with leprosy.** (A) Both
 319 macroscopic and histological features of squirrels infected with either *M. lepromatosis* or *M.*
 320 *leprae* are similar. (B) Histological examination of tissue sections from infected squirrels using
 321 the Ridley-Jopling (RJ) classification following Ziehl Neelsen staining (Mag. x400). LL:
 322 lepromatous leprosy, BL: borderline lepromatous leprosy. (C) Summary of main macroscopic
 323 and microscopic findings from squirrels infected with *M. leprae* (n=8) or *M. lepromatosis*
 324 (n=4).

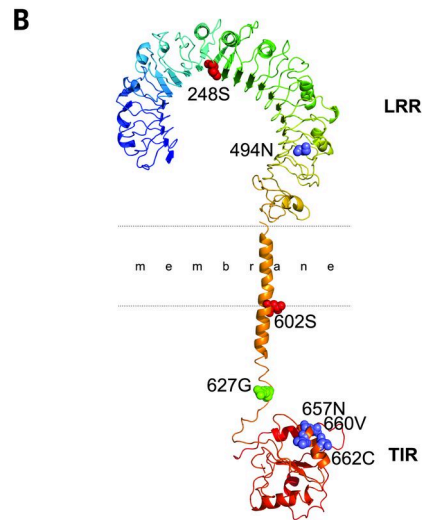
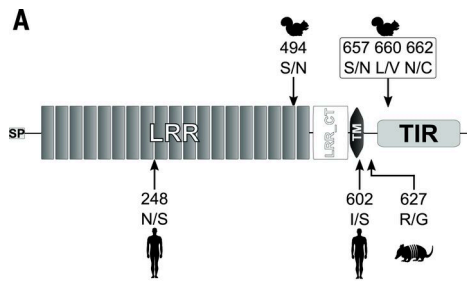


325

326 **Fig. 3. Phylogeny of leprosy bacilli.** (A) Bayesian phylogenetic tree representation of nine
 327 *M. lepromatosis* genome sequences obtained from squirrels (bold) or humans, upper and
 328 lower parts, respectively, calculated by BEAST 1.8.2 (24) using the mutation rate of *M.*
 329 *leprae* and inferred from 432 genome-wide variable positions. Squirrel sample prefixes: Ir,
 330 Ireland; Iow, Isle of Wight; with all others from Scotland. Both human strains were from
 331 Mexico. (B) Bayesian phylogenetic tree representation of *M. leprae* inferred from 498
 332 genome-wide variable positions, calculated as in (A). Squirrel samples (bold): Brw denotes
 333 Brownsea Island cluster with red labeling indicating ancient strains for which radio-carbon
 334 dating information was available (3). For both trees, divergence time intervals are shown on
 335 each node in years before present, with the 95% HPD range in brackets. Posterior
 336 probabilities for each node are shown in grey.



337
 338 **Fig. 4. Organization, structure and polymorphisms in TLR1 associated with leprosy in**
 339 **humans, armadillos and red squirrels..** (A) Schematic representation of TLR1 and its
 340 domains (drawn to scale). SP = Signal peptide, LRR = Leucine-rich repeats, LRR_CT =
 341 Leucine-rich repeat C-terminal, TM = transmembrane domain, TIR = Toll/interleukin-1
 342 receptor. (B) Structural model of the red squirrel TLR1. Protein is colored in a rainbow
 343 spectrum from N-terminus (blue) to C-terminus (red).



344

345 **Supplementary Materials:**

346 Materials and Methods

347 Figures S1-S5

348 Tables S1-S14

349 References (31-51)

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