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Red squirrels in the British Isles are infected with leprosy bacilli

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

2   **bacilli**

3   **Authors:** Charlotte Avanzi, [1†] Jorge del-Pozo, [2†] Andrej Benjak, [1†] Karen Stevenson, [3]

4   Victor R. Simpson, [4] Philippe Busso, [1] Joyce McLuckie, [3] Chloé Loiseau, [1‡] Colin

5   Lawton, [5] Janne Schoening, [6] Darren J. Shaw, [2] Jérémie Piton, [1] Lucio Vera-Cabrera, [7]

6   Jesùs S. Velarde-Felix, [7] Fergal McDermott, [6] Stephen V. Gordon, [6,8,9,10] Stewart T.

7   Cole, [1*] Anna L. Meredith [2*]

8   **Affiliations:**

9   [1]Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne,

10   Switzerland.

11   [2]Royal (Dick) School of Veterinary Studies and The Roslin Institute, The University of

12   Edinburgh, Easter Bush Campus, Roslin, Scotland, UK..

13   [3]Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh, Scotland,

14   UK.

15   [4]Wildlife Veterinary Investigation Centre, Chacewater, Cornwall, UK.

16   [5]School of Natural Sciences, Ryan Institute, National University Ireland, Galway,

17   Ireland.

18   [6]UCD School of Veterinary Medicine, University College Dublin, Belfield, Dublin,

19   Ireland.

20   [7]Laboratorio Interdisciplinario de Investigación Dermatológica, Servicio de

21   Dermatología, Hospital Universitario, Monterrey, N.L., Mexico.

22   [8]UCD School of Medicine, University College Dublin, Belfield, Dublin, Ireland.

23   [9]UCD School of Biomolecular and Biomedical Science, University College Dublin,

24   Belfield, Dublin, Ireland.

25 [10]UCD Conway Institute of Biomolecular and Biomedical Research, University

26 College Dublin, Belfield, Dublin, Ireland.

27 *Corresponding authors: Email: stewart.cole@epfl.ch; Anna.Meredith@ed.ac.uk

28 [†]These authors contributed equally to this work.

29 [‡]Present address: Department of Medical Parasitology and Infection Biology, Swiss

30 Tropical and Public Health Institute, 4002 Basel, Switzerland.

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

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*, Pl-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

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

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

References and Notes

1. WHO, Global leprosy update on the 2012 situation *Weekly epidemiological record* **88**, 368–380 (2013).

2. H. D. Donoghue *et al.*, A migration-driven model for the historical spread of leprosy in medieval Eastern and Central Europe. *Infect. Genet. Evol.* **31**, 250-256 (2015)

3. V. J. Schuenemann *et al.*, Genome-wide comparison of medieval and modern Mycobacterium leprae. *Science*. **341**, 179–183 (2013).

4. A. Alter, A. Grant, L. Abel, A. Alcaïs, E. Schurr, Leprosy as a genetic disease. *Mamm. Genome Off. J. Int. Mamm. Genome Soc.* **22**, 19–31 (2011).

5. S. H. Wong *et al.*, Leprosy and the Adaptation of Human Toll-Like Receptor 1. *PLOS Pathog.* **6**, e1000979 (2010).

6. N. Fulton, L. F. Anderson, J. M. Watson, I. Abubakar, Leprosy in England and Wales 1953–2012: surveillance and challenges in low incidence countries. *BMJ Open*. **6**, e010608 (2016).

7. R. Sharma *et al.*, Zoonotic Leprosy in the Southeastern United States. *Emerg. Infect. Dis.* **21**, 2127–2134 (2015).

8. R. Truman, Leprosy in wild armadillos. *Lepr. Rev.* **76**, 198–208 (2005).

9. R. W. Truman *et al.*, Probable zoonotic leprosy in the southern United States. *N. Engl. J. Med.* **364**, 1626–1633 (2011).

10. X. Y. Han *et al.*, A new Mycobacterium species causing diffuse lepromatous leprosy. *Am. J. Clin. Pathol.* **130**, 856–864 (2008).

11. P. Singh *et al.*, Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium lepromatosis. *Proc. Natl. Acad. Sci.*, 201421504 (2015).

12. M. Carey, G. Hamilton, A. Poole, C. Lawton, "The Irish Squirrel Survey 2007," *Dublin* (COFORD, 2007).

13. S. Harris, G. B. Corbet, Mammal Society, *The Handbook of British mammals* (Published for the Mammal Society by Blackwell Scientific Publications, 3rd ed., 1991).

14. D. M. Tompkins, A. W. Sainsbury, P. Nettleton, D. Buxton, J. Gurnell, Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines. *Proc. R. Soc. B Biol. Sci.* **269**, 529–533 (2002).

15. E. Stokstad, Red squirrels rising. *Science*. **352**, 1268–1271 (2016).

16. Council of Europe, Convention on the Conservation of European Wildlife and Natural Habitats - ETS No 104 - Appendix III (1979).

17. A. Meredith *et al.*, Leprosy in red squirrels in Scotland. *Vet. Rec.* **175**, 285–286 (2014).

18. V. Simpson *et al.*, Leprosy in red squirrels on the Isle of Wight and Brownsea Island. *Vet. Rec.* **177**, 206–207 (2015).

223   19.   Methods are available as supplementary materials on Science Online.

224   20.   J. S. Spencer, P. J. Brennan, The role of Mycobacterium leprae phenolic glycolipid I
225   (PGL-I) in serodiagnosis and in the pathogenesis of leprosy. *Lepr. Rev.* **82**, 344–357
226   (2011).

227   21.   J. S. Velarde-Félix, G. Alvarado-Villa, L. Vera-Cabrera, "Lucio's Phenomenon"
228   Associated with Mycobacterium lepromatosis. *Am. J. Trop. Med. Hyg.* **94**, 483–484
229   (2016).

230   22.   L. Vera-Cabrera *et al.*, Mycobacterium lepromatosis Infections in Nuevo León, Mexico.
231   *J. Clin. Microbiol.* **53**, 1945–1946 (2015).

232   23.   M. Monot *et al.*, Comparative genomic and phylogeographic analysis of
233   Mycobacterium leprae. *Nat. Genet.* **41**, 1282–1289 (2009).

234   24.   A. J. Drummond, A. Rambaut, BEAST: Bayesian evolutionary analysis by sampling
235   trees. *BMC Evol. Biol.* **7**, 214 (2007).

236   25.   B. P. Vieira, C. Fonseca, R. G. Rocha, Critical steps to ensure the successful
237   reintroduction of the Eurasian red squirrel. *Anim. Biodivers. Conserv.* **38**, 49–58 (2015).

238   26.   S. R. Krutzik *et al.*, Activation and regulation of Toll-like receptors 2 and 1 in human
239   leprosy. *Nat. Med.* **9**, 525–532 (2003).

240   27.   C. de S. Marques *et al.*, Toll-like receptor 1 N248S single-nucleotide polymorphism is
241   associated with leprosy risk and regulates immune activation during mycobacterial
242   infection. *J. Infect. Dis.* **208**, 120–129 (2013).

243   28.   L. B. Adams *et al.*, Insights from animal models on the immunogenetics of leprosy: a
244   review. *Mem. Inst. Oswaldo Cruz.* **107 Suppl 1**, 197–208 (2012).

245   29.   P. G. Jessamine *et al.*, Leprosy-like illness in a patient with Mycobacterium
246   lepromatosis from Ontario, Canada. *J. Drugs Dermatol. JDD*. **11**, 229–233 (2012).

247   30.   P. Lurz, "Red squirrel: Naturally Scottish" (Scottish Natural Heritage, Scotland, 2010).

248   31.   V. R. Simpson, J. Hargreaves, H. M. Butler, N. J. Davison, D. J. Everest, Causes of
249   mortality and pathological lesions observed post-mortem in red squirrels (Sciurus
250   vulgaris) in Great Britain. *BMC Vet. Res.* **9**, 229 (2013).

251   32.   D. S. Ridley, W. H. Jopling, Classification of leprosy according to immunity. A five-
252   group system. *Int. J. Lepr. Mycobact. Dis. Off. Organ Int. Lepr. Assoc.* **34**, 255–273
253   (1966).

254   33.   A. M. Phillippy, X. Deng, W. Zhang, S. L. Salzberg, Efficient oligonucleotide probe
255   selection for pan-genomic tiling arrays. *BMC Bioinformatics*. **10**, 293 (2009).

256   34.   A. M. Bolger, M. Lohse, B. Usadel, Trimmomatic: A flexible trimmer for Illumina
257   Sequence Data. *Bioinformatics*, btu170 (2014).

258   35.   B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. *Nat. Methods*.
259   **9**, 357–359 (2012).

260   36.   S. M. Kiełbasa, R. Wan, K. Sato, P. Horton, M. C. Frith, Adaptive seeds tame genomic
261   sequence comparison. *Genome Res.* **21**, 487–493 (2011).

262 37. D. C. Koboldt *et al.*, VarScan 2: Somatic mutation and copy number alteration
263   discovery in cancer by exome sequencing. *Genome Res.* **22**, 568–576 (2012).

264 38. K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: Molecular
265   Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729 (2013).

266 39. M. Nei, S. Kumar, *Molecular Evolution and Phylogenetics* (Oxford University Press,
267   2000).

268 40. C. M. Johnson *et al.*, Cutting edge: A common polymorphism impairs cell surface
269   trafficking and functional responses of TLR1 but protects against leprosy. *J. Immunol.*
270   *Baltim. Md 1950*. **178**, 7520–7524 (2007).

271 41. Practical Statistics for Medical Research. *CRC Press* (1990), (available at
272   https://www.crcpress.com/Practical-Statistics-for-Medical-
273   Research/Altman/p/book/9780412276309).

274 42. L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, M. J. E. Sternberg, The Phyre2 web
275   portal for protein modeling, prediction and analysis. *Nat. Protoc.* **10**, 845–858 (2015).

276 43. M. Biasini *et al.*, SWISS-MODEL: modelling protein tertiary and quaternary structure
277   using evolutionary information. *Nucleic Acids Res.* **42**, W252–258 (2014).

278 44. M. S. Jin *et al.*, Crystal structure of the TLR1-TLR2 heterodimer induced by binding of
279   a tri-acylated lipopeptide. *Cell*. **130**, 1071–1082 (2007).

280 45. Y. Xu *et al.*, Structural basis for signal transduction by the Toll/interleukin-1 receptor
281   domains. *Nature*. **408**, 111–115 (2000).

282 46. K. Zhou, R. Kanai, P. Lee, H.-W. Wang, Y. Modis, Toll-like receptor 5 forms
283   asymmetric dimers in the absence of flagellin. *J. Struct. Biol.* **177**, 402–409 (2012).

284 47. I. Botos, D. M. Segal, D. R. Davies, The structural biology of Toll-like receptors. *Struct.*
285   *Lond. Engl. 1993*. **19**, 447–459 (2011).

286 48. A. Krogh, B. Larsson, G. von Heijne, E. L. Sonnhammer, Predicting transmembrane
287   protein topology with a hidden Markov model: application to complete genomes. *J.*
288   *Mol. Biol.* **305**, 567–580 (2001).

289 49. L. L. C. Schrödinger, The PyMOL molecular graphics system, version 1.8 (2015).

290 50. R. P. Schuring *et al.*, Polymorphism N248S in the Human Toll-Like Receptor 1 Gene Is
291   Related to Leprosy and Leprosy Reactions. *J. Infect. Dis.* **199**, 1816–1819 (2009).

292 51. M. Ben-Ali *et al.*, Functional characterization of naturally occurring genetic variants in
293   the human TLR1-2-6 gene family. *Hum. Mutat.* **32**, 643–652 (2011).

294 Acknowledgments

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
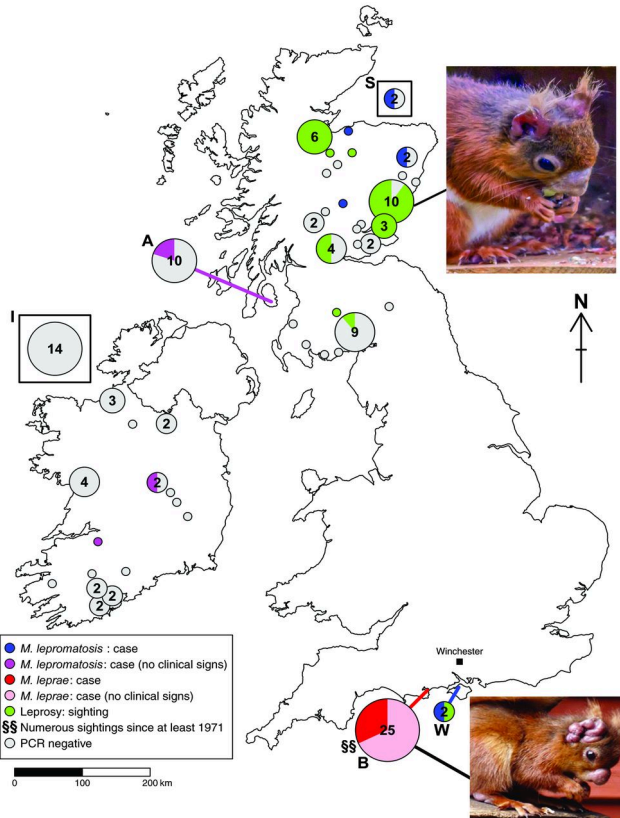
Fig. 2. Gross histopathological features of red squirrels with leprosy. (A) Both macroscopic and histological features of squirrels infected with either *M. lepromatosis* or *M. leprae* are similar. (B) Histological examination of tissue sections from infected squirrels using the Ridley-Jopling (RJ) classification following Ziehl Neelsen staining (Mag. x400). LL: lepromatous leprosy, BL: borderline lepromatous leprosy. (C) Summary of main macroscopic and microscopic findings from squirrels infected with *M. leprae* (n=8) or *M. lepromatosis* (n=4).
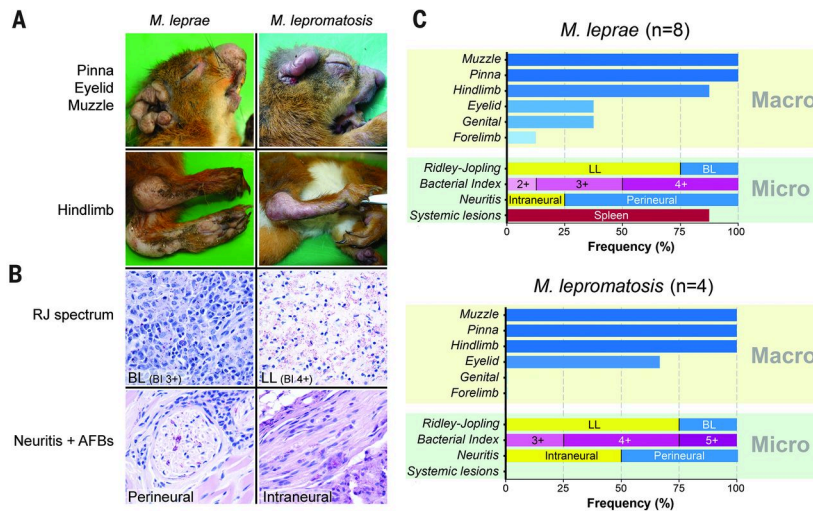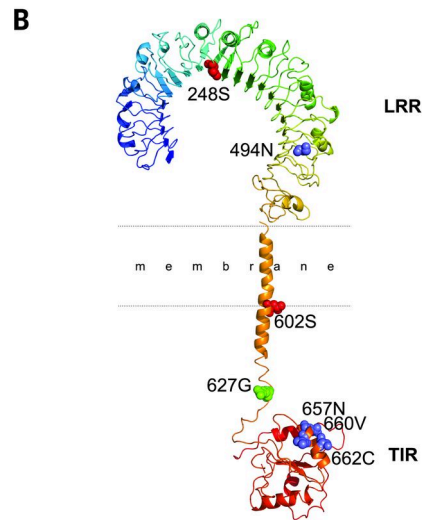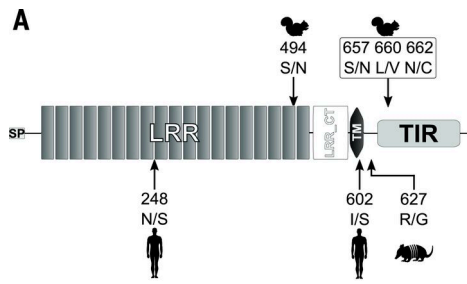
326 **Fig. 3. Phylogeny of leprosy bacilli.** (**A**) Bayesian phylogenetic tree representation of nine

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.
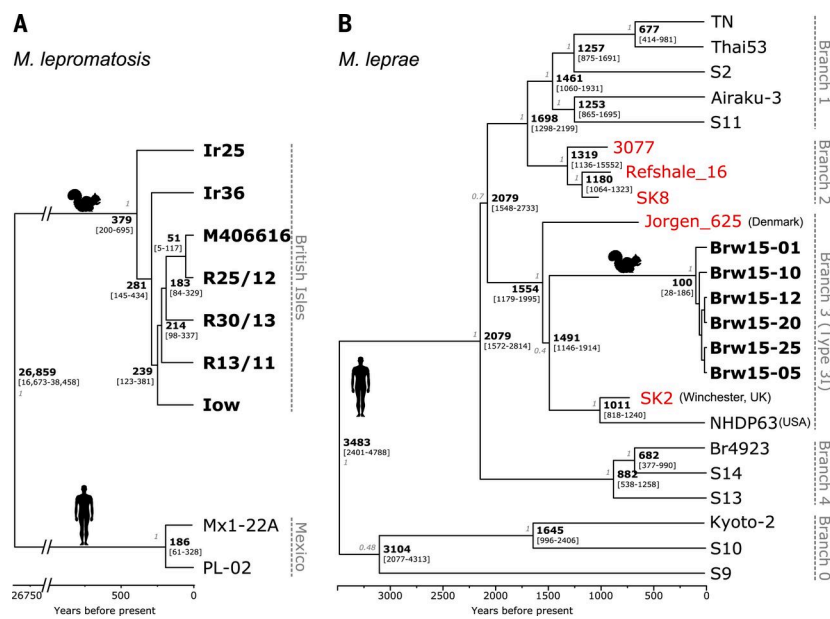
338 **Fig. 4. Organization, structure and polymorphisms in TLR1 associated with leprosy in**

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).

13

A

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S/N

657 660 662
S/N L/V N/C

SP — LRR — LRR_CT — TIR

248
N/S

602
I/S

627
R/G

B

248S

494N

LRR

m e m b r a n e

602S

627G

657N
660V
662C

TIR

344

345 **Supplementary Materials:**

346 Materials and Methods

347 Figures S1-S5

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349 References (*31-51*)

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