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Acknowledgements

I thank C. Lively, K. Quinlan and M. Wade for comments. A. Kondrashov suggested using the multilocus approach to this problem. S. Otto pointed out that the analytical solution is exact by use of equation (6). This work was supported by NSERC Canada.

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PCR amplification of the Irish potato famine pathogen from historic specimens

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Late blight, caused by the oomycete plant pathogen *Phytophthora infestans*, is a devastating disease of potato and was responsible for epidemics that led to the Irish potato famine in 1845 (refs 1–5). Before the 1980s, worldwide populations of *P. infestans* were dominated by a single clonal lineage, the US-1 genotype or Ib mitochondrial DNA (mtDNA) haplotype, and sexual reproduction was not documented outside Mexico, the centre of diversity of the pathogen[6,7]. Here we describe the amplification and sequencing of 100-base-pair fragments of DNA from the internal transcribed spacer region 2 from 28 historic herbarium samples including Irish and British samples collected between 1845 and 1847, confirming the identity of the pathogen. We amplified a variable region of mtDNA that is present in modern Ib haplotypes of *P. infestans*, but absent in the other known modern haplotypes (Ia, IIa and IIb)[8]. Lesions in samples tested were not caused by the Ib haplotype of *P. infestans*, and so theories that assume that the Ib haplotype is the ancestral strain need to be re-evaluated[4,7]. Our data emphasize the importance of using historic specimens when making inferences about historic populations.

During the nineteenth and early twentieth centuries, scientists collected and preserved potato and tomato leaves infected with *P. infestans* from the Irish potato famine, and these specimens can be used today to answer questions about the population biology of the pathogen[9] (Fig. 1). Disputes about nomenclature, phylogenetics, function and evolution of genes, and origins of populations can be addressed using herbarium collections[10–17]. We used polymerase chain reaction (PCR) amplification and sequencing of ribosomal DNA (rDNA) and mtDNA of *P. infestans* from herbarium specimens to determine whether the Ib mtDNA haplotype was the clonal lineage responsible for historic epidemics.

Dried potato and tomato lesions sampled from the Royal Botanic Gardens Mycological Herbarium, Kew, UK, and the US National Fungus Collection, Beltsville, Maryland, were aseptically removed from specimen envelopes and placed in sterile microfuge tubes. Initial DNA extractions from British and European samples were conducted in the Jodrell Laboratory at Kew in a laboratory without a history of work with the pathogen. Non-infected potato leaves and leaves infected with *P. infestans* from modern epidemics were pressed and dried in the Mycological Herbarium in the Department of Plant Pathology, North Carolina State University. All manipulations of DNA from modern samples were performed in our laboratory in the Department of Plant Pathology. Work with the herbarium specimens was performed in the North Carolina State University Phytotron Containment Facility Laboratory, which was equipped with separate supplies, reagents and equipment and has no history of research involving *P. infestans*.

DNA was extracted from herbarium samples according to a modification of a cetyltrimethylammonium bromide (CTAB) procedure[18]. DNA was diluted 1:10 and used with PCR primers PINF and HERB1 and PINF and ITS3 from 123 herbarium samples. Thirty-nine samples (31%) yielded the expected product (around 100 base pairs (bp) in size) when amplified with primers PINF and HERB1 (see Supplementary Information). Only three samples (2.4%), all from the 20th century, yielded a product approximately 300 bp in size when amplified with primers PINF and ITS3. These results are expected because DNA tends to degrade in ancient materials and primers that amplify smaller product sizes (<200 bp) are more effective in these types of specimen[13]. DNA of *P. infestans* was amplified from 39 samples, including four samples of *Solanum tuberosum* collected in Britain and France in 1845, four samples collected in Ireland and Britain in 1846, and one sample collected in Britain in 1847 (Fig. 2; and see Supplementary Information). We also amplified DNA of *P. infestans* from a sample of *Anthocercis ilicifolia,* a solanaceous evergreen shrub native to Western Australia

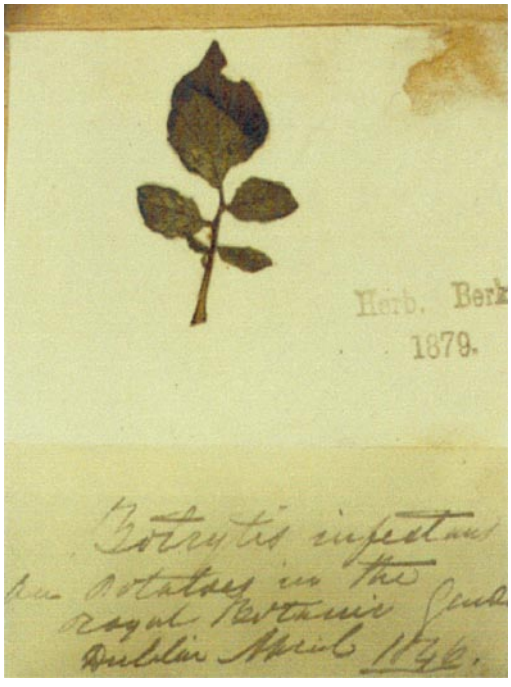

Figure 1 Specimen of potato infected with *P. infestans*. This sample was collected by J. Lindley in 1846 in the Royal Botanic Gardens, Dublin, Ireland and identified by M. J. Berkeley. It is deposited in the collections at the Royal Botanic Gardens, Kew, UK and is one of the oldest known specimens of potato from the potato famine epidemics.

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that was introduced into the National Botanic Gardens at Glasnevin in Dublin in 1842 and collected in 1846 by David Moore. This is the earliest known definitive diagnosis of an alternative solanaceous host for *P. infestans*[19].

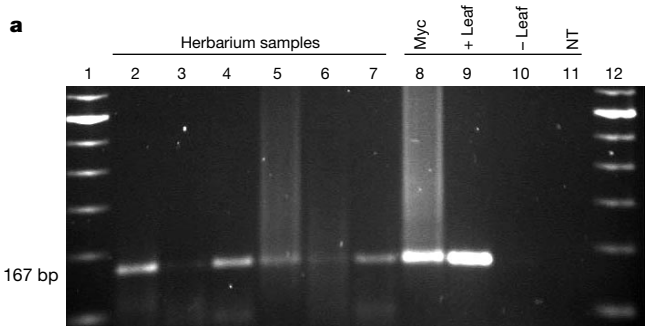
We sequenced the DNA from internal transcribed spacer (ITS) region 2 that was amplified by the PINF and HERB1 primers from 12 of the herbarium specimens. Duplicate PCR and sequencing reactions were performed in both the forward and reverse directions to confirm the DNA sequence and to minimize error. Sequences were aligned with Clustal W, version 1.8 (ref. 20) and have been submitted to GenBank (see Supplementary Information). Analysis of the DNA sequences confirmed that the targeted region of pathogen ITS DNA was successfully amplified from the lesions. This region of ITS DNA is highly conserved among extant isolates of *P. infestans* and provides a diagnostic region for identification of the pathogen in potato tissue[18]. Our molecular data confirm that these historic samples were infected with *P. infestans*.

Mitochondrial haplotypes have been designated in *P. infestans* using both PCR approaches and restriction fragment length polymorphism (RFLP) analysis of mtDNA[8,21–23]. Four haplotypes have been designated using a PCR-based approach: Ia, Ib, IIa and IIb[8]. Some of the DNA sequence mutations that generated the mtDNA polymorphisms in the four haplotypes have been identified[8,23–25]. We have sequenced the P2 and P4 regions from three modern isolates of each of the four known mtDNA haplotypes and have confirmed the sequence variation that has been reported (see Supplementary Information)[8,24,25].

The mtDNA primer set P2F4 and P2R4 amplified a variable region near the 3′ end of the *Nad4* gene in the P2 region of the mitochondrial genome[8,21,24]. A 167-bp PCR product and sequence data were obtained from ten of the herbarium samples that were previously positive for the PINF and HERB1 primer set (Fig. 3a; and see Supplementary Information). Modern isolates of the Ib mtDNA haplotype contain an *Msp*I restriction site in this region, whereas the other three modern haplotypes have a single base-pair change from C to T and thus lack this restriction site (Fig. 3b)[8,23]. None of the sequences from the herbarium specimens contained the *Msp*I restriction site, indicating that the lesions in the samples we examined were not caused by the Ib haplotype of *P. infestans*. It is possible that the ancestral strain belonged to the Ia haplotype, and that the larger insertion sequence found in group II haplotypes occurred more recently from a Ia haplotype[21]. We are investigating this possibility.

Isolates of *P. infestans* that display the typical RFLP fingerprint of the US-1 genotype are the Ib mtDNA haplotype[8,21–23,26–28]. The Ib haplotype has been reported in modern isolates from Ireland, Equador, Brazil, the UK and the US[21,23]. The Ia mtDNA haplotype was the dominant haplotype found in populations of *P. infestans* in Mexico, in addition to the IIa and IIb haplotypes[22,23]. The Ib haplotype has not been found among present-day isolates of the pathogen in Mexico, even though this haplotype is proposed to be the ancestral type[21,23]. In studies on the origin of human populations, all major mtDNA lineages occur in Africa, and only a subset occur in the rest of the world, thus suggesting an African origin of humans[29]. As all the known modern mtDNA haplotypes of *P. infestans* do not occur in Mexico, this might suggest an alternative centre of origin for the pathogen, perhaps in South America, which is the ancestral home of the potato[1,2]. Mexico is clearly a centre of diversity for the late blight pathogen[4,5], but the pathogen's centre of diversity may not coincide with its centre of origin.

This is the first time, to our knowledge, that ITS DNA and mtDNA of a plant pathogen have been amplified and sequenced from lesions of samples from historic epidemics. Sequence data from the mtDNA indicated that the Ib haplotype did not cause the



b

Isolate	Haplotype	Date	*Msp*1 restriction site
93-3	Ia	1993	AATTTCTCCAACAAAACTACTTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA
188.1.1	Ib	1996	AATTTCTCCAACAAAACTACTTGAACCCGGAATAGACATATTTGCTAATACATAAATAAA
18-94	IIa	1996	AATTTCTCCAACAAAACTACTTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA
94-52	IIb	1994	AATTTCTCCAACAAAACTACTTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA
Herbarium specimen Number		Date	
6		1847	AATTTCTCCAACAAAA-T-CTT-AACCTGGAATAGACATATTTGCTAATACATAAATAAA
22		1875	AATTTCTCCAACAAAACTACTTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA
33		1886	AATTTCTCCAACAAAACTACTTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA
31		1884	NATTTCTCCACC-NAANTT-TTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA
186882		1902	AATTTCTCC-ACA---CC-CTTGAAC---G-ATTGACATAT-TGCTAAT-CAT--ATAAA
186890		1906	AATTTCT-CNAC-AA-CC-CTTGANCCTGGAATAGACATATTTGCTNATACATAAATAAA
186875		1916	AATTTCTCCAACA-AACTAC-TGNACNTGGNAT-GACATATTTGCTAATACATAAATAAA
186864		1919	AATTTCT-C-AC-AAACTACTT-AACCTNGAAT-GACATATT--CTA--NCAT-N-T-AA
186869		1922	AATTTCTCCN-CAAAA-A-CTTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA
186872		1928	AATTTCTCCAACAAAACTACTTGAACCTGGAATAGACATATTTGCTAATACATAAATAAA

Figure 3 Amplified PCR products (P2F4/P2R4) from a variable region near the 3′ end of the *Nad4* gene in the P2 region of mtDNA from herbarium specimens infected with *P. infestans*. **a**, Herbarium specimens from England: 22, Vize, 1875; 31, Grove, 1884; 33, Vize, 1886 (lanes 2–4); and from Connecticut: 186875, 186869 and 186872 collected by G. P. Clinton between 1902 and 1928 (lanes 5–7); positive controls: mycelium of *P. infestans* (Myc) and dried infected modern lesion (+ leaf); negative controls: dried non-infected leaf (– leaf) and non-template (NT) control (lanes 8–11); 100-bp ladders in lanes 1 and 12. **b**, MtDNA sequences around the *Msp*I restriction site in P2 region. This restriction site is present in the Ib haplotype and absent in other extant haplotypes. See Supplementary Information and GenBank for full sequences.

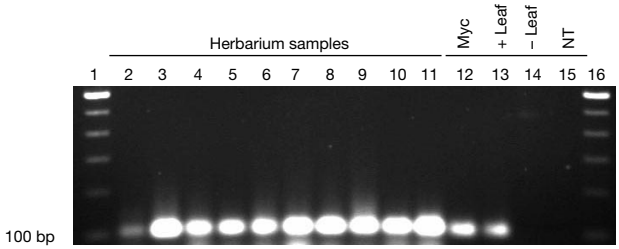


Figure 2 Amplified PCR products obtained using HERB1 and PINF from historic herbarium samples infected with *P. infestans* and collected between 1845 and 1886. PCR products from specimen: number 16, 71, 7, 47, 6, 53, 24, 22, 43, 33 (lanes 2–11); positive controls from mycelium of *P. infestans* (Myc), dried infected modern lesion (+ leaf) and negative controls from dried non-infected leaf (– leaf) and non-template (NT) control (lanes 12–15); 100-bp DNA molecular ladder (lanes 1 and 16). See Supplementary Information for specimen list and sequence data.

lesions in the samples assayed. Our data are not consistent with theories that the US-1 genotype was solely responsible for epidemics of potato late blight during the Irish potato famine and later in the 19th and early 20th centuries in the US and Europe[4,7,23]. Work is in progress to determine whether the lesions were caused by one of the other three extant haplotypes or some unique haplotype of *P. infestans*. Herbarium collections have not been used previously to understand epidemics of the past and track pathogen migrations. In fact, many important natural history collections have recently been critically underfunded and are in danger of being lost. Our work indicates that these historic collections can be valuable to epidemiologists and population geneticists who study plant diseases. Misleading data can be derived from studies that examine only extant phylogeographic patterns of genotypes of plant pathogens. It is important to use historic specimens when making inferences about historic populations. □

Methods

DNA extraction

Small pieces (2–3 mm^2) of dried tissue were placed in sterile 1.5-ml tubes, 150 ml extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.005 M EDTA, pH 7.5, 0.02 M sodium bisulphite) was added and tubes were vortexed. Nuclei lysis buffer (150 μl) containing 0.2 M Tris, 0.05 M EDTA, pH 7.5, 2.0 M NaCl and 2% CTAB (hexadecyltrimethylammonium bromide) was added followed by 60 μl of 5% sarkosyl (*N*-lauryl sarcosine). Tubes were vortexed then incubated at 65 °C for 15–30 min. One volume of chloroform:isoamyl alcohol (24:1) was added to each tube and centrifuged for 15 min at 13,000*g* at room temperature. The aqueous phase was removed to a new tube and the chloroform extraction was repeated. DNA was precipitated overnight at −20 °C in 0.1 volumes of 3 M sodium acetate, pH 8.0, and two volumes of cold 100% ethanol. The supernatant was discarded and the pellets were washed with 70% ethanol and then dried under vacuum centrifugation. DNA was resuspended in Te (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

PCR reactions

HERB1 (CGGACCGCCTGCGAGTCC) and PINF (CTCGCTACAATAGGAGGGTC) amplify a 100-bp region of rDNA in spacer region two, and PINF and ITS3 amplify an ~300-bp region of 5.8S and spacer region 2 DNA (see Supplementary Information). PCR reactions with PINF and HERB1 or PINF and ITS3 were conducted in 50-μl reaction volumes consisting of 1 μl of template DNA (1:10 dilution of original DNA extract in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)), 5 μl of 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 36.5 μl of sterile distilled water, 2 μl of 2.0 mM dNTPs (0.08 mM, Amersham Pharmacia), 2 μl of 10 mM $MgCl_2$ (0.4 mM, Sigma), and 2 μl each of a 10-mM primer (0.4 μM final concentration), and 0.4 μl of Taq (5 U μl^{-1}) DNA polymerase (2 units, Life Technologies). Cycling parameters were initial denaturation at 96 °C for 2 min, followed by 35 cycles consisting of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min followed. Aerosol-protected pipette tips and negative controls (master mix and no template DNA, or non-infected dried potato leaves) were used. All manipulations of positive control DNA (modern DNA of *P. infestans* from a pure culture or modern dried lesions on potato) were performed in a lab separate from the Phytotron lab where the herbarium samples were manipulated using the same master mix reagents. Amplified products were visualized on 1.6% agarose gels run in 1× TBE (0.5 μg ml^{-1} of ethidium bromide). All PCR reactions were repeated twice.

PCR amplifications of mtDNA with P2F4 (5′-ACCAATTGTTGCGAAAACAG-3′) and P2R4 (5′-ATTACGGCGGTTTAGCACAT-3′) were conducted in 30-μl reaction volumes consisting of 5 μl of template DNA (1:10 dilution of original DNA extract in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)), 3 μl of 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 9.77 μl of sterile distilled water, 3 μl of 2.0 mM dNTPs (200 μM) (Amersham Pharmacia), 8.25 μl of 10 mM $MgCl_2$ (2.75 mM) (Sigma), and 0.15 μl each of a 65-μM primer (0.325 μM), 0.48 μl of bovine serum albumin (10 mg ml^{-1}) and 0.2 μl of 5 U μl^{-1} Taq DNA polymerase (1 U) (Life Technologies). Positive and negative controls were used as described above. Cycling parameters were initial denaturation at 94 °C for 90 s, followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 1 min, and extension at 72 °C for 90 s. A final extension at 72 °C for 10 min followed. Amplified products were electrophoresed on 1.6% agarose gels containing 0.5 μg ml^{-1} of ethidium bromide with 1× TBE running buffer. All PCR reactions were repeated twice.

Received 29 November 2000; accepted 30 March 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

This research was supported in part by a grant from the National Geographic Society and in part by the North Carolina State University Agricultural Research Service and the North Carolina State University Office of International Programs. We thank K. May for producing the gels for final publication; J. Thomas, NCSU for use of the Phytotron Facility Laboratory; M. Chase, Jodrell Laboratory, Royal Botanic Gardens, Kew, England, for use of his lab facilities; and D. Pegler and B. Spooner, Mycology Herbarium, Royal Botanic Gardens, Kew, England; A. Rossman, US National Fungus Collections, USDA; M. Jebb, National Botanic Gardens, Glasnevin, Dublin, Ireland; and D. Pfister, Farlow Herbarium, Harvard University for providing access to specimens. Statistical advice from J. Thorne is appreciated.

Correspondence and requests for materials should be addressed to J.B.R. (e-mail: Jean_Ristaino@ncsu.edu). DNA sequence data can be found in GenBank under accession nos AF004277–AF004280 (ITS and rDNA of four modern isolates); AY027652–AY027655, AF349576–AF349587 (PINF/Herb amplification of spacer region 2 from four modern isolates and herbarium specimens); AY003908–AY003919 (mtDNA P2 region for three modern isolates of each haplotype); AF348598–AF348609 (mtDNA P4 region for three modern isolates of each haplotype); and AF349588–349601 (P2F4/R4 amplification of mtDNA from four modern haplotypes and herbarium specimens).