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Molecular characterization of *Ralstonia solanacearum* strains from Ethiopia and tracing potential source of bacterial wilt disease outbreak in seed potatoes

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Bacterial wilt, caused by *Ralstonia solanacearum*, is emerging as a major threat to potato production in Ethiopia, reaching epidemic proportions in the Chencha district recently, with a prevalence of 97% of potato fields in 2015. The recent disease outbreak in the district coincided with a significant introduction of seed potatoes. This research was therefore initiated to genetically characterize the pathogen so as to trace its source, identify its relationship with outbreaks in the rest of the country, and make intervention recommendations. *Ralstonia solanacearum* isolates were sampled both from seed and ware potato fields in Chencha and from seed potato fields in production regions suspected of being potential sources of the pathogen. Multiplex PCR and phylogenetic analysis of partial endoglucanase gene sequences identified all of the isolates as phylotype IIB sequevar 1. VNTR sequence analysis distinguished 11 different haplotypes, nine of which were unique to the Chencha district. However, one of the haplotypes was common to all seed potato producer regions of Ethiopia except for the Shashemene area. The unique and diverse VNTR haplotypes of the pathogen in Chencha indicates that it is well established in the district. When a geographical map of the VNTR haplotypes was superimposed with the main cross-regional seed potato distribution pattern of the country, it became evident that the pathogen was being disseminated via latently infected seed from the Holeta-Jeldu area in the Central Highlands of Ethiopia. Identification of largely uninfected highland districts and multiplication of high-grade seed potato exclusively in those districts should be given priority.

Keywords: molecular epidemiology, multilocus VNTR, *Ralstonia solanacearum*, seed potato

Introduction

Ralstonia solanacearum is a bacterial plant pathogen that causes lethal vascular wilt disease in a wide range of crops of economic importance (Hayward, 1994). It is considered one of the most destructive phytobacteria due to its lethality, persistence, wide host range and broad geographical distribution (Elphinstone, 2005; Mansfield *et al.*, 2012). Bacterial wilt of potato (*Solanum tuberosum*), caused by *R. solanacearum*, was first reported in Ethiopia in 1956 by Stewart (cited by Gorfu *et al.*, 2013) and is currently becoming the most important potato disease after late blight, caused by *Phytophthora infestans* (Lemaga *et al.*, 2005).

In the absence of a formal seed certification scheme and lack of regional quarantine measures in Ethiopia, bacterial wilt is becoming a very serious concern, threatening the potato industry (Gorfu *et al.*, 2013; Kassa & Chindi, 2013). This is unfortunate given the fact that the potato crop has great potential in achieving food security for an ever-growing human population in the country, which is expected to double by 2043 at the current yearly growth rate of 2.5% (World Bank, 2015). The disease is encroaching on the highlands that are supposed to serve as ideal niches for seed potato production, putting at risk the recent efforts by the International Potato Center (CIP) and the National Potato Program to introduce a Quality Declared Seed (QDS) scheme as an alternative to a formal seed certification scheme for smallholder seed producers (Schulz *et al.*, 2013). This scheme, which has already been legislated into a seed law, envisages zero tolerance for the bacterial wilt disease.

The Chencha district in the Gamo-Gofa Administrative Zone of the Southern Nations, Nationalities and Peoples Region of Ethiopia was selected recently to serve as a pilot for the 'Potato Centre of Excellence' initiative by Vita, an Irish NGO, in seed potato production, and to serve as a benchmark for best production practices. Potato is a very important crop in the Chencha district, with every

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household allocating a small plot of land for the crop every cropping season. Potato is produced in two production seasons in Chencha: the Belg season (short rain season: March to June), and the Meher season (long rain season: July to November), of which the Belg season constitutes the bulk of potato production, as the Meher season is conducive for late blight development.

Since 2010, hundreds of tonnes of seed tubers of improved varieties have been introduced to Chencha and the surrounding districts by aid agencies. However, in the last 2 years (2014–15), the bacterial wilt disease has appeared in epidemic proportions in almost all seed potato producers' fields, with increasing trends of both the prevalence and severity of the disease. The recently introduced potato varieties appear to be more severely affected than the local varieties, suggesting that bacterial wilt in these areas might have been introduced via seed of introduced potato varieties. Understanding the genetic diversity of the strains involved in the disease epidemic is important for epidemiological inferences.

Based on phenotypic properties, *R. solanacearum* has been historically divided into five races (related to the ability of the bacterium to wilt specific plant species) and six biovars (related to the ability of the bacterium to metabolize three sugar alcohols and three disaccharides; Fegan & Prior, 2005; Wicker *et al.*, 2012). Based on this classification scheme, only race 1 biovar 1 (with ability to wilt the Solanaceae family), race 3 biovar 2 (the potato brown rot strains), and race 4 biovar 3 (ginger wilting strains) were reported to be present in Ethiopia (Yaynu, 1989; Lemessa & Zeller, 2007; Kifelew *et al.*, 2015), race 3 biovar 2 being the dominant genotype infecting the potato crop. However, this classification system is deemed neither predictive nor phylogenetically meaningful for epidemiological inferences (Fegan & Prior, 2005; Siri *et al.*, 2011; N'Guessan *et al.*, 2013; Parkinson *et al.*, 2013). Race 4 biovar 3 strains, which are in the phylotype I hierarchical scheme of Fegan & Prior (2005), have recently been reclassified to *R. pseudosolanacearum* genospecies along with phylotype III strains of African origin (Safni *et al.*, 2014).

Lemessa *et al.* (2010) studied the genetic diversity of *R. solanacearum* strains of Ethiopia by repetitive element palindromic PCR (rep-PCR). The rep-PCR analysis identified two groups, corresponding to biovar 1 and biovar 2 strains at 55% similarity, and resolved Ethiopian biovar 2 isolates into four subgroups and biovar 1 isolates into a single group at 90% similarity. Lemessa *et al.* (2010) inferred that the biovar 2 and biovar 1 strains of Ethiopia belonged to phylotype II and phylotype III, respectively, of the hierarchical classification of Fegan & Prior (2005). However, despite the high repeatability and reproducibility within a laboratory (Louws *et al.*, 1994), rep-PCR is sometimes not reproducible among laboratories (Roumagnac *et al.*, 2006; Ramsubhag *et al.*, 2012), limiting the utility of the rep-PCR data of Lemessa *et al.* (2010) for this study. There are no other reports from Ethiopia relevant to understanding the molecular epidemiology of the current outbreak of bacterial wilt of potato.

It is not clear if the recent potato bacterial wilt disease outbreak in the Chencha district is due to a recent introduction of the pathogen along with seed tubers of improved varieties or to endemic disease. Endemic is used as defined by Porta (2008): 'the constant presence of a disease or infectious agent within a given geographic area or population group'. Such research questions warranted an objective answer to prevent further spread of the pathogen in the region. In this regard, N'Guessan *et al.* (2013) and Parkinson *et al.* (2013) have recently demonstrated the ability of multilocus variable number tandem repeat (VNTR) analysis to discriminate between the monomorphic sequevar 1 strains of *R. solanacearum*. The objective of this study was, therefore, to identify and genetically characterize *R. solanacearum* strains involved in the current disease outbreak, and possibly trace their source(s).

Materials and methods

Assessment of bacterial wilt disease prevalence and tuber sampling in Chencha

In order to assess the overall prevalence of the bacterial wilt disease and to obtain better epidemiological insight, the district was systematically divided into two categories prior to the field survey, in consultation with senior agronomists of the Chencha Office of Agriculture who were very familiar with the cropping history of the district: five *kebeles* (the smallest administrative unit with two or more villages) known for large-scale introduction of improved varieties and five *kebeles* known for production of mainly local farmers' potato varieties. Data were collected on the disease prevalence (defined as number of potato fields with at least a single wilted potato plant with characteristic bacterial wilt symptoms), and severity (defined as a proportion of wilted potato plants per potato field crop expressed as a percentage). Individual potato fields were selected by randomly walking through the selected *kebeles*. A total of 20 tubers, two from each of 10 wilting plants, were sampled per field in a single paper bag, changing gloves between potato fields, for laboratory confirmation and isolation of the pathogen.

Potential sources of latently infected seed potatoes, and tuber sampling

Historically, Holeta Agricultural Research Centre, along with the surrounding Holeta-Jeldu area in the Central Highlands and Haramaya University in the east, are known to be the main source of seed potato distribution in the country. Shashemene in the south serves as the main hub of ware potato distribution and is also long known as a hot spot of bacterial wilt of potato. However, the Holeta-Jeldu area plays by far the most important role in supplying seed potato across all regions of the country. Hence, survey and sampling of potato fields in this study for the isolation of *R. solanacearum* focused on those regions: seed potato fields in Haramaya, Kersa and Weter in the east, seed potato fields in Holeta, Jeldu and Wenchi in the Central Highland region, ware potato fields in Shashemene, and Tilili-Adet area in the northwestern part of Ethiopia. The tubers were sampled from visibly wilted potato plants/hills in separate paper bags and kept in cool dry conditions until isolation of the pathogen.

Isolation of *R. solanacearum*

Potato tuber samples from each sampled field were separately washed in tap water and then surface sterilized by dipping in 70% ethanol (EPPO, 1990; Sanchez Perez *et al.*, 2008). After surface disinfection, strips along the vascular ring were removed from the stolon end of each tuber and immediately immersed in extraction buffer (phosphate buffer pH 7) containing antioxidant (tetrasodium pyrophosphate, 1 g L^{-1}) in a maceration bag. After maceration, the samples were left to stand for 15 min to allow any bacteria present to be released into the extraction buffer. Bacteria present were then concentrated by centrifugation at 100 ***g*** at 4 °C for 10 min, followed by recentrifugation of the supernatant at 10 000 ***g*** at 4 °C for 10 min. The concentrated pellet was then resuspended in phosphate buffer (pH 7.2) and 500 μL of the resuspended pellet was used for streak plating on modified semiselective media (M-SMSA) (Elphinstone *et al.*, 1996) and incubated at 30 °C for 48 h. Presumptive *R. solanacearum* colonies were purified on tetrazolium chloride (TZC) solid media following proper aseptic techniques.

DNA extraction

DNA was extracted from the presumptive *R. solanacearum* pure cultures using DNeasy Blood and Tissue kit (QIAGEN) following manufacturer's recommendations. Of those extracts that were overgrown by other competing bacteria on the SMSA agar medium, total DNA was extracted from the resuspended bacterial pellets described above using the PowerSoil DNA Isolation kit (Mo Bio Laboratories, Inc.). The DNA isolation kit protocol was fully followed except that the samples in this case were resuspended bacterial pellets (20 μL) extracted from potato tubers instead of soil samples described in the kit protocol.

PCR detection and identification of *R. solanacearum*

The isolates were detected by using *R. solanacearum* species complex-specific primer pairs 759/760 (0.5 μM each per reaction; Opina *et al.*, 1997). PCR was performed in a total 50 μL reaction using 25 μL of Fermentas 2× PCR enzyme mix (Thermofisher Scientific), 5 μL of Q solution (5×; QIAGEN) and 25 ng gDNA on a 2720 thermal cycler (Applied Biosystems) with the following cycling conditions: initial denaturation for 15 min at 95 °C; 30 cycles of 30 s at 94 °C, 1 min at 59 °C and 1 min at 72 °C; and final extension of 10 min at 72 °C.

Identification at phylotype level was performed using multiplex PCR, combining the four phylotype-specific primer combinations (0.2 μM each per reaction) of Fegan & Prior (2005) and 759/760 (0.2 μM each) and 10 μL gDNA (130 ng) using the Quantitect Multiplex PCR kit (QIAGEN) on a 2720 thermal cycler. Thermal cycling conditions were the same as stated above for species detection. Representative reference strains of phylotype II and III obtained from Fera Science Ltd were included in the test and the phylotype of each isolate was determined according to the size of the amplified DNA (Table S1) after separating PCR amplicons on 1.5% agarose gel electrophoresis stained with GelRed.

For sequevar typing of the isolates, a 750 bp virulence-associated *endoglucanase* (egl) gene fragment was amplified from gDNA using Endo-F and Endo-R primers (Fegan & Prior, 2005). The PCR was run using Fermentas PCR mix (2×), 0.5 μM each Endo-F and Endo-R, and 25 ng gDNA in a total of 50 μL. The PCR was run on a Tprofessional Basic Gradient thermal cycler (Biometra) with the following cycling conditions: initial denaturation of 9 min at 96 °C; 30 cycles of 1 min at 95 °C, 1 min at 70 °C, 2 min at 72 °C; and final extension of 10 min at 72 °C. The PCR product was then cleaned using Wizard SV Gel and PCR Clean-up system (Promega) following the kit protocol before being sent to a sequencing service (Eurofins Genomics). The same Endo-F and Endo-R primers were used for sequencing.

The generated sequences from both strands were assembled and manually edited, combined into a multifasta file along with phylotype II *egl* reference sequences from the NCBI database, covering known genetic diversity within *R. solanacearum* genospecies (formerly phylotype II), and aligned with CLUSTALW multiple alignment tool in BIOEDIT sequence alignment software. The aligned sequences were then used to generate a phylogenetic tree in MEGA V. 6 (Tamura *et al.*, 2013). The phylogenetic tree was constructed from the genetic distance data using the neighbour-joining method (Saitou & Nei, 1987) with 1000 bootstrap resampling of data to test tree topology (Felsenstein, 1985). The evolutionary distances were computed using the maximum composite likelihood method of Kumar *et al.* (2004). Assignment to sequevars was done by comparing test strain clustering with a range of identified sequevar reference strains of Wicker *et al.* (2012). In addition to the phylogenetic tree, sequevar 1 assignment of the study isolates was also confirmed by running individual *egl* sequences on a recently published computer program of Stulberg & Huang (2016).

Determination of VNTR profiles

A PCR and sequencing approach described by Parkinson *et al.* (2013) was used to determine six tandemly repeated sequences from five different loci (L504, L539, L540, L563 and L578) and two additional loci (IPO100, IPO4134) of N'Guessan *et al.* (2013). Each of the first five loci was amplified separately with 25 ng gDNA and 0.3 μM each of forward and reverse primers of Parkinson *et al.* (2013), except for the reverse primer of L540 (Table S2). Long PCR enzyme mix (ThermoFisher Scientific), and Fermentas PCR Master mix (ThermoFisher Scientific) were used for amplifying L504, L539, L563, and L540, L578, respectively. The PCR was run on a 2270 thermal cycler with cycling conditions of initial denaturation of 2 min at 94 °C; 34 cycles of 45 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C; and final extension of 10 min at 72 °C. The two additional VNTR loci IPO100 and IPO4134 of N'Guessan *et al.* (2013) were amplified in 50 μL reactions using 25 μL of Fermentas PCR Master Mix, 0.3 μM each of forward and reverse primers, and 25 ng gDNA in a total of 50 μL. PCR was run on a C1000 thermal cycler (Bio-Rad) with cycling conditions of initial denaturation of 5 min at 96 °C; 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C; and final extension of 10 min at 72 °C. The amplified PCR products were cleaned with Wizard SD gel and PCR clean-up system and sequenced by a sequencing service (Eurofins Genomics) using sequencing primers indicated in Table S2.

The tandem repeat sequences were counted manually from the determined sequences using the standard start and finish reference sequences defined by Parkinson *et al.* (2013), except for the pre-repeat sequence at L540; all of the study strains including the reference strain NCPPB 4212 generated the pre-repeat sequence of TCGGCTGAGGCGGCCG at this locus.

Results

Bacterial wilt disease prevalence in Chencha

The average field plot area in the district planted with potato in the survey season was 565 m^2. Out of the 100

potato fields surveyed, the prevalence of the disease was 97%. The overall incidence of the disease in the district ranged from less than 0.06% in a village where there was no significant introduction of recently introduced potato varieties, to 96.6% in one of the first recipients of the new seed. On average, potato fields planted with seed potatoes recently introduced from the Holeta-Jeldu area were more severely affected, with 7.97% of plants per field wilted at flowering stage compared to 1.16% for local varieties.

Detection and identification of *R. solanacearum* isolates

Multiplex PCR for the four phylotypes of the species complex (Fegan & Prior, 2005) along with species complex specific 759/760 primer pairs of Opina *et al.* (1997) identified all the tested isolates collected from Chencha as well as all the major seed potato producing regions of Ethiopia as *R. solanacearum* phylotype II (Fig. 1).

Sequevar typing

Phylogenetic analysis of the partial *egl* sequences clustered all the phylotype II isolates with reference sequences of phylotype II B sequevar 1 strains (Fig. 2) with a high bootstrap value (96%) to support the typology of the tree with long branch length from the nearest clusters. The isolates' partial *egl* sequence was also individually verified as belonging to sequevar 1 by the computer program of Stulberg & Huang (2016).

VNTR sequence analysis

For meaningful epidemiological inferences about the likely geographical transmission pattern of the highly monomorphic sequevar 1 strains (Wicker *et al.*, 2012; N'Guessan *et al.*, 2013; Parkinson *et al.*, 2013), isolates representing historically known main hubs of seed potato distribution in Ethiopia were included in the VNTR analysis, namely Holeta Research Centre and its surrounding districts in Central Ethiopia, Haramaya University and nearby districts in eastern Ethiopia, and Shashemene district in southern Ethiopia. An isolate was also included from northwestern Ethiopia isolated from an irrigated potato crop.

The five VNTR loci containing six tandemly repeated sequences of Parkinson *et al.* (2013) and VNTR locus IPO100 of N'Guessan *et al.* (2013) resolved the sequevar 1 strains into 11 haplotypes (Table 1). Eight of these haplotypes (P1, P3, P4, P5, P6, P7, P8 and P9), and haplotype P11 were unique to isolates collected from Chencha and Shashemene districts, respectively. However, haplotype P2 was common for all the regions surveyed for this study across the country except for Shashemene district. Locus IPO4134 of N'Guessan *et al.* (2013) generated the target sequence in all Ethiopian strains in this study but not in tandem repeats as reported in the article, hence was not useful in discriminating the strains.

Discussion

The genetic diversity of *R. solanacearum* isolates involved in the current bacterial wilt disease outbreak among seed potato growers across Ethiopia was studied at sequential levels of molecular analysis, from phylotype identification with multiplex PCR for the species complex, phylogenetic analysis for sequevar typing using partial *egl* sequences, and multilocus VNTR sequence analysis to discriminate between the otherwise clonal (monomorphic) sequevar 1 strains (Wicker *et al.*, 2012; N'Guessan *et al.*, 2013; Parkinson *et al.*, 2013).

The identification of all *R. solanacearum* isolates in this study as phylotype II B sequevar 1 strains is in agreement with reports of Yaynu (1989), Lemaga *et al.* (2005) and Kassa & Chindi (2013) who reported race 3 biovar 2 from Ethiopia which belongs to phylotype II, but in contrast to Lemessa & Zeller (2007) who also reported race 1 biovar 1 from Ethiopia. Fegan & Prior

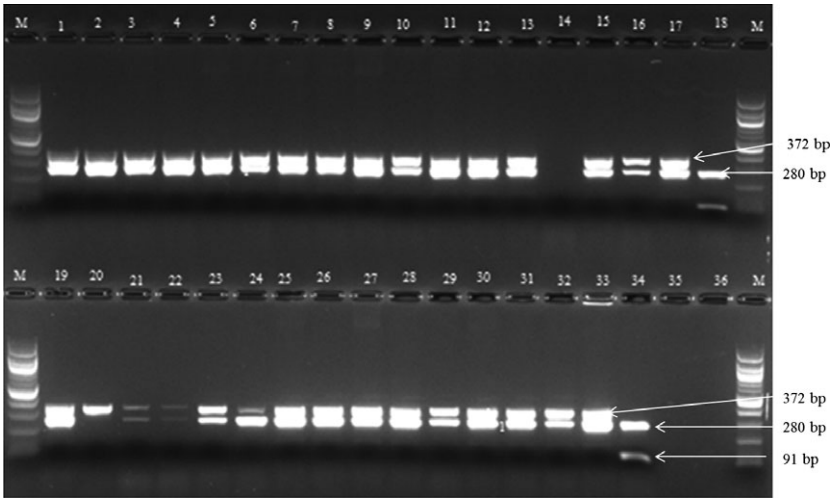

Figure 1 Detection and identification of *Ralstonia solanacearum* strains. Lane M, molecular marker (100 bp; New England Biolabs); lane 36, negative control; lanes 17 and 33, phylotype II (NCPPB4212) positive control; lanes 18 and 34, phylotype III (NCPPB 1018) positive control; lanes 1–16, 19–32, Ethiopian isolates (this study).

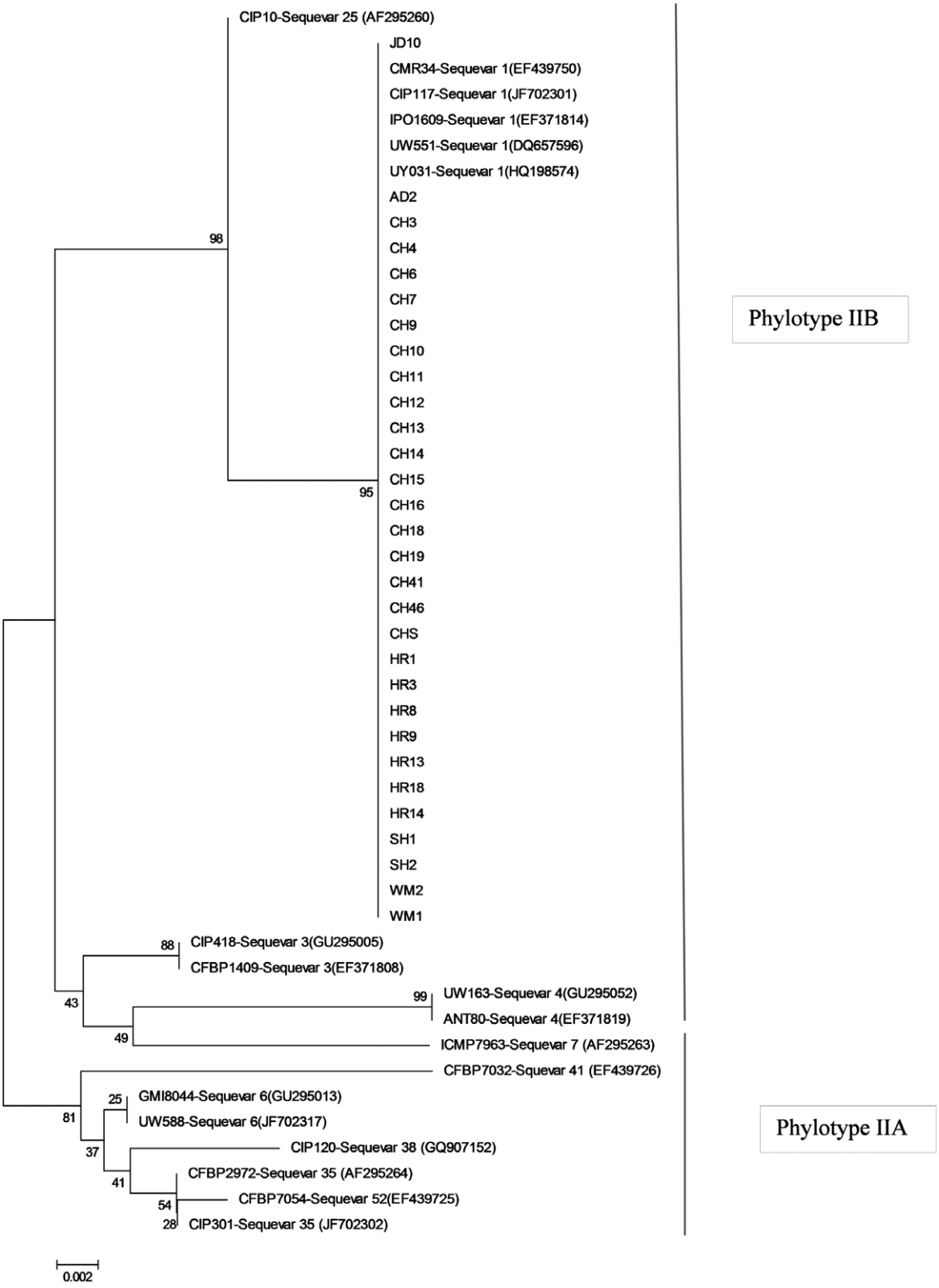

Figure 2 Phylogenetic neighbour-joining tree of partial endoglucanase (*egl*) sequences of 30 *Ralstonia solanacearum* isolates from Ethiopia and 18 reference strains from *R. solanacearum* obtained from NCBI database. The numbers at branch points are bootstrap percentages of 1000 resamplings in which the clusters were found. The scale bar represents 0.2% sequence dissimilarity (one nucleotide substitution per 500 nucleotides). Sequevar typing based on Wicker *et al.* (2012) and Siri *et al.* (2011).

Table 1 VNTR haplotypes of *Ralstonia solanacearum* phylotype IIB sequevar 1 isolates from Ethiopia

Isolate	Origin	Host	Year	Tandem repeat copy number[a]								VNTR haplotype
				L504	L539	L540	L563	L578-L	L578-R	IPO100	IPO4134	
Ch3	Chencha, south Ethiopia	Potato	2015	8	5	12	7	2	6	5	1	P1
ChS	Chencha, south Ethiopia	Soil	2015	8	5	12	7	4	6	5	1	P2
Ch6	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
Ch10	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
HR1	Haramaya, east Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
Ch14	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
JD3	Jeldu, central Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
JD10	Jeldu, central Ethiopia	Potato	2015	8	5	12	7	4	6	6	1	P10
WM1	Holeta, central Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
Ch12	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
HR20	Haramaya, east Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
AD2	Tilili, northwest Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
Ch16	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
Ch4	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	4	1	P3
Ch19	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
Ch41	Chencha, south Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
HR13	Water, east Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
HR14	Haramaya, east Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
WM2	Holeta, central Ethiopia	Potato	2015	8	5	12	7	4	6	5	1	P2
Ch7	Chencha, south Ethiopia	Potato	2015	8	?	12	8	4	6	5	1	P4
Ch15	Chencha, south Ethiopia	Potato	2015	7	5	12	8	4	6	6	1	P5
Ch18	Chencha, south Ethiopia	Potato	2015	8	6	12	6	4	6	4	1	P6
Ch11	Chencha, south Ethiopia	Potato	2015	8	4	12	6	4	6	5	1	P7
Ch13	Chencha, south Ethiopia	Potato	2015	8	4	10	7	4	6	5	1	P8
Ch17	Chencha, south Ethiopia	Potato	2015	8	5	10	7	4	6	5	1	P9
Sh1	Shashemene, south Ethiopia	Potato	2015	7	3	12	7	4	6	5	1	P11
Sh2	Shashemene, south Ethiopia	Potato	2015	7	3	12	7	4	6	5	1	P11
N4212[b]	Kenya	Pelargonium	2001	9	5	12	7	5	6	ND	ND	ND
P6056[c]	UK	Bittersweet	1994	8	5	12	7	4	6	ND	ND	ND
P3854[c]	UK	Potato	1992	8	5	12	6	4	6	ND	ND	ND

ND, not determined.

[a]Repeat sequence L504: CTTGCCG; L539: GCTGCCCTGCGCATT; L540: TCGGTGAG; L563: TCTAGCC; L578-L: CCCAAG; L578-R: TCCGAG; IPO100: AGTGCCC; IPO4134: GTAGCC. Post-repeat sequence IPO100: GGTGCCCGGTGCCCGGTGCCC; IPO4134: TTCCGGGCTGAC.

[b]Reference strain (NCPPB, Fera, UK).

[c]Parkinson *et al.* (2013).

(2005) grouped biovar 1 strains into phylotype II/sequevar 3–7 and phylotype III/sequevar 19, 22 and 23. However, Lemessa *et al.* (2010) deduced that the Ethiopian biovar 1 strains they identified should probably be placed in phylotype III, which was recently placed into the *R. pseudosolanacearum* genospecies (Safni *et al.*, 2014). The assertion that the Ethiopian biovar 1 strains belong to phylotype III needs further investigation as it is also probable that those isolates were from phylotype IIA; phylotype IIA being the more likely case if they were in fact a result of introduction of potato genotypes by CIP, as argued by Lemessa & Zeller (2007). However, either way, the present study is indicative that race 1 biovar 1 strains were not the key players in the current wide-scale bacterial wilt disease epidemic on surveyed seed potato fields across Ethiopia.

Differentiation of strains of monomorphic bacterial pathogens mainly relies on molecular markers on repetitive DNA rather than sequence data (Reyes *et al.*, 2012). VNTR typing is based on short sequences that are repeated tandemly and are located throughout bacterial genomes. VNTR loci mutate by either increasing or decreasing these repeats, probably mainly driven by DNA replication folk error (DNA replication slippage; Reyes & Tanaka, 2010). The epidemiological inference from VNTR typing is based on the consideration that identical molecular fingerprints from different isolates are epidemiologically linked (Reyes & Tanaka, 2010).

Sequevar 1 strains of *R. solanacearum* are reported to be clonal (monomorphic) across the globe (Wicker *et al.*, 2012; N'Guessan *et al.*, 2013; Parkinson *et al.*, 2013). The six tandemly repeated sequence markers of Parkinson *et al.* (2013) resolved all sequevar 1 isolates in this study into nine VNTR haplotypes, in which the most common haplotype was identical to P6056 isolated from bittersweet (*Solanum dulcamara*) in the UK (Parkinson *et al.*, 2013). However, the addition of VNTR locus IP0100 of N'Guessan *et al.* (2013) increased the discriminatory power of VNTR loci by further resolving the most common VNTR haplotype into three (Table 1).

This is in agreement with the assertion that the higher the number of loci used, the higher the discrimination among isolates, resulting in a number of smaller clusters of identical profiles in a sample (Aandahl *et al.*, 2012).

Shashemene district in the south of Ethiopia has long served as the main hub of seed and ware potato marketing (Emana & Nigussie, 2011) and has long been implicated in the dissemination of bacterial wilt of potato, given the apparent prevalence of the disease in the district. It is remarkable that *R. solanacearum* strains with VNTR profile P11 isolated from the most widely grown potato variety (Nech Abeba) in Shashemene district (Emana & Nigussie, 2011; Kolech *et al.*, 2015) were not isolated from other sampled regions in this study.

The unique diversity of the pathogen within phylotype IIB sequevar 1 in Chencha district suggests that it evolved independently of other strains from other regions of the country and hence is an endemic pathogen in the area, given the long tradition of potato growing in the district. It is also likely that there were multiple introductions of the pathogen, as the district was one of the first locations to receive potato genotypes introduced by CIP in the mid-1970s (Kidane-Mariam, 1979) and known for introduction of seed potatoes by aid agencies for years. However, the clear pattern of the higher severity of wilt disease in the villages known for the newly introduced varieties, coupled with a high frequency (0.47) of VNTR haplotype P2 isolates in the district, suggests that there were also recent introductions of haplotype P2 strains with latently infected seed.

Interestingly, VNTR haplotype P2 is shared between seed potato fields of the key potato breeding and dissemination institutions of the country: Haramaya University and its surroundings in the east and Holeta Research Centre and its surroundings in the Central Highlands of Ethiopia. There are two hypotheses as to why they share the same strains of *R. solanacearum* linked with the current bacterial wilt disease outbreak across the country. Haramaya University used to receive advanced potato clones from Holeta Research Centre, hence probably introducing the pathogen by latently infected tubers. The pathogen might have also been transferred from Haramaya to Holeta with tubers when the potato breeding programme was transferred from Haramaya to Holeta more than three decades ago with germplasm transfer (T. Tsegaw, Haramaya University, Ethiopia, personal communication). The first hypothesis is more likely because there is an active seed potato transfer from Holeta to the surroundings of Haramaya but not vice versa. This is further strengthened by the fact that early field generations of the supposedly 'high quality' seed potatoes at Holeta Research Centre tested positive for *R. solanacearum* at high infection rate during this study. The disease might have established long ago in the centre, as they have been practising roguing wilted plants from seed lots for years, whilst continuing to be the main source of seed for multiplication by seed cooperatives across the country. This becomes very apparent when the geographical map of the VNTR haplotypes is superimposed with the main cross-regional seed potato distribution pattern of the country (Fig. 3).

The Holeta-Jeldu area in central Ethiopia and Haramaya and its surrounding districts in eastern Ethiopia are the main suppliers of quality seed potato to small-scale seed potato producers elsewhere in the country. To the authors' understanding, the recently legislated QDS

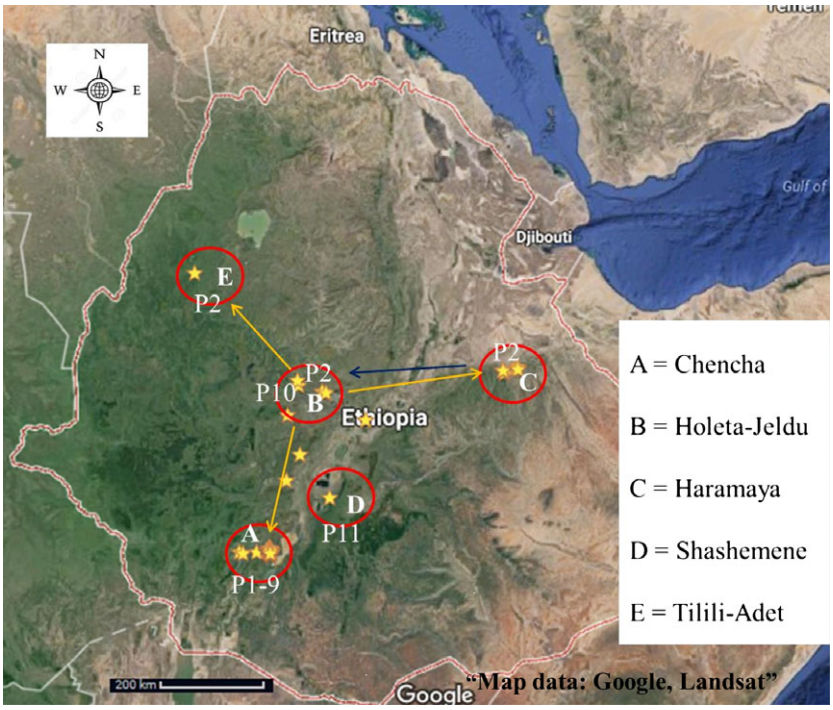

Figure 3 Geographical map of *Ralstonia solanacearum* VNTR haplotypes. Yellow arrows indicate the current main cross-regional seed potato distribution pattern while the blue arrow represents historical potato germplasm transfer to Holeta. A, Chencha; B, Holeta-Jeldu area; C, Haramaya; D, Shashemene area; E, Tilili-Adet area. The stars represent sampling sites for the *R. solanacearum* isolates in this study. P1–P11, VNTR haplotypes.

scheme envisages the use of either those seed cooperatives as seed source in those key regions or new seed producers with seed supplied from those regions. However, the feasibility of the scheme is questionable as the disease was confirmed in 92% of surveyed seed potato producer cooperatives (Parker *et al.*, 2016) and in research institutions that are supposed to supply high-grade seed to those cooperatives.

It is very difficult to produce pathogen-free seed potatoes in places where bacterial wilt of potato is widespread, i.e. where the pathogen is already established and long crop rotations with either non-host and/or *R. solanacearum*-suppressive crops are unlikely. Cleaning of the basic seed stock from the pathogen at the research institutions should be given priority along with identification of pathogen-free highland districts and/or sites for quality seed potato multiplication. In the short term it is also advisable (safer) to exclusively restrict high quality seed production to the newly identified pathogen-free districts.

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References

Aandahl RZ, Reyes JF, Sisson SA, Tanaka MM, 2012. A model-based Bayesian estimation of the rate of evolution of VNTR loci in *Mycobacterium tuberculosis*. *PLoS Computational Biology* **8**, e1002573.

Elphinstone JG, 2005. The current bacterial wilt situation: a global overview. In: Allen C, Prior P, Hayward AC, eds. *Bacterial Wilt Disease and the* Ralstonia solanacearum *Species Complex*. St Paul, MN, USA: APS Press, 9–28.

Elphinstone JG, Hennessy J, Wilson JK, Stead DE, 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *EPPO Bulletin* **26**, 663–78.

Emana B, Nigussie M, 2011. *Potato Value Chain Analysis and Development in Ethiopia: Case of Tigray and SNNP Regions*. Addis Ababa, Ethiopia: International Potato Center (CIP-Ethiopia).

EPPO, 1990. EPPO Standards PM 3/26 Phytosanitary Procedures: *Ralstonia solanacearum*, inspection and test methods. *EPPO Bulletin* **20**, 255–62.

Fegan M, Prior P, 2005. How complex is the *Ralstonia* species complex? In: Allen C, Prior P, Hayward AC, eds. *Bacterial Wilt Disease and the* Ralstonia solanacearum *Species Complex*. St Paul, MN, USA: APS Press, 449–61.

Felsenstein J, 1985. Phylogenies and the comparative method. *The American Naturalist* **125**, 1–15.

Gorfu D, Woldegiorgis G, Kassa B, 2013. Bacterial wilt: an emerging threat to Ethiopian potato industry. In: Woldegiorgis G, Schulz S, Berihun B, eds. *Seed Potato Tuber Production and Dissemination Experiences, Challenges and Prospects: Proceedings of the National Workshop on Seed Potato Tuber Production and Dissemination 12–14 March 2012*. Bahir Dar, Ethiopia: EIAR/ARARI, 211–22.

Hayward AC, 1994. The hosts of *Pseudomonas solanacearum*. In: Hayward AC, Hartman GL, eds. *Bacterial Wilt: The Disease and its Causative Agent,* Pseudomonas solanacearum. Wallingford, UK: CAB International, 9–24.

Kassa B, Chindi A, 2013. Seed tuber cycle and latent infection for the spread of potato bacterial wilt *Ralstonia solanacearum* (Smith) – a threat for seed production in Ethiopia. *Asian Journal of Plant Pathology* **7**, 74–83.

Kidane-Mariam H-M, 1979. Preliminary assessment of the responses of potato genotypes in the eastern, southern and central regions of Ethiopia. *Ethiopian Journal of Agricultural Sciences* **1**, 41–7.

Kifelew H, Kassa B, Sadessa K, Hunduma T, 2015. Prevalence of bacterial wilt of ginger (*Z. officinale*) caused by *Ralstonia solanacearum* (Smith) in Ethiopia. *International Journal of Research Studies in Agricultural Sciences* **1**, 14–22.

Kolech SA, Halseth D, De Jong W *et al.*, 2015. Potato variety diversity, determinants and implications for potato breeding strategy in Ethiopia. *American Journal of Potato Research* **92**, 551–66.

Kumar S, Tamura K, Nei M, 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**, 150–63.

Lemaga B, Kakuhenzire R, Kassa B, Ewell PT, Priou S, 2005. Integrated control of potato bacterial wilt in Eastern Africa: the experience of African Highland Initiative. In: Allen C, Prior P, Hayward AC, eds. *Bacterial Wilt Disease and the* Ralstonia solanacearum *Species Complex*. St Paul, MN, USA: APS Press, 145–57.

Lemessa F, Zeller W, 2007. Isolation and characterisation of *Ralstonia solanacearum* strains from Solanaceae crops in Ethiopia. *Journal of Basic Microbiology* **47**, 40–9.

Lemessa F, Zeller W, Negeri D, 2010. Genetic diversity among strains of *Ralstonia solanacearum* from Ethiopia assessed by repetitive sequence-based polymerase chain reaction (rep-PCR). *Ethiopian Journal of Agricultural Science and Technology* **1**, 17–26.

Louws FJ, Fulbright DW, Stephens CT, De Bruijn FJ, 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* **60**, 2286–95.

Mansfield J, Genin S, Magori S *et al.*, 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology* **13**, 614–29.

N'Guessan CA, Brisse S, Le Roux-Nio A-C, Poussier S, Koné D, Wicker E, 2013. Development of variable number of tandem repeats typing schemes for *Ralstonia solanacearum*, the agent of bacterial wilt, banana Moko disease and potato brown rot. *Journal of Microbiological Methods* **92**, 366–74.

Opina N, Tavner F, Hollway G *et al.*, 1997. A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *Asia-Pacific Journal of Molecular Biology and Biotechnology* **5**, 19–30.

Parker ML, Ochieng B, Atieno E, Sharma K, Schulte-Geldermann E, 2016. Bacterial wilt: the major threat to potato in sub-Saharan Africa. In: *Proceedings of the 6th International Bacterial Wilt Symposium 3–7 July 2016*. Toulouse, France: Laboratory of Plant Microbe Interaction, 15.

Parkinson N, Bryant R, Bew J *et al.*, 2013. Application of variable-number tandem-repeat typing to discriminate *Ralstonia solanacearum* strains associated with English watercourses and disease outbreaks. *Applied and Environmental Microbiology* **79**, 6016–22.

Porta M, 2008. *A Dictionary of Epidemiology*. New York, NY, USA: Oxford University Press.

Ramsubhag A, Lawrence D, Cassie D *et al.*, 2012. Wide genetic diversity of *Ralstonia solanacearum* strains affecting tomato in Trinidad, West Indies. *Plant Pathology* **61**, 844–57.

Reyes JF, Tanaka MM, 2010. Mutation rates of spoligotypes and variable numbers of tandem repeat loci in *Mycobacterium tuberculosis*. *Infection, Genetics and Evolution* **10**, 1046–51.

Reyes JF, Chan CHS, Tanaka MM, 2012. Impact of homoplasy on variable numbers of tandem repeats and spoligotypes in *Mycobacterium tuberculosis*. *Infection, Genetics and Evolution* **12**, 811–8.

Roumagnac P, Gagnevin L, Pruvost O, Achtman M, 2006. Insights into structure and evolution of bacterial species that are revealed by molecular methods. In: Tibayrenc M, ed. *Encyclopedia of Infectious Diseases*. Hoboken, NJ, USA: John Wiley & Sons Inc, 475–94.

Safni I, Cleenwerck I, De Vos P, Fegan M, Sly L, Kappler U, 2014. Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **64**, 3087–103.

Saitou N, Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–25.

Sanchez Perez A, Mejia L, Fegan M, Allen C, 2008. Diversity and distribution of *Ralstonia solanacearum* strains in Guatemala and rare occurrence of tomato fruit infection. *Plant Pathology* **57**, 320–31.

Schulz S, Woldegiorgis G, Hailemariam G, Aliyi A, Van De Haar J, Shiferaw W, 2013. Sustainable seed potato production in Ethiopia: from farm-saved seed to quality declared planting material. In: Woldegiorgis G, Schulz S, Berihun B, eds. *Seed Potato Tuber Production and Dissemination Experiences, Challenges and Prospects: Proceedings of the National Workshop on Seed Potato Tuber Production and Dissemination 12–14 March 2012*. EIAR/ARARI: Bahir Dar, Ethiopia, 60–71.

Siri MI, Sanabria A, Pianzzola MJ, 2011. Genetic diversity and aggressiveness of *Ralstonia solanacearum* strains causing bacterial wilt of potato in Uruguay. *Plant Disease* **95**, 1292–301.

Stulberg MJ, Huang Q, 2016. A computer program for fast and easy typing of a partial endoglucanase gene sequence into genospecies and sequevars 1 & 2 of the *Ralstonia solanacearum* species complex. *Journal of Microbiological Methods* **123**, 101–7.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA 6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–9.

Wicker E, Lefeuvre P, De Cambiaire J-C, Lemaire C, Poussier S, Prior P, 2012. Contrasting recombination patterns and demographic histories of the plant pathogen *Ralstonia solanacearum* inferred from MLSA. *The ISME Journal* **6**, 961–74.

World Bank, 2015. Population growth: Ethiopia overview. [http://data.worldbank.org/indicator/SP.POP.GROW?locations=ET]. Accessed 27 January 2016.

Yaynu H, 1989. Characteristics of isolates of *Pseudomonas solanacearum* in Ethiopia. *Ethiopian Journal of Agricultural Sciences* **11**, 7–13.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1 Primers used in the phylotype specific multiplex PCR.

Table S2 Primers used for amplifying and sequencing VNTR loci.