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DNA Analysis of *Ralstonia solanacearum* Based on 270-bp PCR-amplified Fragment from the Lowlands and Highlands of Kenya

E. K. Kago[1*], Z. M. Kinyua[2], J. M. Maingi[1] and P. O. Okemo[1]

[1]Department of Microbiology, Kenyatta University, P.O.Box 43844-00100, Nairobi, Kenya.
[2]Plant Pathology Section, KALRO-NARL Kabete, P.O.Box 57811-00200, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration between all authors. Author EKK did the sample collection, Isolated, analyzed data and prepared the first draft. Author ZMK provided the working protocols for genetic analysis and guided (Supervised) the activity. Authors JMM and POO provided general guidance on the experiment and edited the manuscript. All authors read and approved the manuscript.

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Original Research Article

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ABSTRACT

Aims: Bacterial wilt, caused by *Ralstonia solanacearum*, is a devastating disease resulting in tremendous losses of economic crops such as plants in the Solanaceae. Studies have shown that *R. solanacearum* is spreading from the lowlands to the highlands. This has seen increased need for understanding the genetic diversity of *R. solanacerum* strains common in these areas as a basis for better strategies in their control.
Methodology: Sixty-nine bacteria isolates obtained from various wilting plant hosts (Tomato, capsicum and potato) from 11 different sites in Nyeri, Nyahururu, Kirinyaga, Kiambu, Nakuru Murang'a and Embu were subjected to molecular analysis.
Results: All the bacteria isolates were confirmed to be *R. solacearum* following PCR amplification of about 270-bp fragment using specific primers OLI 1-F and Y2-R. Based on the targeted 16 S rDNA sequences using primers OLI 1 and Y2, the 69 bacteria isolates had 98 to 100% identity with other

**Corresponding author: E-mail: kagoelizabeth96@gmail.com;*

DNA sequences for *R. solanacearum* isolates deposited in the NCBI database. Analysis of genetic differentiation showed there were total of 26 haplotypes from the 11 studied populations. The total number of segregating sites in all populations was 225.

Conclusion: Through this study, it was realized that the main causative agent of bacterial wilt in potatoes, tomatoes and capsicum grown in the lowland and highland regions in Kenya is *R. solanacearum*. The isolates are in two main groups (Cluster A and B) that represent mainly the phylotypes I and II respectively.

Keywords: Bacterial wilt; haplotypes; Kenya; phylotype; polymerase chain reaction.

ABBREVIATIONS

0°C : Degrees Celsius
AFLP : Amplified Fragment Length Polymorphism
BLAST : Basic Local Alignment Test
CPG : Casamino acid-Peptone-Glucose (CPG) medium
NCBI : National Center for Biotechnology Information
PCR : Polymerase Chain Reaction
RFLP : Restriction Fragment Length Polymorphisms
REP-PCR : Repetitive Extragenic Palindromic
SMSA-E : Selective Medium South Africa - Elphinstone)

1. INTRODUCTION

Ralstonia solanacearum is a gram- negative bacteria found in the soil that causes wilting disease in crops that are of economic importance in the tropics. The bacteria infect over 50 plant families and causes bacterial wilting of more than 250 plant species [1,2,3]. *R. solanacearum* infects the plants through the roots and has the ability to survive in the soil, water and infected plants [3]. They have a wide geographical distribution and host range and pathogenicity [4].

Ralstonia solanacearum, widely distributed in tropical, subtropical, and temperate regions of the world, is a complex species with considerable diversity [5-6]. Members of *R. solanacearum* belong to a species complex (*R. solanacearum* species complex (RSSC)) with a varied population of isolates [7]. Earlier studies have focused on the global genetic diversity of the pathogen. Early attempts have subdivided the *R. solanacearum* strains into five races and six biovars. Based on the host range, *R. solanacearum* has been phenotypically classified into five races: Race 1 (solanaceous vegetables), Race 2 (banana), Race 3 (potato and tomato from temperate regions), Race 4 (ginger), and Race 5 (mulberry) [8-9]. *R. solanacearum* species have been grouped into four phylotypes based on their geographic origin, which include phylotype I from Asia, phylotype II from America, phylotype III from Africa, and phylotype IV from Indonesia-Japan [7].

To improve the understanding of the genetic relationship among the *R. solanacearum* species complex, DNA-based analyses have been used. These include restriction fragment length polymorphisms (RFLP), polymerase chain reaction (PCR) -RFLP, 16S rRNA gene sequence analysis, repetitive extragenic palindromic (REP)-PCR, and amplified fragment length polymorphism (AFLP) [10,11,12,13,14].

Some reports have shown that *R. solanacearum* is spreading from the lowlands to the highlands and cold areas as a result of the changed climate, ecological environment, and planting structure adjustment [15-16]. As examination of the diversity of the strains increased, it became clear that the *R. solanacearum* strains had four major phylotype sub-divisions [17]. Understanding local pathogen diversity is the foundation for a successful breeding and integrated management programme [18]. The objective of this study was to carry out DNA analysis of *R. solanacearum* based on 270-bp pcr-amplified fragment from the lowlands and highlands of Kenya.

2. MATERIALS AND METHODS

2.1 Sample Collection

Plant samples for *R. solanacearum* isolation were collected from areas where various solanacea crops are highly cultivated for both subsistence and commercial production in Kenya**.** These areas included the highland and lowlands of Embu (Embu lowland-EBL, Embu highland-EBL), Nyeri (Nyeri lowland-NYEL), Kiambu (Kiambu lowland-KBL, Kiambu highland – KBH), Nyandarua (Nyandarua highland - NYRH, Nyandarua low land-NYRL), Murang'a

(Murang'a lowland-MRL, Murang'a highland - MRH), Nakuru (Nakuru highland –NKRH) and Kirinyaga (Kirinyaga highland –KRH, Kirinyaga lowland –KRL) counties (Fig. 1). The solanaceae crops sampled in this study include; Tomato, potato, capsicum and black night shade.

2.2 Isolation, Identification, Purification of *R. solanacearum*

The wilted plant stem segment samples and diseased tubers collected from the fields were washed under running tap water to remove adhering soil. The stem of the infected potato plants and diseased potato tubers were surface sterilized with 70% alcohol and cut into two halves and the cut ends were dipped into water in test tubes and beakers respectively. After half an hour bacterial ooze from the infected stem into the water was observed. To culture the bacterium a loop full of water was streaked on SMSA-E (Selective Medium South Africa - Elphinstone) media. The plates were then incubated at 28°C for 24 hours. After isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Casamino peptone agar (CPG) Basal media.

2.3 DNA Extraction

Pure Bacteria cultures growing on solid Casamino acid-Peptone-Glucose (*CPG*) medium were used for DNA extraction. Ten loops full of pure colonies of the bacteria culture were put in 500 µl of normal saline in 1.5 ml Eppendorf tubes. This was followed by vortexing at maximum speed for 30 s. The samples were then centrifuged at 13000rpm for 10 minutes followed by decanting of the normal saline. Four hundred microliters of CTAB lysis buffer was then added followed by vortexing until the cells were uniformly mixed. The samples were then incubated in a water bath at 65°C for 1 hour with intermittent inversions. After one-hour, chilled Chloroform Isoamyl (Ratio 24:1) was added and the samples mixed gently. They were then incubated at -20°C for 20 minutes. This was followed by centrifugation of the samples at 13000 rpm for 10 mins. After centrifuging, the supernatant was transferred to a new sterilized 1.5 ml Eppendorf tube. This was followed by addition of 400 µl chilled absolute ethanol for DNA precipitation. The samples were then centrifuged at 13000 rpm for 8 mins and the liquid phase decanted off gently. 400 µl of chilled 70% ethanol was then added followed by gentle mixing to wash the pellets. The samples were then centrifuged at 13000 rpm for 2 minutes. The liquid phase was then decanted and the Eppendorf tubes inverted on sterilized paper towels to dry the DNA pellet. The DNA pellet was eluted by adding 50 µl of TE and the pellet dissolved by gentle tapping. Quantity and quality of the DNA samples was determined using agarose gel electrophoresis. The DNA was resolved in 1% agarose gel. The DNA samples were then stored at 4ºC.

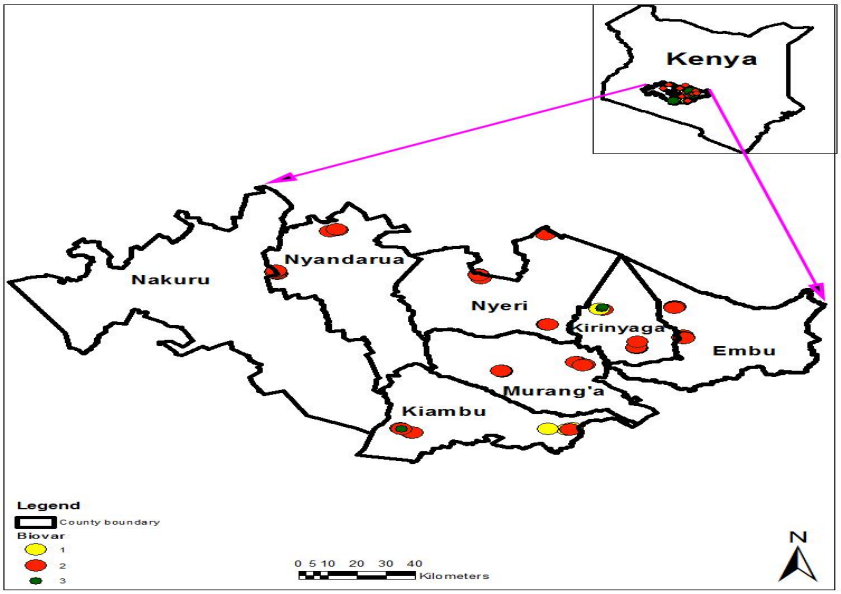

Fig. 1. Map showing *R. solanacearum* isolate origin as obtained in this study

2.4 PCR Reaction and Sequencing

PCR amplification of a 270-bp region of 16S *rDNA* was performed by using the primer pair OLI1 (5'GGGGGTAGCTTGCTACCTGCC-3') and Y2 (5'-CCCACTGCTGCC TCCCGTAGGAGT-3'). The reaction mixture (total volume, 25 µl) contained PCR buffer (QIAGEN), 0.3 mM $MgCl_2$, 10 µM of each dNTP, 10 pmol of each primer, 2 µl of bacterial DNA suspension as the template, and 1 U of Taqman DNA polymerase (Applied Biosystems). PCR was performed using a BIO-RAD T100 thermocycler using the following protocol: (i) initial denaturation at 94°C for 5 min; (ii) 35 cycles, with 1 cycle consisting of 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min; and (iii) a final extension step of 72°C for 10 min. Quality of PCR products were examined by agarose gel electrophoresis using 1% agarose gel in TBE buffer, and bands were revealed by staining in 0.5 µg ml^{-1} ethidium bromide. The PCR products were cleaned and sequenced by Iqbaba- Biotech (South Africa) using primer OLI-F or Y2-R.

2.5 Data Analysis

The sequence data were analyzed using several sequence analysis platforms. Base calling for sequenced data were done using Chromas lite software. Consensus sequences were created using CLC genomics software. The sequenced data was then aligned using Basic Local Alignment Test (BLAST) tool in NCBI database. Alignment with reference was done using Mussle in Mega 7 software. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan and Goldman + Freq. model. The genetic diversity of the ralstonia isolates based on sequence data was determined using DnaSP5 software [19].

3. RESULTS AND DISCUSSION

3.1 Determination of Pathogen Identity

Sixty-nine bacteria isolates obtained from various wilting plant hosts (Tomato, capsicum and potato) from 11 different sites were subjected to molecular analysis. All the bacteria isolates were confirmed to be *R. solanacearum* following PCR amplification of about 270-bp fragment using specific primers OLI 1-F and Y2-R (Plate 1). The OLI 1 and Y 2 primers targeted part of the 16S rDNA sequence of *R. solanacearum*.

BLAST analysis of the 16S rRNA gene sequences revealed that all the isolates had high identity with *R. solanacearum* isolates from different places of the world and different hosts (Table 1). About $950 million is lost annually to *R. solanacearum* which occurs in highlands in the tropical, sub-tropical and warm-temperate areas in more than 30 countries [20]. Several management strategies advocated for the control of the disease had very limited success. The genetic diversity of the pathogen might be one of the reasons for the difficulties encountered. According to Xue et al. [21], genetic diversity of *R. solacearum* can cause difficulties in its management. This necessitates the need to carry out research to determine the genetic diversity of *R. solacearum* for formulation of good management strategies.

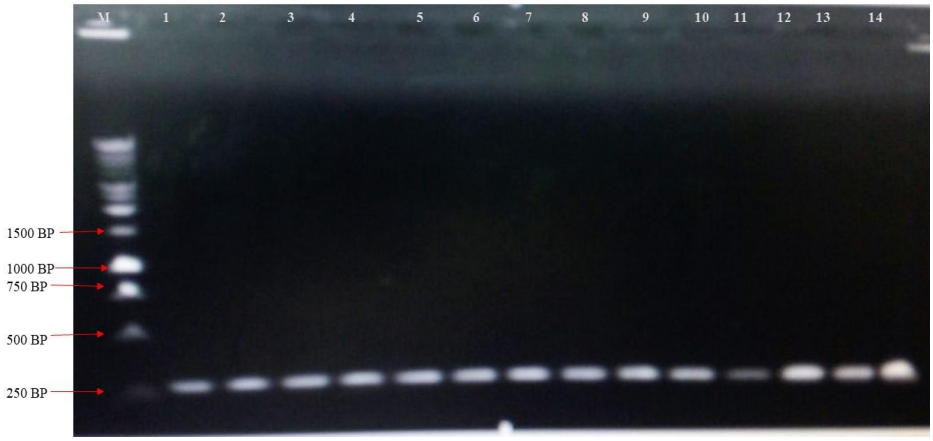

Plate 1. Agarose gel electrophoresis of PCR amplified 16S rDNA of the bacteria isolates from Embu low land (EBL) were stained with ethidium bromide and ran on 1.4 % agarose gel. M – 1 kb DNA ladder from Promega. Lanes 1-14 - amplified 16S rDNA of 14 bacteria isolates

Table 1. Isolate reference matches from the NCBI data base

Isolate	Match	Isolate source/ host	Origin
ELIZK2tpt111	MG266201.1 *Ralstonia solanacearum strain* DIBER119	*Solanum lycopersicum*	India
	NC_003295.1 *Ralstonia solanacearum* GMI1000	Tomato It also has a wide host range.	French- Guyana
ELIZK2ttm115	CP025985.1 *Ralstonia solanacearum strain* RSCM	*Cucurbita maxima*	China
ELIZK2tpt42	MG266201.1 *Ralstonia solanacearum strain* DIBER119	*Solanum lycopersicum*	India
ELIZK2ttm49	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2tpt56	EU795336.1 *Ralstonia solanacearum* strain Col71	Soil, from banana growing region of columbia.	Columbia
ELIZK2tpt72	CP016612.1 *Ralstonia solanacearum* FJAT-91	*Solanum lycopersicum*	Fujian – China
ELIZK2tpt73	CP021448.1 *Ralstonia solanacearum* strain SEPPX05	*Sesame seedling*	China
ELIZK2tpt93	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
	KY594786.1 *Ralstonia solanacearum* strain RS380	*Cucurbita moschata stem*	China
ELIZK2tpt106	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt107	CP016914.1 *Ralstonia solanacearum* strain CQPS-1	*Nicotiana tabacum*	China
ELIZK2tcap109	MG266201.1 *Ralstonia solanacearum* strain DIBER119	*Solanum lycopersicum*	India
ELIZK1132ttm	EU795336.1 *Ralstonia solanacearum* strain Col71	Soil from banana growing region of columbia.	Columbia
ELIZK1142ttm	KT783476.1 *Ralstonia solanacearum* strain UPMT09	Tomato Plants	Malaysia
ELIZK2tpt117	CP025986.1 *Ralstonia solanacearum* strain RSCM	*Cucurbita maxima*	China
ELIZK2tpt118	MF996766.1 *Ralstonia solanacearum* strain TM2	*Solanum lycopersicum*	China
ELIZK2tpt119	MF996766.1 *Ralstonia solanacearum* strain TM2	*Solanum lycopersicum*	China
ELIZK2tpt120	MG266193.1 *Ralstonia solanacearum* strain DIBER115	*Solanum lycopersicum*	India
ELIZK2tpt42	MF996766.1 *Ralstonia solanacearum* strain TM2	*Solanum lycopersicum*	China
ELIZK2tpt72	MF996766.1 *Ralstonia solanacearum* strain TM2	*Solanum lycopersicum*	China
ELIZK3pt88	CP021448.1 *Ralstonia solanacearum* strain SEPPX05	Sesame seedling	China
ELIZK2ttm93	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt101	EU795336.*1 Ralstonia solanacearum* strain Col71	Soil from banana growing region of columbia.	Columbia
ELIZK2tpt103	EU795337.1 *Ralstonia solanacearum* strain Col48	Soil from banana growing region of columbia.	Columbia
ELIZK2ttm112	EU795336.1 *Ralstonia solanacearum* strain Col71	Soil from banana growing region of columbia.	Columbia
ELIZK1pt15	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt16	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tm17	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2ttm18	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2ttm19	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India

Isolate	Match	Isolate source/ host	Origin
ELIZK2tpt26	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2tpt27	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2abns29	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK1pt85	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK3pt86	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK3pt87	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK3pt88	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK3pt89	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2ttm91	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2ttm92	EU795336.1 *Ralstonia solanacearum* strain Col71	Soil, from banana growing region,	Uraba-Colombia
ELIZK2ttm94	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2ttm95	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt96	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2tpt98	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt101	EU795336.1 *Ralstonia solanacearum* strain Col71	Soil from banana growing region of columbia.	Columbia
ELIZK2tpt27	EU795337.1 *Ralstonia solanacearum* strain Col48	Soil from banana growing region of Columbia	Columbia
ELIZK1pt12	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2tpt1	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2tbns30	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt34	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2tpt33	CP022702.1 *Ralstonia solanacearum* strain 10319	*Solanum lycopersicum*	Alberta- Canada
ELIZK2tpt36	EU795337.1 *Ralstonia solanacearum* strain Col48	Soil from banana growing region of Columbia	Columbia
ELIZK2tpt44	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt45	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2ttm47	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2ttm50	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India
ELIZK2tpt51	CP025986.1 *Ralstonia solanacearum* strain RSCM	*Cucurbita maxima*	China
ELIZK2tpt5	EU795333.1 *Ralstonia solanacearum* strain Col11	Soil from banana growing region of Columbia	
ELIZK2apt8	MG266202.1 *Ralstonia solanacearum* strain DIBER116	*Solanum lycopersicum*	India

Key: Embu lowland-EBL, Embu highland-EBL, Nyeri lowland-NYEL, Kiambu lowland-KBL, Kiambu highland – KBH, Nyandarua highland -NYRH, Nyandarua low land-NYRL, Murang'a lowland-MRL, Murang'a highland -MRH, Nakuru highland –NKRH, Kirinyaga highland –KRH and Kirinyaga lowland –KRL

3.2 Phylogenetic Analysis

Based on the targeted 16 S rDNA sequences using primers OLI 1 and Y2, the 69 bacteria isolates had 98 to 100% identity with other DNA sequences for *R. solanacearum* isolates deposited in the NCBI database (Table 1). A phylogenetic tree based on the translated 16 S rDNA sequences clustered the bacteria isolates into two main clusters (cluster A and B) (Fig. 2). Cluster A isolates grouped together with NC_003295.1 *R. solanacearum* GMI1000, CP016612.1 *R. solanacearum* FJAT-91 among other isolates which are pathogenic to the solanacea family of plants (Fig. 2). Cluster B had two main sub-clusters (1 and 2). Sub-cluster 1 isolates grouped with EU795337.1 *R. solanacearum* strain Col48. Sub-cluster 2 bacteria isolates grouped together with MG266201.1 *R. solanacearum* strain DIBER119 which is pathogenic to tomato plants, CP025986.1 *R. solanacearum* strain RSCM which is pathogenic to cucurbita maxima among other isolates (Fig. 2).

The *R. solacearum* isolates were obtained from different hosts including tomato, pepper and potato plants, however; the similar matches from the NCBI database were mainly from tomato plants except isolate RS380 whose origin host was *Cucurbita moschato*. Similar findings have been reported by Li et al. [4] who observed that *R. solacearum* has a wide host range and genetic diversity, which complicates its control and to high economic losses. Phylotype I isolates have also been reported to be prevalent in both moderate highlands and lowlands especially those that are pathogenic to the potato plants that have reported in highland elevations in India [22]. Phylotype I is one of the most diversifying subspecies of ralstonia because it has a huge variety of hosts, dissemination pattern and ability to recombine [23]. The striking genetic diversity among phylotype I strains is a major cause of the substantial pathogenic variability in host range and aggressiveness, local adaptation to diverse biotic and abiotic factors. This genetic variation presents a major challenge for biocontrol strategies and should thus be considered in the development of biological control agents.

The ralstonia isolates also matched with isolates from Uraba, Colombia in cluster B. They clustered with strains; EU795336.*1* *R. solanacearum* strain Col71 and EU795333.1 *R. solanacearum* strain Col11. These isolates were isolated from a banana-growing region in Columbia. They are in Phylotype II and believed to cause Moko disease in bananas. Cardozo et al. [23] reported that moko disease causing *R. solanacearum* had different origins and some of the genotypes had close relationship to isolates that cause wilting disease in tomato and potato. This was also reported by Ramsubhag et al. [24] who observed that *R. solanacearum* isolates causing Moko disease in bananas were closely related to *R. solanacearum* isolates that were pathogenic to potato and banana based on egl gene sequences. Occurrence of *R. solanacearum* isolates related to more than one phylotype has been observed in different countries and is usually credited with efficient mechanisms of dispersion [25].

Generally, conventional PCR is performed on presumptive *R. solanacearum* strains and isolates with the primers OLI-1 and Y-2 of the standard protocols of Ishii and Sadowsky [26]. The primers targeted against a conserved region of the 16S rDNA gene to yield a 292-bp product. This test is considered the 'Gold Standard' for identification of *R. solanacearum* isolates and is used as a tool for investigation of disease epidemiology and is a recommended method in Europe for identification, Council Directive 2006/63/CE (amending EC directives 98/57/EC and 95/4/EC). The main advantage of this test is its speed, as fast identification is of utmost importance in quarantine laboratories.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan and Goldman + Freq. model. The tree with the highest log likelihood (-258.39) is shown. The percentage of trees in which the associated taxa clustered is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 200.0000)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 76 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 21 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

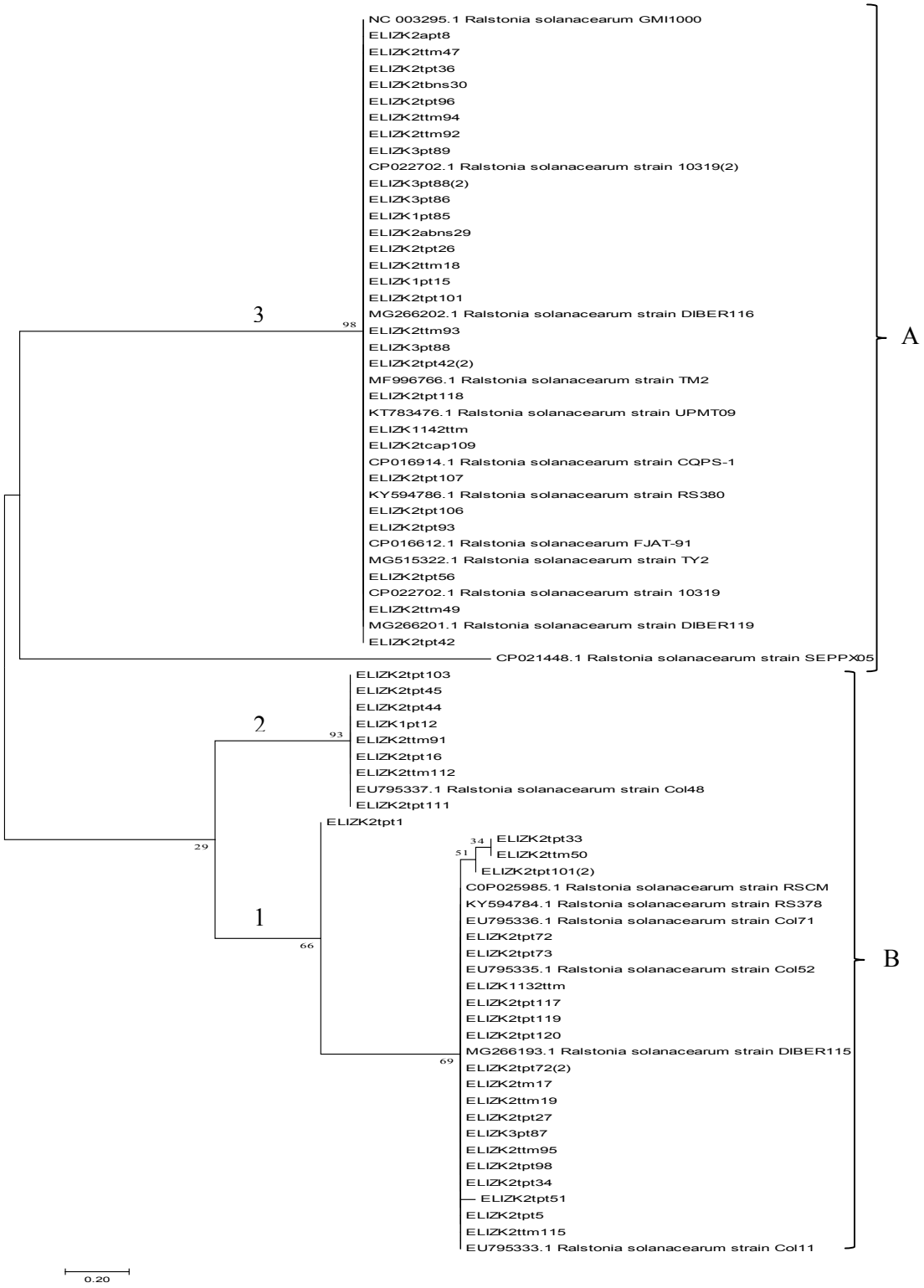

Fig. 2. Molecular phylogenetic analysis by maximum likelihood method

3.3 Genetic Differentiation of the *R. solanacearum* Isolates among Populations

Analysis of genetic differentiation showed there were total of 26 haplotypes from the 11 studied populations. Population EBL had the highest number of haplotypes, with 6 haplotypes while population NKRH and MRL had the least number of haplotypes with 2 haplotypes each. The highest haplotype diversity of 1.00 was observed in populations; KBH, NKRH and MRL while the lowest (0.00) haplotype diversity was observed in NYEL population. Total nucleotide diversity (pi) across the 11 populations was 0.621. Population NKRH had the highest nucleotide diversity of 0.791. NYEL population had the lowest or no nucleotide diversity (Table 2). The total number of segregating sites in all populations was 225. There were no segregating sites in sequences from NYEL population isolates but isolates from EBL population had the highest number (225) of segregating sites.

3.4 Regional Distribution of the *R. solanacearum* Isolates

Cluster analysis based on the genetic distance between the various R. *Solanacearum* isolates was drawn using neighbour joining method using MEGA 7 software (Fig. 3). The isolates were grouped into 2 main clusters (A and B).

Table 2. Genetic differentiation of the *R. solanaceaurum* isolates

Population	Number of isolates	Segregating sites	Haplotypes	Haplotype diversity	Nucleotide diversity pi
KBH	3	212	3	1.000	0.757
NYRH	6	224	5	0.857	0.647
NKRH	2	178	2	1.000	0.791
MRL	2	162	2	1.000	0.720
MRH	5	209	3	0.700	0.522
EBL	9	225	6	0.833	0.623
EBH	6	201	3	0.600	0.427
KRH	6	205	4	0.800	0.523
KRL	7	223	4	0.714	0.532
KBL	7	224	5	0.904	0.679
NYEL	3	0	5	0.000	0.000
Total	56	225	26	0.860	0.621

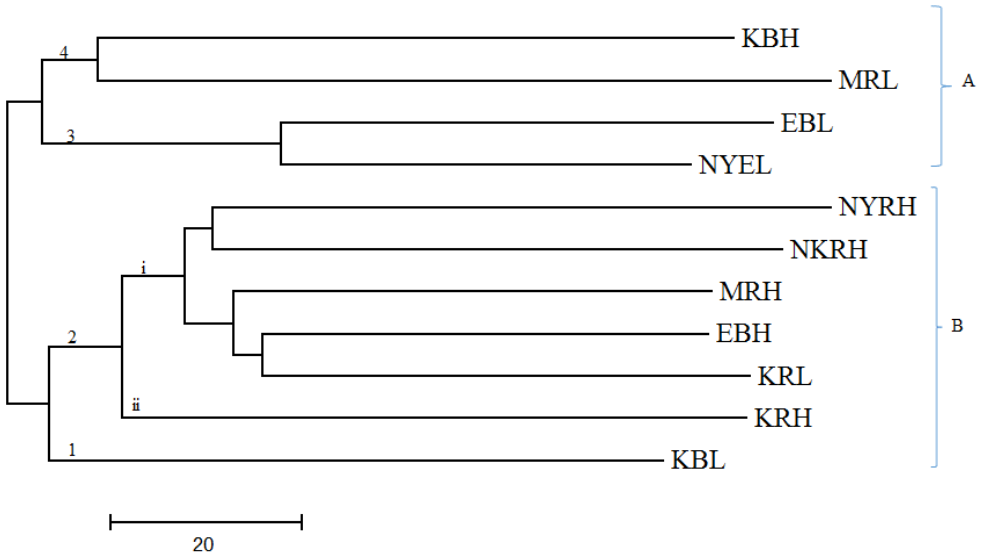

Fig. 3. Cluster analysis showing the regional distribution of the *R. solanacearum* isolates based on genetic distance and neighbour joining method

Cluster B included 7 populations while cluster A had 4 populations. Cluster A had two main sub-clusters (3 and 4). Populations MRL and KBH clustered in sub-cluster 4 while population EBL and NYEL clustered in sub-cluster 3 (Fig. 3). Cluster B had two sub-clusters; 1 and 2. Sub-cluster 1 only had one population (KBL) which was distant from the other populations in this cluster. Sub-cluster 2 had two main groups (i and ii). Populations NYRH and NKRH clustered in group i, while MRH, EBH and KRL were closely related and group i. Group ii only included KRH population (Fig. 3).

A maximum likelihood neighbour joining phylogenetic tree based on the genetic sequences generated using primers Y-1 and OLI-1 grouped the ralstonia isolates into two main groups. Cluster A included isolates with high similarity to *R. solanacearum* strains; GM 1000, DIBER 116, TM2, RS380, DIBER119, FJAT 91 and UPMT 09 as obtained from the NCBI database. The ralstonia isolates from the various parts of Kenya in cluster A, had similarity with *R. solanacearum* isolates from other parts of the world including, China, India, Canada, Columbia and France. This could possibly demonstrate that bacterial wilt disease incidence observed among different solanacea crops in Kenya could be because of importation of infected planting material into the country. Related findings were reported by Seal et al. [27] who observed great similarity of *R. solanacearum* isolates from India with isolates from other parts of the world. The matching isolates in this cluster (GM 1000, DIBER 116, TM2, RS380, DIBER119, FJAT 91, UPMT 09 and TY2) are all in phylotype I as reported by various researchers [28-30]. Broad genetic diversity of phylotype III strains has been reported in a few countries in Sub-Saharan Africa (Angola, Burkina Faso, Cameroon, Guinea, the Ivory Coast, Kenya, and Zimbabwe) and the southwest Indian Ocean (Madagascar and Reunion) [30]. N'Guessan et al. [31] suggest that features of free recombination, broad host range, patterns of dissemination and plastic virulence endow phylotype I with a higher evolutionary potential to spread quickly over long distances.

4. CONCLUSION

Through this study, it was realized that the main causative agent of bacterial wilt in potatoes, tomatoes and capsicum grown in the lowland and highland regions in Kenya is *R. solanacearum*. The isolates are in two main groups (Cluster A and B) that represent mainly the phylotypes I and II respectively.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Appendix 1: Gene flow

Population 1	Population 2	Hs	Ks	Kxy	Gst	DeltaSt	GammaSt	Nst	Fst	Dxy	Da
KBH	NYRH	0.88095	152.9667	157.0952	0.04977	0.07084	0.11607	-0.12111	-0.0053	0.6982	-0.0037
KBH	NKRH	1	173.4	138.3333	0.00826	0.07905	0.14637	0.29911	-0.25904	0.61481	-0.15926
KBH	MRL	1	167	143.6667	0.00826	0.09896	0.18132	0.04106	-0.15661	0.63852	-0.1
KBH	MRH	0.775	137.3125	153.3333	0.08665	0.10324	0.18649	-0.09904	0.06141	0.68148	0.04185
KBH	EBL	0.85417	147.7083	149.037	0.0597	0.04994	0.08444	-0.0236	-0.04169	0.66239	-0.02761
KBH	EBH	0.68	120.7778	160.6111	0.11902	0.12609	0.2373	-0.06649	0.17088	0.71383	0.12198
KBH	KRH	0.84	135.2667	156.3889	0.06118	0.09986	0.17871	0.01917	0.07901	0.69506	0.05491
KBH	KRL	0.7619	134.8333	156.7619	0.08596	0.09094	0.16201	0.05087	0.07518	0.69672	0.05238
KBH	KBL	0.92063	158.0333	154.6667	0.0389	0.06052	0.09772	-0.04409	-0.04449	0.68741	-0.03058
KBH	NYEL	0.5	85.16666	130.6667	0.33333	0.1642	0.39419	-0.04316	0.34821	0.58074	0.20222
NYRH	NKRH	0.85714	152.7407	124.7143	-0.00599	0.02742	0.05017	0.14418	-0.29706	0.55429	-0.16466
NYRH	MRL	0.85714	149.1852	159.7143	0.07182	0.08734	0.14592	-0.09194	0.03727	0.70984	0.02646
NYRH	MRH	0.79821	133.8472	125.2286	-0.01994	0.03427	0.06445	-0.25556	-0.05017	0.55657	-0.02793
NYRH	EBL	0.84325	142.5104	165.4286	0.08364	0.08917	0.13864	0.28106	0.13651	0.73524	0.10037
NYRH	EBH	0.74286	122.6667	119.7143	-0.00924	0.03832	0.0765	-0.17819	-0.00875	0.53206	-0.00466
NYRH	KRH	0.83175	132.6974	140.881	0.05686	0.06507	0.11521	0.05105	0.06568	0.62614	0.04112
NYRH	KRL	0.78571	132.5714	127.4082	-0.01987	0.03061	0.05715	-0.14493	-0.04053	0.56626	-0.02295
NYRH	KBL	0.88095	149.1429	147.0408	-0.00193	0.04268	0.06986	-0.08482	-0.0143	0.65351	-0.00934
NYRH	NYEL	0.71429	101.8667	168	0.2953	0.19718	0.33692	0.52811	0.56689	0.74667	0.42328
NKRH	MRL	0	170	130.75	0	0.10167	0.21205	-0.01834	-0.30019	0.58111	-0.17444
NKRH	MRH	0.7	134.7857	119.3	-0.04798	0.05043	0.10919	0.32087	-0.23847	0.53022	-0.12644
NKRH	EBL	0.83333	147.0455	163	0.08305	0.07432	0.12401	0.67985	0.02403	0.72444	0.01741
NKRH	EBH	0.6	116.5	115.4167	-0.03067	0.05153	0.12354	0.11915	-0.187	0.51296	-0.09593
NKRH	KRH	0.8	132.8	138.0833	0.04673	0.07421	0.14838	0.67834	-0.07085	0.6137	-0.04348
NKRH	KRL	0.71429	132.5926	122.6429	-0.01408	0.04129	0.08538	0.26159	-0.21336	0.54508	-0.1163
NKRH	KBL	0.90476	158.3704	144.1429	0.01385	0.0525	0.08853	0.41571	-0.14734	0.64063	-0.09439
NKRH	NYEL	0	71.2	164	0.37824	0.25493	0.61704	1	0.45732	0.72889	0.33333
MRL	MRH	0.7	130.2143	170.1	0.09915	0.14984	0.27189	0.11195	0.17842	0.756	0.13489
MRL	EBL	0.83333	144.1364	161.5556	0.08305	0.0777	0.13032	0.16051	0.06482	0.71802	0.04654
MRL	EBH	0.6	112.5	173.3333	0.12953	0.15472	0.30255	0.46554	0.25577	0.77037	0.19704
MRL	KRH	0.8	128.8	165.5	0.07901	0.12657	0.23284	0.04679	0.15488	0.73556	0.11393
MRL	KRL	0.71429	129.037	171.5	0.1016	0.1225	0.21996	0.38911	0.17895	0.76222	0.1364

Population 1	Population 2	Hs	Ks	Kxy	Gst	DeltaSt	GammaSt	Nst	Fst	Dxy	Da
MRL	KBL	0.90476	154.8148	167.4286	0.06313	0.09442	0.15058	0.06949	0.06001	0.74413	0.04466
MRL	NYEL	0	64.8	159	0.37824	0.2528	0.6371	0.43656	0.49057	0.70667	0.34667
MRH	EBL	0.79333	132.0714	151.5111	0.08321	0.08615	0.14569	0.2937	0.14968	0.67338	0.10079
MRH	EBH	0.64286	105.7727	99.26667	-0.03913	0.02703	0.0658	-0.11619	-0.07539	0.44119	-0.03326
MRH	KRH	0.75714	117.6273	125.4	0.07186	0.06467	0.13133	-0.0557	0.06207	0.55733	0.03459
MRH	KRL	0.70893	118.7361	105.2571	-0.05434	0.01511	0.0332	-0.19801	-0.12638	0.46781	-0.05912
MRH	KBL	0.82798	138.0694	129.8571	-0.01624	0.03757	0.06816	-0.16341	-0.04061	0.57714	-0.02344
MRH	NYEL	0.525	73.4375	135.4	0.32907	0.18417	0.4136	0.47097	0.5661	0.60178	0.34067
EBL	EBH	0.74848	122.5	165.3148	0.15723	0.13444	0.22079	0.41713	0.28571	0.73473	0.20992
EBL	KRH	0.82121	131.1933	153.0741	0.06256	0.08901	0.14942	0.3512	0.1576	0.68033	0.10722
EBL	KRL	0.78373	131.1771	156.1746	0.09379	0.09322	0.15432	0.4382	0.16828	0.69411	0.11681
EBL	KBL	0.8631	145.6771	144.5238	0.00324	0.03666	0.06082	0.05679	-0.01343	0.64233	-0.00862
EBL	NYEL	0.72917	105.125	94.55556	0.1	0.05377	0.11462	-0.62812	0.25881	0.42025	0.10877
EBH	KRH	0.7	106.8667	126.6111	0.11888	0.08346	0.17414	0.07486	0.15595	0.56272	0.08775
EBH	KRL	0.66349	108.7179	97.92857	-0.04828	0.01472	0.0347	-0.11405	-0.1009	0.43524	-0.04392
EBH	KBL	0.76931	126.5641	134.4524	0.03626	0.06403	0.11824	0.05988	0.07491	0.59757	0.04476
EBH	NYEL	0.48	64	169.6667	0.44186	0.25613	0.51936	0.70858	0.71709	0.75407	0.54074
KRH	KRL	0.75238	118.7487	128.3095	0.07586	0.06183	0.1216	0.11958	0.07508	0.57026	0.04281
KRH	KBL	0.8582	136.5949	140.6667	0.02993	0.05775	0.1009	0.11601	0.03852	0.62519	0.02408
KRH	NYEL	0.64	78.48889	136.1667	0.27273	0.17207	0.37183	0.55251	0.56769	0.60519	0.34356
KRL	KBL	0.80952	136.1905	133.2653	-0.00847	0.03673	0.06612	-0.0006	-0.02195	0.59229	-0.013
KRL	NYEL	0.59524	83.73333	146.5714	0.3051	0.1779	0.35804	0	0.59194	0.65143	0.38561
KBL	NYEL	0.75397	106.9333	122.5714	0.16159	0.10659	0.20739	-0.16102	0.37685	0.54476	0.20529

Appendix 2: Semi-Selective Medium, South Africa (SMSA)

Bactopeptone (Difco)	10 g
Glycerol	5 ml
Casamino acids (Difco)	1 g
Bacto agar (Difco)	15 g
Distilled water	1000 ml

Sterilize for 15 minutes at 121°C.

Add to 250 ml of melted medium at a temperature of 50°C:

1% Polymyxin B sulphate (Sigma) (Final, conc., 100 ppm)	2.5 ml
1% Crystal violet (Final conc., 5 ppm)	125 µl
1% Tetrazolium salts (Sigma) (Final conc., 50 ppm)	1.25 ml
1% Bacitracin (Sigma) (Final conc., 25 ppm)	625 µl
0.1% Penicillin (Sigma) (Final conc. 0.5 ppm)	125 µl
1% Chloramphenicol (Sigma) (Final conc., 5 ppm)	125 µl

When additional inhibition of fungal contaminants or soil inhabitants is desired add:

1% Cycloheximide (Sigma) (Final conc., 100 ppm).	2.5 ml

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