Diversity and Phylotype Analysis of *Ralstonia solanacearum* Strains Causing Tomato and Potato Bacterial Wilt in Ethiopia

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Abstract

Ralstonia solanacearum is the causative agent of bacterial wilt on economically important crops. The occurrence and importance of the disease has been recognized in Ethiopia. The pathogen is known for its high variability and adaptability to various geographical regions of the world. The biovar and race classification system has been considered as insufficient to encompass the diversity within the *R. solanacaerum* species complex. A classification scheme based on phylotype specific multiplex PCR from an internal transcribed spacer (ITS) region and an endoglucanse gene sequencing has been reported to fill the limitation. The study was intended to characterize *R. solanacearum* strains from Ethiopia based on both conventional approaches and determination of phylotypes. On the basis of the conventional approach *R. solanacearum* strains from Ethiopia were identified as biovars 1 and 2 complementing the recent report of biovar 1 in Ethiopia. Rep-PCR analyses also revealed three clonal lines at about 80 % similarity level and overall 9-fingerprint types at 100 % similarity level among the studied strains. Phylotype grouping revealed the occurrences of phylotype II and III and further confirmed by partial endoglucanse gene sequencing. The association of biovar and phylotyping indicate that phylotype II consists of only biovar 2 and phylotype III comprises biovar 1 and 2. This is the first formal report on the occurrence of phylotypes II and III in Ethiopia. The genetic variability of *R. solanacearum* requires proper consideration in future research work in the country. The findings also would contribute to update the existing information on *R. solanacearum* strains in Ethiopia and can help to discriminate and assess emerging pathogens or strains that could be potentially introduced into the country.

Keywords: Bacterial wilt, Ethiopia, Multiplex PCR, Phylotype, *Ralstonia solanacearum*

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum* and, more recently, *Burkholderia solanacearum*) (Yabuuchi *et al.* 1995) is a lethal vascular disease in the family Solanaceae, attacking economically important crops such as potato, tomato, pepper and eggplant (Hayward 1991; 1994). The importance of the disease has been widely recognized in tropical, subtropical and warm temperate regions of the world. *Raltonia solanacearum* differ in host range,

geographical distribution, pathogenicity, epidemiological relationships, and physiological properties (Buddenhagen and Kelman 1964). Earlier investigations on this pathogen mainly employed a binary system using two different approaches; one placing emphasis on host range characterization and the other making use of selected biochemical properties as the basis for the separation into biovars (Hayward 1991). Thus, five races have been described and designated according to the host or hosts primarily affected (Buddenhagen and Kelman 1964; He *et al.* 1983) and five biovars according to metabolisation of different carbon sources (Hayward 1994).

The occurrence and importance of bacterial wilt in Ethiopia, mainly on tomato and potato, was reported as early as in the 1970's (Stewart and Dagnatchew 1967). Occurrences of biovar 2 corresponding to race 3 (Yaynu 1989) and biovar 1 race 1 Lemmessa and Zeller (2007) have been identified by biochemical and physiological methods from Ethiopia. However, the races and biovars grouping system has been considered as informal grouping at the infrasubspecific level that is not governed by the Code of Nomenclature of Bacteria (Lapage *et al* .1975). Furthermore, the biovar classification is a special purpose classification which is primarily applied in the context of epidemiology rather than taxonomy and therefore, insufficient to provide a reliable taxonomic separation of the *R. solanacearum* species complex (Hayward 1994).

A classification scheme, based on analysis of restriction fragment length polymorphisms (RFLP) at the hyper sensitive response and pathogenicity (*hrp)* locus and additional loci from the core genome (Cook *et al.* 1989; Cook and Sequeira 1994) revealed the existence of two evolutionary divisions, corresponding to division I, also named "Asiaticum", comprising strains mainly isolated from Asia and Australia, and division II known as "Americanum", with strains mainly originating from South and Central America. The genetic classification based on geographical origin thus correlates nicely with the biovar classification because strains from division I (Asiaticum) match biovars 3, 4 and 5 and strains from division II (Americanum) match biovars 1, 2 and N2. The diversification of *R. solanacearum* into two major divisions later was confirmed using additional molecular criteria addressing various elements of the core genome including polymerase chain reaction based on restriction fragment length polymorphism (PCR-RFLP) analysis of polygalacturonase (*pglA)* and *hrp* genes (Gillings *et al.* 1993; Poussier *et.al.* 1999). Comparisons of rRNA sequences (Li *et al.* 1993; Taghavi *et al.* 1996, Poussier *et al.* 2000b) by amplified fragment length polymorpnism (AFLP) analysis on genomic DNA (Poussier *et al.* 2000b), and comparison of a partial nucleotide sequence of the hyper sensitive response and pathogenicity (*hrpB)* and endoglucanase genes *(eglA)* (Poussier *et al.* 2000a) have confirmed the diversification of the pathogen. Some of these studies allowed the identification of a third division for strains originating from Africa (Poussier *et al.* 1999; 2000a; 2000b). Over the years, various genetic analyses have been carried out by many authors and have confirmed the genetic variability of the pathogen both between and within the population of *R. solanacearum* (Horita and Tsuchiya 2001; Grover *et al.* 2005). Genomic fingerprints using repetitive DNA based PCR (Rep-

PCR) has been employed to determine the clonal line of phytopathogenic bacteria including *R. solanacearum* (Fegan and Prior 2005; Louws *et al.* 1994; 1995).

Fegan and Prior (2005) introduced a hierarchical classification scheme, which redefines *R. solanacearum* as a species complex and subdivides the species complex into four phylotypes corresponding to the four genetic groups. The phylotype classification scheme has confirmed that phylotype I and II are equivalent to divisions I and II defined by Cook *et al.* (1989). Phylotype III contains strains mainly from Africa and phylotype IV contains Indonesian strains (biovars 1, 2 and 2T) (Villa *et al.* 2005). Furthermore, taxonomic classifications of *R. solanacearum* strains to sequevar and infrasubspecific groups have been based on an endoglucanse gene sequencing (Fegan and Prior 2005). The phylogenetic analyses and phylotyping classification is believed to make a stable and meaningful taxonomy that defines subspecific groups of *R. solanacearum* that are at least related to geographic origin (Denny 2006).

Characterization and knowledge of the genetic structure of the pathogen population is basically required to device effective diseases management options. However, information on the types of Phylotype in Ethiopia is lacking. Hence, this study was intended to characterize *R. solanacearum* strains from Ethiopia based on both conventional approaches and the phylotype classification scheme introduced by Fegan and Prior (2005). Additionally attempt has been made to assess the genetic diversity using rep-PCR analysis (Louws *et al.* 1994; 1995) and to evaluate the phylogentic relationships of the strains based on partial endoglucanase gene sequencing (Fegan *et al.* 1998; Villa *et al.* 2005) along with some reference strains.

Materials and Methods

Bacterial strains, storage and culture condition

Ralstonia solanacearum strains (Table 1, 1-6) were isolated from sample specimens collected during field surveys from September to December 2005, May to June 2006 and March to April 2008. Presumptive identification was done and maintained in distilled sterile water at room temperature. Six *R. solanacearum* strains were further identified by species-specific PCR test from among 46 presumptive isolated strains. Additional R. *solanacearum* strains were initially isolated and identified from Ethiopia by Lemessa and Zeller (2007) and reference strains from Australia, China, Kenya and Thailand were obtained from culture collection at Institute of Plant Disease and Plant Protection, Leibniz Universität Hannover, Germany. Bacterial cultures were maintained in distilled sterile water at room temperature and cultivated on tetrazolium chloride (TTC) agar medium (Kelman 1954).

Table 1. List of *R. solanacearum* strains from Ethiopia and reference strains and their descriptions

No	*Strain code	Host	Locality	Year of isolation	Biovar	Phylotype**
1	Tomzy8	Tomato	Ziway	2005	2	II
2	TomZy9	Tomato	Ziway	2006	2	III
3	TomAw2	Tomato	Tikurwuha	2006	2	II
4	TomNa3	Tomato	Nacha	2006	1	III
5	TomGr6	Tomato	Gudar	2006	2	II
6	TomBk4	Tomato	Bako	2006	2	III
7	Tom1II	Tomato	Holeta	2003	2	II
8	Tom 6II	Tomato	Holeta	2003	1	III
9	Tom 88	Tomato	Ziway	2003	2	II
10	Tom768	Tomato	Ziway	2003	2	III
11	Pep 7	Pepper	Mutulu	2003	1	III
12	Pot 1	Potato	Mutulu	2003	1	II
13	Pot 2 JU	Potato	Jimma	2003	1	II
14	Pot 5II	Potato	Holeta	2003	2	III
15	Pot 10II	Potato	Holeta	2003	2	III
16	Pot 10III	Potato	Bako	2003	2	III
17	Pot 16 III	Potato	Bako	2003	2	II
18	Pot 20III	Potato	Arjo	2003	2	III
19	Pot 21II	Potato	Arjo	2003	2	III
20	Pot 34	Potato	Gedo	2003	2	III
21	Pot 42	Potato	Jeldu	2003	1	III
22	Pot 48	Potato	Ginchi	2003	1	III
23	Pot 50	Potato	Ginchi	2003	1	III
24	Pot 60	Potato	Shashemene	2003	2	III
25	Pot 62	Potato	Awassa	2003	1	III
26	Pot 70	Potato	Dedo	2003	3	III
27	Pot 84	Potato	Ambo	2003	2	III
28	Pot 91	Potato	Shashemene	2003	1	III
29	Pot 92	Potato	Shashemene	2003	2	II
30	Toudk2	Tomato	-	-	3	I
31	GSPB2690	Pepper	-	-	3	I
32	GSPB2695	Tomato		-	2	III
33	UQRS 348	Ginger	-	-	4	I
34	UQRS 559	Ginger	-	-	4	I
35	UQRS 584	Ginger	-	-	4	I

*1-29 = Ethiopian strains, 30-31 = Kenyan strains, 32 = Australia, 33 = China and 34 = Hawaii;
** = Phylotype determined by this study and - = not indicated

Identification and Confirmation of Specificity of *R. solanacearum* Species

The species specificity of *R. solanacearum* strains were identified and confirmed by polymerase chain reaction (PCR) using two primer pairs (Invitrogen, Germany), the forward primer 759 (5'-GTC GCC GTC AAC TCA CTT TCC-3') and the reverse primer 760 (5'-GTC GCC GTC AGC AAT GCG GAA TCG-3') which amplify a 281 base pair (bp) DNA fragment (Opina *et al.* 1997, Villa *et al.*, 2003). PCR reactions were carried out in a total volume of 25 µl each containing 200µM dNTPs (Carl Roth GmbH & Co., Karlsruhe, Germany), 1.0 unit of *Taq* DNA polymerase (Fermentas Life science, Germany), 1x PCR Buffer II (100mM Tris-HCl, pH = 8.3, 500 mM KCl) and 1.5 mM $MgCl_2$ (Fermentas Life Science, Germany), and 25 picomol of the primers. *Ralstonia solanacearum* colonies

were suspended in 1 ml distilled sterilized water and two micro liters of bacterial suspensions were added as DNA templates. For positive control *R. solanacearum* strain Toudk2, from Thailand (standard reference strain at Institute of Plant Diseases and Plant Protection, Leibniz Universität Hannover), for the negative control, 2 µL of sterile de-mineralized water were used. PCR amplifications were performed in a PTC-200™ Programmable thermal controller (MJ Research Inc., Watertown, Massachusetts, USA) which was programmed for an initial denaturation at 95 °C for 3 min, annealing 53 °C for 1 minute and extension 72 °C for 1.5 minutes, followed by 29 cycles of denaturation at 94 °C for 15s, annealing at 59 °C for 30s and extension at 72 °C for 30s. A final extension step was at 72 °C for 10 minutes and the final reaction was held at 11 °C until collected.

Six micro liter PCR products were mixed with 2 µl 6× Orange G Loading Dye Solution (Fermentas Life Science, Germany) and electrophoresed at 80 V for 1 hour onto a 2% (w/v) agarose gel containing 0.2 µg/ml ethidium bromide (Sigma, Germany) where the gel was prepared in 1× TAE (Tris-Acetate-EDTA buffer). The size of DNA fragment was monitored by loading 3 µl of 100 base pair (bp) DNA ladder (Fermentas life science, Germany) along the PCR product. After electrophoresis, the result was visualized on UV trans-illuminator (Spectroline TVL-312A, Germany).

PCR positive strains were further tested for their virulence on the susceptible tomato genotype (*Solanum esculentum* Mill. 'L-390' (AVRDC, Taiwan) in a growth chamber (with 30 °C day-time, 27 °C night temperature, 85% relative humidity, 30,000 lux) with a 14-hour photoperiod. The tomato genotype was sawn on plastic trays placed in the glasshouse with 20 °C day/night temperature, 14 hours of light per day, 30,000 lux and 70% relative humidity, and were watered daily with tap water. Four weeks after sowing, the seedlings were transplanted individually in plastic pots (12 cm diameter) containing about 260 g of soil ('Fruhstorfer' soil (Type P, with 150 mg/l N, 150 mg/l P_2O_5 and 250 mg/l K_2O) (Industrie-Erdenwerk Archut GmbH & Co KG, Lauterbach-Wallenrod, Germany). Each strain was grown on TTC agar medium for 48 hours at 30 °C and bacterial suspension was prepared in distilled sterilized water. A bacterial suspension was adjusted to optical density (OD) of 0.06 at 620 nm corresponding to 10^8 CFU/ml (colony forming units/ml). Inoculation was done on the day of transplanting by soil drenching with 26 ml of bacterial suspension. Each strain was inoculated on 7-10 seedlings and after inoculation, pots were carefully watered. Virulence of each strain was measured by assessing the severity of wilt using the five wilt severity classes of 0-5 modified from Winstead and Kelman (1952), where 0 = no wilt symptoms, class 1 = one leaf wilted, class 2 = two or more leaves wilted, class 3 = all leaves except the tip wilted, class 4 = whole plant wilted and class 5 = death (collapse) of the plant. Disease incidence was assessed as percentage of wilted plants within each treatment.

Phenotypic, biochemical and physiological characterization

Phenotypic characteristics of *R. solanacearum* species such as morphological growth, color, shape,

accumulation of poly-ß-hydroxybutyrate inclusions (Hayward 1994) and physiological characteristics such as oxidase test, oxidation/fermentation test, solubility in potassium hydroxide (KOH) and growth in 1% and 2% of sodium chloride (NaCl) concentration were evaluated according to procedures described by Lelliott and Stead (1987) and Sands (1990). Colony growth, fluidity classes and production of diffusible brown pigment were studied by streaking on TTC and nutrient glucose agar (NGA) media in replicate after incubating at 30 ^{0}C for 48 hours. Fluidity classes were evaluated on the bases of four fluidal classes (Leykun 2003), where 1 = non-fluidal, 2 = lowly fluidal, 3 = fluidal and 4 = highly fluidal.

Determination of biovars was performed on the basis of the ability of the strains to oxidize lactose, maltose, and cellobiose and utilization of the hexose alcohols mannitol, sorbitol and dulcitol (Denny and Hayward 2001). The test involved 5 ml of Hayward's medium containing 10 g L^{-1} filter-sterilized of each carbon sources dispensed into 25 ml test tubes. Hayward's medium without a carbon source and an un-inoculated test tube served as negative controls. Each test tube was inoculated with 3 µl of a 1×10^8 CFU ml^{-1} cell suspension prepared from 48 hours old colonies grown on TTC medium without tetrazolium salt. Assays included as positive control strain Toudk2 (biovar 3). The cultures were incubated at 30 °C for 3 weeks and color development was recorded every 2 days. Each test was replicated three times.

Assessment of genetic diversity by Rep PCR analysis

Rep-PCR protocol described by Louws *et al.* (1994) was employed using BOXAIR primer (5'-CTACGGCAAGGCGACGC TG ACG-3') (Invitrogen, Germany) to assess the genetic diversity among the Ethiopian *R. solanacearum* strains. Amplification was performed in 25 µl reaction volumes containing 200 µM each of deoxynucleoside triphosphates (dNTP), 2 mM $MgCl_2$, primer at 50 pmol/µl, 1.0 U of Taq DNA Polymerase (Fermentas Life Sciences, Germany) and 2 µl suspension of the bacterial strain. The amplification step include an initial denaturation phase for 5 min at 95 ^{0}C and 30 cycles of denaturation at 94 ^{0}C for 1 minute, annealing at 53 ^{0}C for 1 min, and extension at 65 ^{0}C for 8 minutes, with a final extension at 65 ^{0}C for 16 minute and a final held at 11 ^{0}C. Seven to ten micro-liters of the PCR products were mixed with 2 µl 6X Loading Dye Solution (Fermentas Life Sciences, Germany), to apply to an agarose gel electrophoresis (2% (w/v) in 1X TAE buffer [40 mM Tris, 4 mM sodium acetate, 1 mM EDTA, pH 7.9]) containing ethidium bromide (0.2µg/ml) and run at 80V for 3 hour. After electrophoresis the banding patterns of fingerprints were visualized under a UV transilluminator and images were taken with computer-attached camera. To check reproducibility of the result, the analyses were repeated at least twice. Generated fingerprints by BOX- PCR were converted to a two-dimensional binary matrix (1 = presence of a PCR product; 0 = absence of a PCR product). For data scoring, photographs of gels were enlarged (8 by 10 inches or ca. 20 by 25 cm) in Adobe Photoshop soft ware (Adobe® photoshop®7.0) and the PCR

products were scored manually by visual pair-wise comparisons of adjacent lanes. A similarity matrix was generated from the binary data using SIMQUAL module of NTSCpc 2.10q Applied Biostatistics, Inc. Computer Software (Rohlf, 2000). Dice (1945) and Nei and Li (1979) coefficient was used to derive similarity among the strains. The similarity matrix thus generated and was used for cluster analysis by un-weighted pair-group method of arithmetic average (UPGMA) under SAHN (Sequential agglomerative, hierarchical. Nested clustering) module of NTSYpc 2.10q. The output data are graphically represented as a dendrogram.

Determination of phylotype

Determination of phylotypes was performed following the method of Fegan and Prior (2005) with Multiplex PCR. The PCR mixture containing species specific primers 759/760 (Opina *et al.* 1997) as an internal marker and a set of four phylotype-specific primers; Nmult [21: 1F [5'-CGTTGATGAGG CGCGCAATTT-3'], Nmult:21:2F:[5'-AAGTTATGGACGGTGGAAGTC-3'], Nmult:22: InF[5'-TGCCA-AGACGAGA GAAGTA-3'], and Nmult: 23: AF [5'-ATTACGAGAGCA-ATCGAAAGATT-3']) with a unique and conserved reverse primer (Nmult: 22:RR [5'-TCGCTTG ACCCTATA-ACGAGTA-3']), targeted to the 16S-23S intergenic spacer region (internal transcribed spacer). The reaction products includes phylotype specific PCR products of a 144-bp amplicon (phylotype I strains), a 372-bp amplicon (phylotype II strains), a 91-bp amplicon (phylotype III strains) and a 213-bp amplicon (phylotype IV strains) (Fegan and Prior 2005). DNA templates for the test were prepared according to Villa *e .al.* (2003) and Weller *et.al.* (2000). A single colony of each strain was transferred into 100 µl of sterile distilled water, vortexed, heated to >96 °C for 10 minutes, and placed on ice immediately. Samples of each strain were diluted in 900 µl of the sterile water and stored at -20 °C until required. Two microliters of bacterial suspensions was used per test. The reactions were carried out in a total volume of 25 µl containing 200 µM dNTP's (Carl Roth GmbH & Co., Karlsruhe, Germany) 1X PCR Buffer (100 mM Tris-HCl, [pH 8.8], 500 mM KCl), 2 mM $MgCl_2$, 5% DMSO (Dimethylsulfoxid), 6 picomol of each primer described above and 1 unit of *Taq* DNA Polymerase (Fermentas Life Science, Germany) and 2 µl bacterial suspension. PCR amplifications were performed in a PTC-200™Programmable Thermal Controller (MJ Research Inc, USA) programmed for an initial denaturation at 95°C for 5 minutes, followed by 29 cycles of denaturation at 93 °C for 30 s, annealing at 59°C for 1 min 30 s and extension at 72 °C for 1 min 30 s. A final extension step was at 72 °C for 5 minutes. The reaction was held at 11 °C. Seven micro-liters of multiplex PCR products were mixed with 2 µl 6X Loading Dye Solution [(10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60 mM EDTA)] (Fermentas Life Sciences, Germany) and electrophoresed at 80V for 1 hour onto a 2% (w/v) agarose gel containing 0.2 µg/ml ethidium bromide (Sigma), visualized after electrophoresis on an UV trans-illuminator, and images were captured with computer-attached camera. The correct amplicon size of the product monitored by loading 3 µl of 100 bp DNA ladder (Fermentas, Life Sciences, Germany).

Endoglucanase gene amplification, purification and partial Sequencing

R. solanacearum strains from Ethiopia representing phylotypes II and III (Table 2), identified by multiplex PCR and reference strains from Thailand and Kenya were used for endoglucanse gene amplification and sequencing. The amplification step was performed using a forward primer EndoF (5'-ATGCATGCCGCTGGTCGCCGC-3') and a reverse primer EndoR (5'-GCGTTGCCC GGC ACG AAC ACC-3') which amplify a 750 bp internal fragment of the endoglucanse (egl) gene (Poussier *et al.* 2000a; Fegan *et al.* 1998). For the reaction mixture, a total volume of 50 µl was used, containing 0.25 µM of each primer (Invirogen, Germany), 200 µM of dNTPs (Carl Roth GmbH & Co., Karlsruhe, Germany), 1.25 units of *Taq* DNA Polymerase, 1X PCR buffer [67mM Tris HCl (PH 8.8), 16.6mM(NH_4 ($S0_4$, 0.45% (Vol/Vol/) Triton X- 100, 200 µg gelatine per ml], 1.5 mM $MgCl_2$ solution (Fermentas Life Sciences, Germany) and 4 µl bacterial cell suspensions prepared as described for Multiplex PCR. Negative controls contained all ingredients for PCR except the bacterial cell suspension template. The amplifications steps were set with an initial denaturation step of 95 °C for 5 minute, 34 cycles of denaturation at 94 °C for 1 minute, annealing at 69 °C for 1 minute and extension at 72 °C for 2 minute followed by a final extension at 72 °C for 10 minute using PTC-200™ Programmable Thermal Controller (MJ Research Inc., Watertown, Massachusetts, USA) and the reaction was held at 11 °C. Gel electrophoresis was performed with each 5 µl of the PCR products mixed with 1 µl 6X Loading Dye Solution (Fermentas Life science, Germany) on a 2% (w/v) agarose gel prepared in 1X TAE buffer [40 mM Tris, 4 mM sodium acetate, 1 mM EDTA, pH 7.9]) containing 0.3µg/ml ethidium bromide at 80V for 2 hours. A 100 bp DNA ladder (Fermentas life science, Germany) was used as size marker. Amplified PCR products were purified using the QIA quick PCR purification kit (Qiagen, Germany) according to the manufacturer's protocol. Successes of the purification step were checked by taking 2 µl volume of each purified PCR product and by agarose gel-electrophoresis method described above. Purified PCR products were sent for sequencing at QIAGEN Sequencing service Center, Germany. All the DNA sequences of endoglucanase genes made in this study have been deposited at the EMBL/GenBank databases under the accession numbers (Table 2).

Table 2 List of *Ralstonia solanacearum* strains used for endoglucanase gene amplification and sequencing and their accession numbers

No	Strain code	Host	Locality	Country	Biovar	Phylotype	EMBL accession number
1	GSPB 2690	Pepper	*NI	Kenya	2	III	FM883230
2	Toudk2	Tomato	*NI	Thailand	3	I	FM883231
3	Pot 92	Potato	Shashemene	Ethiopia	2	II	FM883232
4	TomAw2	Tomato	Awassa	Ethiopia	2	II	FM883233
5	Tomzy8	Tomato	Ziway	Ethiopia	2	II	FM883234
6	TomGr 6	Tomato	Gudar	Ethiopia	2	II	FM883235
7	Tom1 II	Tomato	Holeta	Ethiopia	2	II	FM883236
8	GSPB 2695	Tomato	*NI	Kenya	3	I	FM883237
9	TomBk4	Tomato	Bako	Ethiopia	2	III	FM883238
10	TomZy9	Tomato	Ziway	Ethiopia	2	II	FM883239
11	Pot 48	Potato	Ginchi	Ethiopia	1	III	FM883240
12	Pot 42	Potato	Jeldu	Ethiopia	1	III	FM883241
13	Pot 84	Potato	Ambo	Ethiopia	2	III	FM883242
14	TomNa3	Tomato	Nacha	Ethiopia	1	III	FM883243
15	Pot 5II	Potato	Holeta	Ethiopia	2	III	FM883244
16	Pot2JU	Potato	Jimma	Ethiopia	1	II	FM883245
17	Pot 91	Potato	Shashemene	Ethiopia	1	III	FM883246
18	Pep 7	Pepper	Gudar	Ethiopia	1	III	FM883247
19	Pot16III	Potato	Bako	Ethiopia	2	II	FM883248
20	Pot10II	Potat	Holeta	Ethiopia	2	III	FM883249

*NI = Not indicated

Endoglucanase gene sequence analysis

For the analysis of sequences, all ambiguous and gap sequences at both ends of nucleotides of the endoglucanse gene sequence were first removed and about 650-700 base pair (bp) nucleotide positions were compared. Each sequence was systematically compared against the complete genome sequence of *R. solanacearum* database using BLAST program (Basic Local Alignment Search Tool) (Tatusova and Madden 1999) on the BLAST network service (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). For the construction of the phylogenetic tree of the phylotyping scheme of *R. solanacearum* species, the endoglucanse genes sequences of this study and the data base endoglucanse gene sequences that represent the four known phylotypes were retrieved (Table 3). The phylogenetic tree was constructed by aligning the sequences in the multiple alignment option of ClustalX 2.0 window interface (Thompson *et.al.* 1997) and the distances between all pairs of sequences from the multiple alignments were first calculated. The neighbor-joining (Saitou and Nei 1987) option was then applied to draw phylogenetic tree. The resulting tree was viewed using tree view drawing software (Page 1996).

Table 3. Lists of *R. solanacearum* strains retrieved from the database for endoglucanase gene sequence comparisons

No	Strain	Phylotype	Biovar	Host	Origin	Database accession	References
1	MAFF211266	I	4	L.esculuntum	Japan	AF295250	A*
2	GMI1000	I	3	*L.esculuntum*	Guyana	AF295251	A
3	NCPPB3190	I	4	*L.esculuntum*	Malaysia	AF295253	A
4	JT523	I	3	*S.tuberosum*	Reunion Island	AF295252	A
5	CFBP2972	II	1	*S.tuberosum*	Martinique	AF295264	A
6	CFB2047	II		*L.esculuntum*	United States	AF295262	A
7	CFBP2958	II	1	*L.esculuntum*	Guadeloupe	AF295266	A
8	CFBP712	II	1	*S.melongena*	Burkina Faso	AF295267	A
9	ICMP7963	II	1	*S.tuberosum*	Kenya	AF295263	A
10	NCPPB283	III	1	*S.pandaratome*	Zimbabwe	AF295275	A
11	CFBP734	III	1	*S.tuberosum*	Madagascar	AF295274	A
12	JT528	III	1	*S.tuberosum*	Reunion Island	AF295273	A
13	JT525	III	N2	*S.tuberosum*	Kenya	AF295279	A
14	WP20	IV	N2	*S.tuberosum*	Philippines	AY464988	B**
15	28MF	IV	N2	*S.tuberosum*	Philippines	AY464989	B
16	R780	IV	N2	*S.tuberosum*	Indonesia	AY465004	B
17	MAFF2112	IV	N2	*S.tuberosum*	Japan	AY465000	B
18	R230	IV	BDB	*Musa spp*	Indonesia	AF295280	B

* = Poussier *et.al.* (2000b); ** = Villa *et.al.* (2005)

Results

Identification and confirmation of specificity of *R. Solanacearum* species

The six strains listed in (Table 1, list number 1 to 6) were identified as *R. solanacerum* species among 46 presumptive samples collected from Ethiopia by species-specific PCR test. The test enabled to produce a single 281 bp DNA fragment revealing the specificity of *R. solanacearum* species. All the six strains were also found pathogenic on the susceptible tomato genotype L-390, causing an initial wilting within 3 to 4 days post- inoculation. There was a significant difference in virulence to tomato among the tested strains where strain *TomNa3* isolated from tomato showing the highest virulence causing 100% mortality within 7-10 days post inoculation (DPI) (Table 2).

Phenotypic, biochemical and physiological characteristics

On TTC medium all strains identified by PCR test revealed a similar morphological growth as irregular, large, elevated, fluidal colonies with a white periphery and a pale or light red center. On NGA medium, colonies were irregular, smooth, creamy white and fluidal. The colony sizes varied in diameter from 2-5 mm, but were not significantly different among the strains. On the basis of colony morphology, shape and appearance, it was not possible to differentiate the strains. Two fluidal types were observed where the majority was grouped into the fluidal class and one strain in the highly fluidal class. The strains were Gram-negative, oxidase

positive, in 1% tetramethylphenylene on filter paper yielded purple coloration within 10-15 seconds, but negative for oxidation/fermentation test. All tested strains were observed to have a cellular reserve poly-ß-hydroxybutyrate (PHB). The strains tolerated and grew in 1% sodium chloride solution (NaCl), but little or no growth was observed in 2% NaCl. Presence and absence of diffusible brown pigment production was observed where some produced and others did not (Table 2). Positive cultures changed the culture medium from green to yellow. In terms of biovar classification, the strains were identified as biovar 2, except one strain identified as biovar 1. The summary of phenotypic and biochemical characteristics of *R. solanacearum* strains studied is presented in Table 4.

Table 4. Phenotypic, biochemical and physiological characteristics of *R. solancearum* strains isolated from tomato in Ethiopia

	R. solanacearum strains from Ethiopia						Reference strain
Characteristic tested*	*Tomzy8*	*TomZy9*	*TomAw2*	*TomNa3*	*TomGr6*	*TomBk4*	*Toudk2*
Fluidity on TTC/NGA**	+++	+++	+++	++++	+++	+++	++++
Solubility in KOH	+	+	+	+	+	+	+
Gram stain	-	-	-	-	-	-	-
PHB inclusion	+	+	+	+	+	+	+
Growth in 1% NaCl	+	+	+	+	+	+	+
Growth in 2% NaCl	-	-	-	-	-	-	-
Oxidase test	+	+	+	+	+	+	+
Pathogenicity test	+	+	+	+	+	+	+
Virulence (%)	90	85	95	100	86	85	100
Utilization/Oxidation							
Mannitol	-	-	-	-	+	-	+
Sorbitol	-	-	-	-	+	-	+
Dulcitol	-	-	-	-	+	-	+
Lactose	+	+	+	-	+	+	+
Maltose	+	+	+	-	+	+	+
Cellobiose	+	+	+	-	+	+	+
Diffusible brown pigment	-	+	-	-	+	+	-
Biovar	2	2	2	1	2	2	3

* = Phenotypic, biochemical and physiological test, **+++ = fluidal, ++++ = highly fluidal, + = positive and - = negative

Genetic diversity based on Rep-PCR Analysis

The BOX-PCR showed 7 to 11 distinct PCR products per strain, ranging from 400-2000 base pair (bp) in size (Figure 1). A similarity matrix from Dice (1945) similarity coefficient was calculated and the result produced three unique genotypic groups among Ethiopian *R. solanacearum* strains at about 80 % similarity level and 9 fingerprint types at about 100 % similarity level (Figure 2). Strains belonging to the same phylotype were found grouped in the same cluster. However, strains belonging to phylotype III were clustered in two different groups. The reference *R. solanacearum* strains belonging to phylotype I, originated from Thailand and Kenya, were grouped into an independent group. The two strains share about 66 % similarity with the Ethiopian strains and about 69 % between themselves (Figure 2). The other reference strain originated

from Kenya (GSPB2695) identified as biovar 2 /race 3 showed a similar banding pattern with Ethiopian strains and clustered together.

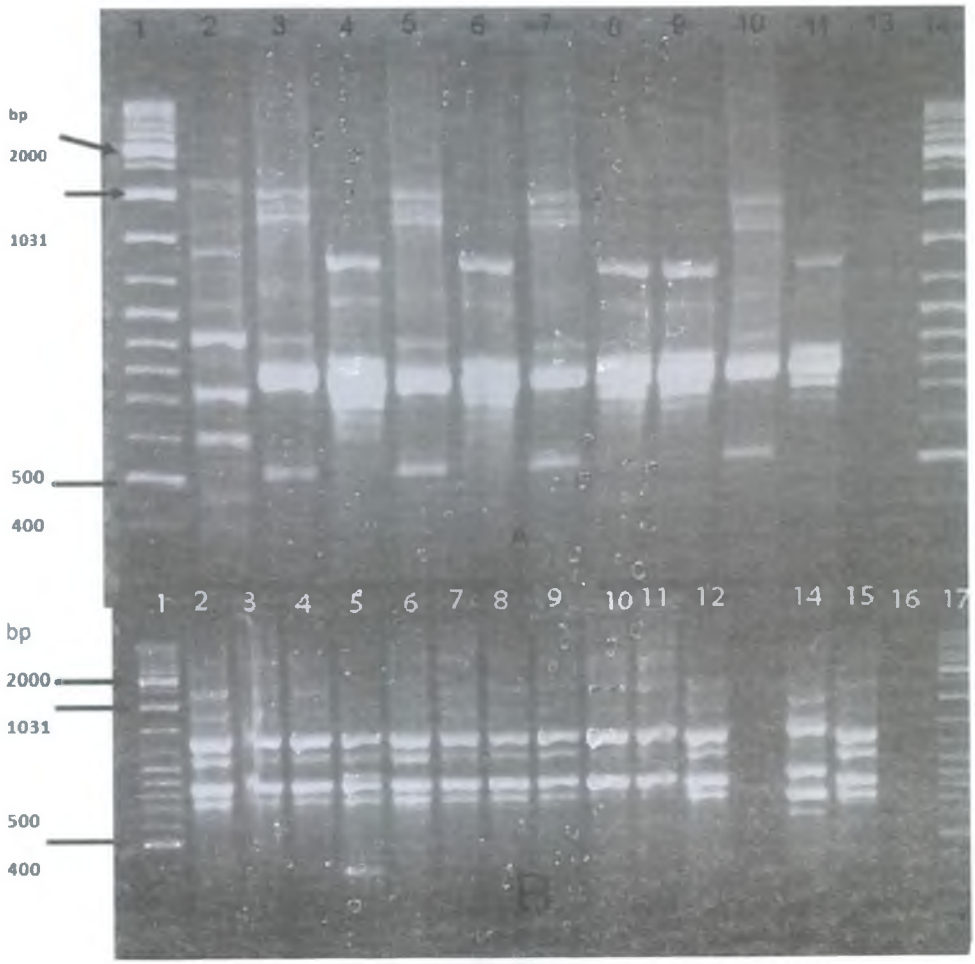

Figure 1. BOX-PCR-based DNA fingerprinting patterns of representative *R.solanacearum* strains from Ethiopia and reference strains. (A) above: Lanes 1 & 14 = 1 Kb DNA ladder, 2 = Toudk2, 3 = TomZy8, 4 = TomZy9, 5 = TomAw2, 6 = TomNa3, 7 = TomGr6, 8 = TomBk4, 9 = Tom1II, 10 = Tom3, 11 = Tom6II, 13 = control. (B) below: Lanes: 1 &17=1 Kb DNA ladder, 2= Pep7, 3=Pot5II, 4=Pot10II, 5 = Pot16III, 6 = Pot29JU, 7 = Pot34, 8 = Pot42, 9 = Pot48, 10 = Pot70, 11 = Pot84, 12 = Pot91, 14 = GSPB2690, 15 = GSPB2695 and 16 = control.

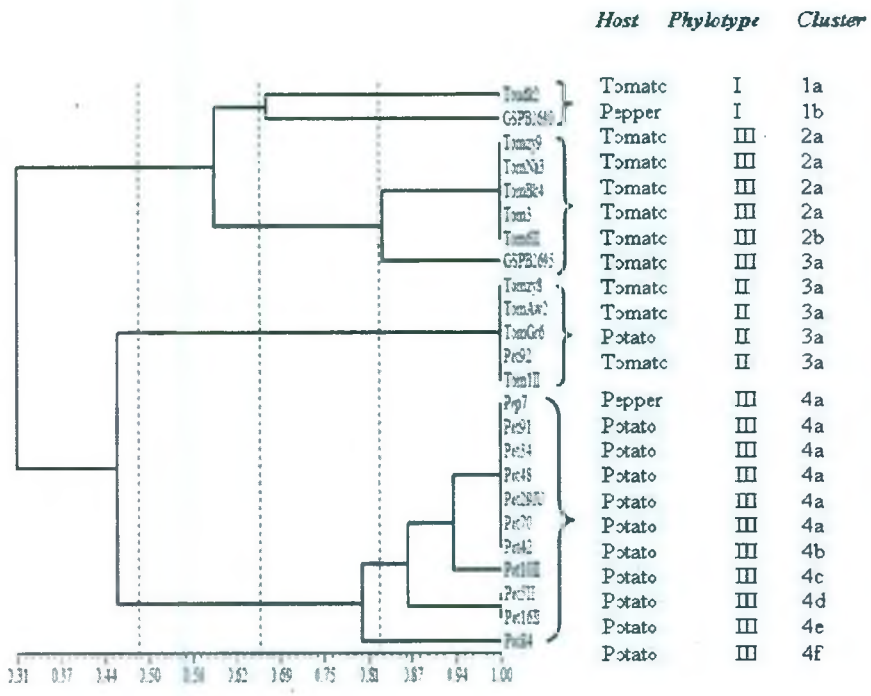

Figure 2. Dendrogram constructed from BOX-PC R fingerprints of *R. solanacearum* strains from Ethiopia and reference strains by Dice (1945) method based on Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering in NTSYS2.1. Host, Phylotype and cluster groups are indicated on the right

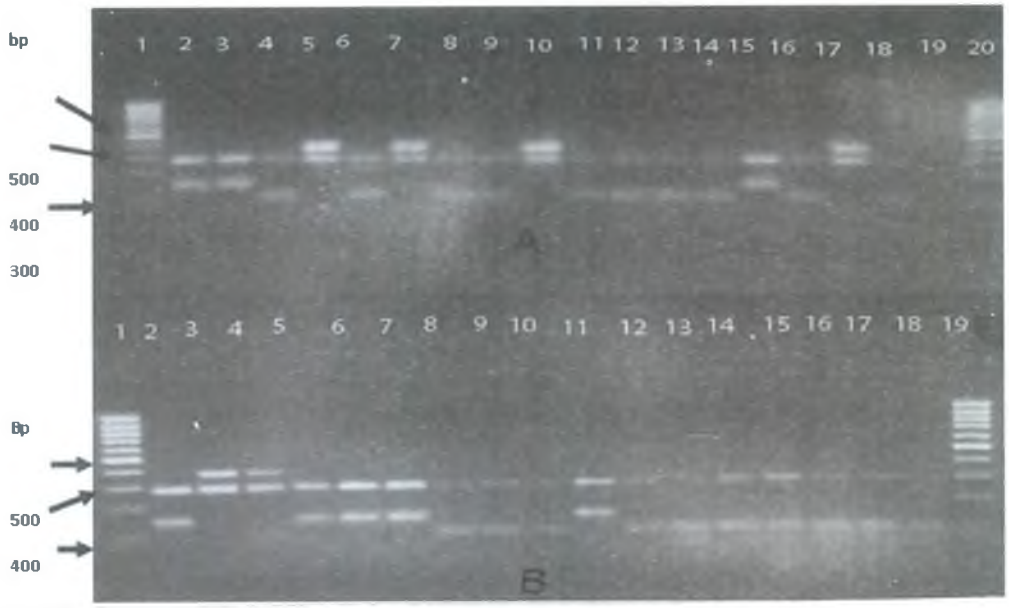

Figure 3. Sample of multiplex PCR gel showing the different phylotypes of *R. solanacearum* strains from Ethiopia and reference strains. Figure 3 (A) above: lane 1 = 100bp DNA ladder, 2 = UQRS348, 3 = Toudk2, 4 =TomZy9, 5 =TomAw2, 6 = Tomna3, 7 = TomGr6, 8 = TomBK4, 9 = Tom1II, 10 = Tom3, 11 = Tom6II, 12 =Tom56, 13 = Tom58, 14 = Tom768, 15 = UQRS559, 16 = Pep7, 17 = Pot92, 18 = Pot91, 19 = Control, 20 =Marker (100bp). Figure 1 (B) below: Lane,1 = Marker,2 = UQRS 559,3 =TomZy8, 4 = Tom88,5 = Pot1,6 = Toudk2,7 = Pot2 JU.8 = Pot 5II,9 = Pot10II,10 = Pot 10II, 11 = Pot16III, 12 = Pot34,13 = Pot42,14 =Pot48, 15= Pot50,16 = Pot60,17 = Pot62,18 = Pot70. & 19 = 100bp DNA ladder. Phylotype III: Lanes 4, 6, 8 ,9, 11-14, 16 & 18 (A); Lane 8, 9,10 &12-18 (B); Phylotype II: Lane 5, 7, 10, 17 (A) & Lane 3, 4(B), Phylotype I, Lane 2, 3, 15 (A) & Lane 2,5,6,7,11(B)

Identification of phylotypes

Based on multiplex PCR the *R. solanacearum* strains belonged to phylotypes I, II and III, identified from the molecular ladder producing an amplicon size of 144, 372 and 91 bp, respectively (Figure 3). As expected the reference strains from Asia were found to belong to Phylotype I corresponding to an amplicon size 144bp (Figure 3). The proportion of Ethiopian *R. solanacearum* strains identified by multiplex PCR account for about 31 % and 69 % of tested strains belonging to phylotype II and III, respectively. Strains identified as phylotype II consisted only biovar 2 and phylotype III comprised strains belonging to biovars 1 and 2.

Phylotype II included biovars 1, 2 and 2T which were isolated from America and *R. solanacearumrace* race 3, potato pathogen, which is distributed worldwide.

Amplification, purification and sequence analysis of endoglucanase gene

An endoglucanse gene was successfully amplified with a PCR amplification step resulting in a 750 base pair band (Figure 4, A). The same amplification products were further purified and success of the purification was checked and resulted in the same banding pattern (Figure 4, B).

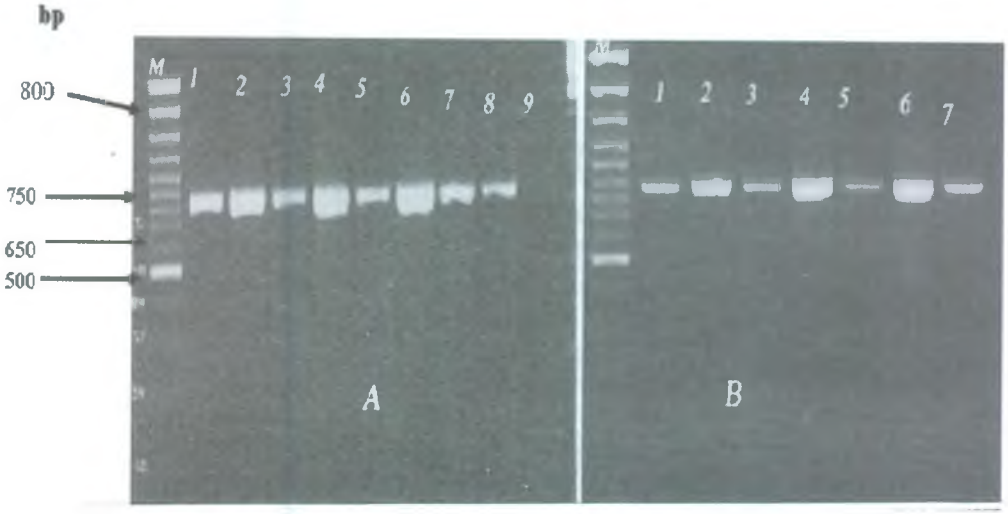

Figure 4. Gel electrophoresis showing an amplified endoglucanase gene in the region of 750 bp of *R. solanacearum* strains: Before purification (A) and after purification (B). Lane M =100bp DNA ladder: Lane 1 = Toudk2, 2 = TomZy8, 3 = TomZy9, 4 = TomAw2, 5 = TomNa3, 6 = TomGr6, 7 = TomBk4, 8 = GSPB2605, 9 =control (A) and lanes 1 = Toudk2, 2 = TomZy8, 3 = TomZy9, 4 = TomAw2, 5 = TomNa3, 6 = TomGr6, 7 = TomBk4 (B)

Sequences of partial endoglucanse gene sequences of all strains in this study were submitted to Basic Local Alignment Search Tool (BLAST) one by one. All sequences aligned with *R. solanacearum* database endoglucanse gene sequences and revealed a mean maximum identity ranging from 92 to 97%.

Phylotypes and phylogenetic relationships from partial endoglucanase gene sequence analysis

For identification of phylotypes and construction of phylogenetic relationship of *R. solanacearum* strains from Ethiopia and reference strains, 12 sequences of this study and 18 database sequences were used in the multiple alignment option of ClustalX 2.0 window interface. The distances among all pairs of sequences from the multiple alignments were first calculated; then the neighbor-joining option was applied to draw the phylogenetic tree. The phylogenetic tree from these sequences resulted in four clusters grouping of strains belonging to the same phylotype (Figure 5). The Ethiopian strains identified as Phylotype II and III by multiplex PCR also grouped into the respective phylotypes from the endoglucanase gene sequences. The reference strains from Thailand (Toudk2) and Kenya (GSPB2690) were also grouped in Phylotype I in cluster 1.

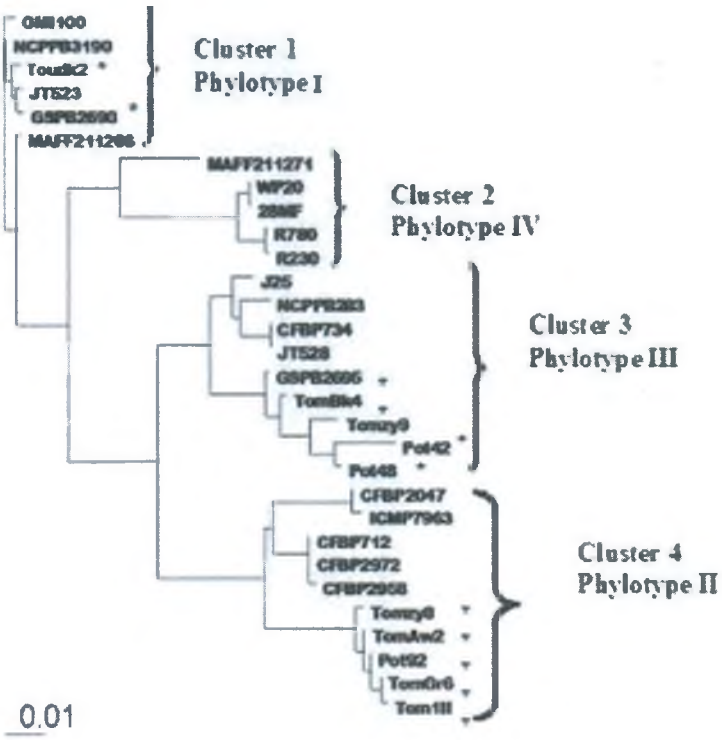

Figure 5. Phylogenetic tree based on comparison of partial endoglucanse gene sequences from *R. solanacearum* strains of Ethiopia, reference strains and selected database sequences. The phylogentic tree was constructed by the neighbor joining methods in ClustalX 2.0 and tree was view with Tree Draw Program. Bar indicates one nucleotide change per 100 nucleotide positions.* = indicates strains sequenced by this study.

Discussion

Strains of *R. solanacearum* identified by species specific PCR from Ethiopia were found to belong to biovars 1 and 2 complement the report of Lemessa and Zeller (2007). The latter report and an earlier report by Yaynu (1989) indicated that *R. solanacearum* strains belonging to biovar 2. Phenotypic, biochemical and physiological characteristics of strains of *R. solanacearum* of this study was similar with the studies from Ethiopia by Yaynu (1989) and Lemessa and Zeller (2007). The same was found in other studies and other countries by Kelman (1954) and He *et al.* (1983). Although all the strains were identified as highly pathogenic on the susceptible tomato genotype, strain *TomNa3* belonging to biovar 1/race 1 Phylotype III was the most virulent strain causing 100 % wilt incidence. The same strain was observed for its highly fluidal characteristic, typical for strains of high virulence. A similar result was reported by Leykun (2003) who observed a positive correlation between colony fluidity and virulence.

The phylotyping scheme of *R. solanacearum* strains with multiplex PCR enabled to clearly identify two phylotypes from Ethiopia, namely, Phylotype II and III. The occurrences of the two phylotypes proposed by Fikre *et. al.* (2010) where *R. solanacaerum* strains identified from Ethiopia as biovar 1 indicated the likely occurrences of phylotype III and those biovar 2 strains may fall in phylotype II. This study has clearly confirmed the occurrences of the two phylotypes. Furthermore, the result is complementing the occurrence of three of the four known phylotypes of *R. solanacearum* on the African continent (Elphinstone 2005). As described by Fegan and Prior (2005) multiplex PCR test is highly discriminative, flexible and accurate in differentiating the population of *R. solanacearum* strains. The reference strains that originated from Asia and Africa belonging to biovar 3 were also accurately identified as Phylotype I. The strains identified as phylotype II consisted of biovar 2 only, and phylotype III comprised strains belonging to biovars 1 and 2. The result is in agreement with Fegan and Mark (2005) where phylotype III contains strains that belong to biovars 1 and 2T primarily isolates from Africa and nearby Islands. Phylotype II included biovars 1, 2 and 2T from America and *R. solanacearum* race 3, which were isolated from potato and distributed worldwide.

Analyses from partial endoglucanse gene sequences, however, enabled to identify only the two phylotypes namely Phylotype II and Phylotype III. Similar results were reported by Villa *et.al.* (2005) where endoglucanase gene sequence analysis showed differentiation of *R. solanacearum* in to the four phylotypes so far described.

Assessment of genetic variability within *R. solanacearum* strains mainly collected from Ethiopia revealed the existence of a variable population structure. Similar result has been reported from Ethiopia (Fikre *et. al.*2010) where strains exhibited different grouping. As described by Fegan and Prior (2005) a test based on finger printing methods such as Rep-PCR enable to define clonal lines. Hence the fingerprint pattern based on BOX-PCR defines the tested Ethiopian *R. solanacearum* strains into three clonal lines at about 80 % similarity level identified 9 fingerprint types at about 100 % similarity where six fingerprint types are among Ethiopian strains. Grouping from Rep-PCR reveals strains belonging to the same phylotype were grouped in same cluster. However, strains belonging to Phylotype III were clustered in two different clusters. The reference *R. solanacearum* strains were grouped in to an independent group, which shared about 66 % similarity to the Ethiopian strains. Therefore, BOX-PCR procedures have been successfully used for discerning unique fingerprint profiles and identifying the diversity and evolutionary lines of *R. solanacearum* strains from Ethiopia and reference strains. The other reference strain which originated from Kenya (GSPB2695) and identified as biovar 2 race 3 showed a similar banding pattern among Ethiopian strains and clustered together. Earlier studies by Smith *et al.* (1995) have identified the similarity/homology of Kenyan and Ethiopia strains. Similarly, Rep-PCR has been successfully used in revealing the fingerprint of strains of *R. solanacearum* from different origin (Tsuchiya 2004), where strains belonging to the same race mostly grouped in the same cluster, and the same race further clustered into sub-

clusters based on the existing similarity/dissimilarity.

Ethiopian *R. solanacearum* strains were highly variable as revealed by Rep-PCR. A high variability was also reported from their reaction on different hosts, where three pathogenic groups were observed (Lemessa and Zeller 2007). Therefore, the existence of the three pathogenic groups identified by finger printing pattern and the two phylotypes identified by multiplex PCR and partial endoglucanse gene sequence is probably related to the difference in virulence of the strains.

In summary, the use of multiplex PCR and partial endoglucanase gene sequences have enabled to identify the occurrences of two phylotypes, namely Phylotype II and Phylotype III. Rep-PCR fingerprint enabled to define the existence of variability among *R. solanacearum* strains from Ethiopia. These findings contribute to update the existing information on *R. solanacearum* strains in Ethiopia and can help to discriminate and assess emerging pathogens or strains that could be potentially introduced into the country. It will also be useful in the development of molecular methods for practical diagnosis and establishing new strategies for disease control.

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