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Genetic diversity among strains of *Ralstonia solanacearum* from Ethiopia assessed by repetitive sequence-based polymerase chain reaction (rep-PCR)

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ABSTRACT

The genetic diversity among Ethiopian strains of *Ralstonia solanacearum*, causal agent of bacterial wilt, was assessed by repetitive sequence-based polymerase chain reaction (rep-PCR) method with BOX and ERIC primer sets. The study comprised 62 strains collected from potato, tomato and pepper, grown in Ethiopia (43 were identified as biovar 2 race 3, and 19 as biovar 1 race 1) and five reference strains obtained from different countries. The rep-PCR defined two major groups (1 and 2) among Ethiopian strains at 55% similarity level, each matching to a single biovar. Group 1 comprised biovar 2 and group 2 biovar 1 strains. At 90% similarity level, biovar 2 strains were grouped into five and biovar 1 into one. Third group, biovar 3, was formed by two of the reference strains at 55% similarity level. Comparative analysis of rep-PCR indicated that Ethiopian biovar 2 strains may fall in phylotype II and be American origin, while most of the biovar 1 strains may fall in phylotype III and be African origin. The study showed diversity in Ethiopian *R. solanacearum* populations which is valuable information and could help in designing disease control strategies.

Keywords: Bacterial wilt, biovar, Ethiopian *Ralstonia solanacearum* strains, race, rep-PCR

INTRODUCTION

Ralstonia solanacearum (Yabucchi *et al.*, 1995) a causal agent of bacterial wilt of several crops like potato, tomato, pepper, tobacco, etc. is one of the most important disease causing organism in tropical, subtropical and warm temperate regions of the world (Hayward, 1991). *R. solanacearum* embraces a diverse array of populations that differ in host range, geographical distribution, pathogenicity, genetic characteristics and physiological properties. To describe this intra-specific variability, binary classification systems are used. Accordingly, the pathogen is divided into five races (He *et al.*, 1983) based on host range and five biovars (Hayward, 1964; He *et al.*, 1983) based on utilization of three disaccharides and three hexose alcohols. There is considerable genetic variation among strains within each race or biovar (Cook *et al.*, 1989; Poussier *et al.*, 1999). The delineation of bacterial populations is a prerequisite to study the epidemiology of pathogens and, ultimately the development of control strategies.

More recently, genomic finger printing protocols have been used to differentiate microorganisms. The DNA-based analysis such as restriction fragment length polymorphism (RFLP), 16S rDNA sequence repetitive sequence-based polymerase chain reaction (rep-PCR), pulsed-field gel electrophoresis (PFGE) have been found effective in identifying the diversity and genetic relationship among strains of *R. solanacearum* (Smith *et al,*. 1995; Poussier *et al.,* 1999; Dookun *et al.,* 2001; Horita and Tsuchiya, 2001; Horita *et al.,* 2005). Prior and Fegan (2005) in their study analyzed the 16S-to-23S internal transcribed spacer region and *mutS*, *hrpB,* and *egel* gene sequences, together with amplified fragment length polymorphism/RFLP typing data (Poussier *et al.,* 2000a; Poussier *et al.,* 2000b) and the 16S rRNA gene sequence (Taghavi *et al.,* 1996) to develop a phylogeny-based scheme. This hierarchical classification is partitioned into four phylotypes (genetic groups), each of which is further subdivided into smaller groups. The phylotypes correlate with the geographical origin of strains: phylotype I include strains originating primarily from Asia, phylotype II from America, Phylotype III from Africa and surrounding islands in the Indian Ocean, and phylotype IV from Indonesia (Schell, 2000; Castillo *et al.,* 2007).

In Ethiopia *R. solanacearum* is an important pathogen that affects particularly potato and tomato (Yaynu, 1989). Recently, Lemessa and Zeller (2007) also reported it is attacking pepper plant in some areas of Ethiopia. This strain is characterized generally by physiological and biochemical tests. However, no study has been done to investigate genetic variability of this pathogen. It was therefore found important to investigate genetic variability amongst the various strains using molecular techniques in order to better understand the population of this bacterium in Ethiopia.

The rep-PCR method uses primer sets referred to as enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (REP) and BOX that yield genomic fingerprints specific to pathovars and strains of gram negative bacteria (Horita and Tsuchiya, 2001; Horita *et al.,* 2005). The BOX and ERIC primer sets generate a robust, reproducible and a highly discriminatory fingerprint (Rademaker *et al.,* 1998). In our study therefore BOX and ERIC primer sets were used for studying genetic variation in Ethiopian *R. solanacearum* strains. The objective of this study was, therefore, to assess the genetic variation of Ethiopian strains and predict their geographical origin based on repetitive fragment method.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains used in this study are shown in Table1. A total of 56 Ethiopian isolates were obtained from diseased potato (*Solanum tuberosum),* tomato (*Solanum lycopersicum)*, and pepper (*Capsicum annuum*) plants and potato tubers from various locations of Ethiopia and six were obtained from the culture collection of the Plant Protection Research Centre (PPRC), Ambo, Ethiopia. Moreover, five strains of *R. solanacearum* from the Göttingen Phytobacteriology Collection (GSPB) were included for comparison. Of the five reference strains, originally two were from Peru, two from Kenya and one from Cameroon. All strains were maintained in long-term storage as suspensions in sterile distilled water at room temperature (Wullings *et al.,* 1998). For further use, they were revived by plating on tetrazolium chloride (TTC) medium (Kelman, 1954) or on casamino acids peptone glucose (CPG) agar (Smith *et al.,* 1995).

Identification of strains and biovar and race testing

The identity of each Ethiopian strain was confirmed at Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Biological Control, Darmstadt, Germany, by pathogenicity test on tomato seedlings and species specific PCR primers (759/760) (Opina *et al.,* 1997; Ito *et al.,* 1998). Biovar verification was done as previously described by Haywrad (1964). Race was determined according to Buddenhagen *et al*. (1962) based on host range.

DNA extraction

Bacterial strains were grown on TTC agar and single colonies of the strain were transferred to CPG broth and grown overnight at 28°C on rotary shaker (150 rpm). One ml of the culture (OD_{600} = 0.4) was used for extraction of genomic DNA. Bacterial DNA was extracted with a commercially available kit (Qiagen GmbH, Hilden, Germany) and used as a template for PCR.

PCR analysis

The primer sets: ERIC, ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′); and BOX, BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) used in this study have been described previously by Versalovic *et al.* (1991) and used by several other authors (e.g., Horita *et al.,* 2005; Shutt *et al.,* 2005; Zhao *et al.,* 2000). Primers were synthesized by Metabion International Ag, Martinsried, Germany. PCR amplification was performed in a final volume of 25 µl, consisting of 1 × PCR reaction buffer (Qiagen GmbH, Hilden, Germany), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of each primer, 2.5 U of DNA polymerase Takra *Taq* polymerase, and 1µl of the extracted DNA (about 25 ng template DNA). The PCR amplification was performed with an automated thermal cycler, Mastercycler®, (Eppendorf AG, Hamburg, Germany) programmed as follows: an initial denaturation step of 95°C for 2 min, followed by 30 cycles of 94°C for 30 s (ERIC) or 94°C for 1 min (BOX), 50°C for 1 min (ERIC) or 52°C for 1 min (BOX), and 65°C for 4 min with a final extension of 65°C for 5 min (ERIC) or 65°C for 2 min (BOX), followed by hold time at 4°C until samples were collected. Amplified products (10 µl) were resolved by gel electrophoresis in 1.5% agarose gel in 1×TAE (10 mM Tris-HCl and 1mM EDTA) buffer, pH 8.0, at 120 voltages. The gel was stained with ethidium bromide (10 mg/ml) and the DNA fragments (bands) were detected by a UV trans-illumination and photographed under UV light using Polaroid 667 films. For confirmation of banding patterns, PCR experiments were repeated at least two times.

Data analysis

Rep-PCR finger print profiles were used to measure genetic similarity among strains. Each band with a different electrophoretic mobility was assigned a position number and scored as either 1 or 0 based on the presence or absence of the band, respectively for this position. Variations in intensity of bands among isolates were not considered as differences. Similarity coefficients for all possible pairs of strains based on the finger print groups were estimated by Dice method (Dice, 1945). Dendrogram was generated from the similarity coefficient data by the unweighted pair group method with arithmetic averages (UPGMA) clustering (Sneath and Sokal, 1973).

Table 1. Strains of *Ralstonia solanacearum* used in the study

Strain name	Geographical origin	Original host	Race	Biovar	rep-PCR group
IBC Pot 1	Guder, Ethiopia	*Solanum tuberosum*	3	2	1a
IBC Pot 5	Guder, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 34	Gedo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 5 II	Holeta, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 9II	Holeta, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 10II	Holeta, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 15II	Guder, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 4JU	Jimma, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 6 JU	Jimma, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 6III	Kejo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 10III	Kejo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 20III	Arjo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 21 III	Arjo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 40	Ginchi, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 56	Shashemene, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 57	Shashemene, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 61	Awassa, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 65	Jimma, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 66	Jimma Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 68	Jimma, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 70	Jimma, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 71	Jimma, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 81	Kombolcha, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 84	Ambo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 86	Kejo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 92	Shashemene, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 93	Shashemene, Ethiopia	*Solanum tuberosum*	3	2	
IBC Tom 3	Guder, Ethiopia	*Lycopersicon esculentum*	3	2	
IBC Tom 1II	Holeta, Ethiopia	*Lycopersicon esculentum*	3	2	
IBC Tom 56	Adam Tulu, Ethiopia	*Lycopersicon esculentum*	3	2	
IBC Tom 58	Guder, Ethiopia	*Lycopersicon esculentum*	3	2	
IBC Tom 88	Ziway, Ethiopia	*Lycopersicon esculentum*	3	2	
IBC Pot 1076PPRC*	Kombolcha, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 1079PPRC*	Qarsa, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 262APPRC*	Ambo, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 1091PPRC*	Agaro, Ethiopia	*Solanum tuberosum*	3	2	
IBC Tom 768PPRC*	Ziway, Ethiopia	*Lycopersicon esculentum*	3	2	
GSPB 2791**	Peru	*Solanum tuberosum*	3	2	
IBC Pot 16III	Bako, Ethiopia	*Solanum tuberosum*	3	2	1b
IBC Pot 29JU	Jimma, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 60	Shasemene, Ethiopia	*Solanum tuberosum*	3	2	
GSPB 2695**	Kenya	*Lycopersicon esculentum*	3	2	1c
IBC Pot 17III	Bako, Ethiopia	*Solanum tuberosum*	3	2	1d
IBC Pot 9JU	Jimma, Ethiopia	*Solanum tuberosum*	3	2	1e
IBC Pot 1080PPRC*	Qarsa, Ethiopia	*Solanum tuberosum*	3	2	
IBC Pot 2JU	Jimma, Ethiopia	*Solanum tuberosum*	1	1	2
IBC Pot 4II	Holeta, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 8 JU	Jimma, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 31 JU	Jimma, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 46	Jeldu, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 48	Ginchi, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 50	Ginchi, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 55	Shashemene, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 58	Shashemene, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 59	Shashemene, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 94	Shashemene, Ethiopia	*Solanum tuberosum*	1	1	
IBC Tom 6II	Holeta, Ethiopia	*Lycopersicon esculentum*	1	1	

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Strain name	Geographical origin	Original host	Race	Biovar	rep-PCR group
IBC Tom 53	Shashemene, Ethiopia	*Lycopersicon esculentum*	1	1	
IBC Pep 58	Guder, Ethiopia	*Capsicum annum*	1	1	
IBC Pep 61	Guder, Ethiopia	*Capsicum annum*	1	1	
IBC Pep 7	Guder, Ethiopia	*Capsicum annum*	1	1	
IBC Pot 42	Jeldu, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 62	Awassa, Ethiopia	*Solanum tuberosum*	1	1	
IBC Pot 91	Shashemene, Ethiopia	*Solanum tuberosum*	1	1	
GSPB 2792**	Cameroon	*Solanum tuberosum*	1	3	
GSPB 2690**	Kenya	*Capsicum annum*	1	3	3a
GSPB 2709**	Peru	*Solanum tuberosum*	1	3	3b

All strains were collections of this study except those with * which were obtained from Plant Protection Research Centr (PPRC), Ambo, Ethiopia, and those with ** were procured from Göttingen Collection of Phytopathogenic Bacteria (GSPB), Göttingen, Germany.

RESULTS AND DISCUSSION

Rep-PCR genomic fingerprints of 62 Ethiopian strains and five reference strains were generated by BOX and ERIC primer sets and representative patterns of Ethiopian strains and the five reference strains is shown in Fig. 1a and b. Similarity coefficients were calculated based on the fingerprints and a dendrogram was constructed (Fig. 2). Both BOX and ERIC primer sets gave clear genomic PCR profiles that were highly reproducible. BOX primer produced 4 to 10 bands per isolate within the range of 0.5 to 5 kb molecular size, whereas the REP primers generated 3 to 11 bands per isolate within a range of approximately 0.15 to 3 kb. Polymorphic bands were clearly observed by both primer sets helping differentiation into groups. Only a single monomorphic band was observed with ERIC primer sets at about 0.7 kb, while no monomorphism was detected with BOX primer.

After duplicate analysis, both BOX and ERIC-PCR defined three major groups at 55% similarity level (Fig. 2). The number of strains in groups 1, 2, and 3 was 45, 20 and 2, respectively (Table 1). Each group contained strains belonging to the same biovar, with the exception of group 2, which included strains of biovar 1 and 3 (only one strain from the latter). Group 1 contained all biovar 2 strains, group 2 all biovar 1 strains and one biovar 3 strain (the exception), and group 3 two strains from biovar 3. Average similarities within groups 1, 2 and 3 were more than 57%, 94%, and 55%, respectively. On the contrary, average similarity between group 1 and 2 was 37%, 2 and 3, 31%, and 1 and 3, 31%. Race wise, all group 1 strains were race 3, while all group 2 and 3 strains were race 1.

At a similarity level of 90%, group 1 strains were distributed over five sub groups (1a, 1b, 1c, 1d and 1e) with majority (84%) of the strains in sub group 1a. Group 2 strains were grouped together and group 3 strains were grouped into two sub groups (3a and b) with one strain in each group. Generally, Ethiopian strains were put under group 1 (the majority) and group 2. In group 3 were only two reference strains; one from Kenya and the other from Peru.

Rep-PCR analysis has been used to differentiate a great number of species and strains of phytopathogenic bacteria (Horita and Tsuchiya, 2001; Horita *et al.*, 2005). Here we also used rep-PCR (BOX and ERIC) analysis to detect genetic diversity of Ethiopian strains along with five reference strains. Each primer set (BOX and ERIC) gave repeatable and distinct patterns (Fig. 1a and b) enabling differentiation of strains into groups. In

the dendrogram based on the rep-PCR finger prints three main groups were found, each group representing a biovar except one strain (Fig. 2). Accordingly, all biovar 2 strains from Ethiopia were grouped into group 1, biovar 1 strains into group 2, and biovar 3 strains into group 3 (except one strain which was grouped to group 2 while it is biovar 3).

In a study with strains from many parts of the world, Smith *et al.* (1995) and Poussier *et al.* (1999) have shown that biovar 2 is the most homogenous group and gather in one group by RFLP and rep-PCR. In our investigation also most of biovar 2 strains were pooled in one subgroup (1a) at 90% similarity level. However, some of the strains formed separate cluster in contrast to what has been mentioned by Smith *et al.* (1995) and Poussier *et al.* (1999). Although the biovar testing in our study did not identify if the strains belonged to biovar 2T, it is possible that some of the strains listed as being biovar 2 can be biovar 2T which may explain the diversity of genomic finger prints of the strains in comparison to previous work. It has been indicated that potato isolates collected in Kenya, Nigeria and Cameroon were found to have the biovar 2T phenotype (Smith *et al.,* 1995). Castillo *et al.* (2007) has also indicated that there is a weaker subdivision of phylotype II into two subgroups.

As opposite to biovar 2, biovar 1 strains were reported to be heterogeneous population by several authors (Horita and Tsuchiya, 2001; Poussier *et al.,* 1999; Smith *et al.,* 1995). In the present study, however, biovar 1 strains were clustered together indicating homogeneity of the population. In a recent study by Lemessa and Zeller (2007), it was indicated that biovar 1 strains are a new introduction to Ethiopia. Thus, the lack of diversity in Ethiopian biovar 1 strains may be due to close genetic basis of biovar 1 strains introduced to Ethiopia. Nevertheless, as biovar 1 was represented only by 19 strains from Ethiopia in this study, it would be crucial to consider large number of strains from this group to substantiate this result. Horita and Tsuchiya (2001) indicated that biovar 3 strains have low average similarity and fall into five groups. Moreover, Poussier *et al.* (1999) has shown that biovar 3 strains cluster into two groups. Similarly, our cluster analysis grouped biovar 3 strains into three sub groups (3a, 3b, and 2) at 95% similarity level indicating their diversity. Prior and Fegan (2005) assessed genetic diversity of *R. solanacearum* strains and revealed four different types of phylotypes. Phylotype I included all strains belonging to biovar 3, 4, and 5 and strains are isolated primarily from Asia (Asiaticum). Phylotype II included strains belonging to biovar 1, 2, and 2T isolated primarily from America. Phylotype III had strains primarily isolated from Africa and surrounding islands. Strains in this group belong to biovar 1 and 2T. Phylotype IV contains strains isolated primarily from Indonesia belonging to biovar 1, 2 and 2T. Similarly, Castillo *et al.* (2007) and Guidtot *et al.* (2007) confirmed the existence of four types of phylotypes matching with those reported by Prior and Fegan (2005).

This indicates that the Ethiopian biovar 2 strains all of which were identified to be race 3 (Group 1) (Fig 1) fall into phylotype II of Prior and Fegan (2005). This further implies that the Ethiopian biovar 2 strains that were put in group 2 (Fig. 1) are American origin. Berga *et al.* (1994) indicated that Ethiopia has introduced several thousands of potato genotypes mainly from International Potato Centre (CIP), Peru, in South America to improve potato productivity in the country. Thus introduction of the disease to Ethiopia may be through latently infected potato tubers. *R. solanacearum* can be widely disseminated on vegetative propagating material

(tomato, *Heliconia*, banana, and potato), and bacterial wilt of potato is spread locally and internationally by latently infected potato tubers (Hayward 1991). Buddenhagen (1985) suggested that race 3 (biovar 2) originated in the Andean region of South America where potato also originated and that the occurrence of race 3 outside South America was the result of human distribution of infected potato tubers. Several reports (Smith *et al.* 1995; Poussier *et al.* 1999; Horita and Tsuchiya 2001) using DNA-based techniques (RFLP, rep-PCR, PFGE) support this hypothesis.

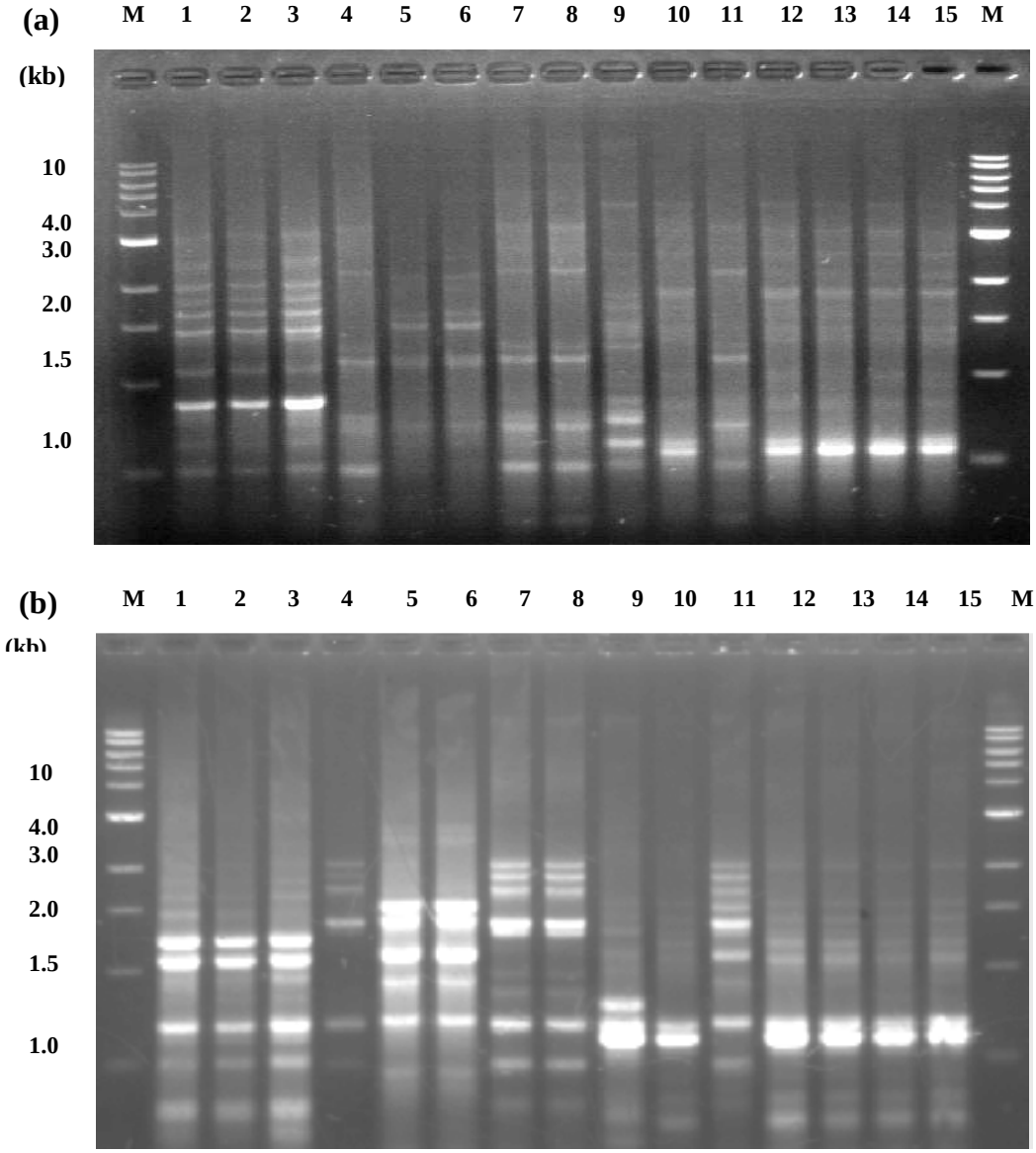

Figure 1. Agarose gel bands showing representative patterns of Ethiopian *Ralstonia solanacearum* strains and five reference strains from Göttingen Collection of Phytopathogenic Bacteria (GSPB), Göttingen, Germany, generated by repetitive sequence-based polymerase chain reaction with (a) BOX and (b) ERIC primers. Lane M, DNA molecular size marker (kilo base DNA ladder); Sizes are indicated on the left in kilo base pairs (kb); lanes: 1, IBC Pot 34; 2, IBC Tom 58; 3, GSPB 2791; 4, GSPB 2695; 5, IBC Pot 9JU; 6, IBC Pot 1080PPRC; 7, IBC Pot 29JU; 8, IBC Pot 60; 9, GSPB 2690; 10, GSPB 2709; 11, IBC Pot 17III; 12, GSPB 2792; 13, IBC Pot 91; 14, IBC Pot 53; 15, IBC Pep 61.

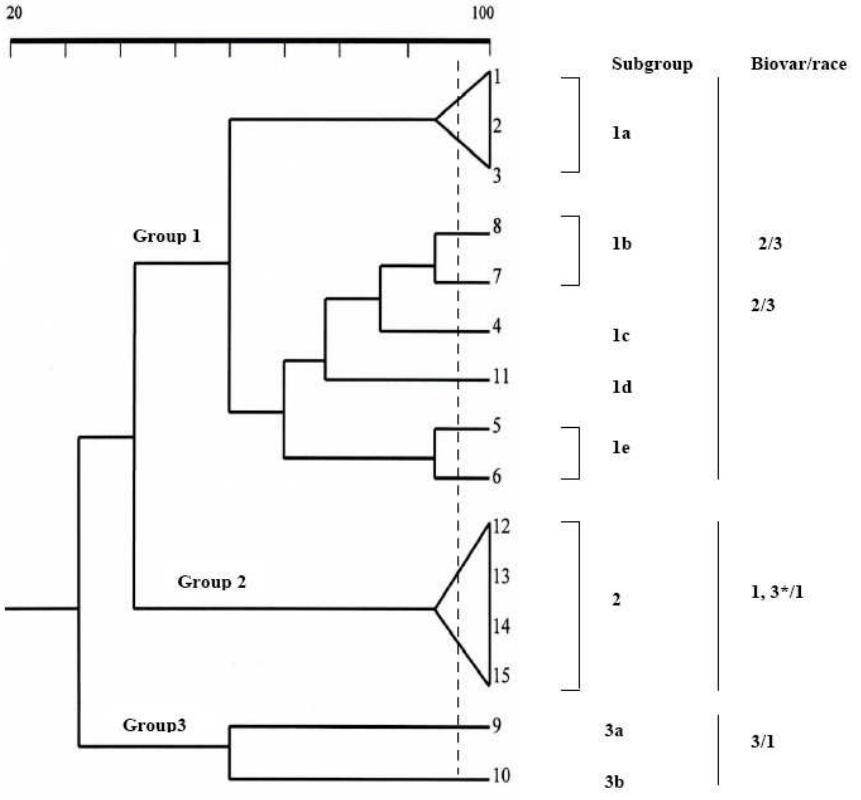

Figure 2. Genetic diversity of Ethiopian *Ralstonia solanacearum* strains and five reference strains from Göttingen Phytobacteriology Collection (GSPB), Göttingen, Germany, on the basis of rep-PCR. A similarity coefficient was calculated based on fingerprints using the Dice (1945) coefficient. A dendrogram was constructed by unweighted pair group method with arithmetic average (UPGMA) clustering. Numbers at the right tips of dendrogram show lane numbers presented in Fig.1. Sub group, biovar and race are given on the right. The * shows that only one strain from biovar III has been put in the group.

Furthermore, according to Prior and Fegan (2005), biovar 1 strains can be categorised into phylotype II, III, or IV. Nevertheless, research findings indicate that strains from Africa belonging to biovar 1 mostly fall under phylotype III (Castillo *et al.*, 2007; Guidot *et al.*, 2007; Prior and Fegan, 2005). This suggests that most of the Ethiopian biovar 1 strains which were reported as new introduction to Ethiopia (Lemessa and Zeller, 2007) likely belong to phylotype III of Prior and Fegan (2005). In general, the data presented here show diversity in Ethiopian *R. solanacearum* population as they cluster into two groups (1 and 2) at 55% and into six (1a to1e and 2) at 90% similarity level (Fig. 2). This suggests that control strategies may not be equally effective for all the grouped bacterial strains. Thus the information can be considered as valuable for designing control strategies for Ethiopian conditions.

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