Genetic diversity of bacterial wilt caused by Ralstonia solanacearum as assessed by PCR

Mansoor, Muhammad; Abbas, Waseem; van Elsas, J. D.; Bashir, Muhammad R.; Atiq, Muhammad

Published in:
Pakistan Journal of Phytopathology

DOI:
10.33866/phytopathol.029.01.0341

Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

GENETIC DIVERSITY OF BACTERIAL WILT CAUSED BY *RALSTONIA SOLANACEARUM* AS ASSESSED BY PCR

[a]Muhammad Mansoor, [b]Waseem Abbas*, [a]J. D V. Elsas, [b]Muhammad R. Bashir, [c]Muhammad Atiq

[a]Department of Microbial Ecology, University of Groningen, the Netherlands.
[b]Ayub Agricultural Research Institute, Jhang road, Faisalabad, Pakistan.
[c]Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan.

A B S T R A C T

The current research was conducted to investigate genetic diversity of *Ralstonia solanacearum* for comparison of different strains that were collected mainly from Netherlands as well as from Bangladesh, Brazil, Kenya, Egypt, Pakistan and Palma. Forty six strains were included in contemporary studies whereas main biovars for these strains included biovar-2 except GMI1000 that belonged to biovar 3. Genetic diversity of bacterial wilt disease caused by *R. solancearum* was assessed by focusing mainly on three genes i.e. *mutL, cbhA* and *dps.* All the genes seem to be conserved but in case of *mutL* some strains showed divergence. Multi Locus Sequence Typing (MLST) scheme was used in this contemporary research. It was concluded that polymerized chain reaction (PCR) is the most imperative and appropriate modern tool of molecular biology to find genetic diversity in *Ralstonia solanacearum* causing bacterial wilt.

Keywords: Genetic diversity, bacterial wilt, *Ralstonia solanacearum,* polymerized chain reaction (PCR)

INTRODUCTION

Ralstonia solanacearum is one of the most destructive bacterial pathogens, cause disease on at least 200 different host species (Hayward, 1991). It affects a wide range of plants worldwide, including herbaceous plants, shrubs, and trees. *R. solanacearum* also affects ornamental plants such as tomato, potato, banana, peanut and eggplant (Hayward, 1964; Williamson *et al.,* 2002). This gram-negative bacterium typically inhabits subtropical and tropical regions and recently has spread to the temperate regions of Europe (Genin *et al.,* 2004). *R. solanacearum* is the most pivotal plant pathogens among other yield limiting factors such as *Pseudomonas solanacearum* Buddenhagen (1986). A comprehensive analysis of pathogen diversity is essential for development of diagnostic tests of universal value. Early classification of *R. solanacearum* divides the species into three races and at least seven subgroups of strains distinguished by pathogenesity on various hosts, colony morphology, biochemical type, lysotype, serotype and bacteriocin production (Buddenhagen *et al.,* 1964).

Oxidation of six key carbon sources separated the species into four major biochemical types (biovars) that have been used to characterize strains worldwide (Hayward, 1964). Both classifications lack an exact concordance with the genetic background of the complex members. Recently, Fegan and Prior (2005) analyzed the16S-to-23S internal transcribed spacer region and *mutS*, *hrpB*, and *egl* gene sequences, together with amplified fragment length polymorphism/restriction fragment length polymorphism typing data (Poussier *et al.,* 2000) and the 16S rRNA gene sequence (Taghavi *et at.,* 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 named sequevars. Each phylotype reflects the geographic origin of strains: phylotype I and II are composed of Asian and American strains, respectively, whereas phylotype III members are

* Corresponding Author:
Email: waseemabbas1894@yahoo.com

African, and phylotype IV isolates, including *R. syzygii* and BDB, are from Indonesia, Japan, and Australia (Prior *et al.,* 2005).

The wide diversity of *R. solanacearum* is reflected in the bacterium's considerable variability in host range, aggressiveness (Jaunet *et al.,* 1999) and the adaptation to different climates that is often influenced by host genotype, natural habitat, and agricultural practices (Hayward, 1991). The fraction of phylotype II commonly known as race 3/biovar 2 (R3B2) infects tomato and common solanaceous weeds and causes brown rot, a serious disease of potato. This group is adapted to lower temperatures than other races; therefore, it constitutes a serious threat to agricultural production in temperate regions of the world (Williamson *et al.,* 2002). *R. solanacearum* is organized into two large circular replicons called the chromosome (the larger replicon) and the megaplasmid. Both replicons contain essential and pathogenicity genes, the same dinucleotide relative abundances and codon usage, and similar distribution and composition of simple sequence repeats (Salanoubat *et al.,* 2002). Thus, the two replicons in this bacterium have likely coevolved over a long time span. However, the evolutionary driving mechanism that shapes the chromosome and megaplasmid of *R. solanacearum* is still unclear. Genome sequence analysis provides clues about the evolution of essential virulence genes such as those encoding the Type III secretion system and related pathogenicity effectors. It is the dire need of the hour to find out resistant gene through molecular biology. Therefore, in the current research the diversity of bacterial wilt was found through polymerized chain reaction.

MATERIALS AND METHODS

Strains of *Ralstonia solanacearum* used in the current research: Forty six strains were used that were mostly taken from the Netherlands by different sources but few strains were also collected from other countries like Bangladesh, Brazil, Kenya, Egypt, Pakistan and Palma. Main host used in other countries for the collection of strains was Potato.

Extraction of DNA: DNAs were extracted by using Ultra Clean® MOBIO Microbial DNA isolation kit. The Ultra Clean® Microbial DNA Isolation Kit is designed to isolate high-quality genomic DNA from microorganisms. A variety of microorganisms, including bacterial and fungal spores, have been tested successfully with this kit. Microbial cells, re-suspended in bead solution were added to a bead beating tube containing beads, followed by lysis solution. The principle is to lyse the microorganisms by a combination of heat, detergent, and mechanical force against specialized beads. The cellular components were lysed by mechanical action using a specially designed MOBIO Vortex Adapter on a standard vortex. From the lysed cells, the released DNA was bound to silica Spin Filter. The filter was washed and the DNA was recovered in certified DNA-free Tris buffer.

PCR amplification: Seven genes were amplified but mainly three genes were focused such as *cbhA, mutL* and *dps*. Amplification conditions for these genes were 94°C (5min) for denaturation and 94°C (45sec) and 58°C (45sec) for annealing and extension, respectively. A preceding denaturation step and a final extension step were carried out at 72°C for 50 sec and 5 min, respectively. Gene *SpoT* was excluded from the study because it gives no product after PCR amplification. Different programs were used for this gene but it was useless. PCR products were resolved using agarose 1% (wt/vol) gel electrophoresis.

Cleaning of PCR products: PCR products were cleaned with the Sephadex™ cleaning method using Sephadex™ G-50 Fine. The cleaned products were checked for concentration on 1% agarose gel.

DNA sequencing: Sequencing reactions were performed in the PCR machines, GeneAmp® 9700 and the MyCycler using this program: 96˚C for 4 minutes → 25*(96˚C for 10 seconds → 50˚C for 5 seconds → 60˚C for 4 minutes) → 4˚C. DNA sequencing was performed in Applied Biosystems 3130×l Genetic analyzer using forward and reverse primers. Raw sequences from both strands were assembled with Sequence scanner v1.0 and Chromas v2.23.All ambiguous and terminal sequences were trimmed before data analysis. Inconsistencies were solved by re-sequencing.

Table 1. List of primers and their sequences with complete genome size

Primer	Sequence (5'—3')	Size gene(bp)
mutLfw	*Acgtccagcacctgtacttc*	1944
mutL rev	*Cgcatcatcgccaggtattc*	
cbhA fw	*Agctgcctcactactaactg*	1728
cbhA rev	*Ccggctgtagttccttgaat*	
dps fw	*Tcctggaacggcacgtaagc*	954
dps rev	*Gctgtcggtcgccatcaaga*	
Rpos fw	*Aagccgccacgtccgctaat*	1146
Rpos rev	*Tcctgcacctcctcggtagt*	
efe fw	*Ccggtctgacgacattccat*	1044
efe rev	*Cggtcaggaattgcaggaag*	

Sequences were aligned in MEGA v4.0 using Clustal W (Thompson *et al.,* 1994). Before alignment all the strains with short sequences were discarded. After the alignment the data were put through another program

within MEGA v4.0 to make phylogenetic trees per gene. Neighbor-joining trees were made with a 1000 replica's and using the Tajima-Nei model. After making the phylogenetic trees with actual strains, out groups were also tried by using the data from NCBI.

Table 2. *Ralstonia solanacearum* strains characterized with their sampling location, year and source

Strains	Location/Country	Year of isolation	Source[a]
GMI1000	Brasil	2004	Potato
Bra1	Brasil	2004	Potato
Bra3	Brasil	2004	Potato
UW551	Kenia	2006	Geranium
715	Bangladesh	Unknown	Potato
715	Pakistan	2010	Potato
1609	The Netherlands	1995	Potato
KZR-1	KZR	2004	S
KZR-2	KZR	2004	S
KZR-3	KZR	2004	S
KZR-5	KZR	2004	S
PA1	A	2004	S
PA2	A	2004	S
PA4	A	2004	S
PA5	A	2004	S
PA8	A	2004	S
RA9	A	2004	R
RA12	A	2004	R
RA13	A	2004	R
RA16	A	2004	R
RA18	A	2004	R
WA19	A	2004	Water
WA20	A	2004	Water
SA31	A	2004	Sediment
WB48	B	2004	Water
WB49	B	2004	Water
SB63	B	2004	Sediment
WC76	C	2004	Water
WC78	C	2004	Water
RA05-9	A	2005	R
RA05-10	A	2005	R
RA05-11	A	2005	R
RA05-12	A	2005	R
RA05-13	A	2005	R
PA05-16	A	2005	S
PA05-17	A	2005	S
PA05-18	A	2005	S
PA05-21	A	2005	S
PA05-22	A	2005	S
WA05-6	A	2005	Water
PB05-28	B	2005	s
RC06-06	A	2004	R
RC06-49	A	2004	R
RC06-50	A	2004	R
UW23	Egypt	Unknown	Potato
9.47	Acquitaine	Unknown	Tomato
1602-1	Palma	1995	Potato

R. solanacearum cells were isolated from either stems (s) or roots (R) of S dulcamara

RESULTS

DNA extraction: DNAs were extracted by using MOBIO kit and tested them for the organism *Ralstonia solanacearum*. The typical growth of *Ralstonia solanacearum* on BGT medium plates can be checked in Figure 3. In the first step, a Box-PCR was performed to see if there was indeed DNA of the organism. It was concluded by using specific primers that it is indeed *Ralstonia solanacearum*. For BOX genomic finger printings, we used a twofold concentrated PCR buffer. Amplicons were analyzed by electrophoresis on 1.5% agarose gels.

PCR amplification: Three genes were mainly studied named as *mutL, cbhA* and *dps*. These three genes and other two that were not sequenced well and we were unable to include them in our results were *Rpos* and *efe* but another exceptional case was the gene *spot.* For *spoT* gene we tried two times but it gives no PCR product at all. We also changed programme and also used new primer for that gene but all was useless. All other five genes gave very nice PCR product and amplified well for further process. The results from these PCR runs that were eventually good enough for sequencing are visualized in the next Figure 1.

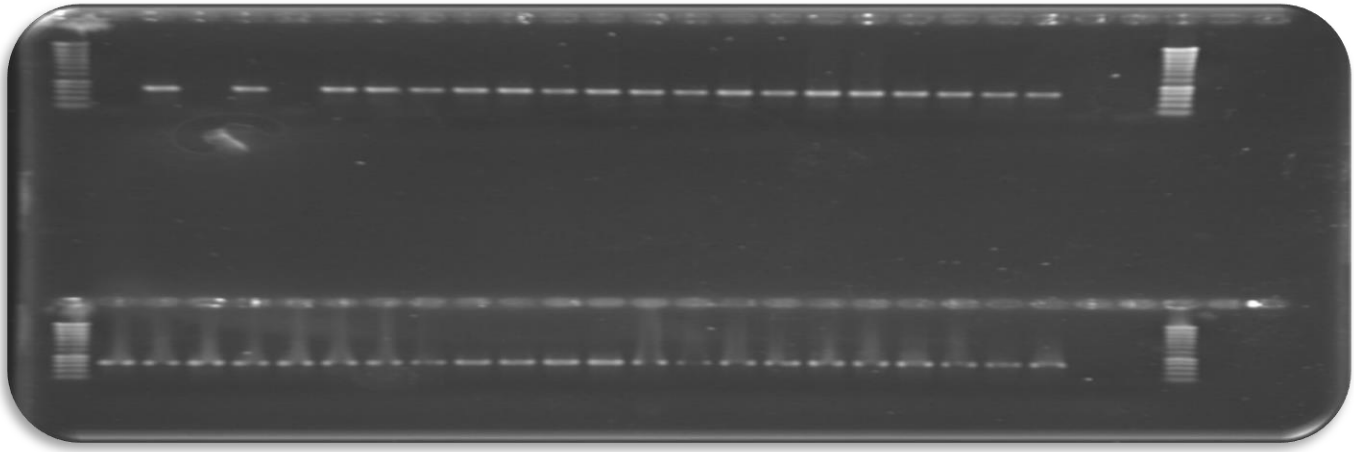

Figure 1. PCR product of Rpos (top) and mutL (bottom)

Clean PCR Product

Sephadex™ cleaning method was used to get clean PCR product (Figure 2).

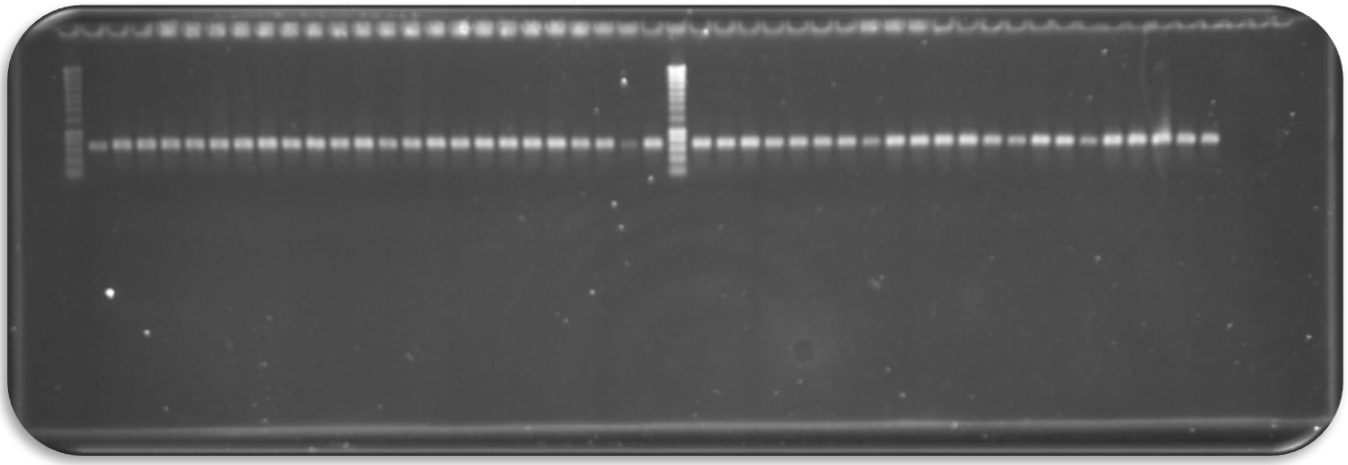

Figure 2. Clean PCR product of mutL

DNA sequencing: Sequence PCR was run as a first step. The sequences were checked by eye with programmes, Sequence scanner v.1.0 and Chromas v.2.23. All the sequences that seem to be very short and in other case bad sequences were not included for alignment. For MLST there should be reasonable long sequences as above 900bp but in our case only the long sequences were above 400bp but it was also

before alignment. Sequence size that was used in alignments can be checked in Table 2.

Table 3. Genes with their base pairs used in alignments and for making Phylogenetic tree

Genes	Base pairs used in alignment
mutL	412 bp
dps	334 bp
cbhA	294 bp

Table 4. List of strains with successfully sequenced genes

Strains	Genes to be sequenced		
	mutL	Dps	cbhA
GMI1000			+
Bra1		+	+
Bra3		+	+
UW551	+	+	+
715	+		+
1609	+	+	+
KZR-1	+		+
KZR-2	+	+	
KZR-3	+	+	+
KZR-5		+	+
PA1		+	+
PA2		+	+
PA4	+	+	
PA5		+	
PA8	+	+	
RA9	+		
RA12	+	+	
RA13	+	+	
RA16	+	+	+
RA18	+		+
WA19	+	+	
WA20		+	
SA31	+	+	
WB48	+	+	
WB49	+	+	+
SB63	+	+	+
WC76		+	+
WC78	+	+	+
RA05-9	+	+	+
RA05-10	+	+	+
RA05-11	+	+	+
RA05-12	+	+	+
RA05-13		+	+
PA05-16		+	+
PA05-17	+	+	+
PA05-18	+	+	
PA05-21	+	+	
PA05-22	+		+
WA05-6	+	+	+
PB05-28		+	
RC06-06	+	+	
RC06-49	+	+	
RC06-50		+	+
UW23		+	
9.47		+	
1602-1		+	+

With the use of alignments Phylogenetic trees were made. These trees demonstrated that how many strains were more or less diversified. General conclusion from these trees was the conservation of genes. It seems that genes were conserved and there was not much diversification between strains. The exceptional case was the gene *mutL* in which strain 18 shows very different place than other Dutch strains and this strain was also out of Dutch strains cluster in gene *mutS*. These two genes showed a different behaviour than other genes. Overall view was that all the Dutch strains had same genetic makeup but this view was little bit different in the gene *dps* in which two strains 11&36 were out of Dutch strain cluster. Another approach was used in this study to make out groups for all the phylogenetic trees. Two other organisms *Burkholderia pseudo mallei* and *Sorangium cellulosum* were used for this purpose and it was interested that strain 18 was more closed with *Burkholderia pseudo mallei* and it seems to be horizontal gene transfer (HGT) but this was not the case when this strain was checked with outgroup in *mutS* gene. So it was confusable for that strain to have a HGT phenomenon. All the genes that were involved in this study was housekeeping genes and these genes should be more conserved than auxiliary genes and same result was in our study. Genes were conserved and not reasonable diversification seems in strains that were ultimately suggest that Dutch strains were not genetically changed. Out of 46 strains approximately 30 strains worked for every gene. Table 3 shows the list of strains used and number of genes sequenced against each strain.

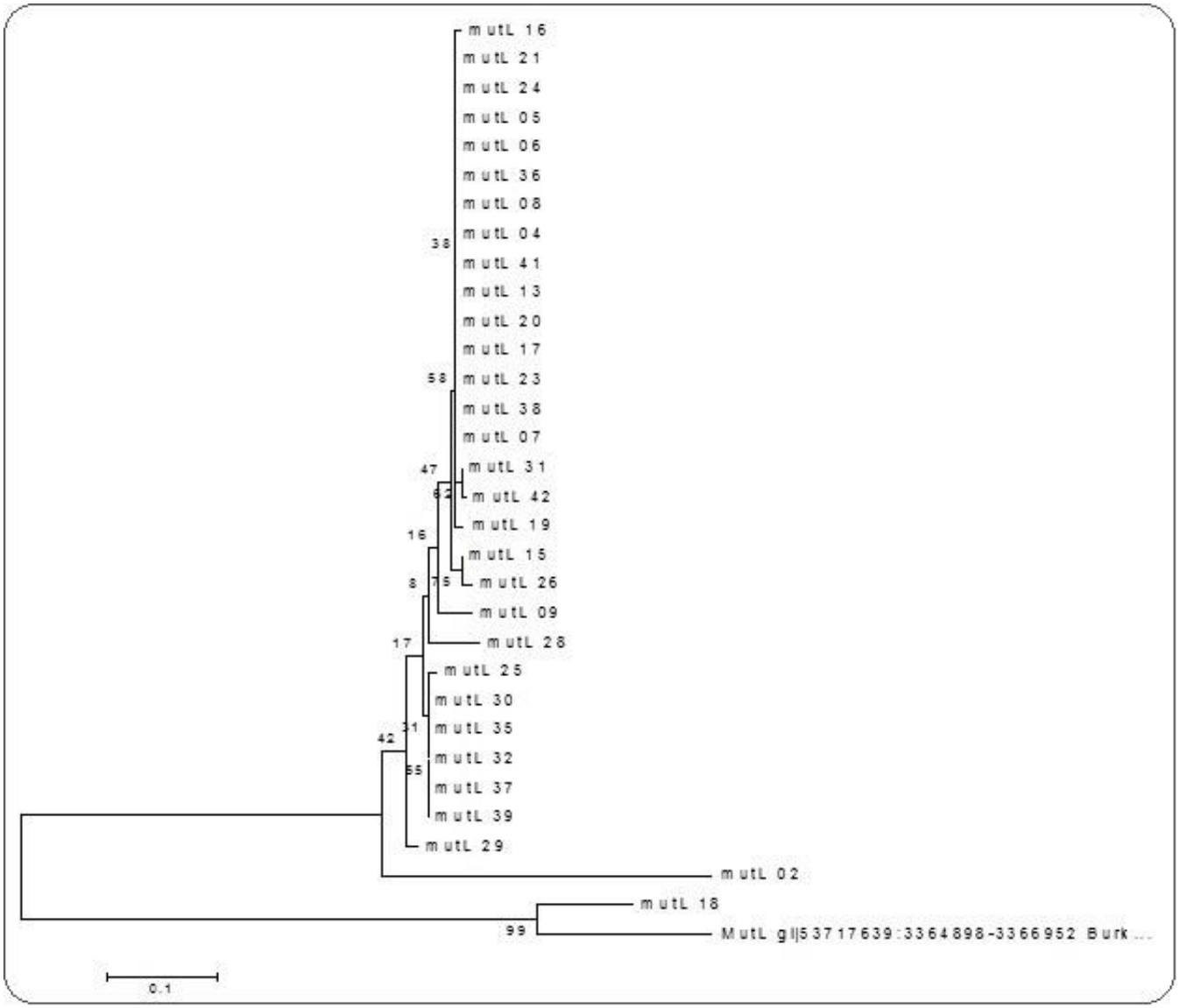

Figure 4. A phylogenetic neighbor-joining tree of mutL. 30 strains were used in this analysis. The gene seems to be conserved. The only striking deviation is strain 18 and strain 02

Figure 3. Growth of *Ralstonia solanacearum* strain 1609 on BGT medium

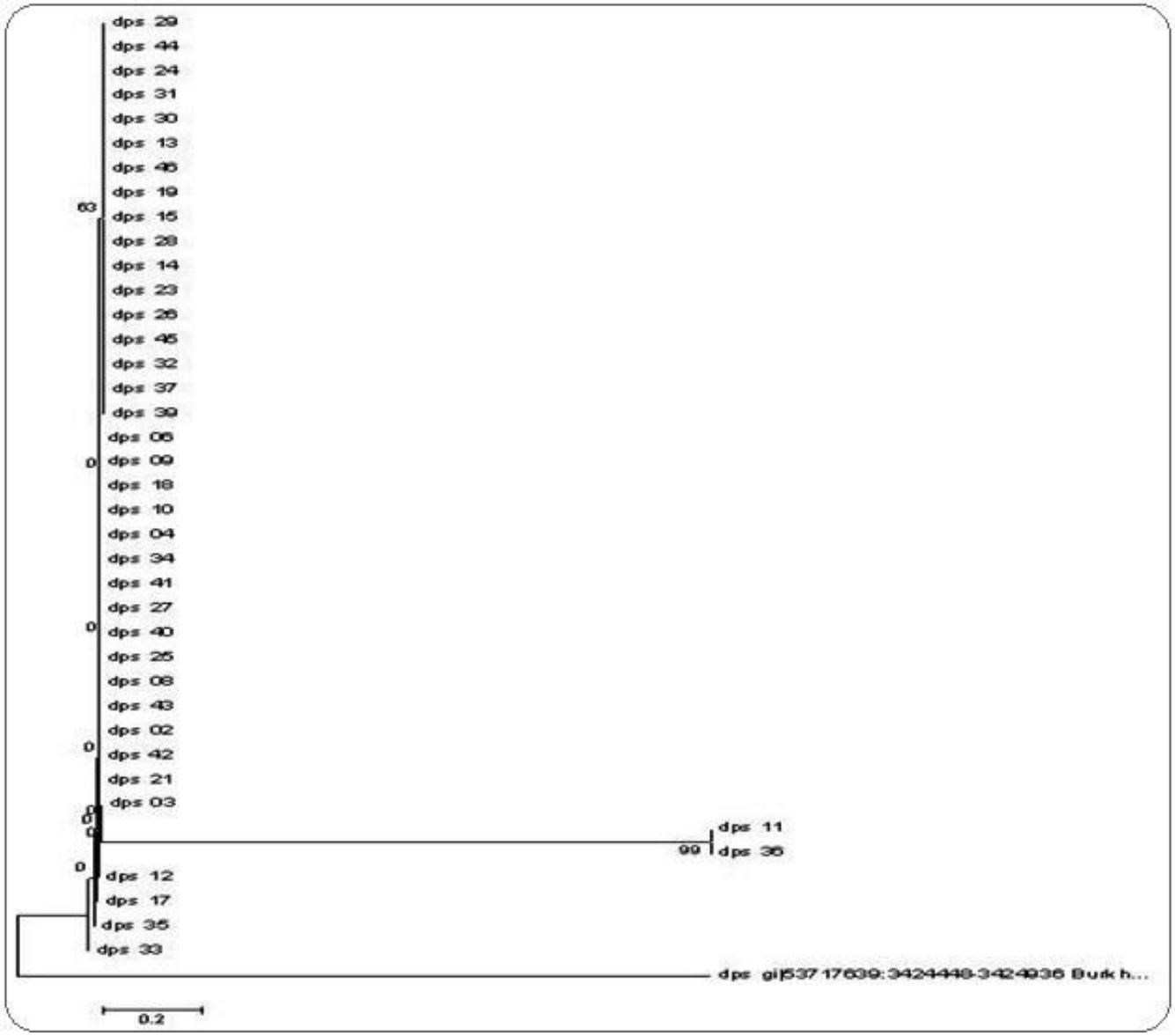

Figure 5. A phylogenetic neighbor-joining tree of dps 40 strains were used in this analysis. The two strains 11and 36 shows divergence

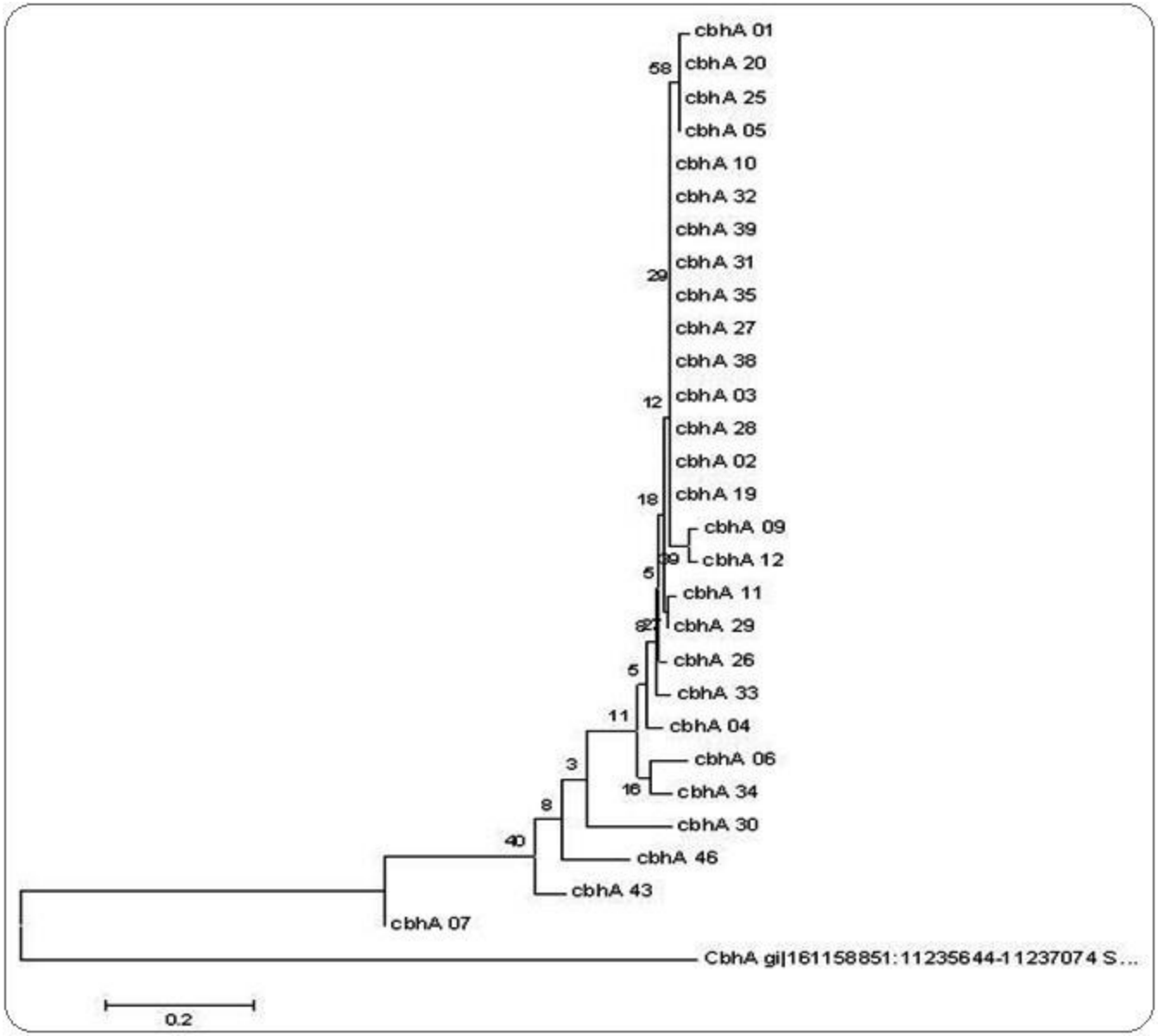

Figure 6. A phylogenetic neighbor-joining tree of cbhA 28 strains were used in this analysis. The gene seems to differentiate a bit more than other genes

DISCUSSION

Multilocus sequence typing (MLST) study provided some valuable results exhibiting that genes were conserved. Multilocus sequence typing is a recently devised method for identifying strains of bacteria based solely on nucleotide sequence differences in a small number of genes (Peter *et al.,* 2013). For this type of study, a long sequence is a pre-requisite. After getting good clean PCR products for almost all the genes, it was expected that a nice sequences works upto 400bp that may be owing to mechanical error in machine 3130xl Genetic analyzer because of any kind of disturbance in the programming of machine. The significant results were obtained through cleaning method that was Sephadex™ cleaning method. There was not diversification in strains and all the strains belonging to Dutch climate made one cluster for most of genes. It seems that there is no genetically adaptation of Dutch strains in temperate climate. Some strains showed different behaviour in two genes, *mutL* and *dps*. Strain 18 was totally deviated from Dutch strain cluster in *mutL* gene. Strain 18 was taken from Local climate and this strain showed closeness with out-group and we suspected that may be there is HGT but after our discussion it was concluded that impurity of the strain

can be the cause of deviation. Strain 02 was also out of big cluster in *mutL* gene but this strain was collected from Brazil and it was an idea for this strain to have a different genetic make up from rest of strains but it was not same in all the genes. Strain 11 and 36 also gave different results in *dps* gene and this time also, these two strains made different cluster from Dutch strains. In *cbhA* gene strains 07 and 30 were out of place and these strains were deviated from Dutch strains. Strains 43 and 46 were also diversified in *cbhA* gene but these strains were collected from Egypt and Palma respectively. There were no reliable clusters in phylogenetic trees because different strains made different clusters for every gene. There were no grouping with the originate of strains or with host specificity but the only conclusion was that the genes were more or less conserved and there was major deficiency of long sequences in our study. We were only able to get short sequences which influence the phylogenetic data. MLST method clearly offers an excellent opportunity for strain typing and cataloguing diversity within a bacterial species. It's relatively easy study but we should devote more time for this type of study and should be more efficient in sequencing. If there is enough data and enough time to analyze it then it can be a good way to get insight in the adaptation and evolution of microorganisms. With our phylogenetic study we can say that *Ralstonia solanacearum* has not genetically adapted to temperate climate and there is no host specificity between the strains that were collected from tropical (Bangladesh) and temperate (Netherlands) climates. It is concluded that there is no diversification between the strains that were collected from local climate (Dutch) and from tropical climate.

REFERENCES

Buddenhagen, I. W. 1986. Bacterial wilt disease in Asia and the South Pacific. Bacterial wilt revisited. J. Plant Dis., 10: 126-143.

Buddenhagen, I. W. and A. Kelman. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol 12:203-230

Fegan, M. and P. Prior. 2005. Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological society, St. Paul,MN. 20:449-461.

Genin, S. and C. Boucher. 2004. Lessons learned from the genome analysis of *Ralstonia solanacearum*. Annu. Rev. Phytopathol. 42:107-134.

Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol. 7: 265-277

Hayward, A. C. 1991. Biology and epidemiology pf bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29:65-87.

Jaunet, T. X. and J. F. Wang. 1999. Variation in genotype and aggressiveness of *Ralstonia solanacearum* race1 isolated from tomato in Taiwan. Phytopathology 89:320-327.

Peeters, Nemo, Guidot, Alice, Vailleau, Fabienne, & Valls, Marc. (2013). Ralstonia solanacearum, a widespread bacterial plant pathogen in the post-genomic era. *Molecular plant pathology, 14*(7), 651-662.

Peter, Nemo, Guidot, Alice, Vailleam, Fabienne and M. Valls. 2013. *Ralstonia solanacearum* a widespread bacteria plant pathogenin the post-genomic era . Mol. Plant. Pathol. 14(7) 651-662.

Poussier, S., D. T. Demery., P. Vandewalle., B. Goffinet., J. Luisetti and A. Trigalet. 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis and identification of an African subdivision. Microbiology. 146:1679-1692.

Prior, P. and M. Fegan. 2005. Recent developments in the phylogeny and classification of *Ralstonia solanacearum*. Acta Hortic. 695:127-136.

Salanoubat, M., S. Genin., F. Artiguenave, J. Gouzy, S. Mangeno, M. Arlat, A. Billaul., P. Brottier, J. Camus, L. Cattolico., M. Chandler., N. Choisne., C. C. Renard, S. Cunnac, N. Demang, C. Gaspin, M. Lavie., A. Moisan., C. Robert., W. Saurin., T. Schie, P. Siguier, P. Thebault, M. Whalen, P. Wincker, M. Levy, J. Weissenbach and C. A. Boucher. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature. 415:497-502.

Taghavi, M., C. Hayward., L. I. Sly and M. Fegan. 1996. Analysis of the phylogenetic relationships of strains of *Burkholderia solanacearum, Pseudomonas syzygii* and the blood disease bacterium of banana based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. 46:10-15.

Thompson, J. D., D. G. Higgins and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4637-4680.

Williamson, L., B. D. Hudelson and C. Allen. 2002. *Ralstonia solanacearum* strains isolated from geranium belong to race 3 and are pathogenic on potato. Plant Dis. 86:987-991.