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Analysis of the Molecular Diversity of Common Bacterial Blight (*Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*) Strains from Ethiopia Revealed by Rep-PCR Genomic Fingerprinting

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Abstract

Common bacterial blight (CBB) disease of the common bean (*Phaseolus vulgaris*) caused by *Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*, is one of the most damaging foliar diseases of common bean production in Ethiopia. CBB causes economic losses due to reduction in seed quality and yield in common bean producing regions of Ethiopia. Currently, information on the genetic diversity of CBB strains in Ethiopia has been lacking. Here, for this specific study common bean bacterial blight strains were obtained from infected leaves collected from diverse bean growing areas. The collected strains of CBB were characterized to study the genetic diversity and relatedness of the CBB strains using repetitive extragenic elements polymerase chain reaction (rep-PCR) genomic fingerprinting technique. Analysis of Molecular Variance (AMOVA) revealed the existence of genetic diversity among bacterial strains and confirmed the presence of genetically distinct strains in Ethiopia. CBB pathogens are seed-borne so the lack of geographic differentiation among the six-different common bean growing localities could be the result of the distribution of one or some limited bacterial genotypes. Common bean improvement programs that develop CBB-resistant bean varieties for higher production should consider this information to determine the relevance and extent of resistance of improved bean cultivars.

Keywords: Rep-PCR fingerprinting; Genetic diversity; Common bacterial blight, Disease resistance

Introduction

Common bean (*Phaseolus vulgarise L.*) is one of the major food and cash crops in Ethiopia. It has considerable national economic significance and is also a traditional food security crop in Ethiopia [1-3]. However, production of this important crop is limited by both biotic and abiotic factors. The most important and widely distributed common bean fungal and bacterial diseases include rust (*Uromyces appendiculatus*), angular leaf spot (*Pseudocercospora griseola*, previously *Phaeoisariopsis griseola*), anthracnose (*Colletotrichum lindemuthianum*), common blight (*Xanthomonas campestris* pv. *phaseoli*) and halo blight (*Pseudomonas syringae* pv. *phaseolicola*) [4,5]. Among the disease, common bacterial blight (CBB) is an important and significant seed borne disease of common bean which is caused by *Xanthomonas campestris* pv. *phaseoli* and the brown-pigmented variant *X. campestris* pv. *phaseoli* var. *fuscans* [6,7]. Both strains cause identical symptoms but *Xanthomonas phaseoli* var. *fuscans* has been reported to be more aggressive. CBB was first described in the Castilla Leon region of Spain in 1940 and is now a major constraint on common bean production all over the world, including in eastern Africa [8]. The disease is widespread throughout Africa's bean growing regions and is favoured by warm to high temperatures and high humidity. CBB ranked among the most important and a widespread disease of common bean in Ethiopia and it was reported by many researchers as the main constraint to common bean production throughout the country [4,9,10]. The development of molecular tools for genetic finger printing of bacteria has allowed for the examination of genetic diversity among *X. campestris* strains. Recently, scientists reported variability among common bacterial blight strains using genetic fingerprinting and correlate this variability with biological properties like pathogenicity [6,7].

Assessment of the genetic diversity and identification of bacterial plant pathogen using primers corresponding to specific repetitive sequences like enterobacterial repetitive intergenic consensus (ERIC), repetitive extra genic consensus (Rep), and repetitive BOX elements (BOX), which are dispersed throughout the bacterial genome, has well been documented [11,12]. Multiple amplicons of different sizes can be resolved by electrophoresis, establishing DNA-fingerprint specific patterns for bacteria strains [13]. The pattern of distribution of these repetitive sequences varies from one bacterium to another and can be used to characterize differentiation in bacterial populations. PCR based on these repetitive sequences (rep-PCR) was found to be effective in identification of bacterial pathogens even at race level [8]. The primers corresponding to the Rep region are rep-1and rep-2, to the ERIC regions ERIC1R and ERIC2 and the BOX element regions are BOX-A1R primer, respectively. The Palindromic Units (PU) Repetitive Extragenic Palindromes (REP) constitutes the characterized family of bacterial repetitive sequences. PU are present in about 500-1000 copies in the chromosome of *Escherichia coli* and of *Salmonella typhimurium*. PU sequences consist of a 35-40 bp inverted repeat and

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are found in clusters. A second family of repetitive elements, called IRU (Intergenic Repeat Units) or ERIC (Enterobacterial Repetitive Intergenic Consensus), has been described [13]. IRU are 124–127 bp long in which successive copies (up to six) are arranged in alternate orientation [14]. Both PU and IRU families are similarly located in non-coding, probably transcribed, regions of the chromosome. Repetitive Element Polymorphism REP-PCR fingerprinting has become a frequent method to discriminate bacteria species analyzing the distribution of repetitive DNA sequences in several prokaryotic genomes. REP-PCR is based on the observation that outwardly facing oligonucleotide primers, complementary to interspersed repeated sequences, enable the amplification of differently sized DNA fragments, consisting of sequences lying located between these elements. Multiple amplicons of different sizes can be resolved by electrophoresis, establishing DNA fingerprint specific patterns for bacteria strains. Several of these interspersed repetitive elements are conserved in diverse genera of bacteria and, therefore, enable single primer sets to be used for DNA fingerprinting in many different microorganisms [15,16]. The effectiveness of genetic resistance is highly dependent on the interactions among host, pathogen, and environmental conditions [17]. So far, there was no any information regarding the genetic diversity of CBB in Ethiopia. Therefore, knowledge of CBB pathogen diversity is a key prerequisite for developing CBB-resistant cultivars adapted to a specific area. The analysis of genetic variation at molecular level may also facilitate the detection and investigations on the taxonomy and epidemiology of the CBB pathogen.

This study was aimed to assess the genetic diversity of 40 strains of common bacterial blight collected from diverse common-bean growing regions of Ethiopia, based on ERIC, REP and BOX sequences, and determine the genetic variability among the bacterial strains.

Materials and Methods

Collection of common bacterial blight strains

Strains of common bacterial blight were recovered from infected leaves of common bean which were collected from field surveys conducted during 2015 and 2016 main seasons from six geographical diverse major common-bean growing areas of Ethiopia (Wolaita, Gurage, Sidama, Gamogofa, Jimma, and Arisi) strains of CBB obtained from each location were considered as populations (Table 1).

Code	Strain	Geographic Origin	Collection zone	Altitude m.a.s.l	Year of collection	Host
1	*et xa 001*	Ethiopia	Arisi	1785	2015	Mesoamerican
2	*et xa 002*	Ethiopia	Gurage	1835	2015	Andean
3	*et xa 003*	Ethiopia	Arisi	1999	2015	Mesoamerican
4	*et xa 004*	Ethiopia	Sidama	1820	2015	Mesoamerican
5	*et xa 005*	Ethiopia	Jimma	1216	2016	Mesoamerican
6	*et xa 006*	Ethiopia	Gurage	1903	2015	Mesoamerican
7	*et xa 007*	Ethiopia	Sidama	1823	2015	Mesoamerican
8	*et xa 008*	Ethiopia	Sidama	1835	2015	Mesoamerican
9	*et xa 009*	Ethiopia	Jimma	1713	2016	Andean
10	*et xa 010*	Ethiopia	Jimma	1700	2016	Andean
11	*et xa 011*	Ethiopia	Jimma	1216	2016	Mesoamerican
12	*et xa 012*	Ethiopia	Wolaita	2058	2015	Mesoamerican
13	*et xa 013*	Ethiopia	Arisi	1999	2015	Mesoamerican
14	*et xa 014*	Ethiopia	Arisi	1835	2016	Mesoamerican
15	*et xa 015*	Ethiopia	Sidama	1823	2015	Mesoamerican
16	*et xa 016*	Ethiopia	Wolaita	1260	2015	Mesoamerican
17	*et xa 017*	Ethiopia	Jimma	1383	2016	Mesoamerican
18	*et xa 018*	Ethiopia	Wolaita	1606	2015	Mesoamerican
19	*et xa 019*	Ethiopia	Wolaita	1257	2015	Mesoamerican
20	*et xa 020*	Ethiopia	Jimma	1823	2016	Mesoamerican
21	*et xa 021*	Ethiopia	Wolaita	1865	2015	Mesoamerican
22	*et xa 022*	Ethiopia	Jimma	1920	2016	Mesoamerican
23	*et xa 023*	Ethiopia	Wolaita	1262	2015	Mesoamerican
24	*et xa 024*	Ethiopia	Wolaita	1909	2015	Mesoamerican
25	*et xa 025*	Ethiopia	Gurage	1893	2015	Mesoamerican
26	*et xa 026*	Ethiopia	Gurage	1835	2015	Mesoamerican
27	*et xa 027*	Ethiopia	Wolaita	1835	2015	Mesoamerican
28	*et xa 028*	Ethiopia	Wolaita	1835	2015	Mesoamerican
29	*et xa 029*	Ethiopia	Gurage	1903	2015	Andean
30	*et xa 030*	Ethiopia	Gurage	1903	2015	Andean
31	*et xa 031*	Ethiopia	Wolaita	1262	2015	Andean
32	*et xa 032*	Ethiopia	Wolaita	1709	2015	Mesoamerican
33	*et xa 033*	Ethiopia	Gamogofa	1266	2015	Mesoamerican
34	*et xa 034*	Ethiopia	Gurage	1855	2015	Mesoamerican
35	*et xa 035*	Ethiopia	Wolaita	1862	2015	Mesoamerican
36	*et xa 036*	Ethiopia	Gamogofa	1862	2015	Mesoamerican
37	*et xa 037*	Ethiopia	Wolaita	1699	2015	Mesoamerican
38	*et xa 038*	Ethiopia	Wolaita	1687	2015	Mesoamerican
39	*et xa 039*	Ethiopia	Gurage	1861	2015	Mesoamerican
40	*et xa 040*	Ethiopia	Sidama	1695	2015	Mesoamerican

Table 1: strains of common bacterial blight collected from diverse regions of Ethiopia.

During surveys representative common bean field were selected and investigated for CBB symptoms. Leaves showing typical symptoms of CBB with water-soaked spots and irregular necrotic lesions with yellow border were collected and dried with paper bags. From the collected leaf sample, tissues were removed from the lesion margin, placed in a drop of distilled water on a microscope slide, and macerated. Loopful of macerate were streaked onto nutrient agar (NA) and the plates were incubated at 28°C for 24 h. Yellow, mucoid, *xanthomonad*-like colonies were selected from each leaf sample and sub cultured on NA.

DNA extraction

Genomic DNA was extracted using a protocol described by [18] with minor modification. Bacterial cells were harvested after 24 h of incubation on Yeast extract-dextrose-$CaCO_3$ (YDC) medium, collected by centrifugation at 5000 × g for 5 min, and washed twice with 1 M NaCl and two times with sterile distilled water. The bacterial cell pellet was re-suspended in warm (55°C) extraction buffer (0.2 M Tris HCl pH 8.0; 10 mM EDTA, pH 8.0, 0.5 m NaCl, 1% SDS) containing Proteinase K (10 µg/ml). After 60 min at 55°C, 0.5 vol. of 7.5 M ammonium acetate were added, gently mixed and left to stand for 10 min at room temperature. Following centrifugation, the supernatant was transferred to a fresh tube and the DNA was precipitated by adding 1 vol. of ice-cold isopropanol. The pellet was washed with 70% ethanol, dried and re-suspended in 1X TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) containing 10 mg/ ml RNase A. Tubes were incubated at 37°C for an hour, and the DNA were precipitated with 1/10 vol. of 3 M NaAc, pH 5.2, and 2 vol. of 95% ethanol. The pellet was dried and finally suspended in 0.1X TE buffer. The quality of extracted DNA was determined by electrophoresis on 0.7% agarose gels.

Rep-PCR fingerprinting

Rep-PCR was done using the primer pairs REP1R-I and REP2-I and ERIC 1 and ERIC2 (Table 2). The reproducibility of rep-PCR was tested by amplifying DNA from three randomly chosen strains two times. Optimal PCR conditions for each of the primer sets were used as described by [15]. PCR amplifications were performed with applied bio system thermal cycler model ABI2720 using Taq DNA polymerase with 25 units/ml concentrations. The PCR products were electrophoresed in a 1.5% agarose gel for 1 h at a constant voltage of 55 V in 1×TAE buffer (40 mMTris–Acetate, 1 mM EDTA, pH 8.0). The rep-PCR profiles were visualized under UV light after staining of the gel with 0.5 µl/ml concentration of ethidium bromide, and digital image capturing was done using a Canon powers hot SX150 digital photo camera mounted on a hood.

Data Analysis and Interpretation

Analysis of molecular variance (AMOVA) was used to partition the genetic diversity among and within the bacterial strain populations and tested whether there is a hierarchy of rep-PCR sequence variation among individuals. The genetic structure of common bacterial blight isolates was obtained from the infected common bean leaves by the DNA finger printing using rep-PCR. Each unit of band pattern generated by rep-PCR (ERIC, BOX and REP PCR primers) and the fingerprints were decoded into a binary matrix (1,0) where 1 represents presence of band and 0 absence. The genetic relationships between strains were evaluated using a matrix of genetic distances constructed using the complement of the Jaccard similarity coefficient (CSJ), which does not consider negative similarities and the absence of the product. The binary matrix was used to drive a distance matrix using Jaccard's matrix from which an average linkage (UPGMA or unweighted pair group method with arithmetic averages) dendrogram was derived. Sources of genetic differentiation were analyzed using Analysis of Molecular Variance (AMOVA) which were performed using GenAlEx6.1to assess genotypic variations across all the populations studied (Tables 3 and 4) [19,20]. The analysis included partitioning of total genetic variation into within-groups and among-groups variance components; hence it provided a measure of intergroup genetic distance as proportion of the total variation residing between populations. The significance of analysis was tested using 999 random permutations. Principal coordinate analysis (PCA) was computed with individual isolates using GenAlEx.

Results

Results of molecular characterization with DNA fingerprinting techniques (Figures 1 and 2) indicated that genetic diversity exists among isolates of common bacterial blight collected from diverse bean growing areas (Wolaita, Gurage, Sidama, Gamogofa, Jimma, and Arisi) of Ethiopia (Table 1). AMOVA was used to partition the genetic diversity among the populations and tested whether there is

Genetic markers	SEQUENCES 5' to 3'	T_a °C	GC %	Number of nucleotide
REP 1	IIIICGICGICATCIGGC	49	52.9	18
REP 2	IIICGNCGNCATCNGGC	58	52.9	17
ERIC 1	ATGTAAGCTCCTGGGGATTCAC	58	50	22
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	42	54.5	22
BOX AIR	CTACGGCAAGGCGACGCTGACG	50	68.2	22

Table 2: Molecular markers used to amplify PCR product of strains of common bacterial blight.

Source	df	SS	MS	Est. Var.	%
Among Pops	5	33.782	6.756	0.338	7%
Within Pops	34	158.068	4.649	4.649	93%
Total	39	191.850		4.987	100%
Stat	**Value**	**P (rand > = data)**			
PhiPT	0.068	0.020			

Table 3: Analysis of molecular variance (AMOVA) within and among bacterial strain populations collected from Ethiopia.

Axis	1	2	3
%	24.44	13.89	10.71
Cumulative		38,33	49.04

Table 4: Percentage of variation explained by the first 3 axes.

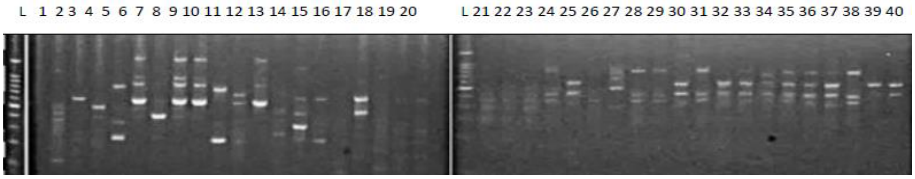


Figure 1: Agarose gel showing polymerase chain reaction fingerprinting pattern of 40 bacterial strains with ERIC molecular marker and repetitive palindromic regions genomic DNA extracted from diverse strains of Common Bacterial Blight (*Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*) Strains from Diverse Geographical Regions of Ethiopia.

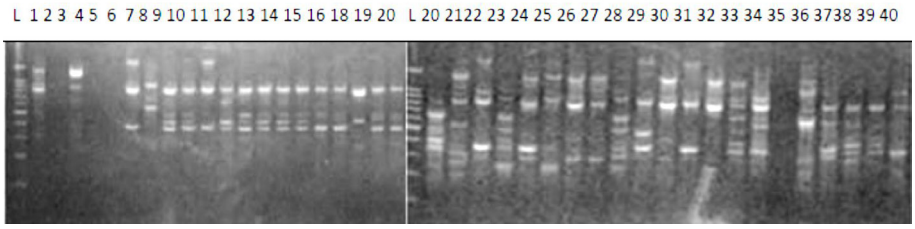


Figure 2: Agarose gel showing polymerase chain reaction fingerprinting pattern 40 with BOX A1R molecular marker and repetitive palindromic regions genomic DNA extracted from diverse strains of Common Bacterial Blight (*Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*) Strains from Diverse Geographical Regions of Ethiopia.

any hierarchy of rep-PCR fingerprinting variation among individuals. The analysis of 40 CBB isolates revealed that 7% of the genetic variation were distributed among populations while 93% of the genetic variations were within the groups (Table 3). The common bacterial blight strains showed no geographic differentiation. The first and the second principal coordinates account for 24.44% and 13.89% of the variations respectively. The result indicates that gene flow is common at the group level.

Discussion

The principal mechanism of gene flow was the informal bean seed system operating in the areas and the predominant seed born nature of the pathogen [21,22]. Gene flow or migration refers the exchange of genetic information among geographic population through the movement of gamete [23]. This is consistent with the fact that several genomic regions were shared by samples of CBB isolates from different populations (Figure 3) which was indicated by the heat map of the red color and the blue. This indicated that gene flow occurred between populations. Genetic differences among common bacterial blight strains within populations might be the result of gene flow between populations and this were indicated by the principal component analysis (Figure 4) where individuals within the same populations marked with the same symbols. The first and the second principal coordinates account for 24.44% and 13.89% of the variations respectively. Out of one hundred forty-three local collocations genetic variation of common bacterial blight strains were also reported by [24]. The same authors reported that both RAPD and AFLP analyses revealed high frequency of DNA polymorphism among isolates and could distinguish between *Xap, Xapf* and a non-pathogenic isolate. Differences between *Xap* and *Xapf* isolates demonstrated the existence of two distinct groups of bacteria [25]. Rep-PCR genomic fingerprinting has proven to have high discrimination power and reproducibility for bacterial diversity study (Figures 1 and 2). It generated estimation of genetic relatedness among the common bean bacterial blight strains. The rep-PCR genomic fingerprinting has been shown to be very valuable tool in molecular biology in studying the classifications and diversity of microbial isolates. The result from our genetic study

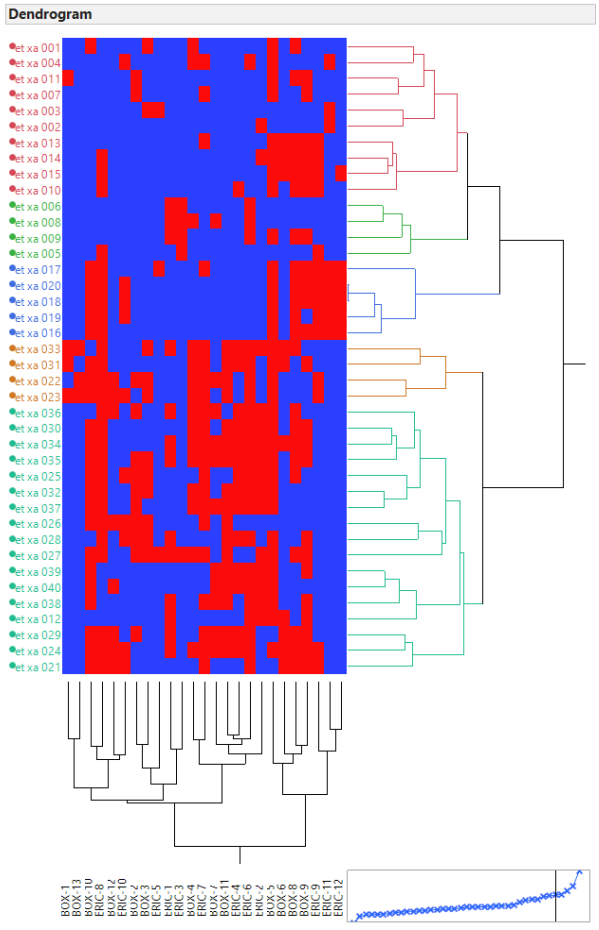
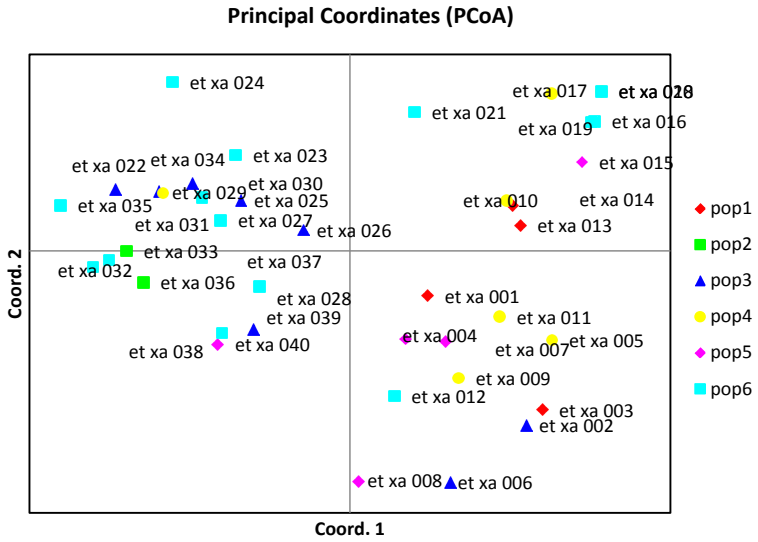


Figure 3: Dendrogram obtained from UPGMA showing the genetic relationship between 40 bacterial strains collected from diverse bean growing regions of Ethiopia using rep-PCR genomic the red and blue colours of the heat map show genomic region shared by the common bacterial isolates.



Pop1: strains of CBB populations obtained from Arsi, pop2=CBB strains from Gamogofa, pop3=CBB strains from Gurage, pop4=CBB strains from Jimma, pop5=CBB strains from Sidama, and pop6=CBB strains from Wolaita

Figure 4: Principal Coordinate Analysis.

based on the combined ERIC and BOX-PCR fingerprinting data with computer-based clustering analysis method, revealed the existence of genetic diversity between the bacterial strains. This confirmed the presence of genetically dissimilar strains of common bean bacterial blight in Ethiopia. The resulted differences might be between *Xap* and *Xapf* isolates representing the existence of two distinct groups of bacteria. Similar distinction between these two groups was also reported by [26], using RFLP's. Non-pathogenic *Xanthomonas* commonly associated with beans could be distinguished from *Xap* and *Xapf* using both RAPD and AFLP techniques. Knowledge on the existence of variability in CBB isolates populations is important for plant breeding and program. Hence the common bean improvement program that designs developing disease resistance common bean varieties for wider production should consider this information (presence of diverse strains) during evaluation process for better achievements. Rep-PCR to be very useful for studying plant pathogen population structure and a powerful tool for the molecular genetic analysis of bacteria. In other reports with rep-PCR techniques, strains of different *Xanthomonas* species were differentiated. The potential of rep-PCR in discrimination the strains and the potential of rep-PCR patterns obtained with ERIC, BOX and REP primers showed polymorphism among Brazilian *X. campestris* pv. strains [27]. The rep-PCR is an effective method in determining genetic diversity among populations of many bacterial pathogenic genera, including *Xanthomonas* and *Pseudomonas* [28,29]. In the present study, the genetic diversity of CBB strains collected from bean growing areas of Ethiopia was determined. With combined analysis of rep-PCR patterns obtained showed the existence of genetic diversity among the Ethiopian CBB strains. Although pathogenicity test was under investigation the molecular characterization revealed the existence of different genotypes of common bean strains in Ethiopia. *X. axonopodis* pv. phaseoli is genetically diverse. *X. axonopodis* pv. *phaseoli* strains were grouped in at least 4 genetic lineages spanning over two different homology groups defined by [7]. Genetic heterogeneity within non-fuscous strains of *X. axonopodis* pv. *phaseoli* was also revealed by [16] Moreover, our cluster analysis of the pairwise similarity values performed using UPGMA confirmed differences in fingerprint patterns. CBB pathogens are seed-borne so the lack of geographic differentiation among the six-different common bean growing localities could be the result of the distribution of one or some limited bacterial genotypes. In current study, CBB isolates obtained from the same common bean growing area present different rep-PCR pattern and clustered differently. The existence of two distinct clusters among CBB isolates in Ethiopia could suggest existence of both CBB strains.

Conclusion

These findings can help to provide better understanding of the CBB strains for disease resistance in the common bean breeding program. The authors also suggest the information obtained could be complimented with pathotype characterization so that the bean breeding program should use the most virulence pathotypes during developing and screening of resistance bean lines.

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