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ORIGINAL ARTICLE

Molecular characterization of *Cucumber mosaic virus* subgroup IB infecting Cavendish banana plants in Ethiopia

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Abstract

Banana is the major fruit crop produced in Ethiopia. Since *Cucumber mosaic virus* (CMV) is one of the most devastating plant viruses infecting banana, the present study was undertaken to survey and identify CMV strains infecting banana plants in Ethiopia. Dot immune-binding assay (DIBA) and reverse transcription-polymerase chain reaction (RT-PCR) revealed the presence of CMV in all of the symptomatic samples tested. The results of sequence and phylogenetic analysis revealed that the isolate under study was a CMV isolate from the IB subgroup. Multiple sequence alignment revealed a three nucleotide sequence variation that could be used to distinguish CMV subgroups. Selection pressure analysis showed the CMV-RNA1 region undergoing positive selection pressure. Tajima`s test of neutrality revealed a positive value of 0.86468 indicating CMV population contraction. To the best of our knowledge, this is the first report and molecular characterization of CMV IB subgroup isolate infecting banana plants in Ethiopia.

Keywords: *Cucumber mosaic virus*, CMV detection, in silco analysis, 1a protein

Introduction

Cucumber mosaic virus (CMV), an aphid transmitted *Cucumovirus* in the family of Bromoviridea, is one of the most economically important plant viruses worldwide (Roossinck *et al.* 1999). Since its first report on cucumber and melon from the USA in 1916, and later from Europe and Africa (Prince 1934) and other parts of the world, CMV has been spreading globally and at present, it is the only virus known to infect more than 1300 plant species (Palukaitis *et al.* 1992; Garcia--Arenal and Palukaitis 2008; Zitter and Murphy 2009). In terms of significance, CMV ranked fourth among the top 10 economically important plant viruses (Rybicki 2015) and was next to *Banana bunchy top virus* (BBTV) among viruses infecting banana (Basavaraj *et al.* 2017). CMV is characterized by yellow mosaic and stripes on the leaf, leaf distortion, and stunting symptoms on the plant (Basavaraj 2017; Kebede and Majumder 2020).

CMV consists of tri-segmented, single stranded positive sense RNAs (RNA1, RNA2 and RNA3) and two sub-genomic RNAs (RNA4A and RNA4) derived from RNA2 and RNA3, respectively (Kumari *et al.* 2013; Bujarski 2021). CMV encodes for five proteins. RNA1 is the only mono-cistroinic RNA segment encoding for 1a protein. The 1a protein is primarily involved in the replication of the viral genome along with the 2a protein and a number of host factors (Palukaitis and Garcia-Arenal 2003; Seo *et al.* 2009). The 1a protein contains two functional domains: the N-terminal methyltransferase and the C-terminal helicase domain (Rozanov *et al.* 1992). CMV 1a protein is not only involved in replication, but it also determines systemic infection (Kang *et al.* 2012) and hence plays an important role in CMV's life cycle. The 1a protein is the largest protein of CMV with almost 1,000 amino acid residues (Seo *et al.* 2009).

In Ethiopia, studies related to CMV are very few. Very recently, CMV IA subgroup infecting banana and tephrosia plants has been reported (Kebede and Majumder 2020; Kumar *et al.* 2020). CMV species are categorized into three subgroups: subgroup IA, IB and II with about 25% nucleotide sequence divergence between them (Roossinck *et al.* 1999). The present investigation aimed at molecular identification and in silico analysis of the CMV IB subgroup infecting Cavendish banana plants in Ethiopia.

Materials and Methods

Sample collection

Severe, CMV-like mosaic symptoms on several banana plant leaves were observed in a field survey conducted in Shara kebele, Arba minch woreda, Southern Nations, Nationalities, and People`s Region, the main banana growing areas in Ethiopia during 2019/20. Fifteen infected banana plants showing mild to severe leaf mosaic accompanied with leaf curling, leaf deformation, and irregular shapes of the leaves were collected (Fig. 1).

Dot immuno-binding assay (DIBA)

Initially, the collected banana leaf samples were screened for the presence of CMV using Dot immuno-binding assay (DIBA) as described by Kebede and Majumder (2020). Briefly, 100 mg of banana leaf samples were ground in 1 ml carbonated buffer containing 2% polyvinylpyrrolidone (PVP) and centrifuged at 8,000 rpm. Ten µl of the supernatant was spotted on strips of nitrocellulose membrane (NCM) (MDI, Gurgaon) in triplicate for each sample. The nitrocellulose membrane was incubated in blocking solution PBS-T (8.0 g of NaCl, 1.5 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 0.2 g of KCl dissolved in 1 l of distilled water; pH 7.4) containing 3% bovine serum albumin (BSA) for 1 h at room temperature. After washing with PBS-T, 10 µl of anti CMV antibody (Agdia Inc., USA) dissolved in 1 l PBS-T at pH 7.4 was spotted on the nitrocellulose sample spots and incubated at 37°C for 1 h. After another washing, 10 µl alkaline phosphatase (AP)-conjugated secondary antibody (Sheep anti-rabbit IgG) (Sigma-Aldrich, USA) diluted (1 : 2,000) in PBS-TPO was dotted on the nitrocellulose sample spot and incubated at 37°C for 1 h. After the final washing, the nitrocellulose membrane was incubated in NBT/BCLP (Sigma-Aldrich, USA) solution prepared as per the manufacturer's instructions for 15 min so that positive samples developed visible purple spots indicating the presence of CMV infection while negative samples were colorless or green.

RNA isolation and RT-PCR

The total RNA was isolated from 100 mg naturally infected banana plant leaf samples using RNeasy plant Mini kit (Qiangen, Germany) according to the

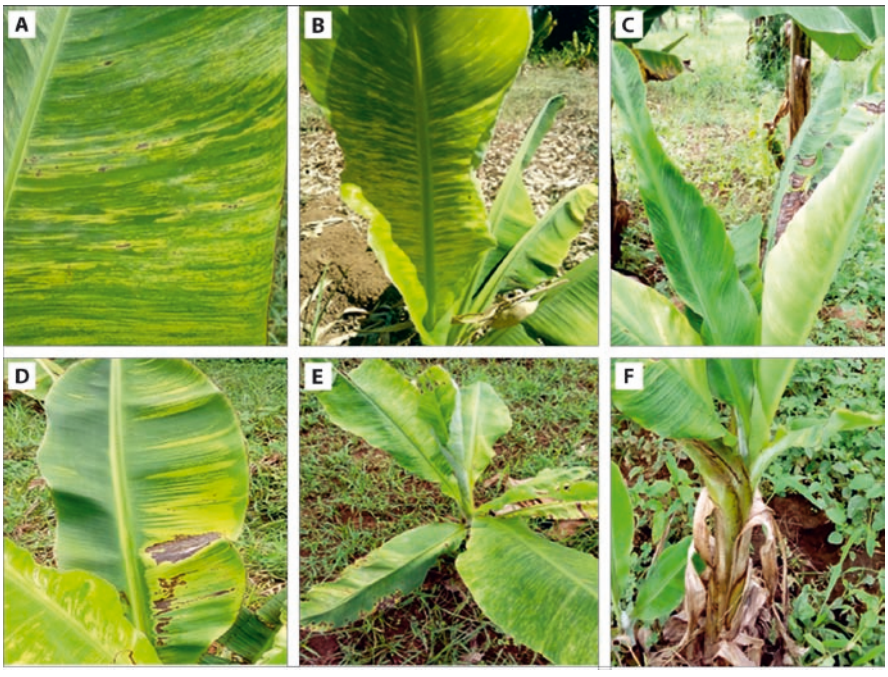

Fig. 1. Banana plant showing CMV symptoms: A – banana leaf showing mosaic; B and C – banana leaf showing yellow stripes, curling of the leaf and leaf margin; D and E – banana plant showing stunting appearance as well as irregular deformation of the leaf

manufacturer's instructions. The quality and quantity of the isolated total RNA were checked using a UV visible double beam Spectro-photometer (Shimadzu, Japan). Forward (CMV RNA IF CAAGAGCGTACGGTTCA ACCCCTGCCT) and reverse (CMV I 947R TCCCA ATCATGAACATAAGAC) primers to amplify 960 bp fragment from methyltransferase domain of RNA1 of CMV were designed in the present study and synthesized at Sigma, Bangalore, India. The first-strand cDNA synthesis was performed in a total volume of 20 µl reaction mixtures containing 10 µl of total RNA, 0.2 µM reverse primer, 4 µl of 5XRT buffer, 0.3 mM dNTPs, 20U M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, USA), and the final volume was made with nuclease free water. The reaction mixture was incubated at 42°C for 45 min whereas the enzyme was inactivated by heating at 70°C for 10 min to stop the reaction. The obtained cDNA was subjected to PCR amplification. The PCR was performed in Bio-Rad T100 Thermocycler with a reaction volume of 50 µl containing 10 µl of the first-strand cDNA, 2 µM of each forward and reverse primer, 1.5 mM of $MgCl_2$, 5 µl of 10X reaction buffer, 0.2 mM dNTPs and 5U of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, USA), and 26 µl of nuclease-free water. The temperature profile of the reaction was: denaturation step at 94°C for 5 min, followed by 30 cycles of 45 s at 94°C, 30 s at an annealing temperature of 50°C, 1 min at 72°C, and one final extension step at 72°C for 10 min. Tissue culture-raised, RT-PCR-tested healthy banana leaves were used as a negative control.

Gel electrophoresis and sequencing

Ten microliters of the PCR products were separated on 1% agarose gel stained with 2 µl ethidium bromide and photographed under UV illumination with an imaging system (Bio-Rad XR documentation system). Three amplicons from the same plant were sent for sequencing with both forward and reverse primers to Barcode Bioscience, Bangalore, India.

Sequence analysis: sequence identity and phylogenetic study

The obtained sequences from both forward and reverse reactions were aligned and joined together to form a full-length sequence using Bio Edit program. The aligned sequence was subjected to BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with the exiting sequences of CMV isolates available in the GenBank data base to confirm its identity. Thirty CMV RNA 1 sequences from subgroups IA, IB, and II including one from this study (Table 1) were used to study sequence identity and its phylogeny using Bio Edit program version 7.0 and MEGA version 7.0, respectively. Multiple sequence alignment (MSA) of the nucleotides and deduced amino acids using Expasy translation tools (https://web.expasy.org/translate/) were performed using CLUSTAL W implemented in Bio Edit program. The phylogenetic trees were constructed using MEGA version 7.0 program through the Neighbor-Joining (NJ) method with 1,000 replicates bootstrapping for both nucleotides and deduced amino acid sequences.

Recombination and selection pressure analysis

The recombination of CMV isolates considered in this study was analyzed using Recombination Detection Program version 4 (RDP4) software (Martin *et al.* 2015). The analysis of recombination events, major and minor parents as well as recombination breakpoints was performed using different algorithms vis., RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3Seq, LARD, and PhylPro implemented in RDP4 software package. The program was run using the default parameters setting. The recombination events were considered significant if three or more methods had $p < 0.01$. On the other hand, to assess the selection pressure imposed upon the CMV-RNA1 region, synonymous (*d*S, silent) and non-synonymous (*d*N, amino acid altering) substitution rate was estimated using Codon-based Z-test of selection method implemented in MEGA7. The selection pressure was considered neutral evolution (*d*N = *d*S), negative or purifying (*d*N < *d*S), and positive or adaptive selection (*d*N > *d*S) for the data set. The analysis was performed using the Nei-Gojobori method where in the analysis, values of $p < 0.05$ were considered significant at 5% level. The variance of the difference was computed using the bootstrap method with 1,000 replicates.

Tajima's test of neutrality

The population genetic analysis of CMV-RNA1 sequences was performed using a total of 30 nucleotide sequences retrieved from NCBI including one sequence from this study. To evaluate if CMV-RNA1 nucleotide sequences showed evidence of deviation from neutrality, the population genetic analysis, including nucleotide diversity, haplotype diversity, and Tajima's D test of neutrality tests, was performed with default setting of DnaSPv6 (Rozas *et al.* 2017).

Results

Field survey

A survey was conducted for CMV-like symptoms on Cavendish banana plants in the study area. The diseased plants exhibited severe yellowing mosaic, yellow

Table 1. CMV isolates used for phylogenetic analysis (Ns – not assigned in GenBank)

Origin of CMV	Accession number	Protein Id	Host	Subgroup
Hungary	AJ580953	CAE45702.1	*Nicotiana glutinosa*	Ns
Italy	MH748551	QCC72885.1	*Phaseolus vulgaris*	Ns
Poland	MG882753	AYP19435.1	*Cucurbita pepo*	IA
South Korea	AJ276479	CAB77386.1	–	IA
Turkey	LC066516	BAW81929.1	*Rapistrum rugosum*	Ns
Brazil	MK387172	QFO48943.1	*Arachis pintoi*	Ns
China	KX660756	ASV48209.1	maize	Ns
China	EF213023	ABN05375.1	*Brassica chinensis*	IB
China	KT350980	AMR72000.1	maize	IB
Egypt	KT921314	ALP32184.1	tomato	Ns
Ethiopia	**MW021453**	**–**	**banana**	**IB**
India	MK482376	QBM19860.1	*Plectranthus amboinicus*	IB
India	KM272277	AJK26759.1	*Capsicum annuum*	Ns
Malaysia	JN054636	AER35120.1	cucumber	Ns
Rwanda	MG470798	AUS29443.1	*Capsicum* sp.	Ns
South Korea	KC527795	AGN56133.1	*Capsicum annuum*	Ns
South Korea	KC527783	AGN56121.1	*Capsicum annuum*	Ns
South Korea	MN422333	QJR84058.1	*Capsicum annuum*	Ns
South Korea	LC390004	BBE52279.1	*Zinnia elegans*	Ns
South Korea	KC527819	AGN56157.1	*Capsicum annuum*	Ns
South Korea	MN422336	QJR84063.1	*Capsicum annuum*	Ns
South Korea	LC510819	BBP10675.1	*Capsicum annuum*	Ns
Tanzania	MK330845	QDQ03559.1	*Ociumum basilicum*	Ns
Uganda	MG021460	AXL94184.1	*Xanthosoma* sp.	IB
Uganda	MG021457	AXL94178.1	*Xanthosoma* sp.	IB
Australia	AF198101	AAM10536.1	*Lupinus angustifolius*	II
Australia	KX525730	AGS99521.1	*Capsicum* sp.	II
China	EF202595	ABN03945.1	*Lycopersicon esculentum*	II
Germany	MN399744	QJX15759.1	legume	Ns
India	HE650150	CCF55378.1	*Cucumis sativus*	Ns

Isolate from the present study was marked in bold

stripes, curling of the leaf margin, leaf deformation, irregular shapes of the leaf, and a stunted appearance compared to healthy plants (Fig. 1). The disease incidence was about 10–30% in the three fields surveyed.

Severe mosaic symptoms were observed in 20% of the banana (*Musa acuminate*) plants growing in the study area.

Dot immuno-binding assay (DIBA)

The dot immuno-binding assay was performed in this study using CMV-specific antibodies. The study results revealed that all the symptomatic samples collected in this study were CMV positive (Fig. 2). The association of CMV with banana leaf samples was further confirmed with the RT-PCR method.

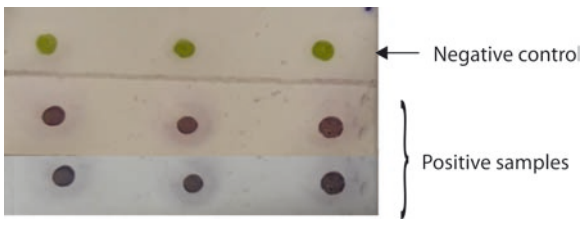

Fig. 2. Dot immuno-binding assay of banana leaf samples for CMV infection

Amplification of CMV-RNA1 fragment

RT-PCR based amplification of the RNA1 using CMV--RNA1 gene specific primers revealed an amplicon of the expected size (~960 bp) in positive samples while no amplification was obtained from the negative controls (Fig. 3). The presence of CMV was detected in all 15 (100%) symptomatic samples examined for CMV. The obtained PCR product from one sample was sequenced three times using F and R primers and the obtained sequences were aligned and joined together. The direct sequencing of three PCR products produced ~940 bp long nucleotide sequences corresponding to the partial RNA1 segment of CMV. The consensus sequence was submitted to GenBank (accession number MW021453).

Sequence identity and phylogenetic study

BLASTn analysis of the sequenced data revealed the identity of our isolate. The BLASTn result revealed the highest sequence identity of 98.25% with RNA1 of CMV MG021454, MG021457 and MG021460 isolates of *Xanthosoma* sp. from Uganda, with 94.86–98.00% sequence identity with other CMV isolates available in the GenBank. Similarly, BLASTp of the deduced amino acid sequence of the isolate under study shared maximum identity of 100% with CMV isolates from Uganda with protein id AXL94178.1 and AXL94184.1 and 97.21–99.6% with other isolates available in the GenBank. Furthermore, multiple sequence alignment (MSA) revealed the isolate under study shared the highest sequence identity of 95.0–98.2% and 96.0–100% with isolates in subgroup IB, with 93.1–94.2% and 95.6–97.6 sequence identity with CMV isolates in subgroup IA, and the lowest sequence identity of 83.9–84.3% and 92.4–92.8% with isolates in subgroup II at both nucleotide and amino acid levels, respectively (supplementary Table 1). In addition, multiple sequence alignment (MSA) further revealed that CMV isolates of subgroup I (IA and IB) including our isolate had three nucleotide deletions at nucleotide positions 39, 40 and 41 that corresponds to nucleotide position 90, 91 and 92 in the complete RNA1 sequence.

The phylogenetic tree constructed using Mega7, using nucleotide along with amino acid sequence of our isolate as well as other isolates retrieved from the National Center for Biotechnology Institute (NCBI) database revealed the three distinct groups of CMV isolates, showing consistent results with previous studies (Kim *et al.* 2014). The isolate under study clustered with CMV isolates in subgroup IB (Fig. 5) showed a close relation with CMV (MG021457) and CMV (MG021460) isolates of *Xanthosoma* sp. from Uganda at both nucleotide and deduced amino acid levels which was consistent with the results of percent identity analysis. Both trees generated using nucleotide and deduced amino acid were topologically identical.

Recombination and selection pressure analysis

Our analysis of recombination using RDP4 indicated no apparent recombination event between CMV isolates considered in the present study. Other than recombination, selection pressure is also an important evolutionary force, which increases the variation between homologous proteins. Selection pressure analysis revealed CMV-RNA1 region undergoing positive selection pressure with a significant probability (p) value of 0.0000, indicating that the number of nonsynonymous substitution is greater than that of synonymous substitution, indicating amino acid replacement.

Tajima's test of neutrality

In the present study, the overall nucleotide diversity (π) value for CMV-RNA1 was found to be less than one and haplotype diversity (*Hd*) value was found to be 0.996. The Tajima's D test revealed a positive value of 0.86468 (Table 2) suggesting a greater pairwise difference between the sequences, indicating CMV population contraction or a decrease in population size.

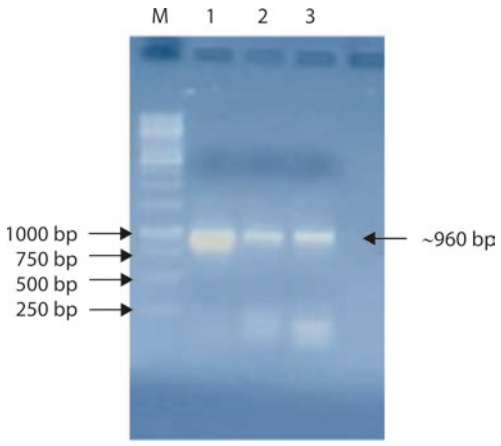

Fig. 3. Electrophoretic separation of PCR reaction products. Lane M – 1 kb DNA marker; Lanes 1, 2 and 3 – CMV

Table 2. Results from Tajima`s neutrality test

m	*S*	*Hd*	π	*D*
30	184	0.984	0.07158	0.86468

m – number of sequences; *S* – number of segregating sites; *Hd* – haplotype diversity; π – nucleotide diversity; D – Tajima test statistic

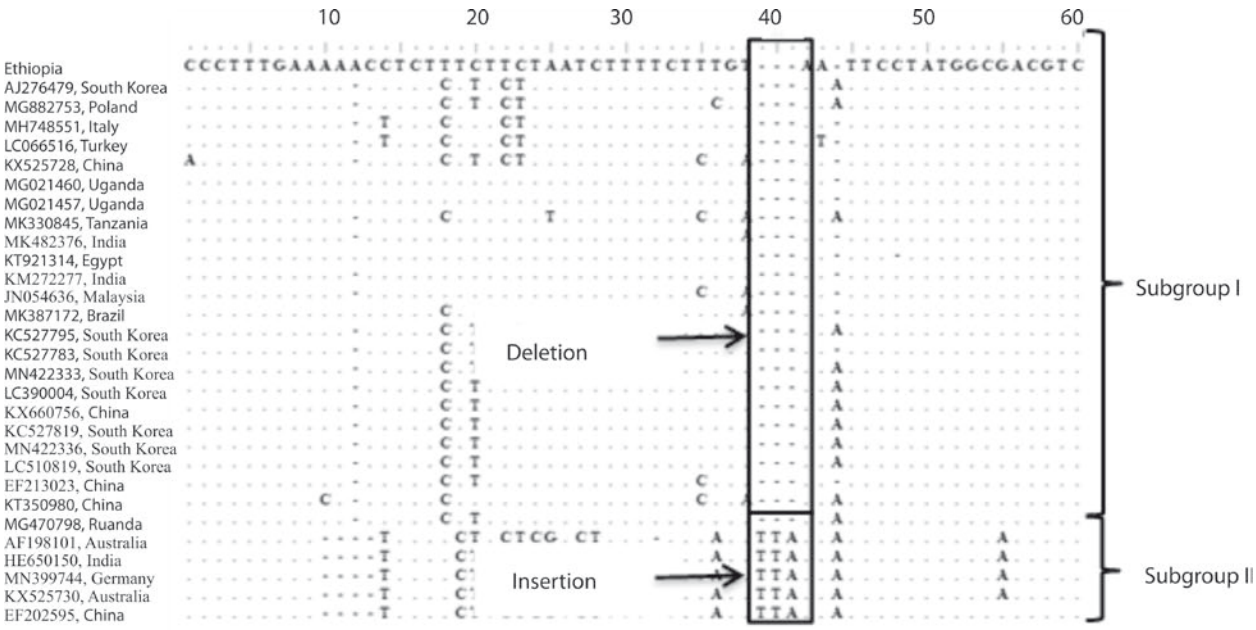

Fig. 4. Portion of multiple sequence alignment of 1a protein sequence of CMV isolates at nucleotide level. The rectangle on the alignment shows deletion and insertion of three nucleotides in CMV subgroup I and II at nucleotide position 39, 40 and 41 in the alignment respectively

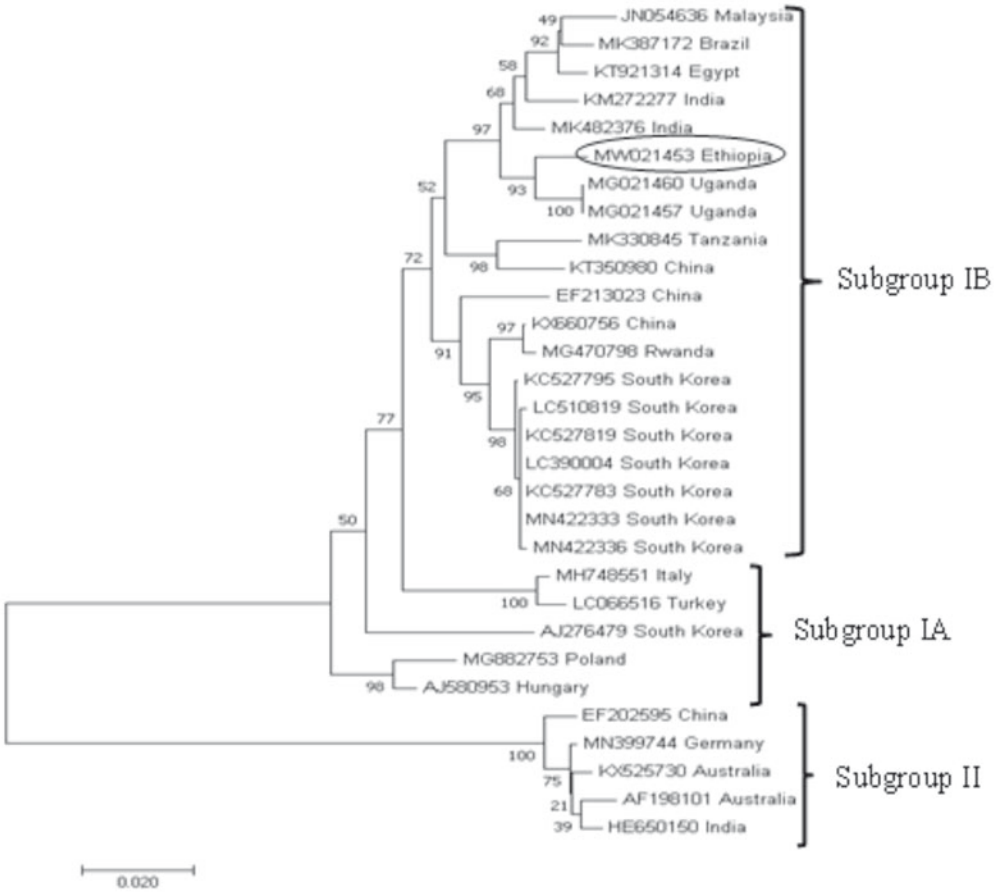

Fig. 5. Neighbor Joining tree based on the nucleotide sequences of partial 1a protein of CMV showing phylogenetic relationships of Ethiopian isolate with other isolates from different part of the world (the ellipse shows the isolate from the present study)

Discussion

Identification of causal agents is one of the most important steps of disease management. In the field, symptoms give the first indication of a disease. The host plant's response to virus infection is induced by interaction between the host and viral factors which result in visible symptoms. In compatible plant-virus interactions the virus invades the whole plant, resulting in systemic infection and provokes the development of systemic symptoms. CMV induces systemic infection and causes mosaic symptoms (Doolittle 1916). Field surveys conducted on three banana farms in the study areas showed, severe mosaic symptoms accompanied with yellow stripes, curling of the leaf margin, leaf deformation, irregular shapes of the leaf and a stunted appearance of the full plants compared to healthy ones (Kebede and Majumder 2020). Severe mosaic symptoms were observed in 20% of Cavendish banana (*Musa acuminate*) plants in the study area indicating how seriously banana production is affected by CMV in the study areas. Khan *et al.* (2011) pointed out that CMV is responsible for 40% yield loss of banana production. In addition to symptoms, CMV infection was further identified by DIBA using CMV-specific antibody and RT-PCR.

After preliminary identification, molecular identity and characterization help pathologists to plan a management module. This study confirmed the molecular identity of CMV infecting Cavendish banana plants based on the CMV-RNA1 segment as well as characterization of the molecule. Molecular characterization contributes to a better understanding of the genetic composition, genetic variation, and the taxonomic position of the virus under study. It aids in understanding the relation of the isolate under study to other isolates reported from elsewhere and their evolution. BLASTn and BLASTp based sequence comparison revealed the identity of the isolate under study. The sequence comparison study at both nucleotide and deduced amino acid levels showed maximum identity with the CMV--RNA1 isolate from Uganda infecting *Xanthosoma* sp., another monocot that is cultivated for its corms, suggesting that the isolate could have been introduced from Uganda due to cross border germplasm movement. Phylogenetic analysis placed the isolate under study in subgroup IB at both nucleotide and deduced amino acid levels, which makes it a new report of CMV IB isolates infecting Cavendish banana plants from Ethiopia. Previous studies indicated the presence of CMV subgroup IA in Ethiopia on Cavendish banana plants and tephrosia, an important medicinal plant and potential feed for livestock (Kebede and Mujumdar 2020; Kumar *et al.* 2020). The prevalence of two subgroups of CMV (IA & IB) on the same host in Ethiopia points towards the requirement that the diversity of the viral strains, their dynamics of interaction and further evolutionary development should be recorded and analyzed cautiously to develop a management strategy in the future. Furthermore, the isolate under study clustered and showed genetic relatedness with CMV isolates from Uganda at both nucleotide and amino acid levels which is consistent with sequence comparison studies. The presence of two distinct isolates of CMV on banana clearly indicates the ability of different groups of CMV to infect banana plants which is in line with an earlier report by Chou *et al.* (2009).

Multiple sequence alignment (MSA) has revealed that CMV isolates of subgroup I (IA and IB) including our isolate consistently had three nucleotide deletions as compared to subgroup II isolates. We suggest that this position of RNA 1 can be used to distinguish between CMV isolates (Fig. 4). However, the functional importance of this variation and its significance on the pathogenicity of CMV strains are yet to be studied.

CMV-RNA1 encodes 1a protein containing two different functional domains, the N-terminal methyltransferase domain and the C-terminal helicase domain that are involved in the capping of the genomic and sub-genomic RNAs, and replication of the virus, respectively (Gorbalenya *et al.* 1989; Rozanov *et al.* 1992; Palukaitis and Garcia-Arenal 2003). The functionality of CMV 1a protein depends on the intra and intermolecular interactions of these domains. Other than replication, it appears that 1a protein is also responsible for systemic movement of the virions in the plant (Canto and Palukaitis 2001). Besides this, RNA 1 is also involved in seed transmission and a hypersensitive response of CMV in tobacco has been mapped to RNA1 (Lakshman and Gonsalves 1985; Hampton and Francki 1992). In addition, studies indicate that CMV RNA1 is involved in severity of symptoms and susceptibility (Roossinck and Palukaitis 1990; Kang *et al.* 2012). Therefore, molecular characterization of CMV--RNA1 is important for better understanding and planning management strategy.

In the present study the genetic structure and diversity of CMV-RNA1 isolates were also studied. The study of genetic and molecular diversity of the viral pathogens provides a better understanding of the biology, ecology and evolution of the viruses. Genetic exchange either by recombination or re-assortment as well as mutation are the major evolutionary forces contributing to divergence and speciation of RNA viruses (Domingo and Holland 1994; Simon and Bujarski 1994). Recombination analysis in the present study revealed no apparent molecular evidence of recombination events in the CMV-RNA1 region, suggesting that it is stable and conserved for this isolate. The lack

of proof-reading ability of RNA polymerases in RNA viruses provides great potential for evolution, genetic variability, and adaption to new environmental conditions due to high mutation rates and the generation of variable populations (Garcia-Arenal and Fraile 2011). In addition to recombination, mutation also plays an important role in the evolution of RNA viruses. Rapid mutation rates, reassortment and RNA-RNA recombination are important driving forces in RNA virus evolution (Domingo and Holland 1994; Simon and Bujarski 1994). The high mutation rates observed in plant viruses result in resistance breaking viral strains (Leach *et al*. 2001). The sequence analysis between the relative abundance of the rate of synonymous substitutions per synonymous site (*d*S) and that of nonsynonymous substitutions per nonsynonymous site (*d*N) revealed that the sequence under study is under positive selection pressure. Tajima's D test also revealed a positive value, indicating population contraction. Population contraction may be due to a 'founder event' since the isolate does not show any similarity to the existing CMV isolate from Ethiopia. Divergence and speciation of RNA viruses occur rapidly and frequently (Roossinck 1997). Currently, no effective management methods of CMV infecting banana are available. CMV evolves rapidly and this ability allows the virus to infect a wide range of hosts (Roossinck 2001). Thus, analyzing the genetic structure of CMV populations of Ethiopia and adjoining countries may facilitate the development of strategies for the management of the virus. Several studies attempting to examine CMV population genetics have been reported so far (Liu *et al*. 2009; Nouri *et al*. 2014). To date, no studies have been conducted on RNA1 of CMV infecting banana from Ethiopia. In the present study, we examined the molecular characteristics of the CMV isolate infecting banana based on analysis of CMV-RNA1 sequence. The phylogenetic relationships and comparisons between each virus group provide an understanding of evolutionary mechanisms. To the best of our knowledge, our study for the first time described the detection and molecular characterization of CMV-RNA1, from the CMV IB subgroup infecting Cavendish banana plants from Ethiopia. This knowledge will help in making informed decisions ensuring CMV free planting material in the country.

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Supplementary Table 1. Amino acid (above the diagonal line) and nucleotide (below the diagonal line) sequence identify matrix of partial RNA 1 sequences of *Cucumber mosaic virus* (CMV)

Seq.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	ID	**95.6**	**96.4**	**96.8**	**97.2**	**96.4**	**100**	**100**	**98.0**	**98.8**	**98.4**	**98.0**	**98.4**	**98.4**	**96.4**	**96.4**	**96.4**	**96.4**	**97.2**	**96.4**	**96.4**	**96.4**	**96.0**	**97.6**	**97.6**	**92.4**	**92.4**	**92.8**	**92.4**	**92.4**
2	93.1	ID	**99.2**	**96.8**	**97.2**	**99.2**	**95.6**	**95.6**	**96.0**	**96.0**	**95.6**	**96.0**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**96.4**	**96.4**	**96.0**	**91.2**	**91.2**	**91.6**	**91.2**	**91.2**
3	94.0	95.3	ID	**97.6**	**98.0**	**99.2**	**96.4**	**96.4**	**96.8**	**96.8**	**96.4**	**96.0**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**97.2**	**97.2**	**96.8**	**90.8**	**90.8**	**91.2**	**90.8**	**90.8**
4	94.2	94.0	94.5	ID	**99.6**	**97.6**	**96.8**	**96.8**	**98.0**	**97.6**	**97.6**	**96.8**	**97.6**	**97.6**	**97.2**	**97.2**	**97.2**	**97.2**	**97.2**	**97.2**	**97.2**	**97.2**	**97.2**	**98.0**	**97.6**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
5	93.7	93.8	94.1	99.2	ID	**98.0**	**97.2**	**97.2**	**98.4**	**98.0**	**98.0**	**97.2**	**98.0**	**98.0**	**97.6**	**97.6**	**97.6**	**97.6**	**97.6**	**97.6**	**97.6**	**97.6**	**97.6**	**98.4**	**98.0**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
6	94.3	95.7	98.5	95.1	94.7	ID	**96.4**	**96.4**	**96.8**	**96.8**	**96.4**	**96.8**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**96.4**	**97.2**	**97.2**	**96.8**	**91.6**	**91.6**	**92.0**	**91.6**	**91.6**
7	98.2	93.2	93.8	94.2	93.8	94.2	ID	100	**98.0**	**98.8**	**98.4**	**98.0**	**98.4**	**98.4**	**96.4**	**96.4**	**96.4**	**96.4**	**97.2**	**96.4**	**96.4**	**96.4**	**96.0**	**97.6**	**97.6**	**92.4**	**92.4**	**92.8**	**92.4**	**92.4**
8	98.2	93.2	93.8	94.2	93.8	94.2	100	ID	**98.0**	**98.8**	**98.4**	**98.0**	**98.4**	**98.4**	**96.4**	**96.4**	**96.4**	**96.4**	**97.2**	**96.4**	**96.4**	**96.4**	**96.0**	**97.6**	**97.6**	**92.4**	**92.4**	**92.8**	**92.4**	**92.4**
9	95.1	93.3	94.0	94.3	94.2	94.3	95.1	95.1	ID	**98.4**	**98.8**	**97.6**	**98.8**	**98.8**	**98.4**	**98.4**	**98.4**	**98.4**	**98.4**	**98.4**	**98.4**	**98.4**	**98.0**	**99.6**	**98.8**	**91.2**	**91.2**	**91.6**	**91.2**	**92.0**
10	97.8	93.6	94.3	95.2	94.8	94.5	97.5	97.5	96.1	ID	**98.8**	**99.2**	**98.8**	**98.8**	**97.6**	**97.6**	**97.6**	**97.6**	**98.4**	**97.6**	**97.6**	**97.6**	**97.2**	**98.8**	**98.8**	**92.0**	**92.0**	**92.4**	**92.0**	**92.8**
11	96.8	93.2	93.8	94.7	94.3	94.2	97.2	97.2	95.1	97.7	ID	**98.0**	**100**	**100**	**97.2**	**97.2**	**97.2**	**97.2**	**98.0**	**97.2**	**97.2**	**97.2**	**96.8**	**98.4**	**98.4**	**91.2**	**91.2**	**91.6**	**91.2**	**92.0**
12	96.8	93.6	95.0	94.9	94.6	95.3	97.0	97.0	95.1	98.4	98.0	ID	**98.0**	**98.0**	**96.8**	**96.8**	**96.8**	**96.8**	**97.6**	**96.8**	**96.8**	**96.8**	**96.4**	**98.0**	**98.0**	**92.0**	**92.0**	**92.4**	**92.4**	**92.8**
13	96.3	92.8	93.3	94.3	94.1	93.7	96.3	96.3	95.5	97.4	98.2	97.4	ID	**100**	**97.2**	**97.2**	**97.2**	**97.2**	**98.0**	**97.2**	**97.2**	**97.2**	**96.8**	**98.4**	**98.4**	**91.2**	**91.2**	**91.6**	**91.2**	**92.0**
14	96.3	93.3	94.0	95.0	94.6	94.1	96.7	96.7	95.5	97.7	98.7	97.5	98.3	ID	**97.2**	**97.2**	**97.2**	**97.2**	**98.0**	**97.2**	**97.2**	**97.2**	**96.8**	**98.4**	**98.4**	**91.2**	**91.2**	**91.6**	**91.2**	**92.0**
15	95.7	94.6	94.5	95.2	95.1	95.3	95.8	95.8	96.0	96.5	95.3	95.7	95.2	95.3	ID	**100**	**100**	**100**	**98.8**	**100**	**100**	**100**	**97.6**	**98.8**	**98.4**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
16	95.7	94.6	94.2	95.4	95.3	95.1	95.8	95.8	95.7	96.4	95.3	95.7	95.2	95.6	99.7	ID	**100**	**100**	**98.8**	**100**	**100**	**100**	**97.6**	**98.8**	**98.4**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
17	95.6	94.7	94.3	95.3	95.2	95.2	95.7	95.7	95.8	96.3	95.2	95.6	95.1	95.5	99.8	99.8	ID	**100**	**98.8**	**100**	**100**	**100**	**97.6**	**98.8**	**98.4**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
18	95.6	94.7	94.3	95.3	95.2	95.2	95.7	95.7	95.8	96.3	95.2	95.6	95.1	95.5	99.8	99.8	100	ID	**98.8**	**100**	**100**	**100**	**97.6**	**98.8**	**98.4**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
19	95.6	94.6	94.5	95.2	95.1	95.3	95.7	95.7	96.1	96.3	95.7	95.8	95.3	95.5	99.0	98.7	98.8	98.8	ID	**98.8**	**98.8**	**98.8**	**97.6**	**98.8**	**99.6**	**91.2**	**91.2**	**91.6**	**91.2**	**92.0**
20	95.6	94.7	94.3	95.3	95.2	95.2	95.7	95.7	95.8	96.3	95.=2	95.6	95.1	95.5	99.8	99.8	100	100	98.8	ID	**100**	**100**	**97.6**	**98.8**	**98.4**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
21	95.5	94.6	94.2	95.2	95.1	95.1	95.6	95.6	95.7	96.2	95.1	95.5	95.0	95.3	99.7	99.7	99.8	99.8	98.7	99.8	ID	**100**	**97.6**	**98.8**	**98.4**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
22	95.5	94.6	94.2	95.2	95.3	95.1	95.6	95.6	95.7	96.2	95.1	95.5	95.0	95.3	99.7	99.7	99.8	99.8	98.7	99.8	99.7	ID	**97.6**	**98.8**	**98.4**	**91.6**	**91.6**	**92.0**	**91.6**	**92.4**
23	95.0	94.5	94.2	94.8	94.4	94.8	95.3	95.3	95.5	96.1	95.1	95.6	95.1	95.5	97.2	97.4	97.3	97.3	97.0	97.3	97.2	97.2	ID	**98.4**	**98.0**	**90.0**	**90.0**	**90.4**	**90.0**	**90.8**
24	95.2	93.6	94.5	94.8	94.7	94.8	95.1	95.1	97.3	96.3	94.8	95.6	95.1	95.1	96.3	96.1	96.2	96.2	96.0	96.2	96.1	96.1	95.8	ID	**99.2**	**91.2**	**91.2**	**91.6**	**91.2**	**92.0**
25	95.6	94.6	94.7	95.5	95.3	95.6	95.7	95.7	96.1	96.3	96.0	96.1	95.6	95.7	98.7	98.5	98.6	98.6	99.7	98.6	98.5	98.5	97.0	96.0	ID	**91.2**	**91.2**	**91.6**	**91.2**	**92.0**
26	84.2	83.1	84.8	83.5	83.0	85.3	83.9	83.9	83.5	83.9	83.2	84.3	82.8	82.9	84.0	83.8	83.9	83.9	83.4	83.9	83.8	83.8	83.5	83.3	83.1	ID	**99.2**	**99.6**	**99.2**	**98.8**
27	83.9	83.5	84.8	83.5	83.0	85.5	83.9	83.9	83.4	83.9	83.2	84.3	82.8	82.9	84.3	84.0	84.1	84.1	83.6	84.1	84.0	84.0	83.6	83.1	83.4	98.9	ID	**99.6**	**99.2**	**98.8**
28	84.3	83.8	84.9	83.6	83.1	85.6	84.0	84.0	83.6	84.3	83.5	84.6	83.1	83.3	84.6	84.4	84.5	84.5	84.0	84.5	84.4	84.4	83.9	83.1	83.8	98.9	99.4	ID	**99.6**	**99.2**
29	84.2	83.8	85.0	83.8	83.3	85.8	83.9	83.9	83.8	84.1	83.4	84.5	83.0	83.2	84.5	84.3	84.4	84.4	83.9	84.4	84.3	84.3	83.8	83.3	83.6	98.6	99.1	99.3	ID	**98.8**
30	84.2	83.5	84.5	84.0	83.5	85.5	83.9	83.9	84.0	84.4	83.4	84.8	83.3	83.2	84.5	84.3	84.4	84.4	83.9	84.4	84.3	84.3	83.8	83.8	83.6	98.4	98.7	98.7	98.3	ID

Note: In the table, accession number and their corresponding country of origin is represented in the following manner: 1. MW021453 (Ethiopia); AJ276479 (South Korea); MG882753 (Poland); MH748551(Italy); LC066516 (Turkey); AJ580953 (Hungary); MG021460 (Uganda); MG021457 (Uganda); MK330845 (Tanzania); MK482376 (India); KT921314 (Egypt); KM272277 (India); JN054636 (Malaysia); MK387172 (Brazil); KC527795 (South Korea); KC527783 (South Korea); MN422333 (South Korea); LC390004 (South Korea); KX660756 (China); KC527819 (South Korea); MN422336 (South Korea); LC510819 (South Korea); EF213023 (China); KT350980 (China); MG470798 (Rwanda); AF198101 (Australia); HE650150 (India); MN399744 (Germany); KX525730 (Australia); EF202595 (China)