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# Molecular identification and characterization of badnaviruses infecting sugarcane in Ethiopia

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**Summary. –** Sugarcane bacilliform virus (SCBV) is an economically important virus limiting sugarcane production worldwide. Although Ethiopia is a major sugarcane producer, and virus-like symptoms are frequently observed in sugarcane fields, there is a complete lack of information as to the occurrence, distribution and molecular properties of SCBV. This study was aimed to identify and characterize SCBV isolates in Ethiopia using molecular methods. Out of 292 leaf samples collected and tested by PCR, 76 samples (26% incidence level) were found SCBV-positive. Nucleotide sequence analysis results showed that three Ethiopian isolates (SCBV-EtS3, SCBV-EtS6 and SCBV-EtC10) shared high level of nucleotide identity (99.5–100%) among themselves and with SCBV isolates from China (accession numbers MH037614 and MH037915). Another isolate, SCBV-EtC2, shared maximum identity of 78% with the other three SCBV isolates from Ethiopia and 99.8% with SCBV isolates from China (KM214357 and KM214307). Based on phylogenetic analysis, isolates from Ethiopia were segregated into two different clusters. Isolates SCBV-EtS3, SCBV-EtS6 and SCBV-EtC10 clustered with SCBV-Q group and SCBV-EtC2 with SCBV-H group. This study provides information on the occurrence of SCBV for the first time in Ethiopia and also contributes to the understanding of the genetic diversity of SCBV.

**Keywords:** Caulimoviridae; RNase H; *Saccharum* spp.; sugarcane bacilliform virus

## Introduction

Sugarcane (*Saccharum spp.*) is an industrial cash crop grown mainly for sugar and ethanol production worldwide. It has been grown in Ethiopia since the 16$^{th}$ century (Tena *et al*., 2016) and accounts for 100% sugar production in the country. Worldwide, the crop is vulnerable to a number of viruses which limit its production with the major ones being sugarcane bacilliform virus (SCBV), sugarcane mosaic virus and sugarcane yellow leaf virus. SCBV belongs to the genus *Badnavirus* (the family *Caulimoviridae*), consisting of circular dsDNA genome of 7.1–9.2 kb with non-enveloped bacilliform-shaped virions measuring 30 nm×130 nm (Geering and Hull, 2012). SCBV virions of up to 500 nm in length have been reported (Lockhart and Autrey, 1988). The virus causes leaf fleck disease showing leaf fleck, mild mottling, foliar streak and mild mosaic symptoms, but many of the infected plants show mild to moderate symptoms and some are even symptomless (Viswanathan *et al*., 1999, 2018). SCBV was first reported in Cuba from sugarcane cultivar B34104 in 1985 (Rodriguez *et al*., 1985) and subsequently in Morocco around 1986 in hybrid Mex. 57-473 (Lockhart and Autrey, 1988). Currently, the virus is reported in all major sugarcane growing regions including several African countries such

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**Abbreviations:** NCBI = National Center for Biotechnology Information; RNase H = ribonuclease H; SCBV = sugarcane bacilliform virus; SCBGA(D)V = sugarcane bacilliform Guadeloupe A (D) virus; SCBMOV = sugarcane bacilliform Morocco virus

**Table 1. Total number of fields surveyed, number of samples collected and PCR results**

| Field location | Region | Zone | District | Symptomatic samples | Asymptomatic samples | Total number of samples collected | Number of SCBV positive samples | Virus incidence (%) |
|---|---|---|---|---|---|---|---|---|
| Wonji[1] | Oromia | East shewa | Adama | 127 | 20 | 147 | 48 | 32 |
| Awash Melkasa[2] | Oromia | East shewa | Adama zuria | 8 | 2 | 10 | 0 | 0 |
| Adulala[2] | Oromia | East shewa | Adama zuria | 4 | 2 | 6 | 0 | 0 |
| Ulaga[2] | Oromia | East shewa | Adama zuria | 13 | 0 | 13 | 0 | 0 |
| Metehara[1] | Oromia | East shewa | Mertti | 84 | 10 | 94 | 17 | 18 |
| Welenchiti[2] | Oromia | East shewa | Welenchiti | 14 | 0 | 14 | 11 | 78 |
| Kessem[1] | Afar | Zone three | Dulecha | 8 | 0 | 8 | 0 | 0 |
| **Total** | | | | **258** | **34** | **292** | **76** | **26** |

[1]Samples collected from sugar factory plantations; [2]samples collected from farmers's field.

as Morocco, South Africa, Mauritius, Madagascar and Malawi (Autrey *et al*., 1995; Viswanathan *et al*., 2018) and perceived as a quarantine risk limiting germplasm exchange worldwide (Autrey *et al*., 1995). SCBV is transmitted by three mealybug vectors namely the pink *Saccharicoccus sacchari*, the gray *Dysmicoccus boninsis* and *Planococcus citri* in a semi-persistent manner (Lockhart *et al*., 1996).

Different isolates of SCBV are classified as different species based on the species demarcation criteria of ICTV (Geering and Hull 2012). This classification is primarily based on the nucleotide sequence difference (>20%) in RT/RNase H region. Accordingly, four distinct species of SCBV, *Sugarcane bacilliform IM virus* from Australia (Geijskes *et al*., 2002), *Sugarcane bacilliform MO virus* from Morocco (SCBMOV, Bouhida *et al*., 1993) and *Sugarcane bacilliform Guadeloupe A virus* and *Sugarcane bacilliform Guadeloupe D virus* from Guadeloupe (SCBGAV, SCBGDV, Muller *et al*., 2011) are recognized by ICTV. Three new species of SCBV (SCBV-BB, SCBV-BO and SCBV-BR) were proposed from sugarcane germplasm collection in India (Karuppaiah *et al*., 2013). Twenty phylogenetic groups (genotypes) of SCBV have been reported worldwide (SCBV-A to SCBV-T), indicating the presence of high genetic diversity of SCBV (Ahmad *et al*., 2019; de Hollanda *et al*., 2019). In Guadeloupe, the presence of five SCBV groups, SCBV-A, -B, -C, -D, and -G, has been reported (Muller *et al*., 2011). Similarly, seven SCBV genotypes are reported from India, SCBV-E, -H, -I, -J, -K, -L, and -M (Karuppaiah *et al*., 2013). Five SCBV genotypes, SCBV-A, -C, -F, -M, and -H, have been identified from germplasm collection in Brazil (da Silva *et al*., 2015). In China, 10 SCBV genotypes, SCBV-G, -H, -L, -N, -O, -P, -Q, -R and two new groups SCBV-S and -T, have been reported (Wu *et al*., 2016; Sun *et al*., 2016, 2018; Ahmad *et al*., 2019).

Sugar industries in Ethiopia are entirely dependent on the introduced exotic sugarcane varieties from different countries which may introduce viruses into the country. While virus-like symptoms were observed in sugarcane fields in the country, there is a complete lack of information on the occurrence of SCBV on sugarcane in Ethiopia. The aim of the present study was therefore to identify and characterize sugarcane bacilliform viruses using molecular techniques. We report here the occurrence, incidence and distribution of SCBV in major sugarcane fields of southeastern and central parts of Ethiopia along with phylogenetic analysis of selected isolates.

## Materials and Methods

*Sample collection.* Symptomatic and asymptomatic leaf samples were collected from seven major sugarcane growing areas in Oromia and Afar regions in Ethiopia (Table 1). The survey was conducted in the dry cropping season of January 2018 and March 2019. A total of 292 samples were collected of which 43 were from small-holder farmers' fields and 249 were from sugar industry fields. Samples were collected approximately from 5-10 km interval wherever sugarcane was available with a random sampling technique in X-fashion. Each sample (about one gram) was kept in 50 ml falcon tube containing silica gel at room temperature until processed.

*Genomic DNA extraction.* Genomic DNA was extracted using a CTAB (cetyl trimethyl ammonium bromide) method as described by Vaze *et al*. (2010) with some modifications. Dried (0.1 g) leaf tissue was ground in liquid nitrogen using mortar and pestle. The powdered leaf was mixed with 800 µl of extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 1.4 M NaCl; 1% PVP-40; 2% CTAB and 0.2% β-mercaptoethanol) in 1.5 ml tubes and mixed well by vortexing. The mixture was incubated at 55°C for 20 min and centrifuged for 5 min at 12,000 x *g*. The supernatant, >500 µl, was subsequently mixed with an equal volume of chloroform: isoamyl-alcohol (24:1) in a new tube and

**Table 2. Primers for amplification of sugarcane infecting badnaviruses**

| Primer sets | Primer name | Sequence (5'–3') | Product size |
|---|---|---|---|
| Generic SCBV primers | SCBV-F | GTTCATCGCHGTNTAYATTGATGAC | 726bp |
| | SCBV-R | GAAGGYTTRTGTTCTVCACTCTTGTTG | |
| Specific SCBV primers | SCBVF5 | TCAAAGTTTGATTTGAAGAGCGGG | 221bp |
| | SCBVF6 | CTCCGAGAAAACCAATATGTCATC | |

**Table 3. Sequences retrieved from the GenBank for pairwise sequence comparison and phylogenetic analysis**

| No. | Name of virus isolate (acronym) | Acc. No. | Country/origin | SCBV groups |
|---|---|---|---|---|
| 1. | Sugarcane bacilliform IM virus (SCBIMV) | NC_003031 | Australia | SCBV-F |
| 2. | SCBV- B51129-26 | FJ439805 | Guadeloupe | SCBV-A |
| 3. | SCBV- R570-67 | FJ439814 | Guadeloupe | SCBV-A |
| 4. | SCBV-CB6 | FJ439782 | Guadeloupe | SCBV-C |
| 5. | SCBV-Aiwa2 | FJ439784 | Guadeloupe | SCBV-C |
| 6. | SCBV- SD8 | FJ439789 | Guadeloupe | SCBV-D |
| 7. | SCBV-Ha11 | FJ439793 | Guadeloupe | SCBV-B |
| 8. | SCBV-Ba14 | FJ439787 | Guadeloupe | SCBV-B |
| 9. | SCBV-BT720231 | FJ439799 | Guadeloupe | SCBV-B |
| 10. | SCBV-GX-ROC5 | KM214353 | China | SCBV-Q |
| 11. | SCBV-GX-GT8182 | KM214340 | China | SCBV-Q |
| 12. | SCBV-CHN1 | KM214357 | China | SCBV-H |
| 13. | SCBV-BO91 | JN377533 | India | SCBV-J |
| 14. | SCBV-Ba33 | FJ439788 | Guadeloupe | SCBV-G |
| 15. | SCBV- HNEa1 | MH142498 | China | SCBV-S |
| 16. | SCBV- LA-CP384 | KM214262 | USA | SCBV-L |
| 17. | SCBV-BT | JN377536 | India | SCBV-K |
| 18. | SCBV-YN-YZ20602 | KM214292 | China | SCBV-N |
| 19. | SCBV-FJ-FN381 | KM214298 | China | SCBV-O |
| 20. | SCBV-QL-Q208 | KM214266 | Australia | SCBV-P |
| 21. | SCBV | GQ385038 | India | |
| 22. | SCBV-HNSs6 | MH142497 | China | SCBV-S |
| 23. | SCBV-HNSb5 | MH142486 | China | SCBV-T |
| 24. | Rice tungro bacilliform virus (RTBV) | NC001914 | Philippines | |
| 25. | SCBV-EtS3 | MT157333 | Ethiopia | SCBV-Q* |
| 26. | SCBV-EtS6 | MT157334 | Ethiopia | SCBV-Q* |
| 27. | SCBV-EtC2 | MT157335 | Ethiopia | SCBV-H* |
| 28. | SCBV-EtC10 | MT157336 | Ethiopia | SCBV-Q* |

*Isolates from this study.

the mixture was centrifuged at 12,000 x g for 2 min. The supernatant was transferred into a new 1.5 ml tube and 50 µl of 7.5 M ammonium acetate was added followed by 500 µl of ice-cold absolute ethanol and mixed by inversion. DNA was pelleted by centrifugation at 12,000 x g for 2 min and the pellets were washed with 70% ethanol, air dried and resuspended in 60 µl of nuclease free water. The integrity of the extracted genomic DNA was checked by electrophoresis using a 0.8% agarose gel.

*PCR screening*. All DNA samples (292) were screened for the presence of sugarcane-infecting badnaviruses with PCR using

the degenerate primers SCBV-F/SCBV-R (Table 2, Wu *et al*., 2016). Samples were also tested with SCBV-specific primers SCBVF5/SCBVF6 (Table 2, Braithwaite *et al*., 1995). Both primer sets target the RT/RNase H region with the expected amplicon of 726 and 221 bp, respectively (Table 2). PCR reaction mixture of 25 µl contained 2.5 µl 10x PCR buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl of 10 µM forward and reverse primer, 0.5 µl Hot start Taq DNA polymerase (Sigma-Aldrich, Germany), 1 µl DNA and nuclease free water to make up the final volume. PCR amplification using SCBV-F/SCBV-R primer pair was performed under the following conditions: initial denaturation of 95 °C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. For SCBVF5/SCBVR6, amplification was performed at 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 45 s and a final extension at 72°C for 10 min. PCR products (4 µl) were visualized by electrophoresis using a 1.2% agarose gel.

*Purification of PCR products and sequencing*. PCR amplicons of four virus isolates from Metehara area were randomly selected and purified using PCR purification kit (Bio Basic, Canada) following the manufacturer protocol. Ten microliters of the purified product were mixed with 5 µl of 10 µM forward or reverse primers in a separate tubes and submitted to Macrogen Inc. for Sanger sequencing (Seoul, South Korea).

*Pairwise sequence comparison and phylogenetic analysis*. Sequences were first analyzed using BLASTn (Basic Local Alignment Search Tool) algorithm from the GenBank nucleotide database to determine the virus identities. Sequences of several previously reported SCBV isolates and *Rice tungro bacilliform virus* isolate (as an outgroup) were retrieved from the NCBI database for evolutionary analysis (Table 3). Sequences were trimmed and multiple alignment on the 529 bp long core sequence of the RT/RNase H coding region was carried out using Clustal W using BioEdit Sequence Alignment Editor version 7. 0. 5.3 (Hall, 1999). PASC (pairwise sequence comparison) was performed using the Sequence Demarcation Tool version 1.2 (Muhire *et al*., 2014). The phylogenetic tree was constructed from Clustal W aligned sequences using MEGA-X version 10. 1. 5 (Kumar *et al*., 2018). Bootstrap values were determined based on 1000 replications using Maximum-Likelihood method and Kimura 2 parameter model. Bootstrap values greater than 50 % are shown, and the scale bar indicates 0.2 substitutions per site.

## Results and Discussion

*Disease symptoms*

During the field survey, a range of symptoms such as mild mosaic, yellowing, chlorotic streaking and mottling were observed on symptomatic leaves. Most of the symptomatic samples were found in Wonji and Metehara sugar factory fields and farmer's field in Welenchiti. Identification of SCBV by visual inspection of symptoms on fields was difficult since symptoms vary from mild to moderate and can even be symptomless. However, in some samples, severe symptom of yellowing to chlorotic streaking and mosaic were observed and the presence of SCBV was confirmed by PCR. Some sugarcane samples with no obvious symptoms of virus infection were also tested positive for SCBV.

The presence of SCBV in asymptomatic and symptomatic samples has also been reported in previous studies (Autrey *et al*., 1995; Rao *et al*., 2014). The lack of visible symptoms in some SCBV-infected sugarcane varieties could possibly be associated with low virus titer, varietal difference, environmental factors and virulence level of the virus isolates (Viswanathan *et al*., 1995). Some symptomatic samples suspected of the virus were tested negative in PCRs for SCBV suggesting that the plants might be infected with viruses other than SCBV such as sugarcane mild mosaic virus and sugarcane yellow leaf virus which are reported from several sugarcane growing countries or other factors (Lockhart *et al*., 1996; Viswanathan *et al*., 2018).

*PCR screening*

Out of the 292 leaf samples screened by degenerate primers (SCBVF/SCBVR) and specific primers (SCBVF5/SCBVF6), 76 samples were tested positive for SCBV with both primer sets (Table 1). Of the 249 samples collected from the three different sugar factory fields, SCBV was detected in 65 of them, 48 from Wonji sugar factory field and 17 from Metehara sugar factory field, whereas all eight samples collected from Kessem sugar factory field were negative (Table 1). Out of the 43 samples collected from different farmers' fields only 11 samples from Welenchiti were tested positive for SCBV, while samples from the other farmer's fields were negative (Table 1). Based on PCR analysis, the virus incidence was 26% (76/292), with the highest of 78% (11/14) at Welenchiti farmer's field followed by SCBV incidence of 32% (48/147) and 18% (17/94) at Wonji and Metehara sugar factory fields, respectively.

*Pairwise sequence comparison and phylogenetic analysis*

RT/RNase H coding region is the most conserved region in the genome of badnaviruses and nucleotide difference of >20% commonly used as a taxonomic marker for species demarcation in the genus *Badnavirus* (Geering and Hull, 2012). The 529 bp core RT/RNase H region has been used to show the evolutionary relationship of badnaviruses (Kidanemariam *et al*., 2018; Yang *et al*., 2003). PASC using the core RT/RNase H showed that the four SCBV isolates

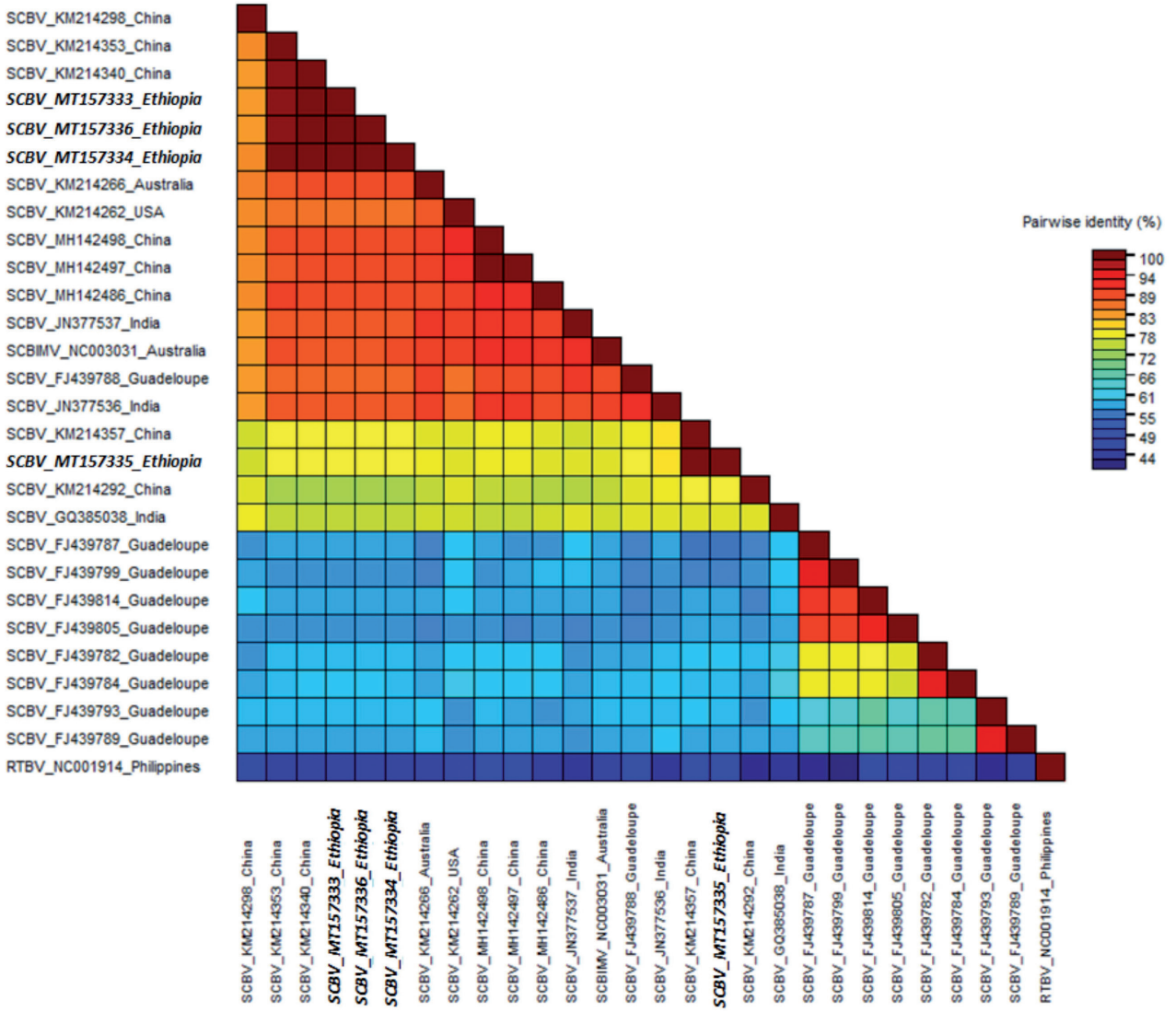


**Fig. 1**

**RT/RNase region pairwise nucleotide sequence comparison**
Pairwise nucleotide sequence comparison of the different SCBV isolates based on the partial RT/RNase region.

from Ethiopia share 78–100% nucleotide identity among themselves and with other SCBV isolates (Fig. 1). Isolates EtS3, EtS6 and EtC10 (MT157333, MT157334 and MT157336, respectively) showed 100% nucleotide identity among themselves and with SCBV isolates from China (KM214353 and KM214340). However, isolate EtC2 (MT157336) had low nucleotide identity (78%) with the other three SCBV isolates from Ethiopia, while having 99.81% identity to SCBV isolates from China (KM214357) (Fig. 1). Isolates from Ethiopia also showed 77–89% nucleotide identity with SCBIMV identified from Australia, 78-80% with SCBMOV from Morocco, and 59–63% with SCBGAV and SCBGDV from Guadeloupe (Fig. 1).

Phylogenetic analysis also revealed that the four SCBV isolates from Ethiopia were grouped into two different phylogenetic groups (Fig. 2). Isolates, MT157333 (EtS3), MT157334 (EtS6) and MT157336 (EtC10) were segregated with isolates from SCBV-Q group (KM214340, KM214353) (Fig. 2). EtC2, MT157335, was clustered with isolates from SCBV-H group (KM214357) (Fig. 2). The high nucleotide difference at the RT/RNase H region among SCBV isolates from Ethiopia and grouping of the isolates in two different clusters suggests that there are probably two different species of SCBV in Ethiopia. Generating and analyzing the full genome sequence of these and other isolates in future studies is expected to provide further insight into

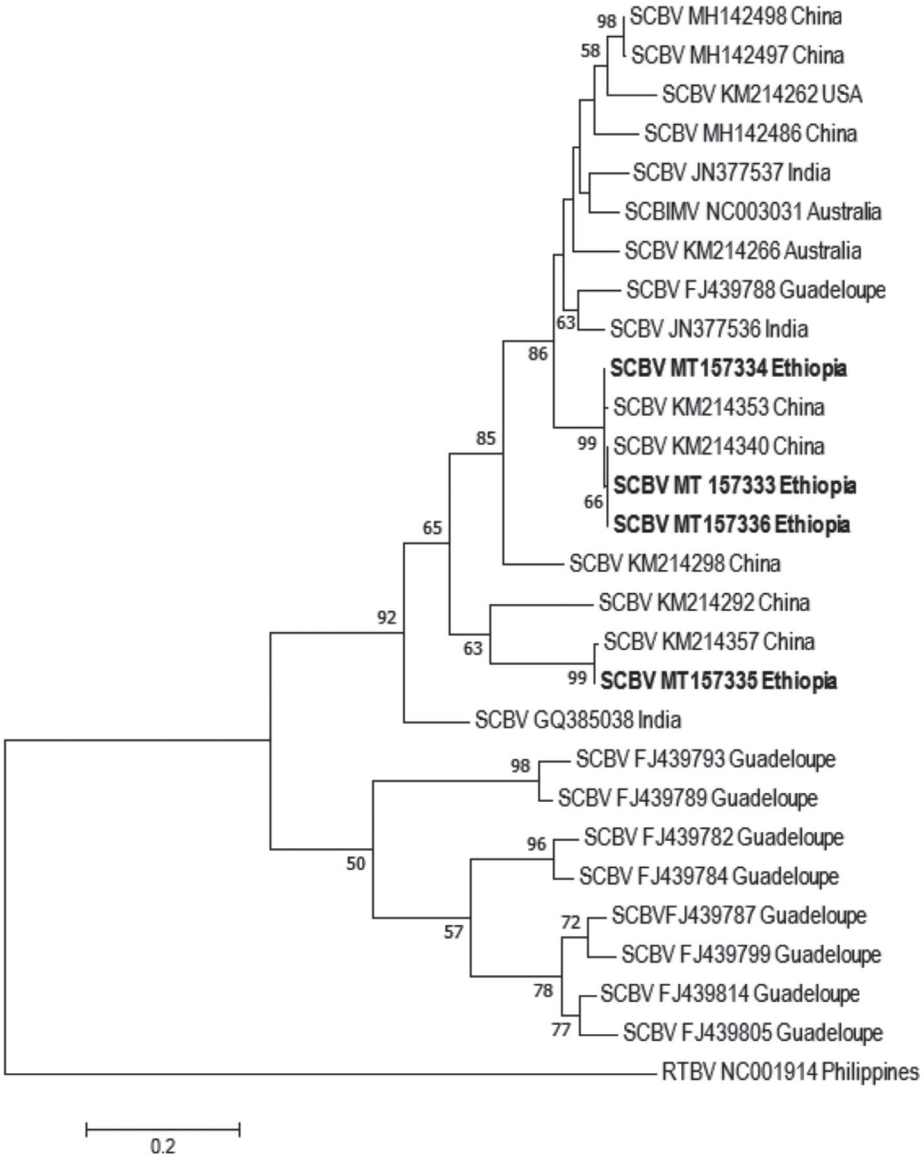


**Fig. 2.**

**Phylogenetic analysis of SCBV isolates from Ethiopia with other representative isolates from NCBI based on the partial RT/RNase region**

Sequences retrieved from GenBank for Phylogenetic analysis are listed in (Table 3). Phylogenetic tree was constructed using maximum likelihood method using Kimura-2 parameter model. The robustness of tree was evaluated by Bootstrap method (1000 replication). Only bootstrap values greater than 50% are shown, and the scale bar indicates 0.2 substitutions per site.

the overall phylogenetic relationship with other SCBV populations.

There are reports indicating that segments of viral genomes from members of the family *Caulimoviridae* could integrate into the host genome (Hohn *et al*., 2008). Also, one report from China (Cai et al. 2009) indicates that SCBV DNA fragment might be integrated into the *Saccharum* interspecific hybrids genome and that further verification would be necessary. However, Geijskes *et al*. (2004) reported that SCBV genome does not appear to be integrated into the host genome. Accordingly, PCR amplification using virus specific primers, often followed by sequence analysis of partial or complete genome has been routinely used to detect and identify SCBV in sugarcane (Braithwaite *et al*., 1995; da Silva *et al*., 2015; Muller *et al*., 2011; Rao *et al*., 2014; Karuppaiah *et al*., 2013; Ahmad *et al*., 2019). Given the extreme diversity of SCBV genotypes (Ahmad *et al*., 2019), it is likely that sequence analysis of

more isolates in the future will reveal genotypes other than Q and H in Ethiopia.

The high incidence and genetic diversity of SCBV in Ethiopia can be considered as a threat to sugarcane industry and calls for attention for its management including the use of disease-free planting materials. Since 2011, many of the Ethiopian sugar estates have been using tissue culture-derived sugarcane planting materials with the belief that disease-free plants can improve the productivity and profitability of sugar industry (Ibrahim *et al.*, 2016). Although some viruses are known to pass through tissue culture and affect the next crop (Fernandez *et al.*, 2020), there has been no information on the identity of viruses occurring in sugarcane fields as well as in tissue culture derived plantlets in Ethiopia. The information generated in this work is expected to alert sugarcane producers in the country on the extent of SCBV in Ethiopia and facilitate the exchange of virus-free planting materials with other countries. It may also encourage researchers to look for other commonly occurring sugarcane viruses ultimately leading to the introduction of virus indexing program in the existing micropropagation schemes.

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