

Research Article

Molecular Detection of *Cucumber Mosaic Virus* and *Tobacco Mosaic Virus* Infecting African Nightshades (*Solanum scabrum* Miller)

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The two viruses *cucumber mosaic virus* (CMV) and *tobacco mosaic virus* (TMV) are among the major viruses that constrain the production of African nightshade (ANS). The ANS provides high proportions of micronutrient content and has medicinal, economic, and agronomic benefits. In this study, we utilized molecular methods to detect these viruses present in ANS at two locations in Kenya: Kisii and Kakamega counties. Ribonucleic acid (RNA) was extracted from leaves of ANS plants exhibiting viral symptoms and sequenced on the Illumina MiSeq platform for phylogenetic studies. The isolates were grouped based on nucleotide and sequence identity. We detected two isolates of CMV and one isolate of TMV in ANS samples. The isolate sequences have been deposited in the GeneBank to obtain accession numbers. *Cucumber mosaic virus* nucleotide sequence closely resembled the Kirinyaga isolates in Kenya classified in subgroup I. *Tobacco mosaic virus* phylogenetic studies revealed close resemblance of isolates KY810785.1 from Britain and AF273221.1 from the USA in clade 1 based on nucleotide and amino acid sequences. This is an indication that the virus is widely distributed across the world. Detection of these two viruses in Kenya suggests that they are prevalent in crop-growing regions and the germplasms. African nightshade could also act as a virus reservoir infecting other plants. This study will inform management options to prevent virus epidemics and be in control of vectors.

1. Introduction

African nightshade is a vital crop cultivated in Western, Eastern, and Southern Africa as well as in India, Indonesia, and China [1]. The crop is rich in protein, vitamin A, iron, and calcium. It is commonly sold in major supermarkets and greengrocery stores as a leafy vegetable in most African countries [2]. The ANS vegetable crop is grown in both highland and lowland areas in most African countries [3]. In Kenya, the consumption has increased tremendously and the African leafy vegetables now account for 30% of all vegetables sold in the market [4]. Viral diseases lead to yield and crop quality losses. Two viruses CMV and TMV have been found as the main epidemic viruses in many parts of Kenya and on different crops [5]. Limited information is available on the incidence and prevalence of these viruses on

ANS. However, there is adequate research concerning these viruses on other solanaceous crops especially tomato and potato.

To date, CMV isolates are classified into subgroups I and II according to their serological relationships and nucleotide sequence identity [6]. Based on recent reports, CMV subgroup I was found to be predominant in tropical and subtropical regions [7]. *Tobacco mosaic virus* belongs to the genus *Tobamovirus* and family *Virgaviridae* [8]. *Tobamoviruses* are transmitted through mechanical contact and at a low percentage through seed and not by insect vectors [9]. The typical viral symptoms are veinal necrosis, mosaic, mottling, yellowing, deformation, shoestring, ring spots, and stunting [10]. Today, new techniques based on DNA analysis have been used and proven to be efficient due to high specificity and sensitivity [11]. Due to its great potential, over

the past 20 years, many PCR-based assays have been reported for the identification of viral pathogens [12]. The molecular tools have promoted efforts to set up assays with specific technical aspects (e.g., specificity, sensitivity, and robustness) and economical demands (e.g., short diagnosis time, high-throughput, minimum taxonomic expertise, and minimum cost) [13]. Next-generation sequencing allows phylogenetic analyses of the complete genome in order to obtain a representative set of sequences reflecting the diversity of strains and geographical distribution [14]. Sequence alignments are performed, gaps and missing data are removed, and rate variation among sites is estimated using gamma distribution [15]. The objective of this study was to detect CMV and TMV infecting ANS in Kenya to understand their distribution as a basis for their management. Information concerning the occurrence of these viruses in ANS and their potential viruses' reservoirs is lacking; hence, there is a need for this study. We hypothesize that CMV and TMV are the major viruses occurring in ANS and constrain its production.

2. Materials and Methods

2.1. Research Sites. African nightshade leaf samples showing virus-like disease symptoms were collected from the sampling sites. These sites are Suneka and Ogembo in Kisii, Lurambi, and Amalemba in Kakamega, Kenya. A global positioning system was used to locate the sites. Suneka is located at a latitude of 0° 40' 43.5" S and a longitude of 34° 42' 27.7" E in upper midland zones AEZ UM 2. The average annual rainfall is 800–1000 mm, temperature ranges from 18 to 21°C, and altitude ranges between 1500 and 2000 meters above sea level (a.s.l.). Ogembo is located at a latitude of 0° 50' 18.8" S and a longitude of 34° 43' 47.6" E in lower highlands zones AEZ LH 2. The average annual rainfall is 1300–1600 mm, temperatures range from 15 to 18°C, and altitude ranges from 2000 to 2500 m a.s.l. Amalemba is located at a latitude of 0° 16' 14.4" N and a longitude of 34° 45' 14.6" E in upper midlands zone AEZ UM 4. The altitude range is 1500–1900 m a.s.l., rainfall is between 1000 and 1600 mm, and temperature ranges from 18 to 21°C. Lurambi is located at a latitude of 0° 17' 42.5" N and a longitude of 34° 4' 47.9" E in the lower midlands AEZ LM 2. The altitude range is 1300–1500 m a.s.l., rainfall ranges from 1500 to 1800 mm, and temperatures range from 20 to 22°C [16, 17].

2.2. Source of Virus Isolates. Leaf material samples (the youngest trifoliate leaves) obtained from 24 plants at each location were flash-frozen in liquid nitrogen and transported to the laboratory at the Kenya Plant Health Inspectorate Service on dry ice for processing.

2.3. Total RNA Extraction. Total RNA was extracted from the plant leaves using the Trizol protocol (Thermo Fisher, Waltham MA). Briefly, it involved extraction and purification of total RNA using Trizol reagents, homogenization of cell suspensions, separation of tissues, RNA precipitation, RNA wash, dissolving the RNA, and determination of RNA

concentration and purity. The protocol was used in the extraction of RNA for all the samples. Finally, the purified RNA was stored at –20°C in a freezer.

2.4. cDNA Production and RT-PCR. Total purified RNAs for TMV and CMV were used for complementary DNA (cDNA) synthesis using a PCR kit (GoTaq, Promega, USA). cDNA was synthesized and incubated following manufacturers' instructions (GoTaq, Promega, USA). Reverse primer for each specific virus was used during synthesis (Table 1). The synthesized cDNA was diluted 1:4 with ddH₂O and stored at –20°C awaiting PCR. The polymerase chain reaction was used to amplify the 3' terminal genomic region of the virus using degenerate primers (Table 1). *Cucumber mosaic virus* and *tobacco mosaic virus* PCR was performed in a 25 µl of a reaction mixture prepared following the manufacturers' protocol (Qiagen, USA).

2.5. Next-Generation Sequencing (NGS). Twenty four samples used earlier were pooled into 14 combinations based on the agroecological zones for sequencing. Fourteen purified PCR products each 15 µl per tube and 12 primers each 10 µl per tube were prepared. Ribosomal RNA was depleted with the kit ribo-Zero rRNA removal kit (Illumina, USA), and the RNA integrity number was measured in a 2200 bioanalyzer (Agilent Technologies, USA). The cDNA library was constructed with the TrueSeq RNA sample preparation kit (Illumina, USA) and NGS performed in Illumina MiSeq 2010 equipment at Inqaba Biotechnology and Genomics Company (South Africa).

2.6. Bioinformatics Analysis. After NGS, low-quality bases were removed from the data set. Sequences from CMV and TMV were identified using BLASTN against a local database containing all viruses infecting plants in the ninth report of the International Committee of Taxonomy of Viruses [10]. The nucleotide sequences of the gene were aligned with those of corresponding viruses deposited in GenBank by using Clustal-LC software. Sequence homology analyses of the gene were performed using Bio-Edit version 7 software. Viral genomes were assembled to reference genomes. Assemblies were verified for inconsistencies and sequencing errors with tablet open reading frames (ORFs) identified using BLASTN. The phylogenetic tree was constructed using MEGA 7.0 software with the neighbor-joining algorithm and 1000 bootstrap replications. Consensus sequences for CMV and TMV were submitted to GenBank to obtain accession numbers.

3. Results

3.1. Viral Leaf Symptoms. The leaf samples obtained from the field had a variety of visible symptoms that were associated with virus infection. These symptoms included yellow-green mosaic, stunting, rugosity, vein clearing, yellowing, leaf curling, and wilting (Figures 1(a)–1(d)).

TABLE 1: Nucleotide sequences of oligonucleotide primers used for molecular testing, genomic locations, and target viruses in African nightshades.

Primers	Sequence (5'–3')	Product size	Target strain
CV (F)	GCCACCAAAAATAGACCG	593 bp	<i>Cucumber mosaic virus (CMV)</i>
CV (R)	ATCTGCTGGCGTGGATTCT		
TMV (F)	CGATGATGATTCGGAGGC	512 bp	<i>Tobacco mosaic virus (TMV)</i>
TMV (R)	GAGGTCCARACCAAMCCAG		

Note. The full-length primer sequences for the specific virus were sourced from the NCBI nucleotide database and manually aligned in a text editor. The primer design software Prifi [18] was used to select primers. CMV: *cucumber mosaic virus*; TMV: *tobacco mosaic virus*; F: forward primer; R: reverse primer; μ l: microliters.

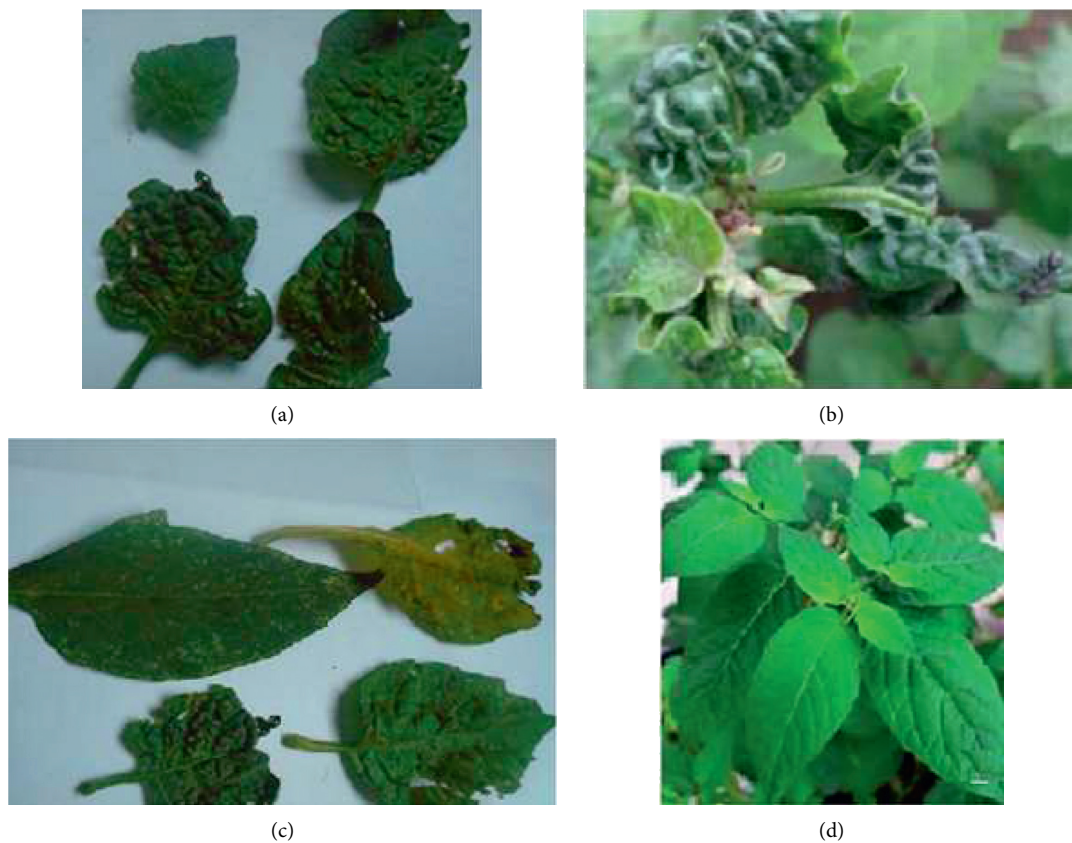


FIGURE 1: ((a)–(d)) Leaf viral-like symptoms in African nightshade observed in the field. (a) Leaf rugosity. (b) Leaf curling. (c) Leaf malformations. (d) A healthy African nightshade plant.

3.2. Detection of Cucumber Mosaic Virus in African Nightshade. African nightshade leaf samples tested positive (Figure 2). Half of the samples from the four sites were positive for the *Cucumber mosaic virus*. Ogembo and Amalemba had more samples positive for CMV at 67% compared to Suneka and Lurambi recording 33%. The virus was present in the four agroecological sites (Figure 2)

3.3. Phylogenetic Studies of Cucumber Mosaic Virus in African Nightshade. Neighbor-joining phylogenetic analysis based on full-length viral genomes was performed with a bootstrap of 1000 replications. The genomic sequence of the 10 CMV isolates (Figure 3) shared genes of 86 and 100% identity with the Kenyan



FIGURE 2: Detection of *Cucumber mosaic virus* in African nightshade leaves from farm fields in different agroecological zones using RT-PCR. L-DNA ladder. Samples 3, 7, and 11: Suneka; 4, 8, and 12: Ogembo; 5, 9, and 13: Amalemba; 6, 10, and 14: Lurambi; 2: healthy control; 1: positive control. CMV = 593 bp.

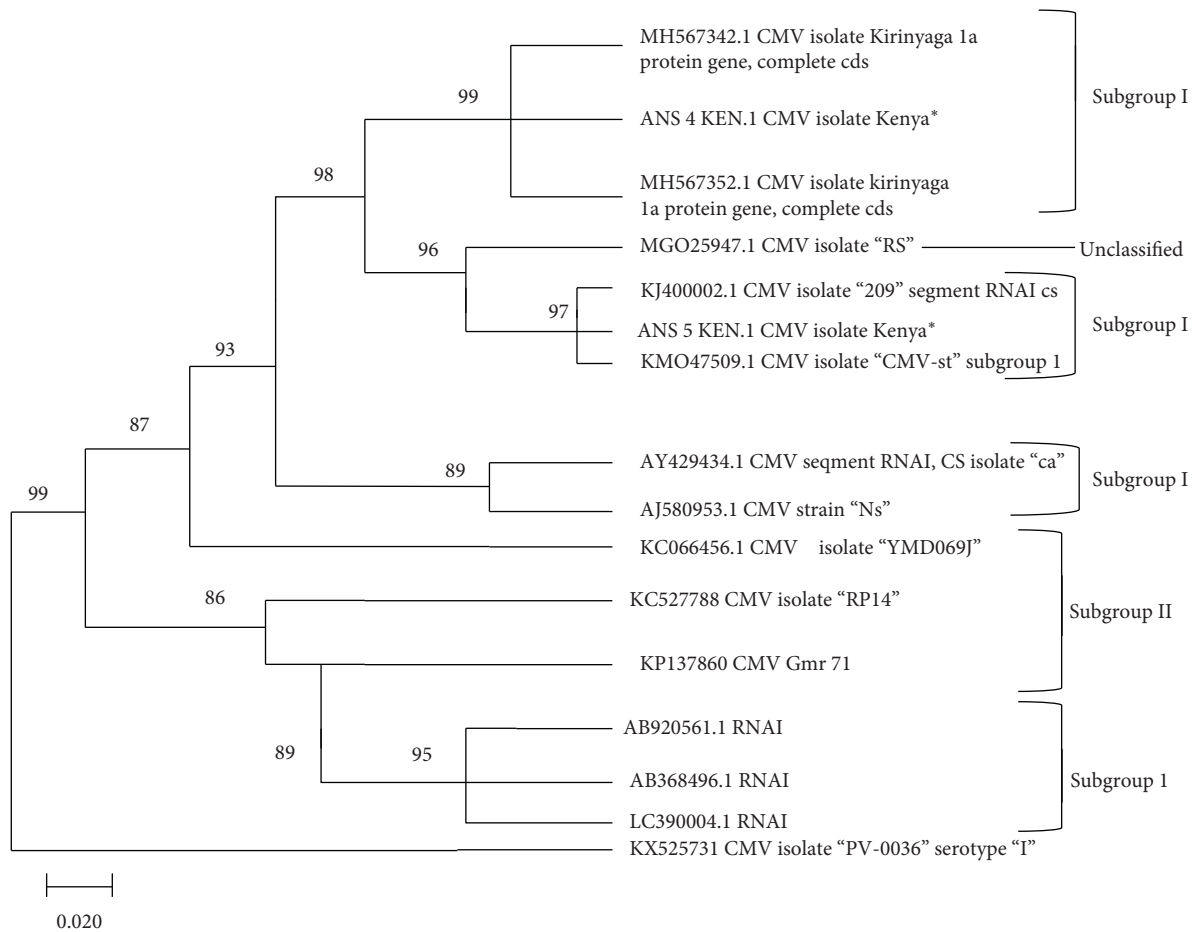


FIGURE 3: Phylogenetic analysis of *Cucurbit mosaic virus* (CMV) isolates, with aligned nucleotide sequences, generated using the neighbor-joining method and MEGA6 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Kenyan ANS isolates. The tree was rooted using KX525731 CMV Isolate "PV-0036" serotype "I" as an outgroup sequence for the phylogenetic analysis.

isolates. The isolates were grouped into groups according to the percentage sequence identity. The ANS isolates were homologous to 10 isolates belonging to subgroup I, three isolates in subgroup II, and one isolate unclassified (Figure 3). Two Kenyan CMV isolates from Kirinyaga under accession numbers (MH567342.1 and MH567352.1) had the highest similarity of 99% and belong to subgroup I. The ANS isolates had a similarity of 96% with MGO25947.1 isolate "RS" from China, KJ400002.1 isolate "209" from South Korea, and KMO447509.1 from South Korea. In addition, it had 89% similarity AYA429434.1 isolate "ca" from China and AJ580953.1 strain "Na" from Hungary in Subgroup I. Isolates L066456, KC527788, and KP137860 had 87% similarity and placed in subgroup II. CMV KC527788 isolate "RP14" from South Korea and KP137860 from Brazil had 86% similarity (Figure 3).

3.4. Detection of Tobacco Mosaic Virus in African Nightshade. Seven pooled leaf samples from agroecological zones UM4, LH2, LM2, and UM2 were positive for *Tobacco mosaic virus* (TMV) (Figure 4). More than half of the samples tested from UM2 (14 and 10), LH2 (17 and 9), and UM4 (16 and 8) representing 67% of the pooled samples were positive for



FIGURE 4: Detection of *Tobacco mosaic virus* in leaf samples of African nightshade in different agroecological zones using RT-PCR. L-DNA ladder. Samples 18, 14, and 10: UM2; samples 17, 13, and 9: LH2; samples 16, 12, and 8: UM4; samples 15, 11, and 7: LM2; 19: healthy control; 20: positive control. TMV = 512 bp.

TMV. Samples from LM2 had the lowest viral load recording 33% compared to other sites (Figure 4).

3.5. Phylogenetic Studies of Tobacco Mosaic Virus in African Nightshade. The phylogenetic analysis with TMV Kenyan isolates confirms the distribution of this plant virus worldwide. TMV KY810785.1 strain FERA 111011 from

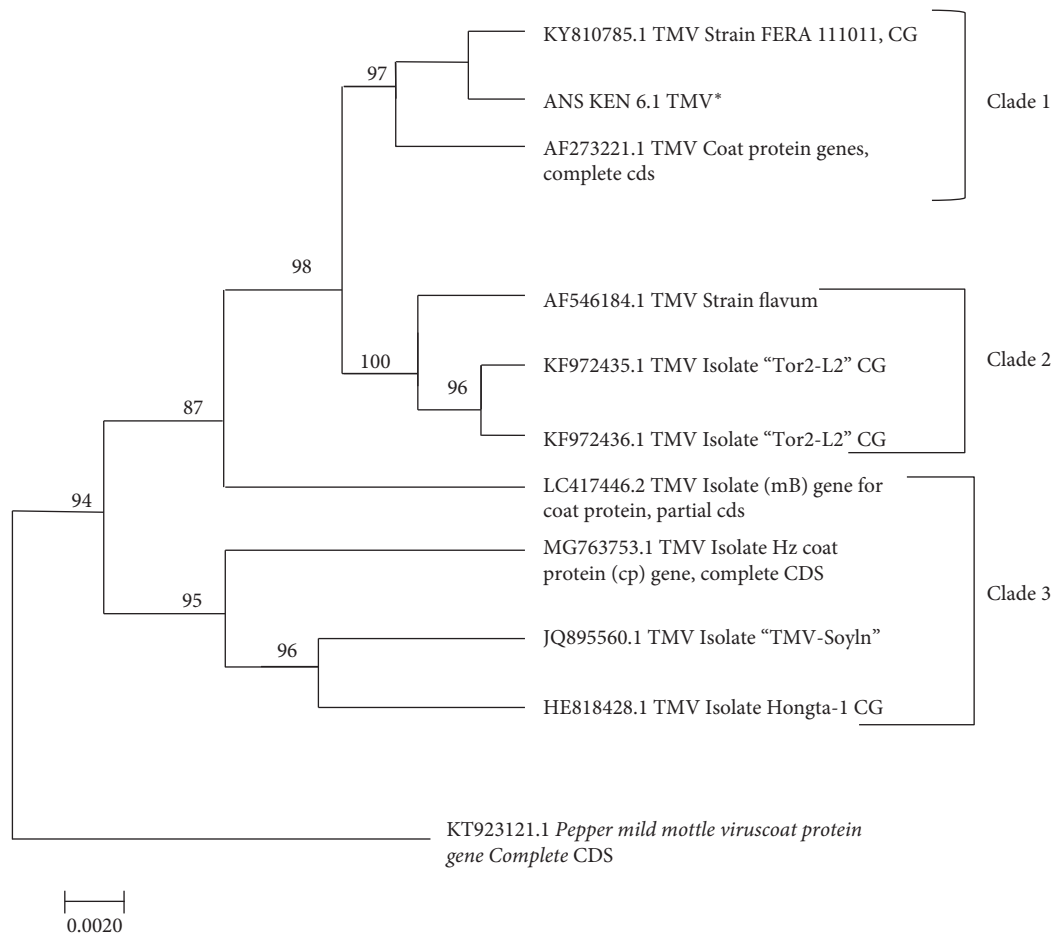


FIGURE 5: Phylogenetic analysis of *Tobacco mosaic virus* (TMV) isolates, with aligned nucleotide sequences, generated using the neighbor-joining method and MEGA6 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Kenyan ANS isolates. The tree was rooted using *Pepper mild mottle virus* (PMMoV) as an outgroup sequence for the phylogenetic analysis.

Britain formed clade 1 with TMV AF273221.1 from the USA and the Kenyan ANS isolate with 97% identity. CMV KF972435.1 TMV isolate “Tor2-L2” from Spain, AF546184.1 from Finland, and KF972436.1 from Spain formed clade 2 and were 96% homologous with Kenyan ANS isolate. In addition, TMV MG763753.1 isolate Hz from China is clustered with isolates TMV JQ895560.1 “TMV-Soyln” from India and TMV HE818428.1 Hongta-1 from China with 95% homology and formed clade 3. Finally, TMV KT923121.1 *Pepper mild mottle virus* from Brazil was used as an outgroup sequence (Figure 5).

4. Discussion

This study has demonstrated that African nightshade is a host to *Cucumber mosaic virus* (CMV) and *Tobacco mosaic virus* (TMV). These viruses were detected in symptomatic African nightshade leaves obtained from farm fields in the agroecological zones using molecular techniques. Although data on the pathogenic potential of the viruses is not readily available, intensive cultivation of the crop might lead to building up inoculum to levels beyond the economic

threshold. The detection of these viruses concurs with studies done on other solanaceous crops like potato, tomato, and tobacco [19]. *Cucumber mosaic virus* belongs to the genus *Cucumovirus* with 60–65% strains identity and is one of the most common plant viruses of major agricultural significance [20]. It is a tripartite virus having three plus sense, single-stranded RNA molecules encased in separate particles [20]. The Kenyan isolates revealed up to 86 to 96% similarity to 10 isolates worldwide. Kenyan CMV isolates caused variable symptoms, including necrotic or chlorotic lesions, mild to severe mosaic, stunting, leaf deformation, and shoestring formation. Similar symptoms were reported by [21]. Phylogenetic analysis revealed isolate homology with 10 different isolates of CMV from Kenya, China, South Korea, South Africa, and Brazil at the nucleotide level. Based on phylogenetic analysis, the CMV isolate in this study was found to be closely related to those of South Korea and China. This study reports a high prevalence of the virus in Kenya and a similar report by [22] concurs. Based on the phylogenetic tree, the isolates under the current investigation could be classified in subgroup I. Subgroup I strains show severity in terms of symptom and disease development

in tobacco [23]. Similar reports show CMV subgroup I to be competitive in its infection on different host plants [23].

Tobacco mosaic virus belongs to the genus *Tobamovirus* and has a very wide host range; it can cause a serious economic impact in many crop families such as cucurbits, brassicas, solanaceous, and ornamental plants [24]. The infected plants showed different types of symptoms which included mosaic, malformation, mottle, and stunting. *Tobacco mosaic virus* infections have been reported in Kenya on solanaceous crops but there are no reports on African nightshade [24]. Phylogenetic analysis of TMV in African nightshade isolates revealed its wide distribution in agroecological zones (UM2, LH2, UM4, and LM2) with 67% of the samples analyzed using RT-PCR testing positive for TMV. It was closely related to isolates from European and Asian countries showing a worldwide distribution. Reference [25] also reported the worldwide distribution of TMV. *Tobacco mosaic virus* identified in the current study is closely related to those isolated from tobacco, tomato, and potato. TMV strains have a genetic variation in different hosts and environmental conditions [26]. *Tobacco mosaic virus* can perpetuate itself through germplasm. Similar studies have reported that *Tobamovirus* can easily be transmitted mechanically through seed and contact between plants, but not transmitted by a vector [25], and that debris can be an important source of inocula in the fields [19].

This study deployed molecular techniques for the detection of viruses associated with edible African nightshade. That was in recognition of the difficulties of using symptoms to identify viruses that usually occur in multiple infections under field conditions.

5. Conclusion

Cucumber mosaic virus and *tobacco mosaic virus* were frequently isolated viruses infecting African nightshade, and phylogenetic analysis revealed that they are closely related to similar viruses isolated worldwide. *Cucumber mosaic virus* caused necrotic and severe mosaic in edible African nightshade. *Tobacco mosaic virus* manifested mosaic, malformation, and mottling. Phylogenetic analysis revealed a close resemblance of two viruses to isolates from solanaceous crops. Molecular methods can be utilized by seed companies and phytosanitary bodies for the screening of seeds before certification. Detection of diseases in plant material especially seeds is essential to ensure safe and sustainable crop production.

Data Availability

The datasets used in the present study are available from the corresponding authors upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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