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Research

Occurrence of a Novel Strain of Moroccan Watermelon Mosaic Virus Infecting Pumpkins in Kenya

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Abstract

The *Potyvirus* Moroccan watermelon mosaic virus (MWMV) naturally infects and severely threatens production of cucurbits and papaya. In this study, we identified and characterized MWMV isolated from pumpkin (*Cucurbita moschata*) intercropped with MWMV-infected papaya plants through next-generation sequencing (NGS) and Sanger sequencing approaches. Complete MWMV genome sequences were obtained from two pumpkin samples through NGS and validated using Sanger sequencing. The isolates shared 83.4 to 83.7% nucleotide (nt) and 92.3 to 95.1% amino acid (aa) sequence identities in the coat protein and 79.5 to 79.9% nt and 89.2 to 89.7% aa identities in the polyprotein with papaya isolates of MWMV. Phylogenetic analysis using complete polyprotein nt sequences revealed the clustering of both pumpkin isolates of MWMV with corresponding sequences of cucurbit isolates of the virus from other parts of Africa and the Mediterranean regions, distinct from a clade formed by papaya isolates. Through sap inoculation, a pumpkin isolate of MWMV was pathogenic on zucchini (*Cucurbita pepo*), watermelon (*Citrullus lanatus*), and cucumber (*Cucumis sativus*) but not on papaya. Conversely, the papaya isolate of MWMV was nonpathogenic on pumpkin, watermelon, and cucumber, but it infected zucchini. The results suggest the occurrence of two strains of MWMV in Kenya having different biological characteristics associated with the host specificity.

Keywords: disease management, tree fruits, viruses and viroids

Moroccan watermelon mosaic virus (MWMV) is a member of the genus *Potyvirus* (McKern et al. 1993), one of the large plant viral groups comprising many economically important plant viruses. At the molecular level, MWMV forms part of the papaya ringspot virus (PRSV) cluster (Yakoubi et al. 2008). The virus has a single-stranded positive sense RNA genome of 9.7 kb, with a single open reading frame that is translated into a large polyprotein, which is cleaved by the virus-encoded proteases into individual functional proteins (Wylie et al. 2017). MWMV was first described in Morocco more than four decades ago as a strain of Watermelon mosaic virus on the basis of host range, having been reported in all commercial cucurbit-producing regions of the country as causing severe damage to cucurbits (Fischer and Lockhart 1974). Using serological techniques, MWMV was reclassified as a distinct *Potyvirus* species (Purcifull and Hiebert 1979). Quiot-Douine et al. (1990) established MWMV to be distantly related to the PRSV *Potyvirus* subgroup based on its biological and serological properties. Using tryptic peptide profiles, McKern et al. (1993) supported the classification of MWMV as a distinct species. Subsequently, sequence analysis of the coat protein (CP) gene and whole genome established MWMV as a distinct member of the genus *Potyvirus* (Lecoq et al. 2001; Yakoubi et al. 2008). Since then, the virus has been detected in many other African countries, including Niger (Yakoubi et al. 2008), South Africa (Ibaba et al. 2016), Sudan (Lecoq et al. 2001), Zimbabwe, Cameroon (Yakoubi et al. 2008), Congo (Arocha et al. 2008), Tunisia (Yakoubi et al. 2008), Tanzania (Menzel et al. 2011), Nigeria (Owolabi et al. 2012), and Kenya (Mumo et al. 2020). Outside Africa, MWMV has been reported in Mediterranean countries including Italy (Roggero et al. 1998), Portugal (Yakoubi et al. 2008), France (Lecoq et al. 2007), Greece (Malandraki et al. 2014), Iraq (Bananej et al. 2018), and Spain (Miras et al. 2019).

MWMV naturally infects and poses a serious production threat to cucurbits and *Carica papaya* L. (Arocha et al. 2008; Ibaba et al. 2016; Kidanemariam et al. 2019; Lecoq et al. 2001, 2007; Mumo et al. 2020; Read et al. 2020; Yakoubi et al. 2008). The virus also infects members of *Chenopodiaceae* through sap inoculation (Yakoubi et al. 2008). The virus is transmitted to cucurbits in a non-persistent manner by a range of aphid species including *Myzus persicae*, *M. persicae* subsp. *nicotianae*, *Aphis gossypii, A. spiraecola*, *A. fabae*, and *A. nerii* (Chatzivassiliou et al. 2016; Owolabi and Ekpiken 2014). However, aphid-mediated transmission of MWMV to papaya is not established yet. But as in the cucurbits, MWMV is most likely transmitted in papaya in a non-persistent manner by several species of aphids because of the presence of highly conserved "RITC" "CSC" "PTR" motifs in the helper component–protease (HC-Pro) and a 'DAG' motif in the CP of papaya isolates of the virus (Mumo et al. 2020). These motifs are associated with aphid transmission in potyviruses (Huet et al. 1994; López-Moya et al. 1999). It was reported that MWMV transmitting aphid species heavily colonize cucurbits (Chatzivassiliou et al. 2016) but not papaya (Martins et al. 2016).

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Symptoms of MWMV infection in papaya include mottling, mosaic, shoe stringing, curling, and puckering on the leaves, and ring spots on the fruits of different sizes and shapes. Other symptoms in papaya caused by MWMV infection include numerous water-soaked or oil-streaked lesions on the upper part of the plants' stems and leaf petioles. As the disease progresses, the infected papaya plants become stunted and rosetted, with fibrous internal trunk (Arocha et al. 2008; Mumo et al. 2020). By contrast, cucurbits infected with MWMV show mosaic, severe filiform and striking interveinal chlorosis, with raised dark green blisters on the leaves. Early infection of these plants leads to severe stunting, resulting in minimal fruit yield or complete crop failure. Infected fruits are misshapen with blistered surfaces (Fischer and Lockhart 1974; Ibaba et al. 2016; Lecoq et al. 2001; Yakoubi et al. 2008).

While sampling for papaya plants to determine viruses associated with papaya ringspot disease (PRSD) in Kenya for a previous study (Mumo et al. 2020), papaya crops were observed to be intercropped with pumpkin. The intercropped pumpkin plants frequently showed symptoms resembling those caused by MWMV infection. The results of the previous study revealed that papaya plants displaying PRSD symptoms were infected with MWMV (Mumo et al. 2020). The goal of this study was to identify and characterize viruses present in the symptomatic pumpkin plants using next-generation sequencing (NGS) and Sanger sequencing approaches. Further, the potential role of pumpkin as an inoculum source for MWMV spread to papaya and vice versa were investigated through sap inoculation experiments. These findings will improve our understanding of MWMV epidemiology and support the improvement of disease management approaches.

Materials and Methods

Sample collection. Leaf tissue samples were collected from two diseased pumpkin plants displaying symptoms of mosaic, puckering, vein clearing, and vein banding (Fig. 1) in two different MWMV-infected papaya fields. The fields were in Meru and Kiambu counties in Kenya. Papaya isolates of MWMV from both fields were sequenced in our previous study and deposited in GenBank with accession numbers MH595741 for isolate Mer (Meru County) and MH595742 for isolate Kia (Kiambu County) (Mumo et al. 2020). The pumpkin leaf samples were preserved in RNAlater solution (Invitrogen) and transported to the Biosciences eastern and central Africa–International Livestock Research Institute (BecA-ILRI) Hub laboratories in Nairobi, Kenya, for NGS analysis on the Illumina MiSeq platform.

RNA sequencing and bioinformatics analysis. Total RNA was extracted from the two pumpkin leaf samples using RNeasy Plant Mini Kit (Qiagen Inc.) according to the manufacturer's instructions and used for cDNA library preparations. The libraries were prepared using the Illumina TruSeq RNA sample preparation protocol (Illumina, San Diego, CA, United States) and sequenced on the Illumina MiSeq system at the BecA-ILRI Hub, Nairobi, Kenya. The obtained 35-151 bp paired-end reads were checked for quality using FastQC, and low-quality reads and sequencing adapters were removed using Trimmomatic v. 0.33 (Bolger et al. 2014). The host genome was removed by mapping all the reads to the *Cucurbita maxima* genome (GenBank accession number GCA_002738345.1) using Bowtie2 v. 2.2.8 (Langmead and Salzberg 2012). The remaining nonhost reads (unmapped) were then assembled de novo to obtain contigs using metaSPAdes V 3.9.0 (Nurk et al. 2017) with default settings. The Krona web-based tool (Ondov et al. 2011) was used for identification and visualization of the assembled virus contigs. The resultant contigs were compared with other sequences in the NCBI nonredundant database (https://www.ncbi.nlm.nih.gov/) (Benson et al. 2012) and the Plant Virus Genome database (Camacho et al. 2009) using BLASTn search. The top hit accession was used for virus identification. Reference assemblies were performed by mapping the de novo sequences against the most similar existing viral genomes using the read mapping module of CLC genomics workbench version 5.5.1 (CLC Bio, Aarhus, Denmark). The de novo sequences and consensus sequences from reference mapping were then compared through visual inspection of individual mappings to ensure that no artifacts were incorporated because of sequencing errors or errors during genome assembly. De novo sequences were, however, preferred over the consensus of reference assembly as a precautionary measure in case the viruses identified had considerably diverged from similar viral genome sequences in the GenBank database.

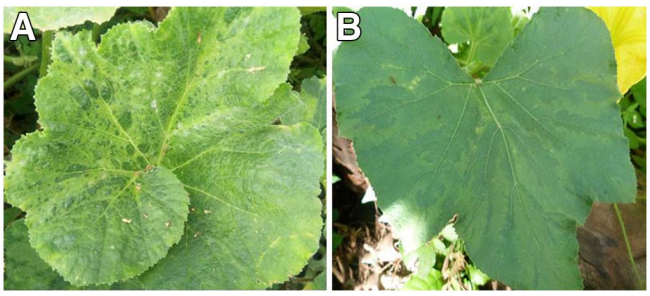

Fig. 1. Disease-affected pumpkin leaf leaves showing symptoms of **A,** mosaic, puckering, and vein clearing and **B,** mosaic and vein banding. The symptomatic plants were observed in an intercropped papaya field in Kenya with a history of Moroccan watermelon mosaic virus (Mumo et al. 2020).

Validation of assembled de novo sequences through RT-PCR and Sanger sequencing. To validate the assembled virus-specific contigs, primers were designed from Illumina-generated sequences using primer 3 (Untergasser et al. 2012), and evaluated for specificity using the NCBI primer-BLAST tool (Ye et al. 2012). The primer sequences designed are shown in Table 1. The primers were used for RT-PCR, and the amplified products were shipped to Macrogen (Netherlands, Europe), for Sanger sequencing. The obtained sequences were assembled using CLC Genomics Workbench (version 5.5.1) and compared with the de novo assembled sequences from the Illumina MiSeq through alignment and visual inspections. The Sanger sequences were also used for BLASTn search in the NCBI nonredundant database.

Sequence and phylogenetic analysis. Open reading frames (ORFs) were determined using ORF finder. The sequence alignment was carried out using in-built program in CLC Genomics Workbench, and protein sequence identities were computed using the Sequence Identity and Similarity (SIAS) tool http://imed.med.ucm.es/Tools/sias.html. Phylogenetic analysis were carried out in MEGA 6 (Tamura et al. 2013) based on complete polyprotein nucleotide sequences of isolates of MWMV and other potyviruses. The phylogenetic tree was constructed using the maximum likelihood method based on the Jones-Taylor-Thornton (JTT) matrix (Jones et al. 1992), as determined in the program Modeltest (Posada and Crandall 1998), using 1,000 replicates for bootstrap analysis. Identification of potential genome recombination sites of the aligned complete genome sequences of isolates of MWMV and several other potyviruses was performed using RDP4 package (Martin et al. 2015), with the default setting. Some of the known recombinant sequences of sugarcane mosaic virus (AF494510, AY149118, AY042184, GU474635, AM110759, EU091075) were included as controls during the recombination analysis (Padhi and Ramu 2011). To determine nucleotide diversity and mutations within MWMV sequences, 22 full genomes available in GenBank (10 genomes isolated from papaya and 12 genomes isolates from cucurbits including two from this study), were analyzed using DNASP V6.11.0 (Rozas et al. 2017).

Sap inoculation and host range. Two virus isolates, from naturally infected pumpkin (this study) and from papaya (Mumo et al. 2020) collected from the same field in Meru County were inoculated and maintained in their natural hosts (papaya and pumpkin) in an insect-free screen house. The sap inoculation experiments were conducted at the Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya. Briefly, symptomatic leaf tissues of papaya and pumpkin plants were separately ground in 1:10 (wt/vol) cold inoculation buffer (0.01M potassium phosphate, pH 7.5), plus 40 g of 600 mesh carborundum (silicon carbide). The crude extract was gently rubbed with a cheesecloth onto two youngest fully expanded leaves of papaya and pumpkin plants, established in a steam-sterilized (2 h

at 121°C) mixture of soil and organic matter. Twenty-eight days after inoculation, the papaya and pumpkin plants were tested for virus presence using RT-PCR. The previously reported forward (5′-TCTCAGCTAGCACGCAACAA-3′) and reverse (5′-CGGTG TTGAGCCAAACGAAG-3′) primer pair based on papaya isolates of MWMV (Mumo et al. 2020) was used to target a 315-bp fragment in a cylindrical inclusion (CI) region of the papaya isolate. For the pumpkin MWMV isolate, the primer pair MWMV18-F/MWMV18-R (Table 1) was used to amplify the 613-bp fragment corresponding to the CI region. The infected papaya and pumpkin plants were maintained in the screen house and used as the source of viral inoculum for subsequent tests.

Seedlings of zucchini, pumpkin, cucumber, watermelon, and papaya were established from seeds in pots containing steam-sterilized soil and maintained in an insect-free screen house. At the two true-leaf stages of growth, the plants were sap-inoculated with virus inoculum of papaya and pumpkin MWMV isolates. One plant from each test species was mock-inoculated with inoculation buffer alone to serve as a control. The inoculated leaves were rinsed thoroughly with sterile water and monitored daily for the development of virus symptoms, which were recorded weekly until 35 days after inoculation. Two independent inoculations were conducted using 10 plants of each crop for each test. All plants without symptoms were tested 14 and 35 days after inoculation by RT-PCR using virus isolate specific primers. For symptomatic hosts, only two plants of each species were tested.

Results

Detection of MWMV in pumpkin plants intercropped with MWMV-infected papaya. The Presence of MWMV was confirmed through homology search against the NCBI nonredundant database of the longest de novo assembled contigs of 9,729 and 9,754 bases with a coverage depth of 1,828 and 1,571, respectively, from the two pumpkin samples. Based on pairwise sequence comparisons, the contigs from pumpkin samples shared 99% sequence identity. The contigs shared 81% sequence identities with MWMV isolates previously reported from papaya in Kenya, GenBank accession numbers MH595736–46 (Mumo et al. 2020). The RT-PCR screening of the pumpkin samples yielded the expected fragment sizes of 613 bp and 455 bp for CI and CP, respectively. Sanger sequencing of the amplified viral amplicons yielded sequences that were 100% identical at the nt/aa levels to those generated de novo based on pairwise comparisons, confirming that the genome sequence assembly of MWMV isolated from pumpkins was accurate. No other virus sequences were found in the pumpkin samples subjected to NGS.

The MWMV viral genome sequences obtained from pumpkins were deposited in GenBank under accession numbers MH713899 and MT497462. The untranslated regions (UTRs) ends were not verified through rapid amplification of cDNA ends (RACE) analysis, but they were almost similar in length to the corresponding sequences of published isolates of MWMV. The MH713899 genome is organized into 158 nt 5′UTR, followed by 9,369 nt polyprotein encoding sequences from which all the proteins of the virus are derived, and 192 nt long 3′UTR. The MT497462 genome, by contrast, is organized into 202 nt in 5′UTR, 9,369 nt of polyprotein, and 180 nt 3′UTR. The MWMV polyprotein of each pumpkin isolate in this study contains 3,122 amino acids (aa), compared with 3,124 aa for published sequences of isolates of papaya and other cucurbits and 3,121 aa for MWMV isolate of *Cucurbita pepo* from Burkina Faso (MN688647). The variability in the N-terminal region of the CP of MWMV isolates from pumpkin samples, compared with those of papaya, was most evident, having a 6 nt deletion. By contrast, MWMV isolate from Burkina Faso had a 6 nt insertion in the same region. Several conserved motifs reported in MWMV isolates in papaya and other cucurbits were also found in MWMV isolates from pumpkins. These include a domain containing five repeats of CAA motifs (CAACACAACACAACAACATTCAA) in the 5′UTR, the nucleotide triphosphate (NTP)-binding motif "GAVGSGKST" in the N terminal region of CI, three active sites of RNA-dependent RNA polymerase motifs: "YCDADGS", "GNNSGQPSTVVDNTLMV", and "NGDDL" in the nuclear inclusion b (NIb). Other motifs like "RITC" and "PTR," both in HC-Pro, and "DAG" in the CP reported in papaya MWMV isolates, were also found in the isolates from pumpkins.

Sequence identities and phylogenetic analysis. The genome sequences of the two pumpkin MWMV isolates were almost identical to each other, sharing >97% nt and aa sequence identities in all encoded proteins (Table 2). However, the pumpkin isolates shared only 79.5 to 79.9% nt (89.2 to 89.7% aa) identity in the polyprotein and 83.4 to 83.7% nt (92.3 to 95.1% aa) identity in the CP region with MWMV isolates from papaya. The P1 was the most variable protein between MWMV isolates from papaya and pumpkins, sharing 69.1 to 69.5% nt and 64.1 to 65.5% aa sequence identities (Table 2). Sequence identities of >80% nt (90% aa) in the polyprotein and 83% nt (>91% aa) in the CP were observed between pumpkin MWMV isolates from this study and virus isolates from *Cucurbita pepo* in Burkina Faso (MN688647), Tunisia (EF579955), and South Africa (KU315176) (Table 2).

Analysis of the aligned complete genome nucleotide sequences using seven different algorithms showed no evidence of recombination involving pumpkin MWMV isolates (data not shown). Based on the full-length genome sequence, sequences of MWMV isolates from cucurbits were more diverse ($\pi = 0.14495$) than those from papaya ($\pi = 0.01939$). The MWMV isolates from papaya had lower numbers of mutations (927) compared with those recorded in MWMV isolates from cucurbits (3,809). The average number of nucleotide substitutions per site between MWMV isolates from cucurbits and those from papaya was 0.20398.

Phylogenetic inferences based on polyprotein nucleotide sequences of selected potyviruses revealed clustering of MWMV isolates from cucurbits in one group, whereas those from papaya formed into a separate cluster with strong bootstrap support of 100% (Fig. 2). As suggested by the phylogenetic analysis, MWMV from papaya and those from cucurbits shared a common ancestor as supported by the 100% bootstrap value. The MWMV isolates from pumpkin sequenced in this study clustered together in one subgroup, with the closest isolate being MG800832, previously sequenced from pumpkin in Kenya (Kidanemariam et al. 2019). The clustering patterns of MWMV sequences from cucurbits correlated well with their geographic origins, with isolates from South Africa (KU315175 and KU315176) forming a separate cluster, those from North Africa and Mediterranean region (EF579955, LN810061, and KY762266) forming another cluster, and those from and East and West Africa

Table 1. List of primers used in the detection for *Moroccan watermelon mosaic virus* isolates from pumpkin in Kenya

Primer[a]	Sequence (5′-3′)	Size (bp)	Target gene	Location (nt)[b]
MWMV18-F	TGCTGTTGGTAGTGGCAAAT	613	Cylindrical inclusions (CI)	4,016–4,035 (Ken-pump) 4,060–4,079 (Ken-Mer)
MWMV18-R	TTCTGTTCGCCCAACTTTCA			4,609–4,628 (Ken-pump) 4,653–4,672 (Ken-Mer)
MWMV20-F	AAACACAAGGGCCACTCAAA	455	Coat protein (CP)	8,969–8,988 (Ken-Pump) 9,013–9,032 (Ken-Mer)
MWMV20-R	ACAATCGAGTGTTTGCACCT			9,404–9,423 (Ken-pump) 9,448–9,467 (Ken-Mer)

[a] F, sense primer; R, antisense primer.
[b] The targeting nucleotide (nt) locations according to the complete genome sequence of MWMV MH713899 (isolate Ken-pump) and MT497462 (isolate Ken-Mer).

(MN688647, MG800832, MH713899, and MT497462) clustering in a different subgroup (Fig. 2).

Host range of MWMV isolates from pumpkin and papaya. The infectivity of the MWMV was determined using a pumpkin (MH595741) and a papaya (MH713899) isolate of the virus (Mumo et al. 2020). The pumpkin MWMV isolate systemically infected and induced symptoms in zucchini, watermelon, cucumber, and pumpkin plants after sap inoculation. In general, symptoms, which started appearing 12 days after inoculation, included mosaic, raised dark green patches, vein clearing, and leaf distortion. The isolate, however, did not induce symptoms when inoculated onto papaya plants (Fig. 3 and Table 3).

The papaya MWMV isolate, by contrast, induced typical viral symptoms on papaya plants similar to those observed in the field, ranging from vein clearing, mottling, ringspots on leaves, leaf distortion, shoe stringing, and water-soaked marks on the stem. The symptoms in the inoculated papaya started appearing 10 days after inoculation. The isolate also caused chlorotic spots on zucchini but did not induce any symptoms on pumpkin, watermelon, and cucumber plants (Fig. 3 and Table 3).

Table 2. Polyprotein and gene-specific nucleotide (nt) and amino acid (aa) sequence identities (%) within Moroccan watermelon mosaic virus (MWMV) isolates from pumpkin in Kenya (MH713899 and MT497462) and between them and global MWMV sequences

Genome segment[a]	MH713899 versus MT497462[b]		MH713899/ MT497462 versus MH595736–46[b]		MH713899/ MT497462 versus MG800832[b]		MH713899/ MT497462 versus MN688647[b]		MH713899/ MT497462 versus KU315176[b]		MH713899/ MT497462 versus EF579955[b]	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Polyprotein	99.4	99.3	79.7–79.9	89.2–89.7	87.9	94.4–94.5	81.4–81.5	89.2–89.3	80.6–80.8	90.8–90.9	80.4–80.5	90.3–90.4
P1	99	98.3	69.1–69.5	64.1–65.5	82.3–82.5	78.8–79.1	74.1–74.5	69.9	73.2–73.7	68.1–68.7	72.7–73.4	66.9–67.5
Hc-Pro	99.8	99.8	78.6–79.3	90.4–91.9	88	98.2–98.5	86.2	95.6	82.5–82.6	93.7	82.8–82.9	94.3
P3	99.4	99.1	81.8–82.5	88.2–89.3	88.1–88.2	92.2	80.7–80.9	88.3	82.0	89	80.1	86.7
6K1	100	100	82.6–83.2	92.3	89.0	92.2	81.9	98.1	83.9	92.3	81.3	92.3
CI	99.3	99.5	80.9–81.3	94.8–95.6	89.7–89.8	98	79.3–79.4	82.7	80.5–80.8	95.9	81.1	95.4
6K2	99.4	100	78.8–79.4	87.7–89.5	85.5–86.5	89.2	77.1–77.6	86.0	76.5–77.1	80.7	78.8–79.4	80.7
Vpg	98.6	97.9	78.7–79.6	86.8–87.9	86.5–87.2	93.1–95.3	83.7–85.1	95.8–97.9	79.8–81.2	91.6–93.7	79.6–80.6	91.1–93.2
Nia	99.7	100	79.2–79.9	92.3–93.2	88.5	96.6	83.8	96.6	79.5–80.0	94.1	81.6–81.8	94.6
Nib	99.5	99.6	82.3–82.7	94.2–94.9	87.6–88.1	97.9	84.2	95.3	82.5–82.6	96.1	81.1	95.7
CP	99.3	98.9	83.4–83.7	92.3–95.1	90.2–90.4	94.3–95.4	86.2	95.4–96.4	83.7	92.1–93.2	83.5	91.8–92.8

[a] HC-Pro, helper component-protease; CI, cylindrical inclusion; Vpg, viral genome-linked protein; Nia. nuclear inclusion A; Nib, nuclear inclusion B; CP, coat protein.

[b] MH713899 and MT497462 (pumpkin isolates sequenced in this study); MH595736–46 (papaya isolates from Kenya); MG800832 (pumpkin isolate from Kenya); MN688647 (squash isolate from Burkina Faso; KU315176 (squash isolate from South Africa); EF579955 (squash isolate from Tunisia).

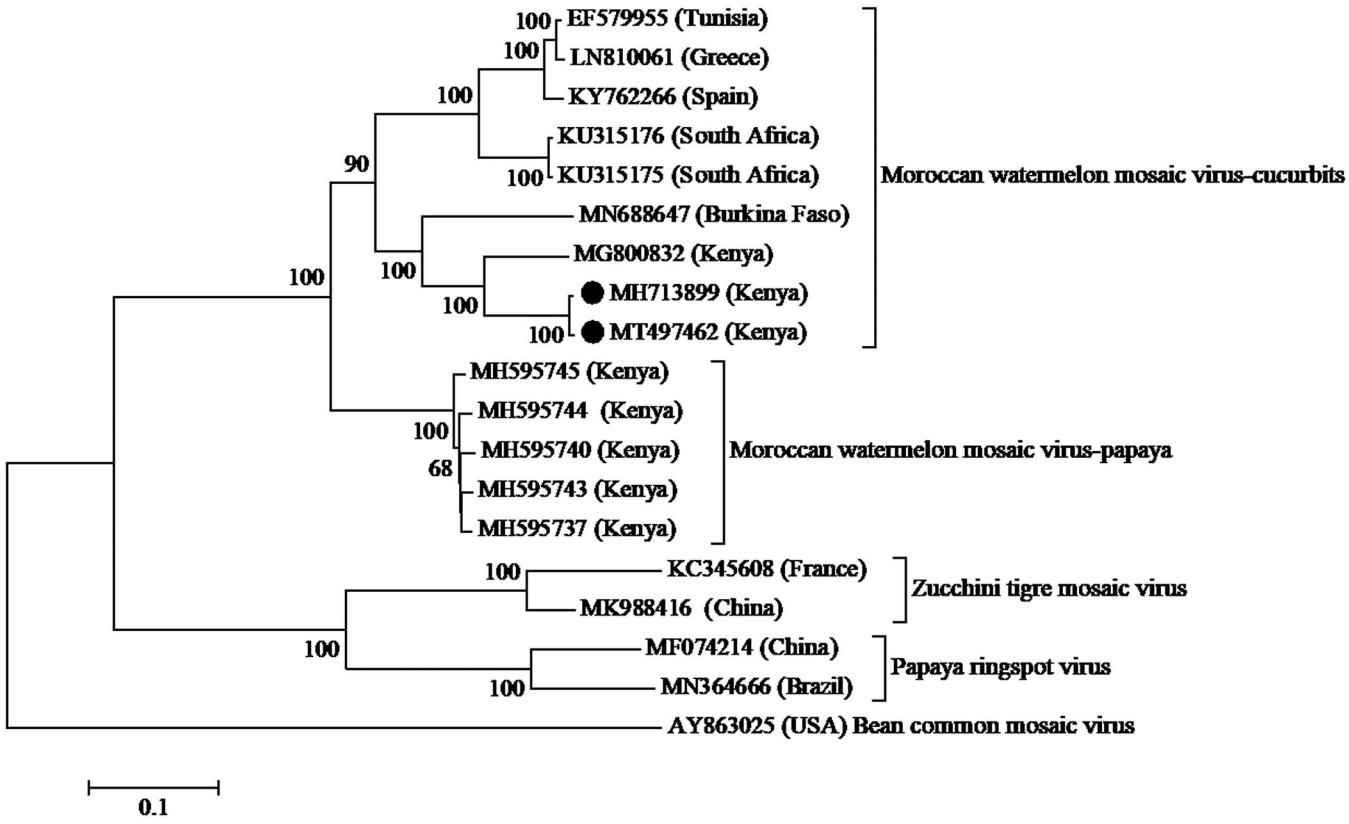

Fig. 2. Rooted phylogenetic tree depicting the evolutionary relationships among Moroccan watermelon mosaic virus (MWMV) isolates from papaya and cucurbits based on analyses of complete polyprotein nucleotide sequences of the virus and corresponding sequences of isolates of other potyviruses. The tree was generated in MEGA 6 (Tamura et al. 2013) using the maximum likelihood method based on JTT matrix-based model (Jones et al. 1992). The scale bar is given in number of nucleotide substitutions per site. Phylogeny was inferred after 1,000 bootstrap replications, and the node values show percentage bootstrap support. The isolates sequenced from pumpkin in this study are shown with black circles (●). Moroccan watermelon mosaic virus–cucurbits refers to MWMV isolates from cucurbits; Moroccan watermelon mosaic virus–papaya refers to MWMV isolates from papaya.

The presence or absence of the MWMV on the test plants was further confirmed through RT-PCR (Fig. 4). Pumpkin, watermelon, zucchini, and cucumber plants inoculated with the pumpkin MWMV isolate showed positive test results for the virus, but the papaya plants inoculated with the same isolate showed negative test results (Fig. 4). The symptomatic papaya and zucchini plants inoculated with a papaya MWMV isolate showed positive test results for the virus by RT-PCR, whereas pumpkin, watermelon, and cucumber plants inoculated with the same isolate showed negative test results (Fig. 4 and Table 3).

Discussion

Papaya in Kenya is grown widely by small scale farmers in mixed cropping systems (Asudi 2010; Rimberia and Wamocho 2014). Intercropping in general has many advantages, including efficient utilization of land resources, enhanced returns per unit area, and insurance against crop failure (Malézieux et al. 2009). The practice, however, may facilitate disease spread because intercrops can serve as alternate hosts or reservoirs of pathogens, a crucial role in the perpetuation of several diseases in different crop species (Ara et al. 2012; Martins et al. 2016; Ocimati et al. 2018).

In this study, MWMV was detected and characterized from field samples of pumpkin intercropped with papaya. The pumpkin MWMV isolate has a genome composition similar to those in previously reported MWMV isolates of papaya from Kenya (Mumo et al. 2020) and *Cucurbita pepo* from Tunisia (Yakoubi et al. 2008). The observed deletion of six nucleotides (two amino acids) in the N terminal region of the CP of the genome sequences of the pumpkin MWMV isolates is reminiscent of reported nucleotide deletions in

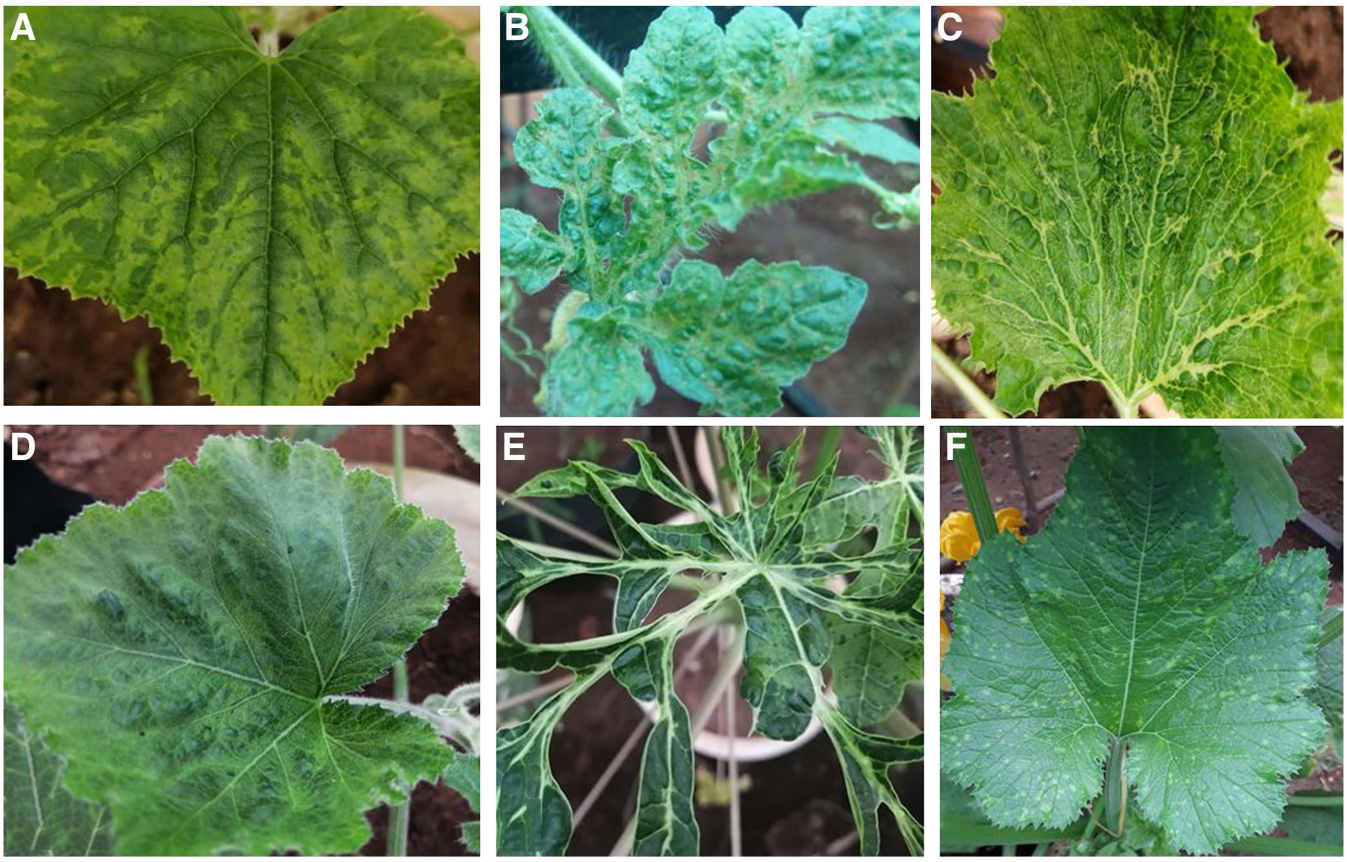

Fig. 3. Symptoms induced by Moroccan watermelon mosaic virus isolates from pumpkin and papaya in various test plants. **A to D,** Symptoms induced by the pumpkin isolate. **E and F,** Symptoms caused by the isolate from papaya. **A,** Mottling on cucumber. **B,** Puckering and leaf distortion on watermelon. **C,** Vein clearing, puckering, and leaf distortion on zucchini squash. **D,** Puckering and mosaic on pumpkin. **E,** Vein clearing, mottling, and leaf distortion on papaya. **F,** Chlorotic spots on zucchini squash.

Table 3. Reaction of several cucurbits and papaya plants to isolates of Moroccan watermelon mosaic virus obtained from pumpkin and papaya based on sap inoculations

Source of virus isolate	Test plants	Symptoms[a]	No. of plants showing symptoms/No. of plants inoculated	No. of plants infected/No. of plants tested (RT-PCR)
Pumpkin	Zucchini	LD, LC, PU, VC	10/10	2/2
	Pumpkin	LD, Mo, MO, PU	10/10	2/2
	Watermelon	PU, LD	10/10	2/2
	Cucumber	LD, Mo, PU	10/10	2/2
	Papaya	NS	0/10	0/10
Papaya	Zucchini	CS	10/10	2/2
	Pumpkin	NS	0/10	0/10
	Watermelon	NS	0/10	0/10
	Cucumber	NS	0/10	0/10
	Papaya	LD, RS, WS,VC, MO, SS	10/10	2/2

[a] Symptom description: CS, chlorotic spots; LD, leaf distortion; LC, leaf curl; ML, mottling; MO, mosaic; PU, puckering; RS, ringspots on the leaves; SS, shoestringing of leaves; WS, water-soaked marks on the stem/petioles; VC, vein clearing; NS, no symptoms. Results presented here were confirmed in a separate experiment.

the N terminal region of a snake cucumber (*C. melo* var flexuosus) MWMV isolate from Sudan (Lecoq et al. 2001). The biological significance of these CP nucleotide deletions is unknown and should be the subject of future studies.

Sap inoculation experiments showed the existence of two strains of MWMV associated with plant specificity. The MWMV strain infecting pumpkin systemically infected several cucurbits species but not papaya plants. By contrast, the strain infecting papaya infected both papaya and zucchini plants. Although in the experiment, insect transmission of the viruses was not evaluated to mimic the natural infection process, it is evident that MWMV host plant specificity exists. The MWMV can be transmitted mechanically or by aphids (Owolabi et al. 2012; Yakoubi et al. 2008). Aphid transmission, which is more efficient, occurs in a non-persistent manner (Yakoubi et al. 2008). In non-persistent transmission, aphids acquire and inoculate virus particles in the epidermal cells within a few seconds because virus particles bind rapidly but loosely to receptors within an aphid's probing mouthparts (stylet) and are released during salivation (Brault et al. 2010; Groen et al. 2017) Similar results in papaya ringspot virus (PRSV), a virus closely related to MWMV, have been reported, where, based on biological properties, there are two strains: PRSV-P, isolated from papaya, which infects several cucurbits, and PRSV-W, isolated from cucurbits, which is unable to infect papaya (Gonsalves 1998; Shukla and Ward 1988). The two PRSV biotypes cannot be distinguished on the basis of divergence in their CP sequences (Bateson et al. 2002; Ventura et al. 2004). For instance, Bateson et al. (2002), studying seven Australian isolates (four P-type and three W-type), found that they shared a high degree sequence homology in the CP gene, ranging from 98.1 to 98.9%. By contrast, Silva-Rosales et al. (2000), studying three Mexican P-type isolates from geographically close areas, observed a lower degree of nucleotide sequence homology, ranging from 93.4 to 98.4% at the CP. Sequences of MWMV infecting papaya and those of pumpkin could be distinguished based on sequence divergence in the CP.

Genomes of plant viruses in the genus *Potyvirus* encode large polyproteins that are cleaved by virus-encoded proteases into 10 mature proteins, namely, P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, Nib, and CP (Adams et al. 2005; Revers and García 2015). P1 is the most variable protein, both in size and sequence (Adams et al. 2005; Valli et al. 2007), and it is alleged that P1 diversification has contributed to the successful adaptation of potyviruses to a wide range of host species (Salvador et al. 2008; Valli et al. 2007). In this study we found that P1 was the least conserved protein between MWMV isolates of papaya and pumpkins in Kenya, sharing 69.1 to 69.5% nt and 64.1 to 65.5% aa sequence identities between them. Similar sequence divergence was observed between MWMV in pumpkins in Kenya and those in *Cucurbita pepo* from Burkina Faso, Tunisia, and South Africa. Therefore, whereas the pattern of P1 divergence between papaya and pumpkin isolates of MWMV in Kenya suggests a possible association with host specificity, this association is less clear when P1 sequences of global isolates of the virus are considered. Furthermore, recombination and mutations are main forces driving plant virus evolution and host adaptation (García-Arenal et al. 2003; Nagy 2008; Valli et al. 2007) and are common in potyviruses (Gell et al. 2015; Moradi et al. 2016; Padhi and Ramu 2011). No recombination was detected, possibly ruling out its involvement to the adaptation of MWMV to either papaya or pumpkin. Analysis of the whole genome sequences of MWMV population from different regions of Africa and the Mediterranean showed that the level of nucleotide diversity and number of mutations were lower in MWMV isolates from papaya than in isolates from cucurbits. Although MWMV genomes from papaya are available only from Kenya, the highest diversity and mutations observed within MWMV isolates from cucurbits suggest that movement of the virus around the world in cucurbits and then mutation to infect papaya could be a factor in the molecular epidemiology of MWMV.

Phylogenetic inferences among the polyprotein regions showed that MWMV isolates from papaya and those from cucurbits are strains of the same virus, sharing a common ancestor. MWMV infects several cucurbit species and has a wide distribution in Africa and the Mediterranean region (Kidanemariam et al. 2019; Lecoq et al. 2001; Owolabi et al. 2012; Yakoubi et al. 2008), and this probably implies that different cucurbit hosts together with local and long-distance movement of the virus may have resulted in variability within MWMV populations. Furthermore, MWMV infection in cucurbits was reported more than three decades (Fischer and Lockhart 1974) before that in papaya (Arocha et al. 2008), indicating that the papaya MWMV isolate might have originated from cucurbit-infecting isolates from where host speciation occurred.

From the results of this study, it is evident that the MWMV isolated from papaya and the MWMV isolated from pumpkin in Kenya are naturally adapted to papaya and pumpkin, respectively. The MWMV isolated from papaya can be transmitted to zucchini, not to other cucurbits, through sap inoculation. Given that the MWMV strain infecting papaya could infect zucchini, this represents a potential inoculum source when papaya and zucchini are intercropped. Furthermore, in the future, more practical questions need to be evaluated, including: 1) Can aphids spread the virus from papaya to cucurbits and then from cucurbits back to papaya? 2) Can they also spread the virus from cucurbits to papaya and then from papaya back to cucurbits? 3) Are there differences in the transmission efficiencies of the virus? Answering these questions will be an important step in the development of appropriate MWMV management strategies for both papaya and cucurbitaceous crops.

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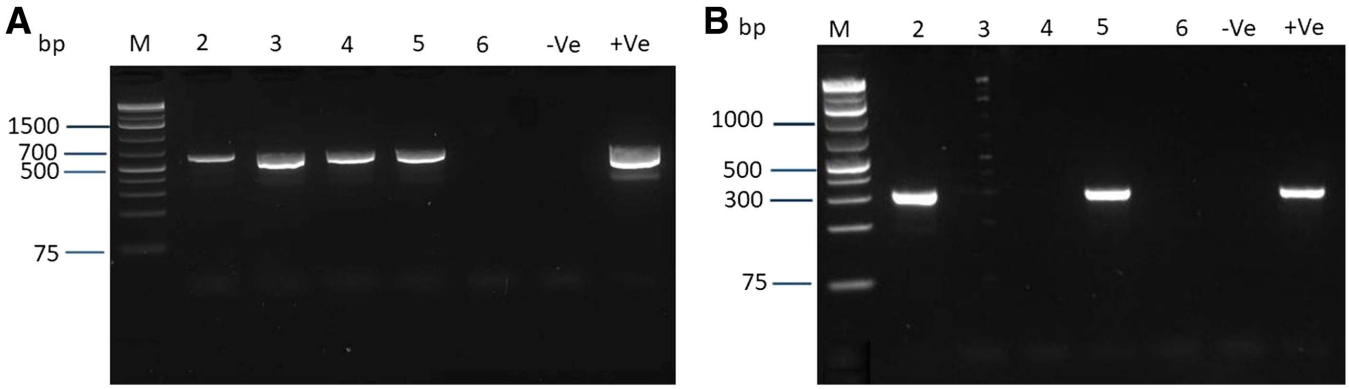

Fig. 4. Agarose gel electrophoresis of PCR products for diagnosis of Moroccan watermelon mosaic virus (MWMV) infections on test plants. **A,** A band at 615 bp shows positive detections after sap inoculation of a pumpkin MWMV isolate onto pumpkin (lane 2), zucchini squash (lane 3), watermelon (lane 4), cucumber (lane 5), and no band for papaya (lane 6). **B,** A band at 315 bp shows positive reactions for papaya (lane 2) and zucchini squash (lane 5), whereas no bands were obtained for pumpkin (lane 3), cucumber (lane 4), and watermelon (6) after sap inoculation of each plant with a papaya MWMV isolate. M indicates the O'GeneRuler 1 kb plus DNA ladder. +Ve is positive control, -Ve is negative control.

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