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Molecular analysis of maize (*Zea mays* L.)-infecting mastreviruses in Ethiopia reveals marked diversity of virus genomes and a novel species

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Abstract

Maize (*Zea mays* L.) is host for more than 50 virus species worldwide with *Maize streak virus* (MSV) (genus *Mastrevirus)* causing significant yield losses in Africa. A survey for viruses infecting maize was conducted in major growing regions of Ethiopia. To test for DNA viruses, in particular mastreviruses, rolling circle amplification was performed for the analysis of virus composition in assayed samples. Following the analysis of the entire virus genomes, three genetic groups, each representing distinct virus species, were identified. The first group was almost identical with the A-strain of MSV. The next sequence-cluster shared 96–98% identity with isolates of *Maize streak reunion virus* (MSRV) confirming the presence of this virus also in continental East Africa. Sequence analysis of additional virus genomes (each 2846 nt) in length revealed only a limited 70–71% nt identity with MSRV isolates and an even lower identity (< 64%) with sequences of mastreviruses described elsewhere. Our analysis suggests a novel virus species, which is tentatively named maize streak dwarfing virus (MSDV). The pairwise comparison of capsid protein and replication-associated protein (Rep) of the novel species revealed a limited identity of 63% and 68% with the respective protein sequences of MSRV. The incidence of the virus species in the maize regions of Ethiopia was studied across 89 samples collected during four growing seasons. PCR analysis with general and specific mastrevirus primers showed that MSV is the most incident virus (39.3%) followed by MSRV (14.6%) and MSDV (12.4%). Identification of three different mastrevirus species in a confined geographical location on the same host, maize, is unprecedented, and suggests that Ethiopia may be one of the potential hot spots for diversity of maize-infecting mastreviruses.

Keywords Diversity · Incidence · Maize · Mastrevirus · Screening

Introduction

Maize (*Zea mays* L.) is the major staple food crop in Sub-Saharan African countries and the leading cereal crop in terms of production in Ethiopia [1]. Its production, however, is constrained by biotic and abiotic factors with virus diseases contributing to significant yield losses. More than 50 virus species can infect maize [2] and among these, *Maize streak virus* (MSV), genus *Mastrevirus* in the family *Geminiviridae* is one of the top ten economically important plant viruses [3].

Geminiviridae have circular single-stranded (ss) DNA genomes and replicate in the nuclei of their host plants via Rolling Circle Replication (RCR) [4]. Geminiviruses fall into viruses with monopartite (one circular ssDNA genome component) or bipartite genomes components (two circular ssDNA genomes component with similar size) [5]. Virus species in the genus *Mastrevirus* have monopartite genomes

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of approximately 2700 nt encapsidated in geminate particles encoding four proteins separated by two intergenic regions (large intergenic region [LIR] and small intergenic region [SIR]). The viron sense strand encodes two proteins; the movement protein (MP), functioning in cell to cell movement, and the coat protein (CP), which encapsidates the virion sense ssDNA and acts as the nuclear shuttle protein (NSP) for viral DNA. The complementary sense strand encodes the replication-associated proteins Rep and Rep A [5].

Virus species in the genus *Mastrevirus* are identified and discriminated by their genome sequences with a nt threshold of < 78% identical sequences to other viruses indicating for distinct virus species and < 94% identical genome sequences considered as strain demarcation threshold [6]. Three *Mastrevirus* species have been reported in maize. MSV and *Maize streak reunion virus* (MSRV) [7] are typical African viruses; the recently described *Maize striate mosaic virus* (MSMV) is present in Brazil [8]. MSV is the most prevalent maize virus in Africa and impacts maize production in Sub-Saharan Africa [9]. Eleven strains of MSV have been described [10] with MSV-A strain being most widely distributed in maize production regions of Africa [11]. MSRV only recently described in 2012 from La Réunion Island [7] and later also reported from uncultivated grass species, *Setaria barbata* and *Rottboellia* sp. from Nigeria [12] and in addition from maize in China [13] indicating its wider geographical distribution and host range. In Ethiopia, a range of maize viruses have been described including MSV [14, 15], *Maize chlorotic mottle virus* (MCMV) and *Sugar cane mosaic virus* (SCMV) [16–18] and *Maize yellow mosaic virus* [19]. Limited research has been done on characterizing mastreviruses in Ethiopia with only partial nucleotide sequence available at GenBank for an Ethiopian MSV isolate (X71956, [20]). This study was designed to identify and analyze the genetic diversity of mastreviruses in major maize growing regions of the country.

Materials and methods

Sample collection

Maize samples were collected in major maize growing regions of Ethiopia including Amhara, Benishangul-Gumuz, Oromia, South Nations, Nationalities and Peoples (SNNP) and Tigray (Table S1). Samples with symptoms indicating for maize streak disease, broken or parallel streaks, vein clearing, fine streaks, and dwarfing were collected and dried over anhydrous calcium chloride. These samples were collected in four survey rounds in August 2015, in July 2016, in November, 2016 and in March 2017.

Detection and analysis of full-length maize mastrevirus sequences by RCA

Total DNA was extracted from each of 89 samples, ~ 20 mg dried maize leaf samples using the Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

For a subset of DNA extracts, an aliquot was subjected to rolling circle amplification (RCA) using Phi29 DNA polymerase (TempliPhi™, GE Healthcare, Germany) essentially as described before [21, 22]. Double-stranded (ds) DNA RCA products were digested with *Bam*HI, *Sph*I or *Pst*I restriction enzymes (New England Biolabs) and separated by electrophoresis. Fragment sizes of about 2.8 kb, indicating for genome size DNA were excised from 1% agarose gels, ligated in a pUC19 vector and subsequently transformed into electrocompetent DH5α cells. Plasmids were extracted from overnight cultures. Recombinant clones were identified by restriction digestion and sequenced bi-directionally at least twice using the universal M13 primers followed by primer walking using the sequencing service at Helmholtz Centre for Infection Research, Genomanalytik, GMAK, Germany. Sequences were edited and assembled using Geneious 10.2.3 (Biomatters LTD, NZ). For sequence confirmation, a pair of abutting primers (F-5′-GAGGATCCCTAGTTGTAATAG GACG-3′ and R-5′-AGGGATCCTCTGTTTTTTGACATG C-3′) was designed for novel genome sequences to amplify the circular genome from DNA extracts. The PCR was made in a 20 µl of final volume containing 10 µl of Phusion Flash Mastermix (Thermo Fisher Scientific), 0.5 µl from each of the forward or reverse primers, 2 µl DNA (approximately 100 ng/µl diluted) and 7 µl nuclease free water. Thermal cycling was programed for 35 cycles of 98 °C for 10 S, 56 °C for 20 S and 72 °C for 3 min preceded by 98 °C of complete denaturation for 30 S. The final extension was adjusted at 72 °C for 5 min.

Phylogenetic relationship

A phylogenetic analysis was conducted with the genome sequences generated and also including mastreviruses reference genomes. Additionally, sequences of geminivirus type species of the genera *Curtovirus* (NC_001412), *Topocuvirus* (NC_003825) and *Begomovirus* (NC_001439) were chosen as out groups to generate a phylogenetic tree. Clustal alignment was done using the online Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) to further subject aligned sequences to MEGA 7.0.26 using the Maximum Likelihood method [23]. Bootstrap with 1000 resampling was applied to generate a phylogenetic

tree. MUSCLE alignment of the sequence demarcation tool (SDTv1.2) [24] was used to generate a color coded matrix from pairwise similarity calculations. The Clustal aligned sequences were scanned with recombination detection program (RDP) 4.39 [25] for possible recombination of novel genome sequences.

Screening for presence of maize mastrevirus

To study the distribution of the three identified mastrevirus and identify any other possible maize-infecting mastrevirus in our samples, primers were designed for universal amplification and specific detection of mastreviruses. An initial universal confirmation for presence of mastreviruses was followed by specific amplification of virus sequences to identify and discriminate species. All the DNA extracts of each sample were diluted to 100 ng/µl prior to PCR amplifications.

A pair of designed general primer (Gen F-5′-GGNGTNARRACBSAGTGGAAGAA-3′ and Gen R-5′-GARGAYTGGHTGAAGGWNATGACTCC-3′) flanking 442 bp spanning the CP coding region of the genome of mastreviruses was used to screen 89 samples collected in different years of survey from major maize regions of Ethiopia (Table S1). The PCR conditions were set for 35 cycles of 94 °C for 10 s, 52 °C for 40 s, and 72 °C for 30 s preceded by 94 °C pre-heating for 5 min and with a final extension at 72 °C for 5 min. Then all the positive samples were separately screened with the three mastrevirus-specific primers (for MSV F-5′-AGCTGATATTTGGAGGACAAGCT-3′ and R-5′-AGCTTGTCCGCCGGGAGT-3′; MSRV F-5′-CCG GCTCCTGATGTACCGG-3′ and R- 5′-GAAACAAAA ACGCAGGTAAGGG-3′; MSDV F-5′-GTGGACAGCAAT TATTAAAGGCA-3′ and R-5′-CCTTTTCGGACGCCTATC TCC-3′). For these primers, the annealing temperature was raised to 57 °C. The regions which were amplified by the primer pairs were 472 bp for MSV, 576 bp for MSRV and 623 bp for MSDV.

Results

Detection and reconstruction of mastreviruses genomes

Ten clones with DNA fragments of sizes similar to full-length genome sequences were sequenced. Annotation of the sequences revealed typical mastrevirus genes, LIR, SIR and the typical nonanucleotide sequence [6]. BLASTN analysis [26] of these sequences resulted in three distinct species of mastrevirus.

Phylogenetic analysis

Phylogenetic analysis of the genome sequences and further 50 reference sequences retrieved from GenBank grouped the virus sequences of this study into three distinct clusters. The first cluster of four clones (MK329306 to MK329309) showed high nt identity with A-strain of MSV (NC_001346) (Fig. S1). These sequences shared a 98–100% identity and similarly were 97–98% identical to the reference sequence of MSV-A. An 80–90% identity with other strains of MSV (MSV-B to MSV-K) and a 56–70% identity with other species confirmed these sequences as MSV-A virus isolates (Fig. S1).

The second sequence cluster comprising two virus sequences (MK329304 and MK329305) was highly identical (96%) with a MSRV genome sequence (NC_017917) indicating that MSRV is present in Ethiopian maize.

Four sequences of virus DNA clones (MK329300 to MK329303) shared 99–100% sequence identity, however only distant sequence relationships were found with other mastreviruses. From BLASTN analysis, we observed that these isolates shared a maximum of 71% identity with Yunnan isolate (KT717933) of MSRV, and similarly, only a 70–71% identity with all the available sequences of MSRV. A pairwise comparison of CP and Rep amino acid sequences showed maximum identities of 63% and 68%, respectively, with isolates of MSRV (data not shown).

The limited (< 78% identical nt) genome identities recorded for these sequences forming a new clade well separated from MSRV (Fig. 1) fulfill the species demarcation criteria proposed by [10] and thus can be considered as distinct mastrevirus species. We also observed streak and dwarfing symptoms during the field survey (Fig. 2a), and this novel virus species is provisionally named as maize streak dwarfing virus (MSDV), therefore.

Three isolates of MSDV have a genome size of 2846 nt (the fourth one 2844 nt). These sequences are distantly related with MSV and MSRV genomes and have typical mastrevirus genome organization. Only the conserved nonanucleotide sequence TAATATT↓AC is diverse in MSDV with nucleotide mutations at the fourth and the eighth positions (underlined, TAACATT↓GC) of this most conserved sequence in geminiviruses. Interestingly this unusual feature is also evident in *Sweet potato symptomless virus* 1 (SPSMV-1) with a nonanucleotide mutation at positions four and eight resulting in TAAGATT↓CC motif [27]. Scanning of aligned sequences with recombination detection program (RDP) 4.39 [25] for any possible recombination failed to reveal any evidence that MSDV is recombinant.

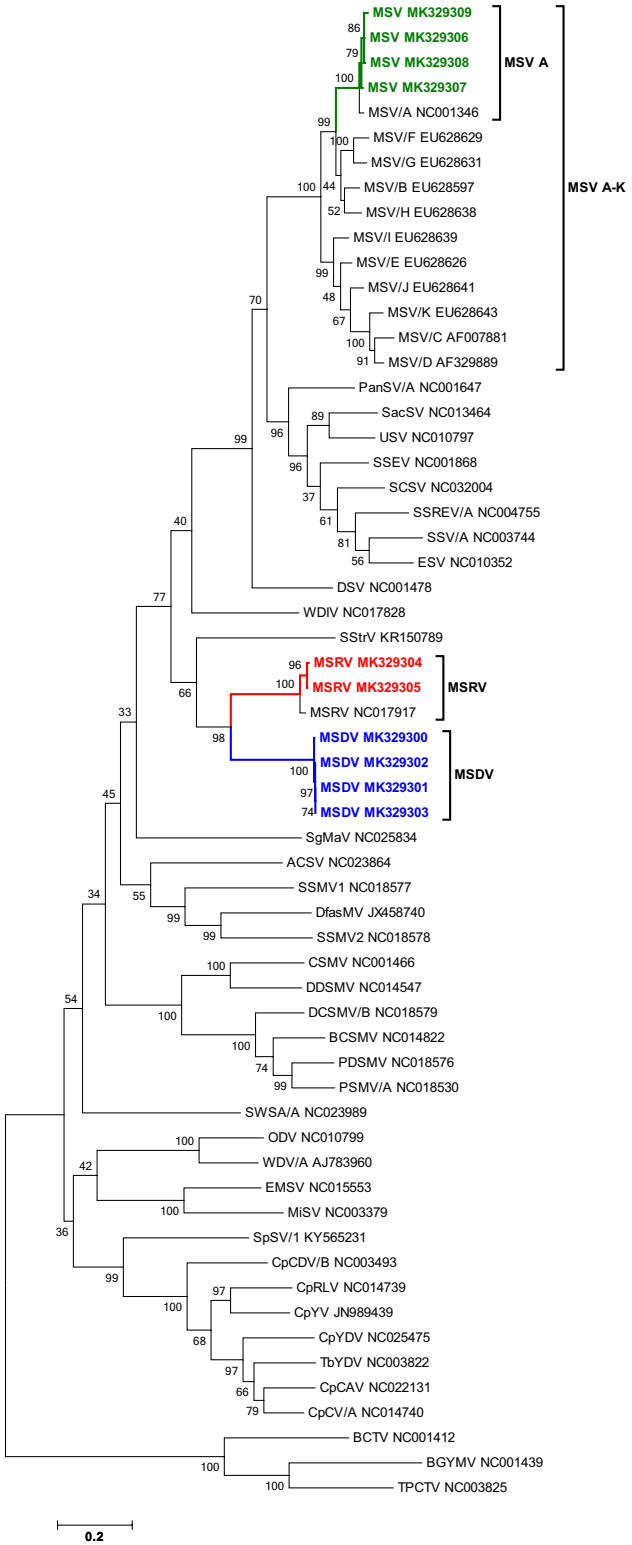

Fig. 1 Phylogenetic tree generated by the Maximum Likelihood method of MEGA 7.0. based on full genome sequence alignments of reference sequences in the genus *Mastrevirus*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Ten isolates determined in this study and 50 isolates (47 mastrevirus and three out groups representing type species from three genera of the *Geminiviridae* family) retrieved from the GenBank were used in the analysis. In those species with more than one strain, only the type species reference sequence was used except the MSV strains. Isolates determined in this study are indicated in colored bold fonts. Species/Strain names and GenBank accession numbers are shown in the phylogenetic tree for each isolate

Distribution and incidence of mastreviruses infecting maize in Ethiopia

Eighty-nine samples collected from August 2015 to March 2017 in major maize growing regions of Ethiopia, Amhara (10), Benishangul-Gumuz (11), Oromia (45), South Nations, Nationalities and peoples (SNNP) (12), and Tigray (11) were subjected to PCR screening.

Of the 89 DNA aliquots screened with general mastrevirus primers, 50 of them were positive. A further screening of these DNA samples with specific primers for MSV, MSRV, and MSDV revealed 35 MSV, 13 MSRV, and 11 MSDV sequences. Viruses were also found in mixed, double, as well as triple infections (Table 1).

The most widely distributed virus, MSV was found in all surveyed regions. MSRV and MSDV were found in samples from Benishangul-Gumuze, Tigray, SNNP and Oromia regions. None of the samples from Amhara region were positive for MSRV and MSDV.

As revealed from PCR results, both MSV (100%) and MSRV (36%) have higher incidence in samples collected during September 2015 (late main rain feed cropping season) but MSDV (15%) was higher in samples collected during July 2016 (middle of main rain feed cropping season). On the other hand, the lower incidence was recorded for MSV (10%) during November 2016 (beginning of dry season, irrigation) and for MSRV and MSDV both 10% during March 2017 (middle of dry season).

When maize plants with clearly identified virus infections were examined for disease phenotypes, there were obvious differences in symptoms from MSV, MSRV and MSDV infections. Seedlings infected with MSV showed clear broken or parallel white streak symptoms (Fig. 2c). Infections with MSRV resulted in milder and less-pronounced yellowish strip symptoms (Fig. 2b) while seedlings infected with MSDV, the newly identified species, were stunted with fine continuous or broken streaks across the leaf lamella and thickening of midveins (Fig. 2a).

Discussion

In this study, streak diseases of maize from Ethiopia were subjected to molecular analysis to identify viruses associated with the disease and characterize their genomes.

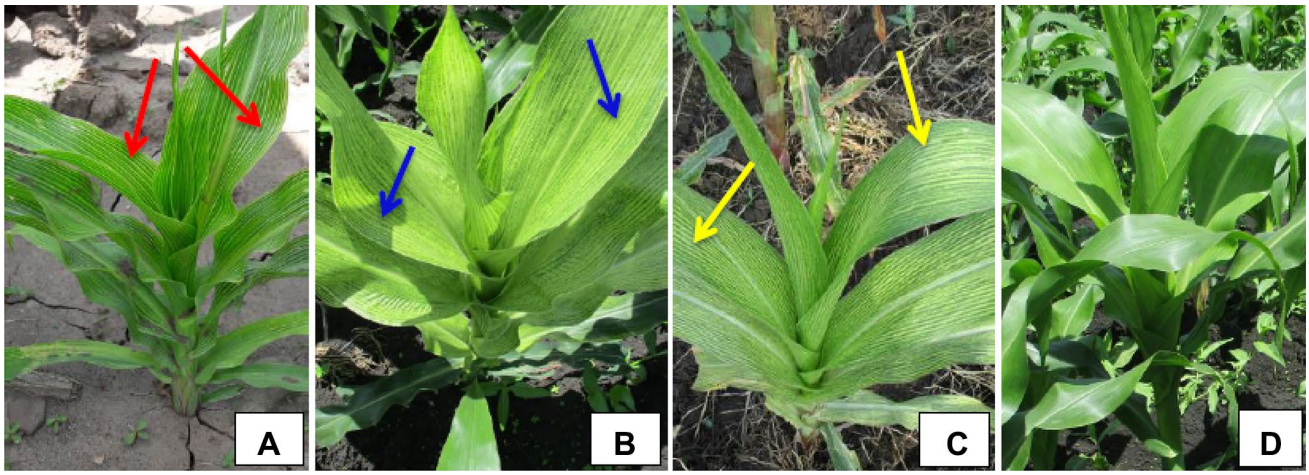

Fig. 2 Symptoms developed by maize seedlings as a result of single infection with MSDV (**a**), MSRV (**b**), MSV (**c**) and negative for the three viruses (**d**) as observed during field survey. Symptoms developed as a result of infections by each virus species are indicated by arrows

The widely distributed MSV-A strain of MSV was also the most prevalent virus in the fields across all regions. As a new disease for Ethiopia, we present the occurrence of MSRV and, in addition, we provide evidence for the presence of a new, hitherto unknown mastrevirus species, tentatively named maize streak dwarfing virus (MSDV). The mastreviruses were found at least in some cases, in sympatry also causing mixed infections in the same host. As this paper is only the first comprehensive report, according to the best of our knowledge, describing different species of viruses causing maize streak disease, we present further investigation on the biology of the viruses to support the assessment on disease management options of a new disease situation.

The identification of MSV from maize in Ethiopia dates back to 1990s, and there is only limited genome sequence information available to date [20]. There is a high degree of sequence identity between the MSV-A isolates determined in our study and that of other reference sequences, indicating highly conserved genomes of MSV-A isolates having a broad geographical distribution [2, 28] and diverse hosts [6, 12, 29].

MSRV after its first report from maize in La Reunion Island [7], was also reported from two uncultivated grass species from Nigeria [12] and from maize in China [13]. Here we report the occurrence of MSRV from Ethiopia and while this is a virus new to maize in Ethiopia, pending biological evidence, it is too early to speculate on impact of this finding to maize cultivation.

Similarly, the new virus tentatively named MSDV is characterized by a divergent and unique genome and symptoms distinct from MSV or MSRV. While present in the region to almost similar extent like that of MSRV, there is no biological evidence to describe its impact on maize, consequences for epidemiology and even more, its origin and fate in the environment.

Interestingly, a unique new mastrevirus species, MSMV has been described from maize in Brazil [8], a virus only distantly related to MSDV (< 61% nt identity). It shows that new viruses appear to continuously emerge thus virus surveys and monitoring is required to keep abreast of new situations.

Although comprehensive surveys were made in other East African countries bordering Ethiopia [30], only A-strain of MSV has been identified. This report shall, therefore, ignite the initiative to assess mastrevirus and other virus species harbored by cereal crops as well as other cultivated and uncultivated grass species in Ethiopia.

Table 1 Incidence and distribution of PCR-screened samples in five major maize-growing regions of Ethiopia

No.	Region	Year of collection	No. Samples	General primer	MSV (+)	MSRV (+)	MSDV (+)	MSV + MSRV(+)	MSV + MSDV(+)	MSRV + MSDV (+)	MSV + MSRV + MSDV (+)
1	Amhara	March 2017	10	1	1						
2	Benishangul-Gumuz	September 2015	7	7	7	3	1	3	1	1	1
		July 2016	4	4	2	2					
3	Oromia	September 2015	4	4	4	1		1			
		July 2016	31	12	6		6				
		November 2016	10	4	1	3	1	1			
4	SNNP	July 2016	12	11	11	2	1	2	1		
5	Tigray	March 2017	11	7	3	2	2				
		Total	89	50 (56%)	35 (39%)	13 (15%)	11 (12%)	7 (8%)	2 (2%)	1 (1%)	1 (1%)

SNNP South Nations, Nationalities and People

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Author contributions DG, AA, KT, SW, and DK conceived and designed the study. DG, AA, and KT collected the samples. DG and DK carried out the experiments. DG and DK performed the data analysis. DG wrote the first draft. AA, KT, SW, and DK reviewed and edited the manuscript. All the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All authors (D. Guadie, K. Tesfaye, D. Knierim, S. Winter, A. Abraham) declare that they have no conflict of interest.

Ethical approval This article does not contain any study with human participants or animals performed by any of the authors.

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