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Serological and Molecular Characterization Detects Unique Rice Yellow Mottle Virus Strains in Kenya

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Abstract: Rice (*Oryza sativa* L.) is an economically important food crop in western Kenya but its production remains very low due to abiotic and biotic constraints. Rice yellow mottle disease (RYMD) caused by *Rice yellow mottle virus* (RYMV, genus: *Sobemovirus*) can cause up to 100% yield loss. This study characterized and determined the genetic diversity of RYMV strains in the ten isolates collected in February 2020 from both symptomatic and asymptomatic plants in western Kenya. The samples from the two rice fields A and B had two major common rice varieties of IR; field A (IR 2793) and field B (IR 2793-8-1). Total RNA was extracted using GeneJET Plant RNA Purification Mini Kit followed by RT-PCR using RYMV CP specific primers. The PCR products were sequenced by Sanger sequencing technology. Phylogenetic analysis was done by MEGA X. RYMV presence was confirmed serologically by DAS-ELISA. Isolates Ke_A1, KeB3 and KeB4 are novel sequences. KeB3 and KeB4 isolates clustered uniquely from the other Kenyan sequences depicting new evolutionary diversity. KeB3 and KeB4 shared 99% sequence identity with Ke105 and Ke101, while isolate Ke_A1 shared 98% identity with Ke101 and 99% with Ke105. Phylogenetic analysis of the CP gene sequences revealed that the isolates from Uganda (Ug), Kenya (Ke) and Tanzania (Tz) clustered together by country, implying that there was a possibility of single introduction of the RYMV CP gene in the three East African (EA) countries once, before further local viral diversity occurred in each country with new recombinations for further research.

Keywords: Genetic Diversity, Novel Sequences, *Rice yellow mottle virus*, Western Kenya

1. Introduction

Rice (*Oryza sativa* L.) is the most important staple food crop worldwide. The rice grain is rich in dietary starch and protein, although it does not contain all the essential amino acids. Rice is a monocot plant of the order *Oryzeae*, family *Poaceae*, and genus *Oryza*. Cultivated rice belongs to two species, *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice). In Kenya, rice is the third staple food crop after maize and wheat. It is grown by small scale farmers as a cash crop and food crop under government owned schemes like Mwea Tebere, Bunyala and Ahero irrigation schemes [1]. Rice cultivated on schemes accounts for about 95% of total domestic production. The remaining 5% is grown under rain-fed conditions in coastal Kenya areas of Kilifi, Kwale, Tana River and in some parts of western Kenya like Bungoma, Busia and Kakamega. Rice (*Oryza sativa* L.) cultivation in western Kenya is one of the food baskets of the country for about 1/3 of the country's population. About 80% of the rice grown in Kenya is from irrigation schemes established by government while the remaining 20% is produced under rain-fed conditions. Rice consumption in Kenya has gone up (300,000 ton/year) while production rates are not commensurate and stands at 110,000 ton/year, catering for only 32% of the rice demand in Kenya [2]. Inability of Kenya

to reach self-sufficiency in rice production is as a result of rice industry facing several constraints, amongst them Rice Yellow Mottle Disease (RYMD). In western Kenya, a survey done in 2012 reported RYMD incidences ranging between 1-40% and can lead to yield loss of up to 100% [2]. There is urgent need to address RYMD genomic diversity and distribution to facilitate breeding for resistant cultivars, and eliminate the trend of over-reliance on imports to meet the ever increasing demand for rice. There is limited information on *Rice yellow mottle virus* (RYMV) strains in western Kenya causing high RYMD incidence and severity. Molecular characterization of RYMV in isolates of western Kenya, will help unravel the unknown strains that will help understand the extent of recombination in RYMV genomic diversity and evolutionary biology for pathological plant breeding.

The RYMV occurrence is restricted only to Africa [3] and its offshore islands of Madagascar [4]. The survey done in neighbouring Uganda in September 2009 to determine the incidence and severity of RYMD, established that RYMD incidence and severity were high with mean values of 72% and 2.3 respectively. The S4 strain of RYMV was the most prevalent with narrow serological and biological variability [5]. In Tanzania, the survey done indicated RYMD incidence of 70% which positively correlated with the vectors (*Chaetocnema* sp. and *Oxya hyla*) suggesting that these vectors are responsible for the new spread of RYMV [6]. Similar additional surveys and molecular analysis revealed a co-existence within the same field of S4, S5 and S6 strains of RYMV in Eastern African region [6]. In Kenya, a survey done in 2012 in western Kenya, reported RYMD incidences ranging from 1-40% [7, 8]. This study unravels RYMV novel genetic diversity as a strong signal to identify the evolutionary mutation rate of the virus and be statistically tested in order to avoid spurious results. This will help in changing rice cultivation in the mainland Africa through molecular genetic breeding for resistance/tolerance. There are six distinct variant strains of RYMV that causes the disease [9], namely; S1, S2 and S3 found in West and Central Africa while S4, S5 and S6 are majorly found in East Africa [10]. The genetic diversity of RYMV in western Kenya isolates is inadequately documented because RYMV is a single-stranded positive-sense RNA species (genus: *Sobemovirus*; family: *Solemoviridae*) with a ca. 4,450-nucleotide-long genome organized into five open reading frames (ORFs) [11]. ORF1 is located at the 5' end of the genome, it encodes a small protein (P1) involved in virus movement and in gene silencing suppression. The ORF2 encodes the central poly-protein that has two overlapping ORFs. ORF2a encodes a serine protease and a viral genome-linked protein that determines the virulence. ORF2b is translated through a ribosomal frame shift mechanism as a fusion protein which encodes an RNA-dependent-RNA-polymerase (RdRp) gene. ORF4 is translated from a sub-genomic RNA at the 3' end of the genome and encodes the coat protein (CP) gene. Research by [12] reported the presence of a fifth ORF (ORFx) conserved in all *Sobemoviruses* which overlaps the 5' end of the ORF2a in the þ2 reading frame. According to [13], there is no sufficient evidence of intra-ORF4 recombination with the exception of a localized strain recently found in western Kenya. The sequence diversity data from this research will help unravel the evolutionary divergence of RYMV RNA genomic strains, helpful in Pathogen-Derived Resistance (PDR) breeding through recombinant DNA technologies and germplasm biofortification.

2. Materials and Methods

2.1. Sample Collection

This study focused on visual symptoms of RYMV in western Kenya. The disease diagnostic survey to determine RYMD occurrence was conducted in two rice fields (A and B) within the Ahero rice irrigation scheme representing the Lower Midland 3 (LM 3) Agro-Ecological Zone (AEZ) in Kisumu County, managed by the government through National Irrigation Board (NIB). The samples from the two rice fields A and B had two major common rice varieties of IR susceptible to RYMV, field A (IR 2793 variety) and field B (IR 2793-8-1 variety). The popular IR rice variety that is susceptible to RYMV was collected from field A (IR 2793 rice type) and from field B (IR 2793-8-1 rice type). The leafy symptomatic and asymptomatic samples were collected in February 2020 from the farmers' fields in a cool box and taken to the laboratory for serological and molecular diagnostics.

2.2. Serological Detection

Serological diagnostics was done using the Double Antibody-Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) protocol according to [14]. Concentration of the virus in the infected leaf was approximated with reference to standardized optical density (OD) of a positive isolate from the Institut de Recherche pour le Développement (IRD) screenhouse. The antibody used was non-discriminatory and worked for all the isolates. An ELISA microplate reader was used to detect the OD values at 405 nm after 2 hours. The isolates that were considered positive were those with OD values of more than twice the value of the negative control [15].

2.3. Molecular Diagnostics

A total of seven isolates from the ELISA samples (Plate 1) were selected to investigate the diversity of RYMV strains. Total RNA was extracted using the GeneJET Plant Purification Mini Kit (Thermo Scientific) according to the manufacturer's protocol. The leaf tissue was homogenized in liquid nitrogen and in lysis buffer provided in the kit. The RYMV F III 5'-CAAAGATGGCCAGGAA-3' and RYMV R M5'-CgCTCAACATCCTTTTCAgggTAg-3' primers at 10 µM were used in transcription and amplification of the targeted Coat Protein (CP) gene [14]. The sense primers were also utilized in the Reverse Transcriptase-Polymerase Chain

Reaction (RT-PCR) to transcribe and amplify genome fragments within the coat protein (CP) gene (nt 3447 to 4166) [14]. Amplification of the two-step RT-PCR products was done using the 2 RYMV specific primers (primer 3' II M 5' III) under the following conditions; Denaturation at 94°C for 5 minutes followed by 30 cycles at 94°C for 1 minute, Hybridization at 55°C for 30 seconds, Elongation at 72°C for 1 minute and final extension at 72°C for 10 minutes. The resultant reaction mixture was stored at 4°C. The PCR products were loaded and visualized in 1% Agarose gel electrophoresis stained with Ethidium Bromide in a X0.5 Tris Acetate EDTA buffer at 100V for 30 minutes to determine the DNA size through visualization under UV light.

2.4. Sanger Sequencing of the Coat Protein (CP) Gene

Sanger sequencing of the coat protein (CP) gene PCR amplicons was done with the Taq terminator sequencing Kit (Applied Biosystems) and followed by analysis of CP sequences on an Applied Biosystem 373A sequencer [14, 16]. Two readings per base (3' to 5' and 5' to 3' directions) led to sequence accuracy of 99.9%. Assembling of sequences was done by Seqman (DNASTAR) and sequence analysis was done by MEGA X software. The sequences of the CP gene with 1000 bp (Figure 2) isolates were compared with reference strains in the GenBank and they were useful in determination of intra-strain diversity.

3. Results

3.1. Serological Detection of RYMV

Seven samples 1, 3, 5, 7, 8, 9 and 10 representing A1, A3, A5, B2, B3, B4 and B5 tested positive for RYMV (Figure 1). Sample 4 (A4) was a healthy leaf sample from western Kenya while sample 6 (B1) was negative control from IRD and sample 9 (B4) was positive control. These results are epidemiologically explained in the viral titre reads (Table 1).

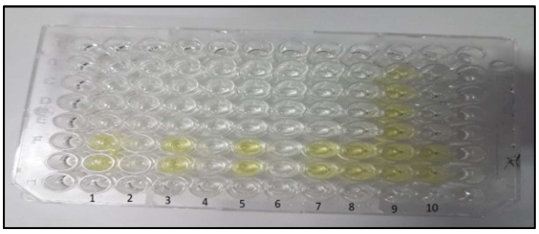

Figure 1. *DAS-ELISA plate of results for RYMV.*

Table 1. *DAS-ELISA reads OD at 405 nm.*

1	2	3	4	5	6	7	8	9	10
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.05	0.00
11	12	13	14	TS	15	17	19	A1	A2
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.60	0.00
A3	A4	A5	AH	B1	B2	B3	B4	TV	B5
1.47	0.00	1.42	0.00	1.55	0.00	1.35	1.39	1.43	1.50

3.2. Two-step RT-PCR Diagnostics

The agarose gel electrophoresis view of RT-PCR products for RYMV isolates.

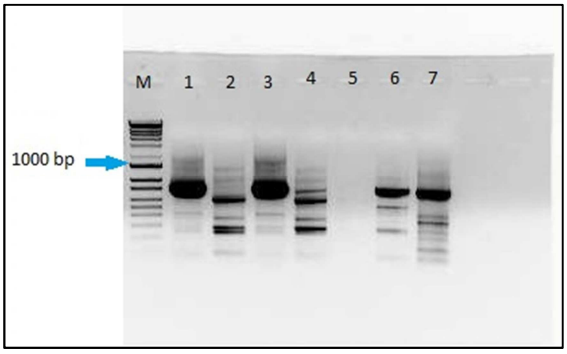

Figure 2. *Gel electrophoresis of RYMV RT-PCR products.*

Lane M- 1kb Ladder, Isolates in lanes 1-4 Ahero, 5-Negative control, 6-Positive control (RT), 7-Positive control (PCR).

3.3. Phylogenetic Analysis of RYMV

Sequences for isolates Ke_A1, KeB3 and KeB4 clustered together with already described Kenyan isolates. Four distinct clusters were formed each one from individual countries of Uganda, Kenya and Tanzania except those from Congo, Rwanda and Burundi which clustered together (Figure 3).

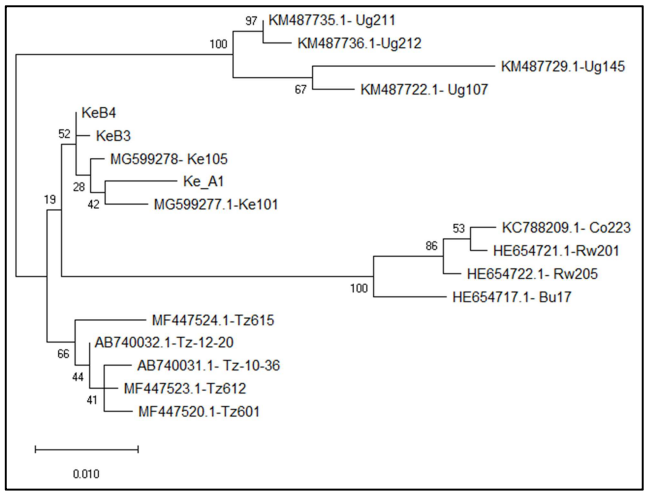

Figure 3. *Phylogenetic analysis of the isolates and sequences selected from the GenBank.*

The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model [17]. The bootstrap values are shown next to the various branches. The phylogenetic tree is drawn to scale, with branch lengths per clade measured in the number of substitutions per site. The

analysis involved 18 nucleotide sequences. Evolutionary analyses was conducted in MEGA X [18]. The sequences for western Kenya RYMV isolates of Ke_A1, KeB3 and KeB4 were deposited in the GenBank with accession numbers LC547477, LC547478 and LC547479 respectively.

4. Discussion

The RYMV symptoms observed in the rice field included yellow leaves, yellow stripes & brown/orange discolouration of older leaves, leaf narrowing, leaf mottling, stunting, leaf necrosis, reduced tillering, crinkling, malformation, incomplete emergence of the panicles and plant death. [19, 2]. All the symptomatic samples tested positive for RYMV by DAS-ELISA. These findings indicate that isolates originating from the same field are similar serologically but with different optical density (OD) values suggesting diversity of the virus strains (Table 1). This disease encounter phenomenon can be unravelled through full genome characterization at both the CP gene and nucleic acid levels. The two-step RT-PCR molecular diagnostic bioassay confirmed presence of RYMV in the seven samples. The PCR amplified fragments produced the expected band size of 850 bp on the gel. The CP gene sequences of the seven isolates had the same base pair length of 720 bp and were found to be of RYMV strains S4, S5 and S6. This confirms earlier findings by [14] that isolates of RYMV strain S4 have been reported to occur solely in the East African region. Since RYMV was first reported at Ahero in Kisumu County in 1966 [19], the virus seems to be gradually evolving into mutant strains resulting into novel strains that causes phylogenetic diversity, which is in agreement with findings by [20, 21].

Phylogenetic analysis of the CP gene sequences revealed that the isolates from Uganda (Ug), Kenya (Ke) and Tanzania (Tz) clustered together by country of origin implying that there was a single introduction of the RYMV CP gene in the three East African (EA) regions once, before further local viral diversity occurred in each specific country. Ke_A1, KeB3 and KeB4 are novel sequences of RYMV isolates from western Kenya. The two isolates of KeB4 and KeB3 shared 99% sequence identity with Ke105 and Ke101, but isolate Ke_A1 shared 98% identity with Ke101 and 99% with Ke105. The Ke_A1 isolate is closely related to Ke101 in the same sub-cluster closer to Ke105 with a more stable clustering relationship. KeB3 and KeB4 isolates clustered together because they might not be similar, but are uniquely different from the other Kenyan sequences depicting new phenomenon in RYMV evolutionary genomics. The isolates from Congo (Co), Rwanda (Rw) and Burundi (Bu) clustered together but they present a likely different bottleneck scenario of having very few sequences, which indicate a possibility of Burundi not to might have had the RYMV CP gene introduced from any of the three EA countries (Ug, Ke and Tz).

5. Conclusion

The high incidence and severity of RYMV in western Kenya is attributed to evolutionary genetic diversity of major RYMV strains among the rice isolates contributing to RYMD. This study has unravelled vital genetic information for crop protection technologies and germplasm screening to curb mixed infections among susceptible IR rice varieties for RYMV resistance/tolerance breeding as the only practical solution. Genomic diversity studies of RYMV strains to be enhanced and identify the evolutionary relationship that exists in western Kenya. This will provide sufficient knowledge for utilization by plant breeders to reduce their frequency and occurrence in rice fields.

Conflict of Interest

All the authors declare no conflict of interest.

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