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Research Application Summary

Serological and molecular detection of rice yellow mottle virus in western Kenya

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Abstract

Rice production remains very low with yields ranging from 1.5-3.5 ton ha^{-1}.The low yields are due abiotic and biotic constraints with pests and diseases being the most important. Rice yellow mottle disease (RYMD), caused by the Rice yellow mottle virus, RYMV, is the greatest challenge causing crop loss of up to 100%. Studies have reported RYMV strain S4-S6 being found in East Africa, however, strains diversity and distribution remain unknown in Kenya. Two diagnostic surveys for RYMD in major rice growing schemes and farms in Western Kenya were conducted in July 2015 and June 2016. Leaf samples were collected from symptomatic and asymptomatic plants for laboratory analysis. Serological analysis was performed by DAS-ELISA. Selected samples (7 in 2015 isolates and 12 in 2016 isolates) representative of location of collection were subjected to molecular analysis. Extraction of total RNA was done using GeneJET Plant RNA Purification Mini Kit followed by RT-PCR detection using RYMV specific primers. Sequencing and sequence analysis of the coat protein gene was done. DAS-ELISA results showed that Western Kenya isolates were serologically similar with very narrow variability in the absorbance readings. Molecular analysis using RT-PCR confirmed the ELISA results. Sequences of the coat protein gene with a length of 720 bp were obtained from sequence analysis which identified RYMV strain S4. The strain of RYMV that exists in Western Kenya is strain S4. The presence of RYMV in samples collected was successful detected by DAS-ELISA and PCR. RYMD is widespread in rice growing areas of Western Kenya. Findings of this study may be useful in integrated management of RYMD in Western Kenya and will assist plant breeders develop resistant rice varieties to RYMV, thereby improve food security, increase smallholder farmers' income, contribute to employment creation in rural areas and reduce the rice import bill.

Key words: DAS-ELISA, Kenya, rice, RYMV, RYMD, PCR, strain

Résumé

La production de riz reste très faible avec des rendements variant de 1,5 à 3,5 tonnes ha^{-1}. Ces faibles rendements sont dus à des contraintes abiotiques et biotiques, les ravageurs et les maladies étant les plus importants. La maladie de la panachure jaune du riz (RYMD), causée par le virus de la panachure jaune du riz (RYMV), constitue le plus grand défi, entraînant des pertes de récolte pouvant atteindre 100 %. Des études ont signalé la présence de la souche S4-S6 du RYMV en Afrique de

l'Est, mais la diversité et la distribution des souches restent inconnues au Kenya. Deux enquêtes de diagnostic du RYMD dans les principaux schémas et fermes de riziculture dans l'ouest du Kenya ont été menées en juillet 2015 et juin 2016. Des échantillons de feuilles ont été collectés sur des plantes symptomatiques et asymptomatiques pour des analyses de laboratoire. L'analyse sérologique a été réalisée par DAS-ELISA. Des échantillons sélectionnés (7 isolats en 2015 et 12 isolats en 2016) représentatifs du lieu de collecte ont été soumis à une analyse moléculaire. L'extraction de l'ARN total a été effectuée à l'aide du mini-kit de purification de l'ARN végétal GeneJET, suivie d'une détection par RT-PCR à l'aide d'amorces spécifiques du RYMV. Le séquençage et l'analyse de la séquence du gène de la protéine d'enveloppe ont été effectués. Les résultats de l'analyse DAS-ELISA ont montré que les isolats du Kenya occidental étaient sérologiquement similaires avec une très faible variabilité des lectures d'absorbance. L'analyse moléculaire par RT-PCR a confirmé les résultats ELISA. L'analyse des séquences du gène de la protéine d'enveloppe, d'une longueur de 720 pb, a permis d'identifier la souche S4 du RYMV comme étant celle qui éxiste au Kenya occidental. Les résultats de cette étude peuvent être utiles dans la gestion intégrée du RYMV dans l'ouest du Kenya et aideront les sélectionneurs à développer des variétés de riz résistantes au RYMV, améliorant ainsi la sécurité alimentaire, augmentant les revenus des petits exploitants, contribuant à la création d'emplois dans les zones rurales et réduisant la facture des importations de riz.

Mots clés : DAS-ELISA, Kenya, riz, RYMV, RYMD, PCR, souche.

Introduction

Rice is a monocot plant and is the most important staple food crop worldwide. In Kenya, rice is the third most important cereal crop after maize and wheat. Rice yields in Kenya have remained very low with yields ranging from 1.5-3.5 ton ha^{-1} and yet yields potential for most rice varieties range from 5.5-13 ton ha^{-1}.Various constraints limit rice production in Kenya and they range from high production costs and decreasing arable land due to population growth as well as agronomic factors. Constraints to rice production include, poor crop management practices, often compounded by drought, weeds, low soil fertility, high production costs and decreasing arable land due to population growth as well as attack by birds and other vertebrate pests, insect pests and diseases (Mughase *et al*., 2010). In Kenya, rice is grown by small scale farmers as a cash and food crop. About 80% of the rice is from irrigation schemes owned by the Government like Mwea Tebere, Bunyala and Ahero established by the Kenya Government while the remaining 20% is produced under rain-fed conditions in Coastal Kenya in areas like Kilifi, Kwale. With accelerated support in rice research from local and international development partners, rice production could be doubled in the near future (Lidejere, 2012; Muhunyu, 2012).

Rice Yellow Mottle Disease (RYMD) caused by Rice Yellow Mottle Virus (RYMV) is the most important viral diseases that causes important losses of up to 100% (Abo *et al*., 1998; Banwo, 2003; Konate *et al*., 2006). The presence of the RYMV in the rice field can be detected by visual observation of symptoms on the plant leaves and biological indexing. However, the use of symptoms for diagnosis is unreliable as symptoms exhibited vary depending on the virus strain, the cultivar, the presence or absence of mixed viral infections, growth stage and stage of infection and finally the environment (Uehara-Ichiki *et al.*, 2013). Biological assay methods are simple and easy to perform without special skills or knowledge (Jones, 1993; Naidu and Hughes, 2001). The use of symptoms to diagnose RYMD has shown some limitations hence there should be more diagnostic tests to confirm the disease.

Among the serological detection techniques, Enzyme Linked Immunorsobant Assay (ELISA) remains the most favored means of RYMV diagnosis. ELISA is simple, easy, quick and sensitive method for detection and quantification of virus level in the plant (Clark and Adams, 1977).

Since its development, modifications have been made to ELISA to improve its sensitivity and reliability. It has advantages over other serological techniques as very low concentration of the virus can be detected, and small amount of antibodies are required, it is suitable for large number of samples, viral titre can be determined/ quantified and it is cheap in terms of cost (Selvarajan and Balasubramanian, 2018).The only limitation of this method is the inability to distinguish mixed virus infections (Thottappily *et al*, 2003).

Polymerase chain reaction (PCR) is a routine molecular diagnostic technique in the laboratory. Advantages of PCR technique over serological technique include high sensitivity, rapid and reproducibility between different labs. For instance, the sensitivity of RT-PCR was 102 -fold higher and more than dot-blot hybridization (Sharma and Dasguta, 2012). Comparison between ELISA and RT-PCR has shown that the sensitivity of real time RT-PCR is 104 fold higher than ELISA (Zhang *et al*., 2008b). The aim of this study was to determine RYMD disease incidence and to diagonise RYMV in Western Kenya.

Material and Methods

Occurrence and distribution of RYMD in western Kenya. Two diagnostic surveys for RYMD were conducted in July 2015 and June 2016 mainly in rice growing schemes and farms in Western Kenya. Five counties were covered in this survey namely Kakamega, Busia, Kisumu, Homa Bay and Siaya. At least four quadrants (1 M2) per rice field were set randomly in a two-way diagonal pattern (Ochola and Tusiime, 2011). Number of plants affected per quadrant (incidence) was calculated which was used to calculate mean incidences. Data obtained (RYMD incidence and severity) were recorded. Symptomatic leaf Samples were collected, put in a cool box and taken to the lab for serological and molecular analysis. Global positioning system (GPS) handset (Magellan®TritonTM) was used to take readings for latitude, longitude and altitude at each sampling site.

Disease incidence and severity determination. Disease incidence was calculated as the percentage of plants showing RYMD symptoms to the total number of plants observed in the field. The average incidence and severity of the sampling points per farm was used as the actual plot disease incidence and severity. The disease incidence was assessed according to IRRI (2002) as the proportion of diseased plants in an area:

$$\text{Disease incidence} = \frac{\text{number of plants infected}}{\text{total number of plants observed}} \times 100$$

Incidence was scored as presence or absence of virus disease using a rating scale according to IRRI (2002) where: low incidence = 1-20%; moderate incidence = 21-49% and high incidence = 50-100%. Disease symptom severity was scored on a scale of 0-3 as previously done (John and Thottappilly, 1987; Ochola and Tusiime, 2011) where 0= No disease symptom, 1= Mild symptoms, 2= Moderate symptoms and 3= Severe symptom (Table 1).

Double Antibody Sandwich ELISA (DAS-ELISA). Serological detection of RYMV was done using DAS-ELISA protocol according to Pinel *et al*. (2000). The RYMV antisera and purified antibodies raised against Madagascar isolate (Ser4) were utilized. Virus concentration in the infected leaf was estimated with reference to a standard optical density of the positive isolate from the IRD green house. The antibody used was nondiscriminatory and worked for all the isolates. A microplate reader was used to measure the values optical density at 405 nm after 1hour and 2 hours and the isolates considered positive were those with optical density values greater than twice the value of a negative control (Ochola *et al*., 2011).

Table 1. Summary of Disease severity scoring scale of 0-3

Symptom appearance	Scale	Symptom Description
	0	No disease symptoms
	1	Sparse yellow spots, oblong yellow linear spots and leaf mottling
	2	Elongated yellow spots parallel to veins, leaf narrowing, Leaf yellowing and mild wilt
	3	Yellow-orange patches/leaf browning, Stunting, leaf necrosis, Extreme wilt and death of rice plants

Severity scale: 0- No disease symptom, 1-Mild disease symptom, 2-Moderate disease symptom, 3-Severe disease symptom.

Molecular Detection by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from 19 frozen isolates/infected rice leaves using the GeneJET Plant Purification Mini Kit, according to manufacturer's instructions. Slight modifications on the speed and time of centrifuging were made on the protocol to optimize the quality and quantity of the total RNA. A pair of primers, the sense primer 3' RYMV II at 10 µM and antisence primer 5' RYMV III at 10 µM was designed to transcribe and amplify the coat protein gene (Pinel *et al*., 2000).

The sense primer 3' RYMV II was used in the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to transcribe and amplify genome fragments containing the coat protein gene (nt 3447 to 4166) (Pinel *et al*., 2000). The PCR amplification of the RT-PCR products was done using the 2 primers (primer 3 II' M 5' III) under PCR conditions of denaturation at 94 °C for 5 minutes followed by 30 cycles of 1 minute at 94 °C, 30 seconds at 55 °C hybridization, 1 minute at 72 °C elongation and final extension of 10 minutes at 72 °C. The mixture was stored at 4 °C. The PCR products were loaded and visualized by the electrophoresis of Ethidium Bromide stained 1% Agarose gel in buffer TAE x 0.5 at 100V for 30 minutes to estimate the size of DNA.

Coat protein gene sequencing and Sequence analysis. Sequencing of the amplicons from the coat protein gene was carried out with the Taq terminator sequencing Kit (Applied Biosystems) and analysed on an Applied Biosystem 373A sequencer (Pinel *et al*., 2000; Fargette *et al*., 2004). Two readings per base (3'to 5' and 5'to 3' directions) led to sequence accuracy of 99.9%. Sequences were assembled by Seqman (DNASTAR) and analysed using lasergene software by DNASTAR for Apple Macintosh computers (Madison). The coat protein gene sequences (720) from Western Kenya isolates were compared with reference strains in the EMBL and were found useful in assigning isolates to a particular strain and to monitor intra-strain diversity (Kanyeka *et al*., 2007).

Results

Occurrence of RYMD in Western Kenya. Typical RYMV symptoms were observed on the rice in the field. They included: yellowing of leaves, stunting, yellow-orange coloration of the field, mottling, and leaf narrowing (Fig.1).

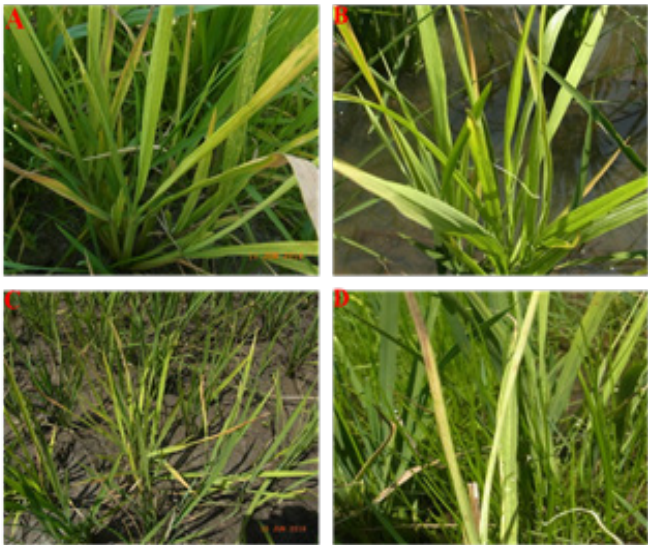

Figure 1. RYMV symptoms observed in rice fields; A: Yellow leaves, yellow stripes & brown/ orange discoloration of older leaves, B: Leaf narrowing & mottling, C: Stunting, D: leaf necrosis and plant death.

Viral symptoms were observed during the survey varied type of cultivar, age of infection and disease incidence. Mean RYMD incidence varied across the counties during the two surveys in June 2015 and in July 2016. In 2015 there was generally low incidence and subsequently low severity levels as compared to 2016. Highest mean incidence was observed in Siaya County (82.7%) in 2016 and lowest in Kakamega County (6%) in 2015. Other counties where the disease incidence was relatively high were Homa Bay (80%), Kisumu (58.1%) and Busia (56.7%) in 2016 survey. In individual farms, the highest incidence (100%) was at Ujuang'a irrigation scheme in Siaya County on a Pishori rice variety. The lowest mean disease incidence (2%) was recorded on various farms in Busia and Kisumu Counties. Furthermore, there was a significant difference in RYMD incidence between counties ($p<0.0001$). Most farms had a severity of 2 with few having 1 and 3. There was a positive correlation between viral incidences and severity ($r=0.8843$, $p< 0.0001$) and therefore severity increased with increase in disease incidence. An ANOVA analysis of incidences revealed there exist a statistically significant difference ($p<0.05$) in the mean for the incidences.

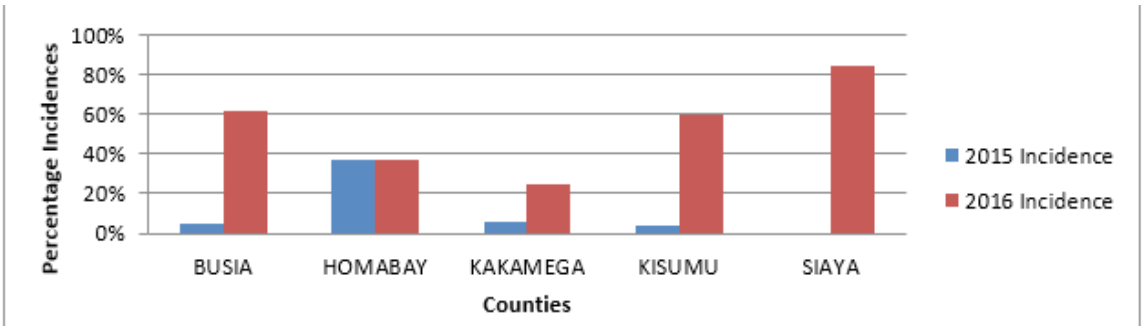

Figure 2. Graph showing RYMD mean incidences in various counties in western Kenya

Detection of RYMV by ELISA. Results showed that 95 samples out of 120 samples were RYMV ELISA positive (Table 2). Kagan area in Homa Bay County had the highest number (33) followed by Bunyala in Busia County and Ahero in Kisumu County at 18 and 13 respectively while all isolates from Kakamega County were ELISA negative (Table 2). All leaf samples collected from Kakamega County were ELISA negative. ELISA result revealed that 79.2% of the total samples tested were positive with ELISA (Table 2). ELISA results in counties showed that Homa Bay

County had the high number (41 out of 46) of ELISA positive samples. High visual incidences in the field resulted into high number of ELISA positive samples.

Table 2. ELISA results of samples collected from various locations

AREA	N	RYMV presence or absence with %			
		RYMV Positive	Percentage RYMV Positive	RYMV Negative	Percentage RYMV Negative
Kagan	37	33	27.5	4	3.3
Bunyala	22	18	15.8	4	2.5
Ujuanga	14	14	11.7	0	0
Nyando	9	5	4.2	4	3.3
Rachuonyo	5	4	3.3	1	0
Muhoroni	5	4	3.3	1	0.8
Nyangweso	4	4	3.3	0	0
Teso south	4	0	0	4	3.3
Koyonzo	3	0	0	3	2.5
Mumias	3	0	0	3	2.5
Alupe	1	0	0	1	0.8
Ahero	13	13	10.8	0	0
Total	120	95	79.2	25	20.8

N=Number of samples

DAS- ELISA revealed Western Kenya isolates are serologically similar with narrow variability in absorbance readings.

RT-PCR detection of RYMV. Amplified fragments of the expected size (1000 bp) were obtained in both 2015 and 2016 isolates after amplification using RYMV specific primers. Nine (9) isolates from 2016 and seven (7) isolates from 2015 group were confirmed to be positive by RT-PCR and were subjected to sequencing (Figure 2).

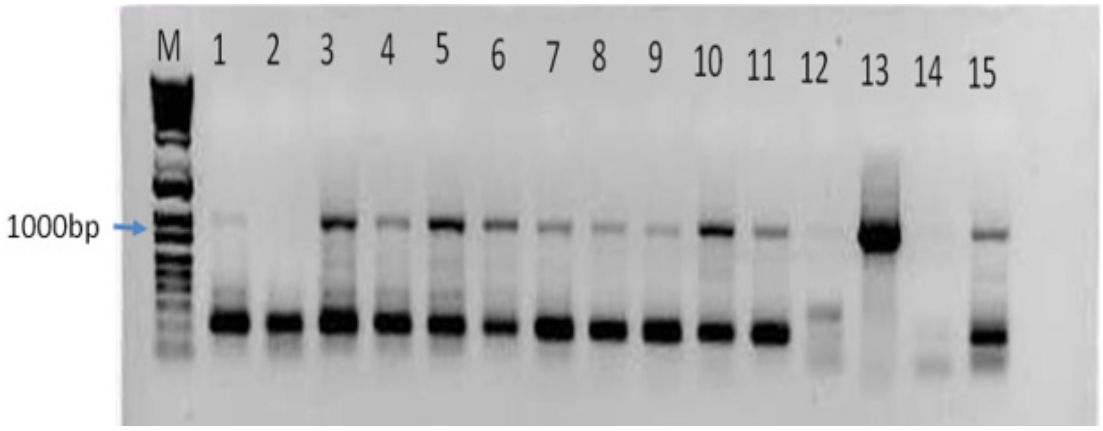

Figure 2 Gel electrophoresis of RT- products of 12 isolates collected in June 2016
Lane M- 2-Log DNA Marker (0.1-10.0 kb), Isolates in lanes; 1-Kagan, 2-Kagan, 3-Nyangweso, 4- Rachuonyo, 5- Rachuonyo, 6- Ahero, 7- Ahero, 8-Ujuanga,9-Ujuanga,10-Bunyalla,11-Bunyalla,12-Koyonzo,Lane 14-Negative control and Lane 15-Positive Control.

Identification and distribution of RYMV strains. The coat protein gene of the 16 isolates (7 from 2015 and 9 from 2016 group) sequenced had the same length of 720 bp. The coat protein gene sequences (720 bp) were found useful for assigning isolates to a particular strain and monitoring diversity of RYMV in W. Kenya. The isolates belong to strain S4. No any other strain was recorded in this study. Assessment of the distribution of the RYMV revealed that strain S4 occurs predominantly in four counties of Western Kenya namely Busia, Siaya, Homa Bay and Kisumu but absent in Kakamega County. Nucleotide sequence analysis revealed that all the sequenced isolates (7 in 2015 and 10 in 2016) in this study had a length of 720 bp (Table 3). These isolates also belonged to strain S4 and had a gap at position 178, 179 and 180 confirming they belong to S4, this particular gap is normally absent in strain S5 and S6 instead bases C,G and C takes up the positions respectively(Table 3).

Table 3. Differences in base pair length (bp) between strain S4, S5 and S6

RYMV strain	Length (bp)	Position-55, 56, 57	Position-178, 179, 180
S4	720	A, A, G	Gap
S5	723(due 3 extra bases)	A, A, G	C, C, G
S6	720	Gap	C, C, G

Phylogenetic tree and RYMV sequence Analysis of Coat protein gene of RYMV isolates from Kenya. The phylogenetic tree of the RYMV sequences using Neighbour joining method separated the 2015 and 2016 isolates into two main clusters when compared with eleven (11) other isolates which had earlier been sequenced (Fig. 3). The first cluster contained majority of the isolates (12) while the second cluster contained five (5) isolates all from the 2016 survey which clustered with two from Uganda (Ug210-2010 and Ug211-2010). This second cluster had two isolates from Busia (ke43-2016 and ke45-2016), two isolates from Siaya (ke25-2016 and ke32b-2016) and one from Ahero in Kisumu (ke23-2016).

The first cluster had 12 isolates of which were from 2015 survey and the other from five 2016 survey. In this cluster two isolates from Muhoroni collected in 2015 (ke215-2015, ke214-2015) grouped closer to one from Ahero (ke-19-2016) and another one (ke105-2012) which was collected in 2012. Two isolates from 2016 (ke6A and ke14D) and two from 2015 survey (ke219 and ke212) occurred close together with two of 2012 (ke106 -2012 and ke101-2012) (Fig. 3).

Discussion

This study has shown that RYMD does occur in all the lowland rice growing areas in Western Kenya, as it was found to occur in the following four counties: Kisumu, Homa Bay, Siaya and Busia. Ocholla and Tusiime (2011) in a similar study in Eastern Uganda found that RYMV was widespread on all the surveyed rice farms; this may be due to continuous cultivation of susceptible varieties. Visual assessment of rice fields revealed that they were infected by RYMV but with varying disease incidences and severity. The symptoms observed in the farms were mainly yellowing of leaves, stunting, a yellow-orange coloration of the field, mottling and leaf narrowing. These resemble major RYMD symptoms that had been earlier reported (Bakker, 1974; Kouassi *et al*., 2005). Majority of symptomatic isolates (79.2%) collected from irrigated rice field that were subjected to ELISA test and tested positive confirming that RYMV is a serious disease in rice ecological systems in Kenya as in other parts of Africa (Abo *et al*.,1998). DAS- ELISA revealed W.Kenya isolates are serologically similar with narrow variability in absorbance readings; this is because they belonged to S4. Finding of this study however revealed that all isolates that were collected from upland rice mainly from Kakamega were negative with ELISA.

In molecular detection, reverse transcriptase polymerase chain reaction (RT-PCR) confirmed the presence of RYMV in 84.2% of all rice samples that were subjected to PCR. The coat protein gene of the 16 isolates sequenced had the same length of 720 bp. The coat protein gene sequences were found useful for assigning isolates to a particular strain S4 and monitoring diversity of RYMV in Western Kenya. The sequencing results revealed that all the sixteen isolates belong to strain S4, which support the earlier reports (Pinel *et al*., 2000) that S4 to occur exclusively in the East Africa region.

This study can report that RYMV strain, S4 found in western part of Kenya is much diverse. For instance, during this study, five (5) isolates in the 2016 group clustered independently together forming a unique cluster with some isolates from Uganda. The isolates were two from Bunyalla (Ke 43 Seq. and Ke 45a seq.), one from Ahero (Ke 23 seq.) and two from Ujuanga irrigation scheme (Ke 32b, and Ke 25 seq.) in Siaya County. This cluster is a new one belonging to S4 strain and has not been reported in the previous studies and it seems to be much older because it is close to the origin of strain, S4.

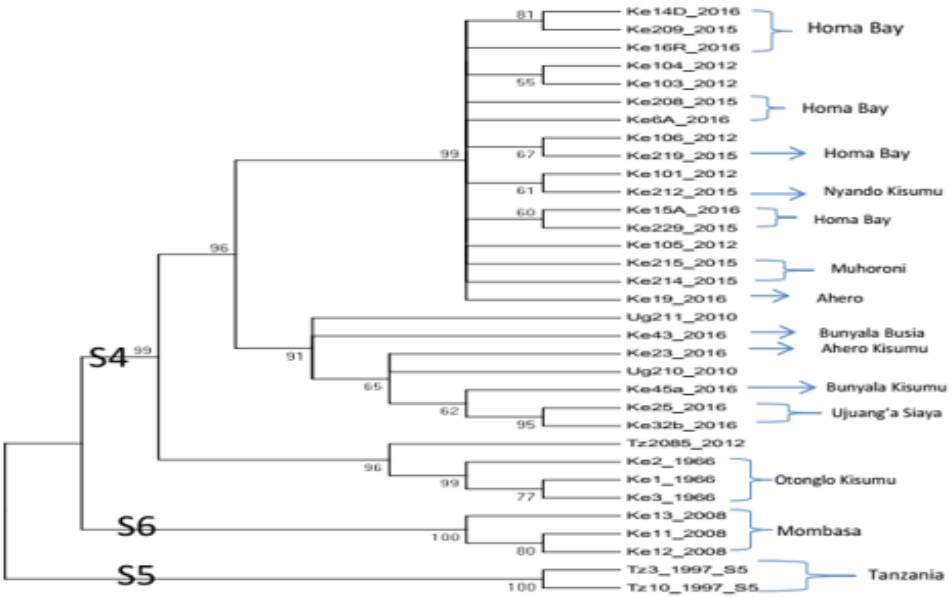

Figure 3. A phylogenetic tree constructed from nucleotide sequence alignments of coat protein genes of RYMV isolates for 2015 and 2016 in comparison to earlier isolates of 1966, 2008 and 2012.

Conclusion

This study has revealed that RYMD is widespread and is a major constraint in farmers' fields in western Kenya. Also, the RYMV strain present in the region is strain S4 and there is a high diversity within strain S4 found in Western Kenya.

Recommendation

This study recommends that farmers use resistant and tolerant rice varieties which may be suitable for cultivating in areas with high RYMD incidences in Western Kenya and should replace highly susceptible rice varieties. There should be capacity building of farmers on integrated management of RYMD. Finally, plant breeders should utilize finding of this research to develop resistant rice varieties suitable for western Kenya.

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