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Rice Blast *Pyricularia oryzae* CAV. Isolates in Kenya Characterized Sequenced and Registered

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Abstract: **Rice (*Oryza sativa* L.) is the third most important cereal crop in Kenya. Globally, rice remains the most favoured grain for human consumption. In Kenya and most of the African countries production is below the world averages. Rice Blast caused by *Pyricularia. oryzae* Cav. has been singled out as one of the main causes limiting production. The control of this disease has remained elusive. Variation of the pathogen in the population is suspect. This research was therefore carried out to analyze the genotypes of different isolates previously described morphologically. Eight Different Kenyan isolates identified from a previous survey were studied using DNA analysis and sequencing of the ITS region from the rice genome. Results revealed variation with respect to colony color, diameter, morphology and conidia shape & size. Sequenced Isolates clustered into four haplo groups (HGs), that is HG1 for isolate 4, HG2 for isolate 6, HG3 for isolate 7a and HG4 for isolate 7b.The new isolates' sequences were submitted to the NCBI GenBank database and the accession numbers assigned as, *Pyricularia oryzae* KY275366, *Pyricularia oryzae* KY275367 and *Pyricularia oryzae* KY275368. The result of the present study demonstrates that there is a certain level of genetic diversity among isolates of *P. oryzae* from various regions of Kenya. The screening for resistance to *P. oryzae* therefore should take into account the different isolates in order to avoid cryptic error.**

Keywords: **Rice Blast, *Pyricularia oryzae* CAV. *DNA* sequencing Variation.**

1. INTRODUCTION

Rice Blast, caused by the fungal pathogen *Pyricularia grisea* (Cooke) Cacc., M*agnaporthe grisea* (Hebert) Barr. (anamorph, *Pyricularia grisea* Sacc.) (*Pyricularia oryzae* Cav.) is a haploid, filamentous, heterothallic Ascomycete in the order Pyrenomycetes (Rossman *et al.*,[1] ,Valent and Chumley,[2]; Indu and Rohini,[3] . Pyricularia belongs to the family Magnaporthaceae. Based on RFLP and DNA sequence analysis, Borromeo *et al.*,[4] and Kato *et al.*,[5] suggested that the Pyricularia isolates from Digitaria sp. and rice represent distinct species. This is economically the most important disease of rice in Kenya and many rice-growing countries of the world. Blast is a key concern in combating global food insecurity given the disease is responsible for approximately 30% of rice production losses globally; these losses increase the global rice price and reduce consumer welfare and food security, Nalley, *et al.*,[6].

Rice Blast has been singled out as one of the main causes limiting high yields Mugambi, 2011[7] of rice in Kenya. This disease is also a constraint to the increased production of many marginal rice growing environments because it is affected by crop management; particularly the use of Nitrogen fertilizer (N). Nitrogen is an essential nutrient element in growth and development of rice, Farnaz *et al*., 2012[8]. Any additional 20 Kg /N produce one ton more of rice grain per hectare, MOA, [9]. But this is applicable up to a maximum application of 2.8 tons/ha, Dobermann1 & White, 2000[10]. However, this positive attribute cannot be fully harnessed as nitrogen beyond certain levels increases crop susceptibility to blast disease ,Kingsolver *et al*., [11]. The most effective control measure of the blast, variety resistance, has not been durable. This non-durability may be as a result of multiple strains. This research thus was initiated to analyze for possible strains in the population. With the major objective of Analyzing differences in the sampled isolates.

2. MATERIALS AND METHODS

To Establish Biological and Molecular Characteristics of Rice Blast Pathogen P. oryzae:

Pathogen Culture Development in the Laboratory: Monoconidial Isolation of the Cultured Fungus:

Leaves with blast lesions resulting from field infections on plants sampled from selected research sites were surface sterilized with 70% ethyl alcohol for 10 s and soaked in distilled water for 2 h to saturate the specimens. Steeped tissues were laid in glass plates containing filter paper plates and subsequently transferring a small sector of the growing mycelia to Petri dishes containing potato dextrose agar; and incubated at 25 to 26°C for 24 to 36 h to induce sporulation of the fungus, Aneja, [12] For culture characterization the Petri dishes with Potato Dextrose Agar (PDA) were further incubated in the chamber for fourteen days. A piece of the young sporulated fungus was picked from slide cultures using a pin, under binoculars microscope. The sector of growing mycelia was placed on a slide with a drop of water, the mycelia was covered by a cover slip and observed under the microscope for classical characterization. Single spores were carefully picked and transferred to PDA in Petri dishes for incubation under 25-26^{0}C for a maximum of 14 days. Replicates of the same were stored as spores aseptically on slides and kept under sealed polythene bags. The mother cultures were preserved as reference cultures.

Characterizations of P. oryzae isolates and mycelia growth on PDA culture media

Colony diameters of each isolate on Petri plates were measured in centimeter in two directions with a ruler at two days intervals and the measurements were recorded in cm at the 12th day of incubation. Mycelial color, type of margin and sporulation were recorded, Barnett, and Hunter, [13].

Microscopic characterization of conidia:

Morphological characterization was done based on conidial features. Each *P .oryzae* isolate was grown on PDA and incubated at 25-26^{O}C for 14days. The morphological characters such as shape, color and size (length) of the conidia were measured on 50 spores for each isolate and the number of septations per conidia was determined under the microscope. Based on these features the eight isolates were identified using key manual developed by Barnett and Hunter [14] The spores were observed on slides after staining with lacto phenol cotton blue under light microscope. The sizes of conidia were measured by using ocular and stage micrometers as described by Meena[15]. The spores were measured using digital solutions for imaging and microscopy, soft image system (BX 51 System microscope Hamburg, Germany). Microphotographs were taken to show the typical spore morphology of the *P. oryzae* isolates.

Molecular Characteristics of rice blast pathogen P. oryzae

Extraction of genomic DNA:

Genomic DNA was extracted using DNeasy$^{(R)}$ Plant mini kit. Isolates were grown in 100 ml of potato dextrose broth for 4 days at 25°C in a rotary shaker at 100 rpm. Mycelia mat was filtered dried and 2g of the mat was ground in pestle and motor to a fine powder using liquid nitrogen. 400 µl Buffer API and 4 µl RNase were added vortexed and incubated at 65°C for 10 min. The tubes were inverted 3 times during incubation. 130µl P3 Buffer was added, mixed and incubated for 5 min. on ice. The lysate was then centrifuged for 5 min. at 20,000 x g (14, 000 rpm). The lysate was pipetted into a QIAshredder spin column placed into a 2 ml collection tube, centrifuged for 2 min. at 20,000 x g (14, 000 rpm). The flow was transferred through into a new tube without disturbing the pellet. 1.5 volumes of Buffer AWI (Ethanol was added to

Buffer AWI and AW2 concentrates (earlier) was added and mixed by pipetting. 650 µl of the mixture was transferred into DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 1 min. at 8000 rpm. The Spin column was placed into a new 2 ml collection tube, 500 µl Buffer AW2 was added and centrifuged for 1 min. at 8000 rpm. The flow through was discarded. Another 500 µl Buffer AW2 was added and centrifuged for 2 minutes 20,000 x g (14, 000 rpm). The spin column was transferred to 2 ml micro centrifuge tube. 100 µl Buffer for elution was added and incubated at 5 min. at room temperature of 25^0C. This last step was repeated once. The suspension was stored at -20^0 C pending use for PCR amplification.

PCR amplification and sequencing:

The internal transcribed spacer (ITS) regions 1 and 2, was amplified in a 25 µL reaction on a GeneAmp 9700 thermal cycler (Applied biosystems) under these reaction conditions: 1 µL of template DNA at a 1 : 20 dilution of the DNA extraction, 0.2 mM each dNTP, 0.2 µL of FastTaq (Applied Biosystems), 0.2 mM each of primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). 2.5 µL of the supplied 103 PCR buffer with $MgCl_2$, and sterile water were added to bring volume to 25 µL. Thermal cycling was initiated by denaturation at 95^0C for 4 min. This was followed by 35 cycles of denaturation at 94^0C for 1 min, annealing at 45^0 C for 1 min, and extension at 72^0 C for 1 min, with a final extension at 72^0C for 7 min. PCR products were analyzed by electrophoresis on 1% agarose gels in TAE buffer (20 mM Tris-Acetate, 1 mM EDTA, pH 8.0) (Sambrook *et. al.*, 1989) and viewed by staining with ethidium bromide. Using 0.5x TBE containing 1mg/mL ethidium bromide with a vertical electrophoresis apparatus. The gel was photographed using Alphaimager 2200 under UV transilluminator. The resolved products were extracted from the gel and purified using the Qiagen DNA purification kit according to the prescribed protocol.DNA quantification was done by using a DNA NanoDrop 2000/2000c Spectrophotometer. A260/280 and A260/230, (oxford gene technology the molecular genetics company™, 2011).Values greater than 1.8 were found to be suitable for analysis. Lower A260/280 values may indicate protein contamination. QIAGEN DNeasy® Kits, was used for incorporating the proteinase K digestion step, to re-purify DNA samples with low A260/280 ratios. Lower A260/230 values indicated contamination with salts or some solvents (e.g. phenol). These were re-purified by ethanol precipitation, re-suspending the DNA in TE buffer. Nucleotides and primers removed with High Pure PCR Product Kit (Roche Molecular Biochemical) according to the manufacturer's instructions. The suspension was stored at -20^0C pending use. Amplified products were sequenced with Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 310 or ABI PRISM 377 automated DNA sequencer. Sequences were determined on both strands with sequencing primers, ITS1 and ITS4 (White *et al.*, 1990).

Phylogenetic analysis of the ITS interspecer region:

The ITS region sequence reads from the sequencer were edited by ChromasLite (http://www.technelysium.com.au/chromas_lite.html) to remove unknown bases and consensus sequences generated from sequence fragments using BioEdit version ver. 7, Hall, [16] and then nucleotide alignments was generated by CLUSTAL W (Thompson *et al.*, [17] implemented in BioEdit ver. 7. The sequence was then loaded in MEGA 6 where the evolutionary history was inferred using the Neighbor-Joining method, Saitou and Nei, [18]. The optimal tree is shown in Figure 4.The bootstrap consensus tree inferred from 1000 replicates , Felsenstein,[19], was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, [19]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches (Felsenstein, [19].

The evolutionary distances were computed using the Jukes-Cantor method , Jukes and Cantor,[17] and are in the units of the number of base substitutions per site. The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 398 positions in the final dataset. Evolutionary analyses were conducted in MEGA5, Tamura *et al.*, [20].

Haplotype list Figure 2 & 3, from the sequences were generated by DnaSP Ver. 5.10.01 software in which sites with alignment gaps and invariable sites were not considered.

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3. RESULTS AND DISCUSSION

Description of the type of culture, their spore, conidia characteristics and morphology group:

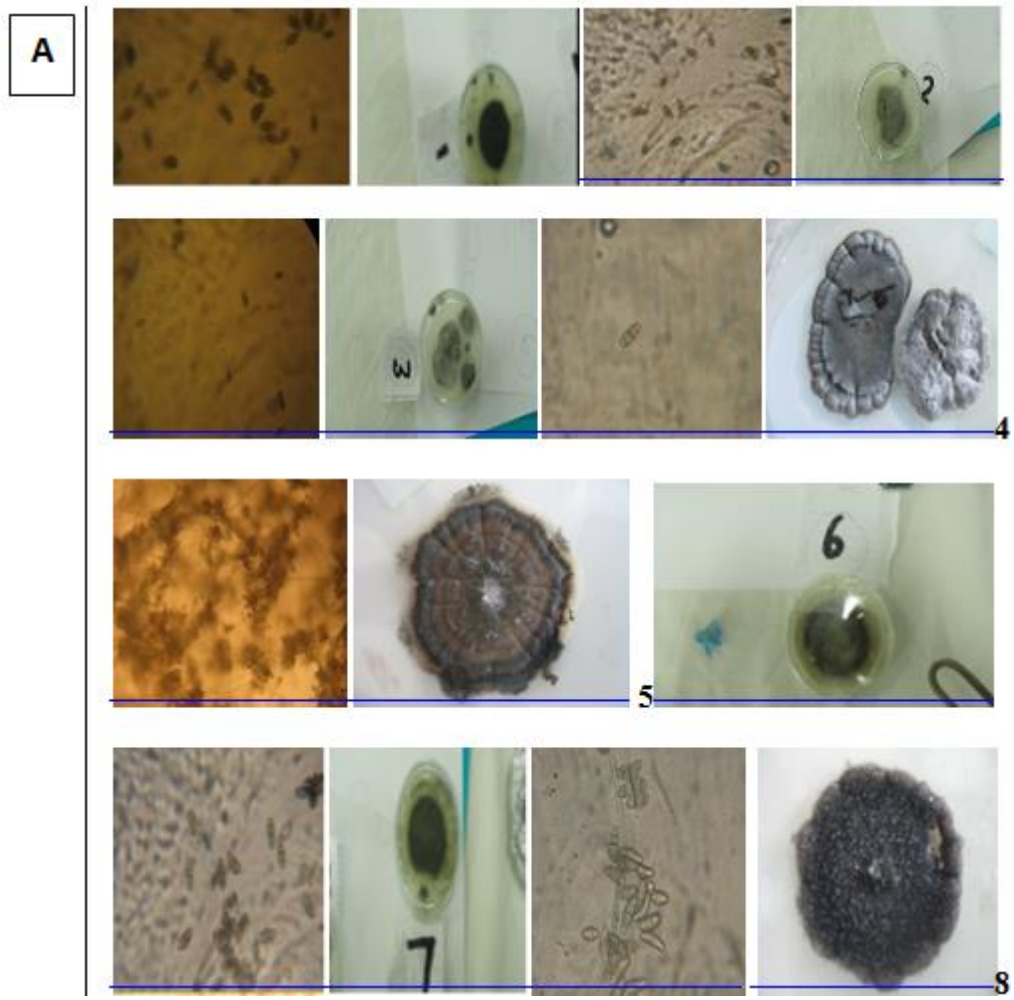

Plate1: Conidia and growth characteristics of the isolates: A, clockwise from top left: 2 weeks old culture of isolate 1, isolate 2, isolate 3, 4.Culture 6. Conidia and growth characteristics of the isolate7 and 8.

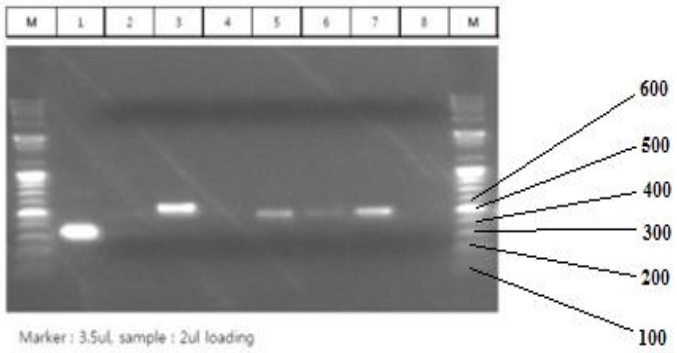

Figure 1: PCR Products of the eight isolates using Primer ITS1 and ITS4:

Electrophoresis of PCR products were observed under UV light. The expected size of the fungal ITS inter-spacer region using ITS1 and ITS4 primers was between 550-600 base pair. The PCR of isolates 4, 6, 7a, 7b were within the target region and therefore further analysis proceeded on these four samples (Plate 1). A phylogenetic analysis of the sequenced products was then carried out.

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The sequence alignment:

The sequence alignment Fig. 2 shows that with the exception of only few positions, most of the loci are not conserved. It is evident that this isolates had undergone various substition mutations generating single nucleotide polymorphisms on very many loci. However, there are only few deletions and insertions as evidenced by the low number of indels.

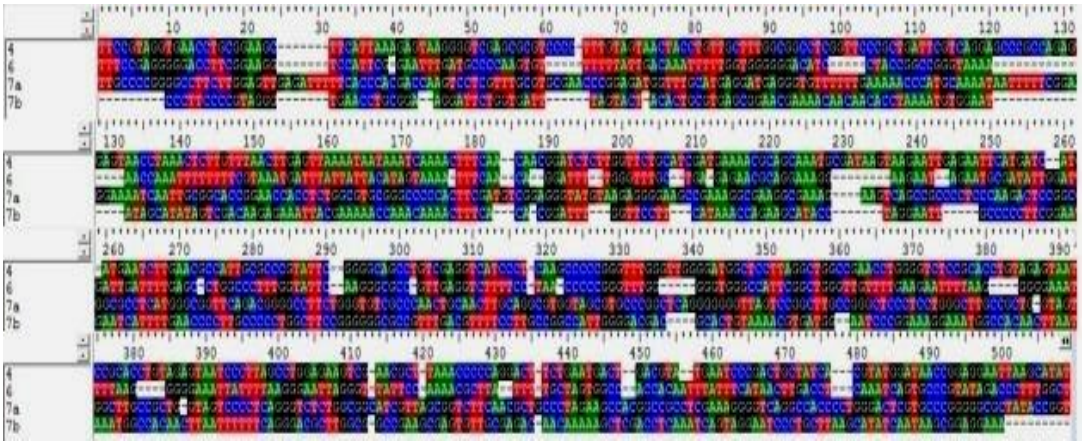

Figure 2: Sequence alignment as viewed in BioEdit.

Conserved locus is indicated by same colour (columns). Different bases in the same locus indicates substitutions while '-' indicates insertions/deletion.

As shown in Fig. 2, the four sequenced isolates generated four haplotypes, each isolate independently generating a single haplotype.

Haplotypes generated from the four sequenced Pyricularia oryzae Cav. races shown in Fig. 6.

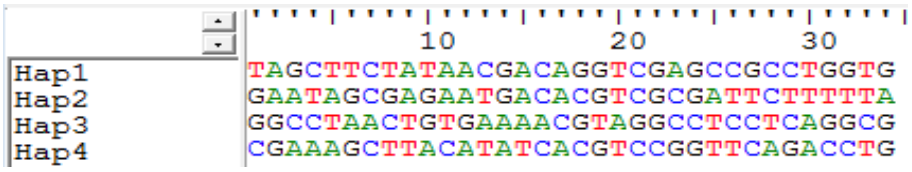

Figure 3: Haplotype list as viewed in Bioedit version 7.

Phylogenetic analysis of the sequenced products:

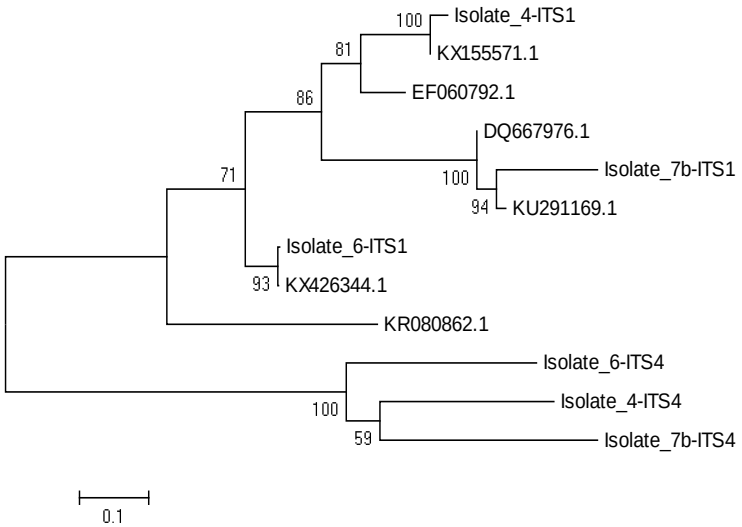

Figure 4: Neighbour joining tree (Dendogram) of blast isolates estimated using a bootstrap analysis with 1000 replications in MEGA 5

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Table 1: Haplotype list as viewed in Bioedit version 7

	Haplogroup	ISOLATE
1:	HG1	4
2:	HG2	6
3:	HG3	7a
4:	HG4	7b

The four strains that were sequenced clustered into four haplogroups (HGs), that is HG1 for isolate 4, HG2 for isolate 6, HG3 for isolate 7a and HG4 for isolate 7b (Table 1). Isolate 4 and 6 were closely related as they shared a branch supported by 99% bootstrap confidence but others formed independent branches (Fig. 4). The highest evolutionary distance was between isolate 6 and isolate 7a (1.597±0.203) while the lowest evolutionary distance was between isolate 4 and isolate 6 (0.638±0.055) as shown in Table 1

The isolates' sequences were submitted to the NCBI GenBank database and the accession numbers assigned to them as shown in table 2.

Table 2: NCBI Accession numbers assigned to the sequenced isolates

Sample name	Organism name	NCBI accession number
4	Pyricularia oryzae	KY275366
6	Pyricularia oryzae	KY275367
7a	Pyricularia oryzae	-
7b	Pyricularia oryzae	KY275368

(Tamura *et al*., 2013). Bootstrap support appears above each branch; depicting the relationships of Isolates of *Pyricularia oryzae* isolate 6,4 ,7b & 7a with the reference races (KX426344.1, DQ667976.1, KU291169.1 and EF060792.1).

4. DISCUSSION

ITS4 sequences were closely related as all the three clustered together on the phylogenetic tree. Isolate_4-ITS4 and isolate_7b-ITS4 seems to have evolved at the same time, from this dendogram, they both branched from isolate_6-ITS4 before evolving into individual 'strains' of *Pryricularia oryzae*. They did not share a branch with other *Pycularia oryzae* relatives from the GenBank. Isolates from ITS1 marker seems to be more diverse as they were distributed into various clades on the phylogenetic tree. Isolate_4-ITS1 shared a branch with GenBank isolate KX155757.1 and the two are phylogenetically related to another GenBank isolate EF060792.1 Isolate_7b-ITS1 formed the same clade with GenBank isolate KU291169.1 through a branch supported by 94% bootstrap confidence. These two are closely related to another GenBank isolate DQ667976.1 through 100% bootstrap confidence. Isolate_6-ITS1 is closely related to GenBank isolate KX426344.1 through 93% bootstrap support. Phylogenetic analysis of the sequenced products showed that with exception of only few positions, most of the loci were not conserved indicating that the isolates had undergone various substitions and or mutations generating single nucleotide polymorphisms on very many loci. Four sequenced isolates generated four haplotypes, each isolate independently generating a single haplotype. The haplotypes were grouped into Figure 3: Haplotype list as viewed in Bioedit version 7.

haplo groups (HGs), that is HG1 for isolate 4, HG2 for isolate 6, HG3 for isolate 7a and HG4 for isolate 7b. Isolate 4 and 6 were closely related as they shared a branch supported by 99% bootstrap confidence but others formed independent branches. This is an indication that *P. Oryzae* fungus species attacking rice in the selected regions is highly diverse. Deepti Srivastava *et al*.,[20], said that the fungus had an ability to overcome resistance within a short time after the release of a resistant cultivar and thus breeding for resistance became a constant challenge in India. Several other countries with similar experience were, Ethiopia, (Mebratu *et al*.,[21] and Brazil, Marta and Sitarama Prabhu [22] . The analysis of genetic variation in plant pathogen populations is an important pre-requisite for understanding co-evolution in the pathosystem (Deepti Srivastava *et al*., [20]. These findings concur that variation has a bearing in breeding for resistance

5. CONCLUSIONS

This research confirms that *P. oryzae* from various rice growing regions in Kenya consists of variable populations based on cultural morphology, and molecular variation.

6. RECOMMENDATIONS

Future screening of resistance varieties to the blast disease of rice in Kenya should take into account all possible 'strains'. Further screening for strains should be carried out using molecular work

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