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5 **Real time portable genome sequencing for global food security**
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Abstract

36 The United Nations has listed Zero Hunger as one of the 17 global sustainable development goals to
37 end extreme poverty by 2030. Plant viruses are a major constraint to crop production globally causing
38 an estimated \$30 billion in damage ¹ leaving millions of people food insecure ². In Africa, agriculture
39 employs up to 50% of the workforce, yet only contributes 15% to the GDP on average ³, suggesting that
40 there is low productivity and limited value addition. This can be addressed through continued
41 innovation in the fields of science and technology as suggested in the Science Agenda for Agriculture in
42 Africa (S3A) ⁴. Sustainable management of plant viruses and their associated vectors must include
43 efficient diagnostics for surveillance, detection and identification to inform disease management,
44 including the development and strategic deployment of virus resistant varieties. To date, researchers
45 have been utilizing conventional methods such as; PCR, qPCR, high throughput sequencing (RNA-Seq,
46 DNA-Seq) and Sanger sequencing for pathogen identification. However, these methods are both costly
47 and time consuming, delaying timely control actions. The emergence of new tools for real-time
48 diagnostics, such as the Oxford Nanopore MinION, have recently proven useful for early detection of
49 Ebola ⁶ and Zika ^{7,8}, even in low resourced laboratories. For the first time globally, the MinION portable
50 pocket DNA sequencer was used to sequence whole plant virus genomes. We used this technology to
51 identify the begomoviruses causing the devastating CMD which is ravaging smallholder farmers' crops
52 in sub-Saharan Africa. Cassava, a carbohydrate crop from which tapioca originates, is a major source of
53 calories for over eight hundred (800) million people worldwide. With this technology, farmers
54 struggling with diseased crops can take immediate, restorative action to improve their livelihoods
55 based on information about the health of their plants, generated using a portable, real-time DNA
56 sequencing device.

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Main

59 The portable DNA technology has great potential to reduce the risk of community crop failure and help
60 improve livelihoods of millions of people, especially in low resourced communities. Plant diseases are a
61 major cause of low crop productivity and viruses such as Tobacco mosaic virus, Tomato mosaic,
62 Tomato spotted wilt, Potato leaf roll, Potato virus X and Y in Potato, Papaya mosaic, Citrus tristeza,
63 Chilli leaf curl, and Banana bunchy top have been implicated. In particular, cassava viruses are among
64 the world's greatest risk to food insecurity. Losses caused by cassava mosaic disease (CMD) and
65 cassava brown streak disease are estimated at US \$2-3 billion annually ⁵. We therefore visited
66 smallholder farmers in Tanzania, Uganda and Kenya (Table 1) who are suffering yield shortages due to
67 cassava virus infections. We utilized the MinION to test infected material and farmers were informed
68 within 48 hours of the specific strain of the virus that was infecting their cassava, and a resistant
69 cassava variety was deployed. The advantages of adopting this technology far outweigh the challenges
70 (see Table 2). Cassava mosaic begomoviruses were in high enough concentration that reads of whole
71 genomes were obtained without an enrichment step (Table 1). As expected the viral reads increased
72 with the severity of the symptoms observed (Table 1). We detected a dual infection for a leaf sample
73 with the severity score of 5 in Uganda. In addition, one asymptomatic plant in Tanzania had one viral
74 read detected. The shortest time to obtain a viral read was 15 seconds (severity score 5) and the
75 longest was 4h11m15s (severity score 1).

76 Additionally, MinION sequencing is superior to traditional methods of PCR identification given
77 its generation of whole genome sequences which enable the identification of the plant virus strain
78 even if it becomes mutated or divergent, as it is not biased using primers that rely on known virus

79 sequences. With regards to cassava, there are three major advantages of this technology. Firstly,
80 improved diagnostics are required and real-time whole genome sequencing will help develop
81 diagnostic primers that are up-to-date. Secondly, this technology will assist with the development of
82 resistant cassava varieties and will allow breeders to immediately test the varieties they are developing
83 against different viral strains. Lastly, it ensures the delivery of the correct healthy uninfected planting
84 material to farmers. In addition, we could detect virus in a plant before it showed symptoms (Table 1).
85 Utilizing traditional PCR methods three samples collected from Asha's field in Tanzania tested positive
86 for EACMVs and none were positive for ACMV. The asymptomatic sample from MARI tested negative
87 for both ACMV and EACMVs. Eight fresh cassava leaf samples from Uganda were dually infected with
88 ACMV and EACMV-UG using conventional PCR primers for ACMV and EACMV-UG. The primers used in
89 this PCR yield products of 1000bp and 1500bp for ACMV and EACMV-UG respectively. Twelve Kenyan
90 samples were tested and all but two (barcode 2 and 3) were positive using conventional PCR (Table 1).
91 Further studies are needed to verify our results regarding the sensitivity of the protocol for early
92 detection of CMD in cassava, but these results are very promising for ensuring farmers receive clean
93 planting material with early detection of viral infection.

94 Nanopore sequencing technology has wide applications globally, but in East Africa these
95 include: (a) crop improvement by screening for virus resistant germplasm and genetic diversity during
96 breeding, (b) indexing of cassava planting materials for virus presence or absence to ensure that only
97 clean materials in multiplication fields are distributed to farmers, (c) detection and identification of
98 alternative plant species for cassava-infecting begomoviruses, so that farmers are advised to remove
99 and/or grow crops away from such plants as a management strategy, (d) virus and biodiversity studies.
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Methods

102 **Sample collection and DNA extraction:** In Tanzania, three cassava mosaic disease (CMD) symptomatic
103 cassava leaf samples (Fig. 1, Table 1) were collected from the smallholder cassava farmer Asha
104 Muhammed's field in Bagamoyo. One more asymptomatic leaf sample was collected at Mikocheni
105 Agricultural Research Institute (MARI), Dar es Salaam. Seven CMD symptomatic plants were collected
106 from Naomi Kutesakwe's farm in Wakiso district in Uganda. Both Tanzanian and Ugandan samples
107 were collected in September 2017. Twelve samples from Kenya were collected in February 2018 from
108 various sources (Table 1). High quality DNA were isolated using CTAB method ⁹. Each DNA sample
109 (Table 1) was quantified and purity checked using a NanoDrop 2000c UV-vis Spectrophotometer
110 (Thermo Scientific, Wilmington, DE, USA) was used to check the purity and quantity of DNA for each
111 sample and results were recorded in Table 1.

112

113 **Nanopore library preparation and sequencing:** In Tanzania and Uganda, the Rapid Barcoding kit SQK-
114 RBK001 and 9.4.1 flow cells were used to process genomic DNA extracted using a standard CTAB
115 method. We utilized the Rapid Barcoding kit SQK-RBK004 with 9.4.1 flow cells in Kenya. DNA was
116 diluted to 700 ng as specified in the library protocol. The SQK-RBK001 (Sept 2017) and/or the SQK-
117 RBK004 (Feb 2018) protocols were performed as described by the manufacturer. In Tanzania and
118 Uganda, the MinION was run for 24 hours instead of the recommended 48 hours, and in Kenya we had
119 a total run time of approximately 17 hours due to power interruptions.

120

121 **Nanopore bioinformatics:** In Tanzania and Uganda, Albacore 2.0.2 was used for base calling. In Kenya,
122 Albacore 2.1.10 was used and the scripts were modified to reflect the newest rapid barcoding kit

123 RBK004. Fastq files were imported into Geneious ¹⁰ and a local blast database of all known cassava
124 mosaic begomovirus whole genomes were downloaded from GenBank and a local blast was performed
125 on each of the reads generated using the nanopore device. Results of the local blast were confirmed
126 using blastx on Magnus supercomputer located in Perth, Australia.

127

128 **Verification of nanopore results:** Traditional PCR was used to verify our nanopore results. In Tanzania
129 and Kenya, two primer pairs: EAB 555F/EAB 555F ¹¹ and JSP001/JSP002 ¹², which amplify 556bp and
130 774bp were used to detect East African cassava mosaic begomoviruses (EACMVs) and African cassava
131 mosaic begomoviruses (ACMVs), respectively. In Uganda, the presence of ACMV and EACMV in each
132 sample was detected using a pair of specific primers for ACMV, ACMV-AL1/F and ACMV-ARO/R and
133 specific for EACMV-UG2, UV-AL1/F and ACMC-CP/R3 ¹³.

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138 **Author contributions:** Designed the study: LMB, JN, TA, FT, PS, MK, AS, EA. Carried out experiments:
139 LMB, AG, BD, JW, MR, JN, PS, CK, DM, JE, HB, TA, GO, PA, JO, JA, EA, BM and SK. Analysed data: LMB,
140 AS, CK, DM, JE, HB, SL, JN, PS, TA, GO, PA, JO, JA, EA, BM. Contributed to the writing: All authors
141 contributed to the writing of the manuscript.

142 **Competing interests:** The authors have no competing interests.

References:

- 1 Subramanya Sastry, K. & Zitter, T. A. in *Plant Virus and Viroid Diseases in the Tropics Vol. 2 Epidemiology and Management* 149–480 (2014).
- 2 Legg, J. P. & Thresh, J. M. Cassava mosaic virus disease in East Africa: a dynamic disease in a changing environment. *Virus Res* **71**, 135-149 (2000).
- 3 AFDB. African Development Bank. Annual Report., (2016).
- 4 FARA. Science agenda for agriculture in Africa (S3A): “Connecting Science” to transform agriculture in Africa. . *Forum for Agricultural Research in Africa (FARA), Accra, Ghana* (2014).
- 5 Scholthof, K. B. *et al.* Top 10 plant viruses in molecular plant pathology. *Mol Plant Pathol* **12**, 938-954, doi:10.1111/j.1364-3703.2011.00752.x (2011).
- 6 Quick, J. *et al.* Real-time, portable genome sequencing for Ebola surveillance. *Nature* **530**, 228-232, doi:10.1038/nature16996 (2016).
- 7 Faria, N. R. *et al.* Mobile real-time surveillance of Zika virus in Brazil. *Genome Med* **8**, 97, doi:10.1186/s13073-016-0356-2 (2016).
- 8 Quick, J. *et al.* Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nat Protoc* **12**, 1261-1276, doi:10.1038/nprot.2017.066 (2017).
- 9 Lodhi, M. A., Guang-Ning, Y., Weeden, N. F. & Reisch, B. I. A simple and efficient method for DNA extraction from grapevine cultivars, Vitisspecies and Ampelopsis. *Plant Mol Biol Rep* **12**, 6-13 (1994).
- 10 Kearse, M. *et al.* Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647-1649, doi:10.1093/bioinformatics/bts199 (2012).

11 Fondong, V. N. *et al.* Evidence of synergism between African cassava mosaic virus and a new double-recombinant geminivirus infecting cassava in Cameroon. *J Gen Virol* **81**, 287-297, doi:10.1099/0022-1317-81-1-287 (2000).

12 Pita, J. S., Fondong, V. N., A, S., RNN, K. & Fauquet, C. Genomic and biological diversity of the African cassava geminiviruses. *Euphytica* **120**, 115-125 (2001).

13 Zhou, X. *et al.* Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *J Gen Virol* **78 (Pt 8)**, 2101-2111, doi:10.1099/0022-1317-78-8-2101 (1997).

Table 1. Nanopore results of cassava mosaic begomovirus-infected cassava leaves from Tanzania, Uganda and Kenya. Disease severity described in Legg et al. 2000 where 1 is healthy and 5 shows severe symptoms of the disease including leaf distortion and stunting of the plant (Figure 1).

Farmer	CMD Severity	Lab ID	Barcode	Location	Concentration ng/uL	Run Time	Time to first geminiviral read	# Nanopore reads	# Viral Reads	PCR Result	Length of 1 st virus read (bp)	Closest Species Match
Tanzania												
Asha	3	3	1	Kiromo-Kitonga Bagamoyo	3,378.40	24 hrs	47s	63,653	950	+	969	EACMV-KE
Asha	2	5	2	Kiromo-Kitonga Bagamoyo	3,385.20	24 hrs	32m57s	8,844	43	+	971	EACMV-KE
Asha	4	6	3	Kiromo-Kitonga Bagamoyo	4,845.20	24 hrs	28s	32,540	338	+	775	EACMV-KE
Asha	1	8	4	MARI	1,752.70	24 hrs	4h11m15s	20,005	1	-	2213	EACMV-KE
Uganda												
Naomi	4	1	1	Wakiso	603.2	24 hrs	15s	9,859	244	+	957	EACMV-UG
Naomi	3	2	2	Wakiso	711.3	24 hrs	43s	8,482	98	+	780	EACMV-UG
Naomi	5	3	3	Wakiso	603.6	24 hrs	17m14s	5,562	298	+	801	EACMV-UG AND ACMV-UG
Naomi	2	4	4	Wakiso	598.2	24 hrs	11s	17,146	371	+	789	ACMV-UG
Naomi	3	5	5	Wakiso	610	24 hrs	33s	7,999	250	+	2264	ACMV-UG
Naomi	1	6A	6	Wakiso	448	24 hrs	2h14m50s	9,972	18	+	920	ACMV-KE
Naomi	3	6B	7	Wakiso	490.9	24 hrs	2m52s	9,662	119	+	1418	ACMV-KE
Naomi	1	7	8	NaCRRI	710.6	24 hrs	-	7,881	none	-	-	none
Kenya												
Rose	2	13	12	Eldoret	44.3	17hr 17min	3h4m9s	30	22	+	754	ACMV-KE
Lungo Mutunga	1	1	1	Ngoliba	2433.4	17hr 17min	20m17s	3,298	1,760	+	1145	ACMV-NG AND EACMV-UG
Lungo Mutunga	1	2	2	Ngoliba	2624.2	17hr 17min	37m23s	3,232	1,838	-	1778	ACMV-UG AND EACMV-UG
Joel Maina	1	3	3	Ngoliba	1812.5	17hr 17min	10m3s	3,787	2,108	-	2723	EACMV-UG
Rainbow Hotel	1	5	4	Ruiru	3066.3	17hr 17min	57m9s	2,536	1,423	+	905	EACMV-UG
JKUAT	3	NF19C2	5	Siaya	767.59	17hr 17min	50s	2,672	1,493	+	1985	ACMV-KE
JKUAT	4	CF13B2	6	Kilifi	1143.7	17hr 17min	28s	3,870	2,221	+	847	EACMV-KE AND ACMV-KE
JKUAT	3	EF23C2	7	Kitui	1213.8	17hr 17min	18m6s	2,964	1,629	+	766	EACMV-KE
JKUAT	3	EF20C1	8	Kitui	1023	17hr 17min	54m2s	5,120	2,906	+	1887	EACMV-UG
JKUAT	3	Wf2S1	9	Vihiga	1111.9	17hr 17min	23m7s	5,095	3,021	+	2233	ACMV-NG AND EACMV-UG
JKUAT	3	WF20C2	10	Busia	929.36	17hr 17min	36m1s	4,652	2,838	+	1966	ACMV-UG AND EACMV-UG
JKUAT	3	CF36C2	11	Taita taveta	1032.3	17hr 17min	55m	4,366	2,541	+	2089	EACMV-KE

Table 2. Advantages and challenges of using the MinION portable DNA sequencer in Tanzania, Uganda and Kenya.

Advantages	Challenges
<ol style="list-style-type: none"> 1. Rapid – obtain results timely to support quick decision making on disease management 2. Expenses on sending samples for sequencing abroad removed 3. High resolution of results and reliability 4. Detection of mixed infections 5. Detection of virus in latently infected plants 6. Discovery of unknown viruses 7. Minimising sample degradation and loss during shipping 8. Virus-indexing for safe movement of germplasm 9. Better plant health regulation, inspection/phytosanitary – improved international trade 	<ol style="list-style-type: none"> 1. Power 2. Internet 3. Cost 4. Computer access 5. Shipping



Figure 1. Cassava plant infected with cassava mosaic begomoviruses. Photo credit: Dr. Ndunguru.