Proposed EPPO validation of plant viral diagnostics using next generation sequencing

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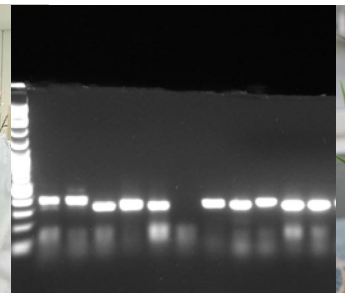
Adoption of Next Generation Sequencing for routine viral diagnosis?

- Cost now competitive with alternative approaches (Sap inoculations, EM, ELISA, Real time PCR, degenerate primers)
- Find the unexpected
- Latest Equipment designed for low staff input (MiSeq)
- Machine derived contamination
- Accreditation?




Not what you expect?

Seed Testing

Pepino mosaic virus, *Tobacco mosaic virus, Cucumber mosaic virus, Tomato bushy stunt virus, Potato leafroll virus*. Novel cavemovirus

Fox, A., Adams, I.A., Hany, U., Hodges, T., Forde, S.M.D., Jackson, L.E., Skelton, A. and Barton, V. (2015), *Seed Sci. & Technol.*, **43**, 3, 1-5. http://doi.org/10.15258/sst.2015.43.3.06

Research Note

The application of Next-Generation Sequencing for screening seeds for viruses and viroids

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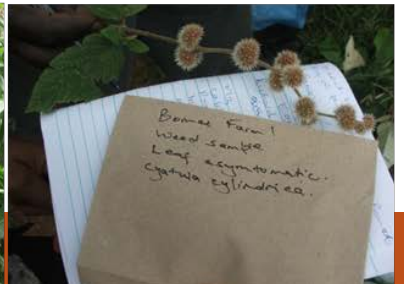
Kenyan Maize virome project

	Crop	samples	known plant viruses	Novel plant viruses	total samples
Farm 1	maize	4	Maize chlortic mottle virus, Sugarcane mosaic virus, Maize yellow dwarf mosaic virus	Tombusvirus, Carmovirus, Foveavirus, Closterovirus, betaflexivirus, positive strand ssRNA virus	30
	others	26	bean comon mosaic virus, Beet pseudoyellows virus, Maize yellow dwarf mosaic virus, SCMV, Potato virus S	Caulumoviridae virus, Chrysovirus,Crinivirus, Potyvirus(es), Tombusvirus, unclassified ssRNA positive strand virus, Varicosavirus, Filoviridae virus	
Farm 2	maize	9	Maize chlortic mottle virus	chrystovirus luteovirus ,Carmovirus, tombusvirus, virus, positive strand ssRNA virus, unclassified virus	29
	others	20	Shallot latent virus, Cauliflower mosaic virus,	Chrysovirus, Crinivirus, Cytorhabdovirus, Waikavirus, Varicosavirus polerovirus, polerovirus associated RNA, Tymoviradae virus, positive strand ssRNA virus	
Farm 3	maize	6	Maize chlortic mottle virus, Maize yellow dwarf mosaic virus	Badnavirus, polerovirus associated RNA, Tymoviradae virus	26
	others	20	Turnip mosaic virus,	Badnavirus, Chrysovirus, Cytorhabdovirus, unclassified ssRNA positive strand virus	
Farm 4	maize	4	Maize chlortic mottle virus, Maize yellow dwarf mosaic virus	none	30
	others	26	Banana streak virus, Apple stem grooving virus, Citrus tristeza virus	Badnavirus, potyvirus(es), Tombusvirus, Rhabdoviridae virus, positive strand ssRNA virus, unclassified virus, Varicosavirus	
				total	115

biosciences

eastern and central africa



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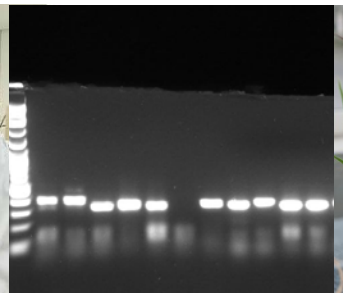


Stream lined process

Infected plant

Total RNA
(Rneasy-Qiagen)

Ribosome subtraction
RiboZero-Eppibio)

epicentre

Library prep
(scriptSeq, Epicentre)

epicentre

Sequence MiSeq 2x300bp V3 (Illumina)

Quality trim sequence (Sickle)

Assemble (Trinity)

RNA-Seq De novo Assembly Using Trinity

BLAST x+n

NCBI BLAST

MEGAN

454: 7 days turnaround (6 days hands on staff time)
MiSeq: 5 days turn around (2 days hands on staff time)

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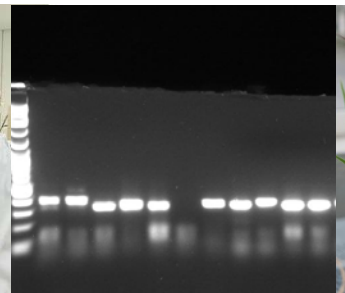


Contamination

Inter-run contamination

Sample sipper fixed in MiSeq

Standard tween wash x3 = 0.02% carry over from previous runs (5000 reads)

Not a problem for genome sequencing as contamination lost in sequencing depth. IS problem for disease diagnostics

- New bleach wash. Now 0.0001% (25 reads)
- Cycle indexes so no index in consecutive runs (48 indexes)

Intra run contamination

Contaminant	sample 1	sample 2 (PVX)	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8	sample 9	sample 10	sample 11	sample 13	sample 14	sample 15	sample 16	sample 17	sample 18	sample 19	sample 20	sample 21	sample 22	sample 23	sample 24	Control
phiX	7015	10161	6716	5686	3466	8957	8062	8226	4089	10659	6598	12031	13495	6268	21547	5460	12294	9452	12243	6897	7538	10382	4173	13036
PVX	3912	1521366	10710	550	2255	3201	63	84	51	40	21	52	44	62	230	15	50	177	65	46	54	43	16	643

- Currently using Real time PCR to confirm any significant results
- Developing solution

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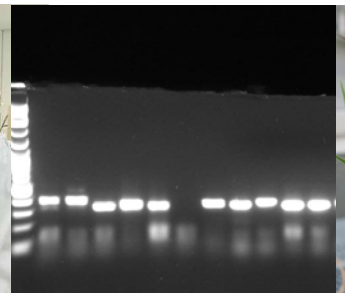



EPPO validation?

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/98 (1)

Diagnostics
Diagnostic

Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity[1]

Specific scope

This guideline includes specific quality management requirements for laboratories preparing for accreditation according to the ISO/IEC Standard 17025 *General requirements for the competence of testing and calibration laboratories* (references to relevant parts of ISO/IEC Standard 17025 are included). It should be noted that in EPPO standards the verb 'should' carries the highest level of obligation.

Specific approval and amendment

First approved in 2009–09.

1. Introduction

Development of quality management systems (also referred to as management systems or quality systems) and accreditation have become a concern for many laboratories in the EPPO region. A Standard PM 7/84 *Basic requirements for quality management in plant pest diagnosis laboratories* was adopted in 2007. PM 7/84 describes basic requirements to assist laboratories conducting plant pest diagnosis in designing their management sys-

2. Scope of accreditation: fixed scope and flexible scope

Historically, the accreditation of laboratories has been usually based on a fixed scope which should define clearly and unambiguously the range of tests covered by the laboratory's accreditation (e.g. immunofluorescence test for the detection of *Ralstonia solanacearum* on potato tubers). However, this does not readily allow new or modified tests to be added to a laboratory's scope,

EPPO validation

- Can’t validate discovery of a new virus, Can validate the methods, controls and monitoring

- Controls
- Analytical sensitivity
- Analytical specificity
- Repeatability
- Reproducibility

Controls

Positive / Negative / extraction

- Negative control / extraction control

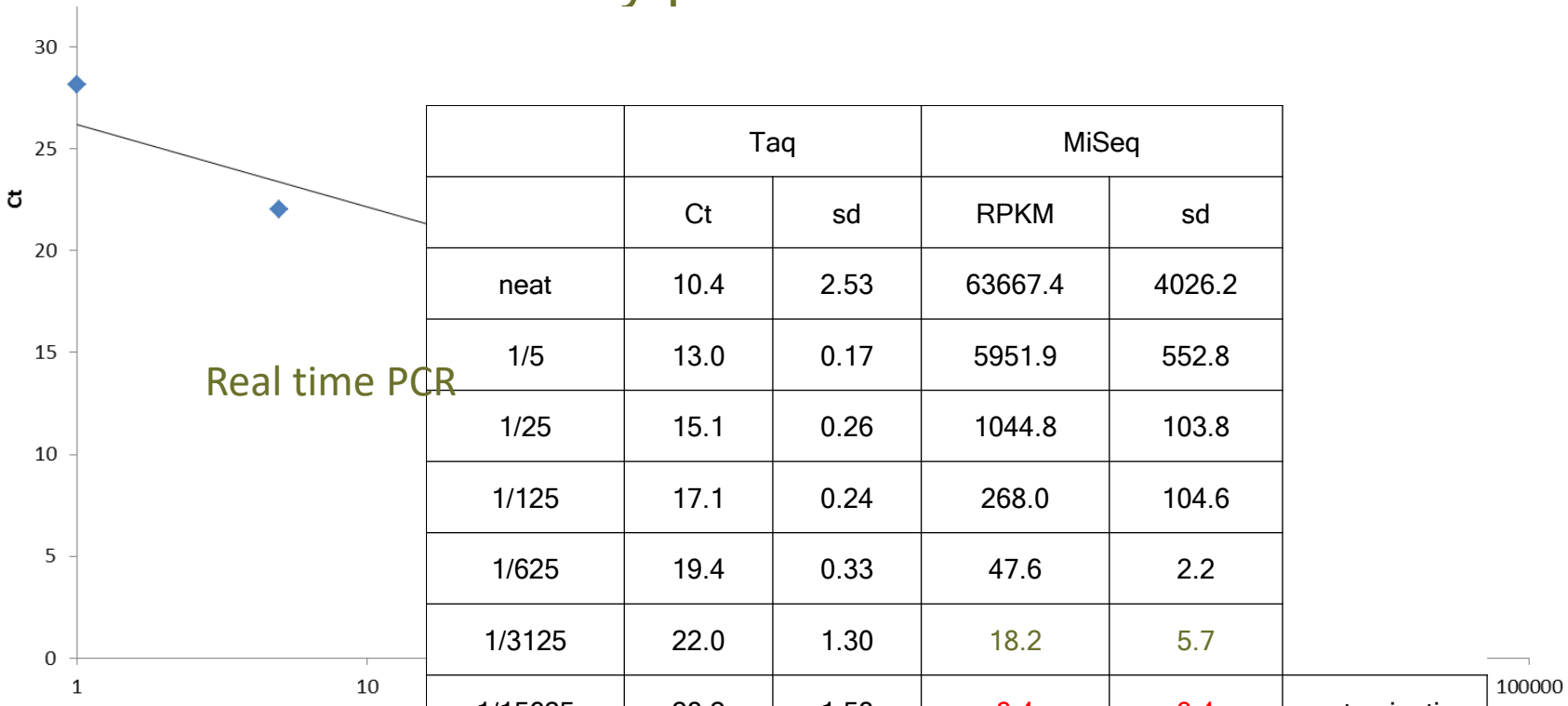
  “healthy plant” taken through whole process

- Positive control

  Difficult to get standard virus positive. Use ERCC spike in control (artificial mix of mRNAs)

sensitivity

Dilution series of virus infected plant in healthy plant

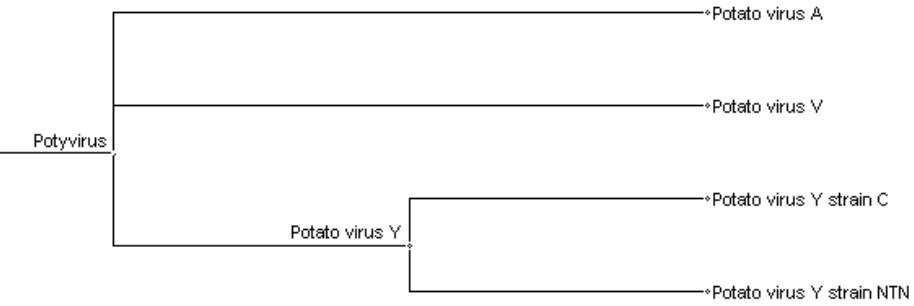


	Taq		MiSeq		
	Ct	sd	RPKM	sd	
neat	10.4	2.53	63667.4	4026.2	
1/5	13.0	0.17	5951.9	552.8	
1/25	15.1	0.26	1044.8	103.8	
1/125	17.1	0.24	268.0	104.6	
1/625	19.4	0.33	47.6	2.2	
1/3125	22.0	1.30	18.2	5.7	
1/15625	28.2	1.58	8.4	3.4	contamination
healthy control	45.0	0.00	10.3	2.6	0.01%

specificity (Targets / Non-targets)

Ability to differentiate different strains:

- PVY is potyvirus with multiple variants
  - PVY-C, PVY-NTN (strain separation)
  - other potyvirus (PVA,PVV) (species separation)
  - Multiple viroids (CLVd, TCDVd) (species separation)



Repeatability

- Repeat PVY / CLVd detection at LOD

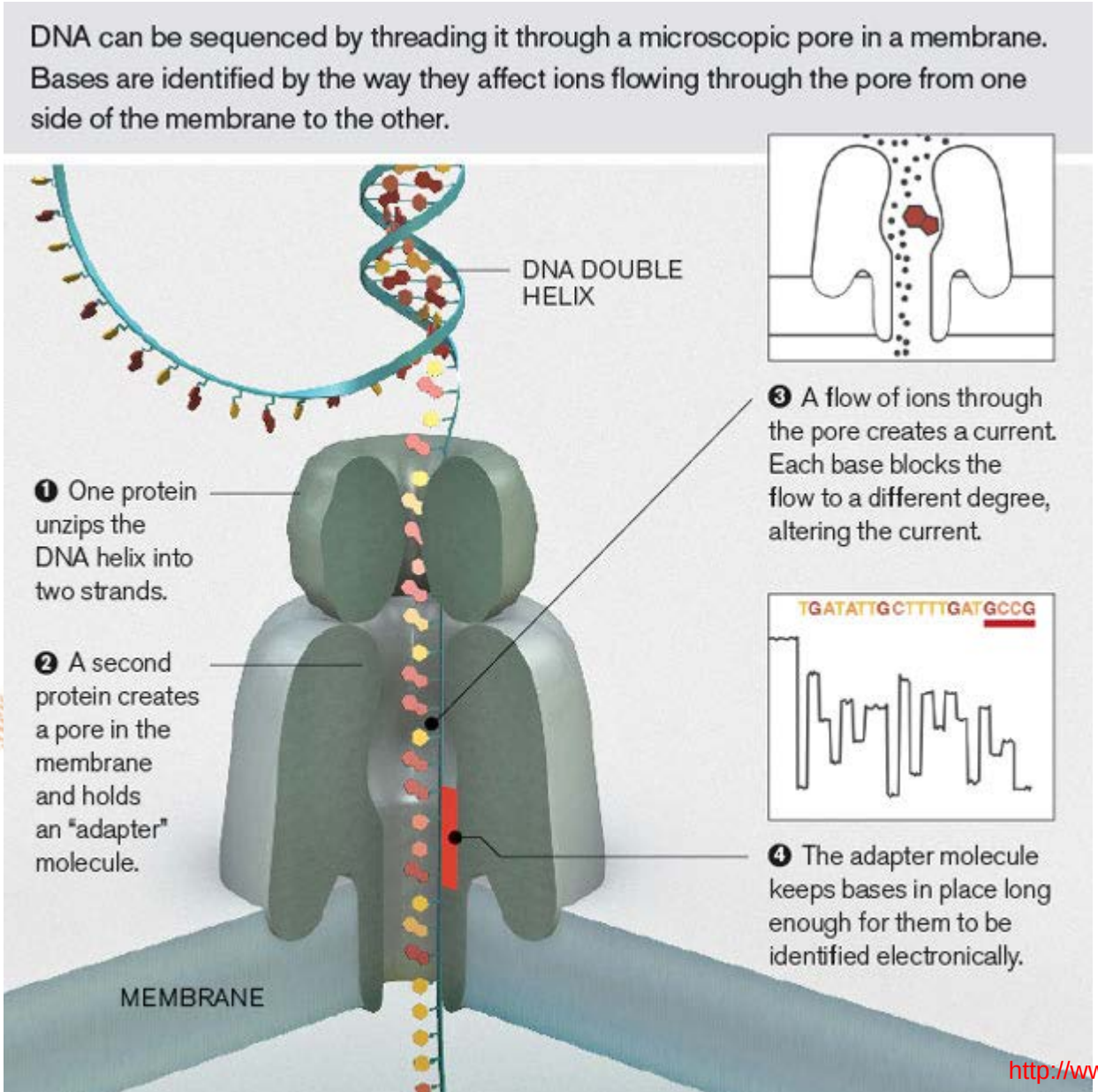
Reproducibility

- Operator: same samples taken through process by two different people
- Machine : Same sample sent to second site for sequencing.
- Bioinformatics : Same sequence dataset run through different servers (repeat analysis on regular basis to confirm server software / database updates haven't affected results)

Conclusion

- Promising method
- Proposed validation method for consideration

Oxford nanopore MinION



Oxford Nanopore MinIon
4-50 kb x thousands

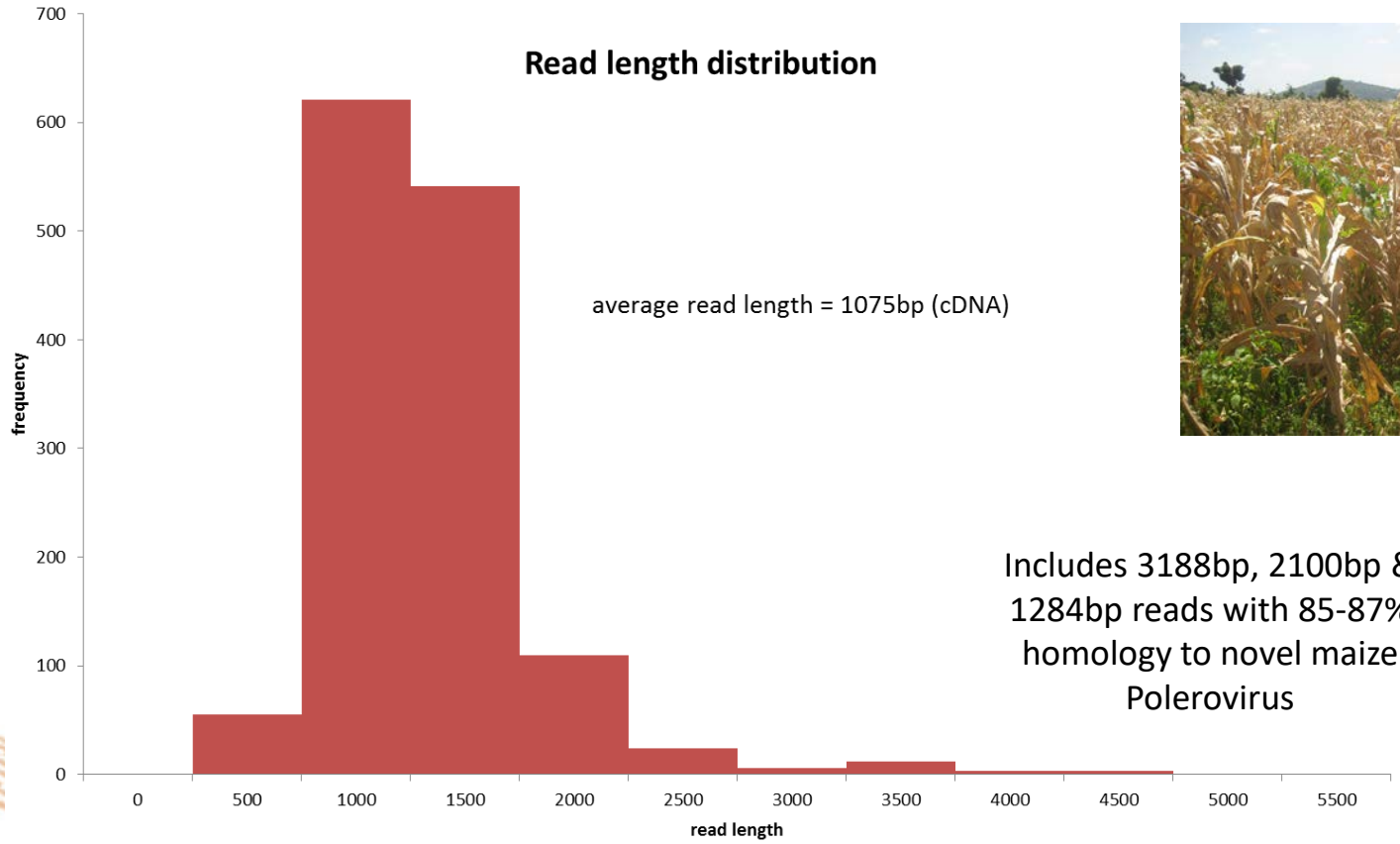
Small, cheap, very long reads, high error rate (15%)

http://www.technologyreview.com.br/article/40246/

What are the plant health applications for the MinION?

- Rapid viral diagnostics (Field*ish*)?
- Finishing bacterial genomes?
- Much longer DNA barcodes?

Maize virus sequencing

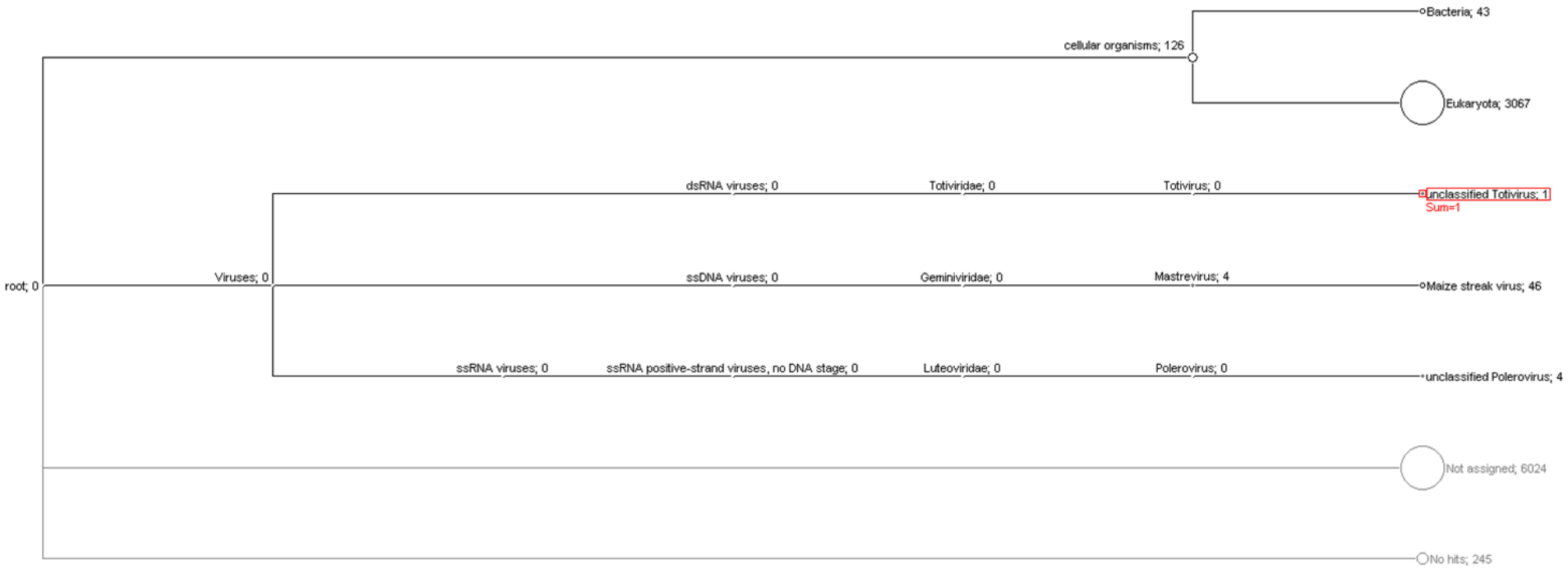


average read length = 1075bp (cDNA)

Includes 3188bp, 2100bp & 1284bp reads with 85-87% homology to novel maize Polerovirus

- MinION identifies know viruses with large genome fragments
- high error rate (15%)

MinION against MiSeq



Analysis	MiSeq	MinION
Virus detection	1 known, **4 novel**	1 know, **2 novel**

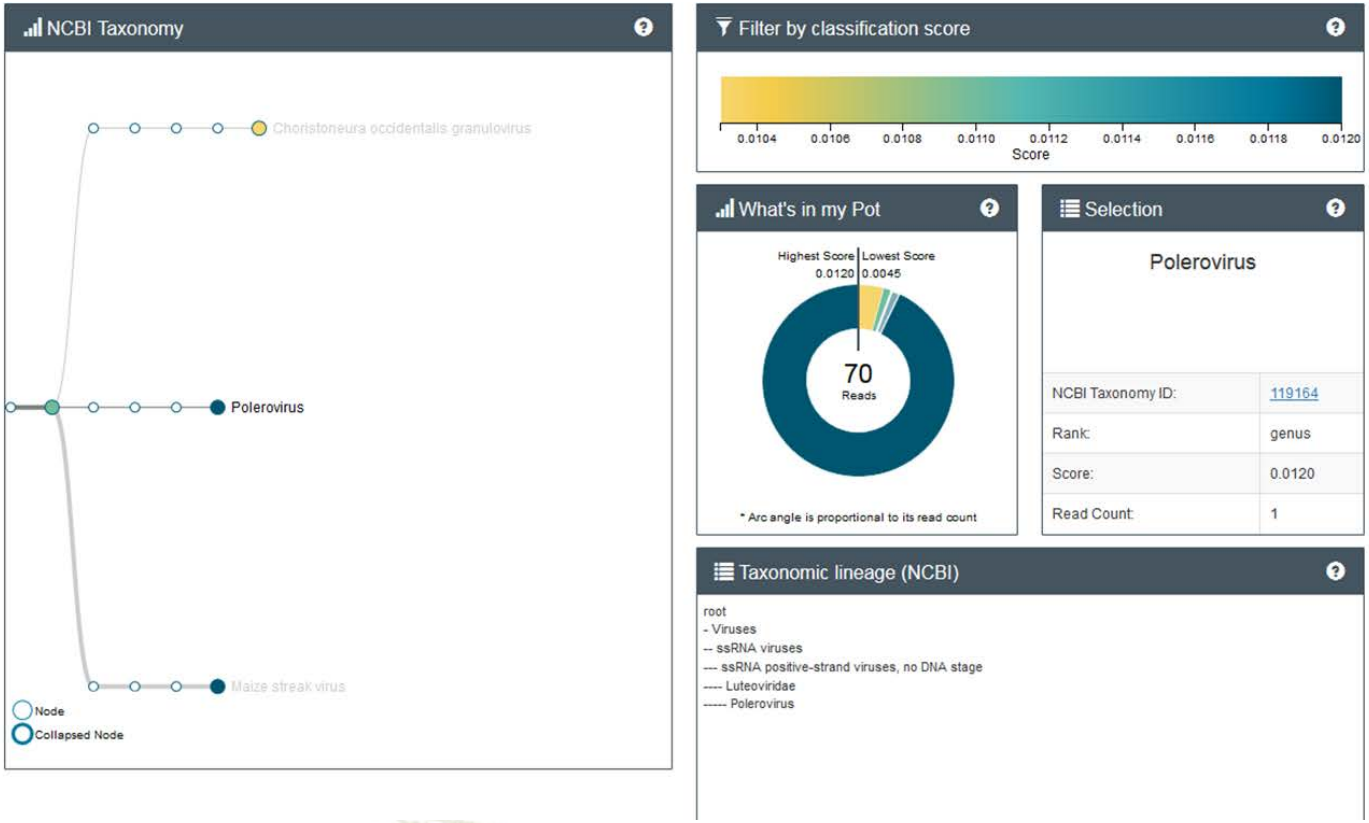
Reference Mapping

MiSeq (BWA) / MinION (LAST)

Virus Sequence	RPKM MiSeq (BWA)	MinION (LAST)
Maize Yellow Streak virus	7615	11289
Novel Polerovirus	347	381
Novel unclassified virus	1220	735
Novel dsRNA virus RNA1	2847	656
Novel dsRNA virus RNA2	24280	5943
Novel Totivirus RNA1	2619	2056
Novel Totivirus RNA2	146	375
Novel Totivirus RNA3	211	291

MinION realtime analysis(WIMP)

- Still early days



Detects: 1 know and 1 novel virus, misses 3 other novel viruses)

Better sample prep?

Current method requires couple of hours in the lab. cDNA method even longer.

VolTRAX-automated sample preparation

VolTRAX, currently in development, is a cartridge designed to convert a complex sample to a form ready for a nanopore sensor, without the need for human intervention. This miniaturised laboratory will use technology common to consumer electronics, adapted for molecular biology. VolTRAX will have the capacity to process multiple samples at a time

Conclusion

- MinION now works (most of the time)
- Error Rate still problem
- Possible to sequence plant viruses
- Real time analysis is under development

Acknowledgements

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