

Development of a Loop-Mediated Isothermal Amplification assay for rapid detection of *Fusarium oxysporum* f.sp. *cubense* tropical race 4 through Diversity Array Technology sequencing

Eppo Meeting Angers; Dec 1, 2015



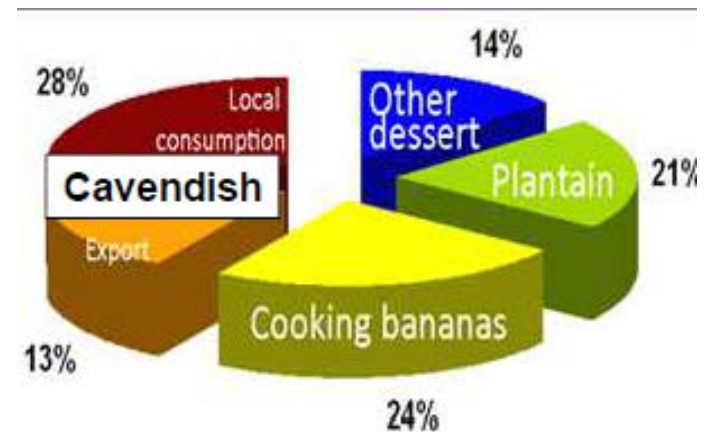
WAGENINGEN UR
For quality of life



PANAMA
DISEASE

Banana: plant at risk

- Banana is the most important food in terms of production value after rice, wheat and maize
- About 107 million tons of bananas produced / year
- >150 countries
 - 13% is exported (>95% Cavendish)
 - 87% is consumed



Fusarium oxysporum complex



Non-pathogenic strains



Pathogenic forms

>150 plant pathogenic,
divided into
formae speciales

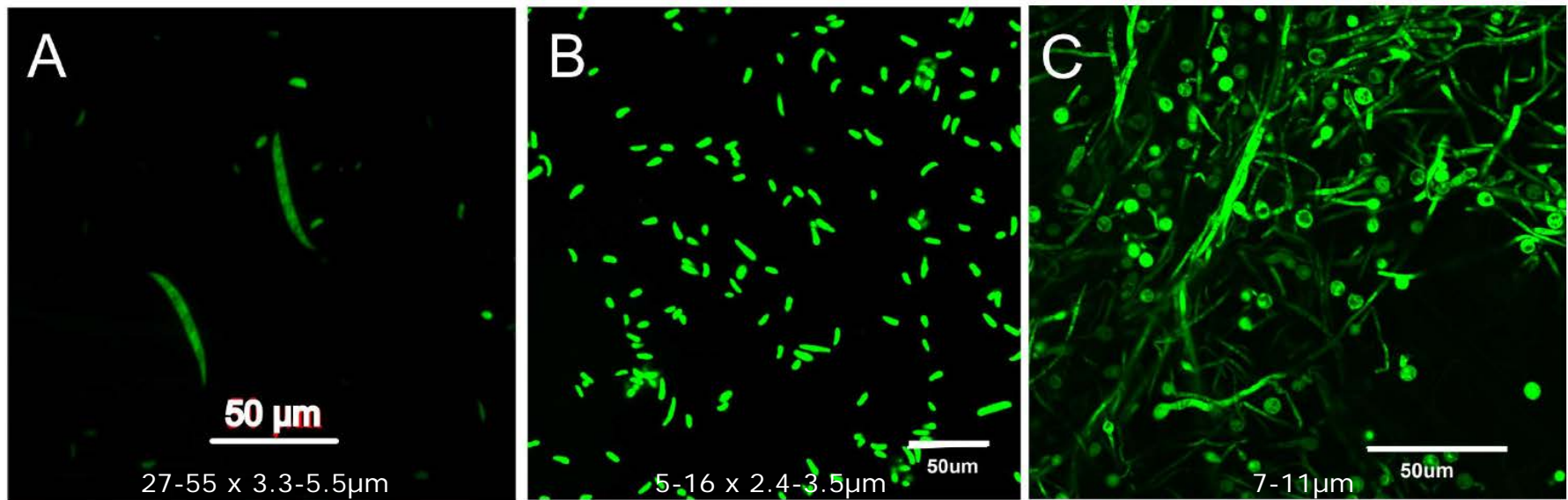


f.sp. cubense



Fusarium oxysporum f.sp. *cubense* (Foc)

- causal agent of Fusarium wilt/Panama disease
- soil-borne (30-50 years in soil)
- asexual fungus (asexual spores)



Guo et al. Plos One, Vol 9:4. April 2014



Fusarium oxysporum diversity

Diversity: Races & Vegetative Compatibility Groups (VCG)

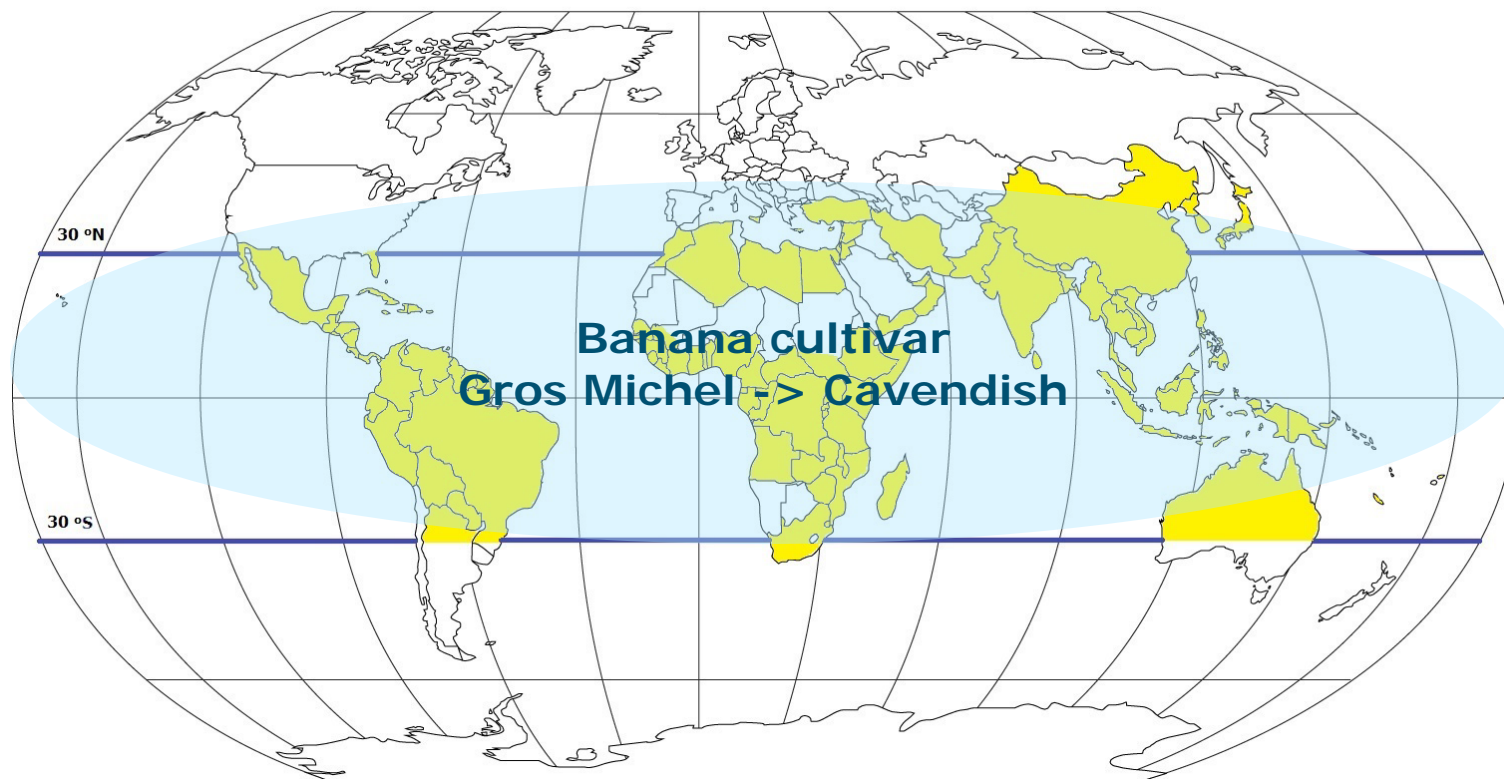
Races, based on specific banana cultivars that they can infect

Banana cultivar	Race 1	Race 2	Race 4	
			ST4	TR4
Gros Michel (AAA)	Susceptible	Resistant	?	Susceptible
Bluggoe (ABB) Cooking type	Resistant	Susceptible	?	Susceptible
Cavendish (AAA)	Resistant	Resistant	Susceptible	Susceptible



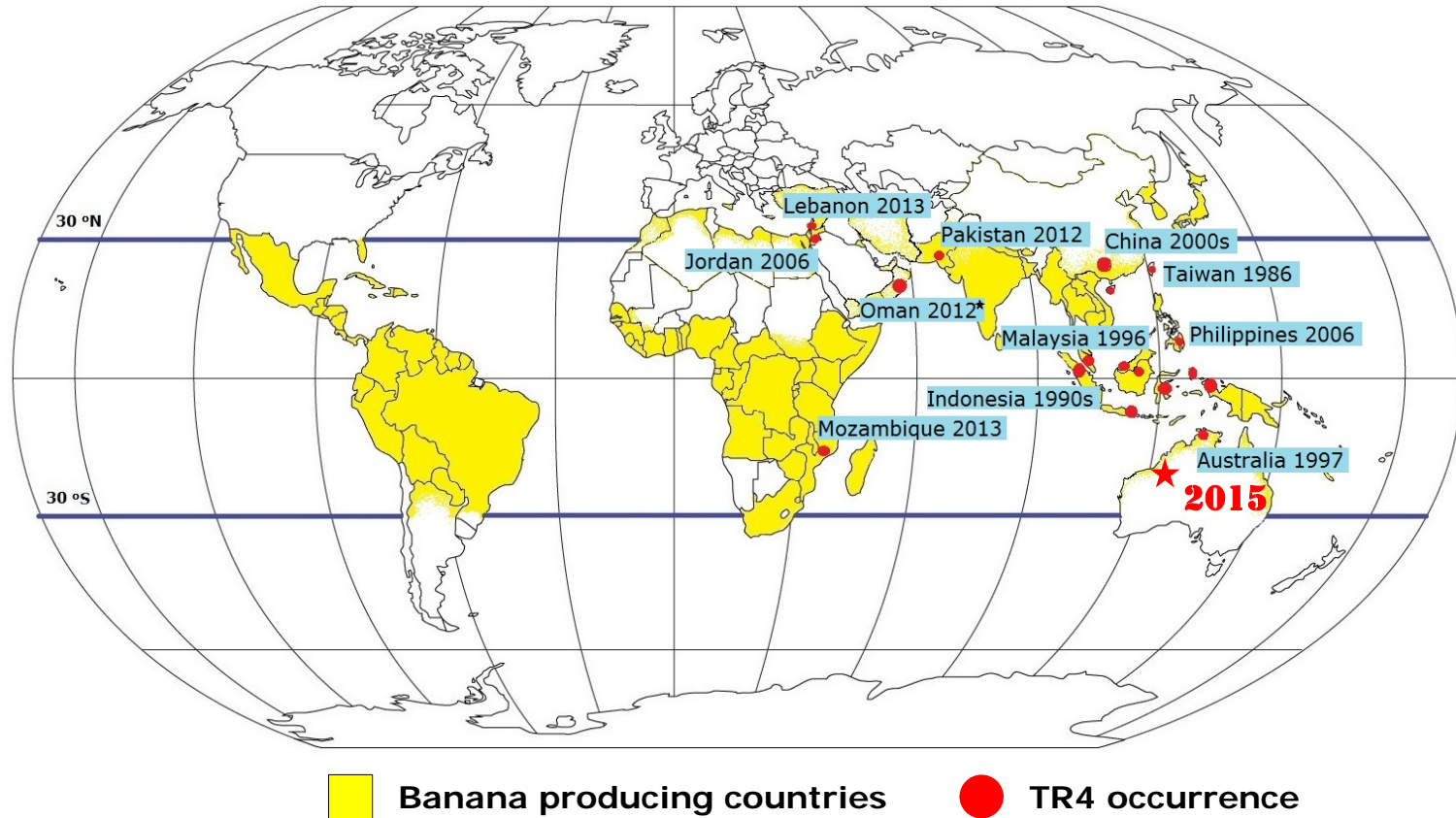
Global distribution of *Fusarium* wilt (Race 1) disease

Threat to Gros Michel Banana Production



Global distribution of *Foc* TR4

Threat to Cavendish Banana Production



Foc TR4 devastation in the Philippines

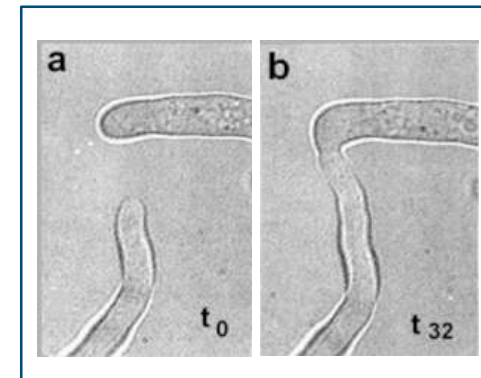
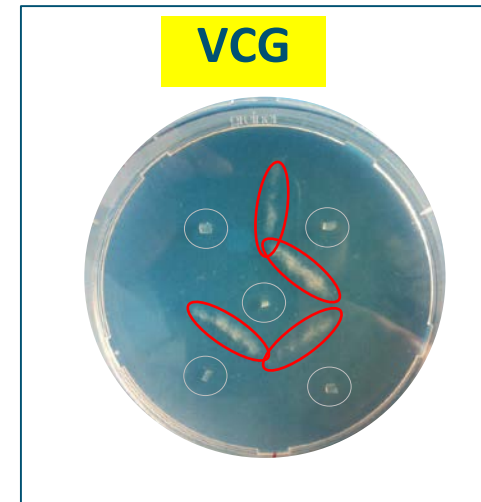


Photos credit to B.N. Corcolon



Fusarium oxysporum f.sp. *cubense* classification

<i>Races</i>	<i>Vegetative Compatibility groups</i> 24 reported VCGs
Race 4 -Cavendish AAA [TR4 ST4	01213/16 0120, 0121, 0124, 0129
Race 1 -Gros Michel AAA	0120, 0124/5, 0125, 0126, 01210
Race 2 -Plantain ABB & cooking banana	0123, 0124 0122, 0128



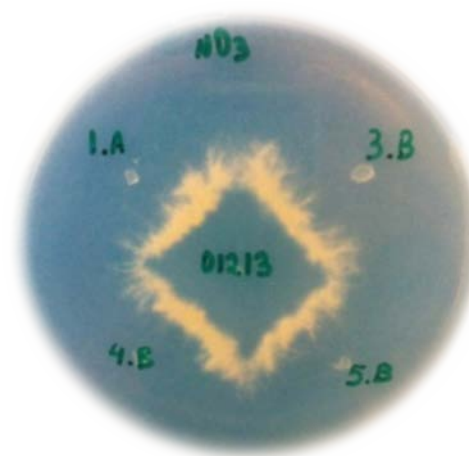
Foc TR4 diagnostics

Visual inspection



Disadvantage
Not accurate diagnosis due to similar symptoms produce by other factors (e.g. Moko disease)

Vegetative Compatibility Groups



Disadvantage

- Time consuming ~1 month
- *nitM* tester not always available
- Self-incompatible isolates

Molecular based probes



Disadvantage

- Laboratory based-methods
- Highly skilled workers
- DNA extraction inhibitors



Aim: Detection of unique genomic regions using DArTseq for *on-site* TR4 specific probe development

DArTseq

- Whole-genome fingerprint tool, without relying on prior sequence data information (e.g. 15% genome coverage for *Foc*)
- Steps includes genome complexity reduction concept, via combination of Restriction Enzymes and sequencing of the representations on Illumina
- Selection of unique and low-copy sequences (e.g. 69bp), by in silico hybridisations

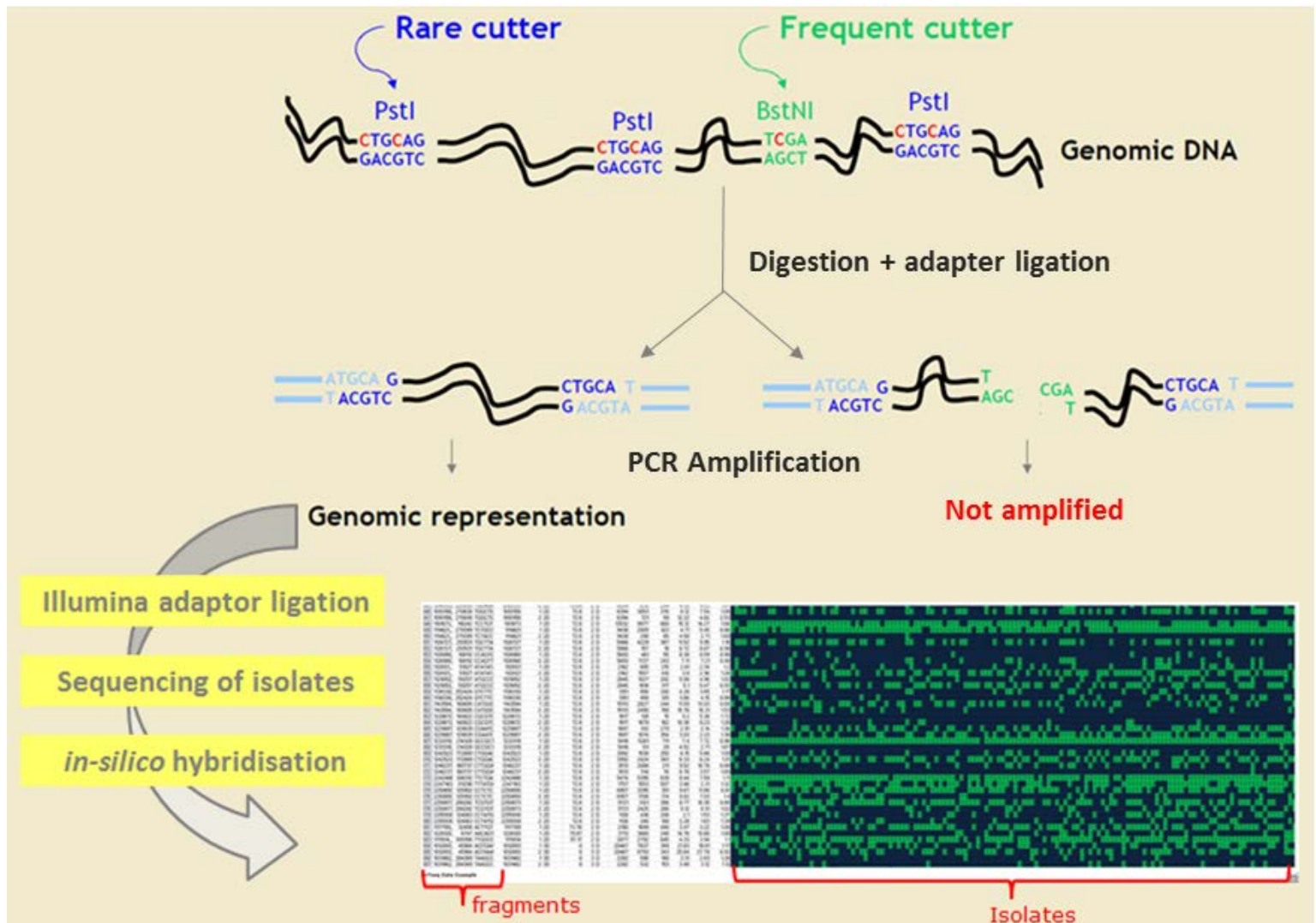
DNA extraction on:
29 *Foc* isolates
All 24 VCGs and 4 races



Data	#Markers
DArTseq	15 905



DArTseq principle

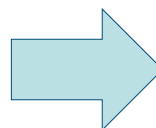


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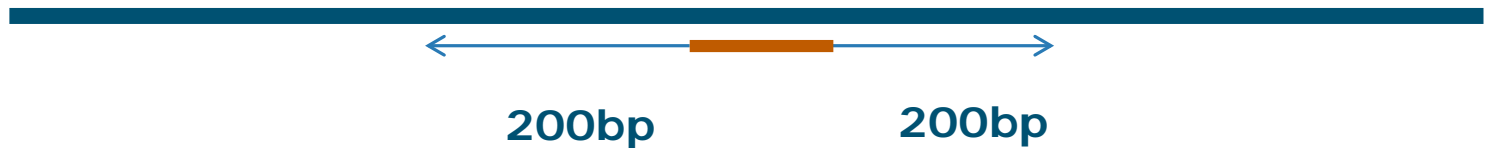


Selection of unique DArTseq TR4 fragments

- 80 Unique DArTseq fragments (69bp each) for TR4 from Silicon data
- After blasting against all Foc and Fo genomes 12 Unique DArTseq TR4
- LAMP primer design relies on a genomic region of around 400 bp

On-Site detection

TR4
genome
sequence

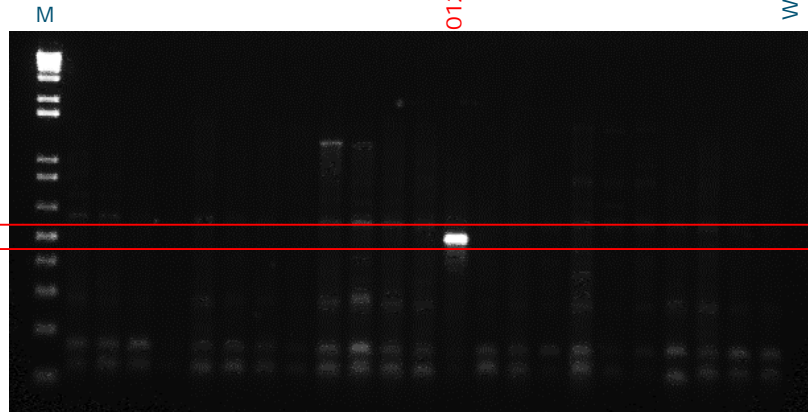


Selection of unique DArTseq TR4 fragments

Seq A

Foc 24 VCGs

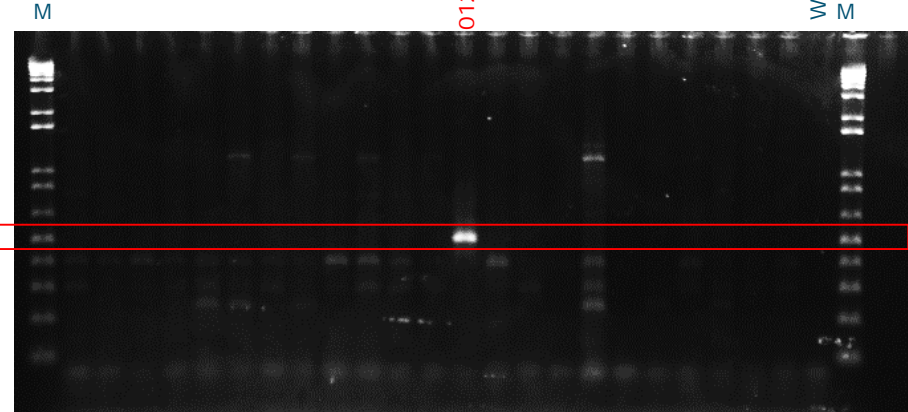
Water (-)



Seq B

Foc 24 VCGs

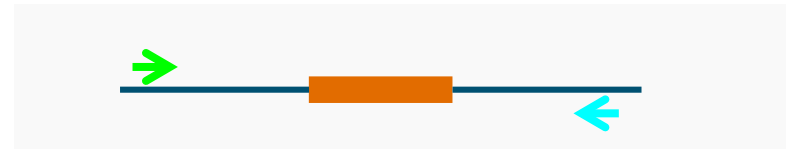
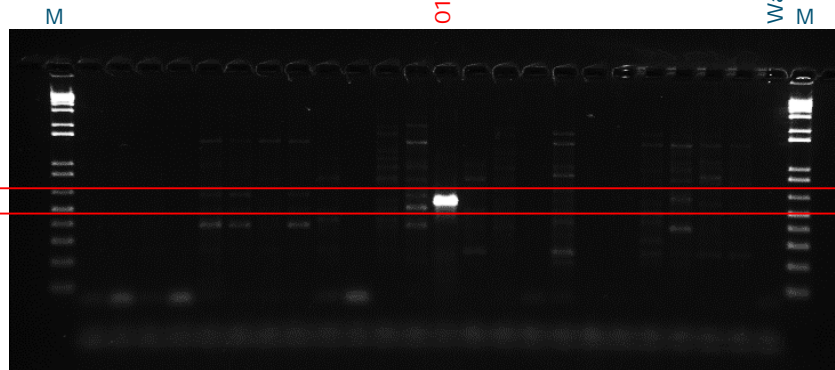
Water (-)



Seq C

Foc 24 VCGs

Water (-)



>F. oxysporum II5 supercont1.16 of Fusarium oxysporum II5 [DNA] 336830-337398 -

```
AGGTACGGCGGAAGAAGAAGACCAAACACAAGGAAATTGAGCAATGGGAGGAAGAACCTTCTAGTATGAAGACGTA
TTCCTTTACCAAAGCCTAGGAGAGGATTCAACATTACATCCCATTCTCTAGAAAGTTGAGCATCTTTACTATT
TATACATTCATTTTACCCGGAAGAAATTAATAACAATAAGTGATACCCCTGCATGCAGTGAGCCAGTAAGCTTTTATTT
TATCAGGTAACCATCACTGCAGCTTGTAAATATGCTATGGGATATTGTCAAGTCAAGCAGGAGAAATAATTACTGCA
ATACTACTAATTGAAAGGGATTCAAAGACCGGACCAAAAGAAATTTATAATAAGGGTCAAGAGATTTGCAGCAAGA
AAACTGGCTTTGTTTTTCATGATGATGTTTTCTCACCATGTAATCCGTTAAGGAAGTTCTATTACATCTTTCATAT
CAAAGTCTATATCCAAGCAATGATAAATCTAAGACAATCCATCAAGCGCCAAAGAACTCATCCGCATCAAAGAATTC
ATCCGATCTTCAACCTCGTCCGAC
```



On-site diagnosis development on unique TR4 region

Loop-mediated Isothermal Amplification-LAMP



- DNA amplification takes place at an isothermal condition
- Specific amplification to recognize 6 distinct regions on the target region
- Costly and time effective
- Not highly skilled personnel required

Validation:

Specificity

23 TR4 isolates

45 non target DNA:

- Other Foc VCGs
- Other *F. oxysporum*
- Moko bacteria
- Banana tissue

Sensitivity

Up to 1 pg pure TR4 DNA detected

After 7 days, TR4 was detected on the corm of Gran Naine banana plants



Foc TR4 on-site field diagnosis

LAMP run on Healthy banana plant

Cultivar: Grand Naine Cavendish

Age: 9th generation - 3 to 4 months old

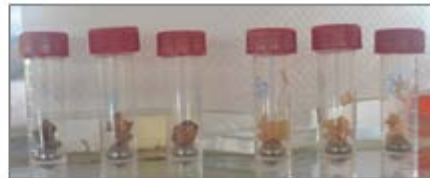
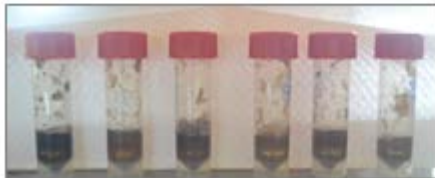
Location: LEF Pantaron, Sto. Tomas, Davao del Norte

GPS reading: N 7°32'17" E 125° 39'44"

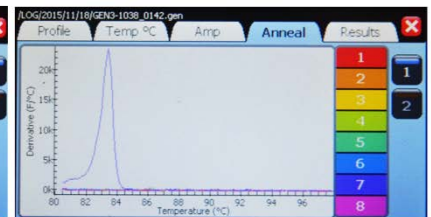
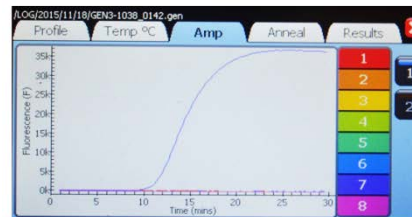
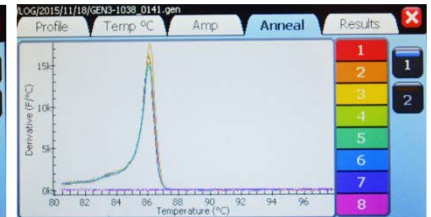
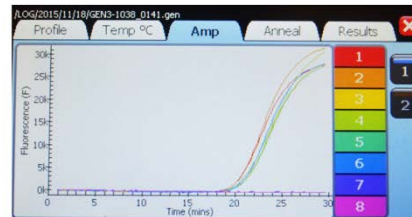


Cavendish banana healthy Corm

Cavendish banana healthy Pseudostem



		Cox	Tr4
Healthy corm	Extraction 1 - Duplo 1	19:30	none
Healthy corm	Extraction 1 - Duplo 2	18:30	none
Healthy corm	Extraction 2 - Duplo 1	17:00	none
Healthy corm	Extraction 2 - Duplo 2	17:15	none
Healthy corm	Extraction 3 - Duplo 1	17:45	none
Healthy corm	Extraction 3 - Duplo 2	23:00	none
Healthy pseudostem	Extraction 1 - Duplo 1	22:00	none
Healthy pseudostem	Extraction 1 - Duplo 2	21:45	none
Healthy pseudostem	Extraction 2 - Duplo 1	22:45	none
Healthy pseudostem	Extraction 2 - Duplo 2	23:00	none
Healthy pseudostem	Extraction 3 - Duplo 1	23:00	none
Healthy pseudostem	Extraction 3 - Duplo 2	22:45	none
Foc TR4 positive control		none	11:45
Foc TR4 positive control		none	12:45
Negative control water		none	none
Negative control water		none	none



Foc TR4 on-site field diagnosis

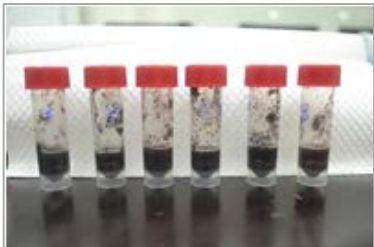
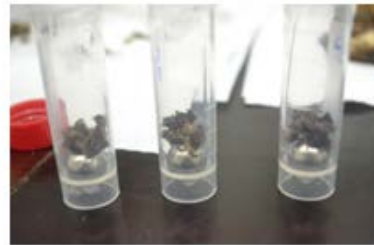
LAMP run on TR4 infected plant

Cultivar: Grand Naine Cavendish

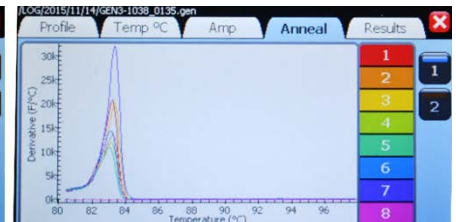
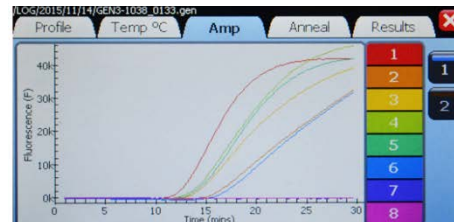
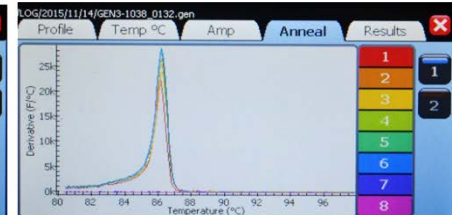
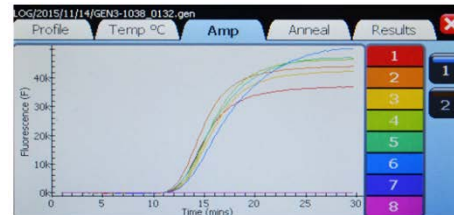
Age: 9th generation - 4 to 6 months old

Location: Sitio San Agustin, Pantaron Gelacio Farm Entrep

GPS reading: N 7°31'21" E 125° 40'17"



		Cox	Tr4
Foc TR4 infected corm	Extraction 1 - Duplo 1	13:30	23:00
Foc TR4 infected corm	Extraction 1 - Duplo 2	13:30	19:45
Foc TR4 infected corm	Extraction 2 - Duplo 1	14:15	18:30
Foc TR4 infected corm	Extraction 2 - Duplo 2	14:15	21:45
Foc TR4 infected corm	Extraction 3 - Duplo 1	14:00	18:30
Foc TR4 infected corm	Extraction 3 - Duplo 2	14:30	19:15
Foc TR4 infected pseudostem	Extraction 1 - Duplo 1	14:00	16:15
Foc TR4 infected pseudostem	Extraction 1 - Duplo 2	17:45	17:45
Foc TR4 infected pseudostem	Extraction 2 - Duplo 1	15:00	17:15
Foc TR4 infected pseudostem	Extraction 2 - Duplo 2	15:00	22:15
Foc TR4 infected pseudostem	Extraction 3 - Duplo 1	15:00	20:15
Foc TR4 infected pseudostem	Extraction 3 - Duplo 2	18:00	18:45
Foc TR4 positive control		none	11:45
Foc TR4 positive control		none	11:30
Negative control water		none	none
Negative control water		none	none



Conclusion

- DArTseq appeared to be a valuable tool for selection of unique fragments without relying on prior sequence data information
- An unique TR4 region (~500bp) was targeted and used for diagnostic development
- LAMP TR4 primer validation was successfully completed on a range of pure DNAs and banana tissue samples
- LAMP TR4 validation under field conditions is ongoing



Acknowledgements

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- **Cees Waalwijk**
- **Gert Kema**
- **Cor Schoen**

- **Andrzej Kilian**

Sequencing of DArTseq unique region in 23 TR4 isolates

Discovery of a ~500bp unique TR4 area highly conserved and most likely an intergenic region

