PHYLOGENETIC ANALYSIS AND APPLIED MOLECULAR DIAGNOSTIC METHODS

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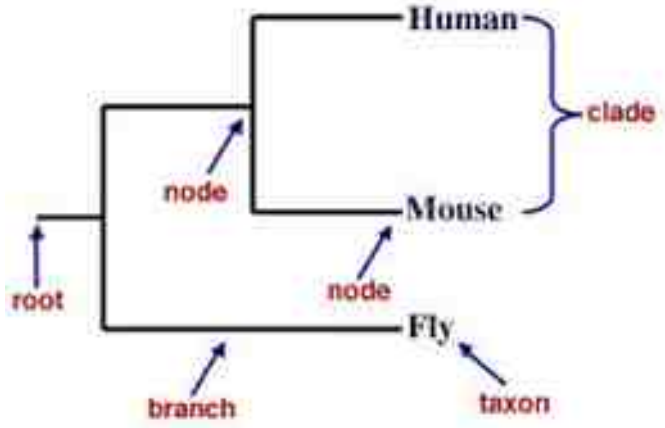
Phylogenetics



"Study of evolutionary relationships among groups of organisms (e.g. species, populations), which are discovered through molecular characterization or sequencing data and morphological data matrices".

Molecular marker (as genetic marker) is a fragment of DNA that is associated with a certain location/function within the genome.

MOLECULAR MARKERS
vs
MORPHOLOGICAL MARKERS

Morphological markers

- Environmental influence
- Low number
- Training y subjectivity

Molecular Markers

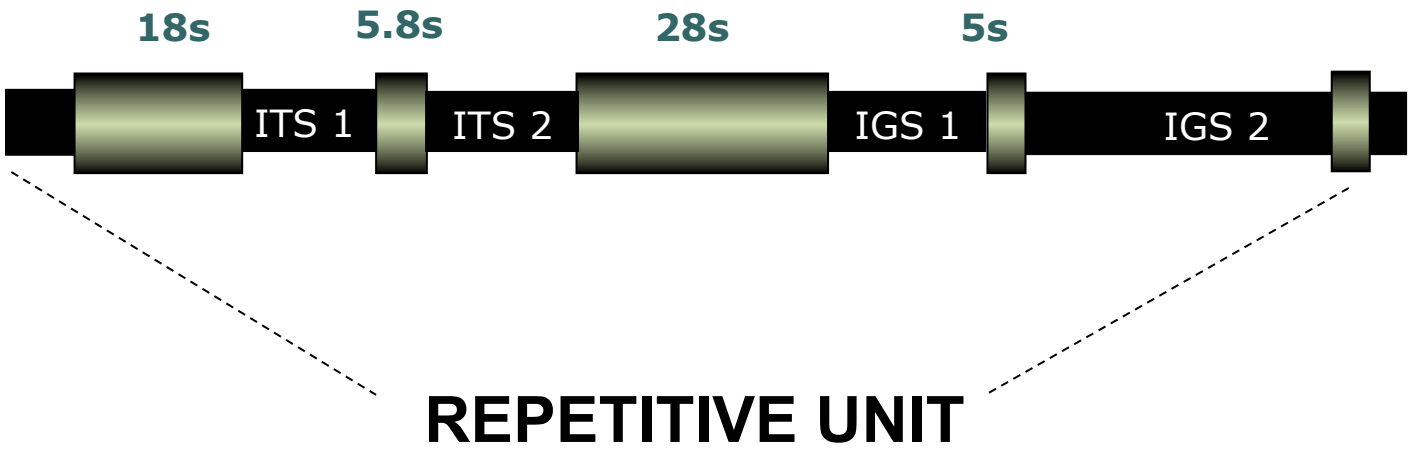
- Without environmental influence
- Unlimited quantity
- Simple, fast and objective

MOLECULAR MARKERS

Genomic regions most frequently used in phylogenetic analysis and characterization of fungi are:

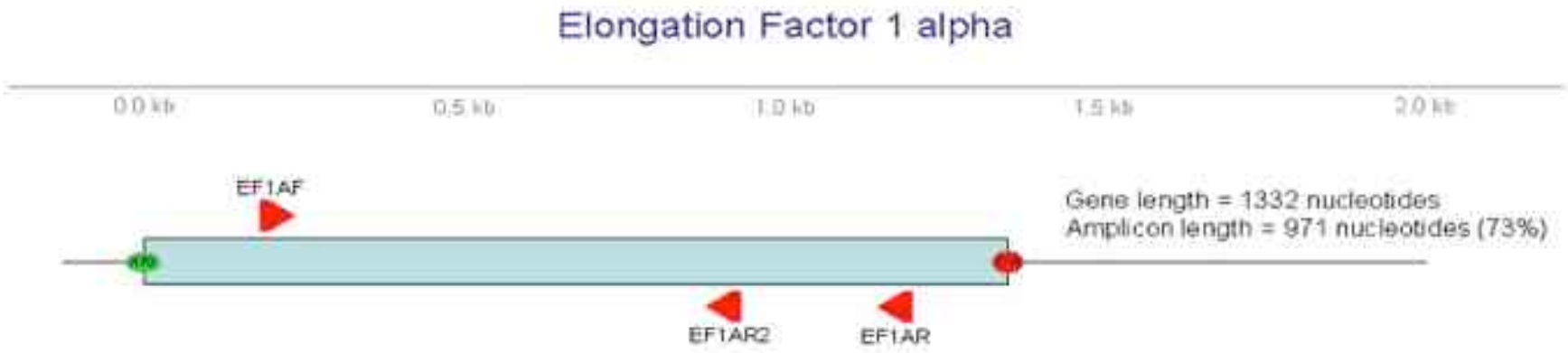
- *DNA ribosomal nuclear genes and its spacer regions* (ITS and IGS).
- *Mithocondrial DNA genes* (mtDNA)
- *ß-tubuline gen*,
- *Elongation factor 1-alpha*(EF-1α or TEF).
- *Other markers* (ERIC sequences and transposable elements).

NUCLEAR RIBOSOMAL DNA GENES



- Regions that codify for 18S, 5.8S y 28S rRNA genes
- **I**nternal **T**ranscribed **S**pacer (ITS1 e ITS2)
- Intergenic spacer;(IGS1 e IGS2)

Elongation factor 1-alpha



- Codify an esential part of the proteic translation machinery,
- Great phylogenetic utility because:

(i) Highly informative at specie level within *Fusarium* genera

(ii) Universal primers have been developed that works trough the genera.

MITHOCONDRIAL GENES

Codify for:

☑ Mitochondrial proteins

☑ tRNA

☑ subunits of rRNA (LSU y SSU ARNr)

Reasons for its widely use :

- Reduce size
- High evolutionary rate,
- Lack of methylate bases,
- High content of adenine-thymine residues (AT)
- Haploid molecule in which most of alleles have the same function and include universally conserved regions

MOLECULAR MARKERS

- Markers based on DNA polymorphism
  1. Detected by Hibridization-(RFLP)
  2. Detected by **amplification-based on PCR**

- **DNA AMPLIFICATION FINGERPRINTING** (DAF)
- **RANDOM AMPLIFIED POLIMORPHIC DNA** (RAPD)
- **SEQUENCE CHARACTERIZED AMPLIFIED REGIONS** (SCAR).
- **AMPLIFIED FRAGMENT LENGTH POLYMORPHISM** (AFLP).
- **CLEAVED AMPLIFIED POLYMORPHIC SEQUENCE** (CAPS)..

MOLECULAR MARKERS APPLICATIONS

- ✓ Genetic diversity studies.
- ✓ Taxonomic studies (phylogenetic relationship).
- ✓ Genealogy establishment.
- ✓ Establishment of hybrid purity
- ✓ Elaboration of genetic maps.
- ✓ Paternity test
- ✓ Cultivar identification – royalties

Diagnostic

Plant Pathogens Diagnostic

Why is so important a fast, specific and reliable diagnostic?

Human health

wrong

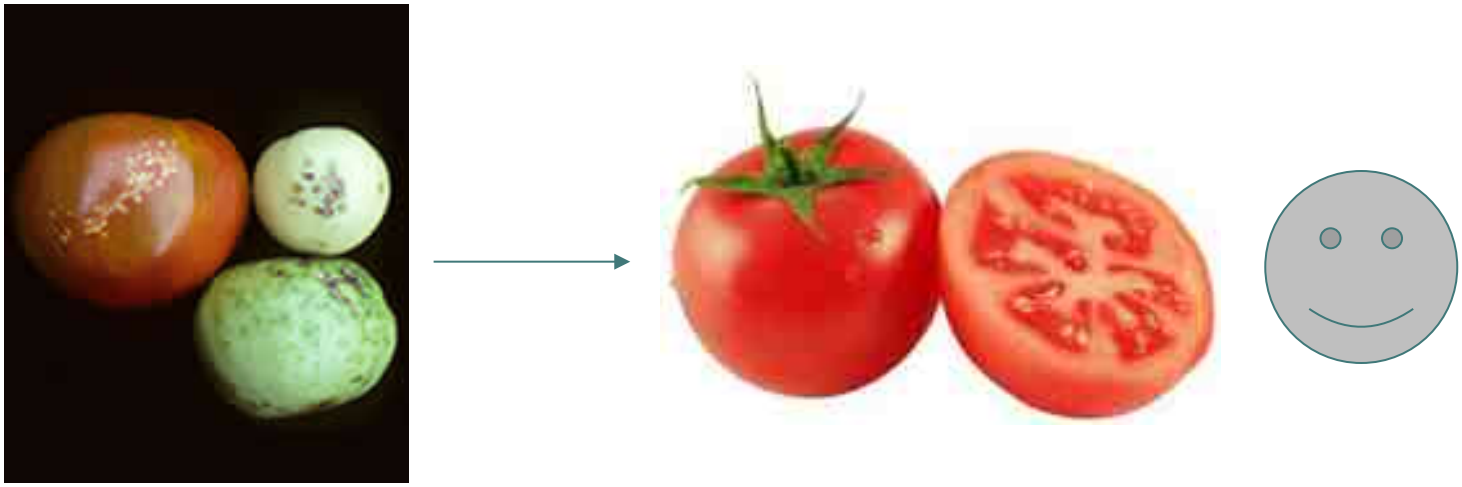
rigth

It is different in Plant Pathology?

PLANT PATHOGENS DIAGNOSTIC

Precise and timely diagnostic: fundamental for manage the problem

- **Generate effective control measures**
- **Allow optimization of resources**
- **Reduction of environmental negative results**



PLANT PATHOGENS DIAGNOSTIC

Until a few years ago, detection methods depended on methods which require high skills and knowledge of microbial taxonomy

PLANT PATHOGENS DIAGNOSTIC

Traditional Diagnosis – Morphological identification

- Microscopically diagnosis: consist in the observation of structures of plant pathogen microorganism.
- This observation could be directly to optic microscope for the case of bacteria, fungi and nematodes or by electronic microscopy for identify virus.

BACTERIAS

BIOCHEMICAL TEST

FUNGI

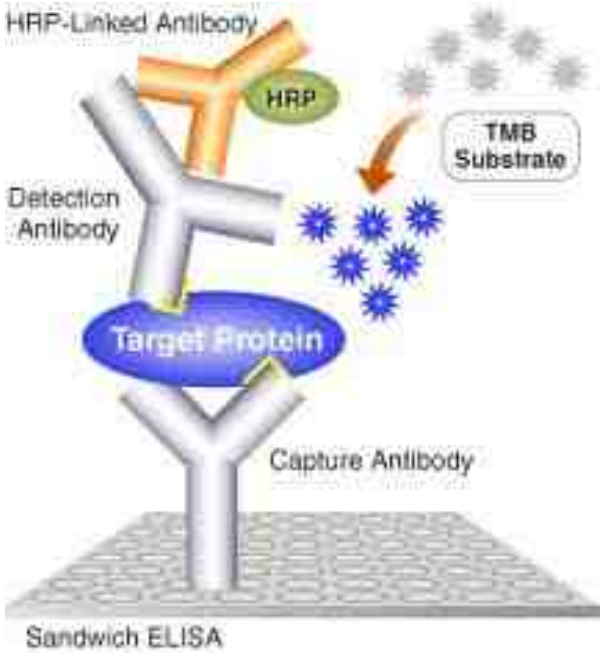
IDENTIFICATION OF STRUCTURES UNDER THE MICROSCOPE

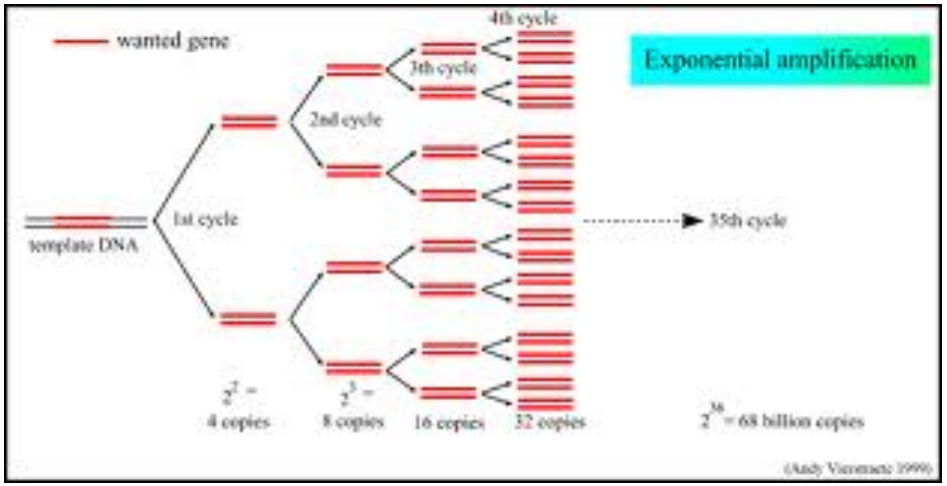
Limitations of diagnostic traditional methods

1. Time-consuming (days, weeks, months).
2. Low detection level.
3. Unable to discriminate related species.
4. Few taxonomists and a great diversity of organisms.

PLANT PATHOGENS DIAGNOSTIC

Modern techniques allow a more efficient detection of pathogenic varieties with higher speed and precision eg. Serological techniques (ELISA) and molecular (PCR)





Materials that can be used for diagnostic

Any vegetable structure (seed, fruit, root, leaf, etc.) with or without symptoms and substrates (soil, water)

Quarantine disease: diagnostic considerations

White mold-*Sclerotinia sclerotiorum*

Symptoms and structures of *S. Sclerotiorum* in several crops

Quarantine disease : diagnostic considerations

Plant Pathology (2009) 58, 324–331 Doi: 10.1111/j.1365-3059.2008.01945.x

Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR

S. L. Rogers, S. D. Atkins and J. S. West*

Rothamsted Research, Harpenden, AL5 2JQ, UK

J Phytopathol 157:465–469 (2009)

doi: 10.1111/j.1439-0434.2009.01543.x

Institute of Biotechnology, Zhejiang University, Hangzhou, China

Detection of *Sclerotinia sclerotiorum in Planta* by a Real-time PCR Assay

YANNI YIN[1], LAISONG DING[1], XIN LIU[1], JINGHUI YANG[2] and ZHONGHUA MA[1]

S. sclerotiorum is polyphague pathogen with a host range extremally large that includes 75 families, 278 genera and y 408 species until 1994 (CABI, 2007).

White mold -*Sclerotinia sclerotiorum*

Yin *et al*., 2009- Detection of *Sclerotinia sclerotiorum* in Planta by a Real-time PCR Assay. *J. Phytopathol* 157:465–469

Amplification Product 1.2 Kb (M13)

Purification, clonage and sequencing

Sequences

Edition and alingment — SEQUENCHER version 4.7 (Genecodes)

Specific primers

PCR o qPCR

SsF (5´-AGTCGAGGGACGGGTACTAA-3´)

SsR (5´-CTTGTCCTCATTGCCGTTT-3´)

©NC State University

White mold -*Sclerotinia sclerotiorum*

Rogers *et al*., 2009- Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. *Plant Pathology,* 58: 324–331.

Sequences of intron rRNA of mtSSU } GenEMBL database

Edition and alingment ↓ BLAST analysis (BLASTN)

Primers for PCR o qPCR

mtSSFor (5´AGGTAACAAGTCAGAAGATGATCGAAAGAGTT-3´)

mtSSRev (5´-GCATTAAGCCTGTCCCTAAAAACAAGG-3')

Specificity Test

White mold -*Sclerotinia sclerotiorum*

Species (isolate)	Detection by PCR	Detection by qPCR
Sclerotinia sclerotiorum (S3)	✓	✓
S. sclerotiorum (M24)	✓	✓
S. sclerotiorum (1999B)	✓	✓
S. sclerotiorum (Phy 1)	✓	✓
S. sclerotiorum (Great Harpenden)	✓	✓
S. sclerotiorum (31)	✓	✓
S. sclerotiorum (M23)	✓	✓
S. sclerotiorum (M1/44)	✓	✓
Botrytis cinerea (IMI 181038)	✓	x
B. cinerea (strawberry06a)	✓	x
B. cinerea (directly from grapes)	✓	x
B. cinerea (directly from *Pelagonium*)	✓	x
B. cinerea (directly from tomato leaf)	✓	x
S. minor (2317)	x	x
S. trifoliorum ('clover')	x	x
S. trifoliorum (R316)	x	x
Penicillium spp.	x	x
Cladosporium spp.(mixed 'rough' and 'smooth' spore types)	x	x
Leptosphaeria maculans	x	x
Pyrenopeziza brassicae	x	x
Brassica napus	x	x

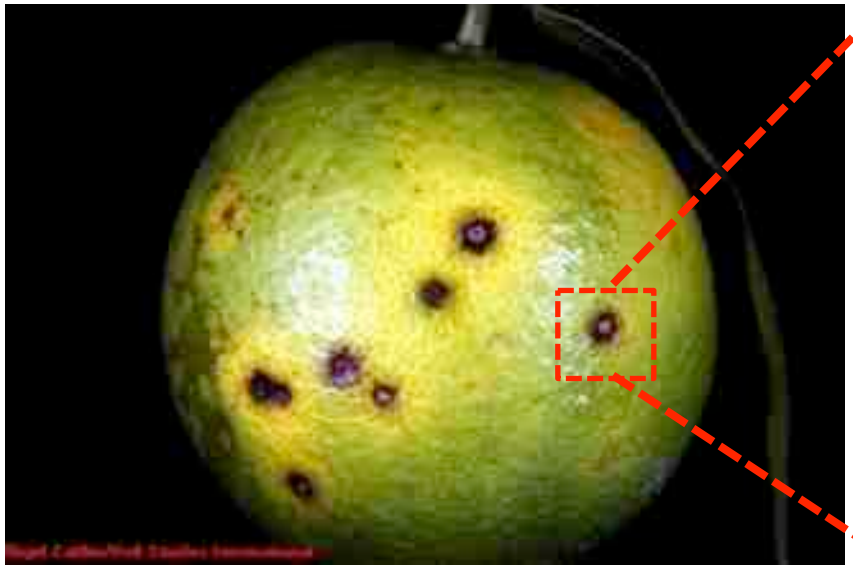
x = not detected within 60 cycles of qPCR.

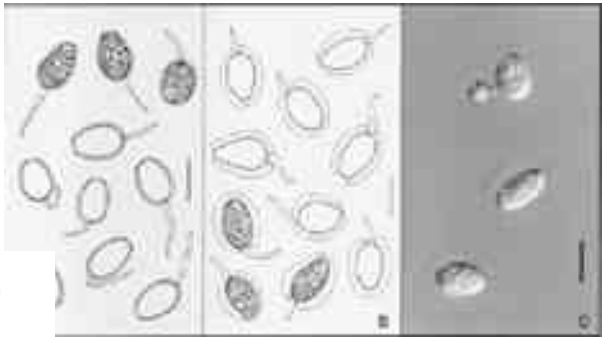
Rogers *et al.*, 2009- Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. *Plant Pathology,* 58: 324–331.

Quarantine disease: diagnostic considerations
Citrus Black spot- *Guignardia citricarpa*

Symptoms of citrus black spot. A: on fruit and B: on leaf

Citrus Black spot- *Guignardia citricarpa*





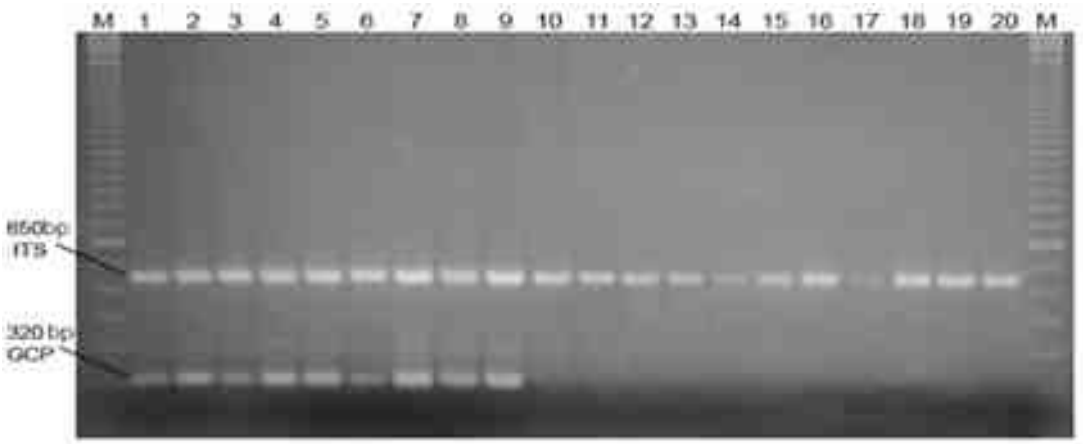
Phyllosticta capitalensis

Phyllosticta citricarpa

Citrus Black spot- *Guignardia citricarpa*

Stringari *et al*., (2009). High Molecular Diversity of the Fungus *Guignardia citricarpa* and *G. mangiferae* and New Primers for the Diagnosis of the Citrus Black Spot. *Brazilian Archieves Biology and Technology,* 52(5):1063-1073.

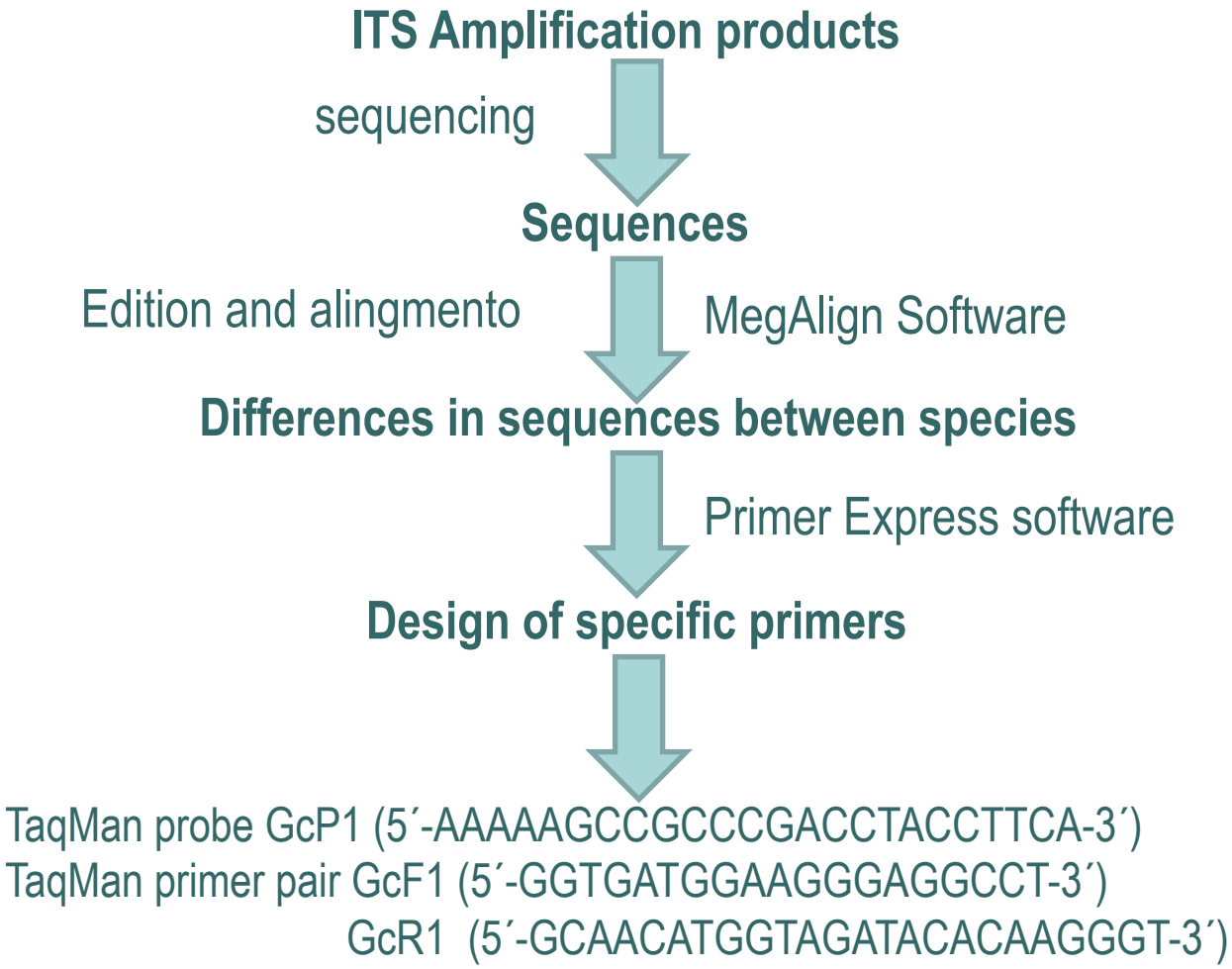
Genetic variability study with RAPDs and developes a SCAR marker to specific diagnostic of *G. citricarpa*.



Electrophoresis in agarose gel of amplification product of *G. citricarpa y G. mangiferae* DNA with GCP1/GCP2 primers. M: Molecular ladder 100 bp. 1-9: *G. citricarpa,* 10 - 20: *G. mangiferae* .

Citrus Black spot- *Guignardia citricarpa*

van Gent-Pelzer *et al*. (2007). A TaqMan PCR Method for Routine Diagnosis of the Quarantine Fungus *Guignardia citricarpa* on Citrus Fruit. *J. Phytopathology,* 155: 357–363.



Sugarcane rust

Puccinia kuehnii

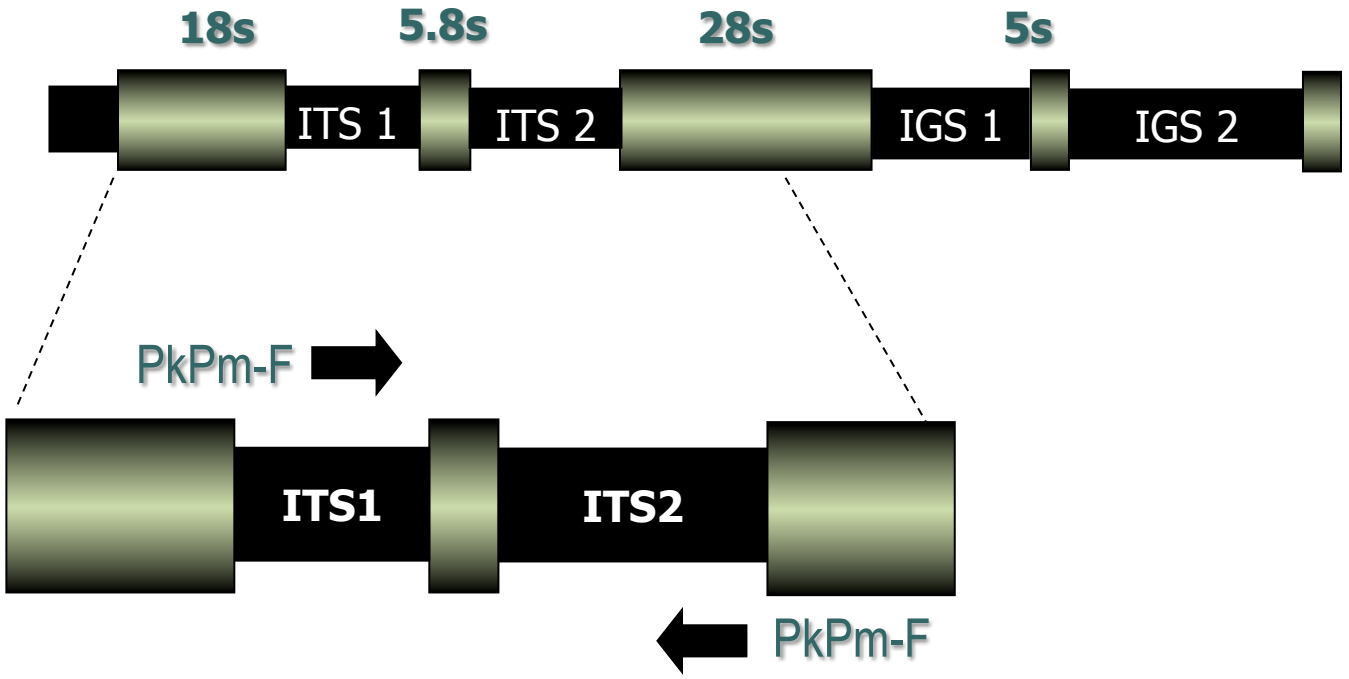
Puccinia melanocephala

Sugarcane rust

Glynn *et al*. (2010). PCR assays for the sugarcane rust pathogens *Puccinia kuehnii* and *P. melanocephala* and detection of a SNP associated with geographical distribution in *P. kuehnii*. *Plant Pathology* ,59:703–711.

PkPmF-AAGAGTGCACTTAATTGTGGCTC
PkPmR-TCCCACCTGATTTGAGGTCT

Analysis of conserved sequences between two species published in GenBank (NCBI-National Center for Biotechnology Information).



Sugarcane rust

Glynn *et al*. (2010). PCR assays for the sugarcane rust pathogens *Puccinia kuehnii* and *P. melanocephala* and detection of a SNP associated with geographical distribution in *P. kuehnii*. *Plant Pathology* ,59:703–711.

PkPmF / PkPmR-Prod. de amplificación

P kuehnii-606 pb
P melanocephala- 585 pb

Purification, clonage and sequencing

Sequences

Edition and aligment

SEQUENCHER version 4.7 (Genecodes)

Specif primers

PCR

P kuehnii- Pk1F/Pk1R-527 pb
P melanocephala-
Pm1F/Pm1R -480 pb

qPCR

P kuehnii- Pk2F/Pk2R-142 pb
P melanocephala- Pm2F/Pm2R -130 pb

MOLECULAR DIAGNOSIC

There are different methods ….

Which one is better?

It depends on:

- Objective,
- Target pathogen
- Sample type
- Availability of equipment
- Number of samples
- Customer requirements

What do we do with the diagnostic?

A diagnostic tool Specific for *Foc* Tropical race 4

Fusarium oxysporum f sp. *cubense* TR4

Diagnosis methodology

Morphology

Fusarium oxysporum

VCG

VCG 01213=*Foc* TR4

Could take months

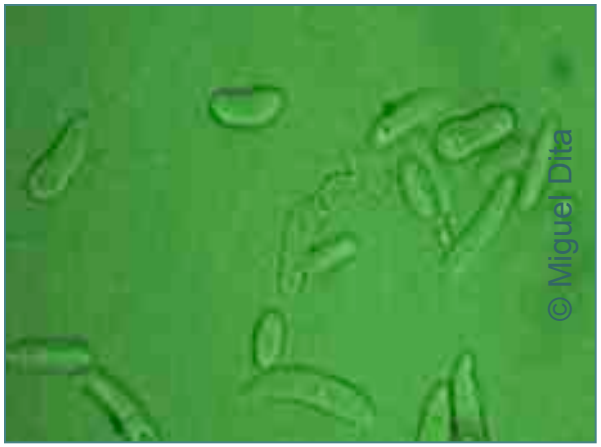
PCR

***Foc* TR4**

6 hours

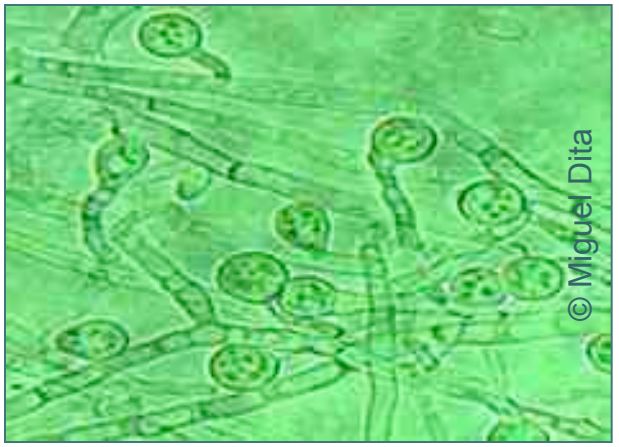
A diagnostic tool for Foc TR4

Structures of *F. o* f. sp. *cubense*



Microconidia are 5 - 16 x 2.4 - 3.5 µm, one- or two-celled, oval- to kidney-shaped, and are borne in false heads

Macroconidia: are 27 - 55 x 3.3 - 5.5 µm, four- to eight-celled and sickle-shaped with foot-shaped basal cell



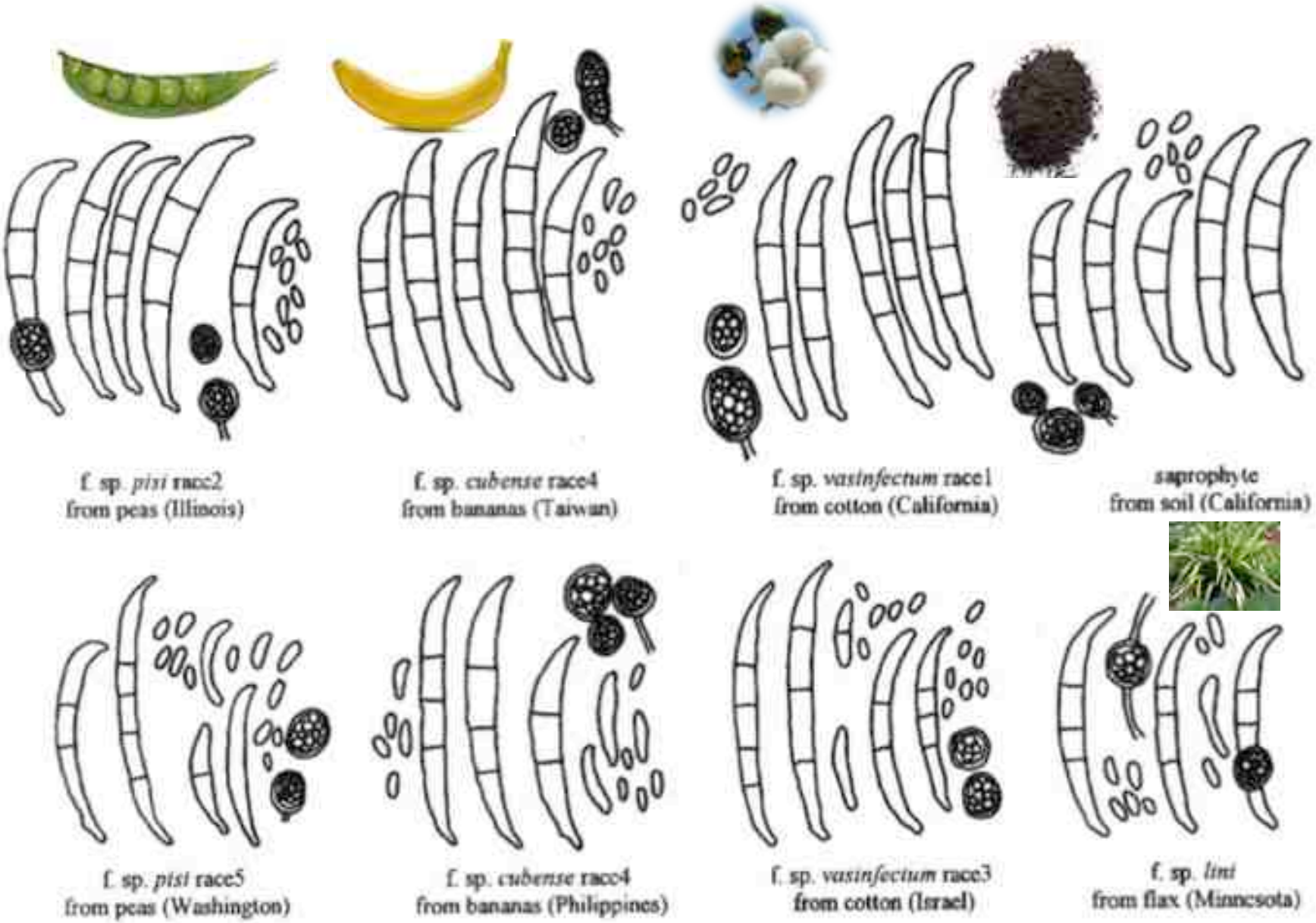
Chlamydospores: Terminal and intercalary are 7 - 11 µm in diameter, usually globose and are formed singly or in pairs in hyphae or conidia

- Fox: ~ 100 *formae speciales* cause wilting in plants

  It contains pathogenic and saprophytic strains that cannot be distinguished morphologically

Source: Ploetz (2000)

Foc can not be distinguished morphologically from other Foxys

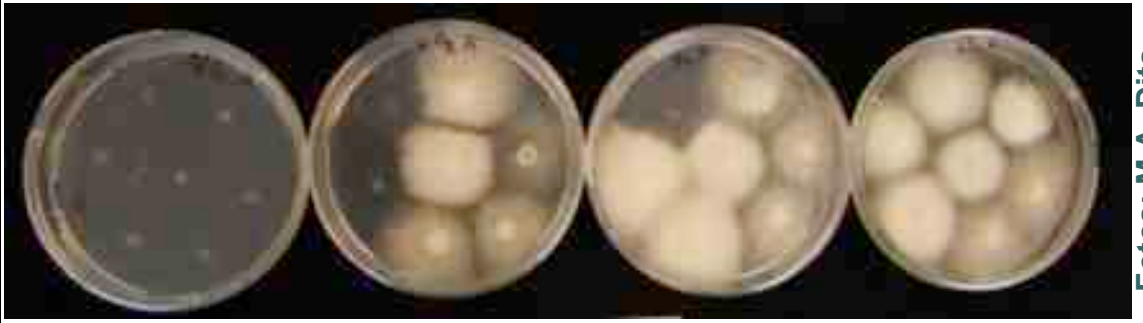


Source: Smith (2007)

VCG classification

Screening of mutants for *nit1*, *nit3* y *nitM*

		nit-?	NO3	NO2	HX	NH4	Result	Code
	1	36105a	-	+	-	+	nitM	36105nitM
A	2	36105b	-	+	+	+	nit1	36105nit1
	3	FocR2a	-	+	+	+	nit1	FocR2nit1
	4	FocR2b	-	-	+	+	nit3	FocR2nit3
	5	FocR2c	-	-	+	+	nit3	FocR2nit3
	6	FocR1a	-	+	+	+	nit1	FocR1nit1
	7	FocR1b	-	+	+	+	nit1	FocR1nit1
B	1	36107a	-	+	+	+	nit1	36107nit1
	2	36107b	-	+	+	+	nit1	36107nit1
	3	36107c	-	+	+	+	nit1	36107nit1
	4	36107d	-	+	+	+	nit1	36107nit1
	5	36107e	-	+	+	+	nit1	36107nit1
	6	36107f	-	+	+	+	nit1	36107nit1
	7	36107g	-	+	+	+	nit1	36107nit1
C	1	36110a	-	-	+	+	nit3	36110nit3
	2	36110b	-	-	+	+	nit3	36110nit3
	3	36110c	-	-	+	+	nit3	36110nit3
	4	36110d	-	-	+	+	nit3	36110nit3
	5	36110e	-	-	+	+	nit3	36110nit3
	6	36110f	-	-	+	+	nit3	36110nit3
	7	36110g	-	-	+	+	nit3	36110nit3
D	1	36111a	-	-	+	+	nit3	36111nit3
	2	36111b	-	+/-	-	+	? Repeat	36111
	3	36111c	-	+/-	+	+	? Repeat	36111
	4	36111d	-	+/-	-	+	? Repeat	36111
	5	36111e	-	+/-	-	+	? Repeat	36111
	6	36111f	-	+/-	-	+	? Repeat	36111
	7	FocR1c	-	+/-	+	+	? Repeat	FocR1
E	1	FocR1d	+	+	+	+	escape? Crn	
	2	36107i	-	+	+	+	nit1	36107nit1
	3	36107h	-	+	+	+	nit1	36107nit1
	4	36110i	-	-	+	+	nit3	36110nit3
	5	36110h	-	-	+	+	nit3	36110nit3



NO3 NO2 Hipoxantina NH4

Fotos: M.A. Dita

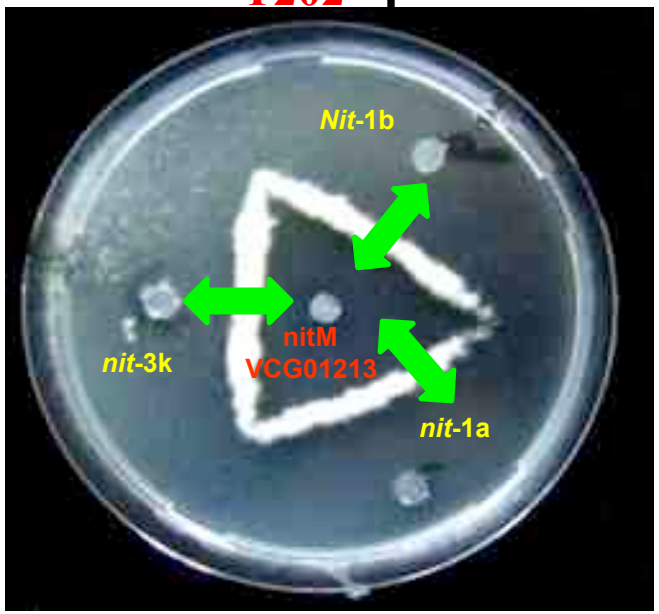
Rep 2

			NO3	NO2	HX	NH4		
D2	1	36111a	-	-	+	+	nit3	36111nit3
	2	36111b	-	-	-	+	?	36111
	3	36111c	-	-	+	+	nit3	36111nit3
	4	36111d	-	-	-	+	?	36111
	5	36111e	-	-	-	+	?	36111
	6	FocR1c	-	-	+	+	nit3	FocR1
	7	FocR1d	+/-	+	+	+	crn	FocR2

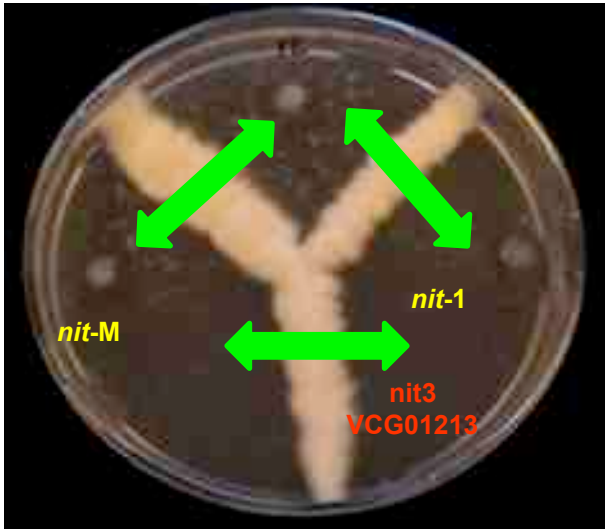
Puhalla,(1985), Correll et al. (1987), Ploetz y Correll (1988)

VCG classification

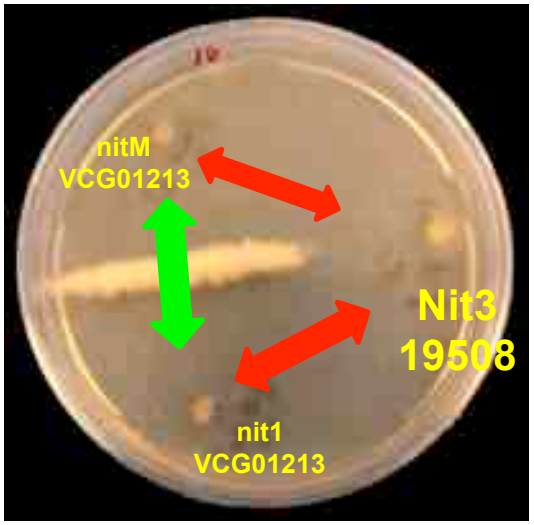
T202



BPS3.1



Foc19508

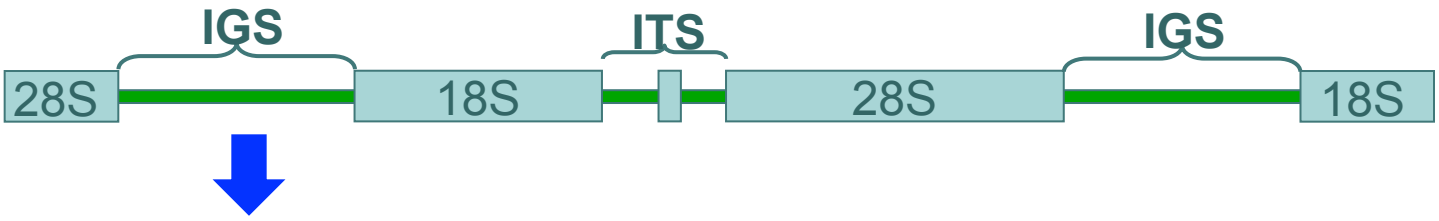


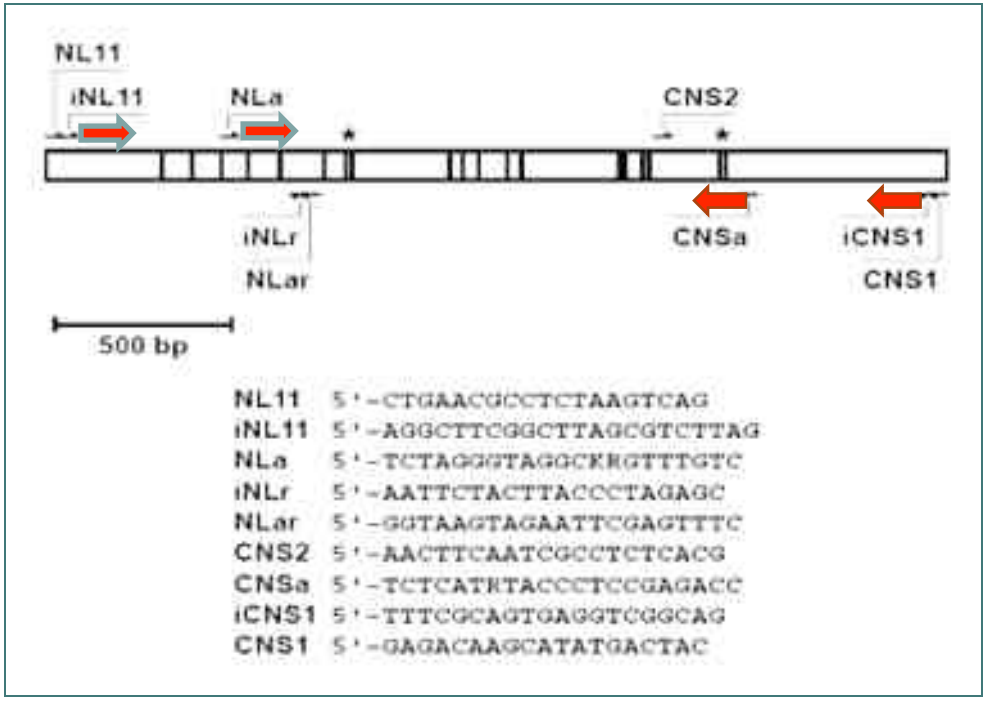
VCG01	Foc19508	BPS3.1	BPS3.2	BPS3.4	BPS1.1	36114
Foc19508	X	N	N	N	N	N
BPS3.1			X	X	X	X
BPS3.2				X	X	X
BPS3.4					X	X
BPS1.1						X
36114						X

Fotos : M.A. Dita

Genetic diversity of *Foc* based on:

Elongation Factor-1a and IGS rDNA sequences



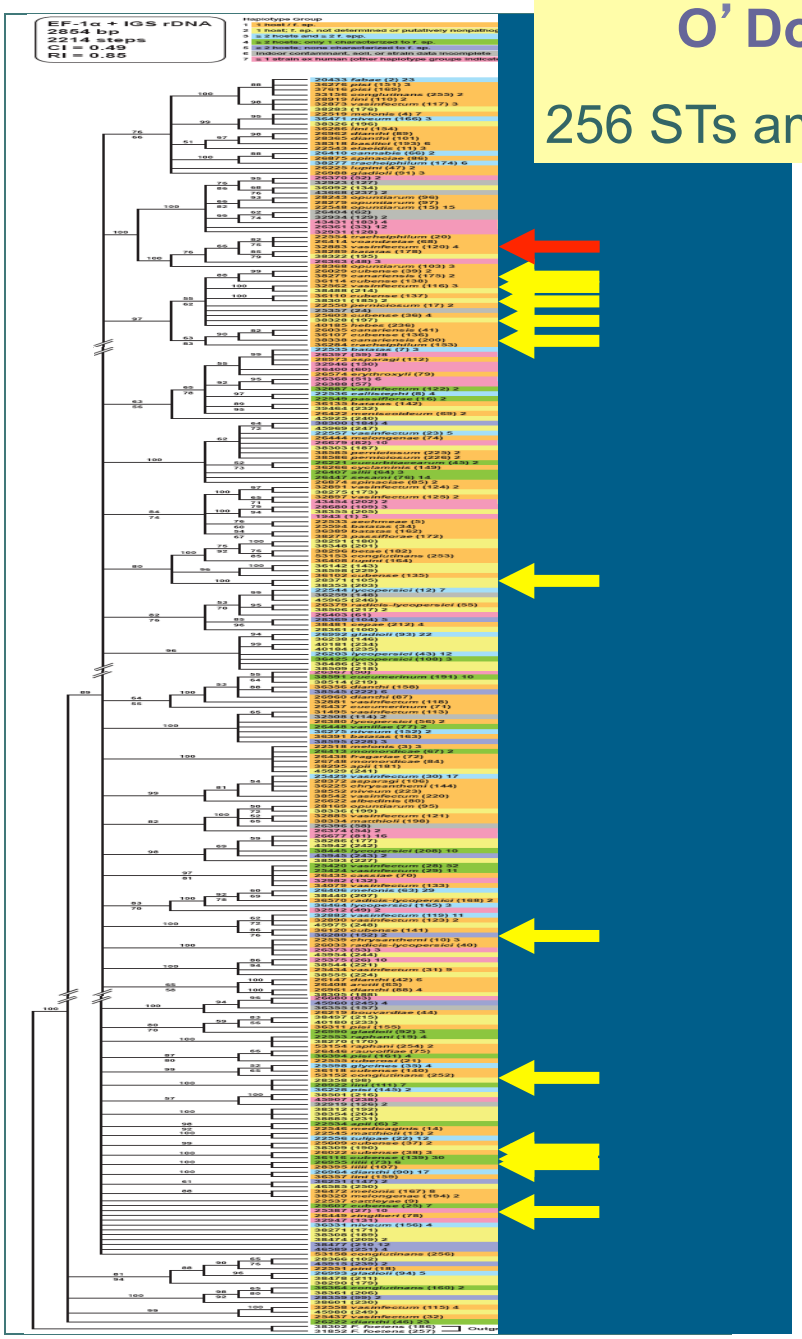


Comparative analyses:

Among *Foc* isolates

- based on *EF-1a gene*
- IGS region
- *EF- 1a* & IGS

TR4

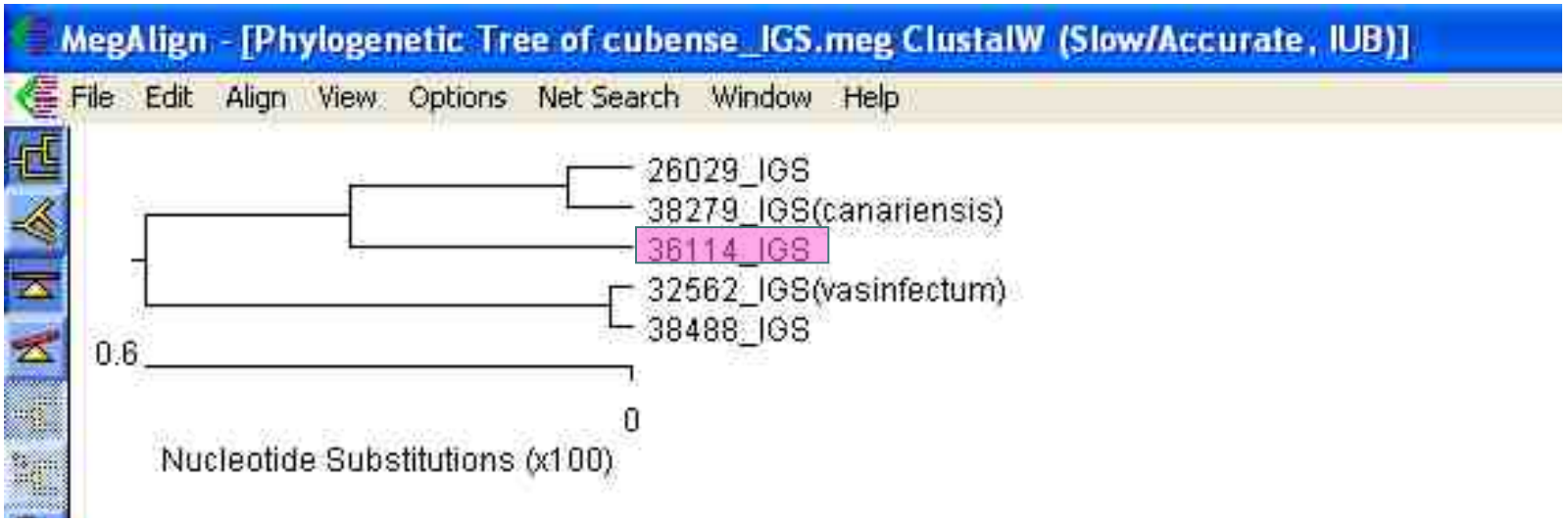


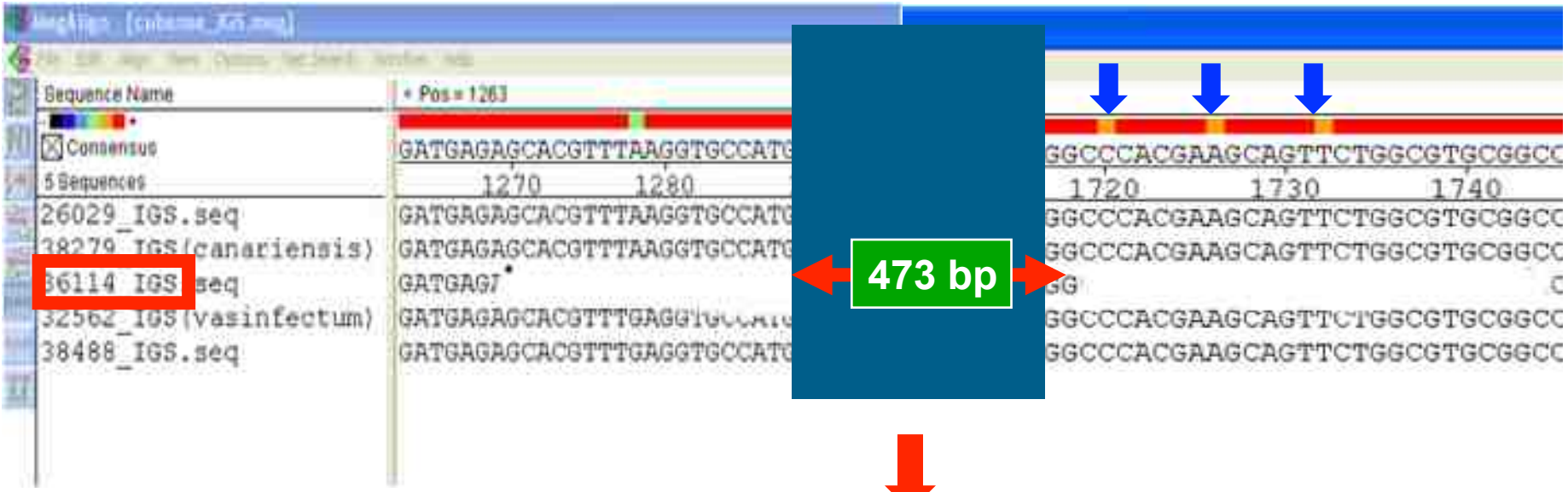
O' Donnell *et al* (in preparation)

256 STs among 850 isolates from > 60 hosts

cubense

- Not enough resolving power in the EF-1α sequences
- IGS sequences revealed higher SNP density





TR4-IGSs – Primer set

PCR with TR4-IGSs primers on PRI *Foc* collection

TR4
36114
VCG01213

VCG:
1. 0120
2. 0121
3. 0122
4. 0123
5. 0124
6. 0125
7. 0126
8. 0128
9. 0129
10. 01210
11. 01211
12. 01212
13. 01214
14. 01215
15. 01218
16. 01221
17. 01222
18. 01223
19. 01224

Unknown VCG No TR4 Regions

TR4
BPS1.1/3.4
VCG01213
Indonesia

VCG-01215 & unknown

VCG:
1. 01220
2. 01210
3. 01214
4. 0126
5. 0124
6. 0128
7. 0124 & Unknown

EF-1a PCR Control

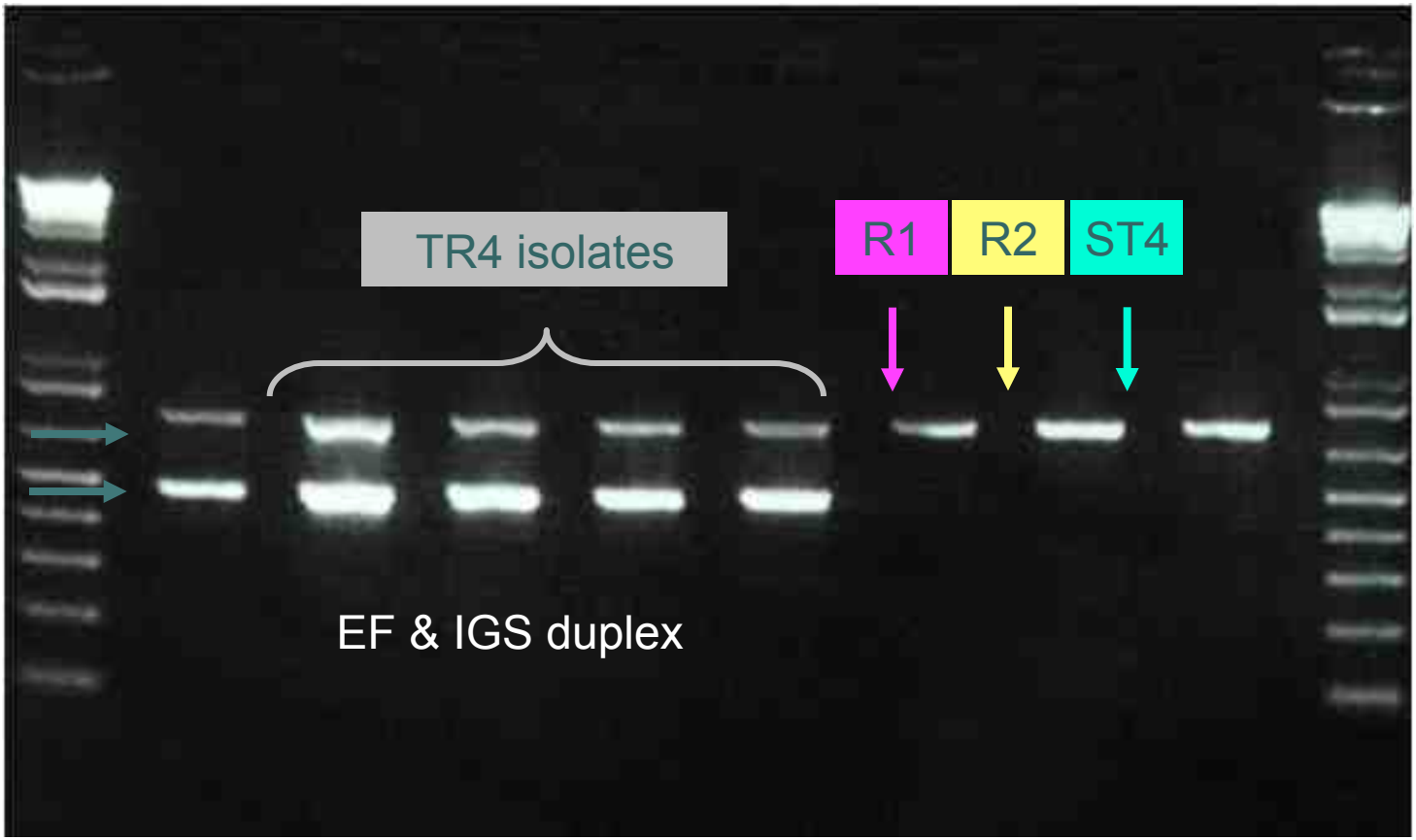
Developing internal controls

- false negative diagnostics

Pathogen

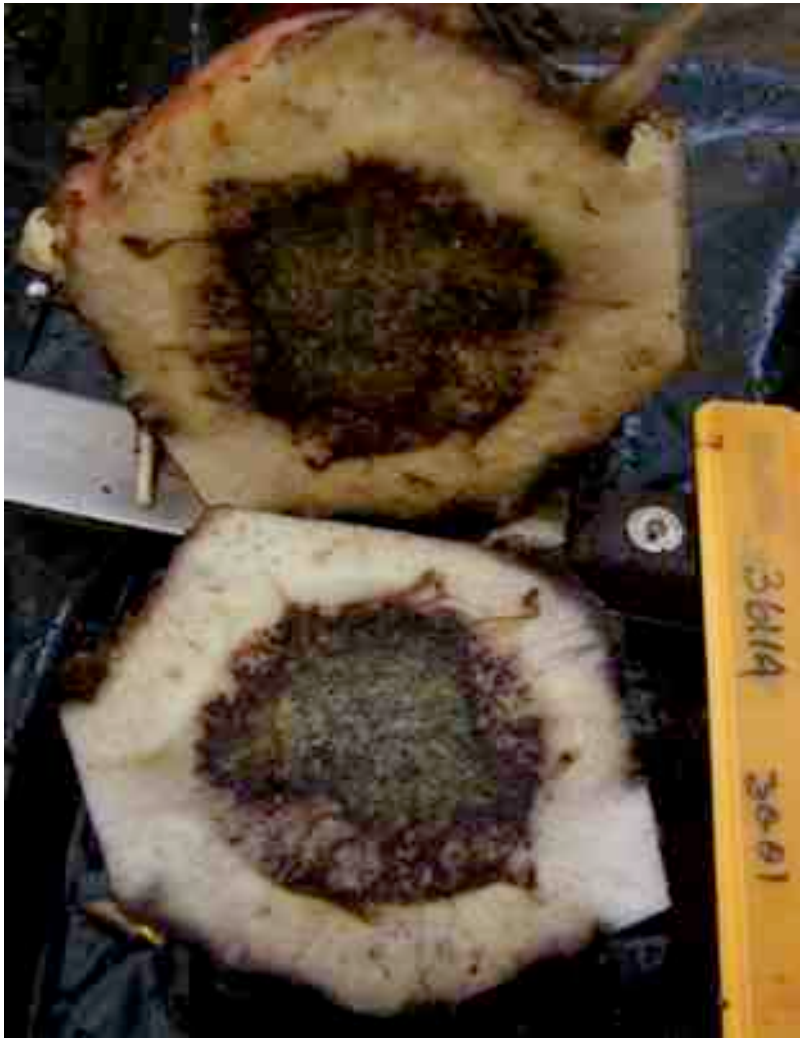
***EF- 1a* primers**

- 650 bp ; Tm= 60 C



in planta detection assay for *Foc* TR4

in planta detection assay for *Foc* TR4



40 days after inoculation

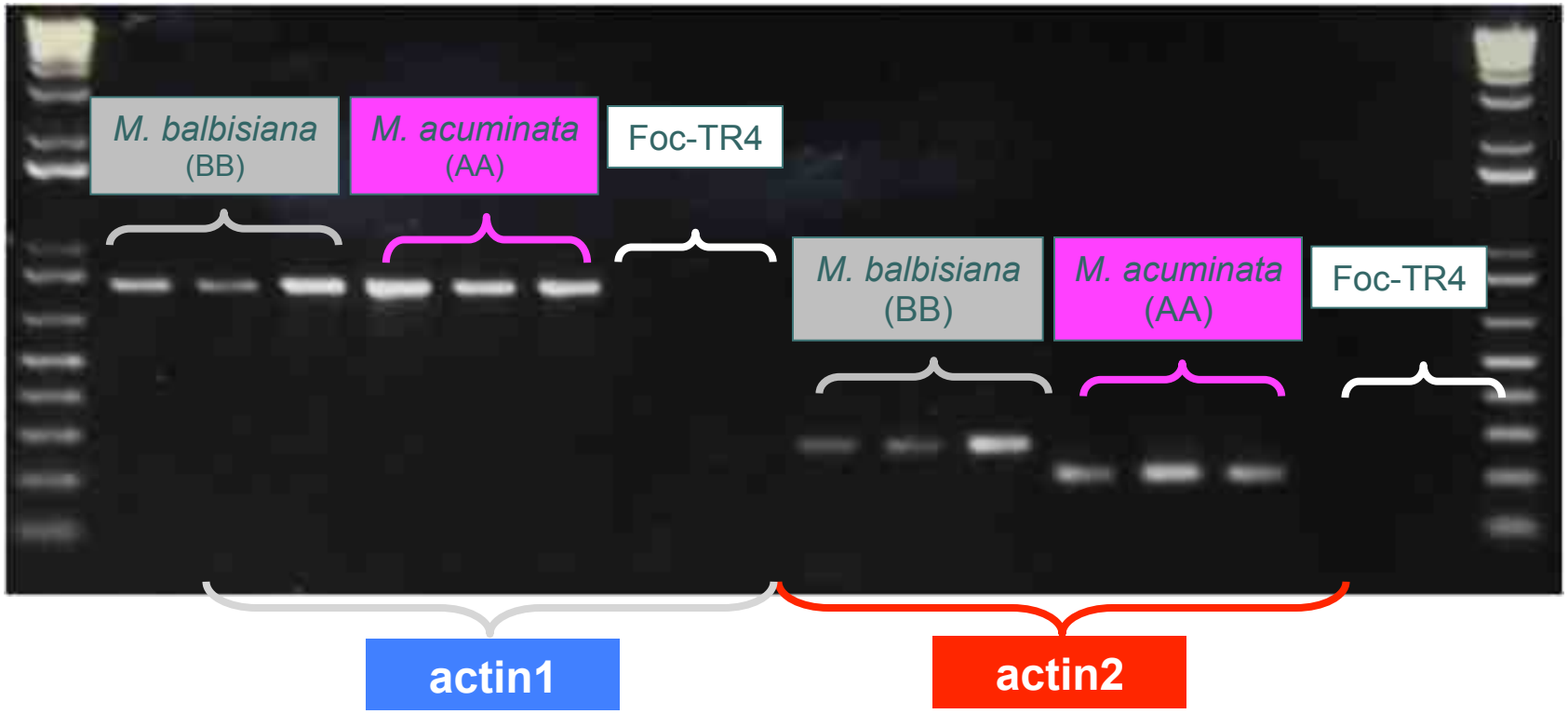
Developing internal controls

- false negative diagnostics

In Planta

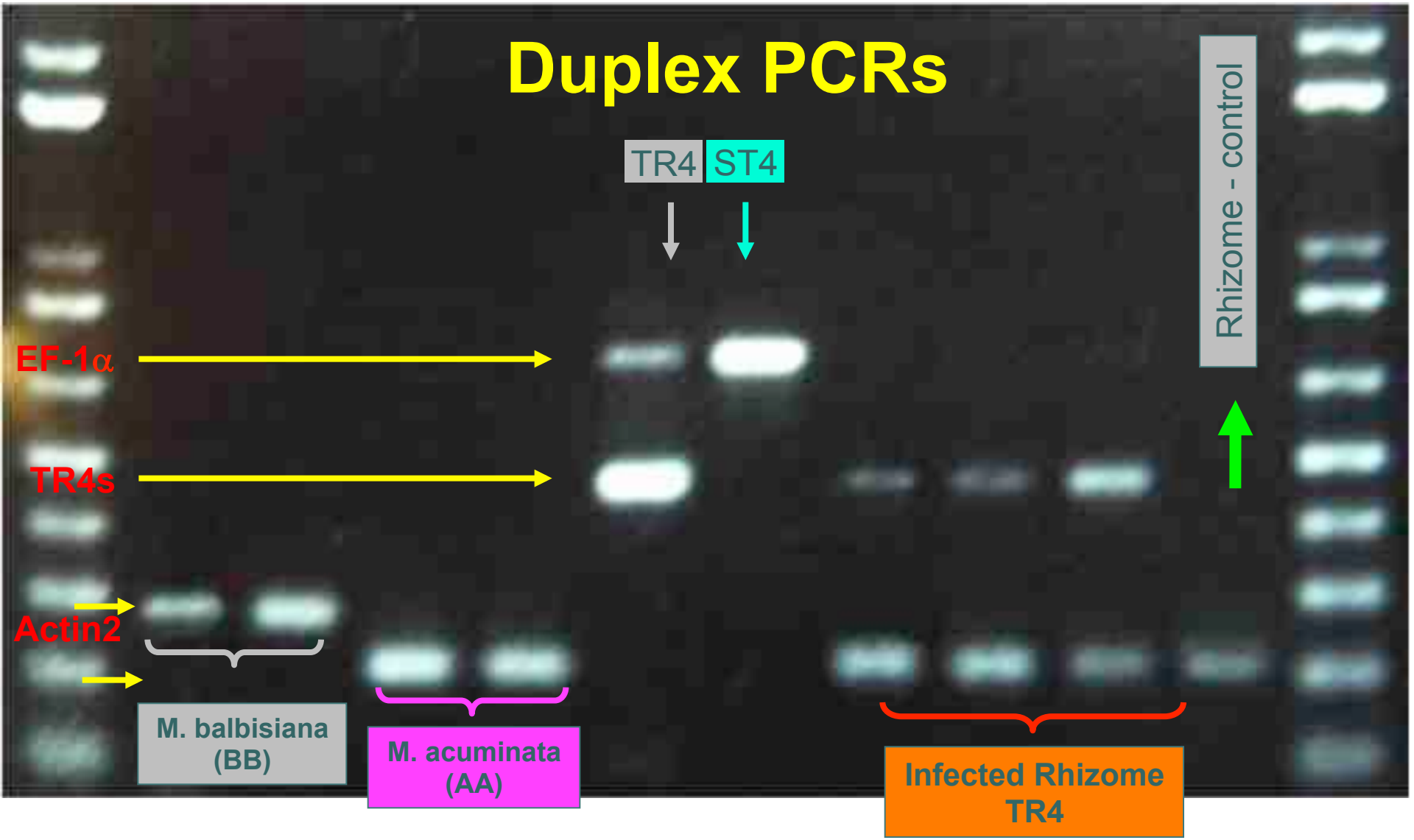
Actin gene primers

- Actin 1 – based on cDNA (716 bp)
- Actin 2 – sapanning Intron (~ 217 bp)



Foc TR4 - *in planta* detection

EF +FocTR4 // Actin2 + FocTR4



Conclusions and Remarks

1- **Diagnostic method specific for TR4 (First?)**

2- E-Factor 1α is not efficient for *Foc* race/VCG discrimination

3. Actin2 gene of *Musa* spp. discriminate AA from BB genotypes

4- Primers set developed, can be used as triplex or duplex

- Plant DNA samples – actin 2 + FocTR4 primers

- Fungi DNA samples – EF-1a + FocTR4 primers

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Development of a molecular marker for specific detection of *Fusarium oxysporum* f. sp. *cubense* race 4

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ST4 + TR4

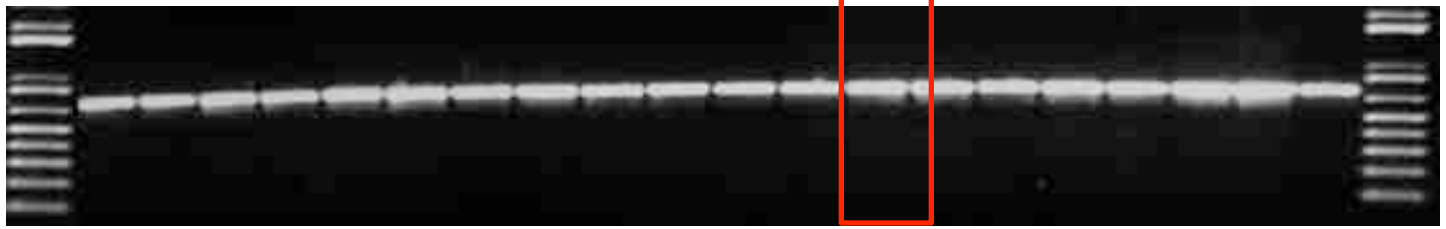
Foc-1/Foc-2
Primer set

Molecular Diagnostic for TR4 (VCG 01213)

Lin *et al*. (2008)- Foc-1/ Foc-2

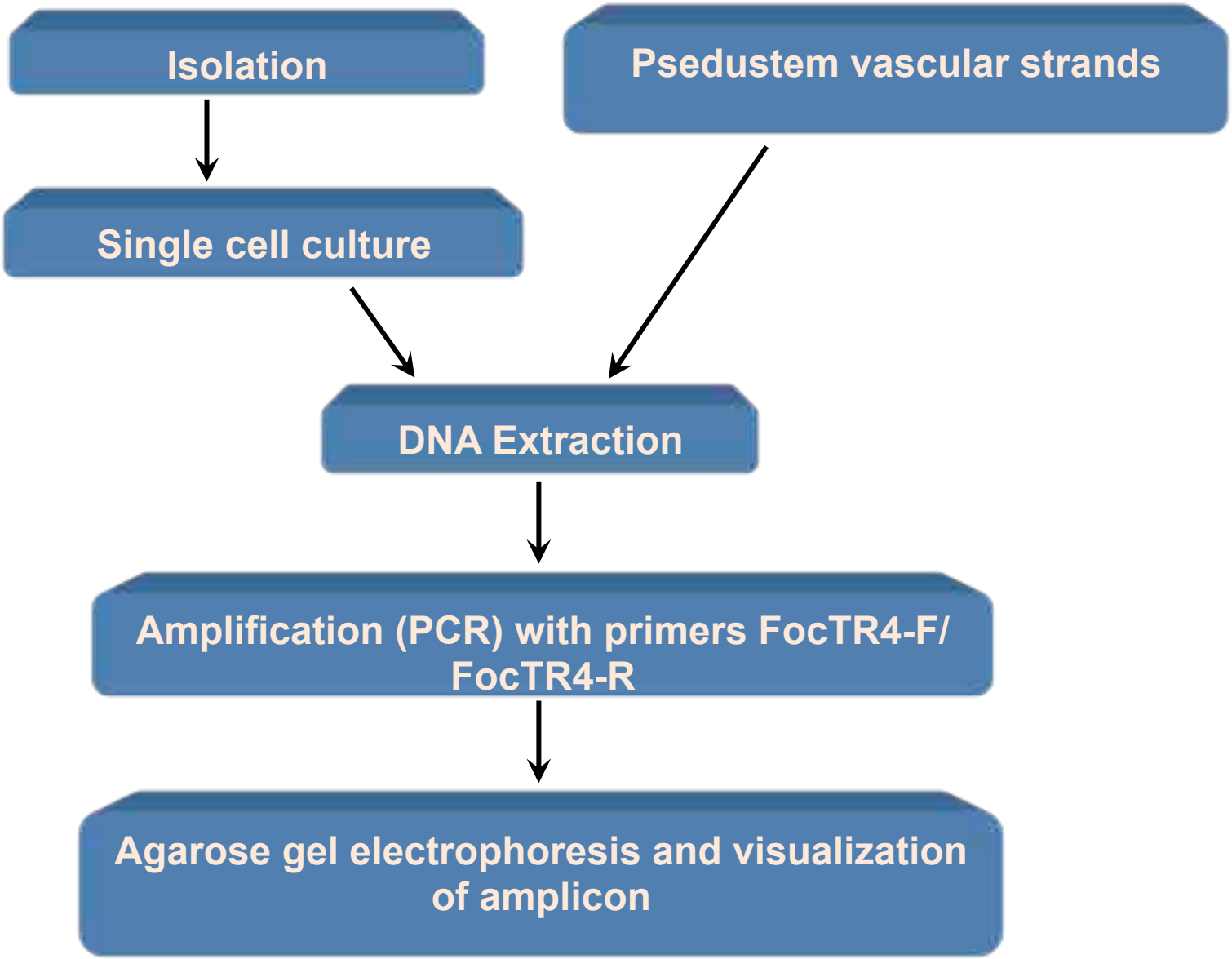
VCG 01213

Primers - FocTR4-F/ FocTR4-F (Dita *et al*. 2010)



Foc1/Foc2: Positive isolate from Honduras, Brazil, Costa Rica!
Australia, Indonesia, Taiwan

Foc RT4 PCR Identification Protocol (Dita *et al*., 2010)



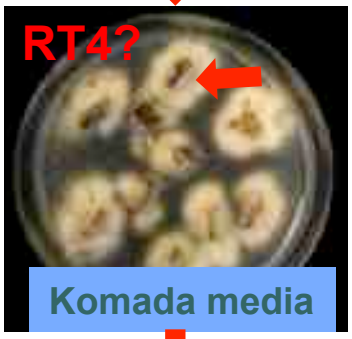
Stages

Sample collection
Pseudostem, petiole etc.
Place , variety, etc

Send samples to a lab with information about plantation

Samples processing Komada media

Fungus Isolation Purification PDA media



DNA Extraction

DNA Extraction

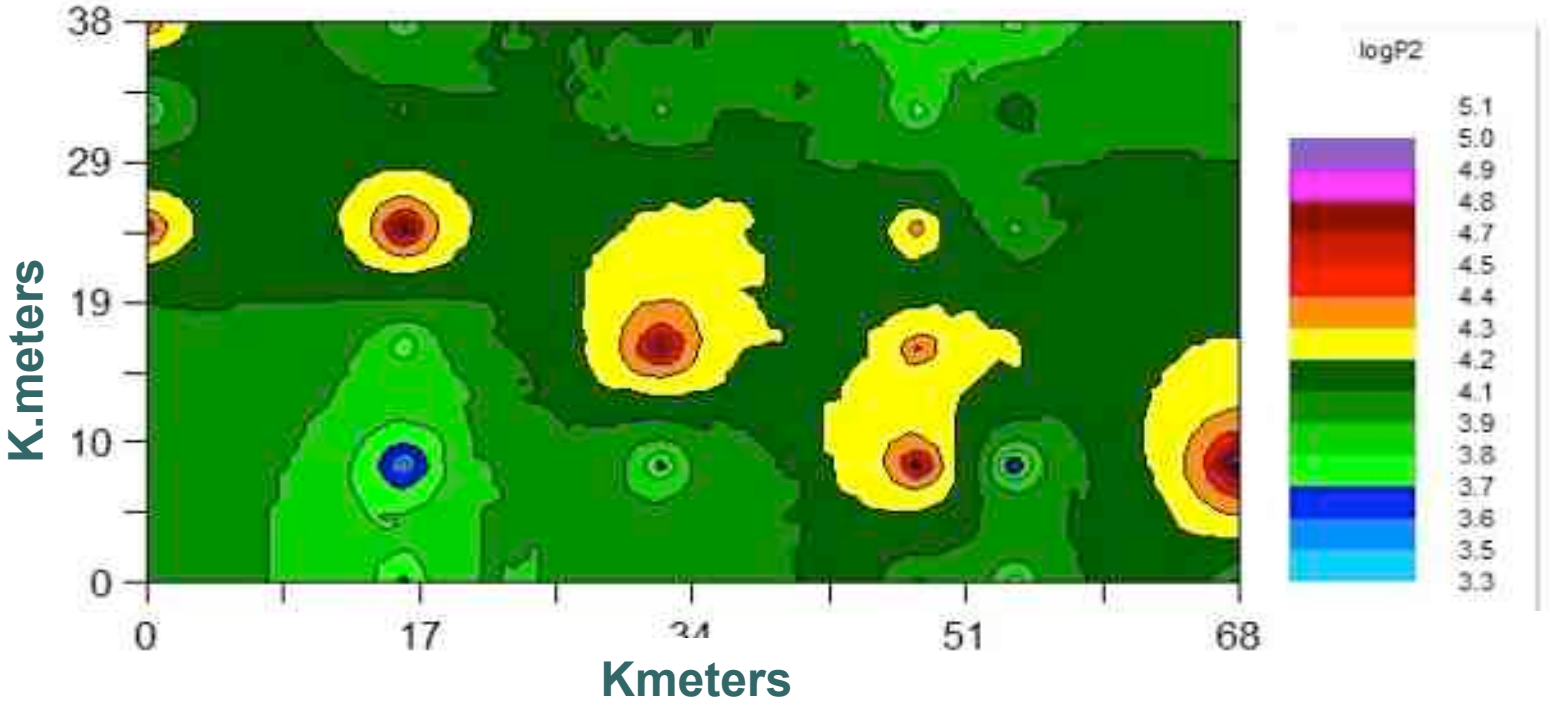
DNA Extraction

PCR

Applications !

1. Quarantine Services
2. Supporting for eradication practices
3. Risk analyses –

Geografic distribution (GS +)



Thank you!

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