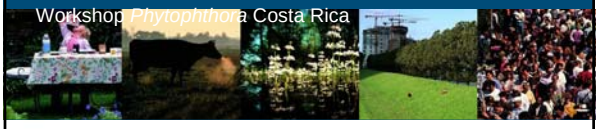
Phytophthora identification/detection: PCR, TaqMan PCR, PCR-RFLP, SSCP, SSR, AFLP, PLP and DNA barcodes

Peter Bonants, 1 July 2010
Workshop *Phytophthora* Costa Rica



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Introduction

- Plant Research International
- Wageningen University & Research Centre
- Netherlands
- *Phytophthora*
- Identification & detection: molecular methods
- Quarantine species

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Introduction

- Identification - Detection (techniques)
- Past / Now / Future
- Qualitative or Quantitative, Single or Multiplex?
- Applications for *Phytophthora*

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Introducción

- Identificación y detección de las técnicas
- Pasado, presente y el futuro
- Cualitativa o cuantitativa, individual o multiplex ?
- Aplicaciones de *Phytophthora*
- Por favor, no dude en hacer preguntas si no entiende

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Clean Material

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What is detection?

- Detection is an activity focused on demonstration of the presence or absence of a certain pathogen, which is suspected to be present in the sample
  - single and multiplex tests
  - specificity, sensitivity
  - diagnostics, monitoring
  - quantitative / qualitative
  - live – dead
  - races – formae speciales

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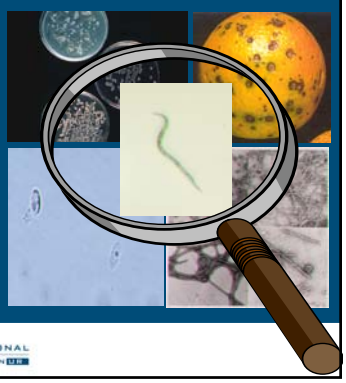
Targets

- Which?
  - Bacteria
  - Viruses
  - Nematodes
  - Fungi
  - Insects
- Where?
  - in plant
  - in water
  - in soil
  - in compost
  - in air



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Techniques for identification/detection:

- Based on physiological characteristics
- Based on biological characteristics
- Based on morphological characteristics (microscopy)
- Based on protein/carbohydrate level:
  - Antisera
  - Isozyme-patterns

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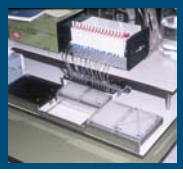
Detection with protein based techniques

- ELISA
- Immuno fluorescence (IF)
- Immuno fluorescence colony staining (IFC)



- Luminex system

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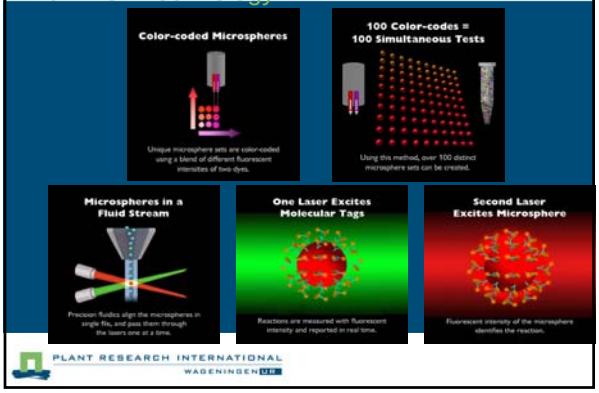
Luminex Technology



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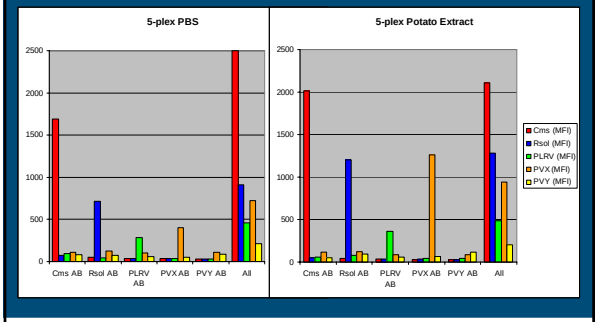
Potato pathogens Luminex 5-plex



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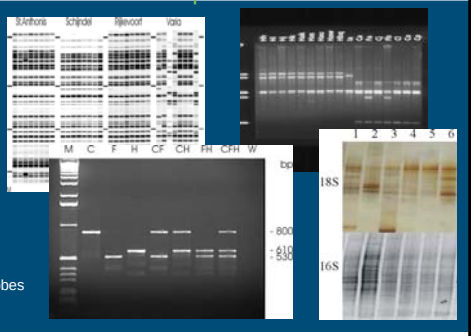
DNA/RNA based techniques

- AFLP
- RAPD-PCR
- SCAR-PCR
- REP-PCR
- ISSR
- SSR
- RFLP
- (RT)-PCR
- DGGE

- NASBA
- TaqMan
- Padlock probes



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Examples *Phytophthora*

- PCR
- TaqMan PCR
- PCR-RFLP
- AFLP
- RAPD-PCR
- ISSR / SSR
- SSCP
- Padlock probes (PLP)

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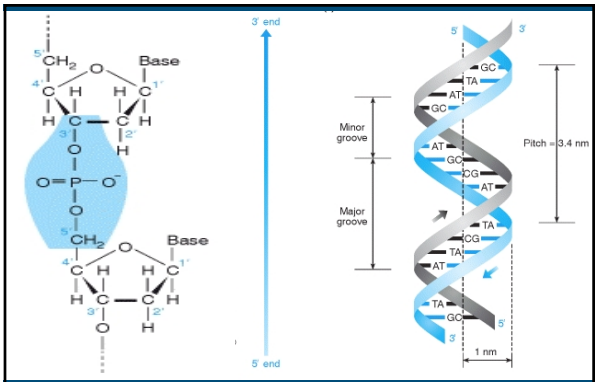

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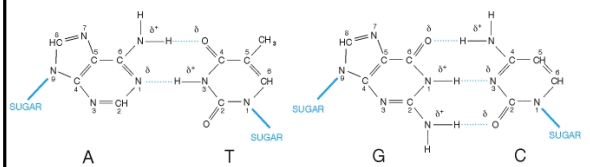
What is DNA?



A: Adenine; T: Thymine; G: Guanine; C: Cytosine

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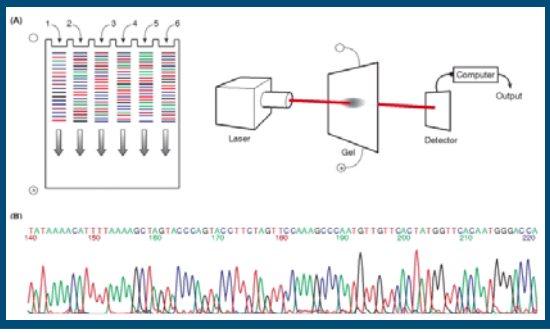
Gene sequence analysis



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Detection techniques: molecular

- Determine DNA sequence of the perpetrator
- DNA sequence differences between target and non-target
  - often ITS, 16S, 18S, 28S, b-tub, EF1a, Cox1

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Detection techniques: molecular

Blair et al. Fungal Genetics and Biology 45: 266-277 (2008)

A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences

Jaime E. Blair [a,*], Michael D. Coffey [b,*], Sook-Young Park [a], David M. Geiser [a], Seogchan Kang [a]

Genes used:

ITS, B-tubulin, Enolase, Heat Shock Protein 90, 60S Ribosoma

Protein L10, LSU rRNA, Cox2, TigA gene fusion, TEF 1a

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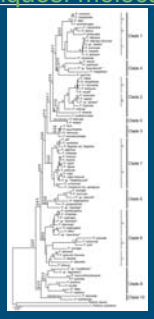
Detection techniques: molecular



Blair et al. Fungal Genetics and Biology 45: 266-277 (2008)

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Detection techniques: molecular

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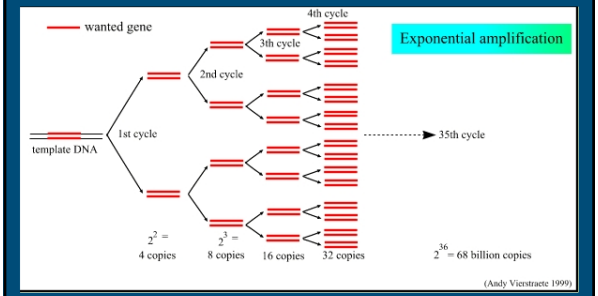
PCR: polymerase chain reaction



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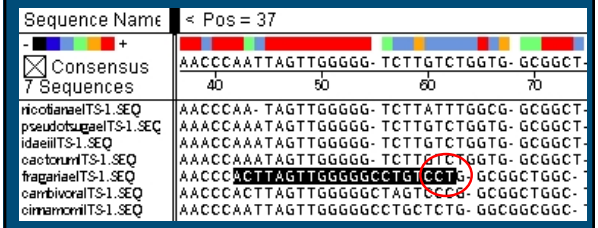
PCR: Sequence difference perpetrator



Specific sequence difference for the perpetrator *Phytophthora fragariae*

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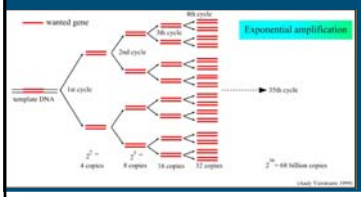
PCR *Phytophthora fragariae*

- ITS scheme for *P. fragariae*

- PCR zoospores *P. fragariae*



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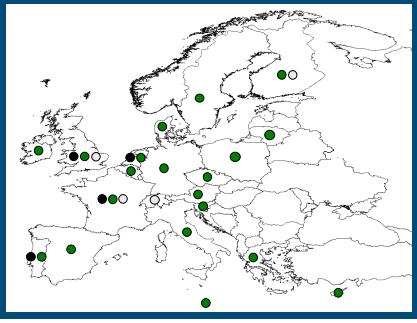
TaqMan PCR: EU project Portcheck



Partners

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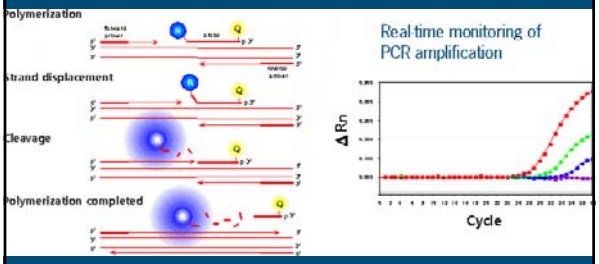
TaqMan PCR



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EU-Portcheck: On-site detection

- TaqMan PCR protocols
- 10 Q-organisms
- DNA extraction protocols
- On-site system

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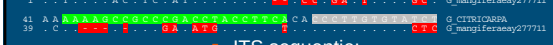
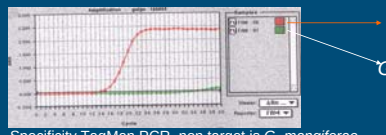
TaqMan PCR *Guignardia citricarpa*

- Symptoms



- ITS sequentie:



Specificity TaqMan PCR, non target is *G. mangiferae*

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3x 3072 PCR reactions in one run; 3x 144 samples

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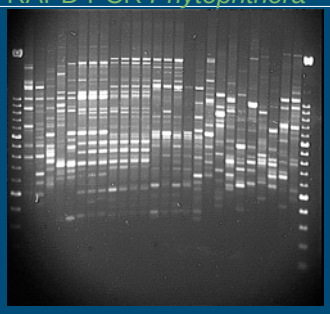
RAPD PCR *Phytophthora*

random amplified polymorphic DNA



PCR with random primers (10-mers)

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Detection techniques: SSCP

Single strand conformation polymorphism

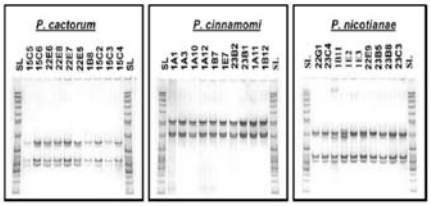

Fig. 2. Examples of single-patterned species include *P. cactorum*, *P. cinnamomi*, and *P. nicotianae*. The names of representative isolates of individual species are indicated on the top of lanes. SL represents the ssDNA ladder developed in this study.

Ping Kong et al. 2003. Fungal Genetics & Biology 39, 238-249

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Identification techniques: SSCP

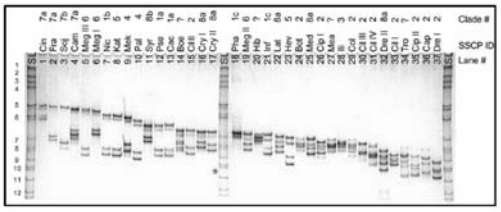

Fig. 3. Distinct SSCP patterns of type isolates of 29 species of *Phytophthora* present in a wide mimigel. Lane number and SSCP pattern identifier are listed on the top of each lane. Phylogenetic clade numbers of respective species as determined by Cooke et al. (2000) are also indicated for reference. SL is the ssDNA ladder. The numbers on the left of gel indicate the position of major fragments in the ladder.

Ping Kong et al. 2003. Fungal Genetics & Biology 39, 238-249

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DNA/RNA based methods

- *Phytophthora ramorum*
- Oak and Rhododendron
- Present in EU and US

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PCR-RFLP *P. ramorum*

restriction fragment length polymorphism

RAATTY *Apo* I

```
P.ramorum OakVS478      AGGTCAAATTCATTTTTGGTT
P.ramorum OakVS479      AGGTCAAATTCATTTTTGGTT
P.ramorum RhodEU233     AGGTCAAATCCATTTTTGGTT
P.ramorum RhodEU98      AGGTCAAATCCATTTTTGGTT
P.ramorum ViburEU474    AGGTCAAATCCATTTTTGGTT
```

Gene sequence analysis Cox-1 *P. ramorum*

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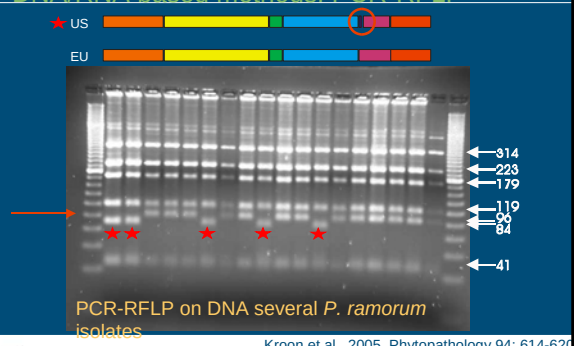
DNA/RNA based methods: PCR-RFLP



PCR-RFLP on DNA several *P. ramorum* isolates

Kroon et al., 2005, Phytopathology 94: 614-620

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Identification techniques: SSR

simple sequence repeats
= microsatellites

AACCTTGAGTGCGGAGAGAGAGAGAGACTGTACGAGCCCGA

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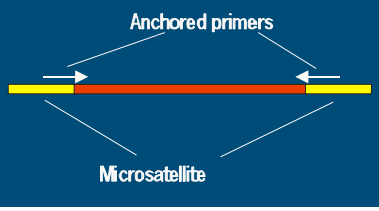
Molecular methods: ISSR and SSR

ISSR : inter-simple sequence repeats

- PCR of ISSR regions with ISSR primers
- Agarose gelelectrophoresis



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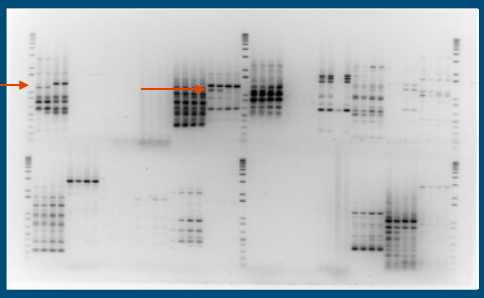
Molecular methods: ISSR *P. ramorum*



ISSR: 2 EU and 2 US isolates with several primers

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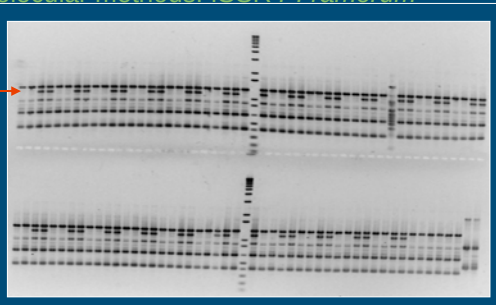
Molecular methods: ISSR *P. ramorum*



ISSR: alternating 2 EU and 2 US isolates with one primer

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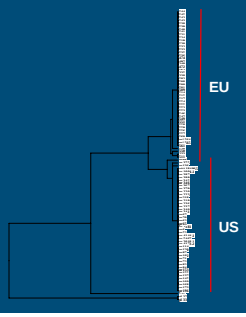
Molecular methods: ISSR *P. ramorum*



ISSR: tree

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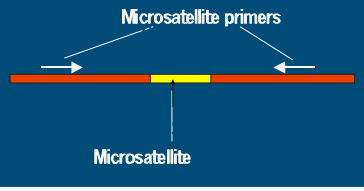
Molecular methods: Microsatellites (SSR)

Microsatellites: SSR (simple sequence repeats), e.g. (AG)n, (TCG)n

- PCR of microsatellite region with SSR primers
- Acrylamide gelelectrophoresis



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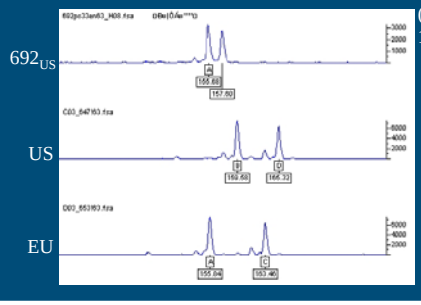
ABI acrylamide gels

Primer 63
$(CT)_{15}$
167 bp



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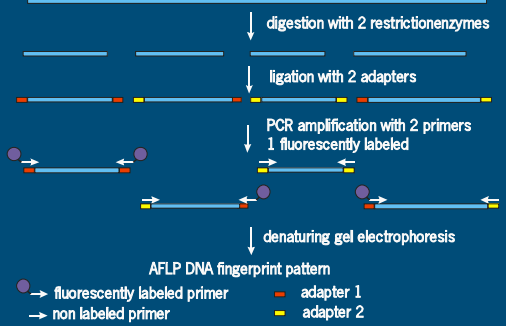
Detection techniques: AFLP

amplified fragment length polymorphism



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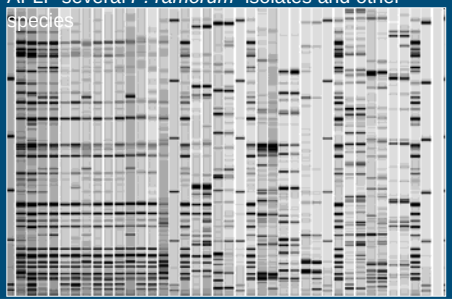
Molecular methods: AFLP

AFLP several *P. ramorum* isolates and other species



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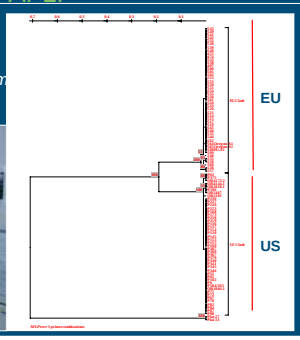
Molecular methods: AFLP

AFLP: 3 primercombinations on many EU and US *P. ramorum* isolates



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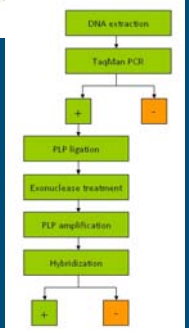
Ivors et al. 2004. Mycol. Res. 108: 378-392.

Puma: Padlock- based Universal Multiplex Array



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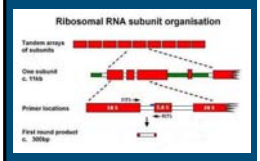
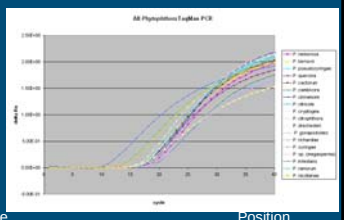
Universal *Phytophthora* TaqMan PCR





Primer	Sequence	Position
Forward: FITS1_15Ph ITS1	TGC GGA AAG GAT CAT TAC CAC ACC	-17 to +7 of
Reverse: RITS1_279Ph	GCG AGC CTA GAC ATC CAC TG	+11 to +30 of 5.8S rRNA
Probe: all_phy 5.8S rRNA	FAM-TTG CTA TCT AGT TAA AAG CA-MGB	-17 to +3 of

Kox et al. 2007. Phytopathology 97: 1119-1129.

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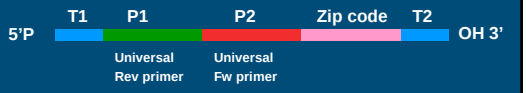
Padlock Probe Ligation



- Circularizing probes
- Long oligonucleotides of ~100bp
- Contain:
  - target complementary regions at both 5' and 3'ends
  - universal primers sites
  - unique sequence identifier (zip-code)

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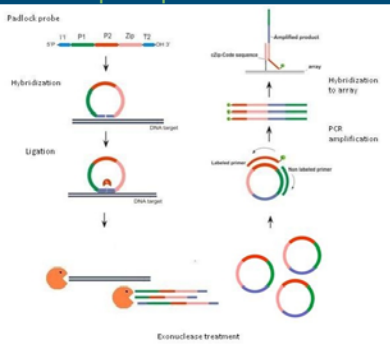
Padlock Probe principle



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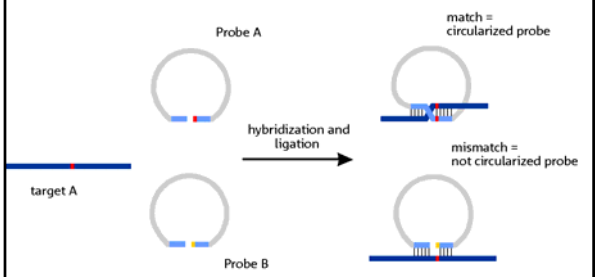
Padlock Probe principle



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Design of the PLP's

- Based on unique sequence motifs in ITS1 region
- Specificity of 5' and 3' arms
- Designed for 22 most important *Phytophthora* species

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List of designed PLP's

Species	Isolate	Padlock probe
Phytophtora alni	CBS 117376	PLP_alni_50
Phytophtora andina	EC 3425	PLP_andid_162
Phytophtora botryosa	CBS 533.92	PLP_botry_193
Phytophtora brassicae	CBS 112277	PLP_bras_78
Phytophtora cactorum	CBS 294.29	PLP_cac_200
Phytophtora cambivora	CBS 114085	PLP_camb_31
Phytophtora capsici	CBS 111333	PLP_caps_150
Phytophtora cinnamomi	CBS 402.48	PLP_cin_30, PLP_cin_46, PLP_cin_55
Phytophtora citricola	CBS 11337	PLP_citri_144
Phytophtora citrophthora	CBS 111338	PLP_citro_154*
Phytophtora cryptogea	CBS 307.62	PLP_cryp_73
Phytophtora fragariae	CBS 309.62	PLP_frag_NAR, PLP_frag_28
Phytophtora hibernalis	CBS 119904	PLP_hybn_96
Phytophtora humicola	CBS 114082	PLP_humicl_129*
Phytophtora infestans	PIC 99189	PLP_infes_161
Phytophtora lateralis	CBS 102608	PLP_lat_93
Phytophtora megasperma	CBS 118733	PLP_megasp_105
Phytophtora multivesiculata	CBS 101593	PLP_multives_232
Phytophtora nicotianae	CBS 304.29	PLP_nic_166, PLP_nic_186
Phytophtora porri	CBS 181.87	PLP_por_100
Phytophtora primulae	CBS 275.74	PLP_prim2_77
Phytophtora ramorum	CBS 101553	PLP_ram_94
Phytophtora rosacearum	CBS 117690	PLP_rosa_136
Phytophtora tentaculata	CBS 412.96	PLP_tentac_171

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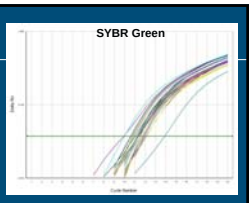
PLP amplification



Primer	Sequence
P1-f19-Biot	5' biotin - CGA GAT GTA CCG CTA TCG T
P2-r20-Phos	5' phosphate - TCA TGC TGC TAA CGG TCG AG

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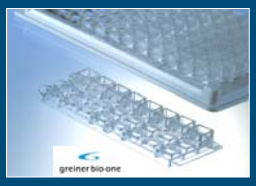
PLP based target signature analysis

Hybridization device



Universal Microarray

- cZipCode oligo's spotted on epoxy-coated slides
- PLP with ZipCode will hybridize on array
- ZipCodes ensure universal hybridization conditio
- ZipCodes provide flexible detection

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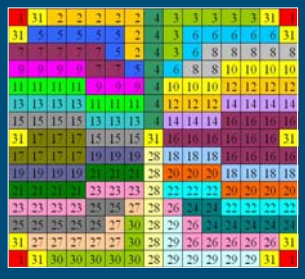
PLP based target signature analysis

Micro-array design



1	Corner Spot	16	PLP_prim2_77
2	PLP_citro_154	17	PLP_bras_78
3	PLP_botry_193	18	PLP_por_100
4	PLP_nic_166	19	PLP_cin_55
5	PLP_tentac_171	20	PLP_cin_46
6	PLP_andid_162	21	PLP_cin_30
7	PLP_infes_161	22	PLP_camb_31
8	PLP_citri_144	23	PLP_alni_50
9	PLP_multives_232	24	PLP_cryp_73
10	PLP_rosa_136	25	PLP_frag_28
11	PLP_humicl_129	26	PLP_frag_NAR
12	PLP_megasp_105	27	PLP_nic_186
13	PLP_hybn_96	28	PLP_caps_150
14	PLP_lat_93	29	PLP_cac_200
15	PLP_ram_94	30	PLP_All_Phyt_2004
		31	CH2 (Immob control)

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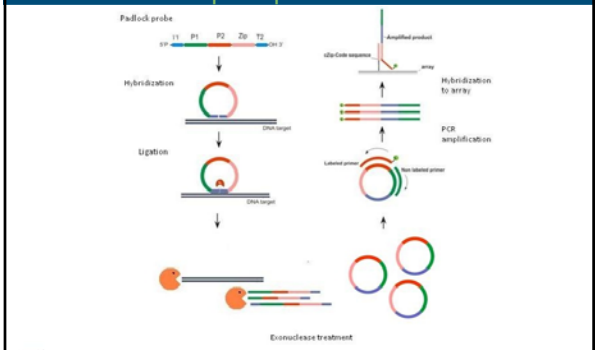
Padlock Probe principle



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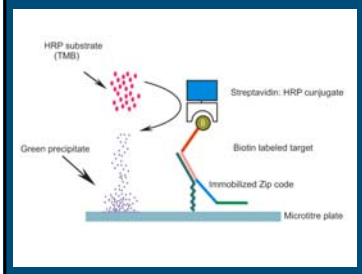
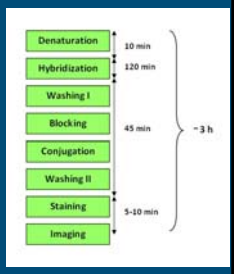
Micro-array staining procedure





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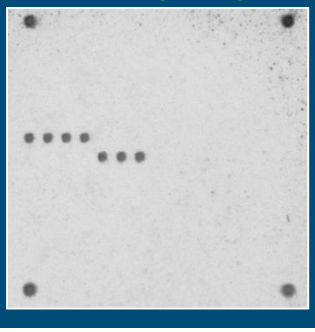
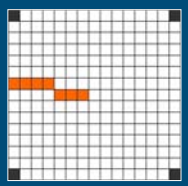
Individual target/single PLP detection



P. ramorum + PLP_ram_94



- Specific PLP ligation
- No cross-hybridization

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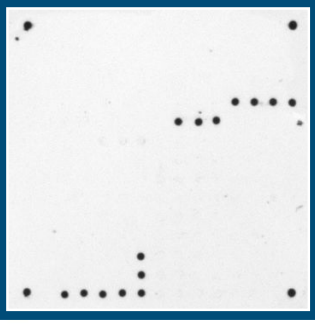
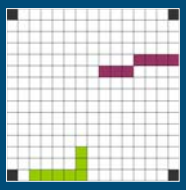
Individual target detection with a mixture of PLP's



P. megasperma



- Specific ligation
- No cross-hybridization
- Unique target signature

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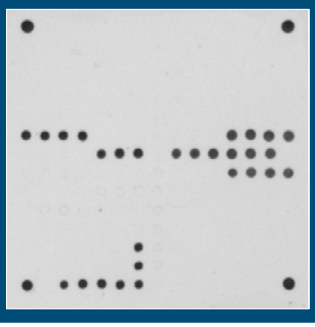
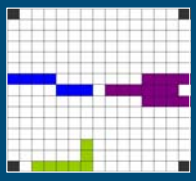
Individual target/mix of PLP's



P. ramorum



- Unique target signature:
  - PLP_ram_94
  - PLP_prim2_77
  - PLP_All-Phyt_2004

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Target signature for:

A: *P. alni*
B: *P. andina*
C: *P. brassicae*
D: *P. cinnamomi*
E: *P. cryptogea*
F: *P. fragariae*
G: *P. megasperma*
H: *P. primulae*

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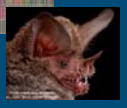
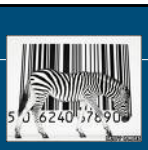
DNA barcodes



DNA barcoding is a new technique that uses a short sequence of a standard region of the genomic DNA as a molecular diagnostic marker for species identification

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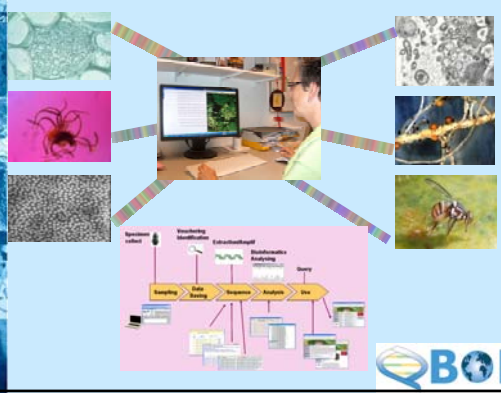
QBOL:

Development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health

www.qbol.org
peter.bonants@wur.nl

QBOL | Barcoding of Life

DNA barcode identification



DNA barcoders

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- Analysis laboratoria
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- National Government
- European Union

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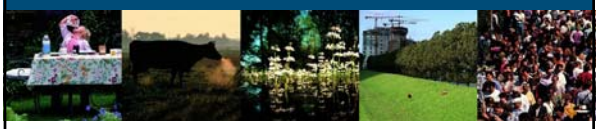
Espero que ustedes hayan comprendido mi presentación

Muchas gracias



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