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# Development of a DNA Macroarray for Detection of Young Vine Decline Pathogens

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Canada

# Development of a DNA Macroarray

## Outline

- Snapshot of BC Vineyards and the Okanagan Valley
- Grapevine Trunk Diseases (GTD) & Young Vine Decline
- DNA Macroarray System
  - a) Macroarray development
  - b) Macroarray diagnostic results
  - c) Macroarray testing
- Macroarray potential applications and future work



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# Okanagan Valley



## Desirable conditions:

- Ideal growing degree days
- Abundant sunshine
- Low rainfall
- Low humidity

## Extremes:

- Short growing season (compact)
- Low and variable winter temperatures (arctic outflow events)

# Okanagan Valley

## Geography

- narrow glaciated valley
- Coastal mountain rain shadow

## Climatic influences

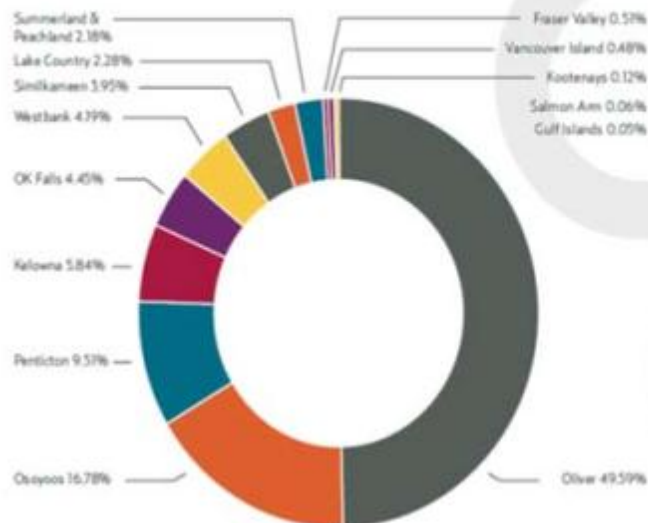
- continental summer heat
- temperature moderated by lakes
- warm Pacific weather systems
- cold arctic outflow events



## RED WINE GRAPE VARIETIES

VARIETY	ACREAGE	% OF WHITES	% OF TOTAL GRAPES
Merlot	1,600.90	31.15%	16.23%
Pinot Noir	948.71	18.46%	9.62%
Cabernet Sauvignon	755.17	14.69%	7.65%
Syrah/Shiraz	546.50	10.63%	5.54%
Cabernet Franc	517.45	10.07%	5.24%
Gamay Noir	153.73	2.99%	1.56%
Maréchal Foch	137.30	2.67%	1.39%
Blettner Reds	97.29	1.89%	0.99%
Malbec	95.96	1.87%	0.97%
Petit Verdot	65.58	1.28%	0.67%
Zweigelt	41.72	0.81%	0.42%
Zinfandel	18.84	0.37%	0.19%
Pinotage	14.90	0.29%	0.15%
Tempranillo	13.91	0.27%	0.14%
Baco Noir	12.45	0.24%	0.13%
Pinot Meunier	11.33	0.22%	0.12%
Lamberger	10.10	0.20%	0.10%
Sangiovese	8.17	0.16%	0.09%
Leon Millot	7.20	0.14%	0.07%
Chancellor	7.10	0.14%	0.07%
Dunkelfelder	6.16	0.12%	0.06%
Agris	5.71	0.11%	0.06%
Cestai	5.32	0.10%	0.05%
Carmenere	4.43	0.09%	0.04%
Grenache	4.18	0.08%	0.04%
Mourvedre	3.82	0.07%	0.04%
Domfelder	3.17	0.06%	0.03%
Berbera	3.13	0.06%	0.03%
Rotberger	2.95	0.06%	0.03%
Michurinetz	1.00	0.02%	0.01%
St Laurent	0.90	0.02%	0.01%
Nebbiolo	0.50	0.01%	0.01%
Miscellaneous Reds	33.70	0.66%	0.34%
<b>TOTAL RED HYBRIDS (ACRES):</b>	<b>280.46</b>	<b>5.5%</b>	<b>-</b>
<b>TOTAL RED VINIFERA (ACRES):</b>	<b>4,858.82</b>	<b>94.5%</b>	<b>-</b>
<b>TOTAL RED GRAPES (ACRES):</b>	<b>5,139.28</b>	<b>100.00%</b>	<b>52.09%</b>

## REPORTED TONNAGE BY PRODUCTION AREA



# Grapevine Trunk Diseases (GTD)

- Caused by fungi (Ascomycetes and Basidiomycetes)
- Infect grapevines through wounds (pruning wounds)
- Endophytes or facultative parasites
- Colonize the vascular tissue (spurs, cordon, trunk, roots)



- Cause either a rapid or slow decline and eventual death
- Significant economic impact on the grapevine industry



# GTD in young vines (< 5-6 years)

## Young Vine Decline Complex

- Petri Disease
- Black Foot Disease



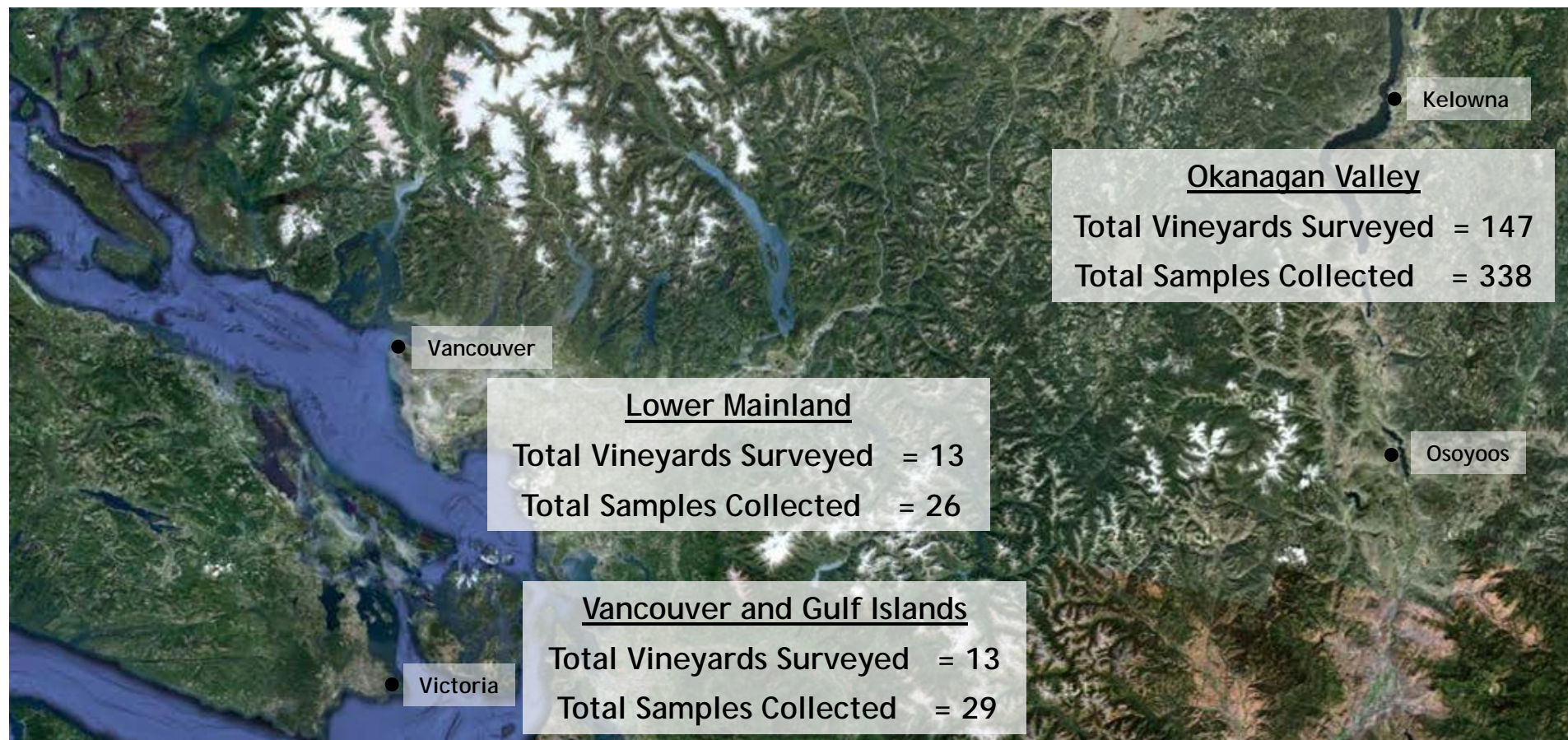
# GTD in mature vines (> 6 years)

- Esca
- Eutypa dieback
- Botryosphaeria dieback
- Phomopsis dieback



# Current status and significance of GTD in B.C.

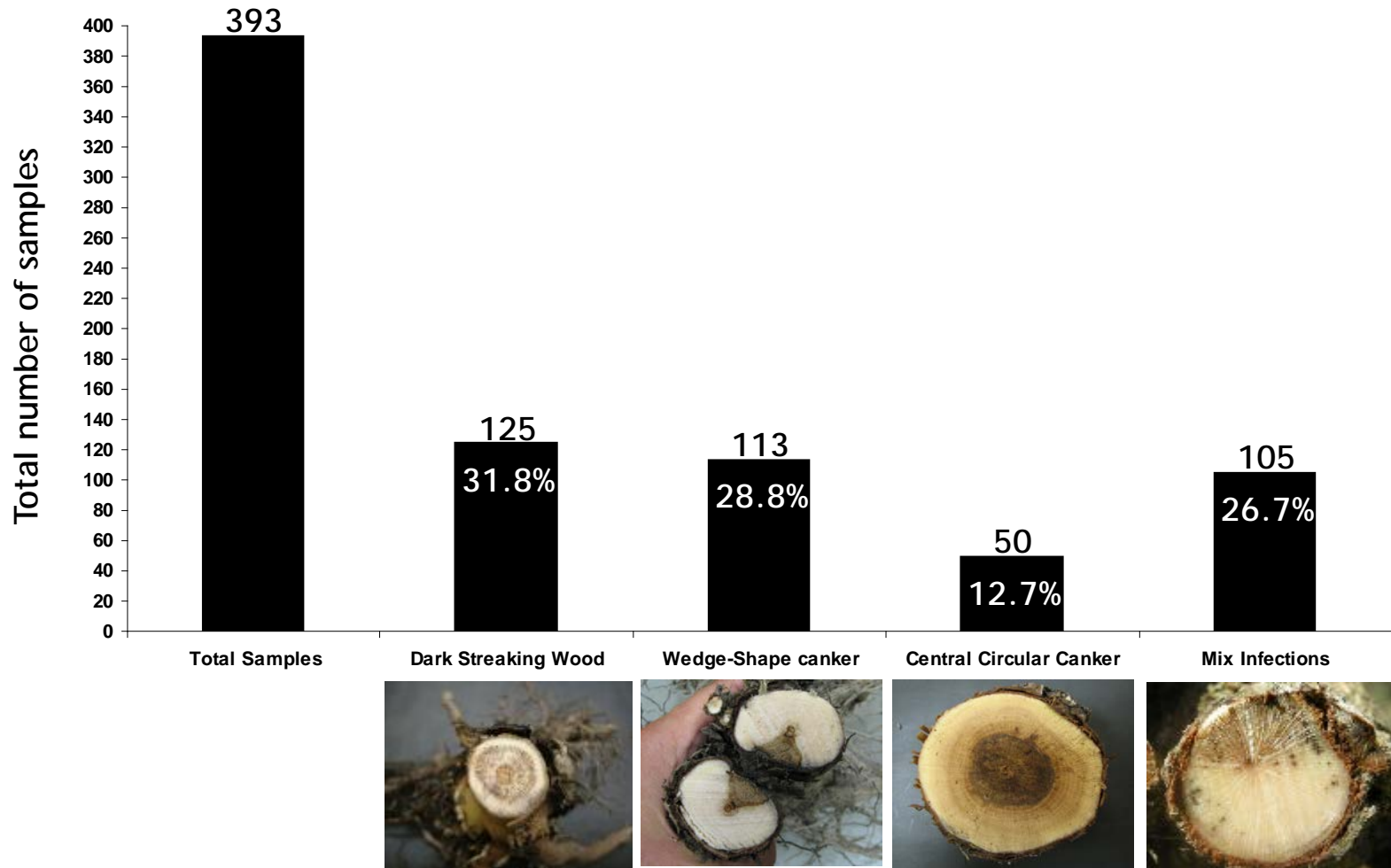
Disease survey: 173 Vineyards surveyed and 393 Samples collected



# Disease survey

## Overall GTD vascular symptoms observed in B.C.

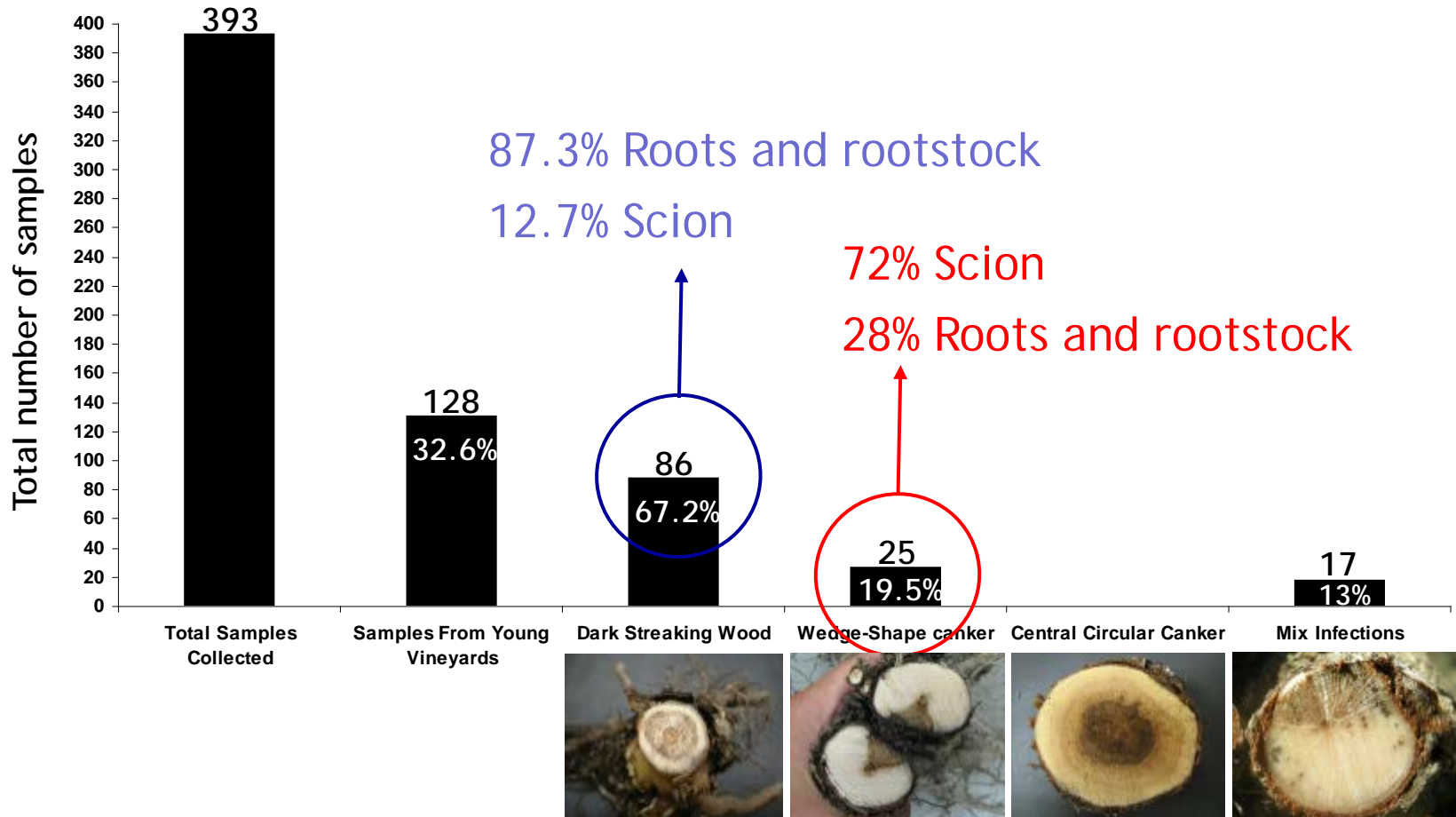
All 173 vineyards surveyed showed GTD infected vines



# Disease survey

## GTD vascular symptoms based on vineyard age

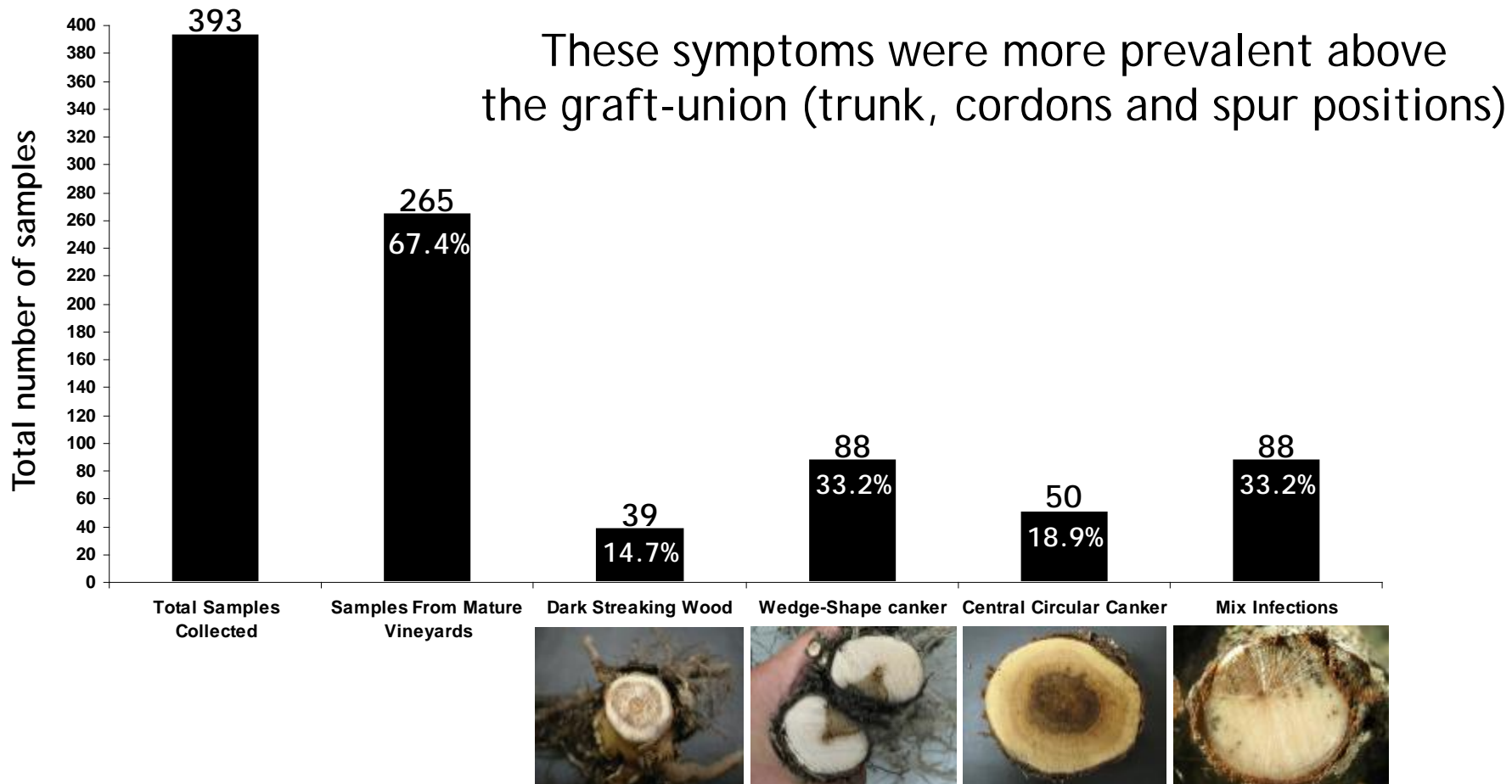
65 young vineyards surveyed (< 6 years)



# Disease survey

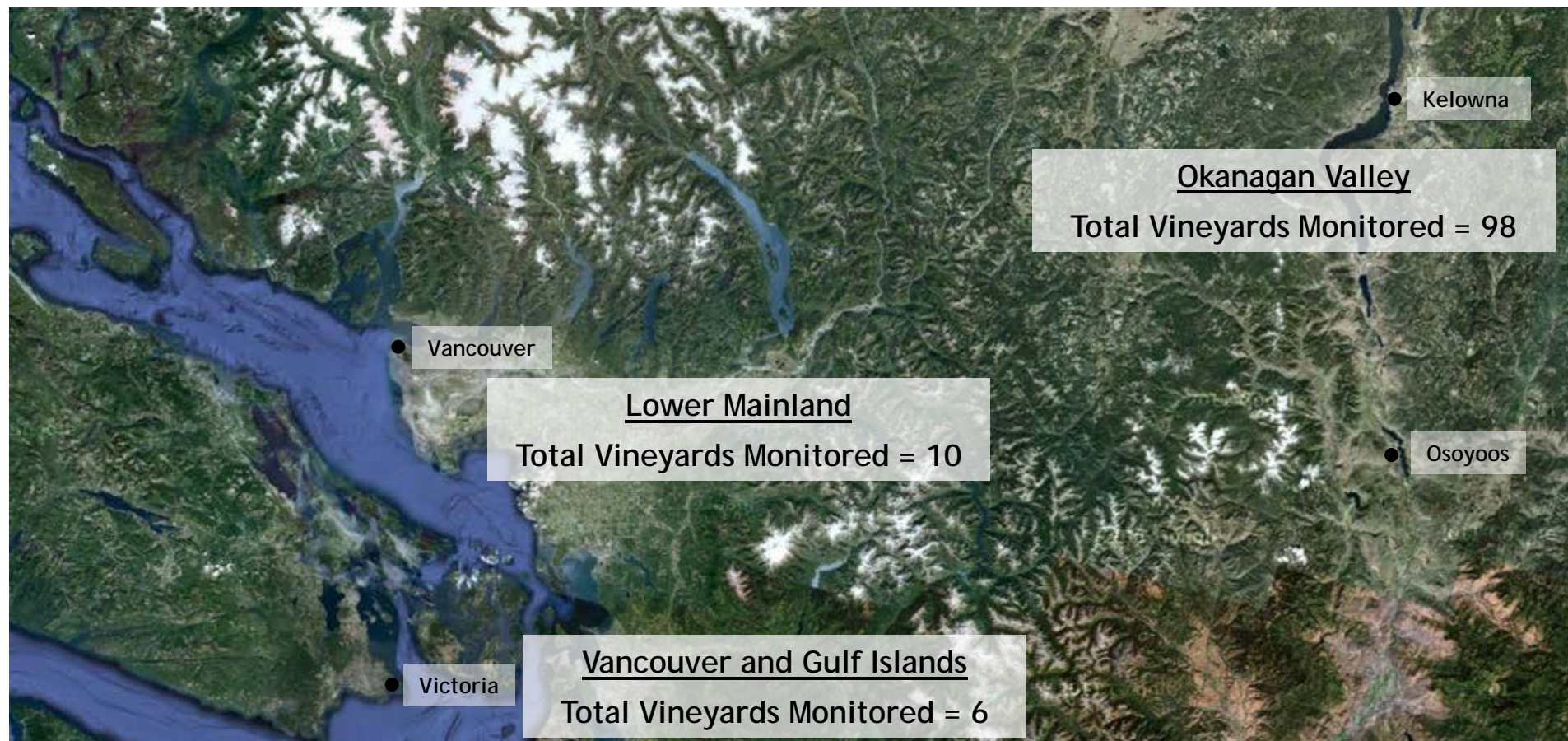
## GTD vascular symptoms based on vineyard age

108 mature vineyards surveyed (> 6-years)



# Disease survey: GTD field symptoms monitoring

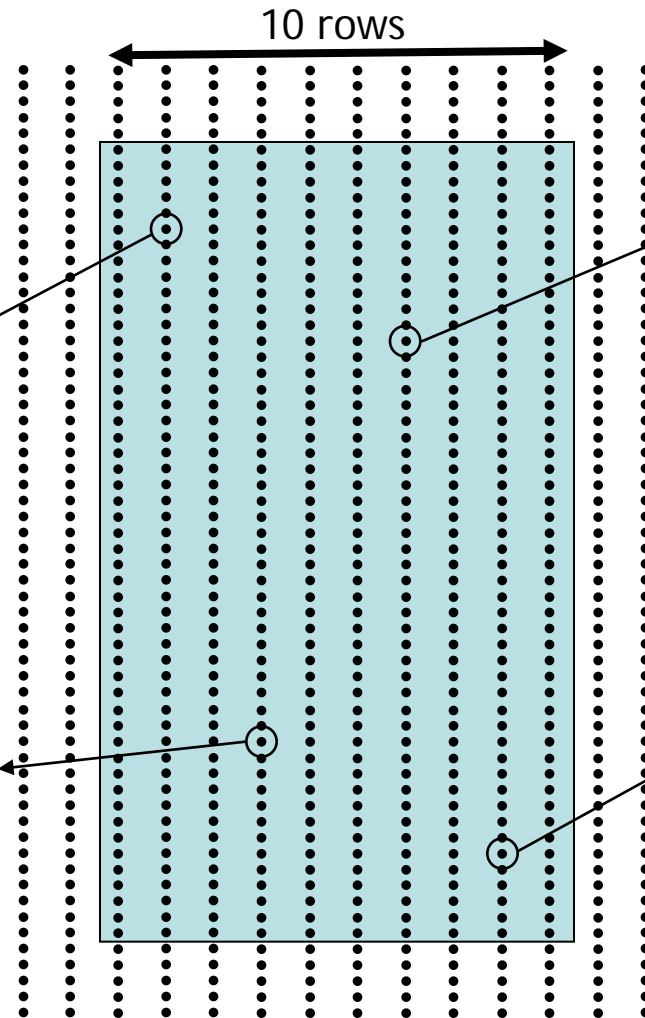
114 Vineyards monitored during the 2011 growing season



# Disease survey: GTD field symptoms monitoring

114 Vineyards monitored during the 2011 growing season

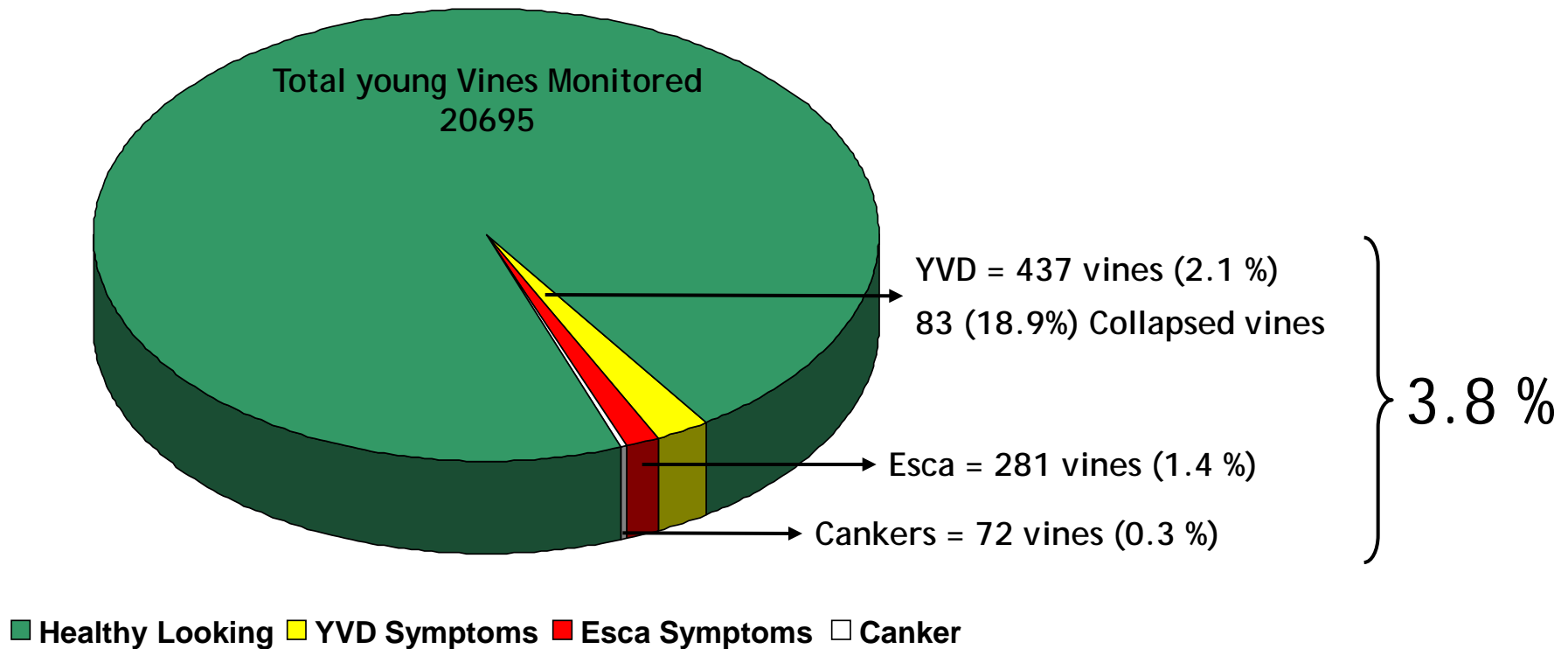
Over 56.000 vines monitored





# Disease survey: GTD field symptoms monitoring

- 44 young vineyards monitored (< 6-years)
- Only 4 out of 44 did not show GTD Symptomatic Vines

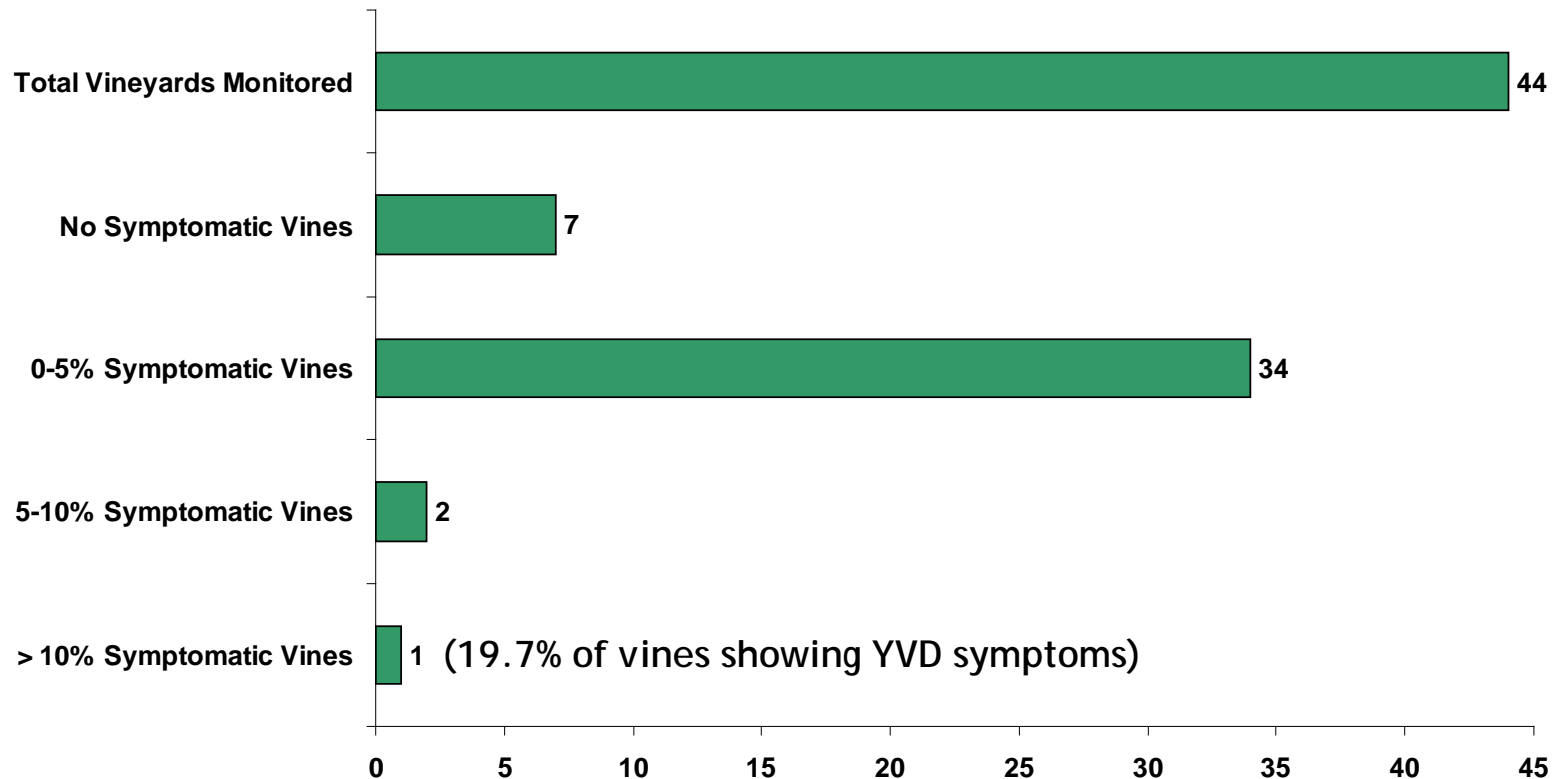


# Disease survey: GTD field symptoms monitoring

44 young vineyards monitored (< 6-years old)

Disease severity varied significantly among vineyards monitored

% of YVD symptomatic vines per vineyard

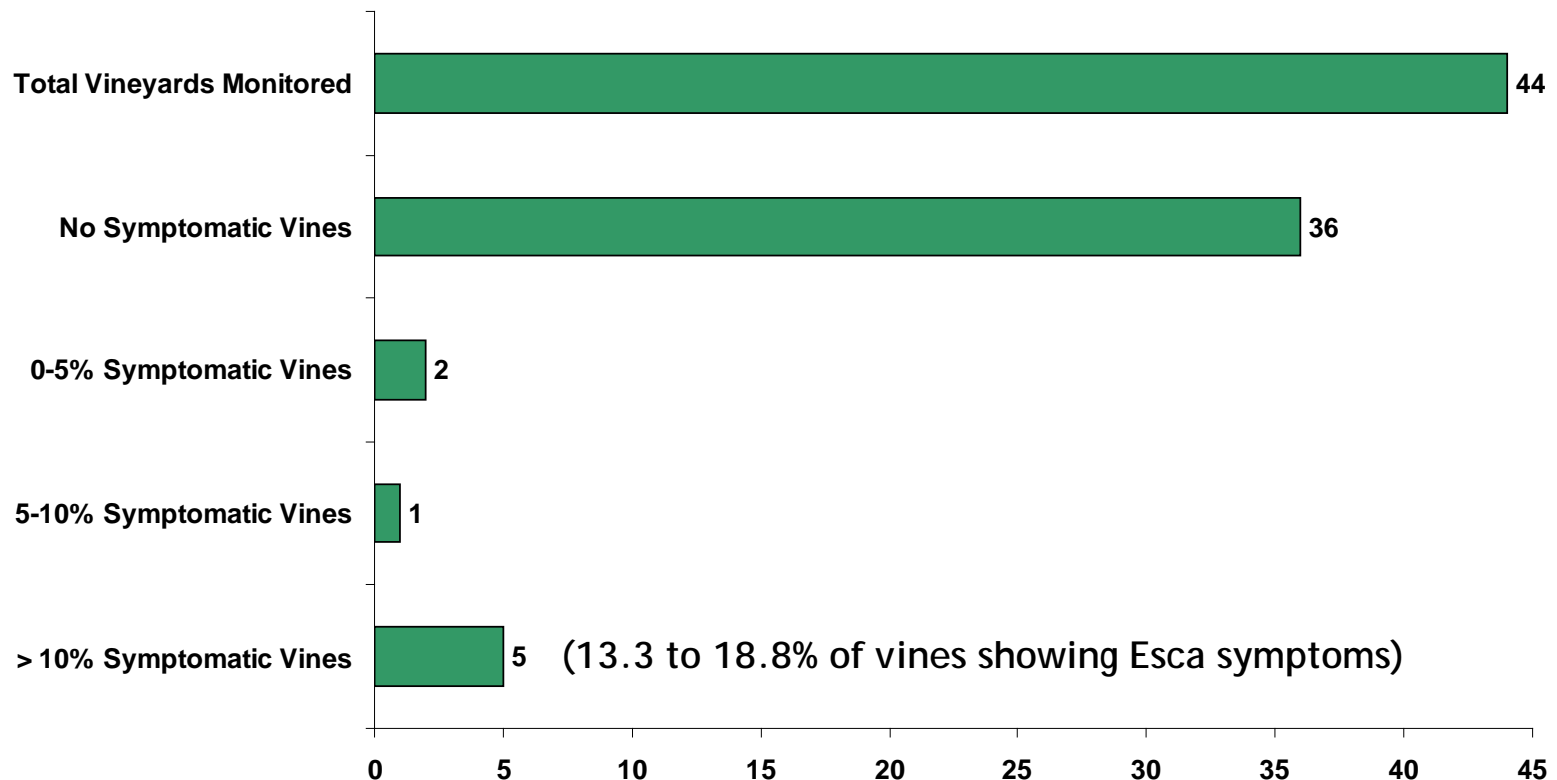


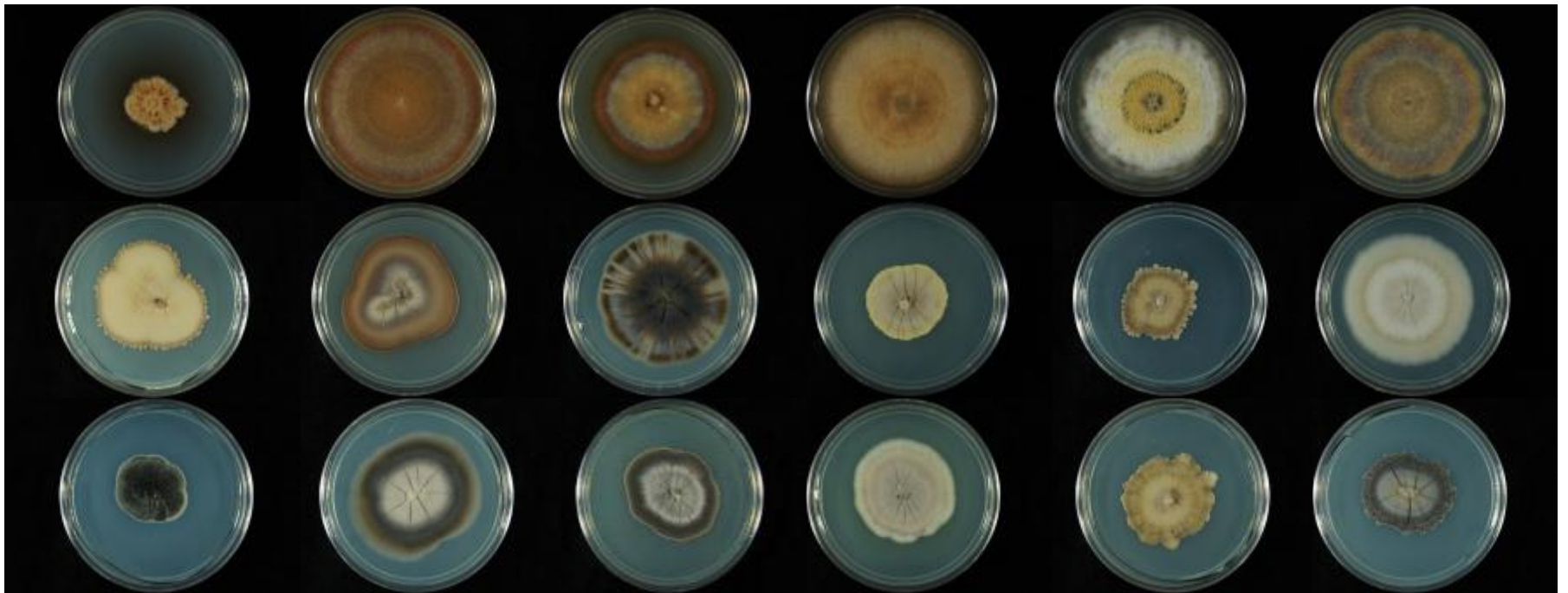
# Disease survey: GTD field symptoms monitoring

44 young vineyards monitored (< 6-years old)

Disease severity varied significantly among vineyards monitored

% of Esca symptomatic vines per vineyard





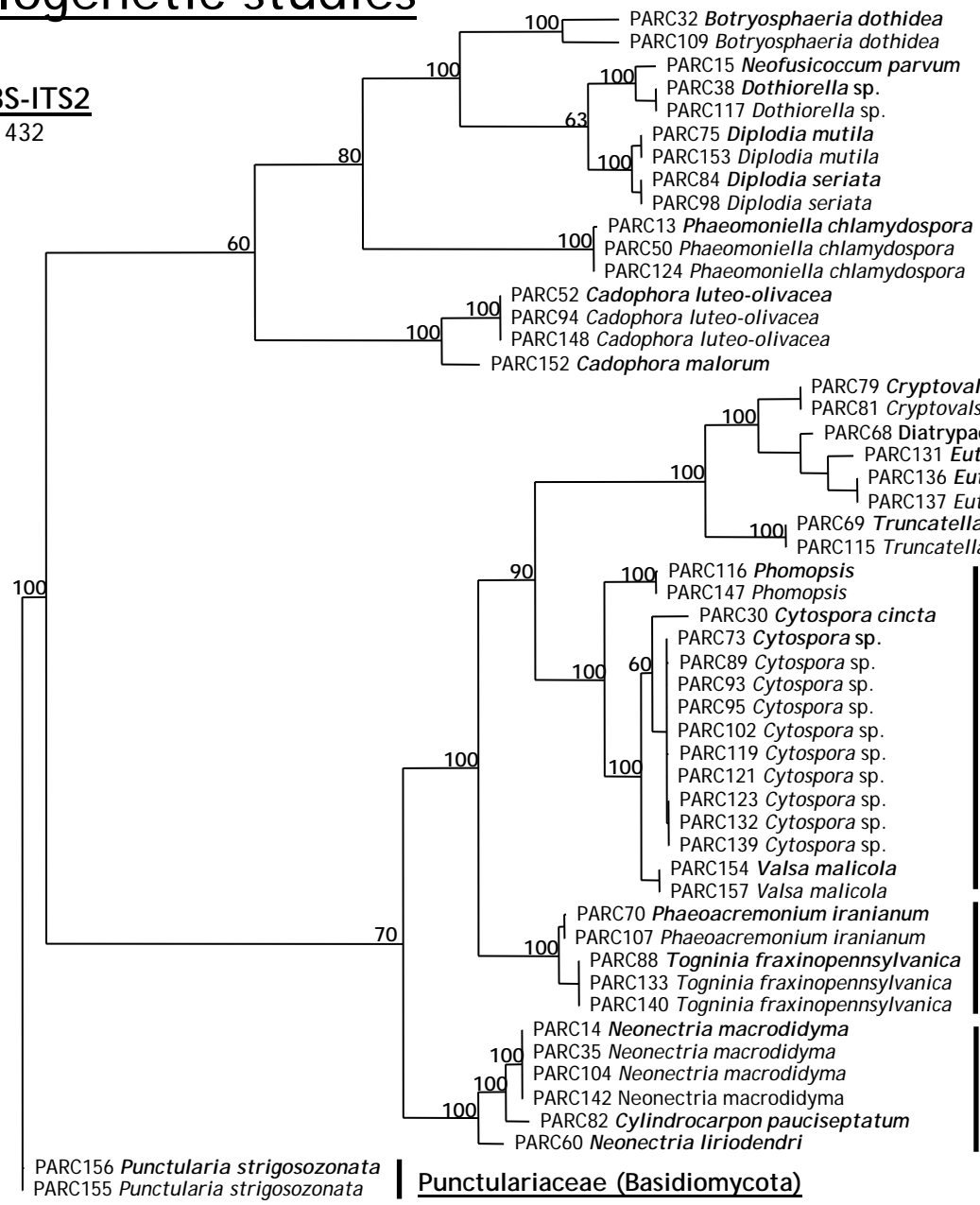
## 2) Molecular identification (DNA - PCR - Sequencing)



# - Phylogenetic studies

## ITS1-5.8S-ITS2

Length = 1432  
 CI = 0.596  
 RI = 0.888  
 HI = 0.404



Botryosphaeriaceae  
 "Botryosphaeria dieback"

Mitosporic Ascomycota  
 "Esca" & "Petri Disease"

Mitosporic Heliotiales  
 "Petri Disease"

Diatrypaceae  
 "Eutypa dieback"

Mitosporic Amphisphaeriaceae  
 Grapevine cankers

Valsaceae  
*Phomopsis viticola*  
 "Phomopsis cane and leaf spot" & "Phomopsis dieback"

*Cytospora* spp.  
 Cankers on fruit trees

Calosphaeriaceae & Mitosporic Calosphaeriaceae  
 "Esca" & "Petri Disease"

Nectriaceae & Mitosporic Neonectria  
 "Black-foot disease"

PARC156 *Punctularia strigosozonata*  
 PARC155 *Punctularia strigosozonata* | Punctulariaceae (Basidiomycota)

# Disease survey: pathogens identification

## 2) Molecular identification (ITS, BTUB, EF, ACTIN)



1. *Cadophora luteo-olivacea*
2. *Phaeomoniella chlamydospora*
3. *Togninia minima*
4. *Togninia fraxinopennsylvanica*
5. *Togninia viticola*
6. *Phaeoacremonium angustius*
7. *Phaeoacremonium iranianum*
8. *Phaeoacremonium* sp. nov.
9. *Cylindrocarpon pauciseptatum*
10. *Ilyonectria liriodendri*
11. *Ilyonectria macrodydima*
12. *Ilyionectria raditicola*
13. *Botryosphaeria dothidea*
14. *Diplodia mutila*
15. *Diplodia seriata*
16. *Dothiorella* sp. nov.
17. *Neofusicoccum parvum*
18. *Neofusicoccum ribis*
19. *Eutypa lata*
20. *Eutypa flavovirens*
21. *Eutypa laevata*
22. *Cryptosphaeria pullmanensis*
23. *Cryptovalsa ampelina*
24. *Diatrype stigma*
25. *Diatrype whitemanensis*
26. *Diatrypella* sp.
27. *Diatrypella* sp. nov.
28. *Phomopsis* sp.

Young Vine Decline (Petri disease / Esca)

Young Vine Decline (Black-foot disease)

Botryosphaeria dieback

Eutypa dieback

Phomopsis dieback

# Disease survey: Pathogens Identification

## 2) Molecular identification (ITS, BTUB, EF, ACTIN)



1. *Cytospora cincta*
2. *Cytospora chrysosperma*
3. *Cytospora* sp. nov.
4. *Valsa* sp. nov.
  
5. *Cadophora melinii*
6. *Cadophora malorum*
7. *Phialophora dancoi*
8. *Neofabraea alba*
9. *Neofabraea malicorticis*
  
10. *Phomopsis amygdali*
11. *Phomopsis quercina*
12. *Phomopsis vacinii*
13. *Phomopsis* sp. nov.
13. *Diaporthe eres*
14. *Diaporthe viticola*
  
15. *Truncatella angustata*
  
16. *Fusarium* spp.

Some of these fungal species are well-known pathogens causing dieback in perennial hosts, including fruit trees (apple, cherry, pear, peach, apricot, and plum) and cane berries (blueberries, blackberries, and raspberries)

# Disease survey: pathogen identification and incidence

## Dark streaking of the wood



1. *Phaeomoniella chlamydospora* (32.3%)
2. *Cylindrocarpon* spp. (21.7%)
3. *Phaeoacremonium* spp. (10.4%)
4. Botryosphaeriaceae spp. (9.8%)
5. *Cytospora* spp. (2.8%)
6. *Phomopsis* sp. (1.8%)
7. *Cadophora* spp. / *Phialophora* spp. (0.8%)
8. *Truncatella angustata* (0.8%)
9. *Verticillium* sp. (0.8%)
10. Other fungi (18.8%)

## Wedge-shape cankers



1. *Cytospora* spp. (27.3%)
2. *Diatrypaceae* spp. (23.3%)
3. Botryosphaeriaceae spp. (23.1%)
4. *Phaeomoniella chlamydospora* (7.4%)
5. *Phaeoacremonium* spp. (6.1%)
6. *Phomopsis* sp. (4.3%)
7. *Truncatella angustata* (3.4%)
8. *Neofabraea* spp. (2.1%)
9. *Cadophora* spp. / *Phialophora* spp. (1.7%)
10. *Cylindrocarpon* spp. (1.3%)



# Disease survey: pathogen identification and incidence

Central necrosis



- |  |         |
|--|---------|
| 1. Diatrypaceae spp.                               | (22.7%) |
| 2. <i>Cytospora</i> spp.                           | (22.1%) |
| 3. <i>Phaeomoniella chlamydospora</i>              | (12%)   |
| 4. <i>Botryosphaeriaceae</i> spp.                  | (11.3%) |
| 5. <i>Phaeoacremonium</i> spp.                     | (10.7%) |
| 6. <i>Truncatella angustata</i>                    | (8.4%)  |
| 7. <i>Neofabraea</i> spp.                          | (4.8%)  |
| 8. <i>Cadophora</i> spp. / <i>Phialophora</i> spp. | (3%)    |
| 9. <i>Phomopsis</i> sp.                            | (1.8%)  |
| 10. <i>Cylindrocarpon</i> spp.                     | (1.2%)  |

# DNA Macroarray Development

- A DNA array (a.k.a. microarray, macroarray, gene chip, or DNA chip,) is a collection of microscopic DNA spots attached to a solid surface
- Each DNA spot contains a specific DNA sequence, known as probes
  - hundreds to thousands spots/probes can be placed in known locations on a single DNA array
  - sample DNA binds specifically to individual probes and is detected by a chemiluminescent signal
- DNA arrays are of great use for:
  - simultaneous pathogen detection (disease complex or multiple infections)
  - detection of many different targets (large number of different pathogen species)

# DNA multiscan

The DNA multiscan: A multiplex method, that means that in one analysis different pathogens can be tested. At this moment Relab den Haan offers only fungal DNA multiscan. This will change soon!! At this moment we are working hard on the development of a membrane with more fungi, bacteria and nematodes.

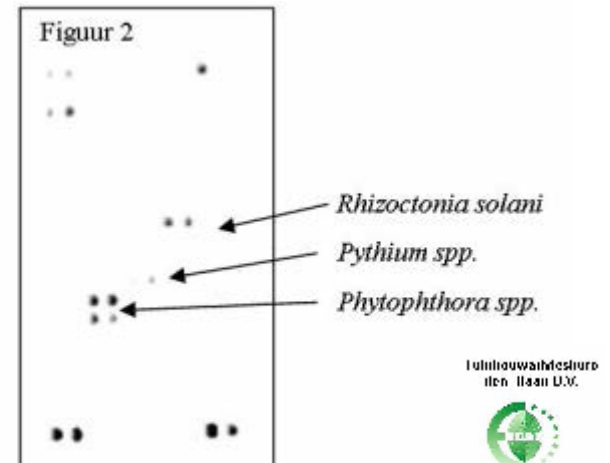
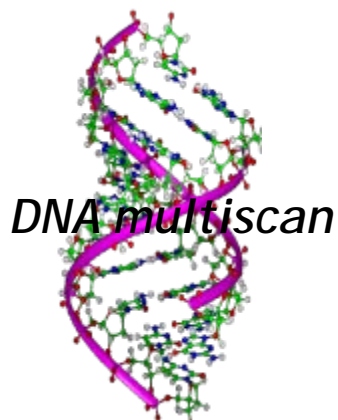
## Fungi detected with the DNA multiscan

- Oomyceten
- *Athelia (Sclerotium) rolfsii*
- *Botrytis cinerea*
- *Colletotrichum spp.*
- *Colletotrichum coccodes*
- *Colletotrichum gleosporioides*
- *Colletotrichum acutatum*
- *Didymella spp.*
- *Fusarium spp.*
- *Fusarium solani*
- *Fusarium oxysporum*

- *Penicillium spp.*
- *Phytophthora spp.*
- *Phytophthora capsici*
- *Phytophthora cinnamomi*
- *Phytophthora drechsleri*
- *Phytophthora cryptogea*
- *Phytophthora infestans*
- *Phytophthora nicotianae*
- *Phytophthora ramorum*
- *Phytophthora fragariae*
- *Phytophthora cactorum*

- *Plectosphaerella cucumerina*
- *Pyrenochaeta lycopersici*
- *Pythium spp.*
- *Pythium aphanidermatum*
- *Pythium dissotocum*
- *Pythium polymastum*
- *Pythium sylvaticum*
- *Pythium ultimum*
- *Pythium irregulare*
- *Rhizoctonia solani*

- *Sclerotinia spp.*
- *Sclerotinia minor*
- *Sclerotinia sclerotiorum*
- *Sclerotinia trifoliorum*
- *Trichoderma spp.*
- *Trichoderma asperellum*
- *Trichoderma harzianum*
- *Trichoderma hamatum*
- *Verticillium spp.*
- *Verticillium albo-atrum*
- *Verticillium dahliae*



# DNA Macroarray Development

## Petri Disease / Esca

1. *Phaeomoniella chlamydospora*
2. *Phaeoacremonium aleophilum*
3. *Phaeoacremonium amstelodamense*
4. *Phaeoacremonium angustius*
5. *Phaeoacremonium argentinense*
6. *Phaeoacremonium armeniacum*
7. *Phaeoacremonium australiense*
8. *Phaeoacremonium austroafricanum*
9. *Phaeoacremonium cinereum*
10. *Phaeoacremonium croatiense*
11. *Phaeoacremonium globosum*
12. *Phaeoacremonium griseolivacea*
13. *Phaeoacremonium fuscum*
14. *Phaeoacremonium hispanicum*
15. *Phaeoacremonium hungaricum*
16. *Phaeoacremonium inflatipes*
17. *Phaeoacremonium iraniamum*
18. *Phaeoacremonium krajdenu*
19. *Phaeoacremonium mortoniae*
20. *Phaeoacremonium nova-zelandae*
21. *Phaeoacremonium occidentale*
22. *Phaeoacremonium pallidum*
23. *Phaeoacremonium prunicola*
24. *Phaeoacremonium scolyti*
25. *Phaeoacremonium sicilianum*
26. *Phaeoacremonium subulatum*
27. *Phaeoacremonium tardicrescens*
28. *Phaeoacremonium theobromatis*
29. *Phaeoacremonium tuscanum*
30. *Phaeoacremonium venezuelense*
31. *Phaeoacremonium viticola*
32. *Phaeoacremonium vivratilis*

32 species of  
*Phaeomoniella* and  
*Phaeoacremonium* spp.

## Black-foot

1. *Ilyonectria liriiodendri*
2. *Cylindrocarpon obtusisporium*
3. *Cylindrocarpon lucidum*
4. *Cylindrocarpon olidum*
5. *Ilyonectria faginata*
6. *Cylindrocarpon theobromicola*
7. *Ilyonectria radiciocla*
8. *Cylindrocarpon ianthothele*
9. *Cylindrocarpon cylindroides*
10. *Cylindrocarpon gamsii*
11. *Cylindrocarpon europaea*
12. *Campylocarpon fasciculare*
13. *Campylocarpon pseudofasciculare*
14. *Ilyonectria macrodydima*
15. *Cylindrocarpon pauciseptatum*
16. *Cylindrocarpon* sp.

16 species of  
*Cylindrocarpon* spp.

# DNA Macroarray Development

- Species-specific oligonucleotide probes design

115 probes (pathogens and related species)

## 86 YVD pathogen-specific probes

12 - *Phaeomoniella*

46 - *Phaeoacremonium*

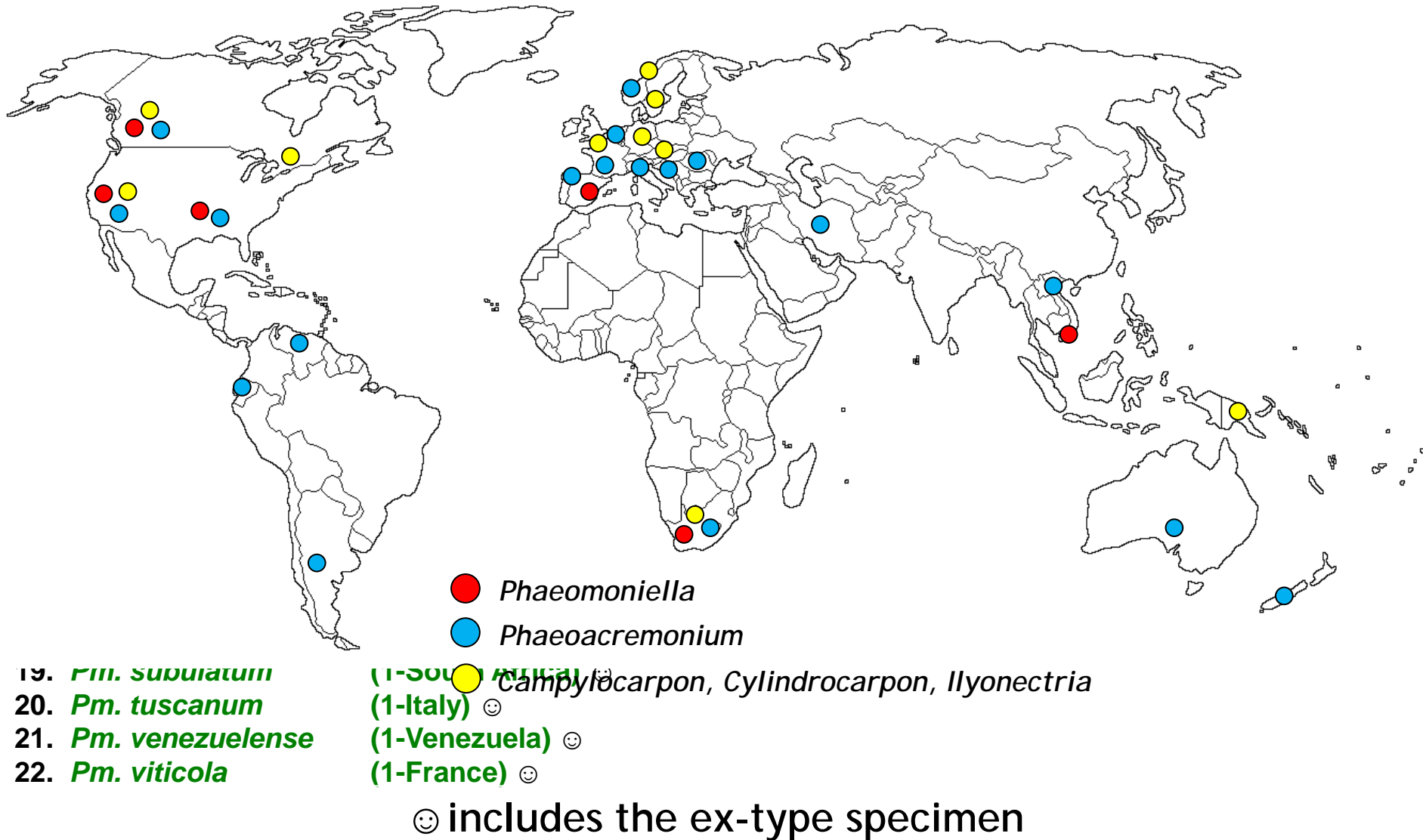
28 - *Cylindrocarpon*, *Campylocarpon*, *Ilyonectria*

6 - Control probes (Bt2a & Bt2b)

- *Fusarium*, *Botrytis*, and *Alternaria* species were included

# DNA Macroarray Development

- YVD fungal pathogens targeted (No. isolates used - Origin)

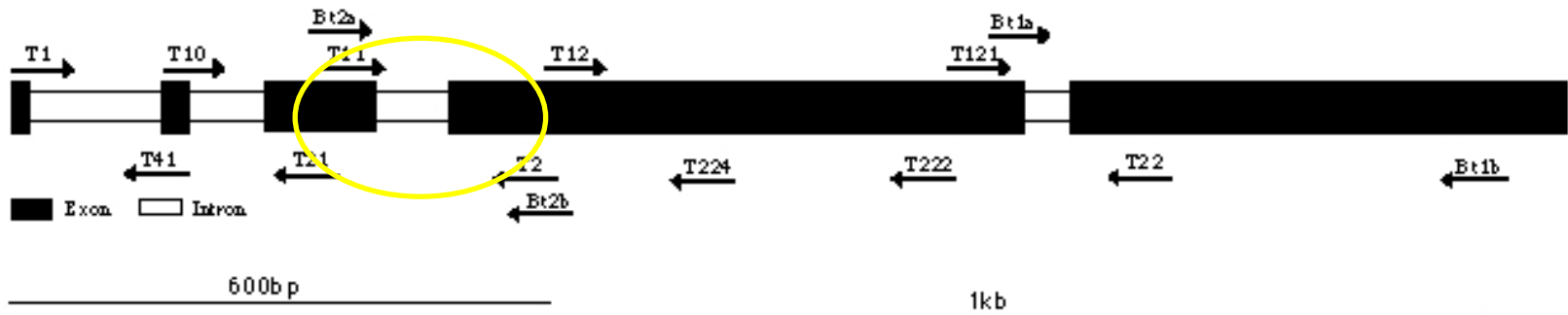


# DNA Macroarray Development

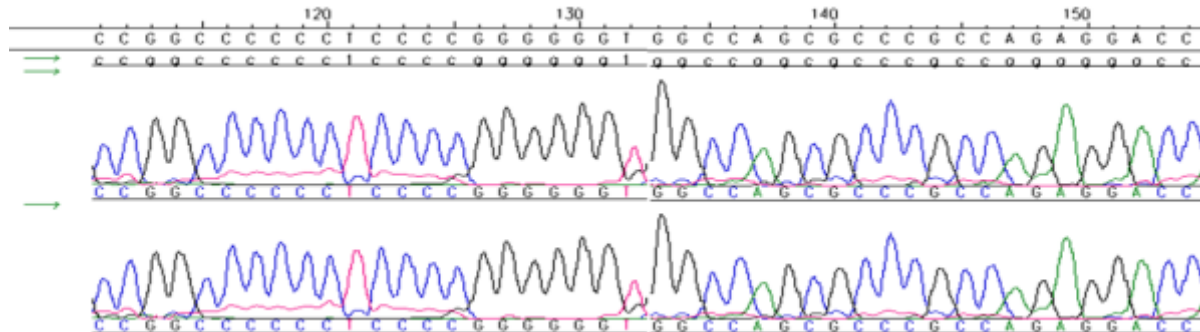
- Pathogen specific oligonucleotide probes design

DNA amplification and sequencing using primers Bt2a & Bt2b

## Beta-tubulin nuclear gene



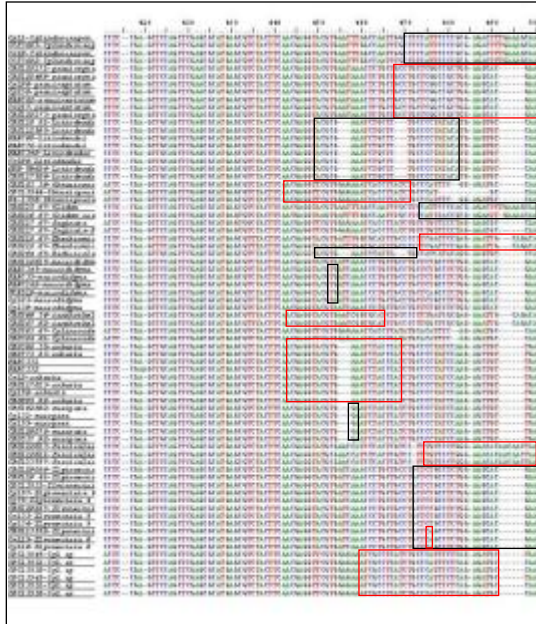
forward and reverse sequences







# DNA Sequence Alignment for Probe Selection and Design

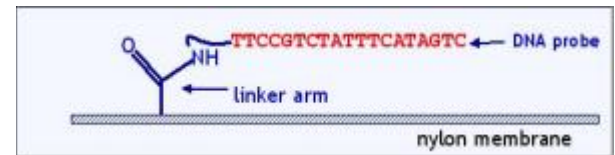


DNA sequence alignment

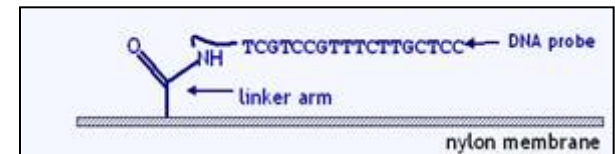
↓ Probe selection

<b>species A(1)</b>	CGCTGTC <b>TTCCGT</b> -- <b>CTATTT</b> CATAGTCGTCCCCA
<b>species A(2)</b>	CGCTGTC <b>TTCCGT</b> -- <b>CTATTT</b> CATAGTCGTCCCCA
<b>species B(1)</b>	CGCGGTCGTCCG----- <b>TTTC</b> --- <b>TTCGT</b> CCCCA
<b>species B(2)</b>	CGCTGTCGTCCG----- <b>TTTC</b> --- <b>TTCGT</b> CCCCA

Probe for **species A**

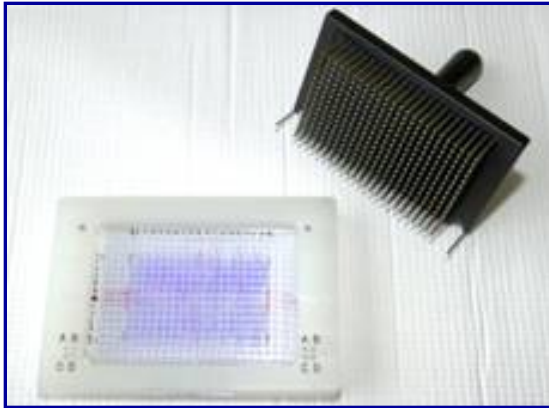


Probe for **species B**

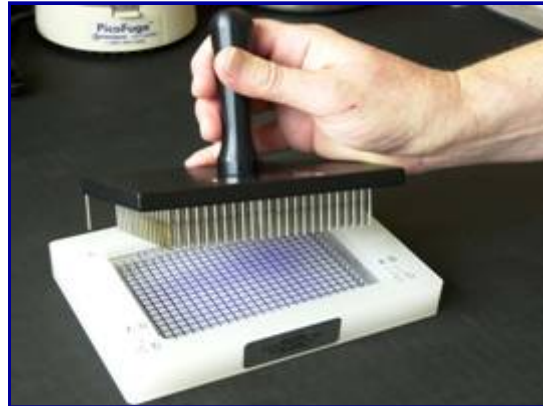


# DNA Macroarray: Attaching probes to a nylon membrane

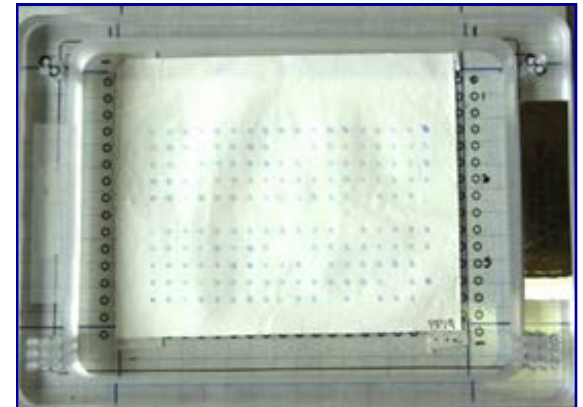
## 2) Membrane printing



384 well plate  
and transfer pins

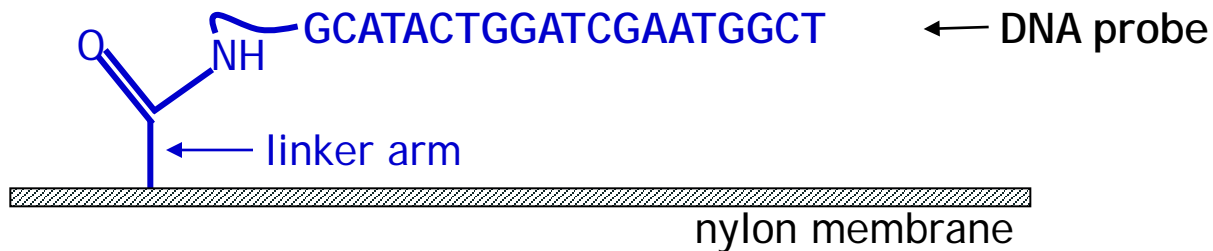


Transferring probe solution  
from the 384 well plate to  
the membrane



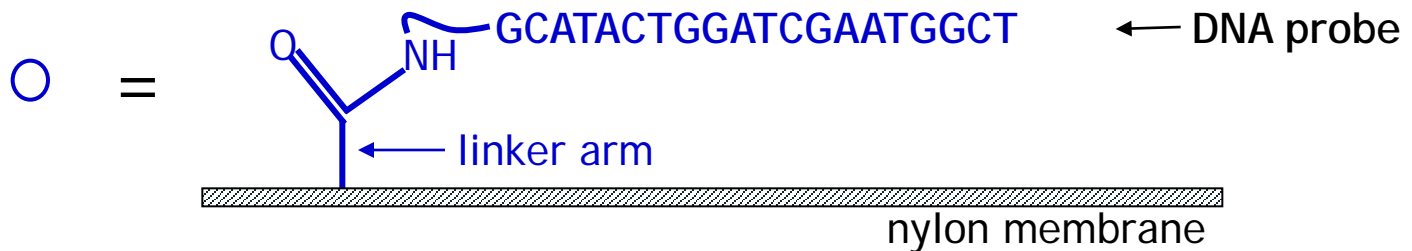
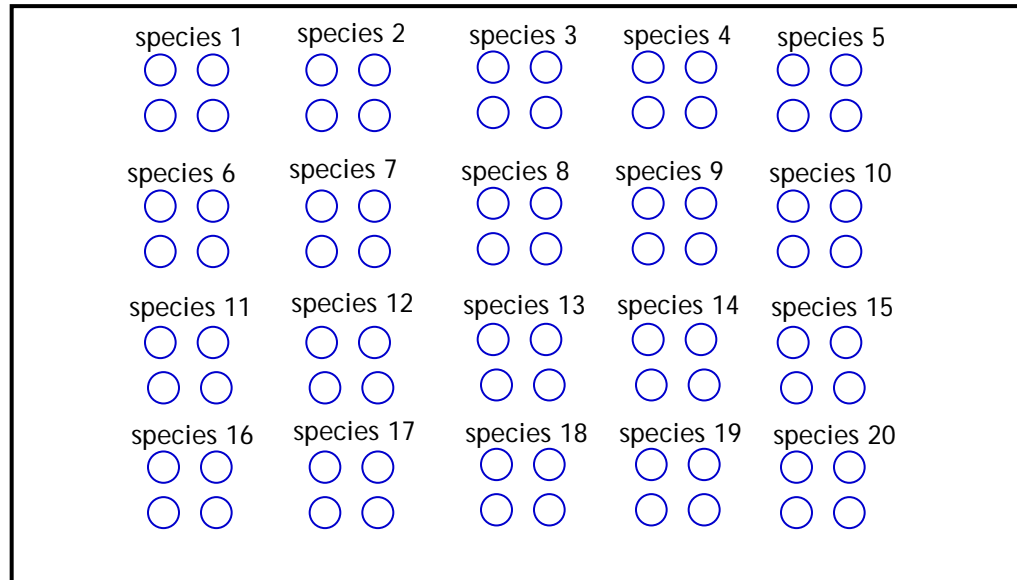
Printed membrane

### Schematic of probe attached to membrane



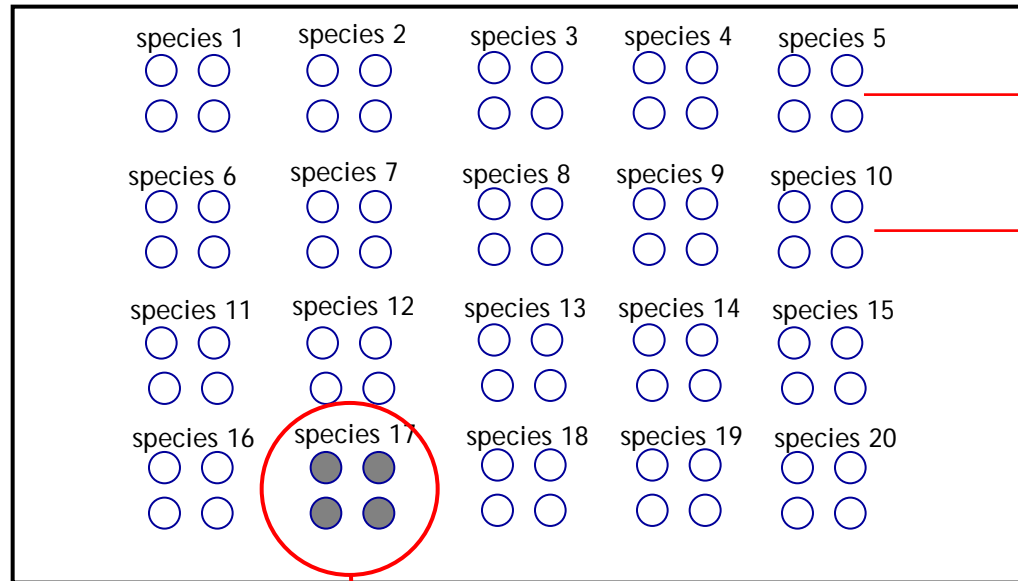
# DNA Macroarray: new membrane

## Example of freshly printed membrane

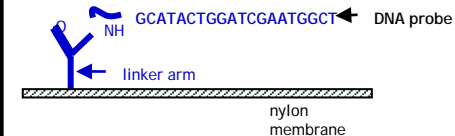


# DNA Macroarray: example of a positive reaction

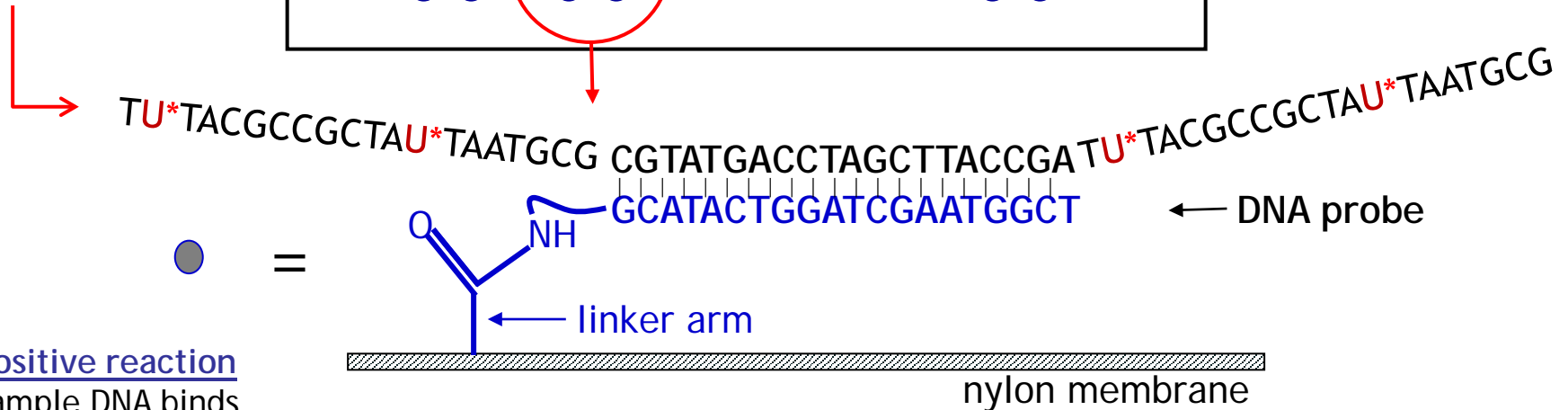
## Positive membrane reaction with sample DNA



Negative reaction  
Sample DNA does not bind to the rest of the probes

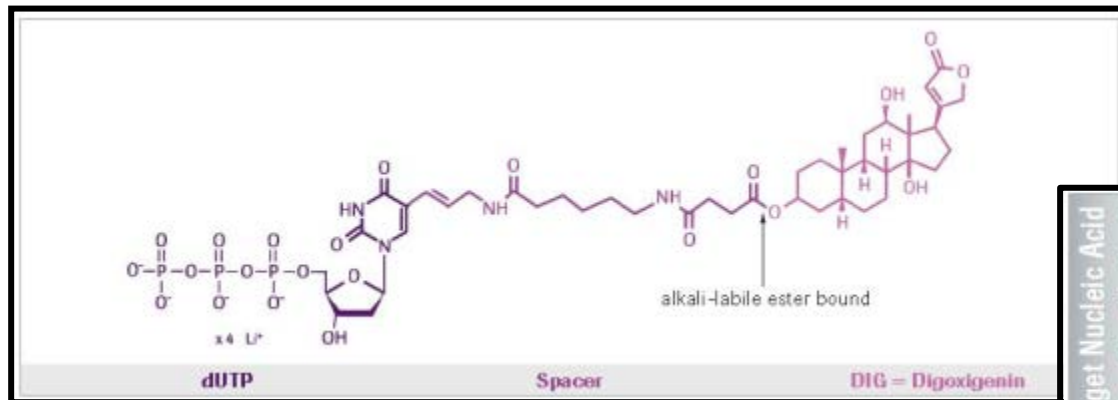


amplified sample DNA

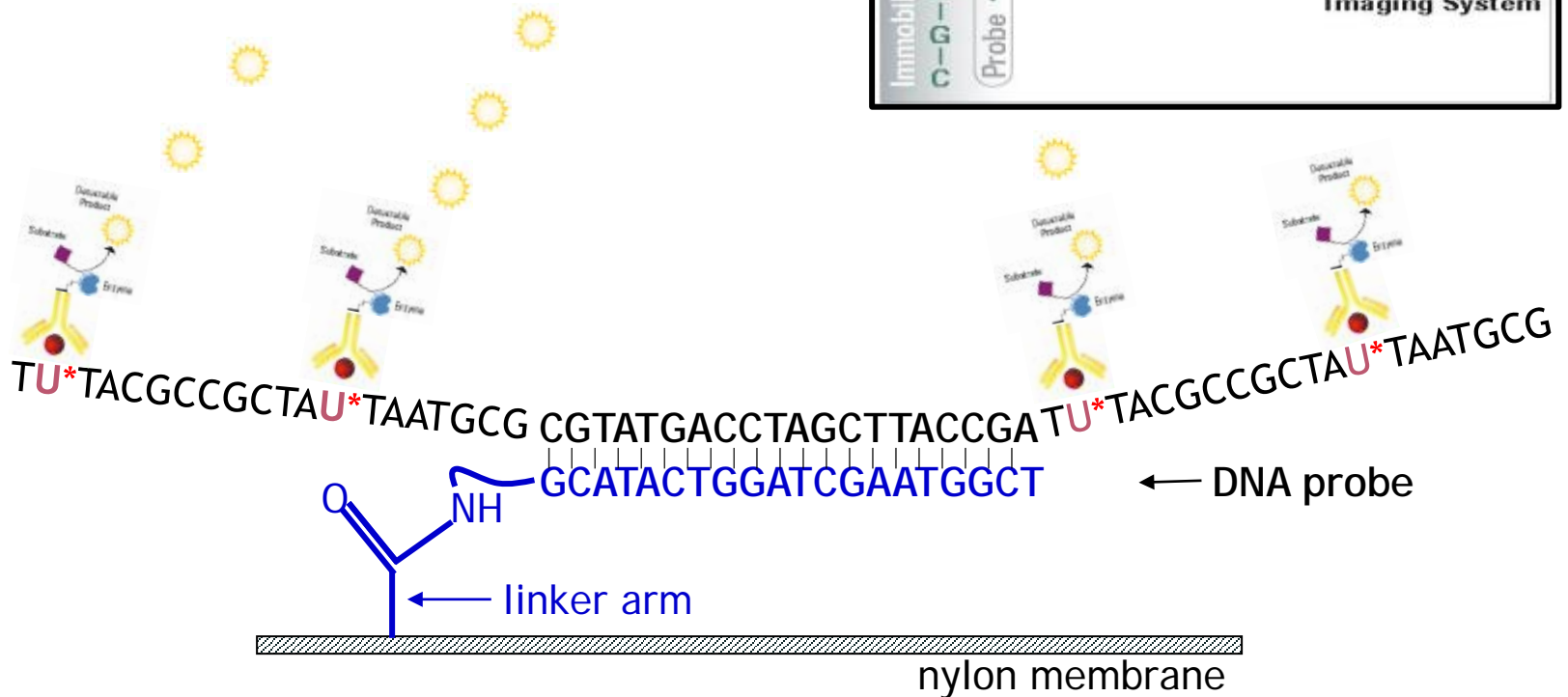
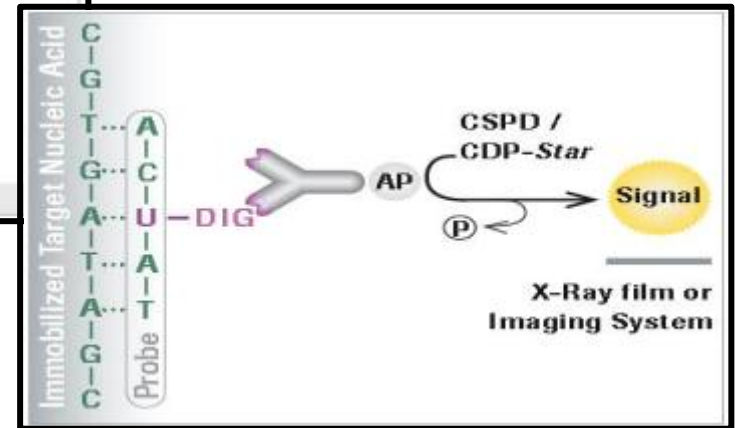


Positive reaction  
Sample DNA binds to specific probe(s)

# DNA Macroarray: positive reaction



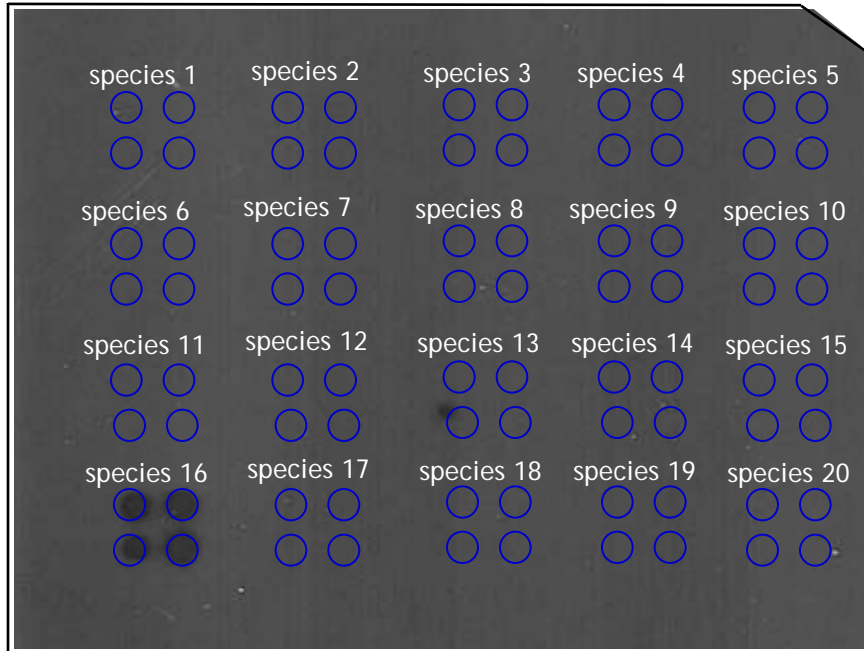
chemiluminescence



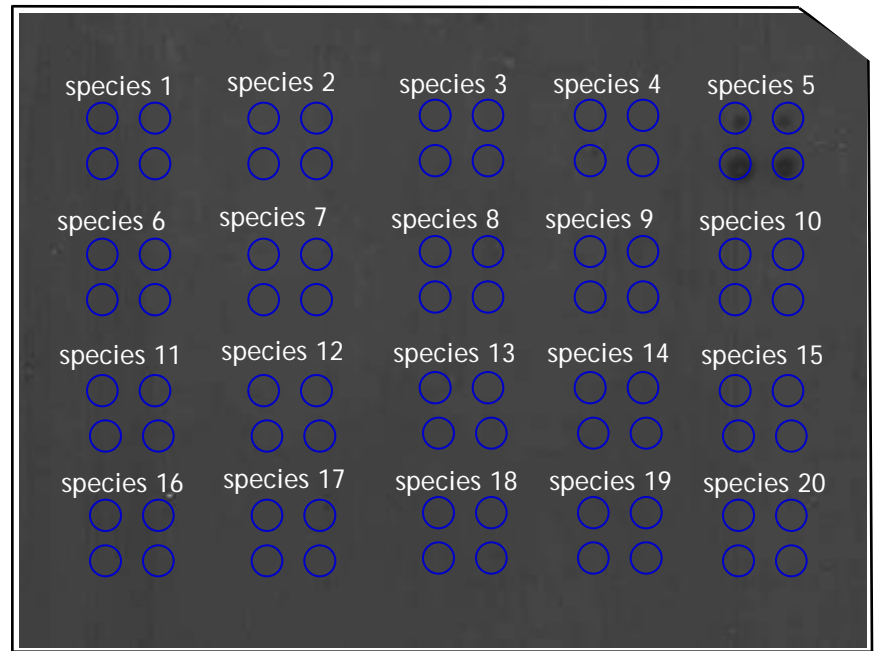
# DNA Macroarray: positive test results

## Petri Disease / Esca pathogens

Positive reaction with sample PARC70  
*Phaeoacremonium iranianum*



Positive reaction with sample ICMP 17037  
*Phaeoacremonium occidentale*

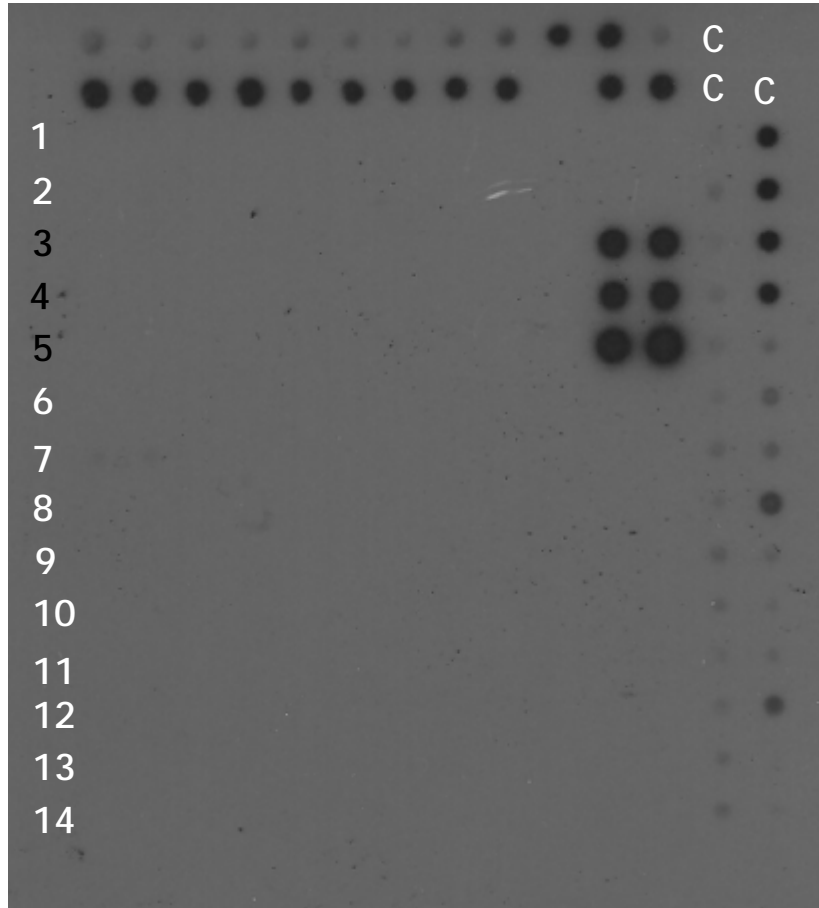


# DNA Macroarray Results

## - Pathogen detection

*Phaeoacremonium aleophilum*

CBS 631.94

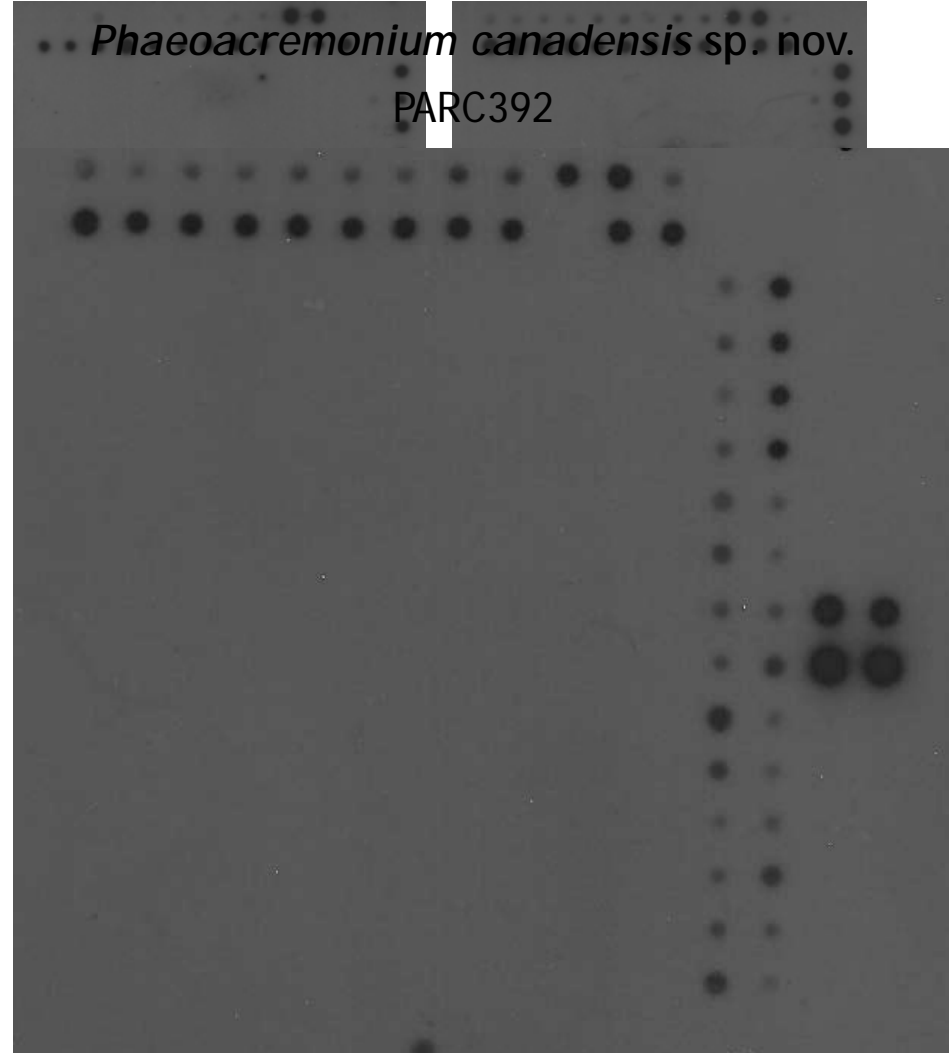


*Pm. griseorubrum*

CBS 111657

*Pm. africanum*

CBS 120863

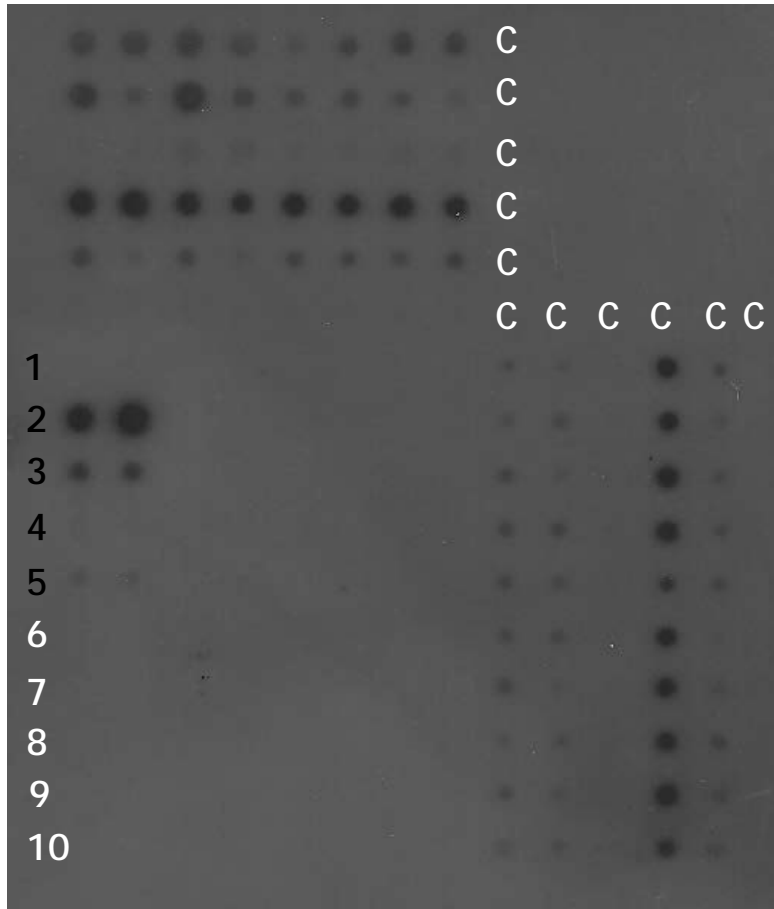


# DNA Macroarray Results

## - Pathogen detection

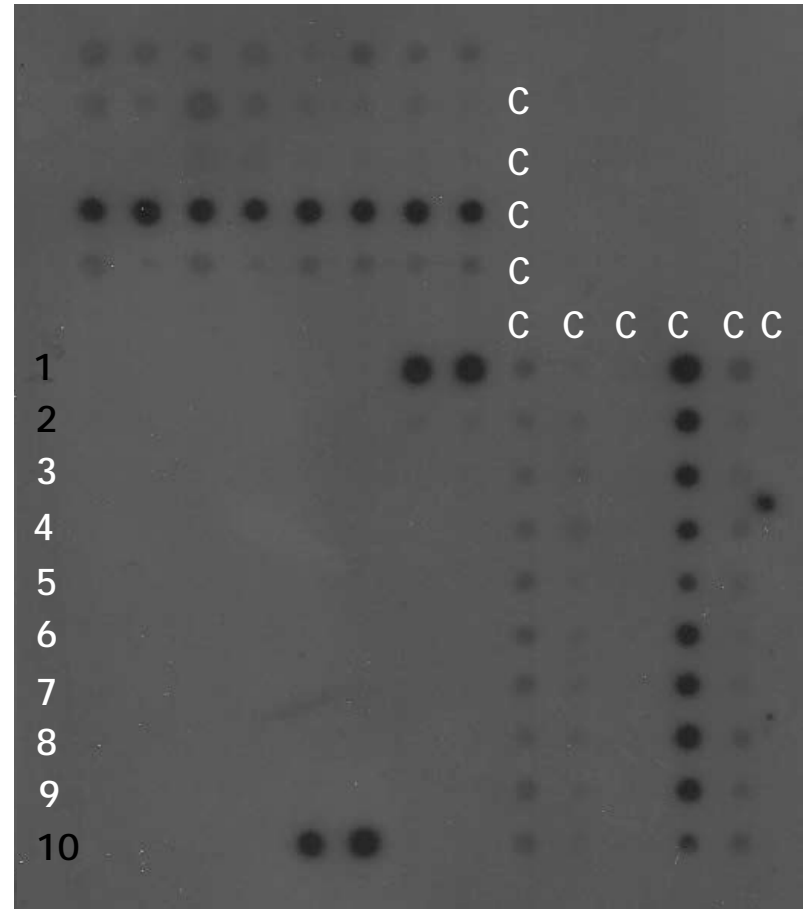
*Ilyonectria liriodendri*

CBS 110.81



*Cylindrocapsa parviusulcata*

CBS 122675





# DNA Macroarray Results

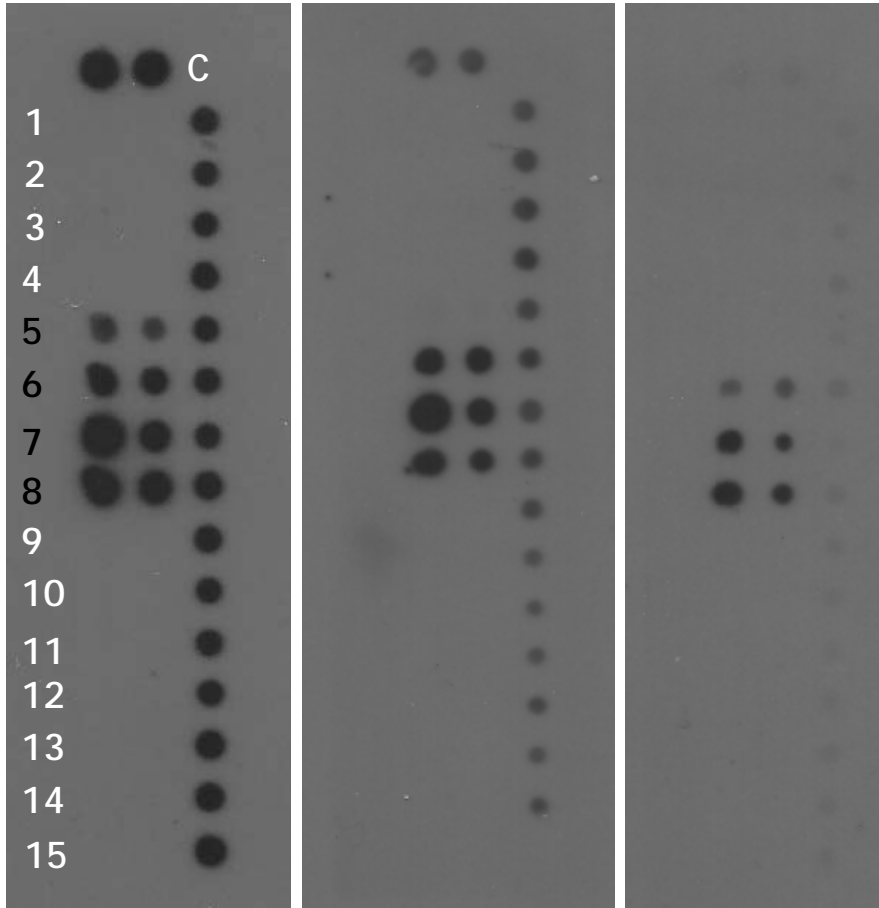
## - Pathogen detection

*Phaeomoniella chlamydospora*

CBS 117179

PARC50

PARC100

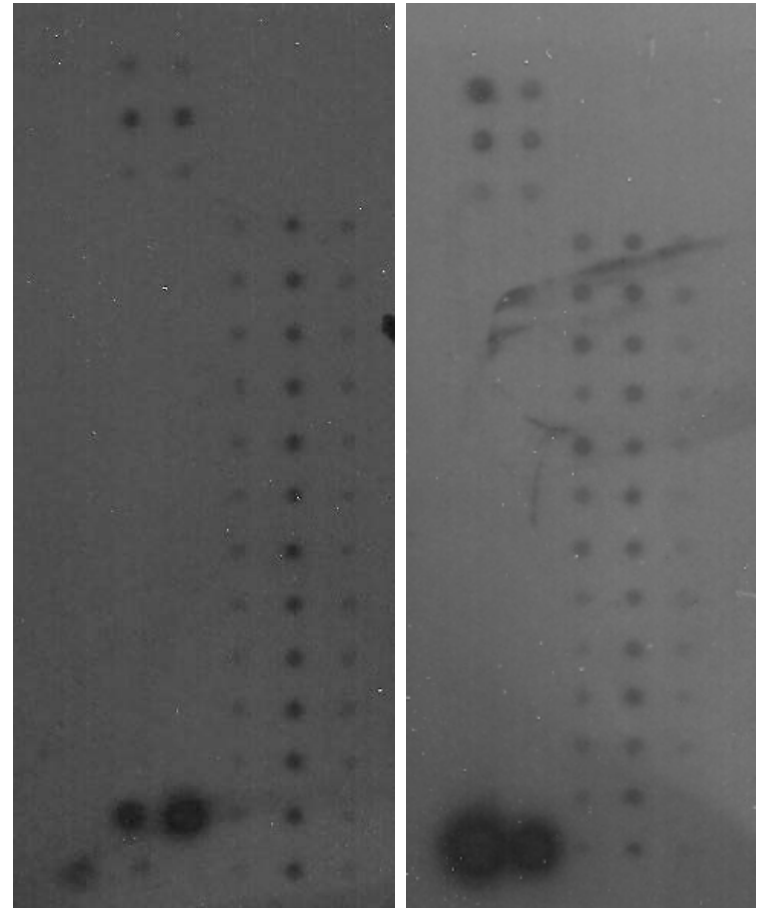


*Pa. tardicola*

CBS 121757

*Pa. zymoides*

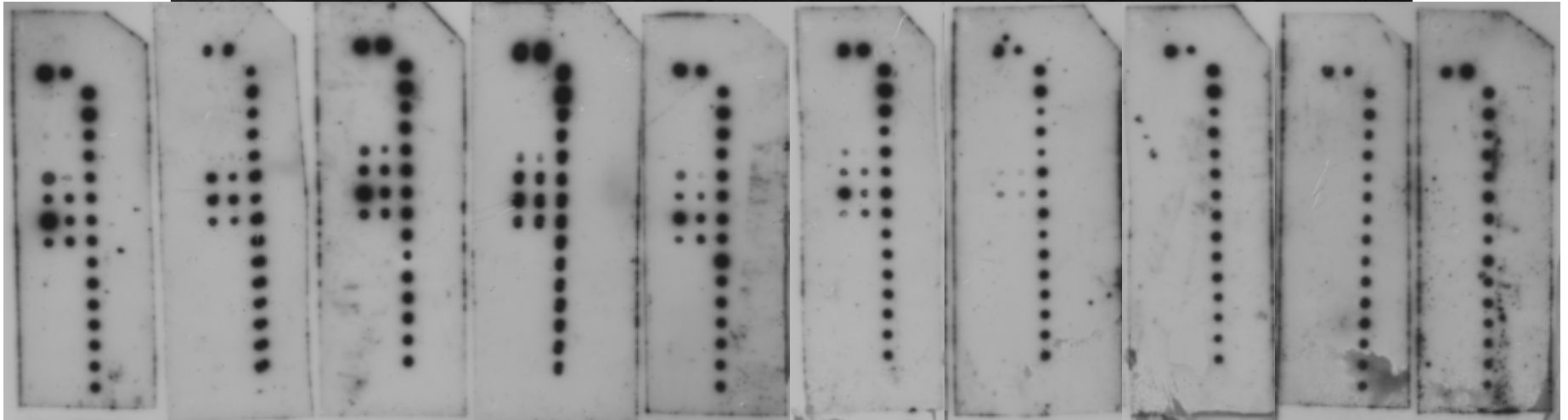
CBS 114904



- DNA

1Kb DNA ladder

1 2 3 4 5 6 7 8 9 10



1 ng/ul 10<sup>-1</sup> ng/ul 10<sup>-2</sup> ng/ul 10<sup>-3</sup> ng/ul 10<sup>-4</sup> ng/ul 10<sup>-5</sup> ng/ul 10<sup>-6</sup> ng/ul 10<sup>-7</sup> ng/ul 10<sup>-8</sup> ng/ul H<sub>2</sub>O Control

*Phaeomoniella chlamydospora* (CBS 117179)  
*Phaeomoniella chlamydospora* (CBS 117179)

DNA Stock Solution

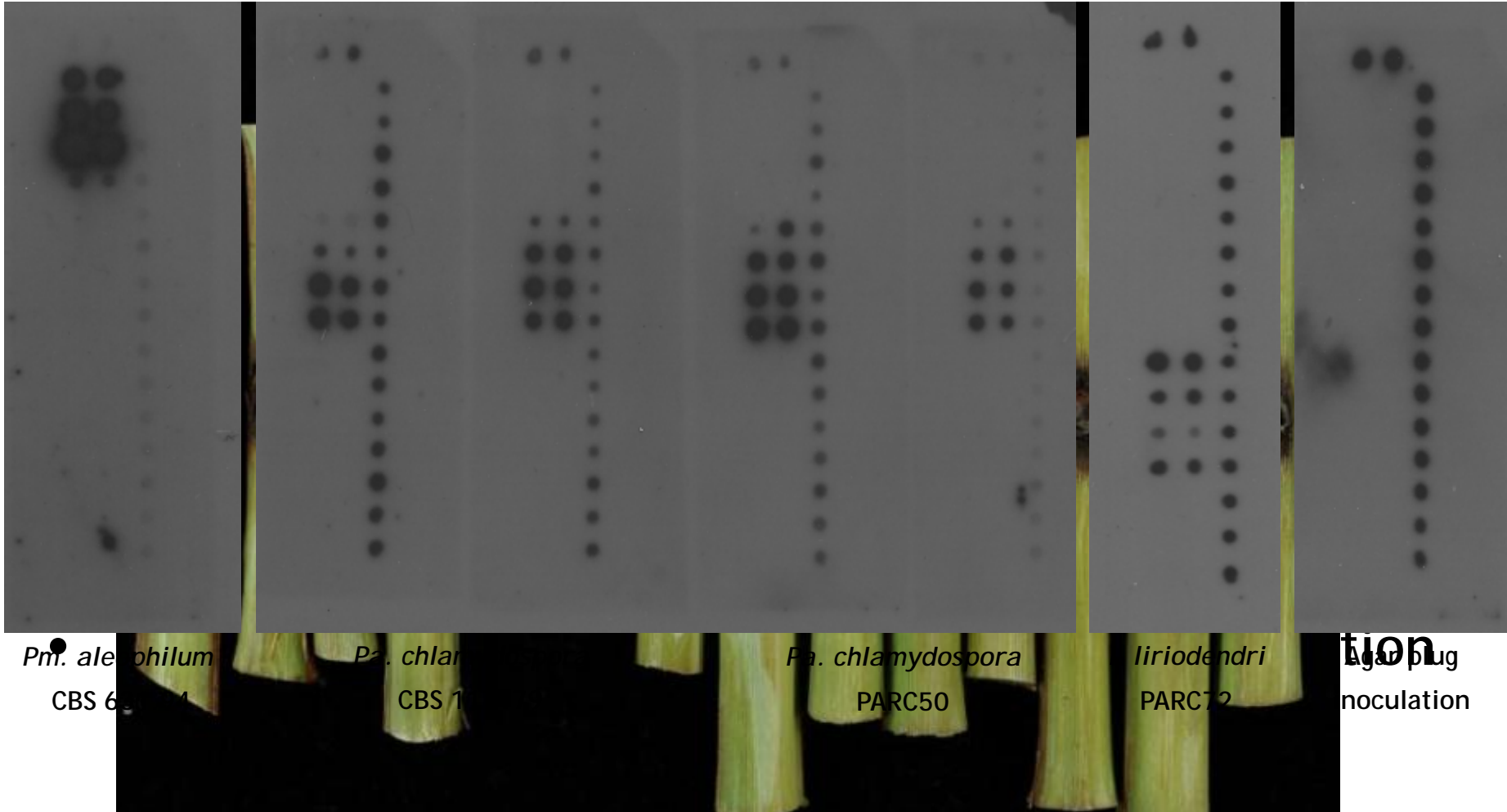
10<sup>-8</sup> ng/ul

<a href="#">deci</a>	d	<a href="#">10<sup>-1</sup></a>	0.1	
<a href="#">centi</a>	c	<a href="#">10<sup>-2</sup></a>	0.01	
<a href="#">milli</a>	m	<a href="#">10<sup>-3</sup></a>	0.001	
<a href="#">micro</a>	μ	<a href="#">10<sup>-6</sup></a>	0.000001	
<a href="#">nano</a>	n	<a href="#">10<sup>-9</sup></a>	0.000000001	
<a href="#">pico</a>	p	<a href="#">10<sup>-12</sup></a>	0.000000000001	
<a href="#">femto</a>	f	<a href="#">10<sup>-15</sup></a>	0.000000000000001	*

# DNA Macroarray Proof of Concept

- Wood tissue inoculation by using mycelium plugs

*Phaeomoniella chlamydospora* (CBS 117179)



# DNA Macroarray Summary

- Identification of 59 different species

*Phaeomoniella*

*Phaeoacremonium*

*Campylocarpon*

*Cylindrocarpon*

*Ilyonectria*

- No cross-reaction seen among the 86 YVD probes designed
- Lower detection limit between  $10^{-5}$  and  $10^{-6}$  ng of DNA
- Positive detection from direct processing of infected wood and soil samples

# Potential Applications

DNA Macroarray: scientific - diagnostic - management tool

Multiple pathogen/disease detection from a single sample with a single test

Diagnostic sample

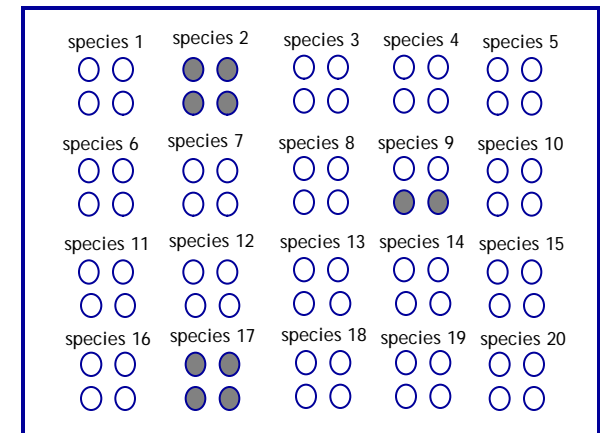


extract  
DNA



amplify and  
label DNA

Hybridize membrane



- taxonomy
- epidemiology
- diagnostics
- plant certification

# DNA Macroarray Potential Applications

## measurement of disease thresholds

A) macro array - screening for multiple pathogens

Diagnostic sample



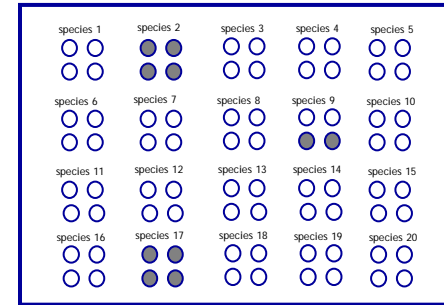
extract DNA



A) DNA macro-array

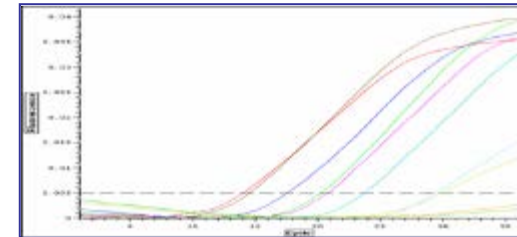
B) QPCR

Detection of multiple pathogens



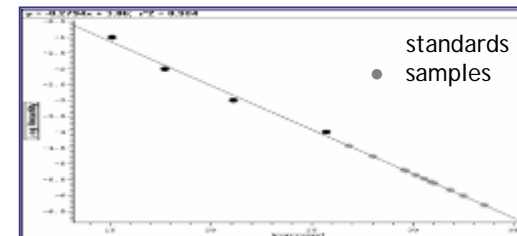
Hybridized membrane

Targeted Detection



B) QPCR - quantification (disease/infection threshold)

Quantification



# DNA Macroarray Ongoing Work

## Development of a DNA Macroarray for Canker Pathogens

Next: develop species specific probes for the canker pathogens

### Botryosphaeria dieback

1. *Botryosphaeria dothidea*
2. *Diplodia corticola*
3. *Diplodia mutila*
4. *Diplodia seriata*
5. *Dothiorella* sp.
6. *Dothiorella americana*
7. *Lasiodiplodia crassispora*
8. *Lasiodiplodia missouriana*
9. *Lasiodiplodia theobromae*
10. *Lasiodiplodia viticola*
11. *Neofusicoccum australe*
12. *Neofusicoccum luteum*
13. *Neofusicoccum macroclavatum*
14. *Neofusicoccum mediterraneum*
15. *Neofusicoccum parvum*
16. *Neofusicoccum ribis*
17. *Neofusicoccum viticlavatum*
18. *Neofusicoccum vitifusiforme*
19. *Phaeobotryosphaeria porosa*
20. *Spencermartinsia viticola*

### Eutypa dieback

1. *Eutypa lata*
2. *Eutypa leptoplaca*
3. *Eutypa laevata*
4. *Eutypella cryptovalsoidea*
5. *Eutypella microtheca*
6. *Eutypella vitis*
7. *Eutypella* sp. 1
8. *Eutypella* sp. 2
9. *Eutypella* sp. 3
10. *Eutypella* sp. 4
11. *Cryptosphaeria lygniota*
12. *Cryptosphaeria pullmanensis*
13. *Cryptovalsa ampelina*
14. *Cryptovalsa rabenhorstii*
15. *Diatrype brunneospora*
16. *Diatrype oregonensis*
17. *Diatrype stigma*
18. *Diatrype whitmanensis*
19. *Diatrypaceae* sp.
20. *Diatrypella verrucaeformis*
21. *Diatrypella vulgaris*

# DNA Macroarray Ongoing Work

- Macroarray for Canker pathogens

*Botryosphaeriaceae* - 20 spp.

*Diatrypaceae* - 21 spp.

*Phomopsis-Diaportha*

*Cadophora - Phialophora*

} ~ 30 spp.

(70 different species)

- Semi-quantification of the signal obtained (ImageQuant TL)

## DNA Macroarray Potential Applications

- Scientific tool - taxonomy, epidemiology, disease thresholds, etc.
- Diagnostic tool - transfer to public/private diagnostic laboratories
- Management tool - nursery propagation, new plantings (soil analysis) and mature vineyards (disease monitoring)



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### Research Team:

Dan O'Gorman - *Biologist*: [daniel.ogorman@agr.gc.ca](mailto:daniel.ogorman@agr.gc.ca)

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Paula Haag - *Research Technician*

Maeghan Sheepwash - *Summer Student*

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