The application of Next Generation Sequencing technology for the detection and diagnosis of non-culturable organisms – an Australian perspective.

Brendan Rodoni[1], Roberto Barrero[2], Lisa Ward[4], Fiona Constable[1], Wycliff Kinoti[1], Rachel Mann[1] and Mark Whattam[3]

[1]AgriBio, La Trobe University, Agriculture Victoria Research, Australia.

[2]Centre of Comparative Genomics, Murdoch University, Australia

[3]Department of Agriculture and Water Resources, Australia

[4] New Zealand Ministry of Primary Industry

PLANT BIOSECURITY

PREPAREDNESS, RESPONSE

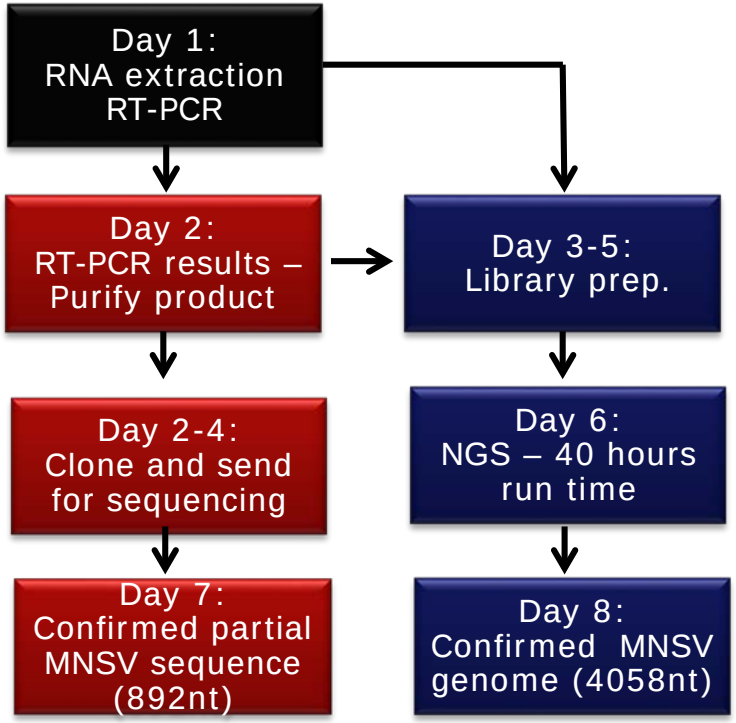
Plant Biosecurity Facts:

•Plant Quarantine is based on the presence of the pathogen and not the disease

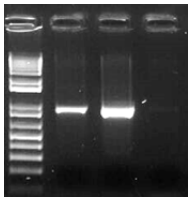
•Trade can be disrupted by the “presence of the pathogen”

Research: Next Generation Sequencing (NGS)

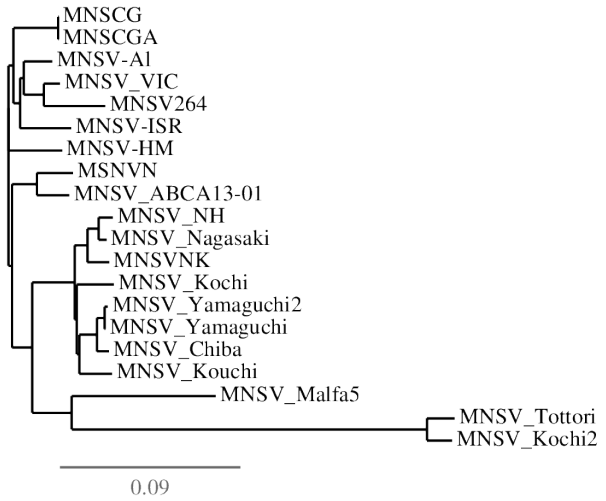
Melon necrotic spot virus (MNSV) "Suspect" to confirmation (8 days)

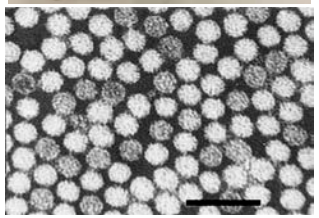


7 days to result: partial sequence



8 days to result (6 days post-PCR +ve)
Near full genome – 98% coverage
Good read depth



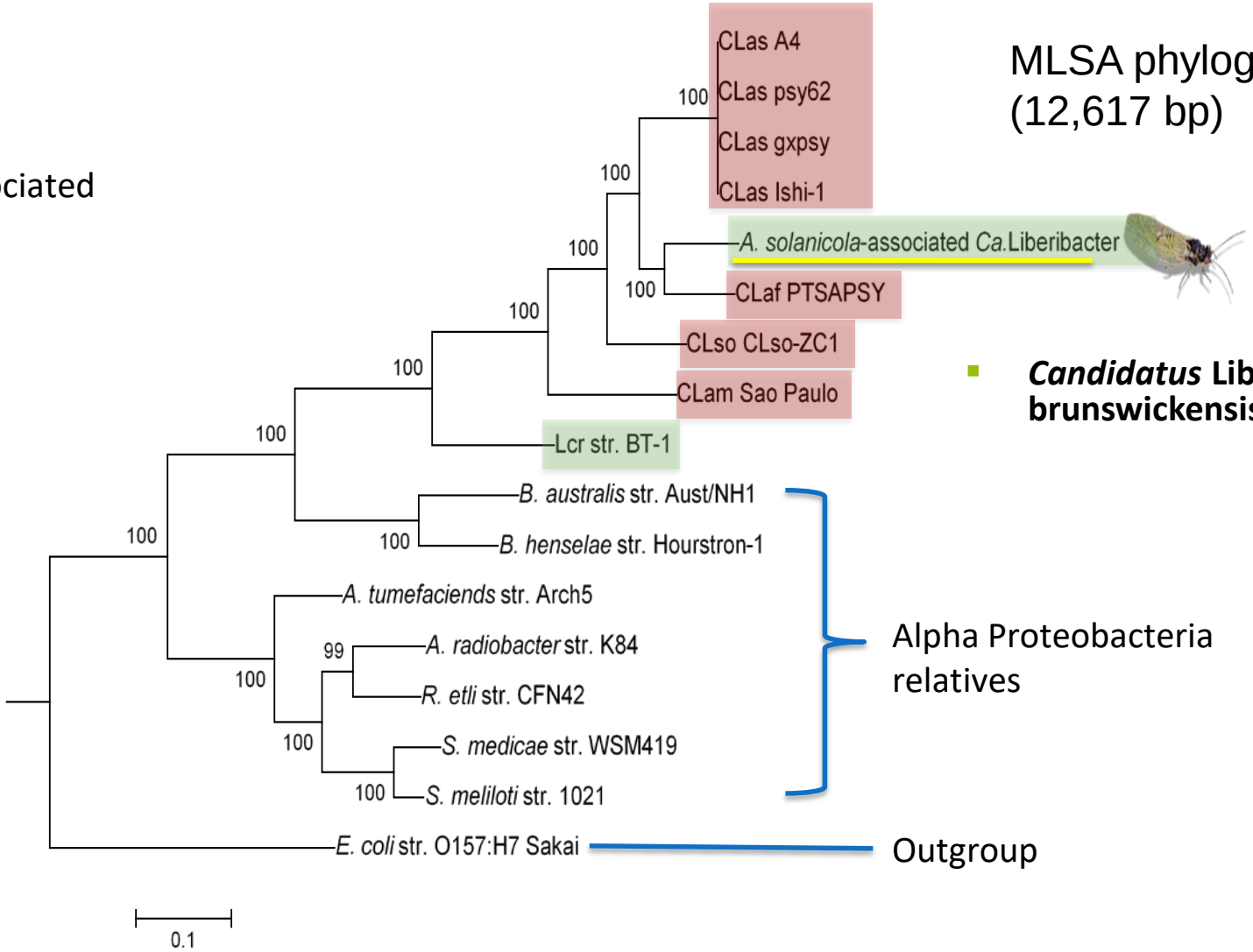


Phylogenetic tree based on full genome sequence

'*Candidatus* Liberibacter brunswickensis' identified in the Australian eggplant psyllid

Disease associated

No disease associated



MLSA phylogeny (12,617 bp)

- ***Candidatus* Liberibacter brunswickensis (CLbr)**

Alpha Proteobacteria relatives

Outgroup

Morris et al., 2017, Microbial Biotechnology

CRC PLANT biosecurity

biosecurity **built on science**

'*Candidatus* Liberibacter brunswickensis' identified in the Australian eggplant psyllid

- A new species of *Ca.* Liberibacter has been detected in *A. solanicola*
- Plant disease associated with the presence of the bacteria has not been observed

- *Candidatus* Liberibacter brunswickensis (CLbr)
- First detection of a *Ca.* Liberibacter species in mainland Australia and from the psyllid genus *Acizzia*

Implication for Diagnostics of High Priority Pests associated with

- Citrus greening
- Zebra Chip

FALSE POSITIVES!!!!!!!

	CLaf HLBaf Li *et al,.* 2006	CLas HLBas Li *et al,.* 2006	CLam HLBam Li *et al,.* 2006	CLso LsoF Li *et al,.* 2009
CLaf	+	-	-	-
CLas	-	+	-	-
CLam	-	-	+	-
CLso	-	-	-	+
CLbr	+	+	-	-

+ false positive!!!!

Morris et al., 2017, Microbial Biotechnology

Surveillance and diagnosis of viruses and viroids using a small RNA next generation sequencing approach

An internet-based bioinformatics toolkit for Plant Biosecurity diagnosis and surveillance of viruses and viroids

Roberto Barrero[1] , Mark Whattam[2] & Lisa Ward[3]

[1]*Centre of Comparative Genomics, Murdoch University, Australia*

[2]*Department of Agriculture and Water Resources, Australia*

[3] *New Zealand Ministry of Primary Industry*

Diagnosis of viruses and viroids at PEQ

Background

- Approx. 500 high risk plants imported annually into Australia (stone/pome fruit, citrus, potato, berry crops, grapevine, etc).
- Current PEQ protocols are time and resource consuming:
  - Visual and biological indicators (herbaceous and woody indicators)
  - Transmission electron microscope (TEM)
  - Serological (ELISA)
  - Molecular (PCR)

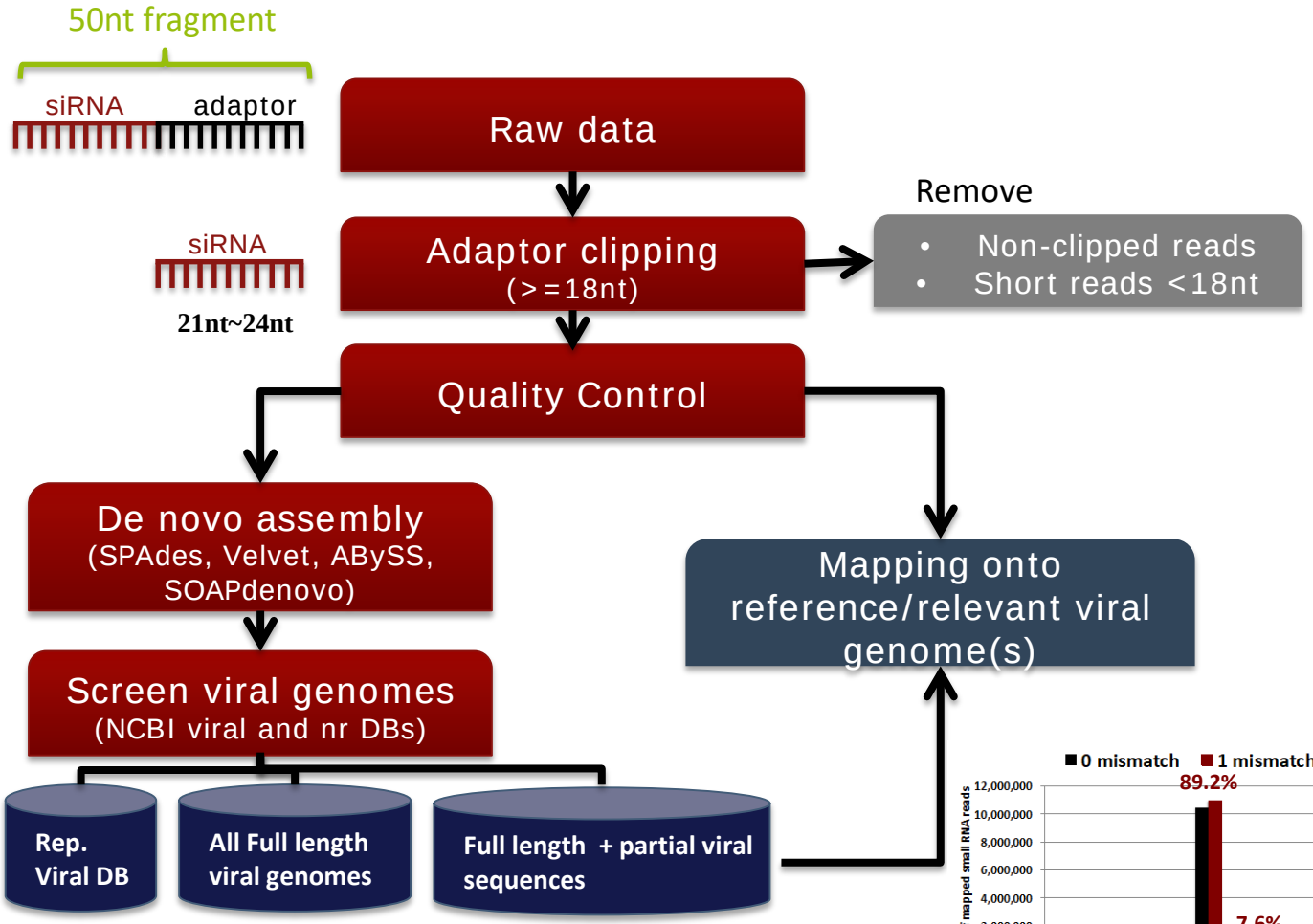
Issues

- Spend 2+ years in PEQ, ambiguous, expensive and declining expertise
- Prolonged delays impact plant industries competitiveness and profitability

Solution

- Need a rapid and robust assay to accelerate quarantine screening
- Implemented **small RNA NGS** approach using specifically host immune response products (21-22 nt siRNAs) detects reliably all known viruses and viroids in a single assay (Barrero et al, 2017).

Overview of bioinfomatics workflows (YABI)



- Dicer4 → 21nt
- Dicer2 → 22nt

Viral pathogen derived reads are mainly 21nt long

(Barrero et al., 2017a)

PCR validation: Side-by-side comparisons

					PEQ				NGS			
Tube label	Project sample identifier	Host	Plant species	Virus	ELISA	S-PAGE Viroids	PCR	Biological indexing	YABI	NGS (VirusDetect)	Confirm	Result
C1	PB64-S093	Citrus	Citrus aurantifolia (Christm.) Swingle	CVEV				✓	×	×		
C1	PB64-S093	Citrus	Citrus aurantifolia (Christm.) Swingle	CEVd			×	×	×	✓	PCR(-)	S
C1	PB64-S093	Citrus	Citrus aurantifolia (Christm.) Swingle	CTV			✓	✓	✓	✓	N/A	E
C1	PB64-S093	Citrus	Citrus aurantifolia (Christm.) Swingle	CVd-III			✓	✓	✓	✓	N/A	E
C1	PB64-S093	Citrus	Citrus aurantifolia (Christm.) Swingle	HSVd			✓	✓	✓	✓	N/A	E
C2	PB64-S094	Citrus	Troyer citrange	CEVd		✓	✓	✓	✓	✓	N/A	E
C2	PB64-S094	Citrus	Troyer citrange	CTV			×	×	×	✓	PCR(-)	S
C2	PB64-S094	Citrus	Troyer citrange	CVd-IV		×	×	×	×	✓	PCR(-)	S
C2	PB64-S094	Citrus	Troyer citrange	HSVd		×	×	×	×	✓	PCR(-)	S
C3	PB64-S095	Citrus	Citrus medica L.	CEVd		×	×	×	×	✓	PCR(-)	S
C3	PB64-S095	Citrus	Citrus medica L.	CTV			✓	✓	✓	✓	N/A	E
C3	PB64-S095	Citrus	Citrus medica L.	HSVd		✓	✓	✓	✓	✓	N/A	E
C4	PB64-S096	Citrus	Rusk Citrange /Swingle Citrumello	CTLV	✓		×	✓	×	×	PCR(-)	E
C4	PB64-S096	Citrus	Rusk Citrange /Swingle Citrumello	CEVd		×	×	×	×	✓	PCR(-)	S
C4	PB64-S096	Citrus	Rusk Citrange /Swingle Citrumello	CitPRV				×	×	✓	N/A	S
C4	PB64-S096	Citrus	Rusk Citrange /Swingle Citrumello	CTV	×		×	×	×	✓	PCR(-)	S
C5	PB64-S097	Citrus	Citrus limon 'Eureka'	Crinkly leaf ilarvirus				✓	×	×		
C5	PB64-S097	Citrus	Citrus limon 'Eureka'	CEVd		×	×	×	×	✓	PCR(-)	S
C5	PB64-S097	Citrus	Citrus limon 'Eureka'	CTV	×		×	×	×	✓	PCR(-)	S
C5	PB64-S097	Citrus	Citrus limon 'Eureka'	HSVd		×	×	×	×	✓	PCR(-)	S
C6	PB64-S098	Citrus	Citrus x sinensis	Citrus ringspot virus/ Psorosis B			×	✓	×	×	PCR(-)	E
C6	PB64-S098	Citrus	Citrus x sinensis	CEVd			×	×	×	✓	PCR(-)	S
C6	PB64-S098	Citrus	Citrus x sinensis	CTV			×	×	×	✓	PCR(-)	S
C6	PB64-S098	Citrus	Citrus x sinensis	CVd-III			✓	✓	✓	✓	N/A	E
C6	PB64-S098	Citrus	Citrus x sinensis	HSVd			✓	✓	✓	✓	N/A	E
C7	PB64-S099	Citrus	Eureka, West Indian Lime	CEVd		×	×	×	×	✓	PCR(-)	S
C7	PB64-S099	Citrus	Eureka, West Indian Lime	CTV	✓		✓	✓	✓	✓	N/A	E
C7	PB64-S099	Citrus	Eureka, West Indian Lime	HSVd		×	×	×	×	✓	PCR(-)	S

× Negative test

✓ Positive test

	Not analysed further
sus	Suspicious test by PCR
NT	not tested
E	Equivalent - all three match
S	Similar - Conventional and YABI match
D	Different
PCR(+/-)	Follow up PCR test was positive or negative

False positive PEQ

False positive VirusDetect

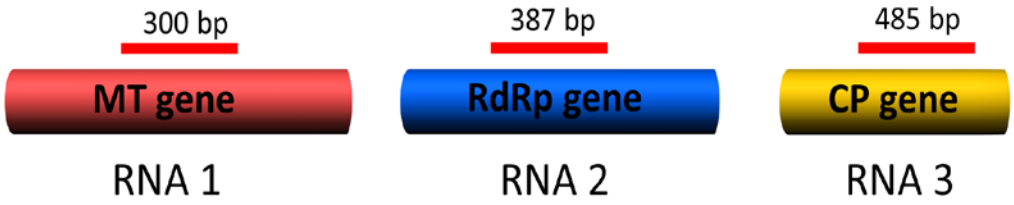
(Barrero, Mackie et al., *in preparation*)

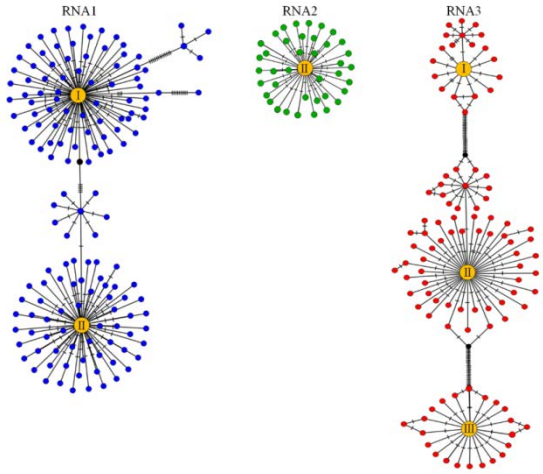
CRC PLANTbiosecurity

biosecurity **built on science**

Amplicon Deep Sequencing to Determine Ilarvirus Species Diversity in Australian Prunus

- Wycliff Kinoti (PhD Candidate)
- NGS of
  - Species specific amplicons (e.g. PNRSV)
  - Genus-specific amplicons (e.g. Ilarvirus)





PNRSV genetic strains identified

Isolate	RNA1 (MT gene)	RNA2 (RdRp gene)	RNA3 (CP gene)
K72	1	1	1
M19	2	2	2
Q15	2	1	3

Generic *Ilarvirus* (RNA2) detection

Isolate	*Ilarvirus* generic amplicon NGS	Sanger sequencing of cloned *Ilarvirus* generic amplicon	RNA2 amplicon region species-specific RT-PCR	RNA1 / 3 species-specific RT-PCR	Metagenomic NGS
K75	ApMV	ApMV	ApMV	ApMV	ApMV full genome
NS9	PDV	PDV	PDV	PDV	PDV full genome
M32	PNRSV	PNRSV	PNRSV	PNRSV	PNRSV full genome
Pch4	PDV	Neg	PDV	PDV	PDV full genome
	PNRSV	Neg	PNRSV	PNRSV	PNRSV full genome
	Ilarvirus-S1	*Ilarvirus*-S1	*Ilarvirus*-S1	Neg	Neg
Q15	APLPV	APLPV	APLPV	APLPV	APLPV full genome
	PNRSV	Neg	PNRSV	PNRSV	PNRSV full genome
	Ilarvirus-S1	Neg	*Ilarvirus*-S1	Neg	Neg
BPch	*Ilarvirus*-S1	*Ilarvirus*-S1	*Ilarvirus*-S1	Neg	Neg
Ch1	*Ilarvirus*-S1	*Ilarvirus*-S1	*Ilarvirus*-S1	Neg	Neg
	Ilarvirus-S2	*Ilarvirus*-S2	*Ilarvirus*-S2	Neg	Neg
FPch	*Ilarvirus*-S1	*Ilarvirus*-S1	*Ilarvirus*-S1	Neg	Neg
Pch2	*Ilarvirus*-S1	*Ilarvirus*-S1	*Ilarvirus*-S1	Neg	Neg
Tas3	*Ilarvirus*-S1	*Ilarvirus*-S1	*Ilarvirus*-S1	Neg	Neg

Kinoti, Wycliff M., et al. *Frontiers in Microbiology* 8 (2017)

Research: R&D4Profit
- National Pest Surveillance

Diagnostic Surveillance Hub, 2017-2022 (5 year project - $22mil)

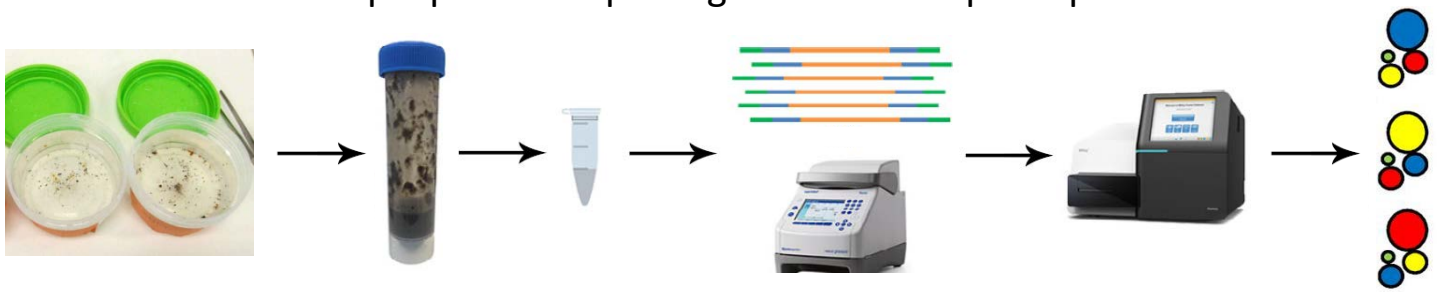
Partnership between: AgVic, CSIRO, SARDI, WA DPIRD

Industries: Grains, Sugar, Cotton, Horticulture, Wine, Forestry

AgVic Role: Metabarcoding

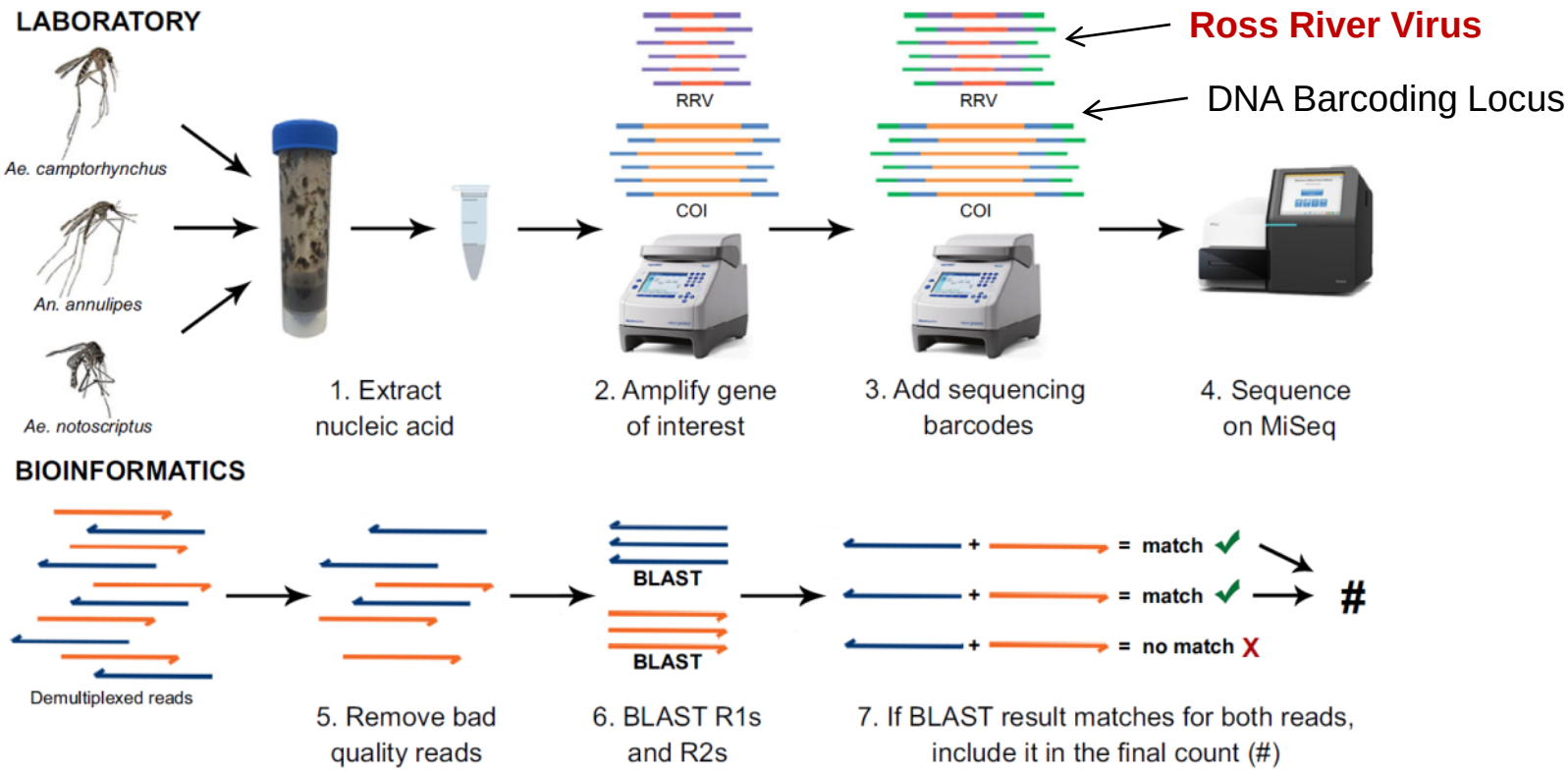
Scope: Utilise molecular methods to detect specific plant pests (e.g. insects) and associated pathogens (e.g. spores / viruses) of concern, including surveillance for potential exotic pests.

Outputs: To develop and deliver new molecular approaches to surveillance, including NGS, for the detection of multiple pests and pathogens within trap samples.



Metabarcoding

Invertebrate Molecular Identification – Mosquitoes

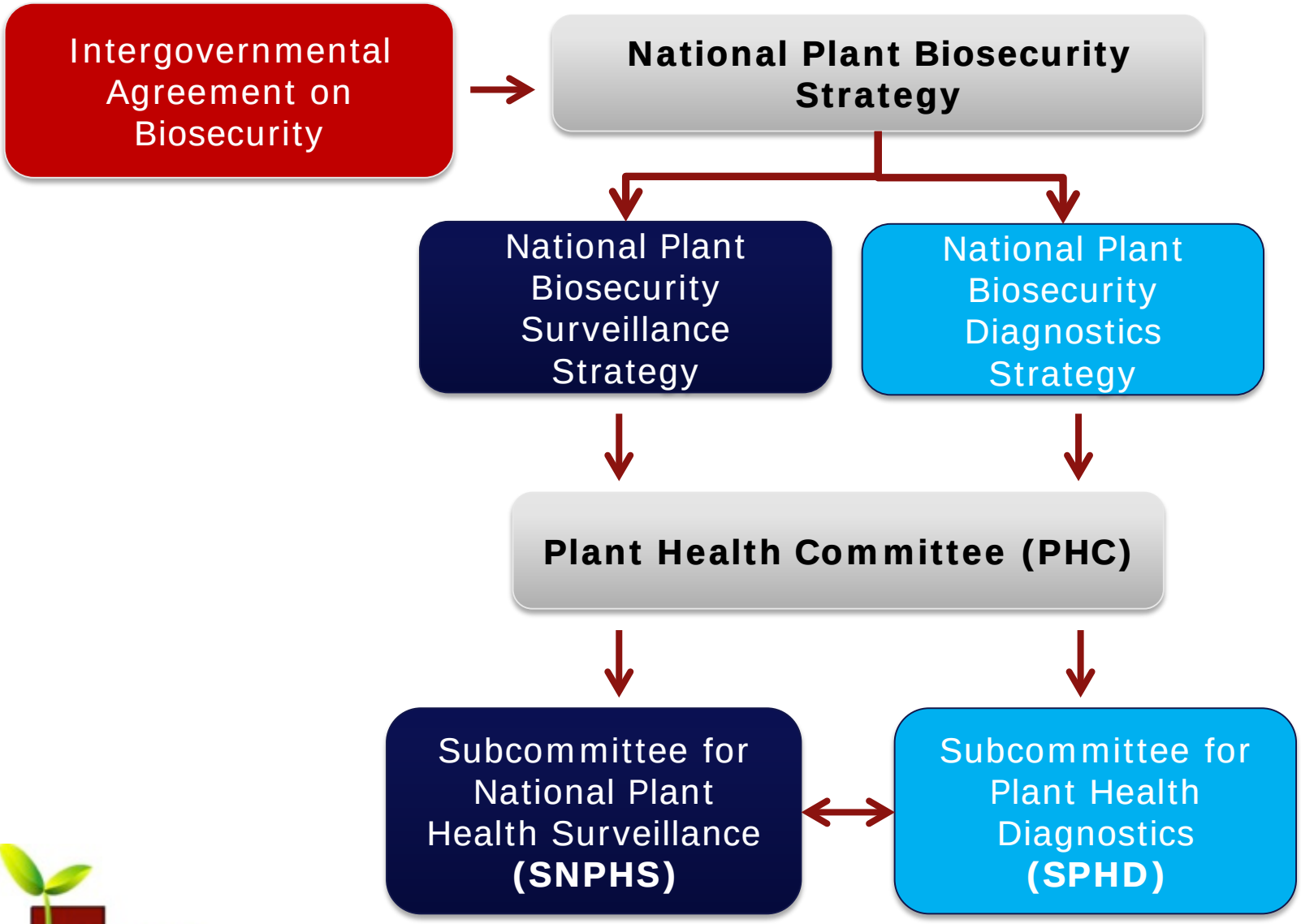


Batovska, Lynch, Cogan, Brown, Darbro, Kho & Blacket (2017)
Ecology & Evolution

CRC PLANT biosecurity

biosecurity **built on science**

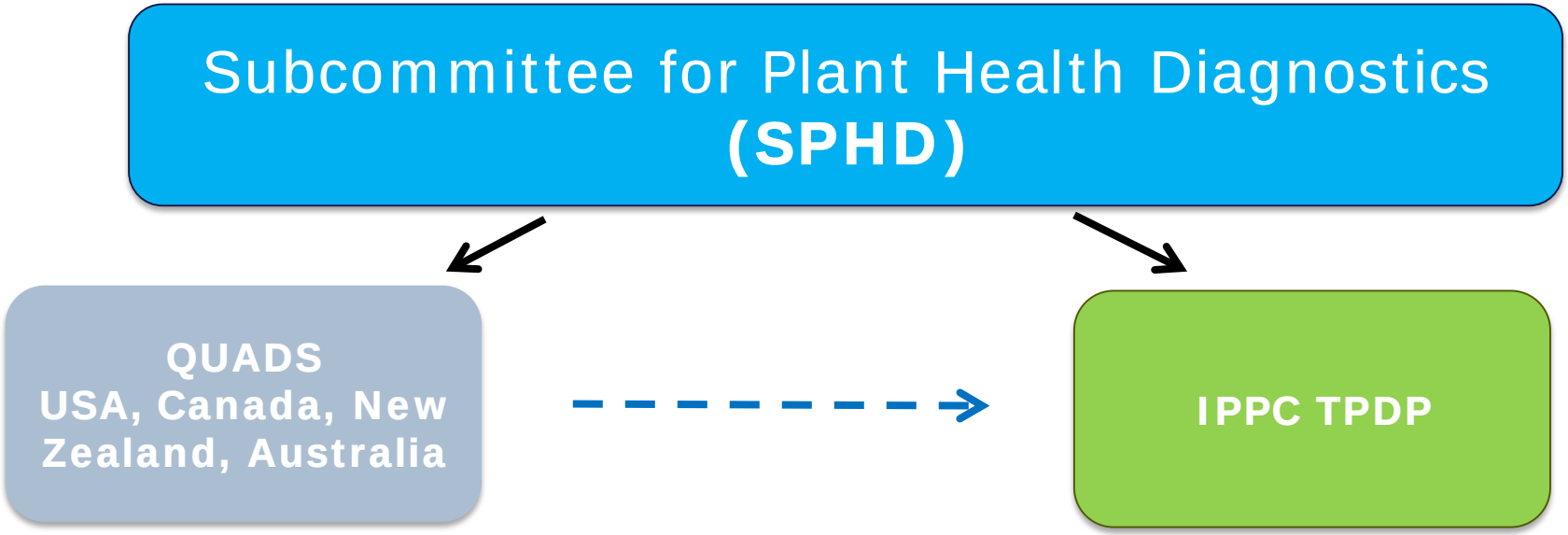
National Plant Biosecurity Strategy



Subcommittee for Plant Health Diagnostics **(SPHD)**

- Facilitate the development of a diagnostic capability and capacity for all High Priority Pests
  - Develop and recommend national standard processes relating to plant pest diagnostics
  - Promote and facilitate the development of National Diagnostic Protocols (NDPs) for EPPs and endemic pests of national significance

- The National Plant Biosecurity Network **(NBPDN)**
  (http://plantbiosecuritydiagnostics.net.au/)
  - SPHD Reference Standard No. 2:
    "Development of Diagnostic Protocols Instructions to Authors"
  - Based on the IPPC ISPM No 27 "Diagnostic protocols for Regulated Pests (IPPC 2006)"



Plant Health Quads 0035 - Diagnostic Tools Collaboration (2008 -)

Geoff Dennis, Patrick Shiel (USA), Mark Nakhla, Laurene Levy (USA);
Pam Rose, Thomas Niederberger (Canada);
Lia Liefting, Lisa Ward (New Zealand);
Brendan Rodoni, Mike Hodda (Australia)

Quads Working group: "Managing regulatory issues arising from new diagnostic technology"

Benedicte Lebas, Rose Souza-Richards (NZ MPI);
Anna-Mary Schmidt, Sarah Brearey (CFIA Canada);
Shailaja Rabindran, Gloria Abad (USDA);
Brendan Rodoni (Australia)

Plant Health Quads 0035 - Diagnostic Tools Collaboration (2008 -)

- Generate a guidance paper on NGS and its application in a diagnostic Laboratory
  - Identify critical control points (CCP) involved in generating NGS data
    - Sampling
    - Nucleic acid extraction
    - Sequence library preperation
    - Sequencing
    - Bioinformatics (data analysis)

  - Formulate guidelines and standards (policy) for each CCP

- A framework for a position paper on NGS and its application in a diagnostic laboratory has been generated. The position paper will be completed in 2018.

- Webinars and seminars from each member country on the application of NGS as a diagnostic tool have been presented to the working group.

Quads position papers related to NGS

Next Generation Sequencing:

- Sampling
- Nucleic acid extraction

"Laboratory best Practice" guidelines generated and aligned with "Instruction

Issues/challenges for Next Generation Sequencing for the detection and diagnosis of non-culturable organisms:

- Baseline data required at Critical Control Points:
  - Accuracy
  - Sensitivity
  - Reproducibility
- Minimum requirements to make a diagnosis on NGS data
  - Provisional Taxonomic Assignment

Taxonomic Assignment:

Acknowledgments

MURDOCH UNIVERSITY
PERTH, WESTERN AUSTRALIA

CENTRE FOR COMPARATIVE GENOMICS

Australian Government
Department of Agriculture and Water Resources

- Matthew Bellgard
- Kathryn Napier
- Adam Hunter
- Tamas Szabo

Plant & Food RESEARCH
RANGAHAU AHUMĀRA KAI

- Simon Bulman
- Sandi Keenan
- Rebekah Frampton
- Arnaud Blouin

- Mark Whattam
- James Cunnington
- Adrian Drinsdale
- Joanne Mackie

MPI

- Michael Robinson
- Jo Luck
- Carla Tadich
- Jane Moran

- Lisa Ward
- Lia Liefting
- Subuhi Khan

CRC PLANT biosecurity

biosecurity **built on science**